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ABSTRACT: Information is provided concerning the new subspecialty certificate in neuromuscular medicine of the American Board of Psychiatry and Neurology and the eligibility requirements for such certification of practicing neurologists and child neurologists. The Accreditation Council for Graduate Medical Education has approved fellowship training in the subspecialty, and it is likely that residents who wish to pursue a career in neuromuscular medicine will select this training option.

CERTIFICATION IN NEUROMUSCULAR MEDICINE: A NEW NEUROLOGIC SUBSPECIALTY

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In 2005 the American Board of Psychiatry and Neurology (ABPN) and the American Board of Physical Medicine and Rehabilitation (ABPMR) received approval from the American Board of Medical Specialties (ABMS) to issue subspecialty certificates in neuromuscular medicine. The ABPN received the original request for recognition from the neuromuscular section of the American Academy of Neurology (AAN). The goals of the certification process are to improve education, research, and patient care in neuromuscular medicine and to acknowledge those ABPN-certified neurologists and child neurologists and ABPMR-certified physiatrists who have developed special expertise in this area.

Neuromuscular medicine includes disorders of the anterior horn cell, peripheral nerve, neuromuscular junction, and muscle. Some of the more important or common conditions embraced by neuromuscular medicine are amyotrophic lateral sclerosis, peripheral neuropathies (e.g., diabetic, inherited, and immune-mediated neuropathies), muscular dystrophies, inflammatory myopathies (e.g., polymyositis), and myasthenia gravis. The new subspecialty reflects the increasing scientific knowledge about the pathophysiology, genetics, diagnosis, and treatment of neuromuscular diseases at a level that is significantly beyond the training and knowledge expected of a general neurologist, child neurologist, or specialist in physical medicine and rehabilitation.

Indicators of scientific progress in this area include the fact that there are now five peer-reviewed journals that focus on neuromuscular medicine, and numerous books related to neuromuscular medicine have been published in the past decade. The AAN’s neuromuscular section has increased from an original membership of 145 in 1998 to almost 1,000 members, making it one of the largest in the Academy. In addition to the AAN, other organizations that supported the proposed subspecialty were the American Association of Neuromuscular and Electrodiagnostic Medicine, the American Board of Electrodiagnostic Medicine, the Amyotrophic Lateral Sclerosis Association, the Child Neurology Society, the Guillain–Barre Syndrome Foundation International, the Muscular Dystrophy Association, the Professors of Child Neurology, and the World Muscle Society.

The purpose of this report is to describe the certification process so that practitioners and residents in training will be aware of the requirements and procedures.

CERTIFICATION PROCESS

It is anticipated that the first subspecialty examination will be administered in 2008, with applications due in early 2008. Each board will credential its diplomates; neurologists and child neurologists will apply to the ABPN, and physiatrists will apply to the
ABPMR. Applicants must submit a completed official application form including all required attachments and the appropriate application and examination fees by the specified deadlines.

For the first 5 years (2008 through 2012), the ABPN will accept applications from ABPN-certified neurologists or child neurologists who possess an unrestricted medical license in a state, commonwealth, territory, or possession of the United States or Canada and who meet one of three criteria: (1) successful completion of training in an Accreditation Council for Graduate Medical Education (ACGME)—accredited neuromuscular medicine fellowship; (2) successful completion of training in a non-ACGME-accredited neuromuscular medicine fellowship; or (3) a minimum of 25% of practice time for a minimum of 2 years devoted to the diagnosis and treatment of persons with neuromuscular diseases. Fellowship training must begin after general residency training in neurology or child neurology, including time spent in combined training programs, is completed. Training or exposure to neuromuscular medicine given to neurology or child neurology residents as part of their basic neurology or child neurology curriculum does not count toward the 1 year of training.

During this period, sometimes referred to as the grandfathering period, fellowship training in clinical neurophysiology can be used in lieu of fellowship training in neuromuscular medicine provided that the clinical neurophysiology training has not been used in the credentialing process for ABPN certification in clinical neurophysiology. If clinical neurophysiology training is used to credential for certification in neuromuscular medicine, it cannot subsequently be used to credential for certification in clinical neurophysiology.

After the 2012 examination, only those neurologists, child neurologists, and physiatrists who have successfully completed an ACGME-accredited fellowship program in neuromuscular medicine will be eligible for certification in this subspecialty. Successful completion of fellowship training in neuromuscular medicine does not meet the training requirement for certification in clinical neurophysiology for which successful completion of ACGME-accredited fellowship training in clinical neurophysiology is required. Likewise, successful completion of fellowship training in clinical neurophysiology will not meet the requirement for certification in neuromuscular medicine.

Like all ABPN certificates, subspecialty certificates in neuromuscular medicine will be valid for 10 years. To retain certification status, diplomates will have to participate in the maintenance of certification process, which has four components: evidence of professional standing, evidence of participation in self-assessment and lifelong learning, evidence of assessment of performance-in-practice, and evidence of cognitive expertise (examination). They will also have to maintain specialty certification.

Following ABMS approval, the ABPN and the ABPMR appointed a test committee, chaired by Janice M. Massey, MD, to develop the content outline for the examination, generate questions, assemble the examination, and set pass/fail standards. In addition to representatives from both specialties, the members of the committee were selected to include a range of expertise in neuromuscular medicine. The examination will consist of approximately 200 multiple-choice questions, and the test will be administered at a national network of computer test centers.

Information about the application requirements and the examination, including the content outline, will be posted on the ABPN Web site (www.abpn.com) and will be available from the ABPN office.

TRAINING REQUIREMENTS

In February 2005, the ACGME approved the program requirements for neuromuscular medicine, and seven accredited programs are listed for the 2006 through 2007 academic year. Other programs have been accredited with future effective dates, and it is expected that the number of accredited programs will increase as awareness grows about this new subspecialty.

Training in neuromuscular medicine requires a minimum of 1 year and occurs after residency training in neurology or child neurology is completed. The requirements are published on the ACGME’s Web site (www.acgme.org). Training in clinical neurophysiology also requires a minimum of 1 year, and at this time there are no RRC-approved programs that provide combined training in these two subspecialties.

SUMMARY

The goal of certification in neuromuscular medicine is to serve the public interest by promoting excellence in teaching, research, and patient care for these costly neurologic diseases. This report provides information for those who want to pursue fellowship training in neuromuscular medicine as well as for neurologists, child neurologists, and physiatrists whose practices are focused on neuromuscular medicine and who may want to pursue board certification.

REFERENCE

INVITED REVIEW

ABSTRACT: Underlying the pathogenesis of chronic disease is the state of oxidative stress. Oxidative stress is an imbalance in oxidant and antioxidant levels. If an overproduction of oxidants overwhelms the antioxidant defenses, oxidative damage of cells, tissues, and organs ensues. In some cases, oxidative stress is assigned a causal role in disease pathogenesis, whereas in others the link is less certain. Along with underlying oxidative stress, chronic disease is often accompanied by muscle wasting. It has been hypothesized that catabolic programs leading to muscle wasting are mediated by oxidative stress. In cases where disease is localized to the muscle, this concept is easy to appreciate. Transmission of oxidative stress from diseased remote organs to skeletal muscle is thought to be mediated by humoral factors such as inflammatory cytokines. This review examines the relationship between oxidative stress, chronic disease, and muscle wasting, and the mechanisms by which oxidative stress acts as a catabolic signal.


OXIDATIVE STRESS, CHRONIC DISEASE, AND MUSCLE WASTING

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Oxidative stress is involved in the pathogenesis of a number of chronic diseases. In a great proportion of these, muscle wasting contributes to morbidity and mortality. This review examines the role of oxidative stress in the pathogenesis of disease, the systemic or muscle-specific mediators of oxidative stress, and the effects of oxidative stress on muscle tissue.

Oxidative stress is a state wherein the normally well-balanced control of oxidant production and antioxidant activity is disturbed. The sources of oxidants are numerous. Most are derived from enzymatic or chemical reactions that produce superoxide anion, hydrogen peroxide (H$_2$O$_2$), or nitric oxide (NO). Once produced, these species undergo conversion to secondary highly reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as hydroxyl radical (OH·) and peroxynitrite (ONOO$^-\$). At basal levels, ROS and RNS serve as important regulators of signal transduction and protein function. However, if left unchecked, elevated levels of ROS or RNS can damage critical cellular components such as membrane lipids, structural and regulatory proteins, and DNA. Antioxidants that neutralize excess oxidant production include enzymes that convert oxidants into less damaging or harmless species, and small molecules that serve as oxidant sinks or scavengers.

There is evidence of oxidative stress in the skeletal muscles of patients with chronic disease.oxidative stress directs muscle cells into a catabolic state and that chronic exposure leads to wasting. Oxidative damage may contribute to skeletal muscle dysfunction and mark myofibrillar proteins for degradation. Concurrently, oxidants may stimulate expression and activity of skeletal muscle protein degradation pathways. These compounding factors of oxidative stress may ultimately lead to muscle wasting in chronic disease.

REDOX HOMEOSTASIS

Oxidant Sources. Figure 1 depicts both intra- and extracellular oxidant sources in muscle. Nitric oxide synthase (NOS) catalyzes oxidation of L-arginine to ROS, Disease, and Wasting
L-citrulline to release NO. There are at least three NOS isoforms: (1) neuronal NOS (nNOS, NOS1); (2) inducible NOS (iNOS, NOS2); and (3) endothelial NOS (eNOS, NOS3). A fourth mitochondrial-specific isoform may also exist (mtNOS). NOS enzymes function in the peripheral and central nervous systems, cardiovascular and immune systems, and skeletal muscle. The predominant isoform in skeletal muscle is an alternatively spliced form of nNOS, nNOSμ. It is localized to the subsarcolemmal region and associated with the dystrophin complex. Skeletal muscle–derived NO affects excitation–contraction coupling, mitochondrial energy production, glucose metabolism, and regulation of blood flow. In addition to regulatory functions, NO has the potential to negatively impact skeletal muscle. For example, iNOS-derived NO from activated immune cells has cytostatic or cytotoxic properties that are normally targeted to pathogens and tumor cells. However, under chronic exposure NO can also damage healthy tissue, including skeletal muscle.

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and myeloperoxidase are plasma membrane and lysosomal enzymes, respectively. In neutrophils, monocytes, and tissue macrophages, these enzymes function in host defense (reviewed by Decoursey and Ligeti). NADPH oxidase is a 5-subunit protein complex that produces O2•− by catalyzing the transfer of one electron from reduced nicotinamide adenine dinucleotide (NADH) or NADPH to molecular oxygen. There are at least seven NADPH oxidase isoforms and they are expressed in a variety of cell types including epithelium, smooth and skeletal muscles, and endothelium. The non-immune isoforms produce low levels of ROS and may provide second messengers for signal transduction. Skeletal muscle NADPH oxidase associates with several cellular compartments. Immunostaining localizes it near the sarcolemma, but NADPH oxidase activity is also associated with the sarcoplasmic reticulum. Myeloperoxidase, found most abundantly in neutrophils, produces hypochlorous acid (HOCl) from H2O2 and chloride anion (Cl−). This toxic oxidant functions as an antimicrobial agent but also has potential tissue-damaging effects when released from the cell. Xanthine oxidase and xanthine dehydrogenase are interconvertible forms of the same gene product, known as xanthine oxidoreductase. A fourth mitochondrial-specific isoform may also exist (mtNOS). NOS enzymes function in the peripheral and central nervous systems, cardiovascular and immune systems, and skeletal muscle. The predominant isoform in skeletal muscle is an alternatively spliced form of nNOS, nNOSμ. It is localized to the subsarcolemmal region and associated with the dystrophin complex. Skeletal muscle–derived NO affects excitation–contraction coupling, mitochondrial energy production, glucose metabolism, and regulation of blood flow. In addition to regulatory functions, NO has the potential to negatively impact skeletal muscle. For example, iNOS-derived NO from activated immune cells has cytostatic or cytotoxic properties that are normally targeted to pathogens and tumor cells. However, under chronic exposure NO can also damage healthy tissue, including skeletal muscle.

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thine oxidoreductase is a cytosolic enzyme found in liver, intestine, kidney, lungs, heart, brain, plasma, erythrocytes, and skeletal muscle. Liver and intestine xanthine dehydrogenase act to detoxify metabolic byproducts. With acute infections, heat stress, respiratory stress, hypercholesterolemia, and cancer, xanthine dehydrogenase is converted to xanthine oxidase and released into the blood stream. Here it has the potential to cause oxidative damage to tissues such as skeletal muscle.

An important source of superoxide is mitochondrial electron transport. Superoxide is generated at both complex I and III of the electron transport chain, but may also be produced by complex II, especially when damaged by oxidative stress or aging. Superoxide generated by mitochondria is much lower due to conversion to \( \text{H}_2\text{O}_2 \) by manganese superoxide dismutase (Mn-SOD) in the mitochondrial matrix and by copper–zinc SOD (CuZnSOD) in the intermembrane space. The resulting \( \text{H}_2\text{O}_2 \) freely diffuses to the cytoplasm and constitutes 20%–30% of the steady-state level. In tissues with a higher aerobic rate, such as skeletal muscle and heart, the contribution can be as much as 96%. This level is estimated to be 10–100 nM in hepatocytes or as much as 1 \( \mu \)M in breast cancer and melanoma cells. Hydrogen peroxide conversion to damaging radicals such as OH\(^-\) is catalyzed by non-enzymatic transition-metal reactions, most notably by Fe\(^{2+}\) (Fenton reaction). Consequently, oxidants produced in the mitochondria can be targeted to specific cellular locations by the availability and distribution of transition metals that together with \( \text{H}_2\text{O}_2 \) give rise to OH\(^-\). This may be a mechanism for precise targeting of ROS to activate signaling pathways or specifically affect myofibrillar components of skeletal muscle cells. Mitochondrial membranes also contain monoamine oxidase. Monoamine oxidase catalyzes oxidative deamination, releasing reactive aldehydes and \( \text{H}_2\text{O}_2 \). Monoamine oxidase expression is upregulated in the presence of glucocorticoids and has been implicated in the pathogenesis of glucocorticoid-induced muscle wasting.

Finally, phospholipase A\(_2\) (PLA\(_2\)) is a family of enzymes that deacetylate phospholipids releasing free fatty acids such as arachidonic acid. Arachidonic acid is converted to inflammatory leukotrienes and prostaglandins by 5-lipoxygenase and cyclooxygenase, respectively. Two PLA\(_2\) isoforms contribute to skeletal muscle ROS. Ca\(^{2+}\)-insensitive PLA\(_2\) is essential for basal production of extracellular ROS by 5-lipoxygenase and is important for force production in unfatigued muscle. Calmodulin-sensitive PLA\(_2\) activity disrupts mitochondrial electron transport, thereby increasing ROS in response to repetitive contraction.

**Antioxidants.** Cellular antioxidants consist of oxidant scavengers and antioxidant enzymes that convert free radicals to more benign molecules. Scavengers include vitamins C and E and carotenoids. These molecules are able to donate an electron and neutralize free radicals but are destroyed upon oxidation. Alternatively, thiol-containing compounds such as glutathione and thioredoxin are oxidized by free radicals and rapidly regenerated. Oxidation reactions are catalyzed by glutathione peroxidase, glutathione transferase, thioredoxin peroxidase, and peroxiredoxin. The end result is the formation of glutathione disulfide and oxidized thioredoxin, which are rapidly converted to their reduced forms by glutathione reductase or thioredoxin reductase using NADPH as a cofactor. Glutathione disulfide is normally less than 1% of total glutathione but is elevated under severe oxidative stress. As an adaptive response, glutathione content increases upon exposure to heavy metals, high glucose, or heat shock. Glutathione can also form disulfides with cellular proteins and this glutathiolation is postulated to have a regulatory function. In addition to control of oxidant balance, thioredoxin also provides a regulatory function by forming disulfides with cellular proteins.

NO has both oxidant and antioxidant properties. It is thought that NO increases the cytotoxicity of \( \text{O}_2^- \) by generation of \( \text{ONOO}^- \); however, reports have shown that NO-releasing compounds protect against the toxic effects of \( \text{O}_2^- \) in fibroblasts and neuron primary cultures. In addition, NO attenuates lipid peroxidation and the metal-catalyzed conversion of \( \text{H}_2\text{O}_2 \) to OH\(^-\) promotes the expression of antioxidant enzymes, and via \( \text{S}^\equiv\text{S} \)-nitrosylation, enhances the antioxidant activity of glutathione and thioredoxin. NO also limits leukocyte adhesion, thereby reducing potential damage from activated leukocytes.

In addition to the glutathione and thioredoxin oxidation/reduction enzymes just mentioned, antioxidant enzymes include SOD, which catalyzes the dismutation of \( \text{O}_2^- \) to \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \). There are three SOD isoforms in humans. CuZnSOD (SOD1) and extracellular SOD (ECSOD, SOD3) use Cu\(^{2+}\) and Zn\(^{2+}\) as cofactors and are found in the cytoplasm and extracellular space, respectively. CuZnSOD is also present in the nucleus, peroxisomes, and...
mitochondrial intermembrane space. ECSOD is secreted from smooth muscle and airway vasculature and has potent anti-inflammatory and ROS-scavenging activity.\(^{71,216,243}\) MnSOD (SOD2) is the mitochondrial isofrom that utilizes Mn\(^{2+}\) as a cofactor.\(^ {270}\) Cellular MnSOD content generally parallels aerobic activity and is induced by chronic hypoxia, cytotoxic drugs, and inflammatory cytokines.\(^ {124}\) Under severe stress, such as lung hyperoxia and renal graft rejection, or upon exposure to ONOO\(^{-}\), nitrotyrosine modification of MnSOD causes a loss of both MnSOD protein and activity.\(^ {45,162}\) A second antioxidant enzyme, catalase, decomposes H\(_2\)O\(_2\) to H\(_2\)O and O\(_2\).\(^ {176}\) Catalase is localized in peroxisomes and functions to remove H\(_2\)O\(_2\) during fatty acid oxidation. It is believed to function in oxidant defense by limiting accumulation of cytosolic H\(_2\)O\(_2\), which diffuses into peroxisomes and is degraded.

**CHRONIC DISEASE**

In addition to muscle-specific diseases such as muscular dystrophy, muscle wasting and cachexia are major complicating factors of certain cancers, chronic heart failure, chronic obstructive pulmonary disease (COPD), rheumatoid arthritis, liver disease, kidney disease, sepsis, and aging (Table 1). Individuals with these conditions exhibit varying degrees of muscle wasting, which often present as part of a cachectic syndrome that includes anorexia, loss of body weight, and decreased adipose tissue.\(^ {28,103}\)

**Muscle-Specific Disease.** Myotonic dystrophy is the most common adult form of muscular dystrophy. The disease causes muscle weakness but also affects the central nervous system, heart, gastrointestinal tract, eyes, and endocrine system. The pathogenesis of myotonic dystrophy is still unclear but it has been described as a premature aging disease due to increased oxidative stress.\(^ {81,113}\) The genetic basis of the disease has been identified as myotonin protein kinase. Cells lacking this kinase show increased susceptibility to oxidative stress.\(^ {258}\) Duchenne muscular dystrophy is a severe genetic disease that affects young boys, with onset between 2 and 6 years of age. There is an increase in oxidative stress in dystrophic muscle as indicated by increased DNA damage, protein carbonyls, and lipid peroxidation.\(^ {111,191,227}\) A gene encoding for the muscle protein dystrophin is the causative factor. The function of dystrophin is still debated, but most agree it stabilizes the sarcolemma during contractions.\(^ {127}\) An alternative hypothesis is that dystrophin prevents excessive generation of free radicals.\(^ {26}\) Dystrophin is thought to anchor nNOS to the sarcolemma. In Duchenne dystrophy, nNOS is either dramatically reduced or absent. Because NO regulates antioxidant levels, dysregulation of nNOS may contribute to the oxidative stress and pathological changes associated with the disease.\(^ {270}\)

Malignant hyperthermia and central core disease are related conditions caused, in most cases, by a mutation of the ryanodine receptor. This mutation causes unregulated Ca\(^{2+}\) release from the sarcoplasmic reticulum that results in muscular rigidity, increased oxygen consumption, and increased temperature, eventually leading to rhabdomyolysis.\(^ {263}\) Central core disease is an autosomal-dominant dis-

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**Table 1.** Redox, inflammatory state, and muscle wasting in chronic disease.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Oxidants</th>
<th>Antioxidants</th>
<th>Inflammation</th>
<th>Prevalence of wasting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myotonic dystrophy</td>
<td>↑ Muscle(^ {256})↑ Muscle(^ {255})↑ Muscle(^ {227})</td>
<td>↑ Muscle(^ {255})↑ Muscle(^ {227})Unknown</td>
<td>↑ H(_2)O(_2)↑ Host(^ {12,155})↑ Tumor(^ {176})</td>
<td>100% (^ {179})100% (^ {227})100% (^ {263})</td>
</tr>
<tr>
<td>Duchenne dystrophy</td>
<td>↑ Muscle(^ {255})</td>
<td>↑ Muscle(^ {255})↑ Muscle(^ {227})Unknown</td>
<td>↑ H(_2)O(_2)↑ Host(^ {12,155})↑ Tumor(^ {176})</td>
<td>100% (^ {179})100% (^ {227})100% (^ {263})</td>
</tr>
<tr>
<td>Malignant hyperthermia/central core disease</td>
<td>↑ Muscle(^ {255})</td>
<td>↑ Muscle(^ {255})↑ Muscle(^ {227})Unknown</td>
<td>↑ H(_2)O(_2)↑ Host(^ {12,155})↑ Tumor(^ {176})</td>
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<td></td>
<td>100% (^ {226})100% (^ {128})100% (^ {293})100% (^ {129})</td>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>100% (^ {179})100% (^ {227})100% (^ {263})</td>
</tr>
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</table>

*NFT, neurofibrillary tangles; *\(\alpha\), \(\beta\)-amyloid.

Up and down arrows indicate increases or decreases in oxidants, antioxidants, and inflammation in chronic diseases. These are systemic changes unless local sites are indicated. The right-most column indicates the percentage of individuals who experience muscle wasting with each disease. Where percentages are not listed, muscle wasting is present in the subpopulation indicated.

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*Source: [Link](https://www.ncbi.nlm.nih.gov) (accessed July 1, 2023).*
Remote Organ Disease. Virtually all patients with COPD, severe sepsis, or chronic kidney disease exhibit some degree of muscle wasting that increases with the severity of disease.69,100,282 Six hundred million people are afflicted with COPD, which is predicted to be the third largest cause of death and fifth most common cause of disability in the world by 2020.159 The disease is defined as a progressive, irreversible airflow limitation and an abnormal inflammatory response of the lung.204 The pathogenesis of COPD is closely linked to oxidative stress. The lungs are exposed to both environmental and cellular oxidants. Environmental oxidants are derived from air pollution, cigarette smoke, and ozone,125 whereas cellular-derived oxidants are produced by inflammatory and epithelial cells within the lung in response to irritants.226 The most important factor driving the pathogenesis of COPD is cigarette smoke, which contains high concentrations of oxidants (1014 molecules/puff).44,125 COPD patients also exhibit significant systemic consequences of inflammation and oxidant imbalance with increased plasma lipid peroxidation, oxidized coenzyme Q10, activated peripheral neutrophils, and decreased plasma antioxidants.218,264

Patients with chronic kidney disease also have increased markers of oxidative stress and uremia. The wasting that accompanies kidney disease parallels the gradual and progressive loss of the ability to excrete waste, concentrate urine, and conserve electrolytes.59,108 Both oxidative stress and wasting are exacerbated by dialysis, perhaps due to dialysis-induced inflammation.206

The prevalence of muscle wasting is also significant in cardiac patients, where approximately 10%–16% of patients are affected.234 Inflammation and oxidative stress contribute to the pathogenesis of chronic heart failure.15,195 Inflammation induces production of tumor necrosis factor (TNF) in both immune cells and myocardium. TNF subsequently suppresses myocardial contractions and induces production of excess oxidants, thus causing further damage to the myocardium.52

Inflammation is a major contributor to the pathogenesis of rheumatoid arthritis, a condition that attacks synovial tissue surrounding the joints, leading to cartilage and bone erosion.199 Oxidants produced by T-lymphocytes cause direct tissue damage and amplify the inflammatory response by inducing production of TNF and interleukin-1 (IL-1).23 Reduced serum antioxidants predispose patients to rheumatoid arthritis.91,105 Two thirds of patients with rheumatoid arthritis exhibit muscle wasting that compromises muscle strength and functional capacity.265

Finally, redox imbalance and inflammation underlie the pathogenesis of ulcerative colitis or inflammatory bowel disease. The radical induction theory of ulcerative colitis146,215 states that H2O2 produced within colonic epithelial cells is converted to OH·, which causes extensive damage to colonic epithelial cells. This damage allows fecal bacteria to invade the submucosal tissue and provoke an immune response. The cause of excess H2O2 is thought to be stress related.87 The first animal model of inflammatory bowel disease was generated by rectal injection of rats with 3% H2O2.235 A more recent model was generated from glutathione peroxidase knockout mice that spontaneously develop a destructive colitis similar to human inflammatory bowel disease.66 The combination of both disturbances in redox balance and the increased inflammatory state suggests that patients with this condition may also be susceptible to muscle wasting. In support of this idea, a recent study by Burnham et al. demonstrated that children and young adults with Crohn’s disease show body composition changes consistent with cachexia, with deficits in both lean and fat mass.35,34

Infectious Disease. Dysregulated inflammation and the accompanying oxidative stress are involved in several infectious conditions including sepsis and infection with human immunodeficiency virus (HIV). In sepsis, the blood-borne infection evokes a massive immune response and excessive oxidant and cytokine production by inflammatory cells. Subsequent oxidant and cytokine exposure of muscle and other affected tissue leads to further oxidant production in these tissues.13,18,245 All patients with severe protracted sepsis exhibit muscle wasting.102 Compounding the sepsis-related wasting, patients often require mechanical ventilation and bed rest. Even in
the absence of sepsis, these conditions, characterized by decreased muscle use, contribute to redox imbalance, muscle dysfunction, and wasting.64,259

Before the use of highly active antiretroviral therapy (HAART), HIV-associated weight loss and muscle atrophy was a major contributor to mortality in the western world and it is still a contributing factor to mortality in regions where HAART is unavailable.89 In effectively treated patients with undetectable plasma HIV RNA, weight is gained. Unfortunately, the weight gain is primarily adipose tissue, and muscle mass is not restored.164,175 HIV-1–infected individuals exhibit a disturbed redox balance and a depletion of antioxidants such as glutathione.7,107 This oxidant imbalance as well as the loss of muscle mass has been attributed to chronic low-grade inflammation.157 However, an alternative hypothesis has developed since the discovery that HIV-1 encodes a homolog of the human antioxidant enzyme glutathione peroxidase.246 Human glutathione peroxidase detoxifies peroxide radicals while oxidizing glutathione. HIV–glutathione peroxidase bears structural similarities with the mammalian homolog and both require a selenium cofactor.288 It is thought that the HIV-1 glutathione peroxidase competes with the host for cofactors and, consequently, compromises host enzyme function while protecting HIV-infected cells against an immune response.76,158

The depletion of glutathione and selenium cofactor could have a systemic effect that also contributes to redox imbalance in skeletal muscle, thus promoting a catabolic program, as discussed later.

Cancer. Many cancer patients (50%–80%) become cachectic and, in 20%, cachexia is the main cause of death.252 Cachexia is a syndrome that includes wasting of body energy reserves. The major affected tissues are adipose and skeletal muscle. The loss of skeletal muscle mass is particularly detrimental and contributes to fatigue, loss of strength, mobility, and quality of life. Cancer patients also have a disturbed oxidant balance that appears to be important for carcinogenesis and tumor progression. Antioxidant activity is increased in a broad range of cancer cells including cervical cancer, non–small-cell lung cancer, pancreatic cancer, and hepatoma. Elevated antioxidants correlate with tumor aggression, and may contribute to tumor resistance to host defense mechanisms.176 Although the cancer cells seem to be effectively protected from oxidative stress, the host is more susceptible and, in animal models, signs of oxidative stress are seen in plasma and other tissues including skeletal muscle.12

Metabolic Disease. Other chronic diseases exhibit oxidant imbalances that are critical for pathogenesis but have a more tenuous connection with muscle wasting. In diabetes, wasting may go unnoticed due to reduced severity or may be attributed to complicating factors such as age. In the United States alone, 20.8 million people are affected with diabetes. Of these, 5%–10% have type 1 diabetes. Models of type 1 diabetes show that insulin-deficient animals have accelerated muscle atrophy and increased protein degradation.182 The remainder and majority of diagnosed individuals have type 2 diabetes. In this case, insulin resistance or inadequate insulin production leads to impaired glucose uptake. Both insulin resistance and type 2 diabetes are associated with muscle wasting in the elderly.92,275 Oxidative stress may promote development of type 2 diabetes.199,237 The most important tissues involved in the pathogenesis of diabetes are muscle and adipose tissue. When caloric intake exceeds energy expenditure, a substrate-induced increase in state 3 respiration or an ADP and oxygen limitation–induced shift to state 4 respiration generates an excess of mitochondrial ROS.22,79 To protect against the harmful effects of ROS, cells may eliminate excess substrate by inhibiting insulin-stimulated glucose uptake.57,109,198 The ability to maintain redox balance may dictate whether muscle wasting manifests in a diabetic state. Consequently, insulin resistance and diabetes have been associated with muscle wasting in the elderly, a population of individuals with reduced oxidant defenses.92,275

Chronic alcohol consumption is also associated with muscle wasting that is evident prior to the onset of liver disease. In a study of 250 alcoholics, 46% exhibited muscle pathology and only 8% had cirrhosis.65 Free radicals generated during ethanol metabolism can damage many tissues including liver and skeletal muscle. Patients with liver disease due to chronic alcohol abuse have a marked inflammation and oxidant imbalance with elevated uric acid and malondialdehyde, and decreased levels of the antioxidant enzymes CuZnSOD (−86%) and glutathione peroxidase (−37%).1,65,91,174,194,205

Aging and Alzheimer’s Disease. Aging can be associated with a loss of muscle.110 A recent study demonstrated that cross-sectional area and specific force are reduced by 16% and 30%, respectively, in the gastrocnemius of elderly men, average age 74 years, compared to young men with an average age of 25 years.187 Low-grade inflammation is associated with this loss, and individuals with elevated interleukin-6 (IL-6) are more likely to have reduced mass and
strength. Age-related increases in oxidative damage are found in organisms ranging from invertebrates to humans. In 1956, Harman proposed that ROS formed during normal oxygen metabolism induce macromolecular damage. He proposed that the accumulation of products of oxidative damage accounts for the progressive deleterious changes of aging, a concept reviewed by Terman and Brunk. This hypothesis was named the free radical theory of aging and has been supported by studies showing that age-related changes accelerate with elevated oxidative stress. For example, mice lacking CuZnSOD display increased oxidative stress and a dramatic acceleration of age-related loss of skeletal muscle mass. These mice have significantly lower muscle mass than wild-type mice as early as 3–4 months of age, and hindlimb muscle mass is nearly 50% lower by 20 months. Alternatively, there is evidence that increasing antioxidant levels can reduce oxidative stress and increase lifespan in nematodes, Drosophila, and mice. SOD mimetics, or Mn- and CuZnSOD overexpression, were found to increase the lifespan of nematodes and Drosophila, respectively. Although increasing SOD in mice has no effect, overexpression of thioredoxin significantly increases lifespan. Mitochondria have been implicated as the primary oxidant source during aging. Correspondingly, overexpression of catalase fused to the leader sequence of ornithine transcarbamylase targets catalase to the mitochondria, reduces markers of oxidative stress, and increases the lifespan of transgenic animals by 17%–21%.

Age-related muscle loss is accelerated with pathological conditions such as Alzheimer’s disease, which affects 5% of Americans over age 65 and 20% over age 80. One of the earliest events in disease pathogenesis is a systemic oxidative stress, indicated by an increase in isoprostanes, lipid peroxides, and oxidized glutathione in cerebrospinal fluid, plasma, and urine. These stress indicators often arise prior to the onset of symptoms. Early intervention with antioxidants reduces the risk of developing the disease. The National Institute of Neurological and Communicative Disorders and Strokes Task Force on Alzheimer’s disease includes weight loss as a “clinical feature consistent with the diagnosis of Alzheimer’s disease.” Loss of body weight is typically associated with reduced muscle mass that compromises mobility. Although this weight loss is not a major focus of disease intervention strategies, it is a problem that contributes to reduced quality of life and may be an additional target of oxidant imbalance.

### Systemic Mediators of Oxidative Stress

As already discussed, chronic diseases of remote organ systems may exert pathological effects on skeletal muscle. It has been proposed that these effects are mediated by the systemic transmission of oxidative stress from remote organs via radical-inducing substances such as cytokines and metabolic byproducts.

#### Humoral Factors

There are multiple humoral factors that induce oxidative stress. These include inflammatory cytokines such as IL-1, IL-6, TNF, and interferon-γ (IFN-γ). Cytokines have been implicated in cancer cachexia and they promote oxidative stress and wasting in muscle via several mechanisms (discussed later). Cytokines induce sickness behaviors, such as listlessness, depression, and anxiety, which promote malnutrition and subsequent oxidant imbalance. They also activate peripheral leukocytes that invade tissues and produce excess oxidants. Correspondingly, neutrophils of COPD patients have enhanced ROS production and HIV-1 transgenic mice that develop muscle wasting have increased leukocyte infiltration of muscle. Oxidants produced by infiltrating immune cells may cause direct injury to muscle tissue or activate catabolic signaling. Alternatively, inflammatory cytokines can interact with muscle receptors to initiate catabolic signaling. In the latter case, there is evidence that ROS act as second messengers.

Other humoral factors that may mediate redox imbalances in muscle include glucocorticoids. Glucocorticoids are thought to be important mediators of starvation-induced atrophy and are elevated with atrophy due to reduced activity and cachexia. Glucocorticoid treatment of cultured human muscle cells results in oxidative stress and mitochondrial dysfunction, while muscles from patients undergoing cortisol treatments have increased oxidative stress, mitochondrial dysfunction, and muscle protein loss. Glucocorticoid levels are also elevated during sepsis and treatment of septic rats with the glucocorticoid receptor antagonist RU 38486 reduces muscle atrophy.

#### Metabolic Byproducts

Byproducts of abnormal metabolic states such as obesity or renal failure may promote oxidative stress in skeletal muscle. For example, overeating leads to glucose and fatty acid overload that...
results in excess muscle-derived ROS. Glucose overload in muscle induces excess ROS from glycolysis and mitochondrial oxidative phosphorylation pathways. In addition, skeletal muscle glucose overload could activate NADPH oxidase. This mechanism exists in smooth muscle where high concentrations of glucose lead to an increase in diacylglycerol, which subsequently induces protein kinase C (PKC) to activate NADPH oxidase. Elevated glucose also contributes to oxidant imbalance in plasma through non-enzymatic interactions with plasma constituents. These oxidation reactions result in the formation of isoprostanes and reactive aldehydes. In addition to increased plasma oxidants, increased plasma acidity is associated with chronic renal failure, diabetic ketosis, sepsis, and COPD. It has been postulated that acidosis mediates muscle wasting. Chronic acidosis is linked to loss of muscle mass. In addition, acute acidosis (blood pH of less than 6.9) in rats and humans fed ammonium chloride is associated with loss of muscle mass. In vitro studies have shown that direct acidification of cultured muscle cells induces protein degradation. However, in vivo measurements in rat models of chronic acidosis have shown that pH is unaltered in skeletal muscle. Therefore, the effects of systemic acidosis on skeletal muscle may be due not to a direct acidification of the muscle, but rather to indirect effects on systemic inflammation or oxidative stress.

In addition, isoprostanes are ROS-catalyzed isomers of arachidonic acid, which circulate in plasma and are excreted in the urine. Isoprostanes are used as markers of oxidative stress and are proinflammatory, affecting both monocyte and neutrophil cytokine release, and may play an important role in various chronic inflammatory diseases. Isoprostanes could activate catabolic pathways in skeletal muscle either directly or through amplification of inflammatory responses.

**External Factors.** There are a number of other complicating factors that contribute to oxidative stress and may amplify a catabolic response. For example, the treatment of cancer with chemotherapy or radiation produces an increase in oxidative stress both directly and through nausea-induced poor nutrition. In conditions such as chronic heart failure, reduced blood flow leads to oxidative stress. In addition, inactivity as a consequence of illness, leads to the adaptive response of muscle atrophy. Atrophy due to inactivity or immobilization is strongly linked to oxidative stress. Both oxidative damage and elevated ROS have been detected in immobilized muscles of animal models.

**EFFECTS OF OXIDATIVE STRESS ON MUSCLE**

**Increased Protein Degradation.** Under normal conditions there is a balanced and continuous degradation and resynthesis of skeletal muscle proteins. With oxidative stress, this balance is disrupted. Most studies have focused on increases in protein degradation. It is widely accepted that the ubiquitin–proteasome pathway is the main route by which proteins are degraded during muscle atrophy. This involves the targeted degradation of proteins via modification by ubiquitin and subsequent proteolysis by the 26S proteasome (reviewed by Robinson and Ardley). In addition, the 20S core proteasome can selectively degrade oxidatively modified proteins in a ubiquitin-independent manner. Proteins targeted by ubiquitin are modified through the actions of three types of ubiquitin-conjugating enzymes—E1, E2, and E3. E1 is a ubiquitin-activating enzyme that maintains ubiquitin in a reactive state. To date, only one E1 isoform has been found. E2 is a ubiquitin-conjugating enzyme that catalyzes attachment of ubiquitin to target proteins. There are dozens of isoforms, including skeletal muscle–specific isoforms such as E2 and UbH2. The specificity of the system is dictated mostly by E3- ligases. There are over 100 E3 isoforms that function in concert with E2 enzymes to add multiple ubiquitins to target proteins. Three E3 proteins appear to mediate in skeletal muscle catabolism—atrogin1/MAFbx, MuRF1, and E3α. Atrogin1 and MuRF1 are upregulated in a number of catabolic conditions including cancer, diabetes, kidney failure, and sepsis.

To dissect the role of oxidative stress and inflammatory mediators on the ubiquitin–proteasome pathway, studies have been performed both in vivo and in isolated cell culture systems, such as mouse-derived C2C12 myotubes. Direct application of H2O2 to C2C12 myotubes increases expression of E2 and atrogin1, MuRF1. These increases correlate with increased ubiquitin-conjugating activity, increased proteasome activity, and decreased myosin protein. This response is mirrored by TNF in mouse diaphragm, where the TNF is injected into the intraperitoneal space, or with direct application to C2C12 myotubes. Consequently, it has been hypothesized that ROS may act as a second messenger in TNF-induced muscle catabolism.

Supporting studies have shown that TNF exposure produces a burst of oxidant activity in C2C12 myotubes, and oxidant levels are elevated in the diaphragm of transgenic mice with cardiac-specific TNF overexpression. The source of TNF-stimulated oxidants has not been confirmed, but inhibitors of mi-
tochondrial electron transport can diminish this response. Alternatively, TNF stimulates phospholipase A₂ activity in skeletal muscle and may also stimulate NAPDH oxidase as it does in other cell types. Finally, TNF stimulates iNOS expression in C2C12 myocytes, but this requires costimulation with INF-γ.

Many catabolic regulatory elements are activated by both TNF and ROS. Figure 2 depicts potential pathways activated by ROS in skeletal muscle. These include transcription factor, nuclear factor-kappaB (NFκB), and mitogen-activated kinase, p38 MAPK. NFκB is a ubiquitous factor activated by ultraviolet light, radiation, heat, inflammatory cytokines, and oxidative stress. NFκB is retained in the cytoplasm by the inhibitor protein, IκB. Upon stimulation, it is released from IκB, translocates to the nucleus, and drives transcription of stress response genes including UbcH2. The evidence that ROS is involved in NFκB activation in skeletal muscle includes the observations that treatment of C2C12 myotubes with H₂O₂ stimulates NFκB activity and pretreatment with catalase inhibits TNF-induced NFκB activation, and dietary N-acetylcysteine reduces NFκB activity in soleus muscles of mice. The mechanism by which ROS activates NFκB is unknown. Studies have shown that NFκB activation by hypoxia or H₂O₂ is concurrent with tyrosine phosphorylation of IκB, which may trigger NFκB release. More recent evidence has demonstrated that the upstream IκB kinase (IKK) is activated by H₂O₂ to phosphorylate IκB on serines 32 and 36, thus targeting it for ubiquitin conjugation and degradation, allowing for release of NFκB. In addition, direct oxidation of NFκB subunits may enhance its activity; oxidation of the p50 subunit of NFκB promotes association with thioredoxin and enhances DNA binding (Fig. 2). Circumstantial evidence supporting this model has shown that thioredoxin localization parallels that of NFκB. It is found in the cytoplasm under basal conditions and translocates to the nucleus when cells are exposed to stimuli that promote oxidative stress. Thioredoxin lacks a nuclear localization signal, and it is therefore postulated that thioredoxin is carried into the nucleus via its association with NFκB.

p38 MAPK is activated in skeletal muscle under catabolic conditions such as type 2 diabetes, aging, or exposure to TNF. Studies with p38 MAPK inhibitors have shown that activation is required for subsequent atrogin1 expression and increased ubiquitin-conjugating activity. The mechanism by which ROS activates p38 MAPK in skeletal muscle is also unknown but, as with NFκB, thioredoxin may be involved. Under normal metabolic conditions, thioredoxin binds and inhibits apoptosis-stimulating kinase 1 (ASK1). ASK1 is required for TNF- and oxidative-stress-induced p38 activation during apoptosis of embryonic fibroblasts. Oxidation of thioredoxin causes disassociation from ASK1, p38 activation, and phosphorylation and activation of p38 (Fig. 2). It is possible that this pathway functions similarly in skeletal muscle. Because thioredoxin is implicated in both TNF- and ROS-responsive pathways, it may be a critical second messenger in oxidative stress–induced muscle wasting.

Finally, Foxo, a member of the forkhead family of transcription factors, is important for expression of atrogin1 and MuRF1 in a variety of muscle-wasting conditions. The extent to which oxidative stress regulates Foxo in skeletal muscle is unknown. However, Foxo activity is modulated by H₂O₂ and by menadione- or heat-shock–induced oxidative stress in mammalian fibroblasts and mouse C2C12 myoblasts. Upon treatment with H₂O₂ or TNF, c-Jun N-terminal kinase (JNK) is activated and phosphorylates Foxo4 on amino acids T447 and T551. This phosphorylation leads to Foxo4 translocation to the nucleus and activation of transcription. In addition, Foxo is negatively regulated by PI-3 kinase/AKT signaling. Stimulation with growth factors activates AKT and results in Foxo phosphorylation and translocation from the nucleus to the cytoplasm. This negative regulation is disrupted by oxidative stress, perhaps via JNK activation, which triggers re-localization of Foxo to the nucleus and subsequent transcription of stress response genes (Fig. 2).

Other protease systems may act in concert with the ubiquitin–proteasome pathway to promote muscle protein loss. For example, lysozomal, calpain, and caspase-3 proteases are activated during muscle atrophy. Both calpain and caspase-3 may play an important role in ubiquitin–proteasome-mediated wasting by promoting release of myofibrillar proteins from the contractile apparatus. The catabolic function of calpain has been demonstrated using exogenous inhibitors, calpeptin and BN82270, or overexpression of calpastatin to inhibit protein breakdown in septic rats and dexamethasone-treated myoblasts. In addition, caspase-3 activation promotes degradation of actomyosin complexes, whereas inhibition of caspase-3 activity suppresses the overall rate of proteolysis in diabetes- and endotoxin-mediated cachexia.

Reduced Protein Synthesis. In concert with increased degradation, ROS-regulated catabolic signaling may reduce protein synthesis. ROS have been shown to reduce translational activity in Chinese
hamster ovary cells; H$_2$O$_2$ treatment decreased the activity of translational regulators including ribosomal S6 kinase (p70S6k) and eukaryotic initiation factor eIF-4E. In animal models of hindlimb unloading and denervation, increased oxidative stress correlates with a significant decrease in both phosphorylated p70S6k levels and protein synthesis. The muscles of rats infused with TNF have reduced eIF-4E activity and reduced protein synthesis.137 Muscle protein synthesis drops as much as 50% in septic rats.135 Acute alcohol exposure also impairs skeletal muscle protein synthesis and p70S6k phosphorylation.136 However, it remains to be tested whether impairment in protein synthesis is mediated by the free radicals generated under these catabolic conditions. Catabolic signaling also alters muscle-specific mRNA expression through the destabilization of MyoD protein.140 MyoD is a muscle-specific basic helix–loop–helix transcription factor that drives expression of genes necessary for induction and maintenance of muscle cell differentiation. Reduced levels of MyoD could lead to impaired myogenesis and muscle repair. Finally, growth hormone resistance and reduced insulin-like growth factor levels are a significant complication of chronic kidney disease.217 These reductions in growth factor effectiveness may contribute to muscle wasting via reduced protein synthesis or lack of inhibition of protein degradation pathways.

INTERVENTIONS

Nutrition. Most treatments of chronic disease do little to address the underlying cachexia. Efforts are being made to find interventions specific for prevention or reduction of cachectic symptoms by promoting appetite and preserving lean body mass. Increased appetite can be effectively achieved with cannabinoids and megestrol acetate. However, these treatments have no measurable effect on lean body mass.202 By contrast, branched-chain amino acid supplementation shows promise (Fig. 3). These essential amino acids (leucine, isoleucine, and valine) stimulate protein synthesis, inhibit protein degradation, and are an important energy source for muscle.98,209 In animal models, they spare lean body mass during weight loss and promote muscle protein anabolism with aging.141,224 Results from clinical trials are mixed. Some findings have shown that leucine administration improves nitrogen balance, reduces skeletal muscle catabolism, increases skeletal muscle protein synthesis, and maintains plasma amino acid concentrations.43 Other trials, however, showed no apparent benefits.43 The reasons for the mixed results are unclear, but branched-chain

![FIGURE 2. Hypothesized pathways for ROS-mediated catabolic signaling in skeletal muscle. Diagram depicts extracellular catabolic stimuli that induce ROS production and hypothesized downstream catabolic signaling pathways in skeletal muscle. Filled arrowheads: known interactions in skeletal muscle; open arrowheads: hypothetical interactions. ROS, reactive oxygen species; RNS, reactive nitrogen species; NFkB, nuclear factor-kappaB; TRX, thioredoxin; Ubch2, ubiquitin-conjugating enzyme E2; Ask1, apoptosis-stimulating kinase; p38, mitogen-activated protein kinase p38; atrogin1, MAFbx, muscle atrophy F-box, muscle-specific F-box protein; Jnk, c-Jun N-terminal kinase; Foxo, forkhead box O transcription factor; MuRF1, muscle-specific RING finger 1.](image1)

![FIGURE 3. Muscle wasting interventions. Diagram shows targets of interventions to alleviate catabolic processes of muscle wasting accompanying chronic disease. Middle: catabolic processes; top: exercise and nutritional interventions mainly target redox imbalances and protein degradation; bottom: pharmaceutical interventions mainly target inflammation and protein degradation.](image2)
amino acid supplementation may only be effective for severe catabolic states.

Other promising dietary interventions include fish oil polyunsaturated fatty acids, melatonin, green tea extract polyphenols, and l-carnitine (Fig. 3). COPD and pancreatic cancer patients treated with fish oil capsules showed decreased inflammatory response, increased body weight, and improved strength. Melatonin acts as a direct oxidant scavenger and stimulates the activity of glutathione peroxidase, SOD, catalase, and NOS. It has been shown to reduce oxidative stress in diabetic patients and has anti-tumor and anti-cytokine effects that improve survival in patients with advanced cancer. In a study of 100 patients with untreated metastatic solid tumor, melatonin decreased circulating TNF and significantly reduced weight loss. In a separate study, melatonin combined with fish oil had an additive positive effect when compared with either agent taken individually; 27% of patients responded with weight stabilization or gain when treated with melatonin alone, 38% responded similarly with fish oil alone, and 63% responded to the combined treatment. Although controlled clinical studies on muscle catabolism are lacking, green tea extract improves muscle function and reduces oxidative stress in mouse models of Duchenne dystrophy. In addition, green tea extract shows promise as a prevention for diseases where oxidative stress is a factor in the pathogenesis, such as Alzheimer’s disease, prostate cancer, and cardiovascular disease.

Reduced l-carnitine levels are associated with the development of cachexia. Accordingly, clinical trials using l-carnitine supplements have been consistently positive (Fig. 3). Of 50 cancer patients treated with l-carnitine (4 g/day for 7 days), 45 showed an improvement of mood and quality of sleep and a significant reduction in their Brief Fatigue Inventory scores, indicators of overall fatigue. A second study demonstrated that cancer patients receiving 6 g/day of l-carnitine for 4 weeks had significantly decreased fatigue and increased lean body mass and appetite. l-carnitine supplementation also significantly decreased fatigue and improved muscle mass in studies with 84 elderly subjects and 122 patients with end-stage renal disease.

**Pharmaceutical Agents.** In addition to dietary interventions, certain pharmaceuticals have been tested for effective prevention of wasting (Fig. 3). Anabolic steroids (oxandrolone and nandrolone) have shown positive effects on muscle mass in patients with HIV infection, COPD, and cancer. Angiotensin-convert-

ing enzyme (ACE) inhibitors (captopril and enalapril) reduce weight loss, perhaps as a consequence of decreased circulating TNF. The β2-adrenergic agonist formoterol alleviates cachectic symptoms in tumor-bearing rats and mice. The mechanism appears to be through stimulation of skeletal muscle protein synthesis and inhibition of the ubiquitin–proteasome pathway. However, there are few clinical trials showing efficacy in humans. Several positive studies have shown that patients with chronic heart failure treated with salbutamol for 3 weeks or clenbuterol for 3 months recovered more skeletal muscle mass and strength than the placebo group. In addition, clenbuterol improved rehabilitation time of strength recovery in patients undergoing knee surgery. Cyclooxygenase-2 (COX2) inhibitors have been effective in animal models of cancer cachexia. Inhibition of COX2 by celecoxib or meloxicam reversed tumor-induced wasting in colon 26 and adenocarcinoma murine models, respectively. In these models, the inhibitors reduced both circulating levels of inflammatory cytokines and protein degradation.

**Anti-Cytokine Therapy.** Because inflammatory cytokines are important mediators of muscle wasting in chronic disease, it is plausible that anti-cytokine antibodies could prove an effective treatment. Anti-IL-6 therapy reduced fever and cachexia in a single trial of patients with HIV/AIDS-related lymphoma. However, in the majority of clinical trials, anti-cytokine therapies have been ineffective. Positive responses have been seen in animal models of cancer where anti-TNF and anti-IFN antibodies partially reverse protein turnover and decrease levels of ubiquitin–proteasome components.

In contrast, the immunomodulatory drug thalidomide possesses powerful anti-TNF properties and selectively destabilizes TNF mRNA (Fig. 3). A recent clinical trial of patients with advanced pancreatic cancer showed significant attenuation of loss of muscle mass and improvement of physical function with thalidomide. These patients gained or maintained weight and arm muscle mass, whereas the placebo group lost 4 kg in weight and 8 cm3 in arm muscle mass. A smaller 2-week trial also showed that thalidomide attenuated weight loss and loss of lean body mass in patients with esophageal cancer.

**Combination Therapy.** Combination therapy has resulted in encouraging patient responses. For example, based on published and clinical observations, Mantovani et al. developed an anti-inflammatory/antioxidant cocktail that included omega-3 fatty ac-
ids (eicosapentaenoic acid and docosahexaenoic acid), medroxyprogesterone acetate, α-lipoic acid, carbocysteine lysine salt, vitamin E, vitamin A, vitamin C, and the COX2 inhibitor celecoxib. Twenty-five patients with cancer cachexia were treated with this combination for 4 months. This regimen resulted in a significant increase in lean body mass and a significant decrease in ROS, IL-6, and TNF, and quality of life comprehensively improved.

An interesting approach yet to be tested for treatment of muscle wasting was recently shown to be effective in reducing plasma lipid peroxidation in humans. The goal of the study was to increase endogenous levels of antioxidant enzymes.168 Because of their greater antioxidant capacity, it was suggested that increases in enzyme activity will be more effective than antioxidant supplements. The investigators used a combination of plant extracts that included milk thistle, green tea, and turmeric (Protandim). These extracts individually are reported to increase SOD and catalase activity while decreasing plasma lipid peroxidation. The Protandim was given to healthy human subjects. After 30 days, markers of lipid peroxidation declined 40% (P < 0.0001). After 120 days, erythrocyte SOD increased 30% (P < 0.01) and catalase increased 54% (P < 0.002).

Exercise. Last but not least, exercise is a critical therapy for alleviating muscle protein loss in many conditions including cancer, heart failure, and rheumatoid arthritis8,106,168 (Fig. 3). The negative effects are few and the benefits are consistent and improve a patient’s physical function and quality of life.6 Exercise-induced increases in muscle mass result in greater strength and reduced susceptibility to fatigue. In addition, exercise reduces inflammatory responses, enhances the activity of antioxidant enzymes, and increases insulin sensitivity, thereby decreasing muscle protein degradation. Endurance training was found to decrease levels of catabolic cytokines in the quadriceps muscle of patients with chronic heart failure.16 Progressive resistance training in rheumatoid arthritis patients, 2.5 times per week for 12 weeks, added approximately 1 kg in lean body mass, with the majority of that affecting leg mass.168 Finally, colorectal cancer survivors, who increased their cardiovascular fitness through moderate exercise three times per week, significantly increased their quality of life.49 Consequently, if tolerated, exercise should be included in nutritional or drug treatment strategies.

This study was supported by NIH Grant HL59878.

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ABSTRACT: The sarcoendoplasmic reticulum (SR) calcium transport ATPase (SERCA) is a pump that transports calcium ions from the cytoplasm into the SR. It is present in both animal and plant cells, although knowledge of SERCA in the latter is scant. The pump shares the catalytic properties of ion-motive ATPases of the P-type family, but has distinctive regulation properties. The SERCA pump is encoded by a family of three genes, SERCA1, 2, and 3, that are highly conserved but localized on different chromosomes. The SERCA isoform diversity is dramatically enhanced by alternative splicing of the transcripts, occurring mainly at the COOH-terminal. At present, more than 10 different SERCA isoforms have been detected at the protein level. These isoforms exhibit both tissue and developmental specificity, suggesting that they contribute to unique physiological properties of the tissue in which they are expressed. The function of the SERCA pump is modulated by the endogenous molecules phospholamban (PLB) and sarcolipin (SLN), expressed in cardiac and skeletal muscles. The mechanism of action of PLB on SERCA is well characterized, whereas that of SLN is only beginning to be understood. Because the SERCA pump plays a major role in muscle contraction, a number of investigations have focused on understanding its role in cardiac and skeletal muscle disease. These studies document that SERCA pump expression and activity are decreased in aging and in a variety of pathophysiological conditions including heart failure. Recently, SERCA pump gene transfer was shown to be effective in restoring contractile function in failing heart muscle, thus emphasizing its importance in muscle physiology and its potential use as a therapeutic agent.


SERCA PUMP ISOFORMS: THEIR ROLE IN CALCIUM TRANSPORT AND DISEASE

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The sarcoplasmic reticulum (SR) is an intracellular membranous network found in muscle cells. Although it is analogous to the endoplasmic reticulum (ER), it can store millimolar amounts of calcium. It serves to initiate muscle contraction by releasing calcium through the ryanodine receptors (RyR) into the cytosol and facilitates muscle relaxation by active reuptake of calcium by the sarcoendoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA). The SERCA pump serves a dual function: (1) to cause muscle relaxation by lowering the cytosolic calcium, and (2) at the same time to restore SR calcium store necessary for muscle contrac-

Abbreviations: α-MHC, α myosin heavy chain gene; CLFS, chronic low-frequency stimulation; E-P, phosphorylated enzyme intermediate; PLB, phospholamban; RyR, ryanodine receptors; SERCA, sarcoplasmic reticulum calcium transport ATPase; SLN, sarcolipin; SR, sarcoplasmic reticulum; T3, triiodothyronine; TG, transgenic mouse

Key words: calcium; endoplasmic reticulum; phospholamban; sarcolipin; sarcoplasmic reticulum; SERCA

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SR Ca\(^{2+}\) ATPase belongs to the P-type ATPase family and is highly conserved in structure

The SR Ca\(^{2+}\) ATPase belongs to the family of P-type ATPases that includes plasma membrane Ca\(^{2+}\) ATPase (PMCA), Na\(^+\)/K\(^+\) ATPase, and H\(^+\), K\(^+\)
cells, fibroblasts, and endothelial cells. SERCA3 isoforms are expressed in a variety of nonmuscle cells, especially in the hematopoietic cell lineages including platelets, mast cells, T cells, epithelial cells, fibroblasts, and endothelial cells.

SERCA3 is known to encode for 6 isoforms, 3a-3f (~999–1052 aa) at the mRNA level. At the protein level there is data only for 3a, b, and c isoforms. SERCA3 isoforms are expressed in a variety of nonmuscle cells, especially in the hematopoietic cell lineages including platelets, mast cells, T cells, epithelial cells, fibroblasts, and endothelial cells.

ATPase. Unlike the Na⁺/K⁺ ATPase, which is a dimer, the SERCA pump is a single polypeptide of molecular mass 110 kDa and is localized both in the ER and SR membrane. A notable feature of P-type ATPases is the transfer of terminal phosphate from ATP to an aspartate residue in the catalytic domain, resulting in a reversible conformational change. P-type ATPases couple the hydrolysis of ATP to the movement of ions across a biological membrane. The SERCA pump utilizes the energy derived from ATP hydrolysis to transport Ca²⁺ across the membrane. The mechanism of the coupling process is such that two Ca²⁺ ions are transported for each molecule of ATP hydrolyzed. SERCA pump activity is additionally regulated by small-molecular-weight proteins, phospholamban (52 aa) and sarcolipin (31 aa), in a tissue-specific manner. PLB in the nonphosphorylated form acts as an inhibitor of the SERCA pump and its inhibition is relieved upon phosphorylation during beta-adrenergic activation of the heart. The mechanism of PLN action on the SERCA pump has been well established. Sarcolipin (SLN) is a novel SR protein and has been shown to regulate SERCA pump activity.  

<table>
<thead>
<tr>
<th>SERCA isoform</th>
<th>Skeletal muscle</th>
<th>Cardiac muscle</th>
<th>Smooth muscle</th>
<th>Non-muscle cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fetal</td>
<td>Adult</td>
<td>Fetal</td>
<td>Adult</td>
</tr>
<tr>
<td>SERCA 1a</td>
<td>–</td>
<td>+ + + + +</td>
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<td>SERCA 1b</td>
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<td>–</td>
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<td>SERCA 3b</td>
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<tr>
<td>SERCA 3c</td>
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</table>

**SERCA GENE FAMILY AND SERCA PUMP ISOFORMS**

The SERCA pump is a 110-kDa transmembrane protein that has been identified both in prokaryotes and eukaryotes. Its widespread occurrence throughout the animal kingdom and in plants delineates the pump as a versatile, evolutionarily conserved protein. The SERCA pump is known to be present in all living organisms, from yeast to mammalian systems.

Although its primary function is to control cytosolic calcium, it plays a vital role in many cellular functions including cell growth and differentiation. In invertebrates, SERCA is encoded by a single gene. In the nematode *Caenorhabditis elegans* the single SERCA gene transcript is alternatively spliced to produce two proteins, CeSERCAa and CeSERCAb. These isoforms resemble the SERCA2a and SERCA2b isoforms of mammals, pointing to an evolutionarily conserved feature of SERCA pump isoform diversity. It has been shown that in C. elegans SERCA is necessary for normal development and muscle function and postembryonic growth and survival.

Similarly, in *Drosophila* the pump is encoded by one gene but is spliced to two isoforms. Studies using conditional mutants of *Drosophila* have shown that SERCA plays a key role in muscle contraction and heartbeat frequency and rhythmicity. The key findings from invertebrate models underscore the evolutionary conservation of SERCA structure and function despite the fact that there is only one gene coding for the pump as opposed to multiple genes in vertebrates.

In vertebrates there are three distinct genes encoding SERCA 1, 2, and 3 that are known to produce more than 10 isoforms, mainly through alternative splicing. SERCA1 is expressed in fast-twitch skeletal muscle and is alternatively spliced to encode SERCA1a (994 aa, adult) and 1b (1011 aa, fetal). SERCA2 encodes SERCA2a (997 aa), which is expressed predominantly in cardiac and slow-twitch skeletal muscle. SERCA2b (1042 aa) is expressed in all tissues at low levels including muscle and nonmuscle cells. Recently, a third isoform, SERCA2c (999 aa), has been reported in cardiac muscle. SERCA3 isoforms are expressed in several nonmuscle tissues but appear to be a minor form in muscle. In humans, SERCA3 is known to encode for six isoforms, 3a-3f (~999–1052 aa) at the mRNA level.
expressed in multiple tissues and cell types. At the protein level there are data only for the 3a, b, and c isoforms (Table 1). SERCA3 isoforms are expressed at high levels in the hematopoietic cell lineages, platelets, epithelial cells, fibroblasts, and endothelial cells.

**SERCA Pump Expression Is Developmentally Regulated.** The regulatory processes that control the expression of SERCA isoforms lend a whole different level of complexity. SERCA expression is not only tissue-specific but undergoes developmental regulation, including switching of isoforms, which represents an important phenotypic change in muscle maturation. Although well documented, the regulatory processes that control these switches are not understood. In rat embryonic development, SERCA2a is absent in the somites but can be detected at the onset of muscle differentiation. During fast skeletal muscle development, both SERCA2a and SERCA1b are coexpressed in fetal/neonatal stages but are completely replaced by SERCA1a in the adult muscle fibers (Table 1). Interestingly, in mammals SERCA2a remains the predominant isoform in slow-twitch skeletal muscle, both in the fetal and adult stages, but disappears from most of the other skeletal muscles (Table 1). By contrast, fast SERCA1 isoforms are neither expressed in slow-twitch muscle fibers nor in cardiac muscle. In mammalian cardiac muscle, SERCA2a remains the predominant isoform during development and in adult stages. The functional significance of isoform switching during development is not clear. One plausible explanation is that the unique Ca$^{2+}$ transport properties of the isoforms determine the characteristics of the muscle or the developmental stage at which they are expressed.

Developmental and age-related changes in myocardial contractility have long been speculated to be associated with alterations in SR function. The overall contractility of the heart could be correlated with the development of the SR during ontogeny. The fetal heart has a very sparse SR network, and a mature network is seen after birth. In chick heart SERCA is present in early embryonic stages even before a functional SR develops, and the increase in SERCA2 mRNA parallels SR maturation. In rat, SERCA2a and 3 mRNA have been detected in the heart tube during early developmental stages (10 days postcoitum) and, as development progresses, SERCA2a persists, whereas SERCA3 is restricted to the endothelial layer of the coronary arteries. It is speculated that the presence of SERCA3, which has a lower sensitivity for calcium, could indicate a different mode of calcium regulation in premature cardiomyocytes. SERCA2b expression is more ubiquitous, neither tissue-specific nor developmentally regulated. Aging-related changes in SERCA levels have been observed both in animal models of aging and in human senescent myocardium. In senescent rat hearts, reductions in SR Ca$^{2+}$ ATPase level (up to 40%) and SR Ca$^{2+}$ uptake have been observed, without any changes in calsequestrin protein level. A decrease in the phosphorylation state of PLB was also observed, suggesting that in the senescent myocardium the beta-adrenergic response is compromised. This finding correlates well with the prolonged calcium transients and delayed relaxation found in senescent hearts.

**Neurohormonal Regulation of SERCA Expression.** Thyroid hormone (T4) is a potent regulator of SERCA pump expression and cardiac muscle contractility. The effect of thyroid hormone on cardiac contractility and gene expression has been studied extensively in rodents. Neonatal hypothyroidism in mice decreases SERCA2a and increases the levels of its inhibitor, PLB, suggesting a role for the increasing thyroid levels during development in regulating SERCA2a expression. In general, hypothyroidism decreases SERCA2a and increases PLB, whereas hyperthyroidism increases SERCA2a levels but reduces PLB expression. In rabbits hyperthyroidism increased the steady-state level of SERCA2a mRNA to 147% after a 4-day treatment with T4 and to 186% after 8 days of treatment. SERCA protein was increased to 199% in the 8-day treatment group. The increases in SERCA expression are consistent with increased velocity of Ca$^{2+}$ uptake and enhanced cardiac function observed in hyperthyroidism in adult hearts. Interestingly, there is a very close correlation between the Ca$^{2+}$ dependence of Ca$^{2+}$ uptake (EC$_{50}$ of SR Ca$^{2+}$ uptake) and the ratio of PLB to SERCA in hypothyroid, euthyroid, and hyperthyroid hearts, and this determines the contractile parameters of the heart. Detailed studies on the SERCA2 gene have identified promoter elements (thyroid response elements) that can bind to thyroid receptors and regulate SERCA expression.

In skeletal muscle, T4 regulation is fiber-specific. Using a rat model, Sayen et al. showed that hypothyroidism produced in soleus a marked decrease in SERCA1 and SERCA2 mRNA levels, whereas in EDL SERCA1 mRNA decreased. These findings are compatible with a hypothyroidism-induced decrease in SR Ca$^{2+}$ ATPase activity and a delay in muscle relaxation. In contrast, in EDL muscle the SERCA2a mRNA (which represents only a small percent of total SERCA mRNA in this muscle)
increased to 175% of control values. This study showed that muscle-specific and SERCA gene-specific changes also occur after acute triiodothyronine (T3) administration to hypothyroid rats. T3 had little effect on SERCA1 or SERCA2 mRNA levels in soleus. In contrast, T3 increases SERCA1 mRNA level about 3-fold from its hypothyroid level in EDL, whereas SERCA2 mRNA decreases to 75% of control levels.

Arai et al.\textsuperscript{4} showed that, in rabbits, T3 administration increased SERCA pump levels in soleus (SERCA2a) and plantaris (SERCA1a and SERCA2a) significantly. These studies emphasize the complex nature of gene regulation by thyroid hormone, which is highly muscle type-specific.

**Effect of Innervation on SERCA Expression.** The expression of fast (SERCA1a) and slow (SERCA2a) isoforms were studied in denervated, regenerated, and dystrophic chicken fast and slow skeletal muscle fibers.\textsuperscript{46} That study demonstrated that denervation of the pectoralis major (a fast muscle) resulted in the reappearance of neonatal myosin heavy chain (MHC) and slow SERCA isoforms that had been expressed previously in the developing muscle but were repressed in the adult stages. However, the denervated muscle continued to express the adult fast MHC and the fast SERCA1 isoforms. Similar to fast muscle, denervated slow muscle expressed the neonatal MHC and the slow isoforms of SERCA (SERCA2a), without a switch in SERCA protein. In dystrophic chicken, the expression of the slow SERCA isoforms in fast muscle continued up to 1 year of age, along with neonatal MHC isoforms. This is in contrast to the normal-maturing fast muscle, where the slow isoform is completely replaced by the fast isoform within 40 days. However, the fast Ca\textsuperscript{2+} ATPase isoform disappeared from slow tonic muscle in normal and dystrophic birds similarly. These studies clearly document that extrinsic factors, such as neural input and disease, influence the pattern of SERCA expression.

Denervation of rat soleus muscle caused a decrease in the expression of SERCA 2a isoform, whereas the level of fast SERCA1a isoform was unchanged.\textsuperscript{30} By contrast, studies in rabbit showed that denervation of soleus muscle induced a slow-to-fast transition of the Ca\textsuperscript{2+} ATPase isoform with a decrease in the expression of SERCA2a and an increase in SERCA1a protein levels.\textsuperscript{30} Studies carried out in 8-day-old rabbits showed that denervation of gastrocnemius caused a decrease in SERCA1a isoform without replacement by SERCA2a.\textsuperscript{72}

**CHRONIC LOW-FREQUENCY STIMULATION**

Pette and colleagues\textsuperscript{35,36,52,79} tested the effect of electrical stimulation on the pattern of expression of SR Ca\textsuperscript{2+} transport proteins, including SERCA. For those studies the tibialis anterior muscle of the rabbit was continuously stimulated at 10 Hz for 4 and 30 days. Chronic low-frequency stimulation (CLFS) resulted in a switch in the expression pattern of SERCA isoforms. Following 30 days of stimulation, SERCA2a levels were upregulated and SERCA1a protein was downregulated. Interestingly, PLB levels were upregulated in these muscles similar to SERCA2a. In addition to switching of isoforms, CLFS also resulted in partial inactivation of the SR Ca\textsuperscript{2+} ATPase and a net decrease in SR calcium transport. A reversal of these changes in SERCA isoform expression was observed if stimulation was discontinued for 30 days. These studies document that the type of innervation or electrical stimulation regulate excitation–contraction properties. In particular, the expression pattern of SERCA isoforms and muscle proteins are influenced by neuronal innervation.

In skeletal muscle there is a good correlation between motor neuron innervation and muscle fiber type. However, a muscle group is often composed of multiple fiber types. It is believed that the hierarchy of motor unit recruitment order might, in part, be responsible for the heterogeneity of muscle fibers within the same muscle. This muscle fiber heterogeneity is largely determined by the consequence of motor unit activity. It is believed that the type of motor neuron innervation regulates gene expression, and electrical stimulation can alter the phenotype of the muscle.

**FUNCTIONAL COMPARISON OF SERCA ISOFORMS**

A notable feature of SERCA isoforms is that their primary structure is highly conserved. SERCA2a protein is about 84% identical to SERCA1a protein, and SERCA3 protein is ~75% identical to either SERCA1 or SERCA2 molecules. Due to this high conservation in their primary structure, all of the SERCA isoforms are predicted to have essentially identical transmembrane topologies and tertiary structures. Another interesting feature is that all of the SERCA isoforms are inhibited by thapsigargin (Fig. 1), a sesquiterpene lactone, derived from the plant *Thapsia garganica*.\textsuperscript{108} However, thapsigargin has no effect on Na\textsuperscript{+}/K\textsuperscript{+} ATPase or other plasma membrane ATPases. The expression pattern of SERCA isoforms to specific stages of development or tissue types strongly suggests that there must be functional differences among the SERCA isoforms, which might
impart unique properties of calcium homeostasis to the tissue or cell type. The finding that SERCA2a, but not SERCA1a, is regulated by PLB also suggests that functional diversity could be additionally established by regulatory molecules of the SERCA pump.

The SERCA1a pump expressed in fast-twitch muscle fibers is encoded by 994 amino acids. The structure and function of SERCA1a has been extensively studied using site-directed mutagenesis and crystallization of the protein, which led to the identification of key domains involved in nucleotide (ATP) binding, hydrolysis, Ca\(^{2+}\) binding, and translocation.\(^{17,58,65,64,68,94–99,108}\) The crystal structure of SERCA confirmed the presence of 10 transmembrane helices (M1–M10), three cytoplasmic domains, an A domain (actuator or anchor domain), a P domain (phosphorylation domain), and an N domain where ATP binds.\(^{63,67,96,105}\) Tg resides in a cavity delimited by the M3, M5, and M7 helices, near the cytosolic surface of the membrane. (Reproduced with permission from Dr. Guiseppe Inesi.\(^{105}\))

**FIGURE 1.** Thapsigargin (Tg) bound to Ca\(^{2+}\)-ATPase embedded in the lipid bilayer based on crystal structure. The crystal structure of SERCA confirmed the presence of 10 transmembrane helices (M1–M10), three cytoplasmic domains, an A domain (actuator or anchor domain), a P domain (phosphorylation domain), and an N domain where ATP binds.\(^{63,67,96,105}\) Tg resides in a cavity delimited by the M3, M5, and M7 helices, near the cytosolic surface of the membrane. (Reproduced with permission from Dr. Guiseppe Inesi.\(^{105}\))
Ca$^{2+}$/H$^{+}$ binding sites lie side by side, near the cytoplasmic surface of the lipid layer. Ca$^{2+}$/H$^{+}$ binding sites are accessible only from the cytoplasm and not from the SR lumen. Phosphorylation of the enzyme by ATP initiates a series of conformational changes that, through long-range interactions, change the conformation of the transmembrane helices so that access of bound calcium to the cytoplasm is lost, whereas access to the lumen is gained, resulting in Ca$^{2+}$/H$^{+}$ transport to the lumen.

During Ca$^{2+}$/H$^{+}$ transport the SERCA pump transitions through multiple steps (Fig. 2). Ca$^{2+}$/H$^{+}$ binding to the high-affinity sites on the cytoplasmic face of the SERCA pump is the first step. Once these sites are occupied, ATP hydrolysis is triggered, the enzyme is phosphorylated, and a series of conformational changes in the enzyme results in the transport of Ca$^{2+}$/H$^{+}$ across the SR. For each molecule of ATP hydrolyzed, the pump transports two molecules of Ca$^{2+}$/H$^{+}$ (Fig. 2). Because the SERCA pump is highly abundant in the SR, Ca$^{2+}$/H$^{+}$ reuptake takes only a few milliseconds, allowing faster rates of relaxation in smaller mammals such as mice, accommodating higher heart rate, as high as 500 beats per minute.

A number of factors including Ca$^{2+}$ ion concentration, ATP level, pH, and ADP and inorganic phosphate level can influence SERCA pump activity at one or more steps, including pump affinity for Ca$^{2+}$/H$^{+}$, rate of phosphorylation, E-P formation (ATP binding and hydrolysis), and decomposition of E-P (Fig. 2). In addition, it has been shown that PLB affects the SERCA pump affinity for Ca$^{2+}$/H$^{+}$ but not the enzyme kinetics, such as the rate of E-P formation and phosphoenzyme decay.

The enzymatic properties of SERCA isoforms, SERCA1a, SERCA2a, and SERCA3a, have been studied using the COS-1 cell expression system and site-directed mutagenesis. The heterologous cell system provides an advantage because the cells do not express high levels of endogenous pump or regulatory molecules such as PLB and SLN. By generating chimeras between the SERCA1, SERCA2, and SERCA3 isoforms, MacLennan and colleagues were able to swap domains and ask whether corresponding domains had similar functions. These chimeric molecules helped to identify two distinct cytoplasmic domains that were involved in PLB interaction. The first is a region between amino acids 336 and 412, which corresponds to a PLB interaction domain; and the second region is the nucleotide binding/hinge domain (amino acids 467–762), which determines high Ca$^{2+}$/H$^{+}$ affinity for SERCA-type pumps. These studies demonstrated that SERCA pumps can be expressed efficiently in a heterologous cell culture system and their functional properties can be studied using isolated microsomes directly. These in vitro studies showed that SERCA1a and SERCA2a isoforms are similar in their properties including SERCA pump affinity for Ca$^{2+}$/H$^{+}$ and the velocity of Ca$^{2+}$/H$^{+}$ uptake ($V_{\text{max}}$). However, later studies proved that there were kinetic differences between the SERCA1a and 2a pumps (see below). Interestingly, PLB inhibited the SERCA2a, SERCA2b, and SERCA1a pumps equally, but not the SERCA3 pump, which is unique in that it lacks the putative interacting domain for PLB (Table 2).

SERCA2a and the ubiquitous SERCA2b isoforms are structurally similar for the first 993 amino acids.

**Table 2.** Functional comparison of SERCA pump isoforms.

<table>
<thead>
<tr>
<th>SERCA isoform</th>
<th>Phospholamban inhibition</th>
<th>Sarcolipin inhibition</th>
<th>Affinity for calcium</th>
<th>Pump velocity</th>
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<td>++</td>
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<td>+</td>
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<tr>
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<td>n/a</td>
<td>+</td>
<td>+</td>
<td>pH 7.2–7.4</td>
</tr>
</tbody>
</table>
as with transgenic animals have shown that cardiac myocytes by Sumbilla et al. when maxi-
pared by expressing these proteins in COS cells and parameters of SERCA2a and SERCA1a were com-
differentials in the C-terminal amino acids have been shown to contribute to the functional differences between SERCA2a and 2b. This finding was further supported by Verboomen et al., who showed that the functional differences between SERCA2a and 2b could be ascribed to the terminal 12 amino acids that are specific to SERCA2b. SERCA2c has recently been shown to have a lower affinity for cytosolic calcium than SERCA2a and 2b, which could be due to the specific C-terminus in SERCA2c. The physiological relevance of SERCA2c is yet to be defined but it is speculated to work in an environment with high local calcium.

Very little is known about the functional properties of the SERCA3 isoforms. SERCA3 when expressed in COS cells shows a 5-fold lower apparent affinity for calcium, a 10-fold higher apparent affinity for vanadate inhibition, and an altered pH optimum (pH 7.2–7.4 vs. pH 6.8–7.0) compared to other SERCA pumps (Table 2). Among the human SERCA3 isoforms, a, b, and c, the length of the alternatively spliced C-terminus tail may contribute to the differences in their enzymatic activity. SERCA3b and 3f have similar enzymatic behavior that could be attributed to their common C-terminal tail.

Experiments with cultured myocytes as well as with transgenic animals have shown that heterologous expression of the SERCA1a isoform can be obtained in cardiac muscle, thereby demonstrating that SERCA1a can support cardiac muscle excitation–contraction coupling. Using adenoviral gene transfer, the Ca\(^{2+}\) transport and the kinetic parameters of SERCA2a and SERCA1a were compared by expressing these proteins in COS cells and cardiac myocytes by Sumbilla et al. When maximally expressed (similar protein levels), SERCA1a Ca\(^{2+}\) transport activity was approximately two times higher than that of SERCA2a. By comparing the exponential decay of the phosphorylated enzyme intermediate of SERCA1a and 2a, that study demonstrated that the SERCA1a isomer is twice as fast as SERCA2a (Table 2). These findings suggest that SERCA1a might provide a unique functional advantage in muscle fibers that require a faster rate of Ca\(^{2+}\) uptake and contractile function.

**MUTATIONS IN SERCA GENES CAUSE HUMAN DISEASE**

Mutations in SERCA genes have been shown to cause human diseases. Brody’s disease is a rare, inherited disorder affecting skeletal muscle function. Patients suffer from severe cramps and manifest exercise-induced impairment of skeletal muscle relaxation. The disease results from mutations in the SERCA1a gene, one mutation affecting the splice donor site of intron 3 and the other mutation gives rise to premature stop codons resulting in a truncated protein. Patients have a normal life span because the loss of SERCA1a expression is partially compensated by upregulation of SERCA2a and other isoforms. The disease, however, has a more heterogeneous origin with reported mutations in genes other than SERCA.

Mutations in the SERCA2 gene are extremely rare. Deletion of both copies of SERCA2 gene is lethal in most species including nematode, Drosophila, and mouse. Missense mutations affecting one copy of the SERCA2 gene (+/0) causes an autosomal-dominant skin disease in humans, Darier’s disease, which results in keratinized squamous epithelial cells. These patients do not manifest any heart disease, showing that a single SERCA2 allele is sufficient to maintain cardiac muscle function. Similarly, in mice a single SERCA2 allele is sufficient to maintain normal cardiac function but the mice showed an increased incidence of epithelial cancer when they aged.

**GENETIC MANIPULATION OF SERCA PUMPS IN ANIMAL MODELS**

The importance of SERCA isoforms and their physiological relevance has been studied extensively using transgenic animals that overexpress SERCA pump in the heart or are deficient in SERCA pump isoforms. Here we briefly summarize the critical findings from these mouse models. TG mouse and rat models overexpressing the SERCA2a isoforms were generated using the α-MHC cardiac gene promoter. Overexpression of SERCA2a in the heart resulted in an increase in the velocity of SR Ca\(^{2+}\) uptake and increased maximal rates of contraction and relaxation of the heart, both in mice and rats. An interesting finding of these TG lines was that the SERCA2a protein level was only modestly increased above the endogenous level (20%–30%) despite a several-fold (2–4-fold) increase in SERCA2a mRNA in the TG hearts. These TG animals did not exhibit structural or functional abnormalities and had a normal life span. Overexpression of SERCA1a
(fast-twitch muscle isoform) in the mouse heart increased SERCA protein levels to 2.5-fold and Ca\(^{2+}\) uptake to \(-2\)-fold.\(^{41,51,56}\) Interestingly, the expression of SERCA1a in the heart led to a 50% downregulation of the endogenous SERCA2a pump, along with decreased levels of PLB. A decrease in SERCA2a suggests competition between SERCA2a and SERCA1a for functional sites in the SR, or a compensatory downregulation of the endogenous isoform. SERCA1a was colocalized with SERCA2a in cardiac SR. Myocytes from SERCA1a hearts showed increased SR Ca\(^{2+}\) stores and an enhanced rate of Ca\(^{2+}\) uptake, with increased rates of contraction and relaxation.\(^{41,51,56}\) These findings suggested that SERCA1a can functionally substitute for the SERCA2a isoform in the cardiac environment, and isoform specificity is not absolute. Despite chronic increases in SERCA1a and SR Ca\(^{2+}\) stores, these mice did not exhibit cardiac pathology or increased mortality. However, in these hearts, increases in SERCA level and Ca\(^{2+}\) uptake were offset by decreased RyR and L-type Ca\(^{2+}\) channel expression, presumably as a compensatory mechanism. Thus, intracellular Ca\(^{2+}\) homeostasis is maintained by balancing the different components involved in Ca\(^{2+}\) handling. These studies suggest that there is crosstalk between the Ca\(^{2+}\) transport machinery and that an abnormal activity of one component alters the activity of the other. The underlying mechanism is not known but it is likely that Ca\(^{2+}\) itself can act as a signal for this remodeling.

All three SERCA genes have been disrupted using homologous recombination in mouse models.\(^{92}\) In mouse, the disruption of both copies of the SERCA2 gene is lethal, whereas hemizygotic mice with a single functional allele live and reproduce well.\(^{40,78}\) SERCA2a protein levels and the maximal velocity of SR Ca\(^{2+}\) uptake were reduced by \(-35\%\). In addition, the peak amplitude of the Ca\(^{2+}\) transients in isolated single myocytes was decreased by more than 50\%, resulting in decreased rates of cell shortening and relengthening in hemizygotic mice. Analyses of in vivo cardiovascular function showed reductions in heart rate, mean arterial pressure, systolic ventricular pressure, and absolute values for both maximal rates of contraction and relaxation. These results demonstrate that two functional copies of the SERCA2 gene are required to maintain “normal” levels of SERCA2a protein, Ca\(^{2+}\) sequestering activity, and Ca\(^{2+}\) homeostasis, and that the loss of the SERCA2 gene depresses cardiac function. Interestingly, hemizygotic mice do not develop cardiac hypertrophy or other signs of heart failure, suggesting that alterations in SERCA pump level can be compensated.\(^{38,40}\) However, when the SERCA2\(^{+/-}\) mice were stressed with an increased hemodynamic load (aortic banding), heart failure occurred more rapidly and frequently in mice with chronically reduced SERCA2 level.\(^{91}\) Pressure overload caused a significant degree of cardiac hypertrophy and dilatation in SERCA2\(^{-/-}\) mice compared to wildtype, which suggests that Ca\(^{2+}\) handling via the SERCA2 pump is not only important for cardiac function but also affects overall cardiac remodeling during disease state.

In contrast to SERCA2, SERCA3 has a limited cell distribution and often overlaps with SERCA2b in its expression profile. Disruption of the SERCA3 gene resulted in normal viable mutant mice.\(^{53}\) The mutant mice did not exhibit any disease phenotype and are indistinguishable from wildtype mice, both in appearance and behavior. These mice showed altered smooth muscle contractility when challenged with acetylcholine.\(^{45}\) Because SERCA3 expression overlaps with SERCA2b, loss of SERCA3 gene could be compensated by the SERCA2b isoforms, which can maintain the normal phenotype.

**SERCA2A Isoform Is Critical for Cardiac Function**

The SERCA2 gene encodes two different protein isoforms: SERCA2a (muscle-specific) and 2b, the ubiquitous isoform. SERCA2a differs from 2b at the C-terminal end and has a higher rate of Ca\(^{2+}\) transport compared to 2b. Ver Heyen et al.\(^{102}\) investigated whether this isoform specificity is required for muscle and nonmuscle function. Gene targeting in mice successfully disrupted the splicing mechanism responsible for generating the SERCA2a isoform. Homozygous SERCA2a\(^{-/-}\) mice displayed a complete loss of SERCA2amRNA and protein resulting in a switch to the SERCA2b isoform. The expression of SERCA2b mRNA and protein in hearts of SERCA2a\(^{-/-}\) mice corresponded to only 50% of wildtype SERCA2 levels. The SERCA2a\(^{-/-}\) mice showed an increase in embryonic and neonatal mortality (40% died), with histopathologic evidence of major cardiac malformations. In those animals that survived to adulthood the hearts showed mild concentric hypertrophy with impaired in vivo relaxation and contraction kinetics, indicating that SERCA2b can only partially substitute for SERCA2a. Ca\(^{2+}\) uptake levels in SERCA2a\(^{-/-}\) cardiac homogenates were reduced by \(-50\%\) and, in isolated cells, relaxation and Ca\(^{2+}\) removal by the SR were significantly reduced. These data indicate that the muscle-specific SERCA2a isoform is essential for normal cardiac devel-
operation and for the cardiac contraction–relaxation cycle.

SERCA Pump Expression and Activity Are Altered in Heart Disease. The importance of the SERCA pump in heart failure has been studied extensively in both animal models of heart failure and in human hearts using explant heart tissues following heart transplants. These studies have shown that SR calcium transport is decreased in heart failure (reviewed elsewhere). SERCA2a mRNA and protein levels are decreased in failing hearts from patients with endstage heart failure of different etiologies. In some studies the expression levels of SERCA were unaltered, whereas in others only an alteration in PLB phosphorylation status was reported. There is considerable heterogeneity in the expression level of SERCA in failing hearts, which may depend on a variety of factors including methodological differences, severity of the disease, drug treatment, age, and gender. Thus, it is difficult to arrive at a consensus from human studies. A common finding is that heart failure is associated with decreased SR Ca\(^{2+}\) uptake and SR Ca\(^{2+}\) content, suggesting that altered Ca\(^{2+}\) transport plays a role in the pathophysiology of heart failure.

SERCA GENE THERAPY CAN BE USED TO TREAT CARDIAC DISEASE AND RESTORE HEART FUNCTION

The use of the SERCA pump as a therapeutic agent to rescue muscle function, specifically in cardiac muscle, has been tried both in vitro and in vivo in animal models. These studies employed adenoviral gene transfer into cardiac myocytes. Del Monte et al. showed that overexpression of SERCA2a in human ventricular myocytes (from patients with endstage heart failure) can increase SERCA pump activity and enhance contraction and relaxation velocity. More recently, a catheter-based technique was developed to transfer adeno- viral SERCA2a into live animals. For this study, an animal model of pressure-overload hypertrophy in transition to failure with reduced SERCA2a levels was used. Overexpression of SERCA2a by gene transfer in vivo restored contractile function to normal levels and normalized the levels of PCR and ATP. The finding that SERCA2a gene transfer improved cardiac energetics was somewhat surprising because an increase in Ca\(^{2+}\) ATPase activity would increase ATP hydrolysis and potentially contribute to the depletion of CrP levels. It could be argued that an increase in SERCA should decrease diastolic calcium and alleviate the pathological effects of Ca\(^{2+}\) overload, including the activation of pathways leading to hypertrophy and apoptotic cell death. The benefits of SERCA2a gene transfer to rescue function in a model of ischemia reperfusion injury was also examined. SERCA2a overexpression by adenoviral gene transfer significantly decreased ventricular arrhythmias during ischemia and reperfusion and 24 h later, and significantly reduced infarct size and decreased thickening of the anterior wall. These data suggested that the decrease in infarct size is most likely due to the survival of cardiomyocytes in the infarcted myocardium. Therefore, restoring Ca\(^{2+}\) transport by increasing the SERCA level is critical for maintaining cardiac contractility and eliminating the pathological effects of Ca\(^{2+}\) overload.

SUMMARY AND CONCLUSIONS

The SERCA pump is encoded by a highly conserved multigene family whose function is to transport Ca\(^{2+}\) ions and regulate intracellular calcium homeostasis. Their presence is ubiquitous and they are the only mechanism to maintain SR/ER calcium stores in muscle and nonmuscle cells. The SERCA pump regulates the cytosolic calcium level tightly and controls many cellular functions, from muscle contraction to cell signaling responsible for growth and differentiation. The diversity of SERCA pump isoforms and pump expression level imparts tissue- and cell-specific Ca\(^{2+}\) transport capacity. Furthermore, this diversity can be better understood in skeletal muscle, where the SERCA1 isoform with faster kinetics is expressed several-fold higher in fast-twitch skeletal muscle, whereas SERCA2a is expressed at a lower level in cardiac and slow-twitch skeletal muscle. Only the SERCA1 gene shows high tissue specificity, whereas SERCA2a isoforms are expressed in many cell types. The SERCA2b isoform has a higher calcium affinity and is expressed at low levels in nonmuscle cells where intracellular calcium ion concentration is low. Often there is considerable overlap between SERCA2b and 3 isoforms. The differences in the function of individual SERCA pump isoforms can be further amplified by their cellular environment and the presence of SERCA regulatory proteins such as PLB and SLN. These inhibitors provide additional regulation of SERCA pump activity and contribute to Ca\(^{2+}\) homeostasis in cardiac and skeletal muscle. Altered SERCA expression in aging and heart failure suggests that the SERCA pump may play important roles not only in muscle physiology, but in the evolution of pathology. In conclusion, SERCA pump isoforms have evolved and adapted to specialized functions in different cellular environ-
ments and alterations in their activity and expression can affect many cellular functions, including skeletal and cardiac muscle physiology.

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REFERENCES


ABSTRACT: Ataxia and peripheral neuropathy are the most common neurological manifestations of gluten sensitivity. Myopathy is a less common and poorly characterized additional neurological manifestation of gluten sensitivity. We present our experience with 13 patients who presented with symptoms and signs suggestive of a myopathy and in whom investigation led to the diagnosis of gluten sensitivity. Three of these patients had a neuropathy with or without ataxia in addition to the myopathy. The mean age at onset of the myopathic symptoms was 54 years. Ten patients had neurophysiological evidence of myopathy. Inflammatory myopathy was the most common finding on neuropathological examination. One patient had basophilic rimmed vacuoles suggestive of inclusion-body myositis. Six patients received immunosuppressive treatment in addition to starting on a gluten-free diet; five improved and one remained unchanged. Among seven patients not on immunosuppressive treatment, four showed clinical improvement of the myopathy with a gluten-free diet. The improvement was also associated with reduction or normalization of serum creatine kinase level. The myopathy progressed in one patient who refused the gluten-free diet. Myopathy may be another manifestation of gluten sensitivity and is likely to have an immune-mediated pathogenesis. A gluten-free diet may be a useful therapeutic intervention.

Myopathy and Gluten Sensitivity

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Gluten sensitivity is a state of heightened immunological responsiveness to ingested gluten in genetically susceptible individuals.10 It represents a spectrum of diverse manifestations, of which gluten-sensitive enteropathy is one. The term “celiac disease” should now be restricted to gluten-sensitive enteropathy characterized by the triad of villous atrophy, crypt hyperplasia, and increase in intraepithelial lymphocytes on histological study of the small bowel. Other manifestations of gluten sensitivity include dermatopathy (dermatitis herpetiformis) and neurological disorders (e.g., gluten ataxia, neuropathy).8 Neurological manifestations can occur even in the absence of an enteropathy.9

A review of all published studies from 1964 to 2000 (single and multiple case reports) of 83 patients with established celiac disease who then developed neurological illness showed that the most common neurological manifestations were ataxia (29 patients) and peripheral neuropathy (29 patients).10

The third most common entity was myopathy (13 patients). Myopathy in the context of gluten sensitivity has not been well characterized. Muscle involvement in the context of gluten sensitivity might be predicted given that antibodies against endomysium are the most sensitive markers for the presence of enteropathy seen in a subgroup of patients with gluten sensitivity (i.e., patients with celiac disease). The antigen that endomysium antibodies recognize is tissue transglutaminase type 2 (TG2).7

We now report the clinical, neurophysiological, and neuropathological characteristics of a series of 13 patients with gluten sensitivity who presented with
symptoms and signs of myopathy. Investigation led
to the diagnosis of gluten sensitivity. We also report
the effect of treatment with a gluten free-diet with
and without additional immunosuppressive treat-
meth.

METHODS

The 13 patients were part of a cohort of 300 patients
referred to and attending our gluten sensitivity/neu-
rology clinic over the last 12 years. All 13 patients
presented to a general neurology clinic primarily
with symptoms and signs of myopathy. Some had, in
addition, other neurological symptoms and signs
(e.g., ataxia, peripheral neuropathy). All patients
were extensively investigated for the underlying
cause of myopathy and the only potential cause
found was that of gluten sensitivity. Only one of the
patients already had a diagnosis of celiac disease
prior to their neurological presentation. In the re-
mainin 12, the diagnosis of gluten sensitivity was
the result of investigating the cause of the myopathy.
Following the diagnosis of gluten sensitivity, all pa-
tients were followed regularly at the gluten sensitiv-
ity/neurology clinic.

Nerve conduction studies and electromyography
(EMG) were performed at least once in all patients.
In most instances nerve conduction studies were
performed as follows: sensory studies in median and
sural nerves; and motor studies in median and per-
oneal nerves. When needle EMG was performed, the
muscles usually examined were deltoid, abductor pollicis brevis or first dorsal interosseous, vastus me-
dialis, and tibialis anterior.

Following the diagnosis of gluten sensitivity on
serological grounds, all patients underwent duode-
nal biopsy. These were taken from the distal duode-
num using biopsy forceps, through a conventional
forward-viewing endoscope (Key-Med, Southend,
UK). Four biopsies were taken from the third part of
the duodenum. The presence of gluten-sensitive en-
teropathy was established by histological examina-
tion for evidence of crypt hyperplasia, villous atro-
phy, and increase in the intraepithelial lymphocytes.

All patients underwent needle or open muscle bi-
opsy at least once. A portion of the muscle biopsy was
frozen and cryostat sections prepared. The following
panel of stains and histochemistry was then performed:
hematoxylin and eosin; Engel’s trichrome; periodic
acid–Schiff; oil red O; acid phosphatase; nicotinamide
adenine dinucleotide–tetrazolium reductase (NADH-
TR); succinate dehydrogenase; and ATPases at acid
and alkaline pH for fiber typing. Remaining tissue was
fixed in formalin for the preparation of paraffin sec-
tions.

RESULTS

Clinical Characteristics. The clinical and serological
characteristics of all 13 patients are summarized in
Table 1. The male:female ratio was 5:8. The mean
age at onset of the myopathic symptoms was 54 years
(range, 16 to 76). Eight patients (62%) had predom-
inantly proximal weakness, four patients (31%) had
both proximal and distal weakness, and one patient
had primarily distal weakness. Two patients had
ataxia and neuropathy in addition to the myopathy,
and one patient had just neuropathy in addition to
the myopathy. The most common additional auto-
immune disease encountered in these patients was
hypothyroidism (2 patients). In addition, three pa-
tients had high thyroid antibody titers with normal
thyroid function tests. Only one patient was found to
be biochemically hypothyroid at the time of presen-
tation of the myopathy (patient 6). This patient was
the only patient known to have celiac disease prior to
presenting with proximal weakness. All patients had
circulating antigliadin antibodies and two patients
also had antiendomysium antibodies. Ten patients
had the HLA-DQ2 genotype, two the HLA-DQ8, and
one the HLA-DQ1. Serum creatine kinase (CK) level
ranged between normal and 4380 IU/L at presenta-
tion (normal, 25–190 IU/L). Enteropathy was iden-
tified following duodenal biopsy in six patients.

Neurophysiological Assessments. Sensory and mo-
tor nerve conduction studies were normal in 10 pa-
tients. Three patients (nos. 2, 7, and 9) had reduced
compound muscle action potentials in the lower
limb. EMG showed typical myopathic features (short-
duration, small-amplitude motor unit potentials) in
at least one proximal leg muscle in 10 patients (nos.
2–5, 7, 8 and 10–13). Fibrillation potentials were also
seen in two patients (nos. 4 and 12). Of the remain-
ing three patients, two showed only long-duration
motor unit potentials (nos. 1 and 9) and in one
patient (no. 6) EMG was entirely normal. Some pa-
tients had further neurophysiological assessments.
In patients 2, 7, 8, and 10, subsequent examinations
showed abnormalities of sensory nerve conduction
suggesting the development of axonal peripheral
neuropathy in addition to myopathy.

Neuropathology. Table 2 contains the details of the
neuropathological examination of muscle in all 13
patients. The most common finding was that of an
inflammatory myopathy with a polymyositis-like pic-
tage (6 patients). One patient (no. 2) also had evidence of basophilic rimmed vacuoles compatible with the diagnosis of inclusion-body myositis. The remaining patients had non-specific myopathic changes including internal nuclei, rounded atrophic fibers, and patchy staining loss for oxidative enzyme activity. None of the biopsies showed changes of neurogenic atrophy.

**Effect of Gluten-Free Diet.** It is difficult to assess in isolation the effect of a gluten-free diet on the myopathy in those six patients (nos. 2–4, 9, 11, and 12) who also received immunosuppressive treatment. Overall, five of these six patients improved and one (no. 11) remained clinically unchanged but with normal serum CK. Of particular interest is the patient with inclusion-body myositis (no. 2). He refused a gluten-free diet initially. Further assessment because of clinical deterioration showed the development of sensory axonal neuropathy. The neuropathy improved while on gluten-free diet alone within 1 year of strict adherence to the diet. Subsequent introduction of prednisolone and azathioprine and continuation of a strict gluten-free diet resulted in normalization of the serum CK and improvement in muscle strength.

Of the seven patients who did not receive immunosuppressive treatment, one patient (no. 6) was already on the gluten-free diet at the time of presentation when she was found to have hypothyroidism. She was the only patient with a preceding diagnosis of gluten-sensitive enteropathy when she developed myopathy. Her adherence to the diet was assessed serologically and was deemed strict as circulating gliadin, endomysium, and transglutaminase antibodies were negative. Her symptoms improved with the introduction of thyroxine, with normalization of serum CK (from 459 to 157 IU/L; normal, 25–190 IU/L). She still has some mild residual proximal weakness.

Another patient (no. 13) was also found to have low levels of vitamin D. There was no evidence of enteropathy on biopsy. He is currently on vitamin D

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**Table 1. Clinical and serological characteristics of the 13 patients with myopathy associated with gluten sensitivity.**

<table>
<thead>
<tr>
<th>Case</th>
<th>Age at onset/gender (duration in years)</th>
<th>Pattern of weakness</th>
<th>Other neurological findings</th>
<th>Additional autoimmune or other diseases</th>
<th>Gluten-related positive serological findings</th>
<th>HLA type</th>
<th>Serum CK* (IU/L)</th>
<th>Duodenal biopsy</th>
<th>Immunosuppressive treatment (on gluten-free diet)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38/M (15)</td>
<td>Global</td>
<td>None</td>
<td>High thyroid antibodies</td>
<td>IgG antigliadin</td>
<td>DQ8</td>
<td>2636</td>
<td>Normal</td>
<td>None (yes)</td>
<td>Improved</td>
</tr>
<tr>
<td>2</td>
<td>64/M (14)</td>
<td>Distal</td>
<td>Neuropathy</td>
<td>High thyroid antibodies</td>
<td>IgG, IgA antigliadin, endomysium</td>
<td>DQ2</td>
<td>959</td>
<td>Enteropathy</td>
<td>Azathioprine (yes)</td>
<td>Improved</td>
</tr>
<tr>
<td>3</td>
<td>49/F (18)</td>
<td>Global</td>
<td>Ataxia, neuropathy</td>
<td>None</td>
<td>IgG antigliadin</td>
<td>DQ2</td>
<td>4380</td>
<td>Enteropathy</td>
<td>Prednisolone (yes)</td>
<td>Improved</td>
</tr>
<tr>
<td>4</td>
<td>53/M (13)</td>
<td>Proximal</td>
<td>None</td>
<td>Inflammatory arthropathy</td>
<td>IgG, IgA antigliadin, endomysium</td>
<td>DQ2</td>
<td>221</td>
<td>Enteropathy</td>
<td>Methotrexate (yes)</td>
<td>Improved</td>
</tr>
<tr>
<td>5</td>
<td>67/F (8)</td>
<td>Proximal</td>
<td>None</td>
<td>Osteoporosis</td>
<td>IgG antigliadin</td>
<td>DQ2</td>
<td>1138</td>
<td>Normal</td>
<td>None (yes)</td>
<td>Improved</td>
</tr>
<tr>
<td>6</td>
<td>39/F (20)</td>
<td>Proximal</td>
<td>None</td>
<td>Hypothyroid</td>
<td>IgG, IgA antigliadin, endomysium</td>
<td>DQ2</td>
<td>459</td>
<td>Normal</td>
<td>None (yes)</td>
<td>Improved</td>
</tr>
<tr>
<td>7</td>
<td>72/F (10)</td>
<td>Global</td>
<td>Neuropathy ataxa</td>
<td>Hypothyroid</td>
<td>IgG, IgA antigliadin</td>
<td>DQ2</td>
<td>247</td>
<td>Normal</td>
<td>None (yes)</td>
<td>Improved</td>
</tr>
<tr>
<td>8</td>
<td>54/F (13)</td>
<td>Proximal</td>
<td>None</td>
<td>MGUS; low vitamin D</td>
<td>IgG antigliadin</td>
<td>DQ2</td>
<td>1156</td>
<td>Normal</td>
<td>None (no)</td>
<td>Progressed</td>
</tr>
<tr>
<td>9</td>
<td>67/F (8)</td>
<td>Proximal</td>
<td>None</td>
<td>None</td>
<td>IgG antigliadin</td>
<td>DQ2</td>
<td>56</td>
<td>Normal</td>
<td>Methotrexate (yes)</td>
<td>Improved</td>
</tr>
<tr>
<td>10</td>
<td>50/F (12)</td>
<td>Global</td>
<td>Neuropathy</td>
<td>None</td>
<td>IgG antigliadin</td>
<td>DQ8</td>
<td>108</td>
<td>Enteropathy</td>
<td>None (yes)</td>
<td>Improved</td>
</tr>
<tr>
<td>11</td>
<td>52/M (8)</td>
<td>Proximal</td>
<td>None</td>
<td>Large T-cell granular leukaemia</td>
<td>IgA antigliadin</td>
<td>DQ1</td>
<td>982</td>
<td>Normal</td>
<td>Methotrexate (yes)</td>
<td>Unchanged</td>
</tr>
<tr>
<td>12</td>
<td>76/F (3)</td>
<td>Proximal</td>
<td>None</td>
<td>High thyroid antibodies</td>
<td>IgA antigliadin</td>
<td>DQ2</td>
<td>446</td>
<td>Normal</td>
<td>Azathioprine (yes)</td>
<td>Improved</td>
</tr>
<tr>
<td>13</td>
<td>16/M (2)</td>
<td>Proximal</td>
<td>None</td>
<td>Low vitamin D</td>
<td>IgG, IgA antigliadin</td>
<td>DQ2</td>
<td>330</td>
<td>Normal</td>
<td>None (no)</td>
<td>Unchanged</td>
</tr>
</tbody>
</table>

*Normal: 25–190 IU/L.

MGUS, monoclonal gammopathy of uncertain significance.
supplementation without clinical improvement. He has refused to go on a gluten-free diet.

The four remaining patients (nos. 1, 5, 7, and 10) adopted strict gluten-free diet without additional immunosuppressive treatment. Myopathic symptoms improved in all of these cases. In patient 1 there was a significant reduction of serum CK following the introduction of a gluten-free diet, with evidence of elimination of antigliadin antibodies (2636 down to 1340 IU/L). The serum CK gradually increased to 4100 IU/L following re-exposure to gluten. The patient is currently on a gluten-free diet and remains asymptomatic but with serum CK elevated to just under 1000 IU/L. Patient 5 also improved significantly following the introduction of a gluten-free diet. Her serum CK was 1086 IU/L at presentation but normalized within a year of commencing the gluten-free diet.

Table 2. Neuropathological findings on muscle biopsy.

<table>
<thead>
<tr>
<th>Case</th>
<th>Fiber atrophy</th>
<th>Architectural abnormalities</th>
<th>Oxidative enzyme pattern</th>
<th>Inflammation</th>
<th>Interstitium</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>Internal nuclei</td>
<td>Normal</td>
<td>None</td>
<td>Normal</td>
<td>Myopathic changes</td>
</tr>
<tr>
<td>2</td>
<td>Rounded and angular atrophy*</td>
<td>Basophilic rimmed vacuoles, internal nuclei, fiber splitting</td>
<td>Patchy loss of reactivity, some core-like areas</td>
<td>Endomysial chronic inflammatory cell infiltrate</td>
<td>Fine fibrosis</td>
<td>Inclusion-body myositis</td>
</tr>
<tr>
<td>3</td>
<td>Atrophic fibers; type 2 predominant</td>
<td>Internal nuclei</td>
<td>Moth-eaten fibers</td>
<td>Endomysial chronic inflammatory cell infiltrate</td>
<td>Normal</td>
<td>Inflammation†</td>
</tr>
<tr>
<td>4</td>
<td>Rounded atrophic fibers*</td>
<td>Internal nuclei, prominent hypertrophic fibers</td>
<td>Patchy staining loss</td>
<td>Endomysial chronic inflammatory cell infiltrate</td>
<td>Fibrosis</td>
<td>Inflammation†</td>
</tr>
<tr>
<td>5</td>
<td>Rounded atrophic fibers*</td>
<td>Internal nuclei</td>
<td>Patchy staining loss</td>
<td>Endomysial chronic inflammatory cell infiltrate</td>
<td>Focal fibrosis</td>
<td>Inflammation†</td>
</tr>
<tr>
<td>6</td>
<td>Rounded atrophic fibers*</td>
<td>Internal nuclei</td>
<td>Patchy staining loss</td>
<td>Endomysial chronic inflammatory cell infiltrate</td>
<td>Focal fibrosis</td>
<td>Myopathic changes</td>
</tr>
<tr>
<td>7</td>
<td>Rounded atrophic fibers*</td>
<td>Internal nuclei</td>
<td>Coarse pattern</td>
<td>Endomysial chronic inflammatory cell infiltrate</td>
<td>Fibrosis, adipose replacement</td>
<td>Inflammation†</td>
</tr>
<tr>
<td>8</td>
<td>Rounded atrophic fibers; nuclear clusters</td>
<td>None</td>
<td>Coarse pattern, lobulated fibers, patchy staining loss</td>
<td>None</td>
<td>Normal</td>
<td>Myopathic changes</td>
</tr>
<tr>
<td>9</td>
<td>Rounded atrophic fibers</td>
<td>None</td>
<td>Coarse architecture</td>
<td>None</td>
<td>Normal</td>
<td>Myopathic changes</td>
</tr>
<tr>
<td>10</td>
<td>Atrophic fibers</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Normal</td>
<td>Myopathic changes</td>
</tr>
<tr>
<td>11</td>
<td>Rounded atrophic fibers; type 2 predominant</td>
<td>None</td>
<td>Internal nuclei</td>
<td>Endomysial chronic inflammatory cell infiltrate</td>
<td>Focal fibrosis</td>
<td>Inflammation†</td>
</tr>
<tr>
<td>12</td>
<td>Rounded atrophic fibers*</td>
<td>Internal nuclei</td>
<td>Patchy staining loss</td>
<td>Endomysial chronic inflammatory cell infiltrate</td>
<td>Focal fibrosis</td>
<td>Inflammation†</td>
</tr>
<tr>
<td>13</td>
<td>Atrophic fibers; type 1 predominance</td>
<td>None</td>
<td>Normal</td>
<td>None</td>
<td>Normal</td>
<td>Myopathic changes</td>
</tr>
</tbody>
</table>

*Not type selective; †polymyositis-like pattern.

supplementation without clinical improvement. He has refused to go on a gluten-free diet.

The four remaining patients (nos. 1, 5, 7, and 10) adopted strict gluten-free diet without additional immunosuppressive treatment. Myopathic symptoms improved in all of these cases. In patient 1 there was a significant reduction of serum CK following the introduction of a gluten-free diet, with evidence of elimination of antigliadin antibodies (2636 down to 1340 IU/L). The serum CK gradually increased to 4100 IU/L following re-exposure to gluten. The patient is currently on a gluten-free diet and remains asymptomatic but with serum CK elevated to just under 1000 IU/L. Patient 5 also improved significantly following the introduction of a gluten-free diet. Her serum CK was 1086 IU/L at presentation but normalized to 51 IU/L while following a gluten-free diet, with serological evidence of elimination of antigliadin antibodies. Subsequent elevations of serum CK up to 727 IU/L were associated with positive antigliadin and tissue transglutaminase antibodies as a result of a less strict gluten-free diet.

Patient 7 presented with falls and unsteadiness. Over a period of 7 years she progressed and developed difficulty in walking up and down stairs. Examination revealed cerebellar dysarthria, gait ataxia, and global weakness. She had evidence of cerebellar atrophy on computerized tomography scan and inflammatory myopathy on muscle biopsy. She refused steroid treatment. She started the gluten-free diet in 2002 with slow but definite improvement in muscle strength and ataxia. Her serum CK normalized within a year of commencing the gluten-free diet.
Patient 10 presented with painful muscles and proximal weakness. Initial neurophysiological assessment was suggestive of myopathy. Repeat assessment several years later showed evidence of a sensory neuropathy in addition to the myopathic abnormalities. At this stage she went onto a gluten-free diet. She has experienced complete resolution of all her neurological symptoms over a period of 2 years on a strict gluten-free diet, with elimination of antigliadin antibodies. Repeat neurophysiological assessment 4...
years after the introduction of a gluten-free diet showed resolution of the neuropathy. EMG was not performed on this occasion.

**DISCUSSION**

The spectrum of abnormalities seen on muscle histology in these 13 patients suggests that gluten sensitivity–associated myopathy may have diverse pathogenic mechanisms but that muscle inflammation is a common feature.

The landmark publication by Cooke et al. on the neurological manifestations in patients with established celiac disease was followed by the publication of a pathological study by the same investigators providing information on muscle and nerve biopsies from 11 of the patients previously described.4,5 The muscle abnormalities reported were predominantly secondary to nerve degeneration as all patients had severe neuropathies. A subsequent study from Sweden15 reported that, of 76 patients with suspected polymyositis investigated at a neuromuscular unit, 17 had a history of gastrointestinal symptoms and signs together with evidence of malabsorption. Fourteen of these patients fulfilled the diagnostic criteria for polymyositis and, of these, five were diagnosed with celiac disease. Not all of the 76 patients, however, were specifically screened for the presence of gluten sensitivity. Given that gastrointestinal symptoms are only present in one of eight patients with celiac disease, it is difficult to derive a prevalence figure of gluten sensitivity in patients with polymyositis based on this report. In our experience with 300 patients presenting with neurological manifestations of gluten sensitivity over the last 12 years, myopathy is an uncommon manifestation compared with ataxia or neuropathy, which are by far the most common neurological presentations of gluten sensitivity.

In some of our 13 cases, an alternative etiology for the myopathy was considered, such as hypothyroidism, vitamin D deficiency, or idiopathic polymyositis. An alternative etiology is likely in one of these patients based on the response of the myopathy to thyroxine supplementation with normalization of the serum CK. Nutritional osteomalacia (vitamin D deficiency) has been reported as a cause of proximal myopathy mainly in developing countries.16 Vitamin D deficiency in the context of gluten sensitivity has been attributed to malabsorption, but neither of our two patients with vitamin D deficiency had any evidence of enteropathy on biopsy nor did they have any other features to suggest malabsorption. It is also unlikely that vitamin D deficiency was the cause of their myopathy because vitamin D supplementation was not associated with clinical improvement.

It is plausible that gluten sensitivity in some of these patients was coincidental, perhaps reflecting the coexistence of more than one autoimmune disease (e.g., idiopathic polymyositis or inclusion-body

**FIGURE 3.** Muscle fiber showing basophilic rimmed vacuoles (patient 1).
Myositis and gluten sensitivity). It is impossible to prove a causal association in those patients who received immunosuppressive treatment in addition to adopting a gluten-free diet. In favor of myopathy being another rare manifestation of gluten sensitivity is the experience of the four patients from these series whose myopathy appears to have responded to a gluten-free diet without the use of immunosuppressive medication. This clinical improvement was associated with reduction or normalization of the serum CK and, in two cases, clinical deterioration following gluten challenge.

One of our patients with inclusion-body myositis had a neuropathy that responded to a gluten-free diet; the myopathy appeared responsive to the subsequent use of immunosuppressive treatment with prednisolone and azathioprine.

Sporadic inclusion-body myositis (IBM) is the most common cause of myopathy in patients over 50 years of age. The neurological manifestations of gluten sensitivity are also commonly seen in patients over the age of 50 years. The etiology of IBM remains obscure but may well have an autoimmune basis. Sporadic IBM is associated with HLA-DQ2 in 79% of cases. HLA-DQ2 has a strong association with other autoimmune diseases and is found in 90% of patients with celiac disease. It has been suggested that a substantial proportion (>50%) of cases labeled as having idiopathic polymyositis may prove to have IBM after re-evaluation with repeat biopsies. We cannot therefore exclude the possibility that more of our patients with gluten sensitivity and a polymyositis-like picture may have sporadic IBM. The strong HLA-DQ2 association and the shared autoimmune pathogenesis suggest that screening for gluten sensitivity should be considered in all patients with IBM but also in patients with otherwise unexplained myopathies. This is particularly important based on our experience that such a subgroup of patients with gluten sensitivity and IBM may respond to a gluten-free diet with or without immunosuppressive treatment. Supporting evidence for this also comes from a case report of a patient with celiac disease, neuropathy, ataxia, and IBM showing clinical and histological improvement of all the neurological manifestations, including the muscle histology following introduction of a gluten-free diet.

The mechanism of muscle damage in these cases remains unclear but is likely to have an immunological basis as is the case in other extraintestinal manifestations of gluten sensitivity. Antibodies against endomysium are the most specific markers of gluten-sensitive enteropathy. Antigliadin antibodies, however, are the most sensitive markers of the whole spectrum of gluten sensitivity. The autoantigen in gluten-sensitive enteropathy has been shown to be TG2. This is the antigen recognized by endomysium antibodies. Transglutaminases are a group of calcium-dependent intracellular enzymes that catalyze the cross-linking of glutamine donor proteins such as gliadins. TG2 is present in normal muscle and tends to be restricted to some endomysial connective tissue elements. The expression of TG2 is increased fourfold in the muscle of patients with IBM, perhaps reflecting increased participation in the formation of insoluble amyloid deposits in the muscles. Another study comparing TG2 expression in other types of inflammatory myopathies (polymyositis and dermatomyositis), normal muscle, and muscle from patients with Duchenne muscular dystrophy demonstrated TG2 expression to be increased only in patients with inflammatory myopathies.

Additional evidence to suggest that muscle can be one of the extraintestinal target organs in gluten sensitivity comes from recent work demonstrating the presence of IgA deposits against TG2 in the small bowel of patients with gluten sensitivity early in the disease course. Such deposits have been demonstrated in extraintestinal target sites such as muscle in a patient with celiac disease and myopathy and cerebral tissue in a patient with gluten ataxia. It therefore appears that transglutaminases play a central role in the pathogenesis of both intestinal and extraintestinal manifestations of gluten sensitivity.

It is as yet unclear what triggers muscle dysfunction in only a small proportion of patients with gluten sensitivity. It has been proposed that the type of transglutaminase may be the key factor determining the type of manifestation seen in the context of gluten sensitivity, for example, enteropathy, dermatomyositis, or neurological manifestations.

We conclude that myopathy may be a manifestation of gluten sensitivity and may have an immunemediated pathogenesis. Patients presenting with myopathy should be screened for the possibility of gluten sensitivity. For those patients with gluten sensitivity and myopathy, a gluten-free diet should be considered as a therapeutic intervention irrespective of the use of immunomodulation.

We thank the Sheffield Hospitals Charitable Trust for financial support.

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ABSTRACT: A detailed understanding of injury mechanisms in peripheral nerve fibers will help guide successful design of therapies for peripheral neuropathies. This study was therefore undertaken to examine the ionic mechanisms of Ca\(^{2+}\) overload in peripheral myelinated fibers subjected to chemical inhibition of energy metabolism. Myelinated axons from rat dorsal roots were co-loaded with Ca\(^{2+}\)-sensitive (Oregon Green BAPTA-1) and Ca\(^{2+}\)-insensitive (Alexa Fluor 594) dextran-conjugated fluorophores and imaged using confocal laser scanning microscopy. Axoplastic regions were clearly outlined by the Ca\(^{2+}\)-insensitive dye, from which axonal Ca\(^{2+}\)-dependent fluorescence changes \(F_{\text{Ca,ax}}\) were measured. Block of Na\(^{-}\)–K\(^{+}\) ATPase (ouabain), opening of Na\(^{+}\) channels (veratridine), and inhibiting energy metabolism (iodoacetate + NaN\(_3\)) caused a rapid rise in \(F_{\text{Ca,ax}}\) to 96% above control after 30 min. Chemical ischemia (iodoacetate + NaN\(_3\)) caused a more gradual increase in \(F_{\text{Ca,ax}}\) (54%), which was almost completely dependent on bath Ca\(^{2+}\), indicating that most of the Ca\(^{2+}\) accumulation occurred via influx across the axolemma. Na\(^{-}\)–Ca\(^{2+}\) exchange inhibition (KB-R7943) significantly reduced ischemic \(F_{\text{Ca,ax}}\) rise (18%). Together our results indicate that the bulk of Ca\(^{2+}\) overload in injured peripheral myelinated axons occurs via reverse Na\(^{-}\)–Ca\(^{2+}\) exchange, driven by axonal Na\(^{+}\) accumulation through voltage-gated tetrodotoxin-sensitive Na\(^{+}\) channels. This mechanism may represent a viable therapeutic target for peripheral neuropathies.


SOURCES OF AXONAL CALCIUM LOADING DURING IN VITRO ISCHEMIA OF RAT DORSAL ROOTS

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Accumulation of excess axoplasmic Ca\(^{2+}\) may be responsible for axonal damage associated with a number of types of injuries including anoxia, diabetes, and neurotoxic chemical exposure (reviewed elsewhere\(^{35}\)). The routes of Ca\(^{2+}\) accumulation in peripheral axons during ischemic injury are not fully elucidated, although past studies have revealed that during anoxia, reverse operation of the axolemmal Na\(^{+}\)–Ca\(^{2+}\) exchanger\(^{13,19}\) plays an important role. Excess Ca\(^{2+}\) entry and subsequent calpain activation has also been shown to play a key role in the degeneration of axons following axotomy.\(^7\) Although a number of sources of deleterious Ca\(^{2+}\) have been identified for damaged central nervous system axons, comparable detailed studies have not been conducted for peripheral axons. In order to investigate mechanisms of Ca\(^{2+}\) entry in injured peripheral nervous system (PNS) axons, we studied the effects of in vitro application of various pharmacological agents during chemical ischemia (induced using iodoacetate and azide) in rat dorsal roots, while measuring axonal [Ca\(^{2+}\)] using confocal laser scanning microscopy.

MATERIALS AND METHODS

Experiments were performed in accordance with institutional guidelines for the care and use of animals. Adult Long–Evans male rats (200–250 g) were anesthetized using a 80% CO\(_2\)/20% O\(_2\) gas mixture.
and decapitated. Following laminectomy a 40-mm section of the lumbar spinal cord was removed and placed in an oxygenated ice-cold zero-Ca²⁺ artificial cerebrospinal fluid (aCSF). Several dorsal roots were gently cut away, then immersed into a zero-Ca²⁺ solution bubbled with 95% O₂/5% CO₂ at room temperature, containing Ca²⁺-insensitive Alexa Fluor 594 dextran 10,000 MW dye (100 μM) and the Ca²⁺ indicator Oregon Green 488 BAPTA-1 dextran, potassium salt, 10,000 MW (100 μM) (Invitrogen, Carlsbad, California). The roots were loaded for 1 h in the above solution and rinsed for 1 h in normal-Ca²⁺ aCSF using techniques well established in our laboratory, where it was shown that such loading stains axon cylinders in isolation. aCSF had the following composition (in mM): 126 NaCl, 3.0 KCl, 2.0 MgSO₄, 26 NaHCO₃, 1.25 NaH₂PO₄, 2.0 CaCl₂, and 10 dextrose (pH 7.4). Zero-Ca²⁺ aCSF had a composition similar to normal aCSF but with 2 mM MgCl₂ replacing 2 mM CaCl₂, and with the addition of 0.75 mM 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA). The tissue was transferred to a custom-built perfusion chamber on a Nikon C1 confocal microscope and imaged every 60 s at 37°C with a 60 1.0 NA dipping lens, itself maintained at 37°C. Chemical ischemia was induced with the glycolytic inhibitor iodoacetate (1 mM) together with NaN₃ (2 mM), a blocker of oxidative phosphorylation, which has been shown to be a reliable and reproducible model of ischemic injury. Pharmacological tests were performed by dissolving the agents [veratridine (in ethanol), ouabain, tetrodotoxin (TTX), KB-R7943 (2-[2-[4-(4-nitrobenzoyloxy)phenyl]ethyl]isothiourea methane-sulfonate), in dimethyl sulfoxide (DMSO) and nimodipine (in DMSO)] into aCSF from the indicated stock solutions as required, and perfusing dorsal roots 15–20 min prior to the start of the ischemic insult.

Individual axonal regions of interest were selected by first manually outlining individual axons (an example is shown in Fig. 1A,B), then setting a threshold based on the strong Alexa Fluor 594 emission (Fig. 1A) to select all pixels above a certain value and enclosed within the selection outline; in this way the axoplasm from a single axon is selected for analysis. From such regions the corresponding Ca²⁺-dependent Oregon Green 488 BAPTA-1 fluorescence was measured. Because emission intensities of the two dyes differed significantly (10–20-fold), fluorescence from each channel was first normalized to baseline values at time 0, then a ratio of normalized green emission against normalized red fluorescence was calculated to compensate for minor signal

![Image](https://example.com/image.png)

**FIGURE 1.** Live myelinated axons from rat dorsal roots were loaded with Alexa Fluor 594 (A), and the Ca²⁺-sensitive Oregon Green BAPTA-1 (B) (both dextran conjugates). An example of a selection outline illustrates how individual axons were chosen for analysis (see Materials and Methods for details). The contrast of these two panels was adjusted for presentation, but quantitative fluorescence analyses were performed on raw unadjusted image data. (C) Mean relative Ca²⁺-dependent fluorescence (ΔF/F₀) from axonal regions expressed as ΔF/F₀ plotted against time. At time 0, dorsal roots were exposed to a combination of ouabain, veratridine, iodooacetate, and NaN₃, designed to poison metabolism and promote Na⁺ and Ca²⁺ accumulation. A large and rapid rise in F_Ca,ax was observed within minutes, confirming our ability to reliably measure axonal Ca²⁺ changes with this method.
losses over time due to bleaching and dye loss (typically <20% after 1 h). Background fluorescence was typically 10 times less than signal in regions of interest, and changed by <5% during any experiment, and therefore could be safely ignored from the calculations. All fluorescence changes cited here were analyzed in this manner using ImageTrak software written by P.K.S. (http://www.ohri.ca/stys/imagetrik). An example of normalized, corrected Ca\(^{2+}\)-dependent fluorescence increase in a poisoned nerve is shown in Figure 1C. Statistical differences within a single treatment group were calculated using paired \(t\) tests, and differences between treatment groups were determined using analysis of variance (ANOVA) with Tukey’s HSD test for multiple comparisons. All errors are reported as standard deviations.

Compound resting membrane potential measurements in normoxic dorsal root axons were measured at 37°C using the grease gap technique as previously described. All drugs were from Sigma Chemical (St. Louis, Missouri) except KB-R7943, which was purchased from Tocris Bioscience (Ellisville, Missouri).

RESULTS

In order to determine whether our recording paradigm could sense a rise in axoplasmic [Ca\(^{2+}\)], we first tested whether a severe insult that was expected to increase [Ca\(^{2+}\)] significantly would be detected as an increase in Oregon Green 488 BAPTA-1 emission intensity. Figure 1A shows a sample image of a dorsal root with axons outlined by the fluorescence of Alexa Fluor 594 dextran. As expected, green fluorescence is very weak in this healthy resting tissue. Dorsal roots were exposed to a combination of veratridine (50 µM) to open Na\(^+\) channels, allowing depolarization and Na\(^+\) entry,\(^2\) Na\(^+\)-K\(^+\) ATPase inhibition with ouabain (1 mM), and chemical ischemia (iodoacetate and the glycolytic inhibitor iodoacetate and the mitochondrial poison sodium azide (NaN\(_3\)), induced a rise in \(F_{\text{Ca,ax}}\) that began almost immediately but was more gradual compared to the mixture shown in Figure 1. The time course of \(F_{\text{Ca,ax}}\) change vs. time is shown in Figure 2A. After 30 min of ischemia, \(F_{\text{Ca,ax}}\) rose by 54 ± 36% above preischemic baseline (\(n = 38\) axons, \(P < 0.0001\), paired two-sample \(t\) test), and continued to rise gradually for the nearly 60 min that the experiment was continued. Addition of DMSO to the perfusing solutions did not alter the \(F_{\text{Ca,ax}}\) response to ischemia (\(F_{\text{Ca,ax}}\) increase at 30 min OGD: 54 ± 39% without vs. 52 ± 39% with 0.05% DMSO); the results using normal and DMSO-containing aCSF were therefore pooled.

To test the contribution of extracellular Ca\(^{2+}\) to the observed ischemic rise of \(F_{\text{Ca,ax}}\), Ca\(^{2+}\) was removed from the bathing solution (with the addition of 0.75 mM BAPTA to chelate any residual Ca\(^{2+}\)) and the roots exposed to this medium for 5–10 min before chemical ischemia was applied. After 30 min of ischemia in 0Ca\(^{2+}\)/BAPTA perfusate, there was no significant rise in \(F_{\text{Ca,ax}}\) (9 ± 20% increase vs. control in Ca\(^{2+}\)-containing aCSF, \(n = 28\), paired \(t\) test); the minimal increase in \(F_{\text{Ca,ax}}\) at 30 min in Ca\(^{2+}\)-free bath was significantly less than in Ca\(^{2+}\)-containing solution (\(P < 0.0001\)). A typical time course of \(F_{\text{Ca,ax}}\) is shown in Figure 2B. In most experiments, switching to 0Ca\(^{2+}\)/BAPTA perfusate resulted in a small drop of \(F_{\text{Ca,ax}}\), suggesting a reduction of resting axoplasmic Ca\(^{2+}\) by the Ca\(^{2+}\)-free bathing solution. A minimal reversal of this depletion was observed as ischemia progressed, but \(F_{\text{Ca,ax}}\) neither fell below nor rose above baseline in a significant manner at any timepoint. Continuing ischemia, but reintroducing Ca\(^{2+}\)-replete perfusate after 30 min, caused a rapid increase in \(F_{\text{Ca,ax}}\), suggesting that conditions promoting Ca\(^{2+}\) entry (likely a rise in axonal [Na\(^+\)]) had accumulated during the initial ischemic phase and were now poised to admit Ca\(^{2+}\) rapidly into the axon after reintroduction of this ion into the perfusate.

In optic nerve and dorsal column anoxia, TTX-sensitive voltage-gated Na\(^+\) channels play an important role in the series of events leading to axonal Ca\(^{2+}\) accumulation and functional injury. Here, dorsal roots were treated with 1 µM TTX during ischemia to block voltage-gated Na\(^+\) channels and axoplasmic [Ca\(^{2+}\)] was measured. Figure 2C shows the typical time course of \(F_{\text{Ca,ax}}\) changes. After 30 min of in vitro ischemia in the presence of TTX, \(F_{\text{Ca,ax}}\) increased to 14 ± 28% (\(n = 79\)), a significant reduction from the rise observed in the absence of TTX (\(P < 0.0001\)). In addition to Na\(^+\) channels, voltage-gated Ca\(^{2+}\) channels have been implicated in the axonal degeneration that occurs in response to axotomy. We examined the contribution of L-type voltage-gated Ca\(^{2+}\) channels in the ischemic Ca\(^{2+}\) rise in dorsal root axons by treating...
with the dihydropyridine nimodipine (10 μM), a specific blocker of L-type Ca\(^{2+}\) channels.\(^6\) At the end of 30 min of ischemia, \(F_{Ca,ax}\) increased by 60 ± 54% above baseline (\(n = 23\) axons), which is not different from drug-free ischemia. In contrast, KB-R7943 (10 μM), a blocker of Na\(^+-Ca^{2+}\) exchange,\(^10\) with additional inhibitory actions at L-type Ca\(^{2+}\) channels,\(^29\) significantly reduced ischemic rise of \(F_{Ca,ax}\) at 30 min (18 ± 26% increase above baseline, \(n = 29\) axons vs. 54 ± 36% in drug-free aCSF, \(P < 0.0001\)). The axonal Ca\(^{2+}\) rise in KB-R7943 was significantly reduced compared to the nimodipine-treated group (\(P < 0.0001\)), but was not different from measurements obtained in 0Ca\(^{2+}\)-CSF.

A bar graph summarizing \(F_{Ca,ax}\) changes at 30 min of ischemia as a function of the various treatments is shown in Figure 3. To exclude an unexpected nonspecific effect of KB-R7943 on persistent Na\(^+\) conductance in axons, which could, like TTX, reduce the ischemic Ca\(^{2+}\) rise indirectly, membrane potential measurements were performed in resting dorsal roots with the addition of this agent (Fig. 4). No hyperpolarizing shift in resting potential was noted (\(n = 4\) roots). In contrast, the Na\(^+\)-channel blocking anesthetic lidocaine caused a detectable hyperpolarization, which is indicative of a resting persistent Na\(^+\) permeability.\(^27,36,41\)

**DISCUSSION**

Cellular Ca\(^{2+}\) overload is a common phenomenon in a variety of tissue injuries, and may indeed represent the “final common pathway” of cellular degeneration and necrosis.\(^31\) Peripheral nerve damage is a
frequent feature in many disorders such as diabetes, ischemia, trauma, and neurotoxicity; such insults may lead to complete disintegration of the axon via Wallerian degeneration, which exhibits a significant Ca-dependent component. Ca overload also leads to breakdown of axonal neurofilament structures and myelin damage. Taken together, these observations emphasize the central role that deregulation of axonal Ca homeostasis plays in irreversible damage to peripheral fibers.

Several reports have described fluorescence-based measurements of axonal Ca in response to electrical and chemical activation. In unmyelinated axons, for example, trains of action potentials elicit axonal Ca rises mainly via activation of voltage-sensitive Ca channels; depending on the preparation, these activity-dependent Ca transients are reduced by Cd, N-type or P/Q-type Ca channel-blocking conotoxins. In addition, certain chemical ligands (e.g., capsaicin, nicotine, and acetylcholine acting on nicotinic receptors) can promote Ca increases in unmyelinated fibers. In contrast, far less is known about the mechanisms of Ca entry into myelinated fibers. Central myelinated optic nerve axons exhibit Ca increases in response to action potential conduction, and although the buffering of such Ca transients depends partly on Na–Ca exchange, the mechanism of activity-dependent Ca increase is unclear.

Injured axons accumulate Ca in response to anoxia/ischemia or axotomy probably to a far greater extent than electrically activated fibers. After transection, axoplasmic Ca accumulation is a key event leading to fragmentation of the axonal cytoskeleton and Wallerian degeneration, with Ca entry proceeding through specific ion transporters (possibly voltage-gated Ca channels), rather than nonspecific leakage into the fiber through the breached axolemma. Using electron probe microanalysis, a more quantitative measurement of elemental deregulation in anoxic myelinated axons revealed that in both central and peripheral nervous systems, axoplasmic Ca increase is heavily dependent on Na influx through TTX-sensitive channels, and is reduced by pharmacological inhibition of the Na–Ca exchange. This closely parallels the improvement in functional recovery observed in anoxic optic nerve exposed to similar pharmacological manipulations.

In the present study, the slight ischemia-induced increase in Ca observed in the presence of TTX (Figs. 2C, 3) was not significant (P > 0.05, paired t-test), so that in our paradigm of chemical ischemia of peripheral myelinated axons, axonal Ca accumulation seemed totally dependent on TTX-sensitive Na channels, at least during the first 30 min.
Unblocked, Na⁺ channels may promote axonal Ca²⁺ rise either directly or indirectly. Voltage-gated Na⁺ channels exhibit some permeability to Ca²⁺ ions, with PCa/PNa ratios as high as ~0.1 in squid axons, but probably nearer to 0.01 in myelinated fibers. This tiny potential Ca²⁺ permeability, coupled with our observation that Na⁺–Ca²⁺ exchange block with KB-R7943 was as effective as TTX in reducing ischemic Ca²⁺ rise, argues strongly against any significant Ca²⁺ influx directly through Na⁺ channels, although a minor component cannot be excluded. Instead, the Ca²⁺ rise more likely secondary to Na⁺ influx through a nonactivating permeability component of TTX-sensitive channels shown to be present in peripheral axons.

Such Na⁺ influx can promote Ca²⁺ entry by at least two means. First, by allowing depolarization, voltage-gated Ca²⁺ channels may be activated. Our present results with nimodipine indicate that at least L-type voltage-gated Ca²⁺ channels did not play a significant role in mediating ischemic axonal Ca²⁺ overload, as nimodipine failed to reduce the ischemic Ca²⁺ rise (Fig. 3). A contribution of other non-L-type Ca²⁺ channels, as observed in unmyelinated axons, cannot be excluded, however. Alternatively, Na⁺ rise may cause Ca²⁺ increase by reverse Na⁺–Ca²⁺ exchange, or even indirectly by promoting neurotransmitter (e.g., glutamate) release via Na⁺-coupled transporters, which could then activate Ca²⁺ permeable, ligand-gated channels. The robust decrease of ischemic Ca²⁺ rise by KB-R7943 suggests that this Ca²⁺ transporter plays an important role in Ca²⁺ entry into ischemic dorsal root myelinated axons. This agent is nonspecific, with inhibitory effects on L-type Ca²⁺ channels, so that a minor parallel contribution from these cannot be excluded, although the negative result with nimodipine argues against this possibility.

The present data, obtained using fluorescent Ca²⁺ reporters and laser-scanning microscopy, offer the advantage of providing continuous information about [Ca²⁺] in real-time in live myelinated fibers. As with previous studies on electrically stimulated axons or those injured traumatically or by axonia, we found that dorsal root axons exposed to in vitro ischemia exhibit Ca²⁺ accumulation mainly from the extracellular space: removal of bath Ca²⁺ almost completely blocked ischemic Ca²⁺ increase, which was not statistically significantly elevated above preischemic control baseline (e.g., Fig. 2B). In some axons, preischemic Ca²⁺ fell slightly after the switch to 0Ca²⁺/BAPTA perfusate (before ischemia), suggesting a basal Ca²⁺ cycling across the axolemma, with steady-state axonal [Ca²⁺] dependent on a continuous, albeit slow, influx of this cation from the extracellular space. The small ischemia-induced rise in axonal Ca²⁺ (8% above baseline) in roots bathed in 0Ca²⁺/EGTA perfusate was not statistically significant; however, fluorescence changes were calculated as a mean pixel intensity change in a long (up to 150 µm) stretch of axon, so any localized release of Ca²⁺ from intracellular stores may have been averaged out. More detailed experiments will be required to establish whether peripheral myelinated axons, like their central counterparts, also exhibit significant Ca²⁺ elevations as a result of release from axoplasmic reticulum.

Such real-time Ca²⁺ imaging also allows analysis not only of temporal, but spatial changes in [Ca²⁺] along the axon; we did not observe any focal or nonuniform accumulation of Ca²⁺, arguing against a localized point of influx such as at nodes of Ranvier. This is consistent with a uniform appearance of Ca²⁺-dependent spectrin breakdown in anoxic optic nerve, which also suggests a more uniform, nonfocal influx of Ca²⁺ in injured CNS myelinated fibers. Nevertheless, because we acquired images every 60 s, it is still possible that focal Ca²⁺ increases occurred early and became nearly uniform through rapid diffusion along the axons, in which case we may have missed any early, focal Ca²⁺ increases.

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ABSTRACT: We conducted a sequential study of quantitative sensory testing (QST) during compression-induced conduction block of the median nerve to determine relative vulnerability of the small and large myelinated nerve fibers. We tested cold (CPT) and vibratory perception thresholds (VPT) of the third digit in 15 healthy subjects during constant, localized compression for 30 min of the median nerve at the wrist. The orthodromic sensory nerve action potentials (SNAPs) recorded at wrist and elbow served to monitor the degree of associated conduction block. After the onset of nerve compression, it took 16 min for CPT to show the first change; VPT remained normal for 26 min. CPT recovered 2 min later than VPT after release of compression. The SNAP amplitude at the wrist diminished immediately at the start of compression and declined progressively, whereas the response at the elbow remained the same initially, showing no latency change for 20 min. A nearly identical time course of SNAP changes in the two experiments justified the comparison of separately tested CPT and VPT as a measure of modality-specific vulnerability. Contrary to the common belief, a focal compression sufficient to produce rapidly reversible conduction abnormalities affects the slow-conducting small myelinated fibers mediating cold perception before the fast-conducting large myelinated fibers transmitting vibration perception. The data document the order of modality-specific vulnerability of sensory nerve fibers to mild compression. The finding suggests that testing CPT, rather than VPT, provides a better QST to delineate rapidly reversible symptoms induced by compression.


QUANTITATIVE SENSORY TESTING OF COLD AND VIBRATION PERCEPTION DURING COMPRESSION OF MEDIAN NERVE AT THE WRIST

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According to the commonly held notion, mechanical pressure applied to the nerve preferentially affects the fast-conducting large myelinated fibers before slow-conducting small myelinated fibers. 10, 23 It is not clear, however, whether this order of susceptibility also applies to a mild compression that produces rapidly reversible conduction abnormalities. In fact, a constant, localized compression of the median nerve initially gives rise to a substantial diminution in size of orthodromic compound sensory nerve action potentials (SNAPs) without a notable change in latency, as shown in our previous study. 32 This finding suggests a preferential vulnerability of slower, rather than faster, myelinated fibers to this type of nerve insult. To further test this hypothesis, we have now used quantitative sensory testing (QST) to study the time course of conduction abnormalities by sequential changes of SNAPs using a previously reported method. 32 Modality-specific sensory changes of cold and vibration perception should help elucidate the order of susceptibility between small (A-delta) and large myelinated sensory fibers (A-beta). 1, 3, 4, 22, 33

MATERIALS AND METHODS

Subjects. We studied orthodromic SNAPs in 15 healthy men who provided informed consent. Of these, three subjects each underwent QST of cold or
vibration perception. The remaining nine participated in the study of both modalities on two different days separated by 4–24 months (mean, 10 months). Thus, we analyzed a total of 12 subjects each for the study of cold perception (C-group; ages 22–34 years, mean, 27) and vibration perception (V-group; ages 21–28 years, mean, 26). We monitored the time course of SNAP changes to verify that the two groups underwent the same conduction abnormalities. The difference in time course of sensory testing would then be attributable to cold and vibration perception per se.

Nerve Compression. The subject lay supine on a table with his left forearm supinated on the base plate of a specially designed external compression device as described previously (Fig. 1). A constant load of 9.8 N was applied percutaneously to the median nerve 4 cm proximal to the distal crease of the wrist through a 10-mm diameter brass hemisphere, maintained for 30 min.

SNAP Study to Monitor Conduction Abnormalities. As detailed previously, we applied supramaximal electrical stimulation (0.1-ms duration, 2 Hz) with a pair of ring electrodes placed around the third digit, 2 cm apart (Dantec 13L 69; Dantec Medical, Skovlunde, Denmark). An 8-channel averager (Dantec Evomatic 8000) allowed simultaneous analysis of orthodromic SNAPs at the compression site, R(w), and at the elbow, R(e) (Fig. 1) after averaging 50 summated potentials recorded with a frequency response of 20 Hz to 5 kHz (3 dB down). The brass hemispheric compressor itself served as the active recording electrode at R(w), and an 11-mm diameter disk electrode (Dantec 13L 29) at R(e). At each recording site another disk electrode was placed 3 cm medially to the active electrode as the reference.

The SNAPs were recorded immediately after the start of compression, which served as the baseline, and at 2-min intervals thereafter during a 30-min period of constant compression. Recording continued for an additional 10 min after release of compression at R(e). The forearm skin temperature was maintained at 35 °C ± 0.5°C with a thermostatically controlled heat lamp coupled to a thermistor probe throughout the experiment. Measurements of SNAPs included: (1) latencies from the stimulus artifacts to the initial positive peaks, and (2) areas (voltage–time integral) of the initial-positive and the negative phases.

Quantitative Sensory Testing. The same investigator (H.T.) carried out QST of the tip of the third digit between successive recording sessions for SNAPs at 2-min intervals with the method of limits. Before the initial test session, sample stimuli were given to acquaint the subject with the procedure. The QST performed immediately after the start of compression served as the baseline to coincide with the beginning of recording of the SNAP used for monitoring the degree of conduction block. Subjects underwent a series of testing for only a single sensory modality of either cold or vibration perception in one experiment, the other modality being tested on a separate day.

Cold Perception Testing. Cold perception thresholds were measured using a thermostimulator (UDH-201; Unique Medical, Tokyo, Japan), which operates on the principle of the Peltier effect. In short, the intensity and direction of the
electric current determine the surface temperature of a 10-mm round stimulating plate, changing it from the preset value of 35°C to 5°C with a linear ramp rate of 0.5°C/s. The subject was instructed to press a button as soon as he perceived a cold sensation from the plate applied to the fingertip of the third digit.

**Vibration Perception Testing.** Vibration perception thresholds were measured using a hand-held vibration testing device (SMV-5; Data Graph, Tokyo, Japan). In this method the examiner presses the button to initiate sinusoidal oscillations of 200 Hz that increase in magnitude linearly from 0 to 300 × 10⁻²g m/s² (g, gravity) per minute until the examiner releases the button. The stimulating probe of a 15-mm disk was applied perpendicularly to the fingertip tested. The vibration perception threshold was determined as the value displayed when the subject gave a verbal signal that vibration was first perceived during an increasing ramp.

**Statistical Analysis.** We used repeated-measures analysis of variance (ANOVA) followed by Dunnett’s test for statistical differences in a series of SNAP and QST data, and unpaired t-tests for evaluating differences of SNAP data between C- and V-groups, which showed a nearly normal distribution. Values are given as mean ± SD and were considered significant at P < 0.05.

**RESULTS**

**SNAPs Recorded at R(w) and R(e).** In the SNAP recorded at R(w), the negative components started to diminish (P < 0.01) in area (N-areas) almost immediately for both groups. The N-areas for the C- and the V-group declined progressively from the initial values of 6.5 ± 1.9 μV.ms and 7.8 ± 3.0 μV.ms to the minimal values of 1.5 ± 1.9 μV.ms and 1.2 ± 1.3 μV.ms at 30 min, showing a reduction of 76% and 84%, respectively (Figs. 2, 3). The progressive reduction in N-area accompanied the progressive enlargement in area of the initial-positive component (P-area) of 3.6 ± 1.2 μV.ms and 4.2 ± 1.4 μV.ms to a maximal value of 5.9 ± 2.9 μV.ms and 7.1 ± 3.6 μV.ms at 30 min, showing an increment of 64% and 67%, respectively. None of these measures showed a significant difference (P > 0.05) for the duration of compression between the C-group and V-group.

In contrast, both N- and P-area of the SNAP recorded at R(e) remained nearly constant for up to 24 min for the C-group and 22 min for the V-group, followed by a steep decline (P < 0.01). The N-areas for the C- and V-group decreased from the initial values of 2.4 ± 1.0 μV.ms and 3.1 ± 1.6 μV.ms to the...
Minimal values of 1.2 ± 1.0 μV.ms (50%) and 1.3 ± 1.2 μV.ms (42%) at 30 min, showing a reduction of 50% and 58%, respectively (Figs. 3, 4). The latency for the C- and V-group showed a small but progressive increase after a delay of 20 min (for both, *P* < 0.01), reflecting a change in conduction velocity from the initial 62.1 ± 4.3 m/s and 62.1 ± 2.1 m/s to 59.8 ± 3.8 m/s and 60.1 ± 2.7 m/s, respectively, at 30 min (Fig. 5). At each duration of compression, the velocities were not significantly different between the two groups. The removal of the compression caused rapid recovery of the SNAP, which returned to the baseline in size at 2 min after release and in velocity at 4 min after release in the C-group, and both in size and velocity at 2 min after release in the V-group (Figs. 3–5).

**Cold Perception Threshold (CPT).** The CPT was 26.8 ± 4.8°C at the onset of compression. The value started to decrease significantly after 16 min (*P* < 0.05), reaching a minimum value of 10.9 ± 5.8°C in 30 min. Removal of the compression caused a rapid recovery of the CPT, which returned to the baseline at 4 min after release (Fig. 6, Table 1).

**Vibration Perception Threshold (VPT).** The VPT was 9.6 ± 3.8 (× 10⁻⁲ g) m/s² immediately after the compression. The value started to increase signifi-

![FIGURE 4. Sequential changes of the orthodromic median nerve SNAPs recorded at the elbow, R(e), during 30 min of compression and another 10 min after release of compression in the same subject as in Figure 2. Both the initial-positive and the negative components remained nearly constant in size for up to more than 20 min, and then steeply declined toward minimal values at 30 min, followed by rapid recovery after release of compression.](image1)

![FIGURE 5. The change in the mean conduction velocity (ordinate) calculated based on the measured distance between the cathode and G1 at R(e) as a function of the duration of compression (abscissa) in 12 healthy subjects each for C-group (top) and V-group (bottom). Changes in conduction velocity had a nearly identical time course, showing no significant difference between the two groups at each point of measurement for the duration of compression. The asterisk indicates that the conduction velocity is significantly smaller than that of the initial response recorded at the onset of compression.](image2)
peak from fast fibers, nor the subsequent negativity. In this case, two opposing effects on the SNAP tend to counter each other, maintaining the SNAP size at nearly the same as the baseline values until conduction block began to involve a greater number of the fast-conducting fibers. Therefore, recording at a distance, as compared to the compression site, sometimes gives rise to a misleading impression of normalcy at the beginning of conduction block.\(^\text{18,30}\)

These SNAP changes had a nearly identical time course for the C- and V-groups, showing no significant difference between the two groups in N-area or conduction velocity at each point of measurement for the duration of compression. These findings indicate that the C- and V-groups developed progressive focal conduction abnormalities to a comparable degree. Thus, disparity of the time course between CPT and VPT delineated in our subjects must have resulted from modality-specific vulnerability of small rather than large myelinated sensory fibers to the experimentally applied focal compression. In this context, our study has documented reduction of CPT 10 min earlier and its recovery 2 min later

\begin{table}
\centering
\caption{Quantitative sensory testing thresholds.}
\begin{tabular}{lcccc}
\hline
 & \multicolumn{2}{c}{Cold perception} & \multicolumn{2}{c}{Vibration perception} \\
\hline
Time after compression (min) & Mean ± SD & P-value\(^*\) & Mean ± SD & P-value\(^*\) \\
\hline
Compress & & & & \\
0.5 & 26.8 ± 4.8 & NA & 9.6 ± 3.8 & NA \\
2 & 26.8 ± 3.7 & NS & 9.8 ± 5.5 & NS \\
4 & 26.9 ± 3.1 & NS & 9.6 ± 5.1 & NS \\
6 & 26.1 ± 5.5 & NS & 11.0 ± 7.7 & NS \\
8 & 26.6 ± 5.8 & NS & 10.3 ± 6.1 & NS \\
10 & 25.7 ± 5.7 & NS & 10.8 ± 5.9 & NS \\
12 & 26.0 ± 5.3 & NS & 11.0 ± 6.8 & NS \\
14 & 22.8 ± 8.5 & NS & 10.5 ± 6.5 & NS \\
16 & 21.4 ± 10.4 & <0.05 & 11.0 ± 7.2 & NS \\
18 & 19.0 ± 10.4 & <0.01 & 11.1 ± 7.1 & NS \\
20 & 16.1 ± 10.5 & <0.01 & 11.9 ± 8.3 & NS \\
22 & 16.4 ± 9.0 & <0.01 & 11.9 ± 7.7 & NS \\
24 & 14.8 ± 8.1 & <0.01 & 13.1 ± 8.1 & NS \\
26 & 15.6 ± 8.6 & <0.01 & 15.8 ± 8.5 & <0.01 \\
28 & 12.4 ± 7.6 & <0.01 & 19.8 ± 12.5 & <0.01 \\
30 & 10.9 ± 7.6 & <0.01 & 22.1 ± 14.5 & <0.01 \\
Release & & & & \\
32 & 18.6 ± 8.3 & <0.01 & 12.1 ± 6.7 & NS \\
34 & 21.9 ± 7.7 & NS & 11.7 ± 8.0 & NS \\
36 & 23.0 ± 8.4 & NS & 11.9 ± 9.1 & NS \\
38 & 24.6 ± 7.6 & NS & 10.9 ± 7.3 & NS \\
40 & 12.5 ± 10.3 & NS & & \\
\hline
\end{tabular}
\end{table}

\(^*\)Repeated measures ANOVA followed by Dunnett’s test comparing each point of measurement with the initial value obtained at the onset of compression.

\(^\text{NA, not applicable; NS, not significant.}\)

In DISCUSSION, we have previously reported that a constant, localized compression of the median nerve produces a progressive focal conduction block, which gives rise to complex waveform changes of the SNAP depending on the recording sites.\(^\text{32}\) The present study duplicated the results under the same experimental setting. Initial findings at R(w) consisted of a nearly immediate reduction in size of the negative component accompanied by progressive enlargement of the initial-positive component. In contrast, the SNAP at R(e) showed no change in size for 22–24 min, with a steep decline thereafter. Its onset latency also remained unchanged for 20 min, indicating at least partial preservation of the fast-conducting fibers. These waveform changes accord with the concept of temporal dispersion, as a reduction in one polarity of constituent nerve fiber action potentials may enhance the other polarity from the loss of physiologic phase cancellation.\(^\text{16,18,30,31}\)

Briefly, blocked fibers continue to produce a robust approaching positivity followed by a substantially reduced negativity at R(w) as the impulse approaches without reaching the recording site,\(^\text{18,30}\) leading to unopposed reduction of the SNAP almost immediately. At R(e), however, initially blocked slow fibers contribute neither the approaching positivity, which would normally partially cancel the negative

\textbf{FIGURE 6.} The change in QST as a function of the duration of compression (abscissa) in 12 healthy subjects each for CPT (bottom) and VPT (top). After the onset of nerve compression, it took 16 min for CPT to show the first change, or 10 min earlier than VPT, which remained normal for 26 min. The removal of the compression caused a rapid recovery of the VPT, which returned to the baseline value 2 min after release (Fig. 6, Table 1).
compared to changes of VPT. Modality differences in central processing for perception may account for the quicker susceptibility of the cold sense, but we are unaware of any report documenting greater central redundancy in the system for vibration sensation. We therefore interpret our finding to indicate a greater susceptibility of the small fibers mediating cold perception than the large fibers mediating vibration perception in accordance with the previous results on the time course of SNAP changes.32

Why, then, does the CPT begin to change with a delay of 16 min from the start of compression, which nearly immediately suppresses the SNAP amplitude recorded at R(w)? Similar to scalp-recorded somatosensory evoked potentials (SEPs), QST assessment involves the entire length of the afferent pathways with complicated synaptic modulation. In such central processing, sensory impulses undergo convergence and divergence at each synapse, compensating for peripheral conduction block.9,14,17 A surprisingly normal SEP recorded over the scalp in patients with a very small peripheral SNAP also attests to this central amplification.5 This phenomenon probably accounts for a delayed onset of CPT change compared to the start of reduction in amplitude of the SNAP recorded at R(w).

The present result confirms the preferential vulnerability of smaller myelinated fibers to mild compression shown in the recording of single fiber action potentials of the cat sciatic nerve.8 Animal studies have also demonstrated that ischemia, similar to mild compression, affects the smaller myelinated fibers before the larger fibers.7,24 Their greater vulnerability to hypoxia may result from a low safety factor of transmission proposed theoretically2 and demonstrated experimentally.6 Viewed in this light, the rapidly reversible nature of our finding supports an ischemic, rather than a mechanical, basis for the rapidly reversible nature of our finding supports a delayed onset of CPT, rather than VPT, is a more sensitive measure of transmission block induced by mild compression.

REFERENCES


ABSTRACT: It is unclear whether there are clinically significant differences in amplitude, duration, and numbers of turns and phases if an electromyographic (EMG) study is performed near to, or far from, the end-plate zone. The effects of temporal dispersion of arriving muscle-fiber action potentials on quantitative motor unit action potential (MUAP) metrics were assessed in simulated and biologic muscles. Two muscle simulation models were studied with electrode recording positions near the motor end-plate zone and 50–75 mm away. When the electrode was moved away from the end-plate zone, averages of 20 MUAPs significantly decreased in amplitude and area, and increased in numbers of turns and phases, but there was no significant change in duration. In biologic muscles (both normal and pathologic), similar changes in average metrics were observed, but to lesser degrees; few were statistically significant. Zones of innervation in biologic muscles are broadly distributed and, during routine electrode studies, distances between random electrode placements and end-plate zones are therefore relatively short, leading to clinically insignificant changes in quantitative MUAP metrics with distance from the end-plate zone. Thus, electrode position within a muscle is unlikely to affect clinical MUAP interpretation.


EFFECTS OF INTRAMUSCULAR NEEDLE POSITION ON MOTOR UNIT ACTION POTENTIAL METRICS

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A major component of electromyography (EMG) is assessment of motor unit action potential (MUAP) waveforms that can be described by metrics of amplitude, duration, turns, and phases. Modeling studies indicate that MUAP amplitude is influenced most by a few fibers within 0.5 mm of the electrode,10 and can be optimized by adjusting the position of the electrode to achieve a rapid rise-time of the main spike component. MUAP duration is more complex and includes both clinically and physiologically defined components.5 The number of turns and phases is largely influenced by temporal dispersion of arriving muscle-fiber action potentials.10 Temporal dispersion is also influenced by the position of the electrode along the length of contributing muscle fibers. A question arises as to whether there are clinically significant differences in amplitude, duration, and numbers of turns and phases if an EMG study is performed near to, or far from, the end-plate zone. The effects of electrode position and the influence of muscle-fiber diameter and fiber density have been studied with respect to single MUAPs.13 We examined the pragmatic implications, from the perspective of quantitative EMG, of the effects of these variables by two methods. First, two muscle simulation models were assessed to determine the effects of electrode position with respect to the end-plate zone on quantitative MUAP metrics. Second, the biologic counterpart was assessed in biceps brachii muscles in normal subjects and those with diseases affecting the motor unit by comparing quantitative MUAP metrics obtained with a decomposition algorithm for electrode positions near the physiologic end-plate zone and at a maximal longitudinal distance away.

MATERIALS AND METHODS

Simulated Data. Two muscle simulation models were studied. The Medtronic EMG Simulator version
3.5 (Karlsson and Stålberg, CD; Medtronic, Skovlunde, Denmark) developed by Karlsson and Ståberg\(^9\) (thus designated the K/S model) was used with default settings for muscle-fiber properties: fiber diameter, 50 ± 5 μm; neuromuscular junction delay, 500 μs; jitter, 50 μs; muscle fibers per motor unit, 100; motor unit radius, 2,500 μm; firing frequencies, 5–30 Hz; frequency variability, ±2 Hz; and contraction force, 30%. An effort was made to distribute motor units randomly in the muscle by manually positioning the center of each unit so that a uniform density of muscle fibers was obtained. Each simulated muscle was restricted to a diameter of 2.5 cm.

Two concentric needle electrodes from the model were used in the simulation studies,\(^9\) one with a large uptake area to simulate a standard-size concentric needle electrode, and one with a smaller uptake area to simulate a pediatric-size concentric needle. Recordings were made from different longitudinal positions along the muscle, resulting in several sets of MUAP data. In this muscle model, the “0” end-plate zone covers an area of ±2 mm, but because the “0” end-plate zone is artificially narrow, sets of 20 MUAPs were recorded starting nominally at ±5 mm from the end-plate zone (±2 mm) and at a position 75 mm distant (maximum distance possible in the model). In the first set of recordings, the radial position of the electrode in the muscle (defined as the distance from the center of the muscle) was constant and the same MUAPs were recorded at both positions. In the second set, the electrode was placed at different radial positions and different MUAPs were recorded at both positions. Overall, 20 MUAPs were recorded at each of the sites. Duration markers were determined by the simulator software algorithm and could not be changed manually. Amplitude values were determined by the simulator software algorithm, but only MUAPs >200 μV were included because 200 μV is a reasonable visual limit during routine EMG studies. Numbers of turns and phases were counted manually using an amplitude criterion of >25 μV for both.

A second muscle simulation model (not commercially available; available from the author at stashuk@pami.uwaterloo.ca) was developed by Hamilton-Wright and Stashuk\(^8\) (thus designated the H-W/S model) and creates whole muscles by taking the number of desired motor units and calculating the muscle diameter needed to contain that number of units. The center of the motor unit is placed within the muscle to simulate the even and random distribution of motor units. Muscle-fiber distribution within the motor unit is performed in a similar manner. Muscle-fiber diameters are selected from a Gaussian distribution specific to each motor unit so that the overall mean fiber diameter is 55 μm with a standard deviation of 9 μm.

A model of a standard concentric needle electrode was placed randomly within the radial plane of the muscle at the end-plate zone and a MUAP train (MUAPT) consisting of a number of motor units within the uptake area of the electrode was created. Similar placements of the electrode in the muscle were made in this model as in the first model to record sets of 20 MUAPTs at the end-plate zone (which represents a distribution ±5 mm from zero) and 50 mm away (maximal distance from the endpoint allowed by the software) and different MUAPTs at the end-plate zone and 50 mm away. Individual MUAPs were extracted from MUAPTs by software algorithms that detect the presence of each motor unit in the train. A total of 17 MUAPs were obtained at each site. Metrics of amplitude and duration of isolated MUAPs were initially determined by algorithms within the software. MUAPs with amplitudes >200 μV were included. Duration markers were occasionally changed. Numbers of turns and phases were manually counted using an amplitude criterion of >25 μV for both.

We also studied a model of a single-fiber electrode (available for the K/S model) to determine the effect of greater conduction distance on the separation of single muscle-fiber action potentials (based on default mean fiber diameter of 50 ± 5 μm). Detection of single-fiber action potentials was established criteria.\(^12\)

**Biologic Data.** This portion of the study was approved by our institutional review board, and informed consent was obtained. Studies were performed in the biceps brachii muscle: five muscles were from normal subjects, one was from a patient with dermatomyositis (DM), and five from patients with amyotrophic lateral sclerosis (ALS). A pediatric-size concentric needle electrode (25-mm long, 0.3-mm diameter, 0.03-mm\(^2\) recording area; Teca-Oxford Instruments, Hawthorne, New York) was used for subject comfort. Data were acquired using decomposition-based quantitative electromyography (DQEMG) software\(^4\) on a Compumedics Neuroscan EMG machine (El Paso, Texas). The physiologic end-plate zone was identified by moving a 10-mm disc cathode electrode (anode electrode placed proximally over the musculocutaneous nerve), using low-intensity shocks to locate the site of maximal muscle contraction. Twenty acceptable MUAPs were obtained with the electrode at the physiologic end-
plate zone and from a position 100 mm proximal to the end-plate zone. At the distal site, care was taken to avoid the tendon by assessing for insertional activity and voluntary motor units as indications of being in muscle. MUAP metrics were determined by the same algorithm used for the H-W/S model.

Statistical Analysis. Analysis was performed on averaged data from 20 MUAPs from each longitudinal site in modeled and biologic muscles. Statistically significant differences were determined by two-tailed t-tests. Significance was assumed at $P$-values $<0.05$ for single comparisons and $<0.02$ for multiple comparisons.

RESULTS

Simulated Data. Average MUAP metrics from modeled muscles (Fig. 1) show that all values change with greater distance from the end-plate zone (specifically, there are decreases in amplitude and area, and increases in duration and numbers of turns and phases). Among the metrics, amplitude values change the most with distance. The results are not different when the same MUAPs (identical radial position) are compared to different MUAPs (random radial position) for all metrics. The same results are found whether electrodes with large or small uptake areas are modeled (Table 1).

Within the model of a single-fiber electrode, there is an average increase in fiber density from 1.4 close to the end-plate zone to 2.4 when recorded 75 mm away.

Biologic Data. Average MUAP metrics from the biceps brachii muscle from normal subjects and those with DM and ALS are shown in Figure 2. With few exceptions among individual subjects, all metrics change in the expected directions with greater distance from the physiologically determined end-plate zone. Exceptions to the expected changes were not distributed in any consistent pattern among individual normal subjects and those with ALS. Few changes are statistically significant ($P < 0.02$ for multiple comparisons), and the magnitude of the changes is such that none are likely to be considered clinically significant. When the results from each muscle group type are combined, no significant differences are found in any metric. When the changes with longitudinal distance from simulated data are compared to biologic data, there are lesser effects of distance on the metrics of amplitude, area, and numbers of turns and phases in biologic data (Table 2).

DISCUSSION

We investigated the effects of longitudinal electrode position within muscle with respect to averaged quantitative MUAP metrics, as would be gathered with quantitative EMG techniques, to assess the clinical effects of temporal dispersion of arriving muscle-
fiber action potentials. Electrophysiologic modeling is useful to enhance understanding of the factors that influence MUAP waveform shape and metric values but it is important to test predictions in normal and diseased muscle to determine which aspects are numerically and clinically important. Two muscle simulation models were used that differ in the assembly of motor units within the muscle, but similar results were obtained with each model. There were no statistical differences whether the same or random MUAPs were compared at the two distances. Previous modeling studies assessed the effects of temporal dispersion on single\textsuperscript{10,13} and multiple\textsuperscript{11} MUAPs and found similar changes. Other studies have investigated gross needle position (at middle and distal sites) within biologic muscle.\textsuperscript{7} We attempted to improve on these by locating the center of the end-plate zone and taking recordings at greater measured distances. By increasing the distances used in previous studies, we exaggerated the degree of dispersion and compared our simulated data to biologic data (in both normal and pathologic muscle).

In modeled muscles, we found a fall in MUAP amplitude distant from the end-plate zone that likely can be attributed to changes in the spatial relation of

<table>
<thead>
<tr>
<th>Site</th>
<th>Variable</th>
<th>Small uptake</th>
<th>Large uptake</th>
<th>Comparison (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mm</td>
<td>Amplitude (μV)</td>
<td>1,174 (360)</td>
<td>1,022 (411)</td>
<td>0.217</td>
</tr>
<tr>
<td></td>
<td>Duration (ms)</td>
<td>4.52 (0.4)</td>
<td>5.40 (0.76)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Area (μVms)</td>
<td>709 (206)</td>
<td>712 (274)</td>
<td>0.971</td>
</tr>
<tr>
<td></td>
<td>Turns</td>
<td>2.95 (0.22)</td>
<td>3.05 (0.5)</td>
<td>0.427</td>
</tr>
<tr>
<td></td>
<td>Phases</td>
<td>3.00 (0.0)</td>
<td>3.00 (0.32)</td>
<td>1.000</td>
</tr>
<tr>
<td>80 mm</td>
<td>Amplitude (μV)</td>
<td>504 (162)</td>
<td>445 (235)</td>
<td>0.359</td>
</tr>
<tr>
<td></td>
<td>Duration (ms)</td>
<td>4.81 (0.32)</td>
<td>5.44 (1.01)</td>
<td>0.033</td>
</tr>
<tr>
<td></td>
<td>Area (μVms)</td>
<td>344 (114)</td>
<td>337 (150)</td>
<td>0.863</td>
</tr>
<tr>
<td></td>
<td>Turns</td>
<td>6.00 (1.69)</td>
<td>5.10 (1.55)</td>
<td>0.081</td>
</tr>
<tr>
<td></td>
<td>Phases</td>
<td>4.10 (1.55)</td>
<td>4.43 (1.72)</td>
<td>0.525</td>
</tr>
</tbody>
</table>

*MUAP, motor unit action potential. **MUAP metrics obtained at two longitudinal sites in the muscle (5 mm and 80 mm from the end-plate zone). Values represent averages with standard deviations in parentheses.
the contributing dipoles to the recording electrode and the effects of phase interaction. The increase in the number of turns and phases can also be attributed to greater temporal separation of contributing muscle-fiber action potentials. MUAP duration is increased, but to a lesser degree than other metrics. A study using shorter interelectrode distances found no difference in average duration. The effect of temporal dispersion on duration is emphasized by using greater interelectrode spacing. It has been noted that the greatest changes in metrics occur over the first 10 mm away from the end-plate zone, but we assessed greater distances from the end-plate zone and found no differences in average metrics when different sizes of end-plate zones were studied.

There are a variety of electrodes to choose from with respect to configuration and size. We chose a pediatric-size concentric needle electrode for subject comfort. In a separate study (submitted for publication), we found no clinically significant difference in MUAP metrics recorded with standard- or pediatric-sized concentric electrodes in biologic muscles. We used models of concentric needle electrodes in the modeled muscle studies, including electrodes with large and small recording uptake radii, and found no significant differences (Table 1). Thus our results are applicable to any size of concentric (and likely monopolar) electrode.

When we studied a model of a single-fiber electrode to determine whether fiber pairs are merged into a single waveform when recorded close to the end-plate zone, we found an average increase in fiber density from 1.4 close to the end-plate zone to 2.4 when recorded 70–75 mm away. This shows the effects of temporal dispersion on the simplest motor unit.

For the study in biologic muscles we chose the biceps brachii because muscle fibers are long and parallel, matching more closely the modeled muscles and permitting comparisons of electrode recording distances up to 100 mm from the end-plate zone. We found similar changes in MUAP metrics recorded at greater distances from the endpoint zone. Duration values were larger, which contrasts with a study comparing both clinical and physiologic duration values from the same MUAP recorded at two sites 16–20 mm apart in the biceps brachii muscle in normal subjects where no differences are noted in either duration value. However, another study based on multi-unit acquisition, and similar in design to our study, found increased duration between recording in the middle third and distal third of the biceps brachii muscle (interelectrode distances not given). Thus, empiric evidence supports an increase in clinical MUAP duration with greater distance from the end-plate zone, but underlying factors remain unexplained.

Comparisons between modeled and biologic data from normal subjects obtained at the end-plate zone revealed higher average amplitude, shorter duration, but similar numbers of turns and phases in modeled muscles. MUAP amplitude in biologic recordings can be optimized by electrode adjustments, but we used multi-motor unit recording techniques that do not optimize amplitude. Greater amplitude in modeled data likely also reflects simplifications in the model, including lack of background activity that could degrade amplitude. MUAP duration values vary among modeled muscles, but are always shorter than in biologic muscle by factors of 2 to 5 times. One factor in modeled MUAPs is the marking algorithm; some duration markers appeared short but could not be manually set and were placed in a consistent fashion. The longer duration values in biologic data reflect different marking algorithms and may also be due to the presence of more fibers in biologic than modeled MUAPs, since duration is proportional to the number of fibers in the electrode recording radius. Other factors are the degree of background noise that is dependent upon the number of waveforms averaged to yield the measured MUAP waveform (52 waveforms are averaged in the decomposition algorithm), and the use of marking algorithms with manual resetting (visual assessment of duration was conservative, likely resulting in shorter rather than longer duration values).

### Table 2. Percent change in MUAP metrics with greater conduction distance in modeled muscle (both models) compared to those from normal subjects and those with ALS.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Amplitude (µV)</th>
<th>Duration (ms)</th>
<th>Area (µVms)</th>
<th>Turns</th>
<th>Phases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modeled</td>
<td>−53.6% (12.1)</td>
<td>31.6% (8.5)</td>
<td>−43.6% (11.7)</td>
<td>96.4% (55.4)</td>
<td>70.1% (51.6)</td>
</tr>
<tr>
<td>Normal</td>
<td>−13.8% (12.3)</td>
<td>16.8% (13.0)</td>
<td>−7.9% (29.2)</td>
<td>16.0% (9.8)</td>
<td>1.4% (6.4)</td>
</tr>
<tr>
<td>ALS</td>
<td>−24.4% (9.7)</td>
<td>4.8% (9.2)</td>
<td>−26.0% (17.4)</td>
<td>43.8% (45.0)</td>
<td>18.4% (25.5)</td>
</tr>
</tbody>
</table>

ALS, amyotrophic lateral sclerosis; MUAP, motor unit action potential.

*Percent values represent average metrics (and standard deviations) measured at the end-plate zone and at a distance (modeled muscles = 50 and 80 mm; biologic muscles = 100 mm).
Comparisons between biologic data in normal subjects and those with myopathic and neuropathic MUAPs reveals similar changes in all metrics with distance but greater variability among metrics within subjects, as is expected with a range of pathologic involvement. Degrees of change in pathologic MUAP metrics with distance are greater for amplitude and number of turns, and relatively less for duration and number phases (Table 2). Pathologic muscles were not modeled because MUAP changes at greater conduction distances are clearly evident in models of normal muscles. Similar discrepancies are noted between modeled and biologic data for numbers of phases, and have been attributed to assumptions about the relationship between muscle-fiber diameter and conduction velocity.10

A major difference in muscle architecture between simulated and biologic muscle is the precision of the size and position of the end-plate zone. In modeled muscles, it is over a zone of ±2 mm (K/S model) or of ±5 mm (H-W/S model). In biologic muscle, an end-plate zone was identified by physiologic methods, but its boundaries were not fully known. Histologic data on the distribution of neuromuscular junctions in the biceps brachii muscle indicate a primary clustering of end-plates in a longitudinally oriented “V” distribution that occupies at least the middle third of the muscle,1 and in the tibialis anterior a diffuse distribution over the entire length.1 Muscle fibers in complex muscles are relatively short, and the distance from the end-plate zone to the electrode will be much less than the 100-mm distances studied here. The zone of innervation for a single motor unit in human muscle is not known and likely spans 5–10 mm. Electrophysiologic data from surface multi-electrode recording techniques indicate that within a muscle the peak amplitudes of single MUAPs are distributed over a relatively broad area.3 Further, clinical experience with recording end-plate activity suggests that the end-plate region may differ widely from person to person. We conclude that true distances for temporal dispersion are actually short in biologic muscle and this largely accounts for the minimal effect of electrode position within a muscle. Thus, electrode position within a muscle is unlikely to affect clinical MUAP interpretation.

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ABSTRACT: Most studies examining the effect of electrical stimulation pattern on the force response of muscle have been done in able-bodied persons. The purpose of this study was to examine the electrically elicited force responses of the paralyzed quadriceps femoris muscles of persons with spinal cord injuries (SCI) to see whether stimulation patterns that increase the force response in non-paralyzed muscle will do so in paralyzed muscle. Thirteen subjects ranging in age from 11 to 24 years old with motor-complete SCI were studied. Isometric muscle performance was tested using 6-pulse constant-frequency trains (CFTs), variable-frequency trains (VFTs), and doublet-frequency trains (DFTs) delivered at mean frequencies of 10, 20, 33, 50, and 100 Hz. In the non-fatigued and fatigued condition, the VFT and DFT peak forces were greater than the CFT peak forces at 10 Hz. In addition, in the fatigued condition the 20-Hz VFT peak forces were greater than the CFT peak forces, and there was a trend for the DFT peak forces to be greater than the CFT peak forces. In the non-fatigued condition, the 33-Hz and 50-Hz DFT force–time integrals were greater than both the CFT and VFT force–time integrals. In the fatigued condition, there was no significant effect of train-type on the force–time integrals. These results differ from those previously reported from studies using able-bodied persons and indicate that findings from studies of the electrically elicited force responses of the muscles of able-bodied persons do not apply to the paralyzed muscles of persons with SCI.

EFFECT OF ELECTRICAL STIMULATION PATTERN ON THE FORCE RESPONSES OF PARALYZED HUMAN QUADRICEPS MUSCLES

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Functional electrical stimulation (FES) can be used to produce functional movements in persons who cannot voluntarily activate their muscles due to neurological dysfunction.27 In addition, electrically elicited muscle contractions have been shown to induce training effects, such as increased strength and fatigue resistance.20 It is well established that the frequency and pattern of the electrical stimulation affect the force responses of the muscle.8,11,12,15,36 Traditionally, electrical stimulation of muscle for FES and training has used constant-frequency trains (CFTs).14,24 However, some researchers have found that variable-frequency trains (VFTs) that begin with two pulses (a doublet) separated by a brief (5–10 ms) interpulse interval, followed by regularly spaced pulses with longer interpulse intervals can take advantage of the catch-like property of skeletal muscle and augment forces compared to CFTs in mammalian single motor units,2,38 including human thenar motor units36 and whole muscles.5,11,35,37 The catch-like property is the force enhancement that occurs when an initial high-frequency burst of pulses is added to the beginning of a subtetanic train of pulses.12 Although not all investigators have found VFT force augmentation in non-fatigued mammalian muscle,7,8,34 all studies that have compared CFT and VFT force production in fatigued muscle have found that VFTs augment force,2,6,8,9,25,30,34

Abbreviations: AB, able-bodied; CFT, constant-frequency train; DFT, doublet-frequency train; FES, functional electrical stimulation; SCI, spinal cord injury; VFT, variable-frequency train

Key words: constant-frequency train; force–time integrals; spinal cord injury; variable-frequency train

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Although VFTs may enhance force production as compared to CFTs, they may not be the best activation pattern for force production. A mathematical model that accurately predicts the force generated in response to brief stimulation trains was developed in our laboratory. The model showed that a type of train consisting entirely of pairs of closely spaced pulses (doublets) separated by longer intervals, which we call a doublet-frequency train (DFT), should produce greater forces than either CFTs or VFTs in non-paralyzed muscle. Doublets occur when the central nervous system activates skeletal muscle and are thought to represent a “functional entity” that augments force production. Experimental data confirmed that DFTs augment force production in non-fatigued, and to a greater extent, fatigued conditions, the train patterns that maximized force production would include the VFT and DFT, and that the force augmentation would vary depending on the mean frequency of the stimulation trains and whether the muscles were non-fatigued or fatigued.

**MATERIALS AND METHODS**

**Subjects.** Data were collected on 13 subjects (2 women) with motor-complete SCI who were recruited and tested at Shriners Hospital for Children in Philadelphia. The subjects were adolescents and young adults (age, 16.9 ± 4.1 years) with mid-thoracic or cervical (n = 4) level injuries. The time since injury was 45.8 ± 43.4 months. Inclusion criteria consisted of a motor-complete SCI, at least 1 year since SCI or neurologically stable, no lower motor neuron involvement of the quadriceps muscle, no history of orthopedic knee injuries or spontaneous lower-extremity fractures, and a passive knee joint flexion of 100° when sitting. Subjects were considered neurologically stable if their sensory and motor function was unchanged upon admission to the hospital at the time of testing in comparison to their most recent previous admission. Additionally, subjects were excluded if they had a history of orthopedic knee injuries, heart disease, peripheral vascular disease, current neoplasms, or neurological disorder (other than SCI) affecting the lower extremities. Participation was voluntary and subjects were free to withdraw from the study at any time. All subjects and the legal representatives of minors signed an informed consent form that was approved by the University of Delaware Human Subjects Review Committee and the Institutional Review Board of Temple University, which served as the oversight committee for Shriners Hospital for Children. In addition, minors signed an assent form that was approved by the oversight committees.

**Experimental Protocol.** Isometric muscle performance was tested using 6-pulse CFTs, VFTs, and DFTs delivered at mean frequencies of 10, 20, 33, 50, and 100 Hz. Figure 1 shows the three train patterns at the 33-Hz frequency. Each subject was tested during a single session that lasted approximately 1 h. Quadriceps muscles were tested with the subjects’ knees flexed to 90° and approximately 75° of hip flexion. Subjects were tested using a computer-controlled dynamometer; the Kincom II fitted with the
approximately 25% of the tetanic forces. Next the subjects used in this study, the twitch forces equaled 20-H stimulation intensity for the session was set using 1-s recticeps muscles of subjects with SCI is elevated. For the that the twitch force produced by the paralyzed quad-
ment, the position of the electrodes was adjusted against the anterior aspect of the lower leg proximal to the lateral malleolus. The muscles were stimulated using a Grass S88 stimulator with a SIU8T stimulus isolation unit (Grass Instrument Co., Quincy, Massachusetts). The stimulation pattern and frequency were controlled by custom-written Labview software (National Instruments, Austin, Texas). Two 7.5 × 12.5 cm self-adhesive electrodes were used for transcutaneous electrical stimulation of the muscle. One electrode was placed distally over the muscle belly of the vastus medialis and the other was placed proximally over the rectus femoris muscle. Smaller (5 × 9 cm) electrodes were used for subjects with small legs. Following placement of the electrodes, 1-s 20-Hz stimulation trains were delivered to test electrode location. A smooth rate of rise and a plateau in force indicated that a consistent population of motor units was being recruited throughout the stimulation train. If a smooth rate of rise and plateau in force were not achieved with the initial electrode placement, the position of the electrodes was adjusted until the desired response was observed.

Following testing for electrode placement, the peak twitch force of the subjects’ quadriceps muscle was recorded when stimulated with a series of single 600-μs pulses delivered at a rate of 1 pulse every 10 s; the stimulator voltage was incrementally increased until the maximum of 150 V. Previous studies have shown that the twitch force produced by the paralyzed quadriceps muscles of subjects with SCI is elevated. For the subjects used in this study, the twitch forces equaled approximately 25% of the tetanic forces. Next the stimulation intensity for the session was set using 1-s 20-Hz stimulation trains. A train was delivered approximately every 10 s as the voltage was adjusted, and the force response of the muscle was monitored. We adjusted the stimulator voltage until the peak force in response to this train was equivalent to the maximum twitch force recorded for that subject. Once the intensity was set, it remained unchanged for the remainder of the testing session.

Non-Fatigued Muscle Testing. Following a 5-min rest, the non-fatigued portion of the testing protocol was begun. There were two non-fatigued protocols that subjects received in a random order separated by a 5-min rest. During both protocols, stimulation trains were delivered at a rate of 1 every 20 s to avoid producing fatigue. One protocol consisted of a sequence of 15 6-pulse testing trains (3 train types at 5 frequencies) delivered in a random order and then repeated in reverse order for a total of 30 non-fatigued testing trains. This protocol generated the data of interest for the present report. In addition, a second protocol involved delivery of a single pulse and 8 1-s CFTs ranging in frequency from 10 to 100 Hz in a random order and then repeated in reverse order. Data from the second protocol are presented in a separate report. For the present report, only the single pulse and 1-s 100-Hz train were used from the second protocol to record the twitch and tetanic responses of the muscles, respectively. All subjects received the same random order of testing trains. We did not potentiate the muscles prior to testing because pilot work showed that it was difficult to produce potentiation without simultaneously producing fatigue.

Fatiguing Stimulation and Fatigued-Muscle Testing. Following another 5-min rest, the fatigue-producing protocol, consisting of 110, 13-pulse, 40-Hz CFTs delivered at a rate of 1 every second (300 ms on, 700 ms off, 30% duty cycle), was begun. Immediately following the last fatigue-producing train, the fatigued-muscle testing started. The same 15 6-pulse testing trains followed by the single pulse and 8 1-s-long CFTs that were delivered in the non-fatigued condition were now delivered to the fatigued muscles. However, in the fatigued condition, the testing trains were separated by two fatigue-producing trains (i.e., the 13-pulse, 40-Hz CFTs). There was a 700 ms off time between each train, and there was only one occurrence of each of the testing trains. The fatigue-producing trains were included prior to each testing train to control for prior activation history of the muscle and to attempt to ensure a consistent level of fatigue throughout the fatigued-muscle testing.

Data Management. The entire force record for each subject in response to the non-fatigued testing proto-
cols, the fatigue-producing protocol, and the fatigued testing protocol were digitized on-line at a sampling frequency of 200 Hz and stored for subsequent analysis. Data were analyzed using custom-written software (Labview 5.0; National Instruments). The primary dependent variables analyzed in this study were the peak forces and force–time integrals in response to the three different train patterns at the five different mean frequencies. For the non-fatigued data, the two occurrences of each testing train were averaged. We also used the peak force responses to the single pulse and the 1-s 100-Hz CFT to calculate the non-fatigued and fatigued twitch and tetanic responses of the muscles. Additionally, we calculated the doublet response of the muscle in the non-fatigued and fatigued conditions from the peak force responses to the first doublet of the 10-Hz DFT.

Data Analysis. The force data from the non-fatigued and fatigued conditions were analyzed separately. One-way, repeated measures ANOVAs were used to analyze the effect of train type on the force responses of the muscles at each frequency tested. Paired t-tests, with a Bonferroni correction for multiple comparisons, were used for post hoc testing. We chose this statistical analysis strategy because, based on previous research in our laboratory and reports in the literature, we assumed there would be a significant main effect on the forces produced by all three factors (train type, frequency, and muscle condition) and significant interaction effects between these factors. In addition, because of the limited number of subjects available to us and more critically, the limited number of subjects with complete data sets (n = 6), we were concerned that a three-way repeated measures ANOVA, although perhaps more appropriate, would leave us underpowered to detect real differences in the force responses to the three train types. Consequently, we decided a priori that we would focus our analysis on the effects of train type at each of the five frequencies tested in the non-fatigued and fatigued condition.

We used the twitch, tetanus (i.e., the 1-s, 100-Hz CFT response), and doublet peak force responses to calculate twitch-to-tetanus, doublet-to-twitch, and doublet-to-tetanus force ratios for each subject in both the non-fatigued and fatigued conditions. Paired t-tests were used to compare the non-fatigued and fatigued force ratios. Statistical significance was accepted at \( P \leq 0.05 \).

RESULTS

All 13 subjects completed the data collection session. However not all subjects had complete data sets.

FIGURE 2. Average (mean ± SE) (n = 13 unless otherwise noted) of the non-fatigued (A) and fatigued (B) peak forces in response to the three train types at each of the five frequencies tested. #Indicates a significant main effect of train type on the force responses. *Indicates CFT different than VFT; †indicates CFT different than DFT; and ‡indicates VFT different than DFT. See Results section for \( P \)-values.

From the non-fatigued testing, due to an error in the data collection program, for four subjects the 20-Hz VFT data were not accurate and therefore could not be analyzed. In the fatigued condition, one subject was not included because spasms throughout the fatigued testing disrupted the force responses. Additionally, four subjects experienced intermittent spasms that interfered with some of the force responses. In these cases, the force responses that were affected by the spasms were eliminated from the analyses. The number of subjects used for each one-way, repeated measure ANOVA is indicated in Figures 2 to 4. For the three force ratio measures, there were complete data sets for 13 subjects in the non-fatigued condition, but only 12 in the fatigued condition. As a result of the paired t-test statistical design employed, we used only the 12 SCI subjects who had complete non-fatigued and fatigued data.

Six of the 13 subjects reported using electrical stimulation regularly for training or FES. However,
there were no differences in either the contraction times or the half-relaxation times (calculated from each subject’s maximum twitch force response), maximum twitch force, or twitch-to-tetanus ratios for the six subjects that used electrical stimulation and the seven subjects that did not; nor was there a difference in the fatigue resistance of the two groups’ muscles. Consequently, we grouped all the SCI subjects together in a single group for the analyses.

**Peak Forces.** In the non-fatigued condition, there was a main effect of train pattern at 10 Hz on the peak forces ($P < 0.001$) (Fig. 2A). Post hoc testing revealed the VFT and DFT peak forces were greater than the CFT peak forces ($P < 0.001$ for both). In the fatigued condition, there was a main effect of train type at 10 Hz ($P < 0.001$), 20 Hz and 50 Hz ($P < 0.001$ for both) (Fig. 3A). Post hoc testing showed that at 20 Hz the DFT force–time integrals were lower than the VFT force–time integrals ($P < 0.05$). The 33-Hz and 50-Hz DFT force–time integrals were greater than both the CFT ($P < 0.001$ for both) and VFT ($P < 0.005$ for both) force–time integrals. There was a trend for there to be a main effect of train type on the force–time integrals produced in the fatigued condition at 10 Hz ($P = 0.07$, Fig. 3B).

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![Graph](image)

**FIGURE 3.** Average (mean ± SE) ($n = 13$ unless otherwise noted) of the non-fatigued (A) and fatigued (B) force–time integrals in response to the three train types at each of the five frequencies tested. #Indicates a significant main effect of train type on the force responses. *Indicates CFT different than VFT; †indicates CFT different than DFT; and ‡indicates VFT different than DFT. See Results section for $P$-values.

**FIGURE 4.** Average (mean ± SE) ($n = 12$) of the twitch-to-tetanus (TtToTet), doublet-to-twitch (DdTt), and doublet-to-tetanus force ratios (DoToTet). *Indicates non-fatigued and fatigued conditions significantly different. See Results section for $P$-values.

**Force–Time Integrals.** There were main effects of train type on the force–time integrals in the non-fatigued condition at 20 Hz ($P < 0.05$), 33 Hz and 50 Hz ($P < 0.001$ for both) (Fig. 3A). Post hoc testing showed that at 20 Hz the DFT force–time integrals were lower than the VFT force–time integrals ($P < 0.05$). The 33-Hz and 50-Hz DFT force–time integrals were greater than both the CFT ($P < 0.001$ for both) and VFT ($P < 0.005$ for both) force–time integrals. There was a trend for there to be a main effect of train type on the force–time integrals produced in the fatigued condition at 10 Hz ($P = 0.07$, Fig. 3B).

**Force Ratios.** There was not a significant difference between the twitch-to-tetanus ratios in the non-fatigued (0.26) and fatigued (0.28) conditions (Fig. 4). There was a significant difference ($P < 0.005$) between the non-fatigued (2.70) and fatigued (1.66) doublet-to-twitch ratios (Fig. 4). In the fatigued condition, the doublet-to-tetanus ratios was 61% of the value in the non-fatigued condition. There was a significant difference ($P < 0.005$) between the non-fatigued (0.61) and fatigued (0.45) doublet-to-tetanus ratios (Fig. 4). With fatigue, the doublet-to-tetanus ratios declined, producing 73% of the non-fatigued value.
**DISCUSSION**

In this study, we have shown that the force augmentation that 6-pulse VFTs and DFTs generate in paralyzed human quadriceps muscles is dependent on the mean frequency of the stimulation train, the condition of the muscle (i.e., non-fatigued or fatigued), and the force measure examined.

**Peak Forces.** Relative to the CFT, the VFT and DFT only augmented the non-fatigued peak forces at 10 Hz. In the fatigued condition, the VFT and DFT, relative to the CFT, augmented the peak forces at 10 Hz and 20 Hz (DFT, $P = 0.07$). We are aware of only one other study that has examined the isometric force responses of paralyzed quadriceps muscles to different patterns of stimulation. Consistent with our findings, Bickel and colleagues showed that for the $\sim 15$ Hz trains they tested, the VFT did not augment the peak torques relative to the CFT of acute (<1 year post-injury) or chronic (>1 year post-injury) subjects with SCI in the non-fatigued condition, but did do so for the subjects with chronic SCI when the muscles were fatigued.

Similar to our findings in this study, a previous report from our laboratory that used 6-pulse CFTs and VFTs to activate the non-paralyzed quadriceps muscles of AB subjects across a wide range of frequencies found that the VFT only augmented peak forces at $\leq 10$ Hz when the muscles were non-fatigued and $\leq 20$ Hz when the muscles were fatigued. In contrast to previous reports using AB subjects from our laboratory, the subjects with SCI in this study did not show a similar augmentation of peak forces by the DFTs. With AB subjects' quadriceps stimulated at 30 Hz, we have observed peak force augmentation by the DFT relative to the CFT in a non-fatigued condition and augmentation relative to both the CFT and VFT in a fatigued condition. The findings from these previous studies and those in this study suggest that the DFT may augment peak force relative to the VFT in the non-paralyzed muscles of AB subjects but not in the paralyzed muscles of subjects with SCI.

**Force–Time Integrals.** Relative to the CFT, the VFT did not augment the force–time integrals at any of the tested frequencies in either the non-fatigued or fatigued condition. The DFT produced greater force–time integrals than the CFT at 33 Hz and 50 Hz in the non-fatigued condition. Bickel and colleagues also failed to observe VFT force–time integral augmentation in subjects with SCI regardless of whether the muscles were non-fatigued or fatigued. Our present observation is similar to our prior study of AB subjects that showed DFT force augmentation relative to both the CFT and VFT at 30 Hz in the non-fatigued quadriceps muscles.

In contrast to this study where we failed to observe augmentation of the force–time integrals by the VFTs or DFTs in fatigued paralyzed muscle, we have previously shown in AB subjects that VFTs augment force–time intervals relative to the CFT at frequencies $\leq 20$ Hz when muscles are fatigued. Additionally, we observed DFT force–time integral augmentation in a fatigued condition relative to both the CFT and VFT at 30 Hz. Consequently, the force–time integral responses of the paralyzed muscles appear similar to those of AB subjects’ quadriceps muscles when the muscles are non-fatigued but not when they are fatigued.

**Force Ratios.** We calculated three force ratios and compared them across conditions to provide information that we thought might provide insight into the anticipated different responses of the paralyzed muscles to the three train types in the different conditions in this study and what has been reported previously in the literature for both SCI and AB subjects. Gerrits and colleagues reported previously that the non-fatigued twitch-to-tetanus ratio of the paralyzed human quadriceps is elevated relative to the non-paralyzed quadriceps. We also observed an elevated twitch-to-tetanus ratio of the paralyzed quadriceps of our SCI subjects compared to AB subjects and showed that it remained elevated when the muscles were fatigued. Griffin and colleagues reported greater doublet responses for paralyzed than non-paralyzed human thenar muscles, and we have also observed greater doublet responses of the paralyzed than non-paralyzed human quadriceps muscles. Paradoxically, we presently observed that the doublet responses of the paralyzed muscles declined relative to both the twitch and tetanic responses when the muscles were fatigued, yet the VFTs and DFTs continued to augment the peak forces at 10 Hz in the fatigued condition as they did in the non-fatigued condition and they augmented the peak forces at 20 Hz in the fatigued condition when they did not in the non-fatigued condition.

Increased $\text{Ca}^{2+}$ release and changes in muscle stiffness have both been proposed as mechanisms to explain the catch-like property of skeletal muscle that results in force augmentation from VFTs and DFTs. It is probable that both mechanisms contribute to the catch-like property. Recently, Abbate and colleagues, using single fibers of the mouse, confirmed that the increase in force from the initial
high-frequency burst of pulses in the VFT is due to increased Ca\(^{2+}\) release.\(^1\) They also showed that the increased force in response to the initial high-frequency burst is sustained beyond the initial elevation of myoplasmic Ca\(^{2+}\). They suggested that changes in Ca\(^{2+}\) sensitivity of the contractile mechanism as a result of the facilitated formation of additional cross-bridges in the vicinity of already attached cross-bridges accounts for this observation.

It is unclear at this time how the physiological changes in paralyzed muscles account for the different responses to electrical stimulation that we observed in SCI subjects and previous reports in the literature from studies of AB subjects. Abbate and colleagues\(^1\) showed that when single muscle fibers of the mouse were tested in a lengthened (i.e., stiffer) position, there was less force augmentation by VFTs, suggesting that increased stiffness decreases the catch-like property. Conversely, Lee and colleagues showed that VFTs augmented the force–time integral response of non-paralyzed quadriceps muscles to a greater extent when muscles were tested at short rather than long lengths, again suggesting that the VFT, and presumably the DFT, are more likely to augment force when muscles are less stiff.\(^26\) Interestingly, Maganaris and colleagues\(^28\) recently showed that the patellar tendons of persons with SCI are less stiff than those of AB subjects. Decreased stiffness of the muscle–tendon complex of the paralyzed quadriceps may explain the greater doublet response that we observed.

Another possibility is that changes in Ca\(^{2+}\) dynamics or Ca\(^{2+}\) sensitivity occur in paralyzed muscle. The loss of contractile proteins due to muscle atrophy that occurs following paralysis may not be accompanied by a similar loss of the Ca\(^{2+}\) available for release in response to stimulation. This should shift the force–pCa\(^{2+}\) curve to the left in paralyzed compared to non-paralyzed muscle, as similar submaximal amounts of calcium would activate a relatively greater percentage of the available cross-bridges relative to the maximum number available in the paralyzed muscle, thus leading to relatively large twitch and doublet responses.

Regardless of the mechanism that accounts for the elevated doublet response in paralyzed muscle, it does not appear that VFT stimulation offers any more augmentation of force in the paralyzed quadriceps muscle than reported for the non-paralyzed quadriceps muscle. In fact, the VFT appears to be less effective in paralyzed muscle, because in our previous study\(^9\) of AB subjects, the VFT augmented the force–time integral of fatigued muscle when stimulated with frequencies \(\leq 20\) Hz, but there was no augmentation of the fatigued force–time integrals in this study. Although the DFT has not been studied across a wide range of frequencies in AB subjects, our studies with 30-Hz DFTs have shown peak force and force–time integral augmentation in both non-fatigued and fatigued conditions relative to both CFTs and VFTs.\(^11,31\) In this study, in contrast, the DFT only augmented the force–time integrals relative to the CFTs and VFTs in the non-fatigued condition at 33 and 50 Hz, suggesting that the DFT is not as effective in augmenting force in paralyzed muscle as it is in non-paralyzed muscle.

Taken together, these observations suggest that when muscles are fatigued, VFTs augment the peak forces similarly in paralyzed and non-paralyzed muscle but do not augment the force–time integrals of paralyzed muscle. Furthermore, in the paralyzed quadriceps muscle, the DFTs appear to offer little advantage relative to the VFT. A possible explanation that may account for these observations is that the physiological cause of fatigue is different in the AB and SCI subjects. In AB subjects, the VFT and DFT are thought to be more effective in augmenting force in the fatigued condition because the increased Ca\(^{2+}\) release in response to the doublets of the stimulation trains overcomes impairments of excitation–contraction coupling that results from reduced Ca\(^{2+}\) release from the sarcoplasmic reticulum. It is possible that the fatigue in the paralyzed muscle of our SCI subjects was not the result of excitation–contraction coupling impairments but rather impairments of the force-generating ability of the cross-bridges, and therefore unlikely to be overcome by additional Ca\(^{2+}\) release in response to the doublets of the VFT and DFT. The results of our study comparing the force–frequency relationships of the same subjects used in this study also suggests that the fatiguing protocol used in this study produced less low-frequency fatigue in the subjects with SCI than the AB subjects.\(^32\) Another possibility suggested by Bickel and colleagues is that the faster contractile times of the paralyzed muscles lead to relatively shorter contractions and thus a lack of force–time integral augmentation despite augmented peak forces from the same trains.\(^4\) The subjects with SCI used in this study had both faster contraction and relaxation times than matched AB subjects when their respective quadriceps muscles were fatigued.\(^32\) Finally, damage to paralyzed muscles as indicated by an elevated T2 signal on magnetic resonance imaging has been shown to occur following electrically elicited contractions.\(^3\) This again suggests that the fatigue we observed in this study may have been less influenced by excitation–
contraction impairments and more so due to impairments at the level of the cross-bridges.

The most important finding of this study is that the relative forces produced by the three stimulation patterns tested are different between subjects with SCI and what has been reported for AB subjects. This demonstrates that it cannot be assumed that the results of electrical stimulation studies with AB subjects are applicable to patient populations. For patients with SCI, this study suggests that VFTs will only be useful to increase the peak force output of the muscle when using stimulation frequencies of approximately \( \pm 10 \) Hz in a non-fatigued condition and \( \pm 20 \) Hz in a fatigued condition.

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ABSTRACT: To characterize and compare electrical myotonia in myotonic dystrophy type 1 (DM1) and type 2 (DM2), 16 patients with genetically confirmed DM1 and 17 patients with DM2 underwent standardized concentric needle electromyography of deltoid, biceps, extensor digitorum communis, first dorsal interosseous, tensor fascia lata (TFL), vastus lateralis (VL), tibialis anterior, and thoracic paraspinal muscles. Eight needle insertions per muscle were made by electromyographers blinded to DM type who recorded the presence and type of myotonia (e.g., classic waxing–waning or less specific waning discharges). Manual muscle testing was performed by a physical therapist. Overall, myotonia was more elicitable in DM1 than DM2; only in VL and TFL was myotonia more elicitable in DM2 than DM1. The major type of myotonia was waxing–waning in DM1, and waning in DM2. Four DM2 (24%), but no DM1 patients had only waning myotonia. In the arms, myotonia was distally predominant in both DM1 and DM2. In the legs, it was distally predominant in DM1, but both proximal and distal in DM2. The severity of myotonia was positively correlated with muscle weakness and with the presence of waxing and waning discharges in DM1, but with neither in DM2. Thus, myotonia is qualitatively and quantitatively different in DM1 than DM2. Except for proximal leg muscles, myotonia is more evocable in DM1 than DM2. It tends to be waxing–waning in DM1 but waning in DM2, thus making electrodiagnosis of DM2 more challenging. Its severity correlates with muscle weakness and the presence of waxing–waning discharges in DM1 but not DM2.


SEVERITY, TYPE, AND DISTRIBUTION OF MYOTONIC DISCHARGES ARE DIFFERENT IN TYPE 1 AND TYPE 2 MYOTONIC DYSTROPHY

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In the era before genetic confirmation of the diagnosis of myotonic dystrophy, a needle electromyographic study of 25 successive patients with classic myotonic dystrophy showed that electrical myotonia (EM) is distally predominant and that the severity of EM correlates with the severity of muscle weakness.21 A more recent study of 27 patients confirmed these findings using similar methodology to estimate the severity of myotonia.14

In 1992, it was reported that the genetic defect underlying classic myotonic dystrophy is an unstable expanded CTG triplet repeat in the untranslated region of the DMPK protein kinase gene on chromosome 19.3 In the middle to late 1990s, a number of investigators6,17,18,23,25 reported an autosomal-dominant systemic disease very similar to classic myotonic dystrophy, except that the distribution of limb-muscle weakness was often more proximal than distal, muscle atrophy was less, and there was no expansion of the CTG triplet repeat sequence. The genetic basis of this second form of myotonic dystrophy, initially referred to as proximal myotonic myopathy (PROMM), or proximal myotonic dystrophy (PMD) by some groups, was found subsequently to be due to an unstable CCTG tetra-repeat expansion in intron 1 of the zinc finger protein 9 (ZNF9) gene.
on chromosome 3,5,15 The current nomenclature of these two diseases is myotonic dystrophy type 1 (DM1) for the classic form and type 2 (DM2) for the more recently recognized form.7

To our knowledge, there are no published systematic studies of EM in DM2, and no studies directly comparing EM in DM2 and DM1. Myotonic discharges have been reported in the majority of patients with DM2, but it has been suggested that EM is less severe and less prevalent in DM2 than in adults with DM1.5,10 In the largest series to date, 90% of 234 affected DM2 patients were found to have EM; an even higher percentage of affected DM1 patients probably manifest EM.5 In other series, EM was found in 23 of 27,18 13 of 14,17 and 13 of 13 patients with DM2.2 Overall, these data do not provide compelling evidence that EM in DM2 is less severe or prevalent than in DM1. Moreover, the distribution of EM in DM2 is also not known. Since muscle weakness tends to be proximally predominant in DM2, it might be expected that EM in DM2 would be more prominent proximally, in contrast to DM1 in which EM and weakness both tend to be distally predominant.

Another point of interest is whether the type of myotonic discharge differs in DM2 and DM1. Some investigators have noted that EM may markedly fluctuate in DM2 patients and that it may be accompanied by complex repetitive discharges, brief runs of high-frequency 180–240-Hz single-spike discharges, and even fasciculation potentials.16,18 EM is usually defined as spontaneous electromyographic (EMG) discharges that wax and wane in frequency and amplitude.1 Spontaneous discharges that only wane in frequency or amplitude (e.g., waning discharges) do not meet strict criteria for EM as they are not as specific as waxing–waning discharges. Still, in our experience, waning discharges commonly occur in myotonic myopathy in general, but the type of spontaneous discharges (e.g., waxing, waning, waxing–waning, or other) has not been systematically investigated in either DM1 or DM2.

The purpose of this study was to: (1) determine and compare the severity, distribution, and type of EM in DM1 and DM2; and (2) test the hypothesis that myotonia is proximally predominant in DM2, distally predominant in DM1, and correlates with muscle weakness in both disorders.

**METHODS**

From July 2001 through December 2005, patients with DM1 and DM2 were prospectively enrolled in a functional genomics study that was approved by the institutional review board at the University of Rochester Medical Center, where neurologic examination, quantitative and manual muscle testing, needle electromyography, and measurement of grip myotonia were performed. Patients were included in the study if they had muscle weakness and percussion or grip myotonia on neurologic examination, and positive genetic testing for DM1 or DM2. The criterion for genetic diagnosis of DM1 was a trinucleotide CTG expansion of over 50 repeats in blood leukocytes,7,24 and the criterion for DM2 was a tetrancleotide CCTG expansion of over 75 repeats in blood leukocytes.5,12 Patients with significant medical, neurologic, or psychiatric comorbidity, or who were receiving antymotonic drug therapy, were excluded.

All DM1 and DM2 patients underwent standardized EMG examination using disposable concentric 25–37-mm electrodes (Viasys Healthcare, Hawthorne, New York) of eight commonly tested, proximal and distal limb and thoracic paraspinal muscles, using a Viking Select EMG machine (Nicolet, Madison, Wisconsin). All patients were resting in bed for at least 10 minutes prior to the examination. Surface temperature was not monitored. In the upper extremity, mid-deltoid (MD), biceps brachii (BB), extensor digitorum communis (EDC), and first dorsal interosseous (FDI) muscles were studied. In the lower extremity, tibialis anterior (TA), vastus lateralis (VL), and tensor fascia lata (TFL) were examined, as were the thoracic paraspinals (TPs). Eight brisk needle insertions per muscle were made into different muscle regions through one skin puncture site by experienced electromyographers (E.L., E.C.) blinded to DM type, who recorded the presence of myotonic discharge and the type of discharge. The electrical activity evoked by each insertion was classified as normal, increased insertional activity, or myotonic discharge, as determined by on-line visual and audio feedback of the EMG discharges observed from onset to termination. Myotonic discharges were required to be at least 400 ms in duration (two successive 200-ms oscilloscope screens using a 10-ms/division sweep speed), to have muscle-fiber action potential morphology (e.g., either a positive wave or fibrillation potential), and a variable firing frequency with a maximal frequency of 20–80 Hz.1

Using the criteria set forth by the American Association of Electrodiagnostic Medicine,1 discharges were further characterized as classic waxing–waning myotonic discharges if the muscle-fiber potentials increased and decreased in both amplitude and frequency, and as less specific waning discharges if they gradually decreased, but never increased, in frequency or amplitude. For each patient, an EM sever-
ity score was calculated for each muscle based on the number of insertions per muscle that evoked a myotonic discharge, with a maximum score of 8 and minimum score of 0. For the DM1 and DM2 patient groups, the severity of EM in each muscle was calculated by determining the percent of insertions that evoked a myotonic discharge.

An experienced physical therapist (S.P.) performed manual muscle testing on all patients enrolled in the study using a modified 10-point Medical Research Council (MRC) scale,13,22 in which muscle strength was categorized as 0, 1, 2, 2.67, 3, 3.67, 4, 4.33, 4.67, or 5. For the purposes of this study, we used the MMT data from the four muscle groups in which we also had concentric-needle EMG data: shoulder abduction (deltoid), elbow flexion (biceps), knee extension (vastus lateralis), and ankle dorsiflexion (tibialis anterior).

At the completion of the study in 2005, the blind was broken and the results were analyzed. Several statistical analyses were performed: (1) A two-tailed chi-square test was used to compare the severity of EM for each muscle in the DM1 and DM2 patient groups (e.g., FDI in DM1 vs. FDI in DM2, etc). Because multiple comparisons were made, one for each of the eight muscles, a Bonferroni adjustment was applied to control for type I error. To reach statistical significance a Bonferroni-adjusted P value of 0.05 was required. (2) A similar chi-square test with Bonferroni adjustment was performed when comparing the severity of myotonia in distal and more proximal muscles within each DM group (e.g., FDI vs. deltoid in DM1). (3) The non-parametric bivariate Spearman correlation coefficient was used to determine the correlation between muscle strength and myotonia, both quantitated with ordinal, discontinuous scales (e.g., the 10-point modified MRC scale, and the 9-point myotonia scale). (4) Similarly, the Spearman coefficient was used to determine whether myotonic discharge type is a function of the overall severity of EM in DM1 and DM2 by calculating the strength of the correlation between severity of myotonia (e.g., percent of insertions evoking a myotonic discharge) and the ratio of waxing–waning to waning discharges for each muscle.

RESULTS

Clinical Features. There were 17 DM2 (7 women, 10 men) and 16 DM1 patients (7 women, 9 men) enrolled in the study. DM2 patients were slightly, but not significantly, older (mean 51 years, range 31–74) than the DM1 group (mean 43 years, range 25–61), P < 0.06. The 16 DM1 patients represented 14 separate kindreds (2 patients were sisters, and 2 were mother and son), and the 17 DM2 patients represented 12 separate kindreds (2 patients were sisters, 2 were uncle and nephew, 2 were uncle and niece, and 3 were first cousins). No congenital DM1 patients were included. All patients were ambulatory and fully independent in all activities of daily living. On neurologic examination, besides evidence of myotonia, all DM1 patients had distally predominant limb weakness, whereas all DM2 patients had proximally predominant limb weakness.

Severity of Myotonia. All DM1 and DM2 patients had EM on at least one insertion from one muscle (Fig. 1A, last column), with the most distal muscles (TA, FDI) most likely to have a myotonic discharge in both disorders. However, the percent of patients with myotonia was not significantly greater in DM1 than DM2 in any of the muscles individually or combined. When the analysis was expanded to include all needle insertions (1024 in DM1, 1088 in DM2), the percent of insertions that successfully evoked myotonia was found to be significantly greater in DM1 (54%) than DM2 (41%) (Fig. 1B, last column). Similarly, on analyzing the muscles individually using all the insertions (128 per muscle in DM1 and 136 in DM2), the percent of insertions evoking myotonia was significantly greater in DM1 than DM2 in all four arm muscles and in the most distal leg muscle (TA; Fig. 1B). In two proximal leg muscles (VL and TFL), the percent of insertions that evoked EM was significantly greater in DM2 than DM1 with myotonic discharges found in 60% of VL and 28% of DM2 TFL insertions compared to 45% and 9% in DM1, respectively. In the TPs, the percent was not significantly different (42% DM1, 45% DM2).

Type of Myotonia. The major myotonic discharge type was waxing–waning in DM1 (61% of discharges; Fig. 2A, Fig. 3A, last column) vs. waning in DM2 (64%; Fig. 2B, Fig. 3B, last column). Notably, 4 DM2 (24%), but none of the DM1 patients, exhibited only waning myotonia on all insertions that evoked myotonic discharges. Even in TFL and VL, the muscles in which EM was significantly more evocable in DM2 than DM1, waning discharges still predominated and accounted for about 60% of EM in DM2 patients (Fig. 3B). The majority of waning discharges in DM2 patients were characterized by decline in both frequency and amplitude of the muscle-fiber potentials. However, some waning discharges were observed in which only frequency, and not amplitude, declined. We categorized these as myotonic discharges of the waning type, but recognize that they are in fact
non-specific and could also be labeled as fibrillation potentials or positive waves, which are known to decline in frequency while maintaining fairly constant amplitude.

It might be argued that, in the spectrum of EM, waning discharges are simply a milder form of myotonia in DM2, and waxing–waning discharges are a more severe form in DM1. We investigated this possibility by plotting the ratio of waxing–waning to waning discharges on the y-axis against severity of myotonia on the x-axis for both DM1 and DM2. We found that, although the ratio was positively correlated with severity of myotonia in DM1 \( (r = 0.81, P < 0.015) \), it was not correlated with myotonia severity in DM2 (Fig. 4). We did not observe complex repetitive discharges, high-frequency single-spike discharges greater than 180 Hz, or fasciculation potentials in our cohort of DM2 patients, as reported by others.\(^{16,18}\)

**Distribution of Myotonia.** In both DM1 and DM2, evocable myotonia was most prominent in distal limb muscles (FDI and TA) and decreased significantly in a distal to proximal gradient in the arm and leg. In the arm, the percent of insertions evoking myotonia in FDI (88%/57% in DM1/DM2) was significantly greater than in deltoid (33%/27%, \( P < 0.0001 \) for DM1, \( P < 0.0001 \) for DM2). In the leg, the percent of insertions evoking myotonia in TA (73%/45% in DM1/DM2) was significantly greater than in TFL (9%/21%, \( P < 0.0001 \) for DM1, \( P < 0.0001 \) for DM2).

Although limb EM declined in a distal to proximal gradient in both DM1 and DM2, the drop-off in evocable myotonia from FDI to deltoid in the arm and from TA to TFL in the leg appeared to be steeper for DM1 and flatter for DM2 patients (Fig.

**FIGURE 1.** (A) The percent of patients in whom EM was evocable in each of the eight muscles in one or more of the eight insertions per muscle is shown. Filled bars: DM1; open bars: DM2. All DM1 and DM2 patients had EM in at least one muscle (last column), particularly in distal limb muscles (TA and FDI). (B) The percent of insertions that evoked myotonia is shown. An asterisk indicates a significant statistical difference between DM1 and DM2. Myotonia is more frequently evoked in DM1 than DM2 in all arm muscles, the TA, and overall. The exceptions are VL and TFL in which EM is more frequently evoked in DM2 than DM1.

**FIGURE 2.** (A) Two-second myotonic discharge in a DM1 patient with typical waxing and waning frequency and amplitude; maximal frequency about 60 Hz, minimal about 8 Hz. (B) Four-second myotonic discharge (two successive oscilloscope sweeps) in a DM2 patient in which frequency and amplitude gradually decline with no waxing component; maximal frequency toward onset about 23 Hz, minimal toward termination about 19 Hz.
1A and 1B), suggesting greater proximal myotonia than expected (relative to the distal myotonia) in the DM2 than DM1 patients. The difference in steepness of drop-off in EM between DM1 and DM2 was not significant in the arm, but in the leg it was; that is, the drop in EM from TA to TFL was significantly less in DM2 than DM1 (P < 0.0001). Even more striking was the absence of a drop-off in EM from TA to VL in DM2, and the presence of a sharp drop in EM from TA to VL in DM1 (Fig. 1B, P < 0.0001).

**Correlation of Myotonia With Muscle Strength.** Correlation of the modified 10-point MMT score with the 9-point myotonia score in the four muscle groups (two in the arm and two in the leg) in which we had both of these data available showed that EM and muscle strength were negatively correlated in DM1 patients, but were not correlated in DM2 (Table 1).

**DISCUSSION**

Our study has the advantages of genetically confirmed diagnoses, blinded investigators, a prospective study design, a standardized concentric-needle EMG examination protocol, a simple method to quantitate EM, and correlation with MMT results. EM was graded as either present (1) or absent (0) in each of 8 EMG needle insertions per muscle for a maximum myotonia score of 8 and a minimum score of 0 for each muscle. We believe this method is somewhat easier to apply than previous methods of quantitating EM on a 1–4 scale in which the electromyographer is required, for example, to differentiate discharges that are present “in 2 or more muscle areas” (grade 2) from those that are present in “most needle locations” (grade 3).21

**Table 1. Correlation of MMT with myotonia scores.**

<table>
<thead>
<tr>
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<th>DM1</th>
<th>DM2</th>
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<tr>
<td>r value</td>
<td>p value</td>
<td>r value</td>
</tr>
<tr>
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**FIGURE 3.** The percent of myotonic discharges that had a waxing and waning pattern (A) versus a waning pattern (B) is shown. Filled bars: DM1; open bars: DM2. An asterisk indicates a significant statistical difference between DM1 and DM2. Myotonic discharges in DM1 are more likely to have a classic waxing–waning pattern, while discharges in DM2 are more likely to have a waning pattern.

**FIGURE 4.** The mean percent of insertions that evoke EM on the x-axis versus the mean ratio of waxing–waning to waning discharges for each muscle. Filled squares: DM1; open triangles: DM2. The two muscles with the highest (FDI for DM1, VL, for DM2) and lowest mean myotonia severity scores (TFL for DM1 and MD for DM2) are also indicated. In DM1, as severity of myotonia increases, the evoked discharges transition from waning to waxing–waning (r = 0.81, P < 0.015). By contrast, in DM2, there is no dependence of myotonic discharge type on severity of EM.
The first conclusion from this study is that for all muscles combined, the severity of EM (as quantitated by the percent of insertions that evoked myotonic discharges) was significantly greater in DM1 than DM2 (Fig. 1B), as suspected by other investigators.\textsuperscript{5,10} This was the case for all tested arm muscles and in the one sampled distal leg muscle (TA). Only in VL and TFL, both proximal leg muscles, was severity of EM greater in DM2 than DM1, but notably TFL, the more proximal of these two muscles, was not actively myotonic in that only 28\% of insertions evoked EM in DM2.

Second, the study has shown that in DM1, classic waxing–waning discharges predominate, whereas in DM2, less-specific waning discharges are the rule. This makes electrical diagnosis of DM2 more challenging, especially in the sizable minority of DM2 patients (24\% in this study) in whom classic myotonic discharges were not elicitable in any of the eight muscles tested and only waning discharges were found. In our experience, needle EMG is decreasingly performed for diagnostic purposes in DM1 patients in whom the diagnosis is usually evident at the bedside and easily confirmed with genetic testing. DM2 patients, by contrast, are more likely to be referred to the EMG laboratory because the diagnosis is often less clear on clinical grounds. Clinical myotonia can be subtle, intermittent, or absent. Facial weakness is often absent, and limb muscle weakness is typically proximal, and sometimes associated with muscle hypertrophy or a pain syndrome.\textsuperscript{5,17,18,23,25} It is important therefore for electromyographers to be aware that EM in DM2 has different characteristics than in DM1, and in particular that less specific waning discharges are common in this disorder. One unifying hypothesis is that waning discharges are a manifestation of a milder form of EM in DM2 and waxing–waning discharges of a more severe form of EM in DM1. Our data do support this hypothesis for DM1, in that, as severity of myotonia increases, the ratio of waxing–waning to waning discharges also increases (Fig. 4). This relationship was not observed in DM2 patients, suggesting that waning discharges in DM2 are not simply indicative of a less severe form of myotonia.

Third, the distribution of EM is surprisingly similar in DM1 and DM2, particularly in the arm. Our hypothesis was that the distribution of EM would conform to the distribution of muscle weakness in the two disorders: more distal in DM1 and more proximal in DM2. In DM1, we confirmed that myotonia is far more evocable distally in the arm and leg, as noted by others,\textsuperscript{14,21} and all our DM1 patients had distally predominant limb weakness on neurologic examination. Contrary to what we expected, myotonia was also worse distally in DM2, at least in the arm, in which the steepness of the drop-off in myotonia from distal to proximal muscles was not significantly different than in DM1. The leg was different in that, although evocable myotonia in the most distal muscle (TA) in DM2 was significantly greater than in the most proximal muscle (TFL), the drop-off in evocable myotonia from TA to TFL and from TA to VL was significantly flatter for DM2 than for DM1 (see Fig. 1B). Thus, in the leg, relative to the severity of distal myotonia in TA, we observed greater proximal muscle myotonia in TFL and VL in DM2 than DM1, consistent with the previous result that absolute severity of myotonia was lower in TA but greater in VL and TFL in DM2 than DM1.

Fourth, the correlation of myotonia and muscle strength was also unexpectedly different in the two disorders. In DM1, the MMT and myotonia scores were negatively correlated, whereas in DM2 they were not. This observation fits with the previous one in which EM was unexpectedly more prominent distally in DM2, although all DM2 patients in this study had proximally predominant limb muscle weakness. It has recently been suggested that myotonia in both DM1 and DM2 is due to a toxic RNA repeat mechanism (CUG in DM1 and CUUG in DM2) leading to sequestration of muscle-blind proteins in myonuclei\textsuperscript{8} and aberrant splicing of the muscle-specific chloride channel.\textsuperscript{4,11} The mechanism of muscle weakness in these diseases is still unclear, but our data suggest that, although the molecular causes of weakness and myotonia may be closely allied in DM1, in which myotonia and weakness are correlated, they may be more disparate in DM2, in which myotonia and weakness are not correlated.

This study has several strengths, but we recognize that there are some weaknesses as well. Although the study benefited from a standardized EMG examination, sampling only eight muscles may not have been enough to unequivocally determine the proximal to distal distribution of limb EM in these two disorders, particularly in the leg in which only three muscles were sampled. However, in order to enroll as many subjects as possible in a study that included multiple components, we were obligated to limit the EMG testing to an essential group of muscles commonly tested in our laboratory in patients with suspected muscle disease in general and myotonic dystrophy in particular. The assumption that severity of EM can be quantitated with a 0–8 myotonia score is potentially problematic because not all portions of a muscle can be examined with 8 insertions and the score itself does not take into account myotonic discharge...
duration or density. Still, we believe that the blinded, prospective nature of the study and use of conservative statistics do protect against spurious results, a conclusion supported by the fact that our severity and distribution data in DM1 are concordant with earlier work on EM in this disorder.21 Finally, there could also be a selection bias in a study of this kind. Because DM2 is more likely to be underdiagnosed in general compared with DM1, the DM2 patients enrolled may be on the more severe end of the DM2 disease spectrum than the DM1 patients on their spectrum. The observation that DM2 patients were slightly older than the DM1 patients in this study may, in part, be a result of such a selection bias. But, to the extent that myotonia is proportional to disease severity in DM1 and DM2, the greater severity of EM in DM1 than DM2 that we observed is likely an underestimate of the differences between the two groups as a result of this kind of bias.

Despite its limitations, this study has identified significant differences in the severity, type, and distribution of EM in DM1 and DM2, as well as in the correlation between muscle weakness and myotonia in the two disorders. The basis for these differences is yet to be determined. Some physiologic clues to them may be drawn from previous reports on the disparate responses of EM in the two diseases to changes in potassium concentration as well as to apamin,17 to short exercise testing,19 and to temperature.20 At the molecular level, we can only speculate that DM1 and DM2 have some splicing abnormalities in common (e.g., muscle-specific chloride channel) that account for the presence of myotonia in both diseases, as well as other, yet to be identified, disease-specific abnormalities that account for the qualitative and quantitative differences in myotonia between the two disorders.

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ABSTRACT: The aim of the present study was to further confirm the validity of measurements for characterizing neuromuscular alterations by establishing their reliability both before and after fatigue. Thirteen men (28 ± 5 years) volunteered to participate in two separate identical sessions requiring the performance of a sustained maximal voluntary contraction (MVC) with the quadriceps muscle for 2 min. MVC and transcutaneous electrical stimulations were used before and immediately after the fatiguing contraction to investigate the reliability of MVC torque, central activation, and peripheral variables (M-wave properties, peak twitch, peak doublet) within and between sessions. Based on previous and present results, we advise the use of (1) voluntary activation level with potentiated doublet as a reference to describe central fatigue, (2) electromyographic activity of vastus lateralis muscle as a surrogate for quadriceps for both voluntary and evoked contraction, and (3) potentiated peak doublet amplitude to investigate contractile fatigue. These findings can be useful in the choice of the parameters describing central and peripheral fatigue of the quadriceps muscle in future studies.

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ASSESSMENT OF THE RELIABILITY OF CENTRAL AND PERIPHERAL FATIGUE AFTER SUSTAINED MAXIMAL VOLUNTARY CONTRACTION OF THE QUADRICEPS MUSCLE

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Muscle fatigue can be defined as an exercise-induced reduction in maximal voluntary muscle force. Although the underlying mechanisms have not been fully described, it is accepted that central fatigue (including alteration in processes located above the neuromuscular junction) can be distinguished from peripheral fatigue. The use of voluntary contraction and noninvasive transcutaneous electrical stimulation combined with surface electromyography allows exploration of the sites of neuromuscular fatigue in humans.

Data from recent studies suggest that voluntary activation level with reference to the potentiated doublet (VALp) is the most valid method based on the twitch interpolation technique to assess the level of muscle activation and therefore central fatigue. However, other authors have used the central activation ratio (CAR) or the voluntary activation level with reference to the resting doublet (VALr). Furthermore, central fatigue can also be investigated as the change in the electromyographic (EMG) signal recorded during the maximal voluntary contraction (MVC) normalized to the compound muscle action potential (M wave). A previous study showed that EMG activity of the vastus lateralis (VL) muscle could be considered as a surrogate of quadriceps muscle EMG activity, but it remains to be determined whether this finding is consistent under fatigue for both voluntary and evoked contractions (M wave). Kufel et al. suggested that the potentiated twitch is better for de-
tecting peripheral fatigue than the unpotentiated twitch. Nevertheless, the amplitude of the unpotentiated mechanical response induced by a single stimulus (twitch) or paired stimuli (doublet) is still considered in a number of studies to describe contractile fatigue. The reliability of these measures during the day and between days has never been examined in the fatigued condition. Therefore the purpose of the present study was to further confirm the validity of the measurements used to characterize neuromuscular alterations by establishing their reliability both before and after fatigue.

**MATERIALS AND METHODS**

**Subjects.** A convenience sample of 13 men (27.8 ± 4.9 years, 177.9 ± 6.1 cm, 72.8 ± 7.4 kg, mean ± SD) without any known neuromuscular disorder volunteered to participate in this study. All subjects were well familiarized with voluntary and evoked contraction of the quadriceps, having participated in several experiments on this muscle in the laboratory during the past 3 years. They were asked to refrain from strenuous physical activity for 24 h prior to each testing session and to maintain their normal physical activity level during the experimental period. The procedures were conducted according to the Declaration of Helsinki. Prior to the study, each subject gave written consent and the University of Burgundy Committee on Human Research approved the study protocol.

**Apparatus.** A Biodex isokinetic dynamometer (Shirley, New York) was adjusted to position the knee joint at 75° of flexion (0° = leg fully extended) and the hip joint at an angle of 90°. Experiments were performed on the dominant (right for all subjects) lower limb. Quadriceps muscle was chosen as (1) it has been extensively used in previous fatigue studies, and (2) EMG activity and muscular torque induced by electrically evoked contractions are easy to record. The axis of the dynamometer was aligned with the knee extension axis, and the lever arm was attached to the shank with a strap. Extraneous movement of the upper body was limited by two crossover shoulder harnesses and a belt across the abdomen. Subjects were asked to cross their arms during the testing procedure. Gravity correction was performed to account for the weight of the limb.

**Protocol.** The experimental procedure consisted of two sessions, separated by 3–5 days. The following design was strictly repeated for the two different days.

**Warm-Up.** Before any measurement, subjects performed a warm-up that included ~10 brief and non-fatiguing submaximal contractions of the knee extensor muscles. This was followed by a 2-min rest period before starting the experimental procedure in order to standardize the protocol.

**Prefatigue and Postfatigue Tests.** A graphic overview of the protocol for one session is provided in Figure 1. A series of neuromuscular tests were applied to each subject. This sequence lasted 33 s and included: (1) three electrically evoked twitches separated by 3 s (total duration of 9 s); (2) one 5-s knee extensors MVC torque assessment with doublet delivered 3 s before (resting doublet), over the isometric plateau (superimposed doublet), and 2 s after the MVC (potentiated doublet) (total duration of 15 s); and (3) three electrically evoked twitches separated by 3 s (total duration of 9 s).

This sequence was performed twice before fatigue with 1 min rest to allow for recovery after the

![FIGURE 1. Graphic overview of the procedures used for one of the two sessions. (A, B) Sequences pre 1 and pre 2; (C) 2-min sustained MVC; (D, E) sequences post 1 and post 2. Small arrow, single stimulation; large arrow, paired stimulation.](image-url)
first MVC and twice after fatigue with a rest period of about 10 s to limit the recovery process. The greatest level of torque achieved by the subjects before the fatiguing exercise was taken as the MVC torque. Before fatigue, the two trials for MVC were within 5% for every subject. The procedures were identical for the second session.

Fatigue Test. After a recovery time of 3 min, subjects had to perform a MVC for 2 min\(^2\) with the quadriceps femoris muscle. Strong verbal encouragement was provided by the investigators throughout the duration of the sustained MVC.

Electrical Stimulation. Transcutaneous electrical stimulation was induced using a high-voltage (maximal voltage 400 V) constant-current stimulator (model DS7, Digitimer, Welwyn Garden City, United Kingdom). The femoral nerve was stimulated using a monopolar cathode ball electrode (0.5-cm diameter) pressed into the femoral triangle by the same investigator (N. P.) during all experiments. The site of stimulation, i.e., the position giving the greatest visible contraction of the whole quadriceps muscle group, was marked on the skin so that it could be utilized again after the sustained contraction. The anode was a 50-cm\(^2\) (10 × 5 cm) rectangular electrode (Compex SA, Ecublens, Switzerland) located in the gluteal fold opposite the cathode. The optimal electrode (center-to-center) distance of 20 mm. The femoral nerve was stimulated using a monopolar cathode ball electrode (0.5-cm diameter) pressed into the femoral triangle by the same investigator (N. P.) during all experiments. The site of stimulation, i.e., the position giving the greatest visible contraction of the whole quadriceps muscle group, was marked on the skin so that it could be utilized again after the sustained contraction. The anode was a 50-cm\(^2\) (10 × 5 cm) rectangular electrode (Compex SA, Ecublens, Switzerland) located in the gluteal fold opposite the cathode. The optimal electrode (center-to-center) distance of 20 mm. The femoral nerve was stimulated using a monopolar cathode ball electrode (0.5-cm diameter) pressed into the femoral triangle by the same investigator (N. P.) during all experiments. The site of stimulation, i.e., the position giving the greatest visible contraction of the whole quadriceps muscle group, was marked on the skin so that it could be utilized again after the sustained contraction. The anode was a 50-cm\(^2\) (10 × 5 cm) rectangular electrode (Compex SA, Ecublens, Switzerland) located in the gluteal fold opposite the cathode. The optimal electrode (center-to-center) distance of 20 mm.

Surface EMG activity of the vastus lateralis (VL), vastus medialis (VM), and rectus femoris (RF) muscles was recorded with pairs of silver chloride, circular (recording diameter, 10 mm) surface electrodes (Controle Graphique Medical, Brie-Comte-Robert, France) positioned lengthwise over the middle of the muscle belly with an interelectrode (center-to-center) distance of 20 mm. These recording sites were determined in pilot testing at the beginning of the first test session by eliciting the largest M wave for each muscle via femoral nerve stimulation and marked so that they could be reutilized for the second test session. Low resistance between the two electrodes (< 5 kΩ) was obtained by abrading the skin, and oil and dirt were removed from the skin using alcohol. The reference electrode was fixed to the patella of the opposite leg. Myoelectrical signals were amplified with a bandwidth frequency ranging from 15 Hz to 5 kHz (common mode rejection ratio, 90 dB; impedance input, 100 MΩ; gain, 1,000), digitized on-line at a sampling frequency of 2 kHz using a computer (IPC 486; Newton PC, Dijon, France) and stored for analysis with commercially available software (Tida; Heka elektronik, Lambrecht/Pfalz, Germany).

Data Analysis. The three trials for the mechanical twitch response were averaged and further analyzed. Peak twitch (Pt), resting peak doublet, and potentiated peak doublet were analyzed. Peak-to-peak amplitude and duration of the M wave were analyzed for VL, VM, and RF muscles with the average of the three trials used for analysis.

Global central activation was determined with the three different calculations most often used when assessing central fatigue.

Central activation ratio (CAR) is equal to peak MVC divided by the sum of peak MVC and superimposed doublet amplitude.\(^{20}\) Maximal voluntary activation level with reference to the resting doublet (VAL\(_r\)) is calculated as \([1 − (superimposed doublet amplitude/resting doublet amplitude)] \times 100.\(^{28}\) Maximal voluntary activation level with reference to the potentiated doublet (VAL\(_p\)) is \([1 − (superimposed doublet amplitude/potentiated doublet amplitude)] \times 100.\(^{4}\)

A correction was consistently applied to the original equation when the superimposed doublet was elicited slightly before or after the real maximal voluntary torque.\(^{49}\) In these cases, VAL was calculated as \([1 − (superimposed doublet amplitude × voluntary torque level just before the superimposed doublet/maximal voluntary torque)/potentiated doublet amplitude] \times 100.\(^{4}\)

Furthermore, EMG activity of the VL, VM, and RF muscles collected during the knee-extensor MVC was quantified as the root mean square (RMS) for a 0.5-s interval at peak force (250-ms interval either side of the peak force). Maximal RMS values for VL, VM, and RF muscles were then normalized to the amplitude of the M wave for the respective muscles, in order to obtain RMS.M\(^{-1}\) ratio. This normalization procedure accounted for peripheral influences including neuromuscular propagation failure and changes in impedance from the EMG recordings.
Statistical Analysis. Statistical tests were conducted for the following variables: Pt, resting peak doublet, potentiated peak doublet, M-wave amplitude and duration of VL, VM, and RF muscles, MVC torque, torque during the sustained MVC, CAR, VALr, VALp, and RMS.M⁻¹ ratio for VL, VM, and RF muscles. A Kolmogorov–Smirnov test revealed that all these variables were normally distributed. Data are reported as means ± standard deviation (SD) within the text and tables.

Separated three-way ANOVAs [sequence (e.g., pre 1 vs. pre 2) × time (pre- vs. post-fatigue) × session (1 vs. 2)] were used to compare dependent variables when appropriate. Post-hoc analyses (Tukey’s honestly significant difference test) were used to test for differences among pairs of means when appropriate. A significance level of P < 0.05 was used to identify statistical significance.

Relative reliability concerns the degree to which individuals maintain their position in a sample with repeated measurements.5 We assessed this type of reliability with the intraclass correlation coefficient (ICC) (2,1), a two-way random effects model with single measure reliability in which variance over the repeated session is considered.38 The ICC indicates single measure reliability in which variance over the measurements/mean of these two measurements (typical error) or as a proportion of the total variance in scores. ICC was not calculated for CAR, VALr, and VALp due to the ceiling effect (poor between-subject variability) associated with these measurements.

Absolute reliability is the degree to which repeated measurements vary for individuals; this type of reliability is expressed either in the actual units of measurement (typical error) or as a proportion of the measured values (coefficient of variation, CV). Both typical error and CV refer to intrasubject variation between two measurements. Typical error (or standard error of measurement) was calculated as standard deviation of the differences between the two measurements/√2. CV was calculated for each subject as standard deviation of the two measurements/mean of these two measurements × 100. Mean CV corresponding to the 13 subjects was then reported.

Bland and Altman’s 95% limits of agreement, another measure of absolute reliability, was used to explore reproducibility of the central activation parameters for which no ICC was calculated (i.e., CAR, VALr, and VALp). This was done in the following manner: (1) the difference between the two measurements for each subject was plotted against their average value of the two trials, and (2) the plots were examined to see whether any data points were beyond 1.96 × SD of the differences (95% confidence limits) above and below the mean of the two trials.13 As recommended by Atkinson and Nevill,5 we also determined from the Bland and Altman plot the presence of heteroscedasticity (when the differences depend on the magnitude of the mean) by using the same criterion as the recent study of Clark et al.13 A correlation (R²) between the absolute differences and the mean values between 0 and 0.1 is considered homoscedastic (no relation between error and the size of the measured variable), and R² values greater than 0.1 are heteroscedastic.13 In this last case, limits of agreement ratio (LOA ratio) was calculated as (1.96 × SD diff/grand mean) × 100, where “SD diff” represents standard deviation of the differences between the two tests and “grand mean” is (mean of test 1 + mean of test 2)/2. In the case of homoscedasticity, systematic bias (mean of the differences between test 1 and test 2) and random error (SD of the differences × 1.96) were determined.5

Statistical analyses were performed using Statistics software for Windows (Statsoft, version 6.1; Statistica, Tulsa, Oklahoma). ICC and typical error analyses were done using a downloadable spreadsheet.19

RESULTS
Fatigue. The torque produced during the 2-min sustained MVC decreased significantly for the two sessions (on average from 263 ± 42 N.m at the beginning of the contraction to 59 ± 19 N.m at the end of the 2 min (P < 0.001), i.e., a loss of 77 ± 8%). No significant difference in torque at start, middle, and end of the fatiguing contraction was found between the two sessions, indicating a comparable effort from the subjects during the fatiguing task of the two sessions.

To explore whether the model we chose induced neuromuscular fatigue of the quadriceps muscle and to determine the potential sites of fatigue, three-way ANOVAs with repeated measures was consistently adopted for each dependent variable. ANOVA showed a decrease for all measurements (P < 0.05), except for M-wave amplitude and duration, which were not affected by the fatiguing task (Table 2). Figure 2 illustrates the decrease in MVC torque and the reduction in peak doublet amplitude after the sustained contraction.

From the different parameters depressed following the sustained MVC, only Pt, peak doublet and potentiated peak doublet started to recover between post 1 and post 2 sequences, and this recovery was found whatever the session (on average by 28% ± 22%, 16% ± 12%, and 14% ± 9% for Pt, resting...
peak doublet and potentiated peak doublet, respectively, $P < 0.05$). A one-way ANOVA showed that Pt recovery was faster than for doublets ($P < 0.05$).

**Reliability.** When comparing a measurement between the two sessions and for the same sequence (i.e., pre 1 vs. pre 1, pre 2 vs. pre 2, post 1 vs. post 1, and post 2 vs. post 2), ANOVA failed to show any significant differences for all parameters (no “session” effect, $P > 0.05$), except for the RMS.M$^{-1}$ ratio of RF muscle ($P = 0.035$).

We chose to present intraday reliability (within the same test session) during the first session for every subject and for measurements performed before fatigue only (Table 1); some parameters partially or totally recovered between the two trials after

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**Table 1.** Intraday reliability of neuromuscular function measures before fatigue (session 1 sequence pre 1 vs. session 1 sequence pre 2).

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Intraday reliability before fatigue</th>
<th>Mean CV (SD)</th>
<th>ICC (95% CI)</th>
<th>Typ. err.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVC (N.m)</td>
<td>272 (46) vs. 276 (51)</td>
<td>0.98 (0.94–0.99)</td>
<td>2.2 (1.2)</td>
<td>6.6</td>
</tr>
<tr>
<td>Central parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle activation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAR (%)</td>
<td>97.2 (1.1) vs. 97.6 (1.2)</td>
<td>Not calculated</td>
<td>0.7 (0.5)</td>
<td>0.8</td>
</tr>
<tr>
<td>VAL (%)</td>
<td>90.8 (3.9) vs. 92.2 (4.1)</td>
<td>Not calculated</td>
<td>2.5 (1.7)</td>
<td>2.6</td>
</tr>
<tr>
<td>VALr (%)</td>
<td>92.9 (2.8) vs. 93.7 (2.0)</td>
<td>Not calculated</td>
<td>1.7 (1.2)</td>
<td>1.9</td>
</tr>
<tr>
<td>RMS.M$^{-1}$ ratio VL</td>
<td>0.054 (0.025) vs. 0.051 (0.026)</td>
<td>0.96 (0.86–0.99)</td>
<td>11.2 (10.0)</td>
<td>0.005</td>
</tr>
<tr>
<td>RMS.M$^{-1}$ ratio VM</td>
<td>0.067 (0.018) vs. 0.064 (0.023)</td>
<td>0.75 (0.32–0.93)</td>
<td>15.3 (12.0)</td>
<td>0.010</td>
</tr>
<tr>
<td>RMS.M$^{-1}$ ratio RF</td>
<td>0.108 (0.051) vs. 0.099 (0.040)</td>
<td>0.85 (0.56–0.95)</td>
<td>10.5 (9.9)</td>
<td>0.018</td>
</tr>
<tr>
<td>Peripheral parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-wave properties</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplitude VL (mV)</td>
<td>12.6 (4.6) vs. 12.6 (5.0)</td>
<td>0.99 (0.96–1)</td>
<td>2.7 (2.8)</td>
<td>0.5</td>
</tr>
<tr>
<td>Amplitude VM (mV)</td>
<td>9.8 (3.0) vs. 9.3 (3.3)</td>
<td>0.86 (0.59–0.96)</td>
<td>6.1 (12.2)</td>
<td>1.2</td>
</tr>
<tr>
<td>Amplitude RF (mV)</td>
<td>5.0 (2.2) vs. 5.0 (2.1)</td>
<td>0.99 (0.96–1)</td>
<td>3.4 (3.7)</td>
<td>0.2</td>
</tr>
<tr>
<td>Duration VL (ms)</td>
<td>7.9 (3.3) vs. 7.9 (3.4)</td>
<td>0.99 (0.98–1)</td>
<td>2.6 (3.2)</td>
<td>0.2</td>
</tr>
<tr>
<td>Duration VM (ms)</td>
<td>6.0 (1.9) vs. 6.1 (2.1)</td>
<td>0.98 (0.94–0.99)</td>
<td>3.4 (2.9)</td>
<td>0.3</td>
</tr>
<tr>
<td>Duration RF (ms)</td>
<td>8.6 (2.0) vs. 8.7 (2.1)</td>
<td>0.97 (0.91–0.99)</td>
<td>3.1 (2.8)</td>
<td>0.3</td>
</tr>
<tr>
<td>Contractile properties</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak twitch (N.m)</td>
<td>45.5 (9.9) vs. 45.2 (10.6)</td>
<td>0.98 (0.95–1)</td>
<td>2.1 (1.9)</td>
<td>1.3</td>
</tr>
<tr>
<td>Rest. Peak doublet (N.m)</td>
<td>84.5 (15.2) vs. 87.3 (16.6)</td>
<td>0.98 (0.93–0.99)</td>
<td>2.7 (2.0)</td>
<td>2.4</td>
</tr>
<tr>
<td>Pot. Peak doublet (N.m)</td>
<td>103.7 (17.2) vs. 102.2 (19.0)</td>
<td>0.94 (0.82–0.98)</td>
<td>3.0 (3.2)</td>
<td>4.3</td>
</tr>
</tbody>
</table>

CAR, central activation ratio; CV, coefficient of variation; ICC, intraclass correlation coefficient; MVC, maximal voluntary contraction; 95% CI, lower and upper confidence intervals; Pot, potentiated; Rest, resting; RMS.M$^{-1}$ ratio, maximal RMS normalized to the amplitude of the M-wave; Typ. err., typical error; VALr, maximal voluntary activation level with reference to the potentiated doublet; VALr, maximal voluntary activation level with reference to the resting doublet; VL, vastus lateralis; VM, vastus medialis.
fatigue and the estimation of the reliability in these conditions would be pointless. For reasons of clarity, interday reliability (between the two testing sessions) is presented before exercise for the first sequence immediately after the sustained MVC to limit the number of data (Table 2). To compare conditions would be pointless. For reasons of clarity, interday reliability after fatigue is presented only for the first sequence pre 1 vs. session 2 sequence pre 1) fatigue.*

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Mean value (SD)</th>
<th>ICC (95% CI)</th>
<th>Mean CV (SD)</th>
<th>Typ. Err.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVC (N·m)</td>
<td>272 (46) vs. 278 (45)</td>
<td>0.90 (0.75; 0.97)</td>
<td>3.5 (4.2)</td>
<td>14.0</td>
</tr>
<tr>
<td>Central parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle activation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAR (%)</td>
<td>97.2 (1.1) vs. 97.9 (1.5)</td>
<td>Not calculated</td>
<td>1.2 (1.0)</td>
<td>1.5</td>
</tr>
<tr>
<td>VAL (%)</td>
<td>90.8 (3.9) vs. 92.8 (4.9)</td>
<td>Not calculated</td>
<td>4.3 (4.4)</td>
<td>4.9</td>
</tr>
<tr>
<td>VALp (%)</td>
<td>92.9 (2.8) vs. 94.1 (4.0)</td>
<td>Not calculated</td>
<td>3.1 (2.7)</td>
<td>3.9</td>
</tr>
<tr>
<td>RMS M$^{-1}$ ratio VL</td>
<td>0.054 (0.025) vs. 0.052 (0.027)</td>
<td>0.78 (0.41; 0.93)</td>
<td>12.1 (10.2)</td>
<td>0.012</td>
</tr>
<tr>
<td>RMS M$^{-1}$ ratio VM</td>
<td>0.067 (0.018) vs. 0.061 (0.011)</td>
<td>0.45 (–0.17; 0.81)</td>
<td>13.1 (10.1)</td>
<td>0.011</td>
</tr>
<tr>
<td>Peak twitch (N·m)</td>
<td>45.5 (9.9) vs. 44.4 (10.3)</td>
<td>0.92 (0.74; 0.98)</td>
<td>5.6 (5.0)</td>
<td>2.9</td>
</tr>
<tr>
<td>Rest. peak doublet (N·m)</td>
<td>84.5 (15.2) vs. 83.5 (18.5)</td>
<td>0.85 (0.55; 0.95)</td>
<td>5.9 (6.2)</td>
<td>6.7</td>
</tr>
<tr>
<td>Pot. peak doublet (N·m)</td>
<td>103.7 (17.2) vs. 98.9 (21.4)</td>
<td>0.93 (0.78; 0.98)</td>
<td>5.2 (4.5)</td>
<td>5.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Interday reliability before fatigue</th>
<th>Interday reliability after fatigue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean CV (SD)</td>
<td>ICC (95% CI)</td>
</tr>
<tr>
<td>180 (54) vs. 193 (49)</td>
<td>0.91 (0.77; 0.97)</td>
</tr>
</tbody>
</table>

Typical errors remained in the same range compared with before fatigue.

**Table 2.** Interday reliability of neuromuscular function measures before (session 1 sequence pre 1 vs. session 2 sequence pre 1) and after (session 1 sequence post 1 vs. session 2 sequence post 1) fatigue.*

*Abbreviations are the same as in Table 1.

**Table 3.** Limits of agreement measures of central activation measured within the same session (intraday) before fatigue and between the two sessions (interday) before and after fatigue.*

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Heteroscedasticity</th>
<th>Systematic bias</th>
<th>Random error</th>
<th>LOA ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraday, pre</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAR (%)</td>
<td>No</td>
<td>0.38</td>
<td>2.14</td>
<td></td>
</tr>
<tr>
<td>VAL (%)</td>
<td>No</td>
<td>1.49</td>
<td>7.08</td>
<td></td>
</tr>
<tr>
<td>VALp (%)</td>
<td>No</td>
<td>0.77</td>
<td>5.27</td>
<td></td>
</tr>
<tr>
<td>Interday, pre</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAR (%)</td>
<td>No</td>
<td>0.62</td>
<td>4.08</td>
<td></td>
</tr>
<tr>
<td>VAL (%)</td>
<td>No</td>
<td>2.09</td>
<td>13.72</td>
<td></td>
</tr>
<tr>
<td>VALp (%)</td>
<td>Yes</td>
<td></td>
<td></td>
<td>11.32</td>
</tr>
<tr>
<td>Interday, post I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAR (%)</td>
<td>No</td>
<td>–0.19</td>
<td>8.72</td>
<td></td>
</tr>
<tr>
<td>VAL (%)</td>
<td>No</td>
<td>–0.91</td>
<td>22.93</td>
<td></td>
</tr>
<tr>
<td>VALp (%)</td>
<td>No</td>
<td>–0.54</td>
<td>18.42</td>
<td></td>
</tr>
</tbody>
</table>

CAR, central activation ratio; LOA, limits of agreement ratio; post 1, sequence post 1; post 2, sequence post 2; pre, sequence pre 1; VAL, maximal voluntary activation level with reference to the potentiated doublet; VALp, maximal voluntary activation level with reference to the resting doublet.

*Systematic bias was not significantly different from 0 ($P < 0.05$, tested with a paired t-test).
Figure 3 and Table 3 showed that CAR was the most reproducible measurement of central activation, with the lowest dispersion of the individual data and the lowest random errors. It also appears that random error and absolute difference dispersion is consistently higher for VALr compared to VALp (Tables 1 and 2).

DISCUSSION

The main objective of this study was to confirm the validity of measures classically used to describe fatigue of the neuromuscular system by establishing their reliability both before and especially after a fatiguing task. We found a high level of reproducibility for central parameters and evoked force, and moderate to high repeatability for measurements involving surface EMG (M wave and RMS.M⁻¹ ratio). By combining previous results on validity and present findings on reliability, the discussion will aim to provide methodological recommendations in the choice of variables that should be used in further studies to describe fatigue along the neuromuscular system.

Origin of Fatigue. The 2-min sustained MVC was chosen in the present study as it induces both central and peripheral fatigue.37 We observed a significant drop in MVC after the fatiguing contraction (−33%). Furthermore, significant central fatigue (−4.1% for CAR, −11.6% for VALr, −9.0% for VALp, and −22.4% for RMS.M⁻¹ ratio on average for the three knee extensor muscles investigated) was reported during the MVC performed immediately after the sustained isometric contraction, suggesting that inability to voluntarily recruit all the motor units of quadriceps femoris muscle, or a sub-optimal discharge rate of the motor units pool,41 accounts for the loss in voluntary force. It might be that central fatigue was underestimated to a similar extent for the two sessions, as superimposed MVCs were conducted a few seconds after the sustained MVC; however, subjects experienced a large tremor near the end of the 2-min contraction, and the superimposition at that time would have led to biased calculations.

We also found an excitation–contraction coupling failure, as shown by the decrease in force evoked by electrical stimulation after fatigue (−30.0% for peak twitch, −19.1% for resting peak doublet, and −96.5% for potentiated peak doublet). The unchanged M wave after the sustained MVC indicated no failure to drive action potentials to the muscle fibers, as previously observed after sustained MVC of hand muscles,9 and suggests that alterations in calcium kinetics beyond the muscle cell-membrane2 might have occurred. However the main aim of the present work was to determine the level of reliability for classic neuromuscular measurements after fatigue, in order to assess whether the same fatiguing exercise induced comparable alterations.
Which Variables Should We Consider When Assessing Neuromuscular Fatigue? We assessed both intraday and interday (3–5 days interval) reliability of selected parameters before fatigue. All variables had a lower level of variability within the same session than between sessions, as confirmed by CV and typical error values. This observation was the same for relative reliability (ICC), which describes the agreement between the repeated measures. We have not provided threshold values for ICC, as no consensus really exists; as suggested by Morrow and Jackson,29 the reader has to determine the “practical significance” of the obtained reliability.

MVC, the “gold standard” of muscle fatigue, was highly reproducible, with ICC > 0.90 and CV < 8%; MVC has already been reported to be highly reproducible without fatigue for quadriceps muscle, with CV <7% within a testing session and equal to 10% across sessions separated by 2–7 days.24,41 Todd et al.41 observed an ICC of 0.79 between sessions, slightly below our 0.90, which could be explained by the larger interval between the two testing sessions in their study (1 week). Fatigue did not alter variability of MVC torque, as typical error after the 2-min sustained MVC was equivalent to that calculated before fatigue (~15 N.m). Furthermore, CV was in line with a previous study which assessed MVC reproducibility after a sustained 4-min isometric MVC of the dorsiflexor muscles.20 Altogether, these findings suggest that performing a MVC after a fatiguing exercise is a reliable measure to detect muscle fatigue.

The validity of three methods based on interpolated twitch technique has been investigated in previous studies. For example, the CAR method has been criticized,39 as the calculation compares a superimposed force with a voluntary contraction in which additional synergistic muscles not recruited by the stimulation could contribute to torque production.7 We observed that this technique led to an overestimation of central activation compared to the VAL method, where the superimposed evoked torque is compared with a resting evoked torque. Furthermore, Bilodeau10 reported that the VAL method is more sensitive to fatigue than CAR. It appears also that the use of a potentiated doublet as a reference is more appropriate, as the superimposed response is also under the influence of potentiation during the MVC.14 Our results showed that the three indexes of central activation based on twitch interpolation were highly consistent before fatigue, with CV and typical error lower than 5%, confirming previous studies.3,6,41 Fatigue resulted in additional but acceptable variability, with CV < 8%. It is noteworthy that CAR was more reliable than VAL, both before and after fatigue. Furthermore, VALp was slightly but consistently more reproducible than VALr. The analysis of limits of agreement revealed that random error (i.e., biological or mechanical variation) could account for the vast majority of the observed variance for central activation parameters, as systematic bias, referring to a general trend for measurements to be different in a particular direction between repeated tests, was not significantly different from 0.5 As for CV and typical error, random error was consistently lower for CAR than for VAL measures and slightly lower for VALp than VALr. As VALp, before fatigue and between sessions was heteroscedastic, the LOA ratio was calculated and it can be said that “any two tests will differ due to measurement error by no more than 11% either in a positive or negative direction.”15 Finally, Figure 2 showed that data dispersion was limited for all measures of central activation, as all the test–retest differences (except one for VALr after fatigue) were within the 95% limits of agreement that were calculated for those differences. However, the results concerning LOA ratio have to be considered cautiously because of potential variability from sample to sample (based on the relatively small sample size). Our results showed a high level of reliability for the three methods to determine muscle inactivation after fatigue; within the context of validity discussed above, we propose that VALp should be used to describe the central component of fatigue.

Although less used in the literature than the twitch interpolation technique, RMS.M−1 ratio is another technique to detect neural alterations due to fatigue.27 This method is based on surface electromyography and thus allows changes in descending drive between the synergists of a muscle group to be distinguished. Few studies have compared the reliability of EMG measurements across the knee extensor muscles. For example, Pincivero et al.31 reported a greater reliance on EMG of the VL than VM and RF muscles to estimate muscle torque at submaximal levels. Mathur et al.25 reported comparable ICC and standard errors of EMG measurements between VL, VM, and RF during sustained isometric contractions at 20% and 80% MVC. Finally, Rainoldi et al.34 observed a slightly higher level of reliability of EMG measurements for VL than VM during sustained isometric contractions at 50% MVC and suggested that this was due to a more critical electrode repositioning pertaining to a lower cross-sectional area18 of the latter compared to the former. We obtained quite variable measurements (CV >10%) with ICC > 0.70, as already noticed by Gondin et al.,18 except for the VM muscle before fatigue between sessions (ICC = main body text...
Fatigue added only minor variability in the measurements. Besides, ANOVA revealed a session effect for RF muscle, suggesting that care should be taken when considering EMG activity of RF muscle. Nonetheless the main advantage of this technique is to compare neural alterations between different synergists investigated after a fatiguing task so that several muscles could be considered; thus our results suggest that studies using RMS.M⁻¹ ratio and more generally surface EMG as an index of voluntary drive should be interpreted with caution.

Although it appeared that neuromuscular transmission or propagation was unchanged after fatigue, we assessed repeatability of peak-to-peak amplitude and duration of M wave for the three knee extensor muscles investigated. Reliability was very good within the same testing day for amplitude and duration with CV < 7% and ICC > 0.85, as previously observed for VL and RF muscles.32 However, when assessed between different test days, M-wave variability increased in the present study (CV often > 10% and typical errors three time higher than within the same testing session), whereas M-wave properties are reliable on separate days.12,13,18 As fatigue did not induce further variance, our finding suggests that M-wave parameters are reliable as with without fatigue and thus that M-wave characteristics could be used to detect failure in neuromuscular propagation. Nevertheless, we observed that ICC were relatively low for M-wave amplitude of the VM muscle, confirming the RMS.M⁻¹ ratio results.

It is appropriate to consider which muscle head can be used as a surrogate for the quadriceps femoris. Previous works showed that the VL muscle is representative of the quadriceps muscle.1,43 The current results confirm this assertion, as VL presented the most reproducible data. Besides, the relatively low levels of reliability for VM suggest that this muscle is of secondary importance when considering EMG of the quadriceps. Finally, the intermediate reliability found for the RF and its unique anatomic function within the quadriceps (both knee extensor and hip flexor) indicates that it would be interesting to consider this muscle to investigate muscular synergies within the quadriceps (e.g., the interaction of mono- and bi-articular muscles).35

Contractile failure was assessed with the amplitude of the electrically evoked responses. In a recent study, Kufel et al.23 observed that potentiated evoked responses are more sensitive for detecting early fatigue than non-potentiated ones. Furthermore, they reported that potentiated responses were as reliable as non-potentiated ones, despite the increased variability associated with potentiated twitches reported by Morton et al.30 In the present study, electrically evoked torques were found to be highly reliable before fatigue with ICC > 0.85 and CV < 10%, in agreement with previous studies both for intraday32 and interday41,42 comparisons; as for the MVC, a slightly higher variability, as revealed by greater typical errors, was found between sessions compared to within sessions. Reliability of Pt, resting peak doublet, and potentiated peak doublet was not influenced by the fatiguing contraction and was very close for each of the three parameters investigated. Interestingly, we observed a fast recovery of evoked torque, but it was twofold less for potentiated peak doublet than for Pt (14% vs. 28%, P < 0.05). As reliability was comparable between the three indexes of peripheral fatigue considered here, the most valid measurement should be adopted. It appears that (1) potentiated peak doublet recovery was slower compared to unpotentiated responses immediately after fatigue, and (2) the influence of a preceding contraction on possible potentiation can be discarded when considering potentiated peak doublet, leading to a more sensitive quantification of peripheral fatigue.25 Consequently, these arguments suggest that potentiated peak doublet should be adopted to characterize both contractile impairment and central fatigue (with VALp).

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REFERENCES

THE EFFECT OF ECCENTRIC EXERCISE ON POSITION SENSE AND JOINT REACTION ANGLE OF THE LOWER LIMBS

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Adequate position sense is required for capable and safe human movement. Disturbed position sense (particularly of the lower limbs) may lead to perturbations in daily activities (such as walking) and ultimately to injuries. Position sense is normally expected to be disturbed after muscle-damaging exercise. Eccentric exercise of the upper limbs disturbs position sense in the exercised arm.3,26,37,39,41

Studies that have examined the effect of eccentric exercise on position sense used muscles of the upper limb.3,26,37,39,41 However, it is a common experience that we feel unsteady on our legs and have difficulty in performing common movements after some activities such as downhill walking. This may relate to diminished position sense of the lower limbs and may increase the risk for injuries. To our knowledge, no study has investigated the effect of eccentric exercise on position sense of the lower limb.

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Muscle reaction time is defined as the time from a stimulus to the beginning of a muscle response, and has been used frequently in the evaluation of motor performance.9,13,24 It is frequently measured by asking a subject to move the limb from a resting position to a reference position as rapidly as possible in response to a visual or auditory stimulus.14,19,20,26 Two studies investigated the effect of eccentric exercise on muscle reaction time of the upper limbs after a light stimulus and produced contrasting results.8–10,26 The stimulus in these studies originated from an external source (vision) rather than the muscle. For the present investigation, a new test was developed that measures the reaction angle of the lower limb in response to a stimulus (free fall) originating from the muscle itself.

To our knowledge, there are no data regarding the effect of eccentric exercise on position sense and muscle reaction time of the lower limbs. As the lower limbs play a major role in body movements during exercise and everyday activities, the primary purpose of the present study was to examine whether position sense and joint reaction angle to release can be affected by eccentric exercise–induced muscle damage. Additionally, we present a new test for measuring joint reaction angle to release, performed in a common isokinetic dynamometer.

### MATERIALS AND METHODS

**Subjects.** Twelve healthy untrained women (age 20 ± 1 years, height 168 ± 1 cm, mass 57 ± 2 kg) took part in the study. Subjects had no experience with eccentric exercise training for at least 6 months prior to the study and were not taking anti-inflammatory drugs. They were instructed to abstain from strenuous exercise for 3 days prior to and during data collection. All volunteers were eumenorrheic (reporting their menstrual cycle as lasting 24–30 days). The eccentric exercise trial fell within the luteal phase, during which estrogen concentrations are more stable and higher than during the follicular phase. Subjects read and signed an informed consent form. The study was approved by our institutional review board.

**Research Design and Measurements.** An isokinetic eccentric exercise protocol was undertaken by all participants in their dominant leg while the other leg served as control. DOMS, isometric peak torque, position sense, and knee joint reaction angle to release were examined before, immediately after, and 24, 48, and 72 h post-exercise. Serum creatine kinase (CK) activity was determined at the same time-points, except for immediately after exercise. Each subject was familiarized with the experimental procedures by performing all assessments prior to formal measurements.

**Isokinetic Exercise Protocol.** An isokinetic dynamometer (Cybex Norm, Ronkonkoma, New York) was calibrated weekly according to the manufacturer’s instructions. Subjects were seated (120° hip angle) with the lateral femoral condyle aligned with the axis of rotation of the dynamometer, and were coupled to the dynamometer by an ankle cuff attached proximal to the lateral malleolus. The position of each subject was recorded and used in follow-up measurements. Each subject’s functional range of motion was set electronically between full extension (0°) and 120° of knee flexion to prevent hyperextension and hyperflexion. Gravitational corrections were made to account for the effect of limb weight on torque measurements. Feedback of the intensity and duration of eccentric exercise was provided automatically by the dynamometer.

Prior to each exercise session, subjects performed a warm-up consisting of 8-min cycling on a cycle ergometer (Monark, Vansbro, Sweden) at 70 rpm and 50 W, followed by 5 min of ordinary stretching exercises of the major muscle groups of the lower limbs. Exercise sessions consisted of eccentric contractions of the knee extensors of the dominant lower limb at an angular velocity of 60°.s⁻¹ (knee range, 0° to 90°), while the other leg served as control limb. In each exercise session, subjects had to accomplish 5 sets of 15 eccentric maximal voluntary contractions in the seated position as previously described.2,15,30 A 2-min rest interval was incorporated between sets.

**Muscle Damage Indices.** Each subject determined soreness of the exercised lower limb by self-palpation of the muscle belly and the distal region of the vastus medialis, vastus lateralis, and rectus femoris in a seated position with the muscles relaxed. Perceived soreness was then rated on a scale ranging from 1 (normal) to 10 (very sore) as previously suggested.7,21

The isokinetic dynamometer was used for the measurement of isometric peak torque of quadriceps femoris at 90° knee flexion.22,23,30–32 The best of three maximal voluntary contractions was recorded. There was a 1-minute rest between isometric efforts. Prior to the maximal voluntary contractions, all subjects were familiarized with the experimental procedure.
Blood samples were drawn from an antecubital vein into plain evacuated test tubes. The blood was allowed to clot at room temperature for 30 min and centrifuged at 1500 × g for 10 min. The serum layer was removed and frozen at −30°C until analyzed. Serum CK was determined spectrophotometrically (Spectronic 401; Milton Roy, Rochester, New York) in duplicate using a commercially available kit (Spinreact, Sant Esteve, Spain). The reference range of serum CK activity for females according to this method is up to 170 U.L⁻¹ at 37°C.

**Position Sense at the Knee.** Subjects sat upright on the isokinetic dynamometer (with the trunk tilted back at a 120° hip angle) while the evaluation was performed with or without visual feedback (i.e., blindfolded) in random order. During the evaluation with visual feedback, the limb was in the visual field of the subjects. All assessments were performed on both lower limbs (the non-dominant limb was the control limb) in random order. The angles were automatically recorded by the dynamometer. During determination of the perception of knee joint angle, the limb was moved from full extension (0°) to 90° knee flexion in order to familiarize subjects with the range of motion. Then the investigator positioned the limb at the reference angle (45°), maintained it for 10 s, and returned the lower limb to the initial position (90°). Afterwards, subjects were asked to remember where the reference position was (45°) and reproduce it from memory. Subjects actively moved their limb to the target angle and, when they were satisfied with the angle they had selected, they would hold it for about 2 s. The direction and degrees deviating from the reference angle were recorded. Four efforts were performed and the two best were recorded. The test–retest reliability in the joint reaction angle to release with or without visual feedback was 0.98 in both cases.

**Knee Joint Reaction Angle to Release.** The isokinetic dynamometer was also used for the new test developed for the evaluation of reaction angle of the lower limb. The subjects sat upright (120° hip angle) while the evaluation was performed with or without visual feedback. All assessments were performed on both lower limbs, with the non-dominant limb serving as the control limb. The angles were automatically recorded by the dynamometer. The lower limb was passively positioned by the investigator at one of the four different angles (0°, 15°, 30°, and 45°) in random order. When muscles of the lower limb relaxed at the predetermined angle, the investigator without warning let the limb fall. The muscle belly was palpated by the investigator to ensure muscle relaxation. The instruction given to the subjects was to stop the fall of the limb as soon as it was perceived as being released. The angle through which the leg moved before the subjects managed to stop the motion was recorded and considered the knee joint reaction angle to release. Four trials were performed and the two best were recorded. The test–retest reliability in the joint reaction angle to release with or without visual feedback (measured in 4 individuals on 5 consecutive days) was 0.98 in both cases.

**Statistical Analysis.** The distribution of all dependent variables was examined by the Kolmogorov–Smirnov test and was found not to differ significantly from normal, except for serum CK values. We used two-way analysis of variance (ANOVA; state × time) with repeated measurements on time to analyze DOMS and isometric peak torque. Serum CK activity was analyzed non-parametrically by Friedman’s test.

**Position sense and knee joint reaction angle to release were analyzed by three-way ANOVA (state × visual feedback × time) with repeated measurements on time.** If a significant interaction was obtained, pairwise comparisons were performed through contrasts analysis. The test–retest reliability of the position sense and reaction angle test were determined by performing the intraclass reliability test. Data are presented as mean ± SEM. The level of significance was set at α = 0.05. SPSS version 13.0 was used for all analyses (SPSS, Inc., Chicago, Illinois).

**RESULTS**

**Muscle Damage Indices.** All muscle damage indices are presented in Figure 1. Isometric peak torque
decreased immediately, and at 24 h and 48 h following eccentric exercise in the test limb \((P < 0.05)\), whereas no changes were noted at any time-point in the control limb. Compared to baseline data, DOMS and serum CK activity increased \((P < 0.05)\) at all time-points after exercise.

**Position Sense at the Knee Joint Angle at Absolute Values.** Eccentric exercise disturbed position sense at the knee joint angle, as measured through degrees (in absolute values) deviating from the reference angle only immediately after exercise \((P < 0.05)\), regardless of whether the trial was performed with visual feedback (Fig. 2). In all trials, subjects placed their limbs in a more extended position (i.e., produced negative degree values). Independent of exercise, there were no significant differences in position sense with or without visual feedback.

**Knee Joint Reaction Angle to Release.** Eccentric exercise increased the knee joint reaction angle to release from 0° and 15° \((P < 0.05)\), independent of visual feedback, at most of the post-exercise time-points (Fig. 4). In contrast, the knee joint reaction angle to release from 30° and at 45° remained unaffected by eccentric exercise, independent of visual feedback (Fig. 5).

**DISCUSSION**

The main purpose of the present study was to determine whether an eccentric exercise session designed to cause muscle damage could affect position sense and reaction angle to release of the knee joint of the lower limb. To this end, a new test performed on a common isokinetic dynamometer measuring knee joint muscle reaction angle to release was developed.
We found that eccentric exercise induced muscle damage and impaired position sense immediately after exercise; indeed the limb was constantly extended relative to the reference angle at 45°. Additionally, eccentric exercise increased the knee reaction angle of the lower limb (measured by the new test) after release from 0° and 15° up to 72 h, but not from 30° and 45°.

The special feature of our new test is that it measures the reaction angle of the knee joint by receiving the signal of release from the very muscles of the knee joint. That is, the knee joint receives information for alteration in its position from proprioceptors. In contrast, in other frequently used tests, subjects respond to a visual or auditory stimulus, receiving information for its position from exteroceptors. There are many situations in real life in which a person has to react to stimuli originating from impulses other than visual or auditory ones. This situation may occur, for example, when a person stumbles. Another advantage of the new test is that it does not require any special construction or equipment except for a common isokinetic dynamometer.

Position Sense at the Knee Joint. We found that eccentric exercise disturbed position sense. That is, after exercise the subjects felt that knee extensors were longer than they actually were and so they adopted a position representing a shorter muscle length, placing the lower limb in a more extended position. The most relevant studies to ours are those that have measured position sense after non-eccentric exercise. In general, these studies also reported disturbances to position sense at the knee after the non-eccentric exercise. Additionally, studies that eccentrically exercised the flexor muscles of the elbow reported (in agreement to our findings) impairment of position sense after eccentric exercise of the elbow flexors with subjects placing the exercised arm in a more extended position.

Overall, our position sense data indicate that the subjects placed their limb in a more extended position relative to the reference angle. Because it is generally agreed that signals from muscle spindles contribute to the sense of position and movement of the limbs, it has been proposed that the rise in passive tension after eccentric exercise can mechanically unload muscle spindles. Unloading of muscle spindles can lower their passive discharge rates, leading subjects to flex their muscles more by extending the knee joint. In contrast, in a recent investigation, eccentric contractions of cat muscle did not appear to affect the function of muscle spindles. Another possible explanation for subjects thinking that their muscles were longer than they really were is that, during the position sense test, the subjects supported their limb themselves, which means that knee extensors were contracting during angle reproduction. Therefore, at least some spindles were activated. After eccentric exercise, more muscle activation would be necessary to support the limb, leading to a greater degree of spindles activation and perhaps to a shorter muscle length. We are not aware of any other comparable study, and more studies are needed to substantiate unequivocally whether the function of muscle spindles is affected by eccentric exercise.

A quite unexpected finding of the present study is that vision did not alter position sense. It has been reported that the ability to determine the position of the upper limb in space tends to degrade during visual occlusion, implying that proprioception drifts when it is not calibrated by vision. By contrast, other studies failed to reveal a deterioration in ability to estimate upper-limb location when vision was occluded. In all these previous studies, position sense was assessed in the upper limbs and not in the...
lower limbs as in this study. Additionally, in all these previous studies, position sense was assessed by matching one-upper limb posture with the other limb whereas, in the present investigation, position sense was investigated by subjects trying to match an angle previously shown in the same lower limb. As a result, due to these methodological dissimilarities, no direct comparisons can be made between the present study and those mentioned earlier.

**Knee Joint Reaction Angle to Release.** Control of a single-joint movement is provided by two types of receptors: proprioceptors, which detect stimuli generated by the system itself, and exteroceptors, which detect external stimuli. With the information provided by these receptors, the single joint is able to organize a rapid response to a disturbance in its position. Proprioceptors include muscle spindles, tendon organs, and joint receptors. Exteroceptors include the eyes, ears, and skin.

We found that eccentric exercise increased the reaction angle to release from 0° and 15°. It has been reported that some sarcomeres are disrupted and therefore non-functioning, so that the number of functioning sarcomeres in series can be decreased acutely after eccentric exercise, leading to reduced ability to maintain muscle tension during contraction. This would slow the speed of shortening of a muscle fiber and thus slow the rate of rise of tension. Therefore, the eccentrically exercised muscle cannot equal the tension developed by the undamaged muscle in response to lower-limb release. In addition, the total series compliance of muscle fibers would be expected to be increased due to the presence of disrupted sarcomeres, leading to delayed tension rise in response to stretch. If quadriceps femoris compliance had increased after the eccentric exercise, perhaps quadriceps femoris needed to be stretched further before spindles signalled the fall of the lower limb. Although muscle spindles are considered the most important peripheral receptors for the sensation of position and movement in humans, joint and skin receptors may also contribute. Therefore, disturbances in the signals provided by receptors at the knee joint and skin after eccentric exercise could also contribute to increases in reaction angle to free fall.

Why was there an increase in muscle reaction time only after release from 0° and 15°, and not after release from 30° and 45°? The knee-joint flexion movement produced by the weight of the calf and...
foot depends on the cosine of knee-joint angle and it is at a maximum level at 0°. The difference therefore may be due to the higher velocity of the fall of the lower limb when it falls from full (at 0°) or almost full extension (15°), as compared to angles in more flexed positions (at 30° and 45°). Therefore, it seems that eccentric exercise affects reaction angle of the lower limb only after a release requiring a high-speed response. Type II muscle fibers are mainly responsible for high-speed contractions, and it is well-described that this fiber type is preferentially damaged during eccentric exercise. Consequently, the need for rapid response after fall at or near extension is considerably impaired after eccentric exercise, due to the relatively greater damage of type II fibers. Another possible reason for this difference is the higher discomfort the subjects feel due to the muscle damage at angles where the limb is in a more extended position (0° and 15° compared to 30° and 45°), because a higher level of contraction is needed to maintain the lower limb in the more extended position.

It is worth noting that joint reaction to release (as was the case also in position sense) is affected by exercise similarly and independently of visual feedback. Hence, based on our findings, it is evident that muscle reacts more rapidly to a stimulus acting on it (free fall) than to a visual stimulus. From a mechanistic point of view, it seems that position sense provided by exteroceptors (i.e., sensors outside of muscle) does not offer a more rapid response than that offered by proprioceptors inside muscle alone. In agreement with our findings, other investigators have found that position sense is not disturbed after visual occlusion.

**Time Course of Changes in Position Sense and Reaction Angle at the Knee to Release.** We observed a different time course of position sense and reaction angle to release from 0° and 15° after eccentric exercise, which points to differences in the potential mechanisms governing position sense and reaction angle. Position sense, either measured in absolute or actual values, was impaired only immediately after exercise. This finding suggests that the primary mechanism responsible is the accumulation of metabolites that occurs shortly after exercise. This mechanism, however, cannot explain the persistent impairment in reaction angle to release for up to 3 days during recovery, at a time when all exercise-induced metabolites would have been removed. These prolonged changes in reaction angle suggest that muscle damage occurring 24–72 h after exercise is the main contributor to this effect.
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ABSTRACT: Neuromuscular electrical stimulation (NMES) involves the use of electrical current to facilitate contraction of skeletal muscle. However, little is known concerning the effects of varying stimulation parameters on muscle function in humans. The purpose of this study was to determine the extent to which varying pulse duration and frequency altered torque production and fatigability of human skeletal muscle in vivo. Ten subjects underwent NMES-elicited contractions of varying pulse frequencies and durations as well as fatigue tests using stimulation trains of equal total charge, yet differing parametric settings at a constant voltage. Total charge was a strong predictor of torque production, and pulse trains with equal total charge elicited identical torque output. Despite similar torque output, higher-frequency trains caused greater fatigue. These data demonstrate the ability to predictably control torque output by simultaneously controlling pulse frequency and duration and suggest the need to minimize stimulation frequency to control fatigue.

IMPACT OF VARYING PULSE FREQUENCY AND DURATION ON MUSCLE TORQUE PRODUCTION AND FATIGUE

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Neuromuscular electrical stimulation (NMES) involves the use of electrical current to facilitate contraction of skeletal muscle. It is commonly used during rehabilitation to accomplish a variety of goals and often prescribed to treat muscle atrophy or impaired motor control associated with orthopedic and neurological damage or joint motion dysfunction. In addition, NMES can serve as a means of compensating for loss of voluntary control of skeletal muscles as well as facilitating exercise to restore or supplement function lost due to disease or injury. Nevertheless, inherent disadvantages surround the application of NMES, including decreased subject tolerance and, most notably, rapid onset of fatigue relative to voluntary contractions. It is the latter of these that results in the greatest limitations when NMES applications are used to elicit functional activities.

The characteristics of electrical stimulation known to impact external torque production include the intensity (i.e., amplitude or voltage), frequency, and duration of pulses. The most widely studied of these characteristics are frequency and intensity. Increasing the intensity results in recruitment of additional motor units, thereby increasing torque output. Subtetanic increases in stimulation frequency increase torque output by maximizing the amount of torque each individual motor unit can produce without affecting recruitment. Increasing stimulation frequency will also increase torque output. This phenomenon holds true to the point that a tetanic contraction is achieved (i.e., any further increase in frequency will not increase torque). Subtetanic increases in stimulation frequency increase torque output by maximizing the rate and level of fatigue during NMES. As such, alternative methods of attaining or maintaining torque output at desired levels using lower activation
frequencies and intensities, thereby potentially altering fatigability, would be beneficial in the design and implementation of optimal stimulation protocols. Previous studies have examined the impact of using reduced frequencies and increasing current intensity to attain a given torque level, and shown improvements in fatigue resistance using this approach. The physiological explanation for these results involves recruiting more motor units at lower firing frequencies to achieve a given torque output. As such, these studies emphasize the importance of stimulation frequency over intensity in causing fatigue. However, to our knowledge, no data exist on the impact of varying pulse duration, either alone or in combination with other parameters, on torque production and fatigability during NMES, even though an understanding of the interaction of these stimulation parameters and their impact on muscle function seems valuable. Therefore, the purpose of this study was to examine the interrelationship of stimulation parameters and their impact on muscle function during NMES, even though an understanding of the interaction of these stimulation parameters and their impact on muscle function seems valuable.

MATERIALS AND METHODS

Subjects. Ten subjects (29.9 ± 6.7 years, 174.2 ± 7.4 cm, 72.7 ± 11.6 kg; 8 men) participated in this study. Criteria for participation included: (1) 18–50 years of age, (2) recreationally active, (3) no history of orthopedic or neurological injury that might affect lower-extremity muscle function, and (4) no known medical conditions that contraindicated NMES. Prior to participating in the study, written informed consent was obtained from all subjects, as approved by our Institutional Review Board.

Isokinetic Dynamometry. Torque measurements were obtained from the quadriceps muscle group using an isokinetic dynamometer (Biodex, Shirley, New York). Subjects were seated in an upright chair with hips and knees flexed to ~80°. The axis of the dynamometer was aligned with the knee joint line and the leg was secured to the lever arm. Proximal stabilization was achieved with straps around the chest, waist, and thigh, as previously described. Prior to data collection, subjects were allowed to perform several warm-up contractions. Next, a value for maximum voluntary isometric contraction (MVIC) was determined. MVIC was defined as the peak isometric torque achieved during three consecutive contractions (~5-s contractions separated by 120 s of rest). In the event that the peak torque values differed by more than 5%, additional contractions were performed. Contraction intensity for subsequent NMES testing was calculated relative to each subjects’ MVIC.

Electrical Stimulation. Bipolar self-adhesive neuromuscular stimulation electrodes were placed over the distal-medial and proximal-lateral portion of the quadriceps muscle group. Stimulation pulses were delivered using a Grass S8800 stimulator with a Grass Model SIU8T stimulus isolation unit (Grass Instruments, Quincy, Massachusetts) and data digitized at 200 samples per second. Intensity was set at a voltage that elicited ~50% of each subjects’ MVIC using a 70-Hz/600-μs pulse train of 500-ms duration. Prior to data collection, five trains (500-ms train duration) were delivered at the aforementioned settings to potentiate the quadriceps muscle group. Next, 500-ms pulse trains were delivered using all possible combinations of frequencies (10, 20, 30, 40, 50, 60, 70, and 100 Hz) and pulse durations (100, 200, 300, 400, 500, 600, and 700 μs) at the predetermined voltage. A minimum of 30 s rest was given between each contraction. Pulses were delivered in random order with the exception that the 70-Hz/600-μs train was given at the beginning, middle, and end of the protocol in an effort to assess fatigability during the testing session. A total of 58 NMES-induced contractions were elicited. In addition, fatigue tests were performed during subsequent sessions separated by 48–96 h. Fatigue tests were performed using different parametric settings of equal total charge (i.e., 50-Hz/200-μs vs. 20-Hz/500-μs) at the same voltage used previously and a 50% duty cycle (1 s on / 1 s off). Fatigue tests were 2 min in duration (i.e., 60 total contractions).

Present Pain Intensity (PPI). Following fatigue tests, subjects rated PPI using a visual analog scale as part of the short-form McGill Pain Questionnaire. The scale ranges from 0 to 100 mm with the zero value representing “no pain” and the 100 mm value representing the “worst possible pain.”

Data Analyses. Torque values obtained for each combination of pulse duration and frequency were used to construct torque–duration and torque–frequency curves. Torque values obtained were normalized to trains elicited using the highest frequency and longest pulse duration (i.e., 70-Hz/600-μs) to construct the normalized torque–duration and torque–frequency curves, respectively. Next, regression analysis was used to determine the relationship between torque produced during parameter titration and total charge (product of pulse duration and pulse frequency; V*s^-6). Tests were used to determine whether differ-
ences existed between torque values elicited by trains of similar total charge yet varying parametric settings. Finally, \( t \)-tests were used to determine whether differences existed in the torque produced or the discomfort reported between contractions elicited by pulse trains of different parametric settings yet equal total charge. For all tests performed, the level of significance was set at \( \alpha = 0.05 \).

RESULTS

Normalized torque data were plotted over a range of pulse frequencies (10–100 Hz) at different durations (100–600 \( \mu \)s) to construct the torque–frequency curve (Fig. 1). In addition, a torque–duration curve was constructed across this same range of pulse durations and averaged across the different frequencies (Fig. 2).

Total charge, the product of pulse frequency and pulse duration (V\( \cdot \)s\(^{-1} \)), is a strong predictor of external torque production during NMES-induced contractions in human skeletal muscle. Total charge explained \( \sim 98\% \) of the variance in external torque production over a range of pulse durations (200–600 \( \mu \)s) and pulse frequencies (20–60 Hz; \( y = -5E-10x^2 + 3E-05x + 0.3262 \), \( R^2 = 0.9812 \); Fig. 3). Of note, no differences were observed in torque production between 70-Hz/600-\( \mu \)s trains delivered throughout the testing protocol. Thus, fatigue of the quadriceps femoris muscle group was not a factor in the relationships found. In this study we limited the range of stimulation frequencies used to determine this relationship so as to avoid those that exist on the plateau of the torque–frequency relationship. Specifically, frequencies where an additional 10-Hz increase did not significantly increase torque production were excluded. In addition, the lowest pulse frequency (10 Hz) and duration (100 \( \mu \)s) were excluded from this relationship. However, as indicated in Figure 3, the relationship between total charge and external torque production plotted using only these lowest parameter values resulted in a separate but similarly strong relationship, with total charge explaining \( \sim 92\% \) of the variance in external torque production (\( y = -2E-09x^2 + 4E-05x + 0.0943 \), \( R^2 = 0.9162 \); Fig. 3).

No differences were found in the amount of torque produced by trains of equal total charge, regardless of stimulation intensity (\( P \geq 0.26 \); Tables 1, 2). In addition, pulse trains of 20 Hz/500 \( \mu \)s (40.3 ± 8.1 ft•lbs) and 50 Hz/200 \( \mu \)s (40.3 ± 4.7 ft•lbs) elicited similar initial torques at the onset of the fatigue tests. However, the stimulation trains utilizing the lower frequency stimulation resulted in less reduction in torque (45.0 ± 4.6\% vs. 62.0 ± 3.6\%; \( P = 0.0005 \)) during fatigue tests. In fact, the lower frequency setting resulted in greater...

---

**FIGURE 1.** Normalized torque–frequency relationship of the quadriceps muscle group averaged over a range of pulse durations (100–600 \( \mu \)s) at constant current amplitude. Values represent mean ± SEM.

**FIGURE 2.** Normalized torque–duration curve averaged over a range of pulse frequencies (10–100 Hz) at constant current amplitude. Values represent mean ± SEM.

**FIGURE 3.** Relationship between normalized torque during a 500-\( \mu \)s stimulation train and total charge (V\( \cdot \)s\(^{-1} \)). Filled symbols represent values over a range of frequencies (20–60 Hz) and pulse durations (200–600 \( \mu \)s). Open symbols represent torque values obtained by stimulation trains of varying frequencies at a 100-\( \mu \)s pulse duration and over a range of pulse durations at a 10-Hz pulse frequency. Values are mean ± SEM.
average torque values beginning with contraction 6 of 60 and continuing throughout the remainder of the test (Fig. 4).

No differences were found in the VAS pain ratings of subjects utilizing pulse trains of 20 Hz/500 μs (15.33 ± 2.96 mm) or 50 Hz/200 μs (20.76 ± 3.75 mm, $P = 0.18$).

**DISCUSSION**

The results of this study demonstrate that the product of pulse duration and pulse frequency, defined as total charge, is an extremely strong predictor of external torque production in healthy human skeletal muscle. Accordingly, pulse trains with equal total charge elicit identical torques across a range of pulse frequencies and durations. Finally, stimulation settings of equal charge that incorporate lower frequencies of activation result in less fatigue with no difference in perceived pain. Of note, a key characteristic of this study is that stimulation intensity was held constant at a relatively high level throughout the entire protocol in an effort to ensure substantial quadriceps muscle activation and was necessary to obtain measurable torque with the low frequency / low pulse duration trains.

The values in Tables 1 and 2 reflect the different combinations of parameters that result in identical total charge and represent some novel findings. These pairs of NMES trains, independent of magnitude, result in equal torque. For example, there was no difference in external torque between the 30-Hz/500-μs and 50-Hz/300-μs trains, both representing trains with a 15,000 V-s total charge. Interestingly, these two frequencies fall on the steep part of the torque–frequency curve (Fig. 1), suggesting that the torques should be

<table>
<thead>
<tr>
<th>Total charge</th>
<th>Mean torque</th>
</tr>
</thead>
<tbody>
<tr>
<td>6000 20 Hz / 300 μs</td>
<td>41.4 (5.9)</td>
</tr>
<tr>
<td>30 Hz / 200 μs</td>
<td>42.8 (6.6)</td>
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<td>8000 20 Hz / 400 μs</td>
<td>46.1 (5.6)</td>
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<tr>
<td>40 Hz / 200 μs</td>
<td>46.3 (6.9)</td>
</tr>
<tr>
<td>10000 20 Hz / 500 μs</td>
<td>50.5 (5.5)</td>
</tr>
<tr>
<td>50 Hz / 200 μs</td>
<td>49.8 (7.3)</td>
</tr>
<tr>
<td>12000 30 Hz / 400 μs</td>
<td>54.6 (6.3)</td>
</tr>
<tr>
<td>40 Hz / 300 μs</td>
<td>52.8 (7.7)</td>
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<tr>
<td>12000 20 Hz / 600 μs</td>
<td>57.8 (7.5)</td>
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<tr>
<td>60 Hz / 200 μs</td>
<td>57.9 (8.4)</td>
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<td>24000 40 Hz / 600 μs</td>
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<td>30000 50 Hz / 600 μs</td>
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</tr>
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<td>60 Hz / 500 μs</td>
<td>74.3 (8.5)</td>
</tr>
</tbody>
</table>

Comparisons for each pair of mean torque values for a given total charge showed no differences ($P > 0.05$).

The values in Tables 1 and 2 reflect the different combinations of parameters that result in identical total charge and represent some novel findings. These pairs of NMES trains, independent of magnitude, result in equal torque. For example, there was no difference in external torque between the 30-Hz/500-μs and 50-Hz/300-μs trains, both representing trains with a 15,000 V-s total charge. Interestingly, these two frequencies fall on the steep part of the torque–frequency curve (Fig. 1), suggesting that the torques should be

<table>
<thead>
<tr>
<th>Total charge</th>
<th>Mean torque</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000 10 Hz / 200 μs</td>
<td>14.1 (3.4)</td>
</tr>
<tr>
<td>20 Hz / 100 μs</td>
<td>14.9 (3.6)</td>
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<tr>
<td>3000 10 Hz / 300 μs</td>
<td>15.3 (3.5)</td>
</tr>
<tr>
<td>30 Hz / 100 μs</td>
<td>16.8 (4.4)</td>
</tr>
<tr>
<td>4000 10 Hz / 400 μs</td>
<td>16.3 (3.9)</td>
</tr>
<tr>
<td>40 Hz / 100 μs</td>
<td>18.4 (5.1)</td>
</tr>
<tr>
<td>5000 10 Hz / 500 μs</td>
<td>17.3 (4.0)</td>
</tr>
<tr>
<td>50 Hz / 100 μs</td>
<td>18.9 (5.0)</td>
</tr>
<tr>
<td>6000 10 Hz / 600 μs</td>
<td>17.6 (3.8)</td>
</tr>
<tr>
<td>60 Hz / 100 μs</td>
<td>21.0 (5.3)</td>
</tr>
</tbody>
</table>

Comparisons for each pair of mean torque values for a given total charge showed no differences ($P > 0.05$).
quite different. However, by simply adjusting pulse duration to normalize total charge, external torque production was the same. A similar finding was seen in the 20-Hz/600-μs and 60-Hz/200-μs trains. These two frequencies are even further apart on the torque–frequency curve, yet when total charge is equalized the trains produced identical torques.

The torque–frequency relationship of the quadriceps muscle group illustrated in Figure 1 is similar to that previously reported.6 The novelty of our data lies in the fact that the curve is generated from normalizing the torque–frequency relationship over multiple pulse durations (100–600 μs), owing to the inherent stability of this physiological phenomenon in nonfatigued muscle. In general, force is relatively low at the lowest frequency due to an inability of the pulses to summate action potentials and result in higher torques. However, torque increases exponentially with increases in frequency until about 60 Hz, where torque seemingly plateaus.22 This plateau is due to calcium uptake being the rate-limiting step while muscle cross-bridge cycling is occurring at a maximal rate, resulting in the maximum torque per muscle fiber in the motor unit.10 Increasing the frequency and providing more calcium does not result in greater torque. Accordingly, energy expenditure will be high during higher frequency stimulation. Interestingly, a higher energy cost of contraction with increased frequencies, even at equal torques, has previously been reported during tetanic contractions.21 In our study, stimulation trains with higher frequencies, and presumably higher energy costs, resulted in greater muscle fatigue than lower-frequency stimulation (Fig. 4). Although an increased metabolic cost at higher frequencies seems the likely mechanism to explain the fatigue response noted, definitive data examining differences in metabolic demand of submaximal contractions warrants future study.

In addition to a predictable response to alterations in frequency of stimulation, skeletal muscle torque production is also affected by altering pulse duration. This relationship is curvilinear in nature and is often referred to as the strength–duration curve.14 The data generated in the present study allowed us to illustrate this relationship, herein referred to as the torque–duration curve given our units of measure (Fig. 2). Similar to the torque–frequency curve, the reproducibility of the torque–duration relationship in the quadriceps muscles group is illustrated by its low variability, despite being generated from the normalized response over eight different frequencies. Although the relationship between muscle output and pulse duration is an accepted phenomenon in skeletal muscle, the mechanisms that dictate this response are understudied. We suspect that during stimulation protocols similar to those in the present study, longer pulse durations increase recruitment by more easily overcoming resistance to current flow, thereby facilitating motor unit recruitment at a given intensity, resulting in greater torque output. In addition, because firing frequency is driving calcium release/reuptake and results in widely different specific tension of muscle fibers,13 the logical assumption is that changing pulse duration results in different numbers of motor units being recruited. In fact, the potential for increasing pulse duration to result in greater muscle recruitment has recently been demonstrated.15

The intriguing part of this study is that the product of pulse duration and pulse frequency seemingly dictates external torque production (Fig. 3). However, stimulation combinations that utilized either the 10 Hz or 100-μs parameter had consistently low torque values. Seemingly, total charge can still predict external torque production at these levels (R² = 0.91) but the low pulse duration and low frequency severely limit the ability of the muscle to produce torque. At 10 Hz there is little to no summation of action potentials, so that peak torque is essentially derived from a single pulse. There may be limitations in motor unit recruitment when using 100-μs pulse durations as it has been shown that when short pulse durations are used it is necessary to increase current in order to reach threshold in peripheral nerves.14

The finding of no difference in pain intensity between the two different stimulation protocols is important. If significantly more pain was present during the stimulation patterns that elicited enhanced muscle function it would be difficult to justify incorporation of such protocols. As it is, perception of pain during NMES in healthy persons is thought to limit the application of this modality. Interestingly, the pain values reported in our subjects were on the low end of the VAS scale and were typically described as “mild” in evaluative assessments on the McGill Pain Questionnaire. Accordingly, these data do not support pain as a factor limiting the application of NMES, even at the relatively high stimulation intensities utilized in our study.

Although few data from human studies exist to explain the response of skeletal muscle to changes in pulse frequency and duration, important information to aid in the development of optimal stimulation strategies can be obtained from muscle modeling studies. Data from these studies suggest that the ability to control multiple parameters of stimulation can improve evoked responses, thus supporting the importance of the current study. Specifically, these studies have indi-
cated that the combination of pulse-frequency modulation with pulse-width modulation can improve the transient response of skeletal muscle over modulation of either parameter alone. However, to our knowledge these results have never matured into a definitive relationship that could govern NMES modulation strategies in vivo. A clear need remains for practical solutions and a better understanding of the underlying mechanisms of muscle responses induced by NMES. We suggest that optimizing stimulation parameters during NMES-induced contractions of skeletal muscles can have a variety of applications. Limitations imposed by suboptimal parametric settings could seemingly limit the desired effects of an NMES training protocol by minimizing muscle activation or torque production as well as fatigability. Given that fatigability of muscle resulting from NMES is a major limitation to its application, the ability to account for or minimize this effect would be invaluable to NMES prescription. However, predicting or compensating for muscle fatigue is a daunting task given the complex nonlinear behaviors exhibited by the muscle response to simultaneous modulation of multiple parameters.

In conclusion, the identification of stimulation patterns that maximize muscle performance would allow more physiologically advantageous activation patterns to be used during electrical stimulation protocols. Our data suggest the importance of minimizing stimulation frequency to achieve a given absolute torque when fatigue is a concern, and demonstrate the ability to predictably control torque output by simultaneously controlling both pulse frequency and duration.

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REFERENCES

Abstract: Patients with mitochondrial myopathies (MM) or myophosphorylase deficiency (McArdle’s disease, McA) show impaired capacity for O₂ extraction, low maximal aerobic power, and reduced exercise tolerance. Non-invasive tools are needed to quantify the metabolic impairment. Six patients with MM, 6 with McA, 25 with symptoms of metabolic myopathy but negative biopsy (patient-controls, P-CTRL) and 20 controls (CTRL) underwent an incremental cycloergometric test. Pulmonary O₂ uptake (VO₂) and vastus lateralis oxygenation indices (by near-infrared spectroscopy, NIRS) were determined. Concentration changes of deoxygenated hemoglobin and myoglobin (Δ[deoxy(Hb + Mb)]) were considered an index of O₂ extraction. Δ[deoxy(Hb + Mb)] peak (percent limb ischemia) was lower in MM (25.3 ± 12.0%) and McA (18.7 ± 7.3%) than in P-CTRL (62.4 ± 3.9) and CTRL (71.3 ± 3.9) subjects. VO₂ peak and Δ[deoxy(Hb + Mb)] peak were linearly related (r² = 0.83). In these patients, NIRS is a tool to detect and quantify non-invasively the metabolic impairment, which may be useful in the follow-up of patients and in the assessment of therapies and interventions.


Impaired oxygen extraction in metabolic myopathies: detection and quantification by near-infrared spectroscopy

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Metabolic myopathies are a heterogeneous group of disorders characterized by derangements of glycogen or lipid metabolism or mitochondrial function due to mutations leading to defects of the main pathways of energy provision in skeletal muscle fibers. In some of these myopathies, such as myophosphorylase deficiency (McArdle’s disease, McA) or mitochondrial myopathies (MM),7,15,26–31 the genetic defect significantly impairs oxidative metabolism. In many of these patients, a reduced exercise tolerance and the associated easy fatigability are often the main (and sometimes the only) symptoms and may significantly affect quality of life. The impairment of oxidative metabolism results in a reduced capacity to increase muscle O₂ extraction, or arteriovenous O₂ concentration difference ([C(a – v)O₂]), during exercise, and leads to a significantly lower than normal maximal (or peak) O₂ uptake (VO₂ peak), even in the presence of normal peak cardiovascular responses.12 These patients also present an exaggerated cardiovascular response to submaximal exercise, that is, higher heart rate (HR), cardiac output (Q), and muscle blood flow (Qm) values, compared to healthy subjects, for the same submaximal VO₂.11,12,16,17,26,29–31 In MM patients the reduced capacity to increase muscle O₂ extraction during exercise has been documented invasively by directly determining C(a – v)O₂ across exercising muscles,17,26 or indirectly by solving Fick’s equation.

Abbreviations: ADP, adenosine diphosphate; AMP, adenosine monophosphate; ANOVA, analysis of variance; A TP, adenosine triphosphate; CaO₂, arterial oxygen concentration; C(a – v)O₂, arteriovenous oxygen concentration difference; CK, creatine kinase; CTRL, controls; Δ[deoxy(Hb + Mb)] and Δ[deoxy(Hb + Mb)], changes in concentration of oxygenated and deoxygenated hemoglobin and myoglobin; ECG, electrocardiography; HR, heart rate; [La]b, blood lactate concentration; McA, McArdle’s disease; MM, mitochondrial myopathies; NIRS, near-infrared spectroscopy; P-CTRL, patient-controls; Pi, inorganic phosphate; Q, cardiac output; Qm, muscle blood flow; R, gas exchange ratio; SaO₂, arterial blood oxygen saturation; VE, pulmonary ventilation; VCO₂, carbon dioxide output; VO₂, oxygen uptake; W, watt; ∆, workload

Key words: mitochondrial myopathy; myophosphorylase deficiency; near-infrared spectroscopy; oxidative metabolism

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NIRS in Metabolic Myopathies

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[\text{VO}_2 = \dot{Q} \cdot \text{C}(a \rightarrow v)\text{O}_2] \text{ after measuring whole-body VO}_2 \text{ and } \dot{Q} \text{ during maximal exercise.}^{29} \text{ A low maximal systemic C}(a \rightarrow v)\text{O}_2, \text{ associated with a reduced VO}_2 \text{ peak, was demonstrated also in McA patients.}^{11,13} \text{ In MM patients the degree of impairment in O}_2 \text{ extraction is quantitatively related to the extent of the genetic defect (as indicated by the degree of heteroplasmy), to the reduction of VO}_2 \text{ peak, and to degree of clinical impairment.}^{29}

Non-invasive methods to determine Q during maximal exercise have been questioned,\textsuperscript{94} whereas the invasiveness of the determination of C}(a \rightarrow v)\text{O}_2 \text{ across exercising muscle precludes serial measurements of this variable in patients. However, a longitudinal perspective is essential in order to define the clinical course of the disease and to evaluate the effects of therapeutic or rehabilitation interventions. At least a partial answer to this need could come from near-infrared spectroscopy (NIRS), a non-invasive method that allows the monitoring of muscle oxygenation based on the principle that the near-infrared (NIR) light absorption characteristics of hemoglobin (Hb) and myoglobin (Mb) depend on their O_2 saturation. Theoretical basis, practical applications, advantages, and limitations of NIRS have been extensively reviewed.\textsuperscript{5,8,19,22} \text{ NIR light absorption changes in muscle reflect changes in oxygenation at the level of small blood vessels (small arterioles and venules), capillaries, and intracellular sites of O}_2 \text{ transport and uptake. Oxygenation indices obtained by NIRS are the result of the balance between O}_2 \text{ delivery (}\dot{Q} \times \text{CaO}_2\text{) and VO}_2 \text{ in the portion of tissue under consideration, being therefore conceptually similar to O}_2 \text{ extraction [or C}(a \rightarrow v)\text{O}_2\text{].}^{6,9,14} \text{ More specifically, an increased muscle oxygenation would indicate an increased VO}_2 \text{ with respect to VO}_2, \text{ that is, a reduced O}_2 \text{ extraction, whereas a decline in muscle oxygenation would indicate an increased VO}_2 \text{ with respect to VO}_2 \text{ (i.e., an increased O}_2 \text{ extraction).}

Previous studies conducted by utilizing NIRS in order to evaluate the reduced capacity of O_2 extraction in metabolic myopathies were either carried out on animals\textsuperscript{21} or, when dealing with humans, were mainly anecdotal reports dealing with a limited number of patients\textsuperscript{1,2} or a single patient.\textsuperscript{25,35} \text{ More importantly, although these studies allowed detection of the impaired capacity to increase O}_2 \text{ extraction by the investigated muscles during exercise, they did not determine whether the degree of impairment was quantitatively related to the reduced exercise tolerance. This aspect is important when considering the utility of NIRS for the functional evaluation of patients. The aim of the present study was to fill this knowledge gap.}

More specifically, we hypothesized that NIRS would allow us to identify and quantify the impairment of muscle O_2 extraction and oxidative metabolism in MM and McA. Indirect evidence in favor of this hypothesis derives from the observation of significant relationships between the NIRS-derived index of O_2 extraction, the reduction of VO_2 peak, and the extent of exaggerated cardiovascular response to submaximal exercise.

**METHODS**

**Subjects.** Four groups of subjects were studied: \text{6 patients with mitochondrial myopathies (MM) and respiratory chain dysfunction due to mutations of the multi-subunit complexes (I, II, III, and IV) or of the mobile electron carriers ubiquinone and cytochrome c.\textsuperscript{15,20,29–31} \text{ 6 patients with myophosphorylase deficiency, or McArdle’s disease (McA)\textsuperscript{3,16} and 25 age- and gender-matched patients with reduced exercise tolerance or other signs or symptoms suggesting a metabolic myopathy [e.g., elevated serum creatine kinase (CK) levels, reduced exercise tolerance, muscle cramps, or pain after exercise], but in whom muscle biopsy did not allow a firm diagnosis of any known myopathy (patient-controls, P-CTRL); and 20 age- and gender-matched healthy untrained controls (CTRL). The patients were recruited from those referred to the Department of Neuromuscular Diseases, Neurological Institute Carlo Besta, Milan. The subjects were fully informed of any risk and discomfort associated with the experiments before giving their written consent to participate in the study, which was approved by the ethics committees of our institutions. All procedures were in accordance with the recommendations of the Helsinki Declaration of 1975.}

Diagnosis of metabolic myopathies was made by standard criteria on homogenates of skeletal muscle samples, obtained from quadriceps by needle or open biopsy under local anesthesia. Exclusion criteria were the presence of neoplastic diseases or other major neurological/psychiatric, orthopedic, rheumatological, endocrine, pulmonary, or cardiovascular disorders. The subjects' age, height, and body mass for the four groups were as follows (mean values ± SE): for MM (5 men, 1 woman), 37.8 ± 6.0 years, 173.2 ± 3.7 cm, 62.4 ± 4.1 kg; for McA (3 men, 3 women), 25.7 ± 3.0 years, 170.5 ± 4.7 cm, 71.8 ± 9.2 kg; for P-CTRL (20 men, 5 women), 31.6 ± 2.8 years, 172.6 ± 1.4 cm; 71.1 ± 2.0 kg; and for CTRL (12 men, 8 women), 32.7 ± 3.0 years.

**Diagnosis of metabolic myopathies**
MM patients were slightly older and McA patients slightly younger than P-CTRL and CTRL; if MM and McA patients are considered together, however, their mean age (31.8 years) was very similar to those of P-CTRL and CTRL. Information related to family and clinical history, as well as the main results obtained by standard methods upon muscle histochemical, biochemical, and DNA analyses (when available), are shown for MM patients in Table 1. McA was indicated if patients had clinical findings of exercise intolerance, muscle weakness, and elevated serum CK levels; vacuolar myopathy with vacuoles positive for periodic acid–Schiff (PAS) at muscle histochemistry; and nearly absent myophosphorylase activity (<1% of the lower limit of the normal range) on biochemical analysis.

Measurements. All tests were carried out under close medical supervision, and subjects were monitored by 12-lead electrocardiography (ECG). An electromagnetically braked cycle ergometer (Cardioline STS 3; Remco Italia, San Pedrino di Vignate, Italy) was utilized. Pedalling frequency was digitally displayed to the subjects. Subjects were allowed time to gain familiarity with the investigators and experimental arrangement, and were carefully instructed about the experimental procedures. Subjects were also familiarized with the experimental protocol by means of short preliminary practice runs. An incremental exercise was performed: after a few minutes of unloaded pedalling, exercise was conducted at 25–50 W for 6 minutes, and thereafter the workload was increased by 10–25 W (according to the subject’s estimated level of physical fitness) every minute until voluntary exhaustion was reached. The latter was defined by: (1) inability to maintain the pedalling frequency (60–80 revolutions/min) despite encouragement by the operators; (2) maximal levels of self-perceived exertion, using the validated Borg’s scale; and (3) heart rate (HR) values 85% of the age-predicted maximum. Values of cardiovascular, ventilatory, gas exchange, and muscle oxygenation variables determined during the last 30 seconds of the exhausting load were considered “peak” values.

Pulmonary ventilation (VE), VO$_2$, and CO$_2$ output (VCO$_2$) were determined breath-by-breath by a computerized metabolic cart (Vmax229; SensorMedics, Bihoven, The Netherlands). Heart rate was determined from the ECG signal. Arterial blood O$_2$ saturation (SaO$_2$) was monitored continuously by pulse oximetry (Biox 3740 Pulse Oximeter; Ohmeda, Trezzano sul Naviglio, Italy) at the earlobe. At

<table>
<thead>
<tr>
<th>MM Patient</th>
<th>Age (years)</th>
<th>Gender</th>
<th>History</th>
<th>Histochemical analysis</th>
<th>Biochemical analysis</th>
<th>DNA analysis</th>
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<tr>
<td>1</td>
<td>32</td>
<td>M</td>
<td>Ptosis; ophthalmoplegia</td>
<td>Ragged-red fibers; Cox depletion</td>
<td>Complex I (11.1) and II (55) reductions*</td>
<td>Heteroplasmic macrodeletion (3000 bp)</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
<td>M</td>
<td>Exercise intolerance; fatigueability; neck flexion weakness</td>
<td>Neurogenic abnormalities; no ragged-red fibers</td>
<td>Complex II (12.1) and SDH reductions*</td>
<td>Not available</td>
</tr>
<tr>
<td>3†</td>
<td>32</td>
<td>M</td>
<td>Ptosis; ophthalmoplegia; long face</td>
<td>Many ragged-red fibers; a few Cox-depleted fibers</td>
<td>Normal</td>
<td>No macrodeletions (by Southern blot) in mitochondrial DNA</td>
</tr>
<tr>
<td>4</td>
<td>33</td>
<td>M</td>
<td>Exercise intolerance; mild neck and proximal lower-limb muscle weakness</td>
<td>Myopathic features; ragged-red fibers; mild lipid increase</td>
<td>Complex III (58) reduction*</td>
<td>Not available</td>
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<tr>
<td>5</td>
<td>42</td>
<td>M</td>
<td>Family history of MM</td>
<td>Ragged-red fibers</td>
<td>Complex II (9), III (40), and IV (75) reductions*</td>
<td>nt 8344 heteroplasmic mutation (60%), associated with MELAS</td>
</tr>
<tr>
<td>6</td>
<td>65</td>
<td>F</td>
<td>Scapular and pelvic girdles, and lower-limb muscle weakness</td>
<td>Myopathic features; ragged-red fibers</td>
<td>Normal</td>
<td>Multiple deletions (&lt;10%)</td>
</tr>
</tbody>
</table>

MM, mitochondrial myopathy; SDH, succinate dehydrogenase; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes.

*Data expressed as multiples of citrate synthase activity (normal values 80–210 nmol/min/mg). Normal values (nmol/min/mg): Complex I, 13–24; Complex II, 15–28; Complex III, 88–167; Complex IV, 120–220; Complex V, 89–221.

†In patient 3, both respiratory chain enzymes analysis and mtDNA analysis indicated no alterations. However, according to Vladutiu, the presence of one major diagnostic criterion (many ragged-red fibers at the histochemical analysis) and two minor diagnostic criteria (some symptoms, Cox-depleted fibers) should make the diagnosis of mitochondrial myopathy “definite” in this patient as well. Respiratory chain function tests were carried out on muscle homogenates, which could have "missed" the Cox-depleted fibers.

178.0 ± 1.0 cm, 77.4 ± 2.0 kg. MM patients were slightly older and McA patients slightly younger than P-CTRL and CTRL; if MM and McA patients are considered together, however, their mean age (31.8 years) was very similar to those of P-CTRL and CTRL. Information related to family and clinical history, as well as the main results obtained by standard methods upon muscle histochemical, biochemical, and DNA analyses (when available), are shown for MM patients in Table 1. McA was indicated if patients had clinical findings of exercise intolerance, muscle weakness, and elevated serum CK levels; vacuolar myopathy with vacuoles positive for periodic acid–Schiff (PAS) at muscle histochemistry; and nearly absent myophosphorylase activity (~1–2% of the lower limit of the normal range) on biochemical analysis.

Measurements. All tests were carried out under close medical supervision, and subjects were monitored by 12-lead electrocardiography (ECG). An electromagnetically braked cycle ergometer (Cardioline STS 3; Remco Italia, San Pedrino di Vignate, Italy) was utilized. Pedalling frequency was digitally displayed to the subjects. Subjects were allowed time to gain familiarity with the investigators and experimental arrangement, and were carefully instructed about the experimental procedures. Subjects were also familiarized with the experimental protocol by means of short preliminary practice runs. An incremental exercise was performed: after a few minutes of unloaded pedalling, exercise was conducted at 25–50 W for 6 minutes, and thereafter the workload was increased by 10–25 W (according to the subject’s estimated level of physical fitness) every minute until voluntary exhaustion was reached. The latter was defined by: (1) inability to maintain the pedalling frequency (60–80 revolutions/min) despite encouragement by the operators; (2) maximal levels of self-perceived exertion, using the validated Borg’s scale; and (3) heart rate (HR) values >85% of the age-predicted maximum. Values of cardiovascular, ventilatory, gas exchange, and muscle oxygenation variables determined during the last 30 seconds of the exhausting load were considered “peak” values.

Pulmonary ventilation (VE), VO$_2$, and CO$_2$ output (VCO$_2$) were determined breath-by-breath by a computerized metabolic cart (Vmax229; SensorMedics, Bihoven, The Netherlands). Heart rate was determined from the ECG signal. Arterial blood O$_2$ saturation (SaO$_2$) was monitored continuously by pulse oximetry (Biox 3740 Pulse Oximeter; Ohmeda, Trezzano sul Naviglio, Italy) at the earlobe. At

Table 1. Characteristics of the MM patients.
rest and at various timepoints (0, 1, 3, 5, and 7 minutes) during recovery after exercise, 20 μl of capillary blood was obtained from a pre-heated earlobe for the determination of blood lactate concentration ([La]b) by an enzymatic method (Biosen 5030; EKF, Eppendorf Italia, Milano, Italy). The highest [La]b value obtained during recovery was considered [La]b peak. [La]b measurements were not carried out in CTRL subjects.

Oxygenation changes in the vastus lateralis muscle were evaluated by NIRS. A portable NIR single-distance continuous-wave photometer (HEO-100; Omron, Kyoto, Japan), which adopts an algorithm based on diffusion theory, was utilized. The instrument provides separate measurements of changes in deoxygenated Hb and Mb concentrations, as well as of oxygenated Hb and Mb concentrations, expressed in arbitrary units. The probe unit, molded in elastic black silicone rubber, has a silicon photodiode as photodetector in the center and two light-emitting diodes (peak wavelengths 760 and 840 nm) on either side. The probe was firmly attached to the skin overlaying the lower third of the vastus lateralis muscle (~10–2 cm above the knee joint) of the dominant limb, parallel to the major axis of the thigh, with a belt secured by Velcro straps and bi-adhesive tape. The skin had been carefully shaved. Pen-marks were made over the skin to indicate the margins of the belt in order to check for any downward sliding of the probe during cycling. No sliding was observed in any subject at the end of each protocol. Black cloths were put around the probe and the skin to prevent contamination from ambient light. The probe was connected to a personal computer for data acquisition, A/D conversion, and subsequent analysis. The sampling frequency was set at 2 Hz.

The distance between each light source and the photodiode was 3 cm. Thus, the penetration depth could be estimated to be at least 1.5 cm, that is, to at least half of the source-detector distance. According to Monte Carlo simulation studies of skin and muscle scattering and absorption characteristics for NIR light, as well as to in vivo measurements, a source-detector spacing of 2 cm is enough for the NIR light passing through the muscle layer, even when the adipose tissue thickness is 15 mm. Skinfold thickness at the site of application of the NIRS probe was determined at the end of the exercise protocol by a caliper (C10 Plicometer Tanner–Whitehouse; Holtain, Ltd., Crymych, UK); the calculated values of skin and subcutaneous tissue thickness were 6.5 ± 1.0 mm (range 2.0–12.5).

The absorption characteristics of light at 760 and 840 nm depend on relative oxygenation of Hb and Mb. Mb has similar absorption spectra to Hb. In human skeletal muscle, however, the ratio [Hb]/[Mb] is higher than 5, so the signal is usually considered as deriving mainly from Hb. Concentration changes of oxygenated Hb + Mb (Δ[oxy(Hb + Mb)]) and deoxygenated Hb + Mb (Δ[deoxy(Hb + Mb)]) were calculated and expressed in arbitrary units. The sum of the two variables (Δ[oxy(Hb + Mb) + deoxy(Hb + Mb)]) is related to changes in the total Hb volume in the muscle region of interest. In the present study, Δ[deoxy(Hb + Mb)] was taken as an oxygenation index, because this variable is relatively insensitive to changes in blood volume. Δ[deoxy(Hb + Mb)] data were expressed as a percentage of the values determined after the exercise by obtaining a maximal deoxygenation of the muscle, by pressure cuff inflation (at about 300 mm Hg) at the root of the thigh (subject in the sitting position on the cycloergometer), for a few minutes until the Δ[deoxy(Hb + Mb)] increase reached a plateau.

**Statistical Analysis.** Values are expressed as mean ± standard error (SE). The statistical significance of the difference between mean values was checked by one-way analysis of variance (ANOVA); a Tukey’s post hoc test was utilized when significant differences emerged upon ANOVA. Regression and correlation analyses were performed by the least-squares residuals method. The level of significance was set at P < 0.05. All statistical analyses were performed by utilizing commercially available software packages (InStat and Prism 4.0; GraphPad, San Diego, California).

**RESULTS**

Average durations of the incremental exercise protocol were ~12 minutes in MM and McA patients, and ~15–17 minutes in P-CTRL and CTRL. Peak values of the main cardiovascular, ventilatory, and metabolic variables are shown in Table 2 for the different groups of subjects. When expressed as a percentage of the age-predicted maximal HR, HR peak values in the various groups were very similar (88% in MM patients, 92% in McA patients, 91% in P-CTRL, and 96% in CTRL). VE peak values were significantly lower in MM, McA, and P-CTRL compared to CTRL, presumably as a consequence of the lower w peak. Indeed, when VE peak was expressed per unit of VO2 peak, that is, as VE peak/VO2 peak, differences between the various groups substantially disappeared. As expected, R peak and [La]b peak
values were lower in McA patients compared to the other groups; more specifically, in all McA patients, \([	ext{La}]_{\text{b}}\) peak values were not substantially higher than \([	ext{La}]_{\text{b}}\) values observed at rest. Two MM patients showed elevated resting \([	ext{La}]_{\text{b}}\), suggesting a significant contribution of “anaerobic” metabolism even at rest. No differences in \([	ext{La}]_{\text{b}}\) peak were observed between MM patients and P-CTRL. In all groups \(\text{SaO}_2\) did not significantly decrease during exercise, suggesting a substantially normal cardiopulmonary function.

\(\text{VO}_2\) peak values obtained in MM, McA, and P-CTRL subjects were ~40%, ~50%, and ~15% lower, respectively, than those obtained in CTRL. Values in MM and McA patients were significantly lower than those in CTRL. Values in McA patients were also significantly lower than those in P-CTRL; no other statistically significant difference was observed between groups.

Individual HR values were plotted as a function of the corresponding \(\text{VO}_2\), and regression lines were drawn. Individual regression lines for patients in groups MM, McA, as well as the calculated averages of the individual regression lines for P-CTRL and CTRL subjects are shown in Figure 1. The average regression line for P-CTRL was shifted slightly to the left (indicating higher HR for the same \(\text{VO}_2\)) compared to that for CTRL. In 3 of 6 MM patients and in 5 of 6 McA patients, regression lines were shifted to the left and had higher slopes than the lines for P-CTRL and CTRL. Mean (+SE) values of the slopes of the HR vs. \(\text{VO}_2\) relationships were 5.44 ± 1.11 in MM, 6.67 ± 0.90 in McA, 3.51 ± 0.16 in P-CTRL, and 3.01 ± 0.16 in CTRL. Values were significantly higher in MM and in McA patients compared to P-CTRL and CTRL; no other significant differences were observed. Whereas higher HR values for the same \(\text{VO}_2\) are generally considered a sign of poor exercise tolerance, the significantly higher slopes of the HR vs. \(\text{VO}_2\) relationship observed in MM and McA patients suggest an enhanced cardiovascular response to exercise.

### Table 2. Peak values of the main cardiovascular, ventilatory, and metabolic variables (resting blood lactate levels also shown).

<table>
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<tr>
<th></th>
<th>(w) (watts)</th>
<th>HR (beats/min)</th>
<th>(\text{VE}) (L/min)</th>
<th>(\text{SaO}_2) (%)</th>
<th>(\text{VO}_2) (L/min)</th>
<th>(\text{VO}_2) (ml/kg/min)</th>
<th>(\text{VCO}_2) (L/min)</th>
<th>(R)</th>
<th>([\text{La}]_{\text{b}}) rest (mM)</th>
<th>([\text{La}]_{\text{b}}) peak (mM)</th>
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<td>74.2</td>
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<td>Mean</td>
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\(w\), power output; HR, heart rate; VE, pulmonary ventilation; \(\text{SaO}_2\), arterial blood oxygen saturation; \(\text{VO}_2\), oxygen uptake; \(\text{VCO}_2\), carbon dioxide output; \(R\), gas exchange ratio; \([\text{La}]_{\text{b}}\) rest, resting blood lactate concentration; \([\text{La}]_{\text{b}}\) peak, peak blood lactate concentration.

*Significantly different compared to CTRL.
†Significantly different compared to P-CTRL.
‡Significantly different compared to McA (all ANOVA plus Tukey’s test).
Individual Δ[deoxy(Hb + Mb)] data, expressed as a percentage of the values obtained during limb ischemia, are presented for MM and McA patients as a function of workload (w) in Figure 2, along with the mean (±SE) values obtained in P-CTRL and CTRL. The w values are expressed in relative terms, that is, as a ratio of peak w (w/peak), in order to allow a comparison of Δ[deoxy(Hb + Mb)] values obtained at the same relative exercise intensity among subjects of different exercise capacity. An increase in Δ[deoxy(Hb + Mb)] indicates an increased O2 extraction by the investigated muscle.6,9,14 Values in P-CTRL were not significantly different from those of CTRL. With the exception of 1 MM patient, MM and McA patients showed Δ[deoxy(Hb + Mb)] values that were substantially lower, at each relative workload, compared to those of P-CTRL or CTRL. In 2 MM and 2 McA patients, Δ[deoxy(Hb + Mb)] values during incremental exercise did not increase compared to the value observed at rest (corresponding to 0% in Fig. 2), suggesting that these patients could not increase their muscle's O2 extraction during exercise with respect to the resting value.

Mean (±SE) values of Δ[deoxy(Hb + Mb)] obtained at exhaustion (Δ[deoxy(Hb + Mb)] peak), expressed as a percentage of the values obtained during limb ischemia, are shown in Figure 3. Signif-
significant differences between groups were detected by ANOVA. Values obtained in MM and McA patients (~20–25%) were significantly lower than those obtained in P-CTRL and CTRL (~65–70%). No significant differences were observed between MM and McA, or between P-CTRL and CTRL.

Individual values of VO$_2$ peak obtained in MM and McA patients are plotted in Figure 4 as a function of Δ[deoxy(Hb + Mb)] peak. Mean (±SE) data obtained in P-CTRL and CTRL are also shown. A highly significant linear relationship with a positive slope was observed between the two variables, suggesting a strong relationship between the NIRS-derived index of impaired maximal O$_2$ extraction and the reduced maximal aerobic power. The observed $r^2$ value indicates that over 80% of VO$_2$ peak variability can be explained in terms of differences in Δ[deoxy(Hb + Mb)] peak. Data obtained from P-CTRL and CTRL subjects lie very close to the regression line and are similar to the highest values obtained in the patient groups. In 1 MM patient, both Δ[deoxy(Hb + Mb)] peak and VO$_2$ peak values were very close to those obtained in P-CTRL and CTRL.

Individual values of the slopes of the HR vs. VO$_2$ relationship, obtained in MM and in McA patients, are plotted as a function of Δ[deoxy(Hb + Mb)] peak in Figure 5. Mean (±SE) data obtained from P-CTRL and CTRL are also shown. A significant linear relationship with a negative slope is observed, suggesting the presence of a relationship between the impaired capacity of maximal O$_2$ extraction and the enhanced cardiovascular response to exercise. The observed $r^2$ value indicates that over 50% of the variability in the slope of the HR vs. VO$_2$ relationship can be explained in terms of differences in Δ[deoxy(Hb + Mb)] peak. Data obtained from P-CTRL and CTRL are very close to the regression line drawn for the patients. Figure 5 also shows that the values obtained in 1 of the MM patients (the same mentioned above, who had substantially normal Δ[deoxy(Hb + Mb)] peak and VO$_2$ peak values) were very close to those obtained in P-CTRL and CTRL.

DISCUSSION

In patients with metabolic myopathies affecting oxidative metabolism, such as mitochondrial myopa-
thies (MM) and McArdle’s disease (McA), we observed highly significant relationships between the NIRS-derived index of O₂ extraction, the reduction of VO₂ peak, and the extent of the exaggerated cardiovascular response to submaximal exercise. These observations provide strong, although indirect, evidence in favor of the concept that in these patients the non-invasive determination by NIRS of a muscle oxygenation index, such as the concentration changes of deoxygenated Hb + Mb (Δ[deoxygen(Hb + Mb)]), allows us to identify and quantify the impaired capacity of O₂ extraction by skeletal muscles during exercise. Such impairment represents, in these patients, the key pathophysiological mechanism responsible for the lower than normal VO₂ peak and for the reduced exercise tolerance. The possibility of quantifying and serially monitoring such impairment by a completely non-invasive tool should be of great interest to clinicians, who need an objective, quantitative, and longitudinal evaluation of the impairment to be used in the follow-up of patients, as well as in the assessment of therapies or other interventions. An obvious limitation of the present approach is that the metabolic impairment should not be so severe as to preclude the conduction of an exercise test on a cycloergometer. The measurements need to be conducted carefully, but are relatively easy to perform, and the instruments are relatively inexpensive. In order to be able to test more severely impaired patients, we are working to implement an incremental exercise protocol to be carried out with small muscle masses (e.g., handgrip exercise).

**Impaired O₂ Extraction, Reduced VO₂ Peak, and Exercise Tolerance.** Whereas in MM patients the cause–effect relationship between the defects of enzymes of the respiratory chain and the reduced capacity to increase O₂ extraction is straightforward, for McA patients the link between the genetic defect (absence or low activity of the myophosphorylase enzyme, which causes an incapacity to break down intramuscular glycogen) and oxidative metabolism may appear less obvious. In these patients, glycolytic flux in muscle fibers is significantly reduced or substantially absent, and lactate production during exercise is low or absent, leading to no or to very low levels of blood lactate accumulation during exhausting exercise (Table 2). The reduced or absent flux of substrates along the glycolytic pathway, however, also significantly impairs one of the two main routes of supply of substrates to the tricarboxylic acids cycle, and disrupts the delicate interplay between carbohydrate and lipid metabolism.

This inevitably leads to a significant impairment of oxidative metabolism, to a reduced capacity to increase C(a – v)O₂ during exercise, and to a lower than normal VO₂ peak.

Previous studies conducted by utilizing NIRS in order to evaluate the reduced capacity of O₂ extraction in metabolic myopathies were either carried out on animals or, when dealing with humans, were mainly anecdotal reports of observations made on one or a few patients. Moreover, in these studies the measurements were conducted during presumably submaximal exercises, and they did not allow the patients’ exercise tolerance and their “peak” capacity of O₂ extraction to be evaluated.

van Beekvelt et al. utilized NIRS to estimate forearm VO₂ in MM patients during handgrip exercise. They found that VO₂ was lower in MM than in controls, and concluded that their approach could discriminate between normal muscle and the muscle of patients with MM. Most of the difference in VO₂ between the two groups in their study, however, could be attributed to the difference in force output. Moreover, it is generally accepted that, in patients with mitochondrial myopathies, VO₂ at the same submaximal load is not significantly different compared to that of healthy subjects, although it is achieved by a higher than normal blood flow in the presence of a lower than normal O₂ extraction.

Lynch et al. evaluated by NIRS the kinetics of forearm VO₂ in MM patients during handgrip exercise. They found that VO₂ was lower in MM than in controls, and concluded that their approach could discriminate between normal muscle and the muscle of patients with MM. Most of the difference in VO₂ between the two groups in their study, however, could be attributed to the difference in force output. Moreover, it is generally accepted that, in patients with mitochondrial myopathies, VO₂ at the same submaximal load is not significantly different compared to that of healthy subjects, although it is achieved by a higher than normal blood flow in the presence of a lower than normal O₂ extraction.

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van Beekvelt et al. utilized NIRS to estimate forearm VO₂ in MM patients during handgrip exercise. They found that VO₂ was lower in MM than in controls, and concluded that their approach could discriminate between normal muscle and the muscle of patients with MM. Most of the difference in VO₂ between the two groups in their study, however, could be attributed to the difference in force output. Moreover, it is generally accepted that, in patients with mitochondrial myopathies, VO₂ at the same submaximal load is not significantly different compared to that of healthy subjects, although it is achieved by a higher than normal blood flow in the presence of a lower than normal O₂ extraction.
ylation, which translates into a reduced capacity to increase O\textsubscript{2} extraction and C(a – v)O\textsubscript{2}, represents the main determinant of the reduced maximal aerobic power.

In the present study the patients with the most severe disease (as shown by the very low VO\textsubscript{2} peak) were substantially unable to increase O\textsubscript{2} extraction (as shown by Δ[deoxy(Hb + Mb)] during exercise; Fig. 4). These patients reached maximal or near-maximal HR values and voluntary exhaustion in the presence of Δ[deoxy(Hb + Mb)] values that were substantially unchanged compared to those determined at rest. Thus, their increases in VO\textsubscript{2} were obtained solely by increasing O\textsubscript{2} delivery to muscles (enhanced cardiovascular response to exercise; Fig. 5). On the other hand, in 1 MM patient Δ[deoxy(Hb + Mb)] peak and VO\textsubscript{2} peak were substantially the same as described for CTRL (Fig. 4). In this patient (No. 5 in Table 1), even in the presence of a firm diagnosis of MM confirmed by biopsy, no limitation of exercise tolerance was observed. The patient was indeed asymptomatic and physically quite active, and also showed a normal cardiovascular response to exercise (Figs. 1 and 5). Ample variability in the degree of clinical and metabolic impairment in MM patients,\textsuperscript{30,31} likely related to the extent of the genetic defect (as indicated by the degree of heteroplasm), has also been recently demonstrated by Taivassalo et al.\textsuperscript{29} As in previous studies,\textsuperscript{11,13,16,29} our MM and McA patients showed a wide range of VO\textsubscript{2} peak values (Fig. 4), spanning from the normal range to severe impairment, corresponding to three to four times the resting metabolic rate. If we consider that only a fraction of this VO\textsubscript{2} peak can be sustained for relatively prolonged periods of time, it seems that in some of these patients several activities of daily living cannot be carried out without significant involvement of non-oxidative metabolism, with obvious negative consequences on exercise tolerance and quality of life. In our study the variability in VO\textsubscript{2} peak was closely related to variability in the NIRS-derived index of peak skeletal muscle O\textsubscript{2} extraction (Δ[deoxy(Hb + Mb)] peak), demonstrating that this NIRS-derived index, although not specific for the disease, seems specific for the degree of metabolic impairment associated with the disease.

Our findings could also have implications in the diagnostic work-up of patients in whom some clinical findings raise the suspicion of a metabolic myopathy (as in the P-CTRL group in the present study). In these patients, the observation of normal Δ[deoxy(Hb + Mb)] peak by NIRS, as well as of a normal HR vs. VO\textsubscript{2} relationship, provides evidence against the diagnosis of a metabolic myopathy, and could be useful, for example, in the selection of patients for undergoing muscle biopsy.

A significant correlation between VO\textsubscript{2} peak and the calculated (by directly measuring Q\textsubscript{peak} and VO\textsubscript{2} peak, and then solving Fick’s equation)\textsuperscript{29} or invasively determined\textsuperscript{17,26} maximal C(a – v)O\textsubscript{2} was previously observed in MM. A low maximal systemic C(a – v)O\textsubscript{2} in exercise, associated with a reduced VO\textsubscript{2} peak, was also demonstrated in McA patients.\textsuperscript{11,15} Non-invasive methods to determine Q\textsubscript{peak} during maximal exercise, however, are questionable,\textsuperscript{34} whereas the invasiveness of the assessment of C(a – v)O\textsubscript{2} across exercising muscles precludes serial measurements of this variable. The obvious advantage of estimating O\textsubscript{2} extraction through the evaluation of Δ[deoxy(Hb + Mb)] by NIRS lies in the non-invasiveness of the measurement.

**Impaired O\textsubscript{2} Extraction and Enhanced Cardiovascular Response.** As mentioned previously, it is well known that patients with metabolic myopathies affecting oxidative metabolism will try to compensate for the limited capacity by their muscles to increase O\textsubscript{2} extraction by increasing blood flow, and hence convective O\textsubscript{2} delivery to muscle.\textsuperscript{10,12,16,17,26,29–31} The feedback link between impaired oxidative phosphorylation and increased convective O\textsubscript{2} delivery appears of general interest, in terms of the regulation of cardiovascular function during exercise.\textsuperscript{12} The signals that could trigger the enhanced cardiovascular response are cellular phosphorylation potential ([ATP]/[ADP] [Pi]), Pi, ADP, AMP, K\textsuperscript{+}, and H\textsuperscript{+}.\textsuperscript{12} Within a complex homeostatic system, such as the control of the cardiocirculatory responses during exercise, it is likely that a redundancy of feedback mechanisms exists. However, the observation that the enhanced cardiovascular response was, in our study, even more pronounced in McA than in MM (Fig. 1) suggests that lactate\textsuperscript{−} and H\textsuperscript{+} accumulation in muscle and blood is not an essential component of this metabolic feedback mechanism. We observed a significant correlation between the slope of the HR vs. VO\textsubscript{2} relationship (a higher slope suggesting an enhanced cardiovascular response to exercise) and Δ[deoxy(Hb + Mb)] peak (Fig. 5). This observation represents further indirect evidence that Δ[deoxy(Hb + Mb)] peak is indeed estimating skeletal muscle O\textsubscript{2} extraction. Because we did not measure Q\textsubscript{peak} as an index of the enhanced cardiovascular response we could not utilize the slope of the Q vs. VO\textsubscript{2} relationship. Although not ideal, the slope of HR vs. VO\textsubscript{2} should substantially yield the same information, for several reasons. First,
patients and controls in the present study were not affected by cardiac diseases (a reduced stroke volume, as a consequence of cardiac diseases, would determine a higher HR by itself). Indeed, although combined myopathy and cardiomypathy is relatively common in mitochondrial metabolic disorders, our MM patients did not have evidence of cardiac involvement. Cardiac muscle normally is spared in McArdle’s disease, due to the presence of cardiac-specific phosphorylase isoenzymes; this appears compatible with the substantially normal peak values determined by previous investigators in McA patients. Moreover, stroke volume is classically considered to reach a plateau for submaximal exercises corresponding to about 40% of VO₂ peak, so that for higher workloads a close linear relationship should exist between HR and Q.

### Physical Deconditioning

In patients with metabolic myopathies, exercise intolerance inevitably leads to a reduced level of habitual physical activity. This phenomenon is associated with physical deconditioning, which might by itself determine lower than normal VO₂ peak and slightly lower than normal maximal O₂ extraction (and hence lower Δ[deoxy(Hb + Mb)] peak) values. In the present study a second group of controls was considered (P-CTRL), composed of patients with reduced exercise tolerance or other signs or symptoms suggesting a metabolic myopathy, but in whom the muscle biopsy did not lead to a diagnosis of any known myopathy. In P-CTRL VO₂ peak and Δ[deoxy(Hb + Mb)] peak values were only slightly lower than those in CTRL, and the HR vs. VO₂ relationship was only slightly shifted to the left compared to that of CTRL; for none of these variables did the observed differences reach statistical significance. This suggests that only a minor portion of the observed differences between MM, McA, and CTRL could be attributed to reduced physical conditioning.

In conclusion, in MM and McA patients, NIRS can detect and quantify the impaired capacity of O₂ extraction by skeletal muscle, which in these patients represents the pathophysiological mechanism responsible for the lower than normal VO₂ peak and exercise tolerance. The possibility to serially monitor such impairment using a non-invasive tool should be of great interest to clinicians, who need a quantitative and longitudinal evaluation of the impairment in the follow-up of patients, as well as in the assessment of therapies or interventions.

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### REFERENCES

ABSTRACT: Patients with implanted cardiac devices and their physicians may defer important electrodiagnostic testing because of anxiety about potential negative effects on the device. To determine the safety of routine nerve conduction studies (NCS) in this population, 10 patients with permanent dual-chamber pacemakers of various types and five patients with implanted cardiac defibrillators (ICD) underwent nerve stimulation at sites commonly used during NCS. The implanted cardiac device was interrogated before and after the study and there was continuous monitoring of the surface electrocardiogram (ECG) and atrial and ventricular electrograms. Electrical impulses generated during routine NCS were never detected by the sensing amplifier and did not affect the programmed settings of the implanted cardiac device. We conclude that routine NCS is safe in patients with implanted cardiac pacemakers with bipolar sensing configurations and defibrillators.

Nerve conduction studies (NCS) and electromyography (EMG) are frequently necessary to establish a diagnosis of neuromuscular disease and to plan an appropriate course of treatment. Many patients with suspected neuromuscular disease suffer from comorbidities that require treatment with implanted cardiac devices, such as cardiac pacemakers and implanted cardiac defibrillators (ICDs). Frequently, patients with implanted cardiac devices and some physicians who treat them defer important electrodiagnostic testing with NCS and EMG because of anxiety about the potential negative effects on the device, despite the prevalent view among electromyographers and cardiac electrophysiologists that NCS poses minimal or no risk to these patients as long as the stimulation is not given in direct proximity to the pulse generator. The fear of some physicians is that the stimulus presented during NCS might be interpreted as cardiac in origin and temporarily inhibit or trigger output, be interpreted as noise and temporarily cause reversion to an asynchronous pacing mode, which could lead to dangerous tachyarrhythmias, or cause inappropriate reprogramming of the device. The possibility that repeated trains of electrical impulses could induce ventricular or atrial fibrillation is also of concern. Less likely, but also a consideration, is the possibility that high levels of current can lead to tissue damage or cause irreversible harm to the pulse generator itself. Large, randomized, blinded trials regarding this subject do not exist and should not be necessary given current implanted cardiac device technology and our knowledge about volume conduction and far-field potentials.

Pacemakers and ICDs function as sensing and stimulating devices. Intracardiac leads detect electrical potentials in the cardiac conduction pathways and stimulate those pathways as necessary. Pacemakers are designed to treat bradyarrhythmic events, whereas ICDs are designed to treat tachyarrhythmic events, particularly ventricular fibrillation. ICDs can also function as pacemakers. Both devices are able to discriminate between interference and signals of cardiac
origin by utilizing a bandpass filter that prohibits the entry of signals that are above or below a certain frequency threshold. Despite this defense against possible sources of interference, such as stimuli delivered during NCS, it is possible that the stimulus could mimic normal or abnormal cardiac activity that could cause antitachycardia pacers to deliver a countershock, or be interpreted as noise, causing suspension of arrhythmia detection, thus leaving the patient unprotected. Fortunately, the sensing systems employed also help to protect against these possibilities.3

Current implanted pacemaker and ICD technology utilizes a bipolar sensing system, which has replaced its predecessor, the unipolar system. However, this nomenclature is a misnomer. Both the unipolar and bipolar systems use a bipolar configuration with an active and reference electrode, but the location of those electrodes has changed in the move from unipolar to bipolar pacemakers. Bipolar pacemakers began replacing unipolar pacemakers 20–25 years ago, and now unipolar pacemakers are nearly nonexistent. By design, all ICDs have a bipolar configuration. In unipolar pacemakers, the active recording electrode, or cathode for stimulation, is the intracardiac lead embedded in the wall of the heart. The reference recording electrode, or anode, which can also be used for stimulation, is the metal can of the pulse generator, in the chest wall. By contrast, the intracardiac leads that are used in the bipolar sensing configuration contain both active and reference electrodes that can function as the cathode and anode for stimulation. These leads are placed ~0.5–1 cm apart, typically in the right ventricular apex and right atrial appendage. This configuration is less susceptible to far-field potentials.

Far-field potentials arise from electrical potentials propagating in constrained compartments, such as the human finger, hand, or arm. They can be recorded when active and reference electrodes are relatively far apart and on opposite sides of the far-field potential, such as in a referential montage, particularly when that montage encompasses an area in which the volume of the compartment suddenly changes direction or dimension (e.g., from finger to hand, or arm to chest). The unipolar sensing configuration of early pacemakers used such a referential montage, with several centimeter separation between recording electrodes, making it theoretically possible to record volume-conducted, far-field potentials from limb NCS. However, the bipolar sensing configuration in use for the past two decades does not use a referential montage, has sensing electrodes very close together, and is not near a volume discontinuity. The electrical impulse generated during NCS is distant, infrequent, and incapable of generating a far-field potential detectable by the intracardiac bipolar sensing mechanism of an implanted cardiac pacemaker or ICD, thus resulting in no risk of affecting the function of these implanted cardiac devices. We therefore proposed to study a series of patients with implanted bipolar cardiac pacemakers and ICDs to prove that the intracardiac electrogram shows no evidence of electrical interference from NCS impulses.

MATERIALS AND METHODS

The patients included in the present study were followed by one of the authors (F.V.C.) at an implanted cardiac device clinic. Patients were initially informed about the study in a letter from their cardiologist (F.V.C.) and again at follow-up visit for pacemaker or ICD interrogation. The patients signed an informed consent form approved by the institutional review board. Since this study examined the effects of an electrical field applied to the body surface on an ICD, we had no specific exclusion criteria regarding the patient’s past medical history, age, sex, ethnicity, and current medications other than that patients with pacemakers using a unipolar sensing mode were excluded. All of the patients had the pulse generator or ICD embedded in the left chest, over the pectoralis muscle. The pacemaker types included Pacesetter models Trilogy 2308L, Synchirhy II 2022L, Entity DR 5326, Affinity DR 5330L, and Trilogy DR 2360L (St. Jude Medical, Inc., St. Paul, Minnesota); Prodigy DR 7860B, Kappa KDR 901, and Minuet 7108 (Medtronic, Inc., Minneapolis, Minnesota), and Discover II 1286 (Guidant Corp., Boston, Massachusetts). The implanted defibrillator types included Atlas + VIR V193, Atlas DR V240 (St. Jude Medical), and Maximo VR, Atlas DR V242 (Medtronic). The range of programmed sensitivity of the pacemakers and ICDs was 0.5–2.0 mV for the atrial amplifier and 2.0–4.0 mV for the ventricular amplifier. Before and after undergoing NCS, the patients’ pacemakers and ICDs were interrogated to ensure normal function. During NCS there was simultaneous display of the patients’ surface ECG and atrial and ventricular electrograms (Fig. 1).

A Counterpoint EMG machine (Dantec, Skovlund, Denmark) was used to perform NCS. Surface bipolar stimulation was applied with a constant-current stimulator. The motor responses to nerve stimulation were recorded to ensure that nerve stimulation actually occurred, thereby making the test situation similar to that in the EMG laboratory. Four
stimulation sites were used: in six patients with pacemakers the left peroneal nerve was stimulated at the left ankle and fibular head with the active surface recording electrode over the left extensor digitorum brevis muscle (EDB), and the left median nerve was stimulated at the left wrist and elbow with the active surface recording electrode over the left abductor pollicis brevis muscle. In four patients with pacemakers and in the five patients with ICDs, median nerve stimulation occurred at the left Erb’s point region instead of the left elbow. Stimulus intensity and duration were increased stepwise at each site: 50 mA at 0.1 ms, 0.3 ms, and 0.5 ms, followed by 100 mA at 0.1 ms, 0.3 ms, and 0.5 ms for a total of six stimulations at each site. The time interval between each electrical stimulus was 10–20 s.

RESULTS

Ten patients with implanted cardiac demand pacemakers and five patients with ICDs with bipolar sensing configuration participated. In all 15 patients, none of the electrical impulses generated during routine NCS of the left peroneal and median nerves (including stimulation at the left Erb’s point region in nine patients) were seen on the surface ECG or detected by the atrial or ventricular sensory amplifiers of the pacemakers or ICD (Fig. 1). In none of the patients was pacemaker or ICD function affected by NCS at any of the stimulation sites, including Erb’s point.

DISCUSSION

In the present study we examined the electrical effects of routine NCS on cardiac pacemaker and ICD models currently in clinical use using stimulus intensities and durations that probably exceed those used during most routine NCS. No electrical impulse was detected by the sensing mechanism of the cardiac pacemakers or ICDs. The failure to detect a far-field potential in the heart from peripheral NCS should come as no surprise, given the close proximity of the active and reference electrodes to each other in the heart, the large volume of the chest, which would rapidly dissipate a volume-conducted potential, and the relatively low sensitivities of the pacemaker and ICD sensing amplifiers (0.5–4 mV). Our results show that there is no risk of peripheral NCS, even at the left supraclavicular fossa, interfering with an implanted cardiac pacemaker or ICD using a bipolar sensing configuration. It is theoretically possible that the pacemaker could be switched to unipolar mode if it is exposed to a larger electromagnetic force (e.g., magnetic resonance imaging, resuscitation paddles). However, this situation is very unlikely to occur; even if it did, the stimulus used in NCS is so low and infrequent that it is unlikely to affect the pacemaker. ICDs are only in bipolar mode.

The literature on the safety of NCS with regard to pacemakers is scarce, and it is nearly nonexistent for ICDs. Based on the case of a 61-year-old woman, whose implanted phrenic nerve stimulator interfered with her cardiac demand-pacemaker, Wicks et al.6 in 1978 examined the effect of phrenic nerve stimulators on pacemakers in a series of patients. Interference was found in 14 of 27 patients, resulting in temporary inhibition or conversion to asynchronous pacing. Twelve of the affected patients had pacemakers using unipolar technology. The phrenic nerve stimulators all had loop antenna and the interference with the pacemakers increased with proximity to the pacemaker device in the chest wall. This case series clearly relates to a different situation than occurs with NCS and is more akin to the interference that may occur from powerful external electromagnetic devices, such as magnetic resonance imaging.

FIGURE 1. A sample recording strip from the ventricular electrogram (bottom tracing) and the surface ECG (top tracing) of a pacemaker patient immediately before, during (dark arrow), and after NCS. A marker channel, showing the detected atrial and ventricular signals, is in the middle channel. There is no extraneous electrical signal recorded from NCS on any channel.
or a magnet placed over the pulse generator. In 1988 LaBan et al. studied 20 patients, 13 of whom had implanted pacemakers with bipolar sensing and seven with unipolar sensing. Only five patients were monitored by ECG during NCS. Pacemaker interference by NCS was judged clinically by pulse palpation and patient reports of untoward side effects. The stimulus duration did not exceed 0.2 ms and stimulus intensities were not reported. All stimulations were “uneventful.” In 1993 O’Flaherty and Adams published a case report of an 89-year-old man with an implanted pacemaker (unipolar configuration), whose plethysmographically measured cardiac output was lost during left facial nerve stimulation performed to quantify neuromuscular blockade, and returned after cessation of nerve stimulation.

There remains a theoretical concern that NCS done directly over or in near proximity to the pulse generator embedded in the chest wall could affect pacemaker function. We were able to stimulate safely over the left supraclavicular fossa, which is as close to the usual site of the pulse generator as NCS should ever be done. Our results by no means address the safety of NCS in patients with temporary transvenous cardiac pacemakers. NCS remain contraindicated in such patients because of the breach in the skin–intravascular barrier, leading to possible propagation of electrical potentials directly along the transvenous pacemaker to the heart.

The number of patients in our study is small but sufficient to demonstrate that there is no effect of NCS on cardiac pacemakers or ICDs given the complete absence of electrical impulse detection in all 15 patients, involving 360 stimuli. This complete and expected absence of detection makes blinding or randomization moot and unnecessary. We did not apply repetitive nerve stimulation (RNS) because of patient discomfort. RNS uses the same stimulation intensities but significantly higher frequencies than those applied here. The detection of external electrical impulses by any cardiac electrical device using bipolar sensing is independent of the frequency of the electrical impulse. Hence, RNS should also be safe.

It is hoped that the results of our study will diminish the anxiety of patients with implanted bipolar cardiac pacemakers or ICDs undergoing routine NCS and at the same time encourage physicians not to exclude such patients from access to this important neurophysiologic test.

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SHORT REPORT

ABSTRACT: We report a patient who experienced progressive diplopia and distal weakness of the upper limbs. Magnetic resonance imaging of the brain showed extensive white matter lesions and analysis of cerebrospinal fluid revealed acute human T-lymphotropic virus type I (HTLV-I) infection. Myasthenia gravis (MG) was evidenced by electromyography (EMG) and antibodies against acetylcholine receptor. This unusual case of MG associated with HTLV-I infection and brain-restricted lesions underscores the possible link between viruses and MG pathogenesis.


MYASTHENIA GRAVIS ASSOCIATED WITH HTLV-I INFECION AND ATYPICAL BRAIN LESIONS

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Myasthenia gravis (MG) is an autoimmune disease mainly caused by autoantibodies directed against the acetylcholine receptor (AChR) located at the postsynaptic neuromuscular junction. In about 90% of cases, no specific origin can be identified but the individual genetic background and environmental factors may precipitate the disease. Although no specific virus has been clearly identified as a direct cause of MG, some reports have described a possible association with herpesvirus, and human immunodeficiency virus (HIV), and human T-lymphotropic virus type I (HTLV-I). HTLV-I is frequently associated with chronic myelopathy, described as HTLV-I–associated myelopathy/tropical spastic paraparesis (HAM/TSP), whereas an association with brain lesions is rare. We report the case of a patient who presented with MG associated with an acute HTLV-I infection of the central nervous system (CNS) and extensive but brain-restricted lesions.

CASE REPORT

A 61-year-old woman with non–insulin-dependent diabetes and a history of thyroid surgery for hypothyroidism was hospitalized for a 1-month history of progressive diplopia, unilateral ptosis, and distal weakness of the upper limbs. She also complained of left upper and lower limb paresthesias. She originated from the Ivory Coast (western Africa) and had visited that country 6 months before hospitalization. The patient was initially referred to an ophthalmologist, who recommended magnetic resonance imaging (MRI) of the brain. The MRI revealed a large right frontal white-matter T2-weighted hyperintensity as well as hyperintensity in the right upper pons (Fig. 1a–d). MRI of the spinal cord was normal. Physical examination on the day of admission revealed vertical diplopia and distal symmetrical weakness (grade 4 on the Medical Research Council scale) of the upper arms. The sensory examination as well as the findings from the remainder of her neurological examination were normal. Routine laboratory tests were normal. Lumbar puncture yielded a clear cerebrospinal fluid (CSF) with protein at 30 mg/dl, white blood cells at 8 × 10^6/L, and IgG oligoclonal bands detected by immunoblot and isoelectrofocalization. Blood serology was negative for HIV and Borrelia burgdorferi but positive for HTLV-I/II; the immunoblot was specific for HTLV-I. According to polymerase chain reaction (PCR) analysis, CSF was negative for JC virus, cytomegalovirus, varicella-zoster virus, and Mycobacterium tuberculosis, but it was positive for HTLV-I (from two independent spinal taps). A peripheral blood smear revealed numerous stimulated lymphocytes and Pfeiffer cells compatible with HTLV-I infection, with no evidence of leukemia or lymphoma. The lymphocyte count by flow cytometry was normal. A detailed neuropsychological evaluation revealed mild cognitive impairment. The following day, the patient developed a generalized tonic–clonic seizure. The EEG showed a left temporal focus with discharges propagating to the midline. A CT scan of the head was normal. On the basis of the clinical and laboratory findings, a diagnosis of acute HTLV-I infection of the CNS was made. The patient was treated with intravenous ganciclovir, which was discontinued after 2 weeks because of adverse effects. She was discharged on valganciclovir and symptomatic treatment.

Abbreviations: AChR, acetylcholine receptor; CNS, central nervous system; CSF, cerebrospinal fluid; CT, computed tomography; EMG, electromyography; HIV, human immuno deficiency virus; HAM/TSP, HTLV-I–associated myelopathy/tropical spastic paraparesis; HTLV-I, human T-lymphotropic virus type I; MG, myasthenia gravis; PCR, polymerase chain reaction

Key words: human T-lymphotropic virus type I–associated myelopathy; tropical spastic paraparesis; human T-lymphotropic virus type I; meningitis; myasthenia gravis

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logical examination revealed visuospatial memory and executive dysfunction. The patient refused a brain biopsy.

The patient initially received high doses of methylprednisolone (1 g/day intravenously for 4 days), but rapidly worsened, with development of an almost complete ophthalmoparesis, bilateral ptosis, bifacial paresis, and a quadriaparesis. She subsequently received lamivudine and zidovudine [Combivir (lamivudine 150 mg and zidovudine 300 mg) once daily], initiated as an off-label treatment\(^7,12\) due to the suspicion of a progressive demyelinating disease of the CNS secondary to HTLV-I infection.

A standard repetitive stimulation test was performed on the left hand using low-frequency (3-Hz) repetitive pulses applied to the ulnar nerve at the wrist. The amplitude of the M response recorded over the abductor digitii minimi muscle was 8 mV, with a 70% decrease in amplitude with the fourth stimulus (Fig. 2a). This decrement was partially reversible after injection of 10 mg of edrophonium chloride (Fig. 2b). MG was confirmed by the presence of acetylcholine receptor (AChR)–specific serum antibodies, quantified at 1.1 pmol/ml (confirmed three times), more than fivefold the upper limit of normal values (<0.2 pmol/ml). A thoracic computed tomography (CT) scan did not reveal any sign of thymoma. The other auto-antibodies testing negative were anti-MuSK, anti–smooth muscle, anti–striated muscle, anti-thyroglobulin, anti-thyroperoxidase, anti-nuclear factor, and anti-nucleosome.

The patient’s symptoms continued to worsen despite plasmapheresis and initiation of pyridostigmine (up to 540 mg/day). She required intensive care observation due to the decrease of vital capacity to 960 ml, but invasive respiratory assistance was not necessary. A favorable response was eventually achieved with a progressive increase of pyridostigmine up to 1350 mg/day, together with intravenous immunoglobulins and immunosuppressive drugs [prednisone (40 mg/day) and azathioprine (150 mg/day)]. Combivir was stopped 2 months after initiation when a control spinal tap revealed a normal white cell count and a decrease of the CSF viral

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**FIGURE 1.** Brain imaging. (a) Unenhanced CT scan at the time of admission shows hypodensity in the right frontal subcortical white matter. (b) MRI on the same day showing a T2-weighted hyperintensity in the right frontal white matter. (c) Coronal fluid-attenuated inversion recovery (FLAIR) image shows the hyperintense lesion extending into the internal and external capsules to involve the basal ganglia, and extending into the mesencephalon and cranial pons. (d) T1-weighted image after administration of gadolinium shows no enhancement of the hypointense lesion. (e) MRI, 1 month later, shows the lesion to remain unchanged on axial FLAIR. (f) T1-weighted imaging showing no enhancement. (g) The coronal FLAIR image shows no progression. (h) Three months after the first MRI, the imaging is still unchanged as shown on this axial T1-weighted post-contrast image.
load by PCR, from 52 HTLV-I copies per reaction to 1 copy per reaction. Repeat MRI performed 1 month and 3 months after onset did not reveal any extension of the described lesions (Fig. 1e–h). Three months after onset, EMG revealed a dramatic improvement of the decrement to 15% (Fig. 2c); the only persistent signs were distal upper-limb paresis (grade 4+/H11001) and vertical diplopia revealed by Simpson’s test.

**DISCUSSION**

We have described an unusual case of MG associated with HTLV-I infection and brain-restricted lesions in the absence of myelopathy. The HTLV-I infection was demonstrated by positive serology, detection of virus DNA in the CSF by PCR, mild pleiocytosis, and the presence of IgG oligoclonal bands in the CSF. MG was established by typical EMG findings, positive anti-AChR antibodies, and the clinical response to an anticholinesterase.

The initial clinical deterioration was attributed to the high dose of methylprednisolone that the patient received in the context of diffuse brain lesions and suspicion of progressive multifocal leukoencephalopathy, acute demyelinating encephalomyelitis, or gliomatosis cerebri. It is noteworthy that patients with MG receiving high doses of corticosteroids may have an exacerbation of their symptoms.9 Combivir was initiated in the context of clinical deterioration associated with HTLV-I infection. This treatment was stopped after the second spinal tap revealed a significant decrease of viral DNA in the CSF, the disappearance of CSF pleiocytosis, and clinical improvement of the patient. Although a possible beneficial effect of Combivir on the disease course cannot be excluded, the clinical improvement was attributed to the specific MG treatments rather than the antiviral therapy.

HTLV-I has a tropism for the CNS but is typically associated with chronic myelitis (HAM/TSP). Rare reports have described the association between HAM/TSP and MG,2,3 but not the association between HTLV-I brain lesions and MG. Recently, molecular mimicry has been proposed as a mechanism linking HTLV-I and CNS autoimmune disease in a study showing that IgG isolated from HAM/TSP patients can specifically bind to and damage neurons.6 In addition, there was a high prevalence of two HTLV-I gene sequences of the thymus gland in a group of MG patients, suggesting a possible link with the pathogenesis of MG through molecular mimicry.8 Other physiopathological explanations could be:

1. virus-induced immunomodulation of CD4+ T cells that control B cells secreting anti-AChR antibodies; and
2. direct virus-induced polyclonal activation of B cells driving the secretion of AChR antibodies. Interestingly, an association between HTLV-I and other autoimmune diseases, such as diabetes5 and thyroid diseases, has been described. The patient studied herein had a history of diabetes and hypothyroidism although no specific auto-antibodies were detected.

In the present case report, the association between brain lesions and HTLV-I was not proven neuropathologically (the patient refused cerebral biopsy). Nevertheless, this association was reinforced by demonstration of acute CSF infection associated with HTLV-I DNA detection and the absence of any previous neurological symptoms. In addition, any brain lesions may have been clinically silent, which is frequent with white-matter lesions. However, the patient’s initial complaints of left upper- and lower-limb paresthesias as well as the neuropsychological
examination suggested a paucisymptomatic rather than silent CNS lesion.

In conclusion, although the biological mechanism remains to be determined, this case study raises the possibility of a causal association between HTLV-I CSF infection and MG, because they occurred at the same time, together with the appearance of acute neurological symptoms. These data reinforce previous hypotheses indicating that viruses such as HTLV-I might play a role, at least in some patients, in MG pathogenesis.

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ABSTRACT: The epidemiology of cauda equina and conus medullaris lesions is not well known, and this study aimed to provide further information on this topic. In the period 1996–2004, patients fulfilling the clinical, electrodiagnostic, and radiological criteria for such lesions were identified. For cauda equina/conus medullaris lesions an annual incidence rate of 3.4/1.5 per million, and period prevalence of 8.9/4.5 per 100,000 population were calculated. The values obtained are probably valid estimates of the incidence and prevalence of these lesions in developed countries.
ate segment of Slovenian population—mean age of patients at the time the lesion occurred] were established. Age- and gender-specific incidence rates were also calculated.

RESULTS

A total of 87 patients (59 men), aged 19–84 years (mean, 46.6 years), with onset of lesion in the period studied, were identified. Most patients had an apparent cause or appropriate imaging abnormalities. Saddle sensory loss was found in 86%, quantitative EMG abnormalities in 68%, electrodiagnostic sacral reflex abnormalities in 72%, urinary/anal incontinence in 51%/48%, and constipation in 56% of patients; erectile dysfunction occurred in 76% of the men. The etiologies consisted of intervertebral disk herniation in 33 (20 men), spinal fracture in 27 (22 men), iatrogenic lesion in 10 (7 men), spinal tumor in 6 (all men), and "other" in 11 (4 men). Lesions at the T12 or L1 levels (conus medullaris lesions) were found in 20 patients; in 16 patients the etiology was spinal fracture. In the remaining 67 patients, segments from L2 to L5–S1 were affected (cauda equina lesions). Two patients with simultaneous involvement above and below the L1–L2 intervertebral disk space level were included in the conus medullaris group.

In the study period, the average annual incidence of cauda equina lesions in Slovenia was 6.8 (from 2 in 2000 to 11 in 2003), and of conus medullaris lesions was 2.9 (from 0 in 2003 to 5 in 1996 and 1999). In the same period the population of Slovenia averaged to 1,989,198. The average annual incidence rate of cauda equina lesions was 3.4, and of conus medullaris lesions 1.5 per million population. The average annual incidence rate was the highest for intervertebral disk herniation (1.8 per million population), followed by spinal fractures (1.5), iatrogenic lesions (0.6), other etiologies (0.6), and spinal tumors (0.3).

In both conditions and almost all age groups, a higher incidence rate was observed in men. Patients with conus medullaris lesions tended to be somewhat younger than those with cauda equina lesions (Fig. 1). The main etiologies in the different age groups were spinal fractures (<40 years, particularly men), disk herniations (40–60 years, both genders), and both disk herniations and iatrogenic lesions (i.e., spinal stenosis surgery; >60 years).

During the study period the average life expectancy in Slovenia was 71.9 years for men and 79.1 years for women. By adjusting life expectancy to the gender ratio in the cauda equina group (men/women = 1.77), a value of 74.5 years was obtained; in the conus medullaris group (men/women = 3.33), the value was 73.6 years. For patients with cauda equina lesions the period prevalence was 8.9, and for conus medullaris lesions it was 4.5 per 100,000 population. The period prevalence adjusted for average age was highest for intervertebral disk herniations (5.2 per 100,000 population), spinal fractures (4.8), other etiologies (1.3), spinal tumors (0.8), and iatrogenic lesions (0.7).

DISCUSSION

To diagnose cauda equina and conus medullaris lesions in Slovenia, in addition to lower-limb EMG, sacral electrodiagnostic studies are performed in all patients. Such studies are available only in the two institutions that served as recruitment centers. Slovenia also seems almost ideal for assessment of the...
epidemiology of these two conditions. The health system is well developed, and the health insurance system covers medical expenses at home but not abroad. People thus do not seek medical assistance for these problems outside the country. Consequently, the annual incidence data were used to calculate the annual incidence rate and period prevalence of cauda equina and conus medullaris lesions in the Slovenian general population. In 20 patients with conus medullaris lesion (mainly due to T12 or L1 vertebral fractures), additional spinal root (i.e., cauda equina) lesions could not be excluded. In the remaining 67 patients with lesions below the L1–L2 intervertebral disk level, isolated cauda equina damage probably occurred.

Previously, a ratio of about 1% between the incidence of all lumbar discectomies and those for severe cauda equina compression was reported. However, using the annual incidence rate of cauda equina lesions due to herniated lumbar intervertebral disks from the present study (1.8 per million) and estimates of the incidence rate of all herniated lumbar intervertebral disks in the USA (150 per 100,000 population), a much lower ratio of 0.12% was obtained. (The U.S. data for the incidence of intervertebral disk herniations were used because no such data are available in Slovenia.) The discrepancy is probably due to a bias toward more severe pathology in operated patients. Furthermore, although every effort was made to recruit all relevant patients, the figures obtained in this study may still be somewhat underestimated. Patients with cauda equina or conus medullaris lesions who were not examined electrodiagnostically were missed. Similarly, the number of conus medullaris lesions was much lower than the number of thoracolumbar (T12 or L1) fractures, because significant spinal cord injury detectable by clinical and electrodiagnostic methods occurred in only a small proportion of these patients.

Nevertheless, if the annual incidence rates obtained in this study are also valid for the USA (2006 estimated population: 299,093,237), 1,016 new cases of cauda equina lesions and 449 of conus medullaris lesions per year would be expected. These figures are lower than those for spinal cord injuries (30 per million population) and benign spinal tumors (10), similar to that for syringomyelia (4), and higher than for malignant primary spinal cord tumors (1).

The higher incidence of conus medullaris lesions in men was caused by a higher incidence of spinal fractures, particularly in young men (Fig. 1). By contrast, a higher incidence of disk herniations and spinal tumors in (middle-aged) men is not widely appreciated, and occurs for less clear reasons.

Although in patients with cauda equina and conus medullaris lesions symptoms may improve partially or completely in the first 2 years after injury, neurological signs and electrodiagnostic abnormalities usually persist. Consequently, all of these were included in period prevalence calculations. Moreover, in the prevalence calculations it was assumed that, although these lesions often have a devastating effect on quality of life, they do not significantly shorten the lifespan. If the reported prevalence rates are valid, it can be calculated that 26,611 patients with cauda equina and 13,455 patients with conus medullaris are currently alive in the USA.

This study was presented in part at the 10th Congress of the European Federation of Neurological Societies, September 2006, Glasgow, UK. The author thanks Professor Maja Primic-Zakelj for the methodological review and Dr. Dianne Jones for language review of the manuscript. The study was supported by the Republic of Slovenia Research Agency (grant no. J3 7899).

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CASE OF THE MONTH

ABSTRACT: We describe a patient who developed an ataxic sensory syndrome associated with xerophthalmia and progressive dysphagia with regurgitation. Electrophysiological findings were consistent with an axonal sensory neuropathy, and superficial peroneal nerve biopsy showed a reduction in number of myelinated fibers with epineurial inflammation. Rheumatoid factor, anti-SSA/SSB and antinuclear antibodies were positive and a diagnosis of Sjogren's syndrome was made. An endoscopic investigation revealed esophageal achalasia. We suggest that there may be a common autoimmune mechanism directed to different targets on the basis of this rare association.


SENSORY ATAXIC NEUROPATHY AND ESOPHAGEAL ACHALASIA IN A PATIENT WITH SJOGREN’S SYNDROME

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Sjogren’s syndrome (SS) is an autoimmune disorder characterized by xerophthalmia and xerostomia, in which extra-glandular disease and systemic immunological phenomena, including vasculitis, are common. Peripheral neuropathy, in particular sensory neuropathy,7,12,16 is a major neurological manifestation that may occur in primary SS in the absence of systemic involvement and in many cases represents the onset of symptoms.9

Dysphagia is another common problem in patients with SS. Although it has been attributed to a lack of saliva, some authors have associated it with esophageal dysmotility.21,23 We are unaware of any reports on an association between SS and esophageal achalasia, a disorder characterized by a failure of relaxation of the lower esophageal sphincter.

CASE REPORT

In May 2004, a 63-year-old woman presented with an atactic gait, dysphagia with frequent regurgitation, and weight loss (16 kg in 3–4 months). She had a 1-year history of paresthesias of the feet, which had gradually spread to the legs and lower abdominal region, the left hand, and the ulnar side of the right hand. She reported a 1-year use of lubricating eye drops and a history of dryness of the mouth. Family history was unremarkable.

On admission, she was unable to walk and was wheelchair-bound. Neurological examination of the cranial nerves showed no abnormality except for bilateral Adie’s pupils. Muscle strength was preserved in all extremities. Deep tendon reflexes were absent in the lower limbs and decreased in the upper limbs. She had ataxia of the trunk and upper limbs and profound loss of touch and pinprick sensation along the ulnar side of the left hand and in the lower limbs from the hips. The appreciation of position and vibration was severely impaired in the lower limbs from the hips, and reduced in the left hand. The patient was catheterized for bladder failure of uncertain cause.

Results of routine blood tests, including of liver, kidney, and thyroid function, and serum levels of vitamins B1, B12, and E were normal. There was no evidence of impaired glucose tolerance. Serological tests for hepatitis B and C were normal. Antinuclear antibodies, C-reactive protein, rheumatoid factor, and anti-SSA and -SSB antibodies were positive. Anti-DNA antibodies and serum immunoglobulins were normal. Antiganglioside, anti–myelin-associated glycoprotein, anti-gliadin, and anti-endomyosial antibodies, and cryoglobulins were undetectable. No monoclonal proteins were found. Tumor markers were normal and antibodies against onconeural antigens (anti-Hu, anti-Yo, and anti-Tr antibodies) were absent.

A Shirmer test showed reduced tear production (<5 mm wetting after 5 min).
Motor conduction studies and F-wave minimal latencies of the median, ulnar, and deep peroneal nerves were normal bilaterally. No temporal dispersion of compound motor action potentials or conduction blocks were observed. Sensory nerve action potentials in the median and ulnar nerves were decreased, with moderately slowed sensory conduction velocity in the upper limbs; they were not evoked in the sural nerves (Table 1). The findings suggested an axonal polyneuropathy.

Magnetic resonance imaging (MRI) showed multiple T2 high-intensity signals predominantly in subcortical and periventricular white matter. MRI of the cervical (Fig. 1A) and thoracic (Fig. 1B) spine showed posterior axial T2-weighted high signal intensity in the posterior columns. Computerized tomography scans of the chest, abdomen, and pelvis were normal.

Biopsy of the superficial peroneal nerve, studied by light and electron microscopy, demonstrated a loss of nerve fibers, which, although diffuse, varied in severity in different fascicles. Indeed, some were less involved, whereas others showed an almost complete absence of fibers. Myelin-devoid axons were also present (Fig. 2A), as was scattered degenerating myelin. No amyloid was detected. Some small vessels had thickened walls. Inflammatory cells were observed surrounding and infiltrating the walls of epineurial vessels (Fig. 2B). Immunohistochemistry revealed some CD8⁺ lymphocytes and scattered macrophages, but no CD3⁺ or CD20⁺ cells.

Endoscopic investigation of the upper gastrointestinal tract was performed by mucosal biopsy, which showed mild esophagitis. Esophageal manometry demonstrated an increase in lower sphincter pressure with incomplete relaxation. Achalasia was treated with botulinum toxin injection, resulting in transient improvement in symptoms. Three months later, a relapse of the esophageal stenosis was resolved definitively with Heller’s myotomy.

A diagnosis of sensory ataxic neuropathy and esophageal achalasia in SS was made on the basis of the clinical, biochemical, and radiological findings. The patient was treated with intravenous immunoglobulins (0.5 g/kg daily for 5 days) in May and June 2004, without improvement of symptoms. Therefore, as of July 2004, monthly pulsed high-dose intravenous prednisolone administration (500 mg/day for 4 days) was begun. This resulted in a progressive improvement of the truncal ataxia, voluntary movements in all limbs, and hypoesthesia, which was most evident in the lower limbs. Further improvement of the symptomatology was noted at the last evaluation, in June 2005, with the patient able to

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| NR, no response; SCV, sensory conduction velocity; SNAP, sensory nerve action potential.

*Normal values in parentheses; abnormal results in bold.

FIGURE 1. MRI of the cervical (A) and thoracic (B) spine demonstrates posterior axial T2-weighted high signal intensity (arrows) in the posterior columns.
Blood tests showed normalization of C-reactive protein, rheumatoid factor, and anti-SS and anti-SSB antibodies.

**DISCUSSION**

Peripheral nervous system involvement is frequent in SS, and the most common neuropathy seems to be a sensory ataxic neuropathy, which represents up to 40% of the neuropathies in this condition. Different pathological features have been observed in SS-associated neuropathy. Indeed, in an autopsied patient with the sensory ataxic form, Mori and colleagues observed the coexistence of perivascular infiltrates in peripheral nerves and reduced sensory and sympathetic ganglion neurons in different spinal segments with ganglionitis. As suggested by the authors, although each neuropathic form has predominant clinical features, different forms may overlap each other on the basis of a common underlying process.

Up to 75% of patients with active peripheral vasculitis also have disease of the central nervous system, which is generally regarded to be involved less frequently than the peripheral nervous system. Our patient had increased signal intensity in T2-weighted images, predominantly in subcortical and periventricular white matter, consistent with a central manifestation of SS.

Dysphagia is a common complaint in SS and does not correlate only with the xerostomia that is a typical finding of the disease. In many papers,

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**FIGURE 2.** Right superficial peroneal nerve biopsy. (A) Blue toluidine–stained semithin sections showing marked reduction in myelinated fibers (×40. Bar, 10 μm). (B) Inflammatory cells surrounding and infiltrating (arrows) epineurial vessels (hematoxylin and eosin, ×20. Bar, 20 μm).
about the gastrointestinal manifestations of SS, a high incidence of esophageal motility disorders has been reported, and dysphagia is thought to be a consequence of both lack of saliva and esophageal dysmotility. We are unaware of reports of achalasia in SS, and the association of esophageal achalasia and SS must be rare. The presence of “sicca syndrome,” neuropathy, esophageal achalasia, and ACTH insensitivity characterizes Allgrove syndrome, a rare hereditary syndrome.

The case reported suggests that the ataxic syndrome in SS probably has an immune basis, with involvement of dorsal root ganglion cells and peripheral nerves. The current understanding in pathogenesis of esophageal achalasia is that a cascade of inflammatory events leads to myenteric plexus alterations following an initial insult. Achalasia is accompanied by autoimmune phenomena such as the presence of circulating autoantibodies against the myenteric plexus, of inflammatory T-cell infiltrates in the myenteric plexus, and increased prevalence of HLA class II antigens that could play a role in its pathogenesis. Although the late onset of achalasia in our patient might be coincidental, it more likely represents the expression of multisystem spread of the pathology to involve the myenteric plexus, with a possible common autoimmune mechanism in pathogenesis of both peripheral and esophageal disease.

Although treatment is often ineffective in sensory ganglionopathies, our patient showed a definite improvement in response to corticosteroid therapy. Whether such treatment would also have helped the achalasia is unclear, as the patient underwent Heller’s myotomy before corticosteroids were introduced.

We are grateful to L. Palmucci, MD, for evaluation of the nerve biopsy and to Ilaria Paolasso, MD, for technical assistance.

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CASE OF THE MONTH

ABSTRACT: Polyglucosan body disease (PBD) is a slowly progressive adult-onset glycogen storage disorder that typically affects upper and lower neurons. Myopathy, as a complication of PBD has been reported rarely and clinically manifests as chronic limb-girdle muscle weakness. We report an unusual case of PBD myopathy presenting as an asymmetric motor syndrome that clinically overlapped with amyotrophic lateral sclerosis, further expanding the phenotype of this disorder.

POLYGLUCOSAN BODY DISEASE MYOPATHY:
AN UNUSUAL PRESENTATION

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Polyglucosan body disease (PBD) is a slowly progressive neurological disorder characterized by involvement of upper and lower neurons that may lead to a spastic quadriaparesis, peripheral neuropathy, extrapyramidal and cerebellar symptoms, neurogenic bladder, seizures, cognitive impairment, or motor neuron disease. PBD myopathy has rarely been reported and presents clinically with symmetrical limb-girdle muscle weakness. We report an unusual case of PBD myopathy with asymmetrical weakness and hyperreflexia, resembling amyotrophic lateral sclerosis (ALS), further expanding the phenotype of this disorder.

CASE REPORT

A 55-year-old, white Anglo-Saxon woman presented with progressive right foot drop of subacute onset, which started 18 months prior to presentation.

Three months prior to presentation, she developed progressive weakness of the right hand. At presentation, there was evidence of left foot drop, left hand weakness, and right proximal upper-limb weakness. She denied any bulbar, extraocular, cerebellar, extrapyramidal, or sensory symptoms. Further, she did not report bowel or bladder dysfunction, especially urinary incontinence. There was no family history of neuromuscular diseases.

On examination, marked atrophy of right forearm and right anterior leg muscles, along with atrophy of the intrinsic hand muscles on both sides, was evident. There was severe and asymmetrical muscle weakness, including global weakness of the right upper limb (distal worse than proximal), distal left upper limb, and distal lower limbs bilaterally, worse on the right. There were no fasciculations. Tone was normal. Deep tendon reflexes were bilaterally brisk. Plantar reflexes were flexor. Large- and small-fiber sensation, cerebellar function, and cranial nerve examination were normal. Higher mental functions as assessed by the mini-mental state examination and the frontal assessment battery were normal.

Laboratory investigations were normal or unremarkable and included blood electrolytes, urea, creatinine, erythrocyte sedimentation rate, liver function tests, complete blood count, creatine kinase, thyroid function tests, cortisol levels, anti-GMI and anti-neuronal antibodies, vasculitic screen (antinu-
clear antibodies, extractable nuclear antigen, anti-neutrophil cytoplasmic antibody, and complement levels), angiotensin-converting enzyme levels, immu-noelectrophoresis, and serology for human immuno-deficiency virus, hepatitis B and C, and syphilis including Venereal Diseases Research Laboratories (VDRL) and Treponema pallidum agglutination assay.

**Neurophysiology.** Nerve conduction studies (NCS) were performed using an Oxford Teca Synergy electromyograph system (Oxford Instruments, Old Woking, England). Temperature of the upper extremities was maintained at 32°C and lower extremities at 30°C. Median and ulnar motor and sensory NCSs, along with tibial, common peroneal, and sural NCSs were performed on the right side and were normal.

Needle electromyography was performed using a disposable 20-gauge concentric needle (Oxford Instruments). There were small-amplitude, brief-duration, polyphasic motor unit potentials with early recruitment in the tibialis anterior muscles on both sides, and triceps, flexor and extensor carpi radialis, abductor pollicis brevis, and abductor digit minimi muscles on the right side. Fibrillation potentials and positive sharp waves, with complex repetitive discharges were evident in these muscles. There were no fasciculation potentials.

**Neuropathology.** An open biopsy was performed on the right quadriceps muscle. The muscle biopsy was divided into two parts for enzyme histochemistry and ultrastructural studies. The tissue subjected to histochemistry was snap frozen in liquid nitrogen–chilled isopentane for 10 s and kept at −20°C until histochemical staining. The routine stains included hematoxylin and eosin (H&E), Gomori trichrome, periodic acid–Schiff (PAS), oil-red O, and the histochemical stains for acid phosphatase, myophosphorylase, succinic dehydrogenase, cytochrome c oxidase, adenosine triphosphatase (pH 9.4 and 4.3), and phosphofructokinase. The tissue prepared for electron microscopy was fixed in cacodylate-buffered 2% glutaraldehyde for 3 days and processed according to the standard protocol.

H&E stains revealed small angulated fibers and groups of fibers containing multiple large vacuoles (Fig. 1A). The vacuoles contained PAS-positive material (Fig. 1B) that remained positive with the diastase–PAS stain, suggesting that the substance in the vacuoles was not normal glycogen. Phosphofructokinase stain was negative. There were no mitochondria in the vacuoles as evidenced by the succinic acid dehydrogenase (SDH) stain, nor were there any myofilaments as indicated by the ATPase stains. Acid phosphatase activity was not increased in the vacuoles. Oil-red O, cytochrome c oxidase, and SDH stains were normal. There was no muscle fiber type grouping or inflammation.

On electron microscopy, polyglucosan bodies (Fig. 2A) were present in scattered fibers, predominantly subsarcolemmal in location but also distributed between myofibrils. The bodies consisted of randomly arrayed short filaments and paired membranes with pointed ends, admixed with finely granular material (Fig. 2B). They were not membrane bound and were frequently rimmed by normal glycogen particles (Fig 2A). Other changes included varying sized, frequently juxtanuclear, “empty” vacuoles, an occasional fiber with myofibrillar disarray, and a few subsarcolemmal tubular aggregates (Fig. 2C). Mitochondria showed no abnormality and there was no increase in lipid or glycogen. No poly-
glucosan bodies were identified in the rare interstitial cells that were present and no nerve fibers were seen in the biopsy.

**Imaging.** T1- and T2-weighted, FLAIR, diffusion-weighted, and post-gadolinium magnetic resonance images of the brain and cervical spinal cord were normal. Specifically there were no white matter abnormalities in the brain or spinal cord. Computerized tomographic scanning of the abdomen was normal. An echocardiogram was normal.

**DISCUSSION**

We report an unusual case of an asymmetric and progressive PBD myopathy with hyperreflexia and absence of other clinical findings typical of PBD, including urinary incontinence, cognitive disturbance, or sensory abnormalities. The diagnosis of myopathy was suggested on needle electromyography (EMG), and confirmed on muscle biopsy, including the demonstration of polyglucosan bodies by electron microscopy.

Polyglucosan body disease, considered by some to be an adult variant of glycogen storage disorder type IV (GSD-IV), has a broad clinical spectrum. Myopathy has been reported infrequently and classically presents as a slowly progressive, symmetrical limb-girdle or predominantly distal myopathy. Further, an ALS-like syndrome with dementia and urinary incontinence has been reported with PBD. Although the present case exhibited clinical features that overlapped with ALS, notably an asymmetric motor presentation and brisk reflexes, the neurophysiological and neuropathological findings confirmed a myopathic process.

In addition to the findings of myopathic motor unit potentials, the presence of abnormal discharges on needle EMG testing, as reported in glycogen storage disorders due to acid maltase and debrancher enzyme deficiency, were also evident in the present case. We found complex repetitive discharges in this case of adult-onset PBD myopathy. The mechanisms underlying the generation of these discharges are unclear, although muscle membrane damage secondary to the accumulated polyglucosan bodies may be an explanation.

![Figure 2](image_url)

**FIGURE 2.** (A) Electron micrograph showing subsarcolemmal polyglucosan bodies (p). The involved muscle fiber contains two vacuoles (v) and its nucleus is seen at n. A capillary (c) is present in the adjacent connective tissue. Bar, 2 μm. (B) At higher magnification, the filamentous (f) and granular (g) structure of a polyglucosan body is evident. Bar, 1 μm. (C) Tubular aggregate composed of parallel bundles of tubules that have been sectioned transversely (t) and obliquely (o) is shown. Bar, 1 μm.
The pathological hallmark of PBD is deposition of small ellipsoid or thread-like structures throughout the nervous system, largely made up of glucose polymers, as evident in this case. Abnormal muscle mitochondria containing crystalline inclusions, although not evident in this case, have been reported in PBD. These mitochondrial abnormalities may be age-related changes and of no clinical significance. Tubular aggregates commonly occur in the periodic paralyses, exertional muscle cramp syndrome, and other disorders but do not seem to have been reported previously in polyglucosan body myopathy.

Given that polyglucosan body inclusions have been reported in GSD-IV, some have suggested that PBD is an adult variant of GSD-IV. Although the clinical phenotype of GSD-IV and PBD are different, the finding of mutations in the glycogen branching enzyme (GBE) gene with accompanying reduction of GBE enzyme activity in some PBD cases, suggests a similar etiology between PBD and GSD-IV. Further, normal GBE activity has been reported in a number of PBD cases. The reasons underlying this phenotypic variability may relate to the presence of genetic heterogeneity with multiple different biochemical abnormalities underlying PBD or to the existence of multiple GBE isoforms that are variably expressed in different tissues. Although the likelihood that the present case resulted from GBE enzyme deficiency is low, given the patient’s ethnic background, the unavailability of GBE enzyme assays precluded definite exclusion of GBE enzyme deficiency in the present case.

In addition to being evident in GSD-IV, polyglucosan body deposits may also occur with phosphofructokinase deficiency, Lafora body disease, double athetosis, and with normal aging. All these conditions were excluded on clinical, neurophysiological, and pathological testing in the present case.

In conclusion, the present study reports an unusual case of PBD myopathy presenting with asymmetric motor weakness and hyperreflexia, with a paucity of other clinical features such as cognitive impairment or urinary incontinence. The finding of complex repetitive discharges in conjunction with “myopathic” motor unit potentials on needle EMG should raise suspicion of polyglucosan body myopathy, with the diagnosis established by demonstrating polyglucosan body inclusions in muscle by electron microscopy.

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Neuromuscular Disease: Evidence and Analysis in Clinical Neurology
by M. Benatar, 483 pp., ill., Totowa, NJ, Humana Press, 2006, $145

A lack of high-quality evidence often forces us to rely on other sources of information, such as clinical experience, expert opinion, and extrapolation from pathophysiology, to make decisions about patient management. After years of practice, we began to accept these “truths” from non-empirical evidence, as is obvious from reliance on textbooks with statements, such as “We have found that...” and “In our hands...” This complacency makes our jobs as clinicians easier, because it obviates self-questioning and doubt, but it comes at a great cost: it impedes progress, the acceptance of new evidence, and the drive to improve the quality of evidence, and our patients ultimately suffer.

Michael Benatar sets out to set the record straight. In his excellent new book, he strips clinical management bare of all non-evidence, revealing the naked uncertainty that remains. The book religiously adheres to empirical evidence from studies of clinical evaluation and treatment of neuromuscular disease. There is nothing here about history, anatomy, or pathology, and no description of how he practices or what he recommends in the absence of empirical data. The book uses many tables and pithy text to review the evidence bared naked. Its writing is crystal clear, with nice summaries in each section and at the end of each chapter. It is very current, as well, with references to findings published even in 2006. Short sections address questions that we might hear asked by residents, fellows, or ourselves. The answers often are not the ones we might like, however, because the data frequently leave the questions unanswered.

As practitioners, we cannot limit our actions to those supported by strong evidence; if we did, our work days would be short. Rather, we must trudge ahead and make the best decisions possible, and this is where the tension of the book rises and its distinct and important function is clarified. It cannot serve as a text for the uninitiated but it is a superb tool in conjunction with traditional teaching or when used by practitioners or teachers. It clarifies, sometimes frighteningly, how little we know and how much work we as researchers need to do. But, it is an outstanding source of the actual evidence, carefully reviewed and discussed, free of flashy distraction. As such, I will turn to it frequently as a trusted reference.

S. Claiborne Johnston, MD, PhD

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LETTER TO THE EDITOR

Δ32 POLYMORPHISM OF THE CHEMOKINE RECEPTOR CCR5 IN A PATIENT WITH MULTIFOCAL MOTOR NEUROPATHY

In the pathogenesis of immune-mediated neuropathies, chemokines and their receptors play a crucial role in recruitment and activation of leukocytes into the peripheral nervous system (PNS). Here we describe a patient with a multifocal motor neuropathy (MMN) and a homozygous mutation for the Δ32 polymorphism of the CCR5 chemokine receptor.

A 51-year-old man presented with progressive difficulties in carrying heavy bags, in fine motor skills with his right hand, and in walking, with a limited walking range of 50 meters. Neurological examination showed marked muscle atrophy of the right shoulder and upper-arm muscles and of the left leg. There was weakness of right arm elevation and extension and of left hip flexion and foot extension. Deep tendon reflexes were generally diminished, with flexor plantar responses. There was no sensory impairment.

Nerve conduction studies showed conduction blocks in both median nerves and in the right ulnar nerve. Serum creatine kinase activity was slightly increased (277 U/L, normal <171 U/L) and anti-GM1 ganglioside antibodies (IgM subtype) tested positive with a titer of over 10,000 units (reference <800 units). Further diagnostic workup excluded other causes for peripheral neuropathy and MMN was diagnosed. Treatment with 0.4 g/kg of intravenous immunoglobulin (IVIg) daily for 5 consecutive days led to a marked improvement of muscle strength and unrestricted ambulation. The good clinical response was maintained by IVIg treatment with the same dose for 3 consecutive days after 8 weeks and by a third IVIg treatment after another 8 weeks with a reduced dose of 0.25 g/kg daily on 3 consecutive days.

Before and after IVIg treatment we investigated the chemokine receptor expression profile on lymphocytes and monocytes by flow cytometry. As CCR5 expression was not detected on any mononuclear cell, we tested for the Δ32 polymorphism of the CCR5 receptor and found a homozygous Δ32 polymorphism mutation (Fig. 1). Expression of the chemokine receptors CCR1, CCR2, CCR4, CCR6, CCR7, and CXCR3 on CD4 and CD8 lymphocytes were not significantly changed by IVIg treatment or different from seven other patients with MMN without a homozygous mutation of the Δ32 polymorphism (Table 1). Expression of CCR5 on CD4 and CD8 lymphocytes in seven other MMN cases without a homozygous mutation of the Δ32 polymorphism did not significantly differ compared to healthy controls or other immune-mediated neuropathies (Table 1).

The Δ32 polymorphism results in expression of a nonfunctional CCR5 protein and therefore individuals with the homozygous mutation are natural human knockouts for CCR5.

FIGURE 1. Polymerase chain reaction amplification of genomic DNA with subsequent restriction enzyme digestion resulted in a 403-basepair and a 332-basepair fragment in wildtype patients, whereas a 371-basepair and a 332-basepair fragment indicated a CCR5 Δ32 mutation.
MMN and a homozygous Δ32 mutation is of particular interest as chemokines and chemokine receptors may play a role in the recruitment and activation of mononuclear cells into the PNS.2,3,5 The finding of MMN in an individual with no functional CCR5 receptors can either be interpreted as indicating that CCR5 has no functional role in the development of MMN or that the redundant chemokine system has substituted the CCR5 function through other chemokine receptors. However, the Δ32 mutation had no influence on the expression profile of other chemokine receptors on circulating lymphocytes. Both the clinical presentation and response to IVIg treatment did not reveal any differences between this patient and cases without the Δ32 polymorphism. Our finding of a homozygous Δ32 mutation in a case with MMN could be a coincidental finding. A larger cohort of MMN patients would need to be tested for the Δ32 polymorphism and correlations made with clinical the course and response to IVIg treatment in order to disclose a possible role of CCR5 in the pathogenesis of MMN.

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Table 1. Chemokine receptor expression profile on peripheral lymphocytes before and after IVIg treatment.

<table>
<thead>
<tr>
<th>Case with Δ32 polymorphism</th>
<th>7 MMN patients without polymorphism</th>
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<tr>
<td></td>
<td>before</td>
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<tr>
<td>CCR1</td>
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<tr>
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</tr>
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<tr>
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<td>ND</td>
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</table>

Percentage of chemokine receptor-positive cells. ND, not detectable. For the 7 MMN cases without polymorphism, mean values ± standard deviation are shown.

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