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Nitric Oxide-Enhancing Therapy: An Evolving Approach in the Management of Heart Failure

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Introduction

Anne L. Taylor, MD,a,* and Clyde W. Yancy, MDb

Treatment with neurohormonal antagonists, including angiotensin-converting enzyme (ACE) inhibitors, angiotensin-receptor antagonists, β-adrenergic blockers, and aldosterone antagonists, has resulted in dramatic, incremental improvements in mortality and morbidity in patients with heart failure. Significant responses to these therapies also have provided insights into the neurohormonal mechanisms responsible for the progressive myocardial remodeling that characterizes heart failure. There is substantial evidence that, in addition to neurohormonal activation, endothelial dysfunction and decreased nitric oxide (NO) availability also occur in patients with heart failure and influence the course of vascular and myocardial remodeling. Thus, therapy that targets enhancement of NO may represent a novel and previously untested therapeutic approach to the treatment of heart failure.

Racial or ethnically-dependent differences in people with heart failure, including prevalence, etiology, morbidity, mortality, and response to therapy, suggest the possibility of multiple pathophysiologic pathways that lead to a common final pathway consisting of left ventricular dysfunction, dilatation, and remodeling. Patients who self-identify as African American are more likely to have hypertension than ischemic disease as a cause of heart failure, and higher rates of hospitalization and death occur in those aged 45 to 64 years. When African American patients are compared with European Americans in hypertension studies, low renin states are more prevalent and adrenergic activation is less common in the African Americans. In addition, some data suggest that NO-mediated responses are less robust in African Americans and may contribute to the higher prevalence and more severe nature of hypertension in this population. Correspondingly, lesser therapeutic responses to monotherapy with ACE inhibitors or β-blockers for hypertension have been observed in African Americans.

Consideration of these data in published studies on hypertension and on ethnic differences in heart failure prompted retrospective analyses of previous heart failure clinical trial databases. The results of these analyses demonstrate a lesser therapeutic response in African Americans with heart failure to ACE inhibitors and to some β-adrenergic blockers while revealing a greater response in this population to combined isosorbide dinitrate–hydralazine, which may enhance NO bioavailability.

This supplement to The American Journal of Cardiology is based on a symposium, held at the annual meeting of the Heart Failure Society of America, September 12–15 2004, convened a panel of experts to review and discuss the evidence supporting a trial of NO-enhancing therapy in heart failure and the benefits this therapy may bring to those self-identifying as African American.

In the first article, Dr. Clyde W. Yancy presents an overview of congestive heart failure in African Ameri-
cans and identifies population differences in the clinical features of heart failure, as well as differences in treatment response.

Dr. Tadeusz Malinski then presents an overview of NO physiology in the heart and describes the use of nanosensors to monitor NO concentrations in the beating heart in vivo. He provides data correlating NO levels with cardiovascular function and provocative data supporting ethnic differences in oxidative stress and NO levels.

In their article, Dr. Andreas Daiber and associates contribute a thorough review of the mechanisms of the actions of nitrates, mechanisms of nitrate tolerance, concepts of oxidative stress related to nitrates, and, finally, interaction of nitrates and hydralazine, which may result in enhanced NO bioavailability.

Next, Drs. Uri Elkayam and Fahed Bitar review clinical data on the use and efficacy of nitrates and hydralazine in heart failure before the African American Heart Failure Trial (A-HeFT).

The supplement concludes with an article by Dr. Anne L. Taylor, who provides an update on A-HeFT, the first heart failure trial to use therapy targeted at enhancing NO bioavailability.

We hope that, together, these articles contribute to an understanding of the potential role of NO in heart failure and clarify the logic supporting the trial of NO enhancement as a novel therapeutic agent.
The demographics of the United States are changing rapidly. Currently, white Americans constitute ≤70% of the population, and it is estimated that by 2050 there will no longer be a racial/ethnic majority population in this country. The burgeoning growth of ethnic populations in the United States necessitates heightened awareness of the influence of cardiovascular disease (CVD) and its responsiveness to medical therapy in these emerging groups. The exploration of CVD in populations defined by racial stratification must be done with deliberate thought and a broad perspective. Further polarization of already disenfranchised groups should not be the end result of increased awareness of ethnic group differences. Rather, the result should be a reduction in the disproportionate burden of heart disease in these groups. To achieve this goal, there must be a full explanation of the peculiarities of CVD that incorporates physiologic, genetic, environmental, and social factors in special populations. These issues are especially pertinent and are clinically relevant in any discourse regarding CVD, especially heart failure, in African Americans.

Despite very real concerns that African Americans experience a disproportionate risk of death from human immunodeficiency virus disease, trauma, cancer, and accidents, the available evidence from the American Heart Association (AHA) 2004 Update on Cardiovascular Disease and Stroke suggests that, similar to most of the population, CVD is the leading cause of death in African Americans (Figure 1).
Figure 1. Leading causes of death for African American men and women. AIDS = acquired immunodeficiency syndrome; CVD = cardiovascular disease; HIV = human immunodeficiency virus. (Adapted from American Heart Association.2)

Table 1
Representation of African Americans in clinical trials

<table>
<thead>
<tr>
<th>Trial</th>
<th>Year</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALLHAT</td>
<td>2002</td>
<td>15,133 (35.6)</td>
</tr>
<tr>
<td>HDFP</td>
<td>1979</td>
<td>4,846 (44.3)</td>
</tr>
<tr>
<td>CONVINCE</td>
<td>2003</td>
<td>1,212 (7.3)</td>
</tr>
<tr>
<td>AASK</td>
<td>2002</td>
<td>1,094 (100.0)</td>
</tr>
<tr>
<td>MRFIT</td>
<td>1985</td>
<td>926 (7.2)</td>
</tr>
<tr>
<td>SHEP</td>
<td>1991</td>
<td>657 (13.9)</td>
</tr>
<tr>
<td>VA (Mono)</td>
<td>1993</td>
<td>620 (48.0)</td>
</tr>
<tr>
<td>HOT</td>
<td>1998</td>
<td>582 (3.1)</td>
</tr>
<tr>
<td>LIFE</td>
<td>2002</td>
<td>533 (5.8)</td>
</tr>
<tr>
<td>EXCEL</td>
<td>1991</td>
<td>462 (8.0)</td>
</tr>
<tr>
<td>SOLVD/Rx</td>
<td>1991</td>
<td>394 (15.3)</td>
</tr>
<tr>
<td>CARE</td>
<td>1996</td>
<td>312 (7.5)</td>
</tr>
<tr>
<td>VALUE</td>
<td>2004</td>
<td>490 (2.7)</td>
</tr>
<tr>
<td>TONE</td>
<td>1998</td>
<td>234 (24.0)</td>
</tr>
<tr>
<td>RENAAAL</td>
<td>2001</td>
<td>227 (15.0)</td>
</tr>
<tr>
<td>TOMHS</td>
<td>1993</td>
<td>177 (19.6)</td>
</tr>
<tr>
<td>UKPDS</td>
<td>1998</td>
<td>87 (7.6)</td>
</tr>
<tr>
<td>MDRD</td>
<td>1995</td>
<td>66 (7.9)</td>
</tr>
<tr>
<td>ABCD/HBP</td>
<td>1998</td>
<td>65 (13.8)</td>
</tr>
<tr>
<td>DCCT</td>
<td>1993</td>
<td>51 (3.5)</td>
</tr>
<tr>
<td>ELITE</td>
<td>1997</td>
<td>34 (4.7)</td>
</tr>
<tr>
<td>CAPTOPRIL-DM</td>
<td>1993</td>
<td>30 (7.3)</td>
</tr>
</tbody>
</table>

AASK = African American Study of Kidney Disease and Hypertension; ABCD/HBP = Appropriate Blood Pressure Control in Diabetes; ALLHAT = Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial; CAPTOPRIL-DM = Captopril in Diabetes Mellitus; CARE = Cholesterol and Recurrent Events; CONVINCE = Controlled Onset Verapamil Investigation of Cardiovascular End Points; DCCT = Diabetes Control and Complications Trial; ELITE = Evaluation of Losartan in the Elderly; EXCEL = Expanded Clinical Evaluation of Losartan; HDFP = Hypertension Detection and Follow-up Program; HOT = Hypertension Optimal Treatment; LIFE = Losartan Intervention for Endpoint Reduction in Hypertension; MDRD = Modified Diet in Renal Disease; MRFIT = Multiple Risk Factor Intervention Trial; RENAAAL = Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan; SHEP = Systolic Hypertension in the Elderly Program; SOLVD = Studies of Left Ventricular Dysfunction; TOMHS = Treatment of Mild Hypertension Study; TONE = Trial of Nonpharmacologic Interventions in the Elderly; UKPDS = United Kingdom Prospective Diabetes Study; VA = Veterans’ Affairs; VALUE = Valsartan Antihypertensive Long-Term Use Evaluation.

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lessons have been learned from the clinical trial experiences that define the state of the art as it pertains to African Americans with heart failure.

**An Overview of Heart Failure in African Americans**

Several consistent observations have been reported with respect to the burden of CVD in African Americans. The African American population has a higher prevalence of heart failure compared with white Americans (3% vs 2%, respectively). When heart failure does occur in African Americans, it has a peculiar natural history. The onset of the disease occurs at an earlier age, and both the degree of left ventricular dysfunction and apparent disease severity are worse at the time of diagnosis. Hospitalization rates are higher in African Americans, and worrisome trends toward decreased survival rates also have been noted. Perhaps the most important observation lies in the epidemiology of the disease. The imputed etiology of left ventricular dysfunction is different in African Americans than in white Americans. There is a smaller likelihood of documented ischemic heart disease as the putative cause of left ventricular dysfunction and a greater likelihood of nonischemic, principally hypertensive disease as the sole potential explanation for left ventricular dysfunction (Figure 2). Importantly, mechanisms that are responsible for the conversion of hypertensive heart disease with intact systolic function to hypertensive heart disease with impaired systolic performance have not been fully elucidated, and thus it is quite difficult to firmly embrace a true causative role for hypertension in the genesis of systolic dysfunction.

**Hypertension as a confounding factor:** Hypertension, as it affects African Americans, is a malignant disease process. The prevalence of hypertension in African Americans is at least 3 to 7 times higher than in white Americans. The rate of end-stage renal disease resulting from hypertension is 2,000% higher, and the rate of stroke and associated fatality is considerably higher. The incidence of left ventricular hypertrophy (LVH) is 3-fold higher, and the pattern of LVH (ie, concentric hypertrophy) is known to be more malignant. Overall, these data suggest that hypertension is a more malignant disease process in African Americans, implying that the vascular response to hypertension is especially injurious.

**Hypotheses for disproportionate disease burden:** A number of plausible explanations for this apparent disease excess have been proposed with no single proven causative theory. The psychosocial burdens of the African American culture are easily recognizable and are undoubtedly important, and no discussion about cardiovascular health and outcomes in African Americans can be complete without acknowledging that healthcare disparities do exist and go far beyond simple issues of patient preference or indication/lack of indication for a given therapy. Ultimately, there are access and system impediments and issues of bias that exert a negative influence on CVD management in African Americans. To have truly effective treatment strategies in place for all patients, these issues of healthcare disparities must be addressed and overcome. Even so, data that control for objective measures of socioeconomic status still reveal CVD excess in African Americans.
of obesity in African Americans does appear to be linked to the presence of hypertension and likely contributes to the complex milieu responsible for excessive CVD in African Americans. As populations of African origin have emigrated from West Africa, through the Caribbean, and into North America, there is a near-linear relation to the increase in mean body mass index and blood pressure.26

Despite these reasonable explanations for excessive CVD in African Americans, the potential presence of important physiologic differences must be considered. An emerging but still incipient database of single nucleotide polymorphisms (SNPs) raises the possibility that within persons self-described as African American, a clustering of unfavorable gene expressions may contribute to the increased incidence of CVD. Candidate genes for which unfavorable SNPs have been described in African Americans include transforming growth factor (TGF)-β1, endothelin, β1-adrenergic receptors, aldosterone synthase, nitric oxide synthase, and the 825T allele of the GNB-3 G protein subunit (Table 2).27–31 There is considerable interest in TGF-β1. This primitive cytokine has been associated with mesangial hypertrophy and LVH. Polymorphism of the gene encoding for TGF-β1 has been described, and the highest levels of TGF-β1 have been seen in African Americans with hypertension.28 TGF-β1 has been noted to stimulate the production of endothelin, which is perhaps the most potent vasoconstrictor. Clearly, this may be associated with more malignant patterns of hypertension. Several SNPs of the adrenergic system also have been described. Substitution of glycine for arginine at position 389 on the β1-adrenergic receptor creates a loss-of-gain function that is consistent with a downregulated sympathetic nervous system and perhaps a decreased response to β-blockers.29,30 This SNP of the β1-adrenergic receptor has been described in African Americans. However, an even more worrisome pattern has been described in the setting of the wild-type β1-receptor in concert with a deletion polymorphism of the α1-receptor. This combination, present almost exclusively in African Americans, leads to an especially malignant course of heart failure.31 These SNPs and the several others listed may be operative in the progression of heart failure, but the distribution of these genetic variations is not homogeneous in the African American population. Nevertheless, genetic signals, which appear to serve as a plausible construct for the excess representation of heart failure in African Americans, do exist.

Although this is an exciting area of investigation, it is evident that any genetic influence is likely to be contextual and quite complex; it will reflect gene–gene interactions, gene–environment interactions, and gene–drug interactions. Thus, race should not be considered a proxy for any genetic substrate because race is too heterogeneous—including persons of various ethnicities and origins—and its designation is completely arbitrary.

**What Have We Learned from Clinical Trials in Heart Failure that Have Included African Americans?**

A number of published trials have reported data as a function of race/ethnicity. The Studies of Left Ventricular Dysfunction (SOLVD) were among the first to suggest differential outcomes as a function of race/ethnicity.12,32,33 Reports from the SOLVD Registry were among the first to indicate that hypertension as a sole associated disease in the setting of heart failure was more likely to occur in African Americans than in white Americans (32% vs 4%, respectively).34 A post hoc analysis of the primary trial results demonstrated that mortality from heart failure was higher in African Americans, with a 1.8-fold increase for African American men and a striking 2.4-fold increase for African American women.12 These data persisted even after adjust-

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**Table 2**

Candidate genes for polymorphism and clinical implications in African Americans

<table>
<thead>
<tr>
<th>Genetic Polymorphism</th>
<th>Clinical Implications</th>
</tr>
</thead>
<tbody>
<tr>
<td>β1-adrenergic receptor; Gly-38929</td>
<td>Subsensitive β1-receptor; decreased affinity for agonist and less cAMP generation</td>
</tr>
<tr>
<td>β1-adrenergic receptor; ARG-389 with α-receptor30</td>
<td>Presence of both polymorphisms is associated with increased risk for heart failure; dual polymorphisms</td>
</tr>
<tr>
<td>eNOS28</td>
<td>Subsensitive NO system</td>
</tr>
<tr>
<td>Aldosterone synthase28</td>
<td>? Excessive fibrosis</td>
</tr>
<tr>
<td>TGF-β127</td>
<td>40% higher TGF-β1 levels; ? higher endothelin levels; ? more fibrosis</td>
</tr>
<tr>
<td>G protein 825-T allele26</td>
<td>Marker of low-renin HTN, LVH, and stroke</td>
</tr>
</tbody>
</table>

ARG = arginine; cAMP = cyclic adenosine monophosphate; eNOS = endothelial nitric oxide synthase; Gly = glycine; HTN = hypertension; LVH = left ventricular hypertrophy; NO = nitric oxide; TGF = transforming growth factor.

ing for educational level and measures of financial stress, which are both crude but quantifiable measures of socioeconomic status. A subsequent reanalysis that adjusted for the degree of left ventricular dysfunction and for trial participation (ie, SOLVD Prevention trial or SOLVD Treatment trial) yielded no differences in mortality, but showed a significantly higher risk (44%) for hospitalization in African American patients than in white patients (p = 0.005) (Table 3). A suggested explanation for this apparent lower responsiveness to the angiotensin-converting enzyme (ACE) inhibitor enalapril was the lack of a blood pressure–lowering response at doses used in the trial in African Americans compared with white Americans. These observations would be consistent with a broad statement that ACE inhibitors are less effective in African Americans. However, these data do not resolve the question of an excess mortality risk, because the matched patient population in this reanalysis was overrepresented by lower-risk patients. Thus, important concerns remain regarding a diminished response to ACE inhibitors in African Americans with heart failure and varied mortality risks.

Conflicting data points have emerged from the clinical trial experience with β-blockers. A recent RAND Corporation (Santa Monica, CA) meta-analysis incorporated data reported by race/ethnicity from the major published β-blocker trials in heart failure. Whereas the aggregate benefit of β-blockers for white Americans was a 31% reduction in mortality, the apparent benefit of β-blockers in African Americans was only 3% (Figures 3 and 4).13,16,17,36 These unfavorable data are heavily influenced by the negative outcomes from the Beta-Blocker Evaluation of Survival Trial. Within this trial that evaluated the effect of bucindolol on survival in patients with advanced heart failure, no apparent benefit was realized in the African American cohort, although a benefit was seen in the white American cohort. However, the magnitude of that benefit was approximately 50% less than that typically seen in previous trials with β-blockers. Subsequent data have since emerged to confirm that bucindolol has partial intrinsic sympathomimetic activity and as such represents an unfavorable β-blocking agent for heart failure. Nevertheless, there was an apparent difference in the benefit of β-blocker therapy in African Americans versus white Americans.

The experience with carvedilol has been quite different and varies substantially from the observations seen with bucindolol. In both the US Carvedilol Heart Failure Trials program and the Carvedilol Prospective Randomized Cumulative Survival Trial, retrospective analyses by ethnicity showed statistically significant benefits with carvedilol.3,38 The US Carvedilol Heart Failure Trials program demonstrated that the combination of an evidence-based β-blocker and an ACE inhibitor yielded similar outcomes in both African Americans and non–African Americans.39 For both groups, the reduction in the progression of heart failure, defined as death due to heart failure, hospitalization for heart failure, or worsening symptoms requiring augmented medical therapy was >50%. These benefits were supported by observations of similar improvements in measures of left ventricular function and similar hemodynamic effects on heart rate and blood pressure. Thus, therapy with the combination of ACE inhibitors and evidence-based β-blockers was demonstrated to be effective in African American patients. It remains unclear whether these benefits are limited to carvedilol. Data from the Metoprolol CR/XL Randomised Intervention Trial in Heart Failure trial are inconclusive because too few African Americans were included in this trial of nearly 4,000 people.16 A summary of major clinical trials in heart failure that reported data as a function of race/ethnicity can be found in Table 4.13,16,32,33,39–41

Neither of the signature trials using aldosterone antagonists for left ventricular dysfunction, either chronic class III–IV heart failure or post–myocardial infarction left ventricular dysfunction, had a sufficient number of African Americans to make any statement regarding subgroup responsiveness.6,7 The Losartan Intervention for Endpoint Reduction in Hypertension (LIFE) trial, a trial performed in hypertensive patients with LVH, suggested that African Americans responded better to an antihypertensive regimen that was based on the β-blocker atenolol than on the angiotensin II receptor antagonist losartan.42 This unexpected observation has been a point of controversy, because once again, data were extracted retrospectively from an under-represented subgroup. The issue regarding responsiveness to angiotensin-receptor antagonists was further complicated by the recent Valsartan Antihypertensive Long-Term Use Evaluation trial (VALUE), which is yet another trial of high-risk hypertensive patients. Only 4.2% of the recruited subjects were African American; thus, a perusal of this

<table>
<thead>
<tr>
<th>Risk Ratio (CI)</th>
<th>p Value</th>
<th>Risk Ratio (CI)</th>
<th>p Value</th>
<th>p Value (p Value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-cause mortality</td>
<td>0.89 (0.69–1.13)</td>
<td>NS</td>
<td>0.95 (0.76–1.18)</td>
<td>NS</td>
</tr>
<tr>
<td>Cardiovascular death</td>
<td>0.92 (0.71–1.20)</td>
<td>NS</td>
<td>0.96 (0.76–1.22)</td>
<td>NS</td>
</tr>
<tr>
<td>Hospitalization for CHF</td>
<td>0.96 (0.74–1.24)</td>
<td>NS</td>
<td>0.56 (0.43–0.73)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Death or hospitalization for CHF</td>
<td>0.91 (0.75–1.12)</td>
<td>NS</td>
<td>0.75 (0.62–0.91)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

CHF = congestive heart failure; CI = confidence interval; NS = not significant.

Adapted from N Engl J Med.32
subgroup would be completely unproductive. There was
even less representation of African Americans in the Heart
Outcomes Prevention Evaluation (HOPE) study; thus, there
are no data from which a statement can be made about the
value of ACE inhibitors in African American patients at
high risk for vascular events.

The clinical trial experience that has prompted the most
intrigue in this arena is the original Vasodilator Heart Fail-
ure Trials I and II (V-HeFT I and II). V-HeFT I is con-
sidered a landmark trial because it established for the first
time that outcomes of heart failure could be slightly im-
proved with medical therapy. Of the 480 patients in V-HeFT
I, 180 were African American. A post hoc retrospective
analysis of this subgroup yielded the striking finding that the
entirety of the survival benefit of combination isosorbide
dinitrate–hydralazine (ISDN-HYD) was seen in the African
American group. The benefit of ISDN-HYD when added
to diuretics and digoxin in African American patients re-
sulted in ≥40% survival benefit. When V-HeFT II was
requiered to determine outcomes as a function of race/ 
nethnicity for therapy with an ACE inhibitor versus the
vasodilating regimen of ISDN-HYD, it was discovered that
white patients responded better to an ACE inhibitor than to ISDN-HYD. African American patients fared equally well receiving ACE inhibitor or vasodilator therapy (Figure 5). Thus, the correct interpretation of this revisit of V-HeFT I and II is not that African Americans failed to respond to ACE inhibitors, but that they had a more robust response to ISDN-HYD.

It is now evident that the combination of ISDN-HYD represents more than a balanced vasodilating regimen. Isosorbide dinitrate is an NO donor, and hydralazine has important antioxidant properties. NO plays an important role in stabilizing vascular endothelium. In an environment where NO is replete, there is less platelet aggregation, reduced sheer forces, less leukocyte accumulation, and a decreased stimulus for thrombosis. In an NO-depleted environment, endothelium is at risk for platelet aggregation, leukocyte adhesion and thrombosis, and presumably vascular events (Figure 6). Given that the production of NO depends on adequate stores of nicotinamide adenine dinucleotide phosphate, a plausible pathologic consideration may be diminished NO along with increased oxidative stress. There are intriguing data to suggest that African Americans have diminished NO bioavailability and increased oxidative stress. There are intriguing data to suggest that African Americans may be diminished NO along with increased oxidative stress. Therefore, this is the basis of the African American Heart Failure Trial. The positive results of this trial confirm the earlier findings of the V-HeFT database and suggest that a novel mecha-

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Table 4
Summary of major clinical trials in heart failure reporting data by race/ethnicity

<table>
<thead>
<tr>
<th>Study</th>
<th>African Americans (%)</th>
<th>Design</th>
<th>Intervention</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>V-HeFT I</td>
<td>29</td>
<td>Double-blind RCT; primary end point: mortality</td>
<td>Placebo vs ISDN-HYD; background: diuretics and digoxin</td>
<td>Annual mortality rate decreased from 17.9% to 9.7%; p = 0.04</td>
</tr>
<tr>
<td>V-HeFT II</td>
<td>27</td>
<td>Double-blind RCT; primary end point: mortality</td>
<td>ISDN-HYD vs enalapril; background: diuretics and digoxin</td>
<td>Annual mortality rate: 12.9% to 12.8%; p = NS</td>
</tr>
<tr>
<td>SOLVD-Treatment</td>
<td>12</td>
<td>Double-blind RCT; primary end point: mortality</td>
<td>Placebo vs enalapril in NYHA class II–III HF</td>
<td>No mortality difference blacks vs nonblacks in a matched population regarding LVEF and clinical trial participation; RR, 0.92 vs 0.95; higher hospitalization rate for blacks; RR, 0.95 vs 0.54; p = 0.005</td>
</tr>
<tr>
<td>SOLVD-Prevention</td>
<td>10</td>
<td>Double-blind RCT; primary end point: mortality</td>
<td>Placebo vs enalapril in NYHA class I–II HF</td>
<td>No difference in the prevention of heart failure using enalapril; statistically significant difference in the incidence of heart failure; RR, 1.81; p &lt;0.001</td>
</tr>
<tr>
<td>BEST</td>
<td>23</td>
<td>Double-blind RCT; primary end point: all-cause mortality</td>
<td>Placebo vs bucindolol in NYHA class III–IV; randomization stratified for women and blacks; background: diuretics, ACE inhibitors; digoxin at investigators’ discretion</td>
<td>Nonsignificant 17% increase in risk of death on bucindolol; p = 0.27</td>
</tr>
<tr>
<td>MERIT-HF</td>
<td>&lt;5</td>
<td>Double-blind RCT; primary end point: mortality</td>
<td>Placebo vs metoprolol succinate in NYHA class II–IV; mostly II–III</td>
<td>Insufficient numbers to ascertain efficacy</td>
</tr>
<tr>
<td>US Carvedilol Trials Program</td>
<td>20</td>
<td>4 concurrent trials; double-blind RCT design; mortality was not a predetermined end point</td>
<td>Placebo vs carvedilol in NYHA class II–IV; mostly II–III with protocol participation determined by 6-min walk time; background: diuretics, ACE inhibitors, and digoxin at investigators’ discretion</td>
<td>Similar efficacy between black and nonblack groups; reduction in death for any cause or hospitalization for any cause: 48%; reduction in worsening of heart failure: 54%</td>
</tr>
<tr>
<td>COPERNICUS</td>
<td>5</td>
<td>Double-blind RCT design; primary end point: all-cause mortality</td>
<td>Placebo vs carvedilol in NYHA class III–IV; LVEF &lt;0.25 (mean 0.19)</td>
<td>Similar efficacy between black and nonblack groups despite small number of blacks</td>
</tr>
</tbody>
</table>

ACE = angiotensin-converting enzyme; BEST = Beta-Blocker Evaluation of Survival Trial; COPERNICUS = Carvedilol Prospective Randomized Cumulative Survival Study; HF = heart failure; ISDN-HYD = isosorbide dinitrate-hydralazine; LVEF = left ventricular ejection fraction; MERIT-HF = Metoprolol CR/XL Randomized Intervention Trial in Congestive Heart Failure; NYHA = New York Heart Association; RCT = randomized controlled trial; RR = relative risk; SOLVD = Studies of Left Ventricular Dysfunction; V-HeFT = Vasodilator Heart Failure Trial.

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Yancy/Heart Failure in African Americans
nism of action may be operative in the setting of heart failure as it affects African Americans.

**Conclusion**

Heart failure in African Americans is likely to be a unique disease entity. Its natural history, epidemiology, morbidity, and perhaps even its mortality vary from heart failure in white patients. The influence of hypertension, and its more aggressive natural history in African Americans, is inescapable and carries with it important messages for disease prevention through effective treatment. Data regarding responsiveness to contemporary evidence-based medical therapy are inconsistent and reflect the inherent inaccuracy of post hoc retrospective analyses of underempowered subgroups. However, no data support avoidance of background therapy including evidence-based strategies, specifically ACE inhibitors and β-blockers. Ongoing efforts to explain the excess CVD burden in African Americans have targeted mechanisms that lead to more malignant varieties of hypertension and genetic profiles that may predispose patients to more advanced left ventricular dysfunction, lower responsiveness to medical therapy, or both. Any statements regarding the influence of genetic factors must be taken in the appropriate context because gene–gene interactions, gene–environment interactions, and gene–drug interactions are all likely to occur.

The newest theory regarding the progression of CVD in African Americans embraces previous observations of unique benefits from vasodilator therapy in this group. The vasodilating regimen has now been identified as one that

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Figure 5. Mortality from congestive heart failure in African American patients in the Vasodilator Heart Failure Trial (V-HeFT). ISDN-HYD = isosorbide dinitrate–hydralazine. (Adapted with permission from *N Engl J Med.*40)

Figure 6. Endothelial function and dysfunction. EDRF = endothelium-dependent relaxing factor; t-PA:PAI-1 = ratio of tissue plasminogen activator to plasminogen-activator inhibitor–1. (Reprinted with permission from *N Engl J Med.*45)
potentially increases NO bioavailability by donating NO. Specific testing of the benefit of this unique combination has been performed in the setting of African Americans with heart failure of moderately severe to severe functional capacity who are receiving standard evidence-based medical therapy. The favorable outcomes seen in the African American Heart Failure Trial are likely to redefine the state of the art for heart failure in African Americans.


Nitric oxide (NO) is well known as an important mediator of many physiologic functions, and its role in the pathogenesis of heart failure is gaining recognition. Data show that African Americans are at greater risk for developing cardiovascular diseases, such as hypertension and heart failure, and are more likely to have complications than their white counterparts.1–3 Furthermore, studies have shown that African Americans and European Americans respond differently to pharmacologic intervention.4,5 Recent data suggest that these disparities are the result of an imbalance of NO, O$_2^-$, and ONOO$^-$ production in the endothelium.6 Pharmacologic interventions aimed at correcting this imbalance in African Americans are being investigated and have shown promising results.7 The purpose of this article is to review how NO is synthesized, to describe a relatively new means of measuring NO in the endothelium using a nanotechnologic approach, to explain the physiologic role of NO in the cardiovascular system, and to illustrate racial/ethnic differences in the production of NO.

Synthesis of Nitric Oxide Oxidative Stress

Endothelial NO modulates vascular tone and blood pressure by cyclic guanosine monophosphate (cGMP)–stimulated smooth muscle relaxation, inhibition of platelet aggregation and adhesion to the endothelium, and prevention of smooth muscle proliferation (prevents vascular wall thickening) (Figure 1).8 In mammals, 3 isoforms of NO synthase (NOS) have been identified: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS).9 These isoforms are products of different genes and have different localization and regulation properties. The kinetics of NO production by these enzymes, and the inhibition of NO production by different inhibitors, is distinctively different. There is 50% to 57% homology among human isoforms of NOS, and all 3 NOS isoforms are found in the human heart. NOS enzymes catalyze 5 electron oxidation of L-arginine to L-citruline (Figure 2). Nicotinamide adenine dinucleotide phosphate (NADPH) is oxidized to NADP$^+$ in this process. Molecular oxygen acts as a cosubstrate for this reaction, and tetrahydrobiopterin (BH4), flavin adenine dinucleotide, flavin mononucleotide, and heme are cofactors involved in the catalytic process.10,11

NO production through nNOS occurs twice as fast as it does through eNOS; however, output by eNOS is significantly higher than output by nNOS.2 Both eNOS and nNOS are constitutive enzymes (cNOS). NO production by eNOS and nNOS is calcium dependent (ie, a calcium/calmodulin complex is needed for NOS activation). iNOS, which is calcium independent, is a very high-output but slow kinetic enzyme. NO is produced by cNOS in a pulsatile manner, whereas the production of NO by iNOS is continuous.12 Unlike nNOS and iNOS, eNOS is acclimated and targeted to the plasmalemma terminal caveole. The interaction of
eNOS with some domains of caveolin-I causes the eNOS to become inactive. However, interaction with the calcium/calmodulin complex with eNOS permits electron transfer through the enzyme and the oxidation of L-arginine.

**cNOS uncoupling leads to oxidative stress:** Dysfunctional endothelium generates a much greater amount of $O_2^-$ than normal endothelium (Figure 2).13–16 Subsaturating concentrations of BH$_4$ or L-arginine promote cNOS uncoupling and 1 electron reduction of (O$_2$) to O$_2^-$. The addition of L-arginine and BH$_4$ restores NO generation and abolishes O$_2^-$ generation. NO and O$_2^-$ react in a rapid, diffusion-controlled reaction to form ONOO$^-$, a highly cytotoxic compound. The probability of a reaction between O$_2^-$ and NO is very high because both molecules can be produced by the same enzyme, cNOS. ONOO$^-$ can be protonated to peroxynitrous acid (ONO0OH). At low concentration, ONOOH can undergo rapid isomerization to form nitrate (NO$_3^-$) and proton (H$^+$) (Figure 3). However, at high concentration, the gradient of ONOOH established between the membrane of the endothelial cell cytoplasm and the blood facilitates its diffusion. During the diffusion process, the ONOOH molecule undergoes homolytic or heterolytic cleavage. The homolytic cleavage leads to production of hydroxyl (OH$^-$) and nitrogen dioxide (NO$_2^-$) radicals. Both of these radicals are very strong oxidants. The heterolytic cleavage of ONOOH produces hydroxyl ion (OH$^-$) and another strong oxidant, nitronium ion (NO$_2^+$). O$_2^-$, ONOOH, NO$_2^-$, OH$^-$, and NO$_2^+$ are major components of oxidative stress generated by the dysfunctional endothelium.

Oxidative stress reduces the bioavailability of NO in the cardiovascular system and causes vasoconstriction of the vessels and increases in blood pressure. Oxidative stress is not only responsible for the decline of diffusible NO but also is responsible for the decrease in the concentration of some cNOS cofactors, such as BH$_4$. The reduced concen-
tration of BH₄ further increases eNOS uncoupling and increases the production of O₂⁻ and ONOO⁻. In addition to the oxidation of NO by O₂⁻, ONOOH can oxidize NO to form nitrite ion (NO₂⁻). The deficiency of NO and BH₄ and high ONOOH or its products of decomposition trigger a cascade of events leading to the oxidation of a wide variety of biologic molecules. Therefore, the formation of ONOOH has 2 important negative consequences in biologic systems: loss of bioactive NO and an increase in oxidative stress. ONOOH is quite indiscriminate; it can transfer oxygen atoms, oxidize protein tyrosine residue, inhibit the activity of enzymes, and initiate lipid peroxidation. Thus, ONOO⁻ formation represents an important means of generation of O₂⁻/NO-mediated oxidative stress (Figure 4). Deficiency of bioavailable NO, O₂⁻ overproduction, high oxidative stress, or a combination of these are common features of

![Diagram of ONOOH isomerization and cleavage](image_url)

**Figure 3.** Isomerization and cleavage of peroxynitrous acid (ONOOH) at low and high concentrations in biologic milieu. H⁺ = proton; NO₂⁻ = nitrogen dioxide radical; NO₂⁺ = nitronium ion; NO₃⁻ = nitrate; OH⁻ = hydroxyl radical; OH = hydroxyl ion. (Adapted with permission from J Physiol Pharmacol.)

![Diagram of oxidative stress induced changes](image_url)

**Figure 4.** Oxidative stress-induced changes in biologic milieu. DNA = deoxyribonucleic acid; eNOS = endothelial nitric oxide synthase; H⁺ = proton; NO = nitric oxide; O₂⁻ = superoxide; ONOOH = peroxynitrous acid.
several vascular and cardiac diseases. Reduced bioactive NO and increased oxidative stress usually precede the clinical manifestation of vascular disease.

### Direct Measurement of Nitric Oxide and Oxidative Species in the Endothelium: Nanosensors/Nanotechnology/Nanomedicine

NO is difficult to measure in vivo because it has a short half-life, is unstable, and its release is affected by changes in the concentration of calcium, chemical agonists (eg, bradykinin, adenosine triphosphate, acetylcholine), physical agonists (eg, shear stress flow, pressure), and other cofactors involved in its production. Therefore, nanotechnology has been used to develop a nanosensor capable of measuring NO in vivo.

**Factors affecting NO production and concentration:** Calcium flux turns on NO biosynthesis by eNOS for several seconds; subsequently, eNOS is turned off by phosphorylation of NOS and its serine residue. Compared with other messengers (neurotransmitters and hormones), NO is an inferior chemical messenger. NO is extremely nonspecific and cannot be stored and released on demand. Its high rate of isotopic diffusion (D = 2.6 × 10^7 cm^2 per sec) requires the generation of high amounts of NO to attain a given concentration at the target cell. The activity of NO at any target site depends on its local concentration, which is determined by a combination of its rate of production and nonenzymatic destruction. The rate of production of NO can be limited by the local concentration of eNOS, calcium, NADPH, BH4, oxygen, and L-arginine. Because the latter 3 substances are depleted by the production of NO, enhanced NO synthesis can lead to an oscillatory NO output, even when the intracellular level of calcium is steady. The level of eNOS can be affected by the level of ferrous ions (Fe^{2+}); eNOS synthesis requires Fe^{2+} but is inhibited by ferric ions (Fe^{3+}). The enzymatic production of NO is down-regulated by NO, which binds to eNOS.

**Table 1**

<table>
<thead>
<tr>
<th>Low</th>
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<tr>
<td>• Hypertension</td>
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<td>• Atherosclerosis</td>
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<td>• Diabetes mellitus</td>
<td>• Meningitis</td>
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<td>• Ischemia</td>
<td>• Rheumatoid arthritis</td>
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**Table 2**

<table>
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<tr>
<th>Biomedical nanosensors used in the diagnosis of endothelial function</th>
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<tr>
<td><strong>Nanosensors</strong></td>
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<tr>
<td>• Nitric oxide</td>
</tr>
<tr>
<td>• Superoxide</td>
</tr>
<tr>
<td>• Hydrogen peroxide</td>
</tr>
<tr>
<td>• Peroxynitrite</td>
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<tr>
<td>• L-arginine</td>
</tr>
<tr>
<td>• Tetrahydrobiopterin</td>
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<tr>
<td>• Carbon monoxide</td>
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<tr>
<td>• Oxygen</td>
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<td>• NADPH</td>
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NADPH = nicotinamide adenine dinucleotide phosphate.

NO concentration is not homogenous in the endothelial cell. Propagation of neutral NO in the hydrophobic cell membrane is much faster (permeability for NO is 26.9 cm⁻¹) than propagation in an equivalent layer of aqueous cytoplasm. The cell membrane does not present a significant barrier to the diffusion of NO and is not rate determining for NO propagation in the tissue. The membrane is a storage reservoir for NO, and the small membrane volume can develop a relatively high concentration within a short period when NO is released by eNOS that is associated with the membrane. The high NO concentration in the membrane creates a high concentration gradient between the membrane and the aqueous phases on both sides of the membrane. This gradient facilitates an efficient diffusion, a controlled supply of NO to the muscle cells and to the blood.

The deficiency of NO on a target may play a role in hypertension, hyperglycemia, atherosclerosis, stroke, myocardial infarction, heart failure, Parkinson disease, and Alzheimer disease, and conversely, excessive NO may play a role in rheumatoid arthritis, hypotension, and septic shock (Table 1). Thus, from a pharmacologic as well as a medical point of view, one should quantify the details of NO production and the production of oxidative species and their diffusion and propagation in abnormal and normal cells and tissues. NO reacts rapidly with cellular components in vitro or in vivo, producing protein nitrosylation and reacting with hemoglobin and oxygen. In addition, in the presence of O₂⁻, NO is rapidly converted to ONOO⁻ (k = 6 × 10^9 M⁻¹ per sec). Consequently, NO has a half-life of 2 to 5 seconds in vivo, and detection of NO in biologic systems has thus proven technically difficult. To study the dynamics of NO and its rate as a messenger, one cannot use the buildup of products (nitrate and nitrite), nor can one use NO swept out into the gas phase, to be determined by chemiluminescence. Also, electron paramagnetic resonance cannot be used because this technique does not detect unbound NO in the biologic milieu. The only technique currently available that can be used for the study of NO release and its propagation is an electrochemical assay of NO by an NO-selective nanosensor.

**Measuring NO concentration:** Nanosensors can have a diameter <200 nm and can be used for in vitro, ex vivo, and...
in vivo measurement of NO at the molecular level in a single endothelial cell. The NO nanosensors are based on specifically designed electrically conductive organic materials (porphyrinic molecular metals) that can oxidize NO selectively and generate a current proportional to its concentration. A response time of NO sensors is approximately 10 to 50 msec; therefore, real-time detection is possible at the level of a single biologic cell or neuron.

A number of nanosensors have been developed (Table 2). All of these nanosensors can be combined into 1 unit or into a sequence of 2 to 4 sensors in 1 unit and used for simultaneous in situ detection and determination of 2 to 4 different molecules. In addition to NO nanosensors, other sensors for detecting molecules, such as O₂⁻⁻, ONOO⁻⁻, hydrogen peroxide, and carbon monoxide, in a single endothelial cell or in tissue, have been developed. This nanotechnologic/nanomedical approach allows for monitoring the function of endothelium on a molecular level in near real-time. Each of the nanosensors can sample pico- to femtoliter and can detect an amount of analyte less than attomol (10⁻¹⁸ mol). At the detection limit of 10⁻⁹ nmol/L, most of the nanosensors require approximately 5,000 molecules to produce a detectable electrical current analytic signal. The sensors can be implanted in the cytosol, but for the diagnosis of endothelial function, the sensors can be positioned in close proximity to the cell surface where the highest NO concentration is expected. Computer-controlled micromanipulators can be used to position the nanosensors a well-defined distance (3 to 50 µm) from the cell membrane. The concentration of NO decreases exponentially with the distance from the membrane and cannot be detected at a distance >100 µm.

For in vivo measurements, the sensors can be implanted with the help of different types of catheters (eg, Swan-Ganz catheter, intravenous catheter). The design of nanosensors mounted on the truncated needle of an intravenous catheter is shown in Figure 5. Initially, the catheter is positioned in a tissue with a normal needle. When in place, the normal needle is replaced by a truncated needle with nanosensors placed on its tip. Microdiameter pores (5 to 10 mm) close to the tip of the catheter will allow free diffusion of the analyte(s) to the sensor. These intravenous protected nanosensors have been used for in vivo measurement of NO, O₂⁻⁻, and ONOO⁻⁻ in a wall of the beating heart or arterial wall with time resolution <100 µsec.

Physiology of Nitric Oxide Release from the Endothelium

The hemodynamic forces resulting from blood flow include 2 components: (1) shear stress, the tangential frictional force produced when blood flows over the endothelial surface; and (2) pressure force acting perpendicular to the vascular wall. The shear stress acting on the endothelium is responsible for flow-induced NO release. In larger arteries, the average wall shear stress is 1 to 20 dynes/cm². At endocardium and bifurcations, peeled wall shear stress may be as high as 100 dynes/cm². Immediate (millisecond to second) responses to shear stress include increases in ionic conductance and intracellular levels of calcium. Delayed (minute to hour) responses to chronic shear stress include altered gene expression, deoxyribonucleic and ribonucleic acid levels, and cell orientation. Shear stress causes transient NO release 3 to 5 seconds after initiation of flow and 1 to 3 seconds after the increase in intracellular calcium. Although the amount (peak rate) of NO release increases as a function of the shear stress (0.08 to 3.80 pmol/sec), because of the concomitant increase in the flow rate (increase in volume), peak NO concentration remains constant (Figure 6A). Maintenance of flow results in additional transient NO release with peak-to-peak intervals of 10 to 15 minutes. During this 10- to 15-minute period, when the cells are unresponsive to shear stress, exogenous adenosine triphosphate (ATP) or calcium ionophore evokes NO release (Figure 6B). The shear stress causes a calcium-mediated, ATP-
independent transient release, but the peak concentration depends on the level of shear stress.

The shear stress forms a thin layer of NO in the lumen next to the layer of the endothelial cell. NO concentration in this layer depends on the diameter of the vasculature and the type of blood flow: laminar versus turbulent. Therefore, the highest concentration of NO (micromolar level) is recorded in the endocardium of the heart, which is exposed to the highly turbulent flow. A deficiency of bioavailable NO in close proximity to endothelial cells increases platelet/leukocyte adhesion and clot formation in the vasculature.

Nitric Oxide in the Beating Heart

The beating heart responds rapidly to local changes in loading conditions, with increased cardiac output observed after simple volume infusion. When enervated after cardiac transplantation, the heart maintains its ability to autoregulate cardiac performance in response to increased venous return. Although several mechanisms underlying increased cardiac contractility in response to passive stretch have been identified, investigators have posited the existence of rapidly acting autoregulatory mechanisms to explain certain aspects of the beat-to-beat reg-

Figure 6. (A) Increased amounts of nitric oxide (NO) release by the endothelium with increased flow (shear stress) and constant NO concentration maintained near the endothelium during change of blood flow; (B) NO released by a single endothelial cell as a result of shear stress. ATP = adenosine triphosphate. (Adapted [A] and reproduced [B] with permission from Circ Res. 22)
ulation of cardiac performance. In particular, the mechanism(s) underlying cardiac memory for mechanical events of preceding beats and load-dependent relaxation is not completely understood.

Recent studies have shown that endogenous NO agonists, exogenous NO donors, or increased intracellular cGMP will hasten myocardial relaxation (positive lusitropy).\(^{26}\) In addition to their relaxation-hastening effects, NO and cGMP appear to depress ventricular contractility directly (negative inotropy). Conversely, the effects of changes in physiologic load on instantaneous cardiac NO synthesis remain unknown. To understand this potentially important cardiac autoregulatory mechanism, which may modulate myocardial lusitropy, inotropy, and flow on a beat-to-beat basis, the impact of altering loading conditions on modulation of endogenous cardiac NO synthesis was considered. With the help of nanomedical methods, it was demonstrated for the first time that altering ventricular filling in beating hearts in vivo or altering mechanical force on ex vivo hearts is followed by a parallel increase or decrease in cardiac NO synthesis.\(^{19}\) This appears to represent a novel autoregulatory mechanism that may be relevant to cardiac physiology as well as to clinical conditions associated with pathologic myocardial distension.

Nanosensors were placed (via apical puncture) in the left endocardium and myocardium. Rapid changes in cardiac NO concentrations were observed. In the heart of a rabbit, each cardiac cycle (period, 326 ± 16 msec) began and ended with an intercycle NO concentration of 0.67 ± 0.16 µmol/L near the endocardium.\(^{19}\) During early systole, NO concentration reached a basal level of 0.62 ± 0.05 µmol/L, followed by a slow increase to a semiplateau. Early diastolic filling was accompanied by a brisk increase of NO with a peak diastolic NO concentration of 2.7 ± 0.1 µmol/L that was attained at 239 ± 17 msec into the cardiac cycle. After this peak, there was a sharp decline to the intercycle NO concentration. A similar cyclic fluctuation of NO concentration was observed in the

Figure 7. The electrocardiogram signal (A), instantaneous nitric oxide (NO) concentration (B), and intracavitary left ventricular pressure (LVP) (C). (Reproduced with permission from Circ Res.\(^ {27}\))
myocardium. The relation among intracavitary left ventricular pressure, the electrocardiographic signal, and instantaneous NO concentration is depicted in Figure 7.

When preload was increased by a rapid intravenous infusion of physiologic saline solution, there was a gradual but significant increase in both peak and basal NO concentration in the beating heart (Figure 8).27 When ventricular filling was reduced by temporary legation of the vena cava, NO concentration decreased gradually over several beats. Total production of NO in the beating heart comprises NO produced by both mechanical and chemical stimulation of cNOS. To determine which cell type within the heart that expresses cNOS activity may be responsible for load-dependent cardiac NO, nanosensor (diameter 150 nm) studies were performed using single, isolated myocardial endothelial and endocardial cells.27 Isolated beating myocardial cells (neonatal rat) did not produce NO after mechanical stimuli. However, these cells produced NO after stimulation with receptor-dependent agonists, such as norepinephrine. Both cardiac endothelial cells and endocardial cells produced NO after both mechanical and chemical stimulation, and both the mechanical transduction of load-dependent cardiac NO and chemical stimulation involved calcium flux.

There have been a growing number of publications addressing the influence of NO on the contractile (isotropic) and relaxation (lusitropic) properties of cardiac myocytes and the heart.25–30 Endothelial shear stress has been shown to increase NO concentration by stimulating cNOS. However, until the use of nanomedical sensors, there was no evidence to indicate that altering cardiac loading conditions independent of coronary blood flow could affect NO synthesis in vivo.26 Measurements obtained with nanosensors implanted into the heart wall demonstrate for the first time that NO is released in a pulsatile fashion from the beating heart and that its synthesis is directly related to ventricular loading conditions in vivo. These results support the existence of a novel cardiac autoregulatory mechanism that may facilitate ventricular filling in times of increased demand. Furthermore, these data may help to explain certain aspects of the beat-to-beat regulation of cardiac performance and flow and provide insights into the pathophysiology of diseases associated with increased myocardial distension, such as valvular heart disease or heart failure.

If the heart were to function as a purely systolic (extrusion) pump, without the ability to transduce changes in preload into parallel changes in NO concentration, then within certain physiologic limits, abrupt changes in preload would produce abrupt changes in cardiac output. Several explanations exist for why this is not so. It has been demonstrated that abruptly increasing or decreasing ventricular preload in vivo is gradually (not abruptly) followed by parallel changes in NO concentration over several cardiac cycles.27 This model provides an explanation for several deviations observed from the Frank-Starling model (a relatively static systolic pump model, which does not reflect the dynamic interplay between systole and diastole).

There is also likely to be an additional beneficial reason for increased cardiac NO concentration under conditions of increased mechanical stimulation. The turbulent blood flow within the beating heart provides a potent stimulus for platelet adhesion and aggregation.31 Because both platelet adhesion to endocardial cells and platelet aggregation are inhibited by NO,32 the unusually high load-dependent local NO concentration observed near the endocardium is likely to be of considerable importance in inhibiting local platelet adhesion and aggregation in the highly turbulent blood flow.
in the heart. A lack of this effect in poorly contracting hearts, artificial hearts, and on the surface of prosthetic cardiac valves may contribute to the prothrombotic diathesis observed under these conditions.

Although different cell types within the heart express cNOS activity, cardiac microvascular endothelial cells appear to be the predominant source of load-dependent cardiac NO synthesis. These cells are abundant within the heart and are located close to all cardiac myocytes. The endocardium itself also contributes significantly to load-dependent cardiac NO synthesis. However, rapid effects caused by endocardial NO in the heart wall are likely to occur only in a narrow zone of adjacent myocardium because of diffusion constraints. Therefore, most of the endocardium-derived NO is rapidly dissipated into the intracavitary flow of ventricular blood, where it inhibits local platelet aggregation; this situation may be relevant to the possibility that hemoglobin may serve as a systemic carrier of NO.

Myocytes also possess calcium-dependent cNOS; however, they do not represent a significant source of NO synthesis, at least in response to an applied load. Spontaneously beating neonatal ventricular myocytes do not release detectable NO concentration in culture in the absence of adrenergic stimulation. This is not surprising, given that the large intracellular calcium concentration gradients during excitation and contraction are likely to dwarf increases in extracellular calcium concentration entry via mechanically gated cation channels. Furthermore, stimulation of the cNOS in intact hearts causes lusitropic effects that are not observed in similarly stimulated ventricular papillary muscle preparations, suggesting a role for cardiac endothelial cells in NO-dependent effects on cardiac muscle.

There are likely to be several physiologic consequences of mechanical transduction of cardiac NO synthesis. Cells within the heart are subjected to tremendous mechanical deformation during filling and beating. In addition, there is substantial evidence that NO directly affects mechanical properties of cardiac myocytes. NO acts directly on myocytes via increases in cGMP to facilitate relaxation and to mediate an acetylcholine-stimulated decrease in contractility (negative inotropy). cGMP concentration in myocytes fluctuates during each cardiac cycle, reaching a sustained maximum immediately after the T wave. This is in excellent agreement with a previous nanomedical study, which observed the peak NO concentration immediately after the T wave.

The use of nanosensors helps to identify load-dependent mechanical transduction of NO synthesis in the beating heart in vivo and to identify phasic changes in NO concentration that occur during the cardiac cycle. Because NO and cGMP have negative inotropic and positive lusitropic actions on the heart, these nanomedical studies suggest that mechanical transduction of cardiac NO synthesis and its short-term accumulation may provide an important autoregulatory mechanism for the modulation of myocardial contractility and relaxation in response to abrupt changes in preload.

Race-Related Difference in Nitric Oxide Production and Oxidative Stress

African Americans have one of the highest rates of hypertension and diabetes mellitus in the world and have disproportionately more cardiovascular complications associated with these diseases than do European Americans. Tandem NO/O$_2^-$/ONOO$^-$ nanosensors allowed for the first time concurrent measurement of molecules in real-time in a single human umbilical vein endothelial cell (HUVEC). This approach is extremely favorable toward understanding the process and mechanism of pathogenesis in vascular diseases at the molecular level. Western blot analysis revealed that eNOS expression in HUVECs from African Americans was approximately twice that of European Americans (Figure 9). The high eNOS expression and high activity of this enzyme (based on conversion of H-L-arginine to H-L-citrulline) indicate that the endothelium of African Americans has a much higher potential to produce NO than the endothelium of European Americans. However, biomedical/biosensing measurements clearly indicate that the concentration of bioavailable NO in African Americans is approximately 400 nmol/L, which is 2 times lower than that observed in European Americans (approximately 800 nmol/L) (Figures 10A and 10B). In both racial groups, stimulated (by calcium ionophore) NO release from a single endothelial cell was associated with concurrent release of O$_2^-$ and ONOO$^-$. In a HUVEC from European Americans, the pattern of ONOO$^-$ release was similar to that of NO (Figure 10A). In contrast, the peak value of ONOO$^-$ concentration recorded in a HUVEC from African Americans was reached approximately 2 seconds before reaching the maximum for NO concentration (Figure 10B). The rate of NO release was approximately 5 times slower in African Americans than in European Americans (94 vs 505 nmol/sec), whereas the rates of release of O$_2^-$ and ONOO$^-$ were approximately 2 and 4 times faster, respectively, in African Americans than in European Americans (9.4 vs 22.1 nmol/L per sec for O$_2^-$ and 209 vs 810 nmol/L per sec for ONOO$^-$). The difference between European Americans and African Americans in the peak NO, O$_2^-$, and ONOO$^-$ concentration was revealed in HUVECs in response to either calcium ionophore, a receptor-independent eNOS agonist, or acetylcholine, a receptor-dependent eNOS agonist. Most O$_2^-$ (approximately 75%) is generated by uncoupled eNOS, and approximately 20% is generated by NADPH oxidase. These data provide the first direct evidence for enzymatic uncoupling of eNOS function in the human endothelium under physiologic conditions and suggest that the degree...
of enzyme uncoupling may operate on a different level in endothelial cells of European Americans and African Americans. The release of NO and O$_2^-$ by these same enzymes produces high concentration of ONOO$^-$, which is the main component of oxidative stress.

Biomedical measurements on the molecular level clearly indicate that endothelial cells from African Americans generate significantly more O$_2^-$ from 2 enzymatic sources: NADPH oxidase and uncoupled eNOS. Increased O$_2^-$ production by NADPH causes an uncoupling of eNOS and potential amplification of O$_2^-$ generated by NOS and a high level of oxidative stress. These findings reveal that NO/O$_2^-$/ONOO$^-$ balance in intact endothelium may operate in a diverted steady state among ethnic groups. Increased levels of NADPH-oxidase protein subunits and total eNOS protein in the endothelial cells of African Americans compared with European Americans, combined with an increase in enzymatic activities in African Americans, contribute to the enhancement of local NO/O$_2^-$/ONOO$^-$ steady-state concentrations in the endothelium of African Americans.

Paradoxically, the greater potency of NO production resulting from eNOS up-regulation in endothelial cells of African Americans is associated with a decrease in the availability of biologically active (diffusible) NO$^{16,37}$. This is a result of increased NO degradation by excess O$_2^-$ predominantly generated by eNOS and NADPH oxidase. The endothelial NO/O$_2^-$/ONOO$^-$ metabolism highlights the high oxidative stress, low bioactive NO, and potential predisposition to endothelial dysfunction, vascular complications, heart failure, and stroke prevalent in African Americans.

**Summary and Clinical Implications**

Nanosensing/nanotechnologic/nanomedical techniques are a relatively new way to monitor the physiology of NO in vivo. These methods involve the application of nanosensors to monitor real-time dynamics of NO production in the heart as well as the dynamics of nitro-oxidative species (oxidative stress) produced in the failing heart. With use of nanosensing/nanomedical techniques, it has been demonstrated that altering mechanical forces on the heart is followed by a parallel increase or decrease in cardiac NO synthesis. This novel cardiac autoregulatory mechanism facilitates ventricular filling in times of increased demand. Dysfunction of this mechanism may explain the pathophysiology of diseases associated with increased myocardial distension, such as

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![Figure 9. Race/ethnicity-related difference in endothelial nitric oxide synthase (eNOS) expression in human umbilical vein endothelial cells (*p < 0.01). (Adapted with permission from Circulation.6)](image_url)
valvular heart disease or heart failure. Cardiac microvascular endothelial cells appear to be the predominant source of load-dependent, mechanically stimulated cardiac NO synthesis. An increasing or decreasing ventricular preload is gradually, not abruptly, followed by parallel changes in NO concentrations. The turbulent blood flow within the beating heart provides a potent stimulus for platelet adhesion and aggregation. The unusually high load-dependent, local NO observed near the endocardium is of considerable importance in inhibiting local platelet adhesion and aggregation. The lack of this effect in poorly contracting hearts and artificial hearts may contribute to the prothrombotic diathesis observed under these conditions. The following potential therapeutic strategies are suggested to prevent or ameliorate damage of the heart during cardiac events (according to their effectiveness): prevention of $O_2^-$ and ONOO$^-$ production (by stabilizing eNOS), supplementation of NO (NO donors), and dismutation or scavenging of $O_2^-$ (antioxidants). The latter 2 strategies have been evaluated in combination in the African American Heart Failure Trial (A-HeFT). Results from this study have shown that the combination of an NO donor (isosorbide dinitrate) and an antioxidant (hydralazine) are effective in treating African
Americans with heart failure, and this topic forms the basis of the remaining articles in this supplement.


Nitroglycerin (NTG) has been one of the most widely used anti-ischemic drugs for more than a century. Organic nitrates are effective for the acute treatment of stable-effort angina, mixed angina, unstable angina, acute myocardial infarction, and chronic congestive heart failure. However, during chronic treatment, the efficacy of nitrates is often blunted secondary to development of nitrate tolerance. In this review, we examine current concepts of the mechanisms underlying nitrate tolerance, particularly the theories of oxidative stress and impaired biotransformation of NTG. We also discuss the mechanisms whereby concomitant nitrate and hydralazine treatment may inhibit tolerance as well as nitrate-induced endothelial dysfunction.

Nitroglycerin: Mechanisms and Biotransformation

Emerging data question long-held beliefs about the mechanism of NTG bioactivation and suggest the need for a paradigm shift.

**Mechanism of action:** The mechanisms underlying NTG-induced smooth muscle relaxation are complex and not completely understood. Cellular effects of NTG include activation of the intracellular nitric oxide (NO) receptor enzyme, soluble guanylyl cyclase (sGC), which increases cyclic guanosine-3′,5′-monophosphate (cGMP) levels and activates cGMP-dependent protein kinases and cyclic nucleotide-gated ion channels. Thus, NTG and organic nitrates are believed to use the same signaling mechanism as en-
endothelial tissue. The notion that NO was the primary active metabolite of NTG became very attractive to the scientific community and led to speculation that NTG may supplant endothelial production of NO, as occurs in coronary heart disease. Results from several studies appear to support the NTG/NO hypothesis by demonstrating the formation of NO in cells and tissues exposed to NTG. However, in all of these studies, NTG was applied in concentrations far exceeding the therapeutic range. Strikingly, none of these studies addressed the mechanism by which low, therapeutically effective concentrations of NTG affect vascular NO levels.

Using 2 novel approaches to test the NTG/NO hypothesis, we recently examined the effect of low concentrations of NTG on NO levels in isolated blood vessels. Vascular NO production was quantified via electron paramagnetic resonance spin trapping of NO using colloidal Fe-diethyl-dithiocarbamate. In addition, we assessed the phosphorylation state of the vasodilator-stimulated phosphoprotein (P-VASP) at serine239, which is a prominent cGMP-dependent kinase (cGK)-I substrate. P-VASP is a reliable biochemical marker of the NO-stimulated cGK-I pathway and integrity of endothelial tissue. In these experiments, we compared the vasodilation and NO donor properties of NTG with a known NO donor (isosorbide dinitrate [ISDN]) and a calcium ionophore (A23187). When compared with ISDN and A23187, NTG exhibited a striking dissociation (3 log U of concentration difference) between its vascular activity (increase in P-VASP and vasorelaxation) and its NO donor properties. In support of this finding, the vasodilator activity of NTG was much less susceptible to inhibition by the NO scavenger carbonic anhydrase inhibitor than was the vasodilator activity of established NO donors. These results challenge the widely accepted NTG/NO hypothesis and suggest that at therapeutically effective concentrations (ie, 10 to 1,000 nmol/L), NTG activates the vascular sGC/cGK-I pathway, resulting in vasorelaxation that is not related to NO bioactivation. Interestingly, in the presence of endothelial tissue, NTG exhibits a lower vasodilator potency, yet higher NO formation. This observation indicates that metabolism of NTG to NO, if it is present at all, occurs preferentially in endothelial cells, but this happens without translation into higher vasorelaxant potency when compared with endothelium-denuded vessels.

Preeminent cGMP effectors are cGK-I, cGK-II, and cyclic nucleotide-gated ion channels. The mechanisms whereby cGK-I, the isoform expressed in vascular smooth muscle, lowers agonist-induced contractile tone, include a cGMP/cGK-I–induced reduction of agonist-induced intracellular free calcium ion (Ca2+) levels in smooth muscle cells and desensitization of contractile elements to Ca2+. NTG can also affect vascular eicosanoid production, which suggests that NTG also may affect vascular tone by this mechanism. Indeed, NTG was shown to activate cyclooxygenase and to lead to enhanced prostacyclin (PGI2) formation in cultured endothelial cells and vascular tissues.

**NTG biotransformation:** NTG is bioconverted by different pathways to active and inactive metabolites. Transformation of NTG to glycerol-1,3-dinitrate (1,3-GDN) and other inactive metabolites occurs via the glutathione reductase (GR) and glutathione-S-transferase (GST) pathways. Bioactive metabolites of NTG include NO, S-nitrosothiols, inorganic nitrite, and 1,2-dinitroglycerin (1,2-GDN). These appear to be distinct bioactivation pathways for low (therapeutic) versus high (supropharmacologic) concentrations of NTG. Interestingly, nanomolar concentrations of NTG, which produce anti-ischemic effects and vasodilatation, do not generate measurable quantities of NO as measured by electron paramagnetic resonance spin trapping.

Mitochondrial aldehyde dehydrogenase isofrom 2 (ALDH2) has been suggested to be the enzyme responsible for bioactivation of NTG. Chen and coworkers demonstrated that ALDH2 generates nitrite (NO2−) and 1,2-GDN from NTG in a dithiol-dependent fashion. During the catalytic cycle, ALDH2 is inactivated as the result of disulfide formation. Dithiols such as dithiothreitol or lipoic acid restore enzyme activity. Nicotinamide adenine dinucleotide (NAD+) accelerates the catalysis, but since the conversion of NTG to 1,2-GDN and nitrite is a reduction, NAD+ cannot function as an electron acceptor but rather increases turnover by steric effects. Nonspecific inhibitors of this enzyme (eg, disulfiram, cyanamide, chloral hydrate) and high concentrations of the substrate acetaldehyde attenuate the vasorelaxing, cGMP-eliciting, and blood pressure-lowering activity of NTG in rats and inhibited organic nitrate reductase activity. Because ALDH2 is confined to the mitochondria, isolated mitochondria can mediate activation of sGC by NTG. Using isolated rat aortic rings, we recently demonstrated a marked attenuation of NTG vasodilator potency after incubation with acetaldehyde, choral hydrate, cyanamide, and the specific ALDH2 inhibitor daidzin. In addition, activation of cGK-I (as assessed by P-VASP) and vasodilation by NTG were greatly inhibited by the more sensitive ALDH2 inhibitor benomyl. In contrast, benomyl did not modify sodium nitroprusside-induced phosphorylation of VASP or vasorelaxation. We also showed that in vivo treatment with NTG or exposure to benomyl reduced NTG bioactivation (1,2-GDN formation). These results confirmed the observations by Chen and associates and pointed to a specific role for ALDH2 in cGMP-mediated NTG-induced vasorelaxation. Depletion of functional mitochondria from endothelial cells (so-called ρ0 cells) resulted in marked attenuation of NTG-stimulated increases in cGMP.
Nitrates Tolerance, Cross-Tolerance, and Endothelial Dysfunction

Nitrates tolerance is a complex phenomenon that involves neurohormonal counterregulation, collectively classified as “pseudotolerance,” as well as intrinsic vascular processes, defined as “true tolerance.” Treatment with nitrates can reduce the vasodilator responses to other organic nitrates, NO donors, and endothelium-derived NO. This phenomenon is referred to as “cross-tolerance.” Cross-tolerance is most often observed after chronic administration of nitrates in experimental models.1,20 In contrast, cross-tolerance is not encountered in vitro where “nitrate tolerance” is produced by short-term exposure (ie, 1 hour) of isolated vascular segments to high concentrations of nitrates (ie, 0.1 to 1 mmol/L).21

Endothelial dysfunction: This has been observed in patients during prolonged nitrates therapy. For example, continuous transdermal administration of nitrates (ie, 5 days) resulted in the paradoxical constriction of large coronary arteries after acetylcholine (ACh) infusion, instead of endothelium-dependent vasodilation. These findings were interpreted to be a surrogate marker for endothelial dysfunction.22 In another study, Gori and colleagues23 used strain-gauge plethysmography to determine if chronic administration of nitrates (ie, 0.6 mg/hr per day for 6 days) impaired endothelial function of the forearm circulation of healthy volunteers. Continuous transdermal administration of nitrates resulted in a marked reduction of ACh-induced increases in forearm blood flow compared with that in controls. Likewise, the vasoconstriction elicited in control subjects by NG-monomethyl-L-arginine (L-NMMA), a NOS inhibitor that unmasks a tonic reduction in vascular tone by basal nitric oxide synthase (NOS III)-derived NO, was significantly blunted in volunteers treated with nitrates. In the lowest concentration, L-NMMA even caused a paradoxical dilation. The investigators concluded that nitrates treatment reduces basal as well as agonist-stimulated vascular NO bioavailability and that this may, at least in part, be the result of abnormalities in NOS function. In other words, Gori and coworkers24 postulated that NOS generates a vasconstrictor agent. Taken together, these findings suggest that chronic nitrates treatment promotes endothelial dysfunction. A causal relation between nitrates and endothelial dysfunction has important clinical implications, because endothelial dysfunction predicts adverse long-term outcomes in patients with coronary artery disease.

Mechanism of nitrates tolerance and cross-tolerance: In 1995, we defined a new molecular mechanism underlying the relation among nitrates tolerance, cross-tolerance, and endothelial dysfunction.20 We found that aortic segments from rabbits exposed to nitrates for 3 days were tolerant to the vasodilator action of nitrates and exhibited cross-tolerance to other cGMP-dependent vasodilators, namely ACh and 3-morpholino sydnonimine (SIN-1), which confirmed earlier reports by Molina and coworkers.3 Upon removal of the endothelium, however, tolerance to nitrates and cross-tolerance to SIN-1 were greatly attenuated. This observation led us to hypothesize that the endothelium is either chronically releasing a vasoconstrictor or that NO is inactivated before it can stimulate sGC in the vascular smooth muscle. In support of the latter hypothesis, we found that the superoxide levels in tolerant vessels were 2-fold higher than in controls and were normalized by removal of the endothelium.20

Subsequently, Skatchkov and coworkers25 demonstrated that nitrates treatment stimulates vascular production of peroxynitrite, a highly reactive oxygen species (ROS) generated from the rapid reaction of NO with superoxide, with limited NO-like bioactivity.26 A stable metabolite of peroxynitrite, nitrotyrosine, is formed by nitration of free or protein-bound tyrosine.27 In vitro and in vivo data indicated that nitrates treatment increased vascular28,29 and urinary nitrotyrosine levels,25 which can be taken as a semiquantitative indicator of increased peroxynitrite formation. Interestingly, we were recently able to demonstrate that in vivo exposure to NTG leads to increased vascular nitrotyrosine formation, which was restricted to the endothelial and subendothelial space.30

Increased vascular peroxynitrite formation may affect the proper function of NOS III and thus induce endothelial dysfunction by several different mechanisms. For example, peroxynitrite could oxidize the NOS III cofactor tetahydrobiopterin (BH4) to dihydrobiopterin (BH2) via intermediate formation of trihydrobiopterin (BH3) radicals. Provided that dihydrobiopterine reductase activity is not sufficiently high, the resulting intracellular BH4 deficiency may lead to dissociation of the dimeric functional NOS III or mislead electron flow to molecular oxygen, resulting in superoxide formation. Monomeric NOS III always transfers electrons to molecular oxygen-yielding superoxide. Consequently, NOS III becomes uncoupled and releases superoxide. Thus, NITG therapy may switch NOS III from an NO- to a superoxide-producing enzyme, which may further increase oxidative stress in vascular tissue in a positive feedback fashion. We recently demonstrated the increased expression of an uncoupled NOS in an animal model of nitrates tolerance, in which an inhibitor of NOS, N-nitro-L-arginine, significantly reduced vascular superoxide production in tolerant vessels.31 In addition, supplementation of NITG-treated rats with BH4 reversed NITG-induced endothelial dysfunction,34 further indicating that endothelial dysfunction induced by chronic NTG treatment is, at least in part, secondary to intracellular depletion of BH4. The clinical relevance of this experimental finding has been recently highlighted.35 In these studies, Gori and coworkers demonstrated that NTG-induced endothelial dysfunction responded well to treatment with folic acid, which is a substrate for BH4 synthesis. In addition, they found that folic acid improved nitrates tolerance in forearm vessels of healthy volunteers.35 Interestingly, recent in vitro studies with the isolated NOS III enzyme indicate that folic acid restores NOS III function by increasing depleted intracellular BH4 levels.36 In another study, Wang and associates...
examined the relation between nitrate tolerance in rats (10 μg/min per 8 hr) and vascular gene expression. They observed a marked reduction in guanosine triphosphate cyclophosphodiesterase I feedback regulatory protein messenger ribonucleic acid, which is a protein that controls the rate of BH₄ synthesis by guanosine triphosphate cyclohydrolase.⁴³ Provided that messenger ribonucleic acid and protein expression are correlated in a 1:1 ratio, which has not been proven, the rate of BH₄ synthesis would be halved in nitrate-tolerant tissue.

The depletion of intracellular l-arginine may be another mechanism accounting for the uncoupling of NOS III.³⁸ Interestingly, incubation of cultured endothelial cells with NTG has been shown to deplete intracellular l-arginine levels by interference with cellular l-arginine uptake³⁹ and to stimulate endothelial production of superoxide and peroxynitrite.³⁹ Because endothelial superoxide production was blocked by NOS III inhibitors, and tolerance improved in rats³⁸ and human⁴⁰ aortas by l-arginine supplementation, the authors concluded that NTG-induced superoxide production may be, at least in part, secondary to NOS III uncoupling.³⁹ However, it is unlikely that endothelial dysfunction is secondary to decreased expression of NOS III, because we observed that NOS III is up-regulated rather than down-regulated in tolerant rat aorta.³³

A third theory explaining the mechanism of NTG-associated endothelial dysfunction relates to protein kinase C (PKC). NTG is a potent, acute stimulus for PKC activation in cultured endothelial cells, and inhibition of PKC prevents in vivo nitrate tolerance.⁴¹ Moreover, acute incubation of cultured endothelial cells with NTG (ie, 10 μmol/L per hr) induced a chronic membrane translocation of PKC-α and PKC-ε, associated with increased tyrosine nitration.³⁹ Tyrosine nitration was acutely blocked by peroxynitrite scavengers (ie, uric acid), superoxide dismutase, N⁵G-nitro-l-arginine methyl ester, and the PKC inhibitor chelerythrine. The authors concluded that NTG-induced activation of specific PKC isoforms triggers intracellular events leading to NOS uncoupling. Incubation of endothelial cells with PKC activators phosphorylates NOS III, leading to an inhibition of NOS activity and NO production,⁴² which may also contribute to NTG-induced endothelial dysfunction. Because PKC activation is induced by superoxide and peroxynitrite, NTG may initiate a vicious cycle that involves mutual activation of PKC, increased ROS production, depletion of intracellular l-arginine and BH₄, and uncoupling of NOS III. NTG-induced increases in oxidative stress may also lead to increased production of endothelin-I within endothelial and smooth muscle cells,⁴³⁴⁵ leading to PKC activation, which in turn may trigger enhanced constrictor responses to almost every receptor-dependent agonist.⁴⁵

The finding that the degree of NTG tolerance was similar in NOS III knockout and wild-type mice⁴⁶ cannot be taken as an argument disqualifying uncoupled NOS III from the mechanism of NTG tolerance, because other investigators have shown that neuronal-type NOS I functionally substitutes for NOS III in NOS III knockout mice.⁴⁷ One could speculate that in these mice, vascular NOS I will be uncoupled in the nitrate-tolerant state.

Until very recently it was not known if nitrate tolerance in the clinical setting is causally related to increased superoxide formation, reduced NTG biotransformation, or both. Sage and colleagues⁴⁸ were the first to assess in vitro superoxide production and formation of NTG metabolites. In this study, rings prepared from the internal mammary artery and saphenous vein of patients treated with a 24-hour infusion of NTG (10 μg/min) before elective bypass surgery were assayed for superoxide. These data were correlated with in vitro assessment of vasodilator responses to NTG, sodium nitroprusside, and A23187. Compared with vessels from control patients, the vessels from NTG-treated patients were tolerant to NTG, exhibited increased superoxide formation as detected by lucigenin chemiluminescence, and generated 40% less 1,2-GDN, the putative metabolite derived from bioactivation of NTG. However, this study failed to demonstrate cross-tolerance to endothelium-dependent (A23187) and endothelium-independent (sodium nitroprusside) vasodilators. Also, an acute 3-fold increase in vascular superoxide production by exposure to the superoxide dismutase inhibitor diethyldithiocarbamic acid did not modify the NTG dose-response relation. Therefore, Sage and colleagues⁴⁸ concluded that impaired NTG biotransformation more likely accounts for tolerance than for vascular superoxide formation, and that endothelial function is preserved in the tolerant state.

In contrast, using higher doses of NTG (ie, 0.5 μg/kg per min) and longer infusion periods (ie, 48 hours), we demonstrated tolerance as well as endothelial dysfunction in the mammary artery and radial artery of patients undergoing coronary bypass surgery and greatly increased superoxide production in these vessels,⁴⁹ which confirmed previous findings in patients with stable coronary artery disease.²² The failure of endothelial dysfunction development in the study by Sage and colleagues,⁴⁸ in contrast to the results from Gori and coworkers,²³³⁵ Caramori and coworkers,²² and our own findings,⁴⁹ are likely explained by the different duration of NTG administration, which was only 1 day in the former versus 2 and 6 days, respectively, in the latter studies. After 1 day of NTG exposure, pseudotolerance may still prevail over vascular tolerance.

Further support for the oxidative stress concept of nitrate tolerance was provided by the demonstration that nitrate tolerance achieved by 7 days of NTG treatment (0.6 mg/hr) in healthy volunteers increased plasma levels of cytotoxic aldehydes and isoprostanes, which are sensitive markers for free radical-induced lipid peroxidation.⁵⁰ A similar conclusion was drawn by McVeigh and coworkers⁵¹ from the observation that nitrate tolerance in healthy volunteers who received 0.4 mg/hr of NTG for 3 days increased formation of esterified 8-epi-PGF₂α in isolated platelets, which is a marker of oxidative stress.
Nitroglycerin Signaling Targets

The following discussion focuses on NTG downstream signaling targets of superoxide and peroxynitrite.

**Effects of NTG on sGC:** NTG tolerance–induced superoxide and peroxynitrite formation may also interfere with nitrovasodilator action at the level of sGC. Both superoxide and peroxynitrite are potent, direct inhibitors of NO-sensitive sGC. Reduced basal and NO-stimulated sGC activities have been detected in peroxynitrite-treated cells and vascular tissues. We also found that very low concentrations (<1 μmol/L) of peroxynitrite nearly abolished NO-dependent as well as NO-independent activation of the purified enzyme (A. Mülsch, unpublished data, 2002). Interestingly, exposure of the purified enzyme to a full inhibitory concentration of peroxynitrite (1 μmol/L) did not induce formation of immunodetectable nitrotyrosine on sGC subunits, suggesting that inhibition is not accomplished by nitration of tyrosine residues. Similarly, sGC in homogenates from NTG-tolerant rabbits was not precipitated by a 3-nitrotyrosine antibody (A. Mülsch, unpublished results), indicating that inhibition of sGC in nitrate tolerance is not mediated by tyrosine nitration, but rather by another probably thiol-dependent oxidative mechanism. Tonic inhibition of sGC in nitrate tolerance may contribute to cross-tolerance to other nitrovasodilators, NO donors, and endothelium-dependent agonists, as observed previously. Our unexpected recent observation that expression of sGC subunits α1 and β1 was increased in nitrate-tolerant vascular tissue may be interpreted as a biologic counter-regulatory mechanism compensating partially for tonic inhibition of sGC activity.

**Effects of NTG on cGK-I:** Another important unanswered question is the mechanism by which NTG-induced stimulation of superoxide and peroxynitrite production influences intracellular cGMP downstream signaling. Recent studies with cGK-I-deficient mice highlighted the crucial role of this enzyme in mediating cGMP-stimulated vasodilation. In cGK-I knockout animals, endothelium-dependent as well as endothelium-independent nitrovasodilators failed to induce significant relaxation of vascular tissue. This observation clearly indicates that the activity or expression of cGK-I could be affected in the setting of nitrate tolerance and endothelial dysfunction and that it is of utmost importance to monitor this parameter. Very recently, P-VASP has been shown to be a useful monitor for cGK-I activity in intact cells. In addition, the findings of other studies by our group indicate that changes in P-VASP inversely follow increased endothelial dysfunction and oxidative stress, suggesting that P-VASP can be used as a novel, biochemical surrogate parameter for vascular NO bioavailability and efficiency of cGMP downstream signaling. Thus, we could not detect any changes in cGK-I expression in aortas from NTG-tolerant rats and rabbits compared with controls but observed a striking reduction of P-VASP when these animals were compared with untreated controls. These findings clearly indicated that the NO/cGMP pathway was functionally inhibited during NTG-induced tolerance, whereas cGK-I and total VASP expression were not modified at all. To address specifically the role of oxidative stress in inhibiting the activity of sGC/cGK-I, the phosphorylation level of VASP was quantified in response to in vitro and in vivo treatment with NTG. In vivo treatment of rats and rabbits resulted in a marked decrease in the degree of phosphorylation of VASP that was compatible with an inhibition of NO/cGMP signaling.

These observations from animal models have been extended to patients in our clinic. We demonstrated that treatment with NTG (ie, 0.5 μg/kg per min for 48 hours) led to a significant inhibition of P-VASP. In all cases, findings were associated with tolerance, a marked degree of endothelial dysfunction, and increased superoxide production, as indicated by dihydroethidine staining of the mammary artery.

Recent data indicate the importance of peroxynitrite in inhibiting NO/cGMP signaling (P-VASP) in NTG-exposed animals. The luminol-enhanced chemiluminescence values were strikingly higher in vessels from NTG-treated animals than in controls (Figure 1). Because luminol detects superoxide, peroxynitrite, and hydrogen peroxide, peroxynitrite quenchers, such as uric acid or ebselen, are required to quantify the contribution of peroxynitrite to the chemiluminescence signal. The uric acid- and ebselen-dependent reduction in the luminol chemiluminescence signal was strikingly stronger in vessels from NTG-exposed animals than vessels from controls, which is compatible with higher peroxynitrite levels (Figure 1A). Interestingly, ebselen and uric acid strikingly increased cGK-I-dependent P-VASP formation, which suggests a crucial role for peroxynitrite in inhibiting NO/cGMP signaling in the setting of tolerance (Figure 1B and 1C). Accordingly, incubation of tolerant tissue with ebselen partly restored NTG sensitivity of tolerant tissue.

**Effects of NTG on PGI2 synthase activity:** NTG-induced production of peroxynitrite may also adversely affect the activity of the PGI2 synthase (PGI2-S). Recent studies have shown that PGI2-S is a preferential nitration target of peroxynitrite. Importantly, tyrosine nitration of PGI2-S resulted in an almost complete inhibition of the activity of the enzyme leading to decreased PGI2 formation. Because the nonmetabolized prostaglandin endoperoxide (PGH2) can activate the thromboxane A2 (TXA2)/PGH2 receptor of vascular smooth muscle cells, thereby counter-acting NTG-mediated vasodilation, peroxynitrite can be considered as a mediator of endothelial dysfunction as well as of nitrate tolerance. Our recent data obtained in nitrate-tolerant rats and rabbits support this concept. In these studies, exposure to NTG increased the luminol-derived chemiluminescence signal in rat and rabbit aorta, which was
effectively inhibited by the peroxynitrite quenchers uric acid and ebselen. These findings are compatible with increased vascular peroxynitrite formation. Western blots of 3-nitrotyrosine immunoprecipitates exposed to a polyclonal antibody directed against PGL2-S detected a single 52-kd protein band and revealed a marked increase in the PGL2−S signal from tolerant aortas (indicating increased levels of nitrated PGL2-S) compared with those in controls, whereas total PGL2-S expression was not modified by NTG tolerance. As a functional consequence of tyrosine nitration of PGL2-S, the conversion of [14C]-PGH2 into 6-keto-prostaglandin F1α (6-keto-PGF1α) was greatly inhibited. We also observed a shift to increased prostaglandin E2 (PGE2) formation in tolerant tissue. These findings suggest that tyrosine nitration accounts for the observed inhibition of the activity of the enzyme in the setting of nitrate tolerance. The functional inhibition of PGL2-S activity in tolerant tissue was indirectly confirmed by experiments using U51605, the dual blocker of PGL2-S and TXA2 synthase, which mimics the vasodilating effects of peroxynitrite. The concentration-response relation for NTG was shifted significantly to the right by U51605. This finding was interpreted as an indication for accumulation of unmetabolized PGH2, which elicits vasoconstriction via activation of the TXA2/PGH2 receptor of the vascular smooth muscle. In contrast, incubation of tolerant tissue with U51605 failed to modify the remaining vasodilator responses to NTG, because tolerance and U51605 rely on PGH2 to counteract vasorelaxation.

**Impaired biotransformation versus the oxidative stress concept:** Within the past few decades, several concepts concerning the mechanisms underlying nitrate tolerance have been intensively discussed. One favorite hypothesis originating from early work by Jakschik and Needleman and later modified by other investigators was that impaired NTG bioactivation leads to decreased NTG sensitivity in the tolerant vasculature and that these processes rely on cellular (especially mitochondrial) thiol levels. The other concept, introduced by our group, claimed that increased oxidative stress and reduced NO bioavailability were the mechanisms underlying nitrate tolerance. Supporters and detractors have voiced their views throughout the years without conclusion. For instance, impaired bioactivation of NTG, as known until very recently, may not explain associated phenomena, such as endothelial dysfunc tion, increased sensitivity to vasoconstrictors, and increased vascular superoxide production. On the other hand, we were forced to abandon the idea of a reduced bioavailability of NTG-derived NO because at therapeutic concentrations, NTG does not generate detectable levels of NO as measured by electron paramagnetic resonance spin trapping.

A clue to these apparently contradicting concepts appeared recently with the identification of ALDH2 as the enzyme responsible for NTG bioactivation. Tolerance-inducing high concentrations of NTG (ie, 0.3 mmol/L for 30 minutes) in vitro resulted in an inhibition of ALDH2 activity and an inhibition of NTG biotransformation to 1,2-GDN (NTG reductase activity), frequently taken as a marker of NTG vasodilatory action, and an attenuation of cGMP increases in response to acute NTG challenge. Nonetheless, the question of whether inhibition of ALDH also accounts for nitrate tolerance in vivo remained unanswered. Therefore, we analyzed the effect of nitrate tolerance on ALDH activity and NTG reductase activity in 3-day NTG-infused rats. The aortas removed from the tolerant rats exhibited reduced NTG vasodilator responses. However, in contrast to nontolerant controls, in vitro treatment of tolerant aortic rings with ALDH2 inhibitors did not cause a further shift in the NTG dose-response curve. Total ALDH activity in vascular homogenates and ALDH activity in mitochondria isolated from tolerant rat aorta and heart were reduced by >50% compared with nontolerant controls, or to a similar extent as achieved by daidzin, the more specific ALDH inhibitor, in control tissues and mitochondria. Furthermore, acute exposure of isolated mitochondria to 500 μmol/L of NTG decreased ALDH activity to a similar extent and stimulated mitochondrial superoxide formation. Inhibition of complex III by antimycin A similarly elicited superoxide formation and inhibition of ALDH activity. In addition, mitochondria isolated from tolerant animals generated ROS at a rate approximately 50% higher than control mitochondria, which were entirely blocked by acute addition of dithiothreitol, uric acid, or ebselen. The effects of nitrate tolerance on classic ALDH activity were mirrored by a similar reduction in NTG reductase activity, as detected by formation of 1,2-GDN. These findings suggest that NTG metabolism within mitochondria may also trigger superoxide production in mitochondria of vascular tissue.

These data confirm previous observations published by Needleman and Hunter showing that incubation of isolated heart mitochondria with high concentrations of nitrates stimulated oxygen consumption and uncoupled oxidative phosphorylation, observations consistent with a mitochondrial source of ROS. Whether NTG stimulates ROS production by blockade of respiration by NTG metabolites, accumulation of toxic aldehydes, initiation of lipid peroxidation, depolarization of mitochondrial membrane potential, or mitochondrial swelling, the loss of ALDH2 activity should cause NTG to accumulate in mitochondria and thus amplify the effect. Chen and associates proposed that oxidation of essential thiol groups in the active site of the enzyme underlies the molecular basis of tolerance, an observation supported by our study. However, it is not known to what degree substrate NTG, ROS, or both mediate ALDH2 inactivation. In studies with purified yeast ALDH2, we found that NTG, superoxide, and peroxynitrite are all capable of directly inhibiting the enzyme (DaiBer and colleagues, unpublished observations, 2004). These observations support the idea that oxidative stress may contribute directly to mechanism-based tolerance, either by oxidative inhibition of ALDH2 or perhaps by oxidizing key
enzyme cofactors from the ALDH2 repair cascade (e.g., lipoic acid, GSH/GSH reductase, or both). Irrespective of the precise sequence of events, incubation of tolerant tissue with various reductants and antioxidants completely restored vascular ALDH activity and simultaneously normalized mitochondrial ROS production. Assuming that nitrate tolerance underlies increased cardiac morbidity (derived from a retrospective meta-analysis), observations of the effect of antioxidants may have therapeutic implications. The fact that ALDH dehydrogenase activity is reduced in nitrate-tolerant patients provides support for the clinical relevance of this mechanism.

Prevention of Nitroglycerin Tolerance

The demonstration of increased endothelial superoxide formation in NTG tolerance suggests that treatment with antioxidants may prevent this phenomenon.

Antioxidants: Experiments published by Bassenge and coworkers, and Bassenge and Fink demonstrate that concomitant treatment with vitamin C preserved the sensitivity of the vasculature to organic nitrates. In these studies, vitamin C completely prevented the development of nitrate tolerance in chronically instrumented dogs. In addition, concomitant treatment with vitamin C and NTG in patients with congestive heart failure completely prevented the development of hemodynamic tolerance.

Recently, we studied the effects of vitamin C on cGK-I signaling in rats treated with NTG. We found that in vitro incubation of tissue from rats exposed to NTG with vitamin C reduced oxidative stress, increased vascular sensitivity to NTG, and improved NO/cGMP signaling by increasing the degree of the phosphorylation of the cGK-I target VASP. In vivo administration of vitamin C was even more effective. It normalized oxidative stress, reversed tolerance, and strikingly increased P-VASP to nearly 200% of control levels. This observation may indicate that, in addition to improving NO/cGMP signaling, antioxidant treatment may partially restore biotransformation of NTG stored in tolerant tissue. Vitamin C has also been characterized as an effective peroxynitrite scavenger. This finding may further point to the crucial role of peroxynitrite as the reactive intermediate responsible for mechanisms underlying tolerance and also the so-called cross-tolerance phenomenon.

Hydralazine: an antioxidant? Favorable interactions between hydralazine and nitrates have been demonstrated in the Veterans Heart Failure Trials (V-HeFT). The combination of hydralazine and ISDN has been shown to have beneficial effects on left ventricular function, exercise capacity, and most notably on survival in a large patient population with severe heart failure. Very recently, a fixed-dose combination of ISDN/hydralazine has been shown to decrease mortality, reduce the incidence of first hospitalization for heart failure, and improve quality of life in the African American Heart Failure Trial (A-HeFT). Hydral-
azine has also been shown to prevent the development of NTG tolerance in both experimental models and patients with congestive heart failure. It is a potent arteriolar dilator that stimulates reflex increases in vasoconstrictor stimuli, including circulating catecholamines and plasma renin activity (reflecting increased circulating angiotensin II levels). This would appear, upon first inspection, to worsen rather than to improve tolerance by enhancing the neurohormonal counterregulatory adjustments to nitrate. The fact that this does not occur indicates that hydralazine may have direct effects on superoxide production of vascular tissue by inhibiting nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, one of the most important superoxide-producing enzymes, via cell membrane hyperpolarization or by a direct free-radical scavenging effect.

An explanation for this apparent paradox may be that administration of hydralazine and NTG to rats completely prevented the NTG-induced increase in vascular superoxide production and tolerance (Figure 2). Further, acute addition of hydralazine in clinically relevant concentrations to segments of aorta from control-and NTG-exposed animals greatly reduced steady-state levels of vascular superoxide. Hydralazine was only effective when administered in vivo or incubated with intact rings but had no effect when added to the vascular homogenates. One explanation for this finding is that in order for hydralazine to exert its effect, possibly via hyperpolarization, the cell must be intact. This possibility is strengthened by the fact that another hyperpolarizing agent, the adenosine triphosphate-sensitive potassium ion channel activator pinacidil, also greatly inhibited vascular superoxide production. In performing relaxation studies, we found that hydralazine normalized tolerance to NTG and cross-tolerance to SIN-1. Considering the importance of ALDH2, the relation between mitochondrial membrane potential and superoxide formation is an important question. It appears that the mitochondrial membrane potential controls mitochondrial matrix volume and intermembrane space, and this in turn controls mitochondrial metabolism. Shrinking of the matrix and increased intermembrane space leads to uncoupling of electron flow and increased mitochondrial superoxide formation.

Another possibility could be that hydralazine, per se, may be able to scavenge ROS. To address this issue, we generated superoxide via the xanthine/xanthine oxidase reaction or peroxynitrite by spontaneous decomposition of SIN-1, and tested the ability of hydralazine to quench in vitro chemiluminescence signals created by both ROS. With these studies we could clearly show a dose-dependent inhibition of the superoxide and peroxynitrite signal in clinically relevant concentrations of hydralazine, which indicates that the molecule, per se, is an effective ROS quencher (Figure 3). We then tested whether hydralazine added in vitro prevented the increase in NTG-induced stimulation of superoxide/peroxynitrite production in mitochondria. The data presented indicate that hydralazine suppressed NTG-induced ROS production in a concentration-dependent fashion in isolated mitochondria from the heart (Figure 4).

In vivo treatment with NTG resulted in a marked decrease in NO/cGMP signaling, as indicated by the marked inhibition of the P-VASP signal of vascular tissue. Previously, we were able to demonstrate that incubation of vascular tissue with vitamin C and ebselen, both effective peroxynitrite quenchers, markedly increased P-VASP levels in vessels from in vivo NTG-treated rats. A similar phenomenon was observed with respect to hydralazine. Incubation of in vitro tolerant tissue with hydralazine increased P-VASP significantly and partly restored organic nitrate sensitivity. Therefore, it is tempting to speculate that these phenomena are, at least in part, caused by the ROS-scavenging effects of hydralazine. In addition, incubation of in vivo hydralazine treatment on vascular superoxide production (A) and vascular nitroglycerin (NTG) sensitivity (B) in control and NTG-treated animals. Treatment with hydralazine significantly reduced superoxide production in vessels from control and NTG-treated animals and simultaneously prevented the development of tolerance. Values are expressed as mean ± SEM of 4 to 12 experiments. *p < 0.01 untreated versus NTG-treated; †p < 0.05 versus without hydralazine treatment. (Adapted with permission from J Clin Invest.)
vivo tolerant tissue with hydralazine partly restored vascular ALDH activity (Daiber and coworkers, unpublished observations, 2005), indicating that hydralazine treatment may beneficially influence both mechanisms underlying nitrate tolerance, namely ROS production and impaired NTG bio-transformation process.

Our present in vitro findings that hydralazine strongly and potently prevented ROS-induced chemiluminescence suggest that hydralazine may be an efficient ROS scavenger. Several recent studies apparently support this conclusion. Leiro and colleagues \(^70\) demonstrated an inhibitory effect of hydralazine on inducible NOS/COX-2 gene and protein expression in rat peritoneal macrophages. Hydralazine at 0.1 to 10 mmol/L inhibited both extra- and intracellular ROS production by inflammatory macrophages, by a ROS-scavenging mechanism probably affecting superoxide generation by xanthine oxidase, and nicotinamide adenine dinucleotide (NADH)/NADPH oxidase. \(^70\) Hydralazine also blocked NOS-2 and COX-2 gene expression, suggesting that it strongly attenuates the macrophage activation by virtue of its antioxidant properties. Recently, the antioxidant properties of hydralazine were linked to alterations in vascular gene expression. Knowles and coworkers \(^71\) tested whether hypoxia-inducible factor (HIF)-regulating proline hydroxylase could be a target of hydralazine. They found that hydralazine inhibited prolyl hydroxylase domain activity and induced nonhydroxylated HIF-1α, evidence for HIF stabilization specifically by inhibition of prolyl hydroxylase domain enzyme activity. Consequently, hydralazine induced rapid and transient expression of HIF-1α and downstream targets of HIF (endothelin-1, adrenomedullin, heme oxygenase 1, and vascular endothelial growth factor) in

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**Figure 3.** Effects of hydralazine on superoxide- or peroxynitrite-induced chemiluminescence (L-012-enhanced chemiluminescence). These data clearly show that hydralazine is an effective scavenger of reactive oxygen species from 1 mM 3-morpholino sydnonimine (SIN)-1 and 10 mU/mL xanthine oxidase (XO)/1 mmol/L hypoxanthine, respectively. Hydralazine, 100 μmol/L, was added to the SIN-1 incubation. Data are expressed as mean ± SEM from 3 independent experiments.

**Figure 4.** Effects of hydralazine on mitochondrial reactive oxygen species production on exposure to nitroglycerin (NTG). Incubation of mitochondria with NTG greatly increased the L-012-enhanced chemiluminescence signal. The addition of hydralazine concentration dependently (0.1 to 100 μmol/L) decreased NTG-induced production of reactive oxygen species in mitochondria. GTN = glyceryl trinitrate.
endothelial and smooth muscle cells and induced endothelial cell-specific proliferation. In vivo, hydralazine induced HIF-1 and vascular endothelial growth factor protein in tissue extracts and elevated plasma vascular endothelial growth factor levels. Thus, hydralazine initiates a proangiogenic phenotype and may be beneficial in ischemic heart disease.

Summary and Clinical Implications

In summary, there is mounting evidence that systemic therapy with organic nitrates induces tolerance and endothelial dysfunction in patients with coronary artery disease and in patients with congestive heart failure, and even in healthy controls. Mechanisms contributing to this phenomenon may be a nitrate-induced stimulation of vascular (mitochondrial) superoxide or peroxynitrite production, or both, and the ensuing inhibition of ALDH2 leading to impaired NTG biotransformation. Treatment of congestive heart failure with the combination of ISDN and hydralazine has been shown to improve exercise capacity and prognosis in these patients, which may be secondary to the inhibitory effects of hydralazine on vascular ROS production. The mechanisms whereby hydralazine exerts its antioxidant effects may include cyclic adenosine monophosphate- or hyperpolarization-induced inhibition of the expression and activity of the NADPH oxidase or direct ROS scavenging effects. The scavenging of peroxynitrite may be of utmost importance, because peroxynitrite has been identified to cause NOS III uncoupling, tyrosine nitration of the PGI₂-S, and inhibition of activity of the NO target sGC (Figure 5).


Figure 5. Schematic diagram depicting mechanisms underlying nitroglycerin (NTG)-induced vasodilation (A) and the vascular consequences of NTG-induced peroxynitrite formation (B). (A) Short-term NTG treatment causes vasorelaxation by releasing nitric oxide (NO) or an NO-related vasoactive metabolite, which in turn stimulates the soluble guanylyl cyclase (sGC) and release of prostacyclin (PGI₂). Activation of sGC and adenylyl cyclase (AC) increases the formation of second messengers such as cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) in tissue extracts and elevated plasma vascular endothelial growth factor levels. Thus, hydralazine initiates a proangiogenic phenotype and may be beneficial in ischemic heart disease.


The initial rationale for use of organic nitrates and hydralazine (HYD) in combination was their complementary “nitroprussidelike” hemodynamic effect caused by the predominant venodilatory action of organic nitrates and the arterial-dilatory effect of HYD. This combination leads to a significant improvement in cardiac function, with a concomitant reduction in right and left ventricular filling pressures and augmentation of cardiac output. Based on this hemodynamic profile, the Vasodilator Heart Failure Trial (V-HeFT) was designed to examine the effect of this drug combination on the outcome of patients with congestive heart failure (CHF). Results from V-HeFT I showed improvements in left ventricular ejection fraction (LVEF), exercise tolerance, and survival in patients treated with isosorbide dinitrate (ISDN) and HYD compared with those treated with placebo. A retrospective analysis of V-HeFT I and V-HeFT II showed that the benefit of ISDN-HYD was seen mainly in African Americans. This observation led to the design of the African American Heart Failure Trial (A-HeFT), which confirmed the benefit of these drugs in combination in African American patients with CHF. There are a number of potential mechanisms responsible for the beneficial therapeutic effects of combination ISDN-HYD in patients with CHF, including favorable hemodynamic effects and improvement in left ventricular systolic function. Data from V-HeFT II showed a significant improvement in LVEF with combination ISDN-HYD, greater than the effect of the angiotensin-converting enzyme inhibitor enalapril. This increase in LVEF was associated with a favorable effect on survival. Prevention of nitrate tolerance with HYD may also be responsible for the favorable therapeutic effects of combination ISDN-HYD. Frequent administration of ISDN has been shown to result in the early development of nitrate tolerance. Concomitant use of HYD with a nitrate, both in an animal model and in patients with CHF, has been shown to prevent the development of nitrate tolerance and maintain the favorable hemodynamic effect of nitrates. © 2005 Elsevier Inc. All rights reserved. (Am J Cardiol 2005;96[suppl]:37i–43i)
the benefit of this therapeutic approach over placebo. The V-HeFT II study (804 patients) was designed to compare the effects of direct vasodilation with ISDN-HYD (160 to 300 mg/day) with those of angiotensin-converting enzyme (ACE) inhibition with enalapril (maximum dose 20 mg/day). The results of this study demonstrated that when given to patients with mild-to-moderate symptomatic CHF, enalapril had a greater effect on survival than the combination of ISDN-HYD. Lower mortality in the enalapril group was the result of a lower incidence of sudden death, whereas no difference was seen between the 2 treatment regimens with respect to mortality from worsening CHF. The results of these studies clearly demonstrate that ACE inhibition in patients with New York Heart Association (NYHA) class II or III CHF provides a stronger survival benefit than direct vasodilation.
Effects of Isosorbide Dinitrate and Hydralazine on Markers of Cardiac Function

In addition to the survival benefit observed in patients with CHF, the combination of ISDN-HYD has been shown to improve other markers of cardiac function, including exercise tolerance and left ventricular ejection fraction (LVEF).

Effect on exercise tolerance: The results of the V-HeFT trials demonstrate a small but significant (p <0.05) improvement in peak oxygen consumption in patients treated with ISDN-HYD.4,5 Improvement in peak oxygen consumption with this drug combination was seen for the first 6 months of the follow-up period and was greater than the effect of the ACE inhibitor enalapril (Figure 2).5

Effect on LVEF: Data from V-HeFT II6 demonstrate a significant improvement in LVEF with the combination of ISDN-HYD (Figure 3). Similar to the effect on peak oxygen consumption, the effect of this drug combination was superior to that of enalapril. A relation was demonstrated between improvement in LVEF and survival in patients treated with ISDN-HYD. In patients whose LVEF increased by >10%, there was a very favorable effect on long-term survival (80% at 5 years), whereas survival in patients whose LVEF remained unchanged or decreased during the first 6 months of the study was only 30% at 5 years.

Thus, although patients with CHF achieved a greater survival benefit with enalapril, the combination of ISDN-HYD improved exercise tolerance and LVEF significantly more than enalapril in these patients.

Figure 2. Mean change from baseline in peak oxygen consumption over 2 years in the Vasodilator Heart Failure Trial (V-HeFT) II. The increase in the isosorbide dinitrate–hydralazine (ISDN-HYD) arm was significant for the first 6 months (p <0.01) and was greater than in the enalapril arm. (Reproduced with permission from N Engl J Med.4)

Figure 3. Mean change from baseline in left ventricular ejection fraction over 2 years in the Vasodilator Heart Failure Trial (V-HeFT) II. ISDN-HYD = isosorbide dinitrate–hydralazine. *p <0.0001 versus baseline; †p <0.05 versus enalapril. (Reproduced with permission from Drugs.6)
Effect of Isosorbide Dinitrate–Hydralazine in African Americans with Congestive Heart Failure in the Vasodilator Heart Failure Trials

African Americans constitute 180 of 642 patients in V-HeFT I, and 215 of 804 patients in V-HeFT II. A retrospective analysis of the outcome of African Americans compared with European Americans in V-HeFT I showed a significant 56% lower annual mortality rate in African American patients who were treated with ISDN-HYD than with placebo (9.7% vs 17.3%; p = 0.04) (Figure 4). Administration of ISDN-HYD was also associated with a greater effect on oxygen consumption, which increased by 1.25 mL/kg per min in African Americans treated with ISDN-HYD compared with a decrease of 0.4 mL/kg per min in African Americans receiving placebo. The difference between the 2 groups was borderline significant.

Despite a superior effect of enalapril on survival in the overall V-HeFT II study population, the effect of ISDN-HYD was comparable to that of enalapril in African American patients (12.9% vs 12.8%; p = NS) (Figure 5). In addition, compared with enalapril, ISDN-HYD significantly improved the quality of life in African Americans (p < 0.043). When peak oxygen consumption was analyzed using a longitudinal model that took into account all data collected during the entire year, ISDN-HYD performed slightly better than enalapril compared with these drugs at baseline (p < 0.067). Collectively, these results demonstrate that ISDN/HYD is more effective than placebo and as effective as enalapril in reducing mortality in African Americans compared with European Americans and is significantly more effective at improving quality of life than enalapril in these patients, despite only modest improvements in oxygen consumption.

Effect of Hydralazine on Nitrate Tolerance

Multiple studies have clearly demonstrated that frequent administration of oral nitrates or continuous administration of intravenous or topical nitrates results in early develop-
ment of nitrate tolerance with marked attenuation of the initial hemodynamic effect.\textsuperscript{8,9} It is therefore not surprising that various strategies have been proposed and attempted for preventing nitrate tolerance. These strategies include concomitant administration of sulfhydryl groups, an ACE inhibitor, or diuretics.\textsuperscript{10} However, these methods have not been proven beneficial in patients with CHF.\textsuperscript{10–12} Intermittent nitrate therapy, allowing a daily nitrate washout interval of at least 12 hours, has been effective in preventing nitrate tolerance,\textsuperscript{8,13} but such a regimen is limited by its inability to provide a continuous and uninterrupted therapeutic effect. Interaction between HYD and nitrates was first reported by Unger and colleagues,\textsuperscript{14} who demonstrated potentiation of nitroglycerin response with HYD incubation in isolated aortic rings rendered tolerant in vivo to nitroglycerin. Münzel and colleagues\textsuperscript{15} later showed the ability of HYD to inhibit vascular superoxide production and prevent nitrate tolerance in vitro. The results of these investigations suggest that the antioxidant properties of HYD may be responsible for preventing nitrate tolerance. In 2 studies performed approximately 10 years ago, in an in vivo animal model of CHF\textsuperscript{16} and in patients with CHF,\textsuperscript{17} it was also demonstrated that concomitant administration of HYD is useful in preventing nitrate tolerance.

In the first study, Bauer and Fung\textsuperscript{16} produced CHF in Sprague-Dawley rats by ligating the left coronary artery, producing a myocardial infarction at the left ventricular free wall and apex. They then allowed the rats to recover for at least 6 weeks. The development of myocardial infarction resulted in elevated venous pressure and reduced cardiac output similar to the hemodynamic changes observed in patients with CHF. Infusion of nitroglycerin to the rats with CHF produced a reduction in left ventricular end-diastolic pressure of 46%\textsuperscript{110} 3%. However, with continuation of nitroglycerin infusion, the initial hemodynamic effect was not maintained, and left ventricular end-diastolic pressure returned to near baseline values within 6 hours as a result of the development of tolerance. Coadministration of HYD, which was given intravenously (2 × 0.1-mg bolus injections) at 1.5 and 2 hours during nitroglycerin infusion, prevented the development of nitrate tolerance (Figure 6), and the initial reduction in left ventricular end-diastolic pressure was maintained throughout the remainder of the NTG infusion. Hemodynamic tolerance to NTG did not occur during coadministration with HYD. Values are expressed as mean ± SEM. *Statistically significant differences from baseline (p <0.05). (Reproduced with permission from \textit{Circulation}.)

Figure 6. Effects of nitroglycerin (NTG) infusion alone (n = 7) or in combination with hydralazine (HYD) (n = 8) on left ventricular hemodynamics in rats with heart failure. NTG was infused at 10 μg/min in both groups. Hydralazine (H) was administered at 1.5 and 2 hours (0.1-mg bolus) during NTG infusion. NTG alone produced initial reductions in left ventricular end-diastolic pressure (LVEDP), but had no effect on left ventricular peak systolic pressure (LVPSP). LVEDP returned to baseline by 8 hours, indicating tolerance development. HYD caused significant reductions in LVPSP, which were maintained throughout the remainder of the NTG infusion. *Statistically significant differences from baseline (p <0.05). (Reproduced with permission from \textit{Circulation}.)
pressure with nitroglycerin was maintained throughout the 10-hour nitroglycerin infusion period. The plasma concentrations of nitroglycerin and dinitrate metabolites before and after HYD dosing were not significantly different. Because of the potential therapeutic value of these study results, a similar experiment to evaluate the effect of oral HYD on the development of nitrate tolerance was conducted in 28 patients with chronic CHF (New York Heart Association [NYHA] functional class III or IV). Patients were randomized to receive a continuous infusion of nitroglycerin for 24 hours either alone (14 patients, group 1) or concomitantly with oral HYD (14 patients, group 2) given at a dose of 75 mg 4 times daily and begun ≥24 hours before the study. Therapy with nitroglycerin was started in both groups at a rate of 20 μg/min. The rate of infusion was increased in increments of 20 to 60 μg/min every 5 minutes to achieve at least a 30% reduction in mean pulmonary artery wedge pressure or until a maximum dose of 560 μg/min was reached. The dose required to achieve the desired hemodynamic response was maintained at the same rate for 24 hours, and hemodynamic measurements were repeated periodically throughout the study. Continuous infusion of nitroglycerin alone resulted in a gradual and significant attenuation of the initial effect of therapy (group 1) on mean pulmonary artery pressure (27% ± 4% at 0 hour vs 10% ± 3% at 24 hours; p < 0.05) and mean pulmonary artery wedge pressure (40% ± 4% at 0 hour vs 16% ± 4% at 24 hours; p < 0.05) (Figure 7). In contrast, in group 2, concomitant administration of oral HYD prevented nitroglycerin-induced hemodynamic tolerance and resulted in persistent effects on mean pulmonary artery and wedge pressures throughout the study period (31% ± 3% at 0 hour vs 27% ± 4% at 24 hours [p = NS] and 37% ± 4% vs 34% ± 6% [p = NS], respectively). In addition, the initial effect on blood pressure reduction was attenuated at 24 hours in group 1 (12% ± 3% at 0 hour vs 5% ± 2% at 24 hours; p < 0.05), but not in group 2 (17 ± 2% at 0 hour vs 15% ± 3% at 24 hours; p = NS). The results of this study support the observations made by Bauer and Fung in animals, indicating the ability of HYD to prevent early development of nitrate tolerance and maintain nitrate-mediated favorable hemodynamic effects.

Figure 7. Mean pulmonary artery wedge pressure measured over time during 24-hour continuous administration of nitroglycerin (NTG) alone or in combination with oral hydralazine (HYD). *p < 0.05 versus 0 hour. (Reproduced with permission from J Am Coll Cardiol.17)

Conclusion

In both in vivo animal models of CHF and patients with chronic CHF, preview reports have demonstrated prevention of nitrate tolerance and preservation of nitrate-induced hemodynamic effects with concomitant administration of HYD. Studies with in vitro models of nitrate tolerance have demonstrated that HYD potentiates the vasorelaxing effect of nitroglycerin and reduces the formation of nitrate-mediated vascular superoxide that leads to nitrate tolerance. These data, in addition to the results of the V-HeFT and A-HeFT studies, which demonstrated a beneficial effect of ISDN-HYD on cardiac function, exercise tolerance, and survival, provide strong support for the combined use of nitrates with HYD in patients with CHF.


Progressive vascular and myocardial remodeling in heart failure is effectively slowed by therapy with neurohormonal antagonists, including angiotensin-converting enzyme inhibitors, angiotensin-receptor blockers, aldosterone antagonists, and adrenergic-receptor blockers. These therapies, along with the correction of hemodynamic abnormalities, have dramatically reduced morbidity and mortality in patients with heart failure. Endothelial dysfunction, increased oxidative stress, and decreased bioavailability of nitric oxide (NO) also occur in heart failure. Data suggest that endothelial dysfunction and reduced NO bioavailability may be more prevalent in populations who self-identify as African Americans. Thus, differences observed in the African American population with respect to prevalence of heart failure, etiology, outcomes, and response to medication may in part be explained by differences in the relative contributions of neurohormonal activation and diminished NO bioavailability to the progression of heart failure. The African American Heart Failure Trial (A-HeFT) was designed to assess the benefit of fixed-dose combination isosorbide dinitrate–hydralazine (ISDN-HYD) in an African American population with advanced heart failure. The A-HeFT enrolled 1,050 African American patients with New York Heart Association (NYHA) class III–IV heart failure with dilated ventricles and low ejection fractions. Patients were randomized to receive either a fixed-dose combination of ISDN-HYD or placebo added to standard neurohormonal blockade. The primary end point was a composite score in which mortality, hospitalization, and quality of life were weighted. On July 19, 2004, the independent Data Safety Monitoring Committee recommended early termination of the trial because of a significant mortality benefit in the cohort receiving fixed-dose ISDN-HYD. The A-HeFT confirms the benefit of fixed-dose ISDN-HYD, which may enhance NO bioavailability in African American patients with NYHA class III–IV heart failure and suggests that NO-enhancing therapy is an effective new treatment strategy for heart failure. In addition, the A-HeFT affirms the critical importance of the inclusion of population subgroups in clinical trials both as a way to probe for pathophysiologic mechanisms of disease and to devise optimal treatment strategies. The rich and unique A-HeFT database will provide new opportunities to understand the pathophysiology and management of heart failure. © 2005 Elsevier Inc. All rights reserved. (Am J Cardiol 2005;96[suppl]:44i–48i)
ferences between self-identified African American populations and European American majority populations.

Decreased responses to stimuli of forearm vasodilation, a NO-mediated effect, have been noted in African American subjects compared with white subjects. Importantly, these responses have been observed in both healthy and hypertensive African American subjects. Furthermore, oxidant stress, which can result in inactivation of NO, may be higher at baseline in African Americans, who also have a higher prevalence of risk factors associated with higher oxidant stress, such as diabetes mellitus, renal insufficiency, hypertension, and obesity.

In addition to differences in NO-mediated processes, African Americans with hypertension have been found to demonstrate less responsive renin-angiotensin and sympathetic nervous system activation. Correspondingly, hypertension trials have revealed a decreased response among African Americans to angiotensin-converting enzyme (ACE) inhibitors and β-blockers when these agents are used as antihypertensive monotherapy. In this context, retrospective analyses by ethnicity of earlier heart failure trials were performed and demonstrated a decreased response to ACE inhibitors in African Americans compared with non–African Americans. Heart failure outcomes in African Americans treated with β-blockers have been mixed, with no ethnic differences in response to carvedilol but significantly decreased response to bucindolol.

Importantly, these analyses revealed a significantly improved mortality benefit in African Americans receiving combination isosorbide dinitrate–hydralazine (ISDN-HYD), which may enhance bioavailability of NO.

Nitric Oxide–Enhancing Therapy in Heart Failure:
A Novel Strategy

Background and rationale: Organic nitrates and HYD have been used as vasodilators for many years; however, recent data have shown a synergistic action between the 2 agents, which may result in enhancement of NO bioavailability. The landmark Vasodilator-Heart Failure Trial (V-HeFT I) was the first to demonstrate the benefits of this combination in heart failure. In V-HeFT I, reduction in blood pressure was shown to be greater in the treatment group receiving prazosin, an α-blocking vasodilator, than in either the combined ISDN-HYD or placebo group. However, no mortality benefit was conferred by the prazosin treatment arm, which suggests that the mortality benefit found in the ISDN-HYD arm could be attributed to mechanisms other than reduction in blood pressure. Subgroup reanalysis by ethnicity of V-HeFT I and V-HeFT II demonstrated that African American patients responded particularly well to ISDN-HYD, with significantly improved mortality. In contrast to white subjects, no survival advantage was demonstrated in African American subjects when treatment with enalapril was compared with ISDN-HYD. Although the findings of these retrospective analyses of relatively small patient cohorts were provocative and hypothesized-generating, a prospective confirmatory clinical trial of the efficacy of ISDN-HYD was required. As a result, the African American Heart Failure Trial (A-HeFT) was based on the following construct: myocardial dysfunction results in neurohormonal activation and diminished NO bioavailability leading to myocardial remodeling, and the relative contribution of impaired NO bioavailability and neurohormonal blockade may vary by ethnicity. Thus, NO enhancement as a treatment strategy in heart failure can improve heart failure outcomes, particularly in African Americans.

Trial design and outcomes: The A-HeFT was a randomized, placebo-controlled, double-blind study designed to evaluate the efficacy of fixed-dose ISDN-HYD added to standard background neurohormonal blockade to improve outcomes in African American patients with moderate-to-severe symptomatic heart failure. In all, 1,050 patients classified as having New York Heart Association (NYHA) class III–IV heart failure with dilated ventricles and low ejection fractions were recruited at 161 centers in the United States and randomized to either placebo or fixed-dose ISDN-HYD, added to standard recommended neurohormonal blockade. Patients were followed for ≥6 months of observation and for a maximum of 18 months of follow-up. The dosing schedule for the active drug was initially HYD 112.5 mg and ISDN 60 mg administered in 3 divided doses. This dose was titrated to HYD 225 mg and ISDN 120 mg in 3 daily divided doses based on the absence of adverse effects as judged by the patient’s physician. Inclusion and exclusion criteria are listed in Table 1. The primary outcome of this trial was a unique composite score weighting death from any cause, hospitalization for heart failure, and change in quality of life at 6 months. The composite scoring system is shown in Table 2. Missing data received the worst-case score for that component of the composite end point. The Minnesota Living with Heart Failure questionnaire was used to evaluate quality of life. This is a 21-item, self-administered questionnaire that serves as a measure of the patients’ perceptions of how heart failure affects their psychologic and physical lives. A scale of 0 to 105 points is used to quantify the extent of impairment and how it is affected by therapeutic intervention; lower scores correspond to better quality of life. Secondary outcomes included the 3 components of the primary composite score in addition to overall quality of life throughout the trial, total number of hospitalizations for heart failure, total number of hospitalizations for any reason, total number of days spent in the hospital, number of unscheduled emergency room and office or clinic visits for heart failure, change in β-type natriuretic peptide levels, newly established need for cardiac transplantation, death from any cause, and changes in myocardial remodeling at 6 months.
Results

The study was initiated in June 2001 and prematurely terminated in July 2004 upon recommendation of the Data Safety Monitoring Committee as a result of the significantly improved survival in the ISDN-HYD group. At the time of trial cessation, mortality in the fixed-dose ISDN-HYD group was 6.2% compared with 10.2% in the placebo group \( (p = 0.02) \). Survival was increased by 43% in the active treatment arm (hazard ratio 0.57; \( p = 0.02 \)). The significant difference in survival became apparent at approximately 180 days and widened thereafter (Figure 1). The primary composite score and all individual components of the primary composite score were significantly and positively impacted by treatment with ISDN-HYD (Table 3). All patients were recalled and offered fixed-dose ISDN-HYD.

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**Table 1**

Inclusion and exclusion criteria for the African American Heart Failure Trial (A-HeFT)

<table>
<thead>
<tr>
<th>Inclusion</th>
<th>Exclusion</th>
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</thead>
<tbody>
<tr>
<td>• Age ≥18 yr</td>
<td>• Pregnant, nursing, or women of childbearing age and not using an effective method of contraception</td>
</tr>
<tr>
<td>• Self-identified as African American</td>
<td>• Acute myocardial infarction, acute coronary syndrome, or stroke within the preceding 3 mo</td>
</tr>
<tr>
<td>• NYHA class III–IV heart failure for ≥3 mo</td>
<td>• Occurrence of likelihood of cardiac surgery or percutaneous coronary intervention during or within the preceding 3 mo</td>
</tr>
<tr>
<td>• Resting LVEF ≤0.35 or LVEF &lt;0.45 with LVIDD ≥2.9 cm/m² or 6.5 cm (by ECHO)</td>
<td>• A history of cardiac arrest or life-threatening arrhythmias within the preceding 3 mo (unless treated with an implantable defibrillator)</td>
</tr>
<tr>
<td>• Receiving standard therapy for heart failure, as determined to be appropriate by physician. Stratified according β-blocker use for ≥3 mo before randomization and nonuse</td>
<td>• Clinically significant valvular heart disease, hypertrophic or restrictive cardiomyopathy, active myocarditis, or uncontrolled hypertension</td>
</tr>
<tr>
<td></td>
<td>• Treatment with parenteral inotropic agents ≥1 mo before randomization</td>
</tr>
<tr>
<td></td>
<td>• Potential need for cardiac transplantation during trial period</td>
</tr>
<tr>
<td></td>
<td>• Symptomatic hypotension</td>
</tr>
<tr>
<td></td>
<td>• Presence of an illness likely to result in death within the study period</td>
</tr>
<tr>
<td></td>
<td>• Inability to complete the quality-of-life questionnaire</td>
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<tr>
<td></td>
<td>• Contraindications to nitrates or hydralazine therapy</td>
</tr>
</tbody>
</table>

ECHO = echocardiogram; LVEF = left ventricular ejection fraction; LVIDD = left ventricular internal end-diastolic diameter; NYHA = New York Heart Association.

Adapted from *J Card Fail* and *N Engl J Med*.

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**Table 2**

Scoring system for the primary composite score end point of the African American Heart Failure Trial (A-HeFT)*

<table>
<thead>
<tr>
<th>End Point</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Death (at any time during the trial)</td>
<td>-3</td>
</tr>
<tr>
<td>Survival to end of trial</td>
<td>0</td>
</tr>
<tr>
<td>First hospitalization for heart failure (adjudicated)</td>
<td>-1</td>
</tr>
<tr>
<td>No hospitalization</td>
<td>0</td>
</tr>
<tr>
<td>Change in quality of life at 6 mo (or at last measurement if &lt;6 mo)</td>
<td></td>
</tr>
<tr>
<td>Improvement by ≥10 U</td>
<td>+2</td>
</tr>
<tr>
<td>Improvement by 5–9 U</td>
<td>+1</td>
</tr>
<tr>
<td>Change by &lt;5 U</td>
<td>0</td>
</tr>
<tr>
<td>Worsening by 5–9 U</td>
<td>-1</td>
</tr>
<tr>
<td>Worsening by ≥10 U</td>
<td>-2</td>
</tr>
<tr>
<td>Possible score</td>
<td>-6 to +2</td>
</tr>
</tbody>
</table>

* Reprinted with permission from *J Card Fail*. 
The African American Heart Failure Trial: Lessons, Opportunities, and Challenges

The new clinical insights emerging from the A-HeFT present both opportunities and challenges. The trial demonstrates that fixed-dose ISDN-HYD added to standard neurohormonal blockade significantly improved survival, decreased heart failure hospitalization rates, and improved quality of life in African American patients with advanced heart failure. This suggests that there are additional mechanisms of heart failure progression, perhaps decreased NO bioavailability not treated by standard neurohormonal blockade, which are favorably impacted by combined ISDN-HYD. Thus, NO-enhancing therapy represents a novel approach to slow the progression of heart failure. There is also opportunity for the potential application of NO-enhancing therapy to other populations with heart failure or other cardiovascular diseases. In addition, the A-HeFT provides resounding affirmation for the critical importance of including adequate numbers of diverse populations in clinical trials. In the absence of population inclusivity, important mechanistic data contributing to the understanding of disease pathophysiology and treatment may remain unrecognized. Clinical trial results based on average effects in large clinical trials with only small subgroup representation may obscure therapeutic efficacy in some subgroups and the absence of efficacy in others. A challenge will be to define the criteria for choosing population subgroups for inclusion in clinical trials. At some point, genetic, metabolic, or biomarker profiles may provide better population definition than race/ethnicity, which is a complex melange of population biology and population-based social factors. The A-HeFT has a rich and unique database, which will allow investigators to probe metabolic and genetic correlates of therapeutic response, effects of sex, and interactions with other heart failure medications.
Lastly, the A-HeFT provides evidence for the use of composite end point scoring systems, which may allow shorter trials with smaller cohorts to probe the efficacy of new therapeutic strategies. A challenge in the use of such scores is the choice of optimal parameters, which will most sensitively measure trial outcomes.


