Basic nutritional investigation

Cinnamon polyphenols regulate S100β, sirtuins, and neuroactive proteins in rat C6 glioma cells

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ABSTRACT

Objective: Increasing evidence suggests that cinnamon has many health benefits when used in herbal medicine and as a dietary ingredient. The aim of this study was to investigate the effects of an aqueous extract of cinnamon, high in type A polyphenols, on molecular targets in rat C6 glioma cells that underlie their protective effects.

Methods: C6 rat glioma cells were seeded in 35-mm culture dishes or six-well plates, then were incubated with cinnamon polyphenols at doses of 10 and 20 μg/mL for 24 h. The targeting protein expression, secretion, and phosphorylation were evaluated by immunoprecipitation/immunoblotting and immunofluorescence imaging.

Results: Cinnamon polyphenols significantly enhanced secretion of S100β, a Ca2+-binding protein, and increased intracellular S100β expression after 24 h of incubation, in rat C6 glioma cells. Cinnamon polyphenols also enhanced protein levels of sirtuin 1, 2, and 3, deacetylases important in cell survival, and the tumor suppressor protein, p53, and inhibited the inflammatory factors, tumor necrosis factor alpha, and phospho-p65, a subunit of nuclear factor-κB. Cinnamon polyphenols also up-regulated levels of phospho-p38, extracellular signal-regulated protein and mitogen-activated protein and kinase-activated protein kinases that may be important for prosurvival functions.

Conclusion: Our results indicate that the effects of cinnamon polyphenols on upregulating prosurvival proteins, activating mitogen-activated protein kinase pathways, and decreasing proinflammatory cytokines may contribute to their neuroprotective effects.

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Introduction

Plant-derived natural polyphenols represent a wide variety of compounds that are enriched in fruits, vegetables, wine, tea, extra virgin olive oil, chocolate, and other plant products. Accumulating evidence suggests that cinnamon has many health benefits when used both in herbal medicine and as a spice [1]. We have isolated and characterized a polyphenol-rich water-soluble extract from cinnamon polyphenols (CP), that is high in doubly-linked polyphenol type-A polymers [2,3] that has insulin-potentiating [2], antioxidant [4], and anti-inflammatory properties [5,6]. In human studies, water-soluble CP have been shown to reduce fasting blood sugar levels in prediabetic patients with metabolic syndrome [7], improve antioxidant status, decrease risk factors associated with diabetes and cardiovascular diseases [4], and improve insulin sensitivity in women with polycystic ovary syndrome [8]. Metabolic syndrome is associated with insulin resistance, elevated glucose and lipids, inflammation, decreased antioxidant activity, increased weight gain, and increased glycation of proteins. CP have been shown to improve all of these variables in in vitro, animal, and/or human studies [1].

CP also have been reported to exert neuroprotective effects, such as inhibiting tau aggregation associated with Alzheimer’s disease [9], reducing cell swelling associated with ischemic injury [10], attenuating mitochondrial dysfunction in ischemic injury [10,11], and improving glutamate uptake following ischemia-like injury in vitro [11]. Moreover, an aqueous extract of cinnamon has been shown to inhibit Aβ oligomer formation and ameliorate Alzheimer’s disease symptoms [12]. Although polyphenols have been reported to exert neuroprotective actions against injury induced by neurotoxins, and display the ability to suppress neuroinflammation [13], it is not clear whether such...
polyphenols exert protective effects by modulating the functions of other prosurvival proteins, including S100β, sirtuins, Bcl-xL, as well as intracellular signaling pathways mediated by mitogen-activated protein kinases (MAPKs), and nuclear factor-κB (NF-κB).

S100β, is a calcium-binding protein primarily expressed in the central nervous system (CNS), and synthesized and secreted by astrocytes [14]. Increasing evidence suggests that S100β plays a double function as an intracellular regulator and an extracellular signal of the CNS. S100β is found in the cytoplasm in a soluble form and also is associated with intracellular membranes, centrosomes, microtubules, and type III intermediate filaments [15]. Within cells, it is involved in the regulation of cell proliferation, differentiation, shape, Ca2+ homeostasis, protein phosphorylation, transcription, enzyme activity, and metabolism [14]. When secreted into the extracellular medium, S100β exerts regulatory effects on neighboring cells including astrocytes, neurons, and microglia. At nanomolar levels, S100β has neurotrophic activity in the extracellular medium, but at micromolar levels it stimulates apoptosis [16,17]. The mechanisms of regulating S100β secretion are not completely understood and appear to be related to many factors [14], such as the proinflammatory cytokines, tumor necrosis factor alpha (TNF-α) [18], interleukin (IL)-1β [19], and metabolic stress [20]. Moreover, a previous study suggested that S100β secretion involves the MAPK pathway and apparently could involve NF-κB signaling [19].

A Sirts activator, resveratrol, has been shown to increase glutamate uptake and S100β secretion in cortical astrocyte cultures [21]. It also has a protective effect against acute H2O2-induced oxidative stress in astrocyte cultures, enhancing the impaired S100β secretion [22]. Sirtuins (Sirt), a family of nicotinamide adenine nucleotide (NAD)-dependent deacetylases, are implicated in energy metabolism and lifespan. Sirtuins are novel targets for Alzheimer’s disease and other neurodegenerative disorders. Sirt1 has been found to protect cell DNA damage and reduce apoptotic death in vitro [23]. Sirt2 activity plays a key role in maintaining the survival of glioma cells [24], and Sirt3 acts as a survival factor playing an essential role to protect neurons under excitotoxic injury [25].

The C6 glioma cell line exhibits many astrocytic features, and has been used widely to characterize the mechanisms of an astrocytic model system, such as the study of neurotrophic actions [26], nerve growth factors [27], and insulin-like growth factors [28]. Here, we investigated the in vitro effects of CP on the intracellular expression and the extracellular secretion of S100β in rat C6 glioma cultures. We also evaluated whether CP regulates Sirtuin 1, 2, and 3 expression. Additionally, the anti-apoptotic protein, Bcl-xL, inflammatory factor, TNF-α, transcription factor, phospho-NF-κB (p-NF-κB) p65, MAPK phospho-p38, extracellular signal-regulated protein (ERK) and mitogen-activated protein and kinase-activated protein (MAPKAPK2) kinases also were investigated.

Materials and methods

Cinnamon polyphenols

The aqueous extract of cinnamon contained 0.74% of a type A tetramer, cinnamitan A, with a molecular weight of 1152 and 2.30% of cinnamantan D1, molecular weight 864, 0.72% of cinnamantan B1, and 0.22 and 0.12% of two type A trimer isomers, whose exact structure is not known, molecular weight 864. The total percent of the type A polymers that were quantitated was 4.03%. Type A polymers were identified using electrospray ionization mass spectrometry analyses using an LCQ ion trap instrument (ThermoElectron Co., San Jose, CA) and high-performance liquid chromatography. The dried CP, Cinnulin PF®, was provided by Integrity Nutraceuticals International (Spring Hill, TN, USA) and also can be prepared as described [2].

Cell culture

C6 glioma cells (CCL-107) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cell cultures were grown in F-12 K medium (Gibco/Invitrogen) supplemented with 10% horse serum and 2% fetal bovine serum and maintained at 37°C with 5% CO2/95% air. Cultures were grown to 85% confluence in 75-mm flasks and, after trypsinization, were seeded in 35-mm culture dishes or six-well plates (0.25 × 10⁶ cells/well) and allowed to confluence during the experimental period. All cultures used in the experiment were between passages 22 and 32.

Extracellular S100β secretion of rat C6 glioma cells and Western blotting analysis

C6 glioma cells were incubated with CP (10 and 20 μg/mL) in the fresh medium, at 37°C. The concentration of the doses of CP was based on our previous studies showing significant effects of CP in primary enterocytes [5], macrophages [6], and C6 gliomas [10]. After 24 h, the cultures were harvested for immunoprecipitation using rabbit anti-S100β immunoglobulin G and Dynabeads® protein A (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s directions. The eluted samples were prepared for Western blotting (a Nikon TE2000-S microscope, Nikon, Tokyo, Japan). Fluorescence intensities from images of five randomly selected microscopic fields of cells were semiquantitatively analyzed by densitometry (Image) software, NIH Image.

Extraction of cell nuclear protein and analysis of phospho-p65, subunit of NF-κB

Following 24-h treatment with CP, the cells were harvested by scraping and washed with cold PBS. The nuclear fractions were isolated using NE-PER® nuclear and cytoplasm extraction reagents (NER and CER) according to the manufacturers’ instructions (Pierce, Rockford, IL). Briefly, the cell suspensions were centrifuged, CER I solution was added to cell pellets and vortexed, and then CER II solution was added and vortexed. After centrifugation, the supernatant cytoplasmic extracts were removed. Nuclear pellets were suspended in 50 μl of NER solution, vortexed every 10 min, incubated in an ice bath for 40 min, and the supernatant containing nuclear extracts collected. The nuclear extracts were analyzed by Western blotting using antibody against p-NF-κB p65.

Statistical analyses

Data were analyzed by one-way analysis of variance followed by post hoc analysis of between groups, according to the least significant difference (LSD) test. P-values < 0.05 were significant for all analyses.

Results

CP enhanced the intracellular expression and the extracellular secretion of S100β in C6 cells

To investigate whether CP increases S100β secretion in C6 cells, extracellular S100β content was measured after exposure to CP for 24 h by immunoprecipitation and subsequent immunoblotting (Fig. 1). After CP incubation, a significant increase in S100β secretion was observed with both concentrations of CP (147% and 243% of controls; P < 0.05, respectively). Increased secretion of S100β also was observed after 4 h of CP treatment (data not shown). Furthermore, intracellular S100β expression
was significantly increased by 4 h (Fig. 2A, B) and 24 h (Fig. 2C, D). Both concentrations of CP (10 and 20 \( \mu \)g/mL) increased the number of S100B-immunopositive cells compared with non-treated control cultures (4-h incubation: 136% and 147% of controls; 24-h incubation: 223% and 200% of controls; \( P < 0.05 \), respectively).

**CP increased sirtuin 1, 2, and 3 expression in C6 cells**

Because sirtuins 1 to 3 have been linked with neuroprotective effects [2,24], Sirt1 to 3 protein levels were measured by immuno-fluorescence in C6 glial cells. Following 24-h treatment, both CP dosages (10 and 20 \( \mu \)g/mL) significantly increased Sirt1 (145% and 297% of controls), Sirt2 (155% and 165% of controls), and Sirt3 (194% and 180% of controls) immunoreactive cells compared with control cultures (Fig. 3; \( P < 0.05 \), respectively).

**CP inhibited TNF-\( \alpha \) protein levels and phospho-p65 in C6 cells**

To assess the antiinflammatory effects of CP, we measured proteins that are important in inflammatory processes, including TNF-\( \alpha \) and NF-\( \kappa \)B. When compared with controls, there was a significant decrease in TNF-\( \alpha \) protein levels in cultures treated with CP (10 and 20 \( \mu \)g/mL) for 24 h (Fig. 4A). The transcription factor, NF-\( \kappa \)B, is a key regulator of inflammation and cell survival. NF-\( \kappa \)B activation increases following cerebral ischemia. As shown in Figure 4B, there was a significant decrease in p-NF-\( \kappa \)B p65 levels in the nuclear fractions upon treatment with CP for 24 h.

**CP inhibited Bcl-xl and induced p53 expressions in C6 cells**

We then examined the effects of CP on Bcl-xl, an antiapoptotic protein of the Bcl-2 family, and p53, a tumor suppressor protein. Cultures were treated with CP (10 and 20 \( \mu \)g/mL) for 24 h. CP treatment significantly decreased Bcl-xl protein levels measured by immunofluorescence (Fig. 5A, B: 50% and 73% of controls; \( P < 0.05 \), respectively) and immunoblot (Fig. 5C). CP increased p53-immunoreactive cells (Fig. 5D: 152% and 174% of controls; \( P < 0.05 \), respectively).

**CP increased phosphorylation of p38, ERK and MAPKAPK2 in C6 cells**

The activation of MAPK has been linked to neuroprotection in various cellular model systems [31,32]. Cultures were treated with CP (10 and 20 \( \mu \)g/mL) for 24 h. As shown in Figure 6, CP enhanced phosphor-p38 and phosphor-ERK levels by Western blot analysis; both doses of CP also stimulated phospho-MAPKAPK2 levels by immunofluorescence (157% and 155% of controls; \( P < 0.05 \), respectively).

**Discussion**

CP have been reported previously to exert neuroprotective effects [9–11,33]. This study adds to mounting evidence that CP increases intracellular S100B expression and extracellular S100B secretion in C6 glial cultures. Additionally, CP also increased the levels of sirtuins, phosphorylation of MAPK, and decreased levels of proinflammatory proteins.

S100B is involved in the regulation of cell proliferation, differentiation, shape, \( \mathrm{Ca}^{2+} \) homeostasis, protein phosphorylation, transcription, enzyme activity, and metabolism [14]. S100B has been also implicated in glia–neuronal communication [34]. At nanomolar levels, extracellular S100B may protect hippocampal neurons against glutamate-induced damage [35]. Antibody anti-S100B addition decreased glutamate uptake without affecting cell integrity or viability. Low levels of S100B stimulate glutamate uptake [36]. In previous studies, CP were shown to attenuate cell swelling and mitochondrial dysfunction following ischemia-like injury and to increase glutamate uptake in glial cultures [10,11]. It is possible that CP-induced S100B secretion, may be associated with increased glutamate uptake [10,11,36,37]. Consistent with this, resveratrol, a plant-derived polyphenol with multiple biological effects including antioxidant and antiinflammatory properties, has been shown to be neuroprotective by increasing glutamate uptake, and it also stimulates S100B secretion. All of this contributes to functional recovery after brain injury [38,39].

Sirtuins are neuroprotective in various disease models and have multiple beneficial activities on metabolic syndrome-related diseases, such as protecting cell DNA damage and reducing apoptotic death [23], maintaining the survival of glia cells [24], and protecting neurons under excitotoxic injury [25]. In this study, our data indicate that CP treatment enhances Sirt1, 2, and 3 protein expressions in C6 glial cells (Fig. 3). Resveratrol, which has neuroprotective effects, also has been identified as a potent pharmacologic agonist of sirtuin activity [40]. An increase in sirtuins in the present study supports the hypothesis that one mechanism by which CP exert their neuroprotective effects is by increasing sirtuin levels. It is also possible that sirtuins are involved in other beneficial effects of CP, including antioxidant [4,41], insulin-potentiating [2,29,42], and antiinflammatory effects [6], as well as improving lipid metabolism [5,43].

At micromolar levels, S100B can induce neuronal apoptosis, mediated by the induction of proinflammatory cytokines, such as IL-1B, TNF-\( \alpha \), and inflammatory stress-related enzymes, such as inducible nitric oxide synthase [44]. There is a crosstalk between proinflammatory cytokines and S100B. Previous studies have reported an increase in cellular S100B in C6 glioma cells after...
24 h of exposure to IL-1β [45], but no change in astrocyte cultures after 48 h [46]. S100β secretion in brain tissue is stimulated rapidly and persistently (at least for 24 h) by intracerebroventricular administration of lipopolysaccharide (LPS) [47]. TNF-α, a potent proinflammatory cytokine with a major role in initiating a cascade of activation of other cytokines and growth factors in inflammatory responses, which is synthesized by microglia, astrocytes, and some populations of neurons, has several important functions in the CNS, including astrocyte activation, glutamatergic transmission, and synaptic plasticity [48]. It has been proposed that TNF-α is able to mediate S100β secretion in astrocytes and decrease S100β secretion in cultured astrocytes after 3 d of exposure to TNF-α [18]. Our data indicate that CP inhibit cellular TNF-α protein expression (Fig. 4), that may be associated with S100β expression and secretion. Our previous studies have shown that CP attenuated TNF-α–induced intestinal lipoprotein apoB48 overproduction by regulating inflammatory, insulin, and lipoprotein pathways [5], and increased antiinflammatory protein expression [6].

NF-κB, a transcription factor, is a downstream signal of the TNF-α pathway and a ubiquitous transcription factor in virtually all cell types; it plays a pivotal role in inflammatory and immune responses [49]. Moreover, the activation of NF-κB is required for the neuroprotective and potentially neuroregenerative effects of S100β. A previous study suggested that very low concentrations of S100β significantly protect primary rat hippocampal neurons against N-methyl-D-aspartate (NMDA) toxicity by activation of transcription factors of the Rel/NF-κB family [50]. NF-κB also partially regulates the expression of Bcl-xl [51], which is an antiapoptotic member of the Bcl-2 family. In the current study, our data suggest that CP decreased Bcl-xl protein levels (Fig. 5) and the p-NF-κB p65 expression (Fig. 4). Cinnamon extract has
Fig. 3. CP stimulated Sirt1, 2, and 3 protein expressions in C6 glioma cells. Cultures were treated for 24 h with PBS or CP (10 or 20 μg/mL). The cells were then fixed, permeabilized, and stained as described in Materials and methods. In the left panels of A, C, and E, the cells were stained with anti-Sirt1 primary antibody (Abcam Inc, Cambridge MA), or anti-Sirt2 antibody or anti-Sirt3 antibody and AlexaFluor 488-conjugated normal mouse IgG (green) or AlexaFluor 594-conjugated normal rabbit IgG (red) secondary antibodies; in the middle panels of A, C, and E, DAPI staining (blue) identifies nuclei; the right: merged images correspond to the overlay of the anti-Sirt1–3 and DAPI staining. A, C, and E: Representative immunofluorescence images for Sirt1–3; B, D, and F: Fluorescence intensities from images were measured and values are mean ± SE of two separate experiments performed in duplicate. *versus control, P < 0.05.

Fig. 4. CP inhibited TNF-α and phospho-p65 expressions in C6 glioma cells. The immunoblots show decreased TNF-α (A) and phospho-NF-κB p65 (B) expression in 24-h CP-treated C6 cells; the blots were reprobed with anti-GAPDH or anti-Lamin a/c as the loading control; the immunoblots shown are representative of three independent experiments.
been shown to strongly inhibit tumor cell proliferation and decrease NF-κB activity and a target gene, Bcl-xl, in vitro and also in vivo in a mouse melanoma model [52]. Epigallocatechin-3-gallate also has been shown to decrease the expressions of Bcl-2 and Bcl-xl by inhibition of NF-κB in human hepatocellular carcinoma [53]. The antiapoptotic protein Bcl-xl has been implicated in both cancer cell and normal cell survival [54]. Recently, S100β has been shown to bind directly to the p53 protein [55], and enhance [56] or downregulate [55] p53 expression. The p53 protein, a non-histone substrate of Sirt1, also activates the transcription of proapoptotic factors, as well as suppresses the transcription of antiapoptotic genes such as Bcl-xl [57]. There are protein–protein interactions between S100β and p53, which are crucial for the proper function of cells. The S100β–p53 interactions are regulated by post-translational modifications [58]. Moreover, the effects of S100 proteins on p53 may be different in various cell types particularly, because S100 proteins themselves are cell-specifically distributed [59]. In this study, our data indicate that CP also increased p53 protein levels (Fig. 5), which are associated with improved S100β secretion and expression.

MAPK pathways constitute a large modular network that regulates a variety of physiological processes, such as cell growth, differentiation, and apoptotic cell death. The activation of MAPK cascades has been linked to neuroprotection in various cellular model systems [31,32], and the phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival [60]. In response to cytokines, stress, and chemotactic factors, MAPKAPK2 is rapidly phosphorylated and activated. MAPKAPK2 also is activated in vitro by the p42/p44MAPK [61]. It has been shown that MAPKAPK2 is a direct target of p38 MAPK [61]. A previous study has shown that very low concentrations of S100β protect neurons against NMDA toxicity, and that S100β-mediated neuroprotection might involve the MAPK pathway [50]. We have reported previously that CP induces phosphor-p38, ERK, and Jun N-terminal kinase

![Image of Fig. 5](image-url)

**Fig. 5.** CP inhibited Bcl-xl and p53 expressions in C6 glioma cells. The experimental conditions described in Figure 2 and in Materials and methods. In the left panels of A, cells were stained with anti-Bcl-xl antibody (Abcam Inc, Cambridge MA), and AlexaFluor® 594-conjugated Normal rabbit IgG (red); In the middle panels of A, DAPI staining (blue) identifies nuclei; the right: merged images correspond to the overlay of the anti-Bcl-xl and DAPI staining. A and D: Representative immunofluorescence images for Bcl-xl and p53; B and E: Fluorescence intensities from images were measured and values are mean ± SE of two separate experiments performed in duplicate; * versus control, P < 0.05.

C: Representative immunoblots show decreased Bcl-xl expression in CP treated C6 cells; the blots were reprobed with anti-GAPDH as a loading control; the immunoblots shown are representative of three independent experiments.
levels in primary enterocytes [62]. In the current study, CP enhanced phospho-p38, ERK, and MAPKAPK2 levels, and these effects were associated with increased S100β secretion and are consistent with the neuroprotective effects of CP.

In summary, in agreement with the protective roles previously demonstrated for CP [10,11], our data show that CP are associated with significant improvements in the intracellular expression and extracellular secretion of S100β in C6 glioma cells. CP also enhanced sirtuins, which have been shown to have therapeutic potential in neurodegenerative diseases such as stroke, ischemic brain injury, Alzheimer’s disease, and Parkinson’s disease [63]. CP also up-regulated multiple prosurvival proteins and increased phosphorylation of MAPK pathways. An interesting new study has suggested that polyphenols from olive tree leaves in primary enterocytes [62]. In the current study, CP enhanced phospho-p38, ERK, and MAPKAPK2 levels, and these effects were associated with increased S100β secretion and are consistent with the neuroprotective effects of CP.

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