In vitro Attenuation of Nitric Oxide Production in C6 Astrocyte Cell Culture by Various Dietary Compounds (44309)

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Abstract. **Excessive nitric oxide (NO) production in the brain has been correlated with neurotoxicity and the pathogenesis of several neurodegenerative diseases. NO production from neuroglial cells surrounding neurons contributes significantly to the pathogenesis of these diseases. The suppression of NO production in these cells may be beneficial in retarding many of these disorders. The present study was designed to evaluate the capacity of dietary-derived polyphenolic compounds, flavonoids, crude extracts, oils, and other food constituents in suppressing the release of NO from lipopolysaccharide (LPS)/gamma-interferon (IFN-γ) stimulated C6 astrocyte cells. In this experiment, 61 compounds were tested, and 36 showed significant suppressive effects of NO production. The results indicate that the following compounds exhibited a dose-dependent suppressive effect of NO production with an IC_{50} less than 10^{-3} *M:* quercetin, (−)-epigallocatechin gallate, morin, curcumin, apigenin, sesamol, chlorogenic acid, fisetin, (+)-taxifolin, (+)-catechin, ellagic acid, and caffeic acid. Compounds, which reduce NO production at less than 300 ppm, include milk thistle, silymarin, grapenol, and green tea. These results demonstrate a possible value for dietary compounds to inhibit the excessive production of NO.** [P.S.E.B.M. 1998, Vol 218]

Many neurological diseases are associated with elevated levels of brain nitric oxide (NO) and inducible nitric oxide synthase (iNOS) mRNA (1). An accumulation of NO in the brain has been correlated to conditions such as brain viral infection (1), Alzheimer's disease (2), multiple sclerosis (3), ischemia, stroke (4, 5) and head injury (6). A large majority of the NO produced in response to neural inflammatory diseases originates from glial cells and hypertrophic astrocytes (7, 8). The neurotoxic properties of NO are primarily due to its ability to bind to heme compounds (9), irreversibly inhibiting cytochrome C oxidase and contributing to the formation of peroxynitrite, which can irreversibly inhibit mitochondrial complexes I–III (10). High levels of NO have been associated with elevated levels of lactate in the brain (11), ADP ribosylation of protein (12), generation of the hydroxyl radical ($OH^{\cdot}$) and nitrogen dioxide free radical ($NO^{2\cdot}$) (13–15)-membrane lipid peroxidation, oxidation of sulfhydryls (14), single strand breaks in DNA, necrosis (16), and inhibition of cellular respiration (17).

Astrocyte NOS is calcium independent, requires L-arginine and NADPH, and is inducible by cytokines or LPS (18). Pharmacotherapy, which reduces damage to the brain after stroke or other inflammatory conditions, may involve the use of NOS inhibitors (5, 9, 19, 20). Therefore, compounds that suppress the NO produced in astrocytes may also be beneficial in retarding neurodegeneration (8).

Many of the compounds tested in the present investigation were chosen due to their known radical trapping ability or inhibitory properties on cellular mechanisms directly associated with glial iNOS induction. These selected compounds have a reported ability to inhibit PKC, tyrosine kinase, cyclic AMP and/or cytokine expression. These specific cellular events are directly involved in iNOS expression and/or NO release (21–23). Therefore, the present investigation was designed to evaluate the suppressive effects of several dietary compounds on NO production in C6 glial cells. These compounds should presumably reduce NO pro-

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This work was supported by grants from the National Institutes of Health (NIH RR 03020 and GM 0811).

Received August 18, 1997. [P.S.E.B.M. 1998, Vol 218]
Accepted April 13, 1998.

0037-9727/98/2184–0390$10.50/0

duction *via* their potent effects on NO regulating cellular mechanisms.

Materials and Methods

Cell Culture. C6 astrocyte cells were cultured according to the instructions provided by the supplier, American Type Culture Collection (Rockville, MD). The cells were cultured in growth medium containing Hams F10 medium (82.5%), horse serum (15%), fetal bovine serum (2.5%), penicillin/streptomycin solution (1%) and glutamine 2 m*M*. Cells were passaged once a week using 0.02% EDTA and 0.25% trypsin, and the media were changed every 2 days. The cells were plated at 7.5×10^5/ml in 96 well plates and incubated for 8–12 hr at 37°C with 5% CO_2/95% O_2 prior to treatment in order to allow for cell adherence. Experimental compounds were dissolved in Hanks balanced salt solution in 2 m*M* HEPES buffer. The stock solution for each treatment was adjusted to a pH of 7.2–7.4 with 1 *N* sodium hydroxide or 1 *N* hydrochloric acid prior to dilution. After the experimental compounds were added to the wells, cells were incubated for an additional 3 hr prior to the addition of the stimulant. The stimulant, consisting of lipopolysaccharide (*E. coli* 0111:B4) 6 μg/ml and INF-γ (100 U/ml) in serum-free medium, was added to the wells and incubated at 37°C at 5% CO_2, 95% air for 24 hr.

Materials. Sulfanilamide, N- (1-napthyl) ethylenediamine dihydrochloride, horse serum, fetal bovine serum, Hams F-10 medium, glutamine, and antibiotics were purchased from Sigma (St. Louis, MO). Shark cartilage was supplied by Nature's Bounty (Bohemia, NY); green tea by Traditional Medicinals (Sebastopol, CA); caffeic acid and taurine, ICN Biomedical Inc. (Irvine, CA); garlic oil, Puritan's Pride (Oakdale, NY); clove oil, Now Natural Foods (Glendale Ht., IL); rosemary leaf, Nature's Answer (Hauppauge, NY); aloe vera juice, Aloe Vera Products Inc. (Kerrville, TX); and milk thistle extract, grape seed extract, Solaray (Ogden, UT). All other supplies were purchased from Sigma. C6 astrocyte cells were purchased from American Type Culture Collection.

Nitric Oxide Determinations. NO was assayed by the Griess reagent (24), which contained 1 part 0.75% sulfanilamide in 0.5 *N* HCl to 1 part .075% N- (1-napthyl) ethylenediamine dihydrochloride in H_2O. The plates were incubated with the Griess Reagent at 25°C under reduced light for 10 min. Absorbance was read at 550 nm using a Packard Multiskan UV spectrophotometer. Protein was determined by the Lowry method (25), and the data were expressed as n*M* of nitrite/mg protein/24 hr. Standards were prepared using dilutions of sodium nitrite in the plating medium. The toxicity of the tested compounds at various dilutions was determined by a (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) toxicity assay (26) and examination of cell morphology using a fluorescence microscope.

Statistical Analysis and Data Handling. A regression analysis was done to determine the linear response curve for each tested compound. Data were collected on six dilutions ranging from 10 m*M* to 10 μ*M* or from 80,000 parts per million (PPM) to 2 PPM ($n = 8$). The IC_{50}s were calculated for each compound exhibiting a dose-dependent reduction in nitrite production. The data were expressed as the R^2 value. A comparison between the treatment groups and controls was reported as the mean ± S.E.M. ($n = 8$). The significance of difference between controls and treatment groups was determined using a one-way ANOVA followed by a Bonferonni *post hoc* test and a Students *t* test.

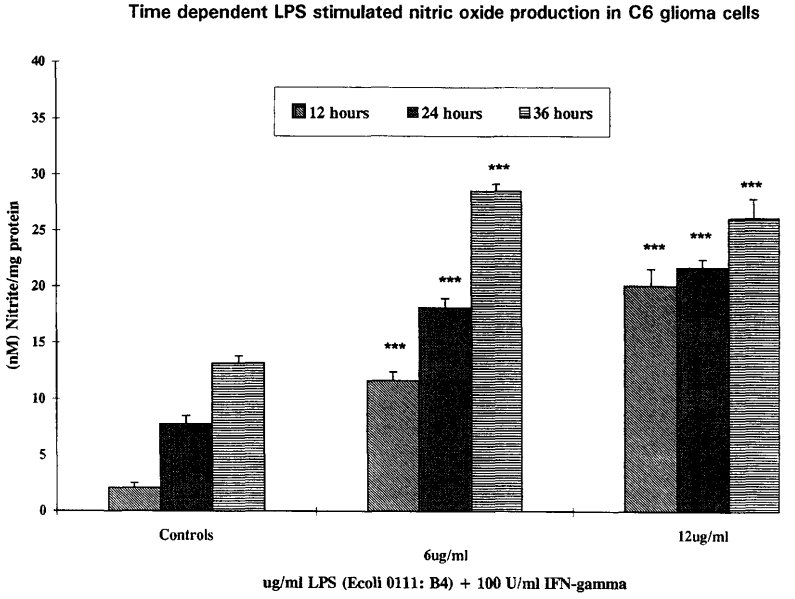

Figure 1. The production of nitric oxide in C6 glioma cells stimulated with lipopolysaccharide from *E. coli* 0111:B4 (6 or 12 μg/ml) + IFN-gamma (100 U/ml) with variation in incubation period at 37°C. The data represent the mean ± S.E.M. ($n = 8$).*** Nitrite production in untreated control was significantly different from the stimulated cells at 12, 24 and 36 hr of incubation. NO levels were significantly lower than the control group ($P < .001$).

Statistical analysis was done using the SPSS statistical package (6.1.3; SPSS Inc., Chicago, IL).

Results

The induction of iNOS in astrocytes was established in the presence of cytokines and lipopolysaccharide isolated from gram-negative bacteria. An experiment was done to determine expected measurable nitrite production with variation in incubation time or concentration of the stimulant (Fig. 1). The controls consisted of cells incubated with serum-free medium in the absence of stimulant. An inherent production of nitrite was detected in controls as would be expected due to basal levels of enzyme activity and L-arginine in the media. Stimulation of C6 cells with LPS at concentrations of either 6 μg/ml or 12 μg/ml with 100 U/ml of IFN-γ was found to produce adequate response.

The cells were incubated for 24 hr in the presence of tested compound in addition to 6 μg/ml LPS plus 100 U IFN-γ. A linear curve was generated for each tested compound effectively producing a dose-dependent reduction in nitrite production. The IC_{50}s were calculated from each curve (Table 1). There was no significant reduction of NO produced after exposure to the following compounds: (+)-limonene, aloe vera juice, rutin-hydrate, allopurinol, β-phenylethylisothiocyanate, melatonin, diosmin, propolis extract, carnitine, caffeine, carvacrol, eugenol, niacinamide, taurine, pentoxifylline, kelp, coenzyme Q_{10}, canola oil, tocopherol, l-deprenyl, azulene, ursolic acid, ferulic acid, ginseng, or green magma extract. There was a significant reduction in NO produced with exposure to quercetin, dexamethasone, (−)-epigallocatechin gallate, N*w*-nitro-l-arginine, morin, curcumin, apigenin, sesamol, chlorogenic acid, fisetin, (+)-taxifolin, (+)-catechin, ellagic acid, genistein, caffeic acid, dipyridamole, hesperetin, thymol, naringin, n-acetyl-cysteine, indomethacin, piperine, hesperidin, myricetin, silymarin, milk thistle extract, grapenol, green tea, shark cartilage, garlic oil, menhaden oil, cod liver oil, rosemary leaves, linseed oil, and clove oil. Commonly consumed compounds in tea and turmeric (curcumin) show impressive suppressive effects, comparable to the range achieved with treatment of the NOS inhibitor N*w*-nitro-L-arginine (Figs. 2–5).

Table I. The Effect of Various Compounds on Nitric Oxide Production in C6 Cells Stimulated with 6 μg/ml LPS + 100 U/ml of IFN-gamma.

Compound	IC_{50}	Unit	R^2
Quercetin	−7.21	Log (M)	0.62
Dexamethasone	−5.01	Log (M)	0.44
EGCG	−4.99	Log (M)	0.94
NW-Nitro-L-arginine	−4.35	Log (M)	0.89
Morin	−4.25	Log (M)	0.80
Curcumin	−4.14	Log (M)	0.96
Apigenin	−3.82	Log (M)	0.79
Sesamol	−3.65	Log (M)	0.93
Chlorogenic acid	−3.53	Log (M)	0.78
Fisetin	−3.52	Log (M)	0.93
(+)-Taxifolin	−3.51	Log (M)	0.82
(+)-Catechin	−3.41	Log (M)	0.90
Ellagic acid	−3.24	Log (M)	0.67
Genistein	−3.23	Log (M)	0.83
Caffeic acid	−3.08	Log (M)	0.71
Dipyridamole	−2.81	Log (M)	0.88
Hesperetin	−2.76	Log (M)	0.94
Thymol	−2.67	Log (M)	0.80
Naringin	−2.25	Log (M)	0.67
N-Acetyl-cysteine	−1.34	Log (M)	0.80
Indomethacin	−1.01	Log (M)	0.68
Piperine	−0.97	Log (M)	0.76
Hesperidin	−0.19	Log (M)	0.80
Myricetin	−0.11	Log (M)	0.76
Milk thistle	25.90	PPM	0.64
Grapenol	50.00	PPM	0.94
Green tea	131.82	PPM	0.74
Silymarin	236.54	PPM	0.97
Shark cartilage	743.74	PPM	0.78
Garlic oil	792.48	PPM	0.75
Menhaden oil	1137.00	PPM	0.76
Cod liver oil	1467.00	PPM	0.30
Rosmary leaf	12116.67	PPM	0.88
Linseed oil	12185.00	PPM	0.71
Clove oil	14515.79	PPM	0.84

Note. The data were expressed as a correlation between concentration of the treatment and nitrite measured for 4–6 concentrations ($n = 8$). The IC_{50}s were calculated using linear regression analysis. PPM: parts per million.

Discussion

Various polyphenolic compounds and dietary oils used in the present study are effective antioxidants and can modify various cellular enzyme systems associated with oxidative stress. However, there is meager data on the use of these compounds against the production of NO, a compound that plays a pivotal role in neurodegenerative diseases. The present study identifies natural compounds, which are effective in suppressing cytokine-stimulated NO production in astrocyte cells. Several of these dietary compounds exhibiting substantial suppressive effects were comparable in strength to other known inhibitors of NO in astrocytes including dexamethasone, N*w*-nitro-L-arginine (NOS inhibitor) and genistein (tyrosine kinase inhibitor).

There are a variety of powerful antioxidants in green tea which include (−)-epicatechin gallate (ECG), (−)-epigallocatechin gallate (EGCG), gallic acid (GA), (−)-epicatechin and (+)-catechin (27, 28). The present study found that green tea was effective in suppressing NO production in astrocytes. It appears that EGCG and (+)-catechin are the major compounds involved in this suppressive effect. Both EGCG and (+)-catechin may offer protection against inflammatory neuronal damage by inhibiting NO production and providing a powerful ability to capture a variety of free radicals. EGCG is a potent antioxidant that can trap either six O_2- or $OH^{\cdot}$ per molecule (29). (+)-Catechin is 100–300 times more potent in its ability to scav-

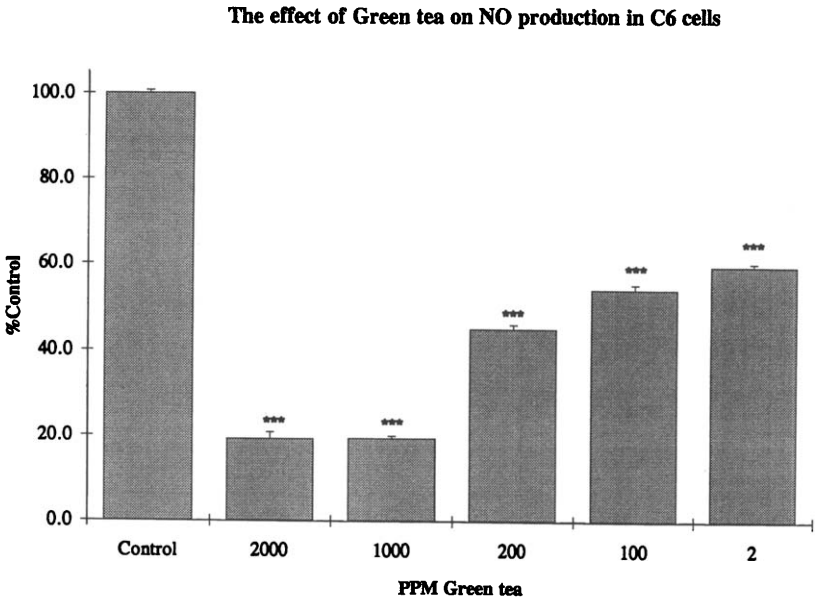

Figure 2. The effect of various concentrations of green tea on nitric oxide production in C6 cells stimulated with 6 μg/ml LPS + 100 U/ml of IFN-gamma. Data were expressed as the mean ± S.E.M. for % Control. Controls were equivalent to 53 ± 1.8 n*M* nitrite/mg protein/24 hr. The significance of difference was determined by a one-way ANOVA followed by a Bonferroni multiple range test and a *t* test for independent samples,*** = $P < .001$. $n = 8$.

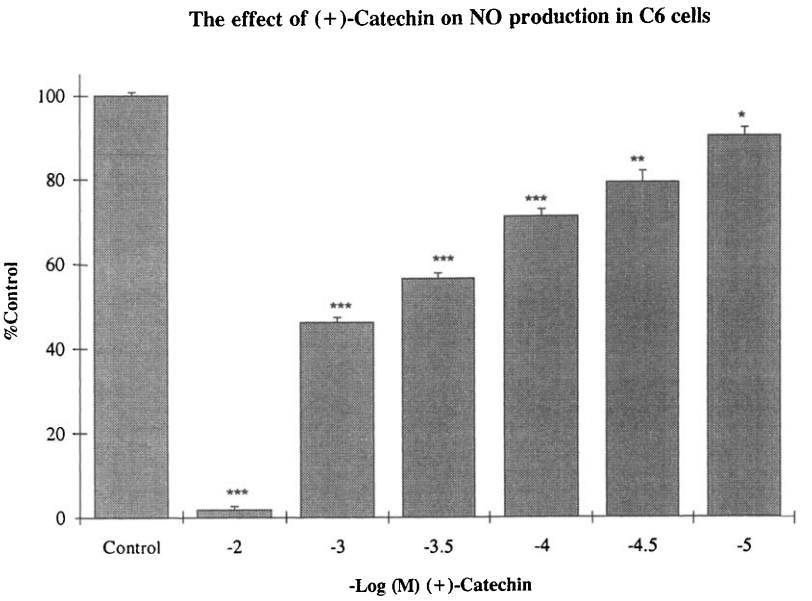

Figure 3. The effect of various concentrations of (+)-catechin on nitric oxide production in C6 cells stimulated with 6 μg/ml LPS + 100 U/ml of IFN-gamma. Data were expressed as the mean ± S.E.M. for % Control. Controls were equivalent to 53 ± 1.8 n*M* nitrite/mg protein/24 hr. The significance of difference was determined by a one-way ANOVA followed by a Bonferroni multiple range test and a *t* test for independent samples * = $P < .05$, ** = $P < .01$, *** = $P < .001$. $n = 8$.

enge Fenton-generated OH radical than mannitol (30). (+)-Catechin has also been reported to provide protection against oxidation of phospholipid liposomes by capturing superoxide radicals and hypochlorous radicals (31). Another indication that catechins may offer protection against brain injury is that they can antagonize a variety of metals that catalyze biological oxidation reactions (32–34). *In vitro* studies show that tea catechins EC and (+)-catechin are protective against oxidative neuronal death in cultured mouse cerebral cells (35) and oxidative cytotoxicity in cultured fibroblasts (36). It is also possible that green tea extract and its components may cross the blood-brain-barrier (BBB). *In vivo* studies show that brain damage from cerebral ischemia and glucose oxidative injection in mice was attenuated by administration of tea catechins (35, 37). Oral administration of a catechin-based antioxidant solution has also proven beneficial in decreasing free radical generation and enhancing free radical scavenging systems after Fe Cl_3 induced damage to the cortex in rats (37). The potential for catechin compounds to downregulate NO in astrocytes most likely involves its ability to downregulate PKC (38). Similarly both EGCG and curcumin have been reported to inhibit peroxynitrite radicals and nitrite produced by LPS/IFN-γ induced mouse peritoneal macrophages by more than 50% (39).

Curcumin bis (4-hydroxy-3-methoxyphenyl)-1,6-heptandiene-3, 5-dione) is a water-soluble, yellow-orange pigment derived from the rhizome of turmeric (*Curcuma longa Linn*). It is used as a coloring agent, a spice, and a medicinal anti-inflammatory agent in India. There are various reasons why this traditional spice component may provide protection against inflammatory disorders. Curcumin

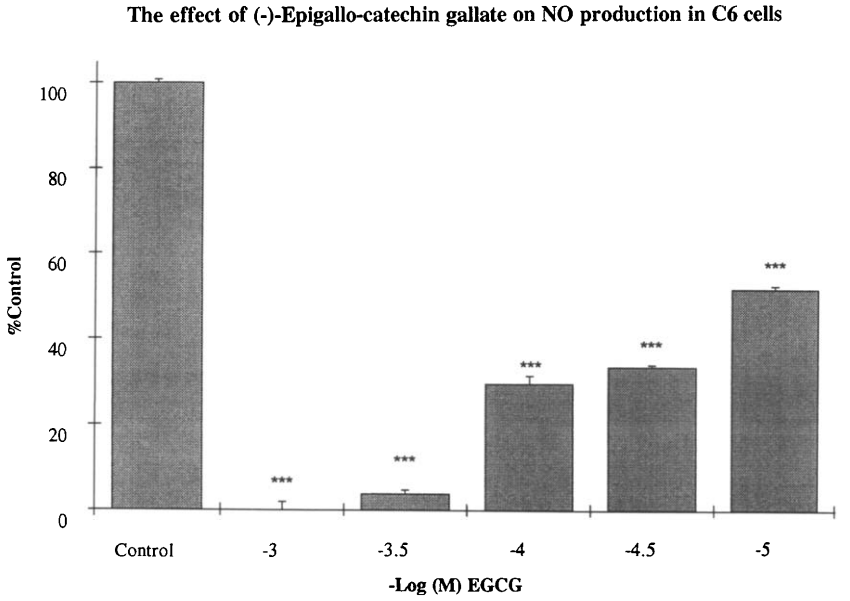

Figure 4. The effect of various concentrations of (–) epigallocatechin-gallate on nitric oxide production in C6 cells stimulated with 6 μg/ml LPS + 100 U/ml of IFN-gamma. Data were expressed as the mean ± S.E.M. for % Control. Controls were equivalent to 53 ± 1.8 n*M* nitrite/mg protein/24 hr. The significance of difference was determined by a one-way ANOVA followed by a Bonferroni multiple range test and a *t* test for independent samples, *** = $P < .001$. $n = 8$.

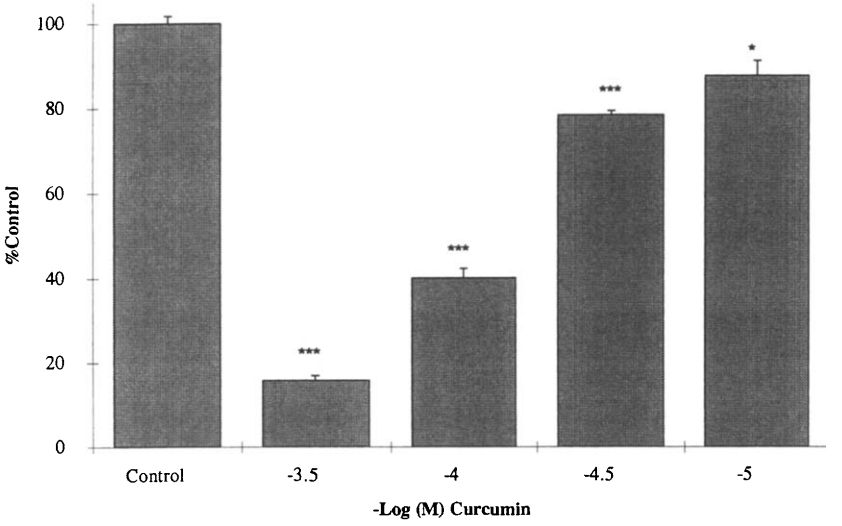

Figure 5. The effect of various concentrations of curcumin on nitric oxide production in C6 cells stimulated with 6 μg/ml LPS + 100 U/ml of IFN-gamma. The data were expressed as the mean ± S.E.M. for % Control. Controls were equivalent to 53 ± 1.8 n*M* nitrite/mg protein/24 hr. The significance of difference was determined by a one-way ANOVA followed by a Bonferroni multiple range test and a *t* test for independent samples, * = $P < .05$, *** = $P < .001$. $n = 8$.

has been found to inhibit lipid peroxidation, arachidonic acid metabolism, PKC activity, and tyrosine kinase activity (40). Curcumin also has potent antioxidant capability (41). It has been found to inhibit lipid peroxidation, superoxide, hydrogen peroxide (42), xanthine-xanthine oxidase superoxide production, and Fe^{+2}-induced generation of hydroxyl radicals (43). Curcumin can completely inhibit nitrite radicals in activated peritoneal macrophage (44). And, *in vivo* studies show that dietary administration of curcumin can lead to the attenuation of free radical production in peritoneal macrophage (45).

Exposure to curcumin can provide protection against lipid peroxidation and ischemic damage in a variety of tissues. Dietary administration of curcumin in rats is effective in minimizing iron-induced hepatic toxicity, serum lipid peroxides, (45) and can prevent necrotic damage to heart tissue after myocardial infarction (46). In the cat, administration of curcumin can prevent ischemia-induced elevation of malonaldehyde production and lactate dehydrogenase activity (47). Another indication that curcumin may provide therapeutic value against neurological injury is that curcumin may chelate iron and reduce peroxidative damage to brain tissue. Curcumin derivatives are more potent than α-tocopherol in inhibiting iron-induced lipid peroxidation in rat brain homogenates (48, 49).

The ability of curcumin to inhibit NO as exhibited in the present study may be based on a number of cellular events. NOS has a similar sequence homology to cytochrome P450. Curcumin has demonstrated a strong affinity for the cytochromes with a capacity to inhibit cytochrome P450 (50, 51). It is also possible that curcumin can suppress NO production by its ability to downregulate the critical

transcription factor NF-kappa β, which directs the expression of iNOS. Curcumin has the ability to inhibit NF-kappa β in human myeloid ML-1a cells treated with tumor necrosis factor; phorbol ester or hydrogen peroxide (52) or LPS activated L929 fibroblast (53). It is also possible that curcumin is acting on second messenger systems involved with NO production. It can inhibit PKC activity and tyrosine kinase activity (40). Not only does curcumin have an ability to inhibit enzyme induction, but it may also antagonize the end product. Curcumin derivatives have been found to inhibit nitrite induced oxidation of hemoglobin due to a reported ability to antagonize nitrogen dioxide (54). Other studies confirm findings similar to the present study including that curcumin can suppress iNOS mRNA and nitrite production in RAW 264.7 macrophages activated with LPS and IFN-γ (55).

Caffeic acid is a phenolic compound derived from hydroxycinnamate acid found in propolis and coffee. Caffeic acid is also isolated from the leaves of Alsophila Spinulosa Hook Tryon, a plant with reputable qualities for aiding in hepatitis, gout, and rheumatism (56). Physiological effects of caffeic acid include its capacity to act as an antioxidant, anti-inflammatory agent, antinocioceptive agent (57), inhibitor of xanthine oxidase (56), metal chelator (58), and a protective agent against hydrogen peroxide induced cytotoxic cell death (59). Given these events, and the capacity to suppress NO in astrocyte cells, perhaps this compound may be beneficial to inflammatory neurological diseases. Studies on caffeic acid and lipid systems indicate a potential for this compound against inflammatory neurodegeneration. Moreover, caffeic acid is effective in attenuating the oxidation of low-density lipoproteins (60, 61) and can inhibit Cu^{2+} catalyzed macrophage LDL oxidation (58).

Taxifolin is a powerful antioxidant, which can inhibit superoxide anion generated by the xanthine oxidase system, microsomal lipid peroxidation, and can protect red blood cells against oxidative damage *in vitro* (62). The present study also confirms its ability to substantially inhibit NO production. Hesperetin was also somewhat effective in inhibiting NO. Hesperitin may provide protective effects against neurological injury, due to its ability to inhibit metal-catalyzed oxidation. Hesperetin is more effective than quercetin and naringenin in its ability to retard Fe^{+2}-induced lipid peroxidation and auto-oxidation of rat cerebral membrane (63). Hesperetin may provide protection against inflammatory conditions due to its inhibitory effects on protein kinase (64). Silymarin is another compound that offers neuroprotective properties. It has been reported to attenuate lipid peroxidation and GSH depletion in hepatocytes (65). The present study also confirmed impressive nitrite-lowering effects for silymarin and milk thistle.

In summary, the present study identified several compounds that are able to reduce NO production dramatically *in vitro* in astrocyte cells. Many of these compounds may provide excellent protection against several neurodegenerative diseases. There is a great deal of evidence to support that some of these compounds, such as catechins, can act centrally and cross the BBB (35, 39); however, the ability of many of these compounds to cross the BBB is unknown. Therefore, there is a need for supportive studies to determine specific mechanisms involved and potential effects on *in vivo* models of neurodegenerative disorders for many of these compounds.

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