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Comparison of Inhibitory Potency of Various Antioxidants on the Activation of BV2 Microglial Cell Lines Induced by LPS

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Antioxidant properties have been proposed as a mechanism for the putative anti-inflammatory effects of phenolic compounds. To reveal the relationship between antioxidant activity and anti-inflammatory effects of various antioxidants, we measured 1, 1-diphenyl-2-picryhydrazyl (DPPH)-reducing activity and examined the inhibitory effects on LPS-induced inflammation-related gene expression in the BV2 microglial cell line. Lipopolysaccharide (LPS) (0.2 μg/ml) was used with or without antioxidants to treat cells, and the regulation of iNOS and cytokine gene expression was monitored using an RNase protection assay (RPA). Although, all tested antioxidants had similar DPPH-reducing activity and inhibited nitrite production, but the curcuminoid antioxidants (ferulic acid, caffeic acid, and curcumin) inhibited LPS-induced gene expression (iNOS, TNF-α, IL-1β, IL-6, and IL-1 Ra) in a concentration-dependent manner. Other tested antioxidants did not exhibit the same effects; N-acetylcysteine (NAC) only began to suppress IL-1β gene expression just below the concentration at which cytotoxicity occurred. Moreover, the antioxidant potency of curcuminoids appeared to have no correlation with anti-inflammatory potency. Only curcumin could inhibit LPS-induced microglial activation at a micromolar level. These data suggest that curcumin may be a safe antioxidant possessing anti-inflammatory activity.

Key Words: Antioxidant, BV2 microglial cells, Nitric oxide

INTRODUCTION

Microglia are considered the resident immune cells of the central nervous system (CNS), and excessive quantities of several factors produced by activated microglia can be deleterious to neurons (Boje & Arora, 1992; Chao et al, 1992; McGuire et al, 2001). When microglia was activated, they undergo morphological and functional transformations. Morphologically, this involves contraction of the long and finely branched processes of resting cells into short and stout processes. Functionally, activated microglia increase NO production and expression of cytokines, such as TNF-α and IL-1β. The immune responses elicited by pro-inflammatory cytokines (e.g., TNF-α and IL-1) and subsequent nitric oxide (NO) generation from microglia in injured tissues have been implicated in neurodegenerative conditions, such as Alzheimer's disease, Parkinson's disease, and ischemic/reperfusion damage (Gonzalez-Scarano & Baltuch, 1999; Kim & Joh, 2006).

Low, physiologically relevant concentrations of reactive oxygen species (ROS) can regulate a variety of key molecular mechanisms such as the immune response, cell-cell adhesion, cell proliferation, inflammation, metabolism, aging, and cell death. Redox-based regulation of gene expression appears to be a fundamental regulatory mechanism in the biological changes mediated by ROS (Haddad, 2002). Because the antioxidant properties have been postulated as a mechanism for the putative anti-inflammatory effects of phenolic compounds (Rahman et al, 2004), antioxidants may exert neuroprotection through anti-inflammatory effects on microglia.

To examine the relationship between anti-inflammatory potency and antioxidant activity of some phenolic compounds, we used the RPA method to assess the DPPH-reducing activity of various antioxidants and the effects of antioxidant treatment on LPS-induced iNOS and cytokine gene expression in the microglial BV2 cell line. NO and cytokine production can be indexes of inflammation and depend on gene expression.

METHODS

Materials

All materials for cell culture were obtained from Gibco (Grand Island, NY). Chemicals that are not otherwise specified were purchased from Sigma (St. Louis, MO).

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ABBREVIATIONS: RPA, RNase protection assay; DPPH, 1, 1-diphenyl-2-picryhydrazyl; ROS, reactive oxygen species.

Cell culture

The immortalized murine BV2 cell line, which exhibits phenotypic and functional properties of reactive microglial cells (Blasi et al, 1990; Bocchini et al, 1992), was obtained from M. McKinney (Mayo Clinic, Jacksonville, FL). The cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37°C in a humidified incubator with 5% CO_2. When the cells became 80~90% confluent, they were subcultured.

DPPH radical scavenging assay

The 1,1-diphenyl-2-picryhydrazyl (DPPH) free radical scavenging activity was measured according to Hwang et al. (Hwang et al, 2001). DPPH (100 μl, 0.2 mM in methanol) was added to each sample (50 μl) in methanol solution in a 96-well microtiter plate. After incubation at room temperature for 30 min, the absorbance of each solution was determined at 517 nm using an ELISA microtiter plate reader (Bio-Rad Laboratories, Hercules, CA). The inhibition percentage (IP) of the DPPH radical was calculated as

$$IP = \frac{(Absorbance_{t=0min} - Absorbance_{t=30min})}{Absorbance_{t=0min}} \times 100$$

The IC_{50} parameter was calculated from the IP data, as the concentration causing a 50% inhibition of the DPPH radical.

Measurement of nitrite release

Accumulated nitrite was measured in the cell supernatant by the Griess reaction (Green et al, 1982). The conditions of cell culture and treatment were same as those for the ELISA described above. Briefly, 100 μl of Griess reagent (mixing equal volumes of 0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid) was added to 96-well microtiter plate and absorbance was read at 540 nm using a plate reader. Sodium nitrite, diluted in culture media at concentrations of 10 to 100 μM, was used to prepare a standard curve.

RNA isolation and RNase protection assay

For the RNase protection assay, total RNA from dissected testes was purified by the acid guanidinium isothiocyanate/phenol-chloroform extraction method (Chomczynski & Sacchi, 1987). RPA was performed using a Riboquant multi-probe RPA kit (PharMingen, San Diego, CA). Total RNA (8 μg) was hybridized to ^{32}P-labeled antisense RNA probes, transcribed using the cytokine multi-probe template set (including iNOS, IL-12, TNF-α, IL-6, IL-1β, IL-1 Ra, TGF-β1, L-32, and GAPDH). After RNase digestion, protected RNA and probe were resolved on a denaturing 8 M urea, 5% polyacrylamide gel. The gel was dried and exposed to autoradiographic film at −70°C with an intensifying screen.

Statistics

Comparisons between groups were made using Student's t-test and ANOVA. A difference between groups of $p<0.05$ was deemed statistically significant.

RESULTS

Effects of various antioxidants on DPPH scavenging activity

The change in absorbance produced by reduced DPPH was used to evaluate the ability of various antioxidants to act as free radical scavengers. DPPH has a deep purple color in methanolic solution and an absorbance maximum at 517 nm, but becomes pale yellow when trapped by an antioxidant. DPPH decolorization was induced by various antioxidants in a concentration-dependent manner. Table 1 shows the IC_{50} value of each antioxidant, as determined from three independent experiments. The data indicate that curcumin, ferulic acid, caffeic acid, NAC, vitamin E, and trolox possess strong antioxidant activity; trolox was the most potent antioxidant tested.

Effects of antioxidants on NO production in BV2 cells

To determine the anti-inflammatory properties of antioxidant compounds, we investigated the inhibitory effects of these compounds on LPS-induced NO production in BV2 microglial cells. The cells were challenged with LPS with or without pretreatment with each compound. When BV2 cells were stimulated with LPS (200 ng/ml) for 16 h, accumulation of nitrite, a stable oxidized product of NO, in the culture medium significantly increased. LPS-induced NO production was significantly suppressed in a concentration-dependent manner by various antioxidants in LPS-challenged BV2 cells (Fig. 1).

Effects of antioxidants on LPS-induced cytokine gen expression in BV2 cells

To determine the anti-inflammatory properties of antioxidant compounds, we investigated the inhibitory effects of these compounds on LPS-induced mRNA expression of

Table 1. DPPH radical scavenging activity of various antioxidants

Compound	IC_{50}±SD (μM)
Curcumin	119.5±6
Farulic acid	193.1±6.1
Caffeic acid	100.5±3.2
NAC	214.7±16
Vit. E	153.5±9
Trolox	91.5±4

The reported IC_{50} value is the concentration of compound required to reduce the absorbance of the DPPH radical at 517 nm to one half of the initial value in 30 min at room temperature. Reported IC_{50} values are the average values from three independent experiments.

inflammatory cytokines in BV2 cells. Levels of cytokine gene expression were measured in LPS-stimulated cells for 6 h by RNase protection assay. Stimulation with LPS resulted in strong increases in iNOS, TNF-α, IL-6, IL-1β, and IL-1 Ra mRNA. Pretreatment with curcuminoid antioxidants (curcumin, ferulic acid, caffeic acid) resulted in decreases in LPS-induced inflammatory cytokine (iNOS, IL-6, IL-1β, and IL-1 Ra) mRNA levels (Fig. 2). Vitamin E and trolox did not exhibit the same effects; NAC only began to suppress IL-1β and IL-1 Ra gene expression at concentrations that were cytotoxic (Fig. 3).

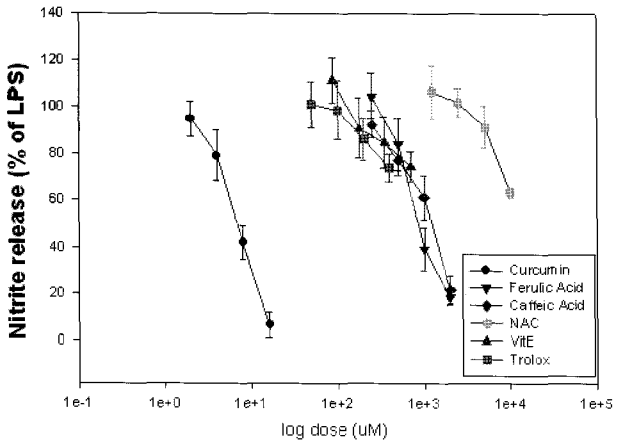

Fig. 1. Effect of antioxidants on nitrite accumulation in BV2 microglia cells stimulated with LPS. Murine microglia cells (BV2) were pretreated with antioxidants for 1 h followed by addition of LPS at 200 ng/ml. After 16 h of incubation, the medium was used to estimate nitrite accumulation by the modified Griess reaction. LPS-induced NO production was significantly suppressed in a concentration-dependent manner by all tested antioxidants in LPS-challenged BV2 microglial cells.

DISCUSSION

Microglia responds rapidly to various kinds of CNS injury through activation. Activated microglia synthesize a variety of potentially harmful soluble factors such as TNF-α, NO, and IL-1β. Studies using cell culture and animal models have demonstrated that excessive quantities of individual factors produced by activated microglia can be deleterious to neurons (Boje & Arora, 1992; Chao et al, 1992; McGuire et al, 2001).

It is believed that inflammation in the brain is closely associated with neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease (Griffin, 2006; Sawada et al, 2006). The production of NO and cytokines from microglia and subsequent neuro-inflammation can contribute greatly to the pathogenesis of these conditions. Suppression of NO and cytokine production from these cells may be beneficial in retarding these disorders (McCarty, 2006).

Lipopolysaccharides (LPS) are the principal component of the outer membrane of Gram-negative bacteria. Although LPS has no known direct toxic effect on neurons, it activates microglia to release a host of neurotoxic factors that can induce neuronal death (Bronstein et al, 1995; Araki et al, 2001; Liu et al, 2002). Thus, LPS is a widely used and powerful tool for the activation of microglia and peripheral immune cells.

Because the activation of transcription factors, such as nuclear factor kappa B (NF-κB) and AP-1 is redox-sensitive, antioxidants that increase antioxidant glutathione levels by induction of g-glutamylcysteine synthetase behave as anti-inflammatory agents through inhibition of NF-κB and AP-1. Thus, antioxidants can reduce the production of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-8.

Curcumin is a phenolic antioxidant responsible for the

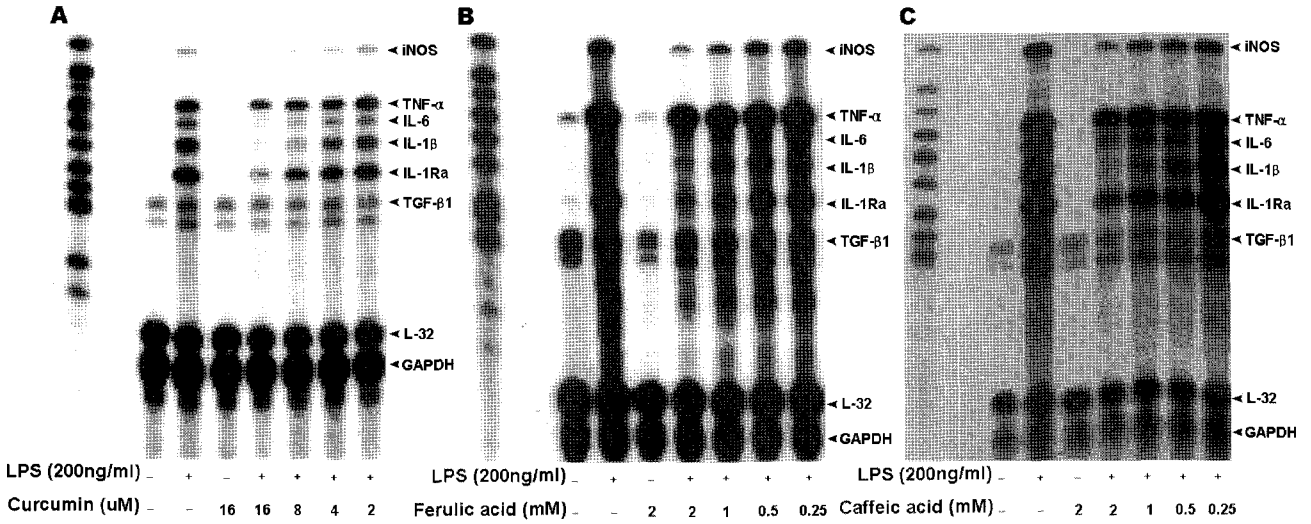

Fig. 2. Effect of antioxidants on LPS-induced cytokine gene expression in BV2 microglial cell lines. Levels of cytokine gene expression were measured in LPS-stimulated cells for 6 h by RNase protection assay. The band corresponding to each cytokine is indicated by arrow. Curcuminoid antioxidants suppressed LPS-induced iNOS, TNF-α, IL-6, IL-1β and IL-1Ra mRNA expression in a concentration dependent manner.

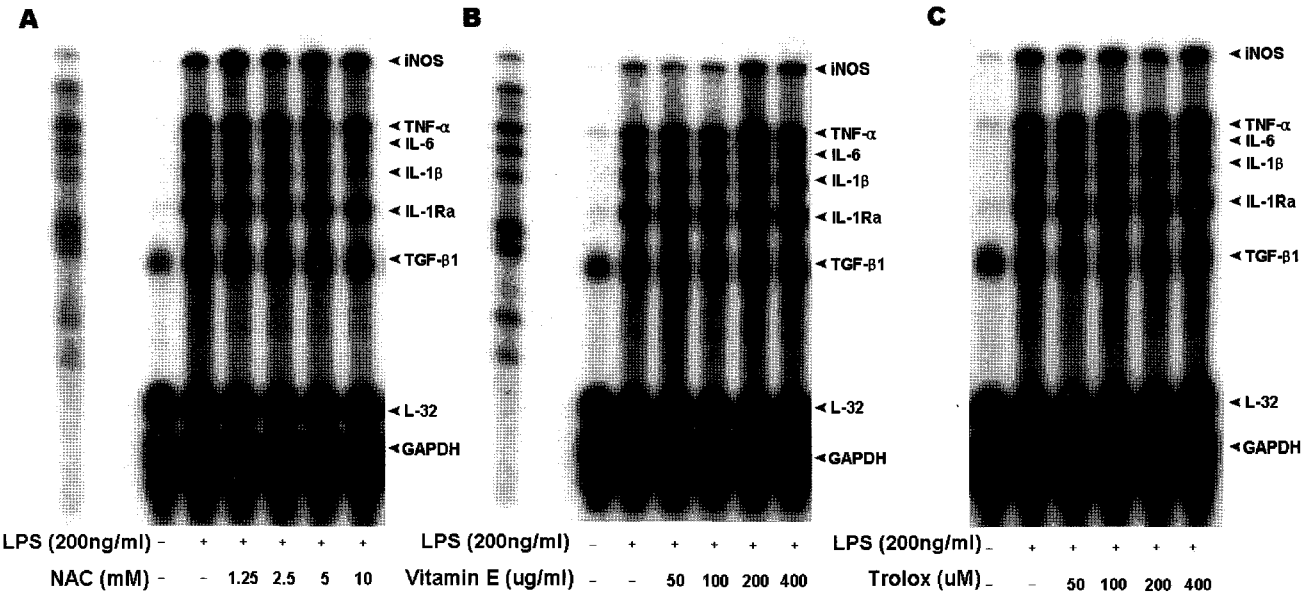

Fig. 3. Effect of antioxidants on LPS-induced cytokine gene expression in BV2 microglial cell lines. Levels of cytokine gene expression were measured in LPS-stimulated cells for 6 h by RNase protection assay. The band corresponding to each cytokine is indicated by arrow. NAC only begin to suppress IL-1β and IL-1Ra gene expression in concentration that cytotoxicity occurs. Vitamin E and trolox did not suppress LPS-induced inflammatory cytokine gene expression.

yellow coloring and the active component of turmeric that has anti-inflammatory and anticancer properties (Huang et al, 1991). In particular, curcumin has been shown to reduce oxidative damage and amyloid pathology in Alzheimer's disease (Frautschy et al, 2001; Lim et al, 2001; Ono et al, 2004).

Here, we report that pretreatment with curcuminoid antioxidants (curcumin, ferulic acid, caffeic acid) resulted in decreases in LPS-induced inflammatory cytokines (iNOS, IL-6, IL-1β, and IL-1 Ra) mRNA levels in a concentration-dependent manner. Although curcumin had similar DPPH reducing activity to caffeic and ferrulic acid, only curcumin exhibited anti-inflammatory activity at the micromolar level. Other types of antioxidants did not show the same phenomenon; indeed, NAC only began to suppress IL-1β and IL-1 Ra gene expression at concentrations that were cytotoxic. As BV2 cells are particularly sensitive to many drugs, higher concentrations of these drugs may usefully inhibit cytokine gene expression in other cell types.

With respect to NO, all antioxidants tested decreased NO production induced by LPS. However, NAC, vitamin E, and trolox could not inhibit iNOS gene expression by LPS at concentrations that were not cytotoxic to BV2 cells. Thus, these antioxidants can reduce iNOS activity at concentrations that do not inhibit iNOS gene expression.

The drugs tested in this experiment had similar antioxidant activity, but the inhibitory potencies for gene expression were different. This may be due to differences in lipid solubility, membrane penetration, interaction with other proteins, or cytotoxicity among the drugs. Curcumin may be a safe antioxidant possessing anti-inflammatory activity.

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