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Inhibitory Effects of Blueberry Extract on the Production of Inflammatory Mediators in Lipopolysaccharide-Activated BV2 Microglia

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Sustained microglial activation in the central nervous system (CNS) has been extensively investigated in age-related neurodegenerative diseases and has been postulated to lead to neuronal cell loss in these conditions. Recent studies have shown that antiinflammatory drugs may suppress microglial activation and thus protect against microglial overactivation and subsequent cell loss. Research also suggests that fruits such as berries may contain both antioxidant and antiinflammatory polyphenols that may be important in this regard. Our previous research showed that blueberry extract was effective in preventing oxidant-induced calcium response deficits in M1 (muscarinic receptor)-transfected COS-7 cells. Extrapolating from these findings, the current study investigated the effect of blueberry extract on preventing inflammation-induced activation of microglia. Results indicated that treatments with blueberry extract inhibited the production of the inflammatory mediator nitric oxide (NO) as well as the cytokines interleukin-1β and tumor necrosis factor-α, in cell conditioned media from lipopolysaccharide (LPS)-activated BV2 microglia. Also, mRNA and protein levels of inducible nitric oxide synthase and cyclooxygenase-2 in LPS-activated BV2 cells were significantly reduced by treatments with blueberry extract. The results suggest that blueberry polyphenols attenuate inflammatory responses of brain microglia and could be potentially useful in modulation of inflammatory conditions in the CNS. © 2007 Wiley-Liss, Inc.

Key words: antioxidants; microglia; neuroinflammation; cytokines

A large body of evidence suggests that inflammation in the central nervous system (CNS) increases during normal aging (Eikelenboom and Veerhuis, 1996; O'Banion and Finch, 1996; Gordon et al., 1997; Rozovsky et al., 1998). This phenomenon is augmented in age-related neurodegenerative diseases. Neuroinflammation is largely mediated by activated microglial cells. Microglia constitute up to 20% of the cell population in certain regions of the brain (Lawson et al., 1990; Dobrenis, 1998). This activation has been attributed to enhanced signal transduction, leading to the induction of inflammatory enzymes such as the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) as well as cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and transcription factors such as nuclear factor-κB (NF-κB; Talley et al., 1995; Allen and Tresini, 2000; Perry et al., 2002; Roy et al., 2002; Schroeter et al., 2002; Yoon et al., 2002; Klein and Ackerman, 2003; Mrak and Griffin, 2005). Activated microglia also generate high levels of reactive oxygen species (ROS), including $O_2^{\cdot-}$ and NO (Darley-Usmar et al., 1995; McGeer and McGeer, 2004b). The inability to balance the production and the elimination of ROS results in oxidative stress (Halliwell, 1996). Oxidative stress-mediated inflammation has been linked to neurodegenerative disorders, including Alzheimer's disease (AD) and Parkinson's disease (PD; Hensley et al., 1995, 1998; Rogers et al., 1996; Munch et al., 1998; Esch et al., 2002; McGeer and McGeer, 2004a). It has been shown that activated microglia overexpressing IL-1 are readily detectable in the brains of AD patients (Griffin et al., 1989). Furthermore, highly activated microglia, not astrocytes, have been found in the substantia nigra of PD brains (Banati et al., 1998).

Our research has shown that supplementation with fruits and vegetables rich in polyphenolics is beneficial in both forestalling and reversing the deleterious effects of aging on neuronal communication and behavior (for review see Joseph et al., 2005). The observed protection may be due to the antioxidant and antiinflammatory properties of the polyphenolic compounds found in

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these fruits and vegetables (Rice-Evans and Miller, 1996). Epidemiological evidence has indicated that dietary antioxidants may inhibit the onset of dementia (Deschamps et al., 2001; Engelhart et al., 2002). Since inflammation and oxidative stress in the CNS are associated with age-related neurodegenerative diseases, it is possible that dietary interventions through consumption of foods rich in antioxidants and antiinflammatory agents might reduce the risk of developing these age-related neurodegenerative diseases.

Blueberries are among the highest in antioxidant capacity among all fruits and vegetables as measured by the oxygen radical absorbance capacity assay (ORAC; Cao et al., 1996; Prior et al., 1998; Prior and Cao, 2000). Blueberries and other polyphenolic-enriched fruits and vegetables have been shown to possess a variety of beneficial properties in protection against inflammation, carcinogenesis, and chronic diseases (Bomser et al., 1996; Knekt et al., 2002; Joseph et al., 2005; Lau et al., 2005b; Yi et al., 2005). Although muscarinic receptors (MAChRs) lose their sensitivity as a function of age and AD (Roth et al., 1995; Fowler et al., 1997), in vitro studies from our laboratory have revealed that blueberry (BB) extract provided protection to MAChR-transfected COS-7 cells against amyloid beta (Aβ)- and dopamine (DA)-induced deficits in calcium homeostasis (Joseph et al., 2004). Moreover, DA-induced up-regulation of signals involved in oxidative stress and inflammation in MAChR-transfected COS-7 cells was attenuated by BB treatment (Joseph et al., 2006).

Thus, it was of interest in the present study to determine whether blueberry extract treatment might reduce activation of proinflammatory mediators in lipopolysaccharide (LPS)-activated murine BV2 microglia. The BV2 cell line was used in the current study because this cell line has been shown to mimic many microglial responses in culture and has been widely used as a model microglial system (Blasi et al., 1990; Bocchini et al., 1992; Murphy et al., 1998). LPS signals through its Toll-like receptor (TLR)-4 (Palsson-McDermott and O'Neill, 2004) and signal transduction by LPS via TLR-4 elicits a cascade of intracellular events such as transcription of inflammatory genes (Hagberg and Mallard, 2005). The model of LPS-activated microglia has been widely used as an in vitro system for the study of mechanisms underlying neuron damage by various mediators released from activated microglia. Therefore, the current in vitro study investigated the inhibitory effects of BB extract on the production of inflammatory mediators at early (IL-1β and TNF-α) and late (iNOS and COX-2) stages of LPS-induced BV2 microglial activation.

MATERIALS AND METHODS

Cell Culture

BV2 murine microglial cell line, developed in the laboratory of Dr. Blasi at the University of Perugia (Perugia, Italy; Blasi et al., 1990), was generously provided by Dr. Van Eldik of Northwestern University (Chicago, IL). Cell culture media, antibiotics, and serum were obtained from Invitrogen (Carlsbad, CA). Cell culture dishes and multiwell plates were purchased from Fisher Scientific (Pittsburgh, PA). LPS and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). BV2 cells were cultured in growth medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified incubator under 5% CO_2. For all experiments, cells were washed twice with warm phosphate-buffered saline (PBS) and incubated in warm serum-free DMEM in the absence of phenol red and antibiotics.

Preparation of BB Extracts

BB extract was prepared as described elsewhere (Joseph et al., 1998; Youdim et al., 2000). Briefly, one part of blueberries in one part of water was homogenized for 2 min. The homogenate was then centrifuged at 26,000*g* for 15 min at 4°C. The supernatant was collected and lyophilized and stored at −20°C. Media supplemented with BB at various concentrations (50–500 μg/ml) were prepared immediately before the experiments. The highest BB dose to which the cells were exposed in this study was equivalent to a 1% BB-supplemented diet used in a rat study (Goyarzu et al., 2004). According to Goyarzu et al., a rat on a 2% BB-supplemented diet consumed about 400 mg/day of lyophilized BB (Goyarzu et al., 2004). Assuming that an average adult rat weighs 400 g, the daily BB dose translates into 1 mg of BB/g of body weight. Because the highest dose used in the current study was 500 μg/ml or 0.5 mg/g of cell medium, it was one-half of the daily dose for an adult rat on a 2% BB-supplemented diet (Goyarzu et al., 2004). BB extract at the concentrations used in this study did not cause cytotoxicity per se (data not shown) as indicated by CellTiter 96 AQueous Assay (Promega, Madison, WI; Malich et al., 1997).

Nitrite Quantification

To assess the production of NO in BV2 cells, the extracellular release of nitrite (NO_2^-), a stable metabolite of NO, was measured by Griess reagent (Promega) according to the instructions provided by the manufacturer, with slight modification. Briefly, cells (2×10^5 cells per well) were seeded in 12-well tissue culture-treated plates (BD Falcon, Bedford, MA) and allowed to grow for 24 hr in growth medium at 37°C and 5% CO_2. Cells were then washed twice with PBS and then pretreated with BB extracts at various concentrations in serum-free, phenol red-free medium for 45 min. After pretreatment, LPS (100 ng/ml) was added, and the cells were incubated at 37°C and 5% CO_2 for 16 hr. After LPS exposure, cell-conditioned media (CCM) were collected. Duplicates of supernatants (100 μl) were mixed with 50 μl of Griess reagent in a 96-well plate and incubated at room temperature for 10 min in the dark. The absorbance was measured with a 548-nm filter by a μQuant microplate spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT). Sodium nitrite standard curve was constructed with nitrite concentrations ranging from 0 to 100 μM. The concentration of nitrite released in the CCM was calculated with the linear equation derived from the standard curve.

Total RNA Isolation and Real-Time Quantitative RT-PCR Analysis

Cell culture conditions and treatments were the same as given in the previous section. Total RNA was extracted from the cells, and residual genomic DNA was digested by RNase-free DNase I with the RNeasy kit from Qiagen (Valencia, CA). RNA concentration was assessed by RiboGreen RNA Assay kit from Invitrogen. One microgram of total RNA was used to synthesize cDNA in a 20-μl total volume with the ImProm-II reverse transcription system from Promega. The resulting cDNA template was subsequently used for optimized real-time PCR. Real-time PCR was carried out for each sample in duplicates on an ABI Prism 7700 Sequence Detection System in a 25-μl total volume with the SYBR Green PCR Master Mix (ABI, Foster City, CA). A dissociation curve was constructed at the end of the cycles to ensure the specificity of amplification. The primers (sequences available upon request) for PCR amplification were designed with Primer3 software (Rozen and Skaletsky, 2000). A comparative threshold cycle (C_T) method was used to analyze the real-time PCR data, where the amount of target, normalized to the endogenous reference of GADPH (ΔC_T) and relative to the calibrator of untreated control ($\Delta\Delta C_T$), was calculated by the equation $2^{-\Delta\Delta CT}$ as described elsewhere (Livak and Schmittgen, 2001).

Detection of iNOS and COX-2 Protein Expression by Immunoblot Analysis

Cell culture conditions and treatments were identical to those used in nitrite assays. After CCM were collected for nitrite measurements, cells were washed twice with ice-cold PBS and 100 μl of CelLytic-M mammalian cell lysis-extraction reagent from Sigma (St. Louis, MO) was added to each well. Cells were lysed at 4°C with rocking for 15 min and centrifuged at 12,000*g* for 15 min at 4°C. Cell lysates were collected, and protein concentration was determined by the DC protein assay from Bio-Rad (Hercules, CA). Equal amounts of denatured protein samples (10 μg per lane) were separated by 12% SDS-PAGE and electrophoretically transferred to PVDF membrane. The reagents for immunoblotting assay, except for the primary antibodies, were from Western-Breeze Immunodetection Kits (Invitrogen). Primary antibody for COX-2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and iNOS was purchased from Upstate Cell Signaling (Waltham, MA). Procedures for immunodetection were as outlined in the instruction manual for the WesternBreeze Immunodetection Kit. Immunoreactivity was visualized and captured by the UVP EC^3 BioImaging system (Upland, CA). The optical density of the antibody-specific bands was analyzed by the LabWorks image acquisition and analysis software (Upland, CA). Membrane was stripped and reprobed with antibody against β-actin (Santa Cruz Biotechnology) to ensure equal loading of protein samples.

Measurement of IL-1β and TNF-α Levels by ELISA

CCM were collected as described in the nitrite measurement section, except that cells were treated with LPS for 4 hr. The levels of IL-1β and TNF-α in the CCM were measured by ELISA as outlined by the manufacturer (Assay Designs, Ann Arbor, MI). Cytokine standards of known amount were included in each assay. The optical density (OD) at 450 nm was measured by a μQuant microplate spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT). A linear equation was obtained from the standard curve and used to calculate the amount in picograms per milliliter of cytokines in CCM.

Intracellular ROS Detection

ROS accumulation in the cells was detected by 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di-acetoxymethylester (DCF-AM) according to the manufacturer (Invitrogen). Briefly, cells were treated in the same manner as for the nitrite assay, except that cells were cultured in black-walled/clear-bottomed tissue culture-treated 96-well Optilux plates (BD Falcon, Bedford, MA) to avoid well-to-well cross-talk during fluorescence measurements. After 16 hr of treatment, cells were washed twice with warm PBS and incubated with 10 μM DCF-AM for 1 hr. After loading with DCF-AM, cells were washed twice with PBS and allowed to recover in phenol red/serum-free medium for 30 min at 37°C in the dark. Fluorescence was measured by a CytoFluor multi-well plate reader (PerSeptive Biosystems, Framingham, MA) with excitation and emission wavelength set at 485 nm and 530 nm, respectively. The bandwidth was set at 20 for both wavelengths. The results were expressed as percentage increase in DCF fluorescence (F) over untreated control.

Statistical Analysis

The results were expressed as mean ± SEM from at least three independent experiments run in duplicate. For multiple-variable comparisons, data were analyzed by analysis of variance (ANOVA), followed by Tukey's post hoc pairwise comparisons in Systat (Systat Software Inc., Point Richmond, CA). The level of statistical significance was set a priori at $P < 0.05$.

RESULTS

Inhibition of NO Production

NO production in conditioned medium from LPS-activated BV2 cells was determined by measuring the concentration of nitrite, a stable metabolite of NO (Griess, 1879; Phizackerley and Al-Dabbagh, 1983). Figure 1 shows that LPS induced an increase in NO production in CCM of activated BV2 cells from 1.19 ± 0.21 μM (control) to 51.90 ± 3.57 μM (LPS alone). BB treatment of LPS-activated BV2 cells significantly inhibited the accumulation of nitrite in the CCM. The concentration leading to a 50% inhibition (IC_{50}) of NO production was about 100 μg/ml, and this inhibitory effect seemed to level off at higher BB concentrations (Fig. 1).

Down-Regulation of iNOS and COX-2 mRNA Expression

The mRNA levels of iNOS, COX-2, and the housekeeping gene GAPDH were measured by quantitative RT-PCR. The mRNA expression of the internal control, GAPDH, was not affected by different treatments ($P = 0.83$). The mRNA expression of iNOS and

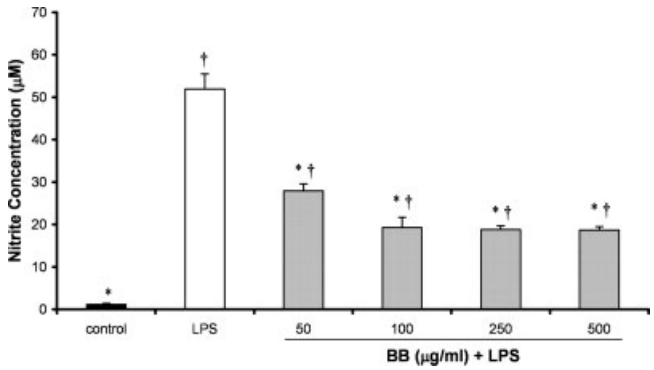

Fig. 1. Inhibition of NO production by BB extract. BV2 cells were pretreated with BB extract at various concentrations as indicated, followed by stimulation with LPS (100 ng/ml) for 16 hr. Cell-conditioned supernatants were collected, and the production of NO was measured by the presence of nitrite in the supernatants using Griess reaction. Values represent mean ± SEM of four independent experiments. $^{\dagger}P < 0.05$ vs. control (solid bar), $^{\star}P < 0.05$ vs. LPS alone (open bar).

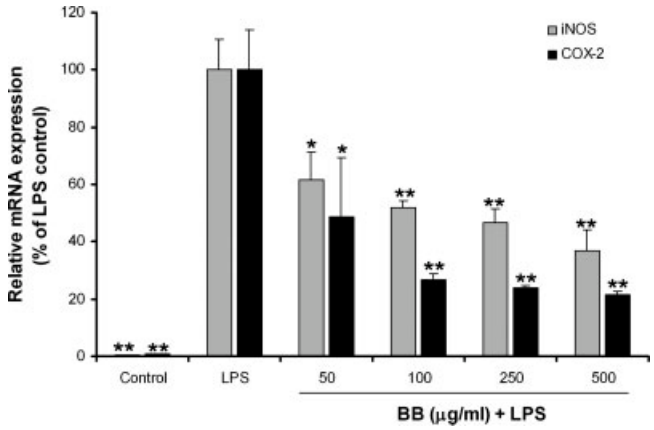

Fig. 2. Effect of BB treatment on the mRNA expression of iNOS and COX-2. BV2 cell culture conditions were the same as those outlined in Figure 1. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as the endogenous reference. Changes in mRNA levels of iNOS and COX-2 relative to GADPH were expressed as percentage of LPS control. Data represent mean ± SEM of three independent experiments. $^{\star}P < 0.05$, $^{\star\star}P < 0.005$ vs. LPS alone.

COX-2 was barely detectable in untreated control cells (Fig. 2). LPS elicited an increase in the mRNA expression of iNOS and COX-2, whereas BB treatment significantly down-regulated the LPS-induced increase in the expression of iNOS and COX-2 mRNA levels as shown in Figure 2. This inhibitory property exerted by BB extract was more effective against COX-2 (IC_{50} of 50 μg/ml) than iNOS (IC_{50} of 100 μg/ml) mRNA expression.

BB Extract Attenuated the Protein Expression of iNOS and COX-2

BB dose responsively inhibited the LPS-induced increase in iNOS and COX-2 protein levels, as illustrated in Figure 3. The protein levels of iNOS and

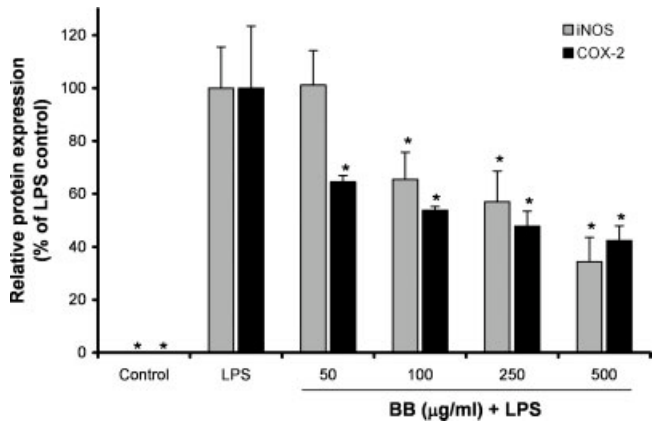

Fig. 3. Effect of BB on iNOS and COX-2 protein expression. BV2 cells were pretreated with BB extract at various concentrations as indicated, followed by stimulation with LPS (100 ng/ml) for 16 hr. Cell lysates were used for Western analysis. The levels of protein expression in iNOS and COX-2 were normalized to that of β-actin. The normalized values were expressed as percentage of LPS control. Values represent mean ± SEM of three independent experiments. $^{\star}P < 0.005$ vs. LPS alone.

COX-2 were virtually undetectable in untreated control cells. Upon LPS stimulation, a dramatic increase in the protein expression of iNOS and COX-2 was observed in the BV2 cells. At the lowest concentration (50 μg/ml), BB treatment was not able to reduce the LPS-stimulated iNOS protein expression. However, at higher concentrations, BB treatment significantly reduced the protein levels of iNOS in LPS-activated cells. In contrast, BB treatment dose responsively suppressed the protein expression of COX-2 at all BB concentrations used in the study. BB treatment was more effective in suppressing COX-2 than iNOS protein expression, as was observed for mRNA expression.

Reduction of the Levels of Cytokines in CCM

The levels of cytokines IL-1β and TNF-α released into the CCM were measured by ELISA. As shown in Figure 4A, the production of IL-1β by BV2 cells was significantly induced by LPS from 6.86 ± 2.18 pg/ml in control medium to 149.17 ± 25.00 pg/ml in LPS-alone medium. The stimulated levels of IL-1β were markedly reduced to 29.97 ± 4.82 pg/ml by treatment with BB at a concentration of 500 μg/ml (Fig. 4A). Similarly, the levels of TNF-α in the CCM were drastically increased by LPS from 66.68 ± 14.16 pg/ml in control medium to 746.55 ± 111.72 pg/ml in the LPS-stimulated medium (Fig. 4B). This increase in the amount of TNF-α by LPS stimulation was attenuated dose responsively by BB treatment. At the highest BB concentration (500 μg/ml), the amount of TNF-α present in the CCM was reduced to levels comparable to the level of the control.

Decrease in the Intracellular ROS Production

Intracellular levels of ROS were increased by LPS stimulation to 67.66% ± 3.72% over the level in the

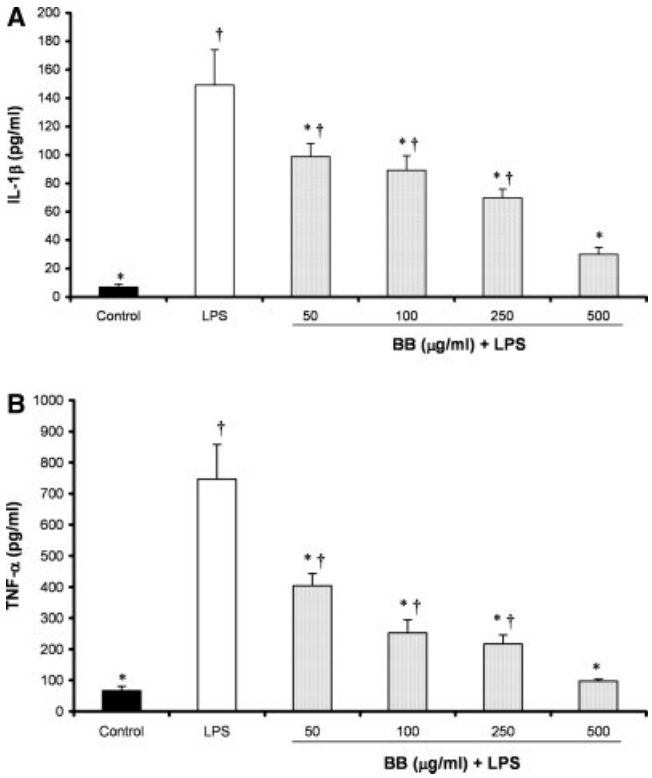

Fig. 4. Attenuation of inflammatory cytokines IL-1β (**A**) and TNF-α (**B**) by BB treatment. BV2 cells were pretreated with BB extract at various concentrations as indicated, followed by stimulation with LPS (100 ng/ml) for 4 hr. Cytokines from BV-2 cell conditioned media were assayed by ELISA. Values represent mean ± SEM of four independent experiments. $^{\dagger}P < 0.005$ vs. control (solid bar), $^{\star}P < 0.005$ vs. LPS control (open bar).

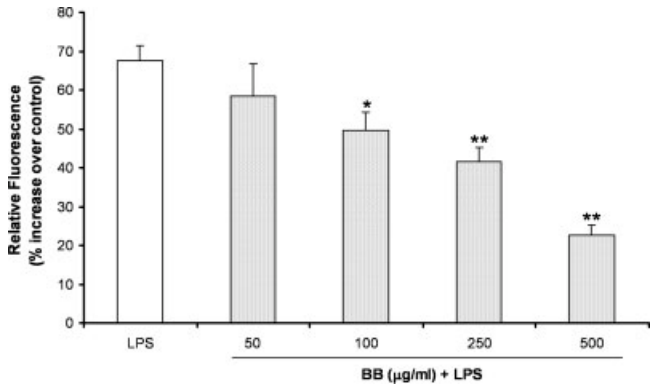

Fig. 5. Suppression of intracellular ROS by BB extract. BV2 cells were pretreated with BB extract at various concentrations as indicated, followed by stimulation with LPS (100 ng/ml) for 16 hr. Cells were loaded with 10 μM DCF for 1 hr, followed by removal of the fluorescent dye. The cells were allowed to recover for 30 min. Fluorescence was measured by a Cytofluor multiwell reader and expressed as percentage increase compared with that of the control. Values represent mean ± SEM of four independent experiments. $^{\star}P < 0.05$, $^{\star\star}P < 0.005$ vs. LPS control (open bar).

control cells. BB treatment significantly lowered the LPS-stimulated rise in intracellular ROS to 22.72% ± 2.60% at a concentration of 500 μg/ml of BB (Fig. 5). However, no inhibition of LPS-stimulated increase in the intracellular ROS levels was observed at the lowest dose of BB (50 μg/ml). Interestingly, the lowest dose of BB also failed to suppress the LPS-stimulated protein expression of iNOS (Fig. 3).

DISCUSSION

Data from the current study showed that LPS elicited a dramatic increase in NO production and that BB treatment attenuated the LPS-induced NO production and iNOS mRNA expression in a dose-responsive manner. The pattern of BB inhibition on NO production and iNOS mRNA expression was essentially the same, with an IC_{50} of about 100 μg/ml. Despite significant down-regulation of iNOS mRNA in response to BB treatment, iNOS protein levels were not significantly inhibited at the lowest concentration of BB (50 μg/ml). However, at higher BB concentrations, a dose-response suppression of iNOS protein was observed. This observation may reflect the steady-state levels of iNOS protein. These findings may suggest that suppression of LPS-stimulated NO production by BB extract was mainly at the transcriptional level, through down-regulating LPS-induced iNOS mRNA expression. This result is important, insofar as it has been reported that LPS induces a respiratory burst accompanied by alterations in gene expression, resulting in the production of ROS, NO, and cytokines as well as the induction of inflammatory enzymes, including iNOS and COX-2 (Zielasek and Hartung, 1996; Mirzoeva et al., 1999; Egger et al., 2003; Chun et al., 2005). Therefore, the concentration-dependent decrease in iNOS protein expression in the BB-treated LPS-exposed BV2 cells suggests that BB polyphenols may inhibit one of the primary steps in this inflammatory stress pathway. This finding might have critical implications for the role of blueberries and possibly other berry fruits as antiinflammatory agents, in that iNOS is inducible in virtually all mammalian cell types by proinflammatory mediators such as LPS and cytokines (Forstermann and Kleinert, 1995; Eiserich et al., 1998).

In this regard, BB treatment also inhibited COX-2 mRNA and protein expression. The level of suppression by BB extract of COX-2 mRNA expression was proportional to that of the protein expression. BB extract seemed to exert greater inhibitory effects on the mRNA expression of COX-2 than that of iNOS. This result is consistent with the observation that apigenin (a flavone found in leafy plants and vegetables) exhibited greater inhibitory effects on COX-2 activity ($IC_{50} < 5$ μM) than on iNOS promoter activity ($IC_{50} < 10$ μM; Liang et al., 1999). It is important to note that COX-2 expression has been shown to be induced by proinflammatory stimuli (Minghetti and Levi, 1998; Egger et al., 2003). Hence, in addition to decreases in iNOS activity, the down-regulation of COX-2 by BB treatment might represent a second site of antiinflammatory activity in the LPS-stimulated cells.

Similarly, treatment of the LPS-exposed BV2 microglial cells with BB extract significantly attenuated the proinflammatory cytokines IL-1β and TNF-α in the CCM. In addition, findings from this study show that BB treatment significantly reduced oxidative stress in BV2 microglial cells through the reduction of intracellular ROS during LPS activation.

Flavonoids found in fruits and vegetables have shown antiinflammatory and antioxidant properties in a variety of models (Robak and Gryglewski, 1988; Middleton et al., 2000; Kim et al., 2004). For example, flavonoids isolated from *Waltheria indica* have been found to suppress the LPS-induced production of NO and cytokines such as TNF-α (Rao et al., 2005). *Ginkgo biloba* extract EGb761 has been shown to inhibit the LPS-induced NO production and iNOS expression (Wadsworth et al., 2001; Wong et al., 2001). Wogonin, a polyphenolic compound isolated from the Chinese herb Huang Qui, has been demonstrated to attenuate the acute production of NO and, at the same time, inhibit the expression of iNOS and COX-2 genes (Chen et al., 2001). Resveratrol, found in the skin of grapes, has been found to reduce intracellular accumulation of ROS (Jang and Surh, 2001). Taken together, results from the current study provide scientific evidence suggesting that BB polyphenols exhibit potent antiinflammatory and antioxidant properties.

These findings, and those from previous experiments, showing a down-regulation of microglial activation, may be especially important insofar as it is well known that microglial activation is the hallmark of inflammation in the CNS (Liu et al., 2002; Orr et al., 2002). Acute activation of microglia by brain injury or infection elicits cellular and metabolic alterations that are believed to be beneficial to the microenvironment of the CNS in host protection and tissue repair (Streit, 2002; Streit et al., 2005). However, pathological activation of microglia has been demonstrated to contribute to the progressive damage in neurodegenerative diseases such as stroke, AD, multiple sclerosis (MS), and human immunodeficiency virus (HIV)-associated dementia (McGeer and McGeer, 2004b). This occurs through the sustained up-regulation of inflammatory genes such as iNOS and COX-2 as well as the prolonged generation of various proinflammatory mediators, including NO, ROS, IL-1β, and TNF-α (Minghetti and Levi, 1998; Akiyama et al., 2000; Luterman et al., 2000; Tarkowski et al., 2003).

The current findings also indicate that there might be dietary/nutritional means for intervention in the aging process. Aging is accompanied by behavioral and neuronal declines, as well as enhanced risk of dementia (Nicita-Mauro, 2002; Joseph et al., 2005; Lau et al., 2005b). These declines may reflect an increasing inability of the organism to protect against oxidative and inflammatory stressors (Halliwell and Gutteridge, 1985; Floyd, 1999; Esposito et al., 2002; Martin et al., 2002; Junqueira et al., 2004), and, consequently, elevated markers of inflammation and oxidative stress have been observed in the CNS (Gordon et al., 1997; Rozovsky et al., 1998; Sloane et al., 1999). Research also indicates that there are morphological changes in microglial cells that may up-regulate their activation markers and lower their activation threshold (Streit, 2005; Conde and Streit, 2006). If blueberries and other fruits and vegetables can prevent microglial overactivation, this may provide evidence to suggest that the neuroprotective effects of BB (for review see Joseph et al., 2005; Lau et al., 2005a,b) that have been demonstrated in rodent studies (Joseph et al., 1999, 2003; Sweeney et al., 2002; Goyarzu et al., 2004; Stromberg et al., 2005; Galli et al., 2006) involve the alteration of microglial activity, subsequent stress signaling cascades, and consequent antiinflammatory activity. Indeed, an in vivo study from our laboratory using the kainic acid (KA)-induced inflammation paradigm showed that rats on a BB-supplemented diet exhibited a decrease in microglial inflammation, as indicated by the reduction in OX-6 (a glial inflammatory marker)-immunoreactive cells in the hippocampus (Shukitt-Hale et al., 2004). Furthermore, this study showed that BB supplementation was able to attenuate the KA-induced up-regulation of NF-κB, IL-1β, and TNF-α mRNA expression in the hippocampus of the KA-stimulated rats (Lau et al., 2004).

Although the precise mechanism underlying the neuroprotective properties of BB is unknown, there is evidence from in vivo and in vitro studies that various plant-derived polyphenols may inhibit NF-κB activation (Kim et al., 2004). We are currently investigating the possible role played by BB polyphenols in NF-κB activation, as well as other stress signaling cascades, such as the mitogen-activated protein kinase (MAPK) pathways (Moreira et al., 2006).

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