Attenuation of iNOS and COX2 by blueberry polyphenols is mediated through the suppression of NF-κB activation

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ABSTRACT

Treatment of BV2 microglial cells with blueberry extracts has been shown to be effective in reducing lipopolysaccharide (LPS)-induced proinflammatory mediators such as nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), inducible NO synthase (iNOS), and cyclooxygenase 2 (COX2). The current study explored the possibility that the down-regulation of iNOS and COX2 by blueberry extracts was mediated through NF-κB signaling pathway. A column-purified fraction of polyphenol-enriched blueberry extract (PC18) was used to treat LPS-activated BV2 cells. The results thus far showed that blueberry polyphenols significantly suppressed iNOS and COX2 promoter activities. In addition, blueberry polyphenols inhibited NF-κB nuclear translocation in LPS-activated BV2 cells. These findings suggested that the beneficial effects of blueberries may involve direct modulation of oxidative stress and/or inflammatory signaling cascades.

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1. Introduction

Accumulating evidence indicates that inflammation in the central nervous system (CNS) increases during normal aging and that neuroinflammation is augmented by age-related neurodegenerative diseases (Eikelenboom & Veerhuis, 1996; O’Banion & Finch, 1996; Rozovsky et al., 1998). Microglial cells, which constitute up to 20% of the neuronal cell population in certain brain regions, have been shown to be activated during neuroinflammation (Dobrenis, 1998; Lawson et al., 1990). Chronic and sustained activation of microglial cells is attributed to enhanced signal transduction (induction of nuclear factor-kappa B, NF-κB) leading to the activation of inflammatory enzymes such as the inducible nitric oxide synthase (iNOS) and the inducible cyclooxygenase-2 (COX2) as well as cytokines such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) (Klein & Ackerman, 2003; Mrak & Griffin, 2005; Shukitt-Hale et al., 2008; Talley et al., 1995). Moreover, activated microglial cells produce high concentrations of reactive oxygen species resulting in an increased burden of oxidative stress on neuronal cells further contributing to the progression of neuroinflammation (Darley-Ustman et al., 1995; McGeer & McGeer, 2004). In fact, several neurodegenerative disorders such as Alzheimer’s disease and Parkinson’s disease have been associated with oxidative stress-mediated neuroinflammation (McGeer & McGeer, 1998).

Plants produce a variety of phytochemicals which are not required per se for their primary cellular functions. These so-called secondary compounds, however, are involved in a wide array of ecological functions to enhance the survival of plants during environmental stress (Winkel-Shirley, 2002). Mounting evidence suggests that the health benefits observed for the consumption of a diet rich in fruit and vegetables are mainly due to the presence of these phytochemicals (Liu, 2003). Polyphenols, which are found in abundance in diets rich in fruit and vegetables, are bioactive compounds with antioxidant,
anti-inflammatory, and anticarcinogenic properties (Surh et al., 2001). Blueberries are high in antioxidant activity as measured by the oxygen radical absorbance capacity assay (ORAC) (Cao et al., 1996; Prior et al., 1998). Blueberries and other polyphenolic-enriched fruit and vegetables have been shown to provide protection against oxidative stress, inflammation, carcinogenesis and chronic diseases (Graf et al., 2005; Lau et al., 2005b; McDougall et al., 2008; Schmidt et al., 2006).

Research has suggested that dietary supplementation with fruit and vegetables rich in polyphenolics is capable of both forestalling and reversing the deleterious effects of aging on neuronal communication and behavior (Lau et al., 2005a). Although the exact mechanisms for the observed beneficial effects are largely unclear, several lines of evidence suggests that the antioxidant and anti-inflammatory properties of the polyphenolic compounds found in these fruit and vegetables may play an important role (Rice-Evans et al., 2000; Rice-Evans & Miller, 1996).

Previously we have shown that crude extract of blueberry is effective in attenuating the production of inflammatory mediators in lipopolysaccharide (LPS) activated murine BV2 microglia (Lau et al., 2006). The current study aimed to determine the effect of a polyphenolic-enriched fraction of blueberry (PC18) on the production of proinflammatory mediators COX2 and iNOS and whether PC18 could affect nuclear factor-kappa B (NF-κB) translocation into the nucleus of BV2 microglia that were activated by the proinflammatory inducer lipopolysaccharide (LPS).

2. Materials and methods

2.1. Reagents

Dimethylsulphoxide (DMSO) and LPS (Escherichia coli serotype O111:B4) were purchased from Sigma (St. Louis, MO). Cell culture media, antibiotics, and serum were obtained from Invitrogen (Carlsbad, CA). Cell culture dishes and multi-well plates were supplied by Fisher Scientific (Pittsburgh, PA). Electrophoretic apparatus and reagents were purchased from Bio-Rad (Hercules, CA).

2.2. Cell culture

The BV2 murine microglial cell line (Blasi et al., 1990) was generously provided by Dr. Van Eldik of Northwestern University (Chicago, IL). BV2 cells were cultured at 37 °C in a humidified incubator under 5% CO₂ in growth medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 μg/ml), and referred as complete medium. 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 at the indicated durations.

2.3. Blueberry PC18 fraction preparation

A blueberry polyphenolic-enriched fraction (PC18) was prepared from a commercially prepared 100% wild blueberry (Vaccinium angustifolium) juice as described by (Wilson et al., 2006). Briefly, blueberry juice was applied to a pre-conditioned C18 column (Waters Canada Ltd., Mississauga, ON, Canada) and washed extensively with 0.1% trifluoroacetic acid (TFA) in water to remove abundant sugars and organic acids. Bound polyphenolics were eluted from the C18 column with 0.1% TFA in methanol. The acidified methanol was removed at 30 °C using a vacuum rotary evaporator (Buchi, Essen, Germany) and the remaining residue was lyophilized. Anthocyanins, which are the most abundant flavonoid polyphenolic in the PC18 fraction, were separated and identified by high performance liquid chromatography (HPLC).

2.4. HPLC elution

The PC18 fraction was analysed using an Agilent 1100 series HPLC (Agilent Technologies Ltd., Mississauga, ON), equipped with a binary pumping system, column compartment and diode array detection (DAD) at 280 and 520 nm. The column was a Zorbax-SB-C18 (Agilent Technologies Ltd, Mississauga, ON) 2.1 x 100 mm 3.5 μm particle size maintained at 26 °C. Solvent A was TFA in water at pH 1.35, and Solvent B (SB) was TFA in acetonitrile at pH 1.35. The flow was 0.4 mL/min and the gradient elution program was as follows: 5% SB initial conditions; 5–10% SB, 0–25 min; 10–20% SB, 25–87.5 min; 20% SB, 87.5–90 min; 100% SB, 90–99 min; 100–5% SB, 99–102 min; 5% SB, and 102–105 min. The column was equilibrated for an additional 5 min prior to each run.

2.5. Cell viability assay

The CellTiter 96® AQueous One Solution Assay (Promega Corp., Madison, WI) was used to determine whether the PC18 concentrations used in the experiment caused any cytotoxicity in BV2 cells. This assay is based on the mitochondrial mediated reduction of a tetrazolium compound (MTS) to a formazan product that did not contain phenol-red or antibiotics. After 16 h, 20 μl of MTS reagent were added to each well and the plates were returned to the incubator. After 4 h incubation, concentration of the MTS formazan product was measured at 490 nm in a 96-well microplate spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT). The assay was performed in quadruplicates. The absorbance values of PC18-treated cells was calculated relative to the values of PBS-treated control and reported as% cell viability.

2.6. iNOS activity

To assess the activity of iNOS, the concentration of extracellularly released nitrite (a stable metabolite of nitric oxide, NO) in cell-conditioned media (CCM) was measured by Griess reagent (Promega Corp., Madison, WI) according to the instructions provided by the manufacturer with slight modifications. Briefly, BV2 cells (2 × 10⁵ cells in 500 μL/well) were seeded in
The murine COX2 and iNOS promoter plasmids were purchased from Promega Corp. (Madison, WI). The reporter plasmids were constructed using standard cloning techniques. Briefly, the COX2 promoter region (containing a 1004 bp fragment, −965 to +39 bp relative to the transcription initiation site) was excised by HindIII–EcoRV digestion, gel purified and then directionally cloned into the unique site of the HindIII–EcoRV linearized pGluc-Basic vector upstream of the Gluc gene resulting in the COX2-pGluc reporter construct. iNOS promoter region (containing a 1749 bp fragment, −1588 to +161 bp relative to the transcription initiation site) was excised by Ael blunt-end restriction enzyme, gel purified and cloned into the EcoRV blunt-end linearized pGluc-Basic vector upstream of the Gluc gene. The orientation of iNOS promoter was verified by asymmetrical restriction digestion with SacI enzyme resulting in the iNOS−pGluc reporter plasmid. The reporter plasmids were maintained and propagated in JM109 cells and purified by Qiagen Plasmid Maxi Kit according to the instructions supplied by the manufacturer (Qiagen, CA). The concentration of the plasmids was measured as described in the Quant-IT DNA Assay Kit by Invitrogen (Carlsbad, CA).

2.7. Immunoblot analysis

Cell culture conditions and treatments for iNOS and COX2 immunoblot were identical to those in iNOS activity measurement using nitrite detection. After CCM were collected for nitrite measurements, cells were washed twice with ice-cold PBS before protein extraction. Protein was extracted using 100 μl/well of Celllytic-M mammalian cell lysis/extraction reagent (Sigma, St. Louis, MO) at 4 °C with rocking for 15 min followed by centrifugation at 12,000 g for 15 min at 4 °C. Cell lysates were collected and protein concentration was determined by the DC Protein Assay Kit according to the instructions furnished by the manufacturer (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 (Bio-Rad, Hercules, CA). Except for the primary antibodies, reagents for immunoblotting assay were from WesternBreeze Immunodetection Kits (Invitrogen, Carlsbad, CA). Primary antibody for COX2 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 WesternBreeze Immunodetection Kit. Immuno-reactivity was visualized and captured by an EC3 Biolmaging system (UVP, Upland, CA). The optical density of the antibody-specific bands was analyzed by the LabWorks image acquisition and analysis software (Upland, CA). PVDF membrane was stripped and reprobed with antibody against β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) to ensure equal loading of protein samples.

2.8. Plasmid construction

The murine COX2 and iNOS promoter plasmids were purchased from Oxford Biomedical Research Inc. (Oxford, MI). The promoterless reporter vector (pGluc-Basic) containing the humanized coding sequence of the secreted Gaussia luciferase as well as enzymes used for restriction digestion and cloning were purchased from New England Biolabs, Inc. (NEB, Ipswich, MA). PCR primers flanking the multiple cloning site and upstream of the Gaussia luciferase (Gluc) gene were also obtained from NEB. Taq PCR Master Mix Kit was purchased from Qiagen (Valencia, CA). Competent cells of strain MJ109 were purchased from Promega Corp. (Madison, WI). The reporter plasmids were constructed using standard cloning techniques. Briefly, the COX2 promoter region (containing a 1004 bp fragment, −965 to +39 bp relative to the transcription initiation site) was excised by HindIII–EcoRV digestion, gel purified and then directionally cloned into the unique site of the HindIII–EcoRV linearized pGluc-Basic vector upstream of the Gluc gene resulting in the COX2-pGluc reporter construct. iNOS promoter region (containing a 1749 bp fragment, −1588 to +161 bp relative to the transcription initiation site) was excised by Ael blunt-end restriction enzyme, gel purified and cloned into the EcoRV blunt-end linearized pGluc-Basic vector upstream of the Gluc gene. The orientation of iNOS promoter was verified by asymmetrical restriction digestion with SacI enzyme resulting in the iNOS−pGluc reporter plasmid. The reporter plasmids were maintained and propagated in JM109 cells and purified by Qiagen Plasmid Maxi Kit according to the instructions supplied by the manufacturer (Qiagen, CA). The concentration of the plasmids was measured as described in the Quant-IT DNA Assay Kit by Invitrogen (Carlsbad, CA).

2.9. Transient transfection and luciferase assay

BV2 cells were seeded in 100 mm dishes in complete medium and incubated until the cells were confluent. The medium was replaced with serum-free Opti-MEM I medium (OMEM, Invitrogen, Carlsbad, CA). Then 60 μl of Lipofectamine 2000 reagent (Invitrogen) were diluted in 1.5 ml OMEM, mixed with 24 μg of reporter construct (COX2-Gluc or iNOS-Gluc) in 1.5 ml OMEM and incubated for 20 min at room temperature to allow the plasmid-Lipofectamine 2000 complexes to form. Confluent BV2 cells were transfected with the COX2-Gluc or iNOS−Glucluciferase activity was recorded as relative light units (RLU) and expressed as % relative to treatment-free control.

2.10. Electrophoretic mobility-shift assay (EMSA)

BV2 cells were seeded in 6-well plates and incubated until the cells were confluent. The media were replaced with serum-free DMEM and cells were treated with LPS (100 ng/ml) and specified concentrations of PC18 for 4 h. Nuclear proteins were isolated with Celllytic NuClear Extraction Kit (Sigma, St. Louis, MO) according to the technical bulletin supplied by the manufacturer. EMSA was performed according to manufacturer’s instructions (Panomics, Redwood City, CA). Briefly, 10 μg of nuclear proteins were mixed with biotinylated
double-stranded oligonucleotide probe containing the NF-κB binding site. The protein–DNA complexes were separated on a 6% non-denaturing acrylamide gel and transferred to nylon membrane. The specificity of NF-κB nuclear protein–DNA binding was confirmed by competition with unlabeled oligonucleotide probes resulting in the absence of the shifted band. The labeled DNA probe was detected by streptavidin-HRP conjugate in the presence of chromogen substrate and visualized with the EC3 BioImaging system (UVP, Upland, CA). The level of statistical significance was set a priori at \( P < 0.05 \).

3. Results

3.1. Effect of blueberry PC18 fraction on cell viability and LPS-induced iNOS activity

Total phenolics and anthocyanins present in the PC18 fraction were analyzed by HPLC. As illustrated in Fig. 1A, the major polyphenol in PC18 is chlorogenic acid. Figure 2A revealed the anthocyanins present in PC18 fraction. The tentative identities of the peaks were summarized in Table 1.

MTS assay was used to examine whether the concentrations of PC18 used in this study caused cytotoxicity in BV2 cell. As demonstrated in Fig. 2, concentrations of PC18 up to 100 \( \mu \)g/ml did not affect BV2 cell viability in the presence of LPS.

![Image](image_url)

Fig. 1 – Chromatograms of total phenolics (A) and anthocyanins (B) found in blueberry PC18 fraction. Blueberry juice was fractionated by C18 column and washed with 0.1% trifluoroacetic acid (TFA) in water to remove abundant sugars and organic acids. Bound polyphenolics were eluted from the C18 column with 0.1% TFA in methanol. The acidified methanol was removed at 30 °C using a vacuum rotary evaporator and the remaining residue was lyophilized. Total phenolics and anthocyanins in the resulting PC18 fraction were identified by standard HPLC procedures and detected at 280 nm and 520 nm.
iNOS activity was assessed by the level of nitrite in the CCM (Griess, 1879; Phizackerley & Al-Dabbagh, 1983). The addition of LPS significantly stimulated BV2 cells causing a surge of nitrite to be released into the medium. Without LPS-activation, there was very little detectable nitrite in the CCM of the vehicle-control group (Fig. 3). However, LPS elicited a drastic increase in nitrite secretion by BV2 cells as indicated by the LPS-alone treatment group. Treatment of LPS-activated BV2 cells with PC18 significantly reduced the level of nitrite in the CCM in a dose-responsive manner (Fig. 3). It has been reported that LPS treatment of cultured microglial cells caused secretion of cytokines, activation of respiratory burst and induction of nitric oxide synthase (Chun et al., 2005; Zielasek & Hartung, 1996).

### 3.2. Inhibitory effect of PC18 on COX2 and iNOS protein expression

The levels of COX2 and iNOS protein were measured quantitatively by immunoblotting and normalized to the protein expression of internal control β-actin. COX2 and iNOS protein expression was hardly detectable in resting BV2 microglia. COX2 and iNOS protein levels were significantly stimulated after LPS-treatment (Fig. 4). This increase in COX2 and iNOS protein expression in BV2 cells after LPS activation was significantly reduced by PC18 treatment at concentrations of 50 and 100 μg/ml (Fig. 4). PC18 at a concentration of 100 μg/ml reduced COX2 protein expression to 50% and iNOS protein expression to 20% as compared to LPS treatment alone (Fig. 4).
3.3. Suppression of LPS-activated COX2 and iNOS promoter activity by PC18 fraction

To determine whether the alteration in COX2 and iNOS protein levels was due to changes in protein synthesis or protein degradation, modulation of COX2 and iNOS promoter activity by PC18 was investigated with COX2 and iNOS luciferase reporter assay. BV2 cells transiently transfected with COX2 or iNOS-luciferase promoter construct and activated with LPS had a three and twofold increase in COX2 and iNOS promoter activity, respectively (Figs. 5A and B). Treatment with PC18 significantly reduced the LPS-stimulated up-regulation of COX2 and iNOS promoter activities in a dose-responsive manner (Fig. 5A and B). This finding indicated that the inhibitory effect of PC18 on COX2 and iNOS protein expression was at the transcriptional level. The result also suggested that PC18 might act upstream of COX2 and iNOS transcriptional regulation by inhibiting the LPS-mediated signal transduction via NF-κB activation.
3.4. Inactivation of LPS-induced NF-κB nuclear translocation by blueberry PC18 fraction

To evaluate the mechanism of PC18-mediated attenuation of COX2 and iNOS, EMSA was conducted to determine whether PC18 was able to suppress NF-κB nuclear translocation. After four hours of LPS stimulation, nuclear NF-κB protein–DNA binding activity was markedly increased as compared to the control (shifted bands, Fig. 6). Incubation with excess unlabelled NF-κB consensus oligo DNA abolished this shifted band indicating the specificity of nuclear NF-κB protein–DNA binding reaction (data not shown). Co-treatment with PC18 decreased the intensity of this shifted band suggesting that PC18 inhibited the translocation of NF-κB to the nucleus (Fig. 6).

4. Discussion

Previous research showed that blueberry supplementation effectively reduced proinflammatory mediators in both in vitro and in vivo experiments (Goyarzu et al., 2004; Lau et al., 2007; Shukitt-Hale et al., 2008). The results from the current study provided significant insight into the underlying mechanisms by which blueberry polyphenols modulate the expression of inflammatory cytokines. This study revealed that a blueberry polyphenolic-enriched fraction, PC18, significantly inhibited the LPS-induced production of proinflammatory mediators NO, iNOS and COX2 in BV2 microglial cell.

It has been shown that inflammation in the CNS produces oxidative stress through respiratory burst of activated microglia (Floyd & Hensley, 2002; Khanna et al., 2001; Rosen et al.,...
1995). This process creates a vicious cycle through the conversion of neuroinflammation-induced oxidative stress to neurotoxic response which in turn further facilitates inflammation (Colton et al., 1994; Rosen et al., 1995). The inducible enzymes COX2 and iNOS as well as the second messenger NO have been shown to be the major factors in mediating inflammatory processes (Surh et al., 2001). Microglial activation is a well characterized hallmark of inflammation in the CNS (Liu et al., 2002). Chronic activation of microglia may lead to loss of neuronal cells thereby contributing to the progressive damage in neurodegenerative disorders including stroke, multiple sclerosis, Alzheimer’s disease and Parkinson’s disease (McGeer & McGeer, 2004; Orr et al., 2002). This pathological microglial activation is achieved through continued stimulation of inflammatory genes such as COX2 and iNOS as well as sustained production of various proinflammatory mediators including NO, ROS, and TNF-α (Akiyama et al., 2000; Luterman et al., 2000; Tarkowski et al., 2003).

The current findings indicated that polyphenols from blueberry extract significantly suppressed inflammation in LPS-activated BV2 microglia which appeared to indicate that polyphenol-enriched diet might be beneficial in alleviating inflammation associated with neurodegenerative diseases, insofar as these polyphenolic compounds are bioavailable in the brain. In fact, it has been demonstrated that polyphenolic compounds were able to cross the blood brain barrier and localized to various brain regions crucial for learning and memory (Andres-Lacueva et al., 2005). It is conceivable to postulate that the absorption and distribution of polyphenols in the brain have an important impact on their bioavailability and bioactivity. Indeed, a positive correlation was observed between Morris water maze performance and the total number of anthocyanin compounds found in the cortex of aged rats supplemented with blueberry extract. These findings strongly suggest that polyphenolic compounds are bioavailable and they deliver their beneficial effects such as antioxidant and signaling properties directly to the CNS thereby forestalling and even reversing the deleterious effects of aging on neuronal communication, memory, and behavior (Andres-Lacueva et al., 2005; Joseph et al., 2005).

Although the major focus of dietary phytochemicals has been on the antioxidant properties, it is apparent that these phytochemicals and their metabolite in vivo may not serve as conventional hydrogen-donating antioxidants. Rather, these dietary phytochemicals may exert their beneficial actions through modulation of signaling cascades (Williams et al., 2004). Polyphenols including flavonoids and their metabolites have been demonstrated to regulate phosphoinositide 3-kinase, Akt/protein kinase, tyrosine kinases, protein kinase C, and mitogen activated protein kinase signaling pathways (Williams et al., 2004). Corroborating with these findings, the current study demonstrated that blueberry polyphenols significantly suppressed COX2 and iNOS promoter activities. This suppression was probably upstream of COX2 and iNOS transcriptional regulation as indicated by the inhibitory action of blueberry polyphenols on NF-κB nuclear translocation. Therefore, blueberry polyphenols may act as modulators of NF-κB signal transduction. The nuclear factor NF-κB is an eukaryotic ubiquitous proinflammatory transcription factor which is sequestered in the cytoplasm under normal conditions by noncovalently bound to inhibitor IκB (Karim and Greten, 2005). Upon activation, NF-κB translocates into the nucleus to initiate the expression of over 200 immune, growth, and inflammation genes (Aggarwal, 2004). Here we showed that the inhibitory effects of blueberry polyphenols on COX2 and iNOS were due to the attenuation of NF-κB nuclear translocation. Thus the observed cell-signaling properties of blueberry polyphenols may be independent of their traditional antioxidant reducing activities.

Given that increased activation of NF-κB associated with the inflammatory processes is involved in the initiation and progression of diverse diseases, inhibition of NF-κB by blueberry polyphenols may present a powerful means for the development of anti-inflammatory nutraceuticals. In this regard, studies further explore the biomedical significance of blueberry polyphenols in the prevention and treatment of inflammation-related diseases are warranted.

**Acknowledgements**

This research was supported, in part, by U.S. Highbush Blueberry Council and Wild Blueberry Association of North America.

**References**


