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Effects of Elderberry Juice from Different Genotypes on Oxidative and Inflammatory Responses in Microglial Cells

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Abstract

Many species of berries are nutritious food and offer health benefits. However, among the different types of berries, information on health effects of American elderberries (*Sambucus nigra* subsp. *canadensis*) has been lacking and little is known about whether elderberry consumption can confer neuroprotective effects on the central nervous system. Microglial cells constitute a unique class of immune cells and exhibit characteristic properties to carry out multifunctional duties in the brain. Activation of microglial cells has been implicated in brain injury and in many types of neurodegenerative diseases. Our recent studies demonstrated the ability for endotoxin (lipopolysaccharide, LPS) and interferon gamma (IFN γ) to induce reactive oxygen species (ROS) and nitric oxide (NO) in murine microglial cells (BV-2) through activating NADPH oxidase and the MAPK pathways. In this study, BV-2 microglial cells were used to examine effects of elderberry juice obtained from different genotypes on oxidative and inflammatory responses induced by LPS and IFN γ . Results show that ‘Wyldeewood’ extract demonstrated antioxidant properties by inhibiting IFN γ -induced ROS production and p-ERK1/2 expression. On the other hand, most juice extracts exerted small effects on LPS-induced NO production and some extracts showed an increase in NO production upon stimulation with IFN γ . The disparity of responses on ROS and NO production from different extracts suggests possible presence of unknown endogenous factor(s) in the extract in promoting the IFN γ -induced iNOS synthesis pathway.

Keywords

LPS; interferon gamma; nitric oxide; reactive oxygen species; ERK1/2; *Sambucus*

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INTRODUCTION

Evidence on the potential of dietary berry fruits in providing health benefits have been well documented (Joseph et al., 2009; Poulouse et al., 2012; Seeram, 2011). Elderberries (*Sambucus* spp.) are widely grown in Europe, Asia, North Africa, and North America. Traditionally, the flowers and berries have been used in folk medicine for centuries (Anonymous, 2005; Grieve, 1931; Moerman, 1998). The berries contain a wide variety of anthocyanins, flavonoids and other polyphenols (Lee and Finn, 2007; Wu et al., 2004) and these bioactive compounds have the potential to interact with stress signaling pathways and/or to upregulate endogenous defense systems (Son et al., 2008).

The hydrophilic antioxidant capacity for elderberry is among the highest measured in fresh fruits/berries (Wu et al., 2004). Different extracts have been shown to possess anti-inflammatory, antiviral, anti-diabetic, anti-carcinogenic and immune-stimulatory activities (Vlachojannis et al., 2010). However, most publications on elderberry include only a few genotypes of *Sambucus* (subspecies *nigra* and/or *canadensis*). Recently, a number of *S. nigra* subsp. *canadensis* cultivars have been developed in the USA, and their anthocyanin and phenolic profiles can be substantially different (Byers et al., 2010; Byers and Thomas, 2011; Lee and Finn, 2007; Thomas et al., 2013). In the present study, elderberries (subsp. *canadensis*) from eight genotypes were selected and used to test their effects on microglial activation.

Microglial cells, the resident macrophages in the central nervous system, are known to play an important role in inflammatory responses in the brain (Block et al., 2007). Microglial activation is associated with the release of reactive oxygen species (ROS), nitric oxide (NO), glutamate, cytokines, phospholipases and proteases (Brown and Neher, 2010). Upon exposure to cytokines and/or lipopolysaccharide (LPS), specific signal transduction pathways are activated which regulate the induction of proinflammatory cytokines, iNOS and other neurotoxic mediators (Glass et al., 2010; Spencer et al., 2012). Several studies, including ours, have demonstrated the critical involvement of NADPH oxidase-dependent redox signaling and the ERK1/2 pathway in oxidative and inflammatory responses in microglial cells (Chuang et al., 2013).

In this study, elderberry juices were obtained from different genotypes harvested from the same location/time. The extracts from different genotypes were used to test for oxidative and inflammatory responses upon stimulation by LPS or IFN γ in microglial cells.

MATERIALS and METHODS

Materials

Elderberries from 8 genotypes including ‘Bob Gordon’, ‘Dallas’, ‘Ocoee’, ‘Ozone’, ‘Sperandio’, ‘Wyldeewood’, ‘York’ (*S. nigra* subsp. *canadensis*) and ‘Marge’ (*S. nigra* subsp. *nigra*) were harvested at the same location of Mt. Vernon (MO, USA) in 2011. Fruit production details are described in Thomas et al. (2015). Berries were immediately frozen upon harvest, and later were thawed, de-stemmed, French-pressed, and the juices centrifuged, filtered, lyophilized, and dissolved in DMSO. Dulbecco’s modified Eagle’s

medium (DMEM), penicillin, streptomycin, 0.05% (w/v) trypsin/EDTA, and phosphate-buffered saline (PBS) were obtained from GIBCO (Gaithersburg, MD, USA). Interferon- γ (IFN γ) was purchased from R & D Systems (Minneapolis, MN, USA). Lipopolysaccharide (LPS) (rough strains) from *Escherichia coli* F583 (Rd mutant) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum was from Atlanta Biologicals (Lawrenceville, GA, USA).

Cell Culture and Treatments

The immortalized murine microglial cells (BV-2) were prepared as previously described (Shen et al., 2005). Cells were cultured in 75 cm² flasks with DMEM (high glucose) supplemented with 10% FBS containing 100 units/ml penicillin and 100 μ g/ml streptomycin, and maintained in 5% CO₂ incubator at 37°C. Cells were subcultured in 12-, 24- or 96-well plates for experiments. Cell viability under different treatment conditions was assessed using the MTT assay protocol. Cells were serum starved for 3 h prior to adding elderberry samples (12.5–200 μ g/ml) for 1 h and then treatment with IFN γ (10 ng/ml) or LPS (100 ng/ml) for 12 h (ROS) or 16 h (NO).

Assessment of Cell Viability

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, Sigma-Aldrich, St. Louis, MO) assay was used to measure cell viability (Sheng et al., 2011). After IFN γ /LPS treatments, medium was removed and 1 ml of MTT reagent (0.5 mg/ml) in serum free DMEM was added into each well. Cells were incubated for 4 h at 37°C, and after dissolving the formazan dye with DMSO, absorption was read at 570 nm using a Synergy4 Plate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

Nitric Oxide Determination in Culture Medium

NO released from cells was converted to nitrite in the culture medium, which was determined using the Griess protocol (Sheng et al., 2011). Cells were cultured in DMEM without phenol red. 16 h after IFN γ /LPS treatments, aliquots (50 μ l) of culture conditioned medium were transferred to 96-well plates and incubated with 50 μ l of reagent A [1% (w/v) sulfanilamide (Sigma-Aldrich, St. Louis, MO, USA) in 5% phosphoric acid] for 10 min at room temperature in the dark. This was followed by incubation with 50 μ l of reagent B [0.1%, w/v, N-1-naphthylethylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, MO, USA)] for 10 min at room temperature in the dark and measurement of the absorbance at 543 nm using the Synergy4 Plate Reader. Sodium nitrite (0–100 μ M), diluted in culture media, was used to prepare the nitrite standard curve.

Measurement of Reactive Oxygen Species Production

ROS production was measured using CM-H₂DCFDA (DCF, Invitrogen, Inc., Grand Island, NY, USA) as described previously (Chuang et al., 2013). Briefly, cells were incubated in serum free DMEM for 3 h, treatment with juice extract for 1 h and stimulation with IFN γ /LPS for 11 h. DCF (10 μ M) was added to each well for 1 h. The fluorescence intensity of DCF was measured using the Synergy4 Plate Reader with an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

Western Blot Analysis

Western blots were performed as described previously (Chuang et al., 2013; Sheng et al., 2011). After treatments, cells were washed twice with ice-cold PBS and harvested in lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl, 0.1% SDS, 1 mM PMSF, 1 mM sodium orthovanadate, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 10 µg/ml aprotinin). The extract was centrifuged at $10,000 \times g$ for 15 min at 4°C. Protein concentration was determined by the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equivalent amounts of protein (5 µg) for each sample were resolved in 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to 0.45 µm nitrocellulose membranes and incubated in Tris-buffered saline, pH 7.4 (TBS) with 0.1% Tween 20 (TBS-T) containing 5% non-fat milk for 1 h at room temperature. The blots were then incubated with iNOS (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or ERK1/2, p-ERK1/2 antibodies (1:2000; Cell Signaling, Beverly, MA, USA) overnight at 4°C. After washing with TBS-T, they were incubated with goat anti-rabbit IgG-horseradish peroxidase (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat anti-mouse IgG-horseradish peroxidase (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Immunolabeling was detected by chemiluminescence (SuperSignal West Pico, Pierce, Rockford, IL, USA). Blots were scanned and the intensity of protein bands was measured as optical density using the Quantity-One program (BioRad, Hercules, CA, USA).

Statistical Analysis

Data are shown as mean $\pm$ SEM from three to five independent experiments. Results were analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests or two-way ANOVA with Bonferroni posttests (V4.00; GraphPad Prism Software Inc., San Diego, CA, USA). Differences were considered significant at $p < 0.05$ for all analyses.

RESULTS

Induction of iNOS is known to involve transcription factors from the NF- κ B and the IFN γ -induced JAK-STAT pathways (Shen et al., 2005). In our recent study, we observed that IFN γ not only stimulates the canonical JAK-STAT pathway, but also the MAPK pathway, including the phosphorylation of ERK1/2 (Chuang et al., 2013). In addition, our study further demonstrated a link between IFN γ -induced p-ERK1/2 with activation of NADPH oxidase, the enzyme responsible for production of ROS. Since ERK1/2 activation plays an important role in mediating a number of metabolic pathways in microglial cells, we tested the ability of elderberry juice extracts to mitigate IFN γ -induced p-ERK1/2. As shown in Figure 1, IFN γ (10 ng/ml) elicited a robust increase in p-ERK1/2, and elderberry juice from the 'Wyldeewood' genotype decreased IFN γ -induced p-ERK1/2 expression at both low (12.5 µg/ml) and high doses (100 µg/ml).

Since IFN γ -induced p-ERK1/2 expression was linked to ROS production through phosphorylation of NADPH oxidase subunits (Chuang et al., 2013), we further tested effects of elderberry juice extracts on IFN γ -induced ROS production in microglial cells. As shown

in Figures 2A and B, elderberry juice, especially from the ‘Wyldeewood’ genotype, effectively inhibited ROS production stimulated by either LPS or IFN γ .

In our earlier studies, several botanicals tested could effectively inhibit LPS- and IFN γ -induced NO (Chuang et al., 2013). In this experiment, however, the elderberry juice extracts exerted little or no inhibitory effect on LPS-induced NO production (Fig. 3A). On the other hand, although the ‘Wyldeewood’ juice extract showed only minimal effects on LPS-induced NO production (Fig. 3A), there was a dose-dependent increase in NO production upon stimulation with IFN γ (Fig. 3B). Western blot analysis also confirmed the correlative increase in IFN γ -induced iNOS protein expression upon pretreatment with ‘Wyldeewood’ (Fig. 3C). Based on results with the ‘Wyldeewood’ juice extract, we further tested other genotypes for their ability to increase NO production in response to treatment with IFN γ . Among the juice extracts from 8 genotypes, extracts from ‘Wyldeewood’, ‘Ozone’, and ‘Sperandio’ genotypes showed significant increases in IFN γ -induced NO production, testing at 50 μ g/ml (Table 1). None of these samples tested influenced cell viability as determined by the MTT assay (data not shown).

DISCUSSION

Our earlier studies demonstrated ability for polyphenols from a number of botanical sources to inhibit LPS- and IFN γ -induced oxidative and inflammatory responses in microglial cells (Chuang et al., 2013). These earlier studies also unveiled an important role for IFN γ , which not only could activate the canonical JAK-STAT transcriptional pathway, but also stimulates the MAPK signaling pathways. In addition, IFN γ -induced phosphorylation of ERK1/2 is linked to activate multiple metabolic pathways including production of ROS through NADPH oxidase and induction of iNOS (Chuang et al., 2013). These studies lead to the conclusion that compounds from plant extracts or phytochemicals that mitigate the IFN γ /p-ERK1/2 pathway can suppress ROS and NO production in microglial cells.

In the present study, elderberry juice extracts could inhibit ROS induced by LPS and IFN γ , and the ‘Wyldeewood’ extract also inhibited IFN γ -induced p-ERK1/2 expression. However, a screening of the different genotypes on LPS- and IFN γ -induced NO production revealed little or no inhibitory effects. In fact, some extracts even showed a small but dose-dependent increase in NO production in response to stimulation with IFN γ . Induction of iNOS is known to require multiple transcription factors, including those from the NF- κ B pathway and the IFN γ -induced JAK-STAT pathways (Shen et al., 2005). However, in microglial cells, LPS or IFN γ alone can induce iNOS, suggesting the presence of a cross-talk mechanism between the NF- κ B and the JAK-STAT pathways (Sheng et al., 2011). The increase in NO production in elderberry juice in response to IFN γ in this study is a surprising finding which needs to be further explored. Since many active components are present in the juice extract, it is possible that an unknown factor(s) in the extract can enhance the IFN γ -NO pathway. There is evidence that factors, such as the granulocyte macrophage-colony factor, activate microglia and increase NO production through action on NF- κ B pathways and MAPKs (Parajuli et al., 2012). Therefore, it is possible that unknown factors capable of interacting with the NF- κ B pathway are present in the ‘Wyldeewood’ and other extracts to enhance

IFN γ -induced NO production. Obviously, more studies are needed to explore this phenomenon and identify the factor(s).

Depending on the cell types and organs, NO is produced by three types of NOS (iNOS, eNOS and nNOS), and is known to serve as an important signaling molecule in mediating a number of physiological events in the body. NO can play a cytotoxic or cytoprotective action by regulating inflammatory and immune responses. On the other hand, although excessive NO production in microglial cells is deemed deleterious and can cause neuronal damage (Brown, 2010), small increases at low levels may offer positive effects instead. Many berry species have been shown to offer antioxidant properties and play a role in modulating a variety of signaling pathways. Based on results obtained from this study, elderberry juice extracts seem to have better anti-oxidative properties than anti-inflammatory effects on microglial cells. However, further research is warranted to test whether the antioxidant effects of elderberry juice can be translated to neuroprotective mechanisms in vivo.

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Fig. 1.

'Wyldewood' elderberry juice inhibits IFN γ -induced activation of p-ERK1/2 in BV-2 microglial cells. Western blot analysis showing a representative experiment of elderberry juice extract pretreatment on IFN γ -induced ERK1/2 phosphorylation in BV-2 microglial cells. Cells were treated with elderberry juice extract (12.5 or 100 µg/ml) for 1 h followed by stimulation with IFN γ (10 ng/ml) for 8 h. Bottom line: The blot was scanned and pERK1/2 versus total tERK1/2 ratios were obtained using IFN γ treatment sample as 100.

Fig. 2. ‘Wyldewood’ elderberry juice inhibits LPS- or IFN γ -induced ROS production in BV-2 microglial cells. Elderberry juice extracts (0 to 100 μ g/ml) were applied to cells 1 h prior to exposure to either LPS (100 ng/ml)(A) or IFN γ (10 ng/ml) (B) for 12 h. ROS production was measured using CM-H2DCFDA as described in the text. Results are expressed as the mean $\pm$ SEM ($n = 3$) and analyzed by two-way ANOVA with Bonferroni posttests. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ indicate significant differences from the respective LPS/IFN γ -stimulated group.

Fig. 3.

'Wyldewood' elderberry juice facilitates IFN γ -induced NO production and iNOS expression in BV-2 microglial cells. A. 'Wyldewood' extracts (0 to 100 µg/ml) were applied to cells 1 h prior to exposure to either (A) LPS (100 ng/ml) or (B) IFN γ (10 ng/ml) for 16 h. Culture media were taken for measurement of NO as described in text. Results are expressed as the mean $\pm$ SEM ($n = 4$) and analyzed by one-way ANOVA with Dunnett's tests. * $p < 0.05$; ** $p < 0.01$ indicate significant differences from the respective LPS/IFN γ -stimulated group. C. Cells were treated with 'Wyldewood' juice extract (12.5 or 100 µg/ml) for 1 h followed by stimulation with IFN γ (10 ng/ml) for 16 h. Representative Western blots of iNOS protein and β -actin.

Table 1

Effects of elderberry extracts (various genotypes) on LPS or IFN γ -induced NO production in BV-2 microglial cells.

Genotype	NO production (% of control $\pm$ SEM)	
	LPS	IFN γ
Ocoee	91.5 $\pm$ 7.0	128.5 $\pm$ 11.7
Marge	95.5 $\pm$ 5.4	115.6 $\pm$ 3.9
Wyldeewood	93.4 $\pm$ 2.0	138.4 $\pm$ 10.9 *
Ozone	109.2 $\pm$ 15.1	151.1 $\pm$ 5.2 **
Dallas	102.4 $\pm$ 5.8	129.2 $\pm$ 3.5
Bob Gordon	91.9 $\pm$ 23.3	112.0 $\pm$ 3.3
Sperandio	90.0 $\pm$ 1.8	158.8 $\pm$ 20.9 **
York	109.0 $\pm$ 12.4	117.3 $\pm$ 3.4

After treatment with elderberry juice extracts (50 μ g/ml each) for 1 h, LPS (100 ng/ml, 16 h) or IFN γ (10 ng/ml) was added for 16 h. NO was determined by the Griess reaction assay. Data are expressed as percentages of the mean value of control (either LPS or IFN γ alone). Values are mean $\pm$ SEM of 3–5 separate experiments.

*
p<0.05,

**
p<0.01 vs. control.