Stilbenes and Anthocyanins Reduce Stress Signaling in BV-2 Mouse Microglia

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Ⓢ Supporting Information

ABSTRACT: Blueberries contain an array of phytochemicals that may decrease both inflammatory and oxidative stress. This study determined if pterostilbene, resveratrol, and two anthocyanins commonly found in blueberries, delphinidin-3-O-glucoside and malvidin-3-O-glucoside, would be efficacious in protecting microglia from inflammatory-induced stress signaling. Microglia that were pretreated with blueberry extract (0.25, 0.5, 1, 2 mg/mL) or its components (1, 10, 20, 30 μM pterostilbene, resveratrol, delphinidin-3-O-glucoside, or malvidin-3-O-glucoside) prior to exposure to lipopolysaccharide (100 ng/mL) demonstrated concentration-dependent reductions in nitric oxide and tumor necrosis factor-alpha release and decreased expression of inducible nitric oxide synthase and cyclooxygenase-2. However, much higher concentrations of the individual components than those found in blueberries were needed to demonstrate the effects. For example, 1 mg/mL blueberry extract significantly reduced LPS-induced nitric oxide release; this concentration of blueberry extract contains 2.6 μM malvidin-3-O-glucoside, but when malvidin-3-O-glucoside was tested individually, 20 μM was necessary to observe a significant reduction in nitric oxide release. Therefore the protective effects of blueberries may not be due to any one component, but rather a synergism of the activity of the compounds tested and/or other blueberry compounds not tested here. These results lend further support that blueberry and its active components are able to combat some of the inflammatory mediators of aging at the cellular level.

KEYWORDS: stilbene, pterostilbene, resveratrol, anthocyanin, blueberry, inflammation, microglia, TNF-α

■ INTRODUCTION

Brain aging, injury, and other pathological processes are associated with the activation of microglia and subsequent neuronal dysfunction. Short-term activation of brain microglia can be beneficial,[1] but microglia that remain in an activated state for a long duration may exacerbate neuronal dysfunction, possibly through the secretion of potentially cytotoxic substances.[2] For example, microglia secrete nitric oxide and tumor necrosis factor (TNF)-alpha (α), which, when produced in excessive amounts, may result in death and dysfunction of neurons.[3,4] Therefore, suppressing microglia activation and the production of cytotoxic intermediates may restore homeostasis in the aged or disease brain.

Previous research has demonstrated that blueberries can decrease activation of microglia in rat brain[5] as well as abrogate the production of nitric oxide, TNF-α, and cyclooxygenase (COX)-2 in BV-2 mouse microglia in vitro.[6] Blueberries contain an array of phytochemicals, such as stilbenes and anthocyanins, which can decrease inflammatory responses and oxidative stress and may be responsible for the effects on microglia. Resveratrol, found in grape skins, peanuts, red wines, and blueberries, is a well-known stilbene[7] that has been shown to be neuroprotective in animal models of neurodegenerative disease.[8−11] Pterostilbene is a stilbenoid derivative of resveratrol and is found in fruits such as blueberries and grapes. Previously it was demonstrated that pterostilbene protected against oxidative stress-induced deficits in calcium buffering in COS-7 cells transfected with oxidative stress sensitive muscarinic receptors[12] and may modulate inflammatory and stress signaling in colon cancer cell models.[7,13] A recent study found that dietary supplementation with pterostilbene was able to prevent cognitive dysfunction in the SAMP8 mouse model of Alzheimer's disease to a greater extent than resveratrol, possibly by preventing down-regulation of peroxisome proliferator-activated receptor α expression.[14] Furthermore, pterostilbene, when fed to aged rats for 12−13 weeks, improved working memory performance in the Morris water maze, which was correlated with pterostilbene levels in the hippocampus.[12]

Additionally, anthocyanins, compounds responsible for the color of some plants, have been shown to have potent antioxidant and anti-inflammatory activities, as well as to inhibit COX-1 and COX-2, enzymes that are responsible for the formation of prostanoids known to be involved in the body's inflammatory response.[15−17] Delphinidin-3-O-β-D-glucoside

and malvidin-3-*O*-β-D-glucoside are two anthocyanins commonly found in blueberries. Research has shown that accumulation of anthocyanins in the brains of aged rats fed a blueberry-supplemented diet was correlated with performance in the Morris water maze.[18]

Given the accumulating evidence that constituents of blueberries may have the potential to mitigate brain alterations associated with aging, we examined whether pterostilbene, resveratrol, delphinidin-3-*O*-glucoside, and malvidin-3-*O*-glucoside are able to reduce the production of cytotoxic intermediates released by microglia exposed to lipopolysaccharide (LPS), which is used in vitro to activate microglia.[19] The ability of these blueberry components to mediate microglia responses would suggest that at least in part they may be responsible for the beneficial effects of blueberry and blueberry extract.

■ MATERIALS AND METHODS

Blueberry Compounds. The blueberry extract was a freeze-dried water-soluble extract of *Vaccinium ashei* cultivar Tifblue (rabbiteye blueberry) that was prepared according to previous methods.[5] The phenolic compounds analyzed in this extract are in Table 1 and were

Table 1. Phenolic Compounds in Blueberry Extract[a]

compound	ng/mg blueberry extract	μM in 1 mg/mL blueberry extract
cyanidin-3-*O*-glucoside	153.00	0.3156
cyanidin-3-*O*-rutinoside	65.10	0.1032
delphinidin-3-*O*-glucoside	98.00	0.1956
malvidin-3-*O*-glucoside	1291.00	2.6163
pelargonidin-3-*O*-glucoside	219.00	0.4343
catechin	190.00	0.6546
ferulic acid	7.38	0.0380
pterostilbene	0.59	0.0023
quercetin	23.90	0.0791
resveratrol	3.83	0.0168
vanillic acid	104.00	0.6185

[a]The components tested in this study are in bold font.

determined by high-performance liquid chromatography according to published procedures.[20] Delphinidin-3-*O*-glucoside (del-3-gluc) and malvidin-3-*O*-glucoside (mal-3-gluc) were purchased from Chromadex (Irvine, CA, USA) and were reconstituted in deionized water prior to dilution in cell media for the experiments. Resveratrol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Pterostilbene was synthesized following published procedures.[12] Resveratrol and pterostilbene were reconstituted in DMSO prior to dilution in cell media; the final concentration of DMSO in the cell culture was ≤0.3%. Chemical structures of del-3-gluc, mal-3-gluc, pterostilbene, and resveratrol are shown in Figure 1.

BV-2 Cell Culture. BV-2 murine microglial cells (generously provided by Dr. Van Eldik, University of Kentucky, Lexington, KY, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal calf serum, 100 μ/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified incubator with 5% CO_2. Prior to treatment, cells were grown in a 12-well plate to approximately 75% confluence. Cells were pretreated with blueberry extract (0, 0.25, 0.50, 1.0, 2.0 mg/mL) or del-3-gluc, mal-3-gluc, pterostilbene, or resveratrol (0, 1, 10, 20, and 30 μM) diluted in serum-free DMEM without phenol red or antibiotics for 1 h and were then stimulated with 100 ng/mL LPS (Sigma-Aldrich) overnight.
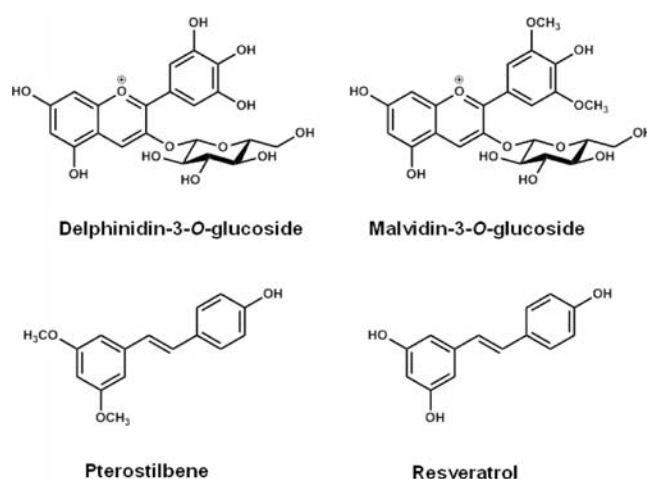

Figure 1. Chemical structures of delphinidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, pterostilbene, and resveratrol.

Nitrite Quantification. To assess the production of nitric oxide from BV-2 cells, extracellular release of nitrite (NO_2^-) was measured by Greiss reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. Cell-conditioned medium (100 μL) was mixed with 25 μL of Greiss reagents in a 96-well plate and incubated at room temperature for 20 min. Absorbance was read at 548 nm, and the concentration of nitrite was calculated with the linear equation derived from the standard curve generated by known concentrations of nitrite.

Western Blots. Western blots were performed as described previously.[21] For inducible nitric oxide synthase (iNOS; 1:1000; EMD Millipore, Billerica, MA, USA) and COX-2 (1:1000; Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA) detection, cells were washed in ice-cold phosphate-buffered saline, resuspended by scraping in a hypotonic lysis buffer (0.25 M sucrose, 5 mM $MgCl_2$, 2 mM EGTA, 2 mM EDTA, 10 mM Tris, pH 7.5, with protease inhibitors), lysed by brief sonication, and centrifuged at 10000g for 5 min at 4 °C to yield the resultant supernatant lysate. Protein concentration of the lysates was quantified with the DC protein assay (Bio-Rad, Hercules, CA, USA). Equal amounts of denatured protein samples were separated by 12% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. Primary antibody incubations were done overnight at 4 °C. Following enhanced chemiluminescence development, the optical densities of antibody-specific bands were analyzed by LabWorks image acquisition and analysis software (UVP, Upland, CA, USA).

Enzyme-Linked Immunosorbent Assay (ELISA). Quantification of TNF-α in cell-conditioned media was performed using ELISA (eBiosciences, San Diego, CA, USA) according to the manufacturer's instructions. TNF-α concentrations were calculated from the linear equation derived from the standard curve of known concentrations of the cytokine.

Data Analysis. Data are represented as the mean ± SEM. Data were analyzed using Systat (SPSS, Inc., Chicago, IL, USA) with two-way ANOVA testing for main effects of pretreatment concentration and LPS condition, as well as an interaction of concentration of pretreatment X LPS (see Supplementary Tables 1 and 2 in the Supporting Information, respectively, for ANOVA results). Significant effects were further analyzed with Tukey's honestly significant difference (HSD) post hoc tests. Note that there were no significant differences in nitric oxide release, iNOS expression, COX-2 expression, and TNF-α production in cells that were incubated with blueberry extract, del-3-gluc, mal-3-gluc, pterostilbene, and resveratrol without subsequent LPS exposure at any concentration tested compared to cells that were treated only with medium.
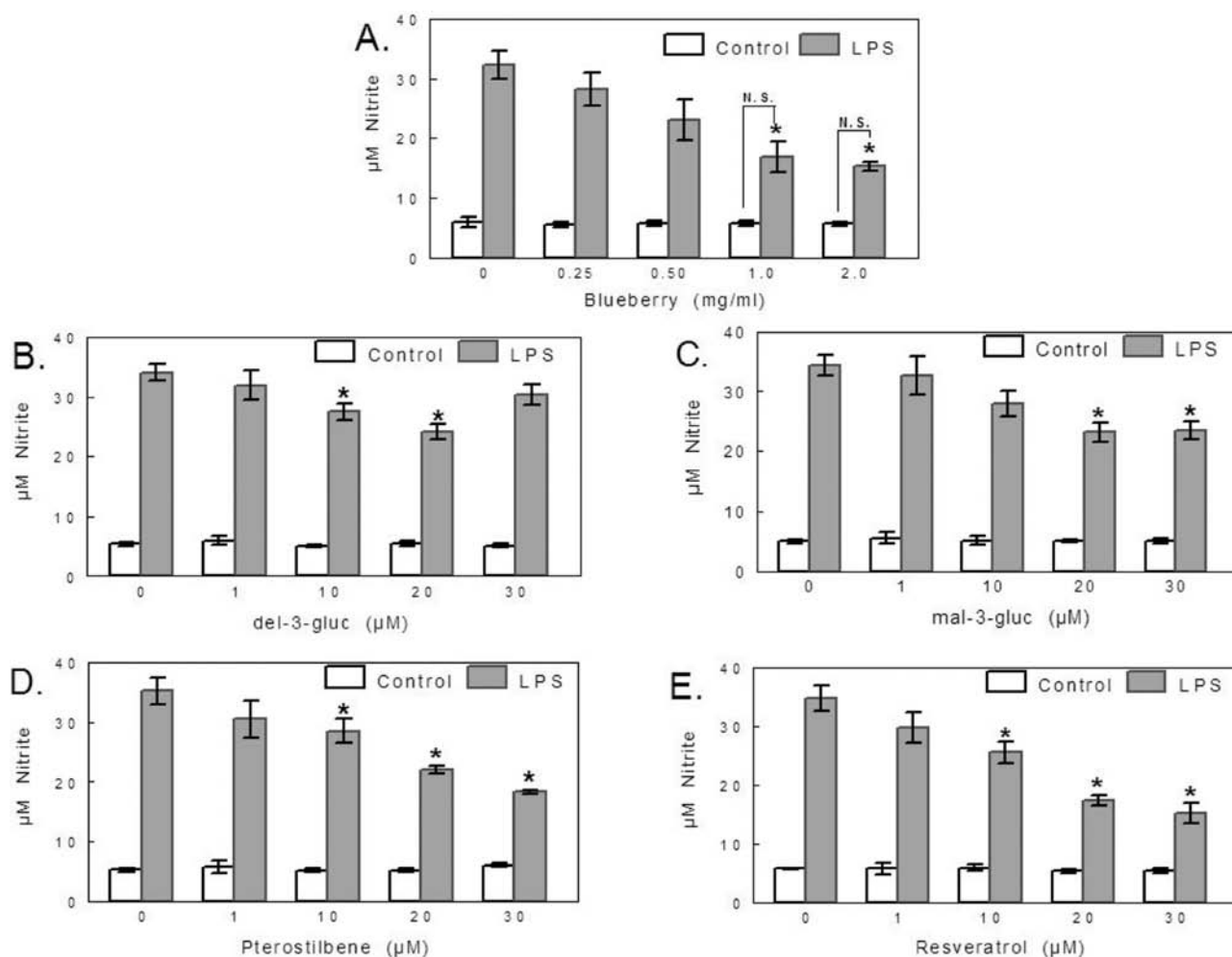
Figure 2. Nitric oxide release in microglia pretreated with blueberry extract (A), delphinidin-3-O-glucoside (B), malvidin-3-O-glucoside (C), pterostilbene (D), and resveratrol (E) prior to LPS exposure. ∗ = different from cells exposed to only LPS, $p < 0.05$, N.S. = not different from concentration-matched control condition, Tukey's HSD.

■ RESULTS

Pretreatment of Microglia with Blueberry Extract, Del-3-gluc, Mal-3-gluc, Pterostilbene, or Resveratrol Attenuated LPS-Induced Nitric Oxide Production. In cells that were exposed to only medium, microglia produced 5.95 ± 0.81 μM nitrite (Figure 2A), which was significantly increased when cells were exposed to LPS (32.23 ± 2.23 μM nitrite; $p < 0.001$ versus control, Tukey's HSD). The 1.0 and 2.0 mg/mL concentrations of blueberry extract significantly attenuated nitrite production (vs LPS control $p < 0.001$). The lower concentrations of blueberry extract, 0.25 and 0.50 mg/mL, did not have a significant effect on nitrite production in cells exposed to LPS.

Pretreatment of microglia cells with 10 and 20 μM, but not 1 or 30 μM, del-3-gluc significantly attenuated LPS-induced nitric oxide release compared to cells that were exposed to only LPS (Figure 2B, $p < 0.05$). Pretreatment of microglia cells with 20 and 30 μM, but not 1 or 10 μM, mal-3-gluc significantly attenuated LPS-induced nitric oxide release (Figure 2C, vs LPS control, $p < 0.001$).

Pretreatment of microglia cells with 10, 20, and 30 μM pterostilbene significantly attenuated LPS-induced nitric oxide release compared to cells that were exposed to LPS only (Figure 2D, $p < 0.05$). This was a concentration-dependent

effect; 10 μM was not as effective as 30 μM pterostilbene at reducing LPS-induced nitric oxide release ($p < 0.05$), but the 20 μM pterostilbene condition was not different from 10 or 30 μM conditions. Pretreatment of microglia cells with 10, 20, and 30 μM resveratrol also significantly attenuated LPS-induced nitric oxide release compared to cells that were exposed to LPS only (Figure 2E, $p < 0.05$). A concentration effect was also apparent, as 20 and 30 μM concentrations of resveratrol were more effective at reducing nitric oxide release than 10 μM resveratrol ($p < 0.01$).

Pretreatment of Microglia with Blueberry Extract, Del-3-gluc, Mal-3-gluc, Pterostilbene, or Resveratrol Reduced the Expression of iNOS Induced by LPS. LPS significantly increased the expression of iNOS compared to control cells (Figure 3A, $p < 0.001$, Tukey's HSD). Expression of iNOS was significantly attenuated when cells were pretreated with 1.0 and 2.0 mg/mL blueberry extract prior to LPS exposure compared to cells that were exposed to only LPS ($p < 0.01$, Tukey's HSD). In concordance with the nitric oxide release data, the lower concentrations of blueberry extract (0.25 and 0.50 mg/mL) did not reduce LPS-induced iNOS expression. Pretreatment of microglia cells with 20 and 30 μM del-3-gluc, pterostilbene, and resveratrol significantly reduced the expression of iNOS induced by LPS (Figure 3B,
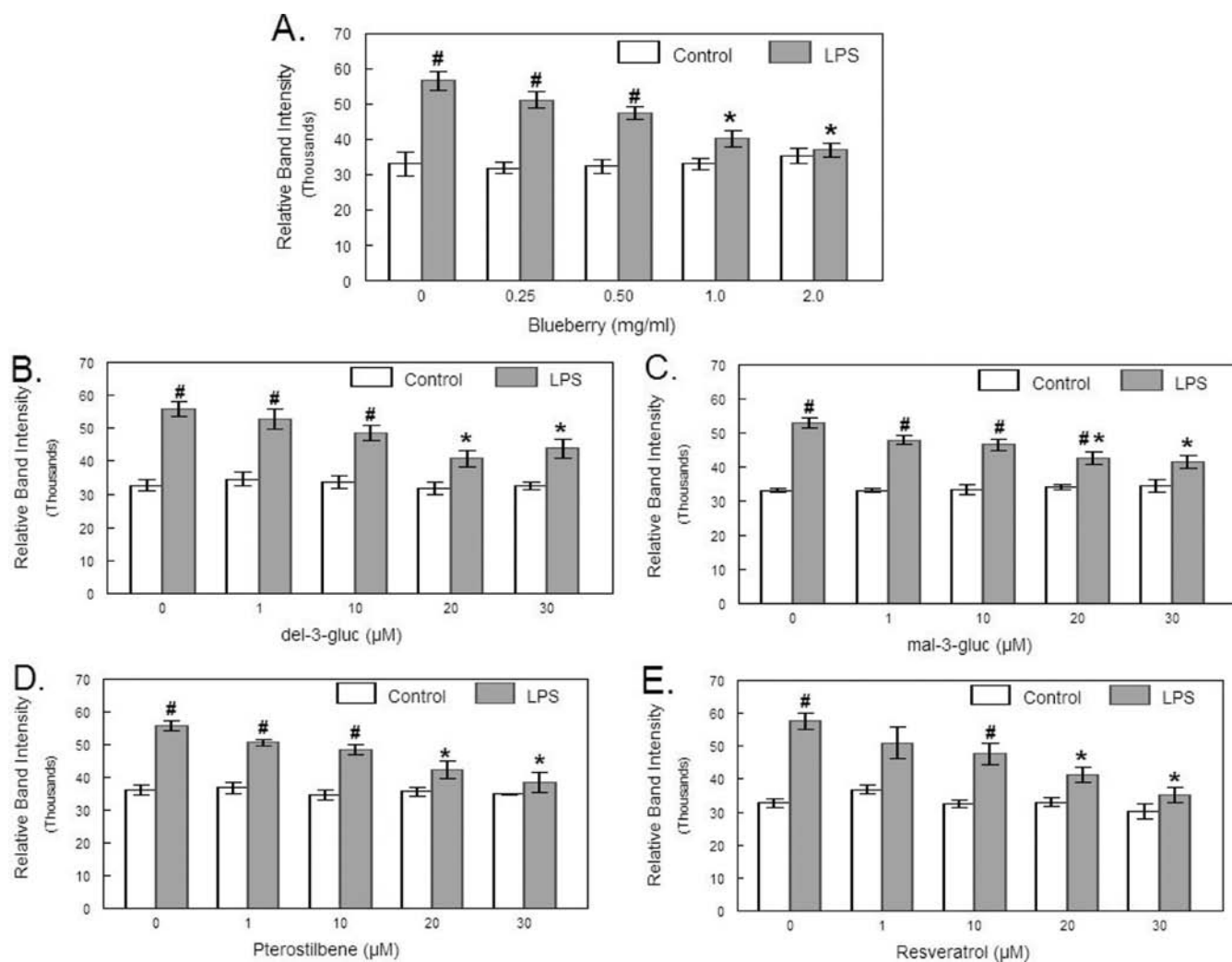
Figure 3. iNOS expression in microglia pretreated with blueberry extract (A), delphinidin-3-*O*-glucoside (B), malvidin-3-*O*-glucoside (C), pterostilbene (D), and resveratrol (E) prior to LPS exposure. ∗ = different from cells exposed to only LPS; # = different from concentration-matched control condition, $p < 0.05$, Tukey's HSD.

$p < 0.05$; Figure 3D, $p < 0.001$; Figure 3E, $p < 0.001$, respectively), whereas only the highest concentration of mal-3-gluc was able to attenuate LPS-induced iNOS expression (30 μM, Figure 3C, $p < 0.001$).

Pretreatment of Microglia with Blueberry Extract, Del-3-gluc, Pterostilbene, or Resveratrol Reduced the Expression of COX-2 Induced by LPS. LPS significantly increased the expression of COX-2 compared to control cells (Figure 4A, $p < 0.001$, Tukey's HSD). Expression of COX-2 was significantly attenuated when cells were pretreated with 0.5, 1.0, and 2.0 mg/mL, but not 0.25 mg/mL, blueberry extract prior to LPS exposure (vs LPS control, $p < 0.001$, Tukey's HSD). Pretreatment of microglia cells with 20 and 30 μM del-3-gluc significantly reduced the expression of COX-2 induced by LPS compared to cells that were exposed to only LPS (Figure 4B, $p < 0.01$). Mal-3-gluc pretreatment did not have a significant effect on COX-2 expression (Figure 4C). Pretreatment of microglia cells with 20 and 30 μM pterostilbene significantly attenuated LPS-induced expression of COX-2 (Figure 4D, $p < 0.01$). This was a concentration-dependent effect, as 30 μM pterostilbene was more effective at reducing the level of COX-2 than 20 μM ($p < 0.05$). Pretreatment of microglia cells with 10, 20, and 30 μM resveratrol also

significantly attenuated LPS-induced COX-2 expression (Figure 4E, vs LPS control, $p < 0.01$).

Pretreatment of Microglia with Blueberry Extract, Del-3-gluc, Mal-3-gluc, Pterostilbene, or Resveratrol Reduced the Production of TNF-α Induced by LPS. LPS significantly increased the expression of TNF-α compared to control cells (Figure 5A, $p < 0.001$, Tukey's HSD). TNF-α production was significantly attenuated when cells were pretreated with 1.0 and 2.0 mg/mL blueberry extract prior to LPS exposure, compared to cells that were exposed to only LPS ($p < 0.001$). Pretreatment of microglia cells with 20 and 30 μM del-3-gluc or mal-3-gluc significantly attenuated LPS-induced production of TNF-α (Figure 5B,C, respectively, $p < 0.001$). Pretreatment of microglia cells with all concentrations of pterostilbene significantly reduced LPS-induced TNF-α production (Figure 5D, $p \leq 0.01$). This was a concentration-dependent effect; 1 μM was not as effective as the higher doses of pterostilbene at reducing LPS-induced TNF-α production, and 10 μM was not as effective as 20 and 30 μM ($p < 0.05$). Pretreatment of microglia cells with all concentrations of resveratrol also significantly reduced LPS-induced TNF-α production (Figure 5E, $p < 0.001$). A concentration effect was also apparent, as 20 and 30 μM resveratrol pretreatments
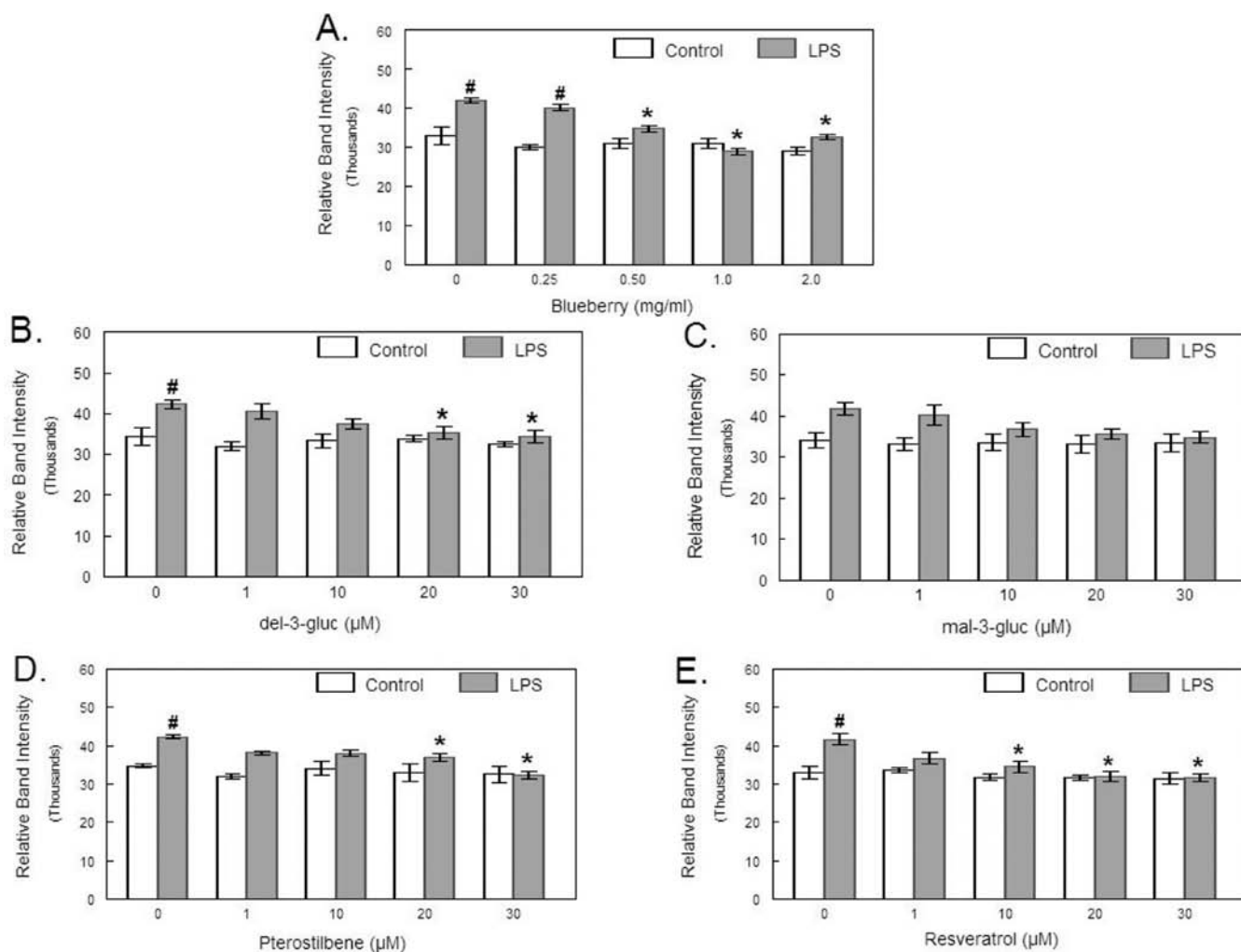
Figure 4. COX-2 expression in microglia pretreated with blueberry extract (A), delphinidin-3-O-glucoside (B), malvidin-3-O-glucoside (C), pterostilbene (D), and resveratrol (E) prior to LPS exposure. ∗ = different from cells exposed to only LPS; # = different from concentration-matched control condition, $p < 0.05$, Tukey's HSD.

were more effective at reducing the amount of TNF-α produced after exposure to LPS than 1 and 10 μM resveratrol pretreatments ($p < 0.001$).

■ DISCUSSION

The current study demonstrates that pretreating BV-2 microglia with blueberry extract or individual components of blueberry desensitized the microglia to the inflammatory actions of LPS. Specifically, blueberry extract, pterostilbene, and resveratrol (two stilbenes found in blueberry) reduced LPS-induced nitric oxide and TNF-α release, as well as decreased the expression of iNOS and COX-2. To a lesser extent the two blueberry anthocyanins, del-3-gluc and mal-3-gluc, also suppressed the secretion of potentially cytotoxic substances. The results demonstrating that blueberry extract inhibited the production of the inflammatory mediator nitric oxide and the cytokine TNF-α are in line with previous research from our laboratory.[6] However, this is the first study to examine the effects of these constituents of blueberries individually on microglia in an attempt to determine which component(s) may be mediating the anti-inflammatory effect of blueberry. This is important because brain aging, injury, and other pathological processes are associated with chronic inflammation, which is thought to be mediated largely by activation of microglia.[2,22,23]

Previous research with stilbenes has demonstrated their potential health benefits. Resveratrol has been shown to be neuroprotective in animal models of neurodegenerative diseases, such as Alzheimer's and Huntington's disease, possibly due to antioxidant activity[8−10] or anti-inflammatory activity.[11] Previously it was demonstrated that pterostilbene reversed the deleterious effects of aging on cognitive performance in rats and also increased the sensitivity of striatal muscarinic receptors.[12] Although this is the first study to explore the effects of pterostilbene on brain microglia, concentrations of pterostilbene similar to the ones used in this study have been shown to modulate inflammatory and stress signaling by reducing, for example, iNOS and TNF-α in colon cancer cell models.[7,13] Moreover, pterostilbene, when fed to aged rats for 12−13 weeks, improved working memory performance in the Morris water maze, which was correlated with pterostilbene levels in the hippocampus.[12] Pterostilbene, but not resveratrol, significantly prevented cognitive dysfunction in the SAMP8 mouse model of Alzheimer's disease.[14] Our data suggest that the beneficial effects of stilbenes demonstrated by these studies, at least in part, may be due to stilbenes reducing or preventing the release of cytotoxic intermediates such as TNF-α by microglia.

For stilbenes to affect microglia cells in vivo, they must be bioavailable to the brain. There is evidence that pterostilbene is
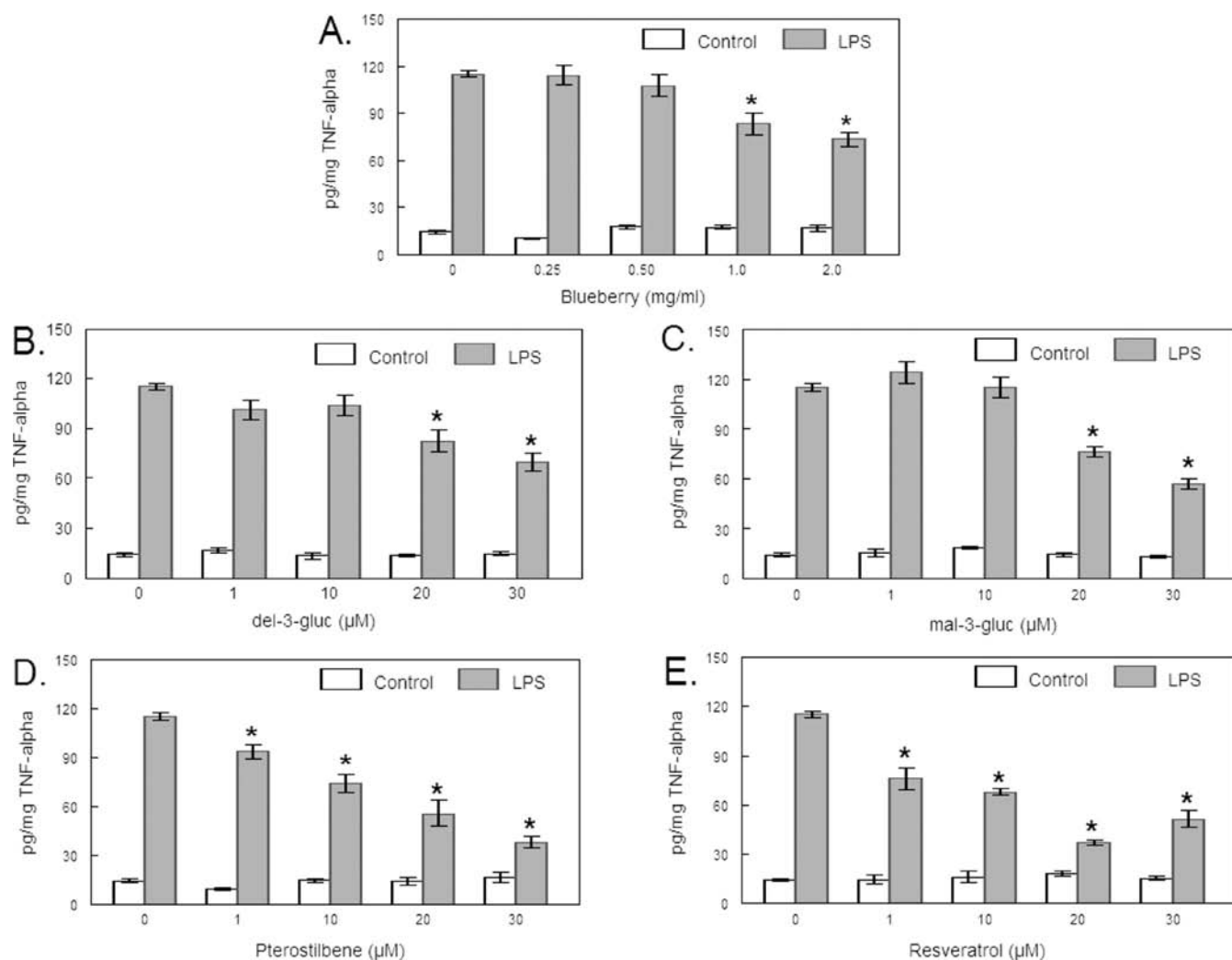
Figure 5. TNF-α expression in microglia pretreated with blueberry extract (A), delphinidin-3-O-glucoside (B), malvidin-3-O-glucoside (C), pterostilbene (D), and resveratrol (E) prior to LPS exposure. All gray bars are different from concentration-matched control conditions; $*$ = different from cells exposed to only LPS, $p < 0.05$, Tukey's HSD.

more orally bioavailable than resveratrol[24,25] and may be more easily transported into cells and more resistant to degradation and elimination in the body.[26] This may be due to the substitution of two hydroxy groups in resveratrol with methoxy groups, which would make pterostilbene more lipophilic and more easily diffusible through the blood−brain barrier.[27] Our data suggest that pterostilbene may have efficacy similar to that of resveratrol in vitro; however, in the context of the whole organism, pterostilbene may be more bioavailable than resveratrol. This difference in bioavailability could result in different levels of protection offered by these two stilbenes and could possibly explain the more significant behavioral effects observed after dietary supplementation with pterostilbene as noted in the study of Chang et al.[14]

Notably, when the individual compounds were tested at similar concentrations found in 1.0 mg/mL (lowest effective blueberry dose), they were not effective (see Table 1). Therefore, the protective effects of blueberry are probably not due to any one compound, but rather a synergism of the activity of the compounds tested, as well as those of other blueberry compounds not tested here, such as cyanidin-3-O-glucoside, catechin, quercetin, and vanillic acid (Table 1). Indeed, these other phenolic compounds found in the extract

have been shown to have anti-inflammatory properties. For example, cyanidin-3-O-glucoside has been reported to suppress the production of the pro-inflammatory cytokines, TNF-α and interleukin (IL)-1β, and the inflammatory mediators, nitric oxide and prostaglandin E2, as well as the gene expression of iNOS and COX-2 in RAW 264.7 cells.[28] In animal studies, catechin ameliorated alcohol-induced liver injury in rats by down-regulating activation of nuclear factor kappa B (NF-κB) and further downstream signaling cascades, including TNF-α, nitric oxide, and reactive oxygen species.[29] In macrophages exposed to LPS, quercetin inhibited the activation of NF-κB and also inhibited the activation of the signal transducer and activator of transcription 1 (STAT-1), another important transcription factor for iNOS.[30] Using a rat model of myocardial ischemia and reperfusion, quercetin was shown to attenuate the expression of both TNF-α and IL-10 and lower the serum levels of inflammatory cytokines.[31] Vanillic acid was demonstrated to exert protective effects in isoproterenol-induced cardiotoxic rats due to its free radical scavenging, antioxidant, and anti-inflammatory properties.[32] Pretreatment with vanillic acid showed significant protective effects on expressions of IL-1β, IL-6, and TNF-α gene in the heart of isoproterenol-induced cardiotoxic rats. In mouse peritoneal

macrophages vanillic acid inhibited LPS-induced production of TNF-α and IL-6, and during the inflammatory process, vanillic acid suppressed both the enhanced levels of COX-2 and production of prostaglandin E2 and nitric oxide.[33]

Our results, similar to those of our previous studies with blueberries, lend further support that blueberry and its active components may contribute to "health span" in aging, as they are able to combat some of the inflammatory and oxidative mediators of aging at the cellular level. Future research should determine other possible mediators of blueberry's beneficial effect, such as other anthocyanins. It must be noted, however, that anthocyanins have generally been reported to have poor bioavailability compared to other flavonoids.[34] In particular, mal-3-gluc, having the highest concentration of the phenols analyzed in the extract, was shown to be poorly absorbed in a human study that involved ingestion of wine.[35]

The mechanisms by which stilbenes and anthocyanins may reduce the release of cytotoxic intermediates from microglia remain to be determined. One possibility is that these compounds could result in the internalization or even antagonization of Toll-like receptor-4 (TLR4). LPS is recognized by this receptor, and activation of TLR4 induces a signaling cascade that activates NF-κB and the production of pro-inflammatory cytokines. It has been demonstrated previously that limiting the interaction of LPS with its receptor reduces the production of cytokines and other cytotoxic intermediates released by microglia.[21] Indeed, resveratrol has been reported to significantly diminish TLR4 expression in LPS-stimulated human intestinal Caco-2 and SW480 cell lines.[36] Another possibility is that these blueberry compounds may directly affect signal transduction cascades.[37] For example, pterostilbene has been demonstrated to exhibit anti-inflammatory activity in colon cancer cells by inhibiting p38 mitogen-activated protein kinase.[13] Studies have shown that blocking p38 activity attenuates the transcriptional activity of the pro-inflammatory transcription factor NF-κB;[38] however, it remains to be determined if this is true for brain microglia, as the activity of a compound may be cell type specific. Additionally, pterostilbene, resveratrol, delphinidin, and malvidin may exert their effects on microglia via different or multiple mechanisms.

In summary, this study reveals that exposure to stilbenes, anthocyanins, or blueberry extract alters the way in which microglia respond to an inflammatory stimulus. This study corroborates previous research on the beneficial effects of blueberry, but also suggests that, although individual components are effective mediators of inflammation, the specific actions of blueberry may be due to a synergism of the activity of multiple components. Although the specific activity of these components remains to be determined and will be assessed in future studies, due to the involvement of activated microglia in the aging process and other neurodegenerative processes, blueberry and its constituents may represent complementary therapeutic options to curtail microglia inflammation in the brain.

■ ASSOCIATED CONTENT

ⓈSupporting Information

Results of two-way ANOVAs (main effects and interactions). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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