JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Anthocyanin-rich Açai (*Euterpe oleracea* Mart.) Fruit Pulp Fractions Attenuate Inflammatory Stress Signaling in Mouse Brain BV-2 Microglial Cells

Shibu M. Poulose,[†] Derek R. Fisher,[†] Jessica Larson,[‡] Donna F. Bielinski,[†] Agnes M. Rimando,[#] Amanda N. Carey,[†] Alexander G. Schauss,[§] and Barbara Shukitt-Hale*[,†]

[†]Human Nutrition Research Center on Aging at Tufts University, Agricultural Research Service, U.S. Department of Agriculture, Boston, Massachusetts 02111, United States

[‡]Friedman School of Nutrition Science and Policy, Tufts University, Boston, Masschusetts 02111, United States

[#]National Center for Natural Products Research, Agriculturral Research Service, U.S. Department of Agriculture, University, Mississippi 38677, United States

[§]AIBMR Life Sciences, Puyallup, Washington 98373, United States

ABSTRACT: Age-related diseases of the brain compromise memory, learning, and movement and are directly linked with increases in oxidative stress and inflammation. Previous research has shown that supplementation with berries can modulate signaling in primary hippocampal neurons or BV-2 mouse microglial cells. Because of their high polyphenolic content, fruit pulp fractions of açai (*Euterpe oleracea* Mart.) were explored for their protective effect on BV-2 mouse microglial cells. Freeze-dried açai pulp was fractionated using solvents with different polarities and analyzed using HPLC for major anthocyanins and other phenolics. Fractions extracted using methanol (MEOH) and ethanol (ETOH) were particularly rich in anthocyanins such as cyanidin, delphinidin, malvidin, pelargonidin, and peonidin, whereas the fraction extracted using acetone (ACE) was rich in other phenolics such as catechin, ferulic acid, quercetin, resveratrol, and synergic and vanillic acids. Studies were conducted to investigate the mitigating effects of açai pulp extracts on lipopolysaccharide (LPS, 100 ng/mL) induced oxidative stress and inflammation; treatment of BV-2 cells with acai fractions resulted in significant ($p < 0.05$) decreases in nitrite production, accompanied by a reduction in inducible nitric oxide synthase (iNOS) expression. The inhibition pattern was emulated with the ferulic acid content among the fractions. The protection of microglial cells by açai pulp extracts, particularly that of MEOH, ETOH, and ACE fractions, was also accompanied by a significant concentration-dependent reduction in cyclooxygenase-2 (COX-2), p38 mitogen-activated protein kinase (p38-MAPK), tumor necrosis factor-α (TNFα), and nuclear factor κB (NF-κB). The current study offers valuable insights into the protective effects of açai pulp fractions on brain cells, which could have implications for improved cognitive and motor functions.

KEYWORDS: *açai pulp, anthocyanins, inflammation, oxidative stress, microglia*

INTRODUCTION

The vulnerability of the central nervous system (CNS) to oxidative and inflammatory stress increases with age and has been postulated to be a leading contributing factor to the alarming rise in many age-related neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, dementia, Huntington's disease, multiple sclerosis, prion disease, and others.[1,2] Due to the impact and immense loss caused by these diseases in terms of quality of life and lifespan as well their skyrocketing economic burden on society, the search for preventative, mitigative, and curative strategies has intensified. Fruits and vegetables rich in polyphenols have been shown to provide protection against an array of human diseases such as cancer and cardiovascular disease, including those involving the CNS.[3−5] Prominent among these polyphenol-rich foods are edible berries (e.g., strawberry or blueberry), known for their high levels of flavonoids, particularly anthocyanins. Berries have been shown to improve age-related motor and cognitive deficits in rat and human studies.[5−8] In previous studies using aged animals we have observed beneficial cognitive improvements associated with a reduction in both inflammatory and oxidative stress in key regions of the brain involved in memory and learning.[4,9−12]

Dietary polyphenols have been shown to modulate a number of cellular processes in the brain, including the activation state of microglia. Microglial activation can result in the generation of cytotoxic intermediates and is associated with a variety of age-related and neurodegenerative conditions.[13] In vitro, microglial activation can be induced with the bacterial cell wall component lipopolysaccharide (LPS). Exposure of microglial cells and aged animals to LPS or a neuroexcitotoxic agent such as kainic acid results in the initiation of intracellular signaling cascades, leading to nuclear translocation of the transcription factor nuclear factor κB (NF-κB) and expression of pro-inflammatory enzymes such as inducible nitric oxide

Received: October 3, 2011
Revised: December 21, 2011
Accepted: January 5, 2012
Published: January 5, 2012

dx.doi.org/10.1021/jf203989k | *J. Agric.Food Chem.* 2012, 60, 1084−1093

synthase (iNOS) and inducible cyclooxygenase-2 (COX-2), as well as inflammatory mediators such as nitric oxide (NO), and cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα).[7,13−16] Studies in our laboratory have demonstrated that blueberries, strawberries, and walnuts attenuate the production of inflammatory mediators in LPS-activated mouse BV-2 microglial cells and that at least part of the berry-induced reductions in stress-mediated signaling might involve decreases in NO production and iNOS expression[7] as well as reduction in IL-1β, TNFα, NF-κB, and IGF-1 in critical regions of stress-induced rat brain.[16]

There are three primary species of the genus *Euterpe* in the Amazon region, that is, *Euterpe precatoria*, *Euterpe edulis*, and *Euterpe oleracea*. These palm tree species bear fruits widely consumed by Amazonian natives and are also exported globally. *Euterpe oleracea* is the most commonly consumed fruit. The pulp of the fruit of *E. oleracea* has received considerable attention among food scientists due to its extraordinarily high antioxidant capacity in vitro when compared to other fruits and berries against all reactive oxygen species, especially the superoxide anion, and hydroxyl, peroxyl, and peroxynitrite radicals. Açai pulp has been extensively studied for its nutritional and phytochemical composition and bioactivity.[17,18] Unlike most fruits and berries, it also has a remarkable number of phytochemicals, in addition to ample amounts of mono- and polyunsaturated fatty acids within its pulp. Phytochemical analyses indicate that, apart from being rich in anthocyanins such as cyanidin, delphinidin, malvidin, pelargonidin, and peonidin, açai pulp also possesses high concentrations of luteolin, quercetin, dihydrokaempferol, and chrysoerial (a unique flavone), along with a number of other polyphenolics.[17−20] In vitro studies showed that açai extracts, as well as individual flavonoids of açai, exhibit potent anti-inflammatory, anticarcinogenic, antioxidant, and certain neuroprotective properties.[20−25] The health benefits of açai pulp consumption have been demonstrated in humans, in a randomized, double-blind, placebo-controlled, crossover study, with healthy subjects from 19 to 52 years of age administered an açai pulp-rich juice in which the pulp had not been clarified or filtered.[26] The study reported significant reductions in lipid peroxidation during oxidative stress, as well as a rapid increase in antioxidant activity in the serum, increasing cellular protection from reactive oxygen species, as measured by the cell-based antioxidant protection in erythrocytes (CAP-e) assay. Apart from being a potent antioxidant and anti-inflammatory agent, studies using the fruit fly (*Drosophila melanogaster)* have shown that when açai pulp is added to a high-fat diet, it extends lifespan via modification of the c-Jun N-terminal kinase (JNK) signaling pathway. Açai may also produce analgesic effects in mice, possibly mediated via modulation of the levels of prostaglandins.[27,28] Furthermore, the effect of açai pulp in reducing pain has been confirmed in an open-label clinical pilot study in humans, where oral consumption of an açai fruit and berry blend significantly reduced inflammation, improving joint motion and altering pain perception.[29] However, the effect of açai fractions on brain cells and the signaling mechanisms involved in interneuronal communications have not been examined.

Thus, in the present study, we have examined the effects of different fractions of açai pulp on BV-2 microglial cells subjected to LPS-induced insults, through the measurement of oxidative- and inflammatory-stress-induced signals, including nitric oxide (NO) release, and levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), mitogen-activated protein kinase (MAPK), tumor necrosis factor α (TNFα), and nuclear factor κB (NF-κB). The study also offers insights on the recovery of microglial cells during chemically induced stress and their tolerance to various polyphenol-rich fractions extracted from açai pulp.

MATERIALS AND METHODS

Açai Fractions. Pasteurized, freeze-dried açai pulp, prepared from naturally ripened açai fruits harvested in the Amazon delta, was kindly donated by AIBMR Life Sciences (Puyallup, WA). Polyphenols from the fruit pulp were fractionated with solvents of various polarities such as ethanol (ETOH), methanol (MEOH), ethyl acetate (ETAC), and acetone (ACE) (Sigma-Aldrich, St. Louis, MO). One hundred grams of finely powdered, freeze-dried açai pulp was initially extracted in 400 mL each of petroleum ether followed by 400 mL of chloroform for 36 h at room temperature with continuous stirring, to remove the oils, lignans, gums, and other petroleum ether-soluble components. The leftover pulp was extracted with 400 mL of ETAC for 36 h at room temperature, with continuous stirring. After removal of the ETAC through vacuum filtration, the remaining pulp was subsequently extracted using 400 mL each of ACE, MEOH, and ETOH, with pulp being in each solvent for 36 h with continuous agitation. Each filtrate was clarified further through vacuum filtration on a Buchner funnel, the solvents were removed via rotary evaporation, and each fraction was freeze-dried to remove all traces of moisture and solvents. ETAC and ACE were used to extract major non-water-soluble flavonoids, anthocyanins and other polyphenolics, whereas ETOH and MEOH were used to extract most of the water-soluble polyphenolic components. The yields of freeze-dried fractions from 100 g of açai pulp were 5.171 g (oily) for petroleum ether, 1.809 g for chloroform (oily), 2.007 g for ETAC, 2.302 g for ACE, 2.817 g for MEOH, and 5.712 g for ETOH. For cell experiments, equal quantities of dried açai fractions were reconstituted in DMSO (Sigma-Aldrich) and diluted in cell culture growth media such that the concentration of DMSO in the treatments was never above 0.01%.

Analysis of Phenolic Compounds in Açai Berry Extracts. Dried/lyophilized açai berry extracts (100 mg) were dissolved in 1 mL of 1% formic acid in MEOH solution and were analyzed for the presence of some known phenolic compounds on an Agilent 1200 series HPLC system (Agilent Technologies, Palo Alto, CA), with a photodiode array detector, using an Inertsil ODS-2 (5 μm, 4.6 × 250 nm) column (GL Sciences, Inc., USA, Torrance, CA). The volume of injection was 20 μL. For analysis of anthocyanins the mobile phase consisted of 5% formic acid in H_2O (A) and H_2O/AcCN/formic acid, 50:45:5 (B), eluted according to the following gradient: initial 20% B, 0−10 min ramp to 25% B, 10−20 min ramp to 33% B, 20−40 min ramp to 100% B, 40−43 min at 100% B. The anthocyanins were monitored at λ520 nm. Under these conditions, the retention times were as follows: delphinidin-3-*O*-glucoside, 10.02 min; cyanidin-3-*O*-glucoside, 13.48 min; cyanidin-3-*O*-rutinoside, 13.54 min; pelargonidin-3-*O*-glucoside, 17.34 min; malvidin-3-*O*-glucoside, 20.05 min; and peonidin-3-*O*-glucoside, 22.08 min. The anthocyanins were quantitated from a calibration curve of a mixture of standards prepared in 0.1% HCl in MEOH/H_2O (80:20).

Other phenolic compounds were analyzed at λ280 nm, eluted according to the following solvent gradient [1% formic acid in H_2O (A), 1% formic acid in MEOH (B)]: 0−3 min, 0−5% B; 3−45 min, 5−70% B; 45−48 min, 70−100% B; 48−51 min, 100% B. Under these conditions, the retention times were as follows: catechin, 18.68 min; vanillic acid, 22.21 min; synergic acid, 23.70 min; ferulic acid, 29.31 min; resveratrol, 34.35 min; and quercetin, 40.82 min. The phenolic compounds were quantitated from a calibration curve of a mixture of standards prepared in 1% formic acid in MEOH. Standards for all anthocyanins and quercetin were purchased from Chromadex (Irvine, CA); catechin, resveratrol, and vanillic acid were purchased from Sigma-Aldrich Co.; ferulic acid was from Indofine Chemical Co., Inc. (Hillsborough, NJ).

Table 1. HPLC Phenolic Content Analysis for Major Anthocyanins and Other Major Polyphenols in Açai Pulp Fractions, Extracted Using Different Solvents[a]

	major anthocyanins (expressed as ng/mg dry extract ± SD)					
açai fraction	cyanidin glucoside	cyanidin rutinoside	delphinidin glucoside	malvidin glucoside	pelargonidin glucoside	peonidin glucoside
ETAC	192.94 ± 53.77	74.53 ± 25.91	trace	trace	13.94 ± 5.09	24.45 ± 3.91
ACE	724.32 ± 91.3	1043.01 ± 176.4	78.36 ± 0.36	110.0 ± 9.5	45.82 ± 2.73	263.51 ± 28.76
MEOH	3461.87 ± 2.08	5851.95 ± 230.9	1064.82 ± 386.1	74.87 ± 2.6	115.2 ± 19.5	774.95 ± 32.68
ETOH	2771.69 ± 7.46	4878.67 ± 8.20	553.16 ± 90.88	66.50 ± 0.72	95.96 ± 4.48	564.09 ± 4.73

	other major polyphenols (expressed as ng/mg dry extract ± SD)					
	flavanol	hydroxycinnamic acid	flavonol	stilbenoid	hydroxybenzoic acid	
açai fraction	catechin	ferulic acid	quercetin	resveratrol	syringic acid	vanillic acid
ETAC	411.08 ± 5.49	1197.54	54.27 ± 1.77	trace	28.47 ± 0.06	135.63 ± 9.04
ACE	5935.8 ± 128.5	2975.40 ± 40.17	2389.8 ± 15.04	889.9 ± 26.6	1135.4 ± 4.8	1499.3 ± 30.51
MEOH	1901.6 ± 193.3	4565.39 ± 207.0	580.67 ± 0.30	42.42 ± 0.88	1312.6 ± 4.8	161.5 ± 100.7
ETOH	235.21 ± 8.21	2304.70 ± 16.79	170.03 ± 10.88	none	609. 73 ± 3.6	57.05 ± 31.02

[a]Major components in each fraction, injected at 100 mg/mL concentration, were identified on the basis of the retention time, using a calibration chromatogram obtained from a mixture of known standards. The data are presented as average ng/mg dry extract from duplicate analyses. Trace refers to a peak observed, but below the limit of quantitation.

Cell Culture. BV-2 murine microglial cells (generously provided by Dr. Van Eldik, University of Kentucky, Lexington, KY) were plated on 12-well plates for immunohistochemical analysis at a density of 50000 cells per well or on 96-well plates for the enzyme-linked immunosorbent assay (ELISA) at a density of 10000 cells per well and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) at 37 °C in a humidified incubator under 5% CO_2. After 16−20 h of incubation, when the cells were nearly 70% confluent, cells were pretreated with either açai extract diluted in media or control media for 4 h and then stimulated with LPS (Sigma-Aldrich) at 100 ng/mL overnight. The açai extract concentrations ranged from 50 to 1000 μg/mL for the MEOH, ETAC, and ACE fractions and from 10 to 250 μg/mL for the ETOH fraction. All treatment groups were assayed in duplicates, in 6six separate experiments.

Cell Viability and Cytotoxicity. Optimal treatment concentration as well as toxicity of different fractions of açai pulp on BV-2 microglial cells was determined using a two-color fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells using live/dead viability/cytotoxicity kits (Molecular Probes−Invitrogen, Carlsbad, CA). The assay measures two recognized parameters of cell viability, intracellular esterase activity and plasma membrane integrity. In brief, BV-2 microglial cells, seeded in 96-well culture plates, were treated with various concentrations (50 μg−10 mg/mL) of açai pulp fractions for 24 h, except for the control and DMSO vehicle controls. At the end of treatment, the cells were stained for 30 min with calcein AM, which is cleaved by esterases in live cells, producing cytoplasmic green fluorescence measured at 530 nm, and ethidium homodimer-1, which stains nucleic acids of the membrane-collapsed or dead cells with red fluorescence measured at 630 nm. Images were captured with a Nikon TE200U inverted microscope. Percent live cells were calculated as the average number of live cells divided by the number of total cells in each field of view, from 10 randomly selected regions in each well.

Nitrite Quantification. To assess the production of nitric oxide (NO) from BV-2 cells, extracellular release of nitrite (NO_2-), which is one of the major, stable, and nonvolatile breakdown products of NO, was measured by the Greiss reagent system (Promega, Madison, WI) according to the manufacturer's instructions. One hundred microliters of cell-conditioned medium supernatant was mixed with 25 μL of Griess reagent (0.1% *N*-1-naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid) 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 sodium nitrite.

Western Blots. Following experimental treatments, cells were washed in ice-cold PBS, and 75 μL of CelLytic-M Cell Lysis Reagent (Sigma-Aldrich), containing mammalian protease inhibitor cocktail at 1:100 dilution (Roche, Basel, Switzerland), PMSF (10 μg/mL), and sodium fluoride (50 mM) (Sigma-Aldrich), was applied to each well. Cells were lysed at 4 °C with agitation for 15 min and centrifuged at 12000*g* for 15 min at 4 °C. The protein concentration of the resulting supernatant lysates was quantified with the DC protein assay (Bio-Rad, Hercules, CA). Equal amounts of denatured protein samples were separated by 8% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. Primary antibodies for iNOS (Upstate-Millipore, Billerica, MA), COX-2 (Santa Cruz Biotech, Santa Cruz, CA), total and phospho NF-κB (Abcam, Cambridge, MA), and p38-MAPK (Abcam) were used at 1:1000 dilution for incubation overnight at 4 °C. After washing in TBST, the signal was detected using an electrochemiluminescence (ECL) detection kit (Amersham Corp., Arlington Heights, IL). Following ECL development, the images of the blots were acquired and band densities were analyzed using a BioImaging System (EC^3 Darkroom, UVP, Upland CA) and LabWorks Imaging Acquisition and Analysis software (version 4.5, UVP). β-Actin was used as a loading control marker.

TNFα ELISA. Quantification of TNFα, a key cytokine in the development of several inflammatory disorders, in cell-conditioned media was performed with an ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Cleavage of membrane-bound TNFα by TACE/ADAM17 releases a 55 kDa soluble trimetric form of TNFα, which can be quantified in the cell supernates spectrophotometrically. In brief, 50 μL of cell-conditioned medium was added to a 96-well plate and incubated for 2 h. Following washing, TNFα conjugate was added to each well and incubated for a further 2 h. Wells were washed again, and substrate solution was added to each well, followed by stop solution and measurement of absorbance at 450 nm. TNFα concentration for each sample was calculated from the linear equation derived from the standard curve of known concentrations of the recombinant TNFα.

Data Analysis. The quantitative data were analyzed by two-way ANOVA with pretreatment (acai fraction + LPS vs LPS alone) and dose as experimental factors. Post hoc comparisons were performed using Fisher's least significant difference (LSD) test, when either the main effect of pretreatment or interaction was significant. All statistical analyses were performed using SYSTAT software (SPSS, Chicago, IL). Results were considered to be statistically significant if the observed significance level with açai pretreatment was $p < 0.05$ (*) or $p < 0.001$ (**) versus LPS. Note that for each dependent measure, those cells treated with LPS alone were statistically higher than the control conditions without LPS, which were not different (data not shown). Additionally, the acai fractions with no LPS did not increase any of the

dependent measures compared to control conditions (data not shown).

RESULTS

Fractional Analysis of Açai Pulp. HPLC analysis to determine the major polyphenolics in different soluble fractions of açai pulp yielded a highly variable distribution of compounds across the fractions as described in Table 1. MEOH and ETOH fractions had substantially higher amounts of anthocyanins, followed by ACE, and relatively low levels in the ETAC fractions. With respect to other polyphenols, such as catechin, ferulic acid, quercetin, resveratrol, synergic acid, and vanillic acids, ACE and MEOH fractions had substantially higher amounts, followed by ETOH and ETAC fractions.

Effect of Açai Pulp Extracts on Viability of Microglial Cells. Cytotoxicity measurements for various açai extracts on BV-2 microglial cells, at extract concentrations ranging from 50 μg to 10 mg/mL, had variable dose−response effects on viability when compared to control (Figure 1). None of the four fractions (ETAC, ACE, MEOH, and ETOH) significantly compromised the viability of the BV-2 microglial cells up to 2.5 mg/mL; however, higher concentrations such as 5−10 mg/mL, particularly of ETAC and ACE fractions, reduced the viability of the cells. On the basis of the viability results, subsequent experiments were carried out at concentrations ranging from 25 to 1000 μg/mL. No differences were observed between cells treated with LPS plus DMSO and LPS without DMSO, verifying that DMSO had no significant effect on the cells. Also, each açai pulp fraction (with no subsequent LPS exposure) had no effect on the microglial cell measurements compared to control medium-treated cells.

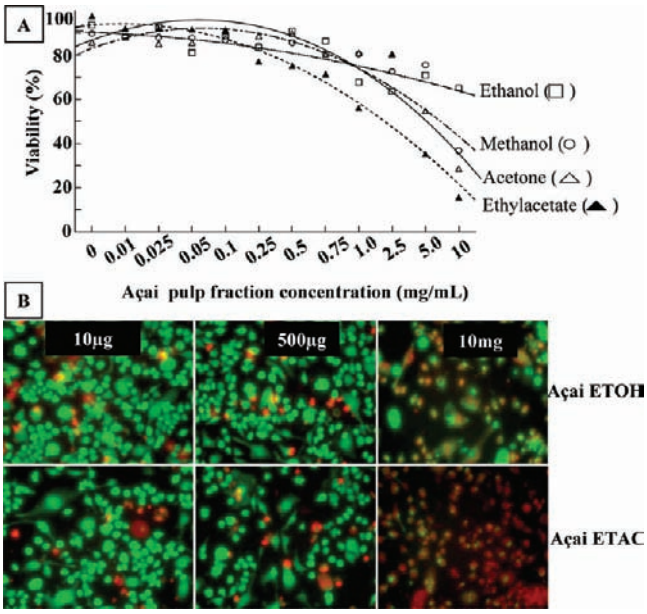

Figure 1. Effect of various solvent fractions of açai pulp on the viability of BV-2 microglial cells assessed using a live/dead viability/cytotoxicity kit (A), which measures the live-cell esterase activity with green fluorescence and membrane-compromised dead cells with red fluorescence. Percent live cells was calculated as the average number of live cells in each field of view from 10 randomly selected regions using a fluorescent microscope. Panel B is the representation of the fluorescent images of BV-2 microglial cells treated with ETOH and ETAC fractions of açai pulp.

Açai Pulp Fractions Attenuate Reactive Nitrogen Species and Pro-inflammatory Mediators. Exposure of brain cells to oxidative stress, primarily caused by the generation of reactive oxygen or nitrogen species, sets off a cascade of events resulting in the death of neurons, ultimately leading to declines in cognitive and motor functions. However, pretreatment of microglial cells with açai pulp fractions significantly attenuated the production of reactive nitrogen species as well as expression of pro-inflammatory mediator proteins. The results are discussed below.

NO Production. Pretreatment of microglia with the individual açai pulp fractions significantly attenuated LPS-induced NO production (Figure 2). Significant main effects were observed for pretreatment ($p < 0.001$), whereas the dose by pretreatment interactions were not significant. The anthocyanin-rich MEOH fraction was the most effective in reducing the extracellular release of NO, a direct measurement for the release of reactive oxygen species (**, $p < 0.001$ for 0.25−1 mg/mL). All other fractions (ETAC, ACE, and ETOH) also significantly (*, $p < 0.05$) inhibited NO production, at concentrations ≥250 μg/mL. A relatively large variability in data points is observed for spectrophotometric-based assays compared to that of Western blot-based analysis. Therefore, except for the MEOH fraction above 250 μg/mL, the level of significance observed is only at $p < 0.05$. Overall, the inhibition of nitrite follows the pattern of relative content of ferulic acid in each of these fractions. It has been well reported that ferulic acid is highly reactive with nitrites and acts as an antioxidant and chemoprotectant by inhibiting the formation of *N*-nitroso compounds in vivo.[30,31]

iNOS. LPS-induced expression of iNOS, which produces the inflammatory mediator NO, was significantly reduced by pretreatment with all fractions of açai pulp (Figure 3). Overall, significant effects were observed for pretreatment and pretreatment by dose interactions at $p < 0.05$. MEOH and ETOH fractions significantly inhibited iNOS expression (**, $p < 0.001$) in a dose-dependent manner. Interestingly, these two fractions have relatively higher concentrations of anthocyanins such as cyanidin glucoside, cyanidin rutinoside, and delphidin glucoside, as well as ferulic acid, compared to ETAC and ACE fractions. Even though a direct dose−response was observed with respect to NO production, the linear relationship with respect to iNOS inhibition was not observed for the ETAC and ACE fractions because of apparent saturation effects on the iNOS enzyme at concentrations >500 μg/mL.

TNFα. A reduction in the release of the inflammatory cytokine TNFα into the media following LPS exposure of microglial cells (Figure 4) was observed by pretreatment with all four açai fractions with varied levels of significance ($p = 0.009$ for ETAC, $p = 0.016$ for ACE, $p < 0.001$ for MEOH and ETOH). However, the pretreatment by dose interaction effects were not significant. There were no significant reductions in TNFα by ETAC or ACE pretreatment when compared to that of LPS alone for any of the concentrations tested. MEOH and ETOH fractions were significant (*, $p < 0.05$) only at concentrations at and above 250 and 50 μg/mL, respectively. One contributing factor for the effectiveness of the MEOH and ETOH fractions can be attributed to the presence of high concentrations of anthocyanins. The large variability of the data observed for ELISA (similar to the nitrite release assay), when compared to those from Western blots, could be the reason for the relatively low response in the inhibition of TNFα release.

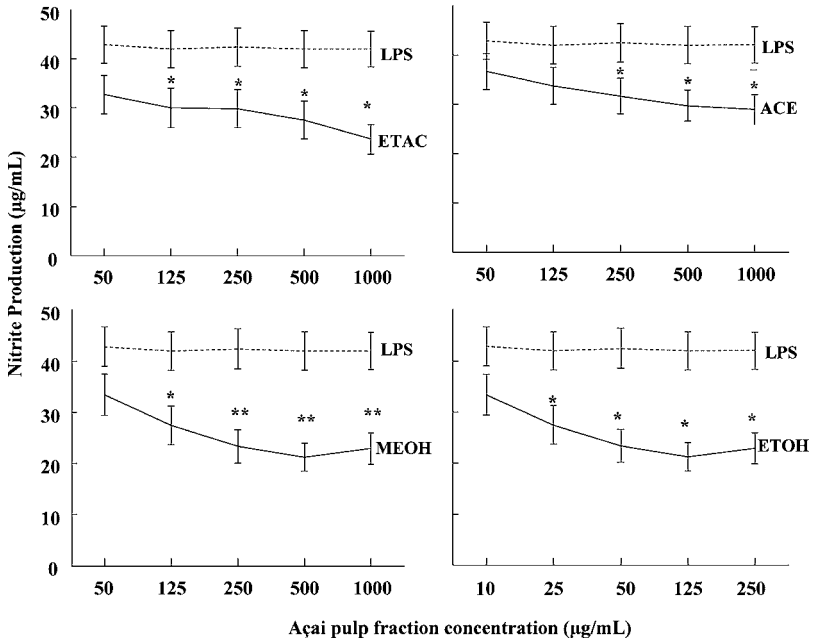

Figure 2. Production of extracellular nitrite (NO_2^-) in BV-2 microglial cells pretreated (4 h) with various fractions of açai pulp extracts and stimulated with LPS (100 ng/mL). Data are expressed as the mean ± SEM and quantified using Greiss reagent. Each açai fraction pretreatment was compared against LPS treatment alone. Comparative post hoc analyses were made by Fisher's LSD with significance at (*) $p < 0.05$ and (**) $p < 0.001$ versus LPS.

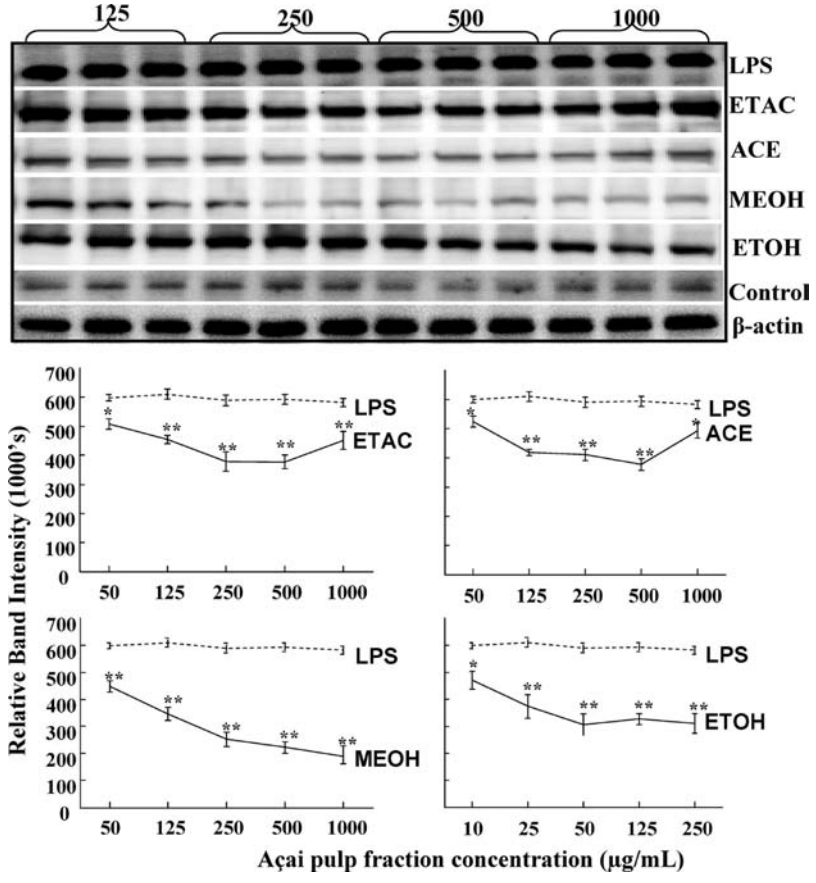

Figure 3. Suppression of iNOS activated by LPS (100 ng/mL) with pretreatment (4 h) of various açai pulp fractions in BV-2 microglial cells. The Western blot immunoreactive band density is expressed as the mean ± SEM with level of significance at (*) $p < 0.05$ and (**) $p < 0.001$ of pretreatment versus LPS alone by Fisher's LSD. The blots for the initial fractions (50 μg/mL ETAC, ACE, and MEOH and 10 μg/mL ETOH) are not shown.

dx.doi.org/10.1021/jf203989k | J. Agric.Food Chem. 2012, 60, 1084–1093

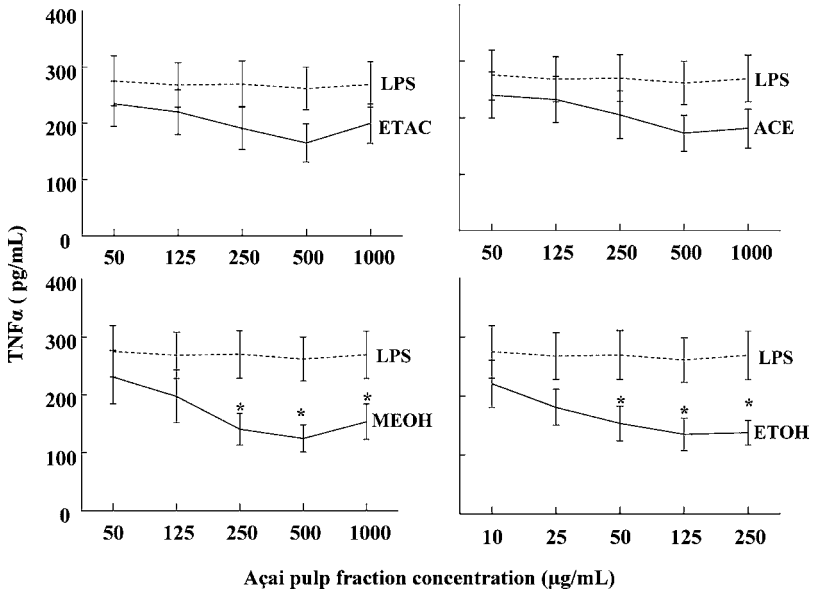

Figure 4. Reduction in LPS-induced TNFα in BV-2 microglial cells pretreated with different açai pulp fractions. Data were assayed by ELISA, and the values are expressed as the mean ± SEM. Cytokines released into the media (pg/mg of protein) by the treatment groups were compared against LPS (100 ng/mL) by Fisher's LSD with level of significance at (∗) $p < 0.05$ and (∗∗) $p < 0.001$ versus LPS alone.

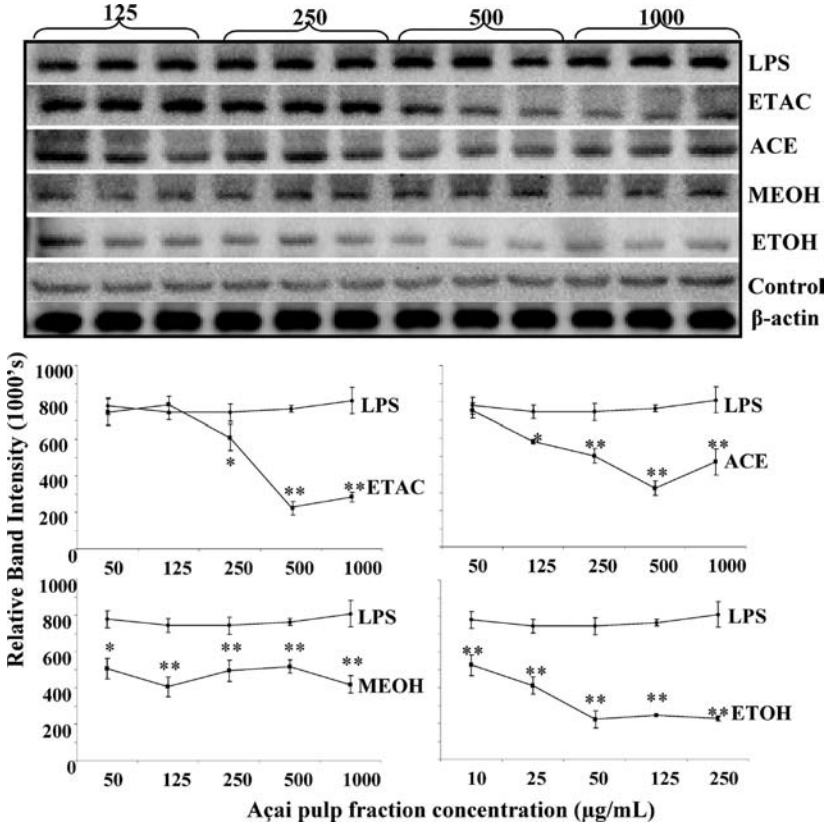

Figure 5. Effect of various açai pulp fractions pretreatment (4 h) in the reduction of LPS-stimulated activation of p38-MAPK in BV-2 microglial cells. The values for each treatment are expressed as the mean ± SEM for the immunoreactive bands with level of significance at (∗) $p < 0.05$ and (∗∗) $p < 0.001$ versus LPS alone (100 ng/mL) by Fisher's LSD. The blots for the initial fractions (50 μg/mL ETAC, ACE, and MEOH and 10 μg/mL ETOH) are not shown.

Açai Pulp Fractions Affect the Signaling Pathways Linked to Inflammation in Microglial Cells. Correlating with the results of suppression of both pro-inflammatory mediators and reactive nitrogen species, pretreatment with açai pulp extracts significantly affected the signaling markers linked to inflammation. The specific effects on key signaling proteins are discussed below.

p38-MAPK. The significant increase in p38-MAPK phosphorylation by LPS was attenuated by pretreatment (4 h) with açai pulp fractions (Figure 5). All four fractions of açai

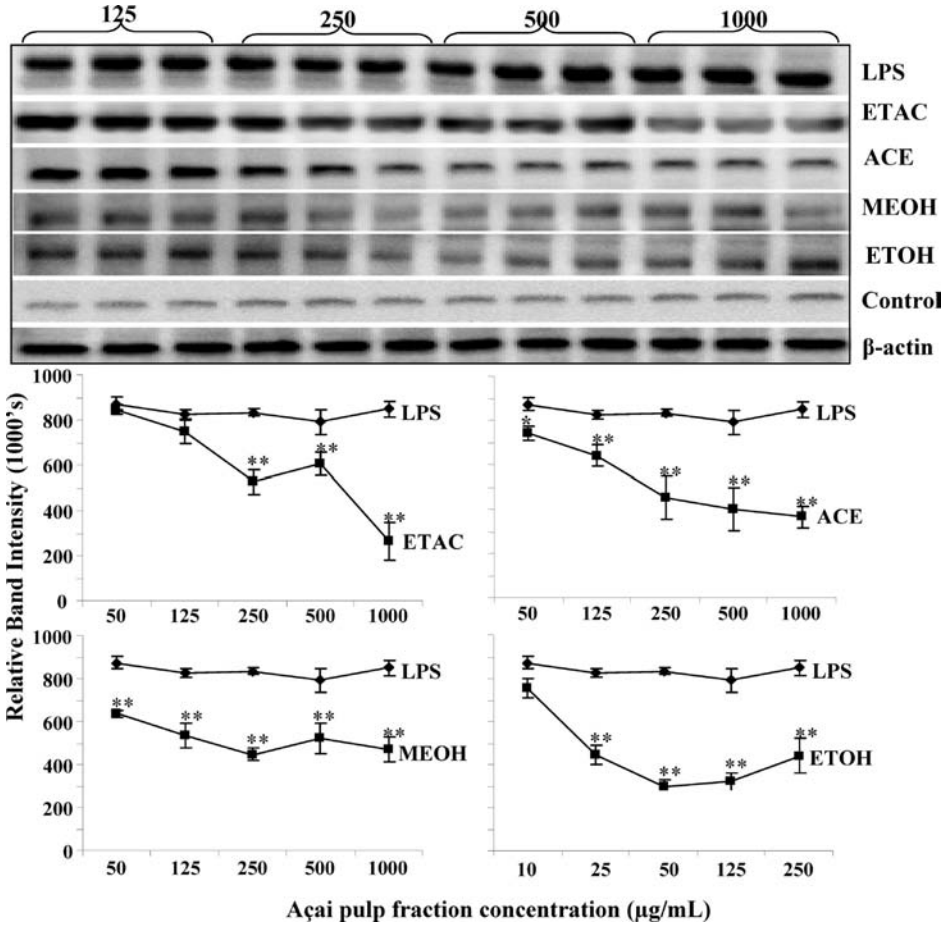

Figure 6. Suppression of phosphorylation of NF-κB (pNF-κB) by açai pulp fraction pretreatment followed by overnight LPS (100 ng/mL) stimulation in BV-2 microglial cells. The values are expressed as the mean ± SEM for the immunoreactive bands of the Western blots with level of significance at (∗) $p < 0.05$ and (∗∗) $p < 0.001$. Statistical comparisons are made between açai pretreatment versus LPS alone. The blots for the initial fractions (50 μg/mL ETAC, ACE, and MEOH and 10 μg/mL ETOH) are not shown.

had significant inhibitory effects (∗∗, $p < 0.001$) on p38-MAPK activation. The inhibition of p38-MAPK phosphorylation by açai fractions exhibited a dose-dependent pattern with the main effects also being significant for pretreatment as well as dose by pretreatment interaction ($p < 0.001$). Following a similar pattern, anthocyanin-rich MEOH and ETOH fractions were highly effective even at the lowest concentrations tested (50 and 10 μg/mL, respectively), whereas the ETAC fraction only began showing the inhibitory effects at 250 μg/mL. Linear dose responses were observed for ACE and ETAC fractions, whereas MEOH and ETOH showed apparent saturation effects.

NF-κB. Phosphorylation of NF-κB results in its translocation into the nucleus, where it binds to DNA, leading to the expression or activation of many inflammatory genes. LPS significantly increased NF-κB phosphorylation compared to control (Figure 6). However, these increases in NF-κB activation were significantly attenuated by pretreatment with açai fractions. Except for the ETAC fraction ($p = 0.148$), all other fractions effectively reduced the phosphorylation of NF-κB in a dose-dependent manner ($p < 0.001$). The main effects were also significant for pretreatment, as well as dose by pretreatment interaction. MEOH and ETOH fractions were highly significant even at low concentrations (50 and 25 μg/mL, respectively), and these effects correlate with the levels of anthocyanins such as glycosides of cyanidin, delphidin, malvidin, pelargonidin, and peonidin. Even though a dose-dependent inhibition of NF-κB phosphorylation was observed for ACE, MEOH, and ETOH fractions, an apparent saturation effect was observed at higher concentrations for MEOH and ETOH fractions.

COX-2. Expression of COX-2 was measured in microglial cells, as it is responsible for the formation of inflammatory mediators called prostanoids. Pretreatment of microglia with the individual açai pulp fractions significantly ($p \leq 0.001$) attenuated LPS-induced increases in COX-2 expression (Figure 7). The dose by pretreatment interactions were significant for all of the fractions except for ETOH. However, the ETOH fraction, which contains the most polyphenolics per unit weight of the pulp, as well as a higher concentration of anthocyanins, was highly significant (∗∗, $p < 0.001$) in inhibiting COX-2 even at 10 μg/mL concentration. The ETAC fraction, which is low in most of the major polyphenolics, was effective only at concentrations ≥250 μg/mL, whereas ACE and MEOH were significantly effective at ≥125 μg/mL.

DISCUSSION

An age-associated increase in neurodegenerative disorders is primarily attributed to excessive neuroinflammation as a result of neurotoxicity induced by reactive oxygen species, trauma, hypoxia/ischemia, toxic agents, and systemic infections by viruses and bacteria.[32−34] Microglial cells, which are derived from myeloid cells and are an important type of resident

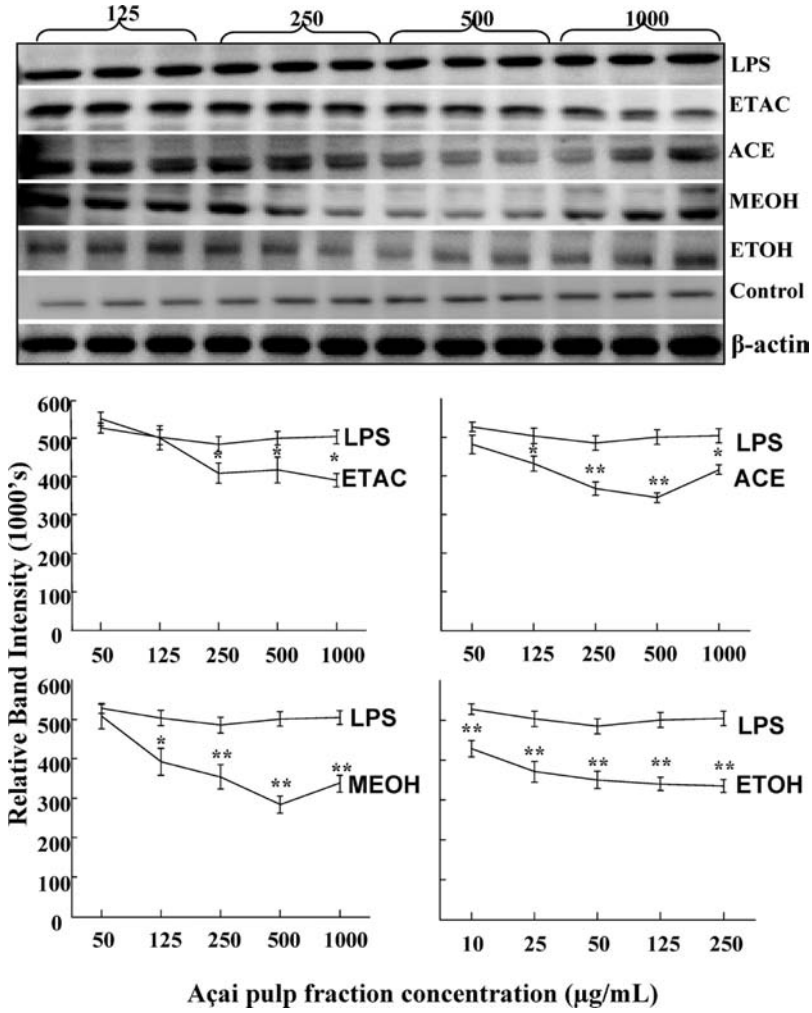

Figure 7. Reduction in COX-2 expression when BV-2 cells were pretreated (4 h) with various açai pulp fractions followed by LPS (100 ng/mL) stimulation. The values are expressed as the mean ± SEM for the immunoreactive bands of the Western blots. Each açai fraction pretreatment was compared against LPS alone with level of significance at (∗) $p < 0.05$ and (∗∗) $p < 0.001$ by Fisher's LSD. The blots for the initial fractions (50 μg/mL ETAC, ACE, and MEOH and 10 μg/mL ETOH) are not shown.

immune cell in the brain, protect neurons from oxidative stress and inflammation.[15] However, under a highly activated state caused by stressors such as LPS, these microglial cells produce inflammatory molecules such as cytokines, superoxide, and nitric oxide, ultimately leading to a cascade of pro-inflammatory proteins, in turn leading to the death of neurons.[14,35]

Bioavailability of natural polyphenols is generally considered to be low in the brain due to the presence of the blood–brain barrier; however, previous reports indicate the presence of anthocyanins, flavonoids such as catechins and quercetin, stilbenoids such as resveratrol, and phenolic acids in brain tissues of animals.[35,36] The results from the current study suggest that pretreatment of BV-2 microglial cells with the individual açai pulp fractions was protective against LPS-induced NO release, iNOS production, COX-2 expression, NF-κB phosphorylation, TNFα release, and p38-MAPK activation. The study is mainly focused on the main effects of each of the açai pulp fractions based on their phytochemical content. The reduction of oxidative stress and inflammation is comparable to what we have observed for walnut, blueberry, strawberry, and other berries, such as cranberry and raspberry, in both in vitro and in vivo studies.[6−12] The stress reduction effects can be attributed to an array of phytochemicals abundant in the açai pulp. Apart from the known major anthocyanins, flavonoids, phenolic acids, and stilbenoid provided in Table 1, açai pulp is also rich in proanthocyanidins, tannins, and phytosterols (β-sitosterol, stigmasterol, Δ5-avenasterol, campesterol), along with 9−12% lipids including oleic acid, palmitic acid, linoleic acid, palmitoleic acid, stearic acids, and other fatty acids in trace amounts.[20] The MEOH and ETOH açai fractions appear to be the most effective against the LPS-induced increases in inflammatory and free radical mediators, as these fractions contain the highest concentrations of most of the anthocyanins and ferulic acid. Also, the MEOH and ETOH fractions contain the most water-soluble components that will be readily amenable to in vitro assays.

Pro-inflammatory enzymes such as COX-2 and iNOS produce mediators such as prostaglandin E_2 (PGE_2) and nitric oxide, respectively.[37] iNOS-derived NO, COX-2-derived PGE_2, and pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα have been identified as key players in inflammatory processes of the neuronal system[38] and are known to activate microglia and astrocytes. Because our anthocyanin-rich fractions (MEOH and ETOH) also contain catechin, resveratrol, and other phenolic compounds, a possible synergetic as well as cell-specific activity

of açai fractions may be contributing to the attenuation of TNFα, parallel to previous results.[39]

LPS-derived inflammation significantly increases NO production, which in turn causes tissue toxicity after reaction with superoxide anions through the production of peroxynitrite anions and peroxynitrous acid.[38] NO is synthesized from L-arginine by NOS either by a constitutive (cNOS) or an inducible (iNOS) form, and iNOS primarily causes the inflammatory effects of NO.[38] The relative effect on the reduction in NO production observed after pretreatment with different açai fractions may be attributed to the relative content of phenolic hydroxycinnamic acids such as ferulic acid. In animal experiments, ferulic acid is known to inhibit the formation of *N*-nitroso compounds, leading to reduced levels of serum *N*-nitrosamines, which are highly carcinogenic in nature.[30,31] In vitro evidence also suggests that ferulic acid can react very rapidly and quantitatively with equimolar quantities of nitrite.[31] Anthocyanins have also been reported to inhibit NO production.[34] Similar to our previous findings using blueberry and strawberry extracts[3−12,16] which also contain high amounts of anthocyanins, açai fruits with their relatively much higher concentrations of similar compounds[19] could possibly boost brain health at much lower quantities.

NF-κB is an important transcription factor, and p38-MAPK pathways are involved in regulating inflammatory gene expression in neurons.[1] p38-MAPK is critical in long-term memory formation through the activation of cyclic-AMP response element-binding (CREB) protein, as well as in synaptic plasticity.[40] However, previous studies indicate that MAPKs are critical for the LPS-induced expression of iNOS and extracellular release of NO in microglial cells.[33,34,41] Earlier studies also suggest that the inhibition of the LPS-derived increase in pro-inflammatory proteins such as iNOS, COX-2, PGE_2, and IL-1β is associated with the negative regulation of p38 MAPK.[38,42,43] COX-2 is a key enzyme for the biosynthesis of PGE_2, which is transcriptionally regulated by NF-κB. Similarly, iNOS activity is also known to be controlled transcriptionally by NF-κB.[43] It has been reported that LPS-induced activation of p38-MAPK can modulate the promoter function of NF-κB, through increased phosphorylation (pNF-κB).[42,44] All of the inhibitory effects were correlated with the relative content of anthocyanins in each of the açai fractions. Therefore, it can be inferred from the current results that anthocyanin-rich açai fractions negatively modulate p38-MAPK signaling, which in turn down-regulates the phosphorylation of NF-κB, transcriptionally suppressing the production of iNOS and COX-2.

Taken together, the results indicate that LPS-mediated up-regulation of p38-MAPK and NF-κB was attenuated by açai pulp fractions, which in turn down-regulated iNOS and COX-2 in BV-2 microglial cells. TNFα can activate NF-κB post-translationally and regulates many pro-inflammatory genes, including cytokines.[45] Therefore, attenuation of TNFα by açai fractions can also be attributed to the NF-κB-derived transcriptional changes observed in other inflammatory mediators. In conclusion, effective reduction in oxidative damage and inflammation in brain cells is likely to be an effective intervention to reduce the incidence of age-related neurodegenerative disorders. These results, similar to those of our previous studies with blueberries, suggest that consumption of açai pulp may contribute to "health span" in aging, as it is able to combat some of the inflammatory and oxidative mediators of aging at the cellular level. To determine if açai fruit is able to reverse or allay age-related motor or cognitive deficits, experiments are being planned to feed aged rats açai-supplemented diets.

AUTHOR INFORMATION

Corresponding Author

*Phone: 1-(617) 556-3118. Fax: 1-(617) 556-3222. E-mail: Barbara.ShukittHale@ars.usda.gov.

Funding

This research is supported by USDA Intramural grants and AIBMR Life Sciences.

ACKNOWLEDGMENTS

We thank Stacey M. Gomes for her time, knowledge, and contributions in editing the paper.

REFERENCES

(1) McGeer, E. G.; McGeer, P. L. Inflammatory processes in Alzheimer's disease. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **2003**, *27*, 741−749.

(2) Van Eldik, L. J.; Thompson, W. L.; Ralay Ranaivo, H; Behanna, H. A.; Martin Watterson, D. Glia proinflammatory cytokine upregulation as a therapeutic target for neurodegenerative diseases: function-based and target-based discovery approaches. *Int. Rev. Neurobiol.* **2007**, *82*, 277−296.

(3) Joseph, J. A.; Shukitt-Hale, B.; Casadesus, G. Reversing the deleterious effects of aging on neuronal communication and behavior: beneficial properties of fruit polyphenolic compounds. *Am. J. Clin. Nutr.* **2005**, *81*, 313S−316S.

(4) Joseph, J. A.; Shukitt-Hale, B.; Denisova, N. A.; Prior, R. L.; Cao, G.; Martin, A.; Taglialatela, G.; Bickford, P. C. Long-term dietary strawberry, spinach, or vitamin E supplementation retards the onset of age-related neuronal signal-transduction and cognitive behavioral deficits. *J. Neurosci.* **1998**, *18*, 8047−8055.

(5) Joseph, J. A.; Shukitt-Hale, B.; Casadesus, G.; Fisher, D. R. Oxidative stress and inflammation in brain aging: nutritional considerations. *Neurochem. Res.* **2005**, *30*, 927−935.

(6) Joseph, J. A.; Shukitt-Hale, B.; Denisova, N. A.; Bielinski, D. F.; Martin, A.; McEwen, J. J.; Bickford, P. C. Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. *J. Neurosci.* **1999**, *19*, 8114−8121.

(7) Lau, F. C.; Bielinski, D. F.; Joseph, J. A. Inhibitory effects of blueberry extract on the production of inflammatory mediators in lipopolysaccharide-activated BV2 microglia. *J. Neurosci. Res.* **2007**, *85*, 1010−1017.

(8) Krikorian, R.; Shidler, M. D.; Nash, T. A.; Kalt, W.; Vinqvist-Tymchuk, M. R.; Shukitt-Hale, B; Joseph, J. A. Blueberry supplementation improves memory in older adults. *J. Agric. Food Chem.* **2010**, *58*, 3996−4000.

(9) Goyarzu, P.; Malin, D. H.; Lau, F. C.; Taglialatela, G.; Moon, W. D.; Jennings, R.; Moy, E.; Moy, D.; Lippold, S.; Shukitt-Hale, B.; Joseph, J. A. Blueberry supplemented diet: effects on object recognition memory and nuclear factor-κB levels in aged rats. *Nutr. Neurosci.* **2004**, *7*, 75−83.

(10) Casadesus, G.; Shukitt-Hale, B.; Stellwagen, H. M.; Zhu, X.; Lee, H.-G.; Smith, M. A.; Joseph, J. A. Modulation of hippocampal plasticity and cognitive behavior by short-term blueberry supplementation in aged rats. *Nutr. Neurosci.* **2004**, *7*, 309−316.

(11) Joseph, J. A.; Shukitt-Hale, B.; Willis, L. M. Grape juice, berries, and walnuts affect brain aging and behavior. *J. Nutr.* **2009**, *139*, 1813S−1817S.

(12) Malin, D. H.; Lee, D. R.; Goyarzu, P.; Chang, Y. H.; Ennis, L. J.; Beckett, E.; Shukitt-Hale, B.; Joseph, J. A. Short-term blueberry-enriched diet prevents and reverses object recognition memory loss in aging rats. *Nutrition* **2011**, *3*, 338−342.

(13) Kreutzberg, G. W. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci.* **1996**, *19*, 312−318.
(14) Qin, L.; Wu, X.; Block, M. L.; Liu, Y.; Breese, G. R.; Hong, J. S.; Knapp, D. J.; Crews, F. T. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia* **2007**, *5*, 453−462.
(15) Sloane, J. A.; Hollander, W.; Moss, M. B.; Rosene, D. L.; Abraham, C. R. Increased microglial activation and protein nitration in white matter of the aging monkey. *Neurobiol. Aging* **1999**, *20*, 395−405.
(16) Shukitt-Hale, B.; Lau, F. C.; Carey, A. N.; Galli, R. L.; Spangler, E. L.; Ingram, D. K.; Joseph, J. A. Blueberry polyphenols attenuate kainic acid-induced decrements in cognition and alter inflammatory gene expression in rat hippocampus. *Nutr. Neurosci.* **2008**, *11*, 172−182.
(17) Kang, J.; Xie, C.; Li, Z.; Wu, T.; Nagaraja, S.; Schauss, A. G.; Wu, X. Flavonoids from açai (*Euterpe oleracea* Mart.) pulp and their antioxidant and anti-inflammatory activities. *Food Chem.* **2011**, *128*, 152−157.
(18) Schauss, A. G.; Wu, X.; Prior, R. L.; Ou, B.; Huang, D.; Owns, J.; Agarwal, A.; Jensen, G. S.; Hart, A. N.; Shanbrom, E. Antioxidant capacity and other bioactivities of the freeze-dried Amazonian palm berry, *Euterpe oleraceae* Mart (açai). *J. Agric. Food Chem.* **2006**, *54*, 8604−8610.
(19) Schauss, A. G.; Wu, X.; Prior, R. L.; Ou, B.; Patel, D.; Huang, D.; Kababick, J. P. Phytochemical and nutrient composition of the freeze-dried Amazonian palm berry, *Euterpe oleraceae* Mart (açai). *J. Agric. Food Chem.* **2006**, *54*, 8598−8603.
(20) Lisbeth, G.; Pacheco-Palencia, A.; Mertens-Talcott, S.; Talcott, S. T. Chemical composition, antioxidant properties, and thermal stability of a phytochemical enriched oil from açai (*Euterpe oleracea* Mart.). *J. Agric. Food Chem.* **2008**, *56*, 4631−4636.
(21) Jensen, G. S.; Schauss, A. G.; Beaman, R.; Ager, D. M. Açai fruit (*Euterpe oleracea* Mart): systematic and collaborative study of the phytochemistry, nutrient composition, and in vitro and in vivo bioactivities of the Amazonian palm fruit in humans. *Altern. Ther. Health Med.* **2009**, *15*, S90−S91.
(22) Schauss, A. G. Açai (*Euterpe oleracea* Mart.): a macro and nutrient rich palm fruit from the Amazon rain forest with demonstrated bioactivities in vitro and in vivo. In *Bioactive Foods in Promoting Health: Fruits and Vegetables*; Watson, R. R., Preedy, V., Eds.; Academic Press: Oxford, U.K., 2010; pp 479−490.
(23) Del Pozo-Insfran, D.; Percival, S. S.; Talcott, S. T. Açai (*Euterpe oleracea* Mart.) polyphenolics in their glycoside and aglycone forms induce apoptosis of HL-60 leukemia cells. *J. Agric. Food Chem.* **2006**, *54* (4), 1222−1229.
(24) Kang, J.; Li, Z.; Wu, T.; Jensen, G. S.; Schauss, A. G.; Wu, X. Antioxidant capacities of seven flavonoid compounds isolated from açai berry (*Euterpe oleracea*). *Food Chem.* **2010**, *122*, 610−617.
(25) Spada, P. D.; Dani, C.; Bortolini, G. V.; Funchal, C.; Henriques, J. A.; Salvador, M. Frozen fruit pulp of *Euterpe oleracea* Mart. (açai) prevents hydrogen peroxide induced damage in the cerebral cortex, cerebellum, and hippocampus of rats. *J. Med. Food* **2009**, *12*, 1084−1088.
(26) Jensen, G. S.; Wu, X.; Patterson, K. M.; Barnes, J.; Carter, S. G.; Scherwitz, L.; Beaman, R.; Endres, J. R.; Schauss, A. G. In vitro and in vivo antioxidant and anti-inflammatory capacities of an antioxidant-rich fruit and berry juice blend. Results of a pilot and randomized, double-blinded, placebo-controlled, crossover study. *J. Agric. Food Chem.* **2008**, *56*, 8326−8333.
(27) Sun, X.; Seeberger, J.; Alberico, T.; Wang, C.; Wheeler, C. T.; Schauss, A. G.; Zou, S. Açai pam fruit (*Euterpe olearacea* Mart.) pulp improves survival of flies on a high fat diet. *Exp. Gerontol.* **2010**, *45*, 243−251.
(28) Ribeiro, J. C.; Antunes, L. M.; Aissa, A. F.; Darin, J. D.; De Rosso, V. V.; Mercadante, A. Z.; Bianchi, M. D. Evaluation of the genotoxic and antigenotoxic effects after acute and subacute treatments with açai pulp (*Euterpe oleracea* Mart.) on mice using the erythrocytes micronucleus test and the comet assay. *Mutat. Res.* **2009**, *695*, 22−28.
(29) Jensen, G. S.; Ager, D. M.; Redman, K. A.; Mitzner, M. A.; Benson, K. F.; Mogle, C.; Schauss, A. G. Pain reduction and improvement of range of motion after consumption of an antioxidant-rich fruit- and berry-juice blend. *J. Med. Food* **2011**, *14*, 702−711.
(30) Rousseau, B.; Rosazza, J. P. Reaction of ferulic acid with nitrite: formation of 7-hydroxy-6-methoxy-1,2(4*H*)-benzoxazin-4-one. *J. Agric. Food Chem.* **1998**, *46*, 3314−3317.
(31) Dikun, P. P.; Ermilov, V. B.; Shendrikova, I. A. Some approaches to prevention of endogenous formation of *N*-nitrosamines in humans. In *Relevance to Human Cancer of N-Nitroso Compounds, Tobacco Smoke and Mycotoxins*; O'Neill, K., Chen, J., Bartsch, H., Eds.; IARC Scientific Publications: Lyon, France, 1991; pp 552−557.
(32) Liu, B.; Gao, H. M.; Hong, J. S. Parkinson's disease and exposure to infectious agents and pesticides and the occurrence of brain injuries: role of neuroinflammation. *Environ. Health Perspect.* **2003**, *111*, 1065−1073.
(33) Ringheim., G. E.; Conant, K. Neurodegenerative disease and the neuroimmune axis (Alzheimer's and Parkinson's disease, and viral infections). *J. Neuroimmunol.* **2004**, *147*, 43−49.
(34) Wang, J.; Mazza, G. Effects of anthocyanins and other phenolic compounds on the production of tumor necrosis factor α in LPS/IFN-γ-activated RAW 264.7 macrophages. *J. Agric. Food Chem.* **2002**, *50*, 4183−4189.
(35) Kalt, W.; Blumberg, J. B.; McDonald, J. E.; Vinqvist-Tymchuk, M. R.; Fillmore, S. A.; Graf, B. A.; O'Leary, J. M.; Milbury, P. E. Identification of anthocyanins in the liver, eye, and brain of blueberry-fed pigs. *J. Agric. Food Chem.* **2008**, *56*, 705−712.
(36) Del Rio, D.; Borges, G.; Crozier, A. Berry flavonoids and phenolics: bioavailability and evidence of protective effects. *Br. J. Nutr.* **2010**, *104*, S67−S90.
(37) Casolini, P.; Catalani, A.; Zuena, A. R.; Angelucci, L. Inhibition of COX-2 reduces the age-dependent increase of hippocampal inflammatory markers, corticosterone secretion, and behavioral impairments in the rat. *J. Neurosci Res.* **2002**, *68*, 337−343.
(38) Floyd, R. A. Neuroinflammatory processes are important in neurodegenerative diseases: an hypothesis to explain the increased formation of reactive oxygen and nitrogen species as major factors involved in neurodegenerative disease development. *Free Radical Biol. Med.* **1999**, *26*, 1346−1355.
(39) Bi, X. L.; Yang, J. Y.; Dong, Y. X.; Wang, J. M.; Cui, Y. H.; Ikeshima, T.; Zhao, Y. Q.; Wu, C. F. Resveratrol inhibits nitric oxide and TNF-α production by lipopolysaccharide-activated microglia. *Int. Immunopharmacol.* **2005**, *5*, 185−193.
(40) Seeram, N. P.; Momin, R. A.; Nair, M. G.; Bourquin, L. D. Cyclooxygenase inhibitory and antioxidant cyanidin glycosides in cherries and berries. *Phytomedicine* **2001**, *8*, 362−369.
(41) Bhat, N. R.; Zhang, P.; Lee, J. C.; Hogan, E. L. Extracellular signal regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor-α gene expression in endotoxin-stimulated primary glial cultures. *J. Neurosci.* **1998**, *18*, 1633−1641.
(42) Choi, Y.; Lee, M. K.; Lim, S. Y.; Sung, S. H.; Kim, Y. C. Inhibition of inducible NO synthase, cyclooxygenase-2 and interleukin-1β by torilin is mediated by mitogen-activated protein kinases in microglial BV2 cells. *Br. J. Pharmacol.* **2009**, *156* (6), 933−940.
(43) Baldwin, A. S. The NF-κB and I κB proteins: new discoveries and insights. *Annu. Rev. Immunol.* **1996**, *14*, 649−683.
(44) Eliopoulos, A. G.; Dumitru, C. D.; Wang, C. C.; Cho, J.; Tsichlis, P. N. Induction of COX-2 by LPS in macrophages is regulated by Tpl2-dependent CREB activation signals. *EMBO J.* **2002**, *21*, 4831−4840.
(45) Jeong, G. S.; Lee, D. S.; Kim, D. C.; Jahng, Y.; Son, J. K.; Lee, S. H.; Kim, Y. C. Neuroprotective and anti-inflammatory effects of mollugin via up-regulation of heme oxygenase-1 in mouse hippocampal and microglial cells. *Eur. J. Pharmacol.* **2011**, *654*, 226−234.