DOI:10.1021/jf100144y

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Differential Protection among Fractionated Blueberry Polyphenolic Families against DA-, Aβ_{42}- and LPS-Induced Decrements in Ca^{2+} Buffering in Primary Hippocampal Cells

James A. Joseph,[†,‡] Barbara Shukitt-Hale,*[,†] Gregory J. Brewer,[§] Karen A. Weikel,[†] Wilhelmina Kalt,[#] and Derek R. Fisher[†]

[†]USDA Human Nutrition Research Center on Aging at Tufts University, Boston, Massachusetts 02111, [§]Southern Illinois University School of Medicine, Springfield, Illinois 62794, and [#]Agriculture and Agri-Food Canada, Kentville, Nova Scotia B4N 1J5, Canada. [‡] In memory of James A. Joseph, who suddenly passed away while the paper was in press. He was a valued colleague and friend.

It has been postulated that at least part of the loss of cognitive function in aging may be the result of deficits in Ca^{2+} recovery (CAR) and increased oxidative/inflammatory (OX/INF) stress signaling. However, previous research showed that aged animals supplemented with blueberry (BB) extract showed fewer deficits in CAR, as well as motor and cognitive functional deficits. A recent subsequent experiment has shown that DA- or Aβ_{42}-induced deficits in CAR in primary hippocampal neuronal cells (HNC) were antagonized by BB extract, and (OX/INF) signaling was reduced. The present experiments assessed the most effective BB polyphenol fraction that could protect against OX/INF-induced deficits in CAR, ROS generation, or viability. HNCs treated with BB extract, BB fractions (e.g., proanthocyanidin, PAC), or control medium were exposed to dopamine (DA, 0.1 mM), amyloid beta (Aβ_{42}, 25 μM) or lipopolysaccharide (LPS, 1 μg/mL). The results indicated that the degree of protection against deficits in CAR varied as a function of the stressor and was generally greater against Aβ_{42} and LPS than DA. The whole BB, anthocyanin (ANTH), and PRE-C18 fractions offered the greatest protection, whereas chlorogenic acid offered the lowest protection. Protective capabilities of the various fractions against ROS depended upon the stressor, where the BB extract and the combined PAC (high and low molecular weight) fraction offered the best protection against LPS and Aβ_{42} but were less effective against DA-induced ROS. The high and low molecular weight PACs and the ANTH fractions enhanced ROS production regardless of the stressor used, and this reflected increased activation of stress signals (e.g., P38 MAPK). The viability data indicated that the whole BB and combined PAC fraction showed greater protective effects against the stressors than the more fractionated polyphenolic components. Thus, these results suggest that, except for a few instances, the lesser the polyphenolic fractionation, the greater the effects, especially with respect to prevention of ROS and stress signal generation and viability.

KEYWORDS: Calcium recovery; blueberry fractions; anthocyanins; proanthocyanidins; reactive oxygen species

INTRODUCTION

Our research has shown that supplementation with fruits and vegetables rich in polyphenolics is beneficial in both forestalling and reversing the deleterious effects of aging on neuronal communication and behavior (*1*). For example, in a previous experiment (*2*) we found that dietary supplementation (for 8 weeks) of blueberry (BB) extracts in rodent diets was effective in reversing age-related deficits in neuronal and behavioral (motor and cognitive) function in aged (19 months) Fischer 344 (F344) rats. We also observed that BB diet reversed age-related dysregulation in Ca^{45} buffering capacity and decreased reactive oxygen species (ROS). Similarly, buffering was decreased in dopamine (DA)- or amyloid beta (Aβ_{42})-exposed cultured primary hippocampal neuronal cells (HNC), and BB pretreatment of the cells prevented these deficits.

The beneficial properties of the BB have been postulated to be due to the polyphenolic makeup of the fruit (*3*). All plants, including fruit- or vegetable-bearing plants, synthesize a vast array of chemical compounds that are not necessarily involved in the plant's metabolism. These secondary properties, of course, would include their antioxidant and anti-inflammatory (INF) properties.

The question remains, however, as to which polyphenolic "family" might be responsible for the beneficial BB effects. It appears that some of these positive benefits may be derived

*Address correspondence to this author at the USDA Human Nutrition Research Center on Aging at Tufts University, 711 Washington St., Boston, MA 02111 [telephone (617) 556-3118; fax (617) 556-3222; e-mail barbara.shukitthale@ars.usda.gov].

 Published on Web 07/02/2010

from flavonoids, possibly from the anthocyanin flavonoids, which are responsible for the colors in fruits such as berry fruits (e.g., strawberries, blueberries). Anthocyanins have been shown to have potent antioxidant/anti-INF activities (*4*, *5*), as well as to inhibit lipid peroxidation and the INF mediators COX-1 and -2 (*6*, *7*). These fruits also contain high levels of proanthocyanidins that have antioxidant effects similar to those of anthocyanins (*8*). Indeed, there is a long history of studies that have described the potent antioxidant activities of numerous flavonoids. As examples, flavonoids have been reported to inhibit lipid peroxidation in several biological systems including mitochondria and microsomes (*9*, *10*), as well as erythrocytes (*11*, *12*) and liver (*13*). They appear to be potent inhibitors of both NADPH and CCl_4-induced lipid peroxidation (*14*) and readily chelate iron (*15*). Thus, the present study was carried out to determine the most effective BB polyphenolic component that would alter DA- or Aβ_{42}-induced deficits in calcium buffering, activation of ROS, increases in signals, and decreases in viability in primary hippocampal cells. Note that the fraction and whole BB concentrations utilized in this study are based on previous cell studies (*16*, *17*) wherein the concentrations were chosen on the basis of the effects on recovery in the calcium imaging parameter. In vivo levels of many of these compounds in plasma and brain are sparse. Our major purpose in this experiment was to determine the mechanistic effects of these fractions on calcium buffering through their putative protective properties with respect to stress signaling.

MATERIALS AND METHODS

Cell Maintenance and Treatment. NeuroPure E18 primary rat hippocampal cells were plated and grown in Neurobasal Medium according to the procedures of Gene Therapy Systems (San Diego, CA) and treated as described by Joseph and colleagues (*16*). Briefly, cells were allowed to differentiate for 4−5 days at 37 °C before being tested. The primary treatment was carried out with frozen whole Tifblue BB (*Vaccinium virgatum*) (as a positive control) prepared as described in previous experiments (see, e.g., ref *18*). Briefly, BBs were homogenized in water (2:1 w/v) for 3 min. The homogenate was centrifuged at 27000*g* for 15 min at 4 °C. The supernatant was collected and lyophilized, and freeze-dried extract was prepared in media for application to the cells. Additional groups of cells were pretreated with BB fractions derived from wild blueberry juice made from *Vaccinium angustifolium* Aiton (see below) and incubated for 45 min at 37 °C. The fraction concentrations reflected the percentage of the amount that was contained in the whole BB fraction (at 0.5 mg/mL) based on phenolic level (see BB Fractions). Secondary treatments included dopamine (DA, 0.1 mM, 2 h), Aβ_{42} (25 μM, 24 h), or LPS (1 μg/mL, 4 h). Following the incubations, cells were evaluated for alterations in calcium parameters, ROS generation, and viability.

BB Fractions. Polyphenolic fractions were obtained using a modified version of a procedure initially reported by Kader and colleagues (*19*). A commercially prepared wild blueberry juice was derived from whole ripe frozen blueberries (*V. angustifolium* Aiton) (PRE-C18) by Van Dyk's Health Juice Products Ltd. (Caledonia, NS, Canada). Berries were thawed, pressed, filtered, and pasteurized. One kilogram of blueberry fruit produced approximately 735 mL of single-strength juice. The anthocyanin content of whole blueberries is approximately 13.96 mg of cyanidin 3-glucoside equiv/g of dry weight, whereas that of the commercial juice was 8.87 mg of cyanidin 3-glucoside equiv/g of dry weight (or 64%). The concentrations of the fractions utilized were determined as a function of their phenolic levels in whole BB as described by Kalt and colleagues (*20*). This study provided a breakdown of the proportions of major phenolic groups in BB. The juice was applied to an activated C18 column (Waters Ltd., Mississauga, ON, Canada), washed with water to remove sugars and organic acids (POST-C18), and then washed with methanol (Fisher Scientific, Mississauga, ON, Canada) to elute the anthocyanins/proanthocyanidins (PAC). The POST-C18 material was lyophilized and then redissolved in a minimum volume of water. The aqueous solution was extracted five times with a 2× volume of ethyl acetate. The aqueous portion was rotovapped to remove traces of ethyl acetate (Fisher Scientific), and then methanol (or ethanol) was added to bring the final solution to 25% methanol. This was applied to a Sephadex LH-20 (Sigma, St. Louis, MO) column. The column was washed with 50% methanol, which eluted anthocyanins (ANTH). The LH-20 column was then washed with 70% acetone (Fisher Scientific) to elute the high molecular weight (HMW) proanthocyanidins. The ethyl acetate fraction from the extraction step was rotovapped to dryness and then resolubilized in a minimum volume of 25% methanol. This was applied to a second LH-20 column. The column was washed with 50% methanol to remove chlorogenic acid (CA) and other hydroxycinnamates and then with 70% acetone to remove low molecular weight (LMW) proanthocyanidins. Chlorogenic acid used in this study was purchased from Sigma. All fractions of interest were dried and resolubilized accordingly. The concentrations of the various fractions that were used to treat the cells were determined by their phenolic levels in the whole BB. These fraction concentrations are shown in **Table 1**.

Table 1. Concentrations of the Whole BB Extract and the Various BB Fractions Used in the Hippocampal Cells (Based upon Phenolic Levels in the Whole BB)

compound	phenolic level (mg/g)	concentration (μg/mL)
blueberry whole extract	10.15	500
PRE-C18	22.70	250
POST-C18	329.37	17.5
anthocyanins	363.50	15
proanthocyanidins (PACs)	414.79	14
low molecular weight PACs	1043.37	5.5
high molecular weight PACs	492.96	11.5
chlorogenic acid	364.0	15

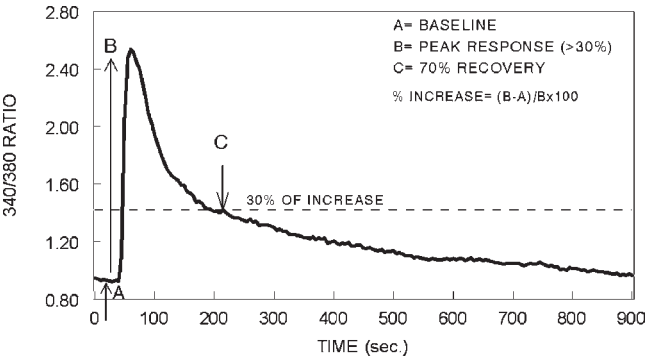

Figure 1. Typical baseline (A), peak depolarization following 30 mM KCl (B), and recovery (C) in control cells that were not treated with DA, LPS, or Aβ_{42}.

Ca^{2+} Imaging. Calcium imaging was carried out as previously described by Joseph and colleagues (*21*). **Figure 1** shows typical baseline, response (to 30 mM KCl, >30%), and recovery (CAR) in control hippocampal cells. Note that for this experiment baselines and responses under the various conditions did not differ. Thus, only the percent recovery is reported.

Viability. Viability was assessed using a LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR). Cells were stained for 30 min with calcein AM, which stains the live cells green, and ethidium homodimer-1 (EthD-1), which stains the dead cells red. Fluorescent images of the cells were then captured with a Nikon TE2000U inverted microscope. The numbers of live and dead cells were then counted for each image, and percent viability was determined.

ROS Generation. ROS production was assessed using an Image-iT LIVE Green Reactive Oxygen Species Detection Kit (Invitrogen, Eugene, OR). Cells were stained for 30 min using carboxy-H2 DCFDA and counterstained with Hoechst 33342. Fluorescent images of the cells were then captured with a Nikon TE2000U inverted microscope. The mean green value of each cell was then measured by circling each individual cell and normalizing for the background.

Immunofluorescence. Cellular changes in the level of activation of a subset of stress signals (Jun kinase, JNK; nuclear factor kappa B, NFκB; P38 mitogen activated protein kinase, P38 MAPK) and a protective signal (MAPK) in the hippocampal control or fraction-supplemented cells exposed to DA under the various conditions were analyzed by fluorescence

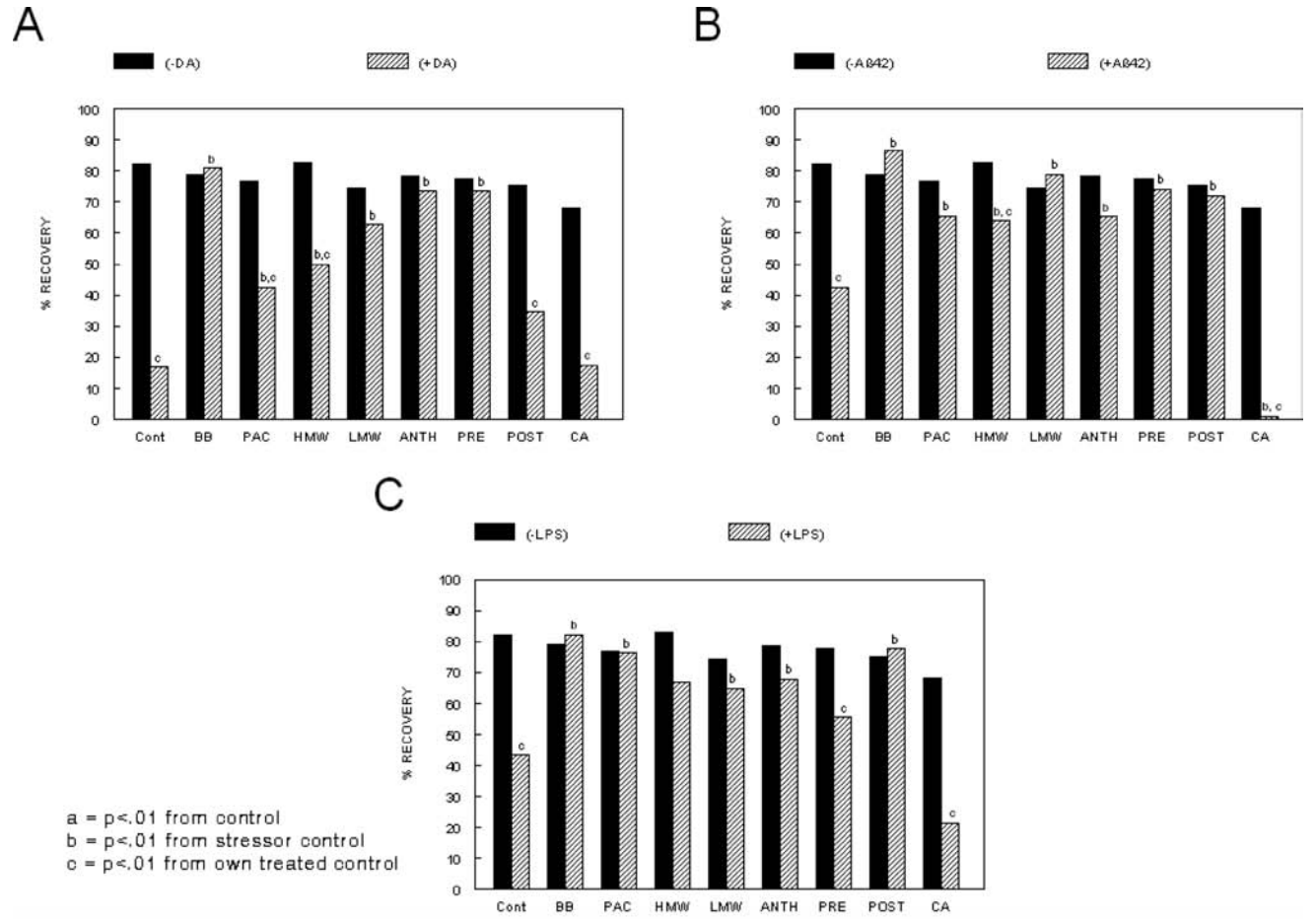

Figure 2. Differences in the values of calcium recovery in whole BB, fraction pretreated, or control (Cont) cells exposed to DA (**A**), Aβ_{42} (**B**), or LPS (**C**). b, $p < 0.05$ from stressor control; c, $p < 0.05$ from own treated control.

immunocytochemistry. Briefly, the differentiated hippocampal cells in 96-well plates were treated with various stressors with or without the fractions as indicated. After treatments, cells were fixed for 15 min in 100% methanol at −20 °C. The fixative was then removed, and the cells were washed twice in 4 °C PBS (5 min/wash). The primary antibody diluted in blocking solution was then applied to the wells, and the cells were incubated for 1 h at room temperature in a humid chamber and then washed four times in PBS. The fluorescence-labeled secondary antibody was added to the wells, and the cells were incubated for 1 h at room temperature as before. The cells were then washed four times with PBS. The cells were mounted with 95% glycerol in PBS, and images were captured with a Nikon Eclipse TE200U inverted fluorescence microscope coupled to a digital CCD camera (Hamamatsu Photonics). The levels of fluorescence intensity, for each individual cell less that of the background, per treatment were analyzed and averaged with Simple PCI software (Compix, Inc., Mars, PA). Note that only DA was used for these determinations, because as seen below, there was greater variation among the fractions with DA used as the stressor than with LPS or Aβ_{42}. The following antibodies were used: phospho P38 MAPK (Cell Signaling, rabbit polyclonal); phospho JNK (Cell Signaling, rabbit polyclonal); phospho NFκB (Abcam, rabbit polyclonal); and phospho MAPK, which detects erk1 and erk2 (Cell Signaling, mouse monoclonal). Negative controls for each antibody were also run by omitting either the primary or secondary antibodies.

Data Analysis. Recovery was analyzed by Kruskal−Wallis one-way analyses of variance (ANOVA) and Mann−Whitney U tests. Viability and ROS generation were analyzed by ANOVA and Fisher's LSD post hoc tests. Alterations in phospho (p)MAPK, pNFκB, pP38 MAPK, and pJNK were assessed via ANOVA and Fisher's LSD post hoc tests.

RESULTS

Ca^{2+} Imaging. Figure 2A shows the differences in the values of recovery in whole BB, fraction pretreated, or control cells exposed to DA (control vs DA, $p < 0.001$). In cells pretreated with the whole BB extract, LMW, ANTH, or PRE-C18, the effects of DA on recovery were antagonized (BB vs BB + DA; LMW vs LWM + DA; ANTH vs ANTH + DA; PRE-C18 vs PRE-C18 + DA, all $p > 0.05$). However, as can be seen from **Figure 2A**, it appeared that in the cells pretreated with PAC, HMW, POST-C18, or CA (PAC vs PAC + DA, $p < 0001$; HMW vs HMW+DA, $p < 0.01$; POST-C18 vs POST-C18 + DA, $p < 0.0001$; CA vs CA+DA, $p < 0.001$), the fractions offered only reduced effects on CAR. Interestingly, though, all of the fractions except for the POST-C18 and the chlorogenic acid fractions showed some protection against DA, because comparisons with the DA alone condition differed from all of the remaining treatments (BB, PAC, HMW, LMW ANTH, or PRE-C18 vs DA under all of these conditions showed increases in recovery, $p < 0.05$).

As shown in **Figure 2B**, and as seen previously, recovery was reduced in the Aβ_{42}-exposed cells (control vs Aβ_{42}, $p < 0.001$). However, unlike the effects seen with DA, a greater number of the fractions were effective in antagonizing the effects of Aβ_{42} (e.g., PAC vs Aβ_{42} + PAC, $p > 0.05$). The only fraction that failed to protect CAR from Aβ_{42} was CA (CA vs CA + Aβ_{42}, $p < 0.001$).

Similar effects were seen with respect to LPS treatment (**Figure 2C**), when pretreatment of the cells with the BB fractions was effective in antagonizing the effects of LPS on CAR. The only fractions that failed to protect CAR from LPS were PRE-C18 (PRE-C18 vs PRE-C18 + LPS, $p < 0.039$) and CA (e.g., CA vs CA + LPS, $p < 0.01$).

Viability. Overall, it appeared that some of the fractions may have lowered the viability of the cells in the absence of the stressor

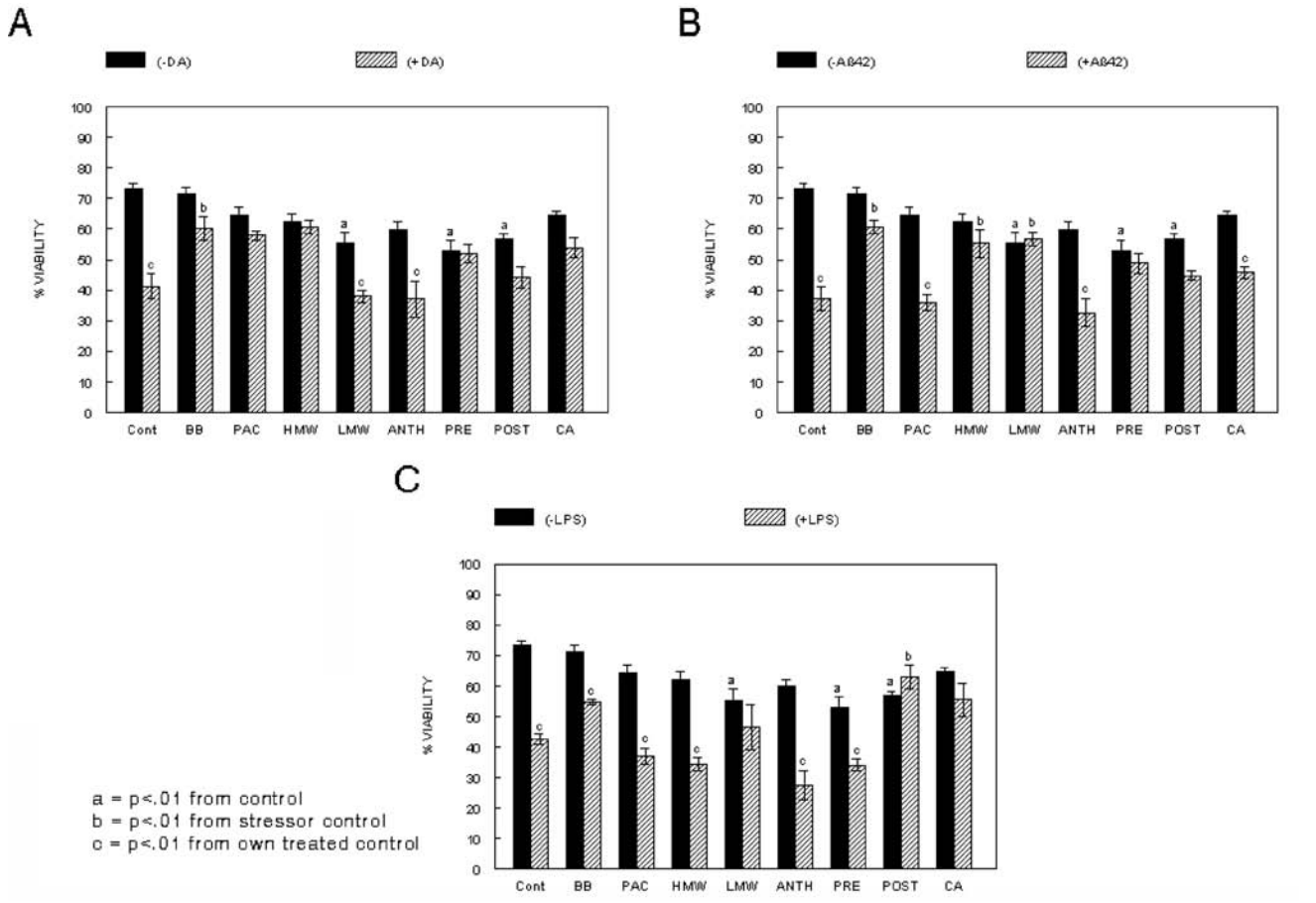

Figure 3. Differences in viability in whole BB, fraction pretreated, or control (Cont) cells exposed to DA (**A**), Aβ_{42} (**B**), or LPS (**C**). a, $p < 0.05$ from control; b, $p < 0.05$ from stressor control; c, $p < 0.05$ from own treated control.

(e.g., control vs LMW, PRE-C18, or POST-C18, $p < 0.011$), whereas BB, PAC, HMW, ANTH, and CA had no significant effect on viability (control vs BB, PAC, HMW, ANTH, or CA, $p > 0.05$, **Figure 3**). As was seen above with respect to recovery, DA also reduced viability in the hippocampal cells (control vs DA, $p < 0.0001$, **Figure 3A**). It also appeared that neither LMW nor ANTH offered protection against DA-induced decreases in viability (LMW vs LMW + DA, $p < 0.024$; ANTH vs ANTH + DA, $p < 0.001$). However, it did appear that although the PRE-C18 fraction lowered the viability of the cells in the absence of DA, no further reductions were seen in the PRE-C18-pretreated cells that were further treated with DA ($p > 0.05$). In fact, the BB + DA cells did not differ from the PRE-C18 cells ($p > 0.05$).

Interestingly, CA, although not protecting against DA-induced deficits in CAR, did protect against the DA-induced deficits in viability (CA vs CA + DA, $p > 0.05$). In fact, only the BB + DA, PAC + DA, and HMW + DA treatments differed from the DA alone condition (e.g., DA vs HMW+DA, $p < 0.004$, **Figure 3A**).

It also appeared that several of the fractions were able to prevent the decreases in viability induced by Aβ_{42}, most notably BB, HMW, LMW, and PRE- and POST-C18 (e.g., all comparisons of fractions with the Aβ_{42} + fractions, $p > 0.05$), whereas ANTH, PAC, and CA were not (PAC vs PAC + Aβ_{42}, $p < 0.0001$; ANTH vs ANTH+Aβ_{42}, $p < 0.001$; CA vs CA + Aβ_{42}, $p < 0.02$) (**Figure 3B**).

Additionally, as seen with DA and Aβ_{42}, LPS reduced the viability of the hippocampal cells; however, almost none of the fractions were effective in preventing the LPS-induced deficits, except for the LMW, POST-C18, and CA fractions (e.g., LMW, POST-C18, or CA vs LMW, POST-C18, or CA + LPS, $p > 0.05$) (**Figure 3C**).

ROS Generation. Figure 4 shows that neither whole BB extract, PAC, PRE-C18, nor POST-C18 enhanced ROS in the absence of a stressor (e.g., control vs BB, PAC, PRE-C18, or POST-C18, $p > 0.05$). Conversely, HMW, LMW, ANTH, and CA increased ROS in the absence of stressor (control vs HMW, LMW, ANTH, or CA, $p < 0.011$). The data also indicate that there were differences among the levels of protection offered by the fractions that were dependent upon the stressor. As shown in **Figure 4A**, only the whole BB extract and the CA fraction were effective in preventing ROS via DA stimulation (DA vs BB + DA or CA + DA, $p < 0.0001$). However, if the cells treated with the extracts are compared to cells treated only with DA + the extract, then PAC vs PAC + DA and PRE-C18 vs PRE-C18 + DA ($p > 0.05$) are also effective in this regard. The results also indicated that HMW, LMW, ANTH, and POST-C18 produced a synergistic effect with DA such that the ROS production was *greater* than that seen in the absence of these fractions (DA vs DA + HMW, $p < 0.01$; DA vs DA + ANTH, DA+ LMW, $p < 0.0001$, **Figure 4A**).

In the case of Aβ_{42}, BB and PAC decreased Aβ_{42}-enhanced ROS (Aβ_{42} vs BB + Aβ_{42} or PAC + Aβ_{42}, $p < 0.0001$). However, similar to the findings seen with DA, there was a significant synergistic effect between Aβ_{42} and the remainder of the fractions in increasing ROS (Aβ_{42} vs Aβ_{42} + HMW, LMW, ANTH or CA, $p < 0.001$; Aβ_{42} vs POST-C18 + Aβ_{42}, $p < 0.012$), where ROS were enhanced to a *greater extent* than seen with Aβ_{42} alone (**Figure 4B**). Interestingly, unlike the findings concerning DA or Aβ_{42}, **Figure 4C** shows that there were no synergistic increases with LPS and the various fractions as compared to the LPS alone

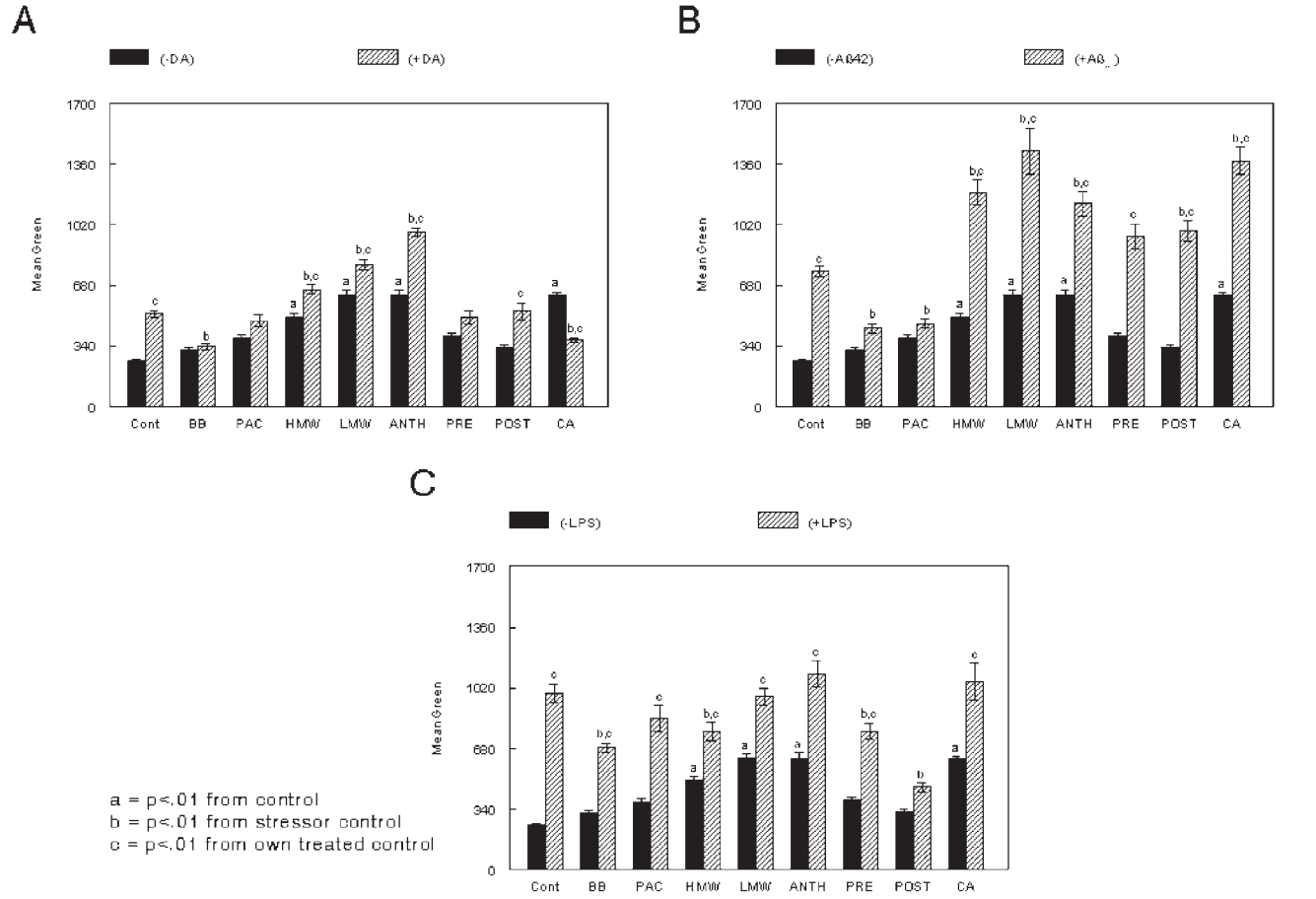

Figure 4. Differences in ROS in whole BB, fraction pretreated, or control (Cont) cells exposed to DA (**A**), Aβ_{42} (**B**), or LPS (**C**). a, $p < 0.05$ from control; b, $p < 0.05$ from stressor control; c, $p < 0.05$ from own treated control.

condition (all LPS vs LPS + ANTH, LMW, or CA comparisons, $p > 0.05$). Additionally, all fractions + LPS (BB, PAC HMW, LMW, ANTH, PRE-C18, CA), except for the POST-C18 fraction, showed ROS greater than their respective fraction pretreatment condition. However, in comparisons between the various fraction treatments in the presence of LPS vs with LPS alone, the results showed that there was some lowering of the LPS-induced increases in ROS [BB (LPS vs BB + LPS, $p < 0.001$), HMW (LPS vs HMW + LPS, $p < 0.018$) PRE-C-18 (LPS vs LPS + PRE-C-18, $p < 0.027$); POST-C18 (LPS vs POST-C18 + LPS, $p < 0.0001$)].

Immunofluorescence. *Phospho (p)MAPK.* In the absence of DA, BB and ANTH significantly raised pMAPK over that seen in control cells (control vs BB, $p < 0.003$; control vs ANTH, $p < 0.0001$) (**Figure 5A**). DA also significantly raised pMAPK ($p < 0.0001$). Each of the fractions except for ANTH significantly increased pMAPK as compared to their respective non-DA-pre-treated, fraction-treated cells (ANTH vs ANTH + DA, $p > 0.05$), but none of the fractions increased pMAPK to the level seen with DA alone.

pJNK. In the absence of DA, neither BB nor any of the fractions increased pJNK to a value greater than that seen in control cells (all comparisons $p > 0.05$ with control). However, in the presence of DA, BB treatment significantly reduced pJNK to a level significantly lower than that seen with any of the other fractions (BB + DA vs PAC + DA, HMW + DA, LMW + DA, ANTH + DA, PRE-C18 + DA, POST-C18 + DA, all comparisons $p < 0.0001$) (**Figure 5B**).

pP38 MAPK. In the absence of DA, all of the fractions significantly increased pP38 MAPK (control vs PAC, HMW, LMW, ANTH, PRE- and POST-C18, all comparisons $p < 0.009$, **Figure 5C**), except for BB (control vs BB, $p > 0.05$). In the presence of DA, BB significantly lowered pP38 MAPK (DA vs BB + DA, $p < 0.0001$). The effects of the other fractions were mixed, with the HMW (DA vs HMW + DA, $p < 0.002$), LMW (DA +LMW +DA, $p < 0.012$), ANTH (DA vs ANTH + DA, $p < 0.0001$), and PRE-C18 (DA vs PRE-C18 + DA, $p < 0.0001$) fractions showing decreases in pP38 MAPK. Conversely, cells treated with DA + PAC or DA+POST-C18 fractions showedr increases in pP38MAPK simila to that seen in the DA-treated cells (all comparisons $p > 0.05$, **Figure 5C**).

pNFκB. DA significantly increased NFκB as compared to control ($p < 0.001$). In the absence of DA, only one of the fractions significantly increased pNFκB (control vs HMW, $p < 0.0001$, **Figure 5D**). In the presence of DA, BB significantly lowered pNFκB (DA vs BB + DA, $p < 0.0001$). The effects of the other fractions were mixed, with the HMW (DA vs HMW + DA, $p < 0.0001$), PRE-C18 (DA vs PRE-C18 + DA, $p < 0.0001$), and ANTH (DA vs ANTH + DA, $p < 0.05$) fractions showing decreases in pNFκB. Conversely, cells treated with DA + PAC, LMW, and POST-C18 fractions showed increases in pNFκB similar to that seen in cells treated with DA alone (all comparisons $p > 0.05$, **Figure 5D**). Note also that the LMW, ANTH, and POST fractions had higher activity in pNFκB with DA than its respective fraction-treated control (e.g., ANTH vs ANTH + DA, $p < 0.01$).

DISCUSSION

Early findings suggested that there was significant neuronal loss and other morphological changes that occur in the aging

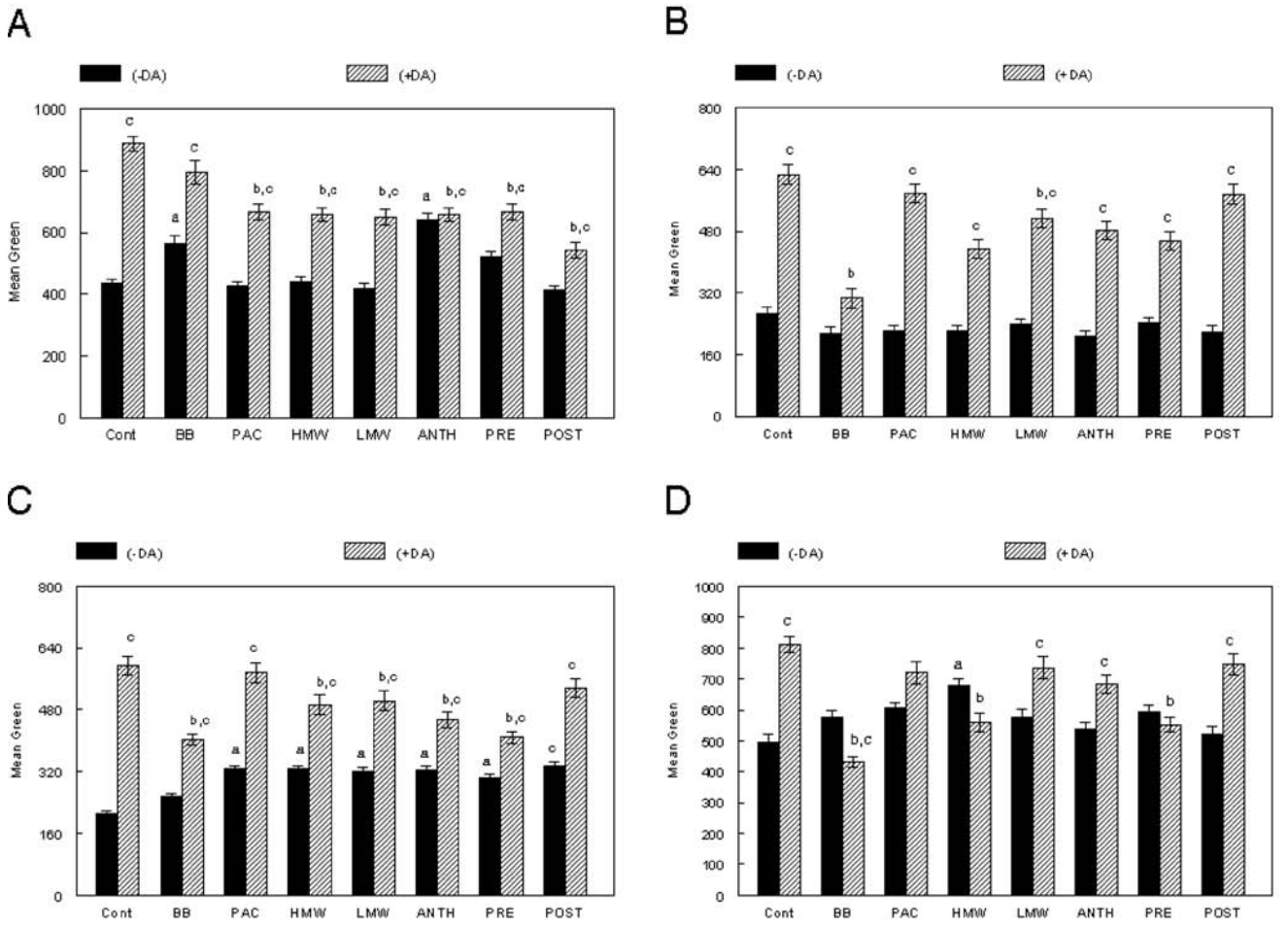

Figure 5. Cellular changes in the level of activation of a subset of protective (MAPK, **A**) and stress signals (JNK, **B**; P38MAPK, **C**; NfκB, **D**) in whole BB, fraction pretreated, or control (Cont) cells exposed to DA and analyzed by fluorescence immunocytochemistry. a, $p < 0.05$ from control; b, $p < 0.05$ from DA control; c, $p < 0.05$ from own treated control.

brain (*22*). However, additional studies indicated that the age-related changes are much more subtle and involve calcium dysregulation (*23*−*25*). Whereas these age-associated changes appear to be dependent upon the particular neurons involved, they all involve various changes in calcium homeostasis and alterations in calcium regulation, with hippocampal and cortical neurons showing the greatest alterations (*26*, *27*). As indicated in the Introduction, however, all of the changes involve some decline in calcium buffering. It has been postulated that these changes are the result of oxidative stress (*28*).

Indeed, the results of the present paper would support these findings, because DA, Aβ_{42}, and LPS all reduced calcium buffering in the hippocampal cells. However, it appeared that, overall, the protective effects of the various fractions on calcium buffering were dependent upon the particular stressor to which they were exposed. Generally, the whole BB extract and PRE-C18 fraction were the most effective in protecting CAR among the stressors, especially with regard to DA and Aβ_{42}, whereas CA offered the least protection. These findings suggest that it may be the synergistic effects among the various polyphenolic families in the least fractionated forms of the blueberry (e.g., BB and PRE-C18) that provided the most generalized protection against DA and Aβ_{42}. However, note that there were differences between the BB extract and the PRE-C18 fraction in protecting CAR against LPS. It appeared that CAR was somewhat impaired when LPS was applied to the PRE-C18-treated cells. This was not seen with the BB extract-treated cells, where CAR decreases were not seen with LPS. These differences were also reflected in the assessments of viability, where it appeared that the PRE-C18 fraction lowered viability in the absence of the stressors and a further lowering was seen with LPS treatment. Again, these alterations in viability were not seen in the cells treated with the BB extract, although there were no differences in viability in DA- or Aβ_{42}-treated cells that were pretreated with the BB extract or the PRE-C18 fraction. There was also some indication that the PRE-C18 fraction was not as protective against increases in ROS with DA or Aβ_{42}. These differences could be the result of differences in concentrations between the two pretreatments (BB or PRE-C18), because the concentrations utilized were based upon the phenolic levels of the compounds in the whole BB. The water-soluble BB extract was utilized at 500 μg/mL, whereas the PRE-C18 fraction utilized was half that of the BB extract (250 μg/mL). These differences could also reflect source variations, because the BB extract was derived from frozen whole Tifblue cultivated BBs, whereas the PRE-C18 fraction was derived from wild blueberry juice. The types of phenolics in the two fractions may differ, making direct comparisons between BB and PRE-C18 difficult. However, because we have previous data using the Tifblue BBs it was necessary to add this condition as a positive control. Additionally, the *V. angustifolium* berries used to produce the juice included literally many hundreds, and possibly thousands, of genotypes harvested from the semicultivated wild stands. This complex mixture would therefore represent the average phenolic composition for the species. This is in contrast to cultivated blueberries

(*Vaccinium virgatum* was used in this study), the commercial juice of which may be produced from far fewer genotypes and, for that reason, may be subject to genotypic variability within the species.

Among the fractions, PAC, the HMW proanthocyanidins, and POST-C18 fractions were not effective against DA in protecting CAR. However, except for CA, which was not effective with any of the stressors, all of the fractions were significantly more protective when LPS or Aβ_{42} was used as the stressor. In fact, overall, fewer differences in protection among the fractions were observed with LPS or Aβ_{42} than that seen with DA.

DA oxidation may be involved in the neuronal toxicity in neurodegenerative diseases such as Parkinson's disease, where DA is easily oxidized to form DA quinones and other ROS species such as 3,4-dihydroxyphenylacetaldehyde or 3,4-dihydroxyphenylethyleneglycolaldehyde (DOPEGAL) and may be responsible for neuronal loss in Parkinson's disease (*29*). One could speculate that reductions of ROS via nutritional supplementation with BBs may reduce DA toxicity. In this respect, in the present experiments when ROS was assessed among the various fractions, the data showed that even in the absence of the stressors (DA, LPS, or Aβ_{42}) some of the fractions raised rather than lowered ROS. These included HMW, LMW, ANTH, and CA. These findings support previous research which has shown that plant polyphenols can act as potent pro-oxidants. For example, research has indicated that resveratrol (*30*), flavonoids in general (*31*), tannins (*32*), and curcumin (*32*) can increase ROS.

It has been suggested that the pro-oxidant effect of plant polyphenols may be an artifact (*33*) of in vitro methods, but it is clear from the present paper that not all of the fractions, at least at the levels assessed here, increase ROS. It is possible that higher concentrations of these fractions may induce ROS increases. These include PAC, POST-C18, and, to some extent, the PRE-C18 fractions, in addition to the whole BB. As alluded to above, these findings suggest that even in vitro the less fractionated the fruit extract is, the less likely that ROS generation is seen in the absence of the stressors.

What is even more important is that when one considers the use of polyphenolic fractions as antioxidants in the presence of the various stressors, the pro-oxidant effects of the treatments may be even greater than that seen with the stressors alone. In the case of Aβ_{42} only the whole BB and PAC treatments reduced the Aβ_{42}-induced ROS effects; virtually all of the other fractions acted synergistically with the Aβ_{42}, whereas HMW, LMW, and ANTH fractions all acted synergistically with DA to increase ROS. Similar pro-oxidant synergistic effects have been reported previously with plant polyphenols (*34*−*36*).

However, when the parameters of calcium buffering and ROS are considered together, except possibly for CA treatment in the presence of LPS or Aβ_{42}, there appears to be little relationship between ROS generation in the presence or absence of the stressor and the ability of the various fractions to mitigate the effects of the stressors on calcium buffering. As mentioned above, most of the fractions were protective, at least under the LPS or Aβ_{42} treatment conditions. These dichotomies suggest that ROS effects may not be reflective of the beneficial effects of the fractions on calcium buffering. It may be that their protective effects on calcium buffering involve alterations in downstream stress signals rather than direct quenching effects on ROS. We have shown in a previous study that the BB extract decreases several stress signals such as calcium response element binding protein (CREB), protein kinase Cγ (PKCγ), and P38 MAPK, among other stress mediators that were enhanced by DA application to hippocampal cells (*16*). In the present experiment, DA, BB, ANTH, and PRE-C18 significantly raised pMAPK over that seen in control cells, and each of the fractions (except ANTH) increased pMAPK in the presence of DA.

BB reductions in pJNK in the presence of DA were greater than those of the other fractions, whereas PAC and POST-C18 increased pJNK in the presence of DA. Although BB and all of the fractions increased pNFκB, in the absence of DA only the whole BB reduced this parameter to a value lower than the control value. Similar findings were seen with DA and BB treatment with respect to pP38 MAPK, where DA alone slightly increased this stress signal but BB prevented the DA-induced increases in pP38 MAPK to a greater extent than any of the fractions. Thus, some of the differences between BB extract and the PRE-C18 fraction that were discussed above are also reflected in stress signal assessments, where DA-induced increases in JNK were greater with PRE-C18 pretreatment than with the BB extract. However, the findings with respect to the other stress signals and pMAPK were similar.

However, despite these differences, taken together these data suggest that the major protective effects of the whole BB extract and to some extent the PRE-C18 fraction involve the reductions of DA-induced increases in stress signal activation (pP38 MAPK and pNFκB). Additional evidence for this hypothesis regarding the various berry extract and fraction pretreatments can be seen when the viability and ROS parameters are compared. In some cases there were parallels between the two parameters (e.g., ANTH increases in ROS under stressor conditions with corresponding decreases in viability, LMW under DA and LPS conditions with decreases in viability, and PAC with decreases in viability and increases in ROS with Aβ_{42} and LPS), but in other cases there was a dichotomy between the two dependent measures (e.g., HMW increases in ROS with Aβ_{42} and DA with no decreases in viability; increases in ROS with PRE-C18 and POST-C18 treatments with no decreases in viability with Aβ_{42}), suggesting alternative forms of protection that may have protected CAR and also prevented losses of viability.

When viability was assessed in the hippocampal cells in the absence of the stressors, some of the fractions decreased viability (LMW, PRE-C18, and POST-C18). One of the most interesting findings concerning viability is that, as with ROS, there seemed to be a dichotomy between the effects of some of the fractions on viability (e.g., CA) and the level of protection provided by these fractions on hippocampal cell calcium buffering (CAR). For example, in the presence of DA there were differences among the fractions regarding the level of protection of CAR. However, there were fewer differences in the efficacy of the fractions when Aβ_{42} or LPS was used as stressor. This was not seen with viability, where there were differences among the fractions regardless of the particular stressor utilized. This would suggest that assessments of viability may not be related to changes in CAR, and the cell, while showing decreases in viability, may still function with respect to CAR as a normal cell. This dichotomous condition is similar to that seen in normal aging, where viability does not seem to explain losses in CAR. For example, it has been suggested that the degree of disruption of calcium buffering may not alter cell function or even viability, but it does make the cell more vulnerable to other stressors (*37*). As mentioned above, in the present study, chlorogenic acid was not protective against CAR decreases induced by any stressor, but offered some protection against viability loss with these stressors. Clearly, however, these relationships are complex and are stressor/fraction/parameter dependent.

Additionally, previous mechanistic studies have also shown that, in addition to their potent antioxidant/anti-inflammatory effects, the beneficial properties of fruit polyphenols such as those

found in BB might occur through alterations in stress signaling mediators such as extracellular signal regulated kinase (ERK), protein kinase C (PKC), cyclic AMP response element binding protein (CREB), and nuclear factor kappa B (NFκB). Indeed, our research has, thus far, shown that the BB protection against Aβ_{42}- or DA-induced decrements in intracellular calcium clearance following oxotremorine-induced depolarization in M1 muscarinic receptor (MAChR)-transfected COS-7 cells or neonatal hippocampal neurons involved reductions in phosphorylated MAPK, PKCγ, and phosphorylated CREB (*17*). Similar findings have also been seen with BB treatment in primary hippocampal cells (*16*). The results showed that BB pretreatment prevented the deficits in calcium buffering, normalized cyclic CREB, and PKCγ associated with ROS signaling and increased expression of protective ERK. Thus, these findings and those of previous studies suggest that the primary mechanisms involved in the beneficial effects of the berries may involve alterations in stress signals. However, because the concentrations utilized here may have been higher than those that occur in vivo, the effects of the treatments will have to be assessed further in whole animal models. Nevertheless, the findings here suggest that one of the mechanisms involved in the beneficial effects of BB may involve reductions in stress signals.

ABBREVIATIONS USED

CAR, calcium recovery; ROS, reactive oxygen species; MAChRs, muscarinic acetylcholine receptors; OS, oxidative stress; INF, inflammation; HNC, hippocampal neuronal cells; DA, dopamine; Aβ, amyloid beta; BB, blueberry; NFκB, nuclear factor kappa B; JNK, jun kinase; MAPK, p38 mitogen activated protein kinase; ERK, extracellular signal regulated kinase; CREB, cyclic AMP response element binding protein; PKC, protein kinase C; LPS, lipopolysaccharide; ANTH, anthocyanin; PAC, proanthocyanidin; HMW, high molecular weight PAC; LMW, low molecular weight PAC; CA, chlorogenic acid; PRE, PRE-C18; POST, POST-C18.

LITERATURE CITED

(1) Lau, F. C.; Shukitt-Hale, B.; Joseph, J. A. The beneficial effects of fruit polyphenols on brain aging. *Neurobiol. Aging* **2005**, *26* (Suppl. 1), 128–132.

(2) Joseph, J. A.; Shukitt-Hale, B.; Denisova, N. A.; Bielinski, D.; 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* (18), 8114–8121.

(3) Lau, F. C.; Joseph, J. A.; McDonald, J. E.; Kalt, W. Attenuation of iNOS and COX2 by blueberry polyphenols is mediated through the suppression of NF-κB activation. *J. Funct. Foods* **2009**, *1* (3), 274–283.

(4) Wang, H.; Nair, M. G.; Strasburg, G. M.; Chang, Y. C.; Booren, A. M.; Gray, J. I.; DeWitt, D. L. Antioxidant and antiinflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. *J. Nat. Prod.* **1999**, *62* (2), 294–296.

(5) 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* (5), 362–369.

(6) Seeram, N. P.; Nair, M. G. Inhibition of lipid peroxidation and structure-activity-related studies of the dietary constituents anthocyanins, anthocyanidins, and catechins. *J. Agric. Food Chem.* **2002**, *50* (19), 5308–5312.

(7) Seeram, N. P.; Zhang, Y.; Nair, M. G. Inhibition of proliferation of human cancer cells and cyclooxygenase enzymes by anthocyanidins and catechins. *Nutr. Cancer* **2003**, *46* (1), 101–106.

(8) Pan, M. H.; Ho, C. T. Chemopreventive effects of natural dietary compounds on cancer development. *Chem. Soc. Rev.* **2008**, *37* (11), 2558–2574.

(9) Bindoli, A.; Cavallini, L.; Siliprandi, N. Inhibitory action of silymarin of lipid peroxide formation in rat liver mitochondria and microsomes. *Biochem. Pharmacol.* **1977**, *26* (24), 2405–2409.

(10) Cavallini, L.; Bindoli, A.; Siliprandi, N. Comparative evaluation of antiperoxidative action of silymarin and other flavonoids. *Pharmacol. Res. Commun.* **1978**, *10* (2), 133–136.

(11) Sorata, Y.; Takahama, U.; Kimura, M. Protective effect of quercetin and rutin on photosensitized lysis of human erythrocytes in the presence of hematoporphyrin. *Biochim. Biophys. Acta* **1984**, *799* (3), 313–317.

(12) Maridonneau-Parini, I.; Braquet, P.; Garay, R. P. Heterogeneous effect of flavonoids on K^+ loss and lipid peroxidation induced by oxygen-free radicals in human red cells. *Pharmacol. Res. Commun.* **1986**, *18* (1), 61–72.

(13) Kimura, Y.; Okuda, H.; Tani, T.; Arichi, S. Studies on Scutellariae radix. VI. Effects of flavanone compounds on lipid peroxidation in rat liver. *Chem. Pharm. Bull. (Tokyo)* **1982**, *30* (5), 1792–1795.

(14) Afanas'ev, I. B.; Dorozhko, A. I.; Brodskii, A. V.; Kostyuk, V. A.; Potapovitch, A. I. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem. Pharmacol.* **1989**, *38* (11), 1763–1769.

(15) Ratty, A. K.; Das, N. P. Effects of flavonoids on nonenzymatic lipid peroxidation: structure−activity relationship. *Biochem. Med. Metab. Biol.* **1988**, *39* (1), 69–79.

(16) Joseph, J. A.; Carey, A.; Brewer, G. J.; Lau, F. C.; Fisher, D. R. Dopamine and abeta-induced stress signaling and decrements in Ca^{2+} buffering in primary neonatal hippocampal cells are antagonized by blueberry extract. *J. Alzheimers Dis.* **2007**, *11* (4), 433–446.

(17) Joseph, J. A.; Fisher, D. R.; Bielinski, D. Blueberry extract alters oxidative stress-mediated signaling in COS-7 cells transfected with selectively vulnerable muscarinic receptor subtypes. *J. Alzheimers Dis.* **2006**, *9* (1), 35–42.

(18) Youdim, K. A.; Shukitt-Hale, B.; Martin, A.; Wang, H.; Denisova, N.; Bickford, P. C.; Joseph, J. A. Short-term dietary supplementation of blueberry polyphenolics: beneficial effects on aging brain performance and peripheral tissue function. *Nutr. Neurosci.* **2000**, *3*, 383–397.

(19) Kader, F.; Rovel, B.; Girardin, M.; Metche, M. Fractionation and identification of the phenolic compounds of highbush blueberries (*Vaccinium corymbosum*, L.). *Food Chem.* **1996**, *55* (1), 35–40.

(20) Kalt, W.; MacKinnon, S.; McDonald, J.; Vinqvist, M.; Craft, C.; Howell, A. Phenolics of *Vaccinium* berries and other fruit crops. *J. Sci. Food Agric.* **2008**, *88* (1), 68–76.

(21) Joseph, J. A.; Fisher, D. R.; Strain, J. Muscarinic receptor subtype determines vulnerability to oxidative stress in COS-7 cells. *Free Radical Biol. Med.* **2002**, *32* (2), 153–161.

(22) Murchison, D.; Griffith, W. H. Calcium buffering systems and calcium signaling in aged rat basal forebrain neurons. *Aging Cell* **2007**, *6* (3), 297–305.

(23) Gibson, G. E.; Peterson, C. Calcium and the aging nervous system. *Neurobiol. Aging* **1987**, *8* (4), 329–343.

(24) Landfield, P. W. Increased calcium-current hypothesis of brain aging. *Neurobiol. Aging* **1987**, *8* (4), 346–347.

(25) Khachaturian, Z. S. Calcium hypothesis of Alzheimer's disease and brain aging. *Ann. N.Y. Acad. Sci.* **1994**, *747*, 1–11.

(26) Toescu, E. C.; Verkhratsky, A. The importance of being subtle: small changes in calcium homeostasis control cognitive decline in normal aging. *Aging Cell* **2007**, *6* (3), 267–273.

(27) Foster, T. C. Calcium homeostasis and modulation of synaptic plasticity in the aged brain. *Aging Cell* **2007**, *6* (3), 319–325.

(28) Esin, M. M. Ageing and the brain. *J. Pathol.* **2007**, *211*, 181–187.

(29) Burke, W. J.; Li, S. W.; Williams, E. A.; Nonneman, R.; Zahm, D. S. 3,4-Dihydroxyphenylacetaldehyde is the toxic dopamine metabolite in vivo: implications for Parkinson's disease pathogenesis. *Brain Res.* **2003**, *989* (2), 205–213.

(30) de la Lastra, C. A.; Villegas, I. Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications. *Biochem. Soc. Trans.* **2007**, *35* (Part 5), 1156–1160.

(31) Rahman, A.; Shahabuddin; Hadi, S. M.; Parish, J. H. Complexes involving quercetin, DNA and Cu(II). *Carcinogenesis* **1990**, *11* (11), 2001–2003.

(32) Hadi, S. M.; Asad, S. F.; Singh, S.; Ahmad, A. Putative mechanism for anticancer and apoptosis-inducing properties of plant-derived polyphenolic compounds. *IUBMB Life* **2000**, *50* (3), 167–171.
(33) Halliwell, B. Biochemistry of oxidative stress. *Biochem. Soc. Trans.* **2007**, *35* (Part 5), 1147–1150.
(34) Bahadorani, S.; Hilliker, A. J. Antioxidants cannot suppress the lethal phenotype of a *Drosophila melanogaster* model of Huntington's disease. *Genome* **2008**, *51* (5), 392–395.
(35) Sanchez, Y.; Amran, D.; Fernandez, C.; de Blas, E.; Aller, P. Genistein selectively potentiates arsenic trioxide-induced apoptosis in human leukemia cells via reactive oxygen species generation and activation of reactive oxygen species-inducible protein kinases (p38-MAPK, AMPK). *Int. J. Cancer* **2008**, *123* (5), 1205–1214.
(36) Shamim, U.; Hanif, S.; Ullah, M. F.; Azmi, A. S.; Bhat, S. H.; Hadi, S. M. Plant polyphenols mobilize nuclear copper in human peripheral lymphocytes leading to oxidatively generated DNA breakage: implications for an anticancer mechanism. *Free Radical Res.* **2008**, *42* (8), 764–772.
(37) Wojda, U.; Salinska, E.; Kuznicki, J. Calcium ions in neuronal degeneration. *IUBMB Life* **2008**, *60* (9), 575–590.

Received for review January 13, 2010. Revised manuscript received May 6, 2010. Accepted May 17, 2010. This study was supported in part by the USDA, the Wild Blueberry Association of North America, and the U.S. Highbush Blueberry Council.