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ARTICLE

Blueberry Treatment Antagonizes C-2 Ceramide-Induced Stress Signaling in Muscarinic Receptor-Transfected COS-7 Cells

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Previous research has shown that muscarinic receptors (MAChRs) show loss of sensitivity in aging and AD and are selectively sensitive to oxidative stress (OS). Thus, COS-7 cells transfected (tn) with MAChR subtype M1 show > OS sensitivity [as reflected in the ability of the cell to extrude or sequester Ca^{2+} following depolarization (recovery) by oxotremorine (oxo) and exposure to dopamine (DA) or amyloid beta (Aβ)] than M3-transfected COS-7 cells. Blueberry (BB) extract pretreatment prevented these deficits. Research has also indicated that C2 ceramide (Cer) has several age-related negative cellular effects (e.g., OS). When these cells were treated with Cer, the significant decrements in the ability of both types of tn cells to initially respond to oxo were antagonized by BB treatment. Present experiments assessed signaling mechanisms involved in BB protection in the presence or absence of DA, Aβ, and/or Cer in this model. Thus, control or BB-treated M1 and M3 tn COS-7 cells were exposed to DA or Aβ_{42} in the presence or absence of Cer. Primarily, results showed that the effects of DA or Aβ_{42} were to increase stress (e.g., PKCγ, p38MAPK) and protective signals (e.g., pMAPK). Cer also appeared to raise several of the stress and protective signals in the absence of the other stressors, including PKCγ, pJNK, pNfκB, p53, and p38MAPK, while not significantly altering MAPK, or Akt. pArc was, however, increased by Cer in both types of transfected cells. The protective effects of BB when combined with Cer generally showed greater protection when BB extract was applied prior to Cer, except for one protective signal (pArc) where a greater effect was seen in the M3 cells exposed to Aβ_{42}. In the absence of the Aβ_{42} or DA, for several of the stress signals (e.g., pNfκB, p53), BB lowered their Cer-induced increases in M1- and M3-transfected cells. We are exploring these interactions further, but it is clear that increases in ceramide, to the same levels as are seen in aging, can have profound effects on calcium clearance and signaling during oxidative stress.

KEYWORDS: Ceramide; aging; oxidative stress; blueberry polyphenols; muscarinic receptors

INTRODUCTION

As described below, numerous studies have suggested that one of the factors that may mediate age-associated changes in vulnerability to oxidative stress (OS) in aging may be ceramide. Ceramide (Cer) is generated intracellularly by the sphingomyelinase-induced cleavage of sphingomyelin (*1*−*3*), and this enzyme is strongly increased during developmental neuronal death in rat brain (*4*), producing increased Cer levels. These increases have been found during apoptosis in neuronally differentiated PC12 cells (*5*) or cortical neurons (*6*) and in the ischemic gerbil hippocampus (*7*). Previous experiments have also demonstrated an association between Cer and cellular senescence, where increased markers of senescence in fibroblasts (*8*) and inhibited cellular growth (*9*, *10*) have been observed. Numerous studies have also suggested that Cer levels are increased by OS (e.g., refs *11*, *12*). Importantly, in this regard, Cer has been shown to enhance the effects of hydrogen peroxide in decreasing calcium buffering following hydrogen peroxide exposure (*13*, *14*).

Additionally, Cutler and colleagues (*15*) have shown significant increases in levels of membrane Cer in neuronal cells in Alzheimer's disease (AD) patients and aged mice that may reflect increased OS and amyloid beta (Aβ) deposition. Research has indicated that samples taken from various Alzheimer brain regions showed four times the levels of Cer as those taken from control brains (*16*−*19*). These increased levels of Cer have been postulated to stabilize the APP-cleaving enzyme 1 (BACE1) and increase Aβ (*20*). Conversely, a reduction of Cer levels appears to reduce the formation of APP and Aβ in human neuroblastoma cells (*21*). Thus, it has been suggested that Cer and Aβ peptides act in concert to induce neuronal death in AD, especially under OS conditions. Studies also suggest that an additional effect of Aβ peptides may be to negatively affect hippocampal functions such as long-term synaptic plasticity in AD transgenic mice and hippocampal preparations (*22*). The Aβ peptide promotes calcium influx and disrupts calcium homeostasis in a manner similar to that seen in aging (*23*, *24*).

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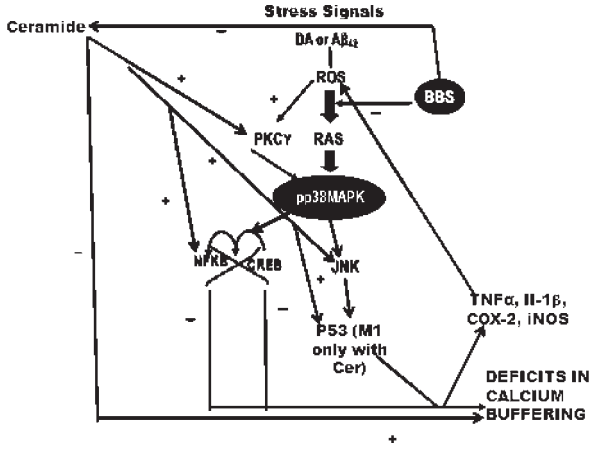

Figure 1. Schematic representation of the effects of the stressors, ceramide, DA and/or Aβ_{42} on stress signals and calcium buffering (from ref *33*).

It appears that, at least to some extent, the mechanisms involved in the deleterious effects of Cer on calcium regulation appear to be related to increases in stress-mediated signaling. Studies have shown, for example, that increases in stress signaling such as Cer-induced nuclear factor kappa B (NFκB) (*5*, *16*, *25*−*27*) can in turn enhance cellular Cer. Additional findings have indicated that Cer can downregulate the neuroprotective extracellular signal regulated kinase (ERK) and upregulate the stress signals jun kinase (JNK) and p38 mitogen activated protein kinase (MAPK) (e.g., ref *28*).

To determine whether Cer would enhance the efficacy of oxidative stressors we assessed the effects of Cer in the presence or absence of DA or Aβ_{42} on calcium baseline, response, and clearance (buffering, recovery) following depolarization in M1- or M3-transfected COS-7 cells (*29*). The rationale for utilizing muscarinic receptor transfected COS-7 cells was to attempt to discern the effects of OS on an important nexus: the receptor in relative isolation. Experiments have shown that aging and Alzheimer disease are associated with a loss of sensitivity in

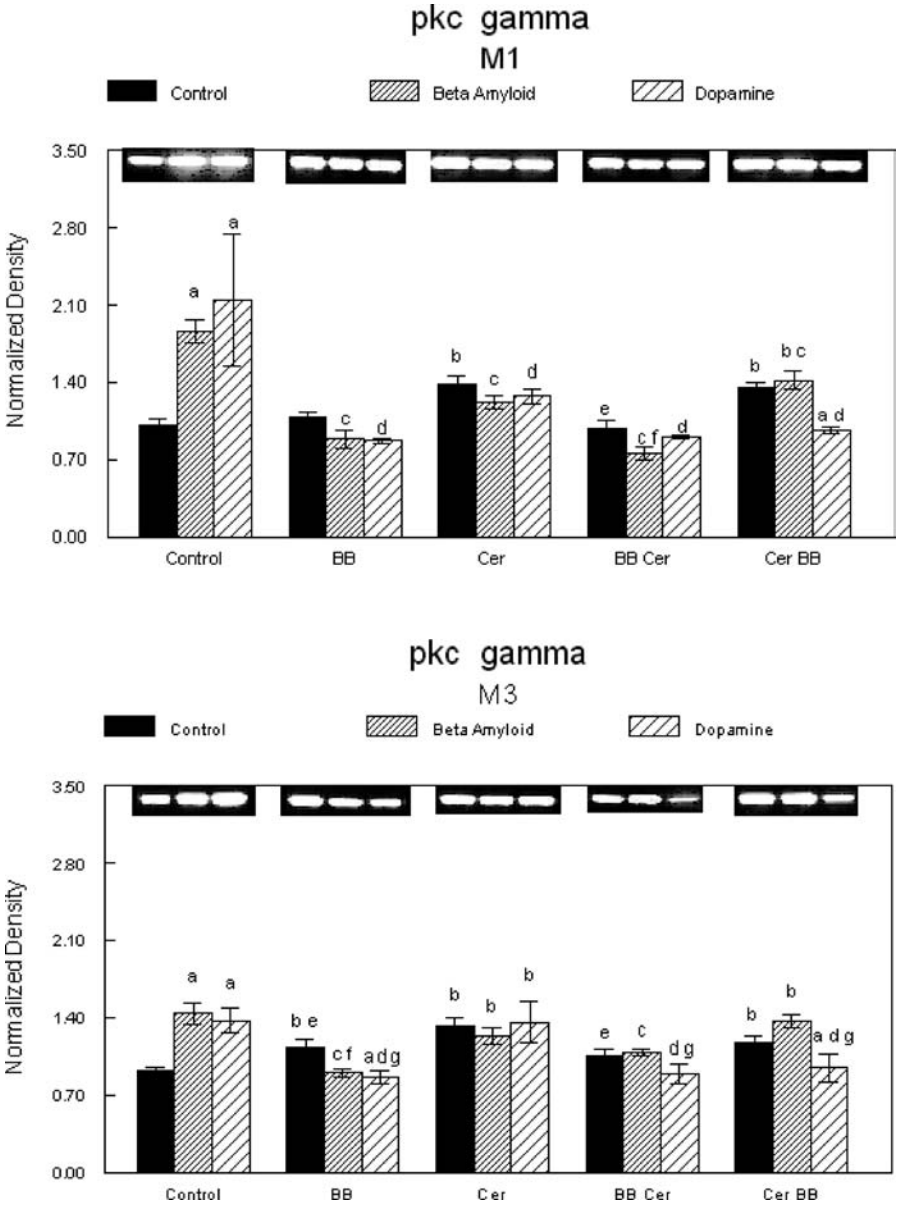

Figure 2. PKC γ immunoreactivity in (**A**) M1- or (**B**) M3-transfected COS-7 cells under the various dopamine (DA), beta amyloid (Aβ_{42}), blueberry (BB), or ceramide (Cer) conditions. a = $p < 0.05$ from own treated control; b = $p < 0.05$ from overall control; c = $p < 0.05$ from overall Aβ_{42} control; d = $p < 0.05$ from overall DA control; e = $p < 0.05$ from Cer control; f = $p < 0.05$ from Cer + Aβ_{42} control; g = $p < 0.05$ from Cer + DA.

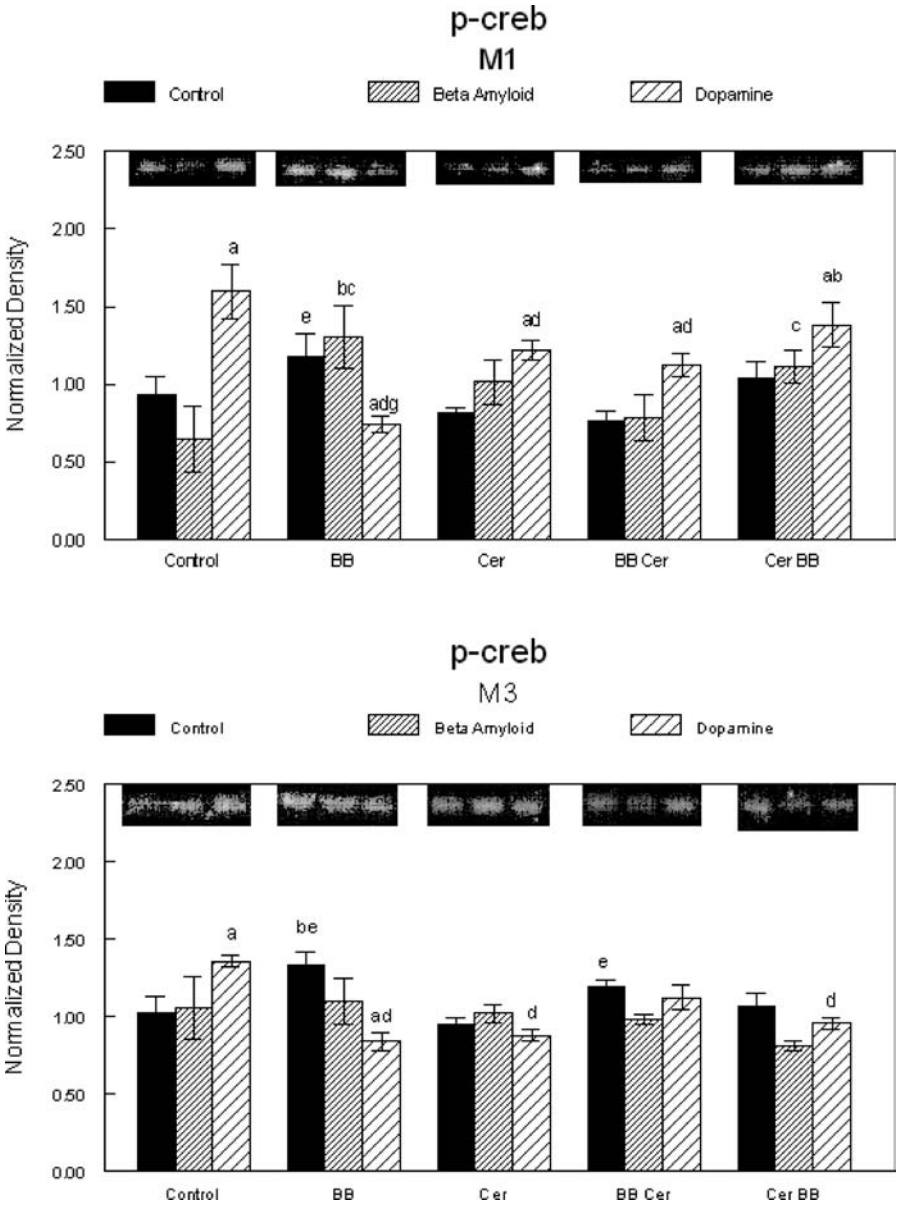

Figure 3. pCREB immunoreactivity in (**A**) M1- or (**B**) M3-transfected COS-7 cells under the various dopamine (DA), beta amyloid ($A\beta_{42}$), blueberry (BB), or ceramide (Cer) conditions. a = $p < 0.05$ from own treated control; b = $p < 0.05$ from overall control; c = $p < 0.05$ from overall $A\beta_{42}$ control; d = $p < 0.05$ from overall DA control; e = $p < 0.05$ from Cer control; g = $p < 0.05$ from Cer + DA.

cognitive relevant muscarinic acetylcholine receptors (MAChR) (*30*, *31*). We have shown that this loss is sensitive both to aging and OS. Additionally, cells transfected with M1-, M2- or M4AChR showed greater disruptions in calcium regulation than those transfected with M3 or M5AChR (*29*, *32*).

In a subsequent study we utilized M1- and M3-transfected COS 7 cells to investigate whether Cer would exhibit differential effects on these cells. The results showed that significant decrements were seen in the ability of M1- and M3-transfected cells to respond initially to a depolarizing agent (oxotremorine) regardless of the particular stressor utilized (DA, $A\beta_{42}$). However blueberry (BB) pretreatment prevented these deficits (*33*), supporting previous studies showing that BB was effective in preventing the deleterious effects of DA or $A\beta_{42}$ in the M1-transfected COS-7 cells (*34*).

Since it is known, as mentioned above, that the mechanism of action of Cer may be to increase stress signals, and since we showed previously that BB protective effects involved antagonizing stress and enhancing protective signals induced by DA and other stressors, it became of interest in the present study to assess whether changes in the alterations in stress signals by Cer could be reduced by BB treatment. Indeed, a study (*35*) in M1-transfected COS-7 cells showed that BB antagonized OS effects induced by DA in M1-transfected cells by lowering activation of two important stress signals [e.g., cyclic AMP response element binding protein (pCREB) and protein kinase C gamma (pPKCγ)], while enhancing protective pMAPK activity. Similar findings were observed in primary hippocampal cells that were pretreated with BB and followed by $A\beta_{42}$ or DA, where stress-induced increases in pCREB and pPKCγ were reduced by the BB pretreatment.

Therefore, in the present study we investigated signals that have been shown to regulate the downstream activation of oxidative and inflammatory stressors (e.g., cytokines). Additionally, we also examined protective signals that show declines in aging including, apoptosis repressor with caspase recruitment (Arc) (*36*), MAPK and protein kinase B (Akt). The latter two are protective against oxidative stress (*37*) and inflammation (*38*).

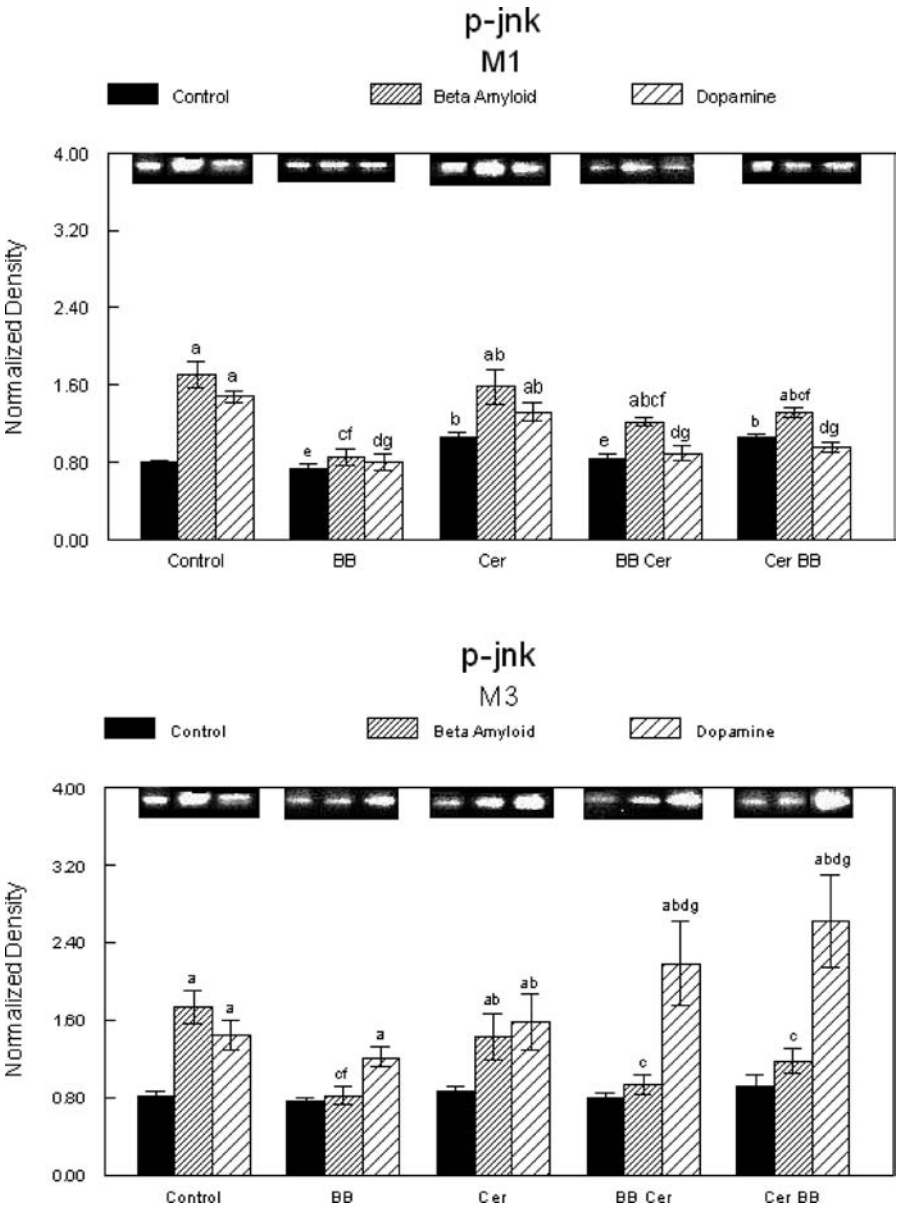

Figure 4. pJNK immunoreactivity in (**A**) M1- or (**B**) M3-transfected COS-7 cells under the various dopamine (DA), beta amyloid ($A\beta_{42}$), blueberry (BB), or ceramide (Cer) conditions. a = $p < 0.05$ from own treated control; b = $p < 0.05$ from overall control; c = $p < 0.05$ from overall $A\beta_{42}$ control; d = $p < 0.05$ from overall DA control; e = $p < 0.05$ from Cer control; f = $p < 0.05$ from Cer + $A\beta_{42}$ control; g = $p < 0.05$ from Cer + DA.

MATERIAL AND METHODS

Treatment and Transfection. COS-7 cells (ATCC) were grown, transfected and maintained as described in Joseph and colleagues (*39*). Cells were pretreated with either blueberry (BB, 2 mg/mL, 45 min), C2 ceramide (75 μM, 30 min), or both in succession then treated with DA (0 or 1 mM, 4 h) or $A\beta_{42}$ (0 or 25 μM, 24 h). The $A\beta_{42}$ was prepared as described in Joseph et al. (*35*). Briefly, the $A\beta_{42}$ was dissolved in distilled deionized water and left at 37 °C overnight. An appropriate concentration of $A\beta_{42}$ was made using the media. No aggregates were formed either overnight or when placed in the media. The concentration of the $A\beta_{42}$ utilized here did not induce cell death in the neurons. The Cer concentration utilized were in the range chosen by previous experiments (e.g., 20 μM (*40*) or 100 μM respectively (*41*)). We chose a shorter time and increased the concentration. The BB concentration reflected the highest amount that did not reduce response or recovery in control transfected COS-7 cells in pilot experiments. The BB extract was prepared as described previously (*23*). Briefly frozen BB homogenized in water (2:1 w/v) for 3 min, centrifuged (13000*g* for 15 min at 4 °C), and then the supernatant was poured into ice trays and frozen at 0 °C. The cubes were then crushed in an ice crusher and then placed on trays and freeze-dried in a lyophilizer for ten days. The resulting powder was reconstituted in growth media as needed for the experiment. The Cer concentration utilized reflected a physiological level that has been reported in membranes of cells from aged animals. The cells were washed and treatments removed before the addition of the next treatment. For example, BB was removed before Cer treatment and vice versa and then the subsequent treatments were removed after their incubation times. The purpose of the treatments was to alter the vulnerability of the cells, not to physically interfere with subsequent applications.

Western Immunoblot Analyses. These assessments were made in the transfected COS-7 cells using Western immunoblotting procedures. The following markers were assessed. Stress signals: phospho JNK, phospho p38MAPK, phospho NFκB, phospho CREB, PKC γ, and p53. Protective signals: Arc, phospho Akt, phospho MAPK. These assessments were made in the transfected COS-7 cells using immunoblotting procedures (*42*, *43*). Briefly, 20 μg of protein samples (2 replicates of each sample were used per run) and pooled protein standard were run on a 12.5% SDS−PAGE gel (*39*). The gel was soaked in protein transfer buffer (TB: 200 mM glycine, 25 mM Tris base, 20% methanol) for 10 min at room temperature, and then electrophoretically transferred to nitrocellulose. Blots were blocked with TBST (140 mM NaCl, 20 mM

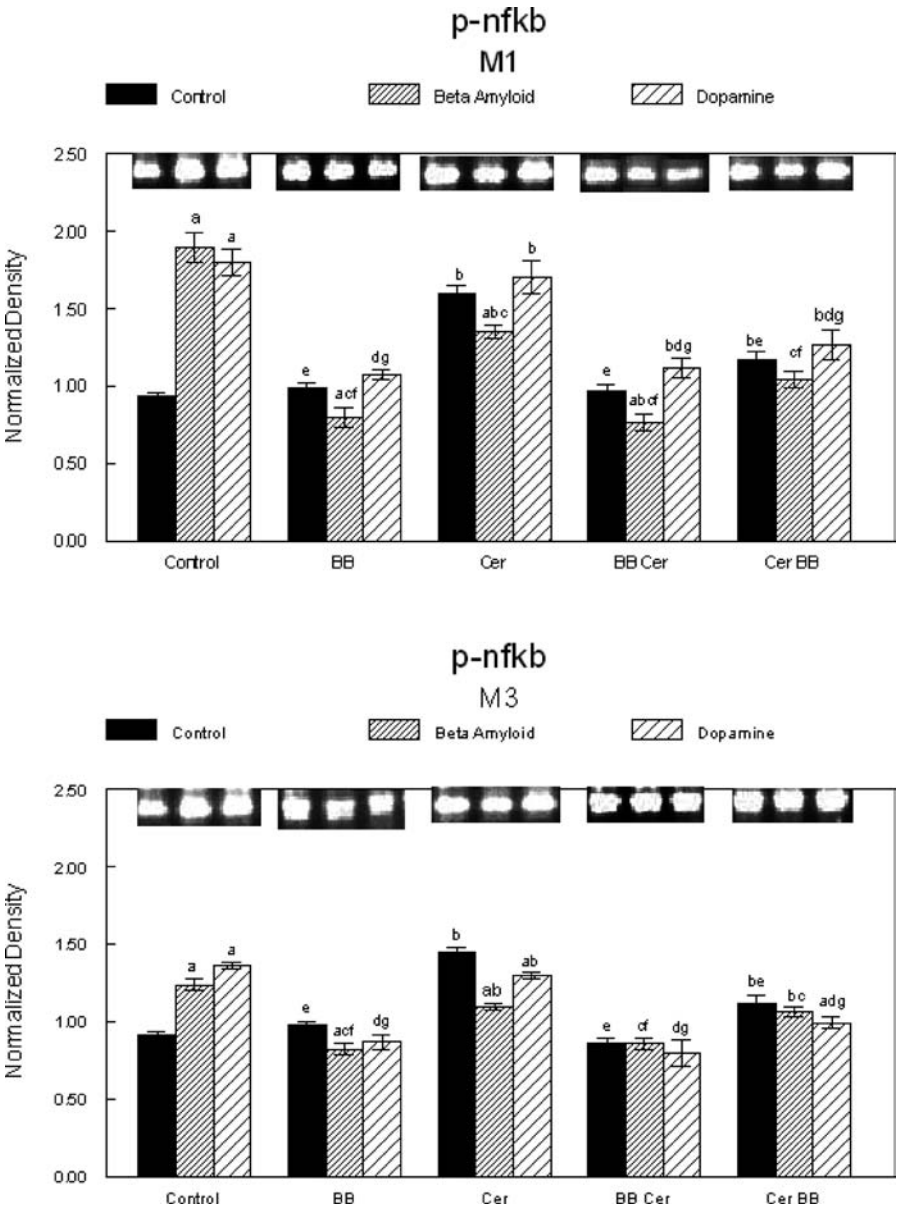

Figure 5. pNFκB immunoreactivity in (**A**) M1- or (**B**) M3-transfected COS-7 cells under the various dopamine (DA), beta amyloid (Aβ_{42}), blueberry (BB), or ceramide (Cer) conditions. a = $p < 0.05$ from own treated control; b = $p < 0.05$ from overall control; c = $p < 0.05$ from overall Aβ_{42} control; d = $p < 0.05$ from overall DA control; e = $p < 0.05$ from Cer control; f = $p < 0.05$ from Cer + Aβ_{42} control; g = $p < 0.05$ from Cer + DA.

Tris, 0.1% Tween-20, pH 7.5) with 5% nonfat dried milk or 3% BSA and incubated in respective primary antibodies in TBST with 5% milk or 3% BSA depending on the antibody, followed by washing with TBST and incubation with the appropriate horseradish peroxidase-conjugated secondary antibody in TBST with 5% milk. After a final washing in TBST, the signal was detected using ECL Plus Reagents (Amersham). The immunoreactive bands, corresponding to the molecular weight of the protein of interest, were visualized with a digital CCD camera (Hamamatsu Photonics) attached to a BioImaging System (EC^3 Darkroom, UVP) and the optical densities were quantified with the LabWorks Imaging Acquisition and Analysis software (version 4.5, UVP). The pooled protein standard was used to normalize the intensities of the antibody-specific bands and the signals were expressed as percent of optical density of the standard for comparison of signal across the blots. Anti-β-actin or anti-GADPH primary antibody was used to check for loading consistency. The following antibodies were used: anti-cPKC γ (Santa Cruz, rabbit polyclonal), anti-phospho-MAPK (Cell Signaling, mouse monoclonal), anti-phospho-CREB (Cell Signaling, rabbit polyclonal), anti-p53 (Abcam, mouse monoclonal), anti-phospho p38 MAPK (Cell Signaling, rabbit polyclonal), anti-phospho NFκB (Abcam, rabbit polyclonal), anti-phospho JNK (Cell Signaling, rabbit polyclonal), anti-Arc (Santa Cruz, rabbit polyclonal), and anti-phospho Akt (Abcam, rabbit monoclonal).

Data Analyses. Differences in immunoblot optical densities were analyzed by ANOVA for each transduction factor under the various conditions. Post hoc comparisons were done by using Fisher's LSD test.

RESULTS

Stress Signals. As can be seen from the Introduction, we assessed a number of stress signals in this study, and in order to try to simplify the presentation of these results, we have organized the signals into stress and protective signals. Generally, the increase of stress signals such as PKCγ may act upstream of CREB and NFκB through p38 MAPK to promote increases in stressors (**Figure 1**).

Analyses of PKCγ showed that in M1-transfected cells both Aβ_{42} and DA significantly increased PKCγ ($p < 0.0001$, for DA and Aβ_{42} compared to control, **Figure 2A**). These effects were antagonized by BB for both treatments ($p < 0.0001$). Cer also lowered the increases that were seen for both treatments under the

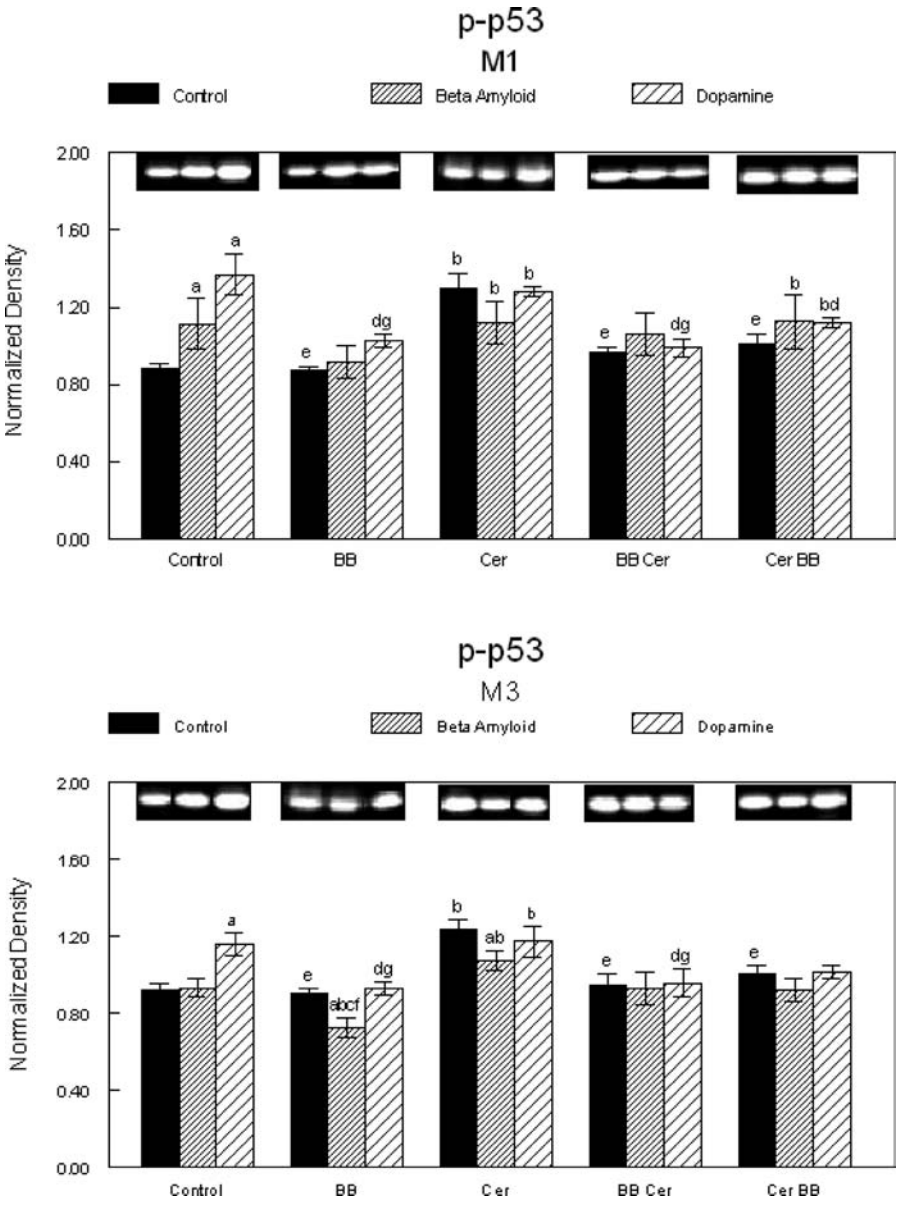

Figure 6. p53 immunoreactivity in (**A**) M1- or (**B**) M3-transfected COS-7 cells under the various dopamine (DA), beta amyloid ($A\beta_{42}$), blueberry (BB), or ceramide (Cer) conditions. a = $p < 0.05$ from own treated control; b = $p < 0.05$ from overall control; c = $p < 0.05$ from overall $A\beta_{42}$ control; d = $p < 0.05$ from overall DA control; e = $p < 0.05$ from Cer control; f = $p < 0.05$ from Cer + $A\beta_{42}$ control; g = $p < 0.05$ from Cer + DA.

control condition, and there were no additional synergistic inhibitor effects under BB/Cer or Cer/BB conditions.

For the M3-transfected cells (**Figure 2B**), there were some increases in PKCγ by both $A\beta_{42}$ and DA ($p < 0.0001$). As was seen with the M1-transfected cells, BB reduced these increases for both DA and $A\beta_{42}$. However, Cer increased PKCγ in the absence of the stressors ($p < 0.003$, Cer vs control). BB was able to reduce these increases induced by Cer, or DA, especially when the cells were treated with BB prior to Cer (e.g., Cer vs BB + Cer, $p < 0.005$; Cer + DA vs BB+ Cer + DA, $p < 0.001$). PKCγ decreases were not seen with Cer vs Cer + $A\beta_{42}$ ($p > 0.05$), but the Cer + $A\beta_{42}$ condition was also not different from the BB + $A\beta_{42}$ + Cer condition ($p > 0.05$).

Similar results were seen with respect to pCREB with DA in both M1- ($p < 0.0001$) (**Figure 3A**) and M3- ($p < 0.0003$) (**Figure 3B**) transfected cells. However, it also appeared that BB alone also enhanced pCREB in both types of transfected cells ($p < 0.002$), but did reduce DA-enhancement of pCREB for both M1- and M3-transfected cells ($p < 0.0001$ for both comparisons). The DA and BB enhancements of pCREB were antagonized in the CER-treated M1-transfected cells (e.g., DA vs Cer + DA, $p < 0.05$), while in the M3-transfected cells, only the DA increases in pCREB were decreased ($p < 0.001$) and BB-induced increases were unaffected (BB vs BB + Cer, $p > 0.05$). $A\beta_{42}$ did not enhance pCREB, but BB did enhance pCREB in $A\beta_{42}$-treated M1- transfected cells ($p < 0.003$). Cer effects were inconsistent on pCREB in the presence of $A\beta_{42}$.

When pJNK was assessed, it appeared that the BB suppression of DA and $A\beta_{42}$ activation of this parameter were greater than that seen with respect to CREB for both M1 and M3 ($p < 0.0001$ for the comparisons in both types of cells) (**Figures 4A** and **4B**) and did not affect baseline pJNK activity ($p > 0.05$). Cer treatment enhanced baseline JNK activity ($p < 0.001$) but did not affect the enhancements in pJNK induced by DA or $A\beta_{42}$ ($p > 0.05$ for both stressors). BB antagonized the Cer/DA and Cer/$A\beta_{42}$ effects on JNK (e.g., Cer + DA vs BB + Cer + DA, $p < 0.0001$) whether the BB was applied before or after Cer in M1 cells (**Figure 4A**).

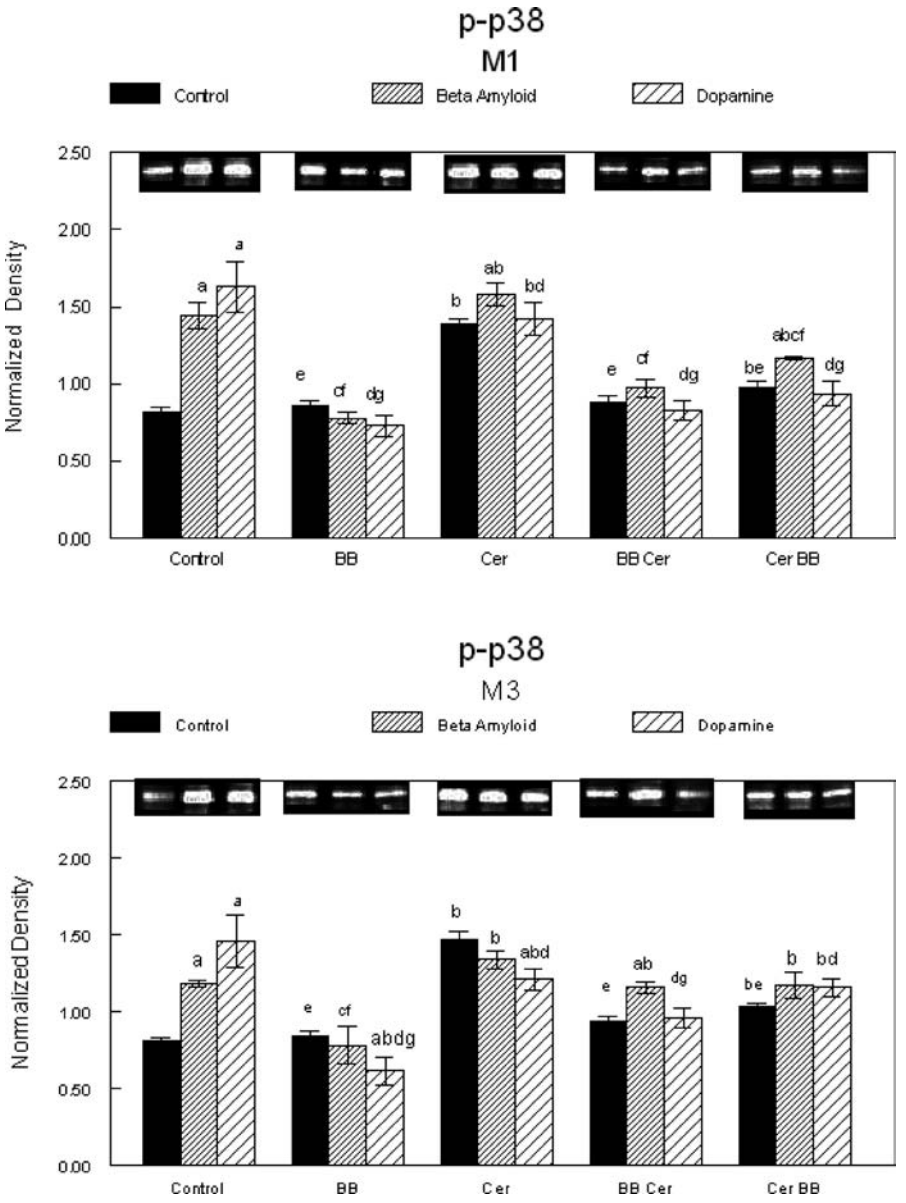

Figure 7. pp38MAPK immunoreactivity in (**A**) M1- or (**B**) M3-transfected COS-7 cells under the various dopamine (DA), beta amyloid ($A\beta_{42}$), blueberry (BB), or ceramide (Cer) conditions. a = $p < 0.05$ from own treated control; b = $p < 0.05$ from overall control; c = $p < 0.05$ from overall $A\beta_{42}$ control; d = $p < 0.05$ from overall DA control; e = $p < 0.05$ from Cer control; f = $p < 0.05$ from Cer + $A\beta_{42}$ control; g = $p < 0.05$ from Cer + DA.

The effects of DA and $A\beta_{42}$ were similar in M3 cells as those seen in M1 with respect to BB or Cer (**Figure 4B**). However, the increases in pJNK were smaller overall, and Cer antagonized the BB-induced decreases in DA by inducing increases in pJNK whether the BB was applied before or after DA (Cer + DA vs BB + Cer + DA, $p < 0.025$; Cer + DA vs Cer + BB +DA, $p < 0.0001$). BB antagonism of $A\beta_{42}$-induced increases in pJNK was unaffected.

In the case of pNFκB it appears that in the M1-transfected cells, both $A\beta_{42}$ and DA increased this parameter ($p < 0.0001$ for both, **Figure 5A**). These increases were significantly reduced by BB treatment in these cells ($p < 0.0001$ for both DA and $A\beta_{42}$). Cer significantly increased pNFκB relative to control ($p < 0.0001$) but reduced the $A\beta_{42}$-induced increases ($p < 0.0001$). Cer did not alter the pNFκB increases in DA ($p > 0.05$). BB reduced the Cer effects whether applied before or after Cer.

As shown in **Figure 5B**, in the M3-transfected cells similar effects were seen with respect to pNFκB, which was increased by both DA and $A\beta_{42}$ ($p < 0.0001$), but the increases were less than those seen in M1-transfected cells. As in the M1-tranfected cells, BB reduced these effects ($p < 0.0001$ for both DA and $A\beta_{42}$), while Cer increased control levels of pNfκB ($p < 0.001$). BB antagonized these effects, primarily when added prior to Cer (e.g., Cer + DA vs BB + Cer +DA, $p < 0.006$).

The effects of the various treatments on p53 are shown in **Figures 6A** and **6B** for M1- and M3-transfected cells, respectively. They were similar for both M1- and M3-transfected cells, except that p53 was increased by both $A\beta_{42}$ and DA ($p < 0.002$ and $p < 0.003$, respectively) for M1 but not M3, where p53 was only increased by DA ($p < 0.002$) and not by $A\beta_{42}$. There was a trend toward reduction of the $A\beta_{42}$ by BB ($p < 0.073$), and BB treatment reduced the DA-induced increases in p53 ($p < 0.003$), while in M3-transfected cells BB reduced p53 activation by either stressors (e.g., $A\beta_{42}$ vs BB + $A\beta_{42}$, $p < 0.017$). Cer treatment increased baseline p53 (control vs Cer + control, $p < 0.0001$) but did not further alter the DA- or $A\beta_{42}$-induced increases in p53 (e.g., $A\beta_{42}$ vs $A\beta_{42}$ + Cer, $p > 0.05$). For M1-transfected cells the BB effects on p53 were blunted somewhat for $A\beta_{42}$ when applied

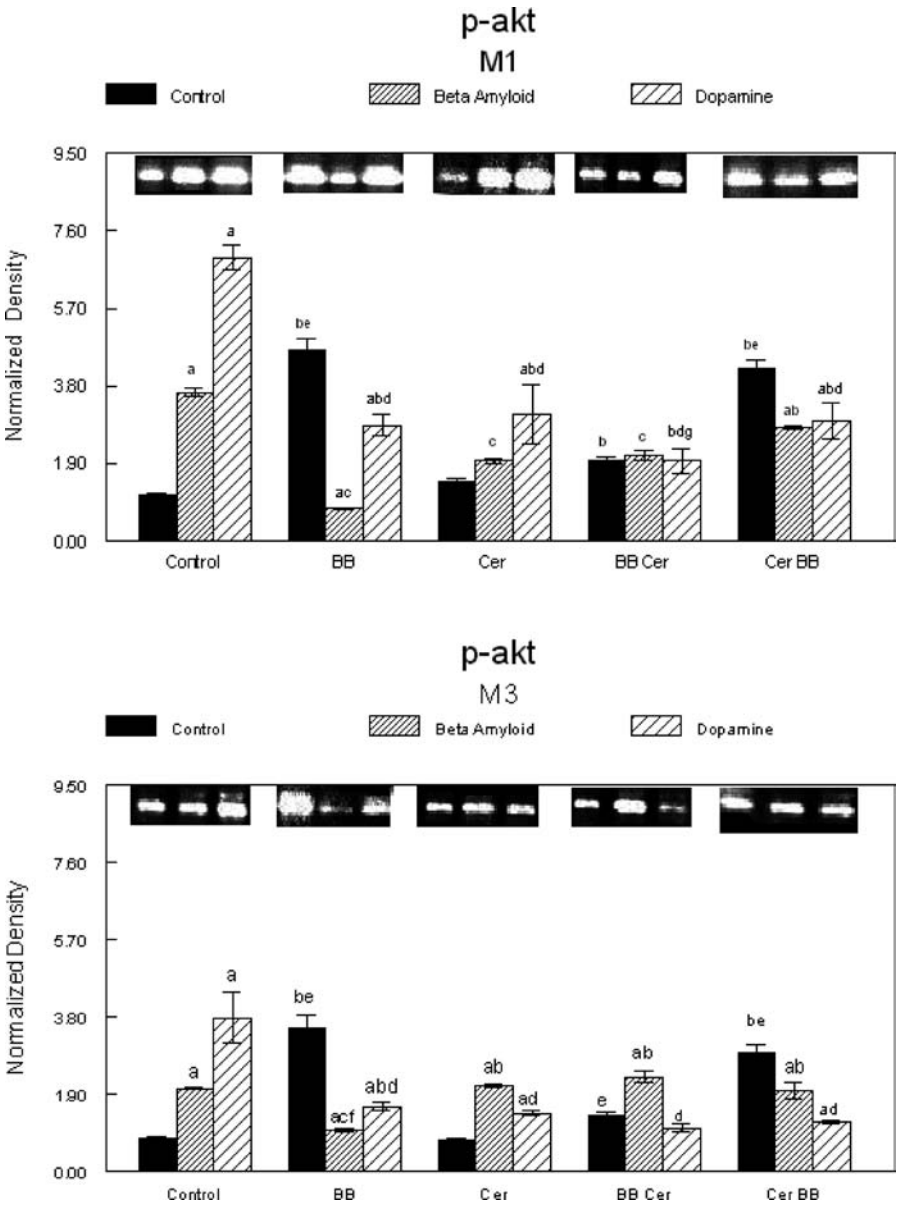

Figure 8. pAkt immunoreactivity in (**A**) M1- or (**B**) M3-transfected COS-7 cells under the various dopamine (DA), beta amyloid ($A\beta_{42}$), blueberry (BB), or ceramide (Cer) conditions. a = $p < 0.05$ from own treated control; b = $p < 0.05$ from overall control; c = $p < 0.05$ from overall $A\beta_{42}$ control; d = $p < 0.05$ from overall DA control; e = $p < 0.05$ from Cer control; f = $p < 0.05$ from Cer + $A\beta_{42}$ control; g = $p < 0.05$ from Cer + DA.

before or after Cer, while it appeared that in the M3-transfected cells p53 was reduced by BB under Cer when either DA or $A\beta_{42}$ were used as stressors.

Further analyses of the stress signals, as indicated in **Figures 7A** and **7B**, respectively, indicated that p38MAPK was significantly increased by both DA and $A\beta_{42}$ in both M1- and M3-transfected cells ($p < 0.0001$ for comparisons with both stressors in both types of transfected cells). BB reduced these increases for both stressors in both transfected cells ($p < 0.0001$). Conversely, Cer significantly increased basal p38MAPK in both types of transfected cells ($p < 0.0001$ for both M1- and M3-transfected cells) but did not further enhance the DA- or $A\beta_{42}$-induced increases. BB reduced the Cer enhancement of p38 MAPK ($p < 0.0001$) and also the increases induced by the stressors in M1-transfected cells, whether applied before or after the stressors ($p < 0.0001$). However, in the case of the M3 receptors, BB suppressed the Cer effects primarily when applied prior to Cer.

Protective Signals. As mentioned in the Introduction it has been shown that pAKT, pMAPK, and pArc are protective against inflammatory and oxidative stressors (*26*, *27*, *29*, *30*). Akt may accomplish this effect through the inactivation of BAD, while MAPK acts through a variety of pathways, one of which is to increase protective bcl-2, which ultimately antagonizes p38 MAPK. Arc acts to decrease caspases as well as p53, to reduce oxidative stress. Again, the major dependent measure that is important here is our functional finding from the previous experiment concerned with calcium dysregulation.

As shown in **Figures 8A** and **8B**, BB reduced the increases in pAkt induced by either DA or $A\beta_{42}$ for both M1- and M3-transfected cells (e.g., M1, DA vs BB + DA, $p < 0.0001$). However, BB alone also increased pAkt in both types of transfected cells as compared to control ($p < 0.0001$ for both M1- and M3-transfected cells). This increase was not seen with Cer, and Cer also reduced both DA- or $A\beta_{42}$-induced increases in pAkt (M1, $p < 0.0001$; M3, $p < 0.03$ for both treatments). Cer reduced the BB-induced increases in pAkt (BB vs Cer + BB, $p < 0.05$, M3, $p < 0.03$, M1) primarily when applied after BB treatment.

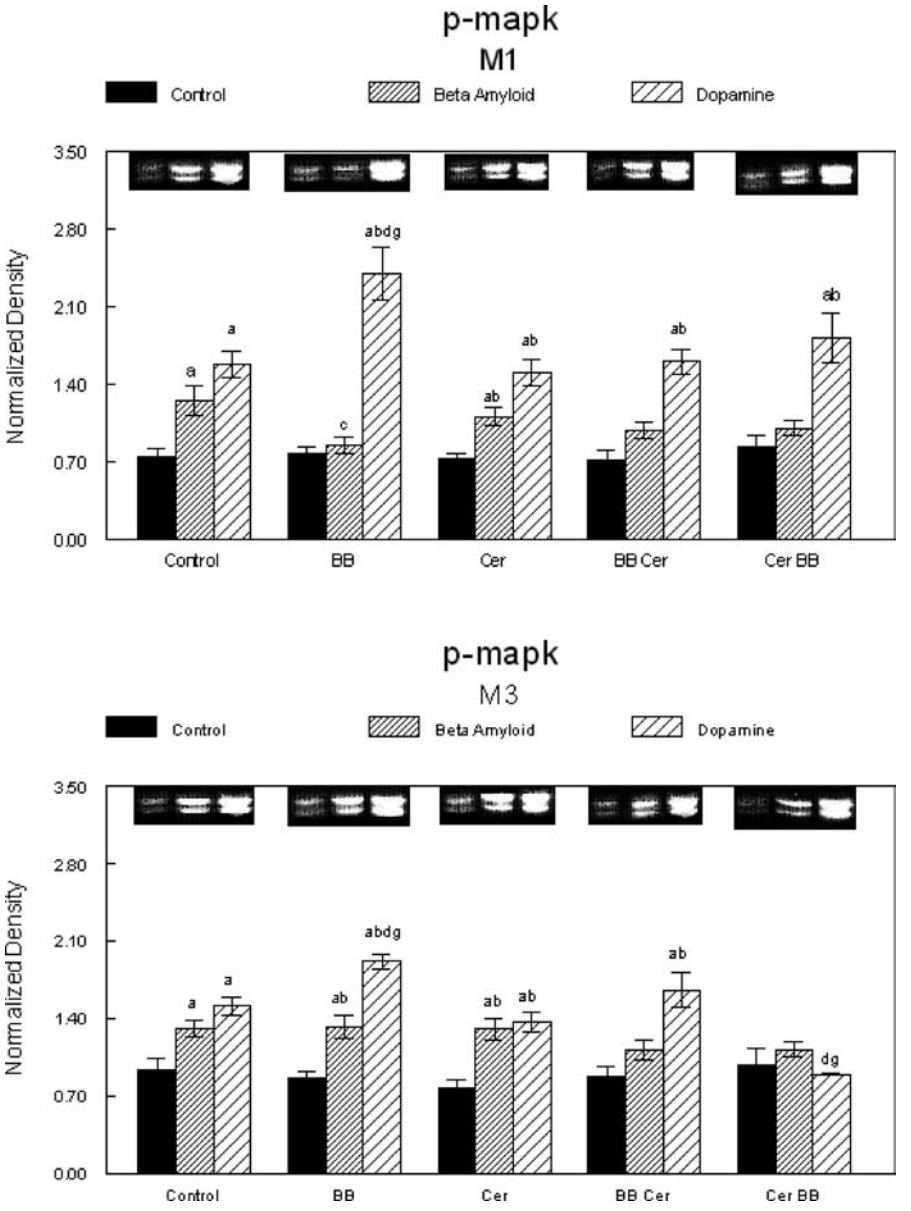

Figure 9. pMAPK immunoreactivity in (**A**) M1- or (**B**) M3-transfected COS-7 cells under the various dopamine (DA), beta amyloid ($A\beta_{42}$), blueberry (BB), or ceramide (Cer) conditions. a = $p < 0.05$ from own treated control; b = $p < 0.05$ from overall control; c = $p < 0.05$ from overall $A\beta_{42}$ control; d = $p < 0.05$ from overall DA control; g = $p < 0.05$ from Cer + DA.

The differences in the values of pMAPK immunofluorescence in the M1 or M3 cells exposed to DA, $A\beta_{42}$, and/or Cer in the presence or absence of BB are shown in **Figures 9A** and **9B** respectively. As was seen above with Akt, both DA and $A\beta_{42}$ raised pMAPK when applied to control M1- or M3-transfected cells (e.g., M1, $p < 0.001$, for both treatments). However, it appeared that the $A\beta_{42}$-induced increases in pMAPK were suppressed by BB in the M1- ($A\beta_{42}$ vs BB + $A\beta_{42}$, $p < 0.001$) but not M3-transfected cells treated with BB where no differences were seen ($p > 0.05$). BB further enhanced DA-induced increases in pMAPK in both M1- and M3-transfected cells (BB + DA vs DA, $p < 0.0001$). Cer did not significantly affect the increases in pMAPK induced by DA or $A\beta_{42}$ in either M1- or M3-transfected cells ($p > 0.05$). It did, however, blunt the effect of BB in enhancing pMAPK with respect to DA (e.g., M1, DA + BB vs BB + Cer + DA, $p < 0.001$), but not with respect to $A\beta_{42}$.

As can be seen from **Figure 10A**, $A\beta_{42}$ induced increases in pArc ($p < 0.0001$) in M1- but not M3-transfected cells, while DA induced decreases in this parameter in M1 cells ($p < 0.0001$). BB reduced the effects of both $A\beta_{42}$ ($p < 0.0001$) but increased DA ($p < 0.002$) in both types of transfected cells. Cer increased pArc alone ($p < 0.0001$) as compared to the non-Cer condition, but did not further increase $A\beta_{42}$-induced increases in pArc. BB was able to antagonize the effects of Cer (Cer vs BB + Cer, $p < 0.0001$) and $A\beta_{42}$ (Cer + $A\beta_{42}$ vs BB + Cer + $A\beta_{42}$, $p < 0.0001$). These effects were diminished when Cer was applied prior to BB.

In the M3-transfected cells (**Figure 10B**), $A\beta_{42}$ did not affect pArc, but as in the M1-transfected cells, DA reduced pArc ($p < 0.002$). BB did not alter $A\beta_{42}$ with respect to pArc ($p > 0.05$), but DA increased pArc in the BB condition ($p < 0.0001$ as in the M1-transfected cells). Cer increased pArc relative to control ($p < 0.0001$) and both stressors (DA and $A\beta_{42}$, both $p < 0.0001$, relative to control). BB reduced these effects but primarily when applied prior to Cer.

DISCUSSION

In this study we attempted to assess the putative signaling mechanisms involved in BB protection against $A\beta_{42}$-, DA- or

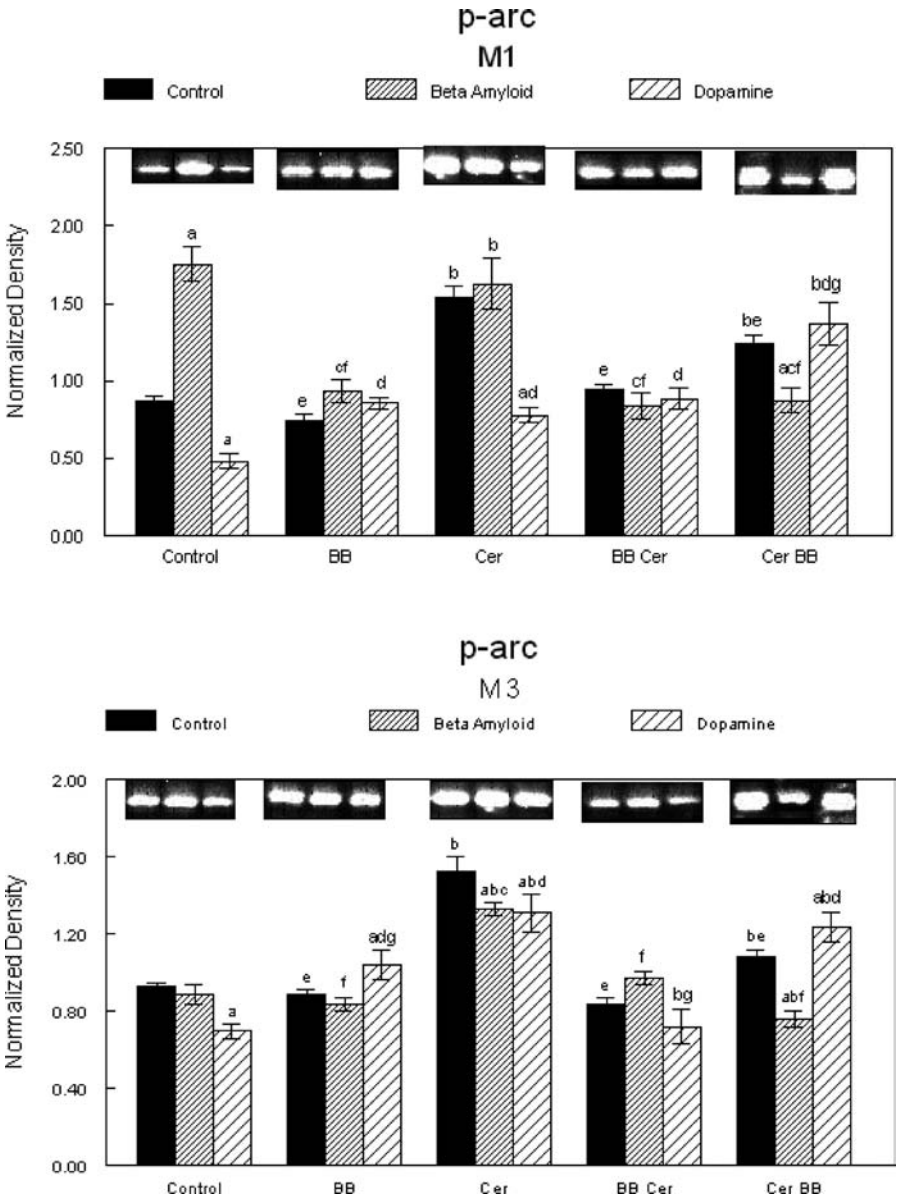

Figure 10. pArc immunoreactivity in (**A**) M1- or (**B**) M3-transfected COS-7 cells under the various dopamine (DA), beta amyloid ($A\beta_{42}$), blueberry (BB), or ceramide (Cer) conditions. a = $p < 0.05$ from own treated control; b = $p < 0.05$ from overall control; c = $p < 0.05$ from overall $A\beta_{42}$ control; d = $p < 0.05$ from overall DA control; e = $p < 0.05$ from Cer control; f = $p < 0.05$ from Cer + $A\beta_{42}$ control; g = $p < 0.05$ from Cer + DA.

Cer-induced dysregulation in calcium function in muscarinic receptor-transfected COS-7 cells that we had observed in a previous study (*39*).

The results showed that, DA or $A\beta_{42}$ increased many of the stress (e.g., pPKCγ, p38 MAPK) and protective (e.g., pMAPK) signals (**Figure 1**). Similarly, Cer also appeared to raise several of the stress and protective signals in the absence of the other stressors. These included pPKCγ, pJNK, pNFκB, p53, and p38 MAPK (**Figure 1**). Importantly, except for pJNK, where enhancement was only seen in the M1-transfected cells, the increases in the stress signals were seen in both M1- and M3-transfected cells. These findings were similar to those showing increases in NFκB (*5*, *16*, *25*−*27*, *44*), as well as additional findings (*44*−*46*) showing increases in p38 MAPK and pJNK with Cer (see also ref *47*). Increases in these stress signals have been associated with increases in cyclo-oxygenase-2 (COX-2) which is important in stress induction and cellular inflammatory processes (*45*). Thus, we would postulate that these processes may be the ones that ultimately lead to increased ROS and calcium dysregulation.

In attempting to discern the possible synergistic effects of Cer in the presence of DA or $A\beta_{42}$, the results indicated that Cer effect might be dependent upon the particular signal assessed and on the subtype of transfected cell. It appeared Cer reduced the DA activation of PKCγ and reduced $A\beta_{42}$-induced PKCγ activation as well as NFκB, in the M1-transfected cells. Conversely, PKCγ activation induced by DA and $A\beta_{42}$ was not altered by Cer in M3-transfected cells. Differences between the transfected cells were also seen primarily with pCREB, which was reduced somewhat in $A\beta_{42}$-treated M1 cells and unaltered in M3 cells, as well as p53 where its activation was unaltered in M1 cells and increased in the M3 cells with Cer and $A\beta_{42}$. Similar effects to that seen with respect to p53 were also seen with Cer /$A\beta_{42}$-treated M1 and M3 cells. In brief, it appears that Cer can enhance stress signals (e.g., pPKCγ, p53, pNFκB) in the absence of exogenous stressors but has rather mixed effects in the presence of stressors such as $A\beta_{42}$ or DA.

Overall, the protective effects of BB when combined with Cer generally reflected those seen previously (*33*) with calcium

dysregulation, with greater protection being seen when BB extract was applied prior to Cer. In the absence of the Aβ_{42} or DA, BB, for several of the stress signals such as pPKCγ, pNFκB, p53, and p38 MAPK, lowered their Cer-induced increases in M1- and M3-transfected cells. Moreover, in the presence of the Aβ_{42} or DA, an absence of Cer as can be seen from the figures, BB lowered virtually all of the stress signals activated by these stressors, except for CREB.

Therefore, we propose that BBs appear to be able to act at multiple points in the pathway to prevent stress signal activation and subsequent calcium disruption (*40*), even in the presence of Cer. In addition, we would also propose that Cer could have multiple effects that could lead to increased OS vulnerability in aging. These could involve age-related changes in the neuronal plasma membrane molecular structure and physical properties that might be important determinants of age-related increases in OS and inflammation vulnerability (*48*, *49*). In fact, previous research suggests that modifications of membrane lipid composition with cholesterol and sphingomyelin, to levels similar to those seen in neuronal membranes in aging, dramatically increased the vulnerability of the cells to OS, as assessed by alterations in viability and impairments in calcium buffering (*14*, *50*), as well as stress and protective signals. This suggests that the previously observed finding that calcium buffering has been shown to be significantly reduced in senescence (*23*, *51*) and AD (*52*) may involve alterations in membrane composition. There is an abundance of data (e.g., ref *53*) showing that polyphenols, such as those from BBs (e.g., anthocyanins, chlorogenic acid, proanthocyanidins, hydroxycinamates, flavonols, catechins), can affect membranes by increasing antioxidant protection against Cer. In addition, they can also lead to the upregulation of receptors and membrane-localized enzymes (*54*−*57*). They can also alter membrane transport of molecules across the membrane (*58*) to possibly enhance calcium regulation. Thus, in addition to decreasing the activation of the stress signals, the polyphenols contained in the BB could also reduce the membrane effects of Cer, as well as its oxidant potential. In this regard, in addition to the effects on stress signaling, the deleterious effects of Cer also may involve alterations in the biophysical properties of the membranes, such as increasing membrane bilayer motion or altering the lipid monolayer curviliniarity (*59*) or lipid rafts (e.g., ref *60*).

Finally, it should also be noted that given the mixed effects of the various stressors and the BBs on the protective signals, the primary benefit of the BBs in the calcium model may be concerned with reduction of stress signals and enhancing membrane function, as well as reducing oxidation. Therefore, it might be suggested that the intake of BBs and possibly other berries may decrease OS vulnerability, reduce stress signaling and increase neuronal calcium regulation, leading to improved neuronal communication in the aged organism. This would have important implications for enhancing cognitive function in aging and possibly in AD. Indeed, recent evidence from a study in elderly people exhibiting symptoms of mild cognitive impairment (MCI) indicated improvements in verbal memory after they were given dietary supplementations of BB juice (up to 12 oz/day, depending upon weight, for 12 weeks (*61*)).

ABBREVIATIONS USED

MAChRs, muscarinic acetylcholine receptors; OS, oxidative stress; oxo, oxotremorine; DA, dopamine; Aβ, amyloid beta; BB, blueberry; Cer, C2 ceramide; NFκB, nuclear factor kappa B; JNK, jun kinase; MAPK, p38 mitogen activated protein kinase; ERK, extracellular signal regulated kinase; pCREB, cyclic AMP response element binding protein; pPKCγ, protein kinase C gamma; TNFα, tumor necrosis factor alpha.

LITERATURE CITED

(1) Hannun, Y. A. Functions of ceramide in coordinating cellular responses to stress. *Science* **1996**, *274* (5294), 1855–9.

(2) Kolesnick, R.; Golde, D. W. The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. *Cell* **1994**, *77* (3), 325–8.

(3) Mathias, S.; Pena, L. A.; Kolesnick, R. N. Signal transduction of stress via ceramide. *Biochem. J.* **1998**, *335* (Pt 3), 465–80.

(4) Spence, M. W.; Burgess, J. K. Acid and neutral sphingomyelinases of rat brain. Activity in developing brain and regional distribution in adult brain. *J Neurochem.* **1978**, *30* (4), 917–9.

(5) Lambeng, N.; Michel, P. P.; Brugg, B.; Agid, Y.; Ruberg, M. Mechanisms of apoptosis in PC12 cells irreversibly differentiated with nerve growth factor and cyclic AMP. *Brain Res.* **1999**, *821* (1), 60–8.

(6) Yang, J.; Liu, X.; Bhalla, K.; Kim, C. N.; Ibrado, A. M.; Cai, J.; Peng, T. I.; Jones, D. P.; Wang, X. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* **1997**, *275* (5303), 1129–32.

(7) Nakane, M.; Kubota, M.; Nakagomi, T.; Tamura, A.; Hisaki, H.; Shimasaki, H.; Ueta, N. Lethal forebrain ischemia stimulates sphingomyelin hydrolysis and ceramide generation in the gerbil hippocampus. *Neurosci. Lett.* **2000**, *296* (2−3), 89–92.

(8) Mouton, R. E.; Venable, M. E. Ceramide induces expression of the senescence histochemical marker, beta-galactosidase, in human fibroblasts. *Mech. Ageing Dev.* **2000**, *113* (3), 169–81.

(9) Venable, M. E.; Blobe, G. C.; Obeid, L. M. Identification of a defect in the phospholipase D/diacylglycerol pathway in cellular senescence. *J. Biol. Chem.* **1994**, *269* (42), 26040–4.

(10) Venable, M. E.; Lee, J. Y.; Smyth, M. J.; Bielawska, A.; Obeid, L. M. Role of ceramide in cellular senescence. *J. Biol. Chem.* **1995**, *270* (51), 30701–8.

(11) Obeid, L. M.; Hannun, Y. A. Ceramide, stress, and a "LAG" in aging. *Sci. Aging Knowl. Environ.* **2003**, *2003* (39), PE27.

(12) Hannun, Y. A.; Obeid, L. M. The Ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind. *J. Biol. Chem.* **2002**, *277* (29), 25847–50.

(13) Denisova, N. A.; Strain, J. G.; Joseph, J. A. Oxidant injury in PC12 cells--a possible model of calcium "dysregulation" in aging: II. Interactions with membrane lipids. *J. Neurochem.* **1997**, *69* (3), 1259–66.

(14) Denisova, N. A.; Fisher, D.; Provost, M.; Joseph, J. A. The role of glutathione, membrane sphingomyelin, and its metabolites in oxidative stress-induced calcium "dysregulation" in PC12 cells. *Free Radical Biol. Med.* **1999**, *27* (11−12), 1292–301.

(15) Cutler, R. G.; Kelly, J.; Storie, K.; Pedersen, W. A.; Tammara, A.; Hatanpaa, K.; Troncoso, J. C.; Mattson, M. P. Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's disease. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101* (7), 2070–5.

(16) Ayasolla, K.; Khan, M.; Singh, A. K.; Singh, I. Inflammatory mediator and beta-amyloid (25−35)-induced ceramide generation and iNOS expression are inhibited by vitamin E. *Free Radical Biol. Med.* **2004**, *37* (3), 325–38.

(17) Cotrina, M. L.; Nedergaard, M. Astrocytes in the aging brain. *J. Neurosci. Res.* **2002**, *67* (1), 1–10.

(18) Puglielli, L.; Ellis, B. C.; Saunders, A. J.; Kovacs, D. M. Ceramide stabilizes beta-site amyloid precursor protein-cleaving enzyme 1 and promotes amyloid beta-peptide biogenesis. *J. Biol. Chem.* **2003**, *278* (22), 19777–83.

(19) Colombaioni, L.; Garcia-Gil, M. Sphingolipid metabolites in neural signalling and function. *Brain Res. Brain Res. Rev.* **2004**, *46* (3), 328–55.

(20) Patil, S.; Melrose, J.; Chan, C. Involvement of astroglial ceramide in palmitic acid-induced Alzheimer-like changes in primary neurons. *Eur. J. Neurosci.* **2007**, *26* (8), 2131–41.

(21) Tamboli, I. Y.; Prager, K.; Barth, E.; Heneka, M.; Sandhoff, K.; Walter, J. Inhibition of glycosphingolipid biosynthesis reduces

secretion of the beta-amyloid precursor protein and amyloid beta-peptide. *J. Biol. Chem.* **2005**, *280* (30), 28110–7.
(22) Xie, C. W. Calcium-regulated signaling pathways: role in amyloid beta-induced synaptic dysfunction. *Neuromol. Med.* **2004**, *6* (1), 53–64.
(23) 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), 8114–21.
(24) Toescu, E. C.; Verkhratsky, A.; Landfield, P. W. Ca^{2+} regulation and gene expression in normal brain aging. *Trends Neurosci.* **2004**, *27* (10), 614–20.
(25) France-Lanord, V.; Brugg, B.; Michel, P. P.; Agid, Y.; Ruberg, M. Mitochondrial free radical signal in ceramide-dependent apoptosis: a putative mechanism for neuronal death in Parkinson's disease. *J. Neurochem.* **1997**, *69* (4), 1612–21.
(26) Hunot, S.; Brugg, B.; Ricard, D.; Michel, P. P.; Muriel, M. P.; Ruberg, M.; Faucheux, B. A.; Agid, Y.; Hirsch, E. C. Nuclear translocation of NF-kappaB is increased in dopaminergic neurons of patients with parkinson disease. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94* (14), 7531–6.
(27) Meydani, S. N.; Wu, D. Age-associated inflammatory changes: role of nutritional intervention. *Nutr. Rev.* **2007**, *65* (12 Pt 2), S213–6.
(28) Jana, A.; Hogan, E. L.; Pahan, K. Ceramide and neurodegeneration: susceptibility of neurons and oligodendrocytes to cell damage and death. *J. Neurol. Sci.* **2009**, *278* (1−2), 5–15.
(29) 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), 153–61.
(30) Roth, G. S.; Joseph, J. A.; Mason, R. P. Membrane alterations as causes of impaired signal transduction in Alzheimer's disease and aging. *Trends Neurosci.* **1995**, *18* (5), 203–6.
(31) Fowler, C. J.; Cowburn, R. F.; Joseph, J. A. Alzheimer's, ageing and amyloid: an absurd allegory? *Gerontology* **1997**, *43* (1−2), 132–42.
(32) Joseph, J. A.; Fisher, D. R. Muscarinic receptor subtype determines vulnerability to amyloid beta toxicity in transfected cos-7 cells. *J. Alzheimer's Dis.* **2003**, *5* (3), 197–208.
(33) Joseph, J. A.; Neuman, A.; Bielinski, D. F.; Fisher, D. R. Blueberry antagonism of C-2 ceramide disruption of Ca2+ responses and recovery in MAChR-transfected COS-7 cell. *J. Alzheimer's Dis.* **2008**, *15* (3), 429–41.
(34) Joseph, J. A.; Fisher, D. R.; Carey, A. N.; Neuman, A.; Bielinski, D. F. Dopamine-induced stress signaling in COS-7 cells transfected with selectively vulnerable muscarinic receptor subtypes is partially mediated via the i3 loop and antagonized by blueberry extract. *J Alzheimer's Dis* **2006**, *10* (4), 423–37.
(35) Joseph, J. A.; Carey, A.; Brewer, G. J.; Lau, F. C.; Fisher, D. R. Dopamine and abeta-induced stress signaling and decrements in Ca2+ buffering in primary neonatal hippocampal cells are antagonized by blueberry extract. *J. Alzheimer's Dis.* **2007**, *11* (4), 433–46.
(36) Shelke, R. R.; Leeuwenburgh, C. Lifelong caloric restriction increases expression of apoptosis repressor with a caspase recruitment domain (ARC) in the brain. *FASEB J.* **2003**, *17* (3), 494–6.
(37) Weinreb, O.; Amit, T.; Bar-Am, O.; Sagi, Y.; Mandel, S.; Youdim, M. B. Involvement of multiple survival signal transduction pathways in the neuroprotective, neurorescue and APP processing activity of rasagiline and its propargyl moiety. *J. Neural Transm. Suppl.* **2006**, *70*, 457–65.
(38) Zhang, W. J.; Wei, H.; Hagen, T.; Frei, B. Alpha-lipoic acid attenuates LPS-induced inflammatory responses by activating the phosphoinositide 3-kinase/Akt signaling pathway. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104* (10), 4077–82.
(39) Joseph, J. A.; Fisher, D. R.; Carey, A.; Szprengiel, A. The M3 muscarinic receptor i3 domain confers oxidative stress protection on calcium regulation in transfected COS-7 cells. *Aging Cell* **2004**, *3* (5), 263–71.
(40) Gubern, A.; Barcelo-Torns, M.; Barneda, D.; Lopez, J. M.; Masgrau, R.; Picatoste, F.; Chalfant, C. E.; Balsinde, J.; Balboa, M. A.; Claro, E., JNK and ceramide kinase govern the biogenesis of lipid droplets through activation of Group IVA phospholipase A2. *J. Biol. Chem.* **2009**.
(41) Furuchi, T.; Suzuki, T.; Sekine, M.; Katane, M.; Homma, H. Apoptotic inducers activate the release of D-aspartate through a hypotonic stimulus-triggered mechanism in PC12 cells. *Arch. Biochem. Biophys.* **2009**, *490* (2), 118–28.
(42) Ferrer, I.; Blanco, R.; Carmona, M.; Puig, B.; Dominguez, I.; Vinals, F. Active, phosphorylation-dependent MAP kinases, MAPK/ERK, SAPK/JNK and p38, and specific transcription factor substrates are differentially expressed following systemic administration of kainic acid to the adult rat. *Acta Neuropathol.* (*Berlin*) **2002**, *103* (4), 391–407.
(43) Mendelson, K. G.; Contois, L. R.; Tevosian, S. G.; Davis, R. J.; Paulson, K. E. Independent regulation of JNK/p38 mitogen-activated protein kinases by metabolic oxidative stress in the liver. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93* (23), 12908–13.
(44) Chen, Y.; Hatalski, C. G.; Brunson, K. L.; Baram, T. Z. Rapid phosphorylation of the CRE binding protein precedes stress-induced activation of the corticotropin releasing hormone gene in medial parvocellular hypothalamic neurons of the immature rat. *Brain Res. Mol. Brain Res.* **2001**, *96* (1−2), 39–49.
(45) Cho, J.; Cooke, C. E.; Proveaux, W. A retrospective review of the effect of COX-2 inhibitors on blood pressure change. *Am. J. Ther.* **2003**, *10* (5), 311–7.
(46) Reunanen, N.; Westermarck, J.; Hakkinen, L.; Holmstrom, T. H.; Elo, I.; Eriksson, J. E.; Kahari, V. M. Enhancement of fibroblast collagenase (matrix metalloproteinase-1) gene expression by ceramide is mediated by extracellular signal-regulated and stress-activated protein kinase pathways. *J. Biol. Chem.* **1998**, *273* (9), 5137–45.
(47) Willaime-Morawek, S.; Arbez, N.; Mariani, J.; Brugg, B. IGF-I protects cortical neurons against ceramide-induced apoptosis via activation of the PI-3K/Akt and ERK pathways; is this protection independent of CREB and Bcl-2? *Brain Res. Mol. Brain Res.* **2005**, *142* (2), 97–106.
(48) Joseph, J. A.; Denisova, N.; Fisher, D.; Bickford, P.; Prior, R.; Cao, G. Age-related neurodegeneration and oxidative stress: putative nutritional intervention. *Neurol. Clin.* **1998**, *16* (3), 747–55.
(49) Joseph, J.; Shukitt-Hale, B.; Denisova, N. A.; Martin, A.; Perry, G.; Smith, M. A. Copernicus revisited: amyloid beta in Alzheimer's disease. *Neurobiol. Aging* **2001**, *22* (1), 131–46.
(50) Ginzburg, L.; Li, S. C.; Li, Y. T.; Futerman, A. H. An exposed carboxyl group on sialic acid is essential for gangliosides to inhibit calcium uptake via the sarco/endoplasmic reticulum Ca^{2+}-ATPase: relevance to gangliosidoses. *J. Neurochem.* **2008**, *104* (1), 140–6.
(51) Landfield, P. W.; Eldridge, J. C. The glucocorticoid hypothesis of age-related hippocampal neurodegeneration: role of dysregulated intraneuronal Ca^{2+}. *Ann. N.Y. Acad. Sci.* **1994**, *746*, 308–321.
(52) Price, K. A.; Crouch, P. J.; Donnelly, P. S.; Masters, C. L.; White, A. R.; Curtain, C. C. Membrane-targeted strategies for modulating APP and Abeta-mediated toxicity. *J. Cell. Mol. Med.* **2009**, *13* (2), 249–61.
(53) Fraga, C. G. Plant polyphenols: how to translate their in vitro antioxidant actions to in vivo conditions. *IUBMB Life* **2007**, *59* (4−5), 308–15.
(54) Conseil, G.; Baubichon-Cortay, H.; Dayan, G.; Jault, J. M.; Barron, D.; Di Pietro, A. Flavonoids: a class of modulators with bifunctional interactions at vicinal ATP- and steroid-binding sites on mouse P-glycoprotein. *Proc. Natl. Acad. Sci.U.S.A.* **1998**, *95*, 9831–9836.
(55) Lancon, A.; Delmas, D.; Osman, H.; Thenot, J. P.; Jannin, B.; Latruffe, N. Human hepatic cell uptake of resveratrol: involvement of both passive diffusion and carrier-mediated process. *Biochem. Biophys. Res. Commun.* **2004**, *316* (4), 1132–7.
(56) Rosenkranz, S.; Knirel, D.; Dietrich, H.; Flesch, M.; Erdmann, E.; Bohm, M. Inhibition of the PDGF receptor by red wine flavonoids provides a molecular explanation for the "French paradox". *FASEB J.* **2002**, *16* (14), 1958–60.
(57) Tachibana, H.; Koga, K.; Fujimura, Y.; Yamada, K. A receptor for green tea polyphenol EGCG. *Nat. Struct. Mol. Biol.* **2004**, *11* (4), 380–1.

(58) Erlejman, A. G.; Verstraeten, S. V.; Fraga, C. G.; Oteiza, P. I. The interaction of flavonoids with membranes: potential determinant of flavonoid antioxidant effects. *Free Radical Res*. **2004**, *38* (12), 1311–20.

(59) Goni, F. M.; Contreras, F. X.; Montes, L. R.; Sot, J.; Alonso, A. Biophysics (and sociology) of ceramides. *Biochem. Soc. Symp*. **2005**, *72*, 177–88.

(60) Worgall, T. S. Regulation of lipid metabolism by sphingolipids. *Subcell. Biochem*. **2008**, *49*, 371–85.

(61) Krikorian, R.; Nash, T. A.; Shidler, M. D.; Shukitt-Hale, B.; Joseph, J. A. Concord grape juice supplementation improves memory function in older adults. *Br. J. Nutr*. **2009**, *23*, 1–5.

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