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Age-related toxicity of amyloid-beta associated with increased pERK and pCREB in primary hippocampal neurons: reversal by blueberry extract☆

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Abstract

Further clarification is needed to address the paradox that memory formation, aging and neurodegeneration all involve calcium influx, oxyradical production (ROS) and activation of certain signaling pathways. In aged rats and in APP/PS-1 mice, cognitive and hippocampal Ca^{2+} dysregulation was reversed by food supplementation with a high antioxidant blueberry extract. Here, we studied whether neurons were an important target of blueberry extract and whether the mechanism involved altered ROS signaling through MAP kinase and cyclic-AMP response element binding protein (CREB), pathways known to be activated in response to amyloid-beta (Aβ). Primary hippocampal neurons were isolated and cultured from embryonic, middle-age or old-age (24 months) rats. Blueberry extract was found to be equally neuroprotective against Aβ neurotoxicity at all ages. Increases in Aβ toxicity with age were associated with age-related increases in immunoreactivity of neurons to pERK and an age-independent increase in pCREB. Treatment with blueberry extract strongly inhibited these increases in parallel with neuroprotection. Simultaneous labeling for ROS and for glutathione with dichlorofluorescein and monochlorobimane showed a mechanism of action of blueberry extract to involve transient ROS generation with an increase in the redox buffer glutathione. We conclude that the increased age-related susceptibility of old-age neurons to Aβ toxicity may be due to higher levels of activation of pERK and pCREB pathways that can be protected by blueberry extract through inhibition of both these pathways through an ROS stress response. These results suggest that the beneficial effects of blueberry extract may involve transient stress signaling and ROS protection that may translate into improved cognition in aging rats and APP/PS1 mice given blueberry extract.

Keywords: Aging; Neurotoxicity; Amyloid-beta; Stress signaling; Oxyradicals; Glutathione

1. Introduction

MAP kinase (MAPK), extracellular regulated kinase (ERK) signaling and transcriptional activator cyclic-AMP response element binding protein (CREB) are required for memory formation in response to an influx of calcium [1] and are involved in ischemia, oxyradical (ROS) stress, aging and neurodegeneration. For example, in neurodegenerative disease, the Alzheimer's disease-associated peptide amyloid-beta (Aβ) stimulates MAPK ERK2 short term while Aβ with ROS-promoting Fe^{+2} stimulates ERK2 long term [2]. Aβ alone [3] or together with glutamate inhibits PKA and its downstream CREB target in embryonic neurons [4]. In a human cell line, intracellular Aβ causes hyperphosphorylation of CREB to block nuclear translocation [5]. This dichotomy between memory creation and disruption is not well understood. It is further complicated by age-related differences in memory, signal processing, and susceptibility to ROS.

A cost-effective and palatable intervention against aging and neurodegeneration that promotes memory may be dietary blueberries, which are rich in phytochemicals. Under oxidative stress, polyphenols contained in tea, red wine or ginkgo biloba not only affect cell signaling by altering ERK activity [6,7] but also reduce protein kinase C (PKC) activity [8,9] and decrease CREB [10]. Berries and fruit phytochemicals are well known for their antioxidant activities. Previously, we have shown that motor and cognitive deficits in aging could be reduced by feeding aged rats a diet containing 2% blueberries or strawberries [11]. Subsequent research has supported these early findings, including a study showing that APP+PS-1 (amyloid precursor protein/presenilin-1) transgenic mice fed a diet containing 2% blueberry extract from 4 to 12 months of age showed no deficits in Y-maze performance when compared to mice fed an unsupplemented NIH-31 diet [12]. Additionally, embryonic

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hippocampal neurons exposed to Aβ showed disruptions in calcium regulation that were prevented by pretreatment of the cells with various fruit extracts [13,14]. Because the reversals in whole animal studies could involve effects on the aging vasculature, inflammatory response, hormonal system or neurons, whether similar protection is possible for isolated old neurons would further clarify the target.

Previous studies have shown that stressors such as Aβ can increase several additional transcription factors associated with oxyradical stress such as CREB [15]. Moreover, acute hypoxia up-regulates CREB (for reviews, see Refs. [16,17]). It has also been shown that CREB is activated by hydrogen peroxide in Jurkat T lymphocytes [18] and by cadmium in mouse neuronal cells [19] as well as during stroke [20]. In a similar manner, PKCγ may be involved in the downstream activation of oxidative stress to activate CREB during protection by treatment with blueberry extract [13]. From these studies, the relationship of Aβ and ROS to stress versus memory signaling and neurotoxicity remains to be clarified.

We have developed a rat neuron model of aging in which neurons from old rats are cultured as easily as middle-age neurons in a common, serum-free defined and optimized medium [21]. As judged by immunostaining, these cultures of middle-age and old neurons are 80% neurons, 10% oligodendrocytes, 5% microglia and 5% astroglia; have the same amount of protein in their regenerated axons and dendrites; take up glucose at similar rates [22]; and have equal levels of resting respiration [23]. Cultured middle-age and old neurons have similar passive membrane properties; both ages fire action potentials spontaneously [24] and have similar resting membrane potential [25]. Although the same numbers of neurons regenerate for these two ages, the old neurons are more susceptible to toxicity from glutamate, lactate or Aβ [21]. The mechanism of cell death involves apoptosis subsequent to caspase activation and ROS generation [26]. In this culture model of brain aging, we can determine whether the protection by blueberry extract in APP transgenic mice against Aβ toxicity and memory loss acts directly on the neurons specifically, avoiding the complexities of the vasculature, the inflammatory response, hormonal system or another uncontrolled target. Here, we determine whether blueberry extract is neuroprotective against Aβ toxicity in old neurons and which kinase pathway is associated with the mechanism of toxicity and protection. We also determine whether blueberry extract lowers the resting rate of oxyradical production.

2. Materials and methods

2.1. Adult neuron culture

Hippocampal neurons were isolated and cultured from 9- to 11- and 22- to 24-month F344 male rats [27,28]. The dissociated cells were plated on 12-mm Assistent glass coverslips (coated with 100 μg/ml poly-D-lysine overnight at room temperature) at a concentration of 320 cells/mm^2. The cells were grown in B27/Neurobasal A medium, 0.5 mM Glutamax and 5 ng/ml human recombinant FGF2 at 37°C, 5% CO_2 and 9% O_2. Embryonic hippocampal neurons were similarly prepared from embryonic rats 18 days in utero and plated at a concentration of 160 cells/mm^2 [29]. After 5 and 12 days in culture, 50% of the medium was changed using B27/Neurobasal A plus 0.5 mM Glutamax and 5 ng/ml FGF2. Lyophilized blueberry extract [12] was dissolved at a concentration of 10 mg/ml in Hibernate A LF (minus phenol red) plus 0.5 mM Glutamax, equivalent to about 125 mg whole blueberry/ml. This solution was sterilized by passage through a 0.22-μm filter. On 13 DIV, 100% of the medium was changed to Neurobasal A (minus phenol red) plus 0.5 mM Glutamax. The blueberry extract was added at a concentration of 0.125 mg/ml from the 10-mg/ml stock. Aged, fibrillized Aβ (1–42) was added from a 500-μM stock in PBS at a concentration of 10 μM. The neurons were allowed to incubate overnight. On 14 DIV, an equal volume of Hibernate A (minus phenol red) was added to the growth medium to stabilize the pH during counting. The dead cells were stained with propidium iodide at a concentration of 4.6 μg/ml from a 4.6-mg/ml stock in Hanks' Balanced Salt Solution. Dead cells were counted through Olympus G1B optics using a ×20 objective. Cells were then fixed for 15 min with 100% methanol at −20°C. Cells were rinsed once with Dulbecco's PBS (DPBS) and were transferred to contact cases containing 0.05% sodium azide in DPBS.

2.2. Immunocytology

Immunostaining was performed on middle-age and old rat hippocampal neurons 14 days in culture after changing 100% of the medium to Neurobasal A plus 0.5 mM Glutamax and treatments as above at 13 days in culture. Cells were first rinsed twice in warm DPBS (Invitrogen) and then placed on ice. Cells were fixed for 15 min at 4°C by addition of −20°C methanol. Cells were then rinsed twice in DPBS before permeabilization and blocking for 5 min in 3% BSA and 0.5% TX-100 in DPBS. Cells were incubated in primary antibodies overnight at 4°C diluted in 3% BSA and 0.05% TX-100 in DPBS. Primary antibodies included rabbit anti-CREB (Abcam #ab30651, Cambridge, MA), mouse mAb anti-CREB (Abcam #ab52844) and rabbit anti-pCREB (Abcam #ab30651), all diluted 1:100, as well as rabbit anti-ERK1/2 (MAPK; Cell Signaling Technology #9102, Danvers, MA) and mouse mAb anti-pERK1/2 (pMAPK; Cell Signaling Technology #9106), both diluted 1:200. Cells were then rinsed four times in DPBS. Secondary antibodies were (red fluorescing) Alexa Fluor 568-conjugated goat anti-mouse IgG (H+L) (Molecular Probes #A-11031, Eugene, OR) and (green fluorescing) Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) (Molecular Probes#A11034), both diluted 1:2000 in block for 60 min at room temperature. Cells were then rinsed four times in DPBS. One microgram per milliliter of bisbenzamide (blue fluorescing) in DPBS was added for 2 min to stain the nucleus. Cells were then rinsed twice in DPBS. Cells were mounted on slides using aqua-mount medium (Fisher). Immunofluorescence was observed through Olympus FITC, TRITC and DAPI optics using a ×60 oil objective. Images were analyzed using Image-Pro Plus from Media Cybernetics, Inc. (Silver Springs, MD).

2.3. Simultaneous live cell monitoring of oxyradicals with DCF and glutathione with MCB

At 13 days in culture, 100% of the medium was changed using Neurobasal A plus 0.5 mM Glutamax. After 24 h, treatments with blueberry extract and Aβ occurred for 0, 10, 20 or 30 min at 37°C, 5% CO_2 and 9% O_2. At 20 min prior to these times, 2′,7′-dichlorofluorescein diacetate (DCF, #D399 Molecular Probes) was added at a final concentration of 20 μM. During the last 5 min of the DCF incubation, monochlorobimane (MCB, #M1381 Molecular Probes) was added at 100 μM. At the end of the DCF and MCB incubation, cells were rinsed twice with Hibernate A LF (BrainBits) plus 0.5 mM Glutamax. Cells on coverslips were placed in a custom microscope chamber with Hibernate A LF plus 0.5 mM Glutamax containing 4.6 μg/ml propidium iodide to stain dead cells. Immunofluorescence was observed through Olympus FITC, TRITC and DAPI optics using a ×40 long working distance objective. Image analysis was done using Image-Pro Plus.

2.4. Statistics

Data are presented as the mean and standard error. Student's *t* test or ANOVA was used to determine the probability of insignificance below a cutoff of .05.

3. Results

Relatively homogeneous neuron cultures were prepared from the hippocampus of embryonic, middle-age (9–11 months old) or old-age Fisher rats (22–24 months old), near the median life span of these rats [30]. To control age-related changes in hormones and other factors, we first prepare cultures in a common serum-free medium (Fig. 1) to serve as a model for testing Aβ toxicity and neuroprotection by blueberry extract.

3.1. Neuroprotection against Aβ toxicity with blueberry extract

Neurons cultured for 13 days were changed to fresh medium without the antioxidants present in B27. Fig. 2 shows the age-related toxicity of fibrillar Aβ (ANOVA for age, $P=.0002$) similar to toxicity with Aβ (1–40) and Aβ (25–35) [21]. Addition of blueberry extract alone to each age of culture kept neuron death at low levels of 12–18% (Fig. 2) (ANOVA vs. Aβ, $P<10^{-4}$), similar to untreated cultures (data not shown). In initial experiments, the addition of blueberry extract was compared at 0.125 and 0.5 mg/ml. Because killing by Aβ was similarly protected at both concentrations, subsequent experiments were performed at the lower dose. We also tested the addition of blueberry extract added 24, 6 or 1 h before or concurrently with the addition of Aβ. Because differences were not significant, in the following experiments, we provide evidence for the simultaneous addition of both blueberry extract and Aβ. The combination addition

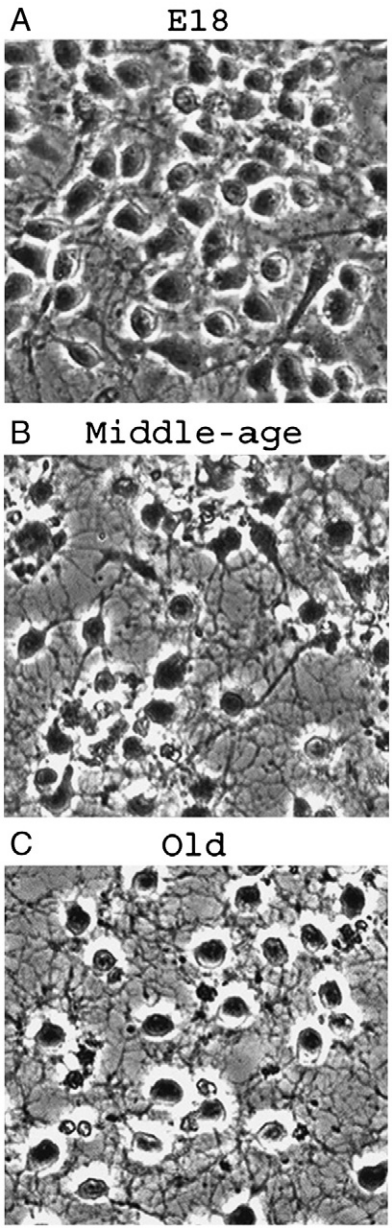

Fig. 1. Neuron culture model of aging with virtually equal yields and regeneration of adult neurons from old brain (24 months) as younger ages. Cultures from hippocampus of (A) Embryonic Day 18, (B) middle-age (9–11 months) and (C) old (22–24 months) rats imaged live at 8 days in culture.

of blueberry extract followed immediately by Aβ significantly lowered cell death compared to treatment with Aβ alone ($P<10^{-4}$).

3.2. Immunocytology for PKCα and PKCγ

We previously found in embryonic neurons that treatment with Aβ significantly increased cellular levels of PKCγ and that blueberry extract blocked this increase [14]. In Fig. 3A, this finding is replicated and extended to adult neurons. Control images from samples treated without primary and with secondary antibodies gave negligible immunofluorescence. In adult neurons, the stimulation of PKCγ immunoreactivity by treatment with Aβ remained, but no reversal of the stimulation by Aβ was observed by coincident treatment with blueberry extract. The opposite

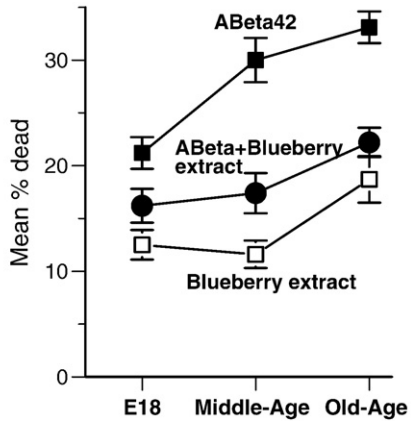

Fig. 2. Concurrent treatment with blueberry extract rescues age-related toxicity of Aβ in adult neurons at 8 days in culture. Compared to cultures treated with blueberry extract alone (0.125 mg/ml, open squares) for 24 h, treatment with fibrillar Aβ (10 μM, filled squares) for 24 h causes an age- and treatment-dependent increase in dead neurons. Co-treatment of cultures with both Aβ and blueberry extract (filled circles) greatly attenuates the killing caused by treatment with Aβ alone (ANOVA Aβ vs. blueberry extract+Aβ: $P<10^{-4}$). $N=12$ fields, 3 from each of 4 cultures at each condition.

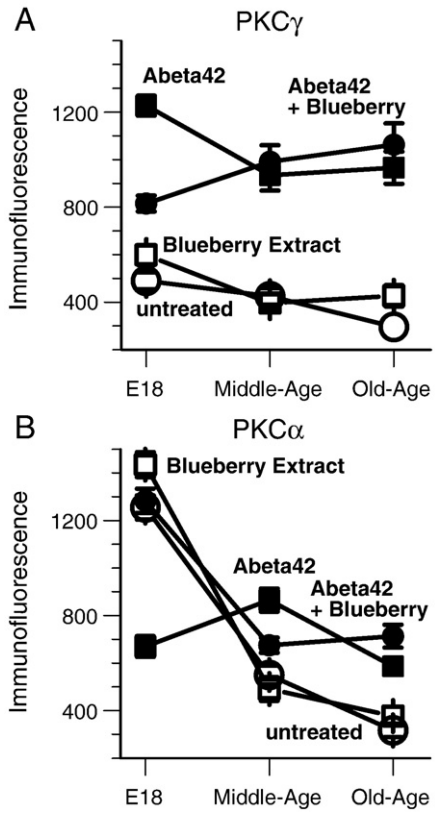

Fig. 3. Age-related increases of neuronal PKCα and PKCγ to Aβ, but failure of blueberry extract to reverse changes that result from treatment of adult neurons with Aβ. (A) Compared to untreated neurons (open circles) or neurons treated with blueberry extract (open squares), treatment with Aβ increased pPKCγ at all ages. Concurrent treatment with Aβ+blueberry extract (filled circles) partially reversed the Aβ-associated increase in embryonic neurons but failed to affect the increase in either middle-age or old neurons. (B) Treatment with Aβ reduces the pPKCα signal in embryonic neurons but raises the signal in adult neurons. Concurrent treatment with Aβ+blueberry extract (filled circles) reversed the Aβ-associated decrease in embryonic neurons, partially reversed the increase in middle-age neurons, but failed to reverse the increase seen in old neurons. Digital analysis of immunofluorescence of 30–90 individual neurons from four cultures of each age and treatment.

relationship was confirmed for PKCα in embryonic neurons (Fig. 3B). Aβ treatment of embryonic neurons lowered PKCα immunoreactivity, which could be reversed by concurrent treatment with blueberry extract. However, middle-age and old neurons had lower resting levels of PKCα that were elevated with Aβ. The further addition of blueberry extract with Aβ failed to reverse elevated PKCα in old neurons like it did in middle-age neurons.

3.3. Immunocytology for pCREB and pERK

To determine the effects of Aβ and blueberry extract on the relative locations of pCREB and pERK in the same neurons, we immunostained with both antibodies simultaneously. Fig. 4A shows the simultaneous immunostaining of untreated neurons for activated forms of CREB, pCREB (green) and the MAPK extracellular signal-regulated kinase pERK (red) with overlap seen as shades of orange and yellow. The axon and dendritic processes show largely distinct staining for pCREB and pERK. In the cytoplasm of the soma, there was considerable overlap of pCREB and pERK immunoreactivity. In the nuclei, distinct puncta of green pCREB labeling were seen on a broader background of overlapping faint yellow pCREB and pERK. Treatment with Aβ for 24 h followed by immunostaining (Fig. 4B) resulted in higher levels of pCREB and pERK in the above three compartments (brighter orange and yellow), with noticeably larger increases in red pERK in the nuclei and the puncta therein. The presence and staining of neurites were also reduced. Simultaneous treatment of old neurons with Aβ and blueberry extract (Fig. 4C) appeared to block the neuritic damage caused by Aβ and reduced overall staining closer to levels seen in untreated neurons.

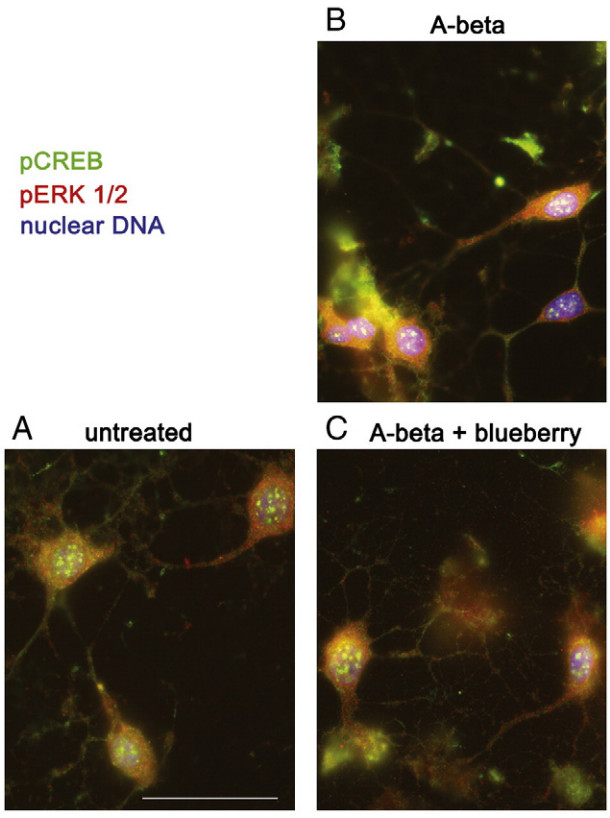

Fig. 4. Immunostain for pCREB (green), pERK1/2 (red) and nuclear DNA (blue) in neurons from old rat brain. (A) Strong cytoplasmic immunoreactivity for pERK (red) and punctate nuclear pCREB (green) in untreated old neurons. Bar = 30 μm. (B) Treatment with Aβ for 24 h increases the red cytoplasmic pERK as well as green pCREB together with red pERK in the nucleus (green+red+blue=white puncta). (C) Concurrent treatment with Aβ reduces the cytoplasmic pERK and nuclear pCREB closer to untreated conditions.

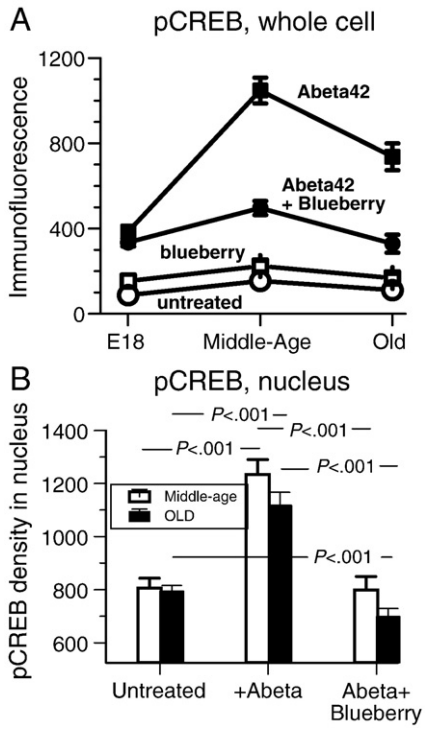

Fig. 5. Digital analysis shows that neuron immunoreactivity for pCREB is elevated with treatment with Aβ (filled squares) but largely restored to baseline (open symbols) by treatment with Aβ+blueberry extract (filled circles). (A) Whole-cell analysis. (B) Nuclear density of pCREB for middle-age (open bars) and old neurons (filled bars).

3.4. Digital analysis of CREB

The immunostaining of each individual cell in 12 adjacent microscope fields was digitally analyzed for mean fluorescence intensity as a measure of intracellular pCREB concentrations. Fig. 5A shows a much larger increase in middle-age and old neurons treated with Aβ than embryonic neurons or treatment with blueberry extract alone. The combination of Aβ+blueberry extract greatly reduced the elevated pCREB levels seen with Aβ alone in middle-age and old neurons but did not change the levels in embryonic neurons. Clearly, regulation of pCREB was very different in adult and embryonic neurons. In separate immunostains of neurons for pCREB and panCREB, the ratio of pCREB to panCREB was unaltered. This indicates that the treatments did not affect overall expression of CREB. To further characterize the pCREB staining specifically in the nucleus, we applied a nuclear mask (based on the bisbenzamide stain). Fig. 5B shows that treatment with Aβ roughly doubled the concentration (density) of immunoreactive pCREB in the nucleus in both middle-age and old neurons. Treatment with blueberry extract plus Aβ returned nuclear pCREB to control levels or below (old).

3.5. Digital analysis of ERK

Fig. 6A shows a large age-related increase in pERK for all three ages of neurons treated with Aβ. Interestingly, treatment with blueberry extract alone caused an age-related decline in pERK. Treatment with Aβ+blueberry extract significantly reduced the elevated pCREB levels seen with Aβ alone. In separate immunostains of neurons for pERK and panERK, Fig. 6B shows that the ratio of pERK to panERK also declines with blueberry extract treatment, indicating that the pERK

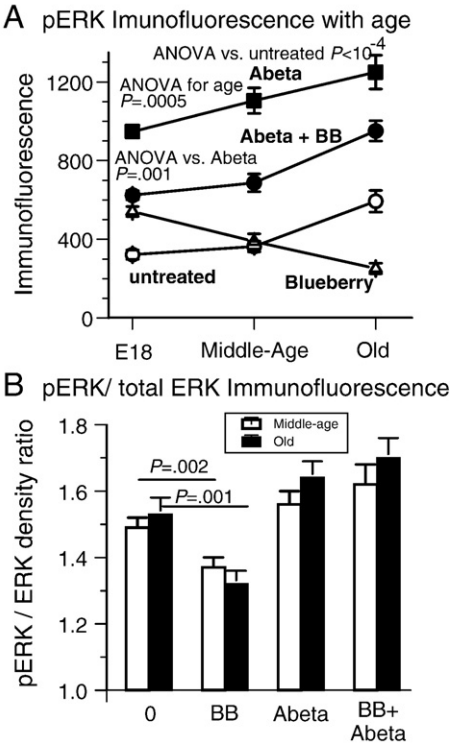

Fig. 6. Digital analysis shows that neuron immunoreactivity for pERK is elevated with treatment with Aβ (filled squares) but partially reversed toward baseline (open symbols) by treatment with Aβ+blueberry extract (filled circles). (A) Whole-cell analysis. (B) Separate immunostains for panERK and pERK for middle-age (open bars) and old (filled bars) neurons show little change in the ratio of the staining, suggesting that the changes seen in Panel A for pERK are not due to more total ERK per cell.

decline is not due to a decline in total ERK. Conversely, with Aβ treatment, the ratio of pERK to panERK also increased, indicating that the pERK rise with Aβ is not associated with as much of a rise in total ERK as a rise in pERK. In Fig. 6A, for treatment with Aβ+blueberry extract, the decline in pERK from the levels for treatment with Aβ alone is associated with no significant change in ratio of pERK/panERK, as seen in Fig. 6B.

3.6. Simultaneous measures of ROS and antioxidant glutathione

To determine whether blueberry extract provided a pro- or antioxidant signal to neurons and the impact on the pro-oxidant Aβ signal [31], we included fluorescent probes for oxyradical production, DCF, and for the cell's primary antioxidant glutathione, MCB. Fig. 7A shows untreated, old, live neuron imaging with negligible green ROS signals in cells visibly labeled blue for glutathione. Treatment with blueberry extract for 30 min produced a small rise in green ROS and a larger increase in blue glutathione (Fig. 7B). Treatment with Aβ for 30 min dramatically increased the green ROS signal as well as cells with higher glutathione, some of which were spared the rise in ROS (Fig. 7C). However, a more revealing mechanism emerges from following the early time course of the ROS responses. Fig. 7D shows a dramatic rise in ROS produced after only 10 min of neuron exposure to blueberry extract. The ROS stimulus quickly declined for middle-age and embryonic neurons by 20 min but was prolonged in old neurons until all ages of neurons declined to near baseline by 30 min. These data suggest that blueberry extract produces a short-lived pro-oxidant pulse to neurons of all ages, which is prolonged in old neurons. Similar to blueberry extract, Aβ also produces a short-lived pulse of ROS in middle-age and embryonic neurons (Fig. 7E). However, treatment of old neurons with Aβ produces a longer, sustained increase in ROS. This finding correlates with increased neuron killing by Aβ in old neurons [21]. Furthermore, treatment with Aβ+blueberry extract (Fig. 7F) produced very little rise in ROS levels over the time examined.

Analogous to a dual-label flow cytometry experiment, but with live adhesive neurons, we correlated the glutathione and ROS signals in individual cells to determine if the mechanism of neuroprotection by blueberry extract against Aβ toxicity correlated with increased levels of the antioxidant glutathione in the same cells. Fig. 8A shows that most old neurons begin with low ROS and low glutathione. Treatment with blueberry extract produced a population with higher glutathione, a few of which have higher ROS. Comparison of the high ROS populations shows a large proportion of neurons after treatment with Aβ, which, after blueberry extract+Aβ treatment, shifted to lower ROS levels associated with a larger proportion with higher glutathione levels. Compared to Aβ treatment alone, Fig. 8B shows that blueberry extract decreased the proportion of neurons with low glutathione and high ROS levels (population 2). Fig. 8C shows the complementary fraction in which blueberry extract increases the percentage of neurons with high glutathione and low ROS levels (population 4). These results suggest that those old neurons that are protected from Aβ toxicity by blueberry extract treatment do so by a mechanism that raises antioxidant glutathione levels.

4. Discussion

As noted in Section 1, Joseph et al. [11] found that dietary supplementation for 8 weeks with spinach, strawberry or blueberry extracts in the rodent diets was effective in reversing age-related deficits in neuronal and behavioral (motor and cognitive) function in aged (19 months) F344 rats. In addition, this study revealed that there were significant increases in neuronal signaling kinases (e.g., muscarinic receptor sensitivity [13]) and that the blueberry extract diet reversed age-related "dysregulation" in Ca^{45} buffering capacity [14]. All of the supplemented groups exhibited significantly less ROS levels than the controls. Subsequent studies have replicated these findings [32]. However, it was clear from these supplementation studies that the effects of blueberry extract on both motor and cognitive behavior were due to more than just antioxidant actions at a single target. Here, we attempted to determine whether the functional site of action was an age-related effect of blueberry extract on neurons in a uniform environment, removed from the aging vascular, hormonal and immune system.

4.1. ROS

In the present study, the target of blueberry extract treatment in protection from $Aβ_{42}$ was clearly shown to include middle-age and old neurons apart from an aging vascular, hormonal and immune system in a defined culture medium. In addition, we probed the ROS-related mechanism involved in the beneficial effects of the blueberry extract against $Aβ_{42}$. The results indicated that old neurons were more sensitive to fibrillar $Aβ_{42}$ toxicity than middle-age or embryonic neurons, shown previously for $Aβ_{40}$ [21]. Additionally, blueberry extract treatment lowered $Aβ_{42}$ toxicity in middle-age and old hippocampal neurons, as might be expected from previous findings of embryonic hippocampal neurons [14]. Importantly, it also appeared that blueberry extract treatment alone increased ROS, perhaps as a hormetic inducer of antioxidants [33,34]. Thus, a small dose of oxidant stressor blueberry extract induces a cellular up-regulation of synthesis of glutathione, a major antioxidant that might be measured as a lower production of cellular ROS causing the blueberry extract to appear as an antioxidant. This was confirmed here by observing that treatment with Aβ lowered levels of the major

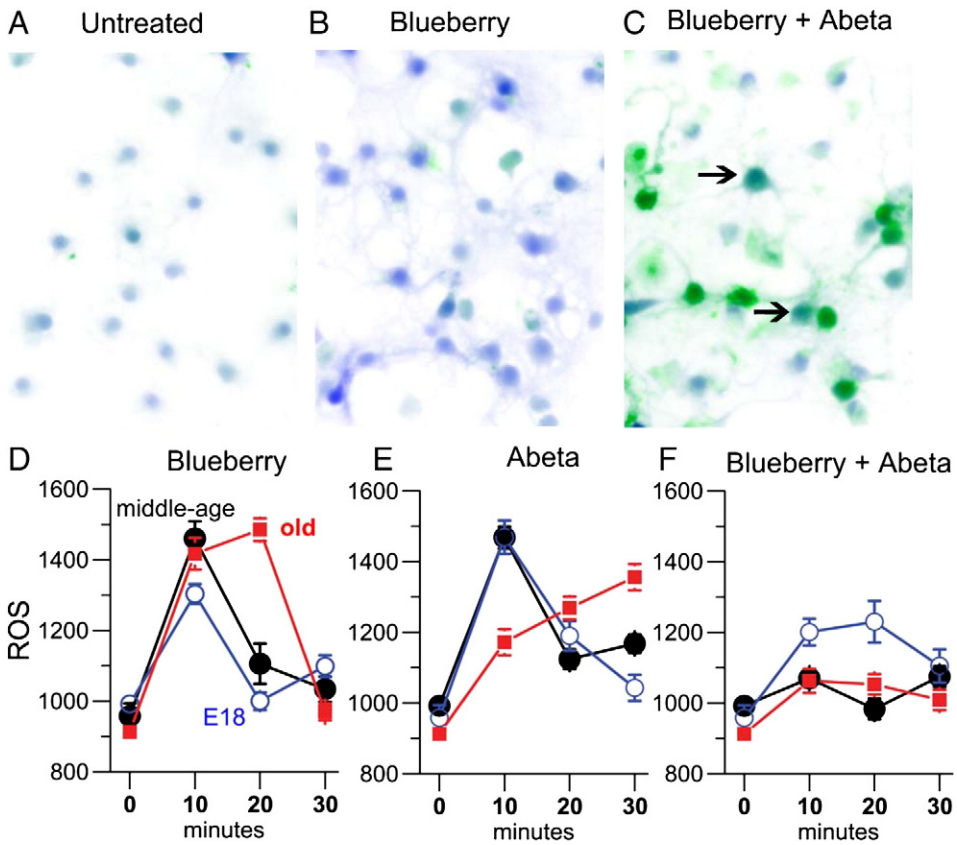

Fig. 7. Treatment with blueberry extract raises neuronal glutathione without raising ROS, while treatment with Aβ raises ROS, mostly without changing glutathione in live neurons. (A) Untreated old neurons with moderate blue stain with MCB for glutathione and low green stain with DCF for ROS (colors in Panels A, B, and C were inverted). (B) Blueberry extract for 30 min raises blue glutathione signal with little change in green ROS. (C) Treatment with Aβ for 30 min greatly elevates ROS levels (green) with only a few neurons resistant to the elevated ROS with associated higher levels of glutathione (arrows). Digital analysis of image at 10-min intervals for neurons treated with blueberry extract (D) rapidly increases ROS levels in all three ages of neurons. Treatment with Aβ (E) causes a rapid but transient increase in ROS in embryonic and middle-age neurons but a prolonged increase in old neurons. In all three ages, the addition of blueberry extract with Aβ (F) reduced ROS production over the 30-min period.

redox buffer, glutathione, consistent with oxidative depletion, but that blueberry extract treatment reversed this loss with increased levels of glutathione.

4.2. ERK

These increases in glutathione with Aβ treatment were accompanied by enhanced pERK signaling in an age-dependent manner with old neurons showing the greatest increase in this MAPK. However, blueberry extract treatment was able to reduce pERK even in the presence of Aβ. This is an important finding since ERK1/2 is essential for protection against neurodegeneration from oxidative stress/inflammation (e.g., Refs. [35,36]). Additionally, ERK1/2 is essential for memory formation [1]. Other studies indicate that activation of ERK and the antiapoptotic bcl-2 play a role in growth factor-mediated neuroprotection from 6-hydroxydopamine toxicity in dopaminergic cells [37], neuropathic pain [38], stroke protection [39] and a variety of oxidative stressors [40]. Similar findings have been reported regarding inflammation [41]. Importantly, in vulnerable Alzheimer's disease brain neurons, pERK is increased in association with oxidative damage [42] but can also be activated in brain neurons from non-demented cases without tau pathology [43]. While these pathology studies cannot distinguish pathological activation from failed neuroprotection, our in vitro viability studies suggest the possibility that too much pERK signaling is pathological but that mid-levels are protective. Thus, at least part of the protective effect of blueberry extract may involve reductions of endogenous pERK overexpression, since the overall oxidative stress load was reduced with blueberry extract and raised with Aβ. As mentioned above in the present experiments, blueberry extract treatment was able to increase glutathione and reduce ROS. Thus, submaximal ERK signaling may reduce endogenous stress.

In neonatal primary hippocampal cells [14] or M1 muscarinic receptor transfected COS-7 cells [44], blueberry extract treatment antagonized the Aβ- or dopamine-induced deficits in calcium buffering following depolarization with KCl or oxotremorine, respectively. These results showed that blueberry extract pretreatment prevented the deficits in calcium buffering as well as increases in the CREB and PKCγ, which were associated with ROS signaling, while increasing ERK, consistent with its protective role in cells. A protective role of the flavonoid fisetin increases Nrf2 expression, which, in turn, elevates glutathione through increased expression of glutamate–cysteine ligase, the first and rate-limiting step in glutathione synthesis, but the signaling is dependent on prior stress [45]. The signaling cascade may depend on the ability of ERK to indirectly activate CREB transcription as well as Nrf2, which, in turn, activates transcription of glutamate–cysteine ligase as in vascular smooth muscle cells [46].

4.3. PKC

Thus, while PKCγ was important for blueberry extract protection in neonatal neurons and COS-7 cells, as confirmed for embryonic neurons here, blueberry extract protection in adult neurons studied

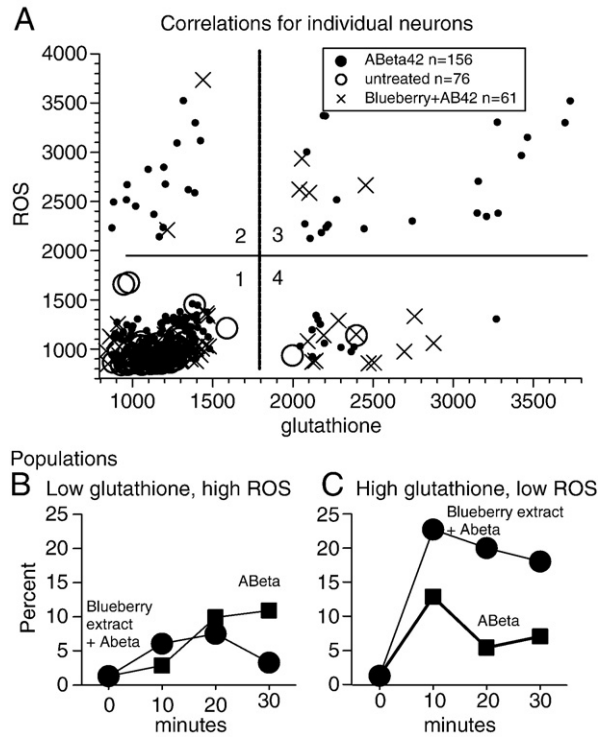

Fig. 8. (A) Tracking individual old neurons for coincident changes in DCF density (ROS) and MCB (glutathione) after 30 min reveals four populations of neurons for each of three treatments. Note that concurrent treatment with Aβ and blueberry extract (x) greatly increases the population of neurons with high glutathione and low ROS (Quadrant 4), in contrast to treatment with Aβ alone (filled circles) with high proportions of ROS with both low and high glutathione levels or untreated neurons (open circles) with low levels of both ROS and glutathione. (B) Time course of population in Quadrant 2 with low glutathione and high ROS shows a sustained increase with time for treatment with Aβ alone (open squares) that is reversed by concurrent treatment with Aβ and blueberry extract. (C) Time course of population in Quadrant 4 with high glutathione and low ROS shows a transient increase with time for treatment with Aβ alone (open squares) that grows into a much larger population by concurrent treatment with Aβ and blueberry extract.

here did not occur via changes in pPKC isoforms α or γ. This is surprising since PKCγ is one of the major forms of PKC that is found in the hippocampus [47,48] and known to be involved in memory formation. The PKC pathway is also part of a major signal transduction system in inflammation [49].

4.4. Creb

CREB activation to pCREB and translocation to the nucleus is a well-established part of the cellular stress response pathway [50]. In the absence of a PKC response to blueberry extract, it appears that the protection from Aβ toxicity by blueberry extract in the adult neurons is associated with a reversal of the elevated levels of total cellular pCREB as well as nuclear pCREB induced by Aβ. A frequent downstream target of pCREB is the activation of nuclear factor kappa B (NFκB). We also investigated age-related activation of NFκB in the same old neuron model and found that the increased neuron killing by Aβ in old neurons relative to middle-age neurons was associated with lower nuclear p50 and the induction of a lower Bcl-2/Bax ratio [51]. The addition of the inflammatory cytokine TNFα further lowered Bcl-2/Bax with a corresponding age-related increase in neuron death. NFκB translocates to the nucleus and mediates the transcription of many inflammatory genes (e.g., COX-2, TNFα, interleukin 1-beta and inducible nitric oxide synthase) to further promulgate inflammatory signals and neuronal degeneration [52]. Thus, the stimulation of TNFα by Aβ could initiate a vicious cycle of runaway inflammation that can be blocked by blueberry extract.

While CREB is very closely associated with learning and memory at the critical synaptic sites affected in Alzheimer's disease [53,54], its actions appear to be dependent upon the experimental conditions and paradigms under study. In embryonic neurons, Aβ failed to stimulate pCREB unless accompanied by depolarizing KCl [3]. Phosphorylation of CREB increases in acute mild hypoxia [16,17]. CREB is activated by hydrogen peroxide in Jurkat T lymphocytes [18] and by cadmium in mouse neuronal cells [19], as well as during stroke [20]. Most relevantly, Arvanitis et al. [5] showed that PC12 cells transfected for overexpression of APP driven by three different promoters raised intracellular Aβ to a higher level that blocked nuclear translocation of pCREB in the case of one promoter, but with the other two promoters that produced lower levels of Aβ, pCREB was translocated to the nucleus. Thus, these studies in PC12 cells at the lower levels of intracellular Aβ (with possibly modest oxidative stress) agree with our findings of Aβ applied exogenously, resulting in an increase in nuclear pCREB in middle-age and old neurons, while the combined stress of blueberry extract and Aβ resulted in blocked nuclear translocation of pCREB. Our observation of reversal of the overstimulation of pCREB by blueberry extract further strengthens the utility of dietary intervention to combat the toxic effects of Aβ.

Thus, it appears that inflammatory and oxidative stressors can elicit a cascade of signals that ultimately result in the generation of additional stressors and possibly reductions in the protective capacity of the neuron in aging. However, our findings and others suggest that blueberry extract can activate protective pathways to reduce the deleterious effects of oxidative stress. Additionally, previous research has shown that under oxidative stress or inflammatory conditions, polyphenols similar to those contained in blueberries (e.g., those found in tea, red wine or ginkgo biloba) altered signaling in ERK activity [14,55], as well as PKC [56,57] and CREB [58]. Although the mechanism of action of various components in blueberry extract is unknown, cyanidins found in the brains of rats fed blueberry extract [59] were shown by Ishige et al. [60] to be among the nearly half of 40 specific flavonoids that protected neuronal cells against glutamate toxicity by mechanisms involving increased glutathione, lowering ROS and preventing the influx of calcium. Interestingly, activities did not correlate with their Trolox equivalent activity concentrations, a measure of antioxidant power, suggesting again that antioxidant mechanisms do not alone explain the beneficial effects of fruit phytochemicals.

4.5. Conclusion

In summary, blueberry extract not only appears to be neuroprotective through pCREB and pERK but should also be considered for evaluation as a low-cost, palatable intervention against the learning and memory deficits elicited by Aβ and oxidative stress in Alzheimer's disease.

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