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Cellular and Behavioral Effects of Stilbene Resveratrol Analogues: Implications for Reducing the Deleterious Effects of Aging

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Research suggests that polyphenolic compounds contained in fruits and vegetables that are rich in color may have potent antioxidant and anti-inflammatory activities. The present studies determined if stilbene (e.g., resveratrol) compounds would be efficacious in reversing the deleterious effects of aging in 19 month old Fischer 344 rats. Experiment I utilized resveratrol and six resveratrol analogues and examined their efficacies in preventing dopamine-induced decrements in calcium clearance following oxotremorine-induced depolarization in COS-7 cells transfected with M1 muscarinic receptors (MAChR) that we have shown previously to be sensitive to oxidative stressors. Experiment II utilized the most efficacious analogue (pterostilbene) from experiment I and fed aged rats a diet with a low (0.004%) or a high (0.016%) concentration of pterostilbene. Results indicated that pterostilbene was effective in reversing cognitive behavioral deficits, as well as dopamine release, and working memory was correlated with pterostilbene levels in the hippocampus.

KEYWORDS: Polyphenolics; pterostilbene; memory; learning; oxidative stress; inflammation

INTRODUCTION

A great deal of research indicates the occurrence of numerous neuronal and behavioral deficits during "normal" aging. These changes may include decrements in calcium homeostasis (*1*) and in the sensitivity of several receptor systems, most notably: (i) dopaminergic (*2*), (ii) muscarinic (*3*), (iii) opioid (*4*), and (iv) adrenergic (*5*). These losses in neuronal function can be expressed, ultimately, as alterations in both cognitive and motor behaviors (*6*).

An abundance of data suggests that one of the most important factors mediating the deleterious effects of aging on behavior and neuronal function is oxidative stress (OS; see ref *7* for a review), wherein the central nervous system (CNS) appears to be especially vulnerable (*8*). Evidence also suggests that inflammatory events in the CNS play an important role in aging. By middle age, there is an increased glial fibrillary acid protein expression (*9*) that later, in the elderly, even occurs in the absence of an inflammatory stimulus (*10*). In fact, it has been suggested that the up-regulation of C-reactive protein may represent one factor in biologic aging (*11*).

Research from our laboratory suggests that the combinations of antioxidant/anti-inflammatory polyphenolics found in fruits and vegetables may show efficacy in aging. Plants, including food plants (fruits and vegetables), synthesize a vast array of "secondary" chemical compounds that, while not involved in their primary metabolism, are important in serving a variety of ecological functions that enhance the plant's survivability. Interestingly, these compounds may be responsible for the multitude of beneficial effects that have been reported for fruits and vegetables on an array of health-related bioactivities. Numerous studies have suggested that their most important benefits may be derived from their antioxidant and anti-inflammatory properties.

To this end, in several previous experiments, we have shown that blueberry (BB) supplementation in senescent rodents reversed several deficits in motor and cognitive behavioral patterns, neurogenesis, and neuronal aborization (*12*−*15* and reviewed in ref *16*). An additional study in APP/PS1 transgenic mice that were given BB supplementations from 4 months of age until 12 months of age showed Y-maze performance similar to that seen in nontransgenic mice and significantly greater than that seen in the nonsupplemented transgenic animals (*17*). Further analyses revealed that the BB-supplemented APP/PS1 mice showed greater levels of hippocampal extracellular signal regulated kinase (ERK), as well as striatal and hippocampal protein kinase C α (PKCα), than those seen in APP/PS1 mice maintained on a control diet. These findings suggest that BB supplementation may actually prevent cognitive deficits by

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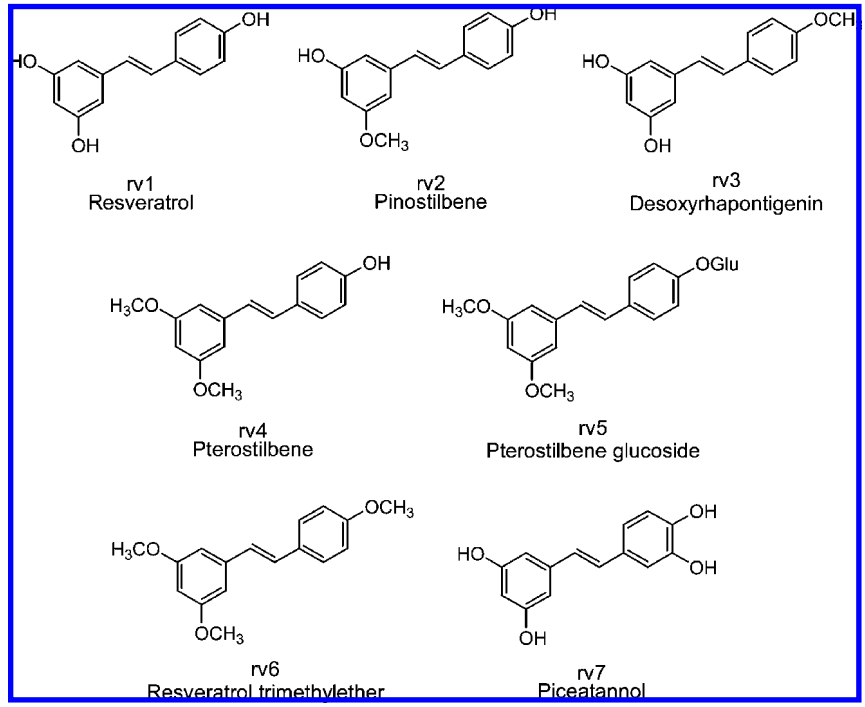

Figure 1. Structures of the stilbenes tested.

directly affecting signaling, subsequently enhancing neuronal signaling, and offsetting any putative deleterious effects of the amyloid deposition. The data also revealed that BB supplements seemed to enhance signaling not only at the level of the kinases but also more directly by increasing the sensitivity of muscarinic receptors (i.e., increasing striatal, carbachol-stimulated GTPase activity).

Additional results, thus far, in COS-7 cells transfected with OS-sensitive muscarinic receptors suggest that BBs may alter several signals in response to oxidative stressors signals such as mitogen activated protein kinases (MAPK) and cyclic AMP response element binding proteins (CREB) (*18*). These findings suggest that at least part of the efficacy of the BB supplementation may be to "strengthen" areas of the brain that may be showing the ravages of time, allowing them to communicate more effectively with other brain regions involved in both motor and memory performance.

Interestingly, an additional study (*19*) suggests that Concord grape juice supplementation has similar effects on cognitive and motor behavior to those seen with BB. Among the polyphenols that are similar between Concord grapes and BB are compounds with a basic stilbene structure such as resveratrol, albeit in low amounts (*20, 21*). Resveratrol has been shown in numerous studies to have anti-inflammatory (*21, 22*) and antioxidant effects (*22, 23*), along with some potent inhibition of pro-inflammatory mediators (e.g., see ref *24* for a review) and enhancement of anti-inflammatory/antioxidant mediators such as peroxisome proliferator-activated receptors (PPARs) (*21, 25*). In several studies, Bastianetto and colleagues have shown that resveratrol has potent neuroprotective properties and shows protection against β-amyloid toxicity (reviewed in ref *26*).

Given the interesting parallel in the pharmacological properties of resveratrol and other compounds with a basic stilbene structure that are found fruits such as BB and Concord grapes and work from Rimando and colleagues (*21, 27*) showing that these compounds affect pPPAR-α and low-density cholesterol (an inflammatory agent), it became of interest in the present experiments to determine whether: (i) experiment 1—these and additional stilbene compounds (**Figure 1**) would be effective in protecting against dopamine (DA)-induced OS deficits in calcium buffering in COS-7 cells transfected with M1 muscarinic receptors, as M1-transfected COS-7 cells have previously been shown to exhibit increased vulnerability to DA and other oxidative stressors such as amyloid β (*28*) and (ii) experiment II—the most effective stilbene compound, pterostilbene, in the cell studies would be effective in reversing cognitive deficits in senescent rodents given a pterostilbene-supplemented diet.

MATERIALS AND METHODS

Experiment I. *Resveratrol Analogue Derivation.* The structures of the stilbenes tested are shown in **Figure 1**. Resveratrol (rv1) was purchased from Sigma-Aldrich (St. Louis, MO). Piceatannol (rv7) was purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). Desoxyrhapontigenin (rv3), pinostilbene (rv2), pterostilbene (rv4), pterostilbene glucoside (rv5), and resveratrol trimethylether (rv6) were synthesized as described in the following procedures.

Synthesis of Pterostilbene. Pterostilbene was synthesized by condensation of 3,5-dimethoxybenzaldehyde and 4-hydroxyphenylacetic acid in acetic anhydride and triethylamine. The reaction mixture was heated (150 °C) under an atmosphere of nitrogen and continuously stirred. After 20 h, the reaction was stopped and cooled to room temperature, and concentrated hydrochloric acid (5 mL) was added. A precipitate formed, and this was dissolved in 50 mL of chloroform and then extracted with 10% aqueous sodium hydroxide. The aqueous extract was acidified to pH 1 with concentrated hydrochloric acid and stirred for at least 6 h, resulting in the precipitation of the intermediate product, α-[(3,5-dimethoxyphenyl)methylene]-4-hydroxy-(αZ)-benzeneacetic acid. This intermediate product was heated with 1.0 g of copper in 10 mL of quinoline (200 °C, 6 h, under nitrogen). The reaction mixture was cooled to room temperature and filtered. To the filtrate was added 5 N hydrochloric acid (25 mL), which was stirred for 1 h and then extracted with chloroform. The chloroform extract containing impure pterostilbene was purified by flash chromatography on a Horizon HPFC system (Biotage, Inc., Charlottesville, VA), using a silica gel column and the solvent system ethyl acetate:hexane (linear gradient from 15:85 to 100% ethyl acetate). Fractions containing pure pterostilbene were combined and concentrated in vacuum. Pterostilbene was recrystallized in hexane, and its structure was confirmed from its spectroscopic data (UV, mass spectrometry, and nuclear magnetic resonance spectroscopy).

Synthesis of Pterostilbene Glucoside. Pterostilbene (20 mg) was mixed with 40 mg of acetobromo-α,D-glucose (Sigma-Aldrich) and potassium hydroxide (5 mg in ethanol) in 1 mL of ethanol and stirred for 2 days in room temperature. Pterostilbene glucoside was purified by preparative layer chromatography using methanol:chloroform as the developing solvent (20:80). The structure of pterostilbene glycoside was determined from its ^{1}H nuclear magnetic resonance spectrum.

Synthesis of Desoxyrhapontigenin, Pinostilbene, and Resveratrol Trimethylether. A solution of resveratrol (150 mg in 3.0 mL of methanol) was treated with diazomethane, dropwise, and the methylation reaction was monitored by thin-layer chromatography. Desoxyrhapontigenin, pinostilbene, and resveratrol trimethylether were purified by preparative layer chromatography using hexane:ethyl acetate (8:2) as the developing solvent (R_f values = 0.2, 0.25, and 0.8, respectively). The structures of these compounds were confirmed by mass spectrometry and nuclear magnetic resonance spectroscopy.

Cell Transfections. COS-7 cells (ATCC) were grown, transfected, and maintained as described previously (*28, 29*). Briefly, COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and containing 100 U/mL penicillin and 100 μg/mL streptomycin sulfate. Twenty-four hours prior to transfection, cells were harvested with trypsin, counted, and plated on 100 mm^2 tissue culture plates at 5×10^6 cells/plate. Cells were transiently transfected with rat muscarinic receptor subtype 1 DNA by the DEAE-dextran method, as previously described (*28*). After transfection, cells were incubated for 2.5 h in growth medium containing 80 μM chloroquine to minimize degradation of the DNA. Transfected cells were then maintained in growth medium for 48 h, harvested with trypsin, plated to coverslips in 35 mm plates for calcium imaging, and incubated overnight.

Cell Treatments. BB extract (2 mg/mL), stilbenes [rv1 (50 μg/mL), rv2 (50 μg/mL), rv3 (25 μg/mL), rv4 (10 μg/mL), rv5 (10 μg/mL), rv6 (10 μg/mL), and rv7 (10 μg/mL)], and DA (1 mM) treatments were carried out as described previously (*29*). The BB and stilbene concentrations utilized in this experiment reflected the highest amount of extract that did not reduce response or recovery in control or nondopamine-treated M1AChR-transfected COS-7 cells in previous (*29*) or pilot experiments. The BB extract and the stilbenes were dissolved in growth media, and cells were subsequently incubated for 45 min at 37 °C with the treated growth medium, followed by DA administration for 4 h. Following these incubations, the cells were washed three times with extract-free growth medium prior to testing.

Calcium imaging was carried out as described previously (*28, 29*) using a Medical Systems Corp. open perfusion microincubator (37 °C) with temperature control that was mounted on the stage of a Nikon Eclipse TE200U microscope and illuminated with a fluorescent light source. Real-time analyses of calcium flux prior to and following 750 μM oxotremorine-induced depolarization were then carried out using Simple PCI software (Compix, Inc., Mars, PA). Response and recovery were then determined for each sample. Response was determined by examining whether a cell showed increases in $[Ca^{2+}]_i$ to oxotremorine by >30% over baseline. Only those cells that showed this magnitude of response were considered for further analysis. Recovery was determined by assessing the time (within 300 s) for the Ca^{2+} levels to return to 20% of the increase following depolarization in the cells that responded. Only the recovery data for the various conditions are presented in this manuscript. Because recovery is a dichotomous variable, data were analyzed by Kruskal−Wallis one-way analyses of variance (ANOVA) and Mann−Whitney U tests.

Experiment II. *Animals.* Forty-two 19 month old male Fischer 344 rats were obtained from the NIA colony (Harlan Sprague Dawley, Indianapolis, IN). The rats were individually housed in stainless steel mesh suspended cages, provided food and water ad libitum, and maintained on a 12 h light/dark cycle. The rats were given 1 week to adjust to their new environment, after which time they were weight matched and then randomly placed on one of three diets for 12−13 weeks total (until euthanized) (n = 14/group): control, low-dose pterostilbene (0.004%, equivalent to 2.5 mg/kg body weight), or high-dose pterostilbene (0.016%, equivalent to 10 mg/kg body weight). All animals were observed daily for clinical signs of disease. During the course of the study, one rat in each group died, and additionally, a high-dose stilbene rat was euthanized due to excessive weight loss. All animals were utilized in compliance with all applicable laws and regulations as well as principles expressed in the National Institutes of Health, USPHS, Guide for the Care and Use of Laboratory Animals. This study was approved by the Animal Use and Care Committee of our Center.

Pterostilbene Diet. The pterostilbene diets were prepared at Harlan Teklad (Madison, WI) by adding crystalline pterostilbene to the control diet, which was a modification of the NIH-31 diet (40 mg/kg diet, 0.004% w/w for low dose; 160 mg/kg diet, 0.016% w/w for high dose). Pterostilbene was the compound found most effective in the cell studies above (rv4). The amount of corn in the control diet was adjusted to compensate for the added quantity of the pterostilbene. The control diet was the same one used in previous studies in which beneficial effects of BB on aging were found (*13−15, 30*). The rats were maintained on either the control or the pterostilbene diet for 9 weeks before cognitive testing at 21 months of age.

Cognitive Testing. The Morris water maze (MWM), an accepted method of testing spatial learning and memory, is an age- (*6*) and diet- (*12−14, 19*) sensitive learning paradigm that requires rats to find the location of a hidden platform (10 cm in diameter) just below the surface (2 cm) of a circular pool of water (134 cm in diameter × 50 cm in height, maintained at 23 °C) based on distal cues and to remember its location from the previous trial. Accurate navigation is rewarded with escape from the water onto the platform, for which the rat uses distal cues to effectively locate. The working memory version of the MWM was performed daily for 4 consecutive days during the ninth week of treatment, with a morning and an afternoon session, two trials each session, with a 10 min intertrial interval between the two trials. Rats were tested in a random manner, with the restriction that one rat from each group be tested in succession. At the beginning of each trial, the rat was gently immersed in the water at one of four randomized start locations. Each rat was allowed 120 s to escape onto the platform; if the rat failed to escape within this time, it was guided to the platform. Once the rat reached the platform, it remained there for 15 s (trial 1; reference memory or acquisition trial). The rat was returned to its home cage between trials (10 min). Trial 2 (the working memory or retrieval trial) used the same platform location and start position as trial 1. Performances were videotaped and analyzed with image tracking software (HVS Image, United Kingdom), which allows measurements of latency to find the platform (s), path length (cm), and swimming speed (cm/s; latency/path length). For a more detailed description of the maze and the paradigm used, see ref *6*.

Extraction and Analysis of Pterostilbene in Serum. Serum samples were collected when the rats were euthanized 2−3 weeks following behavioral testing and stored at −80 °C and thawed in ice prior to extraction. Extraction was performed according to published procedure (*31*), with minor modification. To 300 μL of serum, 240 μL of sodium acetate solution (1 M, pH 5.0) and 60 μL of β-glucuronidase (77770 μ/mL, MP Biomedicals Inc., Fisher Scientific, Norcross, GA) were added. The mixture was kept in an oven at 37 °C for 20 h, then extracted with 1.3 mL of ethyl acetate, and then centrifuged at 4000 rpm for 5 min. The ethyl acetate extract was collected. Ethyl acetate extraction was repeated, and the extracts were combined and blown dry with a stream of nitrogen.

The dried extract was treated with 30 μL of *N*,*O*-bis[trimethylsilyl]-trifluoroacetamide:dimethylformamide (BSTFA:DMF, 1:1; Pierce Biotechnology, Inc., Rockford, IL) and heated at 70 °C for 40 min. The derivatized sample was used for analysis of pterostilbene by gas chromatography−mass spectrometry (GC-MS). GC-MS was performed on a JEOL GCMate II Instrument (JEOL USA Inc., Peabody, MA) using a J&W DB-5 capillary column (0.25 mm internal diameter, 0.25 μm film thickness, and 30 m length; Agilent Technologies, Foster City, CA). The GC was run under the following temperature program: initial 190 °C, increased to 239 at 20 °C/min rate and held at this temp for 3 min, increased to 242 °C at the rate of 0.2 °C/min, then finally increased to 300 °C at the rate of 25 °C/min and held at this temperature for 1.5 min. The carrier gas was ultrahigh purity helium, at 1 mL/min flow rate. The injection port, GC-MS interface and ionization chamber were at 250, 230, and 230 °C, respectively. The volume of injection was 1 μL, splitless injection. The mass spectrum was acquired in positive,

electron impact (70 eV), low-resolution mode. Pterostilbene was determined and quantified from a reconstructed ion chromatogram using *m/z* 256. Quantitation was performed from a calibration curve of a standard sample of pterostilbene (retention time, 12.5 min). GC-MS analyses were in duplicates.

Extraction and Analysis of Pterostilbene in Brain Tissue. Hippocampal brain tissue samples were stored at −80 °C and thawed in ice prior to extraction. Tissues were homogenized in 500 μL of phosphate buffer (pH 7.4) and then centrifuged (7000*g*, 4 °C, 15 min). The supernatant was collected, and homogenization was repeated. The combined supernatant was extracted with ethyl acetate (500 μL × 2). The ethyl acetate extract was dried under a stream of nitrogen. The dried extract was treated with BSTFA:DMF and analyzed for pterostilbene following the same procedures as in serum analysis, except that the mass spectrum was acquired in select selected ion monitoring mode (*m/z* 328, 313, 297, and 147) for better selectivity and sensitivity.

DA Release. We have previously shown that the muscarinic enhancement of K^+-evoked DA release (K^+-ERDA) from superfused striatal slices is an indicator of receptor sensitivity and striatal function and is sensitive to aging and OS (*3*), as well as dietary supplementation (*12*−*14, 19*). The protective capacity of the striatal tissue obtained from the control and supplemented groups was assessed by examining differences in the oxotremorine enhancement of striatal K^+-ERDA. DA release was conducted 2−3 weeks following behavioral testing on freshly dissected and cross-cut (300 μm, McIlwain tissue chopper) striatal slices from the brains of animals in the various groups. The slices were placed in small glass vials containing modified Krebs−Ringer basal release medium (BRM) that had been bubbled for 30 min with 95% O_2/5% CO_2 and which contained (in mM) $NaHCO_3$ (21), glucose (3.4), NaH_2PO_4 (1.3), EGTA (1), $MgCl_2$ (0.93), NaCl (127), and KCl (2.5) (low KCl) (pH 7.4). Half of the tissue was treated with 50 μM H_2O_2 to assess the effect of diet under conditions of OS. The slices were then placed in the perfusion chambers where they were maintained at 37 °C and perfused with the BRM for 30 min. Following this equilibration period, the medium was switched to one containing (in mM) KCl (30) (high KCl), $CaCl_2 \cdot 2H_2O$ (1.26) (in place of EGTA), NaCl (57), and 0 or 500 μM oxotremorine, and then, the enhancement of K^+-ERDA was assessed. DA release was quantitated by HPLC coupled to electrochemical detection and expressed as pmol/mg protein as determined by the Lowry and colleagues procedure (*32*).

Statistical Analyses. For each behavioral measure, between subjects ANOVA models comparing the three groups were performed using Systat (SPSS, Inc., Chicago, IL) to test for statistical significance at the $p < 0.05$ level. Days or trials, when appropriate, were included in the model as a within subjects variable. Posthoc comparisons, to determine differences among diet groups, were performed using Fisher's LSD posthoc analysis. Pearson *r* correlations between MWM performance and hippocampal pterostilbene levels were performed for the high pterostilbene-fed rats using Systat (SPSS, Inc.) to test for statistical significance at the $p < 0.05$ level.

RESULTS

Experiment I. The results indicated that administration of DA significantly reduced recovery in M1-transfected cells (control vs DA-treated cells, $p < 0.001$) (**Figure 2**). However, BB pretreatment protected against the deleterious effects of DA (BB vs BB + DA-treated cells, $p > 0.05$) as has been seen in previous studies (*29*). Several resveratrol analogues (rv4, rv5, rv6, and rv7) also protected against the DA-induced decreases in recovery (e.g., rv4 vs rv4 + DA, $p > 0.05$), while rv1, rv2, and rv3 offered no protection (e.g., rv1 vs rv1 + DA, $p < 0.001$) (**Figure 2**).

Experiment II. There were no differences in weight ($p > 0.05$; average weight ± SEM = 442.54 ± 5.382) or food intake ($p > 0.05$; average food intake ± SEM = 23.40 ± 0.462) between the diet groups over the course of the study. When examining cognitive performance, the pterostilbene groups showed improved performance as compared to the control diet-fed group (**Figure 3**). There were no differences in MWM latency or distance to find the platform for trial 1 or trial 2 between the diet groups. However, separate *t* tests were performed between the two trial latencies or distances for each group for days 3 and 4 (the days which rely more on memory than learning) to determine if the different diet groups significantly improved their performance from trial 1 to trial 2, an indication of improved working memory. We found that the high-dose pterostilbene group showed a significant ($p < 0.05$) difference in latency to find the platform between trial 1 and trial 2, that is, trial 2 latencies were significantly less than trial 1, showing that these rats demonstrated one-trial learning, even

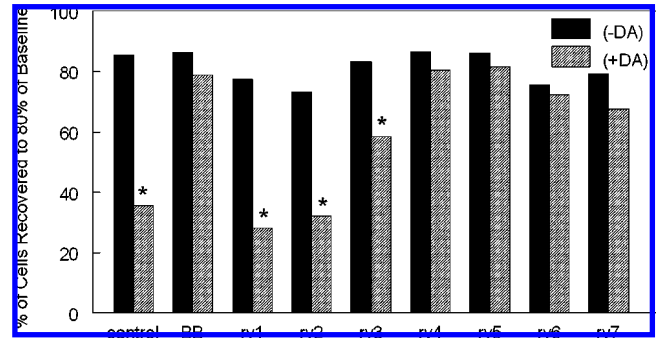

Figure 2. Mean Ca^{2+} recovery (see the Materials and Methods) in M1-transfected COS-7 control cells and those pretreated with BB, rv1 (resveratrol), rv2 (pinostilbene), rv3 (desoxyrhapontigenin), rv4 (pterostilbene), rv5 (pterostilbene glucoside), rv6 (resveratrol trimethylether), or rv7 (piceatannol) following 0 (−DA) or 1 mM DA (+DA) treatment. The asterisk indicates a difference in recovery between non-DA-treated and DA-treated cells for each treatment (*$p < 0.001$).

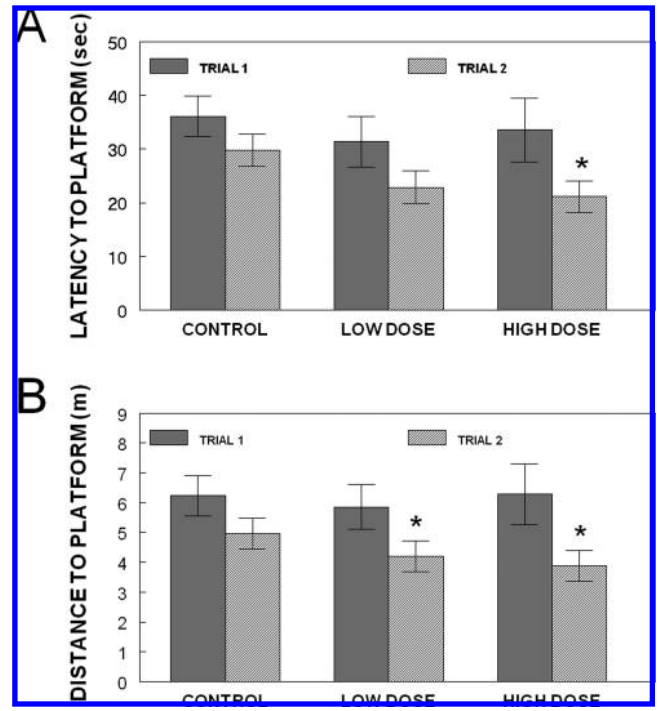

Figure 3. MWM performance assessed as latency in seconds (**A**; mean ± SEM) and distance in meters (**B**; mean ± SEM) to find the hidden platform over days 3 and 4 of testing for animals maintained on the control, low-dose pterostilbene (0.004%), and high-dose pterostilbene (0.016%) diets. The asterisk indicates a difference (i.e., an improvement) between trial 1 and trial 2 performances (*$p < 0.05$), showing improved working memory.

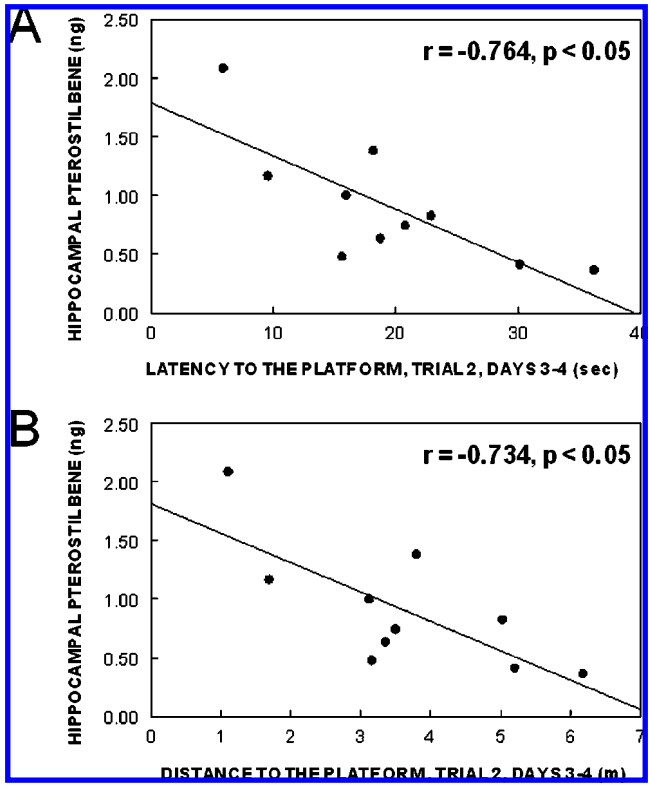

Figure 4. Correlation between hippocampal pterostilbene levels (ng) and working memory performance as measured by latency (**A**) and distance (**B**) to find the hidden platform on trial 2 for days 3 and 4 of testing for rats maintained on the high-dose pterostilbene (0.016%) diet.

with the 10 min retention interval (**Figure 3A**). This one-trial learning was not found in the control group, while the low-dose pterostilbene group tended to improve from trial 1 to trial 2 ($p = 0.09$). Additionally, both the low- and the high-dose pterostilbene groups showed significant ($p < 0.05$) differences in distance to find the platform between trial 1 and trial 2; that is, trial 2 distances were significantly less than trial 1 (**Figure 3B**). The control group did not show this improvement from trial 1 to trial 2 ($p > 0.05$). Therefore, pterostilbene reversed the deleterious effects of aging on cognitive performance, particularly working memory, in a dose-dependent manner. This difference was not due to swim speed as there were no differences between the groups on this parameter on days 3 and 4.

Pterostilbene was undetectable in the serum of control-fed animals. In low-dose pterostilbene rats, the serum levels were 3.951 ± 0.439 ng/mL (mean ± SEM), and for high-dose pterostilbene rats, they were 25.576 ± 5.411 ng/mL. Hippocampal pterostilbene was only detectable in the high-dose pterostilbene group and was 1.352 ± 0.465 ng/hippocampus.

To examine whether there was a relationship between MWM performance of the high-dose pterostilbene rats and pterostilbene levels in the brain, we correlated hippocampal pterostilbene levels with trial 1 and trial 2 latency and distance performance. We examined this brain area because the hippocampus is known for its role in spatial learning and memory, particularly trial 2 working memory. We found a significant negative correlation between hippocampal pterostilbene levels and trial 2 performance on days 3 and 4 for latency ($r = -0.764$, $p = 0.010$) and distance ($r = -0.734$, $p = 0.016$); that is, as pterostilbene levels increased, the latency (**Figure 4A**) and distance (**Figure 4B**) to find the platform decreased. Hippocampal pterostilbene levels were not correlated with trial 1 performance.

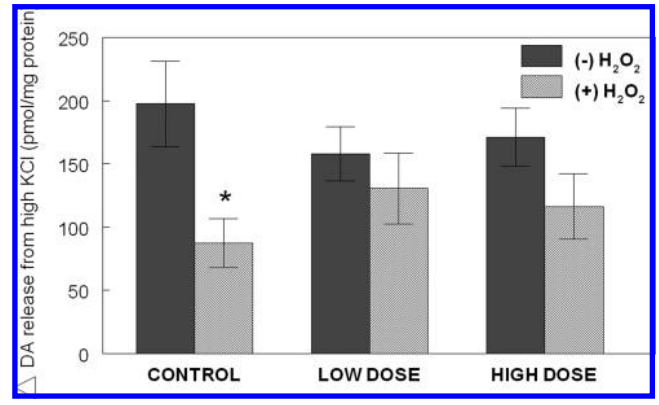

Figure 5. Oxotremorine enhancement of DA release from striatal slices (change in pmol/mg protein, mean ± SEM) obtained and prepared from animals maintained on the control, low-dose pterostilbene (0.004%), and high-dose pterostilbene (0.016%) diets, under basal levels ($-H_2O_2$) and under conditions of OS ($+H_2O_2$, 50 μM) treatment. The asterisk indicates a difference in DA release between non-H_2O_2-treated and H_2O_2-treated striatal slices for each diet group (*$p < 0.01$).

DA release (oxotremorine-enhanced striatal K^+-ERDA) was significantly different among the groups, depending on the diet and H_2O_2 treatment [$F(5,58) = 2.34$, $p < 0.05$] (**Figure 5**). Under basal conditions, there were no differences among the diet groups ($p > 0.05$). However, DA release was significantly reduced in the H_2O_2-treated control diet group as compared to the non-H_2O_2-treated control diet group ($p < 0.05$), whereas no differences were seen following H_2O_2 treatment in the rats fed either low- or high-dose pterostilbene. Therefore, pterostilbene protected against the decrease in DA release following an oxidative stressor.

DISCUSSION

As can be seen from the results, in comparison with the whole BB, it appeared that pterostilbene, pterostilbene glucoside, resveratrol trimethylether, and piceatannol (rv4−7) were the most potent of the compounds in antagonizing the deleterious effects of DA on recovery (calcium flux) in the M1AChR following oxotremorine-induced depolarization. These findings are similar to those that we have reported previously in this model using various fruit extracts [e.g., 2 mg/mL BB, 2 mg/mL black currant, 2 mg/mL boysenberry, 0.5 mg/mL dried plum, 0.5 mg/mL strawberry, 1 mg/mL cranberry, or 0.05 mg/mL grape juice (*29*)]. Importantly, the effects of rv4−7 were seen at much lower concentrations (10 μg/mL).

While specific structural mediators were not assessed in this study, data from this set of stilbenes suggest that the C-3 and C-5 OCH_3 may be important in the protective effects regardless of the other groups that may be present such as OH or O-Glu (see **Figure 1**, rv4−rv6). Additionally, it appears that *ortho*-hydroxyl groups in ring B (rv7) are also protective. Whether these can act as a "free radical sink" or specific modulators of stress signaling remains to be determined. Note also that a recent study (*33*) has indicated that the pharmacokinetics of pterostilbene compounds are very similar to those seen with resveratrol, with similar nonrenal excretion rates and increases in the glucuronidated form. They found that pterostilbene gets metabolized into pterostilbene-glucuronide. The serum concentrations of pterostilbene rapidly decline in the first hour, representing the distribution phase, followed by steady elimination up to 6 h; after 6 h, no pterostilbene was detectable in the serum. The serum half-

life of pterostilbene was determined to be 1.73 ± 0.78 h. Urine samples showed the presence of both the free pterostilbene and the glucuronidated metabolite. The glucuronidated metabolite appeared to be mostly excreted in the urine by 12 h postdose, while the amount of pterostilbene (unconjugated) excreted in the urine steadily increased even at 120 h postdose. The half-life of free pterostilbene in the urine was determined to be 17.3 ± 5.6 h. The authors thought that the discrepancy between the serum and the urine half-life was due to underestimation of the overall half-life of pterostilbene due to assay sensitivity limitations (*33*). However, the brain metabolism of pterostilbenes has not been studied, nor have differences between the various forms utilized here. It is possible that pterostilbene does not accumulate in the brain but rather irrigates it.

It is well-known that muscarinic receptors are important in a variety of parameters including memory, APP processing, and vascular functioning (*29*). OS and age-sensitive deficits in MAChR may result in the cognitive, behavioral, and neuronal aberrations observed in aging that are exacerbated in Alzheimer's disease (AD) and vascular dementia (VaD). Therefore, the finding in experiment I that some stilbenes, particularly the pterostilbene, could alter the M1 sensitivity in aging is extremely critical and may provide starting points for the development of future treatments or preventatives.

This theory has some support in the findings from observed responses in the senescent animals that were given pterostilbene supplementation. As shown in the results, the pterostilbene-supplemented animals showed improved cognitive performance as compared to those given the control diet, especially in the high-dose pterostilbene group in tests that assessed working memory. Pterostilbene also increased the sensitivity of the striatal muscarinic receptors such that greater responses (oxotremorine-induced DA release) were seen in the supplemented groups than in the control group. Age-related alterations in striatal DA release have been shown to be related to spatial memory as well as motor function (*3, 34*). Interestingly, it also appeared that there was a significant correlation between the hippocampal pterostilbene levels and the working memory performance. The findings with respect to behavior are similar to those seen previously in both cell (*29*) and animal models with BB (*12, 13*), suggesting that pterostilbene may increase muscarinic receptor sensitivity and calcium buffering to improve cognitive performance in the aged animals. Moreover, we have also shown in a previous study (*35*) that the greater the number of hippocampal or cortical anthocyanins that were found in animals fed a diet containing 2% BB-supplemented diet, the better the performance on the MWM.

The mechanism of this protection may reflect decreased oxidant effects as was observed in the M1-transfected COS-7 cells. The exact nature of this protection (free radical scavenging or reductions in stress signaling by pterostilbene) requires further study. However, previous research has suggested that resveratrol may enhance sirtuin 1 (Sirt1). Sirt1 has been extensively studied and has been shown to be associated with lifespan extension, trigger lypolysis, and decrease PPAR-γ (*36*). It is becoming increasingly clear, however, that a resveratrol derivative (pterostilbene) may be a powerful tool in forestalling or preventing the deleterious effects of neuronal and behavioral aging and subsequent development of neurodegenerative disease.

Resveratrol has been shown to protect neuronal function in a number of models (e.g., stroke; see ref *37* for a review), and findings from several studies provide evidence that resveratrol can be useful in protecting against downstream activation of inflammatory- and ROS-generated stress increases (see ref *26* for a review). While the upstream signaling mechanisms that may be involved in the resveratrol-induced protection against oxidative and inflammatory stressors are not fully known, it appears that the resveratrol effects may partially involve Sirt 1 inhibition of Nf-κB, FOXO, and p53 (*38*).

Additionally, there is some indirect indication from studies in our laboratory that BB and Concord grapes, which do contain resveratrol albeit in low amounts, may produce their beneficial effects by reducing stress signaling. One of the important stress signals is nuclear factor κ B (NF-κB), and it has been shown that aged male Fischer 344 rats fed a BB-supplemented diet showed reductions of age-induced increases in NF-κB expression as compared to those of aged nonsupplemented controls in the frontal cortex, hippocampus, and the striatum (*30*). Similar decreases in NF-κB by BB supplementation were produced in animals (4 month old rats) given intrahippocampal injections of kainic acid (KA) (*39*). KA is an excitotoxin that produces neuronal lesions and induces an inflammatory response in the brain. Gene expression analysis revealed that BB supplementation normalized NF-κB to control levels and reduced the expression of the cytokines interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) in the hippocampus. BB supplementation also increased the expression of the neuroprotective trophic factor insulin growth factor-1 (IGF-1) in KA-injected animals, suggesting that BB exerts its effect through different cascades with respect to inflammation and neurotrophic events (*39*). These alterations in stress signaling were associated with enhanced behavioral performance (MWM) and reduced microglial activation.

The findings with respect to microglial activation were also seen in in vitro mouse micoglial (BV2) cells treated with BB extract and exposed to lipopolysaccharide (LPS), as they also showed decreases in stress-induced signaling. The study found that incubation with BB significantly and dose dependently inhibited the production of nitrite (a stable metabolite of nitric oxide) in LPS-conditioned media. This reduction was accompanied by a decline in the mRNA and protein expression of inducible nitric oxide synthase (*40*). Furthermore, the pro-inflammatory cytokines (IL-1β and TNF-α from LPS-conditioned media) were reduced in a dose-responsive manner. Intracellular ROS levels were also attenuated by BB treatment (*40*).

It should be noted, however, that there are numerous additional compounds contained in berries such as proanthocyanidins, elagitannins, and anthocyanins that may also have beneficial effects and have been shown to protect against DA or amyloid β-induced decrements in recovery in hippocampal cells (*41*). We have found that BB fractionation products (e.g., anthocyanins and high and low molecular weight proanthocyanidins) show considerable differences in protective ability against DA in primary hippocampal neurons. In fact, some fractionation products (high and low molecular weight proanthocyanidins) were shown to enhance reactive oxygen species (*41*). We are investigating these additional polyphenols and are also assessing the effects of the rv4−7 stilbenes on neuronal signaling in our animal and cell models.

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