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Phytic acid as a potential treatment for Alzheimer's pathology: evidence from animal and *in vitro* models

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Abstract

Alzheimer's disease (AD) causes progressive age-dependent cortical and hippocampal dysfunctions leading to abnormal intellectual capacity and memory. We propose a novel protective treatment for AD pathology with phytic acid (inositol hexakisphosphate), a phytochemical found in food grains and a key signaling molecule in mammalian cells. We evaluated the protective and beneficial effects of phytic acid against amyloid beta pathology in MC65 cells and the Tg2576 mouse model. In MC65 cells, 48–72-hour treatment with phytic acid provided complete protection against amyloid precursor protein-C-terminal fragment-induced cytotoxicity by attenuating levels of increased intracellular calcium, hydrogen peroxide, superoxide, beta amyloid oligomers, and moderately up-regulated the expression of autophagy (beclin-1) protein. In a tolerance paradigm, wild type mice were treated with 2% phytic acid in drinking water for 70 days. Phytic acid was well tolerated. Ceruloplasmin activity, brain copper and iron levels and brain superoxide dismutase and ATP levels were unaffected by the treatment. There was a significant increase in brain levels of cytochrome oxidase and a decrease in lipid peroxidation with phytic acid administration. In a treatment paradigm, 12-month old Tg2576 and wild type mice were treated with 2% phytic acid or vehicle for 6 months. Brain levels of copper, iron, and zinc were unaffected. The effects of phytic acid were modest on the expression of APP trafficking-associated protein AP180, autophagy-associated proteins (beclin-1, LC3B), sirtuin 1, the ratio of phosphorylated AMP-activated protein kinase (PAMPK) to AMPK, soluble A β 1-40, and insoluble A β 1-42. These results suggest that phytic acid may provide a viable treatment option for AD.

Keywords

antioxidant; autophagy; amyloid precursor protein; beclin-1; beta amyloid; phosphorylated AMP-activated protein kinase; sirtuin 1

Introduction

Alzheimer's disease (AD) causes progressive cortical and hippocampal dysfunctions with age and impacts both intellect and memory in affected patients. The amyloid cascade hypothesis suggests that a 4-kDa amyloid beta (A β) peptide is the fundamental cause of AD [1–7], with selective loss of neuronal subpopulations including cholinergic fibers, proliferation of reactive astrocytes and microglia, all progressively leading to mild cognitive impairment (MCI) and ultimately AD [1–3]. Oxidative stress has been suggested as one of the earliest manifestations of MCI and AD pathology [8]. The mainstream research in AD appears to focus on two main therapeutic strategies— prevention of A β accumulation and promotion of A β clearance [8–10]. Currently there are four Food and Drug Administration-approved drugs available for use in patients with AD, but only as symptomatic treatments. There is a huge unmet medical need for therapies that are safe and effective.

In this study, we investigated a novel protective treatment for AD pathology with phytic acid (PA, inositol hexakisphosphate). PA is structurally a myo-inositol sugar ring attached to 6 phosphate molecules. It is found naturally and ubiquitously, as a phosphate-storage phytochemical in unprocessed whole food grains, vegetables, and fruits, and as a key signaling molecule in mammalian cells. The Ca/Mg form of PA found in most plants is known as "Phytin" with its salt form known as "Phytate." Although PA is often described as a metal chelator [11], growing literature indicates that PA influences multiple processes, including antioxidant functions [11–13], anti-apoptotic effects [12], clathrin-coated endocytosis [14–16], DNA repair [17], and mRNA export from the nucleus [18]. Phytic acid also lowers serum cholesterol and triglycerides [19]. These studies suggest that PA possesses much broader functions than simply the originally-presumed metal binding properties.

We hypothesized that some of PA's properties may have a favorable effect on brain aging and AD pathology, by way of: 1) mimicking caloric restriction, 2) promoting autophagy, and 3) modulating clathrin-coated endocytosis of APP and its cleavage products. Caloric restriction (CR) mimetics like resveratrol (which, like PA, is an antifungal in plants) are under study for anti-aging effects [20–22], and resveratrol is already in clinical trials for AD. We hypothesized that PA is a CR mimetic because it has protective functions similar to resveratrol in plants [23–25]. Both CR and resveratrol act to increase levels and activation of SIRT1, an NAD-dependent class III histone and non-histone protein deacetylase which is decreased in AD and other pathologies and extends lifespan in yeasts, worms, flies, and mice [24,26–39]. Since resveratrol acts by way of sirtuin 1 activation, we tested the effect of PA upon SIRT-1 in cell culture and in animal models of AD.

Autophagy is a self-cleaning cellular housekeeping mechanism that plays an important role in numerous pathologies [40]. A heterozygous deletion of the autophagy marker beclin-1 in Tg2576 mice increases intraneuronal A β accumulation, extracellular A β deposits, and neurodegeneration [41], suggesting that autophagy plays a key protective role against AD. Indeed one of the main functions of autophagy is to regulate mitochondrial function by enzymatic degradation of dysfunctional mitochondria and by clearing misfolded proteins within the cell [42–44]. Our previous study in Tg2576 mice provides strong evidence for mitochondrial deposition of A β species and generation of free radicals [45,46]. SIRT1 loss parallels the loss of proteins associated with autophagy (beclin-1 and LC3B) and oxidative stress [47,48], strongly suggesting that SIRT1 regulates autophagy in the brain, and this regulation may be a pathogenic mechanism and therefore a therapeutic target in AD [40,48].

Some recent studies, however, question the ability of resveratrol to activate SIRT1 [49,50]. A SIRT1-independent mechanism mediated predominantly by the metabolic sensor protein

phosphorylated AMP-activated protein kinase (PAMPK) in neurons has been suggested to play an important role in A β peptide metabolism [51,52]. Therefore, we investigated the effects of PA on the PAMPK to AMPK ratio in Tg2576 mice. Furthermore, it has been previously shown that PA suppresses AP180/AP3 protein [14,15], a clathrin-coated vesicle protein, implicated in the regulation of APP trafficking and A β generation [16]. Thus, we sought to clarify the effect of PA on this protein in Tg2576 mice.

The main objective of this study was to clarify the beneficial or harmful effects of PA in the context of AD pathology and determine if clinical trials of phytic acid for AD are justified. The mechanisms considered include metal chelation, SIRT1 activation, autophagy activation, PAMPK activation, and regulation of endocytosis. We employed one *in vitro* (human neuroblastoma/MC65 model of A β neurotoxicity [53–58]) and one animal model (Tg2576 [59]) of AD to test these possibilities. Our *in vitro* model, MC65 cells, are stably transfected with an inducible APP-C99 construct and conditionally express a fusion protein composed of the amino-17 and carboxyl-99 residue-containing fragment of APP [55,56]. Expression is controlled by a tetracycline-responsive promoter whose activity is repressed in the presence of tetracycline. Removal of tetracycline first leads to expression of the C-terminal APP fragment and subsequent processing of this fragment into A β species followed by accumulation of intracellular A β oligomers, and precipitous cell death in 3–4 days [53,55,56]. Our data indicates that PA protected MC65 cells against intraneuronal A β oligomers while up-regulating SIRT1 and the autophagy-associated protein beclin-1. In the animal model, chronic PA administration had no effect on brain levels of metals (copper, iron, zinc), but modulated oxidative stress/ mitochondrial function, autophagy (beclin-1, LC3BI), SIRT1, PAMPK, and endocytosis associated protein AP180 protein, while producing a modest reduction in A β and plaques in Tg2576 mice.

Materials and Methods

Materials

Cell culture media, L-glutamine, and trypsin/EDTA were obtained from Invitrogen (Carlsbad, CA); fetal bovine serum was from Atlanta Biologicals (Lawrenceville, GA); 6E10 monoclonal antibody was obtained from Covance Laboratories (Princeton, NJ); Phytic acid sodium salt hydrate from rice and anti-assembly protein AP180 were from Sigma-Aldrich (St. Louis, MO). Anti- β -tubulin, anti-beclin-1, anti-LC3B, anti-phospho-AMPK α , and anti-AMPK α were from Cell Signaling Technologies, Inc (Danvers, MA); Anti-SIRT1 was from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Unless otherwise mentioned all other chemicals were obtained from Sigma.

Cell culture

MC65 cells were cultured in 75-cm² TC flasks or 100×20 mm petri dishes containing MEMalpha growth medium supplemented with fetal bovine serum (10%), L-glutamine (2 mM) and tetracycline (1 μ g/ml), and maintained in a tissue culture incubator at 37°C and in the presence of 5% CO₂ as described [57,60,61]. We limited our assays to early cell passages.

Cell survival assay

Cell survival was determined by the MTS assay (Promega) simultaneously with calcium assays (described below) on the same cell passages, following manufacturer's protocol and published methods [58]. Cells were harvested from TC flasks at 90% confluence using 0.05% trypsin/EDTA, washed twice with PBS, and plated into 96-well plates at a density of 15×10³ cells/well in OPTIMem medium in the presence (Tet+) or absence (Tet-) of tetracycline (1 μ g/ml) and vehicle, or phytic acid (100 μ M). Cells grown under Tet- condition

usually die after approximately 3–4 days in culture in association with expression of endogenously produced A β oligomers, but the cells grown under Tet+ condition fully survive during this period [58]. The wells containing growth medium without cells served as background control, and Tet+ with vehicle served as a positive control. Each treatment was replicated in 3–6 wells and treatments were applied for periods of 72 hours in each experiment. Absorption was measured at wave length 490 nM using a Spectra Max PLUS plate reader (Molecular Devices Inc). Percent cell survival in different treatments was determined relative to cell survival in Tet+ treatment. Experiments were repeated at least three times.

Intracellular calcium assay

Intracellular Ca²⁺ levels were determined using the Fluo-4 NW Ca²⁺ Assay Kit following the manufacturer's protocol (Molecular Probes) and published methods [62]. Briefly, MC65 cells at a density of 15×10³ cells/well were grown in black, 96-well culture plates with (Tet +) and without (Tet-) tetracycline in either vehicle or phytic acid (100 μ M). The same set of treatments presented in the MTS assay described above were included in the Ca²⁺ assay, and both assays were performed in parallel from the same batch of cells. After the treatment period, Fluo-4 NW dye (100 μ l) containing 2.5 mM probenecid was added directly into each well to prevent the dye efflux from the cells, and the plate was incubated in a TC chamber at 37°C for 30 min, followed by another 30 minutes at the room temperature. Intracellular Ca²⁺ levels for each treatment and control were measured in terms of relative fluorescence units in live cells (RFU) for 10 min at room temperature using the Victor³ 1420 Multilabel Counter (Perkin Elmer) with specific excitation (485/20 nM) and emission (535/20 nM). The fluorescence values of different cultures may be variable depending on the cell density, the time of exposure to the probe, cell loading with the fluorescent dye, and fluctuations in room temperatures. Therefore, special care was taken to calibrate fluorescent values of all other treatments within each experiment relative to the Tet+ treatment; this strategy eliminated errors associated with absolute measurements of fluorescence and assigned relative values meaningful for comparing treatments across different experiments.

Determination of free radicals

Hydrogen peroxide (H₂O₂) levels and intracellular superoxide levels were measured following published methods [60]. Briefly, H₂O₂ levels were determined in the cell media collected from cells treated with Tet+, Tet-, and Tet- PA for 48 hours using the Bioxytech H₂O₂-560TM Quantitative Hydrogen Peroxide Assay (Oxis International, Inc., Foster City, CA) as per manufacturer's instructions. Intracellular superoxide radicals were determined in the cell lysates from the same treatments using the nitroblue tetrazolium (NBT) assay as previously described [63]. Briefly, NBT (Sigma, St. Louis, MO) was added to the cell media at a final concentration of 1 mg/ml and incubated for 3 h at 37°C in a humidified atmosphere containing 5% CO₂. The NBT-containing medium was removed, the cells washed twice with warm PBS followed by methanol, then air-dried. Intracellular blue formazan particles were dissolved by adding 120 μ l/well 2M potassium hydroxide followed by 140 μ l/well DMSO. The absorbance of dissolved NBT was measured at 620 nm using a Spectra Max Plus.

Cell harvesting, protein quantification and western blotting

We followed previously published methods for protein quantification and western blotting [58]. Briefly, MC65 cells were grown under Tet- and Tet+ condition and Tet-PA (100 μ M) in 100×20 mm petri dishes in 10 ml OPTIMem. After 72 h treatment, the medium was removed, and the cells were washed with PBS. Cells were lysed in 300 μ l of lysis buffer (Tris, 62.5 mM, pH 6.8; sodium dodecyl sulfate, 2%; glycerol, 10%) containing protease inhibitor cocktail set VII (Calbiochem, San Diego, CA) without DTT or bromophenol blue

and then scraped and collected into 1.5 ml Eppendorf tubes. The cells were sonicated on ice for 10 sec, boiled at 95°C for 5–10 min, allowed to cool to room temperature, and centrifuged at 14,000 rpm for 5 min. The supernatant was transferred to a new tube, the protein quantity in each sample was determined using BCA Assay (Pierce), and then lysates were stored at –20°C until further use. Cell lysates from different treatments were normalized to the same final concentration of total protein using the lysis buffer, and adjusted to a final concentration of 50 mM DTT and 0.01% bromophenol blue. A 20 µg protein was loaded onto a 10–20% or 16% acrylamide gels containing tricine (Bio- Rad) for fractionation and the proteins transferred to nitrocellulose membranes. The membrane was probed with 6E10 mouse MAb, anti-AP180 mouse MAb, anti-SIRT1 or anti-beclin-1 rabbit polyclonal antibodies; or the loading control anti-β-tubulin antibody. Following treatment with appropriate secondary antibody, the membrane was exposed to enhanced chemiluminescence solutions (Invitrogen), and visualized using the Molecular Imager Gel Documentation System and quantified using Quantity One Software (Bio-Rad).

Animal studies

Two cohorts of mice were used in this study. In the tolerance cohort, all mice were C57BL/6J wild types. In the treatment cohort, Tg2576 mice were generated from a breeding pair originally provided by Dr. Karen Hsiao-Ashe (Mayo Clinic, MN). The transgene was carried on a genetic background of C57BL/6J X SJL. Following weaning, litters were genotyped and group housed at 4–5 animals per cage until the commencement of experiments. All of the mice in this study were female, because males are prone to marked aggression towards cage mates. Mice were maintained in a climate-controlled environment with a 12-hr light/12-hr dark cycle, and fed AIN-93M Purified Rodent Diet (Dyets Inc, Bethlehem, PA). Diet and water were supplied *ad libitum*. All procedures were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Portland VA Medical Center.

Treatments

In the initial tolerance paradigm, female wild type mice (age 9 months) were treated for 70 days with either vehicle/VEH (distilled drinking water, N=10) or 2% PA dissolved in drinking water (N=10). PA was well tolerated as indicated by the fact that average weekly body weights (an indirect measurement of toxicity) were similar for vehicle and PA-treated animals. In the second treatment paradigm, Tg2576 and wild type mice were randomized to receive either 2% PA or vehicle in their drinking water. PA doses were adjusted on a weekly basis in order to assure that body weight did not drop more than 10%. In the treatment paradigm, 12 month-old mice were treated for six months as follows: 22 wild type mice (vehicle N=12, PA N=10) and 15 Tg2576 mice (vehicle N=7, PA N=8). Again, PA was well tolerated in these animals as indicated by their average monthly body weights.

Ceruloplasmin (Cp) assay

In the tolerance paradigm, blood was collected from the saphenous vein every 2–3 weeks from 4–6 animals per treatment group and plasma Cp activity measured using a modified version of the oxidase method [64,65]. Briefly, for each mouse, 7.5 µl of plasma and 75 µl of 100 mM sodium acetate (pH 6.0) were added in duplicate to a 96-well plate and incubated 5 min at 37°C. Aqueous o-dianisidine dihydrochloride solution (30 µl, 2.5 mg/ml, pre-incubated to 37°C) were added to each well and the plate incubated at 37°C for 5 min. An aliquot (56.3 µl) of the mixture was immediately transferred to empty wells. The solution remaining in the original wells (t=5 min) was quenched with 150 µl 9M sulfuric acid. The plate was incubated for an additional 15 min at 37°C, then the secondary wells (t=20 min) were quenched. Absorbance was read at 540 nm and the enzymatic activity of Cp determined from the following equation: Activity (U/ml) = (Abs_{t=20}–Abs_{t=5})(416)

Tissue homogenates and ELISA

Following behavioral analysis, animals were euthanized by CO₂ inhalation and cervical dislocation. Blood and liver samples were collected and frozen and processed as described [65]. Brains were quickly removed and divided. The anterior 3 mm of bilateral frontal cortex and a 1 mm section behind the frontal cortex (“frontal slice”) were dissected and frozen for beta amyloid ELISA and brain metal analysis, respectively. The right hemisphere was immersion fixed in 4% formaldehyde in phosphate buffered saline for histochemical analysis. The contralateral hemisphere was frozen for determination of copper-dependent enzyme activity.

Frontal cortex tissue was homogenized in tris buffered saline (TBS) containing protease inhibitor cocktail (EMD Chemicals, Gibbstown, NJ) and 1% Triton X-100 (Roche, Indianapolis, IL), then centrifuged at 40,000 x g for 30 min at 4°C. The supernatant from this step was labeled “soluble fraction.” The pellet generated in this step was homogenized in TBS, protease inhibitors, and 1% Triton X-100, then centrifuged at 40,000 xg for 30 min at 4°C. The supernatant from this step was discarded as a wash. The pellet was homogenized in 70% formic acid (Sigma), incubated for 1 h at room temperature, and centrifuged at 40,000 xg 30 min. The resulting supernatant was collected and labeled “insoluble fraction.” Aβ1-40 and Aβ1-42 were measured in the soluble membrane and insoluble fractions using commercial ELISA (Invitrogen, Camarillo, CA) according to the manufacturer’s instructions.

Tissue metal assays

Brain iron and copper levels in the tolerance cohort were measured using atomic absorption spectroscopy (AAS). Plasma and brain copper, iron, and zinc levels in the treatment paradigm were measured using both AAS and inductively coupled plasma mass spectroscopy (ICP-MS), respectively. Tissues (approximately 20 – 100 mg wet weight) were digested using a modified version of previously published method [66]. Briefly, samples were incubated overnight in 0.5 – 2 ml of pure HNO₃ (metals grade, Thermo-Fisher), centrifuged at 12,000 x g for 5 min and the supernatants sequentially diluted to yield 8 ml of solutions containing 35% HNO₃. AAS measurements were carried out using an AA6650 spectrometer (Shimadzu, Columbia, MD) equipped with a graphite furnace and ASC6100 autosampler. Samples were diluted with 2% nitric acid to be in the linear absorption range of the calibration curve (1–10 ppb (ng/L, generally between 100–1000)). 20 µl of each sample was injected up to 3 times depending to achieve average data with a low standard deviation. Copper and iron concentrations were derived by comparing the absorption of the samples with a 6 point calibration curve (0, 2, 4, 6, 8, 10 ng/g). ICP MS analysis was performed using an Agilent 7700x system equipped with an ASX 250 autosampler. The system was operated at a radio frequency power of 1550 W, an argon flow rate of 15 L/min, carrier gas flow rate of 1.02 L/min, and helium gas flow rate of 4.3 ml/min (when applicable). Data were quantified using a 5-point calibration curve (0, 1, 10, 100, 1000 ng/g) with external standards for Fe, Cu, and Zn. For the analysis of plasma samples, 30 µl of each sample was diluted in 1.5 ml 1% HNO₃ (Metals grade, Fisher) and measured in He mode to remove interferences. For the analysis of tissue samples, 100 µl of digested tissue solution was diluted into 4 ml of 1% HNO₃ (Metals grade, Fisher). To prevent protein deposition and minimize nebulizer clogging as well as cross contamination the sample probe was washed with 1% HNO₃ for 30 seconds followed by 30 seconds normal wash after each sample.

Assays for copper-dependent proteins, ATP and Lipid peroxidation

Copper-dependent enzyme activity was measured in left hemisphere homogenates (soluble fraction) from the tolerance cohort following our published methods [60,67–69]. Cytochrome c oxidase activity was determined by measuring the oxidation of

ferricytochrome c using the Cytochrome c Oxidase Assay Kit (Sigma, St. Louis, Mo) following manufacturer's instructions. Cytochrome c oxidase and its inhibitor sodium azide were, respectively, used as positive and negative controls. Superoxide dismutase (SOD) activity was determined using the NWLSS Superoxide Dismutase Activity Assay (Northwest Life Science Specialties, LLC, Vancouver, WA) according to manufacturer's instructions. This assay is specific for Cu/Zn, Mn and Fe isoforms of SOD, however Cu/Zn SOD is the most common cytoplasmic form of this enzyme.

ATP levels were measured in the brain homogenates of mice in the tolerance cohort using the ATPlite 1step Luminescence ATP Detection Assay System (Perkin Elmer, Boston, MA) as per manufacturer's instructions and our published methods [60]. Briefly, brain homogenate was mixed with an equal volume of 20% trichloroacetic acid (TCA) and centrifuged for 10 min at 10,000 xg at 4°C. The supernatant was diluted 50% with phosphate buffered saline before adding equal volume of ATP-lite substrate solution. The plate was shaken for 2 min and luminescence read on a Victor³ Multilabel Reader. ATP levels were quantified from an ATP standard curve. ATP levels were normalized to total protein in the homogenate.

Oxidative stress in the brain homogenates of the tolerance cohort was measured by Malondialdehyde (MDA) Colorimetric Assay Kit, an indicator of lipid peroxidation following the manufacturer's protocol (Oxis International, Inc.) [60].

Western blot analysis of tissue samples

Protein expression in the soluble fraction from the treatment cohort was determined by western blot analysis. Briefly, protein quantity in each sample was determined using the BCA assay (Pierce). Protein (20 µg) was loaded onto 4–12% Bis-Tris gels, then separated and transferred onto nitrocellulose using the NuPage® electrophoresis system (Invitrogen). The membrane was probed with antibodies to anti-AP180, anti-beclin-1, anti-LC3B, anti-SIRT1, anti-phospho- AMPK α , and anti-AMPK α , and anti- β -tubulin (Sigma), treated with appropriate secondary antibody, exposed to enhanced chemiluminescence solutions (Invitrogen), visualized using the Molecular Imager Gel Documentation System and quantified using Quantity One Software (Bio-Rad).

Statistical Analysis

Statistical significance of the treatments, treatment durations, and their interaction effects were determined for cell survival and intracellular calcium using one-way or two-way analysis of variance, and with appropriate t-tests. We also conducted the Bonferroni post-hoc test (for comparisons between two samples at a time. The treatment difference is considered significant if $P \leq 0.05$. All statistical analyses were performed using GraphPad Prism 5 software.

Results

Cell culture studies

Effects of phytic acid on free radicals, intracellular calcium, and survival in MC65 cells—Fig.1 shows levels of hydrogen peroxide (H_2O_2), and superoxide (O_2^-), intracellular calcium, and cell survival in MC65 cells cultured in the presence (Tet+) or absence of tetracycline (Tet-), and in the presence of phytic acid in Tet- conditions (Tet-PA; 100 µM). In all instances the measurement in Tet+ was considered 100%, and to which all other treatments were compared. At 48-hour treatment, both H_2O_2 (15% increase; $p \leq 0.05$; Fig. 1A) and O_2^- (2.4-fold increase; $p \leq 0.001$; Fig. 1B) levels increased significantly in Tet- compared to Tet+. PA treatment significantly decreased H_2O_2 levels ($p \leq 0.001$; Fig 1A)

and maintained superoxide at Tet⁺ levels (Fig. 1B). After 72 hour treatment, intracellular calcium levels increased by nearly 5-fold ($p \leq 0.01$; Fig. 1C) and cell survival decreased significantly (80% decrease; $p \leq 0.01$; Fig. 1D) in Tet-compared to Tet⁺ treatment. Phytic acid (Tet-PA) provided near-complete protection against APP-C99-mediated toxicity as shown by greatly attenuated intracellular calcium (Fig. 1C) and superior cell survival (Fig. 1D).

Effect of phytic acid on A β oligomers in MC65 cells—The effect of phytic acid on A β oligomer expression was determined using western blot analysis of MC65 cells 72 hours after tetracycline withdrawal (Fig. 2A & 2B). Products of the transfected C99 construct are located at the low-molecular weight portion of the gel image, especially in Tet⁻ treatment (Fig. 2A). Endogenous APP appears to be present in all three treatments in the higher-molecular weight range of the blot. A β oligomers between approximately 16 and 44 kDa (4-, 6-, and 8-mers as shown in Fig. 2B) were attenuated significantly ($p \leq 0.05$) by PA treatment. The C-terminal 99 amino acid fragment (C99) and an 8-kDa band previously defined as either a dimer (P8) [58] or a combination of unknown protein species with some A β species [53], were unaffected by PA treatment.

Effect of phytic acid on autophagy and sirtuin proteins in MC65 cells—Figs. 2C & 2D show expressions of the autophagy protein beclin-1 and SIRT1 protein, respectively, in MC65 cells following 48-hr exposure to the following four treatments: Tet⁺, Tet⁻, Tet⁻ with resveratrol (RSV; 5 μ M), and Tet⁻ with PA (100 μ M). Resveratrol, a phytochemical known to up-regulate SIRT1 expression and promote longevity in numerous organisms [24, 70], served as a positive control. Beclin-1 expression in RSV and PA-treated cells increased by 1.4 and 1.8-fold, respectively, over the base-level expression in Tet⁺ cells and the effect was significant ($p \leq 0.05$) in the latter (Fig. 2C). SIRT1 expression in RSV and PA-treated cells remained similar to Tet⁺ levels (Fig. 2D).

Animal studies

Tolerance paradigm

Effects of phytic acid on animal body weight, ceruloplasmin (Cp) activity and metal ions in WT mice: Animal body weights taken before and during the 10-week treatment period are shown as weekly averages for each group of vehicle (VEH) and 2% phytic acid (PA) treated wild type female mice in Fig. 3A. Body weights for VEH and PA-treated groups remained similar during most of the treatment period. Fig. 3B shows average Cp activity in the serum of mice treated with VEH or 2% PA. Cp is the main copper transporter in the serum and Cp activity represents an indirect measure of freely available copper [71]. Cp activity was similar in vehicle and PA-treated animals (Fig. 3B). In addition, copper and iron levels were measured in frontal cortex tissue using atomic absorption spectroscopy. There was no significant difference in brain copper and iron levels between vehicle and PA-treated animals (Fig 3C). The activity of the copper-dependent anti-oxidant enzyme Cu/Zn superoxide dismutase (SOD) levels also did not differ significantly between vehicle and PA-treated animals (Fig 3D). These results suggest that when animals are fed a nutritional diet, PA may not have any adverse effects on copper- or iron-dependent metabolisms.

Effects of phytic acid on ATP, COX and MDA levels in WT mice—ATP levels did not differ significantly between vehicle and PA-treated animals (Fig 3D). Levels of cytochrome oxidase (COX), an index of mitochondrial function that declines in AD and aging pathologies [67], significantly ($p < 0.01$) increased (Fig 3D), and levels of malondialdehyde (MDA), an indicator of lipid peroxidation, significantly reduced (Fig 3E) in the frontal cortex homogenates of PA-treated animals. These results suggest that PA

treatment promoted mitochondrial function and attenuated free radical formation in mouse brains.

Treatment Paradigm

Effects of phytic acid on ceruloplasmin (Cp) activity, metal ions and A β species in Tg2576 mice—Body weights (g) of mice measured over 30 times just before and during the treatment period are presented as monthly averages for VEH and PA treated animals in Fig. 4A. No consistent difference exists between animal groups treated with either VEH or PA. Fig. 4B shows terminal plasma Cp activity for four groups of mice in the treatment paradigm. Cp activity was significantly lower in both wild type (mean difference=32.4; $p \leq 0.05$) and Tg2576 (mean difference=31.1; $p \leq 0.01$) mice treated with phytic acid than vehicle-treated animals. Fig. 4C and Fig. 4D show ICP-MS measurements of copper, iron, and zinc in plasma and frontal cortex homogenates, respectively, from the same four groups of mice. Plasma copper levels were significantly lower (Mean difference=144 $\pm$ 57; $p \leq 0.05$) in PA-treated Tg2576 mice than vehicle-treated mice and iron levels were significantly higher (Mean difference=3770 $\pm$ 1577; $p \leq 0.05$) in PA-treated wild type mice than vehicle-treated animals (Fig. 4C). All other plasma comparisons between vehicle and phytic acid-treated animals were non-significant. Interestingly, the levels of all three metal ions (Cu, Fe, Zn) remained similar across the four animal groups in the frontal cortices (Fig. 4D). These results further suggest that PA did not affect the status of brain metal ions when animals were fed a nutritionally balanced diet. Figs 4E, 4F, 4G show soluble A β 1–40, insoluble A β 1–40 and insoluble A β 1–42, respectively, in frontal cortex homogenates of the same four animal groups. A β levels in the WT mice were negligible and soluble A β 1–42 was undetectable in Tg2576 mice; hence the data are not presented. Tg2576 mice treated with PA showed lower but non-significant levels of soluble and insoluble A β 1–40 (Figs 4E & 4F) and insoluble A β 1–42 (Fig 4G) levels in the frontal cortices relative to vehicle-treated mice.

Effects of phytic acid on AP180, autophagy, SIRT1, AMPK, and PAMPK proteins in wild type and Tg2576 mice—Molecular effects of PA treatment on the expression of AP180, beclin-1, LC3BI & II, SIRT1, AMPK, and PAMPK proteins in four animal groups from the treatment paradigm were not statistically significant, hence the results are not presented. Although statistically non-significant, the western blot expression of AP180, an adapter vesicle protein indicative of APP trafficking, showed a slight trend of decline in the PA-treated animals compared to VEH-treated groups. There was a trend towards increased expression of two autophagy proteins, beclin-1 and LC3B (LC3B-II/I ratio), SIRT1 and PAMPK/AMPK ratio in the cortices of animals treated with PA. These results appear to suggest positive roles of SIRT1, PAMPK and autophagy in modulating AD pathology.

Discussion

Our study demonstrates that: (1) PA (100 μ M) has neuroprotective effects in a cell culture (MC65) model of APP-C99 cytotoxicity, (2) PA (2%) is well tolerated by wild type mice in terms of its effects on metal ions and oxidative stress in the brain, and (3) PA (2%) has modest anti-amyloid effects, as well as effects on novel therapeutic targets (SIRT1, PAMPK, autophagy and vesicle proteins) in the Tg2576 mouse model of AD.

Our *in vitro* results show that PA (100 μ M) treatment provided a complete protection against A β cytotoxicity in MC65 cells by attenuating increased intracellular calcium and reducing cellular H₂O₂ and O₂⁻ levels. Furthermore, PA drastically attenuated the production of intracellular A β oligomers in these cells. These results are consistent with those reported for

MC65 cells treated with antioxidant and protein chaperone treatments, which are also associated with markedly less aggregation to higher-molecular weight A β oligomers and preserved cell viability [57,58,72–74]. In addition, PA significantly up-regulates the expression of beclin-1, an autophagy protein essential for cellular housekeeping functions.

Data from the “tolerance” paradigm was important for evaluating the potential risks of phytic acid, which has often been described as an “anti-nutrient.” Phytic acid is available as several different salts; calcium phytate is considered by the Food and Drug Administration to be Generally Regarded As Safe [75,76] and Ca/Mg phytate is currently available without prescription as a food supplement, suggested to be administered orally on an empty stomach in a glass of water, devoid of food and micronutrients such as iron, copper, calcium, magnesium and zinc. However, the usefulness of PA in human and animal nutrition is controversial [77]. Some criticize PA for its “anti-nutrient” or metal chelation property, for it can bind to micronutrients such as iron, zinc, and copper and render them unavailable for animal nutrition [78]. Others have even favored breeding transgenic plants with low PA [78,79], but such a strategy in some plants has lead to increased susceptibility to fungal disease [23] or alterations in the accumulation of anthocyanin pigment or proteins in the seed [80,81]. The presumed anti-nutrient effect of PA may be more relevant to developing nations where diets are relatively deficient in micronutrients and PA may exacerbate this problem. On the contrary in developed nations like the United States, where the diet is predominantly meat-based and relatively well-balanced in terms of micronutrients, PA consumption is considered beneficial to public health [79]. In the tolerance paradigm, PA (2%) administered in the drinking water for 70-days was well tolerated, as indicated by similar average weekly body weights (an indirect measurement of toxicity) for vehicle and PA treated animals. The dose of 2% can be considered mild, as edible legumes, cereals, oil seeds, and nuts naturally provide 1–5% phytic acid in the diet [82]. In this study, all animals were fed AIN-93M Purified Rodent Diet, which contains undetectable level of phytic acid [76]. Our results clearly showed that a 70-day treatment with phytic acid modulated mitochondrial and antioxidant functions without adversely affecting metal (copper, iron) or ATP levels in brains of wild type mice. It appears that the metal chelating property of phytic acid may be of least concern under well-balanced dietary regimens.

Remarkably similar conclusions can be made from the findings of our treatment paradigm, where animal groups were treated with PA (2%) for 6 months. Although longer treatments with PA decreased plasma Cp activity by 33% and plasma copper by 18%, and increased plasma iron by 52–120%, the brain levels of copper, iron, or zinc were unaffected by PA treatment. Soluble A β 1-40 and insoluble A β 1-42 were slightly reduced in the frontal cortices of PA-treated Tg2576 mice relative to vehicle-treated animals. Furthermore, there was a trend towards down-regulation in the expression of AP180, a protein associated with APP trafficking in the vesicle-treated mice, and a moderate up-regulation in autophagy (beclin-1, LC3B), SIRT1, and the PAMPK/AMPK ratio. Based on these results, PA shows a promising trend for attenuating toxic effect of AD pathology. However, the administered PA dose (2%) was likely too mild. Given that intracellular PA could reach up to 100 μ M in mammalian cells [83] and that a 2% PA dose in drinking water is approximately equivalent to 45 μ M plasma concentrations assuming no first-pass metabolism, perhaps a much higher dose of PA starting as early as 2–3 months of animal age is expected to provide a preventive effect on the accumulation of A β plaques. In a previous study, 8.9% PA administered to female Fisher rats through diet for 23 weeks showed no signs of toxicity [84]. Therefore, it is possible to increase the dose without causing any adverse toxic effects.

Our study for the first time identifies potential novel functions of PA—a moderate up-regulation of SIRT1, PAMPK/AMPK ratio, and autophagy proteins. We propose a potential mechanism of protection by PA in the mouse brain as detailed in Fig. 5. PA attenuates

oxidative stress, which in turn may lead to increased SIRT1 function and an increased PAMPK/AMPK. This results in improved autophagy and mitochondrial functions. These events together may have multiple complementary effects in terms of reduced AP180 expression, reduced A β production, attenuation of intracellular calcium increases, and attenuated overall plaque pathology in PA-treated animals. Our *in vitro* studies largely corroborate our *in vivo* findings.

If these findings hold true for higher doses of administered PA, they will have consequences for future research and clinical trials: (1) the up-regulation of SIRT1 benefits not only AD pathology, but also other pathologies where PA modulates this protein; (2) induction of autophagy by PA will have unique implications on mitochondrial research, as autophagy is involved in increasing mitochondrial efficiency by removing dysfunctional mitochondrial debris and attenuating ROS production; (3) PA-induced SIRT1 may functionally imitate CR-induced SIRT1 to promote α -secretase activity by suppressing ROCK1 expression [34,85], and attenuating A β -oligomers (see our preliminary studies); (4) PA-induced up-regulation of the PAMPK/AMPK ratio attenuates mammalian target of rapamycin (mTOR) and promotes autophagy functions [51,52]. (5) PA may suppress AP180/AP3, a clathrin-coated vesicle protein, implicated in the regulation of APP trafficking and A β generation [16]; and (6) for clinical applications, PA is relatively inexpensive, highly tolerated, readily soluble in water, and accumulates several fold greater in the brain than plasma [86]—a scenario ideal for treating CNS disorders such as MCI and AD.

There is a caveat to the findings of this study. In Tg2576 mice, A β accumulation begins at approximately 6 months, well before the ages at which PA treatments started in our studies (9-months & 12-months of animal age). Thus, the results on A β proteins (perhaps for other proteins as well) are not as significant as we expected and the behavioral measurements by the Morris Water Maze were not significant. In addition the administered dose of phytic acid is relatively mild. Perhaps a much higher dose of PA treatment on a larger group of animals that would start as early as 2–3 months of animal age is expected to provide a preventive effect on the accumulation of A β plaques. We are currently exploring a preventive paradigm using PA in the Tg2576 model of AD.

It is important to emphasize that the effects of PA demonstrated here are distinct from those that have been attributed to its “backbone” and metabolite, inositol. Stereoisomers of myo-inositol (the non-phosphorylated backbone of PA) inhibit A β fibril assembly and protect neurons from A β -induced cytotoxicity *in vitro*. The stereoisomer scyllo-inositol inhibits A β aggregation, reduces soluble and insoluble A β , reduces plaque size and inhibits cognitive defects in a transgenic model of AD [87]. Scyllo-inositol (ELND005) has been tested in animals and has entered phase 3 clinical trials for AD by Elan Corporation, plc [87–89]. Recently, however, it had been shown to cause severe adverse drug effects in humans in two arms at doses 1 and 2 gram twice a day leading to death of 9 patients and discontinuation of both arms of this clinical trial. Interestingly, 6–7.4 g/day of phytic acid administered to patients did not have any adverse drug effects for up to 24 months [90]. Our study indicates that phytic acid works by independent mechanisms. Additional support for the hypothesis that phytic acid may have effects beyond those of inositol come from studies showing elevated brain levels of phytic acid in rats fed a high phytate diet, indicating that some unmetabolized phytic acid is delivered to the brain, in addition to other species of phosphorylated inositols [86,91].

Conclusions

This research was intended to determine if clinical trials of phytic acid for AD are justified. A clinical trial of an inexpensive, established, and well-tolerated agent would be of great

significance. Our study has demonstrated an excellent tolerability of phytic acid administered for up to 6 months and identified numerous beneficial effects of this phytochemical in both *in vitro* and *in vivo* studies. Although additional confirmation is required, this study appeared to have identified a novel calorie restriction mimetic, phytic acid, and established experimental paradigms for investigating specific mechanisms (SIRT1, PAMPK/AMPK ratio, and autophagy) and their relevance to AD pathology. Findings from this study may lead to the development of effective protection strategies for amyloidosis, Parkinson's disease, Huntington's disease, ALS, axotomy, brain ischemia, stroke, epilepsy, and other neurodegenerative diseases. It may also have therapeutic relevance for chronic conditions such as cancer, osteoporosis, cardiovascular diseases where autophagy, SIRT1 or PA are known play key protective roles.

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Figure 1.

Protective effects of phytic acid (PA) in human neuroblastoma/MC65 cells, which were grown in the presence (Tet+) or absence (Tet-) of tetracycline and under Tet- with PA (100 μ M) for 48 or 72 hrs, and assessed for: (A) H₂O₂ production, (B) Super oxide production, (C) Intracellular calcium, and (D) Survival. H₂O₂ production and super oxide production was assessed after 48-hour treatment period. Cell survival and the intracellular calcium levels in live cells were assessed as relative fluorescence units (RFU/Live cells) after 72-hour treatment period. Each treatment was replicated on average 3–6 times in each of 3 experiments, and the error bars represent an average percentage of cell survival for the experiments. Pair-wise comparison with Bonferroni post-hoc tests were made on percent survival between Tet+ and other treatments. Statistical significance levels: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 2.

The western blot expression of Aβ oligomers, beclin-1, and SIRT1 proteins in MC65 cells grown under Tet+ and Tet- in the presence of vehicle (Veh) or phytic acid (PA, 100μM). Resveratrol (RSV, 5 μM) served as a positive control. (A) Aβ oligomers, (B) Aβ oligomer densitometry analysis, (C) beclin-1, and (D) SIRT1. Aβ oligomers were fractionated on 16% tricine gels, and beclin-1 and SIRT1 on 10–20% bis-tris gel and transferred to nitrocellulose membranes. The membrane was probed with 6E10 monoclonal antibody to Aβ oligomers, anti-beclin-1, and anti-SIRT1. APP= amyloid precursor protein. Aβ oligomers appear in the range of 16 and 44 kDa. C99 = C-terminal 99 amino acid-fragment, and P8 = an 8-kDa Aβ domain containing fragment. The bars represent average for each band relative to Tet+ Veh band and adjusted for the loading control β-tubulin. Results are summarized from 3 experiments and the error bars=SE±. Statistical significance levels: * p≤0.05.

Figure 3.

Effects of phytic acid (PA) in wild-type female mice. Nine-month-old wild type (WT) mice were treated with distilled drinking water (VEH; N=10) or 1.9% phytic acid (PA; N=10) for 70 days. (A) Body weights (g) of mice were measured 27 times during the treatment period and the weekly averages are presented for VEH and PA treated animals. (B) Celoplasmin activity (Cp) was measured six times in the plasma samples and the average values are presented for each animal group. In addition, the following assessments were made in the frontal cortex (FC) homogenates: (C) Copper/Cu & Iron/Fe, (D) Superoxide dismutase/SOD & ATP, and (E) Cytochrome oxidase/COX & Melondehaldehyde (MDA). Statistical significances: ns, not significant; **, $P < 0.01$; ***, $P < 0.001$.

Figure 4.

Protective effects of phytic acid (PA) in Tg2576 mouse model of AD. Twelve-month-old WT and Tg2576 mice were treated with VEH (N=19) or 2% PA (N=18) for 6 months. Their body weights and terminal assessments are presented. (A) Body weights (g) of mice were measured over 30 times during the treatment period and the monthly averages are presented for VEH and PA treated animals. (B) plasma ceruloplasmin/Cp activity, (C) plasma and (D) brain levels of copper, iron, and zinc measured by ICP-MS, and ELISA measurements of (E) soluble Aβ1–40, (F) insoluble Aβ1–40, and (G) insoluble Aβ1–42. Error bars are ± SEM. Statistical significance levels: * $p \leq 0.05$, ** $p \leq 0.01$.

Figure 5.

A proposed mechanism of protection by phytic acid (PA) in the mouse brain. The schematic diagram shows the down-stream molecular effects of PA. Specific up-regulation of a protein function/activity is shown in blue up-ward arrows and the down-regulation is shown in red down-ward arrows with a magnitude of effect approximately proportional to the thickness of the arrow. Increased brain PA down-regulates oxidative stress and promotes up-regulation of SIRT1 and PAMK/AMPK ratio leading to up-regulation of autophagy and mitochondrial functions, which in turn may attenuate Aβ pathology and perhaps improve behavioral functions.