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## Original Contribution

## Dietary vitamin D deficiency in rats from middle to old age leads to elevated tyrosine nitration and proteomics changes in levels of key proteins in brain: Implications for low vitamin D-dependent age-related cognitive decline

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## ABSTRACT

In addition to the well-known effects of vitamin D (VitD) in maintaining bone health, there is increasing appreciation that this vitamin may serve important roles in other organs and tissues, including the brain. Given that VitD deficiency is especially widespread among the elderly, it is important to understand how the range of serum VitD levels that mimic those found in humans (from low to high) affects the brain during aging from middle age to old age. To address this issue, 27 male F344 rats were split into three groups and fed isocaloric diets containing low (100 IU/kg food), control (1000 IU/kg food), or high (10,000 IU/kg food) VitD beginning at middle age (12 months) and continued for a period of 4–5 months. We compared the effects of these dietary VitD manipulations on oxidative and nitrosative stress measures in posterior brain cortices. The low-VitD group showed global elevation of 3-nitrotyrosine compared to control and high-VitD-treated groups. Further investigation showed that this elevation may involve dysregulation of the nuclear factor  $\kappa$ -light-chain enhancer of activated B cells (NF- $\kappa$ B) pathway and NF- $\kappa$ B-mediated transcription of inducible nitric oxide synthase (iNOS) as indicated by translocation of NF- $\kappa$ B to the nucleus and elevation of iNOS levels. Proteomics techniques were used to provide insight into potential mechanisms underlying these effects. Several brain proteins were found at significantly elevated levels in the low-VitD group compared to the control and high-VitD groups. Three of these proteins, 6-phosphofructokinase, triose phosphate isomerase, and pyruvate kinase, are involved directly in glycolysis. Two others, peroxiredoxin-3 and DJ-1/PARK7, have peroxidase activity and are found in mitochondria. Peptidyl-prolyl *cis-trans* isomerase A (cyclophilin A) has been shown to have multiple roles, including protein folding, regulation of protein kinases and phosphatases, immunoregulation, cell signaling, and redox status. Together, these results suggest that dietary VitD deficiency contributes to significant nitrosative stress in brain and may promote cognitive decline in middle-aged and elderly adults.

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**Abbreviations:** 3-NT, 3-nitrotyrosine; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; 6-PFK, 6-phosphofructokinase; ALP, alkaline phosphatase activity buffer; A $\beta$ , amyloid- $\beta$  peptide; BSA, bovine serum albumin; VitD3, cholecalciferol; 25-OH VitD, calcidiol, 25-hydroxyvitamin D; 1 $\alpha$ ,25-(OH)<sub>2</sub> VitD, calcitriol, 1 $\alpha$ ,25-dihydroxyvitamin D; DI, deionized; DTT, dithiothreitol; FT, Fourier transform; GPx, glutathione peroxidase; IPL, inferior parietal lobule; iNOS, inducible nitric oxide synthase; IPI, International Protein Index; IA, iodoacetamide; IEF, isoelectric focusing; MnSOD, manganese superoxide dismutase; MS, mass spectrometry; NBT, nitroblue tetrazolium; NF- $\kappa$ B, nuclear factor  $\kappa$ -light-chain enhancer of activated B cells; PPIA, peptidyl-prolyl *cis-trans* isomerase A; PrxIII, peroxiredoxin 3; PEP, phosphoenolpyruvate; PK, pyruvate kinase; ROS, reactive oxygen species; TCA, tricarboxylic acid; TPI, triose phosphate isomerase; TGS, Tris-glycine-SDS; TNF $\alpha$ , tumor necrosis factor- $\alpha$ ; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; VitD, vitamin D; VDR, vitamin D receptor

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## Introduction

The steroid hormone vitamin D (VitD) can be produced by the body or obtained through the diet. VitD is synthesized in the skin from the cholesterol precursor 7-dehydrocholesterol and is converted to cholecalciferol (VitD3) upon exposure to sunlight [1]. VitD3 can also be obtained through several dietary sources and is transported in the blood via vitamin D-binding protein. In the liver, VitD3 is converted to calcidiol, 25-hydroxyvitamin D (25-OH VitD), followed by further conversion to calcitriol, 1 $\alpha$ ,25-dihydroxyvitamin D (1 $\alpha$ ,25-(OH)<sub>2</sub> VitD), primarily in the kidneys, where it helps to regulate calcium homeostasis [2,3]. VitD also plays roles in autoimmunity [4], mental health [3,5–8], and inhibition of tumor growth through reductions in proliferation and angiogenesis [9–12].

VitD deficiency has long been associated with osteoporosis, brittle bones, and muscle weakness, but recently low levels of VitD have been linked to increased overall mortality [13,14]. VitD status is typically assessed using serum concentration of 25-OH VitD because it is longer lived than the biologically active  $1\alpha,25\text{-(OH)}_2$  VitD [13,15,16].

VitD deficiency is highly prevalent in Europe and North America [1,17], with the elderly particularly at risk [11,13,18–20]. Current estimates suggest that as many as 40–100% of the elderly populations in these areas are VitD deficient [21]. Poor diet and lower exposure to UV-B from the sun limits VitD synthesis in the skin and an age-related decrease in the VitD synthesis machinery may contribute to the observed lower VitD levels [15].

The elderly represent those at greatest risk of age-related cognitive decline and neurodegenerative disorders [22]. Recent retrospective studies on elderly human subjects provide correlative evidence that those with VitD deficiency have a much higher incidence of cognitive impairment than those with normal VitD levels [23,24]. Thus, it seems that VitD deficiency may accelerate cognitive decline in aging [25]. A recent meta-analysis also shows that patients with Alzheimer disease (AD) typically have lower serum concentrations of VitD [26]. AD is associated with defects in amyloid- $\beta$  ( $A\beta$ ) processing and an upregulation of inflammatory cytokines and nuclear factor  $\kappa$ -light-chain enhancer of activated B cells (NF- $\kappa$ B) [8]. Interestingly,  $1\alpha,25\text{(OH)}_2$  VitD helped to reverse soluble  $A\beta$  and inflammatory issues [8]. In addition to these actions, VitD is neuroprotective against  $\text{Ca}^{2+}$ -mediated excitotoxicity, reduces biomarkers of brain aging associated with  $\text{Ca}^{2+}$  dyshomeostasis [3,5], and helps to regulate levels of glutathione, a primary antioxidant in the brain, by modulating  $\gamma$ -glutamyltranspeptidase activity [27]. VitD also prevents onset of autoimmune demyelination in animal models of multiple sclerosis [28,29].

Here, we manipulated serum VitD status by dietary supplementation with low, moderate/control, or high levels of VitD to identify changes in the VitD-dependent proteome in the brains of rats from middle to old age. Prior studies have shown that cognitively impaired subjects have significant levels of mitochondrial dysfunction and oxidative protein damage. In particular, nitration of protein-resident tyrosine residues is a common marker observed in brain of cognitively impaired subjects [30–34]. Therefore, we tested the hypothesis that manipulating serum VitD levels would alter protein nitration and key protein markers of mitochondrial function. Our results identify several possible targets of VitD action that may mechanistically link circulating VitD levels with risk for age-related cognitive decline.

## Materials and methods

### Chemicals

Criterion precast polyacrylamide gels, Tris–glycine–sodium dodecyl sulfate (SDS) (TGS) and Mes electrophoresis running buffers, ReadyStrip IPG strips, mineral oil, Precision Plus Protein All Blue standards, SYPRO Ruby protein stain, nitrocellulose membranes, dithiothreitol (DTT), iodoacetamide (IA), Biolytes, and urea were purchased from Bio-Rad (Hercules, CA, USA). Chemicals, proteases, protease inhibitors, and antibodies used in this study were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise noted.

### Animals

All animal studies were approved by the University of Kentucky Institutional Animal Care and Use Committee and followed NIH Guidelines for the Care and Use of Laboratory Animals. Middle-aged

(12 months old) male F344 rats, a standard model for studies of brain aging, were obtained from the National Institutes on Aging rodent colony. Four- to five-month dietary manipulation of VitD was carried out using VitD3 added to an isocaloric diet in the following amounts (based on pilot studies intended to mimic the range of human levels): control VitD, 1000 IU/kg food; low VitD, 100 IU/kg food; and high VitD, 10,000 IU/kg food. Each group consisted of nine animals (Table 1). Animals were weighed and food intake was measured two or three times per week. Serum 25-OH VitD levels were monitored as a measure of circulating VitD. Dietary manipulation of VitD resulted in different serum levels of VitD. Upon conclusion of the long-term treatment, the animals were euthanized and samples isolated from the right posterior cortical area. Samples were then stored at  $-80^\circ\text{C}$  until needed for oxidative stress and expression proteomics determinations [35,36].

### Sample preparation

Protein estimation was performed using the bicinchoninic acid (Pierce) assay. Homogenized cortex samples were diluted according to initial protein estimation results: 20  $\mu\text{g}$  sample in 140  $\mu\text{l}$  medium I buffer (0.32 M sucrose, 0.6 mM  $\text{MgCl}_2$ , and 0.125 M Tris, pH 8.0, with protease inhibitors 4  $\mu\text{g/ml}$  leupeptin, 4  $\mu\text{g/ml}$  pepstatin A, and 5  $\mu\text{g/ml}$  aprotinin).

### Slot-blot assay

The slot-blot method was used to determine levels of protein 3-nitrotyrosine (3-NT) in brain as previously described [37,38]. For 3-NT determination, samples were solubilized in Laemmli buffer. Protein (250 ng) from each sample was loaded onto a nitrocellulose membrane in respective wells in a slot-blot apparatus (Bio-Rad) under vacuum. Membranes were blocked in 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) with 0.2% (v/v) Tween 20 for 1.5 h and then incubated in primary antibody (anti-nitrotyrosine produced in rabbit; Sigma–Aldrich) for 2 h, washed three times in PBS with 0.2% (v/v) Tween 20, and then incubated for 1 h with secondary antibody (goat anti-rabbit secondary linked to alkaline phosphatase). Membranes were developed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) dipotassium and nitroblue tetrazolium (NBT) chloride in alkaline phosphatase activity (ALP) buffer, dried, and scanned for analysis. Image analysis was performed using Scion Image (Scion Corp., Frederick, MD, USA). A negative control for nitration was performed by reducing nitrotyrosine to aminotyrosine according to the method of Miyagi et al. [39] as follows. One replicate membrane was treated with 10 mM sodium dithionite in 50 mM pyridine acetate buffer (pH 5.0) for 1 h at room temperature, rinsed thoroughly with Nanopure water, and developed as above. No staining of the membrane was observed (data not shown), indicating that the anti-3-NT antibody was specific for this post-translational modification.

**Table 1**  
VitD dietary manipulation.

	Control	Low	High
Weight at 12 months	480 g	485 g	475 g
Weight at 18 months	538 g	565 g	548 g
Daily food intake	16.5 g/day	16.4 g/day	15.9 g/day
Daily intake of cholecalciferol	16.5 IU/day	1.64 IU/day	159 IU/day
Serum levels of 25-OH VitD*	12.7 ng/ml	5.8 ng/ml	31.7 ng/ml

\*  $p < 0.0001$  (one-way ANOVA).

## Proteomics [35]

### Isoelectric focusing (IEF)

Aliquots (containing 150 µg of protein) of the homogenized cortical samples prepared above were precipitated using cold 100% trichloroacetic acid to obtain a concentration of 15% (v/v) trichloroacetic acid in solution and incubated on ice for 15 min. Samples were centrifuged at 14,000 rpm for 10 min at 4 °C. The resulting pellets were resuspended and rinsed four times in a cold ethanol:ethyl acetate (1:1 v/v) mixture. After being allowed to dry completely at room temperature, the final pellets were rehydrated for 2 h in rehydration buffer (8 M urea, 2 M thiourea, 50 mM DTT, 2.0% (w/v) Chaps, 0.2% Biolytes, bromophenol blue) and then sonicated for 10 s at 20% power. Each entire sample was added to an 11-cm pH 3–10 ReadyStrip IPG strip in a lane of the IEF tray. After 45 min of run time, 2 ml of mineral oil was added to each lane to prevent evaporation. Strips were actively rehydrated at 20 °C for 18 h at 50 V and focused at a constant temperature of 20 °C beginning at 300 V for 2 h, 500 V for 2 h, 1000 V for 2 h, 8000 V for 8 h, and finishing at 8000 V for 10 h rapidly. IPG strips were stored at –80 °C until needed for the second dimension of analysis.

### Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

IEF strips were allowed to come to room temperature (~30 min) and equilibrated for 10 min in the dark in 4 ml equilibration buffer A (50 mM Tris–HCl, pH 6.8, 6 M urea, 1% (w/v) SDS, 30% v/v glycerol, 0.5% DTT) and then reequilibrated for 10 min in the dark in equilibration buffer B (50 mM Tris–HCl, pH 6.8, 6 M urea, 1% (w/v) SDS, 30% v/v glycerol, 4.5% IA). All strips were rinsed in TGS running buffer to remove residual equilibration buffers before being placed onto Criterion precast linear gradient (8–16%) Tris–HCl polyacrylamide gels. Precision Plus Protein All Blue standards and samples were run at a constant voltage of 200 V for 65 min.

### SYPRO Ruby staining

After 2D-PAGE, gels were incubated in 50 ml fixing solution (7% (v/v) acetic acid, 10% (v/v) methanol) for 20 min at room temperature. SYPRO Ruby protein gel stain (50–55 ml) was added to gels and allowed to stain overnight at room temperature on a gently rocking platform. The stain was then removed and gels were rinsed with deionized (DI) water and stored in 50 ml DI water in the refrigerator until scanning. Gels were scanned into Adobe Photoshop 6.0 with a Molecular Dynamics Storm phosphoimager ( $\lambda_{\text{ex}}/\lambda_{\text{em}}$  470/618 nm) and stored in DI water at 4 °C.

### Image analysis: differential expression

Spot intensities from SYPRO Ruby-stained 2D-gel images of cortex samples were quantified according to total spot density using PDQuest software (Bio-Rad). Intensities were normalized to total gel densities. Only low or high VitD samples with normalized spot densities that were significantly increased or decreased by at least 1.4-fold from control were considered for mass spectrometry (MS) analysis. This is a conservative cut-off criterion, but does greatly minimize false positives.

### In-gel trypsin digestion

Protein spots identified as significantly altered in VitD-deficient rat brain relative to normal VitD controls were excised from 2D gels with new, sterilized micropipette tips and transferred to Eppendorf microcentrifuge tubes. Gel plugs were then washed with 0.1 M ammonium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ) at room temperature for 15 min, followed by incubation with 100% acetonitrile at room temperature for 15 min. Solvent was removed, and gel plugs were dried in their respective tubes in a biosafety cabinet at room temperature. Gel plugs were incubated for 45 min in 20 µl of

20 mM DTT in 0.1 M  $\text{NH}_4\text{HCO}_3$  at 56 °C. The DTT solution was then removed and replaced with 20 µl of 55 mM IA in 0.1 M  $\text{NH}_4\text{HCO}_3$  and incubated with gentle agitation at room temperature in the dark for 30 min. Excess IA solution was removed, and the gel plugs were incubated for 15 min with 200 µl of 50 mM  $\text{NH}_4\text{HCO}_3$  at room temperature. Two hundred microliters of 100% acetonitrile was added to this solution in each tube and incubated for 15 min at room temperature. All solvent was removed, and gel plugs were allowed to dry for 30 min at room temperature in a biosafety cabinet. Gel plugs were rehydrated with 20 ng/µl modified trypsin (Promega, Madison, WI, USA) in 50 mM  $\text{NH}_4\text{HCO}_3$  in a shaking incubator overnight at 37 °C. Enough trypsin solution was added to completely submerge the gel plugs (approximately 10 µl).

### Mass spectrometry

Salts and other contaminants were removed from tryptic digest solutions using C18 ZipTips (Sigma–Aldrich), and the digests were reconstituted to a volume of approximately 15 µl in a 50:50 DI water:acetonitrile solution containing 0.1% formic acid. Tryptic peptides were analyzed with an automated Nanomate electrospray ionization (Advion Biosciences, Ithaca, NY, USA) Orbitrap XL MS (ThermoScientific, Waltham, MA, USA) platform. The Orbitrap MS was operated in a data-dependent mode whereby the eight most intense parent ions measured in the Fourier transform (FT) at 60,000 resolution were selected for ion trap fragmentation under the following conditions: injection time 50 ms, 35% collision energy, MS/MS spectra measured in the FT at 7500 resolution, and dynamic exclusion set for 120 s. Each sample was acquired for a total of approximately 2.5 min. MS/MS spectra were searched against the International Protein Index (IPI) database using SEQUEST and the following parameters: two trypsin miscleavages, fixed carbamidomethyl modification, variable methionine oxidation, parent tolerance 10 ppm, and fragment tolerance of 25 mmu or 0.01 Da. Results were filtered with the following criteria:  $X_{\text{corr}} > 1.5, 2.0, 2.5, \text{ or } 3.0$  for +1, +2, +3, or +4 charge state, respectively;  $\Delta\text{CN} > 0.1$ , and  $p$  value (protein and peptide)  $< 0.01$ . IPI accession numbers were cross-correlated with SwissProt accession numbers for final protein identification [36].

### One-dimensional polyacrylamide gel electrophoresis

Sample homogenates were incubated in sample buffer (0.5 M Tris, pH 6.8, 40% glycerol, 8% SDS, 20%  $\beta$ -mercaptoethanol, and 0.01% bromophenol) for 5 min in a water bath at 95 °C and loaded onto precast Criterion TGX (4–15%) or Criterion XT (12% Bis–Tris) precast gels as appropriate for the molecular weight of the protein of interest. Precision Plus Protein All Blue standards and samples were run at 80 V for 15 min, increasing the voltage to 120 V and run for 90 min in TGS or Mes running buffer as appropriate for the gel.

### Western blotting

In-gel proteins were transferred to a nitrocellulose membrane (0.45 µm) using a Trans-Blot Turbo blotting system at 25 V for 30 min (Bio-Rad). After transfer, the membranes were incubated in blocking solution (3% BSA in PBS solution with 0.2% (v/v) Tween 20) at room temperature for 1.5 h. Membranes were then incubated with rabbit anti-inducible nitric oxide synthase (iNOS) antibody (Calbiochem/Millipore, Billerica, MA, USA) or rabbit anti-NF- $\kappa$ B antibody (Enzo, Farmingdale, NY, USA) for 2 h on a gentle rocking platform, followed by three rinses for 5 min each with PBS solution with 0.2% (v/v) Tween 20. Blots were then incubated for 1 h with ECL anti-rabbit IgG horseradish peroxidase-linked whole antibody. The resulting blots were rinsed three times for 5, 10, and 10 min each in PBS solution with 0.2% (v/v) Tween 20 and signals were detected using Clarity Western ECL substrate

(Bio-Rad) and the ChemiDoc MP imaging system (Bio-Rad). Blots were stripped using Re-blot Plus Strong Solution (Millipore) according to package instructions and re-probed with mouse anti-actin antibody (Sigma-Aldrich) or mouse anti-histone H2B antibody (Chemicon, Temecula, CA, USA) for normalization. Analysis was performed using the ImageLab software (Bio-Rad).

### Validation

Proteomics results were validated by IEF and 2D-PAGE as described above followed by Western blot [36] for peroxiredoxin 3 (PrxIII) and 1D-gel electrophoresis followed by Western blot for peptidyl-prolyl *cis-trans* isomerase A (PPIA, or cyclophilin A). PrxIII blots were probed for PrxIII as described above using rabbit polyclonal to PrxIII primary antibody (Abcam, Cambridge, MA, USA), mouse anti-actin primary antibody, and anti-rabbit whole-molecule IgG alkaline phosphatase or anti-mouse whole-molecule IgG alkaline phosphatase secondary antibody, respectively. The resulting blots were developed colorimetrically with a solution of BCIP combined with NBT in ALP buffer (0.1 M Tris, 0.1 M NaCl, 5 mM MgCl<sub>2</sub> · 6 H<sub>2</sub>O, pH 9.5). Developed blots were allowed to dry overnight at room temperature and scanned into Adobe Photoshop 6.0 using a Canon CanoScan 8800 F scanner. The resulting images were analyzed using Image Quant software. PPIA blots were probed for PPIA by incubation with anti-cyclophilin A rabbit antiserum (Upstate/Millipore) primary antibody and ECL anti-rabbit IgG horseradish peroxidase-linked whole antibody and developed for chemiluminescence as described above. PPIA blots

were stripped and re-probed for the actin. Analysis was performed using ImageLab software (Bio-Rad).

### Statistical analysis

All data are presented as means ± SD or means ± SEM, and statistical analyses were performed using a two-tailed Student *t* test or ANOVA where indicated, with *p* < 0.05 considered significant for spot comparison. A Mann-Whitney *U* statistical analysis was performed to determine the significance of differential expression fold-change values, where *p* < 0.05 was considered significant. Significance was also confirmed using a Student *t* test. Only spots that were significant by both tests were further evaluated. Protein and peptide identifications obtained with the SEQUEST search algorithm with a *p* < 0.01 were considered statistically significant. To further validate PDQuest analysis, identities of protein spots were verified matching calculated molecular weight (MW) and isoelectric point (pI) values from MS results and SwissProt database information to spot locations on the gels (Table 2 and Fig. 1).

## Results

### Vitamin D deficiency leads to increased nitrosative protein damage in brain

Tyrosine nitration is a common indicator/biomarker of the aging brain and of age-related neurodegenerative disorders [30,34], both of which typically are accompanied by different extents of cognitive deficit. Here, we tested for indicators of oxidative and nitrosative stress in brain tissue samples from rats in which we manipulated serum VitD levels from middle age to old age. Significantly increased global 3-NT (Fig. 2) in the brains of rats on a low-VitD diet compared to rats on control or high-VitD diets was observed. 3-NT measures of brain samples from rats fed the high-VitD diet were similar to those of the control group. No significant differences were observed for the other oxidative stress parameters measured.

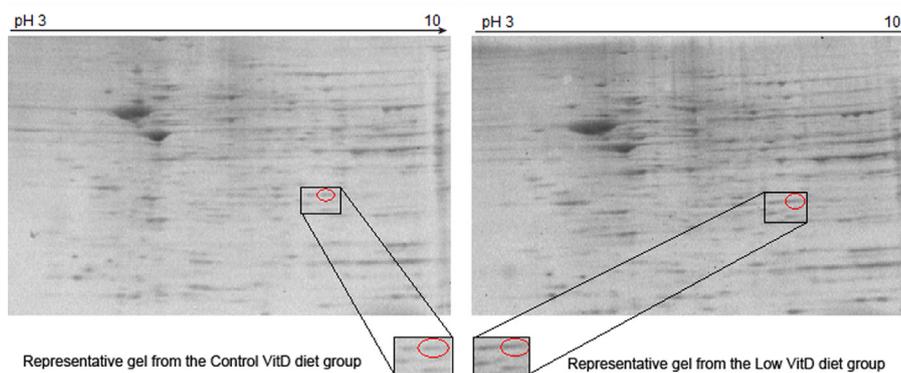
### Vitamin D deficiency elevates peroxidases, glycolytic enzymes, and PPIA

To determine changes from or possible causes, consequences, or mechanisms of the elevated nitrosative stress, expression proteomics experiments [35] were performed on brain of rats

**Table 2**  
Brain protein identifications based on MS/MS results and in-gel spot locations.

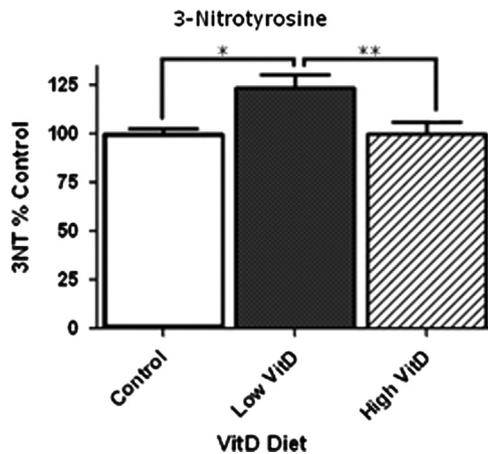
SSP	Protein identification	Score	Coverage	Unique peptides	MW (kDa)	Calcd pI
5201	Triose phosphate isomerase	67.5	48.6	10	26.8	7.24
8103	Pyruvate kinase isozymes M1/M2	241	50.8	21	57.8	7.06
6701	6-Phosphofructokinase type C	69.2	19.5	11	85.7	7.28
3207	Peroxiredoxin 3	155	33.8	6	28.3	7.55
4201	DJ-1/PARK7	61.7	58.2	11	20.0	6.77
8104	Peptidyl-prolyl <i>cis-trans</i> isomerase A	56.2	28.7	7	17.9	8.16

SSP, PDQuest software-generated ID number.



**Fig. 1.** Representative 2D gels used for proteomics identification of differentially expressed proteins. Brain samples of rats fed a control, low-VitD, or high-VitD diet from middle age to old age were separated by IEF using IPG strips pH 3–10 followed by SDS-PAGE using 8–16% Tris-HCl gels. Separated proteins were visualized with SYPRO Ruby protein gel stain. Acquired images were analyzed using PDQuest software and spots showing different intensities were chosen for further analysis. A comparison of the PrxIII spot as a representative of those found with significant expression differences in low vs control VitD groups as identified by PDQuest analysis is shown. Below are expansions of the spot images showing clearly that the PrxIII level is elevated in brain of rats on a chronic low-VitD diet.

fed a low-VitD, control, or high-VitD diet to determine which proteins were altered. PDQuest analysis was performed on all groups as well. Only spots that were statistically different in relative intensity by statistical tests were further evaluated. Compared to control, the high-VitD group was similar to control in terms of protein levels for easily discernible protein spots. In contrast, a number of protein spots with significant differences in intensity were found between the control and the low-VitD groups. Table 3 shows the PDQuest software-generated ID numbers of these protein spots. Spot intensities between control and low-VitD groups were used to calculate fold changes reflecting



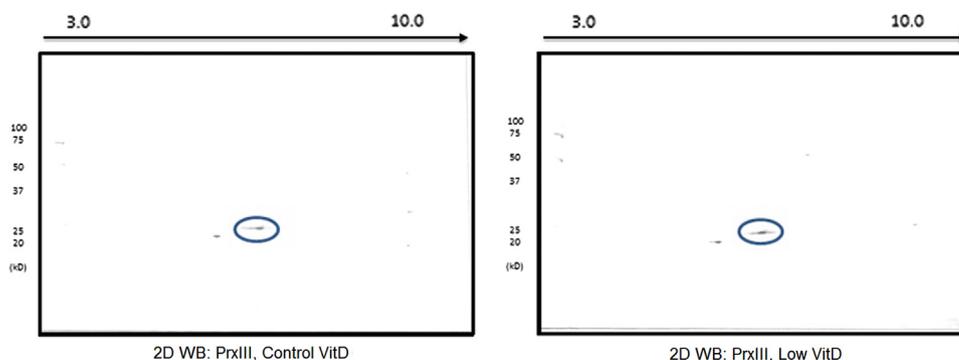
**Fig. 2.** Slot-blot analysis of 3-NT levels in rat brain homogenates. Slot-blot analyses were performed using brain samples of rats fed a control, low-VitD, or high-VitD diet from middle age to old age ( $n=9$ ). Data show a significant increase in 3-NT levels in rat brains between control and low-VitD diet ( $*p < 0.01$ ). No difference was observed in high-VitD diet compared to control. High-VitD diet decreased 3-NT levels compared to low-VitD diet ( $**p < 0.05$ ).

**Table 3**

VitD 2D-gel comparison PDQuest data: spot matching low-VitD (L) vs control (C) levels.

SSP	Fold change	Direction of change L vs C	<i>p</i> value L vs C
6701	1.41	Increased	0.0114
8104	2.49	Increased	0.0130
5201	60.3	Increased	0.0291
3207	1.53	Increased	0.0347
8103	1.48	Increased	0.0373
4201	1.53	Increased	0.0473

SSP, PDQuest software-generated ID number.



**Fig. 3.** Validation of expression proteomics results for PrxIII by 2D Western blot analysis. Depicted are representative blots showing a 1.5-fold increase in PrxIII in brain samples of rats fed the low-VitD diet from middle age to old age compared to those fed the control diet. Circled spots are PrxIII from control-VitD brain samples shown on the left and low-VitD brain samples on the right.

significant increases in protein amounts. These proteins were identified by MS/MS and database interrogation as 6-phosphofructokinase (6-PFK) type C, PPIA, triose phosphate isomerase (TPI), PrxIII, DJ-1/PARK7, and pyruvate kinase (PK) isozymes M1/M2 (Table 3).

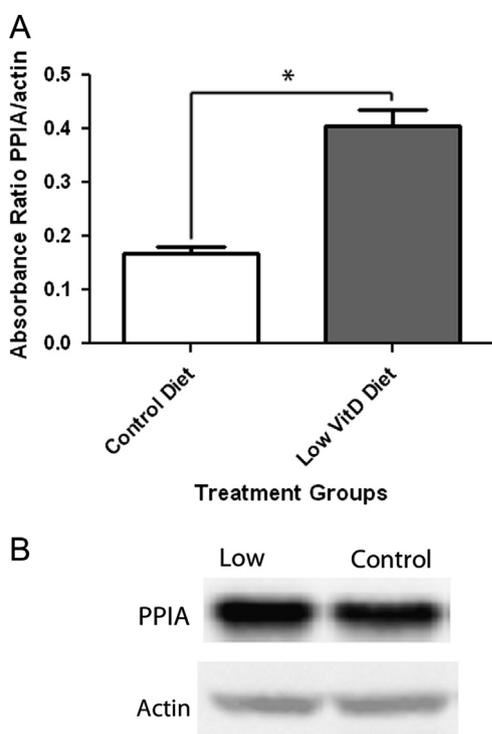
To validate protein identity from proteomics, 2D and 1D Western blots were performed, selecting PrxIII and PPIA as representative proteins. Two-dimensional Western blots showed a 1.5-fold increase in PrxIII levels, confirming the 1.5-fold increase suggested by 2D-PAGE. Further, the position of the PrxIII spot appears at approximately 26 kDa and 7.2 pI on 2D Western blot (Fig. 3). One-dimensional Western blot showed a 2.24-fold increase in PPIA levels, further validating the 2.49-fold increase seen in the proteomics results (Fig. 4). These validation results provide confidence in the proteomics identification of other proteins reported in this study.

#### Vitamin D deficiency leads to NF- $\kappa$ B activation and increased iNOS levels in brain

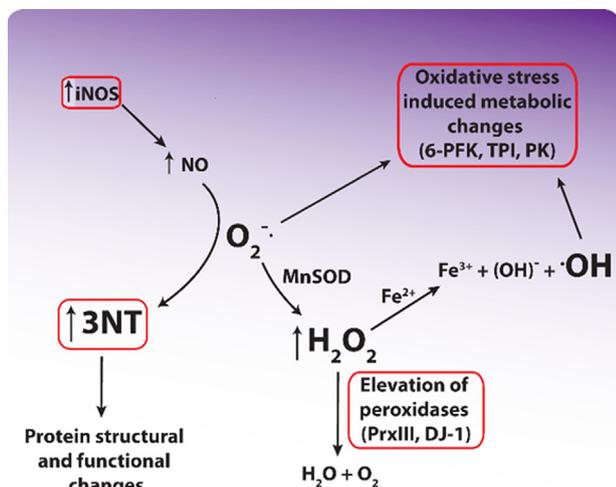
Preliminary results of this study coupled with previous studies in our laboratory and existing literature led us to propose a model that connects the observed increases in nitrosative protein damage and the expression proteomics results showing elevated levels of the above-mentioned peroxidases, glycolytic enzymes, and PPIA to VitD deficiency (Fig. 5). Based on this proposed model, we examined the hypothesis that VitD deficiency would lead to increased NF- $\kappa$ B activation and iNOS levels, resulting in the observed increase in protein tyrosine nitration seen in the low-VitD group compared to control.

NF- $\kappa$ B is a redox-sensitive nuclear transcription factor that can be activated by oxidizing agents such as hydrogen peroxide ( $H_2O_2$ ) [40]. Hypoxic activation of NF- $\kappa$ B and gene transcription has been found to require mitochondrial reactive oxygen species (ROS) [41]. Among the downstream products of NF- $\kappa$ B pathway activation are tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and iNOS [41–43]. Using 1D Western blotting, we investigated whether NF- $\kappa$ B activation of iNOS expression may be a plausible reason for the nitration of tyrosine residues through overproduction of  $NO^*$  [44–48] seen in these samples.

Under normal conditions, NF- $\kappa$ B exists in an inactive form bound to inhibitor of  $\kappa$ B- $\alpha$  ( $I\kappa$ B- $\alpha$ ). Upon activation of a Toll-like receptor by a variety of substrates including lipopolysaccharide and TNF $\alpha$  [49], phosphorylation of  $I\kappa$ B- $\alpha$  occurs through  $I\kappa$ B kinase (IKK), allowing  $I\kappa$ B- $\alpha$  proteasomal degradation and NF- $\kappa$ B activation. NF- $\kappa$ B is translocated to the nucleus, leading to bursts of iNOS activation, subsequent  $NO^*$  elevation [42,47], and further transcriptional activation of the TNF $\alpha$  gene [44,50]. Proinflammatory cytokines such as TNF $\alpha$  lead to further and rapid phosphorylation of  $I\kappa$ B's [51]. Overproduction of  $NO^*$  as a consequence of iNOS



**Fig. 4.** Validation of expression proteomics results by 1D Western blot analysis of PPIA levels. 1D Western blot analysis was used to determine PPIA levels of brain samples of rats fed a control or low-VitD diet from middle age to old age ( $n=9$ ). (A) Analysis using a specific antibody against PPIA showed a 2.24-fold increase in PPIA levels in brain of the low-VitD group compared to control ( $*p < 0.01$ ). This result confirms the increase in PPIA levels found by expression proteomics. (B) Representative lanes from 1D Western blot showing probes for PPIA and the actin loading control.



**Fig. 5.** Potential mechanism for the biochemical consequences of VitD deficiency. Circled areas indicate data collected in this study. Increased tyrosine nitration was found by slot-blot analysis, increased iNOS levels were found by 1D Western blot analysis, and elevation of glycolytic enzymes and peroxidases was found by expression proteomics using brain samples of rats fed a control or low-VitD diet from middle age to old age. Items in large type are the most potentially damaging agents in this diagram.

activation results in protein nitration events and further stimulation of IKK [42,52].  $1\alpha,25\text{-(OH)}_2$  VitD has been shown to interfere with these processes by decreasing  $\text{TNF}\alpha$  levels and  $\text{TNF}\alpha$ -induced inflammatory cytokines [53–55], downregulating Toll-like receptors [56], upregulating  $\text{I}\kappa\text{B-}\alpha$ , decreasing  $\text{I}\kappa\text{B-}\alpha$  phosphorylation,

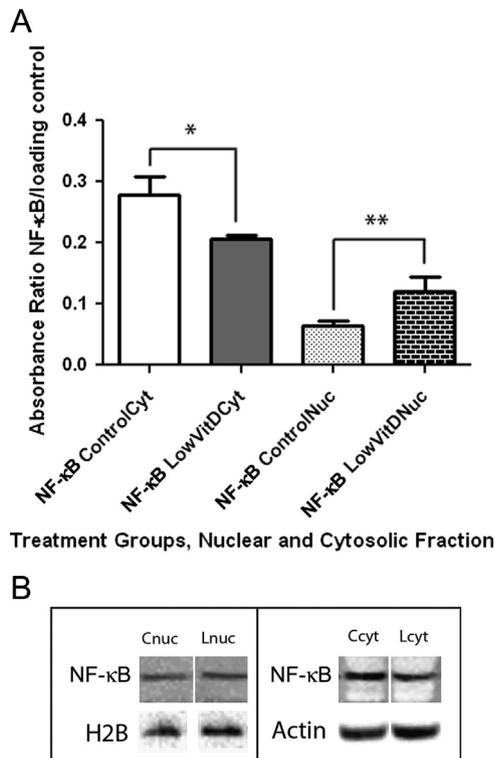
and decreasing translocation of  $\text{NF-}\kappa\text{B}$  [49], thereby reducing many downstream consequences. Further decrease in  $\text{NF-}\kappa\text{B}$  action may occur through the suppression of  $\text{NF-}\kappa\text{B}$ -directed expression by the VitD-responsive element [57]. Thus, VitD can inhibit protein nitration in the brain via its suppressive effects on the  $\text{NF-}\kappa\text{B}$  pathway. VitD deprivation may exacerbate  $\text{NF-}\kappa\text{B}$ -directed nitrosative damage and subsequent neurodegenerative consequences. As an estimate of  $\text{NF-}\kappa\text{B}$  activity, subcellular fractionation was done on control and low-VitD brain samples.  $\text{NF-}\kappa\text{B}$  levels were significantly decreased in the cytosolic fraction and significantly increased in the nuclear fraction of the low-VitD group compared to control (Fig. 6), indicating  $\text{NF-}\kappa\text{B}$  translocation to the nucleus. Our analysis further revealed a significant increase in iNOS levels in the low-VitD samples compared to control (Fig. 7). These  $\text{NF-}\kappa\text{B}$  and iNOS results provide evidence to support the proposed mechanism of protein nitration due to VitD deficiency.  $\text{TNF}\alpha$  is one of several initiators of the  $\text{NF-}\kappa\text{B}$  activation cascade. One-dimensional Western blot analysis of these samples for  $\text{TNF}\alpha$  showed a trend toward increase (data not shown) in the low-VitD group compared to control, consistent with  $\text{NF-}\kappa\text{B}$  activation and iNOS elevation. Fig. 8 depicts a suggested mechanism for the protein nitration regulatory effects of VitD in brain.

## Discussion

We have previously shown that tyrosine nitration occurs early in neurodegenerative processes, i.e., in mild cognitive impairment, arguably the earliest form of AD [31]. Nitration of tyrosine occurs from the reaction of  $\text{NO}^*$  with  $\text{O}_2^{\cdot-}$  through the reactive intermediate  $\text{ONOO}^-$  in the presence of  $\text{CO}_2$  [32,58], leading to tyrosine nitration by the  $\text{NO}_2^*$  radical. Nitrosative stress measures on these cortical samples showed approximately a 25% elevation in 3-NT globally in brain protein in the low-VitD group versus the control- and high-VitD-treated groups.

### Glycolytic enzymes targeted by low VitD

The brain proteins identified by proteomics in this study to be at increased levels (Table 2) fall primarily into the following functional categories: glycolysis, mitochondrial peroxidase activity, and protein folding. Three of these proteins, 6-PFK, TPI, and PK, are enzymes involved in the production of ATP in the cell during glycolysis in response to the need for energy. PK catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate, transferring a phosphate group from PEP to ADP to produce ATP. Pyruvate, NADH, and  $\text{H}^+$  from glycolysis continue onto the tricarboxylic acid (TCA) cycle for further ATP production. PK has been identified by redox proteomics as oxidatively modified in brain regions, such as cortex and hippocampus, during brain aging [22,59] and neurodegeneration, resulting in reduced enzymatic activity [60]. 6-PFK catalyzes a key regulatory step, the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate, at the expense of ATP, committing the path of glucose through glycolysis. This rate-limiting glycolytic enzyme was found by Tang and colleagues [61] to be upregulated in several biological systems as part of the oxidative stress response including the response to  $\text{H}_2\text{O}_2$ -induced oxidative stress. TPI catalyzes the interconversion of the trioses, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Significant increases in TPI have been found by our group using proteomic analysis in the Alzheimer disease hippocampus [62]. Further studies identified TPI as significantly nitrated in the inferior parietal lobe (IPL) in early AD brain [63] and in both AD hippocampus and IPL [64]. Dysfunction of this enzyme leads to the buildup of a toxic species, methylglyoxal, leading to neurodegenerative



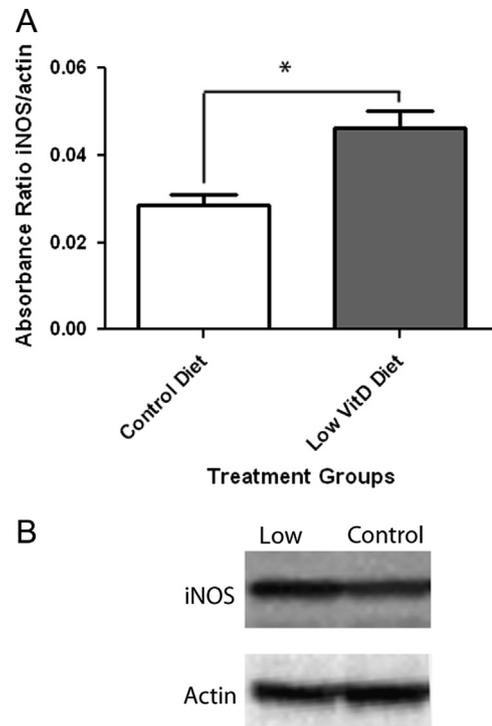
**Fig. 6.** Effect of VitD deficiency on NF-κB subcellular localization in rat brain. 1D Western blots were used to determine the NF-κB (p65) levels in the cytosolic and nuclear fractions of brain samples of rats fed a control or a low-VitD diet from middle age to old age ( $n=9$ ). The NF-κB (p65) subunit is common in the NF-κB signaling pathway. NF-κB (p65) levels in the cytosolic fraction were normalized to  $\beta$ -actin, and NF-κB (p65) levels in the nuclear fraction were normalized to histone H2B. (A) A decrease in NF-κB (p65) in the cytosolic fraction ( $*p < 0.05$ ) and increase in the nuclear fraction ( $**p < 0.05$ ) in brain samples from the low-VitD group compared to control is observed, indicative of NF-κB activation and translocation. (B) Representative bands from 1D Western blot are shown here side by side for comparison. Nuclear and cytosolic fractions were alternated across all gels. Blots were probed for NF-κB (p65), stripped, probed for  $\beta$ -actin, and then stripped and probed for histone H2B.

consequences [65,66]. Thus, metabolic effects in brain are evident from the effect of a low-VitD diet.

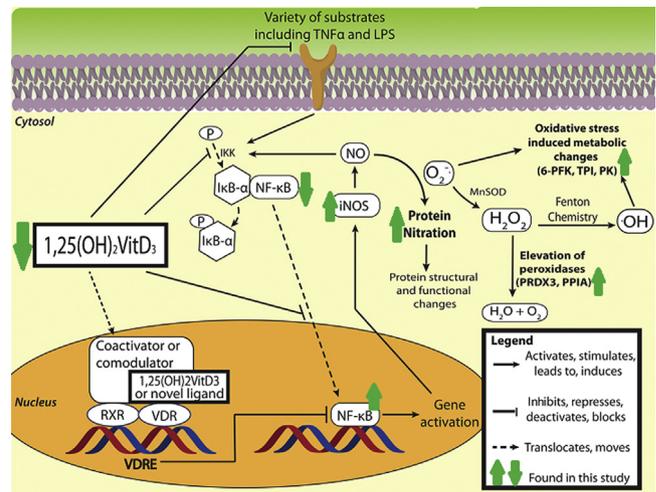
#### Other identified targets of VitD deficiency (DJ-1, PPIA, PRXIII)

Mutations of the DJ-1 gene are believed to be one possibility that leads to Parkinson disease [67]. DJ-1 is thought to be involved in cell cycle regulation, gene transcription, spermatogenesis, and the cellular stress response [68], among other functions. Evidence exists that DJ-1, in response to oxidative stress, may have roles as an antioxidant [69,70] and a redox-sensitive chaperone [71,72]. Accordingly, elevation of DJ-1 in brain could be a consequent cell-stress response to elevated protein nitration.

PPIs regulate the activity of the target proteins to which they bind by catalyzing the *cis-trans* isomerization of proline of these target proteins [73,74], typically a rate-limiting step in protein folding. PPIA, or cyclophilin A, is also known to play a variety of roles from immunoregulation to cellular signaling and proliferation, but more recently has been identified as a potential marker of inflammation in a variety of disease states [75–79] and may play a regulatory role in the NF-κB pathway in some cell types [80,81]. In addition, PPIA treatment was shown to reduce ROS and alleviate some forms of A $\beta$ -induced neurotoxicity in PC12 cell culture while maintaining activities of certain key antioxidant enzymes, including SOD [82]. PPIA binds Prx's and activates their peroxidase activity, acting as an immediate electron donor [83]. PPIA may



**Fig. 7.** Effect of VitD deficiency on iNOS levels in rat brain. 1D Western blot was performed to compare iNOS levels between brain samples of rats fed a control or a low-VitD diet from middle age to old age ( $n=9$ ). iNOS signals were normalized to  $\beta$ -actin. (A) iNOS levels were significantly higher in brain samples from the low-VitD group compared to control ( $*p < 0.01$ ). (B) Representative lanes from 1D Western blot.



**Fig. 8.** Proposed mechanism for the protein nitration regulatory effects of VitD in brain based on recent literature and results from the current study. Results obtained from this study are marked with dark green arrows. The deficient serum levels of VitD in the low-VitD group in brain led to decreased NF-κB in the cytosolic fraction, increased NF-κB in the nuclear fraction, iNOS elevation, increased protein tyrosine nitration, and elevations in mitochondrial and regulatory proteins.

also indirectly stimulate PrxIII, a downstream consequence of TNF $\alpha$  [84].

PrxIII is a mitochondrion-resident, thioredoxin-dependent peroxidase reductase that acts to scavenge as much as 90% of H<sub>2</sub>O<sub>2</sub> in mitochondria, with GPx1, GPx4, and PrxV thought to account for the rest [85–90]. Rhee and colleagues elegantly showed that peroxiredoxin overexpression inhibited NF-κB [85], and overexpression of PrxIII has

been shown to protect against  $H_2O_2$ -dependent apoptosis in cancer cells [91]. Further, PrxIII was shown to be essential in maintaining normal mitochondrial homeostasis [92]. Dysfunctional PrxIII leads to increased ROS and subsequent DNA damage and apoptosis linked to loss of peroxidase activity in mitochondria [93]. Prx's have been shown to detoxify peroxynitrite, thereby decreasing biomolecule damage caused by more reactive products of  $ONOO^-$  and protecting human cells in culture from iNOS-related cell death [94].

Tyrosine nitration can and does have functional consequences on the affected proteins [33,58,95]. Steric hindrance by the  $NO_2$  group in the 3-position of tyrosine may prevent phosphorylation at the 4-position thereby causing dysregulation of activation/deactivation processes in the affected proteins [33,45,95–98]. Such considerations have implications for cognitive dysfunction following low dietary vitamin D.

#### Consequences of low dietary VitD

Based on this experimental evidence, the biochemical consequences of low dietary VitD include increased 3-NT in the brain as well as increases in certain proteins with peroxidase activity. A likely link between these two pathways is  $O_2^{\bullet-}$  (Fig. 5). The high  $O_2$  usage in energy metabolism coupled with age-related decreases in the efficiency of these reactions results in the production and leakage of  $O_2^{\bullet-}$  from the electron transport chain. MnSOD in the mitochondria react with  $O_2^{\bullet-}$  producing  $H_2O_2$ .  $NO^{\bullet}$  reacts with  $O_2^{\bullet-}$  by radical–radical recombination producing the potentially more reactive species,  $ONOO^-$ , which leads to the nitration of tyrosine [32,99–101] residues, hindering protein activity regulation by sterically blocking the phosphorylation site. The findings of this study are consistent with current literature hypothesizing that oxidative damage during the aging process leads to mitochondrial dysfunction [59,61]. Increased glycolytic enzymes could be a compensatory mechanism reflecting changes in energy production, changes in mitochondrial redox status, and oxidative stress [61] reminiscent of the Warburg effect in cancer [102–105].

Collectively, these results reflect changes in mitochondrial redox potential, glucose metabolism, and protein structure. Data from this study are consistent with recent findings that VitD (a) increases SOD activity, (b) decreases levels of endogenous oxidants, (c) attenuates  $H_2O_2$ -induced changes, (d) decreases release of inflammatory cytokines, and (e) may have natural antioxidant and anti-inflammatory properties [106,107]. In contrast, VitD deficiency contributes to inflammation through increased production of inflammatory cytokines, effects that are attenuated by  $1,25(OH)_2$  VitD supplementation [6,13,108,109].

More recently, VitD has been shown to play potential roles in CNS homeostasis [110]. The VitD receptor (VDR) is expressed in microglia, and  $1\alpha$ -hydroxylase, the enzyme that converts 25-OH VitD to  $1,25(OH)_2$  VitD, is present in activated but not resting microglia [28,111–113]. In activated microglia,  $1,25(OH)_2$  VitD suppresses the production of  $NO^{\bullet}$  and the inflammatory mediators  $TNF\alpha$  and interleukin-6 in a dose-dependent manner, suggesting direct anti-inflammatory roles for VitD in the brain [110]. Local conversion of VitD to the active  $1,25(OH)_2$  VitD in the brain may be a direct neuroprotective response to CNS inflammation followed by inhibition of NF- $\kappa$ B-related iNOS induction [112]. NF- $\kappa$ B leads to mitochondrial dysfunction inhibition of MnSOD through nitration via activation of iNOS, an effect that is absent in iNOS knockout animal models [46].

Our data are consistent with existing evidence that sufficient VitD is known to be anti-inflammatory and to suppress the NF- $\kappa$ B cellular stress response pathway [49,57,114–116].  $H_2O_2$  has been shown to modulate NF- $\kappa$ B activity, thereby helping to regulate NF- $\kappa$ B-dependent processes including inflammation [40,117–119].

During inflammatory events in the CNS, iNOS generates excessive amounts of  $NO^{\bullet}$ . This increased activity of iNOS and resulting overproduction of  $NO^{\bullet}$  may occur in brief bursts. In rat models with experimental allergic encephalomyelitis, Garcion and colleagues showed that  $1,25(OH)_2$  VitD inhibited the iNOS increases [120]. VitD restriction led to slight worsening of clinical symptoms [121]. Nitration of protein-resident tyrosine residues also occurs during brain aging, leading to mitochondrial dysfunction and neurodegeneration [45,96,122].

#### Conclusions

This study is the first to demonstrate that a chronic low-VitD diet and consequential low levels of VitD in the bloodstream result in significant increases in tyrosine nitration in brain proteins, alterations in glucose metabolism, and mitochondrial changes in brain of elderly rats, an animal model of brain in older human subjects (Fig. 8). A shift from the TCA cycle to glycolysis may be indicative of metabolic dysfunction. Further, ATP generated from glycolysis is important for maintaining a proper resting membrane potential in neurons (e.g., via  $Na^+/K^+$  ATPase), which is, in turn, important for neurotransmission. The results of this study are consistent with the notion that nitration of brain proteins occurs via NF- $\kappa$ B activation of iNOS. These results provide biochemical evidence to support the conclusions, consistent with other studies, that suggest that higher serum VitD levels may have direct and indirect antioxidant properties and be beneficial to modulate damaging effects of brain aging. Based on evidence from this study and the literature as noted above, it is our opinion that current daily VitD intake in the general adult population is too low and should be increased.

This study used brain tissue from animals as part of a larger study examining the effects of serum VitD status and brain aging. A component of these larger studies examined learning and memory and a preliminary report indicated that low-VitD animals displayed poorer performance, but that performance improved with high VitD (C.S. Latimer et al., personal communication, 2011). Hence, these data support the present findings and conclusions.

As people age, their lifestyles become more sedentary, physically and mentally. Perhaps because of limited access, physical or financial limitations, or lack of motivation, nutritional status often declines. Concurrently, time spent outside decreases. Less sun exposure further decreases circulating VitD levels. Our studies, together with those of others, indicate that higher VitD may be beneficial for older individuals and, thus, it would seem that further studies are warranted to determine whether VitD supplementation can offset some of the changes associated with unhealthy brain aging. Further studies to address this issue are ongoing in our laboratory.

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