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Aluminum modulates brain amyloidosis through oxidative stress in APP transgenic mice

Domenico Praticò*, Kunihiro Uryu†, Syan Sung*, Sei Tang*, John Q. Trojanowski†, and Virginia M.-Y. Lee†

*Center for Experimental Therapeutics and Department of Pharmacology, †Center for Neurodegenerative Disease Research and Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

Corresponding author: Domenico Praticò, Center for Experimental Therapeutics, Department of Pharmacology, University of Pennsylvania, BRB2/3, Room 812, 421, Curie Blvd., Philadelphia, PA 19104. E-mail: domenico@spirit.gcrc.upenn.edu

ABSTRACT

Epidemiological studies have implicated aluminum (Al) exposure in the pathogenesis of Alzheimer's disease (AD); however, other studies have failed to confirm these results. Oxidative stress is a feature of AD, and Al can exacerbate oxidative events. This biological property has been suggested as a possible mechanism by which this metal could influence the onset and/or evolution of the disease. To test this hypothesis, we fed transgenic mice that over express human amyloid precursor protein (Tg2576) with a diet enriched in Al and measured isoprostane levels, sensitive and specific markers of *in vivo* oxidative stress, as well as amyloid β peptide formation and deposition. Here, we show an increase in brain isoprostane levels that correlated with increased amyloid β levels and accelerated plaque deposition in Tg2576 mice but not in wild-type (WT) littermates fed with high dietary Al. Significantly, these *in vivo* effects of Al were reversed by vitamin E, as judged by a reduction of isoprostane production, amyloid β levels, and plaque deposition. These results indicate that dietary Al can modulate *in vivo* AD-like amyloidosis in Tg2576 by increasing brain oxidative stress.

Key words: Alzheimer's disease • Tg2576 transgenic animal model • lipid peroxidation • isoprostanes • amyloid β protein

Alzheimer's disease (AD) is the most common neurodegenerative disorder of the elderly, affecting approximately 6–8% of all individuals over the age of 65 years. It is characterized by the accumulation of protein deposits in the brain as extracellular amyloid β (Aβ) plaques and as neurofibrillary tangles inside neurons (1). Although the initiating molecular events are not known, in recent years it has become evident that environmental and/or nutritional factors may play a protective or disruptive role in AD (2). Dietary Al has been implicated in the pathogenesis of AD based on epidemiological studies (3, 4) as well as the detection of Al in neuritic plaques (5). In vitro studies have also shown that Al exposure can induce several biological effects implicated in AD pathogenesis, including apoptosis (6), mitochondrial gene expression (7), inflammation (8), and oxidative stress (9, 10). However,

conflicting results have also been reported, and the issue of Al in AD pathogenesis is still controversial and unresolved.

Abundant data have linked oxidative stress to the pathogenesis of AD (11), and isoprostanes (iPs) have been shown to be sensitive and specific markers of *in vivo* lipid peroxidation and oxidative stress (12). Previously, we demonstrated that iPs are increased in selective areas of AD brains, as well as in urine, plasma, and cerebrospinal fluid of AD patients (13, 14). These results add support to the notion that oxidative stress occurs in A Dand provide evidence that iPs are reliable markers of brain oxidative damage. Indeed, our recent data demonstrating that an increase in iPs precede the onset of detectable amyloid plaque deposition in transgenic mice over-expressing human amyloid precursor protein (APP) with the double Swedish mutation (Tg2576) (15) provide additional evidence linking brain lipid peroxidation and amyloid deposition (16).

To investigate the relationship between dietary Al, oxidative stress, and AD-like amyloidosis, we measured the levels of a major iP, that is, 8,12-*iso*-iP$F_{2\alpha}$-VI, as well as amyloid-β peptide levels and deposition in Tg2576 mice and WT littermates fed with either regular chow, chow supplemented with Al, or chow supplemented with Al and vitamin E.

MATERIALS AND METHODS

Animals

The genotypic and phenotypic features of the heterozygote APP swe (K670N, M671L) transgenic mice (Tg2576) and WT littermates studied here have been already described elsewhere (15, 16). A total of 30 Tg2576 and 30 WT mice were studied. They were weaned at 4 weeks of age and kept on a regular chow diet until they were 12 weeks old. Starting at 3 months of age, animals were randomized to receive standard rodent chow (5 males, 5 females), a diet enriched in Al (2 mg/Kg diet) (6 males, 4 females), or a diet enriched in Al plus vitamin E (2000 I.U./Kg diet) (4 males, 6 females). Fresh diets were replaced twice per week and continued until the end of the study, that is, 12 months of age. Urine and blood samples were collected before starting the diets (baseline), then at 6 and 9 months, and finally before killing. Animals were checked continuously for any sign of general toxicity or neurotoxicity. Brain tissues were obtained from each group of animals at the end of the study.

Biochemical analysis

Urine samples were extracted on a C_{18} cartridge column, purified by thin layer chromatography, and finally assayed for 8,12-*iso*-iP$F_{2\alpha}$-VI levels by negative ion chemical ionization gas chromatography/mass spectrometry, as previously described (16). An aliquot from each urine sample was used for the measuring creatinine levels (16). Blood samples were anticoagulated with sodium citrate and centrifuged at 3000 rpm for 10 min to obtain plasma. Plasma 8,12-*iso*-iP$F_{2\alpha}$-VI levels were assayed as described for urine. Vitamin E levels were assayed by high performance liquid chromatography, after hexane extraction, as previously described (17). For Al measurement, mice brains and livers were weighed in clean teflon liners and prepared for analysis by microwave digestion in low trace element-grade nitric acid. Samples were then

analyzed for Al content by using inductively coupled plasma mass spectrometry. All tissue handling during analysis took place in a clean room environment by using HEPA air filtration systems to minimize background contamination. All the assays were always performed in a coded fashion.

Tissue preparation

Animals were anesthetized and killed, following the recommendation of the Panel on Euthanasia of the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania. They were perfused intra-cardially for 30 min with ice-cold 0.9% phosphate buffered saline (PBS), containing ethylene diamine tetraacetate (EDTA) (2 mm/L), and butylated hydroxytoluene (20 mM/L), pH 7.4. Brains were removed, and one hemisphere was fixed by immersion in 4% paraformaldehehyde in 0.1 M PBS (pH 7.4) at 4°C overnight, blocked in the coronal plane, and embedded in paraffin as previously described for immunohistochemistry (16). The other hemisphere was gently rinsed in cold 0.9% PBS then immediately dissected in two anatomical regions (cerebral cortex and hippochampus) for iPs, vitamin E and Aβ measurements. For iPs and vitamin E analysis, tissue was homogenized and total lipids were extracted by using Folch solution (chloroform 2 v: methanol 1 v) (16, 17). Total 8,12-*iso*-iP$F_{2\alpha}$-VI and vitamin E levels were measured as described above. Brain tissues of 10 animals from each group were analyzed, always in a coded fashion.

Aβ sandwich ELISA

Sequential extraction of Aβ peptides from tissue samples was performed with high salt buffer and formic acid to isolate soluble and insoluble brain Aβ(x-40) and Aβ(x-42/43), respectively. Cerebral cortex and hippocampus from each animal were serially extracted with high-salt re-assembly buffer [0.1M Tris, 1mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.5mM $MgSO_4$, 0.75M NaCl, 0.02M NaF, pH 7.0] containing protease inhibitor cocktail (pepstatin A, leupeptin, TPCK, TLCK, soybean trypsin inhibitor, 1 µg/ml of each in 5mM EDTA). Brain tissue was chopped into small pieces and sonicated with 10 burst pulses (level 3) of a Fisher Scientific (Pittsburgh, PA) Sonic Dismembrator Model F60. Homogenates were centrifuged at 100,000 x*g* for 1 h at 4°C. Supernatants were removed and pellets were re-suspended in 70% formic acid, re-sonicated and centrifuged at 100,000 x*g* for 1h at 4°C. Supernatants were removed and diluted 1:20 with 1M Tris base. The supernatant obtained in each extraction step was normalized to the original wet weight of the tissue sample and analyzed separately by ELISA. To perform this, samples were diluted in buffer EC [0.02M sodium phosphate, 0.2M EDTA, 0.4M NaCl, 0.2% bovine serum albumin (BSA), 0.05% (3-[(cholamidopropyl)-dimethylamino]-1-propanesulfonate) (CHAPS), 0.4% Block-ace (Dainippon, Suita, Osaka, Japan), 0.05% sodium azide (pH 7.0), and analyzed by using the monoclonal antibody (mAb) Ban50 to capture and MmAbs BA-27 and BC-05 as reporters to detect Aβ1-40 and Aβ1-42/43, respectively, as previously described (16, 18). Results were expressed as pmoles/g tissue. Analyses were performed in duplicate from 10 animals for each group and always in a coded fashion.

Immunohistochemistry

The burden of Aβ deposits were analyzed by immunohistochemistry with the MAb 4G8, as described previously (16, 18). Series of 6-μm-thick coronal paraffin sections were cut throughout each brain. Sections were deparaffinized, hydrated, and subjected to an antigen-retrieval treatment by immersing sections in 88% formic acid for 60 min. Sections were washed in water and endogenous peroxidases were quenched by using 5% hydrogen peroxide mixture of methanol for 30 min. Subsequently, sections were incubated with 2% normal horse serum in Tris buffer, pH7.4, and were incubated overnight at 4°C with primary antibody (4G8). They were then incubated with biotinylated antibody for 1 h (dilution 1:1000), and the Avidin-biotin complex method was used according to instructions of the vendor (Vector Lab, Inc., Burlingame, CA). Immunoreactive products were visualized by using 3,3' diaminobenzidine as the chromogen. Finally, they were dehydrated with ethanol, cleaned with xylene, and coversliped with Cytoseal. As a control, sections were treated in the same manner, except for the primary antibody. Coronal brain sections from levels between the habenula nucleus and the posterior commissure were subjected to quantitative analysis. Amyloid burden was estimated by counting area occupied by 4G8-positive plaques by using five sections from each animal (n=6 for each group), without a counterstain, by using quantitative image analysis as described previously (16, 18). Light microscopic images from somatosensory cortex (SSC), peri-hippocampal cortex (PHC), and hippocampus (HP) were captured from the series of sections by using a Nikon (Tokyo, Japan) Microphot-FXA, with 4× objective lens, attached to a PC. Each image of selected immunoreactive products was analyzed with image analysis software (Image Pro-plus; Media Cybernetics, Inc. Silver Spring, MD). Manual editing was performed to eliminate nonspecific signals. The areas occupied by Aβ immunoreactive products in the regions of interest were measured, and the total area occupied by the outlined structures was measured to calculate 1) total area with selected immunoreactive products, and 2) the percentage of the area occupied by immunoreactive products over the outlined anatomical area in the image. Analyses were always performed in a coded fashion.

Statistical analysis

Data are expressed as the mean ± SEM and were analyzed by ANOVA and subsequently by a Student's unpaired two-tailed t-test. Significance was set at P<0.05. Correlations between parameters were tested by linear regression analysis.

RESULTS

Consistent with our previous results, Tg2576 on regular chow showed a progressive increase in urinary and plasma 8,12-*iso*-iPF$_{2\alpha}$-VI levels as the animals aged from 3 to 12 months (Fig. 1) (16). However, animals receiving a diet supplemented with Al showed a further increase in 8,12-*iso*-iPF$_{2\alpha}$-VI levels that occurred earlier and attained higher levels when compared with Tg mice on regular chow (Fig. 1A). This increase was already significant after 6 months on Al diet and was nearly doubled by 12 months of age (Fig. 1A). Compared with baseline, WT receiving Al-enriched diet also showed an increase in 8,12-*iso*-iPF$_{2\alpha}$-VI levels. However, the values were much lower than the ones observed in Tg animals on chow (0.64 ± 0.02 vs. 1.2 ± 0.1 ng/mg creatinine, P<0.05). Tg mice receiving supplementation of vitamin E to the diet containing Al

had significantly reduced 8,12-*iso*-iPF$_{2\alpha}$-VI levels to values similar to Tg animals on regular chow (Fig. 1A). Similar opposing effects of Al and vitamin E were observed for plasma 8,12-*iso*-iPF$_{2\alpha}$-VI levels (Fig. 1B). Vitamin E treatment also reduced isoprostane levels in WT receiving Al (0.85 ± 0.03 ng/mg creatinine, $P<0.05$). A direct correlation was found between urinary and plasma levels of 8,12-*iso*-iPF$_{2\alpha}$-VI ($r^2=0.95$, $P<0.001$). Compliance with the vitamin E supplementation was documented by analyzing plasma levels of this vitamin, which increased above control levels by the end of the study (21±2 vs. 65±4 μM, $P<0.001$). Inverse correlations existed between plasma vitamin E and both urinary ($r^2 = –0.81$, $P<0.001$) and plasma 8,12-*iso*-iPF$_{2\alpha}$-VI levels ($r^2 = –0.68$, $P<0.001$).

Animals were killed at 12 months of age, neocorteces as well as hippocampi were dissected, and 8,12-*iso*-iPF$_{2\alpha}$-VI levels were assessed. This analysis showed that Tg2576 receiving Al-supplemented chow had significantly higher levels of 8,12-*iso*-iPF$_{2\alpha}$-VI in both cortex and hippocampus, compared with Tg animals on regular chow (Fig. 2). By contrast, Tg mice receiving vitamin E supplementation with the Al-enriched diet had reduced 8,12-*iso*-iPF$_{2\alpha}$-VI levels similar to the ones observed in Tg2576 on regular chow (Fig. 2). WT receiving Al-supplemented diet had also a slight increase in brain iP levels (21±3 vs. 38±2 pg/mg tissue, $P<0.05$), which was reduced by vitamin E (30%). No signs of somatic toxicity (weight loss, red eyes, fur loss) or neurotoxicity (circling) were observed during the entire study in any animal group. To confirm the presence of Al and vitamin E supplements in the treated animals, we assayed their levels in brain and liver. Brain Al levels were increased significantly in Tg mice receiving the Al-supplemented diet (0.2±0.1 vs. 1.5±0.2 ppm, $P<0.001$), and a similar increase was observed in liver (0.4±0.1 vs. 9.3±0.2 ppm, $P<0.001$). Brain Al levels directly correlated with brain iP levels ($r^2=0.67$, $P<0.05$). Vitamin E concentration was increased significantly in brains (40%) and livers (45%) of mice treated with vitamin E, compared with those on chow.

We next assessed Aβ levels and deposition in brains harvested from 12-month-old mice. Levels of A$\beta_{1–40}$ and A$\beta_{1–42}$ in the soluble and insoluble fractions of brain homogenates were measured by a sensitive Aβ sandwich ELISA. Significantly, Tg2576 on Al-supplemented diet had elevated levels of soluble and insoluble A$\beta_{1–40}$ and A$\beta_{1–42}$ in cortex and hippocampus when compared with Tg mice on chow (Fig. 3). By contrast, WT on Al-enriched diet did not show any detectable levels of A$\beta_{1–40}$ or A$\beta_{1–42}$. A direct correlation was observed between insoluble A$\beta_{1–40}$, A$\beta_{1–42}$ and 8,12-*iso*-iPF$_{2\alpha}$-VI levels ($r^2=0.68$, $r^2=0.65$, respectively; $P<0.01$ for both). Remarkably, Tg2576 receiving Al plus vitamin E-supplemented chow showed no significant increase in Aβ levels (Fig. 3).

Analysis of the amount of Aβ brain deposition (i.e., amyloid plaque burden) by using immunohistochemistry further confirmed these observations. In accordance with published data (15–19), we found that, although significant biochemical changes in Aβ levels occur in Tg2576 mice at 12 months of age, only minimal histological evidence of Aβ deposition occurs in these mice on chow at this age (Fig. 4). By contrast, Tg2576 mice receiving chow supplemented with Al showed an appreciable increase in amyloid plaques deposited in the neocortex and hippocampus (Fig. 4). As predicted, no positive reaction was observed in any WT controls (not shown). Remarkably, Tg mice receiving vitamin E with the Al-supplemented diet had significantly reduced the immunopositive areas to a level similar to the Tg2576 on chow (Fig. 4). Quantitative analyses of the positive areas showed consistently that the amyloid plaque burden

was higher in Tg animals receiving diet supplemented with Al than regular chow in all of the three brain regions examined (somatosensory cortex, peri-hippocampal cortex, and hippocampus) (Fig. 5). By contrast, the amyloid plaque burden was reduced in Tg mice administered vitamin E together with Al (Fig. 5).

DISCUSSION

Our study provides compelling evidence that chronic dietary administration of Al increases Aβ levels and accelerates plaque deposition in a model of AD-like amyloidosis. These effects are consistent with an exacerbation of brain lipid peroxidation because inclusion of Al in the diet increased brain 8,12-*iso*-iPF$_{2\alpha}$-VI, Aβ levels as well as amyloid deposition. Conversely, addition of the antioxidant vitamin E at a dosage that reduced 8,12-*iso*-iPF$_{2\alpha}$-VI levels decreased Aβ levels and amyloid deposition.

Evidence has been accumulating to suggest that certain environmental and dietary factors may play a role in sporadic AD (1, 2), and Al could be one such factor. At an average concentration of about 8% in weight, Al is the third most abundant element in the crust of the earth (20). For a long time Al has been thought to exist predominantly in forms not biologically available to humans and animals (21). Over the past two decades, however, the amount of available Al in biological ecosystem has dramatically increased because of the introduction of a variety of chemicals into the atmosphere and, most of all, because of acid rain, both of which have enormously increased the solubility of this metal (22).

Different lines of evidence have implicated a role for Al in the pathogenesis of AD. First, an elevation of Al concentration in brains from AD patients has been reported (5, 23). Some studies, however, did not confirm these results (10, 20). Second, *in vitro* studies of neuronal cell lines have shown that Al can exacerbate oxidative stress and induce inflammatory reactions, two mechanisms that have been suggested to play a key role in AD pathogenesis (8,9). Finally, epidemiological studies have indicated that Al exposure is an independent risk factor for the development of sporadic AD, but other studies failed to confirm this (3, 10). Because of these conflicting results, the issue of whether Al plays a role in the pathogenesis of AD has not yet been resolved.

Our results provide the first *in vivo* evidence to our knowledge that dietary Al can promote Aβ accumulation and accelerate amyloid pathology in an animal model of AD-like amyloidosis. The effect of Al on amyloidosis in Tg2576 mice is consistent with an increased brain oxidative stress since it correlated directly with the augmented 8,12-*iso*-iPF$_{2\alpha}$-VI levels, sensitive markers of *in vivo* lipid peroxidation (12). Abundant data in literature have linked oxidative stress to the pathogenesis of AD (11). For quite some time, however, the ability to implicate this mechanism directly with AD has been hampered by the nonspecific approaches used to measure it. Isoprostanes, stable end-products of free radical oxidation of polyunsaturated fatty acids, are sensitive and specific markers of lipid peroxidation. Previously, we have shown that they are increased in selected AD brain regions (13), in probable AD (14), and in Tg2576 mice, where their levels directly correlate with the amount of Aβ formation and deposition (16). These findings add support to the notion that oxidative stress occurs in AD, and that iPs are reliable markers of brain oxidative stress. In our current study, the fact that the inclusion of vitamin E, a

suppressor of lipid peroxidation, reduces Aβ levels and amyloid deposition induced by dietary Al, significantly supports this idea and provides further insights into the role of brain oxidative stress in AD amyloidosis.

CONCLUSIONS

How these *in vivo* effects, mediated by dietary Al in transgenic mice over-expressing APP, relate to the effects of Al exposure in humans with sporadic AD is not clear at this time. We are aware of the limitations of our study, some of which are related to the non-perfect animal model we used and some also to the high dosage of Al in the diet. Therefore, our results are not immediately transferable to human AD, and further studies are warranted. In conclusion, considering the variables known or suspected that can influence an individual's susceptibility to AD, such as apolipoprotein E allele status, traumatic brain injury, and family history, we speculate that in these conditions a high Al exposure could promote Aβ formation and deposition and with time AD-like amyloidosis.

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Fig. 1

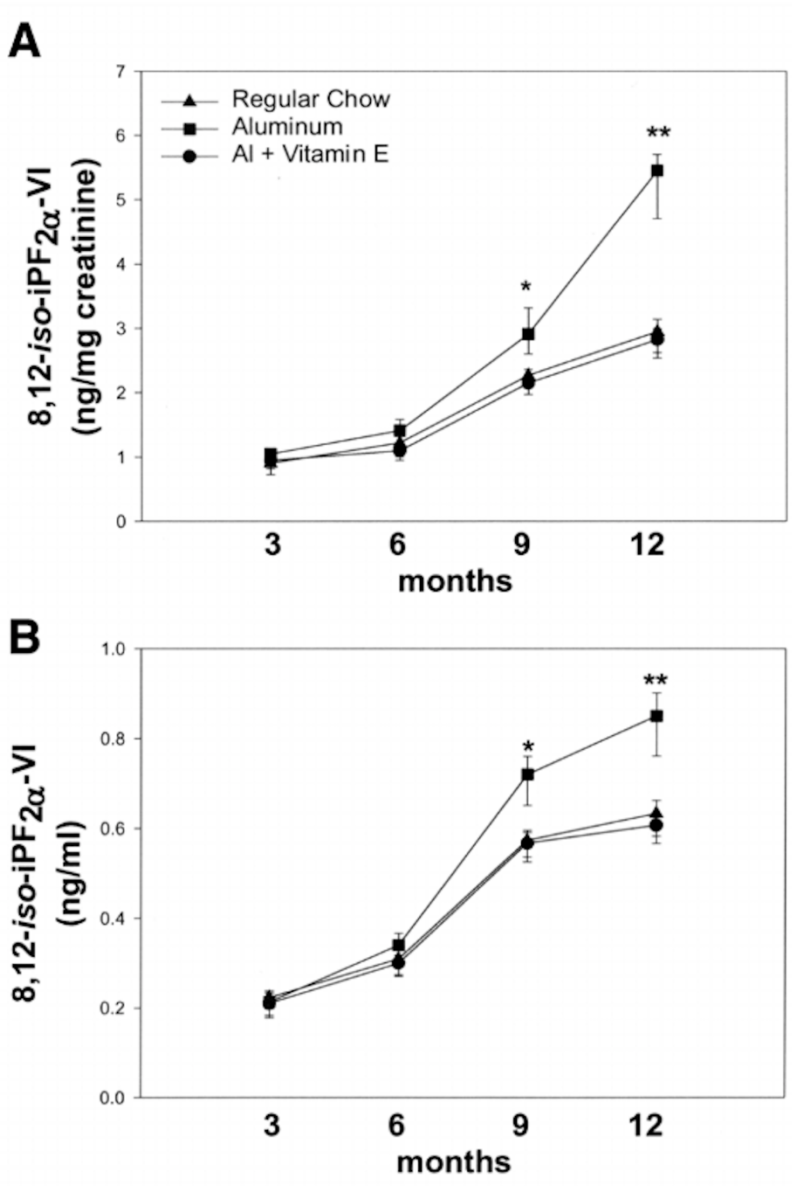

Figure 1. Dietary Al increases urinary and plasma levels of 8,12-*iso*-iPF$_{2\alpha}$-VI. Urinary (**A**) and plasma (**B**) 8,12-*iso*-iPF$_{2\alpha}$-VI levels in Tg2576 mice on a regular chow (triangles), chow supplemented with Al (squares), and chow supplemented with Al plus Vitamin E (circles) at baseline (3 months of age), at 6, 9, and 12 months of age. (*n=10* animals for each group; **P*<0.05, ** *P* <0.001).

Fig. 2

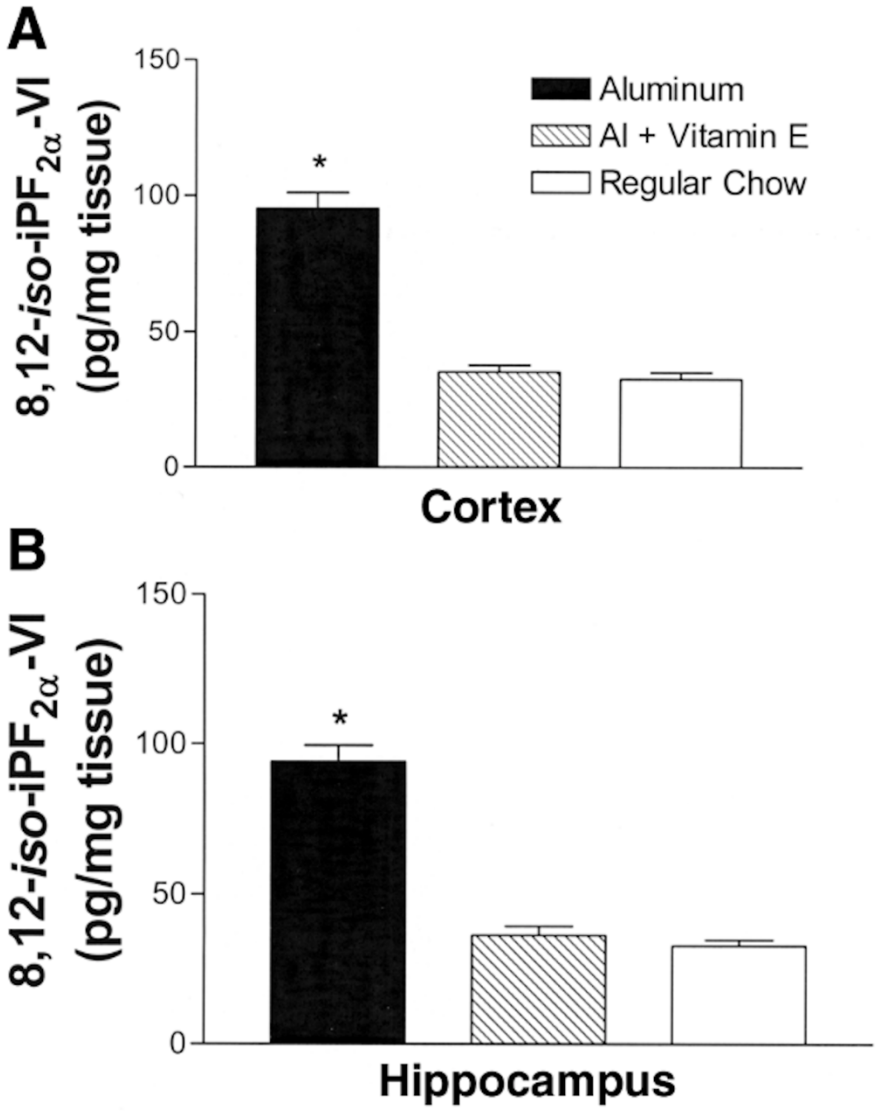

Figure 2. Dietary Al increases brain levels of 8,12-*iso*-iPF$_{2\alpha}$-VI. Total brain cortex **(A)**, and hippocampus **(B)** 8,12-*iso*-iPF$_{2\alpha}$-VI levels in Tg2576 mice on chow supplemented with Al (closed bars), chow supplemented with Al plus Vitamin E (hatched bars), or regular chow (open bars) after 9 months treatments (12 months of age), (*n=10* animals for each group; * $P<0.001$).

Fig. 3

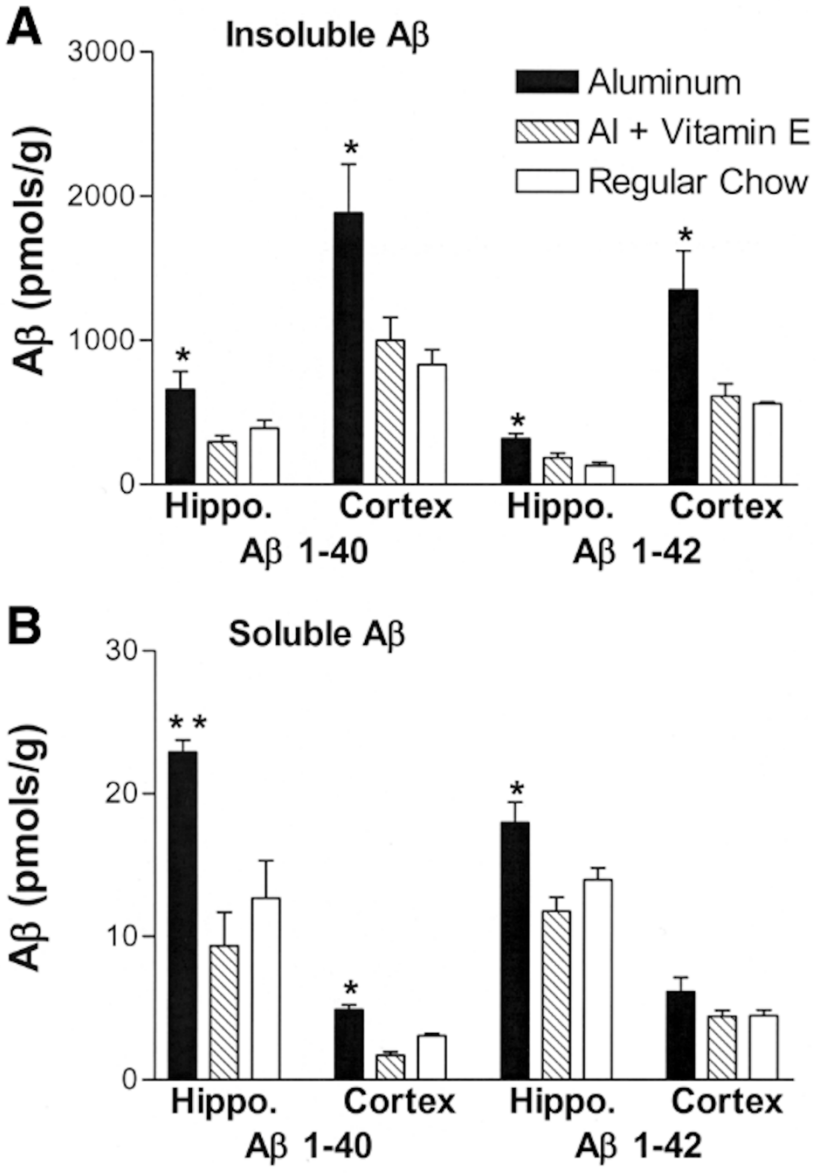

Figure 3. Dietary Al increases soluble and insoluble Aβ levels. Formic acid extraction of insoluble **(A)** and high salt extraction of soluble **(B)** $A\beta_{1-40}$ and $A\beta_{1-42}$ in cerebral cortex and hippocampus of Tg2576 mice on chow supplemented with Al (closed bars), chow supplemented with Al plus Vitamin E (hatched bars), or regular chow (open bars) after 9 months treatments (12 months of age), (*n=10* animals for each group; * $P<0.05$; ** $P<0.001$).

Fig. 4

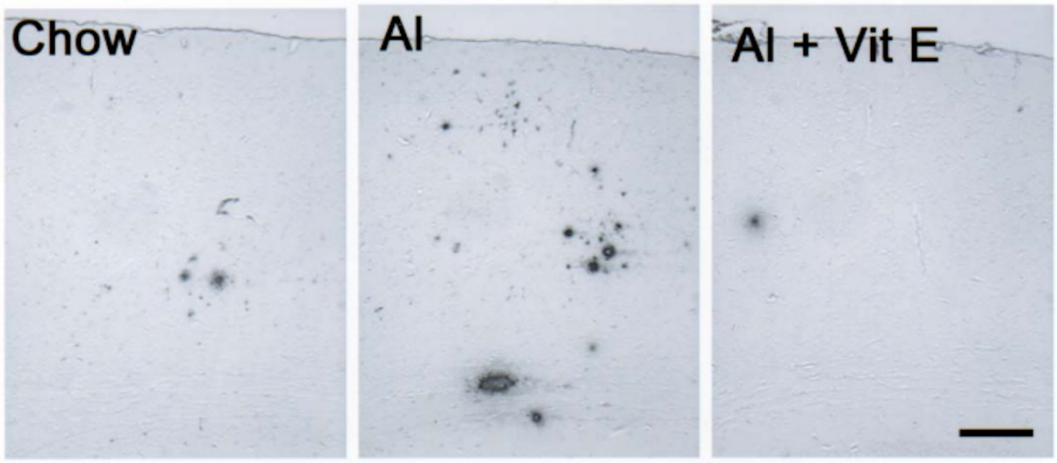

Figure 4. Dietary Al increases amyloid plaque deposition. Representative brain imunohistochemical staining of Tg2576 mice on a regular chow (left), chow supplemented with Al (middle), and chow supplemented with Al plus Vitamin E (right) at 12 months of age. Immunostaining was conducted on the same Tg2576 mice as for brain $A\beta_{1-40}$ and $A\beta_{1-42}$, and 8,12-*iso*-$iPF_{2\alpha}$-VI measurements. All of the panels are at the same magnification. Scale bar, 100 μm.

Fig. 5

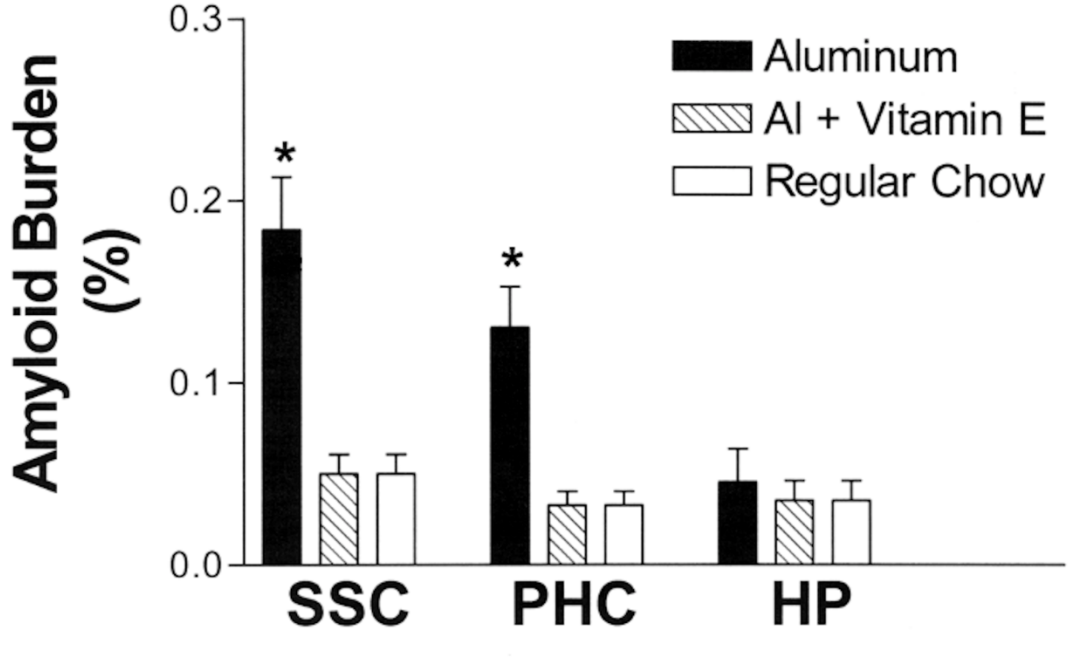

Figure 5. Dietary Al increases amyloid plaque burden. Percentage area of the somatosensory cortex (SSC), perihippocampal cortex (PHC), and hippocampus (HP) occupied by Aβ immunoreactivity deposits as quantified by using the MAb 4G8 in animals on chow supplemented with Al (closed bars), chow supplemented with Al and Vitamin E (hatched bars), or regular chow (open bars) after 9 months treatments (12 months of age), (n=6 animals for each group; *P<0.001).