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Antibodies Against β-Amyloid Reduce Aβ Oligomers, Glycogen Synthase Kinase-3β Activation and τ Phosphorylation In Vivo and In Vitro

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Although active and passive immunization against the β-amyloid peptide (Aβ) of amyloid plaque-bearing transgenic mice markedly reduces amyloid plaque deposition and improves cognition, the mechanisms of neuroprotection and impact on toxic oligomer species are not understood. We demonstrate that compared to control IgG2b, passive immunization with intracerebroventricular (icv) anti-Aβ (1–15) antibody into the AD HuAPPsw (Tg2576) transgenic mouse model reduced specific oligomeric forms of Aβ, including the dodecamers that correlate with cognitive decline. Interestingly, the reduction of soluble Aβ oligomers, but not insoluble Aβ, significantly correlated with reduced τ phosphorylation by glycogen synthase kinase-3β (GSK-3β), a major τ kinase implicated previously in mediating Aβ toxicity. A conformationally-directed antibody against amyloid oligomers (larger than tetramer) also reduced Aβ oligomer-induced activation of GSK3β and protected human neuronal SH-SY5Y cells from Aβ oligomer-induced neurotoxicity, supporting a role for Aβ oligomers in human τ kinase activation. These data suggest that antibodies that are highly specific for toxic oligomer subspecies may reduce toxicity via reduction of GSK-3β, which could be an important strategy for Alzheimer's disease (AD) therapeutics.

Key words: Alzheimer's disease; passive immunization; immunoneutralization; amyloid; neurofibrillary tangles

Alzheimer's disease (AD) is a progressive neurodegenerative disorder leading to dementia during old age. AD is characterized by extracellular amyloid-β (Aβ) plaques and intracellular neurofibrillary tangles (NFTs) comprised of aberrantly phosphorylated τ protein. Although fibrillar Aβ is neurotoxic (Pike et al., 1993; Lorenzo and Yankner, 1994), it is not believed to be the exclusive cause of AD because insoluble Aβ deposits do not predict the severity of dementia in AD patients (McLean et al., 1999). Cognitive deterioration in amyloid precursor protein (APP) transgenic mice precedes extensive plaque deposition and occurs without neuron loss (Moechars et al., 1996; Irizarry et al., 1997; Westerman et al., 2002). Notably, some APP transgenic mice have no insoluble amyloid deposits, but show significant cognitive dysfunction and synaptic damage (Kumar-Singh et al., 2000; Mucke et al., 2000). Recently, soluble Aβ oligomers found in brain extracts from APP transgenic mice and AD patients are increasingly viewed as the major toxic form of Aβ, implicating them in the disease process (Klein et al., 2001; Walsh et al., 2002; Cleary et al., 2005). However, little is known about their impact on τ kinases involved in neurodegeneration.

The relationship between Aβ and τ protein in AD remains unclear. Increasing evidence indicates that Aβ amyloidosis initiates or accelerates τ protein phosphorylation in vivo, even if plaques develop without classic NFTs. Recent studies support a role of Aβ in τ pathology. Bigenic mice expressing mutant τ and APP have enhanced cortical and hippocampal neurofibrillary degeneration (Lewis et al., 2001), whereas injection of Aβ fibrils induced retrograde NFTs in human τ p301L transgenic mice (Gotz et al., 2001). A recent triple transgenic model (mutant APP, PS1, τ) develops both lesions

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in AD-relevant brain regions and anti-Aβ antibody treatment reduced τ accumulation. However, this study did not examine the effect of antibody treatment on τ phosphorylation or τ kinase activation (Oddo et al., 2004). Glycogen synthase kinase (GSK3β) is one strong candidate mediator of Aβ-induced τ protein phosphorylation in vivo. Increased activation of GSK3β was detected in the dystrophic neurites surrounding senile plaque cores in AD (Pei et al., 1999; Hye et al., 2005). The impact of more soluble, diffusible forms of Aβ on phospho-τ (pτ) remains unclear. In this study, using the Tg2576 APP transgenic mouse and human neuronal SH-SY5Y neuroblastoma cells, we investigated the impact of Aβ antibodies and oligomer-specific Aβ antibodies on Aβ deposition and activation of GSK-3β, a crucial signaling pathway involved in Aβ oligomer-induced τ phosphorylation and AD pathogenesis.

MATERIALS AND METHODS

Chemicals and Reagents

Unless otherwise specified, reagents used in this study were obtained from Sigma (St. Louis, MO). Monoclonal antibody used in this experiment was raised against Aβ peptide 1–15 (Yang et al., 1994) whereas control IgG2b was from Sigma. Primary antibodies were: anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-GSK-3α/β[pY279/216] (Biosource, Camarillo, CA) anti-phospho-GSK3β (Ser9) (Cell Signaling, Beverly, MA); anti-GSK3α/β (Biosource); anti-actin (Chemicon International, Temecula, CA); anti-Aβ 6E10 and 4G8 (Signet Labs. Inc., Dedham, MA); anti-phospho-τ AT100 (Innogenetics, Ghent, Belgium). A11 oligomer specific antibody was generously provided by C. Glabe (UC Irvine) and was characterized as described (Kayed et al., 2003). It recognizes only high MW oligomer species, but not dimers, trimers, or tetramers. $A\beta_{42}$ peptide was from (American Peptide, Sunnyvale, CA). The CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit and GelCold SilverSNAP Stain Kit II were from (Promega, Madison, WI) and (Pierce Biotechnology, Rockford, IL), respectively.

Animals and Treatment

Surgical and animal procedures were carried out with strict adherence to the current guidelines set out in the NIH Guide for the Care and Use of Laboratory Animals at the Association for Assessment and Accreditation of Laboratory Animal Care international (AALAC) accredited Greater Los Angeles Healthcare System. Accordingly, all experiments involving animals were approved by the appropriate UCLA and VA Institutional Animal Care and Use Committee (IACUC), Institutional Biosafety (IBC) and Research Development (R&D) Committees. Eleven to twelve-month-old male and female Tg2576 (+) mice were randomly divided into two groups ($n = 6$ for control group; $n = 7$ for treatment group). Mice were anesthetized with 2% Isoflurane and placed into a David Kopf stereotaxic instrument (Tujunga, CA). After placing mice on a Deltaphase isothermal pad (Braintree, MA) and wrapping the body in a Saran Wrap thermoprotector, the surgical site was scrubbed with 4% chlorhexidine and rinsed with 0.5% chlorhexidine tincture in 70% ethanol. Eyes were protected with Duratears (Alcon Upsonic, Fort Worth, TX) and from light with a gauze drape. Using aseptic techniques, a 7-mm incision was made through the scalp skin. After undermining the skin to create a pocket for placement of the osmotic pump, the interior of the pocket was coated with Marcaine, 2 mg/kg, to serve as an adjunct for pain control. A hole was drilled through the skull over the right lateral ventricle at defined stereotaxic coordinates (medial/lateral: +1.0 mm, anterior/posterior: −0.5 mm). Custom length (2.7 mm precut), stainless steel intracerebroventricular (icv) catheters (Plastics One, Roanoke, VA) were implanted to the desired depth (dorsal/ventral: −0.20 mm) and secured with acrylic dental cement (Stoelting Corp., Wood Dale, IL) attached to a screw that did not completely penetrate the skull. The icv cannula was connected to an Alzet osmotic mini-pump (#1002, Durect Corporation, Cupertino, CA) via polyethylene tubing. Control and anti-Aβ IgG2b antibodies were purified with a protein A column followed by an endotoxin-removing column (Pierce Biotechnology) and dialyzed overnight against phosphate buffered saline (PBS). After dialysis, the antibody concentration was determined and diluted to 20 μg/100 μl, which was used to fill the osmotic mini-pumps. The control group received equimolar infusions of non-specific mouse IgG2b. After 14 days, mice were deeply anesthetized with pentobarbital (50 mg/kg bw) and the chest cavity opened, after which cardiac perfusion with protease inhibitor-containing buffer (pH 7.2) was carried out (Lim et al., 2000). After decapitation, the brain was removed and frontal, entorhinal and piriform cortex and remaining ''residual'' cortex from one brain hemisphere, were dissected out and snap frozen as previously described (Lim et al., 2000, 2005). Biochemical measurements of soluble Aβ, insoluble Aβ, Akt, GSK, and τ were carried out on residual cortex as specified. The other brain hemisphere was fixed in 4% paraformaldehyde and processed for immunohistochemistry.

Tissue Preparation

Tissue samples were processed in Tris buffer saline (TBS) (soluble fraction) and lysis buffer (membrane fraction) containing protease inhibitor and phosphatase inhibitor cocktails as previously described (Calon et al., 2004). Briefly, tissue samples were homogenized and sonicated in 10 vol of TBS containing a cocktail of protease inhibitors (1.46 μM pepstatin A, 0.154 μM aprotinin, 2.03 μM leupeptin, 0.5 mM 4-[2-aminoethyl] benzenesulfonyl fluoride hydrochloride [AEBSF], 0.29 mM PMSF) and phosphatase inhibitors (0.05 mM fenvalerate, 0.05 mM cantharidin, 1 mM sodium vanadate, 1 mM sodium pyrophosphate, 50 mM sodium fluoride). Samples were sonicated briefly and centrifuged at 100,000 × *g* for 20 min at 4°C to generate a TBS-soluble fraction. The TBS-insoluble pellet was sonicated in 10 vol of lysis buffer (150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, 1% Triton X-100, 0.5% SDS, and 0.5% deoxycholate) containing the same protease and phosphatase inhibitor cocktails as above. The resulting homogenate was centrifuged at 100,000 × *g* for 20 min at 4°C to produce a lysis buffer-soluble fraction (membrane fraction).

ELISA for Insoluble Aβ Levels

To analyze the detergent-insoluble Aβ, the lysis-insoluble amyloid-laden pellets were sonicated in 8 vol of 5 M guanidine and 50 mM Tris-HCI, and solubilized by agitation at room temperature for 3–4 hr. Insoluble Aβ was measured in the guanidine fraction from control and Aβ antibody-infused animals by sandwich ELISA as described previously (Lim et al., 2005).

Immunocytochemistry

Total number of plaques, including small and large plaques, and plaque burden were assessed using a characterized polyclonal antibody against Aβ 1–13 (DAE) (Lim et al., 2000). Coronal sections were made through anterior (bregma: −1.00 to −1.46 mm), middle (bregma: −1.58 to −2.30 mm), and posterior hippocampus (bregma: −2.46 to −3.16 mm) of mice. Immunolabeling of Aβ positive structures (plaques) was examined in the entorhinal cortex, perirhinal cortex, parietal cortex, retrosplenal-frontal cortex, and hippocampal areas on posterior hippocampal sections of control and Aβ-Ab-infused mice Plaque burden was calculated by dividing total area of Aβ-positive structures by total area of the region analyzed (in square micrometers). Image analysis was carried out as described previously (Lim et al., 2005; Yang et al., 2005). Immunolabeling of GSK3β was examined in various cortical and hippocampal areas of animals as above. Hematoxylin was used as a counterstain.

Preparation of Aβ Oligomers

$A\beta_{42}$ peptide ($A\beta_{42}$) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) in a vial and incubated at room temperature for 20–30 min. The HFIP was removed by gentle streaming of N_2. $A\beta_{42}$ was then dissolved in anhydrous dimethyl sulfoxide (DMSO) and diluted with 10 mM HEPES (pH 7.4) to a final concentration of 110 μM. This solution was incubated at 37°C with a micro stir bar at 500 rpm for 24 hr and centrifuged at 14,000 × *g* for 10 min. The presence of Aβ oligomers was confirmed by silver stain and 4G8 anti-Aβ antibody after its separation on 10–20% Tris-Tricine gradient gels. About 58% of $A\beta_{42}$ converted to 12- and 24-mer, 34% of $A\beta_{42}$ converted to 4-mer, whereas 8% of $A\beta_{42}$ remained in monomer status. These were quantified by scanning silver stained gels and Western blot films using densitometric software (Molecular Analyst II, BioRad, Richmond, CA). The supernatant contained fibril-free oligomers. For cell culture experiments, these oligomers were diluted to 500 nM using growth medium without bovine serum. Protein concentration was determined using the Bio-Rad DC protein assay.

Cell Culture and Treatment

Human neuroblastoma SH-SY5Y cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 2 mM L-glutamine and 10% (v/v) fetal calf serum (FCS). Cells were plated at low density and grown to 80% confluency on 6-well plates at 37°C in a humidified 5% CO_2 atmosphere incubator. Before treatment with A11 anti-Aβ oligomer antibodies (2 ng/ml), cells were rinsed once with serum-free DMEM media. A11 antibodies and serum-free DMEM media were added to cells simultaneously for 1 hr, followed by addition of 500 nM Aβ oligomers directly to cells and incubation for up to 46 hr at 37°C.

Cell Lysate Preparation From SH-SY5Y Cells

SH-SY5Y cells were placed on ice, washed with cold PBS once, scraped with 1 ml of PBS and transferred to a microcentrifuge tube. Cells were centrifuged at 67 g for 5 min at 4°C, washed with cold PBS twice and dissolved in lysis buffer with a cocktail of protease and phosphatase inhibitors. After a brief sonication, the lysate was incubated at 4°C for 30 min, centrifuged at 13,148 × *g* for 10 min, and the supernatants collected.

Cell Viability Assays

The effects of $A\beta_{42}$ oligomers on SH-SY5Y cell viability were determined by measuring the activity of lactate dehydrogenase (LDH) released into the culture medium using a CytoTox 96 Non-Radioactive Cytotoxicity Assay. The assay was carried out in accordance with the manufacturer's protocol and absorption read at 490 nm.

Western Blot Analysis

For Western blot (WB) analysis, protein concentration was determined using the Bio-Rad DC protein assay. Equal amounts of protein per sample were added to Laemmli loading buffer, and boiled for 3 min. Except for Aβ oligomer detection, 30 μg of protein/well was electrophoresed on 10% Tris-glycine gels and transferred to Immobilon-P PVDF membranes (Millipore, Bedford, MA). For Aβ species separation, 4 μg of Aβ oligomer samples were electrophoresed on 10–20% Tris-Tricine gradient gels. Gels were stained with Coomassie blue to ensure equal protein loading. Membranes were blocked for 1 hr at room temperature in 10% non-fat dried milk in PBS, followed by incubation overnight at 4°C with appropriate primary antibodies in PBS containing 0.05% Tween 20 (PBS-T) and 1.5% (W/V) albumin. After being rinsed in PBS-T, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:10,000) or anti-rabbit IgG (1:30,000) in PBS-T with 1.5% albumin for 1 hr. Immuno-labeled proteins were visualized by enhanced chemiluminescence (ECL) detection reagents. Resulting films were scanned and quantified using densitometric software (Molecular Analyst II, BioRad). To strip immunoblots, membranes were incubated for 30 min at 57°C in 100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl (pH 6.8). The membranes were rinsed 3 × 10 min in large volumes of PBS-T, followed by re-probing with appropriate antibodies.

Silver Staining

$A\beta_{42}$ peptide was dissolved in 10 mM HEPES and incubated at 37°C for 24 hr. After centrifugation, the supernatant proteins were separated by 10–20% Tris-Tricine gradient gels and visualized with GelCold SilverSNAP Stain Kit II. The silver staining procedure was carried out in accordance with the manufacturer's protocol.

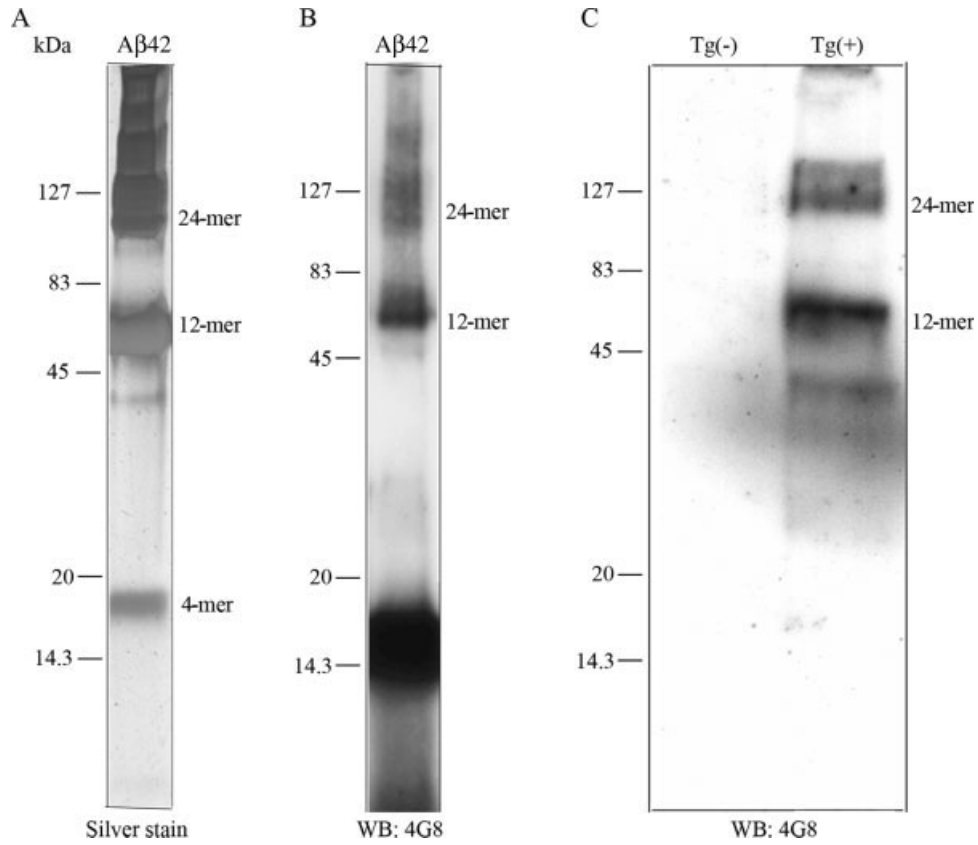

Fig. 1. Silver stain and Western blot comparison of synthetic Aβ oligomer species and natural oligomer species from APPsw Tg2576 mouse. **A:** Silver stain of synthetic $A\beta_{42}$ oligomer in vitro. $A\beta_{42}$ peptides were dissolved in 10 mM HEPES, pH 7.4, and incubated at 37°C for 24 hr. After centrifugation, 4 μg of the supernatant were separated by 10–20% Tris-Tricine gradient gels and visualized with silver stain. The initial $A\beta_{42}$ oligomer preparation showed bands around ~50 kDa (12-mer) and ~110 kDa (24-mer). **B:** The same amount of $A\beta_{42}$ oligomer supernatant solution was loaded on another 10–20% Tris-Tricine gradient gel and visualized by WB with anti-Aβ antibody 4G8. The major bands around ~50 kDa (12-mer) and ~110 kDa (24-mer) of $A\beta_{42}$ oligomers were also clearly detected. **C:** Pooled samples of TBS-soluble cortex fraction from Tg2576 (−) and Tg2576 (+) were separated by 10–20% Tris-Tricine gradient gels and detected by WB with 4G8 antibody. 30 μg of samples per well was electrophoresed. Both ~50 kDa (12-mer) and ~110 kDa (24-mer) were detected in Tg (+) mice, but not in Tg (−) mice.

Statistical Analyses

One-way AVOVA analyses were carried out on all parameters using StatView 5.0 software. *P*-values <0.05 were considered significant. Correlation analysis was carried out by a simple regression test.

RESULTS

Comparison of Aβ Oligomer Species In Vivo and In Vitro

Increases in soluble, oligomeric forms of Aβ have been hypothesized to be very early events in AD and diminish synaptic proteins (Frautschy et al., 2001) and plasticity (Walsh et al., 2002; Cleary et al., 2005). Aβ oligomer species were characterized in vivo and in vitro by silver staining and Western blot analysis in this study. The initial $A\beta_{42}$ oligomer preparation showed prominent silver-stained bands around ~50 kDa (12-mer, dodecamer) and ~110 kDa (24-mer, Fig 1A). The same amount of initial $A\beta_{42}$ oligomer supernatant was immunoblotted with 4G8 (aa 17–24) anti-Aβ antibody. Strong bands of ~50 kDa and ~110 kDa were detected clearly in synthetic $A\beta_{42}$ oligomer preparations (Fig 1B). To examine oligomers in vivo, pooled samples of Tg2576 (−) or Tg2576 (+) mouse cortex (soluble, TBS-fraction) were separated on 10–20% Tris-Tricine gradient gels and detected by WB with anti-Aβ 4G8 antibody, which does not detect sAPPα. Both the ~50 kDa and ~110 kDa bands were detected in Tg (+) mice, not in Tg (−) mice, suggesting that the oligomers found in vivo were similar to the synthetic Aβ oligomers (Fig. 1C).

Anti-Aβ Antibodies Reduce Soluble Aβ Oligomers in Tg2576 Mice

To investigate whether anti-Aβ antibodies reduce soluble Aβ oligomer levels in Tg2576 mice, soluble Aβ

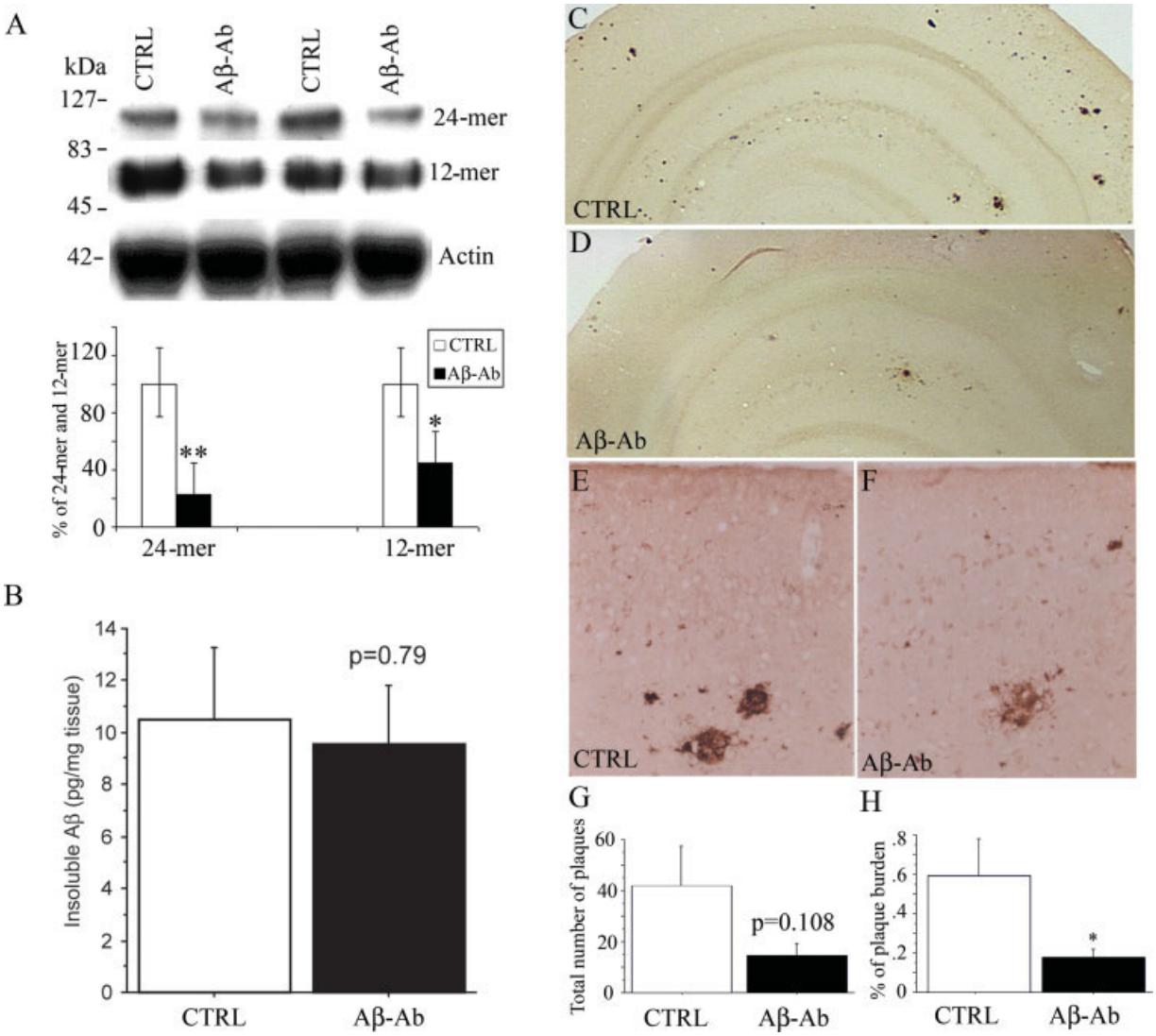

Fig. 2. Reduction or immunoneutralization of soluble Aβ oligomers in the cortex of anti-Aβ infused APPsw Tg2576 mouse. **A:** Aβ ~12-mer and ~24-mer levels in Tg2576 mice from the TBS fraction were determined by WB with 4G8 antibody, which does not recognize the sAPPα, and shown to be significantly decreased in anti-Aβ infused mice by 55% and 76.9%, respectively (*$P < 0.05$; **$P < 0.01$), Aβ oligomers were normalized against actin. **B:** Total insoluble Aβ in Tg2576 mice from guanidine-extracted fraction was assessed by ELISA. Detergent-insoluble Aβ was not reduced in anti-Aβ infused mice (10.5 vs. 9.5 pg/gm tissue) compared to the control IgG2b-infused animals ($P = 0.79$). **C–H:** The morphology of Aβ plaques was analyzed with an Aβ antibody against aa 1–13 in hippocampal areas (C,D) and entorhinal cortex (E,F) from both groups. Anti-Aβ-infused mice had significantly reduced plaque burden ($P < 0.05$; H) compared to control IgG2b-infused mice. A trend toward reducing the total number of plaques was also seen but this reduction did not reach significance ($P= 0.108$; G). Magnification = 4× (C,D); 20× (E,F).

levels from the TBS-soluble fraction of Tg2576 mice were assessed by WB with anti-Aβ 4G8. The results demonstrated that anti-Aβ-infusion significantly reduced levels of Aβ oligomers, including dodecamers by 55% ($P < 0.05$) and 24-mer by 76.9% ($P < 0.01$; Fig. 2A) in Tg2576 mice when compared to IgG2b-infused controls. The ~50 kDa band ''dodecamer'' was confirmed to be an Aβ oligomer and not an IgG heavy chain of the same molecular weight, because it was recognized directly with a biotinylated anti-Aβ antibody (6E10-biotin) without secondary anti IgG antibody (data not shown).

Effects of Anti-Aβ Antibodies on Insoluble Aβ in Tg2576 Mice

Some previous studies have demonstrated that both passive antibody and active peptide immunogen immunization of transgenic mice against Aβ results in markedly reduced insoluble amyloid (Bard et al., 2000; Morgan et al., 2000). Others have shown no reduction

in total insoluble Aβ by ELISA despite decreases in cognitive deficits and plaques (Janus et al., 2000). In our study, detergent-insoluble Aβ was further measured in the guanidine-soluble fraction from cortices of control and Aβ antibody-infused (anti-Aβ infused) animals by sandwich ELISA. Aβ antibody infusion did not reduce detergent-insoluble Aβ (10.5 vs. 9.5 pg/gm tissue) compared to the control IgG2b-infused animals ($P = 0.79$ Fig. 2B). The morphology of Aβ plaques was analyzed with an Aβ antibody against aa 1–13 in hippocampus (Fig. 2C,D) and various cortices (entorhinal cortex shown in Fig. 2E,F) from both groups as described previously (Yang et al., 2005). One-way ANOVA of image analysis data showed a trend toward reduction of total plaques, including small and large plaque in anti-Aβ-infused mice, but the reduction did not reach significance ($P = 0.108$, Fig. 2G), whereas plaque burden was reduced significantly ($P < 0.05$, Fig. 2H), compared to the control IgG2b-infused mice. Plaque burden was calculated by dividing total area of Aβ-positive structures by total area of region analyzed (in square micrometers).

Immunization With Anti-Aβ Antibodies Reduced Aβ Oligomer-Induced Activation of GSK3β: Role of Akt Signaling

The serine-threonine kinase Akt is crucial for neuronal cell survival signaling and inactivates GSK3β via phosphorylation at Ser9. GSK3β phosphorylates τ protein, which in its hyperphosphorylated state is the principal component of NFTs in AD (Alvarez et al., 2002). Therefore, the levels of inactive GSK3β and activated pAkt were assessed by Western blot analysis after passive immunization in Tg2576 mice. Our results indicated that, compared to controls, anti-Aβ infused mice showed increased levels of inactive pGSK3β ($P < 0.05$; Fig. 3A). There was a non-significant increase in pAkt (Ser473) ($P = 0.174$; Fig. 3B) that was highly correlated with pGSK3β ($R^2 = 0.591$, $P < 0.01$, Fig. 3C), consistent with the activation of the "Akt-to-GSK" pathway that exerts inhibitory control over GSK. Active pGSK3β antibody also stained dystrophic neurites around the Aβ plaque cores and neurons in neighboring cortex, and anti- Aβ treatment seemed to reduce the level of neuronal staining that is consistent with Western blot data (Fig. 3D). Total insoluble Aβ by ELISA did not correlate well with pAkt ($R^2 = 0.056$, $P = 0.437$) or phospho-Ser9 GSK3β ($R^2 = 0.098$, $P = 0.297$, data not shown).

Effects of Anti-Aβ Antibodies on Aβ Oligomer-Induced τ Phosphorylation in Tg2576

One prominent amyloid cascade hypothesis for the etiology of AD proposes that the aggregation and deposition of Aβ peptides is the main trigger for τ hyperphosphorylation, NFT formation, synapse degeneration, and neuronal cell death (Hardy and Higgins, 1992; Selkoe, 1999). Recent data, however, support a revision arguing that specific soluble oligomeric forms of Aβ may be the main initiator of aberrant signaling and subsequent neurodegenerative events in AD. Various sizes of Aβ oligomers have been found in soluble brain fraction extracts from human APP mutant transgenic mice and AD patients. We hypothesized that antibodies against N-terminal regions of Aβ would rapidly reduce or immunoneutralize soluble oligomeric forms of Aβ and reduce Aβ-mediated τ phosphorylation in vivo. Therefore, pτ levels were measured from Aβ antibody-immunized Tg2576 mice. WB analysis using the GSK-3β-τ phosphorylation site sensitive antibody, AT100 showed pτ was decreased significantly in Aβ antibody immunized Tg2576 mice, when compared to control IgG2b-infused mice ($P < 0.05$, Fig. 4A). Regression analysis showed that the decrease of pτ correlated significantly with levels of Aβ oligomer species in TBS soluble fractions (Fig. 4B,C). In contrast, total insoluble Aβ by ELISA, a measure of deposited amyloid, was not correlated with AT100 labeled pτ ($R^2 = 0.005$, $P = 0.827$, data not shown).

Oligomer-Specific Antibody Decreased Aβ Oligomer-Induced Activation of GSK3β in SH-SY5Y Cells

To evaluate oligomer involvement in τ phosphorylation, pGSK3β was measured in SH-SY5Y. Cells were pre-incubated with or without A11 antibody (2 ng/ml) for 1 hr followed by treatment with a 500 nM preparation of Aβ oligomers for 1.5 hr, 12 hr, 24 hr, and 46 hr. Cells were collected and inactive pGSK3β levels were assessed by immunoblot. Cells treated with Aβ oligomer alone show significantly decreased levels of inactivated pGSK3β at 12 hr ($P < 0.05$, Fig. 5A). To demonstrate that oligomers and not other forms of Aβ were responsible for this GSK-3β effect, cells were pretreated with A11 anti-oligomer specific antibody to immunoneutralize oligomeric Aβ. The levels of inactive pGSK3β were significantly higher in cells pretreated with A11 antibody when compared to cells treated with $Aβ_{42}$ oligomer alone ($P < 0.01$; Fig. 5A). Total GSK3β (Fig. 5A) and cell viability measured by LDH (Fig. 5B) were unchanged with the increased GSK3β phosphorylation at the end of 12 hr, suggesting that the observed effect was not a result of altered cell viability or other non-specific A11 effects. However, the levels of LDH release in Aβ oligomer-treated cells were significantly increased at 24 hr ($P < 0.01$) and 46 hr ($P < 0.001$), compared to control cells, whereas cells pretreated with A11 antibody significantly prevented the increase of LDH release at 24 hr ($P < 0.01$) and at 46 hr ($P < 0.001$), compared to cells treated with $Aβ_{42}$ oligomer alone (Fig. 5B).

DISCUSSION

We evaluated whether specific reduction in oligomer species without reduction in total insoluble Aβ by ELISA would be sufficient to alter important pathogenic pathways in Tg2576 mice. We demonstrate that a

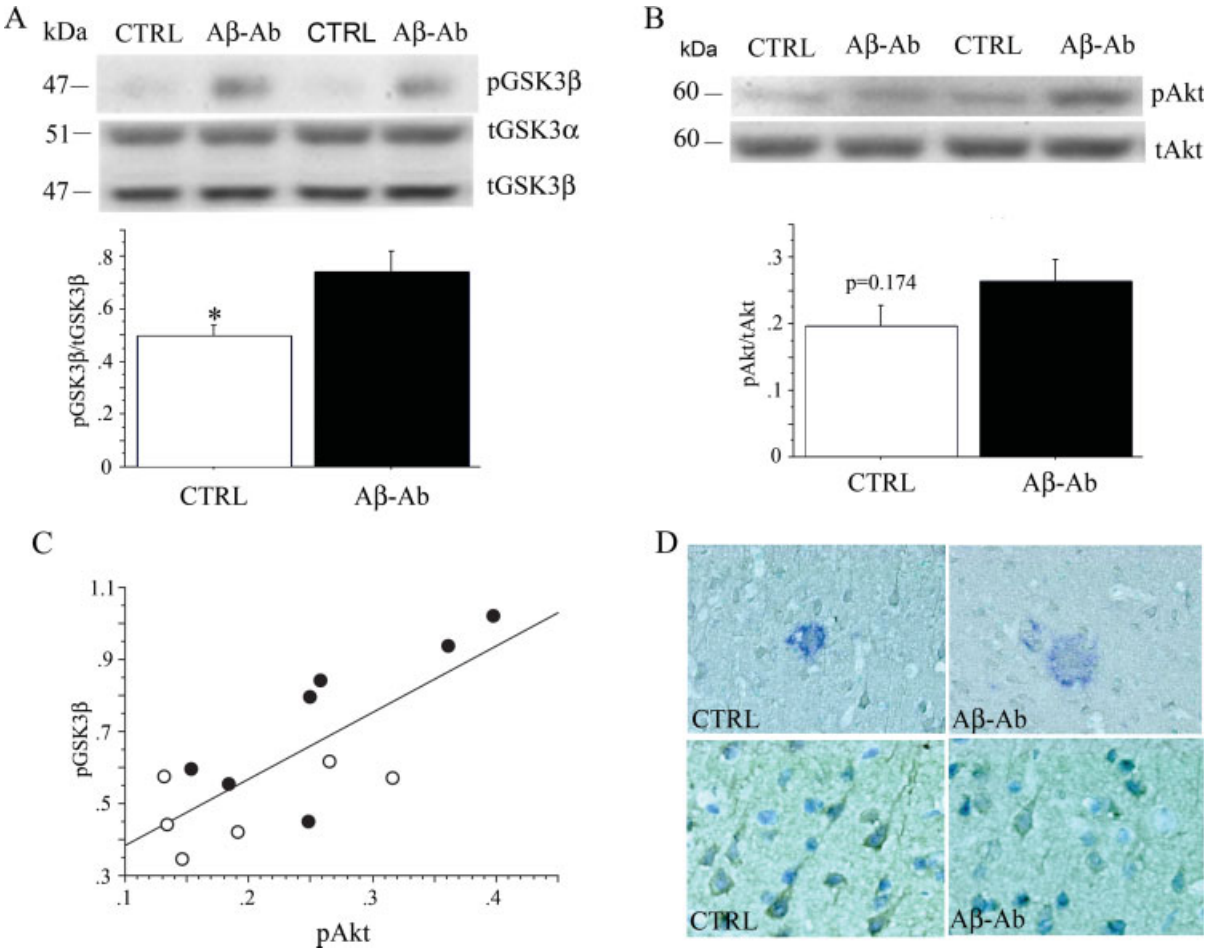

Fig. 3. Passive Aβ immunization reduction in glycogen synthase kinase 3β (GSK3β) activation (increased phospho-GSK3β p-GSK3β), and its correlation with phospho-Akt Ser473 (pAkt) signal transduction in the cortex of anti-Aβ infused APPsw Tg2576 mouse. **A:** Scans and corresponding graphs of pGSK3α and β bands in control- and anti-Aβ infused mice. Anti-Aβ antibodies increased pGSK3β at Ser9 (pGSK3β), the inactive form, in anti-Aβ-infused mice ($*P < 0.05$), when compared to control. pGSK3β was normalized by total GSK3β. Error bars = SEM. **B:** Scans and corresponding graph of pAkt and tAkt bands in control- and anti-Aβ-infused mice. pAkt was non-significantly increased by 34.5% in anti-Aβ-infused mice when compared to control ($P = 0.174$). **C:** Correlation between pAkt at Ser473 and pGSK3β at Ser9 in Tg2576 mice. Increased pAkt at Ser473 significantly correlates with inactive pGSK3β at Ser9 ($R^2 = 0.59$, $P < 0.01$). **D:** Entorhinal cortex regions of brain sections from both groups were double stained with anti-phospho-GSK-3α/β [pY279/216] antibody and 10G4-biotinylated Aβ antibody. Hematoxylin was used to counterstain the nuclei. Anti-GSK3 α/β antibodies labeled dystrophic neurites (dark border) around the Aβ plaque cores and neurons in neighboring cortex, whose staining seems to be reduced with antibody treatment. Magnification = 20× (upper panel); 40× (lower panel).

passive immunization strategy reduced the levels of dodecamers (12-mer) as well as 24-mers of Aβ oligomer species in Tg2576 mice. The reduction in 12-mer and 24-mer was associated with altered AD-associated signaling cascades and did not reduce insoluble amyloid peptides by ELISA. Interestingly, another IgG2b class antibody against Aβ (12A11 and 3A3) as well as IgG1 were less effective than Ig2a in reducing insoluble Aβ perhaps due to their lower affinity for Fc receptors (Bard et al., 2003). These data are in agreement with Bard's contention that antibodies against N-terminus are helpful for neuroprotection but would argue that clearance of insoluble amyloid may not be necessary. In support of this conclusion, Janus et al. (2000) found that Aβ vaccination reduced cognitive deficits without reducing total Aβ by ELISA.

The ability to reduce oligomers without altering total insoluble Aβ may be therapeutically valuable. Although T cells have been implicated in vaccine-related inflammation, vaccine-associated microglial clearance of insoluble amyloid could aggravate the disease process by increasing microglial toxin production, and, in some situations, might be speculated to trigger encephalitis, deaggregate benign sequestered fibrils into toxic oligomers, or increase vascular amyloid deposition.

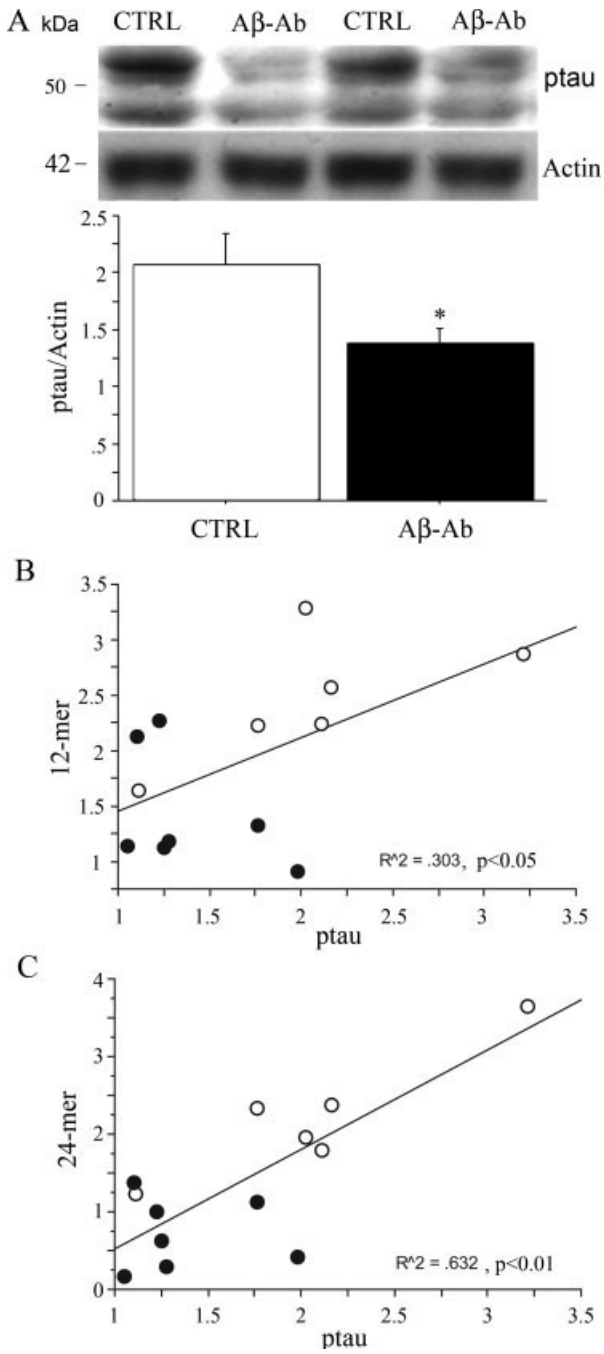

Fig. 4. Reduction of τ phosphorylation in the cortex of anti-Aβ-infused APPsw Tg2576 mouse. **A:** Scans and corresponding graphs of phospho-τ (pτ, AT100) and actin bands of control and anti-Aβ-infused Tg2576 mice. pτ was decreased by 49.6% in anti-Aβ-infused mice, when compared to control IgG2b (*$P < 0.05$). pτ was normalized by actin. Error bars = SEM. **B:** Correlation between AT100 pτ and Aβ dodecamers (*$P < 0.05$, $R^2 = 0.303$). **C:** Correlation between AT100 pτ and 4G8-labelled Aβ 24-mer ($P < 0.01$, $R^2 = 0.632$).

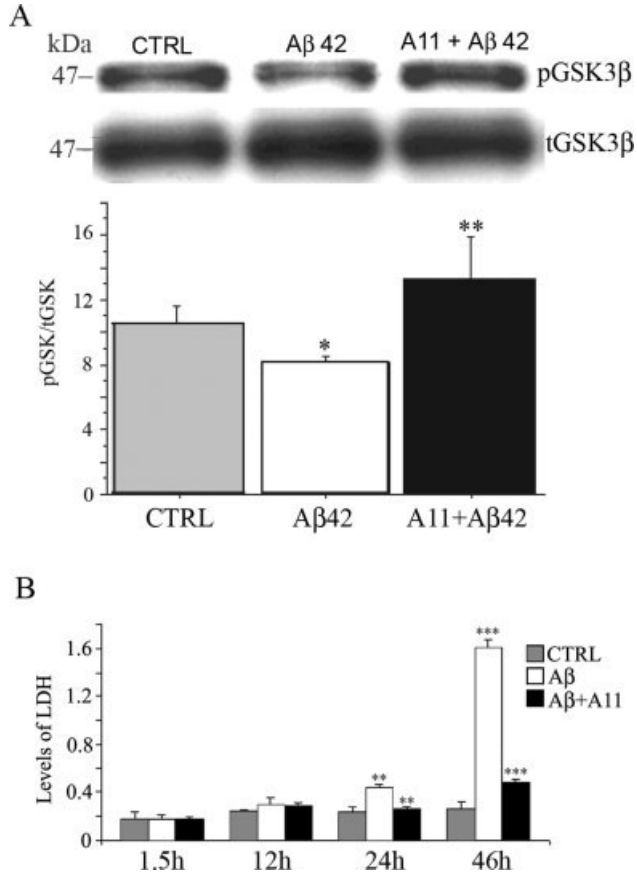

Fig. 5. Aβ oligomer-specific A11 antibody prevents Aβ oligomer-induced activation of GSK3β and neurotoxicity in SH-SY5Y cells. **A:** SH-SY5Y cells were treated with 500 nM of Aβ oligomer, Aβ oligomer plus A11 antibody (2 ng/ml), or control vehicle (rabbit IgG2b) for 1.5 hr, 12 hr, 24 hr, and 46 hr at 37°C. Inactive phospho-GSK3β (pGSK3β) levels were assessed by immunoblot. Cells treated with Aβ oligomer alone show significantly decreased levels of inactivated pGSK3β at 12 hr compared to vehicle ($P < 0.05$), whereas the levels of inactivated pGSK3β were significantly higher in cells pretreated with A11 antibody when compared to cells treated with $A\beta_{42}$ oligomer alone ($P < 0.01$) Total GSK3β levels were unchanged with the increased GSK3β phosphorylation. **B:** LDH release viability assay. Levels of LDH release in Aβ oligomers plus A11 antibody were not significantly different from levels of LDH release with Aβ oligomers alone at 12 hr, demonstrating that the effect on GSK3β occurred before the induction of cell death. However, the levels of LDH release in Aβ oligomer- treated-cells were significantly increased at 24 hr (**$P < 0.01$) and 46 hr (***$P < 0.001$), compared to control cells, whereas cells pretreated with A11 antibody significantly prevented the increase of LDH release at 24 hr (**$P < 0.01$) and at 46 hr (***$P < 0.001$), compared to cells treated with $A\beta_{42}$ oligomer alone (B). Error bars = SEM, $N = 3$ wells/per treatment.

Under abnormal conditions, Aβ is either over-produced or degradation is suppressed, leading to accumulation as specific pathological structures. It remains unclear, however, which forms of Aβ aggregates lead to AD. Previous evidence suggests that various soluble sub-

fibrillar Aβ-derived toxins exist in vivo (Kuo et al., 1996; Funato et al., 1999; Lambert et al., 2001) and correlate well with brain dysfunction and degeneration in humans as well as in transgenic mice (Lue et al., 1999; McLean et al., 1999; Westerman et al., 2002). Recent studies show that Aβ can self-assemble into small, stable, globular assemblies (oligomers) free of fibrils and protofibrils. Denaturing electrophoresis showed that the globular assemblies were comprised of SDS-stable oligomers, ranging from trimers to 24-mers in vitro, and possess neurotoxic properties. However, some species of Aβ oligomers were neither toxic nor recognized by toxicity-neutralizing antibodies, suggesting that Aβ oligomers could assume alternative forms (Chromy et al., 2003). It was demonstrated that Aβ oligomers induced post-synaptic marker loss and cognitive decline in the absence of amyloid deposits (Frautschy et al., 2001). Walsh et al. (2005) demonstrated that the isolated cell-derived Aβ oligomers, but not monomers, block LTP, whereas small molecule hydroxyanaline derivatives RS-0406 and RS-0466 inhibit early Aβ oligomer formation and rescue LTP inhibition. The presence of Aβ oligomers is associated with cognitive dysfunction and may play a critical role in AD. In our study, we demonstrated that the Aβ 12-mer (dodecamer) in Tg2576 was detected as highly stable, soluble molecules and was rapidly reduced by a passive, N-terminal anti-Aβ antibody.

Current evidence suggests that hyperphosphorylation of τ protein is an important pathway driving neurodegeneration in AD. Tau is a microtubule-associated protein (MAP) involved in the regulation of neuronal microtubule assembly and stabilization (Caceres and Kosik, 1990; Avila et al., 1994; Esmaeli-Azad et al., 1994; Harada et al., 1994). GSK3β is a major τ kinase in AD that can efficiently phosphorylate τ protein in vitro (Lovestone et al., 1994; Wagner and Wiederholt, 1996). In AD brains, active GSK3β (phosphorylated at tyrosine 216) shows a similar topographic distribution to hyperphosphorylated τ from very early to late stages of AD neuropathology (Pei et al., 1999). Lithium, a non-specific inhibitor of GSK3β, blocks Aβ-induced τ hyperphosphorylation (Munoz-Montano et al., 1997; Alvarez et al., 1999; Lovestone et al., 1999). Aggregates of Aβ are candidate triggers of τ hyperphosphorylation and the subsequent degeneration of affected neurons (Greenberg et al., 1992; Iqbal et al., 1994; Lovestone and Reynolds, 1997; Spillantini and Goedert, 1998; Lee and Trojanowski, 1999). Several studies have demonstrated that fibrillar Aβ induces τ hyperphosphorylation and cell death in cultured neurons (Busciglio et al., 1995; Takashima et al., 1998). Dual mutant APP and τ transgenic mice display more widespread or accelerated τ pathology (Lewis et al., 2001). In Tg2576 transgenic mouse brain, immunocytochemical staining with both site-specific phosphorylated τ and anti-GSK3β antibodies labeled punctate dystrophic neurites in and around the senile plaque cores (Tomidokoro et al., 2001). Consistent with that, in our study, anti-GSK3β antibodies labeled dystrophic neurites around the Aβ plaque cores and some neurons in neighboring cortex (Fig. 3D). Hence, Aβ aggregates are likely to be involved in τ hyperphosphorylation.

Aβ antibody immunization was shown recently to rapidly reduce plaque-associated dystrophic neurites (Brendza et al., 2005). Our current data show that passive immunization with anti-Aβ antibodies clear soluble 12-mer oligomeric forms of Aβ, while increasing inhibitory phosphorylation of GSK3β and decreasing pτ. The decrease of pτ significantly correlates with decreasing 12-mer and 24-mer of Aβ oligomer species (Fig. 4). We did not detect low MW oligomers along with 12-mer and 24-mer in blots of soluble TBS fractions from Tg2576 treated or untreated mice, hence, treatment effects of these low MW oligomers could not be quantified. We cannot conclude, therefore, whether antibody reduction of low MW oligomers also has an impact on signal transduction. Our in vitro results, however, show that application of preparations, containing 12-mer and 24-mer Aβ oligomers, up-regulated GSK3β activity. The specific conformation dependent anti-oligomer antibody, A11, attenuated this oligomer induction of GSK3β activity in human SH-SY5Y cells because A11 fails to detect monomer and low MW oligomer (Kayed et al., 2003; Lesné et al., 2005). These data provide collective evidence for the hypothesis that high molecular weight Aβ oligomers can induce τ phosphorylation in vitro, explaining our in vivo observation.

In a triple transgenic mouse AD model (APP, PS1, τ) that develops both Aβ plaques and NFTs, passive Aβ immunotherapy led to clearance of early, but not late, hyperphosphorylated τ aggregates (Oddo et al., 2004). The clearance of τ was critically dependent on its phosphorylation state, as late-stage, hyperphosphorylated τ aggregates appeared unaffected by the Aβ antibody treatment. Our results complement these data by showing that passive immunization reduces oligomers, which directly induce GSK3β activation and τ phosphorylation. The mechanism involved may be the loss of inhibitory phosphorylation GSK3β at serine 9 by Akt or integrin linked kinase (ILK). Integrin receptors are potentially involved in Aβ aggregate signaling (Chun et al., 2001; Koenigsknecht and Landreth, 2004) whereas downstream ILK contributes to inhibitory GSK3β phosphorylation (Gary et al., 2003).

Substantial evidence indicates that immunotherapeutic approaches against Aβ markedly improve or slow down the learning and memory deficits in APP transgenic mice and in AD patients (Hock et al., 2003). In Phase I/II clinical trials, however, active Aβ vaccination did not lead to significant titers in all patients and resulted in some AD patients developing CNS inflammation, due apparently to cell-mediated immunity (Gelinas et al., 2004). In contrast with active vaccination, passive immunization with anti-Aβ antibody should circumvent the cell-mediated response while insuring that all patients have functional, high titer antibody levels associated with clinically beneficial outcomes (Hock et al., 2003). In summary, our results strengthen the hypothesis that select soluble oligomeric forms of Aβ

can initiate early events in AD and provide direct evidence that passive immunization with anti-Aβ antibodies rapidly reduce high molecular weight Aβ oligomer species in Tg2576 mice and associated τ phosphorylation by GSK3β. These data support strongly the exploration of specific anti-Aβ oligomer immunogens or antibodies to prevent and treat AD and the use of GSK3β activation state and GSK3β-relevant pτ epitopes, for example in CSF, as functionally relevant endpoints.

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