

showing the most pronounced NH chemical shift movements (greater than 0.02 ppm) were revealed using the Swiss PDB Viewer program (28). These (backbone) regions were labeled with red color and the regions not showing movement with green color.

Cell culture - HEK 293 cells were cultured in 75-cm² flasks (Corning Inc., NY) in Dulbecco's modified Eagle's medium (DMEM)(Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum and incubated in a humidified chamber (85% humidity) containing 5% CO₂ at 37°C. One day before peptide sample treatment, the cell culture medium was replaced with serum-free DMEM, and the cells were trypsinized and re-plated onto coated 96-well plates at a final cell density of 20,000 cells/well.

Cytotoxicity assays - We used to test the toxicity of uncross-linked, cross-linked, cross-linked with Myr, and cross-linked with RA of Aβ42 as assessed in MTT assay. Peptide samples were prepared at Aβ concentrations of 2 μM and 20 μM in 10 mM sodium phosphate, pH 7.4. Aliquots of 50 μl were added to HEK cells to yield final Aβ concentrations of 1 and 10 μM. Twenty hours after the cells were incubated with peptide samples, MTT was added to each well, and the plates were kept in a CO₂ incubator for an additional 2 h. The cells were then lysed by adding lysis solution (50% dimethylformamide, 20% SDS at pH 4.7) and were incubated overnight. The degree of MTT reduction (i.e., cell viability) in each sample was subsequently assessed by measuring absorption at 590 nm at room temperature using a plate reader (PerkinElmer, Turku, Finland). Controls included medium with sodium phosphate ("negative") and 1 μM poly-L-lysine [ave. MW : 75000 Da] (Sigma-Aldrich) ("maximal positive"). Background absorbance values, as assessed from cell-free wells, were subtracted from the absorption values of each test sample. The absorbance measured from the three wells were averaged, and reported as mean ± S.E. percentage of cell viability

$$V = 100 - ((A_{A\beta} - A_{\text{medium}})/(A_{\text{poly-L-lysine}} - A_{\text{medium}})) \times 100$$

where $A_{A\beta}$, A_{medium} , and $A_{\text{poly-L-lysine}}$ were absorbance values from Aβ-containing samples, medium alone, or poly-L-lysine alone, respectively.

Electrophysiology - The fEPSPs were recorded from the CA1 region of acute hippocampal slices derived from C57BL/6 mice (male, 4-5 wk of age). The procedures for slice preparation and electrophysiological recording were described previously (29). Briefly, 300 μm thickness transverse hippocampal slices were placed in a physiological chamber perfused with artificial cerebrospinal fluid (ACSF; 125 mM NaCl, 3.5 mM KCl, 1.25 mM NaH₂PO₄, 25 mM NaHCO₃, 2.0 mM MgSO₄, 2.0 mM CaCl₂ and 20 mM glucose, aerated with a mixture of 95% O₂ and 5% CO₂) at a rate of 1 ml/min at 30°C. Shaffer collaterals/commissural bundle in the CA3 hippocampal subfield were stimulated using a bipolar stainless steel wire electrode at 20-sec intervals throughout the experiment. The fEPSPs were recorded from the stratum radiatum in the CA1 hippocampal subfield using a sharp glass electrode (2-6 MΩ, filled with 2 M NaCl). LTP was induced by tetanic stimulation delivered at 100 Hz for 1 sec. LTD was induced by low frequency 450 paired-pulse stimulation (50 msec paired-pulse interval, 1 Hz) for 7.5 min. LTD was induced only under the presence of cross-linked Aβ42 oligomer in this condition (see Results in detail). Each Aβ42 sample (500 nM in 0.3 mM NaOH in ACSF) or vehicle (0.3 mM NaOH in ACSF) was applied for 20 min so that Aβ42 sample/vehicle application overlapped and coterminated with conditioning stimulation for LTP or LTD. The evoked potential was amplified (x1000), filtered (0.1-1000 Hz), digitized (20 kHz) and stored in a computer for off-line analysis using PowerLab system (AD Instruments, Colorado Springs, CO). LTP and LTD values were presented as the percentage of average fEPSPs slope relative to the mean value of the baseline before conditioning stimulation.

Statistical analysis - One-way factorial ANOVA followed by Tukey-Kramer *post hoc* comparisons were used to determine statistical significance among data sets. These tests were

implemented within GraphPad Prism software (GraphPad Software). Significance was defined as $p < 0.05$.

RESULTS

A β oligomerization - To determine whether the five phenolic compounds blocked formation of low n-order A β oligomers, we used PICUP, a photochemical cross-linking method that is rapid, efficient, requires no structural modification of A β , and accurately reveals the oligomerization state of A β (22). In the absence of cross-linking, only A β 40 monomers (Fig. 2A) and A β 42 monomers and trimers (Fig. 2B) were observed. The A β 42 trimer band has been shown to be an SDS-induced artifact (22). Following cross-linking, and as reported previously (22), A β 40 existed predominately as a mixture of monomers and oligomers of order 2-4 (Fig. 2A,) whereas A β 42 comprised predominately monomers and oligomers of order 2-6 (Fig. 2B).

When 10 μ M Myr was mixed with A β 40 at a peptide:compound ratio of 5:2, oligomerization was blocked slightly (Fig. 2A). The intensity of tetramer band was decreased. When 100 μ M Myr was mixed with A β 40 at a peptide:compound ratio of 1:4, oligomerization was blocked almost completely (Fig. 2A). The effect of Myr on A β 42 oligomerization was equally significant (Fig. 2B). When 10 μ M Myr was mixed with A β 42 at a peptide:compound ratio of 5:2, oligomerization was blocked moderately. The pentamer and hexamer bands disappeared, and the intensity of tetramer band markedly decreased (Fig. 2B). At peptide:compound ratio of 1:4, Myr produced oligomer distributions almost identical to those of untreated A β 42, consistent with an essentially complete inhibition of oligomerization (Fig. 2B). When 10 or 100 μ M RA was mixed with A β 40 or A β 42 at the same ratios as used above, comparable effects were observed on A β oligomerization (Figs. 2A and B).

With A β 40:FA at a 5:2 ratio, no inhibition of oligomerization was observed (Fig. 2A). At a higher concentration of FA (A β 40:FA, 1:4), the trimer and tetramer bands disappeared, and the intensity of dimer band decreased (Fig. 2A). Similarly, with A β 42:FA at a 5:2 ratio, no

inhibition of the oligomerization was observed (Fig. 2B), while at higher concentration of FA (A β 42:FA, 1:4), the pentamer and hexamer bands disappeared, and the intensity of tetramer band weakened (Fig. 2B).

When 10 or 100 μ M NDGA was mixed with A β 40 or A β 42, similar strong effects was observed on A β oligomerization (Figs. 2A and B).

When 10 μ M Cur was mixed with A β 40 at a peptide:compound ratio of 5:2, no inhibition of oligomerization was observed (Fig. 2A). When 100 μ M Cur was mixed with A β 40 at a peptide:compound ratio of 1:4, oligomerization was blocked slightly (Fig. 2A). The intensity of tetramer band was decreased slightly. Similarly, when 10 μ M Cur was mixed with A β 42 at a peptide:compound ratio of 5:2, no inhibition of oligomerization was observed (Fig. 2B). At higher concentration of Cur (A β 42:Cur, 1:4), oligomerization was blocked slightly (Fig. 2B). The intensities of dimer, pentamer, and hexamer band were decreased slightly.

We confirmed dose-dependency of these inhibitions (Fig. S2). The effective concentrations (EC_{50}) of Myr, FA, NDGA, Cur, and RA for the A β 40 oligomerization were 12.4, 76.2, 34.5, 60.8, and 25.6 μ M, respectively. The effective concentrations (EC_{50}) of Myr, FA, NDGA, Cur, and RA for the A β 42 oligomerization were 7.0, 52.6, 38.3, 108.2, and 10.8 μ M, respectively. Taken together, the data indicate that inhibitory activity of A β 40 and A β 42 oligomerization by the phenolic compounds examined in this study may be in the following order: Myr > RA > NDGA = FA \geq Cur.

In the absence of cross-linking, only [Met(O)³⁵]A β 42 monomers were observed (Fig. S3). Following cross-linking, and as reported previously (30), [Met(O)³⁵]A β 42 existed predominately as a mixture of monomers and oligomers of order 2-5 (Fig. S3). When 10 or 100 μ M Myr was mixed with [Met(O)³⁵]A β 42, oligomerization was blocked significantly (Fig. S3). The intensity of trimer, tetramer and pentamer bands disappeared, and the intensity of dimer band markedly decreased. Similar effects were observed when 10 or 100 μ M RA was mixed with [Met(O)³⁵]A β 42 (Fig. S3).

A potential problem relates to the possibility

that the strong inhibition of A β oligomerization could have resulted from an alternative compound, which may form from a possible side-reaction of the inhibitor and the PICUP sensitizer. To evaluate this possibility, cross-linking reactions also were performed on glutathione-S-transferase (GST; ~26 kDa), a positive control for the cross-linking chemistry (31). Uncross-linked GST exhibited an intense monomer band and a relatively faint dimer band (Fig. 2C). Cross-linking produced an intense dimer band, expected because GST exists normally as a homodimer, as well as higher-order cross-linked species. No alterations in GST cross-linking were observed in the presence of Myr, FA, NDGA, Cur, or RA at either of the two protein:compound ratios tested, 5:2 or 1:4 (Fig. 2C). Thus, the significant inhibition of A β 40 and A β 42 oligomerization is from a direct interaction with the phenolic compounds.

A β assembly morphology - To determine the morphology of the small assemblies present following PICUP of A β 40 and A β 42 with or without phenolic compounds, we used AFM. The height of uncross-linked A β 40 was 0.23 ± 0.03 nm (Fig. 2D and Table S1). Following PICUP, the height of A β 40 oligomers became 0.90 ± 0.05 nm (Fig. 2F and Table S1). The height of uncross-linked A β 42 was 0.33 ± 0.02 nm (Fig. 2E and Table S1). Following PICUP, the height of A β 42 oligomers became 1.39 ± 0.09 nm (Fig. 2G and Table S1). These morphologies are not inconsistent with our previous findings (7,23). When A β 40 was cross-linked with Myr at a compound:peptide ratio of 4:1, the height of treated A β 40 decreased to 0.32 ± 0.03 nm (Fig. 2H and Table S1). When A β 40 was cross-linked with RA at a compound:peptide ratio of 4:1, the height of treated A β 40 decreased to 0.42 ± 0.04 nm (Fig. 2J and Table S1). Similarly, when A β 42 was cross-linked with Myr or RA at a compound:peptide ratio of 4:1, the height of treated A β 40 decreased clearly (Figs. 2I, 2K and Table S1).

A β secondary structure dynamics - The above oligomerization studies revealed effects of the phenolic compounds at the initial stages of

peptide self-association. To examine whether the phenolic compounds altered the secondary structure of the A β , we undertook CD studies (Fig. 3). A β 40 and A β 42, incubated alone, produced initial spectra characteristic of statistical coils (SC) (Figs. 3A and B). The major feature of these spectra was a large magnitude minimum centered at ~198 nm. A β 40 displayed substantial secondary structure changes between days 3-5 that were consistent with previously reported SC \rightarrow α -helix/ β -sheet \rightarrow β -sheet transitions associated with monomer \rightarrow protofibril \rightarrow fibril assembly (20). The A β 42 system displayed similar structural changes, although, as expected, these changes occurred at much faster rate (0-2 days). When A β 40 or A β 42 were incubated with Myr at a compound:peptide ratio of 4:1, no such transitions were observed (Figs. 3C and D). Similarly, no such transitions were observed in the presence of RA (Figs. 3E and F). All spectra of A β 40 and A β 42 treated by Myr or RA revealed populations of conformers that were largely statistical coil.

NMR studies - The binding between the phenolic compounds and the A β 42 was explored using NMR spectroscopy, a well-accepted tool for obtaining atomic level aspects of protein structure and ligand binding. The sample preparation protocol and lower NMR probe temperature (5°C) ensured that the A β 42 remained monomeric during the entire data acquisition period (21,32). Standard heteronuclear single quantum coherence (HSQC) spectra were obtained with uniformly 15 N-labeled A β 42, and the compound:peptide was kept at a 20:1 molar ratio. The HSQC experiment detects 1 H signals that are directly bonded to the 15 N atoms, and thus provides a fingerprint of the amide-NH backbone atoms. In the present study, the HSQC data demonstrate that the phenolic compounds do not induce A β Met35^{red} \rightarrow A β Met35^{ox} oxidation (21).

Shown in Fig. 4 are superimposed HSQC spectra of the A β 42 alone (black crosspeaks) and the A β 42 containing RA (Fig. 4A, red crosspeaks) and Myr (Fig. 4B, red crosspeaks). Because the cross peaks of the superimposed spectra in Fig. 4A coincide, this indicates that

RA does not bind with monomeric A β 42. By contrast, the HSQC spectra with Myr show pronounced NH chemical shift movements (labeled peaks in Fig. 4B) indicative of binding. The most pronounced movements (0.02-0.05 ppm) were in the ¹H dimension and were among the R5, V12, H13, K16, L17, V18, F19, F20, A21, E23, D23, I31, and I32 residues. Besides movements, NH peak broadening occurred with R5, V12, K16, and V18, suggesting that the binding may be stronger with these residues. Less peak movements were seen with FA, NDGA, and Cur, and were not localized to any specific peptide region (Fig. S4).

The binding of RA and Myr were further explored using STD experiments, which is a well-established homonuclear NMR technique that permits detection of transient binding of small molecule ligands to macromolecular receptors. Notably, the STD has been used to discriminate ligand binding to monomeric or oligomeric states of the A β that co-exist in solution (24,33,34). In the STD, two separate spectra are obtained and then subtracted, where one spectra involves saturation of a resonance that belongs to the receptor (in this case the A β), while the second spectra involves saturation in a far-removed region that does not contain signals. The presence and strength of signals in the STD is indicative of binding.

Shown in Fig. S5 are three groups of spectral data: A β 40 alone (Figs. S5A and B), A β 40 with RA (Figs. S5C and D), and A β 40 with Myr (Figs. S5E and F). As expected, the STD spectra of A β 40 alone (Fig. S5B) has no signals. By contrast, spectra containing RA (Fig. S5D) has extremely weak, barely-discernable peaks while that containing Myr (Fig. S5F) has obvious peaks. Because solution NMR detects only monomeric A β (21,24), these data demonstrate that Myr binds to monomeric A β while RA does not bind or binds very weakly. Overall, these results are consistent with the above HSQC data.

Highlighted in Fig. 5 are molecular models of the A β 42 that were obtained from a combined molecular dynamics and NMR approach (27). In solution, the A β 42 adopts a rapidly equilibrating ensemble of conformations, which, for the monomeric aggregation state are predominately unstructured (21,32). These models effectively

depict a single, highly-populated conformation that has β -hairpin at residues I31-L34 and G37-I41. The regions showing phenol-induced chemical shifts are colored in red, and because RA did not promote changes (Fig. 4A), only the green unlabeled backbone structure is shown. It is obvious that Myr causes extensive changes throughout the sequence, with continuous 9- and 3-residue spans at K16-V24 and I31-G33. By contrast, FA, NDGA, and Cur interactions are more limited, and interestingly occur at identical residues: R5, S8, G9, H133, K16, D23, and I31 (Cur also causes shifts of L17). With the exception of RA, all phenolic compounds have a proclivity for polar or charged amino acid residues.

Cellular toxicity - The ability of Myr and RA to inhibit formation of low-n A β oligomers suggested that it might be useful in blocking the A β -mediated cellular toxicity. To address this possibility, we performed MTT assays (35) and probed the cellular metabolism of human embryonic kidney (HEK) 293 cells. The MTT assay constitutes a rapid and sensitive method for determination of gross A β toxicity in cultures of dissociated cells (36).

Previous short duration incubation experiments established that the cross-linked A β 40 and A β 42 oligomers were more toxic than uncross-linked oligomers (7,23). In the present study, we explored the immediate consequences (i.e., no incubation time) on the viability of cells. Overall, the results are consistent with the previous study, in that uncross-linked and cross-linked A β 42 oligomers (1 μ M concentration and added immediately to the cells) showed cell viabilities of ~95% and ~79%, respectively. Thus, the cross-linked oligomers were significantly more toxic than uncross-linked oligomers ($p < 0.05$) (Fig. S6). Repeating the experiments with Myr showed almost a complete loss of cell toxicity, with the cross-linked oligomer with Myr producing slightly lower toxicity ($p < 0.05$). Using RA (instead of Myr) also reduced the toxicity to \approx 6%, which was a major reduction relative to cross-linked A β 42 ($p < 0.05$).

Similar observations were made in experiments with A β 42 at higher concentrations

(10 μ M) (Fig. S6). Uncross-linked and cross-linked A β 42 displayed ~53% and ~44% cell viability levels, respectively, revealing a trend towards higher toxicity with cross-linked A β 42. Myr and RA treatment increased cell viability significantly, 69% and 64% ($p < 0.01$) higher, respectively. In summary, regardless of the cross-linked state, Myr and RA are effective in disrupting the cellular toxicity associated with low-n A β 42 oligomers.

Electrophysiology - To obtain an index of A β 42-induced functional alteration of synaptic transmission, we analyzed LTP and LTD in the CA1 region of mouse hippocampal slices. Synaptic current strength was estimated from field excitatory postsynaptic potentials (fEPSP) slope (Figs. 6A and B). The vehicle group indicated LTP by tetanus stimulation ($150 \pm 6.7\%$). Cross-linked A β 42 completely inhibited induction of LTP ($97.5 \pm 7.6\%$) without any alterations in baseline transmission. In contrast, cross-linked A β 42 treated with Myr and RA induced LTP comparable to that of the vehicle alone ($157 \pm 5.1\%$ and $144 \pm 11.1\%$, respectively). Uncross-linked A β 42 also induced LTP comparable to that in the vehicle ($140 \pm 10.1\%$). Fig. 6C shows differences in LTP induction among the five treatment groups. There was a significant group effect on %fEPSP slope [$F(4,22) = 6.295, p < 0.005$]. The post-hoc tests showed that %fEPSPs slope in the cross-linked A β 42 group was significantly lower than those in the other four groups (Fig. 6C, $**p < 0.01$), indicating that cross-linked A β 42 induced LTP suppression, but cross-linked A β 42 treated with Myr and RA did not.

Low frequency conditioning stimulation (1 Hz, 450 paired-pulse) induced a transient decrease in %fEPSP slope beyond the 95% lower confidence limit of the baselines in all groups (Figs. 6D and E). This short-term decrease in synaptic transmission gradually recovered, and the vehicle group showed %fEPSP slope comparable to the baseline level ($104.2 \pm 10.1\%$) during 50-60 min after LTD induction. The cross-linked A β 42 with Myr and RA as well as uncross-linked A β 42 groups also showed %fEPSP slopes comparable to the baseline level ($102 \pm 2.6, 94 \pm 4.2, \text{ and } 105 \pm 9.4\%$, respectively) during 50-60

min after LTD induction. However, the cross-linked A β 42 group showed LTD ($69 \pm 6.6\%$) by low frequency conditioning stimulation. Fig. 6F shows differences in LTD induction among the five treatment groups. There was a significant group effect on %fEPSP slopes [$F(4,20) = 6.925, p < 0.005$]. The post-hoc tests showed that fEPSP in the cross-linked A β 42 group was significantly lower than that in the other four groups (Fig. 6F, $*p < 0.05, **p < 0.01$), indicating that cross-linked A β 42 induced LTD facilitation, but not cross-linked A β 42 treated with Myr and RA.

DISCUSSION

We previously reported that phenolic compounds are effective *in vitro* inhibitors of fA β formation and could destabilize preformed fA β (14-16). Our subsequent *in vivo* work with Tg2576 mice established that Myr and RA reduced the amount of A β oligomers in the brain and prevented the development of AD pathology (18). The purpose of the present study was to unravel chemical and neurophysiological bases for these effects, which could accelerate the development of more effective aggregation inhibitors.

Mechanism of Polyphenol Inhibition to Amyloid Formation - Fig. S7 presents a summary of how the polyphenols interfere with A β aggregation. The monomer \rightarrow soluble oligomer \rightarrow soluble β -sheet oligomer \rightarrow protofibril \rightarrow mature fibril is generally recognized as the normal "on-pathway" process associated with plaque formation of the A β and other amyloid forming proteins. The PICUP studies revealed that all five phenolic compounds dose-dependently inhibited A β 40 and A β 42 later-stage oligomerization, and the PICUP method requires that monomers be in close proximity for covalent cross-linking to occur. The CD studies showed that Myr and RA stabilized A β populations comprising mostly random coil and inhibited SC \rightarrow β -sheet conversion, which was consistent with the PICUP data. However, at the atomic level, NMR showed that Myr and RA behave differently, in that Myr shows significant

binding to monomeric A β 42, whereas RA does not bind to the monomer. This result is intriguing, in that it is possible that RA could prevent aggregation by binding with non-NMR detectable early-formed oligomers (dimers, trimers, etc.)(37), and in doing so prevents neurotoxicity by binding to an exposed toxic structural motif. Other A β binding compounds bind in a similar manner, including human serum albumin (33), apolipoprotein E3 (38), and alcohol dehydrogenase (39). Another possibility is that RA binds to distinct monomer conformers/structures that in turn inhibit oligomerization (40).

The NMR studies also established that Myr, NDGA, FA, and Cur bind to localized or specific monomeric A β peptide regions (Fig. 5), which are R5, S8, G9, H13, K16, D23, and I31. The polyphenol (-)-epigallocatechin gallate prevents α -synuclein (α S) (41) and A β 42 aggregation by also binding to small (localized) amino acid regions. Related work (42) reported that the flavonoid baicalein stabilized a partially folded conformer of α S that existed within oligomeric assemblies. Conway *et al.* (43) showed that dopamine or levodopa inhibits the fibrillization of α S filaments, presumably through stabilization of α S into protofibrillar structures unable to form fibrils. Taniguchi *et al.* (44) reported the formation of tau oligomers in the presence of phenothiazines, polyphenols, or porphyrins. In each of these cases, the inhibitors stabilized oligomeric states, in which the respective protein maintained at least a partial fold. For A β , if the "oligomer cascade" hypothesis is true, (2) aggregation inhibitors that stabilize oligomers could produce peptide populations of enhanced toxicity. The observation that some of the phenolic compounds bind monomeric A β and prevent its oligomerization is a particularly important feature.

Polyphenol Inhibition of Neuronal Toxicity and Relationship to In Vivo Studies - With our previous Tg2576 mice study (18), RA was very promising because it inhibited both HMW A β oligomerization and A β deposition, while FA did not inhibit either process. However, our present *in vitro* studies demonstrate that all

phenolic compounds (including FA) prevent low order (< 10 monomers) oligomer formation. This discrepancy could be due to the non-specific nature of the A11 antibody that was used to detect oligomers in the Tg2576 mice (18), particularly when compared to cross-linked oligomers detected by the PICUP. Similar findings were reported by Necula *et al.*, (45) where dot blot assays with the A11 antibody showed that Myr, NDGA, and Cur inhibited HMW A β oligomerization but not fibrillization.

Myr and RA decreased A β oligomer-induced synaptic toxicities using LTP and LTD assays of hippocampal slices. LTP and LTD are considered important neurophysiological models of memory and learning and are used as experimental models of neuronal plasticity (46). Townsend *et al.* found that A β trimers fully inhibit LTP, whereas dimers and tetramers have an intermediate potency (4). Dimers and trimers from the conditioned medium of APP-expressing CHO cells have been found to cause progressive loss of synapses in organotypic rat hippocampal slices (5). A β oligomers extracted from AD brains potently inhibited LTP and enhanced LTD, indicating disruption of synapse structure and function (6). Our present work is consistent with previous studies (4-6), where A β oligomers suppressed LTP and facilitated LTD induction. Both suppression of LTP and facilitation of LTD induction in the hippocampal CA1 subfield suggests that memory formation is disturbed by A β oligomers. In contrast, A β monomers resulting from reaction with phenolic compounds did not induce such effects. These findings suggest that phenolic compounds have preventive effects on A β -induced memory deficits. Recent work suggested that A β -induced inhibition of LTP was due to a decrease in density of the NR2B subunit of NMDA receptor at the postsynaptic membrane, and facilitation of LTD was ascribed to an increase in glutamate concentration at the synaptic cleft (47,48). These studies suggest that A β toxicity could selectively affect these synaptic functions with usual cell functions relatively intact in brief exposure. Therefore, deficits in A β -induced neuronal plasticity observed in the present study might be related to early neurophysiological alterations

prior to histopathological changes such as neuronal loss and brain atrophy in AD.

Alternative Mechanisms and Benefits of Polyphenol Treatment for AD - The benefits afforded by certain polyphenols to humans or in animal studies (18) may not be just from an anti-amyloid mechanism. It could happen that in some cases the polyphenols may promote $A\beta_{Met35}^{red} \rightarrow A\beta_{Met35}^{ox}$ oxidation, even though we did not detect this in the present *in vitro* studies. Leong *et al.* (49) reported that dopamine could oxidize methionine of αS peptide, and in the process alter the aggregation properties of the protein.

Myr has antioxidant, anti-inflammatory, anticarcinogen, and antiviral activities (50), and can likewise act as a β -secretase inhibitor that reduces $A\beta$ production in a cell cultures (51). FA protects neurons against $A\beta$ -induced oxidative stress and neurotoxicity *in vitro* (52), and long-term administration protects mice against $A\beta$ -induced learning and memory deficits *in vivo*. In fact, it has been now firmly established that all polyphenols have related biological activities, most of which are beneficial *in vivo*.

Another possibility is that the polyphenols could slow down normal age-related events such

as synaptic dysfunction, and in doing so prevent microglial activation and attendant inflammatory responses that lead to neuronal loss and dementia (53). It is also possible that the beneficial effects may not come from the polyphenols themselves, but rather from polyphenol metabolites, which can be generated by microbial enzymes in the colon (54). Once produced, these metabolites could enter the brain and reduce the inflammation associated with AD.

Conclusions - Our results established that the phenolic compounds inhibit the oligomerization and the SC \rightarrow β -sheet conversion of the $A\beta_{40}$ and $A\beta_{42}$, while NMR revealed possible binding interaction sites of the phenolic compounds. In addition, the MTT, LTP and LTD assays established that the phenols inhibit $A\beta$ oligomer-induced cellular and synaptic toxicities. While the exact *in vivo* mechanism behind the benefits of polyphenols remains to be established, the present data, coupled with previously reported antioxidant and ameliorative effects, suggest that phenolic compounds are worthy therapeutic candidates for AD.

REFERENCES

1. Haass, C., and Selkoe, D. J. (2007) *Nat Rev Mol Cell Biol* **8**, 101-112
2. Roychaudhuri, R., Yang, M., Hoshi, M. M., and Teplow, D. B. (2009) *J Biol Chem* **284**, 4749-4753
3. Ono, K., and Yamada, M. (2011) *J Neurochem* **117**, 19-28
4. Townsend, M., Shankar, G. M., Mehta, T., Walsh, D. M., and Selkoe, D. J. (2006) *J Physiol* **572**, 477-492
5. Shankar, G. M., Bloodgood, B. L., Townsend, M., Walsh, D. M., Selkoe, D. J., and Sabatini, B. L. (2007) *J Neurosci* **27**, 2866-2875
6. Shankar, G. M., Li, S., Mehta, T. H., Garcia-Munoz, A., Shepardson, N. E., Smith, I., Brett, F. M., Farrell, M. A., Rowan, M. J., Lemere, C. A., Regan, C. M., Walsh, D. M., Sabatini, B. L., and Selkoe, D. J. (2008) *Nat Med* **14**, 837-842
7. Ono, K., Condron, M. M., and Teplow, D. B. (2009) *Proc Natl Acad Sci U S A* **106**, 14745-14750
8. Orgogozo, J. M., Dartigues, J. F., Lafont, S., Letenneur, L., Commenges, D., Salamon, R., Renaud, S., and Breteler, M. B. (1997) *Rev Neurol (Paris)* **153**, 185-192
9. Truelsen, T., Thudium, D., and Gronbaek, M. (2002) *Neurology* **59**, 1313-1319
10. Flamini, R. (2003) *Mass Spectrom Rev* **22**, 218-250
11. Marambaud, P., Zhao, H., and Davies, P. (2005) *J Biol Chem* **280**, 37377-37382
12. Sharma, R. A., Gescher, A. J., and Steward, W. P. (2005) *Eur J Cancer* **41**, 1955-1968
13. Ganguli, M., Chandra, V., Kamboh, M. I., Johnston, J. M., Dodge, H. H., Thelma, B. K., Juyal, R.

- C., Pandav, R., Belle, S. H., and DeKosky, S. T. (2000) *Arch Neurol* **57**, 824-830
14. Ono, K., Yoshiike, Y., Takashima, A., Hasegawa, K., Naiki, H., and Yamada, M. (2003) *J Neurochem* **87**, 172-181
 15. Ono, K., Hasegawa, K., Naiki, H., and Yamada, M. (2004) *J Neurosci Res* **75**, 742-750
 16. Ono, K., Hirohata, M., and Yamada, M. (2005) *Biochem Biophys Res Commun* **336**, 444-449
 17. Wang, J., Ho, L., Zhao, W., Ono, K., Rosensweig, C., Chen, L., Humala, N., Teplow, D. B., and Pasinetti, G. M. (2008) *J Neurosci* **28**, 6388-6392
 18. Hamaguchi, T., Ono, K., Murase, A., and Yamada, M. (2009) *Am J Pathol* **175**, 2557-2565
 19. Zhao, Y., Hui, J., Wang, D., Zhu, L., Fang, J. H., and Zhao, X. D. (2010) *Chem Pharm Bull (Tokyo)* **58**, 1324-1327
 20. Ono, K., Condrón, M. M., Ho, L., Wang, J., Zhao, W., Pasinetti, G. M., and Teplow, D. B. (2008) *J Biol Chem* **283**, 32176-32187
 21. Hou, L., Shao, H., Zhang, Y., Li, H., Menon, N. K., Neuhaus, E. B., Brewer, J. M., Byeon, I. J., Ray, D. G., Vitek, M. P., Iwashita, T., Makula, R. A., Przybyla, A. B., and Zagorski, M. G. (2004) *J Am Chem Soc* **126**, 1992-2005
 22. Bitan, G., and Teplow, D. B. (2004) *Acc Chem Res* **37**, 357-364
 23. Ono, K., Condrón, M. M., and Teplow, D. B. (2010) *J Biol Chem* **285**, 23186-23197
 24. Narayanan, S., and Reif, B. (2005) *Biochemistry* **44**, 1444-1452
 25. Milojevic, J., Esposito, V., Das, R., and Melacini, G. (2007) *J Am Chem Soc* **129**, 4282-4290
 26. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) *J Biomol NMR* **6**, 277-293
 27. Sgourakis, N. G., Yan, Y., McCallum, S. A., Wang, C., and Garcia, A. E. (2007) *Journal of Molecular Biology* **368**, 1448-1457
 28. Guex, N., and Peitsch, M. C. (1997) *Electrophoresis* **18**, 2714-2723
 29. Soeda, Y., Tsuneki, H., Muranaka, H., Mori, N., Hosoh, S., Ichihara, Y., Kagawa, S., Wang, X., Toyooka, N., Takamura, Y., Uwano, T., Nishijo, H., Wada, T., and Sasaoka, T. (2010) *Mol Endocrinol* **24**, 1965-1977
 30. Bitan, G., Tarus, B., Vollers, S. S., Lashuel, H. A., Condrón, M. M., Straub, J. E., and Teplow, D. B. (2003) *J Am Chem Soc* **125**, 15359-15365
 31. Fancy, D. A., and Kodadek, T. (1999) *Proc Natl Acad Sci U S A* **96**, 6020-6024
 32. Yan, Y., and Wang, C. (2006) *J Mol Biol* **364**, 853-862
 33. Milojevic, J., Raditsis, A., and Melacini, G. (2009) *Biophys J* **97**, 2585-2594
 34. Huang, H., Milojevic, J., and Melacini, G. (2008) *J Phys Chem B* **112**, 5795-5802
 35. Abe, K., and Saito, H. (1998) *Neurosci Res* **31**, 295-305
 36. Ronicke, R., Klemm, A., Meinhardt, J., Schroder, U. H., Fandrich, M., and Reymann, K. G. (2008) *PLoS One* **3**, e3236
 37. Fawzi, N. L., Ying, J., Torchia, D. A., and Clore, G. M. (2010) *J Am Chem Soc* **132**, 9948-9951
 38. Evans, K. C., Berger, E. P., Cho, C. G., Weisgraber, K. H., and Lansbury, P. T., Jr. (1995) *Proc Natl Acad Sci U S A* **92**, 763-767
 39. Yan, Y., Liu, Y., Sorci, M., Belfort, G., Lustbader, J. W., Yan, S. S., and Wang, C. (2007) *Biochemistry* **46**, 1724-1731
 40. Rojas, A., Liwo, A., Browne, D., and Scheraga, H. A. (2010) *J Mol Biol* **404**, 537-552
 41. Ehrnhoefer, D. E., Bieschke, J., Boeddrich, A., Herbst, M., Masino, L., Lurz, R., Engemann, S., Pastore, A., and Wanker, E. E. (2008) *Nat Struct Mol Biol* **15**, 558-566
 42. Zhu, M., Rajamani, S., Kaylor, J., Han, S., Zhou, F., and Fink, A. L. (2004) *J Biol Chem* **279**, 26846-26857
 43. Conway, K. A., Rochet, J. C., Bieganski, R. M., and Lansbury, P. T., Jr. (2001) *Science* **294**, 1346-1349
 44. Taniguchi, S., Suzuki, N., Masuda, M., Hisanaga, S., Iwatsubo, T., Goedert, M., and Hasegawa, M. (2005) *J Biol Chem* **280**, 7614-7623
 45. Necula, M., Kaye, R., Milton, S., and Glabe, C. G. (2007) *J Biol Chem* **282**, 10311-10324
 46. Martin, S. J., Grimwood, P. D., and Morris, R. G. (2000) *Annu Rev Neurosci* **23**, 649-711
 47. Snyder, E. M., Nong, Y., Almeida, C. G., Paul, S., Moran, T., Choi, E. Y., Nairn, A. C., Salter, M. W., Lombroso, P. J., Gouras, G. K., and Greengard, P. (2005) *Nat Neurosci* **8**, 1051-1058

48. Li, S., Hong, S., Shepardson, N. E., Walsh, D. M., Shankar, G. M., and Selkoe, D. (2009) *Neuron* **62**, 788-801
49. Leong, S. L., Pham, C. L., Galatis, D., Fodero-Tavoletti, M. T., Perez, K., Hill, A. F., Masters, C. L., Ali, F. E., Barnham, K. J., and Cappai, R. (2009) *Free Radic Biol Med* **46**, 1328-1337
50. Ong, K. C., and Khoo, H. E. (1997) *Gen Pharmacol* **29**, 121-126
51. Shimmyo, Y., Kihara, T., Akaike, A., Niidome, T., and Sugimoto, H. (2008) *Biochim Biophys Acta* **1780**, 819-825
52. Sultana, R., Ravagna, A., Mohmmad-Abdul, H., Calabrese, V., and Butterfield, D. A. (2005) *J Neurochem* **92**, 749-758
53. Herrup, K. (2010) *J Neurosci* **30**, 16755-16762
54. Kemperman, R. A., Bolca, S., Roger, L. C., and Vaughan, E. E. (2010) *Microbiology* **156**, 3224-3231

FOOTNOTES

This study was supported by Grants-in-Aid for Young Scientists (B) (K.O.), Scientific Research (A) (22240051) (H.N.), Scientific Research (B) (20390242) (M.Y.), and Knowledge Cluster Initiative [High-Tech Sensing and Knowledge Handling Technology (Brain Technology)] (M.Y.) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, Japan, a grant to the Amyloidosis Research Committee from the Ministry of Health, Labour, and Welfare, Japan (K.O. and M.Y.), Novartis Foundation for Gerontological Research (K.O.), Alumni Association of Showa University School of Medicine (K.O.), JSPS Asian Core Program (H.N.), and NIH Grants AG027818 (D.B.T.) and AG027853 (M.G.Z.), and the Jim Easton Consortium for Alzheimer's Drug Discovery and Biomarkers at UCLA (D.B.T.). The authors thank Dr. Chunyu Wang (RPI) for providing models of the A β 42 structures and Ms. Margaret Condron (UCLA) for technical assistance.

Abbreviations used are: A β , amyloid β -protein; ACSF, artificial cerebrospinal fluid; AD, Alzheimer's disease; AFM, atomic force microscopy; APP, amyloid precursor protein; α S, α -synuclein; APS, ammonium persulfate; CD, circular dichroism spectroscopy; Cur, curcumin; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EM, electron microscopy; FA, ferulic acid; fA β , A β fibrils; fEPSPs, field excitatory presynaptic potentials; GST, glutathione S-transferase; HEK, human embryonic kidney; HMW, high molecular weight; HSQC, heteronuclear single quantum coherence; LTD, long-term depression; LTP, long-term potentiation; MTT, 3-(19)-2,5-diphenyltetrazolium bromide; Myr, myricetin; NDGA, nordihydroguaiaretic acid; NMR, nuclear magnetic resonance; PICUP, photo-induced cross-linking of unmodified proteins; RA, rosmarinic acid; RP-HPLC, reverse-phase high-performance liquid chromatography; Ru(bpy), tris(2,2'-bipyridyl)dichlororuthenium(II); SC, statistical coils; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography; STD, saturation transfer difference.

FIGURE LEGENDS

Fig. 1. Structures of myricetin (Myr), ferulic acid (FA), nordihydroguaiaretic acid (NDGA), curcumin (Cur), and rosmarinic acid (RA).

Fig. 2. A β and GST oligomerizations. PICUP, followed by SDS-PAGE and silver staining, was used to determine the effects of 10 and 100 μ M Myr, FA, NDGA, Cur, or RA on oligomerization of A β 40 (A), A β 42 (B) or GST (C). "+" and "-" mean "with cross-linking" and "without cross-linking", respectively. The gel is representative of each of three independent experiments. AFM was performed on 25 μ M un-cross-linked (D, E) and cross-linked (F-K) A β 40 (D, F, H, J) and A β 42 (E, G, I, K) with 0 (F, G), 100 μ M Myr (H, I) or RA (J, K). Scale bars are 100 nm.

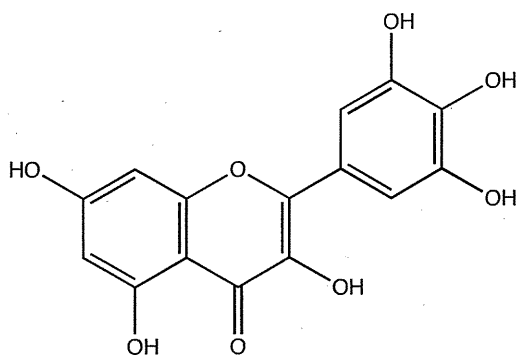
Fig. 3. A β secondary structure dynamics. CD was used to monitor peptide assembly. A β 40 (A, C, E) or A β 42 (B, D, F) were incubated at 37 °C for 6 days in 10 mM phosphate, pH 7.4, in buffer alone (A, B) or in the presence of 100 μ M Myr (C, D) or RA (E, F). Spectra were acquired immediately at the start of the incubation period, d 0 (\circ), and after days 2 (\blacktriangledown), 3 (\square), 5 (\blacksquare), and 6 (\diamond). The spectra presented at each time are representative of those obtained during each of three independent experiments.

Fig. 4. Expanded HSQC spectra of uniformly 15 N-labeled A β 42. The HSQC experiment detects 1 H that are directly bonded to the 15 N atoms, notably the backbone amide 1 H- 15 N. The control spectrum of the A β 42 alone (25 μ M, pH 7.2, 5°C, **black crosspeaks**) is overlaid with the spectra of the A β 42 (25 μ M) containing RA (A) or Myr (B) both at 500 μ M concentrations (**red crosspeaks**).

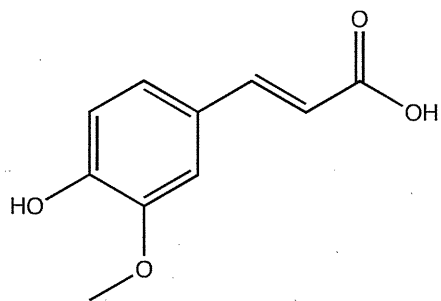
Fig. 5. Representative NMR/MD structural models of A β 42 that show binding locations with the phenolic compounds. The molecular models of the A β 42 that were obtained from a combined molecular dynamics and NMR approach (27). The **green-colored** ribbons depict the backbone atoms, with those regions showing chemical shift changes (indicative of binding to the phenolic compounds) are **red-colored**. Residues at the margins of the binding regions are labeled.

Fig. 6. Effects of myricetine (Myr) and rosmarinic acid (RA) on A β 42-induced alterations of neuronal plasticity (LTP and LTD) in the hippocampal slices. (A and D) Typical fEPSP waveforms of pre- (black lines) and (A) post-tetanic or (D) post-low frequency conditioning (red lines) stimulation in each test group. Thirty waveforms were averaged. (B and E) Time course of %fEPSPs slope. A thick line indicates application of various test compounds. (B) An arrow indicates tetanus stimulation (100 Hz, 1 sec). (E) Dots above the thick line indicate low frequency conditioning stimulation (E, 1 Hz, 450 paired-pulse). (C and F) Comparison of % fEPSP slope among the five groups. Values are shown as the percentage of fEPSP slope relative to the baseline and presented as mean \pm S.E. (n = 5). Differences reaching statistical significance are noted by line segments between samples, along with their associated *p*-values, where * signifies *p* < 0.05 and ** signifies *p* < 0.01.

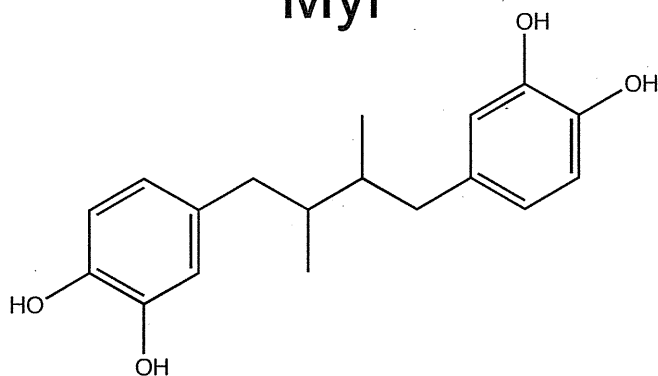
Fig. 1.



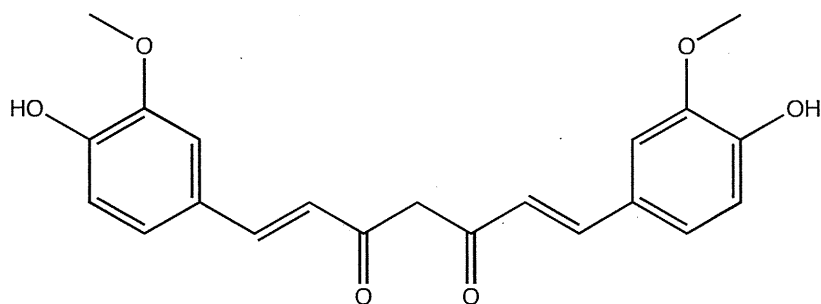
Myr



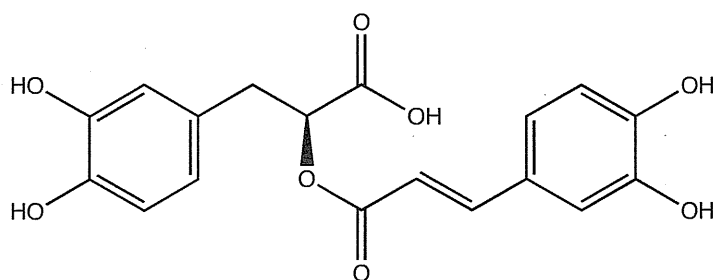
FA



NDGA



Cur



RA

Fig. 2.

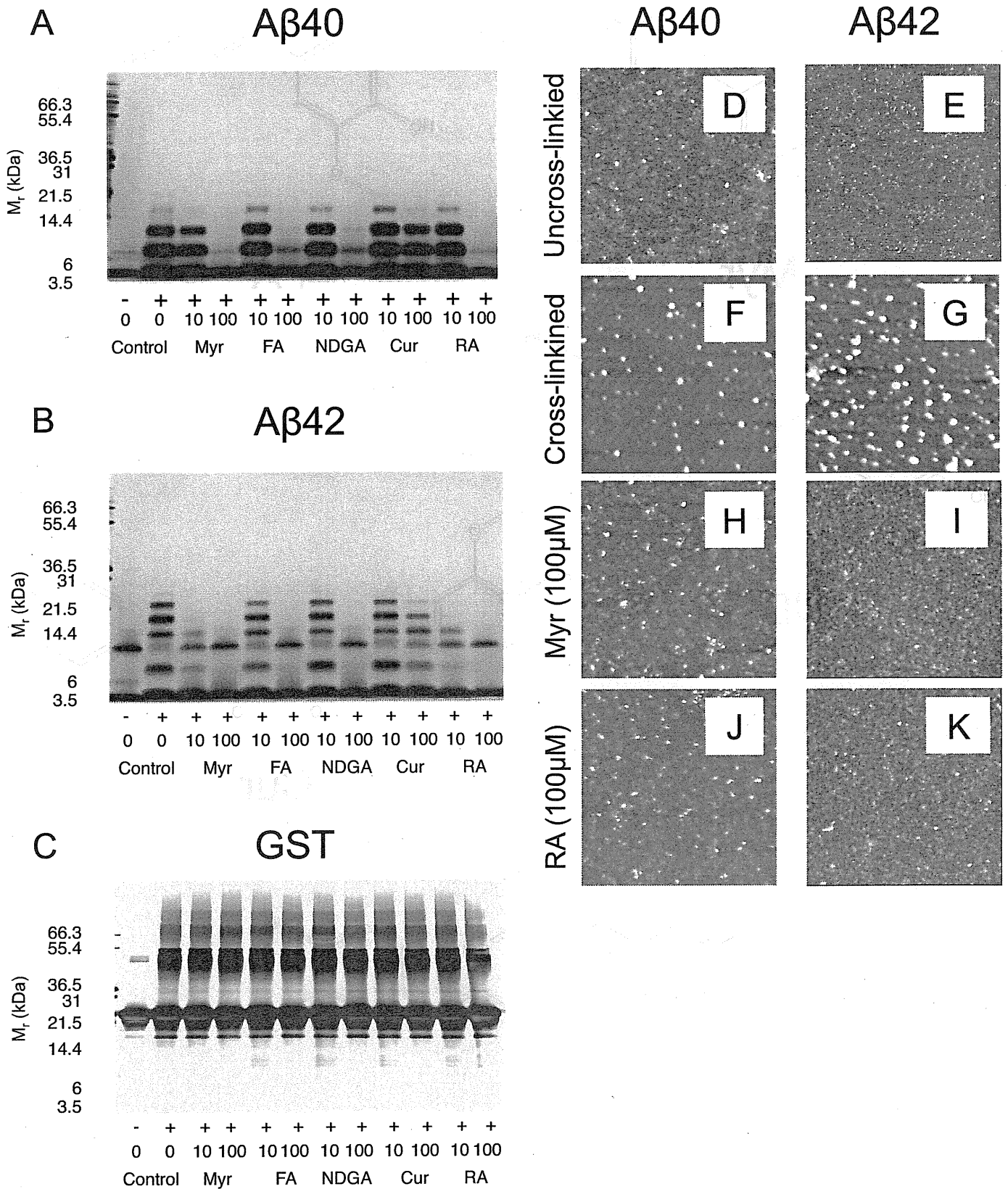


Fig. 3.

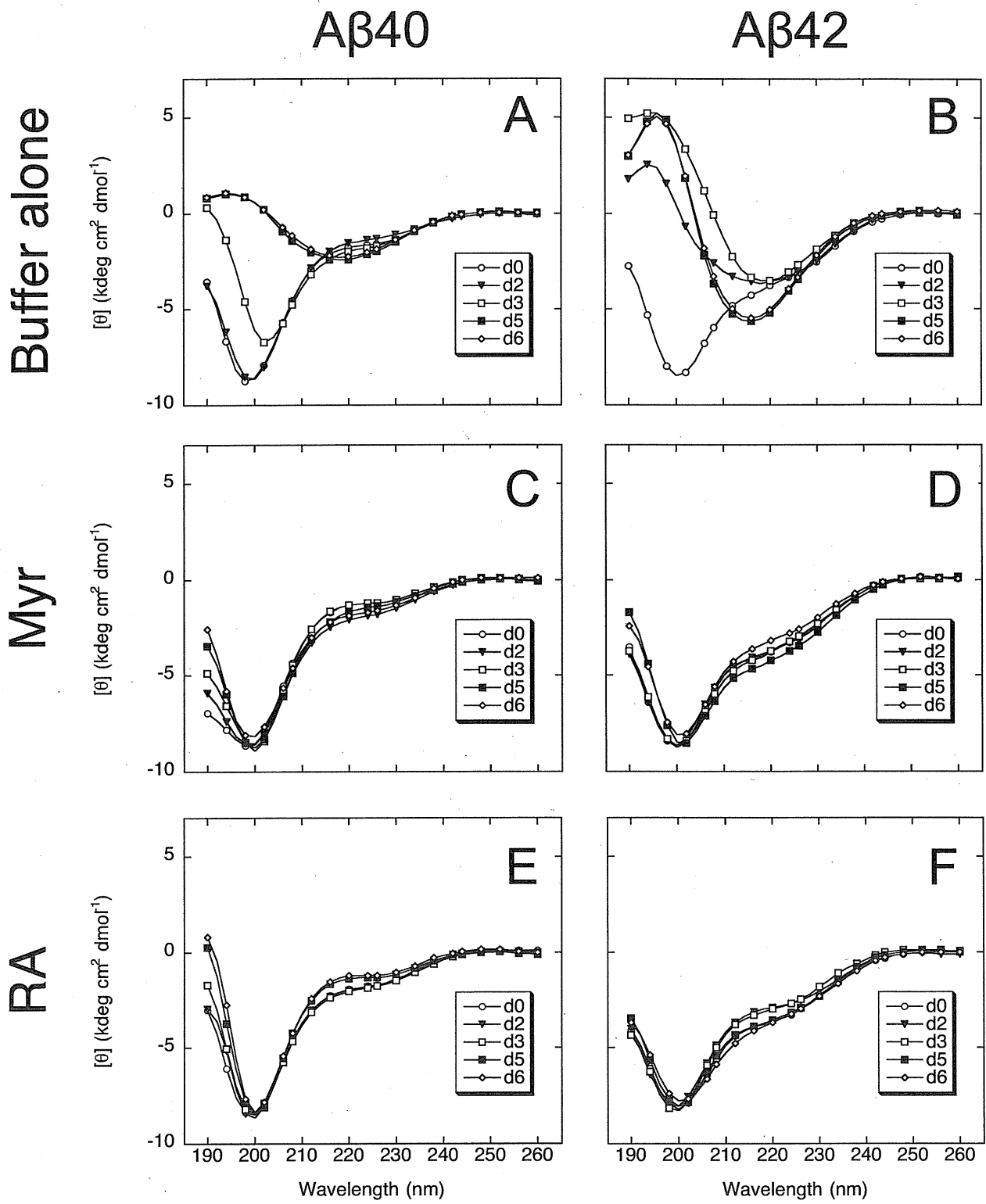


Fig. 4.

A β 42 alone (black cross peaks)
A β 42 plus RA (red cross peaks)

A β 42 alone (black cross peaks)
A β 42 plus Myr (red cross peaks)

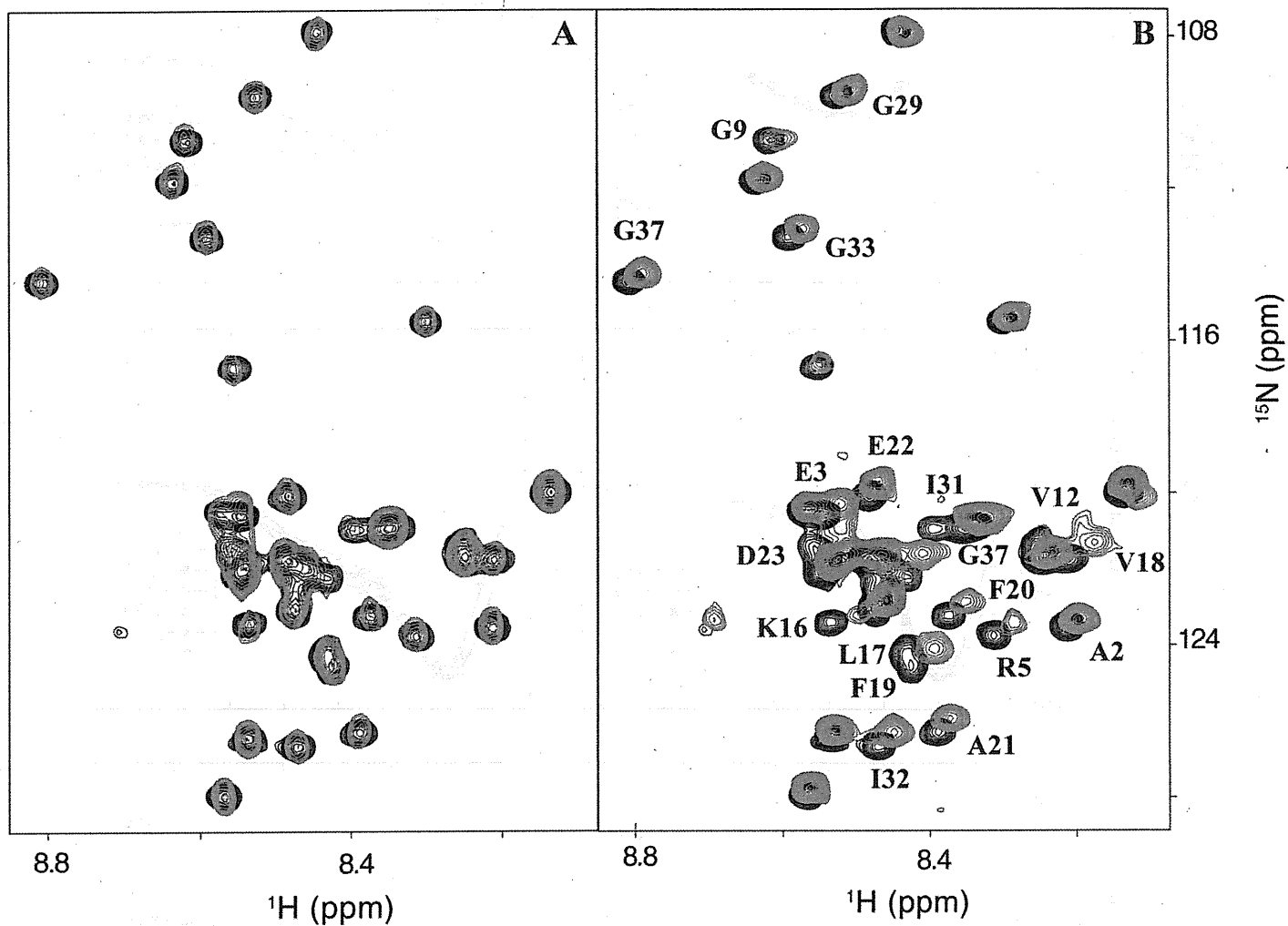


Fig. 5.

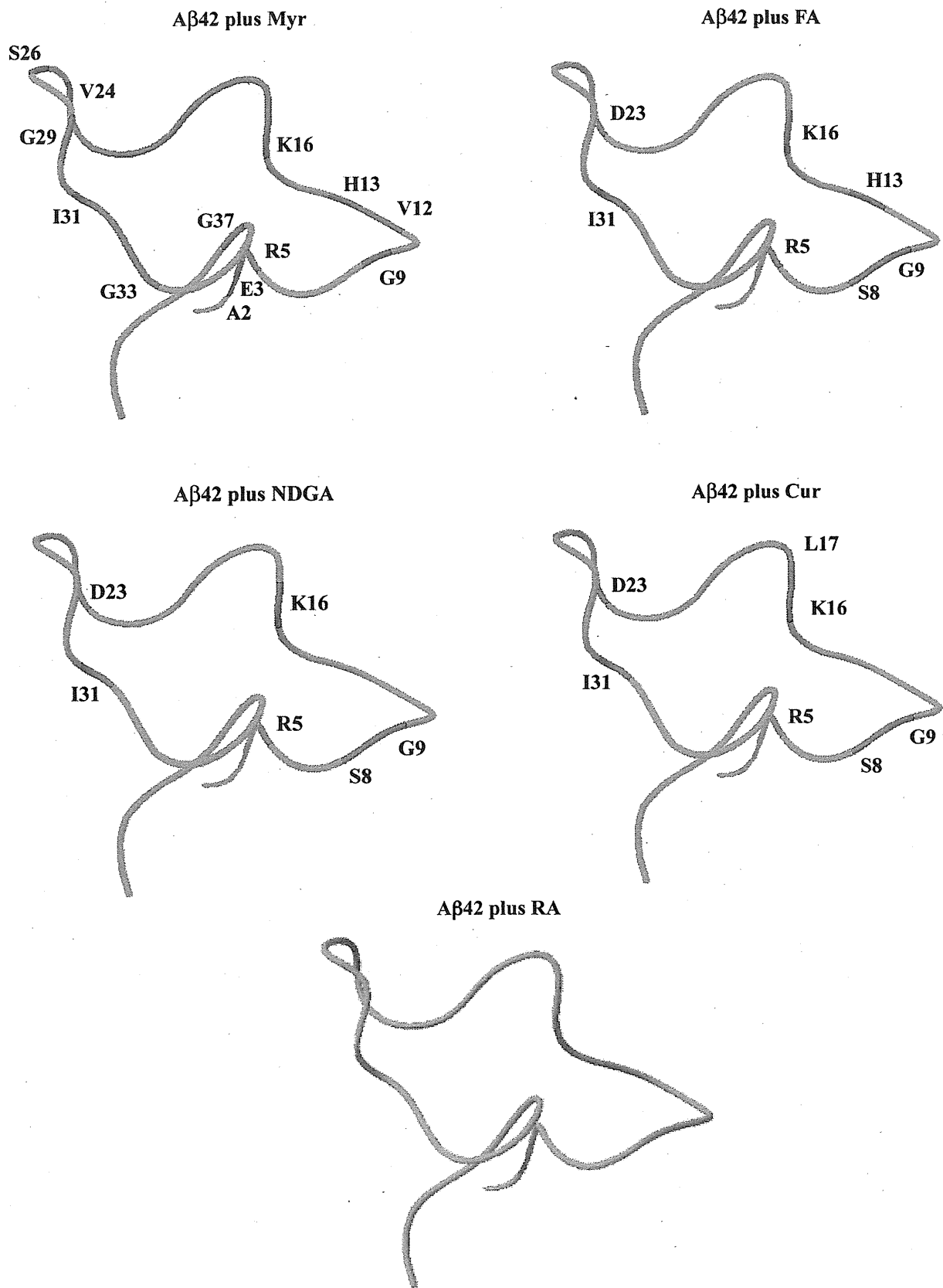
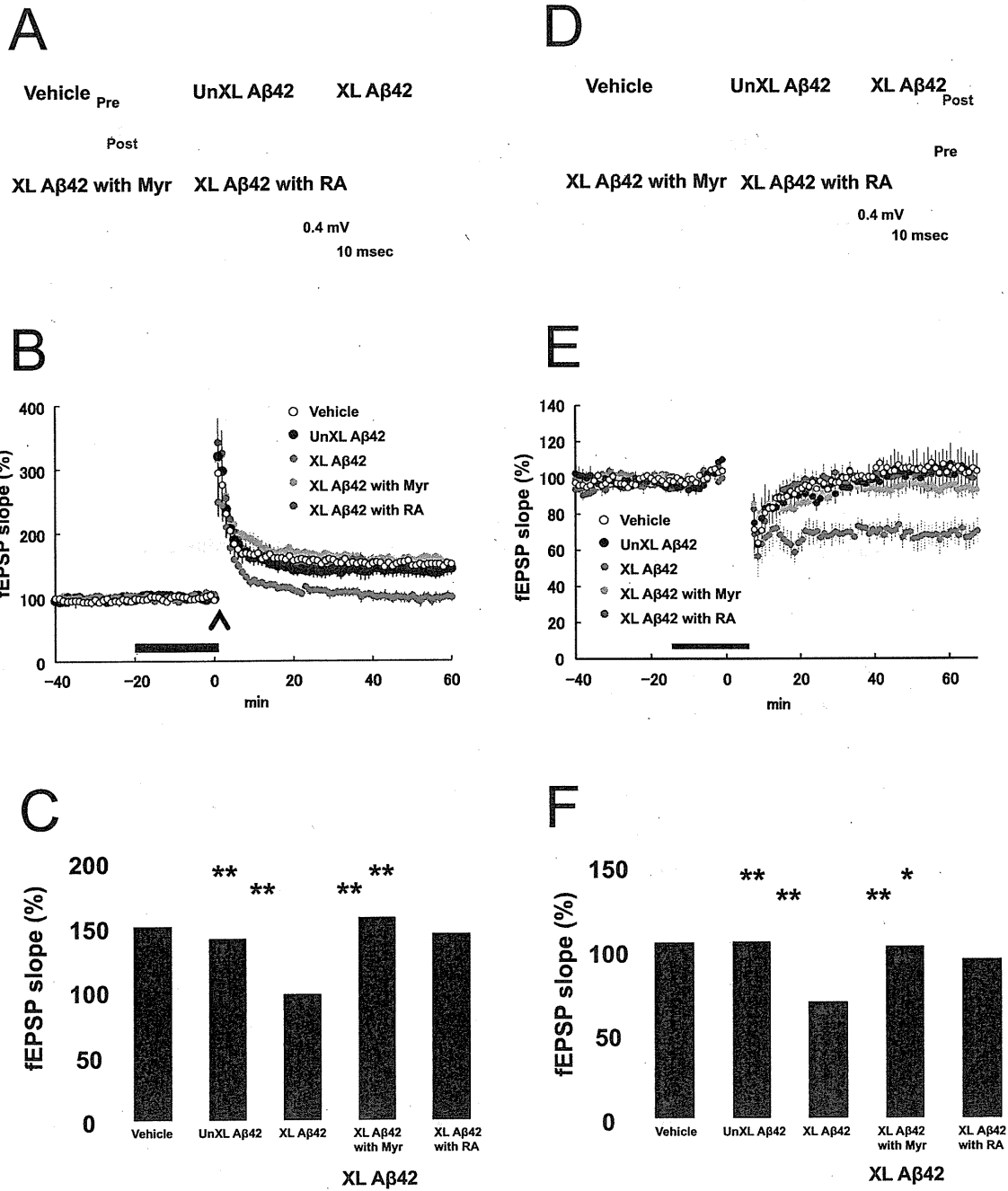


Fig. 6.



Adaptive responses to alloxan-induced mild oxidative stress ameliorate certain tauopathy phenotypes

Yuji Yoshiike,^{1,2} Shunji Yamashita,¹ Tatsuya Mizoroki,¹ Sumihiro Maeda,³ Miyuki Murayama,¹ Tetsuya Kimura,^{1,4} Naruhiko Sahara,⁵ Yoshiyuki Soeda¹ and Akihiko Takashima^{1,4}

¹Laboratory for Alzheimer's Disease, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan

²Alzheimer's Disease Project Team, The Research Institute, National Center for Geriatrics and Gerontology, 35 Gengo, Morioka, Obu, Aichi 474-8511, Japan

³Department of Neurology, Gladstone Institute of Neurological Disease, University of California, San Francisco, CA 94158, USA

⁴Center for Development of Advanced Medicine for Dementia, National Center for Geriatrics and Gerontology, 35 Gengo, Morioka, Obu, Aichi 474-8511, Japan

⁵Department of Neuroscience, Center for Translational Research in Neurodegenerative Disease, College of Medicine, University of Florida, Gainesville, FL 32610, USA

Summary

Oxidative stress is considered to promote aging and age-related disorders such as tauopathy. Although recent reports suggest that oxidative stress under certain conditions possesses anti-aging properties, no such conditions have been reported to ameliorate protein-misfolding diseases. Here, we used neuronal and murine models that overexpress human tau to demonstrate that mild oxidative stress generated by alloxan suppresses several phenotypes of tauopathy. Alloxan treatment reduced HSP90 levels and promoted proteasomal degradation of tau, c-Jun N-amino terminal kinase, and histone deacetylase (HDAC) 6. Moreover, reduced soluble tau (phosphorylated tau) levels suppressed the formation of insoluble tau in tau transgenic mice, while reduced HDAC6 levels contributed to microtubule stability by increasing tubulin acetylation. Age-dependent decreases in HDAC2 and phospho-tau levels correlated with spatial memory enhancement in alloxan-injected tau mice. These results suggest that mild oxidative stress, through adaptive stress responses, operates counteractively against some of the tauopathy phenotypes.

Key words: aging; Alzheimer's disease; memory; microtubule; oxidative stress; tau.

Introduction

The free radical theory of aging posits that aging and age-associated degenerative diseases may be attributed to the deleterious effects of free radicals on various cell components (Harman, 1956). Since the proposal of this theory by Harman in 1956, a great number of studies have supported but not proven this theory (Balaban *et al.*, 2005). Aerobic metabolism and the subsequent generation of reactive oxygen species (ROS) are widely

accepted to cause aging. However, how aerobic metabolism and ROS generation are regulated and how oxidative stress and its targets might affect aging and age-related disorders remain unknown (Balaban *et al.*, 2005).

Macromolecular damage often found in misfolded proteins that accumulate with age is believed to cause aging and degenerative diseases (Kenyon, 2010). A prime candidate for causing age-related damage is ROS produced mainly during respiration in mitochondria, whose integrity has been shown to decline as a function of age (Sigenaga *et al.*, 1994). However, there are a growing number of exceptions to this theory. Some hypothesize that low levels of ROS might play a preventive role against aging, whereas high levels may play a causative role in aging (Gems & Partridge, 2008; Kenyon, 2010). For example, in *Caenorhabditis elegans*, low levels of juglone, which generates ROS, extend lifespan (Heidler *et al.*, 2010). In worms, glucose restriction extends lifespan by inducing mitochondrial respiration and by increasing oxidative stress (Schulz *et al.*, 2007). In yeast, caloric restriction also extends lifespan by inducing elevated levels of hydrogen peroxide, which activate superoxide dismutase (Mesquita *et al.*, 2010). Studies of long-lived species and transgenic (TG) mice with altered expression of antioxidants also suggest that oxidative damage is not directly correlated with lifespan (Van Remmen *et al.*, 2003). Thus, it is perhaps not only the oxidative stress but also the resistance mechanisms against oxidative stress that may determine the rate of aging (Perez *et al.*, 2009). Such adaptive stress responses might underlie hormesis, a phenomenon in which a compound that, at higher levels, produces deleterious effects, but at lower levels, produces beneficial effects (Mattson, 2008; Rattan & Demirovic, 2010). If low levels of ROS could delay aging processes under certain conditions, we surmised that there is also a hormetic condition that exists during oxidative stress that prevents protein misfolding and associated dysfunctions.

One of the normal functions of tau protein is to bind and stabilize microtubules in axons (Ballatore *et al.*, 2007). As a result of modifications such as phosphorylation, tau detaches from microtubules, which become destabilized (Mi & Johnson, 2006; Ballatore *et al.*, 2007). As a cytoskeletal component, microtubules, and importantly their stability, are considered to play substantial roles in neuronal function. Unless free soluble tau is degraded, it starts to aggregate, eventually becoming insoluble and forming intracellular neurofibrillary tangles (NFT), a pathological hallmark of tauopathies such as Alzheimer's disease (AD) (Yen *et al.*, 1995; Mi & Johnson, 2006; Ballatore *et al.*, 2007). The progression of cortical NFT formation is correlated with the degree of cognitive decline in AD (Braak & Braak, 1996). Therefore, tau aggregation, microtubule destabilization, and memory deficits are the critical phenotypes that result from tau misfolding and dysfunction.

Early studies examining the effects of oxidative stress on tau primarily focused on the phosphorylation states of tau and the kinases responsible for phosphorylating tau in acutely treated cells, because tau phosphorylation is considered to regulate the microtubule binding and stabilizing activity of tau (Zhu *et al.*, 2004; Mi & Johnson, 2006; Ballatore *et al.*, 2007). When cells are treated with acrolein, 4-hydroxy-2-nonenal (4-HNE), homocysteine, mercury, and A β peptide, tau phosphorylation increases (Zhu *et al.*, 2004). By contrast, when cells are treated with hydrogen peroxide (H₂O₂), ultraviolet light, menadione, or iron, tau phosphorylation decreases (Zhu *et al.*, 2004). Chronic application of oxidative stress in neuronal cells, *Drosophila*, and mouse models of tauopathy

Correspondence

Yuji Yoshiike, Alzheimer's Disease Project Team, The Research Institute, National Center for Geriatrics and Gerontology, 35 Gengo, Morioka, Obu, Aichi 474-8511, Japan. Tel./fax: +81 562 87 1278; e-mail: yyoshiik@ncgg.go.jp

Accepted for publication 2 October 2011

through genetic manipulations induces neurodegeneration and tau accumulation (Zhu *et al.*, 2004; Dias-Santagata *et al.*, 2007; Kulic *et al.*, 2009). Here, we used a chemical named alloxan to investigate the acute effects of oxidative stress on cellular and mouse models of tauopathy.

Alloxan, which was originally synthesized by Wohler and Liebig, became of interest in diabetes research when Dunn and McLetchie reported its diabetogenic property in animals (Lenzen & Panten, 1988). Alloxan can generate ROS in a cyclic reaction along with its reduction product, dialuric acid (Lenzen & Panten, 1988; Lenzen, 2008). The reduction in alloxan to dialuric acid in cells typically requires the presence of the tripeptide glutathione (GSH) (Lenzen, 2008). Thus, in theory, oxidative stress can be induced by alloxan where a sufficient amount of GSH is available. The brains of alloxan-induced diabetic rats show not only high levels of blood glucose but also increased levels of oxidative stress markers, such as malondialdehyde (MDA) and thiobarbituric acid-reactive substances (TBARS) (Genet *et al.*, 2002; Siddiqui *et al.*, 2005). Taking advantage of accumulative information regarding its mode of action and dose responses *in vivo*, we decided to use alloxan for examining the role of oxidative stress in tau misfolding and related dysfunctions.

Mutations in the tau gene have been identified in familial forms of frontotemporal dementias that are characterized by NFT formation (Hutton, 2000). In the present study, we used a mouse neuronal cell line that stably expresses a mutant form of human tau (P301L) (Hatakeyama *et al.*, 2004) for elucidating the molecular impact of alloxan-generated oxidative stress on tau, tau phosphorylation, and stress-responsive proteins such as c-Jun N-terminal kinases (JNKs) and heat-shock proteins (HSPs). Experiments using H₂O₂, catalase, a HSP90 inhibitor, and a proteasomal inhibitor revealed that the degradation system, which adaptively responded to alloxan-generated ROS, was at least partially responsible for reducing tau and JNK. By injecting alloxan into TG mice expressing human mutant tau (P301L or R406W), we identified conditions that did not raise blood glucose levels but did reduce the formation of insoluble tau in cerebral cortex. In the case of alloxan-injected P301L TG mice, the reduction in insoluble tau was accompanied by a slight increase in the lipoperoxide MDA as well as a decrease in both soluble phosphorylated tau and HSP90. Proteasomes activated by alloxan degraded not only tau but also another HSP90 substrate, histone deacetylase (HDAC) 6, which in turn promoted the acetylation of its substrate, α -tubulin, *in vitro*. Corresponding changes in protein levels were also observed *in vivo*. Further analyses showed that HDAC2, another deacetylase of a different class, was also reduced by alloxan treatment and that age-dependent decreases in HDAC2 and phospho-tau levels were correlated with the enhancement of spatial memory in alloxan-injected P301L TG mice.

Results

Acute effects of alloxan on tau and stress-response proteins *in vitro*

Depending on the experimental conditions, oxidative stress has been known to increase or decrease the phosphorylation of tau in cells treated with ROS-generating compounds (Zhu *et al.*, 2004). We treated murine neuronal cells expressing P301L mutant human tau with alloxan in the presence of GSH. After 1 h of treatment, immunoreactivity against some tau phosphorylation sites increased, but after 5 h of treatment, immunoreactivity decreased and the respective bands shifted (Figs S1 and 1A). In other words, the intensity of phospho-tau band at higher molecular weight was lowered as total tau band migrated more, suggesting that tau was dephosphorylated by 5-h alloxan treatment. These alterations in phosphorylation pattern were mostly consistent with those observed

from H₂O₂-treated cells (Fig. S1). Among the kinases known to phosphorylate tau, JNK and their phosphorylated and thus activated forms decreased with longer treatment periods (Figs S1 and 1A). These results were unexpected, as JNK is also known as stress-activated protein kinase (SAPK) (Zhu *et al.*, 2004; Dias-Santagata *et al.*, 2007). Phospho-JNK levels were increased in cells treated with H₂O₂ (Fig. S1). Indeed, redox reactions of alloxan in the presence of GSH showed that alloxan generated not only H₂O₂ but also other species that might have promoted the inactivation of JNK via an unknown mechanism.

Heat-shock proteins are proteins known to show increased expression during stress. Thus, we also examined the effect of alloxan on HSP expression: alloxan treatment for 5 h increased HSP90 and HSP70 levels (Fig. 1A). This suggests that these chaperones were expressed to prevent unstable proteins such as tau from aggregating. In the meantime, tau levels appeared to decrease as treatment time progressed. Increased polyubiquitination early on during treatment followed by its decrease indicates that the degradation system also responded in order to maintain cellular homeostasis (Fig. 1A).

After overnight treatment with alloxan, we observed that tau, HSP90, HSP40, and HSP27 levels decreased in a dose-dependent manner (Fig. S2A). These decreases were suppressed by adding catalase into the culture medium (Fig. S2B). Similar decreases in protein levels were observed in H₂O₂-treated cells (Fig. S2C). These results suggest that H₂O₂ generated from alloxan in the presence of GSH was responsible for reducing tau and HSP levels. Inhibiting HSP90 through inhibitors such as geldanamycin (GA) reduces the HSP90 substrates, including tau and JNK, in part through degradation (Dickey *et al.*, 2007; Luo *et al.*, 2007; Nieto-Miguel *et al.*, 2008; Novoselova *et al.*, 2008). Reductions in tau, JNK, and phospho-JNK levels were also observed in P301L tau-expressing cells treated with GA (Fig. 1B). The proteasome inhibitor lactacystin partially prevented the alloxan/GSH-induced reductions in tau and JNK levels but not that in HSP levels (Fig. 1C). These results suggest that alloxan-generated H₂O₂ promoted proteasomal degradation of HSP90 substrates, such as tau and JNK, conceivably via reduction in HSP90.

Low-dose alloxan-induced mild oxidative stress in the brain without inducing apparent diabetic symptoms

To test the impact of oxidative stress on the phenotypes associated with tau misfolding and dysfunction *in vivo*, we injected alloxan into TG mice that express human mutant (P301L) tau (Kimura *et al.*, 2010). A single intraperitoneal injection of alloxan at 100 mg kg⁻¹ of body weight, on average, failed to significantly change either blood glucose concentration or body weight (Fig. 2A–D). Moreover, we found no statistically significant differences between the alloxan-treated group and vehicle (saline)-treated group, even after considering blood glucose concentration or body weight values from nontransgenic (NTG) and P301L mice together as a group. The latter indicates that diabetic complications did not develop, at least overall, in the experimental cohort as a result of alloxan treatment at the dosage of 100 mg kg⁻¹. Because significant increases in blood glucose levels (approximately 190 mg dL⁻¹ in fasting mice) and decreases in body weights were observed in mice that had received higher doses of alloxan (400 mg kg⁻¹ by i.p.), it is likely that the alloxan solution used in our experiments was potent enough to generate diabetes in mice (Fig. 2A,B). However, under the condition that clearly did not induce diabetic phenotypes in mice, we observed slight yet significant increases in MDA, a lipid peroxide marker that is normally elevated during oxidative stress in cerebral cortical homogenates from alloxan-treated mice (without separating by genotype) (Fig. 2E,F). Therefore, we assumed that alloxan caused oxidative stress, in the brains of these mice, even though its effect was mild.

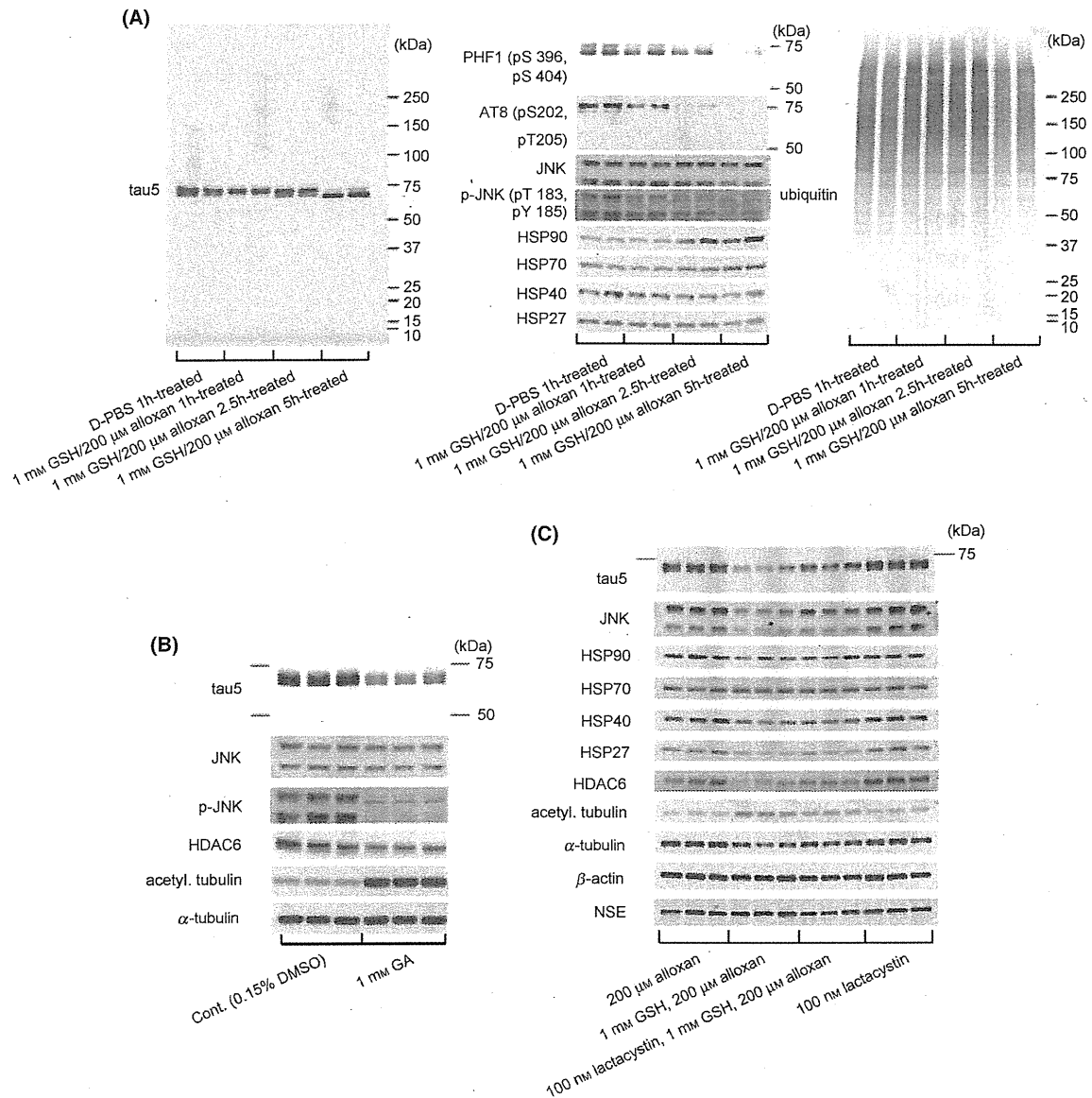


Fig. 1 Changes in protein levels as a result of alloxan treatment *in vitro*. (A) Alterations in the levels of tau, phospho-tau, c-Jun *N*-amino terminal kinase (JNK), phospho-JNK, heat-shock proteins (HSPs), and ubiquitin during alloxan treatment. Neuro2A cells stably expressing P301L tau were treated with alloxan in the presence of glutathione (GSH) for the indicated periods. After treatment, Tris-buffered saline (TBS)-soluble cell lysates derived from cell homogenates were analyzed by Western blotting. (B) Decreases in the levels of tau, JNK, phospho-JNK, and histone deacetylase (HDAC) 6 but increases in the levels of acetylated tubulin by HSP90 inhibition. Tau-expressing cells were treated with geldanamycin (GA), a HSP90 inhibitor, for 24 h, and mRIPA-soluble lysates were prepared and analyzed by Western blotting. (C) Partial blockade of alloxan-induced changes in tau, JNK, HDAC6, and acetylated tubulin protein levels by proteasomal inhibition. Tau-expressing cells were treated for 16 h with alloxan in the presence of GSH with or without lactacystin, a proteasomal inhibitor. TBS-soluble fractions derived from cell lysates were treated with the indicated antibodies except for anti-HDAC6, which was used to treat 2% SDS-soluble fractions containing more nuclear extracts.

Malondialdehyde levels in alloxan-treated mice varied according to the age of the mice (Fig. S3A). Because oxidative stress increases with age (Balaban *et al.*, 2005; Cocheme *et al.*, 2011), it is tempting to speculate that ROS accumulation owing to aging and ROS generated from alloxan were combined, resulting in higher MDA levels in older mice.

Suppression of tau insolubilization in mutant tau transgenic mice injected with alloxan

Next, we examined the levels of tau protein and stress-responsive proteins in cortical homogenates derived from saline- and alloxan-injected

P301L mice (Fig. 3). Quantification of immunoreactivity showed that HSP90 immunoreactivity was inversely proportional to MDA level (Fig. 4A), indicating that HSP90 levels decrease as lipid peroxide levels, and thus oxidative stress, increase. Consistent with the cell culture results, alloxan injections significantly reduced HSP90 levels in P301L mouse brain (Fig. 4B). The HSP90 decrease was directly associated not only with a decrease in sarcosyl-insoluble tau but also with a decrease in JNK (Fig. 4C,D), as observed in the cell culture experiments, which showed that HSP90 substrates, including tau and JNK, were degraded upon oxidative stress (Fig. 1). In addition, the levels of several phospho-tau proteins (tau-phosphorylated at different epitopes) were decreased, as was

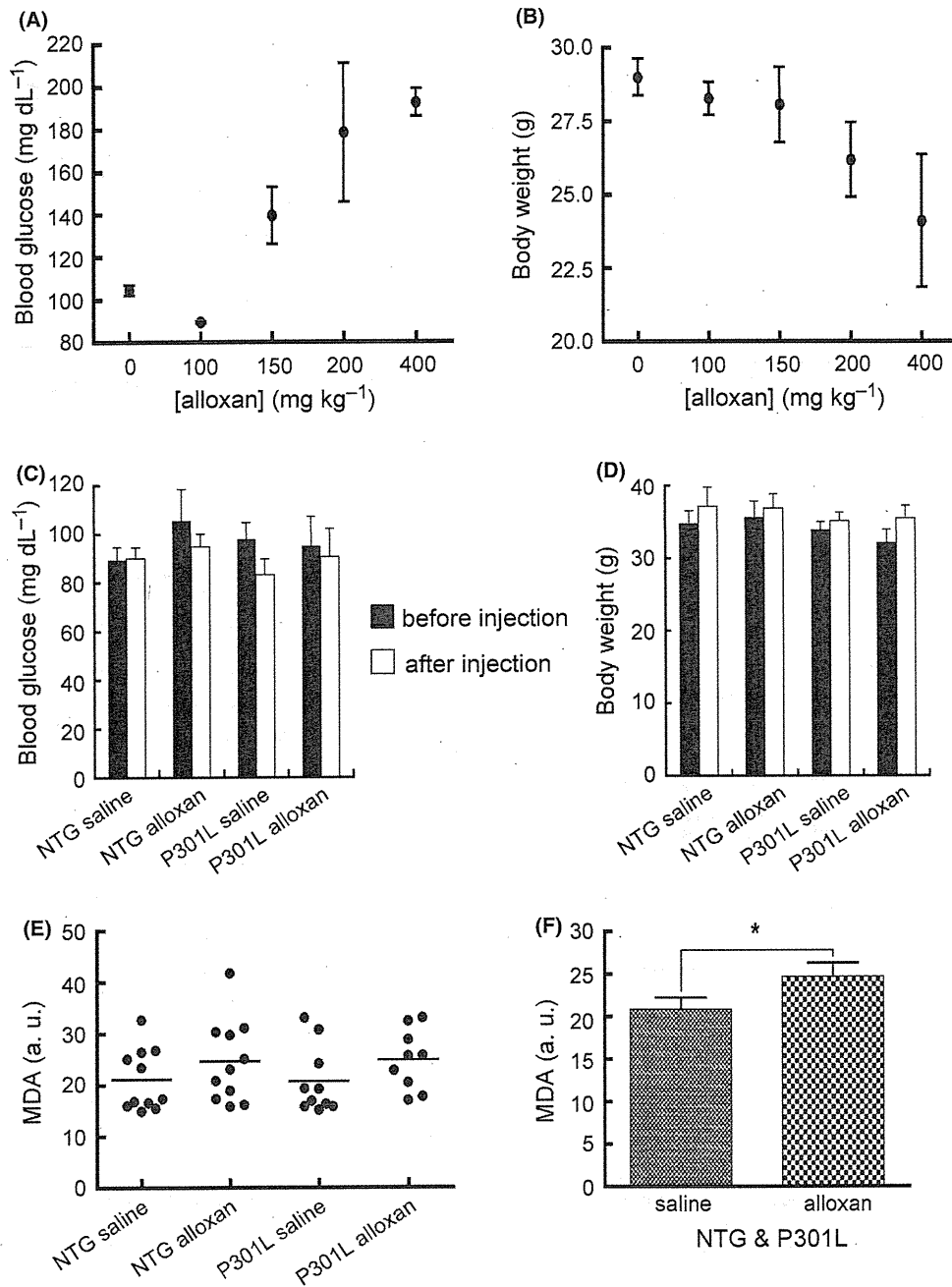


Fig. 2 Low-dose alloxan injection induced no significant changes in blood glucose and body weight but slightly induced oxidative stress in mouse brain. (A) Dose-dependent increase in blood glucose concentrations by alloxan injections in C57BL/6J mice ($P = 0.0417$, Friedman). (B) Dose-dependent decrease in body weight by alloxan injections in C57BL/6J mice ($P = 0.0417$, Friedman). (C) Blood glucose concentrations in nontransgenic (NTG) and P301L transgenic (TG) mice before and after injections of alloxan or saline vehicle. No significant difference between groups after treatment ($P = 0.3750$, Friedman) or by treatment after combining NTG and TG ($P = 0.3486$) was detected. (D) Simultaneous measurement of body weight. No significant difference between groups was detected after treatment ($P = 0.7741$, Friedman) or by treatment after combining NTG and TG ($P = 0.7842$). (E) Variation in malondialdehyde (MDA) values within each group. No significant difference was detected between groups ($P = 0.1623$, Friedman). (F) Significantly higher MDA levels in alloxan-treated mice than in saline-treated mice when MDA level values of NTG and P301L mice were considered together as a group ($P = 0.0461$).

phospho-JNK1 (i.e., activated JNK1), in the alloxan-treated P301L mice (Fig. 4E). Although this decrease at each phosphorylation site was not significant, the overall reduction in phospho-tau proteins was statistically significant when comparing the alloxan-treated group to the saline-treated group (Fig. 4F). In alloxan-injected P301L mice, decreased soluble phospho-tau levels were correlated with a significant decrease in insoluble

tau levels (Fig. 4G,H). An inverse association also existed between MDA levels and insoluble tau levels (Fig. S3B), suggesting that alloxan-generated ROS may play a role in reducing insoluble tau levels (Fig. 4H). These results together with the cell culture results suggest that the reduction in HSP90 levels that occurred in response to alloxan-generated oxidative stress induced the degradation of HSP90 substrates – tau,