

# Oxidative Regulation of Fatty Acid-Induced Tau Polymerization<sup>†</sup>

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**ABSTRACT:** Alzheimer's disease (AD) is characterized by the presence of amyloid-positive senile plaques and tau-positive neurofibrillary tangles. Aside from these two pathological hallmarks, a growing body of evidence indicates that the amount of oxidative alteration of vulnerable molecules such as proteins, DNA, and fatty acids is elevated in the brains of AD patients. It has been hypothesized that the elevated amounts of protein oxidation could lead directly to the formation of neurofibrillary tangles through a cysteine-dependent mechanism. We have tested this hypothesis in an in vitro system in which tau assembly is induced by fatty acids. Using sulfhydryl protective agents and site-directed mutagenesis, we found that cysteine-dependent oxidation of the tau molecule is not required for its polymerization and may even be inhibitory. However, by adjusting the oxidative environment of the polymerization reaction through the addition of a strong antioxidant or through the addition of an oxidizing system consisting of iron, adenosine diphosphate, and ascorbate, we found that oxidation does play a major role in our in vitro paradigm. The results indicated that fatty acid oxidation, the amount of which is found to be elevated in AD patients, can facilitate the polymerization of tau. However, "overoxidation" of the fatty acids can inhibit the process. Therefore, we postulate that specific fatty acid oxidative products could provide a direct link between oxidative stress mechanisms and the formation of neurofibrillary tangles in AD.

The microtubule-associated protein tau is a soluble cytosolic protein thought to contribute to microtubule stability (1, 2). However, in many disease states, tau protein is induced by unknown cellular conditions to self-associate into filamentous structures (3). These filamentous forms of tau can be found in various neurodegenerative disorders such as Alzheimer's disease (AD)<sup>1</sup> (4–6), corticobasal degeneration (CBD) (7), progressive supranuclear palsy (PSP) (8), Pick's disease (PD) (9), Down syndrome (10), and frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17) (11). To elucidate the etiology of AD and other so-called "tauopathies" (12), a much clearer understanding of the molecular mechanisms which lead to the formation of insoluble tau inclusions must be obtained.

Most of the current understanding of tau polymerization derives from in vitro assembly experiments. Relatively mild conditions have been described which result in the polymerization of low concentrations of biochemically purified tau protein (13), and this process can be greatly enhanced by

the addition of polyanionic compounds under oxidative conditions (14–19) or by the addition of free fatty acids under reducing conditions (19–23). Using such techniques, details of the in vitro polymerization of tau are emerging. Extensive phosphorylation of the tau molecule with various protein kinases, which was expected to mimic the hyperphosphorylation in AD and therefore enhance tau association, actually inhibits the induction of polymerization by polyanions (24). In vitro experiments have shown that several of the single-amino acid missense point mutations found in FTDP-17 can lead to increased levels of filament formation (19, 23, 25). We have shown that the tau isoforms have different polymerization characteristics, which may explain the origin of tau pathology in cases of FTDP-17 with altered isoform composition (22). Thus, some of the factors that could lead to tau polymerization in the disease state are gradually being identified. However, links between risk factors associated with the most common neurodegenerative disorder, AD, and an increased level of tau polymerization have remained obscure.

This report attempts to draw a direct correlation between the in vitro induction of tau polymerization by free fatty acids and a putative risk factor in the genesis of AD, oxidative stress (26). Using sulfhydryl protective agents and site-directed mutagenesis, we have shown that the oxidation of the tau molecule through its reactive cysteine residues is not required for the fatty acid induction of tau polymerization. However, strong antioxidants did inhibit the process, probably by preventing the oxidation of the fatty acid inducer. We suggest that the oxidation of fatty acids not only is a prerequisite for the in vitro polymerization of tau in our

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<sup>1</sup> Abbreviations: AD, Alzheimer's disease; FA, fatty acid; AA, arachidonic acid; DHA, docosahexaenoic acid; BHT, butylated hydroxytoluene; EM, electron microscopy; LLS, laser light scattering; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; DTT, dithiothreitol; *i*<sub>s</sub>, intensity of scattered light.

assembly paradigm but also could serve as a possible link between oxidative stress and the formation of the fibrillar pathology in AD.

## EXPERIMENTAL PROCEDURES

**Chemicals and Proteins.** All of the polymerization experiments described herein used the recombinant tau cDNA, ht40 (27), that encodes a 441-amino acid polypeptide containing exons 2, 3, and 10 (2N4R ht40, i.e., two N-terminal exons and four microtubule binding regions) that is polyhistidine-tagged at its amino terminus. The protein was purified as described previously (28). Arachidonic acid (AA) and docosahexaenoic acid (DHA) were purchased from Cayman Chemicals (Ann Arbor, MI) and stored at  $-20^{\circ}\text{C}$ . To control for possible oxidation during storage, the fatty acids were used for a short time (less than 3 months) and then discarded. Butylated hydroxytoluene (BHT) was purchased from Sigma (St. Louis, MO) and was diluted into 100% ethanol to make a 4% stock solution.

**Oligonucleotide-Directed Mutagenesis.** Mutations in ht40 (C291A and C322A) were prepared from pT7c-ht40 using a commercial kit (QuickChange, Stratagene, La Jolla, CA) and synthetic oligonucleotides 5'-agcaacgtccagctccaaggctg-gctcaaaggataatc and 5'-gcaaggtgacctccaaggctggctcattaggcaac (underlined residues encode amino acid residues 291 and 322, respectively). The double mutant pT7c-ht40-C291A/C322A (cysteine-less or "cysless") was created by ligating a *Bst*EII-*Eco*RI fragment from pT7c-ht40-C322A into *Bst*EII-*Eco*RI-digested pT7-ht40-C291A. The doubly mutated protein was purified in the same manner as wild-type 2N4R ht40 (above).

**Polymerization Reaction.** Tau polymerization was induced by incubation in the presence of  $75\ \mu\text{M}$  free fatty acid (FA) in buffer containing 10 mM HEPES (pH 7.4) and 100 mM NaCl (polymerization buffer). Polymerization reactions monitored by laser light scattering were performed at room temperature, and reaction mixtures monitored by electron microscopy were incubated at  $37^{\circ}\text{C}$ . The FAs that were employed were either AA or DHA as indicated in the text. Dithiothreitol (DTT) was used at a final concentration of 5 mM unless otherwise noted. Aliquots for electron microscopy were taken at different time points and fixed with 2% glutaraldehyde.

**Oxidative Conditions.** Under condition 1, the polymerization reaction was performed as stated above. Under condition 2, a 1 h preincubation of  $75\ \mu\text{M}$  FA in polymerization buffer was performed before the addition of 2N4R ht40. Under condition 3,  $50\ \mu\text{M}$   $\text{FeCl}_3$ , 20 mM ADP, and 10 mM ascorbic acid were added to the polymerization buffer before the addition of the fatty acid and 2N4R ht40. Similar conditions have been used previously to generate oxidation products of AA and DHA (29, 30). Under condition 4, a 1 h preincubation of  $75\ \mu\text{M}$  FA in polymerization buffer with an Fe/ADP/ascorbate mixture was performed before the addition of 2N4R ht40. Under the nonoxidative condition, BHT was added to the polymerization buffer at a final concentration of 0.1% before the addition of  $75\ \mu\text{M}$  FA and 2N4R ht40. All incubations were performed at  $37^{\circ}\text{C}$ .

**Laser Light Scattering.** Tau polymerization reaction mixtures ( $250\ \mu\text{L}$ ) in 5 mm fluorometer cells were il-

luminated with 488 nm vertically polarized laser light generated by a Lexel model 65 ion laser at a 5 mW setting. Images were collected at an angle of  $90^{\circ}$  to the incident light and perpendicular to the direction of polarization with an Electrim Corp. model EDC1000HR digital camera with a 25 mm lens controlled by HiCam '95 (written by G. Albrecht-Buehler of Northwestern University Medical School, and available at <http://www.basic.northwestern.edu/g-buehler/hicam.htm>). The intensity of scattered light ( $i_s$ ) was obtained using the histogram feature of Adobe Photoshop 5.0. For simple comparisons between experiments, lines were drawn through the data points that were obtained and are not meant to imply any particular model of polymerization (23).

**Electron Microscopy.** Samples were prepared for electron microscopy by floating a carbon-coated Formvar grid on 10  $\mu\text{L}$  of the sample for 1 min followed by staining with 2% uranyl acetate for 1 min. A JEOL 1220 transmission electron microscope operating at 60 kV was used to view the grids. Images were captured at  $20000\times$  using a MegaPlus model 1.6I AMT digital Kodak camera controlled by the AMT Camera Controller software package. All images were processed and quantified as previously described (22).

**Length Distributions.** Digital electron micrographs were imported into Optimas 6.1 where the lengths of the individual filaments were traced manually using the draw or trace line feature of the program. Filament lengths were then extracted into GraphPad Prism 2.01 software where the relative frequencies of individual filament lengths were determined.

## RESULTS

**Effects of Cysteine Oxidation on Tau Polymerization.** The role of cysteine-mediated oxidation in the AA induction of tau polymerization was investigated by several methods. First, the effect of DTT was established. It has been previously shown that the removal of this sulfhydryl protecting agent from solutions of truncated tau constructs allows cysteine specific dimerization to occur by air oxidation and is required for the induction of polymerization by polyanionic compounds (31). AA was added to solutions of full-length tau in either the presence or absence of DTT. The reactions were monitored by right angle laser light scattering (LLS). The presence of DTT in the tau polymerization reaction mixtures greatly enhanced the amount of LLS when compared to reaction mixtures without DTT (Figure 1A). This suggested that cysteine oxidation of full-length tau protein is not required for tau polymerization induced by AA, and may inhibit the process.

**Elimination of Cysteine Oxidation.** The effect of cysteine oxidation was further tested by changing the two cysteine residues present in the full-length tau molecule (Cys291 and Cys322) into nonreactive alanines through site-directed mutagenesis. The double-mutant form of the protein (hereafter termed cysless) was induced to form filaments upon addition of AA to the solution as indicated by the increase in the level of LLS (Figure 1B). This result further supported the supposition that cysteine oxidation is not required for filament formation. In addition, the cysless mutant protein was no longer dependent on the presence of DTT to achieve filament formation (Figure 1B).

**Cysteine Oxidation Alters the Apparent Rate of Polymerization.** When the x-axes of panels A and B of Figure 1 were

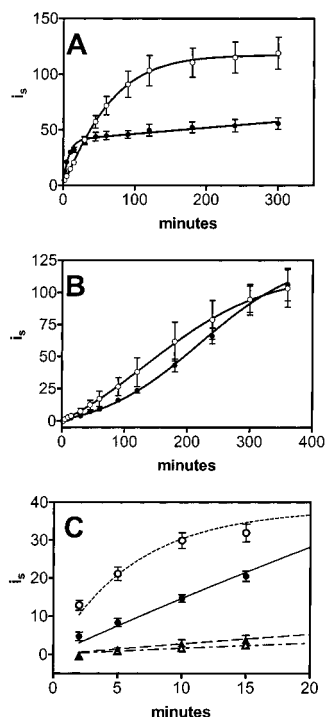


FIGURE 1: Cysteine oxidation inhibits the fatty acid induction of tau filament formation. (A) Wild-type tau (4  $\mu$ M) and 75  $\mu$ M AA in the presence (○) and absence (●) of 5 mM DTT. The data points are the average of eight trials  $\pm$  one standard error of the mean. (B) C291A/C322A, "cysless", double-mutant protein (4  $\mu$ M) and 75  $\mu$ M AA in the presence (○) and absence (●) of 5 mM DTT. The data points are the average of four trials  $\pm$  one standard error of the mean. Reactions were carried out at room temperature and monitored by the increase in the intensity of scattered light ( $I_s$ ) using a laser light scattering assay (LLS). (C) Data from panels A and B on an expanded scale to facilitate comparisons of the apparent initial velocities of polymerization. Early LLS values for httau40 in the absence of DTT (○, short-dash line), httau40 in the presence of DTT (●, solid line), cysless double-mutant protein in the absence of DTT (△, long-dash line), and cysless double-mutant protein in the presence of DTT (▲, long/short-dash line) are shown.

expanded to only include the early time points (first 15 min), major differences in the polymerization of the wild-type protein with DTT, the wild-type protein without DTT, and the cysless mutant protein were more easily discerned (Figure 1C). The wild-type protein polymerized most quickly in the absence of DTT. Also, the apparent rate of polymerization at these early time points for cysless (irrespective of the presence of DTT) was much lower than for the wild-type protein. The apparent velocity of the wild-type tau protein polymerization reaction in the presence of DTT was intermediate between the two extreme conditions for cysteine oxidation.

**Cysteine Oxidation Results in Morphologically Distinct Filaments.** The difference in apparent rates at these early time points prompted an investigation of the morphology of the filaments formed under these conditions. Samples were taken at the end of the polymerization reactions and viewed by electron microscopy (Figure 2A–D). Wild-type protein polymerized most rapidly in the absence of DTT; however, only very short filaments and spherical aggregates were formed (Figure 2B). Moreover, it must be emphasized that even these short filaments and particles of tau are not observed unless AA is added to the polymerization reaction mixture (data not shown). In the presence of DTT, the wild-

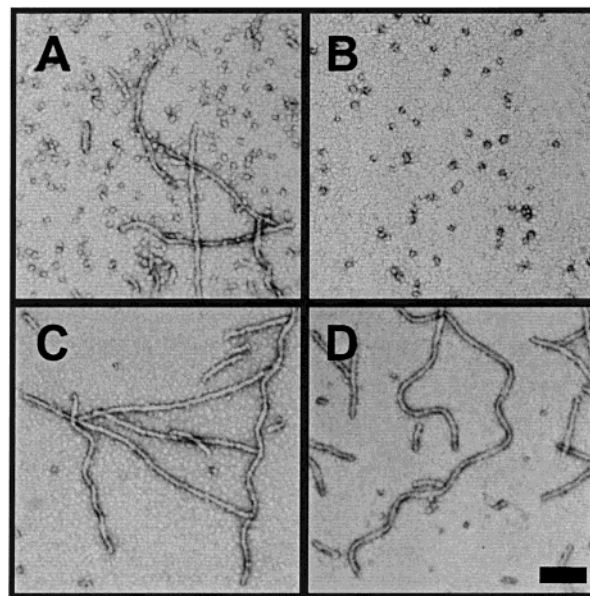


FIGURE 2: Morphological differences among filaments formed under different cysteine oxidative conditions. Wild-type tau protein in (A) the presence or (B) absence of 5 mM DTT and cysless double-mutant protein in (C) the presence or (D) absence of 5 mM DTT were incubated with 75  $\mu$ M AA for 5 h. Samples were removed and viewed by electron microscopy. High-magnification images are shown to facilitate morphological comparison. The bar is 200 nm long.

type protein exhibited an intermediate rate of polymerization and also appeared to form significant populations of both very short and longer filaments (Figure 2A). In contrast, filaments formed from the cysless mutant protein appeared to be much longer than those formed from the wild-type protein whether the polymerization reaction was performed in the presence or absence of DTT (Figure 2C,D).

These apparent morphological differences were confirmed by measuring the mass distribution of filament lengths in each of the four populations;  $91.7 \pm 1.2\%$  of the filament mass formed by the wild-type protein in the absence of DTT consisted of aggregates less than 50 nm in length (Figure 3B). In the presence of DTT, only  $52.4 \pm 14.7\%$  of the filament mass formed by the wild-type protein was in this category (Figure 3A). In stark contrast to these results, the filaments consisting of cysless had only  $11.3 \pm 1.7$  and  $23.1 \pm 7.1\%$  of their filament mass less than 50 nm in length in the presence and absence of DTT, respectively (Figure 3C,D).

**An Antioxidant Can Block the Fatty Acid Induction of Tau Polymerization.** The experiments described above conclusively show that the cysteine-mediated oxidation of the tau molecule inhibits tau polymerization under our assembly conditions. However, the fatty acids that were used in this in vitro polymerization assay are also potential oxidative targets in AD and in our assembly reactions. To test this, polymerization reactions were performed with the two major FAs of phosphatidylethanolamine in human gray matter: 20:4  $\omega$ 6 (AA) and 22:6  $\omega$ 3 (DHA). These studies required the use of BHT, a strong antioxidant that exhibits limited solubility in aqueous solutions. This property necessitated the use of electron microscopy for quantitation, since the presence of BHT in our assembly buffer generated unacceptably high background light scattering.

DHA, which has two more double bonds than AA and therefore four more sites for oxidation, induced the polym-



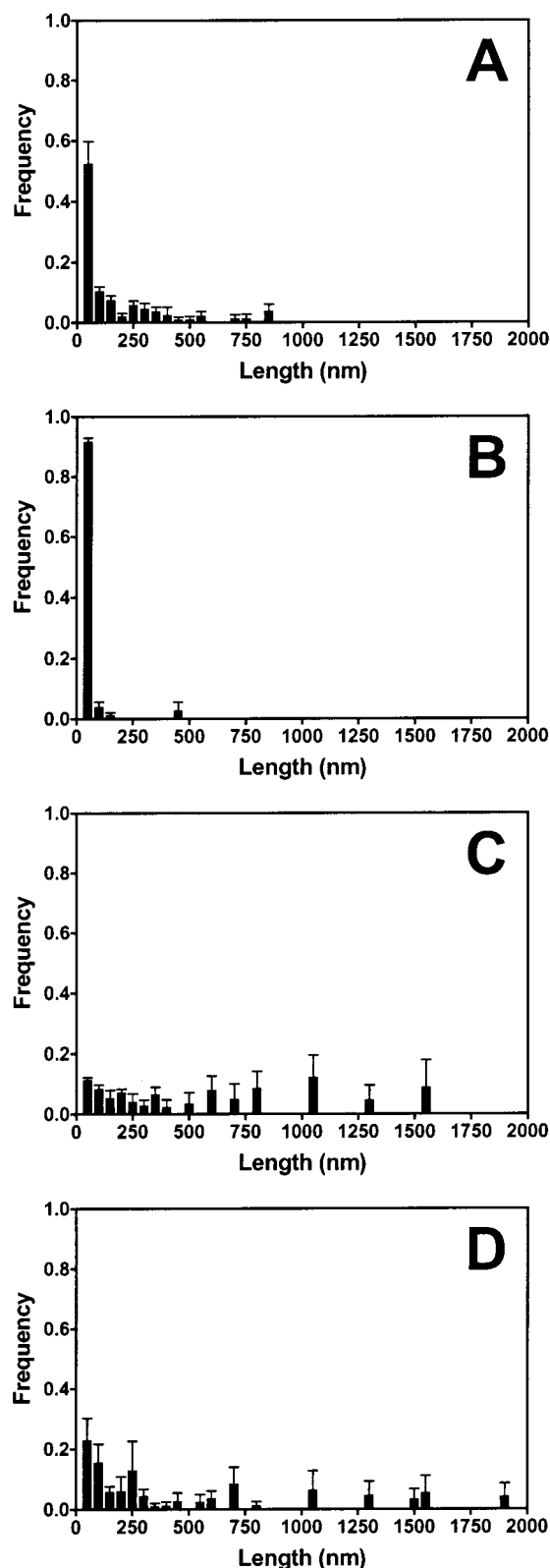


FIGURE 3: Mass distribution of filaments formed from htau40 and the double-mutant protein in the presence and absence of DTT. The relative distribution of filament mass is plotted in histograms (bin size of 50 nm) for (A) htau40 and DTT, (B) htau40 in the absence of DTT, (C) the double-mutant protein and DTT, and (D) the double-mutant protein in the absence of DTT. Filaments from regions of micrographs similar to those shown in Figure 2 were assessed manually using image analysis software. The relative mass distributions of filaments from several micrographs were averaged to generate the histogram (656, 385, 94, and 204 total filaments were assessed for panels A–D, respectively).

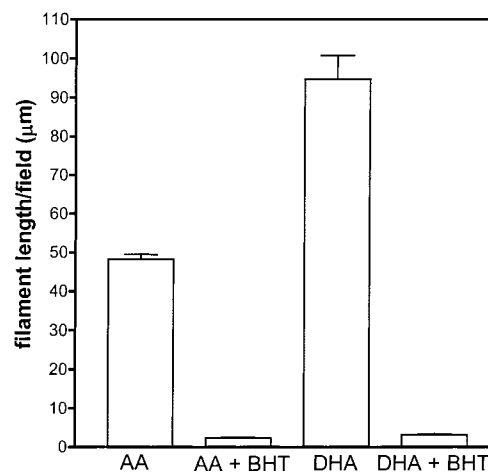


FIGURE 4: Antioxidants reduce the amount of FA-induced tau polymerization. Two different FA inducers, arachidonic acid (AA) and docosahexaenoic acid (DHA), were added to 4  $\mu$ M htau40 solutions at a final concentration of 75  $\mu$ M in either the presence or absence of 0.1% BHT. Samples were analyzed by quantitative electron microscopy since the relative insolubility of BHT interferes with LLS. Error bars represent one standard error of the mean (five representative fields were assessed for each condition). The amount of tau polymerization after 1 h was greatly reduced for both FAs employed in the presence of 0.1% BHT.

erization of tau to a greater degree than did arachidonic acid (Figure 4). In addition, when polymerization reactions with these two FAs were performed in the presence of BHT, the induction of tau assembly by either FA was inhibited (Figure 4). This suggests that oxidation of the FA is a necessary step for the induction of tau filament formation (Figure 4). This conclusion was supported by control experiments in which BHT inhibited cystless polymerization reactions, as well (Figure 5). These data suggest that the effect of BHT on polymerization was due to the inhibition of FA oxidation and had no effect on the tau molecule per se.

*Increases in Oxidative Potential Can Influence Tau Polymerization.* Since the addition of BHT to create a nonoxidative environment inhibited the fatty acid induction of tau polymerization, the effect of increasing the oxidative potential of the polymerization reaction was investigated. Four different oxidative conditions were used. The first condition employed the fatty acid and tau in polymerization buffer alone. These are the same conditions used for the tau polymerization reactions described previously. It can be assumed that this is an oxidative environment since the addition of BHT inhibited the reaction (Figures 4 and 5). The second condition involved increasing the amount of time the fatty acid was in the oxidative environment of the polymerization reaction before the addition of tau. The third condition was created by supplementing the FA- and tau-containing assembly buffer with 50  $\mu$ M  $\text{FeCl}_3$ , 20 mM ADP, and 10 mM ascorbic acid. Similar conditions have been used previously to generate oxidation products of AA and DHA, the  $\text{F}_2$ -isoprostanes and the  $\text{F}_4$ -neuroprostanes, respectively (29, 30). The levels of these compounds have been shown to be elevated in the brain (32) and cerebrospinal fluid (30) of AD patients when compared to age-matched control levels. The fourth condition involved the incubation of the fatty acids in polymerization buffer supplemented with an Fe/ADP/ascorbate mixture for 1 h before the addition of tau.

AA induced tau polymerization under the first oxidative condition (in polymerization buffer). The second (increased

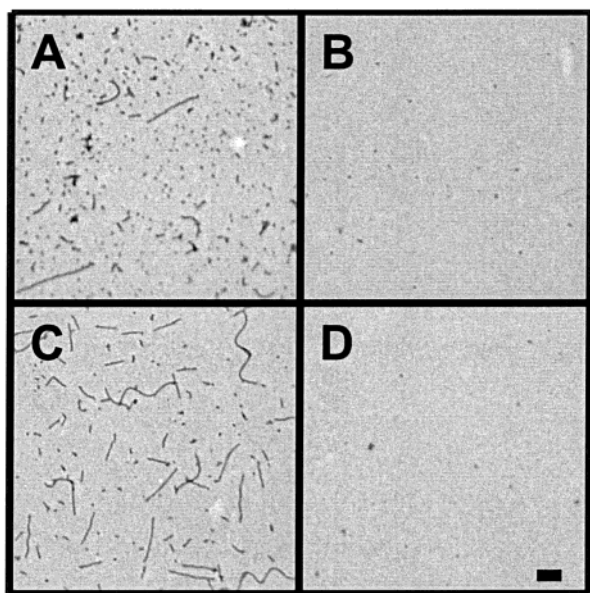


FIGURE 5: BHT inhibits FA-induced tau polymerization in a tau oxidation-independent fashion. (A) Wild-type tau protein and (C) cysless double mutant at concentrations of  $4\ \mu\text{M}$  were incubated in the presence of  $75\ \mu\text{M}$  AA and  $5\ \text{mM}$  DTT for 5 h. Both forms of tau were induced to form filaments. The addition of BHT greatly reduced the amount of filament formation for both (B) wild-type tau and (D) cysless. Lower-magnification images are shown to illustrate differences in the total amount of filaments formed. The bar is  $200\ \text{nm}$  long.

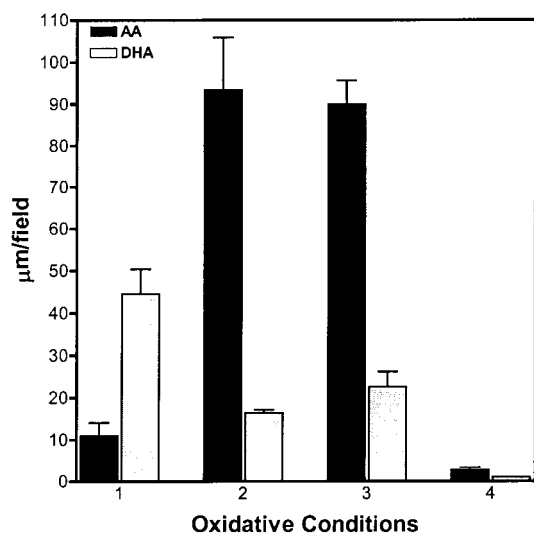


FIGURE 6: Effect of various oxidative environments on the ability of AA and DHA to induce tau polymerization. Tau solutions ( $4\ \mu\text{M}$ ) and fatty acid ( $75\ \mu\text{M}$ ) (AA, black bars; DHA, gray bars) were incubated for 3 h under different oxidative conditions. The conditions are listed on the x-axis and are as follows: condition 1, polymerization buffer; condition 2, 1 h preincubation of FA in polymerization buffer before the addition of tau; condition 3, polymerization buffer and the Fe/ADP/ascorbate oxidation mixture; and condition 4, 1 h preincubation of FA in polymerization buffer and the Fe/ADP/ascorbate oxidation mixture before the addition of tau. Samples were quantified by electron microscopy to be consistent with the BHT inhibition experiment. Error bars represent one standard error of the mean of five representative fields.

incubation time in polymerization buffer) and the third (addition of an Fe/ADP/ascorbate mixture) conditions increased the amount of tau filament formation (Figure 6). If the AA was left in buffer alone for 1 h (condition 2), this would increase the amount of time that it was exposed to

potential oxidizers in the polymerization buffer, thereby increasing the chances that the AA would become oxidized. By the addition of the Fe/ADP/ascorbate system (condition 3), there would be a direct introduction of chemicals that would create free radicals, thereby increasing the chances that the AA would become oxidized. The fourth oxidative environment (preincubation with an Fe/ADP/ascorbate mixture for 1 h prior to the addition of tau), however, greatly diminished the amount of tau filament formation. This suggests that an assembly-inducing oxidative product may be susceptible to further oxidation into compounds incapable of inducing tau polymerization.

DHA behaved differently than AA (Figure 6). Under the first oxidative condition (polymerization buffer alone), DHA induced the formation of tau filaments to a greater extent than did AA under the same conditions. However, the increased oxidative potentials under conditions 2–4 had a detrimental effect on DHA-induced tau filament formation. This reduction in the efficacy of DHA's induction of tau polymerization is most likely due to its increased sensitivity to oxidation since it contains six double bonds as opposed to the four double bonds in AA. Its mere introduction into an aqueous environment would appear to be sufficient to oxidize it into its active form. Further oxidation, as was the case with AA, results in compounds which are incapable of inducing tau polymerization.

## DISCUSSION

*An in Vitro Paradigm for Tau Polymerization.* We have sought to investigate the role of oxidative stress factors using an in vitro paradigm for tau polymerization. In this system, FAs that are targets of oxidative stress in AD brain are used to induce full-length tau molecules to form filamentous structures. These tau filaments form efficiently in a few hours and are morphologically similar to the straight filaments observed in AD and other neurodegenerative diseases (20). In addition, these filaments are similar to tau-positive AD structures in their reactivity to conformationally sensitive antibodies and to thioflavin-S (21). The synthetic filaments may also be structurally related to authentic paired-helical filaments (PHFs), in that PHFs purified from AD brain can be used as "seeds" for synthetic filament formation (21).

*Polymerization of Cysteine-Oxidized Tau.* Previously, the only link between oxidative stress and tau filament formation has come from studies in which the oxidation of the tau molecule is a prerequisite for its polymerization in vitro. These studies show that the oxidation of Cys322 in tau isoforms containing only three microtubule binding repeats results in disulfide-linked dimers and is a necessary first step for the induction of tau filament formation by polyanionic compounds (31). In contrast to this disulfide dimer paradigm of tau polymerization, the removal of a sulfhydryl protective agent, DTT, from FA-induced polymerization reaction mixtures did not result in filament formation, but rather in the formation of small ( $<50\ \text{nm}$ ) structures whose relationship to longer filaments is currently unknown. These structures were present in filament populations that were formed in the presence of DTT, but only accounted for ~50% of the total filament mass. When the reactive cysteines of tau were mutated into nonreactive alanines, the mutants were still capable of filament formation, albeit at a reduced

apparent velocity. The filaments that were formed, however, were on average much longer than those formed from the wild-type protein. This is most likely due to the fact that the extent of formation of the shorter structures (<50 nm) was greatly reduced. When the apparent rate of filament formation is compared with the length distributions of the filaments, it appears that the formation of the cysteine oxidation-enhanced short structures (<50 nm) is energetically favored over the formation of longer, true filamentous tau structures. Therefore, it is possible that this process is actually in competition with filament formation even in the presence of DTT. These structures do not elongate further after several days of incubation (data not shown), suggesting that they are not in a steady-state relationship with filaments but may represent an alternate form of tau aggregate.

The fact that fatty acids stimulate the polymerization of tau in which the cysteines have been mutated to alanines unequivocally demonstrates, at least in this model system and under these experimental conditions, that disulfide bond formation is not essential for filament formation. It is possible that other oxidative changes in the tau molecule, such as carbonyl formation or tyrosine nitration, could affect the AA-induced in vitro polymerization of tau, although these alterations have not been investigated with our assembly paradigm.

Since it is clear that the polyanion induction of tau polymerization in vitro does require cysteine-mediated oxidation (31), at least two possible mechanisms could exist for tau polymerization in vivo: one which requires cysteine oxidation and one which requires unmodified cysteines. It would be possible for such disparate mechanisms to coexist since the inducing molecules are different and the apparent reactive sites within the tau molecule are also different for the two paradigms (33, 34). However, it is our view that the formation of large concentrations of disulfide-based tau dimers as a prerequisite for filament formation is highly unlikely due to the overall reducing environment of the cell.

**Oxidative Stress Factors in Alzheimer's Disease.** Factors which lead to increased amounts of oxidative stress are believed to play a major role in the etiology of AD. These factors may include the normal aging process, head trauma, increased levels of heavy metals (Fe, Al, and Hg), and, especially in the case of AD, aggregation of the  $\beta$ -amyloid protein ( $A\beta$ ). These factors are thought to generate free radicals, possibly in the form of reactive oxygen species, that would then attack biological molecules that are sensitive to oxidation, such as proteins, DNA, lipids, and fatty acids (reviewed in ref 26).

Polyunsaturated fatty acids (FAs) are especially vulnerable to oxidative stress since their double bonds make the removal of  $H^+$  by free radicals relatively easy. Although some reports disagree on the location of FA oxidation in AD brain (reviewed in ref 35), it is clear that the levels of thiobarbituric acid reactive substances (a marker for FA oxidation) are elevated in these patients. In addition, many FA breakdown products, including malondialdehyde (MDA) and hydroxynonenal (HNE), can be detected at greater than normal levels in AD patients. Increased amounts of specific FA metabolites, such as the  $F_2$ -isoprostanes and  $F_4$ -neuroprostanes, can also be found in the affected brain regions of AD patients and even in the cerebrospinal fluid of probable AD patients (36).

**Role of Fatty Acid Oxidation in Tau Polymerization.** As mentioned above, FAs are a major oxidative target in vivo, and therefore could be affected in our in vitro system. The notion that FA oxidation could affect the induction of polymerization is actually not without precedence. Previous experimentation in this laboratory drew a rough correlation between the increased number of sites of unsaturation in FA and the increased efficacy of polymerization (20). In fact, we show in this report that DHA, which contains two more double bonds than AA, stimulates tau filament formation to a greater extent than AA. This is likely indicative of oxidative events facilitating the FA induction of tau polymerization. Consistent with this notion, the addition of a free radical-scavenging antioxidant BHT to the FA-induced tau polymerization reaction mixtures inhibited the process. This phenomenon was most likely due to the inhibition of AA and DHA oxidation, since BHT also inhibited the induction of polymerization of the cystless form of the tau molecule. We therefore conclude that the oxidation of polyunsaturated fatty acids is greatly beneficial or possibly essential to their ability to induce tau polymerization.

Since it was evident that the FA oxidation occurring under normal polymerization conditions was beneficial, we investigated the effects of increasing the potential of the oxidative components of the reaction. We found that the increase in oxidative potential did enhance the AA induction of tau polymerization. DHA, which is more easily oxidized than AA, was a more potent inducer under milder oxidative conditions than AA. However, we also found that the FAs can be overoxidized if the oxidative conditions become too severe. The overoxidation of the FAs resulting in the inhibition of tau assembly is likely due to the additional oxidative changes that inactivate biologically active FA metabolites. Therefore, we speculate that a specific class of FA oxidative products could be responsible for the induction of tau polymerization, and that further alterations of this specific class would have deleterious effects on the assembly reaction. Since this oxidation can presumably occur during storage at  $-20^\circ\text{C}$ , reactions performed on different days or with different lots of FA can result in variable amounts of tau polymerization (compare Figures 4 and 6).

**Biological Implications of the Oxidative Effects on FA-Induced Tau Polymerization.** When the data presented above are considered in the context of the known aspects of neurodegeneration, a potential mechanism for this process becomes evident (illustrated in Figure 7). The most common feature of neurodegeneration is that it is age-dependent, occurring mainly in the elderly. This may be due to the gradual accumulation of oxidative stress factors during the aging process (37). Oxidants such as free radicals and reactive oxygen species are elevated in familial cases of AD, perhaps due to an increased level of deposition of  $A\beta$  resulting from mutations in the amyloid precursor protein or presenilins. However, any source of oxidative stress, such as the accumulation of heavy metals, would be sufficient to propagate the process. The increased levels of oxidants then lead to fatty acid oxidation and the release of FA derivatives such as isoprostanes and neuroprostanes (30, 32, 36).

As our data suggest, the increased intracellular concentrations of such classes of FA oxidation products could then induce tau to form the filamentous structures (Figure 7). Even under the high levels of oxidative stress found in AD that



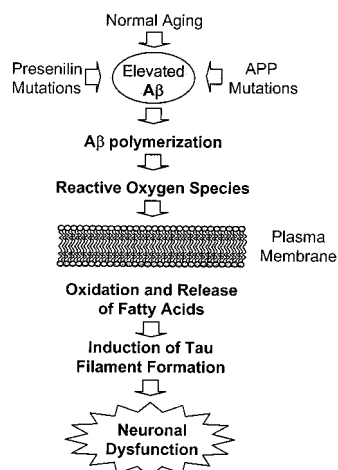


FIGURE 7: Hypothetical model for the oxidative stress induction of tau polymerization and the subsequent neurodegeneration in Alzheimer's disease. See the text for details.

could result in further oxidation into inactive species, a transient influx of assembly-promoting FA metabolites could provide the impetus for tau filament formation since the induction process appears to occur rapidly. In other related neurodegenerative diseases, such as FTDP-17, the absence of A $\beta$  senile plaques indicates a reduced oxidative insult. However, these genetic "tangle-only" diseases may require less FA oxidation for tau polymerization due to the structural alterations in tau generated by point mutations or increased 4R/3R ratios (19, 22, 23, 38). Here, as in AD, we postulate that accumulation of these tau filaments leads to neurodegeneration (Figure 7).

Existing putative protective factors for AD include anti-oxidant vitamins and nonsteroidal anti-inflammatory drugs (reviewed in ref 39) that target the pathways of oxidative stress and FA metabolism. These factors presumably ameliorate the excessive nonenzymatic lipid peroxidation and cyclooxygenase lipid processing associated with AD. The scheme presented here (Figure 7) represents a hypothetical link between these metabolic events and neurodegeneration. Although the actual mechanism is likely more complicated than our representation, such a model may prove useful in charting future research directions.

**Conclusion.** Our data suggest that FA oxidation events, rather than the cysteine-dependent oxidation of tau protein, may lead to the generation of the pathological structures found in AD and other neurodegenerative disorders. Our goal now is to identify the FA metabolite(s) that could be responsible for the induction of filament formation. This is a daunting task since more than 5000 oxidation states of AA are known. Still, such knowledge could provide important clues leading to preventative and/or treatment strategies for AD.

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