THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 10, pp. 6984–6991, March 9, 2007

Effect of Pseudophosphorylation and Cross-linking by Lipid Peroxidation and Advanced Glycation End Product Precursors on Tau Aggregation and Filament Formation*

Received for publication, October 10, 2006, and in revised form, October 30, 2006 Published, JBC Papers in Press, November 1, 2006, DOI 10.1074/jbc.M609521200

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Accumulation of hyperphosphorylated Tau protein as paired helical filaments in pyramidal neurons is a major hallmark of Alzheimer disease. Besides hyperphosphorylation, other modifications of the Tau protein, such as cross-linking, are likely to contribute to the characteristic features of paired helical filaments, including their insolubility and resistance against proteolytic degradation. In this study, we have investigated whether the four reactive carbonyl compounds acrolein, malondialdehyde, glyoxal, and methylglyoxal accelerate the formation of Tau oligomers, thioflavin T-positive aggregates, and fibrils using wild-type and seven pseudophosphorylated mutant Tau proteins. Acrolein and methylglyoxal were the most reactive compounds followed by glyoxal and malondialdehyde in terms of formation of Tau dimers and higher molecular weight oligomers. Furthermore, acrolein and methylglyoxal induced the formation of thioflavin T-fluorescent aggregates in a triple pseudophosphorylation-mimicking mutant to a slightly higher degree than wild-type Tau. Analysis of the Tau aggregates by electron microscopy study showed that formation of fibrils using wild-type Tau and several Tau mutants could be observed with acrolein and methylglyoxal but not with glyoxal and malondialdehyde. Our results suggest that reactive carbonyl compounds, particularly methylglyoxal and acrolein, could accelerate tangle formation *in vivo* and that this process could be slightly accelerated, at least in the case of methylglyoxal and acrolein, by hyperphosphorylation. Interference with the formation or the reaction of these reactive carbonyl compounds could be a promising way of inhibiting tangle formation and neuronal dysfunction in Alzheimer disease and other tauopathies.

Neurofibrillary tangles, composed of bundles of paired helical filaments (PHFs),[3] consist of hyperphosphorylated and aggregated forms of the microtubule-associated protein Tau. Neurofibrillary tangles are major hallmarks of neurodegenerative diseases including Alzheimer disease (AD) (1). Under non-pathological conditions, Tau is a developmentally regulated phosphoprotein that promotes assembly and stability of microtubules and is thus involved in axonal transport (2, 3). In AD and other tauopathies, Tau proteins aggregate and form fibrillar insoluble intracellular inclusions, so-called neurofibrillary tangles. It has been suggested that ionic interactions and covalent cross-linking contribute to pathological Tau aggregation and tangle formation. For example, ionic interactions with glucosaminoglucans (4–7), Mg^{2+} or Ca^{2+} (8), Al^{3+} and Fe^{2+}/Fe^{3+} (9–12), or covalent modifications such as glycosylation (13, 14), glycation (15, 16), or the formation of disulfide bridges (17) have been shown to favor the formation of Tau aggregates *in vitro*.

More recently, reactive carbonyl compounds (RCCs), which are increased under conditions of oxidative stress and in aging (18, 19), have attracted interest as potential bifunctional compounds responsible for promoting cross-linking between proteins and favoring their aggregation. Protein modifications by such RCCs, including the lipid peroxidation products 4-hydroxy-2-nonenal (20), malondialdehyde (MDA) (15, 21, 22), and acrolein (2-propen-1-al) (23) have been detected immunohistochemically in AD brains, particularly in neurofibrillary tangles. Advanced lipid peroxidation end products (ALEs) have also been described to be increased in the cerebrospinal fluid of AD patients (21, 24, 25). Glyoxal, a product of lipid peroxidation or glycation, and methylglyoxal (MG), formed by oxidation of sugars or produced as an intermediate of triosephosphate, threonine, and glycine catabolism, reacts with proteins to form "advanced glycation end products" (AGEs). Immunohistochemical analysis indicates that glyoxal/MG-derived AGE antibodies label predominantly PHF in aggregates (26–28).

* This work was supported by the Interdisziplinäre Zentrum für Klinische Forschung Leipzig at the Faculty of Medicine of the Universität Leipzig (01KS9504, Project N1 and C24), the Hirnliga e.V. (to B. K.), and the Deutsche Forschungsgemeinschaft (HO-2368/1-1, Max Holzer), and a merit research grant by James Cook University (to G. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[3] The abbreviations used are: PHF, paired helical filament; AD, Alzheimer disease; AGE, advanced glycation end product; ALE, advanced lipid peroxidation end product; MDA, malondialdehyde; MG, methylglyoxal; MOPS, 4-morpholinepropanesulfonic acid; RCC, reactive carbonyl compound; CML, carboxymethyllysine.

Hyperphosphorylation is a second important modification, which has been suggested to contribute to Tau aggregation. Tau can be phosphorylated at ~30 sites; however, little is known about which phosphorylation sites actually enhance Tau fibril formation. In *in vitro* aggregation assays, introduction of a negative charge by substitution of serine or threonine by glutamic acid or aspartic acid residues ("pseudophosphorylation") has been shown to mimic the effect of phosphorylation of Tau (29). Such Tau mutants have been used to examine the influence of phosphorylation at distinct sites on Tau aggregation induced by heparin and certain metal ions. It has been shown that these "pseudophosphorylated" Tau mutants, particularly those carrying substitutions in C-terminal regions, have a slightly higher tendency to aggregate than the Tau wild-type protein (30).

In this study, we wanted to elucidate whether the reactive carbonyl compounds acrolein, glyoxal, MG, or MDA are able to induce or accelerate Tau oligomerization, formation of thioflavin T-positive Tau aggregates, and formation of PHFs. Furthermore, we wanted to investigate whether this aggregation process is accelerated by phosphorylation at specific sites using eight different pseudophosphorylated Tau mutants.

MATERIALS AND METHODS

Construction of Expression Plasmids for Tau Mutants and Purification of Recombinant Proteins—Constructs for the Tau mutants were prepared as described previously (30). Briefly, codons for the amino acids representing the potential phosphorylation sites (Thr or Ser) were changed to GAA (glutamate) or GAT (aspartate) using one-tube, two-stage, PCR-directed *in vitro* mutagenesis. Mutant blunt-ended constructs were cloned into the EcoRV site of pZerO-2 and verified by DNA sequencing. For protein expression, Tau constructs were cloned into the EcoRI/NotI sites of pGEX-6P-1 and expressed as glutathione *S*-transferase fusion proteins. pGEX-6P-1 plasmids were transformed into *Escherichia coli* (BL21-CodonPlus(DE3)-RIL) cells. Cells were grown in LB medium supplemented with 50 μg/ml ampicillin and 50 μg/ml chloramphenicol overnight. Cells were grown to late exponential phase (A_{600} = 1.5) and induced with 1 mM isopropyl-β-thiogalactosidase for 2 h at 30 °C. The cell suspension was centrifuged at 7000 × *g* for 10 min at 4 °C and the cell pellet resuspended in 1/20 of the volume of 1× binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3). Cells were then lysed using a sonicator. To facilitate solubilization of protein, 1% (v/v) Triton X-100, lysozyme (4000 units/ml), and benzonase (4 units/ml) were added, and the mixture slowly shaken for 1 h at 4 °C. The clarified lysate was incubated with glutathione-Sepharose 4B for 2 h to bind the expressed fusion proteins. After washing the matrix two times with binding buffer, the recombinant protein was eluted by site-specific proteolysis using PreScission™ protease (10 units/ml) overnight at 4 °C. Protein concentration was quantified according to the method of Bradford (31).

Modification of Tau with Reactive Carbonyl Compounds—Glyoxal (trimer dihydrate) and acrolein (Sigma) were used without further purification. Methylglyoxal was freshly distilled from a 40% solution and then stored at −80 °C before use. Its concentration was determined according to a method described previously (32). Malondialdehyde was generated *in situ* from malondialdehyde bis(dimethyl)acetate by hydrolysis with 1 mM HCl for 5 min. Tau protein (3 mg/ml) was dissolved in 20 mM MOPS (pH 7.0) and incubated with each of the carbonyl compounds (in the concentrations indicated) in a microvessel at 37 °C for up to 96 h.

Determination of Tau Modification and Aggregate Formation by Gel Electrophoresis and Western Blot—Tau samples were boiled for 5 min in Laemmli SDS sample buffer and separated by SDS-PAGE on a 5–20% gradient polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue or for Western blots; Tau samples were transferred to nitrocellulose membrane. Blots were blocked in Roti-Block (ROTH, Karlsruhe, Germany) for 1 h at room temperature. Two different AGE antibodies (6D12 and 4G9) were used. The 6D12 antibody (1:1000) (TransGenic, Inc., Kumamoto, Japan) is an antibody with broad specificity. It has been described to detect AGE-human serum albumin/bovine serum albumin, AGE-α-Tos-Lys, AGE-α-Tos-Lys-o-Me, AGE-β-alanine, AGE-γ-aminobutyric acid, AGE-ϵ-amino capronic acid. But no Schiff bases, Amadori products, unmodified human serum albumin/bovine serum albumin, poly(Lys), unmodified Lys-derivatives, unmodified mono-amino-carbonic acids, FFI, pyrrolaldehyde, and pentosidin) (33, 34) The carboxymethyllysine (CML) antibody 4G9, (1:1000) (Roche Diagnostics) predominantly detects CML residues. Membranes were incubated with the antibodies for 16 h at 4 °C. After five washing steps with TBST (Tris-buffered saline containing 0.1% Tween 20 and thioflavin T (ThT)), a horseradish peroxidase rabbit anti-mouse antibody (Dako, Hamburg, Germany, 1:1500) was applied for 1 h at room temperature. After five washing steps with TBST, the blots were developed on hyperfilms using the ECL kit (Amersham Biosciences AB, Uppsala, Sweden).

Determination of Tau Aggregate Formation by Thioflavin T Assay—Tau (S396D/T403E/S404D) or wild-type Tau (each 1.7 mg/ml) was dissolved in 20 mM MOPS (pH 7.0). Thioflavin T was added (final concentration of 5 μM) and either incubated without or with 1 mM methylglyoxal or acrolein at 37 °C for 72 h. Thioflavin T fluorescence was analyzed in a volume of 100 μl in a 96-well plate (excitation, 400 nm; emission, 485 nm). Fluorescence values were set as 100% for proteins containing ThT, and changes were recorded after the addition of RCCs. As negative controls, buffer samples containing thioflavin T and either acrolein or methylglyoxal were used.

Determination of Tau Aggregate Formation by Electron Microscopy—Filament formation was analyzed by electron microscopy. Samples were placed on a nickel slot grid and stained with 2% uranyl acetate for 1 min. The grids were examined in a 912 OMEGA electron microscope (Zeiss, Germany) equipped with a digital camera (charge-coupled device-slow scan, Proscan). All images were performed at a magnification of 80,000× and processed in Adobe Photoshop (*scale bars* = 100 nm) (Fig. 6).

RESULTS

Formation of Tau Multimers by Reactive Carbonyl Compounds; Detection by SDS-PAGE and Commassie Blue Stain—Recombinant human Tau (full-length of wild-type Tau) and the

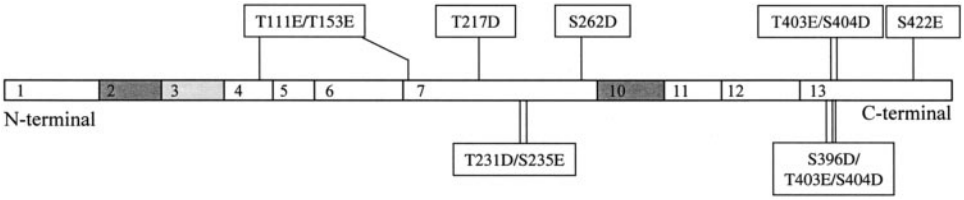

FIGURE 1. **Constructs used for the expression of pseudophosphorylated Tau mutants.** Phosphorylation of Tau at specific sites was simulated by substituting serine or threonine with glutamic or aspartic acids, respectively. Seven pseudophosphorylated mutants were created, the single mutant T217D (in which threonine in position 219 was replaced aspartic acid), S422E and S262D, the double mutants T111E + T153E, T231D/S235E, and T403E/S404, and the triple mutant S396D/T403E/S404D. One more construct used in this study was the one for wild-type Tau (also termed wtt) containing the original sequence without any amino acid replacement.

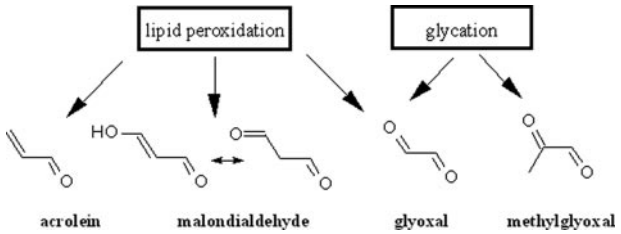

FIGURE 2. **Chemical structures of reactive carbonyl compounds and routes to their formation.** Non-enzymatic degradation of glucose or Amadori products or glucose metabolism yields methylglyoxal or glyoxal, which can react with proteins to form advanced glycation end products (AGEs). Radical-induced lipid peroxidation yields reactive carbonyl compounds such as acrolein, malondialdehyde, or glyoxal, which react with proteins to form advanced lipid peroxidation end products (ALEs).

TABLE 1

Calculation of stoichiometric ratios between lysine and arginine residues of human Tau proteins and different concentrations of RCCs

10 mM RCC represents an excess, which should yield maximally modified Tau proteins.

Tau protein		Reactive side chain		
3 mg/ml = 0.071 mM		*/10 mM RCC*	*/1 mM RCC*	*/250 μM RCC*
40 Lysine residues	2.8 mM	1/3.6	1/0.36	1/0.09
18 Arginine residues	1.3 mM	1/7.8	1/0.77	1/0.19
Reactive residues (Σ)	4.1 mM	1/2.5	1/0.25	1/0.06

following six Tau mutants with the following phosphorylation-mimicking amino acid substitutions were used: (*a*) (single substitution) aspartic acid replacing threonine at position 217 (T217D), aspartic acid replacing serine at position 262 (S262D), and glutamic acid replacing serine at position 422 (S422E); (*b*) (double substitution) glutamic acid replacing threonine both at positions 111 and 153 (T111E/T153E), aspartic acid replacing threonine at position 231, glutamic acid replacing serine at position 235 (T231D/S235E), glutamic acid replacing threonine at position 403, and aspartic acid replacing serine at position 404 (T403E/S404D); (*c*) (triple substitution) aspartic acid replacing serine at positions 396 and 404 and glutamic acid replacing threonine at position 403 (S396D/T403E/S404D) (Fig. 1). For the *in vitro* aggregation assays, the eight recombinant Tau proteins were incubated with methylglyoxal, glyoxal, acrolein, or malondialdehyde (Fig. 2), respectively, and then separated on a reducing SDS-polyacrylamide gel and stained with Commassie Blue.

In the first set of experiments, RCCs were used at a concentration of 10 mM. Because Tau protein, which contains 40 lysine and 18 arginine residues, was used at a concentration of 3 mg/ml, 10 mM RCCs accord to a 2.5-fold excess/reactive basic amino acid, allowing Tau to become "maximally modified" (Table 1).

Reaction with Acrolein—All Tau isoforms were completely converted to high molecular weight aggregates by 10 mM acrolein after 96 h (Fig. 3*A*). In some cases, diffuse bands between 120 and 200 kDa could be detected, which might represent highly modified dimers or trimers. Interestingly, the appearance of such multiple, diffuse Tau protein multimers after polyacrylamide gel electrophoresis has been reported previously for PHFs isolated from AD brains (35, 36). In some cases, *e.g.* in the Tau T403E/S404D mutant, the combined intensity of all bands appears to be far lower than with the other RCCs. Possible explanations for this apparent loss of total Tau protein might be the formation of high molecular weight Tau multimers too large to enter the gel or the low affinity of Coomassie Blue to proteins having lost their positive charges.

Reaction with Glyoxal—Incubation of wild-type Tau, Tau T111E/T153E, Tau T217D, and Tau S422E with 10 mM glyoxal resulted in a weakly stained band at 100–120 kDa (Fig. 3*A*), which may suggest dimerization of these Tau isoforms. In contrast, the incubation of all other Tau mutants with glyoxal only caused a small increase of the molecular weight of the monomer but did not lead to the formation of dimeric or higher oligomeric structures. The triple Tau mutant S396D/T403E/S404D behaved quite unusually, because it produced a high molecular oligomer, whereas the monomeric band at ~50 kDa nearly disappeared.

Reaction with Malondialdehyde—When Tau proteins were incubated with 10 mM MDA, formation of dimers (and trimers in the case of T111E/T153E) could be observed at ~100-120 kDa after 96 h (Fig. 3*A*). Again, the Tau mutant S396D/T403E/S404D behaved quite unusually, because it produced a higher molecular oligomer, whereas the monomeric band at ~50 kDa disappeared (Fig. 3*A*).

Reaction with Methylglyoxal—All seven pseudophosphorylated Tau mutant proteins appeared to have reacted with 10 mM methylglyoxal after 96 h. Bands corresponding to a trimer (150 kDa), a tetramer (200 kDa), and higher molecular weight oligomeric Tau products >250 kDa could be observed (Fig. 3*A*). With wild-type Tau, only a faint band at ~120 kDa could be observed.

Control Incubations without Reactive Carbonyl Compounds—When wild-type Tau or the phosphorylation mimicking Tau mutant proteins were incubated at 37 °C without carbonyl compounds, no Tau dimers, oligomers, or other multimers could be observed. These negative controls are marked with *c* in Fig. 3.

Because the results obtained from the experiments above suggested that the reactivity of the RCCs decreased in this order, acrolein > methylglyoxal > glyoxal ≈ malondialdehyde, only the most reactive compounds, methylglyoxal and acrolein, were used at lower, more physiological concentrations. At concentrations of 1 mM RCCs, only each fourth at 250 μM and each

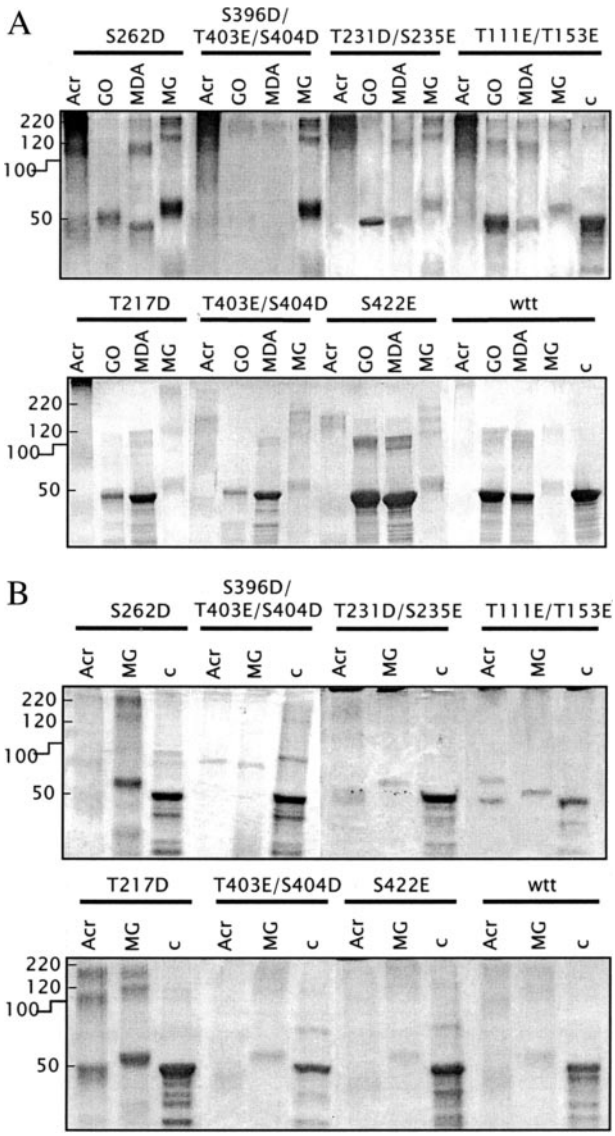

FIGURE 3. *A*, Tau molecular weight changes and formation of Tau oligomers with differing reactive carbonyl compounds. Recombinant Tau proteins (wild-type and pseudophosphorylation mutants) were incubated each with 10 mM acrolein (*Acr*), glyoxal (*GO*), MDA, or MG at 37 °C for 96 h. Proteins were separated by SDS-PAGE and stained with Coomassie Blue. Wild-type Tau and Tau T111E/T153E incubated without any carbonyl served as the controls (*lane c*), whereas the other controls are shown in *B*. *B*, to evaluate the cross-linking reactivity of the most reactive carbonyl compounds, acrolein and methylglyoxal, at lower concentrations, and recombinant Tau proteins were incubated with 1 mM acrolein and 1 mM MG. All Tau proteins incubated without RCCs served as controls (*lane c*).

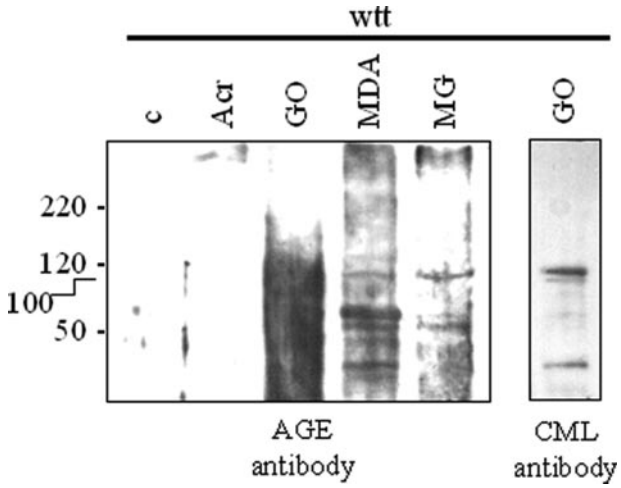

FIGURE 4. **AGE/ALE modification of carbonyl-modified wild-type Tau.** Shown is a Western blot of wild-type Tau incubated without any carbonyl (*lane c*), with acrolein (*Acr*), glyoxal (*GO*), MDA, and MG, each 10 mM, for 96 h at 37 °C. Tau modifications were immunostained by the anti-AGE antibody (clone 6D12), indicating that all carbonyls modify Tau to yield AGEs or ALEs, respectively (*left panel*). Glyoxal modification of Tau could be detected as CML by use of the monoclonal 4G9 antibody (*right panel*).

sixteenth lysine or arginine residue could be modified, respectively (Tab. 1). As shown in Fig. 3*B*, the reaction of Tau with 1 mM acrolein or methylglyoxal also caused the disappearance of Tau monomer and formation of high molecular weight oligomers. In contrast, incubations with 250 μM acrolein or methylglyoxal did not significantly convert Tau monomers into oligomers within the 96 h period, although intensity of monomer bands decreased and a significant increase in the apparent molecular weight of Tau (2–3 kDa) could be observed (data not shown). It was not possible to incubate the Tau preparations for longer time periods than 96 h, because residual protease activity led to obvious protein degradation. It has to be noted that some of the non-modified Tau mutant protein preparations, *e.g.* S262D and S396D/T403E/S404D already contained some traces of oligomers (Fig. 3*B*, *lanes c*). These oligomers disappeared after incubation with 10 mM acrolein or methylglyoxal but not with 1 mM (Fig. 3) or 250 μM (data not shown).

AGE and ALE Modification of Tau Oligomers by Reactive Carbonyl Compounds; Detection by SDS-PAGE and Western Blot—Proteins modified with RCCs eventually form stable end products, which are termed "advanced glycation" or "advanced lipid peroxidation" end products (AGEs or ALEs). To investigate whether higher molecular weight bands of Tau are AGE- or ALE-modified, Western blot analysis was performed with wild-type Tau after incubation with 10 mM acrolein, glyoxal, methylglyoxal, and MDA for 96 h (samples were identical to those shown in Fig. 3*A*). Glyoxal-, MDA-, and methylglyoxal-modified Tau were strongly recognized by the AGE antibody 6D12, whereas acrolein-modified Tau was only weakly recognized (Fig. 4, *left panel*). The specific CML antibody 4G9 detected glyoxal-modified Tau only (Fig. 4) but none of the other carbonyl modifications (data not shown). As expected, non-modified Tau showed no immunoreaction with the AGE antibody (Fig. 4, *lane c*) or the CML antibody (data not shown). These results suggest that most of the Tau oligomers are either AGE- or ALE-modified, assuming that the monoclonal AGE antibody not only detects AGEs but also some chemically similar ALEs. However, it appears that the epitope formed by acrolein is quite unique and cannot be detected by a "conventional" AGE antibody (37).

Formation of Tau Aggregates by Reactive Carbonyl Compounds; Detection by Thioflavin T Fluorescence—Thioflavin T is a fluorescent dye known to interact with fibrillar protein aggregates such as Tau in neurofibrillary tangles. To study the influence of RCCs and pseudophosphorylation on the formation of such aggregates, fibrils were detected by thioflavin T after incubation with 1 mM RCCs for 96 h. The formation of thioflavin T-positive aggregates in the mutants, except the tri-

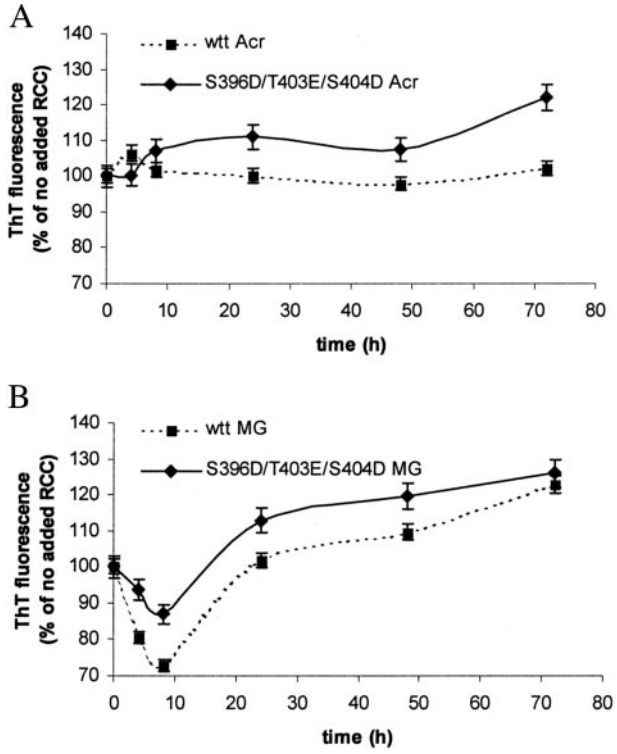

FIGURE 5. **Determination of fibrillar Tau aggregates by ThT fluorescence using wild-type Tau and Tau S396D/T403E/S404D.** Tau proteins were incubated with 1 mM acrolein (*A*) or 1 mM methylglyoxal (*B*), respectively, in the presence of ThT at 37 °C for up to 72 h. Fluorescence of Tau samples without the addition of RCCs was set as 100%. Data are expressed as mean ± S.D. ($n = 2$). * indicates significance ($p < 0.05$, Student's *t* test).

ple mutant S396D/T403E/S404D was not significantly increased compared with wild-type Tau controls (data not shown). In a further experiment investigating the time course of thioflavin T-positive fibril formation, wild-type Tau and the triple mutant were incubated with 1 mM acrolein or methylglyoxal in the presence of 5 μM thioflavin T for up to 72 h. The Tau mutant S396D/T403E/S404D (but not wild-type Tau) showed a slightly accelerated formation of thioflavin T-positive aggregates when reacted with acrolein, which became significant after 24 h (Fig. 5*A*). By contrast, methylglyoxal-induced formation of thioflavin T-positive aggregates initially decreased within the first 8 h of the reaction but continuously increased thereafter in both wild-type Tau and in the triple mutant, with the initial difference in favor of the mutant leveling out after 72 h (Fig. 5*B*). These data suggest that 1 mM methylglyoxal and acrolein generally enhances formation of thioflavin T-fluorescent aggregates (with the exception of wild-type Tau + acrolein) and that ThT-sensitive aggregate formation is slightly favored at certain time points in the triple mutant (30).

Formation of Tau Aggregates by Reactive Carbonyl Compounds; Detection by Electron Microscopy—Further investigations by electron microscopy were performed at this stage to determine whether the reactive carbonyl compounds contribute to the formation of helical structures resembling the characteristic PHFs in Alzheimer disease brains. A further reason for such an experiment was a previous report that such filamentous structures are formed from glucose-modified (glycated) recombinant Tau (35, 38). Analogous to the experiments described above, wild-type Tau and the phosphorylation-mimicking Tau isoforms were incubated with different concentrations of each carbonyl compound for 96 h. In a first set of experiments, wild-type Tau and three pseudophosphorylation-mimicking mutants T217D, T403E/S404D, and S422E were maximally modified by 10 mM acrolein, glyoxal, MDA, and methylglyoxal and studied by electron microscopy using negative staining.

Reaction with Acrolein—Tau incubated with 10 mM acrolein formed PHF-like filaments with lengths of >100 nm with both wild-type Tau and all seven phosphorylation-mimicking Tau isoforms. Fig. 6*A* exemplarily shows the filaments of wild-type Tau, Tau T217D, T403E/S404D, and S422E. The formation of these large fibrillar aggregates further indicates the high reactivity of acrolein. This may also explain why almost no defined small oligomers could be detected in the SDS gel after Coomassie Blue staining.

Reaction with Glyoxal—Tau incubated with 10 mM glyoxal did not form large fibrillar aggregates within 96 h. However, small protofibrils could be detected with wild-type Tau, and some amorphous structures were detected with the Tau mutant T217D (data not shown).

Reaction with Malondialdehyde—Similarly, the incubation of 10 mM MDA with wild-type Tau did not yield fibrillar aggregates, but rather amorphous structures were formed (data not shown). The phosphorylation-mimicking mutants T217D and T403E/S404D yielded only partially ordered rudimental filamentous structures after MDA treatment, but did not show distinctive PHF-like aggregates (data not shown).

Reaction with Methylglyoxal—Methylglyoxal also induced the formation of PHF-like filaments, both in wild-type Tau and in all phosphorylation-mimicking mutants (selected examples are shown in Fig. 6*B*). As summarized in Table 2, 10 mM acrolein and methylglyoxal (but not glyoxal or MDA) induced the formation of Tau oligomers larger than dimers and PHF-like aggregates within the 96-h reaction period.

Control Incubations without Reactive Carbonyl Compounds—All untreated Tau proteins yielded either diffuse or amorphous structures in electron microscopy under the same experimental conditions. Exemplarily, controls for Tau S262D and S396D/T403E/S404D are shown in Fig. 6*C*.

Formation of fibrils by electron microscopy was investigated with the most reactive compounds, acrolein and methylglyoxal, also at lower concentrations. Using 1 mM acrolein or methylglyoxal, only Tau S262D formed distinct filamentous structures (Fig. 6*C*), maybe because it already contained a small amount of oligomers in the control material (see Fig. 3*B*). All other Tau mutants, with the exception of T404E/S404D, yielded small protofibrils that could represent nascent filaments. As an example, Tau S396D/T403E/S404D modified by 1 mM acrolein or MG is shown in Fig. 6*C*. In contrast, Tau mutants not incubated with RCCs yielded only diffuse structures (see Fig. 6*C* for Tau S262D and S396D/T403E/S404D).

In summary, when Fig. 6, *panel C* is compared with *panels A* and *B*, it is obvious that increasing carbonyl concentrations increase the degree of filament formation. Furthermore, when Tau proteins were incubated with any carbonyl at 250 μM for

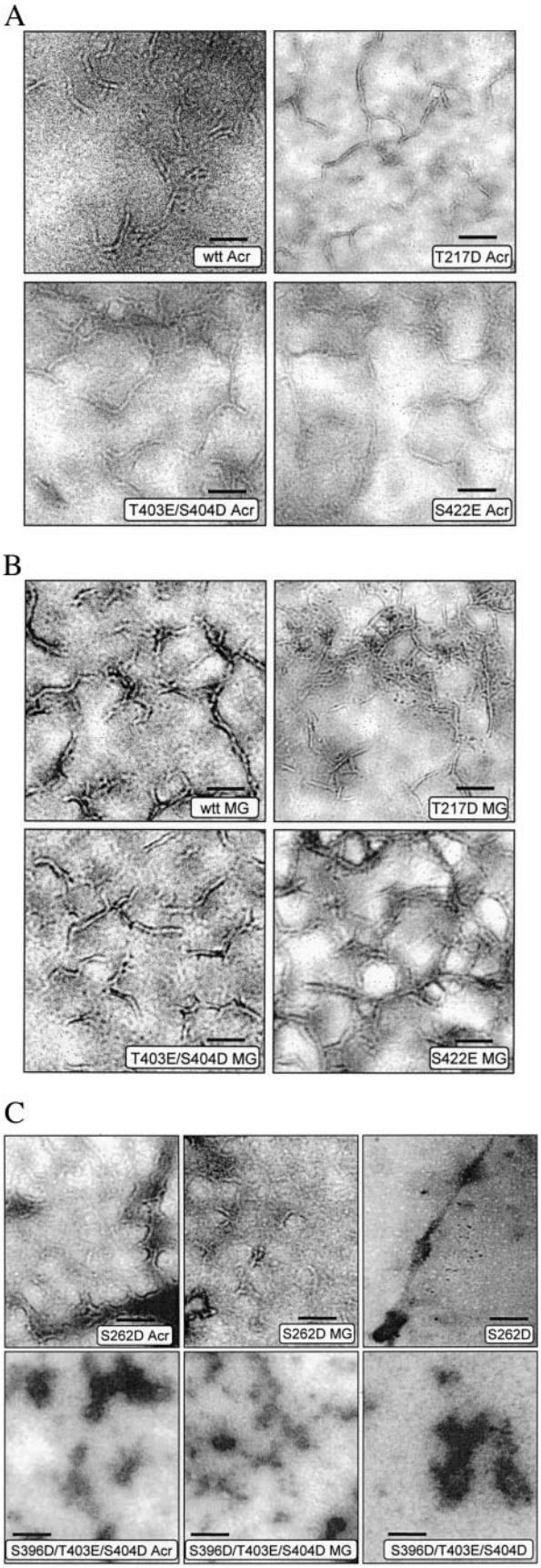

FIGURE 6. **Electron microscopy pictures of acrolein- and methylglyoxal-modified Tau.** Wild-type Tau, Tau T217D, T403E, or S422E were incubated with 10 mM acrolein (*A*) or 10 mM methylglyoxal (*B*). Tau S262D and S396D/T403E/S404D were reacted with 1 mM acrolein or 1 mM methylglyoxal, respectively, or without RCCs (*C*). All reactions were performed for 96 h at 37 °C. *Scale bars* represent a distance of 100 nm.

TABLE 2
Summary of Tau aggregate types upon incubation with 10 mM RCCs, analyzed by SDS-PAGE and EM

	Monomer	Dimers	Oligomers (larger aggregates)	Microscopic fibrils
No RCC	++	−	−	−
Acrolein	−	−	++	++
Methylglyoxal	−	+	+	++
Glyoxal	++	+	−	−
MDA	++	+	−	−

similar time periods, no filamentous structures could be observed (data not shown).

DISCUSSION

Neurofibrillary tangles are major hallmarks of a variety of neurodegenerative diseases, and elucidation of the mechanisms of their formation may provide targets for future therapies. Two main post-translational modifications, which have been found in neurofibrillary tangles, hyperphosphorylation and AGEs and ALEs, may provide clues to unravel this mechanism. Nearly a decade ago, it was suggested that carbonyl-derived post-translational modifications of neurofilaments may account for the biochemical properties of neurofibrillary tangles, possibly as a result of extensive cross-links (22, 39). Because of their relatively long half-life, cytoskeletal proteins are preferred reactants with reactive carbonyl compounds, such as lipid peroxidation products or glucose (40, 41). A first attempt to induce Tau glycation *in vitro* was made by short term incubation with glucose, but this did not result in the formation of PHFs (42). However, glucose is one of the least reactive physiologically relevant carbonyl compounds, and it was not determined in that study (42) to what extent Tau was actually modified by glucose or its degradation products. It also remains an open question whether Amadori products or cross-linking-active AGEs were formed during this short term incubation. By using more reactive carbonyl compounds than glucose in our experiments, we could induce formation of AGE/ALE-modified Tau oligomers and, with acrolein and methylglyoxal, PHF-like structures, which indicated that intensive cross-linking by AGEs or ALEs favor formation of PHFs.

The reactivity of the four RCCs tested decreased in the following order: acrolein > methylglyoxal > glyoxal ≈ malondialdehyde. Interestingly, only acrolein and methylglyoxal, the two most reactive RCCs, formed PHFs in a concentration-dependent manner. However, it is possible that MDA and glyoxal would have also formed oligomers and PHF-like filaments if the reaction had proceeded for a much longer time period. It can be assumed that the reaction of 10 mM acrolein or methylglyoxal with Tau was nearly complete, which could explain the high content of large PHF-like structures that were detected by electron microscopy. When 1 mM acrolein or methylglyoxal were used, more protofibrils and less PHF-like structures were observed. In contrast, the conversion of Tau proteins with 10 mM MDA and glyoxal within 96 h could have been incomplete, and this may be the cause of why only a few small filamentous

structures (protofibrils) could be observed. Such an incomplete reaction may explain why no PHF-like filaments were observed when Tau was incubated with glucose (42). Acrolein immunoreactivity has been observed in more than half of all paired helical filaments (PHF)-1-labeled neurofibrillary tangles in AD cases (23). Acrolein is known as the most reactive among the α,β-unsaturated aldehyde products of lipid peroxidation (~100 times more reactive than hydroxynonenal) and has been shown to be increased in the amygdala and hippocampal/parahippocampal gyrus of AD patients compared with normal controls (18). The reaction of acrolein with nucleophilic amino acids of Tau results in the formation of Michael adducts (43). A further intramolecular reaction of the lysine adducts of acrolein was described yielding N^{ϵ}-(3-formyl-3,4-dehydropiperidino)lysine (44). However, the exact structure of an acrolein-based protein cross-link has not been published so far.

Glyoxal has been shown to form CML via an intramolecular Cannizzaro reaction (45). In AD, CML is co-localized with Tau in neurofibrillary tangles (46, 47). This explains the immunoreactivity of the CML antibody with Tau oligomers in the Western blots. A cross-linking-active AGE formed by glyoxal (*e.g.* present in the dimer at ~100 kDa) could be the imidazolium cross-linked glyoxal-lysine dimer GOLD (48).

In AD brains, MDA has only been described in deposits associated with lipofuscin (21). However, increased levels of MDA have been found in AD patients, particularly in their temporal cortex (49). One of the reaction products of MDA with lysine residues is N^{ϵ}-(2-propenal)lysine (50). It is conceivable that this residue subsequently reacts further with MDA and forms covalent protein-protein cross-links, but to our knowledge such defined cross-links have not yet been described.

The reaction of MG may result in the formation of N^{ϵ}-(1-carboxyethyl)lysine, hydroimidazolone, or argpyrimidine (51). Thus far, specific antibodies for carboxymethyllysine and argpyrimidine, major products in the reaction of methylglyoxal with lysine or arginine, have not been used for immunohistochemical analyses of AD brains. Dimers and oligomers can be formed by an MG-derived C–C cross-link between lysine, arginine, and cysteine residues (52) of Tau or by an imidazolium-AGE cross-link, such as that found in the methylglyoxal-lysine dimers MOLD and MODIC (53). Most of the Tau modifications or oligomers occurred as a double band. This may be due to the fact that Tau polymers themselves can be modified on their protein side chain. The Tau filaments, which were produced by the reaction with acrolein or methylglyoxal (Fig. 6) show some resemblance to PHFs isolated from AD brains (1). These AD filaments are ~530 nm long with characteristic subunits of ~16 × 82 nm (54). However, our synthetic filaments are similar but are straight and lack the twisted appearance of the PHF isolated *in vivo*. In summary, our results obtained with acrolein and methylglyoxal suggest that the formation of covalently linked dimers, trimers, or tetramers may be an important step in filament assembly (17, 55, 56). Consistent with such a suggestion, a Tau tetramer was previously suggested as the smallest building block of AD-derived PHFs when PHF images were analyzed quantitatively from atomic force microscopy and electron microscopy (54).

A further question addressed in this study was the influence of phosphorylation (mimicked by pseudophosphorylated mutants) on carbonyl-induced Tau oligomerization, aggregation, and filament assembly. In terms of non-covalent interactions, *in vitro* studies indicate that phosphorylation of Tau favors self-assembly and the formation of paired helical filaments (57). Using pseudophosphorylated mutants, phosphorylation was simulated by the replacement of serine/threonine residues with glutamic acid or aspartic acid in the C-terminal portion (30). Such mutations mimic the negative charge of $-OPO_3^-$. Self-aggregation induced by heparin and certain metal ions, such as iron and aluminum, is also accelerated in these mutants (30). We asked the question whether pseudophosphorylation influences the reaction with reactive carbonyl compounds and the formation of Tau oligomers. Although the single and double hyperpseudophosphorylated mutants did not significantly form more thioflavin T-positive aggregates (data not shown), the triple Tau mutant S396D/T403E/S404D reacts slightly faster and forms more ThT aggregates in the reaction with acrolein compared with wild-type Tau (Fig. 5*A*). Similarly, modification with methylglyoxal also leads to a higher aggregation rate in the triple mutant than in wild-type Tau, at least at some time points (Fig. 5*B*). Taken together, our data confirm the hypothesis that reactive carbonyl compounds such as methylglyoxal and acrolein induce dimerization, oligomerization, and the formation of PHF-like aggregates and that this reaction may be modestly accelerated in triple pseudophosphorylated Tau compared with wild-type Tau. In summary, our study did not point to particularly crucial sites where phosphorylation would dramatically accelerate Tau cross-linking and aggregation. It might be possible, however, that the bulk effect of a larger number of (pseudo)phosphorylated residues might lead to a dramatically increased susceptibility to aldehyde-induced aggregation.

Furthermore, we have shown that the rate of PHF-like aggregate formation increases with increasing carbonyl concentrations. There are various reasons for the increased carbonyl concentration in the aging brain and in AD. Increased oxidative stress and subsequent peroxidation would result in increased levels of MDA and acrolein (58). In addition, impaired glucose utilization or a decreased activity of triosephosphate isomerase would increase triose phosphates and thus its degradation product methylglyoxal (59). Moreover, a reduced activity of detoxification systems such as aldose reductase or the glyoxalase system and a low level of GSH, the co-factor of both enzymes, may elevate the intra- and extracellular concentration of RCCs (60). Because reduced glutathione is limited under conditions of oxidative stress and inflammation, as is present in AD, supplementation with antioxidants such as vitamin C and E or flavonoids could indirectly strengthen the anti-glycation defense system in AD. In addition, interference with the formation of cross-link-reactive AGEs or ALEs by scavenging RCCs with drugs such as aminoguanidine or tenilsetam could become a novel therapeutic approach to inhibit tangle formation and slow down the progression of AD (61, 62).

Acknowledgment—We thank Dr. Max Holzer for the donation of Tau proteins and for helpful comments.

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