Quinones Facilitate the Self-Assembly of the Phosphorylated Tubulin Binding Region of Tau into Fibrillar Polymers

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ABSTRACT: The fragment of tau containing the first and third tubulin-binding motifs, involved in self-assembly of tau, was phosphorylated by protein kinase A (PKA). In the presence of hydroxynonenal (HNE) or in the presence of quinones such as juglone, 2,3-dimethoxy-5-methyl-1,4-benzoquinone (coenzyme Q_0 or DMM), or menadione, the polymerization of this phosphorylated tau fragment is catalyzed, whereas polymerization of the unmodified fragment takes place in a lesser extent. The quinones coenzyme Q_0 and menadione are found in every cell, including neural cells, and may interact with tau protein to facilitate its assembly into filamentous structures. These tau filaments, assembled in the presence of quinones, have a fibrillar morphology very similar to that of paired helical filaments present in the brains of patients with Alzheimer's disease.

In pathological conditions such as those found in Alzheimer's disease (AD)[1] and other tauopathies, tau protein is observed in a hyperphosphorylated, aggregated form (*1*). Tau aggregates in AD (neurofibrillary tangles, NFT) are composed of paired helical filaments (PHF) (*2*) of which phosphotau is the main component (*3*).

Whether phosphorylation of tau facilitates tau assembly is a fundamental question in study of AD and other tauopathies (*4*). It has been suggested that tau phosphoryl- ation at some specific sites may be a prerequisite for tau assembly (*5, 6*). However, in vitro experiments for tau assembly have shown different results, presumably reflecting differences in experimental conditions. In some cases, phosphotau has a lower assembly capacity (*7*), whereas in other conditions, its capacity for assembly increases (*6*). Phosphorylation of tau is catalyzed by two types of kinases, proline (PDPK) and nonproline (NPDPK) directed protein kinases (*8*). Examples of PDPK are cdk5 and GSK3 (*9- 11*), whereas those of NPDPK are PKA (*12*) and MARK
(*13*). Each of these protein kinases phosphorylates tau in different positions/residues in the molecule. PDPK phosphorylated residues of tau not located in the tubulin-binding region, whereas NPDPK phosphorylated residues present in the tubulin-binding region (*8*).

In the presence of compounds such as hydroxynonenal (HNE), phosphotau, but in less extent unmodified tau, can polymerize into filamentous polymers (*7*). Tau phosphorylation by both GSK3 and PKA appears to be required to facilitate tau assembly (*7*).

In this work, we focus on the phosphorylation of tau protein and how this modification affects its capacity to self-assemble. Our objective was to probe conditions where phosphorylation of tau by PKA increases its self-assembly capacity. Tau phosphorylation by PKA has been previously studied by different authors (*12, 14*), and the possible relationship between levels of cyclic AMP and Alzheimer's disease has been the subject of much speculation (*14, 15*). Although tau can be phosphorylated by several protein kinases, we focused our investigation on the study of the effects of phosphorylation of tau by PKA alone. Since a tau fragment (tau 2R) containing the tubulin-binding region can be modified by PKA and this is the region involved in self-assembly (*16*), we have tested for compounds that can regulate its polymerization. Among them, we have found that HNE, a lipid peroxidation compound (*8*), and also several quinones, such as juglone, coenzyme Q_0, or menadione, increase tau polymerization.

MATERIALS AND METHODS

Materials. 5-Hydroxy-1,4-naphthoquinone (juglone, ref no. H4, 700-3) was obtained from Aldrich. 2-Hydroxy-1,4-naphthoquinone (lawsone, ref no. H0508), 2-methyl-3-phytyl-1 4-naphthoquinone (vitamin K_1, ref no. V-3501), 2-methyl-1,4-naphthoquinone (menadione, ref no. M-5625), 2-methyl-1,4-naphthoquinone (menadione sodium bisulfite, ref no. 5750), 2,3-dimethoxy-5-methyl-1,4-benzoquinone (coenzyme Q_0 or DMM, ref no. D-9150), and 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (decylubiquinone, ref no. D7911) were purchased from Sigma. Acrylamide/bisacrylamide solution was supplied by Bio-Rad. Poly(vinylidene difluoride) membranes (Immobilon Pseq) were from Millipore Corp. The chemiluminescent detection kit (Western Light) was from Tropix. 2′,7′-Dichlorofluorescein diacetate

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[1] Abbreviations: PKA, cAMP-dependent protein kinase; HNE, hydroxynonenal; PHFs, paired helical filaments; AD, Alzheimer's disease; Mab, monoclonal antibody; OD, optical density; EC_{50}, the effective concentration of a compound that is required to promote 50% of maximum aggregation; NFT, neurofibrillary tangles.

(H2DCFDA) was from Molecular Probes (ref no. D-399). (γ-^{32}P)-ATP was purchased from Amersham. ATP was purchased from Boehringer-Mannheim. The catalytic subunit of cAMP-dependent protein kinase (PKA, P 2645) was purchased from Sigma (St. Louis, MO). According to the supplier's specifications, the kinase was purified to greater than 90% homogeneity from bovine heart to a specific activity of 50 U/μg protein. The activity of PKA using p24 as substrate was 20 pmol/min/μg of protein (*17*). Phenylmethylsulfonyl fluoride (PMSF), EDTA, EGTA, 2-mercaptoethanol, heparin, MES, and Tris were obtained from Sigma. DEAE Sephacel-R was purchased from Pharmacia-Biotech (code no. N7-0500-01). The protein phosphatase inhibitor okadaic acid (OkA) was purchased from LC Laboratories (Woburn, MA). Monoclonal antibody 12E8 (*18*), was a kind gift of Dr. Seubert, Athena, San Francisco, CA. Hydroxynonenal (HNE) was partly received as a kind gift of Dr. Perry (Case Western Reserve University, Cleveland, OH) or purchased from Cayman Chemical Co. (cat. no. 32100).

Tau Isolation. Expression and isolation of recombinant protein htau 40 and its fragment tau 2R were performed as described (*19, 20*). Briefly, tau 2R (residues 250-273 linked to residues 305-335), containing the first and third motifs of the largest tau isoform present in central nervous system (*21*), was cloned into vector pRK172 (*20*) for inducible expression in *Escherichia coli*. Protein purification was performed by a protocol involving a heat resistance step in a buffer containing 100 mM Tris, pH 7.5, 0.5 mM $MgCl_2$, 1 mM EGTA,1 mM 2-mercaptoethanol, 100 mM NaCl, and protease inhibitors, 2 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 10 μg/mL pepstatin. This step involves a treatment at 100 °C for 5 min. The soluble protein was isolated after centrifugation, and ammonium sulfate was added. The precipitated fraction between 25% and 50% was taken. The precipitated protein was resuspended in PBS (phosphate-buffered saline) and chromatographed on DEAE Sephacel-R equilibrated in PBS, and the protein was eluted stepwise using 1 M NaCl in PBS.

Tau Phosphorylation by PKA. The phosphorylation of tau and tau 2R by PKA was performed as previously described (*17, 22*). Briefly, purified PKA and 1-4 μg of substrate were mixed in 12 μL total volume buffer solution containing variable ATP concentrations and 0.2 μCi of (γ-^{32}P) ATP. The cAMP kinase protein (100 ng/reaction) was performed in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, and 1 μM okadaic acid buffer at 37 °C. After 20 min of incubation, 4μL aliquots of the supernatant were spotted onto 2 cm $\times$ 2 cm pieces of Whatman p81 phosphocellulose paper. After 20 s, the filter was washed four times (for at least 10 min each time) in 1% phosphoric acid. The dried filters were transferred into scintillation vials, and the radioactivity was measured in a liquid scintillation counter. Also, in other experiments, the phosphorylation was carried out in the presence of 1 mM ATP for 24 h. At this incubation time, maximum phosphorylation was found. To calculate molar concentrations, an estimated relative molecular weight of 44 000 was taken for tau protein and 5500 for tau 2R.

PHF Isolation. PHF fractions were obtained by using the method of Greenberg and Davies (*23*). The presence of PHF was determined by electron microscopy as previously described (*24*).

Assembly of Tau Peptides into Filaments. Filaments were grown by vapor diffusion in hanging drops in the standard way used for protein crystallization as previously indicated (*25*). In a typical experiment, 1-4 μg of peptide was resuspended in 10-15 μL of bufferA (0.1M MES (pH 6.4), 0.5 mM $MgCl_2$, and 2 mM EGTA) containing 50 mM NaCl and 0-3 mM HNE or containing different quinones, such as juglone, at concentrations ranging from 0.25 to 8 mM. In other assays, a similar amount of tau (1-4 μg/mL) but in the presence of 0.5 mg/mL heparin was resuspended in buffer A (*26*). The reservoir in this case contained 0.2 M NaCl in buffer A. Filaments were obtained after incubation for 4 days at 4 °C. The samples were visualized by electron microscopy as described (*26*, *27*), and the amount of polymers was quantified by sedimentation (*7*). Electron micrographs were obtained at a magnification of 80 000 on Kodak SO-163 film. Micrographs were digitized using an Eikonix IEEE-488 camera with a pixel size equivalent to 7 Å in the specimen plane. Processing and measurements were performed using the Digital micrograph 2.1 software from Gatan. Several standards were used for the control of the measurements.

Measurements of Aggregated Tau Protein. The samples were incubated at 37 °C for 1 day to a maximum of 4 days. In some experiments, samples were centrifuged for 30 min at maximal speed in an Airfuge (Beckman), and the protein present in the supernatant and pellet was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed according to the procedure of Laemmli on 10% or 18% polyacrylamide gels (*28*). Gels were stained with Coomassie brilliant blue or analyzed by autoradiography (when the protein was labeled with ^{32}P by phosphorylation with PKA) as the same conditions previously indicated (*22*) or subjected to Western blot analysis (*7*) making use of monoclonal antibody 12E8 to identify phosphotau. The amount of protein was quantified by densitometry of the fractionated protein after Coomassie brilliant blue staining of the gel and after comparison of the results obtained with those found for known amounts of bovine serum albumin, used as a control. Densitometric analysis was also performed on protein samples identified by autoradiography, and the data were processed with an imaging densitometer (GS-6470 model, Bio-Rad). Data were analyzed with Molecular Analyst software. Additionally, when the protein was labeled with ^{32}P, the amount of associated radioactivity was measured and determined by Cerenkov radiation counting.

In the presence of different compounds, for example, juglone, cross-linked tau oligomers can be formed, and tau aggregation can be also measured by determining the amount of those cross-linked tau oligomers.

The above experiments were complemented by doing microscopy analysis in which five fields of view were blindly selected from the grid for processing, by following the indications of Gamblin et al. (*29*).

RESULTS

Isolation of Recombinant Tau and Tau 2R Peptide. Tau and tau 2R were isolated as indicated in the Materials and Methods section by a heat resistance step followed by

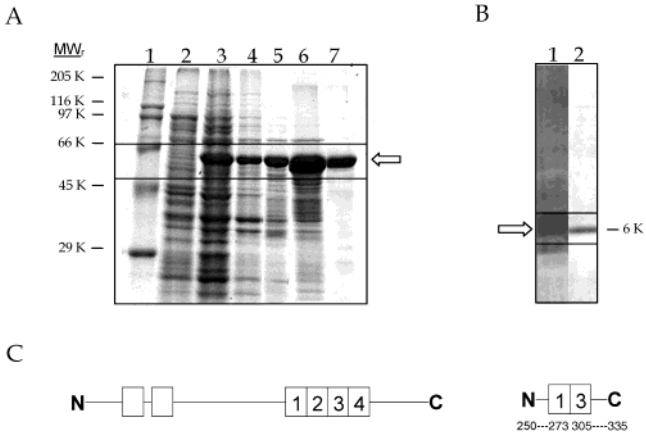

FIGURE 1: Purification of tau and tau 2R fragment. In panel A, lane 1 indicates molecular weight markers, lane 2 indicates *E. coli* cell extracts where the expression of tau was not induced. Lane 3 indicates recombinant tau isolated from *E. coli* cells expressing it (*20*). This recombinant tau was purified by several steps including a heat resistance step (lane 4), ammonium sulfate precipitation (lane 5), DEAE Sephacel-R chromatography (lane 6), and an additional ammonium sulfate precipitation step (lane 7). In panel B, a similar procedure was used for tau 2R, lane 1 being recombinant tau 2R isolated from *E. coli* and lane 2 the final purification step. Panel C provides a diagram of recombinant tau molecule and tau 2R fragment. The numbered boxes indicate the tubulin binding motifs in the tau molecule. The tau 2R fragment contains motifs 1 and 3. Motif 3 contains the tau site for self-assembly. The residues present in tau 2R fragment are indicated.

precipitation with ammonium sulfate and DEAE Sephacel-R chromatography. The purification level was more than 90% in each case (Figure 1, panel A, tau protein, and panel B, tau 2R). Purified polypeptides were used as substrates for in vitro phosphorylation by PKA.

Phosphorylation of Tau and Tau 2R Peptide by PKA. Tau (Figure 2A) and tau 2R (Figure 2B) were phosphorylated by PKA under conditions of increasing ATP concentrations. Figure 2A,B indicates that an incorporation (stoichiometry) of about 2.7 mol of phosphate/mol of tau and 0.8 mol of phosphate/mol of tau 2R was obtained when a concentration of 200 μM ATP was used. Tau 2R peptide (residues 250-273 linked to residues 305-335) has the following sequence: MPDLKNVKSKIG***S***TENLKHQPGGGSVQIVYK-PVDLSKVT*S*KCG*S*LGNIHHKPGGG. Phosphorylation of this peptide by PKA results only in the modification of serine residues (not shown). Those serines having a consensus sequence for PKA have been set in italics (serine 262 of tau protein is bold and italic).

To characterize a site modified by PKA in both phosphotau and phosphotau 2R, these phosphopeptides were incubated with Mab 12E8 that recognizes phosphoserine 262 (in tau protein). As shown in Figure 2C,D, both phosphopeptides bind to Mab 12E8, indicating that ser 262 is modified in both cases. In parallel, it has been shown that the increase in the reaction of modified tau with Mab 12E8 correlates

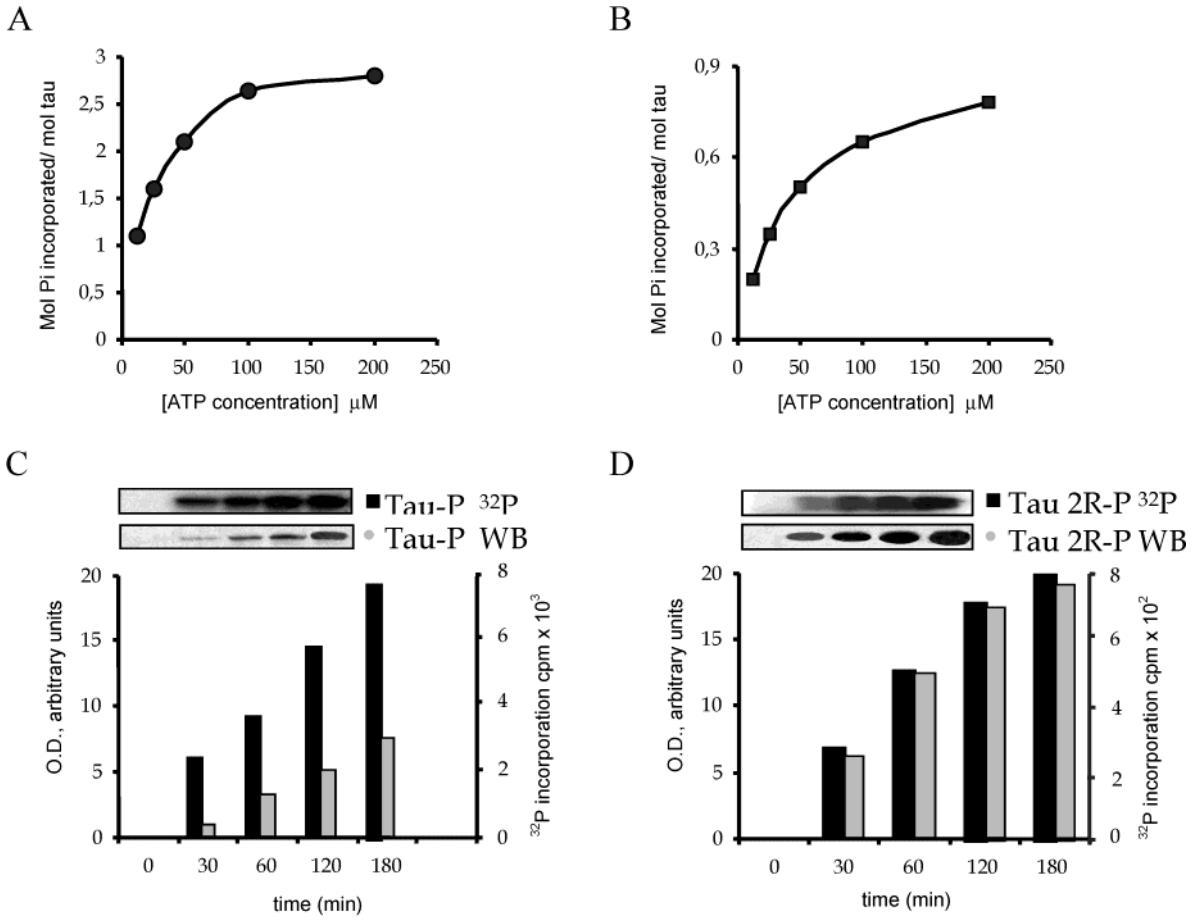

FIGURE 2: Phosphorylation of tau and tau 2R fragment by PKA. To test the extent of tau (A) and tau 2R fragment (B) phosphorylation by PKA, the effect of ATP concentration on phosphate incorporation was measured. Simultaneously, the reaction of tau (C) and tau 2R (D) with Mab 12E8 was measured by Western blotting, followed by densitometry of the obtained band. The values of those densitometries (OD, in arbitrary units) were compared to phosphate incorporation in a time-dependent reaction.

with tau phosphorylation, as measured directly by ^{32}P incorporation. This result suggests that in tau 2R, PKA phosphorylates tau 2R mainly at one site, ser 262. We must indicate that this serine is also modified in tau protein obtained from the paired helical filaments present in the brain of Alzheimer's disease patients (*8*). Nevertheless, we cannot exclude the phosphorylation of other serines, such as ser320 or ser324, although when the peptide KVT*S*KCG*S*LGNIH-HKPGGG (containing both serines) was tested for PKA phosphorylation in the same conditions as those used for than tau 2R, only 0.1 mol of phosphate/mol of peptide (or less) was incorporated.

Aggregation of Phosphotau and Phosphotau 2R. The purpose of the present work was to test for compounds that could favor the aggregation of phosphotau (modified by PKA) compared to that of unmodified tau. In previous studies, it was found that heparin and other polyanions could promote the assembly of unmodified and phosphotau (*16, 30*) with the self-assembly capacity similar or higher for unmodified tau (*6*). On the other hand, in the presence of hydroxynonenal (HNE), a peroxidation product of arachidonic acid, mainly phosphotau (modified by several kinases, including GSK3 and PKA) is assembled, while polymerization was observed to a less extent for unmodified tau (*7*). Both kinases (GSK3 and PKA) were implicated to be necessary for the assembly of modified tau in the presence of HNE (*7*). When the whole tau protein was only modified by PKA and its assembly capacity in the presence of increasing amounts of HNE was measured, a small amount of aggregated tau protein was detected (Figure 3A), probably because tau modification by other kinases (such as GSK3) could also facilitate the assembly (*7*). However, when tau 2R was phosphorylated by PKA (around 1 mol of phosphate/ mol of peptide) and its polymerization in the presence of HNE was tested, a significant increase in phosphotau 2R self-assembly was found (no such increase was found for unmodified tau 2R, Figure 3C). Thus, tau 2R can be used as a useful model to test the influence of PKA phosphorylation on tau assembly. In the absence of HNE less aggregation was found for both unmodified and phosphotau or -tau 2R.

Since tau 2R was able to assemble into polymers, after the single modification of PKA, we tested for other com- pounds that could also favor the assembly of phosphotau 2R. In this empirical study, we tested the role of quinones. Quinones are present in the cytoplasm (where tau protein is also present) and in mitochondria, where they play a role in the respiratory chain. Toxic compounds, such as HNE, products of oxidative stress, could play a role in AD and are also formed in the cytoplasm and mitochondria. Among the different quinones tested, we have found that juglone, 5-hydroxy-1,4-naphthoquinone, a compound that can bind to different molecules (*31-33*), facilitates the assembly of phosphotau 2R to a similar level to that found after HNE addition (Figure 4), as determined by employing centrifuga- tion to isolate the pelleted (aggregated) protein fraction. Unmodified tau 2R assembles to a less extent (Figure 4). Protein characterization of the pelleted protein indicated the presence of tau 2R monomers and also cross-linked tau 2R

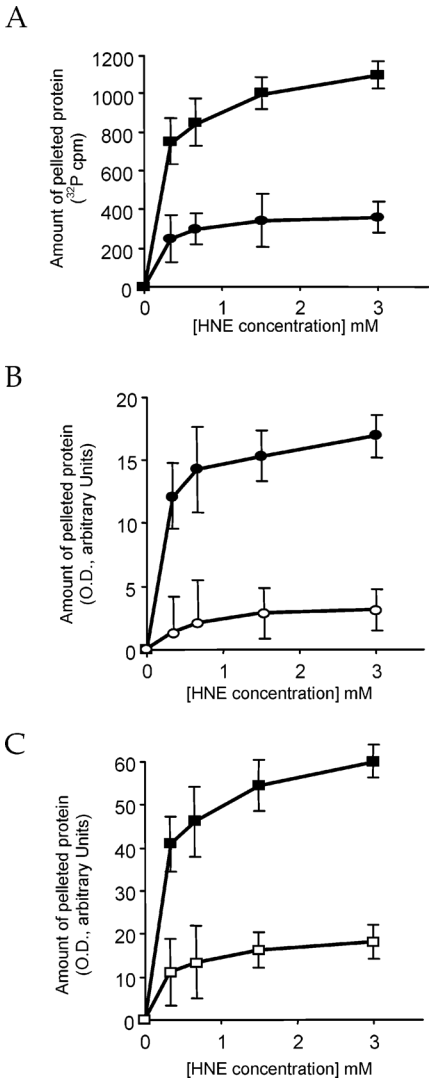

FIGURE 3: Protein aggregation in the presence of increasing HNE concentrations. In panel A, 4 μg of tau (b) and of tau 2R (9) in phosphorylated form labeled with ^{32}P were incubated with different HNE concentrations, and the protein present in aggregated form was determined by centrifugation in a Beckman airfuge, and quantification of the pelleted protein was performed by measuring ^{32}P radioactivity or by autoradiography. The amount of tau (B) or tau 2R (C) in phospho (b, 9) or in unmodified form (O, 0) present in aggregated form was determined in the same way, but the pelleted protein was identified by gel electrophoresis and Coomassie staining followed by densitometry of stained protein. The same amounts of phospho- and unmodified tau (180 μM) where used in the assay. Additionally, the amount of phosphotau (b) or phosphotau 2R (9) was determined by gel electrophoresis, autoradiography, and densitometry of the autoradiographed protein (see Materials and Methods). Error bars, from three different experiments, are shown.

oligomers in a constant ratio (Figure 4B,C). This observation suggests that such protein cross-linking plays a role in tau aggregation but also suggests that monomer tau is part of the assembled polymer. On the other hand, we have calculated that nearly 70% of the total protein could be present in aggregated form upon 8 mM juglone treatment for 1 day. To test for modified residues after juglone treatment, the treated protein was hydrolyzed and an amino acid analysis of the hydrolysate was done. The results (not shown) indicate a decrease (with respect to the untreated

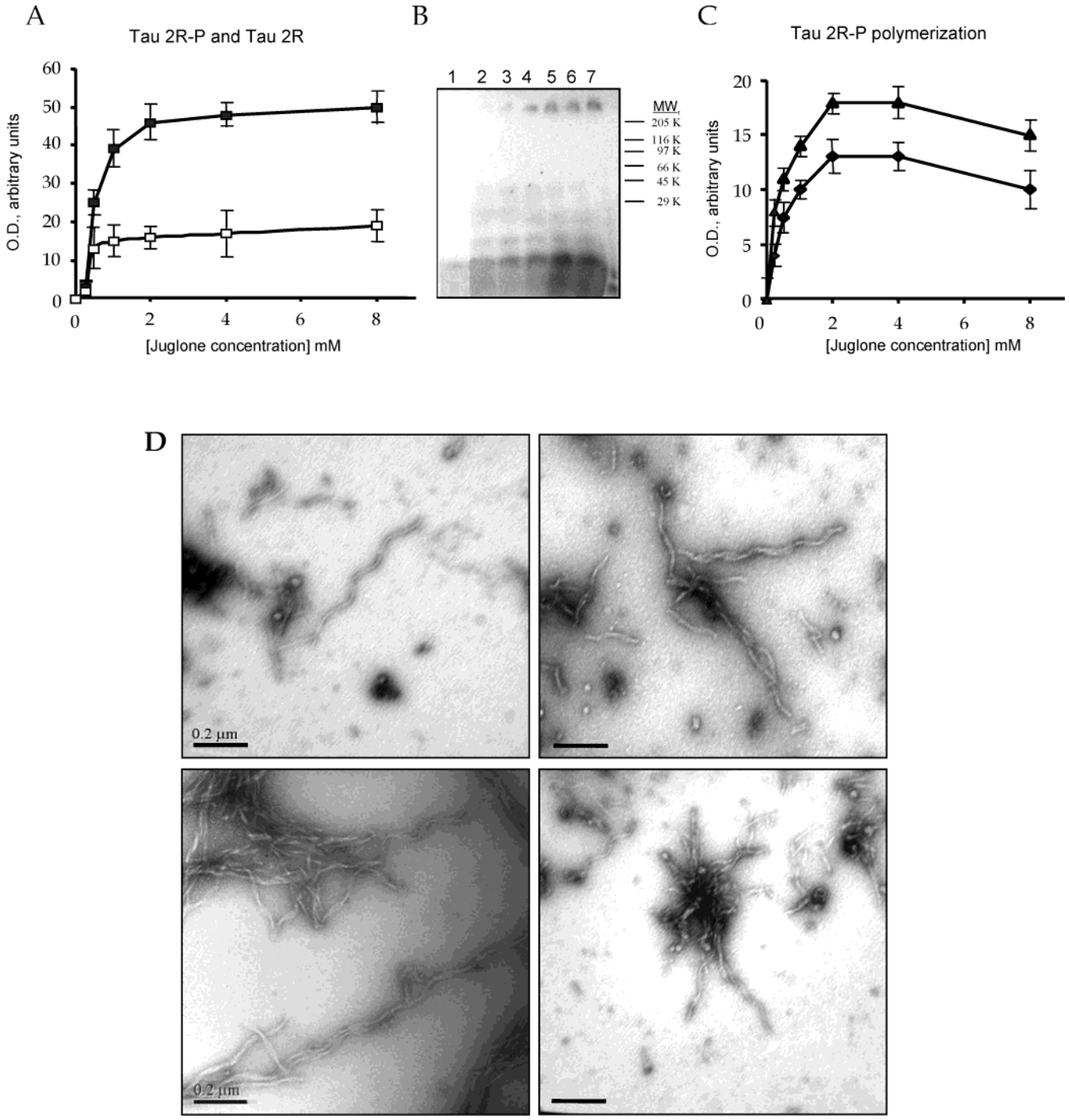

FIGURE 4: Protein aggregation in the presence of increasing amounts of juglone. For panel A, the same amounts (180 μM) of phosphorylated tau 2R (9) (labeled with ^{32}P) or unmodified tau 2R (0) were mixed with increasing concentrations of juglone, and the amount of pelleted protein was determined by centrifugation and by measuring the ^{32}P-associated radioactivity. The determination of the aggregation of tau 2R (0) in unmodified form was carried out as indicated in Materials and Methods. In panel B, the protein after adding increasing juglone concentrations (0, 0.25, 0.5, 1, 2, 4, and 8 mM) present in the pellet fractions was characterized by gel electrophoresis followed by autoradiography. The electrophoretic mobility of molecular weight markers is indicated. In panel C, the proportions of tau 2R-P in monomer (2) or oligomer ([) forms were measured and are indicated with respect to juglone concentration. Panel D shows the morphology of tau 2R aggregates assembled in the presence of juglone. Different samples of phosphotau (modified by PKA) assembled in the presence of juglone are shown. A much lower amount of those filament aggregates were found by electron microscopy when unmodified tau was tested (not shown). Bars indicate 0.2 μm.

sample) mainly of cysteine and histidine. This suggests that these are the main residues modified by juglone.

Phosphotau 2R fibrillar polymers were found when the protein was incubated with juglone (Figure 4D). Theseas-sembled polymers show a morphology similar to that of paired helical filaments present in the brain of AD patients (see Figure 4 and Table 1).

Effects of Other Quinones. Other quinones such as lawsone, vitamin K_1, and decylubiquinone were tested. These quinones, unlike juglone, did not promote phosphotau 2R

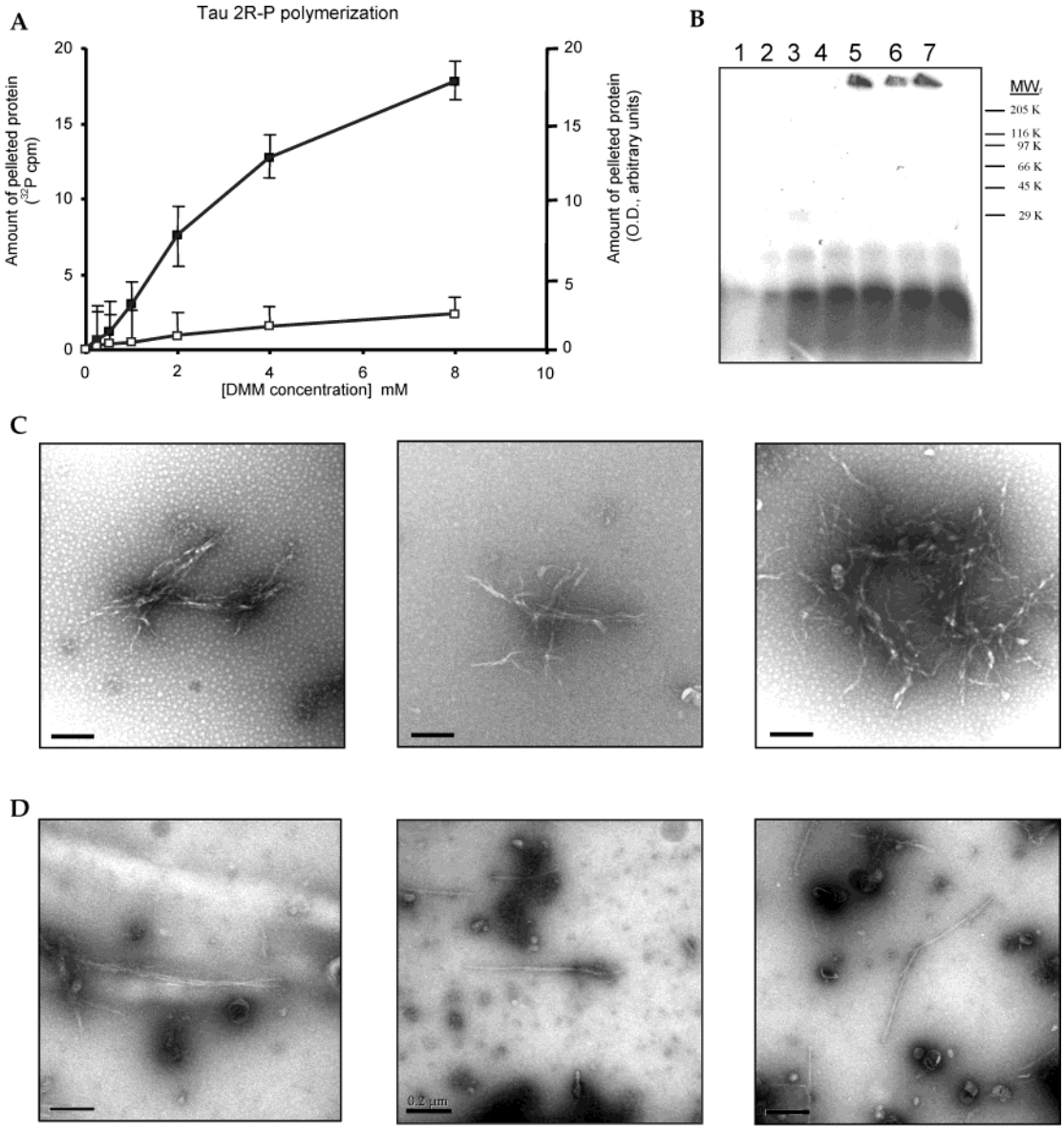

FIGURE 5: Tau 2R aggregation in the presence of DMM. For panel A, phosphotau 2R labeled with ^{32}P was incubated in the presence of increasing concentrations of DMM and the amount of aggregated protein was measured by centrifugation and determination of the ^{32}P-associated radioactivity in the pelleted fraction (9) (see left ordinate). The aggregation of the same amount of unmodified protein (0) (see right ordinate) has been also determined. In panel B, the protein after adding increasing DMM concentrations (0, 0.25, 0.5, 1, 2, 4, and 8 mM) present in the pellet fractions was characterized by gel electrophoresis followed by autoradiography. The electrophoretic mobility of molecular weight markers is also indicated. Panel C shows the filament morphology of different samples of aggregated phosphotau 2R polymerized in the presence of 1 mM DMM. In panel D, different samples of phosphotau assembled in the presence of 1 mM menadione are shown. Bars indicate 0.2 μm.

aggregation. In fact, preliminary experiments suggest that lawsone can partially inhibit the effect of juglone on promoting phosphotau 2R aggregation.

However, the addition of quinone 2,3-dimethoxy-5-methyl-1,4-benzoquinone (DMM or coenzyme Q_0), menadione (Figure 5), or menadione sodium bisulfite (not shown), catalyzed the appearance of phosphotau 2R aggregates in a fashion similar to that of juglone (see below, Figure 5A,B,C). Figure 5 shows that phosphotau 2R aggregation in response to treatment with DMM was dependent on DMM concentration (Figure 5A). The appearance of the assembled tau polymers is shown (Figures 5B,C). Additionally, menadione (a compound that could also regulate tau phosphorylation at PDPK sites (*34*)) can induce the formation of phosphotau filaments (Figure 5D). Menadione sodium bisulfite also

Table 1: Features of Tau 2R-P Polymers Assembled in the Presence of Different Compounds and the Approximate EC_{50} Required for Assembly[a]

sample	compound	width (Å)	EC_{50} (mM)
tau 2R-P	heparin	54 (4	*b*
tau 2R-P	HNE	24 (3	2
tau 2R-P	juglone	77 (5	0.4
tau 2R-P	DMM	31 (4	2
tau 2R-P	menadione	40 (5	5
PHFs		94 (8	

[a] Samples were allowed to polymerize in the presence of different compounds (PHFs were used as control) and observed by electron microscopy. Photographs were taken, and 15 polymers of each sample were selected to measure their width as described in Materials and Methods. The results obtained are shown. Additionally, from the data shown in Figures 3, 4, and 5 and by use of a similar approach for menadione (not shown), the EC_{50} required for assembly was calculated. [b] See ref 20.

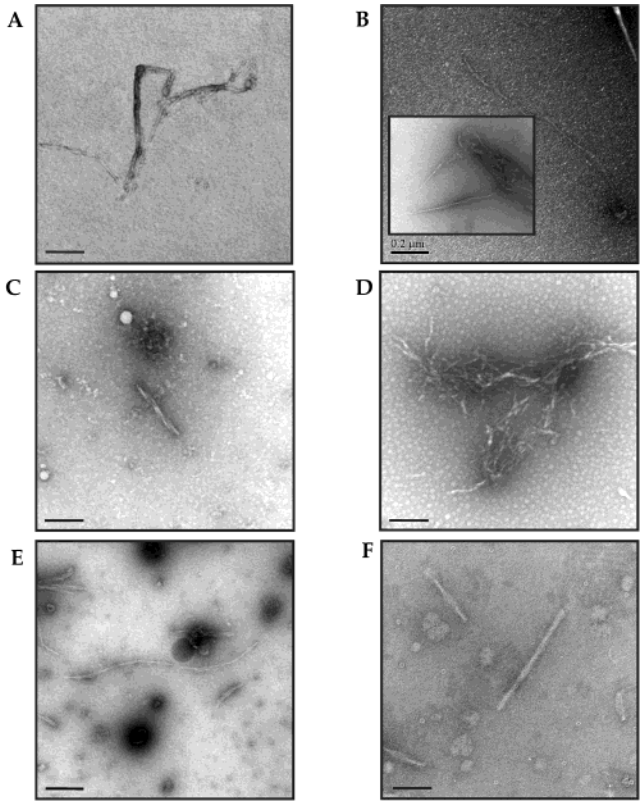

FIGURE 6: Comparison of tau 2R polymers assembled under different conditions. Panel A shows unmodified tau 2R assembled in the presence of heparin. Phosphotau 2R assembly in these conditions is slightly lower (not shown). Panel B shows phosphotau 2R assembled in the presence of HNE (the inset indicates a cluster of filaments). Phosphotau assembled in the presence of juglone (C), DMM (D) and menadione (E), and paired helical filaments (PHFs) isolated from a brain of an Alzheimer's disease patient (F) are also shown. Bars indicate 0.2 μm.

promotes the formation of phosphotau filaments, although to a lesser extent than menadione (not shown).

Comparison of Tau Polymers Assembled in Different Conditions. We have compared the filaments assembled in response to different compounds, HNE, quinones, and heparin, with those filaments isolated from patients with AD (Figure 6). In Table 1 are indicated the different calibers of the different filaments, together with the EC_{50} needed for HNE and the different quinones to promote protein polymerization.

DISCUSSION

In AD and other tauopathies, tau aggregates on filaments that are composed of assembled phosphotau (*35*, *36*). Tau assembly likely requires, or is affected by, the presence of factors that facilitate its polymerization into fibrillar structures. Several factors that promote tau assembly in vitro have been identified. Heparin (and other polyanions) (*16, 30*), as well as a physiological role for heparin, have been suggested because of its presence in NFT (*37*). Additionally, polyglutamate (*16, 38*) and nucleic acids (*39*) facilitate tau assembly. More recently, it has been reported that R-synuclein favors tau aggregation (*40*). Fatty acids, such as arachidonic acid or docosahexanoic acid, promote tau assembly (*29, 41-44*). A role for these fatty acids could be the basis for the reported interaction of tau with membrane components (*45, 46*). A peroxidation product of arachidonic acid, hydroxynonenal (HNE), one that occurs in AD (*47*) and could facilitate tau phosphorylation (*48*), also facilitates the polymerization of phosphotau into fibrillar polymers (*7*). In our in vitro experiments, an effect was found at a HNE concentration of 100 μM; we do not know the concentration of HNE in cells (it cannot be greater to that of arachidonic acid), and therefore, we cannot indicate the possible physiological relevance of HNE effect, until that concentration can be measured in those locations where tau aggregates may assemble.

A common source for fatty acids and their oxidation products could be the mitochondrial membrane. A mitochondrial defect in AD has been suggested (*49*), and this defect could change the redox equilibrium. A peptide related to AD, fJ amyloid peptide, may play a pathological role by impairing mitochondrial function (*50*) related to the electron transport involving redox changes, a process where quinones are involved. In this work, we have shown that in the mitochondria, factors such as quinones that play a role in electron transport could also induce the assembly of tau in a pathological situation.

Mitochondrial quinones, such as coenzyme Q_{12}, could promote aging (*51, 52*), the highest risk factor known for AD. Other important quinones such as vitamin K_3, playing a role in vascular disorders, interfere with mitochondrial oxidative phosphorylation (*53, 54*). We speculate that such quinones could represent a risk for disorders involving oxidative stress, and perhaps AD is a prime example (*55*). Another quinone, juglone, has been widely used to study the function and dysfunction of several proteins (*56-58*). Other quinones, such as idebenone, have used to correct mitochondrial defects (*59*). Juglone is mainly present in plant cells, and little is known about its presence in other cell types. More important could be the action of DMM (or coenzyme Q_0) or that of menadione on tau assembly. DMM and menadione should be present in every cell, including neural cells, as an intermediate compound for the synthesis of other quinones. In this way, intracellular quinones may interact with and catalyze the assembly of tau protein.

Not all the quinones tested facilitate tau polymerization (Figure 7). For example, lawsone does not do it. A single difference between lawsone and menadione is that lawsone has a hydroxyl group in position 2, whereas menadione has a methyl group in that position. This would result in a different reactivity of the carbon in position 3, in each case, with tau protein. This different reactivity is also compatible with the fact that coenzyme Q_0 but not decylubiquinone promotes the assembly of tau protein.

In the present work, we have found that in vitro treatment with juglone, DMM, or menadione promotes phosphotau 2R assembly. This tau fragment contains the minimal region of tau that is able to self-assemble (*16*). This fragment lacks the N-terminal and C-terminal regions, which also could have a regulatory effect on tau assembly (*29, 60, 61*). This tau fragment contains serine 262, a residue that can be phosphorylated by PKA to generate an epitope that can be recognized by Mab 12E8 (18). This antibody reacts with PHF-tau in Western blot analysis (*5*) but not in immunoelectron microscopy analysis (*5*). This indicates that in AD, tau is phosphorylated at that site prior to its assembly (Hernandez et al., *Neurobiology of Aging*, in press), and it

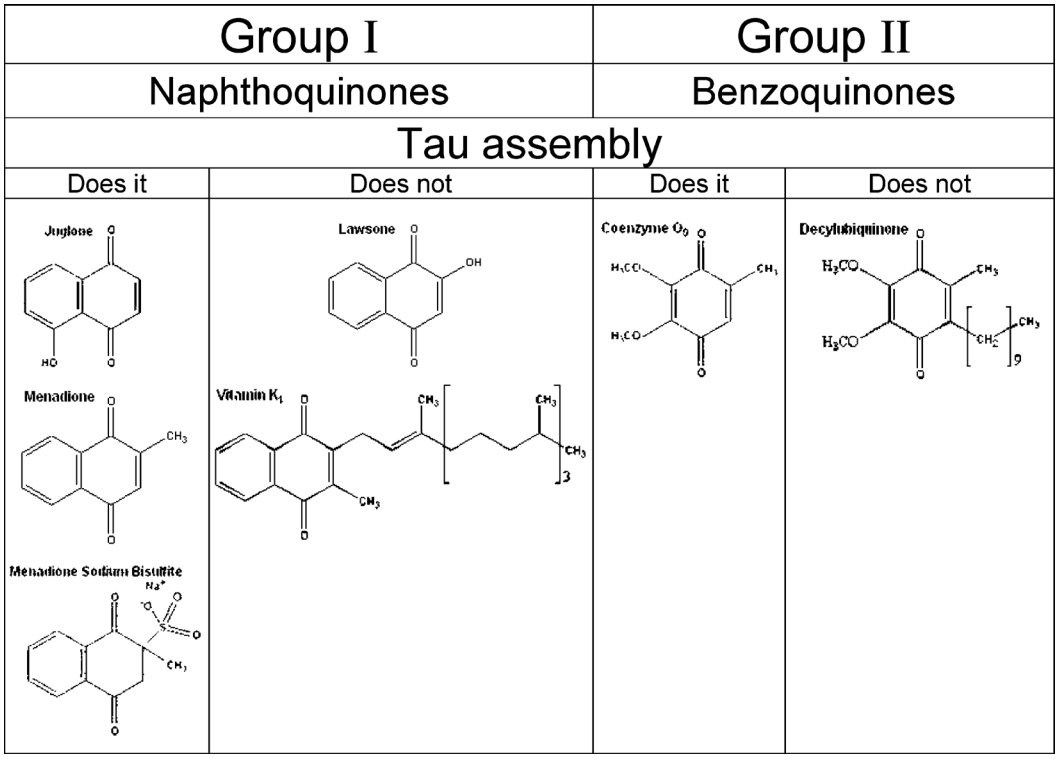

FIGURE 7: Structure of the quinones used in this study indicating their action on tau assembly.

is likely that this region, directly involved in tau-tau interactions, becomes buried in PHF structure (*5*).

By using tau 2R, we have mimicked the step that probably takes place in AD, involving tau phosphorylation by PKA followed by its assembly. This process can be promoted by treatment with compounds such as juglone, DMM, or menadione. It will be important to determine whether these or similar quinones could act inside the cell in a similar way to that found in vitro.

The present work suggests that tau 2R, once modified by PKA, provides a useful model with which to test the influence of PKA phosphorylation on tau assembly. It is not known whether phosphorylation by PKA will be found in every type of tau polymer. Upon the basis of the current, as well as previous, studies, it seems that PKA-catalyzed phosphorylation likely plays an important role in some of these tauopathies. One cannot rule out the possibility that in other cases the assembly of tau into polymers, like PHF, may require the presence of additional factors such as heparin (*17*, *30*) or fatty acids. The assembly of the phosphorylated tau molecule itself may also require the participation of other protein kinases, such as GSK3, that can modify tau conformation (*7*).

In summary, our work suggests the possible involvement of components related to quinone metabolism (or to the incorporation of exogenous quinones that could be present in food) in the formation of tau filamentous polymers.

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