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Phosphorylated, but not native, tau protein assembles following reaction with the lipid peroxidation product, 4-hydroxy-2-nonenal

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Abstract A correlation between hyperphosphorylation of tau protein and its aberrant assembly into paired helical filaments has lead to suggestions that phosphorylation controls assembly, but lacked a mechanistic basic. In this work, we have found that phosphorylated, but not native, tau protein is able to form polymers after the reaction with 4-hydroxy-2-nonenal, a highly toxic product of lipid peroxidation. Phosphorylation of tau by both proline or non-proline directed kinases, was able to assemble it into polymers. © **2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Tau assembly; Phosphorylation; 4-Hydroxy-2-nonenal; Lipid peroxidation

1. Introduction

Alzheimer's disease (AD) is a neurological disorder characterized, histopathologically, by the presence of two aberrant structures, senile plaques and neurofibrillary tangles (NFT). Since the number of NFT in patients' brain has been correlated with the level of dementia [1], an extensive analysis of NFT components has been carried out. NFT are aggregates of bundles of paired helical filaments (PHF), and the major protein component of PHF is a hyperphosphorylated form of the microtubule associated protein, tau factor [2,3].

The formation of aberrant tau polymers, like PHF, requires a conformational change of the protein since there is an increased interaction of PHF–tau, compared to unpolymerized tau, with conformation-dependent antibodies such as Alz50 [4]. Some conformational changes in tau protein occur as a consequence of phosphorylation [5], and it is not coincident that aberrant hyperphosphorylation of tau precedes the formation of NFT in AD [6]. In sum, these findings suggest that tau phosphorylation, its conformational change and its polymerization could be interrelated aspects. However, in vitro analysis, to study the assembly of tau into fibrillar polymers, has indicated that recombinant, unmodified or low phosphorylated tau [7–9] is able to polymerize. Also, it has been found that tau polymerization, in vitro, is favored by the presence of sulfated glycosaminoglycans (sGAG) [10,11], a component of NFT that interacts with tau [12], and that in such conditions, although phosphorylated tau is able to assemble [11] it does with a lower efficiency than unmodified tau [13]. These results could be explained by suggesting the existence of an alternative mechanism to assemble tau into fibrillar polymers, one in which phosphorylated tau could have an increased capacity for polymerization as compared to unmodified tau under certain conditions.

Recently, we found [14] that phosphorylated, but not unmodified tau, could change its conformation, becoming reactive with antibody Alz50, when it reacts with 4-hydroxy-2-nonenal (HNE), a highly reactive product of lipid peroxidation, resulting from oxidative stress [15]. HNE adducts of protein are increased in vivo in AD patients compared to controls [16]. These observations suggest that oxidative damage could play a synergistic role with tau phosphorylation to generate the conformational changes required for PHF formation [14] mediated by the HNE to tau, a binding that was previously described [17]. In this study we tested if phosphorylated tau, following reaction with HNE, is promoted in assembly.

Our results suggest that, by promoting formation of tau-fibrillar polymers of phosphorylated tau, HNE may be the important link between tau phosphorylation and PHF formation.

2. Materials and methods

2.1. Materials

Okadaic acid was purchased from Sigma. HNE was prepared as described [18]. Recombinant tau was isolated as indicated [10]. 7.51 Antibody was a kind gift of Dr. C. Wischik (MRC Cambridge, UK). PHF-1 antibody was a kind gift of Dr. P. Davies (Albert Einstein College, Bronx, NY, USA); 12E8 antibody was a kind gift of Dr. P. Seubert (Athena, San Francisco, CA, USA) and Tau-1 antibody was a kind gift Dr. L. Binder (MGC, IL, USA). H89 was obtained from Calbiochem. Tau peptide KVTSKCGSLGNIHHKPGGG was synthesized as previously described [10].

2.2. Cell culture

Tau-transfected COS-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal calf serum containing glutamine (2 mM) and antibiotics (penicillin/streptomycin). To these cultures, okadaic acid (1 μM) was added for 0.5–1 h to generate hyperphosphorylated tau.

The action of OA was tested by immunofluorescence analysis using tau antibodies (see above) on cultured cells. This was performed as previously described [19].

2.3. Tau isolation

Hyperphosphorylated tau was obtained from tau-transfected COS-1 cell extracts [20], after incubation with okadaic acid. Soluble protein was heat treated and the purification of tau was carried out as previously described [21]. The protein was characterized by gel electrophoresis and immunoblotting.

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To obtain phosphorylated tau lacking the modification promoted by GSK3, or PKA, okadaic acid was added to cultures incubated in the presence of 20 mM LiCl or H89 (10 μM) [22].

2.4. Assembly of tau protein into filaments

Filaments were grown by vapor diffusion on hanging drops containing tau protein (1 mg/ml) in the standard way used for protein crystallization, as previously indicated [10], but in the absence of heparin and in the presence of increasing amounts of HNE. The samples, after incubation for 4 days at 4°C in a final volume of 20 μl, were visualized by electron microscopy as described [23], and the amount of polymers was quantified by sedimentation [10].

3. Results

3.1. Isolation and characterization of phosphorylated tau

Since PHF–tau is in a hyperphosphorylated form in vivo [3], we focused on conditions that promote its polymerization. To isolate hyperphosphorylated tau, the cDNA, expressing the protein (the isoform containing four tubulin-binding repeats [24]), was transfected in COS-1 cells, and the tau-expressing cells were incubated with the phosphatase inhibitor okadaic acid (OA), to increase the level of tau phosphorylation, an increase that can be followed by its decrease in electrophoretical mobility. Fig. 1A shows that tau isolated from tau-expressing cells (and OA treated) has a decreased electrophoretic mobility compared to that of recombinant tau (containing the same number of tubulin-binding repeats).

To characterize some of the residues that have been modified by phosphorylation in tau protein, three antibodies that recognize phosphorytable regions, which are modified in AD, were tested. These antibodies are: ab Tau-1, reacting with serines 199 and 202 in unphosphorylated form; ab 12E8, reacting with phosphoserine 262; and ab PHF-1 reacting with serines 396 and 404 in phosphorylated form. The serines reacting with ab Tau-1 and ab PHF-1, are modified by proline directed protein kinases, like glycogen synthetase kinase 3 (GSK3), whereas that identified by ab 12E8 is phosphorylated by a different type of kinases, like protein kinase A (PKA) or mitogen associated protein kinase (MAPRK). In order to obtain tau phosphorylated in these and other residues, dephosphorylation of tau expressed in cultured cells was prevented by the addition of the phosphatase inhibitor OA. Fig. 1A shows the increased phosphorylation state of tau, reflected by decreased electrophoretic mobility, under OA treatment. Fig. 1B, C, D shows the reaction of tau antibodies with phosphorylated tau (isolated from transfected cells in the presence of OA) and with recombinant tau. It indicates that phosphorylated tau is, at least, phosphorylated in the three tau regions recognized by antibodies Tau-1, 12E8 and PHF-1 (Fig. 1E). This phosphorylated tau was used for polymerization assays.

As previously indicated, tau protein can be modified by proline directed kinases at the region recognized by ab Tau-1 or ab PHF-1; whereas non-proline directed kinases modify the site recognized by ab 12E8. In the second case, different kinases, like PKA or MAPRK (with different characteristics or inhibitors) could be involved in the modification of tau, whereas in the first case it has been indicated that mainly GSK3 is involved in such tau modification [22]. In attempt to inhibit PKA, H89 was used to test if the reaction of ab 12E8 with tau protein was abolished, and it does (Fig. 2). On the other hand, little is known about inhibitors for MAPRK. Since LiCl can inhibit GSK3 activity, we isolated tau from transfected cells incubated with OA and LiCl. In this case, we found reaction of the modified tau with ab 12E8 but not with ab PHF-1 (Fig. 2). This partially modified tau was also tested in polymerization assays (see below).

3.2. Assembly in vitro of phosphorylated tau into fibrillar polymers

It has been previously indicated that the presence of HNE, a product resulting from lipid peroxidation, can change the conformation of phosphorylated, but not unphosphorylated, tau protein, and that such conformational change could be identified by its reaction with the antibody Alz50 [14].

To test if HNE could also promote the assembly of phos-

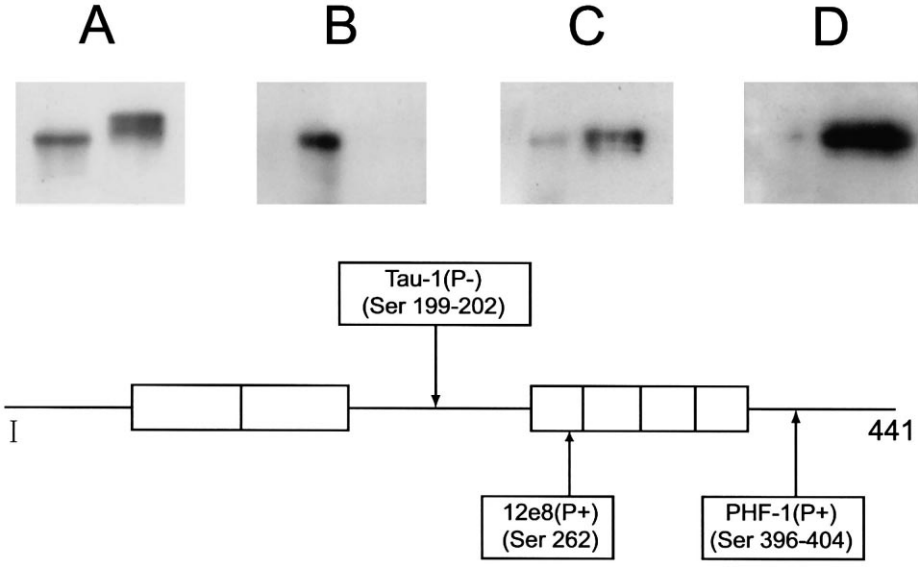

Fig. 1. Phosphorylation of tau protein. A: Phosphorylated tau protein was purified from tau cDNA-transfected COS-1 cells, cultured in the presence of okadaic acid. The modified protein (right) shows a slower electrophoretic mobility than that of recombinant tau upon reaction with tau antibody 7.51. B, C and D indicate the reaction of recombinant (left) and phosphorylated (right) tau protein with tau antibodies Tau-1, 12E8 and PHF-1, respectively. The localization of the modified residues, recognized by ab Tau-1, 12E8 and PHF-1, in tau molecule, is indicated, below in the figure.

phorylated tau, both compounds were mixed and the polymerization of the protein was tested as previously described [10]. Fig. 2 shows that phosphorylated tau (Fig. 2F), but not recombinant tau (Fig. 2E), was able to polymerize when HNE was added. No polymers were found in the presence of OA (Fig. 2G) or H89 (Fig. 2H). This experiment shows, for the first time, a condition in which phosphorylated tau has an increased polymerization capacity, compared to that of unmodified tau. When a HNE concentration-dependent experiment was performed, it was found that the maximum proportion of assembled tau could be obtained at 2 mM HNE (Fig. 3), but only for fully phosphorylated tau. In fact, unphosphorylated or partially (in the absence of GSK3 or PKA modification) phosphorylated tau, is unable to fully assemble under the same conditions (Fig. 3) although a very small amount of tau aggregates could be observed in the absence of PKA modification. These results indicate that phosphorylation of tau by GSK3 or PKA is required for tau assembly in the presence of HNE. Assembled polymers appear in bundled form, probably due to the crosslinking characteristics of HNE [25], a feature that has been also reported in other peroxidant conditions [26,27]. The assembled polymers have an estimated diameter of 2–3 nm, although at high HNE concentrations, straight filaments with a diameter of 10–12 nm were also found (Fig. 3).

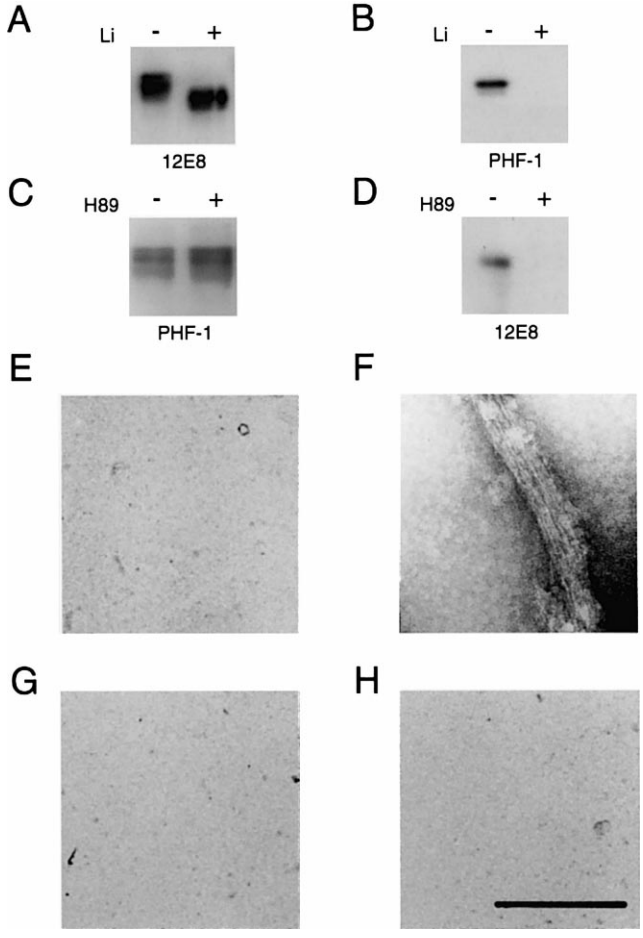

Fig. 2. Assembly of phosphorylated tau into filaments in the presence of HNE. Effect of LiCl in the reaction of tau with ab 12E8 (A) and PHF-1 (B) and that of H89 in the reaction of tau with ab PHF-1 (C) or ab 128E (D). Purified phosphorylated tau was incubated in the presence of different HNE concentrations and the presence of polymers was determined by electron microscopy. The figure shows the samples for recombinant tau+HNE (E), phosphorylated tau+HNE (F), phosphorylated tau (in presence of LiCl)+HNE (G) and phosphorylated tau−HNE (H).

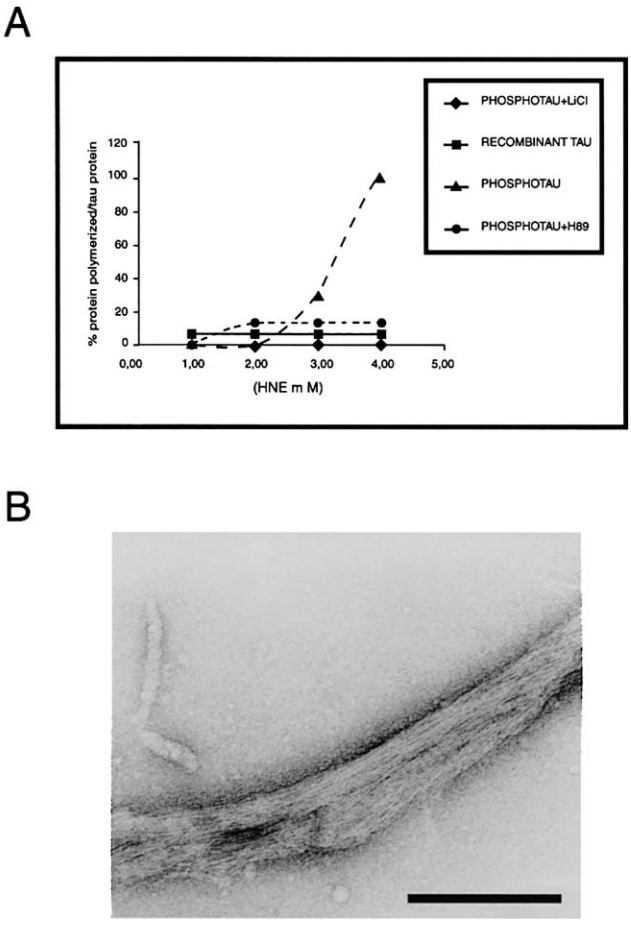

Fig. 3. Effect of HNE concentration on tau assembly. A: Increasing concentrations of HNE were added to unphosphorylated (■), partially phosphorylated (GSK3 modification lithium inhibited) (♦), H89 inhibited (●), or phosphorylated (▲) tau protein, and the amount of polymers assembled at different concentrations was measured by electron microscopy and sedimentation [10]. The morphology obtained for phosphotau polymers in the presence of 1 mM HNE is shown in B. The presence of two types of polymers 2–3 nm or 10–12 nm wide is shown. Bar indicates 200 nM.

3.3. A short tau peptide could be assembled in the presence of HNE and the absence of phosphorylation

In previous works it has been described that a peptide present in the third tubulin-binding repeat of tau protein was able to self-assemble in the presence of sulfate glycosaminoglycans [10]. This peptide is not phosphorylated in normal or pathological conditions.

To test if it could polymerize in the presence of HNE, an experiment similar to that of Fig. 2F was carried out. Our result indicates that 1 mM HNE facilitates the polymerization of this tau peptide, forming 2–3 nm assembled polymers (data not shown).

4. Discussion

For the first time, this work describes conditions in which

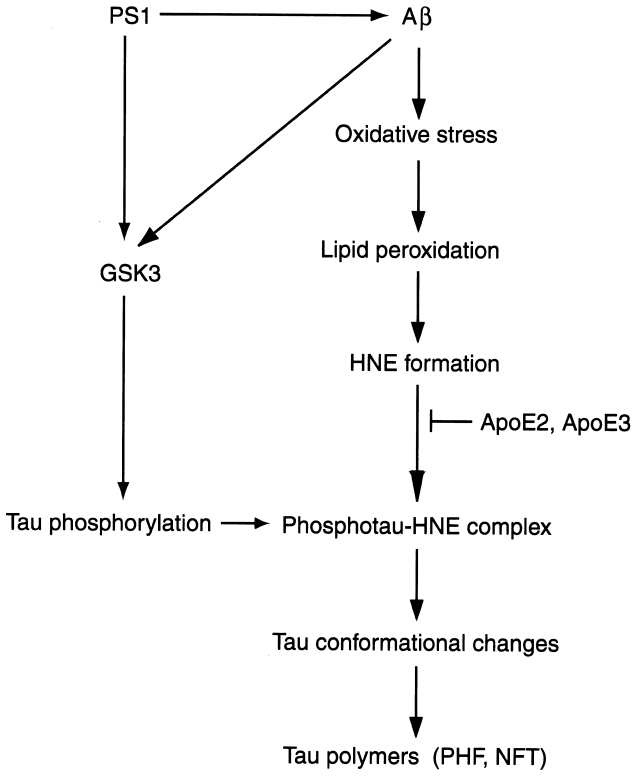

Fig. 4. Model for tau assembly promoted by HNE. A working model indicating the possible pathway, beginning from oxidative stress and finalising in the formation of tau filaments, in which HNE is involved, is indicated. For details, see text.

phosphorylated tau shows a higher capacity to assemble than unphosphorylated tau. It involves the combined effects of tau phosphorylation and reaction of tau with a product of lipid peroxidation generated by oxidative damaged HNE. In a previous report it was indicated that a primary result of both effects was a conformational change of tau protein [14]. Now, it is indicated that such conformational change may be critical to the formation of fibrillar polymers. The phosphorylation of tau protein required for the assembly takes place in at least three of the sites modified in AD [28]; sites that are modified by GSK3 [22] and by a non-proline directed protein kinase, probably PKA [29] or MAPRK [30]. GSK3 and PKA phosphorylation is needed for tau assembly in the presence of HNE.

GSK3 activity is regulated by proteins such as amyloid β or presenilins [31,32] that are directly involved in the onset of the familiar type of AD [33], and, in this way, those proteins could facilitate tau phosphorylation at some proline directed phosphorylation sites, like those recognized by ab Tau-1 (in negative form) or that of ab PHF-1 (in positive form). Lipid peroxidation results from reactive oxygen species reactions with polyunsaturated fatty acids forming secondary reactive aldehydes like HNE [25]. Thus, it can be postulated that in both features, aberrant tau phosphorylation takes place during times of oxidative stress, the formation of tau polymers could also take place.

Consistent with this potential coordination of these events several kinases are known to be increased by oxidative stress, e.g. MAPRK and p38 [34]. The previous points could be discussed by proposing a working model to take into account the role of oxidative damage in inducing the pathological sequels of AD [35] (Fig. 4).

Oxidative stress could occur in aged neurons by the presence of molecules like Aβ that could play a direct role in the induction of oxidative stress [36]. On the other hand, the formation of Aβ could be favored by the presence of mutated PS1 [37]. The oxidative stress could result in oxidative damage to lipids, the presence of lipid peroxidation, and the formation of hydroxynonenal (HNE) and other reactive aldehydes [16,38] and is found as an adduct to proteins of NFts [14,16].

HNE is a highly toxic agent, whose toxicity can be partially blocked by cysteine containing proteins like ApoE2 or ApoE3 but not by ApoE4 [39]. If not blocked HNE can easily pass among neuronal compartments and bind to tau protein [17]. If tau is phosphorylated, the reaction with HNE modifies its conformation [14] and, as shown here, promotes its assembly into fibrillar polymers resembling NFTs [40]. These fibers are similar, in form, to the narrow tau filaments of 2–3 nm found in NFTs [41]. Additionally, wider polymers similar in form to straight filaments of NFTs were found. Phosphorylation could facilitate a tau conformational change that may allow the interaction of HNE with those tau regions mainly involved in polymer formation. One of these regions is the third tubulin-binding motif present in tau molecule. However, a major concern is that the in vitro concentrations of HNE needed to clearly induce tau polymerization are in the millimolar range (although some polymers could be found at a lower concentration), and it is not known if, in vivo, HNE concentration could go that high.

Independently, the formation of tau polymers could also probably occur in pathological conditions if sGAG are present in the cytosol due to leakage of membrane compartments (where sGAG and proteoglycans should be located) [42]. In this way, membrane damage could be very important to the formation of tau polymers, assembled by the interaction of the protein with sGAG [10,11] or with HNE (this work), although, in the second case, tau polymerization could be accelerated by the phosphorylation of the protein. Additionally, the existence of other compounds apart from sGAG or HNE that could facilitate tau assembly in different tau pathologies (taupathies) could not be excluded.

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