Coenzyme Q Induces Tau Aggregation, Tau Filaments, and Hirano Bodies

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Abstract

Tau aggregation is a common feature of tauopathies such as Alzheimer disease (AD). In AD, tau assembles into fibrillar polymers; it may also be present in other aberrant aggregates, including Hirano bodies. The mechanisms leading to tau polymer- ization in vivo are not understood. In this study, we found that coenzyme Q (ubiquinone) facilitates tau aggregation after binding to tau molecules at the region of the tau molecule involved in self- assembly. Consequently, after tau-tau interactions, this region is masked in fibrillar tau polymers. Further in vitro studies showed that ubiquinone facilitates the interaction of tau protein with actin to form structures that are morphologically similar to Hirano bodies. Finally, studies in AD brains show that Hirano bodies react with an antibody raised against ubiquinone, indicating that ubiquinone is a component of Hirano bodies. Taken together, the in vitro models and findings in AD suggest that in the presence of ubiquinone, Hirano bodies may result from the interaction of actin and other proteins, including tau.

Key Words: Actin, Alzheimer disease, Coenzyme Q, Hirano bodies, Tau, Ubiquinone.

INTRODUCTION

Tau is a microtubule-associated protein that in a monomeric form is very soluble in different buffer conditions. In several neurodegenerative disorders known as tauopathies (1), however, tau protein forms insoluble aggregates that are stable even in the presence of detergents and unusual solvents such as 2.5% perchloric acid solution (2).

Tau aggregation resulting in the formation of fibrillar polymers has been reported to take place in vitro (3). This polymerization is facilitated by the addition of assembly inducers such as heparin (4, 5) and other sulfated glycosaminoglycans (6), polyanions (7), fatty acids (8), and quinones (9). It is not clear, however, whether these in vitro inducers also induce tau assembly in vivo (10). Tau polymers in the tauopathies can be present in different brain areas (11), show different morphologies, and have different composition. For example, paired helical filaments (PHFs) and straight filaments are both found in the brains of AD patients, but they are morphologically different. Moreover, PHFs in AD but not Pick bodies are stained with dyes such as thioflavin S (12), indicating different protein composition. In addition, different tau polymers show different solubilities. Thus, tau filaments from patients with progressive supranuclear palsy are more labile than PHFs isolated from patients with AD (13).

Tau can also be associated with other proteins in other structures such as Hirano bodies (14), the actin-rich paracrystalline inclusions found in AD (15). Recently, the formation of structures analogous to Hirano bodies in transgenic flies that overexpress human tau protein was reported (16). The actin in Hirano bodies can associate with 2 actin-binding proteins, actin depolymerizing factor and cofilin (17), and/or with tau protein (16). At present, however, little is known regarding the mechanism underlying the formation of Hirano bodies in vivo, although several mechanisms have been suggested (16, 17). Moreover, it is not known whether different tau-polymerization inducers might be involved in the formation of different tau polymers or of copolymers with other proteins. We previously found that coenzyme Q0 (CoQ0 or ubiquinone) (18) can bind to tau protein and induce its assembly into fibrillar polymers (9), and that CoQ0 and other related quinones (19) can bind to tau not only in noncovalent form but also in a covalent form
(9). The latter observation allows for the search for the modified protein or quinone-tau complex in the aberrant tau structures of tauopathies. Here, we report the presence of quinones in Hirano bodies, and because ubiquinone binds to the tau molecule at its self-assembly region, we suggest that tau-quinone complexes in other tau polymers may be in masked form.

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This work was supported by Grant No. FIS PIo40184 from the Instituto de Salud Carlos III, by grants from the Plan Nacional (Ministerio de Educacion y Ciencia, Spain) and Comunidad de Madrid, Fundacion Botin, and by an Institutional Grant from Fundacion R. Areces.

MATERIALS AND METHODS

Tau Isolation

Expression and isolation of recombinant protein htau 42 (the longest human isoform of tau expressed in the CNS) was performed as previously described (20, 21). Briefly, after induced expression of tau in *Escherichia coli*, protein purification was performed by a protocol involving a heat resistance step in a buffer containing 100 mmol/L of Tris, pH 7.5, 0.5 mmol/L of MgCl2, 1 mmol/L of EGTA, 1 mmol/L of 2-mercaptoethanol, 100 mmol/L of NaCl and protease inhibitors: 2 mmol/L of phenylmethanesulfonyl fluoride, 10 Kg/ml of aprotinin, 10 Kg/ml of leupeptin, and 10 Kg/ml of pepstatin. This step involves a treatment at 100-C for 5 minutes. 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 and chromatographed on DEAE Sephacel-R, equilibrated in PBS, and the protein was eluted stepwise using 1 mol/L of NaCl in PBS.

Assembly of Tau Protein Into Filaments

Filaments were grown by vapor diffusion in hanging drops in the standard way used for protein crystallization as previously described (5). In a typical experiment, 0.5 to 2 Kg of protein tau was resuspended in 10 to 15 KL of buffer A (0.1 mol/L of 2-[*N*-morpholino]ethanesulfonic acid [pH 6.4], 0.5 mmol/L of $MgCl_2$, and 2 mmol/L of EGTA) containing 50 mmol/L of NaCl and 1 mmol/L of CoQ0. The reservoir contained 0.2 mol/L of NaCl in buffer A 5. Filaments were obtained after incubation for 4 days at 4-C. The samples were visualized by electron microscopy as previously described (22). Electron micrographs were obtained at a magnification of 80,000x 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, Inc. (Pleasanton, CA). Several standards were used for the control of the measurements. Alternatively, tau polymers were stained using a tau antibody (ab) and visualized by immunofluorescence as previously described (23).

Assembly of Hirano Body-Like Structures

Tau protein (0.5Y2 Kg), actin (1 Kg; Cytoskeleton, Denver, CO), and CoQ0 (1 mmol/L) were mixed and grown using the hanging drop method as previously described. After 4 days of incubation, samples were analyzed by immunofluorescence using a modified version of the previously described protocol (23). Briefly, a suspension of the samples was adsorbed to gelatinized glass slides and then allowed to dry at 37-C. The protein adsorbed to the slide was processed for double-immunofluorescence experiments with antibodies raised against tau (Tau-5; Calbiochem, Darmstadt, Germany) and CoQ0 (73903, a kind gift of Dr. McDonald et al [24]), and with phodamine phalloidin (1/300; Sigma, Madrid, Spain) to stain polymerized actin, which is incubated at the same time of secondary antibodies. Samples were imaged with a Zeiss Axiovert 200 fluorescent microscope. Fluorescence images were captured through a 100x oil-immersion objective on a high-resolution charge-coupled device camera (SPOT RT Slider, Diagnostic).

Mass Spectrometry Analysis

Mass spectrometry was performed following the method of Reynolds et al (25).

PHF Isolation

Brain samples supplied by Dr. Ravid (Netherlands Brain Bank, Amsterdam, The Netherlands) from AD patients were used to isolate PHFs. They were isolated by preparation of sarkosyl-insoluble extracts, as previously described (26).

Gel Electrophoresis and Western Blot Analysis

Tau protein samples obtained after 0 to 24 hours of incubation of tau protein in the presence of 1 mmol/L of CoQ0 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli on 10% gels (27). The gels were then stained with Coomassie blue R-250. Alternatively, in a similar experiment, tau samples incubated with CoQ0 were analyzed by Western blot using anti-tau (7.51) and anti-CoQ0 (73903) antibodies. Densitometry of the bands was determined using a BioRad GS740 densitometer.

Immunoelectron Microscopy

Immunoelectron microscopy was performed after adsorption of the samples to electron microscopy carbon-coated grids and incubation with the first ab (diluted 1/40) for 1 hour at room temperature. After extensive washing with PBS, the grids were incubated with the secondary ab (diluted 1/40) conjugated with 5-nm-diameter gold particles (Biocell Laboratories, Inc., Rancho Dominguez, CA). Finally, the samples were negatively stained and observed as previously described.

Human Tissue

Tissue samples were from 2 men and 1 woman aged 76, 68, and 74 years, respectively, with AD. The 3 patients had a progressive and severe cognitive impairment. The cause of death was respiratory failure in 2 cases and unknown in the

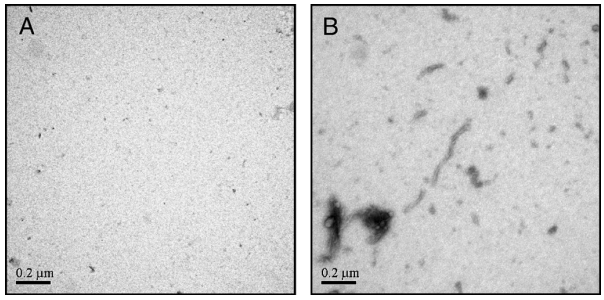

FIGURE 1. Tau assembly in the presence of coenzyme Q0. Electron microscopy micrographs of tau polymers assembled in the absence (A) and presence (B) of coenzyme Q0 are shown. Scale bars = 0.2 Km.

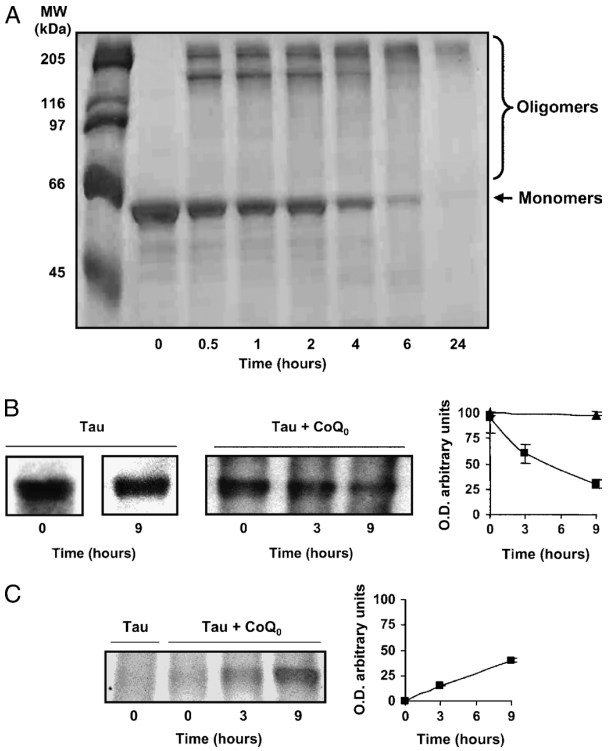

FIGURE 2. Coenzyme Q0 (CoQ0) induces the formation of covalently linked tau aggregates. (A) Tau protein (0. 5 Kg/KL) was mixed with I mmol/L CoQ0, and the mixture was incubated for 0 to 24 hours at 37-C. After that, the protein was characterized by gel electrophoresis, followed by Coo- massie blue staining. The left lane shows the electrophoretic mobility markers. (B) Western blot analysis of the tau monomer species incubated in the presence (■) or absence (r) of CoQ0, as indicated in (A). (C) Just like (B), but the reaction with antibody raised against ubiquinone is tested.

third because the death was not observed. The postmortem delay was 6 and 4 hours and no more than 7 hours in the third case. Neuropathologic analyses were performed on selected paraffin sections of 25 different brain areas immunostained for A-amyloid 1-40 and 1-42, phospho-tau AT8, 3Rtau and 4Rtau, >-synuclein, ubiquitin, >B-crystallin, TAR DNA binding protein 43, glial fibrillary acidic protein, and CD68. The diagnoses of these cases were Braak and Braak AD stages VC and VIC. No other abnormalities, including >- synuclein or TAR DNA binding protein 43 inclusions, were observed in these cases. Small-vessel disease mainly affect- ing the striatum was present in 1 case.

Cases used as controls were 3 women aged 65, 69, and 72 years with no neurologic impairment. The postmortem delay was between 3 and 6 hours. The brains were examined by studying the same corresponding regions with similar immunostaining protocol. No neuropathologic abnormalities, other than mild small-vessel disease in 1 case, were found in the control cases.

Immunohistochemistry

Following the EnVision + system peroxidase procedure (Dako, Carpinteria, CA), 5-Km-thick dewaxed sections of the hippocampus from AD cases and controls were processed for immunohistochemistry. After incubation with methanol and H_2O_2 in PBS and normal serum, the sections were incubated with monoclonal ab to A-actin (Sigma) diluted 1:2500, and rabbit polyclonal anti-CoQ_0 73903 was used at a dilution of 1:200. The peroxidase reaction was visualized with diaminobenzidine and H_2O_2. Control of the immunostaining included omission of the primary ab; no signal was obtained after incubation with only the secondary ab. Sections were counterstained with hematoxylin.

Double Labeling Immunofluorescence and Confocal Microscopy

Dewaxed sections of the hippocampus were stained with a saturated solution of Sudan black B (Merck) for 30 minutes to block the autofluorescence of lipofuscin granules present in nerve cell bodies, rinsed in 70% ethanol, and washed in distilled water. The sections were then incubated at 4-C overnight with the mouse anti-A-actin (Sigma) at a dilution of 1:2500, and rabbit polyclonal anti-CoQ0 73903 (24) was used at a dilution of 1:500. After washing in PBS, the sections were incubated in the dark with the cocktail of secondary antibodies and then diluted in the same vehicle solution as the primary antibodies for 45 minutes at room temperature. Secondary antibodies were Alexa488 anti-rabbit

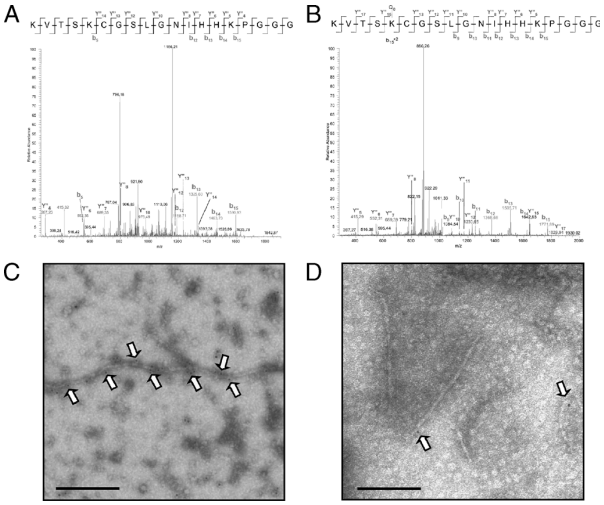

FIGURE 3. Localization of coenzyme Q0 (CoQ0) bound to tau molecule. The major binding sites of CoQ0 on tau protein were analyzed by nanoelectrospray. (A) Tau peptide (used as control without CoQ0 incubation) and its fragmentation peaks. (B) The main modification by CoQ0, sequence, and fragmentation peaks. Essentially, lysine 321 of the whole tau protein is modified. Analysis of the reaction of assembled tau polymers with antibody (ab) raised against tau (ab 134) (C) and CoQ0 (73903) (D). Arrows show gold particles. Scale bar = 200 nm.

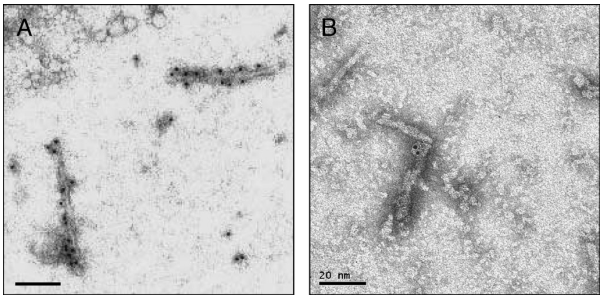

FIGURE 4. Reaction of tau and coenzyme Q0 (CoQ0) antibodies with paired helical filaments from Alzheimer disease patients. (A) Isolated paired helical filaments were incubated with an antibody (ab) raised against tau (ab 134). (B) Just like (A), but in this case, an ab raised against CoQ0 (73903) was tested. Scale bar = 20 nm.

(green) and Alexa546 anti-mouse (red; both from Molecular Probes, Eugene, OR) used at a dilution of 1:400. After washing in PBS, the sections were mounted in an immuno-fluore mounting medium (ICN Biomedicals, Costa Mesa, CA), sealed, and dried overnight. Sections were examined with a Leica TCS-SL confocal microscope.

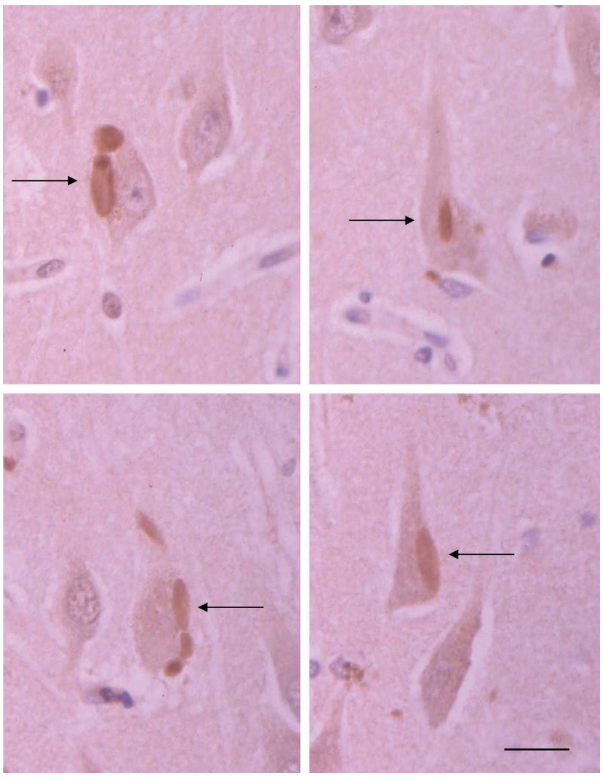

FIGURE 5. Coenzyme Q0 (CoQ0) is present in Hirano bodies. Single label immunohistochemistry shows CoQ0 immunoreactivity in elongated and ovoid-shaped neuronal and perineural bodies (arrows) in the CA1 area of the hippocampus in Alzheimer disease. Paraffin section with slight hematoxylin counterstaining. Scale bar = 25 Km.

RESULTS

CoQ0-Tau Binding and Aggregation

Tau aggregation is a common feature in tauopathies that has been reproduced in vitro. Several tau polymerization inducers, including CoQ0, have been identified. In the presence of CoQ0, tau assembles into fibrillar polymers (Fig. 1). This association of CoQ0 to tau can be in noncovalent or covalent forms. Figure 2A shows that in the presence of CoQ0, the electrophoretic mobility of tau changes, and that the formation of CoQ0 covalently linked tau aggregates occurs. We also tested the effect of CoQ0 concentration on tau aggregation using 1 Kmol/L of tau and determined that aggregates were observed at 0.5 mmol/L of CoQ0, the lowest concentration tested (data not shown). Figure 2A shows the kinetics for the formation of tau aggregates. Additionally, Figure 2B shows a decrease in monomeric tau protein upon the addition of CoQ0 (using ab 7.51 against tau). Figure 2C shows that upon the addition of CoQ0, a reaction with an ab raised against quinone takes place with tau. This reaction is only found for tau in monomeric form, and no reaction is observed for aggregated tau forms.

Localization of CoQ0 Bound to Tau Molecule

Coenzyme Q0 was bound to tau protein, and the complex was isolated by gel electrophoresis (Fig. 2). The

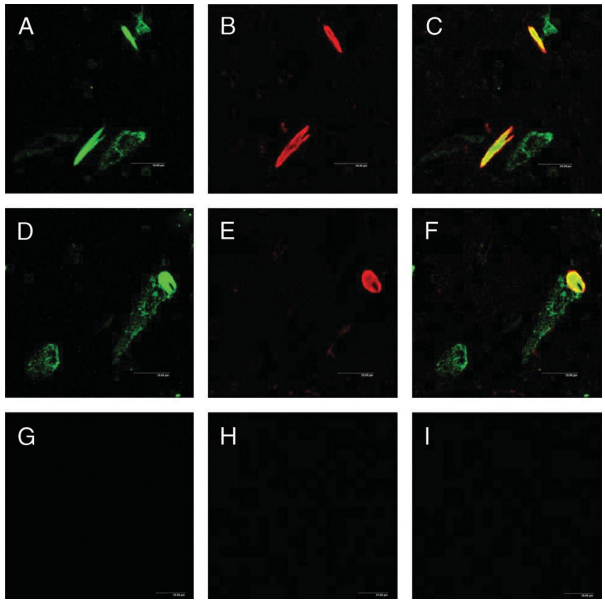

FIGURE 6. Colocalization of actin and coenzyme Q0 (CoQ0). Double label immunofluorescence and confocal microscopy show partial colocalization of CoQ0 (A, D; green) and A-actin (B, E; red) (merge, C, F; yellow) in Hirano bodies. Lower panels (GYYYI) are negative control sections incubated without the primary antibodies.

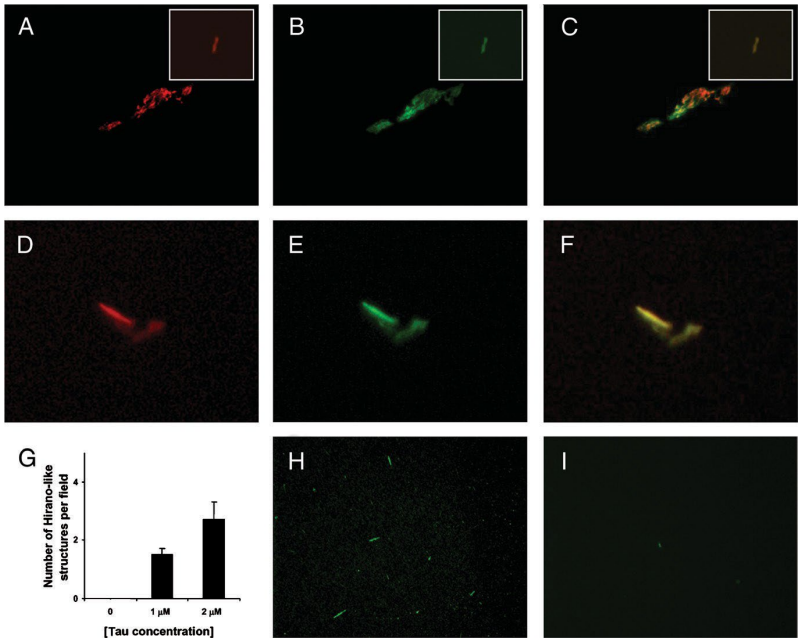

FIGURE 7. Hirano body-like structures. Tau, actin, and coenzyme Q0 (CoQ0) were mixed (see Materials and Methods section), and the formation of Hirano body-like structures (tau-actin-CoQ0 complexes) was analyzed by immunofluorescence (23). Actin-tau-CoQ0 complexes were stained using phalloidin (actin) (A, D) or anti-tau antibody (B, E). The merging of both actin and tau staining is shown in (C) and (F). Inset shows another Hirano body-like structure. In the same manner, tau alone (H) or actin alone (I) were mixed with CoQ0, and the formation of aggregates was analyzed by immunofluorescence using anti-tau antibody and phalloidin. (G) Shows the effect of tau concentration in the formation of Hirano body-like structures.

localization of CoQ0 bound to tau was then determined by proteomic analysis. Figures 3A and B indicate that CoQ0 binds at least to lysine 321, the number corresponding to the largest CNS isoform of the tau molecule. These results indicate that CoQ0 binds to the tau molecule in a region involved in its own interaction (5). Thus, CoQ0 may facilitate tau aggregation, but in the assembled polymer, CoQ0 bound to tau is masked because it occurs at the tau-binding site required for tau self-assembly. As a result, there is only weak staining of tau polymers with the CoQ0 ab (Fig. 3C). Coenzyme Q0 is, however, bound to tau based on characterization of the polymer protein by gel electrophoresis and Western blotting (Fig. 2B).

Our objective was to determine whether CoQ0 can act also as a tau polymerization inducer in vivo. If this was the case, tau aggregates in vivo would contain CoQ0, although only CoQ0 covalent bound to the protein should be observed because noncovalently bound CoQ0 would be detached from tau aggregates during the analysis procedure.

Analysis for the Presence of CoQ0 in Aberrant Aggregates Present in AD

Among the aberrant protein aggregates containing tau protein that are present in AD are PHF and Hirano bodies. The main component of PHF is tau protein, whereas Hirano bodies are neuronal inclusions that are mainly observed in hippocampal neurons and are composed of actin either associated with or not associated with tau (14). Figure 4B shows very weak staining of isolated PHF with CoQ0 ab. This reaction can be compared with that found when a tau ab (134, a kind gift of Dr. Wischik, Aberdeen, UK) was used on PHF (Fig. 4A). As previously indicated, the low interaction observed between

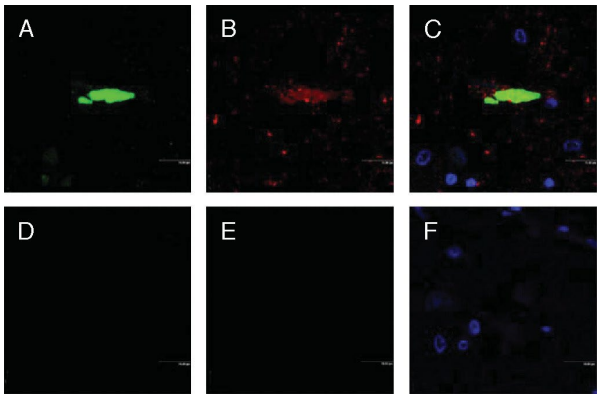

FIGURE 8. Partial colocalization of tau and coenzyme Q0 (CoQ0). Double label immunofluorescence and confocal microscopy show partial colocalization of CoQ0 (A; green) and tau (B; red) (merge; C) in Hirano bodies. Lower panels (DYF) are a negative control section incubated without the primary antibodies. Scale bar = 16 Km.

the CoQ0 ab and PHFs can be explained by the fact that the tau epitope reacting with ab CoQ0 is masked in the PHF structure. In a further step, we purified PHFs from AD patient brains and assessed the reaction of PHF protein with tau ab (PHF-1) and with anti-CoQ0 ab by Western Blot analysis. A weak reaction of the anti-CoQ0 ab with the PHF proteins that reacted with ab PHF-1 was observed (data not shown). Thus, our results suggest that CoQ0 can bind to PHF protein. On the other hand, Hirano bodies showed strong staining with the ab raised against CoQ0 (Figs. 5 and 6). Single immunohistochemistry in AD cases revealed strong Q0 immunoreactivity restricted to perineuronal and neuropil oval-shaped bodies reminiscent of Hirano bodies in the CA1 area of the hippocampus (Fig. 5). No Hirano bodies were found in the matched controls (not shown). Double label immunofluorescence and confocal microscopy showed partial colocalization of Q0 and actin in these structures (Fig. 6), thus demonstrating CoQ0 in Hirano bodies.

Actin and Tau Protein Form Aggregates in the Presence of CoQ0

To determine whether CoQ0 bound to tau can play a role in the formation of Hirano body-like structures in vitro, we mixed tau alone with CoQ0, actin alone with CoQ0, and finally, tau with actin plus CoQ0 and sought to identify possible assembled polymers present in each mixture. Figure 7H shows that when CoQ0 is added to tau, it results in the formation of fibrillar polymers as previously indicated in Figure 1. Only amorphous small aggregates were found upon addition of CoQ0 to actin (Fig. 7I), but aggregates with the rod-shaped ovoids of Hirano body-like structures were found in the mixture of tau, actin, and CoQ0 (Fig. 7AYF). We also tested the tau concentration needed for the formation of Hirano body-like structures, and at a concentration of 1 Kmol/L of tau protein, some of those structures can be found (Fig. 7G). These results are in agreement with the partial colocalization in human Hirano bodies of CoQ0 and tau protein (Fig. 8).

DISCUSSION

Sporadic tauopathies may be the result of various pathogenetic processes that are not understood. One hypothesis is that oxidative stress involving mitochondrial defects or failure promotes tauopathies (28). In support of this hypothesized mechanism are reports that mitochondrial oxidative stress can cause tau hyperphosphorylation (29), and that inhibitors of mitochondrial complexes cause tau pathology in cultured neurons and in mouse models (30). Because aging is the main risk factor for several neurodegenerative disorders, including AD, the presence of mitochondrial quinones (31) might participate in aging and AD pathogenesis.

A member of the coenzyme Q family, CoQ0 (ubiquinone), induces tau polymerization by facilitating intermolecular crosslinks among tau molecules; the tau molecules then form aggregates that act as nucleation centers for further polymerization (9). In the present study, we found that CoQ0 covalently bound to tau at the sites of tau-tau interaction, suggesting that CoQ0 bound to tau may be masked within tau polymers. To determine whether CoQ0 may be associated with tau-containing structures, we used a specific ab that reacts with CoQ0 and observed that CoQ0 bound to PHF from AD patients, suggesting a role for CoQ0 in tau fibrillar assembly in situ. We cannot, however, rule out the possibility that CoQ0 bound tau may mainly facilitate the nucleation, rather than the elongation of tau polymers.

An additional unexpected observation was the presence of heavy staining of the anti-CoQ0 ab with Hirano bodies. It has previously been reported that tau protein is present in some Hirano bodies (14). Thus, actin and tau may be complexed in those structures. Moreover, Hirano bodies contain the actin-binding proteins actin depolymerizing factor and cofilin (32), and these proteins facilitate aberrant actin assembly (33). Therefore, we hypothesize that CoQ0 may play a role in the formation of Hirano bodies. In an attempt to mimic the assembly of Hirano body-like structures in vitro, we found that the mixture of tau, monomeric actin, and CoQ0 results in the formation of rod-shaped structures, whereas the presence of CoQ0 plus tau alone, or of CoQ0 plus actin alone, is not sufficient to form those assemblies. These results suggest that tau and CoQ0 mixed with actin might facilitate the polymerization of Hirano body-like structures, and that tau may mimic the actin assembly proteins in the presence of CoQ0. It has been found, however, that not all the Hirano bodies contain tau protein (14). Thus, tau may be replaced by other proteins that could, together with actin, also promote the formation of Hirano bodies. Although brain tau is a collection of 6 isoforms that may differentially be affected by CoQ, we used a purified recombinant tau protein to avoid possible contaminations from brain-purified tau for our in vitro analysis.

ACKNOWLEDGMENTS

The authors thank Raquel Cuadros for expert technical support and T. Yohannan for editorial assistance.

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