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Microtubule-associated protein tau in bovine retinal photoreceptor rod outer segments: Comparison with brain tau

Akio Yamazaki [a], Yuji Nishizawa [b], Isao Matsuura [c], Fumio Hayashi [d], Jiro Usukura [e], Vladimir A. Bondarenko [f,*]

[a] Kresge Eye Institute and Departments of Ophthalmology and Pharmacology, Wayne State University, Detroit, MI 48201, USA
[b] College of Life and Health Sciences, Chubu University, Kasugai 487-8501, Japan
[c] Division of Molecular and Genomic Medicine, National Health Research Institutes, Zhunan Town, Taiwan
[d] Department of Biology, Graduate School of Science, Kobe University, Kobe 657-8501, Japan
[e] Division of Integrated Project, EcoTopia Science Institute, Nagoya University, Nagoya 464-8603, Japan
[f] College of Osteopathic Medicine, Touro University Nevada, Henderson, NV 89014, USA

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ABSTRACT

Recent studies have suggested a possible involvement of abnormal tau in some retinal degenerative diseases. The common view in these studies is that these retinal diseases share the mechanism of tau-mediated degenerative diseases in brain and that information about these brain diseases may be directly applied to explain these retinal diseases. Here we collectively examine this view by revealing three basic characteristics of tau in the rod outer segment (ROS) of bovine retinal photoreceptors, *i.e.*, its isoforms, its phosphorylation mode and its interaction with microtubules, and by comparing them with those of brain tau. We find that ROS contains at least four isoforms: three are identical to those in brain and one is unique in ROS. All ROS isoforms, like brain isoforms, are modified with multiple phosphate molecules; however, ROS isoforms show their own specific phosphorylation pattern, and these phosphorylation patterns appear not to be identical to those of brain tau. Interestingly, some ROS isoforms, under the normal conditions, are phosphorylated at the sites identical to those in Alzheimer's patient isoforms. Surprisingly, a large portion of ROS isoforms tightly associates with a membranous component(s) other than microtubules, and this association is independent of their phosphorylation states. These observations strongly suggest that tau plays various roles in ROS and that some of these functions may not be comparable to those of brain tau. We believe that knowledge about tau in the entire retinal network and/or its individual cells are also essential for elucidation of tau-mediated retinal diseases, if any.

1. Introduction

Microtubules (MTs), a major component of the cytoskeleton, participate in numerous cellular processes ranging from cell division, organelle positioning, intracellular transport, to neuronal differentiation. The MT-associated protein tau is the best known for its being essential for the assembly and stabilization of MTs in the neuronal system [1–3]. Thus, it is understandable that tau dysfunction is correlated with a variety of neurodegenerative diseases including Alzheimer's disease (AD), front-temporal dementia with parkinsonism linked to chromosome (FTDP-17) and Pick's disease and that these diseases are referred to as 'tauopathies' [4–6].

Expression of tau in the neuronal system is extraordinarily complicated: Multiple tau isoforms are generated from a single gene through alternative mRNA splicing in a developmental stage- and cell type-specific manner [7]. In human adult brain tau, exons 1, 4, 5, 7, 9, 11, 12 and 13 are constitutive and exons 2, 3, and 10 are alternatively spliced [7,8]. Exons 4A, 6, and 8 appear not to be transcribed. Exon 14 is transcribed, but not translated. In turn, matured human brain tau primary transcript gives rise to six mRNA: (Δ6,8), (Δ6,8,10), (Δ3,6,8), (Δ3,6,8,10), (Δ2,3,6,8), and (Δ2,3,6,8,10). These tau isoforms contain repetitive regions serving as MT-binding sites in a domain encoded by exons 9–12. Tau isoforms resulting from encoding exon 10 have four repeat regions, whereas those lacking encoding exon 10 have three repeat regions [5–10]. Various factors such as localization of abnormal tau affect the development of neurodegenerative diseases. Here we emphasize that the splicing also

Abbreviations: AP, alkaline phosphatase; AD, Alzheimer's disease; BSA, bovine serum albumin; Cdk5, cyclin-dependent protein kinase 5; DTT, dithiothreitol; FTDP-17, front-temporal dementia with parkinsonism liked to chromosome; MT, microtubule; MW, molecular weight; PHF, paired helical filament; pI, isoelectric point; PMSF, phenylmethylsulfonyl fluoride; Pγ, the inhibitory subunit of cGMP phosphodiesterase; PDE, cGMP phosphodiesterase; ROS, rod outer segments of retinal photoreceptors; Tα, the α subunit of retinal G protein

* Corresponding author at: College of Osteopathic Medicine, Touro University Nevada, 874 American Pacific Drive, Henderson, Nevada 89014, USA. Tel.: +1 702 777 1806; fax: +1 702 777 1799.
E-mail address: vladimir.bondarenko@touro.edu (V.A. Bondarenko).

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appears to play a role(s) in the pathogenesis of these diseases. In AD, all six isoforms of brain tau have been found in the neurofibrillary tangle of paired helical filaments (PHFs), a hallmark of AD [11,12]. Inclusion bodies in FTDP-17 contain only tau isoforms with four MT-binding regions [13], whereas inclusion bodies in Pick's disease are entirely made from tau isoforms with three MT-binding regions [14,15].

Regulation of tau is also highly complicated: Tau is tightly regulated by phosphorylation [2,10,16]. Remarkably, phosphorylation can occur at 30–40 different sites in tau [17–20]. Normal human brain tau contains 2–3 moles of phosphate per mole of protein, which appears to be optimal for its interaction with tubulin and promotion of MT assembly [21]. However, the level of tau phosphorylation in AD brain is 3- to 4 times higher than that in normal brain [22,23], and, to date, ~20 phosphorylation sites have been identified in tau associated with PHFs [17,24]. Two models have been proposed for the mechanism leading to neurodegenerative diseases by hyperphosphorylation: "toxicity" and "erroneous regulation" [25]. In the former model, hyperphosphorylation leads to formation and accumulation of toxic tau fibers and these fibers cause cell death by virtue of their cytotoxicity [26,27]. In the latter model, hyperphosphorylation converts normal tau to an inhibitory molecule that sequesters normal MT-associated proteins from MTs and, in turn, leads to cell death [25,28]. The following enzymes have been identified as major tau protein kinases: cAMP-dependent protein kinase (EC 2.7.11.11), Ca^{2+}/calmodulin-dependent protein kinase II (EC 2.7.11.17), cyclin-dependent protein kinase 5 (Cdk5) (EC 2.7.11.22), stress-activated protein kinases (EC 2.7.11.24) and glycogen-synthase kinase-3β (EC 2.7.11.26).

The retina, a part of the visual system, contains both tau [29,30] and protein kinases responsible for brain tau hyperphosphorylation [31,32]. Recent studies suggest that abnormal tau may play a role in some retinal degenerative diseases [33–41]. A common viewpoint in these studies is that these retinal diseases may share, at least in part, the mechanism of tau-related brain diseases and that information about these brain diseases may be applied to explain these retinal diseases. This view may use to collectively analyze these retinal diseases. However, the basis of this view may not be strong, *i.e.*, to our understanding, tau in retina (or in individual retinal cells) has not been thoroughly characterized, and the characteristic of retinal tau has not been fully compared with that of brain tau. In addition, the information about retinal tau is based on the tau derived from the whole retina that is composed of various cells, and thus some characteristics may be blurred.

This study collectively examines the common view of these previous studies by characterizing tau in bovine retinal photoreceptor rod outer segments (ROS), *i.e.*, in a single type of retinal cells, and by comparing its characteristics with that of bovine brain tau. We ask three basic questions in the characterization: (1) whether ROS contains tau isoforms identical to those in brain, (2) whether ROS tau shows the phosphorylation pattern similar to that of brain tau, and (3) whether ROS tau interacts with MTs in a manner similar to that of the association of brain tau with MTs. We show that ROS tau is not necessarily similar to brain tau and that the difference from brain tau as well as the similarity with brain tau should be considered in the investigation of tau-related degenerative diseases, if any, in retina.

2. Materials and methods

2.1. Materials

Dark-adapted frozen bovine retinas were obtained from J. A. Lawson Co. (Lincoln NE). Fresh retinas were isolated from dark-adapted calf eyes obtained from a local slaughterhouse. Bovine brain was obtained from a local slaughterhouse. Alkaline phosphatase (AP) (EC 3.1.3.1) from *Escherichia coli* was purchased from Sigma (St. Louis, MO). Immobilon-PSQ transfer membrane was from Millipore (Billerica, MA). Ultra Super Signal chemiluminescent substrate and peroxidase-labeled anti-rabbit IgG and anti-mouse IgG were from Pierce (Rockford, IL). Ampholyte (3–10, 40%), Ampholyte (4–6, 40%), Ampholyte (5–8, 40%), Ampholyte (8–10, 20%) and IPG-strips were obtained from Bio-Rad (Hercules, CA). All other chemicals were purchased from Sigma unless otherwise indicated.

2.2. Antibodies

Rabbit anti-human tau antibody (A0024, 1:5000), obtained from Dako (Carpinteria, CA), was regularly used. Phosphorylation site-specific antibodies, PS^{262} (44750G), PS^{396} (44752G) and PS^{404} (44758G), and their blocking peptides were from Biosource (Camarillo, CA). Anti-α-tubulin monoclonal antibody (T5168, 1:5000) and anti-β-tubulin monoclonal antibody (T5293, 1:200) were from Sigma.

2.3. Preparation of bovine brain and ROS samples

This study used bovine preparations unless otherwise indicated. The reasons for the use of bovine preparations are described in Discussion section. Protein concentration was assayed with bovine serum albumin (BSA) as a standard [42]. Bovine brain (5 g protein) was suspended in 5 volumes of a 1.5 times-concentrated buffer A (10 mM HEPES, pH 8.2, 1 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 5 μM leupeptin, 5 μM pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 9.5 TIU/l aprotinin and 1 μM E-64) and homogenized by using a 18 G needle (×7) and then a 23 G needle (×7). The mixture was divided into 200 μl aliquots per tube and kept at −80 °C. Bovine ROS was obtained from dark-adapted frozen retinas [43] or fresh retinas from dark-adapted calf eyes [44]. After homogenizing in 400 μl of buffer A, ROS (1.0 mg protein) was centrifuged (350,000 ×*g*, 20 min, 4 °C) to obtain membrane and soluble fractions. The soluble fraction was again centrifuged to remove contamination by membranes. The precipitation was suspended in 400 μl of buffer A and used as the membrane fraction.

2.4. Identification of tau isoforms

Bovine tau isoforms are generated from a single gene, and their molecular weights (MWs) are distinctive. Therefore, ROS tau proteins having identical MWs to those of brain tau isoforms are regarded as ROS tau isoforms containing the same exon combination as those in brain tau. Here SDS-PAGE was used to obtain these MWs, *i.e.*, their apparent MWs were compared. The SDS-PAGE system we used has been proven to be excellent for protein isolation [45].

Brain (1.0 mg protein) and ROS (1.0 mg protein) were separately suspended in 400 μl of buffer A. Each preparation was divided into two tubes (150 μl), and to each tube, 10 μl of an AP solution (32 unit/ml AP, 0.32% NaN_3 and 1.6 mM $ZnCl_2$) or 10 μl of a phosphatase-inhibitor solution (160 mM NaF, 1.6 μM okadaic acid, 0.32% NaN_3 and 1.6 mM $ZnCl_2$) was added, and these mixtures were incubated for 18 h at 33 °C. After adjusting the volume of ROS and brain mixtures by adding buffer A to 300 μl and 1200 μl, respectively, 10, 20, 30 and 40 μl of these samples were applied to a 8–16% acrylamide-gradient gel (12 × 17 cm). Proteins were then blotted to a nylon membrane and tau was detected with a rabbit anti-human tau antibody. These proteins are named tau-antibody-sensitive proteins hereafter.

Antibody signal intensities were used for the estimation of tau concentration in ROS, because this allows the concentration to be measured rapidly and simultaneously when multiple tau isoforms are present. However, a linear relationship between the signal intensity and the protein level is difficult to establish. To reduce this ambiguity, four samples containing different protein amounts were applied to a gel and their signal intensities were compared. All