

Proteasome inhibition and Tau proteolysis: an unexpected regulation

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Abstract Increasing evidence suggests that an inhibition of the proteasome, as demonstrated in Parkinson's disease, might be involved in Alzheimer's disease. In this disease and other Tauopathies, Tau proteins are hyperphosphorylated and aggregated within degenerating neurons. In this state, Tau is also ubiquitinated, suggesting that the proteasome might be involved in Tau proteolysis. Thus, to investigate if proteasome inhibition leads to accumulation, hyperphosphorylation and aggregation of Tau, we used neuroblastoma cells overexpressing Tau proteins. Surprisingly, we showed that the inhibition of the proteasome led to a bidirectional degradation of Tau. Following this result, the cellular mechanisms that may degrade Tau were investigated. © 2004 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

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1. Introduction

A large number of neurodegenerative disorders are characterized by intracellular protein aggregates. In Tauopathies, such deposits within neurons are composed of hyperphosphorylated Tau proteins. The knowledge for this abnormal fibrillogenesis of Tau is not completely understood despite that much evidence suggests an hyperphosphorylation of these proteins before their pathological aggregation [1]. In neurons, accumulation of Tau proteins in the cytoplasm may also induce their hyperphosphorylation and aggregation [2,3]. Tau proteins are substrates for many intracellular proteases, including caspases, calpain 1 and the proteasome. Inhibition of one of these proteolytic systems could lead to the intraneuronal accumulation of Tau proteins.

Tau proteins were shown to be sensitive to calpain degradation [4]. This protease, together with its inhibitor, calpastatin, makes up a complex that is thought to be involved in the synaptic impairment of Alzheimer's disease (AD) [5–7]. In apoptotic cells, it was also shown that the degradation of Tau is related to caspase activation [8–10]. The cleavage generates Tau fragments turning Tau itself into an effector

of apoptosis [10,11]. This Tau proteolysis may precede filamentous deposits of Tau within degenerating neurons, which then may participate in the progression of neuronal cell death in AD.

These data indicate that different proteolytic systems could be involved in Tau proteolysis and presumably in Alzheimer's pathology. However, the least documented is the proteasome system. Basically, in Alzheimer's or Parkinson's disease, it was effectively shown that proteasome activity was inhibited [12–14]. In Tauopathies, Tau proteins are hyperphosphorylated and ubiquitinated [1], suggesting that proteasome activity may be implicated in Tau proteolysis within neurons under normal and physiological conditions. Furthermore, in vitro experiments demonstrated the proteasomal-dependent degradation of Tau [15]. It was recently shown that an inhibition of proteasome activity may contribute to the stabilization of aggregates made by hyperphosphorylated Tau in oligodendroglial cells [16]. Despite these observations, the real impact of proteasome inhibition on Tau protein catabolism in neuronal cells remains to be elucidated.

Thus, to test the relationship between Tau proteolysis and proteasome activity in neurons, proteasomal activity was selectively inhibited in neuroblastoma cell lines overexpressing Tau proteins. Surprisingly, we showed that in contrast to in vitro experiments, the inhibition of the proteasome led to Tau proteolysis within cells. Following these results, we then investigated which proteolytic processes might be involved in this phenomenon.

2. Materials and methods

2.1. Antibodies

Phosphorylation-dependent Tau monoclonal antibodies include AD2 directed against phosphorylated Ser396–404 [17], AT270 recognizing phosphorylated Thr 181 [18] and Tau-1 that binds amino-acids 189–207 only when they are dephosphorylated [19]. Abnormal phosphorylation of Tau was assessed with AT100 and AP422 (recognizing phosphorylated T212/S214 and S422, respectively). M19G and Tau-Cter are well characterized antisera (PARP) directed against the first 19 amino acids of the Tau sequence [17] and the carboxy-terminal fragment of Tau (last 15 amino acids), respectively [20]. They both recognize their epitope independently of the Tau phosphorylation state (Fig. 1A).

β -Catenin quantities were determined using the anti- β -catenin H102. Calpain and its inhibitor protein Calpastatin were detected using the anti-calpain1 N19 and anti-calpastatin C19 antibodies (Santa Cruz Biotechnology). Poly-(ADP-ribose)-polymerase (PARP) cleavage was visualized using an Anti-Poly-(ADP-Ribose)-Polymerase antibody (Roche Molecular Biochemical, Mannheim, Germany) and reflected caspase activity. When caspases are activated, the 113 kDa PARP holoprotein is cleaved into 89 and 24 kDa fragments, which could

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Abbreviations: AD, Alzheimer's disease; GSK3 β , glycogen synthase kinase 3 beta; PARP, poly-(ADP-ribose)-polymerase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

serve as an early marker of apoptosis. Finally, the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected using the anti-GAPDH FL-335 (Santa Cruz Biotechnology).

2.2. Cell culture

Stably transfected SY5Y neuroblastoma cells were generated as described earlier [21,22]. Cells were grown in 25 cm² flasks in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 1 mM non-essential amino-acids and penicillin/

streptomycin (Invitrogen SARL, Cergy Pontoise, France) supplemented with G418 (Invitrogen SARL) in a 5% CO₂ humidified incubator at 37 °C.

2.3. Cell treatments and lysates

Protein translation was inhibited by cycloheximide (50 µg/ml) (Sigma–Aldrich SARL, L'isle d'Abeau Chesne, France), as described in [15]. LiCl (10 mM) was used to inhibit glycogen synthase kinase 3 beta (GSK3β) [23]. Lactacystin (10 µM), Proteasome inhibitor (PSI) (10 and 100 µM) and MG132 (40 nM, 200 nM, 3 µM and 10 µM) were used to inhibit ubiquitin-independent (20S) or dependent (26S) proteasome activities, respectively (Calbiochem, Merck Eurolab, Fontenay-sous-bois, France). ALLN (200 nM) (Calbiochem) and Caspase inhibitor Z-VAD-FMK (50 µM) (Promega, USA) were used for inhibiting calpain and caspase activities, respectively. All treatments were done three times in independent experiments.

After each treatment, samples were prepared for biochemical analyses as previously described [22]. Protein concentrations were estimated by the BCA protein assay kit (Pierce) and were adjusted at 1 µg/µl. Proteins were then separated on 10% or 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred and visualized as described previously [3,22].

3. Results

3.1. Tau protein proteolysis after proteasome inhibition

A neuroblastoma cell line SY5Y overexpressing 3R Tau was used to investigate the implication of the proteasome on Tau proteolysis. The proteasome was inhibited by different drugs, including PSI, MG132 (Fig. 1B) and lactacystin (data not shown). β-Catenin was used as a control of proteasome inhibition. Indeed, this protein was shown to be degraded specifically by the proteasome after its prephosphorylation by GSK3β [24,25]. After proteasome inhibition, β-catenin amounts were increased (Fig. 1B) suggesting that under our conditions, the proteasome was efficiently inactivated. This result was also confirmed by the use of cycloheximide (CHX), a translation inhibitor. As expected, by application of CHX, a 50% loss of β-catenin immunoreactivity was observed after 16 h (Fig. 1B). This loss was prevented if CHX was used in the presence of lithium (GSK3β inhibitor), suggesting that in our model GSK3β targets the β-catenin to the proteasome, as described

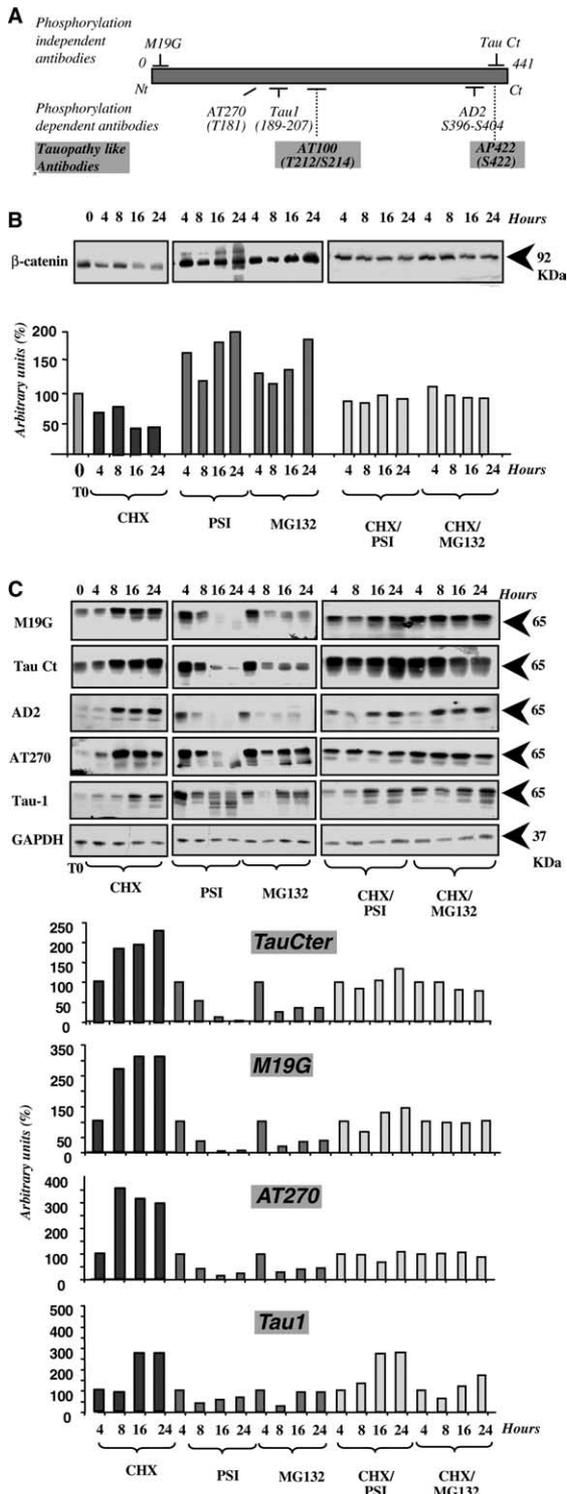


Fig. 1. (A) Schematic representation of anti-Tau antibodies used in the study. M19G and Tau-Cter recognize Tau proteins independently of their phosphorylation state (N and C terminal epitopes, respectively). Three different antibodies were also used to analyze the normal phosphorylation state of Tau (AT270, Tau-1 and AD2). Finally, the abnormal phosphorylation of Tau was assessed with AT100 and AP422 antibodies [22]. (B) Effect of proteasome and/or translation inhibition on β-catenin levels. The translation was inhibited by cycloheximide (CHX, 50 µg/ml). PSI and MG132 (10 µM and 200 nM) were used to inhibit proteasome activity. These inhibitors were used alone or in combination during a time course experiment (4, 8, 16 and 24 h). Note that similar amounts of total proteins were loaded after each treatment. Immunoreactivities obtained after Western Blotting were measured and reported on the relative percentage of β-catenin observed in untreated cells (To). (C) Tau protein degradation and phosphorylation analysis after translation and/or proteasome inhibition. Tau protein degradation and phosphorylation were analyzed for 4, 8, 16 and 24 h after translation (CHX, 50 µg/ml) and/or proteasome (PSI, 10 µM or MG132, 200 nM) inhibition. Immunoreactivities for TauCter, M19G, AT270 and Tau-1, obtained after Western blotting, were quantified and reported on the relative percentage observed in untreated cells (To). It is of note that the same amount of total protein was loaded for each experiment as demonstrated by the Western blot showing GAPDH.

earlier (data not shown). The combination of both treatments (proteasome inhibitors/CHX) showed no modification in cellular quantities of β -catenin, indicating that this protein is mainly proteolyzed through the proteasome pathway.

Conversely to β -catenin, 8 h after the addition of the proteasome inhibitors, Tau proteolysis was observed (Fig. 1C, PSI and MG132). This catabolism of Tau was bidirectional since the loss of Tau-immunoreactivities, obtained with M19G and Tau Ct antibodies, was similar (Fig. 1C, histograms). Under CHX conditions, Tau protein amounts appeared to be increased (Fig. 1C). Tau seems then to be more stable than other intracellular proteins such as β -catenin. When we combined translation and proteasome inhibitors, we also showed that Tau proteolysis was prevented. This result suggested that newly synthesized proteins such as proteases might be implicated in proteolysis of Tau after proteasome inhibition.

As a control, the same set of experiments was performed in native neuroblastoma cells. Treatment of these cells with MG132 (40, 200 nM, 3 and 10 μ M) for 24 h showed similar results to those obtained with the neuroblastoma cell line overexpressing Tau proteins, i.e., a loss in Tau immunoreactivity (data not shown). All these results suggested that the loss of Tau proteins is subsequent to proteasome inhibition in neuroblastoma cell lines.

3.2. Tau protein phosphorylation and proteolysis

It was recently shown that many proteins could be targeted towards the proteasome only if they were previously hyperphosphorylated at many sites [24–26]. Moreover, in Tauopathies, Tau proteins are hyperphosphorylated and aggregated within neurons. In our system, overexpression of Tau proteins leads to their hyperphosphorylation [3,22]. Thus, to investigate if the phosphorylation of Tau is implicated in its degradation, the phosphorylation state of Tau was studied using five phosphorylation-dependent antibodies (Fig. 1A) after proteasome and/or translation inhibition. As detected by biochemical experiments, Tau phosphorylation was not affected after proteasome or translation inhibition. Indeed, similar immunoreactivity profiles were obtained with phosphorylation dependent antibodies (AD2, AT270 and Tau-1) as compared to M19G or Tau-Cter (Fig. 1C, histograms). Moreover, no abnormal phosphorylation of Tau proteins was detected after each treatment (data not shown). These data indicated that during all these treatments, no changes in Tau phosphorylation occurred. It is of note that the use of phosphorylation-dependent antibodies reinforced the idea that during proteasomal inhibition, Tau proteins were degraded from their C-terminal and N-terminal extremities, since the same proteolysis profiles were obtained with all Tau dependent antibodies (Fig. 1C, histograms).

3.3. Tau protease induction after proteasome inhibition

The previous experiments showed that after proteasome inhibition, Tau proteins were degraded only if translation was not prevented. This suggested that a protein synthesis or an indirect activation of proteases might be required for Tau proteolysis after proteasome inhibition. To test this hypothesis, we investigated caspase activity by following PARP cleavage, the intracellular levels of calpain I, and its inhibitor calpastatin. Regarding PARP cleavage, caspase activity was more important after CHX treatments (alone or in combina-

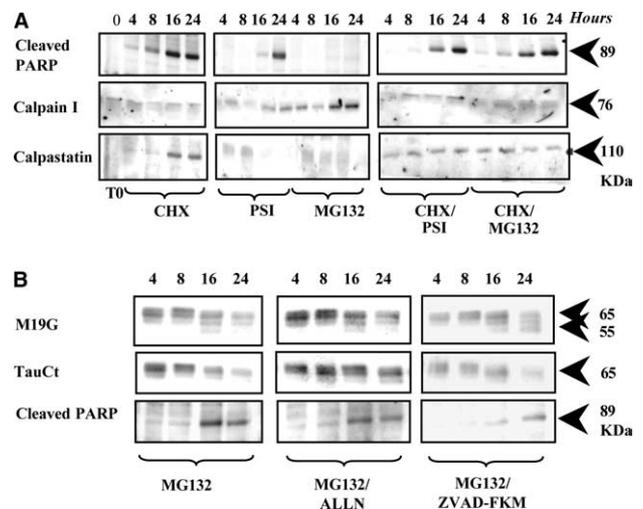


Fig. 2. (A) Tau protease expression after the inhibition of the proteasome. After inhibition of the proteasome and/or the translation for 24 h (same experimental procedures as described for Fig. 1), the amounts of Tau proteases or activity were determined by immunoblotting. Caspase activity was estimated by following PARP cleavage (89 kDa). Calpain I and calpastatin (calpain intracellular inhibitor) amounts were detected directly. Each immunoreactivity was compared to those obtained for untreated cells (T0). (B) Tau protease inhibition assessment during proteasome inactivation. Calpain was inactivated by ALLN, caspases by the caspase inhibitor Z-VAD-FMK and lysosomal activity by NH₄Cl or Chloroquine (data not shown). Caspase activity during each treatment was estimated using an Anti-PARP antibody. Tau protein proteolysis was measured for 24 h by M19G and Tau Cter polyclonal antibodies.

tion with proteasome inhibitors) than when only the proteasome was inhibited (Fig. 2A, PARP). This suggested that caspases were activated after CHX treatment even if no proteolysis of Tau was evident. Conversely, proteasome inhibition led to calpain I accumulation and calpastatin degradation in cells after 8 h (Fig. 2A, panels PSI and MG132). It is of note that Tau protein proteolysis is concomitant with the induction of calpain and the loss of calpastatin (compare Fig. 1C and 2A). Under CHX conditions, a relative increase in calpastatin immunoreactivity was observed, indicating that under these conditions calpastatin was relatively stable. These data indicated that the proteasome may be implicated directly or indirectly in the degradation of calpastatin in cells, thereby regulating the calpain I activity.

Finally, in order to investigate the impact of these proteolytic systems on Tau catabolism when the proteasome was inactivated, specific inhibitors (ALLN (200 nM) for calpain and caspase inhibitor Z-VAD-FMK (50 μ M)) were used. Biochemical analyses showed that only a co-treatment of proteasome inhibitors with ALLN reduced the loss of Tau proteins (Fig. 2B). In contrast, when caspase activity was inactivated during the same time as the proteasome, no change in Tau degradation was seen even if a delay in PARP cleavage was observed.

Taken together, these results suggested that first caspases may not be involved in Tau proteolysis after inhibition of the proteasome. Second, proteasome inhibition led to calpain I stability or synthesis. Third, proteasome inhibition led also to calpastatin proteolysis. This degradation seemed to be indirect and dependent on newly synthesized protein, since calpastatin was not degraded in the presence of CHX.

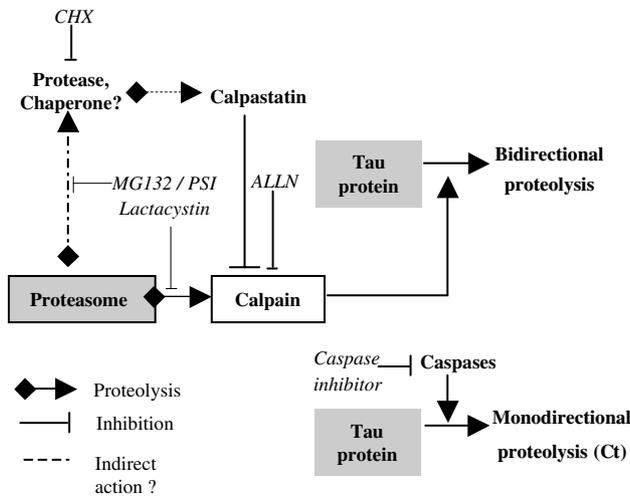


Fig. 3. Schematic representation of Tau proteolysis after proteasome inhibition or induced apoptosis. Proteasome inhibition may lead to calpain activation by decreasing calpastatin levels. This effect is indirect, since the use of cycloheximide inhibits calpastatin degradation. Therefore, some other proteases and/or chaperone proteins may be necessary to inactivate or degrade calpastatin protein. Once calpain is activated, Tau proteins are degraded by a bidirectional process. Under apoptotic conditions, caspases are activated and cleaved Tau by a monodirectional process that begins at their C-terminal extremity [3].

4. Discussion

We have analyzed the implication of proteasome activity in Tau proteolysis in neuroblastoma cell lines and demonstrated that after proteasome inhibition, Tau proteins are catabolized by a bidirectional process, which is protein-translation-dependent. These data also argued that this Tau degradation is independent of its normal and physiological phosphorylation state.

How can we explain the results observed in our cell system, since the proteasome seems to be implicated directly in Tau degradation *in vitro* [15]? In fact, the proteasome is a general degradation system that could act on proteins directly implicated in Tau proteolysis or phosphorylation. Concerning Tau proteases, the proteasome can work on their expression or activity in function of cell type and cell metabolism. Depending on the cell system, the proteasome may lead to an overexpression of caspases or calpain and could therefore act indirectly on Tau protein catabolism. In our system, calpain accumulated after proteasome inhibition while its cellular inhibitor calpastatin was degraded. Thus, if the proteasome is not functional under these conditions, calpain is activated and therefore Tau proteins can be proteolyzed by a bidirectional process (Fig. 3). This effect is not direct and requires presumably protein synthesis, since the Tau proteolysis was not induced if translation was inhibited.

In this study, we also demonstrated that proteasome inhibition did not lead to apoptosis and that caspases seemed unlikely to be involved in Tau degradation under this condition. Indeed, in this cell system, caspase activation leads to a monodirectional (carboxy-terminal) degradation of Tau and not bidirectional as observed after MG132 or PSI treatments [3]. Moreover, one of the most characteristic sign of neuronal apoptosis is the formation of a dephosphorylated Tau fragment of 17 kDa that can be visualized with Tau-1 antibody

[8]. As this fragment never appeared after MG132 or PSI treatments and as a delayed PARP cleavage was seen, it seems therefore that Tau proteolysis is not associated with caspase activity after proteasome inhibition. Finally, it is also of note that Tau protein might also be a substrate for lysosomal proteins such as Cathepsin D [27]. However, it seems that this pathway is not involved in Tau proteolysis after proteasome inhibition, since a co-treatment with MG132 and NH4Cl or Chloroquine (two lysosomal activity inhibitors) failed to reduce the degradation of Tau (data not shown).

5. Conclusion

We demonstrated that proteasome inhibition, in neuroblastoma cell lines, leads to the degradation of Tau proteins by a bidirectional process that involves presumably the calpain/calpastatin system. This study also suggests that under physiological conditions where Tau proteins are normally phosphorylated, the proteasome inhibition does not lead to Tau protein hyperphosphorylation or aggregation but to its degradation. Finally, we demonstrated for the first time that proteasome inhibition could be involved in calpain activation within neurons by altering calpastatin levels. This inhibition could be implicated in neurodegenerative process progression in Tauopathies where calpain was shown to be overactivated. Therefore, calpain activation in AD might not only be dependent on the excitotoxicity or amyloid-beta exposure. This could be an alternative mechanism by which therapeutic approaches could be generated.

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