Modelling Alzheimer-specific abnormal Tau phosphorylation independently of GSK3β and PKA kinase activities

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Abstract In Alzheimer's disease, neurofibrillary degeneration results from the aggregation of abnormally phosphorylated Tau proteins into paired helical filaments. These Tau variants displayed specific epitopes that are immunoreactive with anti-phospho-Tau antibodies such as AT100. As shown in in vitro experiments, glycogen synthase kinase 3 β (GSK3β) and protein kinase A (PKA) may be key kinases in these phosphorylation events. In the present study, Tau was microinjected into *Xenopus* oocytes. Surprisingly, in this system, AT100 was generated without any GSK3β and PKA contribution during the progesterone or insulin-induced maturation process. Our results demonstrate that a non-modified physiological process in a cell model can generate the most specific Alzheimer epitope of Tau pathology.

Key words: Alzheimer's disease; Oocyte maturation; Abnormal phosphorylation; Tau protein

1. Introduction

Abnormal phosphorylation is the major posttranslational modification of Tau proteins aggregated into paired helical filaments (PHF) in Alzheimer's disease (AD). Despite the fact that many phosphorylation sites are common between PHF-Tau and normal Tau in control-biopsy material, there are now evidences of an abnormal phosphorylation in AD (for review see [1]). For instance, phosphorylated Tau aggregated into filaments but not normal Tau can be visualised by AT100 antibody that recognises a conformation-dependent epitope [2]. In vitro, AT100 epitope appears after a long and sequential phosphorylation by glycogen synthase kinase 3 β (GSK3β) and protein kinase A (PKA) in the presence of heparin [2]. In cell models, this phospho-epitope appears only after treatment with okadaic acid (OA) [3,4] suggesting that inhibition of PP2A and or PP1 is necessary for AT100 genesis. To investigate the physiological Tau phosphorylation in vivo, Tau proteins were microinjected into *Xenopus* oocytes as described previously [5].

In fact, *Xenopus* oocytes are arrested at prophase of the first division of meiosis (prophase I). In response to progesterone, oocytes undergo meiosis (also named maturation) [6,7], which can be followed by the appearance of a white spot (WS) at the animal pole. Many kinases are involved in the cytological events that characterise oocyte maturation; some of them have to be activated whereas others must be inhibited (Fig. 1A). First, it is well known that PKA activity is maintained at a high level in immature oocytes and drops following progesterone stimulation [8]. Similarly, it has been recently demonstrated that GSK3β activity follows a similar regulation [9]. Conversely, maturation requires protein kinase activation. For instance, when activated, MAPK and $p34^{Cdc2}$ are responsible for the morphological changes accompanying maturation such as germinal vesicle breakdown (GVBD), chromosome condensation and spindle formation [10,11]. So, *Xenopus* oocytes possess many of the kinases that could be involved in Tau phosphorylation that occurs in AD. Thus, we took advantage of this model to study the AT100 immunoreactivity. Using anti-Tau antibodies directed against phosphorylation-dependent or phosphorylation-independent epitopes, the state of phosphorylation of recombinant Tau proteins was investigated by immunoblotting after their injection into *Xenopus* oocytes. Our results showed that neither GSK3β nor PKA were necessary for AT100 appearance in this system. Therefore, *Xenopus* oocytes could be used as a model to identify the kinases that are involved in the pathological phosphorylation of Tau proteins that are found in AD and several neurodegenerative disorders.

2. Materials and methods

2.1. Experimental conditions

The 2+3–10+ isoform of human brain Tau (ht412) was expressed in *Escherichia coli* from cDNA clone htau46 [12] and purified as described [13]. Recombinant Tau proteins or buffer alone were microinjected in oocytes at a constant volume of 50 nl per stage VI oocyte by the use of a positive displacement digital micropipette (Nichiryo) as previously described [5].

Maturation was induced in ND96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES/NaOH, pH 7.5) by progesterone addition (10 μM) [14] or in K^+-free OR_2 medium [15] in the case of insulin stimulation (1 μM). The hormone was always added 1 h after the injection of ht412 or drug application to allow diffusion in the oocyte due to its sise (1.2 mm in diameter). Maturation was determined either by WS detection or by microscopic examination of dissected heat-treated oocytes (100°C, 3 min).

LiCl (10 or 20 mM) was used to inhibit GSK3β [16], and oocytes were maintained in this medium from 2 h before the microinjection of ht412 until the end of the experiment.

Inhibition of PKA was performed by injection of the PKA regulatory subunit (PKAr) (Promega, France) [17].

2.2. Antibodies

Phosphorylation-dependent monoclonal antibodies included AD2

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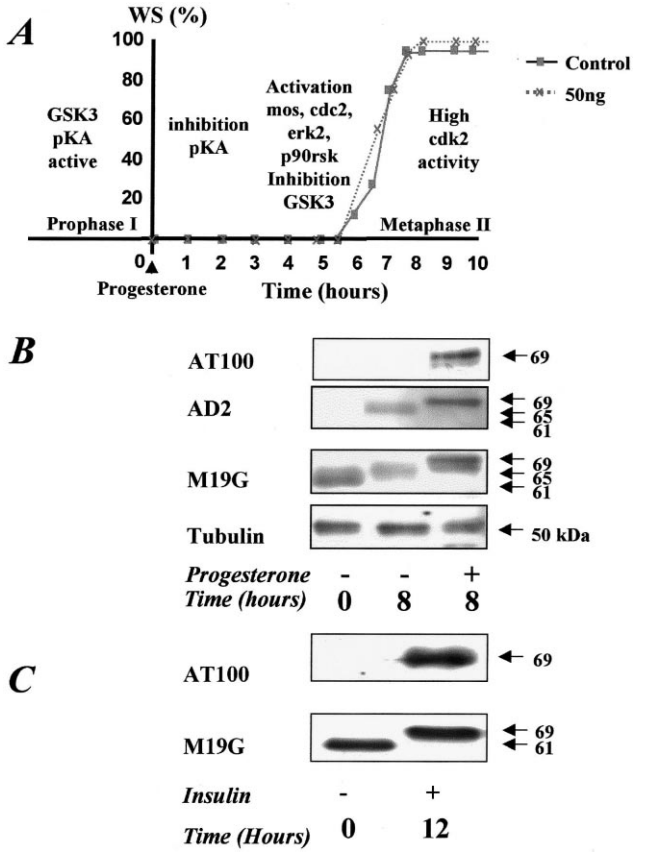

Fig. 1. htau412 phosphorylation during *Xenopus* oocyte maturation. A: Typical representation of progesterone-induced oocyte maturation. The maturation is visualised by the WS appearance with (×) (50 ng) and without (■) tau injection. GSK3β and PKA are both active in prophase I oocytes and inactivated after progesterone stimulation. In meiosis I, a number of kinases are activated including Cdc2 and MAPK. Other kinases may be activated during GVBD such as Cdk2. B,C: Immunoblotting analysis of tubulin and htau412 phosphorylation in prophase I (−) and metaphase II-arrested *Xenopus* oocytes (+). Phosphorylation states of Tau proteins were visualised by M19G, AD2 and AT100 antibodies during progesterone (B)-, or insulin (C)-induced maturation. Note that both Tau proteins and *Xenopus* tubulin are in the same soluble fraction and that insulin maturation is longer (12 h) than with progesterone (8 h). M_rs are indicated at the right side of the figure.

directed against phosphorylated Ser396–404 [18], and Tau-1 that binds amino acids 189–207 only when they are dephosphorylated [19]. AT100 was used to detect abnormal Tau phosphorylation [2]. Finally, M19G is a well-characterised anti-serum, directed against the first 19 amino acids of the tau sequence encoded by exon 1 [18] that recognises its epitope independently of the Tau phosphorylation state. Other antibodies used are a home-made polyclonal anti-tubulin and a polyclonal antibody against human β-catenin (H-102) (Santa Cruz Biotechnology).

2.3. Electrophoresis and Western blotting

Depending on the experiment, 5–20 oocytes were homogenised in homogenisation buffer (MOPS 25 mM pH 7.2 with protease and phosphatase inhibitors [20], 10 μl per oocyte) and centrifuged for 5 min at 13 000 × *g* (4°C) to eliminate yolk platelets. Proteins were then analysed by immunoblotting [5]. Densitometry analyses allowed for the quantification of β-catenin as described for Tau in [5].

2.4. Controls

Hyperphosphorylated ht412 was obtained following htau46 cDNA transfection in COS cells and cell treatment with OA as previously described [4]. This cell lysate and brain homogenate from an Alzheimer patient were used as a positive control for phosphorylation-dependent Tau antibodies.

3. Results

As previously described [5], oocyte injection of 50 ng htau412 did not interfere with maturation (Fig. 1A). This concentration was kept for all further experiments. An analysis of Tau solubility following microinjection revealed that these proteins were found in the 13 000 × *g* supernatant not only in homogenates from prophase I but also in progesterone- or insulin-treated ones (Fig. 1B,C). So, all subsequent analyses were performed in these fractions. Moreover, it should be noted that Tau proteins showed different states of phosphorylation during oocyte maturation. In prophase I-arrested oocytes, Tau proteins exhibited an apparent M_r at about 61 kDa and were detected with the polyclonal anti-Tau antibody (M19G) and Tau-1 monoclonal antibody but not with the phosphorylation-dependent antibodies AD2 or AT100. 8 h after Tau microinjection into oocyte and without progesterone or insulin-induced maturation, Tau displayed an intermediary M_r at 65 kDa as visualised by AD2 and M19G antibodies. However, there was no AT100-immunoreactivity (Fig. 1B). Conversely, in metaphase II oocytes, after progesterone- (8 h) or insulin-induced (12 h) maturation (Fig. 1B,C), htau412 migrated at 69 kDa and was recognised by both anti-

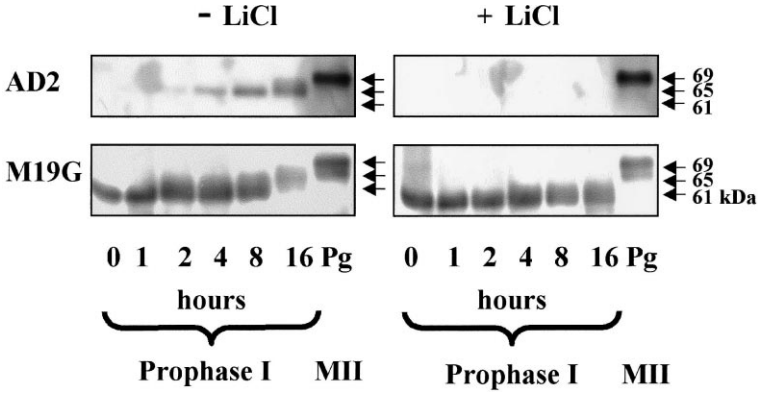

Fig. 2. Effect of the GSK3β inhibitor LiCl on htau412 phosphorylation. A time-course experiment was performed in prophase I oocytes after Tau injection in the presence (+) or absence of LiCl (−). Each experiment was compared to Tau protein in metaphase II extract. AD2 recognises a GSK3β epitope. M19G is a polyclonal anti-Tau antibody.

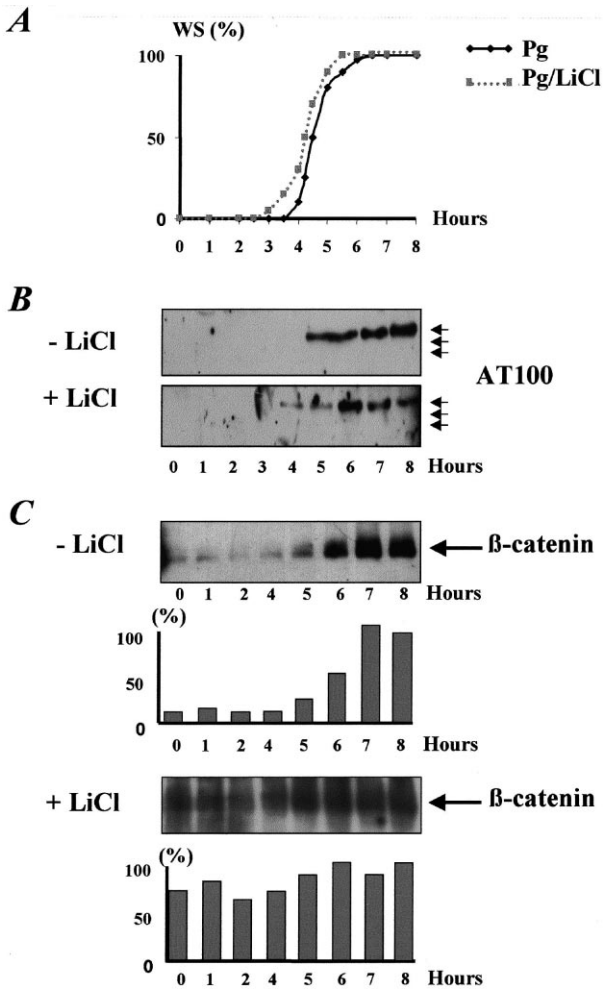

Fig. 3. Effect of the GSK3β inhibitor LiCl on AT100-immunoreactivity during progesterone-induced maturation. A: Effect of LiCl onto oocyte maturation. A time-course experiment of progesterone-induced oocyte maturation was performed after Tau injection in the presence (+) or not of LiCl (−). Maturation was assessed by WS appearance (%). B: Immunoblotting analysis of AT100 immunoreactivity. C: Immunoblotting analysis of β-catenin immunoreactivity and quantification. In B and C, a time-course comparison was performed between control oocytes (−LiCl) and oocytes subjected to LiCl.

bodies AD2 and AT100, indicating that Tau proteins showed an abnormal phosphorylation profile during maturation process. However, there was a complete loss of Tau-1 immunoreactivity (data not shown).

In the *Xenopus* oocyte model, GSK3β and PKA are both active in immature oocytes (prophase I) [8,9]. Moreover, both kinases must be inactivated to allow oocyte maturation. However, not only the 69 kDa shift of htau412 but also the AT100 staining was observed in metaphase II oocytes. Furthermore, insulin was shown to inactivate GSK3β by the pKB/AKT signalling pathway [21]. These results suggested that GSK3β and PKA were not involved in the phosphorylation of htau412 at the AT100 epitope in the *Xenopus* oocyte model. To ascertain that AT100 generation was not related to GSK3β phosphorylation, different conditions where this enzyme is active or inactive were analysed.

To follow Tau phosphorylation by GSK3β, the appearance of the typical phosphorylated AD2 epitope (similar to the PHF-1 epitope) [22] was monitored. Htau412 was firstly injected in prophase I oocyte in the absence of progesterone with or without LiCl, a common GSK3β inhibitor [16]. Appearance of AD2-immunoreactivity first occurred after 4 h and htau412 exhibited an apparent M_r of 65 kDa (Fig. 2, −LiCl). No AT100 immunoreactivity was observed in comparison to metaphase II extract (Fig. 1B). In the presence of 10 mM lithium, AD2 immunoreactivity was never observed (Fig. 2, +LiCl). Furthermore, no 65 kDa variant was visualised using the polyclonal M19G antibody, suggesting that under these conditions, no phosphorylation occurred that may induce conformational changes and higher M_r (Fig. 2, +LiCl). Altogether, these data indicated that *Xenopus* GSK3β is able to phosphorylate human htau412 at AD2 epitope (Fig. 2) but not to generate AT100 epitope.

During oocyte maturation, AT100-immunoreactivity was obtained 5 h after progesterone stimulation (Fig. 3B). Thus, to ascertain that GSK3β is not involved, oocyte maturation was induced in the presence or not of LiCl. In oocytes stimulated by progesterone in the presence of 10 mM LiCl, WS appeared 1 h earlier than in control oocytes (Fig. 3A). This result is in agreement with experiments where oocyte maturation was analysed after microinjection of neutralising anti-GSK3β antibodies [9]. Moreover, analysis of β-catenin showed an accumulation of the protein at GVBD time in control oocytes whereas the level of β-catenin was already

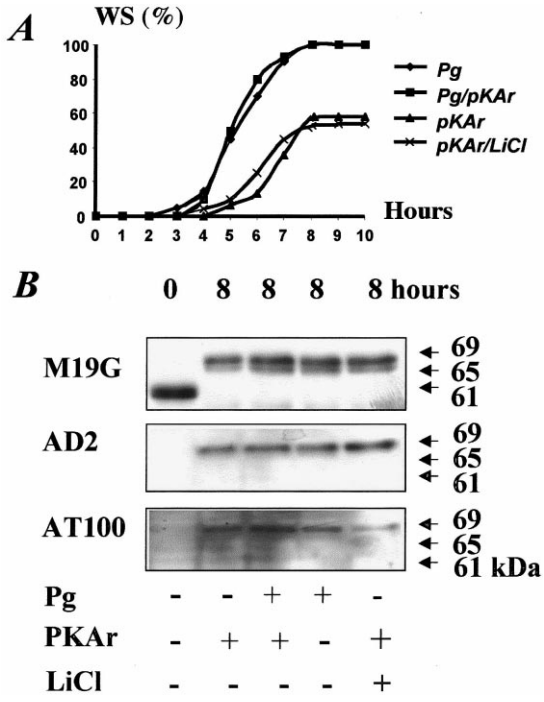

Fig. 4. Effect of PKAr on htau412 phosphorylation. A: Effect of PKAr onto oocyte maturation. Oocytes were injected with PKAr 1 h before htau412 microinjection with or without progesterone-containing medium. Tau proteins were injected into prophase I oocytes. Maturation was assessed by WS appearance (%). Note that PKAr injection did not allow for maturation of all oocytes [17] and that, in the presence of LiCl, PKAr-induced oocyte maturation occurred earlier. B: Htau412 phosphorylation was assessed with AD2 and AT100 antibodies, and compared to metaphase II (pg+/PKAr−) or to prophase I (pg−/PKAr−) oocytes. Killing of both PKA and GSK3β activities was also performed (pg−/PKAr+/LiCl). AT100-immunoreactivity was detected under all conditions of PKA inhibition.

higher after 1 h of hormonal stimulation in LiCl-treated oocytes (Fig. 3C). Both observations were in agreement with a GSK3β inhibition in LiCl-containing medium. Furthermore, when we analysed htau412 phosphorylation in such oocytes, we observed that the events usually observed following progesterone addition still occurred in the presence of LiCl. However, the shift in htau412 revealed with the M19G antibody as well as the AD2 and AT100 staining occurred 1 h earlier than in control oocytes (Fig. 3B, data not shown). So, although GSK3β was inhibited, the AT100 still appeared just after GVBD suggesting that other kinases may be involved in AT100 generation. It should be noted that similar results were obtained using 20 mM LiCl, but at this concentration, oocyte maturation after progesterone treatment occurred 90 min earlier than in control oocytes (data not shown).

Since it was shown that AT100 is generated in vitro after a sequential phosphorylation of GSK3β and PKA, AT100-immunoreactivity was also investigated under different conditions of PKA activation. In prophase I oocytes without progesterone treatment, PKA is active whereas it is inactive after hormonal stimulation. Oocyte maturation is linked to PKA inhibition [8,17]. Moreover, injection of PKAr led to oocyte maturation (Fig. 4A). Under any of the conditions where PKA was inhibited (progesterone stimulation and/or PKAr subunit injection), Tau is highly phosphorylated leading to the appearance of a high M_r variant at 69 kDa and AD2 and AT100 immunoreactivities (Fig. 4B). Finally, even when a killing of both PKA and GSK3β activities was realized at the same time (injection of PKAr in the presence of LiCl), AT100-immunoreactivity was still detected (Fig. 4B). These data indicated that PKA is not necessary for the AT100 epitope in *Xenopus* oocytes.

4. Discussion

Neuropathologically, Alzheimer's disease is characterised by amyloid deposition and neurofibrillary degeneration in which accumulate bundles of PHFs constituted mainly of abnormally phosphorylated Tau proteins. What could be the kinases responsible for the abnormal phosphorylation of Tau proteins and especially at site recognised by one of the only antibodies that specially recognises PHF-Tau, AT100 antibody?

AT100 binds to a conformation epitope including phosphorylated Thr212 and Ser214 [2]. Sequential phosphorylation of Tau protein by GSK3β and PKA has been reported to generate this epitope in vitro [2]. However, in the present study, AT100 was never detected in prophase I-arrested oocytes that contain high levels of PKA and GSK3β activities. In that case, htau412 shifted to an apparent M_r lower than those observed after progesterone stimulation. GSK3β seems to be responsible for this intermediate state recognised by AD2 since no shift and no staining was observed when prophase I-arrested oocytes were incubated in LiCl-containing medium. So, even if GSK3β was able to phosphorylate htau412, AT100 could not appear. Our suggestion that neither GSK3β nor PKA are involved in the genesis of AT100 epitope in our model is heightened by the fact that these kinases are inhibited when oocytes mature, i.e. when AT100 staining appears [8,9]. First, AT100 staining of htau412 was observed when oocyte maturation was induced by the injection of PKAr. This treatment inhibits endogenous PKA activity and is sufficient to trigger maturation without hormonal stimulation [8,17]. Moreover, using lithium, the common GSK3β inhibitor [16], AT100 still appeared when progesterone stimulation was performed. GSK3β inhibition was ascertained by a faster maturation in which β-catenin accumulation occurred earlier than in control oocytes. Furthermore, when oocyte maturation is obtained after insulin stimulation instead of progesterone, AT100-immunoreactivity was still observed. This treatment also leads to GSK3β inhibition since insulin acts through Akt activation [21]. Finally, co-transfection of cDNAs of *tau* and a constitutively active form of GSK3β [22] in COS cells did not allow for AT100 genesis (P.D. and L.B., unpublished data). Altogether, these data clearly indicated that GSK3β is not involved in AT100 epitope.

The present data disagreed with those previously reported from in vitro experiments [2]. The sequential phosphorylation of Tau protein was performed with human Tau 23 and the presence of polyanionic factors such as heparin, tRNA or poly-Glu was absolutely required to generate AT100 epitope [2]. Moreover, several hours were needed for the purified kinases to phosphorylate htau23 at the AT100 site.

Xenopus oocytes might be a more physiological model in which the kinases involved in the abnormal phosphorylation of Tau proteins could be characterised. Indeed, it is one of the rare cells in which the phosphorylation of Tau is very close to those occurring in AD. In fact, even if there is no endogenous Tau protein in this cell, the phosphorylation of injected Tau proteins as well as AT100 labelling occurred following stimulation with progesterone, which is the natural inducer of meiosis. So, *Xenopus* oocytes provide new opportunities to characterise signalling pathways (kinases or phosphatases) leading to abnormal phosphorylation of Tau proteins and so to neurodegeneration observed in AD.

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