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In AβPP-overexpressing cultured human muscle fibers proteasome inhibition enhances phosphorylation of AβPP751 and GSK3β activation: effects mitigated by lithium and apparently relevant to sporadic inclusion-body myositis

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Abstract
Muscle fiber degeneration in sporadic inclusion-body myositis (s-IBM) is characterized by accumulation of multiprotein aggregates, including aggregated amyloid-β (Aβ)-precursor protein 751 (AβPP751), Aβ, phosphorylated tau, and other 'Alzheimer-characteristic' proteins. Proteasome inhibition is an important component of the s-IBM pathogenesis. In brains of Alzheimer's disease (AD) patients and AD transgenic-mouse models, phosphorylation of neuronal AβPP695 (p-AβPP) on Thr668 (equivalent to T724 of AβPP751) is considered detrimental because it increases generation of cytotoxic Aβ and induces tau phosphorylation. Activated glycogen synthase kinase3β (GSK3β) is involved in phosphorylation of both AβPP and tau. Lithium, an inhibitor of GSK3β, was reported to reduce levels of both the total AβPP and p-AβPP in AD animal models. In relation to s-IBM, we now show for the first time that (1) In AβPP-overexpressing cultured human muscle fibers (human muscle culture IBM model: (a) proteasome inhibition significantly increases GSK3β activity and AβPP phosphorylation, (b) treatment with lithium decreases (i) phosphorylated-AβPP, (ii) total amount of AβPP, (iii) Aβ oligomers, and (iv) GSK3β activity; and (c) lithium improves proteasome function. (2) In biopsied s-IBM muscle fibers, GSK3β is significantly activated and AβPP is phosphorylated on Thr724. Accordingly, treatment with lithium, or other GSK3β inhibitors, might benefit s-IBM patients.
Keywords: cultured human muscle fibers, glycogen synthase kinase 3β, inclusion-body-myositis, lithium chloride, phosphorylated amyloid-β precursor protein, proteasome.
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Sporadic-inclusion-body myositis (s-IBM) is the most common progressive muscle disease associated with aging. Its progressive course gradually leads to pronounced muscle weakness and wasting, resulting in severe disability (Askanas and Engel 2008). The exact pathogenesis of s-IBM is not known, and there is no enduring treatment.

An intriguing aspect of s-IBM is that its muscle-fiber phenotype shares several molecular abnormalities with Alzheimer's disease (AD) brain, including accumulation of amyloid-β (Aβ) precursor protein (AβPP) and of its cytotoxic Aβ42 fragment (recently reviewed in Askanas and Engel 2008; Askanas *et al.* 2009). Both s-IBM and AD affect aging individuals, but they are clinically distinct.

Hallmarks of s-IBM muscle fibers are (i) vacuolated muscle fibers having intra-myofiber accumulations – mainly in non-vacuolated cytoplasm – of congophilic, ubiquitinated, multiprotein aggregates that contain, in addition to Aβ, phosphorylated tau in the form of paired helical filaments, and several other 'AD characteristic' proteins and (ii) mononuclear cell inflammation (Askanas and Engel 2001, 2008; Dalakas 2008). Currently, there is no consensus regarding whether the degenerative or inflammatory component plays a more significant role in

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[Correction added after online publication (19-11-09): Occurrences of over expressing and over expression in the text changed to over-expressing and overexpression]

Abbreviations used: Aβ, amyloid-β; AβPP, amyloid-β precursor protein; AD, Alzheimer's disease; AMC, aminomethylcoumarin; CHMFs, cultured human muscle fibers; ER, endoplasmic reticulum; GSK3β, glycogen synthase kinase 3β [EC 2.7.11.36]; s-IBM, sporadic inclusion-body myositis.

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s-IBM pathogenesis. Against a primary role of inflammation is the wide recognition that all types of immunosuppressive treatment fail to reverse, or enduringly halt, disease progression of s-IBM (in contrast to polymyositis). And, there is increasing agreement that Aβ42 is an important pathogenic factor leading to muscle-fiber destruction (Askanas and Engel 2007, 2008; Dalakas 2008). This proposed mechanism has been supported by models involving cultured human muscle fibers (CHMFs) and transgenic mice. For example, long-term overexpression of wild-type full-length 751AβPP in cultured normal human muscle fibers produced in them several aspects of the s-IBM cellular phenotype, including vacuolization, congophilic amyloid inclusions, cytoplasmic 6–10 nm amyloid-like filaments, nuclear paired helical filaments, mitochondrial cytochrome oxidase deficiency, and mitochondrial morphological abnormalities (Askanas *et al.* 1996, 1997). Transgenic-mouse models based on overexpressing AβPP or its fragment in skeletal muscle produced some aspects of the IBM pathology (Fukuchi *et al.* 1998; Jin *et al.* 1998; Kitazawa *et al.* 2006). In s-IBM muscle fibers, there is increased production (Sarkozi *et al.* 1993; Guerin *et al.* 2008) and accumulation of aggregated AβPP751 (Askanas *et al.* 1993), and preferential accumulation of the cytotoxic Aβ42 form (Vattemi *et al.* 2009). Accordingly, we postulate that methods reducing muscle-fiber Aβ accumulation may benefit s-IBM patients.

Furthermore, probably pathogenic oxidative and endoplasmic reticulum (ER) stresses, and proteasome inhibition, also have been demonstrated in s-IBM muscle fibers (Vattemi *et al.* 2004; Fratta *et al.* 2005; Nogalska *et al.* 2006; Terracciano *et al.* 2008).

In AD brain, increased phosphorylation of neuronal AβPP695 on Thr668 has been demonstrated (Lee *et al.* 2003; Shin *et al.* 2007), and in brains of AD mouse models, it was considered to be detrimental by increasing generation of Aβ and inducing tau phosphorylation (Shin *et al.* 2007). Active glycogen synthase kinase 3β (GSK3β) was proposed to have an important role in AD pathogenesis because it was shown to modulate phosphorylation of both tau (Anderton *et al.* 2001) and of AβPP on Thr668 (Aplin *et al.* 1996).

Lithium is a reversible inhibitor of GSK3β (Klein and Melton 1996; Jope 2003; Avila and Hernández 2007). It presumably acts either (i) directly, competing with magnesium in forming a complex with ATP or (ii) indirectly, by increasing phosphorylation of GSK on Ser9, which leads to GSK3β inactivation (Jope 2003). In AD and IBM mouse models, inhibition of GSK3β by LiCl decreased phosphorylated AβPP and phosphorylated tau (Rockenstein *et al.* 2007; Kitazawa *et al.* 2008), respectively.

To explore molecular pathogenic mechanisms in s-IBM, we experimentally modified the cellular micro-environment of CHMFs to mimic various aspects of the s-IBM pathogenesis, thereby producing experimental IBM-CHMFs models. These models have proved very useful in our previous studies (Askanas *et al.* 1996, 1997; Fratta *et al.* 2005; Nogalska *et al.* 2006, 2008; Wojcik *et al.* 2006).

In this study, we asked whether (1) in s-IBM muscle fibers, AβPP is phosphorylated and GSK3β is activated and (2) in the established s-IBM model involving overexpression of AβPP in CHMFs (a) imposed proteasome inhibition or experimental induction of ER-stress influences AβPP phosphorylation and GSK3β activity and (b) treatment with lithium decreases (i) GSK3β activity, (ii) total and phosphorylated AβPP, and (iii) Aβ oligomers.

Experimental procedures

Muscle biopsies

Studies were performed on fresh-frozen diagnostic muscle biopsies obtained (with informed consent) from 12 s-IBM, 2 polymyositis, 1 morphologically non-specific myopathy, 2 amyotrophic lateral sclerosis, 2 peripheral neuropathy, and 12 normal controls age-matched to the s-IBM patients (normal muscle biopsies were of patients who, after all tests were performed, were considered free of muscle disease). s-IBM patients were ages 55–79 years, median age 67; normal control patients were ages 53–84, median age 71. Diagnoses were based on clinical and laboratory investigations, including our routinely performed 16-reaction diagnostic histochemistry of muscle biopsies.

All s-IBM biopsies met s-IBM diagnostic criteria, as described (Askanas and Engel 2001). Not all studies were performed on all biopsies (details below).

Light-microscopic immunocytochemistry

Immunofluorescence was performed as described (Askanas *et al.* 1993; Vattemi *et al.* 2004; Fratta *et al.* 2005; Nogalska *et al.* 2006) on 10-μm thick transverse sections of five s-IBM, two age-matched normal, and seven disease-control muscle biopsies (specified above), using a well-characterized polyclonal antibody specifically recognizing the AβPP751 isoform only when phosphorylated on Thr724 (p-AβPPT724), equivalent to Thr668 of the AβPP695 isoform (Cell Signaling, Danvers, MA, USA), diluted 1 : 50. This antibody was produced by immunizing rabbits with a synthetic phosphopeptide corresponding to the residues surrounding Thr688 of human AβPP695 (corresponding to 724 position on isoform 751) (Ando *et al.* 1999; Muresan and Muresan 2004). To block non-specific binding of an antibody to Fc receptors, sections were pre-incubated with normal goat serum diluted 1 : 10, as described (Askanas *et al.* 1993; Nogalska *et al.* 2006). Controls for staining specificity were (i) omission of the primary antibody or (ii) its replacement with non-immune sera or irrelevant antibody. These were always negative.

Combined immunoprecipitation/immunoblot procedure in s-IBM biopsies

To confirm the specificity of the immunocytochemical reaction in s-IBM muscle fibers, a combined immunoprecipitation/immunoblot technique was performed in three s-IBM biopsies, as detailed previously (Vattemi *et al.* 2004; Fratta *et al.* 2005). In brief, 100 μg of total muscle protein were immunoprecipitated in precipitation

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buffer containing 10 μg of 6E10 antibody (Covance, Princeton, NJ, USA), which on immunoblots recognizes total AβPP and Aβ. The immunoprecipitated complex, containing IgG antibody along with its bound target antigen and all proteins bound to that antigen, was pulled down using Protein G Sepharose 4 Fast Flow (Amersham Biosciences, Piscataway, NJ, USA) during 4 h of incubation at 4°C. The solution was then centrifuged for 5 min (16 000 *g* at 4°C) and the supernatant was removed. The precipitated sepharose immunocomplexes were washed three times with the precipitation buffer by centrifuging 5 min each (16 000 *g* at 4°C). Immunoprecipitates were then electrophoresed and immunoprobed with the rabbit polyclonal anti-p-AβPPT724 antibody, followed by an appropriate secondary antibody, and developed using the Western Breeze anti-rabbit chemiluminescence kit (Invitrogen, Carlsbad, CA, USA). To confirm specificity of the physical association identified by the immunoprecipitation–immunoblot reaction, primary antibodies were omitted from the immunoprecipitation solution (control biopsies were not used for the immunoprecipitation experiments because they do not contain enough AβPP to be demonstrated by this technique).

Immunoblotting

To evaluate whether GSK3β is activated in s-IBM patients, muscle homogenates of six s-IBM and six age-matched control biopsies were immunoblotted, as recently detailed (Vattemi *et al.* 2004; Nogalska *et al.* 2006, 2007; Terracciano *et al.* 2008). In brief, 20 μg of protein were loaded into 4–12% NuPAGE gels (Invitrogen) and electrophoretically separated. After electrophoresis, samples were transferred to a nitrocellulose membrane. To prevent non-specific binding of the antibodies, the nitrocellulose membranes were blocked in Blocking Reagent (Invitrogen). They were then incubated overnight at 4°C with a primary antibody. Antibodies used were against (i) total GSK3β (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1 : 50, (ii) active-GSK3β phosphorylated at Tyr216 (Novus Biologicals, Littleton, CO, USA), diluted 1 : 250, and (iii) inactive-GSK3β phosphorylated at Ser9 (Cell Signaling), diluted 1 : 250. Blots were developed using the Western Breeze chemiluminescent kit (Invitrogen), or Enhanced Chemiluminescence Western Blotting Analysis System (Amersham Biosciences) in combination with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Protein loading was evaluated by the actin band; the results were related to both active and inactive GSK3β and expressed per total GSK3β.

Cultured human muscle fibers and overexpression of AβPP-gene

Primary cultures of normal human muscle were established, as routinely performed in our laboratory (Askanas and Engel 1992), from archived satellite cells of portions of diagnostic muscle biopsies of patients who, after all tests were performed, were considered free of muscle disease. We established 15 culture sets, each from satellite cells derived from a different muscle biopsy. All experimental and control conditions were studied on sister cultures in the same culture set. Not all studies were performed on every set. Twenty days after myoblast fusion was completed, a 3 Kb 751 AβPP-cDNA was transferred into the well-differentiated myotubes using a replication-deficient adenovirus vector at 0.3×10^8 pfu/mL culture medium, as detailed (Askanas *et al.* 1996, 1997; Wojcik *et al.* 2006).

Inhibition of proteasome or ER-stress induction in cultured human muscle fibers overexpressing AβPP

Three days after AβPP gene-transfer, CHMFs were treated with 1 μM epoxomicin (Biomol Research Laboratories, Plymouth Meeting, PA, USA), an irreversible proteasome inhibitor (Meng *et al.* 1999), for 24 h. For comparison, other AβPP-overexpressing cultures were treated for 24 h with ER-stress inducers, either (i) tunicamycin, which inhibits *N*-glycosylation, 4 μg/mL (Sigma Co, St Louis, MO, USA) or (ii) thapsigargin, 300 nM (Sigma Co), which inhibits ER calcium-ATPase (Lee 2005). The doses used were as in our previous experiments (Fratta *et al.* 2005; Nogalska *et al.* 2007, 2008).

Lithium chloride treatment of experimental cultures

Because LiCl is known inhibitor of GSK3β (Klein and Melton 1996; Jope 2003; Avila and Hernández 2007), AβPP-overexpressing cultures in 12 independent experiments were treated with 5 mM LiCl (Sigma Co) for 48 h. Because human muscle material from which those primary cultures are derived is sparse, we were not able to evaluate wide ranges of lithium dosage. In this study, we chose a 5 mM dose, as that dose was shown effective by Yeste *et al.* (2007) and Kim and Thayer (2009). In our preliminary experiments, we evaluated doses of 1, 5, 10, 15, and 20 mM, and found that 5 mM was effective without causing any obvious adverse reactions (the 1 mM dose was not effective).

Immunoblotting of cultured human muscle fibers

After treatments (detailed above and below), experimental cultures and their sister-control cultures were harvested and processed for immunoblotting, as detailed previously (Wojcik *et al.* 2006; Nogalska *et al.* 2007). We evaluated total AβPP, pAβPPT724, and GSK3β with the antibodies described above. 6E10 antibody was used in the concentrations 1 : 1000 for evaluating total AβPP, and 1 : 300 to visualize Aβ oligomers. Protein loading was evaluated by the actin band. Quantification of immunoreactivity was performed by densitometric analysis using NIH IMAGE J-1.310 software (Bethesda, MD, USA).

Measurement of proteasome activity

Proteasome activity was measured as described (Fratta *et al.* 2005). Three main proteasome activities were determined by evaluating the cleavage of specific fluorogenic substrates (Shringarpure *et al.* 2003). Five LiCl-treated and five non-treated AβPP-overexpressing culture sets were homogenized in 20 mmol/L Tris–HCl, pH 7.2, containing 0.1 mmol/L EDTA and 1 mmol/L of fresh dithiotreitol (Meng *et al.* 1999; Demasi *et al.* 2001), centrifuged, the supernatant collected, and protein concentration determined using the Bradford method. Subsequently, 45 μg of protein from cultured muscle were incubated in 100 μmol/L fluorogenic substrates for the three different protease activities: Z-Leu-Leu-Glu-AMC (aminomethylcoumarin) (substrate II) for peptidyl glutamyl-peptide hydrolytic activity (Biomol Research Laboratories); Suc-Leu-Leu-Val-Tyr-AMC (substrate III) for chymotrypsin-like activity (Calbiochem, Gibbstown, NJ, USA); and Z-Ala-Arg-Arg-AMC (substrate VI) for trypsin-like activity (Calbiochem). Fluorescence emission was excited at 355 nm and recorded at 405 nm. Proteasome activities were expressed per 20sβ2 proteasome subunit performed by immunoblotting each culture set using an anti-20sβ2 mouse monoclonal antibody diluted 1 : 500 (Biomol Research Laboratories).

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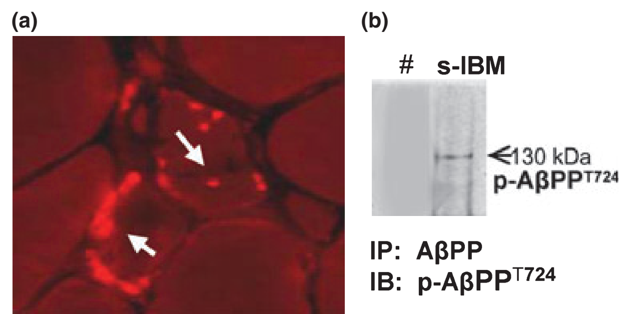

Fig. 1 Phosphorylated AβPP T724 (p-AβPPT724) in sporadic-inclusion-body myositis (s-IBM) muscle fibers. (a) Immunofluorescence using the specific phospho-AβPP antibody illustrates pAβPPT724-immunoreactive aggregates in two muscle fibers (arrows). (b) Immunoprecipitation (IP) of p-AβPPT724 in s-IBM muscle biopsy. IP was performed using 6E10 antibody, which recognizes both AβPP and Aβ; immunoblots (IB) were subsequently probed with the specific anti-p-AβPPT724 rabbit polyclonal antibody. There is a distinct band corresponding to AβPPT724. In (#) the primary antibody was omitted from the immunoprecipitation reaction to determine the specificity of the reaction.

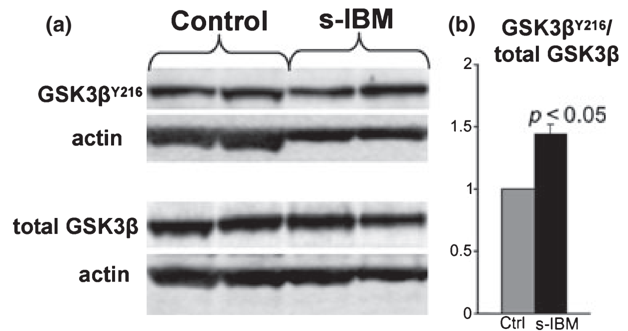

Fig. 2 GSK3β is activated in s-IBM biopsied muscle. (a) Representative immunoblots of normal control and s-IBM muscle biopsies. (b) The ratio of GSK3βY216 to total GSK3β, obtained by densitometric analysis of protein bands, shows that in s-IBM, when compared with controls, GSK3βY216 is significantly increased ($p < 0.05$, two-tail Student's *t*-test), ± SEM.

Statistical analysis

In all experiments these analyses were performed using Student's *t*-test. Significance level was set at $p < 0.05$. For all groups, data are reported as mean ± SEM.

Results

In s-IBM, muscle fibers AβPP was phosphorylated on threonine 724

By immunocytochemistry, pAβPPT724 was accumulated in the form of various-sized aggregates located mainly in the non-vacuolated regions of cytoplasm, of ~80% of s-IBM-vacuolated muscle fibers in all patients (Fig. 1a). None of the normal or disease-control muscle biopsies contained pAβPPT724-immunoreactivity. Endomysial and perivascular mononuclear cells present in s-IBM and polymyositis muscle biopsies did not contain pAβPPT724 immunoreactivity. Muscle blood vessels did not contain pAβPPT724 immunoreactivity.

To confirm the specificity of the immunocytochemical staining in s-IBM, we immunoprecipitated three s-IBM muscle-biopsy homogenates with antibody 6E10 (which on immunoblots recognizes total AβPP as well as Aβ oligomers), and we immunoprobed the blots with the specific anti-pAβPPT724 antibody. Immunoprecipitation/immunoblotting confirmed the existence of AβPP phosphorylated on Thr724 in s-IBM muscle (Fig. 1b).

Active form of GSK3β was increased in s-IBM muscle fibers

Glycogen synthase kinase 3β activity is regulated mainly by phosphorylation on Tyr216 (Y216) which increases its activity, and on Ser9 which inhibits its activity (Bhat *et al.* 2000; Lochhead *et al.* 2006).

In six s-IBM and six age-matched normal control muscle biopsies, we evaluated GSK3β on immunoblots, using antibodies specifically recognizing total GSK3β, p-GSK3βY216, and GSK3βSer9 (studied in duplicate samples) after their normalization to actin. In s-IBM muscle biopsies, total GSK3β was not changed when compared with normal controls, whereas the amount of active p-GSK3βY216 expressed per total GSK3β was increased 1.5-fold ($p < 0.05$) (Fig 2). Inactive p-GSK3βSer9 was decreased 1.6-fold in s-IBM muscle biopsies, but this did not reach *p*-value significance (data not shown).

Proteasome inhibition increased p-AβPP724 and induced activation of GSK3β in our AβPP-overexpressing cultures (human-muscle-culture IBM model)

To explore possible mechanisms involved in AβPP phosphorylation and GSK3β activation in s-IBM muscle fibers, we utilized our experimental model consisting of primary cultures of normal human muscle fibers, with experimental modifications of the intracellular micro-environment to mimic aspects of the s-IBM muscle-fiber milieu. These manipulations involved: overexpressing the AβPP-gene, inhibiting the 26S proteasome, and inducing ER stress.

In 12 independent experiments, immunoblots of CHMFs homogenates, using the phospho-specific antibody recognizing p-AβPPT724, showed that: (i) exposure of the AβPP-overexpressing ($A\beta PP^+$) CHMFs to the proteasome inhibitor epoxomicin caused 6.5-fold ($p < 0.005$) increase of p-AβPPT724, while (ii) ER-stress inducers tunicamycin and thapsigargin had no effect (Fig. 3). Proteasome-inhibition increased total AβPP threefold ($p < 0.01$; data not shown). As in our previous studies, AβPP from our $A\beta PP^+$-Culture IBM model muscle-fibers migrates as a 'mature' 130 kDa band and an 'immature' 115 kDa band.

To determine whether the proteasome-inhibition-induced increase of p-AβPPT724 is associated with GSK3β activa-

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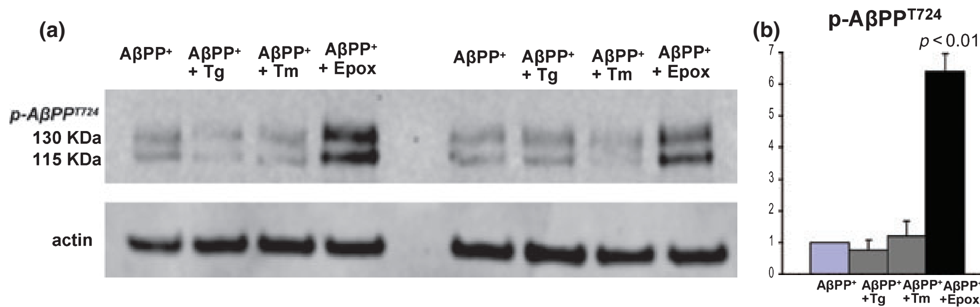

Fig. 3 p-AβPPT724 in (i) proteasome-inhibited and (ii) ER-stress-induced human-muscle-culture AβPP+ IBM model. (a) Representative immunoblots of densitometric analysis in (b). (b) Densitometric analysis based on 12 independent experiments of the pAβPPT724 protein bands relative to the actin bands, expressed in arbitrary units, shows that after treatment with Epoxomicin (Epox) pAβPPT724 is significantly increased (6.5-fold, $p < 0.01$, two-tail Student's *t*-test), while the ER-stress inducers [thapsigargin (Tg) and tunicamycin (Tm)] did not exert this effect, ± SEM.

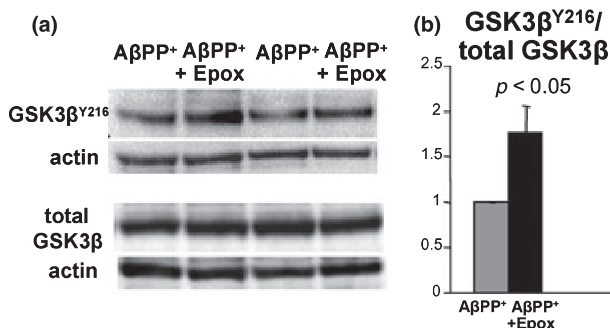

Fig. 4 GSK3β is activated in the epoxomicin-inhibited proteasome in the human-muscle-culture IBM model. (a) Representative immunoblots of densitometric analysis in (b). (b) The ratio of GSK3βY216 to total GSK3β obtained by densitometric analysis of protein bands in six independent experiments shows that in proteasome-inhibited cultures, when compared with controls, GSK3βY216 is significantly increased (1.7-fold, $p < 0.05$, two-tail Student's *t*-test), ± SEM.

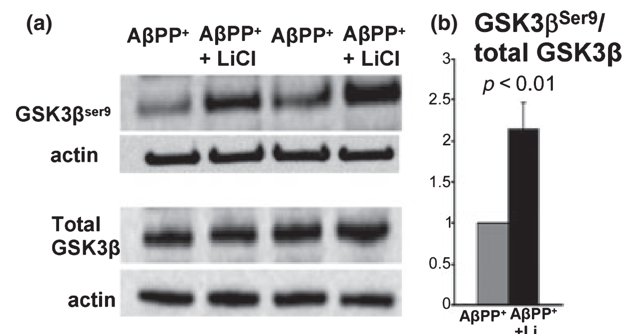

Fig. 5 Inactive form of GSK3β is increased in LiCl-treated culture IBM model. (a) Representative immunoblot of GSK3βSer9 in LiCl-treated and sister-control untreated culture AβPP+ IBM model. (b) Densitometric analysis of GSK3βSer9 bands relative to the actin bands, expressed in arbitrary units, shows that after lithium treatment GSK3βSer9 is significantly increased (2.2-fold, $p < 0.01$, two-tail Student's *t*-test).

tion, we performed immunoblots on six independent tissue-culture experiments using the same three anti-GSK3β antibodies as described above for human muscle biopsies. We found that after proteasome-inhibition active p-GSK3βY216 was increased 1.7-fold ($p < 0.05$) (Fig. 4), while the total GSK3β and GSK3βSer9 were not affected (data not shown). As the ER stress did not influence AβPP phosphorylation, we did not evaluate its influence on GSK3β activation.

Treatment with Lithium inhibited GSK3β, and decreased total AβPP, p-AβPPT724, and Aβ-oligomers in our culture IBM model muscle-fibers

Lithium is known to inhibit GSK3β by increasing its inactive p-GSK3βSer9 form. When, in 12 independent experiments, we treated AβPP-overexpressing CHMFs with 5 mM LiCl, the inactive p-GSK3βSer9 form of GSK3β was increased by 2.2-fold ($p < 0.01$) (Fig. 5), in which lithium treatment decreased total AβPP by 30% ($p < 0.005$), p-AβPPT724 by 50% ($p < 0.0001$), and Aβ oligomers (8–25 kDa) by 25% ($p < 0.05$) (Fig. 6). Because LiCl decreased Aβ oligomers of different molecular weights in different experiments, we calculated all oligomers together.

Lithium induced proteasome activity

In view of the results obtained in our IBM-culture model indicating that proteasome inhibition increased both p-AβPPT724 and total AβPP, we asked whether our observed decrease of them by lithium might be, at least partially, influenced by a lithium enhancement of proteasome activity. Therefore, we studied three main proteasome activities in five independent sets of the IBM-culture models. We found that in lithium-treated cultures, all three proteasome enzymatic activities were increased peptidyl glutamyl-peptide hydrolytic activity twofold ($p < 0.005$), chymotrypsin-like activity 1.3-fold ($p < 0.05$), and trypsin-like activity 1.2-fold ($p < 0.05$), when compared with the sister non-treated controls (Fig. 7).

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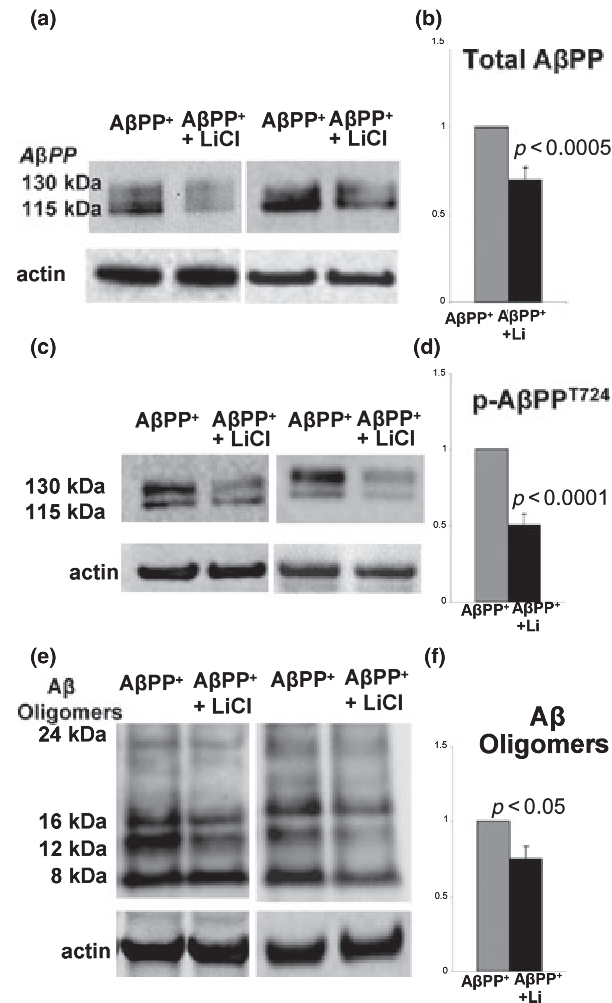

Fig. 6 Lithium decreases the amount of total AβPP, p-AβPPT724, and Aβ-oligomers. (a) Representative immunoblots of total AβPP in LiCl-treated and control untreated culture AβPP+-IBM-model. (b) Densitometric analysis of the total AβPP bands (130 and 115 kDa) relative to the actin bands, expressed in arbitrary units, shows that after lithium treatment AβPP is decreased 30% ($p < 0.0005$, two tail); (c) Representative immunoblots of p-AβPPT724 in LiCl-treated and untreated control culture AβPP+-IBM-model. (d) Densitometric analysis of p-AβPPT724 bands relative to the actin bands, expressed in arbitrary units, shows that after lithium treatment p-AβPPT724 is decreased 50% ($p < 0.0001$, two tail). (e) Representative immunoblots of Aβ oligomers (8, 12, 16, and 24 kDa bands) in LiCl-treated and control untreated AβPP+-IBM-culture-model. (f) Densitometric analysis of Aβ oligomers bands relative to the actin bands, expressed in arbitrary units, shows that after lithium treatment all Aβ bands calculated together were decreased 25% ($p < 0.05$, two-tail Student's *t*-test).

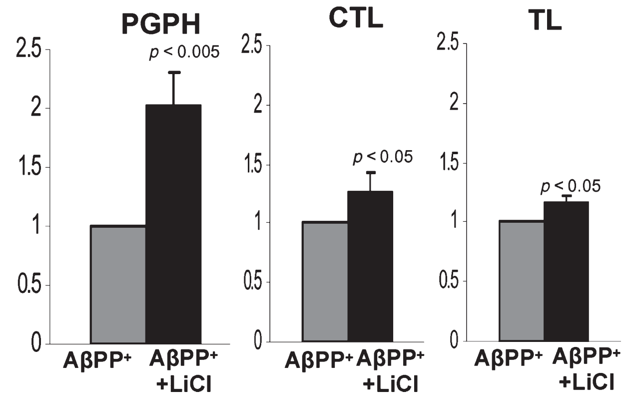

Fig. 7 Three main proteasome enzyme activities – peptidyl glutamyl-peptide hydrolytic (PGPH), chymotrypsin-like (CTL), and trypsin-like (TL) – in LiCl-treated and sister-control untreated culture AβPP+ IBM model. Proteasome activities are expressed per β2 20S proteasome subunit in each culture. After lithium treatment, PGPH activity was increased twofold ($p < 0.005$, two-tail Student's *t*-test), CTL activity was increased 1.3-fold ($p < 0.05$, one-tail Student's *t*-test), and TL activity was increased 1.2-fold ($p < 0.05$, one-tail Student's *t*-test).

Discussion

While AβPP695 is specifically present in neuronal cells (da Cruz e Silva and da Cruz e Silva 2003), the AβPP751 isoform is most abundant in peripheral tissues (Tanaka *et al.* 1989). In s-IBM muscle fibers, AβPP751 is the isoform overproduced and accumulated as aggregates (Askanas *et al.* 1993; Sarkozi *et al.* 1993; Guerin *et al.* 2008). Phosphorylation of AβPP is considered as a regulatory mechanism of AβPP metabolism (da Cruz e Silva and da Cruz e Silva 2003). Phosphorylation on Thr668 of the neuronal isoform AβPP695 (equivalent to threonine at position 724 of AβPP751) was reported (i) to be associated with increased Aβ production (Ando *et al.* 2001; Lee *et al.* 2003) and (ii) to mediate pathological interaction between Aβ and tau (Shin *et al.* 2007). There were reduced levels of Aβ in neuronal cells treated with T688 kinase inhibitors or overexpressing mutant p-AβPPT688 (Lee *et al.* 2003).

Active p-GSK3βY216 has been shown increased in the frontal cortex of AD patients (Leroy *et al.* 2007), and proposed to be a component of AD pathogenesis (Jope 2003; Jope and Johnson 2004). Active p-GSK3βY216 was shown to phosphorylate both AβPP on Thr688 (Aplin *et al.* 1996) and tau protein (Anderton *et al.* 2001).

Glycogen synthase kinase 3β is inactivated by being phosphorylated on Ser9 (Shaw *et al.* 1997; Bhat *et al.* 2000). In AD transgenic mice, overexpression of a dominant negative GSK3β, as well as treatment with lithium or other pharmacological inhibitors, was reported to result in GSK3β inhibition and a subsequent reduction of Aβ, which was associated with improved cognitive performance (Ryder *et al.* 2003; Su *et al.* 2004; Rockenstein *et al.* 2007). GSK3β activity was also found increased in muscle fibers of the transgenic-mouse IBM model (Kitazawa *et al.* 2006); in this model, a decrease of GSK3β activity by lithium correlated with decreased tau phosphorylation (Kitazawa *et al.* 2008).

In this study, we demonstrated for the first time that (i) in biopsied s-IBM muscle fibers and in our AβPP+ CHMFs (AβPP+ human muscle culture IBM model), AβPP is

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phosphorylated on Thr724 and (ii) in s-IBM patients, active GSK3β is significantly increased when compared with the normal aged-matched-control muscle biopsies. In addition, we demonstrated in our IBM culture model that proteasome inhibition significantly enhanced the increase of active GSK3β, which corresponded to the increase of phosphorylated AβPP. Accordingly, we postulate that in s-IBM, phosphorylation of AβPP is influenced by proteasome inhibition, possibly via activation of GSK3β. The increase of total AβPP after proteasome inhibition also suggests that in this model the ubiquitin-proteasome system is involved in the degradation of AβPP.

Our results showed that lithium increased activities of all three proteasome enzymes studied, to various degrees. Lithium is known to have various effects on cells (Phiel and Klein 2001), but to our knowledge it has not been reported to improve proteasome function. However, as AβPP was previously shown to inhibit proteasome function in this culture model (Fratta *et al.* 2005), it is presently not known whether the increase of proteasome function by lithium that we observed in the current study represents its direct influence on the ubiquitin-proteasome system, or it is related to the overall reduction of the AβPP level.

In conclusion, we have demonstrated, apparently for the first time, that in s-IBM muscle fibers GSK3β activity is increased and AβPP is phosphorylated. Our experimental data also strongly suggest that AβPP phosphorylation is increased by GSK3β activation, which is increased by proteasome inhibition.

And, we have shown that in our culture IBM model, lithium treatment significantly decreased the levels of both total and phosphorylated AβPP as well as Aβ oligomers, accompanied by increased proteasome function and decreased GSK3β activation. This is in agreement with the transgenic-mouse IBM model in which lithium treatment decreased both GSK3β activation and tau phosphorylation (Kitazawa *et al.* 2008). Accordingly, treatment with lithium, or other GSK3β inhibitors, might be beneficial for s-IBM patients.

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