Lithium Chloride Increases the Production of Amyloid-β Peptide Independently from Its Inhibition of Glycogen Synthase Kinase 3*

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Glycogen synthase kinase 3 (GSK3) is able to phosphorylate tau at many sites that are found to be phosphorylated in paired helical filaments in Alzheimer disease. Lithium chloride (LiCl) efficiently inhibits GSK3 and was recently reported to also decrease the production of amyloid-β peptide (Aβ) from its precursor, the amyloid precursor protein. Therefore, lithium has been proposed as a combined therapeutic agent, inhibiting both the hyperphosphorylation of tau and the production of Aβ. Here, we demonstrate that the inhibition of GSK3 by LiCl induced the nuclear translocation of β-catenin in Chinese hamster ovary cells and rat cultured neurons, in which a decrease in tau phosphorylation was observed. In both cellular models, a nontoxic concentration of LiCl increased the production of Aβ by increasing the β-cleavage of amyloid precursor protein, generating more substrate for an unmodified γ-secretase activity. SB415286, another GSK3 inhibitor, induced the nuclear translocation of β-catenin and slightly decreased Aβ production. It is concluded that the LiCl-mediated increase in Aβ production is not related to GSK3 inhibition.

Alzheimer disease, the most frequent cause of dementia, is characterized by the presence of typical microscopic lesions in the brain of affected patients. The coexistence of intraneuronal neurofibrillary tangles and extracellular senile plaques allows confirmation of the clinical diagnosis of the disease (1).

Neurofibrillary tangles are made of paired helical filaments (PHFs) containing the microtubule-associated protein, tau (2–4). In Alzheimer disease, tau is hyperphosphorylated, and many serine and threonine residues (5, 6) that are found to be phosphorylated in PHF tau can be phosphorylated by GSK3 in both in vitro and transfected cells (7–9).

Senile plaques contain an amyloid core that is mainly constituted of amyloid-β peptide (Aβ) (10), which is derived from the amyloid precursor protein (APP) (11, 12). The APP gene encodes 10 different APP isoforms (13) with an amino acid content varying from 365 to 770 amino acids. The neuronal APP is a single pass type I transmembrane protein containing 695 amino acids (11) that is processed by amyloidogenic and nonamyloidogenic catabolic pathways. The β-cleavage of APP, catalyzed by the well-characterized aspartyl protease β-site APP-cleaving enzyme 1 (BACE1) (14), produces a C-terminal fragment of APP (βCTF), which is further cleaved by γ-secretase to generate Aβ. The γ-secretase activity is found as a multiprotein complex containing at least four different proteins: Aph-1, nicastrin, presenilin, and Pen-2 (15, 16). APP can also be cleaved within the Aβ sequence by an α-secretase. The α-cleavage of APP generates a soluble N-terminal fragment (sAPP) and a 83-membrane-anchored C-terminal fragment (Cβ3). Experimental evidence indicates that the α-cleavage of APP695 could be performed by members of the desintegrin and metalloprotease family, ADAM10 and ADAM17 (17). The short intracellular C-terminal domain of APP can be phosphorylated in vitro and in vivo by several protein kinases such as cdk5, c-Jun N-terminal kinase, and GSK3 (18–20).

Another substrate of GSK3 is β-catenin, an essential protein of the Wnt signaling pathway. In the absence of a Wnt ligand, GSK3 activity is not inhibited, resulting in the phosphorylation of soluble β-catenin for ubiquitin-proteasome-mediated degradation (21). Alternatively, as a result of GSK3 inactivation by Wnt signaling, intracellular levels of β-catenin increase, allowing its binding to components of the high mobility group family of transcription factors and its translocation into the nucleus.

LiCl, an inhibitor of GSK3 (22), reduces the phosphorylation of tau in rat cultured neurons (23–25). Interestingly, LiCl was recently reported to decrease Aβ production in both transfected cells and transgenic mice (26–29). Therefore, lithium could be a combined therapeutic agent, inhibiting both the phosphorylation of tau and the production of Aβ.

In this study, transfected CHO cells and rat cultured neurons expressing human APP695 and producing human Aβ were treated with LiCl. This GSK3 inhibitor decreased the phosphorylation of tau in neurons and induced the nuclear translocation of β-catenin in both CHO cells and neurons. It was found that LiCl increased the β-secretase activity and consequently increased the amount of βCTF generated from human APP695. The cleavage of βCTF by an unchanged γ-secretase activity led to an overproduction of Aβ. SB415286, another GSK3 inhibitor, induced the nuclear translocation of β-catenin and slightly decreased neuronal Aβ production. Taken together, these results clearly demonstrate that LiCl stimulates the amyloidogenic pathway of human APP independently of its inhibition of GSK3.
EXPERIMENTAL PROCEDURES

Materials and Antibodies—Fetal calf serum, cell culture media, and NuPageTM 4–12% bis-Tris gels were purchased from Invitrogen. Protein A-Sepharose CL-4B was from Amersham Biosciences. Protease inhibitors were from Roche Applied Science. MTT reagent was obtained from Sigma. DAPT, a functional γ-secretase inhibitor (30) was a kind gift from L. Mercken (Aventis, Vitry-sur-Seine, France). LiCl was obtained from Merck (Darmstadt, Germany). SB415286, an ATP-competitive inhibitor of GSK3, was kindly provided by A. Goffinet (University of Bergen, Norway). The mouse monoclonal PHF1 antibody specifically recognizing tau phosphorylated in a phosphorylation independent-manner (31). Mouse monoclonal antibody raised against amino acids 5–8 of human Aβ was purchased from the Genetics Company. Rabbit polyclonal anti-actin serum was purchased from Sigma. B19 rabbit polyclonal serum recognized tau in a phosphorylation independent-manner (31). Mouse monoclonal PHF1 antibody specifically recognizing tau phosphorylated at Ser396/Ser404 was kindly provided by P. Davies and S. Greenberg. Mouse monoclonal β-catenin antibody was purchased from Transduction Laboratories (Lexington, KY). The polyclonal antibody anti-p58 was a kind gift from J. Saraste (University of Bergen, Norway). The rabbit polyclonal antibodies directed against BACE1 were from Oncogene Research (La Jolla, CA). Secondary antibodies were from Amersham Biosciences.

Cell Cultures—Transfected CHO cell lines expressing human APP695 were cultured as described previously (32). Cells were seeded at a density of 3.6 × 10^4 cells/cm² in 6-, 12-, or 96-well culture dishes 24 h prior to treatment. Primary cultures of cortical neurons were prepared from 17-day-old Wistar rat embryos as described previously (33). Cells were plated in 6-, 12-, or 96-well culture dishes (4 × 10^5 cells/cm²) on glass coverslips (1.2 × 10^5 cells/cm²) pretreated with poly-L-lysine (10 μg/ml in phosphate-buffered saline) and cultured for 6 days in vitro in NeurobasalTM medium supplemented with 2% B-27 and 0.5 mM L-glutamine prior to infection with recombinant adenoviruses. Under these conditions, neuronal cultures (up to 98% of neurons) display high differentiation and survival rates (34).

Recombinant Adenoviruses, Neuronal Infection, and Treatment—The construction and purification of adenoviruses encoding human APP695 were performed as described previously (35, 36). After 6 days in vitro, neuronal cultures were infected at a multiplicity of infection of 100 for 4 h in a minimal volume of culture medium. Infection medium was then replaced by fresh culture medium for 4 days before treatment. Under these conditions, at least 75% of neurons stably express the proteins encoded by recombinant adenoviruses (33). Neuronal and CHO cells were treated for 24 h with 5 mM LiCl and/or 25 μM SB415286 (two GSK3 inhibitors) (37) or for 8 h with 250 nM DAPT (a γ-secretase inhibitor) (30).

Survival Assays—Cells survival was measured by the colorimetric MTT assay. Cells grown in 96-well dishes were incubated for 2 h at 37 °C in fresh culture medium containing 0.5 mg/ml MTT. The medium was removed, and dark blue crystals that had formed were dissolved by adding 100 μl/well lysis solution (isopropyl alcohol/0.04 N HCl). Absorbance was measured at 492 nm using a microplate reader (PerkinElmer Life Sciences).

Protein Analysis by Western Blotting and Densitometric Quantification—Culture media and cell lysates were analyzed by Western blotting as described previously (33). Cell lysates (10 μg of protein) and culture medium (15 μl) were separated in NuPageTM 4–12% bis-Tris gels (Invitrogen) and blotted onto nitrocellulose membranes. Membranes were incubated overnight at 4 °C with primary antibody followed by secondary antibody coupled to horseradish peroxidase. Immunoreactive bands were detected by ECL (Amersham Biosciences). For quantification of cellular APP, membranes were stripped and reincubated with an anti-actin antibody. The ratio between immunoreactive proteins and actin was quantified with an electrophoresis Gel Doc 2000 imaging system coupled to a Quantity One™ software (Bio-Rad).

Immunoprecipitation of βCTF Fragment—βCTF production was monitored by immunoprecipitation of cell lysates. Four million neurons or 0.5 million CHO cells were plated in each well of a 6-well plate. Following treatment, cells were washed three times with phosphate-buffered saline and then scraped and pelleted in cold phosphate-buffered saline. Cells were solubilized in radioimmune precipitation assay buffer containing protease inhibitors (1 μg/ml pepstatin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride; purchased from Roche Applied Science). The samples were preincubated overnight at 4 °C with incubation with 5 μg of protein A-Sepharose. After removal of protein A-Sepharose by centrifugation, the supernatants were recovered and analyzed by Western blotting (see above).

Quantification of βAPP Production—βAPP production was monitored by immunoprecipitation of cell culture medium. The quantification of extracellular βAPP (1–40) was performed by ELISA (BIOSOURCE, Camarillo, CA). For both approaches, culture medium was collected, treated with protease inhibitors (1 μg/ml pepstatin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride), and cleared by centrifugation (15,800 × g for 5 min, 4 °C). Supernatants (100 μl) were recovered for βAPP quantification by fluorescent sandwich ELISA assay according to the manufacturer’s instructions. Previous experiments showed that there was no cross-reaction between βAPP (1–40) and βAPP (1–42) recognition (38). Fluorescence emission was measured on an HTS 7000 Plus plate reader (PerkinElmer Life Sciences) at excitation/emission wavelengths of 485 nm/535 nm, respectively. Immunoprecipitation was performed in 1 ml of the culture medium as described previously (33).

Immunocytochemistry—Cells were fixed with 4% (w/v) paraformaldehyde in a phosphate buffer (0.1 M, pH 7.4). The immunohistochemical labeling was performed using the ABC method. Briefly, fixed cells were treated with H2O2 to inhibit endogenous peroxidase and incubated with blocking solution (10% (v/v) BSA, 1% normal horse serum in TBS (0.1M Tris, pH 7.4)). After overnight incubation with the diluted primary antibody, the fixed cells were sequentially incubated with horseradish anti-mouse antibodies conjugated to biotin (Vector, Burlingame, CA) followed by the ABC complex (Vector). Peroxidase activity was revealed using diamobenzidine as chromogen.

β- and γ-Secretase Activity Assay—The β- and γ-secretase activities were measured by FRET-based in vitro assays. CHO cells were washed, scraped in cold phosphate-buffered saline, and pelleted by centrifugation (1,000 × g for 5 min, 4 °C). One hundred μl of extraction buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.01% Triton X-100) was added to the cell pellet (~1.5 × 10⁶ cells). After brief sonication (5 s), cellular extracts were cleared by centrifugation (10,000 × g for 10 min, 4 °C), and enzymatic activities were measured in the supernatant. The β-secretase assay was carried out on ~ 20 μg of proteins in reaction buffer containing the fluorogenic NH2-RE[EDANS]EVLNDAEFK(DAB-CY3)R-OH β-secretase specific substrate (39) according to the manufacturer’s instructions (BioVision, Mountain View, CA). After 1.5 h of
incubated in the presence or absence of 5 mM LiCl. A expression of human APP695 in cell lysates (upper left). Protein loading was controlled by incubating membranes with an anti-actin antibody (lower left). Expression of snAPP (upper right) and detection of extracellular Aβ after immunoprecipitation in the culture medium (lower right) are shown. CHO cells were treated with 0, 1, 2.5, 5, 10, or 20 mM LiCl for 24 h (left) or 48 h (right). B, cell survival was measured using the MTT assay. Results (mean ± S.E.) are given as the percentage of survival of untreated cells (0 mM); n = 16; ***, p < 0.001 when compared with control. C, the extracellular Aβ-(1–40) was quantified by ELISA. Results are expressed as percentage of Aβ-(1–40) produced by nontreated CHO cells (0 mM); n = 4; ***, p < 0.001 when compared with control. D, CHO cells were incubated in the presence or absence of 5 mM LiCl. Aβ-(1–40) was quantified in the culture medium by ELISA before (0 h) or after (24 h) a 24-h incubation at 37 °C. Results (mean ± S.E.) are expressed as the percentage of control (0 h); n = 4; ***, p < 0.001 when compared with control. E, anti-β-catenin immunolabeling of untreated CHO cells (NT, not treated) or cells treated for 24 h with 5 mM LiCl. Scale bars, 10 μm.

incubation at 37 °C in the dark, samples were analyzed in a HTS 7000 Plus fluorescence microplate reader with excitation/emission wavelengths of 360/353 nm, respectively. Positive controls were performed with purified human β-secretase (10 μg/ml, final concentration). The β-secretase inhibitor used in the assays was Z-VLLE-CHO (40). The γ-secretase activity was measured by the QTL Lightspeed™ γ-secretase assay (QTL Biosystems, Santa Fe, NM). Approximately 10 μg of cellular extract was mixed to γ-secretase substrate (GVVIA TVK, flanked by biotin and a fluorescence quencher, 20 μM) in 20 μl (final volume) of assay buffer (50 mM Tris-HCl, pH 7, 2 mM EDTA, 0.05% bovine serum albumin, 2 mM reduced glutathione). After 2 h of incubation in the dark, 40 μl of QTL Sensor™ (41) was added, and the fluorescence was measured in a microplate reader with excitation/emission wavelengths of 430/595 nm, respectively. Results are expressed as δ and γ-secretase activities/μg of cellular protein.

Analytical Subcellular Fractionation—Cells were incubated with 4 μg/ml [125I]transferrin for 5 min, 37 °C. Under these conditions, transferrin is an early endosomal marker. Cells were recovered in 0.25 M sucrose containing 1 mM EDTA, 3 mM imidazole buffered at pH 7.4, and complete protease inhibitors (Roche Applied Science). Cellular suspension was homogenized in a tight Dounce homogenizer. A low-speed nuclear fraction was pelleted at 1,000 × g for 10 min and washed three times by resuspension and sedimentation. Pooled postnuclear supernatants were further sedimented at 100,000 × g for 60 min in a Ti50 rotor (Beckman). The high-speed pellet was resuspended in 0.3 ml of homogenization buffer, mixed with 2.3 μl sucrose to reach 1.28 g/ml in density, and layered at the bottom of a linear sucrose gradient (from 1.10 to 1.24 g/ml in density). After floatation by centrifugation at 200,000 × g for 22 h in a SW40 rotor (Beckman), 12 fractions were collected and analyzed for protein content. Western blotting and quantifications were performed as described above. Results are represented as normalized histograms (42).

Statistical Analysis—The number of samples (n) in each experimental condition is indicated in the legends to Figs. 1, 2, 4, 5, 6, and 7. Unpaired t test were performed to compare two experimental conditions. Otherwise, statistical analysis was performed by one-way analysis of variance followed by Bonferroni’s multiple comparison post-hoc test. Each Western blot presented in Figs. 1, 2, 5, and 6 are representative of at least three independent experiments.

RESULTS

LiCl Increases the Amount of Aβ-(1–40) Produced by CHO Cells Expressing Human APP695—Transfected CHO cells expressing human APP695 were analyzed by Western blotting using the WO-2 antibody. The WO-2 antibody did not detect endogenous APP produced by CHO cells (Fig. 1A), confirming the specificity of this antibody for human APP isoforms (43). Results shown in Fig. 1A indicate that CHO cells expressing human APP695 produced snAPP as well as Aβ in their culture medium.

CHO cells were incubated for 24 h or 48 h in the presence of increasing concentrations of LiCl (0, 1, 2.5, 5, 10, 20 mM), and cell survival was measured by using the MTT assay. The results presented in Fig. 1B indicate that, after 24 h, LiCl induced a significant toxicity at 10 and 20 mM. After 48 h of incubation, 5 mM LiCl also induced a significant cell death. Therefore, LiCl was utilized at a maximal 5 mM concentration during 24 h.
CHO cells were incubated for 24 h in the presence of increasing concentrations of LiCl (0, 1, 2, 5, 50 mM), and the production of extracellular Aβ was quantified by ELISA and normalized to the amount of sAPP. The results presented in Fig. 1C indicate that LiCl increased the production of extracellular Aβ-(1–40) in a concentration-dependent manner. A 24-h treatment of CHO cells with 5 mM LiCl increased the production of Aβ-(1–40) by 67% without detectable cytotoxic effects. The culture medium of CHO cells incubated in the presence or absence of LiCl was recovered, and Aβ was quantified by ELISA before and after a 24-h incubation at 37 °C. Results presented in Fig. 1D show an identical decrease of Aβ in both experimental conditions indicating that LiCl treatment did not affect the turnover of Aβ in the extracellular medium.

To investigate the effect of LiCl on GSK3 activity, we analyzed the cellular distribution of β-catenin. The phosphorylation of β-catenin by GSK3 targets β-catenin for proteasomal degradation, whereas the inhibition of GSK3 allows the binding of β-catenin to transcription factors and its translocation to the nucleus (21). As shown in Fig. 1E, LiCl (5 mM, 24 h) induced the nuclear translocation of β-catenin in CHO cells treated with LiCl, showing that GSK3 activity was inhibited under our experimental conditions.

LiCl Increases the Amount of BCTF Fragments Produced by CHO Cells Expressing Human APP695—The effect of LiCl on the amyloidogenic processing of human APP695 in CHO cells was compared with that of DAPT, a known functional γ-secretase inhibitor (30). DAPT and LiCl did not modify the expression of human APP695 in CHO cells (Fig. 2A). As expected, γ-secretase inhibition by DAPT (250 nM, 8 h) resulted in the accumulation of BCTF detected by Western blotting following its immunoprecipitation with the WO-2 antibody (Fig. 2A). LiCl treatment also induced βCTF accumulation. The effects of DAPT and LiCl on βCTF accumulation were similar (3.2- and 2.8-fold increase, respectively). However, the processing of βCTF that accumulated upon treatment was completely different in CHO cells treated with DAPT or LiCl. Although DAPT strongly inhibited Aβ production (not shown and Ref. 44), LiCl increased by 67% the amount of extracellular Aβ-(1–40) (Fig. 1C). This led us to postulate that the βCTF accumulation induced by LiCl did not result from the inhibition of γ-secretase activity but rather from an increased β-cleavage of APP.

LiCl Does Not Change the Cellular Distribution of APP695 and BACE1—One hypothesis could be that LiCl favors the β-cleavage of APP by changing the cellular distribution of APP and BACE1, the protease known to cleave APP at the β-site (14), so as to favor their encounter. This hypothesis was investigated by analytical subcellular fractionation using floatation in sucrose density gradients. Radiolabeled transferrin internalized for 5 min and p58 were used as markers of early endosomes and intermediate compartment/cis-Golgi, respectively (45, 46). These two markers were clearly resorbed in the density gradients, and their distribution was not altered by LiCl treatment (Fig. 3, A and B). The density distribution of APP695 and BACE1 analyzed in the same fractions (Fig. 3, C and D) was not affected either. These data argued against the possibility that the increase in βCTF production induced by LiCl resulted from a subcellular redistribution of APP695 and BACE1 along the intermediate secretory and/or endocytic pathways.

LiCl Increases β-Secretase Activity in CHO Cells—Alternatively, LiCl-mediated increase of βCTF fragments production could result from a modification of the intrinsic β-secretase activity. This activity was therefore measured in lysates of control and LiCl-treated cells by a FRET-based in vitro assay, using an APP-specific amino acid sequence as substrate. Preliminary controls were performed with purified β-secretase and a β-secretase inhibitor (40) to validate the assay (not shown). Results show that a 24-h treatment with 5 mM LiCl significantly increased the β-secretase activity in CHO cells (Fig. 4A). In addition, we also measured by in vitro assays the effects of LiCl treatment of CHO...
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cells on γ-secretase activity. As shown in Fig. 4B, LiCl did not change γ-secretase activity, whereas DAPT inhibited the γ-secretase activity by 50%. Altogether, these results clearly establish that the treatment of CHO cells with LiCl increased β-secretase activity without modifying γ-secretase activity.

Effects of LiCl on Neuronal Survival and GSK3 Activity—We further analyzed the effects of LiCl on APP processing in neuronal cells. To that end, we infected rat cultured neurons with recombinant adenoviruses encoding human APP695 (33, 44). Four days after infection, high levels of human full-length APP were detected in cell lysates of APP-infected neurons (Fig. 5A, left). In the corresponding medium, sAPP and Aβ were released (Fig. 5A, right).

The effect of LiCl on the neuronal survival was measured by MTT. As shown in Fig. 5B, the LiCl treatment (5 mM, 24 h) had no neurotoxic effects.

To investigate the effect of LiCl on neuronal GSK3 activity, we first analyzed the phosphorylation of the tau protein. Western blotting showed that the expression of tau, either phosphorylated or not phosphorylated, was similar in both controls and neurons treated with LiCl, as detected by the B19 antibody (Fig. 5C, upper panel, and Ref. 5). By contrast, the detection of tau by the PHF1 antibody, which specifically recognizes phosphorylated tau, clearly showed a decrease in signal (Fig. 5C, lower panel) corresponding to a 45% decrease in tau phosphorylation when neurons were incubated with LiCl (Fig. 5D).

Moreover, as shown in Fig. 5E, LiCl induced the nuclear translocation of β-catenin in rat cortical neurons treated with LiCl, confirming the inhibition of GSK3 under our experimental conditions.

LiCl Increases the Amount of βCTF, Aβ, and BACE1 Protein in Neurons—We studied the effect of LiCl on the amyloidogenic processing of human APP695 expressed in rat cultured neurons. Four days after infection with recombinant adenoviruses encoding human APP695, neurons were treated with LiCl (5 mM) for 24 h. Cell lysates and culture media were subsequently analyzed by Western blotting with the WO-2 antibody. LiCl modified neither the amount of cellular human APP695 nor the amount of soluble APP recovered in the culture medium (not shown). The effect of LiCl on the amyloidogenic processing of human APP in rat cortical neurons was compared with that of DAPT. As shown in Fig. 6A and as in CHO cells, DAPT induced a 3-fold increase in βCTF, detected by Western blotting following its immunoprecipitation with the WO-2 antibody. A 2-fold increase in βCTF was also observed in the presence of LiCl (Fig. 6B).

The processing of accumulated βCTF was completely different in neurons treated with DAPT or LiCl. As indicated in Fig. 6C, DAPT completely inhibited Aβ production, as described previously (44). By contrast, LiCl induced a 2-fold increase in extracellular Aβ production. Interestingly, LiCl significantly increased the amount of BACE1 protein in neuronal extracts (Fig. 6D).

Taken together, these results demonstrate that LiCl increases the production of extraneuronal Aβ by increasing the amount of βCTF, which is further processed by an unchanged γ-secretase activity, confirming in neurons the effect of LiCl observed in transfected CHO cells. SB415286, a GSK3 Inhibitor, Does Not Significantly Modify the Production of Extracellular Aβ—Lithium, frequently used as a noncompetitive inhibitor of GSK3, is not really specific and displays a number of other activities. To study whether the LiCl-mediated increase in neuronal Aβ production was related to the inhibition of GSK3, we tested another GSK3 inhibitor. SB415286 selectively inhibits GSK3 in a competitive manner with respect to ATP (37). Rat cultured neurons expressing human APP were incubated in the presence or absence of 25 μM SB415286 for 24 h, and neuronal survival was measured by using the MTT assay. The results presented in Fig. 7A clearly indicate that SB415286 had no detectable neurotoxicity under these conditions. Like LiCl, SB415286 induced the nuclear translocation of β-catenin in neurons (Fig. 7B), confirming GSK3 inhibition. SB415286 did not modify the amount of human APP expressed by rat cultured neurons (not shown). In contrast to LiCl, SB415286 tended to decrease extracellular Aβ production, although the difference was not statistically significant (Fig. 7C). Combined inhibition with LiCl and SB415286 increased Aβ.
production in neurons by 46% as compared with SB415286 alone, indicating cumulative effects. Taken together, these results demonstrate that the LiCl-mediated increase in Aβ production was independent of its inhibition of GSK3.

**DISCUSSION**

Our data indicate that, in both CHO cells and rat cortical neurons each expressing human APP695, treatment for 24 h with 5 mM LiCl was not toxic and inhibited GSK3 activity. In these experimental conditions, LiCl increased the β-secretase activity, generating more βCTF that was processed by an unchanged γ-secretase activity. Consequently, LiCl increased the production of extracellular Aβ.

In contrast to the present study, several reports suggest that LiCl is able to inhibit Aβ production in different cellular models, including cultured neurons (26–29). In some of these studies (26, 28, 29), however, transfected cells are treated with LiCl concentrations that we found to be cytotoxic, which could account for the reported decrease in Aβ production. In mouse cortical neurons in which LiCl is found to block Aβ production (27), expression of human APP695 is driven by recombinant Semliki Forest virus, which allows a very transient expression before inducing severe neuronal toxicity. We have used recombinant adenoviruses that allow long-term APP expression without triggering toxic effects (33).

In APP transgenic mice, lithium is also reported to cause a reduction in Aβ production (27–29). However, lithium ions can affect in vivo several physiological mechanisms involved in APP processing. Therefore, it is difficult to compare these results with those obtained in cultured cells. In addition, it must be stressed that the transgenic mice studied expressed Swedish human APP (swAPP), a mutated form that favors amyloidogenic processing into Aβ. LiCl has also been reported to block Aβ production in various cellular models expressing swAPP. However, it has been shown that the amyloidogenic processing of the wild-type APP is different from that of swAPP, in particular for the β-cleavage (47) that we have demonstrated to be affected by LiCl.

We found that LiCl, contrary to DAPT, does not inhibit γ-secretase activity. Although, LiCl could alter access of APP to the γ-secretase complex (27), our results suggest that LiCl-mediated accumulation of βCTF most likely resulted from increased β-cleavage of APP. We found no effect of LiCl on the subcellular distribution of APP and BACE1. Moreover, using FRET-based in vitro assays, we demonstrated directly that LiCl increases β-secretase activity. Consistent with stimulation by LiCl of APP β-cleavage, this treatment did not affect the production of extracellular Aβ following adenosine expression of C99 in rat cultured neurons (not shown). This is in agreement with previous data indicating that 5 mM LiCl does not significantly decrease the secretion of Aβ in COS cells transiently expressing C99 (26). The mechanisms by which LiCl stimulates β-secretase activity should be further investigated. Treatment of primary cultures of neurons with 5 mM LiCl significantly increased their content in BACE1, suggesting that the increase in β-secretase activity could result from an increase in BACE1 protein.

We confirmed that LiCl efficiently inhibits GSK3 activity in CHO cells and neuronal cultures. The role of GSK3 in Aβ production is not
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FIGURE 7. GSK3 inhibitor (SB415286) does not modify neuronal production of Aβ. Rat cortical neurons expressing human APP695 were incubated for 24 h in the presence or in the absence (NT, no treatment) of 25 μM SB415286. A, the neuronal survival was measured using the MTT assay. Neuronal survival (mean ± S.E.) is represented as the percentage of nontreated controls; n = 24. B, immunocytochemical analysis of untreated neurons (NT) or neurons incubated in the presence of SB415286 with an anti-β-catenin antibody, indicating the LiCl-mediated concentration of β-catenin in nuclei. Scale bars, 10 μm. C, the extracellular Aβ(1–40) was quantified by ELISA in neuronal cultures treated with 5 mM LiCl, 25 μM SB415286 (SB) or co-treated with LiCl and SB415286 (LI + SB) for 24 h. Value (mean ± S.E.) are given as percentage of nontreated controls; n = 6; *p < 0.05 when compared with the control.

clearly understood. Inhibition of GSK3B is believed to reduce Aβ production (28, 29). On the other hand, knocking down by small interfering RNAs demonstrated that the reduction of GSK3α levels reduced Aβ production, whereas the reduction of GSK3β increased Aβ production (27). Because LiCl inhibits both GSK3 isoforms, this suggests that the effect of LiCl on Aβ production could be dissociated from its inhibition of GSK3. In addition to the inhibition of GSK3, lithium is known to display a number of other activities both in vitro and in vivo. Under certain circumstances the cell physiology may shift the balance toward either the suppressive GSK3-dependent or the stimulatory GSK3-independent effect of LiCl on Aβ production.

We showed that SB415286, a more selective inhibitor of GSK3 than is LiCl, only slightly decreased the production of Aβ. Combined treatment of neurons with LiCl and SB415286 showed cumulative effects. Therefore, these results demonstrate that the LiCl-mediated increase in Aβ production is independent of its GSK3 inhibition.

Alzheimer disease is characterized by the presence in the brain of both extracellular senile plaques and intraneuronal neurofibrillary tangles. The amyloid core of senile plaques contains the amyloid-β peptide, and neurofibrillary tangles are made of paired helical filaments containing the hyperphosphorylated microtubule-associated protein tau. To inhibit the formation of these typical lesions, the ideal therapeutic approach should prevent both amyloid and tau pathologies. Lithium was shown to inhibit the phosphorylation of different substrates of GSK3, including tau, as well as to decrease the production of amyloid-β peptide. Therefore, lithium could be considered as a powerful therapeutic agent for the treatment of Alzheimer disease. However, we have demonstrated in this paper that in two cell types expressing human APP695, lithium, when used at noncytotoxic concentrations, actually led to increased production of amyloid-β peptide by increasing the β-secretase activity. The prospect of using lithium in therapeutic approaches therefore needs substantial revision.

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