Neuroprotective effects of SB-415286 on hydrogen peroxide-induced cell death in B65 rat neuroblastoma cells and neurons

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Abstract

Glycogen synthase kinase-3 (GSK-3) is involved in the pathogenesis of several neurodegenerative diseases. In addition, as oxidative stress has been implicated in all neurodegenerative disorders, the inhibition of both pathways offers a potential strategy for preventing or delaying neurodegeneration. We examined the cytoprotective effects of lithium and SB-415286, two inhibitors of GSK-3, using a rat B65 cell line and also in cerebellar granule cells (CGN). H_2O_2 decreased the inactive form of GSK-3 (phospho-GSK-3 at Ser9), as measured by immunoblot experiments involving an antibody against the inactive form of the enzyme. Moreover, lithium inhibited this effect. While SB-415286 exerted a protective effect, lithium did not attenuate the toxic effects of H_2O_2 (1 mM). We then examined those mechanisms implicated in the protective effects of SB-415286. When we analyzed reactive oxygen species (ROS) production using the fluorescent probe 2,7-dichlorodihydrofluorescein diacetate in B65 cells, as well as in CGN, we found that SB-415286 strongly reduced DCF fluorescence. Lithium, however, did not exhibit any antioxidant properties. We conclude that the GSK-3 inhibitor SB-415286 has antioxidant properties, which may explain the cytoprotective effects against H_2O_2 damage. Furthermore, inhibition of GSK-3 activity was not involved in this protective effect.
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Keywords: SB-415286; Lithium; B65 Neuroblastoma cells; Cerebellar granule cells; H_2O_2; Oxidative stress

1. Introduction

Oxidative injury has been linked to a variety of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis, as well as conditions such as ischemia, bipolar disorders and excitotoxicity (Zhu et al., 2006, 2007; Moreira et al., 2005; Pallas and Camins, 2006; Machado-Vieira et al., 2007). Oxidative damage is mediated by ROS, which can be generated by an oxidative burst, or by the presence of excess free transition metals. H_2O_2 is widely used as a toxicant to establish in vitro models of oxidative stress-induced injury (Lee et al., 2007). Since ROS production is a component of neuronal cell demise via necrosis and apoptosis, antioxidant drug research remains an area of interest in neuroscience. Thus, although several clinical studies involving antioxidant drugs (e.g., vitamin E) have been carried out, these proved unsuccessful (Liu et al., 2007). One plausible explanation for the loss of efficacy in these antioxidant drugs is that blocking only a single path is insufficient for achieving effective neuroprotection.

An interesting factor involved in AD pathogenesis is the enzyme GSK-3 (Aghdam and Barger, 2007). Although, originally identified as a regulator of glycogen metabolism, GSK-3 is now known to play a role in the regulation of glucose secretion, tau phosphorylation, gene expression and apoptosis (Linseman et al., 2003; Jope and Johnson, 2004; Xie et al., 2004; Zhong et al., 2006; Jope et al., 2007; Yeste-Velasco et al.,

2007). Moreover, recent data suggest that this enzyme plays a role in the regulation of oxidative stress-induced neuronal cell death (Hongisto et al., 2003; Ghribi et al., 2003; Facci et al., 2003; Goodenough et al., 2004; Schafer et al., 2004; de Vasconcellos et al., 2006). Regarding the latter, several studies have been performed using different neurotoxins and stimuli, including rotenone (an inhibitor of mitochondria complex I) and glutamate (an excitatory amino acid); in both cases, GSK-3 was determined to play a part in cell loss (King and Jope, 2005; Lai et al., 2006). Likewise, GSK-3 has been implicated in the hyperphosphorylation of tau related to AD (Jin et al., 2005; Pallas and Camins, 2006). Consequently, GSK-3 was postulated to play a key role in a Parkinson's disease model involving endoplasmic reticulum stress (Youdim and Arraf, 2004; Yeste et al., 2007). Moreover, studies performed in GSK-3 transgenic mice confirmed the importance of this enzyme in AD pathology (Engel et al., 2006). Furthermore, a recent clinical study proposed the use of lithium for the treatment of AD, since a slight improvement in patient sintomatology was observed (Zhong and Lee, 2007). Although lithium currently remains the only GSK-3 inhibitor clinically administered to humans, this drug has shown undesirable effects. Thus, more specific and newer GSK-3 inhibitors have been synthesised. Those that have shown the most promise include molecules such as maleimide derivatives, namely SB-216763 and SB-415286, which are potent inhibitors of GSK-3. Moreover, recent studies suggest that these compounds could inhibit additional protein kinases (Bain et al., 2007). Indeed, these have exhibited neuroprotective properties *in vitro* against excitotoxicity and other apoptotic stimuli (Cross et al., 2001; Yeste-Velasco et al., 2007).

Therefore, numerous therapeutic agents regulating GSK-3 have been investigated for the treatment of neurodegenerative diseases (Coghlan et al., 2000; Cross et al., 2001; Facci et al., 2003).

In the present study, we examined the protective effects of lithium and SB-415286 on a rat neuroblastoma B65 cell line and also in CGN. We conclude that SB-415286 protects from H_2O_2 toxicity via a new mechanism: its antioxidant properties. This is the first study to describe these properties and to demonstrate that they constitute a protective path independent of GSK-3 inhibition. Finally, we propose that the dual effects afforded by this drug (i.e., its antioxidant and GSK-3 inhibitory properties) may make it suitable for the treatment of neurological disorders.

2. Methods

2.1. B65 neuroblastoma and neuronal cell cultures

Neuroblastoma B65 cell line was purchased from the European Collection of Cell Cultures (ECACC, Alisbury, UK). Cells were placed at 200 cells/mm² and cultured in DMEM media containing 10% FCS.

Primary cultures of cerebellar granule neurons were prepared from postnatal day 7 Sprague–Dawley rat pups as described previously (Verdaguer et al., 2004). Cells were dissociated in the presence of trypsin and DNase I and placed in poly-L-lysine (100 µg/ml)-coated dishes at a density of 8×10^5 cells/cm² in basal Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mg/ml gentamicin, 2 mM L-glutamine and 25 mM KCl. Cytosine-D-arabinofuranoside (10 µM) was added to the culture medium 24 h after plating to prevent the replication of non-neuronal cells. The cultures were maintained at 37 °C in a humidified incubator with 5% CO₂/95% air and left undisturbed until the experiments were performed. All procedures involving animals and their care were approved by the ethics committee of the University of Barcelona, and were conducted in accordance with national (Spanish) laws.

2.2. Assessment of cell viability

B65 cells were used after 24 h of in vitro culture. CGN were used after 7–8 days in vitro (Verdaguer et al., 2004). Lithium and SB-415286 were dissolved in culture media and DMSO, respectively, and added to the neuronal preparation at the precise concentrations, 1 h before addition H_2O_2 (50 µM to 1 mM). To assess the loss in cell viability, we used the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium] method. MTT was added to the cells at a final concentration of 250 µM and incubated for 1 h, allowing the reduction in MTT to produce a dark blue formazan product. Media were then removed, and cells were dissolved in dimethylsulfoxide. Formazan production was measured by the absorbency change at 595 nm using a microplate reader (BioRad Laboratories, CA, USA). Viability results were expressed as percentages. The absorbency measured from non-treated cells was taken to be 100%.

Cell death by LDH method, was determined 24 h after the addition of H_2O_2 using an LDH-cytotoxicity assay kit (Roche diagnostics following manufacture's protocol).

2.3. Measurements of reactive oxygen species

Levels of intracellular ROS were measured using the fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (H₂DCF-DA). Briefly, cells were incubated for 30 min at 37 °C in the presence of 10 µM of H₂DCFDA (added from a 20 mM stock solution in dimethyl sulfoxide). H₂DCFDA diffuses across neuronal membranes, where acetates migrate via intracellular esterases. Oxidation of H₂DCFDA occurs almost exclusively in the cytosol, generating a fluorescent response proportional to ROS generation. After loading the dye, neurons were washed in a Locke's buffer and fluorescence was measured at a 488 nm excitation wavelength and an emission wavelength of 510 nm, using a PerkinElmer Victor 3 fluorometer.

2.4. Western-blot analysis

Aliquots of cell homogenate containing 15 µg of protein per sample were analyzed by Western blot. Briefly, samples were placed in sample buffer (0.5 M Tris–HCl pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) 2-β-mercaptoethanol, 0.05% bromophenol blue) and denatured by boiling at 95–100 °C for 5 min. Samples were then separated by electrophoresis on 10% acrylamide gels, with proteins subsequently transferred to polyvinylidene fluoride sheets (ImmobilonTM-P, Millipore Corp., Bedford, MA, USA) using a transblot apparatus (BioRad). The membranes were blocked overnight with 5% non-fat milk dissolved in TBS-T buffer (Tris 50 mM; NaCl 1.5%; Tween 20, 0.05%, pH 7.5). They were then incubated with primary monoclonal antibodies against ser-9 GSK-3β (from Affinity BioReagents), pAkt (from Cell Signalling) and actin (1:1000, Santa Cruz Biotechnology). After 4 h or overnight, blots were washed thoroughly in TBS-T buffer and incubated for 1 h with a peroxidase-conjugated IgG antibody (Amersham Corp., Arlington Heights, IL, USA). Immunoreactive protein was visualized using a chemiluminescence-based detection kit and following the manufacturer's instructions (ECL kit; Amersham Corp.). Digital images were taken with a Chemidoc XRS (Biorad), which permits semiquantitation of band intensity. The protein load was periodically monitored via immunodetection of actin.

2.5. Statistical analysis

Data are given as the mean ± S.E.M. of at least four experiments involving four to six independent cultures. In all experiments, data were analyzed by ANOVA followed by post hoc Tukey-Kramer multiple comparisons tests. *P*-values lower that 0.05 were considered significant.

3. Results

3.1. Comparative effects of Li$^+$ and SB-415286 against H$_2$O$_2$-induced cytotoxicity

Light microscopic examination revealed that, compared to the untreated control (Fig. 1A), H$_2$O$_2$ caused progressive loss of cell morphology of B65 neuroblastoma cells. B65 cells were exposed to H$_2$O$_2$ (100–1000 μM) for 30 min and cell survival was assessed after 24 h, using the MTT method, which

measures the integrity of the cell membrane. Thus, decreased MTT reduction served as an indicator of cell injury resulting from mitochondrial damage. Specifically, the addition of H$_2$O$_2$ to B65 cells induced a significant decrease in cell survival in a dose-dependent manner, as measured by this method. For example, in the presence of H$_2$O$_2$ (for 24 h), the cell MTT viability values were as follows: 97.51% at 100 μM; 88.01% for 250 μM; 69.83% for 500 μM; 42.75% for 750 μM; 36.33% for 1 mM (Fig. 1B). When B65 cells were treated with H$_2$O$_2$ 1 mM in the presence (applied 30 min prior H$_2$O$_2$) of
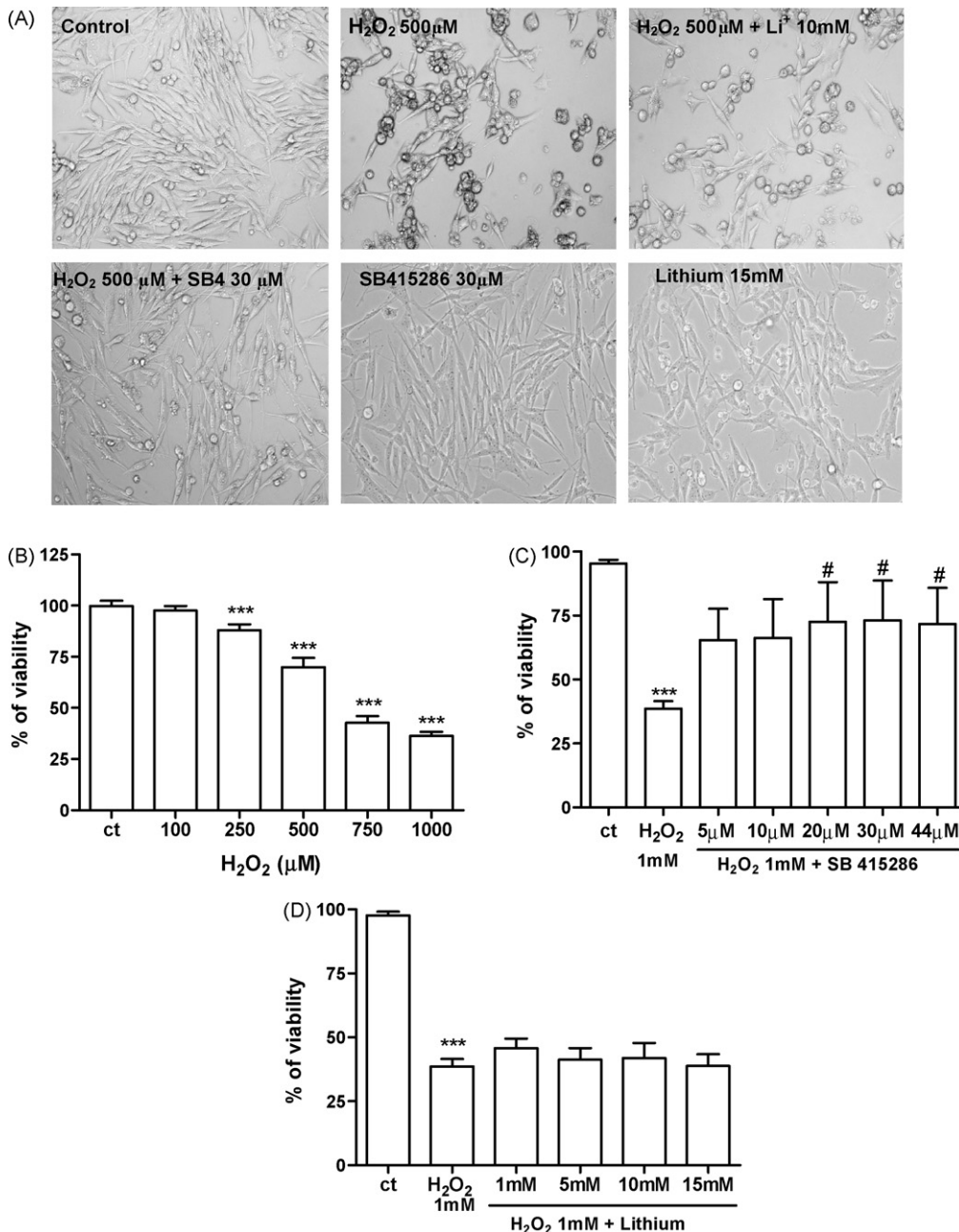

Fig. 1. (A) Morphological changes of B65 cells exposed to H$_2$O$_2$. Phase contrast microscopic analysis of B65 cells after 24 h treatment with 1 mM of H$_2$O$_2$ in the presence of Li$^+$ and SB-415286. Calibration bar, 10 μm. (B) Viability of B65 cells (% respect to the control cells) after treatment with different concentrations of H$_2$O$_2$. B65 cells were treated with H$_2$O$_2$ (100–1000 μM). Viability was assessed after 24 h by an MTT assay. (C) SB-415286 (5–44 μM) attenuated B65 cell loss mediated by 1 mM H$_2$O$_2$. (D) Effects of Li$^+$ (1–15 mM) on 1 mM H$_2$O$_2$-induced cell loss. Data are expressed as the mean $\pm$ S.D. from five independent experiments. $^*P < 0.05$ compared with H$_2$O$_2$-treated cells.

SB-415286 (5–44 μM), our results revealed that SB-415286 exerted a protective effect in a concentration-dependent manner (Fig. 1C). On the other hand, treatment of B65 cells with different concentrations of lithium (1–15 mM) did not protect against H_2O_2 toxicity (Fig. 1D). Furthermore, when we evaluated the protective effects of SB-415286 (5–44 μM) on B65 cells treated with 1 mM H_2O_2 using the LDH leakage method, we did indeed find such an effect (Fig. 2A), with a maximal rescue occurring at a concentration of 44 μM (Fig. 2B). However, Li^+ did not prevent the cytotoxic effects of H_2O_2, in agreement with the results obtained using the MTT method (data not shown).

CGN were treated with H_2O_2 concentrations ranging from 10 to 400 μM for 30 min at the end of which the agent was removed. We examined the cell viability at 24 h. H_2O_2 reduced CGN viability significantly in a concentration dependent manner, compared to the controls ($P < 0.01$) (Fig. 3A). The neuroprotective role of GSK-3 inhibitors against oxidative damage was investigated. Drugs were applied 30 min prior to the subsequent 1 h treatment with H_2O_2 in the continuing presence of the drugs. In the presence of SB-415286 (5–44 μM), the effect of 100 μM H_2O_2 was significantly attenuated ($P < 0.01$) (Fig. 3B). On the other hand, the significant reduction in viability with 100 μM H_2O_2 was unaltered in the presence of Li^+ (5–10 mM) (Fig. 3C).

3.2. Effects of Li^+ and SB-415286 in B65 cells on H_2O_2-induced increase in intracellular reactive oxygen species levels

In H2DCF-DA-loaded B65 neuroblastoma cells treated with H_2O_2 (100–1000 μM), fluorescence intensity increased in a dose-dependent manner, suggesting an increase in the generation of ROS (Fig. 4A). Exposure of B65 cells to the selective GSK-3 inhibitor SB-415286 (5–44 μM) caused a significant ($P < 0.01$) dose-dependent decrease in the fluorescence intensity of DCF (Fig. 4B). This result would indicate that the protective effects of SB-415285 against H_2O_2 toxicity are mediated by their antioxidant ability. On the other hand, treatment of B65 cells with lithium (1–15 mM) failed to prevent H_2O_2-mediated ROS production. These data would suggest that only SB-415286 possesses antioxidant properties (Fig. 4C).

3.3. Effects of Li^+ and SB-415286 in cerebellar granule neurons on H_2O_2-induced increase in intracellular reactive oxygen species

To evaluate whether the antioxidant effects of SB-415286 are cell dependent or stimuli dependent, we studied the effects of H_2O_2 in CGN (Fig. 5A). Thus, in Fig. 5B we show that SB-415286 exerts antioxidant effects in response to this stimulus in a dose-dependent manner. These data confirm the antioxidant properties of this drug obtained in B65 cells. On the other hand, cells treated with lithium (1–10 mM) failed to prevent H_2O_2-mediated ROS production (Fig. 5C).
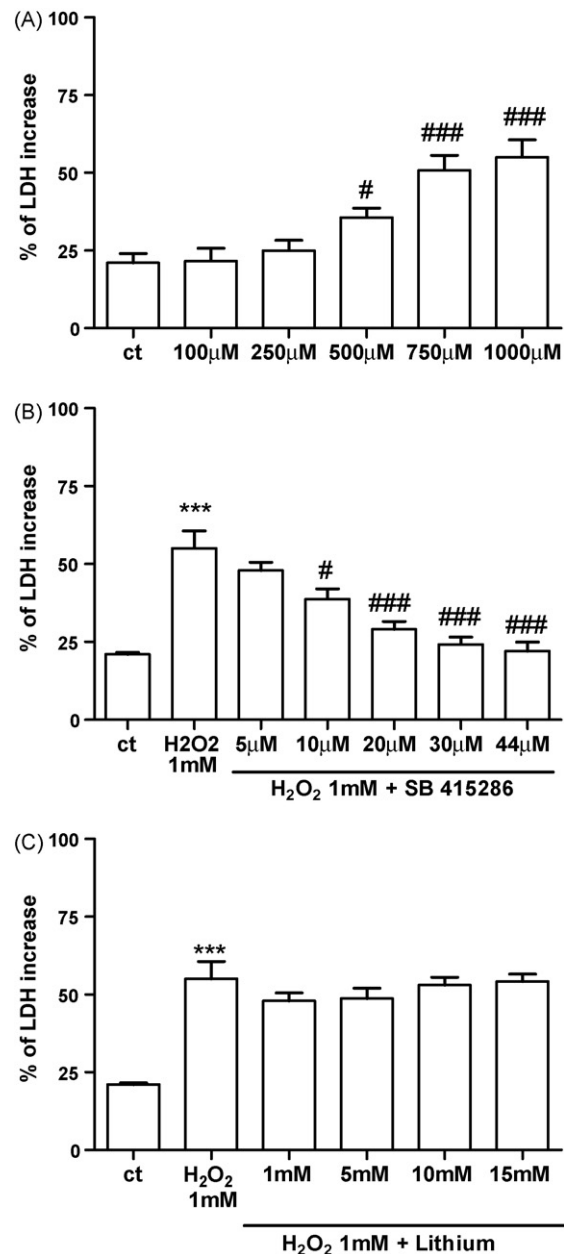

Fig. 2. (A) Viability of B65 cells after treatment with different concentrations of H_2O_2. B65 cells were treated with H_2O_2 (100–1000 μM). Viability was assessed after 24 h by an LDH assay. (B) SB-415286 (5–44 μM) attenuated B65 cell loss mediated by 1 mM H_2O_2. (C) Lithium did not prevent H_2O_2-induced cell loss. Data are expressed as the mean ± S.D. from five independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's tests: $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ compared with H_2O_2-treated cells.

3.4. Role of GSK-3 in H_2O_2-mediated ROS production

To determine whether lithium might inhibit GSK-3 activation in H_2O_2-treated cells, immunoblot experiments were performed using an antibody directed against phospho-GSK-3 (Ser9). This allowed us to detect levels of inactive (phosphorylated) GSK-3β. Following treatment with H_2O_2, phospho-GSK-3β levels decreased significantly within 24 h, which is consistent with rapid and prolonged GSK-3 activation (Fig. 6A). Lithium
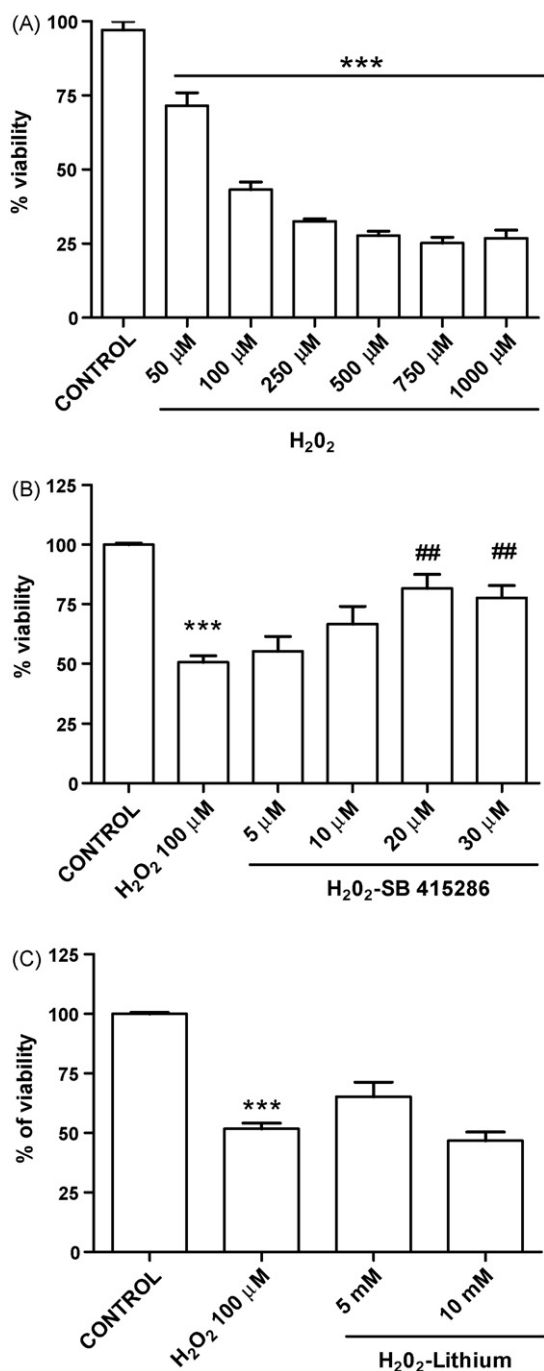
Fig. 3. (A) Viability of CGN after treatment with different concentrations of H$_2$O$_2$. CGN were treated with H$_2$O$_2$ (100–1000 μM). Viability was assessed after 24 h by an MTT assay. (B) SB-415286 (5–44 μM) attenuated cell loss mediated by 1 mM H$_2$O$_2$. (C) Effects of Li$^+$ (5–10 mM) on 1 mM H$_2$O$_2$-induced cell loss. Data are expressed as the mean ± S.D. from five independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's tests: $^{***}P < 0.001$ compared control cells. $^{###}P < 0.001$ compared with H$_2$O$_2$-treated cells.

Fig. 4. (A) ROS production in H$_2$O$_2$-treated B65 cells. ROS accumulation was detected after B65 cells were treated with 1 mM H$_2$O$_2$ for 1 h. ROS production was expressed as the percentage increase of control group. (B) SB-415286 (5–44 μM) attenuated B65 ROS production as mediated by 1 mM H$_2$O$_2$. (C) However, Li$^+$ (1–15 mM) did not attenuate ROS production mediated by 1 mM of H$_2$O$_2$. Data are expressed as the means ± S.E.M. of four independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's tests: $^{###}P < 0.001$ compared with H$_2$O$_2$-treated cells. $^{***}P < 0.001$ compared with control cells.

treatment completely prevented GSK-3 dephosphorylation. When lithium was added to B65 cultures, it was able to induce inhibitory phosphorylation of GSK-3 (Fig. 6B). As expected, SB-415286 failed to attenuate phospho-GSK-3 levels, since GSK-3 inhibition occurs via the ATP inhibition. These data are in agreement with previous studies performed with Li$^+$ in CGN
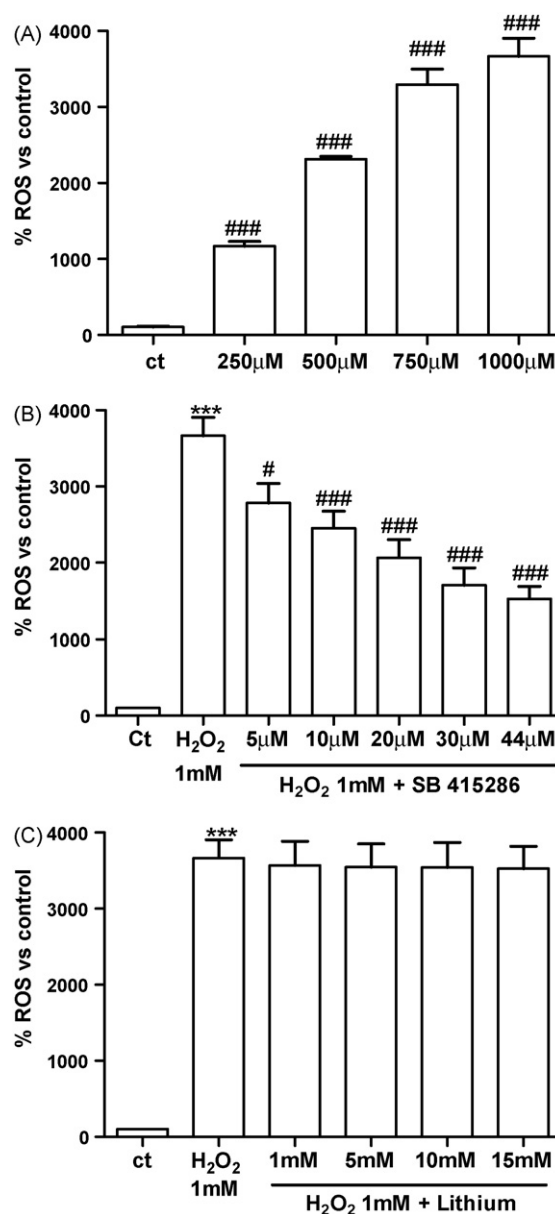
(Yeste-Velasco et al., 2007) Likewise, we also demonstrated that lithium was able to activate the Akt pathway. Therefore, we suggest that this pathway plays no role in the protective properties of SB-415286 (Fig. 7A and B).

4. Discussion

It is widely accepted that oxidative stress is one of the main factors involved in neuronal loss (Zhu et al., 2006, 2007;
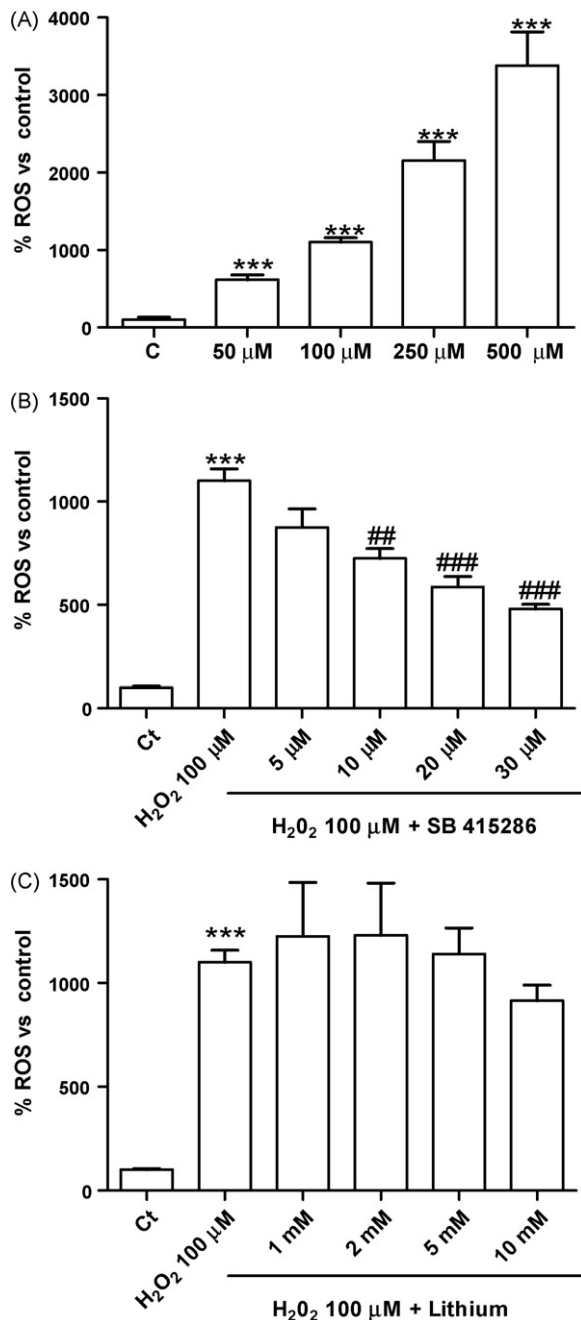
Fig. 5. (A) ROS production in H_2O_2-treated CGN. ROS accumulation was detected after CGN were treated with 1 mM H_2O_2 for 1 h. ROS production is expressed as the percentage increase of control group. Data are expressed as the mean ± S.D. from four independent experiments. $^{***}P < 0.001$ compared control cells. (B) SB-415286 (5–44 μM) attenuated ROS production in CGN mediated by 1 mM H_2O_2. Data are expressed as the means ± S.E.M. of four independent experiments. $^{\#\#}P < 0.05$; $^{\#\#\#}P < 0.001$ compared with H_2O_2-treated cells. (C) However, Li^+ (1–15 mM) did not attenuate ROS production mediated by 1 mM of H_2O_2.

Petersen et al., 2007). Thus, an effective neuroprotective drug must have antioxidant properties (Liu et al., 2007). In the current study, we present a new finding for the selective GSK-3 inhibitor SB-415286. Specifically, we demonstrate its antioxidant properties, which explain the protective effects afforded B65 cells and CGN against H_2O_2.
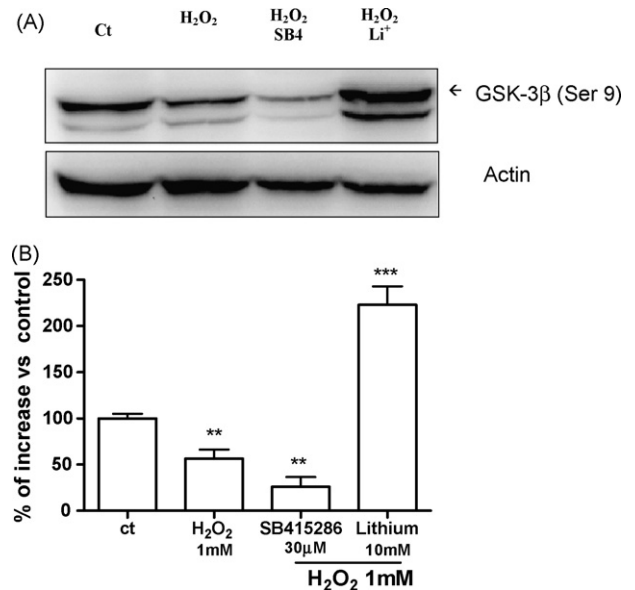

Fig. 6. (A) Treatment of B65 cells with 1 mM H_2O_2-induced GSK-3β activation, which was prevented by 10 mM Li^+. Changes in the band intensities were calculated as percentages of the control band intensity. (B) Columns and bars represent means ± S.E.M. of three or four separate experiments with four different culture preparations. Statistical significance was determined by one-way ANOVA followed by Tukey's tests: $^{***}P < 0.05$ vs. control and H_2O_2 treatment.

These data are of particular relevance since previous studies have widely implicated the activation of GSK-3 in AD pathology (Aghdam and Barger, 2007). Furthermore, research area of high interest in the treatment of neurodegenerative diseases is the synthesis and development of GSK-3 inhibitors.
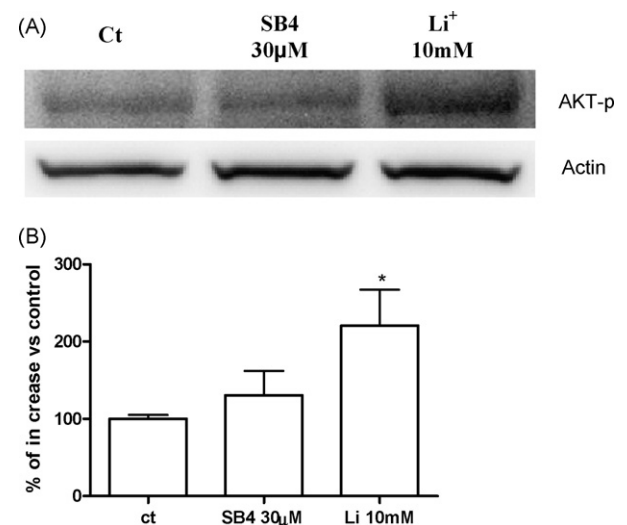

Fig. 7. (A) Western-blot analysis of the levels of loss of Akt phosphorylation, after treatment of B65 with H_2O_2, which was prevented by treatment cells with 10 mM Li^+. Cultures were treated with H_2O_2 alone for 24 h. At the end of the treatments, cells were lysed and the cell lysates were subjected to immunoblot analysis with an antibody directed against Akt-p (see Section 2). (B) Changes in the band intensities were calculated as percentages of the control band intensity. Columns and bars represent means ± S.E.M. of three or four separate experiments with four different culture preparations ($n = 4$). Statistical significance was determined by one-way ANOVA followed by Tukey's tests: $^*P < 0.05$ vs. control.

In this context, GlaxoSmithKline synthesized small selective maleimide derivatives, including SB-415286, which inhibits GSK-3 by preventing ATP binding (Facci et al., 2003). This drug may thus possess two potential mechanisms of action underlying its neuroprotective properties; namely, the inhibition of GSK-3, which not only prevents tau phosphorylation and apoptosis, but which has also shown antioxidant properties.

Under our experimental conditions, however, Li^+ failed to exert any antioxidant effects. Likewise, the antioxidant properties of Li^+, as well as the implication of GSK-3 in the regulation of antioxidant cell levels, remains an area of intensive research (Frey et al., 2006). Previous studies have demonstrated that, following chronic treatment of neuronal and neuronal-like cultures with Li^+, this ion exhibits significant antioxidant effects (Wang et al., 2004; Shao et al., 2005; Lai et al., 2006). Furthermore, this effect stems from the increased expression and activity of antioxidant enzymes and/or proteins. It is widely known that glutathione is the major antioxidant molecule in the brain and plays a key role in defending against oxidative damage. Thus, Li^+ increases glutathione levels in neurons, which may explain the antioxidant properties of this compound (Cui et al., 2007). However, in another study involving SH-SY5Y cells, Li^+ and other inhibitors of GSK-3 exhibited protective properties against complex I inhibitors (intrinsic oxidative stress), although only Li^+ protected against extrinsic oxidative stress (Schafer et al., 2004; King and Jope, 2005). Thus, these data suggest that GSK-3 inhibition is not involved in the antioxidant effects of Li^+ as mediated by H_2O_2.

Other studies involving HT22N neurons demonstrated that Li^+ exerts a protective, albeit slight, property against glutamate and H_2O_2 via the inhibition of GSK-3. In these experiments, acute treatment with Li^+ was carried out, revealing this neuroprotective effect to be independent of antioxidant enzyme modulation. Likewise, in PC 12 cells GSK-3 inhibition was associated with a neuroprotective effect mediated by the GSK-3 inhibitor II (Lee et al., 2007). Moreover, in glial cell lines chronic Li^+ or valproate treatment failed to ameliorate cytotoxicity induced by rotenone and H_2O_2 (Lai et al., 2006). Thus, it appears that the antioxidant properties of Li^+ are both cell- and stimuli-dependent.

In our cellular models, we demonstrated that the antioxidant properties of SB-415286 are independent of cell type and stimuli, since this drug exhibited antioxidant properties in B65 and CGN treated with H_2O_2. Moreover, our study suggests that the antioxidant effects of SB-415286 are probably independent of GSK-3 inhibition. Our hypothesis is based in the fact that while H_2O_2 activates GSK-3, Li^+ – in agreement with previous studies – inhibited the activation of this enzyme, thereby increasing the presence of the inactive form of GSK-3 (ser 9), although this did not prevent B65 cell loss. Another interesting point raised by our study is the evidence that lithium-induced Akt activation was unable to protect B65 cells from H_2O_2 toxicity. It is well known that the protein kinase Akt (also called protein kinase B) not only is activated by inositol lipids, but also is involved in cell survival via its capacity to phosphorylate several substrates, including GSK-3, BAD, CREB and others (Jope et al., 2007).

In summary, our findings have demonstrated a new pharmacological aspect of the protective effects provided by the GSK-3 inhibitor SB-415386: its antioxidant properties.

Acknowledgements

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