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Interleukin-6-induced S100B secretion is inhibited by haloperidol and risperidone

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ABSTRACT

Although inflammation may be a physiological defense process, imbalanced neuroinflammation has been associated with the pathophysiology of brain disorders, including major depression and schizophrenia. Activated glia releases a variety of pro-inflammatory cytokines that contribute to neuronal dysfunction. Elevated levels of S100B, a glia derived protein, have been observed in the serum and CSF of schizophrenic patients suggesting a glial role in the disease. We evaluated whether S100B secretion (in C6 glioma cells and hippocampal slices in Wistar rats) could be directly modulated by the main inflammatory cytokines (IL-1β, TNF-α, IL-6 and IL-8) altered in schizophrenia, as well as the possible involvement of mitogen-activated protein kinase (MAPK) pathways in these responses. We also investigated the effects of typical and atypical antipsychotic drugs on glial cytokine-induced S100B release. Our results suggest that S100B secretion is increased by pro-inflammatory cytokines via MAPK and that oxidative stress may be a component of this modulation. These results reinforce the idea that the S100B protein is involved in the inflammatory response observed in many brain diseases, including schizophrenia. Moreover the antipsychotics, haloperidol and risperidone, were able to inhibit the secretion of S100B following IL-6 stimulation in C6 glioma cells.

1. Introduction

Neuroinflammation plays a critical role in different neurological and psychiatric diseases. Although inflammation may be a physiological defense process, beneficial for repair and recovery of the central nervous system (CNS) (Wee Yong, 2010), imbalanced inflammation has been associated with the pathophysiology of several brain disorders, including Alzheimer's disease, major depression and schizophrenia (Akiyama et al., 2000; Garate et al., 2011; Monji et al., 2009). The inflammatory response in the CNS includes a wide spectrum of complex and integrated cellular responses, such as microglial and astroglial activation. These glial cells, when activated, releases a variety of pro-inflammatory mediators (e.g. cytokines) which can potentially contribute to neuronal dysfunction and result in the progression of the CNS pathology (Khansari et al., 2009).

Cytokines are involved in regulating the communication between immune cells, thus, high peripheral and CNS cytokine levels indicate activation of the inflammatory response. However, these cytokines modulate not only normal CNS function; abnormal cytokine signaling may contribute to major acute and chronic CNS diseases (Tansey, 2010). Increased levels of pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-6, have been found in individuals with epilepsy and autism (Mansur et al., 2012) and levels of IL-2 andIL-8 may also be elevated in schizophrenia (Zhang et al., 2002). In fact, meta-analyses have shown consistent alterations of increased IL-1β, IL6, and TNF-α levels in the serum of schizophrenic patients (Miller et al., 2011; Potvin et al., 2008). Furthermore, animals models using iodinated LPS in pregnant rats show that the development of schizophrenia-like manifestations in offspring rats occurs, most probably, due to the effect of cytokines, since cytokines and not LPS can cross the placenta (Ashdown et al., 2006).

S100B, a calcium-binding protein belonging to the S100 family of proteins in the CNS is predominantly expressed and secreted by astrocytes (Donato, 2001; Donato et al., 2009), exerting paracrine and autocrine effects on neurons and glial cells. This protein may cross the blood–brain barrier and concentrations of S100B determined in serum and/or cerebrospinal fluid (CSF) appear to reflect the degree of injury in brain disorders (Pleines et al., 2001). As such, S100B has been proposed as a marker of brain damage (Goncalves et al., 2008; Marchi et al., 2004). S100B seems to be able to modulate cytokine secretion and may also be modulated by pro-inflammatory cytokines (Edwards and Robinson, 2006; Schmitt et al., 2007). Moreover, some studies have demonstrated a significant correlation between

Abbreviations: CSF, cerebrospinal fluid; DCF, 2′–7′-dichlorofluorescein; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase, one of the three members of the MAPK; GSH, glutathione; GFAP, glial fibrillary acidic protein; IL-1β, interleukin-1β; IL-2, interleukin-2; IL-6, interleukin-6; IL-8, interleukin-8; JNK, c-Jun N-terminal kinase, another of three members of the MAPK; LDH, lactate dehydrogenase; LPS, lipopolysaccharides; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MTT, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NF-κB, nuclear factor kappa B; p38, third member of the MAPK; TNF-α, tumor necrosis factor-α; TRL4, toll-like receptor 4.

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S100B and pro-inflammatory molecules in neurological and psychiatric disorders (Ashraf et al., 1999; Steiner et al., 2009). However, the idea that S100B may be considered to be a cytokine remains controversial.

Our previous reports have suggested that S100B may be an important component in neuroinflammation. We have demonstrated that S100B secretion is induced by IL-1β, mediated by MAPK–ERK signaling, in astrocyte cultures, C6 glioma cells and acute hippocampal slices (de Souza et al., 2009). More recently, we showed that S100B secretion is stimulated by intracerebroventricular lipopolysaccharide (LPS) administration and that LPS directly stimulates the secretion of S100B from astrocytes and hippocampal slices (Guerra et al., 2011).

The presence of elevated S100B levels, observed in the serum and CSF of schizophrenic patients (Lara et al., 2001; Rothermundt et al., 2004; Steiner et al., 2006), as well as the association of the schizophrenia with certain haplotypes of the S100B gene involved in increased S100B expression (Liu et al., 2005), provides further evidence that a dysfunction of glia cells might present a pathogenic factor in schizophrenia. For this reason, C6 glioma cells have been investigated as targets of antipsychotic medication (Nardin et al., 2011; Steiner et al., 2010b). We herein evaluate whether S100B secretion could be directly modulated by the main inflammatory cytokines associated with the pathophysiology of schizophrenia; IL-1β, TNF-α, IL-6 and IL-8 in C6 glioma cells and acute hippocampal slices, as well as the possible involvement of MAPK pathways in these responses. We also investigate the effects of these cytokines on GFAP and oxidative stress. Finally, we explore the effects of typical and atypical antipsychotic drugs on glial cytokine-induced S100B release.

2. Material and methods

2.1. Animals

For the preparation of hippocampal slices, twelve male 30-day old Wistar rats were obtained from our breeding colony (Department of Biochemistry, UFRGS, Porto Alegre, Brazil), were maintained under controlled light and environmental conditions (12 h light/12 h dark cycle at a constant temperature of 22 ± 1 °C), and had free access to commercial chow and water. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23) revised in 1996 and followed the regulations of the local animal housing authorities.

2.2. Materials

Dulbecco's modified Eagle's medium (DMEM) and other materials for cell culture were purchased from Gibco (Belo Horizonte, Brazil). Interleukin-1beta (IL-1β), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), S100B protein, anti-S100B antibody (SH-B1), o-phenylenediamine (OPD), Triton X-100, methylthiazolyldiphenyl-tetrazolium bromide (MTT), Griess reagent (modified), standard glutathione, o-phthaldialdehyde and dichlorofluoresceindiacetate (DCF-DA) were purchased from Sigma (St. Louis, MO, USA). Polyclonal anti-S100B was purchased from DAKO (São Paulo, Brazil), anti-GFAP from rabbit and anti-rabbit linked to peroxidase were purchased from GE (Little Chalfont, United Kingdom). Other reagents were purchased from local commercial suppliers (Sulquímica, Labsul or Biogen; Porto Alegre, Brazil).

2.3. Preparation and incubation of brain slices

Rats were decapitated, their hippocampi were quickly dissected out and transverse sections (300 μm) were rapidly obtained using a McIlwain tissue chopper. One slice was placed into each well of a 24-well culture plate. Slices were incubated in oxygenated physiological medium containing, in mM, 120 NaCl, 2.0 KCl, 1.0 $CaCl_2$, 1.0 MgSO4, 25.0 Hepes, 1.0 KH_2PO_4 and 10.0 glucose, pH 7.4, at room temperature. The medium was changed every 15 min for fresh medium. Following a 120-min equilibration period, slices were incubated in medium plus IL-1β, TNF-α, IL-6 or IL-8 (0.1, 1 or 10 ng/mL) for 1 h at 30 °C.

2.4. C6 glioma cell culture

A rat glioblastoma cell line (C6 cells) was obtained from the American Type Culture Collection (Rockville, Maryland, USA). Late passage cells (i.e. after at least 100 passages) were seeded in 24-well plates at densities of 104 cells/well, and cultured in DMEM (pH 7.4) supplemented with 5% fetal bovine serum, 2.5 mg/mL Fungizone® and 100 U/L gentamicin in 5% CO_2/95% air at 37 °C. After the cells had reached confluence, the culture medium was replaced by DMEM without serum in the absence or presence of IL-1β, TNF-α, IL-6 or IL-8 (0.1, 1 or 10 ng/mL). The inhibitors, PD98059 (10 μM, MEK inhibitor), SB203580 (10 μM, p38 inhibitor), SP600125 (10 μM, JNK inhibitor) or SN-50 (50 μg/mL, NF-κB inhibitor), as well as the antipsychotics, haloperidol (0.3 μM), clozapine (3 μM) or risperidone (70 nM), were added 15 min before cytokine exposure.

2.5. ELISA for S100B

The S100B concentration was determined in the culture medium at 15 min, 1, 6, and 24 h; and in the incubation medium of slices at 1 h. Cells were washed and scraped at 24 h for measurement of intracellular S100B content. S100B levels were determined by ELISA, as described previously (Leite et al., 2008). Briefly, 50 μL of sample plus 50 μL of Tris buffer were incubated for 2 h on a microtiter plate, previously coated with anti-S100B monoclonal antibody (SH-B1, from Sigma). Anti-S100 polyclonal antibody (from DAKO) was incubated for 30 min and then peroxidase-conjugated anti-rabbit antibody was added for a further 30 min. The color reaction with o-phenylenediamine was measured at 492 nm. The standard S100B curve ranged from 0.002 to 1 ng/mL.

2.6. ELISA for GFAP

ELISA for GFAP was carried out by coating the microtiter plate with 100 μL samples containing 20 ng of protein for 24 h at 4 °C. Incubation with a polyclonal anti-GFAP from rabbit for 1 h was followed by incubation with a secondary antibody conjugated with peroxidase for 1 h, at room temperature. A colorimetric reaction with o-phenylenediamine was measured at 492 nm. The standard human GFAP (from Calbiochem) curve ranged from 0.1 to 5 ng/mL.

2.7. Immunocytochemistry and cell morphology

C6 glioma cells were cultured on circular glass cover slips. After 1 h of IL-6 exposure, cells were fixed for 20 min with 4% paraformaldehyde in phosphate buffer (PBS, mM): 2.9 KH_2PO_4; 38 $Na_2HPO_47H_2O$; 130 NaCl; 1.2 KCl, rinsed with PBS and permeabilized for 20 min in PBS containing 0.2% Triton X-100. Fixed cells were then blocked for 60 min with PBS containing 5% bovine serum albumin and incubated overnight with anti-S100B monoclonal antibody from mouse (SH-B1), 1:500 or polyclonal anti-GFAP from rabbit, 1:500. Following incubation with primary antibodies, the cultures were washed in PBS/triton 0.2% (3 × 5 min) and incubated for 2 h with the respective secondary antibody at a 1:1000 dilution: Alexa Fluor 528 (goat anti-mouse-IgG; red fluorescence) and Alexa 488 (goat anti-rabbit-IgG; green fluorescence).

2.8. MTT reduction assay

Cells were treated with 50 μg/mL methylthiazolyldiphenyl-tetrazolium bromide (MTT) for 30 min in 5% CO_2/95% air at 37 °C.

Subsequently, the media were removed and MTT crystals were dissolved in DMSO. Absorbance values were measured at 560 and 650 nm. The reduction in MTT was calculated as (absorbance at 560 nm) − (absorbance at 650 nm).

2.9. Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase assay was carried out in 50 μL of extracellular medium, using a commercial colorimetric assay from Doles (Goiânia, Brazil).

2.10. Evaluation of intracellular reactive oxygen species (ROS) production

Intracellular ROS production was detected using the non-fluorescent cell permeating compound, 2′–7′-dichlorofluorescin diacetate (DCF-DA). DCF-DA is hydrolyzed by intracellular esterases and then oxidized by ROS to a fluorescent compound, 2′–7′-dichlorofluorescein. C6 cells were treated with DCF-DA (10 μM) for 30 min at 37 °C and rinsed with DMEM without serum. Following DCF-DA exposure, the cells were rinsed and then scraped into PBS with 0.2% Triton X-100. Fluorescence was measured with a spectrophotometer plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm.

2.11. Glutathione (GSH) Content Assay

GSH levels (nmol/mg protein) were measured, as described previously (Browne and Armstrong, 1998). C6 glioma cell homogenates were diluted in 10 volumes of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA and protein was precipitated with 1.7% meta-phosphoric acid. Supernatant was assayed with o-phthaldialdeyde (1 mg/mL methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions (0–500 μM).

2.12. Nitric oxide (NO) production

NO was determined by measurement of nitrite (a stable oxidation product of NO), based on the Griess reaction. The Griess reagent was prepared by mixing equal volumes of 1% sulfanilamide in 0.5 N HCl and 0.1% N-(1-naphthyl) ethylenediamine in deionized water. The assay was performed as described (Hu et al., 1996) with modifications. Briefly, cells were cultured on 96-well plates and, after treatment, the Griess reagent was added directly to the cell culture and the incubation was maintained under reduced light conditions at room temperature for 15 min. Samples were analyzed at 550 nm on a microplate spectrophotometer. Controls and blanks were run simultaneously. Nitrite concentrations were calculated using a standard curve prepared with sodium nitrite (0–50 μM).

2.13. Protein determination

Protein content was measured by Lowry's method using bovine serum albumin as standard (Peterson, 1977).

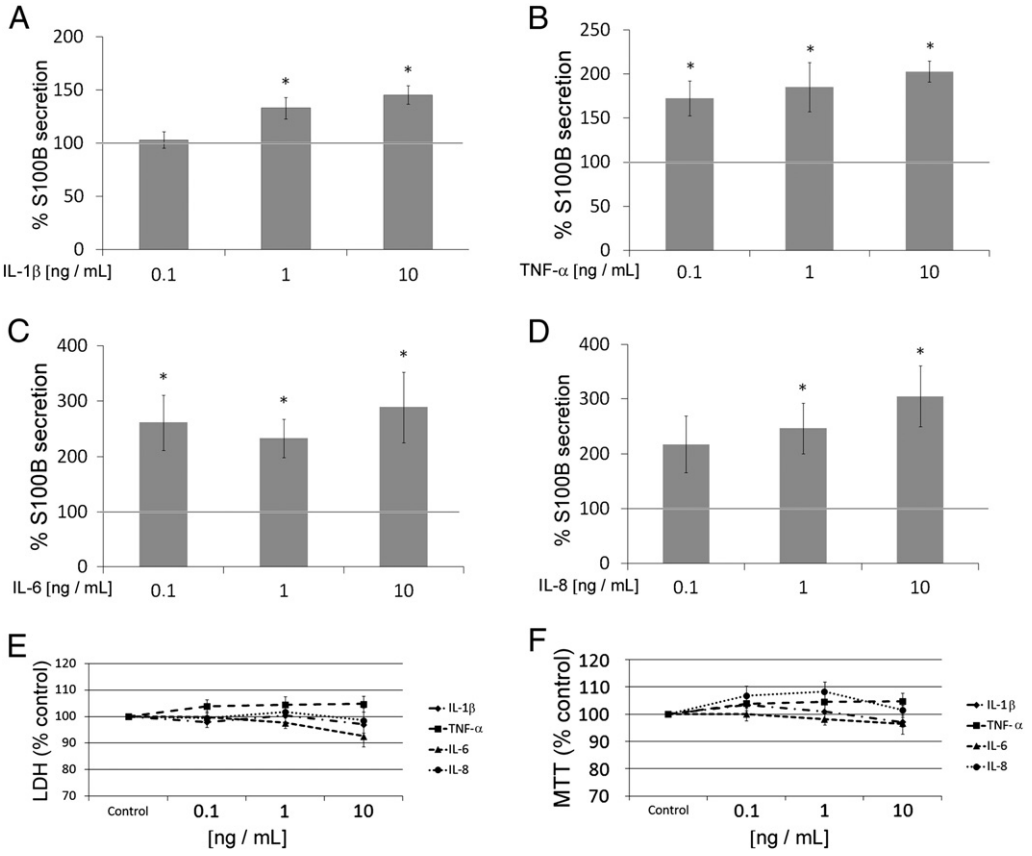

Fig. 1. Cytokines increase S100B secretion in acute hippocampal slice preparations. Hippocampal slices from 30-day old Wistar rats were exposed to IL-1β (A), TNF-α (B), IL-6 (C) or IL-8 (D) at concentrations ranging from 0 to 10 ng/mL for 1 h; S100B was measured by ELISA. The line indicates basal secretion, assumed as 100% in each experiment. The basal levels of S100B ranged from 0.1 to 0.24 ng/mg total protein. Cell integrity and viability, LDH release (E) and capacity for MTT reduction (F) were analyzed, respectively. Each value represents the mean (± standard error) of 6 independent experiments performed in triplicate. * Significantly different from basal secretion (one way ANOVA followed by Dunnett's test, with a significance level of $p<0.05$).

2.14. Statistical analysis

Parametric data are reported as means ± standard error and were analyzed by one-way ANOVA (followed by Tukey's or Dunnett's test). Values of $p<0.05$ were considered to be significant.

3. Results

Cytokines increase S100B secretion in acute hippocampal slice preparations. Different concentrations of IL-1β, TNF-α, IL-6 and IL-8 (from 0.01 to 10 ng/mL) were added to hippocampal slices and extracellular S100B was measured at 1 h (Fig. 1A–D). Both IL-6 and TNF-α were able to increase S100B secretion at concentrations of between 0.1 and 10 ng/mL ($p<0.01$, ANOVA). However, IL-8 and IL-1β increased S100B secretion only at concentrations of 1 and 10 ng/mL ($p<0.05$, ANOVA). No effects were observed at cytokine concentrations of 0.01 ng/mL (data not shown). To make sure that we were determining S100B secretion, we evaluated cell integrity and viability in our preparations by measuring LDH release and capacity for MTT reduction, respectively. No changes in the MTT reduction assay (Fig. 1E, $p=0.58$) or LDH release were observed (Fig. 1F, $p=0.39$).

S100B secretion is also acutely modulated by cytokines in C6 glioma cells. In order to investigate a direct effect of cytokines on glial cells, we added different concentrations of cytokines (from 0.1 to 10 ng/mL) to C6 glioma cell cultures and extracellular S100B was measured at 1, 6 and 24 h (Fig. 2). All cytokines, at higher concentrations (10 ng/mL), increased S100B secretion at 1 h, but not at 6 h or 24 h afterwards. This acute effect also was observed with the cytokine concentration of 1 ng/mL in C6 glioma cells (except with TNF-α) and no effect was observed at the cytokine concentration of 0.1 ng/mL (except with TNF-α). Similar assays were also carried out in all C6 glioma preparations, confirming cell viability and integrity (data not shown).

Cytokine-stimulated S100B secretion occurs via the MAPK pathway. To assess the role of the MAPK signaling pathway in the cytokine-stimulated secretion of S100B, specific inhibitors of this pathway were added to the C6 glioma culture. The ERK pathway inhibitor, PD98059 (Fig. 3) ($p<0.0001$, ANOVA), the p38 pathway inhibitor, SB203580 (Fig. 4) ($p<0.001$, ANOVA), and the JNK pathway inhibitor, SP600125 (Fig. 5) ($p<0.001$, ANOVA), were all able to prevent cytokine-stimulated S100B secretion, without affecting basal S100B secretion. A possible involvement of NF-κB mediating S100B secretion was investigated using a specific inhibitor of NF-κB, SN-50 peptide (Fig. 6). SN-50 was unable to prevent cytokine-stimulated S100B secretion. Importantly, this inhibitor *per se* increased S100B secretion ($p<0.05$, ANOVA) and therefore it is difficult to affirm whether this inhibitor prevented, or not, the effect induced by cytokines.

IL-6 increases S100B and GFAP content at 24 h in glioma cells. S100B and GFAP content in C6 glioma cells were measured after 24 h of exposure to cytokines (IL-1β, TNF-α, IL-6 and IL-8; 10 ng/mL) (Fig. 7A and B, respectively). S100B intracellular content was increased only by IL-6 ($p<0.05$, ANOVA). On the other hand, an increase in GFAP content was observed with all cytokines, except TNF-α ($p<0.01$, ANOVA). The increment in both proteins induced by IL-6 was confirmed by immunocytochemistry, without changes in cell morphology (Fig. 7C).

Signals of oxidative stress are observed in C6 glioma cells exposed to cytokines for 1 h or 24 h. Acute exposure of cytokines (IL-1β, TNF-α, IL-6 and IL-8; 10 ng/mL for 1 h) increased DCF levels (Fig. 8A, $p<0.005$), with the exception of IL-8 ($p=0.077$) and did not change NO levels (Fig. 8B, $p=0.63$). After cytokine exposure for 24 h, we observed a decrease in intracellular content of glutathione in cell cultures treated with IL-1β and IL-8 (Fig. 8C, $p<0.005$).

The increase in S100B secretion, induced by IL-6 in C6 glioma cells, is prevented by antipsychotics. In order to evaluate a protective effect of antipsychotics on S100B secretion induced by IL-6, we added haloperidol (typical antipsychotic), clozapine and risperidone (atypical

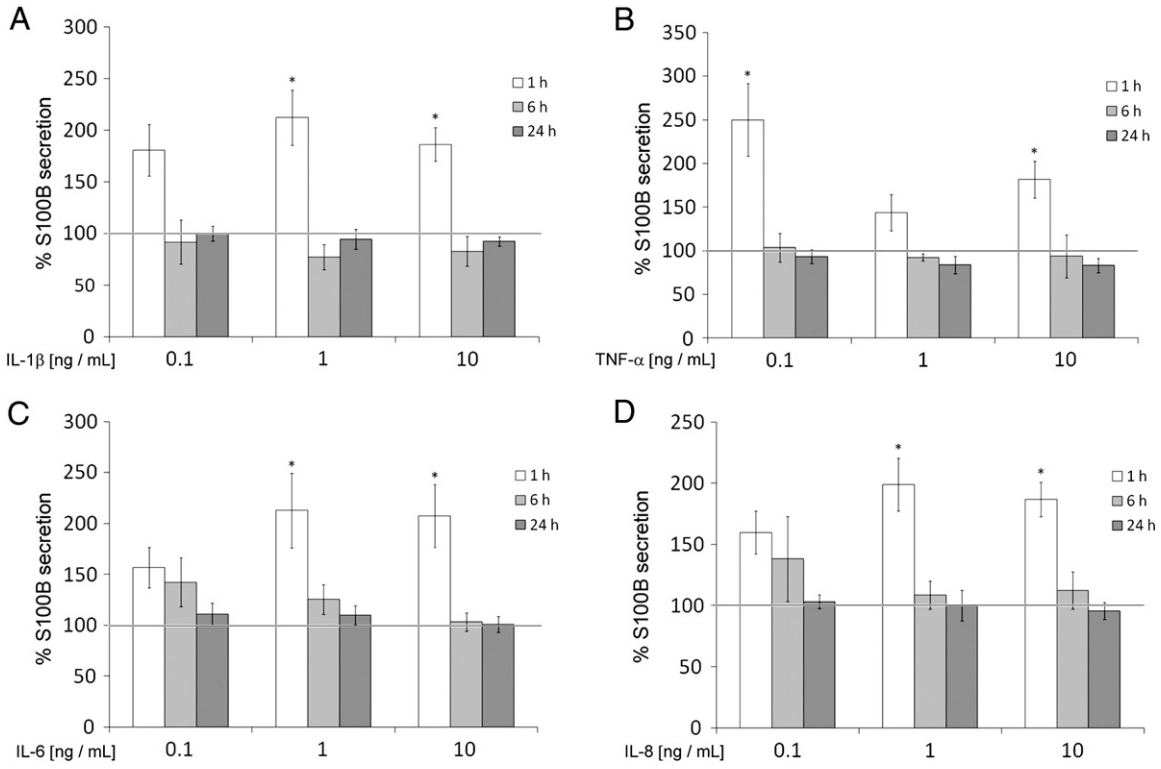

Fig. 2. S100B secretion is also acutely modulated by cytokines in C6 glioma cells. S100B secretion at 1, 6 and 24 h in C6 glioma cells exposed to IL-1β, TNF-α, IL-6 or IL-8 (in A, B, C and D, respectively). The line indicates basal secretion, assumed as 100% in each experiment. The basal levels of S100B ranged from 0.08 to 0.12 ng/mg total protein. Each value is the mean (± standard error) of 6 independent experiments performed in triplicate. * Significantly different from basal secretion (one way ANOVA followed by Dunnett's test, with a significance level of $p<0.05$).

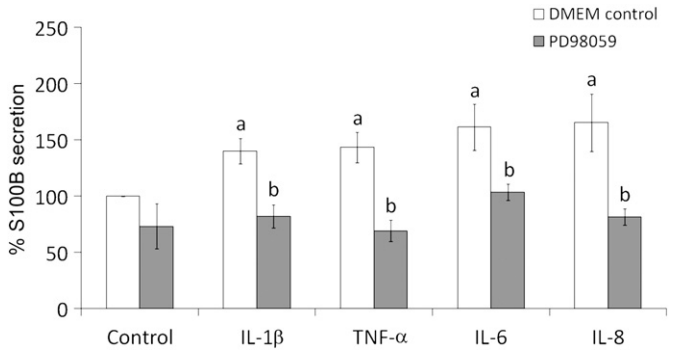

Fig. 3. Effect of a MAPK/ERK pathway inhibitor on cytokine-induced S100B secretion. C6 glioma culture cells were exposed to 10 ng/mL IL-1β, TNF-α, IL-6 or IL-8 for 1 h, in the presence (or not) of 10 μM PD98059 (MAPK/ERK inhibitor), added 15 min before cytokine exposure. S100B content in the medium was measured by ELISA. Each value is the mean (± standard error) of 6 independent experiments performed in triplicate. Means indicated by different letters are significantly different. [a] Significantly different from basal secretion. [b] Significantly different from cytokine-induced secretion (one way ANOVA followed by Tukey's test, with a significance level of $p<0.05$).

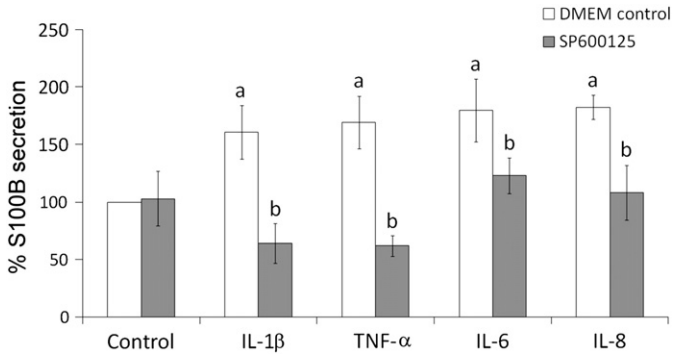

Fig. 5. Effect of a MAPK/JNK pathway inhibitor on cytokine-induced S100B secretion. C6 glioma culture cells were exposed to 10 ng/mL IL-1β, TNF-α, IL-6 or IL-8 for 1 h, in the presence (or not) of 10 μM SP600125 (MAPK/JNK inhibitor), added 15 min before cytokine exposure. S100B content in the medium was measured by ELISA. Each value is the mean (± standard error) of 6 independent experiments performed in triplicate. Means indicated by different letters are significantly different. [a] Significantly different from basal secretion. [b] Significantly different from cytokine induced secretion (one way ANOVA followed by Tukey's test, with a significance level of $p<0.05$).

antipsychotics) (Fig. 9). Both haloperidol and risperidone abolished IL-6-induced S100B secretion ($p<0.0001$). Clozapine did not change the effect of IL-6 on S100B secretion ($p=0.474$). Interestingly, risperidone *per se* increased basal S100B secretion. Moreover, risperidone prevented the IL-6 induced S100B secretion, resulting in secretion to levels that were lower than those at basal conditions.

Acute ROS production induced by IL-6 is prevented by haloperidol. To assess the effect of antipsychotic drugs in intracellular ROS production on C6 glioma cells exposed to IL-6, we added haloperidol, risperidone and clozapine (Fig. 10). Haloperidol abolished IL-6-induced ROS production ($p<0.05$). Neither clozapine nor risperidone were able to prevent the effect of IL-6 on DCF levels ($p=0.59$ and $p=0.32$, respectively). Moreover, risperidone and clozapine *per se* acutely increased basal DCF levels ($p<0.001$ and $p<0.05$, respectively).

4. Discussion

Several lines of evidence indicate that glial dysfunction could be a pathogenic factor in schizophrenia. Levels of S100B, a protein expressed and secreted by glial cells, astrocytes (mainly) and oligondrocytes (Donato et al., 2009; Steiner et al., 2008), are altered in the serum and CSF of schizophrenic patients, reinforcing this idea (Schroeter and Steiner, 2009). The mechanism of S100B secretion is unknown, but neurotransmitters including glutamate (Buyukuysal, 2005; Goncalves et al., 2000; Tramontina et al., 2006), serotonin (Tramontina et al., 2008), dopamine (Nardin et al., 2011) and acetylcholine (P. Lunardi, unpublished data) are able to modulate its secretion in cell cultures and acute brain slices.

However, increments in serum and CSF S100B in schizophrenic patients cannot be explained exclusively by changes in the profile of S100B secretion, caused by altered neurotransmission. For example, assuming that D2 receptors in astrocytes of these preparations are negatively coupled to adenylcyclase, a decrease in S100B secretion would be expected (Nardin et al., 2011). Recent findings, in animal models and human studies, have provided cumulative evidence for the cytokine hypothesis of schizophrenia (Watanabe et al., 2010). In addition, a correlation between S100B and pro-inflammatory molecules has been demonstrated in neurological and psychiatric disorders (Ashraf et al., 1999; Pleines et al., 2001; Potvin et al., 2008). Supporting an inflammatory role for S100B, we have observed changes in S100B secretion in response to interleukin-1β (de Souza et al., 2009) and activation of TLR4 (Guerra et al., 2011). Furthermore, inflammatory cytokines were found to stimulate S100B secretion in hippocampal slices and this effect was confirmed in C6 glioma cells. The effect on S100B secretion was acute, since no effect was observed at 6 or 24 h after cytokine exposure in cell cultures. We previously demonstrated cytokine-mediated modulation of S100B secretion in primary astrocytes and C6 glioma cells (de Souza et al., 2009). Griffin et al. (1998) previously reported an increase in S100B

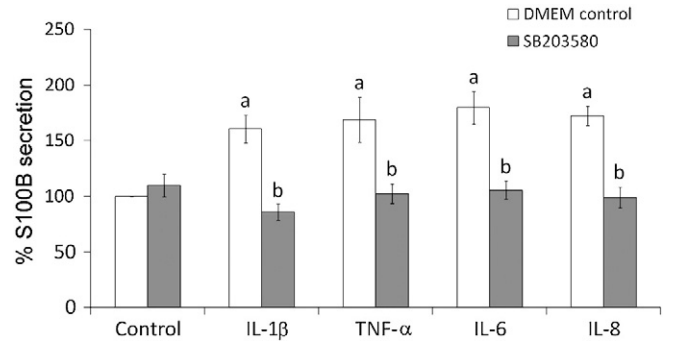

Fig. 4. Effect of a MAPK/p38 pathway inhibitor on cytokine-induced S100B secretion. C6 glioma culture cells were exposed to 10 ng/mL IL-1β, TNF-α, IL-6 or IL-8 for 1 h, in the presence (or not) of 10 μM SB203580 (MAPK/p38 inhibitor), added 15 min before cytokine exposure. S100B content in the medium was measured by ELISA. Each value is the mean (± standard error) of 6 independent experiments performed in triplicate. Means indicated by different letters are significantly different. [a] Significantly different from basal secretion. [b] Significantly different from cytokine induced secretion (one way ANOVA followed by Tukey's test, with a significance level of $p<0.05$).

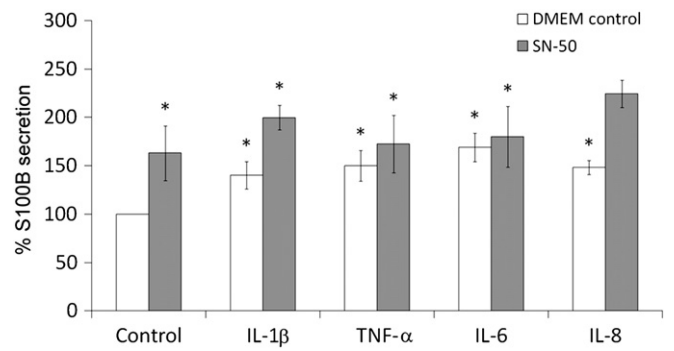

Fig. 6. Effect of a NF-κB inhibitor on cytokine-induced S100B secretion. C6 glioma culture cells were exposed to 10 ng/mL IL-1β, TNF-α, IL-6 or IL-8 for 1 h, in the presence (or not) of 50 μg/mL SN-50 (NF-κB inhibitor), added 15 min before cytokine exposure. S100B content in the medium was measured by ELISA. Each value is the mean (± standard error) of 6 independent experiments performed in triplicate. Means indicated by different letters are significantly different. * Significantly different from basal secretion (one way ANOVA followed by Tukey's test, with a significance level of $p<0.05$).

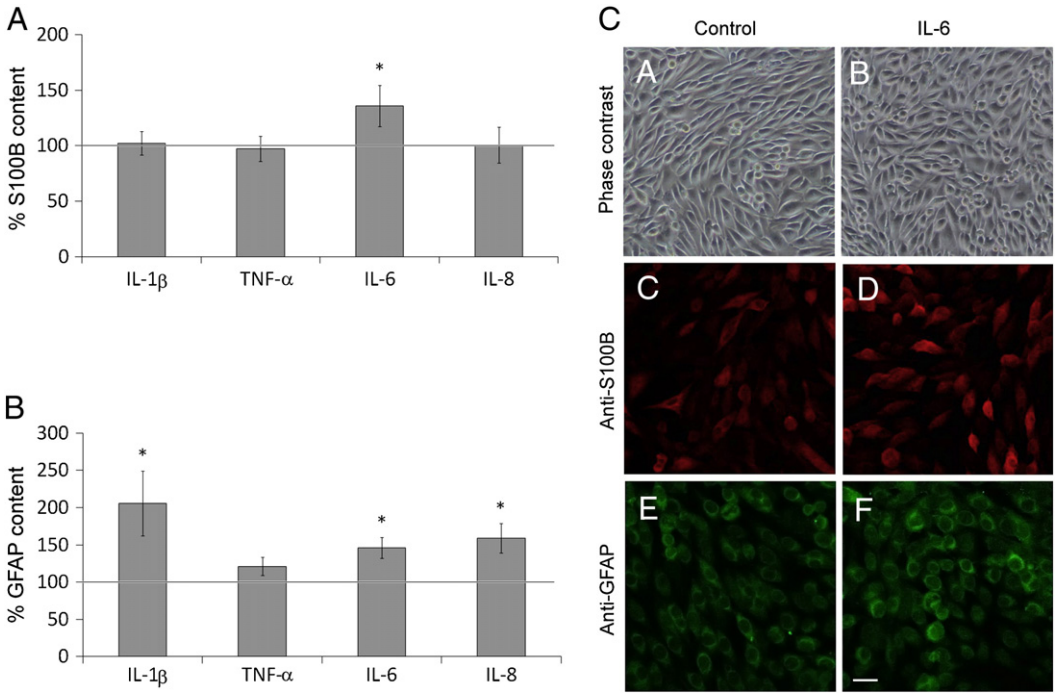

Fig. 7. Intracellular GFAP and S100B content are modified by cytokines in C6 glioma cells. C6 glioma cells exposed to 10 ng/mL IL-1β, TNF-α, IL-6 or IL-8 for 24 h were lysed and intracellular contents of S100B (A) and GFAP (B) were measured by ELISA. The line indicates basal secretion, assumed as 100% in each experiment. In C, C6 glioma cells were exposed to 10 ng/mL IL-6 for 24 h, phase-contrast images from control and IL-6-exposed cells are shown in panels A and B, respectively; immunocytochemistry for S100B from control and IL-6-exposed cells are shown in panels C and D, respectively; and immunocytochemistry for GFAP in control and IL-6-exposed cells are shown in panels E and F, respectively. Each value is the mean (± standard error) of at least 5 independent experiments performed in triplicate.* Significantly different from basal secretion (one way ANOVA followed by Tukey's test, with a significance level of $p<0.05$). Scale bar = 20 μm.

secretion in C6 glioma cells, induced by IL-1beta, as shown by the increase in the intracellular content of this protein. Nonetheless, we know now that an increase in cell content of this protein is not necessarily accompanied by its secretion (de Souza et al., 2009). An increase in extracellular S100B was also observed in cell cultures exposed to TNF-α (Edwards and Robinson, 2006). However, these authors also observed an increase in extracellular GFAP, suggesting that, under the conditions used, TNF-α exposure affected cell integrity. Conversely, S100B appears to modulate expression (and release) of TNF-α and IL-6 (Ponath et al., 2007); suggesting a new role for IL-6 in stimulating S100B release. Our data clearly indicate that S100B secretion is stimulated by the inflammatory cytokines, IL-1β, TNF-α, IL-6 and IL-8, suggesting that the increase in S100B observed in schizophrenic patients could, in part, be explained by cytokine stimulation.

Our next step was to investigate the signaling pathway involved in this mechanism, in particular the MAPK pathway, which is implicated in gene expression as well as in the molecular mechanisms of protein secretion, in response to a diverse range of extracellular stimuli. The MAPK pathway, in fact, encompasses three distinct pathways; the ERK, p38 and JNK pathways. Specific inhibitors of these pathways were able to block the S100B secretion induced by inflammatory cytokines. It is important to mention that these signaling kinases are susceptible to oxidative stress (Gaitanaki et al., 2003). Therefore, the oxidative stress observed in schizophrenic patients and other brain disorders could modulate S100B secretion through MAPK. Moreover, under the conditions used, cytokines were able to induce oxidative stress, as shown by measurements of DCF oxidation and reduced glutathione content. In general, cytokines induced oxidative stress, but not nitrosative stress (based on measurement of nitrate content); however, IL-6 was not able to modify glutathione content and IL-8 did not affect DCF oxidation. These findings may help to explain the presence of markers of oxidative stress and the impaired antioxidant defense system previously found in patients with schizophrenia (Martinez-Cengotitabengoa et al., 2012).

The NF-κB pathway was also investigated using SN-50, an inhibitor for the nuclear migration of this transcription factor. No effect was observed on IL-6-induced S100B secretion. Moreover, SN-50 *per se* caused an increase in S100B secretion. The increase in S100B secretion that we observed, induced by SN-50, could be interpreted as interruption of the tonic inhibition of NF-κB-mediated S100B synthesis. In fact, NF-κB-mediated activation of transcription has been widely characterized, but the mechanisms of NF-κB-mediated repression have not (Tchivileva et al., 2009). However, to our knowledge, a direct involvement of NF-kappaB in S100B expression has not been reported. Moreover, the effects of MAPK and NF-kappa B inhibitors on S100B secretion (measured in 1 h) possibly do not involve protein synthesis. Therefore, the effect of SN-50 may involve target(s) other than NF-κB (Torgerson et al., 1998).

IL-6 was able to induce an increase in GFAP and S100B 24 h afterwards. In fact, IL-6 is an important regulator of neuroinflammation and contributes to astrocytic differentiation of C6 glioma cells (Takanaga et al., 2004). Increased blood levels of IL-6 cytokine in schizophrenia have been described over 20 years (Shintani et al., 1991) and this cytokine has been used in schizophrenia models (Behrens et al., 2008). However, postmortem human brain studies indicate a lack of gliosis in most schizophrenic brains (Arnold et al., 1996; Damadzic et al., 2001). On the other hand, it is possible that cytokine-induced gliosis could be transitory, as observed in animal models (Fatemi and Folsom, 2009). Moreover, it is necessary to consider the effect *per se* on GFAP of chronic antipsychotic administration, which in rats was dependent on type of antipsychotic and brain region (Blazquez Arroyo et al., 2010).

We have chosen IL-6-induced S100B secretion to evaluate the effects of the antipsychotics, haloperidol, clozapine and risperidone. Exposure of C6 and OLN-93 cells to haloperidol and clozapine caused a decrease in basal S100B release (Steiner et al., 2008); the authors of this report suggested that serum S100B elevation in schizophrenic patients is normalized rather than increased by the effects of antipsychotic drugs on

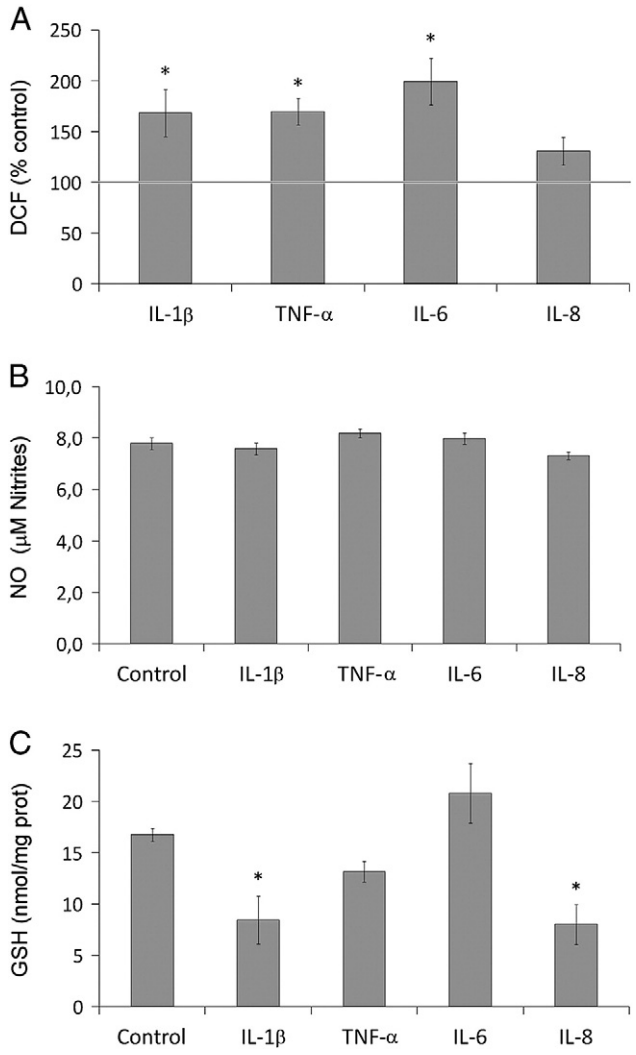

Fig. 8. Signals of oxidative stress are observed in C6 glioma cells exposed to cytokines for 1 h or 24 h. Intracellular ROS production (A) and nitric oxide production (B) were measured in the C6 cell line in the absence and in the presence of 10 ng/mL IL-1β, TNF-α, IL-6 or IL-8 after 1 h of treatment. Glutathione amount (C) was quantified at 24 h. In A, the line indicates basal secretion, assumed as 100%. Each value is the mean (± standard error) of at least 5 independent experiments performed in triplicate.* Significantly different from control (one way ANOVA followed by Tukey's test, with a significance level of $p<0.05$).

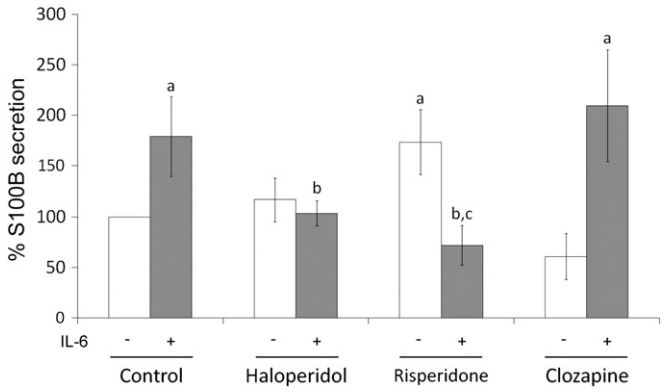

Fig. 9. Increase of S100B secretion induced by IL-6 in C6 glioma cells is prevented by antipsychotics. C6 glioma culture was exposed to 10 ng/mL IL-6 for 1 h, in the presence or in the absence of haloperidol (0.3 μM) or risperidone (70 nM) or clozapine (3 μM), added 15 min before cytokine exposure. S100B content in the medium was measured by ELISA. The line indicates basal secretion, assumed as 100% in each experiment. Each value is the mean (± standard error) of 6 independent experiments performed in triplicate. [a] Significantly different from basal secretion. [b] Significantly different from cytokine induced secretion. [c] Significantly different from risperidone induced secretion (one way ANOVA followed by Tukey's test, with a significance level of $p<0.05$).

glial cells. More recently, we showed a decrease in S100B secretion in hippocampal slices and C6 cells, induced by apomorphine (Nardin et al., 2011); in contrast, antipsychotics (haloperidol and risperidone) were not able to alter this change. In the present study, we demonstrated that S100B secretion, induced by IL-6, is prevented by haloperidol and risperidone, but not clozapine. Only risperidone was able to change basal S100B secretion, confirming a previous study (Quincozes-Santos et al., 2008). Together these data reinforce the idea that antipsychotics help to normalize the elevated serum S100B levels in schizophrenic patients, during acute active phases and that cytokines are underlying these S100B elevations. It should be noted that adipocytes are another important source of serum S100B (Goncalves et al., 2010) and that major metabolic changes in schizophrenic patients are observed in these cells (Steiner et al., 2010a).

However, the effect of antipsychotic drugs on IL-6-induced S100B secretion in C6 glioma cells indicates a modulation via D_2 receptors. It is worth mentioning that atypical antipsychotic drugs, such as clozapine and risperidone, involve more $5HT_{2A}$ than D_2 (Kuroki et al., 2008). Moreover, other receptors such as $5HT_7$, found in astrocytes, are targets of antipsychotic drugs and putatively could underlie the different modulations observed with clozapine and risperidone (Smith et al., 2011). Therefore, these and other receptors are modulating basal and/or cytokine-induced S100B secretion in brain tissue, making it difficult to describe a simple scenario to explain the extracellular changes of this protein. Moreover, the effects of antipsychotic drugs on S100B secretion could be due to changes in cell oxidative status (Donato et al., 2009). However, for haloperidol, in contrast to its *in vivo* long term administration (Pillai et al., 2007), we observed a decrease in ROS levels in glioma C6 cells at 1 h after administration. Moreover, we know that haloperidol, at high concentrations (but not risperidone), is able to induce ROS production after 6 h (Quincozes-Santos et al., 2010) in C6 glioma cells. Therefore, it was not possible to correlate S100B secretion and ROS levels in C6 glioma cells during acute exposure to antipsychotic drugs.

Some limitations of this study should be noted. Firstly, this is an *in vitro* study, useful for studying responses and mechanisms in an isolated manner, but unable to demonstrate the entire complexity of a system *in vivo*. Secondly, levels of S100B secretion found are sub-nanomolar levels compatible with the neurotrophic activity of this protein observed in culture; however, the specific effects of the increased secretion of this protein following the stimulation of neurons and other

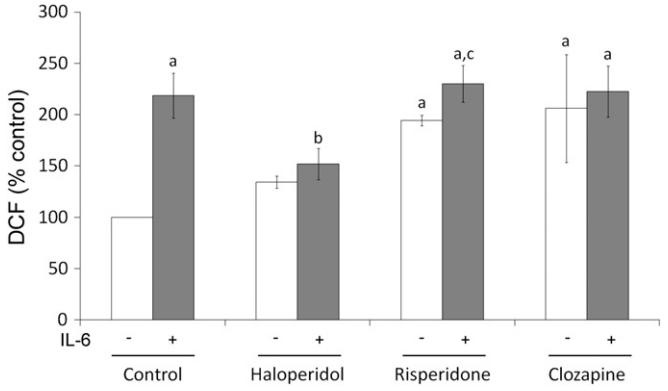

Fig. 10. IL-6-induced intracellular ROS production in C6 glioma cells is prevented by haloperidol. Intracellular ROS production was measured in C6 glioma cultures exposed to 10 ng/mL IL-6 for 1 h, in the presence or in the absence of haloperidol (0.3 μM), risperidone (70 nM) or clozapine (3 μM), added 15 min before cytokine exposure. Each value is the mean (± standard error) of 4 independent experiments performed in triplicate. [a] Significantly different from basal secretion. [b] Significantly different from cytokine-induced secretion. [c] Significantly different from risperidone-induced secretion (one way ANOVA followed by Tukey's test, with a significance level of $p<0.05$).

neuronal cells with pro-inflammatory cytokines should be studied. Finally, it is worth mentioning that although the astrocytes are the main source of extracellular S100B, the contribution of other cells should not be excluded, particularly in slice preparations. However, our ability to reproduce previous results validates our preparations for comparative approaches.

5. Conclusions

Our data provides evidence that S100B secretion is increased by pro-inflammatory cytokines in C6 glioma of cells and hippocampal slices of rats via MAPK, and that oxidative stress may be a component of this modulation. IL-6 induced an increase of S100B and GFAP. These results reinforce the idea that the S100B protein is involved in the inflammatory response observed in many brain diseases including schizophrenia. Moreover the antipsychotics, haloperidol and risperidone, were able to inhibit the secretion of S100B following IL-6 stimulation.

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