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Dual action of chronic ethanol treatment on LPS-induced response in C6 glioma cells

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ABSTRACT

In this study we investigated the anti-inflammatory effects of chronic ethanol (EtOH) treatment on lipopolysaccharide (LPS)-stimulated C6 glioma cells. The cells were chronically treated with 200 mM EtOH; coincubation with LPS and EtOH was obtained upon addition of 2 μg/ml LPS to the incubation medium in the last 24 h of EtOH exposure. We found that EtOH prevented the LPS-induced production of tumor necrosis factor α (TNFα) without decreasing cell viability. Either LPS treated or EtOH plus LPS treated cells presented upregulated glial fibrillary acidic protein (GFAP) and downregulated vimentin levels characterizing a program of reactive astrogliosis. Also, EtOH plus LPS stimulation greatly increased the oxidative stress generation evaluated by DCF-DA measurement, while either EtOH alone or LPS alone was unable to induce oxidative stress. Western blot analysis indicated that either EtOH, LPS or EtOH plus LPS treatments are unable to affect Akt/GSK3β signaling pathway. However, LPS alone and EtOH plus LPS co-treatment inhibited Erk phosphorylation. A dramatic loss of stress fibers was found in EtOH exposed cells, evaluated by cytochemistry using phalloidin-fluorescein. However, LPS alone was not able to disrupt actin organization. Furthermore, cells co-incubated with LPS and EtOH presented reversion of the disrupted stress fibers provoked by EtOH. Supporting this action, RhoA and vinculin immunocontent were upregulated in response to EtOH plus LPS. Interestingly, EtOH suppresses the inflammatory cascade (TNFα production) in response to LPS. Concomitantly it sustains Erk inhibition, increases oxidative stress generation and induces reactive astrogliosis in the presence of LPS, conditions associated with neurotoxicity. The effects observed were not supported by actin reorganization. Altogether, these findings suggest that Erk signaling inhibition could play a role in both suppressing TNFα production and inducing oxidative stress generation and astrogliosis, therefore modulating a dual action of EtOH plus LPS in glial cells.

1. Introduction

Neuroinflammation is a normal defense mechanism aimed at protecting the CNS against insults such as infection, injury or disease. In most cases, it constitutes a beneficial process that resolves on its own once the threat has been eliminated and homeostasis has been restored (Glass et al., 2010). In some instances, however, the insult can persist and/or the inflammatory process might get out of balance, resulting in chronic neuroinflammation, a deleterious process contributing to the pathogenesis of several neurological conditions (Allan and Rothwell, 2003; Glass et al., 2010). Together with microglia (the resident immune cells in the brain), astrocytes are important players in neuroinflammatory processes. This inflammatory reaction has recently received attention as an unexpected potential target for the treatment of diverse diseases such as Alzheimer's disease, Parkinson disease and amyotrophic lateral sclerosis (Block et al., 2007; Philips and Robberecht, 2010).

In response to certain pathological conditions of the brain (Polazzi et al., 1999; Liu et al., 2000; Teeling and Perry, 2009), microglia are readily activated and undergo a dramatic transformation from their resting ramified state into an amoeboid morphology. In their activated state, they can serve diverse beneficial functions essential to neuron survival, which include cellular maintenance and innate immunity. Activated microglia is also involved in regulating brain development and neurogenesis through the release of trophic and anti-inflammatory factors (Hanisch and Kettenmann, 2007). Recent in vitro studies indicate the important impact of activated microglia on astrogliosis (Rohl et al., 2007). This assumption was further supported by the finding that the pro-inflammatory factors released by microglia play an important role as triggers and modulators of astrogliosis (Zhang et al., 2010). Therefore, similar to microglia, astrocytes become activated in response to various stimuli, from subtle changes in their microenvironment to massive tissue damage. This process, known as reactive astrogliosis, far from being an all-or-none event, is a finely graded and non-homogeneous response that varies according to the type, severity, time and duration of the insult (Sofroniew and Vinters, 2010).

Astroglial activation is characterized by a prominent increase in expression of the intermediate filament glial fibrillary acidic protein (GFAP) and the marker aldehyde dehydrogenase 1 family, member

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L1 (ALDH1L1). Astrocytes are not immune cells per se, but can, in specific conditions, contribute to the immune response (Farina et al., 2007). The functions of reactive astrocytes are not well understood, and both harmful and beneficial activities are reported. Reactive astrocytes can protect neurons and neural function secreting trophic factors or produce pro-inflammatory and cytotoxic cytokines that are harmful to neurons or oligodendrocytes in the lesioned brain, which in turn can lead to further damage via, e.g., nitric oxide radicals and TNFα (Oleszak et al., 1998; Zhang et al., 2010).

Lipopolysaccharide (LPS), which constitutes the lipid portion of the outer leaflet of Gram-negative bacteria, is essential for growth (Cipolla et al., 2011). However, LPS facilitates the survival of pathogens by imposing a permeability barrier against antibiotics and antimicrobial peptides. LPS, also termed as endotoxin, functions as a potent inducer of innate immunity. Interception of endotoxin in systemic circulation by immune cells e.g. macrophages is essential to mount surveillance against invading microbes. LPS binding to the host receptor Toll-like receptor 4 (TLR4) triggers an inflammatory reaction characterized by the release of large number of inflammatory mediators that allow the host to respond to the invading pathogen (Ianaro et al., 2009). However, a hyper-activated immune response may lead to the overwhelming production of tissue damaging cytokines TNFα, interleukin (IL)-1, IL-6 and free radicals that may cause multiple organ failures or septic shock syndromes (Bhattacharjya, 2010).

Otherwise, numerous studies suggest a role for ethanol (EtOH) mediating inflammatory processes in leukocytes in rat brain (Altura et al., 2002) or in astroglial cells (Davis and Syapin, 2004; Lee et al., 2004; Blanco et al., 2005), although cellular and molecular mechanisms by which alcohol causes brain damage are not fully understood. In this context, excessive consumption of EtOH interferes with intracellular signaling as well as cytokine responses induced through most TLRs both in vivo and in vitro (Goral and Kovacs, 2005). Also, in vitro experiments using RAW264.7 macrophage-like cell line suggest a novel mechanism of EtOH action that involves interference with TLR clustering, and indicate a potential role for actin filaments in the formation of receptor patches, subsequent activation of macrophages by LPS, and production of TNFα (Dai and Pruett, 2006).

The C6 glioma cell (rat glial tumor cell line) has provided the most appropriate cell line for molecular neurobiologists to study sensitivity of glial cells to various substances and conditions, because of its similarities with primary glial cells in culture (Kumar et al., 1984; Vernadakis et al., 1991). With successive passages (beyond passage 70) C6 glioma cell differentiates into a primarily astrocytic cell like population (Parker et al., 1980) and exhibits many properties of astrocytes, including expression of the astrocyte specific markers GFAP (Bissell et al., 1974) and S100B protein (Nardin et al., 2007). Moreover, we have recently reported the effects of acute treatment with EtOH in C6 glioma cell on actin cytoskeletal organization and we found that this remodeling could be related with oxidative stress (Loureiro et al., 2011).

Since little information is available about the roles of EtOH modulating neuroinflammation under LPS exposure in glial cells, in the present study we used C6 cells to establish the implications of chronic treatment with EtOH on some parameters of the inflammatory response induced by LPS. We used coincubation of C6 cells with EtOH plus LPS focusing on TNFα production, actin reorganization, oxidative stress generation, reactive astrogliosis and activation of signaling mechanisms such as AKT/glycogen synthase kinase 3β (AKT/GSK3β) and mitogen activated protein kinase (MAPK) pathways.

2. Material and methods

2.1. Radiochemicals and compounds

Ethanol was purchased from Merck (Darmstadt, Germany) and was of analytical grade. Lipopolysaccharide (LPS) from *Escherichia coli* serotype 026:B6, L-2654, benzamidine, leupeptin, antipain, pepstatin, chymostatin, acrylamide, bis-acrylamide, phalloidin-fluorescein isothiocyanate, anti-GFAP (clone G-A-5), monoclonal anti-α-tubulin clone B-5-1-2, monoclonal anti-β-actin, anti-vinculin, anti-caspase 3, anti-RhoA, anti-rabbit IgG (whole molecule), F $(ab')_2$ fragment-Cy3, anti-mouse IgG (whole molecule)-FITC, peroxidase-conjugated rabbit anti-mouse IgG, 2′–7′-dichlorofluorescein diacetate (DCF-DA) and material for cell culture were purchased from Sigma (St. Louis, MO, USA). Polyclonal rabbit anti-glial fibrillary acidic protein was obtained from Dako Corp. (CA, USA). Anti-GSK-3β, anti-phospho-GSK-3β, anti-Akt1, anti-phospho-Akt (Thr308), anti-ERK/MAPK, anti-phosphoERK/MAPK, anti-JNK/MAPK and anti-phosphoJNK/MAPK were obtained from Cell Signaling Technology. Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), fungizone and penicillin/streptomycin were purchased from Gibco BRL (Carlsbad, CA, USA). All other chemicals were of analytical grade.

2.2. Maintenance of cell line

The C6 rat glioma cell line was obtained from Rio de Janeiro Cell Bank (Rio de Janeiro, Brazil). We used C6-glioma cells at passage 90, expressing GFAP and vimentin. The cells were grown and maintained in DMEM/F12/10% FBS (pH 7.4) containing 2.5 mg/ml fungizone, 100 mg/ml penicillin/streptomycin and supplemented with glucose (33 mM), glutamine (2 mM) and sodium bicarbonate (3 mM). Cells were kept at a temperature of 37 °C, a minimum relative humidity of 95%, and an atmosphere of 5% CO_2 in air.

After cells reached semi-confluence, the culture medium was removed by suction and the cells were incubated for 96 h at 37 °C in an atmosphere of 5% CO_2/95% air in DMEM/F12 (pH 7.4) containing 2.5% FBS in the presence or absence (controls) of 200 mM ethanol. Thereafter, we incubated the treated cells with 2 μg/ml LPS for 24 h. Morphological studies were performed using cells fixed for immunocytochemistry.

2.3. Lactate dehydrogenase (LDH) assay

The viability was assessed by measuring the release of the cytosolic enzyme lactate dehydrogenase into the medium. C6 cells were incubated in the presence or absence of 200 mM ethanol for 96 h and thereafter added 2 μg/ml LPS for 24 h. LDH measurement was carried out in 250 μl aliquots using the LDH kit from Doles reagents.

2.4. Evaluation of intracellular oxidative stress generation

Intracellular reactive oxygen species (ROS) production was detected using the nonfluorescent cell permeating compound, 2′–7′-dichlorofluorescein diacetate (DCF-DA). DCF-DA is hydrolyzed by intracellular esterases and then oxidized by ROS to a fluorescent compound 2′–7′-dichlorofluorescein (DCF). After treatment with 200 mM ethanol and 2 μg/ml LPS, C6-glioma-cells were treated with DCF-DA (10 μM) for 30 min at 37 °C. Following DCF-DA exposure, the cells were rinsed and then scraped into PBS with 0.2% Triton X-100. The fluorescence was measured in a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm.

2.5. Immunocytochemistry

Immunocytochemistry was performed as described previously (Gomes et al., 1999). Briefly, cultured cells plated on glass coverslips were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min at room temperature. After blocking, cells were incubated overnight with polyclonal anti-GFAP (1:500) and with our monoclonal anti-α-tubulin (1:500) at room temperature, followed by PBS washes and incubation with specific secondary antibody conjugated with Cy3 (1:1000) and

FITC (1:1000) or with fluorescein isothiocyanate (2 g/ml) for 1 h. In all immunostaining-negative controls, reactions were performed by omitting the primary antibody. No reactivity was observed when the primary antibody was excluded. The actin cytoskeleton was observed using phalloidin-fluorescein isothiocyanate and the nucleus was stained with DAPI (0.25 μg/ml). Cells were viewed with a Nikon inverted microscope and images were transferred to a computer with a digital camera (Sound Vision Inc., USA).

2.6. Total protein homogenate

C6 cells were homogenized in 200 μl of a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl, pH 6.8, and 4% SDS. For electrophoresis analysis, samples were dissolved in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, and 50 mM Tris–HCl, pH 6.8, and boiled for 3 min.

2.7. Polyacrylamide gel electrophoresis (SDS-PAGE)

The cytoskeletal fraction and the total protein homogenate from C6 cells were prepared as described above. Equal protein concentrations were loaded onto 10% polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of Laemmli (1970).

2.8. Western blot analysis

Total protein homogenate were separated by 10% SDS-PAGE (50 μg/lane of total protein) and transferred (Trans-blot SD semidry transfer cell, BioRad) to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS). The blot was then washed for 10 min in Tris-buffered saline (TBS) (0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by a 2 h incubation in blocking solution (TBS plus 5% defatted dry milk). After incubation, the blot was washed twice for 5 min with blocking solution plus 0.05% Tween-20 (T-TBS) and then incubated overnight at 4 °C in blocking solution containing one of the following monoclonal antibodies: anti-glial fibrillary acidic protein (clone G-A-5) diluted 1:500, α-tubulin diluted 1:500, actin diluted 1:500, anti-vinculin diluted 1:1000, anti-RhoA diluted 1:1000, anti-ERK/MAPK diluted 1:1000, anti-phosphoERK/MAPK diluted 1:1000, anti-JNK/MAPK diluted 1:1000, and anti-phosphoJNK/MAPK diluted 1:1000. The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in antibody solution containing peroxidase-conjugated rabbit anti-mouse IgG diluted 1:2000. The blot was washed twice again for 5 min with T-TBS and twice for 5 min with TBS. The blot was developed using a chemiluminescence ECL kit (Amersham, Oakville, Ontario).

2.9. S100B measurement

S100B was measured by ELISA, as previously described (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 monoclonal anti-S100B. Polyclonal anti-S100 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.

2.10. Tumor necrosis factor alpha (TNFα) measurement

The assay was carried out in 100 μl of extracellular medium, using a rat TNFα ELISA from eBioscience (San Diego, USA).

2.11. Statistical analysis

Data from the experiments were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Tukey's test when the *F*-test was significant. Values of $P<0.05$ were considered to be significant. All analyses were carried out in an IBM compatible PC using the Statistical Package for Social Sciences (SPSS) software.

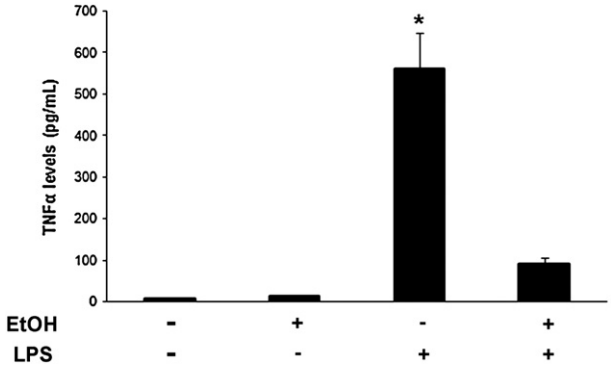

Fig. 1. Effect of chronic ethanol treatment on the ability of LPS in secreting TNFα. Cells were untreated (controls) or treated for 96 h with 200 mM EtOH; coincubation with LPS plus EtOH was obtained upon addition of 2 μg/ml LPS to the incubation medium in the last 24 h of EtOH exposure. The assay was carried out in 100 μl of extracellular medium, using a rat TNFα ELISA from eBioscience. Four independent experiments were performed in triplicate. Data were statistically analyzed by one-way ANOVA followed by Tukey's multiple variation test. Statistically significant differences from controls are indicated: *$P<0.01$.

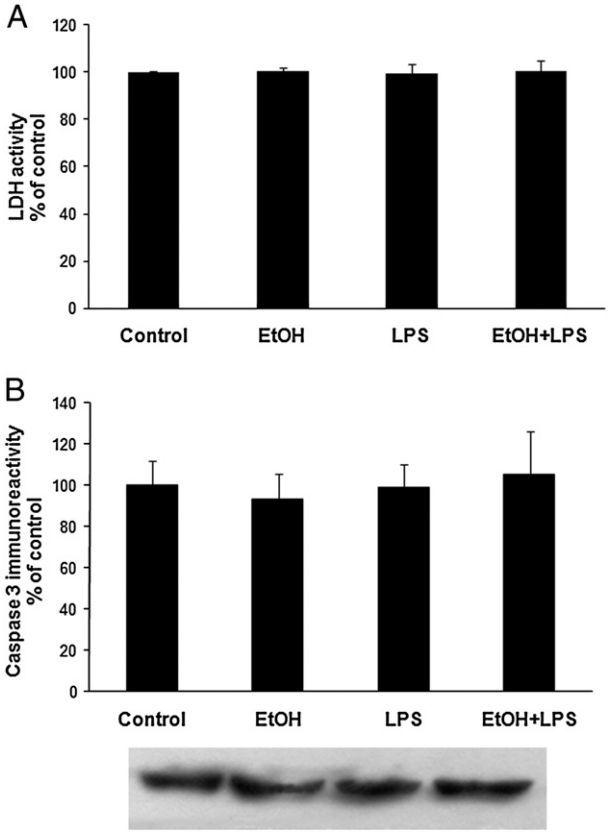

Fig. 2. Effect of chronic ethanol treatment on LPS-induced cell viability. Cells were untreated (controls) or treated for 96 h with 200 mM EtOH; coincubation with LPS plus EtOH was obtained upon addition of 2 μg/ml LPS to the incubation medium in the last 24 h of EtOH exposure. The viability was assessed by (A) measuring the released cytosolic enzyme lactate dehydrogenase (LDH) into the medium. LDH measurement was carried out in 250 μl aliquots using the LDH kit from Doles reagents; and (B) evaluating the caspase 3 immunocontent in cell homogenate. Immunoblot was carried out with monoclonal anti-caspase 3 antibody diluted 1:1000. All lanes received equivalent amount (50 μg) of total protein from cell extract. Representative immunoblot is shown. Four independent experiments were performed in triplicate. The blots were developed using an ECL kit. Representative immunological reactions are shown. Data were statistically analyzed by one-way ANOVA followed by Tukey's multiple variation test.

3. Results and discussion

We initially evaluated the implication of chronic EtOH treatment on the ability of LPS in secreting TNFα. For this, C6 glioma cells were untreated (controls) or treated for 96 h with 200 mM EtOH; co-incubation with LPS plus EtOH was obtained upon addition of 2 μg/ml LPS to the incubation medium in the last 24 h of EtOH exposure. As depicted in Fig. 1, EtOH alone failed to induce C6 cells to secrete this cytokine. This is consistent with Zahr et al. (2010), who described that binge EtOH treatment for 4 days was insufficient to induce the expression of several cytokines, including TNFα in blood, liver, or different brain regions of rats. In addition, our results showed that co-incubation of C6 cells with EtOH plus LPS abrogated LPS-induced TNFα release in these cells. This is in line with Dai and Pruett (2006) who found that acute EtOH exposure suppressed LPS-induced TNFα production in macrophages.

Cell viability was evaluated under the same conditions and results showed that neither mitochondrial damage, evaluated by MTT assay (not shown), nor release of the cytosolic enzyme lactate dehydrogenase (LDH) into the medium (Fig. 2A) was altered. To further evaluate whether chronic EtOH exposure, or EtOH plus LPS stimulation was able to induce apoptosis, we measured the immunoreactivity of caspase 3, one of the main executioner caspases implicated in eliciting apoptosis (D'Amelio et al., 2010). Results showed that either EtOH, LPS or EtOH plus LPS treatment failed to activate this apoptotic enzyme (Fig. 2B). Altogether these findings indicate that the effect of EtOH upon LPS-mediated release of TNFα was not related to cytotoxicity.

The hallmark of neuroinflammation is the activation of glia and the production of cytokines and inflammatory mediators that can trigger neuronal damage (Minghetti, 2005; Ubogu et al., 2006). In line with this, either LPS treated or EtOH plus LPS treated cells presented upregulated GFAP (Fig. 3A) and downregulated vimentin levels (Fig. 3B). GFAP is the major intermediate filament protein in mature astrocytes and forms an important part of the cytoskeleton of these cells. Altered expression of these proteins is considered as part of the spectrum of changes accompanying reactive astrogliosis (Sofroniew and Vinters, 2010; Zhang et al., 2010). Otherwise C6 cells chronically exposed to 200 mM EtOH were not able to become activated, suggesting that EtOH on its own does not activate the program of astrogliosis in C6 cells.

We also attempted to investigate S100B secretion in cells chronically treated with EtOH only, or LPS only, or LPS plus EtOH. S100B is a glial-derived protein, commonly used as a marker of astroglial activation in response to different insults (Funchal et al., 2007). Results showed that either EtOH, LPS or EtOH plus LPS were not able to upregulate S100B production by C6 cells (Fig. 3C). These findings could mean a moderate cell damage, since it has been described that S100B levels generally correlate with the severity of damage and may have predictive value for adverse neurological outcomes (Van Eldik and Wainwright, 2003).

Reactive astrocytes also present increased oxidative stress generation, which potentially mediate neurotoxic effects (Van Eldik and Wainwright, 2003; Farina et al., 2007; Sofroniew and Vinters, 2010). Surprisingly, we found that regarding this parameter, C6 cells were not responsive to the effects of either EtOH alone or LPS alone, evaluated by DCF-DA measurement. Conversely, recently we found that C6 cells acutely treated with 50 to 200 mM EtOH developed increased ROS levels (Loureiro et al., 2011). Otherwise, it is interesting to note that EtOH plus LPS stimulation greatly increased oxidative stress generation, (Fig. 3D), showing that the concomitant presence of EtOH and LPS is able to provoke an overwhelming of the oxidative defense mechanisms of the cell. Although at present we are not able to understand the molecular mechanisms underlying these findings, they highlight the complexity of the cell responses evoked by EtOH and LPS in different experimental conditions.

We next investigated which signaling events were implicated in the EtOH-modulated LPS-induced inflammatory response. Initially we were interested in determining if Akt/GSK-3β signaling pathway

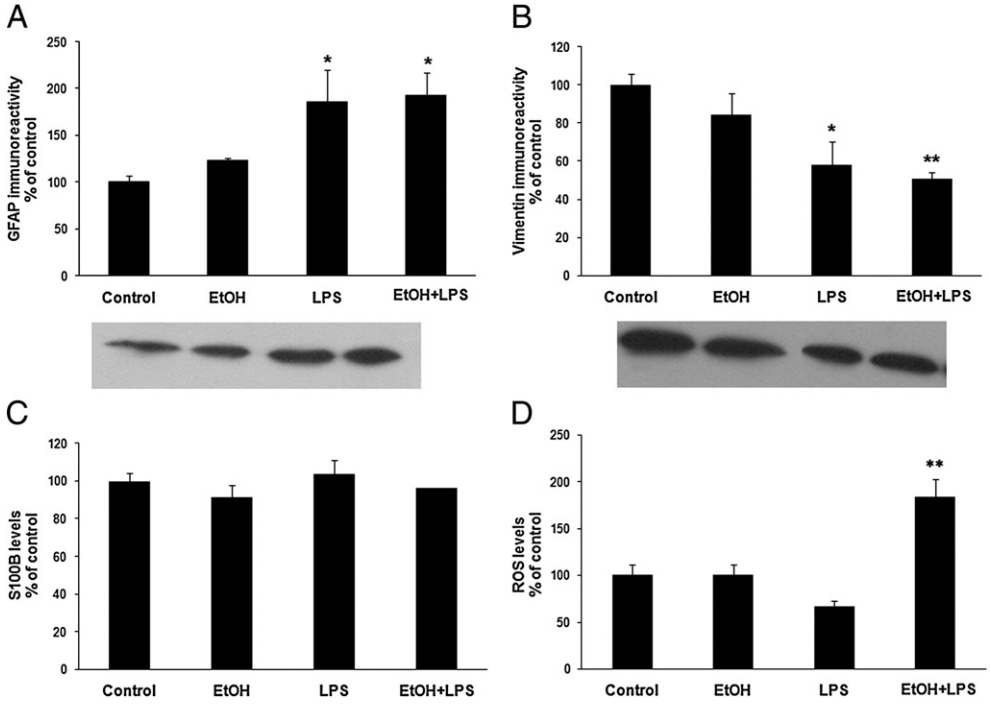

Fig. 3. Effect of chronic ethanol treatment on the LPS effect on GFAP and vimentin immunocontent, S100B levels and ROS production. Cells were untreated (controls) or treated for 96 h with 200 mM EtOH; coincubation with LPS plus EtOH was obtained upon addition of 2 μg/ml LPS to the incubation medium in the last 24 h of EtOH exposure. Immunoblot was carried out with anti-GFAP (clone G-A-5) (A) and anti-vimentin (B) antibodies diluted 1:500. The blots were developed using ECL kit. Representative immunological reactions are shown. All lanes received equivalent amount (50 μg) of total protein from cell extract. (C) S100B was measured by ELISA. Polyclonal antibody anti-S100 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. (D) The intracellular ROS levels were measured with DCF-DA assay. Data were statistically analyzed by one-way ANOVA followed by Tukey's multiple variation test. Statistically significant differences from controls are indicated: *$P<0.05$; **$P<0.01$.

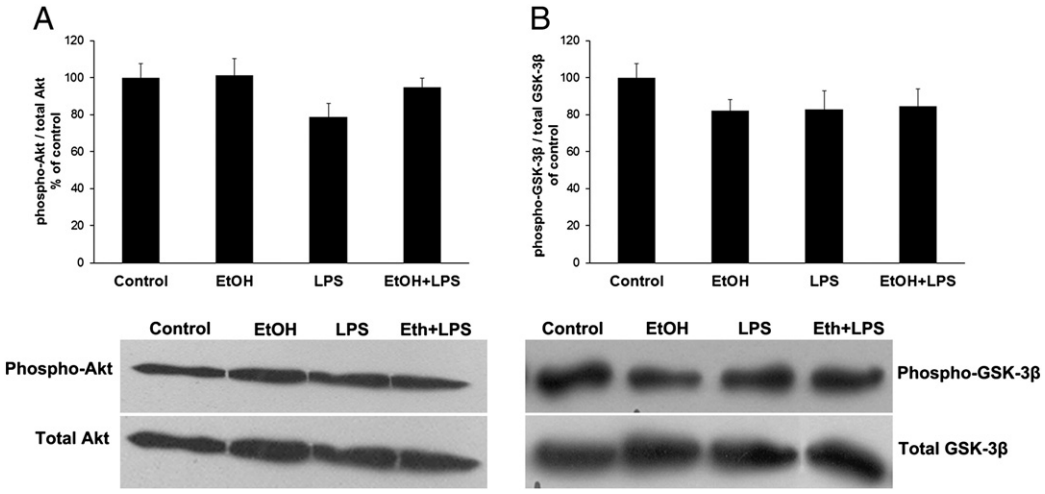

Fig. 4. Effect of chronic ethanol treatment on the LPS effect on Akt/GSK-3β activation. Cells were untreated (controls) or treated for 96 h with 200 mM EtOH; coincubation with LPS plus EtOH was obtained upon addition of 2 μg/ml LPS to the incubation medium in the last 24 h of EtOH exposure. The total and phosphorylated levels of Akt and GSK-3β were evaluated by Western blot with anti-Akt1 and the ratio phospho-Akt (Thr308)/total Akt is depicted in (A); the ratio phosphoGSK-3β/totalGSK-3β is depicted in (B). Antibodies were diluted 1:1000. The blots were developed using ECL kit. Representative immunological reactions are shown. All lanes received equivalent amount (50 μg) of total protein from cell extract. Data were statistically analyzed by one-way ANOVA followed by Tukey's multiple variation test.

(Baki et al., 2004; Lee et al., 2008) could be implicated in these actions in C6 cells. Thus, the protein levels of total Akt, phospho-Akt, total GSK-3β and phospho-GSK-3β were detected by Western blotting. Results depicted in Fig. 4 A indicate that either EtOH, LPS or EtOH plus LPS treatments are unable to affect the ratio phospho-AKT/total AKT. In concert with these results, modulation of GSK-3β phosphorylation is not altered in the inflammatory response (Fig. 4B). Our findings are quite unexpected, since the function of GSK-3β in signaling mechanisms that activate nuclear factor κB (NF-κB), as well as the resulting effects on NF-κB mediated gene expression, indicates that GSK-3β acts as a regulator of inflammation (Sanchez et al., 2003; Buss et al., 2004; Takada et al., 2004), however the potential role and mechanism for this effect are still controversial.

On the other hand, MAPKs play a critical role in regulating LPS-induced inflammation and transcriptional regulation of inflammatory factors (Surh et al., 2001). To examine whether EtOH affected MAPK phosphorylation, we analyzed the phosphorylation levels of Erk and JNK in cells treated with EtOH for the entire duration of the experiment, treated with LPS alone or treated with EtOH plus LPS. Results showed that while EtOH alone was unable to affect the ratio phosphoErk/totalErk, both LPS alone and EtOH plus LPS co-treatment inhibited this ratio (Fig. 5A). Conversely, despite that EtOH exposure has induced decreased phosphoJNK/totalJNK ratio, this kinase seems not to be implicated either in the neuroinflammatory action of LPS or in the effects of EtOH plus LPS co-treatment (Fig. 5B).

Altogether, the insight on some signaling pathways that could be involved in the cell response to LPS and EtOH led us to hypothesize a causal link between inhibition of Erk signal transduction and decreased TNFα production. Also, reactive astrogliosis and oxidative stress observed in EtOH plus LPS treated cells could be a consequence of misregulation of MAPK signaling. Moreover it should be noted that suppression of TNFα production in EtOH plus LPS stimulated cells is apparently not sufficient to inhibit signal transduction leading to cell damage. These findings could characterize a dual action of EtOH,

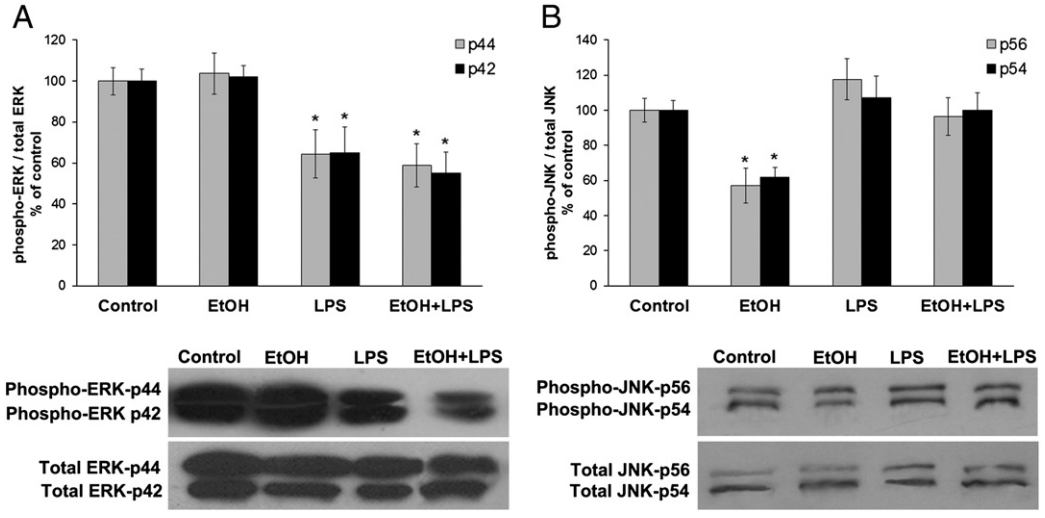

Fig. 5. Effect of chronic ethanol treatment on the LPS effect on ERK and JNK activation. Cells were untreated (controls) or treated for 96 h with 200 mM EtOH; coincubation with LPS plus EtOH was obtained upon addition of 2 μg/ml LPS to the incubation medium in the last 24 h of EtOH exposure. The total and phosphorylated levels of ERK (p44 and p42) and JNK (p56 and p54) were evaluated by Western blot with anti-ERK. The ratio phosphoErk (p44 and p42)/totalErk is depicted in (A); the ratio phosphoJNK/totalJNK is depicted in (B). Antibodies were diluted 1:1000. The blots were developed using ECL kit. Representative immunological reactions are shown. All lanes received equivalent amount (50 μg) of total protein from cell extract. Data were statistically analyzed by one-way ANOVA followed by Tukey's multiple variation test. Statistically significant differences from controls are indicated: *$P<0.05$.

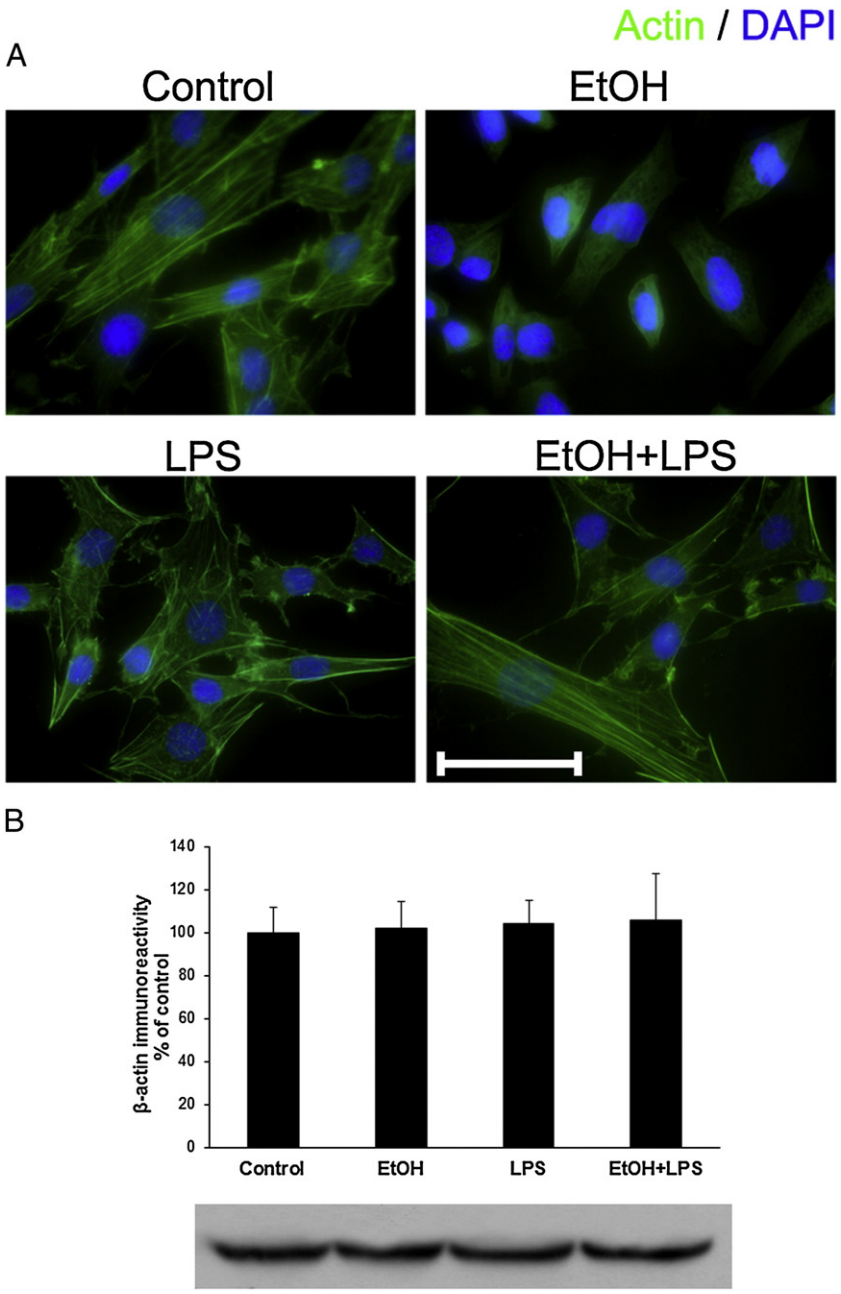

Fig. 6. Effect of chronic ethanol treatment on the effect of LPS on actin reorganization and immunocontent. Cells were untreated (controls) or treated for 96 h with 200 mM EtOH; coincubation with LPS plus EtOH was obtained upon addition of 2 μg/ml LPS to the incubation medium in the last 24 h of EtOH exposure. (A) Representative images of control (untreated) cells; ethanol-treated cells; LPS-treated cell and ethanol plus LPS co-treated cells immunostained with DAPI and phalloidin-fluorescein. Scale bar = 50 μm. (B) Western blot analysis of β-actin immunocontent in cell homogenate. All lanes received equivalent amount (50 μg) of total protein from cell extract. Immunoblot was carried out with monoclonal anti-β-actin antibody diluted 1:1000. The blots were developed using ECL kit. Representative immunological reactions are shown. Data represent mean ± S.E.M. from four independent experiments expressed as percent of controls. Statistically significant differences from controls were determined by one-way ANOVA followed by Tukey's multiple variation test.

on one hand suppressing the production of a neurotoxic cytokine, and on the other hand sustaining a signaling pathway probably implicated in the program of astrogliosis and increased oxidative stress generation.

It is also known that the cell response to LPS is dependent on its interaction with cell surface TLRs achieving the maximum response through TLR clustering (Pfeiffer et al., 2001; Dai and Pruett, 2006), which, in turn, seems to be mediated, at least in part, by rearrangement of the actin cytoskeleton, which is necessary for the formation of a fully active receptor complex on the cell surface (Hao and August, 2005; Dai and Pruett, 2006). Thus, LPS interacts with TLRs before activating downstream signaling pathways, which induce the production of TNFα (Tobias et al., 1989; Schumann et al., 1990; Cheadle et al., 1991). Interestingly, Dai and Pruett (2006) described that, in macrophages, acute EtOH treatment interferes with intracellular signaling elicited by LPS by suppressing LPS-induced actin reorganization, therefore preventing TLR clustering and consequent TNFα release (Pruett et al., 2004; Goral and Kovacs, 2005).

Therefore, to access the effects of chronic EtOH or EtOH plus LPS treatment on actin cytoskeleton of C6 cells, we carried out cytochemical analysis using phalloidin-fluorescein to decorate actin. Results depicted in Fig. 6A show that in untreated cells actin was distributed into numerous well-organized actin stress fibers into the cytoplasm. After EtOH exposure for 96 h, we observed a dramatic loss of stress fibers and more diffuse organization of actin filaments. In line with the implications of actin cytoskeleton in the response of C6 cells to EtOH exposure, chronic EtOH caused alterations in actin organization in rat astrocytes, consisting of the dissolution of stress fibers (Martinez et

al., 2007). In addition, Tomas et al. (2003) registered the appearance of circular filaments beneath the plasma membrane in EtOH-treated astrocytes. Accordingly, we previously described that in C6 cells acutely exposed to 200 mM EtOH elevated ROS levels were related with the EtOH-evoked disruption of actin organization (Loureiro et al., 2011). However, in the present results we show that the action of chronic EtOH exposure on actin reorganization was not mediated by ROS and further experiments will be necessary to elucidate the mechanisms underlying this effect.

Interestingly, in the presence of LPS alone the phalloidin-fluorescein labeling throughout the cytoplasm remained similar to that of untreated cells. Furthermore, when cells were co-incubated with LPS plus EtOH we observed a LPS-induced reversion of the disrupted stress fibers provoked by EtOH. It is interesting to note that actin immunocontent, evaluated by Western blot analysis, was not altered upon the different experimental conditions (Fig. 6B). Supporting restoration of actin architecture, RhoA and vinculin immunocontent were upregulated in extracts of cells treated with EtOH plus LPS, as evaluated by Western blot analysis using specific monoclonal antibodies against these two proteins (results not shown). RhoA is a mononeric GTPase activated by lysophosphatidic acid (LPA), which is a normal constituent of serum and a potent activator of RhoA known to stimulate stress fiber formation (Ridley and Hall, 1992) whereas vinculin allows efficient interaction with the cell surface and promotes actin stress fiber assembly (Le Clainche and Carlier, 2008). Also, decreased content of RhoA suggests inhibition of this monomeric GTPase signaling pathway (Loureiro et al., 2011). In this context, Martinez et al. (2007) have described alterations of the actin cytoskeleton organization and dynamics, as well as downregulation of members of Rho GTPase family in cultured astrocytes chronically exposed to ethanol at moderate (30 mmol/l) and high (100 mmol/l) concentrations. To further investigate the participation of the other cytoskeletal constituents on the action of EtOH on LPS cell response, we also investigated the organization of the microtubules and GFAP networks in C6-glioma cells. Immunocytochemical analysis using anti-α-tubulin or anti-GFAP antibodies showed that in cells chronically exposed to EtOH, in cells stimulated with LPS alone, or co-incubated with LPS plus EtOH, microtubule and GFAP arrays were not altered as compared to untreated cells (results not shown).

Therefore, our present findings lead us to postulate that EtOH and LPS co-treatment is able to abrogate the LPS-induced TNFα production while restoring the stress fibers through activation of RhoA-mediated mechanisms. Thus it is tempting to speculate that the mechanisms involved in the TNFα production following LPS stimulation are not directly related with the reorganization of the cytoskeleton in C6 cell, as it was found in macrophages (Dai and Pruett, 2006).

Otherwise, it is important to emphasize that EtOH suppresses the inflammatory cascade leading to decreased TNFα production in response to LPS, therefore downregulating the inflammatory response. Concomitantly the alcohol sustains ROS production and reactive astrogliosis as well as Erk inhibition induced by LPS, conditions associated with neurotoxicity. Altogether, these findings could mean that EtOH elicits a dual action in glial cell and probably Erk signaling takes part of the signaling pathways leading to cellular injury.

Considering that astrocytes are polyvalent cells implicated in almost all processes that occur in the CNS, and that chronic alcohol consumption is associated with neuroinflammation and neurodegeneration, the vulnerability of glial cells under EtOH influence on neuroinflammatory processes may have important implications for understanding the neurotoxicity linked to several disorders of the CNS.

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