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# Adrenal gland responses to lipopolysaccharide after stress and ethanol administration in male rats

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**Abstract**

All forms of stress, including restraint stress (RS) and lipopolysaccharide (LPS) administration, activate the hypothalamic–pituitary–adrenal (HPA) axis. LPS binds to a recognition protein (CD14) and toll-like receptor 2/4 in different cells and tissues, including the adrenal gland, to induce the production of cytokines and cause upregulation of cyclooxygenase and nitric oxide synthase (NOS) enzymes. Acute ethanol exposure activates the HPA axis, but in some conditions prolonged administration can dampen this activation as well as decrease the inflammatory responses to LPS. Therefore, this study was designed to evaluate the adrenal response to a challenge dose of LPS (50 μg/kg) injected i.p., after submitting male rats to RS, twice a day (2 h each time) for 5 days and/or ethanol administration (3 g/kg) by gavage also for 5 days, twice daily. At the end of the experiment, plasma corticosterone concentrations and adrenal gland content of prostaglandin E (PGE) and NOS activity were measured as stress mediators. The results showed that repetitive ethanol administration attenuated the adrenal stress response to LPS challenge alone and after RS, by preventing the increase in plasma corticosterone concentrations and by decreasing the PGE content and NOS activity in the adrenal gland. Therefore, we conclude that moderate alcohol consumption could attenuate the effects of psychophysical stress and impair an inflammatory response.

**Keywords:** *Adrenal cortex, corticosterone, endotoxin, nitric oxide, prostaglandin E, restraint stress*

## Introduction

The response of an organism to stress is characterized by the activation of autonomic and neuroendocrine system responses. Stressful stimuli, whether physical, metabolic, endotoxic, or psychological, activate the hypothalamic–pituitary–adrenal (HPA) axis (Pacák and Palkovits 2001). Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, stimulates all levels of the HPA axis as indicated by the increased release of brain corticotropin-releasing hormone (CRH), anterior pituitary adrenocorticotropic hormone (ACTH), and consequent increased plasma corticosterone or cortisol concentration (Beishuizen and Thijs 2003; Gadek-Michalska and Bugajski 2004). The adrenal gland adapts to various forms of acute and chronic stress (Pignatelli et al. 1998) by producing several molecules, such as cytokines, nitric oxide (NO), and prostaglandins (PGs) that can modulate corticosterone release (John and Buckingham 2003; Ehrhart-Bornstein and Bornstein 2008). In chronic stress, the adrenal cortex undergoes an adaptation that allows the hypersecretion of glucocorticoids; furthermore, the non-pituitary regulation of adrenocortical function also comes into play (Ulrich-Lai et al. 2006; Bornstein et al. 2008).

Ethanol administration when given acutely can stimulate the HPA axis, but in some conditions, prolonged administration induces a tolerance in the response of the HPA axis (Lee and Rivier 1997; Pruett et al. 2009). Alcohol dependence also leads to a dampened neuroendocrine state (Richardson et al. 2008). Other effects of alcohol intake include decreased inflammatory responses and altered production of cytokines and non-protein inflammatory

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mediators, such as cyclooxygenase (COX) products and reactive oxygen intermediates (Szabo 1999; Kato et al. 2005).

PGs and NO are important signal transducers involved in neurotransmitter and neurohormone secretion during basal and stress conditions (Bugajski et al. 2004; Rettori et al. 2009). PGs are formed from arachidonic acid by phospholipase $A_2$ and COX enzymes and stimulate the secretion of CRH, vasopressin, and ACTH (Gadek-Michalska et al. 2005). They can also stimulate steroidogenesis and the release of corticosterone, by acting directly in the adrenal gland (Wang et al. 2000; Mohn et al. 2005).

Nitric oxide is regarded as a major and ubiquitous modulator of a variety of physiological reactions (Stern 2004). Nitric oxide is formed from L-arginine through the action of nitric oxide synthase (NOS): calcium-dependent neuronal NOS, endothelial NOS, and calcium-independent inducible NOS (iNOS) (Kleinert et al. 2003), each isoform with a specific distribution in the body including the adrenal gland (Kishimoto et al. 1996; Cymeryng et al. 2002; Lai et al. 2005). Nitric oxide, similarly to PGs, can modulate the release of stress hormones such as CRH, vasopressin, ACTH, and corticosterone (Bugajski et al. 2004; Rettori et al. 2009). NOS activity is increased during stress and infection (Gadek-Michalska et al. 2005; Monau et al. 2009). Nitric oxide can regulate cortical and medullary adrenal gland functions such as the secretion of aldosterone (Sainz et al. 2004) and corticosterone (Cymeryng et al. 1998).

LPS action has been linked to the increased production of PGs and NO in some tissues (Wang et al. 2004) and can also elicit direct effects on adrenal gland cells by causing upregulation of COX and NOS enzymes (Cover et al. 2001; Grion et al. 2007). Binding of LPS to adrenal cells has been reported (Enrique de Salamanca et al. 2000), and the expression of toll-like receptors (TLRs) has been described in human and mouse adrenal glands (Bornstein et al. 2004, 2006). Additionally, human adrenal cells release cortisol by direct stimulation with LPS, an effect mediated via COX-dependent mechanisms (Vakharia and Hinson 2005).

On the basis of all these findings and noting that alcohol drinking is common in people when stress is difficult to avoid, we hypothesized that stress may enhance the adrenal response to LPS challenge, while ethanol would eventually attenuate it. Therefore, this study was designed to evaluate the adrenal response to a single challenge dose of LPS as an endotoxic stressor, after 5 days of submitting the rats to repetitive restraint stress (RS) and repetitive ethanol administration. We measured plasma corticosterone and stress mediators such as prostaglandin E (PGE) content and NOS activity in the adrenal gland.

## Materials and methods

Adult male Sprague Dawley rats (250–300 g body weight) were kept in groups (four rats per cage) in an animal room having a photoperiod of 14 h of light (07:00–21:00 h) and room temperature of 22–24°C. Rats had free access to laboratory chow and tap water. They were divided into several experimental groups of 6–8 rats each and then transferred to a separate room during the experiments. The experimental procedures were approved by the Animal Care Committee of the Center of Pharmacological and Botanical Studies of the National Council for Research of Argentina (CEFYBO-CONICET) and were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 85-23, Rev. 1985, National Academy Press, Washington DC, 1996).

### *Treatments*

Ethanol (3 g/kg) was diluted with water to $<20\%$ (v/v) and was administered by gavage twice a day with an interval of 6 h.

The repetitive RS was performed by keeping the rats immobilized in plastic cylinders with several holes along the length of the tubes (diameter = 8 cm, length = 18 cm).

The endotoxin LPS was diluted in sterile saline and injected once i.p. in a single dose of 50 μg/kg rat weight as a challenge at the end of the experiments. This dose of LPS was lower than that injected for immunohistochemical studies, in order to achieve a moderate response to allow the possibility to observe the effects of the previous treatments and to avoid the possibility of endotoxic shock.

### *Experimental groups*

*Control group.* Rats received 5 ml of water by gavage twice a day at 09:00 and 16:00 h for 4.5 days and were killed 1 h after the last water administration between 10:00 and 12:00 h in the morning of the fifth day.

*Repetitive ethanol (EtOH) group.* Rats received ethanol by gavage (3 g/kg) twice a day at 09:00 and 16:00 h for 4.5 days. On the fifth day, rats were killed 1 h after ethanol administration between 10:00 and 12:00 h.

*Repetitive RS group.* Rats were exposed to 120 min of restraint twice a day at 09:00 and 16:00 h for 4.5 days. On the fifth day, rats were killed immediately after the restraint period.

*EtOH and RS group.* Rats in this group received ethanol by gavage (3 g/kg) and were exposed to 120 min of restraint twice a day at 09:00 and 16:00 h

for 4.5 days. Ethanol was administered 1 h after the beginning of RS. Rats were killed on the fifth day immediately at the end of the 2 h of restraint period, 1 h after ethanol administration.

*LPS group.* Rats received 5 ml of water by gavage twice a day at 09:00 and 16:00 h for 4.5 days. On the fifth day after receiving 5 ml of water at 09:00 h by gavage, an i.p. injection of LPS (50 μg/kg rat weight) was given at 10:00 h and the rats were killed 1 h after LPS injection.

*EtOH and LPS group.* Rats received ethanol by gavage (3 g/kg) as described for the EtOH group. On the fifth day, rats were injected with LPS (50 μg/kg, i.p.), 30 min after the administration of ethanol, and were killed 1 h after LPS injection.

*RS and LPS group.* Rats were exposed to 120 min of RS twice a day as described for the RS group. On the fifth day, LPS (50 μg/kg, i.p.) was injected 1 h after the beginning of RS, and the rats were killed immediately after the 2 h of restraint period, 1 h after LPS injection.

*EtOH, RS, and LPS group.* These rats received ethanol by gavage (3 g/kg) and were exposed to 120 min of restraint twice a day as described for the EtOH and RS groups. On the fifth day, ethanol was administered 30 min after the beginning of RS. The LPS (50 μg/kg, i.p.) was injected 1 h after the beginning of RS, and the rats were killed immediately after the restraint period (2 h of restraint, 90 min after EtOH administration, 1 h after LPS injection).

Rats were killed by decapitation, without anesthetic, in an adjoining room, trunk blood was collected into tubes containing sodium and potassium EDTA salts, the samples were centrifuged (800*g* for 20 min), and the plasma was frozen and stored at −20°C for corticosterone measurements. Both adrenal glands were immediately removed, frozen individually in dry ice, and stored at −70°C until used randomly for determinations of total NOS (tNOS) and iNOS activities, PGE content, and COX and NOS protein expression.

*Immunohistochemistry*

In order to determine whether LPS could exert any direct effect in the adrenal gland, we injected a high dose of LPS (5 mg/kg rat weight, i.p.) and observed by immunohistochemistry whether there were any changes in the adrenal gland in LPS receptors and inflammatory enzymes. The rats were killed 5 h after i.p. injection of LPS or 0.9% saline. Adrenal glands were removed, dissected free of adipose tissue, and fixed in 4% paraformaldehyde and 0.4% picric acid in a phosphate-buffered saline (0.05 M, pH 7.4) overnight at 4°C. Paraffin-embedded sections were cut to thicknesses of 5 μm. Endogenous peroxidase activity was inhibited using 0.3% $H_2O_2$ in methanol. Sections were blocked in 5% normal horse serum (Vectastain, Vector Laboratories, Burlingame, CA, USA) and incubated overnight with either rabbit polyclonal immunoglobulin G (IgG) anti-COX-II (1/250, Cayman Chemical, Ann Arbor, MI, USA), anti-iNOS (1/300, Cayman Chemical), anti-CD14 (1/400, Santa Cruz, Heidelberg, Germany), or anti-TLR4 (1/400, Santa Cruz). Indirect immunoperoxidase detection was performed using biotinylated antibody anti-IgG of rabbit, produced in goat, and incubated with avidin–biotin–peroxidase complex (Vectastain Elite ABC, Vector Laboratories). Peroxidase activity was visualized using 0.03% of 3,3′-diaminobenzidine in Tris–HCl (20 mM, pH 7.6) and 0.02% $H_2O_2$. Slides without the first antibody were used as negative controls. Tissue sections were counterstained with hematoxylin. COX-II, iNOS, CD14, and TLR4 were evaluated for the presence or absence of stained cells over the entire section observed at different magnifications. Results were evaluated as positive fields for each section.

*Measurements of NOS activity*

NOS activity was measured by a modification of the [$^{14}$C]-citrulline method (Bredt and Snyder 1990). Calcium-dependent and calcium-independent NOS activities were determined as previously described. In brief, each adrenal gland was homogenized in 520 μl of ice-cold 20 mM HEPES with 1 mM dithiothreitol (DTT), pH 7.4. One aliquot (20 μl) was separated for protein determination by Bradford assay (Bradford 1976). The homogenates were divided into two aliquots of 250 μl each; 250 μl of 20 mM HEPES containing 1 mM DTT plus $CaCl_2$ to a final concentration of 1.25 mM was added to one aliquot for the determination of total NOS activity. In addition, 250 μl of 20 mM HEPES without $CaCl_2$ and with the addition of 2 mM EGTA was added to the other aliquot in order to remove $Ca^{2+}$, which is required for activation of endothelial and neuronal NOS. Since iNOS does not require $Ca^{2+}$ for activation, this aliquot measured iNOS activity. Nicotinamide adenine dinucleotide phosphate (NADPH) (120 μM) and 200,000 dpm of [$^{14}$C]-arginine were added to all tubes. All tubes were incubated for 15 min at 37°C in a Dubnoff metabolic shaker (50 cycles/min and 95% $O_2$/5% $CO_2$ atmosphere). After incubation, the tubes were transferred immediately to a refrigerated centrifuge and spun at 10,000*g* for 10 min at 4°C. The supernatants were immediately applied to individual columns containing 1 ml of Dowex AG 50W-X8 $Na^+$ form and washed with 2 ml of double-distilled water. All effluent fluid from each column was counted for

[$^{14}$C]-citrulline activity in a scintillation counter. Since NOS converts arginine into equimolar quantities of NO and citrulline, the data were expressed as NO produced per milligram of protein per minute.

*Determination of PGE content*

Adrenal glands were homogenized individually in 1.5 ml of 100% ice-cold ethanol, centrifuged at 10,000*g* for 15 min at 4°C, and the supernatants were collected and evaporated in a speed vacuum concentrator. These residues were stored at −20°C for the determination of PGE by RIA. Rabbit antiserum from Sigma-Aldrich (St Louis, MO, USA) was used as previously described (Mohn et al. 2001). The sensitivity of the assay was 12.5 pg/tube. The cross-reactivity between $PGE_2$ and $PGE_1$ was 100%, although other PGs exhibited less than 0.1%. The intra- and inter-assay coefficients of variation for PGE were 8.2 and <12%, respectively. The PGE content was expressed as picograms per milligram weight of adrenal gland.

*Determination of corticosterone*

Plasma corticosterone concentrations were determined quantitatively by RIA (Etches 1976). Plasma samples were incubated with rabbit anti-corticosterone for 30 min at room temperature, [$^3$H] corticosterone was added as tracer and incubated for 1 h at 37°C. The reaction was stopped by the addition of cold dextran-coated charcoal suspension and after incubation for 10 min at 4°C, the tubes were centrifuged at 2000*g* for 15 min at 4°C. Scintillation cocktail was added to the supernatant, and the amount of radioactivity was determined in a beta-counter. The sensitivity and coefficient of variation of the assay were 30 pg/tube and <15%, respectively. The values are expressed as nanograms per milliliter of plasma.

*Western blot for NOS and COX-1 protein measurement*

All adrenal glands were lysed in ice-cold protein extraction buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 1% Triton X-100, and also protease inhibitor cocktail from Sigma-Aldrich). The homogenates were briefly centrifuged, the supernatants removed and stored at −70°C until being used. After total protein determination by the Bradford assay (Bradford 1976), samples were separated by SDS/PAGE and blotted onto nitrocellulose membranes. The blots were probed with anti-iNOS or anti-COX-I rabbit antibody (1/1000; Cayman Chemical) and with anti-rabbit IgG-alkaline phosphatase (1/2000; Santa Cruz Biotechnology) as secondary antibody. After extensive washing, protein bands were visualized with BCPI/NBT Sigma Fast (5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium). Blots were also probed with anti-β-actin (Sigma-Aldrich; 1/3000) to confirm equal loading of protein. Quantification was done using ImageJ 1.42 software package.

*Chemicals*

LPS *Escherichia coli* (serotype 055-B5), $PGE_2$ standard and antiserum, corticosterone standard and antiserum, DLL-DTT, NADPH, and HEPES were purchased from Sigma-Aldrich. Dowex AG 50W-X8 $Na^+$ form mesh 200–400 was obtained from Bio-Rad Laboratories (Hercules, CA, USA), and [U-$^{14}$C]-arginine monochloride, 296 mCi/mmol, was from Amersham Life Science (Buckinghamshire, UK). [1,2,6,7-$^3$H]Corticosterone and [$^3$H] $PGE_2$ were purchased from New England Nuclear TM Life Science Products (Boston, MA, USA).

*Statistics*

Data are expressed as mean ± SEM ($n = 6–8$ rats per group). Student's *t*-test was used for the statistical comparison of two groups. In all other studies, we performed comparisons between groups using two-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test for unequal replicates. All analyses were conducted using Statistica 7 software. Differences with $p$-value $< 0.05$ were considered to be statistically significant.

## Results

*Effect of treatments on plasma corticosterone*

The i.p. injection of LPS (50 μg/kg) increased plasma corticosterone concentration (Student's *t*-test, $p < 0.001$) compared with the control group (Figure 1A). Similarly, RS caused a significant increase in plasma corticosterone concentration [ANOVA $F(1,57):8.7$, $p < 0.001$] (Figure 1B). EtOH treatment did not modify plasma corticosterone concentration but prevented the RS-induced increase in corticosterone concentration [ANOVA $F(1,57):7.9$, $p < 0.001$] (Figure 1B). The LPS-induced increase in plasma corticosterone concentration was completely prevented by EtOH administration alone [ANOVA $F(1,24):33.77$, $p < 0.01$], and concomitantly in rats also submitted to RS [ANOVA $F(1,24):33.77$, $p < 0.001$]. In the rats previously submitted to RS, LPS caused a slight, but not significant increase in plasma corticosterone concentration [ANOVA $F(1,24):3.26$, $p < 0.0713$] (Figure 1C).

*Immunohistochemistry of CD14 protein and TLR4 receptor in the adrenal gland*

Immunohistochemical studies showed the presence of immunostaining for CD14, the LPS recognition protein, in all zones of the adrenal cortex from control rats (Figure 2A, control). The i.p. injection of LPS (5 mg/kg) induced a dramatic increase in the

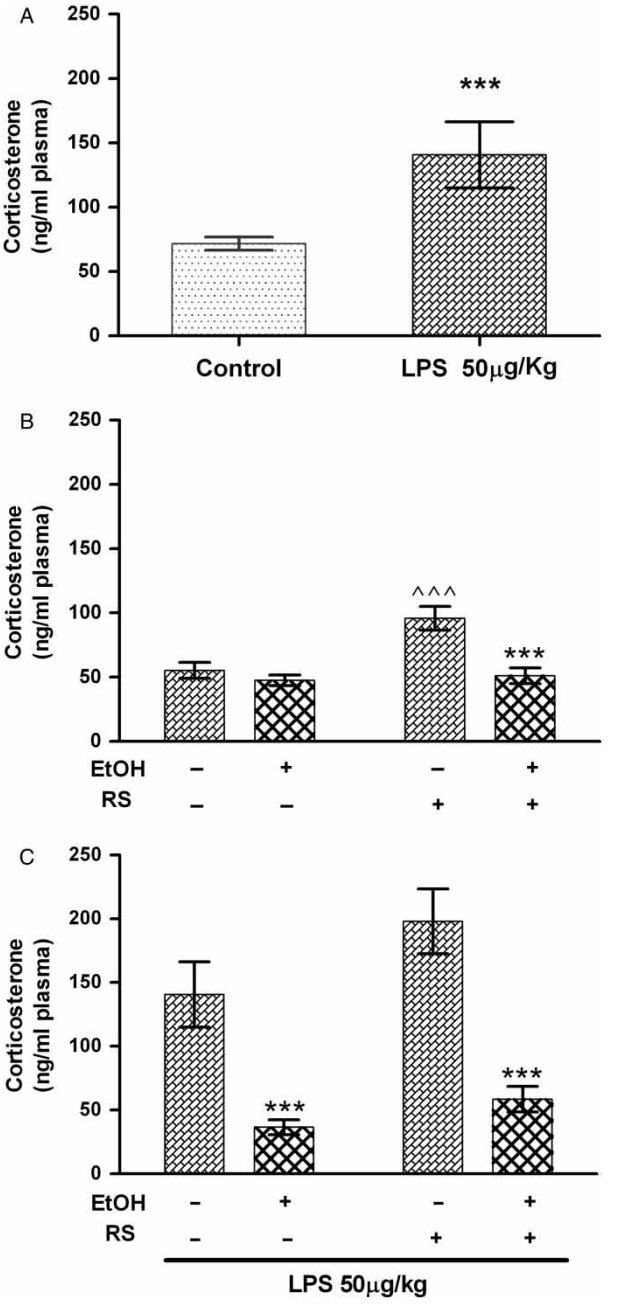


Figure 1. (A) Effect of LPS (i.p. 50 μg/kg) on plasma corticosterone concentrations 1 h after injection. (B) Effect of repetitive ethanol (EtOH) and/or repetitive RS on plasma corticosterone concentrations. (C) Effect of repetitive ethanol (EtOH) and/or repetitive RS on plasma corticosterone concentrations in rats injected with LPS. Values represent mean ± SEM ($n = 6–8$ rats per group). ***$p < 0.001$ vs. respective control group without EtOH. ^^^$p < 0.001$ vs. respective control group without RS. Data were evaluated by (A) Student's *t*-test and (B,C) two-way ANOVA followed by Tukey's post-test.

immunostaining for CD14, predominantly in the glomerular and fasciculata zones of the adrenal gland (Figure 2B, LPS).

Immunohistochemistry for TLR4 performed in adrenals from control rats showed positive immunostaining in the cortex (Figure 2C, control). When LPS (5 mg/kg) was injected, the staining for TLR4 dramatically increased, mainly in the glomerular and fasciculata zones of the adrenal gland (Figure 2D, LPS).

### *Immunohistochemistry of iNOS and COX II in the adrenal gland*

Immunohistochemical studies of adrenal glands of control rats showed the presence of immunostaining for iNOS in all zones of the adrenal cortex (Figure 3A, control). Injection of LPS (5 mg/kg) induced a dramatic increase in the immunostaining for iNOS mostly in the glomerular and fasciculata zones (Figure 3B, LPS).

Immunohistochemistry for COX II performed in adrenal glands from control rats also revealed a scattered immunostaining in the adrenal cortex (Figure 3C, control). The administration of LPS (5 mg/kg) produced an increase in staining of COX II in the cortex of the adrenal gland (Figure 3D, LPS).

### *Effect of treatments on NOS activity and protein expression in the adrenal gland*

The injection of LPS (50 μg/kg, i.p.) increased both tNOS (Figure 4A) and iNOS (Figure 4B) activities (Student's *t*-test, $p < 0.001$) in the adrenal gland. The EtOH treatment prevented the tNOS [ANOVA $F(1,17):21.41$, $p < 0.05$, and $p < 0.01$] (Figure 5A) and iNOS [ANOVA $F(1,16):18.31$, $p < 0.05$] (Figure 5B) increase in activities induced by LPS injection and also when the LPS challenge was applied after RS.

Western blot analysis of iNOS expression in the adrenal gland showed that EtOH prevented the increase in iNOS protein expression elicited by LPS alone [ANOVA $F(4,15):8.631$, $p < 0.05$] or after RS ($p < 0.01$) (Figure 5C).

### *Effect of treatments on PGE content and COX-I protein expression in the adrenal gland*

LPS injection (50 μg/kg) increased significantly the adrenal content of PGE (Student's *t*-test, $p < 0.01$) (Figure 6A). The LPS-increased adrenal content of PGE was prevented by previous administration of EtOH [ANOVA $F(1,26):67.93$, $p < 0.001$]. In contrast, RS produced an additive effect on the LPS-induced increase in PGE content [ANOVA $F(1,26):100.9$, $p < 0.001$], which was suppressed when EtOH was administered concomitantly with RS (Figure 6B).

Western blot analysis of COX-I in the adrenal gland showed that RS increased [ANOVA $F(1,12):15.21$, $p < 0.05$] and EtOH administration diminished [ANOVA $F(1,12):9.57$, $p < 0.05$] the expression of COX-I protein as compared with the control group (Figure 6C).

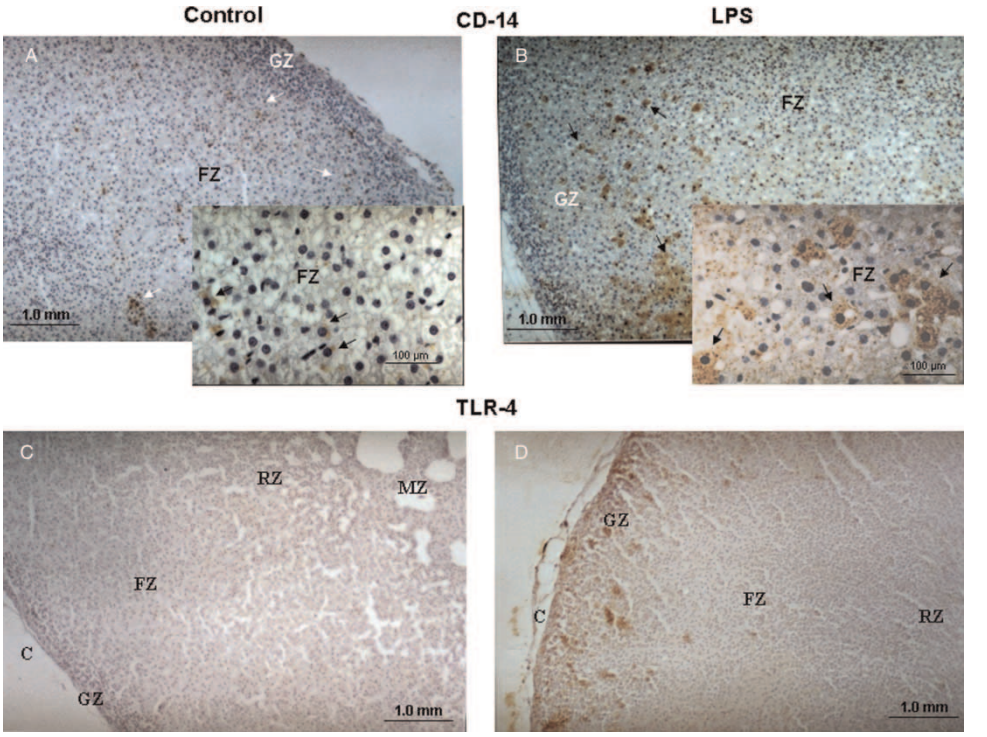


Figure 2. Photomicrographs demonstrating (A,B) the presence and distribution of LPS recognition protein CD14 and (C,D) LPS TLR4 in the adrenal gland. (A,C) CD14- and TLR4-immunoreactive cells are present in the cortex of control adrenals and (B,D) the immunostaining of both increased strongly after LPS (5 mg/kg, i.p.) injection. C, Capsule; GZ, glomerulosa zone; FZ, fasciculata zone; RZ, reticularis zone; MZ, medullary zone. The arrows indicate some of the representative immunostained (dark) cells. Hematoxylin-stained cell nuclei are prominent.



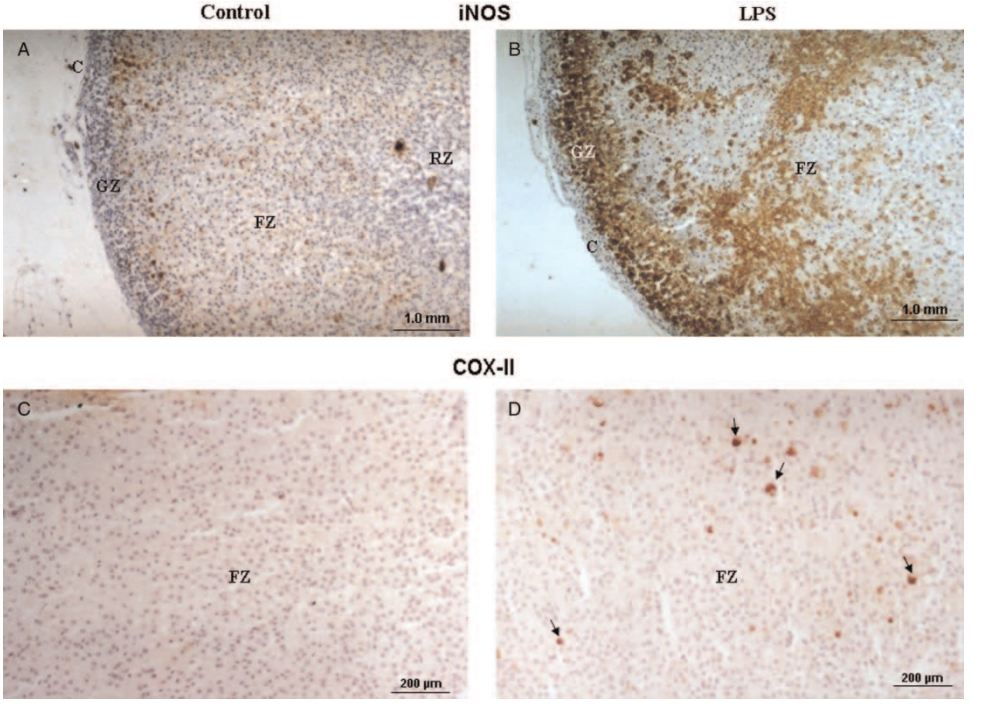


Figure 3. Photomicrographs demonstrating (A,B) the presence and distribution of iNOS and (C,D) COX-II in the adrenal gland. (A) iNOS-immunoreactive cells are abundant, while (C) COX-immunoreactive cells are scarce in the cortex of control adrenals. (B) The iNOS immunostaining increases strongly in LPS-injected rats (5 mg/kg, i.p.) and (D) COX-II immunostaining increase is less pronounced. C, Capsule; GZ, glomerulosa zone; FZ, fasciculata zone, RZ, reticularis zone. The arrows indicate some of the representative immunostained (dark) cells. Hematoxylin-stained cell nuclei are prominent.

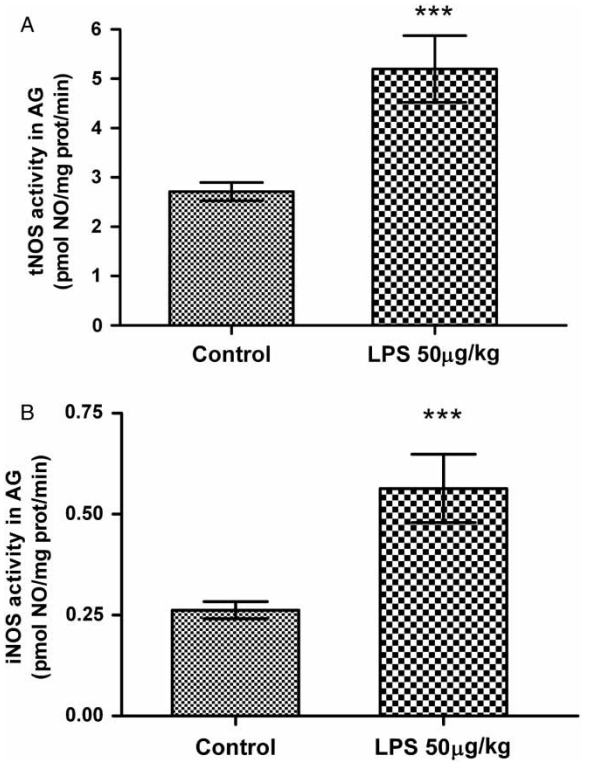


Figure 4. Effect of LPS (50 μg/kg, i.p.) on (A) tNOS and (B) iNOS activity in the adrenal gland (AG). Values represent mean ± SEM ($n = 6–8$ rats per group). Data were evaluated by Student's $t$-test. ***$p < 0.001$ vs. control group.

## Discussion

The present work demonstrates that repetitive administration of ethanol, twice a day for 5 days to rats, prevented the increase in plasma corticosterone concentrations observed when the rats were submitted to repetitive RS and/or received an acute challenge of LPS injection.

It is well known that acute ethanol administration increases plasma corticosterone concentration. We have also observed an increase in plasma corticosterone concentration 1 h after one dose of ethanol (3 g/kg) administered by gavage and compared with a group exposed to RS once for 2 h. The resulting plasma corticosterone concentrations were (mean ± SEM, ng/ml plasma): control, 71.9 ± 7.4; EtOH, 168.1 ± 17.8; RS, 142.9 ± 14.07; $n = 10–16$ per group; both $p < 0.001$ vs. control) (unpublished data). However, repetitive ethanol administration did not elicit any response with regard to plasma corticosterone concentration. This lack of response observed by us agrees with some other authors showing that repetitive alcohol administration for 3 days to rats produced a highly significant decrease in the response of the HPA axis to another dose of alcohol given some days later (Lee and Rivier 1997). Furthermore,

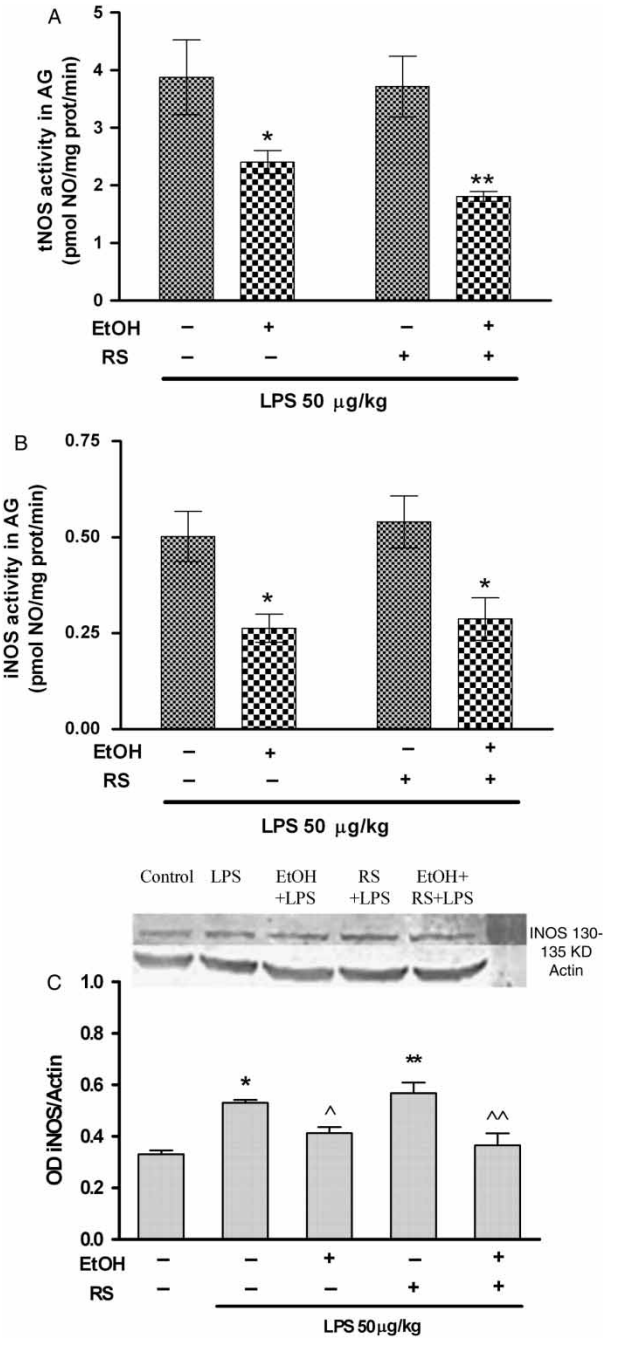


Figure 5. Effect of LPS (50 μg/kg, i.p.) in rats pretreated with repetitive ethanol (EtOH) and/or repetitive RS on (A) tNOS, (B) iNOS activity, and (C) expression in the adrenal gland (AG). Values represent mean ± SEM ($n = 6–8$ rats per group). *$p < 0.05$; **$p < 0.01$ vs. respective control group without EtOH. Data were evaluated by the two-way ANOVA followed by Tukey's post-test. (C) Representative Western blot analysis is shown in the top part of C. Relative protein levels of iNOS were quantified by densitometric scanning and normalized to β-actin and expressed as optic density (OD), shown in the bottom part of C. Values represent mean ± SEM of four independent experiments ($n = 4$ each group). *$p < 0.05$; **$p < 0.01$ vs. control group. ^$p < 0.05$; ^^$p < 0.01$ vs. respective group without EtOH. Data were evaluated by the one-way ANOVA followed by Tukey's post-test.

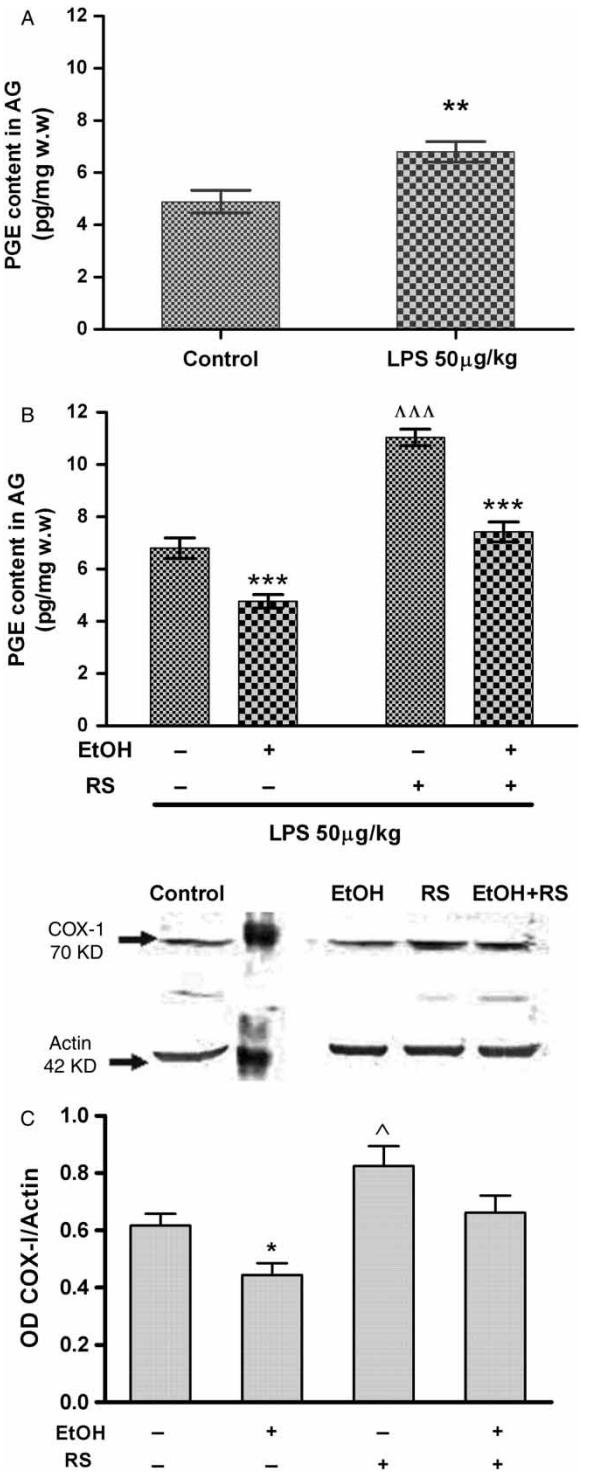


Figure 6. PGE content in the adrenal gland (AG) after LPS (50 μg/kg, i.p.) challenge in control rats (A), and rats pretreated with repetitive ethanol (EtOH) and/or repetitive RS (B). Values represent mean ± SEM ($n = 6–8$ rats per group). $\star p < 0.05$; $\star\star p < 0.01$ vs. respective control group without EtOH. Data were evaluated the by two-way ANOVA followed by Tukey post-test. (C) Representative western blot analysis is shown in the top part of C. Relative levels of COX-I protein were quantified by densitometric scanning and normalized to β-actin and expressed as OD, shown in the bottom part of C. Values represent mean ± SEM of four independent experiments ($n = 4$ each group). $\star p < 0.05$ and $\hat{} p < 0.05$ vs. control. Data were evaluated by the two-way ANOVA followed by Tukey's post-test.

Pruett et al. (2009) described total lack of a corticosterone response to chronic ethanol in mice, indicating probable habituation or tolerance. Other authors have demonstrated that alcohol consumption attenuates the febrile response to LPS in male rats (Taylor et al. 2002). It is quite difficult to compare the results obtained from different experimental designs, but most authors agree that repetitive alcohol ingestion impairs the response of the HPA axis to stress (Richardson et al. 2008).

Our experimental design was aimed to model the stresses of the human working week as repetitive RS, with alcohol ingestion twice per day, and the effect of each or both of these factors on the response to a bacterial endotoxic challenge, induced by LPS injection.

Our first aim was to find out whether LPS could modify the expression of NOS and COX enzymes in the adrenal gland, using a complementary approach to our previous study of LPS effect on NOS and IL-1 beta in the brain, but in which we used *in situ* hybridization (Wong et al. 1996, 1997). In this study, we found by immunohistochemistry that there was a dramatic increase in iNOS and COX immunostaining, as well as in CD14 and TLR4 in the adrenal gland after LPS injection, confirming the possibility of a direct action of LPS in this tissue. These findings agree with those of other authors who have demonstrated the presence of these receptors in human and mouse adrenal glands and their activation by LPS in this tissue, where they can interact directly with the steroid biosynthetic pathway and induce cytokine production (Bornstein et al. 2006; Zacharowski et al. 2006; Sanchez-Lemus et al. 2008).

The secretion of corticosterone is associated with NO and PG production in the adrenal gland. We have previously shown *in vitro* that the rapid release of corticosterone induced by ACTH is mediated by NO acting through PGs (Mohn et al. 2005). The increase of both mediators by LPS and stress in many tissues has been described (Gadek-Michalska et al. 2005; Grion et al. 2007). In agreement with this, in the present work, we found that LPS increased NOS activity, in particular the iNOS isoform that leads to the production of NO and also increased PGE content in the adrenal gland. In rats administered with ethanol, the increase in NOS activity and PGE content after LPS was not observed. The inhibitory effect of ethanol on NOS activity in the adrenal gland demonstrated in the present work agrees mostly with the literature, although this depends on the isoform of NOS, on the tissue studied, and also on the dose of ethanol and the experimental design. It has been reported that acute EtOH treatment inhibits the LPS-induced NO response in Kupffer, hepatic endothelial cells (Spolarics et al. 1993), macrophages (Wakabayashi and Negoro 2002), and microglia (Colton et al. 1998; Davis and Synapin 2004). In

general, both acute and chronic EtOH administration is considered to suppress iNOS induction by LPS in brain glial cells.

The blocking effect of EtOH on the increase in PGE content induced by LPS observed in this study agrees with our previous work where we have shown that repetitive RS increased PGE synthesis in the adrenal gland and that the opposite effect was produced by repetitive EtOH administration (Rettori et al. 2003). We think that the inhibitory effect of EtOH is caused by inhibition of the COX enzyme, but we cannot rule out its effect on other proteins such as ERK (Kato et al. 2005) and phospholipase $A_2$ that could also suppress PG synthesis (Basavarajappa et al. 1998).

RS is a mixture of physical and psychological stress. The magnitude of central stress responses usually diminishes upon exposure to repetitive intermittent RS, most likely reflecting habituation (García-Bueno et al. 2008). In this study, we showed an increase in plasma corticosterone concentrations and COX-I adrenal expression after repetitive RS. These results agree with the work of Pruett et al. (2009) which showed that mice partially adapted to daily restraint, since in these animals corticosterone concentration was increased by a lesser amount after chronic exposure to immobilization.

When the rats subjected to restraint were challenged with LPS, the PGE content in the adrenal gland was increased, suggesting that other factors such as cytokines and other pro-inflammatory molecules induced directly by LPS acting on CD14/TLR4 located in the adrenal gland could be responsible for this increase. This suggests that endotoxin challenge produces different effects on stress mediators as compared with neurogenic stress.

Although we focused on the effects of different kinds of stress on adrenal gland activity considering only some local mediators, it is important to note that the central effects of LPS, RS, and EtOH with all the bidirectional feedback mechanisms take place in this "*in vivo*" experimental design. Therefore, at the beginning of the treatments, both RS and ethanol activated the HPA axis, leading to an increase in corticosterone secretion that acts as a negative feedback mechanism at the hypothalamic–pituitary levels, downregulating the production of factors that participate in the stimulation of corticosterone production and release by the adrenal gland. Also, LPS activates the HPA axis at all levels, central and peripheral. This effect is exerted by activating the neurons in the parvocellular division of the hypothalamic paraventricular nuclei (PVN) which produce CRH and vasopressin (Suzuki et al. 2009). Both neuropeptides stimulate the release of ACTH from the pituitary gland, that is, also a target of LPS. Furthermore, there is a direct effect of LPS on the adrenal gland where it could increase corticosterone secretion (Vakharia and Hinson 2005). It has been shown that NOS and COX are also present in the PVN and mediate CRH action on the sympathetic preganglionic neurons (Yamaguchi and Okada 2009; Yamaguchi et al. 2009). We speculate that repetitive EtOH administration inhibited stimulation of adrenal COX not only at the adrenal gland level but also at the hypothalamic level (Canteros et al. 1995), decreasing the release of CRH and vasopressin and, therefore, diminishing the release of ACTH with the consequent lack of increase in corticosterone release from the adrenal. However, all these comments are largely speculative since we did not measure ACTH secretion or CRH mRNA expression in the PVN.

In summary, this study demonstrates that repetitive ethanol administration twice a day for 5 days attenuates the adrenal cortical stress response in rats submitted to repetitive RS and to an acute endotoxic challenge. Therefore, we conclude that moderate alcohol consumption attenuates the effects of psychological stress; however, since it also impairs the inflammatory response, it alters the host defense mechanisms in a way that may predispose to infectious diseases.

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