

## GENETICS AND CELL BIOLOGY

### The Acute Effect of Ethanol on Adrenal Cortex in Female Rats—Possible Role of Nitric Oxide

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**Abstract** — **Aims:** The present study was designed to investigate a possible role of endogenous nitric oxide (NO) in the adrenal response to an acute alcohol administration in female rats. To this end, *N*<sup>ω</sup>-nitro-L-arginine-methyl ester (L-NAME), a competitive inhibitor of all isoforms of NO synthase, was used. **Methods:** Adult female Wistar rats showing diestrus Day 1 were treated with: (a) ethanol (2 or 4 g/kg, intraperitoneally); (b) L-NAME (30 or 50 mg/kg, subcutaneously) followed by either ethanol or saline 3 h later. Untreated and saline-injected rats were used as controls. The animals were killed 30 min after last injection. Adrenal cortex was analyzed morphometrically, and plasma levels of adrenocorticotrophic hormone (ACTH) and serum concentrations of corticosterone were determined. **Results:** Acute ethanol treatment enhanced the levels of ACTH and corticosterone in a dose-dependent manner. Stereological analysis revealed that acute alcohol administration induced a significant increase in absolute volume of the cortex and the zona fasciculata (ZF). In addition, ethanol at a dose of 4 g/kg increased volume density and length of the capillaries in the ZF. However, other stereological parameters were unaffected by alcohol exposure. Pretreatment with both doses of L-NAME had no effect on ethanol-induced changes. **Conclusion:** Obtained findings indicate that acute ethanol treatment stimulates the activity of the adrenal cortex and that this effect is not mediated by endogenous NO in female rats under these experimental conditions.

## INTRODUCTION

Ethanol is known as a powerful activator of the hypothalamic-pituitary-adrenal (HPA) axis (Rivier, 1996; Budeč *et al.*, 2002; Milovanović *et al.*, 2003) and a modulator of its response to other stressors (Seo *et al.*, 2004). In addition, the HPA axis of female rats is more responsive to ethanol than that of males (Rivier, 1993). It has been shown that acute alcohol administration stimulates the activity of hypothalamic neurons that express corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) (Rivier and Lee, 1996). This observation is extended by finding that ethanol upregulates the expression of CRH gene through cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA)-dependent signal transduction pathway (Li *et al.*, 2005). Furthermore, pituitary receptors CRH type 1 are essential for the response of adrenocorticotrophic hormone (ACTH) to acute alcohol treatment (Lee *et al.*, 2001). At the level of adrenal gland, acute ethanol exposure enhances the expression of steroidogenic acute regulatory (StAR) protein that is paralleled to increases in the plasma levels of pregnenolone, progesterone and corticosterone (Khisti *et al.*, 2003). A more recent study has demonstrated that ethanol-induced steroidogenesis is dependent on the pituitary release of ACTH and synthesis of adrenal StAR protein (Boyd *et al.*, 2010).

There are many examples of the interactions between the ethanol and the unstable gas nitric oxide (NO) (Deng and Deitrich, 2007). NO is a physiologic mediator that is synthesized from L-arginine by three different isoforms of enzyme NO synthase (NOS): two constitutive, neuronal—nNOS (NOS I) and endothelial—eNOS (NOS III) and one inducible—iNOS (NOS II). Ethanol differently affects nNOS, iNOS and eNOS in a variety of cells and tissues. It has been reported that acute ethanol treatment increases plasma levels of NO (Baraona *et al.*, 2002) and on the other hand, NO reacts with ethanol to yield ethyl nitrite (Deng *et al.*, 2004).

Several lines of evidence suggest that NO may be involved in some effects of alcohol (Adams and Cicero, 1998).

It is well recognized that NO plays a pivotal role in the central control of homeostasis acting on neuroendocrine and autonomic outputs within the paraventricular nucleus of the hypothalamus and the supraoptic nucleus (Stern, 2004). Among other neuroendocrine actions, this signal molecule modulates the response of the HPA axis to different stressors, but its functional contribution to the regulation of this axis has not been elucidated (Mancuso *et al.*, 2010). Thus, blockade of NO formation may blunt the release of ACTH to mild electroshocks (Rivier, 1994), and enhance the secretion of ACTH to interleukin-1 $\beta$ , vasopressin and oxytocin, but not CRH (Rivier and Shen, 1994). Also, the inhibition of NOS activity prior to immobilization-induced stress significantly elevates the level of corticosterone (Tsuchiya *et al.*, 1997).

On the basis of all these observations, the current study was initiated to investigate a possible role of endogenous NO in the acute effect of ethanol on adrenal cortex in female rats, using well-known competitive inhibitor of all isoforms of NOS, *N*<sup>ω</sup>-nitro-L-arginine-methyl ester (L-NAME).

## METHODS

### Animals

Female Wistar rats aged 10–12 weeks (obtained from the Breeding Colony of the Medical Military Academy, Belgrade) were housed in groups of five per cage under standard laboratory conditions. Chow for experimental animals (Veterinarski zavod, Subotica) and water were available *ad libitum*. The stages of estrous cycle were monitored every day by vaginal smears, and only the rats showing the diestrus Day 1 were used in this study. To avoid changes associated with the circadian rhythm, the experiments were carried out between 8.00 and 12.00 h. The protocol was in accordance with local institutional guidelines for the care and use of

laboratory animals. The investigation also conformed to the principles and guidelines of Conseil de l'Europe (published in the Official Daily N. L358/1-358/6, 18th December 1986), the US National Institutes of Health (Guide for the Care and Use of Laboratory Animals, NIH publication no. 85–23), and the Canadian Council on Animal Care.

#### Experimental procedure

The animals were weighed and injected with: (a) ethanol (2 or 4 g/kg body weight, intraperitoneally, i.p.), (b) L-NAME (30 or 50 mg/kg body weight, subcutaneously, s.c.) followed by ethanol (4 g/kg, i.p.) 3 h later and (c) L-NAME (30 or 50 mg/kg body weight, s.c.) followed by saline (i.p.) 3 h later. Untreated and saline-injected rats were used as controls.

L-NAME (Sigma Corp.) was dissolved in apyrogenic saline just before use. The choice of dose of L-NAME, regimen of administration and route of injection were based on previous studies (Kim and Rivier, 2000; Seo and Rivier, 2003; Budeč *et al.*, 2007). Ethanol (SUPERLAB, Belgrade, Serbia) was diluted with sterile saline up to 35% (v/v), and the animals were treated with different volumes to reach a concentration of 2 or 4 g/kg.

The rats were killed by decapitation 30 min after last injection. Blood samples were collected and left adrenal glands were promptly removed, fixed in 10% buffered formalin for 24 h and embedded in paraffin.

#### Radioimmunoassay for ACTH and corticosterone

Plasma concentrations of ACTH were determined by a commercially available double antibody radioimmunoassay <sup>125</sup>I hACTH (MP Biomedicals, LLC). The inter- and intra-assay coefficients of variation (CV) were 4.1 and 3.9%.

Serum levels of corticosterone were measured by Amersham Biotrak rat corticosterone [<sup>125</sup>I] assay system using magnetic separation with CV = 5%, per run. The groups for hormone determination consisted of five to seven rats.

#### Morphometric analysis

Using the point-counting method (Weibel, 1979), stereological analysis was carried out on paraffin sections of the adrenal glands, 6 μm thick, stained with Azan. The volume density ( $V_v$ ) of the capsule, the cortex, the zona glomerulosa (ZG), the zona fasciculata (ZF), the zona reticularis (ZR) and the medulla was evaluated by use of the multipurpose test system M42 at an objective magnification of ×20 under Olympus microscope. Assuming that the average specific gravity of the gland is 1.039 g/cm<sup>3</sup> (Swinyard, 1939), absolute volume ( $V_f$ ) of the entire gland and of its components was calculated from the following equation:

$$V_f = V_{vf} \times V_o$$

where  $V_{vf}$  is the volume of fraction and  $V_o$  is the absolute volume of organ.

Further analysis, using an objective magnification of ×100, included numerical density ( $N_v$ ) and volume ( $V$ ) of the adrenocortical cells in all cortical zones. In addition, volume density ( $V_v$ ), length ( $L$ ) and the mean diameter ( $D$ ) of the capillaries in the ZF and the ZR were determined. All these parameters were calculated from stereological equations as described previously (Budeč *et al.*, 2002; Milovanović

*et al.*, 2003). For morphometric analysis, the groups consisted of six to eight animals.

#### Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) of each group. Statistical significance was performed by one-way analysis of variance (ANOVA) with *post hoc* Fisher's least significant differences (LSD) test. The differences were considered significant if  $P < 0.05$ .

## RESULTS

Table 1 illustrates the plasma levels of ACTH following different treatments. ANOVA showed significant difference between the groups ( $F = 12,787$ ,  $P = 0.000$ ). LSD *post hoc* test revealed that acute ethanol administration (4 g/kg) increased the concentrations of ACTH 30 min after injection ( $P < 0.01$ ). Alcohol at a dose of 2 g/kg (Table 1) produced a less profound effect on the release of ACTH compared with that at a dose of 4 g/kg ( $P < 0.01$ ). Subcutaneous pretreatment with 30 or 50 mg/kg of L-NAME did not significantly alter the response of ACTH to ethanol (Table 1). At both doses, the administration of L-NAME followed by saline significantly elevated the concentration of ACTH ( $P < 0.01$ ).

The effect of acute ethanol treatment on serum level of corticosterone is presented in Fig. 1. ANOVA demonstrated significant difference in the level of corticosterone between the groups ( $F = 42.756$ ,  $P = 0.000$ ) with alcohol at a dose of 4 g/kg eliciting more pronounced rise in the concentration of this hormone ( $P = 0.000$ ). As reported, the subcutaneous pretreatment with L-NAME at doses of 30 or 50 mg/kg had no influence on ethanol-induced changes. In addition, both doses of L-NAME (30 and 50 mg/kg) followed by saline significantly increased the level of corticosterone ( $P = 0.000$ ).

Morphometric analysis revealed that absolute volume of the cortex was significantly different between the groups ( $F = 4.5$ ,  $P = 0.002$ ). This parameter was significantly enlarged in the rats injected with: ethanol (2 or 4 g/kg), L-NAME (30 mg/kg) + ethanol (4 g/kg), L-NAME (50 mg/kg) + ethanol (4 g/kg) and L-NAME (50 mg/kg) + saline compared with control animals (Table 2). Also, in all these groups, with the exception of L-NAME (30 mg/kg) + ethanol (4 g/kg), a significant increase ( $F = 6.005$ ,  $P = 0.000$ ) of absolute volume of the ZF was observed (Fig. 2). An increase in this parameter was also detected in the ZR in the groups treated with L-NAME (30 mg/kg) + ethanol (4 g/kg) and L-NAME (30 mg/kg) + saline (Fig. 2). However, no differences were found in volume and numerical density of the adrenocortical cells in the ZG, ZF and ZR between the experimental and control animals (data not shown).

Further analysis showed that stereological parameters of blood vessels were altered in different groups. Thus, the volume density of capillaries of the ZF significantly differ between the groups ( $F = 8.162$ ,  $P < 0.01$ ). As can be seen in Table 3, a significant increase in volume density of capillaries of the ZF was noted in the groups administrated with: ethanol (4 g/kg) ( $P < 0.01$ ), L-NAME (30 mg/kg) + ethanol (4 g/kg) ( $P < 0.05$ ), L-NAME (50 mg/kg) + ethanol (4 g/kg) ( $P < 0.01$ ) compared with controls. In the same experimental groups, a length of the capillaries ( $F = 5.550$ ,  $P < 0.01$ ) was significantly increased in the ZF (Fig. 3). Analysis of

Table 1. Plasma ACTH concentrations in different groups

ACTH (pg/ml)						
Controls	EtOH (2 g/kg)	EtOH (4 g/kg)	L-NAME (30 mg/kg) + EtOH (4 g/kg)	L-NAME (50 mg/kg) + EtOH (4 g/kg)	L-NAME (30 mg/kg) + saline	L-NAME (50 mg/kg) + saline
78.4 ± 5.11	151.17 ± 21.69**	250.20 ± 21.94**##	276.6 ± 21.40**	203.33 ± 22.04**	171 ± 16.06**	156.14 ± 7.78**

EtOH, ethanol.

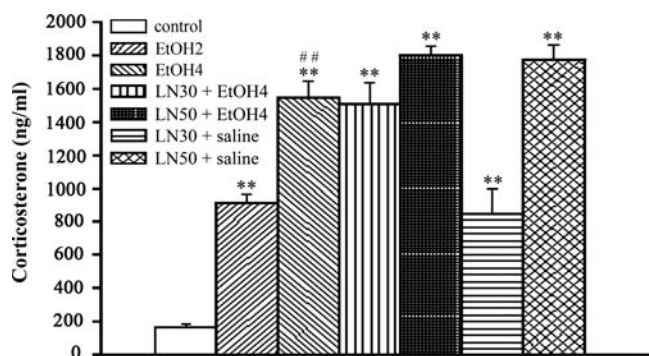
\*\**P* < 0.01 compared with controls.##*P* < 0.01 compared with EtOH (2 g/kg).

Fig. 1. The levels of serum corticosterone in rats under various experimental conditions. \*\**P* < 0.01 compared with controls; ##*P* < 0.01 compared with EtOH2. Data are reported as means ± SEM. (ANOVA, followed by LSD *post hoc* test). EtOH2, ethanol 2 g/kg; EtOH4, ethanol 4 g/kg; LN30, L-NAME 30 mg/kg; LN50, L-NAME 50 mg/kg.

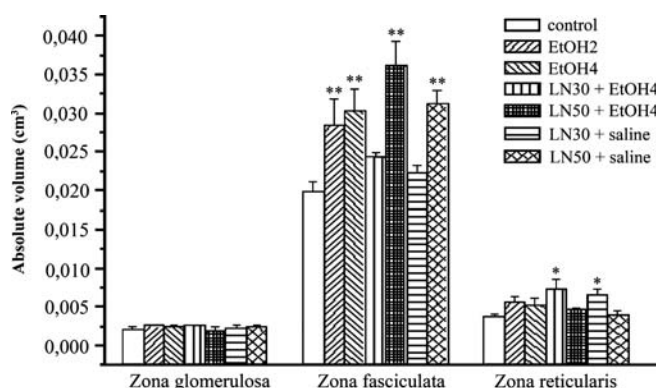


Fig. 2. Mean absolute volume of all cortical zones under various experimental conditions. \**P* < 0.05; \*\**P* < 0.01 compared with controls. Data are reported as means ± SEM. (ANOVA, followed by LSD *post hoc* test). EtOH2, ethanol 2 g/kg; EtOH4, ethanol 4 g/kg; LN30, L-NAME 30 mg/kg; LN50, L-NAME 50 mg/kg.

Table 2. Absolute volume of capsule, cortex and medulla

Groups	Absolute volume		
	Capsule (cm <sup>3</sup> × 10 <sup>-4</sup> )	Cortex (cm <sup>3</sup> × 10 <sup>-4</sup> )	Medulla (cm <sup>3</sup> × 10 <sup>-4</sup> )
Controls	6.76 ± 0.749	238.0 ± 7.7	33.3 ± 3.8
EtOH (2 g/kg)	7.00 ± 0.89	314.2 ± 35.5**	44.7 ± 7.7
EtOH (4 g/kg)	8.86 ± 0.67	326.4 ± 21.5**	40.7 ± 10.3
L-NAME (30 mg/kg) + EtOH (4 g/kg)	6.84 ± 0.19	294.5 ± 13.0**	40.6 ± 5.8
L-NAME (50 mg/kg) + EtOH (4 g/kg)	8.3 ± 0.53	352.4 ± 24.1**	54.8 ± 3.1
L-NAME (30 mg/kg) + saline	6.9 ± 0.75	260.8 ± 25.0	44.3 ± 1.1
L-NAME (50 mg/kg) + saline	7.342 ± 1.01	320.4 ± 15.8**	48.5 ± 9.6

EtOH, ethanol.

\*\**P* < 0.01 compared with controls.

diameter of capillaries demonstrated that ethanol (4 g/kg), applied after both doses of L-NAME, caused dilatation of capillaries in the ZF (Table 3), whereas ethanol alone had no effect. In the ZR, an increase in volume density and diameter of capillaries (Table 3) was observed in the groups injected with: ethanol (2 g/kg) (*P* < 0.01), L-NAME (30 or 50 mg/kg) + ethanol (4 g/kg) (*P* < 0.01) and L-NAME (50 mg) + saline (*P* < 0.05).

## DISCUSSION

We have reported previously that blood ethanol levels, 30 min after intraperitoneal administration at doses of 2 or 4 g/

kg, fulfill the criterion (>21.7 mmol/l) for intoxication (Budeč *et al.*, 2007).

In the current study, a possible role of endogenous NO in the adrenal response to ethanol was investigated by L-NAME in female rats. Since estrous cycle may modulate: (a) the actions of ethanol on ACTH and corticosterone (Rivier, 1993) as well as on the adrenal gland (Milovanović *et al.*, 2003), and (b) the expression of eNOS and iNOS (Ulbrich *et al.*, 2006), female rats showing the same stage of the estrous cycle-diestrus Day 1 were used in our experiment.

Like in our earlier findings (Budeč *et al.*, 2002; Milovanović *et al.*, 2003), a single, hypnotic dose of ethanol (4 g/kg) induced a significant increase in the concentration of ACTH 30 min after injection. In accordance with the results of Ogilvie *et al.* (1997a), this action of alcohol was a dose-related, suggesting its specific effect on the HPA axis. As we had demonstrated previously (Budeč *et al.*, 1996), using the same experimental design, plasma ACTH was still high 180 min after ethanol administration.

It was well established that acute alcohol treatment increased the levels of ACTH and corticosterone in the rat by stimulating CRH and possibly AVP secretion from nerve terminals in the median eminence (Rivier, 1996). Further study suggested the obligatory role of endogenous CRH and AVP in the action of intraperitoneal alcohol injection on proopiomelanocortin synthesis and ACTH release (Lee *et al.*, 2004). Although we were not able to measure plasma AVP, on the basis of the results reported by Ogilvie *et al.* (1997b) after intraperitoneal alcohol treatment (3 g/kg), we could assume that they were decreased in the current study.



Table 3. Volume density and mean diameter of blood vessels

Groups	Volume density of blood vessels		Mean diameter of blood vessels ( $\mu\text{m}$ )	
	Zona fasciculata	Zona reticularis	Zona fasciculata	Zona reticularis
Controls	0.048 $\pm$ 0.005	0.150 $\pm$ 0.014	6.10 $\pm$ 0.25	12.74 $\pm$ 0.75
EtOH (2 g/kg)	0.052 $\pm$ 0.010	0.272 $\pm$ 0.016**	6.72 $\pm$ 0.26	19.98 $\pm$ 0.42**
EtOH (4 g/kg)	0.105 $\pm$ 0.021***	0.210 $\pm$ 0.021#	7.53 $\pm$ 1.14	13.34 $\pm$ 0.96
L-NAME (30 mg/kg) + EtOH (4 g/kg)	0.090 $\pm$ 0.011*	0.264 $\pm$ 0.020**	9.28 $\pm$ 0.86**	18.18 $\pm$ 1.33**
L-NAME (50 mg/kg) + EtOH (4 g/kg)	0.130 $\pm$ 0.018**	0.256 $\pm$ 0.020**	9.77 $\pm$ 0.31**	19.11 $\pm$ 0.99**
L-NAME (30 mg/kg) + saline	0.040 $\pm$ 0.010	0.117 $\pm$ 0.013	7.16 $\pm$ 0.83	13.44 $\pm$ 1.75
L-NAME (50 mg/kg) + saline	0.054 $\pm$ 0.004	0.228 $\pm$ 0.021**	6.47 $\pm$ 0.64	17.33 $\pm$ 1.92*

EtOH, ethanol.

\* $P < 0.05$  compared with controls.

\*\* $P < 0.01$  compared with controls.

# $P < 0.05$  compared with EtOH (2 g/kg).

## $P < 0.01$  compared with EtOH (2 g/kg).

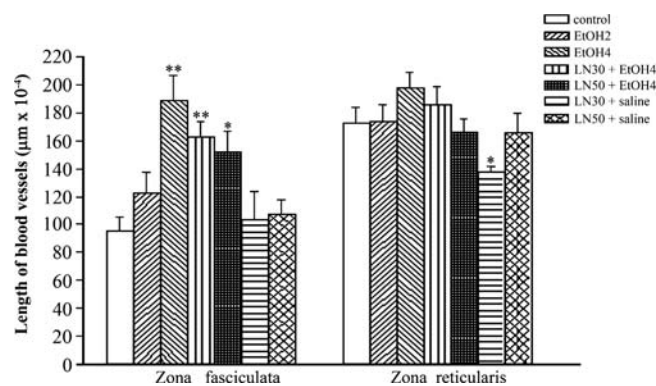


Fig. 3. Length of blood vessels of the ZF and the ZR in experimental and control rats. \* $P < 0.05$ ; \*\* $P < 0.01$  compared with controls. Values are means  $\pm$  SEM. (ANOVA, followed by LSD *post hoc* test). EtOH2, ethanol 2 g/kg; EtOH4, ethanol 4 g/kg; LN30, L-NAME 30 mg/kg; LN50, L-NAME 50 mg/kg.

Because the acute effects of alcohol and blockade of NO synthesis on the adrenal cortex received a relatively scant attention, we evaluated the morphological changes in this gland as well as serum levels of corticosterone in the groups treated with: ethanol and L-NAME followed by either ethanol or saline. Consistent with our previous studies (Budeč *et al.*, 2002; Milovanović *et al.*, 2003), a single, hypnotic dose of ethanol (4 g/kg) produced a significant increase in volume density and length of the capillaries of the ZF as well as in the serum level of corticosterone, indicating an enhanced activity of the adrenal cortex. Both doses of alcohol elevated the concentration of corticosterone, but a dose of 2 g/kg was less effective. Our findings are in accordance with those of Khisti *et al.* (2003), showing that acute ethanol treatment (2 g/kg, IP) increased serum level of corticosterone and expression of adrenal StAR protein. Using the same dose of ethanol, Croft *et al.* (2008) reported elevated plasma levels of corticosterone at different time points, 30, 60 and 120 min following injection. Also, it has been demonstrated that acetaldehyde, the first metabolite of ethanol, is able to elevate the concentration of corticosterone operating through a central mechanism in the rat (Kinoshita *et al.*, 2001). Although both ethanol and acetaldehyde can directly stimulate the adrenal gland *in vitro* (Cobb *et al.*, 1981), the increased levels of ACTH in the ethanol-treated

rats in our study suggest that the action on adrenal cortex is centrally mediated. The more profound effect of ethanol on serum corticosterone, measured 30 min after injection, can be explained by different dynamics of this hormone and ACTH. It has been well documented that maximum secretion of ACTH is usually observed at 15 min, whereas maximum concentrations of corticosterone are usually achieved between 30 and 60 min following exposure to stressor (Armario, 2006). In addition, the adrenal gland can secrete corticosterone in response to relatively low levels of ACTH.

There is evidence that NO may mediate some effects of ethanol. Thus, NO is involved in the action of ethanol on the release of  $\beta$ -endorphin (Boyadjieva *et al.*, 2003), and on the expression of intestinal IgA in female rats (Budeč *et al.*, 2007). Seo and Rivier (2003) reported that NO facilitated the stimulatory influence of alcohol on the release of ACTH in male rats. At variance with these findings, our results showed no effect of blockade of NO synthesis with L-NAME on ethanol-induced changes in the levels of ACTH and corticosterone as well as in the adrenal structure in female rats. Discrepancy between the study of Seo and Rivier (2003) and our study could be due to differences in the rat strain and sex (male Sprague Dawley vs. female Wistar), as well as different route of ethanol administration (intragastric vs. intraperitoneal injection). The influence of sex on the interactions between ethanol and NO was substantiated by our more recent observation (unpublished results) that 7-nitroindazole, selective inhibitor of nNOS, attenuated response of ACTH to acute ethanol treatment in male Wistar rats. Also, sex difference was documented by Uji *et al.* (2007), who showed that NO was involved in the surge of norepineprine to psychological stress in the male Wistar rats, but not in the females. In addition to sex, strain may influence some effects of NO. Although L-NAME increased the levels of corticosterone in BALB/c, C3H/He and DBA-2 mice, it did not affect its concentration in C57Bl/c mice (Giordano *et al.*, 1996). Furthermore, the action of L-NAME on blood pressure was found to differ markedly between Sprague Dawley and Lewis rats (Forster *et al.*, 2001).

Increased levels of ACTH and corticosterone following L-NAME, in the present study, are in concert with earlier investigations (Giordano *et al.*, 1996; Seo and Rivier, 2003). Our results also agree with those of Adams *et al.* (1992), who reported that L-NAME elevated dose-dependently the secretion of corticosterone in rats.

Significantly increased absolute volume of the cortex, volume density and length of the capillaries of ZF in the groups treated with ethanol (4 g/kg), L-NAME (30 mg/kg) + ethanol (4 g/kg) and L-NAME (50 mg/kg) + ethanol (4 g/kg) together with enhanced levels of corticosterone imply the activation of adrenal cortex. In spite of the fact that the concentration of corticosterone was elevated in all experimental groups, these stereological parameters were altered in some of them. A possible explanation for these results was provided by previous findings showing that the threshold level of ACTH that increased adrenal blood flow was higher than the threshold that enhanced the secretion of cortisol (Edwards *et al.*, 1975). High values of volume density and mean diameter of blood vessels in the ZR after treatment with ethanol (2 g/kg) might be due to a retrograde increase in blood pressure in the venous system and the proximity of medulla where, in the same experimental group, a significant increment in these two parameters was observed (data not shown). Our findings obtained by stereological analysis cannot be directly compared with those of others because, to the best of our knowledge, there are no morphometric studies on rat adrenal gland following the treatment with ethanol or L-NAME.

In addition to NO, heme oxygenase (HO)-derived carbon monoxide (CO) modulates the activity of HPA axis (Mancuso *et al.*, 2010). Role of CO in the regulation of HPA axis was supported by findings that inhibition of HO within the central nervous system increased endotoxin-induced release of AVP (Mancuso *et al.*, 1999). There is evidence that the release of hypothalamic neuropeptides is one of several functions in the nervous system, regulated by the interaction between HO and cyclooxygenase (Mancuso *et al.*, 2006). The possible involvement of CO in the action of ethanol on adrenal gland in our experimental model remains a challenging area for the future study.

In conclusion, this study indicates that acute ethanol treatment activates the adrenal cortex in female rats, but it seems that endogenous NO is not involved in this effect under these experimental conditions.

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