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24-Hour rhythm in gene expression of nitric oxide synthase and heme-peroxidase in anterior pituitary of ethanol-fed rats

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Abstract

Chronic exposure of rats to ethanol results in significant changes in pituitary hormone secretion. However, identification of the site(s) and mechanism of action of ethanol to induce these effects remains elusive. Free radical damage at the adenohypophyseal level may play a role in the decline in serum gonadotropin levels in ethanol-fed rats. Since 24-h changes in redox state occurred, we analyzed the 24-h changes in pituitary gene expression of the prooxidant enzymes nitric oxide synthase (NOS) 1 and 2, and of heme oxygenase-1 (HO-1) enzyme, and in plasma NO_2^- and NO_3^- (NO_x) levels, in ethanol and control rats. Male rats, 35-day-old, received a liquid diet for 4 weeks. The ethanol-fed group received a similar diet to controls except for that maltose was isocalorically replaced by ethanol. Animals were killed at six time intervals during a 24-h cycle. Anterior pituitary mRNA levels encoding NOS1, NOS2 and HO-1 were measured by real-time PCR analysis. Plasma NO_x concentration was determined by the Griess reaction. Ethanol feeding of prepubertal rats changed significantly the 24-h pattern of expression of NOS1, NOS2 and HO-1 in the adenohypophysis and augmented NOS2 and HO-1 mRNA levels. Peak values for the three enzymes in ethanol-fed rats occurred at the beginning of the scotophase (i.e., at 21:00 h). Ethanol feeding augmented mean values plasma NO_x levels with a maximum at 13:00 h while in controls a biphasic pattern was observed, with peaks at 09:00 h and 17:00–21:00 h. One of the mechanisms by which ethanol augments oxidative damage in the adenohypophysis may include overproduction of nitric oxide and carbon monoxide.

Keywords: Ethanol toxicity; Circadian rhythms; Adenohypophysis; Nitric oxide synthase; Heme oxygenase-1; Nitrites

Chronic exposure of rats to ethanol results in significant changes in pituitary hormone secretion, the hormones of the hypothalamic pituitary gonadal axis being extensively studied in this respect [8]. However, identification of the site(s) of action of ethanol to induce these effects remains elusive. Both acute and chronic (25–70 days) ethanol exposure are associated with low levels of hypothalamic gonadotropin-releasing hormone (GnRH) and pituitary luteinizing hormone (LH) in adult and peripubertal male rats [3,21]. After chronic ethanol feeding of animals, the circulating levels of prolactin augmented and those of follicle-stimulating hormone (FSH), LH and thyroid-stimulating hormone (TSH) decreased, in addition to significant modifications in the 24-h pattern of pituitary hormone levels in blood [14]. This correlated with significant disruptions of 24-h rhythms in median eminence dopamine (DA), serotonin (5-HT), γ-aminobutyric acid (GABA) and taurine turnover [15].

Recently an analysis was published on the role of free radical damage at the adenohypophyseal level on the decline in serum LH and FSH levels in rats fed with ethanol for 5–60 days [20]. There were increases in pituitary 8-oxo-deoxyguanosine immunoreactivity, a marker of oxidative damage to nucleic acids, and an overall increase in malondialdehyde and 4-hydroxynonenal, markers of lipid peroxidation. Pituitary protein carbonyl formation, a marker of protein oxidation, and tyrosine nitration of proteins, an index of nitrosative stress, increased significantly after 30–60 days of ethanol consumption, respectively [20]. The data provided evidence for ethanol-induced oxidative damage at the pituitary level, presumably contributing to pituitary dysfunction.

Since the above mentioned study on adenohypophyseal oxidative damage was performed at single time points, presumably at morning hours, and in view that 24-h changes in redox

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state occurred in a number of tissues [11,16], we considered it worthwhile to analyze the 24-h changes in pituitary gene expression of the prooxidant enzymes nitric oxide synthase (NOS) 1 and 2 and of heme oxygenase-1 (HO-1), as well as in plasma NO_2^- and NO_3^- (NO_x) levels in ethanol and control rats.

Five-week-old, peripubertal, male Wistar rats were kept under standard conditions of controlled light (12:12 h light/dark schedule) and temperature (22 ± 2 °C). A liquid diet mode of ethanol administration was employed [17]. The diet contained an aqueous suspension of pulverized casein, L-methionine, vitamin mixture, mineral mixture, sucrose, xanthum gum, choline bitartrate, Celufil cellulose, corn oil and maltose. Percent composition of the diet was 35% fat, 18% protein and 47% carbohydrates. The ethanol-fed group received a similar diet except for that maltose was replaced by 96% ethanol. Final ethanol concentration was 6.2% (wt./vol.) and ethanol replacement was isocaloric providing about 36% of the total caloric content of the diet. Saccharin was added to mask the ethanol taste. Control and experimental diets were freshly made each day. Rats were caged in groups of 4 animals/cage and had access to the liquid diet ad libitum. Daily average consumption of diet was 40–50 ml/rat, without significant differences between ethanol and control groups. The liquid diet without ethanol was administered 4 days before the study was begun to allow the animals to become accustomed to the new diet. No difference in the time when the ethanol and control animals started to feed daily was observed.

The care and use as well as all procedures involving animals were approved by the Institutional Animal Care Committee, Faculty of Medicine, Complutense University, Madrid. The study was in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health and the Guide for the Care and Use of Laboratory Animals [13].

Groups of 6–7 rats were killed by decapitation at six different time intervals, every 4 h, throughout a 24 h. At night intervals animals were killed under red dim light. Blood samples were collected from the trunk wound in heparinized tubes and centrifuged at 1500 × *g* for 15 min, the plasma being collected and stored at −20 °C until analyzed. The anterior pituitary gland was quickly removed, weighed and frozen until further assayed.

Total RNA extraction was performed using the RNeasy protect mini kit and was analyzed using QuantiTec SYBR green kit (Qiagen, Hielden, Germany). SuperScript II reverse transcriptase and random hexamers (Invitrogen, Carlsbab, CA) were used to generate template cDNA from total RNA. The house keeping gene β-actin was used as a constitutive control for normalization. Reactions were carried out in the presence of 300 nM of specific primers. Primers were designed using Primer3 software (The Whitehead Institute, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The specificity of the primers was confirmed by running the products of Polymerase Chain Reaction (PCR) amplification in a 2% agarose gel against molecular weight markers and by a BLAST software-assisted search of a non-redundant nucleotide sequence database (National Library of Medicine, Bethesda, MD). After each PCR run, melting curves were performed to assure no contamination in the individual wells of PCR plates [18]. Wells showing more than one peak were excluded from subsequent analysis.

To assess whether ethanol treatment modified the expression of β-actin, PCR with serial dilutions of this housekeeping gene was performed. PCR reactions were carried out in an AbiPrism 7300 (Applied Biosystems, Foster City, CA). The PCR reaction conditions were 15 min at 95 °C followed by 40 cycles of 15 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. Fluorescence data were collected during the 72 °C step. Serial dilutions of cDNA from control pituitaries were used to perform calibration curves in order to determine transcription efficiencies. For the primers used there were no differences between transcription efficiencies, and the amount of initial cDNA in each sample was calculated by the $2^{-\Delta\Delta Ct}$ method [18]. All samples were analyzed in triplicate and in three different measures. Fractional cycle at which the amount of amplified target becomes significant (Ct) was automatically calculated by the PCR device.

Nitrite (NO_2^-) and nitrate (NO_3^-) plasma levels were measured as circulating markers for the activity of NOS. The assay employed was based on the determination of NO_2^- using the Griess reaction. NO_3^- was measured as nitrite after enzymatic conversion by nitrate reductase [10].

Statistical analysis of results was performed by a factorial analysis of variance (ANOVA). Generally, the ANOVA included assessment of treatment effect (i.e., the occurrence of differences in mean values between ethanol- and control-fed rats), of time-of-day effects (the occurrence of daily changes) and of the interaction between treatment and time, from which inference about differences in timing and amplitude could be obtained. Post hoc Bonferroni's multiple comparisons tests in a one-way ANOVA were then employed to show which time points were significantly different within each experimental group to define the existence of peaks. *p*-Values lower than 0.05 were considered evidence for statistical significance.

Fig. 1 depicts the results obtained. As shown in the left upper panel, although mean values for expression of mRNA for NOS1 did not differ significantly between both groups ($p = 0.24$), significant effects of time ($F = 40.7$, $p < 0.0001$) and a significant interaction "treatment × time" ($F = 67.1$, $p < 0.0001$) were found, i.e., NOS1 expression peaked at 21:00 h in ethanol-fed rats and at 01:00–05:00 h in controls.

The 24-h changes in adenohypophyseal NOS2 expression are depicted in the right upper panel of Fig. 1. Ethanol feeding augmented significantly mRNA for NOS2 ($F = 211$, $p < 0.0001$) with a peak at 21:00 h while in controls maximal values were found at 09:00 h ($F = 47$, $p < 0.0001$, for the interaction "treatment × time of day", factorial ANOVA).

In ethanol-fed rats expression of HO-1 augmented significantly ($F = 302$, $p < 0.0001$) with a significant effect of time ($F = 98$, $p < 0.0001$) and a significant interaction "treatment × time" ($F = 89$, $p < 0.0001$) taking place, i.e., HO-1 expression peaked at 21:00 h in ethanol-fed rats whereas a nadir in controls occurred at 13:00 h (Fig. 1, left lower panel).

The right lower panel of Fig. 1 shows the 24-h changes in plasma NO_x levels. Ethanol feeding augmented mean values ($F = 101$, $p < 0.0001$) with a maximum at 13:00 h while in controls a biphasic pattern was observed, with peak values

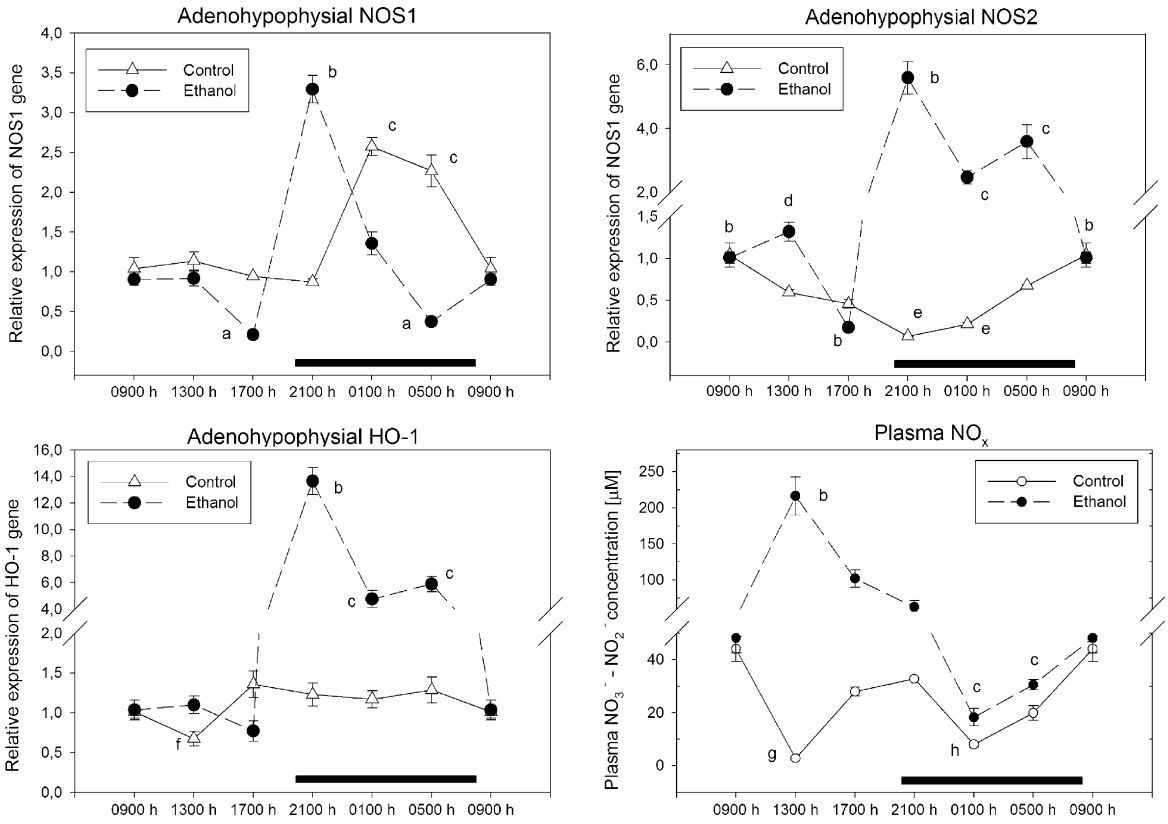

Fig. 1. Effect of chronic ethanol feeding on 24-h changes in expression of mRNA for NOS1, NOS2 and HO-1 in the adenohypophysis and in plasma NO_x concentration. Groups of 6–8 rats were killed by decapitation at six different time intervals throughout a 24-h cycle. Anterior pituitary mRNA levels encoding NOS1, NOS2 and HO-1 were measured as described in the text. Shown are the mean ± S.E.M. of mRNA determination for NOS1, NOS2 and HO-1 as measured by triplicate real-time PCR analyses of RNA samples. Plasma NO_x concentration was determined by the Griess reaction as described in the text. Letters denote significant differences in a one-way ANOVA followed by a Bonferroni's multiple comparison test, as follows: [a] $p < 0.01$ vs. 09:00, 13:00 and 01:00 h; [b] $p < 0.01$ vs. all other means; [c] $p < 0.01$ vs. 09:00, 13:00, 17:00 and 21:00 h; [d] $p < 0.01$ vs. 17:00, 21:00, 01:00 and 05:00 h; [e] $p < 0.01$ vs. 09:00 and 13:00 h; [f] $p < 0.05$ vs. 17:00, 21:00, 01:00 and 05:00 h; [g] $p < 0.01$ vs. 09:00, 17:00, 21:00 and 05:00 h; [h] $p < 0.01$ vs. 09:00, 17:00 and 21:00 h. For further statistical analysis, see text.

at 09:00 and 17:00–21:00 h ($F = 32$ for the interaction "treatment × time", $p < 0.0001$).

The foregoing results indicate that ethanol feeding of prepubertal rats changed significantly the expression of two prooxidant enzymes in the adenohypophysis, i.e., NOS1 and NOS2, and of HO-1, by changing their 24-h pattern and by augmenting NOS2 and HO-1 mRNA levels. For the three enzymes, peak values in ethanol-fed rats occurred at the beginning of the scotophase (i.e., at 21:00 h). This was remarkable in view that control animals maxima in pituitary enzyme's gene expression did not coincide.

One of the mechanisms by which ethanol acts on the adenohypophysis includes nitric oxide (NO), a relevant modulator of pituitary hormones release [6,24,25,27]. NOS, the enzyme converting L-arginine to NO, was first purified and cloned from rat brain (for ref. see [29]). In addition to the neuronal form (NOS1), other isoforms of NOS have been identified in the adenohypophysis like the inducible form (NOS2) [2].

Ethanol affects NO production in different tissues and the possibility has been entertained about the role of NO in ethanol toxicity (for ref. see [4,5]). It is known that NO plays opposite roles in the hypothalamus and the pituitary gland. At hypothalamic level, NO increases pituitary hormones release while, at pituitary level, NO inhibits their release [19,26,28]. Previously we reported that prolactin release was augmented and phase-advanced in rats receiving ethanol [14,15] in correlation with disruptions of 24-h rhythms in median eminence transmitter turnover, particularly DA [15]. Indeed, NO plays an important role in the diurnal changes of tuberoinfundibular dopaminergic neuronal activity and prolactin secretion, since pretreatment of antisense oligodeoxynucleotide against the mRNA of NOS1 was effective in preventing the diurnal changes of DA neuronal activity and prolactin surge while the same treatment of antisense against NOS2 was ineffective [30]. The phase-advance in pituitary NOS1 expression seen in the present study after ethanol administration correlated with a distorted and phase-advanced secretion of prolactin reported in animals similarly fed with ethanol [15].

Ethanol feeding of prepubertal rats increased plasma NO_x significantly, with peak values at 13:00 h, asynchronous to the maxima in NOS1 and NOS2 in the adenohypophysis. Since NO plays a role in the function of nearly all organs in the body, and it is rapidly metabolized to its principal metabolites NO_2^- and NO_3^- that diffuse into the circulation, plasma NO_x, as the sum of NO_3^- and NO_2^- concentration, is an index of the sum total of NO released from all of the organs of the body that would include, besides the anterior pituitary, the brain, heart, vascular system, kidney, adipose tissue and immune cells [7,9]. This may

explain the dissociation between pituitary expression of NOS1 and NOS2 and plasma NO_x levels seen in the present study. Further studies measuring pituitary NO production NO_x levels and NOS expression would shed light on a possible dissociation between enzyme expression and intraglandular production and levels. Such a dissociation would imply that enzyme expression does not always correlate with activity as has been shown in other tissues [12,22,23].

In addition to NO, it has been shown that carbon monoxide (CO) may also affect pituitary hormones secretion [1]. As CO is a product of HO-1 activity, the effect of ethanol on HO-1 gene expression reported herein could partially explain the dysregulation of hormone release caused by ethanol [14]. The present study indicates that expression of the HO-1 gene augmented significantly in the anterior pituitary of ethanol-fed rats.

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