Upregulation of vascular inducible nitric oxide synthase mediates the hypotensive effect of ethanol in conscious female rats

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Clinical and experimental findings have shown that the effect of ethanol on blood pressure follows a J-shaped relationship, depending on the duration and amount of ethanol consumed. Ethanol exerts complex cardiovascular actions, which may influence its net blood pressure effect. Acute administration of ethanol causes increases (8), decreases (25), or no change (1, 44) in blood pressure. Increases (39) and decreases (19, 38) in blood pressure have also been reported after chronic ethanol feeding. The mechanism by which ethanol elevates blood pressure may be related to the associated increase in sympathetic activity, as indicated by the rise in plasma norepinephrine levels (4, 5, 23). Our finding that sympathetic neural activity was higher in ethanol-fed than in control rats provided more direct evidence implicating the sympathetic nervous system in ethanol-induced hypertension (39). The ability of ethanol to attenuate the gain of baroreceptor control of heart rate (HR) may also contribute to its pressor effect (8, 39). On the other hand, ethanol elicits other cardiovascular effects that may counterbalance its sympathoexcitatory effects and result in hypotension, such as direct myocardial depression (26, 51), vasodilation (43), and α-adrenoceptor blockade (2).

Previous reports from our laboratory showed that the hemodynamic effects of ethanol, administered intragastrically, are sexually dimorphic. Ethanol elicits hypotension in female, but not in age-matched male, rats (11), which suggested that the ovarian hormones might be implicated in the hemodynamic effects of ethanol (11). Further support to this notion are the observations that ethanol hypotension in female rats is abolished by ovariectomy and restored after estrogen replacement, highlighting the dependence of ethanol action on estrogen (10). A similar estrogen-dependent hypotension has also been demonstrated in radiotelemetered female rats after long-term (12 wk) ethanol feeding (12). Together, these findings were the first to establish a role for estrogen in the hemodynamic responses elicited by acutely or chronically administered ethanol in female rats.

Although the hypotensive effect of intragastric ethanol in female rats is documented (10, 11), the underlying cellular mechanism remains unknown. In the present study, we hypothesize that upregulation of inducible nitric oxide synthase (iNOS) activity in vascular tissues is implicated in ethanol hypotension. This assumption may be supported by the following observations: 1) ethanol, used in amounts similar to those proposed in this study, enhances the cytokine-evoked induction of vascular iNOS in cultured rat aortic smooth muscle cells (7); 2) ethanol increases the iNOS protein expression and activity in ovarian tissues (41); 3) ethanol elicits no hypotension (11) or iNOS upregulation (17) in male rats; and 4) the hypotensive effect of ethanol is dependent on estrogen (10), which elicits a greater induction of cardiovascular iNOS in female than in male rats (18). To evaluate the role nitric oxide synthase (NOS)-nitric oxide (NO) signaling in the ethanol-evoked hypotension, we undertook experiments to determine the effects of prior treatment with the nonselective NOS inhibitor NG-nitro-L-arginine (NOARG) (6) and the iNOS selective inhibitor aminoguanidine (3) on hemodynamic responses to ethanol (1 g/kg ig) in conscious female rats. Changes evoked by ethanol or equal amount of water in aortic iNOS content in the absence and presence of aminoguanidine were determined by immunohistochemistry and correlated to concomitant changes in blood pressure responses. As a positive control, the effect of lipo-
polysaccharide (LPS; 5 mg/kg iv) on iNOS activity was determined as described elsewhere (49).

MATERIALS AND METHODS

A total of 51 female Sprague-Dawley rats (12–13 wk old, Charles River, Raleigh, NC) were employed in this study. Experiments were carried out in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health.

Intravascular and intragastric cannulations. The method described in our previous studies (10, 11) was adopted. Briefly, the rats were anesthetized with pentobarbital sodium (50 mg/kg ip). Catheters, each consisting of a 5-cm polyethylene-10 tubing bonded to a 15-cm polyethylene-50 tubing, were placed into the abdominal aorta and vena cava via the left femoral vessels for measurement of arterial pressure and intravenous injections, respectively. The polyethylene-10 portion was used for the intravascular segment of the catheter and was secured in place with sutures. The arterial catheter was connected to a Gould-Statham pressure transducer (Oxnard, CA), and blood pressure was displayed on a Grass polygraph (model 7D, Grass Instruments, Quincy, MA). HR was computed from blood pressure waves by a Grass tachograph and was displayed on another channel of the polygraph. Intragastric catheterization was performed by inserting a 20-cm polyethylene-50 tubing into the stomach through a nostril (10, 11). The tubing was bent by heat 6 cm from one end to a 45° angle to fit into the nose. An ∼14-cm portion of the tubing was guided through the esophagus into the stomach. This technique allows intragastric administration of drugs in freely moving rats.

Finally, the catheters were tunneled subcutaneously and exteriorized at the back of the neck between the scapulae. Vascular and nasogastric catheters were flushed with heparin (100 U/ml) and water, respectively, and plugged by stainless steel pins. Incisions were closed by surgical clips and swabbed with povidone-iodine solution. Each rat received intramuscular injections of the analgesic buprenorphine hydrochloride (Buprenex; 30 μg/kg) and penicillin G benzathine and penicillin G procaine in an aqueous suspension (Duraphen, 100,000 U/kg) and was housed in a separate cage. The experiments were performed 2 days later in conscious freely moving rats as in our previous studies (10, 11).

Immunohistochemistry

The technique described in our laboratory’s previous studies and other studies (16, 48) was employed for immunohistochemical measurement of aortic iNOS. Aortas were fixed in 4% paraformaldehyde-PBS solution overnight, transferred to 20% sucrose-PBS solution, and sectioned. Cryostat sections (15 μm thick) were stained with anti-iNOS polyclonal antibody (Transduction Laboratories, Burlingame, CA), according to the manufacturer’s instructions. Sections were immersed in a 0.1% H2O2 for 30 min to block endogenous peroxidase activity, 2) normal animal serum solution containing 0.4% Triton X-100 for 1 h to reduce nonspecific binding, 3) primary iNOS antibody (1:50) solution for 48 h at 4°C, 4) diluted biotinylated secondary antibody solution for 1 h at room temperature, 5) ABC reagent solution for 30 min at room temperature, 6) peroxidase substrate solution until the desired stain intensity developed, and 7) clear and mount. PBS (pH 7.4) was used to dilute each solution and to wash the sections three times after each step. Quantification of the signal was performed using the National Institutes of Health imaging system described in our laboratory’s previous studies (12, 48).

Experimental Groups and Protocols

Experiment 1: hemodynamic effects of ethanol. Two groups of female rats were used in this experiment to investigate the effects of ethanol (1 g/kg, n = 7) or equal amount of water (n = 4) on blood pressure and HR. On the day of the experiment, the arterial catheter was connected to a pressure transducer for the measurement of blood pressure and HR, as mentioned above. A period of at least 30 min was allowed at the beginning of the experiment for stabilization of blood pressure and HR. Ethanol (1 g/kg; 10 ml/kg of 13% ethanol diluted in water) was administered intragastrically as reported in our previous studies (9–11) and others (21). The intragastric administration of similar volume of water (~2–2.5 ml/rat depending on the rat weight) appeared to be well tolerated by the rats because it produced no significant hemodynamic changes in the present or previous studies (9–11). After ethanol or water administration, measurements of blood pressure and HR were made at 10-min intervals for a period of 120 min.

Experiment 2: effect of NOARG on hemodynamic responses to ethanol. This experiment evaluated the effect of the nonselective NOS inhibitor, NOARG, on blood pressure and HR responses elicited by ethanol in female rats. NOARG (5 mg/ml) was continuously infused (Harvard compact infusion pump, Model 975) through the femoral vein at a rate of 13 μl/min. NOARG infusion resulted in an elevation in blood pressure of ∼40–50 mmHg that was maintained throughout the duration of the study. Intragastric ethanol (1 g/kg, n = 4) or equal amount of water (10 ml/kg, n = 6) was administered 10 min after starting NOARG infusion, and hemodynamic variables were monitored for the following 90 min.

To investigate whether the elevated blood pressure by NOARG contributed to its hemodynamic interaction with ethanol, the hemodynamic effect of intragastric ethanol (1 g/kg, n = 5) or equal amount of water (10 ml/kg, n = 7) was evaluated in two additional groups of female rats in which phenylephrine (150 μg/ml) was infused at a rate of 20 μl/min to produce a steady-state elevation in blood pressure comparable to that of NOARG.

Experiment 3: effect of aminoguanidine on hemodynamic responses to ethanol. This experiment examined whether vascular iNOS contributes to the hypotensive effect of intragastric ethanol in female rats. Selective iNOS inhibition was achieved by a single intraperitoneal injection (45 mg/kg) of aminoguanidine as reported elsewhere (3). Intragastric ethanol (1 g/kg, n = 9) or equal amount of water (10 ml/kg, n = 7) was administered 30 min after aminoguanidine, and blood pressure and HR were monitored for 120 min.

Experiment 4: aminoguanidine-ethanol interaction on aortic iNOS. To further characterize the role of vascular iNOS in the hypotensive action of ethanol, the aortic iNOS content was measured by immunohistochemistry in rats treated with ethanol in the absence and presence of aminoguanidine. At the conclusion of experiments 1 and 3, rats were killed and thoracic aortas were harvested for the immunohistochemical measurement of iNOS content. As a positive control, the effect of LPS (5 mg/kg iv) on aortic iNOS content was determined in two rats as described elsewhere (49). Notably, LPS is believed to reduce blood pressure via increasing iNOS activity, and both effects are inhibited by the selective iNOS inhibitor aminoguanidine (20, 40).

Drugs

Phenylephrine hydrochloride, pentobarbital sodium, aminoguanidine hydrochloride, NOARG (Sigma Chemical, St. Louis, MO), povidone-iodine solution (Norton, Rockford, IL), ethanol (Midwest Grain Products, Westfield, MO), Buprenex (Rickett & Colman, Richmond, VA) and Durapen (Vedco, Overland Park, KS) were purchased from commercial vendors.

Data Analysis and Statistics

Values are presented as means ± SE. Mean arterial pressure (MAP) was calculated as diastolic pressure + one-third pulse pressure (systolic − diastolic pressures). Changes in MAP and HR evoked by intragastric ethanol or water in control female rats or in rats pretreated with NOARG, phenylephrine, or aminoguanidine were computed at 10-min intervals. The images generated from immunohistochemical
studies were quantified using a video-based computerized image-analysis system as in our laboratory’s previous studies (12, 48). The integrated densities are computed using the NIH Image software (version 1.62) and a power Macintosh (7300/180). ANOVA followed by a Newman-Keuls post hoc analysis was used to analyze the data. Probability levels <0.05 were considered significant.

RESULTS

Baseline values of MAP and HR were similar in all groups of ethanol and water-treated female rats used in the present study (Table 1).

Hemodynamic Effects of Ethanol

The hemodynamic responses elicited by intragastric ethanol in conscious female rats are shown in Fig. 1. Compared with corresponding water values, ethanol (1 g/kg) produced significant \( P < 0.05 \) decreases in MAP that started at 40 min after its administration and continued throughout the 120-min duration of the experiment (Fig. 1). A maximal hypotension of \( \sim 15 \) mmHg was demonstrated by the end of the experiment (Fig. 1). The HR showed slight increases or decreases by ethanol, but these changes were not statistically different from corresponding values of water-treated rats (Fig. 1).

NOARG-Ethanol Hemodynamic Interaction

Figure 2 depicts changes in MAP and HR evoked by subsequent administration of ethanol (1 g/kg ig) or equal volume of water in female rats infused continuously with the nonselective NOS inhibitor NOARG. Infusion of NOARG (5 mg/ml, 13 \( \mu \)l/min) caused a sustained elevation in MAP (\( \sim 40 – 50 \) mmHg) that was associated with a reduction in HR (Fig. 2). Intragastric administration of ethanol (1 g/kg) during the hypertensive response of NOARG elicited no significant changes in blood pressure during the 90-min observation period. As shown in Fig. 2, changes in MAP evoked by ethanol or water were not statistically different. Similarly, the HR responses to NOARG were not influenced by ethanol administration (Fig. 2).

To eliminate the possibility that the lack of a hypotensive response to ethanol in NOARG-treated rats was due to the existing hypertension, we investigated the hemodynamic effect of ethanol in female rats subjected to a similar elevation in MAP evoked by phenylephrine infusion (150 \( \mu \)g/ml, 20 \( \mu \)l/min). Unlike the case in NOARG-treated rats, the administration of ethanol to phenylephrine-treated rats elicited significant \( P < 0.05 \) decreases in MAP, compared with corresponding control (water) values, at 30, 40, 70, and 80 min (Fig. 3), which indicates the dependence of ethanol hypotension on NOS-derived NO. Furthermore, the arterial baroreceptor-mediated bradycardic response that accompanied the pressor effect of phenylephrine was also significantly \( P < 0.05 \) attenuated by ethanol (Fig. 3).

Aminoguanidine-Ethanol Hemodynamic Interaction

This experiment investigated whether selective inhibition of iNOS by aminoguanidine alters the hemodynamic effects of ethanol in conscious female rats. Aminoguanidine (45 mg/kg ip) did not change baseline MAP (\( +3.2 \pm 1.4 \) mmHg) or HR (\( +2.2 \pm 5.5 \) beats/min). Subsequent administration of ethanol (1 g/kg ig) caused no change in MAP or HR compared with the control (water) values (Fig. 4).

Aminoguanidine-Ethanol Interaction on Aortic iNOS

The effects of intragastric ethanol or water administration to female rats in the absence and presence of aminoguanidine on

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<th>Table 1. Baseline values of mean arterial pressure and heart rate</th>
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<td><strong>Group</strong></td>
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Values are means ± SE; n, no. of subjects. NOARG, \( N^\circ \)-nitro-L-arginine; MAP, mean arterial pressure; HR, heart rate.
the immunohistochemical signal of iNOS in the thoracic aortas are illustrated in Fig. 5. Densitometric analysis showed that ethanol caused a threefold increase \((P < 0.05)\) in aortic iNOS expression compared with water-treated rats (Fig. 5). Amino-guanidine had no effect on iNOS signal in rats subsequently receiving water, but it virtually abolished the ethanol-evoked increase in iNOS activity (Fig. 5). As positive controls, LPS-treated rats exhibited approximately a sixfold increase in aortic iNOS (Fig. 5) and \(-30\)-mmHg reduction in blood pressure (data not shown). Figure 6 shows representative images of iNOS immunohistochemical signals in aortas of different rat groups.

**DISCUSSION**

Reported findings from our laboratory have shown that intragastric administration of ethanol, which simulates human consumption, elicits hypotension in normotensive female rats (10, 11). Because ethanol enhances iNOS-NO signaling (7, 41), we proposed that the upregulation of vascular iNOS activity might contribute to the hypotensive effect of ethanol in female rats. To investigate this possibility, experiments were conducted to determine the effect of prior treatment with NOARG, a nonselective NOS inhibitor, or aminoguanidine, a selective iNOS inhibitor, on the hemodynamic effects of ethanol in conscious female rats. To further characterize the cellular mechanism involved, we evaluated the effect of ethanol, in the absence and presence of aminoguanidine, on iNOS immunohistochemical signal in vascular (aortic) tissues. Three main findings were obtained in the present study. First, intragastric ethanol elicited hypotension in female rats, an effect that was abolished by NOARG pretreatment. Second, the abolition of ethanol hypotension in NOARG-treated rats may not be accounted for by the concomitant NOARG-induced hypertension because 1) ethanol produced hypotension when blood pressure was similarly elevated by phenylephrine infusion, and 2) ethanol elicited no hypotension in rats pretreated with aminoguanidine, which caused no alterations in baseline blood pressure. Third, the lack of ethanol hypotension in aminoguanidine-treated rats was paralleled with the abolition of the ethanol-evoked enhancement of the iNOS activity in thoracic aortas. These findings support the hypothesis that the upregulation of vascular iNOS contributes to the acute hypotensive effect of ethanol in female rats.

In agreement with previous reports (10, 11), the present study demonstrated that intragastrically administered ethanol to female rats elicited significant reduction in blood pressure...
that was maintained for the 120-min duration of the study. The present study also showed, for the first time, that NOARG abolished the acute hypotensive effect of ethanol, suggesting a role for NO in mediating ethanol hypotension. One possible limitation of this conclusion, however, was the demonstration that NOARG caused a sustained elevation in blood pressure. Although the latter finding is consistent with the role of NO in the tonic regulation of blood pressure (33), it is not clear whether the abolition of the hypotensive effect of ethanol by NOARG is due to NOS inhibition or to the associated increase in blood pressure. The latter assumption seems unlikely because ethanol significantly lowered blood pressure in phenylephrine-treated female rats, which attained elevated blood pressure levels similar to those of NOARG-treated rats. Furthermore, previous studies, including those from our laboratory, demonstrated decreases in blood pressure by ethanol in hypertensive rats (22, 38) that were greater in magnitude than those seen in the present study in normotensive rats, which may imply that the hypotensive effect of ethanol is proportional to the preexisting blood pressure levels. Collectively, these findings implicate NOS-derived NO in ethanol hypotension in sexually mature female rats.

It is notable, however, that NOARG is a nonselective NOS inhibitor and may not, therefore, identify the NOS isoform(s) involved in the hypotensive effect of ethanol. Therefore, the present study was extended to investigate the possible involvement of the iNOS isoform in ethanol hypotension. We reasoned that iNOS is a likely candidate because 1) of the delayed onset of ethanol hypotension as demonstrated in the present and previous (10, 11) studies, 2) earlier reports showed that ethanol increases iNOS activity (7, 41), and, 3) like its blood pressure effect (11), ethanol elicits sexually dimorphic effect on iNOS activity in male (decrease) and female (increase) rats (17, 41). Therefore, pharmacological and immunohistochemical studies were undertaken in the present study to investigate the influence of the selective iNOS inhibitor aminoguanidine on ethanol effects on blood pressure and vascular iNOS expression. The results showed that the hypotensive effect of ethanol was associated with an increase in the aortic iNOS expression and that both effects were abolished in rats pre-treated with aminoguanidine. The dose of aminoguanidine (45 mg/kg ip) employed in the present study appeared to selectively inhibit the inducible but not the constitutive NOS isoforms, because aminoguanidine, unlike NOARG, caused no elevation in baseline blood pressure and significantly attenuated ethanol-induced aortic iNOS expression. The reduction in iNOS expression by aminoguanidine is in agreement with previous reports, which highlighted the ability of aminoguanidine to suppress iNOS activity and expression by an as yet unidentified mechanism (42, 46). These findings provide the first experimental evidence that establishes a causal relationship between ethanol hypotension in female rats and the upregulation of iNOS in vascular tissues.

It is important, however, before this conclusion is accepted to comment on two potential limitations of the current study. First, the present results should not be interpreted to rule out a possible modulatory role for constitutive NOS (endothelial NOS and/or neuronal NOS) in the blood pressure response to ethanol because alterations in the activity of these isoforms by ethanol have been demonstrated in previous studies.

![Fig. 4. ΔMAP and ΔHR evoked by intragastric administration of ethanol (1 g/kg) or equal volume of water in conscious female Sprague-Dawley rats pretreated, 30 min earlier, with aminoguanidine (AG; selective inducible nitric oxide synthase inhibitor, 45 mg/kg ip). Values are means ± SE; no. of rats in each group is in parentheses.](image1)

![Fig. 5. Effect of intragastric administration of ethanol (1 g/kg) or equal volume of water to female Sprague-Dawley rats on aortic inducible NOS expression measured by immunohistochemistry in the absence and presence of AG (45 mg/kg ip). Values are means ± SE; no. of rats in each group is in parentheses.](image2)
our own (45, 47, 50). In effect, the relatively longer time required for effective induction of iNOS (7) may possibly implicate constitutive NOS isoforms, at least in the early phase of ethanol hypotension. The second limitation of the present study pertains to the notion that changes in vascular iNOS levels may not precisely explain the cellular mechanism that underlies ethanol hypotension, because previous studies from our laboratory have implicated cardiac (reductions in cardiac output and stroke volume) and not vascular hemodynamics in ethanol hypotension (10, 11). The measurement of cardiac iNOS, therefore, would have been a better alternative, because increased iNOS activity in cardiac tissues is known to depress myocardial activity (42). It is imperative, however, to note that one objective of the present study was to assess the effect of ethanol on vascular iNOS activity as a major source of releasable NO stores that are critically involved in circulatory control (34, 42, 49). Given that ethanol at a dose similar to that used in the present study increases plasma NO activity and nitrite/nitrate levels (21), the modulation of cardiac function by NO of vascular origin is a possibility that remains to be investigated. Importantly, the effect of longer periods (hours) of ethanol treatment on inducible and constitutive NOS isoforms in vascular and cardiac tissues and their possible contribution to the evoked hypotension in female rats are points that should be investigated in future studies.

The mechanism(s) by which ethanol upregulates vascular iNOS activity and subsequently lowers blood pressure in female rats and whether it involves direct or indirect pathways are not clear. One possible explanation may involve facilitation by ethanol of estrogen modulation of iNOS activity. This view is supported by the observations that 1) ethanol hypotension is estrogen dependent (10); 2) ethanol increases plasma estrogen levels (14, 15); 3) estrogen increases iNOS expression (35); and 4) compared with female rats, estrogen elicits a smaller increase in cardiac iNOS in male rats (18), the rat gender in which ethanol causes no hypotension (11). On the basis of these findings, it is likely that ethanol elicits hypotension by facilitating estrogen-evoked increases in iNOS-NO signaling. An alternative mechanism for ethanol upregulation of iNOS may be accounted for by the ethanol-induced endotoxemia. Several studies have shown that ethanol increases intestinal absorption and plasma levels of endotoxin (29), which enhances iNOS gene expression and lowers blood pressure (13, 24). Interestingly, ethanol produces greater increases in plasma endotoxin in female than in male rats (29). More studies are needed, however, to investigate these possibilities.

Published data from our laboratory (30, 39) and others (4, 5) have demonstrated a sympathoexcitatory effect for ethanol in male rats, which appeared to trigger the increase in blood pressure evoked by ethanol (8, 30, 39) or at least served to counterbalance ethanol-induced vasodepressor mechanisms and maintain blood pressure unchanged (1, 44). The issue whether alterations in sympathetic activity played an active part in the blood pressure response to ethanol in female rats has been addressed in a previous study from our laboratory (10), which measured plasma norepinephrine level, as an index of sympathetic activity, at different time intervals (10, 15, 30, 60 min) after ethanol administration. The results showed that, except for a modest sympathoinhibition at 30 min, the hypertensive effect of ethanol was not associated with any change in sympathetic activity, which eliminated a possible modulatory role for sympathetic activity in ethanol hypotension (10). Similarly, Livezey et al. (31) reported that ethanol at a dose (1 g/kg) similar to that used in the present study had no effect on sympathetic activity in female rats. Importantly, the lack of significant changes in HR in response to ethanol in the present study is also indicative of the inability of ethanol to alter sympathetic activity in the female population. As shown in Fig. 1, ethanol produced slight changes (increases followed by decreases) in HR, and these changes were not significantly different from corresponding values of control (water-treated) rats. Interestingly, substantial increases in HR were observed when ethanol was administered during phenylephrine infusion, which might constitute a compensatory baroreflex response to the associated hypotension. It is conceivable, therefore, to assume that the presence of preexisting bradycardia (e.g., after phenylephrine infusion) is a prerequisite for the tachycardic effect of ethanol to be uncovered. The finding, however, that ethanol-evoked tachycardia was not manifested during a similar bradycardic response to NOARG, or aminoguanidine may...
relate to the notion that ethanol-evoked hypotension was not manifested under conditions of NOS inhibition and also to the ability of NOS inhibitors to impair the baroreflex sympathetic gain (32).

The present study yields insights into the role of NOS-NO pathways in the hypotensive effect of moderate amounts of ethanol in female rats. The abolition of the hypotensive action of ethanol by the nonselective NOS inhibitor, NOARG, suggests the possible involvement of NOS-derived NO in ethanol hypotension. A causal relationship between ethanol hypotension and vascular iNOS is evident from the observations that selective inhibition of iNOS by aminoguanidine abolished the ethanol-evoked hypotension and the associated upregulation of iNOS activity in the thoracic aortas. Finally, these findings may be clinically significant given the similarity between the sexually dimorphic blood pressure response to ethanol in our experimental model (11) and humans (27, 28). It is noteworthy that the dose of ethanol used in the present study produces blood ethanol concentrations (10, 11) comparable to those attained in humans after consumption of moderate to intoxicating amounts of ethanol (1, 37).

GRANTS

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