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Facilitation of myocardial PI3K/Akt/nNOS signaling contributes to ethanol-evoked hypotension in female rats

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Abstract

Background—The mechanism by which ethanol reduces cardiac output (CO) and blood pressure (BP) in female rats remains unclear. We tested the hypothesis that enhancement of myocardial phosphatidylinositol 3-kinase (PI3K)/Akt signaling and related nNOS and/or eNOS activity constitutes a cellular mechanism for the hemodynamic effects of ethanol.

Methods—We measured the level of phosphorylated eNOS (p-eNOS) and p-nNOS in the myocardium of ethanol (1 g/kg intragastric, i.g.) treated female rats along with hemodynamic responses (BP, CO, stroke volume, SV, total peripheral resistance, TPR), and myocardial nitrate/nitrite levels (NOx). Further, we investigated the effect of selective pharmacological inhibition of nNOS with N^ω-propyl-L-arginine or eNOS with N⁵-(1-iminoethyl)-L-ornithine on cellular, hemodynamic and biochemical effects of ethanol. The effects of PI3K inhibition by wortmannin on the cardiovascular actions of ethanol and myocardial Akt phosphorylation were also investigated.

Results—The hemodynamic effects of ethanol (reductions in BP, CO, and SV) were associated with significant increases in myocardial NOx and myocardial p-nNOS and p-Akt expressions while myocardial p-eNOS remained unchanged. Prior nNOS inhibition by NPLA (2.5 or 12.5 µg/kg) attenuated hemodynamic effects of ethanol and abrogated associated increases in myocardial NOx and cardiac p-nNOS contents. The hemodynamic effects of ethanol and increases in myocardial p-Akt phosphorylation were reduced by wortmannin (15 µg/kg). On the other hand, although eNOS inhibition by L-NIO (4 or 20 mg/kg) dose-dependently attenuated ethanol-evoked hypotension, the concomitant reductions in CO and SV remained unaltered. Also, selective eNOS inhibition uncovered dramatic increases in TPR in response to ethanol, which appeared to have offset the reduction in CO. Neither NPLA nor L-NIO altered plasma ethanol levels.

Conclusions—These findings implicate the myocardial PI3K/Akt/nNOS signaling in the reductions in BP and CO produced by ethanol in female rats.

Keywords

Ethanol; blood pressure; cardiac output; female rats; nitric oxide synthase

Introduction

Experimental and clinical reports have established that the animal gender and the hormonal status are important factors that determine the hemodynamic effects of ethanol. For instance,

intra-gastric ethanol reduces blood pressure, CO and SV in female, but not in male, rats (El-Mas and Abdel-Rahman, 1999b). The reductions in CO and SV are consistent with a reduction of myocardial contractility elicited by ethanol (El-Mas and Abdel-Rahman, 2007). Further, the hemodynamic effects of ethanol are drastically attenuated in ovariectomized rats and are restored to intact sham-operated levels after estrogen replacement, suggesting a primary role for estrogen in mediating the cardiovascular actions of ethanol (El-Mas and Abdel-Rahman, 1999a). Although the mechanism of the estrogen-dependent hemodynamic effects of ethanol is not fully understood, accumulating evidence suggests that both substances share some cellular effects that might act additively or synergistically to trigger the hypotensive response. These cellular effects include the inhibition of calcium influx (Turlapaty et al., 1979; Zhang et al., 1994), promotion of NOS activity (Greenberg et al., 1993; Weiner et al., 1994), and reduction of α -adrenoceptor responsiveness (Abdel-Rahman et al., 1985; Sudhir et al., 1997).

In a recent study, we provided the first experimental evidence that implicated iNOS in the hypotensive effect of i.g. ethanol in female rats (El-Mas et al., 2006). This conclusion was based on the observations that ethanol-evoked hypotension was associated with significant increases in aortic iNOS protein expression and both effects were abolished after pharmacologic elimination of iNOS (El-Mas et al., 2006). Although this latter study yielded insights into a cellular mechanism of ethanol, the enhanced vascular iNOS signaling does not seem to account for the hypotensive response because reductions in CO and SV, but not TPR, underlie the hypotensive action of ethanol (El-Mas and Abdel-Rahman, 1999a,b). Therefore, in a more recent study we demonstrated an increased iNOS expression in the myocardium of ethanol treated female rats (El-Mas et al., 2007). These previous studies focused on iNOS because we and others have shown that ethanol increases endotoxin level (Rivera et al., 1998; Urbaschek et al., 2001; El-Mas et al., 2007), which causes generalized iNOS activation (Thiemermann, 1997). However, the finding that the hypotensive effect of ethanol appeared within minutes raises the possibility that a rapidly induced signaling mechanism seems to mediate the hypotensive response elicited by ethanol. Notably, while ethanol enhances eNOS/nNOS activity in vitro and in rat cerebellum (Hendrickson et al., 1999; Venkov et al., 1999; Xia et al., 1999), there are no reports on the effects of ethanol on myocardial eNOS or eNOS in female rats.

The objective of the present study was to test the hypothesis that the enhancement of myocardial nNOS and/or eNOS activity (phosphorylation) and its upstream effector PI3K/Akt contributes to the reduction in CO and related hypotensive response elicited by ethanol in female rats. To achieve this goal, we conducted integrative pharmacological studies that were complemented by biochemical, and Western blot studies. Pharmacological evidence that implicates PI3K/Akt/NOS (nNOS or eNOS) in the hemodynamic effects of ethanol was sought by investigating the effect of selective inhibition of nNOS (NPLA), eNOS (L-NIO), or PI3K (wortmannin) on the reductions in CO and BP elicited by intra-gastric ethanol in conscious female rats. To determine whether ethanol increases the level of the active (phosphorylated) form of Akt (p-Akt), nNOS (p-nNOS) or eNOS (p-eNOS), we measured the levels of these proteins in the hearts of the rats that received ethanol as compared to the vehicle (water). We also investigated the effects of the selective eNOS or nNOS inhibition on myocardial NO_x (index of NOS-generated NO) and on myocardial p-nNOS or p-eNOS in the absence or presence of ethanol. Because the ethanol-evoked hypotension is estrogen dependent (El-Mas and Abdel-Rahman, 1999a), all experiments were conducted during the proestrus phase, which exhibits the highest plasma estrogen levels (Marcondes et al., 2001).

Materials and Methods

Female Sprague-Dawley rats (12–13 weeks old, Charles River, Raleigh, NC) were employed in this study. Rats were allowed free access to water and purina chow. Surgical procedures and post-operative care were performed in accordance with, and approved by, the Institutional Animal Care and Use Committee and in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Intravascular and intragastric cannulations

The methods described in our previous studies (El-Mas and Abdel-Rahman, 1999a,b) were adopted. Briefly, rats were anesthetized with pentobarbital (50 mg/kg i.p.). Catheters, each consisting of 5-cm polyethylene-10 tubing bonded to 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 PE-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 Inst. Co., Quincy, MA). Heart rate was computed from blood pressure waveforms by a Grass tachograph and was displayed on another channel of the polygraph. The thermodilution technique described in our previous studies (El-Mas and Abdel-Rahman, 1999a,b) was employed for the measurement of CO (ml/min) and SV (μ l/beat) and TPR (MAP/CO, mmHg/ml/min). Intragastric catheterization was performed by inserting 20-cm polyethylene-50 tubing into the stomach through a nostril (El-Mas and Abdel-Rahman, 1999a,b). The tubing was bent by heat 6 cm from one end to a 45° angle to fit into the nose. An approximately 14 cm portion of the tubing was guided through the esophagus into the stomach. This technique allows i.g. administration of drugs in freely moving rats.

Finally, the catheters and the CO thermistor 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 μ g/kg) and penicillin G benzathine and penicillin G procaine in an aqueous suspension (Durapen, 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 (El-Mas and Abdel-Rahman, 1999a,b).

Western blotting

For the determination of myocardial phosphorylated nNOS, eNOS, and Akt protein levels, the rat ventricle was homogenized on ice in a homogenization buffer [50 mM Tris (pH 7.5), 0.1 mM EGTA, 0.1 mM EDTA, 2 μ M leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.1% (vol/vol) Nonidet P-40, 0.1% SDS, and 0.1% deoxycholate]. After centrifugation (12,000 g for 10 min), protein in the supernatant was quantified (Bio-Rad protein assay system; Bio-Rad, Hercules, CA). Protein extracts (50 μ g per lane) were run on a 4 to 12% SDS-polyacrylamide gel electrophoresis gel (Invitrogen, Carlsbad, CA) and electroblotted to nitrocellulose membranes. Blots were blocked for 120 min at room temperature in tris-buffered saline/tween20 (TBS/T) buffer (100 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% non-fat milk. They were then incubated overnight at 4 °C with rabbit antibodies to phospho-nNOS(Ser1417) (1:200, Affinity BioReagents™, Golden, CO), phospho-eNOS(Ser1177) (1:500, Cell Signaling Technology®, Inc. Danvers, MA), or phospho-Akt (Ser473) (1:1,000, Cell Signaling Technology®, Inc. Danvers, MA) in TBS/T

buffer containing 5% BSA. After 3 washes with TBS/T buffer, the blots were incubated for 60 min at room temperature with the appropriate horseradish peroxidase-linked species-specific anti-IgG (1:2,000, GE Healthcare BIO-Sciences Corp, Piscataway, NJ). After 3 washes with the TBS/T buffer, the blots were detected by enhanced chemiluminescence system and exposed to an X-ray film. Equivalent sample loading was confirmed by stripping membranes with blot restore membrane rejuvenation solution (SignaGen Laboratories, Gaithersburg, MD) and reprobing with anti-actin antibody (Sigma). Bands of phospho-nNOS, phospho-eNOS, or phospho-Akt were quantified by measuring the integrated density (mean density $\times$ area) using the NIH Image software (version 1.62); data was normalized in relation to actin, and expressed as a percent of control (saline+water) as in our previous studies (Wang and Abdel-Rahman, 2002; El-Mas et al., 2006, 2007).

Experimental groups and protocols

Hemodynamic effects of ethanol: Two groups of conscious proestrus female rats were used in this experiment to investigate the hemodynamic effects of ethanol (1 g/kg i.g., n=6) or equal volume of water (n=6). On the day of the experiment, the thermistor was connected to a Cardiomax II for measurement of CO and the arterial catheter was connected to a pressure transducer for measurement of BP and HR as mentioned above. A period of at least 30 min was allowed at the beginning of the experiment for hemodynamic stabilization. Ethanol 1 g/kg (10 ml/kg of 13% ethanol diluted in water) or water (approximately 2–2.5 ml/rat depending on the rat weight) was administered intragastrically as reported in our previous studies (El-Mas and Abdel-Rahman, 1999a,b). After ethanol or water administration, hemodynamic measurements continued for a period of 3 hr.

Effect of eNOS or nNOS inhibition on the hemodynamic actions of ethanol: Eight groups of conscious proestrus female rats (n=6–8 each) were used to investigate the effect of selective inhibition of eNOS or nNOS by L-NIO (4 or 20 mg/kg, i.p.) and NPLA (2.5 or 12.5 μ g/kg, i.p.), respectively, on the hemodynamic effects of subsequently administered ethanol (1 g/kg, i.g.). The rats in each group received one of the following combinations: saline+water, saline+ethanol, L-NIO (20 mg/kg)+water, NPLA (12.5 μ g/kg)+water, L-NIO (4 mg/kg) +ethanol, L-NIO (20 mg/kg)+ethanol, NPLA (2.5 μ g/kg)+ethanol, or NPLA (12.5 μ g/kg)+ethanol. The inhibitor of eNOS or nNOS or equal volume of saline (1 ml/kg, i.p) was administered to rats 30 min before receiving ethanol or water. Hemodynamic measurements continued for a period of 90 min after ethanol or water administration.

For the determination of blood ethanol, 3 blood samples (0.2 ml each) were drawn from each rat via the arterial line at 15, 30, 60 min in addition to a terminal sample drawn at 90 min after ethanol administration. Blood samples were collected into heparinized tubes and centrifuged at 5000 rpm for 5 min. The plasma was aspirated and stored at -80°C till analyzed. The plasma ethanol content was measured at all time points by the enzymatic method as in our previous studies (El-Mas and Abdel-Rahman, 1999a,b). At the conclusion of the experiments, rats were euthanized with an over dose of pentobarbital sodium (100 mg/kg) and the hearts were harvested and stored at -80°C for later measurement of phosphorylated nNOS and eNOS proteins in ventricular tissues. The myocardial NOx content was measured using a fluorometric assay kit in accordance with manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI) and as detailed elsewhere (Misko et al., 1993). Briefly, we added 10 μ l enzyme cofactor and 10 μ l nitrate reductase to 80 μ l sample mixture, incubated at room temperature for 3 hr, then added 50 μ l Griess Reagent R1 and R2 to each well, and read the absorbance at 540 nm.

Effect of PI3K inhibition on the hemodynamic actions of ethanol: This experiment investigated the effect of inhibition of the PI3K/Akt pathway by wortmannin on the

hemodynamic effect of ethanol. Two groups of conscious proestrus rats (n=6 each) instrumented for BP and CO measurements were employed and given wortmannin (15 µg/kg, i.v.)+water or wortmannin+ethanol (1 g/kg, i.g.). Wortmannin was administered to rats 15 min before receiving ethanol or water. Hemodynamic measurements continued for a period of 90 min after ethanol or water administration. At the conclusion of the experiments, rats were euthanized with an over dose of pentobarbital sodium (100 mg/kg) and hearts were harvested and stored at -80 °C for measurement of ventricular phosphorylated Akt.

Drugs: Pentobarbital sodium, wortmannin (Sigma Chemical Co., St. Louis, MO), N^ω-propyl-L-arginine (Tocris Bioscience, Ellisville, MO), N⁵-(1-iminoethyl)-L-ornithine (Biotium Inc., Hayward, CA), povidone-iodine solution (Norton Co., Rockford, IL), ethanol (Midwest Grain Products Co., Weston, MO), Buprenex (Rickitt & Colman, Richmond, VA) and Durapen (Vedco, Inc., Overland Park, KS) were purchased from commercial vendors. Wortmannin was dissolved in DMSO (1 mg/ml) and then diluted with saline to a final concentration of 15 µg/ml. L-NIO and NPLA were dissolved in saline.

Data analysis and statistics: Values are presented as means±SEM. Mean arterial pressure (MAP) was calculated as diastolic pressure + one third pulse pressure (systolic-diastolic pressures). Changes in MAP, HR, CO, SV, and TPR evoked by i.g. ethanol or water in the absence and presence of L-NIO, NPLA, or wortmannin were computed. Data were analyzed by repeated measures two-way ANOVA followed by the Newman-Keuls post-hoc test. This test distinguishes between-subject variability from within-subject variability. These analyses were performed by SAS software Release 6.04 (SAS Institute Inc., Cary, NC). Probability levels less than 0.05 were considered significant.

Results

Hemodynamic effects of ethanol

The hemodynamic responses elicited by intragastric ethanol in conscious female rats are shown in figure 1. Compared with water, ethanol (1 g/kg i.g.) caused significant reductions in MAP that started after the administration of ethanol and continued for at least 75 min (Fig. 1A). Afterwards, similar changes in MAP were observed in ethanol- and water-treated rats (Fig. 1A). The hypotensive effect of ethanol was associated with significant decreases in CO (Fig. 1C) and SV (Fig. 1D) compared with control (water-treated) rat values. The HR was increased by ethanol during the first 45 min of the study (Fig. 1B). TPR was not affected by ethanol during the first 30 min, then it showed significant increases, compared with water-treated values, during the following 2 hr (Fig. 1E).

Effect of eNOS or nNOS inhibition on the hemodynamic actions of ethanol

Baseline MAP, HR, CO, SV and TPR measured in conscious female rats were similar in all groups that subsequently received ethanol or water (control) (Table 1). Pretreatment with NPLA or L-NIO had no significant effect on the measured hemodynamic variables (data not shown). The effects of selective nNOS or eNOS inhibition by NPLA and L-NIO, respectively, on the hemodynamic effects of ethanol in conscious female rats are shown in figures 2 and 3. Prior i.p. injections of NPLA (2.5 or 12.5 µg/kg) or L-NIO (4 or 20 mg/kg) caused dose-dependent reductions in the hypotensive (Figs. 2A, 3A) and tachycardic (Fig. 2B, 3B) effects of ethanol. NPLA significantly and dose-dependently attenuated the ethanol-induced reductions in CO (Fig. 2C) and SV (Fig. 2D) in contrast to no effect for L-NIO (Fig. 3C, 3D). However, the CO and SV of rats that received ethanol following NPLA (2.5 or 12.5 µg/kg) remained significantly lower than the corresponding control (saline+water) values (Fig. 2C, 2D). Overall, the increases in TPR in ethanol-treated rats were similar in rats pretreated with the higher dose of NPLA or saline except for faster onset in the NPLA-

pretreated rats (Fig. 2E). The ethanol-evoked increases in TPR were substantially ($P<0.05$) enhanced in rats pretreated with the 20 mg/kg dose of L-NIO compared with saline throughout the 90 min observation period (Fig. 3E). On the other hand, ethanol caused no changes in TPR in rats pretreated with the lower dose of NPLA (Fig. 2E) or L-NIO (Fig. 3E).

In rats pretreated with saline, NPLA, or L-NIO, i.g. administration of ethanol produced similar blood ethanol concentrations (Table 2). Ethanol significantly increased myocardial NOx content and this effect was abolished in rat pretreated with NPLA or L-NIO (Fig. 4). Also, ethanol caused approximately 4 fold increase ($P<0.05$) in the myocardial p-nNOS protein level, an effect that was virtually abolished in NPLA-pretreated rats (Fig. 5). By contrast, myocardial p-eNOS was slightly ($P>0.05$) increased in ethanol, compared with water, treated rats (Fig. 5). The ability of L-NIO (20 mg/kg) to inhibit myocardial eNOS phosphorylation, particularly in ethanol-treated rats, was evident (Fig. 5).

Effect of PI3K inhibition on hemodynamic actions of ethanol

The effects of the PI3K inhibitor wortmannin on the ethanol-evoked changes in hemodynamics and myocardial Akt activity are illustrated in figures 6 and 7, respectively. Prior treatment with wortmannin (15 μ g/kg) caused significant decreases in the ethanol-evoked reductions in BP (Fig. 6A), CO (Fig. 6C), and SV (Fig. 6D). The increase in TPR caused by ethanol was also reduced in the presence of wortmannin (Fig. 6E). The protein expression of myocardial p-Akt was significantly increased by i.g. ethanol (Fig. 7) and this effect together with the ethanol-evoked increase in myocardial NOx content (Fig. 4) were abolished in preparations pretreated with wortmannin.

Discussion

In this study, we tested the hypothesis that facilitation of myocardial constitutive eNOS and/or nNOS phosphorylation underlies the reduction in CO and the subsequent hypotensive response elicited by ethanol in female rats. The most important findings of the present study are: (i) myocardial p-Akt and p-nNOS, but not p-eNOS, levels were significantly increased along with the reductions in CO and BP in ethanol treated rats; (ii) the enhanced phosphorylation of myocardial nNOS, the increased myocardial NOx content and the reductions in CO and BP elicited by ethanol were virtually abolished in rats pretreated with the selective nNOS inhibitor NPLA; (iii) the hemodynamic effects of ethanol and concomitant increases in myocardial p-Akt were reduced after pharmacologic inhibition of PI3K, (iv) although ethanol caused non-significant increase in myocardial eNOS phosphorylation, selective inhibition of eNOS by L-NIO abrogated the hypotensive response elicited by ethanol, (v) consistent with the cellular response, eNOS inhibition failed to influence ethanol-evoked reduction in CO, and (vi) ethanol caused a substantial increase in TPR in L-NIO pretreated rats, which seems to offset the associated reduction in CO and explains, at least partly, the attenuation of ethanol-evoked hypotension following selective eNOS inhibition. These findings highlight myocardial PI3K/Akt/nNOS as a cellular mechanism that underlies ethanol evoked reduction in CO and BP in conscious female rats.

The present study elucidated the role of myocardial nNOS and/or eNOS signaling in the hypotensive action of ethanol. We focused on myocardial NOS because reductions in CO and SV underlie ethanol-evoked hypotension in female rats (El-Mas and Abdel-Rahman, 1999a,b). Our results showed that selective pharmacologic inhibition of nNOS or eNOS by NPLA and L-NIO, respectively, virtually abolished the hypotensive response elicited by ethanol. These findings, however, should not be interpreted to suggest equal involvement of both NOS isoforms in mediating the BP response to ethanol. In fact, careful analysis of the cellular and hemodynamic data reported here provides convincing evidence that myocardial

nNOS and not eNOS is the likely molecular mediator of the reductions in CO and BP caused by ethanol. This conclusion is supported by several observations. First, Western blot analyses showed that i.g. ethanol elicited remarkable (4-fold) increases in myocardial nNOS phosphorylation in contrast to a non-significant increase in eNOS phosphorylation. The lack of any changes in myocardial eNOS phosphorylation obviously precludes its possible involvement in the mediation of ethanol evoked reduction in CO and associated hypotension. Second, the inhibition of nNOS (NPLA), but not eNOS (L-NIO), significantly attenuated the reductions in CO and SV caused by ethanol and this effect was coupled with the abolition of the ethanol-induced increases in myocardial p-nNOS. Notably, as shown in the present and previous studies (El-Mas and Abdel-Rahman, 1999b), the reductions in CO and SV are probably the underlying cause of the BP lowering effect of ethanol in female rats. This causal relationship is further emphasized by the observations that ethanol elicits no hypotension in male or ovariectomized rats, preparations in which CO and SV are not affected by ethanol administration (El-Mas and Abdel-Rahman, 1999a,b). The increase caused by ethanol in myocardial p-nNOS seems to have been caused by the upregulation of myocardial PI3K/Akt signaling as suggested by the ability of wortmannin, PI3K inhibitor, to reduce the ethanol-evoked hemodynamics and Akt activation. Together, our findings highlight an important role for cardiac PI3K/Akt/nNOS signaling in the reductions in CO and SV, which mediate the hypotensive response to ethanol in female rats.

Because cardiac p-eNOS was not significantly affected by ethanol, the increase in cardiac NO_x content elicited by ethanol might logically be accounted for by the ethanol-evoked rise in p-nNOS phosphorylation. With that said, our finding that pharmacologic inhibition of eNOS by L-NIO abolished the ethanol-evoked increase in myocardial NO_x is surprising. This observation may relate to the dramatic reduction in myocardial p-eNOS expression caused by the L-NIO+ethanol regimen compared with ethanol alone (see figure 5). It is tempting to speculate that the ability of L-NIO to reduce myocardial NO_x in ethanol-treated rats does not functionally correlate with cardiac nitrenergic pathways (possibly nNOS) mediating the effect of ethanol on CO. Notably, the doses of NPLA and L-NIO employed in the current study caused no changes in blood ethanol concentrations or in baseline hemodynamic variables as shown in the present or reported studies (DeWitt et al., 1997; Talman et al., 2007). This was important to circumvent potential confounding effects on the hemodynamic action of ethanol.

It is imperative to note that while the present findings highlight a key role for myocardial nNOS in ethanol-evoked reductions in CO and SV, they do not fully explain the hemodynamic effects of ethanol. We show that ethanol still caused reductions in CO and SV in NPLA-treated rats (Fig. 2A, B). This finding implies that nNOS-independent mechanisms contribute to the ethanol-induced perturbations of cardiac function and contractility. In support of this assumption, the ethanol causes direct myocardial depression (Kelbaek, 1990) and upregulates cardiac iNOS, which contributes to the ethanol-evoked reduction in CO (El-Mas et al., 2007). Further, ethanol produces several other effects within the heart muscle that might explain its cardiac effects such as disruption of calcium homeostasis (Thomas et al., 1994), induction of oxidative stress (Preedy et al., 1993), and elevation of plasma troponin levels, a measure of cardiac cell damage (Patel et al., 2001). The possibility whether these effects contribute to the NOS-dependent hypotensive action of ethanol in female rats is not clear and warrants further investigations.

The ethanol-nNOS hemodynamic and cellular interactions deserve two comments. First, our observation that NPLA abolished the ethanol-evoked increases in nNOS phosphorylation is surprising particularly in view of the information that NPLA produces its pharmacologic activity via competition with L-arginine for a common site on the nNOS molecule with the subsequent inhibition of the NADPH-oxidase activity of the enzyme (Cooper et al., 2000).

Although we are not aware of any study that evaluated the effect of NPLA on nNOS phosphorylation, reported studies including our own demonstrated a reduction in the iNOS protein expression in response to selective pharmacologic iNOS inhibition (Thiemermann, 1997; El-Mas et al., 2006, 2008). The possibility, therefore, remains that NPLA might act directly or indirectly to modify the effect of ethanol on nNOS phosphorylation through as yet unidentified mechanism. More research is clearly needed to investigate this possibility. The second comment pertains to the mechanism through which cardiac nNOS might act to modulate the hemodynamic effects of ethanol. One possible explanation may relate to the ability of nNOS to modulate the cardiac autonomic control. Recent reports have shown that nNOS-derived NO facilitates cardiac vagal neurotransmission via a cGMP-dependent pathway (Herring et al., 2001) and normalizes hyper-responsiveness to β -adrenergic stimulation in the sinoatrial node of spontaneously hypertensive rats (Heaton et al., 2006). The enhancement of nNOS/NO activity might also be responsible for the downplay of the tachycardic response to ethanol in the present study, which possibly results from increased sympathetic drive (Kelbaek, 1990; Murata et al., 1994).

Results of the present study showed a delayed increase in TPR in ethanol-treated rats, which might represent a counter-regulatory mechanism to offset the ethanol-evoked reductions in CO and BP. More importantly, we report, for the first time, that the increase in TPR was substantially enhanced following selective eNOS, but not nNOS, inhibition (Figs. 2E, 3E). This effect, which was observed with the higher dose of the eNOS inhibitor L-NIO, may suggest that vascular eNOS-NO signaling exerts a tonic restraining influence on the increase in TPR caused directly or indirectly by ethanol. Conceivably, the augmentation of the TPR response caused by the withdrawal of eNOS-mediated vascular control might possibly explain the reason for the abolition of ethanol-evoked hypotension in L-NIO-treated rats. As might be expected, eNOS plays a more important role, than nNOS, in vascular tone control (Marin and Sessa, 2007). It should be pointed out that no attempts were made in this study to measure constitutive NOS expression in the vasculature because the main goal of our study was to investigate the cellular mechanisms that underlie the reduction in CO caused by ethanol, which mediates the hypotensive response (El-Mas and Abdel-Rahman, 1999a,b; El-Mas et al., 2007). Nonetheless, the pharmacological and detailed hemodynamic findings with L-NIO suggest that ethanol seems to enhance vascular eNOS activity.

In summary, the ethanol-evoked reductions in BP, CO, and SV were paralleled with significant increases in cardiac Akt and nNOS phosphorylation and myocardial NO_x levels. These hemodynamic, cellular and biochemical effects of ethanol were substantially attenuated after selective pharmacologic inhibition of PI3K or nNOS activity, which implicates cardiac PI3K, Akt/nNOS signaling as a molecular mechanism that underlies the hemodynamic effects of ethanol in proestrus female rats. By contrast, the lack of effect of ethanol on myocardial eNOS phosphorylation and the preservation of ethanol-evoked reductions in CO and SV in L-NIO pretreated rats rule out any significant role for myocardial eNOS-NO signaling in the cardiac effects of ethanol. Notably, the clinical significance of our current findings in proestrus rats is warranted because (i) ethanol causes hypotension in young but not old female subjects (Klatsky, 1990), and (ii) the dose of ethanol used in the present study produced blood ethanol concentrations comparable to those attained in humans following consumption of mild to moderate amounts of ethanol (Ireland et al., 1984; Abdel-Rahman et al., 1987).

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Figure 1.

Changes in mean arterial pressure (Δ MAP), heart rate (Δ HR), cardiac output (Δ CO), stroke volume (Δ SV) and total peripheral resistance (Δ TPR) evoked by intragastric administration of ethanol (1 g/kg) or equal volume of water in conscious female Sprague-Dawley rats. Values are means $\pm$ S.E.M of 6 observations. * P <0.05 vs. respective saline+water values.

Figure 2. Changes in mean arterial pressure (Δ MAP), heart rate (Δ HR), cardiac output (Δ CO), stroke volume (Δ SV) and total peripheral resistance (Δ TPR) 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 N^ω-propyl-L-arginine (NPLA, selective nNOS inhibitor, 2.5 or 12.5 µg/kg i.p.). Values are means±S.E.M of 6–8 observations. *, # and +P<0.05 vs. respective “saline+water”, “NPLA 2.5 µg + ethanol”, and “NPLA 12.5 µg + ethanol” values.

Figure 3.

Changes in mean arterial pressure (Δ MAP), heart rate (Δ HR), cardiac output (Δ CO), stroke volume (Δ SV) and total peripheral resistance (Δ TPR) 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 N^5 -(1-iminoethyl)-L-ornithine (L-NIO, selective eNOS inhibitor, 4 or 20 mg/kg i.p.). Values are means $\pm$ S.E.M of 6–8 observations. *, # and $^+P < 0.05$ vs. respective “saline+water”, “L-NIO 4 mg + ethanol”, and “L-NIO 20 mg + ethanol” values.

Figure 4.

Effect of ethanol on myocardial NOx content in the absence and presence of N^ω-propyl-L-arginine (NPLA, selective nNOS inhibitor, 12.5 μg/kg), N⁵-(1-iminoethyl)-L-ornithine (L-NIO, selective eNOS inhibitor, 20 mg/kg), or wortmannin (PI3K inhibitor, 15 μg/kg). Values are means±S.E.M of 6–8 observations. * and ⁺P<0.05 vs. respective “saline+water” and “saline+ethanol” values.

Figure 5.

Effect of ethanol (1 g/kg i.g.) on myocardial p-nNOS (top panel) or p-eNOS (bottom panel) protein expression in female rats in the absence or presence of N^ω-propyl-L-arginine (NPLA, selective nNOS inhibitor, 12.5 μg/kg i.p.) or N⁵-(1-iminoethyl)-L-ornithine (L-NIO, selective eNOS inhibitor, 20 mg/kg i.p.). Illustrative gels depicting myocardial p-nNOS and p-eNOS protein expression are shown. Values are means ± S.E.M of 6–8 observations. **P* < 0.05 vs. control (saline+water) values, and ⁺*P* < 0.05 vs. “saline+ethanol” values.

Figure 6. Changes in mean arterial pressure (Δ MAP), and heart rate (Δ HR), cardiac output (Δ CO), stroke volume (Δ SV) and total peripheral resistance (Δ TPR) evoked by intragastric administration of ethanol (1 g/kg) or equal volume of water in conscious female Sprague-Dawley rats pretreated, 15 min earlier, with wortmannin (PI3K inhibitor, 15 μ g/kg i.v.). Values are means $\pm$ S.E.M of 6 observations. * and +P<0.05 vs. respective “saline+water” and “wortmannin+ethanol” values.

Figure 7.

Effect of ethanol (1 g/kg i.g.) on myocardial p-Akt protein expression in female rats in the absence or presence of wortmannin (PI3K inhibitor, 15 μ g/kg). Illustrative gels depicting myocardial p-Akt protein expression are shown. Values are means $\pm$ S.E.M of 6 observations. * P <0.05 vs. control (saline+water) values, and ^+P <0.05 vs. “saline+ethanol” values.

Table 1

Baseline values of mean arterial pressure (MAP, mmHg), heart rate (HR, beats/min), cardiac output (CO, ml/min), stroke volume (SV, μ l/beat), and total peripheral resistance (TPR, mmHg/ml/min).

Group	MAP	HR	CO	SV	TPR
Saline+water (n=8)	109.4 $\pm$ 4.8	374.0 $\pm$ 32.4	86.0 $\pm$ 2.4	236.7 $\pm$ 21.2	1.27 $\pm$ 0.01
Saline+ethanol (n=6)	100.5 $\pm$ 3.7	416.0 $\pm$ 8.9	93.6 $\pm$ 6.1	226.7 $\pm$ 20.1	1.09 $\pm$ 0.09
NPLA+water (n=6)	102.9 $\pm$ 4.1	378.3 $\pm$ 16.6	82.8 $\pm$ 2.4	220.8 $\pm$ 10.3	1.23 $\pm$ 0.06
NPLA+ethanol (n=6)	112.3 $\pm$ 6.5	409.2 $\pm$ 17.3	86.0 $\pm$ 1.2	212.6 $\pm$ 11.7	1.30 $\pm$ 0.06
L-NIO+water (n=6)	109.5 $\pm$ 4.6	373.0 $\pm$ 20.2	85.2 $\pm$ 3.0	230.3 $\pm$ 11.1	1.30 $\pm$ 0.09
L-NIO+ethanol (n=6)	125.2 $\pm$ 4.8	331.9 $\pm$ 19.1	83.0 $\pm$ 2.8	238.2 $\pm$ 15.5	1.56 $\pm$ 0.08

Values are means $\pm$ SEM. NPLA, N⁶-propyl-L-arginine; L-NIO, N⁵-(1-iminoethyl)- L-ornithine.

Table 2

Time-course levels of blood ethanol (mg%).

Group	Time (min)			
	15	30	60	90
Saline+ethanol	69.8±4.9	84.1±4.5	62.9±5.1	60.9±4.2
NPLA+ethanol	74.2±4.9	87.7±7.6	61.7±2.6	53.2±2.1
L-NIO+ethanol	67.0±6.4	72.2±2.5	55.1±4.5	51.2±5.2

Values are means±SEM. NPLA, N⁰-propyl-L-arginine; L-NIO, N⁵-(1-iminoethyl)-L-ornithine.