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Ethanol consumption increases blood pressure and alters the responsiveness of the mesenteric vasculature in rats

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Abstract

Chronic ethanol consumption and hypertension are related. In the current study we investigated whether changes in reactivity of the mesenteric arterial bed could account for the increased blood pressure associated with chronic ethanol intake. Changes in reactivity to phenylephrine and acetylcholine were investigated in the perfused mesenteric bed from rats treated with ethanol for 2 or 6 weeks and their age-matched controls. Mild hypertension was observed in chronically ethanol-treated rats. Treatment of rats for 6 weeks induced an increase in the contractile response of endothelium-intact mesenteric bed to phenylephrine, but not denuded rat mesenteric bed. The phenylephrine-induced increase in perfusion pressure was not altered after 2 weeks' treatment with ethanol. Moreover, acetylcholine-induced endothelium-dependent relaxation was reduced by ethanol treatment for 6 weeks, but not 2 weeks. Pre-treatment with indometacin, a cyclooxygenase inhibitor, reduced the maximum effect induced by phenylephrine (E_{max}) in endothelium-intact mesenteric bed from both control and ethanol-treated rats. No differences in the E_{max} values for phenylephrine were observed between groups in the presence of indometacin. L-NNA, a nitric oxide (NO) synthase (NOS) inhibitor, increased the E_{max} for phenylephrine in endothelium-intact mesenteric bed from control rats but not from ethanol-treated rats. Levels of endothelial NOS (eNOS) mRNA were not altered by chronic ethanol consumption. However, chronic ethanol intake strongly reduced eNOS protein levels in the mesenteric bed. This study shows that chronic ethanol consumption increases blood pressure and alters the reactivity of the mesenteric bed. Moreover, the increased vascular response to phenylephrine observed in the mesenteric bed is maintained by two mechanisms: an increased release of endothelial-derived vasoconstrictor prostanoids and a reduced modulatory action of endothelial NO, which seems to be associated with reduced post-transcriptional expression of eNOS.

Introduction

Excessive ethanol intake is a known cardiovascular risk factor in the general population (Kurihara et al 2004). Many studies have reported that chronic alcohol intake is associated with elevated blood pressure, and that this elevation in blood pressure underlies the increased risk of cardiovascular disease (Criqui et al 1981; Strogatz et al 1991). However, the mechanism(s) by which chronic ethanol consumption cause a rise in blood pressure are not yet completely known. Current investigation in this field has centred on areas known to be involved in human hypertension. Several mechanisms have been postulated for the hypertensive response associated with chronic ethanol consumption, such as enhanced secretion of hormones and neurotransmitters, stimulation of the sympathetic nervous system (Chan et al 1985) and a myogenic mechanism, which involves alteration of the contractile properties of vascular smooth muscle (Chan & Sutter 1983). Previous reports have suggested that enhanced vascular reactivity to vasoconstrictor agents (Pinardi et al 1992)

or impairment of vascular relaxation (Kahonen et al 1999) contribute to hypertension associated with chronic ethanol consumption.

Conduit vessels such as the aorta from ethanol-treated rats have been shown to have altered reactivity to vasoconstrictor and vasorelaxant agents. Pinardi et al (1992) found that chronic ethanol consumption significantly increased the contractile response induced by phenylephrine, an α_1-adrenoceptor agonist, in aortic rings with intact endothelium. Likewise, Stewart and Kennedy (1999) demonstrated an ethanol-associated increase in the maximum response to phenylephrine in isolated aortic rings. Ladipo et al (2002) observed that chronic ethanol consumption increased the sensitivity of rat aortic rings to noradrenaline (norepinephrine). We have recently shown that the increased contractile response induced by phenylephrine in isolated aortic rings from chronically ethanol-treated rats involves an increase in the production of thromboxane A_2 (TXA_2) and an increase in calcium influx (Tirapelli et al 2006a). Finally, mesenteric conduit arteries from ethanol-treated rats showed an increased response to noradrenaline after treatment with ethanol (Hatton et al 1992).

Some studies have established a positive correlation between the duration and extent of ethanol intake and the development of hypertension (Abdel-Rahman & Wooles 1987; Strickland & Wooles 1988). Thus, it appears likely that the period of exposure to ethanol is a major factor in the development of hypertension. Recently, we showed increased blood pressure in conscious rats after treatment with ethanol for 2, 6 and 10 weeks (Resstel et al 2006), but the maximum increase in blood pressure did not differ with the three different periods of treatment.

It is also important to note that most of the experiments designed to study the relationship between alterations in vascular functionality and increase in blood pressure induced by ethanol consumption use conduit vessels such as the aorta (Utkan et al 2001). However, little is known about the effect of chronic ethanol consumption on the vascular reactivity of peripheral resistance arteries. While the aorta offers some resistance to flow, vessels of smaller diameter make a much greater contribution to peripheral vascular resistance. The mesenteric circulation of the rat receives approximately one-fifth of the cardiac output (Nichols et al 1985); regulation of this bed therefore makes a significant contribution to the regulation of systemic blood pressure.

On the basis of the aforementioned studies, we hypothesized that increased vascular reactivity within the mesenteric arterial bed contributes to the increased blood pressure induced by chronic ethanol intake. In the present studies we assessed the vascular responses to phenylephrine and acetylcholine of perfused mesenteric beds from rats that had consumed ethanol for 2 or 6 weeks. In addition, we investigated the possible mechanism(s) underlying the effects of ethanol consumption on the reactivity of the mesenteric bed. Our study focused on endothelial mechanisms, since we have previously shown that ethanol consumption alters the reactivity of endothelium-intact, but not denuded, mesenteric bed (Tirapelli et al 2007).

Materials and Methods

Drugs

The following drugs were used: phenylephrine hydrochloride, acetylcholine hydrochloride (Sigma, St Louis, MO, USA), the nitric oxide synthase (NOS) inhibitor *Nω*-nitro-L-arginine (L-NNA) (Sigma/RBI, Natick, MA, USA) and the non-selective cyclooxygenase inhibitor indometacin (Calbiochem, San Diego, CA, USA). Indometacin was dissolved in Tris buffer (pH 8.4). The other drugs were dissolved in distilled water.

Experimental design

Male Wistar rats were housed in plastic cages in a 12 h light–dark cycle (lights on at 06:30) at 24°C. The housing conditions and experimental protocols were in accordance with the Ethical Animal Committee from the Campus of Ribeirão Preto (University of São Paulo). The rats (80–100 days old), initially weighing 300–350 g, were randomly divided into three groups: control, sucrose-treated and ethanol-treated. Control rats received tap water ad libitum. Other groups received food ad libitum and either a 20% (v/v) ethanol solution or an isocaloric sucrose solution (290.50 g L^{-1}) (Tirapelli et al 2006a, b). In order to avoid a considerable loss of animals, the ethanol-treated rats were subjected to a brief and gradual adaptation period: they received 5% ethanol in their drinking water in the first week, 10% in the second and 20% in the third week. The experimental stage began at the end of the third week. After the three-week adaptation period, rats were treated for 2 or 6 weeks. The same procedure was adopted for the sucrose group. In these groups, the caloric content of the liquid diet was adjusted to match that of the ethanol-treated groups. The total caloric contents of ethanol and sucrose are 7.1 and 4 kcal g^{-1}, respectively. We calculated the mass of ethanol per litre (using the density of ethanol of 0.789 g mL^{-1}) on the basis of 200 mL ethanol per litre (20% solution) and thus the amount of sucrose that should be dissolved in 1 litre water to give the same caloric content. The sucrose group was included in the study protocol to evaluate whether alterations in caloric intake following ethanol consumption might explain the effects of ethanol on vascular responses.

The rats were weighed weekly (09:00). Consumption of food and liquid intake were measured weekly. All animals had free access to food (Purina rat chow).

In total, for the 2-week experiments there were 37 control rats, 34 sucrose-treated rats and 53 ethanol-treated rats. For the 6-week experiments there were 71 control rats, 37 sucrose-treated rats and 70 ethanol-treated rats.

In-vivo procedures

In-vivo procedures were performed as described previously (Resstel et al 2006). One day before the experiments, rats were anaesthetized with tribromoethanol, and a catheter (4 cm segment of PE-10 heat-bound to a 13 cm segment of PE-50, Clay Adams, Parsippany, NJ, USA) was inserted into the abdominal aorta via the femoral artery. The catheter was tunnelled under the skin and exteriorised at the animal's

dorsum. The rats were allowed to recover, and baseline mean arterial pressure (MAP) and heart rate (HR) were recorded 24 h later.

During the experiment, rats were kept in individual cages but could move freely. MAP was recorded using an HP-7754A amplifier (Hewlett Packard, Waltham, Massachusetts, USA) connected to a signal acquisition board (Windaq di 190, DATAQ, Akron, OH, USA) and computer processed. Baseline systolic, diastolic and mean blood pressure were measured and expressed as mean ± s.e.m.

Measurement of blood ethanol and serum glucose

Rats were anaesthetized and blood collected from the aorta using a heparinized syringe. A 1 mL aliquot of each blood sample was placed in a 10 mL headspace vial to which was added 1.0 g sodium chloride, 1.0 mL water and 100 μL of the internal standard solution (acetonitrile, 1 mL L^{-1}). Ethanol analysis was performed using a CG-17A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector and an HSS-4A headspace sampler (Shimadzu). Calibration standards (0.10–3.16 mg mL^{-1}) were prepared in similar headspace vials. The results are expressed as mg ethanol per mL blood.

Blood for measurement of glucose concentration was collected during the morning using non-heparinized syringes, taking care to avoid haemolysis. Blood samples were centrifuged at 8000–10 000 g for 10 min at room temperature. The serum was analysed for glucose content using a commercially available kit (Labtest Diagnóstica, São Paulo, Brazil) and an auto-analyser (model ABAA VP, Abbott, Chicago, IL, USA). The results are expressed as mg dL^{-1}.

Perfusion of the mesenteric arterial bed

The rat mesenteric bed isolated and perfused in-vitro was used as a model of vascular resistance, as described previously (Leone & Coelho 2004). Briefly, rats were anaesthetized with 2.5% tribromoethanol (1 mL (100 g body weight)$^{-1}$ i.p.), the abdominal cavity was opened and the intestinal loops were exposed. The superior mesenteric artery was dissected close to its origin in the abdominal aorta and cannulated with a PE-50 polyethylene catheter. The mesenteric bed was perfused with 1 mL Krebs solution (composition, in mmol L^{-1}, 120.0 NaCl, 4.7 KCl, 25.0 $NaHCO_3$, 2.4 $CaCl_2.2H_2O$, 1.4 $MgCl_2.6H_2O$, 1.17 KH_2PO_4, and 11.0 glucose) containing 500 IU heparin. The intestinal loops were removed en bloc, the mesenteric bed was separated by cutting close to the intestinal loops, and the preparation was placed in a moist chamber warmed to 37°C. The cannulated superior mesenteric artery was coupled to a perfusion pump (LKB 2215 Multiperpex pump, Broma, Sweden) and the mesenteric bed was perfused with Krebs solution bubbled with 95% O_2 and 5% CO_2, pH 7.4, at a constant flow of 4 mL min^{-1}. A pressure transducer (R 511A, Beckman Inst., Schiller Park, IL, USA) was coupled in a 'y' arrangement to the system to record perfusion pressure. The pre-amplified and filtered outlet signal was coupled to the data acquisition system (DATAQ DI-150) connected to the RS 232 parallel port of a Pentium II personal computer, and stored for later analysis with the Windaq software, version 2.5 (DATAQ).

Vascular reactivity to vasoconstrictor agents

The mesenteric bed was perfused with Krebs solution and left to rest for 15 min to allow baseline perfusion pressure, which was recorded continuously, to stabilize. Increasing bolus doses of phenylephrine (0.5–80 μg) were then injected using a 50 μL Hamilton syringe. The interval between injections was 5 min, or the time needed for perfusion pressure to baseline. After the control curve, indometacin (10 μmol L^{-1}) or L-NNA (50 μmol L^{-1}), was added to the Krebs solution and the dose–response curve was repeated. In some preparations, the endothelium was removed with a solution of sodium deoxycholate (2 mL bolus of a 1 mg mL^{-1} solution). After 10 min for equilibration, a dose–response curve was constructed using the parameters described previously. At the end of the experiment, the mesenteric bed was pre-contracted with a dose of phenylephrine capable of increasing the perfusion pressure by 60 mmHg. A bolus injection of acetylcholine (10 nmol L^{-1}) was then applied; the total absence of a dilating response indicated effective removal of the vascular endothelium. The tissue was discarded if any degree of relaxation was observed.

Dose–response curves for acetylcholine were also constructed. In order to avoid a possible influence of the pre-contracting levels induced by phenylephrine on acetylcholine-induced relaxation, the increase in perfusion pressure was evoked with a dose of phenylephrine capable of increasing the baseline perfusion pressure with similar magnitude in tissues from control and ethanol-treated rats. Increasing bolus doses of acetylcholine (0.5–50 μg) were then injected using a 50 μL Hamilton syringe. Relaxation was expressed as the percentage change from the phenylephrine-contracted levels.

Reverse transcriptase PCR

Reverse transcriptase (RT) PCR was performed as described previously (Tirapelli et al 2005). Total cell RNA was isolated from mesenteric bed using trizol reagent (Gibco BRL, Life Technologies, Rockville, MD, USA). Total RNA (3 mg per sample) was used for reverse transcription in the presence of 20 U of an RNAase inhibitor (RNAase OUT, Invitrogen Life Technologies, Carlsbad, CA, USA), 200 U Superscript II RR (Invitrogen) and 0.5 μg oligo (dT) 12–18 primer at 42°C for 50 min, according to the manufacturer's specifications. The cDNA was stored at −20°C until required for PCR. PCR primers were designed as follows: eNOS, antisense primer CTGGCCTTCTGCTCATTTTC; sense primer TGACCCTCACCGATACAACA (210-bp fragment); GAPDH, antisense primer CACCACCCTGTTGCTGTA; sense primer TATGATGACATCAAGAAGGTGG (219-bp fragment). GAPDH was used as an internal control for the co-amplification. The following conditions were selected for PCR in a volume of 25 μL: RT products from 3 mg RNA, 1 U platinum Taq DNA polymerase (Invitrogen), 30 cycles of amplification for eNOS and 24 cycles for the GAPDH gene. Amplification was carried out using an initial denaturing cycle at 94°C for 5 min and subsequent cycles as follows: denaturation, 30 s at 94°C;

annealing, 30 s at 60°C for eNOS, 62°C for GAPDH; extension, 45 s at 72°C. PCR products (10 μL per lane) were electrophoresed using 1% agarose gel containing ethidium bromide (0.5 μg mL^{-1}). The gel was subjected to UV light and photographed. The band intensities were measured using commercial software (Kodak Digital Science, Eastman Kodak Company, New Haven, CT, USA).

Western immunoblotting

Mesenteric bed extracts were homogenized in 50 mmol L^{-1} Tris-HCl lysis buffer containing 2 mmol L^{-1} EDTA, 5 mmol L^{-1} EGTA, 1 mmol L^{-1} PMSF, 1 mmol L^{-1} Na_3VO_4 and 2 μL protease inhibitor cocktail (P-8340, Sigma). Equal amounts of protein per 50-μg sample were separated on 7.5% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked with Tris-buffered saline and 0.1% Tween-20 containing 5% skimmed milk powder for 1 h at room temperature. Membranes were then incubated with polyclonal eNOS antibody (1:1000, BD Transduction Laboratories, San Diego, CA, USA) overnight at 4°C. After incubation with secondary antibodies, signals were developed with chemiluminescence, visualized by autoradiography, quantified densitometrically and expressed as relative absorbance of eNOS to the internal standard, α-actin. The exposure period ranged from 15 to 60 s.

Statistical analysis

Results are expressed as mean ± s.e.m. Statistical analyses were performed using Student's *t*-test and one- or two-way (treatment vs. time) analysis of variance (ANOVA), as indicated in the text. Post-hoc comparisons were performed using Bonferroni's multiple comparison test. A P value below 0.05 was considered significant. Statistical analysis was performed using commercially available software (SPSS version 9.0, Chicago, Illinois, USA). The maximum effect elicited by the agonist (E_{max}) and the effective concentration needed to promote 50% of the maximum effect induced by the agonist (EC50) were calculated using a non-linear interactive fitting program (Graph Pad Prism 3.0, GraphPad Software Inc., San Diego, CA).

Results

Body weight, food consumption, liquid intake, blood ethanol and serum glucose concentrations

Before beginning treatment, control rats (n = 88) had a mean body weight of 331 ± 4 g, sucrose-treated rats (n = 71) weighed 316 ± 11 g and ethanol-treated rats (n = 101) weighed 329 ± 8 g. After 2 weeks' ethanol treatment, rats' body weight (437 ± 11 g, n = 53) was lower than that of control rats (487 ± 9 g, n = 37) and sucrose-treated rats (488 ± 12 g, n = 34) ($P < 0.05$; ANOVA). Similarly, 6 weeks' treatment with ethanol reduced the body weight of rats (436 ± 19 g, n = 48) and treatment with sucrose increased the body weight (584 ± 16 g, n = 37) compared with the control group (530 ± 13 g, n = 51) ($P < 0.05$; ANOVA).

Solid food consumption (g per week) was reduced in ethanol- (130 ± 4 g, n = 53) and sucrose-treated rats (131 ± 8 g, n = 34) compared with control animals (253 ± 5 g, n = 37) ($P < 0.05$; ANOVA) in the 2-week treatment protocol. Similarly, treatment for 6 weeks reduced the solid-food consumption of the ethanol-treated (127 ± 3 g, n = 48) and sucrose-treated rats (122 ± 3 g, n = 37) compared with control rats (225 ± 3 g, n = 51) ($P < 0.05$; ANOVA). Liquid intake (mL per week) at week 2 was higher in control rats (459 ± 10 mL) and sucrose-treated rats (463 ± 30 mL) than in ethanol-treated rats (274 ± 9 mL) ($P < 0.05$; ANOVA). Similarly, liquid intake at week 6 was lower in the ethanol group (245 ± 6 mL) compared with control (450 ± 9 mL) or sucrose-treated rats (477 ± 36 mL) ($P < 0.05$; ANOVA).

Blood ethanol levels in the ethanol-treated rats averaged 1.8 ± 0.3 mg mL^{-1} in the second week (n = 10) and 1.9 ± 0.3 mg mL^{-1} in the sixth week (n = 10). No ethanol was detectable in the blood of control or sucrose-treated animals. Blood ethanol levels were not significantly different between the two treatment periods (Student's *t*-test).

After 2 weeks' treatment, serum glucose levels averaged 104.8 ± 8.2, 104.7 ± 4.7 and 110.2 ± 7.6 mg dL^{-1} in the control (n = 12), sucrose (n = 10) and ethanol (n = 13) groups, respectively. No significant differences were found in serum glucose levels after 6 weeks' treatment: 100.4 ± 6.9, 117.6 ± 9.4 and 109.2 ± 8.2 mg dL^{-1} in the control (n = 13), sucrose-treated (n = 12) and ethanol-treated rats (n = 11), respectively (ANOVA). These data indicate that the experimental protocol did not induce an increase in blood glucose levels.

Effect of chronic ethanol consumption on mean arterial pressure and heart rate

Ethanol feeding for 2 weeks increased baseline MAP (120 ± 3 mmHg, n = 5) compared with control and sucrose-treated rats (98 ± 3.5 mmHg (n = 5) and 101 ± 2 mmHg (n = 4), respectively). Similarly, ethanol consumption for 6 weeks increased MAP (control: 97 ± 0.8 mmHg (n = 5); sucrose group 100 ± 1.6 mmHg (n = 4); ethanol-treated 121.6 ± 1.6 mmHg (n = 5). Diastolic arterial pressure increased after treatment for 2 weeks (control 81 ± 3.3 mmHg; sucrose group 82 ± 1.9 mmHg; ethanol-treated 103 ± 2.2 mmHg) and 6 weeks (control 82 ± 1.5 mmHg; sucrose group 81 ± 1.5 mmHg; ethanol group 103 ± 1.7 mmHg) ($P < 0.05$; two-way ANOVA). Likewise, systolic arterial pressure was increased by ethanol feeding for 2 weeks (control 120 ± 3.5 mmHg; sucrose group 123 ± 1.9 mmHg; ethanol group 147 ± 3.6 mmHg) and 6 weeks (control 120 ± 1.3 mmHg; sucrose group 123 ± 2.5 mmHg; ethanol group 152 ± 2.6 mmHg) ($P < 0.05$; two-way ANOVA). There were no differences in HR between the three groups after 2 weeks (control: 333.2 ± 17 beats per min (bpm); sucrose group 341 ± 21 bpm; ethanol group: 320 ± 18 bpm) or 6 weeks (control 323 ± 13 bpm; sucrose 332 ± 14 bpm; ethanol 309 ± 14 bpm) (two-way ANOVA). Values for blood pressure and HR were similar in the sucrose-treated and control groups.

Concentration–response curves for phenylephrine and acetylcholine

The baseline perfusion pressure in tissue from rats treated for 2 weeks with ethanol (19 ± 1.7 mmHg, n = 16) was similar

to that in control (19 ± 0.6 mmHg, n = 14) and sucrose-treated tissues (20 ± 0.6 mmHg, n = 12). Similarly, the baseline perfusion pressure did not differ between the 6-week-treated groups (control 18 ± 1.2 mmHg, n = 36; sucrose 21 ± 1.2 mmHg, n = 13; ethanol 20 ± 1 mmHg, n = 28). The E_{max} induced by phenylephrine in endothelium-intact mesenteric bed was significantly higher in tissue from 6-week ethanol-treated rats compared with tissues from control or sucrose-treated animals, and EC50 was reduced (Figure 1; Table 1). However, no differences in E_{max} or EC50 values for phenylephrine were found between the groups after 2 weeks' treatment. E_{max} and EC50 values for phenylephrine-induced contraction did not differ significantly between the control and sucrose groups after 2 or 6 weeks' treatment (Figure 1; Table 1).

Removal of the endothelium from the mesenteric bed of control or sucrose-treated rats significantly increased the E_{max} and EC50 values for phenylephrine in both 2- and 6-week-treated rats (Table 1), although in the ethanol-treated group these changes were only observed in the 2-week-treated group. After endothelial denudation of the mesenteric bed from both 2- and 6-week-treated animals, E_{max} and EC50 values were similar in the three groups (Figure 1; Table 1).

Acetylcholine-induced endothelium-dependent relaxation did not differ between the 2-week-treated groups (control $46.7 \pm 5.7\%$, n = 6; sucrose $42.8 \pm 5.8\%$, n = 8; ethanol $50.6 \pm 1.8\%$, n = 9). Similarly, no differences were found for the EC50 values (control $5.9 \pm 2.7\,\mu$g, n = 6; sucrose: $6 \pm 2.1\,\mu$g, n = 8; ethanol: $6.4 \pm 2.2\,\mu$g, n = 9). Interestingly, the relaxation induced by acetylcholine in endothelium-intact mesenteric bed from 6-week ethanol-treated rats ($35.4 \pm 4.6\%$, n = 8) was significantly less than that from controls ($49.2 \pm 1.3\%$, n = 11) or sucrose-treated rats ($48.2 \pm 1.9\%$, n = 8) ($P < 0.05$; two-way ANOVA). The EC50 values for acetylcholine in the mesenteric bed from ethanol-treated rats ($5.7 \pm 2.6\,\mu$g, n = 8) was not significantly different from that found in the arteries from controls ($6.7 \pm 2.8\,\mu$g, n = 14) or sucrose-treated rats ($6.2 \pm 2\,\mu$g, n = 9) (Figure 2). The pre-contractile levels induced by phenylephrine were of similar magnitude in the tissues from control (2

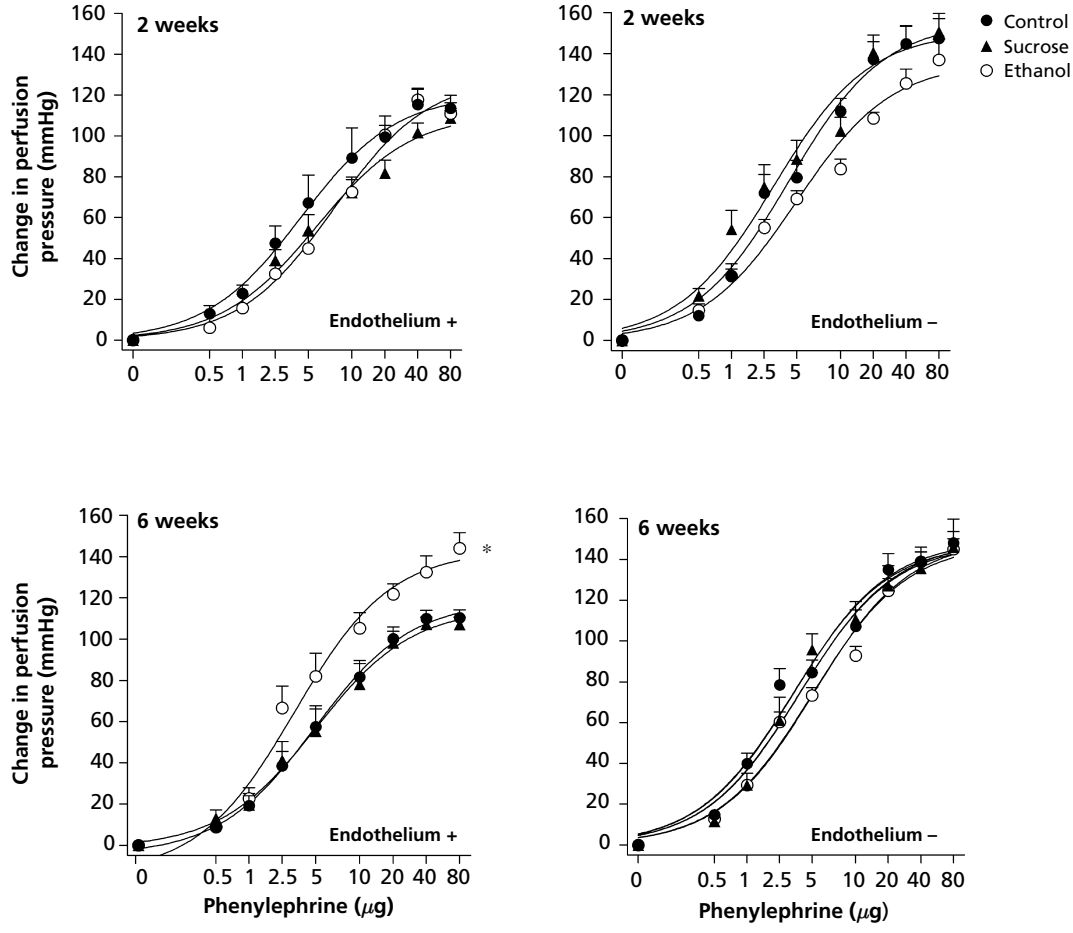

Figure 1 Effect of chronic ethanol consumption on phenylephrine-induced contractile response in rat mesenteric bed. Dose–response curves for phenylephrine were determined in endothelium-intact (Endothelium+, left panels) or endothelium-denuded (Endothelium−, right panels) mesenteric bed from 2- and 6-week ethanol-treated rats and their respective age-matched controls. *Difference in E_{max} (maximum effect induced by phenylephrine) compared with control and isocaloric groups at 6 weeks ($P < 0.05$, two-way ANOVA followed by Bonferroni's comparison test). Data are mean ± s.e.m. of 5–13 independent experiments.

Table 1 Effect of chronic ethanol consumption on the maximum effect induced by phenyleprhine (E_{max}) (mmHg) and EC50 (μg) values (50% of the maximum effect induced by the agonist) for phenylephrine in endothelium-intact (Endo+) or denuded (Endo−) rat mesenteric bed

	Control		Sucrose		Ethanol	
	Endo+	Endo−	Endo+	Endo−	Endo+	Endo−
E_{max}						
2 weeks	113.4 ± 6.4 (9)	147.4 ± 9.7† (5)	108.7 ± 3.7 (6)	137 ± 10† (6)	110.9 ± 5.4 (10)	150.8 ± 8.9† (6)
6 weeks	110.4 ± 3.8 (13)	148.1 ± 5.6† (5)	107 ± 3.7 (6)	145 ± 5.1† (7)	144 ± 7.5* (9)	146 ± 13.6 (5)
EC50						
2 weeks	4.4 ± 1.2	2.2 ± 0.5†	4.3 ± 0.9	2.2 ± 0.6†	4.8 ± 1.2	2.2 ± 0.8†
6 weeks	4 ± 0.3	2.2 ± 0.9†	3.9 ± 0.5	2.3 ± 0.3†	2.9 ± 0.4*	1.9 ± 0.4†

Values are mean ± s.e.m. Numbers in parentheses indicate the number of animals in the group. *$P < 0.05$ vs control and sucrose groups; $^{\dagger}P < 0.05$ vs respective groups with endothelium (two-way ANOVA).

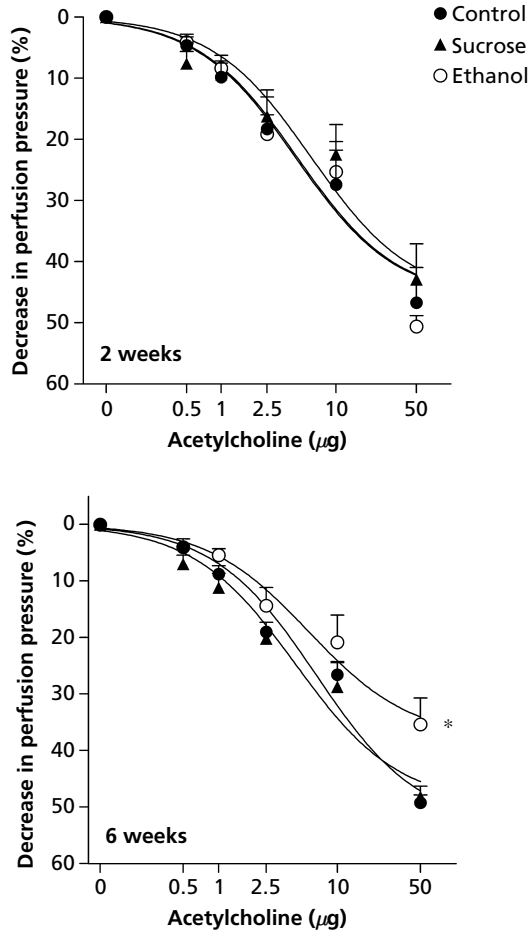

Figure 2 Effect of chronic ethanol consumption on acetylcholine-induced endothelium-dependent relaxation in rat mesenteric bed. Concentration–response curves for acetylcholine were determined in endothelium-intact mesenteric bed from 2- and 6-week ethanol-treated rats and their respective age-matched controls. *Difference in E_{max} (the maximum effect induced by phenylephrine) compared with control and isocaloric groups in 6-week ethanol-treated rats ($P < 0.05$, two-way ANOVA followed by Bonferroni's comparison test). Data are mean ± s.e.m. of 6–14 independent experiments.

weeks: 82.6 ± 5 mmHg; 6 weeks: 81.4 ± 8.3 mmHg), sucrose- (2 weeks: 80.8 ± 3.6 mmHg; 6 weeks: 78.2 ± 7 mmHg) and ethanol-treated rats (2 weeks: 90.2 ± 4.6 mmHg; 6 weeks: 84.2 ± 12 mmHg) (two-way ANOVA).

Investigation of the mechanisms underlying vascular reactivity

Since sucrose feeding did not influence the vascular response induced by phenylephrine or acetylcholine, the following experiments, designed to investigate the mechanisms underlying the effects of ethanol consumption on the vascular reactivity of the mesenteric bed, were carried out in tissues from 6-week ethanol-treated rats and their respective age-matched controls.

Contribution of endothelial NOS and cyclooxygenase

The E_{max} values for phenylephrine were reduced in the tissues from control and ethanol-treated rats in the presence of indometacin (Figure 3B; Table 2). L-NNA significantly increased the maximal contraction induced by phenylephrine in the mesenteric bed from control but not from ethanol-treated rats (Figure 3C; Table 2). When L-NNA and indometacin were added simultaneously, E_{max} and EC50 values for phenylephrine were similar for tissues from control and ethanol-treated rats (Figure 3D; Table 2). L-NNA significantly

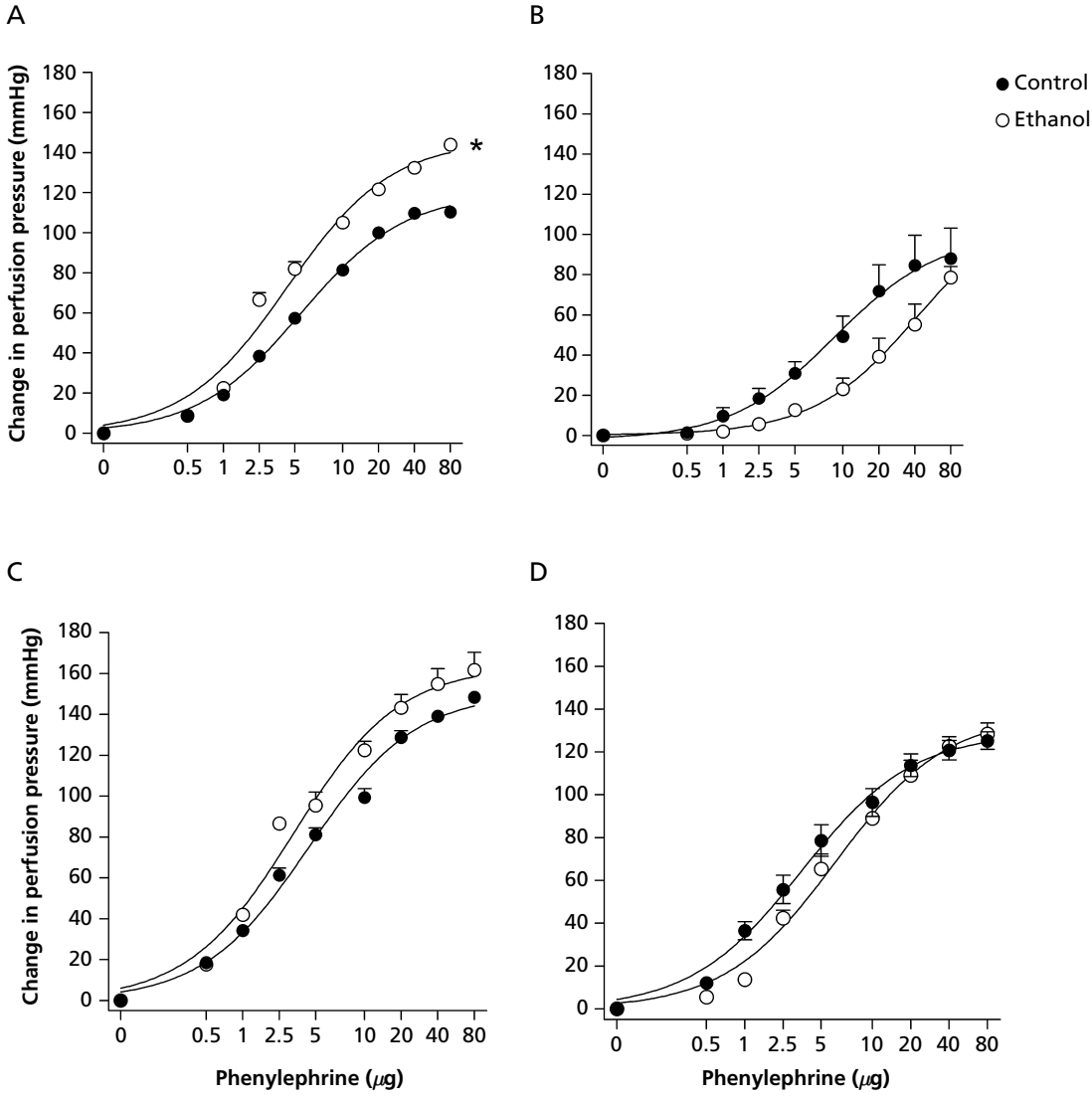

Figure 3 Concentration–response curves for phenylephrine determined in endothelium-intact mesenteric bed from control and 6-week ethanol-treated rats (A), and after 10 min pre-incubation with the cyclooxygenase inhibitor indometacin (10 μmol L^{-1}) (B) the nitric oxide synthase inhibitor *N*ω-nitro-L-arginine (L-NNA; 50 μmol L^{-1}) (C), or L-NNA and indometacin (D). *Difference in E_{max} (the maximum effect induced by phenylephrine) compared with controls ($P < 0.05$, two-way ANOVA followed by Bonferroni's comparison test). Data are mean ± s.e.m. and are representative of 5–13 independent experiments.

Table 2 Effect of L-NNA (50 μmol L^{-1}) (nitric oxide synthase inhibitor) and indometacin (10 μmol L^{-1}) (non-specific cyclooxygenase inhibitor) on the maximum effect induced by phenylephrine (E_{max}) (mmHg) and EC50 (μg) values (50% of the maximum effect induced by the agonist) for phenylephrine in endothelium-intact mesenteric bed from control and 6-week ethanol-treated rats

	Control		Ethanol	
	E_{max}	EC50	E_{max}	EC50
No inhibitor	110.4 ± 3.8 (13)	4 ± 0.3	144 ± 7.5* (9)	2.9 ± 0.5*
Indomethacin	88 ± 15.2† (5)	8.5 ± 1†	78.6 ± 5.6† (5)	10 ± 0.1*,†
L-NNA	148.4 ± 4.2† (5)	2.6 ± 0.3†	161.7 ± 17.3 (4)	2.2 ± 0.5
L-NNA + indomethacin	125.3 ± 10† (6)	4.4 ± 0.3†	128.4 ± 11.8(5)	4.4 ± 0.6†

Values are mean ± s.e.m. Numbers in parentheses indicate the number of animals in the group. *$P < 0.05$ vs respective control group. †$P < 0.05$ vs respective group in the absence of the inhibitors (ANOVA).

reduced, but did not abolish, acetylcholine-induced relaxation in the mesenteric bed from control rats (data not shown) as previously described by Adeagbo and Triggle (1993).

Effect of ethanol consumption on eNOS mRNA expression in the mesenteric bed

The results obtained by RT-PCR show that there was no difference in the expression of mRNA for eNOS between groups (Figure 4A).

Effect of ethanol consumption on eNOS protein in the mesenteric bed

Western immunoblotting showed that levels of eNOS protein in the mesenteric bed was significantly lower in ethanol-treated rats than in control rats (Figure 4B).

Discussion

Mild hypertension following chronic ethanol ingestion was observed in this study, consistent with previous reports (Chan et al 1985; Utkan et al 2001). The present findings are in accordance with previous observations from our group (Resstel et al 2006) showing that ethanol consumption induced an increase in both systolic and diastolic blood pressure. Our data show that ethanol consumption for 2 or 6 weeks did not affect resting HR, indicating that HR enhancement does not play a significant role in mediating the increase in blood pressure in ethanol-treated rats. Similarly, Beilin et al (1992) and Resstel et al (2006) reported that resting HR was not altered by long-term ethanol consumption.

The sucrose-treated groups were included in the present study to evaluate whether alterations in caloric intake with ethanol consumption might explain the effects of ethanol on the cardiovascular responses. Also, vascular responsiveness to vasorelaxant agents has been reported to be caused by elevated glucose (Tesfamariam et al 1991). Sucrose feeding did not affect arterial blood pressure or mesenteric bed reactivity, suggesting that the caloric content of the ethanol diet did not play a significant role in the present findings. Additionally, the level of serum glucose did not differ between the treated groups and their respective age-matched controls. However, it must be borne in mind that sucrose was used to compensate for calories provided by ethanol, not for total caloric intake. Moreover, it is clear from our data that the animals in the ethanol group had lower body weights, which may be associated with their decreased food consumption. Together, these observations indicate that animals in the ethanol-treated groups may have had lower total caloric intake. Thus, reduced intake of essential nutrients in solid food may account for the observed changes in the vascular reactivity.

The mesenteric circulation of the rat receives approximately one-fifth of the cardiac output (Nichols et al 1985); thus, regulation of this bed makes a significant contribution to the regulation of systemic blood pressure. To analyse this further, we evaluated whether alterations in the reactivity of the mesenteric bed could account for the hypertensive state associated with ethanol consumption. Our data demonstrate that chronic ethanol consumption increases responsiveness to phenylephrine in perfused mesenteric bed from 6-week, but not 2-week, ethanol-treated rats. By contrast, using the same experimental protocol, we have previously shown increased response to phenylephrine in isolated aortas from 2-week ethanol-treated rats (Tirapelli et al 2006a). The reasons for these differences are not clear, although they could be explained by the different role of conduit and peripheral arteries in the control of blood flow.

Endothelial denudation of mesenteric bed from control rats increased the contractile response to phenylephrine, indicating that the endothelium partially counteracts the phenylephrine-mediated vasoconstriction. However, this increased responsiveness to phenylephrine in mesenteric bed from ethanol-treated rats was not observed after endothelial denudation of tissues from 6-week ethanol-treated rats. This indicates that chronic ethanol intake alters the modulating effect of endothelium in response to α-adrenoceptor agonists. Moreover, the finding that acetylcholine-induced vasodilatation of mesenteric bed was reduced in vessels from 6-week ethanol-treated rats indicates that chronic ethanol consumption decreased the action of NO on its endothelial cell receptor-stimulated production/release. The vascular endothelium produces contracting and relaxing factors which

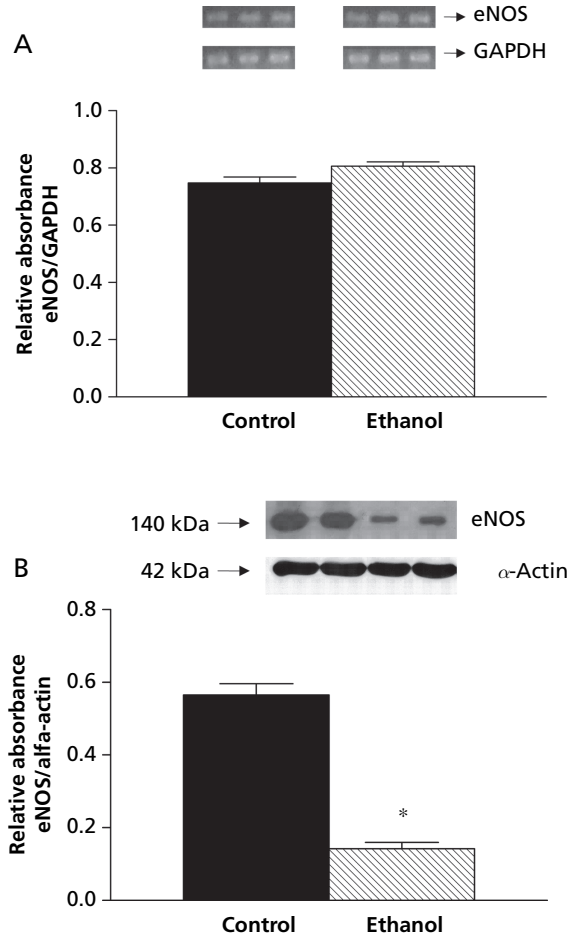

Figure 4 Representative RT-PCR products of 20 ng total mRNA (A) and immunoblotting products of 50 μg total protein (B) extracted from rat mesenteric bed. The bars show the relative absorbance values of endothelial nitric oxide synthase (eNOS) bands in endothelium-intact mesenteric bed from control and ethanol-treated rats. eNOS values were normalized to the corresponding GAPDH values (A) or the corresponding α-actin bands (B) used as internal standards. Data are mean $\pm$ s.e.m. and are representative of four experiments for each group. $^*P < 0.05$ vs control group (Student's *t*-test).

are involved in the control of arterial tonus (Mombouli & Vanhoutte 1999). We first examined the contribution of endothelial-derived prostanoids in the ethanol-induced hyperreactivity to phenylephrine, since these substances have been reported to modulate the reactivity of this tissue to phenylephrine (Leone et al 2004). Inhibition of cyclooxygenase by indometacin reduced the phenylephrine-induced increase in perfusion pressure (E_{max}) and increased the EC50 for this agonist. These observations suggest that activation of α_1-adrenoreceptor by phenylephrine may stimulate the production of vasoconstrictor prostanoids in the mesenteric bed, as previously suggested (Manku & Horrobin 1976; Peredo & Adler-Graschinsky 2000). Interestingly, in the presence of indometacin, the E_{max} for phenylephrine did not differ between tissues from control and ethanol-treated rats, further suggesting the involvment of endothelial-derived vasoconstrictor prostanoids in the increased response to phenylephrine in mesenteric bed from ethanol-treated rats. Using the same protocol for ethanol feeding, we have previously demonstrated that increased production of vasoconstrictor prostanoids (possibly TXA_2) mediates the hyperreactivity to phenylephrine in isolated aortas from ethanol-treated rats (Tirapelli et al 2006a).

Secondly, we investigated the participation of NO in the increased response to phenylephrine induced by ethanol consumption. L-NNA significantly increased the phenylephrine E_{max} in control tissues, suggesting that NO counteracts the contractile response induced by phenylephrine. Since there was a difference in the E_{max} for phenylephrine between control and ethanol-exposed tissues after pre-incubation with L-NNA, we suggest that the increased responsiveness of ethanol-treated mesenteric bed to phenylephrine was due to an impaired modulation of NO on the contractile effect induced by phenylephrine. Thus, chronic ethanol consumption reduced the counteracting effect produced by the activation of the NO pathway on phenylephrine-induced contraction, a fact that might contribute to the increased blood pressure associated with ethanol consumption.

The combination of L-NNA and indometacin prevented the reduction in phenylephrine-induced contraction observed

in the presence of indometacin in tissues from control and ethanol-treated rats. A possible explanation for this observation is that L-NNA inhibited the synthesis of NO, a vasorelaxing agent that counteracts the contractile response induced by phenylephrine. The inhibition of NO synthesis by L-NNA would facilitate the contraction induced by phenylephrine. It is important to note that the effect of the combination of L-NNA and indometacin was slightly different between control and ethanol-treated tissues. This difference could be due to the fact that vasoconstrictor prostanoids make a greater contribution to the contraction induced by phenylephrine in the mesenteric bed from ethanol-treated rats compared with control tissues. For this reason, the combination of L-NNA and indometacin had a more pronounced effect on the mesenteric bed from ethanol-treated rats.

In the process of analysing whether the impaired counteracting action of NO in response to stimulation with phenylephrine could be related to decreased function of the NO pathway, we found that the levels of eNOS protein were markedly reduced by treatment with ethanol, although mRNA expression of the isoenzyme was not changed, suggesting that chronic ethanol consumption down-regulates expression of eNOS at a post-transcriptional level.

The period of exposure to ethanol has been suggested to be a major factor in the development of cardiovascular abnormalities (Abdel-Rahman & Wooles 1987; Strickland & Wooles 1988). The present results demonstrate that chronic ethanol consumption increased the responsiveness of the mesenteric bed to phenylephrine, indicating a relationship between the period of treatment and the magnitude of the enhancement of phenylephrine-induced contraction. Moreover, the endothelium-dependent vasorelaxation induced by acetylcholine decreased after treatment for 6 weeks but not 2 weeks. It is important to note that increased blood pressure was already observed in 2-week ethanol-treated animals, whereas altered responsiveness to phenylephrine and acetylcholine was observed only in 6-week-treated rats. This observation supports the notion that altered responsiveness of the mesenteric bed is not the cause, but rather the consequence, of the increased blood pressure associated with ethanol intake. However, as the mesenteric bed represents only part of the vasculature that contributes to peripheral vascular resistance and control of blood pressure, it is possible that changes to other peripheral vessels or changes to conduit vessels such as the aorta contribute to elevations in blood pressure and may be detected earlier than changes in the mesenteric bed. Thus, although increased blood pressure may contribute to the changes in the mesenteric bed observed in this study, they may also be an effect of chronic ethanol intake.

Conclusion

This work provides a mechanistic explanation for the effects of ethanol consumption on the responsiveness of the mesenteric bed. Chronic ethanol consumption induces an increase in blood pressure and is associated with altered responsiveness of the mesenteric bed to vasoactive agents. Moreover, the increased vascular response to phenylephrine observed in the mesenteric bed is maintained by two mechanisms: an increased release of endothelial-derived vasoconstrictor prostanoids, and reduced post-transcriptional expression of eNOS, which has a modulatory action on endothelial NO.

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