Ethanol potentiates interleukin-1 β -stimulated inducible nitric oxide synthase expression in cultured vascular smooth muscle cells

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Experiments were performed to examine the effect of ethanol on the production of nitric oxide from interleukin-1 β (IL-1 β)-treated cultured rat aortic smooth muscle cells. Incubation of vascular smooth muscle cells with IL-1 β resulted in the release of nitrite and in the intracellular accumulation of L-citrulline. In parallel with this, IL-1 β increased inducible nitric oxide synthase (iNOS) mRNA and protein. Ethanol (6.5–650 mM) potentiated the IL-1 β -mediated stimulation of iNOS mRNA production, the appearance of iNOS protein and the generation of nitrite and L-

citrulline from smooth muscle cells in a concentration-dependent manner. In the absence of IL-1 β , ethanol failed to induce iNOS expression. These results demonstrate that pharmacologically relevant concentrations of ethanol enhance the IL-1 β -induced expression of the iNOS gene in vascular smooth muscle. The ability of ethanol to augment the release of the platelet inhibitor and vasodilator nitric oxide may, in part, contribute to the beneficial cardiovascular effects associated with moderate alcohol consumption.

INTRODUCTION

Nitric oxide is an important signal and effector molecule that is involved in a variety of biological functions [1]. In the circulation, the release of NO by vascular cells plays a pivotal role in regulating blood flow by inhibiting smooth muscle contraction and platelet aggregation [2,3]. NO is synthesized by oxidation of the terminal guanidino nitrogen atom(s) of L-arginine by a family of enzymes designated nitric oxide synthase (NOS). In the vasculature, at least two distinct classes of NOS have been identified. A constitutively active calcium/calmodulin-dependent isoform of the enzyme is present in endothelium, whereas a calcium-insensitive isoform is found in both endothelial and vascular smooth muscle cells [1]. This latter enzyme is termed inducible NOS (iNOS) and has been cloned from rat aortic smooth muscle cells [4,5].

Exposure of vascular smooth muscle cells to interleukin-1 β (IL-1 β) and other inflammatory mediators stimulates the expression of the iNOS gene and the subsequent release of NO from these cells [6–9]. This cytokine-mediated release of NO may have important pathophysiological and pharmacological consequences. The induction of iNOS activity in vascular cells has been implicated in the systemic hypotension observed in patients with Gram-negative sepsis and in patients undergoing anti-neoplastic therapy with IL-2, as well as in local vascular lesions associated with atherosclerosis and balloon angioplasty [10–12]. Furthermore, NO derived from cytokine-treated smooth muscle cells may play an important haemostatic role, since it can inhibit platelet activation at sites of vascular injury [13].

Epidemiological studies indicate that the moderate consumption of ethanol is associated with a significant reduction in the development of coronary artery disease [14–17]. The mechanism of this cardioprotective effect, however, has not been defined.

Although ethanol has been shown to favourably alter the lipid profile, this effect does not fully account for the clinical benefit attributed to ethanol consumption [18,19]. Another proposed mechanism for ethanol's protective action is that it exerts an anti-thrombotic effect by inhibiting vascular tone and platelet reactivity [20]. In addition to its direct effects on vascular smooth muscle and platelets [21,22], ethanol can indirectly modulate the function of these cells by stimulating the release of various vasorelaxing and platelet-inhibitory autacoids. In this regard, ethanol has been shown to stimulate the release of prostacyclin from vascular cells and to increase circulating levels of prostacyclin [23,24]. Since NO is another potent inhibitor of vascular tone and platelet aggregation, the present study examined whether ethanol could also modulate the production of NO by IL-1 β -treated vascular smooth muscle cells.

MATERIALS AND METHODS

Cell culture

Smooth muscle cells were isolated by elastase and collagenase digestion of rat thoracic aorta and characterized by morphological and immunological criteria [25]. Cells were cultured serially in minimum essential medium (MEM) containing Earle's salts, 5.6 mM glucose, 2 mM L-glutamine, 20 mM Tris, 20 mM Hepes, 10 % (v/v) heat-inactivated fetal calf serum, 100 units/ml penicillin and 100 units/ml streptomycin. Smooth muscle cells were passaged twice per week by harvesting with trypsin/EDTA and seeded (1:3 ratio) into 12-well plates (for nitrite and L-citrulline measurements), 6-well plates (for protein isolation) or 100 mm culture dishes (for RNA isolation). Subcultured strains of smooth muscle cells were used in passages 5–35. When cells

Abbreviations used: (i)NOS, (inducible) nitric oxide synthase; IL-1 β , interleukin-1 β ; TNF- α , tumour necrosis factor- α ; MEM, minimum essential medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Rp-cAMP, adenosine 3',5'-cyclic monophosphothioate.

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reached confluence, the culture media were replaced with serum-free MEM containing BSA (0.1 %, w/v) for 24 h. Smooth muscle cells were then exposed to IL-1 β , tumour necrosis factor- α (TNF- α) or their respective vehicle in the presence or absence of ethanol (6.5–650 mM) for various periods of time. In some experiments, smooth muscle cells were washed and the serum-free MEM containing 600 μ M L-arginine was replaced with L-arginine-reduced (100 μ M) serum-free MEM.

Production of nitric oxide from smooth muscle cells

The generation of nitric oxide by vascular smooth muscle cells was determined by measuring the release of nitrite, the stable oxidation product of nitric oxide [26], and by monitoring the intracellular accumulation of L-citrulline. For nitrite determinations, aliquots (400 μ l) of culture medium were mixed with an equal volume of Griess reagent [1 % sulphanilamide/0.1 % *N*-(1-naphthyl)ethylenediamine dihydrochloride in 2 % phosphoric acid], the mixture was incubated at room temperature for 10 min and then the absorbance at 540 nm was measured (Ultraspec III; Pharmacia, Uppsala, Sweden) [27]. Nitrite concentrations were determined relative to a standard curve by using an aqueous solution of sodium nitrite, and background nitrite values corresponding to serum-free medium were subtracted from experimental values.

L-Citrulline formation was monitored by adding L-[14 C]-arginine (0.5 μ Ci) to smooth muscle cells incubated in 500 μ l of L-arginine-reduced (100 μ M) serum-free medium. Following a 6 h incubation, the L-[14 C]arginine containing medium was removed and the cells were washed three times with 2 ml of PBS. A volume (150 μ l) of ice-cold Tris buffer (pH 6.8) containing Triton X-100 (0.01 %) was added to the monolayer; cells were then scraped, vortexed and centrifuged at 1000 *g* for 1 min. Aliquots of the supernatant (10 μ l) were spotted on to TLC plates and developed in the solvent system chloroform/methanol/ammonium hydroxide/water (1:4:2:1, by vol.). After drying, L-arginine products were detected by ninhydrin spray and L-[14 C]citrulline was identified by co-chromatography with unlabelled L-citrulline, scraped and quantified by scintillation counting.

RNA isolation and ribonuclease protection analysis

Total cellular RNA was obtained by the guanidine isothiocyanate/CsCl gradient method [28]. RNA was dissolved in Tris/EDTA buffer, quantified by measuring its absorbance at 260 nm, and stored at -70°C until ready for use. The ribonuclease protection assay was performed essentially as described by Orloff and Cunningham [5], using a rat aortic smooth muscle iNOS (160 bp restriction fragment) cRNA probe, labelled with [α - 32 P]UTP by *in vitro* transcription using T3 RNA polymerase (Ambion Inc., Austin, TX, U.S.A.). A radiolabelled antisense RNA probe for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (316 bp restriction fragment; Ambion Inc.) was also employed to control for variations in the amount of RNA used in each assay. Hybridization reactions were performed with 10 μ g of RNA in hybridization buffer (65 mM sodium citrate, 200 mM sodium acetate, 0.5 mM EDTA and 55 % formamide, pH 6.4) for 16 h at 48°C using $(1.0\text{--}4.0) \times 10^5$ c.p.m. of radiolabelled cRNA per reaction, followed by digestion with ribonucleases A (40 μ g/ml) and T1 (2 μ g/ml) at room temperature for 30 min. Protected RNA was analysed by electrophoresis using a 6 % acrylamide/8 M urea gel. The gel was exposed overnight to X-ray film at -70°C in the presence of intensifying screens. The predicted base pair protected fragments

of iNOS mRNA and GAPDH mRNA were confirmed using a DNA sequence ladder as a molecular size marker.

Protein separation and Western blotting

Following the incubation of smooth muscle cells with the requisite treatments, the culture media were removed, and the cells were washed in warm PBS and scraped into cold PBS. The cells were centrifuged at 500 *g* for 15 min at 4°C and then lysed by the addition of electrophoresis buffer [125 mM Tris/HCl (pH 6.8), 12.5 % glycerol, 2.5 % dithiothreitol, 2 % SDS, and trace Bromophenol Blue], followed by immediate boiling for 5 min. SDS/PAGE was performed on 8 % gels with 20 μ g of protein using the buffer system of Laemmli [29]. The separated proteins were electrophoretically transferred to nitrocellulose membranes as described by Towbin et al. [30]. Nitrocellulose blots were blocked for 1 h in PBS containing 1.0 % Tween 20 and 3 % non-fat milk, and then incubated with anti-(mouse iNOS) IgG (1:500 dilution) in PBS for 1 h. The membrane was then washed in PBS and incubated with anti-mouse horseradish peroxidase-conjugated antibody (1:2500 dilution) for 1 h. After further washing with PBS, blots were detected by the enhanced chemiluminescence method (Amersham Corporation, Arlington Heights, IL, U.S.A.).

Materials

Fatty-acid-free BSA, fetal calf serum, L-glutamine, penicillin, streptomycin, trypsin, sodium nitrite, *N*-(1-naphthyl)ethylenediamine dihydrochloride, sulphanilamide, ninhydrin spray, collagenase and elastase were purchased from Sigma (St. Louis, MO, U.S.A.); guanidine isothiocyanate and CsCl were from Gibco (Gaithersburg, IL, U.S.A.); nitro-L-arginine and TLC plates (silica gel-25) were from Aldrich (Milwaukee, WI, U.S.A.); MEM was from ICN Biomedicals (Costa Mesa, CA, U.S.A.); ethanol was from Pharmco Products (Bayonne, NJ, U.S.A.); TNF- α was from Genzyme Corporation (Cambridge, MA, U.S.A.); adenosine-3',5'-cyclic monophosphothioate (Rp-cAMP) was from Research Biochemicals International (Natick, MA, U.S.A.); Tween 20 was from Promega (Madison, WI, U.S.A.); anti-(mouse iNOS) antibody was from Transduction Laboratories (Lexington, KY, U.S.A.); nitrocellulose was from Bio-Rad Laboratories (Hercules, CA, U.S.A.); anti-mouse horseradish peroxidase conjugated antibody and [α - 32 P]UTP (400 Ci/mmol) were from Amersham; L-[14 C]arginine (55 mCi/mmol) was from Dupont New England Nuclear Research Products (Boston, MA, U.S.A.).

Statistics

Results are expressed as the means $\pm$ S.E.M. Statistical analysis was performed with the use of Student's two-tailed paired *t* test and an analysis of variance when more than two treatments were compared. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Treatment of rat aortic smooth muscle cells with IL-1 β (1 ng/ml for 24 h) stimulated the release of nitrite and the intracellular accumulation of L-[14 C]citrulline (Figure 1). The simultaneous addition of ethanol (6.5–650 mM) to the smooth muscle cells significantly increased both the IL-1 β -induced release of nitrite and the formation of L-[14 C]citrulline in a concentration-dependent manner, without affecting that from untreated cells

Figure 1 Effect of ethanol on the production of NO from cultured rat aortic smooth muscle cells

(a) Effect of ethanol on the release of nitrite from unstimulated (○) and IL-1β-stimulated (1 ng/ml for 24 h) (●) smooth muscle cells. Results are the means ± S.E.M. of five separate experiments, each performed in duplicate. *Statistically significant effect of ethanol on IL-1β-stimulated nitrite release ($P < 0.05$). (b) Effect of ethanol on the intracellular accumulation of L-[¹⁴C]citrulline from unstimulated (○) and IL-1β-stimulated (1 ng/ml for 24 h) (●) smooth muscle cells. Results are means ± S.E.M. of four separate experiments. *Statistically significant effect of ethanol on IL-1β-stimulated L-[¹⁴C]citrulline formation ($P < 0.05$).

(Figure 1). In addition, ethanol (200 mM) significantly elevated smooth muscle cell nitrite production induced by various concentrations of IL-1β (Figure 2). The potentiating effect of ethanol was not associated with any changes in intracellular levels of cyclic AMP in smooth muscle cells (as measured by radioimmunoassay), nor was it affected by pretreating the cells with a specific inhibitor of protein kinase A, Rp-cAMP (500 μM) (results not shown).

Both the IL-1β-stimulated release of nitrite and the ethanol-mediated enhancement of this effect were significantly blocked by the concomitant addition of nitro-L-arginine (500 μM) to smooth muscle cells incubated in L-arginine-reduced culture media (Table 1). Nitro-L-arginine had no effect on smooth muscle cells that had not been exposed to IL-1β (Table 1). In addition, the potentiating effect of ethanol (200 mM) on the release of nitrite induced by IL-1β was dependent on the time at which ethanol was administered. The enhancing effect of ethanol on IL-1β-stimulated nitric oxide production was similar when ethanol was added either at the same time or 2 h after the cytokine (Figure 3). However, the addition of ethanol at 4, 6 or 8 h after the treatment of smooth muscle cells with IL-1β resulted in a progressive decline in the potentiating effect of ethanol (Figure 3). Treating cells with ethanol for up to 1 h followed by its removal prior to the addition of IL-1β did not enhance IL-1β-induced nitrite generation.

Figure 2 Stimulation of nitrite release from cultured rat aortic smooth muscle cells by different concentrations of IL-1β in the absence (○) or presence (●) of ethanol (200 mM)

Cells were incubated with IL-1β for 24 h. Results are means ± S.E.M. of six separate experiments, each performed in triplicate. *Statistically significant effect of ethanol on IL-1β-stimulated nitrite release ($P < 0.05$).

Table 1 Effect of nitro-L-arginine (L-NNA; 500 μM) on the release of nitrite

Cultured rat aortic smooth muscle cells were incubated in L-arginine-reduced (100 μM) media and treated with IL-1β (1 ng/ml for 24 h) or vehicle, in the presence and absence of ethanol (200 μM). Results are the means ± S.E.M. of three separate experiments each performed in triplicate. *Statistically significant increase in nitrite release compared with untreated control cells ($P < 0.05$); †statistically significant effect of L-NNA treatment on IL-1β-stimulated nitrite release ($P < 0.05$).

Cell treatment	Nitrite (μM)	
	– IL-1β	+ IL-1β
None	1.34 ± 0.14	17.11 ± 0.14*
Ethanol	1.45 ± 0.14	27.48 ± 0.91*
L-NNA	0.54 ± 0.45	1.39 ± 0.16†
L-NNA + ethanol	0.91 ± 0.42	2.20 ± 0.16†

Figure 3 Effect of ethanol (200 mM) on the release of nitrite from unstimulated (□) and IL-1β-stimulated (1 ng/ml for 24 h) (■) cultured rat aortic smooth muscle cells

Ethanol was added to the smooth muscle cells either simultaneously or 2, 4, 6 or 8 h after the cytokine. Results are shown as the means ± S.E.M. of three experiments, each performed in triplicate. *Statistically significant decrease in nitrite release compared with that evoked by the simultaneous treatment of cells with ethanol and IL-1β (0 h). ($P < 0.05$).

Figure 4 Effect of ethanol on iNOS mRNA expression and on iNOS protein levels

(a) Ribonuclease protection analysis of iNOS and GAPDH mRNAs from unstimulated (C) and from IL-1 β -stimulated (1.0 ng/ml for 4 h) cultured rat aortic smooth muscle cells in the presence and absence of ethanol (25–200 mM). Similar findings were observed in four separate experiments. (b) Western blot analysis of iNOS protein from unstimulated (C) and from IL-1 β -stimulated (1.0 ng/ml for 24 h) cultured rat aortic smooth muscle cells in the presence and absence of ethanol (25–200 mM). Similar findings were observed in three separate experiments.

The exposure of vascular smooth muscle cells to IL-1 β (1 ng/ml) for 4 h resulted in the expression of iNOS mRNA (Figure 4a). The level of IL-1 β -stimulated iNOS message was increased in a concentration-dependent fashion by the simultaneous addition of ethanol (25–200 mM) to cytokine-treated smooth muscle cells (Figure 4a). However, very high concentrations of ethanol (> 650 mM) inhibited the expression of iNOS transcript following IL-1 β stimulation (results not shown). In untreated smooth muscle cells (Figure 4a) and in cells exposed to ethanol alone (results not shown), no signal for iNOS mRNA could be detected. No significant changes in GAPDH mRNA levels were

Figure 5 Effect of ethanol on iNOS mRNA expression following TNF- α exposure

Nitrite production was measured from unstimulated (○) and TNF- α -stimulated (10 ng/ml for 24 h) (●) cultured rat aortic smooth muscle cells in the presence and absence of ethanol (6.5–650 mM). Results are expressed as the means ± S.E.M. of four separate experiments performed in triplicate. Inset: ribonuclease protection analysis of iNOS and GAPDH mRNA from unstimulated (C) and from TNF- α -stimulated (10 ng/ml for 4 h) cultured rat aortic smooth muscle cells in the presence and absence of ethanol (200 mM). Similar findings were observed in two separate experiments.

observed following the addition of IL-1 β alone or in combination with ethanol (Figure 4a). Treatment of smooth muscle cells with IL-1 β (1 ng/ml) for 24 h also resulted in the appearance of iNOS protein, as revealed by Western blot analysis. Immunoblotting identified a single band with an approximate molecular mass of 125 kDa (Figure 4b), which is the estimated size of iNOS in vascular smooth muscle [31]. The simultaneous treatment of smooth muscle cells with IL-1 β (1 ng/ml) and ethanol (25–200 mM) resulted in an ethanol-concentration-dependent potentiation of iNOS protein levels (Figure 4b). In the absence of IL-1 β , ethanol failed to induce iNOS protein expression (results not shown).

Finally, the simultaneous treatment of smooth muscle cells with ethanol (6.5–650 mM) significantly enhanced the TNF- α -mediated release of nitrite without affecting that from untreated cells (Figure 5). Furthermore, the TNF- α -induced production of iNOS mRNA was increased by the concurrent administration of ethanol (Figure 5, inset).

DISCUSSION

The present study demonstrates that ethanol potentiates the IL-1 β -induced expression of the iNOS gene in rat aortic smooth muscle cells. This is demonstrated by the ability of ethanol to augment the cytokine-mediated stimulation of iNOS mRNA production, the appearance of iNOS protein and the subsequent generation of NO.

Inflammatory mediators, including IL-1 β , stimulate the release of NO by vascular smooth muscle cells [6–9]. Consistent with this, the present study shows that treatment of smooth muscle cells with IL-1 β results in a concentration-dependent increase in nitrite, the stable oxidation product of NO [26], in the incubation medium. The simultaneous addition of ethanol to the cytokine-treated smooth muscle cells enhances nitrite production in a concentration-dependent manner. Furthermore, both the IL-1 β -induced release of nitrite from smooth muscle cells and the capacity of ethanol to increase this release are blocked by the specific NOS inhibitor nitro-L-arginine [32], indicating that NOS activity is responsible for the generation of nitrite.

Ethanol upregulates iNOS expression at an early step in the IL-1 β -stimulated induction cascade. The potentiating effect of ethanol requires the presence of the alcohol during the induction period of NOS, since the addition of ethanol more than 2 h after cytokine addition fails to enhance nitrite release. Furthermore, ethanol markedly increases, in a concentration-dependent manner, the IL-1 β -induced increase in steady-state levels of iNOS mRNA. These results indicate that ethanol enhances the cytokine-stimulated expression of iNOS by increasing the transcriptional activation of the gene and/or by stabilizing the transcript, as has been reported for ethanol with some other mRNA species [33,34]. A direct effect of ethanol on iNOS gene expression is ruled out by the failure of ethanol alone to stimulate iNOS mRNA production, iNOS protein formation or NO release.

Although ethanol enhances the expression of iNOS, its potentiating effects on IL-1 β -stimulated iNOS mRNA and protein levels (approx. 10-fold) are far greater than on nitrite production (approx. 2-fold). The possibility that nitrite is not quantitatively measuring NO synthesis seems unlikely, since ethanol augments IL-1 β -induced L-citrulline formation to the same degree as seen with nitrite generation. Thus it appears that the ethanol-stimulated increase in iNOS gene expression is partially counterbalanced by some other mechanism. Limitations in substrate and/or cofactor supply may also regulate the capacity of ethanol to stimulate NO synthesis in these cells.

The molecular mechanism by which ethanol potentiates the expression of iNOS from IL-1 β -treated smooth muscle cells is not known. The finding that ethanol stimulates adenylate cyclase activity in various cells [35] suggests that cyclic AMP may play a role in mediating the ethanol effect, since it has been recently demonstrated that lipophilic analogues of cyclic AMP and cyclic AMP elevating agents can potentiate iNOS expression in vascular smooth muscle [31,36,37]. However, pretreatment of smooth muscle cells with Rp-cAMP, a selective inhibitor of protein kinase A [38], failed to inhibit the potentiating effect of ethanol. Furthermore, treatment of smooth muscle cells with ethanol did not raise intracellular cyclic AMP levels, indicating that under these conditions the ethanol effect is not mediated by cyclic AMP. Alternatively, ethanol may enhance the induction of iNOS by regulating the levels of endogenous modulators. In this respect, ethanol can inhibit the production of transforming growth factor- β 1 [39], a platelet-derived mitogen which downregulates the expression of iNOS [40,41]. Finally, the potentiating effect of ethanol is not specific for the IL-1 β receptor and/or its transduction mechanism, since ethanol is also able to enhance the induction of iNOS evoked by TNF- α .

Our finding that ethanol can potentiate iNOS expression in cultured rat aortic smooth muscle cells contrasts with a recent study which demonstrates that the *in vivo* administration of ethanol attenuates iNOS expression in the rat liver [42]. The reason for divergent results between these two studies is not known; however, there are several possible explanations. First, the *in vivo* administration of ethanol may result in its biotransformation in the liver to secondary products that can inhibit iNOS induction, which are not formed in our tissue culture experiments. Secondly, iNOS expression was examined in different cells (Kupffer and endothelial cells versus vascular smooth muscle cells), and it has been previously documented that modulators of iNOS induction differentially regulate the iNOS gene in different cell types [43]. Finally, the regulatory effect of ethanol on iNOS expression may be stimulus-dependent. In our investigation IL-1 β and TNF- α were employed, whereas lipopolysaccharide was used in the other study.

The ability of ethanol to upregulate the induction of smooth muscle cell iNOS is of pharmacological relevance. Ethanol concentrations as low as 25 mM potentiate the stimulation of iNOS mRNA levels by IL-1 β , indicating that clinically attainable concentrations of ethanol can regulate iNOS gene expression. Interestingly, high concentrations of ethanol (> 650 mM) have a marked inhibitory effect on cytokine-induced iNOS mRNA formation. This biphasic effect on iNOS expression of ethanol corresponds to its biphasic effect on the incidence of cardiovascular disease, where moderate consumption of ethanol exerts a protective effect while chronic alcohol abuse is associated with a higher incidence of cardiovascular disorders [44].

The capacity of ethanol to enhance the expression of iNOS activity may also have important pathophysiological consequences. Inflammatory mediators, including IL-1 β , are released by mononuclear and vascular cells at sites of vessel wall injury, leading to the induction of iNOS in vascular smooth muscle cells. In the presence of ethanol the induction of iNOS would be further augmented, leading to the release of large amounts of NO into the circulation. This would prevent the adhesion and subsequent release of prothrombotic and constrictor agents from activated platelets and would also relax the underlying smooth muscle resulting in the preservation of blood flow and fluidity at sites of vessel wall damage. An increase in the production of the platelet inhibitor and vasodilator NO by vascular smooth muscle cells may contribute to ethanol's ability to significantly reduce experimentally induced arterial thrombus

formation in various animal models [45,46] and to prolong the bleeding time with alcohol consumption [47,48].

In conclusion, the present study demonstrates that clinically relevant concentrations of ethanol modulate the expression of iNOS in vascular smooth muscle by increasing the IL-1 β -stimulated production of iNOS mRNA. The ability of ethanol to amplify the cytokine-induced release of NO may provide an important mechanism by which ethanol maintains blood flow and fluidity at sites of vascular injury and may, in part, contribute to the reduced risk of coronary artery disease associated with the moderate consumption of alcohol.

We thank Dr. Michael H. Kroll for helpful discussions and suggestions. We also thank Dr. Gregory J. Orloff and Dr. James M. Cunningham for generously providing the cDNA for rat aortic smooth muscle cell iNOS. This work was supported in part by National Heart, Lung and Blood Institute grant HL 36045, the Veterans Affairs Merit Review Board, a grant-in-aid from the American Heart Association, Texas Affiliate, Inc., and an award from the Biomedical Research Support Group of Baylor College of Medicine (PHS RR-05424). R.K.S. is a recipient of a Medical Research Council of Canada Postdoctoral Fellowship.

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Received 22 June 1994/23 December 1994; accepted 9 January 1995