Ethanol Enhances the Endothelial Nitric Oxide Synthase Response to Agonists

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Chronic ethanol consumption is associated with an increased prevalence of hypertension. The mechanisms of this form of hypertension are unknown. Rats fed ethanol for 2 days develop a tolerance to the acute vasoconstrictive effects of ethanol that is believed to be endothelium dependent. We investigated the effects of acute and chronic ethanol exposure on agonist-stimulated nitric oxide synthase activity in bovine pulmonary artery endothelial cells. Exposure of bovine pulmonary artery endothelial cells to ethanol (100 mmol/L) for 20–120 minutes did not change either basal or agonist-stimulated nitric oxide synthase activity measured as the rate of conversion of \( [3H] \)-arginine to \( [3H] \)-citrulline. Chronic exposure of endothelial cells to ethanol (100 mmol/L) for 96 hours significantly increased bradykinin-, adenosine 5′-triphosphate-, and ionomycin-stimulated nitric oxide synthase activity without affecting basal enzyme activity. The ethanol-induced increase in nitric oxide synthase response to agonists was dependent on the duration of ethanol exposure as well as the concentration of ethanol. Moreover, the effect of ethanol was characterized by an increase in the maximal nitric oxide synthase response to adenosine 5′-triphosphate without changes in the EC\( _{50} \). Removal of calcium or addition of N\(^\text{N}\)-nitro-L-arginine completely abolished agonist-stimulated nitric oxide synthase activity in both control and ethanol-treated cells. Our observations support the hypothesis that ethanol enhances nitric oxide synthase response to agonists during early ethanol exposure and may serve in a protective role against its hypertensive effect. (Hypertension 1993;21:939–943)

Key Words • ethanol • endothelium • nitric oxide • endothelium-derived relaxing factor • hypertension, ethanol-related

C hronic ethanol (EtOH) consumption is associated with an increased prevalence of arterial hypertension in humans and experimental animals.1–3 The mechanisms of ethanol-associated hypertension are unknown but may include intermittent withdrawal, alterations in catecholamine production, and direct constrictive effects of ethanol on blood vessels.4 In animal and human studies, the acute administration of ethanol results in vasoconstriction of most vascular beds.4–5 However, chronic ethanol feeding to rats results in the early development of tolerance to the acute vasoconstrictive effect of ethanol,6,7 that is believed to be endothelium dependent.8,9 In addition, studies in ethanol-fed rats have demonstrated augmented relaxation of vascular rings to acetylcholine and adenosine 5′-triphosphate (ATP).10

In 1980, Furchgott and Zawadzki11 reported the obligatory role of the endothelium in vascular relaxation by acetylcholine. Subsequently, several other vasorelaxants such as bradykinin and ATP have been shown to have endothelium-dependent actions by stimulating the release of an endothelium-dependent relaxation factor (EDRF) recently identified as nitric oxide.12 Nitric oxide is synthesized by nitric oxide synthase (NOS) from \( \text{L-arginine} \) in a reaction that produces stoichiometric amounts of \( \text{L-citrulline} \).13 The constitutive endothelial isoform of NOS is calcium/calmodulin dependent and inhibited by N\(^\text{N}\)-nitro-L-arginine (NOARG).14 To examine the relation between ethanol and the L-arginine: nitric oxide system more directly, we investigated the effects of acute and chronic ethanol exposure on NOS in cultured endothelial cells.

Methods

Materials

Bovine pulmonary artery endothelial cells were obtained from American Type Culture Collection, Rockville, Md., at passage 17. Roswell Park Memorial Institute (RPMI)-1640, minimum essential medium (MEM), ethanol, and all cell culture materials were purchased from Fisher Scientific, Orlando, Fla. Ionomycin was obtained from CalBiochem, La Jolla, Calif. ATP, bradykinin, ethanol enzymatic determination kit, and other chemical reagents were purchased from Sigma Chemical Co., St. Louis, Mo. \( [3H] \)-arginine was purchased from Amersham, Arlington Heights, Ill. AG50WX-8

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Determination of Nitric Oxide Synthase Activity

NOS activity in BPAE was determined by measuring the conversion of [3H]L-arginine to [3H]L-citrulline after separation of these amino acids by anion exchange chromatography using a modification of the method of Bredt and Snyder. After a predetermined period of exposure to ethanol, control and treated BPAE cells were incubated in HEPES buffer containing (in mmol/L) HEPES 25 (pH 7.40, 37°C), NaCl 140, KCl 5.4, CaCl2 1.8, MgCl2 1.0, and glucose 5.0 and 3 µCi/mL [3H]L-arginine. Preliminary experiments demonstrated that uptake of [3H]L-arginine by BPAE cells was linear over 20 minutes reaching equilibrium after that (data not shown). Therefore, we chose to radiolabel the cells for 20 minutes before stimulation with the agonists. Agonist stimulation was carried out for 20 minutes at 37°C and terminated by washing the cells with ice-cold Ca2+-free buffer containing 5 mmol/L EDTA and addition of 1 mL of 0.3 mol/L HClO4. After neutralization of the tissue extract with 3.0 mol/L K2CO3, 50-µL aliquots were taken for determination of total uptake of [3H]L-arginine, and 500-µL aliquots were applied to columns containing Dowex AG50WX 8 (Na+ form) added as a 1:1 slurry in H2O. The columns were then washed three times with 2 mL water, and the eluant, whose sole radioactive component is [3H]L-citrulline as determined by thin-layer chromatography, was collected into vials. Ten milliliters of scintillation cocktail was added to the vials, and the radioactivity was quantified by liquid scintillation spectroscopy. NOS activity is expressed as picomoles of [3H]L-citrulline produced per milligram of protein or as percent conversion of [3H]L-arginine to [3H]L-citrulline. Protein determinations were made by the Lowry assay.

Statistical Analysis

Results are presented as mean±standard error of the indicated number of experiments performed in triplicate. Statistical analysis was performed using Student's t test for paired and unpaired data or analysis of variance (ANOVA) and subsequent Scheffé's F test (STATVIEW II, Abacus Concepts, Inc., Berkeley, Calif.) as appropriate.

Results

To investigate the effects of ethanol on NOS, BPAE cells were exposed to 100 mmol/L ethanol for 0, 20, 60, and 120 minutes, followed by measurements of NOS activity under basal conditions and after stimulation with bradykinin (1 µmol/L), ATP (100 µmol/L), or the calcium ionophore ionomycin (2 µmol/L) in the presence or absence of ethanol (unless otherwise indicated these agonist concentrations were used throughout the study). No differences in NOS activity were observed with acute ethanol treatment (data not shown). Conversely, when BPAE cells were exposed to 100 mmol/L ethanol for 96 hours, the response of NOS to agonists was increased by 45±8% after stimulation with bradykinin, 68±8% after ATP, and 37±3% after ionomycin (p<0.05 for each agonist relative to agonist-stimulated control cells, n=6) (Figure 1). Basal NOS activity was not changed by ethanol treatment. The concentration of ethanol at the end of the treatment period was 88±3 mmol/L (n=5). Protein concentrations per well were found to be reduced by 22±4% at the end of 96 hours of ethanol treatment (p<0.001 versus controls, n=12). However, the uptake of [3H]L-arginine per milligram of protein measured over the 40-minute period of the assay (radiolabeling plus agonist stimulation periods) was not affected by ethanol treatment (8.70±0.90 and 8.32±0.84 pmol/mg in control and ethanol-treated cells, respectively, NS, n=6). Therefore, in subsequent experiments we chose to express NOS activity as the rate of conversion of L-arginine to L-citrulline, a ratio that closely correlated with measurements of L-citrulline production per milligram of protein. Osmolarity controls were carried out for up to 96 hours using concentrations of mannitol comparable to the experimental ethanol concentrations. No differences in basal or agonist-stimulated NOS activity were observed between controls and cells exposed to mannitol (data not shown). Thus, the above results indicate that chronic ethanol treatment enhances endothelial NOS response to agonists without changing basal enzyme activity by mecha-
We next examined the time course of the effect of ethanol on NOS. BPAE cells were exposed to 100 mmol/L ethanol for 24, 48, 72, and 96 hours followed by stimulation with ATP or ionomycin. As shown in Figure 2, exposure of endothelial cells to ethanol up to 96 hours produced no change in basal NOS activity. Conversely, the NOS response to ATP and ionomycin was significantly increased after 24 and 72 hours of exposure to ethanol, respectively. Thus, these results indicate that ethanol enhances the response of NOS to agonists in a time-dependent manner. Since the ethanol-induced increase in NOS response to agonists was maximum at 96 hours for the times investigated, we chose this period of ethanol exposure for the remainder of our studies.

To determine the concentration dependency of the effect of ethanol on NOS, BPAE cells were exposed for 96 hours to ethanol at concentrations ranging from 10 to 400 mmol/L followed by stimulation with agonists. As demonstrated in Figure 3, basal NOS activity was unchanged by ethanol. However, the NOS response to stimulation by ATP and ionomycin was significantly increased by ethanol starting at 50 mmol/L (45% and 23% increase, respectively) and up to 400 mmol/L (300% and 115%, respectively), the highest concentration studied. Thus, these results indicate that potentiation of the NOS response to agonists by ethanol is concentration dependent.

To investigate whether the enhanced NOS response to agonists after ethanol exposure was due to increased activity of the constitutive calcium/calmodulin-dependent enzyme, we exposed BPAE cells to 100 mmol/L ethanol for 96 hours followed by agonist stimulation in HEPES buffer devoid of calcium and containing 2 mmol/L ethylenediaminetetraacetic (EDTA), a calcium chelator. As shown in Figure 4, removal of calcium completely abolished the NOS response to agonists, suggesting that ethanol acts mainly on the constitutive calcium/calmodulin-dependent endothelial NOS. In addition, NOARG (300 nM), a known inhibitor of NOS, also abolished agonist-stimulated NOS activation (Figure 4). Agonist-stimulated NOS activation was also completely abolished in control cells by removal of calcium or addition of NOARG (data not shown).

We further characterized the effect of ethanol on receptor-mediated NOS activation by determining concentration–response curves to the purinergic agonist ATP. Ethanol treatment resulted in a significant increase in maximal agonist response compared with controls without significantly altering agonist potency (Figure 5). The maximal NOS response to ATP in ethanol-treated cells was increased by approximately double relative to controls \(p<0.05, \ n=6\), whereas the approximate \(\text{EC}_{50}\) calculated by the method of logits were 58 ± 12 and 28 ± 8 µmol/L for control and ethanol-treated cells, respectively (NS). Thus, these results indicate that in the case of ATP, ethanol enhances the NOS response mainly by increasing agonist efficacy. Although there was also a tendency toward an increase in agonist potency by ethanol treatment, this effect did not reach statistical significance.

**Discussion**

Chronic ethanol exposure results in the development of tolerance to the acute vasoconstrictive effect of ethanol\(^7^\) and augmented relaxation to acetylcholine and ATP.\(^10^\) The mechanism of these changes is unknown but appears to involve changes in EDRF production.\(^8^\) Our study clearly demonstrates that exposure of BPAE cells to ethanol enhances NOS response to agonists in a time- and concentration-dependent manner without affecting basal enzyme activity. This effect appears to occur through changes in the response of the constitutive calcium/calmodulin-dependent isoform of NOS and does not involve changes in oxidant stress or changes in the expression of endothelial nitric oxide synthase (eNOS). These results provide new insights into the mechanisms underlying ethanol-induced vasodilatation and the potential role of NOS in the ethanol-mediated vasodilation.

**Figure 2.** Line graph shows time course of the effect of ethanol on nitric oxide synthase (NOS) activity. Basal and agonist-stimulated NOS activity was measured in control cells (time 0) and in cells exposed to 100 mM ethanol for 24, 48, 72, and 96 hours. ATP, adenosine 5'-triphosphate; Iono, ionomycin. *p<0.05 vs. controls for each agonist, n=6.

**Figure 3.** Line graph shows concentration dependency of the effect of ethanol (EtOH) on nitric oxide synthase (NOS) activity. Basal and agonist-stimulated NOS activity was measured in bovine pulmonary artery endothelial cells exposed to ethanol at concentrations ranging from 0 to 400 mM for 96 hours. Ethanol concentrations of 50 mM and higher resulted in enhanced adenosine 5'-triphosphate (ATP)- and ionomycin (Iono)-stimulated NOS activity relative to ethanol-untreated cells (p<0.05, n=6).
ATP NOARG Calcium

FIGURE 4. Bar graphs show effects of N'-nitro-L-arginine (NOARG) (300 μmol/L) and removal of calcium on adenine 5'-triphosphate (ATP) (panel A) and ionomycin (panel B) stimulated nitric oxide synthase (NOS) activity in bovine pulmonary artery endothelial cells exposed to 100 mM ethanol for 96 hours. Both NOARG and removal of calcium abolished agonist-stimulated NOS activity. *p<0.05 vs. basal, n=6.

FIGURE 5. Line graph shows concentration-response curves for adenosine 5'-triphosphate (ATP) stimulation of nitric oxide synthase activity in control cells and in cells treated with 100 mM ethanol (EtOH) for 96 hours. Ethanol treatment resulted in increased maximal ATP response without significant change in agonist potency (see text).

NOS. Moreover, the effect of ethanol on NOS is observed at concentrations as low as 50 mmol/L and is quite prominent at concentrations of 100 mmol/L. Blood alcohol concentrations in this range are not infrequently observed in chronic alcoholics, and therefore our observations are likely to be relevant to the clinical situation, i.e., alcohol-related hypertension. We did not observe any effect of acute ethanol exposure on either basal or ATP-stimulated NOS activity.

In vitro, acute ethanol exposure results in vasoconstriction in most vascular beds except in the mesentery where it causes vasodilatation, and acute exposure of ethanol to aortic rings or mesenteric artery results in impaired endothelium-mediated relaxation to acetylcholine and ATP. Thus, acute ethanol exposure causes vasoconstriction and impairs the response to vasodilatation by agonists, suggesting a direct role of ethanol in the genesis of hypertension. However, in chronic ethanol animal models, rats fed ethanol for 2–10 weeks develop a tolerance to the acute vasoconstrictive effects of ethanol. These changes also develop as early as 2 days in rats fed ethanol by oral intubation. The mechanism of ethanol tolerance is believed to be mediated by EDRF since the tolerance is diffusible and inhibited by methylene blue or NOARG. Other investigators have demonstrated augmented vasorelaxation to acetylcholine and ATP in rats chronically fed ethanol for 10 weeks suggesting a protective role of this tolerance on the advancement of hypertension. With continued exposure to ethanol, tolerance to the vasoconstrictive effect appears to be lost resulting in the development of more severe hypertension. In fact, Williams and colleagues were not able to demonstrate augmented vasorelaxation to acetylcholine or ATP after hypertension developed in rats fed ethanol for 13 weeks. Other investigators have studied the pressor response in rats chronically fed ethanol. Although the results have varied, a tolerance to the pressor effect of phentylephrine has been demonstrated in rats fed ethanol for 6 to 18 weeks. Interestingly, after 22 weeks of ethanol feeding the tolerance to phentylephrine was lost coincident with the development of worsening hypertension. Thus, it is conceivable that chronic ethanol consumption may eventually lead to endothelial or vascular smooth muscle damage resulting in loss of endothelium-dependent tolerance and the development of hypertension.

The mechanisms responsible for the increased NOS response to agonists after chronic ethanol exposure observed in our present study are unknown. One possibility is that ethanol causes an increase in the synthesis of the enzyme. The increased NOS response to agonists in our study was not observed after 2 hours of exposure to ethanol but became apparent after 24 hours. This observation is consistent with a possible requirement for protein synthesis. Alternatively, ethanol may increase the synthesis of cofactors such as NADPH, FAD, and tetrahydrobiopterin, all of which are necessary for NOS activity, or it may alter the...
sensitivity of NOS to these cofactors or to intracellular calcium. Our study also demonstrates that the enhanced response of NOS to agonists appears earlier for ATP than for ionomycin suggesting that alterations in agonist–receptor interactions or postreceptor signaling events may also contribute to the effect of ethanol. The observation that only small changes in the EC50 for ATP occurred after 96 hours of ethanol exposure suggests that the enhanced NOS response to ATP is not likely due to changes in receptor affinity. Although we did not measure receptor numbers directly, it is unlikely that changes in receptor density are responsible for the effects of ethanol on NOS activity since these changes were also observed during stimulation with ionomycin, a calcium ionophore that acts through receptor-independent mechanisms. Several studies have documented ethanol-induced changes in intracellular signaling mechanisms. Chronic ethanol exposure has been shown to increase intracellular calcium in aorta, brain, and hepatocytes, possibly through an increase in calcium influx via voltage-dependent calcium channels, or through inhibition of plasma membrane Ca2+/Mg2+ ATPase. In addition, ethanol has been shown in isolated hepatocytes and human platelets to activate hormone-sensitive phospholipase C and GTP-dependent proteins and to increase inositol 1,4,5-trisphosphate [Ins(1,4,5)P3], which is formed from Ins(1,4,5)P2, can activate receptor-mediated calcium channels in these cells. Thus, ethanol could increase agonist-stimulated Ins(1,4,5)P3 and Ins(1,3,4,5)P4, formation in endothelial cells, which may in turn lead to increased calcium influx and enhanced NOS response to agonists. Studies are currently underway to explore the potential mechanisms responsible for the effects of ethanol on NOS activity.

In conclusion, chronic ethanol exposure increases the endothelial NOS response to agonists in cells in culture. This finding is consistent with previous observations that chronic ethanol exposure causes augmented relaxation in agonists and tolerance to the vasocostrictive effect of ethanol, effects that are proposed to be mediated by the endothelium and to protect against the development of hypertension. However, animal studies also suggest that the augmented vasoconstriction to agonists and ethanol-induced tolerance to phenylephrine are lost with prolonged ethanol exposure. Loss of these protective mechanisms may reflect alterations in the L-arginine: nitric oxide system and could ultimately contribute to the development of hypertension.

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