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 EC50. Removal of calcium or addition of Nω-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.

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

Chronic ethanol (EtOH) consumption is associated with an increased prevalence of arterial hypertension in humans and experimental animals. 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. In animal and human studies, the acute administration of ethanol results in vasoconstriction of most vascular beds. However, chronic ethanol feeding to rats results in the early development of tolerance to the acute vasoconstrictive effect of ethanol that is believed to be endothelium dependent. In addition, studies in ethanol-fed rats have demonstrated augmented relaxation of vascular rings to acetylcholine and adenosine 5'-triphosphate (ATP).

In 1980, Furchgott and Zawadzki 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. Nitric oxide is synthesized by nitric oxide synthase (NOS) from L-arginine in a reaction that produces stoichiometric amounts of L-citrulline. The constitutive endothelial isoform of NOS is calcium/calmodulin dependent and inhibited by Nω-nitro-L-arginine (NOARG). 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
Dowex (100–200 mesh) was purchased from Bio-Rad, Melville, N.Y.

**Cell Cultures**

Bovine pulmonary artery endothelial cells (BPAE) (passages 18–22) were maintained in RPMI-1640 media containing 20% fetal bovine serum at 37°C in a 5% CO₂–95% air incubator. In preparation for experiments, cells were grown to confluence in 12-well plates and placed in MEM containing 0.5% fetal bovine serum, 0.5% bovine serum albumin, and 725 μmol/L L-arginine for 24 to 96 hours. In ethanol-treated cells, absolute ethanol was diluted and added to the medium to achieve desired concentrations. Cell culture plates were wrapped in parafilm and the medium replaced daily to maintain a stable ethanol concentration. Ethanol concentration in the medium was determined at the end of the period of exposure to cells.

**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 1.40, 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 HClO. After neutralization of the tissue extract with 3.0 mol/L K₂CO₃, 50-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/calcmodulin-dependent isoform of the 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/calcmodulin-dependent endothelial NOS. In addition, NOARG (300 μmol/L), 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 EC50 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 ethanol6-7 and augmented relaxation to acetylcholine and ATP.10 The mechanism of these changes is unknown but appears to involve changes in EDRF production.8-9 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/calcmodulin-dependent isoform of...
FIGURE 4. Bar graphs show effects of Nω-nitro-L-arginine (NOARG) (300 μmol/L) and removal of calcium on adenosine 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.

In vitro, acute ethanol exposure results in vasoconstriction in most vascular beds except in the mesentery where it causes vasodilation, 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 diffusable 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 phenylephrine has been demonstrated in rats fed ethanol for 6 to 18 weeks. Interestingly, after 22 weeks of ethanol feeding the tolerance to phenylephrine 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 β-nicotinamide adenine dinucleotide phosphate, flavin adenine dinucleotide, tetrahydrobiopterin, or calmodulin, 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,30 brain,5 and hepatocytes,22 possibly through an increase in calcium influx via voltage-dependent calcium channels, or through inhibition of plasma membrane Ca²⁺/Mg²⁺-ATPase.21 In addition, ethanol has been shown in isolated hepatocytes23 and human platelets24 to activate hormone-sensitive phospholipase C and GTP-dependent proteins and to increase inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃],22 although voltage-dependent calcium channels have not been conclusively demonstrated in endothelial cells.25,26 Recent studies have shown that inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P₄], which is formed from Ins(1,4,5)P₃, can activate receptor-mediated calcium channels in these cells.27 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 to 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 vasorelaxation 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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References


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