



Original Contribution

Evidence of cardiovascular protection by moderate alcohol: Role of nitric oxide

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Abstract

Epidemiological evidence indicates that moderate alcohol consumption reduces the incidence of heart disease. Endothelial nitric oxide synthase (eNOS) is a key regulator of vascular homeostasis and myocardial functions through the controlled production of nitric oxide (*NO). These studies were conducted to determine if the apparent alcohol-associated cardioprotection is mediated, in part, through modulation of the eNOS protein and activity in the cardiovascular system. Rats were fed alcohol and eNOS protein and *NO production were evaluated at the end of 8 weeks. Myocardial and vascular function was assessed *ex vivo* in a subset of animals. Moderate alcohol improved postischemic myocardial systolic and diastolic function and attenuated the postischemic reduction in coronary vascular resistance. Moderate alcohol also enhanced maximum vascular relaxation by $26 \pm 0.2\%$ and increased plasma *NO production concomitant with a greater than 2.5-fold increase in eNOS protein. Higher levels of alcohol impaired maximum vascular relaxation by $22 \pm 0.1\%$. These results suggest that moderate alcohol improves postischemic myocardial functions and increases *NO production by vascular endothelium. An increase in *NO may explain, at least in part, the cardioprotective benefits of moderate alcohol consumption.

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Introduction

Coronary heart disease (CHD) remains the leading cause of death in both men and women in the United States [24]. In patients with cardiovascular risk factors such as hypercholesterolemia, hypertension, or aging [48] endothelial dysfunction predisposes to the development of structural vascular changes [40] and may play a critical role in acute myocardial infarction (MI) and sudden death. Heavy alcohol consumption has long been associated with vascular as well

Abbreviations: CHD, coronary heart disease; MI, myocardial infarction; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; nNOS, neuronal NOS; iNOS, inducible NOS; ACh, acetylcholine; EC, endothelial cell; HRP, horseradish peroxidase.

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as myocardial complications including hemorrhagic stroke, hypertension, cardiomyopathies, arrhythmias, and coronary heart disease [24]. Paradoxically, several epidemiological studies suggest an inverse association between long-term moderate alcohol consumption and the risk of CHD and MI [44]. Moderate alcohol consumption was also shown to reduce all causes of mortality by almost one-third [14]. Peripheral artery disease (PAD) shares many pathophysiological features with coronary and cerebral atherosclerosis, and similar to CHD, displays an inverse association between moderate alcohol consumption and the risk of vascular complications [10].

The cardioprotection associated with moderate alcohol consumption could be attributed to the effects of low-dose alcohol on various aspects of cardiovascular functions. Moderate alcohol was shown to increase high-density lipoproteins [29], decrease platelet aggregation [38], enhance fibrinolytic activity through the upregulation of tissue plasminogen activator [7], decrease fibrinogen [1,39], and decrease ischemia–reperfusion injury [30]. The antiatherogenic effects of alcohol have been attributed, at least in part, to the antioxidant effects of ethanol and in particular the scavenging of superoxide anion [19]. However, recent data suggest that ethanol does not significantly scavenge superoxide nor increase $\cdot\text{NO}$ through altered reaction of $\cdot\text{NO}$ with superoxide [20]. Despite a growing literature about the effects of alcohol, the detailed molecular mechanisms of the cardiovascular protection remain elusive [8,33].

Nitric oxide produced in the endothelium is a key regulator of vascular homeostasis, including basal vascular tone (blood flow) and blood pressure [35,37]. Nitric oxide also inhibits smooth muscle proliferation, platelet aggregation, and monocyte adhesion, making it an overall anti-thrombogenic agent [26]. Nitric oxide is generated by the action of three isoforms of nitric oxide synthase (NOS). Of these, endothelial NOS is the constitutive form existing primarily in vascular endothelium. Cardiac myocytes also constitutively express eNOS, which contributes to the regulation of myocardial contractility, heart rate [25], and cardiac oxygen consumption [28]. Cardiac eNOS can be activated in both atrial and ventricular myocytes by various stimuli [15] and has been postulated to play a protective role in both congestive heart failure [23] and myocardial ischemia–reperfusion [21,43]. It was the purpose of the present studies to investigate the role of chronic consumption of moderate alcohol in the upregulation of eNOS protein and its effect on vascular and postischemic myocardial function.

In this series of studies, we demonstrate that moderate alcohol consumption enhanced postischemic myocardial systolic and diastolic function as well as attenuated the ischemia-induced increase in coronary vascular resistance. Moderate alcohol consumption also increased the expression of eNOS protein in the vasculature and $\cdot\text{NO}$ metabolites in the blood. The increased eNOS is consistent with the observed improvement in acetylcholine-stimulated vascular relaxation. It is postulated that an increased level of $\cdot\text{NO}$ associated with

upregulation of eNOS protein could account for the enhanced postischemic myocardial function and vascular relaxation thereby implicating $\cdot\text{NO}$ in the cardiovascular protection associated with moderate alcohol consumption.

Materials and methods

Biochemicals

All chemicals, unless specified, were obtained from Sigma Chemical Co. (St. Louis, MO). Polyclonal antibody against eNOS and nNOS was obtained from Transduction Laboratories (Lexington, KY). Polyclonal antibody against eNOS Ser^{1177/1179} was obtained from Cell Signaling Technology, Inc. (Beverly, MA). Polyclonal antibody against eNOS Thr^{495/497} was purchased from Upstate Biotechnologies (Charlottesville, VA). Secondary antibodies were obtained from Amersham-Pharmacia Biotech (Piscataway, NJ).

Animal protocols

All animal protocols were approved by the Animal Review Board of the University of Alabama at Birmingham. All animals were anesthetized prior to surgical procedures with ketamine + Rompun (10 and 1.5 mg/100 g body weight, respectively). Two basic animal alcohol administration protocols were utilized: (1) a liquid Lieber-DeCarli diet and (2) standard chow with drinking water supplemented with ethanol. The oral route of alcohol administration was chosen to mimic the human mode of intake. Weight gain and water intake was recorded 3 times per week. Blood (500 μl) was collected from all experimental animals upon anesthesia before the surgeries and stored at -80°C in a freezer.

Liquid Lieber-DeCarli diet

In a paired feeding paradigm, male Sprague-Dawley rats (225–250 g) consumed an ethanol-containing or equicaloric liquid diet in which ethanol comprised 0, 9, or 18% of total calories for 8 weeks. These diets were prepared from a standard Lieber-DeCarli Diet (Dyets, Inc, Bethlehem, PA) that provides $\sim 36\%$ of the total calories of the animal diet as ethanol. The liquid diet contains 1.0 kcal/ml, 35% of which are fat derived, 47% are derived from carbohydrate, and 18% are derived from protein and is one of the most commonly used diets for alcohol research. The standard diet was mixed with a control diet in which maltose dextrin was substituted for the ethanol in the ratio of 1:1 (18%) or 1:3 (9%).

Water supplementation with alcohol

As a control for the effects of the nonalcoholic components of the liquid diet, some rats were fed a standard laboratory chow with regular water or water supplemented with 7.5% (v/v) ethanol for 8 weeks. For these studies, a modified pair-fed feeding paradigm was designed in which the solid food was weighed and the control animal intake of

solid food was adjusted to that of the treated rat. All experimental protocols included age-paired rats. Ethanol was slowly introduced into the diet over a 10-day period.

Assessment of myocardial function

The protocol used for heart perfusions is as previously described [9,13]. Briefly, hearts from anesthetized rats fed the Lieber DeCarli diet for 8 weeks and controls were quickly extirpated, immersed in ice-cold Krebs-Henseleit buffer, trimmed, and weighed. The aorta was then isolated and cannulated with Teflon tubing, and retrograde perfusion was begun with oxygenated, warmed (37°C) buffer, gradually building up perfusion pressure to 100 mm Hg. A latex balloon was then inserted and anchored in the left ventricle. Water was incrementally added to the balloon via a microliter syringe to increase diastolic pressure to 8–10 mm Hg. Hearts were paced at 420 beats/min with platinum electrodes. After a 20-min stabilization, initial function was determined by recording systolic and diastolic pressures, coronary flow rate, and perfusion pressure at the baseline balloon volume. To characterize left ventricular diastolic function, the end-diastolic pressure was measured following stepwise additions of volume to the balloon (0–20 μ l in 5- μ l increments). Increasing the balloon volume stretched the sarcomeres, resulting in greater force production, as in the Starling curve. As the balloon was inflated, diastolic pressure also increased. The “stiffer” the ventricle (less compliant), the greater the rise in diastolic pressure with balloon inflation. Therefore, diastolic dysfunction was manifested as increased myocardial stiffness or decreased compliance. The balloon was then deflated to baseline volume. Pacing was stopped and global ischemia induced by cessation of perfusion while temperature was maintained. Following 22 min of ischemia, reperfusion was initiated by slowly increasing perfusion pressure to 100 mm Hg over a period of 5 min. After a 30-min reperfusion period, postischemic myocardial function was determined in a manner similar to that described for the preischemic myocardium, allowing paired comparisons within the same heart. The end-diastolic pressure was plotted versus balloon volume with the slope of the line being determined by a regression analysis. The slope has been previously defined as the myocardial stiffness constant and has been equated to the degree of myocardial injury [2,3]. Coronary vascular resistance in this constant pressure system was calculated from the relationship between flow and pressure (expressed as mm Hg*min*ml⁻¹).

Assessment of vascular function

The assessment of vascular function was conducted as previously described [42]. The aortas from rats fed the Lieber-DeCarli diet for 8 weeks and their controls were excised, cut into individual ring segments (2–3 mm width), and suspended from a force-displacement transducer in a water-jacketed tissue bath containing bicarbonate-buffered,

Krebs-Henseleit buffer. Isometric tension was then measured in isolated aortic ring segments of control and ethanol-consuming rats as described [42]. Briefly, a passive load of 2 g was applied to all ring segments and maintained throughout the experiment. At the beginning of each experiment, indomethacin-treated ring segments were depolarized with potassium chloride (KCl, 70 mM) to determine maximal contractile capacity of the vessel. Rings were then thoroughly washed and allowed to equilibrate. Vessels were contracted to 40% of their maximal capacity (40% of KCl response) with phenylephrine (PE, $\sim 3 \times 10^{-8}$ M). When tension development reached a plateau, acetylcholine (ACh, 10^{-9} to 3×10^{-6} M) was added cumulatively to the bath to stimulate endothelial cell (EC) *NO formation via the mobilization of intracellular Ca²⁺, which activated eNOS and invoked EC-dependent relaxation. Dose response profiles for the different experimental treatments were analyzed and tested to determine differences in EC₅₀ and maximum relaxation responses (R_{max}). In a subset of animals, sodium nitroprusside (SNP, 10^{-9} to 3×10^{-6} M) was added to elicit EC-independent relaxation and to ensure that the alterations in the response of the vessel segments to *NO was not a result of mechanical dysfunction. Real time data were collected for all experiments and downloaded to an IBM PC for later analysis using Workbench for Windows (v.3) software.

Detection and quantification of eNOS protein

To determine if chronic moderate alcohol consumption increased eNOS protein expression, rats were supplemented with alcohol (7.5% v/v) in the drinking water for 8 weeks. Thoracic aorta from treated and control rats were either (a) fixed in situ by perfusion with 10% formalin for subsequent immunolocalization of eNOS protein, or (b) snap-frozen in liquid nitrogen and stored at –80°C until analysis by Western blot analysis.

Immunohistochemistry

The thoracic aorta was perfusion-fixed and paraffin-embedded as described in detail for in situ hybridization. The paraffin blocks were sectioned (5 μ m) for immunolocalization of eNOS protein using a specific polyclonal anti-eNOS (1:75 dilution) antibody. After several washes with PBS, a second horseradish peroxidase (HRP)-labeled goat, anti-rabbit IgG antibody (100-fold dilution) was added and the resulting complex visualized by adding a chromophore solution (0.05% 3,3'-diaminobenzadine (DAB) and 0.01% H₂O₂, in 50 mM Tris, pH 7.6) as described [22]. Sections were evaluated by light microscopy by investigators (FZ, DAP) blinded to the experimental protocol.

Western blot analysis

The frozen tissue (see above) was homogenized in a lysis buffer containing 1% Triton X-100 (in TBS, pH 7.5), and then the protein denatured by boiling. Approximately 50–100 μ g

of protein of each sample was separated in a 6% SDS-polyacrylamide gel and then electroblotted onto nitrocellulose membranes. Protein transfer was routinely ensured by staining the membrane with Ponceau S. The membrane was blocked in 5% nonfat dry milk (in TBS), washed, and then incubated with a polyclonal antibody against eNOS, nNOS, or eNOS phosphorylation sites (Ser^{1177/1179} and Thr^{495/497}). A positive control (endothelial cell lysate) was used for comparison and confirmation of molecular weight. Following washing, the blot was incubated with HRP-conjugated antibody and washed extensively. Immunoreactive bands were visualized using an enhanced chemiluminescence kit (ECL) and the resulting radiographic bands quantified by densitometry using Quantity One software (BioRad Corp).

Chemiluminescent detection of nitrite and nitrate in serum samples

Serum samples from treated (7.5% alcohol, v/v, in the drinking water) and control rats were collected at the time of surgery. Measurement of total nitrate and nitrite present in samples involves reduction to ^{*}NO using a VnCl (0.4 g Vn/50 ml 2 M HCl) solution that is boiled (90°C under weak vacuum) and bubbled with helium gas. Samples are injected into the reaction chamber containing VnCl and any gas evolved from the reaction mixture is carried to the chemiluminometer (ANTEK instruments) by a stream of helium. Data are acquired using a PC (Windaq) and analyzed with commercially available software (Origin, Microcal).

Results

Effects of liquid diet and alcohol consumption on weight gain

There was no significant difference in the rate or total amount of weight gain between the control and the experimental groups fed a liquid Lieber-DeCarli diet. There also was no significant difference in weight gain or water consumption between groups fed normal diet with water \pm 7.5% alcohol. The blood alcohol level of ad libitum drinking

of 7.5% ethanol was 3.36 ± 0.33 mM or $\sim 0.02\%$. The diet with 9 or 18% of total calories as alcohol resulted in a blood alcohol of 9.0 ± 0.1 and 13.7 ± 0.1 mM, respectively.

Effects of moderate alcohol consumption on myocardial function

Moderate alcohol treatment using the Lieber-DeCarli diet providing 9% of the total calories as alcohol for 8 weeks resulted in markedly greater recovery of systolic function (developed pressure) following ischemia–reperfu-

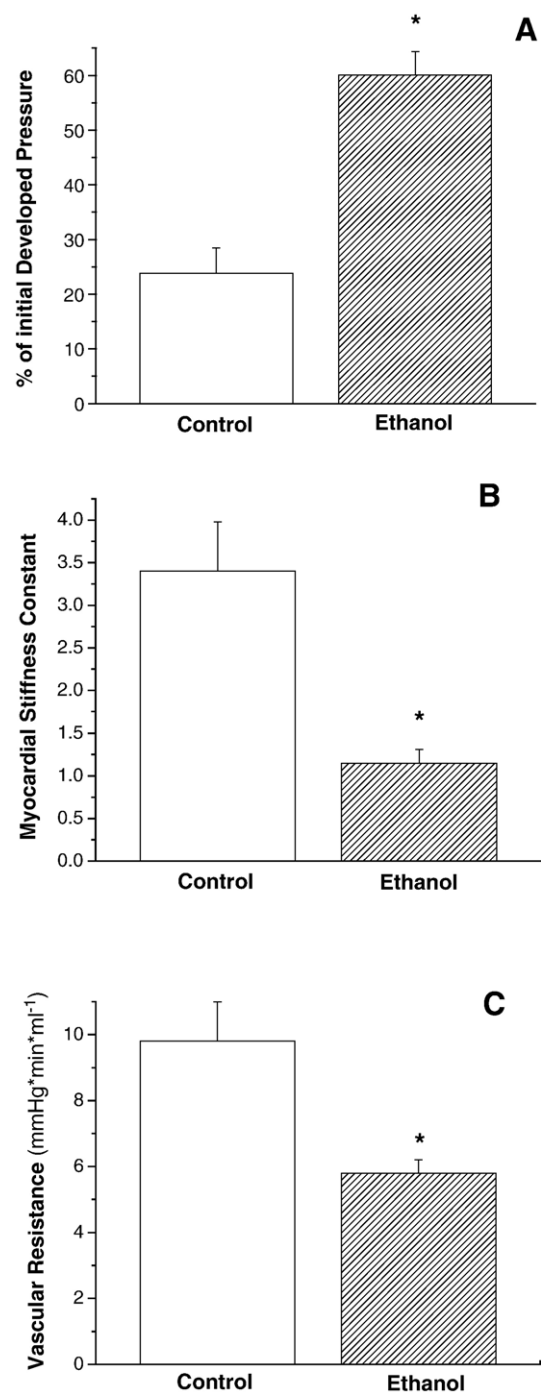


Fig. 1. Improvement of cardiac function in rat hearts from animals treated with moderate alcohol and subjected to ischemia–reperfusion. Alcohol was administered either as part of a liquid Lieber-DeCarli diet (9 or 18% of total calories) or as a supplement to the drinking water (7.5% v/v) for 8 weeks. Hearts were then isolated from the alcohol-treated and untreated control rats and then subjected to 22 min of ischemia and 30 min reperfusion. The percentage postischemic recovery of developed pressure (systolic function) was significantly greater in alcohol-treated hearts vs control hearts (A). Alcohol-treated hearts exhibited a significantly lower myocardial stiffness constant, a parameter that is derived from the slope of the regression line of the end-diastolic pressure versus volume of left ventricular balloon inflation, than hearts from control rats (B). The postischemic increase in coronary vascular resistance (C) was also significantly attenuated in hearts from alcohol-treated rats. Data are represented as means \pm SE with $n = 4–6$ (* $P < 0.05$ vs controls).

sion as compared to hearts obtained from control, untreated rats (Fig. 1A). From the results of a regression analysis of the slope of diastolic pressure vs balloon volume (myocardial stiffness constant) it was evident that the postischemic alcohol-treated hearts exhibited a significantly lower myocardial stiffness constant (were more compliant) than postischemic control hearts (Fig. 1B). While the postischemic control hearts exhibited a markedly increased myocardial stiffness constant, hearts of alcohol-consuming rats were statistically unchanged from preischemic conditions. The postischemic increase in coronary vascular resistance was attenuated by moderate alcohol (Fig. 1C), suggesting interplay between the effects of ethanol on the vasculature and the resulting positive effects on cardiac function.

Effects of moderate alcohol consumption on vascular function

Consumption of moderate levels of alcohol (9% of total calories) in a Lieber-DeCarli liquid diet for 8 weeks significantly ($P < 0.05$) increased the maximal amplitude for vascular relaxation from 93 ± 2 to $117 \pm 2\%$ of control (Figs. 2A and B). The depression in maximal relaxation ($63 \pm 1\%$ of control) in the high (18% of total calories) alcohol exposure groups reflects impairment of the vascular response to *NO . The vascular response to exogenous *NO (sodium nitroprusside) was similar in vessels from control animals and vessels from animals consuming the highest levels of alcohol in the liquid Lieber-DeCarli diet (18%), indicating that the response was not the result of changes in vascular smooth muscle responsiveness (“mechanical dysfunction”) (Fig. 2C).

To determine if components of the liquid diet had any effect on vascular relaxation, additional control studies were conducted in which moderate levels of alcohol were administered in the drinking water (7.5%). Ethanol supplementation of the drinking water resulted in a significant enhancement in maximal relaxation ($118 \pm 1\%$ of control) and was not significantly different from the vascular response exhibited with alcohol in the Lieber-DeCarli diet (Figs. 2A and B). These results are consistent with previous reports indicating that there were no morphologic differences

between alcohol supplementation in water or liquid diets [30,41]. The vascular response to exogenous *NO (sodium nitroprusside) in vessels from control animals was not statistically different from vessels from animals consuming alcohol-supplemented drinking water or alcohol in the liquid Lieber-DeCarli diet (Fig. 2C). In subsequent studies, moderate alcohol was administered only in the water to minimize any potential effect of the diet. These studies indicate that moderate alcohol consumption enhances vascular relaxation in response to an agonist that stimulates endothelial cell *NO formation, consistent with vascular protection.

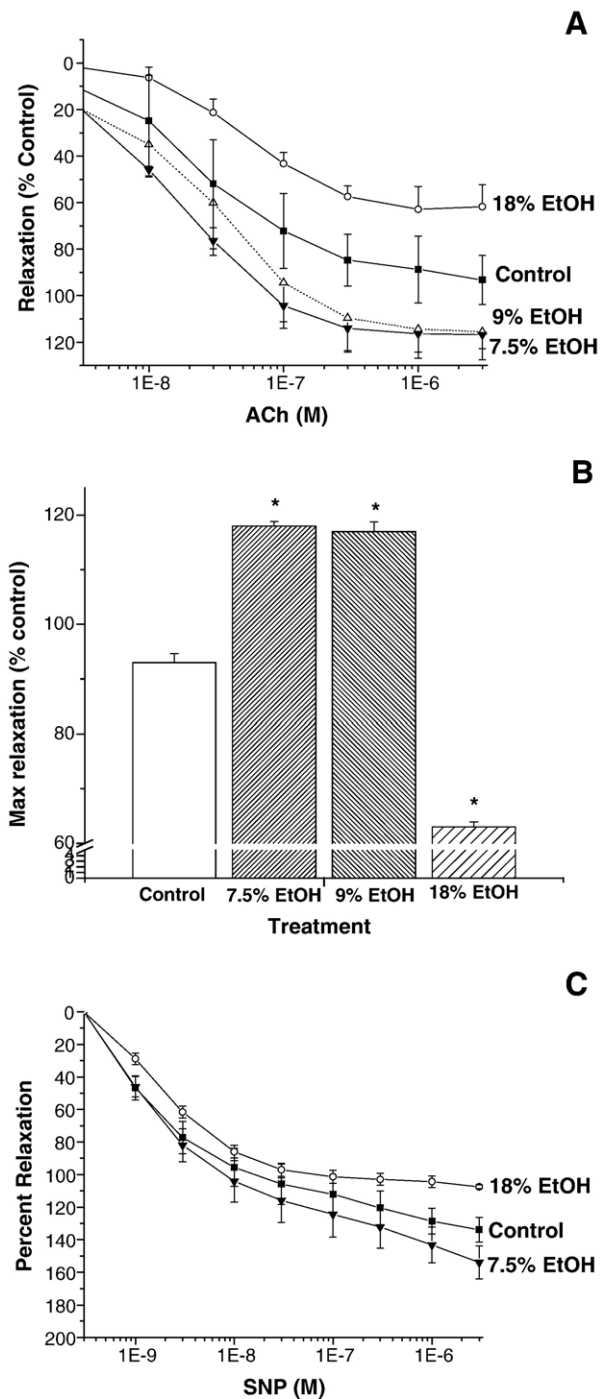


Fig. 2. Enhancement of ACh-induced vascular relaxation by moderate alcohol consumption. Consumption of moderate levels of alcohol in a liquid Lieber-DeCarli diet (9% of total calories) for 8 weeks resulted in augmented ACh-induced vasorelaxation of the thoracic aorta while higher levels of alcohol consumption (18% of total calories) resulted in impairment of vascular relaxation (A). Consumption of alcohol (7.5% v/v) in the drinking water for 8 weeks also resulted in augmented vasorelaxation. The maximum amplitude for relaxation (B) was significantly increased in both the 7.5 and 9% alcohol groups while higher doses of alcohol (18%) significantly decreased maximum relaxation compared to control vessels (B). Addition of 10^{-9} to 3×10^{-6} M sodium nitroprusside, a *NO donor, demonstrated that the response to exogenous *NO was not significant between control vessels and vessels from animals consuming the highest levels of alcohol in the liquid Lieber-DeCarli diet (18%) (C). Data are represented as the mean \pm SE with $n = 6$ ($*P < 0.05$ vs control).

Effects of moderate alcohol consumption on eNOS protein expression in the thoracic aorta

Immunolocalization with specific anti-eNOS antibody revealed that, as anticipated, eNOS was localized predominantly in the endothelium in control rats fed standard laboratory chow and regular drinking water (Fig. 3A). Consumption of water supplemented with moderate alcohol (7.5%) for 8 weeks resulted in markedly increased eNOS protein expression in the vascular endothelium of the thoracic aorta (Fig. 3A). Surprisingly, moderate ethanol consumption (8 weeks) resulted in a very pronounced increase in eNOS immunoreactivity in the smooth muscle cells of the tunica media (Fig. 3B). The effect of moderate alcohol consumption on expression of eNOS protein was confirmed by Western blot analysis using the identical anti-eNOS polyclonal antibody (Fig. 4A). Alcohol significantly increased eNOS protein greater than 2.5-fold compared to vessels from untreated controls (Fig. 4B). The eNOS antibody did not cross-react with either purified nNOS or iNOS protein (data not shown) and cross-reacting protein resulted in the proper MW for eNOS (140 kDa).

Phosphorylation of eNOS at Ser^{1177/1179} or dephosphorylation at Thr^{495/497} has been demonstrated to

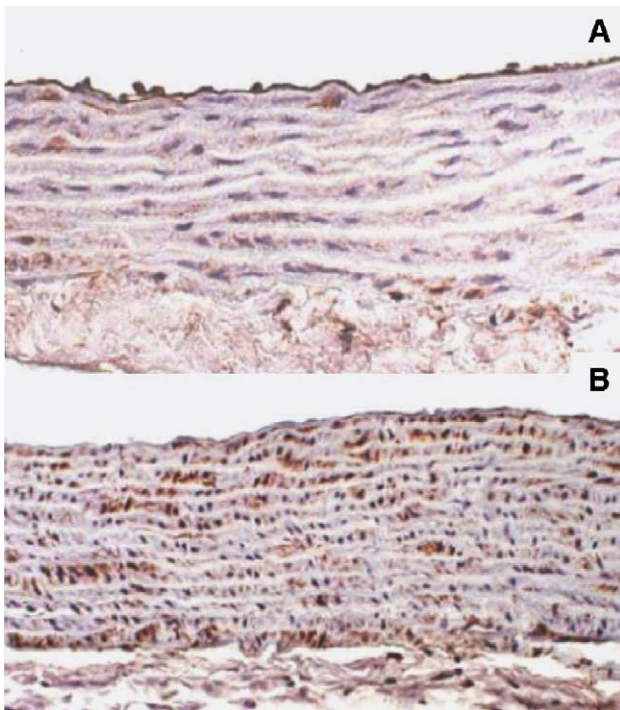


Fig. 3. Increased eNOS protein in thoracic aorta of alcohol-treated rats. Rats ($n = 12$) were fed a standard chow with water or water supplemented with 7.5% (v/v) ethanol for 8 weeks after which the aortas were perfusion-fixed in situ with 10% formalin. In pair-fed control rats (normal aorta, A) eNOS immunoreactivity (immunoperoxidase, brown color) was localized almost exclusively at the endothelial cell surface. The consumption of moderate alcohol dramatically increased immunoreactivity at the endothelial cell surface as well as in the vascular smooth muscle (B) compared to pair-fed controls (A).

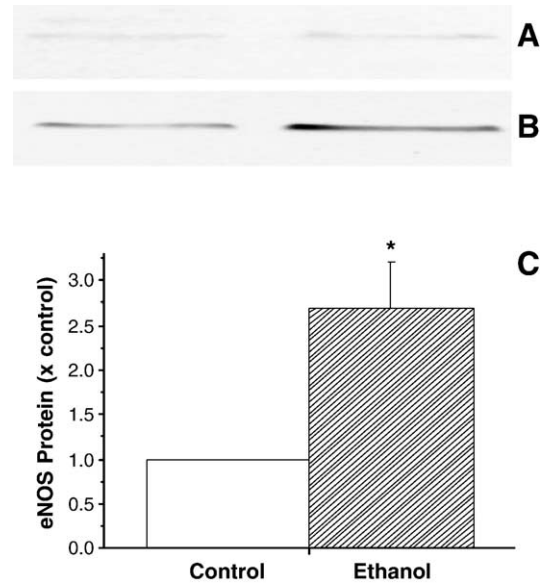


Fig. 4. Moderate alcohol increased eNOS protein in the aorta. Rats were fed 7.5% alcohol in the drinking water for 8 weeks. The thoracic aorta was perfused and immediately frozen for subsequent detection of eNOS total protein and phosphorylation of eNOS at Ser^{1177/1179}. The representative Western blots indicate that there was no significant effect of alcohol on posttranslational modification of eNOS (A) while total eNOS protein was markedly increased by alcohol consumption (B). The intensity of eNOS protein was determined digitally (C). Moderate alcohol (7.5%) consumption resulted in greater than 2.5-fold increase in eNOS protein. Data are represented as the mean \pm SE with $n = 6$ (* $P < 0.05$ vs control).

increase \cdot NO formation without a concomitant increase in eNOS protein [16]. There was a strong band with positive control (endothelial cell lysates) but no detectable differences in phosphorylation of eNOS at Ser^{1177/1179} in vessels from control or ethanol-treated rats (Fig. 4A). There was also no change in Thr^{495/497} phosphorylation in vessels from ethanol-treated rats (data not shown). These data indicate that 8 weeks of alcohol consumption does not affect eNOS phosphorylation (Ser^{1177/1179}) or

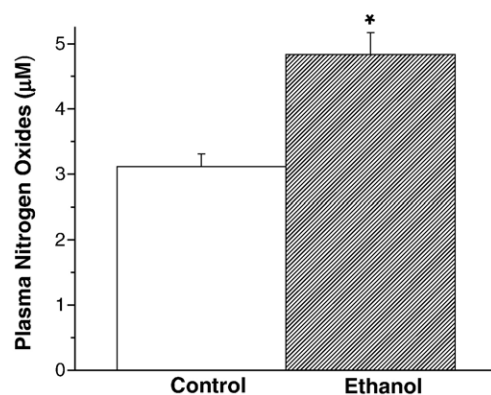


Fig. 5. Increased plasma nitric oxide in alcohol-treated rats by chemiluminescence. Rats were fed 7.5% alcohol in the drinking water for 8 weeks. Plasma samples were obtained prior to assessment of myocardial or vascular function for assessment of nitrogen oxides. Ethanol resulted in a significant increase in plasma NO_x, compared to plasma from untreated rats. Data are means \pm SE with $n = 3$ (* $P < 0.01$ vs control).

dephosphorylation (Thr^{495/497}), suggesting that the increased NO production is likely a result of increased eNOS protein.

Effects of moderate alcohol consumption on plasma nitric oxide levels

Rats were provided standard diet and water or water supplemented with moderate alcohol (7.5%) for 8 weeks as described. Blood was obtained at time of surgery and total nitrates and nitrites (NO_x) were determined using ANTEK instruments. Moderate alcohol consumption for 8 weeks significantly increased ($P < 0.05$) NO_x from 3.1 ± 0.2 to 4.8 ± 0.3 μM (Fig. 5). These data suggest an elevation of *NO levels in the blood of alcohol-treated rats consistent with an increase in functional eNOS protein.

Discussion

In the present study, we provide evidence that moderate alcohol consumption (equivalent to 1–2 glasses of wine on a per kg basis) for 8 weeks improved the recovery of systolic and diastolic function as well as attenuated the increase in coronary vascular resistance associated with myocardial ischemia–reperfusion. Expression of constitutive eNOS protein was markedly increased at the endothelial cell surface and as well as in the smooth muscle of the thoracic aorta following consumption of moderate alcohol for 8 weeks as shown by immunohistochemistry. Western blot analysis quantified the increase in eNOS protein to be greater than 2.5-fold as compared to controls ($P < 0.05$). There was no significant increase in the extent of eNOS phosphorylation at Ser^{1177/1179} or dephosphorylation at Thr^{495/497}, suggesting that the increase *NO was due to changes in eNOS protein. Total plasma NO_x increased significantly from 3.1 ± 0.2 to 4.8 ± 0.3 μM following 8 weeks of supplementation of the water with ethanol, as determined by chemiluminescence. These data suggest that the increased level of *NO may be responsible for the enhanced vascular relaxation and improved postischemic myocardial function, implicating *NO in the cardiovascular protection associated with moderate alcohol consumption.

Nitric oxide is produced from arginine and oxygen in a variety of mammalian cell types by three distinct NOS isozymes [34,35]; two constitutively transcribed forms: endothelial (eNOS) and neuronal (nNOS), and an inducible form (iNOS). Endothelial NOS is the isoform primarily localized in the endothelial layer of the vasculature and cardiac myocytes. Nitric oxide produced is a key regulator of vascular homeostasis, including basal vascular tone (blood flow) and blood pressure [35,37]. Nitric oxide also inhibits smooth muscle proliferation, platelet aggregation, and monocyte adhesion [26].

Decreased eNOS protein and inadequate *NO production is an early and persistent feature of arteriosclerosis and other

vascular injuries [11]. It was recently demonstrated that eNOS is downregulated in the peripheral vasculature (conduit and resistance arteries) following myocardial infarction (MI) [17]. Decreased *NO production due to downregulated eNOS could result in impaired vascular function, vasoconstriction, platelet aggregation, smooth muscle cell proliferation, and leukocyte adhesion [6]. The pivotal role of eNOS in cardiovascular homeostasis is illustrated by the observation that adenoviral transduction of eNOS can improve the vascular dysfunction that is associated with atherosclerosis [32] and MI [17] as well as limiting thrombosis and vascular smooth muscle proliferation in angioplasty-injured vessels [47].

Endothelial NOS is induced by a variety of extracellular signals, including fluid shear stress [4,5,36,49]. There may also be changes in the distribution of eNOS in the vasculature with cardiac disease (congestive heart failure), which results in decreased eNOS in the endothelial cell and increased eNOS in the cardiac smooth muscle [12]. The observation in the present studies that expression of eNOS protein in the endothelium and vascular smooth muscle cells is increased by moderate alcohol consumption is particularly intriguing. The observed increase in eNOS immunoreactivity in the smooth muscle *in vivo* following moderate consumption of alcohol warrants further investigation. It has been reported that a moderate upregulation of eNOS may be associated with beneficial cardiovascular effects [27]. Acute exposure of coronary arteries to red wine polyphenols for 30 min has been reported to result in posttranslational modification of eNOS which could increase *NO and vascular relaxation without a concomitant increase in eNOS protein [31]. In contrast, the present study demonstrated that long-term administration of alcohol at physiologic concentrations did not result in significant posttranslational modification of eNOS.

A limited number of studies address the cardioprotection by wine and other alcoholic beverages, and even fewer studies showed the benefits of wine on eNOS protein levels and activity. Alcohol was shown to enhance basal and flow-stimulated eNOS activity by activating an inhibitory guanine nucleotide-binding protein without a concomitant increase in protein [18]. In contrast, the present studies demonstrated a greater than 2.5-fold increase in protein by Western blot. The reason for the discrepancy might be in the dosage of the alcohol used. In the present studies, the peak blood alcohol level was ~ 3 mM, well within the range of moderate alcohol consumption and considerably less than the 40 mM used in the previous study [18]. Studies with red wine have also been demonstrated to increase eNOS protein levels; however, no specific components of the red wine were implicated [46]. Studies with a red wine polyphenol, resveratrol, have also been recently demonstrated to upregulate eNOS mRNA, protein, and activity [45]. Whether the protective effects of wine are mainly due to its alcohol content or the polyphenol contents remains to be seen. Effects of alcohol and

polyphenols on eNOS could be additive, synergistic, or independent and these issues should be addressed in future studies.

The data described herein suggest that consumption of moderate levels of alcohol results in cardiovascular protection due to increased production of *NO through the upregulation of eNOS. The alcohol-dependent increase in eNOS and the associated increase in *NO may be cardioprotective through a mechanism that leads to restoration of a *NO deficit resulting from cardiovascular disease. This is analogous to the therapeutic strategies of restoring a defect in *NO by administration of *NO donors such as glyceryl trinitrate and amyl nitrite. Even though the molecular mechanism of the alcohol effects on eNOS protein is not yet clear, it will be interesting to see the level of regulation and the possible changes in signal transduction pathways that can mediate such an effect.

In summary, these data indicate that moderate alcohol induces eNOS expression in vascular endothelium and that the associated increased *NO level may be the cause of improved cardiac and vascular function after ischemia–reperfusion injury. Increased functional eNOS can explain, in part, the cardioprotective benefits of alcoholic beverage consumption.

Acknowledgments

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