Original Article

Effects of Antihypertensive Drugs on Alcohol-Induced Functional Responses of Cultured Human Endothelial Cells

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Alcohol-induced endothelial changes might contribute to an increase in blood pressure in regular alcohol consumers. Some antihypertensive drugs affect oxidative stress and endothelial function and might counteract the effects of alcohol at the cellular level. The aim of this study was to investigate *in vitro* the effects of three different types of antihypertensive agents on alcohol-induced endothelial responses and oxidative stress. Cultured human endothelial cells were exposed to increasing concentrations (1, 10, 60 μmol/L) of zofenoprilat, carvedilol, and lacidipine in the absence and in the presence of ethanol (140 mmol/L). Concentrations of endothelin (ET) and nitric oxide (NO) were measured in the culture media as markers of endothelial function, and malondialdehyde (MDA) and intracellular glutathione (GSHi) were measured as markers of oxidative stress. Exposure to alcohol increased the levels of ET, NO, and MDA, and decreased GSHi. Carvedilol and zofenoprilat were more effective than lacidipine in counteracting the effects of alcohol on ET production. Alcohol-induced NO production was enhanced by carvedilol, whereas zofenoprilat and lacidipine did not have a significant effect. The alcohol-induced increase in MDA concentrations was blunted by all three drugs, but only carvedilol restored a normal response. All three drugs increased GSHi levels, with the effect being greater for carvedilol and lacidipine than zofenoprilat. Carvedilol is more effective than zofenoprilat and lacidipine in counteracting alcohol-induced endothelial responses *in vitro* and in decreasing oxidative stress. These effects might be particularly beneficial in patients with alcohol-related hypertension. (*Hypertens Res* 2008; 31: 345–351)

***Key Words*: carvedilol, endothelium, lacidipine, oxidative stress, zofenoprilat**

Introduction

Many cross-sectional and prospective epidemiological studies have reported that regular intake of alcoholic beverages is closely associated with increased prevalence of hypertension (*1*). This association occurs in both sexes at different ages (*2*) and has been observed across various types of alcoholic beverages (*3*) in patients of different ethnicity (*4–6*). Intervention studies performed in moderate to heavy drinkers with either normal (*7*, *8*) or high (*9*, *10*) blood pressure have generally demonstrated that withdrawal of alcohol consumption exerts a significant hypotensive effect.

It is known that the vascular endothelium generates many biologically active substances that contribute to the regulation of vascular tone (*11*), and data obtained in recent years have clearly indicated that alcohol affects endothelial function (*12*). Experiments performed in animal models and cultured endothelial cells indicate that alcohol modulates both endothelin (*13*, *14*) and nitric oxide (*14*, *15*) production, an effect

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Received May 10, 2007; Accepted in revised form September 2, 2007.

that might be related to the demonstration of an impaired endothelium-dependent vasodilatation in chronic alcohol abusers (*16*). In both heavy alcohol consumers and cultured endothelial cells, we have recently demonstrated that alcohol-induced endothelial changes are associated with activation of oxidative stress (*14*), as ascertained by detection of decreased intracellular glutathione and increased malondialdehyde concentrations.

Beneficial effects on oxidative stress and endothelial function have been reported for some drugs belonging to different classes of antihypertensive agents such as zofenoprilat (*17*), carvedilol (*18*), and lacidipine (*19*). The effects of these drugs might contribute to a reduction of blood pressure and organ protection and might be particularly beneficial in patients with alcohol-related hypertension. The present study was designed to compare *in vitro* the effects of zofenopril, carvedilol, and lacidipine on alcohol-induced endothelial responses and oxidative stress.

Methods

Endothelial Cell Culture

Experiments on cultured human endothelial cells were carried out with a procedure that has been described in a previous publication, to which readers are referred for further details (*14*). Briefly, aortic endothelial cells were isolated from a segment of the human ascending aorta, characterized as described previously (*20*), and maintained as a stable cell line. Cells were originally isolated after incubation of the minced aortic tissue for 2 h in a sterile tube, in the presence of a solution containing collagenase–trypsin–chicken serum (CTC), serum containing 30 U/L collagenase (CLS-2, 287 U/mg; Worthington Biochemical Corp., Freehold, USA), 0.75 g/L trypsin (trypsin 1-300, porcine pancreas, 683 U/mg; ICN Biochemicals, Cleveland, USA), and 20 mL/L heat inactivated and dialyzed chicken serum. After incubation, fragments were centrifuged at 2,000× *g* for 5 min and resuspended in HECM (human endothelial cell medium) containing 60% of 199 medium (Sigma Chemical Co., St. Louis, USA), 40% Coon's modified HAM'S F12 (Life Technologies, Paisley, UK), 0.36 g/L glucose, 0.53 g/L $CaCl_2$, 0.049 g/L $MgCl_2$, 8% fetal calf serum (Intergen Company, Purchase, USA), 1 μg/mL insulin (Elanco, Indianapolis, USA), 10^{-8} nmol/L hydrocortisone, 5 μg/mL transferrin, 10 ng/mL glycin-L-histidyl-L-lysine acetate, 10 μg/mL somatostatin, 50 mg/mL pituitary extract (Pel Freez Biologicals, Rogers, USA), and 75 mg/mL hypothalamus extract (Pel Freez Biologicals) (*21*). The obtained cell cultures were amplified *in vitro* and characterized by staining for Factor VIII–related antigen (A082) and for CD31 (Dako Corp., Carpinteria, USA).

Five × 10^5 endothelial cells were cultured in 6 mL of HECM medium and experiments were performed with cells in a stationary state. Two days after plating, the media were supplemented with three different concentrations (1, 10, and 60 μmol/L) of zofenoprilat (the active form of zofenopril) (Menarini Ricerche S.p.A., Florence, Italy), carvedilol (Roche Pharma, Mannheim, Germany), or lacidipine (Glaxo-SmithKline, Durham, UK) in the absence or the presence of 140 mmol/L ethanol. The concentrations of zofenoprilat (*22*), carvedilol (*23*), and lacidipine (*24*) that were used in these experiments were on the same order of magnitude as the concentrations reached by administration of therapeutic doses of the same drugs. These concentrations did not significantly affect the growth of endothelial cells, and cell availability at the end of the culture was comparable in all sets of experiments. The ethanol concentration was chosen as the concentration in the middle of a dose-response curve previously obtained, in the same cells, in dose-titration experiments (*14*). In these experiments, concentrations of ethanol below 30 mmol/L did not show any significant effect on endothelial cells, whereas concentrations greater than 570 mmol/L had lethal effects on the majority of cultured cells. This concentration is of the same order of magnitude as the ethanol concentration that we previously found activated endothelial cells *in vivo* in heavy alcohol consumers (*14*). Cells were then cultured for 2 weeks, during which the media were changed every 24 h in all dishes. On day 15, the media were collected for measurement of endothelin, NO, and malondialdehyde. For this purpose, the cultured cells were collected in a Ca^{2+}- and Mg^{2+}-free Hanks' solution after exposure to a solution of CTC. The duration of cultures was chosen because previous experiments had shown that the effects of ethanol on endothelial cells increase with time and reach a plateau after approximately 2 weeks (*14*). Endothelial cell viability after culture was checked with trypan blue staining and was above 92%. Cells were counted and used to test the intracellular glutathione level.

Laboratory Measurements

After collection, the media obtained from cell culture plates were centrifuged and the supernatants were frozen at −80°C. Cultured endothelial cells were collected after centrifugation and counted using a microscopic camera, and 5 × 10^6 cells were suspended in 500 μL of a 5% (w/v) metaphosphoric acid solution. Pellets were then homogenized by means of a Teflon pestle. The assay was performed on 100-μL aliquots of supernatants obtained by centrifugation at 3,000× *g* for 8 min at 4°C, according to the manufacturer's instructions. All measurements were performed in duplicate by use of colorimetric assays (Nitric Oxide Assay Kit: Calbiochem-Novabiochem Corp., San Diego, USA; Endothelin-1 Elisa Biotrak System: Amersham Pharmacia Biotechnology, Piscataway, USA; Malondialdehyde Bioxytech LPO-586 Assay: Oxis International, Portland, USA; Glutathione-420, Wak-Chemie Medical GMBH, Steinbach/Ts, Germany).

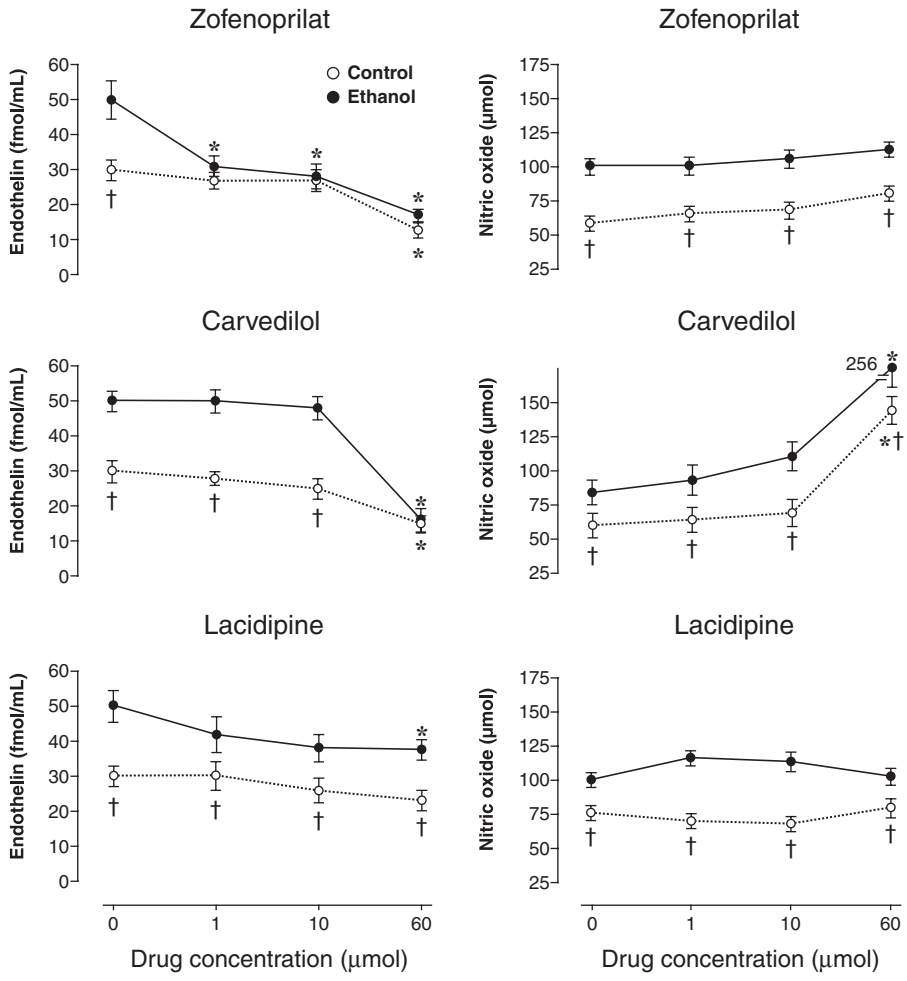

Fig. 1. *Graph showing the effects of increasing concentrations of zofenoprilat, carvedilol, and lacidipine on concentrations of endothelin (left) and nitric oxide (right) in the supernatants of human endothelial cells that were cultured for 14 days in the absence of ethanol (Control) and in the presence of 140 mmol/L ethanol (Ethanol). *$p < 0.05$ vs. absence of drug (concentration 0 µmol). †$p < 0.05$ for the ethanol-enriched vs. the ethanol-free culture condition at the respective drug concentrations.*

Statistical Analysis

Results are presented as the means ± SEM of three independent experiments. Comparisons were done by ANOVA. All tests were two-sided and p values less than 0.05 were considered statistically significant.

Results

Human aortic endothelial cells were cultured in the presence of three different concentrations of zofenoprilat, carvedilol, and lacidipine in alcohol-free media or in the presence of ethanol. Figure 1 illustrates the levels of endothelin and nitric oxide in the culture media. In the absence of antihypertensive drugs, the concentrations of both endothelin-1 ($p < 0.01$) and nitric oxide ($p < 0.01$) were significantly greater in supernatants of cells cultured in the presence of ethanol as compared to the controls. In the absence of ethanol, zofenoprilat decreased endothelin levels only at the highest concentration, whereas increasing concentrations of zofenoprilat progressively decreased endothelin levels in the supernatants of endothelial cells that were cultured in the presence of ethanol, reaching values comparable to those measured in alcohol-free conditions across the drug concentration range. Nitric oxide concentrations were not affected by zofenoprilat in either the alcohol-free or alcohol-enriched conditions, remaining significantly higher in the latter across the drug concentration range. Carvedilol significantly decreased endothelin production only at the highest concentration, reaching comparable levels in the presence and in the absence of ethanol. There was a dose-dependent relationship between carvedilol concentrations and nitric oxide production, a relationship that was more evident in the presence of ethanol, under which condition the concentration of carvedilol remained signifi-

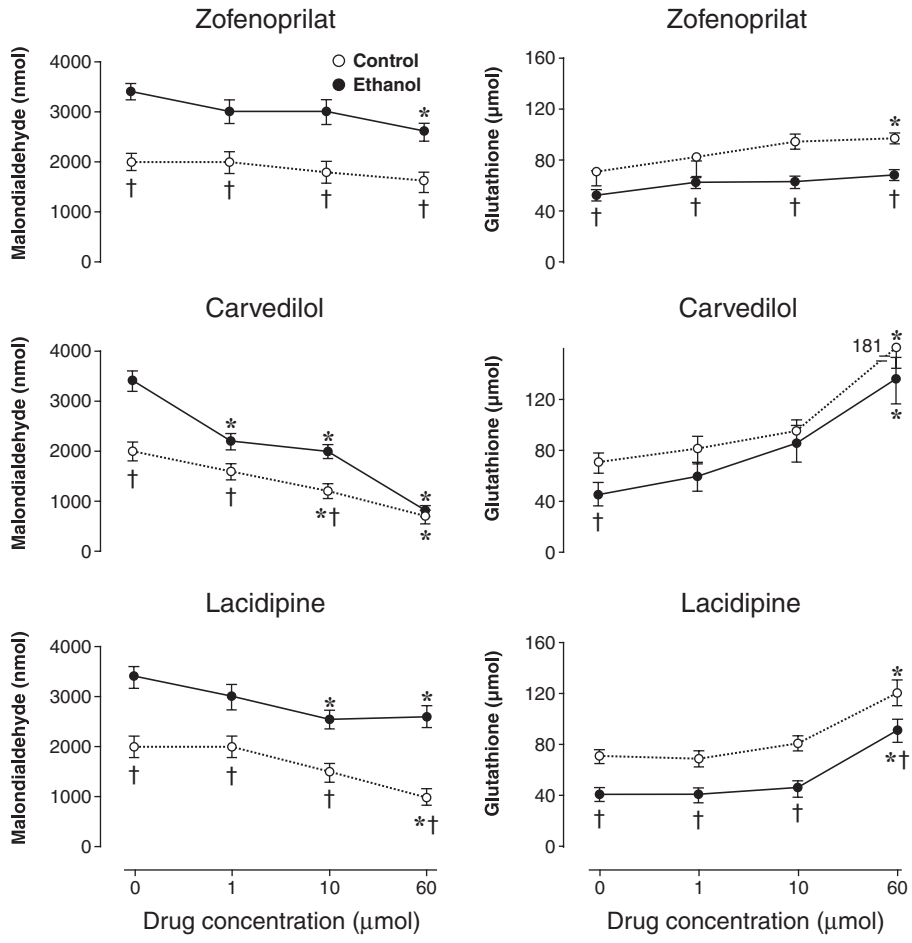

Fig. 2. *Graph showing the effects of increasing concentrations of zofenoprilat, carvedilol, and lacidipine on concentrations of malondialdehyde (left) and intracellular glutathione levels (right) in the supernatants of human endothelial cells that were cultured for 14 days in the absence of ethanol (Control) and in the presence of 140 mmol/L ethanol (Ethanol). *$p < 0.05$ vs. absence of drug (concentration 0 μmol). †$p < 0.05$ for the ethanol-enriched vs. the ethanol-free culture condition at the respective drug concentrations.*

cantly higher across the drug concentration range. Lacidipine induced a weak although significant decrease of endothelin concentrations *vs*. baseline only at the highest concentration in the presence of ethanol. The endothelin concentrations remained significantly higher in the presence of ethanol across the lacidipine concentration range. No effect on nitric oxide production was observed with this drug, and the concentrations were significantly greater in the presence of ethanol across the experiment concentrations.

To evaluate the possible association between changes in endothelial function and oxidative stress, we measured two markers of reactive oxygen species generation: malondialdehyde and intracellular glutathione concentrations (Fig. 2). In the absence of antihypertensive drugs, concentrations of malondialdehyde in the culture media were significantly greater ($p<0.01$) and intracellular glutathione levels were significantly lower ($p<0.01$) in the presence of ethanol as compared to the controls. Only at the highest dose, zofenoprilat decreased malondialdehyde production in the presence of ethanol and increased intracellular glutathione under the alcohol-free condition. Across the zofenoprilat concentration range, both malondialdehyde and intracellular glutathione remained significantly higher and significantly lower, respectively, in the presence of ethanol as compared to the alcohol-free condition. Carvedilol induced a dose-dependent decrease of malondialdehyde and dose-dependent increase of intracellular glutathione, both of which effects were more pronounced in the presence of ethanol. Lacidipine induced a dose-dependent decrease of malondialdehyde and increased the levels of intracellular glutathione at the highest dose, and these effects were comparable in the absence and in the presence of ethanol. Both malondialdehyde concentrations and intracellular glutathione remained significantly different between the presence and absence of ethanol across the lacidipine concentration range.

Discussion

We have compared the effects of three different antihypertensive agents with antioxidant properties on the biochemical response of endothelial cells that had been chronically exposed to ethanol *in vitro*. The results demonstrated that ethanol activated endothelial production of endothelin and nitric oxide, an effect that was associated with evidence of increased oxidative stress. Zofenoprilat, carvedilol, and lacidipine significantly decreased alcohol-induced endothelin production, but only the first two drugs completely reversed the effects of ethanol. Carvedilol was the only drug that further enhanced the production of nitric oxide in the presence of ethanol. All three drugs induced a dose-dependent decrease of malondialdehyde and increase in intracellular glutathione levels, indicating a significant reduction of oxidative stress. Overall, carvedilol appears to be the most effective of the three drugs tested in this study in decreasing both alcohol-induced endothelial responses and oxidative stress.

Although the link between alcohol and hypertension has been investigated extensively, a clear mechanism for the pressor effects of ethanol has not yet been established. Past studies have linked the effects of chronic alcohol consumption to activation of the sympathetic nervous system (*25*, *26*), stimulation of the renin-angiotensin-aldosterone system (*25*), activation of the hypothalamic-pituitary-adrenal axis with increased cortisol secretion (*27*), impairment of peripheral sensitivity to insulin (*28*), and increase of arteriolar tone (*29*), but none of these potential mechanisms has been conclusively demonstrated (*30*). It is known that the vascular endothelium generates many biologically active substances that contribute to the regulation of vascular tone (*11*) and, in the last decade, the study of alcohol-induced endothelial functional modifications has received much attention because the complex relationships between alcohol and blood pressure might result from opposite influences of different alcohol intakes on the vascular endothelium. Previous investigations have been unable to fully clarify these influences, and inconsistent results have been reported by studies using different means—*i.e.*, experimental models or human subjects, acute or chronic exposure, and different doses of alcohol (reviewed in Puddey *et al*. (*12*)). More recent data obtained in humans and experimental animal and cellular models have clearly indicated that the effects of alcohol on endothelial function might contribute to an increase in blood pressure in chronic alcohol consumers, and substances released by endothelial cells together with oxidative stress appear to contribute substantially to endothelial dysfunction in these subjects (*12*). We have demonstrated that normalization of blood pressure after withdrawal of alcohol consumption in hypertensive heavy drinkers is associated with a significant decrease of plasma endothelin concentrations (*10*). Consistently, demonstration of increased endothelin release has been obtained *in vitro* in cultured human endothelial cells after acute (*13*) and chronic (*14*) exposure to ethanol. Furthermore, data obtained in animal models and cultured endothelial cells indicate that alcohol increases nitric oxide bioavailability (*15*, *31*), suggesting an interplay between alcohol-induced endothelial effects and oxidative stress. In agreement with the findings of previous studies, our present results demonstrate increased generation of both endothelin and nitric oxide as a result of chronic exposure of endothelial cells to ethanol. Although these effects should translate into opposite functional responses in terms of vasomotility, they indicate an overall functional activation of endothelial cells that occurs in conjunction with oxidative stress.

Because endothelial dysfunction and oxidative stress may contribute to the pathophysiology of alcohol-related hypertension, their correction could be particularly beneficial in this context. It has been shown that some antihypertensive agents can restore or improve endothelial function depending upon their ability to counteract the underlying cellular mechanisms (*32*). Some angiotensin-converting enzyme (ACE) inhibitors improve the endothelial response in epicardial, renal, and subcutaneous vessels, but are ineffective in the forearm vessels of hypertensive patients (*32*). Zofenoprilat is a sulfhydryl-containing ACE inhibitor that has already been shown to decrease oxidative stress both *in vitro* (*17*, *33*) and *in vivo* (*34*), suggesting that this molecule has hydroxyl-radical scavenging properties. In the present study, zofenoprilat decreased the consumption of intracellular glutathione induced by ethanol and the production of malondialdehyde by endothelial cells. In addition, zofenoprilat restored to normal the ethanol-stimulated production of endothelin.

Intrinsic antioxidant properties have also been reported for carvedilol (*35*), a non-selective β-adrenergic receptor blocker with favorable effects on cardiovascular disease. This drug has been shown to prevent the depletion of endogenous antioxidants such as glutathione (*36*), increase nitric oxide release (*18*), and suppress endothelin liberation (*37*) from endothelial cells. This study shows that carvedilol can effectively counteract ethanol-induced endothelial dysfunction and oxidative stress.

Lacidipine is a calcium-channel blocker of the dihydropiridine class that has been shown to prevent endothelial dysfunction in hypertensive rats (*38*), restore endothelium-dependent vasodilation in hypertensive patients (*39*), and decrease oxidative stress (*19*). In this study, lacidipine reduced endothelin and malondialdehyde production and increased intracellular glutathione levels, although none of the effects induced by exposure of endothelial cells to ethanol were corrected.

Studies on antihypertensive drug treatment of alcohol-induced hypertension are limited because, in this context, withdrawal or significant reduction of alcohol consumption are the most effective interventions (*10*) and drug treatment may mask some of the adverse cardiovascular effects of alcohol (*40*). This is why pharmacologic treatment is commonly withheld for the first couple of weeks of abstinence (*41*).

Among the few studies that have investigated antihypertensive treatment in chronic alcoholism, some have suggested possible benefit of drugs that interfere with the endothelial function (*42*). Subsequent to demonstration of activated renin-angiotensin-system and increased vascular reactivity due to increased intracellular calcium in alcohol-related hypertension, it was suggested that ACE inhibitors or calcium-channel antagonists might be particularly effective (*41*), but this has never been demonstrated in clinical studies. Finally, old β-blockers, such as propranolol, do not seem to modify the hemodynamic response to alcohol in hypertensive patients (*43*). Thus, the overall clinical evidence favoring use of specific antihypertensive drug classes in alcohol-related hypertension is as yet very weak. Based on our *in vitro* observations, we might speculate that the new classes of β-blockers might prove particularly beneficial in this context, but this will have to be tested in appropriately designed studies.

Although the differences we observed among the drugs tested in this study could be attributed to different intracrine and autocrine effects on endothelial cells, potential limitations due to the specific aortic endothelial cell system that was used should be appropriately considered before we extend these results to other vascular beds. As already stated, different vasoregulatory systems contribute to regulation of vascular tone in different vascular systems (*32*), and therefore comparisons of drug effects in experiments such as those performed here could depend substantially on the types of cells and must be interpreted with caution. Moreover, because in our experiments we have examined the effects of three drugs that have been reported to have antioxidant properties in addition to their respective major pharmacological actions, the findings obtained here can not be extrapolated to all members of the respective classes of antihypertensive agents to which they belong.

In conclusion, zofenoprilat, carvedilol, and lacidipine were here shown to modulate functional responses and oxidative stress in cultured human aortic endothelial cells. In these cells, carvedilol was the most effective drug in counteracting the endothelial response and decreasing the oxidative stress induced by chronic exposure to ethanol. These drugs might be particularly beneficial in patients with alcohol-related hypertension, and further studies will be required to test this possibility.

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