Zofenopril Inhibits the Expression of Adhesion Molecules on Endothelial Cells By Reducing Reactive Oxygen Species

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Hypertension and coronary artery disease are intimately connected. The migration of circulating monocytes into the subendothelial occurs through the expression of some adhesion molecules on endothelial cells. The nuclear factor (NF)-κB, a redox-sensitive element, plays a key role in adhesion molecule gene induction. In this study we have compared the effects of two different angiotensin converting enzyme (ACE) inhibitors, one possessing an active sulfhydryl group (zofenopril) and one lacking this group (enalapril) on the cellular redox state (monitored by measuring intracellular reactive oxygen species and thiol status), expression of adhesion molecules, and activation of NF-κB in human umbilical vein endothelial cells (HUVECs). Zofenoprilat, the active form of zofenopril, significantly and dose dependently reduced the intracellular reactive oxygen species (ROS) and superoxide formation induced by oxidized low-density lipoprotein (ox-LDL) (P < .001) and tumor necrosis factor-α (TNF-α) (P < .001). Enalaprilat, the active form of enalapril, was ineffective. Zofenoprilat but not enalaprilat also decreased the consumption of the intracellular GSH induced by ox-LDL (P < .01) and TNF-α (P < .01). Although zofenoprilat significantly and dose dependently reduced the expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1), and E-selectin induced by ox-LDL (P < .01) and TNF-α (P < .01) on HUVECs, enalaprilat did not. Ox-LDL and TNF-α increased the activation of NF-κB and the preincubation of HUVECs with zofenoprilat, but not with enalaprilat, dose dependently reduced its activation (P < .001). The conclusion is that the sulfhydryl (SH)-containing ACE inhibitors may be useful in inhibiting foam cell formation and thus slow the development of atherosclerosis. Am J Hypertens 2002;15:891–895 © 2002 American Journal of Hypertension, Ltd.

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human umbilical vein endothelial cells (HUVECs) induced by prooxidant signals; 2) to evaluate the ability of the drugs to preserve the intracellular glutathione (GSH); and 3) to study the effect of the two drugs on the activation of the transcription factor NF-κB and on the expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1), and E-selectin induced by prooxidant signals.

**Methods**

The HUVECs were isolated and cultured as previously described. Zofenopril, zofenoprilat (the active form of zofenopril), and enalaprilat (the active form of enalapril) (courtesy of Menarini Ricerche, Firenze, Italy) were dissolved in dimethyl sulfoxide and in M-199. Increasing amounts of zofenopril (30 to 60 μmol/L), zofenoprilat (3 to 6 mmol/L), and enalaprilat (0.4 to 4.4 mmol/L) were first added to HUVEC monolayers in 2 mL of M-199 for 30 min at 37°C with the aim of measuring intracellular drug concentrations. For comparison purposes α-tocopherol (5 μmol/L) was also added to HUVECs under the experimental conditions mentioned above. Cells were harvested by careful scraping and immediately added to 2 mL of M-199, centrifuged, washed twice with phosphate buffer saline containing N-ethylmaleimide (NEM), and lysed by the addition of 800 μL of phosphate buffer (pH 5) containing NEM. The determination of zofenopril, zofenoprilat, and enalaprilat in cells was performed by liquid chromatography coupled with tandem mass spectrometry as previously described. The α-tocopherol was measured in cell extracts by liquid chromatography as previously described.

On the basis of preliminary results, zofenoprilat, enalaprilat, and α-tocopherol were then incubated to give intracellular concentrations ranging from 0.27 to 1.08 fmol/cell. The low-density lipoprotein (LDL) was isolated by sequential flotation in sodium bromide and oxidized as described. The extent of LDL oxidation procedure was as reported. Protein was measured by the Pierce bicinchoninic acid protein assay reagent.

Cell adhesion molecule expression was evaluated by flow cytometry, as previously described. The HUVEC monolayers grown to confluence in six-well plates were preincubated with increasing concentrations of zofenoprilat and enalaprilat for 30 min at 37°C. Human recombinant tumor necrosis factor-α (TNF-α) (2 ng/mL) (Sigma Chemical Co., St. Louis, MO) or oxidized LDL (ox-LDL) (100 μg protein/mL) were then incubated with HUVEC monolayers, respectively, for 6 and 8 h at 37°C.

Electrophoretic mobility shift assay (EMSA) was performed as previously described and following the method of Dignam et al. The NF-κB band in electrophoretic gel was quantified by means of a laser densitometer (LKB, Bromma, Sweden).

The ROS and superoxide were measured by using the flow cytometry methods of 2',7'-dichlorofluorescin diacetate (DCFH-DA) and of hydroethidine as previously described. Data on ROS, superoxide, and on adhesion molecules, are given as percentage variation (Δ%) ± standard deviation of mean fluorescence intensity (MFI) relative to ox-LDL alone; data on intracellular GSH are given in nanomoles per milligram of protein.

Statistical analysis was performed by analysis of variance and subsequently by post hoc analysis, using the SYSTAT program and statistical software manual (SYSTAT Inc., Evanston, IL) for Macintosh computers.

**Results**

As shown in Fig. 1, zofenoprilat but not enalaprilat significantly reduced the intracellular ROS and superoxide formation induced by ox-LDL. Similarly, after induction with TNF-α, zofenoprilat (1.08 fmol/cell) reduced intracellular ROS and superoxide concentration to 53.7% ± 5.1% and to 50.9% ± 5.4%, respectively, of the control (TNF-α alone) (P < .01). Enalaprilat was ineffective. The α-tocopherol (1.08 fmol/cell) decreased intracellular ROS and superoxide concentration to 18.7% ± 2.1% and to 22.9% ± 2.4%, respectively, of the control (ox-LDL alone) (P < .01).

In a time-course experiment, the presence of zofenoprilat but not of enalaprilat in HUVECs significantly decreased the consumption of the intracellular GSH induced...
by ox-LDL (Fig. 2). Similarly, when HUVECs were exposed to TNF-α, GSH values reduced from 48.2 ± 2.4 nmol/mg protein to 33.3 ± 2.6, 37.1 ± 2.3, and 43.4 ± 2.7 nmol/mg protein, respectively, after 1, 5, and 10 min. In the presence of zofenoprilat (1.08 fmol/cell), GSH values reduced from 48.3 ± 2.7 nmol/mg protein to 44.4 ± 3.1, 46.2 ± 2.5, and 48.3 ± 2.7 nmol/mg protein (significantly higher at any time than TNF-α alone, P < .01). In the presence of enalaprilat (1.08 fmol/cell), GSH values were never significantly different from TNF-α alone.

As shown in Fig. 3, zofenoprilat significantly and dose dependently reduced the expression of E-selectin, ICAM-1, and VCAM-1 induced by ox-LDL on HUVEC.

FIG. 3. Effect of increasing amounts of zofenoprilat (from 0.27 to 1.08 fmol/cell) on ox-LDL-induced E-selectin, ICAM-1, and VCAM-1 expression on HUVECs. The results are given as percentage variation (∆%) of mean fluorescence intensity (MFI) versus ox-LDL alone and are the mean ± SD of experiments performed in triplicate on six separate occasions. *Values differ from ox-LDL alone (P < .01); †Values differ from E-selectin and ICAM-1 (P < .01). ICAM-1 = intercellular cell adhesion molecule-1; VCAM-1 = vascular cell adhesion molecule-1; other abbreviations as in Figs. 1 and 2.

FIG. 2. Effect of zofenoprilat and enalaprilat (1.08 fmol/cell) on the time course variations of GSH in HUVECs exposed to ox-LDL. The results are given in nanomoles per milligram of protein (prot) and are the mean ± SD of experiments performed in triplicate on five separate occasions. *Values differ from control (no zofenoprilat and enalaprilat; P < .01). GSH = glutathione; other abbreviations as in Figs. 1 and 2.

ICAM-1, and VCAM-1 induced by ox-LDL on HUVEC. Similarly, at concentrations of 0.27, 0.54, and 1.08 fmol/cell, zofenoprilat significantly and dose dependently decreased the expression of E-selectin (to 82.5% ± 7.1%, 64.9% ± 6.8%, and 22.7% ± 3.8% of the control, P < .01), ICAM-1 (to 81.6% ± 7.5%, 72.8% ± 6.9%, and to 18.6% ± 2.1% of the control, P < .01) and VCAM-1 (to 73.2% ± 7.0%, 45.9% ± 4.1%, and to 2.5% ± 0.3% of the control, P < .001). The percentage in which VCAM-1 decreased was always significantly greater than ICAM-1 and E-selectin (P < .01). At the same concentrations, enalaprilat was ineffective after both ox-LDL or TNF-α stimulation. Fig. 4 shows the effect of zofenoprilat and enalaprilat at the highest concentrations on E-selectin, ICAM-1, and VCAM-1 expression stimulated by ox-LDL on HUVECs.

Finally, we examined the activation of the transcription factor NF-κB, induced by TNF-α and ox-LDL. The TNF-α and ox-LDL increased the activation of NF-κB and the preincubation of HUVECs with zofenoprilat, but not with enalaprilat dose dependently reduced its activation (Fig. 5). Densitometrically the decrease induced by zofenoprilat varied from 24% ± 6% to 64% ± 8% (P from < .01 to < .001) for TNF-α, and from 21% ± 4% to 54% ± 7% (P from < .01 to < .001) for ox-LDL.

Discussion

Angiotensin converting enzyme inhibitors are a relatively new class of cardiovascular drugs. They have become established treatments for hypertension and chronic heart failure. Recently, some of these drugs have also been reported to attenuate reperfusion-induced myocardial damage.22 The exact mechanism of cell toxicity after reperfusion is not known but it is suggested that ROS22 and reduction of intracellular GSH concentration23 may play a crucial role. In this context there is agreement that
only the SH-containing ACE agents are capable of protecting cells against free radicals\(^9,11\) and ischemic stress.\(^{24}\)

In this study we showed that the preincubation of HUVECs with zofenoprilat and enalaprilat determined a dose-dependent increase in the intracellular concentrations of the two drugs. Zofenoprilat, because of its high lipophilia\(^8\) and the efficient mechanism of its conversion,\(^{25}\) was found at concentrations 30 times higher inside the cells than enalaprilat. When exposed to similar concentrations zofenoprilat only, however, prevented in a dose-dependent manner the intracellular increase in ROS and superoxide concentration induced by ox-LDL and TNF-\(\alpha\). It is likely that this effect is related to the antioxidant activity of zofenoprilat. In this context it has been demonstrated that the protective effects of the SH agents correlate with their direct radical scavenging ability.\(^{26}\) In particular, those with a thiol or SH group are supposed to behave like the endogenous free radical scavenger GSH.\(^{22,26,27}\) The results of this study are in agreement with this conclusion, as zofenoprilat, but not enalaprilat, an ACE inhibitor lacking the SH group, significantly decreased the consumption of the intracellular GSH induced by prooxidant stimuli, suggesting that the drug cooperates in the cellular defense against free radicals. Similar findings were demonstrated for captopril\(^{24}\) and other SH agents, including GSH,\(^{23}\) but not for enalaprilat,\(^{24}\) in protecting cells against ischemia-induced injury.

There is still dispute over the type of free radicals scavenged by SH-containing ACE inhibitors.\(^9,22\) In our study we demonstrated that zofenoprilat not only reduces the intracellular concentration of general free radicals as evaluated with DCFH-DA,\(^5,28\) but also significantly counteracts the presence of superoxide as measured very specifically with hydroethidine.\(^{20,28}\) This specificity of action may have implications in vivo because oxidative inactivation of nitric oxide by superoxide is regarded as an important cause of its decreased biologic activity,\(^{29}\) which contributes to the impairment of endothelium-dependent relaxation.\(^{30}\) Actually, Creager and Roddy\(^{31}\) failed to demonstrate an efficacy of captopril, the prototype of SH-containing ACE inhibitors, on endothelium-dependent vasodilation in essential hypertensive patients. Captopril, however, was demonstrated to preferentially scavenge general free radicals with the same potency of zofenoprilat.\(^{32}\) This lack of efficacy on superoxide may at least in part explain the negative results on endothelium-dependent vasodilation.

In this study we also showed that zofenoprilat, but not enalaprilat, dose dependently reduced the expression of E-selectin, ICAM-1, and VCAM-1 \((P < .01)\) and NF-\(\kappa B\) activation induced by ox-LDL and TNF-\(\alpha\) on HUVECs. Our results agree with those of Gonzalez et al\(^{13}\) who demonstrated that zofenoprilat reduced monocyte adherence to endothelium, and NF-\(\kappa B\) activation in the aortas of nitro-L-arginine methyl ester-treated rats. Because the increase in the production of ROS and superoxide is a common pathway for a variety of NF-\(\kappa B\) inducers,\(^4\) the results of this study suggest that the effect of zofenoprilat on adhesion molecule expression and on NF-\(\kappa B\) activation induced by TNF-\(\alpha\) and oxidized LDL can be attributed to its antioxidant activity. The fact that zofenoprilat exerted a preferential effect on VCAM-1 expression agrees with this suggestion, as it is now well established that only the activation of endothelial cell VCAM-1 gene expression is regulated by a signal transduction mechanism fully sensitive to inhibition by antioxidants.\(^{34}\) This conclusion is supported by the finding that enalaprilat was ineffective on NF-\(\kappa B\) activation and adhesion molecule expression. Because low thiol levels promote NF-\(\kappa B\) activation,\(^{6,35}\) and in this study we also showed that zofenoprilat, but not enalaprilat, reduces GSH consumption induced by oxidative stimuli, another conclusion of this study is that the effect exerted by this new SH-containing ACE inhibitor on NF-\(\kappa B\) activation and, therefore, on adhesion molecule expression, may also be mediated by its effect on intracellular GSH concentration.

Because hypertension and coronary artery disease are intimately connected, and because monocyte recruitment into the vascular wall after adhesion to endothelial cells is a crucial step in the pathogenesis of atherosclerosis,\(^3\) the results of this study demonstrate that the SH-containing ACE inhibitor zofenoprilat, at a concentration comparable to the maximal concentration found in healthy volunteers circulation,\(^{15}\) may be useful in inhibiting foam cell formation and thus slow the development of atherosclerosis. This conclusion is limited by the fact that at present no data support a clinical superiority of ACE inhibitors possessing an active SH group as compared to ACE inhibitors lacking this group.
References


