Sulfhydryl angiotensin-converting enzyme inhibition induces sustained reduction of systemic oxidative stress and improves the nitric oxide pathway in patients with essential hypertension

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Background Essential hypertension is associated with enhanced LDL oxidation and impaired endothelium-dependent vasodilation. The antioxidant status is linked to the nitric oxide (NO) pathway. Sulfhydryl angiotensin-converting enzyme (ACE) inhibitors inhibit oxidative stress and atherogenesis in experimental models; therefore we tested whether this beneficial antioxidant activity could also be clinically relevant in patients with essential hypertension.

Methods Plasma LDL oxidizability was investigated initially in untreated normocholesterolemic patients with moderate essential hypertension without clinically evident target organ damage (n = 96) and in control normotensive subjects (n = 46). Patients were then randomly assigned into two age- and sex-matched groups to receive the new sulfhydryl ACE inhibitor zofenopril (15 to 30 mg/d; n = 48) or enalapril (20 mg/d, n = 48). LDL oxidizability was evaluated (generation of malondialdehyde, MDA) and systemic oxidative stress was evaluated by isoprostanes (8-isoPGF2α). Asymmetrical dimethyl-L-arginine (ADMA), a competitive inhibitor of endothelial NO synthase, and plasma nitrite and nitrates (NOx) were also measured.

Results LDL from hypertensive subjects had enhanced susceptibility to oxidation in vitro compared with that in control subjects (P < .05). Similarly, isoprostanes were significantly increased (P < .01) in hypertensive subjects versus control subjects. After 12-week treatment, MDA levels were significantly reduced by zofenopril (P < .05) but not enalapril treatment (P = not significant). Isoprostanes were normalized after zofenopril treatment (P < .03), whereas enalapril was ineffective. After treatment with both ACE inhibitors, plasma NOx concentrations were significantly reduced (P < .05). Similarly, hypertension increased ADMA concentration compared with the normotensive state, whereas ACE inhibition elicited a significant decrease. However, the reduction of ADMA concentration was significantly higher in patients receiving sulfhydryl ACE inhibition (P < .05 vs enalapril).

Conclusions The sulfhydryl ACE inhibitor zofenopril reduces oxidative stress and improves the NO pathway in patients with essential hypertension. If confirmed in a large multicenter clinical trial, our data suggest a possible vasculoprotective effect of the compound in retarding vascular dysfunction and atherogenesis that often develops rapidly in hypertensive patients. (Am Heart J 2004;148:e5.)
epitopes in the arterial wall, and atherogenic lesion formation in experimental models. 14–19

The antioxidant status of the living organs is linked to the nitric oxide (NO) pathway contributing to vessel homeostasis. 20 NO inhibits vascular smooth muscle tone and growth, platelet aggregation, and leukocyte adhesion to the endothelium. 20 Asymmetrical dimethyl-l-arginine (ADMA), an analogue of l-arginine, is a competitive inhibitor of endothelial NO synthase. 21 ADMA is produced by endothelial cells and is present in plasma and urine of rats and human subjects. 21 It is known that ADMA is elevated during hypertension. 22 In the current study, we tested whether the beneficial antioxidant activity of sulfhydryl ACE inhibitors could be also clinically relevant in patients with essential hypertension, and, in addition, the role of NO/ADMA in such patients.

Methods

Patients and study design

Plasma indexes of oxidative stress and the NO/ADMA balance were investigated initially in a group of untreated normocholesterolemic patients with moderate essential hypertension without clinically evident target organ damage (n = 96; ambulatory blood pressure, 165 ± 5/97 ± 3 mm Hg) and in a group of control healthy normotensive subjects (n = 46; ambulatory blood pressure, 120 ± 6/71 ± 3 mm Hg) recruited from the outpatient clinic. After this step, hypertensive patients were randomly assigned into two age- and sex-matched groups to receive the new sulfhydryl ACE inhibitor zofenopril (15 to 30 mg/d; n = 48; 56 ± 12 years; 31 men and 17 women) or the non sulfhydryl ACE inhibitor enalapril (20 mg/d; n = 48; 55 ± 11 years; 31 men and 17 women). Zofenopril, a derivative of the amino acid proline, is effective in reducing cardiovascular events after myocardial infarction and as an antihypertensive drug. 23–25 The study consisted of two periods: (1) basal evaluation in control subjects and in hypertensive patients before random assignment and (2) 12 weeks after random assignment with zofenopril or enalapril. Plasma LDL oxidizability, systemic oxidative stress, and the NO/ADMA balance were evaluated (see below). Each patient had a complete clinical workup to exclude secondary forms of hypertension and hepatic, renal, or endocrine diseases. Diabetes was excluded on the basis of the following criteria: unawareness of the disease, absence of hypoglycemic treatment, and fasting plasma glucose values <6 mmol/L on at least 2 recent occasions. All subjects were on a weight-maintaining diet. All subjects were kept on a similar intake of dietary salt (150 mEq sodium chloride/d) and protein (1 g/kg body wt per day) the entire week before the study. Spinach, a food with high nitrate content, and tobacco smoke, able to alter nitrate levels, were not permitted during this week. The purpose and the potential risks of the study were explained to all patients before their informed consent was obtained. The study protocol was approved by the Institutional Review Board of the Pellegrini Hospital.

LDL peroxidation and systemic oxidative stress

The study begun at 8:30 AM after an overnight fast, with the subject lying supine in a quiet room at a constant temperature of 21°C to 24°C. To perform all measurements related to oxidative stress and NO/ADMA (see below), each blood sample was divided into 3 aliquots: two aliquots of 1 mL were collected and immediately centrifuged, and plasma was isolated; 1 mL was collected into heparinized syringes. LDL oxidation was evaluated as the oxidizability in vitro (generation of malondialdehyde [MDA] by using the thiobarbituric acid assay) as previously described in detail, 26 and systemic oxidative stress was evaluated by isoprostanes (8-isoPGF 2α) as described. 27

Evaluation of plasma NOx and ADMA concentrations

Plasma nitrite and nitrate (NOx) levels were measured by means of the classic Griess method. After being passed through 50-kDa ultrafilters, 40 μL of the plasma was diluted with 240 μL assay buffer and mixed with 10 μL cofactor and 10 μL nitrate reductase (NOx colorimetric assay kit, Cayman Chemical Co, Ann Arbor, Mich). After the plasma had been kept at room temperature for 3 hours to convert nitrate to nitrite, total nitrite was measured at 540 nm absorbance by reaction with Griess reagent (sulfanilamide and naphthalene-ethylene diamine dihydrochloride). Amounts of plasma nitrite were estimated by a standard curve obtained from enzymatic conversion of NaN 3 to nitrite.

Plasma (1 mL) was mixed with 2 mL of 10% trichloroacetic acid, put on ice for 10 minutes, and centrifuged at 2500 g for 15 minutes. The supernatant was evaporated under vacuum to dryness and was then loaded to a Bond Elut PRS column. After washing with 10 mL of 1 mol/L pyridine, ADMA was eluted with 10 mL of 3 mol/L ammonia and was again evaporated under vacuum to dryness. The extract was incubated with 20 μL phenylthiocarbamoyl solution for 20 minutes at room temperature. The dried samples were applied to a reverse-phase, high-performance liquid chromatography column and 60 mmol/L acetic buffer (pH 6.5)/0.05% trifluoroacetic acid elution with a linear gradient of acetonitrile ranging from 6% to 60% over a period of 25 minutes at a flow rate of 1 mL/min, as previously described. 26 Amounts of ADMA in the plasma were estimated from a standard curve of synthetic ADMA (Sigma Chemical Co, Milan, Italy). In our experimental conditions, the variability of the method was <7%, and the detection limit of the assay was 0.15 mmol/L.

Statistical analysis

Each set of measurements within each study period (basal and after treatment) was averaged, and t test analysis was then used to compare data within the same study period or different study periods. This choice was based on the consideration that all of the comparisons were actually made within the same subject. Analysis of variance (ANOVA) for doubly repeated measures was also carried out on the mean values.

Results

As expected, both antihypertensive treatments were effective in reducing blood pressure. Indeed, 12-week
treatment with zofenopril (134 ± 3/70 ± 3 mmHg; P < .05 vs before treatment) or enalapril (131 ± 6/68 ± 5 mmHg; P < .05 vs baseline) significantly reduced blood pressure. Throughout the study, both drugs showed no effect either on the results of physical examination or on liver transaminases, creatine phosphokinase, creatinine, or potassium. Both treatments had no effect on plasma total cholesterol or LDL cholesterol levels as compared with values obtained before and after the end of the study (data not shown).

LDL was isolated from plasma samples obtained from patients before treatment and from control subjects. We compared the susceptibility of the patient LDL and the control LDL with oxidation. At baseline, LDL from hypertensive subjects exhibited enhanced susceptibility to oxidation in vitro in comparison to healthy control subjects (Figure 1,* P < .05 by ANOVA for repeated measures). Similarly, isoprostanes were significantly increased in hypertensive patients when compared with values obtained before and after the end of the study (data not shown).

Table I shows the plasma NOx and ADMA concentrations during the study in a subset of patients. As expected, in the normotensive control subjects, the plasma NOx concentrations were significantly lower than those of hypertensive subjects at the baseline of the study. After treatment with both ACE inhibitors, plasma NOx concentrations were significantly reduced. However, the magnitude of the reduction achieved with the sulfhydryl ACE inhibitor zofenopril was higher than that obtained with enalapril. Similarly, essential hypertension resulted in a significant increase in the plasma ADMA concentration in comparison to normotensive state, whereas ACE inhibition elicited a significant decrease. The reduction of plasma ADMA concentration was significantly higher in patients treated with sulfhydryl ACE inhibition.

**Discussion**

We used a therapeutic intervention that was directed at reducing oxidative stress and for improving the NO/ADMA balance in a group of patients with essential hypertension. Enhanced oxidative stress in hypertensive patients is due to an excessive production of O2 radicals and possibly other radical species.4,6,7 If the hypothesis under study (ie, that only sulfhydryl ACE inhibitors possess clinical relevant antioxidant activity) is true, patients treated with zofenopril would benefit from such an intervention. In the current study, LDL oxidizability and systemic oxidative stress were directly assessed in the patients by measuring two different indexes of radical-induced damage.4,6 In our experience, reproducibility of data is easier when two different indexes of oxidative status are estimated. The finding that long-term treatment with the sulfhydryl ACE inhibitor zofenopril but not with the nonsulfhydryl ACE inhibitor enalapril improved the oxidative balance documents its sustained antioxidant activity in
Table I. Plasma NOx and ADMA during the study protocol

<table>
<thead>
<tr>
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<th>Healthy subjects (n = 38)</th>
<th>Hypertensive subjects before treatment (n = 76)</th>
<th>Hypertensive subjects +12 week zofenopril (n = 38)</th>
<th>Hypertensive subjects +12 week enalapril (n = 38)</th>
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<tbody>
<tr>
<td>Plasma NOx (μmol/L)</td>
<td>33.5 ± 8.2</td>
<td>53.8 ± 12.5*</td>
<td>40.8 ± 10.2†</td>
<td>44.9 ± 13.7‡</td>
</tr>
<tr>
<td>ADMA (μmol/L)</td>
<td>0.42 ± 0.01</td>
<td>0.59 ± 0.02*</td>
<td>0.45 ± 0.01†§</td>
<td>0.51 ± 0.02‡</td>
</tr>
</tbody>
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ADMA, Asymmetrical dimethyl-L-arginine; NOx, plasma nitrite and nitrate.
*P < 0.001 versus healthy subjects.
†P < 0.01 versus Hypertensive subjects before treatment.
‡P < 0.05 versus Hypertensive subjects before treatment.
§P < 0.05 versus Hypertensive subjects +12 week enalapril.

Antiproliferative effects, including antioxidant activity of sulfhydryl ACE inhibitors. The rate constant for the reaction of vitamins E and C is approximately 10,000-fold (5000- to 10,000-fold) than the rate constant for the reaction between NO and superoxide radicals. Therefore, to scavenge radicals, antioxidants must be administered in very high concentrations to reach arterial compartments where superoxide radicals are formed. When high concentrations of vitamins are used, however, pro-oxidative effects may come into play. This may at least partly explain the contradicting results of trials with antioxidants to improve prognosis in patients with coronary heart disease. ACE inhibitors also appear to have a beneficial influence on the prognosis in patients with coronary heart disease in the large Heart Outcomes Prevention Evaluation (HOPE) trial. To be clinically relevant, however, any positive antioxidant effect of sulfhydryl ACE inhibitors on atherosclerotic lesion progression should be demonstrated in long-term studies, and this effect should be related to clinical and/or instrumental signs of atherosclerotic lesion progression. Antioxidant protection could be related to the higher sulfhydryl group-mediated scavenging activity of free radicals by the highly lipophilic drug zofenopril. In general, zofenopril showed a good tolerability and a very fast conversion from prodrug to the active metabolite (zofenoprilat). Sulphydryl compounds are a major class of protective agents against radicals generated by radiations by neutralizing radicals by either a hydrogen atom-donating or electron-transferring reaction. The mechanism of oxygen radical repair mediated by sulphydryl compounds may involve carbon-centered radicals and/or correlates with their hydroxyl radical scavenging abilities. Moreover, ACE inhibition would reduce angiotensin II-mediated macrophage lipid peroxidation. Consistently, the non-sulphydryl ACE inhibitor enalapril did not exhibit any plasma antioxidant effects. More important, there is the lack of protection afforded by enalapril in atherosclerotic mice. Interestingly, a very recent study shows that the clinical outcome of patients with diabetes, a condition associated to increased oxidative stress, and non-thrombolized myocardial infarction can be significantly improved by treatment with sulphydryl ACE inhibition. NO is rapidly inactivated by O2-reactive species, but NO synthesis (at least when induced by acetylcholine) is associated with O2 radical generation. Thus, the increased oxidative stress seen in hypertensive patients could make NO more susceptible by “quenching” the local generation of O2 radicals. The therapeutic mod-
ulation of NO release is able to induce vascular protection.54 We previously suggested an NO-related activity on platelets after treatment with zofenopril in Watanabe rabbits.15 In the current study, we show that ACE inhibitors, especially the sulfhydryl ACE inhibitor zofenopril, improved the NO/ADMA balance. NOx measurement appears to be a clinically direct method for measurement of NO production. The main drawback of the use of total nitrate as a measure of NO synthesis is that nitrate may arise from sources other than the metabolism of NO. In human cells, however, NO is synthesized from the guanido nitrogen atoms of the amino acid L-arginine, and this is the only known route by which these nitrogen atoms may be incorporated into nitrate. Therefore, the measurement of total nitrate can be considered a specific indicator of total body NO synthesis. However, the measurement of endogenously generated nitrate may be confounded by dietary assumption of nitrates and nitrites inhaled through tobacco smoke. We showed also that ADMA plasma levels were significantly reduced by treatment with ACE inhibitors. Accordingly, the degree of ADMA reduction was significantly higher in patients receiving zofenopril. This effect could be due to its antioxidant properties, NO-related beneficial effects, or to other unknown effects of sulfhydryl ACE inhibition.55 Abnormal NO-dependent vascular relaxation is commonly observed in patients with essential hypertension.56 Since the Prevention of Atherosclerosis with Ramipril (PART-2) collaborative research group57 suggested that beneficial effects of ACE inhibitors on major coronary events could be due to reversal of endothelial dysfunction, this must be assessed in future investigations. In addition, the results of the HOPE and the Study to Evaluate Carotid Ultrasound changes in patients treated with Ramipril and vitamin E (SECURE) studies indicate that ramipril might improve endothelial dysfunction and atherosclerosis (Reference 46, and reviewed in Reference 58).58 By using the reversed-phase liquid chromatographic method, the related substances of 2-[N-{(S)-1-Ethoxycarbonyl-3-phenylpropyl}-L-alanyl]-L,3S,5S-2-azabicyclo[3.3.0]octane-3-carboxylic acid (ramipril) was determined in Altace capsules.59 Four of the related substances are ramipril diastereomers, but they did not contain sulfhydryl groups.59 However, the vasculoprotective properties of certain non-sulfhydryl-containing ACE inhibitors might be related to unexpected antioxidant actions.60 Finally, we need to acknowledge the reduction of ischemic events in the Studies Of Left Ventricular Dysfunction (SOLVD)61 by the nonsulfhydryl ACE inhibitor enalapril.

Conclusions

The oxidative stress in patients with essential hypertension is improved by chronic administration of therapeutic doses of the sulfhydryl ACE inhibitor zofenopril but not with the nonsulfhydryl ACE inhibitor enalapril. This effect was coupled to an improvement of the NO/ADMA balance. Although interesting and sound, these findings should be tested in a large multicenter clinical trial. We suggest a possible protective effect of the sulfhydryl ACE inhibition in retarding vascular dysfunction and atherogenesis that often develops rapidly in hypertensive patients.

References

lipoprotein oxidation in Watanabe heritable hyperlipidemic rabbits. Gen Pharmacol 1999;33:467–77.


