Enalapril and captopril enhance glutathione-dependent antioxidant defenses in mouse tissues

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De Cavanagh, Elena M. V., Felipe Inserra, León Ferder, and César G. Fraga. Enalapril and captopril enhance glutathione-dependent antioxidant defenses in mouse tissues. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R572–R577, 2000.—The effect of enalapril and captopril on total glutathione content (GSSG + GSH) and selenium-dependent glutathione peroxidase (Se-GPx) and glutathione reductase (GSSG-Rd) activities was investigated in mouse tissues. CF-1 mice (4-mo-old females) received water containing enalapril (20 mg/l) or captopril (50 mg/l) for 11 wk. Enalapril increased GSSG + GSH content (P < 0.05) in erythrocytes (14%), brain (112%), and lung (67%), and captopril increased GSSG + GSH content in erythrocytes (190%) and brain (132%). Enalapril enhanced Se-GPx activity in kidney cortex (42%) and kidney medulla (23%) and captopril in kidney cortex (30%). GSSG-Rd activity was enhanced by enalapril in erythrocytes (21%), brain (21%), liver (18%), and kidney cortex (53%) and by captopril in erythrocytes (25%), brain (19%), and liver (34%). In vitro erythrocyte oxidant stress was evaluated by thiobarbituric acid-reactive substances (TBARS) production (control 365 ± 11, enalapril 221 ± 26, captopril 206 ± 17 nmol TBARS·g Hb·h−1·h−1; both P < 0.05 vs. control) and phenylhydrazine-induced methemoglobin (MetHb) formation (control 66.5 ± 3.5, enalapril 52.9 ± 0.4, captopril: 56.4 ± 2.9 μmol MetHb/g Hb; both P < 0.05 vs. control). Both angiotensin-converting enzyme inhibitor treatments were associated with increased nitric oxide production, as assessed by plasma NO3 + NO2 level determination (control 9.22 ± 0.64, enalapril 13.7 ± 1.9, captopril 17.3 ± 3.0 μmol NO3 + NO2/l plasma; both P < 0.05 vs. control). These findings support our previous reports on the enalapril- and captopril-induced enhancement of endogenous antioxidant defenses and include new data on glutathione-dependent defenses, thus furthering current knowledge on the association of ACE inhibition and antioxidants.

oxygen radicals; antioxidants; angiotensin-converting enzyme inhibitors; hypertension; aging

AEROBIC ORGANISMS continuously generate reactive oxygen species (ROS) in the course of physiological processes. ROS can oxidize biomolecules leading to modifications of cell functions. ROS-mediated damage to biomolecules has been involved in the pathophysiology of a variety of disease conditions (20). Cells have evolved several antioxidant strategies aimed at the detoxification of ROS. One of the major protective systems against oxidant damage is the glutathione redox cycle, composed of the enzymes glutathione peroxidase (GPx) and glutathione reductase (GSSG-Rd) and the cosubstrates glutathione and NADPH (32). Glutathione is the most abundant nonprotein intracellular thiol, its concentration frequently being in the millimolar range (21). GSH has a multiple role as an antioxidant agent. It functions as a scavenger of ROS, including hydroxyl radicals, singlet oxygen, nitric oxide, and peroxyxynitrite. In addition, GSH is a cosubstrate for the detoxification of peroxides by GPx and of toxic metabolites by glutathione-S-transferases (21). Finally, GSH could be involved in the regeneration of ascorbic acid from its oxidized form (dehydroascorbate), and it functions directly, or via ascorbate, in the reduction of the tocopherol radical (28). As a result of its antioxidant action, GSH is oxidized to GSSG. GSSG, whose accumulation in tissues causes a variety of noxious effects, is reduced back to GSH by GSSG-Rd through the reducing power of NADPH provided by the pentose phosphate pathway (21).

Angiotensin-converting enzyme inhibitors (ACEI) are widely prescribed for the treatment of hypertension and congestive heart failure. They also delay the progression of chronic renal failure and of diabetic nephropathy (31). In addition, they have been shown to retard the development of atherosclerosis in experimental models (6) and to improve endothelial dysfunction in patients with coronary artery disease (25). The mechanisms underlying these pharmacological effects of ACEI are not fully understood. Various experimental evidences support the involvement of hemodynamic effects and/or the stimulation of cytoprotective prostaglandins (35). The potentiation of bradykinin (18) and a free radical scavenger action (7) by ACEI have also been postulated. In a previous study in mice (12), we found that chronic administration of enalapril attenuates age-associated myocardial and glomerular sclerosis and increases survival. Subsequent work showed that 11-wk enalapril or captopril treatments increase antioxidant enzymes and nonenzymatic antioxidant defenses in several mouse tissues (4, 5). To further investigate the effect of ACEI administration on antioxidant defenses, in the present study we measured total glutathione content (GSSG + GSH), selenium-dependent glutathione peroxidase (Se-GPx), and GSSG-Rd...
activities in several mouse tissues. Lipid-soluble antioxidants (α-tocopherol and ubiquinol-9) were determined in plasma. Oxidant stress was evaluated in erythrocytes by measuring freeze/thaw-induced thiobarbituric acid reactive substances (TBARS) production, and methemoglobin (MetHb) formation after exposure of the cells to phenylhydrazine. To evaluate the effect of ACEI on nitric oxide production, we determined nitrates and nitrites in plasma.

**MATERIALS AND METHODS**

Animals and chemicals. Female CF-1 mice, 4 mo old, were randomly separated into three groups that were administered either water (pH 7.1; controls) or water containing 20 mg/l enalapril maleate (pH 7.1) or 50 mg/l captopril (pH 7.1) for 11 wk. The duration of the treatments was chosen according to the results obtained for liver (5). Animals had free access to a Purina type II diet (Cargill, Buenos Aires, Argentina). Blood pressure was measured by tail plethysmography (IITC model 29 Amplifier, Life Science Laboratory, Woodland Hills, CA). All reagents were of analytical grade (Sigma Chemical, St. Louis, MO). Captopril was obtained from Bristol-Myers Squibb (Princeton, NJ) and enalapril from Merck, Sharp & Dohme (West Point, PA).

Sample preparation. At the end of the treatment period the mice were anesthetized with chloroform and subjected to a laparotomy. Blood samples were drawn from the inferior vena cava into heparinized tubes, and plasma and erythrocytes were separated by centrifugation. The brain, heart, liver, kidneys, and lungs were excised after perfusion with 150 mM NaCl at 4°C. The tissues were homogenized with 10 (for Se-GPx determination) or 4 (for GSSG-Rd determination) vols of 120 mM KCl-30 mM potassium phosphate, pH 7.4, and centrifuged at 600 g for 10 min. The supernatant, hereafter referred to as homogenate, was used for enzyme activity determinations. Protein content was determined according to Bradford (2) using bovine serum albumin as standard.

Determination of GSSG + GSH. Erythrocytes were deproteinized with 15 vols of 0.33 M HClO4. After neutralization of the supernatant with 1.75 M K2HPO4, an aliquot was taken for the measurement of GSSG + GSH using the 5,5'-dithiobis(2-nitrobenzoic acid) spectrophotometric assay (34). Results are expressed as micromoles of GSH equivalents (GSH + 2 GSSG) per gram of hemoglobin. For the determination of GSSG + GSH in other tissues, homogenates were prepared with 4 vols of 0.33 M HClO4 and centrifuged at 5,000 g for 10 min and the supernatant was used for the neutralization step as described for erythrocytes. Results are expressed as micromoles of GSH equivalents per gram of wet tissue. Considering that fasting reduces organ glutathione content, animals were not fasted overnight. To reduce variability derived from diurnal fluctuation in organ glutathione content, animals were killed between 9:00 and 10:00 AM.

Enzyme assays. Se-GPx activity was determined according to Günzler et al. (19), by following NADPH oxidation (molar extinction coefficient at 340 nm = 6.22 mM-1 cm-1). Homogenate samples were incubated at 30°C for 1 min in the presence of 5 mM GSH, 0.15 mM NADPH, 1 mM NaN3, 0.125 U/ml glutathione reductase, and 0.5 mM H2O2, in 50 mM potassium phosphate, pH 7.7. One unit of Se-GPx was defined as the amount of enzyme that oxidizes one micromole of NADPH per minute. For the determination of enzyme activity in erythrocytes, the samples were prepared as previously described (13). GSSG-Rd activity was determined as described by Carlberg and Mannervik (3), with modifications, by following NADPH oxidation at 340 nm. Homogenate samples were incubated in the presence of 0.5 mM GSSG and 0.15 mM NADPH, in 10 mM Tris-HCl buffer, pH 7.0. One unit of GSSG-Rd was defined as the amount of enzyme that catalyzes the oxidation of one micromole of NADPH per minute.

Evaluation of nitric oxide production. Nitric oxide production was monitored in plasma. The levels of the lipid-soluble antioxidants α-tocopherol and total ubiquinol-9 in plasma were measured using HPLC with electrochemical detection (30). Total ubiquinol-9 measurement includes ubiquinone (CoQ0) and its reduction product, ubiquinol (CoQ10). Plasma aliquots (200 µl) were added with 500 µl of methanol, vortexed, and added with 4 ml of n-hexane. The mixtures were vortexed for 1 min and then centrifuged for 5 min at 1,000 g. A 3-ml aliquot of the hexane layer was dried under N2. The residue was dissolved in 0.2 ml of ethanol-methanol (1:1, vol/vol) and filtered through a 0.22-µm-pore nylon membrane. The samples were subjected to HPLC on a 8-C reversed-phase column, and the antioxidant levels were measured by electrochemical detection (BAS LC4C amperometric detector with glassy carbon working electrode at applied potential of +0.6 V). Commercial standards were used for calibration.

TBARS production in erythrocytes. Erythrocytes were subjected to one freeze-thaw cycle. Suspensions containing 15 mg hemoglobin/ml were prepared with 120 mM KCl-30 mM potassium phosphate, pH 7.4. Oxidation products formed during a 1-h incubation at 37°C were evaluated as TBARS by spectrofluorescence (15). Aliquots of freeze-thawed erythrocyte suspensions (500 µl) were added with 100 µl of butylhydroxytoluene (4% wt/vol in ethanol) before TBARS measurement. Results are expressed as nanomoles of TBARS (malondialdehyde equivalents) per gram of hemoglobin per hour. Malondialdehyde standards were prepared from 1,1,3,3-tetramethoxypropane. Hemoglobin concentration was measured using Drabkin’s reagent.

Formation of MetHb in erythrocytes exposed to phenylhydrazine. Erythrocytes from control, enalapril-, or captopril-treated mice were washed three times with 123 mM NaCl-28 mM Na-K phosphate buffer, pH 7.4. The cells were diluted with the same buffer to a 50% (vol/vol) suspension and incubated in the presence of 1 mM phenylhydrazine over 30 min at 37°C. After incubation, the erythrocytes were lysed by the addition of 15 mM potassium phosphate buffer, pH 7.4, containing 0.01% (wt/vol) digitonin. After centrifugation at 500 g, the supernatant was used to evaluate spectrophotometrically the formation of MetHb at 560, 577, and 630 nm (40).

Evaluation of nitric oxide production. Nitric oxide production was evaluated by measuring NO2- + NO3- in plasma samples. After reduction of NO3- to NO2- (Nitrate Reductor, World Precision Instruments, Sarasota, FL), NO3- + NO2- was determined by spectrophotometry using the Griess reagent (38).

Statistical analysis. Values are means ± SE. Nonparametric Mann-Whitney or Kruskal-Wallis statistics, available in Statview SE+Graphics (Abacus Concepts, Berkeley, CA), were used to establish the significance of between-group differences. P values <0.05 were considered significant.

**RESULTS**

Animal outcome. The 11-wk enalapril or captopril treatment had no effect on body weight (control 33.6 ± 0.8, enalapril 35.1 ± 0.8, captopril 35.7 ± 1.1 g), food intake (control 0.210 ± 0.02 g·day-1·g body wt-1), or blood pressure (control: systolic 112 ± 1, diastolic 81 ± 1 mmHg). Values represent data from at least eight animals.
GSSG + GSH content. In enalapril-treated mice, GSSG + GSH content was significantly higher in erythrocytes (147%), brain (112%), and lung (67%) (P < 0.05, Table 1) compared with control values. Enalapril had no effect on liver, heart, and kidney GSSG + GSH content. Captopril-treated mice had higher levels of GSSG + GSH in erythrocytes (190%) and brain (132%), whereas lung, liver, heart, and kidney GSSG + GSH contents were similar to those in controls (P < 0.05, Table 1). The determination of GSSG + GSH levels was performed in whole kidney homogenates, and not in kidney cortex and medulla separately, because of the very rapid degradation of glutathione by γ-glutamyltranspeptidase during the process of dissecting the kidneys.

Antioxidant enzyme activities. In the enalapril group, Se-GPx activity was higher in liver (90%), kidney cortex (42%), and kidney medulla (23%) (P < 0.05, Table 2) compared with controls. No effect was observed in erythrocytes, brain, lung, and heart. In captopril-treated mice Se-GPx activity was higher in liver (33%) and kidney cortex (30%) relative to controls (P < 0.05, Table 2), but there was no effect in erythrocytes, brain, lung, heart, and kidney medulla.

After 11 wk of enalapril treatment, the activity of GSSG-Rd in tissues from mice treated with enalapril or captopril for 11 wk

Oxidant stress in erythrocytes. Erythrocytes were used as a model system to investigate whether there were any differences among the study groups relative to their oxidant damage status after exposure to oxidative conditions. TBARS production was significantly lower in freeze-thawed erythrocytes from either enalapril (−40%) or captopril (−44%)−treated mice, relative to controls (P < 0.05, Table 4). MetHb formation in erythrocytes incubated with phenylhydrazine was significantly lower in cells obtained from either enalapril (−21%) or captopril (−15%)−treated mice, compared with controls (P < 0.05, Table 4).

Evaluation of nitric oxide production. To investigate whether the effects of ACEI on antioxidant levels and enzyme activities might be associated with increased nitric oxide production, the levels of NO2− and NO3− in plasma were determined. Plasma NO2− and NO3− contents were elevated in enalapril (48%) and captopril (88%)−treated mice relative to controls (P < 0.05, Table 5).

DISCUSSION

We showed previously (4, 5) that enalapril and captopril treatments increase antioxidant enzymes [superoxide dismutases (SOD), GPx] and nonenzymatic antioxidant defenses (integrally evaluated) in several mouse tissues. Here we report that enalapril and captopril, administered over 11 wk, were also able to
Table 4. Oxidant stress parameters in erythrocytes from mice treated with enalapril or captopril for 11 wk

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<tr>
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<th>Control</th>
<th>Enalapril</th>
<th>Captopril</th>
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<tbody>
<tr>
<td>TBARS, mmol-g Hb⁻¹-h⁻¹</td>
<td>365 ± 11</td>
<td>221 ± 26*</td>
<td>206 ± 17*</td>
</tr>
<tr>
<td>MetHb, µmol/g Hb</td>
<td>66.5 ± 3.5</td>
<td>52.9 ± 0.4*</td>
<td>56.4 ± 2.9*</td>
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Values are means ± SE; n = 6 animals/group. For measurement of thiobarbituric acid-reactive substances (TBARS) production, erythrocytes were subjected to 1 freeze-thaw cycle and suspensions containing 15 mg Hb/ml were incubated for 30 min at 37°C before TBARS evaluation. For measurement of methemoglobin (MetHb) formation, erythrocyte suspensions (50% vol/vol) were incubated in presence of 1 mM phenylhydrazine for 30 min at 37°C before MetHb determination. *P < 0.05 vs. control.

Enhance total glutathione content and Se-GPx and GSSG-Rd activities in various mouse tissues. Furthermore, in erythrocytes the augmentation of antioxidants by ACEI was associated with protection against oxidant damage.

The doses of ACEI used in this study were chosen 1) to match those prescribed for humans, considering that the specific metabolic rate is close to 10 times higher in mice than in humans, and 2) to have no effect on mouse blood pressure. Because, as expected, both ACEI did not modify mouse blood pressure, it is possible to assume that the effects of enalapril and captopril treatments on the antioxidant defenses are not secondary to their action on blood pressure.

Enalapril treatment increased total glutathione content in erythrocytes, brain, and lung, whereas the same effect was elicited by captopril in erythrocytes and brain. Se-GPx activity was increased in liver and kidney cortex by the enalapril and captopril treatments. Enalapril also increased Se-GPx activity in kidney medulla, whereas captopril had no effect in this tissue. The effects of enalapril or captopril treatment on Se-GPx activity in the liver and kidney medulla were in agreement with earlier results (4, 6), thus supporting current data in other organs. GSSG-Rd activity was enhanced by enalapril in erythrocytes, brain, liver, and kidney cortex, whereas this enzyme activity was augmented by captopril in erythrocytes, brain, and liver. Apparently, the enalapril- or captopril-induced enhancement of enzymatic and nonenzymatic antioxidant defenses was treatment and tissue dependent.

This lack of pattern in organ response to ACEI treatments might be explained by different tissue metabolism and/or penetration of ACEI, which could arise from 1) the capacity of a particular tissue to transform enalapril (a prodrug) into its active form (enalaprilat); 2) the redox status of the tissue, which can limit captopril bioavailability by favoring the formation of disulfurs through captopril dimerization or reaction with a different thiol compound; 3) the differences in molecular size, ionization coefficient, and lipophility between enalapril and captopril; and 4) the existence of organ renin-angiotensin systems with different levels of expression and/or activity of ACE. Furthermore, it is necessary to consider that an effect of ACEI on the antioxidant defenses might have occurred in certain cell types but not in others and failed to be detected because the determinations were carried out in whole tissue homogenates.

It is also shown that enalapril and captopril can induce a substantial increase of erythrocyte total glutathione content and a moderate increase of erythrocyte GSSG-Rd activity. In addition, we previously reported (4) that both ACEI treatments can increase erythrocyte CuZn-SOD activity. Considering that glutathione can channel radicals to superoxide (39), a concerted action of glutathione and SOD would be necessary for the effective control of oxidant stress. The concurrent ACEI-induced enhancement of glutathione, GSSG-Rd, and SOD in erythrocytes might explain the higher protection against oxidant damage displayed by erythrocytes from enalapril- and captopril-treated mice. Furthermore, erythrocytes serve as vehicles for the transport of GSH from the liver and kidney to other organs, which would be GSH consumers (8). In sum, the maintenance of higher levels of antioxidant defenses in erythrocytes could also be protective against systemic oxidant stress, considering that these cells are circulated through organs containing higher levels of H₂O₂ and that they may act as sinks for H₂O₂ and superoxide anion generated in other tissues (17). Erythrocytes have been shown to take up extracellular dehydroascorbate, a toxic ascorbate oxidation product, and to reduce it back to ascorbate, largely depending on GSH (24). Thus it is possible that erythrocytes may contribute to the removal of dehydroascorbate generated at sites of elevated ROS production in the vascular bed. Again, this underscores the relevance of increasing GSH content as a mechanism of protection for erythrocytes as well as for other tissues.

The free radical theory of aging proposes that senescence-related loss of function is caused by the accumulation of damage inflicted on biomolecules by ROS. Aging has been associated with low glutathione levels in the blood and tissues of several animals as well as in human blood (23). In addition, GSH supplementation has been shown to reverse the age-associated decline in immune responsiveness in mice (16) and to improve survival in Drosophila and in mice (37). We previously showed (12) that ACEI ameliorate several age-associated parameters and improve survival in mice. This could be attributed, at least partially, to the ACEI-induced augmentation of tissue glutathione content in brain and lung and particularly in erythrocytes.

It has been indicated that cellular glutathione may decrease the oxidation of low-density lipoprotein (27), which is thought to underlie the development of atherosclerosis (9). ACEI have been shown to retard the progression of atherosclerosis (6, 25). This might be related, at least in part, to the ACEI-induced enhance-
increase of human antioxidant defenses and the understating encourage further research on the ACEI-induced results obtained in mice, preliminary data from our induced enhancement of erythrocyte antioxidants and antioxidants and report the association between ACEI-

In this study we extend those observations to other increase antioxidant defenses in several mouse tissues. Also, we showed that enalapril and captopril

The mechanism(s) underlying the enhancement of glutathione and glutathione-related enzymes by ACEI remains unknown. Tissue glutathione levels and GSSG-Rd and GPx activities have been shown to increase in response to experimentally induced oxidative stress (14). ACEI decrease angiotensin II formation as well as endogenous bradykinin degradation. As a result, long-term ACE inhibition promotes the accumulation of the latter substance (1). Bradykinin is a potent vasodilator known to stimulate the release of nitric oxide (18).

The data presented in this study show a concurrence of increased antioxidant defenses and nitric oxide levels in blood. In different systems, nitric oxide was able to either increase (22, 29, 32) or decrease (24) antioxidant defenses. Therefore, the participation of nitric oxide as a direct or indirect modulator of the observed increase of glutathione-dependent antioxidant defenses associated with ACEI treatment is a subject that deserves further investigation.

In summary, considering the relevance of the glutathione one system as a cellular antioxidant mechanism, the present findings add to our previous proposal suggesting that by increasing antioxidant defenses in several tissues, enalapril and captopril might protect cells from ROS-mediated damage. The enhancement of the antioxidant defenses might explain, at least in part, the ancillary beneficial effects shown by ACEI in various pathologies as well as during aging.

Perspectives

ACEI have been shown to retard the progression of chronic renal failure, diabetic nephropathy, and atherosclerosis in animals as well as in humans. Excess levels of ROS have been associated with these conditions. Hence, an increase of antioxidant defenses might attenuate the development of those disease states. We previously reported that enalapril attenuates age-related nephrosclerosis and myocardial sclerosis in mice. Also, we showed that enalapril and captopril increase antioxidant defenses in several mouse tissues. In this study we extend those observations to other antioxidants and report the association between ACEI-induced enhancement of erythrocyte antioxidants and protection against oxidant stress. In addition to the results obtained in mice, preliminary data from our laboratory show that ACEI increase antioxidant defenses in human plasma and erythrocytes. These findings encourage further research on the ACEI-induced increase of human antioxidant defenses and the underlying mechanism(s). The elucidation of those mechanisms might help to develop new strategies aimed at increasing the endogenous antioxidant defenses, as opposed to less effective nondietary antioxidant supplementation. The achievement of a sustained elevation of endogenous antioxidant agents could be useful in delaying the progression of degenerative conditions related to oxidant-induced damage.

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