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−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. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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 peroxynitrite. 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 tocopheryl 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 nitrites and nitrates 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 I1 diet (Cargill, Buenos Aires, Argentina). Blood pressure was evaluated 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 Günzlir 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.

Determination of lipid-soluble antioxidants 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 (CoQ9H2). 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 spectrofluorimetry (15). Aliquots of freeze-thawed erythrocyte suspensions (500 µl) were added with 100 µl of a 4% (wt/vol) solution 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 NO3⁻ and NO2⁻ 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.
ACEI ENHANCE GLUTATHIONE-RELATED ANTIOXIDANT DEFENSES

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 was significantly higher in erythrocytes (20%), brain (21%), liver (18%), and kidney cortex (53%) (P < 0.05, Table 3) relative to control values. GSSG-Rd activity was unchanged in lung, heart, and kidney medulla. In captopril-treated mice GSSG-Rd activity was higher than in controls in erythrocytes (25%), brain (21%), liver (18%), heart (21%), and kidney cortex (42%), and kidney medulla (23%) (P < 0.05, Table 3), whereas in lung, heart, kidney cortex and kidney medulla, enzyme activity was not different from controls (Table 3). In captopril-treated mice, liver GSSG-Rd activity was 19% higher than in enalapril-treated mice (P < 0.05, Table 3)

Lipid-soluble antioxidant concentrations in plasma. Enalapril or captopril treatment had no effect on either plasma α-tocopherol (control 10.2 ± 1.3, enalapril 11.3 ± 1.8, capto- pril 11.3 ± 0.8 μM) or ubiquinol-9 (control 0.273 ± 0.115, enalapril 0.211 ± 0.041, capto- pril 0.270 ± 0.037 μM) content.

Table 1. GSSG + GSH content in tissues from mice treated with enalapril or captopril for 11 wk

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>Enalapril</th>
<th>Captopril</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes, μmol/g Hb</td>
<td>7.2 ± 0.8</td>
<td>17.8 ± 4.1*</td>
<td>20.9 ± 3.3*</td>
</tr>
<tr>
<td>Brain, μmol/g wet tissue</td>
<td>0.25 ± 0.04</td>
<td>0.53 ± 0.05*</td>
<td>0.58 ± 0.07*</td>
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<tr>
<td>Liver, μmol/g wet tissue</td>
<td>2.57 ± 0.39</td>
<td>2.74 ± 0.41</td>
<td>3.48 ± 0.25</td>
</tr>
<tr>
<td>Lung, μmol/g wet tissue</td>
<td>0.18 ± 0.02</td>
<td>0.30 ± 0.05†</td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td>Heart, μmol/g wet tissue</td>
<td>0.43 ± 0.03</td>
<td>0.41 ± 0.05</td>
<td>0.50 ± 0.04†</td>
</tr>
<tr>
<td>Kidney, μmol/g wet tissue</td>
<td>30.3 ± 4.5</td>
<td>27.3 ± 2.7</td>
<td>28.4 ± 3.6</td>
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</table>

Values are means ± SE of 6-12 animals. *P < 0.05 vs. control; †P < 0.05 vs. captopril; ns, not significant.

Table 2. Se-GPx activity in tissues from mice treated with enalapril or captopril for 11 wk

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>Enalapril</th>
<th>Captopril</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes, μmol/g Hb</td>
<td>33.4 ± 1.8</td>
<td>36.5 ± 1.6</td>
<td>29.3 ± 2.5</td>
</tr>
<tr>
<td>Brain, μmol/g protein</td>
<td>14.7 ± 3.0</td>
<td>12.9 ± 2.3</td>
<td>15.6 ± 2.4</td>
</tr>
<tr>
<td>Liver, μmol/g protein</td>
<td>29.0 ± 2.1</td>
<td>55.1 ± 2.9*</td>
<td>38.6 ± 1.9*</td>
</tr>
<tr>
<td>Lung, μmol/g protein</td>
<td>27.8 ± 8.5</td>
<td>24.7 ± 6.2</td>
<td>50.5 ± 7.7*</td>
</tr>
<tr>
<td>Heart, μmol/g protein</td>
<td>2.8 ± 0.1</td>
<td>2.7 ± 0.2</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>Kidney cortex, μmol/mg protein</td>
<td>74.1 ± 4.5</td>
<td>105.5 ± 4.3*</td>
<td>96.7 ± 8.0*</td>
</tr>
<tr>
<td>Kidney medulla, μmol/mg protein</td>
<td>12.8 ± 0.6</td>
<td>15.8 ± 0.6*</td>
<td>13.9 ± 1.0</td>
</tr>
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</table>

Values are means ± SE of 4-12 animals. Se-GPx, selenium-dependent glutathione peroxidase. *P < 0.05 vs. control; †P < 0.05 vs. captopril.

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). MethHb 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− + NO3− in plasma were determined. Plasma NO2− + 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

<table>
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<th>Control</th>
<th>Enalapril</th>
<th>Captopril</th>
</tr>
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<tbody>
<tr>
<td>TBARS, nmol·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>
</tr>
</tbody>
</table>

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.

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 under-
nings encourage further research on the ACEI-induced
laboratory show that ACEI increase antioxidant de-
results obtained in mice, preliminary data from our
protection against oxidant stress. In addition to the
induced enhancement of erythrocyte antioxidants and
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In this study we extend those observations to other
mice. Also, we showed that enalapril and captopril
related nephrosclerosis and myocardial sclerosis in
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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 de-
fenses in human plasma and erythrocytes. These find-
ings encourage further research on the ACEI-induced
increase of human antioxidant defenses and the under-
lying mechanism(s). The elucidation of those mecha-
nisms might help to develop new strategies aimed at
increasing the endogenous antioxidant defenses, as
opposed to less effective nondietary antioxidant supple-
mentation. The achievement of a sustained elevation of
endogenous antioxidant agents could be useful in delay-
the progression of degenerative conditions related to
oxidant-induced damage.

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