Modulation of Oxidative Stress by a Selective Inhibition of Angiotensin II Type 1 Receptors in MI Rats

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EXPERIMENTAL STUDY

OBJECTIVES
To examine whether blocking of the renin-angiotensin system (RAS) at the angiotensin II type 1 (AT\textsubscript{1}) receptor site is accompanied by changes in the oxidative stress parameters.

BACKGROUND
Congestive heart failure in rats after myocardial infarction (MI) has been shown to correlate with a decrease in antioxidant enzyme activities and an increase in oxidative stress. Inhibition of the RAS with captopril improves cardiac function and survival in MI rats with a reduction in oxidative stress.

METHODS
Myocardial infarction in rats was produced by ligation of the left coronary artery. At four weeks after surgery, animals from the sham as well as MI groups were treated with losartan (2 mg/ml in drinking water daily). At 16 weeks after surgery, the animals were examined for hemodynamic function and the hearts were analyzed for antioxidant enzyme activities (superoxide dismutase, glutathione peroxidase and catalase) and oxidative stress (lipid hydroperoxides, reduced and oxidized glutathione and redox ratio).

RESULTS
Congestive heart failure was characterized by dyspnea, depressed hemodynamic function and presence of lung and liver congestion. This was also associated with a decrease in the myocardial catalase (−25%), glutathione peroxidase (−38%) and superoxide dismutase (−42%) activities. An increase in oxidative stress in these hearts was indicated by an increase in lipid hydroperoxides (+67%) and reduction in the redox ratio (−75%). Hemodynamic function was better maintained and there were no indications of dyspnea or lung or liver congestion in the losartan-treated MI rats. In these animals, myocardial oxidative stress was markedly reduced and glutathione peroxidase and catalase activities were significantly improved compared with the untreated MI group.

CONCLUSIONS
Blocking of RAS at the AT\textsubscript{1} receptor site without the inhibition of angiotensin-converting enzyme modifies heart failure after MI, and this beneficial effect is associated with a decrease in oxidative stress. This study suggests a newer role for losartan in the treatment of heart failure. (J Am Coll Cardiol 2001;37:1461–6) © 2001 by the American College of Cardiology

In patients surviving a myocardial infarction (MI), the heart undergoes a remodeling process characterized by hypertrophy, which can lead to heart failure. Although hypertrophy is considered an early response to preserve cardiac function, numerous studies have suggested that the long-term process of remodeling has a deleterious effect (1). Although the mechanisms involved in the transition of the compensatory phase to the failure stage are poorly understood, chronic activation of the sympathetic and renin-angiotensin system (RAS) appears to play a role (2). In this regard, angiotensin-converting enzyme inhibitors have been shown to improve cardiac function and prolong life in both experimental and clinical studies (3,4). Recent data from animal studies (5,6) as well as patients (7,8) support the role of increased oxidative stress in the pathogenesis of congestive heart failure following MI.

Using the rat coronary artery ligation model, our laboratory has reported depressed myocardial endogenous antioxidant reserve and increased oxidative stress, which were associated with poor cardiac function (4–6). Inhibition of the RAS by inhibiting angiotensin-converting enzyme with captopril in MI rats not only improved hemodynamic function but also maintained the antioxidant reserve and decreased oxidative stress (4). Because captopril also possesses some free-radical scavenging property (9), and inhibition of angiotensin-converting enzyme is also known to influence bradykinin metabolism (10,11), the exact property of captopril that offers protection is not clear. Losartan is the first of the new class of angiotensin II antagonists that selectively and completely blocks angiotensin II type I (AT\textsubscript{1}) receptor (12). Losartan has been reported to reduce blood pressure and improve cardiac function and survival (13) without decreasing levels of angiotensin II or other humoral factors such as bradykinin, vasopressin and prostaglandin metabolism (10,12). Thus, a selective inhibition of AT\textsubscript{1} receptor provides an ideal approach for dissecting...
the role of RAS versus other humoral changes during angiotensin-converting enzyme inhibition in improving the prognosis in heart failure after MI and associated changes in oxidative stress.

We used a rat MI model of coronary artery ligation to explore the effects of RAS inhibition at the AT1 receptor site by losartan on myocardial antioxidants as well as oxidative stress changes in relation to the hemodynamic function in congestive heart failure. Myocardial antioxidant enzyme (superoxide dismutase, glutathione peroxidase and catalase) activities and changes in oxidative stress (lipid hydroperoxide contents, reduced glutathione [GSH] and oxidized glutathione [GSSG] and GSH/GSSG ratio) were recorded at 16 weeks post-MI with and without losartan. The treatment was started at four weeks post-MI and continued for 12 weeks.

**MATERIALS AND METHODS**

**Animal model and study groups.** Male Sprague-Dawley rats weighing 150 ± 10 g were maintained on standard rat chow and water ad libitum. Myocardial infarction was produced by occlusion of the left anterior descending coronary artery and the sham control animals were similarly treated, except that the suture around the coronary artery was not tied and the thread was passed only through the muscle as described before (4). Sham and animals with MI were divided into four groups as follows: 16-week sham control (without coronary ligation and without drug); 16-week post-MI (with coronary ligation but no drug treatment); 16-week sham + losartan (no coronary ligation; the drug was given in drinking water) and 16-week post-MI + losartan (coronary-ligated group with the drug treatment). The treatment with losartan (2 mg/ml in drinking water daily) was started at four weeks after the surgery and continued for 12 weeks. Animals were monitored daily for general behavior, food, water intake and clinical signs of heart failure. Daily average water consumption in control and experimental animals was about 30 ml.

**Hemodynamic and tissue weight determinations.** Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.). A miniature pressure transducer catheter (Millar Micro-Tip, model PR 249, Houston, Texas) was inserted into the right carotid artery and then advanced into the left ventricle. Left ventricular end-diastolic (LVEDP) and left ventricular peak systolic (LVPSP) pressures were recorded for an online analysis. After these assessments, the rats were killed and the heart and other organs removed for further studies.

In order to obtain the wet/dry weight ratio of the lungs and liver, these organs were removed and freed from adhering tissue. In each case, the sample tissue was weighed, chopped into smaller pieces and placed in the oven (65°C) until a constant weight was obtained, which was usually after 24 h.

**Biochemical assays for antioxidants and oxidative stress.** For all biochemical assessments, atria, scar and the adhering tissue from the heart were removed and only the viable ventricular tissue was used.

**Antioxidants. Catalase.** Catalase activity was determined as previously described (4) and was expressed as μmoles of H₂O₂ consumed per minute per milligram protein. Glutathione peroxidase (GSHPx) activity was determined as previously described (4). Conversion of nicotinamide adenine dinucleotide phosphate reduced (NADPH) to nicotinamide adenine dinucleotide phosphate (NADP) was monitored by continuous recording of the change in absorbance at 340 nm at 1-min intervals for 5 min. Glutathione peroxidase activity was expressed as nanomoles of reduced NADPH oxidized to NADP per minute per milligram protein, with a molar extinction coefficient for NADPH at 340 nm of 6.22 × 10⁵. Superoxide dismutase (SOD) activity was determined by a previously described method (4). Superoxide dismutase activity was expressed as units per milligram protein derived from an SOD standard curve of pyrogallol oxidation obtained in the presence of commercially available SOD.

**Oxidative stress.** Oxidative stress was assessed by studying myocardial GSH and GSSG glutathione and their ratio (GSH/GSSG) as well as by quantitating lipid hydroperoxides.

**GLUTATHIONE AND REDOX RATIO.** Concentration of total glutathione (GSSG + GSH) was measured in the myocardium by the glutathione reductase/5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) recycling assay as described before (5). The rate of DTNB formation was followed at 412 nm and is proportional to the sum of GSH and GSSG present. Reduced glutathione values were calculated as the difference between total (GSSG + GSH) and GSSG concentrations.

**LIPID HYDROPEROXIDES.** This assay was done with a commercial kit that specifically detected lipid hydroperoxides (LPO-CC assay, Kamiya Biomed Co., Seattle, Washington) as described earlier (14). This procedure uses a derivative of methylene blue (10-N-methylcarbonyl-3,7-dimethylamino-10 H phenothiazine), which is specifically cleaved by lipid hydroperoxides to yield methylene blue dye, that can be quantified spectrophotometrically at 675 nm and compared with standard curve based on the same reaction with cumene hydroperoxide (15).

**Abbreviations and Acronyms**

- **AT₁** = angiotensin II type 1
- **GSH** = reduced glutathione
- **GSHPx** = glutathione peroxidase
- **GSSG** = oxidized glutathione
- **LVEDP** = left ventricular end-diastolic pressure
- **LVPSP** = left ventricular peak systolic pressure
- **MI** = myocardial infarction
- **RAS** = renin-angiotensin system
- **SOD** = superoxide dismutase

**Hemodynamic and tissue weight determinations.**
Proteins and statistical analysis. Proteins were determined by the method described by Lowry et al. (16). All results are expressed as mean value ± SEM. For statistical analysis of the data, group means were compared by one-way analysis of variance (ANOVA) followed by the Bonferroni test. A value of p < 0.05 was considered significant. Two-way ANOVA test (SPSS System, Windows 10, Chicago, Illinois) was also performed using both the drug and the surgery as the interaction term.

RESULTS

General characteristics, body weight and mortality. Sham control and MI rats, both from the untreated and losartan-treated groups, were monitored periodically. In terms of general appearance and behavior, nothing unusual was noted in any of the four groups. The body weight gain in both the treated and untreated MI groups was slightly lower than in their respective sham control groups, but the differences were not significant (p > 0.05). Mortality in the coronary-artery-ligated animals during or immediately after the surgery was about 20%. Another ~15% of the animals died within 24 h following the surgery. The untreated MI rats appeared lethargic, showing signs of dyspnea in the later part (12 to 16 weeks) of the study. In contrast, these signs were not present in the losartan-treated MI rats.

Tissue weights and hemodynamics. Results from the lung and liver wet/dry weight ratios and the hemodynamic assessments are summarized in Table 1. The ratio of wet to dry weight in lung and liver of MI rats was significantly higher in the 16-week untreated group. Treatment with losartan ameliorated the increase in these ratios.

Animals were assessed for LVSP and LVEDP pressures. A significant increase in the LVEDP as well as a significant decline in the LVSP were noted in the MI rats compared with their controls. Losartan treatment attenuated the rise in LVEDP in the MI group. Left ventricular peak systolic pressure in the losartan-treated MI group was no longer different from the losartan-treated control group (Table 1).

Antioxidant enzyme activities. Myocardial catalase, GSHPx and SOD activities were examined in the viable myocardium at 16 weeks with and without losartan treatment (Fig. 1A to C). Catalase activity was significantly decreased (~25%) in the 16-week untreated MI group compared with its respective sham control group (Fig. 1A). Glutathione peroxidase activity was depressed by about 38% relative to sham control values (Fig. 1B). A similar trend was seen with respect to SOD activity (~42%) (Fig. 1C). The catalase activity in the 16 W MI group treated with losartan showed some improvement; however, this change was not statistically significant. Losartan treatment in the 16 W C animals resulted in a significant increase in the catalase activity compared with the untreated MI group (Fig. 1A).

Table 1. Lung and Liver Wet/Dry Weight Ratios and Hemodynamic Parameters at 16 Weeks Post-MI With and Without Losartan Treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Untreated 16 WC</th>
<th>Untreated 16 MI</th>
<th>Losartan-Treated 16 WC</th>
<th>Losartan-Treated 16 MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung (ratio)</td>
<td>4.5 ± 0.16</td>
<td>6.13 ± 0.26*</td>
<td>4.6 ± 0.13</td>
<td>4.46 ± 0.10</td>
</tr>
<tr>
<td>Liver (ratio)</td>
<td>3.16 ± 0.10</td>
<td>4.01 ± 0.2*</td>
<td>3.0 ± 0.18</td>
<td>2.97 ± 0.04</td>
</tr>
<tr>
<td>LVSP (mm Hg)</td>
<td>124.3 ± 1.9</td>
<td>89.58 ± 2.9*</td>
<td>117 ± 12.66</td>
<td>95.27 ± 3.9</td>
</tr>
<tr>
<td>LVEDP (mm Hg)</td>
<td>3.38 ± 0.4</td>
<td>26.11 ± 1.5*</td>
<td>2.83 ± 0.4</td>
<td>8.62 ± 1*</td>
</tr>
</tbody>
</table>

Values are mean ± SE of 6 to 8 animals. *Significantly different (p < 0.05) from the respective control (16 WC) group. †Significantly different from the respective untreated MI group.

LVEDP = left ventricular end-diastolic pressure; LVSP = left ventricular peak systolic pressure; 16 WC = 16-week sham control; 16 MI = 16-week post-myocardial infarction (Post-MI).

Figure 1. Myocardial antioxidant enzyme activities in the 16-week control (CONT) and myocardial infarction (MI) rats with and without losartan treatment. Data are mean ± standard error of the mean, from 5 to 7 animals, with each assay done in duplicate. (A) catalase; (B) glutathione peroxidase; (C) superoxide dismutase. *Significantly different from the respective control group; †Significantly different (p < 0.05) from the respective untreated control and MI groups.
activity (Fig. 1A). Losartan treatment resulted in significant improvement in GSHPx activity in the MI group as compared with the 16 W untreated MI group (Fig. 1B). In the 16 W MI group treated with losartan, SOD activity remained unchanged compared with the 16 W MI untreated group (Fig. 1C).

Glutathione (reduced and oxidized). Myocardial GSH and GSSG contents were examined in the 16-week sham and MI groups with and without losartan treatment (Table 2). Myocardial GSH content at 16 weeks in the untreated MI group was significantly (p < 0.001) decreased by about 40% compared with its respective sham control group. Oxidized glutathione content was increased by about 114% in the 16 W MI group (Table 2). The GSH content in the losartan-treated C as well as MI groups was significantly improved. Oxidized glutathione content in the losartan-treated MI group was decreased and the values no longer were different from the respective sham control group. Redox ratio was also assessed in the 16 W sham control and MI group with and without losartan treatment; the results are presented in Figure 2A. The redox ratio was significantly depressed in the 16 W MI group compared with its respective sham control group. This ratio was significantly improved in both the control and MI groups treated with losartan (Fig. 2A).

Lipid hydroperoxides. In order to assess the degree of lipid peroxidation during heart failure, lipid hydroperoxide formation was determined (Fig. 2B). Lipid hydroperoxide content was significantly higher (p < 0.05) in the 16 W MI group compared with its respective sham control group. This increase in the lipid hydroperoxide content was significantly attenuated (p < 0.05) in the 16 W MI group treated with losartan (Fig. 2B).

Analysis of the data using two-way ANOVA further confirmed that losartan had an effect on all parameters in the surgery (MI) group except for LVPSP, SOD and catalase.

**DISCUSSION**

Heart failure and remodeling. Animals in the 16-week untreated MI group exhibited congestive heart failure as indicated by labored breathing, an increase in LVEDP, decrease in LVPSP and the presence of lung and liver congestion. Losartan treatment modulated the increase in LVEDP. Furthermore, there was no lung or liver congestion in the losartan group. This study also demonstrates for the first time that losartan, in addition to reducing cardiac remodeling and improving survival as reported by other investigators (13,17,18), also reduces oxidative stress and maintains myocardial endogenous antioxidants in the MI model of heart failure.

Renin-angiotensin system and heart failure: angiotensin-converting enzyme inhibition and AT₁ blockade. Angiotensin II (AII) exerts its physiological effects by binding either to AT₁ or AT₂ receptors located on the plasma membrane of various tissues including the heart (12). Angiotensin-converting enzyme inhibitors have a proven role in the management of patients (3) and animals with heart failure (4). However because of the limited efficacy of angiotensin-converting enzyme inhibitors in blocking the renin-angiotensin system, angiotensin II receptor blockers offer an attractive alternative for heart failure therapy.

**Table 2.** Myocardial Reduced (GSH) and Oxidized (GSSG) Glutathione Levels in Sham Control and Post-MI Rats at 16 Weeks With and Without Losartan Treatment

<table>
<thead>
<tr>
<th>Post-MI Duration</th>
<th>GSH (μmol/g tissue wt)</th>
<th>GSSG (μmol/g tissue wt)</th>
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<tbody>
<tr>
<td>C Post-MI</td>
<td>71.53 ± 1.32</td>
<td>42.68 ± 1.3*</td>
</tr>
<tr>
<td>16 weeks</td>
<td>88.66 ± 4.1†</td>
<td>77.73 ± 7.1†</td>
</tr>
<tr>
<td>16 weeks + losartan</td>
<td>8.63 ± 0.51</td>
<td>18.51 ± 1.2*</td>
</tr>
<tr>
<td>Post-MI</td>
<td>7.25 ± 0.4</td>
<td>8.12 ± 1.32</td>
</tr>
</tbody>
</table>

Values are mean ± SE of 5 to 7 hearts. *Significantly different (p < 0.001) from the respective control (C) group. †Significantly different from the respective untreated control or myocardial infarction (MI) group.

GSH = reduced glutathione; GSSG = oxidized glutathione.

**Figure 2.** Redox ratio (A) and lipid hydroperoxide content (B) in the 16-week control and MI rats with and without losartan treatment. Data are mean ± SEM from 8 to 10 animals with each assay done in duplicate. *Significantly different (p < 0.05) from the respective control group; †Significantly different from the respective untreated control or myocardial infarction group.
RAS and their additional effects on bradykinin and prostaglandin metabolism (10, 19, 20), AII receptor blockers are considered an attractive alternative to angiotensin-converting enzyme inhibitors.

Losartan selectively and specifically inhibits AT$_1$-mediated actions of AII irrespective of the pathway by which AII is formed (12), and it is considered to be about 10,000 times more selective for AT$_1$ receptors (21). The effects of losartan on the RAS have been examined before (17). Furthermore, losartan is known to have no inhibitory effects on vasopressin, bradykinin or prostaglandin metabolism (10). Numerous studies have demonstrated reversal of left ventricular hypertrophy, reduced fibrosis, improvement in coronary flow and cardiac function following losartan treatment (18, 22). It is known that captopril also improves the prognosis (3) as well as myocardial SOD, losartan treatment (18, 22). It is known that captopril also improves the prognosis (3) as well as myocardial SOD, losartan treatment (18, 22).

**Oxidative stress and heart failure.** Myocardial antioxidants are dynamic in nature and have been reported to change in various physiological and pathological conditions, including hypertrophy (23), exercise (24) and adriamycin-induced cardiomyopathy (25). It is also known that different enzymatic and nonenzymatic antioxidants respond uniquely in a variety of oxidative stress conditions. For example, hypoxia resulted in a reduction in MnSOD and GSHPx and catalase activities following MI (4). Losartan, in the present study, improved GSHPx activity. Because both angiotensin-converting enzyme inhibition with captopril (4) and AT$_1$ blockade with losartan in this study modulated heart failure, we suggest that the beneficial effects of angiotensin-converting enzyme inhibition are indeed due to the blockade of all effects rather than any other humoral changes seen with angiotensin-converting enzyme inhibition.

Another study showed AII increases in hypertensive rats mediated actions of AII irrespective of the pathway by which AII is formed (12), and it is considered to be about 10,000 times more selective for AT$_1$ receptors (21). The effects of losartan on the RAS have been examined before (17). Furthermore, losartan is known to have no inhibitory effects on vasopressin, bradykinin or prostaglandin metabolism (10). Numerous studies have demonstrated reversal of left ventricular hypertrophy, reduced fibrosis, improvement in coronary flow and cardiac function following losartan treatment (18, 22). It is known that captopril also improves the prognosis (3) as well as myocardial SOD, losartan treatment (18, 22). It is known that captopril also improves the prognosis (3) as well as myocardial SOD, losartan treatment (18, 22).

The severe heart failure stage in this study was accompanied by depressed levels of GSH and increased levels of GSSG. A significant decrease in the redox ratio (GSH/GSSG) indicated an increase in the oxidative stress (29) in the viable myocardium of the MI rats as compared with the control. In addition to the redox ratio, the lipid hydroperoxide content was also significantly higher in the MI rats, further supporting the observation of an increase in oxidative stress in these hearts. The increased concentration of lipid hydroperoxides in cellular membranes is indicative of actual damage mediated by oxygen radicals (15). Changes in the GSH levels as well as lipid hydroperoxides in the losartan-treated groups could not be attributed to any direct interference by the drug itself. This is due to the fact that the half-life of losartan is about 2 h (30), whereas the assays for glutathione and lipid hydroperoxides in this study were done at 48 h after the last injection of the drug. Thus, there will hardly be any residual activity of losartan, and the changes seen in these parameters are most likely the result of in vivo effects of the drug.

Oxidative stress was significantly reduced by treatment with losartan, as indicated by an increase in the redox ratio and decreased lipid hydroperoxide content in the MI group. We have previously reported that heart failure after MI in rats correlated with depressed antioxidant reserve and increased oxidative stress (5). Diet supplemented with biological antioxidant vitamin E improved endogenous levels of vitamin E and cardiac function, suggesting a cause-and-effect relationship between increased oxidative stress and cardiac dysfunction (6).

A recent study on atherosclerosis Apo-E deficient mice documented that treatment with losartan reduced low density lipoprotein peroxidation in the atherosclerotic lesion area (31). Losartan treatment in rabbits normalized vascular superoxide anion ($\text{O}_2^-$) production in a dose-dependent manner and prevented tolerance to nitroglycerine (32). Another study showed AII increases in hypertensive rats that were associated with increased vascular smooth-muscle cell $\text{O}_2^-$ production via NADH/NADPH oxidase activation and losartan treatment in these rats normalized $\text{O}_2^-$ production (33). It has also been reported that in oxidative stress conditions, there is an increase in superoxide anion, which reacts with nitric oxide (a potent vasodilator) to produce peroxynitrite that causes vasoconstriction (34). Thus, losartan appears to modulate free radical production, increase antioxidants and reduce oxidative stress.

**Arrhythmias and sudden death.** Catecholamine-induced arrhythmias and sudden death due to excessive catecholamines are suggested to be mediated by increased oxidative stress (35). The reduced incidence of sudden death due to losartan, reported in the Evaluation of Losartan in the Elderly (ELITE 1) Study (13), may partly be explained by a decrease in production of catecholamines due to AT$_1$ blockade with this drug (36), as well as a reduction in oxidative stress because of an inhibition of RAS seen both in this and in earlier (4) studies.

**Conclusions.** This study documents for the first time that inhibition of the AT$_1$ receptor by losartan, not only improves cardiac function but also causes an improvement...
in the myocardial antioxidant reserve and decreased oxidative stress in MI rats, suggesting a new role for losartan in the treatment of heart failure. The precise mechanism(s) for decrease in oxidative stress and improvement in antioxidant reserve after losartan treatment is unclear at this time. The study also emphasizes that modulation of the RAS, either by angiotensin-converting enzyme inhibition or by AT1 receptor blockade, is beneficial and may not involve other humoral changes seen after angiotensin-converting enzyme inhibition.

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