Temocapril treatment ameliorates autoimmune myocarditis associated with enhanced cardiomyocyte thioredoxin expression

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Received 25 January 2002; accepted 25 March 2002

Abstract

\textbf{Objective}: Thioredoxin (TRX) is a redox regulatory protein that protects cells from various stresses. Angiotensin-converting enzyme (ACE) inhibitor was reported to enhance endogenous antioxidant enzyme activities. This study was carried out to investigate whether temocapril, a novel non-sulfhydryl-containing ACE inhibitor, reduces the severity of myocarditis via redox regulation mechanisms involving TRX. \textbf{Methods and Results}: In normal rat myocytes in vitro and in vivo, Western blot showed that temocapril enhanced cytosolic redox regulatory protein TRX expression, but that neither mitochondrial TRX2 nor antioxidant enzymes, such as copper–zinc superoxide dismutase (Cu/Zn-SOD) or manganese superoxide dismutase (Mn-SOD) expression, was up-regulated by the preconditioning treatment. In rats with experimental autoimmune myocarditis (EAM), the severity of myocarditis and the protein carbonyl contents were less increased in temocapril treatment (10 mg/kg/day, orally) from day 1 to day 21, but not in temocapril treatment from day 15 to day 21. An immunohistochemical study showed that TRX stain was enhanced in infiltrating inflammatory cells and in damaged myocytes. Considering the characteristics of this model that myocardial inflammation begins around day 15 and increases until day 21, temocapril treatment for 3 weeks might be thought of as a preconditioning treatment. \textbf{Conclusions}: The results suggest that TRX and the redox state modified by TRX may play a crucial role in the pathophysiology of EAM. Temocapril ameliorates myocarditis associated with inducing TRX up-regulation in a preconditioning manner, although the mechanism of TRX up-regulation by temocapril remains to be elucidated. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: ACE inhibitors; Free radicals; Infection/inflammation; Myocarditis

1. Introduction

In humans, acute myocarditis is a potentially lethal disease, and frequently precedes the development of dilated cardiomyopathy (DCM). The autoimmune giant cell myocarditis in rats mimics human fulminant myocarditis in the acute phase [1]. In acute myocarditis, an imbalance between the occurrence of reactive oxygen species (ROS) and the cellular antioxidant defense mechanism plays a key role in myocardial injury [2,3]. Increased ROS are able to induce severe cardiovascular dysfunction by their direct attack on intercellular biomolecules such as contractile proteins or ion channels [4]; also, the imbalance of intracellular oxido-reductive state (redox) may lead to the activation of stress sensitive signaling pathways [5], increasing apoptosis [6], and potentially contributing to the development of heart failure [7].

Cells have developed elaborate defence systems against oxidative stresses. Among those, a pivotal role of the thiol-mediated redox systems has been recognized. Thioredoxin (TRX) is known to contain a redox-active disulfide/dithiol within the conserved active site sequence Cys-Gly-Pro-Cys, and exhibits reducing activity of oxidized thiol groups on proteins [8]. Recently, we reported that TRX is
up-regulated by acute inflammatory stimuli and may play an important protective role in the pathogenesis and development of myocarditis [9].

Angiotensin-converting enzyme (ACE) inhibitors, frequently used in the treatment of hypertension and cardiac dysfunction, have been clarified to possess other pharmacological effects: they attenuate post-ischemic myocardial dysfunction [10], inhibit complement-mediated myocardial injury [11], and prevent endothelial dysfunction induced by oxidized low-density lipoprotein [12,13]. It has been widely postulated that ROS are causally involved in these conditions. It was reported that ACE inhibitors enhanced myocardial endogenous antioxidant enzyme activities such as copper, zinc superoxide dismutase (Cu/Zn-SOD) and selenium-dependent glutathione peroxidase in normal mice [14]. Moreover, ACE inhibitors reduced myocardial inflammation and necrosis in murine viral myocarditis [15,16]. These findings prompted us to investigate whether temocapril, a non-sulfhydryl containing ACE inhibitor, reduces the severity of myocarditis by redox regulating mechanisms. In this study, we investigate whether temocapril treatment induces cardiomyocyte TRX expression and if so, whether the enhanced TRX expression by temocapril treatment has a beneficial effect on myocarditis.

2. Methods

2.1. Culture of neonatal rat cardiomyocytes and temocapril treatment

Cardiac ventricles from 1- to 4-day-old Lewis rats were minced and dissociated with 0.125% trypsin (Sigma). The dispersed cells were incubated for 60 min at 37 °C. Nonattached myocytes were collected and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Nissui Pharmaceutical Co.) supplemented with 10% fetal calf serum (FCS), in a humidified atmosphere of 5% CO₂ at 37 °C. Bromodeoxyuridine (BrdU, 100 μmol/l) was added during the first 48 h to prevent proliferation of nonmyocytes. Spontaneously beating myocyte-rich cultures (final cell density, 10⁵ cells/cm²) were then incubated for 48 h without BrdU, followed by a final incubation in 10 ml fresh DMEM containing 10% FCS. During this final incubation, myocytes were treated with temocapril (10⁻⁷ to 10⁻⁵ mol/l or absence) for 24 h. Cells were lysed for assaying the TRX and SOD protein expressions. Temocapril was kindly provided by Sankyo Co. (Tokyo, Japan).

2.2. Treatment protocol

2.2.1. Preconditioning treatment

To investigate the inductive effect of temocapril treatment on TRX and SOD expression in vivo, 6- to 7-week-old Lewis rats (SHIMIZU Laboratory Supplies, Japan) were randomly separated into the temocapril-treated (n=8) or temocapril-untreated (n=8) groups. Temocapril-treated rats were administered water containing 0.1 mg/ml temocapril (10 mg/kg/day) [17] and killed on day 8 (n=4) or day 15 (n=4). Temocapril-untreated rats were administered tap water alone and killed on day 8 (n=4) or day 15 (n=4).

2.2.2. Immunization and treatment

EAM was induced as previously described [9]. Porcine cardiac myosin (Sigma) was injected subcutaneously in the foot pads with 0.1 ml of myosin (10 mg/ml) mixed with an equal volume of Freund’s complete adjuvant (FCA) supplemented with Mycobacterium tuberculosis H37Ra (Difco) on days 1 and 8. Temocapril-treated rats (n=10) were administered temocapril (10 mg/kg/day) from day 1 to day 21. Temocapril-untreated rats (n=10) were administered tap water. In experiment II, temocapril-treated rats (n=10) and temocapril-untreated rats (n=9) were administered temocapril or tap water separately from day 15 to day 21. Additional groups were control rats (without myosin injection) treated from day 1 to day 21 in experiment I (temocapril-treated, n=4; temocapril-untreated, n=4) and from day 15 to day 21 in experiment II (temocapril-treated, n=4; temocapril-untreated, n=4). All rats were killed on day 22 under ether anesthesia. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.3. Histopathology

At sacrifice, macroscopic findings were graded according to the following criteria: 0 (normal appearance), 1 (a focal discolored area), 2 (multiple or diffuse discolored areas and not exceeding 1/3 of the heart), 3 (diffuse discolored areas not exceeding 2/3 of the heart), and 4 (diffuse discolored areas exceeding 2/3 of the heart). Pericardial effusion was graded: 0 (none), 1 (mild), and 2 (massive). After macroscopic examination, the ventricles were fixed in 10% formalin, transversely sliced, embedded in paraffin, and stained with hematoxylin and eosin, to examine the degree of myocardial damage and infiltration of inflammatory cells. Microscopic findings were graded: 0 (normal), 1 (lesion extent not exceeding 1% of a transverse section), 2 (not exceeding 25%), 3 (not exceeding 50%), 4 (exceeding 50%). We measured the lesion area using a square lattice scale in an eye lens of a microscope.

2.4. Immunohistochemistry

To analyze the immunohistochemical stains of TRX and TRX2, we used the immunoperoxidase technique [9]. Briefly, deparaffinized slides were incubated with 3% H₂O₂ for 10 min to inactivate the endogenous peroxidase activity. The primary antibodies (rabbit anti-mouse TRX,
rabbit anti-mouse TRX2) or non-immune IgG were added and incubated overnight. The rabbit anti-mouse TRX and rabbit anti-mouse TRX2 antibodies cross-react on rat [9]. Biotinylated and affinity purified anti-rabbit IgG (Dako) was used as a secondary antibody and incubated for 30 min. An avidin–biotin complex was sequentially added for 5 min incubation with substrate 0.1% 3′,3′-diaminobenzidine, followed by hematoxylin nuclear counterstaining.

2.5. Western blotting

The hearts were homogenized and lysed for 30 min. Equal amounts of protein (10–20 μg protein/lane), estimated by the Bradford method (Bio-Rad), were electrophoresed on a 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and sequentially electrophoretically transferred to a polyvinylidene difluoride microporous membrane (Millipore). After blocking with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 0.05% Tween 20 at 4°C overnight, the membrane was incubated with the primary antibody, and then with a peroxidase-linked secondary antibody (Amersham). Chemiluminescence was detected with an ECL Western blot detection kit (Amersham) and semiquantitatively analyzed using an NIH Image system.

2.6. Detection of oxidized proteins in myocardium

Oxidative inactivation of enzymes and oxidative modification of proteins by metal-catalyzed oxidation reactions are accompanied by the generation of protein carbonyl derivatives. Oxidized proteins were detected using an oxidized protein detection kit (OxyBlot, Oncor) [18]. The OxyBlot provides reagents for sensitive immunodetection of carbonyl groups, which is a hallmark of the oxidation status of all proteins. The carbonyl groups in the protein side chains are derivatized to 2,4-dinitrophenylhydrazine (DNP-hydrazone). The DNP-derivatized protein samples were separated on a 12% SDS–PAGE followed by Western blotting. The filters were incubated with a primary antibody specific to the DNP moiety of the proteins, followed by incubation with a peroxidase-linked secondary antibody. The amount of protein carbonyl groups extracted from 20 μg of sample proteins was measured using an image analyzer and compared with 2.5 μl of the DNP-protein standard mixture (an internal control for OxyBlot).

2.7. Statistics

All values were expressed as means±standard deviation (S.D.). One-way analysis of variance (ANOVA), following by Fisher’s protected least significant difference test, was performed. A value of P<0.05 was considered statistically significant.

3. Results

3.1. The effects of temocapril treatment on redox proteins TRX and TRX2, and antioxidant enzymes Cu/Zn-SOD and Mn-SOD in cultured cardiomyocytes in vitro and in normal rats in vivo

To examine the inductive effects of temocapril on redox proteins TRX and TRX2, and antioxidant enzymes Cu/Zn-SOD and Mn-SOD expressions in vitro, we treated cultured neonatal cardiomyocytes with temocapril for 24 h, followed by Western blotting as described in Section 2.1. As shown in Fig. 1, temocapril treatment enhanced TRX protein expression by 1.9-fold, but it had no effects on TRX2, Cu/Zn-SOD or Mn-SOD protein expression.

In the in vivo study, normal rats were treated with temocapril by preconditioning for 1 or 2 weeks as described in Section 2.2. The Western blotting showed that the expression of TRX on day 15, but not on day 8, was enhanced in temocapril-treated rats relative to that in the temocapril-untreated group. TRX2, Cu/Zn-SOD and Mn-SOD expression had not been significantly changed throughout the entire period compared with the temocapril-untreated group (Fig. 2). These findings showed that the induction of TRX expression by temocapril took at least longer than 15 days.

3.2. Effects of temocapril on rats with EAM

EAM was induced in all the immunized rats. The survival in each group was 100% in this study. On day 22 at sacrifice, the hearts in rats without temocapril treatment showed severe and diffuse discoloring (the arrow, Fig. 3A) with massive pericardial effusion (the arrowhead, Fig. 3A). Extensive injuries to myocytes with various kinds of inflammatory changes, such as fragments of necrotic myocardial fibers, mononuclear cells, polymorphonuclear neutrophils, eosinophils, and multinucleated giant cells (arrows, Fig. 3B) were observed. To determine whether the up-regulation of TRX by temocapril preconditioning treatment has a beneficial effect on subsequent myocarditis, we designed two experiments. In experiment I, temocapril administration from day 1 to 21, myocarditis was ameliorated to a certain extent: some hearts showed mild and focal discolor macroscopically and a few infiltrating inflammatory cells microscopically (Fig. 3C, E, and F). The severity of myocarditis in the temocapril-treated group was decreased moderately but significantly as assessed by measuring HW/BW, macroscopic and microscopic scores (Table 1). In experiment II, temocapril administration from day 15 to 21, the severity of myocarditis was not decreased significantly compared with those of temocapril-untreated rats (Table 1 and Fig. 3G and H). These results suggested that the up-regulation of TRX could protect rats from subsequent myocardial inflammation.

No abnormalities were shown in cardiac pathology in
control rats with/without temocapril-treatment in experiments I and II.

3.3. TRX and TRX2 expressions in rats with myocarditis

Our previous study showed that TRX expression was up-regulated in acute myocarditis and that the TRX expression corresponded well to the severity of myocarditis [9]. In the present study, Western blotting showed that TRX expression was up-regulated 2.7-fold in rats with EAM compared with normal controls (Fig. 4). But TRX2 expression was not significantly changed. It was suggested that TRX but not TRX2 was induced by myocardial inflammation.

Immunohistochemistry was performed to determine the histological localization of TRX and TRX2 in the hearts of EAM. TRX (Fig. 5A) was strongly stained in both infiltrating inflammatory cells (arrows) in the necrotic areas

Fig. 1. Effects of temocapril on TRX, TRX2, Cu/Zn-SOD, and Mn-SOD protein expressions in cultured neonatal rat cardiomyocytes. Cultured myocytes (1×10⁷/cm²) were treated with temocapril (10⁻⁷ to 10⁻⁶ or absence) for 24 h. (A) Western blot analysis. Ten micrograms of protein of each sample were loaded. (B) Densitometric analysis of relative protein levels. Temocapril treatment enhanced TRX protein expression 1.9-fold, but it had no effects on TRX2, Cu/Zn-SOD or Mn-SOD protein expression. Values were derived from four independent experiments, represented as the percentage of the absence of temocapril treatment. *P<0.05 vs. absence of temocapril treatment.

Fig. 2. Effects of temocapril on myocardial TRX, TRX2, Cu/Zn-SOD, and Mn-SOD protein expression in normal rats. (A) Western blot analysis. Twenty micrograms of protein of each sample were loaded. In lanes 1 and 2, lysates were loaded from two different hearts of temocapril-untreated rats (control); in lanes 3 and 4, and lanes 5 and 6, lysates were loaded from two hearts of temocapril-treated rats and killed on day 8 and day 15, respectively. (B) Densitometric analysis of relative protein levels. The expression of TRX on day 15 was enhanced in temocapril-treated rats compared with those in the temocapril-untreated group. TRX2, Cu/Zn-SOD and Mn-SOD expression had not been significantly changed throughout the entire period compared with the temocapril-untreated group. Values were derived from four animals, represented as the percentage of the control value. *P<0.01 vs. controls.
and damaged myocytes (arrowheads) in the perinecrotic lesions. TRX2 (Fig. 5B) stains were not enhanced in inflammatory lesions or in damaged myocytes. These results, in addition to our previous study [9], suggested that TRX but not TRX2 has a crucial role in the pathophysiology of EAM.

Table 1
Histological analysis and heart weight/body weight ratio (means±S.D.)

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>HW/BW (mg/g)</th>
<th>Pericardial effusion score</th>
<th>Macroscopic score</th>
<th>Microscopic score</th>
</tr>
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<tr>
<td>Experiment I (temocapril treatment from day 1 to day 21)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocarditis/temocapril(−)</td>
<td>10</td>
<td>5.18±0.24</td>
<td>2.0±0</td>
<td>3.8±0.2</td>
<td>3.8±0.2</td>
</tr>
<tr>
<td>Myocarditis/temocapril(+)</td>
<td>10</td>
<td>4.58±0.31**</td>
<td>1.4±0.3*</td>
<td>2.9±0.4**</td>
<td>2.7±0.4**</td>
</tr>
<tr>
<td>Controls/temocapril(−)</td>
<td>4</td>
<td>3.07±0.31</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Controls/temocapril(+)</td>
<td>4</td>
<td>2.98±0.29</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Experiment II (temocapril treatment from day 15 to day 21)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocarditis/temocapril(−)</td>
<td>9</td>
<td>4.85±0.48</td>
<td>1.7±0.5</td>
<td>3.4±1.3</td>
<td>3.4±0.7</td>
</tr>
<tr>
<td>Myocarditis/temocapril(+)</td>
<td>10</td>
<td>4.81±0.67</td>
<td>1.5±0.7</td>
<td>3.3±0.7</td>
<td>3.2±1.0</td>
</tr>
<tr>
<td>Controls/temocapril(−)</td>
<td>4</td>
<td>2.85±0.31</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Controls/temocapril(+)</td>
<td>4</td>
<td>2.81±0.26</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Myocarditis/temocapril(+), rats with autoimmune myocarditis treated with temocapril; myocarditis/temocapril(−), rats with autoimmune myocarditis untreated with temocapril; controls/temocapril(+), normal rats treated with temocapril; controls/temocapril(−), normal rats untreated with temocapril; HW/BW, the ratio of heart weight and body weight. *P<0.05, **P<0.01 vs. myocarditis/temocapril(−).
It was suggested that cellular protein oxidative damage by increased ROS occurred in myocarditis. In rats with non-myocarditis, the protein carbonyl contents were not significantly changed by the temocapril treatment (P=ns vs. controls). In rats with myocarditis, the protein carbonyl contents were decreased by the temocapril treatment in experiment I (P<0.01, Fig. 6) but not in experiment II (P=ns) compared with the untreated rats (data not shown), suggesting that temocapril prevented cellular proteins from oxidation in a preconditioning manner.

4. Discussion

The present study provided evidence that temocapril treatment ameliorated EAM and prevented cellular proteins from oxidation in rats, associated with enhanced cardiomyocyte redox regulatory protein TRX expression. The protein oxidation is an early intracellular indicator of tissue damage resulting from ROS. Oxidative inactivation of enzymes could lead to the disruption of cellular metabolism and seriously impair the ability of cells to repair this damage. In the present study, protein carbonyl content in myocarditis was increased, suggesting that oxidative modification of cellular proteins occurred due to increased ROS during the acute myocardial inflammation.

TRX is a stress-inducible protein [19]. It has been shown that TRX is involved in cellular protective mechanisms against various types of stress, such as ischemia/reperfusion, X-ray irradiation, hydrogen peroxide and inflammatory cytokines [19]. Moreover, TRX is a scavenger of ROS, and recombinant human TRX has protective activity against H$_2$O$_2$. Overexpression of TRX in transgenic mice showed a protective activity against post-ischemic reperfusion injury in brain [20]. In the present study, TRX expression was enhanced in infiltrating

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In rats with myocarditis, the protein carbonyl contents were markedly increased (P<0.01 vs. control rats, Fig. 6).

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Fig. 6. Changes in the protein carbonyl contents of the myocardium by temocapril treatment in rats with or without myocarditis. (A) OxyBlot analysis. Twenty micrograms of protein of each sample were loaded. C, normal control rat; T, normal rat with temocapril treatment; M, rat with myocarditis without temocapril treatment; M+T, rat with myocarditis with temocapril treatment from day 1 to 21 in experiment I. All rats were killed on day 22. (B) Densitometric analysis of relative protein carbonyl contents. In rats with non-myocarditis, the protein carbonyl contents were not significantly changed by temocapril treatment. In rats with myocarditis, the protein carbonyl contents were markedly increased, and the increasing of protein carbonyl contents were suppressed by temocapril treatment in experiment I, but not in experiment II (data not shown) compared with the untreated rats. Values were derived from four animals and represented as the percentage of the DNP-protein standards. *P<0.01 vs. normal controls; †P<0.01 vs. rats with myocarditis without temocapril treatment.

Inflammatory cells in the necrotic areas and in damaged myocytes in perinecrotic lesions. Taken together, the upregulated TRX may act as protection against ROS-mediated myocardial injury. In the preconditioning experiment, it was shown that temocapril enhanced TRX protein expression in cultured myocytes and in normal hearts. In rats with EAM, temocapril treatment for 3 weeks reduced the severity of myocardial inflammation and necrosis in experiment I where the improvement by the drug was small but clear, but not in experiment II with no preconditioning treatment. In addition, the protein carbonyl contents were less increased in experiment I, but not in experiment II, suggesting that temocapril may prevent cellular proteins from oxidation by enhancing redox regulatory protein TRX expression. Considering the characteristics of this model that myocardial inflammation begins around day 15 and increases until day 21, temocapril treatment for 3 weeks starting simultaneously with myosin immunization may be thought of as a preconditioning treatment. Indeed, the induction of TRX by temocapril was
positive at 15 days, but not at 8 days, as shown in the preconditioning experiment (Fig. 2). Also, preliminary study showed that the induction of TRX by temocapril took longer than 15 days. Enhanced myocardial TRX expression by this procedure may be beneficial for protection from subsequent myocardial inflammation.

Mitochondrial localized TRX2, a recently cloned novel TRX whose function was unclear [21], was not up-regulated in rats with EAM in this study. The function of TRX2 in myocarditis is still unknown and further studies might be needed regarding the role and regulation of TRX2.

It has been shown that captopril and enalapril, other ACE inhibitors, increased Cu/Zn-SOD, Mn-SOD and glutathione peroxidase activities [13,22], and glutathione content in mouse tissues [23]. In the present study, the preconditioning treatment of temocapril in rats and in cultured cardiomyocytes up-regulated TRX expression, but temocapril treatment did not change the myocardial Mn-SOD and Cu/Zn-SOD protein expressions in the present protocol, although we have not assayed their enzymatic activities. Long-term ACE inhibition may increase and stimulate bradykinin production which in turn results in the release of nitric oxide. It was proposed that the over-production of nitric oxide by ACE inhibitor may cause a situation of oxidative stress intense enough to trigger an increase in antioxidants, but not substantial enough to cause oxidative damage [24]. We recently reported that TRX was induced by nitric oxide donors [20] and by prostaglandin E1 [25] in vitro. Accordingly, TRX was actually up-regulated by temocapril in the present study, although the mechanisms of TRX up-regulation by temocapril remain unclear. Considering this notion, ACE inhibitors may represent a novel antioxidant strategy that targets oxidative stress at its source. It is unclear whether temocapril has effects other than as an ACE inhibitor on cardiac myocytes, for example, immunomodulating effects upon myocytes, although examinations of the effect of structurally dissimilar ACE inhibitors on TRX expression are currently under investigation.

In summary, temocapril treatment enhances cardiomyocyte TRX protein expression in vitro and in normal rats in vivo. The treatment prevented cellular proteins from oxidation and ameliorated myocarditis in rats by inducing TRX up-regulation in a preconditioning manner in vivo. The present study provided evidence that temocapril ameliorated EAM associated with enhanced cellular TRX up-regulation.

Acknowledgements

We thank Drs. Toru Tanaka and Fumihito Hosoi, Institute for Virus Research, Kyoto University, for supplying TRX2 antibody.

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and glutathione peroxidase activities are increased by enalapril and captopril in mouse liver. FEBS Lett 1995;361:22–24.