Role of intracellular antioxidant enzymes after in vivo myocardial ischemia and reperfusion

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In vivo ischemia and reperfusion of the heart induce profound myocardial inflammation that results in cellular damage and tissue dysfunction (3). During this inflammatory process, reactive oxygen species (ROS) are formed and may significantly contribute to myocardial injury (19). Although the exact cellular sources are uncertain, neutrophilic NADPH oxidase, endothelial xanthine oxidase, and mitochondrion-derived oxidants are putative sources of ROS. Similarly, there are many intracellular and extracellular enzymes that catalyze the detoxification of ROS, including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). Subsequently, to the discovery of these and other antioxidant enzymes, numerous investigators have sought to attenuate the extent of myocardial injury by the administration of various pharmacological agents that mimic the actions of endogenous antioxidant enzymes.

However, previous studies of the role of antioxidant enzymes in the pathophysiology of myocardial ischemia and reperfusion (MI/R) have yielded conflicting results. Some studies found various forms of SOD to be cardioprotective, whereas others have reported no effect. These studies provide valuable insight into a possible therapeutic value for antioxidant enzymes, but may underestimate the true importance of native, endogenous antioxidant enzymes. More specifically, the potential benefit of modification of the various intracellular antioxidant enzymes may be more important to the ultimate cardioprotective value of any agent. The recent advent of transgenic (Tg) and knockout (KO) mice has allowed further investigation into the possible roles of SOD and other antioxidant enzymes in MI/R injury. In the present study, we sought to determine whether genetic modification of various antioxidant enzymes could affect the extent of myocardial injury after ischemia and reperfusion. Unlike previous investigations, the present study compares four genetic modifications of antioxidant enzymes in a single in vivo model of MI/R injury. Furthermore, we ascertained whether any resultant cytoprotection was associated with preservation of cardiac function after extended reperfusion.

METHODS

Mice. Copper-zinc SOD deficient (Cu/ZnSOD-KO; Ref. 35), Cu/ZnSOD-Tg (9), GPx-Tg (37), and manganese (Mn)SOD-Tg (6) mice were used in the present studies. The Cu/ZnSOD-Tg mice expressed threefold more enzyme activity than their wild-type littermates (9). The GPx-Tg mice expressed approximately eightfold more enzyme activity than their respective wild-type littermates (37). The MnSOD-Tg mice

expressed approximately threefold more enzyme activity than their littermates (6). Wild-type littermates were used as control mice in all experiments. All mice were provided by Dr. Ye-Shih Ho (Wayne State University), except the Cu/ZnSOD-Tg animals, which are commercially available (Jackson Laboratory). All animal experiments complied with the Guide for the Care and Use of Laboratory Animals [DHEW Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205] and with state and federal regulations. All experimental procedures were approved by the Louisiana State University Health Sciences Center Animal Care and Use Committee.

MI/R protocol. Littermate, nonmutant, wild-type (total n = 48), GPx-Tg (n = 12), Cu/ZnSOD-KO (n = 8), Cu/ZnSOD-Tg (n = 9), and MnSOD-Tg (n = 14) mice were used for the in vivo MI/R experiments. There was uniform gender distribution among all of the groups (overall, 50.5% male). The surgical protocol and infarct-size determination were performed similar to methods described previously (16, 28). Mice were anesthetized with injections of pentobarbital sodium (50 mg/kg ip) and ketamine hydrochloride (50 mg/kg ip), then orotracheally intubated with polyethylene-90 tubing, connected to a rodent ventilator (model 683, Harvard Apparatus) via a loose joint, and supplemented with oxygen. The ventilator was set to a tidal volume of 2.5 ml and a rate of 120 strokes/min. Body temperature was maintained at 37°C and a rate of 120 strokes/min. Body temperature was monitored with an infrared heating lamp. After a median sternotomy was performed, the chest wall was retracted to provide access to the heart. LV percent fractional shortening (%FS) was calculated according to the following equation: LV %FS = ([LVEDD – LVEDS]/LVEDD) × 100, where LVEDD and LVEDS are the LV end-diastolic and end-systolic diameters, respectively. End diastole was identified by the QRS wave from the electrocardiogram tracing and coincided with the portion of the M-mode wave immediately before the initiation of systole. End systole was measured at the locus of the waveform, in which the anterior and posterior walls were in closest proximity. Stroke volume (SV) was calculated from the product of the aortic cross-sectional area ([AoD/2]² × π) and the AoVTI. Cardiac output (CO) was calculated from the product of the SV and HR. The CO values were corrected for the animals’ weights (in µl·min⁻¹·g⁻¹).

RESULTS

Myocardial area at risk and infarct size. As shown in Table 1, all Tg mice exhibited similar body weights, ages, and left ventricle-to-body weight ratios compared with wild-type (non-Tg) littermates. Mice aged 8 wk at the time of MI/R protocol.

LV echocardiography. Transthoracic echocardiography of the left ventricle using a 15-MHz linear array transducer (15L8) interfaced with a Sequoia C256 echocardiography system (Acuson) was performed as described previously (15). M-mode (sweep speed, 200 mm/s) echocardiograms were captured from parasternal, short-, and long-axis two-dimensional views of the left ventricle at the midpapillary level. Left ventricular (LV) chamber diameter, aortic diameter (AoD), aortic velocity time integral (AoVTI), and heart rate (HR) were measured before ischemia and after 24 h of reperfusion in MnSOD-Tg (n = 6) and wild-type (n = 7) mice. For measurement of the AoVTI, angle correction of the Doppler signal was incorporated to account for the difference between the ultrasound beam and the aortic flow (≈90°). LV percent fractional shortening (%FS) was calculated according to the following equation: LV %FS = ([LVEDD – LVEDS]/LVEDD) × 100, where LVEDD and LVEDS are the LV end-diastolic and end-systolic diameters, respectively. End diastole was identified by the QRS wave from the electrocardiogram tracing and coincided with the portion of the M-mode wave immediately before the initiation of systole. End systole was measured at the locus of the waveform, in which the anterior and posterior walls were in closest proximity. Stroke volume (SV) was calculated from the product of the aortic cross-sectional area ([AoD/2]² × π) and the AoVTI. Cardiac output (CO) was calculated from the product of the SV and HR. The CO values were corrected for the animals’ weights (in µl·min⁻¹·g⁻¹). Anterior and posterior wall dimensions were also assessed in diastole and systole for both groups of mice. All data were calculated from 10 independent cardiac cycles per time point per experiment.

Statistical analysis. All experimental studies and analyses were performed in a blinded fashion. All findings were analyzed with Student’s unpaired t-test or ANOVA using StatView 4.5 software (Abacus Concepts). Values are reported as means ± SE with significance set at P < 0.05.

Table 1. Age, body weight, left ventricle weight-to-body weight ratio values for wild-type littermate and gene-targeted mice in the present study

<table>
<thead>
<tr>
<th>Gender, % male</th>
<th>Age, wk</th>
<th>Body Wt, g</th>
<th>Left Ventricle Wt/Body Wt Ratio, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>13</td>
<td>46</td>
<td>22.9 ± 0.7</td>
</tr>
<tr>
<td>GPx-Tg</td>
<td>12</td>
<td>42</td>
<td>25.0 ± 0.7</td>
</tr>
<tr>
<td>Wild type</td>
<td>12</td>
<td>58</td>
<td>20.9 ± 0.7</td>
</tr>
<tr>
<td>Cu/ZnSOD-KO</td>
<td>8</td>
<td>50</td>
<td>18.1 ± 1.9</td>
</tr>
<tr>
<td>Wild type</td>
<td>10</td>
<td>60</td>
<td>22.7 ± 1.5</td>
</tr>
<tr>
<td>Cu/ZnSOD-Tg</td>
<td>9</td>
<td>44</td>
<td>25.2 ± 0.6</td>
</tr>
<tr>
<td>Wild type</td>
<td>13</td>
<td>46</td>
<td>18.6 ± 0.7</td>
</tr>
<tr>
<td>MnSOD-Tg</td>
<td>14</td>
<td>57</td>
<td>19.9 ± 0.7</td>
</tr>
</tbody>
</table>

Values are means ± SE. GPx, glutathione peroxidase; Tg, transgenic; Cu/ZnSOD, copper-zenmperoxide dismutase; KO, knockout (deficient); Mn, manganese.
tion was significantly depressed in non-Tg but not Tg mice. This decrement in anterior wall motion was also associated with a significant decrease in %FS in the non-Tg group (Fig. 4). In addition, HR values were not different between the two groups (Fig. 5A). However, CO values (Fig. 5B) were maintained near baseline levels in the Tg group, whereas the non-Tg group experienced a significant decrease from baseline levels (and compared with MnSOD-Tg littermates).

**DISCUSSION**

It is widely accepted that MI/R induces the production of ROS (12, 38, 39). Furthermore, ROS reportedly contribute to the injurious process of MI/R. Consequently, many investigators have demonstrated cardioprotective effects of various antioxidant enzymes (24, 29) or oxidant scavengers (4) in the presence of MI/R injury. In contrast, other studies have failed to demonstrate protective effects of antioxidants or oxygen radical scavengers. In the present study, we provide primary evidence that intracellular antioxidant enzymes vary in capacity to attenuate myocardial injury after ischemia and reperfusion.

Numerous studies have addressed the contribution of postischemic oxidant production and consequent myocardial injury (8). Typically, these studies would involve the administration of SOD alone or in conjunction with another antioxidant enzyme or inhibitor of an oxidant-producing enzyme. However, it is widely appreciated that such previous studies yielded conflicting results. In many animal studies, it was found that administration of an antioxidant entity such as allopurinol (33), recombinant human SOD (2), bovine SOD

Table 2. Left ventricle chamber size in wild-type and MnSOD-Tg mice before 30 min of myocardial ischemia and after 7 days of reperfusion

<table>
<thead>
<tr>
<th></th>
<th>Chamber Diameter, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diastole</td>
</tr>
<tr>
<td><strong>Wild type (n = 7)</strong></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>3.34 ± 0.08</td>
</tr>
<tr>
<td>Post-MI</td>
<td>3.29 ± 0.10</td>
</tr>
<tr>
<td><strong>MnSOD-Tg (n = 6)</strong></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>3.67 ± 0.20</td>
</tr>
<tr>
<td>Post-MI</td>
<td>3.67 ± 0.30</td>
</tr>
</tbody>
</table>

Values are means ± SE. Baseline, values before myocardial ischemia (MI); post-MI, values after reperfusion.
(34), polyethylene glycol-conjugated SOD (7), a SOD mimetic (17), SOD plus catalase (14, 23), adenoviral extracellular SOD (21), and adenoviral MnSOD (1) attenuated myocardial injury and/or dysfunction after ischemia and reperfusion. However, in other studies where similar models, agents, and dosing regimens were used, little or no protective effects of antioxidant enzymes/agents (usually SOD) were found (11, 25–27, 30, 31). Although many reasons may be given for the disparate results of these two groups of studies, the roles of specific antioxidants and enzymes in the protection of the myocardium presently remain uncertain.

Subsequent to the numerous reports of protective effects of antioxidant enzymes in experimental models, clinical trials of SOD therapy were initiated. Several studies examined the role of SOD in preserving ventricular function after acute myocardial infarction in patients (10, 22). However, the results of these trials were largely negative. Although Murohara et al. (22) found SOD to have possible beneficial effects on ventricular arrhythmias, their study did not demonstrate a significant beneficial effect in terms of ventricular function. Similarly, treatment of patients undergoing coronary angioplasty failed to demonstrate any improvement in ventricular function (10). These clinical findings provide important insights regarding the ultimate efficacy of antioxidant interventions in treating acute coronary syndromes.

It is possible that the lack of a clear cardioprotective effect in the aforementioned studies results from the location of the antioxidant enzymes in question. In the previous studies of antioxidants, such intravascular agents would be unable to gain access to potentially significant intracellular sources of oxidative stress in cardiac myocytes. Specifically, mitochondria are likely to be the most pathologically significant source of oxidative stress after MI/R. This is a likely possibility, given the previous demonstration of lethality in MnSOD-deficient mice (18). Such findings (18) are especially interesting considering that MnSOD accounts for a minority of the total superoxide activity within a cell (13). These previous findings (13, 18) in conjunction with data presented in the present study support the idea that mitochondria are the most pathologically significant source of ROS after ischemia and reperfusion. Cu/ZnSOD demonstrated no effect in excess or total deficiency. Conversely, overexpression of the mitochondrial MnSOD isoform demonstrated cardioprotective effects in vivo, which is in excellent agreement with a previous report by Chen et al. (5). Although these data may represent important findings, further investigation of the role of mitochondrial targeted overexpression of antioxidant enzymes is required to elucidate the precise process that follows ischemia. Novel vectors for targeting agents to the mitochondria will be needed for such studies and potential future pharmacotherapies.

Previous studies have addressed the potential role of genetic modification of intracellular antioxidant enzymes using isolated perfused-heart preparations. In all of these studies, genetic overexpression of each antioxidant enzyme investigated resulted in significant cardioprotection of the ischemic myocardium. Specifically, overexpression of catalase (20), GPx (37), or Cu/ZnSOD (5, 32) attenuated postischemic injury and/or dysfunction in isolated perfused mouse hearts. In addition, Chen et al. (6) demonstrated that overexpression of MnSOD attenuated myocardial injury in vivo and dysfunction in vitro. Conversely, deficiency of GPX (36) or Cu/ZnSOD (35) exacerbated the extent of...
myocardial injury and/or dysfunction. Although iso-
lated perfused-heart studies provide valuable infor-
mation, these studies cannot be accepted independent of
further in vivo investigation for several reasons. Re-
moval of the hearts from the animals removes the
input of the central nervous system and other organs in
the body. More importantly, the heart is perfused with
crystalloid solutions, which have compositions that are
dramatically different from circulating blood (e.g., no
plasma proteins, leukocytes, or erythrocytes). Particu-
larly germane to this area is the capacity for crystalloid
solutions to allow Fenton/Haber-Weiss reactions (pro-
duction of hydroxyl radical) to take place. Even ex-
tremely small quantities of free metals (e.g., iron) can
induce Fenton/Haber-Weiss reactions in crystalloid sol-
lutions. This is owing to the absence of iron-binding
proteins that in vivo are immediately scavenged due to
a highly reactive (and dangerous) nature. It follows
that antioxidant genetic interventions are more likely to
be effective in such preparations, because the role of
ex vivo oxidants may confound the situation.

We presently demonstrate that the significant reduc-
tion of myocardial injury in MnSOD-Tg mice is associ-
ated with improvement in myocardial function after 7
days of reperfusion. Neither %FS nor CO values were
significantly altered compared with baseline levels in
MnSOD-Tg mice, whereas both indices of cardiac func-
tion were significantly impaired in the non-Tg litter-
mates. Although the mechanism for improved function
in the Tg group was not a focus of this study per se, the
beneficial effect appears to be related to infarct size
reduction. Posterior wall motion was comparable be-
tween the MnSOD-Tg and non-Tg groups. However,
ankterior wall thickening was impaired in the non-Tg
but not the MnSOD-Tg group. Anterior wall-thicken-
ing deficits are consistent with significant anterior wall
myocardial infarcts. It is reasonable to conclude that
the amount of necrosis in the non-Tg but not the
MnSOD-Tg group was sufficient to induce a relatively
long-term regional wall deficit. Ultimately, this re-

gional wall impairment may have led to the global
decrement in function as indexed by the %FS and CO
values.

Application of our data to human disease is difficult
for a number of reasons. Contrary to the situation with
patients, the duration of ischemia in our study was
precisely controlled. The use of healthy mice in our
studies presented another limitation, because actual
patients suffer from numerous risk factors, such as
hypertension, diabetes mellitus, hypercholesterolemia,
and obesity. Although no other genetic abnormalities
have been found in the mice used in the present study,
genetic modification of mice may induce clandestine
artifacts that could affect experimental find-
ings. Finally, we know that the response to MI/R is
variable among species, and this may clearly be the
case when comparing mice and humans. Nevertheless,
our study does provide some novel mechanistic insights
into the isoform specificity of SOD-mediated cardiopro-
tection in the ischemic myocardium.

In summary, overexpression of MnSOD protects the
murine myocardium from posts ischemic injury. Despite
the protective effect of MnSOD, neither GPx nor Cu/
ZnSOD appear to be important determinants of the
extent of myocardial injury in the present in vivo model.
However, we did not examine the effect of GPx
deficiency in the present model. Considering the simi-
lar kinetics but different cellular loci of Cu/ZnSOD and
MnSOD, these data support the idea that the location
of the antioxidant intervention is critical in inducing
cardioprotective effects in vivo. If this proves to be the
case, future investigators will be challenged to target
therapeutic interventions to specific intracellular loci
such as the mitochondria.

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