Transgenic Mice With Increased Copper/Zinc–Superoxide Dismutase Activity Are Resistant to Hepatic Leukostasis and Capillary No-Reflow After Gut Ischemia/Reperfusion

Yoshinori Horie, Robert Wolf, Sonia C. Flores, Joe M. McCord, Charles J. Epstein, D. Neil Granger

Abstract—The objectives of this study were to (1) determine whether transgenic (Tg) mice overexpressing copper/zinc–superoxide dismutase (CuZn-SOD) are protected from the deleterious effects of gut ischemia/reperfusion (I/R) and (2) compare the effectiveness of Tg SOD overexpression in attenuating I/R injury to intravascularly administered CuZn-SOD or manganese (Mn)-SOD. The accumulation of fluorescently labeled leukocytes and number of nonperfused sinusoids were monitored by intravital microscopy in livers of wild-type mice (C57BL/6), CuZn-SOD Tg mice, and wild-type mice receiving either CuZn-SOD or Mn-SOD. All parameters were measured for 1 hour after release of the occluded (for 15 minutes) superior mesenteric artery. Gut I/R in wild-type mice led to an increased number of stationary leukocytes, while reducing the number of perfused sinusoids (capillary no-reflow). All of these responses were significantly blunted in CuZn-SOD Tg mice, with a corresponding attenuation of liver enzyme release into plasma. Exogenously administered SOD had little or no effect on gut I/R-induced leukostasis or capillary no-reflow in the liver. These observations suggest a role for superoxide in gut I/R-induced leukostasis and hypoxic stress in the liver. Furthermore, the findings suggest that cellular localization of SOD activity is an important determinant of the protective actions of this enzyme in experimental models of I/R injury. (Circ Res. 1998;83:691-696.)

Key Words: leukocyte adhesion ■ tissue hypoxia ■ hepatocellular injury ■ inflammation ■ oxygen free radical

Superoxide has been implicated in the microvascular dysfunction and parenchymal cell injury associated with reperfusion of ischemic tissues, including the intestine and liver.1-3 There are several potential cellular sources of superoxide in these tissues, including endothelial cells, parenchymal cells (eg, hepatocytes), resident macrophages (eg, Kupffer cells), and infiltrating leukocytes, all of which produce increased quantities of superoxide after exposure to either hypoxia-reoxygenation or venous plasma draining postischemic tissues.4-5 The ability of exogenously administered superoxide dismutase (SOD) to preserve organ function and minimize cell necrosis in some experimental models of ischemia/reperfusion (I/R) injury provides strong support for superoxide’s critical role in this pathological process. Although leukocytes have also been proposed as mediators of I/R injury, there is evidence that links the recruitment and activation of leukocytes into postischemic tissues with the production of superoxide by vascular endothelial cells.1-3 In this regard, different forms of SOD that are normally localized in specific intracellular compartments, ie, cytosolic copper/zinc (CuZn)-SOD and mitochondrial manganese (Mn)-SOD, have proven to be effective inhibitors of the I/R-induced inflammatory responses (leukocyte–endothelial cell adhesion and albumin extravasation) when administered extracellularly.6,7 The protective effect of SOD in different experimental models of I/R injury to the liver has not been consistent between studies, despite the tremendous capacity of this tissue to produce superoxide via xanthine oxidase and its large resident population of macrophages (Kupffer cells). In some studies, the postischemic liver responds to intravenous SOD with an attenuated leukocyte recruitment, preservation of capillary (sinusoidal) perfusion and hepatic energy stores (ATP), and reduced hepatocellular injury.8-11 Similarly, it has been reported that SOD prevents the hepatocellular injury, without affecting hepatic leukostasis in a model of gut I/R-induced liver injury.12 However, there are also reports of exogenously administered SOD not affecting reperfusion-induced impairment of protein and energy (ATP) metabolism13 and enzyme release14 after liver ischemia. The relative inability of SOD to attenuate I/R-induced leukocyte recruitment and cellular necrosis in some models has been attributed to either inadequate6,9,15,16 or excessive15,16 administration of the enzyme or the limited accessibility of SOD to the intracellular compartment.17

Transgenic (Tg) mice that overexpress CuZn-SOD represent a novel alternative approach to intravenously administered SOD for addressing the contribution of intracellular superoxide to the pathogenesis of I/R injury. This genetic
approach provides a unique opportunity to assess the ability of an increased intracellular SOD activity to modulate the inflammatory and microvascular responses normally elicited by I/R. It also allows for a comparison of the beneficial effects of different forms (CuZn-SOD and Mn-SOD) of exogenously administered SOD with genetic overexpression of CuZn-SOD. The present study was designed to address these issues in a murine model of gut I/R-induced liver injury that allows for an intravital microscopic assessment of the effects of I/R on leukostasis in the sinusoids of different regions of the liver lobule, leukocyte adherence in postsinusoidal venules, and the number of perfused sinusoids.18–20

Materials and Methods

Animals
All mice (8 to 12 weeks old) used in this study were of a C57BL/6 background. Wild-type (control) mice were obtained from Jackson Laboratory (Bar Harbor, Me), and the heterozygous Tg mice (C57BL/6-TgN[SOD1]3Cje) carrying CuZn-SOD genes (SOD-1)21 were bred (at LSU Medical Center) on the C57BL/6 background. The Tg mice were identified by qualitative demonstration of CuZn-SOD using nondenaturing gel electrophoresis followed by nitroblue tetrazolium (NBT) staining.21,22 While the nitroblue tetrazolium assay is simpler and more rapid than other methods, it yields very similar results to other published approaches.23 All of the mice were maintained on standard mouse chow until 18 hours before the experiment.

Surgical Procedure
After administration of atropine sulfate (0.04 mg/kg body weight, IP), mice were anesthetized with ketamine hydrochloride (150 mg/kg body weight, IM) and xylazine (7.5 mg/kg body weight, IM). The right carotid artery was cannulated, and systemic arterial pressure was measured with a Statham P23A pressure transducer (Gould) connected to the carotid artery cannula. Systemic blood pressure and heart rate were continuously recorded with a physiological recorder (Grass Instruments Co). The left jugular vein was also cannulated for drug administration. After laparotomy, both renal arteries and veins were ligated to prevent excretion of injected SOD. The superior mesenteric artery was occluded with a microvascular clip for 0 (sham) or 15 minutes. After the ischemic period, the clip was gently removed. Leukocyte accumulation and the number of nonperfused sinusoids were measured 15 minutes after reperfusion and every 15 minutes for 45 minutes thereafter, tie, for 60 minutes after reperfusion. In some experiments, the mice were given yeast CuZn-SOD (8 mg/kg; Pentapharm Ltd) or recombinant human Mn-SOD (8 mg/kg; BioTechnology General) intravenously at 15 minutes before ischemia, and the same protocol as described above was followed.

The initial experiments on CuZn-SOD Tg mice were performed primarily on females due to the greater availability of this sex. Consequently, some additional experiments (summarized in Table 2) were performed using the above protocol to determine whether the livers of male and female wild-type mice responded differently to gut I/R.

Circulating Leukocytes
The circulating leukocyte count was determined from a 50-μL blood sample obtained from the carotid artery before ischemia. Leukocytes were stained by mixing the blood sample with 440 μL of 3% acetic acid and 10 μL of 1% crystal violet. Polymorphonuclear cells and mononuclear cells were counted with the aid of a hemacytometer (Reicher-Jung, Cambridge Instruments).

Analysis of Leukocyte Accumulation and Sinusoidal Perfusion in Liver Microcirculation
Leukocytes were labeled in vivo with rhodamine-6G (2 mg was dissolved in 5 mL of 0.9% saline) using a previously described method.18–20 It has recently been shown that rhodamine-6G selectively stains white blood cells and platelets, but not endothelial cells.24 Thus, the fluorochrome allows for differentiation between adherent leukocytes and endothelial cells. Rhodamine-6G (0.4 mL/100 g body weight) was injected before gut reperfusion with subsequent injections every 30 minutes. Rhodamine-6G–associated fluorescence was visualized by epi-illumination at 510 to 560 nm, using a 590-nm emission filter. The number of stationary leukocytes was determined offline during playback of videotape images. A leukocyte was considered stationary within the microcirculation (sinusoids and terminal hepatic venules [THVs]) if it remained stationary for more than 10 seconds. The sinusoid was considered to be perfused if the labeled white blood cells or platelets were observed moving through it. The percentage of nonperfused sinusoids was calculated as the ratio of the number of nonperfused sinusoids to the total number of sinusoids per viewing field.

Experimental Protocols
The superior mesenteric artery was occluded with a microvascular clip for 0 (sham) or 15 minutes. After the ischemic period, the clip was gently removed. Leukocyte accumulation and the number of nonperfused sinusoids were measured 15 minutes after reperfusion and every 15 minutes for 45 minutes thereafter, tie, for 60 minutes after reperfusion. In some experiments, the mice were given yeast CuZn-SOD (8 mg/kg; Pentapharm Ltd) or recombinant human Mn-SOD (8 mg/kg; BioTechnology General) intravenously at 15 minutes before ischemia, and the same protocol as described above was followed.

The initial experiments on CuZn-SOD Tg mice were performed primarily on females due to the greater availability of this sex. Consequently, some additional experiments (summarized in Table 2) were performed using the above protocol to determine whether the livers of male and female wild-type mice responded differently to gut I/R.

Statistics
Standard statistical analyses, ie, 1-way ANOVA and Scheffé’s (post hoc) test were applied to the data. All values are reported as
mean±SEM, with at least 1 mice per group. Statistical significance was set at \( P<0.05 \).

**Results**

Table 1 summarizes the SOD activities measured in liver, small intestine, erythrocyte lysate, and plasma samples obtained from CuZn-SOD Tg mice and in wild-type mice receiving an intravenous injection of SOD. SOD activities in the small intestine and erythrocytes were significantly increased in CuZn-SOD Tg mice. Although plasma SOD activities in wild-type and CuZn-SOD Tg mice were undetectable (no significant difference from 0), intravenous administration of either CuZn-SOD or Mn-SOD yielded significant plasma SOD activities. In addition, there was no difference in the number of circulating leukocytes between SOD Tg mice and Mn 1 I/R mice. Table 2 shows the basal levels of stationary leukocytes, nonperfused sinusoids, and plasma ALT activities in wild-type mice receiving exogenous SOD and in CuZn-SOD Tg mice. There was no significant difference in basal levels of any responses among these groups.

Figure 1 summarize the changes in leukocyte accumulation that occur in sinusoids of the midzonal and pericentral regions of the liver lobule, within the THV, and the entire liver lobule (sinusoids+THV; panel B) of wild-type and CuZn-SOD Tg mice after exposure of the gut to ischemia and reperfusion. Also shown are the effects of intravenous administration of CuZn-SOD and Mn-SOD on these responses in wild-type mice. Exogenous CuZn-SOD did not attenuate the leukostasis elicited by gut I/R in any vascular regions. Although Mn-SOD did not affect the increase in total number of stationary leukocytes elicited by gut I/R, it did attenuate leukocyte retention in the midzonal region. In CuZn-SOD Tg mice, gut I/R-induced leukostasis in the liver was attenuated in all vascular regions (the total number of stationary leukocytes was 16.0±0.9 in wild-type versus 7.0±0.8 in CuZn-SOD Tg mice).

**Table 1. SOD Activities in Liver, Small Intestine, Erythrocytes, and Plasma Obtained From CuZn-SOD Transgenic Mice and Wild-Type Mice Receiving Either CuZn-SOD or Mn-SOD**

<table>
<thead>
<tr>
<th></th>
<th>Erythrocytes</th>
<th>Plasma</th>
<th>Liver</th>
<th>Small Intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>120.1±5.0</td>
<td>UD</td>
<td>7811±882</td>
<td>1446±120</td>
</tr>
<tr>
<td>+ CuZn</td>
<td>130.0±6.5</td>
<td>82.5±25.1</td>
<td>7973±1395</td>
<td>1934±424</td>
</tr>
<tr>
<td>+ Mn</td>
<td>136.2±8.8</td>
<td>78.1±21.2</td>
<td>9280±1754</td>
<td>1927±468</td>
</tr>
<tr>
<td>I/R (untreated)</td>
<td>111.5±6.0</td>
<td>UD</td>
<td>6829±420</td>
<td>1398±259</td>
</tr>
<tr>
<td>+ CuZn</td>
<td>130.0±6.5</td>
<td>70.6±21.0</td>
<td>7973±1395</td>
<td>1792±345</td>
</tr>
<tr>
<td>+ Mn</td>
<td>136.6±7.4</td>
<td>67.0±18.5</td>
<td>9074±2106</td>
<td>1969±189</td>
</tr>
<tr>
<td>CuZn-SOD Tg mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOD Tg</td>
<td>209.9±12.7*</td>
<td>UD</td>
<td>11104±770</td>
<td>4102±315*</td>
</tr>
<tr>
<td>SOD Tg+I/R</td>
<td>198.2±10.8†</td>
<td>UD</td>
<td>11610±941</td>
<td>3592±302†</td>
</tr>
</tbody>
</table>

UD indicates undetectable level. Values for erythrocytes and plasma represent SOD, 525 U/mL of extracted samples. Values for liver and small intestine represent SOD, 525 U/g wet tissue.

* \( P<0.05 \) vs wild-type.
† \( P<0.05 \) vs wild-type + I/R.

**Table 2. Stationary Leukocytes, Nonperfused Sinusoids, and Plasma ALT Activities in Male and Female Mice After Either Sham Operation or Exposure to Gut I/R**

<table>
<thead>
<tr>
<th></th>
<th>Midzonal</th>
<th>Pericentral</th>
<th>THV</th>
<th>Total</th>
<th>NPS, %</th>
<th>ALT Activities, IU/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>13</td>
<td>2.1±0.1</td>
<td>1.0±0.1</td>
<td>0.6±0.3</td>
<td>3.8±0.4</td>
<td>9.2±1.3</td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>1.2±0.3</td>
<td>1.2±0.2</td>
<td>0.4±0.2</td>
<td>2.8±0.6</td>
<td>8.3±0.7</td>
</tr>
<tr>
<td>I/R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>15</td>
<td>4.5±0.2*</td>
<td>5.2±0.4*</td>
<td>6.9±0.8*</td>
<td>16.6±1.0*</td>
<td>31.9±2.4*</td>
</tr>
<tr>
<td>Female</td>
<td>7</td>
<td>4.0±0.3*</td>
<td>4.8±0.6*</td>
<td>5.7±0.9*</td>
<td>14.5±1.6*</td>
<td>27.5±2.0*</td>
</tr>
</tbody>
</table>

NPS indicates nonperfused sinusoids.

* \( P<0.05 \) vs sham operation.
Figure 2 illustrates the changes in percentage of nonperfused sinusoids elicited by gut I/R in wild-type (in the presence or absence of exogenous SOD) and CuZn-SOD Tg mice. Gut I/R resulted in an increase in the percentage of nonperfused sinusoids. This response was unaffected by exogenously administered CuZn- or Mn-SOD. However, the gut I/R-induced increase in the percentage of nonperfused sinusoids was significantly attenuated in CuZn-SOD Tg mice (30.5 ± 1.8 in wild-type versus 14.5 ± 1.1 in CuZn-SOD Tg mice).

Figure 3 shows the plasma ALT changes elicited by gut I/R in wild-type (±exogenous SOD) and CuZn-SOD Tg mice. In untreated wild-type mice, gut I/R resulted in a significant elevation in plasma ALT. A significant attenuation of this response was observed in the CuZn-SOD Tg mice, but not in wild-type mice receiving either CuZn-SOD or Mn-SOD.

Discussion

SOD likely represents the most frequently tested reagent in experimental models of I/R injury. Consequently, there are numerous reports of SOD-mediated protection against the vascular dysfunction, inflammatory cell infiltration, and cellular necrosis that result from I/R.1 There is, however, a similarly large body of literature that fails to show a beneficial effect of SOD, with some reports describing deleterious actions of the enzyme when administered at high doses.15,16 This inconsistency of action of SOD in I/R injury can also be demonstrated within a given organ or model system. The liver, for example, has yielded inconsistent responses to SOD after exposure of either the gut12 or liver per se8–11 to I/R.
negative responses to SOD in the liver and other organs have been attributed to an absence of involvement of superoxide in the pathogenic process, inadequate or excessive levels of exogenously administered enzyme, and/or inaccessibility of SOD to intracellular sites of superoxide production. In the present study, Tg mice that overexpress CuZn-SOD were used to directly address the concern that SOD may not afford protection against I/R injury unless it has direct access to intracellular sites of superoxide generation.

Genetically engineered mice are gaining widespread use for studies on the pathogenesis of inflammatory disorders of the circulation, including I/R injury,18 endotoxemia,26 pulmonary oxygen toxicity,27 and atherosclerosis.28 Tg mice that overexpress CuZn-SOD have been employed to study the role of intracellular superoxide in some of these models. For example, it has been shown that CuZn-SOD Tg mice have smaller brain infarct volumes and fewer neurological deficits after focal ischemia than their wild-type counterparts.29,30 The kidneys of SOD Tg mice also appear to be resistant to I/R injury, provided that the duration of ischemia is brief (<30 minutes).27 However, endotoxemia, which has been implicated in the pathogenesis of I/R injury in some organ systems,31 results in a similar mortality rate in CuZn-SOD Tg and wild-type mice.26 While these findings suggest that an elevated intracellular SOD activity reduces the cellular necrosis caused by I/R, they do not address whether the inflammatory cell infiltration and microvascular perfusion abnormalities that also result from I/R respond more favorably to an elevated intracellular (versus extracellular) SOD activity.

The results of the present study clearly indicate that the livers of CuZn-SOD Tg mice are more resistant to the deleterious effects of gut I/R than wild-type mice, even those that receive an intravenous dose of SOD. Relative to their wild-type controls, CuZn-SOD Tg mice exhibit marked reductions in hepatic leukostasis, sinusoidal malperfusion, and hepatocellular necrosis (plasma ALT). While exogenous administration of SOD (using a dose that has been previously employed in other studies) did attenuate leukocyte retention in specific regions (midzonal) of the liver lobule and reduce the capillary no-reflow normally imposed by gut I/R, the magnitude of these responses was relatively small compared with those observed in the CuZn-SOD Tg mice. Hence, these observations indicate that an elevated intracellular activity of SOD is far more effective than an acute increase in extracellular SOD activity in protecting the liver from the inflammatory and microvascular alterations elicited by gut I/R.

Our previous work with this model of liver injury has revealed that the sinusoidal malperfusion, tissue hypoxia, and hepatocellular enzyme leakage elicited by gut I/R are leukocyte-dependent events. This contention is based on results showing an attenuation of the aforementioned liver injury responses in mice that are genetically deficient in adhesion molecules that mediate leukocyte–endothelial cell adhesion.18 For example, mutant mice that do not express either ICAM-1, P-selectin, or the β2-integrin CD11/CD18 exhibit a 75% to 90% reduction in adherent leukocytes in THVs and a 30% to 50% reduction in both the number of nonperfused sinusoids and NAD(P)H autofluorescence (hypoxic stress) after gut I/R compared with responses observed in livers of wild-type mice. Although the magnitude of the attenuation (~65%) in leukocyte retention observed in CuZn-SOD Tg mice was slightly less than that previously observed in the adhesion molecule–deficient mice, the sinusoidal malperfusion (capillary no-reflow) elicited by gut I/R was similarly diminished in the different mutant mouse (adhesion molecule–deficient versus CuZn-SOD Tg mice). These observations suggest that the protective effect of SOD overexpression in this model may result from the significantly attenuated leukocyte sequestration, which in turn helps maintain sinusoidal perfusion and thus prevents tissue hypoxia. This interpretation is consistent with previous reports that SOD is a potent inhibitor of leukocyte–endothelial cell adhesion in some models of I/R-induced inflammation.1,2,6,7 Although the mechanisms underlying SOD’s antiadhesion effect have not been precisely defined, it has been proposed that SOD may prevent the destruction of endothelial cell–derived nitric oxide, which has been shown to act as an endogenous inhibitor of leukocyte–endothelial cell adhesion.32 This possibility is supported by our observation that inhibition of nitric oxide synthase elicits changes in the liver microcirculation that are very similar to those observed after gut I/R.19

There are some potential alternative explanations for the protective action against gut I/R-induced liver injury that was observed in CuZn-SOD Tg mice. It is conceivable that the elevated enzyme activity in the intestine of CuZn-SOD Tg mice renders this tissue resistant to I/R-induced production of the inflammatory agents that ultimately mediate leukocyte retention in the downstream liver microvessels. This possibility is supported by the observation that SOD activity was significantly elevated (relative to wild-type mice) in the gut, but not the liver, of our CuZn-SOD Tg mice. Further support is provided by a recent report that demonstrates diminished lipid peroxidation and neutrophil infiltration in postischemic intestine of mice overexpressing CuZn-SOD.33 Another possibility is that overexpression of CuZn-SOD resulted in compensatory changes in the activity of other antioxidant...
Superoxide Dismutase and Reperfusion Injury

enzymes, which in turn conferred the protection observed in our gut I/R-induced liver injury model. In a recently developed CuZn-SOD Tg mouse, it was demonstrated that alkaline phosphatase, myeloperoxidase, catalase, and glutathione activities in tissues are very similar to those measured in tissues of wild-type mice. The possibility should also be considered that SOD activity in the Tg mice was significantly elevated in only certain populations of liver cells that contribute to gut I/R-induced leukocyte retention. Since SOD activity is normally 3 times higher in hepatocytes than endothelial cells of the liver, it appears likely that an increased endothelial cell SOD activity (which would oppose leukocyte–endothelial cell adhesion) may not be detected in whole liver homogenates. A similar concern could be raised for liver macrophages (Kupffer cells), which we have shown to significantly contribute to the inflammatory and microvascular responses elicited by gut I/R. Kupffer cells are capable of producing large quantities of superoxide when activated. Macrophages isolated from Tg mice that overexpress CuZn-SOD exhibit a diminished capacity to release superoxide into extracellular fluid and also produce smaller quantities of nitric oxide. Hence, the impaired macrophage function that occurs in CuZn-SOD Tg mice could account for the blunted responses of the liver to gut I/R.

Acknowledgments

This study was supported in part by grants from the National Heart, Lung, and Blood Institute (HL26441) and the National Institute on Aging (AG08938).

References

Transgenic Mice With Increased Copper/Zinc–Superoxide Dismutase Activity Are Resistant to Hepatic Leukostasis and Capillary No-Reflow After Gut Ischemia/Reperfusion
Yoshinori Horie, Robert Wolf, Sonia C. Flores, Joe M. McCord, Charles J. Epstein and D. Neil Granger

Circ Res. 1998;83:691-696
doi: 10.1161/01.RES.83.7.691

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/83/7/691

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/