Intestinal ischemia and reperfusion injury in transgenic mice overexpressing copper-zinc superoxide dismutase

DEVENDRA R. DESHMUKH, OLEG MIROCHNITCHENKO, VIKRAM S. GHOLE, DOREEN AGNESE, PRITESH C. SHAH, MICHAEL REDDELL, ROBERT E. BROLIN, AND MASAYORI INOUYE

Departments of Surgery and Biochemistry, University of Medicine and Dentistry of New Jersey, New Brunswick, New Jersey 08903-0019


Superoxide dismutase (SOD) scavenges oxygen radicals that are implicated in the pathogenesis of intestinal ischemia-reperfusion injury. The effect of intestinal ischemia and reperfusion was investigated in transgenic mice overexpressing human Cu-Zn SOD. Ischemia was induced by occluding the superior mesenteric artery. Myeloperoxidase activity was determined as an index of neutrophil infiltration, and malondialdehyde levels were measured as an indicator of lipid peroxidation. Forty-five minutes of intestinal ischemia followed by 4 hr of reperfusion caused an increase in intestinal levels of malondialdehyde in both nontransgenic and transgenic mice, but the concentration of malondialdehyde was significantly greater in nontransgenic mice. Intestinal ischemia-reperfusion also caused an increase in intestinal and pulmonary myeloperoxidase activity in nontransgenic and transgenic mice, but the transgenic mice had significantly lower levels of myeloperoxidase activity than nontransgenic mice. Transgenic mice had higher levels of intestinal SOD activity than nontransgenic mice. There were no significant differences in the catalase or glutathione peroxidase activities.

In conclusion, our study demonstrates that the overexpression of SOD protects tissues from neutrophil infiltration and lipid peroxidation during intestinal ischemia-reperfusion.

reactive oxygen species; small intestine; lung myeloperoxidase

In intestinal ischemia and reperfusion injury in transgenic mice overexpressing copper-zinc superoxide dismutase, reactive oxygen species (ROS) are implicated in the pathogenesis of intestinal ischemia-reperfusion injury (16, 20, 25, 30). The hypoxanthine-xanthine oxidase system appears to be an important source of ROS during I/R (18, 25). ROS react with nucleic acids, proteins, carbohydrates, and lipids to produce damage to these biological molecules. In addition to their direct tissue-damaging effect, ROS trigger the accumulation and activation of neutrophils. Activated neutrophils adhere to the endothelial cells, drag the capillaries, and release more ROS, prostaglandin metabolites, and various enzymes including myeloperoxidase, elastase, and protease (11, 12, 18, 24, 27). These metabolites and enzymes also cause tissue damage. ROS also contribute to the cardiac and pulmonary dysfunction observed during intestinal I/R (9, 11, 12, 28).

Antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase, and catalase protect tissues from reperfusion injury by destroying ROS (9). SOD is a key enzyme that eliminates free radicals by converting superoxide anions into hydrogen peroxide, which is then removed by glutathione peroxidase and catalase. Among three forms of SOD (intracellular Cu-Zn SOD, extracellular SOD, and mitochondrial Mn-SOD) that exist in eukaryotes, intracellular Cu-Zn SOD constitutes 85–90% of the total cellular SOD. During reperfusion, the endogenous levels of SOD may not be enough to counteract the surge of ROS. A previous study (10) suggested that intravenous administration of Cu-Zn SOD provides some protection against ROS-induced tissue damage. However, the role and mechanism of action of ROS and SOD in intestinal I/R are difficult to investigate because ROS are transient in nature and exogenously administered SOD has a short half-life and poor cellular uptake (24, 27). Transgenic mice are useful for these studies because they offer a direct means for constitutively overexpressing SOD in the tissues. Transgenic mice overexpressing SOD have been shown to protect mice from cerebral I/R injury (6, 35) and from pulmonary oxygen toxicity (32). In the present study, we used transgenic mice overexpressing the human Cu-Zn SOD gene to test the hypothesis that increased levels of SOD ameliorate intestinal I/R injury.

MATERIALS AND METHODS

Animals. To direct expression of the human SOD gene in transgenic mice, the non-tissue-specific mouse hydroxymethylglutaril-CoA reductase gene promoter was used (17). The founder mice were bred with C57BL/6 × CBA/J mice to produce transgenic offspring carrying the SOD gene. Transgenic mice were identified by demonstrating human SOD activity with nondenaturing gel electrophoresis followed by tetrazolium staining. Ten- to twelve-week-old heterozygous transgenic mice weighing 25–30 g were used, and their negative littersmates served as nontransgenic control mice. All animal experiments were performed with Institutional Animal Care and Use Committee approval.

Effects of intestinal ischemia on mortality. We investigated the effect of intestinal ischemia (without reperfusion) on...
mortality in nontransgenic and transgenic mice. The animals were fasted for 16 h and anesthetized with pentobarbital sodium (50 mg/kg intraperitoneally). The mice were kept under heating lamps on a warm blanket. A laparotomy was performed, and intestinal ischemia was induced by occluding the superior mesenteric artery with a microbulldog clamp. Satisfactory occlusion was confirmed by the absence of Doppler signals. The abdomen was covered with warm, moist gauze during this period. Age-matched control mice underwent a sham operation in which the superior mesenteric artery was exposed but not occluded.

Effect of reperfusion on oxidative stress. Preliminary experiments were carried out in nontransgenic mice to determine the reperfusion period at which maximum oxidative stress occurred. Intestinal ischemia was induced in mice by occluding their superior mesenteric arteries as described in Effects of intestinal ischemia on mortality. After 45 min, the clamp was released, the incision was closed with a silk suture in a single layer, and the mice were returned to their cages. The reperfusion was allowed for 2, 4, 6, 8, or 24 h. At the end of the reperfusion period, the mice were anesthetized with pentobarbital sodium (50 mg/kg ip), and the small intestine was removed. The tissues were rinsed with saline and immediately stored at -70°C until further analysis. The oxidative stress was evaluated by determining the time-dependent changes in malondialdehyde concentration and myeloperoxidase activity in the small intestine.

Effect of I/R in nontransgenic and transgenic mice. The nontransgenic and transgenic mice were divided into three groups, with at least six animals in each group: group 1, control (sham operated); group 2, 45 min of ischemia; and group 3, 45 min of ischemia plus 4 h of reperfusion. Intestinal ischemia was induced in mice from groups 2 and 3 by occluding the superior mesenteric artery as described in Effects of intestinal ischemia on mortality. After 45 min of ischemia, mice from groups 1 and 2 were killed, and their intestines and lungs were rinsed with saline and stored at -70°C. The animals in group 3 underwent 45 min of ischemia. At the end of ischemic period, the clamp was removed to initiate reperfusion, the incision was closed with a silk suture in a single layer, and the mice were returned to their cages. Four hours after reperfusion, the mice were reanesthetized with pentobarbital sodium (50 mg/kg ip), and their intestines and lungs were removed.

The intestines were homogenized (10% wt/vol) in ice-cold N-2-hydroxyethylpipperazine-N'-2-ethanesulfonic acid buffer (50 mM, pH 7.0). The lungs were homogenized in phosphate buffer (25 mM, pH 6.0) containing 5 mM EDTA and 0.5% hexadecyltrimethyl ammonium bromide. The tissue homogenates were sonicated for 15 s and centrifuged at 3,000 g for 30 min. The supernatants were used for the enzymatic and biochemical assays described in Assays of antioxidant enzymes and biochemical assays.

Assays of antioxidant enzymes. SOD activity was determined at 37°C by the nitrite method (19). This method is based on the inhibition of nitrite formation from hydroxylamine in the presence of superoxide generators. Catalase activity was assayed at 25°C by measuring the rate of hydrogen peroxide consumption at 240 nm (1). Glutathione peroxidase activity was determined at 25°C in a coupled assay with glutathione reductase and by measuring the rate of NADPH oxidation at 340 nm (7).

Biochemical assays. The malondialdehyde levels in the intestine were determined by the thiobarbituric acid method (4). The neutrophil sequestration in the tissues was assessed by determining myeloperoxidase activity (15). Intestinal alkaline phosphatase activity was determined spectrophotometrically with p-nitrophenyl phosphate as the substrate (14). Protein concentration was determined by the protein dye-binding assay of Bradford (3) with bovine serum albumin as the standard.

RESULTS

The transgenic mice described in the present study have been shown to overexpress SOD in various tissues including liver, kidney, brain, and lungs (17). Figure 1 confirms that human SOD was expressed in the small intestine of transgenic mice. Quantitative analysis of SOD activity in the nontransgenic and transgenic mice is described in Effect of intestinal I/R.

Effect of ischemia on mortality. None of the sham-operated animals died during the mortality experiment. Clinically obvious changes in the bowel color (pale, blue, or black) were noted in all mice during the period of ischemia. Sixty minutes of intestinal ischemia were associated with a 55% (12/22) mortality in the nontransgenic mice and 60% (6/10) mortality in the transgenic mice. There was no statistically significant difference in mortality due to ischemia between the nontransgenic and transgenic mice (P > 0.05). All of these deaths occurred between 50 and 60 min of ischemia. Hence, to eliminate ischemia per se as a cause of death, the mice underwent 45 min of intestinal ischemia in subsequent experiments.

Time-dependent changes in oxidative stress. Preliminary experiments were carried out in the nontransgenic mice to determine the reperfusion time at which maximum oxidative stress occurred. The malondialdehyde levels in the small intestine were significantly elevated during the 4 h after the 45 min of intestinal ischemia (72.8 ± 8.5 vs. 24.6 ± 3.4 nmol/g; n = 6). The malondialdehyde levels remained elevated until 6 h and returned to normal levels 8 h postischemia. The intestinal myeloperoxidase activity was also significantly elevated after 4 h of reperfusion (2,184 ± 153 vs.

![Fig. 1. Expression of Cu-Zn superoxide dismutase (SOD) activity in small intestine.](http://apc.cellphysiology.org/)
236 ± 35 μmol·min⁻¹·g⁻¹; n = 6). Therefore, the nontransgenic and transgenic mice underwent 4 h of reperfusion in the subsequent experiment.

Effect of intestinal I/R. We compared the effect of intestinal ischemia on the alkaline phosphatase activity in the nontransgenic and transgenic mice because intestinal alkaline phosphatase activity has been suggested to be a specific marker for reperfusion injury (26). The baseline levels of intestinal alkaline phosphatase activity were not statistically different between the nontransgenic and transgenic mice (Fig. 2). Forty-five minutes of intestinal ischemia caused a significant decrease in alkaline phosphatase activity in the nontransgenic mice, whereas the decrease was not statistically significant in the transgenic mice. After 4 h of reperfusion, alkaline phosphatase activity returned to normal levels in both the nontransgenic and transgenic mice.

The extent of lipid peroxidation was determined by measuring the malondialdehyde concentration in the small intestine (Fig. 3). There were no differences in the intestinal malondialdehyde levels between the nontransgenic and transgenic mice in the control group. Intestinal ischemia without reperfusion did not change malondialdehyde levels in either the nontransgenic or transgenic mice. Four hours of reperfusion caused a small but insignificant increase in malondialdehyde levels in the transgenic mice. Conversely, the nontransgenic mice exhibited a large and significant increase in malondialdehyde levels after 4 h of reperfusion. These levels were significantly greater compared with the transgenic mice.

The effect of intestinal I/R on myeloperoxidase activity in the intestine is shown in Fig. 4. Intestinal myeloperoxidase activity in the control group was not significantly different between the nontransgenic and transgenic mice. Forty-five minutes of ischemia produced a small but statistically significant increase in intestinal myeloperoxidase activity in the nontransgenic and transgenic mice. Four hours of reperfusion caused a further increase in the intestinal myeloperoxidase activity only in the nontransgenic mice. These levels were significantly higher compared with the transgenic mice.

Because intestinal I/R produces lung injury (12, 13, 28), we investigated the effect of intestinal I/R on lung myeloperoxidase activity (Fig. 4). There was no significant difference in lung myeloperoxidase activity between the nontransgenic and transgenic mice in the control group. Intestinal ischemia caused a significant increase in lung myeloperoxidase levels compared with the transgenic mice.
increase in lung myeloperoxidase activity in the nontransgenic mice. On the contrary, a small but statistically insignificant increase in lung myeloperoxidase activity was observed in the transgenic mice after intestinal ischemia. After 4 h of reperfusion, lung myeloperoxidase activity remained significantly higher in the nontransgenic mice.

Figure 5 shows the activity of three antioxidant enzymes in the transgenic vs. nontransgenic mice. There was a twofold increase in the baseline levels of intestinal SOD activity in the transgenic mice compared with the nontransgenic animals. Intestinal ischemia caused a significant decrease in SOD activity only in the transgenic mice. Four hours of reperfusion returned SOD activity to normal levels. SOD activity remained elevated in the transgenic mice during both ischemia and reperfusion.

There was no statistically significant difference in the activities of catalase or glutathione peroxidase between the nontransgenic and transgenic mice in the control group. Forty-five minutes of intestinal ischemia caused a significant decrease in the catalase activity in the nontransgenic mice. Although catalase activity also decreased in the transgenic mice after ischemia, the decrease was not statistically significant. There was no statistically significant difference in the catalase activity between the nontransgenic and transgenic mice in the reperfusion group (Fig. 5). Intestinal ischemia produced a small decrease in the glutathione peroxidase activities in the nontransgenic and transgenic mice, but the decrease was statistically significant only in the transgenic mice. Reperfusion did not alter glutathione peroxidase activity in either the nontransgenic or transgenic mice (Fig. 5).

DISCUSSION

A previous study (25) suggested that the intestine is extremely susceptible to I/R injury. It is the richest source of the xanthine dehydrogenase-oxidase enzyme system, which is needed for the production of ROS (21). Although ROS have been implicated in the pathogenesis of intestinal I/R, their exact role in intestinal I/R is difficult to evaluate because they have short biological half-lives (23–25).

SOD is a naturally occurring, highly specific enzyme that plays a central role in protecting cells and tissues against oxidant stress. The administration of SOD could prove beneficial in the study and treatment of intestinal I/R injury. However, there are several drawbacks to this approach. First, the circulating half-life of SOD is only 6 min, and, therefore, a continuous infusion of the enzyme may be required (11, 24). Second, SOD has a poor cellular uptake. Therefore, the administration of SOD would fail to increase its levels intracellularly, a site where ROS are produced in high concentrations (11, 24, 25). On the contrary, SOD is produced constitutively in transgenic mice and therefore can destroy intracellularly produced ROS. Another disadvantage of exogenously administered SOD is that it may produce anti-SOD antibodies, which is circumvented in transgenic mice. Hence our studies in transgenic mice overexpressing SOD provide a new means for investigating the role of ROS and SOD in intestinal I/R.

The intestinal villi are extremely susceptible to ischemic damage, and their necrosis is one of the earliest histological changes that occur during intestinal ischemia (33, 34). Intestinal alkaline phosphatase activity, which is localized in the villus cells (5), was significantly decreased during ischemia in the nontransgenic mice but not in the transgenic mice (Fig. 2), indicating that the overexpression of SOD in the transgenic mice provides protection against ischemia without reperfusion. The precise mechanism of decreased alkaline...
phosphatase activity during intestinal ischemia is not known. One possibility is that the alkaline phosphatase may be decreased due to mucosal sloughing during intestinal ischemia. Sisley et al. (26) suggested that a loss in intestinal alkaline phosphatase occurs during reperfusion and that intestinal alkaline phosphatase may be a specific marker for reperfusion injury. In our study, reperfusion injury did not alter alkaline phosphatase activity in the nontransgenic or transgenic mice.

Polyunsaturated fatty acids in the membranes are especially susceptible to oxidative damage and are broken down into the peroxidation product malondialdehyde (4). Intestinal ischemia did not produce significant changes in the malondialdehyde levels in the intestines of the nontransgenic and transgenic mice, presumably because pure ischemia does not induce oxidative stress. The transgenic mice had a significantly lower concentration of malondialdehyde than the nontransgenic mice after intestinal I/R (Fig. 3), indicating that the magnitude of lipid peroxidation was significantly lower in the transgenic mice. Hence transgenic mice overexpressing SOD appear to be protected from the tissue damage induced by reperfusion.

An early imbalance in the supply and demand of oxygen, especially in the intestine, appears to be a common event for initiating multiple organ system failure. Intestinal I/R has also been shown to produce lung injury, which appears to be mediated by neutrophils (13, 22, 28). It has been proposed that adherence of neutrophils to the endothelium creates a condition in which polymorphonuclear neutrophil-derived oxidants and proteases are released and lead to the cellular injury. Loss of microvascular integrity results, migration of activated polymorphonuclear neutrophils occurs, and pulmonary dysfunction follows (28). To determine whether the overexpression of SOD in transgenic mice protects them against lung injury, we compared the effect of intestinal ischemia on lung injury between SOD-transgenic and nontransgenic mice. We assessed the neutrophil sequestration in the intestine and lungs by determining myeloperoxidase activity, which correlates with the number of neutrophils present.

The extent of neutrophil infiltration in the intestine and lungs after I/R was significantly lower in transgenic vs. nontransgenic mice (Fig. 4). The fact that I/R produced significantly lower levels of malondialdehyde and reduced intestinal and pulmonary myeloperoxidase activities in transgenic vs. nontransgenic mice indicates that ROS play an important role in the pathogenesis of intestinal I/R. These results also indicate that the overexpression of SOD protects tissues from neutrophil infiltration and lipid peroxidation during intestinal I/R. Whether the observed protection in lungs is due to the overexpression of SOD in the intestine or in the lungs remains to be determined.

Intestinal ischemia caused a small decrease in SOD activity only in the transgenic mice. The decrease in SOD activity could be due to the inhibition or inactivation of SOD activity, decrease in SOD protein, or decrease in SOD expression. Excess SOD during oxidative stress should lead to the overproduction of hydrogen peroxide. Inactivation of Cu-Zn SOD by hydrogen peroxide, its reaction product, is a well-known phenomenon (29). The major difference between nontransgenic and transgenic mice is the presence of different species of SODs. Both nontransgenic and transgenic mice express mouse SOD. However, transgenic mice also express human SOD as well as a human-mouse hybrid. Because ischemia did not alter SOD levels in nontransgenic animals, it is possible that the small differences in SOD between nontransgenic and transgenic mice may be due to differences in the properties of either human SOD or hybrid SOD vs. mouse SOD. Total SOD activity was determined in the present study. Whether intestinal I/R alters Mn-SOD, intracellular Cu-Zn SOD, or extracellular Cu-Zn SOD in nontransgenic and transgenic mice remains to be elucidated.

Glutathione peroxidase and catalase rapidly destroy hydrogen peroxide and protect tissues from the ROS-induced injury (8). To determine whether antioxidant enzymes other than SOD are altered during intestinal I/R, we investigated the effect of intestinal I/R on catalase and glutathione peroxidase activities in nontransgenic and transgenic mice. There were no significant differences in the glutathione peroxidase or catalase activities between nontransgenic and transgenic mice in the control or I/R groups. SOD was the only antioxidant enzyme with significantly higher activity in transgenic mice. Therefore, a protective effect against intestinal I/R observed in transgenic mice appears to be due to enhanced SOD activity.

Another possible mechanism for the observed protection from intestinal I/R in transgenic mice may involve nitric oxide. Previous studies have shown that superoxide anion rapidly reacts with nitric oxide to form peroxynitrite, a strong and highly reactive oxidant that can participate in several oxidative reactions including oxidation of sulfhydryl groups, nitration of tyrosine, and peroxidation of lipids (2). Thus transgenic mice may produce less peroxynitrite than nontransgenic animals, which could contribute to the observed protection from intestinal I/R.

Although transgenic mice that underwent I/R had lower levels of malondialdehyde and myeloperoxidase activities than similarly treated nontransgenic animals, the levels were slightly greater than those seen in the control animals. These results suggest that the overexpression of Cu-Zn SOD in transgenic mice offered some protection against intestinal I/R-induced damage. It is possible that the overexpression of Cu-Zn SOD may yield increased amounts of hydrogen peroxide that can cause damage to the tissue. Whether overexpression of glutathione peroxidase or catalase, either alone or in combination with overexpression of Cu-Zn SOD, can provide additional protection against intestinal I/R injury remains to be investigated.

In conclusion, our study indicates that transgenic mice overexpressing Cu-Zn SOD are protected from intestinal I/R injury. These results also support the hypothesis that ROS play an important role in the pathogenesis of intestinal I/R injury. Although other
studies have documented a protection against intestinal I/R by exogenously administered SOD (31), our study is the first to demonstrate that the overexpression of SOD by genetic manipulation is beneficial in intestinal I/R injury.

Address for reprint requests: D. R. Deshmukh, Dept. of Surgery, Division of General Surgery, Univ. of Medicine and Dentistry of New Jersey, Medical Education Bldg., One RWJ Place, CN 19, New Brunswick, NJ 08903-0019.

Received 30 September 1996; accepted in final form 28 May 1997.

REFERENCES