Inhibition of Posthemorrhagic Transfusion-Induced Gastric Injury by a Long-Acting Superoxide Dismutase Derivative (43173)

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Abstract. **Although oxygen-free radicals have been postulated to play an important role in the pathogenesis of gastric mucosal injury induced by posthemorrhagic blood transfusion, direct evidence supporting this hypothesis is lacking. Superoxide dismutase (SOD) has been shown to inhibit oxygen toxicity *in vitro* in various types of cell injury. However, in some cases, oxidative tissue injury cannot be decreased efficiency predominantly due to its rapid elimination by renal glomerular filtration. To overcome such frustrating situations, we have synthesized a SOD derivative that circulates bound to albumin with a half-life of 6 hr. When blood was withdrawn from the rat (22 ml/kg) for 30 min followed by transfusion of the extracted blood, marked gastric mucosal lesions occurred within 30 min after transfusion. Intravenously injected SOD derivative markedly decreased gastric mucosal injury. Kinetic analysis using ^{125}I-labeled albumin revealed that the vascular permeability of the stomach increased significantly after transfusion by a SOD derivative inhibitable mechanism. Thus, superoxide radical and/or its metabolite(s) play a critical role in the pathogenesis of posthemorrhagic transfusion-induced gastric injury.** [P.S.E.B.M. 1991, Vol 196]

Hemorrhagic shock followed by blood transfusion often induces gastric mucosal lesions (1–4). Recent studies suggested that oxygen-free radicals play an important role in the pathogenesis of postischemic reflow-induced tissue injury (1, 2, 5, 6). Thus, superoxide dismutase (SOD) and other radical scavengers have been expected to attenuate postischemic reflow injury of the stomach. However, intravenously injected SOD is removed rapidly by the kidney with a half-life of 5 min. (7, 8). Hence, reflow-induced gastric injury could not be inhibited by a single dose of SOD. We have synthesized an SOD derivative (SM-SOD) covalently linked with poly-(styrene-co-maleic acid butyl ester) (SM), a hydrophobic anion with high affinity for albumin (7, 8); SM-SOD circulates bound to albumin with a half-life of 6 hr and accumulates in tissues that have a decreased local pH (8). Due to such properties of SM-SOD, postischemic reflow arrhythmias (8, 9), traumatic brain edema (10), and stress-induced gastric mucosal lesion (11) were inhibited efficiently by intravenous administration of SM-SOD. The present work demonstrates that posthemorrhagic transfusion-induced injury of the stomach is also inhibited by a single dose of SM-SOD.

Materials and Methods

Materials. Rat serum albumin was purchased from Sigma Chemical Co. (St. Louis, MO). SM (mol wt = 1600) was obtained from Kurare Co. (Kurashiki, Japan). ^{125}I-Labeled Bolton-Hunter reagent (2.2 Ci/μmol) was from New England Nuclear (Boston, MA). Other reagents used were of analytical grade. SM-SOD was synthesized as described previously (7, 8).

Animals. Male Sprague-Dawley rats, 250–310 g, were fed laboratory chow and water *ad libitum*. Before experiments, they were fasted overnight. Posthemorrhagic transfusion injury of the stomach was induced essentially as described previously (1–4). Under pentobarbital anesthesia (35 mg/kg body wt), the left carotid artery was cannulated with polyethylene tubing to withdraw and transfuse blood. After ligation of the esophagus at 5 mm proximal to the gastroesophageal junction and the duodenum at 10 mm distal to the pylorus, the stomach was infused with 0.05 *N* HCl (11 ml/kg body

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wt). Blood (22 ml/kg body wt) was slowly withdrawn into a syringe containing sodium citrate (final concentration of 4 mg/ml) over a period of 3 min. After 30 min, the blood was transfused through the carotid artery over a period of 3 min. Animals were divided into four groups. Animals in Group 1 were intravenously administered 0.5 ml of saline just before phlebotomy. SM-SOD was administered intravenously (10 mg/kg body wt) either 1 min before (Group 2) or after phlebotomy (Group 3). Animals in Group 4 were administered the same dose of SM-SOD 1 min before transfusion. During the experiments, body temperature was maintained at 36–37°C with a tungsten lamp, and the mean arterial blood pressure and the heart rate were monitored as described previously (12). Thirty minutes after transfusion, animals were exsanguinated by bleeding through the left femoral artery. The stomach was removed and fixed with 10 ml of 1% Formalin overnight. The ulcer index (mm) was expressed by measuring the total length of linear mucosal lesions of the stomach.

Effect of SM-SOD on Vascular Permeability of the Stomach. Thirty minutes after phlebotomy, the blood was slowly transfused with ^{125}I-labeled albumin (5×10^5 cpm/kg body wt). After 30 min, animals were exsanguinated by bleeding through the left femoral artery. Then gastric juice was collected by puncture of the stomach. Perchloric acid was added to the gastric juice to give a final concentration of 5%. The stomach was homogenized in 2 ml of 5% perchloric acid by a Polytron homogenizer for 1 min. One milliliter of blood sample was also added with 5% of the acid solution. The acidified samples were centrifuged for 10 min at 4000 rpm in a Kubota KR-40 centrifuge. The acid-soluble and insoluble fractions were determined for radioactivity in a Packard autogamma scintillation spectrometer (model 5130).

Effect of Ischemia and Transfusion on Chemiluminescence. At the indicated times during the experiments, 0.1-ml blood samples were collected from the carotid artery into test tubes containing 400 μl of minimum essential medium and 2 m*M* luminor. Polymorphonuclear leukocyte-dependent chemiluminescence intensity of the blood was measured by adding 50 μl of 12.5 mg/ml of opsonized zymozan at room temperature in an Aloka luminescence reader as described previously (12).

Statistical Analysis. All values were expressed as means ± SE derived from 3 to 15 animals. Comparison of vascular permeability of the stomach and chemiluminescence was analyzed by Student's *t* test, and the ulcer index by variance.

Results

Change in Hemodynamics. Figure 1 illustrates the changes in blood pressure and the heart rate during the experiments. Before phlebotomy, the mean arterial blood pressure was 122 ± 3 mm Hg as measured at the right carotid artery. It decreased to a significantly low level (27 ± 2 mm Hg) immediately after phlebotomy. However, the decreased blood pressure returned to 103 ± 33 mm Hg after transfusion. The heart rate was also decreased by phlebotomy from 422 ± 18 to 347 ± 18 beats/min; it decreased transiently after transfusion and remained unchanged thereafter.

Gastric Mucosal Lesion. Figure 2 shows gastric mucosa 30 min after blood transfusion. Gastric mucosal lesions were seen as linear erosion (Fig. 2, left). The mucosal lesion was markedly decreased in animals that were given SM-SOD just before transfusion. Figure 3 shows the ulcer index of the stomach from animals that were administered SM-SOD just before (Group 2) or after phlebotomy (Group 3) or just before transfusion (Group 4). Mucosal injury was inhibited similarly by SM-SOD regardless of the time of its administration. Thus, superoxide radicals generated after blood transfusion might play a critical role in the occurrence of gastric mucosal injury.

Effect of Postischemic Transfusion on Vascular Permeability. Figure 4 shows the effect of SM-SOD on the vascular permeability of the stomach as measured by ^{125}I-labeled albumin. Data were expressed as percentage of the control level found in the sham-operated animals. The amount of radioactivity found in the stomach and the gastric juice increased significantly after blood transfusion, and administration of SM-SOD decreased the radioactivity found in the stomach and the gastric juice; the decrease in radioactivity was more marked with the gastric juice than with the stomach. Most of the radioactivity found in the gastric juice was recovered from the acid-soluble fractions, whereas that in the stomach was recovered predominantly from the acid-precipitable fractions.

Change in Chemiluminescence Intensity. Figure 5 shows the effect of hemorrhagic shock and blood transfusion on polymorphonuclear leukocyte-dependent chemiluminescence intensity of the blood. Before phlebotomy, chemiluminescence intensity was 603 ± 176 cpm/ml of the blood; it decreased to 208 ± 43 cpm/ml immediately after bleeding and this low level remained unchanged for 15 min. However, it returned to normal levels after 30 min of phlebotomy. Chemiluminescence intensity slightly increased after transfusion and then decreased slowly thereafter. Thus, chemiluminescence intensity of the circulating blood from the transfused animals did not show higher levels than that from the control animals at any time point tested.

Discussion

Inhibition of posthemorrhagic transfusion-induced gastric mucosal injury has been reported in animals that received continuous infusion of a large dose of SOD or in animals whose renal vessels were ligated to

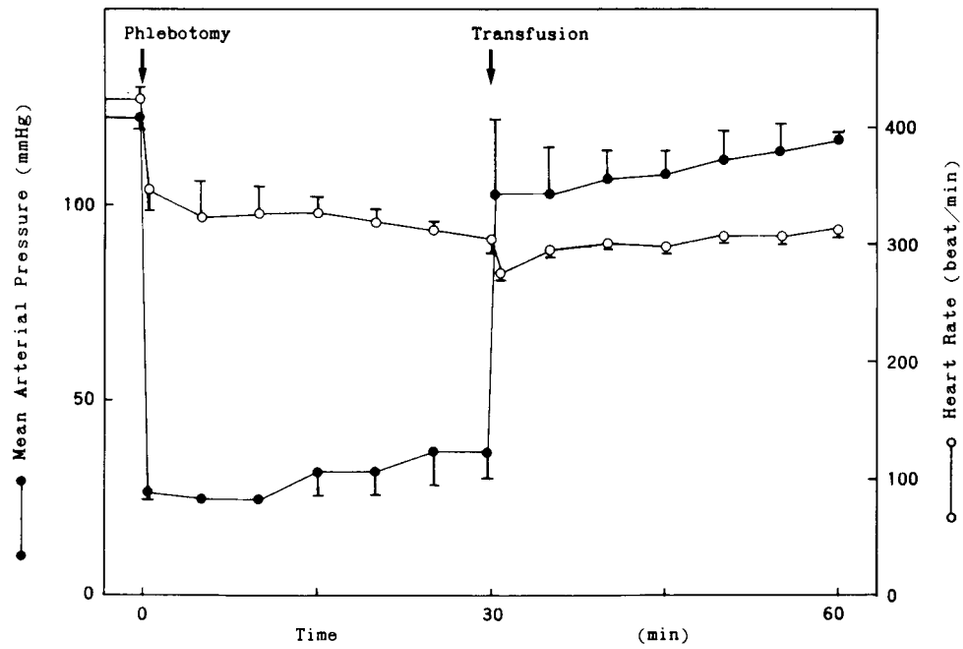

Figure 1. Change in hemodynamics during ischemia and reflow. Under pentobarbital anesthesia (35 mg/kg), blood (22 ml/kg body wt) was withdrawn from the left carotid artery into a citrated syringe (4 mg/ml). After 30 min, the blood was transfused through the left carotid artery. During 30-min ischemia and reflow, the mean arterial blood pressure and the heart rate were measured. Data show mean ± SE derived from three animals. Open circles, heart rate; closed circles, mean arterial blood pressure.

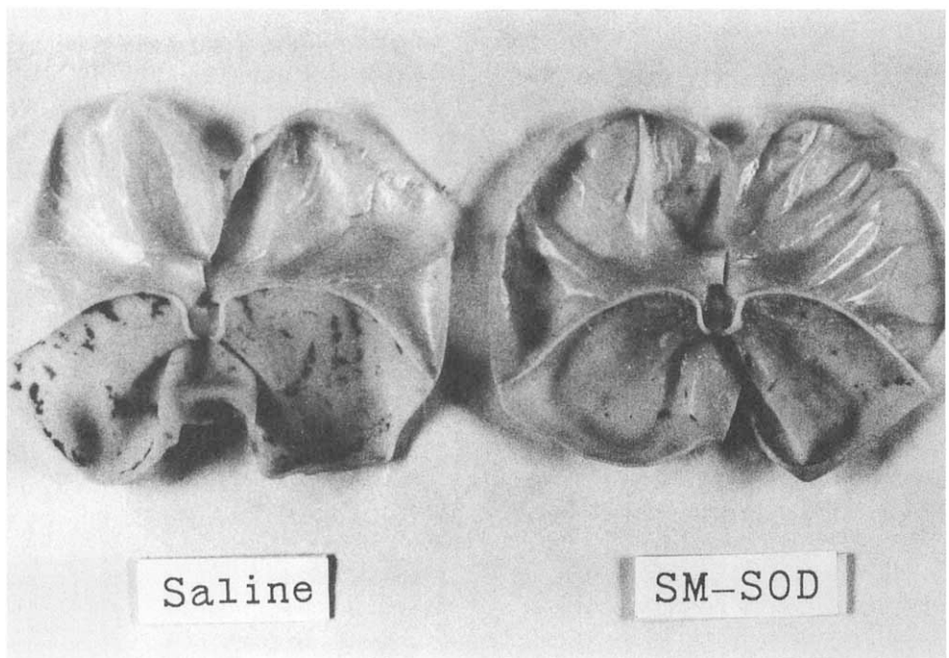

Figure 2. Gastric mucosal injury induced by posthemorrhagic blood transfusion. Thirty minutes after blood transfusion, animals were exsanguinated by bleeding from the left femoral artery. The stomach was removed and fixed with 10 ml of 1% Formalin overnight. The left panel shows the stomach of the animal that was intravenously administered 0.5 ml of saline 1 min before transfusion. The right panel shows the stomach of the animal that was administered SM-SOD (10 mg/kg body wt).

inhibit its glomerular filtration (1). However, infusion of a large volume of SOD solution as well as ligation of the renal vessels might significantly perturb the circulatory status of animals. Since the posthemorrhagic transfusion injury of the stomach might be induced by a marked change in circulatory status, experimental conditions by which the circulatory status was further modified should be avoided to understand the patho-

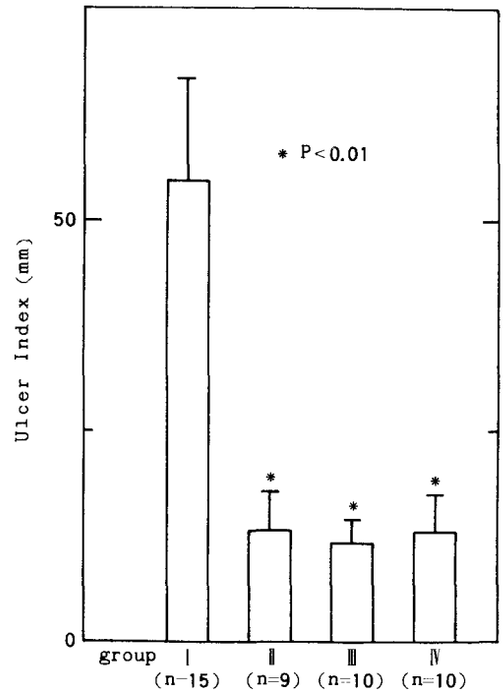

Figure 3. Effect of SM-SOD on gastric mucosal injury induced by posthemorrhagic blood transfusion. Animals in Group 1 were intravenously administered 0.5 ml of saline just before phlebotomy. SM-SOD (10 mg/kg body wt) was also administered intravenously either 1 min before (Group 2) or after phlebotomy (Group 3). Animals in Group 4 were administered the same dose of SM-SOD 1 min before blood transfusion. Total length of the linear mucosal lesions was measured and expressed as ulcer index. Data show mean ± SE derived from 9 to 15 animals. Other conditions were the same as in Figure 2. *Significantly different from Group 1 ($P < 0.01$).

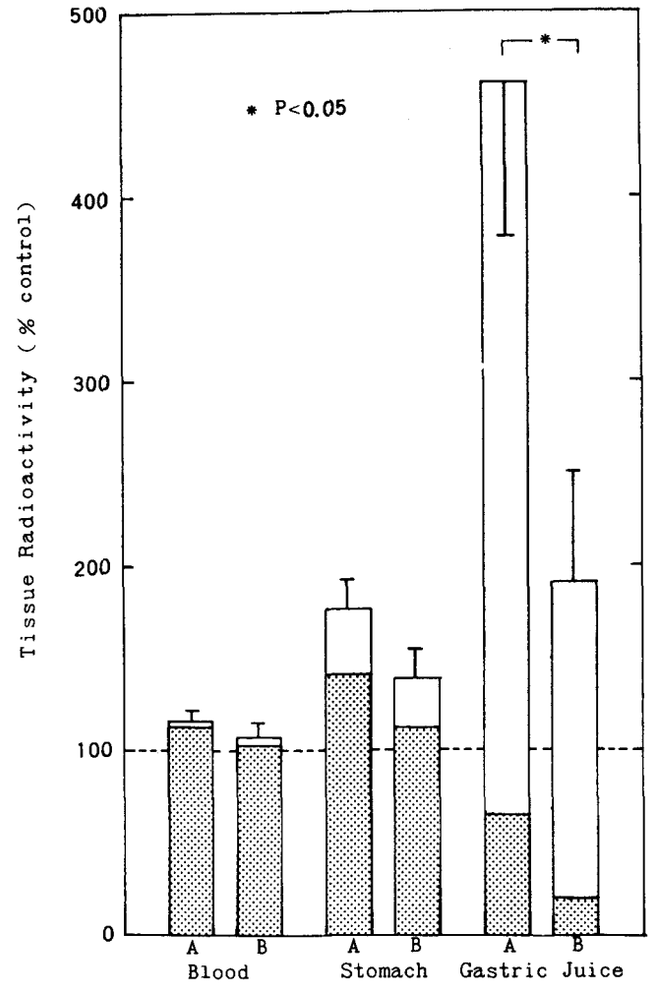

Figure 4. Effect of SM-SOD on vascular permeability of the stomach. Thirty minutes after phlebotomy, animals were intravenously injected with 0.5 ml of saline or 10 mg/kg of SM-SOD 1 min before transfusion. Then, the blood was transfused with ^{125}I-labeled albumin (5×10^5 cpm/kg body wt). After 30 min, animals were exsanguinated by bleeding through the femoral artery. A 1-ml blood sample was collected and perchloric acid was added to give a final concentration of 5%. Gastric juice was collected by puncture of the stomach and added with 5% perchloric acid. The acidified samples were centrifuged for 10 min at 4000 rpm. The acid-soluble and insoluble fractions were determined for radioactivity. Data show percentage of control levels in sham-operated animals that were administered ^{125}I-labeled albumin 60 min after ligation of the left carotid artery. A, Saline-treated group; B, SM-SOD-treated group. Open columns, acid-soluble fractions; spotted columns, acid-precipitable fractions. Data show mean ± SE derived from five animals. *$P < 0.05$, saline-treated group versus SM-SOD-treated group.

genesis of gastric injury. The present work demonstrates that posthemorrhagic transfusion-induced gastric injury was inhibited by a single dose of SM-SOD that circulates bound to albumin with prolonged *in vivo* half-life.

Leung *et al.* (4) demonstrated that, for inducing posttransfusion injury of the stomach, the mean arterial blood pressure should be decreased to 20–30 mm Hg during ischemia. In our experiments, the mean arterial pressure was maintained to be 27 ± 2 mm Hg during hemorrhagic shock. Since the heart rate did not increase during ischemia, the gastric blood flow would be decreased significantly. Thus, the pathologic condition of the stomach seems to mimic the circulatory status for inducing postischemic reflow injury of other tissues, such as heart (13), lung (14), brain (15), and intestinal mucosa (16, 17).

Reactive oxygens have been suggested to play an important role in the pathogenesis of tissue injury during reflow rather than during ischemia (16). It should be noted that the similarly efficient protection of the stomach was achieved by administering SM-SOD before or after phlebotomy. Thus, dismutation of superoxide radicals during and/or after transfusion would be sufficient for inhibiting the gastric injury.

It has been known that a high level of hemoglobin is cytotoxic (18). The phlebotomized blood samples were kept at room temperature in a citrated syringe for 30 min, which may result in hemolysis. Thus, posttransfusion injury of the stomach may possibly be induced by some constituent(s) of blood cells, such as hemoglobin. However, no detectable hemolysis of the phlebotomized blood samples occurred during the incubation for 30 min as measured spectrophotometrically (data not shown).

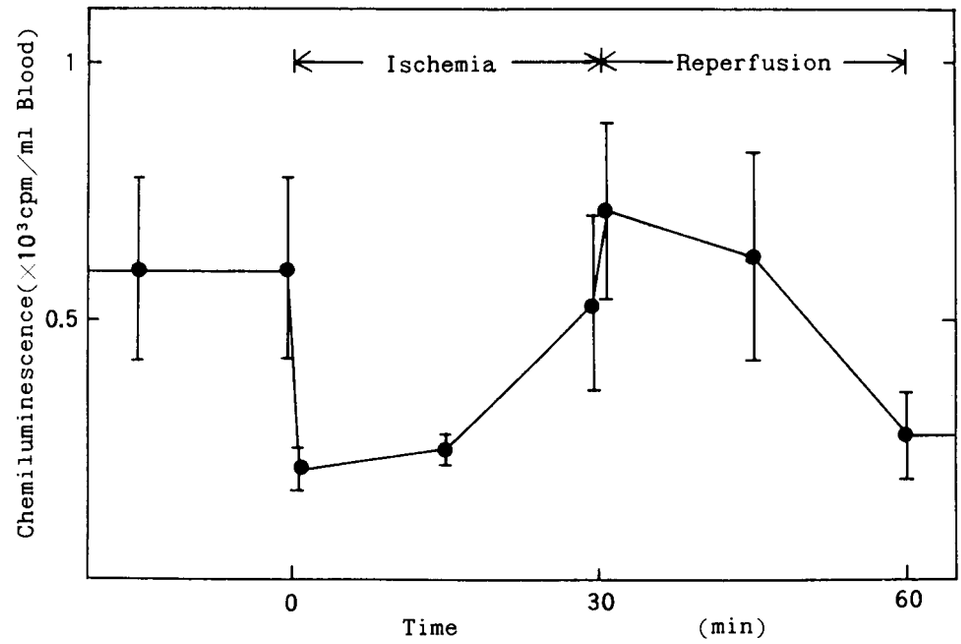

Figure 5. Effect of hemorrhagic shock and blood transfusion on chemiluminescence intensity of the circulating leukocytes. At the indicated times during ischemia and reflow, 0.1-ml blood samples were collected from the carotid artery into test tubes containing 400 μl of minimum essential medium and 2 m*M* luminor. Chemiluminescence intensity of blood samples was measured by adding 50 μl of opsonized zymozan (12.5 mg/ml) at room temperature. Data show mean ± SE derived from five animals.

Kinetic analysis using ^{125}I-labeled albumin revealed that the radioactivity found in the gastric juice was 4.6-fold higher in the transfused group than in the sham-operated group. Since the radioactivity found in the stomach and the gastric juice was significantly decreased by SM-SOD, superoxide radical and/or its metabolites might increase the vascular permeability of the stomach, particularly during the reflow period. It should be noted that the accumulation of radiolabeled albumin in tissues could also be influenced by a number of other factors, such as vascular filtration rate, perfused capillary density, interstitial volume, and lymph flow. Furthermore, the accumulation of radioactivity in gastric juice could be affected by the permeability of gastric vasculature and epithelium. Thus, the mechanism for lumenal occurrence of radioactivity should be studied further. Since most of the radioactivity found in gastric juice was recovered from the acid-soluble fractions, ^{125}I-labeled albumin appearing in the gastric lumen would rapidly be degraded by proteases, such as pepsin.

Previous studies in this laboratory (7) revealed that the intravenously injected SM-SOD predominantly localized in extracellular compartments, such as in plasma. Thus, extracellular superoxide radicals might principally be responsible for posttransfusion injury of the stomach. It is known that neutrophils play an important role in the propagation of inflammatory processes by releasing both reactive oxygens and various lysosomal enzymes (19). The present study revealed that chemiluminescence intensity of the blood was lower than that in the control animals at any time point tested (see Fig. 5). This suggested that the circulating neutrophils might not be the major source for superoxide radicals during ischemia and/or reflow. Engler *et al.* (20) and Smith *et al.* (21) suggested that, when the circulating neutrophils attached on endothelial cells, they increased vascular resistance and significantly perturbed the microcirculation of an injured tissue. Such a change in microcirculatory status may possibly be involved in the pathogenesis of posttransfusion-induced gastric injury. Parks *et al.* (22) demonstrated that a xanthine oxidase-catalyzed reaction was responsible for the formation of superoxide radicals during reflow of the ischemic bowel. Since xanthine dehydrogenase is localized in vascular endothelial cells, and is converted to xanthine oxidase during ischemia (23, 24), such an enzymic conversion may possibly be involved in the generation of superoxide radicals in the reflowed stomach whose vascular permeability was increased significantly.

It has generally been assumed that hydrogen peroxide is more toxic than superoxide radicals. Since SM-SOD effectively dismutates superoxide radicals to hydrogen peroxide, oxygen toxicity of the stomach would be inhibited more efficiently by the combined use of SM-SOD and catalase. However, preliminary experiments revealed that protective effect of SM-SOD did not increase even if the enzyme was injected with catalase (50,000 units/kg). It should be noted that hydrogen peroxide is an amphipathic molecule that easily penetrates membrane/lipid bilayers and, hence, extracellular hydrogen peroxide would efficiently be de-

graded by intracellular catalase and/or glutathione peroxidases (25). In fact, the toxic effect of extracellular hydrogen peroxide on cultured myocardial cells was inhibited by adding either catalase or erythrocytes in culture medium (26). Furthermore, in various disease models, such as postischemic reperfusion injury of the heart (9) and liver (12), the protective effect of SM-SOD *in vivo* was similar to that of SM-SOD plus catalase. Thus, extracellular hydrogen peroxide generated by SM-SOD would have been degraded intracellularly by endogenous catalase and/or peroxidases via some metabolic sink mechanism described above. Another possibility is that superoxide radical *per se* might be responsible for the reflow-induced tissue injury.

Apart from the source for superoxide radicals and the mechanism for degradation of hydrogen peroxide, SM-SOD might seem to be a useful tool for studying the pathogenesis of various diseases in which superoxide radical and/or its metabolite(s) plays critical roles. The molecular mechanism by which gastric microcirculation was perturbed by reactive oxygens is under our current investigation.

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