Mechanism for Enterohepatic Injury Caused by Circulatory Disturbance of Hepatic Vessels in the Rat (43300)

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Abstract. **We reported previously that a transient occlusion followed by reperfusion of the portal vein and the hepatic artery of the rat significantly decreased the transhepatic transport of a cholephilic compound, and that this decrease was prevented by pretreating animals with poly(styrene co-maleic acid butyl ester)-conjugated superoxide dismutase (SM-SOD). To elucidate the mechanism for oxidative injury of the liver and the site for the generation of superoxide radicals, the effect of a portosystemic bypass on the liver function was examined in the rat whose hepatic vessels were temporarily occluded. A portosystemic bypass inhibited the reperfusion-induced decrease in hepatic transport of bromosulfophthalein as effectively as did SM-SOD. Kinetic analysis using ^{125}I-labeled albumin revealed that the permeability of the small intestine markedly increased after a transient occlusion. The increase in intestinal permeability was also inhibited either by SM-SOD or by the portosystemic bypass. Xanthine oxidase activity in portal plasma markedly increased during occlusion and reperfusion, while it remained within normal ranges in the bypassed group. Thus, superoxide radical, and/or its metabolite(s), might play a critical role in increasing the intestinal permeability and in the pathogenesis of reperfusion-induced liver injury.** [P.S.E.B.M. 1991, Vol 198]

Postischemic reperfusion injury of the liver is one of the major obstacles encountered in transplantation and other radical operations. Occlusion of the portal vein might result in several pathologic events, such as congestive injury of the intestine and metabolic disorder of the liver and other organs. In order to minimize such hazardous events, venovenous bypass has been introduced during the so-called "anhepatic phase" in orthotopic liver transplantation (1). Although this method has been assumed to increase the survival rate of the recipients (2), the apparent advantage of this technique remains to be elucidated (3, 4).

Oxygen-derived free radicals play critical roles in ischemia and reperfusion-induced tissue injury (5–8). Although oxidative injury of cells is effectively inhibited by Cu/Zn-superoxide dismutase (SOD) *in vitro*, the protective effect of the enzyme *in vivo* is fairly low, predominantly because of its short half-life (5 min) in the circulation (9). To study the pathologic significance of oxygen radicals *in vivo*, we have synthesized an injured-site-directed-SOD derivative (poly[styrene co-maleic acid butyl ester]-conjugated SOD [SM-SOD]) that circulates bound to albumin with a half-life of 6–8 hr (10, 11). We also reported that a transient occlusion followed by reflow of the portal vein and the hepatic artery impaired the ability of the liver function to transport cholephilic organic anions, such as bile acids and bromosulfophthalein (BSP), by a SM-SOD-inhibitable mechanism (12). To elucidate the mechanism for liver injury and the site for generation of hepatotoxic oxygen metabolite(s), we tested the effect of the portosystemic bypass on postischemic reperfusion injury of the liver induced by a transient occlusion of hepatic vessels.

Materials and Methods

Materials. BSP and bovine serum albumin were purchased from the Sigma Chemical Co. (St. Louis, MO). SM-SOD was synthesized from human Cu/Zn-type SOD by covalently linking 2 mole of poly(styrene co-maleic acid butyl ester) to 1 mole of the enzyme, as described previously (10, 11). ^{125}I-labeled albumin was

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prepared using ^{125}I-labeled Bolton-Hunter reagent, as described previously (13); specific radioactivity of the labeled albumin sample was 5×10^5 cpm/mg protein. Other reagents used were of analytical grade.

Animals. Male Wistar rats, weighing 200–300 g, were fasted for 16 hr prior to the experiments. Control and bypassed animal groups were used. The portal veins of the second group were bypassed to the systemic circulation by sc transposition of the spleen 3 weeks before experiments (14). This bypass method forms anastomosis between the spleen and the anterior abdominal wall and has been used to eliminate congestive injury of the intestine, such as increased vascular permeability and edema, during the occlusion of the portal vein. Under pentobarbital anesthesia (50 mg/kg), the common bile duct of the heparinized rat (500 units/kg) was cannulated with polyethylene tubing. Control animals were injected in the right femoral vein with 0.25 ml of saline or 5 mg/kg of SM-SOD solution. The bypassed group was injected with saline. After 15 min, hepatic ischemia was elicited by occluding the portal vein and the hepatic artery 0.5 cm distal to the hepatic hilum. After 20 min of occlusion, the hepatic vessels were reperfused for 120 min. The body temperature of the anesthetized animals was kept constant at 37°C by a tungsten lamp during the experiments.

Measurement of Systemic Arterial Blood Pressure and Hepatic Blood Flow. Systemic arterial blood pressure was continuously monitored at the right femoral artery by a transducer (Nihon Koden AP600G; Tokyo). Hepatic blood flow was measured during experiments by a laser-meter (Advance ALF-2100; Tokyo) (15).

Hepatic Transport Activity of BSP. After 60 min of reperfusion of the hepatic vessels, 0.2 ml of BSP solution (5 μmol/kg) was injected into the right femoral vein over a period of 5 sec. Bile samples were collected from the common bile duct during 12 consecutive 5-min collection periods for 60 min. BSP levels in bile were determined spectrophotometrically at 580 nm in 0.1 M NaOH, as described previously (16).

Distribution of Radioactive Albumin. Sixty minutes after reperfusion, radioactive albumin (100,000 cpm/rat) was administered intravenously. After 15 min, animals were exsanguinated from the right femoral artery, and the tissue-associated radioactivity was determined in a Packard autogamma-scintillation spectrophotometer (model 5130).

Assay of Plasma Xanthine Oxidase Activity. At timed intervals, blood samples (0.2 ml) were collected from the right femoral vein and the portal vein hepatodistal to the occlusion site. Xanthine oxidase activity in plasma was determined by measuring the rate of urate synthesis (17).

Neutrophil-Dependent Chemiluminescence Intensity. At indicated times, 0.1-ml blood samples from the right femoral vein were collected into test tubes containing 0.8 mg of citric acid. Neutrophil-dependent chemiluminescence was measured by the luminol method using opsonized zymosan (18) in an Aloka BLR-201 luminescence meter (19). Blood samples from the control group were also collected 120 min after reperfusion. Then, neutrophil-dependent chemiluminescence was determined in the presence of 20 units of either SOD, SM-SOD, catalase, SOD + catalase, or 1 mM of NaN_3.

Statistics. In most cases, results were expressed as the mean ± SD. Differences were evaluated for statistical significance using the Student's t test.

Results

Effect of Transient Occlusion on Hemodynamics and Bile Flow. To know the effect of a transient occlusion of the portal vein and the hepatic artery on hemodynamics, changes in hepatic blood flow and systemic blood pressure were monitored during the experiments. Immediately after occlusion of the hepatic vessels, the blood pressure and the hepatic blood flow decreased markedly in the control group (Fig. 1). They rapidly returned to normal levels after reperfusion. During

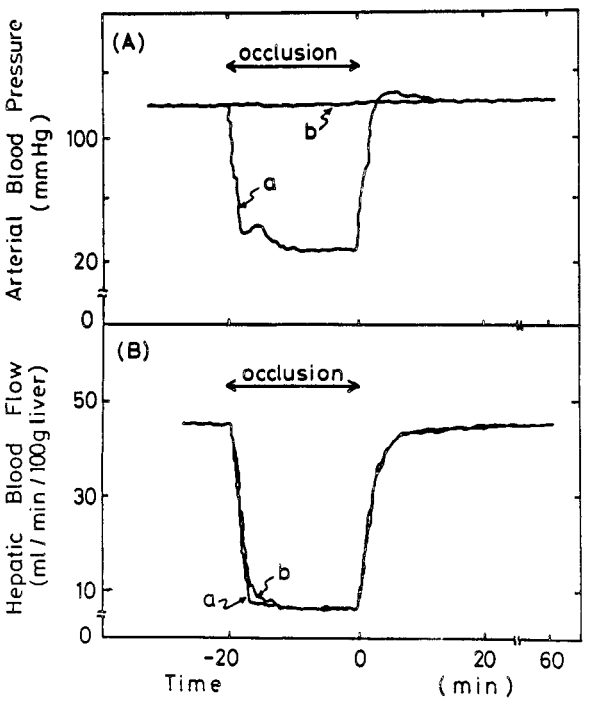

Figure 1. Changes in hemodynamics induced by occlusion and reperfusion of hepatic vessels. Under pentobarbital anesthesia (50 mg/kg), animals were intravenously injected with 0.25 ml of saline. Then, the portal vein and the hepatic artery were transiently occluded for 20 min. During the experiments, (A) systemic blood pressure and (B) hepatic blood flow were monitored. In some experiments, the portal vein was bypassed to the systemic circulation 3 weeks before the experiments. Data show typical changes in the circulatory status of animals during occlusion and reperfusion (n = 8). Arrows show the period of transient occlusion of the portal vein and the hepatic artery. [a] control group; [b] bypassed group.

occlusion and reperfusion, the hepatic blood flow in the bypassed group also changed in a manner similar to that in the control group. However, the blood pressure remained unchanged during the experiments. To know the effect of a transient occlusion of the hepatic vessels on the liver function, the change in bile flow was determined. Bile flow decreased markedly immediately after occlusion. However, it returned to normal levels within 60 min after reperfusion in both the control and bypassed groups. Administration of SM-SOD did not affect the change in bile flow induced by transient occlusion of hepatic vessels (Fig. 2).

Effect of Circulatory Disturbance on Hepatic Transport Activity. Since BSP undergoes transhepatic transport from plasma to bile via some carrier-mediated mechanism (20), the biliary BSP level rapidly reached its maximum within 10 min after administration: 97.5 ± 4.1% of the dose was recovered in the bile within 60 min after injection (Fig. 3A). Biliary secretion of BSP decreased significantly in the reperfused group: 48.7 ± 6.8% of the injected dose was recovered in the bile within 60 min. Consistent with our previous observation (12), the biliary secretion of BSP did not decrease in animals that were pretreated with SM-SOD: 91.4 ± 4.4% of the dose was recovered within 60 min after administration. Hydrogen peroxide-inactivated SM-SOD (50 mg/kg) failed to protect the liver dysfunction in control groups (data not shown). These results suggest that a superoxide radical might play a critical role in decreasing BSP transport in the reperfused group. In contrast, BSP transport occurred normally in the bypassed group (Fig. 3B): 94.2 ± 3.5% of the dose was recovered in the bile within 60 min after administration. Interestingly, BSP transport activity remained unchanged in the bypassed group, even after occlusion followed by reperfusion: 91.9 ± 4.9% of the dose was recovered within 60 min after administration. Thus, pathologic metabolism occurring in the congested intestine rather than hepatic ischemia might be important for the decrease in BSP transport.

Distribution of Radioactive Albumin. Radiolabeled albumin has been used for evaluating circulatory status in animals (21). To know the change in circulatory status of animals after transient occlusion of hepatic vessels, ^{125}I-labeled albumin was injected intravenously 60 min after reperfusion. Compared with sham-operated animals, the radioactivity associated with the small intestine of the reperfused group was increased markedly by an SM-SOD-inhibitable mechanism (Fig. 4). Hydrogen peroxide-inactivated SM-SOD (10 mg/kg) had no such inhibitory action (data not shown). However, the intestinal radioactivity in the bypassed group was similar to that in the sham-operated group. Thus, transient occlusion of hepatic vessels might increase the permeability of the small intestine for macromolecular compounds in the control group, but not in the bypassed group.

Xanthine Oxidase Activity in Plasma. Figure 5 shows the change in xanthine oxidase activity in plasma samples obtained from portal and femoral veins. The enzyme activity in portal vein plasma increased markedly during occlusion and the elevated levels remained unchanged during reperfusion (Fig. 5). Xanthine oxidase activity in plasma samples from the femoral vein also increased during occlusion, though the extent of increase was smaller with femoral vein plasma than

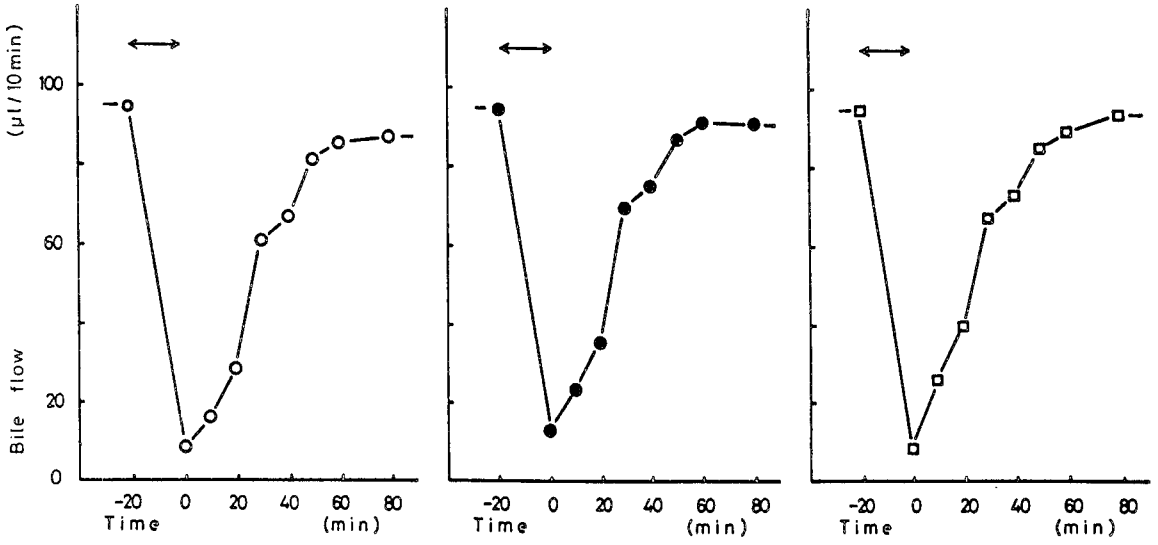

Figure 2. Changes in bile flow during experiments. Control animals were intravenously injected with 0.25 ml of saline (open circles) or 5 mg/kg of SM-SOD (open squares) 15 min before occlusion. Then, the hepatic vessels were occluded for 20 min and reperfused for 60 min. Bile samples were collected from the common bile duct for nine consecutive 10-min collection periods. The bypassed animals (closed circles) were also injected with 0.25 ml of saline 15 min before occlusion. Arrows show the time for occlusion. Other conditions were the same as in Figure 1.

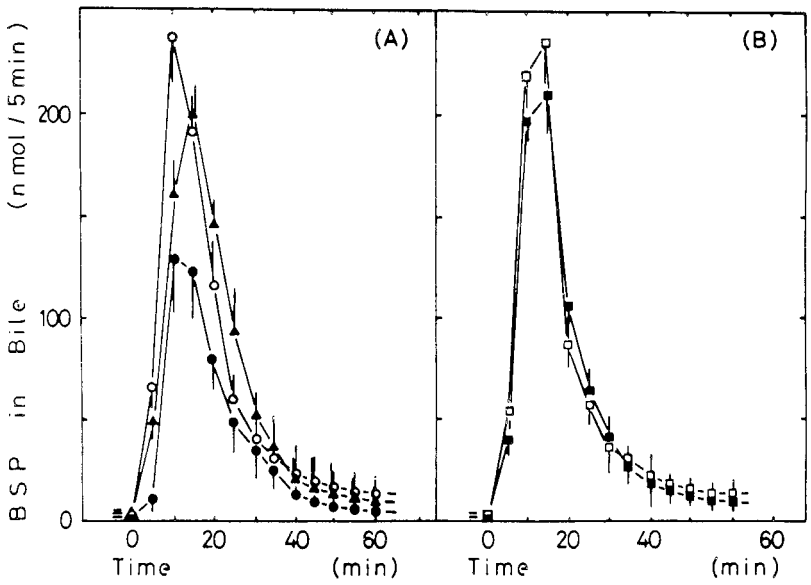

Figure 3. Effect of transient occlusion on BSP transport. Control animals were intravenously injected with 0.25 ml of saline (circles) or 5 mg/kg of SM-SOD (triangles) 15 min before occlusion of the hepatic vessels. The bypassed animals were injected with saline. Hepatic vessels of the (A) control and (B) bypassed groups were occluded for 20 min and reperfused for 60 min (closed symbols). Then, BSP was injected in the right femoral vein (5 μmol/kg) over a period of 5 sec. At the indicated times after BSP administration, bile samples were collected for 12 consecutive 5-min collection periods. BSP levels in the bile were determined as described in Materials and Methods. Bile flow remained constant (1.0 ~ 1.5 μl/min/g liver) during the experiments. Each point represents the mean ± SD derived from six to seven animals. Other conditions were as described in Materials and Methods. Hepatic vessels, indicated by open symbols, were not occluded.

with portal vein plasma. SM-SOD failed to inhibit the increase in xanthine oxidase activity in the control group, as determined by the rate of uric acid synthesis (17). The oxidase activity in plasma samples from the bypassed group remained unchanged during the experiment.

Neutrophil-Dependent Chemiluminescence Intensity of the Blood. When neutrophils are stimulated by various ligands, they produce reactive oxygen species (22). Luminol-dependent chemiluminescence has been used for determining the activity of neutrophils to generate reactive oxygen species (18). To elucidate whether transient occlusion of the hepatic vessels affected the property of circulating leucocytes, the intensity of chemiluminescence of the peripheral blood was measured during the experiments (Fig. 6). Although the chemiluminescence of blood samples from normal animals was of significantly low intensity, it increased markedly after transient occlusion of hepatic vessels. In contrast, the chemiluminescence remained unchanged in intensity during the experiments in the bypassed group. Intravenous administration of SM-SOD significantly inhibited the increase in the chemiluminescence intensity of blood samples from the reperfused group. To know the reactive species responsible for the increase in chemiluminescence, the effects of SOD, SM-SOD, catalase, and NaN_3 were also tested *in vitro* (Fig. 7). When stimulated by zymosan, the chemiluminescence intensity of blood samples from the reperfused group increased markedly. In contrast to the marked inhibition of chemiluminescence by intravenously injected SM-SOD, the zymosan-stimulated chemiluminescence was inhibited only slightly by SOD, SM-SOD, and SOD + catalase added in the blood samples. In contrast, NaN_3, a potent inhibitor of myeloperoxidase, markedly inhibited the chemiluminescence. These results suggested that HClO, rather than superoxide and hydrogen peroxide, might be predominantly responsible for the increase in chemiluminescence.

Discussion

The present study demonstrates that a portosystemic bypass inhibited the decrease in BSP transport by the reperfused liver. Occlusion of the portal vein might induce various pathologic events, such as the decrease in plasma volume leading to systemic hypoperfusion and hypovolemic shock. In fact, the systemic blood pressure decreased markedly during occlusion and the hematocrit increased from 0.45 to 0.65 in the control group. It has been reported that dogs and rats have died of circulatory disturbances when their portal veins were occluded for longer than 45 min (23, 24). Endotoxin often induces microcirculatory disturbances leading to disseminated intravascular coagulation, particularly when the portal vein was occluded for longer than 60 min (25, 26). However, plasma endotoxin levels remained within normal ranges under the present experimental conditions (less than 3 pg/ml), as determined by the method of Obayashi *et al.* (27).

Reactive oxygen species may arise from a number

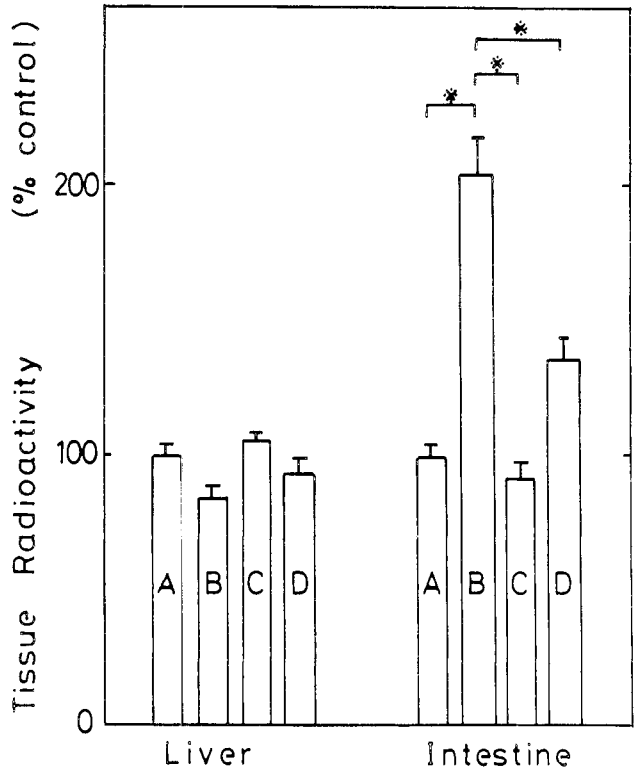

Figure 4. Distribution of radioactive albumin. Control animals were intravenously injected with 0.25 ml of saline or SM-SOD (5 mg/kg). Then, the hepatic vessels of the control and the bypassed groups were occluded for 20 min and reperfused for 60 min. After reperfusion, 1 mg/kg of ^{125}I-labeled albumin (100,000 cpm/rat) was injected into the right femoral vein. After 15 min of administration, animals were exsanguinated from the right femoral artery. Then, tissues were perfused with 10 ml of ice-cold saline through the abdominal aorta. The levels of radioactivity in liver and small intestine were expressed as a percentage of those found in the sham-operated group. Data show the mean ± SD derived from five animals. (A) Sham-operated group; (B) control group; (C) bypassed group; (D) SM-SOD-treated control group. ※ $P < 0.01$, versus control group.

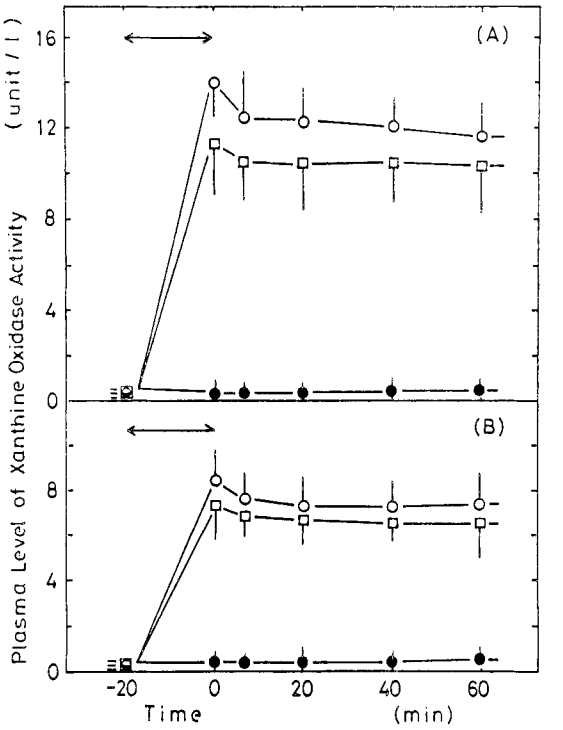

Figure 5. Effect of transient occlusion on xanthine oxidase activity in plasma. Control animals were intravenously injected with 0.25 ml of saline or SM-SOD (5 mg/kg). The bypassed groups were injected with saline. After 15 min, the hepatic vessels were occluded for 20 min (arrows) and reperfused for 60 min. At the indicated times, 0.1-ml blood samples were obtained from the (A) portal and (B) right femoral veins. The oxidase activity in plasma was determined by measuring the rate of uric acid synthesis. Each point represents the mean ± SD derived from five animals. (Open circles) Control group; (closed circles) bypassed group; (open squares) SM-SOD-treated control group.

of sources, such as NADPH-oxidase in activated leucocytes and xanthine oxidase in injured endothelial cells (28, 29). Vascular endothelial cells in the liver and intestine are highly enriched with xanthine dehydrogenase, an enzyme that can be converted to xanthine oxidase (30). Miyagawa (31) demonstrated that a transient occlusion followed by reperfusion of the portal vein destroyed vascular endothelial cells in the intestine of normal dogs, but not in the intestine of animals with a portosystemic bypass. The present study also demonstrates that both xanthine oxidase activity in plasma and the intensity of chemiluminescence of circulating leucocytes increased markedly in the control group by a mechanism which was inhibited by a portosystemic bypass. The prominent increase in the oxidase activity in portal plasma might reflect the destruction of endothelial cells of the injured intestine. Since xanthine oxidase activity in the peripheral blood increased after reperfusion, the circulating oxidase would be of enterohepatic origin. We reported previously that SM-SOD apparently inhibited the increase in xanthine oxidase activity in the peripheral blood of the reperfused animals, as determined by the nitroblue tetrazolium method (12). Since this method is affected significantly by the presence of endogenous and exogenous SOD, another method applicable to plasma samples that contain SOD was introduced in the present experiments. Although SM-SOD failed to inhibit endothelial cell injury of the intestine as determined by plasma levels of xanthine oxidase, hepatic transport of BSP occurred normally in the SM-SOD-treated group. Thus, SM-SOD might degrade superoxide radicals in the circulation, thereby inhibiting reperfusion injury of the liver. The mechanism which causes reperfusion injury of the liver should be studied further.

Since NaN_3, but not SOD + catalase, added to the reaction mixture inhibited the chemiluminescence, HClO generated by myeloperoxidase might be responsible for zymosan-stimulated chemiluminescence. Though SM-SOD failed to inhibit the increase in the intensity of chemiluminescence *in vitro*, the increase was inhibited by an intravenous injection of SM-SOD. Since the intensity of chemiluminescence of blood sam-

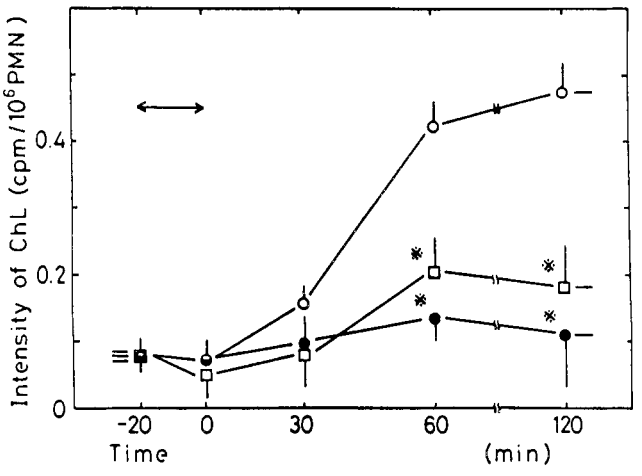

Figure 6. Effect of transient occlusion on neutrophil-dependent chemiluminescence. Control animals were intravenously injected with 0.25 ml of saline (open circles) or 5 mg/kg of SM-SOD (open squares). Then, the hepatic vessels of the control and the bypassed groups (closed circles) were occluded for 20 min (arrow) and reperfused for 120 min. At the indicated times, 0.1 ml of blood was collected from the right femoral vein into test tubes containing 0.8 mg of citric acid. The intensity of zymosan-induced chemiluminescence (ChL) of neutrophils (PMN) was determined, as described in Materials and Methods. Each point represents the mean ± SD derived from five animals. Other conditions were the same as in Figure 1. ※$P < 0.01$, versus control group.

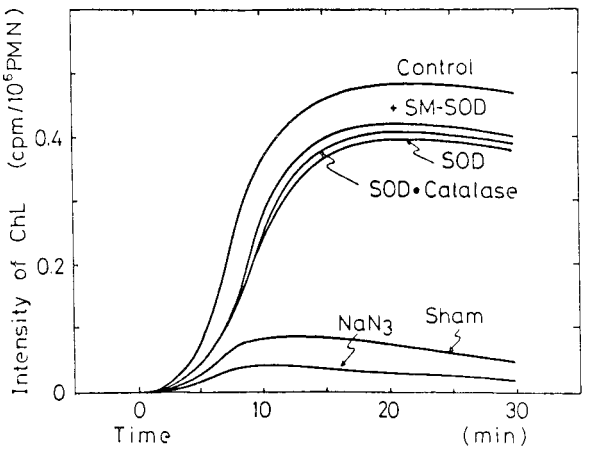

Figure 7. Effect of SOD and catalase on neutrophil-dependent chemiluminescence. Hepatic vessels of control animals were occluded for 20 min and reperfused for 120 min. Then, 0.1 ml of blood was collected from the right femoral vein into test tubes containing 0.8 mg of citric acid. Then, zymosan-induced chemiluminescence intensity (ChL) of neutrophils (PMN) was determined either in the absence (control) or the presence of 20 units of SOD, SM-SOD, or SOD + catalase, or 1 μmol of NaN_3. "Sham" refers to blood samples from sham-operated group.

ples remained unchanged in the bypassed group, the neutrophils would have been activated by some O_2^--dependent mechanism. In fact, a significant amount of xanthine oxidase was released from the endothelial cells of the congested intestine. Activated neutrophils may also participate in decreasing transport function of the reperfused liver.

We also tested the effect of prolonged ischemia (60 min) on the liver function of animals with a portosystemic bypass. Postischemic reperfusion decreased the biliary excretion of BSP in the bypassed group by 42.5 ± 5.6% ($n = 4$). Again, SM-SOD inhibited the reperfusion injury of the liver in the bypassed group; within 60 min, 72.4 ± 6.1% of the injected BSP appeared in the bile of the SM-SOD-treated group ($n = 4$). These findings suggest that the liver also generates reactive oxygen species under prolonged ischemia. Since the permeability of the intestine for radioactive albumin increased markedly after occlusion of the portal vein, some part of the free and albumin-bound BSP would have escaped from the circulation into the extravascular space of the injured intestine. In fact, intravenously injected Evans blue dye (20 μmol/kg) markedly accumulated in the small intestine of the reperfused group by an SM-SOD-inhibitable mechanism; 36.5 and 16.1% of the injected dose of the dye was found to accumulate in the intestine of the control and SM-SOD-treated groups, respectively. In addition to the decrease in hepatic transport activity, extravasation of BSP into the extravascular space of the injured intestine might be responsible for, at least in part, the decrease in the biliary recovery of BSP. Although SM-SOD failed to inhibit the increase in plasma xanthine oxidase levels, efficient dismutation of superoxide radical by SM-SOD seemed to inhibit the increase in the permeability of the congested intestine. Consistent with this notion is the finding that SM-SOD markedly inhibited the increase in vascular permeability in various types of tissue injury, such as cold-induced brain edema (32) and stress-induced gastric mucosal injury (33). The mechanism by which intestinal permeability is controlled by superoxide radicals should be studied further.

Since the portal vein in humans forms an extensive collateral network, occlusion of the portal vein might elicit fewer problems than it does in other species (4). However, Shaw *et al.* (3) reported that a venovenous bypass allowed a longer time for careful dissection of the recipient's liver and preparation of the vessels for anastomosis. Thus, a portosystemic bypass, as well as SM-SOD, might permit longtime occlusion of the portal vein for radical operation of the liver, without causing enterohepatic injury.

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