Free Radical Formation during Splanchnic Artery Occlusion Shock

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Free radical (FR) formation in the rat intestinal lumen was measured using the spin-trapping technique and electron paramagnetic resonance spectroscopy. Intestinal ischemia was produced by occluding the celiac and the superior mesenteric arteries for 30 min followed by reperfusion. The lumen was filled with a solution of PBN (N-tert-butyl-α-phenyl-nitrone) and the intestine was squeezed to enhance the interaction between the PBN solution and the intestinal mucosal cells. Free radicals were produced upon reperfusion, with peaks at 5 and 90 min. Post-ischemic treatment with superoxide dismutase (20 $mg \cdot kg^{-1}$) inhibited the increase of FR production during the second peak by 36%. In a single study in a group of leucocytopenic rats (WBC < $1500/mm^3$), the increase of FR production during the second peak was decreased by 80%. However, these treatments did not inhibit the FR production during the first peak in either group. In contrast, pretreatment with allopurinol (40 and 100 $mg \cdot kg^{-1}$ injection at 24 and 3 hours before ischemia, respectively) inhibited the FR production during the first peak by 76%, but did not inhibit during the second peak. The changes in lipid peroxidation in the intestinal mucosa, specific gravity of the intestine and in the hematocrit were correlated to the FR production in the second peak. These results suggest that a major cause of tissue injury after reperfusion in the ischemic intestine may largely be produced by neutrophils. (Key words: free radical, spin-trapping, ischemia-reperfusion, lipid peroxidation, neutrophil, electron paramagnetic resonance)

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It has been well-recognized that in many organs ischemia-reperfusion induces severe injury and that one of the most important mechanisms may be linked to the production of oxygen free radicals[1,2]. Oxygen free radicals have been shown to attack vital cellular components, such as lipids[3], proteins[4] and nucleic acids[5]. However, the evidence for the production of such radicals in ischemia-reperfusion injury has been indirect. Because of the unstable nature of free radicals, direct demonstration of their production has been difficult. Generally, the forma-

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tion of free radicals has been studied through changes in the level of lipid peroxidation, the alteration of membrane phospholipid components, the changes of the levels of endogenous radical scavengers and through the tissue protection provided by free radical scavengers or antioxidants.

Recently, using the spin-trapping technique and electron paramagnetic resonance (EPR), the formation of oxyradicals during ischemia-reperfusion injury was directly demonstrated in several organs *in vivo*[6–9]. It has been proposed that the sources of oxygen free radicals during ischemia-reperfusion injury may include the stimulation of the xanthine-xanthine oxidase system[1,10], electron leakage and redox state alteration of CoQ in mitochondria[11], activated neutrophils[12], arachidonic acid metabolism and catecholamine oxidation. In the intestinal ischemic model, there is increasing evidence that oxygen free radicals generated from xanthine oxidase in the ischemic cells and NADPH oxidase from activated phagocytosis are mainly responsible for ischemia-reperfusion injury[13–15].

In this study, we measured the free radical formation in the small intestinal lumen by using a spin trapping agent, PBN, and evaluated the effects of human-recombinant superoxide dismutase (SOD) and a xanthine oxidase inhibitor (allopurinol) on free radical formation. The role of neutrophils was also evaluated using leucocytopenic rats. Lipid peroxidation, specific gravity of intestine and the hematocrit value were measured as the indices of tissue injury. The relationship between free radical formation and these indices were discussed.

Methods

Experimental protocol

The present study consists of two types of experiments. The first involved establishing baseline measurements of free radical formation in the intestinal lumen, lipid peroxidation of the intestinal mucosa, specific gravity of intestine and the hematocrit value during ischemia-reperfusion injury in the rat intestine. The second involved observations of effects of SOD, allopurinol (4-hydroxypyrazol (3,4-d) pyrimidine (10% (w/v) suspension at pH 10); Sigma Chemical Co., St. Louis, MO) and the depletion of neutrophils on the above mentioned parameters.

In the first experiments, the rats were divided into 8 groups according to the reperfusion time (when ischemia was produced, the time was 30 min; the numbers in the parenthesis indicate numbers of animals); no ischemia control(10), ischemia only(7), 5 min(10), 10 min(8), 30 min(7). 60 min(7), 90 min(10), and 120 min(7) of reperfusion.

In the SOD treated group, 20 $mg{\cdot}kg^{-1}$ of human recombinant SOD (350,000 U/75 mg, Nippon Kayaku Co. Ltd., Tokyo) was injected i.v. starting at 5 min prior to the onset of reperfusion for a period of 5 min. The dose was based upon a previous study[16]. The rats were exposed to 30 min ischemia, followed by either 5 min reperfusion (N=6) or 90 min reperfusion (N=7).

The allopurinol treatd group received 40 and 100 $mg{\cdot}kg^{-1}$ of allopurinol intraperitoneally 24 and 3 hours before experiment, respectively, according to the method of Roldan et al.[17]. The rats were exposed to 30 min of ischemia, and then to either 5 min reperfusion (N=6) or 90 min reperfusion (N=7).

Leucocytopenic rats were prepared basically by the method by Shasby et al.[18] This study used 1.5 $mg{\cdot}kg^{-1}$ of nitrogen mustard (Mechlorethamine hydrochloride, Sigma Chemical Co.) injected i.v. 72 hours prior to the

experiment. Leucocytopenia (< 1500 cells·mm^{-3}) occurred reproducibly. The rats were exposed to 30 min ischemia, and then to either 5 min (N=6) or 90 min reperfusion (N=7).

Surgical procedure

Male Sprague-Dawley rats (Zivic Miller Lab. Inc., PA), weighing 300–325g, were used for all experiments. They were allowed pellets and water ad libitum. Following 6 hours starvation, anesthesia was induced with sodium pentobarbital (40 mg·kg^{-1}) administered intraperitoneally. All animals were subjected to the same surgical procedure. After tracheal intubation, 24 gauge teflon catheters were inserted into the tail vein for drug administration and into left carotid artery for recording arterial pressure (using Statham P23Dd trasnsducer) and for blood sampling. An electrocardiograph (Hewlett Packard 1511B) was used for measurement of the heart rate. Rectal temperature was monitored (using a Yellow Springs Model 41TA) and maintained 36.5 ± 0.5°C by heat lamps throughout the experiment. After a middle laparatomy, the celiac and the superior mesenteric arteries were isolated. The jejunum was ligated with 6–0 suture without injuring vessels at 10 cm, 25 cm and 32 cm from the Treitz' ligament for making two segments. These segments were used for subsequent measurements of spin adduct formation and lipid peroxidation. After completion of surgery, the animals were held for a 30 min stabilization period prior to the experiment. After administration of heparin (500 U·kg^{-1}, i.v.), the celiac and the superior mesenteric arteries were occluded using soft clips for 30 min. Following the ischemic period, the clips were removed and the intestine was reperfused for desired time periods. SOD or its vehicle (0.9% NaCl, 1.0 ml·kg^{-1}) alone was infused starting from 5 min prior to the start of reperfusion for a 5 min period. At the end of each experiment, the free radical formation, lipid peroxidation, and specific gravity were measured. The hematocrit value was measured prior to and at the end of the experiment.

Detection of PBN spin adducts

The method was essentially similar to that of Sakamoto et al.[8] In brief: PBN, chloroform and methyl alcohol were obtained from Aldrich Chemicals Co. (Milwaukee, WI). PBN was dissolved to a 130 mM concentration in deionized water (prepared through Milli Q cartridges, Millipore Co., Bedford, MA) and was treated with activated charcoal according to the method of Buettner et al.[19] until EPR signals from impurities were eliminated. The final concentration of PBN was determined using the molar extinction coefficient of 16,700 at 294 nm in ethanol. The PBN solution was mixed with an ice-cold deoxygenated Krebs-Ringer phosphate (KRP) buffer (120 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$ and 10 mM Na_2HPO_4) to make a final concentration of 80 mM PBN. To the mixture, 0.1 mM dietylenetriaminepentaacetic acid (DTPA, Aldrich) was added to eliminate the undesirable possibility of trace metal-induced radical formation[20]. At the end of each experiment, PBN-KRP mixture was injected intraluminally into the first intestinal segment using a 26 gauge needle for 15 seconds. Then, the mixture and the mucosal lining of the intestine were extruded by squeezing the intestinal contents into a 6 ml ice-cold, deoxygenated chloroform-methanol solution (2:1). The Folch extraction[21] was performed and the solvent was evaporated. The residue was redissolved into a 0.1 ml chloroform-methanol solution and was transferred into a glass capillary pipet tip (I.D., 1.5 mm; length, 90

mm; one-side had been flame-sealed). EPR measurement was performed using a Varian E-109 spectrometer (Varian Techtron Pty. Limited, Victoria, Australia) at the following settings: gain, 2.5×10^4: microwave power, 20 mW; modulation amplitude, 0.2 mT; time constant, 0.5 second; scan range, 10 mT; scan time, 8 minutes; the temperature was $8 \pm 0.5°C$.

Measurement of Lipid peroxidation

At the end of each experiment, intestinal mucosa of the second intestinal segment was homogenized in 2.5 ml of KRP buffer solution without PBN (20 mg protein/ml. Protein concentration was determined by the Biuret method). The formation of thiobarbituric acid reactive substances (TBAR) was determined as described by Buege and Aust[22] with some modifications. To the 0.15 ml homogenate, 0.5 ml of ice-cold 0.8 N HCl containing 12.5% trichloroacetic acid was added to stop the reaction. Then 0.4 ml of H_2O containing 50 μM desferrioxamine was added to prevent iron-catalyzed TBAR formation. Thiobarbituric acid (Sigma, final concentration 0.67%) was finally added, and the samples were boiled in the presence of 0.05% butylated hydroxytoluene for 20 min. The samples were then cooled, centrifuged at 1500 $\times$ g for 15 min, and the absorbance of the supernatant was read at 532 nm. Quantitation was based upon a molar extinction coefficient of 1.56×10^5.

Measurement of the specific gravity of intestine

The specific gravity (SG) of the intestine was determined according to the method of Tengvar et al.[23] with some modifications. A heavy solution (dimethyl phthalate, SG=1.1866) and a light solution (toluene, SG=0.9660) were mixed to produce solutions with different SG's. The SG was determined using a 10 ml picnometer. The SG's ranged from 1.020 to 1.050 with increments of 0.001. At the end of each experiment, a portion of the intestine 32 to 34 cm from the Treitz' ligament was extracted, sliced circularly every 1–2 mm and the slices were put into solutions with different SG's. The SG of the intestine was determined by the SG of a solution in which the slices neither floated nor sank.

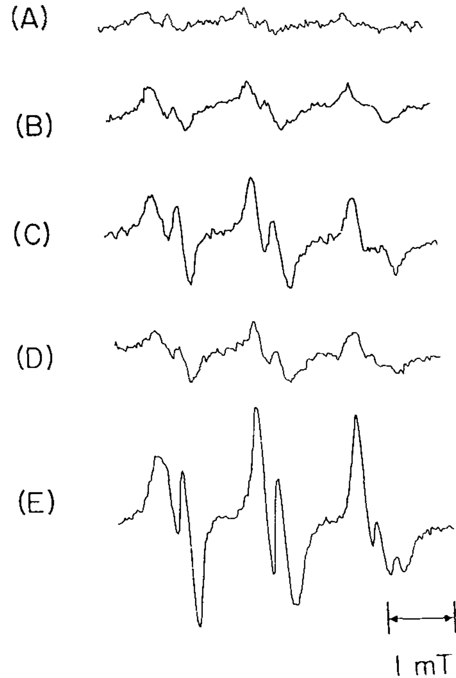

Fig. 1. Representative examples of electron spin resonance spectra of spin adducts: (A) no ischemia control; (B) 30 min ischemia – no reperfusion; (C) 30 min ischemia – 5 min reperfusion; (D) 30 min ischemia – 10 min reperfusion; (E) 30 min ischemia – 90 min reperfusion.

Statistical analysis

Unless otherwise indicated, all values were expressed as the mean ± standard deviation. Data were analyzed by ANOVA. The student's t-test was used to determine the significant differences

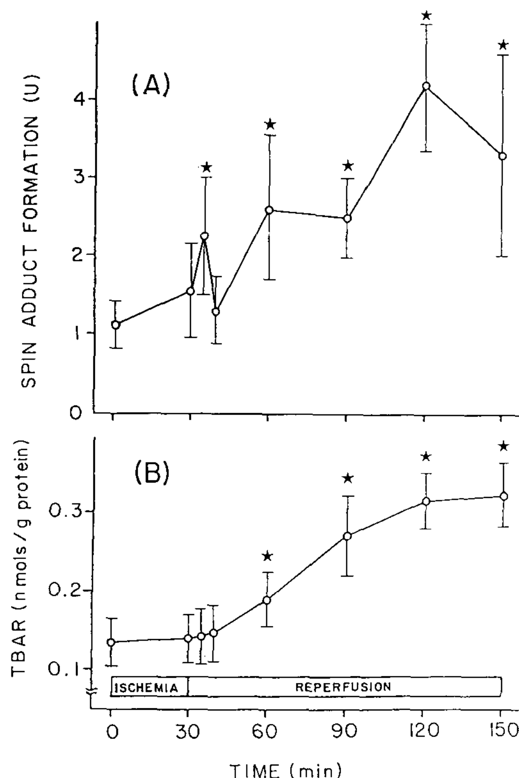

Fig. 2. (A) Time course of the formation of PBN spin adducts during ischemia – reperfusion of the intestine. (B) The level of TBAR from the intestinal mucosa during ischemia – reperfusion of the intestine. *indicates $P < 0.05$.

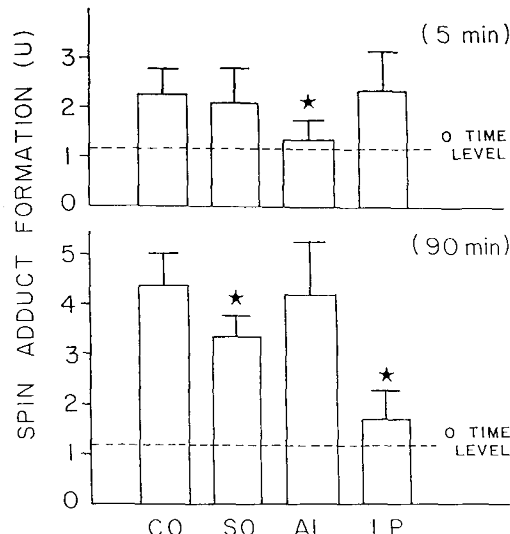

Fig. 3. The effects of SOD, allopurinol and leucocytopenia on the spin adduct formation at 5 min and 90 min of reperfusion time. CO: control; SO: SOD-treated rats; AL: allopurinol-treated rats; LP: leucocytopenic rats. The dotted lines indicate the level of no ischemia control. *indicates $P < 0.05$.

between 2 groups. The differences was considered significant when $P < 0.05$.

Results

Physiological parameters

The pre-ischemic mean arterial blood pressure (MAP) in the different experimental groups ranged from 118 ± 13 to 121 ± 18 mmHg and there were no significant differences among groups. All ischemia experimental group exhibited a similar increase in MAP (about 25 mmHg) after the arterial occlusion, followed by a decrease to pre-ischemic values by the end of ischemia. After the onset of reperfusion, a rapid hypotension (to about 60 mmHg) was observed, followed by an increase in MAP (to about 95 mmHg) by 15 to 20 min of the reperfusion time. Then, there was a gradual secondary decrease in MAP. At 90 min of the reperfusion time, the MAP's were; non-treated group (68 ± 18), SOD-treated group (85 ± 15), allopurinol-treated group (70 ± 14) and leucocytopenic group (86 ± 17 mmHg). There were no significant differences among groups.

EPR measurement

Typical EPR spectra of spin adducts are shown in figure 1. They are (A) no ischemia control; (B) 30 min ischemia no reperfusion; (C) 30 min ischemia-5 min reperfusion; (D) 30 min ischemia-10 min reperfusion and (E) 30 min ischemia-90 min reperfusion. No signal was detected in the solution without samples (data not shown). As shown

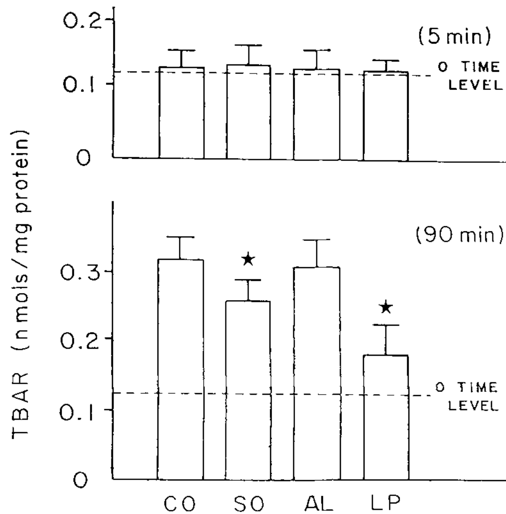

Fig. 4. The effects of SOD, allopurinol and leucocytopenia on the level of TBAR at 5 min and 90 min of reperfusion time. CO: control; SO: SOD-treated rats, AL: allopurinol-treated rats; LP: leucocytopenic rats. The dotted lines indicate the level of no ischemia control. *indicates $P < 0.05$.

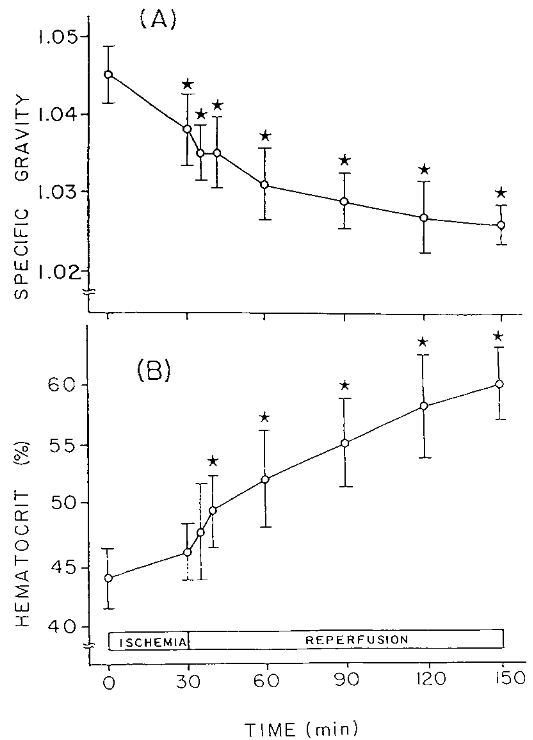

Fig. 5. (A) The time course of the specific gravity during ischemia-reperfusion of the intestine. (B) The level of the hematocrit during ischemia-reperfusion of the intestine. *indicates $P < 0.05$.

in the figure, all signals demonstrated 3 doublets. The spectra had parameters of a_N=14.3G and a_H^β=2.8G. It was assumed that the free radical formation is proportional to the averages of the low-fieled doublet of the spin adduct spectrum[8]. The time course of the production of spin adducts was expressed in U (arbitrary unit) (fig. 2A). The figure shows that upon reperfusion, spin-adduct formation increased and peaked at 5 min, but, returned to the pre-reperfusion level at 10 min. Then, the formation increased again and reached another peak at 90 min. From this result, it was decided to use 5 and 90 min of reperfusion times to measure the effects of SOD, allopurinol and neutrophil-depletion on the free radical formation. As shown in figure 3, at 5 min of reperfusion, allopurinol inhibited reperfusion-induced spin adduct formation by 76%, however, SOD and neutrophil-depletion showed no inhibition. In contrast, at 90 min of reperfusion, SOD and depletion of neutrophil inhibited the increase of spin adduct formation by 36% and 80%, respectively, while allopurinol did not inhibit the formation.

Lipid peroxidation

In non-treated rats, TBAR increased significantly after 30 min of reperfusion and increased progressively with the reperfusion time (fig. 2B). As shown in figure 4, at 5 min of reperfusion, there was no difference among non-treated and drug-treated groups. However, at 90 min of the reperfusion time, SOD and neutrophil-depletion inhibited the reperfusion-induced TBAR formation by 28% and 74%, respec-

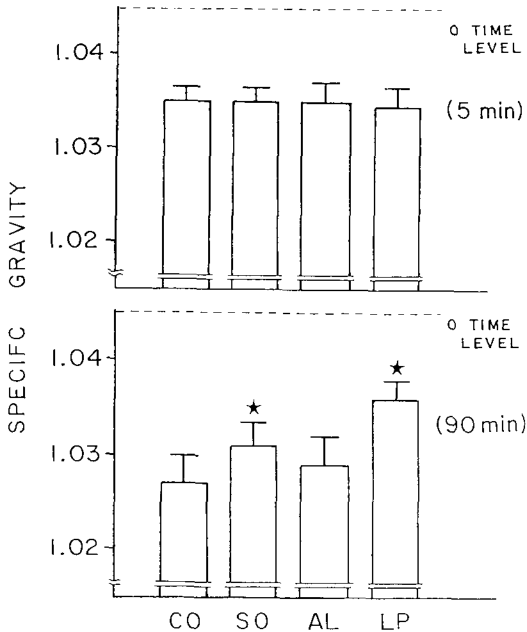

Fig. 6. The effecrts of SOD, allopurinol and leucocytopenia on the change of specific gravity of the intestine at 5 min and 90 min of the reperfusion time. CO: control; SO: SOD-treated rats; AL: allopurinol-treated rats; LP: leucocytopenic rats. The dotted lines indicate the level of no ischemia control. *indicates $P < 0.05$.

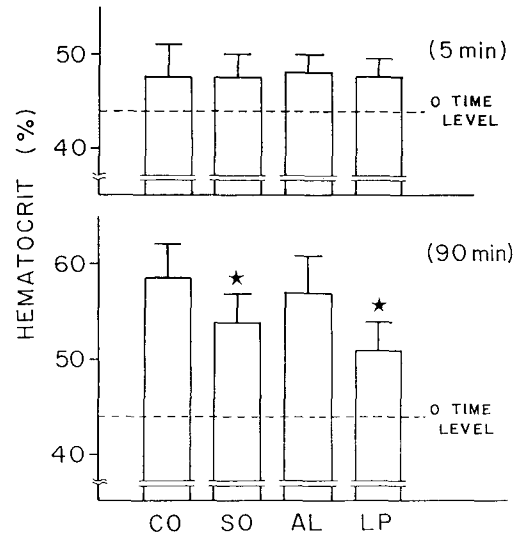

Fig. 7. The effects of SOD, allopurinol and leucocytopenia on the change of the hematocrit value at 5 min and 90 min of reperfusion time. CO: control; SO: SOD-treated rats; AL: allopurinol-treated rats; LP: leucocytopenic rats. The dotted lines indicate the level of no ischemia control. *indicates $P < 0.05$.

tively. On the other hand, allopurinol showed no inhibition.

Specific gravity

Figure 5(A) shows the time course of the specific gravity in non-treated rats. After 30 min ischemia, the specific gravity decreased significantly. It further decreased with the time of reperfusion. As shown in figure 6, at 5 min of reperfusion time, there was no difference among non-treated and drug-treated groups. However, at 90 min of the reperfusion time, SOD and neutrophil depletion inhibited the decrease in specific gravity. Allopurinol had no effect.

Hematocrit

Figure 5(B) shows the time course of the hematocrit value in non-treated rats. After reperfusion, the hematocrit increased with the time of reperfusion. As shown in figure 7, at 5 min of the reperfusion time, there was no difference among non-treated and drug-treated groups. At 90 min of reperfusion time, SOD and neutrophil-depletion inhibited the increase of hematocrit significantly. Allopurinol had no effect.

Discussion

It has been shown that the intestinal mucosa is vulnerable to ischemic insult. Long-term regional ischemia produces the decreases both in the mucosal thickness and in the villus height[24,25]. It also causes epithelial necrosis and crypt epithelial necrosis without injury to the muscularis propria or deeper bowel layers. It is also known that neutrophils accumulate in the mucosa during reperfusion

of the ischemic intestine[13]. These data suggest that intestinal lumen may be a good system to study the mechanism of ischemia-reperfusion injury. In ischemia-reperfusion injury, the productin of oxygen free radicals has been considered to play an important role, however, the demonstration of free radical formation has been indirect, and a more direct method has been desired.

We have recently succeeded in demonstrating the production of oxygen free radicals in brain ischemia *in vivo*[8]. Thus, we undertook this work to study the mechanism of ischemia-reperfusion injury of the intestine by demonstrating the formation of free radicals using the spin-trapping technique. Our method of filling up the intestinal lumen with 2.5 ml PBN solution (into a 15 cm portion of the intestine) seemed to loosen the structure of the mucosa, which in turn allowed PBN to effectively trap free radicals in the mucosal layer. An ischemia time of 30 min is known to produce severe injury, because 90% of the animals died within 24 hours with this model[16]. It was also reported that 30 min of ischemia is enough to produce prolonged functional and structural changes in the rat intestine[26].

In order to study the role of neutrophils, we used nitrogen mustard to prepare leucocytopenic rats. It was shown that intravenous injection of nitrogen mustard causes a selective depletion of circulating neutrophils and monocytes with little or no effect on platelets or lymphocytes[27]. Takahashi et al. used the same method for making leucocytopenic rats in their acute respiratory distress syndrome study[28]. They used 1.75 $mg \cdot kg^{-1}$ of the drug and made a severely leucocytopenic model. However, in our hands, that much of dose sometimes affected systemic conditions (such as loss of appetite). Therefore, we used 1.5 $mg \cdot kg^{-1}$ and were able to obtain mild leucocytopenia (< 1500 $cells \cdot mm^{-3}$) reproducibly.

In this study, the spin trapping technique demonstrated that free radicals were formed in the intestinal lumen during reperfusion. There seemed to be peaks at 5 min and 90 min after the onset of reperfusion. Although the shapes of the EPR signals were similar to those of adducts of oxygen-centered or carbon-centered species, we could not identify the type from our results. According to Buettner, hydroxyl radical adducts give $a_N=15.6G$ and $a_H^\beta=2.7G$ while superoxide anion produces with $a_N=14.3G$ and $a_H^\beta=2.25G$[20]. According to Thornally et al., super oxide anion adducts give $a_N=14.3G$ and $a_H^\beta=2.25G$[29]. Weglicke et al. suggested that oxygen-centered adducts has $a_N=13.6G$ and $a_H^\beta=1.56G$, while carbon-centered adduct $a_N=15.2G$ and $a_H^\beta=3.85G$[30]. Comparing the values of hyperfine splitting constants of our EPR signals with those in the literature, it is possible that our adducts might consist of a mixture of oxygen and carbon-centered adducts.

The production of free radicals at the first peak was inhibited by the pretreatment with allopurinol, but was not inhibited by SOD or by leucocytopenia. In contrast, the production at the second peak was inhibited by SOD and by leucocytopenia, but was not inhibited by allopurinol. These results suggest that the free radical formations in these two peaks were derived from different origins.

The sites of production of oxygen free radicals may be divided into two compartments; intra- and extracellular. In the intracellular compartment, xanthine oxidase is considered to produce superoxide anion by the transmission of one electron to the ground state of oxygen. There have been many reports supporting this theory using *in vivo* models[1,2,25,31,32]. Most reports

were based on the protective effect of a xanthine oxidase inhibitor (allopurinol or oxipurinol) or of the prevention of xanthine oxidase activity (as demonstrated by the improvement of the mortality rate or microvascular permeability). Using chemiluminescence, Roldan et al. reported that allopurinol inhibited the increase of free radical formation in *in vivo* rat intestine in the early reperfusion phase[17]. In our study, we demonstrated that the same dose of allopurinol inhibited the first peak of free radical formation. These results agree well with the view that oxygen radicals derived from the xanthine oxidase reaction is involved in the free radical formation immediately after reperfusion.

In the extracellular component, phagocytes such as leucocytes, macrophages and monocytes continuously produce superoxide anions through the membrane NADPH oxidase. In an intestinal ischemic model, it was shown that there are over 10 million neutrophils in a gram of tissue during ischemia-reperfusion injury (measured by the mucosal myeloperoxidase activity[13]). Hernandez et al. showed that both the neutrophil depletion and the prevention of neutrophil adherence attenuated the reperfusion-induced increase in microvascular permeability. They speculated that the adherence of neutrophils to endothelium is a main factor in reperfusion-induced microvascular injury[14].

To date, the formation of free radicals by neutrophils have not been demonstrated in *in vivo* models. Recently, free radicals formation was suggested during reperfusion of the ischemic intestine by EPR[9] and by low-level chemiluminescence[33]. These results suggest that there is a burst of free radical formation immediately after reperfusion, which lasts for 2–5 min. However, nobody has studied the possible formation of free radicals in a later phase of reperfusion. Recently, Kuzuya et al. reported in the EPR study of their *in vivo* myocardial infarction model that there was an increased level of free radical formation in a later phase of reperfusion (1–3 hours), and that neutrophils may be one of the possible sources of these radicals[7]. In the intestine, we demonstrated that leucocytopenia did not influence the first peak of free radical formation, but significantly inhibited the production of radicals in the second peak (by 80%). These resutls demonstrate that neutrophils may play an important role in free radical formation during a later phase of ischemia-reperfusion injury.

In the intestinal ischemia-reperfusion injury, it has been reported that SOD improved the survival rate[16], prevented increase in microvascular permeability[34], prevented mucosal ulceration[2] and inhibited the depression of water absorption[35]. In our study, SOD prevented the second peak of free radical formation, however, it did not inhibit the first peak. SOD may not penetrate the cell membranes because of its high molecular weight. It has been proposed that SOD scavenges the free radicals produced in the extracellular space[16]. From our results, it is possible that SOD may prevent the extracellular free radical formation caused by adhered neutrophils.

It is hypothesized that splanchnic ischemia causes hypoxia and acidosis of the pancreas and the small intestine. These changes could release lysosomal hydrolases, enhance proteolysis and the production of cardiodepressant substances[36]. Several endogenous substances including histamine, prostaglandins and bacterial endotoxins may also play roles in the pathogenesis of intestinal ischemia-reperfusion injury. However, since fluid therapy has been known to produce significant improvement in the survival

rate[16,37], a major consequence in the pathogenesis of this injury may be an enhanced transcapillary filtration, interstitial edema formation, and ultimately, a net fluid movement into the lumen of the bowel. Therefore, in order to study these aspects of the injury, we measured the specific gravity of intestine as an interstitial edema formation, and the hematocrit value as an index of net blood volume changes. We also measured the TBAR formation as an index of lipid peroxidation (a direct impairment caused by free radicals). Our results demonstrated that the degree of ischemia-reperfusion injury as measured by the changes of these parameters were correlated with the height of the second peak of radical formation. We also observed that the magnitude of second peak was much larger than that of the first peak. Thus, it is possible that in this shock model, neutrophils mediated the free radical formation and triggered tissue impairment. Since activated neutrophils not only produce free radicals but also secrete a variety of enzymes (myeloperoxidase, elastase and protease), they can injure parenchymal cells and the microvasculature[38], and thus exacerbate the injury.

There is an argument as to the role of xanthine oxidase in forming free radical or causing the adherence and activation of neutrophils. Granger et al. hypothesized that xanthine oxidase produces superoxide and hydrogen peroxide, which in turn form highly cytotoxic hydroxyl radicals by the iron-catalyzed Harber-Weiss reaction. This may initiate a process of lipid peroxidation, stimulating the release of chemoattractants, and causing subsequent activation and recruitment of neutrophils[15]. However, our study demonstrated that allopurinol inhibited the first peak in the free radical formation, but did not inhibit the second peak. Tissue impairment, evaluated by various parameters, such as TBAR formation, specific gravity and the hematocrit value, was not inhibited by allopurinol either. The discrepancy seen in the effect of allopurinol between this and previous investigations may be caused by differences either in the experimental protocol or in the dose of allopurinol. In this study, we used allopurinol as pre-treatment (24 and 3 hours before ischemia). It is possible that allopurinol itself acts as a free radical scavenger[39] when it is administered just before or after ischemic insult. Especially when a large dose of allopurinol is used, the effect may be caused by its scavenging action, but not by xanthine oxidase inhibitor. Further study is necessary to solve this issue.

In conclusion, using a spin-trapping technique and EPR, we were able to directly detect the production of free radicals in the small intestinal lumen during ischemia-reperfusion injury. Free radical formation was shown to have two peaks; one at 5 min and a second at 90 min of reperfusion time. The first peak may be partly related to xanthine oxidase-produced free radicals because it was inhibited by allopurinol. However, the height of the first peak seems to be unrelated to the subsequent tissue injury. In contrast, the second peak may be related to neutrophil-produced free radicals. SOD was found to prevent the formation of radicals in the second peak, but not in the first one. The adherence of neutrophils produced a larger amount of free radicals, which resulted in tissue injury.

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