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RADICAL TRAPPING BY PBN DURING REPERFUSION IN RABBIT GASTRIC MUCOSA

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Oxygen free radicals have been considered as a cause of ischemia-reperfusion injury in several organs, but this injury in a stomach, containing acid, may progress to severe damage. Thus, we examined the effect of ischemia-reperfusion and milk-intake on rabbit gastric mucosa. The gastric mucosal blood flow was increased after milk-intake, and gastric rupture was detected. Superoxide dismutase activity measured by an improved nitroblue tetrazolium reduction method and thiobarbituric acid reactive substances in serum is increased during ischemia-reperfusion and milk-intake. By using α-phenyl N-*tert*-butyl nitrone (PBN) as a spin trap and electron paramagnetic resonance (EPR), we detected lipidic radicals from tissue samples in chloroform-methanol solvent only during reperfusion and milk-intake period; no signal was detected before. The EPR signal of spin adducts obtained in the sample after ischemia-reperfusion and milk-intake would be a mixture of peroxyl and alkoxyl radicals from the analysis of coupling constants.

KEY WORDS: Ischemia-reperfusion, gastric rupture, superoxide dismutase, lipid peroxidation.

INTRODUCTION

In recent studies, the reperfusion injury in several organs is often attributed to the contribution of free radicals and active oxygen species derived from molecular oxygen. It has been revealed by the electron paramagnetic resonance (ESR) spectroscopy that there is a burst of oxy-radical generation during the early phase of reperfusion.[1-5] A number of studies have suggested that post-ischemic reperfusion injury may be mediated in part by the generation of reactive oxygen species such as superoxide anion radical, hydrogen peroxide, and hydroxyl radical. Thus, it has been shown that recovery from reperfusion injury is enhanced by agents that either scavenge oxygen metabolites or prevent their generation. It has also been suggested that oxygen radicals contribute to the ischemia-reperfusion injury in the stomach.[6-8] Ischemia-reperfusion in a stomach may produce severe gastric mucosal injury because the acid in this organ could be a promoter of this damage.

In this study, we examined the relationship between the change of superoxide dismutase (SOD) activity or thiobarbituric acid reactive substances in serum and the radical generation in the rabbit stomach during ischemia-reperfusion and milk-intake.

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MATERIALS AND METHODS

Adult New Zealand white rabbits ($n = 40$) weighing 3 kg were anesthetized (pentobarbital) and instrumented with a snare around the celiac artery and a Doppler flow velocity probe around the vessel distal to the snare to be rendered ischemic. The site of occlusion was carefully selected to ensure that no branches were present. The selected artery was occluded for 60 min and then reperfused for 60 min. After the ischemia-reperfusion dry milk solution was injected into the stomach. The dry milk for human baby containing 72.1% of milk components, 24.3% of fats and 3.6% of dextrin with vitamine A, B1, B2, B6, B12, C, D, E, K and small amounts of metal ions was purchased from Morinaga Milk Inc., Tokyo. 40 ml of dissolved milk (13%) was injected into the stomach every experiment. The gastric mucosa was carefully examined macroscopically and microscopically, and the extent of the gastric mucosal lesion was expressed by the total area of errosion, or the ulcer index after the experiment.

The blood flow in the gastric mucosa was measured by a laser Doppler flowmeter (ALF 2100, Advance Co., Tokyo). 200–300 mg of gastric mucosal tissue samples were obtained from the center of the area at risk before occlusion, during ischemia, just before reperfusion, and at various times (immediately after, 10 min, 30 min, and 60 min) during reperfusion and after milk-intake, followed by rapid freezing in liquid nitrogen. Frozen samples were homogenized into 3 ml of α-phenyl N-*tert*-butyl nitrone (PBN) solution (33 mg/ml in chloroform-methanol = 2:1) to trap lipidic radicals. A chloroform layer separated was subjected to EPR spectroscopy using an X-band spectrometer (JEOL, FE-1-X) in a flat quartz cell. EPR spectra were obtained using 20 mW microwave power, 1.25 G modulation amplitude, 100 kHz modulation frequency, 1.0 response, and 8 min scanning time. PBN was purchased from Sigma Chemical Co., St. Louis, MO.

In some experiments, human recombinant superoxide dismutase (h-SOD, 30,000 units/kg) was administered continuously until the end of the experiment.

Thiobarbituric acid reactive substances (TBA reactants) in rabbit serum were measured as nmol of malondialdehyde by fluorometry (excitation; 515 nm and emmision; 553 nm). Superoxide dismutase (SOD) activity in serum was also measured by the improved nitroblue tetrazolium (NBT) reduction method. The improved NBT reduction method was performed using 5 M of hydrogen peroxide (H_2O_2) to inactivate SOD in serum, and then SOD-like activity and real SOD activity in the sample could be calculated. Briefly, 0.5 ml of serum was incubated with 0.1 ml of 5 M H_2O_2 for 20 min, and the reaction mixture was treated with catalase (2,000 units). SOD-inactivated serum (as SOD-like activity) or the original serum was added to the mixture of hypoxanthine (2 mM in 0.1 M-phosphate buffer, pH 7.8), xanthine oxidase (0.55 units/ml of same buffer) and NBT solution (0.24 mmol/l). The rate of NBT reduction was measured at 560 nm.

RESULTS

As shown in Figure 1, the gastric mucosal blood flow was reduced to about 10% of the usual level by the celiac artery occlusion. However it immediately recovered to the 100% level with reperfusion, and increased to the 110% level with milk-intake.

TBA reactants in serum were slightly increased by the celiac artery occlusion and

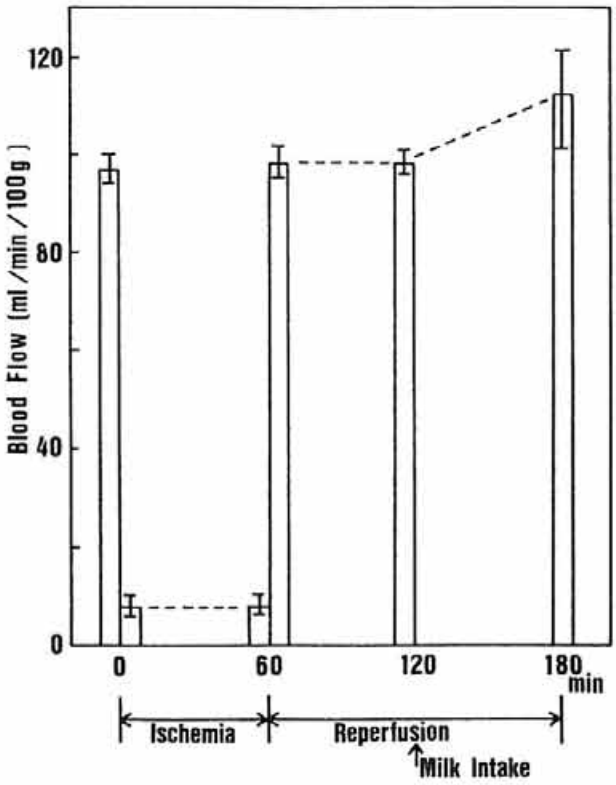

FIGURE 1 Changes of blood flow in rabbit gastric mucosa before occlusion, 60 min-ischemia, 60 min-reperfusion, and after milk-intake. Each value indicates the mean ± SD of 40 rabbits.

significantly increased during reperfusion for 60 min and the following milk-intake (Figure 2).

The improved SOD assay is a new method to distinguish the real SOD activity from the SOD-like activity in serum. By pre-incubating the serum sample with 5 M-H_2O_2 at 37°C, for 20 min, Cu, Zn-SOD (below 100 units/ml) and Mn-SOD (below 35 units/ml) are completely inactivated (Figure 3A). Then, the following equation could be used;

[Real SOD activity in serum] = [Total SOD activity by the original serum] − [SOD-like activity by the inactivated serum]

The value of SOD-like activity calculated from the inactivated serum would be the rate of the inhibition of NBT reduction in the xanthine-xanthine oxidase system by serum components. When this method was checked by sera added standard SOD solution, both the real SOD activity and the SOD-like activity could be measured precisely (Figure 3B). As shown in Figure 4, SOD activity in serum of rabbits ($n = 40$) was increased by the celiac artery occlusion, and raised to the high value after reperfusion and milk-intake.

The total area of errosions, the ulcer index that showed the gastric injury was

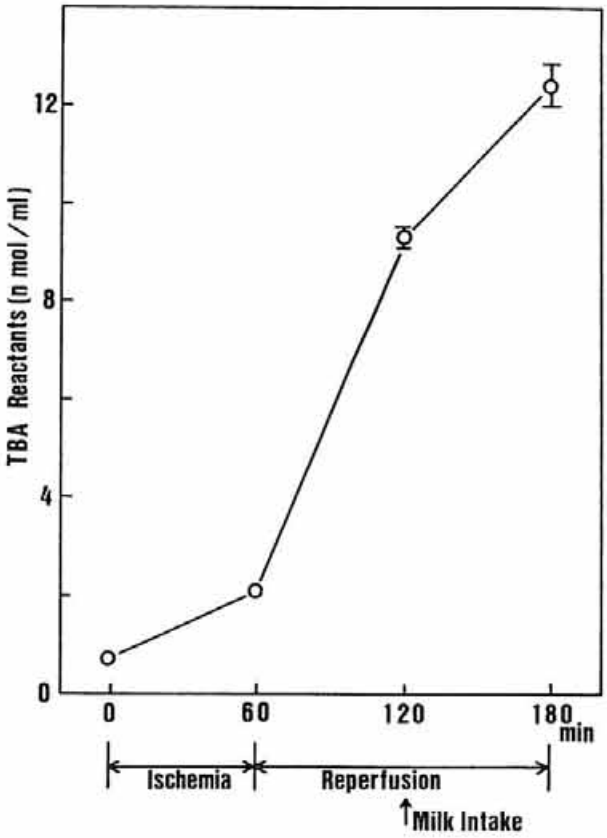

FIGURE 2 Changes of TBA reactive substances in rabbit serum before occlusion, 60 min-ischemia, 60 min-reperfusion, and after milk-intake. Each value indicates the mean ± SD of 40 rabbits.

increased by the celiac artery occlusion, and significantly increased after milk-intake (data not shown). 65% of examined rabbits ($n = 40$) developed the gastric rupture after ischemia-reperfusion and milk-intake. However the development of gastric rupture was completely inhibited by treatment with SOD if the vein of kidney was occluded to maintain SOD level in blood.

The EPR signal of PBN adducts from the gastric tissue sample of milk-intake (Figure 5D) was characterized by a triplet of doublets, with the coupling constants about $a^N = 14.6 \pm 0.2$ G and $a_\beta^H = 3.5 \pm 0.2$ G. However no EPR signal was detected in the gastric tissue sample of the before occlusion (Figure 5A) or of the ischemia (Figure 5B). A weak signal can be observed in the sample collected during reperfusion (Figure 5C).

DISCUSSION

The role of free radical-mediated injuries have been accepted in many disease states.[9-10] Interest has focused on the role and the source of oxygen free radicals

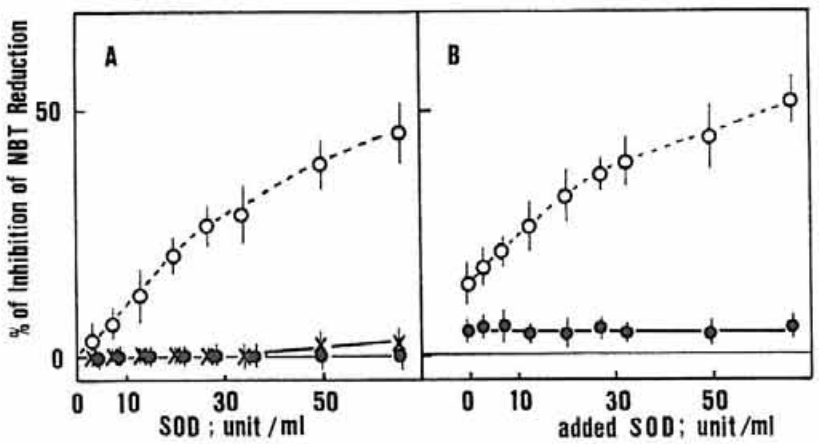

FIGURE 3 The improved SOD assay. A; calibration curve for SOD solution (○--○), % Inhibition of NBT reduction by the H_2O_2-treated Cu, Zn-SOD solution (●—●), and % Inhibition of NBT reduction by the H_2O_2-treated Mn-SOD solution (X—X). Each value indicates the mean ± SD (n = 5). B; % Inhibition of NBT reduction by the sera added the standard SOD solution (○--○), and % Inhibition of NBT reduction by the H_2O_2-treated SOD-added sera (●—●). Each value indicates the mean ± SD (n = 10).

in tissues subjected to ischemia-reperfusion, where the injury has been convincingly linked to free radical production in several organs.[11-14] This study shows that the gastric mucosal injury has been produced during ischemia-reoxygenation, and the gastric rupture could be detected, especially after milk-intake; the acid in stomach may be a promoter of stomach injury. TBA reactive substances and SOD activity in serum also significantly increase with these treatments. We have employed the new improved SOD assay to distinguish the real SOD activity from the SOD-like components in serum because serum components such as bilirubin or cholesterol could scavenge superoxide anion radical.[15] We afford the supplementary support to these data by direct evidence of free radicals formation using PBN spin-trapping. PBN spin trapped radicals described in our report would be a mixture of peroxyl and alkoxyl radicals by the analysis of the coupling constants. A^N values (14.6 ± 0.2 G) in chloroform-methanol as solvent are slightly larger than those generally observed with oxygen-centered radical adducts of PBN (<14.5 G), whereas a_β^H values (3.0 ± 0.2 G) are smaller than those given for carbon-centered radical adducts of PBN (3.25–3.5 G). These adducts would be derived from membrane lipids by attack of active oxygens during the reoxygenation along with the increased blood flow. The EPR signal we detected resembles to the lipid radical adducts of PBN reported in other systems.[13,16]

These results indicate that lipid peroxidation would be induced by oxygen radicals produced in tissues for the duration of reperfusion and milk-intake. An increase of the real SOD activity and TBA reactive substances in serum would be a good monitor for the gastric mucosal injury. The gastric mucosal injury caused by ischemia-reperfusion would progress to the gastric rupture after milk-intake, or the increase of blood flow.

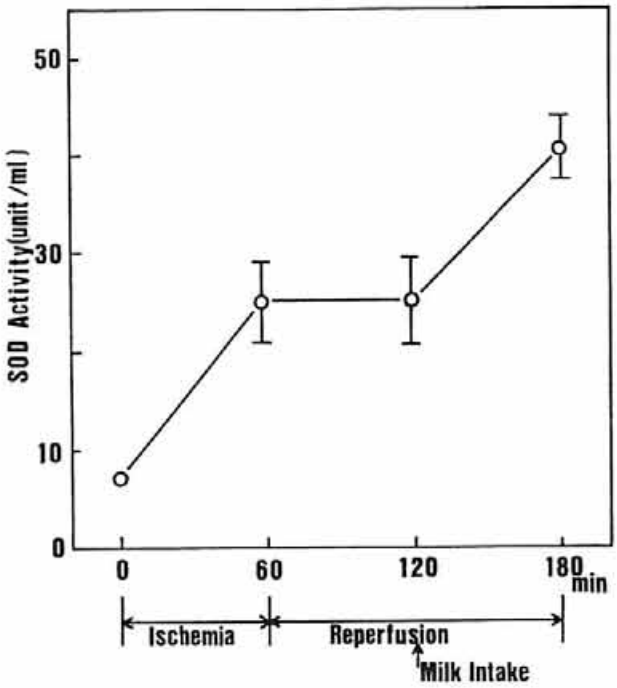

FIGURE 4 Changes of real SOD activity in rabbit serum before occlusion, 60 min-ischemia, 60 min-reperfusion, and after milk-intake. Each value indicates the mean ± SD of 40 rabbits.

Acknowledgements

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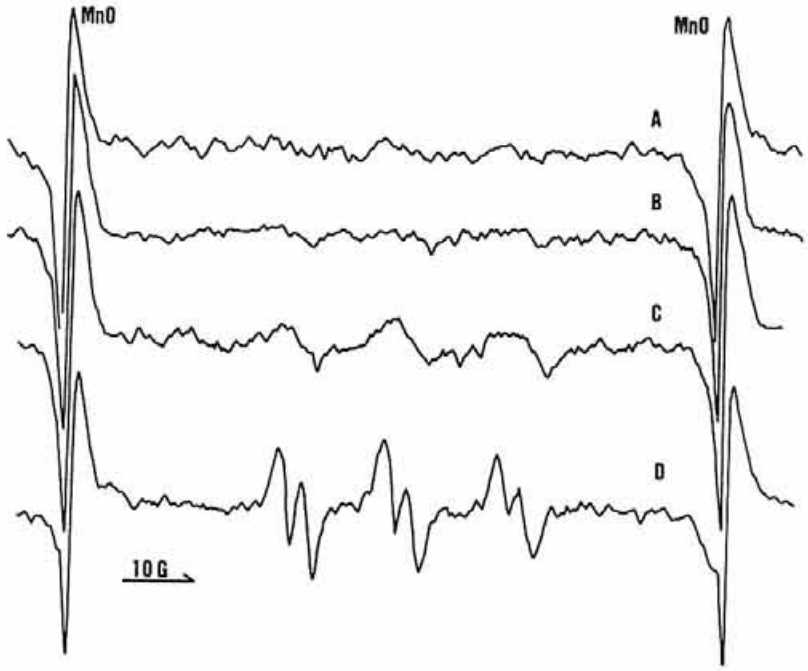

FIGURE 5 EPR spectra of PBN spin adducts in the gastric mucosa subjected to ischemia-reperfusion and milk-intake. A; before occlusion, B; under ischemia, C; 10 min after reperfusion, D; 40 min after milk-intake.

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