Programmed cell death induced by ischemia-reperfusion in rat intestinal mucosa

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Noda, Takahiro, Ryuichi Iwakiri, Kazuma Fujimoto, Shuzo Matsu, and Tak Yee Aw. Programmed cell death induced by ischemia-reperfusion in rat intestinal mucosa. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G270–G276, 1998.—Apoptosis after ischemia-reperfusion (I/R) was characterized in rat small intestine. Under halothane anesthesia, the superior mesenteric artery in the rat was occluded for 15 or 60 min, followed by reperfusion. Rat biopsies were treated with 32P-labeled deoxyribonucleic acid (DNA) to examine the relationship between intestinal apoptosis and ornithine decarboxylase (ODC) activity. The percentage of fragmented DNA significantly increased just after ischemia and peaked at 1 h after reperfusion in the jejunum and ileum. These increases were significantly higher in the 60-min ischemia group compared with the 15-min ischemia group. This increase decreased 6 h after reperfusion. The results were corroborated by histological evaluations of the intestine under the same conditions. DFMO, completely abolished elevation of ODC activity 6 h after reperfusion but did not change the percentage of fragmented DNA. Apoptosis in rat small intestine was induced by ischemia of the gut, and this process was exacerbated by reperfusion. The changes in apoptosis were independent of ODC activity.

Materials and Methods

Surgery. Male Sprague-Dawley rats (280–330 g) were used in this study. Animals were housed in wire-bottomed cages placed in a room illuminated from 0800 to 2000 (12:12-h light-dark cycle) and maintained at 21 ± 1°C. Rats were allowed access to water and chow ad libitum. Under halothane anesthesia, a laparotomy was performed. We previously demonstrated that occlusion of the SMA markedly reduced blood flow to the jejunum and the ileum but not to the duodenum (10); therefore, the SMA was occluded for 15 or 60 min with a micro-bulldog clamp. At the end of the ischemic period, the clamp was released and three drops of lidocaine were applied directly on the SMA to facilitate reperfusion.

Collection of intestinal mucosa. The animals were anesthetized and then euthanized. The entire small intestine was carefully removed and placed on ice. The oral 5 cm was taken as the duodenum, and the rest of the intestine was divided into two equal segments, representing the proximal (jejunum) and distal (ileum) segments. Each segment was rinsed thoroughly with normal saline (0.9% NaCl) and opened longitudinally to expose the intestinal epithelium. The mucosal layer was harvested by gentle scraping of the epithelium, using a glass slide.

DNA fragmentation assay. The mucosal scrapings were processed immediately after collection to minimize nonspecific DNA fragmentation. The amount of fragmented DNA was determined as previously described (2), with modification. Mucosal scrapings of the different intestinal segments were homogenized in 10 vol of a lysis buffer (pH 8.0) consisting of 5 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, 20 mM EDTA, and 0.5% (wt/vol) Triton X-100 (Sigma, St. Louis, MO). Aliquots (1 ml) of each sample were centrifuged...
for 20 min at 27,000 g to separate the intact chromatin (pellet) from the fragmented DNA (supernatant) (29). The supernatant was decanted and saved, and the pellet was resuspended in 1 ml of Tris buffer (pH 8.0) with 10 mM Tris-HCl and 1 mM EDTA. The pellet and supernatant fractions were assayed for DNA content, using diphenylamine reaction as previously described (4). The results are expressed as the percentage of fragmented DNA.

Purification of mucosal DNA and agarose gel electrophoresis. DNA was extracted from the 27,000 g fraction (29). The fragmented DNA from the various fractions was extracted with a phenol-chloroform-isomyl alcohol mixture (25:24:1, vol/vol/vol) sequentially to remove protein. The protein-free DNA extracts were treated with 100% ethanol in 0.1 M sodium acetate at -20°C overnight to purify the DNA. The precipitated DNA was washed with 70% ethanol and resuspended in Tris buffer (pH 8.0) with 10 mM Tris-HCl and 10 mM EDTA. DNA samples were incubated with 100 µg/ml ribonuclease for 15 min at 37°C to remove RNA. Resolving agarose gel electrophoresis was performed with 1% gel using 1.5% gel strength containing 10 µg/ml ethidium bromide. Depending on the experiment, 20 µg DNA per well was loaded. DNA standards (0.5 µg per well) were included to identify the size of the DNA fragments. Electrophoresis was performed for 2 h at 70 V, and DNA was visualized by ultraviolet fluorescence.

ODC assay. ODC activity was assayed by a radiometric technique (27). Mucosal scrapings were placed in 2 ml of 0.1 M Tris buffer (pH 7.4) containing 1 mM EDTA, 50 µM pyridoxal 5-phosphate, and 5 mM dithiothreitol. The tissues were homogenized twice with a Polytron tissue homogenizer for 15 s and centrifuged at 30,000 g for 30 min. Protein content was determined, and a 200-µl aliquot of the supernatant was incubated in stoppered vials in the presence of 3.5 nmol of L-[1-14C]ornithine (52.3 mCi/mmol; New England Nuclear, Boston, MA) for 15 min at 37°C. The 14CO2 liberated by the decarboxylation of ornithine was trapped on filter paper impregnated with 20 µl of 2 N NaOH, which was suspended above the reaction mixture. The reaction was stopped by the addition of 0.3 ml 10% trichloroacetic acid. Radioactivity of the 14CO2 trapped in the filter paper was measured in an aqueous miscible scintillant (Opti-Fluor; Packard Instruments, Downers Grove, IL). The samples were counted for 5 min in a liquid scintillation spectrometer (460 CD; Packard Instruments). Results are expressed as picomoles CO2 per milligram protein per hour.

Immunohistochemical staining. Tissue samples were removed from the jejunum and ileum and immediately fixed in 10% neutral buffered Formalin. The samples were then embedded in paraffin and sectioned. Fragmented DNA was stained by the terminal deoxynucleotidyl transferase (TdT)-mediated diUDP-biotin nick end labeling (TUNEL) method (12) with modification using the ApopTag kit (Oncor, Gaithersburg, MD). Specimens were dewaxed and immersed in phosphate-buffered saline containing 0.3% hydrogen peroxide for 10 min at room temperature and then incubated with 20 µg/ml proteinase K for 15 min at room temperature. Seventy-five microliters of equilibration buffer were applied directly onto the specimens for 10 min at room temperature, followed by 55 µl of TdT enzyme and incubation, which were then incubated at 37°C for 1 h. The reaction was terminated by transferring the slides to prewarmed stop/wash buffer for 30 min at 37°C. The specimens were covered with a few drops of anti-digoxigenin peroxidase and incubated for 30 min at room temperature. Specimens were then soaked in Tris buffer containing 0.02% diaminobenzidine and 0.02% hydrogen peroxide for 1 min to achieve color development. Finally, the specimens were counterstained by immersion in hematoxylin.

**RESULTS**

Experiment 1. Effect of I/R on apoptosis in the small intestine. Results on the percentage of fragmented DNA in the duodenal mucosa before and after I/R are shown in Fig. 1A. Neither ischemia nor reperfusion elicited duodenal DNA damage, and there was no difference between the 15-min and 60-min ischemia groups.

Figure 1B shows the ratio of fragmented DNA to total DNA after I/R in the jejunal mucosa. In the 15-min and 60-min ischemia groups, the percentage of fragmented DNA increased just after ischemia (15 min, 6.9 ± 3.4%; 60 min, 8.8 ± 2.9%) compared with the values before ischemia (15 min, 1.4 ± 0.2%; 60 min, 1.7 ± 0.4%; P < 0.05 in each group), indicating that the percentage of fragmented DNA increased during ischemia. In the 60-min ischemia group, the percentage of fragmented DNA peaked at 1 h after reperfusion (20.7 ± 2.1%; P < 0.05, compared with the value just after 60 min ischemia), and by 6 h after reperfusion, the percentage of fragmented DNA returned to baseline levels (2.0 ± 0.3%). In comparison, 15 min ischemia also resulted in enhanced DNA fragmentation, which peaked between 0 and 1 h (7.8 ± 2.1%) during reperfusion (P < 0.05 vs. the value before ischemia). However, the magnitude of DNA damage at these times was less than that in the 60-min ischemia group (P < 0.05). As it was for the 60-min ischemia group, DNA fragmentation in the 15-min ischemia group returned to baseline levels at 6 h reperfusion (1.7 ± 0.3%).

Figure 1C shows the results on DNA fragmentation in the ileal mucosa. In the 15-min and 60-min ischemia groups, the percentage of fragmented DNA significantly increased just after ischemia (15 min, 4.9 ± 1.0%; 60 min, 12.3 ± 2.0%) compared with the value before ischemia (15 min, 1.5 ± 0.2%; 60 min, 2.4 ± 0.4%; P < 0.05 in each group). Increases in the percentage of fragmented DNA peaked at 1 h after I/R in both groups (15 min, 10.0 ± 1.0%; 60 min, 25.3 ± 4.8%; P < 0.05 in each group compared with the value just after
ischemia), and this increase returned to the value before the ischemia at 6 h after I/R (15 min, 2.8 ± 0.4%; 60 min, 3.1 ± 0.4%). The increase in the percentage of fragmented DNA in the 60-min ischemia group was larger than that in the 15-min ischemia group (P < 0.05), similar to that seen in the jejunum (Fig. 1B).

Resolving agarose gel electrophoresis was performed to evaluate the nature of the fragmented DNA in the jejunal and ileal mucosa after I/R. As shown in Fig. 2, A and B, agarose gel electrophoresis of the fragmented DNA obtained from the jejunal mucosa at 15- and 60-min ischemia, respectively, revealed distinct DNA ladders. These ladders are characteristic of apoptosis (29), although they are not useful for quantitative analysis because the same doses of fragmented DNA were loaded onto each lane. Similar DNA laddering on agarose gel electrophoresis was obtained from ileal mucosa of animals subjected to 15 and 60 min I/R (data not shown).

The immunohistochemical staining (TUNEL) of jejunal segments before and after I/R is shown in Fig. 3 (15 min ischemia) and Fig. 4 (60 min ischemia). Before I/R, few apoptotic cells were observed at the villus tips (Figs. 3A and 4A). Immediately after ischemia, the

![Figure 1](image1.png)

**Fig. 1.** Percentage of fragmented DNA in the intestinal mucosa after ischemia-reperfusion (I/R). Values are means ± SE. A: duodenum. B: jejunum. C: ileum. Superior mesenteric artery (SMA) was occluded for either 15 min (○) or 60 min (●) and then reperfused. *P < 0.05 compared with corresponding control value. *P < 0.05 compared with value at end of ischemic period. *P < 0.05 compared with 15-min ischemia group.

![Figure 2](image2.png)

**Fig. 2.** Agarose gel electrophoresis of fragmented DNA from jejunal mucosa after I/R. A: jejunal mucosa of 15-min ischemia group. B: jejunal mucosa of 60-min ischemia group. Twenty micrograms of fragmented DNA were loaded. Lanes 1–4, DNA before ischemia and 0, 1, and 6 h after reperfusion, respectively. The ladder, which is characteristic of apoptosis, was clearly shown on each lane in both the 15-min and 60-min ischemia groups. Lanes 5 and 6 contained marker DNA from φ×174 Hae III and Lambda EcoR III digest (Wako Pure Chemical), respectively.
number of apoptotic cells increased in both the 15-min ischemia group (Fig. 3B) and the 60-min ischemia group (Fig. 4B). The number of apoptotic cells increased at 1 h after I/R (15-min ischemia group, Fig. 3C; 60-min ischemia group, Fig. 4C). Interestingly, only a few apoptotic cells were seen in the jejunal mucosa 6 h after I/R in both groups (15-min ischemia group, Fig. 3D; 60-min ischemia group, Fig. 4D). These histological findings on I/R-induced apoptosis corroborated the data obtained using biochemical determination of DNA fragmentation.

The histological evaluations further revealed that damage to the small intestine in the 15-min ischemia group was small, with slightly edematous villus tips just after the ischemic period and throughout the period 1-6 h after I/R. In contrast, the histology of the jejunal segment of the 60-min ischemia group showed substantial destruction of the mucosal layer at 1 h
postischemia. Additionally, mucosal integrity was partially restored at 6 h after the I/R insult.

Experiment 2. Effect of DFMO on ODC activity and apoptosis after I/R. Table 1 summarizes the ODC activity and the percentage of fragmented DNA in the jejunum before and 6 h after I/R. The ODC activity markedly increased at 6 h after I/R in the vehicle-treated control group, and this increase of ODC activity was completely inhibited by administration of DFMO. In contrast to differences in ODC activity, the percentage of fragmented DNA did not differ significantly between vehicle-treated controls and DFMO-treated animals, indicating that increased ODC activity was not correlated with induction of apoptosis after I/R.

**DISCUSSION**

Although it is well known that the intestinal mucosa is highly sensitive to I/R (13), the relationship between I/R-induced apoptosis and tissue injury in the small
The injured mucosa. The reason for this interesting indication is consistent with previous findings that intestinal cell death by the process of apoptosis is likely to be controlled by a variety of factors (18). Thus we hypothesized that increased polyamine levels may be responsible for modulating apoptosis because ODC activity increased markedly after an I/R stimulus. Previous studies have documented that some growth factors inhibit apoptosis in many organs (28). The effect of polyamines or ODC on apoptosis appears to be contradictory. After fasting, apoptosis of the rat intestinal mucosa increased when ODC activity was suppressed (17). Circadian variation of apoptosis in the rat intestinal mucosa mirrored ODC activity, i.e., apoptosis was induced in the light period (17), whereas ODC activity was increased in the dark period (8). Desiderio et al. (7) showed that the ODC activity was transiently elevated and polyamine levels rapidly fell below control values in apoptotic thymocytes, whereas ODC elevation was long-lasting and increases of polyamines were persistent in proliferating thymocytes. Packham and Cleveland (23) reported that, in vitro studies using cultured cells, ODC mediated c-myc-induced apoptosis that was blocked by DFMO. In our current study, the increase in ODC activity in the small intestine after I/R was completely ablated by DFMO. However, the percentage of fragmented DNA was not affected by DFMO treatment, indicating that suppression of increased ODC activity by DFMO did not enhance apoptosis. Thus these results indicate that ODC activity did not influence apoptosis in the small intestine after I/R.

The mechanism of I/R-induced apoptosis and the factors that control the ischemic vs. the reperfusion phases are not known. Several studies have shown that free radicals can cause apoptosis (5, 25), which would be consistent with enhanced oxi radical generation during I/R. However, other reports show the lack of an association of oxygen radicals with enhanced apoptosis (22). What is clear is that the regulation of intestinal cell death and proliferation is highly complex and is likely to be controlled by a variety of factors (18). Thus further study is needed to identify and delineate these regulatory factors. In particular, the mechanism of I/R-induced apoptosis and subsequent tissue repair warrants further exploration. Regardless of the mechanism, the present study shows that I/R substantially causes intestinal cell death by the process of apoptosis and that the injured intestinal mucosa is capable of rapid and significant recovery during reperfusion.

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