A apoptotic pathway in the rat small intestinal mucosa is different between fasting and ischemia-reperfusion

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The small intestine is involved in digestion, nutrient absorption, and immunity. Homeostasis of intestinal epithelial architecture in the small intestine is very important for these functions. To maintain the physiological state, the intestinal epithelium keeps steady-state turnover of enterocytes (8, 16, 28). Morphological homeostasis of the small intestinal epithelium is precisely regulated by both cell proliferation and cell death including apoptosis (23).

We previously demonstrated that ischemia from occlusion of the superior mesenteric artery (SMA) followed by reperfusion caused injury and cell death that was characterized by apoptosis (18). An intestinal ischemia-reperfusion (I/R) model has often been used to study apoptosis by many investigators (18, 31). Our previous study suggested that I/R induced mucosal cell apoptosis via a Fas ligand (FasL)-mediated caspase-9-activating pathway (31) and that inducible nitric oxide synthase, inflammatory cytokines, and platelet-activating factor promoted I/R-induced apoptosis (31, 32). Other investigators reported many mediators that were involved in I/R of the intestine such as free radicals (11), xanthine oxidase (12), endothelins (19), heat shock proteins (26), polymorphonuclear neutrophils (5), and heme oxygenase (17).

Our previous studies showed that intestinal mucosal cell apoptosis exhibited a circadian rhythm, and that fasting induced mucosal cell apoptosis (14). Although mechanisms of I/R-induced apoptosis as a pathological state in the rat intestinal mucosa have been well studied, regulation of fasting-induced apoptosis is less well documented. Elucidation of mechanisms of fasting-induced apoptosis might help in understanding the physiological regulation of apoptotic processes because of the relationship between circadian rhythms of apoptosis in the rat small intestinal mucosa and feeding behaviors (14). This study aimed to compare apoptotic pathways following fasting as a physiological condition with those in I/R as a pathological condition in the rat small intestine.

**MATERIALS AND METHODS**

*Animals and surgery.* The experimental protocol and design was approved by the Saga University Animal Experimentation Committee and performed according to Saga University Guidelines for Animal Experimentation. Male Sprague-Dawley rats were used in this study. Rats were housed individually in wire-bottomed cages to prevent coprophagia and were placed in a room illuminated from 8:00 AM to 8:00 PM (12:12-h light-dark cycle). Animals were allowed free access to water and food ad libitum. At 7 wk of age, rats were divided into four groups: 1) ad libitum, 2) I/R, 3) 24-h fast, and 4) 48-h fast. In all groups, experiments were performed at 8 wk of age, and six rats were tested in each group. In the fasting group, food was removed for 24 or 48 h until the start of the experiment. In the I/R study, a laparotomy was performed under halothane anesthesia. We previously demonstrated that occlusion of the SMA markedly reduced blood flow to the jejunum and ileum (9) and that apoptosis in the intestinal mucosa peaked during 60-min reperfusion after 60-min occlusion of the SMA (18). In this study, the SMA was occluded for 60 min with a micro-bulldog clamp, followed by a 60-min reperfusion as previously described (9, 18).

For evaluation of mitochondrial dehydrogenase activity in the small intestinal mucosa, another six rats were tested in each group. Under halothane anesthesia, during reperfusion in the I/R group and 1 h before the end of fasting in the fasting group, a 10-cm segment of ileum was isolated, and both ends were tied off. A plastic tube was inserted into the lumen, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) dissolved in physiological saline (1 mg/ml) was placed into the lumen during the
second half of the reperfusion period and for 30 min before the end of the fasting period.

Collection of intestinal mucosa. After fasting or I/R, animals were anesthetized and then euthanized. The entire small intestine was carefully removed and placed on ice. The oral 10-cm part of the intestine was treated as the duodenum, and the rest of the intestine was divided into two equal segments representing proximal (jejunum) and distal (ileum) segments. Some pieces, ~2 cm in length, were resected from the middle portion of each segment and fixed in 10% neutral buffered formalin for measurement of mucosal height and for immunohistochemistry [terminal deoxynucleotidyl transferase (TdT)-mediated dUDP-biotin nick-end labeling (TUNEL) assay]. Each segment was rinsed thoroughly with physiological saline and opened longitudinally to expose the intestinal epithelium. The mucosal layer was harvested by gentle scraping of the epithelium with a glass slide, as previously described (18). Half of the specimen was used for DNA fragmentation assay and agarose gel electrophoresis, and the remaining half was used for evaluation of apoptotic proteins and for the death ligand/receptor assay with Western blot analysis.

DNA fragmentation assay. Mucosal scrapings were processed immediately after collection to minimize nonspecific DNA fragmentation. Amounts of fragmented DNA were determined as previously described (2, 18) with some modifications. Briefly, mucosal scrapings from different intestinal segments were homogenized in 10 vol of lysis buffer [5 mM Tris-HCl, 20 mM EDTA, and 0.5% (wt/vol) Triton X-100, pH 8.0]. One-milliliter aliquots of each sample were centrifuged for 20 min at 27,000 g to separate the intact chromatin (pellet) from fragmented DNA (supernatant). The supernatant was decanted and saved, and the pellet was resuspended in 1 ml Tris buffer (pH 8.0) with 10 mM Tris-HCl and 1 mM EDTA. Pellet and supernatant fractions were assayed for DNA content using a diphenylamine reaction as previously described (3). Results are percentages of fragmented DNA.

Purification of mucosal DNA and agarose gel electrophoresis. DNA was quantitatively extracted from total mucosal homogenates. Briefly, total DNA from various fractions was sequentially extracted using a phenol-chloroform-isoamyl alcohol mixture [25:24:1 (vol/vol/vol)] to remove proteins. 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 A for 15 min at 37°C to remove RNA. Resolving agarose gel electrophoresis was performed using a 1.5% gel containing 1.0 µg/ml ethidium bromide. Depending on the experiment, 20 µg DNA/well were loaded. DNA standards (0.5 µg/well) were included to identify sizes of DNA fragments. Electrophoresis was performed for 2 h at 70 V, and DNA was visualized using ultraviolet fluorescence.

Mucosal height and immunohistochemical staining. Tissue samples were removed from the jejunum and ileum and were immediately fixed in 10% neutral-buffered formalin. Samples were then embedded in paraffin and sectioned. Specimens were stained with hematoxylin and eosin. Mucosal length (villus height plus crypt depth) was measured with a light microscope using micrometer scale standards for ad libitum-fed and fasted rats. Fragmented DNA was stained using the TUNEL method (10) with some modifications using an Apop Tag kit (Oncor, Gaithersburg, MD). Specimens were dewaxed and immersed in PBS 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 milliliters of equilibration buffer were applied directly onto the specimens for 10 min at room temperature, followed by 55 µl of TdT enzyme and incubation at 37°C for 1 h. The reaction was terminated by transferring slides to prewarmed stop/wash buffer for 30 min at 37°C. Specimens were covered with a few drops of rabbit serum, incubated for 20 min at room temperature, and finally covered with 55 µl antidigoxigenin 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 for color development. Finally, specimens were counterstained with methyl green. A minimum of 50 crypts were randomly selected for apoptotic index analysis, and the number of apoptotic cells was calculated. The apoptotic index was determined by dividing the numbers of cells by the total number of cells in the crypt column and multiplying by 100.

Measurement of intestinal mucosal mitochondrial dehydrogenase activity. Mucosal cellular mitochondrial dehydrogenase activity was assessed by measuring mitochondrial dehydrogenase-dependent reduction of MTT to its formazan derivative (MTT-FZ) (32). After animals underwent I/R and fasting, 10 ml cold physiological saline was used to wash out remaining MTT from ileal segments. Segments were opened, and the mucosal layer was collected and placed into 3 ml cold PBS (pH 7.4). After centrifugation (2,500 g for 10 min at 4°C), the supernatant was removed, and the pellet was homogenized in 3 ml lysis buffer (pH 8.0) consisting of 5 mM Tris-HCl, 20 mM EDTA, and 0.5% (wt/vol) Triton X-100. One-milliliter aliquots of the tissue homogenate were transferred into two Eppendorf tubes (one to be used for the MTT-FZ assay and another for the DNA assay). MTT-FZ contained in mucosal cells was solubilized with 2 ml DMSO. Aliquots were sonicated (Typ T25-S1 Sonic Membra) and centrifuged at 9,200 g for 10 min at 4°C. The supernatant (1.5 ml) was drawn out and placed into a cuvette, and its absorbance was determined at 540 nm by a diphenylamine reaction. Results are the ratio of MTT-FZ to DNA absorbance per milliliter aliquot of tissue homogenate.

Protein purification. Mucosal scrapings were immediately washed twice with ice-cold PBS (pH 7.4) and centrifuged at 1,000 g for 5 min at 4°C. Pellets were then resuspended with 2 vol of buffer A and lysed at 4°C for 30 min (32). Buffer A consisted of 250 mM sucrose (Sigma), 20 mM HEPES (Sigma)-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupatin. Homogenates were centrifuged at 1,000 g for 10 min at 4°C to remove cell nuclei, and supernatants were again centrifuged at 10,000 g for 15 min at 4°C to remove mitochondria. Supernatants of the 10,000-g spin were further centrifuged at 1,000,000 g for 1 h at 4°C. The resulting supernatant represented the soluble cytosolic fraction, and pellets were the membranous fraction. Supernatant and pellet fractions of resuspended solutions in buffer were divided into multiple samples and were frozen at −80°C until immunoblot analysis.

Western blot analysis of caspases and death ligand/receptors. FasL, Fas, TNF receptor (TNFR)1, and TNFR2 were determined in samples and were frozen at −80°C overnight to purify cell nuclei, and supernatants were again centrifuged at 10,000 g for 15 min at 4°C to remove mitochondria. Supernatants of the 10,000-g spin were further centrifuged at 1,000,000 g for 1 h at 4°C. The resulting supernatant represented the soluble cytosolic fraction, and pellets were the membranous fraction. Supernatant and pellet fractions of resuspended solutions in buffer were divided into multiple samples and were frozen at −80°C until immunoblot analysis.

Mucosal mitochondrial dehydrogenase activity was assessed. Mitochondrial dehydrogenase activity was measured via a diphenylamine reaction (32). Results are the ratio of MTT-FZ to DNA absorbance per milliliter aliquot of tissue homogenate.
Statistical analysis. Results are means ± SE. Data were evaluated by analysis of variance in which multiple comparisons were performed by the least-significant difference method. Differences were considered significant if probability of the difference occurring by chance was <5 in 100 (P < 0.05).

RESULTS

DNA fragmentation in intestinal mucosa of ad libitum-fed, fasted (24 and 48 h), and I/R rats. As shown in Fig. 1, percentages of fragmented DNA in jejunal and ileal mucosa in fasted and I/R rats were significantly higher than that in ad libitum-fed rats (P < 0.05 for each). Increases in fragmented DNA were enhanced in 48-h-fasted rats compared with 24-h-fasted rats (P < 0.05). Agarose gel electrophoresis of total DNA revealed a distinct ladder of DNA fragments from the intestinal mucosa of fasted and I/R rats, indicating apoptosis.

Fig. 1. Effects of fasting and ischemia-reperfusion (I/R) on the percentages of fragmented DNA in rat intestinal mucosa. I/R and fasting induced fragmentation of DNA in rat small intestine. Values are means ± SE. Six rats were tested in each group. *P < 0.05 compared with ad libitum-fed rats; †P < 0.05 compared with 24-h-fasted rats; ‡P < 0.05 compared with 48-h-fasted rats; §P < 0.05 compared with I/R rats. Closed bars, jejunum; open bars, ileum.

RESULTS

Fig. 2. Effects of 24- and 48-h fasting on jejunal mucosal height in the rat small intestine. A: jejunum. B: ileum. Intestinal mucosal height in fasted rats was significantly shorter than in fed-ad libitum rats. This decrease in mucosal height was mainly due to a decrease in villous height. Values are means ± SE. Six rats were tested in each group. *P < 0.01 compared with ad libitum-fed rats; †P < 0.01 compared with 24-h-fasted rats.

Fig. 3. Light micrographs of the jejunum stained using the terminal deoxynucleotidyl transferase (TdT)-mediated dUDP-biotin nick-end labeling (TUNEL) method. A: ad libitum-fed. B: 24-h fasting. C: 48-h fasting. Magnifications: ×100. D: apoptotic index. TUNEL staining of the jejunum reveals a few apoptotic cells (apoptotic nuclei are stained dark brown) that are present at the villous tips in the ad libitum-fed rats. An increase in the number of apoptotic cells in 24- and 48-h-fasted rats was observed with significant localization of apoptotic cells in the upper third of the intestinal villi. Values are means ± SE. Fifty crypts were tested in each group. *P < 0.01 compared with ad libitum-fed rats; †P < 0.01 compared with 24-h-fasted rats.

Fig. 4. Effects of fasting and I/R on small intestinal mucosal cellular mitochondrial dehydrogenase activity in the ileal mucosa. Mitochondrial dehydrogenase activity was impaired only in the I/R rats compared with control rats. Values are means ± SE. Six rats were tested in each group. *P < 0.05 compared with ad libitum-fed rats.
jejenum and ileum in the 24- and 48-h-fasted rats. In rats fed ad libitum, profiles of jejunal and ileal DNA fragments were indistinct (data not shown). A distinct DNA ladder was also observed in the jejunum and ileum of I/R rats (data not shown). These data indicate that enhanced intestinal mucosal apoptosis is induced by fasting and I/R.

Mucosal height and in situ detection of apoptosis by immunohistochemical staining. Jejunal mucosal height in 24- or 48-h-fasted rats was significantly shorter than that of ad libitum-fed rats ($P < 0.01$ for each), and this was mainly due to a decrease of villous height (Fig. 2A). Decrease in mucosal height was enhanced in 48-h-fasted rats compared with 24-h-
fasted animals ($P < 0.01$). Decreases in mucosal height were observed in the ileum and in the jejunum (Fig. 2B).

TUNEL staining of the jejunum showed that a few apoptotic cells were present in villous tips in rats fed ad libitum (Fig. 3A), and an increase in the number of apoptotic cells in 24- and 48-h-fasted rats was observed with a significant localization of apoptotic cells in the upper third of the intestinal villi (Fig. 3, B and C). After I/R, the number of apoptotic cells increased with marked destruction of structure with mucosal erosion and edema, and most of the upper portion of villi was detached from mucosal structure following villous denudation (Fig. 3D). As indicated in Fig. 3E, the apoptotic index increased in the 24-h-fasted rat, and this increase was enhanced in the 48-h-fasted rat. The increase in the apoptotic index in the I/R rat was equivalent to that in the 48-h-fasted rat.

Effects of fasting and I/R on small intestinal mucosal cellular mitochondrial dehydrogenase activity. Mitochondrial dehydrogenase activity in rat intestinal mucosa was significantly impaired in I/R rats compared with rats fed ad libitum (Fig. 4, $P < 0.05$). Mitochondrial dysfunction was not observed in 24- or 48-h-fasted rats.

Western blot analysis of apoptotic proteins. Results for Western blot analysis in ad libitum-fed, I/R, and 48-h-fasted rats are shown in Fig. 5. In cytosolic fractions, we evaluated cleavages of caspase-3 and caspase-6, which activate the final execution pathway. Expression of cleaved caspase-3 (17 and 12 kDa) was significantly increased in the 48-h-fasted group compared with rats fed ad libitum (Fig. 5A, $P < 0.05$), and expression of procaspase-3 significantly decreased in the 48-h-fasted group compared with rats fed ad libitum (Fig. 5A, $P < 0.05$). In contrast, no significant increase or decrease was observed in I/R rats compared with rats fed ad libitum (Fig. 5A). Expressions of cleaved caspase-6 and procaspase-6 were significantly increased in the I/R group compared with rats fed ad libitum (Fig. 5F, $P < 0.05$). In contrast, no significant changes were observed in I/R rats compared with rats fed ad libitum (Fig. 5F). Caspase-8, which exists upstream of caspase-3 in both receptor-mediated and mitochondria-mediated apoptotic pathways, and caspase-9, which exists upstream of caspase-3 in the mitochondria-mediated apoptotic pathway, were examined. In both the 48-h-fasted group and I/R rats, procaspase-8 was decreased compared with rats fed ad libitum (Fig. 5B, $P < 0.05$) and was more cleaved than in the ad libitum-fed group (Fig. 5B, $P < 0.05$). Expression of cleaved caspase-9 significantly increased only in I/R group (Fig. 5C, $P < 0.05$). In addition, we investigated release of cytochrome c to the cytosol and Bcl-2, which is an antiapoptotic member of the Bcl-2 superfamily. Expression of cytochrome c significantly increased only in the I/R group compared with rats fed ad libitum (Fig. 5D, $P < 0.05$), and Bcl-2 decreased only in the I/R group (Fig. 5E, $P < 0.05$).

Fas and FasL levels in membranous fractions (Fig. 5G) were increased in I/R and 48-h-fasted rats compared with rats fed ad libitum ($P < 0.05$ for each). TNFR1 increased significantly in I/R and 48-h-fasted rats ($P < 0.05$ for each), and this increase was significantly higher in I/R compared with fasting rats (Fig. 5H, $P < 0.05$). In contrast to TNFR1 patterns, neither I/R nor 48-h fasting affected TNFR2 (Fig. 5H).

DISCUSSION

Although many apoptotic stimuli and signal transduction pathways have been demonstrated, only two principal apoptosis pathways are well recognized: the type I pathway activated by extrinsic stimuli and type II pathway activated by intrinsic stimuli. These signals result in binding of specific ligands to death receptors such as Fas and TNFRs. Downstream signal transduction involves formation of a death-inducing signal complex (DISC) (6), whose primary components are Fas, FADD, and procaspase-8. After the formation of the DISC, procaspase-8 is cleaved. As shown in Fig. 6, cleaved caspase-8 can directly cleave procaspase-3 followed by cleavage of DNA fragmentation factor (DFF) in the type I pathway (22). In the type II pathway, cleaved caspase-8 can truncate Bid to tBid (15), which leads to mitochondrial release of cytochrome c into the cytoplasm to form a complex (apoptosome) with APAF-1 and caspase-9 (20). The apoptosome may cleave procaspase-3, which then activates the joint final execution pathway.

Fig. 6. Schematic summary diagram of apoptotic pathways for I/R- and fasting-induced apoptosis in rat small intestine. Fasting-induced apoptosis in apical epithelial cells occurs via a receptor-mediated type I apoptotic pathway, whereas I/R-induced apoptosis occurs via a mitochondria-mediated type II apoptotic pathway.
The summary of data obtained in this study on apoptotic pathways is illustrated in Fig. 6 (Fig. 6A, I/R conditions; Fig. 6B, fasting conditions). In I/R-induced small intestinal apoptosis, we found that I/R induced expression of FasL, Fas, TNFR1, cytosolic cytochrome c, cleaved caspase-9, and cleaved caspase-6 and suppressed expression of Bcl-2 in the small intestinal mucosa. These results indicated that I/R induced small intestinal apoptosis via the type II pathway. Furthermore, in this study, we assessed and demonstrated that I/R induced mitochondrial respiratory dysfunction, using an assay based on conversion of MTT to its formazan derivative. This mitochondrial dysfunction accompanied by a decrease in Bcl-2 expression might be partly involved in the apoptotic pathway in I/R-induced small intestinal apoptosis. In contrast, fasting induced expressions of FasL, Fas, TNFR1, cleaved caspase-8, and cleaved caspase-3. These results indicate that fasting induces small intestinal apoptosis via the type I pathway.

It is not clear why apoptotic pathways are different between I/R and fasting conditions. Such differences were also reported in other studies where decisions for type I or type II apoptotic pathways were determined by tissue specificity. In Bid knock-out mice, apoptosis of hepatocytes might be following a type II pathway, and apoptosis of thymocytes might be through a type I pathway (34). Moreover, a study reported that thymocytes lacking Apaf1, which is involved in the type II apoptotic pathway, were resistant to apoptotic stimuli but were sensitive to Fas-mediated cell death (35). Jejunal apoptosis induced by I/R was not different between ad libitum-fed rats and 24-h-fasted rats (data not shown), indicating that fasting had no additive increment in jejunal apoptosis caused by I/R.

In this study, we focused on fasting as a physiological condition. We previously demonstrated that apoptosis in the rat small intestine was controlled by several other physiological factors including leptin (29) and the feeding center such as the ventromedial hypothalamus (25). Results from our previous studies suggested that feeding might be an important factor for regulation of apoptosis in the small intestine, which warranted further explorations. We focused on I/R as a pathological condition. The reperfusion injury frequently exceeded the original ischemic damage (21). During the reperfusion period, molecular oxygen reintroduced into the tissue and produced a burst of reactive oxygen species (12). Previous studies demonstrated that I/R injury in the intestinal mucosa was mainly due to reperfusion and not due to ischemia (11, 12, 18, 21). These studies were supported by the result of the present experiment that ischemia for 60 min without reperfusion slightly increased percentage of fragmented DNA in the jejunum but had no influence on expression of cleaved caspase-9 and cytochrome c (data not shown).

Here, we demonstrated that TUNEL-positive apoptotic cells were mainly located in villous tips of the rat small intestine during fasting as demonstrated previously (14). Hyoh et al. (13) indicated that activity of caspase-3 increased in the top of villi, which supports our results that apoptosis during fasting is induced in the top of villi by activation of caspase-3 following a type I pathway. This location of apoptotic cells during fasting might be due to an increase in absorbing area in the villi during feeding. In contrast to apoptosis induced by fasting, apoptotic cells in the intestinal mucosa after radiation was reported to mainly locate in the base of crypts (23, 24). In these studies, apoptosis might be occurring mainly in multipotent progenitor cells for prevention of radiation-induced mutations. This hypothesis was indicated in a study using insulin-like growth factor-I (IGF-I) transgenic mice in which the antiapoptotic effect of IGF-1 was more potent in crypt base cells compared with cells in the upper portion of villi including villous tips (30). Judging from these results, apoptosis might be induced in the bottom of villi after I/R as a pathological condition. Previous studies evaluated mucosal damage with a decrease in villous height and an increase in injury score in I/R injury in the intestine (4, 21, 27). In this study, I/R induced marked destruction of structure with mucosal erosion and edema with a decrease in villous height, but the site of apoptosis after I/R was not identified because of mucosal damage.

In conclusion, fasting induced apoptosis in rat intestinal mucosa via a type I receptor-mediated apoptotic pathway, and I/R induced intestinal apoptosis via a mitochondrial-mediated type II apoptotic pathway.

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