Fasting-induced intestinal apoptosis is mediated by inducible nitric oxide synthase and interferon-γ in rat

Junta Ito, Hiroyuki Uchida, Takayuki Yokote, Kazuo Ohtake, and Jun Kobayashi

Division of Pathophysiology, Department of Clinical Dietetics and Human Nutrition, Faculty of Pharmaceutical Science, Josai University, Saitama, Japan

Submitted 19 October 2009; accepted in final form 5 April 2010

Fasting-induced intestinal apoptosis is mediated by inducible nitric oxide synthase and interferon-γ in rat. Am J Physiol Gastrointest Liver Physiol 298: G916–G926, 2010. First published April 8, 2010; doi:10.1152/ajpgi.00429.2009.—Nitric oxide (NO) is associated with intestinal apoptosis in health and disease. This study aimed to investigate the role of intestinal NO in the regulation of apoptosis during fasting in rats. Male Wistar rats were divided into two groups and subcutaneously injected with saline (SA) or aminoguanidine (AG), followed by fasting for 24, 48, 60, and 72 h. At each time point, the jejum was subjected to histological evaluation for enterocyte apoptosis by histomorphometric assessment and TUNEL analysis. We performed immunohistochemistry for inducible NO synthase (iNOS) expression in the jejunum and measured tissue nitrite levels using HPLC and 8-hydroxydeoxyguanosine aduct using ELISA, indicative of endogenous NO production and reactive oxygen species (ROS) production, respectively. Jejunal transcripational levels of iNOS, neuronal NO synthase (nNOS), and interferon-γ (IFN-γ) were also determined by RT-PCR. Fasting caused significant jejunal mucosal atrophy due to attenuated cell proliferation and enhanced apoptosis with increase in iNOS transcription, its protein expression in intestinal epithelial cells (IEC), and jejunal nitrite levels. However, AG treatment histologically reduced apoptosis with inhibition of fasting-induced iNOS transcription, protein expression, and nitrite production. We also observed fasting-induced ROS production and subsequent IFN-γ transcription, which were all inhibited by AG treatment. Furthermore, we observed reduced transcriptional levels of nNOS, known to suppress iNOS activation physiologically. These results suggest that fasting-induced iNOS activation in IEC may induce apoptosis mediators such as IFN-γ via a ROS-mediated mechanism and also a possible role of nNOS in the regulation of iNOS activity in fasting-induced apoptosis.

aminoguanidine; reactive oxygen species; intestinal epithelial cells; neuronal nitric oxide synthase

The gastrointestinal epithelium is a dynamic tissue characterized by a high cellular turnover rate with a balance between cell proliferation and cell apoptosis, consequently leading to renewal of the entire intestinal epithelium every 3–5 days (9, 19). This single layer of cells lining the gut lumen acts as a barrier against harmful intraluminal entities, including foreign antigens, microorganisms, and toxins, and also acts as a selective filter allowing the translocation of essential dietary nutrients, electrolytes, and water. However, these physiological functions are occasionally impaired under pathological conditions such as inflammation and ischemia-reperfusion and are accompanied by intestinal histological changes, including apoptosis.

Address for reprint requests and other correspondence: J. Kobayashi, Josai Univ., 1-1 Keyaki-Dai, Sakado, Saitama, Japan 350-0295 (e-mail address: junkoba@josai.ac.jp).

Fasting is likely a physiological challenge evoking a response to the absence of luminal nutrients by functional and morphological changes, including intestinal epithelial apoptosis (8, 13, 21). However, prolonged fasting not only impairs intestinal physiological functions but also brings about intestinal barrier dysfunction, including increased epithelial permeability and impaired tight junctions, subsequently leading to bacterial translocation in patients receiving a prolonged course of total parenteral nutrition (TPN) (57). Recent studies also show that proinflammatory cytokine-induced apoptosis causes loose epithelial tight junctions and an increase in paracellular permeability, raising a possibility of apoptosis-mediated intestinal barrier dysfunction (3, 44).

Among the many factors affecting the regulation of apoptosis, considerable attention has been given to the possible contribution of nitric oxide (NO) in the process of intestinal apoptosis (4, 35, 54, 58) and barrier dysfunction. Recent reports describing the link between NO and intestinal barrier functions demonstrated that inhibition of inducible NO synthase (iNOS) reduces bacterial translocation with a decrease in enterocyte apoptosis in LPS-induced rat intestinal injury (12) and improves intestinal barrier dysfunction induced by prolonged oral TPN feeding in rats (23).

According to recent knowledge, apoptosis is regulated through two distinct pathways converging to sequential activation of cysteine proteases of the caspase family, leading to the common final events of DNA fragmentation (14, 33, 50). The intrinsic type 2 pathway occurs in response to diverse stresses, including ischemia-reperfusion via a mitochondria-mediated pathway following the release of cytochrome c from damaged mitochondria. On the other hand, fasting promotes intestinal mucosal apoptosis through ligand-mediated trimerization of death receptors of the tumor necrosis factor (TNF) family, that is, the so-called extrinsic pathway (type 1 apoptotic pathway). Although most reports have investigated the relationships of caspase activity and cytokine levels on intestinal apoptotic morphology in fasting animal models (2, 9, 16, 27), the NO contribution to fasting-mediated intestinal mucosal apoptosis remains to be elucidated, especially in the early phase of fasting. Therefore, the aim of the present study was to investigate the mechanism of fasting-induced intestinal apoptosis with respect to the role of NOS in the regulation of intestinal mucosal apoptosis during the early phase of fasting in rats.

MATERIALS AND METHODS

Animals and experimental design. The experimental protocol and design were approved by the Institutional Animal Care and Use Committee at the Life Science Center of Josai University and were consistent with the Guide for the Care and Use of Laboratory Animals published by the NIH. Male Wistar rats weighing 200–220 g (at 9 wk

G916 0193-1857/10 Copyright © 2010 the American Physiological Society http://www.ajpgi.org
Fasting-induced apoptosis mediated by iNOS and IFN-γ

of age) were purchased from SLC (Shizuoka, Japan), and were housed individually in wire-bottomed cages to prevent coprophagia, in a room illuminated from 7:00 AM to 7:00 PM (12:12-h light-dark cycle). The rats were allowed free access to deionized water and standard rat chow (CE-2, CLEA Japan) ad libitum until the study began. At 10 wk of age, seventy rats were randomly divided into two groups (Fig. 1): saline (SA) group and aminoguanidine (AG; a relatively selective iNOS inhibitor) group. Each group was further divided into five subgroups as follows: 1) SA + ad libitum (normally fed control), 2) SA + 24-h fast, 3) SA + 48-h fast, 4) SA + 60-h fast, 5) SA + 72-h fast, 6) AG + ad libitum (normally fed control), 7) AG + 24-h fast, 8) AG + 48-h fast, 9) AG + 60-h fast, 10) AG + 72-h fast. All rats received SA or AG subcutaneously, 6, 30, and 54 h after fasting. The large block arrows represent treatment with SA (open block arrow) and AG (closed block arrow). At the appointed time after fasting, intestinal tissues were collected. Seven rats were tested in each group.

38-40 rats were allowed free access to deionized water and standard rat chow (CE-2, CLEA Japan) ad libitum until the study began. At 10 wk of age, seventy rats were randomly divided into two groups (Fig. 1): saline (SA) group and aminoguanidine (AG; a relatively selective iNOS inhibitor) group. Each group was further divided into five subgroups as follows: 1) SA + ad libitum (normally fed control), 2) SA + 24-h fast, 3) SA + 48-h fast, 4) SA + 60-h fast, 5) SA + 72-h fast, 6) AG + ad libitum (normally fed control), 7) AG + 24-h fast, 8) AG + 48-h fast, 9) AG + 60-h fast, 10) AG + 72-h fast. All groups were subdivided into 5 subgroups, including a normally fed control group, according to fasting durations in each group: 1) SA + ad libitum (normally fed control), 2) SA + 24-h fast, 3) SA + 48-h fast, 4) SA + 60-h fast, 5) SA + 72-h fast, 6) AG + ad libitum (normally fed control), 7) AG + 24-h fast, 8) AG + 48-h fast, 9) AG + 60-h fast, 10) AG + 72-h fast. All rats received SA or AG subcutaneously, 6, 30, and 54 h after fasting. The large block arrows represent treatment with SA (open block arrow) and AG (closed block arrow). At the appointed time after fasting, intestinal tissues were collected. Seven rats were tested in each group.

Fig. 1. Experimental design. Seventy male Wistar rats were divided into 2 groups. Thirty-five rats in each group received saline (SA) or aminoguanidine (AG; 20 mg/kg) subcutaneously for 18 h before fasting and were randomly subdivided into 5 subgroups, including a normally fed control group, according to fasting durations in each group: 1) SA + ad libitum (normally fed control), 2) SA + 24-h fast, 3) SA + 48-h fast, 4) SA + 60-h fast, 5) SA + 72-h fast, 6) AG + ad libitum ( normally fed control), 7) AG + 24-h fast, 8) AG + 48-h fast, 9) AG + 60-h fast, 10) AG + 72-h fast. The large block arrows represent treatment with SA (open block arrow) and AG (closed block arrow). At the appointed time after fasting, intestinal tissues were collected. Seven rats were tested in each group. Open bars, SA-treated rats; solid bars, AG-treated rats.

Fig. 2. Body weight changes expressed as the ratio of after to before fasting in SA-treated rats and AG-treated rats. Fasting caused gradual decreases in body weight in both SA- and AG-treated groups. There was no difference in weight loss between the SA and AG treatment at each fasting period. Values are means ± SE. *P < 0.05 compared with the ad libitum-fed rats in SA-treated group. Seven rats were tested in each group. Open bars, SA-treated rats; solid bars, AG-treated rats.

Fig. 3. Effect of fasting and AG treatment on jejunal mucosal height. Although fasting caused gradual decreases in jejunal mucosal height in both SA- and AG-treated rats, there were significant differences (P < 0.05) in jejunal mucosal height between the SA- and AG-treated rats at 48, 60, and 72 h fasting periods. Values are means ± SE. *P < 0.05 compared with the ad libitum-fed rats in SA-treated group. bP < 0.05 compared with the respective SA-treated group by the fasting period. Seven rats were tested in each group. Open bars, SA-treated rats; solid bars, AG-treated rats.

Fig. 4. Effect of fasting and AG treatment on cell proliferation index in the jejunum. The jejunal crypt cell proliferation was histologically assessed by 5-bromo-2’-deoxyuridine (5-BrdU) incorporation to identify cells in the S phase of the cell cycle. The number of labeled cells in at least 10 well-oriented longitudinal crypts in each sample was determined and expressed as cell proliferation index (5-BrdU-positive cells/10 crypts). The decrease in cell proliferation along with fasting was observed both with and without AG. Values are means ± SE. *P < 0.05 compared with the ad libitum-fed rats in SA-treated group. Seven rats were tested in each group. Open bars, SA-treated rats; solid bars, AG-treated rats.
The oral 10-cm part of the intestine was treated as the duodenum, and the rest of the intestine was divided into two segments representing proximal (jejunum) and distal (ileum) ends. The segments used for several analyses were the jejunum, from 3 to 5 cm distal to the duodenum (16). Some pieces, ~3 cm in length, were fixed in 10% neutral buffered formalin for measurement of mucosal height and for immunohistochemistry. Other segments were snap frozen in liquid nitrogen and stored at ~80°C until use.

Histopathological analysis, including apoptotic index, terminal deoxynucleotidyl transferase (TdT)-mediated dUDP-biotin nick-end labeling (TUNEL) staining, and cell proliferation. Tissue samples fixed in 10% neutral buffered formalin were then embedded in paraffin and sectioned. The specimens were stained with hematoxylin and eosin (HE). Mucosal height (villous height plus crypt depth) was measured using a microscope (Olympus BX41) and a digital camera system (Pixera Penguin 150CL) for ad libitum-fed and fasted rats. Mucosal height was measured in at least 30 villi per animal.

To detect enterocyte apoptosis in the jejunal villus, both TUNEL staining and TUNEL-positive rate in the jejunal villus. TUNEL staining is easy to interpret over all images for apoptosis; however, because of its nonspecific staining (28), representative apoptotic changes were also detected for the analysis of AI by conventional light microscopy of HE-stained specimens. We followed the method described by Dahly et al. (11). In brief, jejunal sections were examined for apoptotic enterocytes in a blinded manner by an experimental pathologist (H. Uchida) on the basis of the characteristic findings of apoptotic cells, including condensed chromatin, nuclear fragmentation, intensely eosinophilic cytoplasm, and formation of apoptotic bodies (Fig. 6A). Fifty villus columns were assessed per rat (n = 7 rats per subgroup). For each villus column assessed (i.e., one side of the villus in a longitudinal cross section), the number and position of apoptotic cells as well as the total number of cells in the villus column were recorded. To account for the effects of fasting and AG treatment on apoptosis, the ratio of apoptotic cells to one villus column and AI were determined. The mean number of apoptotic cells per villus column was calculated by dividing the total number of apoptotic cells in the well-oriented villus cell columns by 50 for each rat. In addition, AI was quantified by counting the total number of apoptotic cells in the 50 well-oriented villus columns and expressing this as the percentage of the total number of cells in the 50 villus columns for each rat.

To identify locations of apoptosis along the villus, AI distribution curves were constructed on the basis of group means that plotted cell

![Fig. 5. Effect of fasting and AG treatment on terminal deoxynucleotidyl transferase-mediated dUDP-biotin nick-end labeling (TUNEL) staining and TUNEL-positive rate in the jejunal villus. Apoptotic assessment of the jejunal villus was performed by use of TUNEL staining and TUNEL-positive rate in the jejunal villus. TUNEL-positive cells were observed in the tip of the jejunal villus of SA-treated rats with increasing TUNEL-positive rate. A: TUNEL staining of representative jejunal section. TUNEL-positive cells over the indicated fasting periods (A-1, A-2, C-1, C-2, E-1, E-2, G-1, G-2), which were all inhibited by AG (B-1, B-2, D-1, D-2, F-1, F-2, H-1, H-2). A-1, A-2: ad libitum-fed with SA treatment. B-1, B-2: ad libitum-fed with AG treatment. C-1, C-2: 24-h fasted with SA treatment. D-1, D-2: 24-h fasted with AG treatment. E-1, E-2: 48-h fasted with SA treatment. F-1, F-2: 48-h fasted with AG treatment. G-1, G-2: 72-h fasted with SA treatment. H-1, H-2: 72-h fasted with AG treatment. Magnification: left side ×20, right side ×80. B: TUNEL-positive rate was determined by dividing the number of TUNEL-positive cells by the total number of cells in the villus and multiplying by 200. Fasting significantly induced jejunal cell apoptosis, which was completely inhibited by AG over the indicated fasting periods. Values are means ± SE. *P < 0.05 compared with the ad libitum-fed rats in SA-treated group. **P < 0.05 compared with the respective SA-treated group by fasting period. Seven rats were tested in each group. Open bars, SA-treated rats; solid bars, AG-treated rats.](http://ajpgi.physiology.org/doi/10.1093/ajpgi/gf017)
position vs. AI at each position. AI, in this case, was defined as the percentage of the total number of cells counted at that cell position.

Fragmented DNA was stained by the TUNEL method (17), with an Apoptosis In Situ Detection Kit (Wako). The specimens were dewaxed and incubated with 20 μg/ml proteinase K for 20 min at 37°C, and then the reaction was terminated with buffer containing bovine serum, and finally the specimens were incubated for 5 min. PBS was applied directly on the sections for 10 min at room temperature. Subsequently, 100 μl of TdT reaction solution was added, and the mixture was incubated at 37°C for 25 min. In additional control sections, TdT reaction solution was omitted. The specimens were incubated successively with PBS containing 3% hydrogen peroxide for 20 min at room temperature to inactive endogenous peroxidases, and then were covered with 100 μl of horseradish peroxidase-conjugated antibody solution, and incubated for 10 min at 37°C. The specimens were soaked in PBS for 10 min and then covered with 100 μl of 3.3’diaminobenzidine tetrahydrochloride (DAB) solution for 2 min at room temperature for color development. Finally, the specimens were counterstained with hematoxylin and examined with a light microscope. A minimum of 30 villi were randomly selected for TUNEL staining, and the number of TUNEL-positive cells was calculated. The TUNEL-positive rate was determined by dividing the number of cells by the total number of cells in the villi and multiplying by 200.

Cryostat sections were prepared by using 5-bromo-2’-deoxyuridine (5-BrdU) incorporation to identify cells in the S phase of the cell cycle (49). 5-BrdU immunohistochemistry staining was performed by an anti-5-BrdU monoclonal antibody and a VECTASTAIN Elite ABC Kit (Vector Laboratories). Rats were given intraperitoneal injections of 100 mg/kg 5-BrdU 60 min before euthanasia. The specimens were dewaxed and then covered with 100 μl of 3.3’diaminobenzidine tetrahydrochloride (DAB) solution for 2 min at room temperature for color development. Finally, the specimens were counterstained with hematoxylin and examined with a light microscope. The number of labeled cells in at least 10 well-oriented longitudinal crypts in each sample was determined under a light microscope. The result is shown as the number of 5-BrdU-labeled cells among the crypt cells.

Immunohistochemistry of iNOS. Immunohistochemical staining of iNOS was performed with an anti-iNOS polyclonal antibody and a VECTASTAIN Elite ABC Kit (Vector Laboratories) (32). The specimens obtained from the jejunum after 24-h, 48-h, and 72-h fasting as well as ad libitum rats were dewaxed and treated for antigen retrieval by boiling in 10 mM citrate buffer (pH 6.0) for 20 min at 95°C (44). After being washed with PBS, sections were incubated in 6% hydrogen peroxide for 1 h and washed again with PBS. Nonspecific binding was blocked with a 20% goat serum solution in PBS for 10 min at room temperature. Sections were incubated with rabbit anti-iNOS polyclonal antibody (1:100; BD Transduction Laboratories). In additional control sections, the primary antibody was omitted. A biotinylated goat anti-rabbit IgG (1:200; VECTASTAIN) was used as a secondary antibody. Sections were then treated with VECTASTAIN Elite ABC Kit, and reaction products were detected by color development at room temperature in a substrate medium containing a 0.05% DAB-H2O2 solution. Finally, the specimens were counterstained with hematoxylin and examined with a light microscope and a digital camera system.

Nitrite concentrations in jejunum. Tissue samples stored at −80°C were measured for nitrite concentrations via a dedicated HPLC system (ENO-20; EiCom). This method is based on the separation of nitrate and nitrite by ion chromatography, followed by online reaction of nitrate to nitrite, postcolumn derivatization with Griess reagent, and detection at 540 nm. Proteins in each sample were removed by centrifugation at 10,000 × g for 5 min following methanol precipitation (jejunum-methanol = 1:2 weight/volume, 4°C) (26).

Analysis of iNOS, nNOS, and iNγ RNAs by RT-PCR. Tissue samples stored at −80°C were extracted using TaKaRa RNAiso Reagent (TaKaRa Bio) according to the manufacturer’s instructions. Total RNA concentrations were quantified by spectrophotometry at 260 nm. RT-PCR was performed with 1 μg of total RNA by use of an RNA PCR kit (AMV) Ver. 3.0 (TaKaRa Bio) according to the manufacturer’s instructions: 1 cycle at 42°C for 30 min, 99°C for 5 min, and 5°C for 5 min for reverse transcription; and 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min for PCR. PCR was performed with a RoboCycler 96 gradient temperature cycler (Stratagene). The oligonucleotides used as primers were synthesized by TaKaRa Bio. The primer pairs were designed as follows (product size and Primer ID provided by TaKaRa Bio are shown in parentheses): iNOS forward primer, 5’-ctcaatggtggtgccactaca-3’; and iNOS reverse primer, 5’-gggcttcgggctcgagattga-3’ (101 bp, RA008296). In addition, because intraepithelial lymphocyte (IEL)-

### Table 1. Enterocyte apoptosis of the jejunal villus by conventional light microscopy of hematoxylin and eosin-stained specimens

<table>
<thead>
<tr>
<th></th>
<th>Ad Libitum</th>
<th>24-h Fast</th>
<th>48-h Fast</th>
<th>60-h Fast</th>
<th>72-h Fast</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells per villus column, n</td>
<td>84 ± 1</td>
<td>72 ± 1*</td>
<td>70 ± 2*</td>
<td>70 ± 2*</td>
<td>68 ± 1*</td>
</tr>
<tr>
<td>Apoptotic cells per villus column, n</td>
<td>0.04 ± 0.01</td>
<td>0.24 ± 0.04*</td>
<td>0.29 ± 0.02*</td>
<td>0.29 ± 0.02*</td>
<td>0.29 ± 0.04*</td>
</tr>
<tr>
<td>Apoptotic index, %</td>
<td>0.05 ± 0.01</td>
<td>0.34 ± 0.07*</td>
<td>0.42 ± 0.03*</td>
<td>0.42 ± 0.02*</td>
<td>0.43 ± 0.05*</td>
</tr>
<tr>
<td><strong>AG</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells per villus column, n</td>
<td>85 ± 1</td>
<td>75 ± 1</td>
<td>76 ± 3</td>
<td>75 ± 1</td>
<td>75 ± 2</td>
</tr>
<tr>
<td>Apoptotic cells per villus column, n</td>
<td>0.04 ± 0.01</td>
<td>0.17 ± 0.02*</td>
<td>0.15 ± 0.01†</td>
<td>0.19 ± 0.03†</td>
<td>0.17 ± 0.03†</td>
</tr>
<tr>
<td>Apoptotic index, %</td>
<td>0.05 ± 0.01</td>
<td>0.22 ± 0.02†</td>
<td>0.20 ± 0.02†</td>
<td>0.25 ± 0.04†</td>
<td>0.23 ± 0.04†</td>
</tr>
</tbody>
</table>

Significant decreases in cell numbers per villus column of fasting rats were noted compared to that of the ad libitum-fed rats over the indicated time periods. Conventional light microscopy detecting representative apoptotic changes showed significant increases both in ratio of apoptotic cells to one villus column and in apoptotic index (AI) (6.8- to 8.6-fold increase: *P < 0.05) in fasted rats with saline (SA) and also showed significant inhibitory effects of aminoguanidine (AG) on fasting-induced apoptosis over the indicated fasting periods. Values are means ± SE; n = 7 per group. *P < 0.05 compared with the ad libitum-fed rats in SA-treated group. †P < 0.05 compared with the respective SA-treated group at each fasting period. AI in the villus is defined as the total number of apoptotic cells expressed as a percentage of the total number of cells counted in 50 well-oriented villus columns.
RESULTS

Body weight changes. Figure 2 shows body weight changes expressed as the ratio of after to before fasting in SA-treated and AG-treated rats. Gradual decreases in body weight in both SA- and AG-treated groups were observed along with fasting. Rats fasted for 72 h with SA and AG treatment showed an average of 19% (P < 0.05) and 18% (P < 0.05) body weight loss, respectively, compared with ad libitum-fed control rats. There was no significant difference in weight loss at each fasting period between the SA and AG treatment, suggesting AG has no impact on fasting-induced body weight loss.

Histological characterization of jejunal mucosal atrophy in fasted rats treated with SA or AG. Decreasing jejunal mucosal height in both SA- and AG-treated rats was observed along with fasting. However, significant differences (P < 0.05) in jejunal mucosal height were found between the SA- and AG-treated rats at 48, 60, and 72 h fasting periods, suggesting that AG significantly inhibited progressive jejunal mucosal atrophy in fasted rats (Fig. 3).

To evaluate how reduced cell proliferation and/or increased cell apoptosis contributes to jejunal mucosal atrophy, we assessed BrdU incorporation to the cells and TUNEL staining of the jejunum, indicative of cell proliferation and apoptosis in the jejunum, respectively. Although decreases in cell proliferation along with fasting were observed irrespective of the presence or absence of AG (Fig. 4), fasting remarkably induced jejunal cell apoptosis, which was completely inhibited by AG over all of the fasting periods as observed by TUNEL staining and TUNEL-positive rate (Figs. 5, A and B). Conventional light microscopy for detecting representative apoptotic changes also showed an increased ratio of apoptotic cells to one villus column and AI (6.8- to 8.6-fold increase: P < 0.05) in fasted rats with SA, as well as significant inhibitory effects of AG on fasting-induced apoptosis over the indicated fasting periods (Table 1). For better identification of locations of apoptosis along the villus compartments, AI distribution curves were constructed by detecting representative apoptotic changes by conventional light microscopy of HE-stained specimens. Although many TUNEL-positive cells were observed in the tip of the jejunal villus in fasted rats (Fig. 5A), AI distribution curves showed increased apoptosis throughout the whole jejunal villus of fasted rats, with peak incidence of apoptosis in the bottom half of the villus compared with ad libitum-fed rats (Fig. 6B). Similar to the inhibitory effect of AG on AI, decreases in apoptosis by AG treatment were also observed in the AI distribution curves (Fig. 6B).

Nitrite levels in the jejunum. Similar to the inhibitory effect of AG on intestinal apoptosis, AG, a selective iNOS inhibitor, also significantly inhibited the increasing accumulation of intestinal nitrite, a stable oxidation product of endogenous NO, especially in

Fig. 6. Effect of fasting and AG treatment on apoptotic index in the jejunal villus. The representative apoptotic changes by conventional light microscopy of hematoxylin and eosin (HE)-stained specimens (48-h fasted rat with SA treatment) are shown in A. The boxed areas (a, b, c) in a low-power view of jejunal villi on the left (×20) are seen at higher power on the right (×80). Apoptotic cells in the villus are indicated by an arrow showing an intensely eosinophilic cytoplasm and condensed chromatin (a and b). B: apoptotic index (AI) in the villus of ad libitum and the indicated fasting periods (24, 48, 60, and 72 h) for SA treatment (top row) and AG treatment (bottom row). AI is defined as the total number of apoptotic cells at each cell position expressed as a percentage of the total number of cells counted at that cell position. Cell position 1 is defined as the cell at the crypt-villus junction. Seven rats were tested in each group.
fasting (Fig. 7), suggesting that iNOS-induced NO might act as an important mediator of intestinal apoptosis in fasting rats.

**Intestinal transcriptional expression of iNOS and IFN-γ.** Next, we tried to demonstrate the causative effect of iNOS on intestinal apoptosis by evaluating transcriptional levels of iNOS during fasting. Figure 8 shows that fasting induced iNOS mRNA, which was suppressed by AG. Because of intimate anatomical localization of IEL located at basolateral surfaces of intestinal epithelial cells (IEC), possibly implicating a functional dialogue between the two cells, recent reports indicated phenotypic changes of IEC during administration of total parenteral nutrition via IEL-derived cytokines such as IFN-γ (52, 55, 56). We therefore investigated the effects of fasting and AG treatment on transcriptional expression of IFN-γ.

**Intestinal transcriptional expression of nNOS.** Because there are reports showing that nNOS plays an important role in the regulation of iNOS expression in the small intestine of fasting animal models (18, 39), intestinal transcriptional levels of nNOS were also investigated to evaluate the effect of fasting on nNOS transcription in the jejunum. In contrast to the fasting-induced increase in iNOS mRNA and protein expression (Figs. 8 and 10), a reduced nNOS transcription after fasting was observed (Fig. 11), possibly suggesting a reverse relationship between nNOS and iNOS at least at the transcriptional level.

**Intestinal DNA oxidative damage.** The 8-OHdG levels were also investigated to evaluate the effect of fasting on the ROS generation in the jejunum (Fig. 12). In SA-treated groups, fasting increased jejunal 8-OHdG levels at 24-, 48-, and 72-h fasting periods compared with the ad libitum-fed rats (P < 0.05). AG significantly (P < 0.05) inhibited the fasting-
induced increases in the jejunal 8-OHdG levels at 48 and 72 h fasting periods compared with the respective SA-treated group at each fasting period. Considering the AG inhibitory effect on increased IFN-γ induction and ROS production after fasting (Figs. 9 and 12), iNOS-induced NO might play a central role upstream in the process of fasting-induced apoptosis in which IFN-γ and ROS might be involved.

**DISCUSSION**

Gut mucosal homeostasis depends on a balance between cell proliferation and cell death (15, 27, 37). In the present study, incremental fasting resulted in jejunal mucosal atrophy caused by both decreased crypt stem cell proliferation and increased villus cell apoptosis. We applied TUNEL staining for detection of apoptotic cells (9). TUNEL staining allows easy observation of apoptotic distribution over tissue sections; however, because of its nonspecific staining (28), we also used conventional light microscopy for detection of characteristic apoptotic changes in HE-stained specimens. This method is extremely precise and presently considered the reference standard (36) if representative morphological changes are observed (22). Consistent with the results from TUNEL-positive rate, we observed fasting-induced apoptosis mediated by iNOS and IFN-γ.
induced apoptosis and its improvement with AG by using this histomorphometric assessment. However, the distribution of apoptotic cells is spread throughout the whole villus with predominant localization at the bottom one-half of the villus in the histomorphometric assessment (Fig. 6B), whereas apoptosis is confined to the top one-half of the villus in TUNEL staining (Fig. 5A), despite use of carefully controlled techniques on rapidly fixed tissues to avoid nonspecific staining. It has been suggested by Merritt et al. (31) that senescent cell suicide particularly evident on the villus tip may have different temporal and even genetic regulation from that of damage-induced apoptosis observed in the crypts treated with irradiation or TPN (11, 31). The difference in methodology between histomorphometric assessment and TUNEL staining might have some influence on the localizations of apoptotic cells in the villi, as observed in the present study. The precise mechanism involved still remains to be elucidated, and further investigation will be required to interpret this issue.

In the present study, we indicated that fasting-induced apoptosis might be mediated by iNOS, which is supported by increasing tissue levels of nitrite (Fig. 7), the stable oxidation product of endogenous NO especially in fasting, and the increase in both transcriptional and protein levels of iNOS in atrophied jejunal mucosa observed as early as 24 h after fasting (Figs. 8 and 10). To examine how iNOS-induced NO may be related to fasting-induced apoptosis, an iNOS inhibition study was also carried out using AG, a selective iNOS inhibitor. Decreasing jejunal mucosal height with progressive fasting was significantly inhibited by AG (Fig. 3), which suppressed mucosal cell apoptosis without having any effects on the decreasing cell proliferation index (Fig. 4). We are convinced that iNOS plays a central role in fasting-induced intestinal apoptosis, because AG treatment histologically improved apoptosis (Figs. 5 and 6) accompanied with inhibition of increased iNOS transcription and protein expression (Figs. 8 and 10), increased ROS production (Fig. 12) and increased IFN-γ transcription (Fig. 9) following fasting, suggesting that iNOS-induced NO might be working upstream in the process of fasting-induced apoptosis (Fig. 13).

The causative effect of NO on apoptosis has been well documented. Depending on cell types and NO concentrations at the site where NO is produced, NO is a bifunctional regulator of apoptosis (7, 29). Low levels of NO exposure through the activation of endogenous NOS and slow release rates from NO donors are associated with antiapoptotic effects, whereas under some conditions such as inflammation or neurodegenerative diseases, NO-dependent apoptosis (proapoptotic) has been observed (6). Recently, it has been clearly demonstrated that ischemia-reperfusion-induced apoptosis as a
pathological state is related to the type 2 apoptosis pathway, in which cytochrome c release from the mitochondria triggers intrinsic apoptosis by opening mitochondrial permeability transition pores (16, 53).

Although the role of NO in the regulation of fasting-induced apoptosis has been less well documented so far, we considered that this apoptosis might be mediated by the type 1 death pathway, mainly on the basis of the previous observation by Fujise et al. (16) using the same experimental setting as in our present study. From our present results, we cannot determine whether NO works via type 1 death pathway-mediated apoptosis. However, if we pay attention to IFN-γ, we are able to hypothesize that ROS-mediated IFN-γ might induce intestinal apoptosis through the type 1 death pathway, because ROS is a main inducer of IFN-γ from IEL in the intestine. We therefore performed additional experiments examining a possible contribution of ROS in iNOS-induced apoptosis by measuring 8-OHdG levels, consequently showing an increased intestinal ROS production following fasting, which was reversed by AG treatment (Fig. 12), indicating a ROS-involved mechanism in this iNOS-induced apoptosis pathway (Fig. 13). Recent evidence shows that IEL-derived IFN-γ evokes enterocyte apoptosis via upregulation of Fas/FasL, leading to the increase in sensitivity of epithelial cells to Fas-mediated apoptosis (30, 56) through the type 1 pathway (56). It has been suggested that complex reciprocal interactions exist between IEC and IEL (20, 56); therefore, it is likely that IEC and IEL form a reciprocal feedback loop in which iNOS-released NO, physiologically present in IEC, stimulates IFN-γ production in IEL through a ROS-mediated mechanism (1, 5, 38), then IFN-γ stimulates further iNOS-derived NO production, resulting in further IFN-γ production and subsequent IEC apoptosis (Fig. 13).

Since AG, a selective iNOS inhibitor, generally inhibits iNOS activity via binding to its active center according to its crystal structural analysis (10), the inhibition may act at the posttranslational rather than at the transcriptional level. Our present study, however, showed that AG inhibited fasting-induced iNOS in both mRNA and protein levels, accompanied with the decrease in transcription of IFN-γ. As mentioned above, taking into consideration the close proximity and interaction between IEL and IEC, there could be a situation in which NO induction by IEC and IFN-γ by IEL affect each other via a reciprocal mechanism (Fig. 13). Therefore, iNOS inhibition by AG suppresses NO-mediated IFN-γ transcription, followed by the decrease in iNOS transcription as well.

Another important issue to be discussed is how fasting induces intestinal iNOS expression. Although little has been confirmed so far regarding the cause-and-effect relationship between fasting and iNOS, Qu and colleagues (39) recently showed nNOS involvement in the regulation of iNOS expression in the rat small intestine. They demonstrated that nNOS, the predominant form (>90%) expressed in the rat small intestine, physiologically suppresses the gene expression of constitutive iNOS in IEC through NF-κB downregulation and also that nNOS suppression leads to IkBa degradation, followed by NF-κB activation and a subsequent increase in iNOS expression. Gronnet and David (18) further showed that the intestinal nNOS expression is drastically reduced by fasting in the piglet jejunum. Consistent with these reports, we observed a reduced nNOS transcription after fasting (Fig. 11) with an increase in iNOS transcription (Fig. 8), suggesting a possible nNOS-involved mechanism underlying fasting-induced iNOS expression in our model (Fig. 13). However, although several other physiological factors, including leptin (51) and the feeding center such as the ventromedial hypothalamus (42), absence of luminal nutrients (16, 25) and mechanical stimuli (27), and reduced intestinal blood flow (16, 34), have also been reported to be involved in the regulation of apoptosis in the rat small intestine, further studies are needed to clarify the causative mechanism of iNOS expression in a fasting rat model.

In conclusion, these results suggest that fasting induces intestinal apoptosis through an iNOS-mediated mechanism and that a close interaction could exist between iNOS and IFN-γ in the intestinal mucosa, affecting each other and leading to intestinal apoptosis in fasting rats.

GRANTS
This work was supported by The Ministry of Education, Culture, Sports, Science and Technology of Japan under Exploratory Research Grant-In-Aid 19659347 (to H. Uchida).

DISCLOSURES
No conflicts of interest are declared by the author(s).

REFERENCES
15. Fujimoto K, Iwakiri R, Wu B, Fujise T, Tsuchida S, Ootani A. Homeostasis in the small intestinal mucosa balanced between cell prolif-


