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Suppression of Apoptosis Is Responsible for Increased Thickness of Intestinal Mucosa in Streptozotocin-Induced Diabetic Rats

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Intestinal mucosal growth is a common, but uncharacterized, observation associated with diabetes mellitus. Epithelial homeostasis is balanced by regulation of cell proliferation and cell death. To determine the contribution of apoptosis to the overall maintenance of intestinal growth, we examined intestinal apoptosis in the well-characterized streptozotocin (STZ)-induced diabetes rat model. Rats were injected with STZ (75 mg/kg body weight), thereafter they were allowed free feeding or restricted feeding for 3 weeks. Food intake and intestinal mucosal height were evaluated. In a second experiment, additional groups of animals were injected with STZ and were fed ad libitum for 1 or 3 weeks. Ornithine decarboxylase (ODC) activity, ratio of fragmented DNA to total DNA, electrophoresis of fragmented DNA, and Western blot analysis of caspase-3 were examined. Food intake gradually increased in free-feeding rats after induction of diabetes. Intestinal mucosal height in free-feeding diabetic rats was approximately 25% longer than controls, but this increase in mucosal height was not observed in restricted-fed diabetic rats (25 g/d). ODC activity in intestinal mucosa in diabetic rats did not differ from that of control rats. Percent fragmented DNA of diabetic rats 1 week after STZ injection was significantly lower than that of control rats, and this decrease returned to the control level 3 weeks after STZ treatment. Active form of caspase-3 was attenuated 1 week after drug treatment. Attenuated effect of diabetic rats on intestinal apoptosis did not affect increased apoptosis after ischemia-reperfusion. Suppression of apoptosis in the early days of STZ-induced diabetes was responsible for the increased mucosal height in the small intestine in STZ-induced diabetic animals.

IT IS WELL KNOWN that diabetes mellitus causes functional and morphologic changes in many organs. Many studies have shown that streptozotocin (STZ)-induced diabetes induces hyperphagia, body weight loss, and intestinal mucosal growth in rats.[1-3] However, there remains controversy over whether hyperphagia is a major factor of the intestinal mucosal growth in STZ-induced diabetic rats.[4-7]

Some investigators have considered mechanisms of intestinal mucosal growth in STZ-induced diabetic rats from the perspective of factors, which were related to epithelial cell proliferation, such as gastrin,[8] growth hormone,[9] ornithine decarboxylase (ODC),[10] and glucagon-like peptide 2.[11] Regulation of epithelial growth in the small intestine, however, depends on not only cell production, but also on cell death. Recent studies show that apoptosis plays an important role in physiologic and pathologic intestinal epithelial cell death.[12-15] In this regard, epithelial cell apoptosis in the small intestine is necessary to maintain of mucosal homeostasis. Feeding is one of many important factors in the regulation of apoptosis in the small intestine.[16-19]

Despite many reports on diabetic changes and apoptosis in the small intestine, there are no studies that focused on apoptosis of intestinal epithelial cells in the diabetic state. The objectives of this study were to determine: (1) whether the amount of food intake is a main factor governing intestinal growth in STZ-induced diabetic rats, and (2) whether apoptosis and ODC activity in the small intestine were involved in epithelial growth in diabetic rats.

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MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were used in this study. Animals were housed in wire-bottomed cages placed in a room illuminated from 8 AM to 8 PM (12-hour light-dark cycle). All rats were fed with standard pellet chow and were allowed free access to water. Diabetes was induced by intravenous injection of STZ (75 mg/kg body weight; Sigma Chemical, St Louis, MO) dissolved in the citrate buffer (pH 4.5) under halothane anesthesia. Blood glucose levels increased to above 350 mg/dL within 1 week of STZ injection. All rats were killed at week nine.

Experiment 1: Effect of STZ-Induced Diabetes on Food Intake, Body Weight, and Small Intestinal Mucosal Height

Rats were injected with STZ or vehicle 3 weeks before euthanasia. STZ-injected rats were divided into 2 groups: a free-feeding diabetic group and a restricted-feeding group. Rats were given 25 g of the standard chow at 8 PM every day. In the control group, rats were injected with vehicle only and allowed free access to standard chow.

In all groups, rats were weighed at the beginning of the experiment and 3 weeks after STZ injection. The amount of chow taken during the light and dark periods was measured every day after STZ or vehicle injection. Rats were killed under halothane anesthesia at 11 AM to noon at 3 weeks after the injection of STZ. The oral 5-cm segment of the small intestine, which was fixed in retroperitoneum and regarded as duodenum, was removed, and the rest of the intestine was divided into 2 equal segments, representing the proximal (jejunum) and distal (ileum) segments. Some pieces, about 2 cm in length, were resected from the middle portion of each segment and fixed in 10% neutral buffered formalin. Thereafter, they were transversely cut into smaller samples, embedded in paraffin, and sectioned. Specimens were stained with hematoxylin and eosin. Mucosal length (villous height plus crypt depth) was measured with light microscope using micrometer scale standards. Six animal were studied in each group.

Experiment 2: Effect of STZ-Induced Diabetes on ODC Activity and Apoptosis in the Small Intestinal Mucosa

Eighteen rats were divided into 3 groups. Rats in the first group were euthanized 1 week after STZ injection. Rats in the second group were euthanized 3 weeks after STZ injection. Rats in the third group (control group) were injected with vehicle only. In all groups, rats were allowed free access to chow and water. Six animal were studied in each group.

Collection of intestinal mucosa. Rats were killed under halothane anesthesia at 11:00 AM to 12:00 PM. The small intestine was carefully removed and placed on ice, and thereafter cut into 2 equal segments, the jejunum and ileum. 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.

ODC assay. ODC activity was assayed by a radiometric technique.[20] Mucosal scrapings were placed in 2 mL of 0.1 mol/L Tris buffer (pH 7.4), containing 1 mmol/L EDTA, 50 mol/L pyridoxal 5-phosphate, and 5 mmol/L dithiothreitol. The tissues were homogenized twice with a polytron tissue homogenizer for 15 seconds and centrifuged at 30,000*g* for 30 minutes. 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-^{14}C]ornithine (52.3 mCi/mmol; New England Nuclear, Boston, MA) for 15 minutes at 37°C. The $^{14}CO_2$ 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 $^{14}CO_2$ trapped in the filter paper was measured in an aqueous miscible scintillant (Opti-Flour; Packard Instruments, Downers Grove, IL). The samples were counted for 5 minutes in a liquid scintillation spectrometer (460 CD; Packard). Results are expressed as picomoles CO_2 per milligram protein per hour.

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.[15] Mucosal scrapings of the different intestinal segments were homogenized in 10 times volumes of a lysis buffer (pH 8.0) consisting of 5 mmol/L tris hydrochloride (Tris-HCl), 20 mmol/L ethylenediaminetetraacetic acid (EDTA), and 0.5% (wt/vol) t-octylphenoxypolyethoxyethanol (Triton X-100; Sigma). Aliquots (1 mL) of each sample were centrifuged for 20 minutes at 27,000*g* to separate the intact chromatin (pellet) from the fragmented DNA (supernatant).[21] The supernatant was decanted and saved, and the pellet was resuspended in 1 mL of Tris buffer (pH 8.0) with 10 mmol/L Tris-HCl and 1 mmol/L EDTA. The pellet and supernatant fractions were assayed for DNA content, using diphenylamine reaction as previously described.[22] 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. The fragmented DNA from the various fractions was extracted with a phenol-chloroform-isoamyl alcohol mixture (25:24:1, vol/vol/vol) sequentially to remove protein. The protein-free DNA extracts were treated with 100% ethanol in 0.1mol/L 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 mmol/L Tris-HCl and 10 mmol/L EDTA. DNA samples were incubated with 100 μg/mL ribonuclease for 15 minutes at 37°C to remove RNA. Resolving agarose gel electrophoresis was performed with 1.0% gel strength containing 1.0 μg/mL of 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 hours at 70 V, and DNA was visualized by ultraviolet fluorescence. Distinct DNA ladder was considered as a characteristic of apoptosis.[15,21]

Western blot analysis of caspase-3. The mucosal scrapings were immediately homogenized in 10 times volumes of a lysis buffer (pH 7.6) consisting of 50 mmol/L Tris-HCl, 300 mmol/L NaCl, 0.5% Triton X-100, 10 μg/mL aprotinin, 10 μg/mL leuptin, 1 mmol/L phenylmethylsulfonyl fluoride, and 1.8 mg/mL idoacetamide and lysed at 4°C. Insoluble material was removed by centrifugation at 4°C for 15 minutes

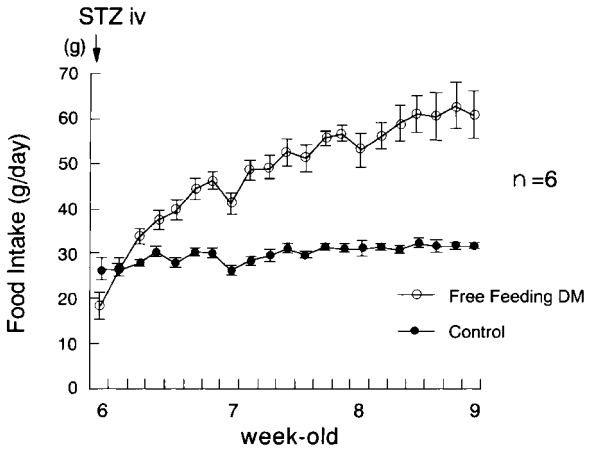

Fig 1. Food intake after STZ injection. Food intake in free-feeding diabetic (DM) rats (○) increased with each passing day after STZ injection. On the other hand, food intake in control nondiabetic rats (●) did not increase and was significantly lower compared with the diabetic rats ($P < .01$).

Table 1. Effect of STZ-Induced Diabetes on Body Weight and Intestinal Mucosal Height in Rats

	Control Nondiabetic Rats	Free-Feeding Diabetic Rats	Restricted-Feeding Diabetic Rats
Body weight (g)			
Before STZ injection	214 ± 3	215 ± 2	216 ± 5
3 weeks after STZ injection	374 ± 3	319 ± 7*	214 ± 7*†
Mucosal height (μm) 3 weeks after STZ injection			
Jejunum	1,294 ± 29	1,632 ± 31*	1,167 ± 40†
Ileum	1,093 ± 77	1,374 ± 63*	913 ± 21†

NOTE. Values are means ± SE.

* $P < .01$ compared with the corresponding value of control nondiabetic rats treated with vehicle only.

† $P < .01$ compared with the corresponding value of free-feeding diabetic rats.

at 14,000*g*, and the protein concentration of the soluble fraction was determined. Equal quantities (20 μg) of lysates were resolved by 15% sodium dodecyl sulfate-polyacrylamide gel and electroblotted to a nitrocellulose membrane (Trans-Blot; Bio-Rad, Hercules, CA). After blocking with phosphate-buffered saline containing 0.1% polyoxyethylene sorbitan monolaurate (Tween-20; Sigma) and 5% dry milk at 4°C overnight, the membrane was incubated with polyclonal rabbit anti-caspase-3 antibody (1:100; PharMingen, San Diego, CA), which recognizes both procaspase-3 (inactive form) and cleaved caspase-3 (active form)[23] for 2 hours. Antigen-antibody complexes were detected with horseradish peroxidase-conjugated antirabbit IgG (1:100; Amersham, Piscataway, NJ). Detection of chemoluminescence was performed with an ECL Western blotting detection reagents (Amersham), according to the supplier's recommendation.

Experiment 3: Effect of Ischemia-Reperfusion on Intestinal Apoptosis of STZ-Induced Diabetic Rats

Rats were injected with STZ or vehicle 1 week before ischemia-reperfusion. In the diabetic and control groups, rats were allowed free access to standard chow. Under halothane anesthesia, a laparotomy was performed. The superior mesenteric artery was occluded for 60 minutes with a micro-bulldog clamp. At the end of the ischemic period, the clamp was released, and 3 drops of lidocaine were applied directly on the superior mesenteric artery to facilitate reperfusion. After 60 minutes of reperfusion, the animals were anesthetized and then euthanized for evaluation of percent fragmented DNA in the small intestine as described in Experiment 1. Five rats were tested in each group.

Statistics

Results are expressed as means ± SE. Data were evaluated by analysis of variance in which multiple comparisons were performed by the method of least significant difference. Differences were considered significant if the probability of the difference occurring by chance was less than 5 in 100 ($P < .05$).

RESULTS

Experiment 1: Effect of STZ-Induced Diabetes on Food Intake, Body Weight, and Small Intestinal Mucosal Height

The results on food intake in free-feeding diabetic rats and control nondiabetic rats are shown in Fig 1. Food intake gradually increased with each passing day after STZ injection in the free-feeding diabetic group. On the other hand, daily food intake remained in the control group injected with vehicle only (≃25 to 30 g/d). Percentages of food intakes during the dark period were 83.1% ± 2.2% in free-feeding diabetic rats and 86.2% ± 2.6% in control nondiabetic rats. There was no significant difference between the 2 groups.

Table 1 shows body weight and mucosal height in each group. Before STZ or vehicle injection, there were no significant differences in body weight among the 3 groups. Three weeks after STZ or vehicle injection, the body weight in the free-feeding diabetic rats was significantly lower than that in the control group ($P < .01$). The body weight in the diabetic rats fed with 25 g/d was significantly lower than that in the control nondiabetic rats and the free-feeding diabetic rats ($P < .01$ in each). Jejunal mucosal height in the free-feeding diabetic rats was significantly longer than that in the control rats ($P < .01$), but this increase in the intestinal mucosal height in the diabetic rats was not observed in the restricted-feeding diabetic rats. The changes in mucosal height in the ileum in diabetic

Table 2. Effect of STZ-Induced Diabetes on ODC Activity and Percent Fragmented DNA in the Rat Small Intestine at 11 AM

	Control Nondiabetic Rats	1 Week After STZ Injection	3 Weeks After STZ Injection
ODC activity (CO_2 pmol/h/mg protein)			
Jejunum	3.5 ± 1.1	4.1 ± 1.8	3.8 ± 1.3
Ileum	3.5 ± 0.5	3.7 ± 1.5	3.3 ± 1.2
Percent fragmented DNA (fragmented DNA/total DNA)			
Jejunum	7.6 ± 1.6	3.0 ± 0.7*	8.5 ± 2.0
Ileum	10.9 ± 1.2	4.2 ± 0.4*	8.7 ± 0.9

NOTE. Values are means ± SE.

* $P < .05$ compared with the corresponding value of control nondiabetic rats treated with vehicle only.

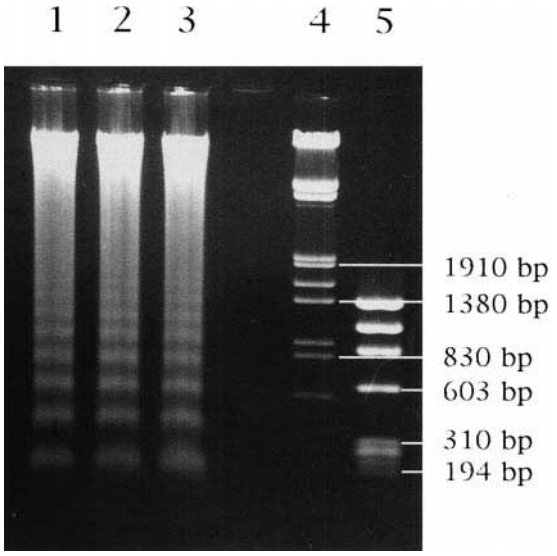

Fig 2. Agarose gel electrophoresis of fragmented DNA from the jejunal mucosa. A total of 20 μg DNA (fragmented DNA) was loaded. Lanes 1 to 3 represent DNA of the control nondiabetic group, the diabetic group 1 week after STZ injection, and the diabetic group 3 weeks after STZ injection, respectively. The ladder, which is characteristic of apoptosis, was clearly shown on each lane. Lanes 4 and 5 contained marker DNA from X174 *Hae*III and Lamda *Eco*RIII digest (Wako, Japan), respectively.

verses nondiabetic rats with or without food restriction were the same as those in the jejunum.

Experiment 2: Effect of STZ-Induced Diabetes on ODC Activity and Apoptosis in the Small Intestinal Mucosa

Table 2 summarizes the results on ODC activity and apoptosis in the rat small intestine at 11 AM. There were no significant differences in ODC activity among the 3 test groups in the jejunum and ileum. ODC activity at 11 PM, when ODC activity is high in free-feeding rats, did not differ among the 3 test groups in the jejunum (controls, n = 5, 62.2 ± 6.8 CO_2 pmol/h/mg protein; 1 week after STZ injection; 65.2 ± 7.3; 3 weeks after STZ injection, 66.6 ± 6.5). Percent fragmented DNA in jejunal and ileal mucosa in rats 1 week after STZ injection was significantly lower than that in the control nondiabetic animals ($P < .05$ in each). The mucosal height in the jejunum and ileum in the diabetic rats 1 week after STZ injection (jejunum, 1,408 ± 29 μm; ileum, 1,264 ± 2 μm) were significantly longer than those in the control group (jejunum, 1,281 ± 32 μm; ileum, 1,095 ± 64 μm; $P < .05$ in each). The decrease in the mucosal apoptosis in the diabetic rats returned to the control level 3 weeks after STZ injection. The suppression of the intestinal mucosal apoptosis in the free-feeding diabetic rats 1 week after STZ injection was not observed in the restricted-feeding diabetic rats (data not shown).

Resolving agarose gel electrophoresis was performed to evaluate the nature of the fragmented DNA in the jejunal and ileal mucosa. As shown in Fig 2, agarose gel electrophoresis of the fragmented DNA obtained from the jejunal mucosa of each group showed distinct DNA laddering characteristic of apoptosis. Because of the same volume of fragmented DNA was loaded in each lane, electrophoresis did not indicate quantitative differences. Similar DNA laddering on agarose gel electrophoresis was obtained from ileal mucosa from each group (data not shown).

Seventeen kilodalton-cleaved caspase-3 (active form of caspase-3) band in the diabetic rats 1 week after STZ injection was less than those in the control group, but this decrease returned to the control level 3 weeks after STZ injection in the jejunum (Fig 3A) and ileum (Fig 3B). Regarding procaspase-3, there was no significance difference among the 3 experimental group. These results indicated that apoptosis signal of the small

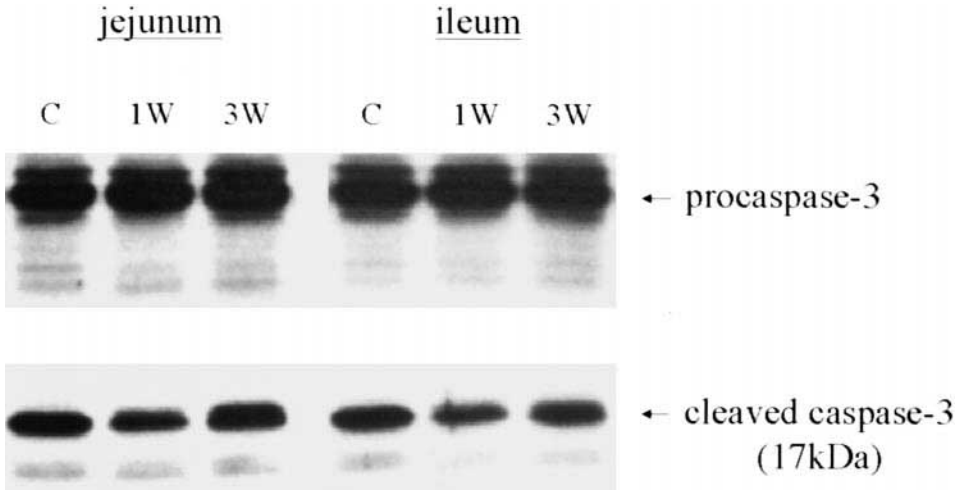

Fig 3. Processing of caspase-3 in the jejunal and ileal mucosa. A total of 20 μg of protein from the rat intestinal mucosa was subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with a polyclonal rabbit anticaspase-3 antibody. Expression of 17-kd subunit of caspase-3, which is an active form of caspase-3, in the diabetic rat 1 week after STZ injection (1W) was suppressed as compared with those in the control group (C) both in the jejunum (A) and ileum (B). This suppression was not observed 3 weeks after STZ injection (3W).

Table 3. Effect of Ischemia-Reperfusion on Percent Fragmented DNA of the Small Intestine in STZ-Induced Diabetic Rats

	Control Nondiabetic Rats	1 Week After STZ Injection
Percent fragmented DNA (fragmented DNA/total DNA)		
Jejunum	35.8 ± 4.6	34.3 ± 3.8
Ileum	33.1 ± 7.4	30.8 ± 6.9

NOTE. Values are means ± SE. Control nondiabetic rats treated with vehicle only. Rats of both groups were fed ad libitum.

intestinal epithelial cell, 17 kd-cleaved caspase-3, was suppressed in the diabetic rats 1 week after STZ injection compared with the control group, which corroborated the results of percent fragmented DNA.

Experiment 3: Effect of Ischemia-Reperfusion on Intestinal Apoptosis of STZ-Induced Diabetic Rats

Ischemia-reperfusion in the rat small intestine was induced by 60 minutes superior mesenteric artery occlusion followed by 60 minutes reperfusion. As shown in Table 3, % fragmented DNA in the rat jejunal and ileal mucosa increased both in the STZ-induced diabetic rats, but there were no significant differences between the 2 tested groups.

DISCUSSION

It is generally accepted that STZ-induced diabetic rats show body weight loss, hyperphagia, and intestinal mucosal hyperplasia.[1-3] However, there still remains controversy over whether hyperphagia is a major factor contributing to the intestinal mucosal hyperplasia observed in STZ diabetic rats. Some investigators reported that hyperphagia caused increase of the intestinal cell growth,[4,5] while others showed that hyperphagia was not a primary factor of increased intestinal growth.[6,7] Our current experiments show loss of body weight and increased intestinal mucosal height in the free-feeding STZ diabetic rats. The increased intestinal mucosal height in the diabetic rats was reversed by restricted feeding (25 g/d). These results indicate that hyperphagia is essential for the increase in intestinal mucosal height in the STZ-induced diabetic rats.

An increase in mucosal hyperplasia correlates with either an increase in cell proliferation or a decrease in cell death. ODC, a rate-limiting enzyme of polyamine synthesis, is a well-characterized proliferating factor in the small intestine. In the present study, total mucosal ODC activity did not increase in the STZ diabetic rats. In comparison, a previous study reported that ODC activity in STZ diabetic rats was high in the lower villus, and this increase in the ODC activity was not observed in the villous tip.[10] The discrepancy between the 2 studies was not clear, but 1 of the reasons is that we did not determine ODC activity in the different cell populations along the intestinal villous. Moreover, intestinal ODC activity has been shown to possess a circadian variation,[24] and we measured ODC activity at 11 AM when ODC activity is lowest, and at 11 PM when ODC activity is high. Under these assay conditions, we found no difference in ODC activity between the diabetic and nondiabetic rats.

Factors modulating intestinal apoptosis have been under investigation in recent years.[13,14,25,26] Several investigators have shown profound effects of dieting factors on intestinal mucosal apoptosis.[16-19] Holt et al[17] reported that calorie restriction increased the number of apoptotic epithelial cells in the jejunum in aging rats without altering the numbers of jejunal epithelial cells. Moreover, this effect of calorie restriction-induced intestinal apoptosis was not observed in young rats. Raab et al[18] showed that high energy substrates and purines in the diet increased apoptosis of the intestinal epithelium. In the present study, apoptosis of intestinal mucosa decreased 1 week after STZ injection, although there was no change in mucosal height or apoptosis 1 day after STZ injection. The apoptotic change returned to control level 3 weeks after STZ injection. This decrease in apoptosis during the early phase of hyperphagia may, at least in part, be responsible for the increased mucosal height in the small intestine. However, the reason why a decrease in apoptosis was not observed at 3 weeks after STZ injection, when the intestinal mucosal height increased in the STZ diabetic rats, is not clear. There are several possibilities. Prolonged hyperphagia for 3 weeks is known to enhance intestinal growth factors, which counteract the apoptotic signals. Moreover, downregulation of apoptotic pathways during long-term hyperphagia may appear to be transient. In this regard, growth effect of hyperphagia appears to be transient, ie, the growth changes are greatest during early phase of hyperphagia, thereafter the intestinal villi may grow at the same rate as that before hyperphagia.

Previous reports showed that ischemia-reperfusion induced by occlusion of superior mesenteric artery enhanced apoptosis in intestinal mucosa.[15,27,28] The effect of STZ-induced diabetes on apoptosis of intestinal mucosa was opposite the effect of ischemia-reperfusion. In the present experiments, we performed the additional experiment to examine whether the STZ-induced diabetes attenuated the increase in intestinal apoptosis after ischemia-reperfusion injury. STZ-induced diabetes had no effect on increased apoptosis after ischemia-reperfusion, which suggested that there might be different mechanisms to regulate intestinal apoptosis between STZ-induced diabetes and ischemia-reperfusion–induced injury.

In conclusion, the present study shows that hyperphagia is important for increased intestinal mucosal growth in the STZ diabetic rats, and that suppression of apoptosis during the early phase of hyperphagia was responsible for this hyperproliferative response of the intestinal mucosa.

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