Polyamines and Intestinal Epithelial Hyperplasia in Streptozotocin-Diabetic Rats

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Abstract. **We measured specific activity of ornithine decarboxylase (ODC) and contents of putrescine and of the polyamines (spermidine and spermine) in isolated villus and crypt enterocytes from the jejunum of adolescent streptozotocin-diabetic and weight-matched control rats and diabetic and control rats treated with difluoromethyl ornithine (DFMO) 10 days after induction of diabetes. Consistent with previous observations by others of elevated ODC activity and contents of putrescine and of the polyamines in the intestinal epithelium undergoing hyperplasia, our studies showed elevated ODC activity and contents of putrescine and spermidine, but not of spermine, in the hyperplastic intestinal epithelium of diabetic rats. As in previous studies, suppression of ODC activity by DFMO prevented not only the jejunal epithelial hyperplasia in the diabetic rats, but also retarded jejunal epithelial growth in the control rats. DFMO administration lowered ODC activity by over 80% in both diabetic and control rat enterocytes and prevented the rise in enterocyte contents of putrescine and spermidine in the diabetic rat. The observation that, in both diabetic and control rats, treatment with DFMO lowered spermidine content in the crypt enterocytes but had no similar consistent effect on contents of putrescine or spermine suggested that spermidine could have been responsible for the intestinal epithelial hyperplasia in the diabetic rats and for the normal growth of the intestinal epithelium in control rats.** [P.S.E.B.M. 1993, Vol 202]

Intracellular polyamines (spermidine and spermine) and their precursor putrescine are necessary for cellular replication and differentiation (1). Activity of the enzyme ornithine decarboxylase, (ODC) which catalyzes the conversion of ornithine to putrescine, and the contents of the polyamines are increased during intestinal mucosal hyperplasia that occurs after refeeding 72-hr starved rats and during lactation and after massive small bowel resection (2–5). Administration of difluoro-α-methylornithine (DFMO), which inhibits ODC activity, retards or prevents the mucosal hyperplasia noted in the above conditions.

Experimentally induced insulin-dependent (Type I) diabetes in rats is known to alter gastrointestinal function and development (6, 7). After a few days of induction of diabetes, the intestinal epithelium undergoes hyperplasia, with villi becoming taller and crypts deeper (8). The activity of the intestinal mucosal disaccharidases (9) and the absorption of glucose are enhanced (10). The relationship of these developmental and hyperplastic changes to intestinal epithelial ODC activity and contents of the polyamines has thus far not been studied in diabetes and is the subject of this investigation.

In the present study, streptozotocin-induced diabetes in rats caused intestinal epithelial hyperplasia, which was associated with increased enterocyte (villus and crypt) activity of ODC, as well as increased concentrations of putrescine and spermidine but not of spermine. Administration of DFMO significantly reduced enterocyte activity of ODC and the contents of putrescine and spermidine and prevented the epithelial hyperplasia in the diabetic rats and retarded the normal epithelial growth in control rats.

Methods and Materials

Rats. Male, albino, Sprague-Dawley, 140–150 g rats (Herlemn, Indianapolis, IN) were divided into

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weight-matched diabetic and control groups. Regular rat chow (Purina, Indianapolis, IN) and water were allowed *ad libitum*. Rats were housed individually in raised wire-mesh-bottom cages in a room at 25 ± 1°C with a 12:12-hr light:dark cycle. Amounts of food and water consumed by the rats and their body weight were measured daily.

Induction of Diabetes Mellitus. Diabetes was induced by intraperitoneal injection of streptozotocin (Sigma Chemical Co., St. Louis, MO), 100 mg/kg body wt, dissolved in citrate buffer (pH 4.3), as described before (9). Control rats were injected with a similar volume of vehicle. Diabetes was confirmed from hyperglycemia (blood glucose >250 mg/dl), glucosuria (4+), polyphagia, and polydipsia. Blood glucose was determined at the end of the study using a Glucometer II (Ames, Miles, NJ). Urine glucose was determined every other day using Strip Test (Ames). Diabetic rats lost weight after induction of diabetes, whereas control rats gained weight (Table I).

Administration of DFMO. Diabetic and control rats were divided into two groups each. One group of diabetic and control rats received DFMO (a gift from Merrell Dow Pharmaceuticals, Cincinnati, OH) as a 2 g/100 ml solution in drinking water. Because of the large volume of water consumed by the diabetic rats, the concentration of DFMO was reduced to around 0.7 g/100 ml in the drinking water so that these diabetic rats consumed a daily amount of DFMO similar to the control rats. DFMO was administered 24 hr after induction of diabetes and continued for the duration of the study.

Rats were studied on the 10th day after induction of diabetes. At the time of study, nonfasted rats were anesthetized with an intraperitoneal injection of pentobarbital (20 mg/100 g body wt. After a midline abdominal incision, 60 cm of the proximal small bowel (jejunum) distal to the ligament of Treitz was washed with ice-cold 0.9% NaCl solution containing 50 mg/dl of dithiothreitol (Sigma) and 20 mg/dl of Na azide (Sigma).

Isolation of Villus and Crypt Enterocytes. Intestinal villus and crypt enterocytes were isolated according to the method described by Weiser (11), modified as indicated below. Incubation in buffer A was for 20 min and was discarded. To obtain the enterocytes from the upper half of the villi, the first incubation period in buffer B was prolonged to 15 min. The next incubation period in buffer B was for 20 min, during which the enterocytes from the lower half of the villi were harvested. Crypt enterocytes were harvested in the next 30-min incubation period. The incubates were centrifuged at 4°C at 1800 rpm for 5 min and the supernatant was discarded. The enterocyte pellet was weighed and then frozen at −72°C until time of analysis. Isolated enterocytes were over 90% viable as determined by tryphan blue exclusion. At the end of the incubation periods, binocular evaluation of representative jejunal samples at 50 times magnification revealed no villi, but very few or no crypts present in the wall of the incubated intestinal segments.

Distinction of Villus from Crypt Enterocytes. Crypt enterocytes were distinguished from villus enterocytes from the high thymidine kinase and low or negligible sucrase activity in the crypt enterocytes. In homogenates of the isolated enterocytes, thymidine kinase activity was determined by the method described by Hansjorg and Wolfgang (12) and sucrase activity as described by Dahlqvist (13). Activity was expressed on the basis of protein content of the homogenates. Protein was assayed by the Lowry method (14).

Assessment of Jejunal Epithelial Hyperplasia. In five rats in each group, 1- to 2-cm long segment of the distal jejunum was fixed in buffered 10% formalin and processed for routine histologic evaluation after hematoxylin and eosin staining. In well-oriented, representative, 100× magnified fields, villus height and crypt depth were measured using a lens with a micrometer. In representative groups of diabetic and control rats after rinsing with cold 0.9% NaCl solution, a 5-cm segment of the distal jejunum was opened along the mesenteric line, and the mucosa was scraped with the edge of a microscope slide and weighed to the nearest mg and the wt:cm ratio was calculated.

Determination of ODC Activity. ODC specific activity was determined in the pellet of the isolated enterocytes by a method similar to that described by Yang *et al.* (4). The pellet was homogenized in 100 m*M* phosphate buffer (pH 7.4), then sonicated for 60 sec and centrifuged at 1×10^5 *g*. The supernatant was suspended in 500 μl of 0.1 *M* phosphate buffer (pH 7.2) containing 20 mg/dl of 23-laurylether, 5 m*M* NaF, 10 μ*M* EDTA, 2 m*M* dithiothreitol, and 100 μ*M* pyridoxal phosphate. The above methods and reactions were performed at 4°C. ODC activity was measured by release of CO^2 from L-[1-^{14}C]ornithine (58 mCi/mmol; New England Nuclear, Boston, MA) and expressed as dpms of $^{14}CO_2$ released/hr/mg protein content. Supernatant (300 μl) was incubated with 15 μl of ^{14}C-labeled ornithine, 150 μl of 100 m*M* phosphate buffer (pH 7.30), 25 μl of 5 m*M* pyridoxal phosphate, and 10 μl of 10 m*M* L-ornithine. The incubation took place in glass scintillation vials at 37°C for 120 min in a shaker water bath at room temperature. The reaction was stopped by the addition of 300 μl 5 *N* sulfuric acid. The $^{14}CO_2$ liberated by the decarboxylation of ornithine was trapped in 200 μl of 1 *N* hyamine hydroxide (Sigma) placed in a center well. Blanks were run simultaneously by using the vehicle instead of the supernatant. The hyamine hydroxide with the trapped $^{14}CO_2$ was placed in the scintillation vial and 10 ml of Packard Permaflour V scintillation fluid was added. Radioactivity was

counted in a Packard liquid scintillation spectrometer (Packard 1600CA).

Determination of Putrescine and the Polyamines. Polyamine content was determined in enterocyte pellets treated with 2 ml/dl of perchloric acid by a method similar to that described by Luk and Yang (3). The acidified homogenate was sonicated for 40 sec and centrifuged at 1400*g* for 30 min at 4°C. The supernatant was added to 1 ml of 400 mg/ml Na_2CO_3. Samples were then dansylated; a solution (300 μl) containing 40 mg of densyl chloride (Sigma) dissolved in 1 ml of acetone was added to 100 μl of the supernatant and incubated in the dark at room temperature for 18 hr. A total of 100 μl of L-proline (150 mg/ml) were added and putrescine and the polyamines were separated using high-performance liquid chromatography (Spectra Physics, Picataway, NJ) on a cation exchange column (P/N 30832; Dionex, Sunnyvale, CA). Polyamines were eluted from the columns under increasing acetonitrile concentrations and quantitated using a UV detector (250 n*M*). Reference standards of pure putrescine, spermidine, and spermine were chromatographed between every 10th and 20th sample.

Statistics. Mean and SE were calculated for all parameters determined. Data from at least five separate rats were used to calculate mean values for a given parameter. Significance of differences between mean values was determined using analysis of variance. Values of $P < 0.05$ were considered as indicating statistical significance between the corresponding mean values.

Results

Data pertaining to the rats studied are shown in Table I.

Body Weight. Diabetic and DFMO-treated diabetic rats lost weight, whereas control and DFMO-treated control rats gained weight during the 10 days of the experiment. Treatment with DFMO did not alter change in body weight significantly in either diabetic or control rats. Blood glucose was around 400 mg/dl in diabetic rats and <250 mg/dl in control rats and not significantly altered by treatment with DFMO in either control or diabetic rats.

Jejunal Weight. Weight of the 60-cm jejunum and wt:cm length ratio of the jejunal mucosa were greater in diabetic than control rats ($P < 0.01$). Jejunal weight and mucosal wt:cm ratio were significantly lower in the DFMO-treated diabetic and control rats than in the corresponding nontreated rats ($P < 0.05$).

Food and Water Consumption. Daily food consumption was 1.5-fold and daily water consumption was over 5-fold greater in diabetic than in control rats. Administration of DFMO did not significantly affect food or water consumption in either diabetic or control rats.

Table II compares height of villi and depth of crypts and specific activity of sucrase and thymidine kinase in the rats studied. Villus height and crypt depth were significantly greater in diabetic than control rats ($P < 0.01$). DFMO significantly ($P < 0.01$) reduced villus height in diabetic and control rats, but reduced crypt depth only in diabetic rats ($P < 0.01$).

Sucrase Activity. Specific activity of sucrase was over 5-fold and significantly greater ($P < 0.001$) in villus enterocytes of diabetic than control rats. Sucrase activity was not different in upper and lower villus cells. DFMO had no significant effect on enterocyte sucrase specific activity either in diabetic or control rats. Crypt enterocytes in diabetic rats showed slightly higher sucrase activity than expected.

Thymidine Kinase Activity. Specific activity of thymidine kinase was greater in crypt than in villus enterocytes of both diabetic and control rats ($P < 0.01$). The slightly lower thymidine kinase activity in the crypts of the DFMO-treated than in the untreated diabetic or control rats did not achieve statistical significance ($P < 0.1$).

Ornithine Decarboxylase Activity. Specific activity of ODC (Table III) appeared to be greater in both villus and crypt enterocytes of diabetic than control

Table I. Body Weight, Jejunal Weight, and Jejunal Mucosal Weight:CM Ratio and Daily Consumption of Food and Water[a]

	Rats			
	D	D + DFMO	C	C + DFMO
Body wt change (g/day)	−0.8 ± 0.2	−1.4 ± 0.4	7.9 ± 0.5[b]	7.2 ± 0.2
Jejunal wt (g)	5.6 ± 0.2	4.2 ± 0.3[c]	4.9 ± 0.1[b]	4.2 ± 0.2[c]
Jejunal mucosal wt (mg/cm)	73 ± 3	50 ± 4[c]	59 ± 4[b]	47 ± 3[c]
Food consumed (g/day)	38 ± 3	34 ± 2	24 ± 3[b]	26 ± 2
Water intake (ml/day)	110 ± 3	98 ± 3	40 ± 2[b]	43 ± 2
Blood glucose (mg/dl)	400 ± 9	397 ± 6	168 ± 7	169 ± 9

[a] Data are for daily change in body weight, jejunal weight, and jejunal mucosal wt:cm ratio and daily consumption of food and water in the diabetic (D), control (C), and DFMO-treated diabetic (D + DFMO) and control (C + DFMO) rats studied. Values are mean ± SE.
[b] Mean values in control rats significantly different than corresponding mean values in diabetic rats ($P < 0.01$).
[c] Mean values in DFMO-treated rats significantly different than corresponding mean values in the nontreated rats ($P < 0.05$).

Table II. Jejunal Villus Height and Crypt Depth and Specific Activity of Sucrase and Thymidine Kinase[a]

	Rats			
	D	D + DFMO	C	C + DFMO
Jejunal				
Villus height (μM)	1221 ± 75	530 ± 102[b]	815 ± 70[c]	350 ± 60[b]
Crypt depth (μM)	242 ± 350	146 ± 26[d]	150 ± 22[c]	123 ± 20[d]
Sucrase activity[e]				
Upper villus	83.1 ± 7.3	69.0 ± 5.4	13.7 ± 1.1[c]	13.7 ± 0.6
Lower villus	62.6 ± 6.3	52.8 ± 11.1	10.2 ± 0.7[c]	13.6 ± 1.6
Crypt	21.1 ± 0.8[d]	15.3 ± 0.8[d]	0.5 ± 0.1[c,d]	0.2 ± 0.1[d]
Thymidine kinase activity[f]				
Upper villus	2.1 ± 0.7	1.4 ± 0.3	1.4 ± 1.1	1.5 ± 0.8
Middle villus	0.9 ± 0.1	1.5 ± 0.3	2.3 ± 1.6	3.3 ± 1.6
Crypt	15.6 ± 1.2[d]	13.8 ± 1.6[d]	16.6 ± 1.3[d]	13.2 ± 1.8[d]

[a] Data are for jejunal villus height and crypt depth and specific activity of sucrase and thymidine kinase in the isolated enterocytes from the upper villus, lower villus, and crypts of the jejunum in diabetic (D), control (C), and DFMO-treated diabetic (D + DFMO) and control (C + DFMO) rats studied. Values are mean ± SE.
[b] Mean values in DFMO-treated diabetic or control rats significantly different than corresponding mean value in nontreated rats ($P < 0.05$).
[c] Mean values in control rats significantly different than corresponding mean values in diabetic rats ($P < 0.01$).
[d] Mean values in crypt enterocytes significantly different than corresponding mean values in villus enterocytes ($P < 0.01$).
[e] Sucrase specific activity expressed as μmol of glucose released/min/mg protein from sucrase by the enterocyte homogenates.
[f] Thymidine kinase specific activity expressed as [^{3}H]methyl-released ([^{3}H]dpm/mg protein/hr) from methyl-[^{3}H]thymidine (2 Ci/mM; Amersham, Chicago, IL) by the enterocyte homogenates.

Table III. Specific Activity of Ornithine Decarboxylase in Jejunal Enterocytes[a]

	Rats			
	D	D + DFMO	C	C + DFMO
ODC specific activity[b]				
Upper villus	1532 ± 275	311 ± (183)[c]	1250 ± 312	259 ± 46[c]
Lower villus	3852 ± 456[d]	63 ± 25[c]	1545 ± 208[e]	223 ± 43[c]
Crypt	1629 ± 230	187 ± 106[c]	1048 ± 140	129 ± 52[c]

[a] Data indicate specific activity of ornithine decarboxylase in jejunal enterocytes isolated from the upper villus, lower villus, and crypts of diabetic (D), control (C), and DFMO-treated diabetic (D + DFMO) and control (C + DFMO) rats. Values are means ± SE.
[b] $^{14}CO_2$ released (^{14}C dpm/mg protein/hr) from ^{14}C-labeled L-ornithine by enterocyte homogenates.
[c] Mean values in DFMO-treated diabetic and control rats significantly different than corresponding mean values in the nontreated diabetic and control rats ($P < 0.001$).
[d] Mean value of lower villus enterocytes significantly greater than corresponding mean values of the upper villus or crypt enterocytes in diabetic rats ($P < 0.05$).
[e] Mean value in lower villus enterocytes of control rats significantly lower than corresponding mean value in diabetic rats ($P < 0.05$).

rats; however, only in the lower villus enterocyte did ODC activity in diabetic rats significantly exceed that in control rats ($P < 0.05$). In diabetic rats, ODC activity of lower villus enterocytes was greater than that of upper villus or crypt enterocytes. This difference between villus and crypt enterocytes was not observed in the control rats. Administration of DFMO significantly reduced ODC activity in villus and crypt enterocytes of both diabetic and control rats by over 80% ($P < 0.001$).

Contents of putrescine, spermidine, and spermine in the isolated jejunal enterocytes of the diabetic and control rats are shown in Table IV.

The concentration of putrescine in both villus and crypt enterocytes was significantly greater in diabetic than in control rats ($P < 0.01$). Administration of DFMO significantly reduced putrescine concentration in crypt enterocytes of diabetic rats but not in crypt enterocytes of control rats.

The concentration of spermidine in upper villus enterocytes was consistently less than that in lower villus and crypt enterocytes in all four rat groups studied. In lower villus and crypt enterocytes, spermidine concentration was significantly higher in diabetic than in control rats ($P < 0.05$). DFMO significantly reduced ($P < 0.05$) the concentration of spermidine in crypt but not in villus enterocytes of both diabetic and control rats.

The concentration of spermine in upper villus enterocytes was less than that in the lower villus or crypt enterocytes in the diabetic rats ($P < 0.05$), but not in the control rats. In villus enterocytes, spermine content was not significantly different in diabetic and control rats. Administration of DFMO significantly increased concentration of spermine in crypt ($P < 0.05$) but not villus enterocytes of diabetic rats, but did not signifi-

Table IV. Concentration of Putrescine, Spermidine, and Spermine in Isolated Villus and Crypt Enterocytes of the Jejunum[a]

	Rats			
	D	D + DFMO	C	C + DFMO
Putrescine				
Upper villus	10.1 ± 3.0	7.1 ± 1.0	1.9 ± 0.4[b]	1.7 ± 0.5
Lower villus	11.7 ± 3.1	6.5 ± 2.0	3.0 ± 0.5[b]	4.2 ± 1.0
Crypt	16.4 ± 3.5	6.6 ± 1.9[c]	2.2 ± 0.3[b]	1.6 ± 0.2
Spermidine				
Upper villus	26.8 ± 3.6[d]	23.5 ± 4.6[d]	20.4 ± 4.0[d]	12.5 ± 2.2[d]
Lower villus	177.9 ± 32.8	91.2 ± 29.6	46.0 ± 8.4[b]	25.5 ± 7.0
Crypt	214.1 ± 37.3	103.8 ± 23.7[c]	58.9 ± 7.9[b]	21.1 ± 5.3[c]
Spermine				
Upper villus	22.4 ± 8.9[d]	33.4 ± 4.0[d]	47.7 ± 3.9	39.2 ± 6.0
Lower villus	66.8 ± 18.0	173.2 ± 54.0	60.2 ± 12.6	35.7 ± 8.4
Crypt	60.6 ± 20.7	211.7 ± 32.5[c]	67.0 ± 15.4	26.6 ± 6.3[c]

[a] Data show concentration (nmol/g protein) of putrescine, spermidine, and spermine in isolated villus and crypt enterocytes of the jejunum in diabetic (D), control (C), and DFMO-treated diabetic (D + DFMO) and control (C + DFMO) rats. Values are mean ± SE.
[b] Mean values in control rats significantly lower than corresponding mean values in diabetic rats ($P < 0.05$).
[c] Mean values in DFMO-treated diabetic or control rats significantly different than corresponding mean values in nontreated diabetic or control rats ($P < 0.05$).
[d] Mean values in upper villus enterocytes significantly different than the corresponding mean values of lower villus or crypt enterocytes ($P < 0.05$).

cantly alter spermine concentration in enterocytes of the control rats.

Discussion

The observations of significantly greater jejunal villus height, crypt depth, jejunal weight, and jejunal mucosal wt:cm ratio in diabetic rats than in control rats are consistent with results of previous studies indicating small intestinal epithelial hyperplasia (8, 9) secondary to increased enterocyte production rate in experimentally induced diabetes in rats (8). Similar to results of previous experiments, specific activity of sucrase was also severalfold greater in jejunal epithelium of diabetic than control rats (6, 7, 9).

Although administration of DFMO prevented jejunal epithelial hyperplasia in diabetic rats and suppressed epithelial growth in control rats, it did not seem to have an adverse effect on body growth of either the diabetic or control rats. There was also no apparent effect of DFMO on severity of diabetes, since change in body weight, blood glucose concentration, and food and water consumption were not significantly different in diabetic and DFMO-treated diabetic rats. Villus enterocyte sucrase activity was also unaffected by DFMO, which suggests that putrescine and the polyamines may not be involved in the biochemical mechanisms that control the enhancement of sucrase activity in the diabetic intestinal epithelium. Whether polyamines alter other functional aspects of the intestine that are enhanced with diabetes, such as rate of glucose absorption, remains unknown.

The elevated ODC activity and contents of putrescine and spermidine in the enterocytes of the hyperplastic intestinal epithelium of diabetic rats were similar to those noted in other conditions associated with intestinal mucosal hyperplasia, such as growth after refeeding 72-hr fasted rats, during adaptation to lactation, and after massive small bowel resection (2–5). The inhibition of jejunal epithelial hyperplasia in diabetic rats and the normal epithelial growth in control rats by DFMO indicated that putrescine and/or polyamines were needed for the processes that induce enterocyte proliferation, not only under stimulated situations when epithelial growth is enhanced, but also under normal conditions.

The question as to whether it was putrescine, spermidine, or spermine or a combination of two or more of these that were responsible for the jejunal epithelial hyperplasia in diabetic rats and normal growth in control rats remains unknown. The fact that DFMO lowered the content of spermidine significantly in the crypt enterocytes but not in the villus enterocytes of both control and diabetic rats, and had no consistent effect on content of putrescine or spermine in both villus and crypt enterocytes, suggests that the elevated content of spermidine in the crypt enterocytes where enterocyte proliferation occurs may have been the major factor responsible for the proliferation of the enterocyte observed in both diabetic and control rats.

The significantly elevated levels of spermine in crypt enterocytes of the DFMO-treated diabetic rats were unexpected and need further investigation. One possibility for the elevated spermine levels in the diabetic rats is that DFMO suppressed activity of the enzyme, *N*-spermine-acetylase, that catalyzes the conversion of spermine to spermidine, or that spermine

concentrations could have been elevated by some other mechanisms that are unknown at this time.

In conclusion, the intestinal epithelial hyperplasia in streptozotocin-diabetic rats, as in other conditions that are associated with intestinal epithelial hyperplasia, seemed to be dependent upon the enhanced activity of ODC and increased contents of putrescine and spermidine in the isolated villus and crypt enterocytes. DFMO suppressed activity of ODC and contents of putrescine and spermidine and the epithelial hyperplasia in diabetic rats, and also appeared to retard the normal epithelial growth in control rats. The fact that DFMO had no effect on villus enterocyte sucrase activity suggests that ODC activity and the polyamines do not regulate sucrase activity. Because the crypt enterocyte concentration of spermidine, but not putrescine or spermine, was consistently suppressed by DFMO in diabetic and control rats, it appeared possible that spermidine, rather than putrescine or spermine, may have been responsible for the enterocyte proliferation in both the diabetic and control rats.

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