Polyamine metabolism of rat gastric mucosa after oral administration of hypertonic sodium chloride solution

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THE GASTROINTESTINAL MUCOSA has one of the most rapid turnover rates of any tissue in the body and correspondingly high rates of cell growth and differentiation. It has been reported that polyamines are involved in growth and differentiation of gastrointestinal and other types of cells (13, 16, 19). In the stomach luminal polyamines stimulate repair of gastric mucosal injury (36), and putrescine injection stimulates exocytic mucosal growth (20). Oral administration of hypertonic NaCl solution causes gastric mucosal damage, followed by induction of ornithine decarboxylase (ODC), the rate-limiting enzyme of polyamine biosynthesis, mucosal restitution, and cell growth (1, 11, 35). The processes of mucosal restitution and cell growth require polyamines.

Monaocetyl spermidine is known to be present in Escherichia coli. Tabor (31) showed that monoacetyl spermidine accumulated in bacterial cells when they were harvested at 4°C or when large amounts of endogenous polyamines were formed (32). Heat shock and certain types of chemical stress have also been shown to induce the accumulation of monoacetyl spermidine (6). However, the physiological function of the acetylation of polyamine is not well understood. Recently, evidence has been obtained that spermidine/spermine N1-acetyltransferase (SSAT) and ODC play important roles in the regulation of intracellular polyamine levels. N1-acetyl polyamines produced by SSAT are quite readily excreted from cells, resulting in a decrease in spermidine and spermine levels (8). In addition, N1-acetyl derivatives of spermine and spermidine are good substrates of polyamine oxidase and are converted to spermine and putrescine, respectively (3, 22). SSAT thus participates in the production of putrescine from preexistent spermidine or spermine. In the liver, conversion of spermidine to putrescine occurs after treatment with carbon tetrachloride (14) and after treatment with growth hormone and thyroxine or partial hepatectomy (21). SSAT plays a more important role than ODC in increasing the putrescine level of duodenal mucosa in chicks with vitamin D deficiency induced by administration of 1α,25-dihydroxyvitamin D3, and this increase in putrescine level may affect calcium absorption (30). However, the physiological role of SSAT in the stomach is still unclear. In this study, we attempted to determine whether administration of hypertonic NaCl solution induces SSAT activity and examined the relationship between SSAT activity and DNA synthesis in rat gastric mucosa. We found that oral administration of hypertonic NaCl caused a biphasic elevation of SSAT activity and a peak in ODC activity, which was located between the first and second peaks of SSAT activity. We also found that elevation of SSAT activity rather than ODC activity is required for early DNA synthesis after gastric mucosal damage.

MATERIALS AND METHODS

Materials. L-1-14C]ornithine (2.07 GBq/mmol), [acetyl-1-14C]acetate CoA (2.00 GBq/mmol), and [methyl-3H]thymidine (2.22–3.33 TBq/mmol) were obtained from Moravek Biochemicals (Brea, CA), and [α-32P]UTP (29.6 TBq/mmol) was from DuPont-NEN (Boston MA). Putrescine, pepstatin A, bovine serum albumin, and calf thymus DNA were obtained from Sigma Chemical (St. Louis, MO). N,N′-bis(2,3-butadienyl)-1,4-butanediamine (MDL-72527) and α-difluoromethylornithine (DFMO) were kindly supplied by Marion Merrell Dow Research Institute (Cincinnati, OH). The other reagents used were products of special grade from Wako Chemicals (Osaka, Japan).

Animals and experimental procedure. Male Wistar rats weighing 200–220 g were purchased from CLEA Japan (Tokyo, Japan) and were fed standard chow and tap water ad libitum. The study protocol was approved by the Animal Ethics Committee of Osaka City University.
Research Committee of Osaka City University, and care of animals was in accordance with the standards of this Institution (Guide for Animal Experimentation, Osaka City University). The animals were fasted for 24 h before the experiments but had free access to drinking water. They were given 1.0 ml of NaCl solution by gastric tube and killed by exsanguination via the abdominal aorta under ether anesthesia at various times after NaCl administration. The stomach was excised open along the great curvature and washed thoroughly in ice-cold phosphate-buffered saline (PBS). The antrum was removed, and the oxyptic gland mucosa of the stomach was scraped away from the underlying smooth muscle with a glass slide. The collected tissues were divided into three portions: the first was used for examination of polyamine levels, the second for assays of SSAT and ODC activities, and the third for analysis of SSAT mRNA.

MDL-72527 (1 mg/100 g body wt) dissolved in 0.2 ml of 0.15 M saline and DFMO (50 mg/100 g body wt) dissolved in 0.4 ml of 0.15 M saline were administered intraperitoneally 1 h and 15 min respectively, before intragastric 3.42 M NaCl administration. Putrescine (10 µmol/100 g body wt) dissolved in 0.5 ml of distilled water was administered intragastrically 3 h after NaCl administration. Control rats were given 0.15 M saline and DFMO (50 mg/100 g body wt) dissolved in 0.4 ml of 0.15 M saline were administered intraperitoneally 3.42 M NaCl intragastrically, respectively, with intraperitoneal administration of the vehicle. Animals were killed 5 h after NaCl administration, and DNA synthesis and intracellular putrescine level were measured. When the effects of MDL-72527 and DFMO on putrescine level and DNA synthesis 16.5 h after NaCl administration were examined, the inhibitors were supplemented with the same doses 7.5 h after NaCl administration.

Assay of ODC activity. ODC activity was assayed by the release of 3H from [1-14C]ornithine as previously described (25). In brief, tissues were washed twice with ice-cold PBS and suspended in 400 µl of 500 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.5) containing 250 µM pyridoxal phosphate, 0.1 mM EDTA, 2.5 mM dithiothreitol, 1.5 mM pepstatin A, and 292 mM phenylmethylsulfonyl fluoride. The tissues were disrupted by three cycles of freezing and thawing and centrifuged at 30,000 g for 20 min at 4°C. Then 90 µl of the supernatant were added to a glass tube containing 9.25 GBq of [acetyl-1-14C]acetyl CoA (50 µl), 0.25 GBq of [1-14C]ornithine (5 µl), and 1.48 GBq of [acetyl-1-14C]acetyl CoA in a final volume of 50 µl at 37°C for 10 min. The reaction was terminated by chilling and the addition of 20 µl of 1 M NH4OH, and the reaction mixture was placed in boiling water for 3 min. Aliquots of 50 µl of the reaction mixture were then spotted onto a Whatman P81 paper disk (2.4 cm in diam). The paper disk was washed with tap water and then five times with 1 ml of ethanol on a filter, dried, and transferred to a vial containing 5 ml of toluene scintillation fluid, and radioactivity was measured with a Beckman liquid scintillation counter.

Isolation of RNA. Total RNA was extracted from scraped gastric mucosal specimens by the acid guanidinium thiocyanate-phenol-chloroform method (9). RNase protection assay. Portions of SSAT cdNA and 18S rDNA were obtained by reverse transcripase-polymerase chain reaction (PCR) amplification using total RNA extracted from rat liver as a template. Reverse transcription was primed using random hexanucleotides as described previously (34). The primers 5′-CTGAACGTCTGCTGCCTATCA-3′ and 5′-CTTGGATGTTGCCTATTTG-3′ were used for amplifying the 83-bp 18S rDNA. The primers 5′-GAAGATGTTTTTGAGAGCA-3′ and 5′-TCTGTACCTATGCGAAAGCC-3′ were used for amplifying the 198-bp SSAT cdNA. The thermal cycle profile was as follows: denaturation for 1 min at 94°C, annealing of primers for 1 min at 55°C, and extension of primers for 1 min at 72°C. The number of PCR cycles was 25. These PCR products were cloned into the Srf I site of pCRII-Script SK(+) plasmid vector (Stratagene, La Jolla, CA), sequenced, and used as templates for production of antisense RNA probe with the ribonuclease (RNase) protection assay. The antisense RNA probe for SSAT mRNA and 18S rRNA were synthesized according to the manufacturer’s instructions (MAXI script T7 in vitro transcription kit and MEGA script T7 in vitro transcription kit; Ambion, Austin, TX). The specific activities of [α-32P]UTP used to label the RNA probe for SSAT mRNA and 18S rRNA were 5.92 GBq/mmol and 88.8 kBq/mmol, respectively. RNase protection assay (17) was performed according to the manufacturer’s instructions (RNase protection kit; Boehringer Mannheim, Mannheim, Germany). The RNase digestion products were subjected to denaturing polyacrylamide gel electrophoresis on 4.5% polyacrylamide-7 M urea gel. The amounts of protected RNA probes were measured using a bioimaging analyzer (BAS 2000II; Fujix, Tokyo, Japan). To normalize the amounts of RNA applied to the gel, the amounts of SSAT mRNA relative to those of 18S rRNA were calculated.

Measurement of polyamine contents. The tissues were washed with ice-cold PBS and disrupted by ultrasonication in 750 µl of 0.4 N perchloric acid. After centrifugation at 18,000 g for 20 min, the supernatant was stored at −20°C until assay for polyamines. The pellet was used for measurement of DNA content. The concentration of polyamine in the acid extract was determined by high-performance liquid chromatography (Shimadzu LC-6A; Shimadzu, Kyoto, Japan) equipped with a fluorescence detector. Polyamines were separated on an STR ODS-II column (4.6 × 150 mm, particle size 5 µm, Shimadzu Techno-Research) as described previously (23). Polyamine contents were expressed as picomoles per milligram of DNA.

Assay of DNA content. DNA precipitated with 0.4 N perchloric acid was washed and solubilized by heating at 75°C for 20 min in 0.4 N perchloric acid. After centrifugation at 18,000 g for 20 min, DNA of the supernatant was measured by diphenylamine reaction (5) using calf thymus DNA as a standard.

Assay of DNA synthesis. DNA synthesis was assayed by the incorporation of [3H]thymidine into the acid-insoluble fraction using the method of Furihata et al. (12) with some modifications. Briefly, five specimens weighing about 20 mg each were obtained from the oxyptic gland mucosa with a 3-mm dermatological punch. These specimens were then incubated for 1 h at 37°C in 2 ml of Eagle’s minimum essential

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medium containing [3H]thymidine (370 kBq/ml) with gentle shaking. DNA was precipitated with 0.4 N perchloric acid and centrifuged at 18,000 g for 20 min. The precipitate was washed with 0.4 N perchloric acid, and DNA was solubilized from the precipitate as previously described. The radioactivity of the supernatant was measured with a liquid scintillation counter, and the amount of DNA was measured as previously described. Incorporation of [3H]thymidine into DNA was expressed as counts per hour per microgram of DNA.

Measurement of protein content. Protein content was assayed by the method of Bradford (4) using reagent from Bio-Rad Laboratories (Richmond, CA) and bovine serum albumin as a standard.

Statistical analysis. Results obtained are expressed as means ± SD. The significance of differences between mean values was determined by one- or two-way analysis of variance (ANOVA). Differences between means were evaluated by ANOVA, with specific differences tested using Scheffé’s F-test as a post hoc test. Differences with P < 0.05 were considered significant.

RESULTS

Effects of oral administration of NaCl solution on gastric SSAT and ODC activity. Effects of the administration of various concentrations of NaCl on gastric SSAT are shown in Fig. 1. Compared with physiological saline solution, 3.42 M of NaCl solution increased SSAT activity significantly, whereas concentrations of NaCl higher and lower than 3.42 M had less effect on SSAT activity. The changes in SSAT activity after oral administration of 3.42 M NaCl solution are shown in Fig. 2. SSAT activity increased biphasically, with a first peak at 5 h and a second at 7 h after NaCl administration. ODC activity was also increased by NaCl solution and peaked at 6 h, when the first peak of SSAT activity had already declined (Fig. 3).

Effect of oral administration of NaCl solution on gastric SSAT mRNA. SSAT mRNA level was measured at the times indicated after 3.42 M NaCl administration. It increased about threefold by 3 h after NaCl administration and peaked at 7 h (Fig. 4).

Effects of oral administration of NaCl on gastric polyamine levels. Polyamine levels of gastric mucosa were measured 5, 6, and 7 h after NaCl administration (Fig. 5). Putrescine level was increased and spermidine

Fig. 1. Spermidine/spermine N1-acetyltransferase (SSAT) activities of oxyntic gland mucosa after 5 h exposure to various concentrations of NaCl. One milliliter of various concentrations of NaCl solution was administered intragastrically. Rats were then killed, and the stomach was removed. SSAT activity of gastric mucosa was measured as described in MATERIALS AND METHODS. Each value represents mean ± SD for 3–4 rats. **P < 0.01 compared with 0.15 M NaCl-treated group.

Fig. 2. Changes in SSAT activities of oxyntic gland mucosa after 3.42 M NaCl administration. Rats were administered 3.42 M NaCl solution and were killed at times indicated. SSAT activity of gastric mucosa was assayed as described in MATERIALS AND METHODS. ○, 0.15 M NaCl-treated group; ●, 3.42 M NaCl-treated group. Each value and bar represent mean ± SD for 4–7 rats. **P < 0.01 compared with 0.15 M NaCl-treated group; ††P < 0.01 compared with 0.15 M NaCl-treated and immediately killed group.

Fig. 3. Changes in ornithine decarboxylase (ODC) activities of oxyntic gland mucosa after 3.42 M NaCl administration. ○, 0.15 M NaCl-treated group; ●, 3.42 M NaCl-treated group. ODC activity was measured as described in MATERIALS AND METHODS. Each value and bar represent mean ± SD for 4–7 rats. **P < 0.01 compared with 0.15 M NaCl-treated group; ††P < 0.01 compared with 0.15 M NaCl-treated and immediately killed group.
Effects of inhibitors of ODC and polyamine oxidase on putrescine level. Putrescine level was increased 5 h after administration of solutions with higher NaCl concentrations, and this increase was inhibited by MDL-72527 but not by DFMO (Fig. 9). However, the

and spermine levels were decreased by administration of solutions with higher NaCl concentrations.

Effects of oral administration of NaCl solution on gastric DNA synthesis. The changes in incorporation of \[^{3}H\]thymidine into the acid-insoluble fraction after NaCl administration are shown in Fig. 6. Incorporation was increased at 5 h after higher NaCl administration and continued to increase until 23 h. The increase in the incorporation 5 h after administration of solutions with higher NaCl concentrations was inhibited by MDL-72527, a specific inhibitor of polyamine oxidase, and this inhibition was reversed by putrescine administration (Fig. 7). The ODC inhibitor DFMO had no significant effect on DNA synthesis at 5 h. DNA synthesis at 16.5 h was inhibited by both MDL-72527 and DFMO, and the inhibition caused by the combination of these two agents was greater than that caused by either alone (Fig. 8).
increase in putrescine level at 16.5 h was inhibited by both MDL-72527 and DFMO, and the combination of these two reduced the putrescine level more than did either of the inhibitors alone (Fig. 10).

DISCUSSION

It has been reported that oral administration of hypertonic NaCl solution increases ODC activity in the gastric mucosa (11, 35). We found that administration of solutions with higher NaCl concentrations caused biphasic induction of SSAT activity in addition to induction of ODC activity, with the first peak of SSAT activity occurring earlier than that of ODC activity.
Intracellular putrescine level increased and spermidine and spermine levels decreased after administration of solutions with higher NaCl concentrations. Furthermore, a polyamine oxidase inhibitor, but not an ODC inhibitor, inhibited the increase in putrescine level 5 h after NaCl administration, indicating that SSAT and polyamine oxidase participate in putrescine formation from preexistent spermidine and spermine early after NaCl administration. Banan et al. (1) recently reported that DFMO inhibited the increase in gastric mucosal putrescine level 4 h after 3.4 M NaCl treatment. Their results are not consistent with our own, even though the dose and method of DFMO administration they used were the same as in our study. The reason for these differences in findings is unclear at present.

DNA synthesis induced by mucosal injury began 5 h after NaCl administration and continued until 16.5 h with administration of solutions with higher NaCl concentrations. This finding is consistent with those reported previously by Furihata et al. (10), who showed that the proportion of S-phase cells detected with 5-bromo-2′-deoxyuridine staining in oxyntic gland mucosa of rat stomach increased over 6–24 h after 2.6 M NaCl administration. Our results showed that the effects of MDL-72527 and DFMO on DNA synthesis 5 h (Fig. 7) and 16.5 h (Fig. 8) after NaCl administration were very similar to those of the inhibitors on increase in putrescine level at the same times (Figs. 9 and 10). These findings suggest that putrescine plays a very important role in DNA synthesis and that SSAT rather than ODC is involved in putrescine formation during the initial phase after NaCl administration but that both enzymes are involved in this formation in the later phase.

SSAT mRNA level was elevated 3 h after NaCl administration and peaked at 7 h. Treatment with α-amanitin, an inhibitor of RNA polymerase II, inhibited the increase in SSAT activity and SSAT mRNA levels at 3, 5, and 7 h after NaCl administration (data not shown), suggesting that NaCl administration stimulates transcription of the SSAT gene. However, the increase in SSAT enzymatic activity at the second peak was less than that in SSAT mRNA level, suggesting that in addition to transcriptional regulation, posttranscriptional regulation is involved in the control of SSAT enzymatic activity, as reported previously (26).

In addition to ODC activity, SSAT activity has been found to be elevated in tumors (24, 33) and proliferating cells stimulated by various types of growth factors (21). These findings suggest that both SSAT and ODC are involved in cell growth. However, Pegg and Erwin (28) found that SSAT activity was increased after ODC induction and that intracellular accumulation of spermidine and spermine induced SSAT activity, suggesting that SSAT plays a role in the control of intracellular spermidine and spermine levels by conversion of these polyamines to N-acetylated derivatives, which were excreted extracellularly, resulting in a decrease in spermidine and spermine levels (8). Basu et al. (2) and Casero et al. (7) reported that polyamine analogs that induced SSAT activity decreased intracellular polyamine levels and inhibited cell growth. Ignatenko and Gerner (15) also showed that SSAT mRNA level increased as human colon tumor-derived HCT116 cells traversed the log phase and entered the plateau phase. The overexpression of SSAT activity in E. coli transfected with a plasmid containing SSAT gene reduced cell growth (27). These findings suggest that SSAT causes growth arrest by decreasing spermidine and spermine levels. On the other hand, it has been reported that SSAT activity is increased before ODC induction. Höltta et al. (14) reported that putrescine level increased in association with a decrease in spermidine level and that this was followed by ODC induction; this finding is consistent with that of Matsui et al. (22) that treatment of rats with carbon tetrachloride increased hepatic SSAT activity. These findings also suggest that SSAT has a physiological role other than preventing overaccumulation of polyamines. However, the role of the SSAT activity induced earlier than ODC activity has not been elucidated. Seidel and Snyder (29) reported that the trophic response of gastrointestinal mucosa to treatment with pentagastrin was associated with SSAT induction but not with ODC induction, suggesting that the polyamine interconversion pathway plays a role in DNA synthesis. Löser and Fölsch (18) reported that simultaneous administration of DFMO and MDL-72527 resulted in significant inhibition of camostate-induced increases in rat pancreatic putrescine and DNA. However, because they did not administer camostate plus MDL-72527 alone, it was not proved that putrescine formed by SSAT and polyamine oxidase was responsible for DNA synthesis. Our findings suggest that SSAT plays an important role in putrescine formation early after NaCl treatment and triggers DNA synthesis and that both SSAT and ODC are required...
for the continuation of DNA synthesis in repair after gastric mucosal damage.

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