MORPHOLOGIC AND MORPHOMETRIC ANALYSIS OF CELLULAR AND SUBCELLULAR STRUCTURES OF RAT GASTRIC MUCOSA AFTER ADMINISTRATION OF E3810, A NOVEL PROTON PUMP INHIBITOR

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Abstract: To further understand gastric mucosal alterations caused by E3810, a novel proton pump inhibitor, in parietal cells, morphometry was applied to gastric tissue of rats administered E3810 subcutaneously at doses of 5, 10, and 20 mg/kg/day for 2 or 5 weeks. Plasma gastrin levels in the 2-week treatment groups were significantly elevated at all doses of E3810, whereas in the 5-week treatment groups, a significant increase in the level was found in rats administered 20 mg/kg E3810 only. By light microscopy, the fundic mucosal thickness dose-dependently increased in the 5-week treatment groups. The number of chromogranin-immunopositive enterochromaffin-like (ECL) cells also increased at the dose of 20 mg/kg in the 2-week treatment groups and at all doses in the 5-week treatment groups. By electron microscopy of parietal cells, no distinct vacuolization appeared in parietal cells even in rats administered 20 mg/kg E3810 for 5 weeks. Morphometrically, the volume and surface density of tubulovesicles did not change in both the 2- and 5-week treatment groups, whereas those of microvilli on secretory canaliculi significantly decreased in the 5-week treatment groups. These results suggest that the increases in mucosal thickness and number of ECL cells after treatment with E3810 are due to hypergastrinemia induced by its prolonged pharmacological effects. Moreover, the changes in morphometric parameters of microvilli on secretory canaliculi in parietal cells may result from the inhibitory effect of E3810 on H+/K+-ATPase. However, the morphologic and morphometric changes in parietal cells after E3810 treatment were slight, compared with those reported previously for omeprazole at the same dose levels. (J Toxicol Pathol 9: 121–130, 1996)

Key words: E3810, Proton pump inhibitor, Gastrin, ECL cell, Morphometry

Introduction

E3810, a substituted benzimidazole derivative, is a potent inhibitor of H+/K+-ATPase (proton pump inhibitor, PPI) localized on the membranes of tubulovesicles and microvilli of the intracellular canaliculi in parietal cells1,2. Omeprazole and lansoprazole, of the same class of compounds as E3810, are known to induce morphologic changes in the gastric mucosa of rats by repeated dosing3–9. The most pronounced changes appearing in rat parietal cells after treatment with omeprazole or lansoprazole is vacuolization3–6. In preclinical toxicologic studies in rodents and dogs, however, E3810 did not induce vacuolization in parietal cells in any of the species10–13. In pharmacological study measuring gastric acid secretion in dog stomach14, the duration of action of E3810 was distinctly shorter than that of omeprazole. The shorter pharmacological actions of E3810 suggests that the mechanism of inhibition of
H⁺/K⁺-ATPase by omeprazole is different from that by E3810; the partial reaction that was the most differently affected by PPIs was the conformational change for omeprazole and the luminal K⁺-dependent dephosphorylation for E3810. However, little is known about the precise effects of E3810 on the morphological features and morphometric parameters of gastric mucosa.

Furuhashi et al. (1992) have shown ultrastructural and morphometric changes in parietal cells of rats treated with omeprazole for 2 or 5 weeks; omeprazole induces marked decreases in the morphometric parameters of tubulovesicles and microvilli of intracellular canaliculi and increases in those of lysosomes. Moreover, these changes in subcellular structures of parietal cells were more prominent in the 5-week than in 2-week treatment groups. Therefore, morphological and morphometric approaches may be important to further understand the effects of E3810 on gastric mucosa.

The present study examined the precise alterations in cellular and subcellular structures of rat gastric mucosa after treatment with E3810 using light and electron microscopic morphometric techniques.

Materials and Methods

Animals and experimental design

Animals: Male Wistar rats (6 weeks of age) were purchased from Japan SLC Co. Ltd., Shizuoka, Japan. They were housed in stainless steel cages (five rats per cage) kept in a well-ventilated room (temperature 22–24°C with relative humidity adjusted to 50–60%), and food (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and water were given ad libitum. The lighting conditions consisted of a 12:12 light/dark cycle (light period: 07:00 hr–19:00 hr) and air change was performed at the rate of 12 times per hour. After 2 weeks of quarantine, rats were divided into four groups (Groups I to IV) (20 rats per group).

Administration of E3810: Rats in Groups II to IV were subcutaneously injected once a day with 5 (Group II), 10 (Group III), and 20 (Group IV) mg/kg E3810, which was dissolved in 0.9% physiological saline at concentrations of 0.25, 0.5, and 1.0% (W/V), respectively. Rats in Group I were administered physiological saline (vehicle alone) once a day and used as controls. Rats in each group were subdivided into two groups (10 rats per subgroup) according to the administration period: 2 weeks and 5 weeks. Animals in each subgroup were killed after 2 or 5 weeks of administration.

Determination of plasma gastrin levels

For the determination of plasma gastrin levels, blood sampling was performed 24 hr after the last administration of E3810. Anesthesia was induced by intraperitoneal injection of sodium pentobarbital, and blood was collected from the abdominal aorta using a heparinized plastic syringe. The blood samples were frozen at −80°C until use. Radioimmunoassay of plasma gastrin levels was performed using a gastrin radioimmunoassay kit (Gastrin RIA Kit II, Dinabot, USA).

Morphometry

Light microscopy

After blood sampling, the stomachs of 7 rats from each group were excised, opened along the greater curvature, and rinsed in physiological saline. They were then pinned out on a cork board and fixed with 10% phosphate buffered formalin (pH 7.2–7.4) for about 24 hr. Fixed stomachs were dehydrated with graded alcohols, embedded in paraffin, and sectioned at 2 μm. Sections were stained with hematoxylin and eosin and used for the measurement of fundic mucosal thickness. As the mucosal thickness, the perpendicular distance was measured from the junctional region of the lamina propria with the muscularis mucosa to the tip of the gastric glands in sections showing well oriented glands, using an image analyzer (Nippon Avionics Co. Ltd., Tokyo, Japan). Ten fields in sections of the fundic region along the greater curvature from each rat were chosen randomly and the thickness was measured.

The number of enterochromaffin-like (ECL) cells per visual field was also analyzed. It has been shown that ECL cells comprise 65% of all neuroendocrine cells seen in rat oxyntic mucosa, most of which are immunopositive for chromogranin. In the present study, the chromogranin-immunopositive cells were estimated as ECL cells. For this, sections were immunostained with anti-bovine chromogranin (SP1, INCSTAR Corporation, Minnesota, USA) using ABC method, and then counter-stained with...
hematoxylin. To count the number of ECL cells under a light microscope, a rectangular sampling frame covering the whole lamina propria of the fundic mucosa was used and cells showing immunoreactivity for chromogranin with visible nuclei were counted as ECL cells.

**Electron microscopy**

Three rats from each group anesthetized with pentobarbital and perfused via the aorta first with 50 ml physiological saline and then with 200 ml 2% paraformaldehyde-2% glutaraldehyde buffered with 0.1 M cacodylate-HCl buffer, pH 7.2. The gastric mucosa of the fundic region was quickly excised, cut into small pieces, and placed in the same fixative at 0°C for 24 hr. After washing thoroughly in the same buffer containing 7.5% sucrose, the blocks were postfixed with 2% OsO4 buffered with 0.1 M cacodylate-HCl, pH 7.2, containing 7.5% sucrose for 2 hr. The blocks were then dehydrated using graded alcohols and embedded in epoxy resin. Silver sections were cut with an ultramicrotome (Ultracut N, Reichert-Nissei, Tokyo, Japan), and, after staining with uranyl acetate and lead citrate, were observed using a Hitachi H-600 electron microscope.

Ten fields of parietal cells for each rat were chosen randomly and photographed at original magnifications of ×1,560 and ×3,640, calibrated using a carbon grating replica with 2,000 lines per mm. Three to four blocks from each rat were used.

The morphometric method followed was as previously reported. Briefly, measurements were made of volume and surface density (Vv and Sv) of the rough endoplasmic reticulum (rER), tubulovesicles, microvilli, mitochondria, and lysosomes in parietal cells.

Vv of various cytoplasmic organelles was analyzed at 10 times the original magnification with a universal projector (Olympus), using a double lattice system with 1.5 cm spacing (test points; 234). For estimation of Sv, the number of intersection points of cytoplasmic membranes with the test line was counted on the same screen of the instrument at the same magnifications.

After morphometric analyses of photographs, the values of morphometric parameters for each component in each group were calculated for 10 electron micrographs (30 electron micrographs per group). The mean, standard deviation, and standard error of the mean (SE) were obtained for all values calculated from each photograph. Corrections of systematic errors occurring in Vv and Sv of various components were performed according to Weibel.

**Statistical analysis of data**

For statistical analysis of plasma gastrin levels, fundic mucosal thickness, and ECL cell number, homogeneity of variance was analyzed using Bartlett's test. If variance was homogeneous, Student's t-test was carried out, and if not, Aspin-Welch's t-test was carried out between the control and E3810-treated groups. For electron microscopic morphometry, statistical analysis was performed by analysis of variance (ANOVA) using Tukey's Honestly Significant Difference (HSD) test.

**Results**

**Plasma gastrin levels**

In the 2-week treatment groups of E3810, plasma gastrin levels were significantly elevated, compared with that in the control group (Table 1). In the 5-week treatment groups of E3810, plasma gastrin levels showed an increasing tendency in a dose-dependent manner, but a significant increase in the level was discerned in Group IV (20 mg/kg/day) only.

**Light microscopic morphometry**

The fundic mucosal thickness in the 2-week treatment groups of E3810 displayed no significant change, compared with that in the control group (Table 2). In the 5-week treatment groups, the thickness significantly increased in a dose-dependent manner.

**Table 1. Plasma Gastrin Levels after E3810 Administration to Rats (pg/ml)**

<table>
<thead>
<tr>
<th>Dose of E3810</th>
<th>2-week group</th>
<th>5-week group</th>
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<tbody>
<tr>
<td>0 (Control)</td>
<td>408.8 ± 47.3</td>
<td>616.8 ± 84.6</td>
</tr>
<tr>
<td>5 mg/kg/day</td>
<td>1,185.2 ± 80.9**</td>
<td>856.5 ± 119.3</td>
</tr>
<tr>
<td>10 mg/kg/day</td>
<td>1,239.2 ± 142.1**</td>
<td>1,113.1 ± 223.2</td>
</tr>
<tr>
<td>20 mg/kg/day</td>
<td>908.5 ± 205.8*</td>
<td>1,568.2 ± 216.4*</td>
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Mean±S.E., N=10 rats/group/measured point
*p<0.05, **p<0.01 compared with control group
Table 2. Light Microscopic Morphometric Changes in Fundic Mucosa after E3810 Administration

<table>
<thead>
<tr>
<th>Dose of E3810</th>
<th>Fundic mucosal thickness (μm)</th>
<th>Density of ECL cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2-week group</td>
<td>5-week group</td>
</tr>
<tr>
<td>0 (Control)</td>
<td>506.9 ± 15.7</td>
<td>451.8 ± 13.7</td>
</tr>
<tr>
<td>5 mg/kg/day</td>
<td>534.2 ± 16.3</td>
<td>522.1 ± 13.3**</td>
</tr>
<tr>
<td>10 mg/kg/day</td>
<td>492.0 ± 19.6</td>
<td>535.3 ± 26.2*</td>
</tr>
<tr>
<td>20 mg/kg/day</td>
<td>539.6 ± 25.9</td>
<td>560.9 ± 19.5**</td>
</tr>
</tbody>
</table>

Mean ± S.E., N = 7 rats/group/measured point

*No. of chromogranin-positive cells per visual field in fundic mucosa

*p < 0.05, **p < 0.01 compared with control group

ECL cells were predominantly localized in the basal third of the fundic mucosa in the control rats (Fig. 1a), whereas they were increased in number and densely localized throughout the mucosa in particular in rats belonging to Group IV (20 mg/kg/day) (Fig. 1b). In the 2-week treatment groups of E3810, a significant increase in the number of ECL cells per visual field was seen only in Group IV (20 mg/kg/day) (Table 2). In the 5-week E3810 treatment groups, the number significantly increased in all groups in a dose-dependent manner.

Electron microscopy and morphometry

In the 2-week E3810 treatment groups, the subcellular structures of parietal cells did not clearly change, compared with those in the control group. In the 5-week treatment groups, the number and length of microvilli on the membranes of intracellular
canaliculi slightly decreased, although profiles of tubulovesicles seemed similar to those in the control even in Group IV (20 mg/kg/day). However, no changes in rER, Golgi complex, mitochondria, and lysosomes were discerned in parietal cells of E3810-treated rats. In some cases, small vacuole-like structures were seen in the cytoplasm of parietal cells in Group IV of the 5-week treatment period (Figs. 2a, b).

No changes in Vv and Sv of tubulovesicles were seen in both the 2- and 5-week E3810 treatment groups, except for Group III of the 5-week treatment.

Fig. 2. Electron micrographs of parietal cells obtained from a control rat (a) and an E3810-treated rat (20 mg/kg) after treatment for 5 weeks (b). Parietal cells in both the control and E3810-treated group demonstrate numerous mitochondria and several lysosomes (a and b). Microvilli on the membranes of intracellular canaliculi are well developed in a control parietal cell (a), whereas their profiles are slightly decreased on the membranes of the canaliculi and facing the secretory lumen in an E3810-treated cell (b). Note that a small vacuole-like structure can be seen in the cytoplasm of the E3810-treated parietal cell. ×3,000.
Fig. 3a Vv (µm³/µm³) of tubulovesicles.

Fig. 3b Sv (µm²/µm³) of tubulovesicles.

Fig. 3c Vv (µm³/µm³) of microvilli.

Fig. 3d Sv (µm²/µm³) of microvilli.

Fig. 3e Vv (µm³/µm³) of tubulovesicles and microvilli.

Fig. 3f Sv (µm²/µm³) of tubulovesicles and microvilli.

Mean ± S.E., N = 3, *p<0.05, **p<0.01 compared with concurrent controls

Fig. 3.
Fig. 3. Changes in volume density ($\mu m^3/\mu m^3$) and surface density ($\mu m^2/\mu m^3$) of tubulovesicles (a, b), microvilli (c, d), tubulovesicles and microvilli (e, f), mitochondria (g, h), lysosomes (i, j), and rough endoplasmic reticulum (k, l) in parietal cells of control and E3810-treated rats after 2 or 5 weeks of treatment.
period (10 mg/kg/day), in which Sv was significantly decreased (Figs. 3a, b). Vv and Sv of microvilli on the membranes of intracellular canaliculi were significantly decreased in most E3810 treatment groups; in particular, Sv in both the 2- and 5-week E3810 treatment groups distinctly decreased (Figs. 3c, d). When combined values of Vv and Sv of tubulovesicles and microvilli, their Vv significantly decreased in Group IV of the 2-week treatment period and Groups II and IV of the 5-week treatment period. Clear-cut decreases in Sv were demonstrated in both the 2- and 5-week treatment groups (Figs. 3e, f).

Other cytoplasmic organelles: Vv and Sv of rER, mitochondria and lysosomes exhibited no significant alterations in both the 2- and 5-week treatment groups, although Sv of mitochondria significantly decreased in Group IV of the 5-week treatment period (Figs. 3g-1).

Discussion

The present study demonstrated morphologic and morphometric alterations of gastric mucosa in rats treated with E3810 for 2 or 5 weeks.

Recent studies have shown that repeated administration of PPIs and potent H₂-receptor antagonists induces various histological as well as cytological changes in gastric mucosa: fundic mucosal hypertrophy/hyperplasia, eosinophilic chief cell, and ECL cell hyperplasia. PPIs and potent H₂-receptor antagonists inhibit gastric acid secretion, this situation stimulating secretion of gastrin from G cells located in the pyloric mucosa. Prolonged inhibition of gastric acid secretion by these drugs results in hypergastrinemia, which induces fundic mucosal hypertrophy and increased the number of ECL cells due to the trophic effects of gastrin. Plasma gastrin levels were elevated in the E3810-treated groups. In the present study, increases in the fundic mucosal thickness and ECL cell density were distinctly demonstrated in the 5-week E3810 treatment groups.

Different from the action of H₂-receptor antagonists, PPIs inhibit the final stage of acid production, that is, H⁺/K⁺-ATPase on the membranes of microvilli of the intracellular canaliculi. Parietal cells exhibit morphological differences between resting and stimulated states. In the resting state, parietal cells are characterized by small secretory canaliculi with few short microvilli and numerous profiles of tubulovesicles in the cytoplasm. In cells in the stimulated state, marked expansion of secretory canaliculi with an increase in the number and length of microvilli occurs concomitantly with a decrease in profiles of tubulovesicles. Structural features of parietal cells induced by omeprazole have been shown to differ from those induced by cimetidine, an H₂-inhibitor. Pharmacologically, H₂-receptor antagonists induce resting parietal cells in various animals. The morphological and morphometric features of parietal cells after omeprazole treatment differ from those of resting and stimulated parietal cells; omeprazole induces significant decreases in volume and surface density of both microvilli on the membranes of intracellular canaliculi and tubulovesicles in the cells. By electron microscopy, after omeprazole treatment, the profiles of microvilli on the membranes of intracellular canaliculi and tubulovesicles are poorly developed, although no difference in the profiles of intracellular canaliculi is discerned between parietal cells of omeprazole-treated and non-treated rats. The present study confirmed that E3810 induces similar morphological and morphometric changes in parietal cells to those induced by omeprazole. However, ultrastructurally numerous intact microvilli could be seen on the membranes of intracellular canaliculi after E3810 treatment, whereas the morphometric parameters decreased slightly. Moreover, no distinct difference in morphometric parameters of tubulovesicles was seen after E3810 treatment, although they showed a tendency to decrease in the 5-week treatment groups.

One of the characteristic alterations in parietal cells after treatment with omeprazole or lansoprazole is vacuolization of cells. In the present study, however, no vacuolization appeared in the cells, although small vacuole-like structures occasionally appeared in the cytoplasm of the cells in Group IV of the 5-week treatment period (20 mg/kg/day). Moreover, in preclinical toxicology studies of E3810 in rodents and dogs, no vacuolization appeared in parietal cells. One distinct change in parietal cells after E3810 treatment in the present study was significant decreases in morphometric parameters of microvilli on the membranes of intracellular canaliculi. At present, it remains unknown how
vacuoles are formed in parietal cells after omeprazole treatment. From their ultrastructural findings, it was speculated that vacuoles appearing in parietal cells after omeprazole treatment are related to altered intracellular canaluli. Under normal conditions, membranes are recycled between tubulovesicles and the plasma membrane, corresponding to acid secretion. It has been suggested from immunocytochemical and morphometric studies that lysosomes actively degrade membranes of tubulovesicles after omeprazole treatment; this disrupts the membrane recycling between tubulovesicles and the plasma membrane, resulting in marked decreases in profiles of tubulovesicles and microvilli on the membranes of the canaluli. After E3810 treatment, no significant changes were discerned in the volume and surface densities of lysosomes and mitochondria, while tubulovesicles also did not show any morphological and morphometric change, except for decrease in the surface density of tubulovesicles in Group III of the 5-week treatment period. In these situations in parietal cells, the membrane dynamics between tubulovesicles and the plasma membrane may still function. Moreover, it seems likely that the membrane system containing the proton pump in parietal cells after E3810 treatment is not damaged as much as to form vacuoles, although it remains unknown whether the vacuole formation is related to altered secretory canaluli.

Fujisaki et al. (1992) have shown that the reversal of H⁺/K⁺-ATPase inhibition by E3810 and omeprazole is achieved both by de novo enzyme synthesis and by endogenous glutathione (GSH). Moreover, the binding of E3810 with H⁺/K⁺-ATPase is more rapidly reversed in vitro by GSH than that of omeprazole. This suggests that E3810 has a shorter duration of pharmacological action than omeprazole. Furthermore, the difference in the duration of pharmacological action may explain the differences in morphological and morphometric alterations in parietal cells between E3810 and omeprazole; E3810 induces mild morphologic as well as morphometric alterations in parietal cells.

From these results, it is concluded that there certainly exist morphological as well as morphometric differences between the effects of E3810 and omeprazole on rat parietal cells. After E3810 treatment, no distinct vacuolization appeared in parietal cells, while the most remarkable changes in the cells were decreases in morphometric parameters of microvilli on the membranes of intracellular canaluli, although their profiles seemed to be intact. These slight alterations in the morphology of parietal cells may possibly be due to the reversible binding of E3810 with H⁺/K⁺-ATPase.

References
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