Ethenzamide Exerts Protective Effects against Ibuprofen-Induced Gastric Mucosal Damage in Rats by Suppressing Gastric Contraction

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Received June 7, 2020; accepted December 14, 2020; advance publication released online December 19, 2020

Nonsteroidal anti-inflammatory drugs (NSAIDs) are known to cause gastric mucosal damage, in which gastric hypermotility has been reported to play a primary role. The antipyretic analgesic drug ethenzamide (ETZ) is widely used in combination with other NSAIDs and, in a recent study, was found to possess 5-hydroxytryptamine (5HT)2B receptor antagonistic activity. Therefore, the inhibition of gastric contraction via 5HT2B receptor blockade by ETZ might contribute to ETZ’s protective effect against NSAIDs-induced gastric mucosal damage. In the present study, we examined the effects of ETZ on gastric contraction and ibuprofen (IBP)-induced gastric mucosal damage in rats. We found that ETZ suppressed both 5HT- and α-methyl-5HT (5HTα receptor agonist)-induced contractions of rat-isolated gastric fundus in a concentration-dependent manner. This suppressive effect of ETZ was not seen for either high-KCl- or acetylcholine-induced contractions. Furthermore, ETZ was confirmed to decrease ibuprofen-induced gastric mucosal damage in a dose-dependent manner in rats. Similarly, clonidine is known to reduce gastric motility, and methysergide (a 5HT2 receptor antagonist) is known to inhibit 5HT-induced contractions of the gastric fundus, which also decreases IBP-induced gastric mucosal damage, respectively. Although further research on other possible sites or mechanisms of action would be needed, these results suggest that ETZ exerts a protective effect against IBP-induced gastric mucosal damage and that suppressing the gastric contraction may play an important role in the gastroprotective effect of ETZ.

Key words  ethenzamide; analgesic; gastric contraction; gastroprotective; ibuprofen

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen (IBP), loxoprofen, and aspirin, are widely used for the relief of several pain symptoms. The suppression of prostaglandin (PG) production by both cyclooxygenase (COX)-1 and -2 inhibition is thought to be the major analgesic mechanism of NSAIDs.1 However, endogenous PG deficiency caused by NSAIDs leads to gastric mucosal damage, and several functional events including gastric hypermotility have been reported as possible elements in this pathogenesis.2

Ethenzamide (ETZ) is an antipyretic analgesic categorized as an NSAID and is widely used as an OTC drug in combination with other NSAIDs. While the combined use of two or more analgesics with different mechanisms has been suggested to have a synergetic analgesic effect, a reduction in adverse effects, such as gastric mucosal damage, can also be expected.3 In this regard, for example, acetaminophen has been reported to exert a protective effect against IBP-induced gastric mucosal damage in rats via the suppression of matrix metalloproteinase-13.4

Recently, we reported that ETZ does not have both COX-1 and COX-2 inhibiting effects.5 Furthermore, ETZ was confirmed to exert an antagonistic action on the 5-hydroxytryptamine (5HT)2B receptor in an in vitro cellular functional assay.5 The 5HT2B receptor is found throughout the gastrointestinal tract, including the smooth muscle of the stomach fundus,6,7 and is known to mediate the contractile response to 5HT.8

Therefore, from these mechanistic perspectives, we hypothesized that ETZ could inhibit stomach hypermotility and exert a protective effect against gastric mucosal damage induced by NSAIDs with COX-inhibiting action. In the present study, to explore the effect on stomach motility, we first examined the effects of ETZ on 5HT- and α-methyl-5HT-induced contractions of rat-isolated gastric fundus. Then, the effect of ETZ on IBP-induced gastric mucosal damage was evaluated in rats. In addition, the effects of clonidine5,6 and methysergide,10 both of which are known to inhibit gastric motility, on IBP-induced gastric mucosal damage were also evaluated to confirm the pathogenic role of stomach motility.

MATERIALS AND METHODS

Materials Potassium chloride (KCl; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 5-hydroxytryptamine hydrochloride (5HT; Sigma-Aldrich Japan, Tokyo, Japan), α-methyl-5HT hydrochloride (Sigma-Aldrich Japan), acetylcholine chloride (acetylcholine; Sigma-Aldrich Japan), Krebs–Henseleit Buffer Modified with 2000mg/L glucose without calcium and sodium bicarbonate (Sigma-Aldrich Japan), calcium chloride dihydrate (FUJIFILM Wako Pure Chemical Corporation), sodium hydrogen carbonate (FUJIFILM Wako Pure Chemical Corporation), sodium chloride hydrate (FUJIFILM Wako Pure Chemical Corporation), sodium bicarbonate (FUJIFILM Wako Pure Chemical Corporation), ethenzamide (ETZ; SHIZUOKA COFFEIN, Shizuoka, Japan), ibuprofen (IBP; BASF, Ludwigshafen, Germany), clonidine hydrochloride (clonidine; Sigma-Aldrich Japan), methysergide maleate salt (methysergide; Tocris Bioscience, Bristol, U.K.), 100mM

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hydrochloride acid (HCl; FUJIFILM Wako Pure Chemical Corporation), dimethyl sulfoxide (DMSO; KOKUSAN CHEMICAL Co., Ltd., Tokyo, Japan), gum arabic (FUJIFILM Wako Pure Chemical Corporation), and polyethylene glycol 400 (FUJIFILM Wako Pure Chemical Corporation) were purchased from the indicated manufacturers.

**Animals** Male Sprague-Dawley rats were purchased from Charles River Japan (Kanagawa, Japan). The rats were all housed under conditions of controlled temperature (23 ± 3°C), humidity (50 ± 20%) and lighting (lights on from 07:00 to 19:00h) and were used in the study at an age of 7–8 weeks old. All the rats were given access to food and tap water ad libitum. All the experimental procedures were conducted with the approval of the Animal Care Committee at Taisho Pharmaceutical Co., Ltd., in accordance with the company’s guidelines for the Care and Use of Laboratory Animals.

**Tissue Preparation and Measurements of Mechanical Activity** The rats were euthanized by CO₂ asphyxiation.

Whole stomachs were obtained, and strips of longitudinal muscle were dissected from the greater curvature of the fundus; the mucosa was then removed from the muscle layers. The strips of the gastric fundus were placed in 10 mL of tissue organ bath containing oxygenated (95% O₂/5% CO₂) Krebs–Henseleit solution (d-glucose, 11.1 mM; MgSO₄, 1.2 mM; KH₂PO₄, 1.2 mM; KCl, 4.7 mM; NaCl, 118.1 mM; CaCl₂·2H₂O, 2.5 mM; NaHCO₃, 25.0 mM) at 37°C. In the organ baths, the strips were mounted between stainless hooks to apply approximately 1 g of resting tension during the 60-min equilibration period. Mechanical responses were recorded using isometric transducers (TD-612T; Nihon Kohden, Tokyo, Japan) coupled to a polygraph system (RMT-1000; Nihon Kohden).

**Effect of ETZ on the 5HT- or α-Methyl-5HT-Induced Contraction of Tissue Strips** At first, 60 mM KCl was applied to the strips, and cumulative concentrations of 5HT (3 × 10⁻¹⁰ to 10⁻⁵ M) or α-methyl-5HT (3 × 10⁻¹⁰ to 3 × 10⁻⁶ M) were then applied to obtain a stable contraction. Subsequently, to evaluate the effect of ETZ on 5HT- or α-methyl-5HT-induced contractions, the strips were exposed to 5HT (3, 10, 30, and 300 μM) or the vehicle (0.1% DMSO) 10 min before and during the measurement of the concentration–response curves for 5HT or α-methyl-5HT, as described above. The amplitudes of the contractions induced by 5HT or α-methyl-5HT were represented as values relative to that initially induced by KCl. This procedure was conducted 8 times (N = 8).

**Effect of ETZ on the KCl-Induced Contraction of Tissue Strips** KCl (60 mM) was applied to the strips twice to obtain a stable contraction. Subsequently, to investigate the effect of ETZ on 5HT- or α-methyl-5HT-induced contractions, the strips were exposed to 100 μM ETZ or vehicle (0.1% DMSO) 10 min before and during the measurement of the concentration–response curves of acetylcholine, as described above. The amplitudes of the contractions were represented as values relative to that initially induced by KCl. This procedure was conducted 8 times (N = 8).

**Effect of ETZ on the Acetylcholine-Induced Contraction of Tissue Strips** KCl (60 mM) was applied to the strips, and then cumulative concentrations (10⁻⁹ to 10⁻⁴ M) of acetylcholine were applied to obtain a stable contraction. Subsequently, the strips were exposed to 100 μM ETZ or vehicle (0.1% DMSO) 10 min before and during the measurement of the concentration–response curves of acetylcholine, as described above. The amplitudes of the contractions were represented as values relative to that initially induced by KCl. This procedure was conducted 8 times (N = 8).

**Effect of ETZ, Clonidine, and Methysergide on IBP-Induced Gastric Mucosal Damage** ETZ (50, 150, or 500 mg/kg for oral administration, 50 or 100 mg/kg for intraperitoneal administration), clonidine (0.1 mg/kg), or methysergide (3 or 10 mg/kg), or the same dose of solvent for each drug was administered at the same time as the IBP, followed by the measurement of gastric mucosal damage as described above. ETZ was suspended in 5% gum arabic solution and orally administered at volume of 5 mL/kg (N = 10). ETZ was suspended in solvent consisting of 40% polyethylene glycol, 10% ethanol and distilled water and intraperitoneally administered at volume of 2 mL/kg (N = 8), clonidine was dissolved in saline and subcutaneously administered at volume of 2 mL/kg (N = 8), and methysergide was dissolved in saline and intraperitoneally administered at a volume of 2 mL/kg (N = 10).

**Effect of 100 mM HCl on the Protective Action of ETZ against IBP-Induced Gastric Mucosal Damage** ETZ (500 mg/kg) and 100 mM HCl, or the same dose of solvent for both test substances were administered at the same time as the IBP, followed by the measurement of gastric mucosal damage as described above (N = 8). ETZ was suspended in 5% gum arabic solution and orally administered at volume of 5 mL/kg, 100 mM HCl was orally administered at volume of 5 mL/kg, and distilled water as solvent for HCl was orally administered at volume 5 mL/kg.

**Data Analysis** Data were presented as the means ± standard error of the mean (S.E.M.). For the gastric mucosal damage results, statistical analyses were performed using Steel test, Aspin–Welch t-test and Student’s t-test. From data of the concentration-dependent contractile responses of stomach tissue strips by 5HT or α-methyl-5HT, each EC₅₀ value in the presence and absence of ETZ were obtained by calculating the non-linear regression line based on the least-squares method using SAS (System version 9.2, SAS Institute Inc.), and then, the concentration ratio (CR) was calculated using the following equation: CR = (mean of the individual EC₅₀ values in the presence of ETZ) ÷ (mean of the individual EC₅₀ values in the absence of ETZ). The slopes of liner regression and pA₂ values were obtained from a Schild plot of the CR values.

**RESULTS**

**ETZ Inhibited 5HT- or α-Methyl-5HT-Induced Contractions of Tissue Strips** To investigate the effect on stomach motility, the effects of ETZ on 5HT- or α-methyl-5HT-induced...
contractions of rat-isolated gastric fundus were examined. 5HT (Fig. 1a) or α-methyl-5HT (Fig. 1b) resulted in the contraction of the tissue strips in a concentration-dependent manner. The 5HT- or α-methyl-5HT-induced contractions were suppressed by ETZ. The concentration–response curves of 5HT or α-methyl-5HT were shifted to the right in a concentration-dependent manner (Figs. 1a, b). In addition, the slopes of the Schild regression of ETZ in 5HT and α-methyl-5HT were 1.11 and 1.32, respectively. The pA₂ values of ETZ in 5HT and α-methyl-5HT were 4.63 and 4.36, respectively.

**ETZ Did Not Inhibit KCl or Acetylcholine-Induced Contractions of Tissue Strips**

To confirm that ETZ does not have a nonselective action on gastric smooth muscle, its effect on KCl or acetylcholine-induced contractions of tissue strips was examined. While 60 mM KCl (Fig. 2a) or a cumulative concentration (10⁻⁹ to 10⁻³ M) of acetylcholine (Fig. 2b) resulted in the contraction of the tissue strips, 100 µM ETZ did not suppress these contractions (Figs. 2a, b).

**ETZ Suppressed IBP-Induced Gastric Mucosal Damage**

To investigate the effect of ETZ on IBP-induced gastric mucosal damage, the area of the hemorrhagic lesion was measured.

The oral administration of IBP-induced hemorrhagic lesions in the gastric mucosa, whereas the co-treatment (oral administration) of ETZ reduced the area of hemorrhagic lesions in a dose-dependent manner (Fig. 3a). Similarly, the intraperitoneal administration of ETZ also reduced the area of IBP-induced hemorrhagic lesions in the gastric mucosa (Fig. 3b).

**100mM HCl Had No Effect on the Protective Action of ETZ against IBP-Induced Gastric Mucosal Damage**

To clarify whether the gastroprotective action of ETZ is not derived from inhibition of basal gastric acid secretion, influence of exogenously applied 100 mM HCl on protective action of ETZ against IBP-induced gastric mucosal damage was investigated. As a result, ETZ significantly suppressed the area of IBP-induced hemorrhagic lesion in the gastric mucosa, whereas the damage score was similar in the group of co-administered with 100 mM HCl (Fig. 3c).

**Clonidine and Methysergide Suppressed IBP-Induced Gastric Mucosal Damage**

To consider the possible involvement of the suppression of stomach motility in the gastroprotective action of ETZ, the effects of clonidine and
methysergide on IBP-induced gastric damage were investigated. IBP-induced gastric mucosal damage was inhibited by the subcutaneous administration of clonidine (Fig. 4a) and the intraperitoneal administration of methysergide (Fig. 4b) in dose-dependent manners.

**DISCUSSION**

In our recent study, ETZ was confirmed not to possess COX-1 and COX-2 inhibiting actions and to have a 5HT2B receptor blocking action as its analgesic mechanism. The 5HT2B receptor is known to be expressed in the smooth muscle of the stomach fundus and to mediate the contractile response to 5HT. Meanwhile, stomach hypermotility has been reported as a possible element of gastric mucosal damage induced by
non-steroidal anti-inflammatory drugs (NSAIDs) with COX inhibiting actions.\(^2\) Hence, we postulated that ETZ might inhibit stomach motility, reducing COX inhibitor-induced gastric mucosal damage.

In this study, we first found that ETZ inhibited 5HT- or α-methyl-5HT (5HT\(_2\) receptor agonist)-induced contractions of rat-isolated gastric fundus (Figs. 1a, b), whereas no inhibitory effects on KCl- or acetylcholine-induced contractions were observed (Figs. 2a, b). In addition, the slope of the Schild regression of ETZ in 5HT was close to unity (from 0.8 to 1.2,\(^2\)) see Results), indicating that ETZ competitively antagonized at least 5HT-induced contractions. Recently, we demonstrated that ETZ has some selectivity for the 5HT\(_{2B}\) receptor, compared with other 5HT receptor subtypes.\(^5\) Consistent with our data, RS-127445\(^1\) (a selective 5HT\(_{2B}\) receptor antagonist) and tegaserod\(^1\) (a potent 5HT\(_{2B}\) receptor antagonist and 5HT\(_4\) receptor agonist) have been reported to inhibit 5HT-induced contractions of the rat-isolated stomach fundus. Therefore, ETZ may inhibit contractions of the strips of gastric fundus by blocking the 5HT\(_{2B}\) receptor.

Secondly, as expected, the oral administration of ETZ suppressed IBP-induced hemorrhagic lesions in the gastric mucosa in a dose-dependent manner (Fig. 3). The orally administered test solution could potentially behave as a physical barrier\(^4\) or mild irritant\(^5,6\) acting directly on the surface of the stomach mucosa to prevent lesion formation; however, we also found that intraperitoneally administered ETZ suppressed the IBP-induced gastric mucosal damage (Fig. 3b), suggesting that the gastroprotective effect of ETZ is not directly exerted on the mucosa, but instead induces a functional event such as a reduction of stomach motility. Whether ETZ suppresses stomach motility in vivo was not shown in the present study; however, tegaserod, which possesses an inhibitory effect on the contraction of the gastric fundus in vitro via 5HT\(_{2B}\) receptor blockade (similar to ETZ), has been reported to reduce stomach motility in vivo\(^1\). Furthermore, from a pharmacokinetic point of view, a previous study\(^7\) suggested that the rat plasma ETZ level in this investigation could be expected to reach 30 to 300 μM of concentration at which inhibitory effect on the contraction of the isolated gastric fundus was observed (Figs. 1a, b). Thus, ETZ is highly likely to reduce stomach motility in vivo, and this suppressive action is thought to play a role in its gastroprotective effect against IBP-induced mucosal damage. Additionally, protective action of ETZ against IBP-induced mucosal damage was not affected by increasing the acidity in the stomach by extrinsic administration of 100 mM HCl (Fig. 3c). Furthermore, we recently found that ETZ has no affinity at least for histamine H\(_2\) receptor,\(^5\) suggesting ETZ is unlikely to exert gastroprotective action by suppressing basal gastric acid secretion. The dose of ETZ used in this study (up to 500 mg/kg) is considered to appropriate, since ETZ is taken up to 500 mg at a time as an OTC antipyretic drug in Japan (as well, IBP is taken up to 200 mg at a time). Further, when evaluating the effect of analgesic drug in animal experiments, it is common to administer 60 times the dose used in human experiments, it is common to administer 60 times the dose used in humans. Further, when evaluating the effect of analgesic drug in animal experiments, it is common to administer 60 times the dose used in human studies.

Finally, similar to ETZ, we found that clonidine (CLO) and methysergide (MSG) showed suppressive effects on IBP-induced gastric damage (Figs. 4a, b). Both CLO\(^9\) and MSG\(^1\) are known to inhibit contractions of isolated gastric fundus via the activation of α\(_2\)-adrenoceptor and probably the blockade of the 5HT\(_{2B}\) receptor, respectively. Furthermore, CLO has been shown to prevent gastric damage induced by acetylated ethanol\(^9\) or stress,\(^9\) while MSG prevents gastric damage induced by repeated applications of 5HT\(^20\) or compound 48/80.\(^2\) Since multiple and closely interacting elements are involved in the formation of gastric damage in each of these models, the identification of the primary mechanism of the gastroprotective action of these compounds is expected to be difficult. However, at least for NSAIDs-induced gastric damage, hypermotility of the stomach induced by endogenous prostaglandin deficiency has been reported to be a primary element in this pathogenesis\(^2\); therefore, we considered that the inhibitory effect on gastric motility by CLO and MSG probably plays an important role in the suppression of IBP-induced gastric damage. From these data, the pathogenic role of stomach motility in IBP-induced gastric damage was reconfirmed, and the suppression of stomach motility was considered to contribute to the gastroprotective action of ETZ.

CLO has been shown to suppress gastric damage more strongly than ETZ or MSG (Figs. 3a, b, 4a, b), suggesting another gastroprotective mechanism including the inhibition of peptidase and acid secretion.\(^2\) Furthermore, since MSG is known to be metabolized to methylergometrin, which possesses several pharmacological actions,\(^2\) it cannot be concluded that gastroprotective mechanism of MSG and also ETZ is exerted via 5HT\(_{2B}\) receptor blockade in this study. Further research would be needed to determine the precise mechanism of gastroprotective effect of ETZ.

In summary, we have provided evidence suggesting that the antipyretic analgesic ETZ exerts a protective effect against IBP-induced gastric mucosal damage at least by suppressing the gastric contraction. We propose that an appropriate combination of ETZ and other NSAIDs might be useful not only for its synergic analgesic effect, but also for reducing gastric symptoms.

**Conflict of Interest** The authors declare no conflict of interest.

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