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Identification of a unique nsaid, fluoro-loxoprofen with gastroprotective activity☆,☆☆

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ABSTRACT

We previously proposed that direct cytotoxicity of NSAIDs due to their membrane permeabilization activity, together with their ability to decrease gastric prostaglandin E_2, contributes to production of gastric lesions. Compared to loxoprofen (LOX), fluoro-loxoprofen (F-LOX) has much lower membrane permeabilization and gastric ulcerogenic activities but similar anti-inflammatory activity. In this study, we examined the mechanism for this low ulcerogenic activity in rats. Compared to LOX, the level of gastric mucosal cell death was lower following administration of F-LOX. However, the gastric level of prostaglandin E_2 was similar in response to treatment with the two NSAIDs. Oral pre-administration of F-LOX conferred protection against the formation of gastric lesions produced by subsequent administration of LOX and orally administered F-LOX resulted in a higher gastric pH value and mucus content. In the presence of a stimulant of gastric acid secretion, the difference in the ulcerogenic activity of F-LOX and LOX was less apparent. Furthermore, an increase in the mucus was observed in gastric cells cultured in the presence of F-LOX in a manner dependent of increase in the cellular level of cAMP. These results suggest that low ulcerogenic activity of F-LOX involves its both low direct cytotoxicity and protective effect against the development of gastric lesions. This protective effect seems to be mediated through an increase in a protective factor (mucus) and a decrease in an aggressive factor (acid).

1. Introduction

The balance between aggressive and defensive factors determines the development of gastric lesions, with either a relative increase in aggressive factors or a decrease in protective factors resulting in lesions. The gastric mucosa can be challenged by a variety of both endogenous and exogenous factors, including gastric acid, reactive oxygen species, ethanol, *Helicobacter pylori* and non-steroidal anti-inflammatory drugs (NSAIDs) [1]. In order to protect the mucosa, the body relies on defence systems such as the production of surface mucus and bicarbonate, and the regulation of gastric mucosal blood flow. Prostaglandin E_2 (PGE_2) also exerts a strong protective effect, inhibiting the secretion of gastric acid and stimulating the production of mucus [2].

NSAIDs, such as indomethacin, comprise a therapeutically valuable family of drugs [3]. An inhibitory effect of NSAIDs on cyclooxygenase (COX) activity is responsible for their anti-inflammatory actions, COX being an enzyme that is essential for

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Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)-2NH_4; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; COX, cyclooxygenase; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; EIA, enzyme immunoassay; ELLA, enzyme-linked lectin-binding assay; F-LOX, fluoro-loxoprofen; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H & E, hematoxylin and eosin; IBMX, 3-isobutyl-1-methylxanthine; LOX, loxoprofen; NSAID, non-steroidal anti-inflammatory drug; PG, prostaglandin; RGM1, rat normal gastric epithelial cell line; SD, Sprague-Dawley; SBA, soybean agglutinin; TCA, trichloroacetic acid; TUNEL, terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling.

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the synthesis of prostaglandins, which have a strong capacity to induce inflammation. However, as described above, NSAID use is also associated with gastrointestinal complications [4–7], which was thought to result from the inhibition of COX and a decrease in gastric PGE_2 level. In fact, NSAIDs have been reported to stimulate the secretion of gastric acid and inhibit the production of mucus through decreasing gastric PGE_2 level [8,9]. However, it is now believed that the production of gastric lesions by NSAIDs involves additional mechanisms, given that the increased incidence of gastric lesions and the decrease in PGE_2 levels induced by NSAIDs do not always occur in parallel [10,11]. We have recently demonstrated that NSAIDs induce cell death (apoptosis) in cultured gastric mucosal cells and at the gastric mucosa in a manner independent of COX inhibition [12–16]. With regards to the molecular mechanism governing this apoptosis, we have proposed the following pathway. Permeabilization of cytoplasmic membranes by NSAIDs stimulates Ca^{2+} influx and increases intracellular Ca^{2+} levels, which in turn induces the endoplasmic reticulum stress response [12,17,18]. During the course of this response, an apoptosis-inducing transcription factor, C/EBP homologous transcription factor, is induced and, as we have previously shown, this protein is essential for NSAID-induced apoptosis [13,19]. Furthermore, we have proposed that both COX inhibition and gastric mucosal cell death are important for the formation of NSAID-induced gastric lesions *in vivo* [16,20].

In 1991, two subtypes of COX, COX-1 and COX-2, which are responsible for the majority of COX activity at the gastrointestinal mucosa and in tissues subject to inflammation, respectively, were identified [21]. It is therefore not surprising that a reduced incidence of gastroduodenal lesions has been reported following treatment with selective COX-2 inhibitors [22–24]. However, a recently raised issue concerning the use of selective COX-2 inhibitors is the potential risk of cardiovascular thrombotic events [25,26]. This may be due to the fact that prostacyclin, a potent anti-aggregator of platelets and a vasodilator, is mainly produced by COX-2 [27–29]. Therefore, in order to minimize clinical complications, gastric safe NSAIDs other than selective COX-2 inhibitors need to be developed. Based on the hypothesis outlined above, we believe that NSAIDs with lower membrane permeabilization activity would represent an efficacious alternative, even if they had no selectivity for COX-2 [14].

In order to investigate this possibility, we screened for such compounds from a range of clinically used NSAIDs without COX-2 selectivity, and found that the membrane permeabilization activity and direct cytotoxicity of loxoprofen (LOX) (Fig. 1A) was relatively lower than that of the other NSAIDs tested [30]. LOX is a leading NSAID on the Japanese market, being widely used because clinical studies have suggested that it is safer than other traditional (non-selective) NSAIDs [31,32]. LOX is a pro-drug, which is converted (by reduction of the cyclopentanone moiety) to its active metabolite (the *trans*-alcohol metabolite, LOX-OH) by aromatic aldehyde-ketone reductase (Fig. 1A) [33]. We therefore synthesized a series of LOX derivatives, demonstrating that fluoroloxoprofen (F-LOX) (Fig. 1A) has much lower membrane permeabilization and gastric ulcerogenic activities than LOX, but similar anti-inflammatory activity, suggesting that it is likely to be a therapeutically viable drug [34]. We suggested that F-LOX is also a pro-drug, which is converted to its active metabolite (the *trans*-alcohol metabolite, F-LOX-OH (Fig. 1A)), because the inhibitory effect of F-LOX-OH on COX is much more potent than that of F-LOX *in vitro* [34]. Although we concluded that the low membrane permeabilization activity of F-LOX is responsible for its low ulcerogenic activity [34], it remained possible that other mechanisms could also be involved. In this study, we therefore examined the mechanism governing the low ulcerogenic activity of F-LOX. Our results suggest that this effect is mediated not only by the low direct cytotoxicity due to its low membrane permeabilization activity, but also by protection of the gastric mucosa. In contrast to LOX and other NSAIDs, oral administration of F-LOX led to an increase in gastric pH value and mucus, suggesting that these effects are also involved in the low ulcerogenic activity of this drug.

2. Experimental procedures

2.1. Chemicals and animals

LOX, LOX-OH, F-LOX and F-LOX-OH (Fig. 1A) were synthesized in our laboratory as previously described [34]. Methylcellulose and RPMI1640 were obtained from Wako Pure Chemical Industries (Osaka, Japan). Formaldehyde, paraformaldehyde, Alcian blue 8GX, mucin, cycloheximide, histamine, fetal bovine serum (FBS), Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham, 2-methyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine-3-acetonitrile (SCH 28080; an inhibitor of gastric H^+,K^+-ATPase), 3-isobutyl-1-methylxanthine (IBMX), omeprazole, forskolin and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Mayer's hematoxylin, 1% eosin alcohol solution and mounting medium for histochemical analysis (malinol) were from MUTO Pure Chemicals (Tokyo, Japan). Terminal transferase was obtained from Roche Diagnostics (Mannheim, Germany). Biotin 14-ATP and streptavidin-conjugated Alexa Fluor 488 were purchased from Invitrogen (Carlsbad, CA). Mounting medium for the TdT-mediated biotinylated UTP nick end labeling (TUNEL) assay (VECTASHIELD) was from Vector Laboratories, Inc. (Burlingame, CA). 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM) were obtained from Dojindo (Kumamoto, Japan). SQ22536 was from CALBIOCHEM (San Diego, CA). ONO-8711 and ONO-AE2-227 were from our laboratory stocks. The prostaglandin E_2 enzyme immunoassay (EIA) kit was purchased from Cayman Chemical (Ann Arbor, MI). cAMP complete ELISA kit was from Enzo Life Sciences (Farmingdale, NY). Horseradish peroxidase-labeled soybean agglutinin (SBA-HRP) was from Seikagaku Biobusiness Co. (Tokyo, Japan). 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)-2NH_4 (ABTS) was obtained from KPL (Gaithersburg, MD). The RNeasy Mini kit was from QIAGEN (Valencia, CA), the first-strand cDNA synthesis kit was obtained from Takara (Kyoto, Japan), and iQ SsoFast EvaGreen Supermix was purchased from Bio-Rad (Hercules, CA). Wistar rats (3-week-old males) and Sprague-Dawley (SD) rats (6-week-old males) were obtained from Charles River Laboratories Japan (Yokohama, Japan). Guinea pigs (3-week-old males) were obtained from Japan SLC (Shizuoka, Japan). Animals were housed under conditions of controlled temperature (22–24 °C) and illumination (12 h light cycle starting at 8:00 AM) for 1 week before experiments. The experiments and procedures described here were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health, and were approved by the Animal Care Committee of Keio University and Kumamoto University. Totally, we used 287 Wistar rats, 182 SD rats and 4 guinea pigs for all experiments in this study.

2.2. Gastric and small intestinal damage assay

The gastric ulcerogenic response was examined as described previously [20,35] with some modifications. Wistar or SD rats fasted for 18 h were orally administered each NSAID and, after 8 h or 4 h, respectively, the animals were sacrificed, their stomachs were removed, and the gastric mucosal lesion area measured by an observer unaware of the treatment that the animals had received. For Wistar rats, calculation of the scores involved measuring the area of all the lesions in square millimeters and summing the