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Pharmacological inhibition of soluble epoxide hydrolase or genetic deletion reduces diclofenac-induced gastric ulcers

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ABSTRACT

Aims: This research was conducted to evaluate the hypothesis that gastric ulcers caused by the NSAID diclofenac sodium (DCF) can be prevented by the soluble epoxide hydrolase inhibitor TPPU.
Main methods: Mice were administered a single dose of 10, 30 or 100 mg/kg of DCF. Once an ulcerative dose of DCF was chosen, mice were pretreated with TPPU for 7 days at 0.1 mg/kg to evaluate anti-ulcer effects of the sEH inhibitor on anatomy, histopathology, pH, inflammatory markers and epithelial apoptosis of stomachs.
Key findings: Diclofenac caused ulceration of the stomach at a dose of 100 mg/kg and a time post dose of 6 h. Ulcers generated under these conditions were associated with a significant increase in the levels of TNF-α and IL-6 in serum and increased apoptosis compared to control mice. Pretreatment with TPPU resulted in a decrease of ulceration in mice treated with DCF with a significant decrease in the level of apoptosis, TNF-α and IL-6 in the serum in comparison to diclofenac-treated mice. TPPU did not affect the pH of the stomach, whereas omeprazole elevated the pH of the stomach as expected. A similar anti-ulcer effect was observed in sEH gene knockout mice treated with DCF.
Significance: The sEH inhibitor TPPU decreases the NSAID-induced stomach ulcers.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) have a long history of use worldwide for the treatment of pain, inflammation and fever. However, the gastrointestinal injury is an important use-limiting side effect of NSAIDs [1]. Approximately 50% of the patients who regularly use NSAIDs display gastric erosion and it is estimated that 15–30% have ulcers. Clinical treatment of this gastrointestinal toxicity is required for 3–4.5% of these patients [2]. Over 30 million people use NSAIDs daily [3], making gastrointestinal toxicity a major health issue. Though many drugs are available to alleviate NSAID-induced ulceration, inhibitors of soluble epoxide hydrolase (sEH), which decrease pain [4] and inflammation [5] hold promise to decrease such ulcers [1,6].

The prostanoid PGE_2 is an important potent lipid mediator of pain and inflammation. However, in the gastrointestinal system, its key function is to protect the stomach from acid secreted by parietal glands through stimulating the production of bicarbonate and mucus. The bicarbonates neutralize acid while the mucus covers the stomach mucosa and prevents contact of acid with mucosal cells. NSAIDs decrease the production of prostanoids, in particular, PGE_2, but this lack of protective actions of PGE_2 then leads to gastric lesion i.e. erosion and/ulcers of gastric cells. Clinical treatment of gastric ulcers typically involves the use of agents which either decrease the secretion of acid or mimic the actions of PGE_2 (prostaglandin analogs). Omeprazole (OME), the widely used inhibitor of proton pump (H^+K^+-ATPase) decreases the secretion of acid in the stomach [1]. Despite the wealth of knowledge on the effects of NSAIDs, the mechanistic aspects of NSAID-induced gastric ulcers are still not fully understood. In addition to PGE_2, multiple other biological pathways seem responsible for the formation of NSAID-induced ulcers [7–11]. While NSAIDs suppress inflammation, NSAID-induced ulcers are associated with increases in the levels of inflammatory markers such as TNF-α and IL-6 [8,12]. Although a downstream consequence of COX-2 activity, TNF-α is proposed to play an important role in ulcer pathways activated by prolonged use of NSAID. Anti-TNF-α antibodies for example significantly decrease necrotic and apoptotic lesions associated with ulcers in rats [13,14] and TNF-α knockout mouse is more resistant to NSAID-induced ulceration [8].

In addition to its pro-inflammatory functions, TNF-α is known to be a strong inducer of apoptosis through at least several mechanisms [14]. Furthermore, a role for apoptosis in ulceration is supported in numerous

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studies [15–17]. Consequently, agents which decrease NSAID-induced apoptosis also decrease stomach ulcers [17]. NSAID-induced apoptosis of gastric cells seems to be driven by not only TNF-α but also by activated endoplasmic reticulum (ER) stress responses to an increase in intracellular Ca^{2+} concentrations [18].

The FitzGerald hypothesis suggests adverse effects of NSAIDs are due, in part, to an alteration in the ratio of thromboxane B2 (TxB2) and prostaglandin E_2 (PGE_2) [19]. Inhibitors of sEH not only synergize the anti-inflammatory and analgesic effects of NSAIDs but also balance the TxB2/PGE_2 altered by treatment with some COX inhibitors [20]. Similarly, genetic deletion of sEH enzyme also leads to alterations in the level of TxB2 [21]. Previously published data suggest sEH inhibitors should protect animals from NSAID driven gastric ulcers [6,22]. Several sEH inhibitors are in human clinical trials [23,24] and these molecules display synergistic effects with NSAIDs [5]. Earlier, we demonstrated decreased NSAID-induced intestinal ulcers in mice treated with diclofenac and an inhibitor of sEH [6,22]. NSAID-induced intestinal ulcer [6] is a serious problem in man. It is more likely that sEH inhibitor would decrease the dose of NSAID and mitigate adverse effect such as ulceration. Therefore, understanding the interactions of inhibition of sEH with NSAID-induced ulceration is important. Here, we evaluated the effects of inhibition of sEH on NSAID-induced gastric ulcers using a mouse model.

The sEH inhibitor *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (*t*-AUCB) was shown to decrease piroxicam (a COX inhibitor)-induced inflammatory bowel disease [22]. The sEH inhibitors also decrease apoptosis of cells [25] and ER stress [26,27]. Therefore, we evaluated the prophylactic effects of N-[1-(1-oxopropyl)-4-piperidinyl]-N′-[4-(trifluoromethoxy)phenyl]-urea (TPPU) on diclofenac-induced gastric ulcers and the effect of this treatment on markers of inflammation (TNF-α and IL-6), and apoptosis. We selected the most commonly used sEH inhibitor in the field, TPPU, which is highly potent, has high oral availability and a longer half-life than the previously used *t*-AUCB in mice [28,29]. Commercial availability of TPPU from several vendors should enable other laboratories to follow up on our results. Ulu et al. [30] recently demonstrated that when given in drinking water TPPU had high oral availability and quickly reached a steady state. Blood levels linearly correlated with the dose in drinking water. Here the effects of TPPU on diclofenac-induced gastric ulcers are reported. In addition, the effect of treatment was studied on pH of the stomach to find out whether sEH inhibitors influence ulceration through modulating pH. Omeprazole, a proton pump inhibitor (PPI) was used as a standard for comparison.

2. Materials and methods

2.1. Materials

Diclofenac (Sigma-Aldrich Co., St. Louis, MO), Mouse TNF-α and IL-6 ELISA kits (Thermo Scientific, Rockford, IL), Pierce® BCA protein assay reagent (Thermo Scientific, Rockford, IL), TACS® 2 TdT-DAB in situ apoptosis detection kit (Trevigen Inc., Gaithersburg, MD) were purchased. TPPU was synthesized previously in the laboratory and is available from commercial sources [28]. All other reagents were of analytical grade.

2.2. Animals

Male Swiss Webster mice, C57BL6 and sEH knockout mice in a C57BL6 background weighing 34–41 g were used for the research. Animals were 4 months old. The Institutional Animal Care and Use Committee (IACUC) of the University of California, Davis reviewed and approved the animal protocol. The National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) guidelines has been followed to conduct animal experiments. The animals were maintained in 12 h dark and 12 h light cycle with unlimited access to mouse chow and water. However, animals were kept fasting for 12 h before sacrifice to assess effects of treatment on stomach ulcers. In the cage, animals were kept 2.5 cm above bedding, by use of a wire mesh, to prevent them from eating their feces and bedding during fasting.

The sEH knockout mice were provided originally by Dr. Christopher J. Sinal from the Dr. Frank J. Gonzalez lab (Laboratory of Metabolism, Division of Basic Sciences, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA) and a colony of knockout mice has been maintained at the University of California, Davis under Mouse Biology Program with repeated back breeding into C57BL6 mice from Jackson Laboratories [31,32,33]. Therefore, C57BL6 mice were also used as controls for sEH (−/−) mice.

2.3. Induction and scoring of gastric ulcers

Diclofenac-induced ulceration was studied in mice using 3 doses i.e. 10, 30 and 100 mg/kg. Six hours after diclofenac dosing, mice were anesthetized with isoflurane. The abdominal cavity was opened to expose the stomach, which was opened along the greater curvature for evaluating the gastric damage. Ulcer indexes of stomachs exposed to different treatments were calculated as described by Naidu et al. [34]. The images of stomachs were acquired with a digital camera (MA-1000, AmScope, Irvine, CA 92606, United States) mounted on SterioZoom 6 Plus Leica microscope (Leica Microsystems, Buffalo Grove, IL 60089, United States) at 7× magnification. Briefly, normal colored stomachs and pink colored stomachs were scored as 0 and 0.5 respectively. Spot ulcers were given a score of 1, whereas hemorrhagic streaks were given a score of 1.5. Numbers of ulcers exceeding 3 but <5 were scored as 2, and the number of ulcers ≥5 were given a score of 3. The final ulcer index was obtained by adding the scores for colored stomach, spot ulcer, hemorrhagic streak and full ulcers. The maximal score, warranted by the ulcer index, is 6. Ulcers were graded by a person not aware of details regarding the dosing. TPPU (0.1 mg/kg/day, 7 days in drinking water) and omeprazole (50 mg/kg/day, p.o., 5 days) were used to pre-treat the mice before dosing with DCF to test if these agents can decrease the ulcerative effect of the NSAID. The last doses of TPPU and omeprazole were administered 1 h before dosing with DCF. TPPU and OME were administered in 1% PEG400 solution in water. Since TPPU has high oral availability, and its efficacy in reducing intestinal ulcer as a function of oral dose in drinking water has been previously studied [6], this route of administration was selected as one minimizing trauma to the mice.

2.4. Sample collection and tissue processing

Prior to opening the stomach, it was washed with 0.5 mL of saline and the solution was stored in microcentrifuge tubes to measure the pH of gastric contents. Blood samples were collected via cardiac puncture to determine the levels of drugs, TxB2 and PGE_2 in the plasma, and levels of cytokines in the serum. After scoring ulcers in stomachs, the tissues were placed in a neutral buffered formalin solution for 48 h and then transferred to 70% ethanol for further histopathological processing. The tissues were embedded in paraffin, cut into 5 μm size sections and stained (with hematoxylin and eosin) for histopathological evaluation.

2.5. Quantification of pH in gastric content

The pHs of gastric contents were measured using pH indicator strips (Catalog number 9590, EMD Chemicals Inc., Gibbstown, NJ) in the range of 1–14 pH units [35].

2.6. Quantification of drugs in blood

Blood samples were collected in tubes containing K_2EDTA solution (final concentration of 1.2 mg/mL EDTA in the blood) and subsequently mixed and stored on ice until centrifugation. Blood samples were centrifuged at 4000 rpm for 10 m at 4 °C. Plasma samples were then collected and stored at −80 °C until analyzed by LC-MS/MS [6].