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Attenuation of gastric mucosal inflammation induced by indomethacin through activation of the A_{2A} adenosine receptor in rats

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Abstract

Background—Nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin induce gastric mucosal lesions in part by the activation of inflammatory cells and the production of proinflammatory cytokines. The activation of adenosine A_{2A} receptors inhibits inflammation by increasing cyclic AMP in leukocytes and reducing both the production of various proinflammatory cytokines and neutrophil chemotaxis. The aim of present study was to determine whether administration of an orally active adenosine A_{2A} receptor agonist (4-[3-[6-amino-9-(5-cyclopropylcarbamoyl)-3,4-dihydroxy-tetrahydro-furan-2-yl]-9H-purin-2-yl]-prop-2-ynyl]-piperidine-1-carboxylic acid methyl ester; ATL-313) ameliorated indomethacin-induced gastric mucosal lesions in rats.

Methods—Gastric lesions were produced by oral gavage of indomethacin (30 mg/kg). ATL-313 (1–10 µg/kg) was given orally just before the indomethacin administration.

Results—The ulcer index induced by indomethacin was significantly (>50%) reduced by pretreatment with ATL-313 and this effect was blocked completely by the addition of equimolar ZM241385, a selective A_{2A} receptor antagonist. The gastric content of myeloperoxidase (MPO)

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and proinflammatory cytokines was significantly reduced by 10 µg/kg ATL-313, but gastric mucosal prostaglandin 2 (PGE₂) was not affected.

Conclusion—We conclude that ATL-313 does not inhibit the mucosal damaging effect of indomethacin, but it does block secondary injury due to stomach inflammation. A_{2A} agonists may represent a class of new therapeutic drugs for NSAID-induced gastric ulcers.

Keywords

NSAIDs; Gastric ulcer; Adenosine A_{2A} receptor

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin are the most widely used anti-inflammatory analgesic agents [1,2]. However, gastrointestinal injury is a serious adverse effect of NSAIDs, and effective strategies to protect the gastrointestinal mucosa are being sought. Many previous studies have investigated the mechanisms of the development of NSAID-induced gastric mucosal lesions [3–5]. NSAIDs may cause gastric lesions by inhibiting cyclooxygenase (COX) and reducing prostaglandin (PG) production. However, the exact pathogenic mechanism remains to be elucidated [6]. Intraperitoneal injection of anti-neutrophil serum or the immunoneutralization of adhesion molecules on the cell surfaces of neutrophils and endothelial cells significantly attenuates the gastric mucosal injury induced by NSAIDs [7,8]. Therefore, the activation and infiltration of neutrophils into the stomach appears to contribute to the gastric mucosal lesions induced by NSAIDs.

Adenosine is a primordial signaling molecule that elicits numerous physiological responses in all mammalian tissues. The receptor-mediated effects of adenosine are mediated by four G-protein-coupled receptors (A₁, A_{2A}, A_{2B}, and A₃) [9,10]. They are variably expressed on immune cells, depending on cell type and species. A_{2A} receptors are found on bone marrow-derived cells, including neutrophils, monocytes, macrophages, lymphocytes, platelets, and some mast cells [11]. The activation of A_{2A} receptors on immune cells produces a series of responses that, in general, can be categorized as anti-inflammatory [12]. In vivo studies have demonstrated that the activation of A_{2A} receptors attenuates ischemia/reperfusion injury in heart, lung, liver, and kidney by reducing lymphocyte activation and the subsequent expression of adhesion molecules on endothelial cells, neutrophil adhesion and extravasation, and superoxide generation [13–16]. This occurs in part due to the activation of A_{2A} receptors on human or mouse monocytes and macrophages which inhibit the secretion of the proinflammatory cytokines, interleukin (IL)-12 and tumor necrosis factor (TNF)-α [17,18]. We have reported that systemic administration of the selective adenosine A_{2A} agonist, ATL-146e, reduces gastric mucosal lesions induced by stress and aspirin [19,20]. On the basis of this evidence, we hypothesized that the activation of adenosine A_{2A} receptors with an orally active A_{2A} agonist might reduce the gastric mucosal injury induced by NSAIDs. In the present study, we used ATL-313, a new adenosine A_{2A} agonist that, unlike ATL146e, is orally active. [21]. We now report that the oral administration of ATL-313 to rats inhibits TNF-α, IL-1β, and growth related oncogene/cytokine-induced neutrophil chemoattractant-1 (GRO/CINC-1) production and neutrophil accumulation following the gastric injury induced by indomethacin, without affecting mucosal prostaglandin E₂ (PGE₂) concentration.

Materials and methods

Animals

Male Sprague–Dawley rats weighing 250–300 g were fed on a standard laboratory diet and water ad libitum, and kept in cages in a temperature- and humidity-controlled room with a 12-h dark-light cycle before and during the experiment. Prior to the administration of indomethacin, the animals were deprived of food for 24 h but had free access to water. This experimental protocol was approved by the Akita University Animal Care Committee.

Chemicals

4-[3-[6-Amino-9-(5-cyclopropylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl]-piperidine-1-carboxylic acid methyl ester (ATL-313) was synthesized and provided as a gift from the Adenosine Therapeutics Group of PGxHealth (Charlottesville, VA, USA) [21]. ATL-313 was dissolved in a small volume of dimethylsulfoxide and then diluted more than 100-fold with physiological saline just prior to administration.

Effect of ATL-313 on indomethacin-induced gastric mucosal injury model—

Indomethacin-induced gastric injury was produced by the intragastric administration of indomethacin (30 mg/kg). Indomethacin was dissolved in 3% sodium bicarbonate solution, and 0.5 ml of the solution was administered. ATL-313 (1, 2.5, 5, 10 µg/kg; $n = 5$) or vehicle was given by oral gavage, immediately followed by indomethacin administration. Control rats were given physiological saline. The animals were killed by stunning and cervical dislocation 6 h after indomethacin administration and the stomach was removed. The gastric mucosal lesions were measured by two blinded observers. The ulcer index was calculated as the sum of the lengths of all lesions [22]. In some experiments rats received ATL-313 (10 µg/kg) and ZM-242385, a selective A_{2A} antagonist (10 µg/kg) to determine whether the effects of ATL-313 were specifically mediated via the A_{2A} receptor.

Effect of ATL-313 on myeloperoxidase concentration in the gastric mucosa—

An assay of gastric myeloperoxidase (MPO) concentration was used to quantify the degree of neutrophil infiltration. A modification of the method described by Krawisz et al. [23] was used. Briefly, 100 mg of gastric tissue was placed in a polypropylene tube in 2 ml of 0.5% (vol/vol) hexadecyltrimethylammonium bromide (HTAB) buffer in 50 mmol/l phosphate buffer, pH 6.0. Samples were homogenized for 2 s, and then the homogenates were centrifuged at 10,000g for 10 min. One hundred microliters of supernatant was combined with 2 ml of 50 mmol/l phosphate buffer, pH 6.0. The change in absorbance was measured by spectrophotometry at 460 nm for up to 2 min. One unit of MPO is defined as the amount of enzyme that will degrade 1 mol of hydrogen peroxide per min at 25°C, pH 6.0.

Effect of ATL-313 on gastric concentrations of proinflammatory cytokines—

One hundred milligrams of scraped mucosa was homogenized for 30 s with a Polytron homogenizer (PT 1200; Kinematica, Littau, Switzerland) in 1.0 ml of ice-cold potassium phosphate buffer (pH 7.4). Following centrifugation at 10,000g for 10 min, aliquots of homogenate supernatants in phosphate-buffered saline (PBS) were collected. The total protein was measured by Bradford's method [24]. The concentration of TNF- α , IL-1 β , and GRO/CINC-1 in the supernatant of mucosal homogenates was determined with an enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. After color development, optimal density was measured with a microplate reader.

Effect of ATL-313 on mucosal content of PGE₂—A portion of fundic mucosa (about 100 mg) was excised for the determination of PGE₂. The samples were weighed, finely minced with scissors for 15 s, then suspended in 1.0 ml of 10 mM sodium phosphate buffer (pH 7.4). The samples were then incubated in a shaking bath (37°C) for 29 min, followed by centrifugation (9000g for 30 s). The supernatant was frozen for subsequent determination of PGE₂ by radioimmunoassay, using a PGE₂ [¹²⁵I] RIA kit (Dupont/NEN, Boston, MA, USA).

Statistical analysis

All data values were expressed as means $\pm$ SEM. Statistical significance was determined by the Mann–Whitney *U* test, using the Statview-J 5.0 statistics program (Abacus Concepts, Berkeley, CA, USA). *P* values of <0.05 were considered statistically significant.

Results

Effect of ATL-313 on indomethacin-induced gastric lesions

Administration of indomethacin resulted in the appearance of linear and punctate erosions in the gastric mucosa of vehicle-treated rats. In contrast, pretreatment with ATL-313 resulted in smaller erosions. The total length of gastric erosions (ulcer index) in control rats was 50.8 ± 6.68 mm. The ulcer index in rats pretreated with 1 $\mu\text{g/kg}$ of ATL-313 did not show significant suppression, but the index was suppressed to 39.8 ± 4.78 mm with 2.5 $\mu\text{g/kg}$ ($P < 0.05$), 23.3 ± 3.38 mm with 5.0 $\mu\text{g/kg}$ ($P < 0.001$), and 23.2 ± 7.4 mm with 10.0 $\mu\text{g/kg}$ ($P < 0.001$). The selective adenosine A_{2A} antagonist ZM241385 inhibited the effect of ATL-313, to reduce the ulcer index (Fig. 1). The protective effect of ATL-313 was also confirmed histologically. Indomethacin administration resulted in large areas of epithelial crypt loss, predominant neutrophilic infiltration, erosions, and mucosal bleeding. Treatment with ATL-313 reduced these pathological changes (Fig. 2).

Effect of ATL-313 on MPO activity in the gastric mucosa

Tissue MPO concentration in the gastric mucosa increased 6 h after indomethacin administration. The MPO activity in normal control animals (1.85 ± 0.27 U/mg protein) was increased in vehicle-treated rats to 2.69 ± 0.37 U/mg protein. The increase in MPO activity in the gastric mucosa induced by indomethacin was significantly suppressed by treatment with ATL-313 to 2.10 ± 0.26 U/mg protein with 2.5 $\mu\text{g/kg}$ ($P < 0.05$), 1.76 ± 0.21 U/mg protein with 5 $\mu\text{g/kg}$ ($P < 0.01$), and 1.75 ± 0.26 U/mg protein with 10 $\mu\text{g/kg}$ ($P < 0.01$) compared with vehicle-treated rats (Fig. 3a). The increase in MPO above control levels caused by indomethacin was reduced to near normal levels by 5 and 10 $\mu\text{g/kg}$ ATL-313.

Effects of ATL-313 on gastric concentrations of proinflammatory cytokines

Gastric mucosal concentrations of TNF- α and IL-1 β were significantly increased 6 h after the administration of indomethacin. ATL-313 at doses of 5.0 and 10 $\mu\text{g/kg}$ significantly suppressed the increase in tissue IL-1 β and TNF- α in the gastric mucosa following indomethacin administration. The increase in IL-1 β above control levels caused by indomethacin was reduced by 25.0 and 52.0%, respectively, by 5.0 and 10 $\mu\text{g/kg}$ ATL-313 (Fig. 3b). The increase in TNF- α above control levels caused by indomethacin was reduced by 37.0 and 42.0%, respectively, by 5.0 and 10 $\mu\text{g/kg}$ ATL-313 (Fig. 3c). The gastric mucosal concentration of GRO/CINC-1 increased significantly, from a basal concentration of 0.095 ± 0.07 pg/mg protein to 1.89 ± 0.28 pg/mg protein, 6 h after the administration of indomethacin. The concentration of GRO/CINC-1 was also suppressed by ATL-313, to 0.78 ± 0.27 pg/mg protein with 5 $\mu\text{g/kg}$ ($P < 0.05$) and 0.74 ± 0.31 pg/mg protein with 10 $\mu\text{g/kg}$ ($P < 0.05$) compared with vehicle-treated rats (Fig. 3d).

Effect of ATL-313 on mucosal content of PGE2

The concentration of PGE2 was 242 ± 20.3 pg/mg in vehicle-treated rats and it was reduced to 10.3 ± 1.8 pg/mg in indomethacin-treated animals (95.8% reduction; $P < 0.01$). ATL-313 (10 μ g/kg) administration did not interfere with the reduction of gastric PGE2 concentration induced by indomethacin (Fig. 4).

Discussion

This study has demonstrated that the oral administration of the adenosine A_{2A} agonist, ATL-313, reduced by more than 50% the extent of gastric mucosal lesions induced by indomethacin. This protection was reversed by cotreatment with an equimolar concentration of the selective A_{2A}R antagonist, ZM-241385. Gastric protection is correlated with the inhibition of neutrophil infiltration into the gastric mucosal tissue and the inhibition of proinflammatory cytokine production in gastric mucosal tissue. Previous studies have shown that gastric mucosal MPO activity, a biochemical indicator of neutrophil infiltration, increases with the development of gastric mucosal lesions [23] and that depletion of neutrophils reduces injury. These findings suggest that the component of indomethacin-induced gastric injury that is susceptible to inhibition by ATL-313 may correspond to the secondary injury that occurs as a result of gastric inflammation. These findings are consistent with recent studies reporting that gastric mucosal lesions can be reduced by the administration of antibodies against TNF- α [25]. Therefore, we postulate that the infiltration of neutrophils in rat gastric mucosa after indomethacin administration could have occurred in response to the gastric production of proinflammatory cytokines that recruit neutrophils to the injured mucosa, exacerbating injury.

Adenosine has been identified as an endogenous anti-inflammatory agent, because activation of the A_{2A} receptor is known to increase intracellular cAMP levels and to reduce diverse leukocyte inflammatory functions [26]. Ross et al. [14] demonstrated that the selective A_{2A} receptor agonist, ATL-146e, protected lung from reperfusion injury by reducing neutrophil sequestration. Our recent study reported that systemic ATL-146e administration inhibited water-immersion stress- and aspirin-induced gastric injury, neutrophil accumulation, and proinflammatory cytokine production [19,20]. ATL-146e is rapidly metabolized in rodents and is not orally active. On the other hand, ATL-313 is not metabolized and is orally active [27]. These findings led us to examine whether the oral administration of ATL313 could reduce NSAID-induced gastric mucosal injury by reducing gastric inflammation. In the present study, we demonstrated that MPO activity, which is an index of tissue-associated neutrophil accumulation, was increased in the gastric mucosa 6 h after indomethacin administration. The increase in MPO activity was significantly inhibited by treatment with ATL-313.

TNF- α is a proinflammatory cytokine and has recently been shown to be a major contributor to NSAID-induced gastric mucosal injury [28]. Also, TNF- α is a cytokine that strongly stimulates neutrophil adherence, by inducing the synthesis and expression of adhesion molecules on endothelial cells and neutrophils [29,30]. TNF- α augments neutrophil-derived superoxide generation, upregulates the expression of adhesion molecules on neutrophils and endothelium, and stimulates the production of IL-1 β , GRO/CINC-1, and other cytokines, leading to neutrophil accumulation [29,30]. Furthermore, studies with experimental models have shown that intravenous administration of TNF- α produces extensive neutrophil infiltration within the microvasculature of the digestive tract. In addition, portal infusion of TNF- α caused gastric and small-intestinal damage in rats [31]. Several studies have shown that proinflammatory cytokine production is inhibited through the activation of the adenosine A_{2A} receptor by ATL-313 in vivo and in vitro [32–34]. The present study has demonstrated that ATL-313 treatment inhibited the increase in TNF- α , IL-1 β , and GRO/

CINC-1 concentrations in the gastric mucosa after indomethacin administration. These findings strongly support the hypothesis that the oral administration of ATL-313 attenuates NSAID-induced neutrophil accumulation by inhibiting the production of proinflammatory cytokines in the gastric mucosa.

Andrews et al. [6] reported that the expression of intercellular adhesion molecule (ICAM)-1 on endothelial cells was increased by NSAIDs and that adhesion molecules played an important role in the pathogenesis of NSAID-induced gut injury. Okusa et al. [13] reported that an adenosine agonist inhibited ischemic reperfusion injury of the kidney not only by reducing neutrophil accumulation but also by reducing the expression of the adhesion molecules P-selectin and ICAM-1 on the reperfused vascular endothelium. In addition to being found on inflammatory cells, adenosine receptors are also found on endothelial and epithelial cells, but these are predominantly A_{2B} [35,36]. Moreover, the tissue-protective effects of A_{2A} agonists in liver, heart, and kidney have been attributed to bone-marrow derived cells. Therefore, the anti-ulcer effects of A_{2A} receptor agonists likely are predominantly mediated by receptors on inflammatory cells that regulate neutrophil trafficking to the gastric mucosa.

It is well known that various anti-secretory agents, such as H₂-receptor antagonists and proton pump inhibitors, prevent gastric lesions [37]. In a previous study, we reported that ATL-146e significantly increased gastric acid secretion, and we noted that the protective effect of the adenosine A_{2A} receptor agonist was not due to reduced gastric acid secretion [20]. In the present study, ATL-313 also stimulated gastric acid secretion (data not shown). Further experiments have to be performed to elucidate the precise mechanisms of stimulation of acid secretion underlying the actions of these adenosine A_{2A} receptor agonists.

It has been reported that PGE₂ prevents gastric mucosal damage by NSAIDs in humans and animals [38]. The protective effect of ATL-313 did not depend on gastric mucosal PG synthesis, since pretreatment with ATL-313, which reduced gastric damage, had no effect on the gastric mucosal PG concentration. Therefore, the inhibition of mucosal lesions by ATL-313 cannot be attributed to gastric acid inhibition and PG synthesis.

In conclusion, the potent and selective adenosine A_{2A} receptor agonist, ATL-313, significantly inhibited the acute gastric mucosal injury induced by indomethacin in rats. This effect may be due, in part, to a reduction of neutrophil infiltration into the gastric mucosa and the inhibition of proinflammatory cytokine production. Our results demonstrate, for the first time, that activation of adenosine A_{2A} receptors inhibits the pathogenesis of indomethacin-induced gastric injury. The modulation of adenosine A_{2A} receptor activity by the oral administration of specific agonists may be clinically useful to reduce NSAID-induced gastric mucosal damage.

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Fig. 1.

Ulcer index of rats 6 h after indomethacin administration with and without treatment with 4-[3-[6-amino-9-(5-cyclopropylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl]-piperidine-1-carboxylic acid methyl ester (*ATL-313*; 1, 2.5, 5, or 10 µg/kg; $n = 5$ in each group); * $P < 0.05$, ** $P < 0.001$, significant difference compared with the indomethacin-alone treated group

Fig. 2. Microscopic findings of gastric mucosa 6 h after administration of indomethacin with and without treatment with ATL-313 (10 $\mu\text{g/kg}$, i.p.). **a** Hemorrhagic mucosal erosions and inflammatory cell infiltration developed in the glandular stomach of rats 6 h after the administration of indomethacin. **b** Pretreatment with ATL-313 at a dose of 10 $\mu\text{g/kg}$ markedly inhibited these changes. **a** and **b** H & E $\times 200$

Fig. 3.

a Effect of ATL-313 on myeloperoxidase (MPO) activity in the gastric mucosa ($n = 5$ in each group); $*P < 0.05$, $**P < 0.01$, significant difference compared with the indomethacin-alone treated group. **b** Effect of ATL-313 on the increase in interleukin 1β (IL- 1β) concentration in the gastric mucosa induced by indomethacin administration ($n = 5$ in each group); $*P < 0.05$, $**P < 0.01$, significant difference compared with indomethacin-alone treated group. **c** Effect of ATL-313 on the increase in tumor necrosis factor α (TNF- α) concentration in the gastric mucosa induced by indomethacin administration ($n = 5$ in each group); $*P < 0.05$, $**P < 0.01$, significant difference compared with indomethacin-alone treated group. **d** Effect of ATL-313 on the increase in growth related oncogene/cytokine-induced neutrophil chemoattractant-1 (GRO/CINC-1) concentration in the gastric mucosa induced by indomethacin administration ($n = 5$ in each group); $*P < 0.05$ significant difference compared with indomethacin-alone treated group

Fig. 4. Gastric mucosal concentrations of prostaglandin E2 (PGE2) in untreated rats (control group), in rats treated with 30 mg/kg indomethacin alone, and in rats treated with 10 µg/kg ATL-313 ($n = 5$ in each group); $P < 0.001$, significant difference compared with control group; *N.S.* no significant differences between groups