Co-administration of Water Containing Magnesium Ion Prevents Loxoprofen-Induced Lesions in Gastric Mucosa of Adjuvant-Induced Arthritis Rat

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Non-steroidal anti-inflammatory drugs (NSAIDs) comprise one of the most frequently used classes of medicines in the world; however, NSAIDs have significant side effects, such as gastroenteropathy, and rheumatoid arthritis patients taking NSAIDs are more susceptible to NSAID-induced gastric lesions as compared to patients with other diseases. In Asian countries, loxoprofen has been used clinically for many years as a standard NSAID. We demonstrate the preventive effect of the co-administration of water containing magnesium ion (magnesium water, 1–200 µg/kg) on the ulcerogenic response to loxoprofen in adjuvant-induced arthritis (AA) rats. Oral administration of loxoprofen (100 mg/kg) caused hemorrhagic lesions in the gastric mucosa of AA rats 14 d after adjuvant injection, and, following loxoprofen administration, the lesion score of AA rats was significantly higher than that of normal rats. The expression of inducible nitric oxide synthase (iNOS) mRNA and nitric oxide (NO) production in the gastric mucosa of AA rats were also increased by the administration of loxoprofen, and the increase in lesions and NO were prevented by the administration of aminoguanidine, an iNOS inhibitor. The co-administration of magnesium water decreased the ulcerogenic response to loxoprofen in AA rats. In addition, the co-administration of magnesium water attenuated the increase in iNOS mRNA expression and NO production in AA rats receiving loxoprofen. These results suggest that the oral co-administration of magnesium water to AA rats has a potent preventive effect on the ulcerogenic response to loxoprofen, probably by inhibiting the rise in iNOS and NO levels in the gastric mucosa.

Key words magnesium ion; gastric lesion; loxoprofen; adjuvant-induced arthritis; nitric oxide

Rheumatoid arthritis (RA) is a complex chronic inflammatory disease dependent on multiple interacting environmental and genetic factors, such as T cells, neutrophils, activated T lymphocytes, macrophages, eicosanoids and cytokines, making it difficult to understand its pathogenesis and thereby to find effective therapies. In treatments for RA, the focus is on the reduction of pain, inflammation and joint damage. The principal pharmacological agents are nonsteroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs, glucocorticoids and specific inhibitors of the mediator response. Gastroenteropathy is the most common side effect among patients taking NSAIDs, and RA patients taking NSAIDs are more susceptible to NSAIDs-induced gastric lesions in comparison with other patients. Therefore, the development of NSAIDs that do not cause gastroenteropathy is highly requested. We are attempting to develop a method of administration of NSAIDs that will not lead to gastroenteropathy.

Cyclooxygenase-1 and 2 (COX-1 and COX-2) are responsible for the majority of COX activity at the gastrointesinal mucosa and in tissues with inflammation, respectively, and it is reasonable to speculate that selective COX-2 inhibitors can maintain anti-inflammatory activity without gastrointestinal side effects. It has been reported that selective COX-2 inhibitors (such as rofecoxib and celecoxib) provide a greatly reduced incidence of gastroduodenal lesions. However, a recently raised issue concerning the use of selective COX-2 inhibitors is their potential risk for inciting cardiovascular thrombotic events, and rofecoxib has been withdrawn from the worldwide market. Therefore, NSAIDs exhibiting gastrointestinal safety, other than selective COX-2 inhibitors, are clinically important.

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Loxoprofen has been used clinically for many years as a standard NSAID in Japan. Loxoprofen is a pro-drug that is converted (by reduction of the cyclopentanone moiety) to its active metabolite (the trans-alcohol metabolite of loxoprofen) by aromatic aldehyde-ketone reductase only after absorption into the gastrointestinal tract, and loxoprofen has relatively lower membrane permeabilization activities and cytotoxic effects on gastric mucosal cells than other NSAIDs. Based on these findings, loxoprofen is suggested to be safer than other NSAIDs, such as indomethacin. However, even loxoprofen causes gastroenteropathy as a clinical side effect. Recently, we reported that the co-administration of magnesium ion and indomethacin suppresses hemorrhagic lesions in the gastric mucosa of adjuvant-induced arthritis (AA) rats. Therefore, it is of interest to test whether loxoprofen produces lesions in the gastric mucosa, similar to indomethacin. We demonstrate the preventive effect of water containing Mg2+ on the ulcerogenic response to loxoprofen in AA rats.

MATERIALS AND METHODS

Animals and Materials The rats used were male Dark Agouti (DA) rats, aged 6 to 13 weeks, provided by Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). They were housed under standard conditions (12 h/d fluorescent light (07:00–19:00), 25°C room temperature) and given a commercial diet (CE-2, Clea Japan Inc., Tokyo, Japan) and water.

Magnesium water, including only magnesium, was prepared by adding MgSO4 to purified water (PW). Aminoguanidine (Sigma-Aldrich Japan, Tokyo, Japan) was dissolved in saline. Loxoprofen sodium (loxoprofen) was purchased from Wako (Osaka, Japan). All other chemicals used were of the highest purity available.

Animals and Materials

The authors declare no conflict of interest.

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commercially available purity. All animal experiments were performed in accordance with the Kinki University School of Pharmacy Committee for the Care and Use of Laboratory Animals.

**Induction of Arthritis in DA Rats** Arthritis was induced by the injection of 50 µL of adjuvant, a suspension of 10 mg/mL heat-killed *Mycobacterium butyricum* (Difco, Detroit, MI, U.S.A.) in Bayol F oil, into the plantar region of the right hind foot and tail of DA rats. The control group received 50 µL of Bayol F oil (normal rats). In this study, inflammation during the development of AA was assessed by measuring paw edema, one of the parameters of inflammation. The paw edema of arthritis was assessed by measuring the paw volume by plethysmometry, and was quantified by the following equation (Eq. 1):

\[
paw\text{ edema} (\Delta mL) = paw\text{ volume of arthritis rat} - paw\text{ volume of normal rat}
\]

(Eq. 1)

**Evaluation of Gastric Mucosal Lesions** Loxoprofen was suspended in PW or magnesium water containing 1.0% carboxymethyl cellulose (Nacalai Tesque, Kyoto, Japan). The rats were fasted for 18 h before the experiments, but had free access to water. The rats were co-administered PW, magnesium water or aminoguanidine (20 mg/kg)(1) and loxoprofen (100 mg/kg) orally, and killed under deep ether anesthesia 6 h later. The stomachs were excised, washed and fixed in 10% formalin solution, and the area of gastric glandular mucosal lesions was observed in digital photographs and quantified with an image processing program (Win Roof, Fukui, Japan). The lesion area is expressed as a percentage of the total area of glandular stomach except the fundus.

**RNA Preparation** Total RNAs were extracted from the gastric mucosa of rats by the acid guanidium thiocyanate-phenol-chloroform extraction method(25) using Trizol reagent (Life Technologies Inc., Rockville, U.S.A.) according to the manufacturer’s instructions. The purity and concentrations of RNA were determined spectrophotometrically. The OD260/OD280 values of all RNA samples used were greater than 1.8, which indicates low protein contamination and high RNA purity.

**Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)** The RT reaction was performed using an RNA PCR kit (AMV Ver. 2.1, Takara Bio Inc., Shiga, Japan). One microgram of total RNA was mixed with 3 µL of 10 mM Tris–HCl buffer (pH 8.3) containing 5 mM MgCl2 and 50 mM KCl. The following components were then added to give a final volume of 10 µL: 1 unit/µL RNase inhibitor, 10 mM deoxynucleotide triphosphate, 2.5 units/µL reverse transcriptase, and 0.125 µM oligo dT-adaptor primer. The RT reaction was performed at 42°C for 15 min, followed by 5 min at 95°C. PCR reactions were performed using LightCycler FastStart DNA Master SYBR Green I according to the manufacturer’s instructions (Roche Diagnostics Applied Science, Mannheim, Germany). Briefly, 2 µL of cDNA was mixed with 2 µL of reaction mixture, LightCycler FastStart DNA Master SYBR Green I Reaction Mix, containing FastStart Taq DNA Polymerase, reaction buffer, MgCl2, SYBR Green I dye, and deoxynucleotide triphosphate mix. The following components were then added to give a final volume of 20 µL containing specific primers for inducible nitric oxide synthase (iNOS) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 10 pmol each). The following primers were used: 5’-GGA GAGATTGTTTCGACGACC-3’ and 5’-CCATGCTA ATTTGGACTTGCAGA-3’ for iNOS (GenBank accession No. NM_012611); and 5’-ACGCCAGCTCAGGCTGAGA-3’ and 5’-CGTCCTCTGAA GATGGT GAT-3’ for GAPDH (GenBank accession No. NM_017008). The PCR conditions were 95°C for 10 min, 50 cycles of 95°C for 10 s (denaturing), 60°C for 10 s (annealing), and 72°C for 5 s (extension). The quantities of the PCR products were measured fluorometrically in a real-time manner using a LightCycler DX 400 (Roche Diagnostics Applied Science, Mannheim, Germany). After completion of the PCR reactions, dissociation curves of the PCR products were generated using the LightCycler Software Version 4.0 program to detect nonspecific amplification, including primer-dimers, and to ascertain the quality of the amplification data. The differences in the threshold cycles for GAPDH and iNOS were used to calculate the levels of mRNA expression in the rats.

**Measurement of Protein** Protein levels in the gastric mucosa of rats were determined according to the method of Bradford(26) using a Bio-Rad Protein Assay Kit (BIO-RAD, California, U.S.A.) with bovine serum albumin as the standard.

**Measurement of Nitric Oxide (NO) Level** The gastric mucosae of rats were excised, and stored at −80°C until used. The gastric mucosae were homogenized in saline on ice, and the homogenates were centrifuged at 10000 rpm for 15 min at 4°C. The resultant supernatants were used for measurements of NO level. A concentric microdialysis probe (A-1-20-05, 5 mm length; Eicom, Kyoto, Japan) was placed in the supernatant and perfused with Ringer’s solution (140 mM NaCl, 4 mM KCl, 1.26 mM CaCl2, and 1.15 mM MgCl2, pH 7.4) at a constant flow rate of 2 µL/min using a micro syringe pump (ESP-64, Eicom, Kyoto, Japan). NO2− and NO3− in the supernatant were separated on a reverse-phase separation column packed with polystryrene polymer (NO-PAK, 4.6×50 mm, Eicom, Kyoto, Japan); NO2− was reduced to NO3− in a reduction column packed with copper-plated cadmium filings (NO-RED, Eicom, Kyoto, Japan). NO2− was mixed with Griess reagent to form a purple azo dye in a reaction coil, and placed in a column oven set at 35°C. The absorbance of the colored product dye at 540 nm was determined by a flow-through spectrophotometer (NOD-10, Eicom, Kyoto, Japan) with a mobile phase consisting of 10% methanol containing 0.15 mM NaCl–NH4Cl and 0.5 g/L Na2-ethylenediaminetetraacetic acid (EDTA) delivered by a pump at a flow rate of 0.33 mL/min. The Griess reagent, 1.25% HCl containing 5 g/L sulfanilamide with 0.25 g/L N-naphthyllylenediamine, was delivered at a rate of 0.1 mL/ min. In this paper, NO amounts reflect the level of the NO2− metabolite, which is produced from NO.

**Assay of Plasma Loxoprofen Concentration** On the day before loxoprofen administration, a cannula filled with 30 µg/mL heparin (silicone tubing; i.d. 0.5 mm, o.d. 1.0 mm) was inserted into the right jugular vein of AA rats under pentobarbital anesthesia (30 mg/kg, intraperitoneally (i.p.)). One milliliter per kilogram of loxoprofen solution (1.2, 100 mg/kg) suspended in PW or magnesium water (Mg2+, 200 µg/kg) was administered orally to the AA rats 14 d after adjuvant injection. Venous blood (200 µL) was collected at 0, 5, 15, 30, 60,
90, 120, 180 and 240 min after the oral administration of loxoprofen from the jugular vein through the cannula. The blood was centrifuged at 3000 rpm for 20 min at 4°C, and the plasma obtained was stored at −80°C until loxoprofen analysis by the following HPLC method. Fifty microliters of plasma was added to 100 µL methanol containing 1 µg·n-butyl·p-hydroxybenzoate (internal standard), and the mixture was filtered through a Chromatodisk 4A (pore size of 0.45 µm, Kurabo Industries Ltd., Osaka, Japan). The filtrate (10 µL) was injected into an Inertsil ODS-3 (3 µm, column size: 2.1 mm×50 mm) column (GL Sciences Inc., Tokyo, Japan) using a LC-Net II/ADC system (JASCO Co., Tokyo, Japan). The mobile phase consisted of acetonitrile–water–acetic acid (40:60:1 by vol.) at a flow rate of 0.25 mL/min; the wavelength for detection was 225 nm.

The loxoprofen concentration in the serum after a single injection of 0.3 mL of loxoprofen solution (1.2 mg/kg) into the femoral vein was analyzed by Eq. 2:

\[ C_{\text{LOX}} = C_0 \cdot e^{-k_e t} \]  

where \( C_{\text{LOX}} \) is loxoprofen concentration in the serum, \( C_0 \) is the initial concentration of loxoprofen in the serum (2.47±0.03 µg/mL), \( k_e \) is the elimination rate constant for loxoprofen from the serum. The \( k_e \) and distribution volume (\( V_d \)) data obtained from 5 experiments were 0.90±0.081 h\(^{-1}\) and 347.2±9.6 mL/kg, respectively. In addition, linearity was observed for \( C_0 \) up to 120 µg/mL (in a single injection of 60 mg/kg loxoprofen solution).

The serum loxoprofen concentration data after the oral administration of 0.3 mL of loxoprofen solution were analyzed by Eq. 3:

\[ C_{\text{LOX}} = \frac{k_a \cdot F \cdot D}{V_d(k_0 - k_e)} (e^{-k_e t} + e^{-k_t t}) \]  

where \( C_{\text{LOX}} \) is the loxoprofen concentration in the serum, \( D \) is the dose of loxoprofen administered (1.2, 100 mg/kg), \( k_0 \) is the absorption rate constant, \( t \) is time (0–4 h) after loxoprofen administration, \( V_d \) is the distribution volume, \( F \) is the fraction of loxoprofen absorbed. A nonlinear least-squares computer program (MULTI) was employed for the calculation.

The area under the loxoprofen concentration–time curve (\( AUC_{\infty} \)) was calculated according to the following equation (Eq. 4):

\[ AUC_{\infty} = \int_0^{4h} C_{\text{LOX}} dt + \frac{C_{\text{LOX at 4h}}}{k_e} \]  

Briefly, \( AUC_{\infty} \) was determined according to the trapezoidal rule up to 4 h, which was the time of the final loxoprofen concentration measurement.

**Statistical Analysis** All values are represented as mean± standard error of the mean (S.E.). Statistical differences in multiple groups were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison. \( p \) values less than 0.05 were considered significant.

**RESULTS**

**Gastric Ulcerogenic Response to Loxoprofen in AA Rats**

Figure 1 shows the changes in the volume of paw edema in the right (A) and left (B) hind feet of AA rats. Paw edema in the right hind foot injected with adjuvant appeared on the 1 day following injection, and reached a maximum 14 d after adjuvant injection. On the other hand, no paw edema in the left hind foot, into which adjuvant was not injected, was observed during the first 7 d after adjuvant injection, but clearly increased from 10 d (0.44±0.06, means±S.E. of 10 independent rat), and reached a maximum 14 d after adjuvant injection. Figure 2 shows the changes in the gastric ulcerogenic response to loxoprofen in AA rats. The oral administration of loxoprofen caused hemorrhagic lesions in the gastric mucosa of normal and AA rats; the gastric mucosal lesions scores in AA rats at 7, 21, 28, 35 and 42 d after adjuvant injection were approximately 2.8-fold higher than those of normal rats. The loxoprofen-induced lesion scores of AA rats 14 d after adjuvant injection were higher than in normal rats at any time and AA rats at 7, 21, 28, 35 or 42 d after adjuvant injection; the lesion scores were approximately 7.4-fold higher than those in normal rats. Figure 3 shows the changes in lesion (A), iNOS mRNA (B) and NO (C) levels in loxoprofen-administered AA rats following treatment with or without aminoguanidine. The iNOS mRNA and NO levels in normal and AA rats were increased by the administration of loxoprofen, and were significantly higher in AA rats than in normal rats. The expression of iNOS mRNA showed no difference by treatment with or without aminoguanidine. On the other hand, aminoguanidine attenuated the increases in gastric mucosal lesions and NO
production in normal and AA rats administered loxoprofen.

Preventive Effect of the Co-administration of Magnesium Water on Gastric Lesions in Loxoprofen-Administered AA Rats  
Figure 4 shows the preventive effect of the co-administration of magnesium water on gastric lesions in loxoprofen-administered AA rats 14 d after adjuvant injection. The lesion scores in AA rats co-administered 1 µg/kg Mg²⁺ were similar to those in rats co-administered PW and loxoprofen; however, the co-administration of 10–200 µg/kg Mg²⁺ decreased the area of gastric lesions in AA rats administered loxoprofen, with the area of gastric lesions in AA rats co-administered 25–200 µg/kg Mg²⁺ and loxoprofen being approximately 35.5% that of AA rats administered loxoprofen in suspension in PW. Figure 5 shows the expression of iNOS mRNA (A) and NO production in the gastric mucosa of AA rats administered loxoprofen. iNOS mRNA expression and NO production in the gastric mucosa of AA rats co-administered 1 µg/kg Mg²⁺ and loxoprofen were similar to those in rats co-administered PW and loxoprofen; however, the co-administration of 10–200 µg/kg Mg²⁺ attenuated the increases in iNOS mRNA expression and NO production in AA rats administered loxoprofen. Figure 6 shows the plasma loxoprofen concentration following the oral co-administration of magnesium water (Mg²⁺, 200 µg/kg) and loxoprofen at a therapeutic dose (Fig. 6A, 1.2 mg/kg) or excessive dose (Fig. 6B, 100 mg/kg) in AA rats 14 d after adjuvant injection. The fates of the blood loxoprofen following the administration of both the therapeutic and excessive doses of loxoprofen co-administered with magnesium water were similar to those following the administration of loxoprofen alone. Table 1 shows the pharmacokinetic parameters for the combination of magnesium water and loxoprofen. The elimination rate constant for loxoprofen from the serum (kₑ) is approximately 0.99 h⁻¹, not significantly different than the value (0.90±0.081 h⁻¹, mean±S.E., n=5) obtained for the loxoprofen concentration data in serum after a single injection of loxoprofen solution (1.2 mg/kg) into the femoral vein. In addition, there were no significant differences in the pharmacokinetic parameters (absorption rate constant (kₛ), kₒ, fraction of loxoprofen absorption (F) and AUCₜₐ₀) observed between the co-administration of loxoprofen and magnesium water the administration of loxoprofen alone.

DISCUSSION

L oxoprofen has been used clinically for many years as a standard NSAID in Japan, even though gastroenteropathy is a side effect. Recently, we reported that the oral administration of Mg²⁺ to AA rats prevents the development of inflammation, and that the co-administration of Mg²⁺ and indomethacin suppresses hemorrhagic lesions in the gastric mucosa of AA rats. In this study, we investigated the preventive effect of water containing Mg²⁺ on loxoprofen-induced gastric lesions in AA rats.

When studying agents to protect against RA, the selection of the experimental animal is very important. The AA rat is an animal model in which arthritis is induced by the injection.
of an adjuvant. Inflammatory pain during the development of AA is assessed by measuring paw volume (paw edema).\(^{28,29}\) Paw edema in AA rats is known to involve two inflammatory processes: primary and secondary inflammation. Primary inflammation starts from the day following the injection of adjuvant into the right hind foot. Secondary inflammation is observed beginning 7 days after adjuvant injection, and reaches a maximum 14 days after adjuvant injection into the right and/or left hind foot.\(^{10,11}\) In addition, AA rats taking NSAIDs are more susceptible to NSAID-induced gastric lesions than normal rats.\(^{10,11}\) It is noteworthy that changes in the biological characteristics of AA rats correspond to those that occur in human RA.\(^{10,11,28,29}\) Therefore, AA rats provide a useful model for use in the study of NSAID-induced gastric lesions in RA.

Paw edema in the hind foot injected with adjuvant appears on the day following injection, and reaches a maximum 14 days after adjuvant injection (Fig. 1). The area of the gastric mucosal lesions in AA rats administered loxoprofen (100 mg/kg) was higher than in normal rats; however, the areas of the gastric mucosal lesions induced by the administration of loxoprofen did not differ significantly among AA rats at 7, 21, 28, 35 and 42 days after adjuvant injection (Fig. 2). At 14 days after adjuvant injection, the oral administration of loxoprofen (100 mg/kg) caused severe hemorrhagic lesions in the gastric mucosa of AA rats, and the lesion score of AA rats administered loxoprofen was significantly higher than of normal rats administered loxoprofen (Fig. 2). In addition, the lesion scores for AA rats increased with time (0–24 h) after the oral administration of 100 mg/kg loxoprofen [3 h, 2.55±0.69; 6 h, 5.93±1.00; 9 h, 6.53±1.38; 12 h, 6.30±1.96; 24 h, 7.30±2.04 (% mean±S.E.\(n=3–5\)). On the other hand, the loxoprofen-induced gastric lesion scores for AA rats were lower than in the case of indomethacin-induced gastric lesions [lesion scores 6 h after the administration of 40 mg/kg indomethacin: 2.9±0.53; 100 mg/kg indomethacin: 10.7±0.95 (% mean±S.E. of 3–4 independent rats)]. These results suggest that the enhanced ulcerogenic response to loxoprofen in AA rats may occur during the progression of secondary inflammation, and that loxoprofen is safer than indomethacin. Therefore, we decide on time to induce gastric mucosal lesions by a loxoprofen in 6 h, and used AA rats 14 days after adjuvant injection to demonstrate the preventive effects of the co-administration of magnesium water on the ulcerogenic response to loxoprofen.

The pathogenesis of NSAID-induced gastric lesions is generally considered to involve the depletion of endogenous prostaglandins (PGs) caused by the inhibition of cyclooxygenase activity.\(^{30–32}\) However, it has been reported that NSAIDs induce necrosis and apoptosis in cultured gastric mucosal cells and in the gastric mucosa in a manner independent of COX inhibition,\(^{33–37}\) and that the total inhibition of PG production

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**Fig. 4. Gastric Images (A) and Lesion Score (B) of the Gastric Mucosa of AA Rats Co-administered Magnesium Water and Loxoprofen**

AA rats 14 days after adjuvant injection were orally administered loxoprofen (100 mg/kg) in PW or magnesium water, and killed 6 h later. The data are presented as means±S.E. of 3–5 independent rats. *p<0.05 vs. PW and loxoprofen-administered rats.
in the stomach causes very little gastric damage in rats. Therefore it is now believed that the inhibition of COX by NSAIDs is not the sole explanation for the gastrointestinal side effects of NSAIDs, and it is assumed that other mechanisms in addition to PG deficiency are involved in the gastric ulcerogenicity of NSAIDs. Recently, Mizushima et al. clearly showed that the primary targets of NSAIDs in the induction of necrosis and apoptosis are the cytoplasmic membranes. In addition, Kato et al. reported that excessive NO production via iNOS is related to an increased ulcerogenic response to NSAIDs including loxoprofen and indomethacin. We show that the expression of iNOS mRNA and NO production in the gastric mucosa of normal and AA rats are increased by the administration of loxoprofen, and that these increases are significantly higher than those that occur in normal rats administered loxoprofen. In addition, the lesions and NO levels in AA rats administered loxoprofen are prevented by the administration of aminoguanidine, a selective inhibitor of iNOS (Fig. 3). These results suggest that the oral administration of loxoprofen causes excessive NO production via iNOS in gastric mucosa, and that excessive NO relates to the gastric ulcerogenicity of loxoprofen.

It is very important to prevent the ulcerogenic response to loxoprofen, and we have demonstrated the protective effect of magnesium water on loxoprofen-induced gastric lesions in AA rats. Yokoyama et al. reported that Mg\(^{2+}\) deficiency causes

**Table 1. Pharmacokinetic Parameters for Plasma Loxoprofen Concentration after Oral Co-administration of Magnesium Water and Loxoprofen to AA Rats**

<table>
<thead>
<tr>
<th>Loxoprofen</th>
<th>Magnesium ion</th>
<th>(k_a (h^{-1}))</th>
<th>(k_e (h^{-1}))</th>
<th>(F) (%)</th>
<th>(AUC_\infty (\mu g\cdot h/mL))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2 mg/kg</td>
<td>without</td>
<td>9.91±0.71</td>
<td>1.00±0.09</td>
<td>57.3</td>
<td>1.82±0.15</td>
</tr>
<tr>
<td></td>
<td>with</td>
<td>10.10±0.80</td>
<td>0.96±0.08</td>
<td>56.2</td>
<td>1.84±0.16</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>without</td>
<td>11.30±1.02</td>
<td>0.98±0.09</td>
<td>43.5</td>
<td>130.4±6.7</td>
</tr>
<tr>
<td></td>
<td>with</td>
<td>10.93±1.08</td>
<td>1.04±0.09</td>
<td>44.5</td>
<td>127.0±7.2</td>
</tr>
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</table>

One mL/kg of loxoprofen solution (1.2 or 100 mg/kg) with or without magnesium water (Mg\(^{2+}\), 200 µg/kg) was administered orally to AA rats 14 d after adjuvant injection. The absorption rate constant \(k_a\), elimination rate constant \(k_e\), fraction of loxoprofen absorption \(F\)/distribution volume \(V_d\) and \(AUC_\infty\) were calculated using Eqs. 2–4. The \(V_d\) was analyzed using the loxoprofen concentration data in the serum after a single injection of loxoprofen solution (1.2 mg/kg) into the femoral vein; \(F\) was determined based on the \(V_d\) value (347.2 mL/kg). The data are presented as means±S.E. of 5 independent rats.
iNOS activation in rat macrophages. Our previous report also showed that the co-administration of 200 µg/kg Mg²⁺ prevents indomethacin-induced gastric lesions. In addition, we showed that the iNOS gene promoter region containing a nuclear factor kappa B is related to the change in iNOS gene expression in response to Mg²⁺ levels. In this study, the hemorrhagic lesions area, iNOS mRNA and NO levels of AA rats co-administered 1 µg/kg Mg²⁺ were all similar to the PW controls. However, the co-administration of 10–200 µg/kg Mg²⁺ with loxoprofen suppressed the formation of hemorrhagic lesions in the gastric mucosa of AA rats 14 d after adjuvant injection, and iNOS mRNA expression and NO production in the gastric mucosa of AA rats were also decreased by the co-administration of 10–200 µg/kg Mg²⁺ (Figs. 4, 5). Moreover, the preventive effect against gastric mucosal lesions by Mg²⁺ was observed following the administration of 40 mg/kg loxoprofen [PW, 1.87±0.30; 1 µg/kg Mg²⁺, 1.57±0.28; 10 µg/kg Mg²⁺, 0.41±0.13; 100 µg/kg Mg²⁺, 0.39±0.15 (%), means±S.E., n=3]. Taken together, these findings suggest that Mg²⁺ decreases NO production via iNOS, resulting in the prevention of the ulcerogenic response to loxoprofen in AA rats, and that a magnesium dose over 10 µg/kg Mg²⁺ is needed to achieve this prevention. The results support the previously reports showing that loxoprofen has relatively lower membrane permeabilization activities and cytotoxic effects on gastric mucosal cells than other NSAIDs, since the gastric lesion score and concentration of Mg²⁺ to prevent loxoprofen-induced gastric lesions in AA rats are lower than those of indomethacin. In addition, we have demonstrated the effect of magnesium ion on loxoprofen absorption in AA rats. Since loxoprofen is a pro-drug that, after absorption in its unchanged form from the gastrointestinal tract, is transformed into its active metabolite (the trans-alcohol metabolite of loxoprofen) by aromatic aldehyde-ketone reductase, we investigated whether the co-administration of loxoprofen with magnesium would change the plasma concentration of the pro-drug (unchanged form). At both a therapeutic dose (1.2 mg/kg) and an excessive dose (100 mg/kg), the fate of loxoprofen in the blood after co-administration was similar to that when loxoprofen was administered alone, with no significant differences in the pharmacokinetic parameters (absorption rate constant (kₐ), elimination rate constant (kₗ), fraction of loxoprofen absorption (F) and AUCₜₐₗ, Table 1 and Fig. 6). It is likely that the co-administration of magnesium water does not affect the absorption of loxoprofen from the gastrointestinal tract, and may provide an effective administration method for RA patients taking NSAIDs.

Further studies are needed to determine the precise mechanisms for the production of NO in the gastric mucosa of AA rats administered loxoprofen. In addition, it is important to clarify the therapeutic effect of the combination of Mg²⁺ and loxoprofen, since the oral administration of Mg²⁺ alone is also effective in controlling inflammation. Therefore, we are now investigating the changes in inflammation and the plasma active metabolite (the trans-alcohol metabolite of loxoprofen) using AA rats co-administered Mg²⁺ and loxoprofen.

In conclusion, the present study demonstrates that the co-administration of magnesium water with loxoprofen prevents the ulcerogenic response to loxoprofen in AA rats, probably by inhibiting the increase in iNOS and NO levels in the gastric mucosa. These findings provide significant information that can be used to design further studies to optimize the administration method of loxoprofen.

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