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# Intestinal absorption of lysozyme, an egg-white allergen, in rats: Kinetics and effect of NSAIDs

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ABSTRACT

The absorption pathway(s) of a representative food allergen, lysozyme, and the mechanisms of lysozyme absorption facilitated by non-steroidal anti-inflammatory drugs were examined by intestinal closed-loop and re-circulating perfusion methods in rats. The absorption rate of fluorescein isothiocyanate (FITC)-labeled lysozyme in the proximal intestine was higher than that for a marker of non-specific absorption, FD-10, and was suppressed by colchicine (endocytosis inhibitor). Aspirin increased the absorption of FITC-lysozyme in the proximal intestine with no effects on tissue accumulation. Diclofenac facilitated FITC-lysozyme absorption, but meloxicam and loxoprofen exerted no effects on absorption. Co-administration of misoprostol (synthetic prostaglandin-E1 analog) with aspirin significantly ameliorated the aspirin-facilitated absorption of FITC-lysozyme to the same level as that seen with controls. Thus, lysozyme absorption was mediated by endocytic and paracellular pathways in the proximal intestine, and was facilitated by aspirin and diclofenac after impairment of the paracellular pathway. Misoprostol may suppress the allergen absorption facilitated by aspirin.



## 1. Introduction

Food-dependent exercise-induced anaphylaxis (FDEIA) is a peculiar form of food allergy induced by exercise after ingestion of foods that cause anaphylaxis [1,2]. Patients with FDEIA have specific immunoglobulin (Ig) E antibodies to the causative food allergens, and typically present with generalized urticaria, dyspnea and anaphylactic shock induced by a type-I allergic reaction [3,4]. Intake of non-steroidal anti-inflammatory drugs (NSAIDs), especially aspirin, can induce FDEIA symptoms without exercise [5]. Increased absorption of allergen from the intestinal tract by exercise or aspirin is considered to be a key factor in the development of FDEIA symptoms [5–7]. Thus, understanding the absorption mechanisms of allergens in the intestine as a trigger of FEDIA symptoms is important.

Orally ingested macromolecules (e.g., food allergens, protein-based drugs) cannot be absorbed readily in an intact form from the intestinal lumen. However, small amounts of macromolecules are known to be absorbed in the intact form from the intestinal lumen to blood. Ingested food allergens are transported *via* the specialized microfold (M) cells of Peyer's patches or across epithelial cells through the paracellular pathway and transcellular pathway by endocytosis [8]. In normal, the paracellular pathway is regulated by tight junctions and restricts the permeation of macromolecules. Aspirin reduces the production of prostaglandins by inhibiting cyclooxygenases (COXs) and can cause gastrointestinal damage [9,10]. Thus, it is hypothesized that aspirin intake also enhances the permeability of allergens through the paracellular pathway. Recently, Takahashi et al. [11] reported that a synthetic analog of prostaglandin E1, misoprostol, prevents allergic reactions upon the provocation test in FDEIA patients. However, the mechanisms of intestinal absorption of allergens facilitated by aspirin are incompletely understood.

NSAIDs are used widely as anti-pyretic, pain-relieving and anti-inflammatory agents, so avoiding intake of allergens and NSAIDs (even in subjects with FDEIA) is difficult. Therefore, investigating the facilitation of NSAIDs for allergen absorption and finding prophylactic drugs for FDEIA is important.

In the present study, we first investigated the absorption pathways of the egg-white lysozyme as well as the mechanisms of

*Abbreviations:* FITC, fluorescein isothiocyanate; FD, fluorescein isothiocyanate-labeled dextran; FDEIA, food-dependent exercise-induced anaphylaxis; Ig, immunoglobulin; NSAIDs, non-steroidal anti-inflammatory drugs; COX, cyclooxygenase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CBB, coomassie brilliant blue; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; AUC, area under the plasma concentration–time curve.

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lysozyme absorption facilitated by NSAIDs in rat intestines. Next, we evaluated the effects of conventional doses of NSAIDs such as diclofenac, meloxicam and loxoprofen on intestinal absorption of lysozyme. The prophylactic effect of misoprostol on lysozyme absorption facilitated by aspirin was also evaluated.

## 2. Materials and methods

### 2.1. Materials

Lysozyme chloride from egg white and colchicine were obtained from Nacalai Tesque (Kyoto, Japan) and Tokyo Kasei (Tokyo, Japan), respectively. Fluorescein isothiocyanate (FITC), FITC-dextrans (FD-10, average molecular weight, 9.4 kDa; FD-150, average molecular weight, 167 kDa), diclofenac, meloxicam and misoprostol were purchased from Sigma–Aldrich (St. Louis, MO, USA). Aspirin and loxoprofen were from Wako Pure Chemicals (Osaka, Japan). All other chemicals used were of the highest purity available.

### 2.2. Animals

Male Sprague–Dawley (SD) rats aged 7–8 weeks were obtained from Japan SLC, Inc. (Shizuoka, Japan). Experiments involving animals were carried out in accordance with the Guide for Animal Experimentation from the Committee of Research Facilities for Laboratory Animal Sciences of Hiroshima University (Hiroshima, Japan).

### 2.3. Preparation of FITC-labeled lysozyme

Labeling of lysozyme with FITC was undertaken as described previously [12]. Briefly, 2 mg FITC and 200 mg lysozyme were dissolved in 0.1 M borate buffer (pH 9.0). After incubation for 60 min at room temperature, pH was adjusted to 7.5 with 0.1 M boric acid. The solution was dialyzed using cellulose membranes with a molecular-weight cutoff of 3.5 kDa for 48 h at 4 °C and concentrated by freeze drying. When the lyophilized proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), a single band was observed by coomassie brilliant blue (CBB) staining and a Fluoroimage Analyzer (Typhoon FLA-7000, GE Healthcare, Little Chalfont, UK). The band size was the same as that with authentic lysozyme (14.3 kDa), suggesting no degradation of lysozyme during FITC labeling.

### 2.4. Absorption of FITC-lysozyme and FITC-dextrans from the intestinal closed loop

Rats were fasted overnight and anesthetized with pentobarbital (30 mg/kg, i.p.). Anesthetized rats were cannulated with polyethylene tubing (PE-50) at the femoral artery for blood sampling. The proximal intestine (a 20-cm long segment from 5 cm below the opening of the bile duct) was flushed with 20 ml of saline prewarmed at 37 °C and ligated to make a closed loop. Vehicle alone (phosphate-buffered saline (PBS) at pH 6.5 containing 1% (v/v) dimethyl sulfoxide (DMSO)) or aspirin (167 μmol/kg) were administered into the closed loop. Thirty minutes later, FITC-lysozyme (0.7 μmol/kg) was also administered into the same loop. Blood (0.25 ml each) was collected at a designated time interval for 60 min *via* the cannula inserted at the femoral artery. At 60 min, the amount of FITC-lysozyme in the mucosal tissue was measured. The intestinal lumen was washed rapidly with ice-cold, FITC-lysozyme-free PBS. Mucosal tissues of the everted intestine were collected by scraping with a cover glass. Absorption of FD-10 (1.6 μmol/kg) and FD-150 (0.18 μmol/kg) were evaluated in the same manner as that described for FITC-lysozyme. Concentrations of FITC-lysozyme and FITC-dextrans in biological samples were determined using a Microplate Fluorometer (PerkinElmer, Waltham, MA, USA) at a wavelength of 500 nm for excitation and 520 nm for emission.

### 2.5. In situ re-circulating perfusion study of FITC-lysozyme

The intestinal absorption of FITC-lysozyme was evaluated in a re-circulating perfusion manner as described previously with slight modification [13]. Briefly, rats were fasted overnight and anesthetized with pentobarbital (30 mg/kg, i.p.). The proximal (a 20-cm-long segment from 5-cm below the opening of the bile duct) and distal small intestine (a 20-cm-long segment from the ileocecum) were used to elucidate regional differences in the absorption of FITC-lysozyme. Proximal and distal segments were pre-perfused with PBS (pH 6.5 and 7.4) warmed at 37 °C for 15 min, respectively. Then, each intestinal segment was perfused in a re-circulating manner at 1 ml/min with 10 ml of each type of PBS containing FITC-lysozyme (0.35 μM) and 1% DMSO. To examine the effects of modulators, colchicine (100 μM), aspirin (16.7 mM), diclofenac (314 μM), meloxicam (80 μM), loxoprofen (789 μM) and/or misoprostol (1 μM) were added to the perfusate, respectively. The intestinal perfusate (200 μl) was collected periodically to determine the concentrations of FITC-lysozyme. The rate of absorption of FITC-lysozyme was estimated from the rate of disappearance by measuring the FITC-lysozyme remaining in the perfusate between 0.5 h and 1 h. The rate of absorption of FD-10 (0.35 μM) was estimated in the same way as that described for FITC-lysozyme.

### 2.6. Stability of intact FITC-lysozyme in biological samples

The "intactness" of FITC-lysozyme in biological samples was evaluated using Fluoroimage Analyzer FLA-7000 after separation by SDS–PAGE. Plasma, perfusates, and mucosal homogenates were added in loading buffer comprising 65 mM Tris–HCl, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 3% SDS and 0.01% bromophenol blue. These samples were applied to each lane of a 12.5% polyacrylamide gel, and SDS–PAGE conducted. Fractioned proteins were visualized by CBB staining and the Fluoroimage Analyzer. Images were analyzed using ImageQuant TL software (GE Healthcare).

### 2.7. Statistical analyses

Data are the mean ± standard error of the mean (SEM). Differences among mean values between groups were assessed by the Kruskal–Wallis test or ANOVA test followed by a *post hoc* test (Tukey test) or the Student's *t*-test. $P < 0.05$ was considered significant.

## 3. Results

### 3.1. Effect of aspirin on the absorption of FITC-lysozyme and FITC-dextrans from the intestinal closed loop

To evaluate the aspirin-facilitated absorption of FITC-lysozyme into blood, we examined the effect of aspirin on the intestinal absorption of FITC-lysozyme by an *in situ* loop method using the proximal intestine (Fig. 1). Plasma levels of FITC-lysozyme increased gradually with time and reached almost constant levels at 30 min (Fig. 1A). The area under the plasma concentration–time curve from 0 min to 60 min ($AUC_{0-60\ min}$) of FITC-lysozyme in aspirin-treated rats increased significantly by ≈1.8-fold compared with that in controls (Table 1). Accumulation of FITC-lysozyme in mucosal tissue 60 min after intra-luminal administration was not

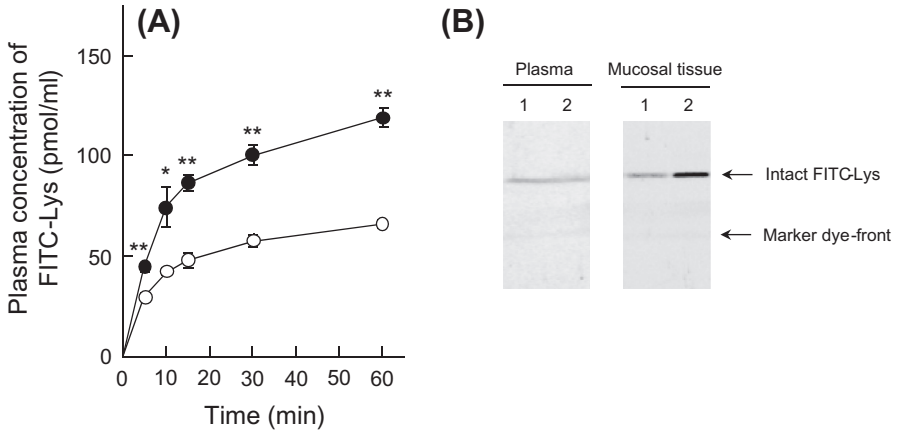


**Fig. 1.** Effect of aspirin on plasma concentrations (A) of FITC-lysozyme and intactness in each biological sample (B) 60 min after administration into the proximal intestinal loop in rats. Aspirin was administered into the closed loop at 167 μmol/kg at 30 min before FITC-lysozyme (FITC-Lys) administration (0.7 μmol/kg). (A) Open and closed circles represent the control and aspirin treatment (167 μmol/kg), respectively. Each value represents the mean ± SEM for four rats. $^{*}P < 0.05$, $^{**}P < 0.01$: significantly different from the values for control. (B) Intactness of FITC-lysozyme in biological samples obtained at 60 min was evaluated by a Fluoroimage Analyzer after separation by SDS–PAGE. Lane 1 is intact FITC-lysozyme and lane 2 is the biological sample, respectively.

**Table 1**
Effect of aspirin on $AUC_{0-60\ min}$ values and mucosal accumulation of FITC-Lys, FD-10 and FD-150 at 60 min after intraluminal administration in the proximal intestine.

| | FITC-Lys | | FD-10 | | FD-150 | |
|---|---|---|---|---|---|---|
| | Control | +Asp | Control | +Asp | Control | +Asp |
| $AUC_{0-60\ min}$ (pmol min/ml) | 3128 ± 134 | 5503 ± 185** | 1092 ± 94 | 1585 ± 136* | 11.8 ± 0.9 | 20.3 ± 0.4** |
| Mucosal accumulation (nmol/g tissue) | 515 ± 27 | 512 ± 57 | n.d. | n.d. | 16.9 ± 1.8 | 19.2 ± 4.3 |

FITC-Lys, FITC-lysozyme; +Asp, aspirin treatment; n.d.; not determined. The doses of FITC-Lys, FD-10 and FD-150 were 0.7, 1.6 and 0.18 μmol/kg, respectively. Aspirin was administered into the closed loop at 167 μmol/kg at 30 min before administration of FITC-Lys, FD-10 or FD-150. Each value represents the mean ± SEM for four rats.
* $P < 0.05$, ** $P < 0.01$: significantly different from the values for the control.

affected by aspirin treatment. In plasma samples 60 min after intraluminal administration, intact FITC-lysozyme was observed but smaller molecules were also observed near the top of the gels, suggesting that FITC-lysozyme was absorbed into blood in an intact form (Fig. 1B).

Next, the effect of aspirin on intestinal absorption of FITC-dextrans was evaluated (Table 1). It has been reported that FITC-dextrans with high molecular weight (≥70 kDa) appear to be transported primarily *via* non-paracellular pathways, such as fluid-phase endocytosis [14]. Treatment with aspirin increased the $AUC_{0-60\ min}$ of the preferential paracellular marker FD-10 (9.4 kDa) by ≈1.5-fold compared with that in controls (Table 1). The increased $AUC_{0-60\ min}$ of FD-150 (167 kDa), a marker of non-specific absorption (fluid-phase endocytosis and paracellular pathway), was also observed in aspirin-treated samples without affecting mucosal accumulation (Table 1).

### 3.2. Characteristics of absorption of FITC-lysozyme in rat intestines by an in situ re-circulating method

In the intestinal loop method, a high concentration of FITC-lysozyme was used to measure plasma concentrations. If endocytosis is involved in lysozyme absorption, it may not be detectable because endocytosis could be saturated under a high concentration of FITC-lysozyme. Thus, intestinal absorption of FITC-lysozyme was also examined at a much lower concentration by an *in situ* re-circulating method. The rate of intestinal absorption of FITC-lysozyme was ≈2.5-fold greater in the proximal intestine than that in the distal intestine (Fig. 2), a finding that was in good agreement with another report [12]. The rate of absorption of FITC-lysozyme in the proximal intestine was also greater than that of FD-10 and was decreased by colchicine (a general inhibitor of endocytosis) by ≈62% that seen with controls (Figs. 2B and 3A). Absorption of FITC-lysozyme in the distal intestine was almost identical to that seen with FD-10 and was not affected by colchicine (Figs. 2B and 3B). As in the closed-loop method, in the re-circulating method, aspirin increased the rate of absorption of FITC-lysozyme by approximately 1.6- and 2.5-fold more than that in the proximal and distal intestine, respectively (Fig. 3).

### 3.3. Effects of NSAIDs and misoprostol on intestinal absorption of FITC-lysozyme

The effects of clinical doses of diclofenac, meloxicam and loxoprofen on intestinal absorption of FITC-lysozyme were evaluated by an *in situ* re-circulating perfusion method (Fig. 4A). Diclofenac increased the absorption of FITC-lysozyme by ≈1.6-fold compared with that in controls in the proximal intestine, whereas meloxicam and loxoprofen did not affect its absorption. Next, we investigated the prophylactic effect of misoprostol on intestinal absorption of FITC-lysozyme facilitated by aspirin (Fig. 4B). Misoprostol treatment reduced the aspirin-facilitated absorption of FITC-lysozyme almost to the same levels as that seen in control rats.

## 4. Discussion

Previous reports have shown that the development of FDEIA symptoms are correlated with serum levels of allergens, and that allergen absorption facilitated by exercise and/or aspirin is a key factor causing FDEIA symptoms [5–7]. Here, we investigated the absorption pathway(s) of lysozyme as well as the mechanism of enhancement of absorption by aspirin in rat intestines. We also evaluated the effects of various NSAIDs and misoprostol on the intestinal absorption of lysozyme in rats.

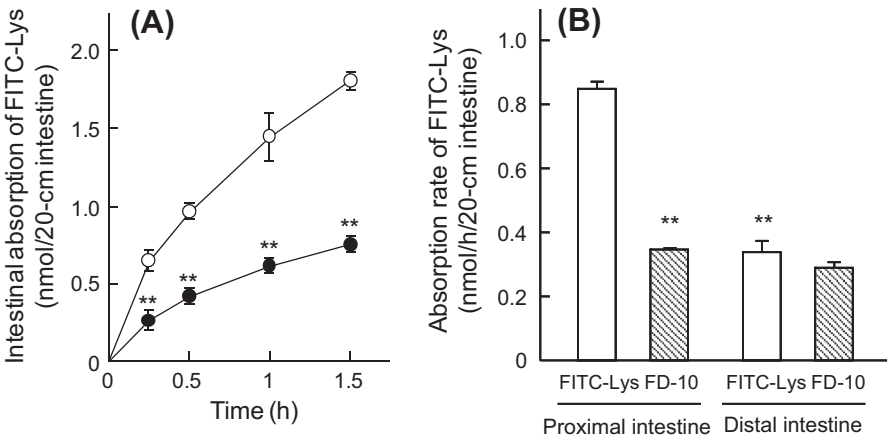


**Fig. 2.** Time-courses for the intestinal absorption of FITC-lysozyme (A) and rates of absorption of FITC-lysozyme and FD-10 (B) in the proximal and distal intestine as evaluated by an *in situ* re-circulating perfusion study. The amounts of FITC-lysozyme (FITC-Lys) and FD-10 absorbed were assumed to be of the same magnitude as the amount that disappeared from the perfusate. Open and closed circles represent the proximal and distal intestine, respectively. Each value represents the mean ± SEM for four rats. $^{**}P < 0.01$: significantly different from the values for the proximal intestine.

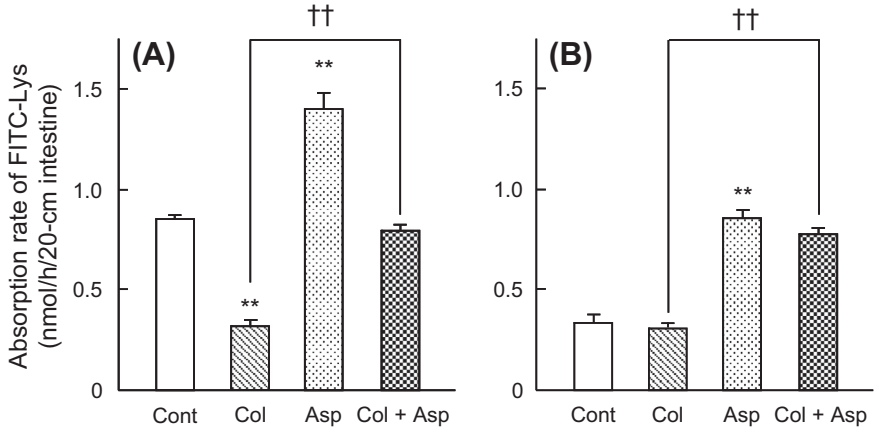


**Fig. 3.** Effects of colchicine and aspirin on the intestinal absorption of FITC-lysozyme in the *in situ* re-circulating perfusion study using the proximal (A) and distal intestine (B). The amount of FITC-lysozyme (FITC-Lys) absorbed was assumed to be of the same magnitude as the amount that disappeared from the perfusate. Concentrations of colchicine (Col) and aspirin (Asp) in the perfusate were 100 μM and 16.7 mM, respectively. Each value represents the mean ± SEM for four rats. $^{**}P < 0.01$ and $^{††}P < 0.01$: significantly different from the values for control and colchicine treatment, respectively.

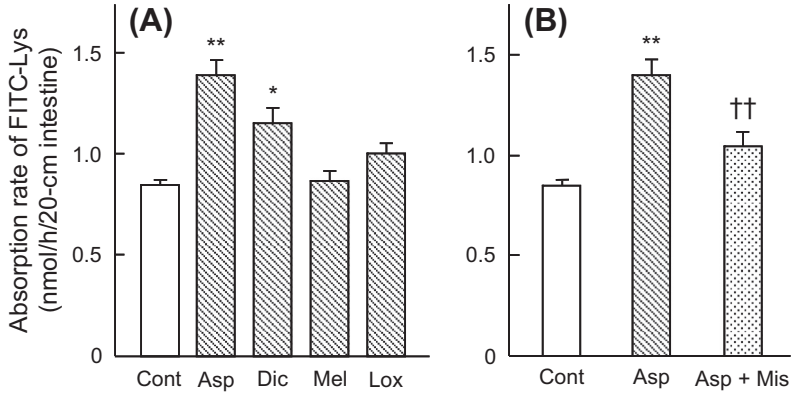


**Fig. 4.** Effects of various NSAIDs (A) and misoprostol (B) on the intestinal absorption of FITC-lysozyme in an *in situ* re-circulating perfusion study using the proximal intestine. The amount of FITC-lysozyme (FITC-Lys) absorbed was assumed to be of the same magnitude as the amount that disappeared from the perfusate. Concentrations of aspirin (Asp), diclofenac (Dic), meloxicam (Mel), loxoprofen (Lox) and misoprostol (Mis) in the perfusate were 16.7 mM, 314 μM, 80 μM, 789 μM and 1 μM, respectively. Each value represents the mean ± SEM for four rats. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{††}P < 0.01$: significantly different from the values for control and aspirin treatment, respectively.

Lysozyme is an allergen in the egg white of hens and has been shown to induce allergic symptoms in a mouse model of FDEIA [15]. However, the mechanisms of absorption of lysozyme are not clear. The rate of absorption of FITC-lysozyme in the proximal

intestine was higher compared with that in the distal intestine (Fig. 2A). SDS–PAGE showed FITC-lysozyme in the perfusate to be stable, suggesting that regional differences in the rates of absorption of FITC-lysozyme were independent of their degradation (data not shown). FD-10, which has a similar molecular size to that of lysozyme, was used as a marker of the paracellular pathway in the present study. A higher rate of absorption of FITC-lysozyme was observed compared with that of FD-10 in the proximal intestine, but not in the distal intestine (Fig. 2B). The rates of absorption of FITC-lysozyme in the proximal intestine were inhibited significantly by colchicine (Fig. 3). Takano et al. [12] demonstrated, in an inhibition study with unlabeled lysozyme, that saturable and non-saturable processes were involved in the absorption of FITC-lysozyme in the proximal intestine. Those results showed that specific mechanism(s), such as adsorptive or receptor-mediated endocytosis, may be involved in the absorption of FITC-lysozyme in the proximal intestine.

In the closed-loop study, aspirin increased the absorption of FITC-lysozyme and FD-150 in the proximal intestine without eliciting significant effects on mucosal accumulation (Fig. 1 and Table 1). These findings suggested that aspirin could facilitate the paracellular (but not the transcellular) pathway of FITC-lysozyme. Pals et al. [16] showed, using the lacturose and rhamnose differential urinary test, that prolonged or intense exercise increases the intestinal permeability of macromolecules *via* the paracellular pathway in humans. Berin et al. [17] reported that specific sensitization increased the absorption of allergens *via* the paracellular pathway in a mast cell-dependent manner. Those reports support our findings that aspirin facilitates allergen absorption *via* the paracellular pathway. With respect to the molecular mechanism of aspirin-induced intestinal toxicity, suppression of prostaglandin synthesis by COX-1 [18], oxidative stress [19] and modulation of tight junctional proteins such as zonula occludens-1 [20] and claudin-7 [21] have been reported. Misoprostol ameliorated absorption of FITC-lysozyme facilitated by aspirin (Fig. 4B). In addition, benzoate (which does not exhibit COX-inhibitory activity) did not affect FITC-lysozyme absorption (data not shown). Thus, aspirin-induced functional disturbances of tight junctions might be ascribed to the suppression of prostaglandin synthesis in the intestinal tract.

The physiological volume of fluid in the human small intestine under fasting conditions has been reported to be 50–1100 ml (mean, 500 ml) [22]. Thus, it could be speculated that the initial concentration of NSAIDs in the intestinal lumen reached the concentrations used in the present study. A significantly higher rate of absorption was observed in diclofenac-treated rats compared with those of control rats (Fig. 4A). Thus, diclofenac, as well as aspirin, may increase the risk of development of allergic symptoms in FDEIA. Diclofenac is known to enhance the absorption of wheat allergens in humans and to induce allergic responses in peanut-sensitized mice [7,23]. Meloxicam and loxoprofen did not facilitate the absorption of FITC-lysozyme from rat intestines. Meloxicam is a preferential COX-2 inhibitor and loxoprofen is a prodrug, so they have fewer adverse effects in the gastrointestinal tract compared with aspirin. This could be why meloxicam and loxoprofen fail to facilitate lysozyme absorption.

In conclusion, we showed that intestinal absorption of lysozyme exhibited appreciable regional differences in rat intestines, and that lysozyme was absorbed preferentially from the proximal region by endocytosis and the paracellular pathway. Among the four NSAIDs evaluated, aspirin and diclofenac facilitated the intestinal absorption of FITC-lysozyme *via* the paracellular pathway, suggesting that intake of these drugs may induce allergic reactions in individuals with FDEIA. Misoprostol is a prophylactic agent for FDEIA because it ameliorates the intestinal absorption of allergens facilitated by aspirin. These findings may help to clarify the pathophysiology of FDEIA and aid its treatment.

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