Characterization of Ovalbumin Absorption Pathways in the Rat Intestine, Including the Effects of Aspirin

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Ingested proteins are absorbed from the intestinal lumen via the paracellular and/or transcellular pathways, depending on their physicochemical properties. In this study, we investigated the absorption pathway(s) of ovalbumin (OVA), an egg-white allergen, as well as the mechanisms of aspirin-facilitated OVA absorption in rats. In situ intestinal re-circulating perfusion experiments showed that the absorption rate of fluorescein isothiocyanate (FITC)-labeled OVA in the distal intestine was higher than that for a marker of non-specific absorption, FITC-dextran (FD-40), and that colchicine, a general endocytosis inhibitor, suppressed OVA absorption. In the distal intestine, baflomycin A1 and phenylarsine oxide inhibited the OVA absorption rate, whereas methyl-β-cyclodextrin exerted no significant effects. Thus, OVA is preferentially absorbed from the distal intestine via the paracellular and receptor- and clathrin-mediated endocytic pathways. Furthermore, aspirin increased OVA absorption in the presence or absence of colchicine, indicating that aspirin facilitated OVA absorption by inducing intestinal barrier disruption and paracellular permeability.

Key words ovalbumin; aspirin; clathrin-dependent endocytosis; paracellular pathway; absorption

Food-dependent exercise-induced anaphylaxis (FDEIA) is a peculiar form of food allergy induced by physical exercise in combination with causative food ingestion. In addition to exercise, intake of non-steroidal anti-inflammatory drugs (NSAIDs), especially aspirin (ASP), is another well-documented trigger for FDEIA symptoms. Patients with FDEIA typically exhibit generalized urticaria, dyspnea, and anaphylaxis induced by a type I allergic reaction. Many kinds of foods appear to be responsible for FDEIA symptoms, including wheat, shrimp, vegetables, and eggs. Several previous reports have shown that increased intestinal absorption of allergens is considered a key factor in the development of FDEIA symptoms. Hanakawa et al. suggested that the symptom development depends on the amount of ingested allergen. Kohno et al. reported that an increased serum level of gliadin, a major FDEIA allergen from wheat flour, was required to elicit allergic reactions in patients with a wheat-FDEIA provocation test. Thus, it is important to elucidate the intestinal absorption mechanisms of allergens that trigger allergic reactions in patients with food allergy including FDEIA.

Orally ingested dietary proteins are not readily absorbed from the intestinal lumen in intact form owing to their enzyme digestibility and poor membrane permeability. Most ingested proteins are degraded by luminal gastric and pancreatic proteases as well as brush-border proteases, including aminopeptidases. However, a small amount of intact protein, which has escaped protease degradation, is absorbed via specialized microfold (M) cells in Peyer’s patches and across epithelial cells by transcytosis (i.e., apical endocytosis and basolateral exocytosis). Endocytic pathways are divided into two types: clathrin-dependent and clathrin-independent (e.g., caveolae-mediated endocytosis). Intact proteins are absorbed across intestinal epithelial cells through paracellular as well as transcellular pathways. Although normal tight junctions limit absorption of macromolecules through the paracellular pathway, strenuous exercise and activation of intestinal mast cells enhances macromolecule permeability following impairment of the paracellular pathway. NSAIDs also facilitate intestinal absorption of food allergens owing to reduced prostaglandin production by inhibiting cyclooxygenases (COXs). We previously reported that the absorption rate of lysozyme, an egg-white allergen from rat proximal intestine, was higher than that from distal intestine, and ASP enhanced lysozyme absorption. However, the absorption properties of ovalbumin (OVA), another important allergen in egg-allergy and egg-FDEIA, are poorly understood because macromolecule absorption pathways depend on their physicochemical properties, such as molecular size and electric charge. OVA has a molecular mass of 45 kDa and a pI of 4.7, and belongs to the serine-protease inhibitor family. Although it has been shown that intact OVA is absorbed into peripheral blood and lymph from the gastrointestinal lumen, how ASP effects OVA absorption pathways may be different from that of lysozyme, with a molecular mass of 14 kDa and pI of 11. In this study, we investigated OVA absorption pathway(s), and the effects of ASP on OVA absorption in rat intestine.

MATERIALS AND METHODS

Materials OVA (Grade V), bovine serum albumin (BSA; Cohn fraction V), fluorescein isothiocyanate (FITC), FITC-dextran40 (FD-40; average molecular weight, 40 kDa), and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). ASP, phenylarsine oxide (PAO), and methyl-β-cyclodextrin (MCD) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Colchicine (COL) and baflomycin A1 (BAF) were obtained from Nacalai Tesque (Kyoto, Japan) and LC laboratories (Shizuoka, Japan), respectively. All chemicals used were of the highest purity available.

Animals Male Sprague-Dawley (SD) rats aged 7–8 weeks were obtained from Japan SLC, Inc. (Shizuoka, Japan). Rats

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were fed a standard laboratory diet and water ad libitum for more than 1 week prior to the experiments. 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).

Preparation of FITC-Labeled OVA

Labeling of OVA with FITC was undertaken as described previously. Briefly, 1 mg FITC and 100 mg OVA were dissolved in 0.1 M borate buffer (pH 9.0). After incubation for 3 h at room temperature, pH was adjusted to 7.5 with 0.1 M boric acid. The reaction mixture was subjected to gel filtration using the Superdex HiLoad 26/600 75 prep grade column (GE Healthcare, Little Chalfont, U.K.) to remove free FITC. The solution collected was dialyzed using cellulose membranes with a molecular-weight cutoff of 3.5 kDa overnight 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 imaging with a Fluorimaging Analyzer (Typhoon FLA-7000, GE Healthcare, Little Chalfont, U.K.). The band size was the same as that with authentic OVA (45 kDa), suggesting no degradation of OVA during FITC labeling.

In Situ Re-circulating Perfusion Study of FITC-OVA

The intestinal absorption of FITC-OVA was evaluated in a re-circulating perfusion manner as described previously, with slight modifications. Briefly, rats were fasted overnight and anesthetized with pentobarbital (30 mg/kg, i.p.), and cannulated with polyethylene tubing (PE-50) at the femoral artery for blood sampling. Vehicle alone (PBS at pH 7.4) or ASP (167 µmol/kg) were administered orally using a stainless steel feeding tube. OVA (1.1 µmol/kg) dissolved in PBS was administered orally 30 min after the treatment. Blood (0.25 mL each) was collected at a designated time interval for 3 h via the cannula inserted at the femoral artery, and centrifuged to collect plasma. To estimate the extent of absorption, OVA was injected intravenously at a dose of 1.0 nmol/kg via the femoral vein. Blood was then collected from the femoral artery periodically for 3 h to measure plasma OVA concentrations, which were determined by a sandwich enzyme-linked immunosorbent assay kit (Morinaga Institute of Biological Science, Yokohama, Japan) using OVA as a standard. The detection limit of OVA was 2.2 to 556 pm under these conditions. The extent of oral OVA absorption was estimated as the absorbed fraction of oral OVA (FA_{p.o.}) by comparing the OVA area under the plasma concentration–time curve (AUC) after oral administration, with the OVA AUC after intravenous bolus injection using the following equation:

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FA_{p.o.} = \frac{AUC_{p.o.}}{AUC_{i.v.} \times D_{i.v.} / D_{p.o.}}
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where \( AUC_{p.o.} \) and \( AUC_{i.v.} \) are AUCs of OVA after oral and intravenous administration, respectively; \( D_{p.o.} \) and \( D_{i.v.} \) are the OVA doses in the respective experiments.

Characteristics of Absorption Pathway(s) of FITC-OVA

Absorption of OVA after Oral Administration

Rats were fasted overnight and anesthetized with pentobarbital (30 mg/kg, i.p.), and cannulated with polyethylene tubing (PE-50) at the femoral artery for blood sampling. Vehicle alone (PBS at pH 7.4) or ASP (167 µmol/kg) were administered orally using a stainless steel feeding tube. OVA (1.1 µmol/kg) dissolved in PBS was administered orally 30 min after the treatment. Blood (0.25 mL each) was collected at a designated time interval for 3 h via the cannula inserted at the femoral artery, and centrifuged to collect plasma. To estimate the extent of absorption, OVA was injected intravenously at a dose of 1.0 nmol/kg via the femoral vein. Blood was then collected from the femoral artery periodically for 3 h to measure plasma OVA concentrations, which were determined by a sandwich enzyme-linked immunosorbent assay kit (Morinaga Institute of Biological Science, Yokohama, Japan) using OVA as a standard. The detection limit of OVA was 2.2 to 556 pm under these conditions. The extent of oral OVA absorption was estimated as the absorbed fraction of oral OVA (FA_{p.o.}) by comparing the OVA area under the plasma concentration–time curve (AUC) after oral administration, with the OVA AUC after intravenous bolus injection using the following equation:

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Statistical Analyses

Data are displayed as the mean ± standard error of the mean (S.E.). Mean value differences between groups were assessed by Kruskal–Wallis or ANOVA tests, followed by a post hoc Tukey or Student’s t-test. A p < 0.05 was considered significant.

RESULTS

Characteristics of Absorption Pathway(s) of FITC-OVA

Absorption of OVA after Oral Administration

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hibitor of endocytosis), relative to that seen with controls (Fig. 1B). These results indicate that both the paracellular pathway and specific-endocytosis mechanism(s), including adsorptive and/or receptor-mediated endocytosis, may be involved in FITC-OVA absorption in the distal intestine. The FITC-OVA absorption rate in the proximal intestine was 1.7-fold higher than that of FD-40, and was affected slightly by COL (Fig. 1B). In the perfusate samples, 90 min after re-circulating perfusion, intact FITC-OVA was observed, suggesting that FITC-OVA was essentially stable in the intestinal perfusate during the time course of the perfusion (Fig. 1C).

Characteristics of Endocytic Pathway(s) of FITC-OVA in Rat Intestine We examined the effects of various concentrations of unlabeled OVA and BSA on the absorption of

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FITC-OVA in the distal intestine to characterize the specificity of FITC-OVA intestinal absorption. As shown in Fig. 2A, intestinal absorption of FITC-OVA was inhibited by unlabeled OVA in a concentration-dependent manner. The specific (endocytic) absorption pathway of FITC-OVA appeared to be inhibited completely in the presence of 2 µM of unlabeled OVA, because the absorption rate decreased to the same level as seen for COL treatment and the absorption rate of FD-40 as shown in Fig. 1B. The IC50 value of unlabeled OVA was 0.61 µM (γ=2.30). Notably, the FITC-OVA absorption rate decreased in the presence of BSA to almost the same level as seen for unlabeled OVA treatment (Fig. 2B).

To further elucidate the molecular mechanisms underlying the endocytic pathways of FITC-OVA absorption, the effects of BAF (an inhibitor of endosomal acidification), PAO (an inhibitor of clathrin-dependent endocytosis) and MCD (an inhibitor of caveolae-mediated endocytosis) on FITC-OVA absorption were evaluated (Fig. 3). The FITC-OVA absorption rate was significantly inhibited by pretreatment with BAF or PAO, decreasing to the same level seen in COL treatment. In contrast, no significant inhibitory effect was observed for MCD.

Effect of ASP on Intestinal Absorption Rate of FITC-OVA The effects of clinical doses (16.7 mM) of ASP on FITC-OVA intestinal absorption were evaluated by an in situ re-circulating perfusion method (Fig. 4). ASP increased the FITC-OVA absorption rate by approximately 2.0- and 1.7-fold more than that in the proximal and distal intestine, respectively. In COL-treated rats, ASP increased the FITC-OVA absorption rate in both intestinal regions. Thus, ASP modulates intestinal OVA absorption through a paracellular but not endocytic pathway.

Effect of ASP on Oral Absorption of OVA OVA given intravenously disappeared from plasma rapidly, following a first order rate constant (Fig. 5A). The relevant biological and kinetic parameters are shown in Table 1. The apparent distribution volume of OVA was ≈170–210 mL/kg body weight, which corresponds to the extracellular space volume (Table 1). Next, we sought to evaluate the ASP-facilitated absorption of OVA into blood by examining the effect of ASP on OVA intestinal absorption following oral administration (Fig. 5B). Oral OVA was absorbed gradually with time, and plasma concentrations displayed flip-flop kinetics, indicating that the elimination rate was greater than the absorption rate (Table 1). Plasma kinetics showed that ASP treatment increased OVA oral absorption (F40p.o values) by ≈2.6-fold compared with controls (F40p.o: control, 0.00158±0.00047%; ASP-treated rats, 0.00404±0.00030%, **p<0.01).

DISCUSSION

In this study, we investigated the absorption pathway(s) and in vivo kinetics of OVA, and the effects of ASP on OVA absorption in rat intestine. FITC-OVA, with a molecular mass
Oral intestine. The inhibitory effect of BAF on FITC-OVA absorption was also observed in the distal intestine (Fig. 3). BAF is a potent inhibitor of vacuolar H+−ATPase. Thus, FITC-OVA is absorbed via non-specific and specific pathways in the distal intestine. The inhibitory effect of BAF on FITC-OVA absorption was also observed in the distal intestine (Fig. 3). BAF is a potent inhibitor of vacuolar H+−ATPase that localizes in the endosomal membrane. The acidification of endosomes by the vacuolar H+− ATPase is an essential process for the dissociation of ligands and receptors during receptor-mediated endocytosis. Thus, FITC-OVA is absorbed via receptor-mediated endocytosis in the distal intestine. To characterize the receptor ligand specificity responsible for FITC-OVA absorption, we examined the effect of BSA on FITC-OVA absorption. As shown in Fig. 2B, BSA inhibited the absorption of FITC-OVA, suggesting that the receptor(s) involved in FITC-OVA absorption in the distal intestine also recognize BSA. Several studies have reported that BSA is recognized as a ligand by the megalin-cubilin complex receptor, which is expressed predominantly in the distal intestine. Thus, there is a possibility that OVA is absorbed from the distal intestine by megalin-cubilin-mediated endocytosis.

We therefore examined the possibility that the endocytic pathway of FITC-OVA in the distal intestine may be involved. PAO inhibits clathrin-dependent endocytosis by reacting with vicinal sulphydryls to form stable ring structures. The absorption of FITC-OVA was inhibited by PAO in the distal intestine, indicating that clathrin-dependent endocytosis is involved in FITC-OVA absorption in the distal intestine (Fig. 3). Several clathrin-independent endocytic pathways have been reported, including caveolea-dependent endocytosis. We found that MCD, which disrupts caveolea integrity by cholesterol sequestration, did not affect FITC-OVA absorption in the distal intestine. Thus, clathrin-dependent, but not caveolea-dependent endocytosis is involved in the FITC-OVA absorption in rat distal intestine. In addition to megalin-cubilin, the gp18, gp30 and gp60 were also identified as endocytic receptors for albumin in endothelial cells. However, the characteristics of intestinal OVA absorption observed in this study cannot be explained by these receptors because these receptors-mediated endocytosis of albumin are reportedly caveolea-dependent pathway in contrast to our results (Fig. 3).

Takano et al. reported that lysozyme was also absorbed.
by clathrin-dependent endocytosis. However, the lysozyme absorption rate in the proximal intestine was higher than in the distal intestine, which is opposite to that observed for OVA absorption.\(^{16}\) That is, a higher FITC-OVA absorption rate was observed in the distal intestine compared with that in the proximal intestine (Fig. 1A). Because we used protease inhibitors to evaluate the absorption of intact OVA, and lysozyme was stable in the perfusion experiments without protease inhibitors, regional differences in the absorption rates between FITC-OVA and lysozyme were thought to be independent of their degradation (Fig. 1C). Thus, OVA intestinal absorption mechanisms are different from those of lysozyme.

We previously reported that ASP enhanced the intestinal absorption of lysozyme and FITC-dextran with different molecular weight (FD-10, average molecular weight, 9.4kDa; FD-150, average molecular weight, 167kDa) without affecting the intestinal accumulation by impairment of the enterocytic tight junction.\(^{16}\) In this study, ASP increased the extent of oral absorption of OVA in the presence or absence of COL (Fig. 4). Moreover, Sugita et al.\(^{34}\) showed that oral ingestion of spermine increased OVA absorption by modulating the paracellular pathway. These results strongly indicate that ASP facilitates the paracellular pathway and allergen absorption, and that augmentation is not affected by allergen properties such as molecular size and electric charge.

In conclusion, we show that intestinal OVA absorption exhibits regional differences in the rat intestine, with OVA preferentially absorbed from the distal intestine by paracellular and clathrin-dependent endocytic pathways. In addition, ASP facilitated FITC-OVA intestinal absorption via the paracellular pathway. Although further studies are necessary to identify the receptors underlying OVA intestinal absorption, these findings shed new light on the pathophysiological mechanisms of FDEIA and common food allergies.

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