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Original Article

Intestinal absorption of the wheat allergen gliadin in rats

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Abbreviations:
$AUC_{0-3\ h}$, the area under the concentration −time curve from 0 to 3 h; CBB, Coomassie brilliant blue; C_{max}, a peak plasma concentration; COX, cyclooxygenase; EBD, Evans blue dye; ELISA, enzyme-linked immunosorbent assay; FD, fluorescein isothiocyanate-labeled dextran; FDA, Food and Drug Administration; FDEIA, food-dependent exercise-induced anaphylaxis; FITC, fluorescein isothiocyanate; FITC-gliadin, fluorescein isothiocyanate-labeled gliadin; HMW, high molecular weight; HRP, horseradish peroxidase; IAP, intestinal alkaline phosphatase; Ig, immunoglobulin; LMW, low molecular weight; MW, molecular weight; ND, not detected; OVA, ovalbumin; PBS, phosphate-buffered saline; SD, Sprague−Dawley; S.E., standard error of the mean; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; t-TG, tissue transglutaminase; WDEIA, wheat-dependent exercise-induced anaphylaxis; ZO, zonula occludens

ABSTRACT

Background: Aspirin enhances food allergy symptoms by increasing absorption of ingested allergens. The objective of this study is to elucidate the role of aspirin in facilitating intestinal absorption of the wheat allergen, gliadin, in rats.

Methods: Plasma concentrations of gliadin were determined after oral administration by gavage or administration into a closed intestinal loop in rats. We used an *in situ* intestinal re-circulating perfusion experiment to examine the effect of pepsin on aspirin-facilitated gliadin absorption. Fluorescein isothiocyanate (FITC)-labeled dextran-40 (FD-40) was used as a marker of non-specific absorption. The molecular size of gliadin and its allergenicity in plasma were examined using immunoblot analysis and intradermal reaction tests with Evans blue dye (EBD) extravasation, respectively.

Results: Aspirin increased plasma concentrations of gliadin after oral administration but had no effect in the closed intestinal loop study. An *in situ* intestinal re-circulating perfusion study showed that FITC-labeled gliadin was absorbed similarly to FD-40. Aspirin increased absorption of both intact and pepsin-digested gliadin, with a more significant effect on absorption of pepsin-treated gliadin. Immunoblotting showed that most gliadin was absorbed in intact form. When the gliadin fraction was extracted from rat plasma after gavage and injected intradermally into gliadin-sensitized rats, EBD extravasation was observed at injection sites in a gliadin dose-dependent manner.

Conclusions: Aspirin increased the absorption of intact and pepsin-digested gliadin via the paracellular pathway, maintaining their allergenicity. Moreover, the effect of aspirin on gliadin absorption was enhanced by modification and digestion of gliadin in the stomach.

Introduction

Wheat is a basic staple food all over the world, but can also cause food allergies ranging in severity. Severe wheat allergy can result in life-threatening anaphylaxis. Several clinical types of wheat allergy

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occur, including immediate-type wheat allergy, wheat contact dermatitis, bakers' asthma, and wheat-dependent exercise-induced anaphylaxis (WDEIA).[1] WDEIA is a specific type of wheat allergy that develops after ingestion of wheat products followed by physical exercise. Patients with WDEIA typically present with skin manifestations including generalized urticaria, dyspnea and anaphylactic shock induced by immunoglobulin (Ig) E-mediated mast cell degranulation.[2,3]

The total protein content of wheat flour ranges from 8% to 12%, and wheat proteins can be classified as water/salt-soluble proteins (albumin and globulin) and water/salt-insoluble proteins (gluten).[4] Gluten is composed of more than two proteins, including those of the gliadin and glutenin families. Gliadin-family proteins are monomeric and can be genetically classified into α/β, γ-, ω1,2- and ω5-gliadins. The glutenin family proteins are divided into low molecular weight (LMW)- and high molecular weight (HMW)-glutenins.[4] Gliadin is a major causative allergen of various types of wheat allergy including WDEIA.[1,5–9]

Previous reports have demonstrated that the dose of food allergen absorbed via the intestine is one of the essential factors in determining the severity of IgE-mediated allergic symptoms.[10–12] Since the enzymatic digestibility and intestinal permeability of allergens may affect their allergenicity, it is important to understand the effects of these factors on allergen absorption and the pathophysiology of food allergies including WDEIA. Most ingested proteins including allergens are degraded by gastric and pancreatic proteases as well as brush-border proteases in the intestinal lumen. However, a small amount of intact protein escapes protease degradation and is absorbed via specialized microfold (M) cells in Peyer's patches and via transcytosis across epithelial cells through endocytic and exocytic pathways. Previous reports have shown that several types of endocytic pathways mediated allergen absorption, depending on the properties of allergen proteins.[13–15] For example, we previously reported that an egg allergen, lysozyme [molecular weight (MW) of 14 kDa and p*I* of 11] was absorbed in the proximal intestine via endocytic pathways.[13] By contrast, an egg allergen, ovalbumin (OVA; MW of 45 kDa and p*I* of 4.7) was preferentially absorbed in the distal intestine via carrier-mediated endocytic pathways.[14] In addition to transcellular pathways, intact proteins are absorbed across intestinal epithelial cells through the paracellular pathway. Although normal enterocytes form tight junctions, preventing the absorption of macromolecules through the paracellular pathway, various factors (strenuous exercise, some drugs, and activation of intestinal mast cells) can enhance macromolecule permeability following impairment of tight junctions.[16,17]

Aspirin is used clinically as an anti-inflammatory agent. However, aspirin elicits and exacerbates allergic symptoms including anaphylaxis in patients with WDEIA.[10,16] Previous reports showed that allergic reactions elicited in provocation tests in patients with WDEIA were dependent on elevation of serum gliadin levels after exercise and/or aspirin intake.[10,11,16] We previously reported that aspirin increased intestinal absorption of intact OVA via the paracellular pathway, resulting in development of allergic symptoms in an OVA-sensitized rat model.[14,17] These findings suggested that aspirin-induced facilitation of intestinal absorption of allergens could be an underlying mechanism of WDEIA pathogenesis. However, the mechanisms of gliadin absorption as well as the facilitation of gliadin absorption by aspirin are poorly understood. In this study, we elucidated the pathways of gliadin absorption and the effects of aspirin on gliadin absorption in rat intestine. Furthermore, we also examined the effects of pepsin digestion since this might affect gliadin absorption and allergenicity prior to reaching the small intestine.

Methods

Materials

Gliadin was obtained from Tokyo Chemical Industry (TCI, Tokyo, Japan). Fluorescein isothiocyanate (FITC), FITC-dextran 40 (FD-40; average MW of 40 kDa), pepsin and Evans blue dye (EBD) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Aspirin was from Wako Pure Chemicals (Osaka, Japan). Rabbit anti-wheat gliadin antibody (BYA2520-1) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (ALI3404) were obtained from Accurate Chemical & Scientific Co. (Westbury, NY, USA) and BioSource International (Camarillo, CA, USA), respectively. Alum adjuvant (Imject® Alum) was from Thermo Fisher Scientific (Waltham, MA, USA). All chemicals 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 were provided with a standard laboratory diet (MF, Oriental Yeast, Tokyo, Japan) and water *ad libitum*. Rats were maintained in a temperature- and light-controlled environment for more than 1 week prior to experiments. All 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 (approval No. A16-138, Hiroshima, Japan).

Oral administration study

After overnight fasting, SD rats were anesthetized with pentobarbital (30 mg/kg, *i.p.*) and cannulated with polyethylene tubing (PE-50) at the femoral artery for blood sampling. Vehicle alone [phosphate-buffered saline (PBS), pH 7.4] or vehicle containing aspirin (30 mg/kg) were administered orally using a stainless-steel feeding tube. Gliadin (100 mg/kg) suspended in olive oil was administered orally 30 min after treatment. Blood (0.25 mL) was collected at designated time intervals for 3 h via the cannula to determine the plasma concentrations of gliadin. At the end of the study (3 h), whole blood was collected via the abdominal aorta using a heparinized syringe to evaluate the molecular size and allergenicity of ingested gliadin circulating in plasma. Each blood sample was centrifuged and the plasma sample was stored at −30 °C until use.

Intestinal closed loop study

After overnight fasting, SD rats were anesthetized with pentobarbital (30 mg/kg, *i.p.*) and cannulated with polyethylene tubing (PE-50) at the femoral artery. The common bile duct was ligated with surgical sutures. The proximal intestine (a 20-cm long segment beginning 10 cm below the pylorus) was flushed with 20 mL of saline solution prewarmed to 37 °C and ligated to make a closed loop. Vehicle alone (PBS, pH 7.4) or vehicle containing aspirin (30 mg/kg) were administered into the closed loop. In our previous report, pH 6.5 of PBS containing 1% (v/v) dimethyl sulfoxide (DMSO) was used to aid the aspirin solubility because the solubility of aspirin is low at pH 6.5.[13] DMSO is often used to dissolve the water-insoluble compounds, but DMSO may affect the intestinal absorption of gliadin to disrupt the intestinal barrier function. Thus, in this study, we used pH 7.4 of PBS without DMSO to dissolve aspirin. Thirty min later, gliadin (100 mg/kg) suspended in olive oil was administered into the same loop. Blood (0.25 mL) was collected at designated time intervals for 1 h via the cannula. Each blood sample was centrifuged and the plasma sample was stored at −30 °C until use.

Measurement of plasma gliadin concentration

The plasma concentration of gliadin was determined using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Yokohama, Japan) according to the procedure described by Matsuo *et al*. with slight modification.[16] The gliadin concentration in each plasma sample was calculated using known concentrations of gliadin as standards. The detection limit of gliadin was between 0.78 ng/mL and 50 ng/mL under these conditions.

Preparation of FITC-labeled gliadin (FITC-gliadin)

Labeling of gliadin with FITC was conducted as described previously with slight modification.[13] Briefly, 200 mg gliadin were dissolved in 0.1 M borate buffer (pH 9.0) containing 50% (v/v) ethanol and the solution was centrifuged at 3000×*g* for 10 min. The gliadin solution was reacted with 2 mg of FITC dissolved in 0.1 M borate buffer (pH 9.0) for 2 h at room temperature. After incubation, the pH was adjusted to 7.5 with 0.1 M boric acid. The mixture was dialyzed using a cellulose membrane with a molecular-weight cutoff of 3.5 kDa overnight at 4 °C and then concentrated by freeze-drying. After the lyophilized proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), bands corresponding to α/β-, γ- and ω1,2-gliadins (~30–50 kDa) were observed after Coomassie brilliant blue (CBB) staining and imaging with a Fluoroimage Analyzer (Typhoon FLA-7000, GE Healthcare, Little Chalfont, UK), as reported previously.[14]

Pepsin digestion of gliadin

Pepsin digestion of FITC-gliadin was performed according to a previously-described method with slight modification.[18,19] Briefly, FITC-gliadin (512 μg/mL in 0.1 M HCl, pH 1.5) was incubated for 1 h at 37 °C with pepsin (417 IU/mg) at an enzyme:substrate ratio of 1:100 (w/w). The digestion was terminated by addition of 1 M NaOH solution at designated time intervals for 1 h. Digestion of FITC-gliadin was confirmed using Fluoroimage Analyzer FLA-7000 after electrophoretic separation by SDS-PAGE in the same manner as for intact FITC-gliadin. For the *in situ* re-circulating perfusion experiment, we used FITC-gliadin digested with pepsin for 0.75 h. Digested FITC-gliadin was lyophilized and stored at −30 °C until use.

In situ re-circulating perfusion study

Intestinal absorption of intact and pepsin-digested FITC-gliadin was evaluated in a re-circulating perfusion experiment as described previously, with slight modifications.[13] Briefly, after overnight fasting, SD rats were anesthetized with pentobarbital (30 mg/kg, *i.p*. injection) and affixed supine under an incandescent lamp to maintain their body temperatures around 36 °C. The common bile duct was ligated with ligating string. The proximal intestine (a 20-cm long segment beginning 10 cm below the pylorus) was flushed with saline solution prewarmed to 37 °C and a loop was constructed. The intestinal segment was pre-perfused with PBS, pH 6.5, warmed at 37 °C for 15 min, then perfused by re-circulation of 10 mL of PBS containing intact or pepsin-digested FITC-gliadin (12.8 μg/mL) at 2 mL/min. To examine the effects of aspirin, aspirin (3 mg/mL) was added to the perfusate. Intestinal perfusate samples (100 μL) were collected periodically and mixed with 0.25 M NaOH solution (100 μL) to determine FITC-gliadin concentration. The absorption of FD-40 (0.4 μM) was estimated in the same way as for FITC-gliadin. Concentrations of FITC-gliadin and FD-40 in perfusate were determined at a wavelength of 500 nm for excitation and 520 nm for emission using a Microplate Fluorometer (PerkinElmer, Waltham, MA, USA). Stability of FITC-gliadin in perfusate was evaluated using Fluoroimage Analyzer FLA-7000 after separation by SDS-PAGE as reported previously.[14]

Immunoblot analysis of plasma gliadin after oral administration

Immunoblot analysis of ingested gliadin circulating in plasma was performed as described previously with slight modification.[9,20] Briefly, 2.5 mL of plasma was mixed with 0.5 mL of distilled water and 7 mL of absolute ethanol. After vortex mixing for 1 min, the mixture was centrifuged at 20,000× *g* for 5 min at 20 °C. The supernatant was transferred to a new tube and evaporated. The residue was dissolved in 0.1 mL of gel loading buffer containing 5% (w/v) 2-mercaptoethanol. Samples (15 μL) were applied to each lane of a 12.5% polyacrylamide gel. Western blot analysis was performed using an anti-gliadin antibody (diluted 1:1000) followed by a HRP-conjugated anti-rabbit IgG (diluted 1:30,000). The blots were developed using a chemiluminescent substrate (Western Lightning®Ultra, PerkinElmer, Waltham, MA, USA) and imaged using a LAS-4000 mini instrument (GE Healthcare, Little Chalfont, UK) as reported previously.[9]

Evaluation of gliadin allergenicity in plasma after oral administration

To evaluate the allergenicity of circulating gliadin in plasma, intradermal reaction tests were performed using the EBD extravasation method in a gliadin-sensitized rat model as described previously with slight modification.[17] Briefly, rats were sensitized by intraperitoneal injection with 1 mL of physiologic saline containing 5 mM acetic acid, 1 mg of gliadin and Imject® Alum [10 mg $Al(OH)_3$ and 10 mg $Mg(OH)_2$] at weekly intervals for 6 weeks. Six weeks after the first immunization, blood (0.2 mL) was collected from the jugular vein to assess plasma levels of gliadin-specific IgE by ELISA. Rats with high gliadin-specific IgE titers were used in this study. Plasma levels of gliadin-specific IgE (absorbance at 450 nm) prior to gliadin sensitization and 6 weeks after the first gliadin immunization were 0.00 ± 0.00 and 0.06 ± 0.01, respectively. The fur on the backs of these rats was shaved using an electric clipper (Thrive 2100; Daito Electric Machine Industry, Osaka, Japan). Twenty-four h after shaving, rats with no wounds were anesthetized with pentobarbital (30 mg/kg, *i.p*.), and cannulated with polyethylene tubing (PE-50) at the femoral vein. Gliadin was spiked into rat plasma containing no gliadin at a concentration of 150 ng/mL. Reference and ingested gliadin were extracted from plasma (30 μL) as described for immunoblot analyses. After evaporation, the residue was dissolved in 150 μL of 17.5 mM acetic acid. Rats were injected intradermally with 100 μL of each gliadin sample. Thirty min later, EBD (5 mg) was injected intravenously via the cannula. As a positive control, histamine dissolved in 100 μL of physiologic saline (150 μg/mL) was simultaneously injected intradermally. Thirty min later, the skin was stripped and photographed. The injected sites were punch-biopsied using a dermal punch for EBD quantitation. Each skin sample (0.1 g) was immersed in formamide (5 mL) to extract EBD at 20 °C for 24 h. Another skin sample (0.1 g) was dried at 60 °C for 24 h. The concentration of EBD was determined at a wavelength of 620 nm using Multiskan GO (Thermo Fisher Scientific) and represented as μg/g dry tissue weight.

Statistical analyses

Data were displayed as the means ± standard errors of the mean (S.E.). Mean value differences between groups were assessed using

Kruskal–Wallis or ANOVA tests, followed by post hoc Tukey or Student's *t*-tests. $P < 0.05$ was considered statistically significant.

Results

Effect of aspirin on gliadin absorption after oral and intra-intestinal loop administration

The effects of aspirin on plasma gliadin concentrations were evaluated after oral and intra-intestinal loop administration in SD rats. When gliadin was administered orally, it was absorbed into blood gradually over time and reached a peak plasma concentration (C_{max}) of 3.23 ± 0.99 ng/mL 1 h after gavage (Fig. 1A). Aspirin significantly increased the gliadin C_{max} and the area under the concentration–time curve from 0 to 3 h ($AUC_{0-3\ h}$) by ~8.6-fold (3.23 ± 0.99 ng/mL vs 27.7 ± 7.96 ng/mL, $P < 0.05$) and ~11.3-fold (5.15 ± 0.47 ng-h/mL vs 58.0 ± 16.2 ng-h/mL, $P < 0.05$), respectively. By contrast, when gliadin was administered into the intestinal loop, C_{max} reached 18.5 ± 8.75 ng/mL 10 min after administration (Fig. 1B). In the intestinal loop study, aspirin did not affect plasma concentrations (or $AUC_{0-3\ h}$) of gliadin.

Pepsin digestion of gliadin

Hydrolysis of gliadin by pepsin was evaluated by SDS-PAGE (Fig. 2). Bands corresponding to the molecular sizes of α/β-, γ-, and ω1,2-gliadins (30–50 kDa) were observed in reagent gliadin in the presence or absence of pepsin, but ω5-gliadin (~60 kDa) was not observed. In our preliminary study, we also could not obtain the clear band of ω5-gliadin by western blot analysis using anti-gliadin antibody. Thus, the content of ω5-gliadin may be much lower than those of the other gliadin in reagent gliadin. The electrophoretic profiles of gliadin showed no changes during incubation in the absence of pepsin. By contrast, gliadin was gradually hydrolyzed over time by pepsin into peptides with MWs of ~25 kDa and completely degraded into peptides after 0.75 h. Thus, we used FITC-gliadin digested with pepsin for 0.75 h in an *in situ* re-circulating perfusion experiment.

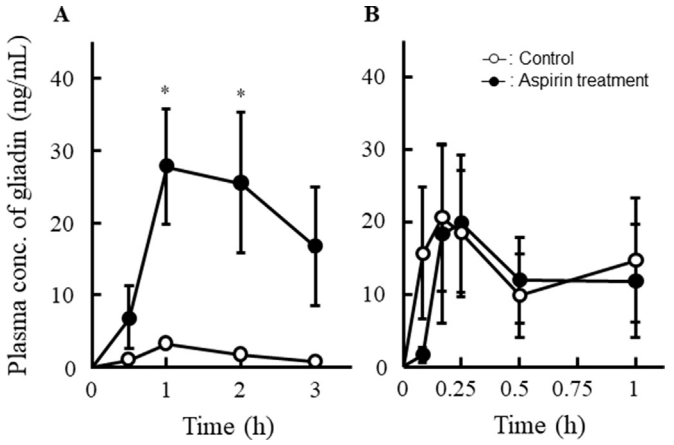

Fig. 1. Effect of aspirin on plasma concentrations of gliadin after oral **(A)** and intra-intestinal loop **(B)** administration in SD rats. Aspirin (30 mg/kg) was administered 30 min before oral or intra-intestinal loop administration of gliadin at a dose of 100 mg/kg. Open and closed circles represent the control (vehicle alone) and aspirin treatment, respectively. Each value represents the mean ± S.E. of five **(A)** and seven **(B)** rats. $^{*}P < 0.05$ with respect to control. In these studies, two rats, a control rat and an aspirin treatment rat, were evaluated simultaneously. The oral **(A)** and intra-intestinal loop **(B)** administration studies were independently performed five and seven times, respectively and we confirmed the results were almost same among these independent trials.

Absorption of intact and pepsin-digested FITC-gliadin in rat intestine and effect of aspirin on gliadin absorption

To investigate the absorption pathways of intact and pepsin-treated gliadin as well as the effect of aspirin on absorption, we performed an *in situ* re-circulating perfusion study. Approximately 20% of the initial dose of intact FITC-gliadin was absorbed from perfusate 1.5 h after initiation of perfusion, similar to absorption of FD-40, a marker for non-specific absorption through paracellular pathways (Fig. 3A). Aspirin increased absorption of intact FITC-gliadin by ~1.7-fold. These results suggested that aspirin enhanced intestinal gliadin absorption through the paracellular pathway. In perfusate samples obtained 1.5 h after initiation of perfusion, intact FITC-gliadin was observed by SDS-PAGE analysis (data not shown). Thus, FITC-gliadin might be absorbed from the intestinal perfusate over the time course of the perfusion. Next, we examined the intestinal absorption of pepsin-digested gliadin and the effect of aspirin on absorption using the same perfusion method. When pepsin-digested FITC-gliadin was perfused into the rat intestinal lumen, ~20% of the initial dose was absorbed 1.5 h after imitation of perfusion. Aspirin increased intestinal absorption of pepsin-digested FITC-gliadin by ~2.5-fold (Fig. 3B). Thus, pepsin digestion enhanced the aspirin-induced absorption of gliadin.

Molecular sizes of gliadin absorbed into plasma after oral administration

To determine the molecular sizes of ingested gliadin, we extracted gliadin from plasma with 70% ethanol 3 h after oral administration and then evaluated their sizes by western blot analysis (Fig. 4). In reagent gliadin, bands of ~35–45 kDa, corresponding to α/β-, γ-, and ω1,2-gliadins were observed but ω5-gliadin (~60 kDa) was not observed. Bands of ~35–45 kDa were also detected in plasma samples containing 17.1 and 47.1 ng/mL gliadin. Furthermore, LMW bands (<35 kDa) and HMW bands (≥64 kDa) were detected in these plasma samples. These results suggested that gliadin can be absorbed in intact form, although some types of gliadin were degraded by enzymes in the intestinal lumen and tissues.

Allergenicity of gliadin absorbed into plasma after oral administration

To evaluate the allergenicity of gliadin absorbed into plasma, an intradermal reaction test was performed using the EBD extravasation method (Fig. 5). When histamine was injected intradermally into gliadin-sensitized rats, extravasation of EBD was observed. When gliadin extracted from the plasma of rats after ingestion was injected intradermally into gliadin-sensitized rats, EBD extravasation was observed. Thus, we confirmed that this rat model is suitable to assess the allergenicity of gliadin. The extent of EBD extravasation depended on plasma gliadin concentration. By contrast, the extracted material from plasma containing no gliadin did not induce EBD extravasation. These results indicated that gliadin with allergenic activity was absorbed from the intestine.

Discussion

The amount of allergen absorbed into blood is a key factor in determining the development and severity of food-dependent exercise-induced anaphylaxis (FDEIA) symptoms. Thus, it is important to elucidate the mechanisms of intestinal absorption of allergens, as well as the effects of agents that disrupt intestinal homeostasis, to understand the pathophysiology of FDEIA. In this study, we characterized the mechanisms of gliadin absorption

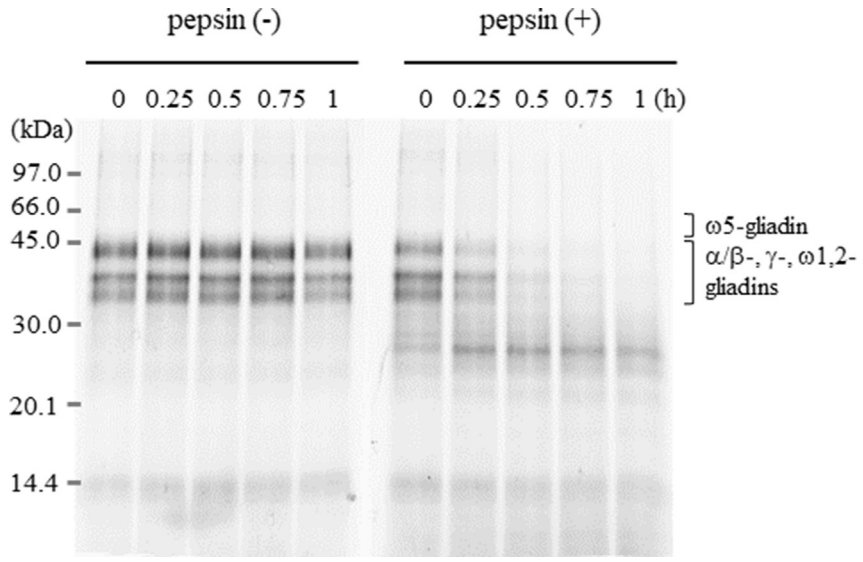

Fig. 2. SDS-PAGE analysis of pepsin-digested gliadin. FITC-gliadin was incubated for 1 h at 37 °C in the absence [pepsin (−)] or presence [pepsin (+)] of pepsin under acidic conditions (pH 1.5). FITC-gliadin was detected using a Fluoroimage Analyzer FLA-7000 after separation by SDS-PAGE. This trial was repeated three times independently to confirm the reproducibility.

including its absorption pathways, as well as the effects of pepsin digestion on aspirin-facilitated absorption of gliadin in rat intestine.

When gliadin was administered orally to aspirin-treated rats, plasma concentrations of gliadin were significantly higher than those in vehicle-treated controls (Fig. 1). This result agreed with our previous finding that aspirin enhanced absorption of ingested gliadin in healthy human volunteers.[16] In this study, we administered gliadin at a dose of 100 mg/kg. According to the Food and Drug Administration (FDA), dose of compounds in rats should be divided by coefficient factor (6.2) to convert to human equivalent dose based on the body surface area.[21] Thus, a 100 mg/kg dose of gliadin in rats corresponds to 16.1 mg/kg in humans. Since wheat flour is composed of ~6% gliadin, we estimate that the dosage of gliadin used in this study corresponds roughly to ~0.27 g/kg of wheat flour in humans.[22] In wheat challenge tests, the mass of ingested wheat flour is typically ~70–135 g.[16] In addition, the dosage of aspirin used in this study (30 mg/kg) was estimated to be equivalent to ~4.8 mg/kg in humans.[21] In clinical settings, aspirin is used as an anti-inflammatory drug at a dose of 0.5–1.5 g. Assuming human body weight is ~50 kg, clinical dose of aspirin is estimated to 10–30 mg/kg which corresponds to 62–186 mg/kg in rats. Thus, the dosages of gliadin and aspirin used in this study may have been smaller than those used clinically, but not significantly so.

Aspirin did not increase gliadin absorption after intra-intestinal loop administration while it did so after gavage. The reason underlying the difference in aspirin facilitation of gliadin absorption

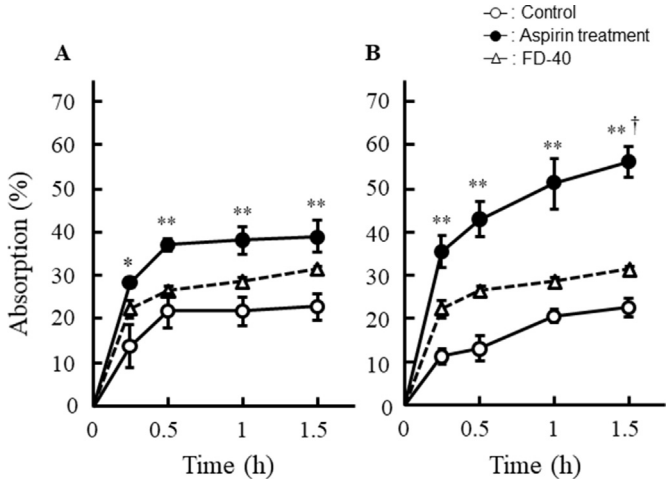

Fig. 3. Effect of aspirin on intestinal absorption of intact **(A)** or pepsin-digested **(B)** FITC-gliadin in the *in situ* re-circulating perfusion study. The amount of FITC-gliadin absorbed was assumed to be equivalent to the amount eliminated from perfusate. The concentration of aspirin in the perfusate was 3 mg/mL. Open and closed circles represent the control (vehicle alone) and aspirin treatment, respectively. Open triangles represent absorption of FD-40. Each value represents the mean ± S.E. of four rats. $^{*}P < 0.05$ and $^{**}P < 0.01$ with respect to control. $^{\dagger}P < 0.01$ with respect to intact FITC-gliadin. In the re-circulating perfusion study, five rats, a control and an aspirin treatment rat for intact FITC-gliadin **(A)**, a control and an aspirin treatment rat for pepsin-digested FITC-gliadin **(B)**, and a rat for FD-40, were evaluated simultaneously. This experiment was independently performed four times and we confirmed results were almost same among these independent trials.

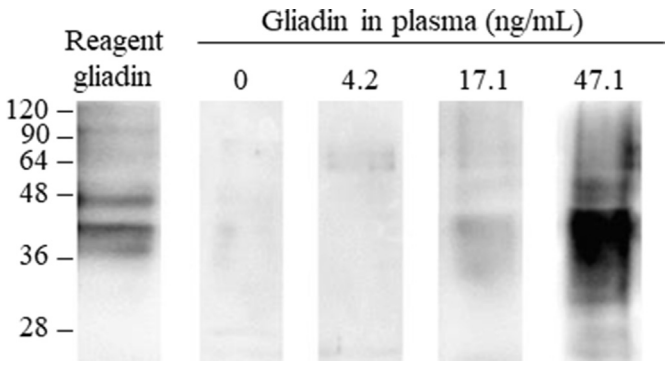

Fig. 4. Western blot analysis of gliadin circulating in plasma after oral administration in rats. Aspirin (30 mg/kg) was administered 30 min before oral administration of gliadin at a dose of 100 mg/kg (Gliadin in plasma). The sample "0" was obtained from one rat administered with aspirin and olive oil (vehicle for gliadin). Each plasma sample was collected from one (individual) rat used in the four independent oral administration studies. As a reference, gliadin was spiked into rat plasma containing no gliadin at a concentration of 150 ng/mL (Reagent gliadin). Gliadin was extracted with 70% ethanol from plasma 3 h after oral gliadin administration. Each gliadin sample was separated by 12.5% SDS-PAGE and blotted onto a PVDF membrane. The membrane was incubated with anti-gliadin antibody. Plasma concentrations of gliadin were determined by ELISA. Western blot analysis was repeated three times independently to confirm the molecular sizes of ingested gliadin.

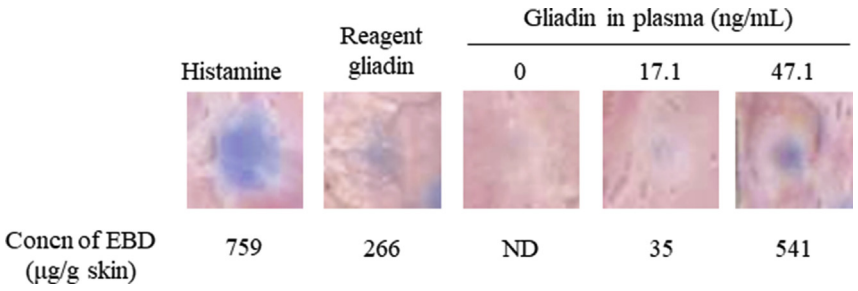

Fig. 5. Allergenicity of gliadin circulating in plasma after oral administration in rats. Aspirin (30 mg/kg) was administered 30 min before oral administration of gliadin at a dose of 100 mg/kg (Gliadin in plasma). The sample "0" was obtained from one rat administered with aspirin and olive oil (vehicle for gliadin). Each plasma sample was collected from individual rat used in the three independent oral administration studies. As a reference, gliadin was spiked into rat plasma containing no gliadin at a concentration of 150 ng/mL (Reagent gliadin). Gliadin was extracted with 70% ethanol from plasma 3 h after oral gliadin administration. Reagent gliadin was spiked into rat plasma containing no gliadin at a concentration of 150 ng/mL. As a positive control, 100 μL of histamine dissolved in physiologic saline (150 μg/mL) was simultaneously injected intradermally. Intradermal reaction test was repeated three times independently to confirm the allergenicity. ND represents the value which bellows the detection limit (concentration of Evans blue dye: 1.78 μg/mL) in the assay.

between these studies is not clear at present. However, two possible hypotheses may explain this finding. First, gliadin might be modified by acid in the stomach. Gliadin is a water/salt-insoluble protein and its solubility in water was reportedly ~0.3 mg/mL.[18] In our intestinal loop study, plasma concentrations of gliadin displayed flip-flop kinetics, indicating that the elimination rate was greater than the absorption rate due to the poor solubility of gliadin (Fig. 1). Furthermore, aspirin increased the intestinal absorption of FITC-gliadin dissolved at a much lower concentration in an *in situ* re-circulating study, unless gliadin did not pass through the stomach (Fig. 3). Gliadin contains a large number of glutamine residues that can be easily deamidated to glutamic acid by acidic hydrolysis. Kumagai *et al.* reported that the solubility of deamidated gliadin was ~1.6-fold higher in water than that of unmodified gliadin.[18] Thus, the solubility of gliadin may be related to its oral absorption and thus to the effect of aspirin. Second, gliadin might be degraded by acid and pepsin in the stomach. Our perfusion study showed that aspirin increased the intestinal absorption of pepsin-digested FITC-gliadin (~25 kDa) by ~2.5-fold and that of intact FITC-gliadin (~30–50 kDa) by~1.7-fold (Fig. 3). These results suggest that degradation of gliadin by pepsin enhances the aspirin-facilitated absorption of gliadin. However, we also showed that more than 0.5-h digestion with pepsin was needed for degradation of gliadin into peptides (Fig. 2). Mori *et al.* reported that the recovery of FD-10S from the stomachs of fasting rats was ~20% of the initial dose 0.5 h after oral administration as a powder.[23] These results suggest that most gliadin may be excreted from the stomach before pepsin digestion. In general, wheat is eaten after heating, and heating may affect the digestibility of gliadin by pepsin. However, we did not examine the effect of heating on gliadin digestion by pepsin in this study. Further studies are necessary to clarify the role of acid and pepsin in modification and degradation of gliadin in the stomach using unheated and heated gliadin. In addition to pepsin digestion, gliadin is reportedly digested by pancreatin, containing trypsin and chymotrypsin.[18] However, the effects of pancreatin digestion can be disregarded since the digestibility of gliadin by pancreatin is minimal compared with pepsin. Therefore, we surmise that the effects of degradation by digestive enzymes on aspirin-facilitated absorption of gliadin after oral administration are negligible. In fact, our results showed that most gliadin was absorbed into blood from the intestinal lumen in intact form retaining its allergenicity, despite the fact that some gliadin was degraded (Fig. 4, 5).

In immunoblot analysis, binding of anti-gliadin antibody to HMW proteins (≥65 kDa) was observed for samples containing high gliadin content (Fig. 4). The identities of HMW proteins were not assessed in this study. Previous reports have shown that gliadin-gliadin complexes with HMWs were formed as a result of cross-linking by tissue transglutaminase (t-TG) in the intestine.[24,25] We speculate that HMW species can result from rearrangement of intact and/or degraded gliadins due to cross-linking between a lysine residue and a glutamine residue by t-TG. In the perfusion study, we used FITC-labeled gliadin. Because FITC is bound to lysine residues in gliadin, it is considered that FITC labeling may inhibit the formation of rearranged HMW gliadins during the perfusion. Further studies are necessary to clarify the effect of FITC labeling on the rearrangement of HMW gliadins.

Intact FITC-gliadin was absorbed from perfusate to the same extent as FD-40, indicating that gliadin was absorbed through the paracellular pathway (Fig. 3A). We previously reported that aspirin enhanced the intestinal absorption of FDs with different molecular weights (FD-10, average MW of 9.4 kDa; FD-150, average MW of 167 kDa), without affecting intestinal accumulation, by impairment of enterocytic tight junctions.[13] Moreover, we reported that aspirin increased the oral absorption of FD-40 by ~2.0-fold compared with that in controls.[17] These reports suggested that aspirin induced gliadin absorption through the paracellular pathway following disruption of the intestinal barrier by suppression of prostaglandin synthesis by cyclooxygenase (COX)-1,[26] oxidative stress[27] and modulation of tight junctional proteins such as zonula occludens (ZO)-1[28] and claudin-7.[29] In addition, Hamarneh *et al.* reported that loss of intestinal alkaline phosphatase (IAP) expression increased intestinal permeability to FD-4 and FD-10 via impaired intestinal barrier function.[30] Kaur *et al.* reported that aspirin decreased IAP activity.[31] Taking into account all of these results, aspirin may promote absorption via the paracellular pathway by impairment of intestinal barrier function due to inhibition of IAP activity.

When plasma sample containing gliadin was injected intradermally into gliadin-sensitized rats, gliadin caused EBD extravasation in a dose-dependent manner (Fig. 5). However, the amount of EBD extravasation by injection of plasma containing 47.1 ng/mL of gliadin was higher than that by injection of reagent gliadin spiked into plasma at concentration of 150 ng/mL. The reason why the amount of EBD extravasation did not depend on the dose of gliadin between plasma sample and reagent is not clear at present. We speculate that specific IgE antibodies to modified gliadin may be produced in gliadin-sensitized rats and these IgE may react with modified gliadin in plasma sample. Further studies are necessary to clarify the difference in IgE reactivity between ingested (modified) and reagent (intact) gliadins.

In conclusion, we showed that aspirin increased absorption of intact and pepsin-digested gliadin via the paracellular pathway

while maintaining its allergenicity. Furthermore, the effect of aspirin on gliadin absorption was enhanced by gliadin modification and pepsin digestion in the stomach. Thus, oral absorption of gliadin may be modulated by various factors such as drugs and meals which alter gastric emptying rate, stomach pH and gliadin solubility. These findings shed new light on the pathophysiological mechanisms underlying WDEIA.

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Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

TY, TF and HM conceived and designed the experiments. TY, KH, NN and RO performed the experiments and analyzed the data. TY, TT and HM wrote the manuscript. All authors read and approved the final manuscript.

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