Food allergens affect the intestinal tight junction permeability in inducing intestinal food allergy in rats

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Summary

Background: The intestinal tract plays an important role in food allergy and the intestinal mucosa barrier is critical for maintenance of its function. The underlying mechanisms of how food allergens modulate the intestinal permeability in inducing intestinal food allergy remain elusive.

Objective: The aim of this study was to explore the mechanism of how food allergens influence the function of intestinal barrier and induce intestinal food allergy.

Methods: Ovalbumin (OVA) was chosen to establish intestinal food allergy models in juvenile and adult rats that were confirmed by IgE and IgG assay. Intestinal tissue morphology was analyzed by HE staining. Intestinal permeability was dynamically monitored using a Lactulose (L)-Mannitol (M) assay. The morphology of the tight junctions in the intestinal mucosa barrier were analyzed under TEM. The expression of key molecules in tight junction regulation was evaluated by Real-time PCR.

Results: We found: 1) The sensitization rate in juvenile rats was higher than in adult rats; 2) Intestine fluff erosion was more serious in juvenile rats than in adult rats in the duodenum and ileum; 3) Intestinal permeability was severely damaged, according to the results of the Lactulose (L)-Mannitol (M) assay; 4) Tight junction damage on the mucosal barrier was observed; Real-time PCR results showed that the expression of some key molecules that are involved in tight junction regulation was also affected. Conclusions: Our data suggested that the allergy sensitization rate of Ovalbumin (OVA) in the juvenile group is higher than in adults and food allergens may increase intestinal mucosal permeability through intestinal tight junction regulation in inducing intestinal food allergy. (Asian Pac J Allergy Immunol 2014;32:345-53)

Keywords: Food allergen, ovalbumin, allergy, intestinal permeability, tight junction

Introduction

Food allergy is a serious public health problem,1,2 which cause severe symptoms in the gastrointestinal tract, skin, respiratory tract and other organ systems, and even anaphylactic shock and death.3-5 Epidemiological studies have reported that the incidence of food allergies (FA) has been increasing. Previous research has contributed a lot of information about food allergy. Its incidence is higher in children (6% to 10%) than in adults (just 1% to 2%).3 and its pathogenesis is still not well understood.

As the mainly organ involved in digestion, the intestinal tract is considered to be a barrier to food allergens in cases of food allergy. The intestinal barrier, which can be divided into the mechanical barrier, microbial barrier, immunological barrier and chemical barrier etc., plays an important role in maintaining intestinal function to prevent bacteria, endotoxins and other toxic substances passing into the underlying tissues.6,7 Studies suggested that abnormal intestinal permeability is also associated with diabetes, celiac disease, inflammatory bowel disease and other diseases besides food allergy.8,9 Increased intestinal permeability is also believed to

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be significantly related to the pathogenesis of these diseases and more and more studies are focusing on this relationship.

It seems that the food allergy morbidity varies in different age groups, which is possibly associated with the development and functional status of the intestinal mucosal barrier. Permeability increase and functional damage can be found in the intestinal barrier when food allergy occurs. It is reported that intestinal permeability increases in the early stages of food allergy. Yet the molecular basis of the structural changes in the intestinal barrier associated with food allergy remain unknown. The aim of our study was to explore the molecular mechanisms of how food allergens affect and damage the morphology and function of the intestinal mucosal barrier to induce intestinal food allergy, through OVA induced food allergy models in juvenile or adult rats.

**Methods**

**Animals**

4 week (considered Juvenile) and 12 week old (considered Adult) BN (Brown Norway) rats were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) and raised in SPF facilities. The animal studies were approved by the School of Medicine of Shenzhen University, and carried out as previously reported.

Briefly, the juvenile and adult rats were randomly divided into a sensitization group and a control group. The sensitization group was administrated 1mg/mL OVA (in PBS, phosphate-buffered saline) (Sigma/Flu/Ald, USA) orally everyday for 48 days, while the control group was administrated 1 mL PBS. All the animals were challenged on the 49th day with 100mg/mL OVA after the last sensitization and sacrificed 8hrs later (Figure 1). Blood and intestinal tissue samples were collected for further study.

**Intestinal permeability evaluation (Lactulose/Mannitol assay)**

Intestinal permeability was evaluated by the ratio of lactulose and mannitol (M/L) in urine excretion as previously reported. Rats were put into metabolic cages and given (i.g.) 100mg lactulose and 50 mg mannitol (dissolving in 1mLPBS) orally once a week. Rat urine was collected during a 24hrs fasting period (but with water). 5% acetic acid was added to the urine samples after centrifugation. The samples were then boiled, centrifuged and the filtered through a 0.22μm Millipore filter to remove impurities (such as urine proteins). Afterwards the specimen was applied to HPLC analysis to determine the ratio of lactulose and mannitol (L/M) [20μL was taken for analysis (mobile phase: acetonitrile/water (80%/20%); flow rate: 1mL/min; column temperature: 35°C)]

**Serum OVA-IgE/OVA-IgG analyzing**

Blood was collected from the rats’ tail vein every week. OVA-sIgE and OVA-sIgG were assayed by ELISA as previously reported. In brief, undiluted serum samples were detected using OVA pre-coated plates (10 μg/mL OVA of 100 μL/well) and HRP-labeled rat-anti-mouse IgE or IgG (1:1000) as the secondary antibody (Southern Biotechnology Associates Inc., Birmingham, AL). After the incubation and reaction with TMB (3,3’,5,5’-Tetramethylbenzidine), the final OD value was detected at 450 nm wavelength using a RT-2100C Microplate reader (Rayto Life and Analytical Sciences Co., Ltd., China).

**HE staining**

Intestinal samples were collected immediately after rat sacrifice. The specimens were fixed with 4% paraformaldehyde for 24 hours and stained with Hematoxylin-Eosin stains after dehydration,
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Embeding and slicing. The structural and morphological changes were observed and analyzed under a OLYMPUS BX51 microscope.

**Electron microscope study**

Rat intestinal samples were collected immediately after sacrifice. Samples were cut into 1.5mm×1.5mm×2 mm pieces and put into a fixation solution (provided by the electron microscopy room of the School of Medicine at Sun Yat-sen University). Samples then were kept at 4 degrees C overnight and sent to the electron microscopy room of the School of Medicine at Sun Yat-sen University for analysis. The structural and morphological changes (of epithelial cells, tight junction, etc.) were examined under the electron microscope.

**Real-time PCR**

50-100 mg of intestinal tissues were collected and ground in liquid nitrogen, then moved into a 1.5mL EP tube. The total RNA was extracted using a TRIzol Reagent (15596018, invitrogen/Gibco/MP) and reverse transcribed using a Reverse Transcriptase Kit (Fermentas). Real-time PCR was performed using C1000™ Thermal Cyclers (Bio-Rad, USA) with SYBR green fluorescence. Samples were run in triplicate and the cycling parameters were: 95°C for 3 min, followed by 40 cycles of 95°C for 25s, 60°C for 20s, and a detection step at 72°C for 30 s. Human β-actin transcript was used as an internal control for results standardization (2-△△CT) by eliminating variations in mRNA quantity and each RNA sample was arranged in triplicate. All the primers were synthesized by BGI Cooperation (Shenzhen, China) as list:

**ZO-1:** Forward, 5'-ACCCACGAAGTTATGAGCA AG-3'; Reverse, 5'-AGACTGTGGTTTCATTGC TGG-3';

**Ocludin:** Forward, 5'-ATTCCTCTGACCTTGTC CGTG-3'; Reverse, 5'-CCTGTGCTGTAGTCTG GTTITCA-3';

**Claudin-2:** Forward, 5'-ATTCCCTCTGACCTTGTC CGTG-3'; Reverse, 5'-AGCCAACCGCCGTCAC AATG-3';

**Claudin-8:** Forward, 5'-TGTTCATGTGTTGAGAA CCGCTGGG-3'; Reverse, 5'-ACGGACGCAC GACACATCAGTC-3';

**Claudin-9:** Forward, 5'-TTCCACTGGGCGCTTG AACTCTTCG-3'; Reverse, 5'-GCTGTGTGCGCCAATGAAGGCCTG-3';

**Claudin-15:** Forward, 5'-AACTGCTGGGACTT CCGCTCCAT-3'; Reverse, 5'-TCGATTGTGCCC ACGTTGTCG-3';

**β-actin:** Forward, 5'-GTCTCACACCACACTGGCA TTGTG-3'; Reverse, 5'-TCTCAGCTGTGTTGGTG GTGAAG-3'.

**Statistical analysis**

The data were analyzed using SPSS v13.0 software. All values in the study are presented as mean±SD from at least three independent experiments. Statistical analysis of experiments in cell lines was evaluated using Student's t test. Values of P < 0.05 were considered to be statistically significant.

**Result**

Juvenile rats were more prone to allergy to OVA induction compared with the adults.

Successful food allergy model induction was determined according to a significant serum IgE rise and behavior observations (e.g., diarrhea). We found that it was easier to induce allergy in juvenile rats (with a sensitization rate 69.23%) to OVA, compared with their adult counterparts (Table 1). The serum OVA-IgE began to rise significantly growing in both the juvenile and adult groups from the 4th week (Figure 2A); The serum OVA-IgE level of juvenile rats was higher than that in adult rats at the 6th week (Figure 2A). Also, the serum OVA-IgG increased from the 2nd week in both the juvenile and adult rats, compared with controls (Figure 3A), though there is no statistically significant difference between them (Figure 2B).

Further HE staining studies showed that the intestinal villi of all OVA sensitized rats were damaged at certain levels and the mucosa in juvenile rats was more seriously ulcerated (Figure 2C and 2D). These data together showed that it was easier to establish food allergy to OVA in juveniles, and they more prone to get intestinal damage.

**Table 1.** Rate of successfully induced intestinal food allergy by OVA in the juvenile and adults rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Rat in Total</th>
<th>Rat got Allergy</th>
<th>Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Juvenile</strong></td>
<td>13</td>
<td>9</td>
<td>69.23</td>
</tr>
<tr>
<td><strong>Adult</strong></td>
<td>12</td>
<td>4</td>
<td>33.33</td>
</tr>
</tbody>
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OVA induces damage in duodenum, jejunum and ileum mucosal to affect intestinal permeability

Our results further suggested that in juvenile rats OVA induced damage exists not just in duodenum (Figure 3B), but also in the villi of jejunum (Figure 3C) and ileum (Figure 3D) in juvenile rats. Compared with the control group, damage and erosion could be found more frequently in the intestinal villi and mucosa of OVA-treated rats, with a certain amount leakage of mucus and fibrous protein (Figure 3B, 3C and 3D).

We then used the Lactulose/Mannitol assay to assess the intestinal permeability change. As shown in Figure 3E, the intestinal permeability was greatly increased, with the L/M value rising significantly from the 2nd week, indicating OVA induced damage in the duodenal, jejunal and ileal mucosa.

OVA causes damage to tight junctions of the intestinal villi epithelial cells

Compared with the control group, conspicuous damage can be seen in ileum villus in the OVA-treated group (Figure 4A, 4B and 4C). Moreover, we could see the gap expansion as well as tight junction damage in intestinal villus epithelial cells (marked with two black arrows) and swelling of mitochondria (Figure 4B and 4C). It’s worth mentioning that the epithelial cells also swelled (Figure 4D) and we could see the increase and aggregation of eosinophils (Figure 4F), indicating that the basement membrane might be damaged causing osmosis, inflammation and aggregation of immune cells.

Figure 2. Juvenile rats were easier to get allergy under OVA induction compared with the adults.
A. Serum OVA-IgE levels rose in both juvenile and adult groups; B. Serum OVA-IgG level increased significantly, both in the juvenile and adult rats; C&D. HE staining of intestinal damage in the upper (C) or lower (D) jejunal villi of OVA sensitized rats. *, p < 0.05, **, p < 0.01 versus the control; respectively (Magnification: A1, A2, 400×; B1, B2, 400×).
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Further studies with Real-time PCR indicated that OVA could regulate the expression of key proteins in tight junctions. As shown in Figure 5, tight junction regulating proteins such as ZO-1, and Claudin-2, Claudin-8, Claudin-15’s expression were significantly down-regulated, while Claudin-9 appeared remarkably up-regulated (Figure 5A), indicating that OVA may be able to regulate the tight junction permeability.

Discussion

In the present study, by using the rat food allergy model we found that it is easier to induce sensitization to a food allergen (OVA) in juveniles.
than in adults. Moreover, OVA can significantly influence the expression of key proteins of the tight junctions and in turn the intestinal permeability, to cause allergen absorption and induce food allergy.

Epidemiology studies have shown that the incidence of food allergy is higher in children than in adults. We also found that juveniles were more susceptible to allergy, which is consistent with the epidemiology studies (Figure 2 and Table 1). Food allergy can be divided into IgE dependent (type I hypersensitivity), or non-IgE dependent, in which specific IgE plays a central role. According to ELISA data, serum OVA-IgE increased weekly in our experiments. The OVA-IgE levels of the juvenile group had risen by the fourth week and reached a peak at the sixth week (Figure 2A). This is in agreement with a study from Taiwan which showed that the symptoms in skin, and the

Figure 4. OVA induce intestinal damage in tight junction and inflammation in Juvenile rats. A&B&C. OVA causes damage to intestinal tight junctions (black arrows) and mitochondria (hollow arrows); D. Swollen intestinal epithelial cells following OVA sensitization in juvenile rats; E. Aggregation of eosinophils (indicative of inflammation) in intestinal mucosa of OVA-sensitized juvenile rats [(Magnification: A, 18500×; B, 13500×; C, 26500×; D, 9700×; E, 13500×); (A, control; B,C&D, OVA-treated)].
respiratory and gastro-intestinal tracts are obviously different between adults and children (i.e., more common in children than in adults) with allergy and their sensitization rate was obviously higher than the adult group (Table 1). That might be related to the immaturity of the intestinal mucosa barrier and mucosal immunity in children (as well as in the juvenile rats).

It is reported that food specific IgG levels may rise in food allergy patients. Our results also showed that OVA specific IgG rose at an early stage. Serum OVA-IgG increased both in juvenile and adults rats (Figure 2B). We hence verified that food allergy is not just type I IgE-mediated allergy, though the underlying mechanisms still need exploring.

The intestinal tract is the main digestion organ. Most patients have alimentary symptoms such as abdominal pain, distension, diarrhea, and other irritable bowel symptoms during food allergy. The structure and function of the intestinal mucosa may change when allergy occurs. Intestinal villus cells of the duodenum or the lower ileum were swollen, damaged or even eroded after OVA sensitization in our study (Figure 2 and 3, Figure 4D). Moreover, inflammatory cell infiltration was observed under electron microscope, indicating inflammation in intestinal tract during allergy induction by OVA (Figure 4E), accompanied by swelling of mitochondrial (Figure 4C).

Intestinal mucosa is the first defense barrier to prevent the intake of harmful substances in cases of food allergy. It is reported that the intestinal permeability may increase when the intestinal mucosal barrier function is damaged during allergy. Our lactulose/mannitol assay showed that the intestinal permeability increased after OVA sensitization (Figure 3E), which was further confirmed by the electron microscopy which showed that the intercellular tight junctions of intestinal villus epithelial cells were damaged with expanded gaps (Figure 4B and 4C).

Tight junctions play important role in intestinal permeability maintenance, which is considered to be the barrier which determines selective cellular absorption. Tight junctions consist of multiple proteins as a functional complex in which transmembrane proteins, the ZO protein family and cytoskeleton proteins are the most important. Transmembrane proteins (including occludin, claudins, JAMs and CAR and etc.) are the structural proteins arranged in a linear structure, while the cytoplasmic adhesion proteins, ZO-1, ZO-2 and...
ZO-3, are the foundations or supporting structure and are responsible for the connection of transmembrane proteins to the cytoskeleton.\(^{28-30}\)

We further explored the underlying mechanisms that are responsible for intestinal barrier damage and tight junction permeability induced by OVA. Our Real-time PCR study showed that OVA could down-regulate the expression of ZO-1, Claudin-2, Claudin-8 and Claudin-15 (Figure 5A). ZO-1 plays an important role in the regulation of tight junction permeability.\(^{27,31}\) Studies have shown that the inflammatory bowel disease, chylous diarrhea, is closely related to ZO-1 expression.\(^{32}\) Celiac patients have been found to have reduced ZO-1 expression in the duodenal mucosa.\(^{27,31}\) Claudins also play an important role in intestinal barrier function regulation.\(^{32-35}\) Abnormal expression of Claudins (e.g., claudins-3, -5, and -8) can lead to Crohn’s disease.\(^{33}\) Transgenic Claudin-1 knockout mice may die rapidly because of a serious lack of transdermal water loss.\(^{33}\) Our results suggested that OVA induces damage to the intestinal barrier and tight junction permeability was related to the expression and regulation of ZO-1. Claudins and other key tight junction proteins (Figure 5B). However, Claudin-9 appeared to be significantly up-regulated, which may be due to its differential expression style between the adults and juveniles\(^{33}\) and there were no significant changes in Oclcluding expression (Figure 5A).

In summary, our study has demonstrated that juvenile rats were more easily sensitized by OVA induction; OVA could modulate the intestinal permeability through tight junction (key proteins expressions) regulation, which might cause intestinal mucosal leakage and induce intestinal food allergy (Figure 5B). Though the exact underlying molecular mechanism remains to be clarified, these findings to some extent add new knowledge to our understandings of intestinal food allergy and provide new insights for food allergy treatment or prevention.

Conflict of interest

The authors confirm that there are no conflicts of interest.

Acknowledgement

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References

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