



Impaired activation of mast cells upon IgE-mediated antigen stimulation in a stroke-prone spontaneously hypertensive rat strain, SHRSP.Z

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ABSTRACT

We investigated IgE-mediated allergic responses in a metabolic syndrome model rat strain, SHRSP.Z, which develops obesity and hypertension to cast light on the relationship between metabolic disturbances and allergic responses. IgE-mediated cutaneous anaphylactic responses were severely attenuated in this strain regardless of the presence of *fa/fa* mutation, compared with the parental WKY/Izm strain. Furthermore, in the peritoneal mast cells of both the SHRSP.Z and SHRSP/Izm strains, IgE-mediated activation, such as degranulation and protein tyrosine phosphorylation, was severely impaired whereas no significant differences were found in morphology and number of peritoneal mast cells. Immunoblot analyses revealed that phosphorylation levels of Syk upon IgE-mediated antigen stimulation were significantly decreased and basal expression of linker for activation of T cells (LAT) was down-regulated in peritoneal mast cells of the SHRSP strains. These results suggest that attenuated cutaneous allergic responses in the SHRSP.Z strain might be attributed to impaired FcεRI-mediated signal transduction in mast cells.

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1. Introduction

A series of common metabolic disturbances, such as obesity and elevated blood pressure, have been focused on, since they were found to be closely associated with type 2 diabetes and cardiovascular diseases [1–3]. Metabolic syndromes have been associated with chronic inflammatory changes, indicating that these disturbances should modify systemic immune responses. Visceral obesity, in particular, was found to exert a strong influence on systemic immune regulation through the release of various adipocytokines, such as leptin, adiponectin, TNF- α , and IL-6. Indeed, epidemiologic analyses and basic studies with animal models have supported the hypothesis that obesity is a risk factor for asthma [4].

Since metabolic disturbances display polygenic features in general, attempts to identify the responsible genes were only modestly successful [5]. On the other hand, phenotype-driven approaches have developed a variety of useful experimental animal models, such as the spontaneous hypertensive rat (SHR) [6]. The SHRSP.Z strain, which was established by crossing stroke-prone SHR (SHRSP/Izm) to Zucker fatty (ZF) rats that harbor a missense

mutation (*fa/fa*) of the leptin receptor, was found to develop obesity and hypertension simultaneously, and is regarded as a good model of human metabolic syndrome [7]. In this study, we investigated the IgE-mediated immediate allergic responses using the SHRSP.Z strain, to clarify how hypertension and/or obesity affect the IgE-mediated anaphylactic responses.

2. Materials and methods

2.1. Animals

Specific pathogen-free, 16-week-old male WKY/Izm (WKY/Izm), stroke-prone spontaneously hypertensive rat (SHRSP/Izm) (SHRSP/Izm) and SHRSP.Z-*Lepr^{fa}/IzmDmcr* (SHRSP.Z) rats were provided by the Disease Model Cooperative Research Association (Kyoto, Japan). The SHRSP.Z^{*fa/fa*} strain was established by introducing the *fa* mutation of *Lepr* into the original SHRSP/Izm strain, whereas the SHRSP.Z^{*+/+*} strain was the congenic wild type strain established simultaneously. The SHRSP.Z^{*+/+*} strain might be genetically identical to the SHRSP/Izm strain. This study was approved by the Committee on Animal Experiments of Mukogawa Women's University.

2.2. Cutaneous allergic response

In passive cutaneous anaphylaxis (PCA) experiments, rats were sensitized with an anti-DNP IgE (50 ng/site, clone SPE-7, Sigma,

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St. Louis, MO) by intradermal injection into ear pinna 24 h before antigen stimulation. The rats were then challenged by intravenous injection of the antigen/dye solution (2.5 mg DNP-conjugated human serum albumin (HSA), Sigma, and 5 mg Evans blue in 1 ml saline). In analyses of compound 48/80-induced cutaneous responses, rats were intradermally injected with compound 48/80 (0.6 µg/site, Sigma) immediately after the intravenously injection with the dye solution (5 mg Evans blue in 1 ml saline). The rats were sacrificed 30 min after the injection. The ear pinna was dissolved in 2 ml of 3 M potassium hydroxide and then neutralized with 1 ml of 1.24 M phosphoric acid. Evans blue dye was extracted with 3 ml of acetone and the OD₆₂₀ value was measured. The dye amount was calculated using the standard curve.

2.3. Measurement of protease activities and histamine

Ear tissues or peritoneal cells were lysed in PBS containing 2 M NaCl and 0.5% Triton X-100, and incubated for 30 min on ice. The lysate was centrifuged at 10,000 × g for 30 min at 4 °C. Activities of three different types of granule proteases and histamine content in the resultant supernatants were measured as previously described [8,9].

2.4. Degranulation

Peritoneal cells suspended in PIPES buffer (25 mM PIPES–NaOH, pH 7.4 containing 125 mM NaCl, 2.7 mM KCl, 5.6 mM glucose, 1 mM CaCl₂, and 0.1% bovine serum albumin) were sensitized with an anti-DNP IgE (1 µg/ml) for 1 h, twice washed with the buffer, and then stimulated with DNP-conjugated HSA. Degranulation was evaluated by measuring the amount of histamine.

2.5. Flow cytometry

Surface expression of FcεRI in peritoneal cells was measured using FACSCalibur (Becton Dickinson) equipped with CELLQUEST software as described previously [8].

2.6. Purification of peritoneal mast cells

Purification of peritoneal mast cells was performed with Histo-denz (Sigma) as previously described [10]. The purity of mast cells was >90% based on the staining with Safranin-O.

2.7. Measurement of cytosolic Ca²⁺ concentrations

Cytosolic Ca²⁺ concentrations were measured using Fura-2/AM as described previously [9].

2.8. Enzyme-linked immunosorbent assay (ELISA) for total serum IgE

Total serum IgE concentrations were measured by a ELISA kit according to the manufacturer's instruction (Shibayagi, Gunma, Japan).

2.9. Immunoblot analyses

Immunoblot analysis was performed as described previously [9] using antibodies raised against phosphotyrosine (clone 4G10, Millipore, Billerica, MA), LAT, phospholipase C-γ1 (Millipore), Lyn, Fyn (Santa Cruz, CA), phosphorylated Syk (Tyr525/526), phosphorylated p44/42 MAP kinase (phosphorylated ERK, Thr202/Tyr204), phosphorylated LAT (Tyr171), Syk, p44/42 MAP kinase (Cell Signaling Technology, Danvers, MA), and actin (Chemicon, Temecula, CA).

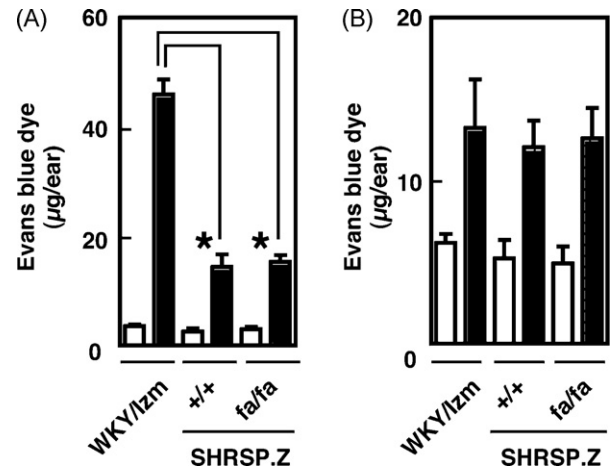


Fig. 1. Attenuated responses of the SHRSP.Z strains to IgE-mediated passive cutaneous anaphylaxis. Rats were challenged with intravenous injection of the antigen and Evans blue 24 h after sensitization with (closed columns) or without (open columns, saline alone) an anti-DNP IgE (SPE-7, 50 ng/site, s.c.) in IgE-mediated passive cutaneous anaphylaxis (A). Compound 48/80 (0.6 µg/site, closed columns) or saline (open columns) was subcutaneously injected right after the intravenous injection of Evans blue dye for comparison (B). The levels of extravasation were evaluated by measuring the amount of dye in the ear tissues. Values are presented by the means ± SEM ($n = 6-11$). The values of * $P < 0.05$ are regarded as significant by one way ANOVA (Dunnett's post hoc test, vs. WKY/lzm).

3. Results

3.1. Hyporeactivity of the SHRSP.Z strain to IgE-mediated passive cutaneous anaphylaxis

We first investigated the immediate cutaneous responses in the SHRSP.Z strains. Significant decreases in dye extravasation levels were found in the SHRSP.Z strain upon IgE-mediated passive cutaneous anaphylaxis (PCA) responses, whereas no significant differences in dye extravasation levels were observed upon compound 48/80-induced cutaneous responses among the WKY/lzm and SHRSP.Z strain (Fig. 1).

3.2. Characterization of the peritoneal mast cells in the SHRSP.Z strain

We then characterized the peritoneal mast cells to clarify how IgE-mediated activation of mast cells is impaired in the SHRSP.Z strain, since it is difficult to collect a large number of intact dermal mast cells from the skin tissues. The number and morphology of peritoneal mast cells were not different among the WKY/lzm and SHRSP.Z strains (Fig. 2A and B). We measured the granule protease activities and histamine content in the peritoneal cells. The peritoneal cells from the SHRSP.Z strain had significantly higher chymotryptic activity than those from the WKY/lzm strain (Fig. 2C). The peritoneal cells from the SHRSP.Z^{fa/fa} strain exhibited higher carboxypeptidase A activity compared with those from the WKY/lzm and SHRSP.Z^{+/+} strains (Fig. 2D). We could not detect tryptic activity in the peritoneal cells of any strain (data not shown). The histamine content in the peritoneal mast cells from the SHRSP.Z^{+/+} strain was as half as that from the WKY/lzm strain, whereas that from the SHRSP.Z^{fa/fa} was comparable to that from the WKY/lzm strain (Fig. 2E). We then measured the serum IgE concentrations and found that the serum IgE levels were significantly decreased in the SHRSP.Z strains (WKY/lzm; 30.9 ± 6.10 , SHRSP.Z^{+/+}; 4.96 ± 0.301 , SHRSP.Z^{fa/fa}; 13.7 ± 6.07 ng/ml, $n = 6$). Since IgE bound to FcεRI was found to stabilize and up-regulate the surface expression of FcεRI [11], it was possible that the surface expression of FcεRI was down-

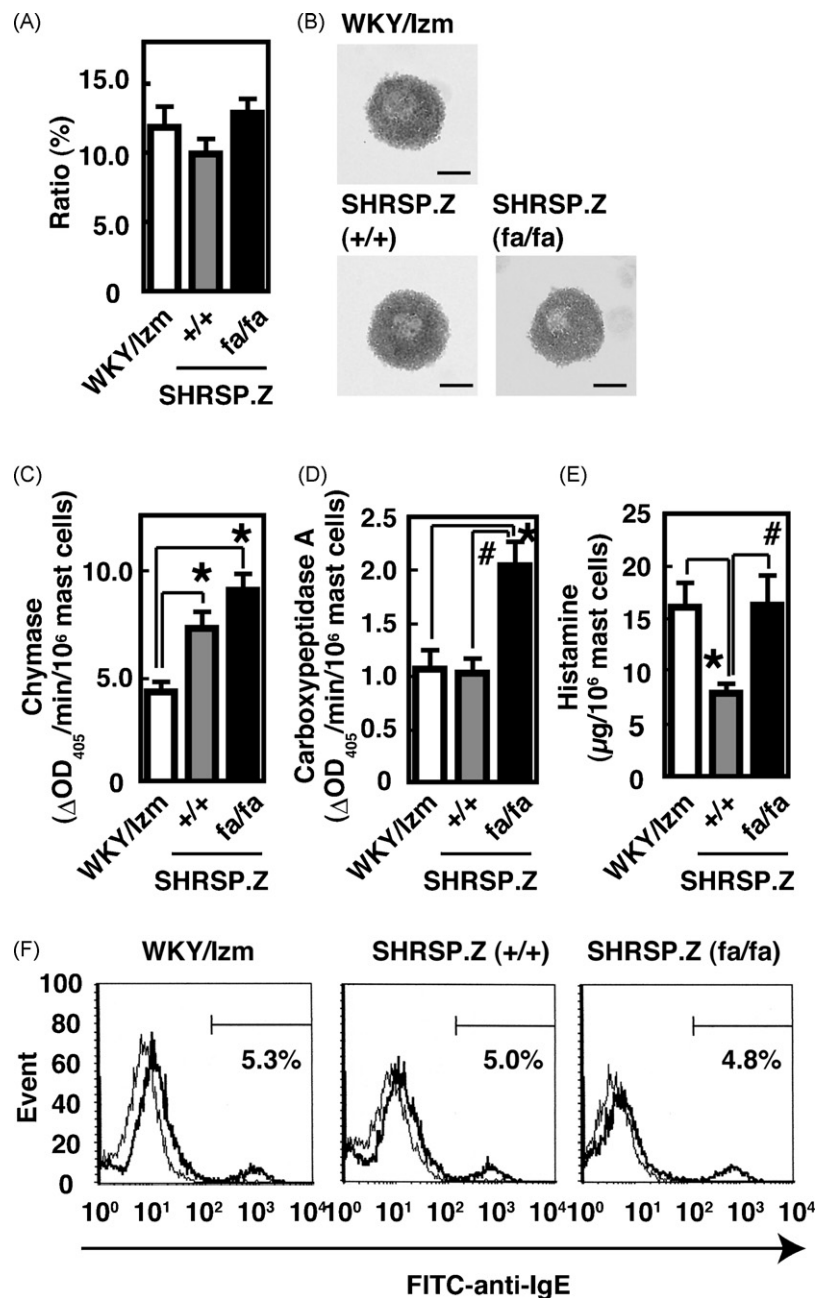


Fig. 2. Characterization of peritoneal mast cells. (A) The number of Safranin-positive cells was counted and presented as the ratio in the total peritoneal cells. Values are presented by the means $\pm$ SEM ($n = 7-8$). (B) Images of Safranin staining of peritoneal mast cells derived from each strain are presented. Bar = 10 μm . (C-E) Granule protease activity and histamine content were presented. Values are presented by the means $\pm$ SEM ($n = 7-8$). The values of * P , # $P < 0.05$ are regarded as significant by one-way ANOVA (Dunnett's post hoc test, vs. *WKY/lzm or #SHRSP.Z^{+/+}). (F) The surface expression of Fc ϵ R1 in total peritoneal cells was measured by flow cytometry. Percentages of the cells showing high levels of IgE binding (mast cells) are indicated. This is a representative panel of three independent experiments showing similar results.

regulated in mast cells in the SHRSP.Z strains. However, the surface expression levels of the Fc ϵ R1 in the peritoneal cells were not significantly changed among the WKY/lzm and SHRSP.Z strains (Fig. 2F).

3.3. Impaired degranulation of the peritoneal mast cells of the SHRSP.Z strain upon IgE-mediated antigen stimulation

We then investigated the degranulation of the peritoneal mast cells. Drastic decreases in histamine release upon IgE-mediated antigen stimulation were observed in the peritoneal mast cells from the SHRSP.Z strain, whereas a slight increase in sensitivity to compound 48/80 was observed (Fig. 3). Since these *in vitro* observations

were consistent with the results of the cutaneous responses, we further investigated the mechanism, under which antigen-induced degranulation is impaired.

3.4. Attenuated tyrosine phosphorylation upon IgE-mediated antigen stimulation in peritoneal mast cells of the SHRSP/lzm and SHRSP.Z strains

We then measured tyrosine phosphorylation of various signaling molecules and intracellular Ca^{2+} mobilization using the purified peritoneal mast cells. Tyrosine phosphorylation upon IgE-mediated antigen stimulation was totally attenuated in the peritoneal mast cells from the SHRSP.Z and SHRSP/lzm strains compared with that

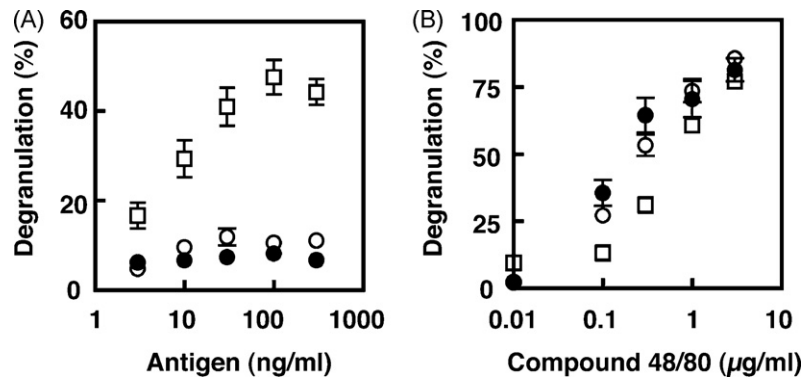


Fig. 3. Impaired degranulation upon IgE-mediated antigen stimulation in peritoneal mast cells of the SHRSP.Z strains. (A and B) Peritoneal cells were collected from WKY/lzm (open squares), SHRSP.Z^{+/+} (open circles), and SHRSP.Z^{fa/fa} (closed circles) rats, respectively. A part of the collected cells was stimulated with the indicated concentrations of DNP-conjugated HSA 1 h after sensitization with an anti-DNP IgE (1 μg/ml, A), whereas the residual part was stimulated with the indicated concentrations of compound 48/80 (B). Degranulation of mast cells was determined by measuring the amount of histamine. Values are presented by the means ± SEM ($n = 5-7$).

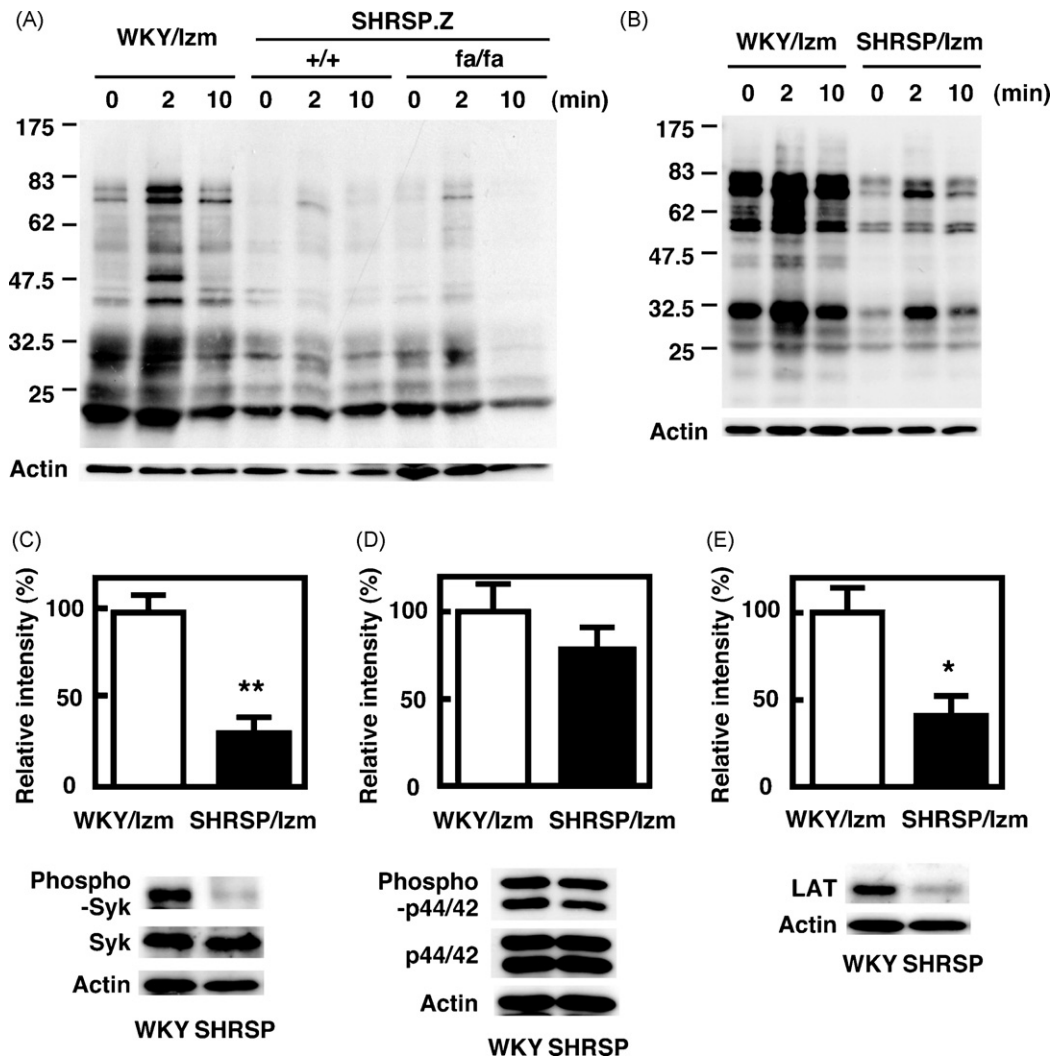


Fig. 4. Attenuated responses upon IgE-mediated antigen stimulation in peritoneal mast cells purified from the SHRSP.Z and SHRSP/lzm strains. (A and B) Peritoneal mast cells purified from WKY/lzm (A and B), SHRSP.Z^{+/+} (+/+), SHRSP.Z^{fa/fa} (fa/fa) (A), and SHRSP/lzm (B) rats were stimulated with DNP-conjugated HSA (100 ng/ml) 1 h after sensitization with an anti-DNP IgE (1 μg/ml). Immunoblot analyses with an anti-phosphotyrosine antibody (clone 4G10) was performed. Expression levels of actin were investigated as the loading control. (C and D) Peritoneal mast cells purified from the WKY/lzm (WKY) and SHRSP/lzm (SHRSP) strains were stimulated as described above. Immunoblot analyses were performed using an anti-phosphorylated Syk antibody and an anti-phosphorylated p44/42 MAP kinase (anti-phosphorylated ERK) antibody. The intensities of immunoreactive bands were densitometrically determined and are expressed as the relative value using the mean intensity in the WKY/lzm rats as the standards (100%). The relative band intensities are presented by the means ± SEM ($n = 3$). The value of $^{**}P < 0.01$ is regarded as significant by the Student's *t*-test. (E) Basal expression levels of LAT in the purified peritoneal mast cells were determined by the immunoblot analyses. The values are expressed as described above. The relative band intensities are presented by the means ± SEM ($n = 3$). The value of $^{*}P < 0.05$ is regarded as significant by the Student's *t*-test.

from the WKY/Izm strain (Fig. 4A and B). On the other hand, the levels of Ca^{2+} mobilization were marginally decreased in the peritoneal mast cells from the SHRSP.Z strain, whereas no detectable changes were observed in compound 48/80-induced Ca^{2+} mobilization (data not shown). Severe decreases in the total tyrosine phosphorylation levels raised the possibility that the signal transduction immediately downstream of the $\text{Fc}\epsilon\text{RI}$ is impaired in the mast cells from the SHRSP.Z and SHRSP/Izm strains. We then compared the signal transduction pathway in the peritoneal mast cells activated upon IgE-mediated antigen stimulation between the WKY/Izm and SHRSP/Izm strains. Upon antigen stimulation, tyrosine phosphorylation of Syk was significantly impaired in the mast cells of the SHRSP/Izm strain, whereas phosphorylation of extracellular signal-regulated kinase (ERK) was slightly but not significantly attenuated (Fig. 4C and D). Basal expression levels of Syk, ERK, Lyn and Fyn were not changed in the peritoneal mast cells between both strains (Fig. 4C and D and data not shown), whereas those of LAT were significantly decreased in peritoneal mast cells of the SHRSP/Izm and SHRSP.Z strains (Fig. 4E and data not shown).

4. Discussion

We demonstrated in this study that the IgE-mediated PCA responses were severely attenuated in the SHRSP.Z strain. Kwasniewski et al. [12] previously demonstrated that in the spontaneously hypertensive rat (SHR) strain IgE-mediated PCA responses were attenuated and degranulation of the mesenteric mast cells upon immunological challenge was impaired, which is consistent with our results. Although they further excluded the possibility that endogenous nitric oxide is involved in impaired degranulation of mesenteric mast cells in the SHR strain [13], it remains unknown why IgE-mediated activation of mast cells is impaired in the SHR strain. Our approaches with the purified peritoneal mast cells allowed us to investigate the signal transduction in detail.

We found that tyrosine phosphorylation of Syk was significantly impaired in the peritoneal mast cells of the SHRSP/Izm strain upon IgE-mediated antigen stimulation. Since accumulating evidence indicates that Syk plays a critical role in the signal transduction in mast cells upon antigen stimulation [14,15], it is likely that the drastically impaired tyrosine phosphorylation upon antigen stimulation should be attributed to insufficient activation of Syk in peritoneal mast cells of the SHRSP/Izm strain. We further investigated the status of upstream tyrosine kinases, Lyn, since phosphorylation of Syk was found to be drastically impaired in the mast cells lacking Lyn, not in those lacking Fyn [16], but could not determine it using the commercially available antibodies whether the enzymatic activity of these kinases is impaired in the SHRSP/Izm strains.

We also found that basal expression levels of LAT were significantly decreased in peritoneal mast cells in the SHRSP/Izm and SHRSP.Z strains. Accumulating evidence suggests that LAT plays a critical role in $\text{Fc}\epsilon\text{RI}$ -mediated signal transduction [17,18]. The phenotypic changes observed in the SHRSP.Z strains were reminiscent of the LAT deficient mice. Profound defects in degranulation after cross-linking of the $\text{Fc}\epsilon\text{RI}$ were observed in bone marrow-derived cultured mast cells (BMMCs) lacking LAT, whereas Ca^{2+} mobilization upon antigen stimulation was marginally changed [19,20]. Furthermore, IgE-mediated PCA responses were found to be abolished in the LAT deficient mice. However, it remains to be clarified how the down-regulation of LAT in the SHRSP strains is involved in impaired degranulation, since activation of one of the downstream signaling molecules, ERK, was only marginally affected. Insufficient activation of Syk may contribute to impaired degranulation of peritoneal mast cells in the SHRSP/Izm strain in a LAT-independent manner, since phosphorylation of Syk remained unchanged in

BMMCs lacking LAT [19]. To investigate the effects of forced expression of LAT in the peritoneal mast cells on degranulation and protein tyrosine phosphorylation should provide important clues to the mechanism, although no established methods have been reported.

It is interesting to note that human LAT gene is located on chromosome 16p11.2, which is in close proximity to the regions that are identified as hypertension-related quantitative trait locus (QTL) [5,21]. Previous studies suggested that immunologic dysfunction including depressed T cell function in the SHR strain resulted in hypertension [22]. Our findings raised the possibility that a decrease in LAT expression results in impaired T cell function, which triggers hypertension. Further in-depth analyses should be required to verify this possibility.

We demonstrated significant increases in chymotryptic activity in peritoneal mast cells in the SHRSP.Z strain. Accumulating evidence suggests that angiotensin II formation is mediated by two pathways; the angiotensin-converting enzyme (ACE)-dependent and the chymase-dependent pathway. Involved chymases are divided into two categories; chymases expressed in mast cell granules, and a vascular smooth muscle chymase. Rat vascular smooth muscle chymase (RVCH) was found to be up-regulated in the SHR strain and the transgenic strain that over-expressed this enzyme in the smooth muscle cells exhibited hypertension [23,24]. These findings suggested that RVCH plays a critical role in hypertension in the SHR strain, although involvement of chymases in blood pressure regulation was disclaimed by pharmacological approaches with specific inhibitors for ACE and chymases [25]. Since expression levels of mast cell chymases have not been investigated in the SHR strain and the involvement of them in hypertension is controversial, our findings raised the possibility that up-regulation of chymotryptic activity in mast cells is at least in part involved in hypertension in the SHRSP.Z strain.

We could find no highlighted differences between the lean substrain (+/+) and the obese substrain (*fa/fa*) of the SHRSP.Z strain except in carboxypeptidase A activity and histamine content in peritoneal mast cells. A significant increase in granule histamine storage raised the possibility that inflammatory responses are exacerbated in the obese substrain. However, dye extravasation induced upon non-immunological stimuli were comparable between both substrains. It should require further analyses using the mutant strains without hypertensive background, such as the Zucker strain, to determine precisely the effects of the *fa/fa* mutation on histamine synthesis in mast cells.

In summary, these results suggest that attenuated cutaneous allergic responses in the SHRSP.Z strain might be attributed to impaired activation of mast cells, in which expression of LAT was significantly down-regulated.

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