

# Inhibition of degranulation and cytokine production in bone marrow-derived mast cells by hydrolyzed rice bran

Yuka Hoshino · Naohide Hirashima ·  
Mamoru Nakanishi · Tadahide Furuno

Received: 18 April 2009 / Revised: 29 January 2010 / Accepted: 2 February 2010 / Published online: 5 March 2010  
© Springer Basel AG 2010

## Abstract

**Objective** We investigated the effects of hydrolyzed rice bran (HRB), an arabinoxylan extracted from rice bran, on mast cell degranulation and cytokine production.

**Materials and methods** HRB was obtained by treating rice bran with an extract obtained from shiitake mushrooms. Bone marrow-derived mast cells (BMMCs) were prepared by culturing bone marrow cells from BALB/c mice in the presence of interleukin-3 and stem cell factor for 4 weeks. BMMCs were pretreated with HRB (0–3 mg/ml) for 30 min and were then antigen activated.

**Results** Pretreatment of BMMCs with HRB significantly inhibited antigen-induced degranulation and cytokine production (tumor necrosis factor- $\alpha$  and interleukin-4) in a dose-dependent manner. HRB also diminished membrane fusion between liposomes in which soluble *N*-ethyl maleimide-sensitive factor attachment protein receptors were reconstituted. Phosphorylation of RelA and mitogen-activated kinases after antigen stimulation was suppressed by pretreatment of BMMCs with HRB.

**Conclusions** These findings suggest that HRB may have an anti-inflammatory effect by inhibiting mast cell degranulation and cytokine production.

**Keywords** Rice Bran · Mast Cell · Degranulation · Cytokine · Phosphorylation

## Introduction

MGN-3/biobran, a modified arabinoxylan obtained from the treatment of rice bran with carbohydrate-hydrolyzing enzymes from shiitake mushrooms, is known to be a potent immunological modifier, producing effects such as enhancing activities of natural killer (NK) cells, T cells, B cells, and macrophages [1]. With regard to anti-cancer effects, MGN-3 has been reported to increase yeast-induced apoptosis of human breast cancer cells and increase susceptibility to death receptor-induced apoptosis of human T leukemia cells [2]. It has also been reported to have a beneficial effect against cisplatin-induced weight loss in mice [3]. In addition, oral administration of MGN-3 has been found to significantly reduce symptoms of toluene-diisocyanate-induced asthma in mice (personal communication).

Like MGN-3, hydrolyzed rice bran (HRB) is also derived from rice bran. It is obtained from the treatment of water-soluble rice bran with carbohydrate-hydrolyzing enzymes from shiitake mushrooms for a longer period, and is composed of polysaccharides of lower molecular size, compared to MGN-3. Oral administration of HRB (500 mg/day) has been reported to decrease scores in symptoms of common cold syndrome and shorten the average duration of symptoms in the elderly [4]. HRB does not affect the disease rate of symptoms and the NK cell activity, suggesting that HRB might have an anti-inflammatory effect rather than an immunomodulatory effect. However, it is not yet understood how HRB influences functional roles of inflammatory cells such as mast cells.

---

Responsible Editor: M. Katori.

---

Y. Hoshino · N. Hirashima  
Graduate School of Pharmaceutical Sciences,  
Nagoya City University, 3-1 Tanabe-dori,  
Mizuho-ku, Nagoya 467-8603, Japan

M. Nakanishi · T. Furuno (✉)  
School of Pharmacy, Aichi-Gakuin University,  
1-100 Kusumoto-cho, Chikusa-ku, Nagoya 464-8650, Japan  
e-mail: furuno@dpc.agu.ac.jp

Mast cells express high-affinity IgE receptors (FcεRI) on their surface and play an important role in allergic disorders. Antigen-mediated crosslinking of FcεRI induces the activation of several protein tyrosine kinases, including Lyn, Fyn, Syk, and Btk, and the phosphorylation of Akt and phospholipase Cγ (PLCγ). The activation of PLCγ leads to the production of inositol 1,4,5-triphosphate and diacylglycerol that elicit increased intracellular  $\text{Ca}^{2+}$  concentrations ( $[\text{Ca}^{2+}]_i$ ) and protein kinase C (PKC) activity, respectively [5, 6]. The antigen activation of mast cells also induces the release of inflammatory mediators from secretory granules and the production of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-4 (IL-4) [7]. In releasing mediators, membrane-membrane fusion proteins, termed soluble N-ethyl maleimide-sensitive factor attachment protein receptor (SNAREs), control the exocytosis of granules in mast cells [8–12]. There are distinct SNAREs on plasma and granule membranes. The fusion of these membranes is induced by the binding of SNAREs on the granule membrane (v-SNAREs) to SNAREs on the plasma membrane (t-SNAREs). With respect to cytokine production, mitogen-activated protein (MAP) kinases, nuclear factor κB (NF-κB), and nuclear factor of activated T cells (NF-AT) regulate cytokine gene transcription [7]. The activation of their signaling pathway follows the increase in  $[\text{Ca}^{2+}]_i$  and the activation of PKC, and stimulates the transcription of several cytokines.

In this paper, to investigate anti-inflammatory effects of HRB, we examined effects of HRB on the activation of bone marrow-derived mast cells (BMMCs) induced by FcεRI ligation. Although BMMCs belong to mucosal type mast cells (MMC) that contain fewer granules than connective tissue mast cells (CTMCs), they are well-characterized primary culture mast cells and are established counterparts of MMCs. We established that pretreatment of BMMCs with HRB suppressed degranulation and cytokine production in a dose-dependent manner, probably by inhibiting the activation of NF-κB and MAP kinases as well as the fusogenic activity of plasma and granule membranes. Our results suggest that HRB may be useful for the prevention and treatment of allergic diseases mediated by mast cells.

## Materials and methods

### Materials

BMMCs were obtained by culturing bone marrow cells from 10 to 15 week-old BALB/c mice (Japan SLC, Shizuoka, Japan) in Dulbecco's modified Eagle medium

(DMEM) supplemented with 10% fetal calf serum (Roche, Mannheim, Germany), 0.1 mM non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml recombinant murine IL-3 (Peprotech, London, UK), and 2 ng/ml recombinant murine stem cell factor (Peprotech) [13]. After 4 weeks of culture, >95% of the viable cells were confirmed to be mast cells, as assessed by staining with acidic toluidine blue. BALB/c mice were housed under standard conditions and in compliance with Aichi Gakuin University guidelines for the use of experimental animals, and all animal experiments were performed with approval by the Animal Care and Use Committee of Aichi Gakuin University. Mouse anti-dinitrophenyl (DNP) IgE (IgE-53-569) was originally provided by Prof. T. Kishimoto (Osaka University). DNP-conjugated bovine serum albumin (DNP-BSA) was prepared using methods described previously [14]. In our present experiments, six DNP groups, on an average, were conjugated with a BSA molecule.

### HRB

HRB, which was a gift from Daiwa Pharmaceutical Co., Ltd. (Tokyo, Japan), was obtained from defatted extracts of rice bran modified by digestion with glucosidases obtained from shiitake mushroom (*Lentinus edodes*) mycelia. HRB is a polysaccharide that primarily contains arabinoxylan hemicelluloses. The molecular mass distribution of HRB is divided into two groups of approximately 5,400 (main peak) and 1,800. The procedure for obtaining HRB is repeatable. Acute and sub-chronic toxicity tests of HRB revealed an LD50 >5,000 mg/kg in rats and the no-observed-adverse effect levels of HRB were 1,301 and 1,562 mg/kg/day for male and female rats, respectively. The Ames test for mutagenicity was negative.

When BMMCs were cultured overnight in the presence of HRB (0–3 mg/ml), >90% of BMMCs were confirmed to be viable and morphologically normal by the MTT assay and trypan blue test. BMMCs were pretreated with HRB for 30 min and then were stimulated in the presence of HRB.

### Assay for degranulation

Degranulation of BMMCs was monitored by measuring the activity of the granule-stored enzyme β-hexosaminidase secreted into cell supernatants [15, 16]. BMMCs ( $5 \times 10^5$  cells) were sensitized with 1 μg/ml anti-DNP IgE for 30 min at 37°C, and then washed twice with Hepes buffer [10 mM Hepes (pH7.4), 140 mM NaCl, 5 mM KCl, 1 mM  $\text{CaCl}_2$ , 0.6 mM  $\text{MgCl}_2$ , 0.1% bovine serum albumin, and 0.01% sulfinpyrazone]. BMMC suspension (200 μl) was

incubated with 500 ng/ml DNP-BSA for 30 min at 37°C. Aliquots of supernatants were transferred to a 96-well plate (20 µl/well) and incubated with 20 µl of substrate solution [2 mM *p*-nitrophenyl-*N*-acetyl- $\beta$ -D-glucosaminide (Sigma-Aldrich, St Louis, MO, USA) in 100 mM citrate (pH4.5)] for 1 h at 37°C. After terminating the reaction with 160 µl of 167 mM Na<sub>2</sub>CO<sub>3</sub>-NaHCO<sub>3</sub> buffer, the absorbance at 405 nm was measured using a microplate reader (Model 680, Bio-Rad Laboratories, Tokyo, Japan). The release activity relative to the total  $\beta$ -hexosaminidase content of cells was determined by disrupting cells with 0.1% Triton X-100.

#### Assays for cytokine production

BMMCs ( $5 \times 10^5$  cells) were sensitized with 1 µg/ml anti-DNP IgE for 30 min at 37°C, and then washed twice with Hepes buffer and stimulated with DNP-BSA (500 ng/ml) for 4 h at 37°C. Supernatants were collected and absolute amounts of TNF- $\alpha$  and IL-4 released by cells were determined using a mouse TNF- $\alpha$  immunoassay system and a mouse IL-4 immunoassay system (R&D Systems, Minneapolis, MN, USA), respectively [16].

#### Binding of IgE to Fc $\epsilon$ RI

Assays for determining the binding of IgE to Fc $\epsilon$ RI were performed using a procedure described previously [17]. Briefly, anti-DNP IgE (1 µg/ml) was added to the BMMC suspension ( $1 \times 10^5$  cells/ml). After incubation for 4 h at 37°C, BMMCs were washed twice with Hepes buffer. FITC-conjugated goat anti-mouse IgE antibody (1 µg/ml; Bethyl Laboratories, Montgomery, TX, USA) was added to the BMMC suspension. After incubation for 15 min at 4°C, BMMCs were washed twice with Hepes buffer. FITC fluorescence intensity was measured and analyzed with FACSsort (Becton-Dickinson, Franklin Lakes, NJ, USA) after filtering the BMMC suspension through a nylon mesh ( $\phi = 35$  µm).

#### [Ca<sup>2+</sup>]<sub>i</sub> measurement

BMMCs ( $5 \times 10^5$  cells) were loaded with 1 µM Fura 2-AM (Molecular Probes, Eugene, OR, USA) and sensitized with 1 µg/ml anti-DNP IgE for 30 min at 37°C, and then washed twice with Hepes buffer. Fluorescence intensities at 500 nm using excitation wavelengths of 340 and 360 nm were measured at 37°C, and the F340/F360 ratio was calculated using a spectrofluorometer equipped with a personal computer (RF-5300PC; Shimadzu, Kyoto, Japan). Ratios were converted to Ca<sup>2+</sup> concentrations by a procedure described previously [15, 16].

#### Western blotting

To prepare whole cell lysates, BMMCs ( $1 \times 10^6$  cells) were suspended in cold lysis buffer (20 mM Hepes [pH7.9], 0.1% NP-40, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10 mM Na<sub>3</sub>VO<sub>4</sub>, 10 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10% glycerol) and allowed to stand on ice for 30 min [16, 18, 19]. The suspension was clarified by centrifugation (15,000 rpm, 20 min) at 4°C. After this, supernatants were solubilized by treatment with Laemmli buffer [0.125 M Tris-HCl (pH6.8), 10% 2-mercaptoethanol, 4% SDS, 10% sucrose, and 0.004% bromophenol blue] for 3 min at 100°C.

Prepared proteins were separated by electrophoresis in an SDS-polyacrylamide gel. Electrophoresed proteins were transferred to a PVDF membrane with an electroblotter. After blocking with 0.5% casein, membranes were probed with rabbit anti-phospho Akt (Thr308) antibody (C31E5; 1:1,000 dilution), rabbit anti-Akt antibody (C67E7; 1:1,000 dilution), rabbit anti-phospho I $\kappa$ B kinase (IKK)  $\alpha/\beta$  (Ser176/180) antibody (16A6; 1:1,000 dilution), rabbit anti-phospho I $\kappa$ B $\alpha$  (Ser32) antibody (14D4; 1:1,000 dilution), rabbit anti-phospho RelA (Ser536) antibody (93H1; 1:1,000 dilution), rabbit anti-IKK $\alpha$  antibody (1:1,000 dilution), mouse anti-I $\kappa$ B $\alpha$  antibody (L35A5; 1:1,000 dilution), rabbit anti-RelA antibody (C22B4; 1:1,000 dilution), rabbit anti-phospho extracellular signal-regulated kinase (ERK)1/2 (Thr202/Tyr204) antibody (D13.14.4E; 1:1,000 dilution), rabbit anti-phospho p38MAP kinase (Thr180/Tyr182) antibody (3D7; 1:1,000 dilution), rabbit anti-phospho c-Jun N-terminal kinase (JNK) (Thr183/Tyr185) antibody (81E11; 1:1,000 dilution) (Cell Signaling Technology, Beverly, MA, USA), or mouse anti- $\beta$ -actin antibody (1:4,000 dilution) (Sigma-Aldrich) and treated with 1:1,000 dilution HRP-labelled anti-rabbit IgG or 1:1,000 dilution HRP-labelled anti-mouse IgG (cell signaling technology). Immunoreactivity was detected by enhanced chemiluminescence (ECL; GE Healthcare, Buckinghamshire, UK) with a LAS-3000mini (Fuji Film, Tokyo, Japan) and analyzed by Image Gauge (Fuji Film).

#### Assay for membrane fusion by SNARE liposomes

To isolate and purify SNAREs, full-length genes for syntaxin-3, vesicle associated membrane proteins (VAMP-8), and synaptosomal-associated protein 23 kDa (SNAP23) were expressed in *Escherichia coli* (BL21 Star; Invitrogen, Grand Island, NY, USA) as histidine (His)-tagged fusion proteins or glutathione S transferase (GST)-tagged fusion proteins by a procedure described previously [20].

To prepare proteoliposomes, purified SNAREs were incorporated by a detergent dialysis method based on

procedures reported previously [21–23]. 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC), 1,2-dioleoylphosphatidylserine (DOPS), N-(7-nitro-2,1,3-benzoxadiazole-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine (NBD-DPPE), and N-(lissamine rhodamine B sulfonyl) 1,2-dipalmitoyl phosphatidylethanolamine (rhodamine-DPPE) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). In brief, for v-SNARE proteoliposomes, 3 mM premixed lipid solution in chloroform [POPC:DOPS:NBD-DPPE:rhodamine-DPPE = 82:15:1.5:1.5 (mol ratio)] was vacuum dried with a rotary evaporator to obtain a lipid film. Following this, v-SNARE solution (5  $\mu$ M VAMP-8, 0.8% n-octyl-D-glucoside, 100 mM KCl, 10% glycerol, 1 mM DTT, and 25 mM Hepes) was added to the film, and the film was gently agitated to dissolve the lipid. Proteoliposomes were prepared by adding 200  $\mu$ l of reconstitution buffer (100 mM KCl, 10% glycerol, 1 mM DTT, and 25 mM Hepes) and were subsequently dialyzed against reconstitution buffer containing 1 g/l of SM2 beads (Bio-Rad) for 16 h to remove n-octyl-D-glucoside. Vesicles were recovered and concentrated by flotation in a Nycodenz step gradient (Sigma-Aldrich). For t-SNARE proteoliposomes, 15 mM premixed lipid solution in chloroform [POPC:DOPS = 85:15 (mol ratio)] was dried to obtain a lipid film. Following this, t-SNARE solution (5  $\mu$ M syntaxin-3, 5  $\mu$ M SNAP23, 0.8% n-octyl-D-glucoside, 100 mM KCl, 10% glycerol, 1 mM DTT, and 25 mM Hepes) was added to the film. Proteoliposomes were prepared and concentrated by the procedure described above.

To monitor membrane fusion, the fluorescence resonance energy transfer (FRET) assay was performed as described previously [20]. Membrane fusion increased the distance between the two fluorescent lipids NBD-DPPE and rhodamine-DPPE resulting in an attenuated FRET efficiency. The two types of proteoliposomes, v-SNARE donor vesicles and t-SNARE acceptor vesicles, were mixed in reconstitution buffer and added to a quartz microcuvette. The cuvette was placed in a spectrofluorometer (RF-5300PC) at 37°C. Fluorescence intensity of NBD (525 nm) excited at 480 nm was measured. Liposomes with v-SNARE and t-SNAREs were mixed at time 0. Membrane fusion was estimated by the following formula: membrane fusion =  $100 \times [F(t) - F(0)]/[F_{\max} - F(0)]$ ; where  $F(t)$  is the fluorescence intensity of NBD at time  $t$ ,  $F(0)$  is the fluorescence intensity at time 0, and  $F_{\max}$  is the maximal fluorescence intensity when Triton X-100 was added to the cuvette [20].

### Statistical analysis

Results are expressed as means  $\pm$  standard deviation (S.D.). A one-way ANOVA was used to compare differences between groups. A  $P$  value  $<0.05$  was considered significant.

## Results

### Inhibition of degranulation and cytokine production by HRB

MGN-3, a compound related to HRB, has been reported to enhance apoptosis of human breast cancer cells and augment macrophage phagocytosis at a concentration of 0.5 mg/ml in vitro [2, 24]. We first examined whether HRB could directly affect mast cell functions, such as degranulation and cytokine production, at appropriate concentrations. Pretreatment of BMMCs with HRB (0–3 mg/ml) for 30 min resulted in the significant inhibition of  $\beta$ -hexosaminidase release after antigen stimulation in a dose-dependent manner (Fig. 1). The results indicated suppressed antigen-induced degranulation by HRB, because HRB itself did not elicit the release of  $\beta$ -hexosaminidase (Fig. 1), and treatment with HRB for 30 min did not affect the total amount of  $\beta$ -hexosaminidase in BMMCs.

Similarly, secretion of TNF- $\alpha$  and IL-4 by BMMCs after antigen stimulation were also dose-dependently inhibited by pretreatment with HRB (Fig. 2a). When BMMCs were permeabilized with Triton X-100 (0.1%) to measure the amount of cytokine synthesis after antigen stimulation, antigen-induced TNF- $\alpha$  and IL-4 syntheses were decreased in BMMCs pretreated with HRB (Fig. 2b). These results suggested that HRB significantly suppressed both degranulation and cytokine syntheses (TNF- $\alpha$  and IL-4) in BMMCs after Fc $\epsilon$ RI ligation.

### Effects of HRB on the binding of IgE to Fc $\epsilon$ RI and increased $[Ca^{2+}]_i$

To study the inhibitory mechanism of HRB on mast cell activation, we investigated the upstream events in Fc $\epsilon$ RI

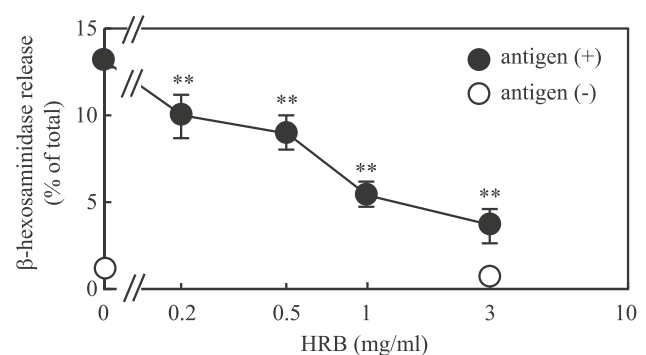
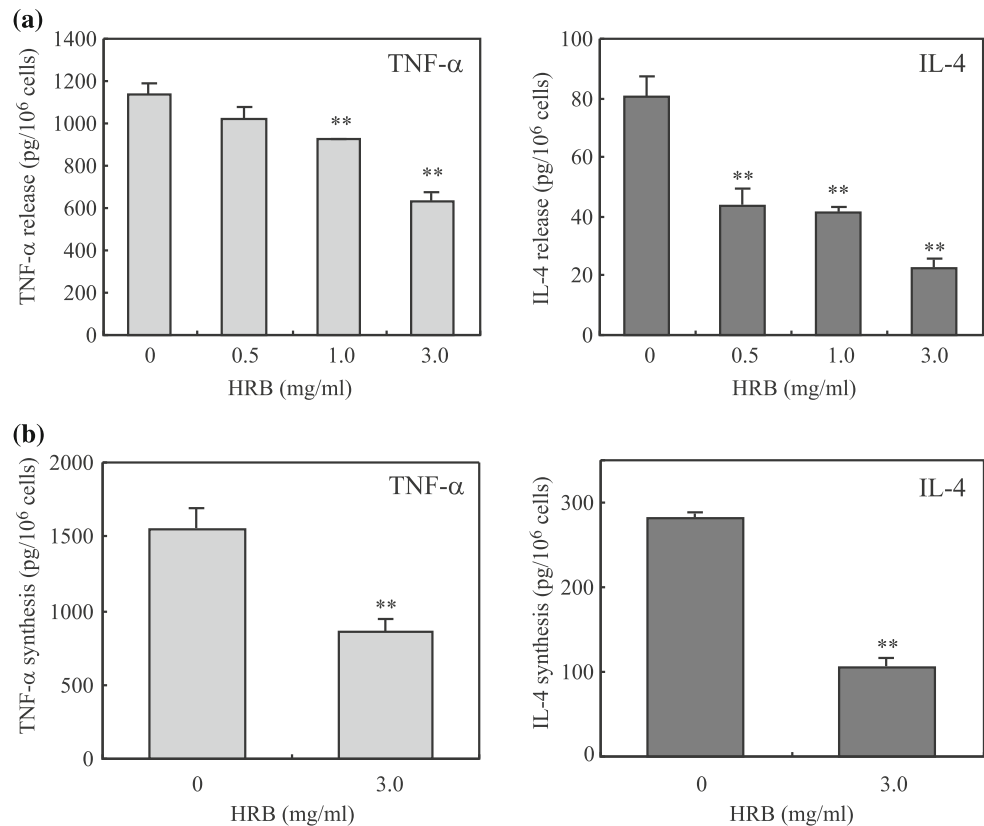


**Fig. 1** Effect of hydrolyzed rice bran (HRB) on  $\beta$ -hexosaminidase release from bone marrow-derived mast cells (BMMCs) upon antigen stimulation. Anti-dinitrophenyl (DNP) IgE-sensitized BMMCs were pretreated with the indicated concentrations of HRB for 30 min and stimulated with antigen (DNP-conjugated bovine serum albumin (DNP-BSA); 500 ng/ml) for 30 min or without antigen. Values are plotted as percentages of the total enzyme activity obtained by the treatment with Triton X-100 (mean  $\pm$  S.D.) ( $n = 3$ ). \*\* $P < 0.01$  compared with the absence of HRB

**Fig. 2** Effect of HRB on production of tumor necrosis factor (*TNF*)- $\alpha$  and interleukin (*IL*)-4 in BMMCs upon antigen stimulation. Anti-DNP IgE-sensitized BMMCs were pretreated with the indicated concentrations of HRB for 30 min and stimulated with antigen (DNP-BSA; 500 ng/ml) for 4 h. **a** The amounts of cytokines released from BMMCs. Cytokines contained in supernatants of BMMCs were measured. **b** The amounts of cytokines synthesized in BMMCs. Cytokines in supernatants of BMMCs after permeabilizing cells by the treatment with Triton X-100 were measured. Values are mean  $\pm$  S.D. ( $n = 3$ ).  $**P < 0.01$  compared with the absence of HRB



signaling. First, we examined the effect of HRB on the binding of IgE to Fc $\epsilon$ RI using flow cytometry. Pretreatment with HRB (3 mg/ml) did not affect the amount of IgE bound to Fc $\epsilon$ RI (Fig. 3a). Next, we measured  $[Ca^{2+}]_i$  of BMMCs after antigen stimulation. The resting level of  $[Ca^{2+}]_i$  was  $\sim 60$  nM in BMMCs, both with and without HRB pretreatment, indicating that HRB had no effect on resting level of  $[Ca^{2+}]_i$ . After antigen stimulation,  $[Ca^{2+}]_i$  of BMMCs increased to  $\sim 200$  nM. The increase in  $[Ca^{2+}]_i$  in BMMCs pretreated with HRB (3 mg/ml) was similar to that without pretreatment with HRB (Fig. 3b).

#### Inhibition of mast cell activation induced by A23187 and phorbol myristoyl acetate (PMA)

Results described above suggested that HRB affected the downstream pathway for increased  $[Ca^{2+}]_i$  rather than the earlier steps in the signaling pathway of BMMCs. To confirm this hypothesis, we investigated whether HRB could suppress mast cell activation induced by A23187 and PMA, which are a calcium ionophore and a PKC activator, respectively. Costimulation with A23187 and PMA elicited degranulation and cytokine production (TNF- $\alpha$  and IL-4) in BMMCs. Pretreatment of BMMCs with HRB (0–3 mg/ml) significantly inhibited degranulation and cytokine production in a dose-dependent manner (Fig. 4). This suggested

that HRB inhibited a signaling pathway following the increase in  $[Ca^{2+}]_i$  and PKC activation in BMMCs.

#### Effect of HRB on phosphorylation of Akt

To investigate whether HRB did not affect the early events in signal transduction mediated by Fc $\epsilon$ RI ligation, we evaluated the phosphorylation of Akt in BMMCs after the addition of antigen. Phosphatidylinositol 3-kinase (PI3 K) phosphorylates the inositol ring of membrane-associated phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) and converts it to phosphatidylinositol-3,4,5-triphosphate (PIP<sub>3</sub>). It is known that PIP<sub>3</sub> activates Akt by phosphoinositide-dependent kinase 1 (PDK1) activation and that the PI3 K-Akt pathway is essential for optimal mast cell activation [25]. Phosphorylation of Akt was observed after antigen addition, but pretreatment with HRB did not significantly inhibit the phosphorylation of Akt upon antigen stimulation (Fig. 5). This result corresponded to the results that HRB did not affect the antigen-induced increase in  $[Ca^{2+}]_i$  (Fig. 3b). Thus, it was proposed that HRB influenced the distal events, and not early events, in mast cell signal transduction. Therefore, we studied the effects of HRB on membrane fusion and a transcription factor that is necessary for degranulation and cytokine production, respectively.

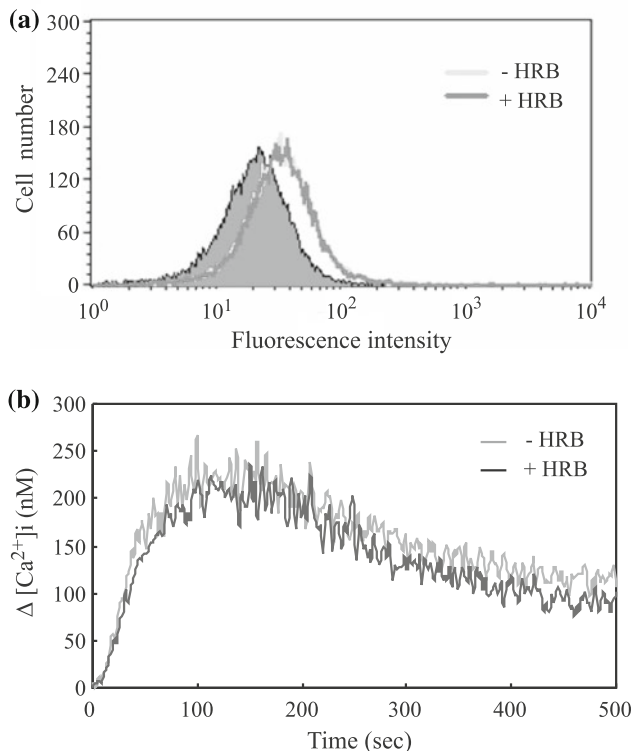


**Fig. 3** Effects of HRB on the binding of IgE to high-affinity IgE receptors (FcεRI) and the increase in intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ). **a** The binding of IgE to FcεRI on BMMCs. The IgE bound to FcεRI on BMMCs were detected by FITC-conjugated anti-IgE antibody using flow cytometry. BMMCs were pretreated with anti-DNP IgE (1  $\mu\text{g}/\text{ml}$ ) and HRB (3  $\text{mg}/\text{ml}$ ) for 4 h and then incubated with FITC-conjugated goat anti-mouse IgE antibody (1  $\mu\text{g}/\text{ml}$ ). The left grey peak means background fluorescence (autofluorescence). **b** The mobilization of  $[\text{Ca}^{2+}]_i$  in BMMCs after the addition of antigen. Anti-DNP IgE-sensitized BMMCs were pretreated with HRB (3  $\text{mg}/\text{ml}$ ) for 30 min and stimulated with antigen (DNP-BSA; 500  $\text{ng}/\text{ml}$ ). Representative data were presented among at least three separate experiments

#### Effect of HRB on membrane fusion of SNARE liposomes

To examine the HRB inhibitory mechanism of degranulation, we monitored the effect of HRB on membrane fusion between SNARE liposomes since HRB could possibly affect membrane fusion by residing in the plasma membrane of mast cells. Several kinds of SNARE proteins are expressed by mast cells and are involved in mast cell exocytosis. Syntaxin-3, syntaxin-4, and SNAP23 are possible candidates for t-SNAREs, while VAMP-2, -7, and -8 are possible candidates for v-SNAREs. Sakiyama et al. previously demonstrated that the combination of SNAP23/syntaxin-3/VAMP-8 had the most efficient fusogenic activity between liposomes in a cuvette [23]. Thus, we prepared liposomes containing VAMP-8 to serve as a v-SNARE and liposomes containing SNAP23 and

syntaxin-3 to serve as t-SNAREs (Fig. 6a). Following this, the fusogenic activity between these liposomes was monitored using FRET between NBD- and rhodamine-conjugated lipids in v-SNARE liposomes.

Before the addition of t-SNARE liposomes, two fluorophores on v-SNARE liposomes resided close enough to transfer energy from NBD to rhodamine, resulting in low intensity NBD fluorescence. Once t-SNARE liposomes were added, the distance between these two fluorophores increased and the efficiency of FRET decreased, resulting in increased NBD fluorescence and decreased rhodamine fluorescence due to membrane fusion. In this model system of membrane fusion using liposomes, HRB (3  $\text{mg}/\text{ml}$ ) significantly inhibited membrane fusion between liposomes mediated by SNARE proteins (Fig. 6b).

#### Effect of HRB on phosphorylation of NF-κB and MAP kinase

NF-κB is a dimeric transcription factor comprised of Rel family proteins, such as RelA and p50, and plays an important role in the production of cytokines by mast cells [26]. The activation of NF-κB occurs via IKK-mediated phosphorylation of inhibitory molecules, including  $\text{I}\kappa\text{B}\alpha$ . The phosphorylated  $\text{I}\kappa\text{B}$  proteins are ubiquitinated and subsequently degraded by the proteasome [27]. Degradation of  $\text{I}\kappa\text{B}$  proteins induces the translocation of NF-κB into the nucleus and an increase in transcriptional activity. To examine the HRB mechanism for suppression of cytokine production, we studied the effect of HRB on the activation of the NF-κB signaling pathway after FcεRI ligation on BMMCs. Pretreatment of BMMCs with HRB (3  $\text{mg}/\text{ml}$ ) inhibited the phosphorylation of  $\text{IKK}\alpha/\beta$ ,  $\text{I}\kappa\text{B}\alpha$ , and RelA after antigen addition (Fig. 7).

In addition, we examined the effect of HRB on the activation of subfamily member proteins of MAP kinases (ERK, JNK, and p38). These MAP kinases are known to be phosphorylated in mast cells after antigen addition and are known to activate transcription factors [28]. Pretreatment of BMMCs with HRB (3  $\text{mg}/\text{ml}$ ) inhibited the phosphorylation of ERK and JNK, but not p38, after antigen addition (Fig. 8).

These results suggested that HRB suppressed the syntheses of proinflammatory cytokines, such as  $\text{TNF-}\alpha$  and IL-4, by inhibiting the NF-κB and MAP kinase (ERK and JNK) activation pathways.

#### Discussion

Both HRB and MGN-3 are denatured hemicelluloses derived from rice bran, and their main chemical structure is an arabinoxylan, with a xylose in the main chain and an

**Fig. 4** Effect of HRB on  $\beta$ -hexosaminidase release and cytokine production in BMMCs upon costimulation with A23187 and phorbol myristoyl acetate (PMA). BMMCs were pretreated with the indicated concentrations of HRB for 30 min and stimulated with A23187 (1  $\mu$ M) and PMA (50 ng/ml). **a** The  $\beta$ -hexosaminidase release from BMMCs stimulated for 30 min. The averaged rate of  $\beta$ -hexosaminidase release in BMMCs untreated with HRB was 26.2% of total enzyme. **b** The TNF- $\alpha$  secretion from BMMCs stimulated for 4 h. **c** The IL-4 secretion from BMMCs stimulated for 4 h. Values are mean  $\pm$  S.D. ( $n = 3$ ). \*\* $P < 0.01$  compared with the absence of HRB

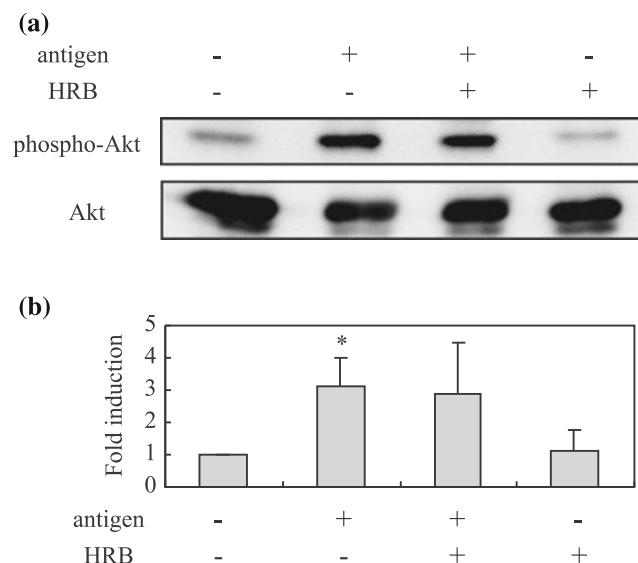
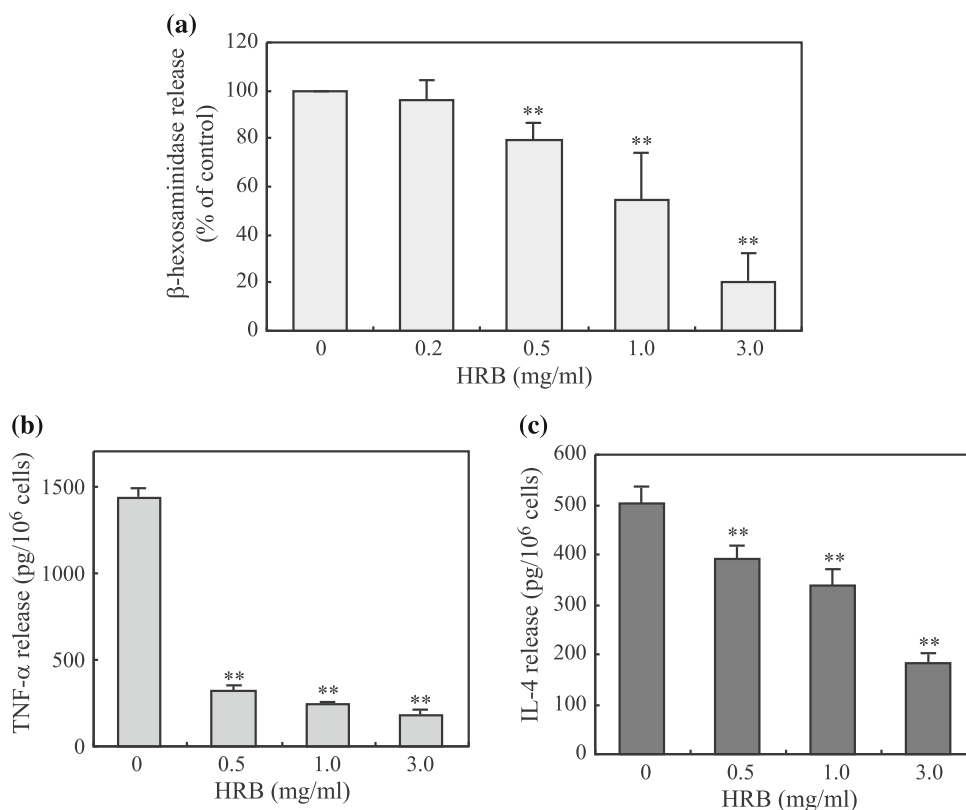


**Fig. 5** Effect of HRB on phosphorylation of Akt in BMMCs upon antigen stimulation. **a** Anti-DNP IgE-sensitized BMMCs were pretreated with HRB (3 mg/ml) for 30 min and stimulated with antigen (DNP-BSA; 500 ng/ml). The phosphorylation levels of Akt in whole cell lysate prepared from BMMCs at 5 min after antigen addition were determined by Immunoblot analysis. **b** The amounts of pixel densities in bands derived from phospho-Akt. The data were normalized to the value in the absence of antigen and HRB after the correction to amount of total protein level. Values are mean  $\pm$  S.D. ( $n = 3$ ). \* $P < 0.05$  compared with the antigen(-)/HRB(-)

arabinose polymer in a side chain [29]. The molecular size of HRB is smaller than that of MGN-3 because HRB is treated with carbohydrate-hydrolyzing enzyme for a longer period than MGN-3. MGN-3 is known to have anti-cancer effects by upregulating activities of NK cells, T cells, B cells, and macrophages, and by inducing apoptosis to cancer cells [1, 2, 24, 30]. The molecular mechanism by which MGN-3 induces the anti-cancer effect is not fully understood, but the promotion of cytokine production, adhesion molecule expression, and caspase activation are considered to be involved in this effect [1, 2, 24, 30]. On the other hand, HRB is reported to decrease scores and shorten duration of cold syndrome symptoms without affecting the disease rate and the NK cell activity [4]. This suggested that HRB may have an anti-inflammation effect, as distinct from MGN-3. However, it has been unknown whether HRB affects activation of inflammatory cells.

Here we have elucidated that HRB can significantly inhibit both mast cell degranulation and cytokine production in a dose-dependent manner. HRB did not influence the early events in signal transduction during mast cell activation, such as the binding of IgE to Fc $\epsilon$ RI and the increase in [Ca<sup>2+</sup>]<sub>i</sub> after ligation of Fc $\epsilon$ RI. Rather, it suppressed events downstream of the increase in [Ca<sup>2+</sup>]<sub>i</sub> and PKC activation, including NF- $\kappa$ B and MAP kinase (ERK and JNK) activation. HRB also inhibited the membrane

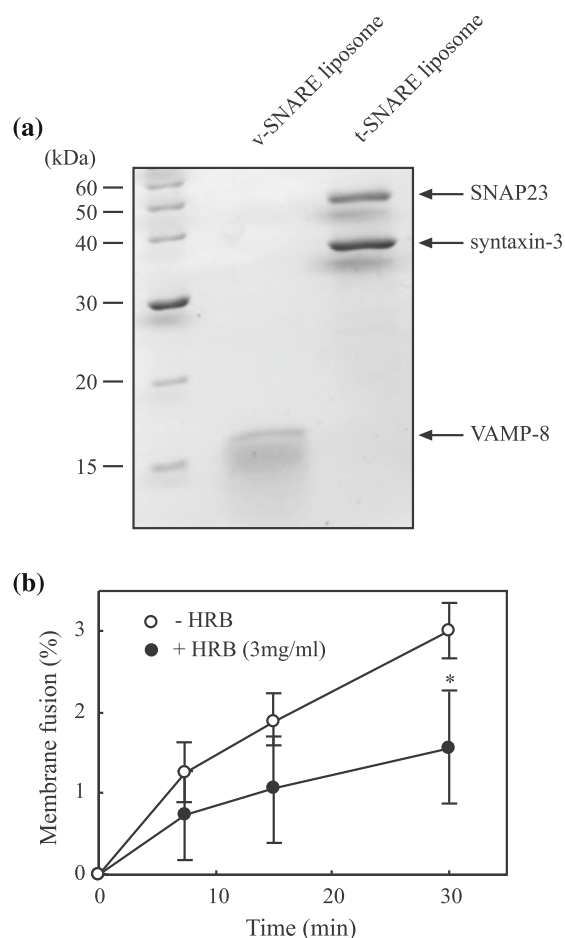


**Fig. 6** Effect of HRB on membrane fusion of liposomes mediated by soluble *N*-ethyl maleimide-sensitive factor attachment protein receptor (SNARE) proteins. **a** SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified SNAREs incorporated into liposomes. Vesicle associated membrane protein (VAMP)-8 was detected in v-SNARE liposomes and synaptosomal-associated protein 23 kDa (SNAP23) and syntaxin-3 were detected in t-SNARE liposomes. **b** Membrane fusion between liposomes containing v-SNARE and t-SNARE. v-SNARE liposomes and t-SNARE liposomes were mixed at time 0. Membrane fusion activity was calculated as the fluorescence intensity of N-(7-nitro-2,1,3-benzoxadiazole-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine (NBD-DPPE) at indicated times relative to that in the presence of Triton X-100. Values are mean  $\pm$  S.D. ( $n = 3$ ). \* $P < 0.05$  compared with the absence of HRB

fusion between liposomes mediated by SNARE proteins. These results indicated that inhibition of degranulation and cytokine production by HRB appeared to be involved in the suppression of membrane fusion and transcriptional activity. In addition, HRB had no cytotoxic effects under these conditions, as the antigen-induced increase in  $[Ca^{2+}]_i$  was normal in BMMCs pretreated with HRB.

Pretreatment of BMMCs with HRB (3 mg/ml) for 30 min inhibited  $\beta$ -hexosaminidase release after antigen addition by  $\sim 70\%$  (Fig. 1). Incubation with HRB

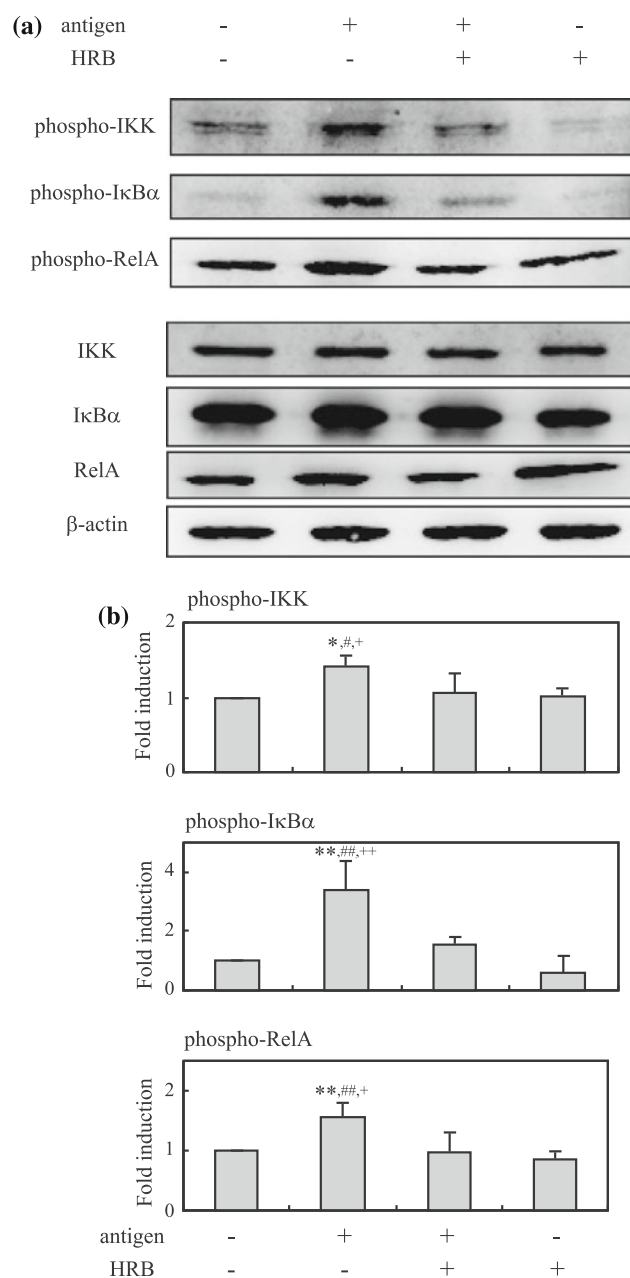


**Fig. 7** Effects of HRB on phosphorylation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) signaling pathway in BMMCs upon antigen stimulation. **a** Anti-DNP IgE-sensitized BMMCs were pretreated with HRB (3 mg/ml) for 30 min and stimulated with antigen (DNP-BSA; 500 ng/ml). The phosphorylation levels of I $\kappa$ B kinase (IKK) $\alpha/\beta$ , I $\kappa$ B $\alpha$ , and RelA in whole cell lysate prepared from BMMCs at 5 min after the addition of antigen were determined by Immunoblot analysis. **b** The amounts of pixel densities in bands derived from phospho-IKK, -I $\kappa$ B $\alpha$ , and -RelA. The data were normalized to the value in the absence of antigen and HRB after the correction to amount of total level of each protein (IKK, I $\kappa$ B $\alpha$ , or RelA). Values are mean  $\pm$  S.D. ( $n = 3$ ). \*\* $P < 0.01$  compared with the antigen(-)/HRB(-), antigen(-)/HRB(+), and antigen(+)/HRB(+), respectively. \* $P < 0.05$  compared with the antigen(-)/HRB(-), antigen(-)/HRB(+), and antigen(+)/HRB(+), respectively.

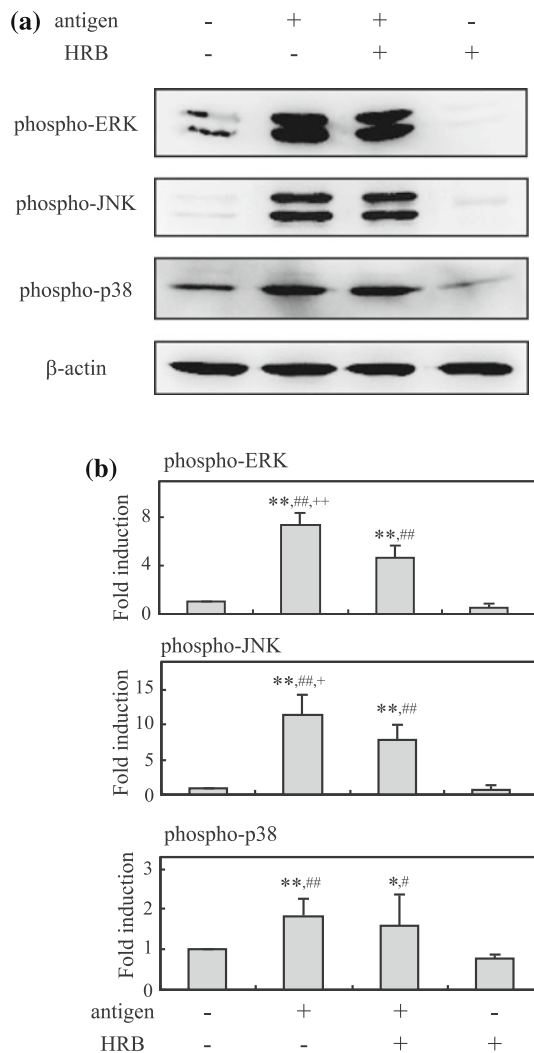


**Fig. 8** Effects of HRB on the phosphorylation of mitogen-activated protein (MAP) kinase subfamily in BMMCs upon antigen stimulation. Anti-DNP IgE-sensitized BMMCs were pretreated with HRB (3 mg/ml) for 30 min and stimulated with antigen (DNP-BSA; 500 ng/ml). The phosphorylation levels of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 in whole cell lysate prepared from BMMCs at 5 min after the addition of antigen were determined by Immunoblot analysis. **b** The amounts of pixel densities in bands derived from phospho-ERK, -JNK, and -p38. The data were normalized to the value in the absence of antigen and HRB after the correction to amount of total level of each MAP kinase. Values are mean  $\pm$  S.D. ( $n = 3$ ).  $^{**},^{###},^{+++}P < 0.01$  compared with the antigen(-)/HRB(-), antigen(-)/HRB(+), and antigen(+)/HRB(+), respectively.  $^{*},^{*#},^{*++}P < 0.05$  compared with the antigen(-)/HRB(-), antigen(-)/HRB(+), and antigen(+)/HRB(+), respectively.

(3 mg/ml) for 30 min also suppressed the membrane fusion between SNARE liposomes by  $\sim 50\%$  (Fig. 6), suggesting that HRB directly affected fusion between plasma and granule membranes. In addition, because the inhibitory effect of HRB on degranulation of BMMCs was greater, to some extent, than membrane fusion between liposomes, it

was possible that HRB suppressed the function of proteins leading to degranulation.

During degranulation, the association of v-SNAREs and t-SNAREs is required to form a ternary complex that triggers membrane fusion and exocytosis. It has also been reported that phosphorylation of SNAP23 (t-SNARE) modulates exocytosis [31, 32]. Recently, it was reported that IKK2 (also called IKK $\beta$ ) phosphorylates SNAP23 and increases exocytosis of mast cells upon Fc $\epsilon$ RI ligation [33]. When we examined whether pretreatment with HRB influenced the phosphorylation of SNAREs in BMMCs after antigen addition, we observed no significant inhibition of the phosphorylation of SNAP23, syntaxin-3, or VAMP-8 (data not shown) despite inhibition of IKK phosphorylation. This suggested that HRB suppressed the activation of intracellular signaling molecules.

The signaling pathway that activates NF- $\kappa$ B and the subfamily of MAP kinases are known to play important roles in the synthesis of cytokines in mast cells [26, 28]. We found that HRB significantly decreased the production of TNF- $\alpha$  and IL-4 by inhibiting the activation of RelA, ERK, and JNK in BMMCs (Figs. 2, 7, and 8). Pretreatment with HRB did not inhibit the increase in  $[Ca^{2+}]_i$  (Fig. 3) or the phosphorylation of tyrosine residues in the early steps (data not shown) mediated by crosslinking of Fc $\epsilon$ RI on BMMCs. In addition, HRB inhibited degranulation and cytokine production induced by co-stimulation with A23187 and PMA (Fig. 4). Thus, HRB did not inhibit the earliest activation events, such as the phosphorylation of tyrosine and the increase in  $[Ca^{2+}]_i$ , but suppressed later processes, such as degranulation, NF- $\kappa$ B activation, MAP kinase activation, and cytokine production.

Although we do not have direct evidence to show that HRB crosses the plasma membrane and influences signaling molecules in the cytoplasm, our results suggest that HRB directly affects signaling molecules involved in later processes in the cytoplasm. Therefore, there is a possibility that HRB resides in the cytoplasm and inhibits not only the activation of the intracellular signaling pathway but also the fusogenic activity of plasma and granule membranes. Although detailed molecular mechanisms by which HRB diminishes the activation of NF- $\kappa$ B and MAP kinases remain unclear, it is probable that the suppression of NF- $\kappa$ B and MAP kinase activation inhibits the production of cytokines.

As described above, we have studied the effect of HRB on mouse BMMC activation. Mouse mast cell phenotypes are widely divided into two groups: MMCs and CTMCs. There are significant differences between MMCs and CTMCs in morphology, histochemical characteristics, contents of mediators, and sensitivity to growth factors and drugs [34, 35]. In addition, some characteristics of mast

cells differ between humans and rodents. However, the signaling pathway through Fc $\epsilon$ RI is common to most mast cells regardless of their types. Furthermore, phenotypes of BMMCs developed from knockout mice are generally consistent with characteristics reported in CTMCs and human mast cells. Therefore, it is likely that HRB has a suppressive effect on different types of mast cells other than MMCs.

In conclusion, our results provide evidence that HRB can directly inhibit mast cell activation, and it may have anti-inflammatory effects making it suitable for treating allergic and inflammatory diseases. Although additional studies are necessary to elucidate the efficacy of HRB in treating allergic patients, the results described here indicate that HRB may be effective against allergic and inflammatory disorders.

**Acknowledgment** We wish to acknowledge Dr. Yoshikazu Inoh (Aichi Gakuin University) and Satoshi Tadokoro (Nagoya City University) for technical support.

## References

- Ghoneum M, Abedi S. Enhancement of natural killer cell activity of aged mice by modified arabinoxylan rice bran (MGN-3/Biobran). *J Pharm Pharmacol*. 2004;56:1581–8.
- Ghoneum M, Gollapudi S. Modified arabinoxylan rice bran (MGN-3/Biobran) enhances yeast-induced apoptosis in human breast cancer cells in vitro. *Anticancer Res*. 2005;25:859–70.
- Endo Y, Kanbayashi H. Modified rice bran beneficial for weight loss of mice as a major and acute adverse effect of Cisplatin. *Pharmacol Toxicol*. 2003;92:300–3.
- Maeda H, Ichihashi K, Fujii T, Omura K, Zhu X, Anazawa M, et al. Oral administration of hydrolyzed rice bran prevents the common cold syndrome in the elderly based on its immunomodulatory action. *Biofactors*. 2004;21:185–7.
- Rivera J. Molecular adapters in Fc $\epsilon$ RI signaling and the allergic response. *Curr Opin Immunol*. 2002;14:688–93.
- Galli SJ, Kalesnikoff J, Grimaldeston MA, Piliponsky AM, Williams CMM, Tsai M. Mast cells as “tunable” effector and immunoregulatory cells: recent advances. *Annu Rev Immunol*. 2005;23:749–86.
- Abramson J, Pecht I. Regulation of the mast cell response to the type 1 Fc $\epsilon$  receptors. *Immunol Rev*. 2007;217:231–54.
- Guo Z, Turner C, Castle D. Relocation of the t-SNARE SNAP-23 from lamellipodia-like cell surface projections regulates compound exocytosis in mast cells. *Cell*. 1998;94:537–48.
- Hibi T, Hirashima N, Nakanishi M. Rat basophilic leukemia cells express syntaxin-3 and VAMP-7 in granule membranes. *Biochem Biophys Res Commun*. 2000;271:36–41.
- Paumet F, Le Mao J, Martin S, Galli T, David B, Blank U, et al. Soluble NSF attachment protein receptors (SNAREs) in RBL-2H3 mast cells: functional role of syntaxin 4 in exocytosis and identification of a vesicle-associated membrane protein 8-containing secretory compartment. *J Immunol*. 2000;164:5850–7.
- Blank U, Rivera J. The ins and outs of IgE-dependent mast-cell exocytosis. *Trends Immunol*. 2001;38:1341–5.
- Stow JL, Manderson AP, Murray RZ. SNAREing immunity: the role of SNAREs in the immune system. *Nat Rev Immunol*. 2006;6:919–29.
- Suzuki A, Suzuki R, Furuno T, Teshima R, Nakanishi M. N-cadherin plays a role in the synapse-like structures between mast cells and neurites. *Biol Pharm Bull*. 2004;27:1891–4.
- Teshima R, Suzuki K, Ikebuchi H, Terao T. Possible involvement of phosphorylation of a 36,000-dalton protein of rat basophilic leukemia (RBL-2H3) cell membranes in serotonin release. *Mol Immunol*. 1986;23:279–84.
- Kato N, Nakanishi M, Hirashima N. Cholesterol depletion inhibits store-operated calcium currents and exocytotic membrane fusion in RBL-2H3 cells. *Biochemistry*. 2003;42:11808–14.
- Nakamura R, Furuno T, Nakanishi M. The plasma membrane shuttling of CAPRI is related to regulation of mast cell activation. *Biochem Biophys Res Commun*. 2006;347:363–8.
- Gustin SE, Thien CB, Langdon WY. Cbl-b is a negative regulator of inflammatory cytokines produced by IgE-activated mast cells. *J Immunol*. 2006;177:5980–9.
- Furuno T, Hirashima N, Onizawa S, Sagiya N, Nakanishi M. Nuclear shuttling of mitogen-activated protein (MAP) kinase (extracellular signal-regulated kinase (ERK) 2) was dynamically controlled by MAP/ERK kinase after antigen stimulation in RBL-2H3 cells. *J Immunol*. 2001;166:4416–21.
- Chikamatsu S, Furuno T, Kinoshita Y, Inoh Y, Hirashima N, Teshima R, et al. Effects of Cot expression on the nuclear translocation of NF- $\kappa$ B in RBL-2H3 cells. *Mol Immunol*. 2007;44:1490–7.
- Sakiyama H, Tadokoro S, Nakanishi M, Hirashima N. Membrane fusion between liposomes containing SNARE proteins involved in mast cell exocytosis. *Inflamm Res*. 2009;58:139–42.
- Arora N, Williamson LC, Leppla SH, Halpern JL. Cytotoxic effects of a chimeric protein consisting of tetanus toxin light chain and anthrax toxin lethal factor in non-neuronal cells. *J Biol Chem*. 1994;269:26165–71.
- Martinez-Arca S, Rudge R, Vacca M, Raposo G, Camonis J, Proux-Gillardeaux V, et al. A dual mechanism controlling the localization and function of exocytic v-SNAREs. *Proc Natl Acad Sci USA*. 2003;100:9011–6.
- Proux-Gillardeaux V, Raposo G, Irinopoulou T, Galli T. Expression of the Longin domain of TI-VAMP impairs lysosomal secretion and epithelial cell migration. *Biol Cell*. 2007;99:261–71.
- Ghoneum M, Matsuura M. Augmentation of macrophage phagocytosis by modified arabinoxylan rice bran (MGN-3/biobran). *Int J Immunopathol Pharmacol*. 2004;17:283–92.
- Kim MS, R  dinger M, Gilfillan AM. The multiple roles of phosphoinositide 3-kinase in mast cell biology. *Trends Immunol*. 2008;29:493–501.
- Marquardt DL, Walker LL. Dependence of mast cell IgE-mediated cytokine production on nuclear factor- $\kappa$ B activity. *J Allergy Clin Immunol*. 2000;105:500–5.
- Perkins ND. The Rel/NF- $\kappa$ B family: friend and foe. *Trends Biol Sci*. 2000;25:434–40.
- Ishizuka T, Chayama K, Takeda K, Hamelmann E, Terada N, Keller GM, et al. Mitogen-activated protein kinase activation through Fc $\epsilon$  receptor I and stem cell factor receptor is differentially regulated by phosphatidylinositol 3-kinase and calcineurin in mouse bone marrow-derived mast cells. *J Immunol*. 1999;162:2087–94.
- Ghoneum M. Anti-HIV activity in vitro of MGN-3, an activated arabinoxylane from rice bran. *Biochem Biophys Res Commun*. 1998;243:25–9.
- Ghoneum M, Gollapudi S. Modified arabinoxylan rice bran (MGN-3/Biobran) sensitizes human T cell leukemia cells to death receptor (CD95)-induced apoptosis. *Cancer Lett*. 2003;201:41–9.
- Lin RC, Scheller RH. Mechanism of synaptic vesicle exocytosis. *Annu. Rev. Cell Dev. Biol*. 2000;16:19–49.

32. Hepp R, Puri N, Hohenstein AC, Crawford GL, Whiteheart SW, Roche PA. Phosphorylation of SNAP-23 regulates exocytosis from mast cells. *J Biol Chem.* 2005;280:6610–20.
33. Suzuki K, Verma IM. Phosphorylation of SNAP-23 by I $\kappa$ B kinase 2 regulates mast cell degranulation. *Cell.* 2008;134:485–95.
34. Welle M. Development, significance, and heterogeneity of mast cells with particular regard to the mast cell-specific proteases chymase and tryptase. *J Leukoc Biol.* 1997;61:233–45.
35. Caughey GH. New developments in the genetics and activation of mast cell proteases. *Mol Immunol.* 2002;38:1353–7.