

Hyaluronic acid targets CD44 and inhibits FcεRI signaling involving PKCδ, Rac1, ROS, and MAPK to exert anti-allergic effect

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Abstract

Effects of hyaluronic acid (HA) on allergic inflammation were investigated. HA exerted negative effects on β-hexosaminidase secretion and histamine release in antigen-stimulated rat basophilic leukemia (RBL2H3) cells. HA inhibited interaction between IgE and FcεRI and between FcεRI and PKCδ. HA inhibited CD44 interaction with PKCα, indicating that HA targets CD44. PKCα and -δ were responsible for increased Rac1 activity and expression of p47^{phox}, p67^{phox}. HA inhibited phosphorylation of PKCα and -δ. Rac1 was responsible for increased ROS, and NADPH oxidase was the main source for ROS. The inhibition of PKC prevented antigen from increasing phosphorylation of ERK and p38 MAPK. ERK, p38 MAPK, and ROS, were responsible for secretion of β-hexosaminidase, histamine release, and induction of chemokines. HA suppressed induction of chemokines, such as MIP-2 and Sprr-2a. CD44 mediated effect of antigen on phosphorylation of ERK, p38MAPK, ROS production, secretion of β-hexosaminidase, and histamine release. GPCR did not mediate allergic function of antigen or affect anti-allergic function of HA. *In vivo* anti-allergic effect of HA was investigated using Nc/Nga mice model of DNFB-induced atopic dermatitis. HA reduced skin lesions in Nc/Nga mice treated with DNFB, decreased expression levels of MIP-2, Sprr-2a, and serum IgE level. In conclusion, hyaluronic acid exerts negative effect on allergic inflammation by targeting CD44 and inhibiting FcεRI signaling.

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Keywords: HA; Anti-allergic effect; CD44; FcεRI

1. Introduction

RBL2H3 cells are mucosal mast cells that express the immunoglobulin receptor FcεRI. Stimulation of IgE-sensitized RBL2H3 cells with specific antigen triggers a cascade of events leading to degranulation, mediator release, MAPK activation,

tyrosine kinase and phospholipase C activation, increased ROS, calcium influx, and cytokine production (Reth, 1989; Cambier, 1995). This cascade of events suggests that mast cells are ideal targets for controlling various allergic diseases, including atopic dermatitis, allergic rhinitis, and asthma (Kobayashi et al., 2000; Williams and Galli, 2000; Pawankar et al., 1997).

HA is a glycosaminoglycan found in the extracellular matrix of connective tissues. Several studies suggested the efficacy of HA in modulating acute and chronic inflammation. High-molecular weight HA was shown to inhibit macrophage proliferation and cytokine release, leading to decreased inflammation in the early wound of a preclinical postlaminectomy rat model (Schimizzi et al., 2006). HA enhances proteoglycan synthesis, reduces the production and activity of proinflammatory mediators and matrix metalloproteinases, and alters the behavior of immune cells (Moreland, 2003).

Abbreviations: HA, hyaluronic acid; PKC, protein kinase C; MIP-2, macrophage inflammatory protein-2; Sprr-2, small proline-rich protein-2; ROS, reactive oxygen species; RBL2H3, rat basophilic leukemia; Phox, NADPH oxidase; IgE, immunoglobulin E; siRNA, small interfering RNA; MAPK, mitogen-activated protein kinase; GPCR, G-protein-coupled receptor; DNFB, dinitrofluorobenzene; DNP-HSA, dinitrophenyl-human serum albumin; Rho GDI, rho GDP dissociation inhibitor.

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A role for CD44, HA receptor, in the regulation of inflammation *in vivo* has been shown by studies in which anti-CD44 treatment inhibited the development of collagen-induced arthritis (Nedvetzki et al., 1999), murine arthritis (Mikecz et al., 1995), and optimal contact allergic responses (Camp et al., 1993). CD44 has been shown to be responsible for the development of pulmonary eosinophilia (Kato et al., 2003).

In this study, we investigated effect of HA on allergic inflammation *in vitro* and *in vivo*. HA exerted anti-allergic effects *in vitro* and *in vivo*. HA targets CD44 to inhibit activation of PKC α by antigen stimulation. HA inhibited Fc ϵ RI signaling involving PKC δ , Rac1 activity, ROS, and MAPK. HA decreased skin lesions in atopic model of DNFB-treated Nc/Nga mice. It also decreased chemokines, such as MIP-2 and Srr-2a, and serum IgE level in DNFB-treated Nc/Nga mice.

2. Materials and methods

2.1. Mice

Nc/Nga mice 4–5 weeks of age were purchased from SLC Japan (Shizuoka, Japan) and maintained in SPF condition. All animal experiments were approved by Institutional review Board for animal studies of Kangwon National University.

At day 25, blood samples were drawn for the measurement of serum immunoglobulin levels.

2.2. β -Hexosaminidase secretion assay

Degranulation was determined by measuring the release of β -hexosaminidase. The assays were carried out according to the standard procedures (Matsubara et al., 2004). To determine effect of ROS, ERK, or p38 MAPK on β -hexosaminidase secretion, IgE-sensitized RBL2H3 cells were pretreated with NAC (100 μ M), PD98059 (20 μ M), or SB203580 (10 μ M) for 15 min. Cells were then stimulated with DNP-HSA (100 ng/ml) for 1 h. Secretion of β -hexosaminidase was then measured. To determine effect of CD44 on β -hexosaminidase secretion, the IgE-sensitized RBL2H3 cells were incubated with anti-CD44 antibody (2 μ g/ml) for 1 h, followed by stimulation with DNP-HSA (100 ng/ml).

2.3. Histamine release assay

Histamine release in antigen-stimulated RBL2H3 cells was analyzed by protocol supplied by manufacturer (Immunotech, France).

Histamine release assays in Guinea pig mast cells were also carried out. In brief, Guinea pig mast cells were isolated, and purified by enzyme digestion and rough and discontinuous percoll density gradient method. Mast cells (4×10^5 cells) were passively sensitized by anti-OVA antibody, and challenged by 1.0 μ g/ml OVA. Samples were added 5 min before antigen challenge. Histamine in supernatant was determined by fluorometric analyzer.

2.4. Measurement of ROS

IgE-sensitized RBL2H3 cells were incubated with 5 μ M of 2,7-dichlorodihydrofluorescein diacetate for 30 min. For the last 5 min of incubation, DNP-HSA (100 ng/ml) was added. After incubation, cells were immediately observed by a laser scanning confocal microscope (LSM410, Carl Zeiss). The IgE-sensitized RBL2H3 cells were incubated with HA (200 μ g/ml) for 15 min in the presence of 5 μ M of 2,7-dichlorodihydrofluorescein diacetate. For the last 5 min of incubation, DNP-HSA (100 ng/ml) was added and ROS measurement was carried out as above. To determine effect of PKC on ROS production, RBL2H3 cells on coverslips were transiently transfected with control vector (2 μ g), dominant negative PKC α (2 μ g), or dominant negative PKC δ (2 μ g). At 24 h after transfection, cells were sensitized with DNP-specific IgE antibody (100 ng/ml) for 16 h. Cells were then stimulated with DNP-HSA (100 ng/ml) for 30 min. To determine effect of CD44 on ROS production, IgE-sensitized RBL2H3 cells were incubated with anti-CD44 antibody (2 μ g/ml) for 1 h, followed by stimulation with DNP-HSA (100 ng/ml) for 30 min.

2.5. NADPH oxidase activity assays

NADPH oxidase activity was measured according to the instruction manual provided by the manufacturer (Stratagene). Assay medium, comprising 100 μ M luminol and enhancer, was added to IgE-sensitized RBL2H3 cells pretreated without or with HA (200 μ g/ml) for 30 min prior to stimulation with DNP-HSA (100 ng/ml). The chemiluminescence was measured continuously in a luminometer. To examine effect of downregulation of p47^{phox} and p67^{phox} on NADPH oxidase, cells were transiently transfected with 10 nM each of control siRNA, p47^{phox} siRNA, or p67^{phox} siRNA. 24 h after transfection, RBL2H3 cells were sensitized with DNP-specific anti-IgE antibody for 16 h, followed by stimulation with DNP-HSA (100 ng/ml) for 30 min.

2.6. Preparation of siRNA duplexes

The siRNA duplexes were constructed with the following target sequences. p47^{phox}, sense (5'-AAATGTTCCCTATTGAG-GCAGCCTGTCTC-3'); antisense (5'-AACTGCCTCAATAG-GGAACATCCTGTCTC-3'); p67^{phox}, sense (5'-AAGTACA-CGGTAGTCATGAAGCCTGTCTC-3'); antisense (5'-AACTTCATGACTACCGTGTACCCTGTCTC-3'); control, sense (5'-AATTCTCCGAACGTGTCACGTCCTGTCTC-3'); antisense (5'-AAACGTGACACGTTCCGAGAACCTGTCTC-3'). The construction of siRNA was carried out according to the instruction manual provided by the manufacturer (Ambion, Austin, TX).

2.7. Western blot analysis

For Western blot analysis, cells were solubilized by lysis buffer (62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 50 mM DTT, 0.01% (w/v) bromophenol blue, 10 mM

NaF, 1% (v/v) protease inhibitor cocktail, 1 mM sodium orthovanadate). The samples were boiled for 5 min, and equal amounts of protein (20 μ g/well) were analyzed on 10% SDS-PAGE.

2.8. RT-PCR

Total RNA was isolated by Trizol and converted into cDNA using Superscript reverse transcriptase. The following primer pairs were used for PCR. MIP-2, 5'-CTA-CAT-TGG-TGC-AGA-CTG-TG-3' and 5'-CGT-ATG-TAG-CAA-AGA-TGC-AG-3'; Sprr-2a, 5'-TGT-CTT-ACT-ACC-AGC-AGC-AG-3' and 5'-TGC-TCA-TAG-CAC-ACT-ACA-GG-3'; β -actin, 5'-TAA CCA ACT GGG ACG ATA TG-3' and 5'-ATA CAG GGA CAG CAC AGC CT-3'. The PCR conditions were as follows: MIP-2: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and 30 cycles; Sprr-2a: 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min and 30 cycles; β -actin: 94 °C for 60 s, 58 °C for 45 s, 72 °C for 45 s and 20 cycles.

2.9. Rac1 activity assays

IgE-sensitized RBL2H3 cells were treated without or with HA (200 μ g/ml) for 15 min prior to antigen stimulation. Cells were quickly rinsed in phosphate-buffered saline and lysed using 500 μ l lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$) plus protease inhibitors. Cell lysates were centrifuged for 5 min at 14,000 rpm. 400 μ l of each cell lysate was incubated with PAK (CRIB)-GST beads for 1 h with rocking at 4 °C. Samples were centrifuged for 2 min at 2000 rpm and supernatant was discarded. The beads were washed three times with wash buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, and 10 mM $MgCl_2$). Finally, 50 μ l 2 $\times$ Laemmli buffer was added to the beads and boiled for 10 min. The levels of total Rac1, Rac1-GTP, were detected by Western blotting using monoclonal anti-Rac1 antibody. Approximately 10 μ g of total lysates were subjected to western blot analysis. To determine effect of PKC on Rac1 activity, IgE-sensitized RBL2H3 cells were pretreated with rottlerin (10 μ M) or Go6976 (1 μ M) for 1 h. Cells were then stimulated with DNP-HSA (100 ng/ml) for various time intervals.

2.10. DNFB-induced dermatitis

Allergic dermatitis was induced by repeated topical application with DNFB. Briefly, 100 μ l of 0.15% DNFB in acetone/olive oil (3:1) was applied to shaved back side mice twice at 7 day intervals. DNFB was also applied to each side of right ears of mice twice at 7 day intervals. Ear thickness was measured once a week using a digital thickness gauge. Increase in ear thickness was determined by subtracting ear thickness before the first painting from that of each time point. To determine effect of HA, 100 μ g of HA was added along with DNFB. 10 μ M of PD98059 was added along with DNFB to determine effect of ERK on DNFB-induced atopic dermatitis.

2.11. Histological analyses

Skin samples were fixed in 10% (v/v) buffered formalin, embedded in paraffin, sectioned at 4 μ m, and then stained with hematoxylin and eosin. Ear samples were fixed and stained as was done with skin samples.

2.12. Serum IgE measurement

Blood samples were collected and IgE concentration in the serum was measured using mouse IgE EIA kit (Immunology Consultants laboratory, Inc., USA).

3. Results

3.1. Hyaluronic acid decreases secretion of β -hexosaminidase and histamine release in antigen-stimulated RBL2H3 cells

We examined whether hyaluronic acid affected allergic inflammation *in vitro*. Hyaluronic acid significantly decreased secretion of β -hexosaminidase from antigen-

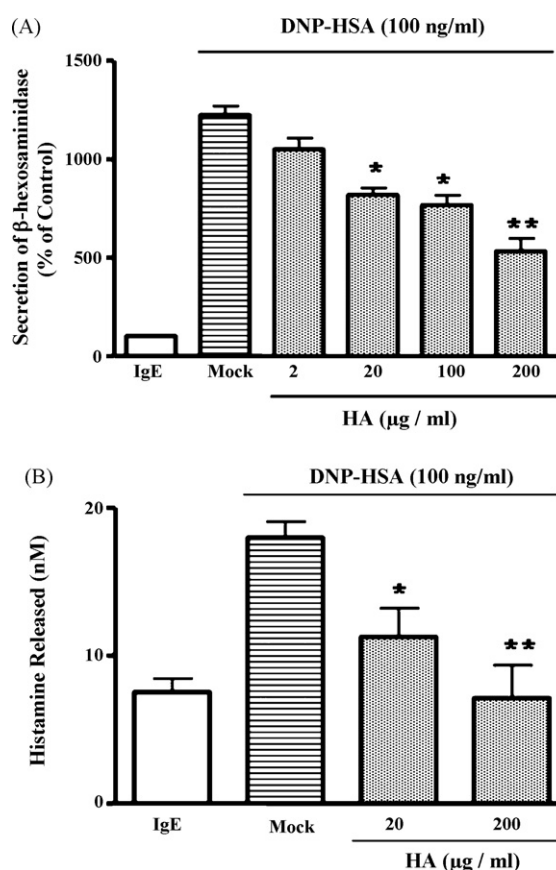


Fig. 1. HA decreases secretion of β -hexosaminidase and histamine release from antigen-stimulated cells. (A) The IgE-sensitized RBL2H3 cells were treated with ketotifen fumarate (100 μ M) or various concentrations of HA (1 MDa) for 15 min, followed by stimulation with DNP-HSA (100 ng/ml). The enzyme activities of β -hexosaminidase were measured. (B) Same as (A) except that histamine release assay was carried out. Statistical significance was determined at the $p < 0.01$ (*) and $p < 0.001$ (**) levels using a Student's t test.

stimulated RBL2H3 cells, in a dose-dependent manner (Fig. 1A). HA with different size (1 MDa, 100 kDa, 6 kDa) decreased secretion of β -hexosaminidase from antigen-stimulated cells (Supplementary Fig. 1A). Hyaluronic acid significantly decreased release of histamine from antigen-stimulated RBL2H3 cells, in a dose-dependent manner (Fig. 1C). HA with different size HA with different size (1 MDa, 100 kDa, 6 kDa) decreased release of histamine from antigen-stimulated cells (Supplementary Fig. 1B).

3.2. Hyaluronic acid exerts negative effect on Fc ϵ RI-mediated signaling and targets CD44 to exert anti-allergic effect

It has been known that Fc ϵ RI cross-linking results in the activation of Lyn kinase (Furumoto et al., 2004). HA decreases phosphorylation of Lyn in antigen-stimulated RBL2H3 cells (Fig. 2A, upper panel) by inhibition of interaction between Fc ϵ RI β and Lyn (Fig. 2A, lower panel). HA inhibits interaction between IgE and Fc ϵ RI γ in antigen-stimulated RBL2H3 cells (Fig. 2B). Fc ϵ RI has been shown to bind to PKC in response to antigen stimulation (Liu et al., 2001). HA inhibits interaction between Fc ϵ RI γ and PKC δ (Fig. 2B). CD44 interacts with PKC α , but not with PKC δ (Fig. 2C). Activated form

of PKC α binds to CD44 after antigen stimulation (Fig. 2C). HA inhibits interaction between CD44 and PKC α (Fig. 2C). Blocking of CD44 by anti-CD44 antibody inhibits phosphorylation of PKC α , but not PKC δ (Fig. 2D), suggesting that CD44 is responsible for the phosphorylation of PKC α by antigen.

3.3. HA decreases phosphorylation of PKC and Rac1 activity in antigen-stimulated RBL2H3 cells

Antigen stimulation increased Rac1 activity (Fig. 3A). This increase in Rac1 activity was accompanied by an increased phosphorylation of PKC α and δ (Fig. 3A). Inhibition of PKC α by Go6976 and that of PKC δ by rottlerin decreases Rac1 activity (Fig. 3A), suggesting that PKC regulates Rac1 activity in antigen-stimulated RBL2H3 cells. Inhibition of PKC α and δ prevented antigen from increasing expression of p47^{phox} and p67^{phox} (Fig. 3A). HA significantly decreased phosphorylation of PKC α , δ , and Rac1 activity in antigen-stimulated RBL2H3 cells (Fig. 3B). HA decreases phosphorylation of rhoGDI, and expression levels of p47^{phox} and p67^{phox} in antigen-stimulated RBL2H3 cells (Fig. 3B). PKC and Rac1 have been shown to be necessary for allergic reactions in mast cells (Cho et al., 2004a,b).

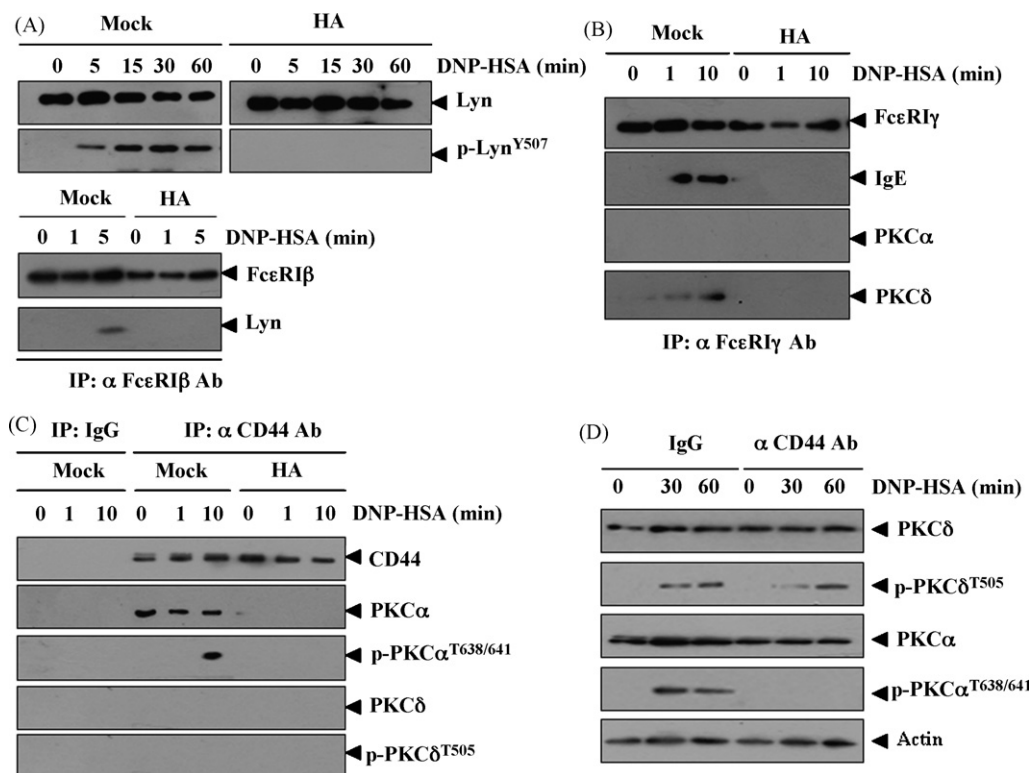


Fig. 2. HA inhibits recruitment of Lyn, PKC δ , and targets CD44 to inhibit interaction between CD44 and PKC α . (A) The IgE-sensitized RBL2H3 cells were pretreated without or with HA (200 μ g/ml) for various time intervals, followed by stimulation with DNP-HSA (100 ng/ml). Cells lysates were subjected to Western blot analysis. (B) The IgE-sensitized RBL2H3 cells were pretreated without or with HA (200 μ g/ml) for 1 h, followed by stimulation with DNP-HSA (100 ng/ml) for various time intervals. Cell lysates were immunoprecipitated with anti-Fc ϵ RI γ antibody (5 μ g). The immunoprecipitates were then subjected to Western blot analysis. (C) The IgE-sensitized RBL2H3 cells were pretreated without or with HA (200 μ g/ml) for 30 min, followed by stimulation with DNP-HSA (100 ng/ml) for various time intervals. Cell lysates were immunoprecipitated with anti-CD44 antibody (5 μ g). (D) The IgE-sensitized RBL2H3 cells were preincubated with control antibody (2 μ g/ml) or anti-CD44 antibody (2 μ g/ml) for 2 h, followed by stimulation with DNP-HSA (100 ng/ml) for various time intervals. Cell lysates were subjected to western blot analysis.

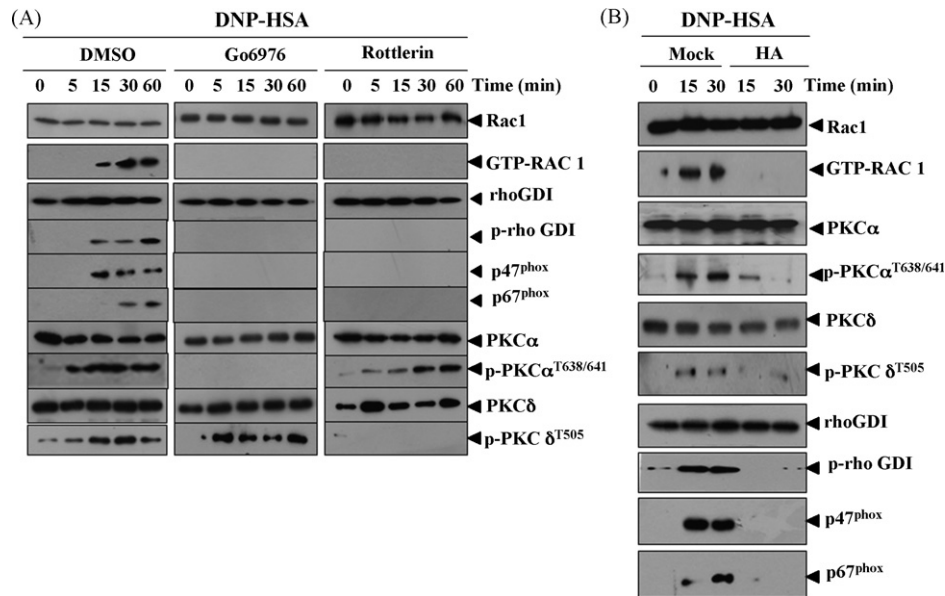


Fig. 3. HA inhibits PKC signaling, which are responsible for the increased Rac1 activity, expression of p47^{phox} and p67^{phox} in antigen-stimulated RBL2H3 cells. (A) IgE-sensitized RBL2H3 cells were pretreated without or with rottlerin (10 μ M) or Go6976 (1 μ M) for 1 h, followed by stimulation with DNP-HSA (100 ng/ml) for various time intervals. Cell lysates were subjected to GST-pull down assays for measuring GTP-Rac1. 10 μ g of total lysates was subjected to Western blot analysis. (B) Same as (A) except that the IgE-sensitized RBL2H3 cells were pretreated without or with HA (200 μ g/ml). Cell lysates prepared were subjected to GST-pull down assays for measuring GTP-Rac1. 10 μ g of total lysates was subjected to Western blot analysis.

3.4. PKC α , - δ , and Rac1 are responsible for the increased ROS in antigen-stimulated RBL2H3 cells

The inhibition of PKC δ and - α by each dominant negative construct decreased ROS production in antigen-stimulated RBL2H3 cells (Supplementary Fig. 2A). Inactivation of Rac1 by dominant negative Rac1 mutant construct decreased ROS production (Supplementary Fig. 2C). Rac1 has been shown to be responsible for the increased ROS production (Patil et al., 2004). Inactivation of Rac1 by dominant negative Rac1 N17 did not affect phosphorylation of PKC α or - δ (Supplementary Fig. 2D). Inactivation of Rac1 prevented antigen from inducing expression of p47^{phox} and p67^{phox} (Supplementary Fig. 2D).

3.5. ROS, ERK, and p38 MAPK are responsible for the decreased β -hexosaminidase secretion and histamine release

Antigen stimulation significantly increased ROS (Fig. 4A) and NAC, an inhibitor of ROS, significantly suppressed it (Fig. 4A). Ketotifen fumarate, an anti-allergic drug, decreased ROS from antigen-stimulated cells (Fig. 4A). HA decreased ROS in antigen-stimulated RBL2H3 cells (Fig. 4B). HA with different size decreased ROS from antigen-stimulated RBL2H3 cells (Fig. 4B). MAPK signaling has been known to be target of PKC, and involved in allergic inflammation (Cho et al., 2004a,b). Inhibition of PKC α and - δ by each dominant negative construct decreased phosphorylation of ERK and p38 MAPK by antigen stimulation (Fig. 4C). NAC, an inhibitor of ROS, decreased secretion of β -hexosaminidase (Fig. 4D), suggesting that ROS are necessary for secretion of β -hexosaminidase. Inhibition of ERK and p38 MAPK decreased secretion of β -

hexosaminidase (Fig. 4D). Inhibition of ROS, ERK, and p38 MAPK decreased release of histamine from antigen-stimulated RBL2H3 cells (Fig. 4E).

3.6. NADPH oxidase is the main source of ROS in antigen-stimulated RBL2H3 cells

Based on the fact that antigen stimulation increased expression of p47^{phox}, p67^{phox} (Fig. 3A), it is likely that NADPH oxidase is responsible for the increased ROS in antigen-stimulated RBL2H3 cells. Antigen stimulation increased NADPH oxidase activity (Supplementary Fig. 3A). PMA, an activator of NADPH oxidase, increased NADPH oxidase activity (Supplementary Fig. 3A). DPI, an inhibitor of NADPH oxidase, prevented antigen from increasing ROS in RBL2H3 cells (Supplementary Fig. 3A), implying that NADPH oxidase is responsible for the increased ROS. The downregulation of p47^{phox} and p67^{phox} decreased NADPH oxidase activity (Supplementary Fig. 3B), and prevented antigen from increasing ROS in RBL2H3 cells (Supplementary Fig. 3D). These results indicate that NADPH oxidase is mainly responsible for the increased ROS in antigen-stimulated RBL2H3 cells.

3.7. Induction of chemokines by antigen is mediated by CD44, ROS, ERK, and p38 MAPK

In experimental allergic asthma model, expression of MIP-2 and Sprr-2 was shown to be induced by Ovalbumin stimulation (Zimmermann et al., 2005; Xiao et al., 1997). Antigen stimulation increased expression of both MIP-2 and Sprr-2 (Fig. 5A). Induction of these chemokines required ROS, ERK, and p38 MAPK (Fig. 5A). HA suppressed induction of MIP-2 and Sprr-2

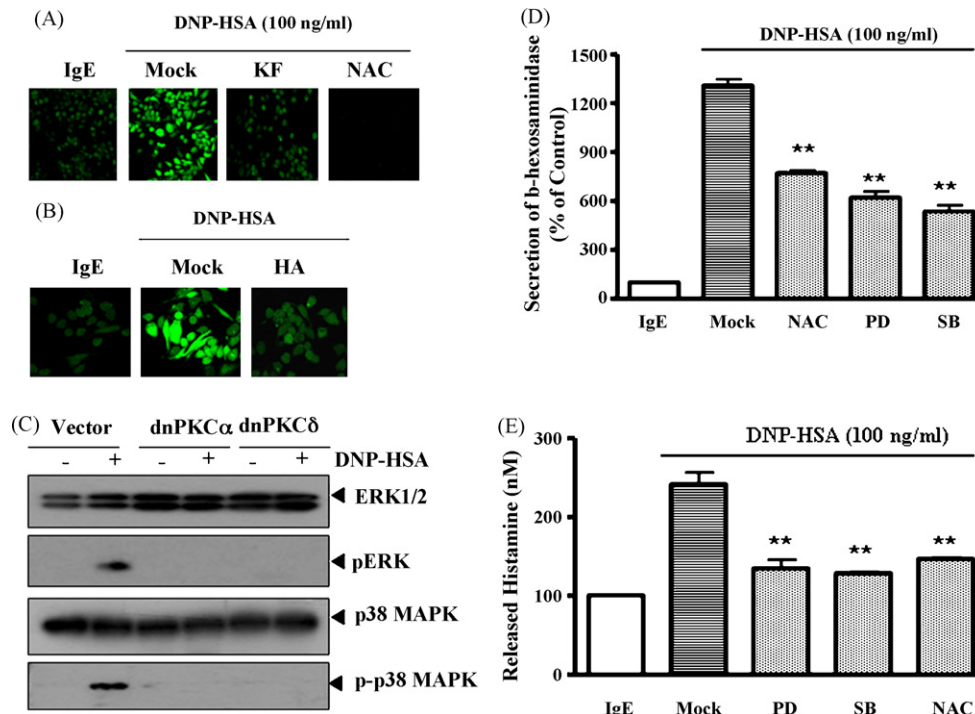


Fig. 4. ROS, ERK, and p38 MAPK are responsible for the secretion of β -hexosaminidase and histamine release. (A) IgE-sensitized cells were incubated with 5 μ M of 2,7-dichlorodihydrofluorescein diacetate. For the last 5 min of incubation, DNP-HSA (100 ng/ml) was added. For the last 15 min of incubation, HA (1 MDa) was added before stimulation with DNP-HSA. (B) IgE-sensitized cells were incubated with 5 μ M of 2,7-dichlorodihydrofluorescein diacetate. For the last 5 min of incubation, DNP-HSA (100 ng/ml) was added. For the last 15 min of incubation, HA (200 nM) with different size was added before stimulation with DNP-HSA. ROS measurement was carried out as described. Secretion of β -hexosaminidase was measured as described. (C) RBL2H3 cells were transiently transfected with control vector (2 μ g), dominant negative PKC α (2 μ g), or dominant negative PKC δ (2 μ g). The next day, cells were sensitized with DNP-specific anti-IgE antibody (100 ng/ml) for 16 h, followed by stimulation with DNP-HSA (100 ng/ml) for 1 h. (D) The IgE-sensitized RBL2H3 cells were treated with PD98059 (20 μ M), SB203580 (10 μ M), or NAC (100 μ M) for 15 min, and stimulation with DNP-HSA (100 ng/ml) was followed. Secretion of β -hexosaminidase was measured. (E) Same as (D) except that histamine release assay was carried out. Statistical significance was determined at the $p < 0.01$ (*) and $p < 0.001$ (**) levels using a Student's t test.

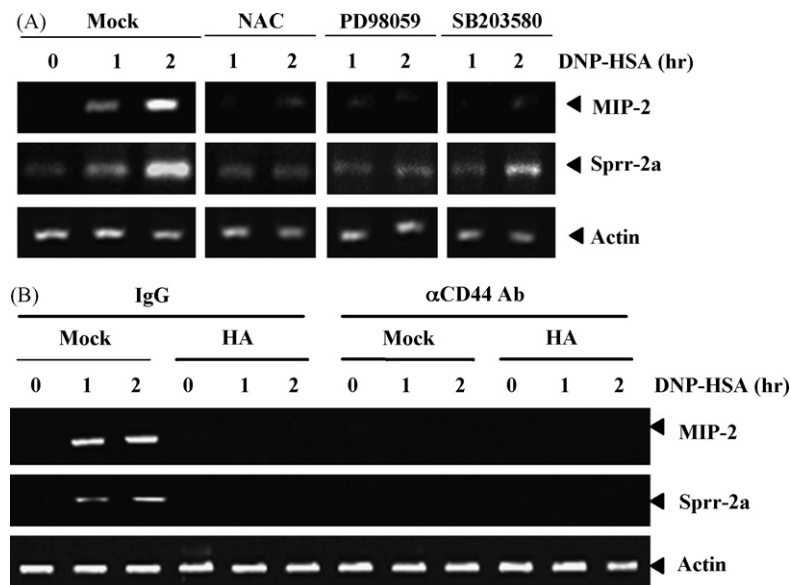


Fig. 5. HA decreases induction of chemokines in a CD44-dependent way in antigen-stimulated RBL2H3 cells. (A) The IgE-sensitized RBL2H3 cells were pretreated without or with NAC (100 μ M), PD98059 (20 μ M), or SB203580 (10 μ M) for 15 min. Cells were then stimulated with DNP-HSA 100 ng/ml for various time intervals. RT-PCR analysis was carried out. (B) IgE-sensitized RBL2H3 cells were pretreated with HA along with anti-CD44 antibody (2 μ g/ml) or IgG antibody (2 μ g/ml) for 1 h, prior to stimulation with DNP-HSA (100 ng/ml). At each time point, total RNAs were isolated and subjected to RT-PCR.

in antigen-stimulated RBL2H3 cells (Fig. 5B). Anti-CD44 antibody prevented antigen from inducing expression of MIP-2 and Sprr-2 (Fig. 5B), suggesting that CD44 signaling is necessary for mediating allergic inflammation.

3.8. CD44 is at least partially necessary for mediating allergic inflammation

We further examined whether CD44 was necessary for mediating allergic inflammation. The blocking of CD44 prevented antigen from increasing phosphorylation of ERK and p38 MAPK (Fig. 6A), ROS production (Fig. 6B), secretion of β -hexosaminidase (Fig. 6C), and histamine release (Fig. 6D) in RBL2H3 cells. Taken together, these results indicate that CD44 is at least partially responsible for mediating allergic inflammation.

3.9. In vivo anti-allergic effect of HA

The Nc/Nga mouse has been used as a useful model for atopic dermatitis (AD). Nc/Nga mouse model has been used for evaluation of the anti-allergic effects of functional foods (Kotani et al., 2000). Nc/Nga mice develop human AD-like lesions mediated by elevated levels of serum IgE as a result of the application of dinitrofluorobenzene (DNFB) (Matsuda et al., 1997; Tomimori et al., 2005). *In vivo* anti-allergic effect of HA was examined. DNFB-treated Nc/Nga mice showed skin lesions (Fig. 7A). HA treated along with DNFB greatly reduced skin lesions (Fig. 7A). It was shown that antigen-stimulation increased phosphorylation of ERK (Fig. 4C). Therefore, the effect of ERK on DNFB-induced atopic dermatitis was examined. The inhibition of ERK

by PD98059 reduced skin lesions in Nc/Nga mice treated with DNFB (Fig. 7A). HA and PD98059 significantly reduced ear thickness of DNFB-treated with Nc/Nga mice (Fig. 7B). However, the inhibition of p38 MAPK by SB203580 did not affect ear thickness (Fig. 7B). Reportedly, MIP-2 was involved in antigen-induced eosinophilia and neutrophilia in mouse model (Zimmermann et al., 2005; Xiao et al., 1997). Total RNAs prepared from skin lesions of Nc/Nga mice were subjected to RT-PCR. DNFB-induced expression of MIP-2 and Sprr-2a while HA suppressed induction of these chemokines (Fig. 7C). HA and PD98059 decreased serum IgE levels of Nc/Nga mice treated with DNFB (Fig. 7D).

3.10. Histological analyses of DNFB-treated Nc/Nga mice

In the skin lesions at 4 weeks, thickening of epidermis and cell infiltration was more evident in DNFB-treated Nc/Nga mice than untreated control (Fig. 8A and B). Both HA and PD98059 attenuated effect of DNFB (Fig. 8A and B).

4. Discussion

We investigated whether HA affected allergic inflammation in a rat mucosal mast cells, RBL2H3. Based on the fact that high-molecular weight HA reduced levels of pro-inflammatory cytokines (Moreland, 2003), we hypothesized that HA would enhance allergic inflammation. HA decreased both β -hexosaminidase secretion (Fig. 1A and Supplementary Fig. 1A) and histamine release (Fig. 1C and Supplementary Fig. 1B). HA inhibited secretion of β -hexosaminidase and histamine release, regardless of size (Supplementary Fig. 1A and

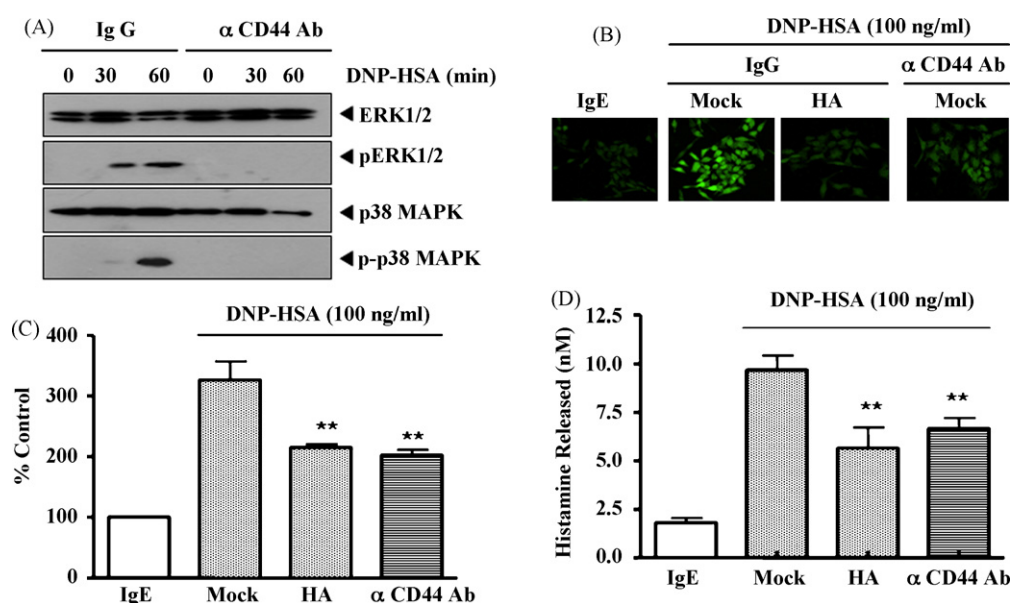


Fig. 6. CD44 is necessary for mediating allergic inflammation. (A) The IgE-sensitized RBL2H3 cells were preincubated with control IgG (2 μ g/ml), or anti-CD44 antibody (2 μ g/ml) along without or with HA (200 μ g/ml) for 1 h. Cells were then stimulated with DNP-HSA (100 ng/ml) for various time intervals. Cell lysates were subjected to Western blot analysis. (B) IgE-sensitized RBL2H3 cells pretreated with HA (200 μ g/ml), anti-CD44 antibody (2 μ g/ml), or HA along with anti-CD44 antibody (2 μ g/ml), were stimulated with DNP-HSA (100 ng/ml). ROS measurement was carried out. (C) IgE-sensitized RBL2H3 cells pretreated with HA (200 μ g/ml) or anti-CD44 antibody (2 μ g/ml), were stimulated with DNP-HSA (100 ng/ml). β -Hexosaminidase secretion was measured. (D) Same as (C) except that histamine release assay was carried out. Statistical significance was determined at the $p < 0.01$ (*) and $p < 0.001$ (**) levels using a Student's *t* test.

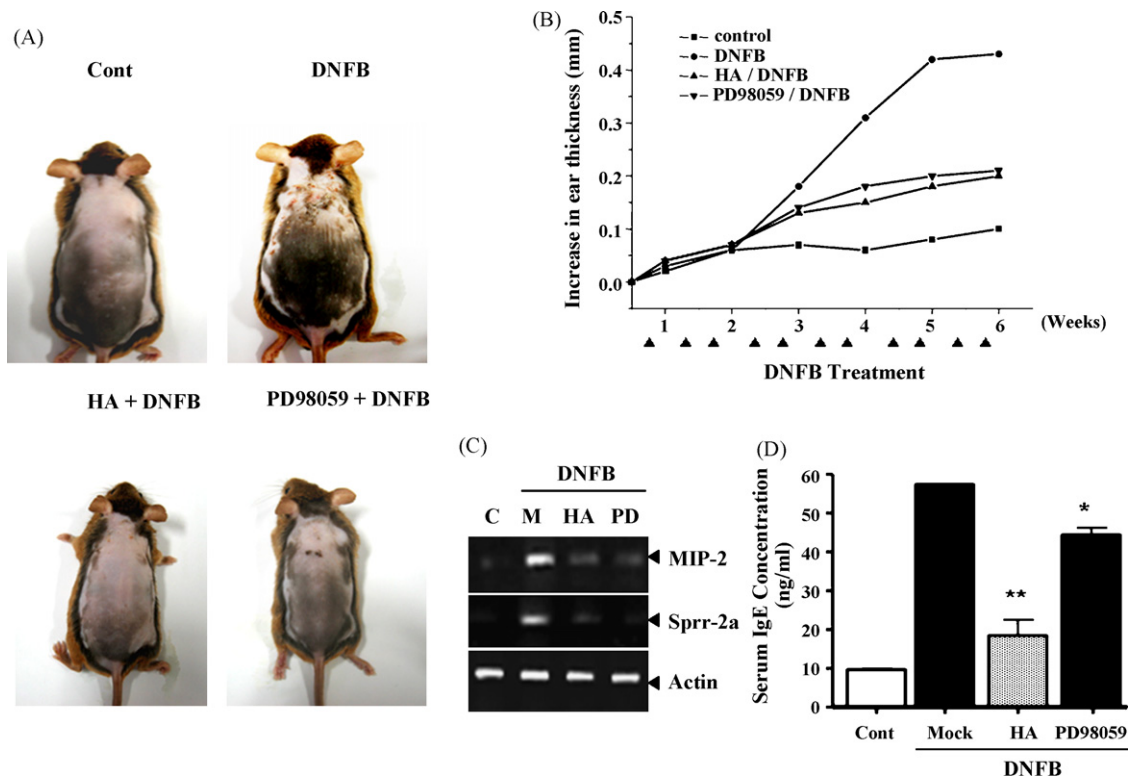


Fig. 7. *In vivo* anti-allergic effect of HA. (A) Skin features of the DNFB-treated regions in Nc/Nga mice. (B) Ear thickness of Nc/Nga mice treated with DNFB, measured once a week for 6 weeks. Nc/Nga mice treated with DNFB along with HA or PD98059 shows reduced ear thickness. Data are average values of three independent experiments. (C) Expression level of MIP-2 and Sprr-2a in DNFB-treated Nc/Nga mice. Nc/Nga mice treated with DNFB in the presence of HA or PD98059 shows reduced expression of MIP-2 and Sprr-2a. (D) DNFB-treated Nc/Nga mice showed reduced serum IgE level. Statistical significance was determined at the $p < 0.01$ (*) and $p < 0.001$ (**) levels using a Student's *t* test.

B). Reportedly, both low and high-molecular weight HA effectively decreased inflammatory genes, such as PGE2 and IL-6, in lipopolysaccharide-challenged fibroblast-like synovial cells (Schimizzi et al., 2006). Antigen stimulation through cell-bound IgE leads to the phosphorylation of multiple substrates, including Lyn, Btk, Syk, and PKC θ (Liu et al., 2001; Kovarova et al., 2001; Meltzer et al., 2005; Wahl et al., 1997). Fc ϵ RI signaling has been shown to involve phosphorylation of PKC θ by

antigen (Liu et al., 2001). We detected phosphorylation of only PKC α and δ , but not others in antigen-stimulated RBL2H3 cells. Fc ϵ RI phosphorylation in response to antigen stimulation results in histamine release and calcium signaling (Teshima et al., 1994). We studied effect of HA on Fc ϵ RI signaling. It has been shown that phosphorylation of Fc ϵ RI β , in response to antigen stimulation, results in phosphorylation of Lyn (Andrásfalvy et al., 2005). The phosphorylation of Lyn by antigen stimulation

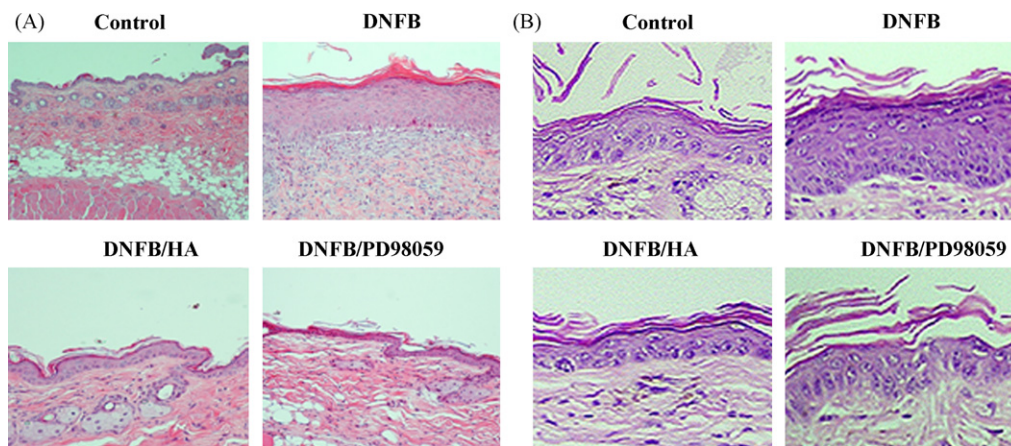


Fig. 8. Histological analyses of skin lesions in DNFB-treated Nc/Nga mice. (A) Skin inflammation was induced by topical application of DNFB, along without or with HA or PD98059, to shaved back side twice at 7 day intervals. Skins were excised at 4 weeks, and the skin sections were stained with hematoxylin and eosin. (B) Same as (A) except that ears were excised at 6 week. Skin sections were stained with hematoxylin and eosin.

was inhibited by HA (Fig. 2A). HA inhibits interaction between FcεRIβ and Lyn (Fig. 2A), suggesting that FcεRIβ is responsible for the phosphorylation of Lyn. HA inhibited interaction between FcεRIγ and PKCδ (Fig. 2B), suggesting that FcεRIγ is responsible for the activation of PKCδ. Since HA interacts with CD44, it was necessary to examine the role of CD44 in allergic inflammation. HA inhibited interaction between CD44 and PKCα, suggesting that HA targets CD44 to exert anti-allergic effect (Fig. 2C). Blocking of CD44 inhibited phosphorylation of PKCα, but not that of PKCδ (Fig. 2D). In our data, CD44 did not show interaction with FcεRI (data not shown). This implies that CD44 and FcεRI constitute separate signaling pathways to mediate allergic inflammation. These data suggest that HA exerts its negative effect on secretion of β-hexosaminidase and histamine release by inhibiting FcεRI signaling and targeting CD44.

The role of Rac1 in the generation of ROS was previously reported (Furumoto et al., 2004). ROS have been known to be involved in allergic inflammation (Springer et al., 2007; Dharajiya et al., 2007). Antigen stimulation increased Rac1 activity, and this was accompanied by an increased phosphorylation of PKCα and -δ (Fig. 3A). Inhibition of PKCα and -δ decreased Rac1 activity in antigen-stimulated RBL2H3 cells (Fig. 3A), suggesting that PKCα and -δ are responsible for the activation of Rac1.

PKCα and -δ are known to be regulators in mediating activation of Rac1 (Ugolev et al., 2006; Pan et al., 2005). Both PKCα and -δ have been shown to be critical for histamine release in human mast cells (Sandler et al., 2005). Rac1 complexes with rhoGDI in cytosol and is translocated into the membrane upon activation (Patil et al., 2004). Phosphorylation of rhoGDI regulates Rac1 activity (Patil et al., 2004). Antigen stimulation increased phosphorylation of rhoGDI (Fig. 3A). Inactivation of Rac1 by dominant negative construct did not affect phosphorylation of PKCα or -δ (Supplementary Fig. 2D), suggesting that PKC activates Rac1 to increase ROS. Reportedly, antigen stimulation leads to increased ROS in RBL2H3 cells (Ogasawara et al., 1986). ROS play an important role in mast cell functions, including β-hexosaminidase secretion (Son et al., 2006) and histamine release in antigen-stimulated RBL2H3 cells (Kim et al., 2005; Boldogh et al., 2005). Antigen stimulation increased ROS, and ketotifen fumarate, an anti-allergic drug, prevented antigen from increasing ROS (Fig. 4A). The inhibition of ROS by NAC significantly decreased β-hexosaminidase secretion (Fig. 4D) and histamine release (Fig. 4E). Inhibition of ERK and p38 MAPK also exerted negative effects on β-hexosaminidase secretion and histamine release (Fig. 4D and E). Reportedly, MAPK signaling plays important role in allergic inflammation (Duan et al., 2004; Tigani et al., 2003).

NADPH oxidase complex is composed of three cytosolic proteins (p40^{phox}, p47^{phox}, and p67^{phox}), catalytic subunit gp91^{phox}, and p22^{phox}. NADPH oxidase was shown to be required for generation of ROS in a PKC zeta-dependent manner (Frey et al., 2006). HA inhibits activation of PKCα, -δ and Rac1 by antigen stimulation (Fig. 3B). Rac1 supports NADPH oxidase activity (Cheng et al., 2006). Reportedly, Rac1 is necessary for mast cell degranulation (Hong-Geller et al., 2001). Although antigen

stimulation increases ROS, such as H₂O₂, main source of ROS is still not clear. DPI, an inhibitor of NADPH oxidase, decreased ROS in antigen-stimulated RBL2H3 cells (Supplementary Fig. 3A). However, DPI is not a specific inhibitor of NADPH oxidase. The downregulation of p47^{phox} and p67^{phox} decreased ROS in antigen-stimulated RBL2H3 cells (Supplementary Fig. 3B), suggesting that NADPH oxidase is indeed responsible for the increased ROS. CXC chemokine such as MIP-2 was shown to modulate IgE secretion and pulmonary inflammation (McKinley et al., 2005). Upregulation of MIP-2 was reported in allergic inflammation (Shahan et al., 1998). Sprr-2 has been known to be induced in the small intestine after induction of allergic gastrointestinal inflammation, in a STAT6-dependent way (Zimmermann et al., 2005). Antigen stimulation led to upregulation of MIP-2 and Sprr-2a, which was reversed by HA (Fig. 5B). Induction of these chemokines required ERK and p38 MAPK (Fig. 5A). ERK has been shown to regulate expression of various chemokines, such as MIP-2, in lung inflammation (Lee et al., 2006). HA-binding CD44 has been known to be involved in leukocyte migration in allergy dermatitis (Mummert et al., 2000; Gonda et al., 2005), and antigen-induced pulmonary eosinophilia (Schimizzi et al., 2006). CD44 has been known to mediate leukocyte adhesion to extracellular matrix to exert its effect on allergic inflammation (Katoh et al., 2007). The blocking of CD44 prevented induction of MIP-2 and Sprr-2a (Fig. 5B). The blocking of CD44 prevented antigen from increasing phosphorylation of ERK and p38 MAPK (Fig. 6A), secretion of β-hexosaminidase (Fig. 6B), ROS production (Fig. 6C), and histamine release (Fig. 6D). This suggests that CD44 is at least partially responsible for allergic inflammation. G protein-coupled receptors (GPCRs) have been shown to mediate responses throughout the immune, nervous, and muscular systems that might contribute to the pathogenesis of allergic processes and asthma (Johnson and Druey, 2002). It has been shown that GPCR signaling is closely linked to PKC activation (Dho et al., 2006; Rey et al., 2004). Suramine, an inhibitor of GPCR, did not affect secretion of β-hexosaminidase (Supplementary Fig. 4A), histamine release (Supplementary Fig. 4B), ROS production (Supplementary Fig. 4C), or phosphorylation of ERK and p38 MAPK (Supplementary Fig. 4D) in antigen-stimulated RBL2H3 cells. This suggests that GPCR is not necessary for mediating allergic inflammation. Anti-allergic effect of HA *in vivo* was demonstrated in atopic model of DNFB-treated Nc/Nga mice (Figs. 7A and 8A). In conclusion, we found that HA exerted negative effect on allergic inflammation in both *in vitro* and *in vivo*. Based on data presented in this study, it is apparent that antigen stimulation activates both CD44 and FcεRI, which in turn activates PKCα and -δ, respectively. PKCα and -δ converge to activate Rac1 signaling involving ROS and MAPK. ROS and MAPK were shown to be responsible for the increased secretion of β-hexosaminidase, histamine release, and induction of MIP-2 and Sprr-2a.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [10.1016/j.molimm.2008.01.008](https://doi.org/10.1016/j.molimm.2008.01.008).

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