

Inhibitory Effect of *N*-Acyl Dopamines on IgE-Mediated Allergic Response in RBL-2H3 Cells

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Abstract Recently, endogenous *N*-acyl dopamines have been found to show anti-inflammatory and immunomodulatory activities. However, the effect of the *N*-acyl dopamines on allergic responses was not reported. In this study, we investigated whether *N*-acyl dopamines might inhibit immunoglobulin E-mediated degranulation in RBL-2H3 cells. When RBL-2H3 cells were exposed to palmitoyl dopamine (NP-DA), oleoyl dopamine (NO-DA) or arachidonoyl dopamine (NA-DA) at micromolar levels, all these compounds significantly inhibited the release of β -hexosaminidase, a marker of degranulation, as well as tumor necrosis factor (TNF)- α . In comparison, NP-DA, potently suppressing the release of β -hexosaminidase (IC₅₀, 3.5 μ M) and TNF- α (IC₅₀, 2.2 μ M), was more potent than NO-DA or NA-DA. Additionally, NP-DA markedly suppressed the formation of prostaglandin E₂, prostaglandin D₂ and leukotriene C₄, corresponding to pro-inflammatory lipid mediators in asthma. In the mechanistic analyses, where the effect of NP-DA on the Fc ϵ RI cascade was examined, NP-DA significantly

inhibited the phosphorylation and expression of Syk, but not Lyn. And, NP-DA also suppressed phosphorylation of ERK1/2 and Akt. Further, NP-DA decreased the phosphorylation of cPLA₂ and 5-lipoxygenase (5-LO), but not cyclooxygenase-2 (COX-2). Based on these results, it is suggested that NP-DA exert anti-allergic effect on allergic response through suppressing the activation of Syk, ERK1/2, Akt, cPLA₂ and 5-LO. Besides, a strong inhibition of COX-2 activity by NP-DA may be additional mechanism for its anti-allergic action. Such an anti-allergic action of *N*-acyl dopamines may contribute to further information about biological functions of *N*-acyl dopamines.

Keywords RBL-2H3 cells · *N*-Acyl dopamine · β -Hexosaminidase · TNF- α · PGD₂ · LTC₄

Abbreviations

Akt	Protein kinase B
COX-2	Cyclooxygenase-2
cPLA ₂	Cytosolic phospholipase A ₂
ERK1/2	Extracellular signal-regulated kinase 1/2
Fyn	Feline yes-related protein
HSA	Human serum albumin
IgE	Immunoglobulin E
LTC ₄	Leukotriene C ₄
Lyn	Src family protein kinase
NA-DA	<i>N</i> -Arachidonoyl dopamine
NO-DA	<i>N</i> -Oleoyl dopamine
NP-DA	<i>N</i> -Palmitoyl dopamine
PGE ₂	Prostaglandin E ₂
PGD ₂	Prostaglandin D ₂
Syk	Spleen tyrosine kinase
TNF	Tumor necrosis factor
5-LO	5-Lipoxygenase

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Introduction

Allergic responses are classified into various types of models including type I (anaphylactic shock) [1]. In the allergic response of type I, the activation of immunoglobulin E (IgE)-mediated FcεRI receptor, known as the high affinity IgE receptor, on the plasma membrane of mast and basophilic cells is known to release β-hexosaminidase, a common marker of degranulation, as well as various allergic mediators including histamine, cytokines, prostaglandins and leukotrienes [2]. Especially, arachidonic acid metabolites including prostaglandins and leukotrienes mediate acute and chronic allergic reactions [3, 4]. In the progression of allergic diseases such as asthma, allergic rhinitis and inflammatory arthritis [5], mast cells are known to be mainly responsible for allergic reactions. RBL-2H3 cells [6] have been generally used for the study about IgE–FcεRI interactions involving the pathway of intracellular signaling in degranulation [7–9]. Moreover, RBL-2H3 cells have been employed as a useful tool for in-vitro screening for candidates of the anti-allergic drugs [7–9].

Previously, endocannabinoids, well known to be lipid mediators in nervous systems [10], had been observed to show additional biological actions such as anti-inflammatory and immunosuppressive actions [11]. Recently, endocannabinoids such as arachidonoyl dopamine (NA-DA), oleoyl dopamine (NO-DA), palmitoyl dopamine (NP-DA) and stearoyl dopamine have been reported to be present in the brain as well as other tissues [12]. We mentioned briefly about some biological actions of endocannabinoids; NA-DA, one representative of *N*-acyl dopamines, was reported to be a ligand of cannabinoid 1 [13] and transient receptor potential vanilloid 1 receptor [14], a potent inhibitor of 5-lipoxygenase (5-LO) [15] and a mild inhibitor of fatty acid amide hydrolase [13], responsible for the regulation of the endocannabinoid level. In addition, NA-DA possesses some biological actions such as increased immobility, decreased body temperature or delayed reaction to a thermal stimulus in mice [13, 16], and has shown anti-inflammatory and immunosuppressive actions [17, 18]. Nonetheless, there has been no report concerning the anti-allergic action of *N*-acyl dopamines.

In this respect, we examined the anti-allergic action of *N*-acyl dopamines in RBL-2H3 cells stimulated by an IgE–antigen complex, and elucidated the mechanism for the anti-allergic action of *N*-acyl dopamines. Here, it is suggested that *N*-acyl dopamines may exert a suppressive action in allergic responses involving IgE–FcεRI interactions.

Materials and Methods

Reagents

The MEM medium, penicillin, streptomycin and fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Grand Island, NY, USA). 4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate (WST-1) was obtained from Dojindo (Kumamoto, Japan). Specific antibodies against, phospho-Syk (#2711), Syk (#2712), phospho-Lyn (#2731), Lyn (#2796), cyclooxygenase-2 (COX-2; #4842), phospho-ERK1/2 (#9101), ERK1/2 (#9102), Akt (#9272), phospho-Akt (#9271), cPLA₂ (#2832), phospho-cPLA₂ (#2831) and β-actin (#4970) were purchased from Cell signaling Technology (Beverly, MA, USA). The enzyme-linked immunosorbent assay (ELISA) kit for tumor necrosis factor-α (TNF-α) was obtained from e-Bioscience, Inc. (Science Center Drive, San Diego, USA). Specific antibodies against 5-LO (160402) and phospho-5-LO (10007820), arachidonoyl dopamine, oleoyl dopamine, palmitoyl dopamine and enzyme immunoassay (EIA) kits for prostaglandin E₂ (PGE₂), prostaglandin D₂ (PGD₂) and leukotriene C₄ (LTC₄) were purchased from Cayman Chemical (Ann Arbor, MI, USA). 4-Nitrophenyl *N*-acetyl-β-D-glucosaminide (p-NAG), dinitrophenyl-immunoglobulin E (DNP-IgE) and DNP-human serum albumin (DNP-HSA) were obtained from Sigma-Aldrich (St Louis, MO, USA). All other chemicals were of analytical grade.

Cell Culture

RBL-2H3 cells were cultured in MEM medium containing 5 % (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5 % CO₂ as previously described [19].

Cytotoxicity Assay

Cell respiration, an indicator of cell viability, was determined by measuring the mitochondrial-dependent reduction of WST-1 to the water-soluble tetrazolium salt [20]. Briefly, RBL-2H3 cells were seeded on a 96-well plate (1 × 10⁴ cells/well) in MEM with 5 % FBS at 37 °C overnight. The cells were washed, and incubated with DNP-IgE (1 μg/ml) for 24 h. The cells were incubated with *N*-acyl dopamines (0–30 μM) for 1 h. Both DNP-HSA (25 ng/ml) and WST-1 reagent (10 μl) were simultaneously added to the above, and incubated for another 4 h. To measure cell viability, the absorbance was measured at 450 nm using a micro-plate reader (Emax, Molecular Devices, Inc. Sunnyvale, CA, USA).

β -Hexosaminidase Release Activity

RBL-2H3 cells were incubated in a 24-well plate (1×10^5 cells/well) at 37 °C overnight. The above cells were washed with $1 \times$ PBS, and then incubated with DNP-IgE for 24 h. The cells were incubated with *N*-acyl dopamines (0–30 μ M) for 1 h. They were spiked with DNP-HSA, and incubated for another 4 h. To measure the amount of β -hexosaminidase activity released from the cells, and then the cultured media were transferred, and centrifuged ($17,000 \times g$ for 10 min) at 4 °C. The supernatant (25 μ l) was mixed with 50 μ l p-NAG (10 mM) in 0.1 M sodium citrate buffer (pH 4.5) into a 96-well plate, and incubated for 1 h at 37 °C. The reaction was terminated by stop buffer (0.1 M sodium carbonate buffer, pH 10.0). The β -hexosaminidase activity was determined by measuring the difference of absorbance at wavelength 405 nm.

Enzyme-Linked Immunosorbent (ELISA) Assay of TNF- α

To measure TNF- α level in cultured media, all cultured media were centrifuged ($17,000 \times g$ for 10 min) at 4 °C, and the samples were stored at –80 °C until assaying. The concentration of TNF- α was detected by ELISA kits (e-Bioscience, Inc.) according to the manufacturer's instructions.

Enzyme Immunoassay Analysis of PGE₂, PGD₂ and LTC₄

To determine the levels of PGE₂, PGD₂ and LTC₄ in cultured media, all cultured media were centrifuged ($17,000 \times g$ for 10 min) at 4 °C, and the samples were stored at –80 °C until analyzed. The concentrations of PGE₂, PGD₂ and LTC₄ were detected by EIA kits (Cayman Chemical, Inc.) according to the manufacturer's instructions.

Reverse Transcription-Polymer Chain Reaction

Total RNA was extracted with TRI Reagent (SolGent, Inc., Daejeon, Korea) according to the manufacturer's protocol and quantified by spectrophotometry at 260 nm. The total RNA (1 μ g) was used as the template for cDNA synthesis and PCR using the DiaStar™ 2 $\times$ OneStep RT-PCR Pre-Mix kit (SolGent, Inc.). The following primers were used: TNF- α forward 5'-CAA GGA GCA GAA GTT CCC AA-3', TNF- α reverse 5'-CGG ACT CCG TGATGT CTA AG-3' (500 bp); β -actin forward 5'-AGC CAT GTA CGT AGC CAT CC-3', β -actin reverse 5'-TCT CAG CTG TGG TGG TGA AG-3' (227 bp) [21]. The denaturation, annealing, extension conditions, and number of cycles were 94 °C for

30 s, 60 °C for 60 s, 72 °C for 60 s, and 40 cycles, respectively. PCR was performed in a My Genie32 Thermal Block (Bioneer, Inc., Daejeon, Korea). The PCR products were electrophoresed on a 2 % agarose gel and visualized by ethidium bromide staining and UV illumination. The levels of target mRNAs were compared to those of a loading control (β -actin), and the results were expressed as a ratio of density of each mRNA, which was identified by a DNA standard size marker. The density of each band was determined using ImageJ software (version 1.46r for Windows; NIH, USA).

Immunoblotting Analysis

RBL 2H3 cells were seeded on a 6-well plate (5×10^5 cells/well) in MEM with 5 % FBS at 37 °C overnight. The cells were washed, and incubated with DNP-IgE (1 μ g/ml) for 24 h. Then the cells were incubated with palmitoyl-dopamine (0–30 μ M) for 1 h, and then stimulated with DNP-HSA (100 ng/ml) for 10 min or 4 h. The harvested cells were lysed, and the protein of interest was resuspended in a protein lysis buffer (0.2 % NP-40, 10 mM HEPES, 15 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA and 0.1 mM PMSF). The cell lysates were separated on SDS-PAGE containing 10 % acrylamide gel, and then transferred to PVDF membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membranes were then incubated with a 1:1,000 dilution of specific antibodies against phospho-Syk, phospho-Lyn, Syk, Lyn, phospho-ERK1/2, ERK1/2, phospho-Akt, Akt, phospho-cPLA₂, cPLA₂, COX-2 and β -actin (Cell signaling Technology, Inc.) and antibodies against phospho-5-LO and 5-LO antibodies (Cayman Chemical, Inc.). The blots were washed with TBS-T, and then incubated with a 1:5,000 dilution of horseradish peroxidase-conjugated IgG secondary antibody (Cell signaling Technology, Inc.). The proteins on membranes were detected using a chemiluminescent reaction (ECL plus kit, Amersham Pharmacia Biotech, Buckinghamshire, UK), followed by exposure of the membranes to Hyperfilm ECL (Amersham Pharmacia Biotech, Buckinghamshire, UK). The levels of target proteins were compared to those of loading controls (β -actin or non-phosphorylated protein), and the results were expressed as a ratio of density of each protein, which was identified by protein standard size marker. The density of each band was determined using ImageJ software.

Statistical Analysis

The experimental results were expressed as means $\pm$ SD. One-way analysis of variance (ANOVA) was used for multiple comparisons (GraphPad Prism version 4.03 for Windows, San Diego, CA, USA). If there was a significant

variation between treated groups, the Dunnett test was applied. Differences at the $*P < 0.05$ and $**P < 0.01$ levels were considered statistically significant.

Results

Inhibitory Effect of *N*-Acyl Dopamines on IgE-Mediated Degranulation in RBL-2H3 Cells

To compare the effect of *N*-acyl dopamines on an immunoglobulin E (IgE)–antigen complex reaction, we examined RBL-2H3 cells were preincubated with 1 $\mu\text{g}/\text{ml}$ of dinitrophenyl-IgE (DNP-IgE) and exposed to palmitoyl dopamine (NP-DA), oleoyl dopamine (NO-DA) or arachidonoyl dopamine (NA-DA) of different concentrations (0–30 μM) for 1 h, and stimulated with 25 ng/ml of DNP-HSA for 4 h. All the *N*-acyl dopamines significantly inhibited the release of both β -hexosaminidase, a common marker of degranulation, and TNF- α , a pro-inflammatory cytokine, in a concentration-manner (Fig. 1). In addition, these chemicals dose-dependently reduced the expression of mRNA encoding TNF- α in IgE-mediated activation of RBL-2H3 cells (Fig. 2). However, the concentration-dependent effect of NP-DA on

TNF- α mRNA level was not consistent with that of NP-DA on protein amounts in repeated experiments. Although this difference is not explained in the present experiments, it is supposed that *N*-acyl dopamine may interfere with the process of the translation or maturation of TNF- α . Especially, the IC_{50} value of NP-DA was smaller than that of NO-DA or NA-DA for degranulation and TNF- α formation (data not shown). These results indicate that NP-DA, NO-DA or NA-DA can inhibit both degranulation and allergic inflammation in mast cells stimulated by an IgE–antigen complex. Moreover, NP-DA has more potent inhibitory effect than NO-DA or NA-DA.

Inhibitory Effect of Palmitoyl Dopamine on the Release of β -Hexosaminidase and TNF- α from RBL-2H3 Cells Stimulated by an IgE–Antigen Complex

Next, we reexamined suppressive effect of NP-DA, a stronger inhibitor than NO-DA or NA-DA, on the release of β -hexosaminidase and TNF- α , and then determined each IC_{50} values in reducing β -hexosaminidase activity and the TNF- α level. When RBL-2H3 cells were preincubated with NP-DA of different concentrations (1–30 μM) for 1 h before challenge with antigen, NP-DA was found to inhibit

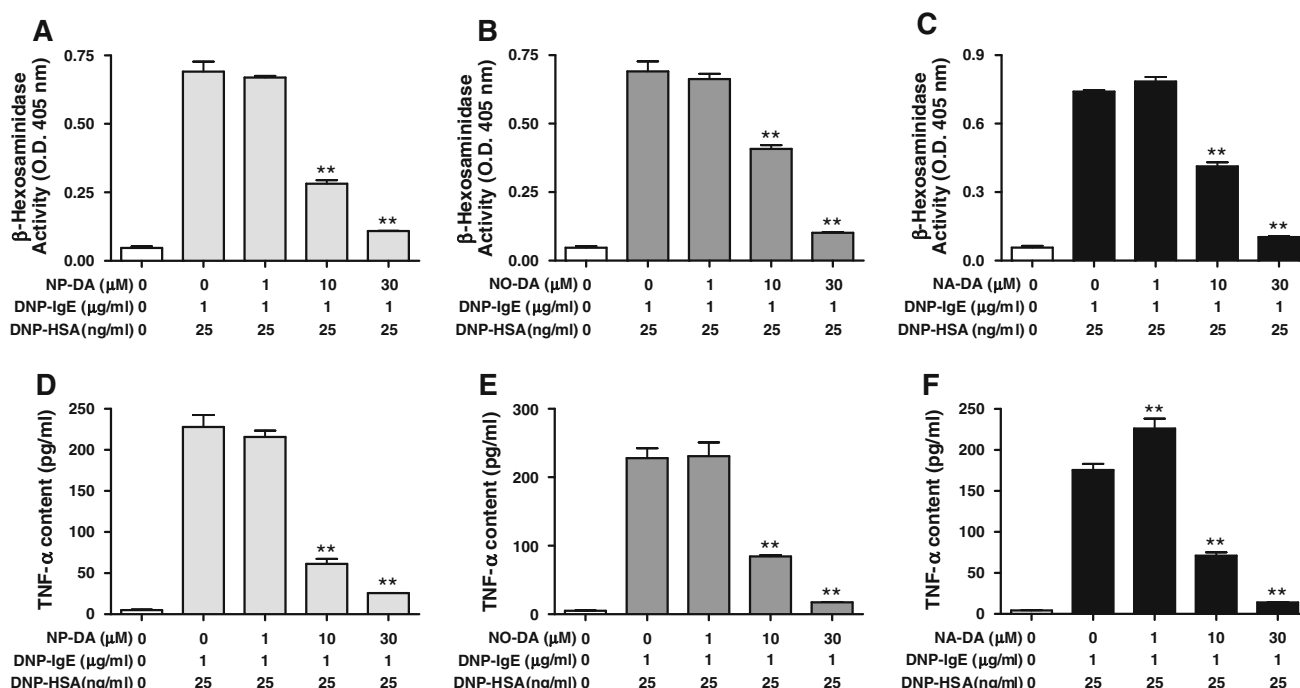


Fig. 1 Inhibitory effect of *N*-acyl dopamines on activity of β -hexosaminidase and level of TNF- α released from RBL-2H3 cells stimulated by an IgE–antigen complex. RBL-2H3 cells were seeded on a 24-well plate (1×10^5 cells/well) in MEM with 5 % FBS at 37 °C overnight, and then the cells were washed, and further incubated with DNP-IgE for 24 h. The cells were incubated with

N-acyl dopamines (0–30 μM) for 1 h, and then stimulated by DNP-HSA (25 ng/ml) for 4 h. β -Hexosaminidase activity (a–c) and TNF- α level (d–f) were determined as described in “Materials and Methods”. Data are listed as mean $\pm$ SD values of triple determinations. $**P < 0.01$ versus DNP-HSA-treated group

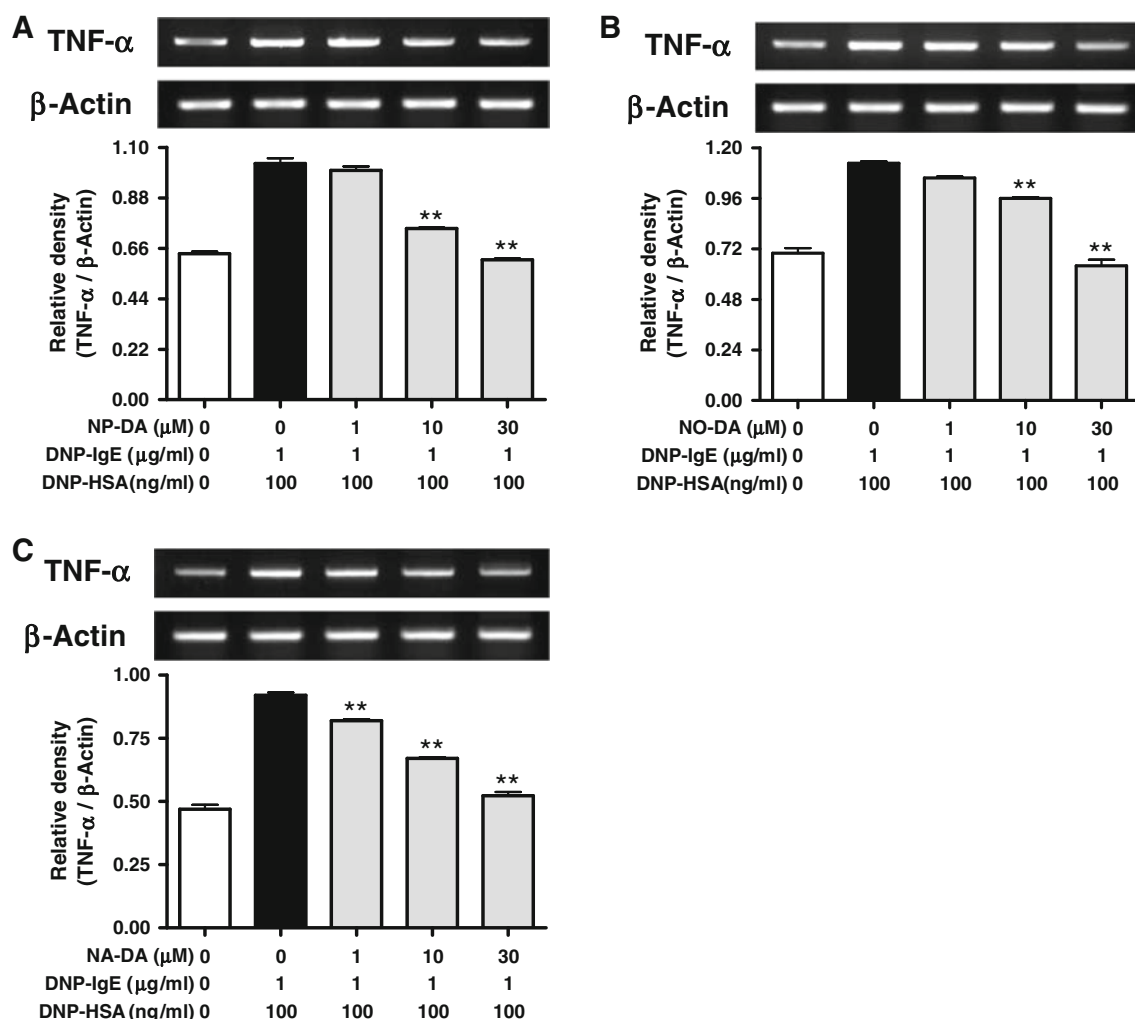


Fig. 2 Inhibitory effect of *N*-acyl dopamines on mRNA expression of TNF- α in RBL-2H3 cells stimulated by an IgE–antigen complex. RBL-2H3 cells were seeded on a 6-well plate in MEM with 5 % FBS at 37 °C overnight, and then the cells were washed, and further incubated with DNP-IgE for 24 h. The cells were incubated with *N*-acyl dopamines (0–30 μ M) for 1 h, and then stimulated by DNP-HSA

(100 ng/ml) for 4 h. The cells were rinsed with 1 $\times$ PBS, and lysed with TRI reagent. The mRNA levels of TNF- α were determined as described in “Materials and Methods”. Similar results were obtained in three independent experiments. ** $P < 0.01$ versus DNP-HSA-treated group

the release of β -hexosaminidase (IC_{50} , 3.49 μ M) and TNF- α (IC_{50} , 2.22 μ M) in a concentration-dependent manner (Fig. 3a, b). Furthermore, NP-DA had no cytotoxicity at concentration range of anti-degranulation effect (Fig. 3c). Taken together, these findings suggest that NP-DA can exhibit stronger anti-allergic action at non-cytotoxic concentrations.

Inhibitory Effect of Palmitoyl Dopamine on the Release of Pro-Inflammatory Lipid Mediators

Next, we examined the effect of NP-DA on the formation of PGE₂, PGD₂ and LTC₄, pro-inflammatory lipid mediators implicated in an allergic response [2–4, 22, 23].

RBL-2H3 cells were preincubated with NP-DA (1–30 μ M) before stimulation with antigen, and the amount of PGE₂, PGD₂ and LTC₄ was measured by EIA kit assay. As shown in Fig. 4, NP-DA markedly inhibited the formations of PGE₂ (IC_{50} , 1.30 μ M) and PGD₂ (IC_{50} , 0.69 μ M). On the other hand, NP-DA insensitively suppressed the formation of LTC₄ (IC_{50} , 2.94 μ M) to a less extent than that of PGE₂ or PGD₂. These results suggest that NP-DA can block acute as well as chronic allergic inflammation induced by PGE₂, PGD₂ or LTC₄. Especially, NP-DA has a greater suppressive effect on the formation of PGD₂ compared to PGE₂ or LTC₄. Thus, it may indicate that NP-DA directly inhibits an enzyme implicated in the formation of PGD₂.

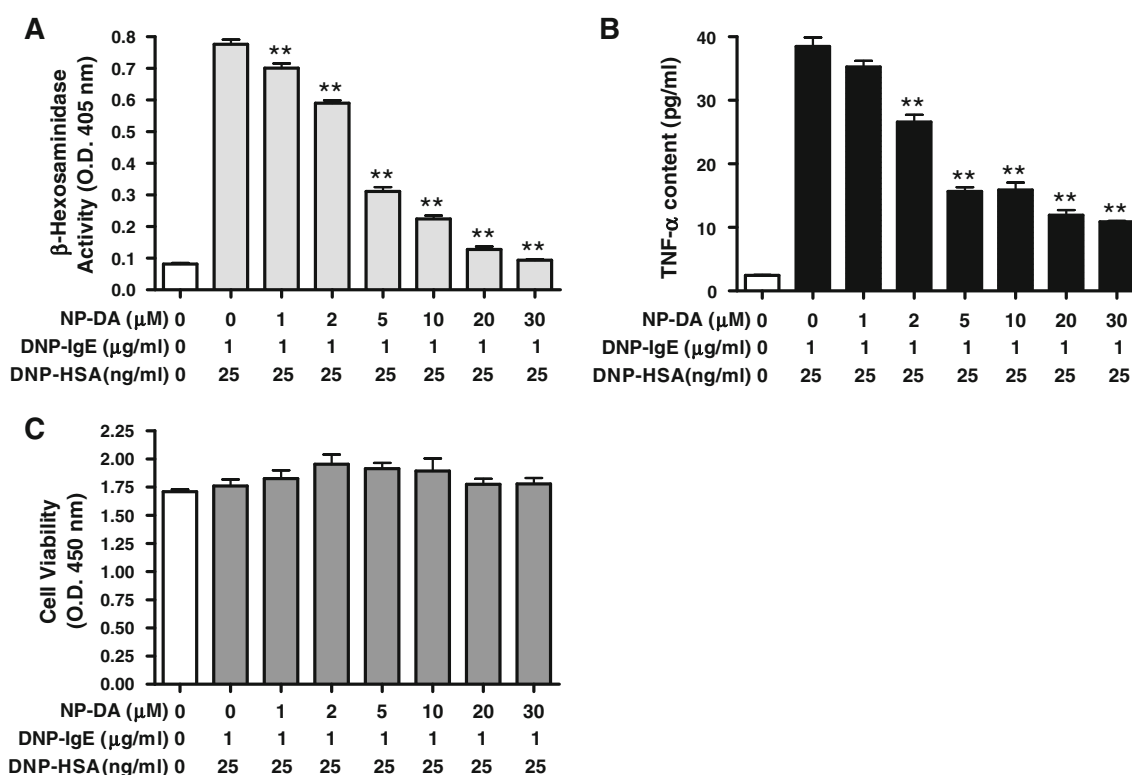


Fig. 3 Inhibitory effect of palmitoyl dopamine on activity of β -hexosaminidase and level of TNF- α released from RBL-2H3 cells stimulated by an IgE-antigen complex. RBL-2H3 cells were seeded on a 24-well plate in MEM with 5 % FBS at 37 °C overnight, and then the cells were washed, and further incubated with DNP-IgE for 24 h. The cells were incubated with palmitoyl dopamine (0–30 μ M) for 1 h, and then stimulated by DNP-HSA (25 ng/ml) for 4 h. β -Hexosaminidase activity (a) and TNF- α level (b) were determined as described in “Materials and Methods”. Data are listed as mean $\pm$ SD

values of triple determinations. RBL-2H3 cells were seeded on a 96-well plate (2.5×10^4 cells/well) in MEM with 5 % FBS at 37 °C overnight, and then the cells were washed, and further incubated with DNP-IgE for 24 h. The cells were incubated with palmitoyl dopamine (0–30 μ M) for 1 h, simultaneously treated with DNP-HSA (25 ng/ml) and WST-1 reagent (10 μ l), and then incubated for 4 h. Cell viability (c) was determined as described in “Materials and Methods”. Data are listed as mean $\pm$ SD values of octuple determinations. ** $P < 0.01$ versus DNP-HSA-treated group

Regulatory Effect of Palmitoyl Dopamine on Fc ϵ RI Signaling and Arachidonate Cascade

Next, we turned to the mechanism for the anti-allergic action of NP-DA. The activation of the Fc ϵ RI receptor is known to be associated with phosphorylation of Lyn and Syk, which cause degranulation of mast cells [24]. In this respect, NP-DA, showing a strong anti-degranulation action, was supposed to affect phosphorylation of Lyn or Syk as well as Fc ϵ RI receptor cascade. When RBL-2H3 cells were preincubated with NP-DA (1–20 μ M) for 1 h before antigen challenge, and the incubation was extended for another 10 min, NP-DA significantly inhibited phosphorylation and expression of Syk in a dose-dependent manner, but neither Lyn nor Fyn (Fig. 5). Although Fyn has been reported to be important for the activation of mast cells [28], it did not inhibit the phosphorylation of Fyn. Besides, NP-DA dose-dependently suppressed the phosphorylation of ERK1/2 and Akt. In addition, when RBL-2H3 cells were preincubated with NP-DA at different

concentrations for 1 h before stimulation with DNP-HSA, and the incubation was extended for another 4 h, NP-DA at 20 μ M diminished phosphorylation of cytosolic phospholipase A₂ (cPLA₂), an enzyme of the limiting step for the arachidonate cascade, but not expression of cPLA₂ (Fig. 6). Similarly, NP-DA was able to decrease phosphorylation of 5-LO, an enzyme of the limiting step for leukotriene biosynthesis. However, NP-DA could not inhibit the expression of cyclooxygenase-2 (COX-2), an enzyme of the limiting step for prostaglandin biosynthesis (Fig. 6). These findings indicate that NP-DA directly inhibits both activation and expression of Syk. Additionally, NP-DA also decreases activation of other targets such as ERK1/2, Akt, cPLA₂ and 5-LO in the Fc ϵ RI signal cascade.

Discussion

Previously, *N*-acyl dopamine have been reported to possess some biological actions; they increase immobility, reduce

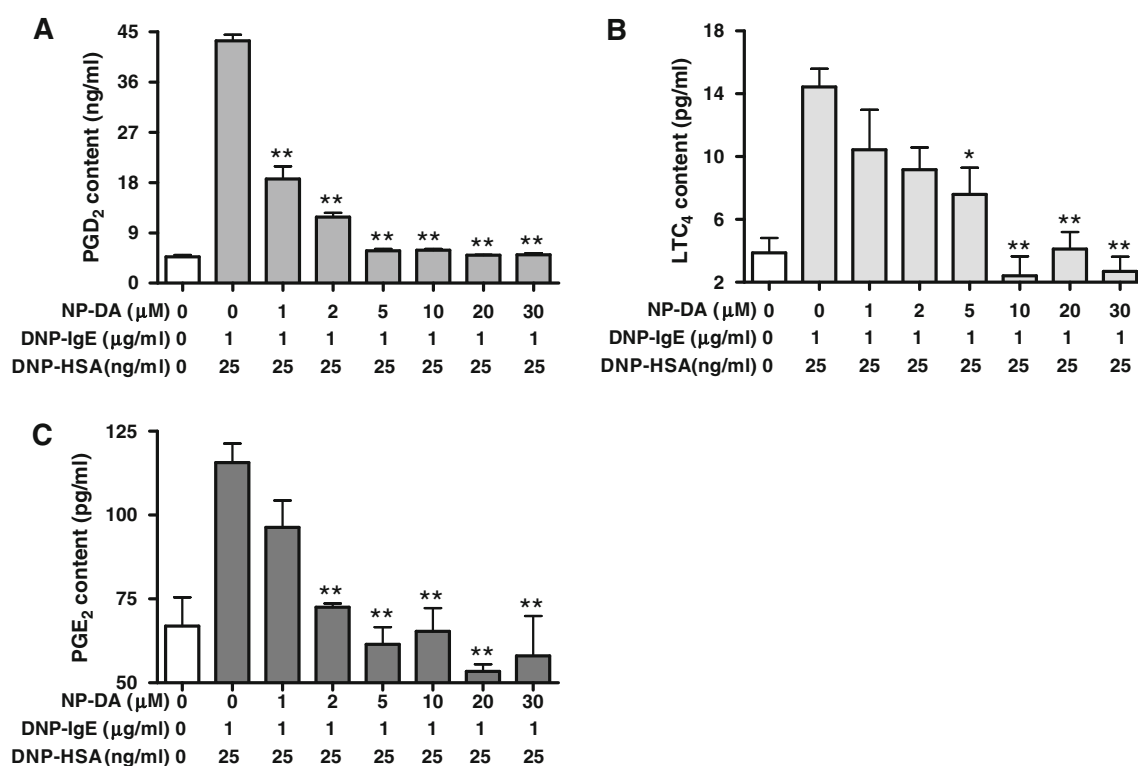


Fig. 4 Inhibitory effect of palmitoyl dopamine on formation of PGE₂, PGD₂ and LTC₄ in RBL-2H3 cells stimulated by an IgE-antigen complex. RBL-2H3 cells were seeded on a 24-well plate in MEM with 5 % FBS at 37 °C overnight, and then the cells were washed, and further incubated with DNP-IgE for 24 h. The cells were

incubated with palmitoyl dopamine (0–30 μM) for 1 h, and then stimulated by DNP-HSA for 4 h. The amounts of PGD₂ (a), LTC₄ (b) and PGE₂ (c) were determined as described in “Materials and Methods”. Data are listed as mean ± SD values of triple determinations. **P* < 0.05 and ***P* < 0.01 versus DNP-HSA-treated group

body temperature and locomotor activity, and delay the reaction to a thermal stimulus in mice [13, 16]. Also, they reduce opioid-induced emesis in ferrets [25], and thermal allodynia in rat [12, 14] and monkeys [26, 27]. Nonetheless, there is no report concerning an anti-allergic action of *N*-acyl dopamines.

Arachidonoyl dopamine (NA-DA), one of *N*-acyl dopamines, was reported to be a potent inhibitor of 5-LO, an enzyme of the limiting step in leukotriene biosynthesis [15]. Leukotrienes, metabolites of 5-LO, are known to be strong inducers of allergic response and inflammation [3, 4, 23]. Thus, it was hypothesized that NA-DA might inhibit the degranulation of mast cells induced by IgE. Here, it is demonstrated that NA-DA, NO-DA or NP-DA have anti-allergic actions, based on the release of β-hexosaminidase, a marker of degranulation, from RBL-2H3 cells induced by an IgE-antigen reaction. Especially, NP-DA is a stronger inhibitor than NO-DA or NA-DA in IgE-mediated degranulation in RBL-2H3 cells. In support of this, NP-DA significantly inhibits the levels of some allergic mediators such as

TNF-α, PGE₂, PGD₂ and LTC₄. Thus, among *N*-acyl dopamines used, NP-DA is the most effective in expressing anti-allergic actions.

Concerning the mechanism for anti-allergic activity of NP-DA, one possible mechanism may be associated with a direct inhibition of the FcεRI signal cascade. The IgE-mediated degranulation in mast cells is correlated with activation of the FcεRI receptor, and the activation induces the liberation of various mediators containing TNF-α, leukotrienes and prostaglandins through phosphorylation of the Fyn or Lyn/Syk pathway [28–30]. As a result, the activation of Syk leads to the increase in intracellular Ca²⁺ as well as the activation of the MAP kinase family [31, 32]. Thus, Fyn, Lyn and Syk are important intracellular mediators in the early signaling pathway of FcεRI receptor activation. In our present study, the inhibition of Syk activation by NP-DA may support the notion that a primary target of NP-DA may be Syk. In support of this, NP-DA significantly decreased the activation of Akt and ERK1/2, which belong to down-stream proteins in the FcεRI receptor cascade.

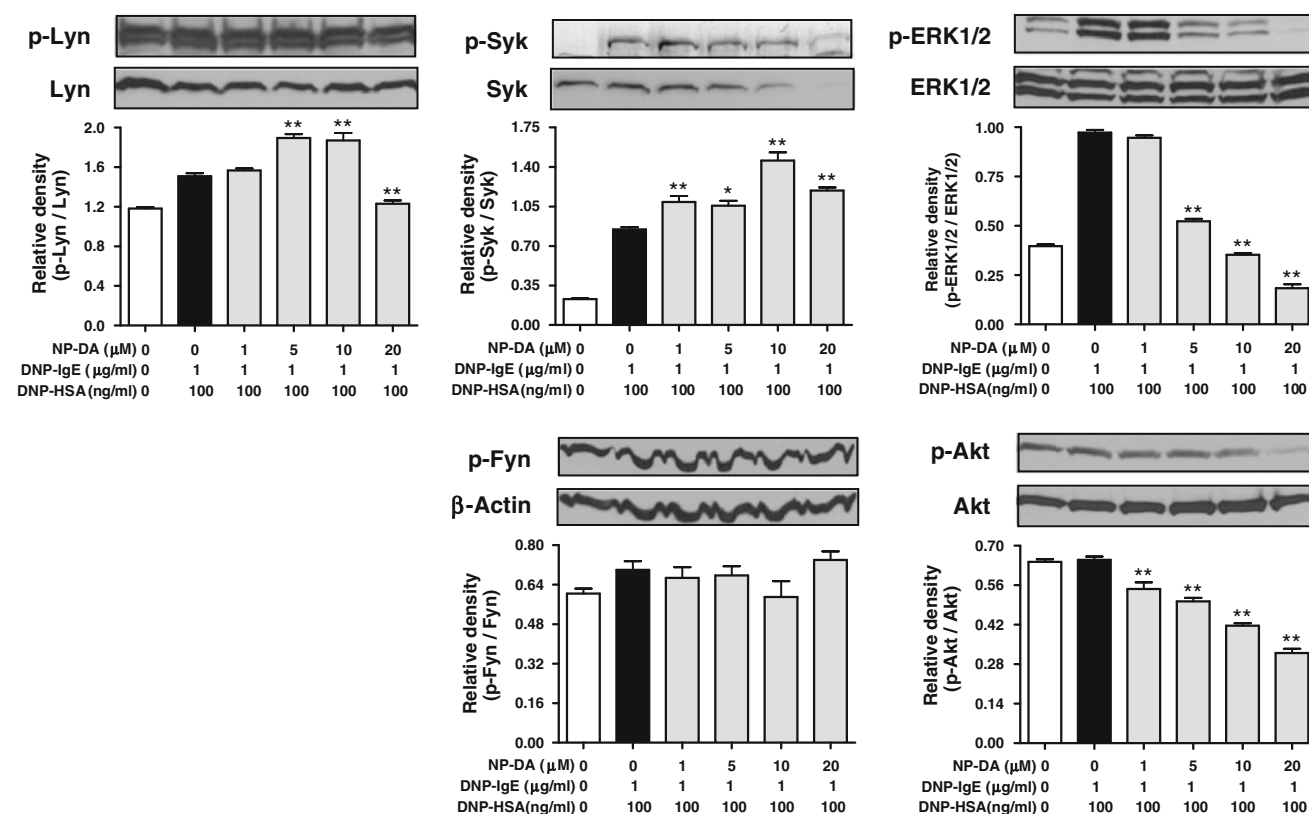


Fig. 5 Effect of palmitoyl dopamine on the early stage of the FcεRI signal cascade in RBL-2H3 cells stimulated by an IgE–antigen complex. RBL-2H3 cells were seeded on a 6-well plate (5×10^5 cells/well) in MEM with 5 % FBS at 37 °C overnight, and then the cells were washed, and further incubated with DNP-IgE for 24 h. The cells were incubated with palmitoyl dopamine (0–20 μM) for 1 h, and

then stimulated by DNP-HSA (100 ng/ml) for 10 min. The cells were rinsed with $1 \times$ PBS, and lysed with a cell lysis buffer. The expression of p-Lyn, Lyn, p-Syk, Syk, p-ERK1/2, ERK1/2, p-Akt, Akt and β-actin was determined as described in “Materials and Methods”. Similar results were obtained in three independent experiments. * $P < 0.05$ and ** $P < 0.01$ versus DNP-HSA-treated group

Although NP-DA could not inhibit COX-2 expression, it dramatically reduced the levels of PGE₂ and PGD₂, corresponding to the COX-2 product, which are known to be enhanced in activated immune cells including mast cells [22, 23, 33, 34]. Recently, it has been reported that NP-DA suppresses the formation of PGE₂, but not COX-2 expression in nerve cells [18]. In addition, *N*-acyl dopamines induced the expression of COX-2 in brain endothelial cells [35]. Thus, these findings suggest that NP-DA may directly inhibit COX-2 activity in prostaglandin biosynthesis. Such a suppressive effect of NP-DA on PGE₂ formation may contribute to the increase in its anti-allergic action, since PGE₂ is considered to contribute to asthma development and inflammation associated with IL-4 and IL-5, which are produced by helper T cells [34]. Also, the inhibitory effect of NP-DA on PGD₂ formation may add to the anti-allergic action, since PGD₂ is known to cause bronchoconstriction, vasodilation, increases in capillary permeability, and mucous production in asthma [22]. Furthermore, NP-DA also inhibits activation of 5-LO, actually

reducing the level of LTC₄. Thus, NP-DA directly inhibits the activation of 5-LO in leukotriene biosynthesis. Such an effect of NP-DA on 5-LO activation may improve its anti-allergic action, because LTC₄ is known to be a potent spasmogenic and chemotactic biochemical that increases the permeability of capillaries and other small vessels, and it plays a role in promoting and maintaining the allergic inflammatory reaction in allergic diseases via activation of cysteinyl-LT receptors [23]. Taken together, it is suggested that NP-DA can reduce allergic reactions through suppression of Syk, cPLA₂ and 5-LO activation as well as inhibition of COX-2 activity. Further, such an effect of NP-DA may be extended to an anti-inflammatory effect on other cells or tissues. Meanwhile, several cytokines may play critical roles in allergic inflammation [36]. Especially, TNF-α, secreted from mast cells in an IgE–antigen binding reaction [37], is known to play an important role in allergic reactions [36]. Moreover, expression of TNF-α is associated with ERK1/2 activation of the FcεRI receptor cascade in RBL-2H3 cells stimulated by an IgE–antigen complex

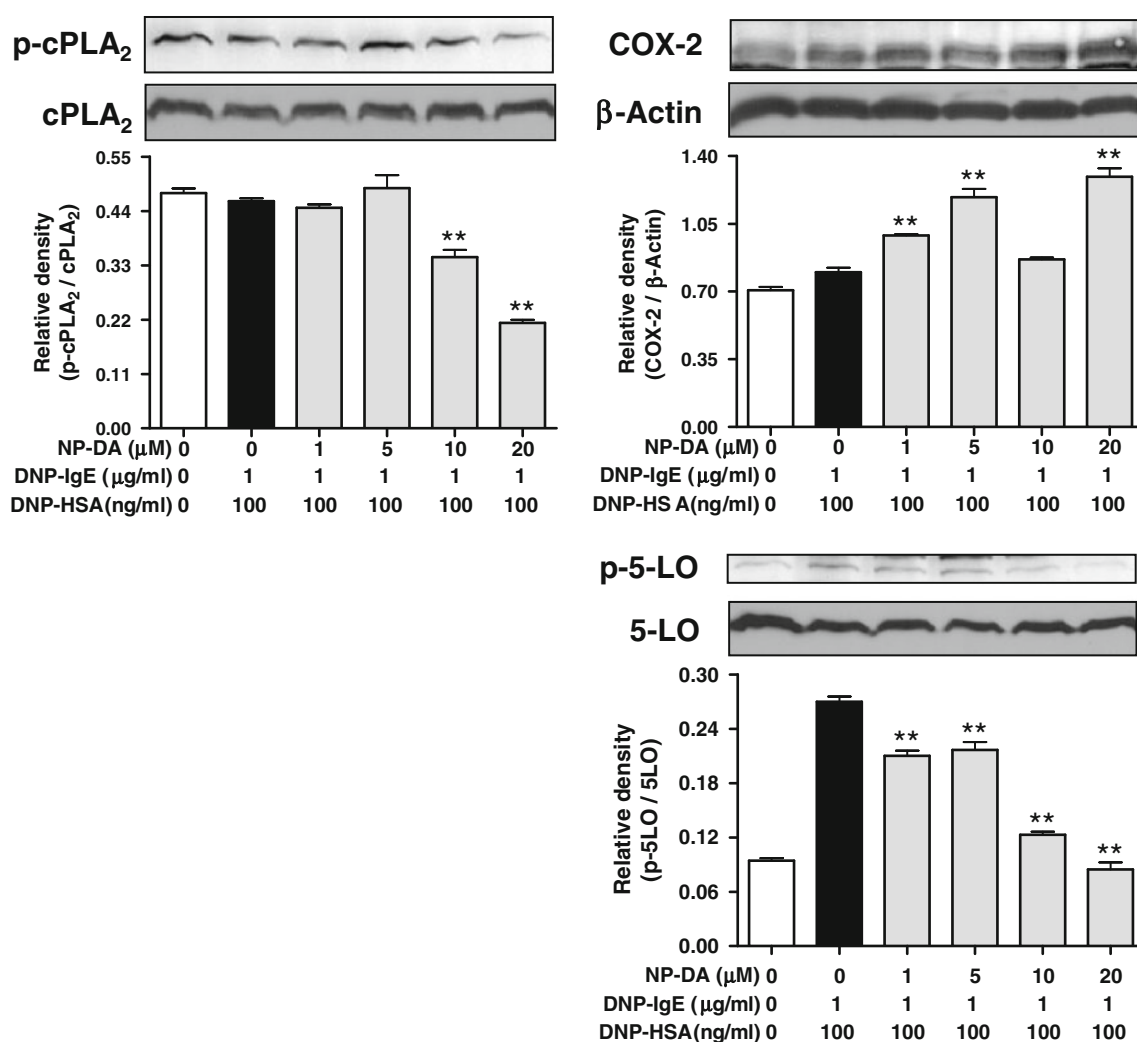


Fig. 6 Effect of palmitoyl dopamine on a late stage of the FcεRI signal cascade in RBL-2H3 cells stimulated by an IgE–antigen complex. RBL-2H3 cells were seeded on a 6-well plate in MEM with 5 % FBS at 37 °C overnight, and then the cells were washed, and further incubated with DNP-IgE for 24 h. The cells were incubated with palmitoyl dopamine (0–20 μM) for 1 h, and then stimulated by

DNP-HSA for 4 h. The cells were rinsed, and lysed with a cell lysis buffer. The expression of p-cPLA₂, cPLA₂, p-5-LO, 5-LO, COX-2 and β-actin was determined as described in “Materials and Methods”. Similar results were obtained in three independent experiments. ***P* < 0.01 versus DNP-HSA-treated group

[32]. In support of this, NP-DA significantly inhibited ERK1/2 activation and reduced both protein and mRNA level of TNF-α. Therefore, the reduction of TNF-α formation by NP-DA may provide an additional advantage of NP-DA as an anti-allergic agent.

In summary, this study demonstrates that NP-DA possesses anti-allergic functions in RBL-2H3 cells stimulated by an IgE–antigen complex. These findings reveal a novel feature of NP-DA in allergic response. The mechanisms for its anti-allergic action may include multiple targets including Syk, Akt, ERK1/2 and cPLA₂ as well as 5-LO. Such effects may provide further information for the application of *N*-acyl dopamine as a therapeutic or preventive agent.

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Conflict of interest No potential conflicts of interest were disclosed.

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