

3-*O*-(2,3-Dimethylbutanoyl)-13-*O*-decanoylingenol from *Euphorbia kansui* Suppresses IgE-Mediated Mast Cell Activation

Satoshi NUNOMURA,^a Susumu KITANAKA,^b and Chisei RA*^a

^a Division of Molecular Cell Immunology and Allergology, Nihon University Graduate School of Medical Sciences; 30-1 Oyaguchikami-cho, Itabashi-ku, Tokyo 173-8610, Japan; and ^b College of Pharmacy, Nihon University; 7-7-1 Narashino-dai, Funabashi, Chiba 274-8555, Japan.

Received September 7, 2005; accepted November 7, 2005; published online November 11, 2005

Aggregation of the high affinity receptor for IgE (FcεRI) on mast cells by antigen and IgE complex induces release of chemical mediators, leading to acute allergic inflammation. We recently found that 3-*O*-(2,3-dimethylbutanoyl)-13-*O*-decanoylingenol (DBDI), purified from the *Euphorbia kansui* L., inhibits degranulation in rat basophilic leukemia 2H3 cells upon aggregation of the FcεRI. In the present study, we demonstrated that the DBDI significantly inhibits release of β-hexosaminidase, synthesis of eicosanoids, and mobilization of intracellular Ca²⁺ in the bone marrow-derived mouse mast cells stimulated with IgE and antigen. Furthermore, we revealed that phosphorylation of Syk, phospholipase C-γ₂, and extracellular signal-related kinase 1/2 is significantly suppressed in the DBDI-treated mast cells. These findings suggest that the DBDI may have a therapeutic potential for allergic diseases by inhibiting intracellular signaling pathways for activation and chemical mediator release in mast cells.

Key words diterpene; mast cell activation; FcεRI; signal transduction; allergy; *Euphorbia kansui* L.

Mast cells express the high affinity receptor for IgE (FcεRI) on the cell surface which plays a pivotal role in the development of allergic inflammation. FcεRI is mainly expressed on mast cells and basophils as a tetramer of one α, one β and γ chain homodimers. Aggregation of the FcεRI on mast cells by bound IgE and multivalent antigens induces rapid release of preformed intragranular mediators such as histamine and heparin, newly synthesis of arachidonic acid metabolites, such as prostaglandins (PGs) and leukotrienes (LTs).¹⁾ Activation of intracellular signaling is essential for these acute allergic reactions through FcεRI. Namely, upon FcεRI cross-linking, tyrosine phosphorylation of the β chain via activated-Lyn and subsequent phosphorylation of γ chain and Syk by Lyn are immediately induced, which in turn leads to activation of phospholipase C (PLC)-γ and linker for activation of T cells (LAT), intracellular Ca²⁺ mobilization, activation of protein Kinase C (PKC) and mitogen activated protein (MAP) kinase family.²⁾ A large number of studies revealed that suppression of Ca²⁺ responses, PKC and MAP kinase family members has substantial inhibitory effects on release of histamine and generation of arachidonic acid metabolite in mast cells.^{3,4)}

The dried roots of *Euphorbia kansui* L. (*E. kansui*) are known as “Kan Sui” in Chinese medicine and have been used as herbal remedy for cancer or edema in the mainland of China.⁵⁾ As for anti-tumor activity of *E. kansui*, it was reported that numerous diterpenes with an ingenol skeleton from the roots of the *E. kansui* suppress progression of cell cycle related to proliferation of cancer cells.⁶⁾ Recently, we found that 3-*O*-(2,3-dimethylbutanoyl)-13-*O*-decanoylingenol (abbreviated thereafter as DBDI) from *E. kansui* has a potent inhibitory effect on release of β-hexosaminidase in rat basophilic leukemia 2H3 cells (RBL-2H3) following FcεRI cross-linking (unpublished data).

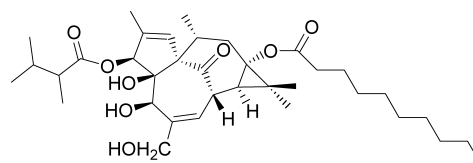
In this study, we investigated effects of DBDI on the activation of intracellular signaling pathways and the release of inflammatory chemical mediators in the bone marrow-derived mouse mast cells upon FcεRI stimulation. Here, we re-

port that DBDI-treated mast cells show a significant reduction of β-hexosaminidase release and eicosanoids synthesis when compared with non-treated cells. These suppressive actions of DBDI may be caused by inhibiting of intracellular signaling including Syk, PLC-γ₂, Ca²⁺ responses and extracellular signal regulated kinase (ERK) 1/2. Our findings of the present study may provide useful information for anti-allergic action of the DBDI against mast cell activation through FcεRI.

MATERIALS AND METHODS

Chemical DBDI (Fig. 1) was purified from the dried roots of *E. kansui* as described previously.⁷⁾ Briefly, the dried roots of *E. kansui* (15.1 kg) were extracted twice with 60% ethanol under reflux. Evaporation of the solvent from the combined extract under reduced pressure gave the 60% ethanol extract. The extract was dissolved and suspended in water and partitioned with chloroform, ethyl acetate, and *n*-butanol. DBDI was purified from chloroform fraction employing column chromatography and HPLC. The purified-DBDI was dissolved in 100% Dimethyl Sulfoxide (DMSO) (Wako Chemicals, Japan) at −20 °C.

Reagents Anti rat β-chain mAb (the hybridoma (clone JRK) was kindly gifted by Dr. Juan Rivera at NIH, U.S.A.) was prepared in our laboratory. Polyclonal Abs to ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), phospho-PLC-γ₂



3-*O*-(2,3-dimethylbutanoyl)-13-*O*-decanoylingenol (DBDI)

Fig. 1. The Chemical Structure of 3-*O*-(2,3-Dimethylbutanoyl)-13-*O*-decanoylingenol (DBDI)

* To whom correspondence should be addressed. e-mail: fcericra@med.nihon-u.ac.jp

(Tyr1217) and phospho-Syk (Tyr525/526) were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). Anti-trinitrophenyl (TNP) monoclonal IgE was purchased from Pharmingen (San Diego, CA, U.S.A.) and was labeled with fluorescein isothiocyanate (FITC). 4-Amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP1) was purchased from Calbiochem (San Diego, CA, U.S.A.).

Mice Four-week-old C57BL/6j mice were purchased from Charles River Japan Inc. (Tokyo, Japan). All experiments were performed in accordance with the guide for the care and use of laboratory animals of Nihon University.

Cell Cultures Bone marrow-derived mouse mast cells (BMMC) were prepared from femur of C57BL/6j mice. Cells were cultured in the RPMI1640 (Sigma, St. Louis, MO, U.S.A.) supplemented with 10% FBS, and 5 ng/ml recombinant IL-3 (Peprotech, London, U.K.). After 4-week, BMMC were confirmed FcεRI expression on the cell surface and then used for experiments.

Cell Stimulation BMMC (5×10^6) were sensitized with anti-TNP IgE (1 μg/ml) in the absence of recombinant IL-3 at 37 °C for 4 h. The IgE-sensitized cells were treated with 0–12.5 μg/ml of DBDI at 37 °C for 30 min, and then washed twice with 1×Tyrode's buffer (20 mM HEPES, pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 0.05% BSA). Cells were stimulated with 0–30 ng/ml of TNP-BSA (LSL, Tokyo, Japan) for indicated time periods.

Immunoblotting The stimulated cells (5×10^6) were washed twice with ice-cold PBS and lysed in Tris Buffer Saline containing 1% Nonidet P-40, 60 mM Octyl-β-glucoside, 2 mM Phenylmethylsulfonylfluoride (PMSF), 10 μg/ml aprotinin, 2 μg/ml leupeptin and pepstatin A, 50 mM NaF and 1 mM sodium orthovanadate, for 30 min on ice. The lysates were centrifuged for 15 min at 14000×*g*. The samples were then boiled, separated on 10 or 12% SDS-PAGE, and transferred on to Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.). The membrane was incubated with primary antibody and an appropriate secondary horseradish peroxidase-conjugated antibody. Signals were detected by enhanced chemiluminescence (Amersham Bioscience, Little Chalfont, U.K.). The immunoreactive bands were scanned to produce digital images that were quantified employing NIH Image software, and then fold induction was calculated by the relative amount of phospho-Syk, phospho-PLC-γ₂ protein, phospho-ERK1/2 to each loading control proteins.

Measurement of [Ca²⁺]_i Mobilization The IgE-sensitized cells (5×10^5) were incubated with 0–12.5 μg/ml of DBDI for 30 min, and then washed twice with 1×Hank's balanced salt solution (HBSS). The cells were loaded with 16 μM Fluo-3AM and 8 μM Fura-RedAM (Invitrogen, Groningen, The Netherlands) in 1×HBSS for 20 min at 37 °C. The cells were resuspended in 1×Tyrode's buffer, and then changes of dye fluorescence upon addition of TNP-BSA (30 ng/ml) were evaluated employing flow cytometry. The [Ca²⁺]_i mobilization expressed as the ratio of Fluo-3AM/Fura-RedAM (Dojindo, Kumamoto, Japan) fluorescence intensity.

β-Hexosaminidase Release Assay Degranulation of mast cells was determined by β-hexosaminidase release. Briefly, cells (5×10^5) were stimulated in 0.2 ml 1×Tyrode's buffer at indicated concentrations of antigen at 37 °C for 30

min. The cells were lysed with 1% Nonidet P-40 in the above-mentioned medium. The supernatants and the total cell lysates were incubated with 1.3 mg/ml of *p*-nitrophenyl-*N*-acetyl-β-D-galcopyranoside (Sigma) in 0.1 M sodium citrate buffer (pH 4.5) at 37 °C for 40 min. The reaction was terminated by addition of 0.2 M glycine buffer (pH 10.7). The release of the product 4-*p*-nitrophenol was evaluated by optical absorbance at 405 nm.

Measurements of Eicosanoids Production Cell culture supernatants after FcεRI stimulation were analyzed for LTC₄, PGD₂ and PGE₂ by specific ELISA kits (Cayman Chemical, Ann Arbor, MI, U.S.A.) according to the manufacture's instructions.

Flow Cytometry For the evaluation of FcεRI expression on the cell surface, BMMC (5×10^5) were incubated with 1 μg/ml FITC-conjugated anti TNP IgE for 60 min at 4 °C, and then with 12.5 μg/ml of DBDI or 0.1% DMSO for another 30 min at 37 °C. In order to monitor apoptosis, cells (1×10^5) were incubated with 1 μg/ml of FITC-labeled annexin V (Pharmingen) and 2.5 μg/ml of propidium iodide (Pharmingen) at room temperature for 15 min in the dark. Flow cytometric analyses were performed employing FACSCalibur (Becton-Dickinson, San Jose, CA, U.S.A.).

Statistical Analysis Williams multiple comparisons test was performed to determine statistical significance among the experimental groups; $p < 0.05$ was considered significant.

RESULTS

Inhibition of β-Hexosaminidase Release and Eicosanoids Synthesis by DBDI To examine effects of the DBDI on release of preformed mediators from mast cells, we prepared BMMC from C57BL/6j mice. In the DBDI-treated BMMC, β-hexosaminidase (one of the preformed intragranular enzymes) release induced by FcεRI stimulation was inhibited in a dose-dependent manner (Fig. 2A). We next investigated whether DBDI suppresses secretion of LTC₄, PGD₂ and PGE₂, which are newly synthesized inflammatory mediators. As shown in Table 1, production of LTC₄, PGD₂ and PGE₂ induced by FcεRI stimulation was also significantly inhibited with treatment of the DBDI. In DBDI-treated resting BMMC, production of LTC₄ and PGD₂ was also inhibited at 12.5 μg/ml. These results suggest that the DBDI has severe suppressive effects on both the release of preformed mediators and the newly synthesis of eicosanoids in mast cells. Since ingenane-type diterpenes have been reported to induce apoptosis of intact murine thymocytes and Jurkat human T lymphocytes,^{8,9} we further evaluated cytotoxic effects of DBDI on BMMC. BMMC were cultured with 12.5 μg/ml of DBDI or 0.1% DMSO for 24 h. As shown in Fig. 2B, fraction of dead cells (R2 gate: double positive of annexin V and propidium iodide) was almost at same levels in the DBDI-treated cells and the control cells (DMSO-treated), suggesting that inhibitory effects of DBDI are independent of cytotoxicity.

Suppression of Intracellular Ca²⁺ Mobilization by DBDI The ingenane-type diterpenes are structurally related to phorbol ester such as phorbol 12-myristate 13-acetate (PMA). In non-adherent RBL-2H3 cells treated with the PMA, intracellular Ca²⁺ concentration ([Ca²⁺]_i) increase and β-hexosaminidase release are suppressed in a dose-depend-

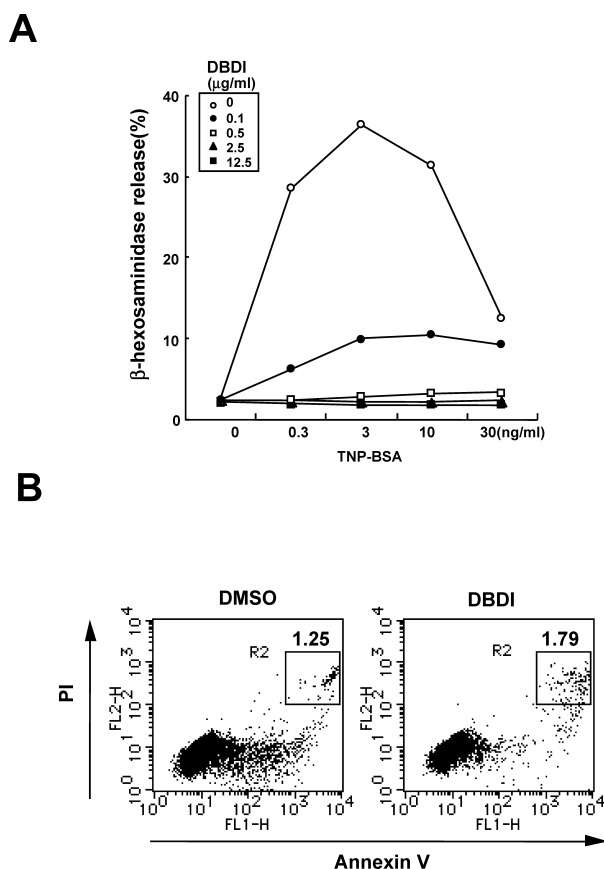


Fig. 2. Effects of DBDI on β -Hexosaminidase Release and Cell Viability

(A) DBDI inhibited β -hexosaminidase release from BMMC in a dose-dependent manner. BMMC ($5 \times 10^5/\text{ml}^{-1}$) were sensitized with $1 \mu\text{g}/\text{ml}$ of anti-TNP IgE in the absence of recombinant IL-3 for 4 h at 37°C . The IgE-sensitized cells were treated with indicated concentrations of DBDI for 30 min at 37°C . The cells were then stimulated with TNP-BSA at indicated concentrations for 30 min. One of representatives of three independent experiments with similar results is demonstrated. (B) DBDI does not affect cell viability. BMMC ($1 \times 10^5/\text{ml}^{-1}$) were cultured with $12.5 \mu\text{g}/\text{ml}$ of DBDI or 0.1% DMSO for 24 h at 37°C . The cells were incubated with $1 \mu\text{g}/\text{ml}$ FITC-labeled annexin V and $2.5 \mu\text{g}/\text{ml}$ propidium iodide at room temperature for 15 min in the dark. The R2 region (annexin V+/propidium iodide+) represents the fraction of dead cells.

ent manner,¹⁰⁾ we therefore examined effects of DBDI on $[\text{Ca}^{2+}]_i$ in BMMC upon Fc ϵ RI engagement. Interestingly, DBDI-treated BMMC showed a significant time lag of $[\text{Ca}^{2+}]_i$ elevation in a concentration-dependent manner (0 – $0.1 \mu\text{g}/\text{ml}$) (Fig. 3A), and this chemical completely blocked $[\text{Ca}^{2+}]_i$ increase in the Fc ϵ RI-stimulated mast cells at concentrations of 0.5 – $12.5 \mu\text{g}/\text{ml}$. This result appears in accordance with effects of DBDI entirely inhibiting β -hexosaminidase release and eicosanoids secretion at concentrations of 0.5 – $12.5 \mu\text{g}/\text{ml}$. We next examined effects of DBDI on Fc ϵ RI expression on BMMC because PMA can induce a rapid and significant decrease in Fc ϵ RI expression on RBL-2H3 cells as an alternative pharmacological effect.¹¹⁾ As shown in Fig. 3B, DBDI had no effect on the cell surface Fc ϵ RI expression (Fig. 3B). It is thereby concluded that DBDI has no potential to reduce the Fc ϵ RI expression on BMMC at protein level, although effect of the compound on the gene expression for Fc ϵ RI of the RBL-2H3 cells is unknown. Based on the above results shown in Figs. 2 and 3, we hypothesized that DBDI directly suppresses activation of a variety of intracellular signals, which are necessary for $[\text{Ca}^{2+}]_i$ increase and mediators secretion induced by Fc ϵ RI

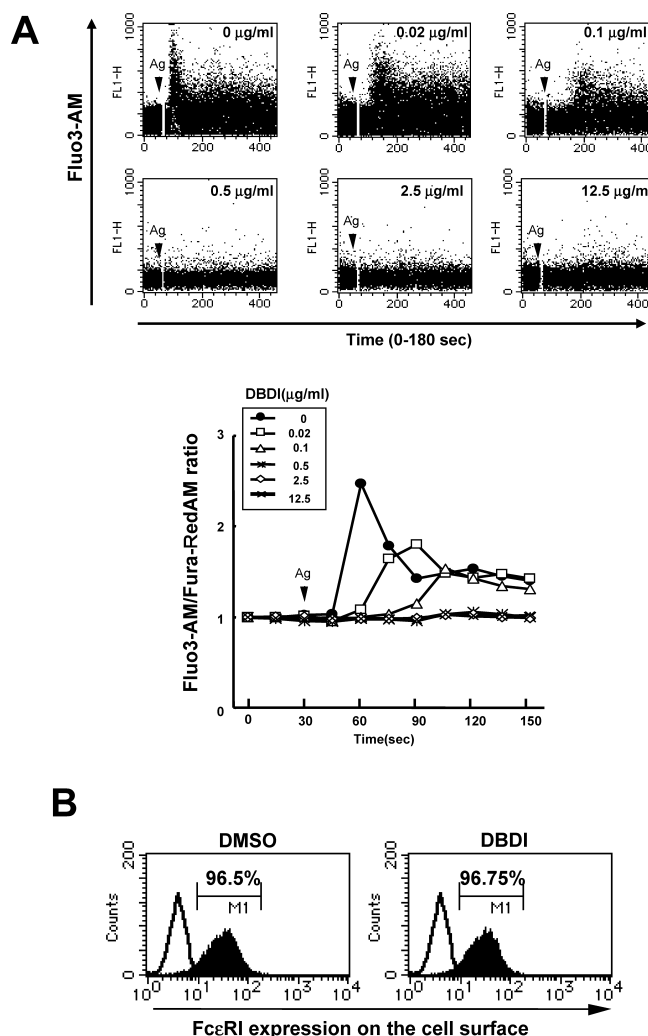


Fig. 3. Effects of DBDI on the $[\text{Ca}^{2+}]_i$ Mobilization and the Fc ϵ RI Expression

(A) Inhibition of $[\text{Ca}^{2+}]_i$ mobilization by DBDI. The IgE-sensitized BMMC ($5 \times 10^5/\text{ml}^{-1}$) were treated with DBDI at indicated concentrations for 30 min, and then cells were incubated with $16 \mu\text{M}$ Fluo-3AM and $8 \mu\text{M}$ Fura-RedAM for 20 min at 37°C . Changes of dye fluorescence were determined employing flow cytometry after stimulation with $30 \text{ ng}/\text{ml}$ of TNP-BSA at room temperature. The flow cytometric pattern of Fluo-3AM was shown in the upper panel. $[\text{Ca}^{2+}]_i$ mobilization until 150 s is shown as ratio of Fluo-3AM/Fura-RedAM fluorescence intensity, and displayed in the bottom panel. One of representatives of three independent experiments with similar results is demonstrated. (B) DBDI does not affect Fc ϵ RI expression on the cell surface. BMMC ($5 \times 10^5/\text{ml}^{-1}$) were incubated with $1 \mu\text{g}/\text{ml}$ FITC-conjugated anti TNP IgE for 60 min at 4°C , and then with $12.5 \mu\text{g}/\text{ml}$ of DBDI or 0.1% DMSO for another 30 min at 37°C . Fc ϵ RI expression was assessed by percentage of M1 region (black area). Unstained cells are employed as negative control (white area). All flow cytometric analyses were performed with FACSCalibur. Similar results were obtained from four independent experiments.

cross-linking.

DBDI Suppresses Activation of Syk and PLC- γ_2 in Mast Cells Protein-tyrosine phosphorylation is early intracellular signaling event^{12,13)} that initiates activation cascade resulting in inflammatory mediator release. We first examined effects of DBDI on whole cellular protein tyrosine phosphorylation in BMMC. In the BMMC treated with $0.5 \mu\text{g}/\text{ml}$ of DBDI, decrease of whole protein-tyrosine phosphorylation was not so much, but tyrosine phosphorylation of some proteins (marked with asterisks) was significantly reduced when compared with that of control cells (Fig. 4A). Compared to PP1, DBDI inhibited tyrosine phosphorylation more strongly. Thus, DBDI is likely to have direct effects on mast

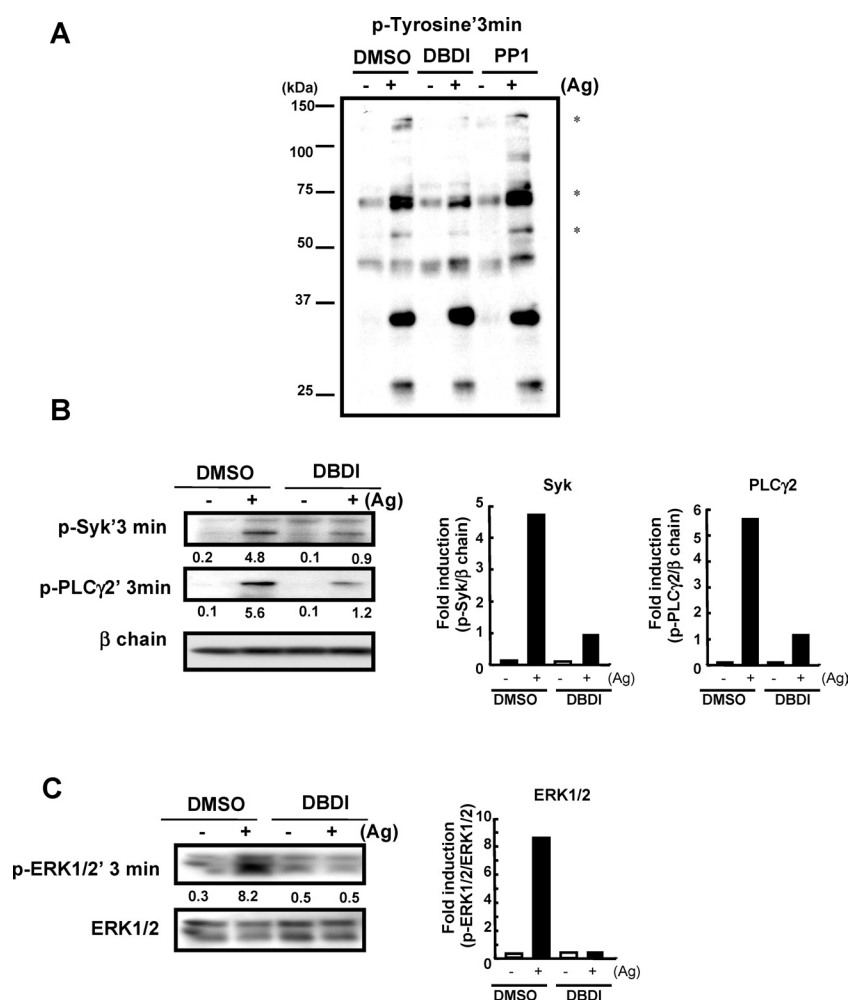


Fig. 4. DBDI Suppresses Intracellular Signal Transduction Pathways

The IgE-sensitized cells ($5 \times 10^5/\text{ml}^{-1}$) were cultured with 0.5 $\mu\text{g}/\text{ml}$ of DBDI, 0.1% DMSO or 20 μM PP1 for 30 min at 37°C, and then stimulated with 30 ng/ml of TNP-BSA for 3 min and lysed with lysis buffer. Whole cell lysates were analyzed by immunoblotting with anti-phospho tyrosine (A), anti-phospho-Syk and anti-phospho-PLC- γ_2 (B), anti-phospho-ERK1/2 (C).

Table 1. Inhibition of Eicosanoids Secretion by DBDI

DBDI ^{a)}	Ag(-)			Ag(+) ^{b)}		
	LTC ₄ (pg/ml)	PGD ₂ (pg/ml)	PGE ₂ (pg/ml)	LTC ₄ (pg/ml)	PGD ₂ (pg/ml)	PGE ₂ (pg/ml)
0 $\mu\text{g}/\text{ml}$	6.3 $\pm$ 3.2	202.6 $\pm$ 2.9	109.5 $\pm$ 28.9	9189.1 $\pm$ 279.5	2502.1 $\pm$ 203.8	238.2 $\pm$ 9.0
0.1 $\mu\text{g}/\text{ml}$	5.2 $\pm$ 2.1	186.2 $\pm$ 7.8	89.42 $\pm$ 4.87	725.9 $\pm$ 5.8*	2692.3 $\pm$ 288.5	117.6 $\pm$ 12.7*
0.5 $\mu\text{g}/\text{ml}$	4.5 $\pm$ 1.4	158.21 $\pm$ 32.2	100.2 $\pm$ 10.5	3.1 $\pm$ 1.2*	399.8 $\pm$ 27.7*	88.4 $\pm$ 5.3*
2.5 $\mu\text{g}/\text{ml}$	2.5 $\pm$ 1.5	143.5 $\pm$ 17.5	120.3 $\pm$ 22.8	2.4 $\pm$ 1.2*	328.6 $\pm$ 26.9*	90.4 $\pm$ 6.0*
12.5 $\mu\text{g}/\text{ml}$	1.0 $\pm$ 0.4*	91.43 $\pm$ 14.8*	101.9 $\pm$ 16.18	0.4 $\pm$ 0.4*	317.7 $\pm$ 35.1*	90.0 $\pm$ 12.0*

a) IgE-sensitized BMMC were treated with DBDI at indicated concentrations for 30 min. b) DBDI-treated cells were stimulated with 10 ng/ml of TNP-BSA for 30 min at 37°C. * Statistical analyses were performed by Williams multiple comparisons test. * $p < 0.05$ relative to maximum secretion of each DBDI non-treated cells. The values are means $\pm$ S.D. ($n = 3$).

cell signaling cascades.

To investigate the effects of DBDI on intracellular signaling of mast cells in more details, we further analyzed phosphorylation of Syk and PLC- γ_2 which play important roles for $[\text{Ca}^{2+}]_i$ responses of mast cells.^{14,15)} Western blotting analyses employing phospho-specific antibodies revealed that, upon Fc ϵ RI cross-linking, tyrosine phosphorylation of Syk and PLC- γ_2 was obviously reduced in the DBDI-treated BMMC when compared to that of the control cells (Fig. 4B).

Effects of DBDI on Activation of ERK1/2 Activation

of extracellular signal regulated kinase (ERK) 1/2 as well as $[\text{Ca}^{2+}]_i$ increase is required for eicosanoids synthesis in mast cells,⁴⁾ and the activation is mainly induced through Syk-mediated signaling pathway.¹⁶⁾ Figure 4B therefore suggests that ERK1/2 activation in the DBDI-treated mast cells might be reduced due to decrease of Syk activation, we examined phosphorylation of ERK1/2 in the DBDI-treated mast cells upon Fc ϵ RI cross-linking. As expected, the DBDI-treated BMMC showed significant decrease of ERK1/2 phosphorylation when stimulated with IgE and antigen (Fig. 4C).

DISCUSSION

In the present study, we revealed that DBDI, one of ingenane-type diterpenes from *E. kansui*, significantly inhibits $[Ca^{2+}]_i$ increase β -hexosaminidase release, and synthesis of PGD_2 , PGE_2 and LTC_4 in the BMMC stimulated with IgE and multivalent antigen complex. Moreover, we demonstrated that DBDI inhibits activation of intracellular signaling molecules, including Syk, PLC- γ_2 and ERK1/2. It is noteworthy that the inhibition of β -hexosaminidase release was observed only in the DBDI-treated RBL-2H3 cells when we performed screening of 11 ingenane-type diterpenes obtained from *E. kansui* (data not shown).

Increase of $[Ca^{2+}]_i$ is thought essential for preformed mediators release of mast cells upon Fc ϵ RI engagement.^{3,17)} As shown in Fig. 3A, DBDI suppressed $[Ca^{2+}]_i$ increase induced by Fc ϵ RI stimulation in a dose-dependent manner. Based on the results of the present and previous other studies, we concluded that inhibition of β -hexosaminidase release from granules in the DBDI-treated mast cells is caused by inhibition of $[Ca^{2+}]_i$ increase. Generally, $[Ca^{2+}]_i$ increase is totally dependent on release of Ca^{2+} from intracellular storage and subsequent influx of extracellular Ca^{2+} through Ca^{2+} channel. It is known that depletion of intracellular Ca^{2+} storage evoked by inositol 1,4,5-triphosphate (IP3) which is generated from phosphatidylinositol 4,5-bisphosphate by activated-PLC- γ s.¹⁵⁾ Both $[Ca^{2+}]_i$ increase and mediators release are significantly attenuated in the absence of extracellular Ca^{2+} ¹⁸⁾ or in the presence of Ca^{2+} channel blocker¹⁹⁾ and IP3 receptor blocker.²⁰⁾ Although we do not know whether DBDI affects functions of Ca^{2+} channel itself in BMMC, we speculate that suppression of $[Ca^{2+}]_i$ increase by DBDI is due in part to reduction of extracellular Ca^{2+} influx by inhibiting depletion of intracellular Ca^{2+} storage, because DBDI clearly suppressed tyrosine phosphorylation of Syk and PLC- γ_2 , which leads to IP3 production (Fig. 4B).

In mast cells, Syk is thought to stimulate not only Ca^{2+} responses via PLC- γ but also Syk-LAT-ERK1/2 pathway. In fact, Syk-deficient mast cells fail to activate ERK1/2 when stimulated through Fc ϵ RI.²¹⁾ Therefore, we consider that inhibition of ERK1/2 phosphorylation by DBDI might depend on suppression of Syk activation. Generally, ERK1/2 as well as $[Ca^{2+}]_i$ play essential roles for activation of Ca^{2+} dependent-cytosolic phospholipase A₂ (cPLA₂), leading to arachidonic acid generation.^{22,23)} Because DBDI significantly inhibits both Ca^{2+} responses and ERK1/2 activation of mast cells, it is conceivable that DBDI may suppress arachidonic acid generation by inhibiting cPLA₂ activation.

The primary target of DBDI in a series of mast cell signaling upon aggregation of the Fc ϵ RI is currently unknown. In the Fc ϵ RI-stimulated human basophils, significant time lag of $[Ca^{2+}]_i$ mobilization is caused by inhibiting Lyn activation with a specific inhibitor, PP1.²⁴⁾ In addition, Lyn-deficient mast cells also show a delayed $[Ca^{2+}]_i$ mobilization when compared with wild-type mast cells.²⁵⁾ In the present study, it was similarly observed that DBDI induces a significant time lag of $[Ca^{2+}]_i$ mobilization of BMMC at low concentrations. Collectively, it is suggested that DBDI may also suppress Lyn kinase activity in relation to initiation of mast cell signaling. These findings of the present study suggest that

DBDI acts as a potent inhibitor for IgE-mediated production of inflammatory chemical mediators *in vitro*.

However, DBDI itself induces a harmful effect such as convulsion in mice when 25 mg/kg of DBDI was administered by intravenous injection (data not shown), we therefore consider that administration of DBDI to experimental animals should be carefully performed for *in vivo* analyses of DBDI. It will be necessary that optimal dose and methods of DBDI administration into experimental animals are further examined in future investigations.

Acknowledgements This work was supported by grant from Ministry of Health, Labour, and Welfare and the Grants-in-Aid for Scientific Research from the Nihon University, and a grant from the Uehara Memorial Foundation.

REFERENCES

- Gould H. J., Sutton B. J., Beavil A. J., Beavil R. L., McCloskey N., Coker H. A., Fear D., Smurthwaite L., *Annu. Rev. Immunol.*, **21**, 579—628 (2003).
- Nadler M. J., Matthews S. A., Turner H., Kinet J. P., *Adv. Immunol.*, **76**, 325—355 (2000).
- Ozawa K., Szallasi Z., Kazanietz M. G., Blumberg P. M., Mischak H., Mushinski J. F., Beaven M. A., *J. Biol. Chem.*, **268**, 1749—1756 (1993).
- Zhang C., Baumgartner R. A., Yamada K., Beaven M. A., *J. Biol. Chem.*, **272**, 13397—13402 (1997).
- Liang A., *Zhongguo Zhong Yao Za Zhi*, **20**, 3—4 (1995).
- Okouchi T., Abe T., Araki S., Arai S., Iida T., Wang L. Y., Kitanaka S., Miyata S., *Bioorg. Med. Chem.*, **13**, 3847—3851 (2005).
- Wang L. Y., Wang N. L., Yao X. S., Miyata S., Kitanaka S., *J. Nat. Prod.*, **65**, 1246—1251 (2002).
- Asada A., Zhao Y., Kondo S., Iwata M., *J. Biol. Chem.*, **273**, 28392—28398 (1998).
- Blanco-Molina M., Tron G. C., Macho A., Lucena C., Calzado M. A., Munoz E., Appendino G., *Chem. Biol.*, **8**, 767—778 (2001).
- Wolfe P. C., Chang E. Y., Rivera J., Fewtrell C., *J. Biol. Chem.*, **271**, 6658—6665 (1996).
- Ra C., Furuichi K., Rivera J., Mullins J. M., Isersky C., White K. N., *Eur. J. Immunol.*, **19**, 1771—1777 (1989).
- Benhamou M., Gutkind J. S., Robbins K. C., Siraganian R. P., *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 5327—5330 (1990).
- Kawakami T., Inagaki N., Takei M., Fukamachi H., Coggeshall K. M., Ishizaka K., Ishizaka T., *J. Immunol.*, **148**, 3513—3519 (1992).
- Costello P. S., Turner M., Walters A. E., Cunningham C. N., Bauer P. H., Downward J., Tybulewicz V. L., *Oncogene*, **13**, 2595—2605 (1996).
- Wen R., Jou S. T., Chen Y., Hoffmeyer A., Wang D., *J. Immunol.*, **169**, 6743—6752 (2002).
- Hirasawa N., Scharenberg A., Yamamura H., Beaven M. A., Kinet J. P., *J. Biol. Chem.*, **270**, 10960—10967 (1995).
- Aketani S., Teshima R., Umezawa Y., Sawada J., *Immunol. Lett.*, **75**, 185—189 (2001).
- Hohman R. J., Hultsch T., Talbot E., *J. Immunol.*, **145**, 3876—3882 (1990).
- Beaven M. A., Rogers J., Moore J. P., Hesketh T. R., Smith G. A., Metcalfe J. C., *J. Biol. Chem.*, **259**, 7129—7136 (1984).
- Oka T., Sato K., Hori M., Ozaki H., Karaki H., *Br. J. Pharmacol.*, **135**, 1959—1966 (2002).
- Simon M., Vanes L., Geahlen R. L., Tybulewicz V. L., *J. Biol. Chem.*, **280**, 4510—4517 (2005).
- Currie S., Roberts E. F., Spaethe S. M., Roehm N. W., Kramer R. M., *Biochem. J.*, **304**, 923—928 (1994).
- Hirasawa N., Santini F., Beaven M. A., *J. Immunol.*, **154**, 5391—5402 (1995).
- MacGlashan D., Jr., Lavens-Phillips S., *J. Leukoc. Biol.*, **69**, 224—232 (2001).
- Nishizumi H., Yamamoto T., *J. Immunol.*, **158**, 2350—2355 (1997).