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Full paper

Suplatast tosilate alleviates nasal symptoms through the suppression of nuclear factor of activated T-cells-mediated IL-9 gene expression in toluene-2,4-diisocyanate-sensitized rats

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ABSTRACT

Histamine H_1 receptor (H1R) gene is upregulated in patients with pollinosis; its expression level is highly correlated with the nasal symptom severity. Antihistamines are widely used as allergy treatments because they inhibit histamine signaling by blocking H1R or suppressing H1R signaling as inverse agonists. However, long-term treatment with antihistamines does not completely resolve toluene-2,4-diisocyanate (TDI)-induced nasal symptoms, although it can decrease H1R gene expression to the basal level, suggesting additional signaling is responsible for the pathogenesis of the allergic symptoms. Here, we show that treatment with suplatast tosilate in combination with antihistamines markedly alleviates nasal symptoms in TDI-sensitized rats. Suplatast suppressed TDI-induced upregulation of IL-9 gene expression. Suplatast also suppressed ionomycin/phorbol-12-myristate-13-acetate-induced upregulation of IL-2 gene expression in Jurkat cells, in which calcineurin (CN)/nuclear factor of activated T-cells (NFAT) signaling is known to be involved. Immunoblot analysis demonstrated that suplatast inhibited binding of NFAT to DNA. Furthermore, suplatast suppressed ionomycin-induced IL-9 mRNA upregulation in RBL-2H3 cells, in which CN/NFAT signaling is also involved. These data suggest that suplatast suppressed NFAT-mediated IL-9 gene expression in TDI-sensitized rats and this might be the underlying mechanism of the therapeutic effects of combined therapy of suplatast with antihistamine.

1. Introduction

Pollinosis is a seasonal allergic rhinitis caused by hypersensitivity to tree or grass pollens and affects >36 million people in the United States and approximately 30% of the Japanese population (1, 2). It is characterized by paroxysmal repetitive sneezing, watery rhinorrhea, and nasal blockage. Histamine is a major chemical mediator of the allergic reaction, especially of the early-phase reaction; its action is mainly mediated through the activation of histamine H_1 receptor (H1R).

Previously, we demonstrated that H1R gene expression is strongly correlated with the allergic symptom severity in patients with pollinosis (3) and that suppression of the upregulation of H1R gene expression alleviated allergic symptoms in toluene-2,4-diisocyanate (TDI)-sensitized rats (4–7). Furthermore, we showed that histamine and phorbol-12-myristate-13-acetate (PMA) stimulation increased H1R at both the mRNA and protein levels through H1R activation in HeLa cells endogenously expressing H1R (8), and

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the protein kinase C-δ (PKCδ) signaling pathway was involved in PMA-induced upregulation of HIR gene expression in HeLa cells (9). Consequently, antihistamines are widely employed as the first-line treatment for nasal symptoms of allergic rhinitis because they inhibit histamine signaling by blocking H1R or suppressing H1R signaling as inverse agonists. However, we found that long-term treatment with antihistamines alone could not completely resolve TDI-induced nasal symptoms, although it decreased TDI-induced upregulation of H1R gene expression to the basal level (10), suggesting that other types of signaling are also responsible for the pathogenesis of the allergic symptoms.

Suplatast tosilate (IPD-1151T) is an immunomodulator that curbs eosinophil infiltration, IgE production, and allergic inflammation by suppressing the IL-4 and IL-5 production (11, 12). Previously, we reported that treatment with suplatast alleviated TDI-induced nasal symptoms and H1R and IL-4 mRNA elevation in TDI-sensitized rats although suplatast did not curb histamine-induced upregulation of H1R gene expression in HeLa cells (5). Thus, it is probable that the anti-allergic activity of suplatast is not through PKCδ signaling suppression but through an unknown signaling pathway suppression, and uncovering the unknown signaling pathway could result in a new strategy for overcoming the pathogenesis of this allergy.

In the present study, we investigated suplatast effects on allergic symptoms in combination with antihistamines that suppress PKCδ signaling. We found that combined suplatast and antihistamine treatment markedly suppressed nasal symptoms compared with treatment with single drug. We also identified the previously unknown signaling pathway suppressed by suplatast as being calcineurin (CN)/nuclear factor of activated T-cells (NFAT) signaling. These data suggest the molecular mechanism of suplatast in the combined therapy with antihistamine is the suppression of NFAT-mediated IL-9 gene expression.

2. Materials and methods

2.1. Materials

Suplatast and its major metabolite M-1 were gifts from Taiho Pharmaceutical and Sawai Pharmaceutical (Osaka). Monoclonal anti-dinitrophenyl (DNP) IgE (clone SPE-7) and minimum essential medium (MEM; with Earle's salts, L-glutamine, and nonessential amino acids without $NaHCO_3$) were from Sigma–Aldrich (St. Louis, MO, USA). RPMI-1640 medium was from Gibco BRL Life Technology (Grand Island, NY, USA), and RNAiso Plus, PrimeScript RT Reagent Kit, and Premix ExTaq were from Takara Bio Inc. (Kyoto). All other chemicals were of analytical grade.

2.2. Toluene-2,4-diisocyanate-sensitized rats experiments

Six-week-old male Brown Norway rats (200–250 g; Japan SLC, Hamamatsu) were used in this experiment. They were allowed free access to water and food and were kept in a room at 25 °C ± 2 °C and 55% ± 10% humidity with a 12-h light–dark cycle. They were divided into three groups of four rats each: a control group, a group sensitized with TDI (Wako Pure Chemical Industries Ltd., Tokyo), and a test group. Sensitization with TDI was performed by the method described by Shahriar et al. (Fig. 1) (5). In brief, 10 μl of a 10% solution of TDI in ethyl acetate was applied bilaterally on the nasal vestibule of each rat once a day for five consecutive days. This sensitization procedure was then repeated after 2 days. Nine days after the second sensitization, TDI solution was again applied to the nasal vestibule to provoke nasal symptoms. The control group was sensitized and provoked with 10 μl of ethyl acetate using the same procedure. Epinastine (24 mg/kg/day) and/or suplatast (80 mg/kg/day) were administered orally once a day for 2 weeks, starting 7 days after the first sensitization with TDI (Fig. 1). In accordance with the findings from our previous studies, we used these doses (80% of the dose that we used in our previous studies) to produce a clear combination effect of these two drugs. TDI-induced nasal symptoms were measured over the 10-min period immediately after TDI provocation, including the number of sneezes and the nasal score, which takes into account the extent of watery rhinorrhea, swelling, and redness, measured on a scale ranging from zero to three (Table 1). All experimental procedures were performed in accordance with the guidelines of the Animal Research Committee of Tokushima University.

2.3. Cell culture

Jurkat cells were cultured at 37 °C under a humidified 5% CO_2/95% air atmosphere in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 120 IU/mL penicillin (Sigma–Aldrich) and 120 μg/mL streptomycin (Sigma–Aldrich). RBL-2H3 cells were cultured in MEM containing 10% FBS, 120 IU/mL penicillin, and 120 μg/mL streptomycin.

2.4. Real-time quantitative reverse transcription polymerase chain reaction

Jurkat cells and RBL-2H3 cells cultured to 70% confluency in six-well dishes were treated with suplatast (36 h) or M-1 (24 h) before ionomycin (1 μM) + PMA (50 nM) (for Jurkat cells) or ionomycin (1 μM for RBL-2H3 cells) stimulation. After a 6-h (for Jurkat cells) or 2-h (for RBL-2H3 cells) treatment, the cells were harvested and total RNA was prepared using RNAiso Plus. For the IgE stimulation experiments, RBL-2H3 cells were stimulated with 100 ng/mL of anti-DNP IgE for 4 h. To determine the mRNA in the rats, the nasal mucosa was collected in RNAlater 4 h after provocation with TDI and stored at −80 °C until used. The nasal mucosa was homogenized using a Polytron (Model PT-K; Kinematica AG, Littau/Luzern, Switzerland) in 10 volumes of RNAiso Plus. The RNA samples were reverse transcribed to cDNA using a PrimeScript RT Reagent Kit. TaqMan primers and the probe were designed using Primer Express (Applied Biosystems, Foster City, CA, USA). Real-time PCR was conducted using a GeneAmp 7300 Sequence Detection System (Applied Biosystems). The sequences of the primers and TaqMan probe are listed in Table 2. To standardize the starting material, endogenous control human GAPDH and rodent GAPDH control reagents (Applied Biosystems) were used, and data were expressed as a ratio of GAPDH mRNA.

2.5. Preparation of nuclear DNA-bound proteins

Nuclear DNA binding proteins were isolated using the subcellular protein fractionation kit (Thermo Fisher Scientific, Foster City, CA, USA) according to the manufacture's instructions. Briefly, Jurkat cells cultured to 70% confluency in 100 mm dishes were treated with M-1 (24 h) or cyclosporin A (1 h) before ionomycin (1 μM)/PMA (50 nM) stimulation. After a 30-min treatment with ionomycin/PMA, the cells were harvested and dissolved in cytoplasmic extraction buffer provided by the kit, then, cytoplasmic, membrane, nuclear soluble, nuclear DNA-bound, and cytoskeletal protein extracts were fractionated. The nuclear DNA-bound proteins were separated by 12% SDS-PAGE. Immunoblot analysis was conducted using NFATc1 antibody (sc-13033, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Goat anti-rabbit IgG (H+L)-HRP conjugate (Bio-Rad, Richmond, CA, USA) was used as the secondary antibody, and proteins were visualized with an Immobilon Western Chemiluminescent HRP substrate (Merck Millipore, Billerica, MA, USA).

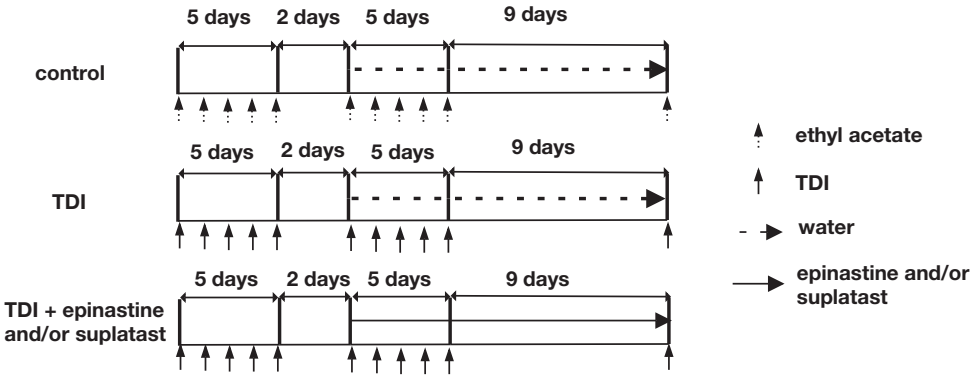

Fig. 1. Experimental protocol. The rats were sensitized by intranasal application of 10 μl of 10% toluene-2,4-diisocyanate (TDI) in ethyl acetate for 2 weeks. After 1 week, the nasal symptoms were further provoked using 10 μl of 10% TDI. The control group was sensitized with ethyl acetate only. Epinastine (24 mg/kg/day) and/or suplatast (80 mg/kg/day) were administered orally once a day for 2 weeks starting 7 days after the first sensitization with TDI. The drugs were administered 1 h before the rats were treated with TDI.

Table 1
Criteria for grading the severity of toluene-2,4-diisocyanate-induced nasal responses in rats.

Nasal response	Score			
	0	1	2	3
Watery rhinorrhea	–	At the nostril	Between 1 and 3	Drops of discharge from the nose
Swelling and redness	–	Slightly swollen	Between 1 and 3	Strong swelling and redness

Table 2
Nucleotide sequences for the primers and probes used in this study.

Primer/probe name	Sequence
Rat IL-5 mRNA	
Sense primer	5′-CAGTGGTGAAAGAGACCTTGATACAG-3′
Anti-sense primer	5′-GAAGCCTCATCGTCTCATTGC-3′
Probe	FAM-TGTCACTCACCGAGCTCTGTTGACG-TAMRA
Rat IL-9 mRNA	
Sense primer	5′-GACGACCCATCATCAAAATGC-3′
Anti-sense primer	5′-CTGTGACATTCCCTCCTGGAA-3′
Probe	FAM-TTGTGCCTCCCCATCCCATCTGAT-TAMRA

Rat IL-13 mRNA and human IL-2 mRNA were determined using TaqMan gene amplification primers and probes (Rn00587615_m1 for IL-13 and Hs00174114_m1 for IL-2; Applied Biosystems).

2.6. Statistical analysis

The results are shown as the mean ± S.E.M. Statistical analysis was performed using unpaired *t*-tests or ANOVA with Dunnett's test using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of epinastine and/or suplatast on TDI-induced nasal symptoms in TDI-sensitized rats

Application of TDI for 3 weeks caused nasal symptoms in TDI-sensitized rats (Fig. 2). As reported previously (10), pretreatment with epinastine alone significantly reduced TDI-induced sneezing and the nasal score. Pretreatment with suplatast alone also significantly reduced nasal symptoms but was less effective than epinastine. Combined treatment with epinastine and suplatast markedly reduced TDI-induced sneezing and the nasal score (Fig. 2). The control rats showed no nasal symptoms after TDI provocation.

3.2. Effect of suplatast on TDI-induced upregulation of Th2 cytokine gene expression

Previously, we demonstrated that suplatast alleviated nasal symptoms by inhibiting histamine signaling in TDI-sensitized rats through the suppression of histamine- and IL-4-induced H1R gene expression by the inhibitions of HDC and IL-4 gene transcriptions, respectively (5). Thus, we investigated the effect of suplatast on TDI-induced upregulation of other Th2 cytokine gene expressions in TDI-sensitized rats. In addition to IL-4 gene, suplatast significantly suppressed TDI-induced upregulation of IL-5, IL-9, and IL-13 gene expressions (Fig. 3).

3.3. Effect of suplatast on ionomycin/PMA-induced upregulation of IL-2 gene expression in Jurkat cells

As CN/NFAT signaling was involved in the regulation of Th2 cytokine gene expression (13–15), we investigated the effect of suplatast on CN/NFAT signaling. Previous studies established that CN/NFAT signaling is involved in IL-2 gene expression in T-cells, in which NFAT activates IL-2 transcription in concert with AP-1 (14, 15). Therefore, to identify the signaling suppressed by suplatast, we examined the effect of suplatast on ionomycin/PMA-induced upregulation of IL-2 gene expression in Jurkat cells. Treatment with ionomycin/PMA increased IL-2 mRNA expression in Jurkat cells (Fig. 4). This ionomycin/PMA-induced IL-2 mRNA elevation was significantly suppressed by inhibitor of NFAT-CN association-6 (INCA-6) and cyclosporin A (Fig. 4), which are both known to inhibit CN/NFAT signaling (13, 16, 17), confirming the involvement of CN/NFAT signaling. Pretreatment with suplatast or its major metabolite M-1 (18) suppressed ionomycin/PMA-induced upregulation of IL-2 mRNA expression in a dose- and time-dependent manner (Fig. 4). Our time-course studies also suggested the possibility that suplatast is a “prodrug” and that its metabolite M-1 may be the active form (Fig. 4A and C).

3.4. Effect of suplatast on ionomycin-induced upregulation of IL-9 gene expression in RBL-2H3 cells

IgE alone activates several signaling pathways that lead to cytokine production, including IL-4 and IL-13 (19). We found that stimulation with IgE increased IL-9 gene expression in RBL-2H3 cells (Fig. 5A). As it was reported that stimulation with monomeric IgE increased intracellular Ca^{2+} concentration (20, 21), we

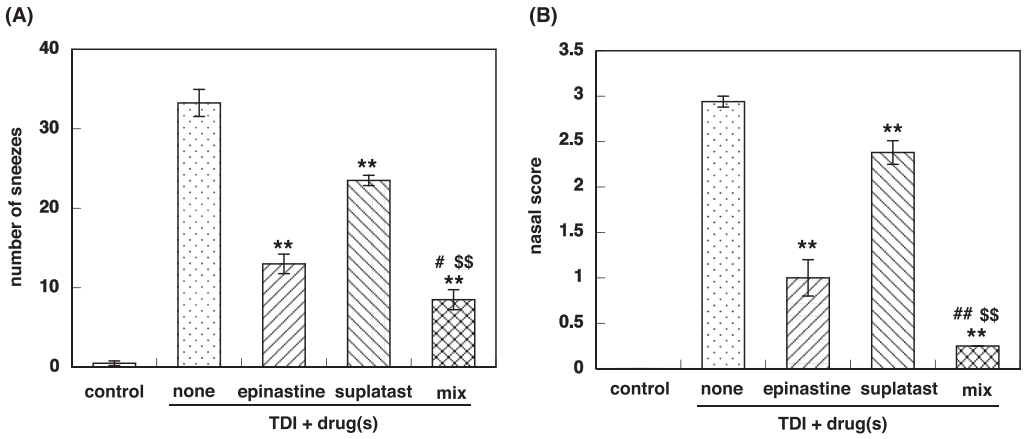

Fig. 2. Effect of epinastine and/or suplatast on toluene-2,4-diisocyanate (TDI)-induced nasal symptoms in TDI-sensitized rats. The rats were sensitized and provoked as described in the Materials and Methods. The numbers of sneezes (**A**) and the nasal score (**B**) were measured over the 10-min period immediately after TDI provocation. The data are expressed as means ± S.E.M. ($n = 4$). $^{**}p < 0.01$ vs. TDI; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. epinastine; $^{\$\$}p < 0.01$ vs. suplatast.

investigated the effect of ionomycin on IL-9 gene expression in RBL-2H3 cells. A 2-h treatment with 1 μM ionomycin increased IL-9 mRNA expression in RBL-2H3 cells. This IL-9 mRNA elevation was significantly suppressed by cyclosporin A and INCA-6 (Fig. 5B and C), suggesting the involvement of CN/NFAT signaling. Pre-treatment with suplatast or M-1 suppressed IL-9 mRNA elevation in a dose- and time-dependent manner (Fig. 5D–G). These data suggest that suplatast and M-1 suppress IL-9 gene upregulation by inhibiting CN/NFAT signaling in IL-9 gene expression in RBL-2H3 cells.

3.5. *Effect of suplatast on CN/NFAT signaling in Jurkat cells*

In CN/NFAT signaling, NFAT is activated through its dephosphorylation by CN. Activated NFAT, then, translocated into the nucleus, where they can cooperate with multiple transcriptional partners such as AP-1 to regulate gene expression. We investigated the effect of suplatast/M-1 on dephosphorylation of NFAT and subsequent its nuclear translocation. Suplatast or M-1 did not inhibit NFAT dephosphorylation by CN (data not shown). They did not suppress ionomycin-induced NFAT translocation into the nucleus in RBL-2H3 and Jurkat cells, either (data not shown). These data suggest the possibility that suplatast/M-1 inhibit binding of NFAT to the promoter DNA. As described above, our data suggest that suplatast is a “prodrug” and M-1 may be the active form. So, we, next, investigated the effect of suplatast/M-1 on the binding of NFAT to DNA using M-1. We obtained the chromatin-bound nuclear extract from PMA/ionomycin-stimulated Jurkat cells using the subcellular protein fractionation kit. This fraction contains nuclear proteins bound to chromatin in response to PMA/ionomycin stimulation. Because M-1 did not inhibit activation or nuclear translocation of NFAT, NFAT should be recovered in this fraction and immunoblot analysis of this fraction using anti-NFAT antibody can assess if M-1 inhibits DNA binding of NFAT. Immunoblot analysis after subcellular protein fractionation demonstrated that M-1 inhibited DNA binding of NFAT (Fig. 6).

4. Discussion

In the present study, we showed that treatment with suplatast in combination with antihistamines markedly alleviates nasal symptoms in TDI-sensitized rats. We demonstrated that the expression level of the H1R gene is highly correlated with the severity of the allergic symptoms in patients with pollinosis (3) and that PKCδ signaling is involved in histamine-induced upregulation of H1R gene expression in HeLa cells (8, 9). However, suppression of

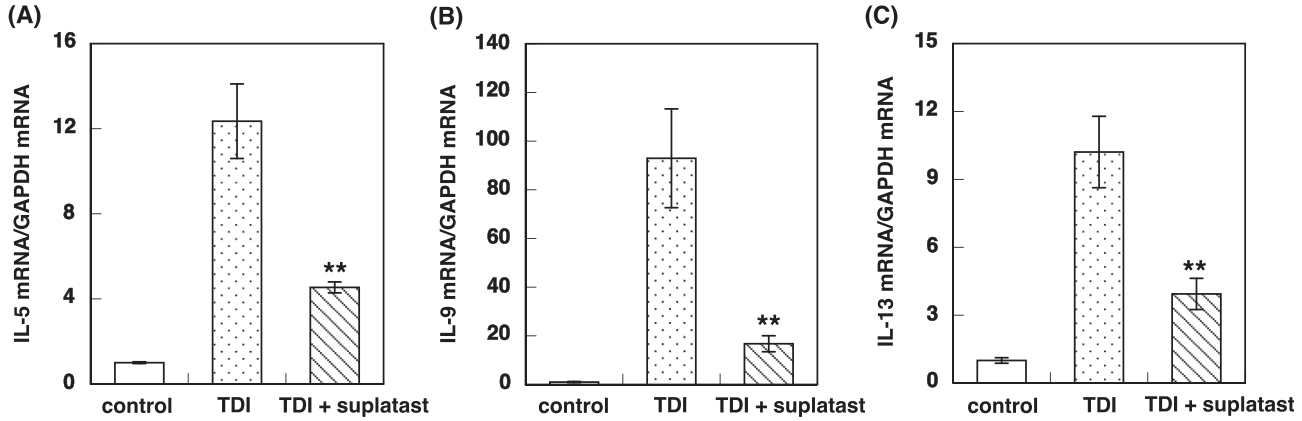

Fig. 3. Effect of suplatast on toluene-2,4-diisocyanate (TDI)-induced upregulation of Th2 cytokine gene expression in TDI-sensitized rats. The rats were sensitized and provoked as described in the Materials and Methods. The rats were sacrificed 4 h after provocation with TDI, and total RNA was isolated. The mRNA levels of IL-5 (**A**), IL-9 (**B**), and IL-13 (**C**) were determined by real-time quantitative RT-PCR. The data are expressed as means ± S.E.M. ($n = 4$). $^{**}p < 0.01$ vs. TDI.

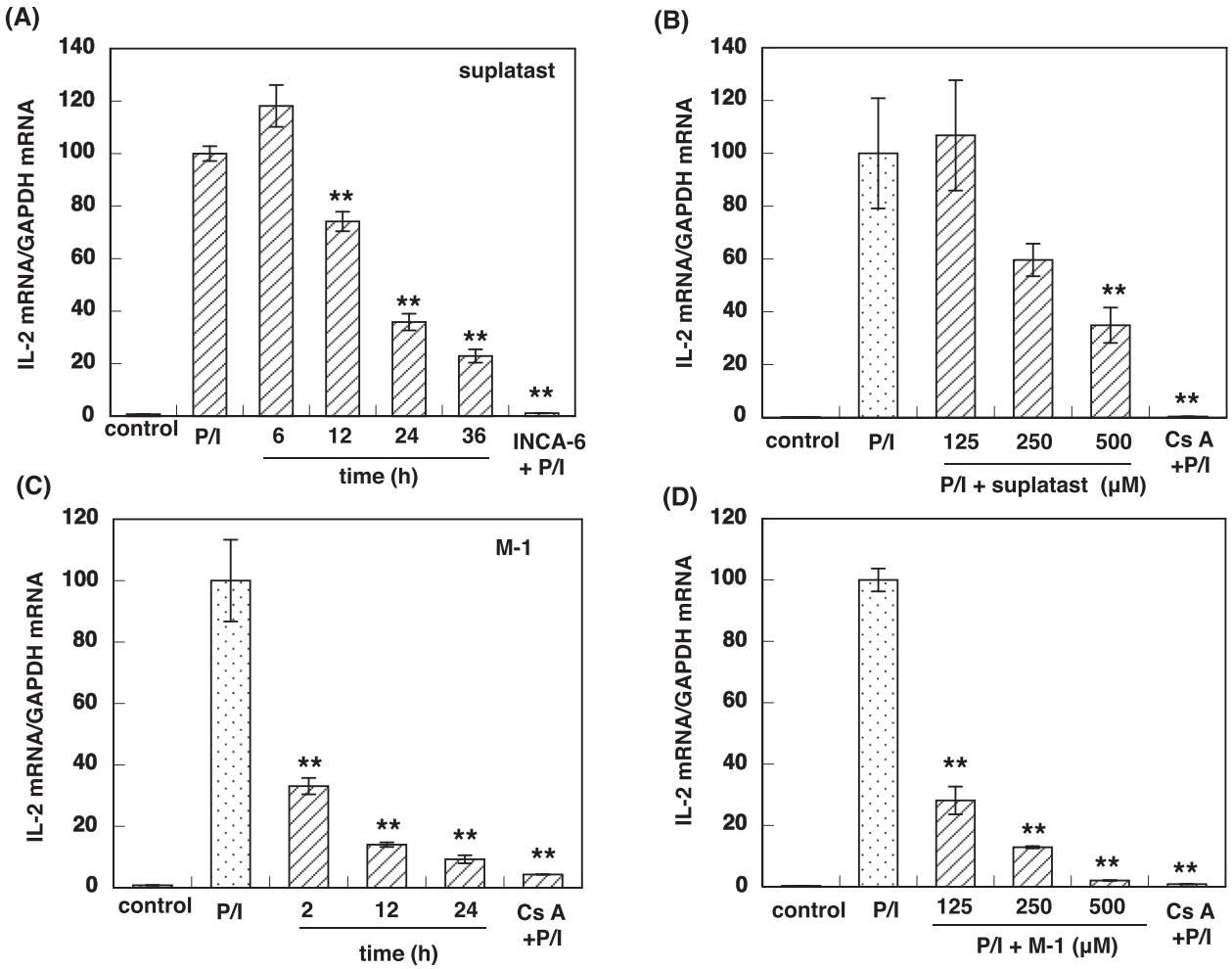

Fig. 4. Effect of suplatast (**A** and **B**) and its metabolite M-1 (**C** and **D**) on ionomycin/phorbol-12-myristate-13-acetate (PMA)-induced upregulation of IL-2 gene expression in Jurkat cells. In **A** and **C**, Jurkat cells were treated with 500 μM of suplatast (**A**) or M-1 (**B**) at the given time intervals before ionomycin/PMA stimulation. In **B** and **D**, Jurkat cells were treated with various concentrations of suplatast (**B**) or M-1 (**D**) 36 h (suplatast) or 24 h (M-1) before ionomycin/PMA stimulation. After 6 h of stimulation, the Jurkat cells were harvested, and IL-2 mRNA was determined by real-time quantitative RT-PCR. As a positive control, Jurkat cells were treated with 50 μM of inhibitor of NFAT-CN association-6 (INCA-6) (**A**) or 0.5 μM of cyclosporin A (**B**–**D**) 1 h before ionomycin/PMA stimulation. CsA, cyclosporin A; P/I, PMA/ionomycin. The data are expressed as means ± S.E.M. ($n = 3$–4). $^{**}p < 0.01$ vs. ionomycin/PMA.

PKCδ signaling by antihistamines failed to completely alleviate the nasal symptoms in TDI-sensitized rats, suggesting the existence of additional signaling responsible for the pathogenesis of the nasal symptoms.

In addition to histamine, Th2 cytokines, including IL-4, IL-5, IL-9, and IL-13, also play important roles in the pathogenesis of allergic inflammation (22). Further, the existence and importance of crosstalk of histamine with Th2 cytokines in allergic inflammation have also been reported, in which histamine influences the expression and action of Th2 cytokines; Th2 cytokines, in turn, modulate the production and release of histamine (23, 24). We demonstrated that prophylactic treatment with antihistamines suppressed TDI-induced upregulation of both H1R and IL-4 mRNAs in TDI-sensitized rats (10). We also showed that direct administration of IL-4 into the nasal cavity of normal non-TDI-treated rats upregulated the expression of H1R mRNA. Moreover, intranasal histamine application caused an increase in IL-4 mRNA elevation in the normal rats (5). In addition, we showed that the H1R expression level was strongly correlated with that of IL-5 in patients with pollinosis (25). These findings suggest that gene expressions of H1R and Th2 cytokines are correlated and suppression of H1R gene expression could affect the expression level of the Th2 cytokines. In TDI-sensitized rats, effects of suplatast and/or epinastine on the nasal symptoms did not completely parallel with that on gene expression. This could be the reason why we can see the strong suppression of symptoms even if suppression of mRNA expression in each protein is moderate.

Suplatast tosilate inhibits the production of Th2 cytokines such as IL-4 and IL-5 (11, 12). Previously, we demonstrated that suplatast suppressed TDI-induced upregulation of HDC gene expression in TDI-sensitized rats, which decreased histamine synthesis and resulted in the suppression of H1R mRNA elevation (5). Suplatast also inhibited TDI-induced histamine release in the nasal lavage fluid. In addition, suplatast inhibited IL-4-induced upregulation of H1R gene expression although it did not directly suppress PKCδ signaling (5). Here, we have shown that suplatast strongly suppressed TDI- or ionomycin-induced upregulation of IL-9 gene expression in TDI-sensitized rats (Fig. 3B) or in RBL-2H3 cells (Fig. 5D and E), respectively. IL-9 is a pleiotropic cytokine produced by activated helper T cells and activated mast cells (26–28), and it promotes Th2-specific allergic responses, allergic inflammation, and asthmatic symptoms (29, 30). Immunohistochemical analysis showed stimulation with TDI induced infiltration of rat mast cell protease-2 positive mast cells (data not shown), suggesting

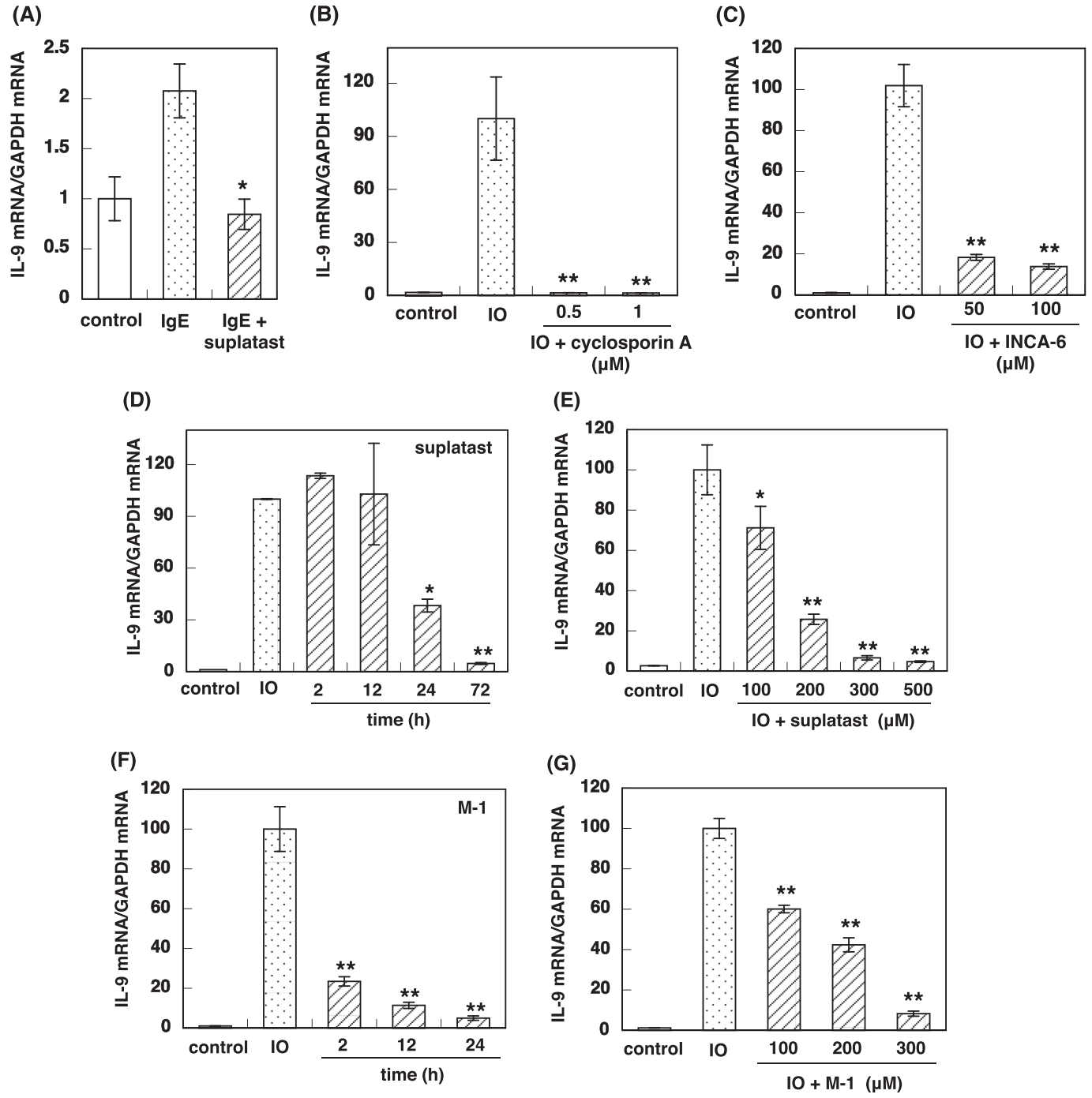

Fig. 5. Effect of suplatast and its metabolite M-1 on monomeric IgE-stimulated (**A**) or ionomycin-induced (**B**–**G**) upregulation of IL-9 gene expression in RBL-2H3 cells. (**A**) RBL-2H3 cells were stimulated with 100 ng/mL of anti-DNP IgE. After 4 h of stimulation, the cells were harvested, and IL-9 mRNA was determined by real-time quantitative RT-PCR. RBL-2H3 cells were treated with suplatast (500 μM) 72 h before IgE stimulation. In **B** and **C**, RBL-2H3 cells were treated with 0.5 μM of cyclosporin A (**B**) or 50 μM of inhibitor of NFAT-CN association-6 (INCA-6) (**C**) 1 h before ionomycin stimulation. In **D** and **F**, RBL-2H3 cells were treated with 500 μM of suplatast (**D**) or 300 μM of M-1 (**F**) at the given time intervals before ionomycin stimulation. In **E** and **G**, RBL-2H3 cells were treated with various concentrations of suplatast (**E**) or M-1 (**G**) 72 h (for suplatast) or 24 h (for M-1) before ionomycin stimulation. After 2 h of stimulation, the RBL-2H3 cells were harvested, and IL-9 mRNA was determined by real-time quantitative RT-PCR. IO, ionomycin. The data are expressed as the mean ± S.E.M. ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. ionomycin.

increase in local IL-9 mRNA level in TDI-sensitized rats. As IL-9 upregulates IL-4, IL-5, and IL-13 (31), suppression of IL-9 expression by suplatast may affect the expression levels of these Th2 cytokines. These findings suggest that the suppressive effect of suplatast on the nasal symptoms is partially due to the indirect suppression of PKCδ signaling, however, most of the effect is derived from the suppression of the signaling responsible for Th2 cytokine gene expression (Fig. 4).

CN/NFAT signaling plays a central role in inducible gene transcription during immune response (32, 33). NFAT was first identified as a factor that binds to the distal antigen-receptor response element of the human IL-2 promoter (34–36). In

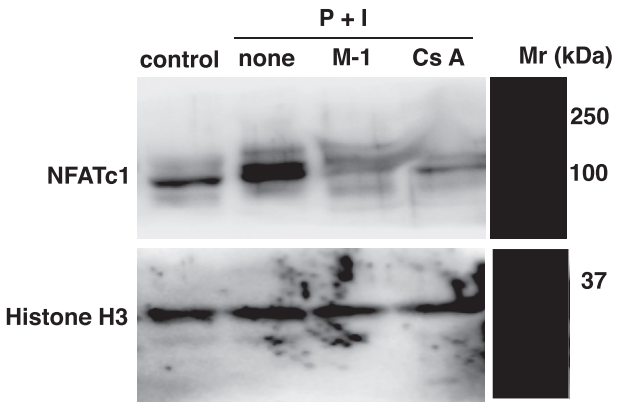

Fig. 6. Effect of M-1 on PMA/ionomycin-induced DNA binding of activated NFAT. Jurkat cells were treated with M-1 (24 h) or cyclosporin A (1 h) before ionomycin (1 μM)/PMA (50 nM) stimulation. After a 30-min treatment, the cells were harvested and nuclear DNA-bound protein extracts were prepared using the subcellular protein fractionation kit. The proteins were separated by 12% SDS-PAGE and immunoblot analysis was performed using NFATc1 antibody. P + I, PMA + ionomycin.

addition, it has also been reported that NFAT binds to the promoter region of many cytokines, including IL-4, IL-5, and IL-13 (13–15). As suplatast suppressed TDI-induced upregulation of Th2 cytokine gene expression in our previous study, we speculate that the suppressive effect of suplatast is through the suppression of CN/NFAT signaling. Suplatast suppressed ionomycin/PMA-induced upregulation of IL-2 gene expression in Jurkat cells. NFAT and AP-1 have been reported to cooperatively regulate transcription of the IL-2 gene (14, 15). As suplatast does not suppress histamine-induced upregulation of H1R gene expression in HeLa cells, in which the activation of AP-1 is indispensable for gene transcription (5, 9), it is unlikely that suplatast suppresses the activation of c-Fos, one of the subunits of AP-1, in Jurkat cells. To understand the mechanism of action of suplatast, we investigated the effect of suplatast on CN/NFAT signaling in Jurkat cells. Suplatast/M-1 did not inhibit dephosphorylation of NFAT by CN and subsequent its nuclear translocation (data not shown). However, immunoblot analysis using M-1 after subcellular protein fractionation demonstrated that M-1 inhibited binding of NFAT to DNA (Fig. 6). This data strongly suggest that suplatast/M-1 suppress CN/NFAT signaling although it is not clear, so far, if suplatast inhibits binding of NFAT to DNA directly or through modification of chromatin architecture. It was reported that combination with suplatast could reduce the dose or even negate the need for using tacrolimus ointment, which inhibits CN/NFAT signaling (37). Data that suplatast inhibits CN/NFAT signaling may explain why suplatast can use as a substitute for tacrolimus in treating atopic dermatitis.

Although involvement of CN/NFAT signaling in IL-9 gene expression has not been fully elucidated, our data suggest that the CN/NFAT signaling pathway is possibly involved in ionomycin-induced upregulation of IL-9 gene expression in RBL-2H3 cells. Recently, it was reported that NFAT and NF-κB synergistically enhance IL-9 transcription in $CD4^+$ cells, during which NFAT creates an accessible chromatin platform for the recruitment of NF-κB onto the IL-9 promoter (38). Although we cannot exclude the additional involvement of NF-κB in the ionomycin-induced IL-9 upregulation in RBL-2H3 cells, the suppressive effect of suplatast may be exerted through the inhibition of CN/NFAT signaling.

These data suggest that suplatast suppressed NFAT-mediated IL-9 gene expression in TDI-sensitized rats and this might be the underlying mechanism of the therapeutic effects of combined therapy of suplatast with antihistamine.

Conflicts of interest

N.O. is an employee of Taiho Pharmaceutical Co. Ltd. The other authors declare no financial conflicts of interest.

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