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Inhibitory Effect of a Potent and Selective Cytosolic Phospholipase $A_2\alpha$ Inhibitor RSC-3388 on Skin Inflammation in Mice

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Key Words

Cytosolic phospholipase $A_2\alpha$ · Prostaglandin · Leukotriene · Cytokine · Chemokine · 2,4,6-Trinitro-1-chlorobenzene · *Dermatophagoides farinae*

Abstract

Cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) preferentially hydrolyzes membrane phospholipids containing arachidonic acid, resulting in the biosynthesis of eicosanoids such as prostaglandins and leukotrienes. To examine the contribution of $cPLA_2\alpha$ to skin inflammation, we evaluated the effect of (E)-N-[(2S,4R)-4-[N-(biphenyl-2-ylmethyl)-N-2-methylpropylamino]-1-[2-(2,4-difluorobenzoyl)benzoyl]pyrrolidin-2-yl]methyl-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl) phenyl]acrylamide (RSC-3388), a potent and selective $cPLA_2\alpha$ inhibitor, on 2,4,6-trinitro-1-chlorobenzene (TNCB)-induced ear inflammation and mite antigen-induced dermatitis in mice. Topical application of RSC-3388 showed a significant inhibitory activity against TNCB-induced ear swelling and eicosanoid production in mice. Comprehensive expression analysis using Gene-Chip technology and subsequent experiments concerning mRNA and protein expression demonstrated that RSC-3388 clearly reduced the levels of interleukin-1β, macrophage inflammatory protein-1α (MIP-1α) and MIP-1β in a TNCB-induced mouse model. In addition, RSC-3388 ointment significantly alleviated atopic dermatitis-like skin lesions induced by repeated application of mite antigen. Furthermore, increased expression of $cPLA_2\alpha$, assessed by anti-phospho-$cPLA_2\alpha$ antibody, was observed in the skin lesions of mite-antigen-induced dermatitis. These results indicate that $cPLA_2\alpha$ is involved in the development of skin inflammation in mice, and RSC-3388 is expected to be useful for the treatment of inflammatory skin disorders such as atopic dermatitis.

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Introduction

Cytosolic phospholipase A_2 ($cPLA_2$) is a member of a diverse superfamily of PLA_2 enzymes including secretory PLA_2, calcium-independent PLA_2 and other members. $cPLA_2$s are classified into at least four isoforms, α, β, γ and δ [1–4]. Among these $cPLA_2$s, $cPLA_2\alpha$ is thought to mainly contribute to the biosynthesis of eicosanoids, including prostaglandins (PGs) and leukotrienes (LTs), by releasing arachidonic acid from cell membranes, because it has been shown that the ability of $cPLA_2\beta$ and δ to release arachidonic acid is much lower than that of $cPLA_2\alpha$ [2–4]. Several studies using $cPLA_2\alpha$-deficient mice have indicated that this enzyme is involved in various inflammatory conditions, such as allergen-induced airway inflammation, acute lung injury, and brain ischemia/reperfusion injury [5–8].

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Atopic dermatitis is a chronic inflammatory skin disease accompanied by severe pruritus. The levels of eicosanoids such as PGE_2 and LTB_4 are elevated in the skin lesions of patients with atopic dermatitis, as compared to nonlesional skin in patients and healthy volunteers [9]. It has also been demonstrated that PGE_2 induces not only vasodilation, but also pruritus in patients with atopic dermatitis [10]. Furthermore, it is suggested that LTB_4 is involved in keratinocyte hyperproliferation, which is an important phenomenon in the pathogenesis of atopic dermatitis [11–13]. Therefore, $cPLA_2\alpha$ inhibitors, which inhibit the biosynthesis of both PGs and LTs, are promising candidates as agents for attenuating inflammation in atopic dermatitis.

Recently, we have discovered nonpeptide and low-molecular-weight inhibitors of $cPLA_2\alpha$ [14]. (E)-N-[(2S,4R)-4-[N-(biphenyl-2-ylmethyl)-N-2-methylpropylamino]-1-[2-(2,4-difluorobenzoyl)benzoyl]pyrrolidin-2-yl]methyl-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)phenyl]acrylamide (RSC-3388) is one of the $cPLA_2\alpha$ inhibitors established in our laboratories, which has potent inhibitory activity in in vitro enzyme and cellular assays. In the present study, to clarify the role of $cPLA_2\alpha$ in skin inflammation, we examined the effect of RSC-3388 in models of chronic atopic dermatitis induced by hapten or mite antigen in mice. Topical application of RSC-3388 exhibited an anti-inflammatory effect on chronic dermatitis in mice, through suppressing eicosanoid production and reducing cytokine and chemokine levels in the skin, indicating that $cPLA_2\alpha$ contributes to atopic dermatitis-like skin lesions in mice. These results suggested that RSC-3388 is useful for the treatment of skin inflammation, such as atopic dermatitis.

Materials and Methods

Mice

Female Balb/c and NC/Nga mice were purchased from Charles River Japan (Yokohama, Japan). These mice were all housed under conditions of controlled temperature (20–26°C), humidity (30–70%) and lighting (lights on from 08:00 to 20:00 h). Food and tap water were provided ad libitum. Balb/c mice were used at 6 weeks of age and NC/Nga mice at 13 weeks of age. The animal experiments in this study were approved by the Animal Care and Use Committee of Shionogi Research Laboratories.

Drugs and Chemicals

RSC-3388 was synthesized in our laboratories. Other drugs and chemicals used were as follows: indomethacin and acetone (Sigma-Aldrich, St. Louis, Mo., USA), 2,4,6-trinitro-1-chlorobenzene (TNCB; Tokyo Kasei, Tokyo, Japan), tacrolimus ointment (Protopic ointment 0.1%; Fujisawa Pharmaceutical, Osaka, Japan) and ibuprofen picnol ointment (Staderm ointment 5%; Torii Pharmaceutical, Tokyo, Japan).

Mites

Dermatophagoides farinae (Df) were cultured, and mite bodies were separated from the feces and culture media by flotation on a saturated water solution of sodium chloride [15]. Afterwards, Df body (Dfb) extract was purified according to the method of Yamaguchi et al. [16]. In brief, floating mites were pooled, and collected mite bodies were homogeneously suspended in phosphate-buffered saline (PBS) using an agate mortar and pestle. The suspension was stirred overnight, followed by centrifugation. The supernatant was dialyzed and lyophilized. The freeze-dried extracts were mixed with hydrophilic petrolactum (Maruishi, Osaka, Japan) at a concentration of 10 mg Dfb extract/g hydrophilic petrolatum, referred to as Dfb ointment.

TNCB-Induced Ear Inflammation Model

TNCB-induced ear inflammation was elicited according to the method described by Kitagaki et al. [17], with slight modifications. Briefly, TNCB was dissolved in acetone (0.5%), and 10 μl of solution was repeatedly applied to both the inner and outer surface of each ear in Balb/c mice. TNCB application was performed on days 0, 7, 9, 11, 14, 16 and 18. To assess the effect of RSC-3388, 0.3, 1 and 3% RSC-3388 dissolved in acetone in a volume of 10 μl of solution, or 3% RSC-3388 ointment, tacrolimus ointment and ibuprofen picnol ointment in a weight of 100 mg were applied to both the inner and outer surface of each ear twice daily (tacrolimus ointment and ibuprofen picnol ointment once daily) from day 7 to 20, respectively. Ear thickness was measured just before each TNCB application and on day 21 with a dial thickness gauge (SM-112; Teclock, Nagano, Japan).

On day 21, the mice were sacrificed. Each ear was fixed with 10% neutral formalin, embedded in paraffin, and sectioned at 4 μm. Sections were stained with hematoxylin-eosin. Other skin tissue was used for the measurement of eicosanoid levels. The skin tissue extracts for eicosanoid enzyme immunoassay were prepared as described by Kuwabara et al. [18]. Briefly, the skin samples were homogenized with 80% cold ethanol, then PGE_2 and LTB_4 in the supernatants were extracted using Sep-pak C18 column (Waters, Milford, Mass., USA). Then, PGE_2 and LTB_4 levels in the eluate were quantified by eicosanoid enzyme immunoassay kits (Cayman Chemical, Michigan, Ill., USA) according to the manufacturer's instructions.

Gene Expression Analysis and Quantitative PCR

TNCB was applied on days 0, 7, 9, 11, 14 and 16, and drugs were administered twice daily from day 7 to 17. The ear tissue was excised at 6 (day 16), 24 (day 17) and 48 h (day 18) after TNCB challenge. Total RNA was extracted from each ear tissue using Isogen (NipponGene, Tokyo, Japan), and was treated with RNase-free DNase and purified using RNeasy Mini Column (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

For microarray analysis, samples excised at 6 h (day 16) after TNCB challenge were pooled per group in equal amount of total RNA and labeled using Agilent Low RNA Input Fluorescent Linear Amplification Kit (Tokyo, Japan). The hybridization to Mouse Oligo Microarray (Agilent) and scanning were performed, and the data were analyzed using Genespring (Bio Matrix Research, Chiba, Japan).

For quantitative PCR, cDNA was synthesized from each sample of total RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, Calif., USA). The cDNAs were amplified on the Gene Amp 5700 Sequence Detection System (Applied Biosystems, Foster, Calif., USA), with gene-specific primers and SYBR Green PCR Master Mix (Applied Biosystems) to confirm the gene expression changes. Amplification conditions were as follows: 95°C for 10 s, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Mouse GAPDH was used as reference to normalize the expression levels, and data were calculated by the $2^{-\Delta\Delta Ct}$ method.

Measurement of Cytokine and Chemokine Production in Mouse Ears

The levels of cytokines and chemokines in the ear tissue were determined by ELISA, as described by Kitagaki et al. [19]. Briefly, at 6 (day 16), 24 (day 17) and 48 h (day 18) after TNCB challenge, ears were resected and homogenized with a 10-fold volume of 0.1% Tween-20 in PBS. The homogenate was quickly frozen in liquid nitrogen, thawed in a 37°C water bath, sonicated for 15 s, and centrifuged at 13,000 *g* for 5 min. The levels of IL-1β, macrophage inflammatory protein (MIP)-1α and MIP-1β in the supernatants were assayed by ELISA kits (Genzyme, Cambridge, Mass., USA), according to the manufacturer's instructions.

Mite Antigen-Induced Dermatitis Model

NC/Nga mice were anesthetized with diethyl ether and the hair on the upper back was shaved with a clipper and a shaver. Elicitation was performed by topical application of 100 mg Dfb ointment onto the shaved dorsal skin and both surfaces of each ear. Barrier disruption was achieved by 150 μl 4% sodium dodecyl sulfate on the shaved dorsal skin and both surfaces of each ear, 3 h before the Dfb ointment application. Mice were treated with barrier disruption and Dfb ointment elicitation twice a week for 3 weeks. To investigate the effect of RSC-3388 on chronic dermatitis, mice having demonstrated dermatitis with scores of over 3 for 2 weeks, were treated with 100 mg RSC-3388 ointment or ointment base on the shaved dorsal skin and both surfaces of each ear twice a day for 13 days. Tacrolimus ointment was applied once daily. The mice were sacrificed on day 13 after the start of each treatment. Portions of dorsal skin were fixed with 10% neutral formalin, embedded in paraffin, and sectioned at 4 μm. Sections were stained with hematoxylin-eosin. The dermatitis score was defined as the sum of the individual scores, graded as 0 (none), 1 (mild), 2 (moderate) and 3 (severe) for each of the four symptoms (erythema/hemorrhage, scarring/dryness, edema, excoriation/erosion).

Immunohistochemical Analysis

The lesional skin of mite antigen-induced mice, having demonstrated dermatitis with scores of over 3 for 2 weeks, and the nonlesional skin of mice treated with barrier disruption and hydrophilic petrolatum (control mice) for 3 weeks after first elicitation were removed, fixed in 10% phosphate-buffered formalin, and embedded in paraffin. Six-micrometer-thick sections were fixed in acetone for 10 min. After pretreatment with a solution of 0.3% hydrogen peroxide for 15 min, the preparations were washed with blocking reagent (nonspecific staining blocking reagent; Dako, Glostrup, Denmark). Rabbit anti-phospho-$cPLA_2$ antibody (Cell Signaling, Denvers, Mass., USA) was applied for 45 min in PBS with 1% bovine serum albumin. After washing, ENVISION kit/HRP (Dako) was overlaid for 30 min. Visualization of the reaction products was performed with diaminobenzidine solution (Dako).

Statistical Analysis

Data were expressed as the mean ± SE. Statistically significant differences were assessed by Student's t test or Dunnett's test, except for the dermatitis score. Statistical analysis for the dermatitis score was performed by pairwise comparisons between ointment base and each of the other treatment groups, based on the repeated measurement ANOVA with a mixed effects model. p values from the pairwise comparisons were adjusted by employing Bonferroni's procedure. For all tests, the level of significance was set at 0.05 (two-sided).

Results

Effect of RSC-3388 in Acetone Solution on TNCB-Induced Ear Inflammation

Balb/c mice were sensitized on both surfaces of each ear with TNCB, and then were elicited 7 days later on the sensitized ear with TNCB at 2- to 3-day intervals. Repeated challenge with TNCB for 21 days induced a dramatic increase in ear thickness compared with normal mice (fig. 1a). To determine the effect of RSC-3388, 0.3, 1 and 3% RSC-3388 dissolved in acetone was applied to both ears twice daily from day 7 to 20. RSC-3388 (0.3–3%) significantly inhibited ear swelling from day 9 to 21. The inhibition rates for 0.3, 1 and 3% RSC-3388 on day 21 were 28, 46 and 60% ($p < 0.01$), respectively. In the histological examination, RSC-3388 inhibited epidermal and dermal thickness and inflammatory cell infiltration (fig. 1b).

Effect of RSC-3388 on Eicosanoid Production in TNCB-Induced Ear Inflammation

We examined the effect of RSC-3388 on PGE_2 levels in TNCB-induced ear inflammation (fig. 2). On day 21, PGE_2 level in the TNCB + acetone-treated group was significantly elevated compared to that in normal mice. RSC-3388 (3%) significantly inhibited PGE_2 production on day 21. The level of LTB_4 was quite low and increased minimally in the TNCB + acetone-treated mice compared to normal mice (data not shown).

Differential Gene Expression Revealed by GeneChip Analysis and Quantitative PCR

To investigate whether RSC-3388 affected cytokine or chemokine levels in TNCB-induced dermatitis, we compared the gene expression profile in the ears of TNCB +

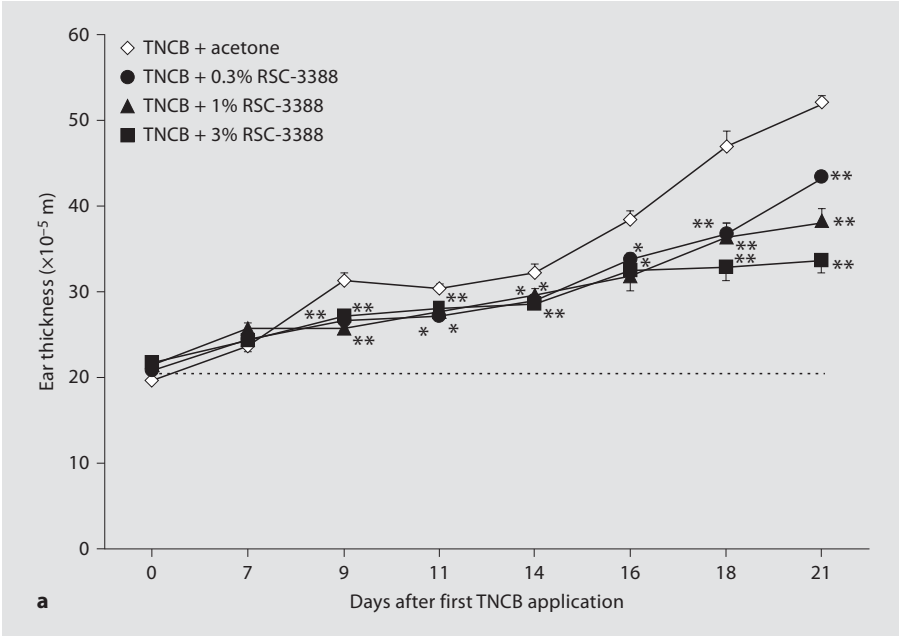
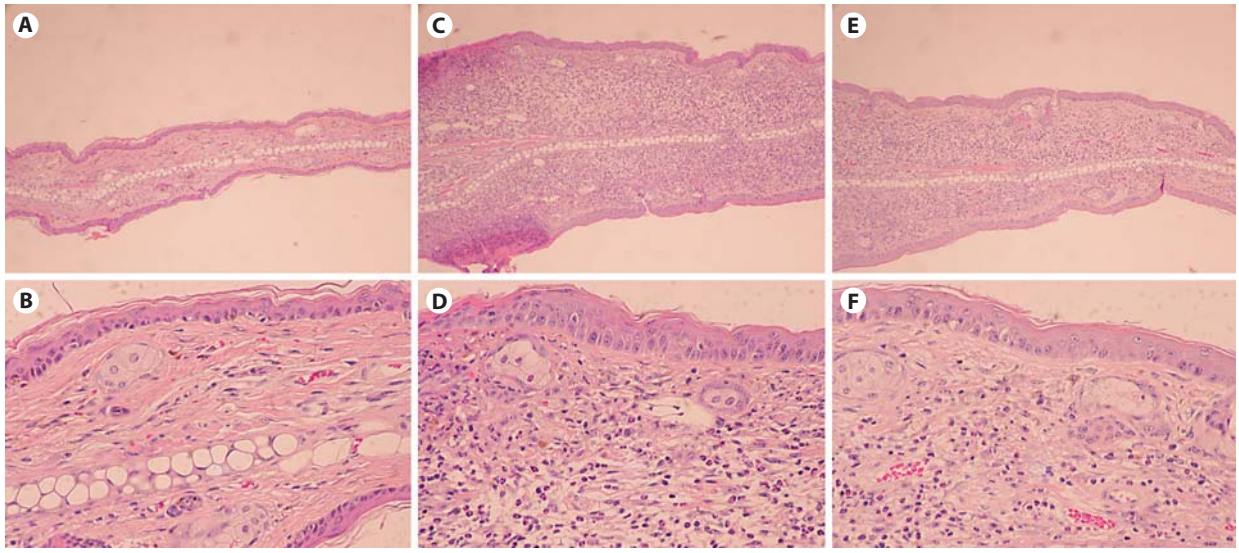


Fig. 1. a Effect of RSC-3388 acetone solution on TNCB-induced ear inflammation. TNCB was applied on days 0, 7, 9, 11, 14, 16 and 18, and drugs were administered twice daily from day 7 to 20. Ear thickness was measured just before each TNCB application and on day 21. Each value represents mean ± SE. Ear thickness of normal mice was indicated by the dotted line. * $p < 0.05$, ** $p < 0.01$ versus vehicle (Dunnett's test). **b** Histological changes in the ears in mice treated with TNCB + acetone or TNCB + 3% RSC-3388. Ear tissues of normal mice (**A**, **B**), TNCB + acetone-treated (**C**, **D**) and TNCB + 3% RSC-3388-treated mice (**E**, **F**) were stained with hematoxylin-eosin. Original magnification ×100 (**A**, **C**, **E**) and ×400 (**B**, **D**, **F**).

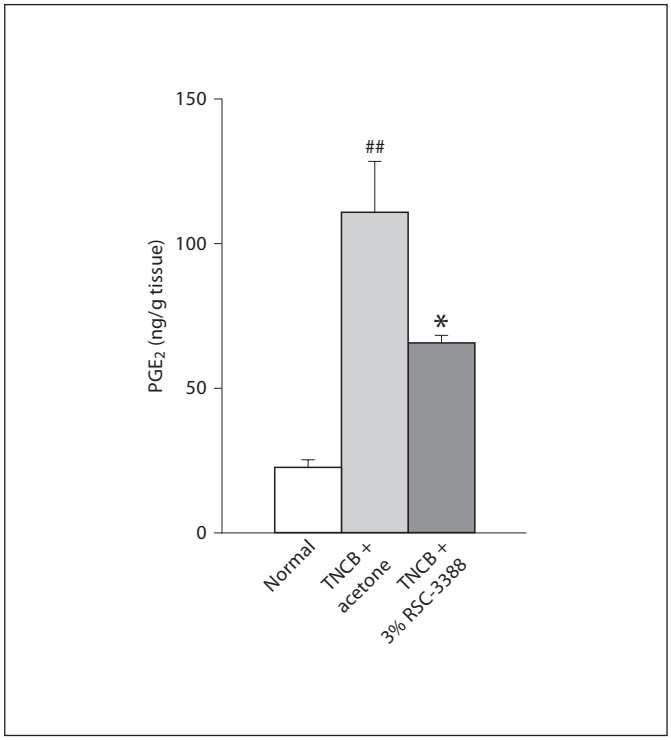


RSC-3388-treated and TNCB + acetone-treated mice at 6 h after TNCB challenge on day 16, because the increase in ear thickness was the most evident after TNCB challenge on day 16. Among 20,000 genes examined, 365 increased more than twofold in TNCB-treated compared to normal mice. The increased expression of twenty out of 365 genes was decreased by RSC-3388 (table 1). Since RSC-3388 inhibited inflammatory cell infiltration into the lesional skin, as seen by histological examination, we focused on CC chemokines MIP-1α and MIP-1β among the twenty genes, and investigated any changes in their expression levels in TNCB + RSC-3388-treated and TNCB + acetone-treated mice at 6 (day 16), 24 (day 17) and 48 h (day 18) after TNCB challenge, using quantitative real-time PCR. MIP-1α and MIP-1β expression decreased 3.5-fold and 3.9-fold in the TNCB + RSC-3388-

Table 1. Expression profile by GeneChip analysis

GenBank	Description	Fold change		Ratio
		3388	vehicle	3388/vehicle
NM_007560	bone morphogenetic protein receptor type 1B (Bmpr1b)	1.0	4.5	0.2
L38281	immune-responsive gene 1 (Irg1)	3.2	10.6	0.3
NM_010819	C-type lectin, superfamily member 8 (Clecsf8)	4.3	11.8	0.4
NM_009263	secreted phosphoprotein 1 (Spp1)	1.8	4.8	0.4
NM_008039	formyl peptide receptor, related sequence 2 (Fpr-rs2)	1.8	4.8	0.4
NM_009705	arginase type II (Arg2)	2.0	4.9	0.4
NM_009841	CD14 antigen (Cd14)	1.7	4.1	0.4
NM_013652	small inducible cytokine A4 (Scya4)	10.3	22.8	0.4
NM_008230	histidine decarboxylase cluster (Hdc)	5.0	10.5	0.5
NM_023380	SH3 domain and nuclear localization signals, 1 (Samsn1)	1.2	2.4	0.5
NM_019932	platelet factor 4 (Pf4)	1.2	2.3	0.5
NM_009114	S100 calcium-binding protein A9 (calgranulin B)	36.2	68.1	0.5
M92417	cysteine proteinase inhibitor (MS1)	8.8	16.3	0.5
NM_030701	interferon-gamma-inducible gene	2.1	3.9	0.5
NM_007482	arginase 1, liver (Arg1)	2.1	3.9	0.5
NM_011660	thioredoxin 1 (Txn1)	1.8	3.3	0.6
NM_008059	G0/G1 switch gene 2 (G0s2)	0.9	1.7	0.6
NM_010809	matrix metalloproteinase 3 (Mmp3)	0.9	1.7	0.6
AI787571.1	EST	5.2	9.2	0.6
NM_011337	small inducible cytokine A3 (Scya3)	7.2	12.8	0.6

TNCB applied on days 0, 7, 9, 11, 14 and 16, and drugs were administered twice daily from day 7 to 15. Samples, which were excised at 6 h (day 16) after TNCB challenge, were pooled per group in equal amount of total RNA and used for microarray analysis. 3388 = TNCB + RSC-3388; vehicle = TNCB + acetone.



treated group compared to the TNCB + acetone-treated group at 24 h after TNCB challenge, respectively (fig. 3a). As IL-1β, a multifunctional inflammatory cytokine, is known as one of the inducers of MIP-1α and MIP-1β, we also examined its expression level. IL-1β induced by TNCB challenge also decreased 2.4-fold following RSC-3388 treatment.

Effect of RSC-3388 on Cytokine and Chemokine Production in TNCB-Induced Ear Inflammation

To confirm whether RSC-3388 inhibited IL-1β, MIP-1α and MIP-1β production at the protein level, we examined their production by ELISA (fig. 3b). Repeated application of TNCB resulted in marked elevation of IL-1β,

Fig. 2. Effect of RSC-3388 on eicosanoid production in TNCB-induced ear inflammation. TNCB was applied on days 0, 7, 9, 11, 14, 16 and 18, and drugs were administered twice daily from day 7 to 20. The ear tissue was excised on day 21. Each value represents mean ± SE. $^{\#\#}$ $p < 0.01$ versus normal mice (Student's t test). * $p < 0.05$ versus TNCB + acetone group (Student's t test).

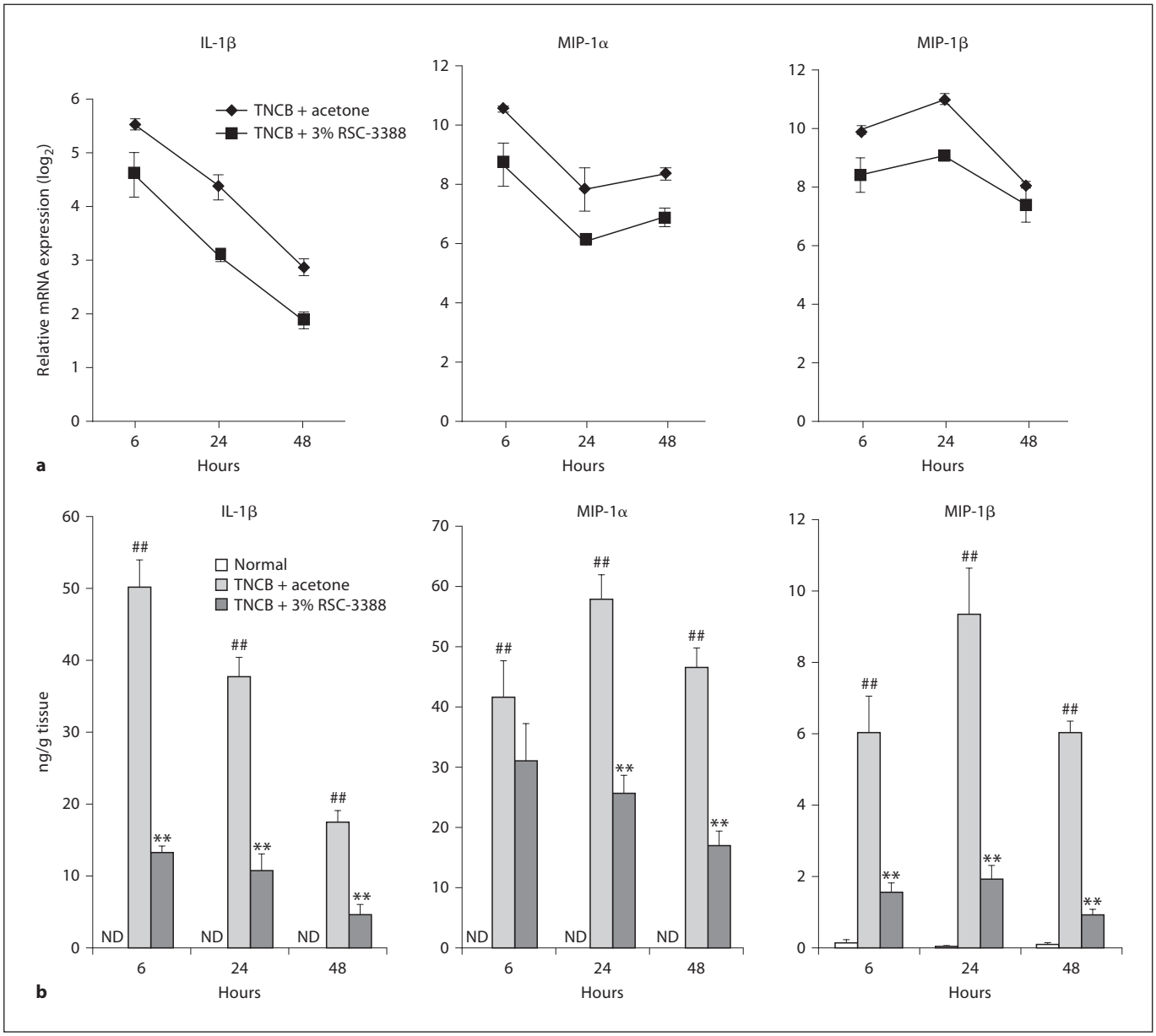


Fig. 3. Effect of RSC-3388 on IL-1β, MIP-1α and MIP-1β mRNA expression (**a**) and production (**b**) in ear tissue of mice with TNCB-induced inflammation. TNCB was applied on days 0, 7, 9, 11, 14 and 16, and drugs were administered twice daily from day 7 to 17. The ear tissue was excised at 6 (day 16), 24 (day 17) and 48 h (day 18) after TNCB challenge. Each value represents mean ± SE. $^{\#\#}$ $p < 0.01$ versus normal mice (Student's t test). ** $p < 0.01$ versus TNCB + acetone group (Student's t test).

MIP-1α and MIP-1β levels in the inflamed ear at 6 (day 16), 24 (day 17) and 48 h (day 18) after TNCB challenge, compared with those in the normal mice. RSC-3388 (3%) significantly inhibited production of these cytokines and chemokines.

Effect of RSC-3388 Ointment on TNCB-Induced Ear Inflammation

Next, we prepared RSC-3388 ointment for comparison with tacrolimus ointment, a calcineurin inhibitor, and ibuprofen picnol ointment, a cyclooxygenase inhibitor, which are used as therapeutic agents for atopic dermati-

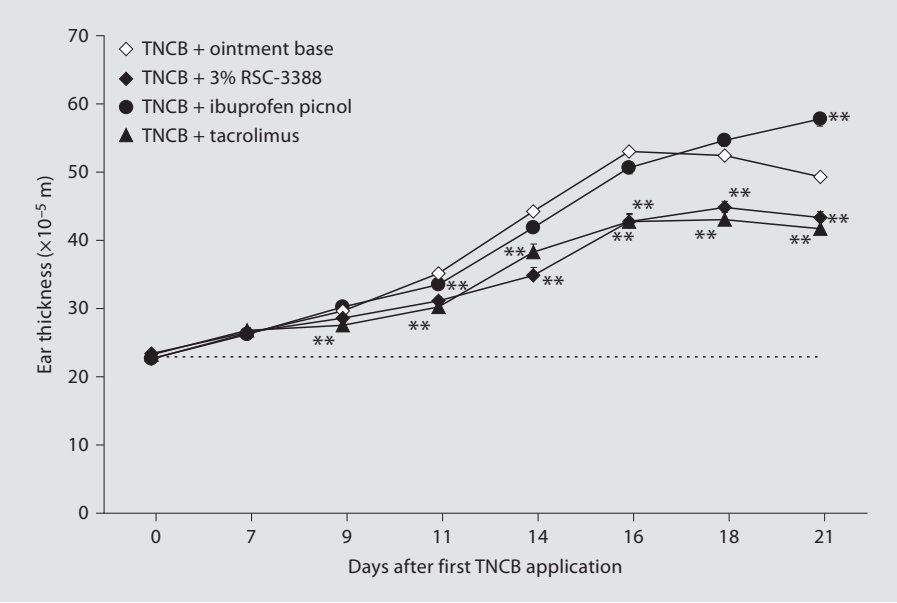


Fig. 4. Effect of RSC-3388 ointment on TNCB-induced ear inflammation. TNCB was applied on days 0, 7, 9, 11, 14, 16 and 18. RSC-3388 ointment was administered twice daily, and tacrolimus ointment and ibuprofen picnol ointment were administered once daily from day 7 to 20. Ear thickness was measured just before each TNCB application and on day 21. Each value represents mean ± SE. Ear thickness of normal mice is indicated by the dotted line. ** $p < 0.01$ versus TNCB + ointment base group (Dunnett's test).

tis. RSC-3388 (3%) ointment significantly inhibited ear swelling from day 11 to 21 (fig. 4). Tacrolimus ointment also inhibited ear thickness in this model. The potency of 3% RSC-3388 ointment was comparable to that of tacrolimus ointment in this model. Ibuprofen picnol ointment had no effect and finally aggravated the ear swelling on day 21.

Effect of RSC-3388 Ointment on the Mite Antigen-Induced Dermatitis Model

Repeated application of Dfb ointment with barrier disruption caused atopic dermatitis-like chronic skin lesions. The skin inflammation in mice with a dermatitis score of over 3 for 2 weeks persisted for at least for 2 weeks, without further application of Dfb ointment. We evaluated the efficacy of RSC-3388 ointment using the mice that exhibited chronic dermatitis. In the RSC-3388 ointment- and tacrolimus ointment-treated groups, the dermatitis score was reduced immediately after the ointment application, and significant suppression was observed from day 1 to 13 after the start of treatment in both groups, compared with the ointment base-treated group (fig. 5a). Histologically, in the RSC-3388 ointment-treated group, epidermal and dermal thickening were decreased compared with those in the ointment base-treated group (fig. 5b). Treatment with tacrolimus ointment also suppressed mite antigen-induced histological changes.

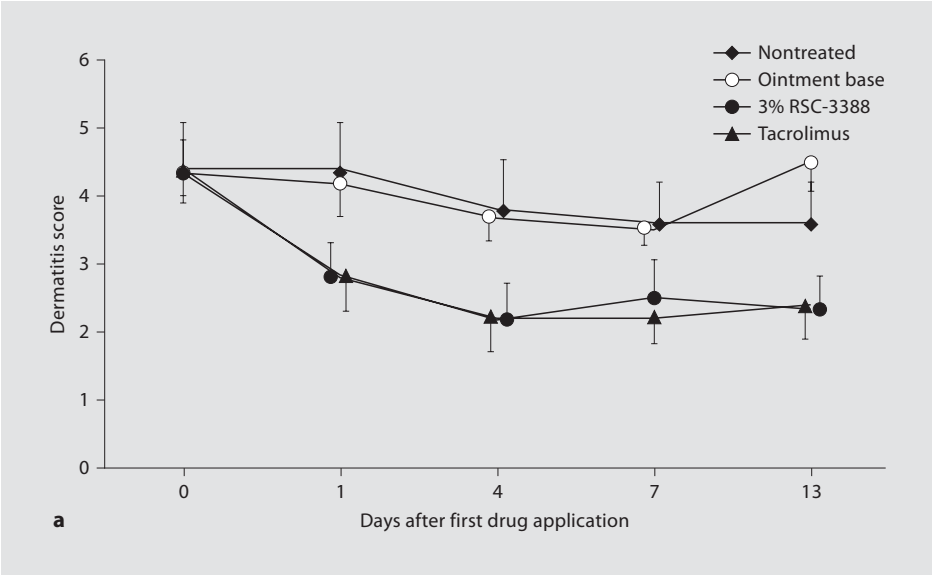
Expression of Phospho-cPLA$_2$ in the Skin of Mite Antigen-Induced Dermatitis

Since the active form of cPLA$_2$α is known to be phospho-cPLA$_2$ [20], immunohistochemical analysis was performed using anti-phospho-cPLA$_2$ antibody. The expression of phospho-cPLA$_2$ was clearly increased in the lesional skin of mite antigen-induced dermatitis compared with that in the skin of control mice (fig. 6). Major populations of cells expressing phospho-cPLA$_2$ in the skin lesions were epidermal keratinocytes.

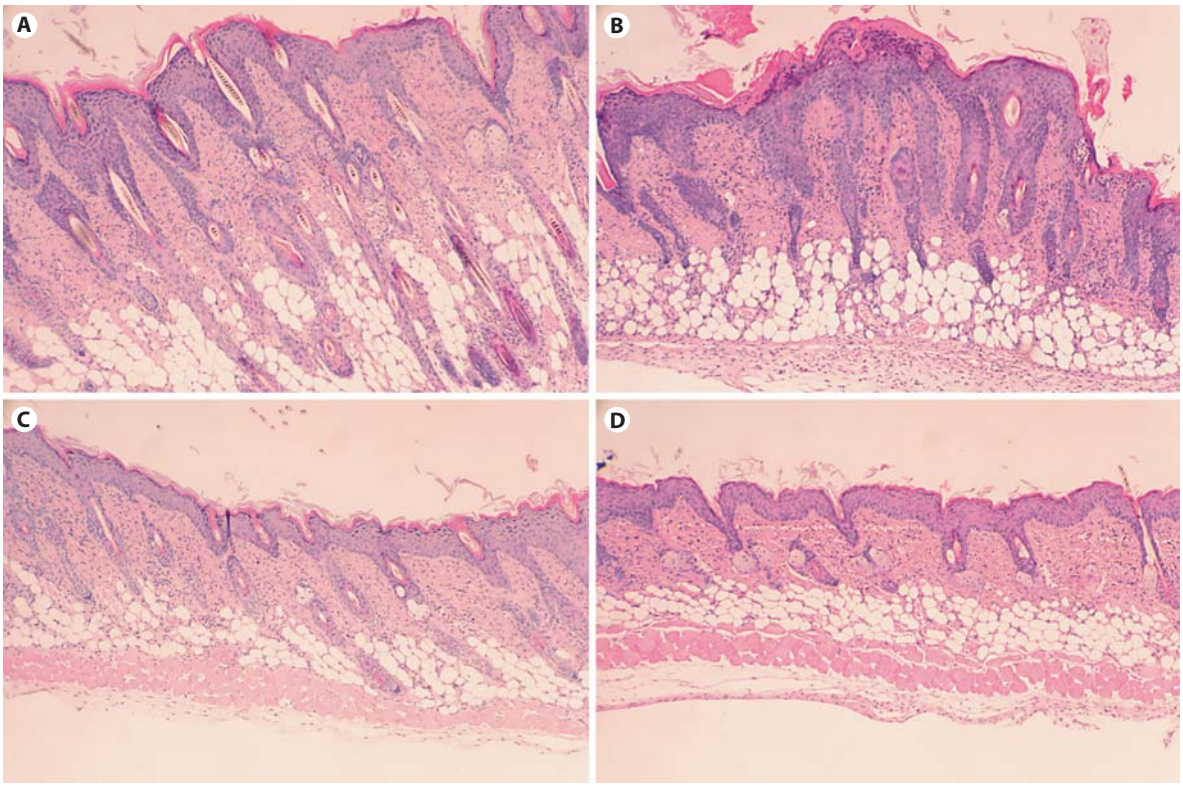
Discussion

In the present study, we demonstrated the efficacy of cPLA$_2$α inhibitor RSC-3388 acetone solution and ointment in TNCB-induced skin inflammation. RSC-3388 inhibited eicosanoid production and decreased the levels of cytokines and chemokines in the inflamed ear. In addition, RSC-3388 alleviated atopic dermatitis-like skin lesions induced by repeated application of mite antigen. Furthermore, we confirmed increased expression for cPLA$_2$α in the skin lesions of mite antigen-induced model compared to that in control mice, by immunohistochemical analysis using anti-phospho-cPLA$_2$α antibody. These findings suggest that RSC-3388 is useful for the treatment of skin inflammation, such as atopic dermatitis.

cPLA$_2$α preferentially hydrolyzes membrane phospholipids containing arachidonic acid, and plays a key



a



b

Fig. 5. a Effect of RSC-3388 and tacrolimus ointments on an established dermatitis model. NC/Nga mice with a dermatitis score >3 for 2 weeks were used. Ointment base and RSC-3388 ointment were applied twice daily to shaved dorsal skin and both surfaces of each ear. Tacrolimus ointment was applied once daily. Nothing was applied to the nontreated group. Data represent mean ± SE. Statistical analysis was performed by pairwise comparisons versus ointment base-treated group based on the repeated measurement ANOVA with a mixed-effects model. Multiple comparisons were performed using Bonferroni's procedure. p values versus ointment were as follows: RSC-3388 ointment, p = 0.0065; tacrolimus ointment, p = 0.0115. **b** Histological changes in the dorsal skin in mice treated with RSC-3388 or tacrolimus ointment. Dorsal skin from nontreated (**A**), ointment base-treated (**B**), RSC-3388 ointment-treated (**C**) and tacrolimus ointment-treated (**D**) mice was stained with hematoxylin-eosin. Original magnification ×100 for all photomicrographs.

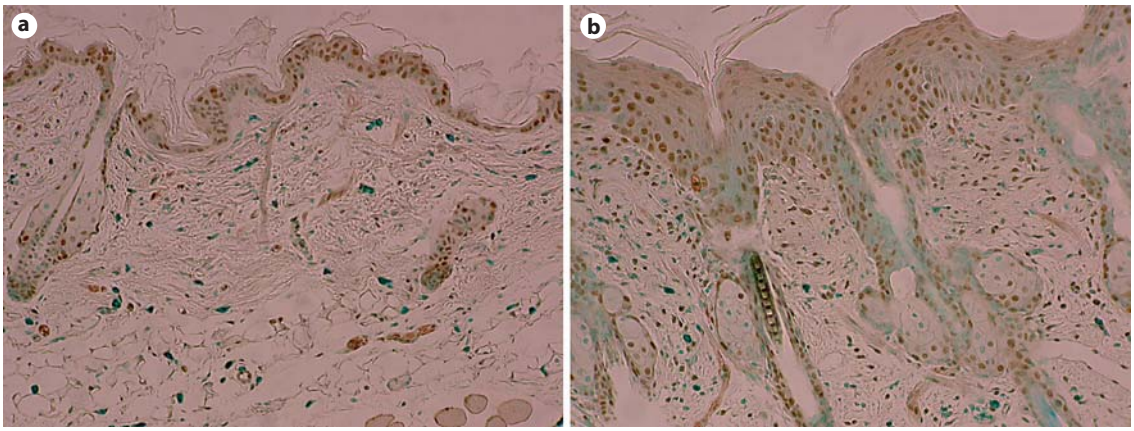


Fig. 6. Immunohistochemical analysis of phospho-cPLA$_2$ in the non-lesional skin of control mice treated with barrier disruption and hydrophilic petrolatum (**a**), and the lesional skin of the mite antigen-induced dermatitis model (**b**). Original magnification ×400 for both photomicrographs.

role in the biosynthesis of eicosanoids such as PGs and LTs. It has previously been reported that BMS-229724, a cPLA$_2$α inhibitor, exerts an anti-inflammatory effect on TPA-induced ear swelling and ultraviolet B-induced skin erythema [21]. Therefore, it is plausible that cPLA$_2$α inhibitors are potential therapeutic targets for the treatment of inflammatory skin diseases such as atopic dermatitis and psoriasis. Recently, we have synthesized several hundreds of pyrrolidine inhibitors of cPLA$_2$α [14], and finally discovered a highly potent and selective cPLA$_2$α inhibitor, coded RSC-3388. In an in vitro study, RSC-3388 clearly suppressed calcium ionophore-induced arachidonic acid release, and PGE$_2$ and LTC$_4$ production from human monocytic THP-1 cells, with IC$_{50}$ values of 0.022, 0.031 and 0.013 μmol/l, respectively [14], and these inhibitory activities of RSC-3388 were much more potent than those of BMS-229724 (data not shown). In the present study, we examined the effect of RSC-3388 on two different mouse models of atopic dermatitis.

Repeated application of TNCB to the ears of Balb/c mice can induce skin inflammation, changing from a typical delayed-type to an immediate-type hypersensitivity, followed by a late reaction [18]. This skin inflammation is associated with epidermal thickness, accumulation of large numbers of mast cells and CD4+ T cells in the upper dermis, and elevated serum levels of antigen-specific IgE. Thus, many features in this model are similar to the events observed in patients with chronic skin inflammation, such as atopic dermatitis. RSC-3388 had a significant inhibitory activity against TNCB-induced ear swelling in mice. Also, RSC-3388 significantly inhibited PGE$_2$ production in the ear on day 21. On the other hand, the level of LTB$_4$ only increased minimally in the TNCB-treated compared to the normal mice on the day. Possibly LTB$_4$ production might be observed immediately after TNCB has been applied to the ears. We demonstrated that ibuprofen picnol ointment, a cyclooxygenase inhibitor, had no effect and finally aggravated ear inflammation in this model. In addition, Inoue et al. [22] have demonstrated that this cyclooxygenase inhibitor, applied to the ears therapeutically, exacerbates ear edema and does not prevent rebound phenomena, following withdrawal of corticosteroid therapy in TNCB-induced skin inflammation. Furthermore, we have confirmed the inhibitory effect of RSC-3388 on both PGE$_2$ and LTB$_4$ production in mouse skin in vivo induced by phorbol ester (data not shown), indicating that the decrease in both PGs and LTs should contribute to the anti-inflammatory effect of RSC-3388 in this model. However, to clarify the time-course changes in LTB$_4$ level and the effect of RSC-3388 on LTB$_4$ production in this model, further studies are required.

We investigated whether RSC-3388 affected other pro-inflammatory mediators, except for lipid mediators. By comprehensive expression analysis using the GeneChip technology, we identified 20 transcripts in which expression was upregulated in the ear of TNCB + acetone-treated mice relative to that in normal mice, and downregulated in the ear of TNCB + RSC-3388-treated mice relative to that in TNCB + acetone-treated mice. As RSC-3388 inhibited inflammatory cell infiltration into the lesional skin, we particularly focused on CC chemokines MIP-1α

and MIP-1β, which are monocyte and lymphocyte chemoattractants [23, 24], and confirmed that RSC-3388 significantly abrogated these chemokines at the protein level. Furthermore, RSC-3388 significantly inhibited IL-1β expression and protein levels. Since it has been reported that the expression and production of MIP-1α and MIP-1β are induced by IL-1β in vitro [25, 26], and $cPLA_2$ inhibitors inhibit expression of the IL-1β gene at the level of transcription and promoter activation in human monocyte cell line [27], RSC-3388 would have been expected to inhibit IL-1β production, followed by suppression of MIP-1α and MIP-1β levels in this model. Although it remains unclear whether RSC-3388 inhibits IL-1β production directly or indirectly, and we must confirm whether RSC-3388 suppresses the expression or production of IL-1β in vitro, these results suggest that RSC-3388 can show potent inhibitory activity against TNCB-induced ear inflammation, through suppressing eicosanoid production and reducing cytokine and chemokine levels in the skin.

Recently, we have developed a novel animal model for atopic dermatitis by repeated application of Df extract ointment, together with barrier disruption to NC/Nga mice [28]. All the clinical, histological and immunological features in this model are similar to the events observed in patients with atopic dermatitis. Increased expression of $cPLA_2$ was observed in the skin lesion, particularly in the epidermal keratinocytes, suggesting that $cPLA_2$ is involved in the pathogenesis of this model. RSC-3388 inhibited the dermatitis score immediately after drug application, and significant suppression was observed for 13 days. The reduction of the dermatitis score by RSC-3388 was accompanied by improvement of histological changes, such as epidermal and dermal thickening. The efficacy of RSC-3388 ointment was as potent as that of tacrolimus ointment in this model.

In conclusion, RSC-3388, a highly potent and selective $cPLA_2\alpha$ inhibitor, should be useful for the treatment of inflammatory skin disorders such as atopic dermatitis, through the suppression of not only arachidonic acid and eicosanoid synthesis, but also cytokine and chemokine production.

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