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Oxysterol represses high-affinity IgE receptor-stimulated mast cell activation in Liver X receptor-dependent and -independent manners

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ABSTRACT

Oxysterols activating liver X receptors (LXRs) repress expression of pro-inflammatory genes and have anti-inflammatory effects. Here, we show for the first time that bone marrow-derived murine mast cells (BMMCs) predominantly express LXR β . 25-hydroxycholesterol, a representative LXR activating oxysterol, suppressed IL-6 production and degranulation response in BMMCs following engagement of high-affinity IgE receptor (Fc ϵ RI). Interestingly, 25-hydroxycholesterol reduced cell-surface Fc ϵ RI expression by inhibiting assembly of Fc ϵ RI α and Fc ϵ RI β . We demonstrate that LXR activation was involved in the suppression of IL-6 production in BMMCs, but that reduced Fc ϵ RI expression and degranulation response was mediated in an LXR-independent manner.

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1. Introduction

Mast cells express the high-affinity IgE receptor (Fc ϵ RI) on the cell surface which plays crucial roles in the development of allergic inflammation [1]. Fc ϵ RI is mainly expressed on mast cells and basophils as a tetramer comprised of an IgE-binding α -chain, two types of signaling subunits, a β -chain, and a disulfide-linked homodimer of γ -chains [2]. The IgE-bound Fc ϵ RI is engaged by multivalent antigens for mast cell activation, and triggers the release of preformed intragranular chemical mediators, synthesis of eicosanoids, and the production of pro-inflammatory cytokines.

Liver X receptors (LXR) α and β are known as representative nuclear receptors and were recently reported to negatively regulate expression of pro-inflammatory genes by inducing trans-repression [3]. Some oxysterols were identified as natural ligands for LXR [4], and have anti-allergic activity; e.g., topical application of

LXR-activating oxysterols in mice reduces irritant and allergic cutaneous inflammation [5]. More recently, Heine et al. reported that activation of LXRs reduces IgE production from B cells stimulated with anti-CD40 and IL-4 [6].

In the present study, we found that bone marrow-derived murine mast cells (BMMCs) predominantly express LXR β but not LXR α . Thus, we further investigated employing BMMCs whether 25-hydroxycholesterol (25-OHC), 22(R)-hydroxycholesterol (22(R)-OHC), and 24(S),25-epoxycholesterol (24(S),25-EC), all of which are LXR activating oxysterols, suppress Fc ϵ RI-dependent mast cell activation including production of a representative pro-inflammatory cytokine, IL-6.

2. Materials and methods

2.1. Antibodies and other reagents

Mouse anti-Fc ϵ RI β monoclonal antibody (mAb) (clone JRK; the hybridoma was a kind gift from Dr. Juan Rivera, NIH, USA) was prepared in our laboratory. Rabbit anti-Fc ϵ RI γ polyclonal antibody (#06-727) was purchased from Upstate (Lake Placid, NY, USA). Armenian hamster anti-mouse Fc ϵ RI α mAb (MAR-1) conjugated with or without FITC and armenian hamster IgG isotype control (#16-4888) was purchased from eBioscience (San Diego, CA, USA). Horseradish peroxidase (HRP) conjugated secondary polyclonal antibodies to rabbit IgG, armenian hamster IgG, and

Abbreviations: BMMCs, bone marrow-derived mast cells; Fc ϵ RI, high-affinity IgE; LXR, liver X receptor; LDLR, LDL receptor; 25-OHC, 25-hydroxycholesterol; 22(R)-OHC, 22(R)-hydroxycholesterol; 24(S),25-EC, 24(S),25-epoxycholesterol; 22(S)-hydroxycholesterol, (22(S)-OHC); ER, endoplasmic reticulum; SREBP, sterol regulatory element-binding protein

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mouse IgG, were purchased from Cell Signaling Technology (Beverly, MA, USA), Santa Cruz Biotechnology (Santa Cruz, CA, USA), and GE Healthcare Bio-sciences (Little Chalfont, UK), respectively. Anti-TNP IgE (IgE-3) and FITC-conjugated anti-mouse IgE (R35-72) mAbs were purchased from BD Pharmingen (San Diego, CA, USA). The anti-TNP IgE was labeled with Alexa488 (Molecular Probes/Invitrogen, Tokyo, Japan) in our laboratory. LY295427 was purchased from Calbiochem (San Diego, CA, USA). 25-hydroxycholesterol (25-OHC) was purchased from Sigma (St. Louis, MO, USA). 22(R)-hydroxycholesterol (22(R)-OHC) and T0901317 were purchased from Cayman (Ann Arbor, Michigan, USA), and 24(S),25-epoxycholesterol was purchased from Biomol (Plymouth Meeting, PA, USA). 22(S)-hydroxycholesterol (22(S)-OHC) was purchased from Steraloids (Newport, RI, USA).

2.2. Preparation of BMMCs

Following approval by the committee of animal care and use in Nihon University, BMMCs were prepared from the femurs of 4- to 6-week-old C57BL/6J mice as previously described [7]. BMMCs were used for experiments after 4–5 weeks of culture.

2.3. Treatment of BMMCs with oxysterols

BMMCs (5×10^5 /ml) were incubated in culture medium containing oxysterols (1, 5, or 10 μ M) for 48 h.

2.4. Degranulation assay

The degranulation response was evaluated by β -hexosaminidase release as previously described [7]. Briefly, IgE-sensitized BMMCs (1×10^6 /ml) were stimulated with or without 1 ng/ml TNP-BSA (LSL, Tokyo, Japan) at 37 °C for 30 min. Whole cell lysates with 0.5% TritonX-100 were used to quantify the total β -hexosaminidase activity. The supernatant or the whole cell lysates were

incubated with *p*-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside 3.8 μ M for 40 min at 37 °C. The reaction was stopped by the addition of glycine 0.2 M (pH 11.0), and then the absorbance was measured at 415 nm wave length. The percentage of net β -hexosaminidase release was calculated as follows: (supernatant optical density of the stimulated cells – supernatant optical density of the unstimulated cells)/(total cell lysate optical density of unstimulated cells – supernatant optical density value of unstimulated cells) $\times$ 100.

2.5. Measurement of IL-6

IgE-sensitized BMMCs (1×10^6 /ml) were stimulated with or without 1 ng/ml TNP-BSA for 6 h. The production of IL-6 was analyzed with a specific ELISA kit (Biosource International, Camarillo, CA, USA)

2.6. Flow cytometry

Binding of IgE to Fc ϵ RI was evaluated by employing IgE-Alexa488. Briefly, BMMCs (2×10^5) were incubated with 0.1 μ g/ml of IgE-Alexa488 at 4 °C for 30 min. To evaluate Fc ϵ RI expression at the cell surface, BMMCs (2×10^5) were labeled with 0.1 μ g/ml of IgE at 4 °C for 30 min. The cells were stained at 4 °C for 30 min with 0.1 μ g/ml anti-IgE mAb conjugated with FITC. Alternatively, BMMCs (2×10^5) were directly labeled with 0.1 μ g/ml FITC-conjugated anti-mouse Fc ϵ RI α mAb, or with isotype control mAb at 4 °C for 30 min. These cells were analyzed using a FACSCalibur (Becton Dickinson Biosciences, San Jose, CA, USA).

2.7. Quantitative real-time PCR

cDNA was reverse transcribed from 1 μ g of total RNA. For quantitative real time PCR, cDNAs was analyzed in the ABI PRISM 7000 sequence detection system using Power SYBR Green PCR Master

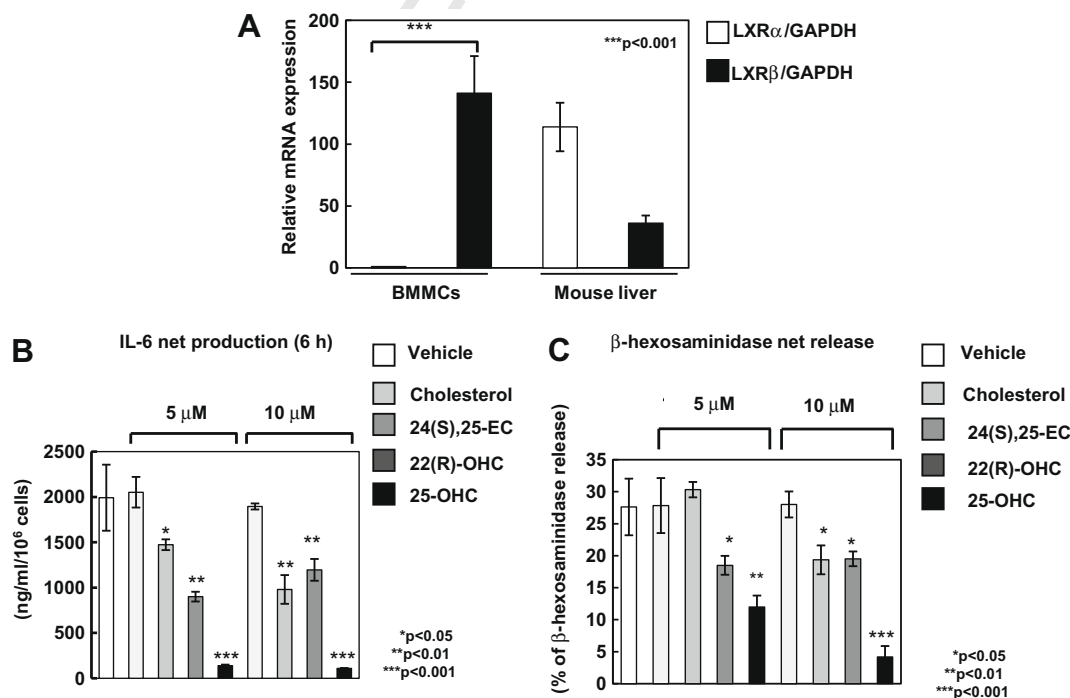


Fig. 1. 25-OHC reduces IL-6 production and degranulation response in mast cells upon Fc ϵ RI engagement. (A) Quantitative real-time PCR analyzes for expression of LXR α and β mRNA in BMMCs. Mouse liver was used as a positive control. Data shown are the mean $\pm$ S.D. of samples obtained from three separate experiments. BMMCs (5×10^5 /ml) were treated with or without oxysterols for 48 h and then stimulated with IgE and antigen. (B) Effects of oxysterols on IL-6 net production. (C) Effects of oxysterols on β -hexosaminidase net release. In (B) and (C), data shown are the mean $\pm$ S.D. of triplicates. Similar results were obtained from three independent experiments. Student's *t*-test was performed to analyze differences between the cells treated with vehicle and the other cells.

Mix (Applied Biosystems, Foster City, CA, USA). Primer sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), LXR α , LXR β , ABCA1, and LDL receptor (LDLR) were described as follows: 5'-TGCAACCACTGCTTAG-3' and 5'-GATGCAGGGATGATGTTCC-3' (GAPDH); 5'-AGGAGTGTCTGACTTCGAAA-3' and 5'-TGTTCTCTCTCTGCGCTT-3' (LXR α); 5'-AACATCTTCTCAGCCGATCG-3' and 5'-TTCATGAGCATGCGTGGGAA-3' (LXR β); 5'-ATTGCCAGACGGAGCCG-3' and 5'-TGCCAAAGGGTGGCACA-3' (ABCA1); 5'-AGAGGACGAGCTCCACATT-3' and 5'-TCATGCCACATCGTCCTCA-3' (LDLR). The amplified RNA values were normalized to the level of GAPDH mRNA.

2.8. Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed as previously described [8]. Briefly, cells (1×10^7) were lysed for 30 min on ice in lysis buffer (Tris-buffered saline containing 0.25% Triton-X100, 2 mM PMSF, 10 μ g/ml aprotinin, 2 μ g/ml leupeptin and pepstatin A, 50 mM NaF and 1 mM sodium orthovanadate). The lysates were centrifuged for 15 min at 15 000 $\times$ g, and the supernatant was collected. Anti-mouse Fc ϵ RI α mAb (10 μ g) and isotype control (10 μ g) was added to the supernatant and incubated for 3 h at 4°C. The immune complex was precipitated with 10 μ g of protein-G-sepharose and then resuspended in an equal volume of 2 $\times$ sample buffer without reducing agent. The samples were then boiled for 5 min at 100 °C, separated by 10–20% gradient SDS-PAGE, then transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was incubated with the primary antibody and an appropriate secondary horseradish peroxidase-conjugated antibody. Signals were detected using

enhanced chemiluminescence (GE Healthcare Bio-sciences). The immunoreactive bands were scanned to produce digital images that were quantified with SCION Image software.

2.9. Statistical analysis

The data shown are the means $\pm$ S.D. of at least triplicate samples. Statistical analysis was performed using Student's *t*-test. Probability values of less than 0.05 were considered as statistically significant.

3. Results

3.1. Treatment of mast cells with 25-OHC reduces IL-6 production and degranulation response upon Fc ϵ RI engagement

In preliminary experiments, we observed that BMMCs express both LXR α and β constitutively (data not shown). Analyses employing quantitative real-time PCR show that the level of LXR β expression was much higher than that of LXR α expression (Fig. 1A). Next, we examined the effects of three oxysterols on Fc ϵ RI-dependent IL-6 production and degranulation response of mast cells. BMMCs were cultured in the presence of 5 or 10 μ M oxysterols for 48 h. Cholesterol was used as a control. Under the experimental conditions, cell viability was unaltered (data not shown). As shown in Fig. 1B, all oxysterols, particularly 25-OHC, significantly suppressed IL-6 production. On the other hand, 25-OHC and other oxysterols considerably or slightly suppressed the degranulation response, respectively (Fig. 1C). Spontaneous release from unstimulated cells was below 3% of the total content of the

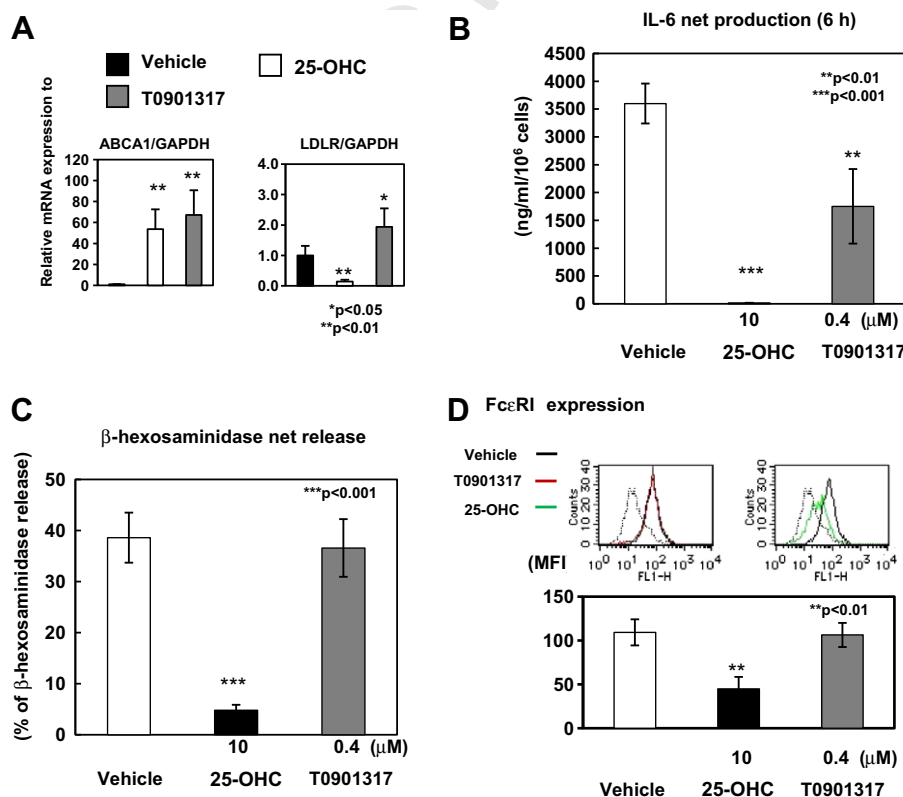


Fig. 2. LXR activation is involved in suppression of Fc ϵ RI-mediated IL-6 production. BMMCs (5×10^5 /ml) were treated with 25-OHC or T0901317 for 48 h. (A) Quantitative real-time PCR analyzes for expression of ABCA1 and LDLR mRNA. (B) IL-6 net production. (C) β -hexosaminidase net release. (D) Cell-surface expression of Fc ϵ RI was analyzed with flow cytometry, using mouse IgE and anti-mouse IgE mAb FITC. Data shown are the mean $\pm$ S.D. of triplicates (B), or quadruplicates (C). Similar results were obtained from three independent experiments. In (A) and (D), data shown are the mean $\pm$ S.D. of samples obtained from three independent experiments. Student's *t*-test was performed to compare cells treated with vehicle to the other cells.

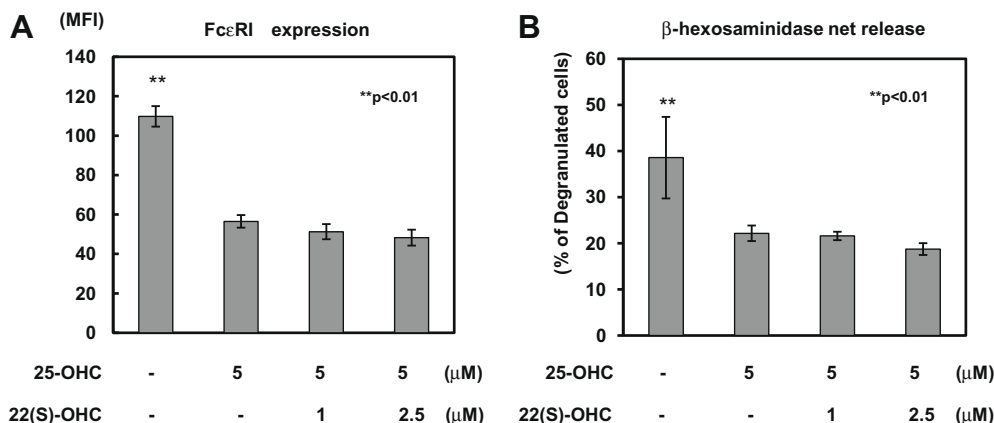


Fig. 3. An LXR antagonist does not reverse suppression of FcεRI expression and degranulation response. BMMCs (5×10^5 /ml) were treated with 5 μM 25-OHC for 48 h in the presence or absence of 22(S)-OHC. (A) Cell-surface expression of FcεRI was analyzed with flow cytometry, using mouse IgE and anti-mouse IgE mAb FITC. Data shown are the mean $\pm$ S.D. of samples obtained from three separate experiments. (B) β-hexosaminidase net release. Data shown are the mean $\pm$ S.D. of triplicates. Similar results were obtained from three independent experiments. In (A) and (B), Student's *t*-test was performed to compare cells treated with 5 μM 25-OHC to the other cells.

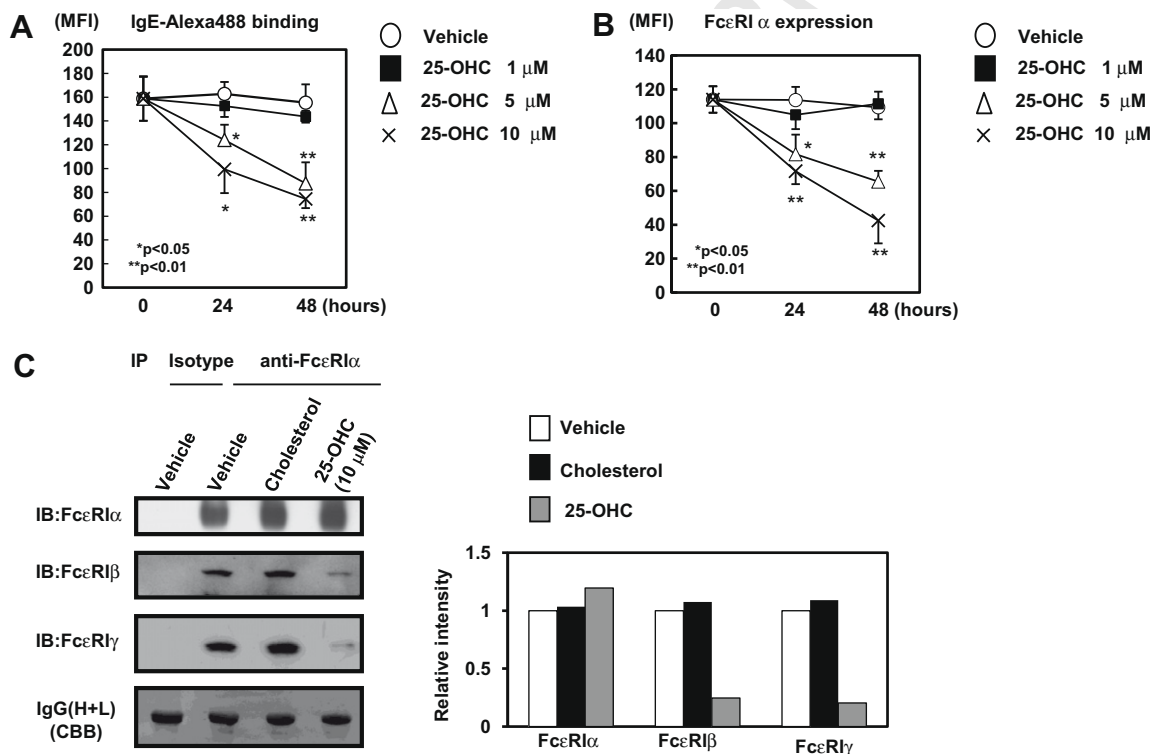


Fig. 4. 25-OHC decreases assembly of FcεRIα and FcεRIβγ in mast cells. BMMCs (5×10^5 /ml) were treated with 25-OHC at the indicated concentrations for 48 h. (A) Effects of 25-OHC on IgE-Alexa488 binding to FcεRI. (B) Effects of 25-OHC on cell-surface FcεRIα expression. (C) Cells (1×10^7 /ml) were lysed in lysis buffer as described in Section 2. Whole cell lysates were incubated with anti-FcεRIα mAb (10 μg) or isotype control (10 μg). The immunoprecipitates were separated on 10–20% SDS-PAGE under non-reducing condition, and then FcεRIβ or FcεRIγ co-precipitated with FcεRIα was analyzed by immunoblotting with anti-FcεRIα mAb, anti-FcεRIβ mAb, or anti-FcεRIγ antibody. Coomassie brilliant blue (CBB) staining of the anti-FcεRIα mAb showed comparable sample loading. In (A) and (B), data shown are the mean $\pm$ S.D. of samples obtained from three independent experiments. In (C), the results shown are representative of three independent experiments with similar results.

whole cell lysates. Also, basal IL-6 secretion from unstimulated cells was below threshold of the minimum detectable concentrations (data not shown). These data indicate that LXR-activating oxysterols, principally 25-OHC, act as potent inhibitors of FcεRI-dependent mast cell activation.

3.2. LXR activation is not responsible for suppression of the degranulation response

To explore the participation of LXR activation in the suppression of IL-6 production and the degranulation response, we examined

the effects of a synthetic LXR agonist T0901317 on IL-6 production and the degranulation response. Fig. 2A shows that 0.4 μM T0901317 as well as 10 μM 25-OHC induced mRNA expression of ABCA1, a LXR-target gene, in BMMCs. 10 μM 25-OHC reduced LDLR mRNA expression but the synthetic LXR ligand did not reduce the expression. As shown in Fig. 2B, treatment of BMMCs with 0.4 μM T0901317 and 10 μM 25-OHC significantly reduced FcεRI-induced IL-6 production. On the other hand, the same treatment with T0901317 failed to reduce the degranulation response (Fig. 2C). These results suggest that alternative machineries are involved in 25-OHC-mediated inhibition of the degranulation response. We

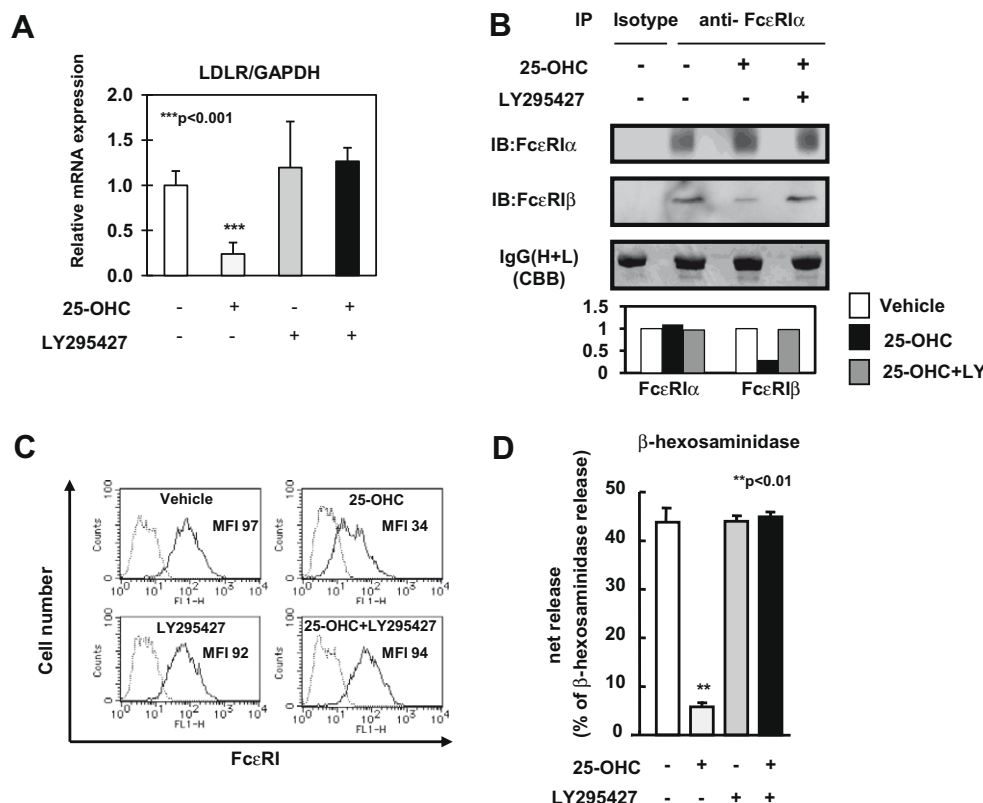


Fig. 5. LY295427 restores decreased FcεRI assembly and degranulation response in mast cells treated with 25-OHC. BMMCs (5×10^5 /ml) were treated with 10 μM 25-OHC or with 25-OHC plus 20 μM LY295427 for 48 h. (A) Quantitative real-time PCR analyzes for expression of LDLR mRNA. Data shown are the mean $\pm$ S.D. of samples obtained from three independent experiments. (B) Effects of LY295427 on FcεRI assembly. The immunoprecipitates were prepared from cells (1×10^7 /ml) as in Fig. 4C. FcεRIβ co-precipitated with FcεRIα was analyzed by immunoblotting with anti-FcεRIα mAb or anti-FcεRIβ mAb. (C) Effects of LY295427 on cell-surface FcεRIα expression. (D) Effects of LY295427 on degranulation response. In (B) and (C), the results shown are representative of three independent experiments with similar results. In (D), data shown are the mean $\pm$ S.D. of triplicates. Similar results were obtained from three independent experiments. In (A) and (D), Student's *t*-test was performed between the cells treated with vehicle and the other cells.

observed that 25-OHC, but not T0901317, considerably down-regulates expression of cell-surface FcεRI (Fig. 2D).

3.3. 22(S)-OHC fails to antagonize 25-OHC-mediated inhibition of FcεRI expression and degranulation response

We employed 22(S)-OHC, a competitive LXR antagonist [9], to clarify the participation of LXR activation in the 25-OHC-mediated suppression of FcεRI expression and the degranulation response. Fig 3A and B show that the LXR antagonist did not reverse the suppression of FcεRI expression and degranulation response in mast cells treated with 25-OHC.

3.4. 25-OHC decreases assembly of FcεRIα and FcεRIβγ in mast cells

Because cell-surface FcεRI expression is a critical element in the FcεRI-dependent mast cell response [10], we examined the mechanism for down-regulation of FcεRI by 25-OHC. As shown in Fig. 4A, 25-OHC reduced binding of IgE-Alexa488 to the FcεRI in a concentration-dependent manner. Consistent with this observation, FcεRIα expression on the cell surface was decreased in BMMCs treated with 25-OHC (Fig. 4B). However, the expression of FcεRIα, β, and γ were unaltered at both the mRNA and protein levels (data not shown). Because the appropriate assembly of FcεRI components is indispensable for cell-surface FcεRI expression [11], we examined whether 25-OHC affects assembly of FcεRI in BMMCs. Biochemical analyzes using immunoprecipitation with anti-FcεRIα mAb show that the association between FcεRIα and

FcεRIβγ was significantly decreased in BMMCs treated with 10 μM 25-OHC (Fig. 4C).

3.5. LY295427 reverses suppression of FcεRI assembly and degranulation response in mast cells treated with 25-OHC

Sterol regulatory element-binding proteins (SREBPs) regulate the expression of LDLR at the level of transcription [12]. As demonstrated in Fig. 2A, 25-OHC significantly downregulated the expression of LDLR mRNA in BMMCs, suggesting that 25-OHC decreased the efficacy of SREBPs in mast cells. LY295427 restores the dysregulation of SREBPs at the endoplasmic reticulum (ER) induced by 25-OHC, resulting in up-regulation of SREBP target genes including LDLR [13]. In addition, it was reported that FcεRIα assembles with FcεRIβ in the ER [14]. We therefore considered that LY295427 may reverse the inhibitory actions of 25-OHC on FcεRI expression and the degranulation response in mast cells. Fig. 5A shows that 20 μM LY295427 restored the decrease in LDLR mRNA expression in BMMCs treated with 10 μM 25-OHC. As shown in Fig. 5B and C, decreases in cell-surface expression of FcεRI as well as assembly of FcεRIα and FcεRIβ in 25-OHC loaded cells, were nearly reversed by treatment with 20 μM LY29542. Furthermore, suppression of the degranulation response was also restored (Fig. 5D).

4. Discussion

In the present study, we demonstrated that inhibitory actions of 25-OHC on FcεRI-mediated mast cell activation occur via

LXR-dependent and -independent mechanisms. With regard to LXR-dependent inhibition, we showed that LXR activation mainly contributed to 25-OHC-mediated repression of IL-6 production. However, we believe that the LXR-independent mechanism is also involved in the reduction of IL-6 production observed in mast cells treated with 25-OHC given that the inhibitory effect of the specific LXR ligand T0901317 on IL-6 production was much less than that of 25-OHC (Fig. 2B). In macrophages, trans-repression through nuclear receptor co-repressor is responsible for LXR-mediated repression of pro-inflammatory gene expression in response to lipopolysaccharide [3], but it is unclear whether the same mechanism is responsible for LXR-mediated repression of the FcεRI-mediated IL-6 production in mast cells. Therefore, we are currently investigating the underlying mechanism of this trans-repression in mast cells.

Figs. 2D and 3A collectively indicate that one of the LXR-independent effects of 25-OHC on mast cells is down-regulation of FcεRI at the cell surface. Murine FcεRIα does not assemble with FcεRIγ in the absence of FcεRIβ [14]; we thus concluded that decreased cell-surface FcεRI expression is principally due to reduction of the association between FcεRIα and FcεRIβ. LY295427 nearly reversed the effects of 25-OHC on assembly between FcεRIα and FcεRIβ (Fig. 4B). We could not elucidate how SREBPs maintain FcεRI assembly in BMMCs. Because biological functions of SREBPs are affected by ER stress including the accumulation of unfolded proteins [16–18], we believe that SREBPs might participate in ER quality control, which is indispensable for the appropriate assembly of FcεRI.

As compared to 25-OHC, 22(R)-OHC showed slight suppressive effects on IL-6 production and degranulation response of mast cells although they are structurally very similar. A part of the differences may be explained by a previous report, which demonstrated 25-OHC is stronger repressor for function of SREBPs than 22(R)-OHC [19]. Alternatively, different LXR ligand-complex formations might lead to different biological actions including selective modulation. In tissues or cells, 25-OHC is synthesized from cholesterol by cholesterol 25-hydroxylase [20]. In general, however, low levels (several nM) of 25-OHC are present in human plasma [21]. Therefore, administration of 25-OHC may be one of practical approaches for the treatment of allergic inflammation. In addition, a previous study reported that T0901317 attenuates IgE synthesis by human and murine B cells at 10^{-8} M. On the other hand, T0901317 reduced FcεRI-dependent cytokine production from BMMCs at 10^{-6} M. Since IgE production is maximally inhibited in B cells treated with 10^{-6} M T0901317, at the concentration of the LXR agonist, it might exhibit synergistic anti-allergic effects.

In conclusion, we addressed novel actions of oxysterols on FcεRI-dependent mast cell activation, and our findings in the present study provide useful information for future therapeutic strategies for the prevention of allergic inflammation.

5. Uncited reference

[15].

Acknowledgements

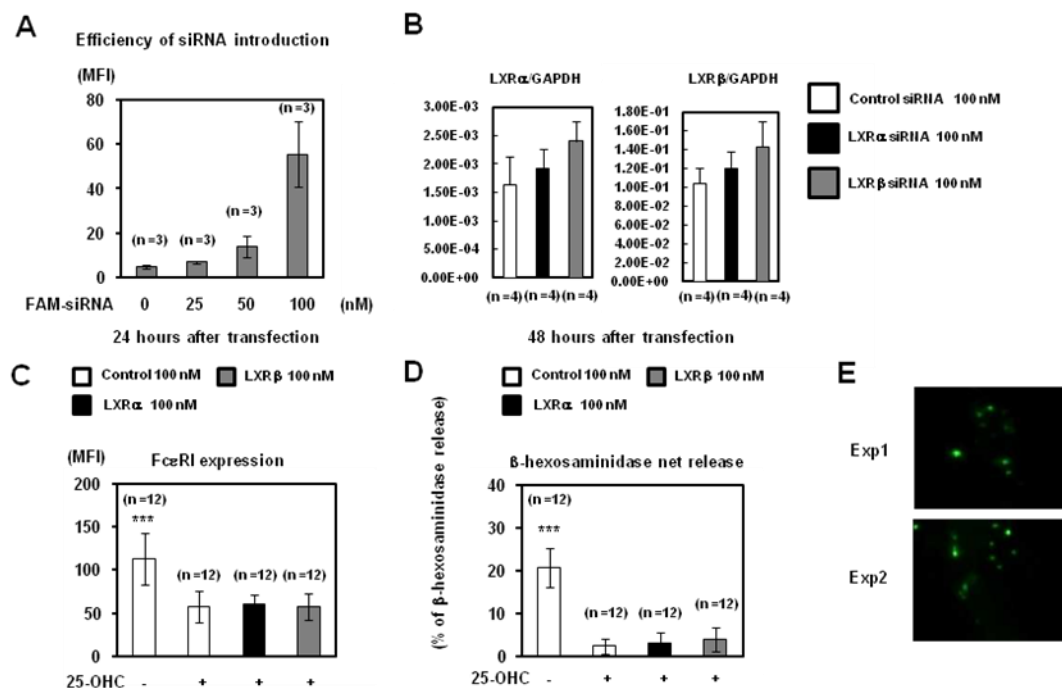
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.02.006.

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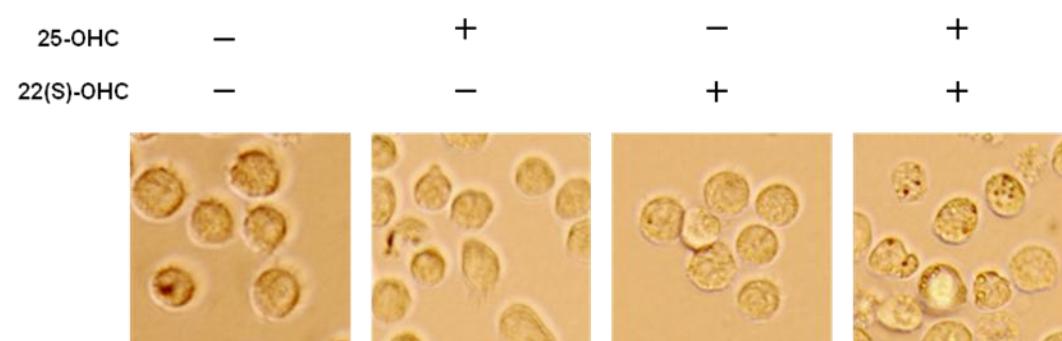
Supplemental data1. (A) BMMCs (5×10^5) were cultured with FAM-labeled siRNA at the indicated concentrations for 24 hours. (B) 48 hours after transfection, quantitative PCR analyses for LXR α and LXR β were performed. In A and B, the data shown are mean $\pm$ SD obtained from 3 or 4 separate experiments. After siRNA transfection, the cells were treated with or without $10 \mu\text{M}$ 25-OHC. (C) Fc ϵ RI expression was analyzed by Flow cytometry using IgE and anti-IgE FITC. (D) Degranulation response was analyzed. In C and D, the data shown are mean $\pm$ SD ($n = 12$) obtained from 4 separate experiments. (E) Intracellular distribution of FAM-labeled siRNA in BMMCs 48 hours after transfection.

Supplemental data1

Introduction of siRNA

Silencer Select Pre-designed siRNAs for murine LXRs (#s75786, #s202340) and negative control siRNA (#AM4611) were purchased from Applied Biosystems. 2 μ l of TransIT-TKO reagent (Mirus, Madison, WI) was diluted in 50 μ l of Opti-MEM. Ten minutes later, 1 μ l of 50 μ M siRNA was added to the diluted reagent and further incubated for 20 min. The siRNA solution was added to 5×10^5 BMSCs in 500 μ l of medium and then cultured for 48 hours.

Supplemental Methods



Supplemental data2. BMSCs (5×10^5) were cultured with or without 5 μ M 25-OHC in the presence or absence of 5 μ M 22(S)-OHC for 48 hours.

Supplemental data2