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Involvement of 15-Lipoxygenase in the Inflammatory Arthritis

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ABSTRACT

15-Lipoxygenase (15-LOX) is involved in many pathological processes. The aim of this study is to examine the role of 15-LOX in the matrix metalloproteinase (MMP) expression and inflammatory arthritis. It was found that treatment of 15-LOX downstream product of 15-(S)-HETE (15-S-hydroxyeicosatetraenoic acid) increased the mRNA and protein levels of MMP-2 in rheumatoid arthritis synovial fibroblast (RASF) derived from rheumatoid arthritis patients. The enhancement effect of 15-(S)-HETE was antagonized by the addition of LY294002 (PI3K inhibitor) and PDTC (NF-κB inhibitor). Treatment of 15-(S)-HETE increased the phosphorylation of AKT, nuclear translocation of p65 and the breakdown of IκBα. TNF-α and IL-1β are the key cytokines involved in arthritis and also increase the activity of MMP-2 in RASF, which was antagonized by pretreatment with 15-LOX inhibitor PD146176 or knockdown of 15-LOX. It was also found that these two cytokines increased the expression of 15-LOX in RASF. Treatment of glucocorticoid but not NSAIDs inhibited 15-(S)-HETE-induced expression of MMP-2. In comparison with wild-type mice, adjuvant-induced arthritis and MMP-2 expression in synovial membrane were markedly inhibited in 15-LOX knockout (KO) mice. These results indicate that 15-LOX plays an important role in the disease progression of arthritis and may be involved in the inflammatory action induced by TNF-α and IL-1β. 15-LOX is thus a good target for developing drugs in the treatment of inflammatory arthritis. J. Cell. Biochem. 113: 2279–2289, 2012.

KEY WORDS: ARTHRITIS; LIPOXYGENASE; METALLOPROTEINASE; TNF-α; IL-1β

Inflammatory arthritis often causes the damage of joint. Many kinds of cells infiltrate into the joint cavity during arthritis, including immune cells (such as macrophage, T cells, B cells, etc.) and erosive cells [such as osteoclasts and synovial fibroblasts (SFs)]. Among these, the SFs play a critical role in the destructive process, which become invasive and hyperproliferative during inflammation. The rheumatoid arthritis synovial fibroblasts (RASFs) are described as tumor-like because they are relatively resistant against apoptosis [Korb et al., 2009]. At the site of arthritic lesion, RASFs invade bone and cartilage to form the inflammatory pannus, where many kinds of immune cells and erosive cells can interact with each other to cause vicious cycle.

Within the synovial pannus, secretion of proinflammatory cytokines and expression of matrix metalloproteinase (MMP) lead to the deterioration of joint destruction. MMPs are the family of zinc-dependent proteinases, which play a major role in the cartilage destruction, bone degradation, and regulation of inflammatory response [Murphy and Lee, 2005]. It has been reported that RASFs can secrete collagenase (MMP-1, MMP-8, MMP-13) and gelatinase (MMP-2, MMP-9) [Konttinen et al., 1999]. Compared with healthy control, MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13 are upregulated in RA patients [Itoh et al., 2002; Mahmoud et al., 2005; Chang et al., 2008]. Collagenases and gelatinases are higher in joint fluid of RA patients than that in osteoarthritis (OA) [Kim et al., 2011]. Although MMP-1 and MMP-13 are the major proteinases for the cartilage degradation [Muller-Ladner and Gay, 2002], all other MMPs have also been detected in RA synovium [Konttinen et al., 1999; Pap et al., 2000; Yoshihara et al., 2000]. Of these MMPs, the

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gelatinase, especially MMP-2, plays an important role in tissue invasion [Kleiner and Stetler-Stevenson, 1999] and may be involved in the pannus invasion in RA. In several malignancies, levels of active MMP-2 can predict the mortality and disease severity [Cockett et al., 1998; Kleiner and Stetler-Stevenson, 1999]. It has also been reported that patients with erosive arthritis have significantly higher levels of active MMP-2 in synoviocytes than patients without erosions [Goldbach-Mansky et al., 2000]. In addition, MMPs are important in angiogenesis and MMP-2 can modulate the angiogenic activity of FGF [Moses, 1997], which is prominent in RA [Walsh, 1999]. Numerous cytokines are highly expressed in RA synovial tissue, such as TNF and interleukin family [McInnes and Schett, 2007]. IL-1β and TNF-α play a critical role in the pathogenesis of RA through a complex regulatory network, including the stimulation of MMPs synthesis [Migita et al., 1996; Eberhardt et al., 2000; Catrina et al., 2002]. In human synoviocytes, mechanical compression increased the MMP-2 production, which was synergistically upregulated by the co-existence of TNF-α [Wang et al., 2010].

The synovial fluid normally contains extremely low concentration of lipoprotein and apolipoprotein, which is enhanced in arthritis patients [Prete et al., 1995]. The lipid is mainly from blood [Navarro et al., 2000] and arachidonic acid (AA) is a key inflammatory intermediate from the lipid composition. In response to a variety of stimuli, AA is released from membrane phospholipid by phospholipase. It has been reported that phospholipase A2 (PLA2) activity is increased in arthritis [Pruzanski et al., 1985], and IL-1 and TNF-α, the most abundant cytokines in synovial fluids, can stimulate the activity of PLA2 [Bomalaski and Clark, 1993; Gilman et al., 1988]. Both cyclooxygenase (COX) and lipoxygenase (LOX) pathways are involved in the inflammatory pathway related to AA [Laufer, 2003]. The COX pathway produces prostaglandins, which are well-known inflammatory mediators; and the LOX pathway is another eicosanoid family that plays a role in chemoattraction, inflammation and immune function. LOXs are dioxygenases that insert oxygen into polyunsaturated fatty acids, resulting in the formation of hydroxyeicosatetranoic acid (HETE), leukotriene (LT), and lipoxins [Kim et al., 2008]. 15-Lipoxygenase (15-LOX) is involved in many pathological processes, such as asthma [Andersson et al., 2008], heart failure [Kayama et al., 2009], atherosclerosis [Wittwer and Hersberger, 2007], and insulin resistance in adipocytes [Chakrabarti et al., 2009]. However, the role of 15-LOX in arthritis is still not clear.

Here we found that the 15-LOX downstream product of 15-(S)-HETE (15-S-hydroxyeicosatetraenoic acid) increased the activity of MMP-2 through AKT and NF-κB pathway in RASF. TNF-α and IL-1β, which are important pathological cytokines in arthritis, could increase the MMP-2 activity via the activation of 15-LOX in RASF. Furthermore, inflammatory arthritis was markedly inhibited in 15-LOX KO mice, indicating that 15-LOX plays an important role in inflammatory arthritis.

MATERIALS AND METHODS

CELL CULTURES

Human SFs were isolated by collagenase treatment from synovial tissue obtained from knee replacement surgeries of patients with RA after approval by the local ethic committee. Fresh synovial tissues were minced and digested in a solution containing collagenase and DNase. Isolated fibroblasts were filtered through 70 μm nylon filters. The cells were grown on the plastic cell culture dishes in 95% air–5% CO_2 with RPMI 1640 (Gibco, Grand Island, NY), which was supplemented with 20 mM HEPES and 10% heat-inactivated FBS, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml; pH adjusted to 7.6). The cells were grown on the plastic cell culture dishes in 5% CO_2.

ZYMOGRAPHY AND ELISA

MMP-2 activity was analyzed by gelatin zymography. The RASFs were starved in 1% FBS medium, and then treated with 15-(S)-HETE (Cayman Chemical Company) for 24 h. The media were collected and mixed with SDS gel non-reducing loading buffer. The samples were run in the 10% polyacrylamide gel containing 2 mg/ml gelatin. The gel was then incubated in developing buffer (1% Triton X-100, 10 mM $CaCl_2$ in 50 mM Tris–HCl buffer) overnight at 37°C. Finally, the gel was stained with Coomassie blue buffer (0.12% Coomassie blue, 50% methanol, 10% acetic acid) and destained.

Cells were treated with 15-(S)-HETE in 1% FBS medium for 24 h, and the supernatant was collected for the ELISA analysis of total MMP-2 (R&D Systems, Minneapolis, MN). Briefly, 100 μl Assay Diluent was added to each well, and then incubated with 100 μl supernatant for 2 h at room temperature. After aspirating and washing four times, 200 μl conjugate was added and incubated for 2 h. The washing step was repeated and 200 μl substrate solution was added for 20 min. Finally, the Stop Solution was added and read at 450 nm.

THE QUANTITATIVE AND SEMI-QUANTITATIVE RT-PCR

RASFs were treated with 15-(S)-HETE for indicated time intervals, and mRNA was then analyzed by reverse transcription polymerase chain reaction (RT-PCR) or real-time PCR. Total RNA was extracted from RASF by using TRIzol kit (MDBio, Inc.). RNA was analyzed by using two-step SuperScriptIII and Taq polymerase. In semi-quantitative RT-PCR, human MMP-2, 15-LOX, TNF-α, and IL-1β primer sequences were used as following.

MMP-2, forward: 5′-TTCAGACAACCTGAGTCCTT-3′, reverse: 5′-ACTTCAGGCTCTTCTCCTTT-3′ (NM_004530.4); 15-LOX, forward: 5′-GCCAAGGGGCTGGCCGACCT-3′, reverse: 5′-TGGTGGGGATCC-TGTGCGGGGCA-3′ (NM_001140.3); TNF-α, forward: 5′-CGAAT-CGCCGTCTCCTACC-3′, reverse: 5′-GGGAAGGTTGGATGTTCGTCC-3′ (NM_000594.2); IL-1β, forward: 5′-GATGAAGTGCTCCTTCCAG-G-3′, reverse: 5′-GGAGAACACCACTTGTTGCT-3′ (NM_000576.2).

The reaction started with a denaturation step at 95°C for 45 s. Annealing was performed at 55°C (for MMP-2), 62°C (for 15-LOX), 56°C (for IL-1β), or 54°C (for TNF-α) for 45 s and elongation at 72°C for 45 s. Amplification was accomplished at 28–37 cycles. PCR products were then separated electrophoretically in a 2% agarose DNA gel and stained with ethidium bromide. In quantitative real-time PCR, MMP-2 probe (Hs00234422, Applied Biosystems, Foster City, CA) was used, and the condition was: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, and 60°C for 1 min. Reactions were normalized to copies of *GAPDH* mRNA within the same sample

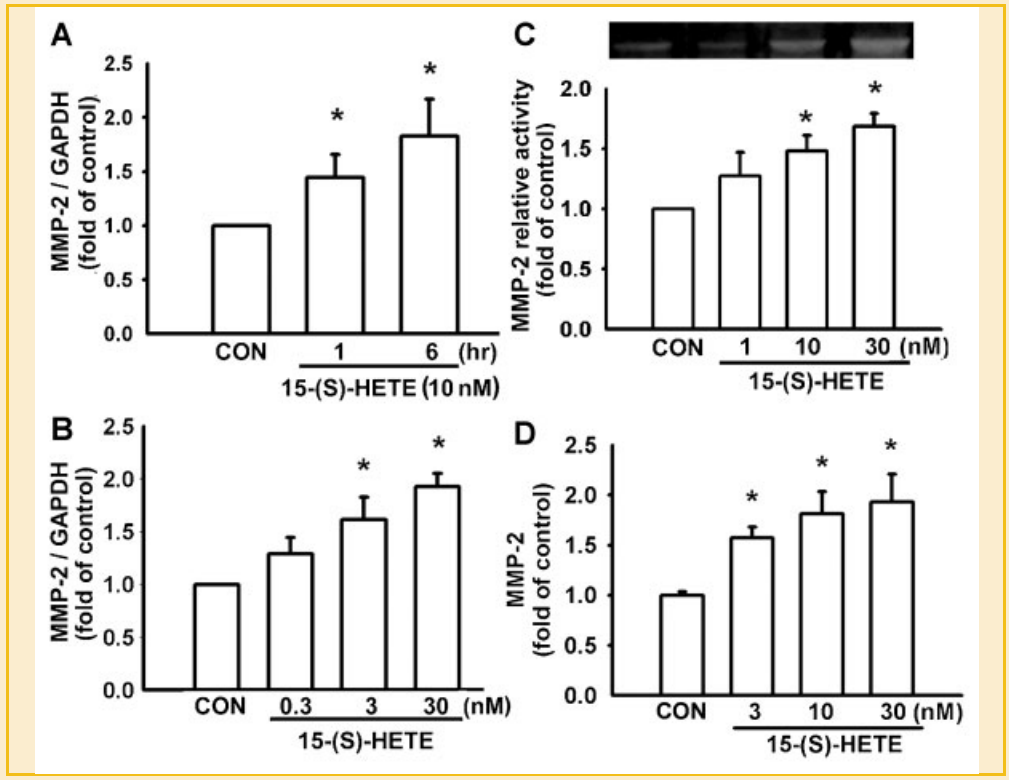

Fig. 1. Upregulation of MMP-2 expression by 15-(S)-HETE in human synovial fibroblasts. A: Quantitative PCR shows that treatment of 15-(S)-HETE (10 nM) for the indicated time intervals increased the mRNA expression of MMP-2 in RASF. B: Treatment of different concentrations of 15-(S)-HETE for 6 h, the MMP-2 mRNA increased in a concentration-dependent manner in RASFs. RASFs were treated with various concentrations of 15-(S)-HETE for 24 h, and the supernatant was collected for the analysis of MMP-2 activity by zymography (C) or ELISA (D). Data are presented as Mean ± SEM (n = 3). $^{*}P < 0.05$ compared with control (CON).

using the $^{-\Delta\Delta}C_T$ method. The levels of mRNA are expressed as the fold change in expression level compared with that of controls.

shRNA TRANSFECTION

RASFs were cultured on 6-well plate at 50% confluence and transfected with 15-LOX shRNA (sequence: GCTATCAAAGACTCTC-TAAAT) using Oligofectamine reagent (Invitrogen, Carlsbad, CA) in serum- and antibiotic-free media for 6 h at 37°C. Cells were allowed to recover with serum containing medium without removal of the transfection mixture and incubated for additional 24 h. Cells were treated with TNF-α or IL-1β in 1% FBS medium, and the RNA were then collected at the indicated time intervals.

WESTERN BLOTTING

Protein lysates were prepared from cell culture or mice paw. Protein concentration was determined using the bicinchoninic acid protein assay. Thirty micrograms of protein extract were loaded in each lane of a 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and run at 120 V. Protein was transferred to a nitrocellulose membrane at 22 V (80 mA) overnight at 4°C. After blocking, the membrane was incubated with an MMP-2 antibody (1 μg/ml, Abcam, Cambridge, UK) overnight at 4°C and then washed by phosphate buffered saline with Tween-20. After washing, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, followed by three

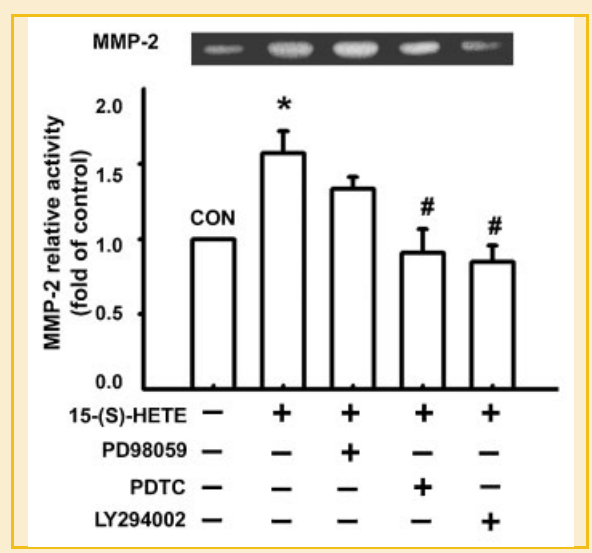

Fig. 2. PI3K and NF-κB pathways are involved in 15-(S)-HETE-induced MMP-2 upregulation. Human synovial fibroblasts were pretreated with PD98059 (ERK inhibitor), PDTC (NF-κB inhibitor), or LY294002 (PI3K inhibitor) for 30 min, and then treated with 15-(S)-HETE (10 nM) for another 24 h. The supernatant was collected for the zymography analysis. The summarized results were shown in the lower panel. Data are presented as Mean ± SEM (n = 3). $^{*}P < 0.05$ compared with control (CON). $^{\#}P < 0.05$ compared with 15-(S)-HETE treatment alone.

15-min washings. The labeling was visualized by the addition of chemiluminescence reagent.

NUCLEAR/CYTOSOLIC EXTRACTION

For the analysis of NF-κB p65 translocation, nuclear and cytosolic fractions were separated by using NE-PER kit (Thermo Scientific, Pierce, Rockford, IL). Briefly, RASF cells were suspended in tube with ice-cold CER I and vortex vigorously for 15 s. Ten minutes after chilling, ice-cold CER II were added, and vortexed for 5 s. One minute after chilling, tube was vortexed for 5 s and centrifuged for 5 min. The supernatant was collected as cytoplasmic extract, and the pellet was transferred to another tube with ice-cold NER. The tube was vortexed for 15 s and chilling for 15 s every 10 min, for a total of 40 min. The tube was then centrifuged for 10 min, and the supernatant was colleted as nuclear extract.

IMMUNOFLUORESCENT CONFOCAL MICROSCOPY

Cells were fixed with 4% paraformaldehyde and blocked with 1% BSA and 0.1% Triton X-100 in PBS for 15 min. Cells were then incubated with anti-NF-κB p65 primary antibody (1 μg/ml). After 2 h, cells were washed and incubated with the secondary antibody conjugated with Alexa flour 488 (4 μg/ml, Invitrogen) for 1 h. To confirm the location of nucleus, cells were washed and incubated with 0.1% DAPI for 10 min. The slides were then mounted and detected by immunofluorescence confocal microscope (Leica, TCS SP5).

INDUCTION OF ARTHRITIS IN C57/B6 MICE

All protocols complied with institutional guidelines and were approved by Animal Care Committees of Medical College, National Taiwan University. The complete Freund's adjuvant containing heat-killed *Mycobacterium tuberculosis* H37Ra (Difco) was injected into the right hind paw of C57/B6 mice (The Jackson Laboratory), and the contralateral left side was injected with saline for comparison. The volume of the mice paw was evaluated by paw volume meter every week, and the mice were sacrificed at week-5. The calculation of the percent of swelling was shown as

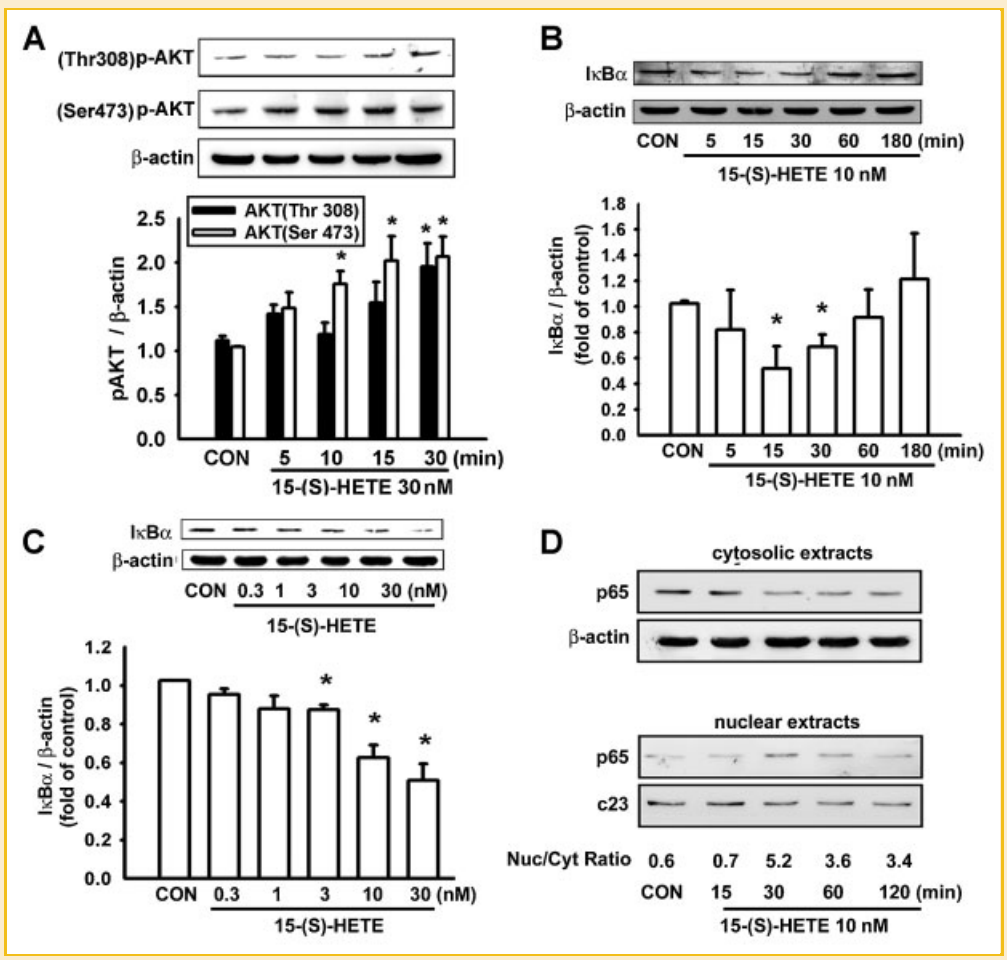

Fig. 3. Activation of AKT and NF-κB by 15-(S)-HETE in human synovial fibroblasts. A: Treatment of 15-(S)-HETE (30 nM) increased the phosphorylation of AKT at both sites of Thr 308 and Ser 473 in a time-dependent manner. B: Treatment of 15-(S)-HETE (10 nM) caused the degradation of IκBα time-dependently in RASF. C: Treatment of 15-(S)-HETE for 30 min enhanced the degradation of IκBα in a concentration-dependent manner. D: After treatment of 15-(S)-HETE (10 nM) for the indicated time intervals in RASFs, the cytosolic (Cyt) and nuclear (Nuc) proteins were then separated for the Western blot analysis of p65. Actin and c23 were used as the cytosolic and nuclear internal control, respectively. Note that 15-(S)-HETE increased the translocation of p65 into the nucleus. CON, control.

following:

$$\left[\left(\frac{R_{Wn}-R_{W0}}{R_{W0}}\right)-\left(\frac{L_{Wn}-L_{W0}}{L_{W0}}\right)\right]\times 100\%$$

R_{Wn} is the volume of right paw at week n, R_{W0} is the volume of right paw at week 0, L_{Wn} is the volume of left paw at week n, and L_{W0} is the volume of left paw at week 0.

After perfusion with PBS to remove blood, the paw was fixed in 4% paraformaldehyde for 2 days, and decalcified in 10% EDTA for 2 weeks. The slices of 5 μm thick were used for immunohistochemistry.

IMMUNOHISTOCHEMISTRY

The paraffin embedded tissues were used for the immunohistochemical analysis of MMP-2 or CD45 expression in the paw joint of mice. Briefly, slices at 5 μm thick were deparaffinized and rehydrated, and then treated with 3% H_2O_2 in methanol for 10 min. After retrieving with 0.05% protease XIV at 37°C for 10 min, the slices were washed and blocked with 5% BSA and 1% Triton X-100 in PBS for 1 h. The slices were then incubated with the primary antibody at 4°C overnight (MMP-2 or CD45 antibody 5 μg/ml, Abcam). After washing with PBS, the slices were incubated with secondary antibody (Vector, CA) for 1 h. Staining was performed using ABC kit (Vector) and DAB reaction.

STATISTIC ANALYSIS

The values given are Means ± SEM. The significance of difference between the experimental group and control was assessed by ANOVA and Student's *t*-test. The difference is significant if the *P*-value is less than 0.05.

RESULTS

UPREGULATION OF MMP-2 EXPRESSION BY 15-(S)-HETE IN RASF

RASFs were starved overnight with 1% serum, and then treated with 15-(S)-HETE (30 nM) for different time periods. The quantitative PCR results show that MMP-2 mRNA increased in a time-dependent manner (1.8 ± 0.3-fold of control at 6 h, Fig. 1A). Treatment of different concentrations of 15-(S)-HETE for 6 h, mRNA was then extracted from RASF (Fig. 1B). MMP-2 mRNA was also increased by 15-(S)-HETE in a concentration-dependent manner (1.6 ± 0.2 and 1.9 ± 0.1-fold of control at 3 and 30 nM, respectively). The supernatant was collected 24 h after addition of 15-(S)-HETE for gelatin zymography (Fig. 1C). The zymography (Fig. 1C) and

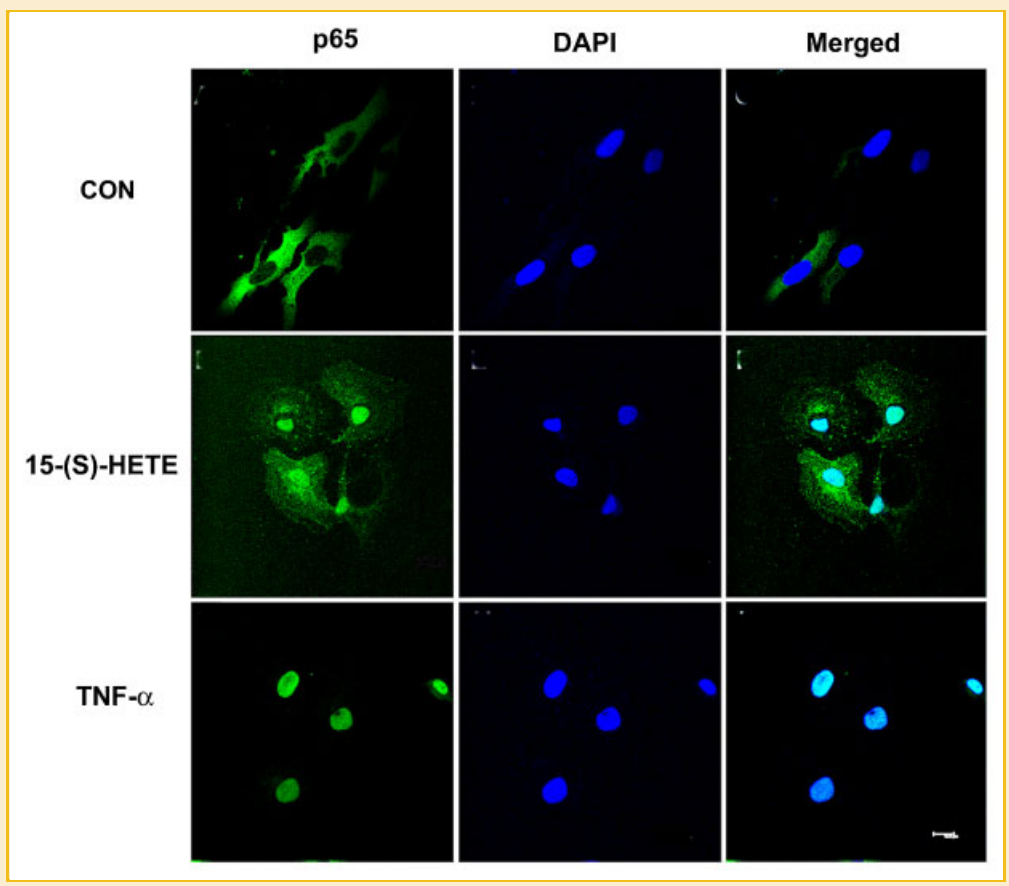

Fig. 4. Nuclear translocation of NF-κB p65 subunit is enhanced by 15-(S)-HETE in RASF. RASFs were treated with 15-(S)-HETE (10 nM) for 30 min. Nuclear translocation of the NF-κB p65 subunit was assessed by immunofluorescent confocal microscopy. The nuclear location was confirmed by DAPI fluorescence. TNF-α treatment was used as the positive control. Note that p65 translocated into nucleus after 30 min treatment of TNF-α and 15-(S)-HETE. Scale bar: 15 μm. CON, control.

ELISA analysis (Fig. 1D) showed that 15-(S)-HETE increased the release of MMP-2 from RASF concentration-dependently, whereas, the contents of MMP-1 and MMP-9 were not detectable by using respective ELISA kits. Since MMP-2 was much more prominent in RASFs, the effect of 15-(S)-HETE on the expression of MMP-2 was thus examined in the following experiments.

15-(S)-HETE INCREASES MMP-2 EXPRESSION VIA PI3K AND NF-κB SIGNALING PATHWAYS

To examine which signaling pathway is involved in 15-(S)-HETE-mediated upregulation of MMP-2, several signaling inhibitors were used. RASFs were starved overnight with 1% serum, and then pretreated with PD98059 (MEK1 inhibitor, 20 μM), PDTC (NF-κB inhibitor, 10 μM), or LY294002 (PI3K inhibitor, 20 μM) for 30 min.

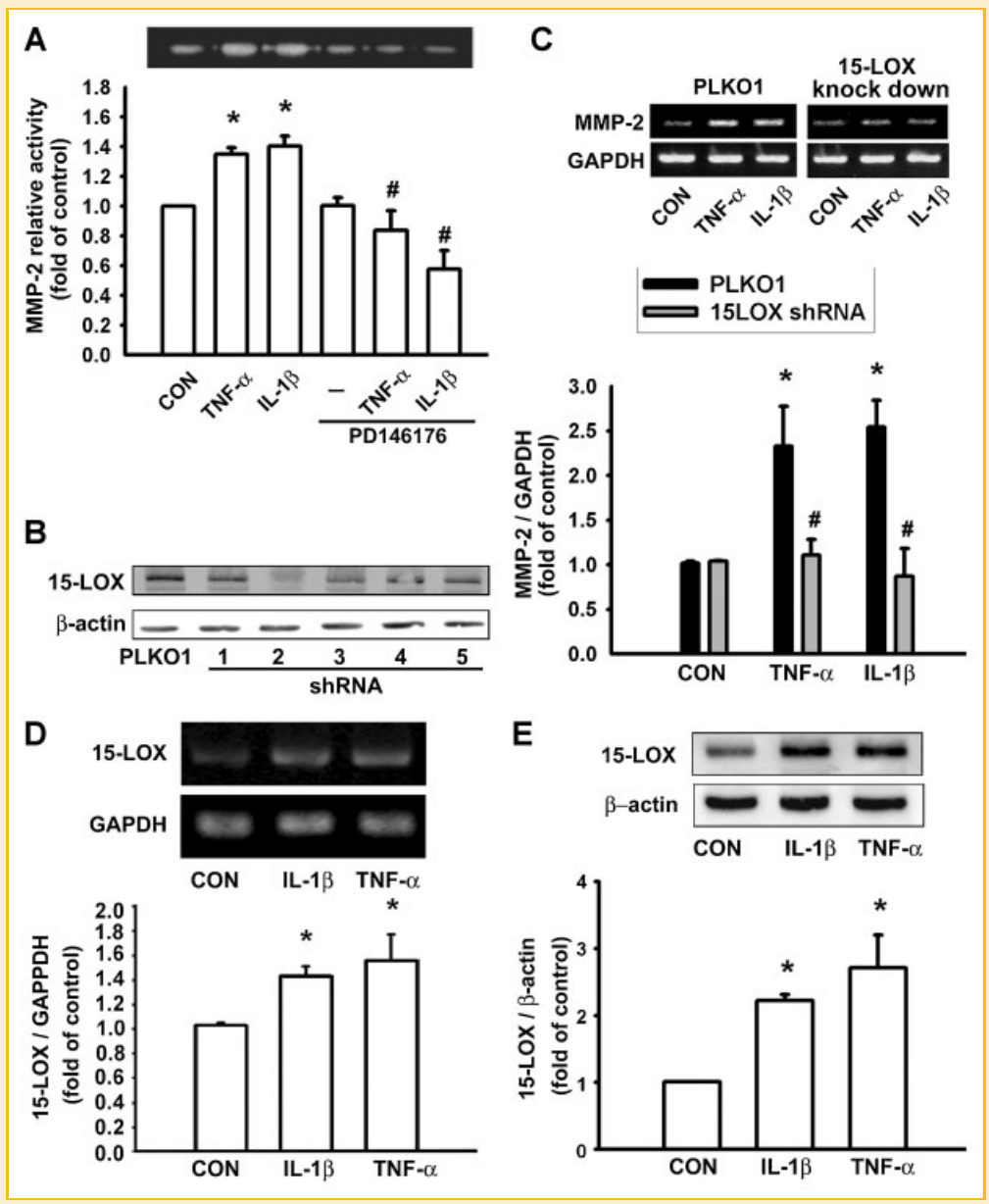

Fig. 5. 15-LOX is involved in TNF-α- or IL-1β-induced upregulation of MMP-2 expression in human synovial fibroblasts. A: Treatment of TNF-α (10 ng/ml) or IL-1β (10 ng/ml) for 24 h increased the zymography activity of MMP-2 in the supernatant of RASF. Pre-treatment of 15-LOX inhibitor PD146176 for 30 min antagonized the potentiating action on MMP-2 release by TNF-α and IL-1β. B: RASFs were transfected with control shRNA (PLKO1) or 15-LOX shRNA (sh1–sh5) for 24 h, and the cell lysate were collected for Western blotting. 15-LOX shRNA2 (sh2) was selected for the following experiments. C: RASFs were transfected with 15-LOX shRNA 2 for 24 h, and TNF-α or IL-1β was then added. After 3 h, the RNA was extracted. Note that MMP-2 upregulation by these two cytokines was inhibited by the knockdown of 15-LOX. The summarized results were shown in the lower panel. Human synovial fibroblasts were stimulated with TNF-α (10 ng/ml) or IL-1β (10 ng/ml). For RT-PCR experiment (D), mRNA was isolated 12 h later, and for Western blot experiment (E), total protein was extracted 24 h later. Both TNF-α and IL-1β increased the mRNA and protein levels of 15-LOX in RASF. Data are presented as Mean ± SEM (n = 3). $^{*}P < 0.05$ compared with control (CON). $^{\#}P < 0.05$ compared with cytokine treatment alone.

The MMP-2 upregulation effect of 15-(S)-HETE in RASF was markedly antagonized by PDTC or LY294002, indicating that PI3K and NF-κB pathways were involved in the action of 15-(S)-HETE (Fig. 2).

Since PI3K and NF-κB inhibitors markedly antagonized the effect of 15-(S)-HETE, we then examined the activation of AKT and NF-κB by 15-(S)-HETE. As shown in Figure 3A, application of 15-(S)-HETE (30 nM) increased the phosphorylation of AKT-Ser 473 time-dependently, and AKT-Thr 308 was mainly phosphorylated at 30 min. In addition, 15-(S)-HETE increased the degradation of IκBα time-dependently (Fig. 3B). Treatment of 15-(S)-HETE for 30 min concentration-dependently enhanced the degradation of IκBα (Fig. 3C). Furthermore, the cytosolic and nuclear proteins from 15-(S)-HETE-treated RASF were then separated for the Western blot analysis. The results show that the nuclear/cytosolic ratio of p65 was increased up to 5.2-fold at 30 min treatment of 10 nM 15-(S)-HETE (Fig. 3D). To further confirm the NF-κB activation by 15-(S)-HETE in RASF, we examined the nuclear translocation of p65 subunit by using immunofluorescent confocal microscopy. In control group, p65 subunit remained in the cytoplasm, and treatment of 15-(S)-HETE (30 nM) for 30 min induced the translocation of p65 into the nucleus in RASF (Fig. 4). TNF-α (10 ng/ml) treatment was used as positive control.

15-LOX IS INVOLVED IN TNF-α- AND IL-1β-INDUCED UPREGULATION OF MMP-2 IN RASF

TNF-α and IL-1β are the most common cytokines involved in the disease progression of inflammatory arthritis. RASFs were thus treated with these two cytokines for 24 h, and the supernatant was collected for zymography. It was found that MMP-2 activity was increased by these two cytokines, and the effect was antagonized by pretreatment for 30 min with 15-LOX inhibitor PD146176 (Fig. 5A). The MTT assay shows that the survival rate was not affected by the addition of PD146176 (data not shown), indicating that the decrease of MMP-2 was not resulting from the decrease of cell number. We further examined the role of 15-LOX in the action of TNF-α and IL-1β by the transfection with 15-LOX shRNA for 24 h. Five sequences of shRNA were tested and the Western blotting showed that the expression of 15-LOX was markedly reduced by shRNA 2 (Fig. 5B). The shRNA 2 was thus used for further experiments. TNF-α- or IL-1β-induced upregulation of MMP-2 in RASF was markedly inhibited by the transfection with 15-LOX shRNA (Fig. 5C). The summarized results were shown in the lower panel. It was found that MMP-2 mRNA was increased to 2.3 ± 0.4- and 2.5 ± 0.3-fold of control by IL-1β and TNF-α, respectively. Knockdown of 15-LOX markedly inhibited the cytokine-induced MMP-2 mRNA expression. These results indicate that 15-LOX is involved in the MMP-2 upregulation by TNF-α and IL-1β. Since the TNF-α- or IL-1β-induced upregulation of MMP-2 was antagonized by 15-LOX inhibition, we further investigated whether the expression of 15-LOX in SFs is affected by these cytokines. RASFs were treated with TNF-α (10 ng/ml) or IL-1β (10 ng/ml) and total RNA or protein in the cell lysate were collected 12 or 24 h later, respectively. The RT-PCR (Fig. 5D) and Western blot (Fig. 5E) show that 15-LOX was upregulated in RASF by these two cytokines (protein expression: 2.2 ± 0.1-fold and 2.7 ± 0.5-fold of control for TNF-α and IL-1β, respectively).

INHIBITION OF 15-(S)-HETE-INDUCED UPREGULATION OF MMP-2 BY DEXAMETHASONE BUT NOT BY NSAID

NSAIDs and glucocorticoids are commonly used drugs for the treatment of inflammatory arthritis. Here we further investigated the effect of dexamethasone (1 μM), indomethacin (10 μM), and sulindac (10 μM) on the 15-(S)-HETE-induced upregulation of MMP-2 in RASF. The results of zymography show that pretreatment of dexamethasone (inhibition by $86.0 \pm 0.3\%$) but not NSAID (indomethacin and sulindac) antagonized the stimulatory effect of 15-(S)-HETE on MMP-2 expression in RASF (Fig. 6).

15-LOX KNOCKOUT INHIBITS THE INDUCTION OF INFLAMMATORY ARTHRITIS

To examine the in vivo role of 15-LOX in arthritis progression, the Freund's Complete Adjuvant H37Ra (FCA; containing 1 mg/ml *Mycobacterium tuberculosis* H37Ra, 50 μl) injection was used as the model of inflammatory arthritis in C57/B6 mice. Saline or FCA was injected into the right hind paw of mice, and the left hind paw was injected with saline for comparison. The paw volume was measured every week using paw volume meter, and the percent of swelling induced by FCA was compared between wild-type and 15-LOX knockout (KO) mice. The outlook appearance shows that the adjuvant-injected paw in wild-type was more swollen than the 15-LOX KO mice (Fig. 7A), the percent of swelling increased by 50% at

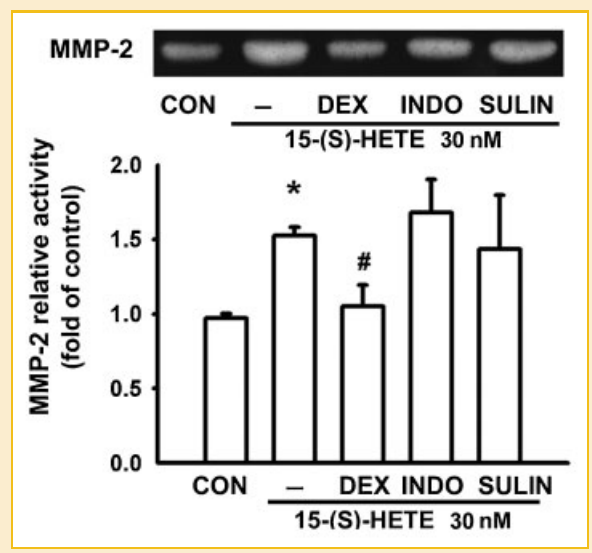

Fig. 6. Inhibition of 15-(S)-HETE-induced upregulation of MMP-2 by glucocorticoid in human synovial fibroblasts. Human synovial fibroblasts were pretreated with dexamethasone (DEX 1 μM), indomethacin (INDO 10 μM), or sulindac (SULIN 10 μM) for 30 min, and then treated with 15-(S)-HETE (10 nM) for another 24 h. The supernatant was collected for the zymography analysis. Note that dexamethasone but not NSAIDs inhibited the MMP-2 release from synovial fibroblasts. Data are presented as Mean ± SEM ($n = 3$). $^{*}P < 0.05$ compared with control (CON). $^{\#}P < 0.05$ compared with 15-(S)-HETE treatment alone.

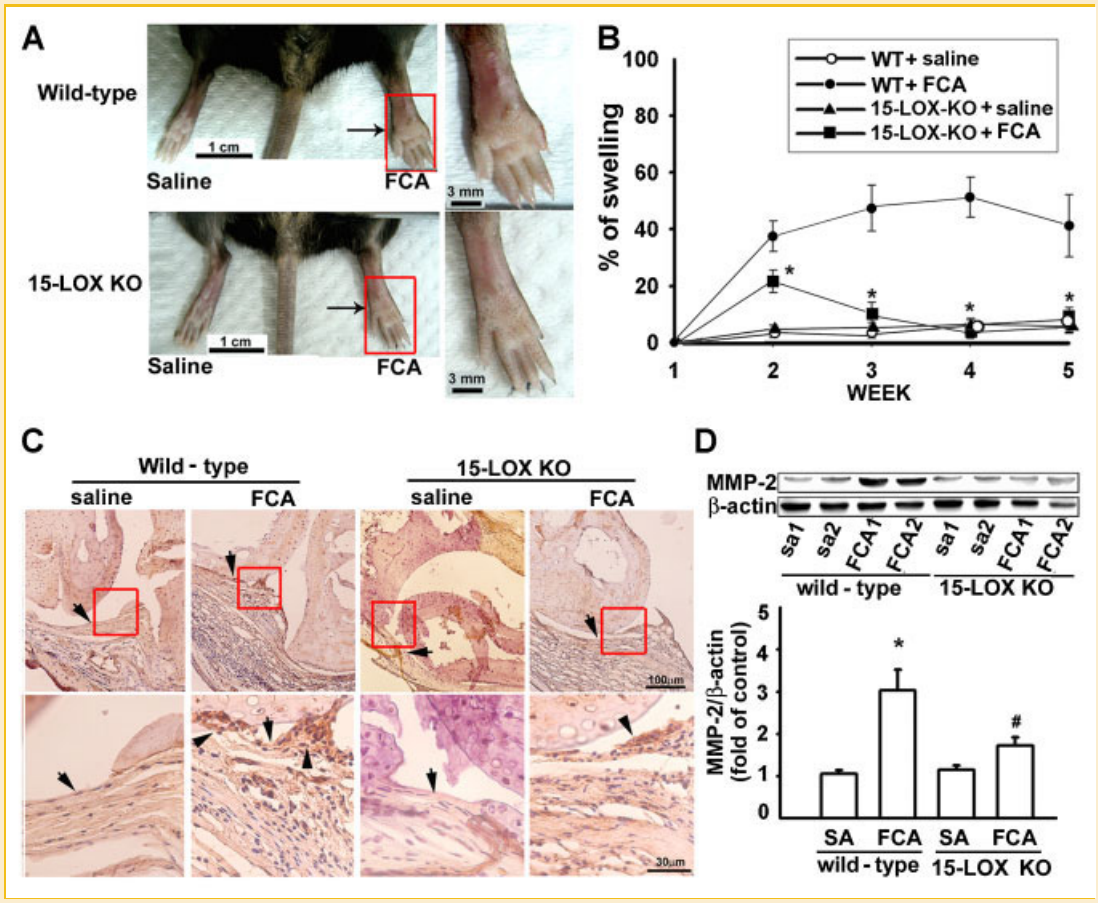

Fig. 7. Inhibition of arthritis induction in 15-LOX knockout mice. Arthritis was induced by injection of FCA into right paw and saline was injected into left paw for comparison. A: The mice were sacrificed at week 5. The enlargement of the red frame was shown in the right side. The swelling of the FCA-injected side was more prominent in wild-type mice than in 15-LOX knockout mice. B: The volume of Freund's complete adjuvant-injected right paw (FCA) and saline-injected left paw was measured weekly, and the percent of swelling was evaluated. Note that 15-LOX knockout markedly inhibited the induction of arthritis. C: Immunohistochemistry of MMP-2 in the paw joint of wild-type and 15-LOX KO mice was performed. The lower panels show the enlarged image of the frame in the upper panels. The synovial membrane (arrows) expressed higher MMP-2 staining (arrowheads) induced by FCA in wild-type than in 15-LOX KO mice. D: MMP-2 Western blotting was performed on paw tissue lysates prepared from wild-type and 15-LOX knockout mice. The representative samples from two mice were shown here. Sa, saline-injected left paw; FCA, FCA-injected right paw. Data are presented as Mean ± SEM ($n = 5$). $^{*}P < 0.05$ comparison of saline and FCA-injected paw in wild-type mice. $^{\#}P < 0.05$ comparison of FCA-injected paw between wild-type and 15-LOX KO mice. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

week-4 (Fig. 7B). However, there was only a 20% transient swelling in 15-LOX KO mice at week-2, and the swelling then gradually declined (Fig. 7B). Immunohistochemistry shows that there was more FCA-induced MMP-2 expression in wild-type mice than that in 15-LOX KO mice (Fig. 7C). MMP-2 also appeared in synovial membrane of wild-type mice following arthritis. In addition, the HE staining shows that more cells infiltrated into inflammation area in wild-type mice after FCA treatment. Protein lysate prepared from the mice paw were subjected to Western blotting, and the results show that MMP-2 was upregulated more prominently in wild-type mice than that in 15-LOX KO mice (Fig. 7D). To evaluate the cartilage damage caused by FCA-induced inflammation, the Safranin O staining shows that there was more destruction in the joint of wild-type mice than that in the 15-LOX KO mice (as shown by arrow in Fig. 8A). In addition, the CD45 immunohistochemistry staining shows that more leucocytes infiltrated into inflammation area in wild-type mice after FCA treatment (Fig. 8B,C).

DISCUSSION

It was found here that the 15-LOX downstream product of 15-(S)-HETE increased the expression of MMP-2 in human SFs derived from rheumatoid arthritis patients. 15-LOX is one of the LOXs involved in the inflammatory disorders such as asthma and arthritis. Treatment of IL-1β and IL-4 in SFs could increase the production of 15-(S)-HETE [Liagre et al., 1999]. In addition, treatment of 15-(S)-HETE enhances the differentiation of osteoclasts [Kronke et al., 2009b], which implicates the substantial role of 15-LOX in the pathogenesis of arthritis. There are many activated T helper cells in RA synovium [Aarvak and Natvig, 2001] and Th1 cells are able to promote RA [Rooney et al., 1989]. Th1 cells can also produce the proinflammatory cytokine of macrophage migration inhibitory factor (MIF) [Bacher et al., 1996], which is correlated with the disease activity of RA [Morand et al., 2002] and involved in the upregulation of MMP-2 in RASF [Pakozdi et al., 2006]. It has also been reported

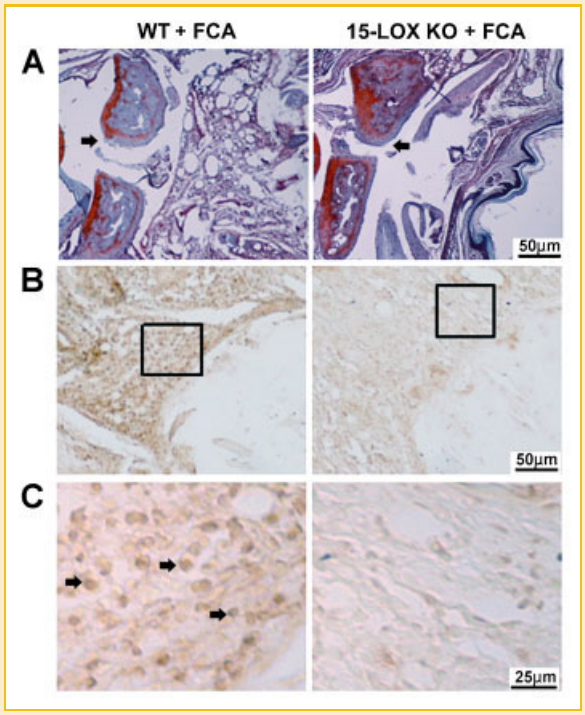

Fig. 8. Inhibition of inflammatory arthritis-induced joint destruction and leucocytes infiltration in 15-LOX knockout mice. A: Staining of Safranin O around the metacarpophalangeal joint in the FCA-induced wild-type mice (left panel) and 15-LOX KO mice (right panel). The arrow shows that the cartilage destruction was more severe in wild-type mice than that in 15-LOX KO mice. B: Immunohistochemistry of CD45 in the finger of wild-type mice (left panel) and 15-LOX KO mice (right panel). The enlarged image of the frame in the upper panels was shown in (C). CD45 is expressed on leucocytes, and the arrows show that there were more CD45-positive cells in wild-type mice than in 15-LOX-KO mice.

that 15-(S)-HETE is an important mediator in the virus-induced Th1 allergic inflammation [Jeon et al., 2009].

We further examined the signaling pathways mediating 15-(S)-HETE-induced MMP-2 production in RASF. It was found that PI3K and NF-κB pathways were involved in the potentiating action of 15-(S)-HETE. 15-(S)-HETE can enhance the phosphorylation of AKT within 30 min. In human synovial tissue, NF-κB is activated in the early stage of joint inflammation [Gilston et al., 1997] and NF-κB DNA binding activity is also increased in RA patients [Asahara et al., 1995]. Here we found that 15-(S)-HETE increased the IκBα degradation and the nuclear translocation of NF-κB subunit.

Cytokines play an important role in the inflammatory events. TNF-α and IL-1β are the causative cytokines leading to deterioration process of rheumatoid arthritis. These two cytokines can stimulate the release of AA from adherent synoviocytes [Angel et al., 1993; Bidgood et al., 2000] and cause the activation of MMP-2 in RASF [Migita et al., 1996; Honda et al., 2001; Wang et al., 2010]. In addition, inhibition of secretory phospholipase A_2 ($sPLA_2$) suppresses the interleukin 1β-induced MMP production in RASF [Thwin et al., 2009]. In human fetal lung fibroblasts, IL-1β-dependent induction of cytosolic $PLA_2\alpha$ mRNA expression is positively regulated by 15-LOX [Walters et al., 2011]. Here we found that MMP-2 release was enhanced by TNF-α or IL-1β in RASF, which was antagonized by inhibiting the 15-LOX pathway. Furthermore, the expression of 15-LOX was upregulated by these two cytokines, indicating that 15-LOX is involved in MMP-2 upregulation by TNF-α or IL-1β in RASF.

NSAIDs and glucocorticoid are often used as symptomatic relief drugs in treating inflammatory arthritis. Here we found that dexamethasone but not NSAIDs of indomethacin and sulindac inhibited the 15-(S)-HETE-induced upregulation of MMP-2 in RASF. Dexamethasone is also reported to inhibit the IL-4-induced 15-LOX expression in human bronchial epithelial cells [Jayawickreme et al., 1999] and IL-4- or IL-13-induced 15-LOX expression in human orbital fibroblast [Chen et al., 2006]. These results further demonstrate that glucocorticoid exerts more pronounced anti-inflammatory action than NSAIDs.

According to the genetic linkage studies for adjuvant arthritis-linked quantitative trait loci (QTL), QTLs for adjuvant-induced arthritis overlap with QTLs for human rheumatoid arthritis [Kim and Moudgil, 2009], and hence adjuvant-induced arthritis is the widely-used animal model for human inflammatory arthritis studies [Pearson and Wood, 1963]. It is reported that an orally synthetic MMPs inhibitor, FR217840, which inhibits collagenase and gelatinase, can suppress bone destruction rather than affect paw swelling in rat adjuvant-induced arthritis model [Ishikawa et al., 2005]. In our in vivo experiment, the symptom of adjuvant-induced arthritis and cartilage destruction was markedly inhibited in 15-LOX KO mice. Furthermore, the MMP-2 upregulation and leucocytes infiltration in the mice paw was also suppressed in 15-LOX KO mice. These results indicate that 15-LOX plays an important role in the inflammatory arthritis. A number of studies have also shown the functional role of 15-LOX in chronic inflammation [Dobrian et al., 2010]. In contrast to our results, however, it is reported that 12/15 LOX plays a protective role in inflammatory arthritis [Kronke et al., 2009a]. The authors use the TNF transgenic mice and K/BxN serum transfer as the animal model, which cause arthritis by over-expressing TNF-α [Li and Schwarz, 2003] or producing the self-antigen glucose-6-phosphate isomerase (GPI) [Ditzel, 2004], respectively. In the adjuvant-induced arthritis, the experiment probably mimic the infectious response, and compared with other disease, the rate of infections is increased in RA patient [Carmona et al., 2010]. As for anti-TNF-α treatment, 30% of RA patients show limited response to the drug, and the therapy would be more effective in early RA [Li and Schwarz, 2003]. Moreover, 64% of RA patients have increased amount of anti-GPI IgG in the serum and synovial fluid, which cause inflammatory arthritis through antigen–antibody complex-mediated immunological effects [Chang and Wei, 2011]. In Kronke's experiment [Kronke et al., 2009a], 12/15 LOX deficient mice shows minor symptom after induction of K/BxN serum transfer arthritis, while 12/15 LOX was also highly expressed in the synovial tissue of wild-type mice. These results demonstrate the complex inflammatory process. Since RA is triggered by multiple factors, such as genetic effects, infections, and other environmental risk, the different animal model may influence the inflammation through a variety of pathways.

In summary, 15-LOX plays a crucial role in promoting inflammation and arthritis progression. The risk factors of

inflammatory arthritis include cytokines of TNF-α and IL-1β, and 15-LOX is involved in these abnormal states. 15-LOX is thus a good target for the drug development in treating inflammatory arthritis.

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