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Original Research Article

7-Ketocholesterol induces ROS-mediated mRNA expression of 12-lipoxygenase, cyclooxygenase-2 and pro-inflammatory cytokines in human mesangial cells: Potential role in diabetic nephropathy

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ABSTRACT

7-Ketocholesterol (7-KCHO) is a highly proinflammatory oxysterol and plays an important role in the pathophysiology of diabetic nephropathy (DN). Lipoxygenases (LOXs) and cyclooxygenases (COXs) are also involved in the development of DN. The aim of this study was to clarify the effects of 7-KCHO on mRNA expression of LOXs and COXs as well as pro-inflammatory cytokines in human mesangial cells (HMC). We evaluated cell viability by WST-8 assay and measured mRNA expression by reverse transcription-polymerase chain reaction. Intracellular reactive oxygen species (ROS) production was evaluated by flow cytometry. Although 7-KCHO did not affect cell viability of HMC, 7-KCHO stimulated significant increases in mRNA expression of 12-LOX, COX-2 and pro-inflammatory cytokines. 7-KCHO also induced an increase in ROS production, while *N*-acetylcysteine partially suppressed the increase. The 12-LOX and COX-2 inhibitors also suppressed mRNA expression of cytokines. These findings may contribute to the elucidation of the molecular mechanism of the pathophysiology of DN.

1. Introduction

Diabetic nephropathy (DN) is a major cause of end-stage renal failure worldwide, and further contributes substantially to the overall morbidity and mortality of diabetic patients [1]. Despite significant advances in knowledge about diabetes, the precise pathological mechanisms and molecular events of DN remain incompletely understood. Specific inhibitors of various pathways are currently available and these new pharmaceutical interventions may have implications for the prevention and treatment of DN [2,3]. Nevertheless, the mainstay of therapy has been limited to achieving optimal blood glucose and blood pressure control to delay the progression of DN [4].

Oxidation of lipoproteins is considered to play an important role in atherosclerosis of diabetic patients [5]. As low-density lipoprotein (LDL) is oxidized in vitro, various changes in lipid composition occur, including substantial loss of free and esterified cholesterol and co-occurrence of cholesterol oxidation products (oxysterols) [6,7]. Oxysterols have potent biological effects, some of which are suggested to have a role in the initiation and/or progression of atherosclerosis [5].

7-Ketocholesterol (7-KCHO), an oxysterol, is a representative compound of oxidized cholesterol [8–10]. 7-KCHO has been demonstrated to exhibit strong cytotoxicity through enhancing reactive oxygen species (ROS) production in various cell lines [11–13]. We previously reported that blood 7-KCHO level is elevated in type 2 diabetic patients [14]. Moreover, Murakami et al. [15] reported an elevation of blood 7-KCHO level in patients with DN, suggesting that 7-KCHO may be involved in the pathophysiology of DN. However, the molecular mechanism by which 7-KCHO contributes to the development of DN has not been sufficiently elucidated.

Lipoxygenase (LOX) and cyclooxygenase (COX) are enzymes that metabolize arachidonic acid. Lipoxygenases (LOXs) are a family of iron-containing enzymes that mainly oxidize arachidonic acid to produce hydroxyeicosatetraenoic acids (HETEs). LOXs are classified as 5-, 8-, 12-, and 15-LOX according to the carbon atom of arachidonic acid at

Abbreviations: 7-KCHO, 7-ketocholesterol; DN, diabetic nephropathy; LOX, lipoxygenase; COX, cyclooxygenase; HMC, human mesangial cells; RT, reverse transcription; PCR, polymerase chain reaction; ROS, reactive oxygen species; NAC, *N*-acetylcysteine; LDL, low-density lipoprotein; LOXs, lipoxygenases; HETEs, hydroxyeicosatetraenoic acids; 5-HpETE, 5-hydroperoxyeicosatetraenoic acid; COXs, cyclooxygenases; PG, prostaglandin; SD, standard deviation; IL, interleukin; PBS, phosphate buffered saline; CAD, coronary artery disease

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which oxygen is inserted [16,17]. There are three major isoforms of 12-LOX; platelet-type, macrophage- or leukocyte-type (12/15-LOX), and epidermal-type [18]. Human and rabbit 15-LOXs as well as the leukocyte-type 12-LOX share high sequence homology, and are classified as 12/15-LOX since they form both 12-HETE and 15-HETE from arachidonic acid [18].

12-HETE, a major product of 12-LOX-mediated metabolism of arachidonic acid, has pro-inflammatory effects and is implicated in diabetic vascular complication [19,20]. For example, high glucose-induced 12-HETE production is linked to leukostasis via an intracellular adhesion molecule-1 dependent pathway in vascular endothelial and smooth muscle cells [21–23]. 12-LOX-mediated 12-HETE production is also elevated in glomeruli of diabetic rats [19], and inhibition of 12/15-LOX prevents the elevation in renal 12-HETE production in streptozotocin-induced diabetic mice [24]. Arachidonic acid is metabolized to 5-hydroperoxyeicosatetraenoic acid (5-HpETE) by 5-LOX, and subsequently to leukotriene A4. Leukotriene A4 is metabolized to leukotriene B4 by leukotriene A4 hydrolase. Leukotriene B4 is a known leukocyte attractant, and generation of leukotriene B4 has been linked to ROS generation, cytokine activation, and apoptosis [25–27]. 15-LOX expression is higher than 12-LOX expression in human carotid plaque macrophages [28,29], and 15-LOX has been suggested to play an important role in the initiation and development of atherosclerosis in humans.

Cyclooxygenases (COXs), also known as prostaglandin (PG) synthase, consist of two major isoforms. COX-1 is expressed constitutively in many tissues, but COX-2 is expressed in many organs at the time of inflammation [30,31]. COX-2 is highly expressed in podocytes, mesangial cells as well as macula densa cells in diabetic rat. Furthermore, COX-2 inhibition attenuates proteinuria and delays DN progression [32].

Although excessive activation of LOXs and COXs is involved in the development of DN, the mechanism by which LOXs and COXs are regulated is not fully elucidated. Therefore, we propose that 7-KCHO may influence inflammation via expression of LOXs and COXs in the kidney. The aim of this study was to clarify the effect of 7-KCHO on mRNA expression of LOXs and COXs as well as inflammatory markers in human mesangial cells (HMC).

2. Materials and methods

2.1. Cell culture and reagents

HMC were purchased from Lonza (Basel, Switzerland) and were cultured in DMEM medium containing 10% FBS at 37 °C with 5% CO_2. We used the cells between the three and eighth passages. For mRNA experiment, 8 mL of cell suspension at a density of 2.0×10^4/L was plated in a 10 cm Petri dish. 7-KCHO, *N*-acetylcysteine (NAC), PD146176, celecoxib and other reagents were purchased from Sigma (St. Louis, Missouri). HMC were pre-incubated overnight with or without 5.0 mM of NAC, and then incubated with different concentrations of 7-KCHO for defined times. Each experiment was repeated at least twice, and the data of three independent experiments were analyzed.

2.2. Measurement of HMC viability by the WST-8 method

Cell viability was determined by the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl) -5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt] assay. HMC (2.0×10^4 cells in a final volume of 100 μL) were incubated in a 96-well microtiter cell culture plate in a humidified atmosphere of 5% CO_2. After 5-day incubation, 10 μM of WST-8 solution was added to each well, and the plate was incubated for an additional 1 h at 37 °C. A micro-spectrophotometer (GloMax Multi Detection System; Promega BioSystems Sunnyvale, Sunnyvale, CA, USA) was used to measure the absorbance of each well at 450 nm. The percentage of viable cells was calculated by comparison to a control well. Data are expressed as mean ± standard deviation (SD) of triplicate cultures.

2.3. Reverse transcription-polymerase chain reaction

Total cellular RNA was extracted using an RNeasy kit (Qiagen, Hilden, Germany), and complementary DNA was synthesized using a reverse transcription-polymerase chain reaction (RT-PCR) kit (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. RNA concentrations were determined by measuring absorbance at 260 nm. Then, RT-PCR was performed using 2 μg of reverse transcribed total RNA. Expression of the housekeeping gene L32 was used as an internal standard. The primers used for COX-2, 5-LOX (ALOX5), 12-LOX (ALOX15), 15-LOX (ALOX15B), interleukin (IL)-6, IL-1β, L32 were as follows: COX-2, sense 5′-TGAAACCCACTCCAAACACAG-3′, antisense 5′-TCAGCATTGTAAGTTGGTGGAC-3′; 5-LOX, sense 5′-GTGCGTTCCA GTGACTTCCA-3′, antisense 5′-AGGCCACACTCGCAGATGA-3′; 12-LOX, sense 5′-CAGCTGGAGAAGGAGCTGGA-3′, antisense 5′-CTGGCTACAG AGAATGACGTTGG-3′; 15-LOX, sense 5′-CCACAGCCAAGAATGCC AAC-3′, antisense 5′-CTCCTGCCAGTGCTCAAATG-3′; interleukin (IL)-6 sense 5′-GAAAGCAGCAAAGAGGCACT-3′, antisense 5′-GCTTGTTCCTC ACTACTCTC-3′; IL-1β sense 5′-TGAAGCAGCCATGGCAGAAG-3′, antisense 5′-GGTCGGAGATTCGTAGCTGGA-3′; and L32, sense 5′-TTCCTG GTCCACAACGTCAAG-3′, antisense 5′-TGTGAGCGATCTCGGCAC-3′. RT-PCR was run on a Stratagene Mx3005P quantitative PCR System (Agilent Technologies, California, USA) for 40 cycles. Each PCR cycle consisted of denaturation at 95 °C for 5 s, followed by annealing and extension at 60 °C for 30 s each.

2.4. Detection of intracellular reactive oxygen species production in HMC by flow cytometry

After culturing in 6-well plates under various experimental conditions, cells were incubated with 3 μM 2′,7′-dichorodihydrofluorescein diacetate (Invitrogen Corp. Carlsbad, CA, USA) for 30 min. After incubation, cells were washed with phosphate buffered saline (PBS), trypsinized, and resuspended in PBS solution. Then, samples were run on a Becton Dickinson FACSCalibur (Immunocytometry Systems, San Jose, CA, USA) equipped with a 15 mW, 488 nm argon laser and filter configuration. Cell samples (2.0×10^4 cells) were analyzed using Cell Quest Pro software (BD Biosciences).

2.5. Statistical analysis

All data are expressed as mean ± SD. Statistical analyses were performed using SPSS software (version 11.5, Chicago, IL, USA). Treatment effects were evaluated using a one-way ANOVA followed by Bonferroni multiple comparison test. P values less than 0.05 were considered significant.

3. Results

3.1. Effect of 7-KCHO on cell viability

Fig. 1 shows the effect of 7-KCHO on cell viability of HMC evaluated by WST-8 assay. Exposure of HMC to 10 or 30 μM of 7-KCHO did not affect cell viability at 24 h, and 30 μM of 7-KCHO also did not affect the viability of HMC at 24 and 48 h.

3.2. Effect of 7-KCHO on LOXs and COXs mRNA expression

Fig. 2 shows the temporal changes of mRNA expression of LOX and COX stimulated by 7-KCHO at 30 μM. Messenger RNA expression of 5-LOX or 15-LOX was not detected by stimulation with 7-KCHO at 30 μM (data not shown). On the other hand, 7-KCHO significantly stimulated

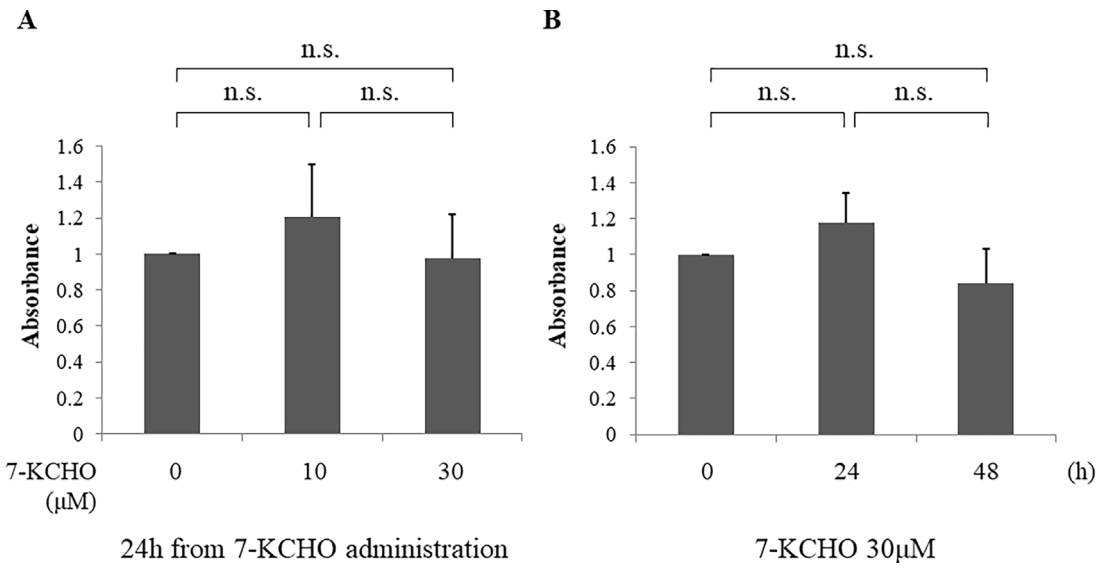

Fig. 1. Effect of 7-ketocholesterol (7-KCHO) on viability of human mesangial cells (HMC). (A) HMC were seeded in 96-well plates (2.0×10^4 cells per well) and cultured until reaching 90–95% confluence. 7-KCHO was added at indicated concentrations and cell viability was assayed using the WST-8 assay at 24 h after addition of 7-KCHO. (B) Cell viability was also evaluated from 0 h to 48 h after addition of 30 μM of 7-KCHO. Data are presented as mean ± SD from three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni multiple comparison test.

mRNA expression of COX-2 and 12-LOX at 24 and 48 h, with significantly higher expression at 24 h than at 48 h for both enzymes.

Fig. 3 shows the dose-dependent effect of 7-KCHO on mRNA expression of LOX and COX at 24 h. Although mRNA expression of 5-LOX or 15-LOX was not detected by stimulation with 30 μM of 7-KCHO (data not shown), mRNA expression of COX-2 and 12-LOX was significantly enhanced by 7-KCHO at 30 μM.

3.3. Effect of 7-KCHO on IL-6 and IL-1β mRNA expression

The temporal changes of mRNA expression of IL-6 and IL-1β stimulated by 30 μM of 7-KCHO are shown in Fig. 4A and B. Messenger RNA expression of IL-6 was significantly stimulated at 24 h, and although the expression decreased at 48 h, the level was still significantly higher than control. 7-KCHO stimulated IL-1β mRNA expression in a time-dependent manner, but the level of expression was lower than that of IL-6. The dose-dependent effect of 7-KCHO on IL-6 and IL-1β mRNA expression at 24 h are shown in Fig. 4C and D. Both cytokines were significantly stimulated by 7-KCHO at 30 μM, but stimulation was stronger for IL-6 than for IL-1β.

3.4. Effect of 7-KCHO on reactive oxygen species production

ROS production in HMC was assessed by flow cytometry. As shown in Fig. 5A, a rightward shift of the FL1 histogram was observed depending on the concentration of 7-KCHO, indicating 7-KCHO dose-dependent increase in ROS production in HMC. Pre-incubation with NAC 5 mM caused a decrease in ROS production as shown by a leftward shit of the histogram (Fig. 5B). We analyzed the roles of ROS in 7-KCHO-stimulated increases in mRNA expression of 12-LOX, COX-2, IL-6 and IL-1β using NAC, a known scavenger of ROS (Fig. 6). NAC alone had no effect on mRNA expression of the four compounds. However, when co-treated with 30 μM of 7-KCHO, NAC partially suppressed 7-KCHO-stimulated increases in mRNA expression for 12-LOX, COX-2 and IL-6, but not for IL-1β (Fig. 6A–D).

3.5. Effects of specific inhibitors of 12-LOX and COX-2 on 7-KCHO-stimulated IL-6 and IL-1β mRNA expression

We examined the roles of COX-2 and 12-LOX in 7-KCHO-stimulated mRNA expression of IL-6 and IL-1β using selective inhibitors of 12-LOX and COX-2 (Fig. 7). PD146176, a specific inhibitor of 12-LOX, significantly suppressed the increases in mRNA expression of IL-6 and IL-

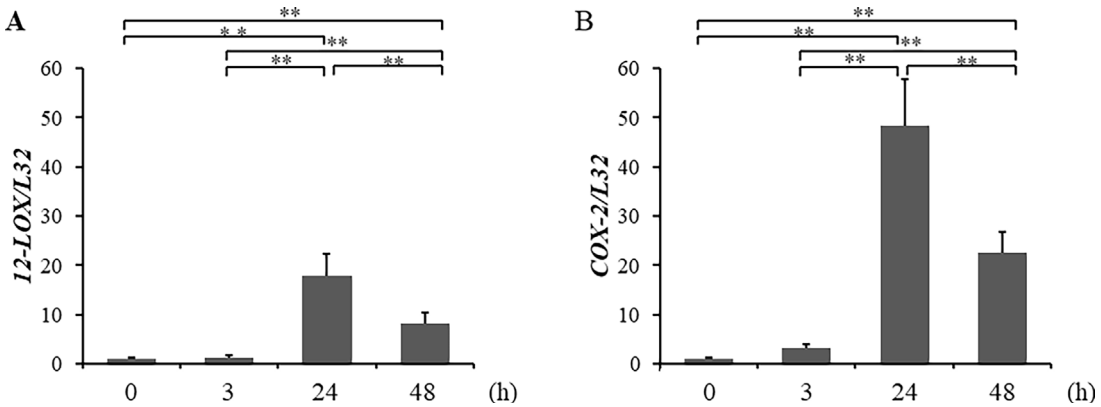

Fig. 2. Effect of 7-ketocholesterol (7-KCHO) on 12-lipoxygenase (LOX) and cyclooxygenase (COX)-2 mRNA expression from 0 to 48 h after addition of 7-KHO. Human mesangial cells were seeded into 10 cm diameter Petri dishes (2.0×10^4 cells per dish), and cultured until reaching 90–95% confluence. The target mRNAs were evaluated by RT-PCR. Data are presented as mean ± SD from three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$; one-way ANOVA followed by Bonferroni multiple comparison test.

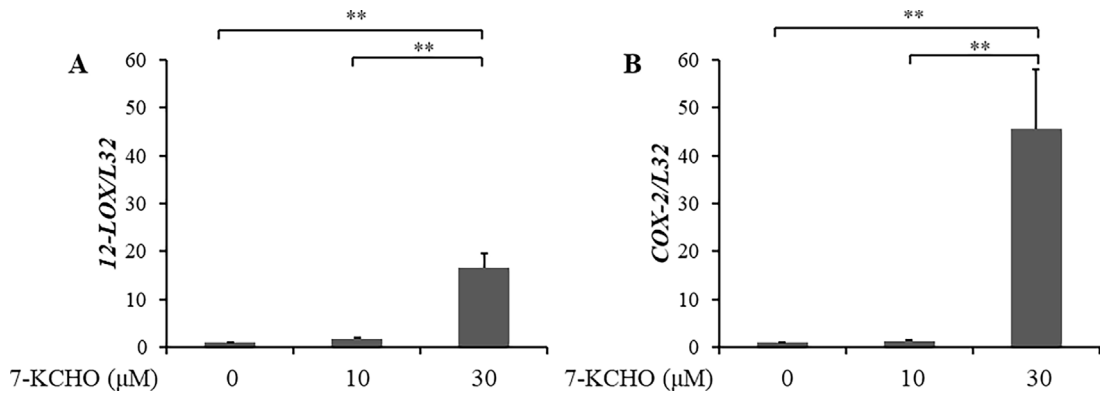

Fig. 3. Effect of various concentrations of 7-ketocholesterol (7-KCHO) on 12-lipoxygenase (LOX) and cyclooxygenase (COX)-2 mRNA expression. Human mesangial cells were seeded into 10 cm diameter Petri dishes (2.0×10^4 cells per dish), and cultured until reaching 90–95% confluence. The target mRNAs were evaluated by RT-PCR. Data are presented as mean ± SD from three independent experiments. $*p < 0.05$, $**p < 0.01$; one-way ANOVA followed by Bonferroni multiple comparison test.

1β stimulated by 30 μM of 7-KCHO (Fig. 7A and B). On the other hand, celecoxib, a specific inhibitor of COX-2, significantly suppressed 7-KCHO-stimulated increase in mRNA expression of IL-1β, but not IL-6 (Fig. 7C and D).

4. Discussion

In the present study, we demonstrated that 7-KCHO enhanced mRNA expression of LOXs and COXs as well as pro-inflammatory cytokines at least partially mediated by ROS in HMC. Studies using specific inhibitors indicated possible involvement of 12-LOX and COX-2 pathways in 7-KCHO-induced increases in IL-6 and IL-1β mRNA expression.

Oxidation of cholesterol produces oxysterol [33]. 7-KCHO, an oxysterol, has been demonstrated to exist in human circulation at the highest concentration among the oxysterols. 7-KCHO has been shown to be a highly inflammatory substance both in *in vitro* [34,35] and *in vivo* studies [36]. Recent studies have reported that 7-KCHO is related to the pathophysiology of various diseases including atherosclerosis [34,37,38]. Blood 7-KCHO level is high in diabetic patients with coronary artery disease (CAD) [38] and is particularly high in patients with DN [15], suggesting an association with the pathophysiology of these clinical conditions.

In the present study, we examined the effects of 7-KCHO on mRNA expression of LOXs and COXs in HMC. Changes in the expression levels of LOXs and COXs, the regulatory enzymes of the arachidonic cascade, are involved in the pathophysiology of DN. In *in vivo* studies, urinary excretion of 12-HETE, a metabolite produced by LOX, was accelerated in insulin-independent diabetic patients [39], and production of 8-iso-PGF2 alpha which is an indicator of COX-mediated inflammation was accelerated in type 2 diabetic patients [40]. In rat mesangial cells, hyperglycemia and angiotensin II that are associated with the

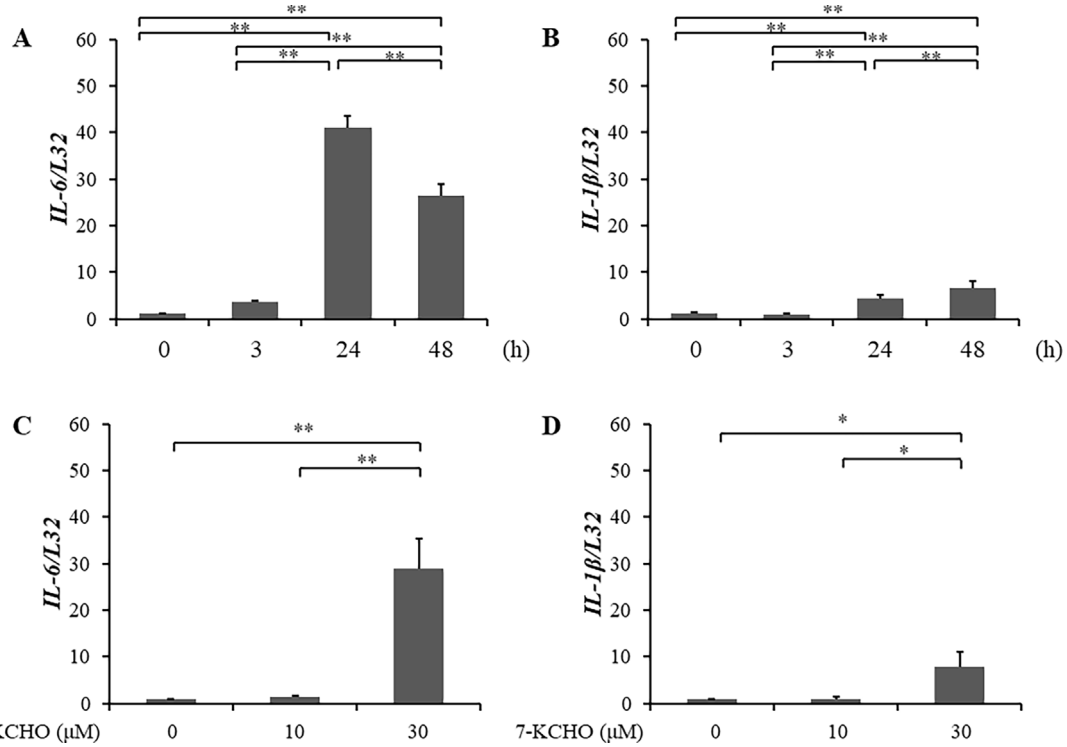

Fig. 4. Effect of 7-ketocholesterol (7-KCHO) on IL-6 and IL-1β mRNA expression (A and B) from 0 to 48 h after addition of 30 μM of 7-KCHO, and (C and D) for different concentrations of 7-KHO at 24 h after addition. Human mesangial cells were seeded into 10 cm diameter Petri dishes (2.0×10^4 cells per dish), and cultured until reaching 90–95% confluence. The target mRNAs were evaluated by RT-PCR. Data are presented as mean ± SD from three independent experiments. $*p < 0.05$, $**p < 0.01$; one-way ANOVA followed by Bonferroni multiple comparison test.

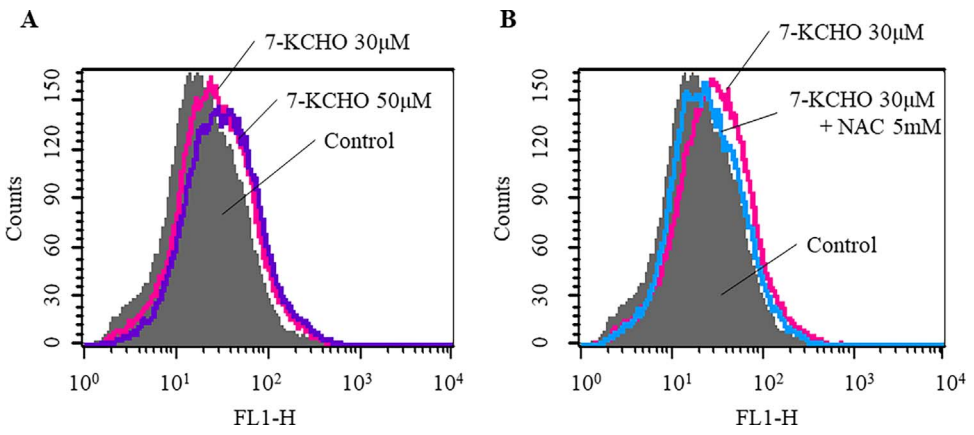

Fig. 5. Effect of 7-ketocholesterol (7-KCHO) on production of reactive oxygen species (ROS) in human mesangial cells (HMC), with or without *N*-acetylcysteine (NAC). HMC were seeded in 6-well plates (2.0×10^4 cells per well) and cultured until reaching 90–95% confluence. The cells were pre-incubated with or without NAC for 24 h. Then 7-KCHO was added at indicated concentrations. ROS production was analyzed using 2′,7′-dichorodihydrofluorescein diacetate followed by flow cytometry. Changes in ROS are shown in FL1 histograms.

pathophysiology of DN enhanced the expression of COX-2 and leukocyte-type 12-LOX [41–44].

Activities of LOXs in organs are regulated by tissue distribution and cell type. In general, 5-LOX expression is primarily limited to bone marrow-derived cell types, and has been studied in a variety of contexts with respect to inflammation, as the enzyme is required for downstream production of proinflammatory leukotrienes [45]. 12-LOX is broadly expressed in virtually all metabolically active cell types [46]. 15-LOX is expressed mainly in the skin and other epithelial cells, and is involved in atherosclerosis, neuronal disorder, immune modulation, skin diseases, and maintenance of the epithelium [47]. The cell-specific expression of LOXs is also reflected in the results of our experiments. In this study, 5-LOX and 15-LOX mRNA expression was not detected in HMC and not induced by 7-KCHO. On the other hand, 12-LOX and COX-2 mRNA expression was detected at low levels in untreated HMC and was enhanced by exposure to 7-KCHO.

There are numerous reports regarding the critical roles of inflammation on the pathophysiology of DN. Serum IL-6 level is elevated in patients with type 2 DM [48], and IL-6 expression is detected and elevated in kidney biopsy tissues in patients with DN [49]. Furthermore, renal expression of IL-1β was reported to increase in an experimental model of DN [50,51]. In the present study, we clearly demonstrated that 7-KCHO stimulated mRNA expression of both IL-6 and IL-1β in HMC, suggesting the important roles of these pro-inflammatory cytokines in the pathogenesis of 7-KCHO-induced renal damage.

Regarding the underlying mechanism of 7-KCHO-induced inflammation, studies have demonstrated that 7-KCHO promotes intracellular ROS production, causing strong oxidative stress in various cells [11–13]. In particular, 7-KCHO exists in human arteriosclerotic lesion at high concentration, and induces ROS-mediated apoptosis of smooth muscle cells [52]. In this study, we first demonstrated that 7-KCHO induced ROS production in HMC, and then, the mRNA increment of IL-6 and IL-1β as well as 12-LOX and COX-2 induced by 7-KCHO were suppressed by the addition of ROS inhibitor NAC. These data

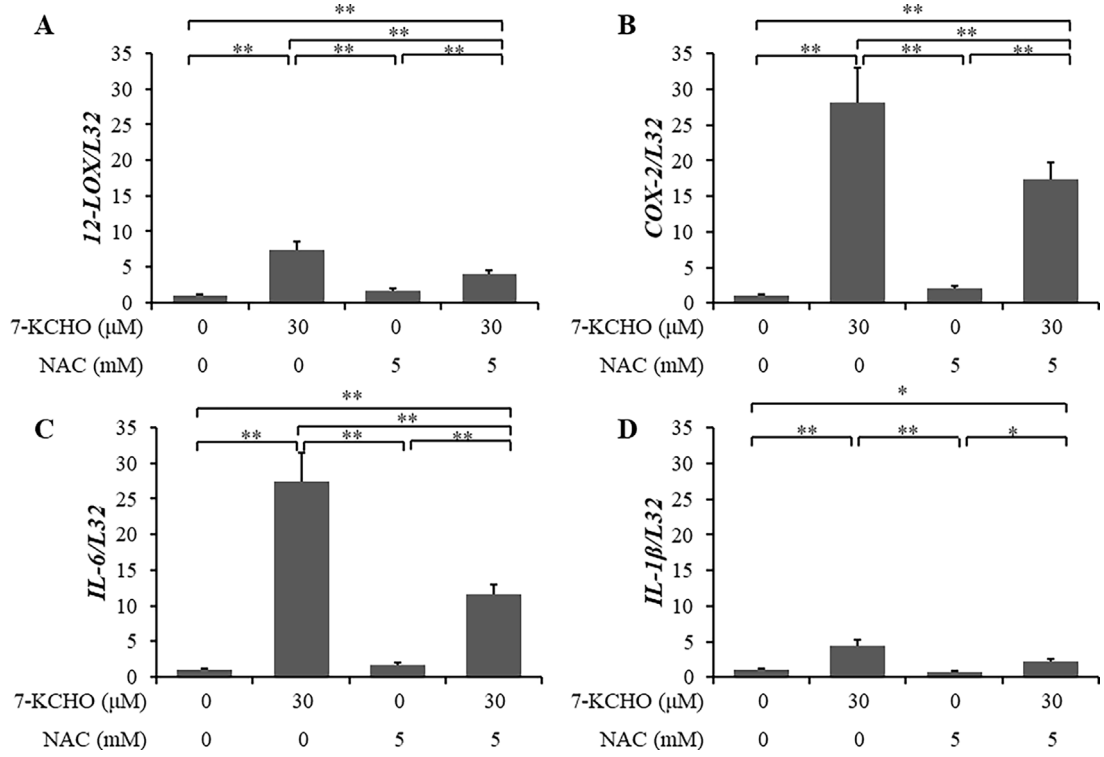

Fig. 6. Effects of 7-ketocholesterol (7-KCHO) on 12-lipoxygenase (LOX), cyclooxygenase (COX)-2, IL-6 and IL-1β mRNA expression in human mesangial cells (HMC) with or without NAC. HMC were seeded into 10 cm diameter Petri dishes (2.0×10^4 cells per dish), and cultured until reaching 90–95% confluence. The cells were pre-incubated with or without 5 mM of NAC for 24 h. Then, 30 μM of 7-KCHO was added. The target mRNAs were evaluated by RT- PCR. Data are presented as mean ± SD from three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$; one-way ANOVA followed by Bonferroni multiple comparison test.

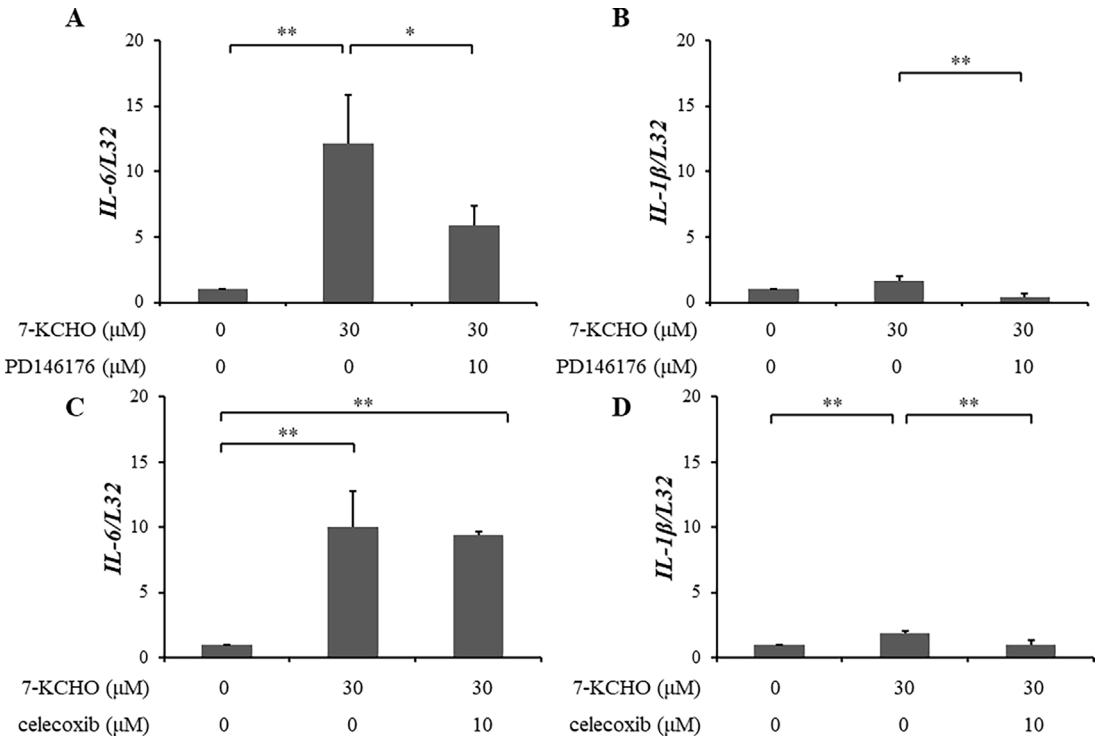

Fig. 7. Effects of PD 146176 (specific inhibitor of 12-LOX) and celecoxib (specific inhibitor of COX-2) on 12-lipoxygenase (LOX), cyclooxygenase (COX)-2, IL-6 and IL-1β mRNA expression in human mesangial cells (HMC) treated with 30 μM of 7-KCHO. HMC were seeded into 10 cm diameter Petri dishes (2.0×10^4 cells per dish), and cultured until reaching 90–95% confluence. The target mRNAs were evaluated by RT-PCR. Data are presented as mean ± SD from three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$; one-way ANOVA followed by Bonferroni multiple comparison test.

clearly indicate that 7-KCHO increases mRNA expression of not only 12-LOX and COX-2, but also IL-6 and IL-1β through ROS-dependent mechanisms. Although there may be a possibility that 12-LOX and COX-2 enhancement lead to increased ROS production due to subsequent change in the eicosanoids, our experiments showed that the increase of 12-LOX and COX-2 expression by 7-KCHO was suppressed by the addition of NAC with the cancellation of ROS production. Onodera et al. [53] reported that oxidative stress induces phosphorylation of MAPKs and NF-κB through TAK1 activation and resulted in increased COX-2 and prostaglandin E2 expression using bovine synovial fibroblasts, and ROS-induced COX-2 expression was inhibited by supplementation of NAC. With these results, ROS may be upstream of LOX, COX.

To elucidate the relation between the arachidonic cascade through increased 12-LOX and COX-2 expression and the 7-KCHO-induced increases in pro-inflammatory cytokines of IL-6 and IL-1β, we used specific inhibitors of 12-LOX and COX-2. Surprisingly, both inhibitors suppressed 7-KCHO-induced mRNA expression of both inflammatory cytokines, suggesting that the metabolites of 12-LOX and COX-2 may control 7-KCHO-induced pro-inflammatory cytokines of IL-6 and IL-1β. These metabolites could include prostaglandins and eicosanoids that are produced from metabolism of fatty acids by LOX and COX. Specific COX-2 inhibitors have been reported to delay progression of proteinuria and improve nephropathy in diabetic rats [32,54]. Moreover, 12-LOX inhibitor reduced proteinuria with a transient suppression of 12/15-LOX in a rodent model of DN [19]. About the mechanism by which 7-KCHO increases the cytokines, Larrayoz et al. [55] reported three kinase signaling pathways, NF-κB, p38 MAPK, and ERK. In our study, the inhibition of 12-LOX and COX-2 partially canceled IL-6 and IL-1β production, and this suggests that the eicosanoid pathway mediated by LOX and COX may be also partially involved in the cytokine production by 7-KCHO. Thus, 12-LOX and COX-2 pathways might be involved in the renal damage.

5. Limitation

The present study has some limitations. First, this study examined the effect of 7-KCHO in mesangial cells only, but kidney contains various cell types such as endothelial cells, podocytes, tubular epithelium, fibroblasts as well as macrophages and immune cells. Since the detailed mechanisms by cell–cell interactions in the kidney are clarified in recent years [56–58], further investigation would be required to investigate how 7-KCHO affects cells other than mesangial cells and how our findings in mesangial cells link to the pathogenesis of DN. Second, 7-KCHO-induced enhancement of IL-6 and IL-1β expression was partially suppressed by 12-LOX and COX-2 inhibitors, suggesting the important roles of other pathways. Finally, the results of this *in vitro* study cannot be extrapolated directly to *in vivo* situation. Further studies are required to confirm the roles of 7-KCHO using animal model and human subjects.

6. Conclusion

In HMC, 7-KCHO enhances mRNA expression of pro-inflammatory cytokines through enhancing ROS production, and the 7-KCHO-induced inflammatory pathway is partially regulated by 12-LOX and COX-2. These findings may contribute to the elucidation of the molecular mechanism in the pathophysiology of DN.

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