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Effect of *trans*-10, *cis*-12 Conjugated Linoleic Acid on Production of Prostaglandin E_2, Cyclooxygenase-2 and 5-lipoxygenase in Lipopolysaccharide-Stimulated Porcine Peripheral Blood Mononuclear Cells

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Abstract : The objective of this study was to examine the effect of *trans*-10, *cis*-12 conjugated linoleic acid (t10c12-CLA) on the expression of cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) pathway in lipopolysaccharide (LPS)-stimulated porcine peripheral blood mononuclear cells (PBMCs). t10c12-CLA was treated with different concentrations in culture medium of LPS-naïve and LPS-stimulated PBMCs. The mRNA expressions of prostaglandin E_2 (PGE_2)-synthase, COX-2 and 5-LOX were measured using quantitative real-time PCR. In addition, the production levels of PGE_2 and 5-LOX in culture supernatant from PBMCs with or without LPS were assessed by ELISA. In LPS-naïve PBMCs, treatment of t10c12-CLA significantly ($p < 0.05$) increased the mRNA expressions of PGE_2 synthase and 5-LOX compared to vehicle control. Expression of COX-2 mRNA did not show significant difference compared to vehicle control by t10c12-CLA treatment in LPS-naïve PBMCs. However, the addition of LPS in PBMCs markedly ($p < 0.05$) increased the mRNA expression of COX-2, PGE_2 synthase and 5-LOX, and also significantly ($p < 0.05$) enhanced the production of PGE_2 and 5-LOX relative to LPS-naïve PBMCs, respectively. However, the addition of t10c12-CLA significantly ($p < 0.01$) suppressed the LPS-induced excessive expression of COX-2, PGE_2 synthase, and 5-LOX compared to those of PBMCs treated with LPS alone. The production levels of PGE_2 and 5-LOX in culture supernatant from LPS-stimulated PBMCs were also significantly ($p < 0.05$) inhibited by the treatment of t10c12-CLA compared to LPS alone. These results suggested that t10c12-CLA has an anti-inflammatory effect via dual inhibition of COX-2 and 5-LOX with gene expression and production level in LPS-stimulated porcine PBMCs. Therefore, it was thought that t10c12-CLA can attenuate the inflammatory response by down-regulation of eicosanoids production.

Key words **:** *trans*-10, *cis*-12-conjugated linoleic acid, COX-2, PGE_2, 5-LOX, lipopolysaccharide, pigs.

Introduction

Trans-10, *cis*-12 conjugated linoleic acid (t10c12-CLA) modulates immune responses via modulation of pro-inflammatory cytokine secretion and has therapeutic effects in inflammatory disorders (19). It can also downregulate inducible eicosanoids in inflammatory environment (28). Pro-inflammatory and anti-inflammatory mediators derived from arachidonic acid (AA) regulate peripheral inflammation and resolution (2). Cyclooxygenase-2 (COX-2), as the rate-limiting enzyme, is required to produce prostaglandins (PGs) from arachidonic acids (14,22). Among the multiple COX-2 downstream signaling pathways, PGE_2, signaling is considered to be a major mediator of inflammatory reactions (12). In previous study, CLA showed anti-inflammatory effect in RAW 264.7 macrophage by reduction of LPS-induced mRNA and protein expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) as well as subsequent production of nitric oxide and prostaglandin E_2, respectively (7).

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5-Lipoxygenase (5-LOX), in the other pathway of arachidonic acid metabolism, is mainly recognized for the role in formation of leukotrienes with pro-inflammatory functions (17). 5-LOX and its metabolite are crucial marker for chronic inflammation and tumorigenesis. Leukotrienes (LTs) act as the inflammatory mediators for various tissues (17). LTs were considered as pathophysiological mediators in asthma, rhinitis, and other chronic inflammatory disease, and their structures and biosynthesis have been elucidated in many studies (27). It was demonstrated that n-3 polyunsaturated fatty acid (PUFA) ingestion can ameliorate methacholine-induced respiratory distress in an asthmatic population by measuring the level of urinary leukotriene (3). CLA isomers decreased 5-hydroxyeicosatetraenoic acid (5-HETE) production in 5-LOX pathway by competition with arachidonic acid and the reduction of 5-lipoxygenase activating protein (FLAP) expression in breast tumor cell (16).

In this study, we focused on the down regulatory effect of t10c12-CLA to the arachidonic acid metabolism. We figured out whether t10c12-CLA suppresses the production of both COX and LOX pathways in lipopolysaccharide (LPS)-stimulated porcine peripheral blood mononuclear cells by measuring the levels of PGE_2, COX-2 and 5-LOX.

Materials and Methods

Chemicals and reagents

t10c12-CLA (96% purity) and dimethyl sulfoxide (DMSO) were commercially purchased (Sigma-Aldrich Co., St. Louis, MO, USA). t10c12-CLA was dissolved in DMSO to a final concentration of 50 mM prior to being passed through a 0.45 μm membrane filter (Millipore Co., Bedford, MA, USA) as stock solution and stored at −70°C until used. LPS from Escherichia coli 0127:B8 (Sigma-Aldrich Co.) was diluted with phosphate buffered saline (PBS) to a final concentration of 100 μg/ml as stock solution and used with the concentration of 1 μg/ml.

Isolation of porcine peripheral blood mononuclear cells (PBMCs)

Healthy 6 months crossbred pigs in slaughterhouse (Farm story, Cheongju, Korea) were used as blood donors. Porcine PBMCs were isolated as described previously (13,20). Peripheral blood drawn in heparinized tube from anterior vena cava was layered 1:1 on Percoll™ solution (Sigma-Aldrich, Co.; specific gravity, 1.080). After centrifugation at 400 g for 45 min at room temperature, the cells in interface between plasma and Percoll™ solution were harvested and added EDTA-PBS before centrifugation at 900 g for 5 min. Residual erythrocytes were lysed by brief treatment with 0.83% NH4Cl. All cells were resuspended in RPMI 1640 medium (Sigma-Aldrich, Co.) with 2 mM L-glutamine supplemented with 1% penicillin and streptomycin (Hyclone Lab, Logan, UT, USA) and 5% Fetal bovine serum (FBS; Hyclone lab) at 37°C under a 5% CO_2-humidified atmosphere.

Culture supernatants

The PBMCs at a density of 3×10^6 cells/ml in a 24-multiwell plate (Corning Incorporated., Corning, NY, USA) was incubated with a concentration of 10, and 20 μM of t10c12-CLA and/or LPS (1 μg/ml) for 24 h at 37°C under 5% CO_2-humidified atmosphere. The supernatant was collected by centrifugation at 900 g for 10 min and stored at −70°C until used for assay.

Production of PGE_2 and 5-LOX assay

Levels of PGE_2 and 5-LOX were detected using quantitative sandwich ELISA kit (MyBioSource., San Diego, CA, USA) following the manufacturer's protocol. Briefly, 50 μl of culture supernatants were added to ninety-six plates with standard reagents in duplicate manner. Then, 100 μl of HRP-conjugate reagents were added to each well and incubated for 60 min at 37°C. After washing the plate 4 times, chromogen solution A 50 μl and chromogen solution B 50 μl were added to each well successively and incubated for 15 min at 37°C protected from direct light. Finally, 50 μl of stop solution were added to each well and optical density was determined using an automated microplate reader (Epoch, BioTek® Instruments, VT, USA) set at 450 nm within 15 min.

RNA extraction and real-time quantitative PCR

Total RNA was extracted using Trizol reagent (Invitrogen Co., Carlsbad, CA, USA) according to the method outlined in the protocol, and the concentration of total RNA was determined by measuring the absorbance at 260 nm. A first strand complementary DNA (cDNA) was prepared by subjecting total RNA (1 μg) to reverse transcription using Moloney murine leukemia virus (mMLV) reverse transcriptase (Invitrogen Co.) and random primers (9-mers; TaKaRa Bio Inc, Otsu, Shiga, Japan). cDNA template (2 μL) was added to 10 μL of nucleic acid dye, 2 × SYBR Premix Ex Taq (TaKaRa Bio), containing 10 pmol of the specific primer pairs. Reactions were carried out for 40 cycles. At the end of the extension phase of each cycle, fluorescence intensity was measured. The threshold fluorescence intensity for all samples was set manually. The reaction cycle at which the PCR products exceeded this threshold was identified as the threshold cycle (CT) of the exponential phase of PCR amplification. The expressions of PGE_2 synthase, COX-2, and 5-LOX were quantified relative to that of 1A, the cytochrome c oxidase subunit 1, which is a key enzyme for aerobic metabolism. The oligonucleotides for PGE_2 synthase, COX_2, 5-LOX, and 1A gene, which were based on the cDNA sequence, were described below.

PGE_2 synthase
sense 5'-AAA TAC TGG CTC ATG CTG GA-3'
antisense 5'-TTG CCC TCC TTG ACA ATG TA-3'
Cyclooxygenase-2
sense 5'-TCC TGC CCT TCT GGT AGA AA-3'
antisense 5'-CTG AAT CGA GGC AGT GTT GA-3'
5-lipoxygenase
sense 5'-ATT TTC CTC CCT TCG GAT GC-3'
antisense 5'-ACC AGG AGC TTG AAA ATG GG-3'
1A
sense 5'-CAC CGT AGG AGG TCT AAC G-3'
antisense 5'-GTA TCG TCG AGG TAT TCC G-3'

Data for each sample were analyzed by comparing cycle threshold (CT) values at constant fluorescence intensity. The amount of transcript was inversely related to the observed CT, and for every two-fold dilutions of the transcript, the CT was expected to increase by one increment. Relative expression (R) was calculated using the equation: $R = 2^{-[\Delta CT \text{ sample} - \Delta CT \text{ control}]}$.

Statistical analysis

All experiments were replicated more than three times. All data were analyzed by one-way analysis of variance (ANOVA) using SPSS 19.0 software (Statistical Package for Social Science, IBM, NY, USA) to determine the statistical significance of the differences between the control and treatment groups followed by Duncan and Tukey's test. Comparisons between two groups were made using 2 sample t-test. P value of < 0.05 was considered statistically significant. Data were represented as mean ± standard deviation (SD).

Results

Effect of t10c12-CLA on production of PGE_2 and expression of PGE_2 synthase mRNA in porcine PBMCs

To investigate the effect of t10c12-CLA on PGE_2 production by LPS-naïve and LPS-stimulated porcine PBMCs,

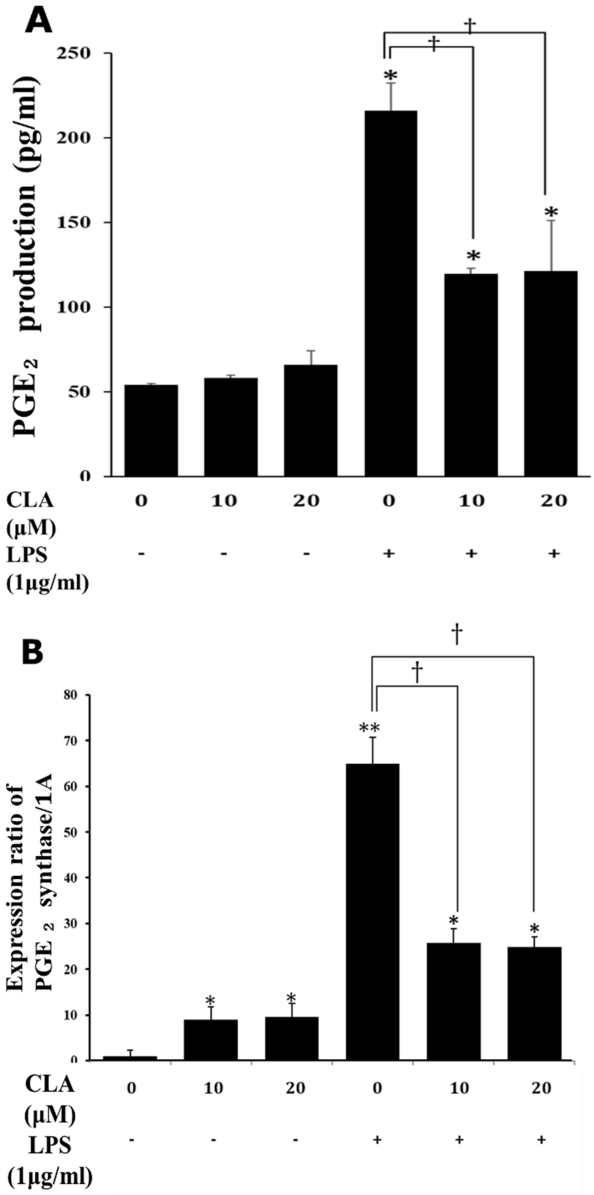

Fig 1. The effect of t10c12-CLA on production of PGE_2 (A) and expression of PGE_2 synthase (B) in LPS-naïve and LPS-stimulated PBMCs. (A) PBMCs (3×10^6 cells/ml) were treated with indicated concentrations of t10c12-CLA (0, 10 and 20 μM) and/or LPS (1 μg/ml) for 24 h to determine the amount of PGE_2 in the culture supernatant using ELISA. (B) To measure the expression level of PGE_2 synthase by quantitative real-time PCR, cells were incubated for 8 h with t10c12-CLA (0, 10 and 20 μM) and/or LPS (1 μg/ml). PGE_2 synthase mRNA expression was expressed as ratio compared to that of the 1A gene. The data represent means ± SD of three experiments. $*p < 0.05$, $**p < 0.001$ vs. vehicle-treated control group. One-way ANOVA followed by Tukey and Duncan's test. †$p < 0.01$ as determined by 2-sample t-test.

PBMCs (3×10^6 cells/ml) were incubated with t10c12-CLA (10 and 20 μM) and /or LPS (1 μg/ml) for 24 h. The amount of PGE_2 in the culture supernatant from PBMCs treated with t10c12-CLA and/or LPS was measured. As shown in Fig 1A, the treatment of t10c12-CLA had no effect on the production of PGE_2 compared with vehicle control. However, treatment

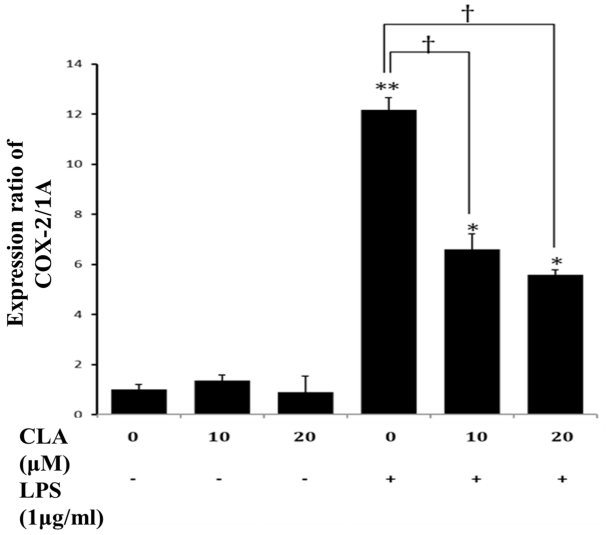

Fig 2. The effect of t10c12-CLA on expression of COX-2 mRNA in LPS-naïve and LPS-stimulated PBMCs. To investigate the expression level of COX-2 mRNA using quantitative real-time PCR, PBMCs (3×10^6 cells/ml) were incubated for 8 h with t10c12-CLA (0, 10 and 20 μM) and/or LPS (1 μg/ml). Expression of COX-2 mRNA was described as ratio compared to that of the 1A gene. The data represent means ± SD of three experiments. $*p < 0.05$, $**p < 0.001$ vs. vehicle-treated control group. One-way ANOVA followed by Tukey and Duncan's test. †$p < 0.01$ as determined by 2-sample t-test.

of PBMCs with LPS significantly ($p < 0.05$) increased the production of PGE_2 when compared with that of vehicle control. This LPS-induced PGE_2 production was significantly ($p < 0.01$) decreased by the addition of t10c12-CLA. To evaluate the expression of PGE_2 synthase by quantitative real-time PCR, cells were incubated for 8 h with t10c12-CLA (10 and 20 μM) and/or LPS (1 μg/ml) (Fig 1B). Treatment of t10c12-CLA in PBMCs significantly ($p < 0.05$) increased expression of PGE_2 synthase mRNA compared to vehicle control. In addition, treatment of PBMCs with LPS remarkably ($p < 0.001$) increased PGE_2 synthase mRNA expression relative to vehicle control. This LPS-stimulated PGE_2 synthase expression was significantly ($p < 0.01$) reduced by the treatment of t10c12-CLA .

Effect of t10c12-CLA on mRNA expression of COX-2 in porcine PBMCs

The expression of COX-2 mRNA in PBMCs was measured using quantitative real-time PCR. PBMCs (3×10^6 cells/ml) were incubated for 8 h with t10c12-CLA (10 and 20 μM) and/or LPS (1 μg/ml). As shown in Fig 2, t10c12-CLA did not induce the increase of the level of COX-2 mRNA expression relative to vehicle control. However, LPS significantly ($p < 0.001$) increased the expression of COX-2 mRNA compared to vehicle control. However, in LPS-stimulated PBMCs, t10c12-CLA significantly ($p < 0.01$) reduced COX-2 mRNA expression in comparison with LPS alone.

Effect of t10c12 -CLA on production and mRNA expression of 5-LOX in porcine PBMCs

To examine the effect of t10c12-CLA of 5-LOX produc-

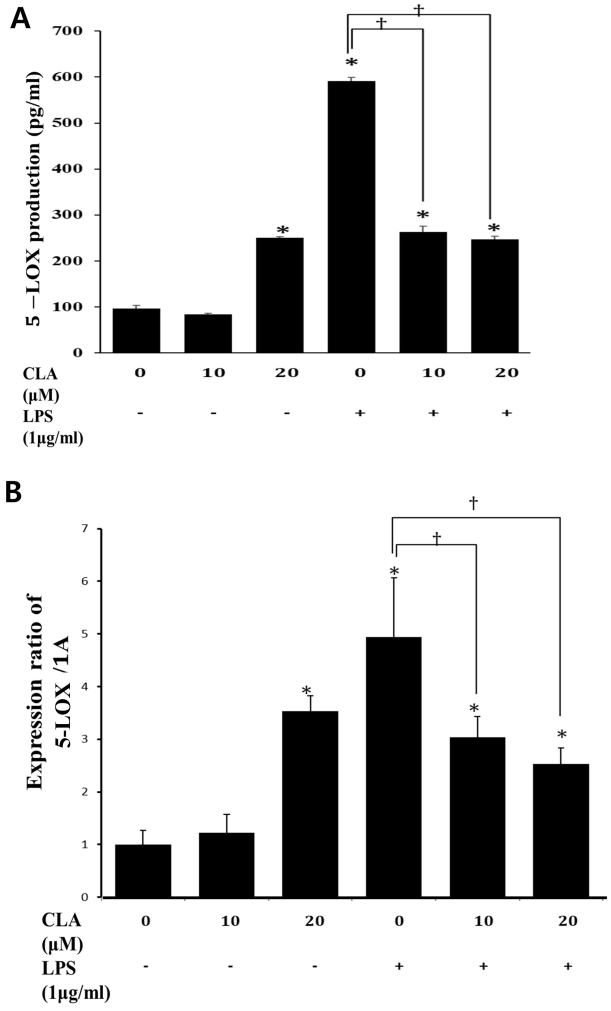

Fig 3. The effect of t10c12-CLA on production of 5-LOX (A) and expression of 5-LOX (B) in LPS-naïve and LPS-stimulated porcine PBMCs. (A) Cells (3×10^6 cells/ml) were treated with indicated concentrations of CLA (10 and 20 μM) and/or LPS (1 μg/ml) for 24 h. The amount of 5-LOX in the culture supernatant was determined using ELISA. (B) To examine the expression of 5-LOX by quantitative real-time PCR, cells were incubated for 8 h with t10c12-CLA (0, 10 and 20 μM) and/or LPS (1 μg/ml). 5-LOX mRNA expression was expressed as ratio compared to that of the 1A gene. The data represent means ± SD of three experiments. *$p < 0.05$ vs. vehicle-treated control group. One-way ANOVA followed by Tukey and Duncan's test. †$p < 0.01$ as determined by 2-sample t-test.

tion in LPS-naïve and LPS-stimulated PBMCs, PBMCs (3×10^6 cells/ml) were incubated with t10c12-CLA (10 and 20 μM) for 24 h. Production of 5-LOX in PBMCs treated with t10c12-CLA of 20 μM but not 10 μM was significantly ($p < 0.05$) increased compared to that of vehicle control. 5-LOX production in LPS-stimulated PBMCs was also significantly increased ($p < 0.05$) relative to vehicle control. In addition, 5-LOX production in PBMCs increased by LPS was significantly ($p < 0.05$) reduced by addition of t10c12-CLA (Fig 3A). Expression levels of 5-LOX mRNA in LPS-naïve and LPS-stimulated PBMCs were measured using quantitative real-time PCR. Cells were incubated for 8 h with t10c12-CLA (10 and 20 μM) and/or LPS (1 μg/ml). Treatment of t10c12-CLA (20 μM) in LPS-naïve PBMCs significantly ($p < 0.05$) increased 5-LOX mRNA expression when compared to that of vehicle control. Moreover, treatment of PBMCs with LPS significantly ($p < 0.05$) stimulated 5-LOX expression than that of vehicle control. This LPS-induced 5-LOX mRNA expression was significantly ($p < 0.01$) reduced by treatment of t10c12-CLA (Fig 3B).

Discussion

In this study, we investigated whether t10c12-CLA can regulate the eicosanoid metabolism in porcine PBMC with or without LPS. Eicosanoids are signaling molecules and classed as prostaglandin, e.g. prostacyclins, thromboxanes, and leukotrienes. Prostaglandins and leukotrienes are produced by cyclooxygenase (COX) and 5-LOX from phospholipase-released arachidonic acid (AA), respectively (11). In the inflammatory disease, excessive-expression and over-production of their products can aggravate the clinical symptoms. However, eicosanoid production can be decreased by n-3 polyunsaturated fatty acid (27). In addition, some studies revealed that CLA can attenuate the eicosanoid secretion related to COX-2 and 5-LOX (4,28). We demonstrated that t10c12-CLA can modulate arachidonic acid metabolism, especially in eicosanoid synthesis with COX-2 and 5-LOX in LPS-stimulated porcine PBMCs.

LPS is a prototypical endotoxin derived from bacterial cell walls. COX-2 is crucial in the eicosanoid metabolism during inflammation (1). It is normally absent in most cells but highly inducible in certain cells in response to inflammatory stimuli resulting in enhanced prostaglandin (PG) release. PGs formed by COX-2 primarily mediate pain and inflammation (27). PGE_2 is a major pro-inflammatory cytokine and exerts its function like inducing fever and increasing vascular permeability. Thus, we used LPS originated from E. *coli* to induce inflammatory environment on porcine PBMCs.

In the present study, production of PGE_2 was measured in LPS-naïve PBMCs and LPS-stimulated PBMCs. In LPS-naïve PBMCs, there was no significant difference between vehicle control and t10c12-CLA treatment group. In addition, t10c12-CLA at concentrations of 10 μM and 20 μM also have no significance in LPS-naïve PBMCs. However, expression of PGE_2 synthase mRNA was increased in LPS-naïve PBMCs. We measured the expression of PGE_2 synthase 8 hours after incubation. But production of PGE_2 was measured 24 hours after incubation. Therefore, it can be thought that mRNA expression for PGE_2 synthase and its reflection on quantity of product, PGE_2, had the difference on time and expression level according to different incubation time.

On the other hand, treatment of higher dose (20 μM) of t10c12-CLA also showed no significance in COX-2 mRNA expression in LPS-naïve PBMCs. It was, therefore, thought that the expression of COX-2 mRNA was not affected by t10c12-CLA in LPS-naïve PBMCs. However, similarly, although treatment of capsaicin inhibited the production of LPS-induced PGE_2 and the enzyme activity of COX-2 in peritoneal macrophage, expression of COX-2 mRNA was not affected by capsaicin (15). Therefore, additional study is needed to evaluate the activity of COX-2 with treatment of

t10c12-CLA.

In LPS-stimulated PBMCs, we observed remarkable increase in production of PGE_2 and expression of PGE_2 synthase mRNA. Treatment of LPS increased the release of PGE_2 in human peripheral blood monocytes (23). LPS also increased the expression of prostaglandin in RAW264.7 cells (21). COX-2 mRNA expression, upper enzyme associated with PGE_2, was also increased with the stimulation of LPS. However, t10c12-CLA decreased the overproduction of PGE_2 in accordance with suppression of COX-2 and PGE_2 synthase expression in LPS-stimulated porcine PBMCs. Inhibitory effect by concentration differences (10 and 20 μM) of t10c12-CLA on the production of PGE_2 and the expression of PGE_2 synthase and COX-2 mRNA was not observed. Therefore, it was suggested that t10c12-CLA has an anti-inflammatory effect via COX-2 pathway in porcine PBMCs stimulated with LPS. In addition, both CLA isomers, c9t11-CLA and t10c12-CLA, increased phagocytosis of human macrophages through inhibition of COX-2 expression and PGE_2 production by inactivation of the NF-kB pathway. Reduced PGE_2 can enhance phagocytic capacity of macrophage in human (24). Thromboxane B_2 (TXB_2) was also reduced with PGE_2 by treatment of t10c12-CLA in human macrophages in inflammatory condition (25). It can be assumed that t10c12-CLA reduces not only PGE_2 but also other eicosanoid within COX-2 pathway in macrophages in inflammatory condition. Therefore, we need to investigate the effect of t10c12-CLA on other products of arachidonic acid metabolism according to the type of immune cells. Additional studies are also required to confirm the relation among COX-2 pathway, TNF-α, and phagocytic capacity. Furthermore, overexpression of COX-2 in prostate and breast cancer cell was suppressed with the treatment of CLA, which can contribute to anti-tumorigenic or anti-proliferative effect in tumor cells (9,18).

5-LOX is another pathway of arachidonic acid metabolism, which is responsible for production of leukotrienes (LTs). LTB_4 generated by 5-LOX is responsible for chemotaxis of leukocytes (5). In this study, treatment of t10c12-CLA in LPS-naïve PBMCs increased 5-LOX production and mRNA expression. This indicates that t10c12-CLA has an immuno-stimulatory effect, which increases the 5-LOX production and mRNA expression in LPS-naïve PBMCs. But lower dose of t10c12-CLA appeared to be not enough to express the 5-LOX because treatment of 10 μM t10c12-CLA did not affect 5-LOX expression. It is, therefore, needed to evaluate the minimal effective dose of t10c12-CLA to exert immuno-stimulatory effect associated with 5-LOX pathway. However, stimulation of LPS in PBMCs remarkably increased 5-LOX production and mRNA expression. This excessive increase with production and mRNA expression of 5-LOX was suppressed by t10c12-CLA. 5-LOX and its mRNA were increased in duodenal and colonic biopsies in dog with chronic enteropathies (10). In addition, minocycline, a tetracycline antibiotic, inhibited brain inflammation by the inhibition of 5-LOX expression and enzymatic activation in rats (8). Leukotriene B4 from the exudate cells, splenic cells, and LTC4 from lung cells were reduced in response to the dose of dietary CLA level, which demonstrated the effect of CLA on allergic response (26). These findings indicate that CLA can modulate the release of product of 5-LOX, leukotriene. It can be thought that t10c12-CLA exerts anti-inflammatory effect via inhibition of 5-LOX pathway in LPS-stimulated porcine PBMCs.

It was revealed that dual COX/5-LOX (cyclooxygenase/5-lipoxygenase) inhibitors can act as a valuable alternative to classical non-steroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors for the treatment of inflammatory diseases since these latter show diverse side effects such as gastrointestinal (GI) problem, which are less or absent in dual-acting agent (6). From this study, we could observe the possibility of t10c12-CLA as an alternative therapeutic agent of inflammation. t10c12-CLA is thought to have an anti-inflammatory effect through dual inhibition of COX-2 and 5-LOX pathway from gene expression to production level in LPS-stimulated porcine PBMCs. Therefore, it can be concluded that t10c12-CLA attenuate the inflammatory response by down-regulation of eicosanoids production.

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