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Trans-10, *cis*-12 Conjugated Linoleic Acid Modulates Tumor Necrosis Factor-α Production and Nuclear Factor-κB Activation in RAW 264.7 Macrophages Through Formation of Reactive Oxygen Species

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Abstract : The aims of this study were to explore the effects of conjugated linoleic acid (CLA) on reactive oxygen species (ROS) production in lipopolysaccharide (LPS)-naïve and LPS-stimulated RAW 264.7 macrophages and to examine whether these effects affect the regulation of tumor necrosis factor-alpha (TNF-α) production, and nuclear factor-kappa B (NF-κB) and peroxisome proliferator-activated receptor gamma (PPARγ) activation. *Trans*-10, *cis*-12 (*t*10*c*12)-CLA increased the production of ROS, as well as TNF-α in LPS-naïve RAW 264.7 cells. The CLA-induced TNF-α production was suppressed by treatment of diphenyleneiodonium chloride (DPI), a NADPH oxidase inhibitor. In addition, CLA enhanced the activities of NF-κB and PPARγ in LPS-naïve RAW 264.7 cells, and this effect was abolished with DPI treatment. LPS treatment increased ROS production, whereas CLA reduced LPS-induced ROS production. LPS increased both TNF-α production and NF-κB activity, whereas *t*10*c*12-CLA reduced TNF-α production and NF-κB activity in LPS-stimulated RAW 264.7 cells. DPI treatment suppressed LPS-induced ROS production and NF-κB activity. Moreover, DPI enhanced the inhibitory effects of *t*10*c*12-CLA on TNF-α production and NF-κB activation in LPS-stimulated RAW 264.7 cells. However, neither *t*10*c*12-CLA nor DPI affected PPARγ activity in LPS-stimulated RAW 264.7 cells. Taken together, these data indicate that *t*10*c*12-CLA induces TNF-α production by increasing ROS production in LPS-naïve RAW 264.7 cells, which is mediated by the enhancement of NF-κB activity via PPARγ activation. By contrast, *t*10*c*12-CLA suppresses TNF-α production by inhibiting ROS production and NF-κB activation via a PPARγ-independent pathway in LPS-stimulated RAW 264.7 cells. These results suggest that *t*10*c*12-CLA can modulate TNF-α production and NF-κB activation through formation of ROS in RAW 264.7 macrophages.

Key words **:** conjugated linoleic acid, lipopolysaccharide, tumor necrosis factor-alpha, nuclear factor-kappa B, reactive oxygen species, peroxisome proliferator-activated receptor gamma, RAW 264.7 macrophages.

Introduction

Reactive oxygen species (ROS) include molecules such as hydrogen peroxide, hypochlorous acid, and singlet oxygen, which are all capable of forming free radicals in the extra- and intracellular environments (8). ROS cause tissue damage via a variety of mechanisms by activating nuclear factor-kappa B (NF-κB) (28). Tumor necrosis factor-alpha (TNF-α) is a protein synthesized and secreted by mononuclear phagocytes in response to bacterial endotoxin and other inducers. It mediates a wide range of effects such as regulation of immune function, inflammatory responses, and induction of apoptosis of certain tumor cells (11).

Conjugated linoleic acid (CLA), a mixture of positional and geometric conjugated dienoic isomers of linoleic acid, is naturally found in ruminant foods and dairy products (2). CLA has received considerable attention due to its unique properties when tested in rodent models, including anti-cancer (3), anti-atherogenic (15), and anti-diabetic effects (10). CLA isomers increased and decreased the DNA-binding activity of peroxisome proliferator-activated receptor gamma (PPARγ) and NF-κB, respectively, in vascular smooth muscle cells (24). CLA reduces the interferon (IFN)-γ-induced expression of mRNA for inflammatory mediators such as cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), and TNF-α. Also, CLA reduces the production of prostaglandin (PG) E_2, TNF-α, nitric oxide (NO), and other pro-inflammatory cytokines such as interleukin (IL)-1β and IL-6 (34). *Trans*-10, *cis*-12 (*t*10*c*12)-CLA reduces the expression of COX-2 mRNA and the subsequent production of PGE_2 in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells, which is attributed to the inhibition of NF-κB both *in vitro* and *in vivo* (17). CLA prevents LPS-induced body weight loss, and inhibits NO production and TNF-α release (6,33).

There is no doubt that CLA affects immune and inflammatory responses. However, its role in ROS production remains unclear. Therefore, the aims of the present study were to

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investigate the effects of CLA on ROS production in LPS-naïve and LPS-stimulated RAW 264.7 cells and to examine whether these effects influence the regulation of TNF-α production, and NF-κB and PPARγ activation.

Materials and Methods

Chemicals and reagents

*T*10*c*12-CLA (96% purity), LPS from *Salmonella enterica* serotype Enteritidis, and diphenyleneiodonium chloride (DPI), a nicotinamide adenine dinucleotide phosphate (NADPH) inhibitor, were purchased from Sigma-Aldrich (St. Louis, MO, USA). *T*10*c*12-CLA was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) to yield a final concentration of 50 mM prior to being passed through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA).

RAW 264.7 cell culture

RAW 264.7 cells, a mouse macrophage cell line, were obtained from the Korean Cell Line Bank (Seoul, Korea) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Hyclone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone Lab.), 100 U/mL penicillin, 100 μg/mL streptomycin (Hyclone Lab.), 4 mM L-glutamine, and 450 mg/dL glucose with sodium pyruvate, at 37°C in a 5% CO_2-humidified atmosphere.

Measurement of intracellular ROS using confocal microscopy

ROS levels were measured using the Image-iT® LIVE green ROS detection kit (Invitrogen, Carlsbad, CA, USA). RAW 264.7 cells (1×10^2 cells/mL) were seeded at into a cover glass-bottomed 35 mm cell culture dish for 12 h at 37°C. Cells were gently washed with warm Hank's balanced salt solution (HBSS) (Sigma-Aldrich) and then pretreated with or without DPI (10 μM) for 30 min. To stain ROS, plates were incubated with carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA, 10 μM) for 30 min at 37°C followed by incubation with *t*10*c*12-CLA (20 μM) alone, LPS (100 ng/mL) alone, or LPS (100 ng/mL) plus *t*10*c*12-CLA (20 μM) for 20 min. After the indicated treatments, the cells were washed three times with HBSS and visualized under a confocal microscope (TCS SP2 AOBS; Leica Microsystems, Wetzlar, Germany). The fluorescence was measured using a standard fluorescein filter set at 493-542 nm. ROS fluorescence (green) was quantified using LSM ZEN imaging software (Zeiss, Jena, Germany).

Culture supernatants

RAW 264.7 cells (1×10^6 cells/mL) were seeded into 24-multi-well plates (Nunc, Naperville, IL, USA) and cultured for 12 h at 37°C. DPI (10 μM) was added (or not) for the last 30 min of culture. Next, the cells were incubated with *t*10*c*12-CLA (20 μM), LPS (100 ng/mL) or LPS (100 ng/mL) plus *t*10*c*12-CLA (20 μM), with control cells receiving the same amount of DMSO only, for 12 h. All culture supernatants were collected after centrifugation at 14,000 g for 5 min and stored at −71°C until used for TNF-α analysis. Pellets were harvested and stored at −71°C until nuclear protein extraction and analysis of NF-κB p65 and PPARγ transactivation.

Nuclear protein extraction

The nuclear fractions from RAW 264.7 cells plated on 24-multi-well plates were isolated using a nuclear extract kit (Active Motif, Carlsbad, CA, USA) as described elsewhere (12).

Measurement of TNF-α production

Culture supernatants were collected following 12 h culture. The amount of TNF-α was measured in a direct sandwich ELISA (mouse TNF-α ELISA kit, Thermo scientific, Rockford, IL, USA) according to the manufacturer's protocol. All samples, standards and controls were assayed in duplicate. The optical absorbance values were calculated by subtracting the 550 nm values from the 450 nm values in an automated microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The concentration of TNF-α was quantified using four titration points from standard curves prepared using lyophilized recombinant mouse TNF-α.

NF-κB p65 transcription factor assay

NF-κB activity was determined using the TransAM® NF-κB transcription factor assay kit (Active Motif) according to the manufacturer's protocol. Nuclear extracts were added to the wells of 96-well plates coated with an immobilized oligonucleotide containing the NF-κB consensus site. The optical density was measured in an automated microplate reader (BioTek instruments, Inc.) set at 450 nm with a reference wavelength of 655 nm.

PPARγ transactivation assay

Activation of the PPARγ transcription factor was detected using an ELISA-based TransAM® PPARγ transcription factor assay kit (Active Motif) according to the manufacturer's protocol. Briefly, nuclear extracts were added to wells of 96-well plates pre-coated with immobilized oligonucleotides containing elements which PPARγ specifically binds to. The optical density was determined in an automated microplate reader (BioTek Instruments, Inc.) set at 450 nm with a reference wavelength of 655 nm.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The one-way analysis of variance followed by Dunnett's test for each pair for multiple comparisons was used to determine the statistical significance of the differences between the control and treatment groups. Comparisons of two groups were made by two-sample *t*-test. *P*-values of < 0.05 were considered to be statistically significant. All data are expressed as mean ± standard errors (SEM).

Results

CLA increases ROS formation

As shown in Fig 1A, the ROS level was significantly increased ($P < 0.05$) by *t*10*c*12-CLA treatment in a dose-

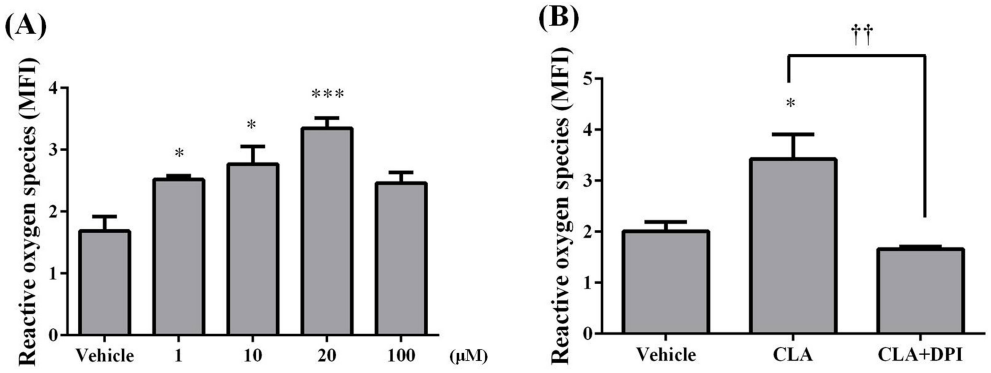

Fig 1. (A) Effect of *t*10*c*12-CLA on ROS production in RAW 264.7 cells. Cells (1×10^2 cells/mL) were incubated with *t*10*c*12-CLA (20 μM) for 20 min at the indicated concentrations. (B) Effect of DPI (10 μM) on ROS production in cells treated with *t*10*c*12-CLA (20 μM). The carboxy-H_2DCF green fluorescence was quantified by image analysis (see Materials and methods section) and values were expressed as mean ± SEM (n = 3). **P* < 0.05, ****P* < 0.001 (one-way ANOVA); ††*P* < 0.05 (as determined by two-sample *t*-test). MFI, mean fluorescence intensity.

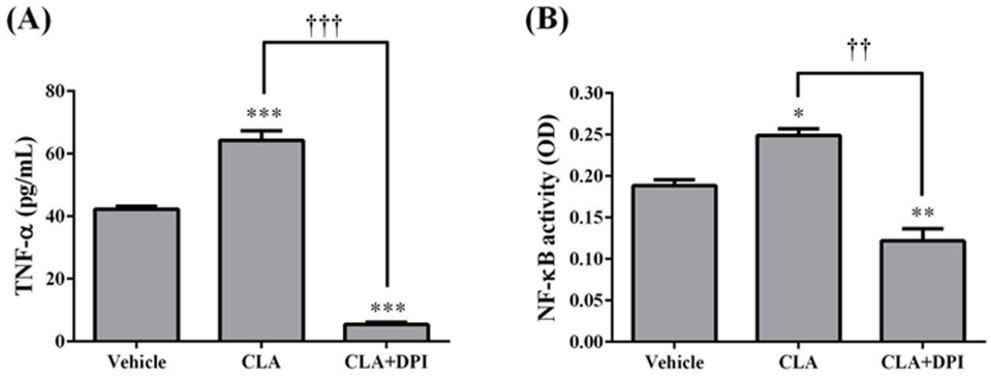

Fig 2. Effect of *t*10*c*12-CLA on TNF-α production and NF-κB activation in RAW 264.7 cells. Cells (1×10^6 cells/mL) were pre-treated with or without DPI (10 μM) for 30 min followed by treatment with *t*10*c*12-CLA (20 μM) for 12 h. (A) The concentration of TNF-α (pg/mL) in the culture supernatant and (B) NF-κB p65 activation in nuclear extracts was measured as described in the Materials and methods section. Values are expressed as mean ± SEM (n = 3). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (one-way ANOVA); ††*P* < 0.01, †††*P* < 0.001 (as determined by the two-sample *t*-test). OD, optical density.

dependent manner and peaked at a *t*10*c*12-CLA concentration of 20 μM (*P* < 0.001) compared with vehicle (DMSO)-treated controls. We next tested whether DPI, a NADPH inhibitor, inhibits *t*10*c*12-CLA-induced ROS production. Cells were pre-treated with DPI (10 μM) for 30 min prior to incubation with *t*10*c*12-CLA (20 μM) for 20 min. Treatment with DPI significantly suppressed (*P* < 0.05) the *t*10*c*12-CLA-mediated increase in ROS level (Fig 1B).

CLA increases TNF-α production and NF-κB activity through the production of ROS

As shown in Fig 2A, *t*10*c*12-CLA significantly increased (*P* < 0.001) TNF-α production compared with vehicle-treated controls. The *t*10*c*12-CLA-mediated increase in TNF-α production was reversed (*P* < 0.001) by the addition of DPI. The concentration of TNF-α was lower (*P* < 0.001) than that of vehicle-treated controls. NF-κB p65 DNA-binding activity was also significantly increased (*P* < 0.05) by *t*10*c*12-CLA treatment compared to that in vehicle-treated controls, and this effect was abolished by DPI (*P* < 0.001). NF-κB activity in DPI-treated cells was lower than that in vehicle-treated controls (*P* < 0.01) (Fig 2B).

CLA induces PPARγ activation through the production of ROS

The ROS-dependent effect of *t*10*c*12-CLA on PPARγ activation was tested by pre-treating the cells with DPI (10 μM) for 30 min, followed by incubation with *t*10*c*12-CLA (20 μM) for 12 h. As shown in Fig 3, *t*10*c*12-CLA significantly increased PPARγ activity compared with vehicle-treated controls (*P* < 0.001) and this effect was significantly inhibited (*P* < 0.001) by the addition of DPI.

LPS increases ROS production

To examine the time taken for LPS to induce inflammatory stimuli in RAW 264.7 cells, the cells were incubated with carboxy-H_2DCFDA (10 μM) for 30 min, followed by incubation with LPS (100 ng/mL) for indicated times. As shown in Fig 4, ROS levels were significantly higher (*P* < 0.05) in LPS-stimulated cells (peaking at 20 min) than in the vehicle (DMSO)-treated controls.

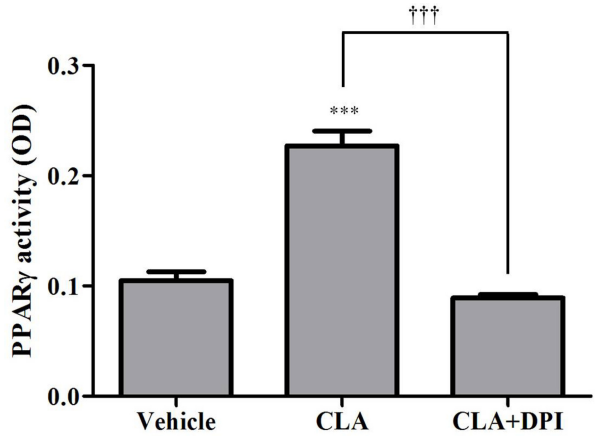

Fig 3. Effect of *t*10*c*12-CLA on PPARγ activation in RAW 264.7 cells. Cells (1×10^6 cells/mL) were pre-treated with or without DPI (10 μM) for 30 min prior to treatment with *t*10*c*12-CLA (20 μM) for 12 h. PPARγ activity was assayed in nuclear extracts as described in the Materials and methods section. Values are expressed as mean ± SEM (n = 3). ***$P < 0.001$ (one-way ANOVA); †††$P < 0.001$ (as determined by two-sample *t*-test). OD, optical density.

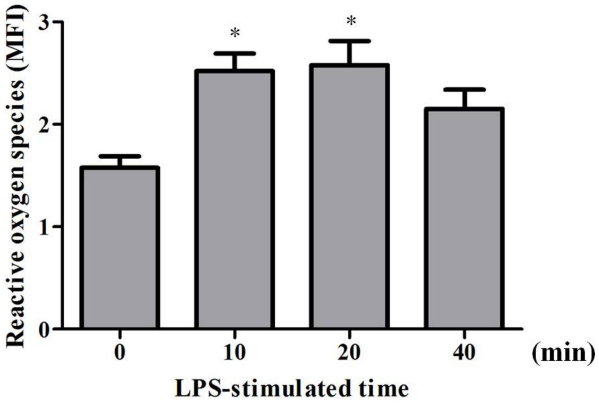

Fig 4. Time-dependent ROS production in LPS-stimulated RAW 264.7 cells. Cells (1×10^2 cells/mL) were incubated with carboxy-H_2DCFDA (10 μM) for 30 min, followed by treatment with LPS (100 ng/mL) for the indicated times. The carboxy-H_2DCF fluorescence was quantified by image analysis (see Materials and methods section) and values are expressed as mean ± SEM (n = 3). *$P < 0.05$ (one-way ANOVA followed by Dunnett's post-test). MFI, mean fluorescence intensity.

CLA decreases LPS-induced ROS production

Next, we asked whether *t*10*c*12-CLA affects LPS-induced ROS production in RAW 264.7 cells. Compared with vehicle-treated controls, there was a significant increase in ROS production in cells treated with LPS or LPS plus *t*10*c*12-CLA (Fig 5; $P < 0.001$). However, the level of ROS was significantly lower in LPS/CLA-treated cells than in only LPS-treated cells ($P < 0.05$). ROS levels in cells treated with LPS ($P < 0.01$) or LPS/CLA ($P < 0.01$) were also significantly reduced by pre-treatment with DPI. However, the DPI-mediated suppression of ROS production in LPS-stimulated cells was not significantly different from that seen in cells treated with LPS plus CLA.

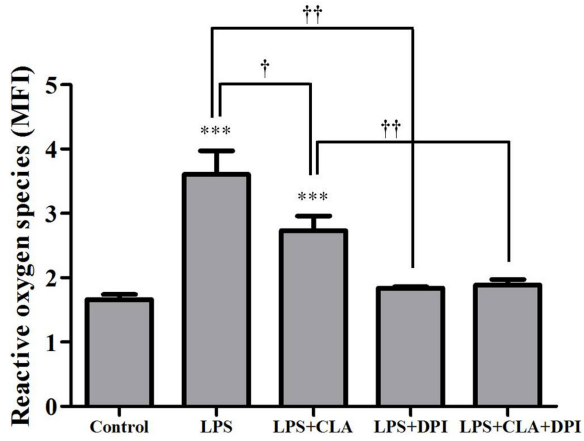

Fig 5. Effect of *t*10*c*12-CLA on ROS production in LPS-stimulated RAW 264.7 cells. Cells (1×10^2 cells/mL) were pretreated with or without DPI (10 μM) for 30 min and then incubated with carboxy-H_2DCFDA (10 μM) for 30 min, followed by treatment with LPS (100 ng/mL) plus *t*10*c*12-CLA (20 μM) for 20 min. The carboxy-H_2DCF fluorescence was quantified by image analysis (see Materials and methods section) and values are expressed as mean ± SEM (n = 3). ***$P < 0.001$ (one-way ANOVA followed by Dunnett's post-test); †$P < 0.05$ and ††$P < 0.01$ (two-sample *t*-test). MFI, mean fluorescence intensity.

CLA reduces ROS-induced TNF-α production and NF-κB p65 DNA-binding activity in LPS-stimulated RAW 264.7 cells

Compared with vehicle-treated controls, both LPS and LPS/*t*10*c*12-CLA significantly increased TNF-α production ($P < 0.001$). However, TNF-α production in vehicle-treated control cells was significantly reduced ($P < 0.001$) when cells were treated with DPI prior to exposure to LPS/*t*10*c*12-CLA. Thereafter, *t*10*c*12-CLA significantly reduced TNF-α production in LPS-stimulated RAW 264.7 cells when compared with only LPS-treated cells ($P < 0.01$), and this effect was amplified by pre-treatment with DPI ($P < 0.001$). In addition, the levels of TNF-α in LPS-stimulated RAW 264.7 cells were significantly reduced by pre-treatment with DPI alone ($P < 0.001$), and were further suppressed by the addition of *t*10*c*12-CLA ($P < 0.01$) (Fig 6A).

As shown in Fig 6B, NF-κB p65 DNA-binding activity in LPS-stimulated cells was significantly higher than that in the vehicle-treated controls ($P < 0.01$). The LPS-mediated increase in NF-κB activity was reversed by treatment with *t*10*c*12-CLA ($P < 0.01$). The NF-κB activity in LPS-stimulated cells pre-treated with DPI was significantly reduced ($P < 0.05$) when compared with that in vehicle-treated controls. NF-κB binding activity in cells stimulated with LPS alone was significantly reduced by pre-treatment with DPI ($P < 0.05$). Although the NF-κB binding activity in cells treated with LPS plus *t*10*c*12-CLA was not significantly different from that in cells pretreated with DPI, treatment of *t*10*c*12-CLA in LPS-stimulated cells pre-treated with DPI led to further suppression of NF-κB binding activity ($P < 0.001$).

CLA has no effect on PPARγ activation in LPS-stimulated RAW 264.7 cells

To find out whether the effects of *t*10*c*12-CLA in LPS-

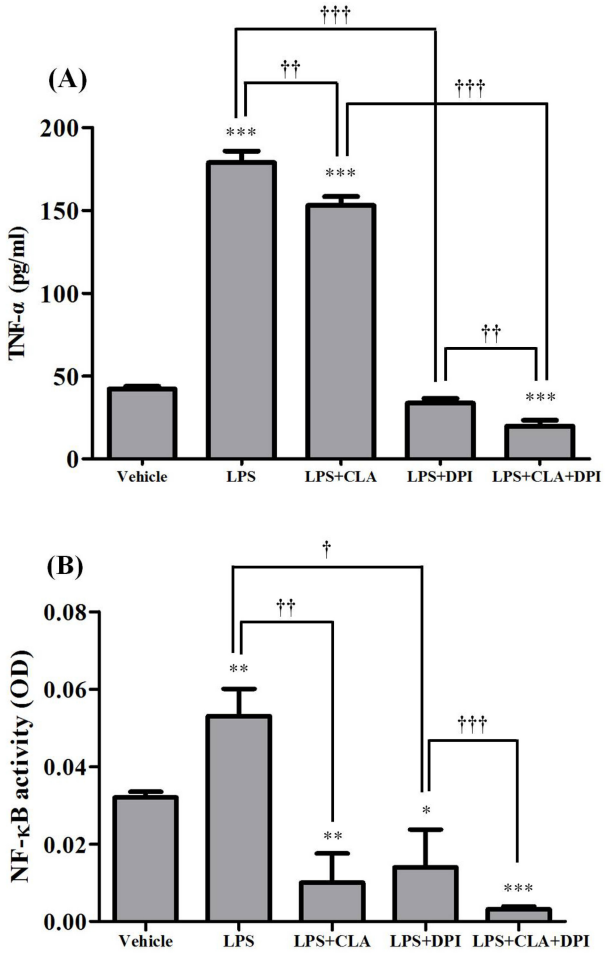

Fig 6. Effect of *t*10*c*12-CLA on ROS-induced TNF-α production and NF-κB activation in LPS-stimulated RAW 264.7 cells. Cells (1×10^6 cells/mL) were pretreated with or without DPI (10 μM) for 30 min followed by treatment with LPS (100 ng/mL) plus *t*10*c*12-CLA (20 μM) for 12 h. (A) The concentration of TNF-α (pg/mL) in the culture supernatant and (B) NF-κB p65 activation in nuclear extracts was measured as described in the Materials and methods section. Values are expressed as mean ± SEM (n = 3). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (one-way ANOVA followed by Dunnett's post-test); $^\dagger P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ (two-sample *t*-test). OD, optical density.

stimulated cells are associated with PPARγ activation, we treated cells with LPS (100 ng/mL) or LPS (100 ng/mL) plus *t*10*c*12-CLA (20 μM) in the presence or absence of DPI for 12 h. Representative results are shown in Fig 7, which depicts the level of PPARγ activation in LPS-stimulated cells subjected to the indicated treatments. There was no significant difference in the level of PPARγ activity between each of the treatment groups and the vehicle-treated control.

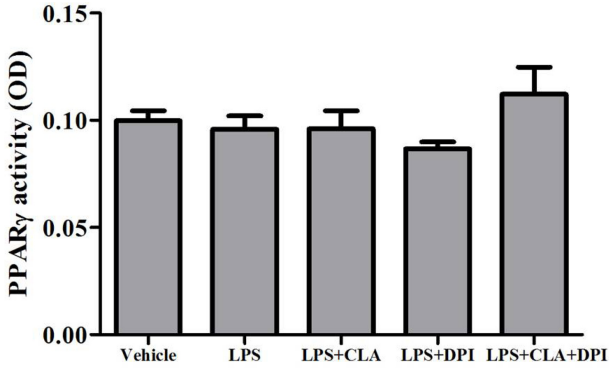

Fig 7. Effect of *t*10*c*12-CLA on PPARγ activation in LPS-stimulated RAW 264.7 cells. Cells (1×10^6 cells/mL) were pretreated with or without DPI (10 μM) for 30 min prior to treatment with LPS (100 ng/mL) plus *t*10*c*12-CLA (20 μM) for 12 h. PPARγ activity was assayed in nuclear as described in the Materials and methods section. Values are expressed as mean ± SEM (n = 3). OD, optical density.

Discussion

The results of the present study revealed that *t*10*c*12-CLA increased TNF-α production in LPS-naïve RAW 264.7 cells, which is consistent with previous reports (16). The *t*10*c*12-CLA-induced enhancement of TNF-α production in RAW 264.7 cells up-regulated the phagocytic capacity of these cells (27). We found that *t*10*c*12-CLA increases ROS production in LPS-naïve RAW 264.7 cells, and similar increases in ROS synthesis have been reported in human macrophages (29). DPI, a nonspecific inhibitor of a NADPH oxidase, suppressed the CLA-mediated enhancement of TNF-α production. These findings suggest that enhancing effects of CLA on TNF-α production in LPS-naïve RAW 264.7 cells could be mediated by ROS.

LPS promotes the secretion of pro-inflammatory cytokines by many cell types, particularly macrophages (31). It induces TNF-α production which is partially caused by stimulation of ROS production (25,36). Here, we used LPS to examine the effects of *t*10*c*12-CLA on the inflammatory responses. In the present study, LPS increased both ROS and TNF-α production in RAW 264.7 cells. Interestingly, *t*10*c*12-CLA then reduced ROS production by LPS-stimulated cells. Several studies identified that CLA reduces oxidative stress and increases the oxidative stability of the liver, suggesting that CLA protects against oxidative stress (9,21). However, another study showed that CLA actually increases oxidative stress (30). Therefore, we used DPI to examine whether CLA-mediated ROS reduction is associated with NADPH oxidase activity. The results showed that DPI further suppressed *t*10*c*12-CLA-induced a decrease in ROS production in LPS-stimulated RAW 264.7 cells. These findings suggest that CLA can help to protect these cells against oxidative stress in inflammatory condition.

In the present study, we found that *t*10*c*12-CLA down-regulated LPS-induced TNF-α production in RAW 264.7 cells. This is in agreement with a previous study showing that *t*10*c*12-CLA down-regulates the production of pro-inflammatory cytokines, including TNF-α (4,33,34), IL-10 (13), and IL-12 (18). Therefore, we presume that the decrease in TNF-α production in *t*10*c*12-CLA-treated, LPS-stimulated cells in the present study might be also related to both an increase in IL-10 and a decrease in IL-12 production. TNF-α increases the expression of various NADPH oxidase components, which then contribute to increased oxidase activity. This is because TNF-α induces superoxide generation by activating NADPH oxidases (14). However, activation of p38 mitogen-

activated protein kinase (MAPK) is required for TNF-α expression under LPS-stimulated conditions, and increased ROS production is a prerequisite for the activation of p38 MAPK (26). The results presented herein revealed that DPI suppresses LPS-induced TNF-α production, and that DPI suppresses the production of TNF-α in *t*10*c*12-CLA-treated, LPS-stimulated RAW 264.7 cells to a greater extent than in cells stimulated with LPS alone. These suggest that CLA can also inhibit LPS-induced inflammatory events, which is mediated by ROS.

The CLA-mediated activation of NF-κB p65 DNA binding in LPS-naïve RAW 264.7 cells observed in the present study has also been reported in peripheral blood mononuclear cells (PBMCs) (12), human adipocytes (5), and human umbilical vein endothelial cells (20). The activation of NF-κB is followed by the transcription of various pro-inflammatory genes including TNF-α (32). These observations imply that CLA may participate in TNF-α production in LPS-naïve RAW 264.7 cells by stimulating NF-κB activation. ROS activate NF-κB by inducing the release of TNF-α from T lymphocytes (25). Certain NF-κB-regulated genes play a major role in regulating the amount of ROS in cells; ROS have various inhibitory or stimulatory roles in NF-κB signaling (1,19). In our study, *t*10*c*12-CLA-mediated increase in NF-κB p65 activity in LPS-naïve RAW 264.7 cells was abolished by the addition of DPI. These observations indicated that CLA could participate in the NF-κB-mediated activation through a ROS-dependent mechanism.

We found that LPS induced NF-κB p65 DNA-binding activity in RAW 264.7 cells. DPI suppressed LPS-induced NF-κB activation, indicating that LPS increases NF-κB activities via ROS production. We also found that *t*10*c*12-CLA suppressed NF-κB activity in DPI-pretreated, LPS-stimulated cells. Collectively, these results suggest that *t*10*c*12-CLA suppresses TNF-α production from macrophages by inhibiting NF-κB activation associated with ROS in LPS-stimulated conditions. CLA prevents TNF-α gene expression by inhibiting NF-κB-binding activity under inflammatory conditions (35). Therefore, CLA may have anti-inflammatory activity and help to prevent oxidative stress by down-regulating ROS-mediated NF-κB activation and the subsequent production of inflammatory cytokines such as TNF-α.

*T*10*c*12-CLA and *c*9*t*11-CLA increased both PPARγ and NF-κB activities in PBMCs (12) and human umbilical vein endothelial cells (20). In the present study, PPARγ activity in LPS-naïve RAW 264.7 cells was enhanced by t10c12-CLA treatment. These findings support the idea that the activation of PPARγ by *t*10*c*12-CLA can modulate TNF-α production through the up-regulation of NF-κB activity. We found that DPI reversed the effects of *t*10*c*12-CLA on PPARγ activation in RAW 264.7 cells. These results imply that the effects of CLA on PPARγ activation may be dependent on ROS.

However, we found that neither *t*10*c*12-CLA nor DPI affected PPARγ activation in LPS-stimulated RAW 264.7 cells. CLA is functionally similar to PPARγ ligands. For example, 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2), a PPARγ agonist, exerts anti-inflammatory effects by reducing the production of inflammation-associated molecules. Indeed, 15d-PGJ_2 suppresses NF-κB activity via both PPARγ-independent and PPARγ-dependent mechanisms (23). By contrast, 15d-PGJ_2 suppresses NF-κB and activator protein (AP)-1 activity in a PPARγ-dependent manner (22). In case of CLA, the regulatory effects of CLA on iNOS transcription are dependent on PPARγ transcription (34). CLA up-regulates PPARγ activity and mRNA expression in porcine PBMCs, regardless of LPS stimulation (12). By contrast, CLA down-regulates the expression of PPARγ and its target genes in adipocytes (7). These findings suggest that CLA-induced PPARγ activation may depend upon the status and type of cells, and that the anti-inflammatory effects of CLA under LPS-stimulated conditions may also be mediated by a PPARγ-independent pathway in RAW 264.7 cells.

In conclusion, we provide evidence that *t*10*c*12-CLA induces TNF-α production by increasing ROS production in LPS-naïve RAW 264.7 cells, which is mediated by the enhancement of NF-κB activity. This effect of CLA is mediated by PPARγ activation. In contrast, *t*10*c*12-CLA exerts anti-inflammatory effects by inhibiting ROS production in LPS-stimulated RAW 264.7 cells, which regulates TNF-α production and NF-κB activation via a PPARγ-independent pathway.

Acknowledgments

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RAW 264.7 세포에 있어 *t*10*c*12-CLA의 ROS를 통한 TNF-α 생산 및 NF-κB 활성 조절

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요 약 : 본 연구는 염증상태에서의 CLA의 효과와 작용기전을 알아보기 위해 LPS-자극 RAW 264.7 macrophages에 있어 ROS 생성과 TNF-α 생산, NF-κB 및 PPARγ 활성을 검토하였다. *t*10*c*12-CLA는 LPS로 자극하지 않은 비염증시의 RAW 세포에서는 ROS 생성을 증가시켜 TNF-α 생산을 유도하였으며, 이 효과는 PPARγ 활성화에 의존해서 NF-κB 활성 증가에 의해 매개되었다. 반면, LPS로 자극한 염증조건의 RAW 세포에서는 *t*10*c*12-CLA가 PPARγ 활성화에 의존하지 않는 경로로 ROS 생성 및 과도한 TNF-α 생산을 억제하였다. 본 결과로부터 CLA는 ROS 생성을 통해 TNF-α 생산 및 NF-κB 활성을 염증 유무에 따라 조절하는 것으로 사료되었다.

주요어 : CLA, LPS, TNF-α, NF-κB, ROS, PPARγ, RAW 264.7 세포