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Effect of *trans*-10, *cis*-12 Conjugated Linoleic Acid on Calcium-Dependent Reactive Oxygen Species and Nitric Oxide Production and Nuclear Factor-κB Activation in Lipopolysaccharide-Stimulated RAW 264.7 Cells

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Abstract : *Trans*-10, *cis*-12-conjugated linoleic acid (*t*10*c*12-CLA) has been shown to participate in the regulation of anti-inflammatory effects. The objectives of this study were to examine the effects of *t*10*c*12-CLA on reactive oxygen species (ROS) and nitric oxide (NO) production and nuclear factor-kappaB (NF-κB) activation in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells and to determine whether these effects were associated with change of intracellular calcium ion (Ca^{2+}). ROS production was increased in LPS-stimulated RAW 264.7 cells, and this effect was suppressed by 1,2-bis-(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA/AM), a calcium chelator. *t*10*c*12-CLA suppressed ROS production in LPS-stimulated RAW 264.7 cells, which was further more decreased by treatment with BAPTA/AM. These indicated that *t*10*c*12-CLA decreases Ca^{2+}-dependent ROS production in LPS-stimulated RAW 264.7 cells. Similarly, NF-κB p65 DNA binding activity and NO production were decreased by treatment with either *t*10*c*12-CLA, BAPTA/AM, or *t*10*c*12-CLA and BAPTA/AM combination. However, there were no differences between *t*10*c*12-CLA and BAPTA/AM treatment in NO production of LPS-stimulated RAW 264.7 cells. These data indicate that *t*10*c*12-CLA inhibits the increases in ROS and NO production and the NF-κB activation in LPS-stimulated condition. These results suggested that CLA exerts potent anti-inflammatory effects by suppression of LPS-induced ROS and NO production, and NF-κB activationn via Ca^{2+}-dependent pathway.

Key words **:** conjugated linoleic acid, lipopolysaccharide, reactive oxygen species, nuclear factor-kappaB, nitric oxide, calcium ion.

Introduction

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid with conjugated double bonds. CLA is found mainly in dairy products and meats (5). CLA has been shown to have many potential health benefits, such as antidiabetes (27), anti-inflammation (30), anti-carcinogenesis (23) and anti-artherogenesis effects (16). CLA can stimulate or inhibit immune cell function. Among CLA isomers, *trans*-10, *cis*-12-CLA (*t*10*c*12-CLA) has shown to participate in the modulation of pro- or anti-inflammatory cytokine secretion (17,19).

Inflammation is a complex process regulated by a cascade of cytokine, nitric oxide (NO) and prostaglandin produced by activated macrophages (26). Macrophages are activated by pro-inflammatory cytokines (2) and bacterial lipopolysaccharides (LPS) (33). LPS-mediated inflammation is highly dependent on reactive oxygen species (ROS). LPS, a highly conserved outer membrane component of gram-negative bacteria, triggers many biological response such as fever, septic shock, and even death (22). LPS activates cell signaling through the Toll-like receptor (TLR)-4 (13).

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It has been underscored the notion that the Ca^{2+} and ROS signaling systems are intimately integrated and that Ca^{2+}-dependent regulation of ROS homeostasis might influence intracellular redox balance, and *vice versa* (3,11). ROS are actively involved in the production of pro-inflammatory mediators, such as NO and TNF-α (24,25). ROS play an important role in physiological cellular functions by activating several enzymatic cascades and transcription factors (8). NO is signaling molecule that displays anti-inflammatory characteristics under normal physiological conditions. But when produced at excessive levels it possesses pro-inflammatory properties (28). NO is synthesized from the amino acid l-arginine by isoforms of nitric oxide synthase (NOS) enzymes. A large amount of NO, particularly synthesized by inducible NOS (iNOS), induces an inflammatory response to inhibit the growth of invading microorganism and tumor cells. Cells, especially macrophages, can be induced to produce NO upon exposure to stimulants such as bacterial LPS and/or cytokines (6).

Nuclear factor-kappaB (NF-κB), a nuclear transcription factor, exists in the cytoplasm of the majority of cell types as homodimers or heterodimers of a family of structurally related proteins (21). NF-κB is a ubiquitously expressed family of transcription factors, which controls the expression of numerous genes involved in inflammatory and immune

response, and cellular proliferation (10). ROS cause tissue damage via a variety of mechanisms by activating NF-κB (29).

In this study, we examined the effect of *t*10*c*12-CLA on ROS and NO production and NF-κB activation in LPS-stimulated RAW 264.7 cells and determined whether this effect is associated with change of intracellular Ca^{2+}.

Materials and Methods

Chemicals and reagents

Trans-10, *cis*-12-conjugated linoleic acid (*t*10*c*12-CLA) (96% purity), 1,2-bis-(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA/AM), a calcium chelator, and lipopolysaccharide (LPS) from *Salmonella enterica* serotype Enteritidis were purchased (Sigma-Aldrich Co., St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich Co.). *T*10*c*12-CLA was dissolved to a final concentration of 50 mM prior to being passed through a 0.45 μm membrane filter (Millipore Co., Bedford, MA, USA).

RAW 264.7 cells culture

The BALB/C mouse macrophage cell line RAW 264.7 was obtained from the Korea Cell Line Bank (Seoul, Korea) and maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone Lab., Logan, UT, USA) supplemented with 4 mM L-glutamine, 450 mg/dl glucose with sodium pyruvate, 10% fetal bovine serum (FBS; Hyclone Lab.), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO2.

Determination of intracellular ROS level

ROS levels were assayed using the Image-iT® LIVE green reactive oxygen species detection kit (Invitrogen Company, Carlsbard, CA, USA). RAW 264.7 cells were seeded at 1×10^3 cells/μl into a coverglass-bottomed 35-mm cell culture dish for 12 h in the presence or absence of BAPTA/AM (200 μM) for final 1 h, and then cells were gently washed with warm Hank's balanced salt solution (HBSS, Sigma-Aldrich Co.). Cells 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), LPS (1 μg/ml), and *t*10*c*12-CLA in combination with LPS for final 20 min. After the indicated treatments, cells were washed three times with HBSS and visualized using a confocal microscope (TCS SP2 AOBS; Leica Microsystems, Wetzlar, Germany). The fluorescence from the dyes was measured using a standard fluorescein filter set at 493-542 nm.

Culture supernatants and nuclear protein extraction

RAW 264.7 cells were seeded into 24-multiwell plates (Nunc Co., Naperville, IL, USA) at a density of 1×10^6 cells/ml for 12 h in the presence or absence of BAPTA/AM (200 μM) for final 1 h. After 12 h incubation at 37°C under a 5% CO_2 condition, *t*10*c*12-CLA (20 μM), LPS (1 μg/ml), and *t*10*c*12-CLA in combination with LPS were added to the RAW 264.7 cells culture media, except for the control cells to which the same amount of DMSO was added. All culture supernatants were collected after centrifugation at 14,000 g for 5 min and stored at −71°C until use for the NO analysis. And nuclear protein was extracted using nuclear extract kit (Active motif., Carsbad, CA, USA). Ice-cold phosphate buffered saline (PBS)/phosphatase inhibitors were added into 24-well plate. All samples were centrifuged at 13 g for 5 min at 4°C, and retrieved pellets were treated with hypotonic buffer and detergent, followed by centrifugation at 14,000 g for 30 sec at 4°C. After centrifugation, nuclear pellets were lysed and incubated for 30 min on ice in a rocking platform set. Following centrifugation at 14,000 g for 10 min at 4°C, the supernatants (nuclear fraction) were collected, divided into aliquots and stored at −71°C until it was further used for NF-κB p65 transcription factor assays.

NF-κB p65 transcription factor assay in the nuclear fraction

NF-κB activity was determined using transcription factor kits for NF-κB p65 (Thermo Scientific, Rockford, IL, USA) following the manufacturer's protocol. The nuclear extracts were added to 96-well plate. And 50 μl of working binding buffer were added to each well, and then incubated for 1 h with mild agitation. Each well was washed three times with wash buffer and 100 μl of primary antibody diluted with distilled water were added. After 1 h of incubation, the wells were washed three times with wash buffer. Next, 100 μl of diluted secondary antibody was added to each well. After 1 h of incubation without agitation, each well was washed four times with wash buffer. As final step, chemiluminescent substrates were added and chemiluminescence was measured using a charge-coupled device (CCD) camera system.

Nitric oxide assay in the culture supernatant fraction

Nitrite concentration as an index for nitric oxide (NO) synthesis was determined from supernatants using nitric oxide assay kit (Thermo Scientific). Reagent diluents, a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) based buffer containing detergent and preservatives, were added to 96 well plate where supernatants, additional reagent diluents, and Griess reagent were added to each well subsequently. After incubation at room temperature for 10 min, optical density was determined using an automated microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) set at 540 nm.

Statistical analysis

All statistical analyses were performed using Graphpad prism 6 (GraphPad Software, Inc. La Jolla, CA, USA). Results were compared by one-way analysis of variance (ANOVA) with Dunnett's post hoc test or two-way ANOVA with Bonferroni's post hoc test, as appropriate. Comparisons between two groups were performed using a *t*-test. A *p*-value of < 0.05 was considered to be significant. Data were expressed as mean ± standard deviation (SD).

Results

CLA and/or BAPTA/AM decrease LPS-induced ROS production

ROS production in LPS-stimulated RAW 264.7 cells was significantly increased ($P < 0.001$) compared to vehicle-treated

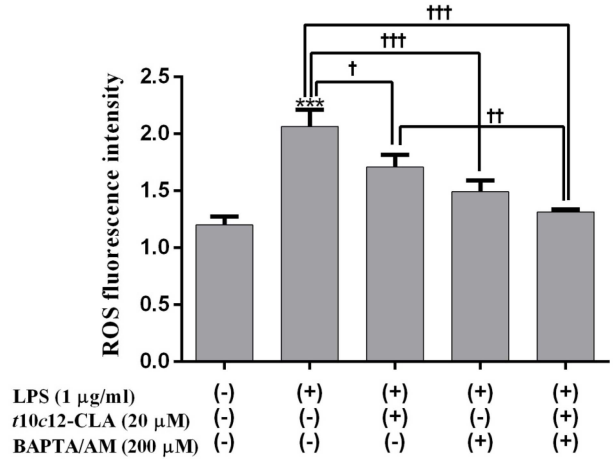

Fig 1. Effect of *t*10*c*12-CLA and/or BAPTA/AM on ROS production in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (1×10^3 cells/μl) were seeded into coverglass-bottomed 35-mm cell culture dish for 12 h in the presence or absence of BAPTA/AM (200 μM) for final 1 h and incubated with carboxy-H_2DCFDA (10 μM) for 30 min followed by treatment with *t*10*c*12-CLA (20 μM) and LPS (1 μg/ml) for last 20 min. The carboxy-H_2DCF green fluorescence was quantified by image analysis (see Materials and Methods section). The data represent means ± SD (n = 3). ***$P < 0.001$ (one-way ANOVA). Two-way ANOVA was used for statistical analysis in *t*10*c*12-CLA treatment in the presence of BAPTA/AM. †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$ (two-sample *t*-test).

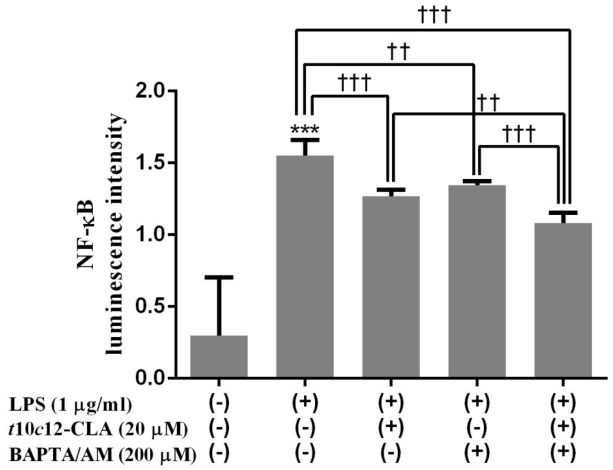

Fig 2. Effect of *t*10*c*12-CLA and/or BAPTA/AM on NF-κB p65 DNA binding activity in LPS-stimulated RAW 264.7 cells. Cells (1×10^6 cells/ml) pre-treated with or without BAPTA/AM (200 μM) for 1 h were incubated with *t*10*c*12-CLA (20 μM), LPS (1 μg/ml), or *t*10*c*12-CLA in combination with LPS for 12 h. The activity of NF-κB in the nuclear fraction was measured as described in the Materials and Methods section. The data represent means ± SD (n = 3). ***$P < 0.001$ (one-way ANOVA). Two-way ANOVA was used for statistical analysis in *t*10*c*12-CLA treatment in the presence of BAPTA/AM. ††$P < 0.01$, †††$P < 0.001$ (two-sample *t*-test).

(dimethyl sulfoxide) cells. This increased ROS were suppressed ($P < 0.05$) by *t*10*c*12-CLA treatment. BAPTA/AM, a calcium chelator, also suppressed ($P < 0.001$) the increase of ROS production in LPS-treated cells. *t*10*c*12-CLA-mediated decrease in ROS production was also further significantly reduced ($P < 0.001$) by addition of BAPTA/AM (Fig 1).

CLA and/or BAPTA/AM reduce LPS-induced nuclear NF-κB p65 DNA binding activity

NF-κB p65 DNA binding activity was significantly increased ($P < 0.001$) in LPS-stimulated RAW 264.7 cells compared to vehicle-treated cells. This increased NF-κB p65 DNA binding activity in LPS-stimulated RAW 264.7 cells was suppressed ($P < 0.01$) by BAPTA/AM. *t*10*c*12-CLA suppressed ($P < 0.001$) NF-κB p65 DNA binding activity increased by LPS treatment, which was also more significantly reduced ($P < 0.001$) by addition of BAPTA/AM (Fig 2).

Effect of CLA on nitrite production

Nitrite production, an index for nitiric oxide (NO), was significantly decreased ($P < 0.001$) by treatment with *t*10*c*12-CLA for 12 h in LPS-naïve RAW 264.7 cells compared to vehicle treatment control (Fig 3A). To determine the appropriate incubation time of LPS for nitrite production, RAW 264.7 cell was seeded for 24 h and treated with LPS (1 μg/ml) for final 1 h, 2 h, 6 h, and 12 h, respectively. As shown in Fig 3B, nitrite production was significantly ($P < 0.001$) increased after 6 h and 12 h of incubation comparable to

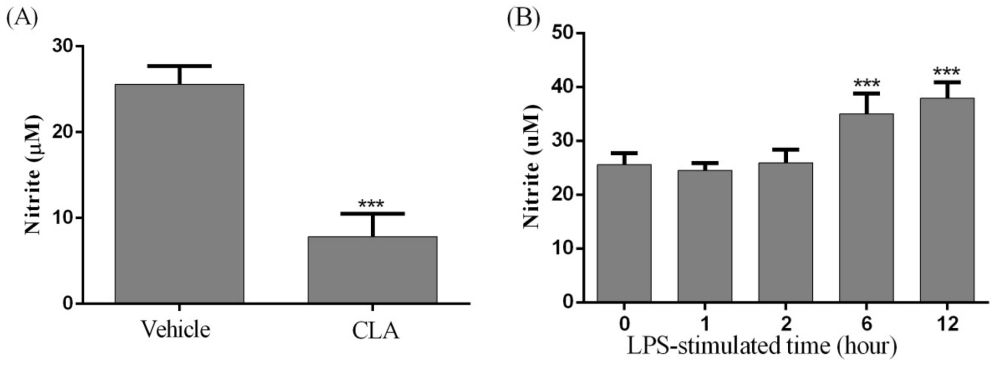

Fig 3. Effect of *t*10*c*12-CLA on nitrite production. (A) LPS-naïve RAW 264.7 cells (1×10^6 cells/ml) were treated with *t*10*c*12-CLA (20 μM) for 12 h. (B) Cells (1×10^6 cells/ml) were incubated with LPS for indicated times. Nitrite production in supernatant was assayed, as described in the Materials and Methods section. The data represent means ± SD (n = 3). ***$P < 0.001$ (one-way ANOVA followed by Dunnett's test).

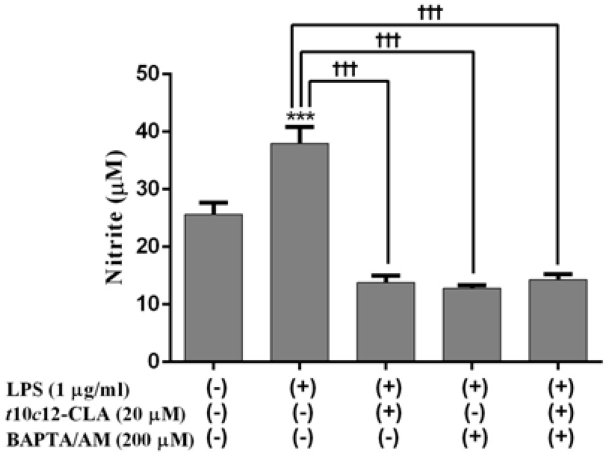

Fig 4. Effect of *t*10*c*12-CLA and/or BAPTA/AM on nitrite production in LPS-stimulated RAW 264.7 cells. Cells (1×10^6 cells/ml) pre-treated with or without BAPTA/AM (200 μM) for 1 h were incubated with LPS (1 μg/ml), *t*10*c*12-CLA (20 μM), and *t*10*c*12-CLA in combination with LPS for 12 h. Nitrite production in supernatant was assayed, as described in the Materials and Methods section. The data represent means ± SD (n = 3). ***$P < 0.001$ (one-way ANOVA). Two-way ANOVA was used for statistical analysis in *t*10*c*12-CLA treatment in the presence of BAPTA/AM. †††$P < 0.001$ (two-sample *t*-test).

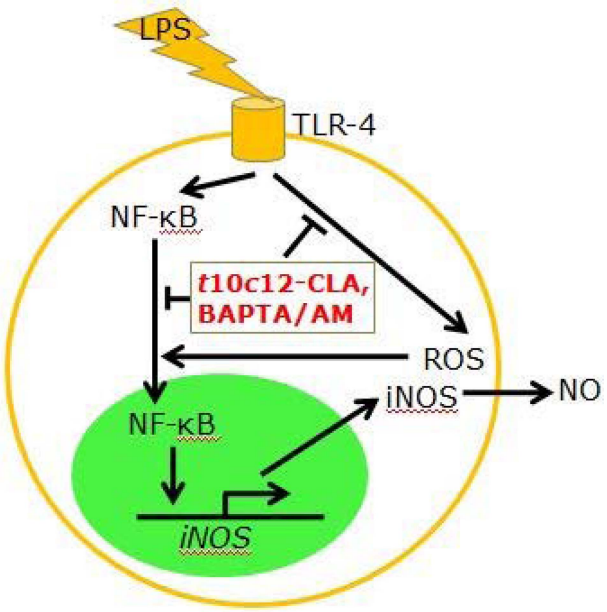

Fig 5. The scheme for the mechanism by which *t*10*c*12-CLA inhibits calcium-dependently the increases in ROS and NO production and NF-κB p65 DNA binding activity by LPS stimulation.

LPS-naïve cells (0 h).

CLA and/or BAPTA/AM reduce LPS-induced nitrite production

Nitrite production was significantly increased ($P < 0.001$) in LPS-stimulated RAW 264.7 cells compared to vehicle treated controls. The increased level of nitrite was significantly decreased ($P < 0.001$) by treatments with *t*10*c*12-CLA, BAPTA/AM, or *t*10*c*12-CLA and BAPTA/AM combination. But there were no significant differences among *t*10*c*12-CLA, BAPTA/AM, *t*10*c*12-CLA plus BAPTA/AM in LPS-stimulated RAW 264.7 cells (Fig 4).

Discussion

Macrophages are key players in the immune response to foreign invaders such as infectious microorganisms and LPS. LPS triggers the generation of ROS, activation of NF-κB, and finally, production of NO. In this study, we used a bacterial LPS for the induction of inflammatory response in RAW 264.7 macrophages. Our results showed an increase in ROS and NO production and NF-κB activity when RAW 264.7 cells were treated with LPS.

Macrophages produce ROS during phagocytosis or stimulation with wide variety agents through activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (9). The primal ROS made by electron transport chains is O_2^-, which is changed by H_2O_2 either by spontaneous dismutation or catalyzed by superoxide dismutase (SOD). Ca^{2+} might increase ROS generation in LPS-stimulated RAW 264.7 cells by enhancing metabolism (32). LPS-triggered inflammatory responses induce ROS production via the activation of NADPH oxidase in macrophages (7). We observed that *t*10*c*12-CLA reduced an increase in ROS production in LPS-stimulated RAW 264.7 cells, which was further suppressed by addition of BAPTA/AM. This finding suggested that *t*10*c*12-CLA has an anti-inflammatory effect in LPS-stimulated cells by Ca^{2+}-dependent pathway.

ROS participate in the modulation of NF-κB activation. NF-κB activation is a key step for COX-2 and iNOS expression in LPS-activated macrophages (18,31). Thus, inhibition of NF-κB activation is likely to suppress the expression of COX-2 and iNOS in activated macrophage as observed in CLA-treated cells (12). It is already shown that CLA modulating the NF-κB activation and therefore negatively regulating expression of inflammatory mediators (4). In the present study, we found that *t*10*c*12-CLA reduced an increase in NF-κB p65 DNA binding activity by LPS treatment. This effect of CLA was more suppressed by treatment with BAPTA/AM. ROS stimulates the redox-based activation of NF-κB and pro-inflammatory cytokines gene transcription (14). Therefore, inhibition of ROS production by *t*10*c*12-CLA was effected on down-regulation of NF-κB p65 DNA binding activation in LPS-stimulated condition.

Nitric oxide plays an important role in diverse physiological processes, including immune responses, inflammatory and neurotransmission. Low level of NO play a role as neurotransmitter, and high level of NO induces host cell death and inflammatory tissue damage (15). Promoter of iNOS contains 2 binding sites for NF-κB and NF-κB plays a critical role in LPS-induced iNOS expression (20,31). It is evident that LPS-stimulated NO release from RAW macrophage is in NF-κB dependent (1). It is already known that treatment with CLA mixture (isomers 9, 11 and 10, 12) down-regulated inducible NO synthase and cyclooxygenase-2 expression, as well as the subsequent production of NO and PGE_2 in LPS-stimulated RAW 264.7 macrophages through

the inhibition of NF-κB p65 DNA-binding activity (4). To investigate the effect of *t*10*c*12-CLA and BAPTA/AM on NO production, we measured the nitrite, the stable metabolite of NO. We found that *t*10*c*12-CLA reduced LPS-stimulated increase in NO production as well as the production of LPS-naïve macrophages. These powerful inhibiting effects of *t*10*c*12-CLA and/or BAPTA/AM were correlated with down-regulations of NF-κB p65 DNA binding activity and ROS production. This finding strongly also indicated that *t*10*c*12-CLA has an anti-inflammatory effect in LPS-stimulated cells through Ca^{2+}-dependent pathway.

Our data provided novel information concerning the mechanism by which CLA inhibits calcium-dependently LPS-induced ROS and NO production and NF-κB activation in RAW 264.7 cells (Fig 5). Further studies are required to elucidate the action mechanism and therapeutic potential of CLA.

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LPS 자극 RAW 264.7 세포에 있어서 칼슘의존성 ROS와 NO 생산 및 NF-κB 활성에 대한 CLA의 억제효과

최태원 · 강병택 · 강지훈 · 양만표[1]

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요 약 : 염증상태에서의 CLA의 효과와 작용기전을 알아보기 위해 LPS로 자극한 RAW 264.7 세포에 있어서 ROS와 NO생산 및 NF-κB 활성에 대한 *t*10*c*12-CLA의 효과를 검토하였다. 또한 이러한 효과가 세포질 내 칼슘이온의 변화와 관련이 있는지도 알아보았다. LPS 자극으로 ROS 생산은 증가하였고 이러한 증가는 칼슘결합제인 BAPTA/AM에 의해 감소하였다. *t*10*c*12-CLA 또한 LPS 자극 RAW 264.7 세포의 ROS 생산 증가를 억제시켰으며 BAPTA/AM과 함께 처리시 더욱 억제되었다. NO의 생산과 NF-κB p65 활성도 *t*10*c*12-CLA, BAPTA/AM, *t*10*c*12-CLA와 BAPTA/AM의 동시처리 모두에서 현저하게 억제되었다. 이상의 결과로부터 염증 조건에서 CLA는 과도한 ROS와 NO의 생산 및 NF-κB의 활성을 칼슘 의존적으로 억제하여 항염증 효과를 발휘하는 것으로 생각되었다.

주요어 : CLA, LPS, ROS, NF-κB, NO, 칼슘이온