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Effect of Conjugated Linoleic Acid on Nuclear Factor-κB Activation and Tumor Necrosis Factor-α Production in RAW 264.7 Cells Exposed to High Concentration of Glucose

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Abstract : Diabetes-related complications in human and veterinary medicine have been shown to be associated with hyperglycemia-induced inflammation. It has been recently suggested that the onset of insulin resistance may be caused by over-production of inflammatory cytokines such as tumor necrosis factor (TNF)-α from immune cells. Conjugated linoleic acid (CLA) regulates inflammatory response through modulation of TNF-α expression. The objective of this study was to examine the effect of CLA on nuclear factor kappaB (NF-κB) p65 binding activity, inhibitory kappaB (IκB)-α expression, and TNF-α production from high glucose-treated RAW 264.7 cells. CLA was added to RAW cells that had been previously cultured with low or high concentration of glucose. The levels of TNF-α protein in the culture supernatant of RAW cells exposed to high concentrations of glucose were higher than those of cells exposed to low concentrations of glucose. The treatment with the high concentration of glucose in RAW cells increased levels of NF-κB p65 binding activity and the decreased IκB-α expression when compared with those of low glucose. The treatments in combination with CLA and glucose (low and high) glucose in RAW cells increased TNF-α production when compared with that glucose alone. These treatments with CLA increased TNF-α production in high glucose-treated RAW cells than those with low glucose. These treatments of CLA also showed higher NF-κB p65 binding activity and lower IκB-α expression in high glucose than those in low glucose condition. This suggests that CLA can increase NF-κB p65 binding activity and TNF-α production from high glucose-treated RAW 264.7 cells and is likely to promote hyperglycemia-induced inflammation.

Key words **:** conjugated linoleic acid, high glucose, tumor necrosis factor-α, nuclear factor (NF)-κB, inhibitory kappaB (IκB)-α, RAW 264.7 cells.

Introduction

Tumor necrosis factor (TNF)-α is a cytokine related to systemic inflammation. TNF-α has been recently identified as a potential causative factor of type 2 diabetes mellitus (DM) involved in insulin resistance (10). It was reported that circulatory TNF-α is elevated in type 2 DM due to consequence of adipocyte-dependent TNF-α production (24) or adipose tissue associated macrophages-dependent TNF-α production (31). Excessive production of TNF-α is related to insulin resistance through activation of nuclear factor (NF)-κB leading to decreased glucose uptake and causes several secondary complications such as cardiovascular diseases, atherosclerosis, nephropathy, and neuropathy (25,27,30,39) in DM.

Conjugated linoleic acid (CLA) has been known to have many potential health benefits, including anti-carcinogenic (23) and anti-atherosclerotic activity (27) as well as anti-adipogenic (34), anti-inflammatory effects (42), and anti-diabetogenic effects (32). However, studies have reported that CLA may also have serious adverse effects, especially related to diabetes and metabolic syndrome. In a study of obese men with signs of metabolic syndrome, CLA supplementation reduced body fat and weight but also increased insulin resistance and blood sugar, and dropped high-density lipoprotein (HDL) levels (28). Similarly, it was demonstrated that the tans-10, cis-12 (t10c12)-CLA isomer, not cis-9, trans-11 (c9t11)-CLA, promotes insulin resistance by acceleration of NF-κB activation in human adipocyte (4). Effects of CLA on anti-inflammation are controversial. It was observed that CLA decreases the production of prostaglandin (PG) E_2, nitric oxide (NO), inflammatory cytokines such as interleukin (IL)-1, IL-6 and TNF-α in RAW 264.7 cells (42). On the other hand, CLA increases lipid peroxidation, oxidative stress and production of cytokine such as TNF-α and IL-6 in rat spleen lymphocytes (29,40). The effect of CLA on TNF-α production through NF-κB activation depends on LPS stimulation. Particularly, t10c12-CLA promotes or reduces TNF-α release in peripheral blood mononuclear cells (PBMCs) without LPS or with LPS, respectively (15).

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It is widely recognized that inflammation is an important factor for insulin resistance and diabetic complications (10). Moreover, the presence of hyperglycemia increases inflammation (18,37). However, there has been little research into effect of CLA under hyperglycemia. Thus, the aim of this study is to investigate the effects of CLA on TNF-α production via NF-κB pathway in RAW 264.7 cells exposed to high glucose conditions.

Materials and Methods

RAW 264.7 macrophages culture

RAW 264.7 macrophages were obtained from the Korean 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, 100 mg/dl glucose, 1 mM sodium pyruvate, 10% fetal bovine serum (FBS; Hyclone Lab), 1,000 U/ml penicillin and 1,000 μg/ml streptomycin (Hyclone Lab) at 37°C in a humidified atmosphere containing 5% CO_2.

Reagents

c9t11-CLA, t10c12-CLA (98% purity; Matreya Inc., Pleasant Gap, Pennsylvania, USA) were purchased commercially and were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 50 mM. The solution was filtered through a 0.22 μm membrane filter (Millipore Co, Bedford, MA, USA) before use. D-glucose was purchased from Sigma-Aldrich Company (St Louis, MO, USA), IκB-α antibody, actin antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), anti-rabbit IgG, HRP-linked antibody (Cell signaling Technology Inc, Danvers, MA, USA), caffeic acid phenethyl ester (CAPE) (Sigma-Aldrich Co) were purchased commercially.

Culture supernatant

The RAW 264.7 cells were seeded into in 24-well tissue culture plates (Nunc Co, Naperville, Illinois, USA) at a density of 1×10^5 cells/ml and allowed to adhere for 24 h in serum free DMEM with low glucose (100 mg/dl) or high glucose (1,000 mg/dl) (20) at 37°C and 5% CO_2. After 24 h incubation, the non-adherent cells were removed by washing cells two times with warm DMEM. C9t11-CLA or t10c12-CLA was added to cell culture media with a minimal volume (< 0.1%) of DMSO as the solvent and the same amount as vehicle DMSO was added to control cells. RAW 264.7 cells were incubated with c9t11-CLA (10 μM) or t10tc12-CLA (10 μM) for 24 h at 37°C under a 5% CO_2 atmosphere. All culture supernatants were collected after centrifugation at 5,000 × g for 30 min and stored at −70°C until use.

Nuclear protein extraction

The nuclear fraction from RAW 264.7 cells was isolated by using a nuclear extract kit (Active Motif, Carlsbad, California, USA). Media was aspirated and cells were rinsed ice-cold phosphate buffered saline (PBS)/phosphatase inhibitors. Cells were removed by scraping and then were centrifuged at 400 × g at 4°C for 5 min to harvest cell pellet. Cells were resuspended in hypotonic buffer and centrifuged at 14,000 × g at 4°C for 30 sec to obtain supernatant (cytoplasmic fraction) and nuclear pellet. Nuclear pellet was resuspended in complete lysis buffer and it was centrifuged for 10 min at 14,000 × g. The supernatant (nuclear fraction) was divided into a pre-chilled microcentrifuge tube and store at −70°C until ready to use.

NF-κB p65 transcription factor assay

NF-κB p65 DNA binding activity was determined using an ELISA-based TranseAM® NF-κB transcription factor assay kit (Active Motif) following the manufacturer's protocol. In brief, standard and experimental samples were added to 96-well plates pre-coated with oligonucleotide and incubated for 1 h at room temperature with mild agitation. Each well was washed three times with wash buffer, then incubated at 25°C for 1 h in the presence of diluted NF-κB antibody (1:1,000). Wells were washed three times and diluted HRP-conjugated antibody (1:1,000) was added for 1 h. After washing, developing solution was added and incubated 30 sec to 5 min. The reaction was blocked by adding stop solution and assay of absorbance was performed by using an automated microplate reader (BioTek Instruments, Inc, Winooski, Vermont, USA) at 450 nm with an optional reference wavelength of 655 nm.

TNF-α assay

TNF-α was measured by a sold phase sandwich ELISA using the BD OptEIA™mouseTNF-α immunoassay kit (BD Biosciences, San Jose, California, USA) according to the manufacturer's protocol. All samples, standards and controls were tested in triplicate. Standards and samples were added to a monoclonal antibody specific for mouse TNF-α coated on a 96-well plate and incubated for 2 h at room temperature. The wells were washed and bioninylated polyclonal anti-mouse TNF-α antibody was added for 1 h. After a second washing, enzyme working reagent was added. The wells were again washed and TMB substrate solution was added, which produces a blue color in direct proportion to the amount of TNF-α present in the initial sample. Addition of the stop solution changed the color from blue to yellow and optical density was determined using an automated microplate reader (BioTek Instruments, Inc) at 450 nm.

Determination of IκB-α expression

Proteins were extracted by using PRO-PREP™ Protein extraction solution (iNtRon Biotechnology, Seoul, Korea) according to the manufacture's suggestions. Protein concentration was determined using PRO-MEASURE protein measurement solution (iNtRon Biotechnology) according to the manufacture's protocol at absorbance 595 nm. Electrophoresis was performed through a verticalgel of 12% polyacrylamide loading gel with 5% stacking gel in a tris-glycine buffer system under denaturing conditions. All samples were mixed

with an equal volume of sample buffer, boiled, and applied to the gel. Protein were transferred (Trans-Blot® Cell; Bio-Rad Lab, Hercules, California, USA) onto 0.2 μm nitrocellulose membranes (GE Healthcare Bio-sciences AB, Bjoerkgatan, Uppsala, Sweden) overnight at 30 V. Non-specific bind sites were blocked for 1 h with 3% bovine serum albumin (BSA) in tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST), rinsed briefly three times with TBST. Membranes were subsequently incubated for 3 h in 0.5% BSA-TBST containing IκB-α antibody (1:500; Santacruz Biotechnology Inc) or actin antibody (1:500; Santacruz Biotechnology Inc). The membranes were rinsed briefly three times with TBST, incubated for 1 h in 0.5% BSA-TBST containing anti-rabbit IgG, horseradish peroxidase (HRP)-linked antibody (1:2,000; Cell signaling Technology Inc). The membranes were rinsed briefly three times with TBST, incubated for 1 min with West-One™ solution (iNtRon Biotechnology). IκB-α protein or actin levels were quantified by the Chemi Doc XRS system using Quantity One Software (Bio-Rad Lab) and standardized against the actin levels of each samples.

Statistical analyses

All statistical analysis was performed using GraphPad prism 5 software (GraphPad Software, San Diego, California, USA). Comparisons of the two groups were analyzed by Student's t test. P value of under 0.05 was considered statistically significant. All data are expressed as means ± standard deviation (SD).

Results

Effect of high glucose treatment on TNF-α production from RAW 264.7 cells

To examine the effect of high glucose treatment on TNF-α production in RAW 264.7 cells, cells (1×10^5 cells/ml) adapted by low concentration of glucose (100 mg/dl) were incubated with low concentration (100 mg/dl) or high concentration (1,000 mg/dl) of glucose for 48 h. As shown in Fig 1, the treatment with high glucose showed significantly the increased TNF-α production when compared with that of low glucose ($P < 0.01$).

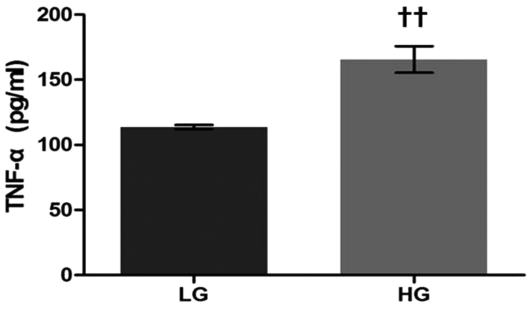

Fig 1. Effect of glucose on TNF-α production in RAW 264.7 cells. RAW 264.7 cells (1×10^5 cells/ml) adapted by low concentration of glucose (100 mg/dl) were incubated with low concentration (100 mg/dl) or high concentration (1,000 mg/dl) of glucose for 48 h. The data represent mean ± SD (n = 4). ††P < 0.01 vs. LG as determined by 2-sample t-test. LG, low glucose; HG, high glucose.

Effect of CLA on TNF-α production in RAW 264.7 cells exposed to low or high glucose culture media

To examine the effect of CLA treatment on TNF-α production in RAW 264.7 cells exposed to low or high glucose, cells were pre-incubated with low (100 mg/dl) or high (1,000 mg/dl) glucose media for 24 h and then were incubated with c9t11t-CLA (10 μM) or t10c12-CLA (10 μM) for additional 24 h (Fig 2). The level of TNF-α in the culture supernatant from RAW 264.7 cells treated with vehicle plus high glucose significantly ($P < 0.001$) increased when compared with treatment of vehicle plus low glucose. c9t11-CLA ($P < 0.01$) but not t10c12-CLA plus high glucose also significantly increased TNF-α production in RAW 264.7 cells compared with c9t11-CLA but not t10c12-CLA plus low glucose treatment. Treatment with t10c12-CLA but not c9t11t-CLA plus low glucose resulted in a significant increase ($P < 0.05$) in TNF-α production in RAW 264.7 cells compared with vehicle plus low glucose-treated cells (control). c9t11-CLA ($P < 0.01$) or t10c12-CLA ($P < 0.05$) plus high glucose treatments significantly increased TNF-α production when compared with vehicle plus high glucose-treated control.

Effect of CLA on NF-κB activity in RAW 264.7 cells exposed to low glucose or high glucose

To investigate the effect of CLA treatment on NF-κB binding activity in RAW 264.7 cells exposed to low or high glucose, cells were pre-incubated with low (100 mg/dl) or high (1,000 mg/dl) glucose media for 24 h and then were treated with c9t11-CLA (10 μM) or t10c12-CLA (10 μM) for additional 6 h (Fig 3). The level of NF-κB p65 DNA-binding

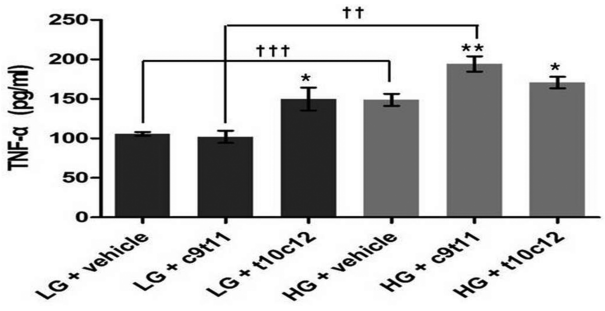

Fig 2. Effect of CLA isomers on TNF-α production in RAW 264.7 cells exposed to low or high glucose media. RAW 264.7 cells (1×10^5 cells/ml) were pre-incubated with low (100 mg/dl) or high (1,000 mg/dl) glucose for 24 h and then c9t11-CLA (10 μM) or t10c12-CLA (10 μM) was supplemented and incubated for additional 24 h. The data represent mean ± SD (n = 4). *P < 0.05, **P < 0.01 vs. LG or HG + vehicle, ††P < 0.01, †††P < 0.001 as determined by 2-smaple t-test. LG, low glucose; HG, high glucose.

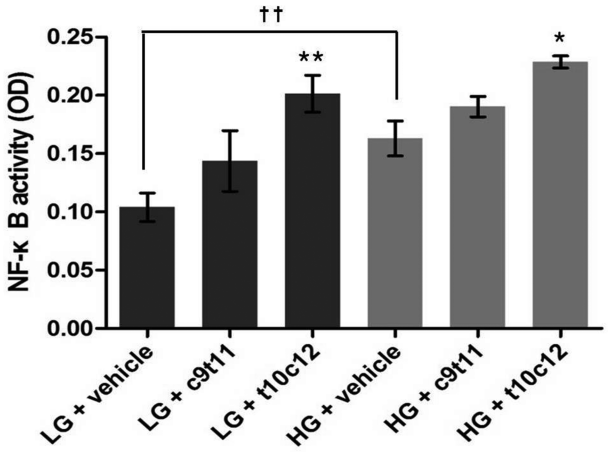

Fig 3. Effect of CLA isomers on NF-κB binding activity in RAW 264.7 cells exposed to low glucose or high glucose. RAW 264.7 cells (1×10^5 cells/ml) were incubated with low (100 mg/dl) glucose or high (1,000 mg/dl) glucose for 24 h and c9t11-CLA (10 μM) or t10c12-CLA (10 μM) was treated for additional 6 h. NF-κB p65 binding activity was measured in nuclear extracts using an ELISA-based Trans AMNF-κB p65 transcription factor assay kit, as described in the materials and methods section. The data represent mean ± SD (n = 4). *P < 0.05, **P < 0.01 vs. vs. LG or HG + vehicle. ††P < 0.001 as determined by 2-smaple t-test. LG, low glucose; HG, high glucose; OD, optical density.

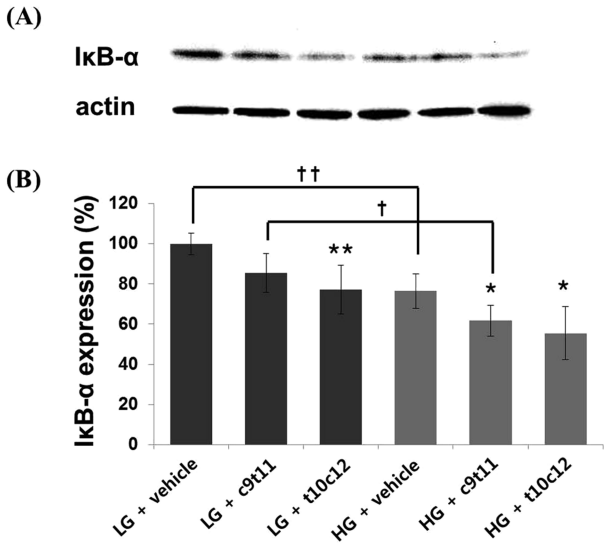

Fig 4. Effect of CLA isomers treatment on Western blots analysis of IκB-α expression (A) and bar graph of IκB-α expression (B) in RAW 264.7 cells exposed to low glucose or high glucose. RAW 264.7 cells (1×10^5 cells/ml) were incubated with low (100 mg/dl) glucose or high (1,000 mg/dl) glucose for 24 h and then c9t11-CLA (10 μM) or t10c12-CLA (10 μM) was supplemented for additional 4 h. (A), the IκB-α expression was analyzed by Western blot assay as described in materials and methods. (B), bar graph shows the densitometry measurements for IκB-α, which is expressed as the ratio of IκB-α to actin. The data represent mean ± SD (n = 4). *P < 0.05, **P < 0.01 vs. LG or HG + vehicle. †P < 0.05, ††P < 0.01 as determined by 2-smaple t-test. LG, low glucose; HG, high glucose.

activity in RAW 264.7 cells exposed to vehicle plus high glucose was significantly (P < 0.01) increased when compared with treatment of vehicle plus low glucose. However, there was no significant difference in treatments of between CLA plus high glucose and CLA plus low glucose. NF-κB p65 DNA-binding activity was significantly enhanced by treatment with t10c12-CLA plus low (P < 0.01) or high (P < 0.05) glucose in RAW 264.7 cells compared with vehicle plus low or high glucose-treated control, respectively. c9t11t-CLA plus glucose also increased NF-κB p65 DNA-binding activity although there was no significant difference when compared with those of vehicle plus glucose.

Effect of CLA on IκB-α expression from RAW 264.7 cells exposed to low or high glucose

To examine the effect of CLA on IκB-α expression from RAW 264.7 cells exposed to low or high glucose media, RAW 264.7 cells were pre-incubated with low (100 mg/dl) or high (1,000 mg/dl) glucose media for 24 h and then c9t11t-CLA (10 μM) or t10c12-CLA (10 μM) were added for additional 4 h. As shown in Fig 4, treatment with vehicle plus high glucose decreased IκB-α expression significantly compared with that of vehicle plus low glucose (P < 0.01). Furthermore, treatment with c9t11-CLA plus high glucose group revealed a significant decrease (P < 0.05) of IκB-α expression although t10c12-CLA also showed a tendency of decrease when compared with CLA plus low glucose group. Treatment with t10c12-CLA plus low glucose groups in RAW 264.7 cells significantly (P < 0.01) decreased IκB-α expression compared with that of vehicle plus low glucose. c9t11-CLA plus low glucose also decreased level of IκB-α expression although it was not significant when compared with vehicle plus low glucose. Combination with c9t11-CLA or t10c12-CLA plus high glucose significantly (P < 0.05) decreased IκB-α expression when compared with RAW 264.7 control cells treated with vehicle plus high glucose.

Discussion

Circulatory TNF-α produced mainly by monocytes and macrophages is increased in hyperglycemia (24,31). Our results also showed that TNF-α production was increased in RAW 264.7 cells exposed to high concentrations of glucose. These findings are in agreement with several studies which have shown that high glucose stimulated TNF-α release in different cells, including rat microglia (26), vascular smooth muscle cells (41) and monocyte (9). Thus, high glucose has been recognized as an important mediator for inflammation. There is strong evidence that high glucose induces inflammatory cytokines (6,33), reactive oxygen species (ROS), and NF-κB activity (26,38) in both clinical and experimental systems. Also, there are studies demonstrating the harmful effects of

excess glucose in a variety of cells that include pancreatic β-cells (43), PBMCs (20) and monocytes (7) through a number of signaling pathways.

These results revealed that treatment with a high concentration of glucose in RAW 264.7 cells increased NF-κB p65 binding activity and suppressed IκB-α expression. NF-κB can be activated by sustained high blood sugar (26,41). NF-κB is a proinflammatory master switch that controls the production of a host of inflammatory markers and mediators, including TNF-α, IL-6, and C-reactive protein (CRP) (8). The degradation of IκB-α, which is necessary for NF-κB activation by enabling the translocation of NF-κB p50/p65 from the cytoplasm to the nucleus, requires the phosphorylation of this inhibitor by IκB-α kinase (IKK) (1,36). In addition, activation of the NF-κB induces the expression of many inflammatory cytokines such as TNF-α (26). TNF-α is not only induced by NF-κB but also a strong activator of NF-κB (9,41). It was also found that TNF-α production was accompanied by the increase of NF-κB expression and the suppression of IκB expression in high glucose-treated RAW cells. These findings suggested that TNF-α production in high glucose condition is induced by NF-κB and IκB pathway.

In this study, we provided evidence that either c9t11t-CLA or t10c12-CLA may promote inflammation by increasing TNF-α level and NF-κB activity in RAW 264.7 cells treated with low or high glucose condition. CLA also decreased the level of IκB-α expression which is supported by an increase in NF-κB activity in RAW cells. In contrast, CLA decreases TNF-α release in inflammatory condition such as LPS-stimulated PBMCs (15) and interferon-γ-induced macrophage (42). Thus, CLA is known as an anti-inflammatory mediator which decreases the production of PGE_2, NO, inflammatory cytokines such as IL-1, IL-6 and TNF-α in RAW 264.7 cells (42). CLA also has inflammatory mediator functions in increasing lipid peroxidation, oxidative stress and production of cytokines such as TNF-α and IL-6 in rat spleen lymphocytes (29,40). It was demonstrated that CLA up-regulated the activation of NF-κB in a diversity of cells such as human adipocytes (4), human umbilical vein endothelial cells (19), and porcine PBMCs (15). In these results, the enhanced effect of CLA in TNF-α level and NF-κB activity was observed in RAW cells exposed to high glucose than low glucose condition. These findings implied that CLA can enhance hyperglycemia-induced inflammation as it was synergistic with high glucose.

CLA isomers are synthesized by base isomerization of linoleic acid. These have multiple effects according to biochemical mechanisms. Two isomers of CLA (c9t11-CLA or t10c12-CLA) employed in this study are known to possess biological activity (5,21,22). However, the effects of c9t11-CLA and t10c12-CLA in our results were similar to each other and appeared to be not likely to differ in TNF-α production, NF-κB p65 DNA-binding activity, and IκB-α expression from RAW 264.7 cells.

In vitro and in vivo studies have demonstrated a range of molecular mechanisms through which CLA acts including alteration of eicosanoid synthesis (35), reduction of NF-kB signaling proteins (15), activation of peroxisome proliferator-activated receptors (PPAR)γ (2,14,15) and alteration of cytokine levels (4,16,42). Dietary CLA contributed to delaying the onset of experimental inflammatory bowel disease in a pig (2) and decreased allergic sensitization and airway inflammation in mice (13). In the present study, CLA treatment increased TNF-α production with NF-κB p65 binding activity in RAW 264.7 treated with glucose. Our results have important implications for understanding that the combined effects of high glucose and CLA can promote inflammation. This study suggests that CLA can act as proinflammatory factor and does not prevent high-glucose-induced inflammation pathway.

In conclusion, CLA treatment strongly up-regulated TNF-α production with NF-κB p65 binding activity and down-regulated IκB-α expression in RAW 264.7 treated with high glucose than low glucose. These results may suggest that CLA may not have a beneficial effect on patients who present with hyperglycemia.

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고농도의 당에 노출된 RAW 264.7 세포에서 conjugated linoleic acid의 TNF-α 생산과 NF-κB의 활성 효과

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요 약 : 고혈당으로 야기된 염증은 당뇨병에서 합병증을 일으키는 주된 요인이다. 최근 연구에 따르면 면역세포에서 TNF-α 같은 염증성 cytokine의 과도한 생성은 인슐린 저항성을 야기시킨다고 한다. Conjugated linoleic acid (CLA)는 TNF-α 생산에 관여하여 면역반응을 조절하는 것으로 알려져 있다. 본 연구는 고농도의 당으로 처리한 RAW 264.7 세포에서 TNF-α 생산, NF-κB의 활성과 IκB-α 분해에 대한 CLA 효과를 검토하였다. 고농도의 당에 노출된 RAW세포는 저농도의 당에 노출된 RAW 세포보다 NF-κB의 활성과 IκB-α 분해가 증가되었으며 RAW 세포의 배양 상층액 중에 TNF-α 생산을 증가시켰다. CLA와 고농도 또는 저농도의 당을 같이 처리한 군은 당만 단일 처리한 군보다 TNF-α 생산, NF-κB의 활성 및 IκB-α 분해가 증가되었다. 그리고 고농도의 당과 CLA를 처리한 군에서 저농도의 당과 CLA 처리군에 비해 NF-κB의 활성과 IκB-α 분해가 증가되었으며 이와 더불어 TNF-α의 양이 증가되었다. 이상의 결과로부터, CLA는 고농도의 당에 노출된 RAW 세포에서 NF-κB의 활성을 높이고 TNF-α 생산을 증가시키며 이는 고혈당으로 유발되는 염증반응을 촉진하는 인자로 작용할 수 있음을 시사하였다.

주요어 : conjugated linoleic acid(CLA), 고농도당, TNF-α, NF-κB 활성, IκB-α, RAW 세포