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Upregulation of tumor necrosis factor-α expression by *trans10-cis12* conjugated linoleic acid enhances phagocytosis of RAW macrophages via a peroxisome proliferator-activated receptor γ-dependent pathway

Deok-Ho Song [a], Ji-Houn Kang [a], Geun-Shik Lee [b], Eui-Bae Jeung [b], Mhan-Pyo Yang [a,*]

[a] *Laboratory of Veterinary Internal Medicine, Department of Veterinary Medicine, College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk 361-763, Republic of Korea*

[b] *Laboratory of Veterinary Biochemistry and Molecular Biology, Department of Veterinary Medicine, College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk 361-763, Republic of Korea*

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Abstract

The aim of this study was to examine whether tumor necrosis factor (TNF)-α expression in the phagocytic activity of RAW macrophages by *trans*10–*cis*12 (10t–12c) conjugated linoleic acid (CLA) is associated with peroxisome proliferator-activated receptor γ (PPARγ) activation. 10t–12c CLA induced the TNF-α expression in RAW macrophages. Phagocytic activity of naive RAW macrophages was increased either by recombinant mouse (rm) TNF-α or by culture supernatant from 10t–12c CLA-treated RAW macrophages. This phagocytic activity was inhibited by addition of anti-rmTNF-α polyclonal antibody (pAb). 10t–12c CLA also increased the level of PPARγ protein and mRNA in RAW macrophages. When naive RAW macrophages were incubated with the culture supernatant from RAW macrophages treated with 10t–12c CLA plus GW 9662, a PPARγ antagonist, their phagocytic activity was significantly inhibited. In addition, GW 9662 antagonized the effect of 10t–12c CLA in stimulating TNF-α expression. These results suggest that 10t–12c CLA modulates the phagocytic activity of RAW macrophages by upregulating TNF-α expression via a PPARγ-dependent pathway.

Keywords: *Trans*10–*cis*12 conjugated linoleic acid; Tumor necrosis factor-α; Peroxisome proliferator-activated receptor γ; Phagocytosis; RAW macrophages

1. Introduction

The peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors that belong to the nuclear hormone receptor superfamily and form heterodimers with the retinoid X receptors (RXR) [1,2]. To date, three isotypes of PPARs, α, β, and γ, have been identified in a number of species. PPARγ was initially characterized as a regulator of adipocyte differentiation and lipid metabolism and is found in various cell types [3,4]. PPARγ is activated by polyunsaturated fatty acids (PUFAs), the thiazolidinedione (TZD) class of antidiabetic drugs, a variety of nonsteroidal anti-inflammatory drugs, and endogenous ligands such as 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) [5,6]. 15d-PGJ_2 and troglitazone, PPARγ agonists, inhibit phorbol myristyl acetate (PMA)-induced production of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in peripheral blood monocytes [7]. However, pioglitazone and rosiglitazone, also PPARγ agonists, did not inhibit phorbol ester-induced TNF-α

* Corresponding author. Fax: +82 43 261 3224.
E-mail address: mpyang@chungbuk.ac.kr (M.-P. Yang).

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release from human monocytic THP-1 cells [8]. This PPARγ may regulate inflammatory and immune responses by modulating the activity of monocytes or macrophages.

Dietary fatty acids such as linoleic acid and arachidonic acid may act as PPARγ ligands to modulate lipid metabolism and suppress carcinogenesis, cytokine production, acute inflammation, and lymphocyte proliferation [9–11]. Conjugated linoleic acid (CLA) is a class of positional and geometric isomers of linoleic acid with conjugated double bonds ranging from 6,8 to 12,14 [12,13]. CLA has shown a wide range of biologically beneficial effects such as anti-adipogenic activity, including reduction of body fat and lowering cholesterol level, and anti-diabetogenic, anti-carcinogenic, and anti-atherosclerotic activity [14–16]. CLA can stimulate or inhibit immune cell functions. CLA increased TNF-α and IL-6 secretion and decreased IL-4 secretion by splenocytes [17] and reduced IL-1, IL-6, and TNF-α production by macrophages [18]. CLA modulates expression of genes regulated by PPARγ in muscle and activates PPARγ *in vitro* [19]. In addition, expression of PPARγ is enhanced in CLA-fed pigs [20]. In contrast, CLA down-regulates expression of PPARγ, as well as its target genes, fatty acid binding protein and liver X receptor α in adipocytes [13]. Murine alveolar macrophages, which express high levels of PPARγ, suppress oxidative burst activity in response to PMA, expression of inducible nitric oxide synthase (iNOS) in response to lipopolysaccharide (LPS) and IFN-γ, and production of IL-12 mRNA and protein in response to LPS [21]. These reports indicate that the effects of CLA in modulating immune responses are similar to effects induced by ligands of PPARγ.

Two CLA isomers, the *cis*9–*trans*11 (9c–11t) CLA and *trans*10–*cis*12 (10t–12c) CLA are known to stimulate the immune system [22]. The previous studies [23,24] revealed that 10t–12c CLA has directly no effects on phagocytosis of peripheral blood phagocytes and on chemotaxis of polymorphonuclear cells (PMN). However, 10t–12c CLA was shown to stimulate peripheral blood mononuclear cells (PBMC) to secrete soluble factor(s) that increased the chemotactic and phagocytic activity of PMN, which may be an important mechanism to enhance the innate immune response. The phagocytic activity of PMN was demonstrated to mediate through TNF-α produced by PBMC stimulated with egg white derivatives, one of immunostimulants [25].

We examined whether TNF-α expression in the phagocytic activity of RAW macrophages by 10t–12c CLA is associated with PPARγ activation.

2. Materials and methods

2.1. RAW macrophages

The BALB/c mouse macrophage cell line RAW 264.7 was purchased from the Korean Cell Line Bank (Seoul, Korea) and maintained in Dulbecco's modified Eagle's medium (Gibco Co., Grand Island, NY, USA) with 4.5 mg/ml L-glutamine supplemented with 10% heat-inactivated fetal calf serum (Gibco Co.) and 100 μg/ml streptomycin in a humidified 5% CO_2 atmosphere.

2.2. Reagents

10t–12c CLA (98% purity; Matreya Inc., Pleasant Gap, PA, USA) was purchased commercially. 10t–12c CLA stock solution was prepared by dissolving 10t–12c CLA in dimethyl sulfoxide (DMSO) to final 50 mM and passed through a 0.45 μm-membrane filter (Milipore Co., Bedford, MA, USA) before use. Recombinant mouse (rm) TNF-α, goat anti-rmTNF-α polyclonal antibody (pAb), rabbit anti-goat IgG alkaline phosphatase (AP) conjugate, GW 9662 (Sigma–Aldrich Co., St. Louis, MO, USA), rmPPARγ, rabbit anti-PPARγ pAb, goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Santacruz Biotechnology Inc., Santa Cruz, CA, USA), and goat anti-recombinant human (rh) IL-2 pAb (R&D Systems Inc., Minneapolis, MN, USA) were also purchased commercially.

2.3. Culture supernatant

10t–12c CLA and/or GW 9662, a PPARγ antagonist, were added to RAW 264.7 cells 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 without 10t–12c CLA treatment. The RAW 264.7 cells at a density of 5×10^5 cells/ml in 24-well tissue culture plates (Nunc Co., Naperville, IL, USA) were incubated with 10 μM 10t–12c CLA, in the presence or absence of 0.01–1 μM GW 9662 at 37 °C in a 5% CO_2-humidified atmosphere. After a 24 h-incubation, all culture supernatants were collected after centrifugation at 5000*g* for 30 min, filtered through a 0.45 μm-pore size membrane filter and stored at −70 °C until used. The viability of RAW 264.7 cells was consistently more than 90% at the time of culture supernatant collection, as determined by trypan blue dye exclusion.

2.4. Protein extraction and Western blot

The cultured RAW 264.7 cells were lysed in ice-cold PRO-PREP™ (iNtRon Biotechnology, Seoul, Korea) protein extraction solution, according to the manufacturer's protocol. Protein concentrations of RAW 264.7 cells lysate and culture supernatant were determined using the Bradford assay [26]. Electrophoresis of protein lysates was performed using a 12% polyacrylamide separating gel (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 loaded onto the gel. Proteins were transferred (Trans-Blot® Cell; Bio-Rad Lab., Hercules, CA, USA) onto 0.2 μm-nitrocellulose membranes (Sartorius AG., Gottingen, Germany) at 300 mA for 3 h. Non-specific binding sites were blocked

for 3 h with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST), and washed briefly three times with TBST. Membranes were subsequently incubated for 2 h at room temperature in 0.5% BSA–TBST containing either a rabbit antipeptide pAb raised against a synthetic PPARγ peptide synthesized using the mouse PPARγ sequence, or goat anti-rmTNF-α pAb. After washing three times with TBST, the membranes were incubated with either anti-rabbit IgG conjugated with HRP or anti-goat IgG conjugated with AP in 0.5% BSA/TBST for 2 h. The membranes were washed twice with TBST and once with TBS. The membranes with HRP conjugated antibody were incubated in chemiluminescent substrate and exposed to Kodak X-AR film (Eastman Kodak, Rochester, NY, USA). The membranes with AP conjugated antibody were incubated with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Boehringer Mannheim GmbH, Mannheim, Germany) in alkaline phosphatase buffer containing 0.1 M Tris–HCl (pH 9.5), 0.1 M NaCl, and 5 mM $MgCl_2$. The reaction was stopped by rinsing membranes with 20 mM EDTA in TBS.

2.5. Phagocytic activity analyses

One hundred microliter of RAW 264.7 cells, adjusted to 5×10^6 cells/ml, was added to each well of a 24-well plate. The RAW 264.7 cells were incubated with rmTNF-α and culture supernatant from RAW 264.7 cells (5×10^5 cells/ml) treated with either 10 μM 10t–12c CLA alone or in combination with 0.01–1 μM GW 9662 for 12 h at 37 °C under a 5% CO_2-humidified atmosphere. For neutralization test, various concentrations of anti-rmTNF-α pAb were mixed with 10t–12c CLA-stimulated RAW 264.7 cell culture supernatant. Goat anti-rhIL-2 pAb was used as a control isotype IgG. The samples were mixed and incubated 30 min at room temperature. Ten microliter of fluorescein isothiocyanate (FITC)-latex beads (1×10^9 beads/ml, latex bead size, 2.0 μm; Polysciences Inc., Warrington, PA, USA) were added to each well for the final 2 h. RAW 264.7 cells incubated without FITC-latex beads were used as a negative control. The cultured cells were harvested gently by slow pipetting, centrifuged at 400*g* for 3 min and washed three times with PBS containing 3 mM ethylenediamine tetraacetic acid (EDTA). The supernatant was discarded and replaced with 1 ml PBS containing 1% paraformaldehyde to stabilize the cells. Phagocytic activity was measured by flow cytometry (BRYTE HS, Bio-Rad, Microscience Ltd., England) and surface-adherent FITC fluorescence on RAW 264.7 cells was quenched by addition of 20 μl of a 0.4% trypan blue solution to each tube before flow cytometry analysis. FITC fluorescence was measured between 520 and 560 nm on 5000 RAW 264.7 cells per sample. The cells were gated by forward and side light scatter characteristics. The results were expressed as percentages of absolute phagocytic activities.

2.6. RNA isolation

Total RNA was prepared from RAW 264.7 cells using the TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA). RAW 264.7 cells were mixed with 1 ml TRIzol reagent. Chloroform (200 μl per 1 ml TRIzol reagent) was added and the mixture was vigorously vortexed for 15 s. The lysate was centrifuged for phase separation at 13,000*g* for 10 min. After separation, the aqueous phase was transferred to a fresh tube, 500 μl of isopropyl alcohol was added and the tube gently inverted to mix. The samples were centrifuged for 10 min. The supernatant was removed and the visible pellet was washed with 1 ml of 75% ethanol. The RNA pellet was dissolved in diethylpyrocarbonate (DEPC)-treated water, and the concentration of RNA was determined by absorbance at 260 nm.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA (2 μg) was reverse transcribed into first strand complementary DNA (cDNA) using the Moloney-murine leukemia virus (M-MLV) reverse transcriptase (Ambion Inc., Austin, TX, USA) and a random primer (9 mer). To determine the conditions under which PCR amplification of PPARγ, TNF-α, and cytochrome *c* oxidase subunit I(1A) mRNA are in the logarithmic phase, 1 μl samples were amplified using different numbers of cycles. The 1A gene was PCR amplified to control for RNA degradation and variation in mRNA concentrations in the reverse transcription (RT) reaction. PCR products and amplification cycles were linearly related in PPARγ, TNF-α, and 1A mRNAs. Thirty cycles of PCR for PPARγ and TNF-α and 25 cycles for 1A were used for quantification. The cDNA was amplified in a 20 μl PCR reaction containing 1 unit *Taq* polymerase in PCR buffer (Promega Co., Madison, WI, USA), 1.5 mM $MgCl_2$, 2 mM dNTP, and 50 pmol of specific primers. PCR reactions were denatured at 95 °C for 1 min, annealed at 50 °C for 1 min, and extended at 72 °C for 1.5 min. The cDNA sequences for the detection of transcripts of PPARγ, TNF-α and 1A were obtained by RT using the following primers (Macrogen Inc., Seoul, Korea). The PPARγ primers were based on the deposited cDNA sequence (GenBank Accession No. NM011146): 5′-GAA AAG ACC CAG CTC TAC AAC-3′ (forward) and 5′-GTT CAG CTG GTC GAT ATC AC-3′ (reverse). The TNF-α primers were based on the deposited cDNA sequence (GenBank Accession No. NM013693): 5′-CCT CCC TCT CAT CAG TTC TAT-3′ (forward) and 5′-GAG GTT GAC TTT CTC CTC CTG GT-3′ (reverse). The 1A gene primers (GenBank Accession No. J01420) were: 5′-CAC CGT AGG AGG TCT AAC G-3′ (forward) and 5′-GTA TCG TCG AGG TAT TCC G-3′ (reverse). PCR products (10 μl) were electrophoresed on a 2% agarose gel and stained with ethidium bromide. Photographs of the gels were scanned and analyzed using

a digital analysis program (Gel Doc 1000, version 1.5, Bio-Rad Lab.).

2.8. Data analyses

The one-way analysis of variance followed by Dunnett's test for each pair for multiple comparisons was used in order to determine statistical significance. Comparisons of two groups at each GW 9662 concentration were made by Student's *t*-test. All data expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. TNF-α expression of RAW macrophages by 10t–12c CLA

To examine whether 10t–12c CLA induces the production of TNF-α in RAW 264.7 cells, Western blot analysis of the culture supernatant from RAW 264.7 cells treated with 10t–12c CLA (10 μM) for 24 h was performed using anti-rmTNF-α pAb. A single protein band with a molecular weight of 16–18 kDa was present in culture supernatant from RAW 264.7 cells treated with 10t–12c CLA and rmTNF-α samples, but not medium alone or culture supernatant from RAW 264.7 cells incubated with vehicle (DMSO) instead of 10t–12c CLA (Fig. 1A). 10t–12c CLA (10 μM) stimulation of TNF-α mRNA expression in RAW 264.7 cells was also examined. As shown in Fig. 1B, TNF-α mRNA expression of RAW 264.7 cells was detected by RT-PCR and peaked after 1 h of incubation with 10t–12c CLA.

3.2. Phagocytic activity of RAW macrophages

RAW 264.7 cells (5×10^5 cells/ml) were incubated for 12 h with culture supernatant (3.13–50%) from RAW 264.7 cells (5×10^5 cells/ml) treated with 10 μM 10t–12c CLA for 24 h. The phagocytic activity of RAW 264.7 cells incubated with culture supernatant from RAW 264.7 cells

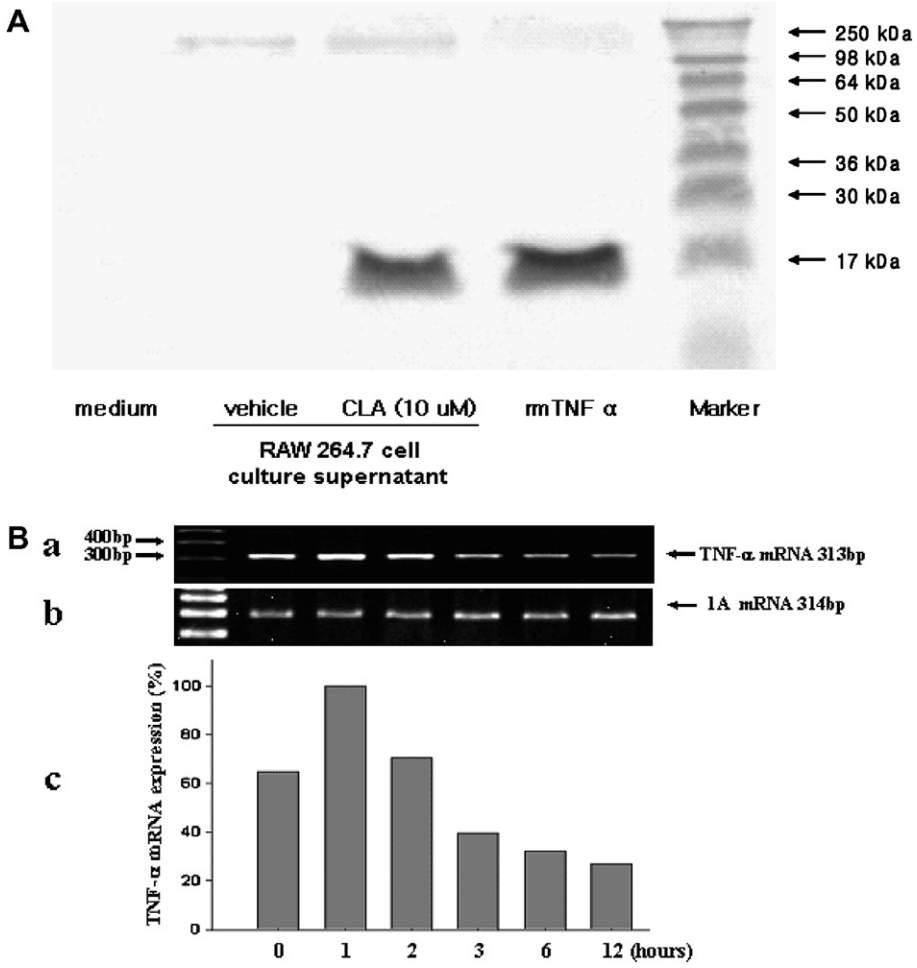

Fig. 1. TNF-α expression of RAW 264.7 cells by 10t–12c CLA. (A) Culture supernatant from RAW 264.7 cells (5×10^5 cells/ml) treated with 10 μM 10t–12c CLA for 24 h or from vehicle-treated cells was loaded onto SDS-PAGE under denaturing conditions (50 μg per lane) and analyzed by Western blotting using anti-rmTNF-α pAb. (B) RT-PCR analysis was performed on TNF-α mRNA in RAW 264.7 cells treated with 10 μM 10t–12c CLA for the indicated times (a). Normalization of the TNF-α mRNA expression with 1A (b). Signals were quantified by a digital analysis program and expressed as a percent of the maximum values (c). The expected product sizes of TNF-α and 1A mRNA are 313 and 314 bp, respectively.

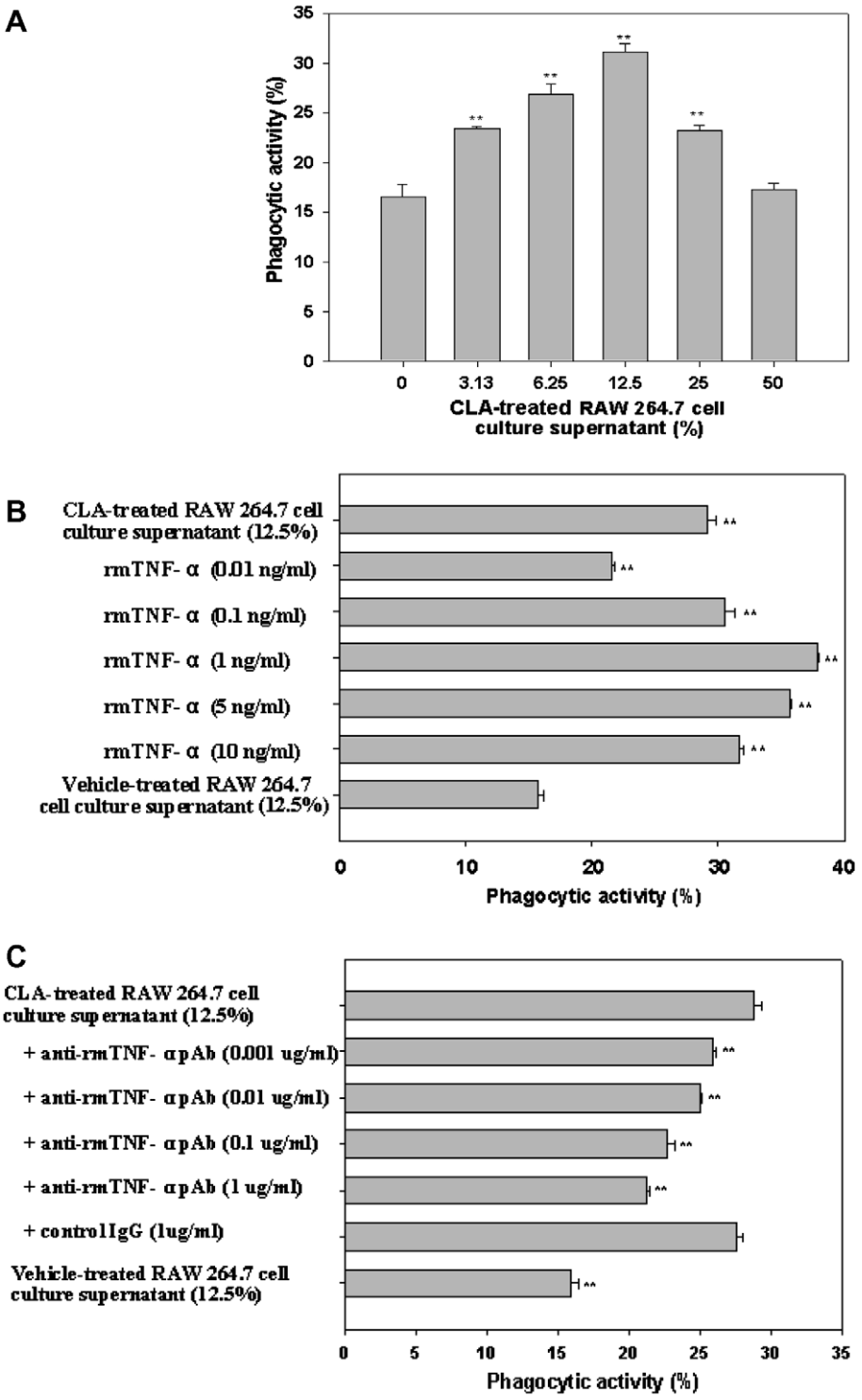

Fig. 2. Phagocytic activity of RAW 264.7 cells. (A) Effect of culture supernatant from 10t–12c CLA-treated RAW 264.7 cells on phagocytic activity of naive RAW 264.7 cells. RAW 264.7 cells (5×10^5 cells/ml) were incubated for 12 h with the culture supernatant from RAW 264.7 cells (5×10^5 cells/ml) that had been treated with 10 μM 10t–12c CLA for 24 h. (B) Effect of rmTNF-α on phagocytic activity of RAW 264.7 cells. RAW 264.7 cells (5×10^5 cells/ml) were incubated for 12 h with rmTNF-α at the indicated concentrations. Culture supernatant (12.5%) from RAW 264.7 cells treated with 10 μM 10t–12c CLA for 24 h was used as the positive control. (C) Neutralization effect of anti-rmTNF-α pAb on phagocytic activity of RAW 264.7 cells stimulated by 10t–12c CLA-treated RAW 264.7 cell culture supernatant. Anti-rmTNF-α pAb at the indicated concentrations and goat anti-rhIL-2 pAb as a control isotype IgG were added to the culture supernatant (12.5%) from RAW 264.7 cells (5×10^5 cells/ml) treated with 10 μM 10t–12c CLA for 24 h. RAW 264.7 cell cultures were incubated with these samples for 30 min. All cultures were supplemented with 10 μl of FITC-latex beads (1×10^9 beads/ml) for the final 2 h. The phagocytic activity of RAW 264.7 cells was measured using flow cytometry. The data are presented as mean ± SEM, $n = 3$, $^{**}P < 0.01$, compared to control (in panel A), vehicle-treated RAW 264.7 cell culture supernatant (in panel B) and 10t–12c CLA-treated RAW264.7 cell culture supernatant (12.5%) (in panel C).

that had been treated with 10t–12c CLA for 24 h was significantly enhanced ($P < 0.01$) in a dose-dependent manner compared to controls and showed the highest level of stimulation at 12.5% culture supernatant (Fig. 2A). The phagocytic activity of RAW 264.7 cells was also significantly increased ($P < 0.01$) in a dose-dependent manner by addition of 0.01–10 ng/ml rmTNF-α compared to the response to culture supernatant (12.5%) of RAW 264.7 cells treated with vehicle (DMSO) instead of 10t–12c CLA for 24 h and was highest at 1 ng/ml rmTNF-α (Fig. 2B). The effect of anti-rmTNF-α pAb on the phagocytic activity of RAW 264.7 cells in response to the culture supernatant from RAW 264.7 cells treated with 10t–12c CLA was examined. The enhanced phagocytic activity of RAW 264.7 cells to culture supernatant (12.5%) from RAW 264.7 cells (5×10^5 cells/ml) treated with 10 μM 10t–12c CLA for 24 h was inhibited ($P < 0.01$) in a dose-dependent manner by addition of 0.001–1 μg/ml anti-rmTNF-α pAb, when compared to the activity induced by the 10t–12c CLA-treated culture supernatant alone (Fig. 2C). In contrast, the enhanced phagocytic activity of RAW 264.7 cells in response to culture supernatant treated with 10t–12c CLA was not inhibited by addition of anti-rhIL-2 pAb (1 μg/ml: control IgG) instead of anti-rmTNF-α pAb.

3.3. Effect of 10t–12c CLA on PPARγ expression of RAW macrophages

RAW 264.7 cells were incubated with 10t–12c CLA and harvested for total protein and RNA isolation. Western blot analysis showed that a 24 h-incubation of RAW 264.7 cells (5×10^5 cells/ml) with 10 μM 10t–12c CLA induced PPARγ protein expression (Fig. 3A). A detectable PPARγ protein was also found in untreated RAW 264.7 when larger amounts of cell lysate (40 μg) were analyzed. RT-PCR analysis revealed that PPARγ mRNA expression in RAW 264.7 cells was induced by 10t–12c CLA treatment and peaked at 1 h-incubation, although minor expression of PPARγ was detected in untreated RAW 264.7 cells (Fig. 3B).

3.4. Effect of a PPARγ antagonist, GW 9662, on the phagocytic activity and TNF-α expression of RAW macrophages

To examine the effect of a PPARγ antagonist, GW 9662 (2-chloro-5-nitrobenzanilide), on the CLA-stimulated RAW 264.7 cells, culture supernatant (12.5%) from RAW 264.7 cells treated with 10 μM 10t–12c CLA plus 0.01–

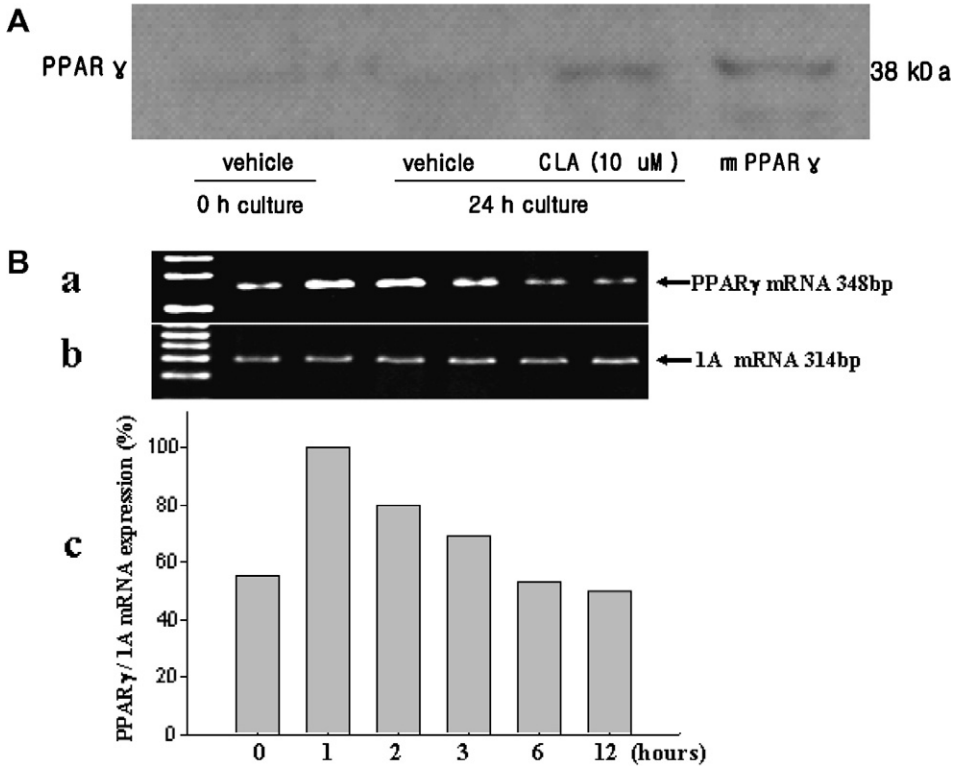

Fig. 3. Effect of 10t–12c CLA on the levels of PPARγ protein and mRNA expression in RAW 264.7 cells. (A) RAW 264.7 cells (5×10^5 cells/ml) were treated with 10 μM 10t–12c CLA or vehicle (DMSO; control) for 24 h. Cell lysate samples (40 μg per lane) were loaded onto SDS–PAGE under denaturing conditions and analyzed by Western blotting with rabbit anti-PPARγ pAb. (B) RT-PCR analysis of PPARγ mRNA expression in RAW 264.7 cells (5×10^5 cells/ml) treated with 10 μM 10t–12c CLA for the indicated times (a). Normalization of the PPARγ mRNA expression with 1A (b). Signals were quantified by a digital analysis program and expressed as a percent of the maximum values (c). The expected product sizes of PPARγ and 1A mRNA are 348 and 314 bp, respectively.

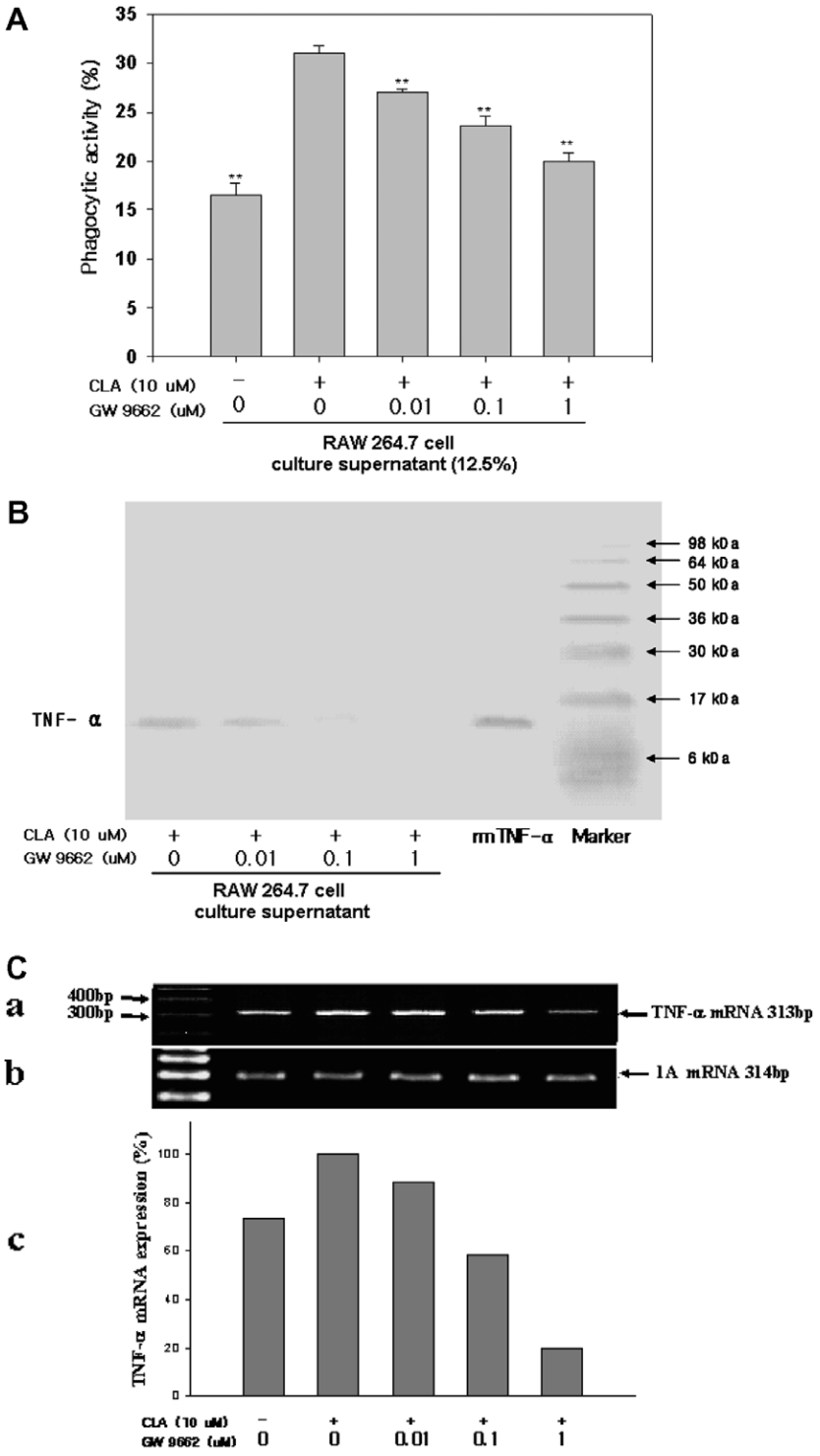

Fig. 4. Effect of GW 9662 on the phagocytic activity and TNF-α expression of 10t–12c CLA-stimulated RAW 264.7 cells. (A) Phagocytic activity of RAW 264.7 cells stimulated by culture supernatant (12.5%) from RAW 264.7 cells (5×10^5 cells/ml) treated with 10 μM 10t–12c CLA plus 0.01–1 μM GW 9662 for 24 h. The data are presented as mean ± SEM, $n = 3$, $^{**}P < 0.01$, compared to 10t–12c CLA-treated RAW 264.7 cell culture supernatant (12.5%). (B) Effect of GW 9662 on TNF-α protein level of 10t–12c CLA-stimulated RAW 264.7 cells. Culture supernatant (25 μg total protein per lane) from RAW 264.7 cells treated with 10 μM 10t–12c CLA plus 0.01–1 μM GW 9662 for 24 h was loaded onto SDS–PAGE under denaturing conditions and analyzed by Western blotting using anti-rmTNF-α pAb. (C) Effect of GW 9662 on TNF-α mRNA expression of 10t–12c CLA-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with 10 μM 10t–12c CLA plus 0.01–1 μM GW 9662 for 1 h. RT-PCR analysis of TNF-α mRNA expression in RAW 264.7 cells treated with 10t–12c CLA plus GW 9662 (a). Normalization of the TNF-α mRNA expression with 1A (b). Signals were quantified by a digital analysis program and expressed as a percent of the maximum values (c). The expected product sizes of TNF-α and 1A mRNA are 313 and 314 bp, respectively.

1 μM GW 9662 for 24 h was prepared. As shown in Fig. 4A, the phagocytic activity of RAW 264.7 cells induced by 10t–12c CLA-treated RAW 264.7 cell culture supernatant was inhibited ($P < 0.01$) in a dose-dependent manner by culture supernatant from RAW 264.7 cells treated with 10t–12c CLA plus GW 9662, compared to 10t–12c CLA-treated culture supernatant alone. In addition, Western blot and RT-PCR analysis showed that 0.01–1 μM GW 9662 antagonized the expression of TNF-α protein (Fig. 4B) and mRNA (Fig. 4C) of RAW 264.7 cells induced by 10t–12c CLA, in a dose-dependent manner.

4. Discussion

Expression of TNF-α is elevated in undifferentiated human monocytes or incompletely differentiated macrophage RAW 264.7 cells in comparison to differentiated human macrophages [27]. High concentrations of the PPARγ ligands troglitazone (30 μM) and 15d-PGJ_2 (10 μM) inhibited the expression of TNF-α as well as decreased the cell viability *in vitro* [28]. In contrast, 10 μM 10t–12c CLA, used in this study, up-regulated TNF-α expression and maintained a high RAW 264.7 cell viability of greater than 90%. The phagocytic activity of RAW 264.7 cells was enhanced by either culture supernatant from RAW 264.7 cells treated with 10t–12c CLA or rmTNF-α. In addition, anti-rmTNF-α pAb neutralized the enhanced phagocytic activity of RAW 264.7 cells stimulated by culture supernatant from 10t–12c CLA treated RAW 264.7 cells. These findings indicate that 10t–12c CLA stimulates RAW 264.7 cells to produce TNF-α, which enhances the phagocytic activity of RAW 264.7 cells.

TNF-α is a natural stimulant which promotes the phagocytic activity of phagocytes [29]. It has beeen reported that the enhanced phagocytic activity of PMN by culture supernatant from PBMC treated with immunostimulating egg white derivatives, was due to TNF-α, but not IL-1 [25]. Rats fed a diet containing 10t–12c CLA have the enhanced phagocytic activity of phagocytes, correlated with an increase in plasma TNF-α [30]. The CLA stimulated tumor cell killing ability of macrophages has been associated with induction of TNF-α expression [31]. It has been also reported that CLA decreases the synthesis of prostaglandin E_2 (PGE_2) [32], and PGE_2 has been shown to have profound down-regulatory effects on various inflammatory cells [33]. PGE_2 has been reported to regulate macrophage TNF-α production through negative feedback [34]. Therefore, one possible explanation for increased TNF-α expression in porcine PBMC treated with t10c12-CLA may be related to decreased PGE_2 regulation of TNF-α.

The results demonstrated that 10t-12c CLA increased the level of PPARγ protein and mRNA in RAW 264.7 cells. This finding is consistent with reports that the expression of PPARγ was increased in CLA-fed pigs [20] and that the supplementation of CLA in the diet enhanced the colonic expression of PPARγ [35]. Since CLA undergoes elongation and desaturation processes similar to those of polyunsaturated fatty acids (PUFAs) such as linoleic acid, Δ6 desaturase metabolites of CLA can also activate PPARγ expression [12]. Thus, the induction of PPARγ expression may occur through direct binding of CLA as well as active metabolites of CLA [36]. These results suggest that CLA acts as a ligand of PPARγ and activates PPARγ.

The present study indicates that 10t–12c CLA stimulates both PPARγ and TNF-α expression in RAW 264.7 cells. We used a specific and high affinity PPARγ antagonist (nanomolar IC_{50}, [37]), GW 9662, to elucidate the role of PPARγ on TNF-α expression in RAW 264.7 cells induced by 10t–12c CLA. GW 9662 antagonized the effect of 10t–12c CLA on TNF-α expression of RAW 264.7 cells as well as their phagocytic activity induced by TNF-α. These results suggest that the effects of 10t–12c-CLA on TNF-α production in RAW 264.7 cells may be dependent on the PPARγ pathway. High affinity PPARγ ligands such as the thiazolidinedione (TZD) antidiabetic agent AD-5075 and the non-TZD insulin-sensitizing agent L-796,499 did not inhibit production of TNF-α and IL-6 in RAW 264.7 cells [38].

In conclusion, the overall results of this study show that the effect of 10t–12c CLA on the phagocytic activity of RAW 264.7 macrophages might be mediated by TNF-α expression via a PPARγ-dependent pathway.

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