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Trans-10, *cis*-12-conjugated linoleic acid modulates NF-κB activation and TNF-α production in porcine peripheral blood mononuclear cells via a PPARγ-dependent pathway

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Abstract

The activation of PPARγ by ligands, including conjugated linoleic acid (CLA) isomers, plays an important role in the immune response. Among CLA isomers, *trans*-10, *cis*-12 (*t*10*c*12)-CLA is known to participate in the modulation of pro-inflammatory cytokine secretion. The aim of the present study was to assess the effect of *t*10*c*12-CLA on PPARγ activation, NF-κB activation and TNF-α expression in lipopolysaccharide (LPS)-naive and LPS-stimulated porcine peripheral blood mononuclear cells (PBMC). In addition, the effect of PPARγ inhibition on NF-κB activation and TNF-α expression in porcine PBMC was examined. *t*10*c*12-CLA was found to increase TNF-α expression and NF-κB activity in LPS-naive porcine PBMC. In contrast, *t*10*c*12-CLA decreased TNF-α expression and NF-κB activity in LPS-stimulated porcine PBMC. *t*10*c*12-CLA up-regulated PPARγ activity and mRNA expression in both LPS-naive and LPS-stimulated porcine PBMC. GW9662, a PPARγ antagonist, completely negated the modulating effects of *t*10*c*12-CLA on TNF-α expression and NF-κB activity in both LPS-naive and LPS-stimulated porcine PBMC. These results suggest that *t*10*c*12-CLA can modulate TNF-α production and NF-κB activation by a PPARγ-dependent pathway in porcine PBMC.

Key words: ***Trans*-10, *cis*-12-conjugated linoleic acid: TNF-α: PPARγ: NF-κB: Pigs: Lipopolysaccharide**

Conjugated linoleic acid (CLA) refers to a group of PUFA that exist as a mixture of positional and stereoisomers of conjugated dienoic octadecadienoate. CLA is found in ruminant food products, such as beef and dairy products(1). CLA has been shown to have many potential health benefits, such as lean body mass deposition(2), antidiabetes(3), anti-inflammation(4), anticarcinogenesis(5) and anti-atherogenesis effects(6).

CLA can stimulate or inhibit immune cell function, and among CLA isomers, *trans*-10, *cis*-12 (*t*10*c*12)-CLA has shown to participate in the modulation of pro- or anti-inflammatory cytokine secretion(7). *t*10*c*12-CLA has also been reported to increase TNF-α and IL-6 secretion in rat spleen lymphocytes(8), as well as the phagocytosis of canine peripheral blood polymorphonuclear cells(9). In contrast, CLA decreased the production of PGE_2, TNF-α and the inflammatory agent NO in RAW cells treated with interferon-γ(10).

PPAR represent a subfamily of nuclear hormone receptors that are activated by a variety of dietary and endogenous fatty acids. PPARγ is one of three PPAR isoforms: α, β/δ and γ(11). Some PPARγ agonists, such as 15-deoxy-$\delta^{12,14}$-PGJ_2 and troglitazone, inhibit the phorbol myristyl acetate-induced production of IL-1β, IL-6 and TNF-α in peripheral blood monocytes(12). CLA isomers enhanced PPARγ activation and attenuated the production of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in weaned pigs challenged with lipopolysaccharide (LPS)(13). PPARγ agonists might also possess pro-inflammatory activity through the inhibition of IL-10 activity mediated by the PPARγ ligand 15-deoxy-$\delta^{12,14}$-PGJ_2(14). Recently, there has been increasing evidence that the expression and activation of PPARγ may participate in the activity of NF-κB. NF-κB is a ubiquitously expressed family of transcription factors, which controls the expression of numerous genes involved in inflammatory and immune

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Abbreviations: CLA, conjugated linoleic acid; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cell.

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responses, and in cellular proliferation(15). The most common form of NF-κB, which is found in virtually all cell types, is composed of two subunits named p50 and p65(16). The NF-κB family of transcription factors exists in the cytoplasm of unstimulated cells as homo- or heterodimers complexed with inhibitory κB proteins. Various stimuli lead to the phosphorylation of inhibitory κB by inhibitory κB kinase, which triggers its degradation and the activation of NF-κB. The activation and translocation of NF-κB to the nucleus is followed by the transcription of various pro-inflammatory genes including TNF-α(17,18). PPARγ ligands such as 15-deoxy-$\delta^{12,14}$-PGJ_2 and ciglitizone have been shown to interfere with the activity of NF-κB in human colon cancer cells(19). Furthermore, CLA isomers have been shown to increase PPARγ DNA-binding activity and decrease the DNA-binding activity of NF-κB in vascular smooth muscle cells(20).

The aim of the present study was to examine the effect of *t*10*c*12-CLA on PPARγ activation, NF-κB activation and TNF-α expression in LPS-naive and LPS-stimulated porcine peripheral blood mononuclear cells (PBMC). In addition, the effect of PPARγ antagonism on NF-κB activation and TNF-α expression in porcine PBMC was examined.

Materials and methods

Chemicals and reagents

*t*10*c*12-CLA (>98 % purity; Matreya, Inc., Pleasant Gap, PA, USA) was purchased commercially. *t*10*c*12-CLA stock solution was prepared by dissolving *t*10*c*12-CLA in dimethyl sulphoxide to a final concentration of 50 mM; the solution was filtered through a 0·45 μm membrane (Millipore Corporation, Bedford, MA, USA) before use. LPS from *Escherichia coli* 0127:B8, bovine serum albumin and GW9662, a PPARγ antagonist, were all purchased from Sigma-Aldrich Company (St Louis, MO, USA).

Isolation of porcine peripheral blood mononuclear cells

All experimental procedures and animal use were approved by the ethics committee of the Chungbuk National University. Heparinised porcine peripheral blood was drawn from the anterior vena cava, diluted with an equal volume of PBS without Ca and Mg, and overlayed 1:1 on a Percoll™ solution (GE Healthcare Bio-sciences AB, Uppsala, Sweden). After centrifugation at 400 ***g*** for 45 min at room temperature, the cells in the interface between the plasma in PBS and the Percoll solution were harvested and treated with 0·83 % NH_4Cl in a tris(hydroxymethyl) aminomethane base buffer (pH 7·2) for 5 min. The resulting PBMC were washed three times with PBS. PBMC were resuspended in Roswell Park Memorial Institute 1640 medium (Sigma-Aldrich) supplemented with 5 % heat-inactivated fetal bovine serum (Gibco Company, Grand Island, NY, USA).

Cell culture

*t*10*c*12-CLA was added to the PBMC culture media with a minimal volume (<1 %) of dimethyl sulphoxide as a solvent, and the same amount of dimethyl sulphoxide was added to control cells without the *t*10*c*12-CLA treatment. The PBMC seeded at a density of 2×10^6 cells/ml in a twenty-four-multiwell plate (Nunc Company, Naperville, IL, USA) were incubated with or without LPS (1 μg/ml) and with *t*10*c*12-CLA (10 μM) or *t*10*c*12-CLA (10 μM) in combination with GW9662 (1 μM) for 24 h at 37°C under a 5 % CO_2-humidified atmosphere. After a 24 h incubation, all culture supernatants were collected after centrifugation at 14 000 ***g*** for 5 min, filtered through a 0·45 μm pore size membrane filter and stored at −80°C until used for the analysis of TNF-α. The cells were harvested and stored at −80°C to extract nuclear protein and test PPARγ and NF-κB activation.

Nuclear protein extraction

The nuclear fraction of PBMC was isolated by using a nuclear extract kit (Active Motif, Carlsbad, CA, USA). After treatment with or without LPS (1 μg/ml) and with *t*10*c*12-CLA (10 μM) or *t*10*c*12-CLA (10 μM) in combination with GW9662 (1 μM) for 24 h, PBMC previously plated on twenty-four-multiwell plates were rinsed and scraped into ice-cold PBS containing phosphatase inhibitors. The cells were centrifuged at 14 000 ***g*** at 4°C for 30 s, suspended in hypotonic buffer and lysed with 0·5 % NP-40. The nuclear pellet was collected after centrifugation of the cell lysates at 14 000 ***g*** at 4°C for 10 min. The suspended nuclear pellet was lysed and centrifuged at 14 000 ***g*** at 4°C for 10 min. The supernatant (nuclear fraction) was collected, divided into aliquots and stored at −80°C until they were used for PPARγ and NF-κB transcription factor assays.

PPARγ transactivation assay

PPARγ activity was assayed using an ELISA-based TransAM® PPARγ transcription factor assay kit (Active Motif) following the manufacturer's protocol. In brief, the nuclear extract was added to each well of ninety-six-well plates pre-coated with immobilised oligonucleotides containing a peroxisome proliferator response element. After 1 h of incubation with gentle agitation, wells were washed three times with wash buffer and then incubated with anti-PPARγ antibody (dilution 1:1000) for 1 h at 20°C. After three successive washes, the extracts were incubated for 1 h with diluted anti-mouse horseradish peroxidase-conjugated antibody (dilution 1:1000) followed by the addition of 100 μl of developing solution. After 5 min of incubation, the reaction was blocked by adding 100 μl of stop solution reagent. Optical density was determined using an automated microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) at 450 nm with a reference wavelength of 655 nm.

NF-κB p65 transcription factor assay

NF-κB activity was determined using the TransAM® NF-κB transcription factor assay kit (Active Motif) following the manufacturer's protocol (see PPARγ transactivation assay). The NF-κB transcription factor assay kit contains a ninety-six-well plate pre-coated with NF-κB consensus binding oligonucleotides.

Measurement of TNF-α production in the culture supernatant fraction of peripheral blood mononuclear cells

The culture supernatant fraction was collected after 24 h incubation. The amount of TNF-α was determined by direct sandwich ELISA using the Quantikine® P porcine TNF-α immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. All samples, standards and controls were assayed in triplicate. Optical density was determined using an automated microplate reader (BioTek Instruments, Inc.) at 450 nm. TNF-α was quantified from eight titration points using standard curves generated with purified porcine TNF-α, and the concentrations were expressed as pg/ml. Lower and upper detection limits were 11·7 and 1500 pg/ml, respectively.

Ribonucleic acid preparation and RT-PCR

Total RNA was extracted using the Trizol reagent (Invitrogen Company, Carlsbad, CA, USA) according to the methods outlined in the protocol, and the concentration of total RNA was determined by measuring the absorbance at 260 nm. First-strand complementary DNA was prepared by subjecting total RNA (1 μg) to reverse transcription using Moloney Murine Leukemia Virus RT (Invitrogen Company) and random primers (9-mers; Takara Bio, Inc., Otsu, Shiga, Japan). To determine the conditions for logarithmic-phase PCR amplification of PPARγ, TNF-α and cytochrome *c* oxidase subunit (1A), mRNA aliquots (1 μg) were amplified using different numbers of cycles. The 1A gene was PCR-amplified to rule out the possibility of RNA degradation and was used to control for variations in mRNA concentration in the RT reaction. A linear relationship between PCR product band visibility and the number of amplification cycles was observed for target mRNA. The 1A and target genes were quantified using twenty-eight and thirty cycles, respectively. Complementary DNA was amplified in 20 μl PCR mixtures containing 1 unit Taq polymerase (iNtRON Biotechnology, Inc., Sungnam, Kyungki, South Korea), 2 mM-deoxyribonucleotide triphosphate and 10 pmol specific primers. PCR mixtures were denatured at 95°C for 30 s, annealed at 58°C for 30 s and extended at 72°C for 30 s. Oligonucleotides for PPARγ were based on the complementary DNA sequence (GenBank accession no. AJ006756) $5'$-CTG GCA AAG CAC TTG TAT G-$3'$ (sense) and $5'$-GGT GTA AAT GAT CTC GTG GA-$3'$ (antisense). Oligonucleotides for TNF-α were based on the complementary DNA sequence (GenBank accession no. X57321) $5'$-CAA GGA CTC AGA TCA TCG TC-$3'$ (sense) and $5'$-CTT GGT CTG GTA GGA GAC G-$3'$ (antisense). The primer for the 1A gene (GenBank accession no. AF03253) was $5'$-CAC CGT AGG AGG TCT AAC G-$3'$ (sense) and $5'$-GTA TCG TCG AGG TAT TCC G-$3'$ (antisense). PCR products (8 μl) were fractionated on a 2·3 % agarose gel, stained with ethidium bromide and photographed under UV illumination. The photograph was scanned using a Gel Doc EQ system (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analyses

All statistical analyses were performed using GraphPad prism 5 software (GraphPad Software, San Diego, CA, USA). One-way ANOVA was used to determine the statistical significance of the differences between the control and treatment groups, followed by a Dunnett test. Comparisons of two groups were done using the *t* test. P values of less than 0·05 were considered to be statistically significant. Data are expressed as means and standard deviations.

Results

Trans-10, *cis*-12 conjugated linoleic acid up-regulates PPARγ activity and mRNA expression in porcine peripheral blood mononuclear cells

To examine whether *t*10*c*12-CLA treatment activates PPARγ in porcine PBMC, cells were incubated with *t*10*c*12-CLA (10 μM) or *t*10*c*12-CLA (10 μM) in combination with the PPARγ antagonist GW9662 (1 μM) for 24 h. To assess the level of PPARγ activation, we used the binding assay as described in the Materials and methods section, although the results of this type of assay may not necessarily reflect transcriptional activation. PPARγ DNA-binding activity was significantly increased ($P<0·05$) by *t*10*c*12-CLA treatment when compared with vehicle (dimethyl sulphoxide) controls. Treatment with GW9662 significantly suppressed ($P<0·05$) the *t*10*c*12-CLA-mediated enhancement of PPARγ DNA-binding activity in PBMC (Fig. 1(A)). RT-PCR analysis also showed that *t*10*c*12-CLA significantly increased ($P<0·05$) PPARγ mRNA expression in PBMC compared with vehicle controls. GW9662 negated ($P<0·05$) the *t*10*c*12-CLA-induced enhancement of PPARγ mRNA expression (Fig. 1(B)).

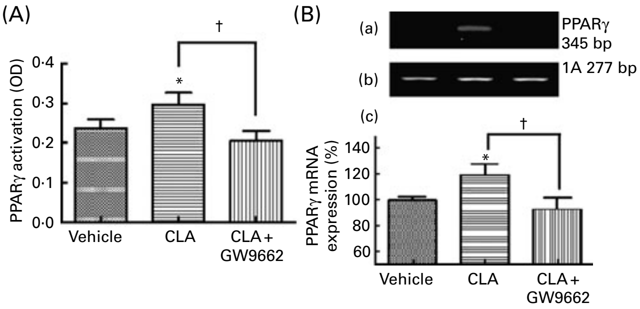

Fig. 1. The effect of *trans*-10, *cis*-12-conjugated linoleic acid (*t*10*c*12-CLA) on PPARγ activation in porcine peripheral blood mononuclear cells (PBMC). (A) Porcine PBMC (2×10^6 cells/ml) were incubated with *t*10*c*12-CLA (10 μM) or *t*10*c*12-CLA (10 μM) in combination with GW9662 (1 μM), a PPARγ antagonist, for 24 h. PPARγ activity was measured in nuclear extracts using an ELISA-based TransAM® PPARγ transcription factor assay kit, as described in the Materials and methods section. (B) RT-PCR analysis of PPARγ mRNA expression in porcine PBMC treated with *t*10*c*12-CLA (10 μM) or *t*10*c*12-CLA in combination with GW9662 (1 μM) for 1 h (a). PPARγ mRNA expression was normalised with 1A (b). Signals were quantified with a molecular analysis program and were expressed as a percentage of the vehicle value (c). Values are means, with standard deviations represented by vertical bars (*n* 3). Mean values were significantly different from that of the vehicle group: * $P<0·05$ (one-way ANOVA); † $P<0·05$ (as determined by the two-sample *t* test). OD, optical density.

Trans-10, cis-12 conjugated linoleic acid increases NF-κB activity in porcine peripheral blood mononuclear cells

To examine the effect of *t*10*c*12-CLA treatment on NF-κB activation in porcine PBMC, *t*10*c*12-CLA (10 μM) was added to the cell culture for 24 h. NF-κB p65 DNA-binding activity was significantly increased ($P<0{\cdot}01$) by *t*10*c*12-CLA treatment in PBMC compared with vehicle controls. GW9662 (1 μM) significantly suppressed ($P<0{\cdot}05$) the *t*10*c*12-CLA-mediated enhancement of NF-κB p65 DNA-binding activity (Fig. 2).

Trans-10, cis-12 conjugated linoleic acid increases TNF-α expression in porcine peripheral blood mononuclear cells

To examine whether *t*10*c*12-CLA induces the expression of TNF-α through the activation of PPARγ in porcine PBMC, these cells were incubated with *t*10*c*12-CLA (10 μM) or *t*10*c*12-CLA (10 μM) in combination with GW9662 (1 μM) for 24 h. As shown in Fig. 3, *t*10*c*12-CLA significantly increased ($P<0{\cdot}01$) TNF-α production in PBMC compared with vehicle-treated controls. The enhancement of TNF-α production by *t*10*c*12-CLA was significantly decreased ($P<0{\cdot}01$) by the addition of GW9662 (Fig. 3(A)). *t*10*c*12-CLA also significantly enhanced ($P<0{\cdot}05$) TNF-α mRNA expression in PBMC compared with controls, and this effect was abolished by GW9962 ($P<0{\cdot}001$; Fig. 3(B)).

Trans-10, cis-12 conjugated linoleic acid up-regulates PPARγ activity and mRNA expression in lipopolysaccharide-stimulated porcine peripheral blood mononuclear cells

The effect of *t*10*c*12-CLA on PPARγ activation in LPS-stimulated porcine PBMC was tested by treating PBMC with LPS (1 μg/ml) and *t*10*c*12-CLA (10 μM) in the presence or absence of GW9662 (1 μM) for 24 h. PPARγ DNA-binding activity in LPS-stimulated PBMC was significantly increased ($P<0{\cdot}01$) by *t*10*c*12-CLA treatment when compared with vehicle plus LPS-treated controls. GW9662 treatment significantly inhibited ($P<0{\cdot}01$) the *t*10*c*12-CLA stimulation of PPARγ DNA-binding activity in LPS-stimulated PBMC (Fig. 4(A)). RT-PCR analysis showed that *t*10*c*12-CLA also significantly increased ($P<0{\cdot}001$) PPARγ mRNA expression in LPS-stimulated PBMC compared with controls (vehicle plus LPS), and this effect was significantly inhibited ($P<0{\cdot}001$) by the addition of GW9662 (Fig. 4(B)).

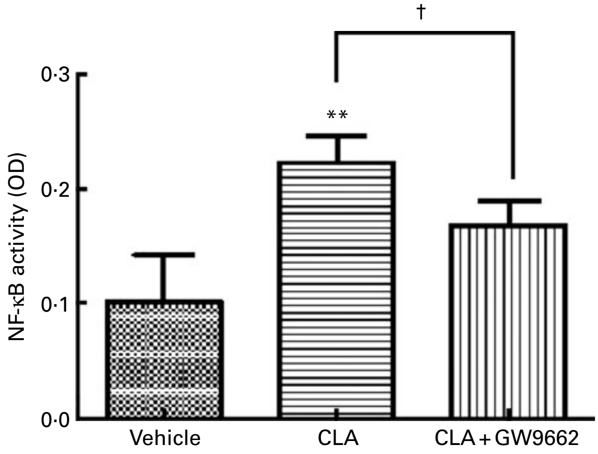

Fig. 2. The effect of *trans*-10, *cis*-12-conjugated linoleic acid (*t*10*c*12-CLA) on NF-κB activation in porcine peripheral blood mononuclear cells (PBMC). Porcine PBMC (2×10^6 cells/ml) were incubated with *t*10*c*12-CLA (10 μM) or *t*10*c*12-CLA (10 μM) in combination with GW9662 (1 μM) for 24 h. NF-κB p65 activation was assayed in nuclear extracts using an ELISA-based TransAM® NF-κB p65 transcription factor assay kit, as described in the Materials and methods section. Values are means, with standard deviations represented by vertical bars (*n* 3). Mean values were significantly different from that of the vehicle group: ** $P<0{\cdot}01$ (one-way ANOVA); † $P<0{\cdot}05$ (as determined by the two-sample *t* test). OD, optical density.

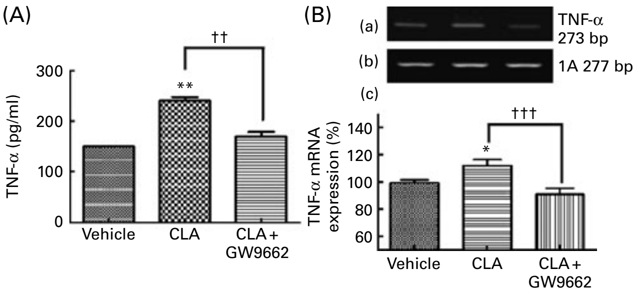

Fig. 3. The effect of *trans*-10, *cis*-12-conjugated linoleic acid (*t*10*c*12-CLA) on TNF-α expression in porcine peripheral blood mononuclear cells (PBMC). (A) Porcine PBMC (2×10^6 cells/ml) were incubated with *t*10*c*12-CLA (10 μM) or *t*10*c*12-CLA (10 μM) in combination with GW9662 (1 μM) for 24 h. The concentration (pg/ml) of TNF-α in the culture supernatant from porcine PBMC was measured by ELISA. (B) RT-PCR analysis of TNF-α mRNA expression in porcine PBMC treated with *t*10*c*12-CLA (10 μM) or *t*10*c*12-CLA (10 μM) in combination with GW9662 (1 μM) for 1 h (a). TNF-α mRNA expression was normalised with 1A (b). Signals were quantified with a molecular analysis program and were expressed as a percentage of the vehicle value (c). Values are means, with standard deviations represented by vertical bars (*n* 3). Mean values were significantly different from that of the vehicle group: * $P<0{\cdot}05$, ** $P<0{\cdot}01$ (one-way ANOVA); †† $P<0{\cdot}01$, ††† $P<0{\cdot}001$ (as determined by the two-sample *t* test).

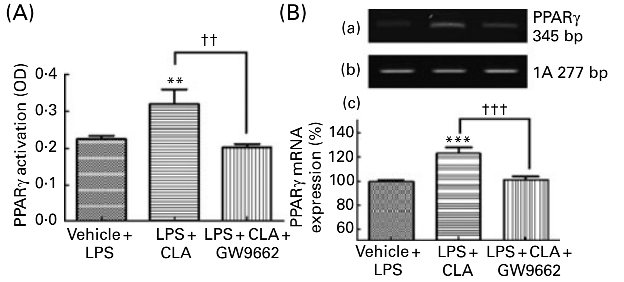

Fig. 4. The effect of *trans*-10, *cis*-12-conjugated linoleic acid (*t*10*c*12-CLA) on PPARγ activation in lipopolysaccharide (LPS)-stimulated porcine peripheral blood mononuclear cells (PBMC). (A) Porcine PBMC (2×10^6 cells/ml) were treated with LPS (1 μg/ml) and *t*10*c*12-CLA (10 μM) or *t*10*c*12-CLA (10 μM) in combination with GW9662 (1 μM) for 24 h. PPARγ activity was assayed in nuclear extracts using an ELISA-based TransAM® PPARγ transcription factor assay kit, as described in the Materials and methods section. (B) RT-PCR analysis of PPARγ mRNA expression in porcine PBMC treated with LPS (1 μg/ml) and *t*10*c*12-CLA (10 μM) or *t*10*c*12-CLA (10 μM) plus GW9662 (1 μM) for 1 h (a). PPARγ mRNA expression was normalised with 1A (b). Signals were quantified with a molecular analysis program and were expressed as a percentage of the vehicle value (c). Values are means, with standard deviations represented by vertical bars (*n* 3). Mean values were significantly different from that of the vehicle plus LPS group: ** $P<0{\cdot}01$, *** $P<0{\cdot}001$ (one-way ANOVA); †† $P<0{\cdot}01$, ††† $P<0{\cdot}001$ (as determined by the two-sample *t* test). OD, optical density.

Trans-10, *cis*-12 conjugated linoleic acid decreases NF-κB activity in lipopolysaccharide-stimulated porcine peripheral blood mononuclear cells

To assess NF-κB activation in response to *t*10*c*12-CLA in LPS-stimulated porcine PBMC, cells were treated with LPS (1 μg/ml) and *t*10*c*12-CLA (10 μM) in the presence or absence of the PPARγ antagonist GW9662 (1 μM) for 24 h. NF-κB p65 DNA-binding activity was significantly suppressed ($P<0·01$) by *t*10*c*12-CLA treatment in LPS-stimulated PBMC compared with controls (vehicle plus LPS); NF-κB DNA-binding activity was restored ($P<0·01$) by the addition of GW9662 (Fig. 5).

Trans-10,*cis*-12 conjugated linoleic acid decreases TNF-α expression in lipopolysaccharide-stimulated porcine peripheral blood mononuclear cells

The effect of *t*10*c*12-CLA on TNF-α expression in LPS-stimulated porcine PBMC was tested by adding LPS (1 μg/ml) and *t*10*c*12-CLA (10 μM) to the PBMC culture in the presence or absence of GW9662 (1 μM) for 24 h. As shown in Fig. 6, *t*10*c*12-CLA significantly decreased ($P<0·05$) TNF-α production in LPS-stimulated PBMC compared with vehicle plus LPS-treated controls, and this effect was reversed ($P<0·05$) by the addition of GW9662 (Fig. 6(A)). In addition, RT-PCR analysis revealed that *t*10*c*12-CLA significantly suppressed ($P<0·001$) TNF-α mRNA expression in LPS-stimulated PBMC compared with vehicle plus LPS-treated controls. This suppression of TNF-α mRNA expression by *t*10*c*12-CLA was reversed ($P<0·05$) by GW9662; however, TNF-α mRNA expression was restored with a level lower than that of vehicle plus LPS-treated controls (Fig. 6(B)).

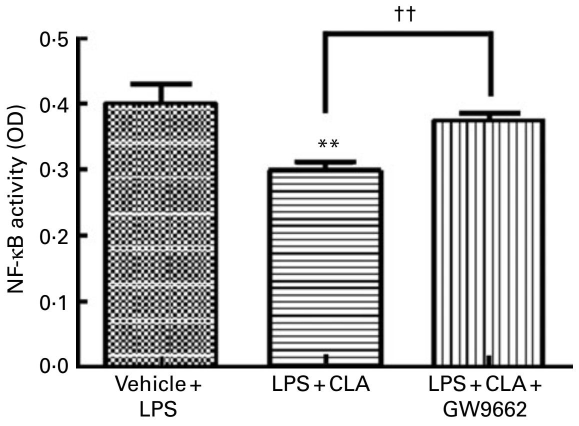

Fig. 5. The effect of *trans*-10, *cis*-12-conjugated linoleic acid (*t*10*c*12-CLA) on NF-κB activation in lipopolysaccharide (LPS)-stimulated porcine peripheral blood mononuclear cells (PBMC). Porcine PBMC (2×10^6 cells/ml) were treated with LPS (1 μg/ml) and *t*10*c*12-CLA (10 μM) or *t*10*c*12-CLA (10 μM) in combination with GW9662 (1 μM) for 24 h. NF-κB activity was assayed in nuclear extracts using an ELISA-based TransAM® NF-κB p65 transcription factor assay kit, as described in the Materials and methods section. Values are means, with standard deviations represented by vertical bars (*n* 3). Mean values were significantly different from that of the vehicle plus LPS group: ** $P<0·01$ (one-way ANOVA); †† $P<0·01$ (as determined by the two-sample *t* test). OD, optical density.

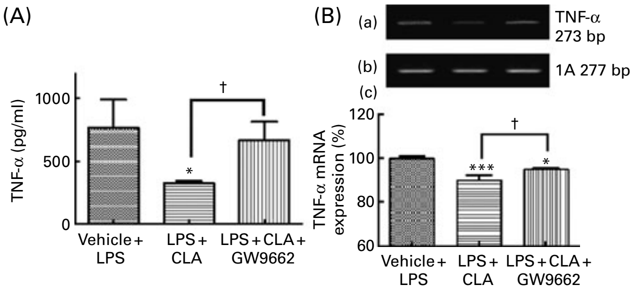

Fig. 6. The effect of *trans*-10, *cis*-12-conjugated linoleic acid (*t*10*c*12-CLA) on TNF-α expression in lipopolysaccharides (LPS)-stimulated porcine peripheral blood mononuclear cells (PBMC). (A) Porcine PBMC (2×10^6 cells/ml) were treated with LPS (1 μg/ml) and *t*10*c*12-CLA (10 μM) or *t*10*c*12-CLA (10 μM) in combination with GW9662 (1 μM) for 24 h. The concentration (pg/ml) of TNF-α in the culture supernatant from porcine PBMC was measured by ELISA. (B) RT-PCR analysis of TNF-α mRNA expression in porcine PBMC treated with LPS (1 μg/ml) and *t*10*c*12-CLA (10 μM) or *t*10*c*12-CLA (10 μM) plus GW9662 (1 μM) for 1 h (a). TNA-α mRNA expression was normalised with 1A (b). Signals were quantified with a molecular analysis program and were expressed as a percentage of the vehicle value (c). Values are means, with standard deviations represented by vertical bars (*n* 3). Mean values were significantly different from that of the vehicle plus LPS group: * $P<0·05$, *** $P<0·001$ (one-way ANOVA); † $P<0·05$ (as determined by the two-sample *t* test).

Discussion

The results of the present study showed that *t*10*c*12-CLA up-regulated TNF-α expression in porcine PBMC. A similar increase in TNF-α mRNA levels in response to *t*10*c*12-CLA treatment has also been reported in isolated adipocytes(21). *t*10*c*12-CLA also enhanced TNF-α and IL-6 gene expression in white adipose tissue(22), and increased TNF-α secretion in mouse splenocytes(23). In porcine PBMC, the *t*10*c*12-CLA-induced enhancement of TNF-α production up-regulated the phagocytic capacity of porcine polymorphonuclear cells(24). In addition, *t*10*c*12-CLA has been found to enhance the chemotaxis of porcine polymorphonuclear cells through IL-8 produced by CLA-treated PBMC(25). These findings suggest that *t*10*c*12-CLA has an immunostimulating effect mediated by the production of pro-inflammatory cytokines, including TNF-α and IL-8.

The *t*10*c*12-CLA-mediated activation of NF-κB p65 DNA binding in porcine PBMC found in the present study has also been reported in human umbilical vein endothelial cells(26). The activation of NF-κB is followed by the transcription of various pro-inflammatory genes including TNF-α(18). *t*10*c*12-CLA increased TNF-α production through the activation of NF-κB in human adipocytes(27). Moreover, *t*10*c*12-CLA directly induced IL-6 secretion in 3T3-L1 adipocytes by an NF-κB-dependent mechanism(22). These observations indicate that *t*10*c*12-CLA may participate in the expression of inflammatory mediators through the activation of NF-κB.

In contrast with its effect on LPS-naive PBMC, *t*10*c*12-CLA down-regulated TNF-α expression in LPS-stimulated PBMC. Similarly, *t*10*c*12-CLA attenuated the production and gene expression of TNF-α in weaned pigs challenged with LPS(13). *t*10*c*12-CLA also decreased pro-inflammatory cytokines including TNF-α in interferon-γ-stimulated macrophages(10), and CLA has been reported to increase IL-10 production and decrease IL-12 production in LPS-stimulated murine dendritic cells(28). IL-10 is known to be an anti-inflammatory cytokine capable of inhibiting the synthesis of pro-inflammatory cytokines and blocking NF-κB activity(29,30). The decrease in TNF-α expression in LPS-stimulated PBMC treated with *t*10*c*12-CLA found in the present study could therefore be related to an increase in IL-10 production. In the present study, *t*10*c*12-CLA suppressed NF-κB p65 DNA-binding activity in LPS-stimulated porcine PBMC. *c*9*t*11-CLA, which is also known to have an anti-inflammatory effect, decreased phorbol ester-induced NF-κB activation in HR-1 mouse skin cells by down-regulating inhibitory-κB degradation(31). *t*10*c*12-CLA prevented TNF-α gene expression by inhibiting NF-κB-binding activity in PBMC from weaned pigs challenged with LPS(32). Also, treatment with a 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 DNA-binding activity(33). These findings suggest that *t*10*c*12-CLA may suppress TNF-α expression in LPS-stimulated porcine PBMC by inhibiting NF-κB activation.

In the present study, PPARγ activity and mRNA expression in porcine PBMC were enhanced by *t*10*c*12-CLA treatment regardless of LPS stimulation. These findings are in agreement with several studies which have shown that CLA stimulated PPARγ gene expression in different tissues and cells, including skeletal muscle(34), adipocytes(35) and macrophages(10). Moreover, treatment with a CLA mixture enhanced PPARγ DNA-binding activity in cardiomyocytes(36). The activity of *t*10*c*12-CLA in PBMC could therefore be associated with both increased levels of PPARγ protein and activation of PPARγ.

PPAR activators have been reported to increase TNF-α production in mouse hepatocytes(37), and *t*10*c*12-CLA, which activates PPARγ, enhanced TNF-α and PPARγ expression in RAW macrophages(38). Furthermore, *t*10*c*12-CLA and *c*9*t*11-CLA increased both PPARγ and NF-κB activity in human umbilical vein endothelial cells(26). In contrast, PPARγ activation inhibited the production of inflammatory cytokines, including TNF-α, in LPS-stimulated dendritic cells(39). The PPARγ agonists troglitazone and PUFA attenuated the activation of NF-κB and the production of IL-6, IL-8 and inducible NO synthase in IL-1β-stimulated intestinal-like Caco-2 cells and in LPS-stimulated human dendritic cells(40). *t*10*c*12-CLA suppressed the production of TNF-α, IL-1β and IL-6, and enhanced PPARγ activation and gene expression in LPS-stimulated PBMC(13). *t*10*c*12-CLA and *c*9*t*11-CLA also inhibited platelet-derived growth factor-induced NF-κB activation in human vascular smooth muscle cells by a PPARγ-dependent mechanism(41). These findings support the idea that the activation of PPARγ by *t*10*c*12-CLA can modulate TNF-α expression through the up- and down-regulation of NF-κB activity in LPS-naive and LPS-stimulated PBMC, respectively.

The present results showed that *t*10*c*12-CLA increased TNF-α expression and NF-κB activation in porcine PBMC. In contrast, in LPS-stimulated porcine PBMC, *t*10*c*12-CLA decreased TNF-α expression and NF-κB activation. *t*10*c*12-CLA also up-regulated PPARγ activity and mRNA expression in both LPS-naive and LPS-stimulated porcine PBMC. To elucidate the role of PPARγ on NF-κB activity and TNF-α expression in CLA-treated porcine PBMC, we used a PPARγ antagonist, GW9662. GW9662 negated the effects of CLA on TNF-α expression and NF-κB activation in both LPS-naive and LPS-stimulated porcine PBMC. These results strongly suggested that the effects of *t*10*c*12-CLA on NF-κB activation and TNF-α expression may be dependent on the PPARγ pathway. In the present study, however, *t*10*c*12-CLA, LPS and GW9662 were added simultaneously, which might preclude mechanistic insights due to the potential for interference or interactions between the various drugs. An experimental method employing sequential additions may be necessary to clarify more precisely the mechanism involved.

Recent nutritional immunology studies have revealed an important role for dietary CLA in the attenuation of inflammation-associated diseases. The preventive administration of a CLA mixture before the onset of porcine bacterial-induced colitis attenuated inflammatory lesion development and growth failure(42). Dietary CLA supplementation (mixture of isomers 9, 11 and 10, 12) up-regulated colonic PPARγ expression and contributed to delaying the onset of experimental inflammatory bowel disease in a pig model(43). It has also been demonstrated that CLA ameliorates dextran sodium sulphate-induced colitis(44) and prevents colorectal tumour formation, partly through a PPARγ-dependent mechanism in PPARγ-null mice(45). *c*9*t*11-CLA reduced allergic sensitisation and airway inflammation in mice, most probably via a PPARγ-related mechanism and by reducing eicosanoid precursors(46). Recently, *t*10*c*12-CLA and *c*9*t*11-CLA have been found to suppress muscle wasting, which is mediated by TNF-α, in a model of human muscle cell inflammation(47). In the present study, we have shown that *t*10*c*12-CLA treatment increases TNF-α expression in naive PBMC but suppresses LPS-induced TNF-α expression. This apparently conflicting result following LPS stimulation may be related directly to NF-κB p65 activity and may be mediated at least partly through a PPARγ-dependent mechanism. Although it is currently difficult to determine why *t*10*c*12-CLA yields such a conflicting effect, the conflict could explain discrepancies in previous reports over whether or not CLA has a beneficial effect on health. Besides the effects on IL-10 and PGE_2 production mentioned above, another factor that might explain TNF-α expression signalling modulated by *t*10*c*12-CLA could be the ubiquitin-editing enzyme A20, also known as TNF-α-induced protein 3(48). Ubiquitin-editing enzyme A20 is a key player in the negative feedback regulation of NF-κB signalling(49), but there is yet no evidence to directly explain the effects of CLA. Recently, it has been reported that the ubiquitin-editing enzyme A20 is required for the termination of NF-κB signalling in response to LPS and TNF-induced NF-κB responses(50). To clarify the effects of *t*10*c*12-CLA, further research into the overall TNF signalling pathway, including the ubiquitin-editing enzyme A20, will be necessary.

In conclusion, *t*10*c*12-CLA has an immunostimulating effect on porcine PBMC, which is mediated by the enhancement of NF-κB activity and TNF-α expression. By contrast, *t*10*c*12-CLA has an anti-inflammatory effect through the suppression of NF-κB activity and TNF-α expression in LPS-stimulated PBMC. These immunostimulating and anti-inflammatory effects of *t*10*c*12-CLA on porcine PBMC are mediated by a PPARγ-dependent pathway.

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