Conjugated linoleic acid decreases production of pro-inflammatory products in macrophages: evidence for a PPARγ-dependent mechanism

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Abstract

Conjugated linoleic acid (CLA) is a dietary fatty acid that has received considerable attention due to its unique properties in rodent models including anti-cancer, anti-atherogenic and anti-diabetic effects. The effects of CLA are similar to those seen with ligands for peroxisome proliferator-activated receptor (PPARs), most notably of the PPARγ subtype. With the recent observation of a role for PPARγ in regulation of immune responses, we suspected that CLA could affect immune function, in particular macrophage activity. The goal of our study was to examine whether this dietary fatty acid has anti-inflammatory properties similar to those reported for PPARγ activators such as 15-deoxy prostaglandin J2 (PGJ2). In reporter assays, various CLA isomers activated PPARγ in RAW264.7 mouse macrophage (RAW) cells. CLA decreased the interferon-γ (IFNγ)-induced mRNA expression of mediators of inflammation including cyclooxygenase 2 (COX2), inducible NOS (iNOS), and tumor necrosis factor α (TNFα). Reporter assays also demonstrated reduced IFNγ-stimulated transcriptional activity of the iNOS and COX2 promoters by CLA. Consequently, CLA decreased the production of PGE2, TNFα and the inflammatory agent nitric oxide (NO) in RAW cells treated with IFNγ. Other pro-inflammatory cytokines such as IL-1β and IL-6 were similarly decreased by CLA treatment of RAW cells. In addition, various CLA isomers induced HL60 cell differentiation along the monocytic lineage as assessed by measuring expression of the cell surface marker CD14. This differentiation process, as well as the regulation of iNOS and COX2 by 15dPGJ2, is believed to involve PPARγ. Mutations of Leu468 and Glu471 to alanine in helix 12 of the ligand-binding domain of PPARγ resulted in a protein with strong dominant-negative activity (dnPPARγ). Transfecting dnPPARγ into RAW cells eliminated the ability of various CLA isomers to regulate the iNOS reporter construct. Taken together, these results suggest that CLA has anti-inflammatory properties that are mediated, at least in part, by the nuclear hormone receptor PPARγ. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Conjugated linoleic acid; Inducible nitric oxide synthetase; Cyclooxygenase; Peroxisome proliferator-activated receptor; Macrophage; Regulation of inflammation

1. Introduction

The naturally occurring fatty acid, conjugated linoleic acid (CLA), is synthesized in vivo from linoleic acid by rumen bacteria and may also be synthesized during heat processing of animal-derived foods [1]. Cooked meat, pasteurized dairy products and processed cheeses are good dietary sources of these fatty acids [1], where its concentration may range from 2.5 to 15.0 mg/g lipid [2]. The term CLA refers to a group of linoleate (c9,c12-octadecadienoic) derivatives that exhibit several possible positional and geometric isomers (9–11, 10–12 in cis/Z and trans/C configurations). At least eight different CLA isomers have been observed [1].

Much attention has been given to CLA due to its unique properties in rodent model systems including anti-cancer, anti-atherogenic and anti-diabetic effects [3,4]. CLA is protective against cancer during both initiation and promotion stages of carcinogenesis and it is more effective than any other fatty acid in inhibiting the development of mammary tumors [5]. A substantial reduction in lung metastases was observed in mice fed the CLA-supplemented diet as compared to the control diet-fed group [6]. CLA has anti-atherogenic effects, partially attributed to a reduction in the level of total and low-density lipoprotein cholesterol and triglycerides [7]. Recently, we have observed that CLA normalizes impaired glucose tolerance and prevents or slows
the development of hyperglycemia in the pre-diabetic ZDF rat [4]. Despite the increasing knowledge about CLA's beneficial effects, little is known about mechanism of action of these fatty acids.

Several of the therapeutic effects of CLA in laboratory animals described above are similar to those of fatty acid-like chemicals called peroxisome proliferators as well as new drugs used to treat diabetes, the thiazolidinediones (TZDs). These chemicals activate a group of steroid hormone receptors, the peroxisome proliferator-activated receptors (PPARs). PPAR is a member of the nuclear receptor superfamily of ligand-dependent transcription factors that includes the receptors for the steroid, retinoid, and thyroid hormones, and its mechanism of action has been well characterized (for reviews see Refs. [8–13]). Three PPAR subtypes, which are the products of distinct genes, have been identified [14]. PPARα is predominantly expressed in liver, kidney, intestine and heart and increases the production of enzymes involved in fatty acid oxidation. PPARα activators include the fibrate class of hypolipidemic drugs and fatty acids, and this receptor subtype is required for peroxisome proliferation in rodent liver. PPARβ is expressed in a wide range of tissues and the biological role of this isoform is not clearly known. The PPARγ subtype is highly expressed in adipose tissue, adrenal gland, colon and macrophage [15]. TZD antidiabetic drugs, 15-deoxy prostaglandin J2 (PGJ2), nonsteroidal anti-inflammatory drugs (NSAIDs) and long chain fatty acids including arachidonic acid and linoleic acid are known PPARγ ligands and activators [16]. PPARγ plays a crucial role in regulation of adipocyte differentiation, fatty acid metabolism and glucose homeostasis [17–19]. In addition, recent research has shown that PPARγ activation has profound effects on macrophage differentiation and activation [12,20,21].

CLA shares functional similarities with ligands of PPARγ. For example, PPARγ activation is associated with a decrease in colon and breast cancer [22,23], and this receptor is essential for adipogenesis and the anti-diabetic properties of TZDs [24]. We have reported that CLA is an activator of PPARα, β and γ in reporter assays, with PPARα showing the highest affinity [4,25,26]. However, we see little evidence of peroxisome proliferation, a PPARα-dependent event, when mice or rats are administered CLA in vivo [25]. Therefore, we propose that CLA exerts some of its biological effects via PPARγ and chose to examine this hypothesis in macrophage cells. Recent literature has documented that activation of PPARγ inhibits production of pro-inflammatory cytokines, such as TNFα and IL-6 [27]. Furthermore, treatment of macrophages with 15dPGJ2 and synthetic PPARγ ligands decreased the expression of the inducible nitric oxide synthase (iNOS) in response to interferon-γ (IFNγ) treatment [12]. We will provide evidence that CLA does indeed act through PPARγ to regulate a certain subset of genes. In addition, as a result of PPARγ activation, a novel beneficial effect of CLA is observed, namely decreased production of pro-inflammatory products such as NO and TNFα. Therefore, CLA may be classified as an NSAID and may be useful in the treatment of inflammatory diseases.

2. Materials and methods

2.1. Materials

Individual CLA isomers (9Z,11E)-CLA (99% purity), (9E,11E)-CLA (98% purity), (9Z,11Z)-CLA (96% purity), (10E,12E)-CLA (98% purity), 8-(5-hexyl-2-furyl)-octanoic acid (Furan) and linoleic acid were from Matreya Inc. (Pleasant Gap, PA). The CLA mixture (C16:0 Palmitic Acid 4.4%, C18:0 Stearic Acid 2.8%, C18:1 Oleic Acid 15.4%, C18:2 c9, c12 Linoleic Acid 1.8%, C18:2, conjugated CLA 75.6%, C18:2, conjugated c9,t11CLA, 34.9%, C18:2, conjugated t10,c12CLA 35.9%) was a generous gift of Pharmaneutrients (Chicago, IL). 15-deoxy-Δ12,14-prostaglandin J2 (15dPGJ2) was purchased from Biomol (Plymouth Meeting, PA). The PGJ3TK luciferase reporter contains three tandem repeats of a PPAR response element (PPRE) and was a gift of B. Staels (INSERM, France) The iNOS promoter driven luciferase reporter was a gift from Dr. Helmut Kleiner (Gutenberg University, Mainz, Germany). The transfection reagent Superfect was obtained from Qiagen (Valencia, CA). All primers were designed using PrimerSelect (DNASTAR, Madison, WI) and purchased from Operon (Alameda, CA). Recombinant murine IFNγ was purchased from Pepro Tech Inc. (Rocky Hill, NJ). Enzymes, cofactors and nucleotides necessary for reverse transcriptase polymerase chain reaction (RT-PCR) and internal standard construction were obtained from Promega Corporation (Madison, WI). Nusieve 3:1 agarose was purchased from FMC Bioproducts (Rockland, ME). All media components and fetal calf serum were purchased from Gibco BRL (Gaithersburg, MD). RAW264.7, COS-1 and HL60 cell lines were purchased from ATCC (Manassas, VA). Other chemicals and reagents were of the highest grade commercially available.

2.2. Cell culture

Murine macrophage RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 50 U/ml penicillin, 50 mg/ml streptomycin, 2 mM l-glutamine, 100 mM sodium pyruvate and 100 µM non-essential amino acids. HL-60 cells were cultured in IMEM containing 20% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin and l-glutamine. COS-1 cells were cultured in αMEM supplemented with 5% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells are all maintained in a 5% CO2 humidified atmosphere at 37 °C.
2.3. Measurement of inflammatory products

The concentration of NO in the media was estimated by the amount of NO2 quantified [28]. Culture supernatants from RAW cells were reacted with an equal volume of the Griess reagent (50 μl of 1.0% sulfanilamide in 5.0% H3PO4 and 50 μl of 0.1% naphthylethylenedimine dihydrochloride) at room temperature for 15 min. The absorbance at 550 nm was determined and the concentrations of NO2 were calculated by comparison with a standard curve prepared using dilutions of NaNO2. Enzyme-linked immunosorbent assay (ELISA) was used to quantify the concentration of various cytokines. Culture supernatant was collected from triplicate wells and concentrations of mouse IL-1α, IL-1β and IL-6 and TNFα were measured by ELISA using protocols supplied by the manufacturer (Endogen, Woburn, MA).

2.4. Reporter gene assays

RAW cells were transfected by Superfect (Qiagen) with a total amount of DNA equal to 2 μg per well of a six-well plate. For each experiment, the total amount of transfected DNA per well was kept constant by using corresponding empty vector. See figure legends for the reporter plasmid and expression plasmids used in each experiment. The pRL-TK plasmid was used in all cases as a transfection efficiency control. The transfection was halted after 3 h and triplicate samples were treated with various CLA isomers in the absence or presence of IFNγ. Luciferase activity was assayed 24 h later using the Dual-luciferase reporter assay system (Promega). Luciferase reporter genes from the regulatory region of iNOS and cyclooxygenase 2 (COX2) were obtained. Activation is quantified as the amount of luciferase activity relative to control plasmid expression (pRL-TK) corrected for protein recovery. Protein concentrations were determined using CoomassiePlus (Pierce, Rockford IL) and bovine serum albumin as a standard.

2.5. Quantitative RT-PCR

Total RNA was isolated from cells using TRI-reagent (Molecular Research Center, Cincinnati, OH) following the provider's protocol. Total RNA extracts were frozen at −80 °C until analyses were performed. Quantitative RT-PCR (qRT-PCR) used recombinant RNA as an internal standard to negate tube-to-tube variability in amplification efficiency. Preparation of synthetic internal standard of recombinant RNA was described elsewhere [29]. Competitive RT-PCR was performed as described by Ref. [30] and modified by Ref. [31]. Reverse transcription in a final volume of 20 μl containing 2.5 units of reverse transcriptase, 2.5 mM oligo(dT), 5 mM MgCl2, 0.1 μg total RNA, and various amounts of rcRNA internal standard was carried out for 15 min at 42 °C and inactivated by heating at 99 °C for 5 min. Subsequently, cDNA products were incubated with PCR master mix in a 50 μl volume containing 4 mM MgCl2, 2.5 units Taq polymerase and 6 pmol forward and reverse primers. The sequences for the primers and products length are shown in Table 1. PCR reactions were electrophoresed on a 2.5% NuSieve/agarose gel (3:1;w/w, FMC BioProducts). PCR fragments were visualized with ethidium bromide and photographed. Quantification of the amount of target mRNA for COX2, iNOS, TNFα and CD36 mRNA were determined as described by Ref. [30] and modified by Ref. [32].

2.6. Differentiation of HL60 cells

Differentiation of HL60 cells along the monocytic lineage was assessed by flow cytometric analysis of CD14 expression. Following culture for 5 days in the presence of DMSO (0.1%, v/v), 9Z11E-CLA, 9Z11Z-CLA, 9E11E-CLA, 10E12Z-CLA, Furan-CLA, LA (150 μM) or 15dPGJ2 (10 μM), HL60 cells were incubated with 1% BSA for 15 min. To block Fc receptors, cells were incubated with Diotin free Fc receptor blocker (Accurate Chemical and Scientific Corp., Westbury, NY) for 20 min. Medium was then washed away and cells were incubated with PE-conjugated anti-human CD14 (Pharmingen, San Diego, CA) for 60 min on

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>TPPS</th>
<th>ISPPS</th>
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<tr>
<td>mCOX2</td>
<td>Forward: ACTCACTCAGTCTTGTGGAGTCATTG</td>
<td>582</td>
<td>440</td>
</tr>
<tr>
<td></td>
<td>Reverse: GATTAGTACTCTAGGTTAATG</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Forward: CCCAGAGAAGCACCAGTCTG</td>
<td>445</td>
<td>335</td>
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<tr>
<td></td>
<td>Reverse: GAAAGAGAGCTGCGCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miNOS</td>
<td>Forward: AATGGCAACTCAGGCGCGCCATC</td>
<td>326</td>
<td>440</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCTGTTGCTACAGAAGGCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mTNFα</td>
<td>Forward: CAGTCCTCTCTGCATC</td>
<td>417</td>
<td>335</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCGTCTCGTTGTTCTT</td>
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</tr>
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TPPS: Target PCR (product size in base pairs).
ISPPS: Internal standard PCR (product size in base pairs).
ice. Purified mouse IgG2a, κ isotype (Pharmingen) was used as a control. Cells were stained with propidium iodide in PBS containing 2% Newborn Calf Serum and analyzed on a Becton-Dickinson FACScan.

2.7. Generation of a dominant-negative PPARγ (dnPPARγ)

Leucine and glutamic acid residues within the mouse PPARγ AF2 motif were mutated to alanine to generate a dominant-negative construct (dnPPARγ) [33,34]. Two oligonucleotide primers containing the desired mutation with each complementary to opposite strands of the vector were designed (forward primer: catgaccttacccggtcggagtcgtacaagaccttg, reverse primer: gtactcggaagtggggcgcaagtgcctgaac). The pSG5-mPPARγ plasmid (obtained from J. Tugwood, AstraZeneca, UK) was used as template. Site-directed mutagenesis was performed using protocols supplied by the manufacturers (Stratagene, La Jolla, CA) and verified by sequencing.

2.8. Statistics

Data was analyzed by one-way analysis of variance using Minitab (State College, PA). If a significant treatment effect was observed (\(P<0.05\)) a Dunnett’s multiple comparison test was performed. Values with an asterisk are different from the appropriate vehicle-treated control, at the \(P<0.05\) level.

3. Results

3.1. PPAR subtype expression in RAW cells

Macrophages play a key role in regulating cell-mediated immune response. In addition to the well-known function of endocytosis, macrophages can be induced to secrete a series of cytotoxic products such as TNFα, IL-1β, IL-6 and nitric oxide (NO) by exposure to various agents, such as IFNγ [35]. For the majority of studies, we utilized RAW264.7, a mouse macrophage cell line that secretes the cytotoxic products described above and is responsive to IFNγ and to PPARγ activators. As shown in Fig. 1, the PPAR subtype expression in this cell line mimic, to some extent, what is seen in primary macrophages [36]. That is, PPARγ is the predominant subtype expressed in RAW cells under the present culture conditions. IFNγ had no effect on PPARγ expression, which is consistent with a previous study [37]. Both PPARα and PPARβ were not detectable in either resting or activated macrophages. Therefore, this model system is appropriate for the examination of CLA’s biological effects via PPARγ, without interference from other PPAR subtypes. However, it should be cautioned that this is a transformed cell line that may not completely recapitulate what has been observed in primary monocyte-derived macrophages, which express detectable levels of PPARα and β [36].

3.2. Activation of PPARγ by CLA isomers in macrophages

To determine whether CLAs can activate a promoter consisting of a PPRE in macrophages, a reporter assay was utilized. RAW cells were transfected with the PPRE-containing reporter gene (PGJ3TK) and treated with various CLA isomers (200 μM) or DMSO (vehicle control) for an additional 24 h. Data is expressed as normalized luciferase activity (firefly/renilla/protein) as a percent of the DMSO control (\(n=3\), mean ± S.E., black bars). Values with an asterisk are statistically different from the DMSO-treated control (\(P<0.05\)). Also shown for comparison is the activity of linoleic acid (200 μM) and PGJ2 (10 μM) (gray bars) done in similar experiments (mean, \(n=2\)).
difference between the isomers. This data clearly indicates that CLA does activate PPAR in macrophages, supporting previous research reported in CV-1 cells transfected with mouse PPARγ [4].

3.3. Decreased IFNγ-dependent nitrite, iNOS production and iNOS promoter activity

In macrophages, NO is produced by iNOS following exposure to IFNγ, TNFα or microbial products including lipopolysaccharide (LPS). The production of NO is determined by assaying culture supernatants for nitrite (NO2), a stable reaction product of NO with molecular oxygen. Increasing levels of 9Z11E-CLA led to a dose-dependent inhibition of IFNγ-dependent NO2 production (Fig. 3A). The known PPARγ activator, 15dPGJ2 was used as a positive control and was compared to its vehicle control, ethanol. The effect of various CLA isomers on iNOS expression was further analyzed by qRT-PCR (Fig. 3C). As expected, IFNγ increased iNOS expression substantially. However, the induction of iNOS mRNA by interferon was effectively inhibited by various CLA isomers as well as 15dPGJ2. The potency of the parental compound LA was relatively less than that of CLAs, although it was also active at reducing iNOS mRNA. To determine whether changes in mRNA levels can be ascribed to differences in promoter activity, RAW cells were cotransfected with iNOS promoter-luciferase reporter DNA. Consistent with the previous observation, treatment of RAW cells with increasing concentrations of 9Z11E-CLA led to a dose-dependent reduction of iNOS promoter activity (Fig. 3B). These studies are consistent with the regulation of iNOS by CLA being at the level of transcription, producing less mRNA and subsequently less active enzyme and product (NO).

3.4. Decreased production of prostaglandin E2 (PGE2), COX2 and COX2 promoter activity

In macrophages, PGE2 is formed from cyclooxygenase (COX) metabolism of arachidonic acid and is a major mediator of vasodilation and pain. As a marker of macrophage activation, PGE2 release to the culture supernatant of CLA-treated RAW cells was measured. IFNγ-induced synthesis of prostaglandin E2 was suppressed in a dose-dependent manner by 9Z11E-CLA and 15dPGJ2 (Fig. 4A). To investigate the role of expression of the inducible form of COX (COX2), mRNA expression in RAW cells treated with various CLA isomers was examined. As shown in Fig. 4C, COX2 mRNA levels induced by IFNγ were reduced by different CLA isomers, as compared to DMSO control. Once again, there was little difference among the isomers and LA. Furthermore, a COX2 promoter-driven luciferase reporter was utilized to investigate the involvement of COX2 promoter activity (Fig. 4B). RAW 264.7 cells were cotransfected with COX2 promoter-luciferase reporter vector and PRLTK (control plasmid) followed by 24 h treatment with IFNγ (10 U/ml) or without IFNγ in the presence of test compounds as indicated. Activation is quantified as the amount of luciferase activity (pRL-TK) corrected for protein recovery. Panel C: Inhibition of IFN-dependent induction of iNOS mRNA expression by CLA. RAW 264.7 cells were cotransfected with iNOS promoter-driven luciferase reporter vector and PRLTK (control plasmid) followed by 24 h treatment with IFNγ (10 U/ml) or without IFNγ in the presence of test compounds as indicated. Activation is quantified as the amount of luciferase activity relative to control plasmid expression (pRL-TK) corrected for protein recovery. Values with an asterisk are statistically different from the appropriate DMSO-treated control ($P<0.05$, comparisons were performed among the control and IFNγ-treated cells). N.D., not determined.

Fig. 3. CLA decreased iNOS activity and expression in macrophages. Panel A: Decreased NO2 production by CLA. RAW cells were treated with 9Z11E-CLA at concentrations of 200, 100 and 25 μM, DMSO, ethanol and 15dPGJ2 in the presence and absence of IFNγ (10 U/ml). Ethanol and DMSO are vehicle control for 15dPGJ2 and DMSO, respectively. The levels of NO2 in the supernatant of RAW cells following 24 h stimulation were measured. This is the mean of three independent experiments, each performed in triplicate. Panel B: CLA decreased iNOS promoter activity. RAW 264.7 cells were cotransfected with iNOS promoter-driven luciferase reporter vector and PRLTK (control plasmid) followed by 24 h treatment with IFNγ (10 U/ml) or without IFNγ in the presence of test compounds as indicated. Activation is quantified as the amount of luciferase activity relative to control plasmid expression (pRL-TK) corrected for protein recovery. Panel C: Inhibition of IFN-dependent induction of iNOS mRNA expression by CLA. RAW 264.7 cells were treated with DMSO, different CLA isomers (200 μM), linoleic acid (200 μM) and 15dPGJ2 (10 μM) in the presence or absence of IFNγ (10 U/ml). Total RNA were isolated in triplicates following 24 h stimulation. iNOS expression was analyzed by quantitative RT-PCR and expressed as percent of DMSO-treated RAW cells. Values with an asterisk are statistically different from the appropriate DMSO-treated control ($P<0.05$, comparisons were performed among the control and IFNγ-treated cells). N.D., not determined.
promoter-reporter DNA. Consistent with decreased COX2 mRNA expression, 9Z11E-CLA effectively inhibited COX2 promoter activity in a dose-dependent manner. The data with COX2 (Fig. 4) is similar to that of iNOS (Fig. 3) in most respects, although the magnitude of the mRNA regulation by CLA and 15dPGJ2 was much less with the former.

3.5. Inhibition of tumor necrosis factor α (TNFα) production by CLA

Activated macrophages are the major source of TNFα, which has broad functions including cytotoxicity, cell growth, differentiation and pro-inflammatory activity. Because of this physiological importance, the effects of CLA on TNFα production and mRNA expression were examined. The TNFα concentration in the supernatant of RAW cells was examined by ELISA following 24 h of treatment with various compounds in the presence of IFNγ. As shown in Fig. 5A, CLA isomers, linoleic acid, Furan and 15dPGJ2 effectively decreased IFNγ-dependent TNFα production compared to the DMSO control. A mixture of CLA isomers (Pharmanutrients) was also able to affect TNFα production. Messenger RNA accumulation was further analyzed by qRT-PCR. Again, IFNγ-dependent TNFα mRNA was suppressed by various CLA isomers, linoleic acid, Furan and 15dPGJ2 (Fig. 5B). The decreased TNFα mRNA expression by CLAs was more pronounced than the decreased final product.
3.6. Suppression of cytokine synthesis

PPARγ agonists have been shown to suppress the production of pro-inflammatory cytokines in human monocytes [27]. It therefore seemed likely that CLA would have similar effects in our mouse macrophage model. In general, all CLA isomers inhibited IFNγ-induced IL-1β and IL-6 production at concentration of 200 μM (Fig. 6). However, due to the amount of variability, and the fact that several measurements were below the limit of detection for this assay, no single isomer resulted in statistically different value than the DMSO control. The inhibition of IL-6 was somewhat greater than that of IL-1β and was less variable. The expression of IL-1α was also examined by ELISA, but was undetectable in this system (data not shown).

3.7. Altered monocyte/macrophage differentiation

To further investigate the effect of CLA on monocyte/macrophage differentiation, a myeloid precursor cell line, the human leukemia cell HL60, was utilized. HL60 cells display controlled differentiation along the granulocyte or the monocyte lineage by exposure to various signaling molecules [38]. Surface markers CD18 and CD11b are increased during differentiation to either granulocytes or monocytes, while expression of the LPS receptor CD14 is only induced during monocytic differentiation [39]. HL60 cells were cultured for 5 days in the presence of different CLA isomers, LA, Furan as well as 15dPGJ2, and CD14 was examined by flow cytometry. Fig. 7A shows that exposure of HL60 cells to CLA isomers induced CD14 expression by 1.5–2.5-fold. The effects of 9Z11E, 9E11E and 15dPGJ2 were more pronounced than that of linoleic acid.

Monocytes/macrophages can further differentiate to a foam cell, a lipid accumulating macrophage. During this process, scavenger receptor type B CD36, which mediates the uptake of oxidized low-density lipoprotein (oxLDL), is selectively induced [40]. The ability of CLA to regulate CD36 expression in macrophages was examined by qRT-PCR. As shown in Fig. 7B, after 24 h of treatment with different CLA isomers and 15dPGJ2, increased CD36 mRNA levels were observed. In this assay, 15dPGJ2, 9Z11E, 9Z11Z and 9E11E CLA had the most apparent effects. These observations are consistent with the ability of CLA to induce monocyte/macrophage differentiation.

3.8. A dnPPARγ inhibits the effects of CLA

The effects observed above are indicative of CLA isomers activating PPARγ, but are not definitive. We therefore used a competitive antagonist of this receptor, bisphe- nol A diglycidyl ether (BADGE) [41], but it proved to be toxic to RAW264.7 and HL60 cells even at low doses (data not shown). Therefore, to determine whether PPARγ is required for some of the effects of CLA in macrophages, we constructed a dominant-negative inhibitor (dnPPARγ) [34]. The ability of dnPPARγ to inhibit PPARγ-dependent transcription was measured in a reporter assay. First, we...
tested the influence of dnPPARγ on PPRE activity in COS1 cells. COS1 cells were cotransfected with increasing amount of dnPPARγ and constant amounts of wtPPARγ and PRLTK together with the reporter gene PGJ3TK, followed by 24 h of treatment with the indicated compounds. As shown in Fig. 8A, at the highest concentration of dnPPARγ plasmid, the induction of PPRE reporter activity we observed in the previous study was reduced to similar level as that of the DMSO control. A similar experiment with iNOS promoter the reporter in RAW cells was conducted. RAW cells were transfected with increasing amounts of dnPPARγ or corresponding empty vector to keep the total amount of transfected DNA per well constant. Cells then were treated with 9Z11E CLA and DMSO for 24 h. With an increasing amount of dnPPARγ, the decreased iNOS promoter activities observed with PPARγ ligands in the presence of IFNγ were restored (Fig. 8B). Taken together, these data suggest that CLA decreases iNOS reporter activity and increases PGJ3TK activity in a PPARγ-dependent manner.

To determine the effect of a dnPPARγ on PPRE activity in macrophages treated with various CLA isomers, RAW cells were transfected with dnPPARγ and PRLTK together with the reporter gene PGJ3TK followed by treatment with 200 μM of the various CLA isomers. In Fig. 9A, the increased PPRE reporter activities by various CLA isomers were substantially reduced. An equivalent experiment with iNOS promoter luciferase vector was also performed. Compared to cells transfected with the dnPPARγ, the reduction of iNOS expression in the presence of IFNγ by CLAs was less than previously observed; However, in virtually every case, the dnPPARγ construct ameliorated the effect on iNOS activity. With the specific chemical inhibitor of PPARγ described above (BADGE) in RAW cells transfected with PGJ3TK, the CLA-induced PPRE promoter activities were reduced to the same level as that of the control group, although...
toxicity was a concern (data not shown). Taken together, these functional studies demonstrating that dnPPARγ reduces the effects of the CLA isomers on iNOS promoter and PPRE reporter activity, allow, perhaps for the first time, the proposal of a definitive target molecule for these important dietary fatty acids.

4. Discussion

Of the numerous beneficial effects of CLA in animal models, the anti-atherosclerosis properties of this fatty acid have gained little notoriety [7,42]. The involvement of the immune system in atherosclerosis is well known and CLA has been shown to have anti-inflammatory activities. For example, CLA significantly decreased antigen-induced histamine and PGE₂ release [43]. Watkins and Seifert [44] showed that CLA isomers have anti-inflammatory activity in bone by regulating prostanoid formation. Though evidence supporting the anti-inflammatory properties of CLA is accumulating, the mechanism behind the action of CLA in the immune system is not clear. Since a similarity exists between the biological effects of CLA and 15d-PGJ₂, we speculated that CLA would bind to PPARγ, activate this receptor to a transcriptionally active form and subsequently regulate gene expression. In fact, CLA has been shown to activate PPARγ in rat adipocytes [4,45,46]. Here, by using a transactivation reporter assay, we demonstrated the ability of various CLA isomers to activate PPARγ in RAW cells. Previous studies have shown that resting primary macrophages express low levels of PPARγ and expression of this receptor was induced in primary macrophages in response to IFNγ [36]. However, based on our data, PPARγ expression level is relatively high in RAW cells and not apparently increased by IFNγ under the present conditions.

The data presented herein is consistent with CLA acting as an anti-inflammatory agent. First, CLA decreased the IFNγ-dependent expression of inducible NOS (iNOS) and iNOS promoter activity in a concentration-dependent manner. The functional consequence of inhibited expression of iNOS by CLA was strengthened by the finding of decreased inflammatory agent NO production in response to IFNγ. Second, treatment of macrophages with various CLA isomers reduced the COX2 mRNA expression, COX2 promoter activity and the end product PGE₂ in response to IFNγ. This inhibition showed a dose–response relationship with the quantity of CLA. Third, CLA attenuated the expression of genes involved in the different steps of the immune response to IFNγ, such as TNFα at both the mRNA and protein level, IL1β and IL-6 at protein level. Our data strongly support the concept that CLA exerts anti-inflammatory effects by negatively regulating the expression of certain pro-inflammatory genes.

PPARγ is involved in monocyte/macrophage differentiation and in the uptake of oxLDL by inducing the expression of scavenger receptor type B CD36 [20]. CD36 is a receptor for oxLDL and the uptake of oxLDL by macrophages promotes intracellular lipid accumulation, resulting in foam cell formation. Foam cells have pathogenic effects in atherosclerosis and are a predominant cell type found in fatty streaks [47]. On the other hand, oxLDL was shown to increase PPARγ expression [20] and two components of oxLDL, 9-HODE and 13-HODE, are PPARγ activators [40]. This has been suggested to form a positive feedback loop leading to foam cell formation [40]. In the present study, various CLA isomers induced macrophage differentiation as assessed by inducing CD36 expression, which seems contradictory to the anti-atherogenic role of CLA in rodent animals. However, pro-inflammatory cytokines, such as IL1β, TNFα and IL-6 are known to exert pathogenic effects in atherosclerosis; thus, the anti-inflammatory effects of CLA could partially explain the anti-atherogenic role of CLA in vivo. Previous studies have shown a substantial increase in CD14 expression in HL 60 cells by the combination of PPARγ and RXR ligands [48]. The ability of CLA to induce both CD14 and CD36 expression suggests PPARγ involvement in the CLA signaling pathway. The predominant forms of CLA, the 9Z11E and 10E12Z isomers, are not RXR activators, as assessed by reporter assays (data not shown), thereby strengthening the possibility that PPARγ is the cognate receptor for CLA in macrophages.

The role of PPARγ in macrophage differentiation has become much more controversial based on some recent research using embryonic stem cells devoid of PPARγ expression [49–51]. For example, in PPARγ-deficient stem cells, myeloid development, phagocytosis and inflammatory cytokine production appear to be normal. PPARγ is required for basal expression of CD36, but not for expression of the other major scavenger receptor responsible for uptake of modified lipoproteins, SR-A. The dominant-negative approach used in the present study showed a role for PPARγ in relatively simple model systems such as iNOS expression in reporter assays. However, this approach does not address the role of PPARγ in differentiation. Rather, in a model system responsive to the differentiation effects of PGJ₂, the HL60 cells, CLA was able to enhance markers of differentiation. In addition, the role of PPARγ in TNFα and IL6 regulation has been an area of contention [52]. There appears to be a dichotomy of effects of PPARγ ligands with endogenous activators such as PGJ₂ being effective at regulating TNFα whereas TZDs are unable to affect this inflammatory product. Based on the results presented herein, CLA acts in a manner more reminiscent of PGJ₂ than that of the synthetic drugs.

PPARγ can directly regulate gene transcription through a PPRE-dependent process. A PPRE is present in the promoter region of scavenger receptor CD36, which can be induced by the classic pathway involving the PPARγ/RXR heterodimer. In addition, PPARγ ligands exert anti-inflammatory effects by interfering with transcription factors NFκB, AP1 and STAT [36,53]. Analysis of the promoter regions of COX2, iNOS and IL-6 show binding sites for
these oxidant stress-related transcription factors [36,54]. Binding sites for NFκB have also been identified in TNFα and IL-1β promoters [55]. Therefore, it is likely that PPARγ ligands decrease the IFNγ-induced COX2, iNOS and IL-1β through a PPRE-independent process by antagonizing the activity of NFκB, AP1 and STAT, while affecting other genes (i.e. CD36) through a PPRE-dependent mechanism.

A major finding of our study was that PPARγ is responsible for some of the biological effects of CLA, a mechanism that has been proposed [45,56,57] but not appropriately tested. Since the PPARγ null animal is not viable [24], other biochemical approaches were used to examine the role of this transcription factor. Upon transfection with a dnPPARγ construct, CLA's effects on certain reporter gene expression in macrophages were greatly diminished. The dnPPARγ may inhibit the endogenous receptor activity through a variety of mechanisms, including inhibition of DNA binding, sequestering of RXR or other proteins, or competition for the coactivator pool [34]. The fact that a dnPPARγ eliminated the inhibitory effects of the CLA isomers on iNOS promoter activity and decreased PPRE activity in RAW cells is a strong indicator of a role for PPARγ in some of CLA's effects. The decreased PPRE-luciferase activity by CLA in the presence of the dominant-negative construct is probably indicative of a decreased ability of this mutant protein to recruit coactivators. The reversal of the repressed iNOS promoter activity by dnPPARγ is less easily explained, as no PPRE element exists in the promoter region of this enzyme. PPARα and γ have been shown to physically interact with c-Jun, P65 and CBP [58,59] and PPARγ activators strongly inhibited the binding of nuclear proteins Fos/Jun to the AP1 consensus oligonucleotide in bovine aortic endothelial cells [36]. If the dnPPARγ protein interferes with the ability of the endogenous receptor to associate with components of AP1 and NFκB complexes, the effects observed on the iNOS promoter are possible. Alternatively, PPARγ may affect the synthesis of components of the NFκB and AP-1 complexes through a PPRE-dependent process; these effects would be reverse by the dnPPARγ construct. For example, PPARα regulates the expression of IkBα [60] as well as c-Jun [61]. Thus, it is possible that CLA and other PPARγ activators can regulate the activity of inflammatory markers in a variety of mechanisms.

Among the various CLA isomers we have used thus far, there is no significant difference in terms of potency in inhibition of pro-inflammatory cytokines. However, in general, the potency of the parental compound linoleic acid was less than that of CLAs, especially in inhibition of IL-1β and IL-6 expression and in enhancing differentiation of monocytes (i.e. CD14 and CD36 expression). The unique structural properties of CLA contribute to the biological effects different from its parental compound, although we cannot rule out the possibility that CLA is metabolized to a more potent PPARγ ligand. Our data showed a similar level of potency of the potential CLA metabolite 8-(n-hexyl-2-furyl)-octanoic acid (Furan) with other CLA isomers with respect of the biological effects on macrophages. In addition to the anti-cancer, anti-atherogenic and anti-diabetic effects of CLA, in present study, we demonstrated that CLA acted as an antioxidant and identified another potential health benefit to humans who consume products rich in CLA. The results of these studies aid in the promotion of meat and dairy products and may be of therapeutic value in treatment of inflammatory diseases, such as inflammatory bowel disease and atherosclerosis in which activated macrophages play a critical role in pathogenesis.

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