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Contribution of Conjugated Linoleic Acid to the Suppression of Inflammatory Responses through the Regulation of the NF-κB Pathway

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Data from a number of researchers have shown that conjugated linoleic acid (CLA) has some beneficial health activities in animal models. Because inflammatory responses are associated with pathophysiology of many diseases, the aim of this study is to explore the effect and mechanism of CLA in the regulation of lipopolysaccharide (LPS)-induced inflammatory responses in RAW 264.7 macrophages. The addition of increasing levels of CLA proportionally augmented the incorporation of CLA in cultures. CLA diminished LPS-induced mRNA and protein expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2) as well as subsequent production of nitric oxide and prostaglandin E_2, respectively. We further examined the effect of CLA on LPS-induced NF-κB activation by Western blot and the electrophoretic mobility shift assay. The addition of CLA at 200 μM significantly diminished LPS-induced protein expression of the cytoplasmic phosphorylated inhibitor κBα and nuclear p65 as well as NF-κB nuclear protein−DNA binding affinity. In conclusion, our data suggest that CLA may inhibit LPS-induced inflammatory events in RAW 264.7 macrophages and this inhibitory activity of CLA, at least in part, occurs through CLA modulating the NF-κB activation and therefore negatively regulating expression of inflammatory mediators.

KEYWORDS: Conjugated linoleic acid; inducible nitric oxide synthase; cyclooxygenase 2; nuclear transcription factor-κB

INTRODUCTION

Three types of nitric oxide synthase (NOS) have been identified in mammalian cells. Two of these, endothelial NOS and neuronal NOS, constitutively express and catalyze relatively small amounts of nitric oxide (NO) synthesis associated with various physiological functions of the nervous and cardiovascular systems (*1*, *2*). The third NOS, the expression of which is induced in stimulated macrophages, neutrophils, and endothelial and smooth muscle cells, catalyzes large amounts of NO production and is named inducible NOS (iNOS). Long-term exposure to such high concentrations of NO is believed to be associated with inflammatory diseases such as rheumatoid arthritis, atherosclerosis, inflammatory bowel disease, septic shock, and glomerulonephritis (*3*−*8*). Like NOSs, cyclooxygenase (COX), the enzyme catalyzing the rate-limiting step of prostaglandin (PG) synthese from fatty acids, contains two isoforms, which are either constitutively expressed or induced in various tissues (*9*). COX1, present in most mammalian tissues, constitutively produces a low level of PGs, which is linked to maintenance of physiological homeostasis in blood flow, gastric secretions, blood platelet aggregation, etc. (*10*). In contrast to COX1, the expression of COX2 is hardly measurable under normal physiological conditions. On the other hand, COX2 can be induced by cytokines, bacterial endotoxins, growth factors, and phorbol esters (*11*) and subsequently catalyzes a large amount of PGE_2 production. In light of the data reported in the literature, expression of COX2 and iNOS is associated with not only chronic inflammatory diseases but also carcinogenesis (*12*−*14*).

In mammalian cells, members of the Rel/nuclear transcription factor-κ B (NF-κB) family of proteins, including p65 (RelA), p50/p105 (NF-κB1), p52/p100 (NF-κB2), RelB, and c-Rel, form homodimers or heterodimers and act as inducible transcription factors. Inappropriate activation of NF-κB at the site of inflammation has been found in diverse diseases, and it is well-recognized that activation of NF-κB can trigger inflammatory responses by transcriptional induction of several inflammatory mediators including iNOS and COX2 (*15*, *16*). It has been established that the activated NF-κB binding to a unique sequence termed NF-κBd in the iNOS promoter is crucial for the bacterial endotoxin lipopolysaccharide (LPS)-induced iNOS gene expression in mouse macrophages (*17*). It has also been

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noted that the COX2 promoter holds two separate NF-κB consensus sequences (*18*). In this regard, the data available support the idea that regulation of NF-κB activation induced by LPS is the major factor, if not all, modulating the expression of iNOS and COX2 and thus production of NO and PGE_2 in murine macrophages. Several agents have emerged as the basis for potential therapeutic approaches for reduction of inflammation because of their ability to modulate NF-κB activity and consequently regulate expression of inflammatory mediators (*14*).

The term conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of octadecadienoic acid with conjugated dienoic double bonds in Δ7,9; Δ9,11; Δ10,12; Δ8,-10; and Δ11,13 positions, in either cis and/or trans configurations (*19*, *20*). Animal products are the principal dietary sources of CLA. Ruminant meats (2.7−5.6 mg CLA/g fat) and dairy products are good dietary sources of CLA (2.9−7.1 CLA mg/g) while seafood and plant oils contain less CLA (0.1−0.7 CLA mg/g). Except for turkey, the meat from nonruminants is low in CLA content. Usually, the CLA content of foods can be increased by heat processing such as dairy pasteurization and pan-frying meat (*21*).

Although the molecular activities of CLA have not been completely documented yet, the beneficial impacts of CLA on disease prevention have been reported elsewhere. In animal model, CLA has been found as a chemopreventive agent in rat mammary tumorigenesis (*22*), rat colon carcinogenesis (*23*), mouse forestomach neoplasia (*24*), mouse prostate cancer (*25*), and mouse skin carcinogenesis (*26*, *27*). In addition, CLA has received much attention in modulating blood sugar and lipid homeostasis, as well as immune function (*28*−*32*). There is no doubt about the ability of CLA to modulate inflammatory responses, but the mechanisms underlying its effect have not been elucidated yet (*33*−*35*). For this purpose, we investigated the role of CLA in the LPS-induced proinflammatory events in RAW 264.7 macrophages and whether this modulation is effected through the regulation of LPS-induced NF-κB activation.

MATERIALS AND METHODS

Reagents. The mouse macrophage-like cell line RAW 264.7 was purchased form the American Type Culture Collection (Menassas, VA), and the fetal bovine serum was from the Biowest (France). RPMI 1640 Media and medium supplements for cell culture were obtained form Gibco BRL (Gaithersburg, MD). LPS was obtained from Sigma Chemical Company (St. Louis, MO), and the CLA mixture (99% purity) was from NuChek Prep, Inc. (Elysian, MN). The specific antibodies for iNOS, COX-2, phosphorylated inhibitor κBα (IκBα), and p65 were purchased form BD Biosciences (Franklin Lakes, NJ), Cayman Chemical Company (Ann Arbor, MI), Cell Signaling Technology, Inc. (Beverly, MA), and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Reagents such as enzymes, cofactors, and nucleotides for internal standard (IS) construction and reverse transcriptase polymerase chain reaction (RT-PCR) were from Promega Co. (Madison, WI) or Gibco BRL. Oligonucleotide primer sequences of genes for RT-PCR were selected by using Primer Select (DNASTAR, Madison, WI). The oligonucleotide primers for RT-PCR as well as the biotin-labeled double-stranded NF-κB consensus oligonucleotide, nonlabeled double-stranded NF-κB consensus oligonucleotide, and a mutant double-stranded NF-κB oligonucleotide for the electrophoretic mobility shift assay (EMSA) were synthesized by MDBio, Inc. (Taiwan). All other chemicals were of the highest quality available.

Cell Culture. The RAW 264.7 macrophages (passage levels between 8 and 13) were maintained in RPMI-1640 media supplemented with 2 mM L-glutamine, antibiotics (100 Unit/mL penicillin and 100 μg/mL streptomycin), and 10% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere of air/CO_2 95:5 (mol %). In this study, cells were plated at a density of 8×10^5 per 35 mm dish and incubated until 90% confluence was reached. For the cell viability assay, measurements of NO synthesis and of iNOS protein expression cells were treated with or without LPS (1 μg/mL) plus methanol vehicle control or 20−200 μM CLA for 18 h. For the remainder of the experiments in this study, cultures were treated with methanol or 20−200 μM CLA for 12 h prior to addition of LPS (1 μg/mL).

Cell Viability Assay. The mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2y-l)-2,5-diphenyltetrazoleum bromide (MTT) to formazan was used to measure the cell respiration as an indicator of cell viability (*36*). After the supernatants were removed for measurements of NO synthesis, cells were incubated in the RPMI medium containing 0.5 mg/mL MTT for 3 h at 37 °C and 5% CO_2 atmosphere. After the medium was aspirated, the 2-propanol was added into the cells to dissolve the formazan. The supernatant of each sample was transferred into 96 well plates and read at the 570 nm by VersaMax Tunable Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA). The absorbance in cultures treated with methanol vehicle control was used as 100% of cell viability.

Determination of Nitrite Synthesis. The nitrate in media was measured by the Griess assay (*37*) and was used as an indictor of NO synthesis in cells. Briefly, an equal volume of the culture supernatants and Griess solution ([1:1 mixture (v/v) of 1% sulfanilamide and 0.1% N-(naphthyl)ethyl-enediamine dihydrochloride in 5% H_3PO_4] was added into 96 well plates at room temperature for 10 min. The absorbances at 550 nm were measured by a VersaMax Tunable Microplate Reader and calibrated by using a standard curve of sodium nitrate prepared in culture media.

Determination of PGE_2 Synthesis. Cultures were treated with methanol or 20−200 μM CLA for 12 h prior to addition of LPS (1 μg/mL) for 6 h. The diluted culture supernatants were used to quantify PGE_2 by the enzyme immunoassay kit (Cayman Chemical Company) according to the protocol provided by the manufacturer.

Fatty Acid Analysis by Gas Chromatography. Cultures were treated with methanol or 20−200 μM CLA for 12 h. The medium was aspirated, and cultures were washed twice with phosphate-buffered saline and frozen at −70 °C until lipid extractions were performed. Thawed cells were scraped into methanol, and lipids were extracted by adding chloroform and 2 M KCl (*38*). Extracts were dried and resuspended with tetramethylguanidine to derive the fatty acid methyl esters of total lipid fractions. The fatty acid methyl esters were quantified by gas chromatography (G-3000, HITACHI, Japan) on a 30 m fused silica column with an internal diameter of 0.25 mm (Supelco, Bellefonte, PA). The flow rate of carrier gas, helium, was 30 mL/min, and the oven temperature was programmed to start at 150 °C for 8 min and then heated to 190 °C at a rate of 3 °C/min. Retention times of fatty acid methyl esters were compared with retention times of authentic standards in order to identify fatty acids.

RNA Isolation and Quantitative RT-PCR. Total RNA was isolated from cells by using Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH) as described by the manufacturer. RNA extracts were suspended in nuclease-free water and frozen at −70 °C until the RT-PCR analyses were performed.

The quantitative RT-PCR was accomplished by using recombinant RNA (rcRNA) templates as ISs to quantitatively monitor mRNA expression, as described previously (*39*). The basis for this method was that 0.1−0.25 μg of total RNA and varying amounts of rcRNA IS were reverse transcribed with M-MMLV reverse transcriptase in a 20 μL final volume of the reaction buffer consisting of 25 mM Tris-HCl (pH 8.3 at 25 °C), 50 mM $(NH_4)_2SO_4$, 0.3% β-mercaptoethanol, 0.1 mg/mL bovine serum albumin, 5 mM $MgCl_2$, and 1 mM each of deoxynucleotide triphosphate, 2.5 units RNase inhibitor, and 2.5 mM oligo $(dT)_{16}$. Each gene has its own specific rcRNA template, which contains a forward and reverse primer sequence for the target gene, and the procedure for generating the rcRNA template for use as an IS is performed as describe by Vanden Heuvel et al. (*40*). For the synthesis of complementary DNA, reaction mixtures were incubated for 15 min at 45 °C and stopped by denaturing the reverse transcriptase at 99 °C for 5 min. To these complementary DNA samples, PCR master mix containing 4 mM $MgCl_2$, 2.5 units Taq polymerase, and 6 pmol of forward and reverse primers was added to a total volume of 50 μL. The sequences for the RT-PCR primers are as follows: 5′-CAGTTCT-GCGCCTTTGCTCAT-3′ (forward) and 5′- GGTGGTGCGGCTG-GACTTT-3′ (reverse) for miNOS, 5′-CTGAAGCCCACCCCAAACA-3′ (forward) and 5′-AACCCAGGTCCTCGCTTATG-3′ (reverse) for mCOX2, and 5′-GACGTGCCGCCTGGAGAAA-3′ (forward) and 5′-GGGGGCCGAGTTGGGATAG-3′ (reverse) for glyceraldehydes-3-phosphate dehydrogense (GAPDH). The reactions of PCR amplification were heated to 94 °C for 3 min and immediately cycled 32 times through a 30 s denaturing step at 94 °C, a 30 s annealing step at optimal temperature (50−60 °C depending on primers used), and a 60 s elongation step at 72 °C. Following the final cycle, a 5 min elongation step at 72 °C was carried out. The amplified PCR products of the IS and target mRNA can be easily visualized and separated by 2.5% agarose (SeakemLE agarose, Biowhittaker Molecular Application, Rockland, ME) gel electrophoresis with ethidium bromide. Gels were photographed, and the intensity of the stained PCR fragments from photographs was quantified through densitometric analysis by Zero-Dscan (Scanalytics, Inc., FairFax, VA).

The amount of target mRNA present was quantified as follows. First, a range-finding study was set to determine the approximate optimum concentration of IS required to display a 1:1 intensity of IS:target mRNA PCR product. Then, RNA samples with a constant amount of optimal IS were examined in triplicate by RT-PCR. To make a standard curve, gradual concentrations of IS and constant concentrations of sample RNA were amplified, and the log(ratio of band intensity) vs log(IS added) was plotted. The ratio of target gene to IS mRNA intensity was used to quantify target gene mRNA level according to this standard curve (*39*).

Western Blot Analysis. Protein content in each sample was quantified by the Coomassie Plus Protein Assay Reagent Kit (Pierce Chemical Company, Rockford, IL). Protein aliquots were denatured and separated on 8−12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis gels and then transferred to poly(vinylidene difluoride) membranes (New Life Science Product, Inc., Boston, MA). The membranes were pretreated with a blocking buffer (3% bovine serum albumin in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20) to block the nonspecific binding sites. The blots were then incubated sequentially with primary antibodies and horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Bio-Rad, Hercules, CA). Immunoreactive protein bands were developed by using 3-3′diaminobenzrdine color-developing solution or enhanced chemiluminescence (ECL) kits (Amersham Life Sciences, Arlington Heights, IL) and then were quantified through densitometric analysis by Zero-Dscan.

Preparation of Nuclear Protein and EMSA. Cultures were treated with methanol or 200 μM CLA for 12 h prior to the addition of 1 μg/mL LPS for 1 h. Nuclear proteins were extracted by the NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce Chemical Company) and were frozen at −70 °C until the EMSA was performed.

The LightShift Chemiluminescent EMSA Kit from Pierce Chemical Co. and synthetic biotin-labeled double-stranded NF-κB consensus oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were used to measure the effect of CLA on NF-κB nuclear protein−DNA binding activity. Nuclear extract (2 μg), poly(dI-dC), and biotin-labeled double-stranded NF-κB oligonucleotide were mixed with the binding buffer (to a final volume of 20 μL) and were incubated at room temperate for 30 min. In addition, the nonlabeled and a mutant double-stranded NF-κB oligonucleotide (5′-AGTTGAGG*C*GACTTTCCCAGGC-3′) were employed to confirm the specific binding and protein binding specificity, respectively. The nuclear protein−DNA complex was separated by electrophoresis on a 6% TBE−polyacrylamide gel electrophoresis and then was eletrotransferred to nylon membrane (Hybond-N+, Amersham Pharmacia Biotech Inc., Pisscataway, NJ). Next, the membrane was treated with streptavidin−horseradish peroxidase and the nuclear protein−DNA bands were developed using Amersham ECL kits.

Statistical Analysis. Data were expressed as means ± SE from at least three independent experiments. Differences among treatments were analyzed by analysis of variance with Scheffe's multiple comparison test (α = 0.05) using the Statistical Analysis System (Cary, NC).

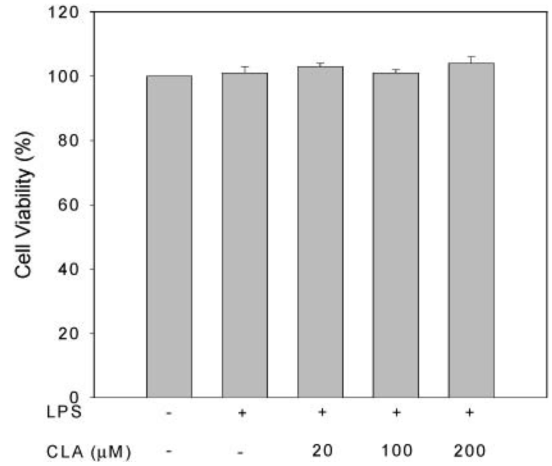

Figure 1. Effect of CLA on RAW 264.7 macrophage viability. Cultures were treated with or without LPS (1 μg/mL) in the absence or presence of CLA at various concentrations for 18 h, and the cell viability was measured by MTT assay. Data are the means ± SE of at least three separate experiments and are expressed as the percentage of methanol vehicle control.

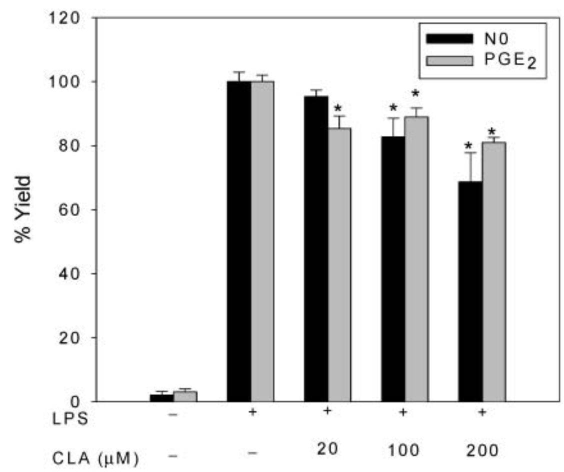

Figure 2. Effect of CLA on LPS-induced nitrite and PGE_2 production in RAW 264.7 macrophages. For nitrite assay, cultures were treated with 1μg/mL LPS alone or with various concentrations of CLA for 18 h. The levels of nitrite in the supernatant of RAW 264.7 macrophages were measured by Griess reaction. For PGE_2 assay, cultures were preincubated with or without various concentrations of CLA for 12 h and then treated with 1 μg/mL LPS for 12 h. The levels of PGE_2 in the supernatant of RAW 264.7 macrophages were measured by the enzyme immunoassay kit from Cayman Chemical Company. The levels of nitrite and PGE_2 are expressed as the percentage of maximal production observed with the LPS alone group. Data are the means ± SE of at least three separate experiments. An asterisk (*) indicates a significant difference from the LPS alone group ($P < 0.05$).

RESULTS

Effect of Exogenous CLA on Cell Viability and LPS-Induced Nitrite and PGE_2 Synthesis. To examine whether the amount of CLA used in this study caused cell toxicity, we used the MTT assay (**Figure 1**). Our results indicated that a concentration of CLA up to 200 μM had no adverse effects on the growth of RAW 264.7 macrophages in the presence of LPS.

The addition of LPS stimulated RAW 264.7 macrophages to cause a substantial release of nitrite and PGE_2 as compared with the methanol vehicle control (**Figure 2**, respectively). CLA treatments, especially at 200 μM, significantly reduced the LPS-

Table 1. Effect of Exogenous CLA on Lipid Composition of RAW 264.7 Macrophages[a]

fatty acid	exogenous fatty acid treatment group			
	control	CLA (20 μM)	CLA (100 μM)	CLA (200 μM)
14:0	2.35 ± 0.09	2.66 ± 0.10	1.75 ± 0.27	1.70 ± 0.01
16:0	33.62 ± 0.63	31.56 ± 1.15	21.10 ± 0.78*	15.80 ± 0.34*
18:0	26.64 ± 0.11	26.71 ± 1.05	19.58 ± 1.37*	15.19 ± 0.08*
18:1;9	18.71 ± 1.95	15.26 ± 0.67	9.72 ± 1.03*	7.31 ± 0.99*
LA	2.92 ± 0.9	2.24 ± 0.11	1.89 ± 0.07	1.34 ± 0.47
CLA	0.0 ± 0.0[b]	6.66 ± 0.02*	34.29 ± 1.75*	48.31 ± 1.11*
20:4; 5, 8, 11, 14	15.75 ± 0.43	14.88 ± 0.82	11.62 ± 1.10*	10.31 ± 0.07*

[a] Values are means ± SE expressed as percent total fatty acid. $N = 3$ dishes per treatment group. Means with an asterisk (*) within the same row were significantly different as compared with control ($P < 0.05$). [b] Not detected in measurable quantities and estimated to account for less than 0.09% total fatty acid.

induced nitrite and PGE_2 production ($P < 0.05$). The inhibitory effect of CLA treatments on LPS-induced nitrite and PGE_2 synthesis was not due to lessening of cell viability.

Effect Of Exogenous CLA on Fatty Acid Composition of RAW 264.7 Macrophages. The amount of CLA (isomers 9,-11 and 10,12) in the total cellular lipid was elevated in a dose-dependent manner by increasing the level of exogenous CLA (**Table 1**). Accompanied by an increasing amount of CLA in cells, the compositions of other fatty acids in the RAW 264.7 macrophage lipid pool were reduced. Increasing the exogenous CLA to 100 or 200 μM significantly reduced the amount of palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1; n9), and arachidonic acid (20:4; n6) in cellular lipids ($P < 0.05$). Although there was no significant difference statistically, the level of cellular linoleic acid (18:2; n6) also became reduced as increasing amounts of CLA were added to cultures.

Effect of Exogenous CLA on LPS-Induced mRNA Expression of iNOS and COX2. The levels of iNOS, COX2, and GAPDH mRNA expression were measured by quantitative RT-PCR. IS was used in competitive RT-PCR to quantify target gene mRNA expression and to minimize the tube to tube variation in an RT-PCR reaction. The mRNA expression of the housekeeping gene, GAPDH, was not influenced by LPS or CLA treatments (data not shown).

In the resting RAW 264.7 macrophages, the expression of iNOS and COX2 mRNA was hardly detectable while it was dramatically induced in cultures treated with LPS. The LPS-induced expression of iNOS and COX2 mRNA was significantly decreased in cultures treated with LPS plus CLA (**Figure 3**, respectively; $P < 0.05$). The expression of iNOS and COX2 mRNA in cultures treated with LPS plus 200 μM CLA was only one-third of that in cultures treated with LPS alone.

Expression of iNOS, COX2, Cytoplasmic Phosphorylated IκBα and Nuclear p65. There was no difference in the protein expression of the internal control, α-tubulin, observed among the different treatments (data not shown). In the resting RAW 264.7 macrophages, the protein expression of iNOS and COX2 was hardly detectable or undetectable and the CLA treatments did not influence the basal level of iNOS and COX2 protein expression (**Figure 4A**,**B**, respectively). On the other hand, the LPS treatment activated RAW 264.7 macrophages and drastically increased the levels of iNOS and COX2 protein expression. The addition of exogenous CLA significantly declined LPS-induced iNOS and COX2 protein expression.

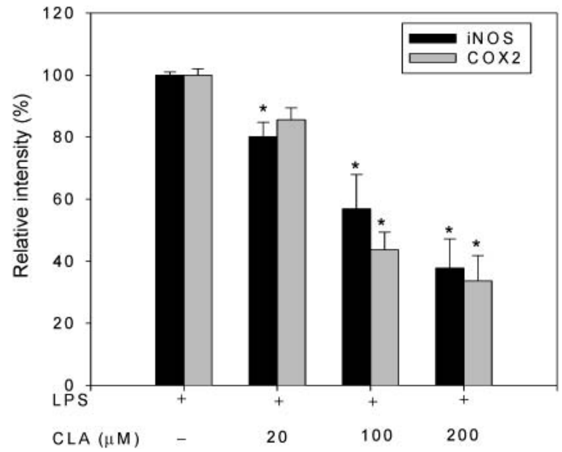

Figure 3. Effect of CLA on LPS-induced expression of iNOS and COX2 mRNA in RAW 264.7 macrophages. For iNOS mRNA expression, cultures were preincubated with or without various concentrations of CLA for 12 h and then treated with 1 μg/mL LPS for 6 h. For COX2 mRNA expression, cultures were preincubated with or without various concentrations of CLA for 12 h and then treated with 1 μg/mL LPS for 3 h. Total RNA was isolated by TRI reagent, and the expression of iNOS and COX2 mRNA was analyzed by quantitative RT-PCR. The expression of iNOS and COX2 mRNA is expressed as the percentage of maximal expression observed with the LPS alone group. Data are the means ± SE of at least three separate experiments. An asterisk (*) indicates a significant difference from the LPS alone group ($P < 0.05$).

Upon LPS treatment, the amount of cytoplasmic phosphorylated IκBα protein increased while the addition of CLA at 200 μM significantly decreased the LPS-induced phosphorylated IκBα protein expression (**Figure 5A**). Note also that the amount of p65 protein in the nuclear fraction of RAW 264.7 macrophages treated with LPS alone was significantly higher than in the cultures incubated with CLA at 200 μM for 12 h prior to stimulation with LPS (**Figure 5B**).

Effect of CLA on LPS-Induced NF-κB Nuclear Protein–DNA Binding Activity. To explore the mechanism of CLA-mediated inhibition of iNOS and COX2 mRNA transcription, EMSA was performed to assay whether CLA could repress NF-κB nuclear protein–DNA binding activity in RAW 264.7 macrophages. Upon treatment with LPS, the DNA binding activity of NF-κB nuclear protein was markedly increased (**Figure 6**, lane 2) as compared to the methanol vehicle control treatment (**Figure 6**, lane 1). Moreover, the band had completely vanished after the addition of excess nonlabeled double-stranded NF-κB consensus oligonucleotide (**Figure 6**, lane 5). In contrast, only a minor change was seen in the DNA binding of NF-κB when mutant double-stranded NF-κB oligonucleotide was added (**Figure 6**, lane 6). These two pieces of data emphasize the specificity of the NF-κB nuclear protein–DNA binding reaction. The reduction of LPS-induced NF-κB nuclear protein–DNA binding activity was found in cultures pretreated with 200 μM CLA (**Figure 6**, lane 3).

DISCUSSION

The unique health benefit properties of CLA have been addressed in numerous studies (*41*). In the present study, we first reported that addition of exogenous CLA in concentrations ranging from 20 to 200 μM had a cytotoxic effect on RAW 264.7 macrophages. We then demonstrated that in RAW 264.7 macrophages, CLA significantly reduced the mRNA and protein expression of iNOS and COX2 induced by LPS and subsequent NO and PGE_2 synthesis, respectively. Furthermore, our data

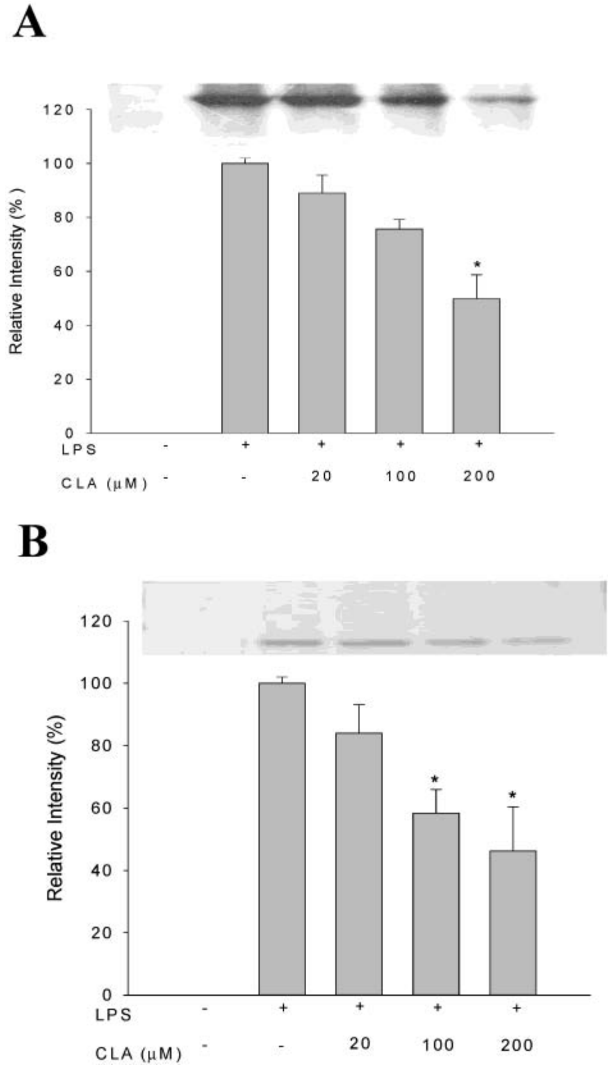

Figure 4. Effect of CLA on LPS induced the protein expression of iNOS and COX2 in RAW 264.7 macrophages. (**A**) For iNOS protein expression, cultures were treated with or without 1 μg/mL LPS in the absence or presence of CLA at various concentrations for 18 h. (**B**) For COX2 protein expression, cultures were preincubated with or without various concentrations of CLA for 12 h and then treated with either vehicle control or 1 μg/mL LPS for 6 h. The whole cell lysates were used to analyze the protein content of iNOS and COX2 by Western blot. The protein expression of iNOS and COX2 was detected by Western blot with anti-iNOS and anti-COX2 antibody, respectively. The relative protein levels of iNOS and COX2 were quantified by scanning densitometry (Zero-Dscan) of the band intensities in immunoblots. The protein expression of iNOS and COX2 is expressed as the percentage of maximal expression observed with the LPS alone group. Data are the means ± SE of at least three separate experiments. An asterisk (*) indicates a significant difference from the LPS alone group ($P < 0.05$).

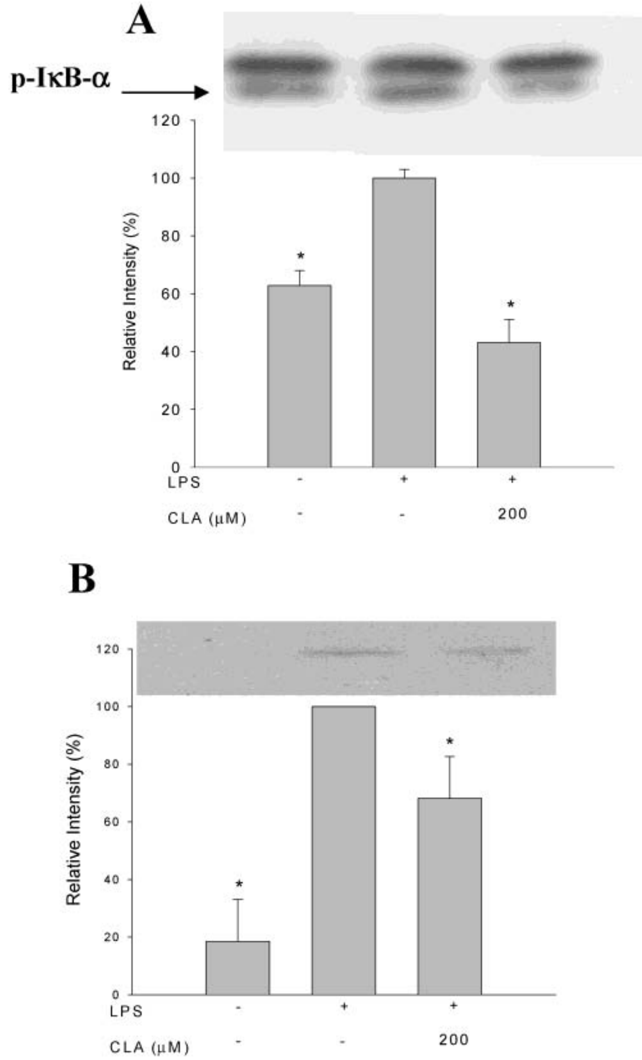

Figure 5. Effect of CLA on LPS induced protein expression of phosphorylated IκBα and p65 in the nuclear portion of RAW 264.7 macrophages. (**A**) For phosphorylated IκBα protein expression, cultures were preincubated with or without 200 μM CLA for 12 h and then treated with either vehicle control or 1 μg/mL LPS for 30 min. The cytoplasmic protein factions were used to analyze the content of phosphorylated IκBα protein by Western blot. (**B**) For p65 protein expression, cultures were preincubated with or without 200 μM CLA for 12 h and then treated with either vehicle control or 1 μg/mL LPS for 1 h. The nuclear protein fractions were used to analyze the content of p65 protein by Western blot. The protein expression of phosphorylated IκBα protein and p65 was detected by antiphosphorylated IκBα and p65 antibody, respectively. The relative protein levels of phosphorylated IκBα and p65 were quantified by scanning densitometry (Zero-Dscan) of the band intensities in immunoblots. The protein expression of phosphorylated IκBα and p65 is expressed as the percentage of maximal expression observed with the LPS alone group. Data are the means ± SE of at least three separate experiments. An asterisk (*) indicates a significant difference from the LPS alone group ($P < 0.05$).

have shown that the underlying antiinflammatory mechanisms of CLA are due, at least in part, to negative regulation of the LPS-induced NF-κB activation.

From the data of gas chromatography analysis, we have stated here that addition of increasing levels of CLA augmented the incorporation of CLA in cultures proportionately. Moreover, increasing the exogenous CLA concentration from 20 to 200 μM significantly reduced the amount of AA in cellular lipids but did not change the LA content of cells. The manner of dietary CLA incorporation into the total lipid pool in RAW 264.7 macrophages was similar to that observed in the murine keratinocyte, HEL-30 (*37*). Previous studies have demonstrated that CLA-mediated alteration of the fatty acid composition of the cellular lipid pool could influence the fatty acid metabolism and eicosanoid synthesis, which are involved in tumor promoter-induced morphological and biochemical changes in mouse epidermis and keratinocytes (*37, 42*). However, little is known about the influence of the alteration of fatty acid composition

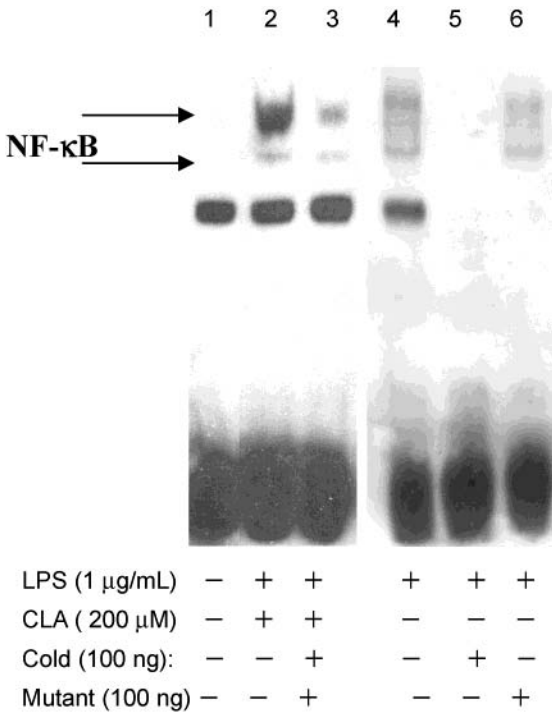

Figure 6. Effect of CLA on LPS induced NF-*κ*B nuclear protein DNA binding activity in RAW 264.7 macrophages. Cultures were preincubated with or without different concentrations of CLA for 12 h and then treated with either vehicle control or 1 *μ*g/mL LPS for 1 h. Nuclear extracts were used to measure the NF-*κ*B nuclear protein DNA binding activity by EMSA. Unlabeled double-stranded NF-*κ*B oligonucleotide (100 ng) was added for the competition assay, and unlabeled double-stranded mutant NF-kB oligonucleotide (100 ng) was added for the specificity assay. Bands for NF-*κ*B nuclear protein–DNA binding were detected by using a streptavidin–horseradish peroxidase and developed by using Amersham ECL kits from Amersham Life Sciences.

in the RAW 264.7 cellular lipid pool by exogenous CLA on the potency of CLA-modulating inflammatory responses.

It is well-established that overexpression of NO and PGE_2 plays a pivotal role in inflammatory responses. The results of the present study and of recent reports (*34*, *35*) have demonstrated that in RAW 264.7 macrophages, CLA decreased LPS or interferon-*γ* (IFN*γ*)-induced NO and PGE_2 synthesis. In addition to RAW 264.7 macrophages, CLA reduced PGE_2 synthesis in rat serum (*32*) and bone (*43*), as well as in sensitized guinea pig trachea (*44*). On the other hand, the ability of CLA to decrease NO synthesis has only been found in RAW 264.7 macrophages so far. In addition to reducing proinflammatory product synthesis in RAW 264.7 macrophages, our data have also established that CLA significantly reduces LPS-induced iNOS and COX2 mRNA expression, which is consistent with the findings of Iwakiri et al. (*35*). In our subsequent experiments, we have also found that CLA dramatically decreases LPS-induced protein expression of iNOS and COX2. On the basis of the above data, we suggest that the effect of CLA in decreasing LPS-induced NO and PGE_2 synthesis is due to CLA diminishing the mRNA and protein expression of iNOS and COX2, respectively.

Recently, the effect of CLA on modulating gene expression has gained a great deal of attention in investigation of the molecular mechanism of CLA. A series of reports from the laboratory of Martha A. Belury have shown that CLA can activate peroxisome proliferator-activated receptors (PPARs) and induces the expression of PPAR response genes (*41*). Yu et al. showed that transfecting RAW 264.7 macrophages with PPAR-*γ* dominant negative plasmid could block CLA, reducing the INF*γ*-induced transcriptional activity of iNOS promoter (*34*). Because no PPAR response element exists in the promoter region of iNOS, it is possible that PPAR-*γ* dominant negative protein interferes with the INF*γ*-induced binding ability of NF-*κ*B to iNOS promoter. NF-*κ*B is ubiquitously expressed in most eukaryotes and is sequestered in the cytoplasm of unstimulated cells by noncovalently binding to a member of inhibitor proteins termed I*κ*B (α, *β*, or *ϵ*) (*45*). Exposure of cells to external stimuli such as inflammatory cytokines, oxidative stress, ultraviolet irradiation, or bacterial endotoxins (*46*, *47*) results in NF-*κ*B activation and then induction of the expression of specific cellular genes associated with host inflammatory and immune responses (*48*), as well as cellular growth properties (*49*). In macrophages, the bacterial endotoxin LPS can induce NF-*κ*B activation by stimulating phosphorylation and degradation of I*κ*Bα (*50*). Then, the activated NF-*κ*B is translocated into the nucleus, thereby binding to the cis-acting *κ*B enhancer element of target genes and activating expression of proinflammatory mediators including iNOS and COX2 (*17*, *18*). Our data have demonstrated that CLA significantly reduces LPS-induced protein expression of cytoplasmic phosphorylated I*κ*Bα and nuclear p65. This result agrees with the finding that NF-*κ*B nuclear protein−DNA binding affinity was significantly attenuated by pretreatment of CLA. It is well-established that the activation of NF-*κ*B is redox sensitive and can be blocked by antioxidant (*51*). Of interest is the opposite effect on oxidation between two main isomers of a CLA mixture. At relatively low concentrations (2 and 20 *μ*M), c9,t11 CLA and t10,c12 CLA possess the antioxidant properties. On the other hand, at a concentration of 200 *μ*M c9,t11 CLA behaves as a strong prooxidant while t10,c12 CLA has antioxidant activity (*52*). Further studies are required to determine whether the oxidation capacity of a CLA mixture influences CLA-modulating activation of NF-*κ*B by LPS.

To our knowledge, this is the first report to show that CLA, at noncytotoxic doses, can modulate LPS-induced NF-*κ*B activation, NF-*κ*B nuclear protein−DNA binding activity, and NF-*κ*B-dependent inflammatory mediator expression. Highly increased activation of NF-*κ*B-inducing inflammatory events is involved in the initiation and progression of diverse diseases. In this regard, control of NF-*κ*B activation, which is associated with regulation of inflammatory mediator expression, could become a promising new target for the design of antiinflammatory drugs. Thus, it would be worthwhile to explore the biomedical importance of dietary CLA in the treatment and prevention of inflammation. Moreover, because inflammation has been documented as a risk factor in carcinogenesis (*12*), it would be of interest to study the importance of CLA in modulating the NF-*κ*B activation on the chemopreventive characteristic of CLA.

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