10t,12c-conjugated linoleic acid inhibits lipopolysaccharide-induced cyclooxygenase expression in vitro and in vivo

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Abstract   Previous data demonstrated that conjugated lino-leic acid (CLA) reduced eicosanoid release from select organs. We hypothesized that one active CLA isomer was responsible for the reduced prostaglandin release and that the mechanism was through the inhibition of inducible cyclooxygenase-2 (COX-2). Here, we examined the effects of 10t,12c-CLA and 9c,11t-CLA on COX-2 protein/mRNA expression, prostaglandin E_2 (PGE_2) production, and the mechanism by which CLA affects COX-2 expression and prostaglandin release. The COX-2 protein expression level was inhibited 80% by 10t,12c-CLA and 26% by 9c,11t-CLA at 100 μM in vitro. PGE_2 production was decreased from 5.39 to 1.12 ng/2 × 10^6 cells by 10t,12c-CLA and from 5.7 to 4.5 ng/2 × 10^6 cells by 9c,11t-CLA at 100 μM. Mice fed 10t,12c-CLA but not 9c,11t-CLA were found to have a 34% decrease in COX-2 protein and a 43% reduction of PGE_2 release in the lung. 10t,12c-CLA reduced COX-2 mRNA expression level by 30% at 100 μM in vitro and by 30% in mouse lung in vivo. Reduced COX-2 mRNA was attributable to an inhibition of the nuclear factor κB (NF-κB) pathway by 10t,12c-CLA.   These data suggested that the inhibition of NF-κB was one of the mechanisms for the reduced COX-2 expression and PGE_2 release by 10t,12c-CLA.—Li, G., D. Barnes, D. Butz, D. Bjorling, and M. E. Cook. **10t,12c-conjugated linoleic acid inhibits lipopolysaccharide-induced cyclooxygenase expression in vitro and in vivo.** *J. Lipid Res.* 2005. **46:** 2134–2142.

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Chronic inflammation is an essential step in the progression of many diseases, such as atherosclerosis (1), cancer (2), and neurodegenerative diseases (3). Inhibition of inflammation by targeting proinflammatory enzymes and cytokines has been repeatedly shown to be beneficial in the prevention and/or treatment of a broad range of diseases. Although novel synthetic inhibitors of proinflammatory enzymes and cytokines are effective in slowing the development of inflammatory diseases, side effects significantly limit their use (4). Hence, natural dietary substances that possess anti-inflammatory properties will be of potential value. Conjugated linoleic acid (CLA), a naturally occurring fatty acid found in ruminant animal fat, has been shown to be anticarcinogenic (5–7) and antiatherogenic (8–10). CLA is a collective term referring to a group of positional and geometrical isomers of *cis*-9,*cis*-12 linoleic acid (LA), of which 10t,12c-CLA and 9c,11t-CLA have received the most study. Of these two isomers, 9c,11t-CLA is the most abundant naturally occurring isomer, accounting for >75% of the total CLA in dairy products (11). However, although the 10t,12c-CLA content in food is low, the amount is biologically relevant (as low as 0.017 g of 10t,12t-CLA per 100 g of diet) (12).

CLA has also been reported to influence immune/inflammatory responses. Dietary CLA was shown to prevent body weight loss induced by lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), or Sephadex (13–15) and to inhibit nitric oxide production and TNF-α release (16). Recent work has demonstrated that dietary CLA inhibited antigen-induced eicosanoid release in a type I hypersensitivity guinea pig model (17, 18). Mixed or purified isomers of CLA have also been demonstrated to inhibit inducible isoforms of cyclooxygenase (COX) mRNA and protein expression in vitro (19–21).

COX is the rate-limiting enzyme in the conversion of arachidonic acid to thromboxanes and prostaglandins. The enzyme exists in two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most cell types and is thought to be responsible for the maintenance of gastrointestinal

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Abbreviations: CLA, conjugated linoleic acid; COX, cyclooxygenase; EIA, enzyme immunoassay; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1, interleukin-1; iNOS, inducible nitric oxide synthase; LA, linoleic acid; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; PGE_2, prostaglandin E_2; TNF-α, tumor necrosis factor-α.

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mucosa, kidney, platelet function, and other housekeeping functions. Although existing constitutively in some tissues, such as brain and kidney, COX-2 is a highly inducible isoform whose expression is regulated by many growth factors, LPS, and cytokines such as interleukin-1 (IL-1), IL-2, and TNF-α (22). Because COX-2 has been shown to play a significant role in many inflammatory diseases, it has been an important pharmacological target for the prevention and/or treatment of arthritis and cancer (23).

One pathway leading to the transcription of COX-2, along with other proinflammatory enzymes and cytokines, such as inducible nitric oxide synthase (iNOS) and TNF-α, is the nuclear factor κB (NF-κB)-dependent pathway (24). NF-κB is a multiple-subunit transcription factor whose activation is involved in carcinogenesis, atherosclerosis, and rheumatoid arthritis (25, 26). Currently, NF-κB family members include p50/p105, p52/p100, c-Rel, RelB, and p65. In resting cells, NF-κB is sequestered in the cytoplasm by the inhibitory proteins IκBα, IκBβ, and IκBε. Upon stimulation by cytokines or LPS, IκBα is phosphorylated and then degraded in a ubiquitin-proteosome-dependent manner. NF-κB is liberated and translocates to the nucleus, where it activates the gene transcription of proinflammatory mediators. Thus, NF-κB represents a central mediator in inflammatory responses (25, 26). It has been demonstrated that nonsteroidal anti-inflammatory drugs such as aspirin (27), steroidal anti-inflammatory drugs such as dexamethasone (28, 29), and fatty acids such as docosahexaenoic acid and eicosapentenoic acid (30) exert anti-inflammatory responses by inhibiting the activation of NF-κB.

Based on the previous results with CLA, this study was designed to investigate the isomer-specific effect of CLA on COX-2 mRNA and/or protein inhibition and whether reduced COX-2 protein expression was attributable to the inhibitory effects on the NF-κB pathway. We found that 10t,12c-CLA was the isomer that inhibited COX-2 protein, mRNA level, and prostaglandin E_2 (PGE_2) release both in vitro and in vivo. The other isomer, 9c,11t-CLA, was only effective at 100 μM in vitro and was not effective in vivo. The 10t,12c-CLA isomer was also found to inhibit IκBα phosphorylation and to reduce the activated level of NF-κB binding to its consensus site in DNA, suggesting an NF-κB-dependent molecular mechanism.

MATERIALS AND METHODS

Materials

LA (>99%) and 10t,12c-CLA and 9c,11t-CLA isomers (>98%) for in vitro studies were purchased from Matreya, Inc. (Pleasant Gap, PA). CLA isomers for feeding trials were from Natural Lipid, Inc. (Hovdebygda, Norway). The composition of the Natural Lipid 10t,12c-CLA isomer was 0.4% oleic acid, 3.7% 9c,11t-CLA, 92.5% 10t,12c-CLA, and ∼3.4% other fatty acids. The composition of the Natural Lipid 9c,11t-CLA isomer was 4.5% oleic acid, 90.3% 9c,11t-CLA, 2.4% 10t,12c-CLA, and 2.8% other fatty acids.

The PGE_2 enzyme immunoassay (EIA) kit and COX-2 antibody were purchased from Cayman Chemical (Ann Arbor, MI). IκBα and phosphorylated IκBα antibodies were purchased from Cell

Signaling Technology (Beverly, MA). The TransAM NF-κB DNA binding ELISA kit was from Active Motif (Carlsbad, CA). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and β-actin antibodies were from Trevigen (Gaithersburg, MD) and Sigma (St. Louis, MO), respectively. LPS (*Escherichia coli* O55:B5) was from Sigma. Premium FBS was from Biowhittaker (Walkersville, MD). LR White resin was from London Resin Co. Ltd. (Berkshire, UK). Alexa Fluor 488 goat anti-rabbit IgG was from Molecular Probes (Eugene, OR). BALB/c mice were from Jackson Laboratory (Bar Harbor, ME).

Cell culture

The Raw264.7 macrophage cell line was maintained in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 incubator. Cells (1×10^6/well) were plated onto 24-well plates. When cells were confluent, free fatty acids dissolved in ethanol, LA, 10t,12c-CLA, 9c,11t-CLA (3.3, 10, 33, and 100 μM), or ethanol were added to DMEM containing 0.5% FBS. Twenty-four hours later, LPS (100 ng/ml or 1 μg/ml) was introduced into the cell culture for various lengths of time. Cell viability was >96% in LA- and CLA-treated cells, and total protein concentration was not affected by LA or CLA treatment (data not shown). The highest ethanol concentration in each sample was <0.19%.

Western blot

Confluent Raw264.7 macrophages were pretreated with CLA isomers for 24 h and then stimulated with LPS for the indicated time for each experiment. Cells were then lysed with radioimmunoprecipitation assay buffer containing proteinase and phosphatase inhibitors (1 mM phenylmethylsulfonyl, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM Na_3VO_4, and 1 mM NaF). Protein concentration was measured by the Bradford assay (31). Denatured proteins were separated with 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. After nonspecific blocking, membranes were incubated with primary COX-2, IκBα, or phosphorylated IκBα antibody and then with a horseradish peroxidase-coupled secondary antibody before being exposed to Fuji medical X-ray film. A preliminary study was conducted to optimize the linear relationship between the band area and the amount of loaded protein. For COX-2, percentage of control was calculated as % control = (COX-2 band area with fatty acids/COX-2 band area with ethanol) × 100. Cell culture medium for the analysis of COX-2 was also collected for PGE_2 release analysis. For IκBα and phosphorylated IκBα, % positive control = (band area in fatty acids and LPS-treated sample/band area in ethanol- and LPS-treated sample) × 100. Lung tissue was ground in liquid nitrogen, and ∼100 μg of powder was dissolved in 300 μl of radioimmunoprecipitation assay buffer with proteinase and phosphatase inhibitors as described above. The powder was further homogenized in the ice bath with an Ultra-turrax homogenizer and then centrifuged at 10,000 *g* for 10 min. The protein concentration in the supernatant was measured by the Bradford assay (31), and Western blotting was conducted as described above. G3PDH or β-actin was also measured as a loading control. The supernatant was also used for PGE_2 analysis.

PGE_2 analysis

PGE_2 was determined using EIA kits obtained from Cayman Chemical. Procedures were followed as indicated in the kit instructions.

Animal treatment

Animal experimentation was approved by the Animal Care Committee of the University of Wisconsin-Madison College of Agricul-

tural and Life Sciences. Two separate feeding trials were conducted to examine the isomer-specific effects of CLA on COX-2 mRNA and protein level. For each trial, 24 BALB/c male mice (4 weeks old) were randomly assigned to four groups: Corn Oil (CO), CO + LPS, 9c,11t-CLA + LPS, and 10t,12c-CLA + LPS. The mice were fed a basal diet (99% semipurified diet + 1% CO) for at least 2 days and then placed on their respective diet treatment for at least 21 days. Diet composition is shown in **Table 1**. At the end of feeding, mice were injected intraperitoneally with LPS (1 mg/kg body weight) or PBS (vehicle). The mice were euthanized with CO_2 after LPS injection, and lungs were dissected and snap-frozen for mRNA isolation and Western blot or placed in PBS for immunocytochemical analysis of COX-2. PGE_2 production from lung homogenates was measured by EIA.

Immunocytochemistry

The procedure followed has been described elsewhere (32). Briefly, the lung was rinsed with PBS, fixed in 3% paraformaldehyde for 1.5 h, washed in 0.1 M glycine in PBS, dehydrated with graded concentrations of ethanol, and incubated with LR White resin. Sections (1 μm thick) were blocked with 5% BSA and then incubated with COX-2 antibody for 1.5 h. Bound antibodies were detected with fluorescent secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG incubated for 1.5 h). Sections were washed with PBS three times and then stored at 4°C until observation by light microscopy with the appropriate filter settings (AxioCam HRm, HBO 100).

Isolation of mRNA and RT-PCR

Confluent cells were treated with CLA isomers for 24 h and then incubated with LPS for 4 h (COX-2) or 6 h (iNOS). mRNA was isolated according to procedures described by Diaz, Crenshaw, and Wiltbank (33). Briefly, cells were dissolved in 100 μl of lysis buffer (4 M guanidium isothiocyanate, 0.5% sarcosyl, 10 mM Tris-HCl, pH 8.0, and 1% dithiothreitol) and passed through a 25 gauge needle to shear DNA. Binding buffer (200 μl; 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 400 mM NaCl) was added, and samples were centrifuged. Supernatants were incubated with Magnetight™ oligo(dT) magnetic particles for at least 5 min. The particles were washed four times with wash buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), and the associated mRNA was rinsed off the beads with 10 μl of elution buffer (2 mM EDTA). Isolated mRNA (2 μl) was reverse-transcribed in the master mix (4.0 μl of reaction buffer, 1 μl of 100 μM random primer, 0.4 μl of 10 mM deoxynucleotide triphosphate, 0.2 μl of reverse transcriptase, and 12.4 μl of water). Polymerase chain reaction was used to amplify individual genes using specific primers. mRNA for the G3PDH gene was amplified as an internal control. Their respective primers were as follows: COX-2 upstream, 5′-CAA-GCA-GTG-GCA-AGG-CCT-CCA-3′; COX-2 downstream, 5′-GGC-ACT-TGC-ATT-GAT-GGT-GGC-T-3′; iNOS upstream, 5′-GGC-TTG-CCC-CTG-GAA-GTT-TCT-CTT-CAA-AGT-C-3′; iNOS downstream, 5′-AAG-GAG-CCA-TAA-TAC-TGG-TTG-ATG-3′; G3PDH upstream, 5′-GGC-ATT-CTC-GGC-TAC-ACT-GA-3′; G3PDH downstream, 5′-CAT-ACC-AGG-AAA-TTA-GCT-TGA-C-3′.

Samples from mouse lung were homogenized in lysis buffer solution, and mRNA was then similarly isolated and amplified as those from Raw264.7 macrophages. Amplification products were separated with 5% PAGE. Band area was analyzed by Quantity One software. Band area was shown to be proportional to the amount of cDNA within the amplification cycles. For mRNA level from Raw264.7 macrophages, percentage control was derived by first normalizing the band area of the amplification product to the band area of its corresponding G3PDH, then dividing the results by the ratio obtained from the positive control and multiplying by 100. mRNA level from lung tissue were directly normalized with its corresponding area of G3PDH.

Determination of NF-κB binding by DNA binding ELISA

Confluent Raw264.7 macrophages were incubated with LA or 10t,12c-CLA (50 or 100 μM) in DMEM containing no FBS. After 24 h, cells were incubated with 1 μg/ml LPS for 4 h. Cells were then harvested for the determination of NF-κB (P50 and P65) binding by ELISA according to the instructions in the TransAM NF-κB DNA binding ELISA kit. In brief, activated P65 and/or P50 binds to its consensus site (5′-GGGACTTTCC-3′) immobilized on the wells. The primary antibody of P65 or P50 recognizes and binds to p65 or p50 only when it is activated and bound to its consensus site. HRP-conjugated secondary antibody converts its substrates for the final determination by spectrophotometry.

Statistical analysis

Data were analyzed with Student's t-test. Differences were considered significant at $P < 0.05$.

RESULTS

Effect of 10t,12c-CLA and 9c,11t-CLA on COX-2 protein expression and PGE_2 production in vitro

COX-2 protein was not detectable in the resting Raw264.7 macrophages. Upon stimulation by LPS, COX-2 protein

TABLE 1. Diet composition

| Ingredient (g/100 g)	Experimental Groups			
	CO	CO + LPS	10t,12c-CLA + LPS	9c,11t-CLA + LPS
Sucrose	47.6	47.6	47.6	47.6
Casein	21	21	21	21
Corn starch	15	15	15	15
Corn oil	6	6	5.75	5.75
Cellulose	5	5	5	5
AIN-76 mineral mix	3.5	3.5	3.5	3.5
AIN-76 vitamin mix	1	1	1	1
Calcium carbonate	0.4	0.4	0.4	0.4
DL-Methionine	0.3	0.3	0.3	0.3
Choline bitartrate	0.2	0. 2	0.2	0.2
Ethoxyquin	0.001	0.001	0.001	0.001
10t,12c-CLA	—	—	0.25	—
9c,11t-CLA	—	—	—	0.25

CLA, conjugated linoleic acid; LPS, lipopolysaccharide.

was significantly induced. After Raw264.7 macrophages were incubated with LA, 10t,12c-CLA, or 9c,11t-CLA isomers for 24 h and stimulated with LPS (100 ng/ml) for 8 h, cell viability (assessed by trypan blue exclusion) exceeded 96%. 10t,12c-CLA decreased COX-2 protein expression significantly. When macrophages were treated with 100 μM 10t,12c-CLA, COX-2 protein levels were only ∼21.6% of the control values (0.19% ethanol + LPS). 9c,11t-CLA also significantly inhibited COX-2 protein expression, decreasing expression to 74.3% of control levels at 100 μM. However, LA did not inhibit COX-2 protein expression even at 100 μM (**Fig. 1A**, **B**).

LPS-induced PGE$_2$ production by Raw264.7 macrophages in the presence of LA, 10t,12c-CLA, or 9c,11t-CLA isomers was also measured (Fig. 1C). It was found that PGE$_2$ release by macrophages treated with 10t,12c-CLA was inhibited in a concentration-dependent manner. PGE$_2$ release by cells treated with 0, 3.3, 10, 33, or 100 μM 10t,12c-CLA was 5.39, 4.65, 3.28, 2.83, or 1.12 ng/2 × 10^6 cells, respectively. 9c,11t-CLA decreased PGE$_2$ production only at 100 μM (PGE$_2$ production was reduced from 5.7 to 4.5 ng/2 × 10^6 cells). In contrast, PGE$_2$ production was

dose-dependently increased by LA and was 5.8, 5.7, 7.3, 8.1, and 11.9 ng/2 × 10^6 cells for 0, 3.3, 10, 33, and 100 μM LA (Fig. 1C).

Effect of 10t,12c-CLA and 9c,11t-CLA on COX-2 protein expression and PGE$_2$ production in vivo

To test whether COX-2 expression and PGE$_2$ production were also decreased in vivo, mice were fed purified CLA isomers. In a preliminary trial, COX-2 protein expression was detected in the lung (**Fig. 2A**) but not in the spleen (data not shown) 8 h after mice were injected with LPS. Although 9c,11t-CLA had no effect on LPS-induced COX-2 protein expression in the lung, 10t,12c-CLA reduced COX-2 protein expression relative to the control diet-fed mice (Fig. 2A, B). The LPS-induced increase of PGE$_2$ release from lung was reduced in 10t,12c-CLA-fed mice but not in 9c,11t-CLA-fed mice relative to LPS-injected mice fed the control diet (Fig. 2C). The increased COX-2 protein expression in the lung attributable to LPS injection and inhibition of the expression by feeding 10t,12c-CLA but not 9c,11t-CLA is shown in Fig. 2D using immunocytochemistry.
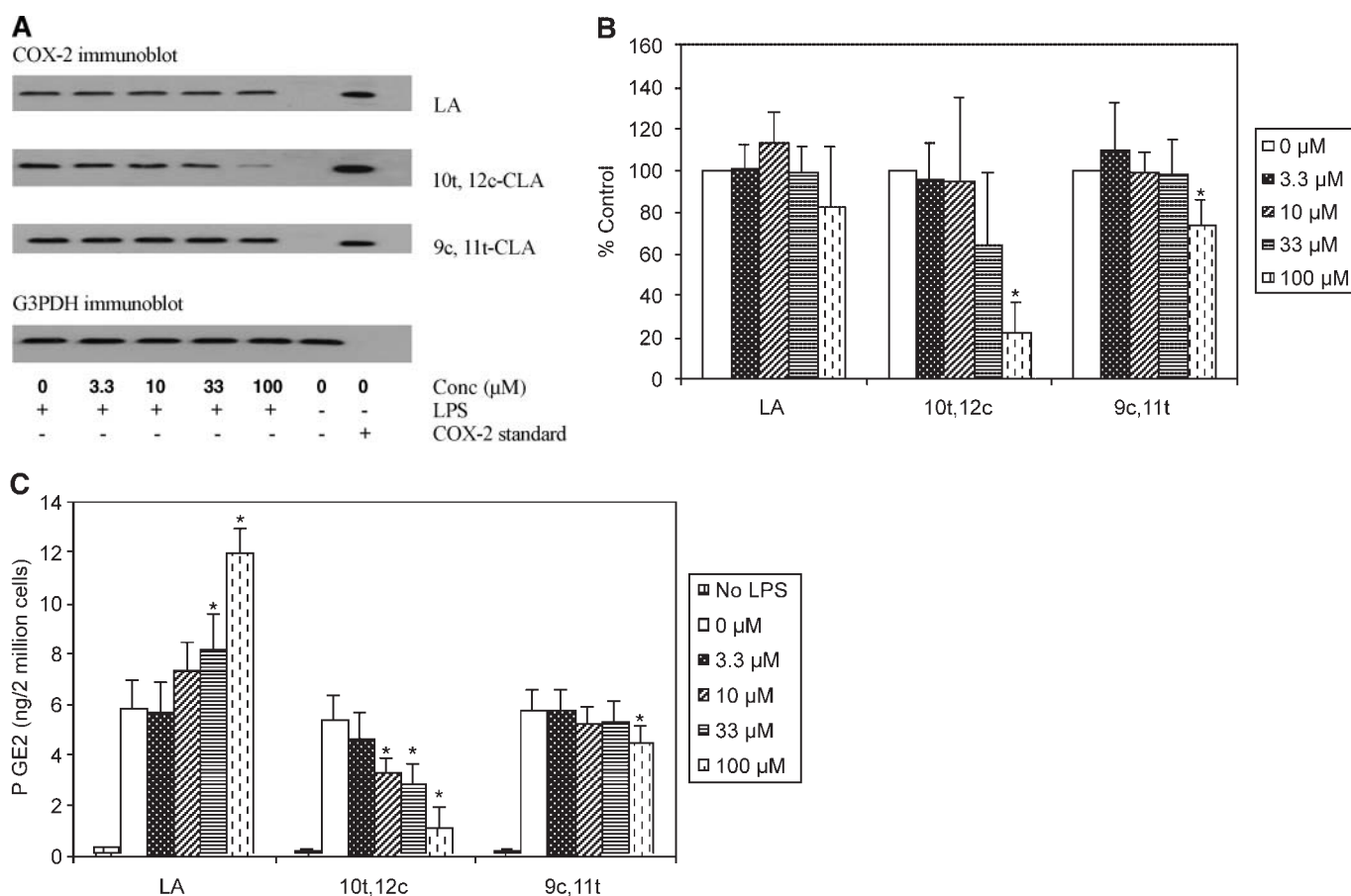
Fig. 1. Effects of 10t,12c-CLA and 9c,11t-CLA on cyclooxygenase-2 (COX-2) protein expression (A, B) and prostaglandin E$_2$ (PGE$_2$) production (C) in Raw264.7 macrophages. Confluent Raw264.7 macrophages were treated with different concentrations of linoleic acid (LA), 10t,12c-CLA, or 9c,11t-CLA (0, 3.3, 10, 33, and 100 μM) or an ethanol solution (control) for 24 h in 0.5% FBS DMEM. Lipopolysaccharide (LPS; 100 ng/ml) was introduced for 8 h to induce the expression of COX-2. The cell lysate and cell medium were harvested to determine the expression of COX-2 and PGE$_2$ production. Data are presented as means ± SD from four replicates. * $P < 0.05$ versus control.
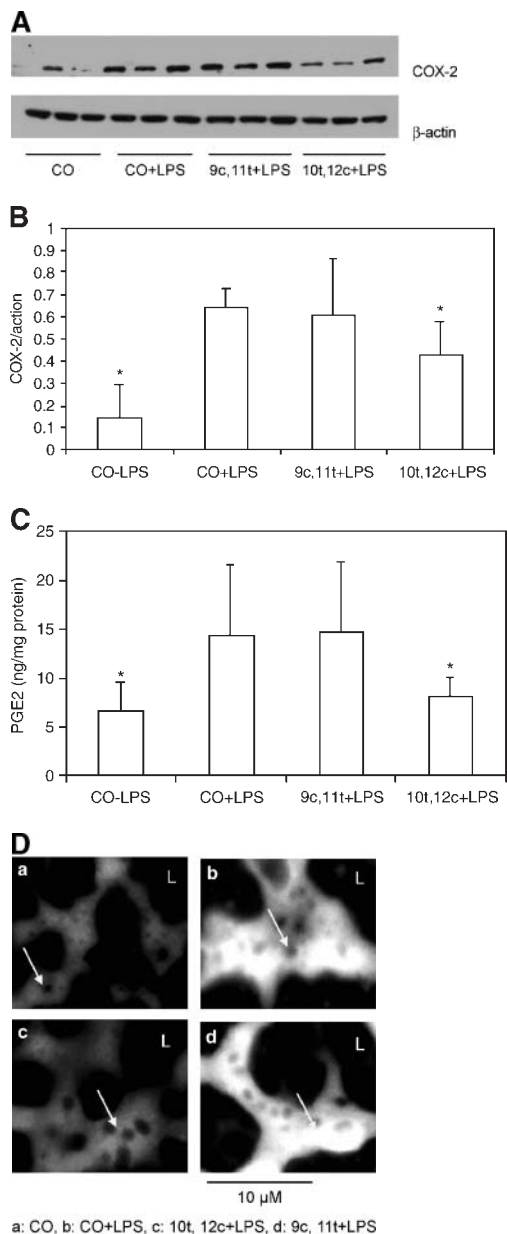
Fig. 2. Inhibitory effects of 10t,12c-CLA on COX-2 protein expression (A, B, D) and PGE$_2$ release (C) in mouse lung. Twenty-four BALB/c male mice (4 weeks old) were randomly assigned to four groups: CO, CO + LPS, 9c,11t-CLA + LPS, and 10t,12c-CLA + LPS. The mice were fed a basal diet (99% semipurified diet + 1% CO) for 11 days and then placed on their respective diet treatment for 26 days. On the 26th day, mice were injected with LPS (1 mg/kg) or vehicle. Mice were euthanized with CO$_2$ at 8 h after LPS injection, and lungs were harvested for Western blot analysis of COX-2. Each band represents data from one mouse (A), and PGE$_2$ concentration was determined by enzyme immunoassay (C). Data are presented as means ± SD, n = 6 per group (data from three animals in each treatment are shown). * $P < 0.05$ compared with the CO + LPS group by one-tailed t-test. D: After the mice were euthanized with CO$_2$ at 8 h after LPS injection, lungs were taken out for immunocytochemical analysis. The immunocytochemistry procedures followed are described in Materials and Methods. Fluorescence was detected with a microscope, and fluorescence intensity (shown as white) reflects the abundance of COX-2 in the cell. The small black areas inside the bright cytoplasm is the nucleus of the cells (arrows), and the large black area surrounding the bright cytoplasm is the lumen of the lung (L).

Effect of 10t,12c-CLA on COX-2 mRNA in vitro and in vivo and on iNOS mRNA in vitro

One pathway by which LPS induces COX-2 expression is through NF-κB activation. If reduced COX-2 protein expression was attributable to the inhibition of the NF-κB pathway, the COX-2 mRNA level should also be inhibited by 10t,12c-CLA. Raw264.7 macrophages treated with 10t,12c-CLA had reduced COX-2 mRNA expression (**Fig. 3A**). Likewise, in a feeding trial, mice fed 10t,12c-CLA had decreased COX-2 mRNA by 29.4% ($P = 0.059$; Fig. 3B). Because gene transcription of iNOS has been shown to be under the regulation of NF-κB, the effects of 10t,12c-CLA on iNOS mRNA were measured. It was shown that iNOS mRNA was reduced by 26% in the presence of 100 μM 10t,12c-CLA isomer (Fig. 3C).

Effect of 10t,12c-CLA on the NF-κB-mediated signal transduction pathway

NF-κB is a transcription factor that binds to inhibitory proteins such as IκBα, IκBβ, and IκBε in the cytoplasm of resting cells. Upon activation by LPS, cytokines, or growth factors, IκBα is phosphorylated and then degraded. NF-κB then translocates to the nucleus and activates the transcription of various proinflammatory cytokines and enzymes (COX-2 and iNOS). Phosphorylation of IκBα represents an important step for the activation of NF-κB in initiating the transcription of inflammatory mediators.

After incubation of Raw264.7 macrophages with 10t,12c-CLA, cells were stimulated with LPS for 4 h. As shown in **Fig. 4A**, phosphorylation of IκBα was inhibited by 10t,12c-CLA, but not by LA, in a concentration-dependent manner. Total IκBα was also determined in cells treated with LA or 10t,12c-CLA and stimulated with LPS for 4 h. Both compounds were shown to have no effect on the total level of IκBα (Fig. 4B).

LPS in the absence of supplemental fatty acids increased the binding of two NF-κB member proteins, P50 and P65, to the DNA consensus sites (Fig. 4C, D). 10t,12c-CLA, but not LA, was shown to decrease the binding of P50 and P65 to the consensus site (Fig. 4C, D).

DISCUSSION

CLA has been shown to prevent immune-induced wasting (13–15), decrease antigen-induced eicosanoid release (17, 18), and inhibit carcinogenesis (5–7) and atherosclerosis (8–10) in several animal models. The multiple functionalities of CLA have been hypothesized to be related to eicosanoid signaling (14). In the present study, the isomer of CLA that downregulates COX-2 protein and mRNA expression in vitro and in vivo was 10t,12c-CLA (active isomer). The data presented here support the hypothesis that 10t,12c-CLA exerts its protective effect against inflammatory diseases through the inhibition of COX-2 protein expression and activity.

This study first examined the effects of these fatty acids on LPS-induced COX-2 expression and PGE$_2$ production in a macrophage cell line. The data demonstrated that after LPS treatment, COX-2 protein expression and PGE$_2$ release by
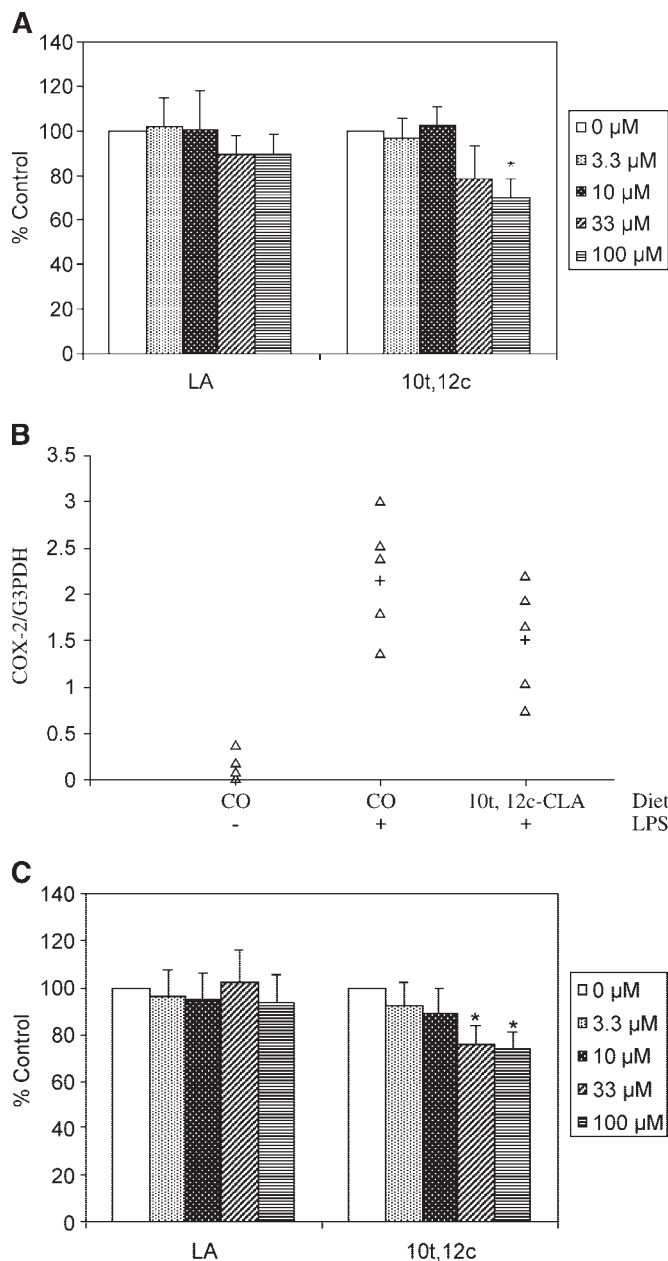
Fig. 3. Effects of LA and 10t,12c-CLA on LPS-induced COX-2 mRNA in vitro (A) and in vivo (B) and on inducible nitric oxide synthase (iNOS) mRNA in vitro (C). A, C: Confluent Raw264.7 macrophages were incubated with LA, 10t,12c-CLA, or 9c,11t-CLA (0, 3.3, 10, 33, and 100 μM) or ethanol (positive control) for 24 h and then were stimulated with LPS for 4 h (COX-2) or 6 h (iNOS) based on a kinetic study. B: Twenty-four BALB/c male mice (4 weeks old) were randomly assigned to four groups: CO, CO + LPS, 9c,11t-CLA + LPS, and 10t,12c-CLA + LPS. The mice were fed a basal diet (99% semipurified diet + 1% CO) for 2 days and then placed on their respective diet treatment for 21 days. On the 22nd day, mice were injected with LPS (1 mg/kg) or vehicle. G3PDH, glyceraldehyde-3-phosphate dehydrogenase. Mice were euthanized with CO_2 at 2 h after LPS injection, and lungs were harvested for mRNA isolation. There are five samples in the 10t,12c group because of an insufficient amount of samples from the sixth mouse. The plus sign represents the average value in each group. Isolation of mRNA, reverse transcription-polymerase chain reaction, and percentage control calculation were performed as described in Materials and Methods. Data are presented as means ± SD of five or six replicates. * $P < 0.05$ versus control.

LPS in macrophages were increased, as shown in previous studies (30, 34). The increase of COX-2 protein and PGE_2 release was inhibited by 10t,12c-CLA in a concentration-dependent manner and by 9c,11t-CLA only at 100 μM (Fig. 1A, B). Although 10t,12c-CLA was shown to reduce COX-2 protein expression and PGE_2 release, the concentration to reduce protein expression was higher than that required to decrease PGE_2 release. CLA has been shown to be a direct inhibitor of COX-2 enzymatic activity (17, 35; unpublished data) and to reduce arachidonic acid level in phospholipids through the inhibition of fatty acid elongase (36). Hence, 10t,12c-CLA-induced decreases in COX-2 product formation could be the results of *1*) decreased COX-2 protein, *2*) decreased substrate availability, and *3*) decreased COX-2 activity.

It has been shown that fatty acids inhibit COX-2 protein expression (30) and COX enzymatic activity (37) in vitro, but the in vivo effects of CLA isomers have not been shown. Feeding trials were conducted to investigate the effects of 9c,11t-CLA and 10t,12c-CLA isomers on COX-2 protein expression and PGE_2 release in vivo. Mouse lung was selected based on known COX-2 expression and PGE_2 release from this organ after inflammatory stimulation (18, 38–40). As shown in Fig. 2, feeding 10t,12c-CLA, but not 9c,11t-CLA, significantly reduced LPS-induced COX-2 protein expression and PGE_2 release compared with that in CO-fed LPS-injected mice. Although it has been reported that mixed isomers of CLA inhibited COX-2 expression and PGE_2 release in vitro (20, 21), this is the first study of the isomer-specific effects of CLA on COX-2 protein expression and PGE_2 release both in vitro and in vivo.

COX-2 expression is regulated at the transcriptional and posttranscriptional levels (see below). To identify whether reduced COX-2 protein by 10t,12c-CLA was attributable to its reduced mRNA level, the effects of 10t,12c-CLA in vitro (macrophage) and in vivo (mouse lung) on LPS-induced COX-2 mRNA were studied. As shown in Fig. 3, COX-2 and iNOS mRNA levels were also decreased by the 10t,12c-CLA in macrophages. COX-2 mRNA was also decreased in lungs of 10t,12c-CLA-fed mice. Interestingly, 10t,12c-CLA inhibited COX-2 protein by 80% and mRNA by only 30% in macrophages. Several pharmacological agents have been shown to participate in the posttranscriptional regulation of COX-2 mRNA, such as dexamethasone (41) and leptomycin B (42). Pyrrolidine dithiocarbamate, an oxidant scavenger, and rotenone, an inhibitor of the mitochondrial electron transport system, have also been reported to inhibit the expression of COX-2 protein expression without affecting COX-2 mRNA level in primary mesangial cell cultures (43). The differential inhibition rate on COX-2 mRNA and protein expression by 10t,12c-CLA may be attributed to the posttranscriptional regulation of the gene product. Although there are no data for 10t,12c-CLA involved in the posttranscriptional regulation of COX-2, polyunsaturated fatty acids have been shown to posttranscriptionally regulate for Δ-9 desaturase-1, glucose transporter-4, and Δ-9 desaturase-2 in adipocytes and lymphocytes (44).

It is well documented that LPS stimulation leads to the activation of NF-κB (45). When cells are not activated, NF-κB is sequestered in the cytoplasm by the inhibitory proteins
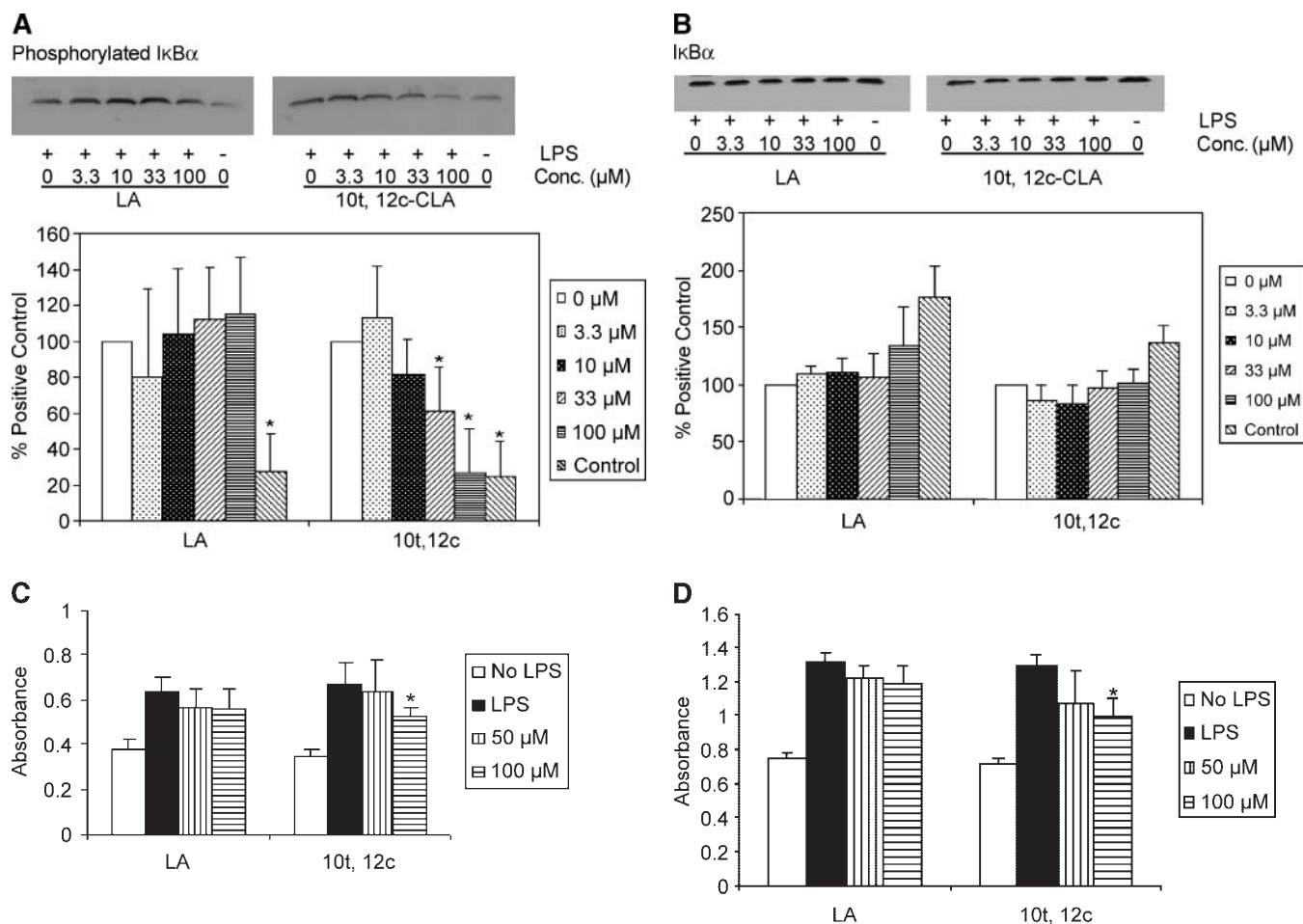
Fig. 4. Effect of 10t,12c-CLA on IκBα phosphorylation (A), total IκBα level (B), nuclear factor κB (NF-κB) p50 binding (C), and NF-κB p65 binding (D) in Raw264.7 macrophages. A, B: The cells were incubated with different concentrations (0, 3.3, 10, 33, and 100 μM) of LA, 10t,12c-CLA, or ethanol for 24 h and then were stimulated with LPS (1 μg/ml) for 4 h. Cell lysate was separated with SDS-PAGE and then probed with phosphorylated IκBα or IκBα antibody. Data are presented as means ± SD of four replicates. * $P < 0.05$ versus a positive control (ethanol + LPS). Percentage positive control = (band area in fatty acids and LPS-treated sample/band area in ethanol- and LPS-treated sample) × 100. C, D: The cells were incubated with different concentrations (50 and 100 μM) of LA, 10t,12c-CLA, or ethanol for 24 h and then were stimulated with LPS (1 μg/ml) for 4 h. Cell lysate was harvested for the determination of p50 and p65 binding according to kit instruction. Data are presented as means ± SD of four (p50) or five (p65) replicates. * $P < 0.05$ versus a positive control (ethanol + LPS).

IκBα, IκBβ, and IκBε. Upon stimulation by growth factors, cytokines, or LPS, IκBα is phosphorylated and then degraded in a ubiquitin-proteosome-dependent manner. Liberated NF-κB translocates to the nucleus and activates the gene transcription of proinflammatory mediators. NF-κB thus is an important transcription factor in the regulation of proinflammatory enzymes and cytokines (26). Pharmaceutical strategies for the prevention of inflammatory diseases and cancer have been designed to target NF-κB (46). Decreased COX-2 and iNOS mRNA was at least partially attributable to the inhibition of the NF-κB-mediated signal transduction pathway. As shown in Fig. 4, 10t,12c-CLA inhibited IκBα phosphorylation after LPS stimulation for 4 h in a concentration-dependent manner, whereas LA had no inhibitory effects on IκBα phosphorylation (Fig. 4A). Inhibition of IκBα phosphorylation would thus inhibit the translocation of NF-κB to the nucleus and subsequent gene transcription under NF-κB regulation. Indeed, the treatment of 10t,12c-CLA significantly decreased the binding

of activated p50 and p65 to its consensus site (Fig. 4C, D). These data suggested that the decreased binding of activated p50 and p65 was at least partially attributable to the inhibition of IκBα phosphorylation. Inhibition of IκBα phosphorylation has also been the proposed mechanism by which n-3 fatty acids reduce TNF-α release from macrophages (47). Interestingly, the total level of IκBα was not affected by LA and 10t,12c-CLA (Fig. 4B), similar to what has been observed with the effects of n-3 fatty acids on IκBα level in macrophages (47). Other NF-κB-regulated products, such as TNF-α and IL-1β, are also affected by CLA. Yang and Cook (16) have demonstrated that CLA reduced TNF-α release in vitro and ex vivo from CLA-treated macrophages and mice, respectively. In another study, 10t,12c-CLA-fed mice were shown to exhibit lower plasma TNF-α compared with CO-fed mice (unpublished data). CLA was also shown to decrease basal and LPS-stimulated IL-1 levels in macrophages of rats fed a diet containing CLA (48). Although the proteins of TNF-α and IL-

1β were shown to be affected by CLA in these studies, its effects on mRNA level warrant further examination. What is not clear from this and other studies is whether the effects of 10t,12c-CLA on IκBα phosphorylation are direct or are the result of an event upstream of IκBα phosphorylation.

One major finding in the current study is that 10t,12c-CLA, a naturally occurring fatty acid, inhibits COX-2 and PGE$_2$ in vivo. Decreased COX-2 protein level and PGE$_2$ production in vivo may help explain some beneficial effects of CLA in a number of animal models, such as its inhibitory effects on carcinogenesis (5–7), atherosclerosis (8–10), and other diseases. Because COX-2 has also been reported to play an important role in the development of systemic lupus erythematosus (49, 50), the prolonged survival after onset of proteinuria could be related to the COX-2 protein and PGE$_2$ inhibition in mice fed a CLA diet (16, 51). Interestingly, it was shown the CLA feeding spared gastrocnemius muscle breakdown in a mouse cancer cachexia model that is also sensitive to COX-2 inhibition (52). Given the role of COX-2 in inflammatory diseases and 10t,12c-CLA's effect on COX-2 and other proinflammatory mediators, it is expected that a diet rich in CLA may help prevent an array of inflammatory diseases.

In summary, this study has shown that 10t,12c-CLA is the active isomer in inhibiting COX-2 protein expression and PGE$_2$ production in both in vitro and in vivo models. COX-2 inhibition was probably mediated at both transcriptional and posttranscriptional levels. Interference with NF-κB activation was one of the potential mechanisms underlying the isomer's effect in decreasing COX-2 and other cytokine gene transcription. These results suggest that some of CLA's beneficial effects may be mediated through inhibitory effects on COX-2 activity/protein expression, and 10t,12c-CLA may represent a naturally occurring nutrient against inflammatory diseases in which COX-2 is significantly involved.

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