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Title Page

Contribution of conjugated linoleic acid to the suppression of inducible nitric oxide synthase expression and transcription factor activation in stimulated mouse mesangial cells

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Abbreviations used: AP-1, activator protein-1; CLA, conjugated linoleic acid; CREB, cAMP response element binding protein; DMEM, Dulbecco's modified Eagle's media; EMSA, eletrophoretic mobility shift assay; eNOS, endothelial nitric oxide synthase; IFN-γ, interferon-γ; IκB-α, inhibitor κB-α; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccaride; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-κB; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; PGE_2, prostaglandin E_2; PMSF, phenyl methyl sulfonyl fluoride; PPAR, peroxisome proliferator-activated receptor; RT-PCR, reverse transcriptase polymerase chain reaction.

Abstract

That both infiltrating macrophages and resident mesangial cells express inducible nitric oxide synthase (iNOS) and produce nitric oxide (NO) excessively is crucial to the progress of glomerulonephritis. Although several reports have mentioned the protective impacts of conjugated linoleic acid (CLA) in stimulated macrophages, the role of CLA in glomerular mesangial cells is unknown. The aim of the present study was to explore the ability of CLA to regulate iNOS expression and NO production in stimulated glomerular mesangial cells. Additionally, we evaluated the effect of CLA on activation of transcription factors which mediate iNOS expression. Exogenous CLA dose-dependently diminished iNOS mRNA and protein expression as well as NO production in lipopolysaccharide (LPS) plus interferon-γ (IFN-γ)–stimulated SV-40–transformed mouse mesangial cells. Electrophoretic mobility shift assay experiments demonstrated that CLA (100 μM) dramatically reduced activation of nuclear factor-κB (NF-κB), activator protein-1 (AP-1) and cAMP response element binding protein (CREB) induced by LPS/IFN-γ. Moreover, addition of 100 μM CLA significantly diminished LPS-IFN-γ–induced protein degradation of inhibitor κB-α (IκB-α) and the protein expression of phosphorylated IκB-α in the cytosolic fraction as well as nuclear p65 expression ($P < 0.05$). In

summary, inhibition of NF-κB, AP-1 and CREB activation by CLA may be associated with the molecular basis for which CLA suppresses iNOS expression and NO production in stimulated mesangial cells.

1. Introduction

Nitric oxide (NO) produced from the oxidation of L-arginine is a free radical gas that acts as a potent regulatory molecule in diverse biological events, including neurotransmission (Garthwaite, 1995), vasodilation (Ignarro et al., 1987), and inflammation and cytotoxicity (Moncada et al., 1991; Nathan, 1992). At least three different isoforms of nitric oxide synthase (NOS), named neuronal NOS (nNOS, or NOS1), endothelial NOS (eNOS, or NOS3), and inducible NOS (iNOS, or NOS2), have been identified as being responsible for NO production in mammalian cells (Förstermann et al., 1995). In general, the small quantity of NO produced by the constitutive forms of NOS, such as nNOS and eNOS, is profitable in certain physiologic functions through activation of soluble guanylate cyclase (Förstermann et al., 1995). On the other hand, expression of iNOS results in relatively high amounts of NO in various cell types, such as macrophages, hepatocytes, smooth muscle cells, and glomerular mesangial cells after exposure to lipopolysaccharide (LPS) and/or inflammatory cytokines (Pfeilschifter and Schwarzenbach, 1990; Knowles and Moncada, 1994; Shultz et al., 1994). This buildup of NO not only causes harmful cellular responses but also affects the pathogenic processes of several diseases, including sepsis, multiple sclerosis, atherosclerosis, cancer, and glomerulonephritis (Ross, 1993; Cook et al., 1994; Wong and Billiar, 1995; Hooper et al., 1997; Takahashi et al., 1997). Although activation of nuclear factor -κB (NF-κB) is important in transcriptional regulation of iNOS expression, additional signals are required for iNOS expression in glomerular mesangial cells (Beck and Sterzel, 1996). Using in vitro transfection assay has identified that inducible transcription factors such as activator protein-1 (AP-1) and cAMP response element binding protein (CREB) are also involved in the iNOS expression (Zhang et al., 1998).

Glomerular mesangial cells are a major player in the regulation of the glomerular filtration rate, matrix synthesis, and glomerular structure (Menè et al., 1989). Apart from their physiologic functions in the kidney, mesangial cells together with infiltrating macrophages manage the overall inflammatory response in the glomerulus through the production of various inflammatory mediators, such as cytokines, eicosanoids, and NO (Radeke and Resch, 1992; Horri et al., 1993; Pfeilschifter et al., 1993; Sedor et al., 1993; Raij and Baylis, 1995). In human glomerulonephritis, iNOS expression is increased in mesangial cells, and the extent of iNOS expression in the glomerulus is positively linked to the degree of glomerular injury (Furusu et al., 1998). The SV40–transformed mouse mesangial cell line, MES13, has morphologic and biochemical characteristics similar to those of primary mesangial cells (MacKay et al., 1988). It is well established that LPS and/or cytokines can induce iNOS expression and NO synthesis in primary rat mesangial cells (Chin et al., 2001; Kaszkin et al., 2004**)** and in the SV40 MES13 cells (Datta and Lianos, 1999; Prabhaker, 2001; Crosby et al., 2005).

The term *conjugated linoleic acid* (CLA) refers to a group of positional and geometric isomers of octadecadienoic acid with conjugated dienoic double bonds in the Δ7,9; Δ9,11; Δ10,12; Δ8,10; and Δ11,13 positions, in either *cis* or *trans* configurations (Lavillonnière et al., 1998; Yurawecz et al., 1998). Ruminant meats and dairy products are the major dietary sources of CLA. Except for turkey, meat from nonruminants as well as seafood and plant oils contain less CLA (Chin et al., 1992). Although the molecular activities of CLA have not been completely documented, the beneficial effects of CLA on disease prevention have been reported elsewhere. In addition to chemoprevention, CLA has received much attention in modulating blood sugar and lipid homeostasis and in immune function and inflammation responses (Belury, 2002). Previous reports from other researchers and

by our group have shown that CLA can decrease LPS or interferon-γ (IFN-γ)–induced pro-inflammatory products in RAW 264.7 macrophages and primary peritoneal macrophages (Iwakiri et al., 2002; Yu et al., 2002; Yang and Cook, 2003; Cheng et al., 2004). Several lines of investigations are now targeting the biological effects of CLA on the kidney. The incorporation of CLA and its metabolites is altered in patients with chronic renal failure, although the clinical effects of these alternations are unknown (Lucchi et al, 2002). Dietary CLA completely abolishes the formation of mutations induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in the kidney of female rats but not in male rats (Yang et al., 2002). Furthermore, dietary CLA significantly decreases prostaglandin E_2 (PGE_2) and parathyroid hormone release as well as interstitial inflammation and fibrosis in the rat model of polycystic kidney disease (Ogborn et al., 2003; Weiler et al., 2004). Although the increasing awareness of the health benefits of CLA in kidney, little is known about the physiologic effect of CLA in glomerular mesangial cells. To explore the role of CLA in glomerular injury, we assessed the efficacy of CLA in modulating iNOS expression and NO production in SV40 MES13 cells. Because the inducible transcription factor activation not only mediates iNOS expression (Xie et al., 1994; Zhang et al., 1998) but also regulates the immune and inflammatory responses (Otten et al., 2000), the effect of CLA on the LPS-IFN-γ–stimulated activation of NF-κB, AP-1 and CREB was also under investigation.

2. Materials and methods

2.1. Materials

SV40–transformed mouse mesangial cell line, MES13, was purchased from the American Type Culture Collection (Manassas, VA), and the fetal bovine serum

was from Highclone (Logan, UT). Dulbecco's modified Eagle's media (DMEM), Ham's F12 media, and medium supplements for cell culture were obtained from Gibco BRL (Gaithersburg, MD). LPS was obtained from Sigma Chemical Company (St. Louis, MO), and IFN-γ was from PeproTech EC Ltd. (London,UK). The specific antibodies for iNOS and p65 were from BD Biosciences (Franklin Lakes, NJ). Monoclonal antibodies for inhibitor κB-α (IκB-α) and phosphorylated IκB-α were purchase from Santa Cruz Biotechnology (Santa Cruz, CA), and α-tubulin antibody were from Transduction Laboratories (Greenland, UK). Reagents such as enzymes, cofactors, and nucleotides for internal standard construction and reverse transcriptase polymerase chain reaction (RT-PCR) were from Promega Co. (Madison, WI). Oligonucleotide primer sequence of iNOS for RT-PCR was selected by using Primer Select (DNASTAR, Madison, WI). The oligonucleotide primers for RT-PCR as well as the biotin-labeled and unlabeled consensus oligonucleotides of NF-κB, AP-1 and CREB and mutant double-stranded NF-κB oligonucleotide for the electrophoretic mobility shift assay (EMSA) were synthesized by MDBio Inc. (Taipei, Taiwan). The 99% purity of CLA which contains c9,t11-,t9,c11-, c9,c11-, t9,t11-CLA and t10, c12-, c10,c12, t10, t12-CLA was from NuChek Prep, Inc. (Elysian, MN). All other chemicals were of the highest quality available.

2.2. Cell culture

The MES13 cells (passage levels between 35 and 39) were maintained in a 3:1 mixture of DMEM and Ham's F12 medium supplemented with 14 mM HEPES, 2 mM L-glutamine, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and 5% 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 were incubated until 90% confluence was reached. For the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, measurements of NO synthesis and of iNOS protein and mRNA expression and the EMSA experiment of CREB activation, cells were treated with or without a mixture of LPS (1 μg/ml) and IFN-γ (5 ng/ml) plus methanol vehicle control or 25 to 100 μM CLA for 18 or 12 h. For remainder of the experiments in the present study, the cultures were treated with methanol or 100 μM CLA for 12 h before addition of the LPS-IFN-γ mixture.

2.3. MTT assay for cell viability

The mitochondrial-dependent reduction of MTT to formazan was used to measure cell respiration as an indicator of cell viability (Denizot and Lang, 1986). After the supernatant fluid was removed for measurement of NO synthesis, the cells were incubated in DMEM and Ham's F12 medium containing 0.5 mg/ml MTT for 3 h at 37°C in a 5% CO_2 atmosphere. After the media were aspirated, isopropanol was

added to the cells to dissolve the formazan. The supernatant fluid from each sample was transferred to 96-well plates, which were read at 570 nm in a VersaMax™ Tunable Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA). The absorbance in the cultures treated with LPS-IFN-γalone was used to indicate 100% of cell viability.

2.4. Determination of nitrite concentration

The nitrite in the media was measured by the Griess assay (Green et al., 1982) and was used as an indictor of NO synthesis in cells. Briefly, equal volumes of the culture supernatant fluid and Griess solution [1:1 mixture (v/v) of 1% sulfanilamide and 0.1% N-(napthyl)ethyl-enediamine dihydrochloride in 5% H_3PO_4] were added to 96-well plates at room temperature for 10 min. Absorbances at 550 nm were measured in a VersaMax™ Tunable Microplate Reader and were calibrated by using a standard curve of sodium nitrite prepared in culture media.

2.5. Western blot analysis

The protein content of each sample was quantified by using a Coomassie® Plus Protein Assay Reagent Kit (Pierce Chemical Co., Rockford, IL). Protein aliquots were denatured and separated on 8–12% SDS-PAGE gels and then transferred to polyvinylidene 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'diaminobenzidine color developing solution and were quantified by densitometric analysis by Zero-Dscan (Scanalytics, Inc. Fairfax, VA).

2.6. RNA isolation and quantitative RT-PCR

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

Quantitative RT-PCR was accomplished by using recombinant RNA templates as internal standards to monitor mRNA expression, as described previously (Belury et al., 1997). In brief, 0.25 μg total RNA and various amounts of the recombinant RNA internal standard were reverse transcribed with M-MMLV reverse transcriptase in a 20-μl final volume of the reaction buffer, which consisted of 25 μM 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 $MgCl_2$, 1 mM of each deoxynucleotide triphosphate, 2.5 units RNase inhibitor, and 2.5 mM oligo$(dT)_{16}$. Each gene has its own specific

recombinant RNA template, which contains forward and reverse primer sequences for the target gene. The procedure for generating the recombinant RNA templates was as described by Vanden Heuvel et al. (1994). For the synthesis of complementary DNA, reaction mixtures were incubated for 15 min at 45°C and were 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 forward and reverse primers was added to a total volume of 50 μl. The sequences for the RT-PCR primers were as follows: 5'-CAGTTCTGC GCCTTTGCTCAT-3' (forward) and 5'- GGTGGTGCGGCTGGACTTT-3' (reverse) for mouse iNOS. The samples for PCR amplification were heated to 94°C for 3 min and immediately cycled 31 times through a 30-s denaturing step at 94°C, a 30-s annealing step at 58.5°C, and a 60-s elongation step at 72°C. After the final cycle, a 5-min elongation step at 72°C was carried out. The amplified PCR products of the internal standard and the target mRNA can be easily visualized and separated by 2.5%-agarose (Seakem®LE agarose, Biowhittaker Molecular Application, Rockland, ME) gel electrophoresis with ethidium bromide. The gels were photographed, and the intensity of the stained PCR fragments was quantified by densitometric analysis with Zero-Dscan.

The amount of target mRNA present was quantified as follows. First, a range-finding study was set to determine the approximate optimum concentration of

internal standard required to display a 1:1 intensity of internal standard:target mRNA PCR product. Then, RNA samples with a constant amount of optimal internal standard were examined in triplicate by RT-PCR. To make a standard curve, gradual concentrations of internal standard and a constant concentration of sample RNA were amplified, and the log (ratio of band intensity) versus log (internal standard added) was plotted. The ratio of target gene to internal standard mRNA intensity was used to quantify the target gene mRNA level according to this standard curve (Belury et al., 1997).

2.7. Preparation of nuclear protein and EMSA

At the time of harvest, the MES13 cells were scraped with cold phosphate-buffered saline, suspended in the hypotonic extraction buffer [10 mM HEPES, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 0.2 mM phenyl methyl sulfonyl fluoride (PMSF), 4 μg/ml leupeptin, 20 μg/ml aprotinin, and 0.5% NP-40] for 15 min on ice, and then centrifuged at 6000×g for 15 min. Pelleted nuclei were resuspended in the hypertonic extraction buffer [10 mM HEPES, 0.4 M KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 4 μg/ml leupeptin, 20 μg/ml aprotinin**,** 10% glycerol], constantly shaken at 4°C for 30 min, and then centrifuged at 10,000×g for 15 min. The supernatant fluid containing nuclear proteins was collected and stored at −70°C until the EMSA was performed.

The LightShift Chemiluminescent EMSA Kit from Pierce Chemical Co. and synthetic biotin-labeled, double-stranded consensus oligonucleotides of NF-κB (5'-AGTTGAGGGGACTTTCCCAGGC-3'), AP (5'-CGCTTGATGACTCAGCCGGAA-3') and CREB (5'-AGAGATTGCCTGACGTCAGAGAGCTAG-3') were used to measure the effect of CLA on NF-κB, AP-1 and CREB nuclear protein DNA-binding activity. Nuclear extract (2 μg), poly(dI-dC), and biotin-labeled double-stranded oligonucleotides were mixed with the binding buffer (to a final volume of 20 μl) and were incubated at room temperate for 30 min. Unlabeled double-stranded oligonucleotides of NF-κB, AP-1 and CREB as well as a mutant double-stranded NF-κB oligonucleotide (5'-AGTTGAGG$_{C}$GACTTTCCCAGGC-3') were also used to confirm specific binding and protein binding specificity. The nuclear protein–DNA complex was separated by 6% Tris/Boric acid/EDTA-polyacrylamide gel electrophoresis and was then transferred to nylon membranes (Hybond N+; Amersham Pharmacia Biotech Inc., Piscataway, NJ). Next, the membrane was treated with streptavidin–horseradish peroxidase, and the nuclear protein-DNA bands were developed with the use of a SuperSignal West Pico kit (Pierce Chemical Co).

2.8. Statistical analysis

Data are expressed as the mean ± SD from at least three independent

experiments. Differences among treatments were analyzed by variance with Scheffe's multiple comparison test ($\alpha = 0.05$) using Statistical Analysis System (Cary, NC).

3. Results

3.1. Effects of CLA on cell viability and LPS-IFN-γ–induced nitrite synthesis and iNOS mRNA expression

Compared with methanol vehicle control, cells treated with mixture of LPS and IFN-γ slightly influenced cell viability measured by MTT Assay (94±3% of control). Cultures treated with test concentrations of CLA of up to 100 μM still had more than 90% of the cell viability of cultures treated with LPS-IFN-γ alone (Fig. 1).

The nitrite concentrations of the cells treated with LPS, IFN-γ, or CLA alone were similar to those of the cells treated with the methanol vehicle control (0.51 ± 0.14 μM). Treatment of mouse mesangial cells with LPS-IFN-γ substantially increased nitrite release (27.62 ± 5.77 μM), and the presence of CLA in LPS-IFN-γ–activated mouse mesangial cells significantly reduced nitrite production ($P < 0.05$; Fig. 1).

In resting mouse mesangial cells and in cells treated with LPS, IFN-γ, or CLA alone, the expression of iNOS mRNA was hardly detectable (data not shown), whereas it was dramatically increased in cultures treated with LPS-IFN-γ alone. The expression of iNOS mRNA in cultures treated with LPS-IFN-γ plus 100 μM CLA was

significantly lower by 63% than that in cultures treated with LPS-IFN-γ alone (LPS-IFN-γ: $2.59\times10^{9} \pm 7.5\times10^{8}$ molecules RNA; LPS-IFN-γ+ 100 μmol/L CLA: $9.5\times10^{8} \pm 2.5\times10^{7}$ molecules RNA, Fig. 1).

3.2. Protein expression of iNOS, cytoplasmic IκB-α, phosphorylated IκB-α, and nuclear p65 in mouse mesangial cells

In resting mouse mesangial cells and in cells treated with LPS, IFN-γ, or CLA alone, the protein expression of iNOS was undetectable. iNOS protein expression was markedly augmented in cultures treated with LPS-IFN-γ alone, whereas the addition of exogenous CLA significantly reduced the LPS-IFN-γ–induced iNOS protein expression ($P < 0.05$; Fig 2).

The inhibitory effects of CLA on LPS-IFN-γ–induced nitrite synthesis and iNOS mRNA and protein expression were not due to a reduction in cell viability.

On treatment with LPS-IFN-γ, the amount of cytoplasmic IκB-α protein was degraded but cytoplasmic phosphorylated IκB-α protein synthesis was induced when compared with the control (Fig. 3A and 3B, respectively). Addition of 100 μM CLA significantly inhibited the LPS-IFN-γ–induced degradation of IκB-α protein and the expression of phosphorylated IκB-α protein in the cytosolic fraction of mesangial cells ($P < 0.05$). Notably, the amount of nuclear p65 protein in cells incubated with 100 μM CLA for 12 h before stimulation with LPS-IFN-γ was significantly lower than

that in cells treated with LPS-IFN-γ alone ($P < 0.05$; Fig. 4).

3.3. Effects of CLA on LPS-IFN-γ–induced NF-κB, AP-1 and CREB DNA binding activities

EMSA experiments were used to evaluate the effect of CLA on activation of NF-κB, AP-1 and CREB (Fig 5A, 5B, and 5C, respectively). In resting mouse mesangial cells and in cells treated with LPS, IFN-γ, or CLA alone, the DNA-binding activities of NF-κB, AP-1 and CREB nuclear proteins were undetectable (data were not shown). On treatment with LPS-IFN-γ, the DNA-binding activities of NF-κB, AP-1 and CREB nuclear proteins were markedly higher than those after treatments with the methanol vehicle control. LPS-IFN-γ–induced NF-κB, AP-1 and CREB nuclear protein DNA binding activities were reduced in cultures treated or pretreated with 100 μM CLA. Moreover, the bands completely vanished after the addition of excess unlabeled, double-stranded consensus oligonucleotides of NF-κB, AP-1 and CREB. In contrast, only a minor change was seen in NF-κB nuclear protein DNA binding activity when mutant, double-stranded NF-κB oligonucleotide was added (Fig 5A). AP-1 and CREB nuclear protein DNA complex was not affected by addition of excess unlabeled, double-stranded NF-κB consensus oligonucleotide (Fig. 5B and 5C, respectively) These data emphasize the specificity of the NF-κB AP-1 and CREB nuclear protein DNA binding reactions.

4. Discussion

Previous data have shown that CLA diminishes the production of inflammatory mediators such as NO, PGE_2, tumor necrosis factor-α, interleukin-1β, and interleukin-6 in activated RAW 264.7 macrophages (Yu et al., 2002, Cheng et al., 2004). In agreement with the role of CLA in RAW 264.7 macrophages, we showed that addition of exogenous CLA to mouse mesangial cells, MES13, significantly reduces LPS-IFN-γ–induced iNOS expression and subsequent NO production. It is no doubt that a large amount of NO production catalyzed by iNOS is involved in the pathogenesis of various diseases such as glomerulonephritis (Pfeilschifter et al., 1993; Cook et al., 1994). It is noteworthy, with respect to the data present here and previously, that the ability of CLA to modulate iNOS expression in different cell types could be a view to exam the biologic and physiologic effect of CLA in disease prevention.

In mesangial cells, the increased NF-κB activation is associated with not only expression of a variety of pro-inflammatory genes (Ghosh et al., 1998) but also regulation of mesangial cell proliferation and apoptosis (Barkett and Gilmore, 1999). NF-κB is ubiquitously expressed in most eukaryotes and acts as an inducible transcription factor by forming homodimers or heterodimers with proteins of the NF-κB family, including p65 (RelA), p50/p105 (NF-κB1), p52/p100 (NF-κB2), RelB,

and c-Rel. In unstimulated cells, NF-κB is sequestered in the cytoplasm by noncovalently binding to an inhibitor protein termed IκB (α, β or ε) (Baldwin, 1996). After cells are exposed to external stimuli—such as inflammatory cytokines, oxidative stress, ultraviolet irradiation, or bacterial endotoxins (Bours et al., 2000; Wang et al., 2002)—NF-κB is activated by stimulating phosphorylation and degradation of IκBα (Henkel et al., 1993). Then the activated NF-κB is translocated into the nucleus, and leads to transcriptional expression of genes associated with host inflammatory and immune responses (Ghosh et al., 1998) and cellular growth properties (Barkett and Gilmore, 1999). Our data show that in mouse mesangial cells CLA hinders NF-κB activation by reducing LPS-IFN-γ–induced protein degradation of IκB-α and the protein expression of phosphorylated IκB-α in the cytosolic fraction and of nuclear p65. Furthermore, the ability of CLA to inhibit the NF-κB nuclear protein DNA binding affinity is compatible with CLA's preventive effect on NF-κB activation.

Excessive NO production catalyzed by iNOS in mesangial cells is associated with several forms of glomerular injury (Aiello et al., 1998; Furusu et al., 1998; Trachtman, 2004). In addition to NF-κB, activation of AP-1 and CREB is also involved in the transcriptional expression of iNOS gene in mesangial cells stimulated by cytokines and /or LPS (Sakurai et al., 1997; Eberhardt et al., 1998). Among the putative hypotheses proposed for the unique health-benefit properties of CLA, the role

of CLA in the molecular regulation of gene expression has attracted a great deal of attention (Iwakiri et al., 2002; Yu et al., 2002; Cheng et al., 2004). It is well established that CLA is a potent activator of peroxisome proliferator-activated receptors (PPARs) and can induce the expression of PPAR response genes (Belury, 2002). Our data showed that exogenous addition of CLA not only could suppress NF-κB activation but also could modulate AP-1 and CREB nuclear protein DNA binding activities in stimulated mesangial cells. These results suggested that CLA suppressed iNOS expression at least in part through inactivation of NF-κB, AP-1 and CREB. The aptitude of CLA for lessening the inducible transcription factor activation and then modulating gene expression in mesangial cells provides a new direction to explore the feature of CLA during glomerular injury.

In addition to decreasing PGE_2 production in the rat model of polycystic kidney disease (Ogborn et al., 2003), CLA modulating LPS-IFN-γ–induced events in mesangial cells, such as iNOS synthesis and transcription factor activation, is a new piece of evidence to prove the therapeutic aspect of CLA in renal disease. Accumulating evidence demonstrates the pathogenic role of NF-κB, AP-1 and CREB in chronic inflammation-related degenerative diseases (Chen et al., 1999; Otten et al., 2000; Calixto et al., 2004); therefore, the inhibitory effect of CLA on activation of

these transcription factors is worthwhile to study the biomedical importance of CLA not only in the glomeruli of kidney but also in overall human health.

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Fig. 1

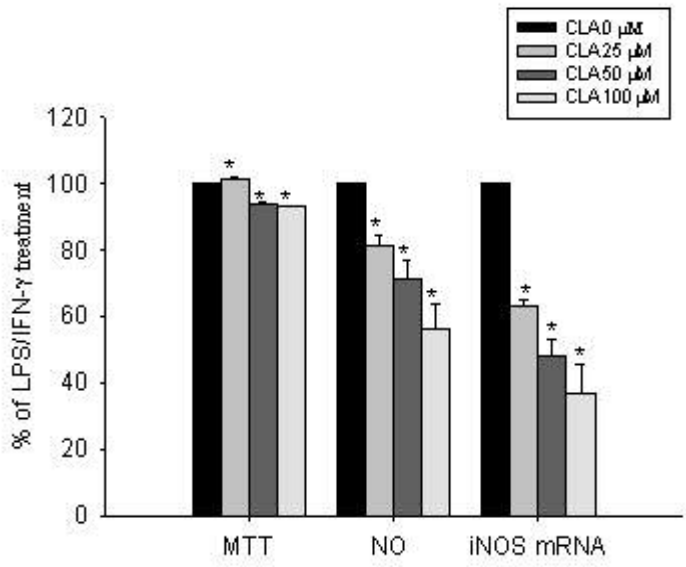


Fig. 1. Effect of CLA on cell viability and LPS-IFN-γ–induced nitrite synthesis and iNOS mRNA expression in mouse mesangial cells. Cultures were treated for 18 h (for cell viability and nitrite synthesis) or for 12 h (for iNOS mRNA expression) with a mixture of LPS (1 μg/ml) and IFN-γ (5 ng/ml) in the absence or presence of CLA at various concentrations. Cell viability was measured by use of the MTT assay, nitrite by the Griess reaction, and mRNA by the quantitative RT-PCR. Data are the mean ± SD of at least three separate experiments and are expressed as the percentage of the culture treated with LPS-IFN-γ alone. An asterisk indicates a significant difference from treatment with LPS-IFN-γ alone ($P < 0.05$).

Fig. 2

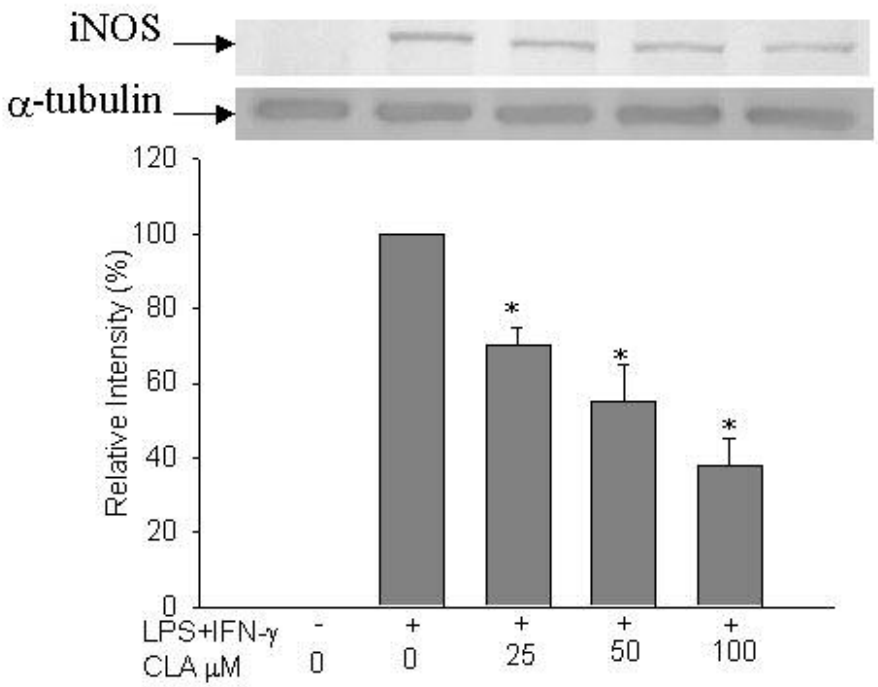
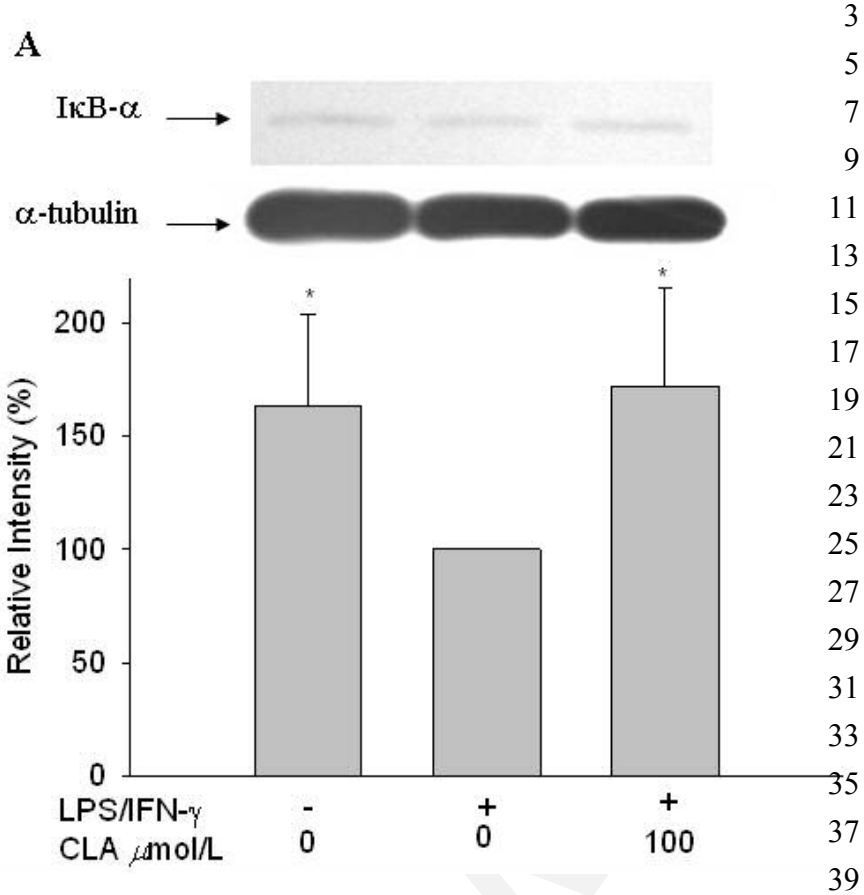


Fig. 2. Effect of CLA on LPS-IFN-γ–induced protein expression of iNOS in mouse mesangial cells. Cultures were treated for 18 h with a mixture of LPS (1 μg/ml) and IFN-γ (5 ng/ml) in the absence or presence of CLA at various concentrations. Whole-cell lysates were used to analyze the protein content of iNOS by Western blot. The protein expression of α-tubulin was used as an internal control. Relative protein levels were quantified by scanning densitometry and are expressed as a percentage of the maximal band intensity in the culture treated with LPS-IFN-γ alone. Data are the mean ± SD of iNOS/α-tubulin at least three separate experiments, and an asterisk indicates a significant difference from treatment with LPS-IFN-γ alone ($P < 0.05$).

Fig. 3



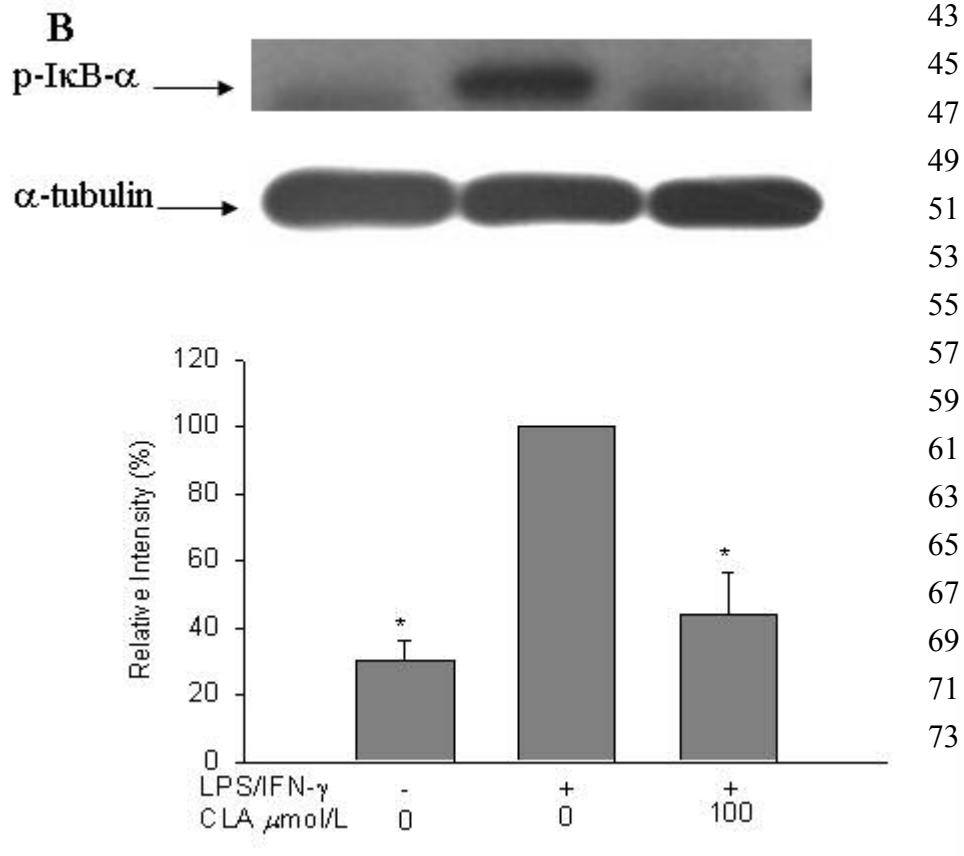


Fig. 3. Effect of CLA on LPS-IFN-γ–induced IκB-α protein degradation (A) and phosphorylated IκB-α protein expression (B) in mouse mesangial cells. Cultures were preincubated with or without 100 μM CLA for 12 h and were then treated with either methanol vehicle control or a mixture of LPS (1 μg/ml) and IFN-γ (5 ng/ml) for 20 min. The cytosolic factions were used to analyze the protein content of IκB-α and phosphorylated IκB-α by Western blot. The protein expression of α-tubulin was used as an internal control. Relative protein levels were quantified by scanning densitometry and are expressed as a percentage of the maximal band intensity in the culture treated with LPS-IFN-γ alone. Data are the mean ± SD of IκB-α/α-tubulin or phosphorylated IκB-α/α-tubulin at least three separate experiments, and an asterisk indicates a significant difference from treatment with LPS-IFN-γ alone ($P < 0.05$).

Fig. 4

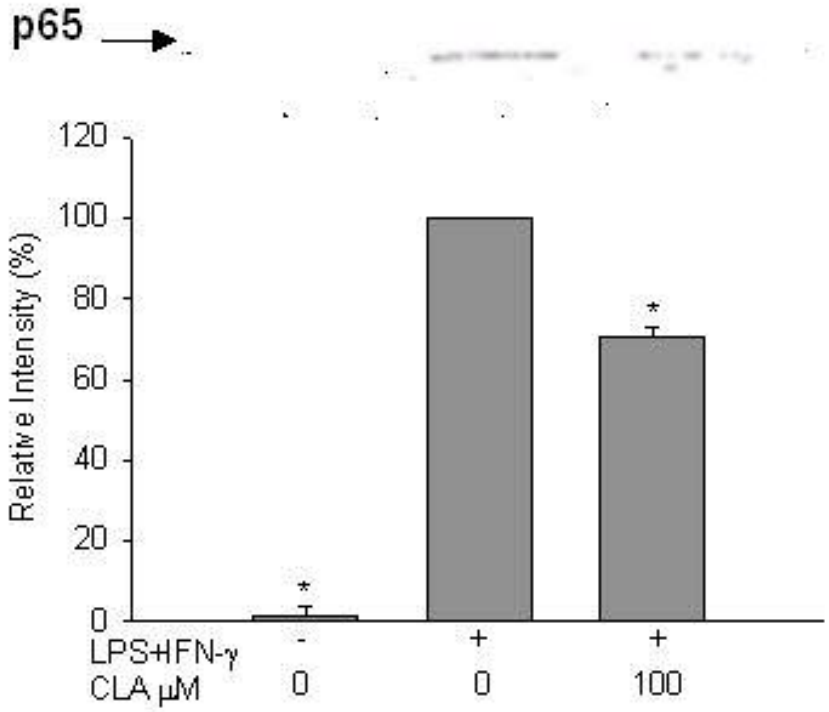
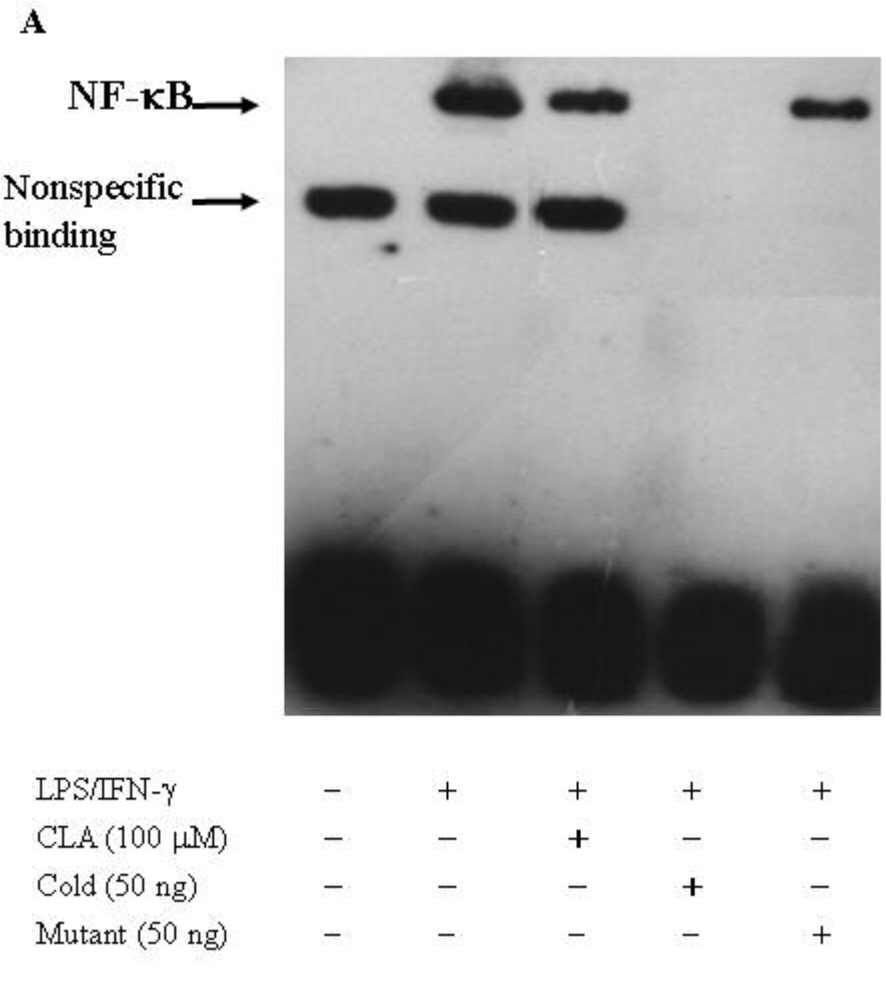
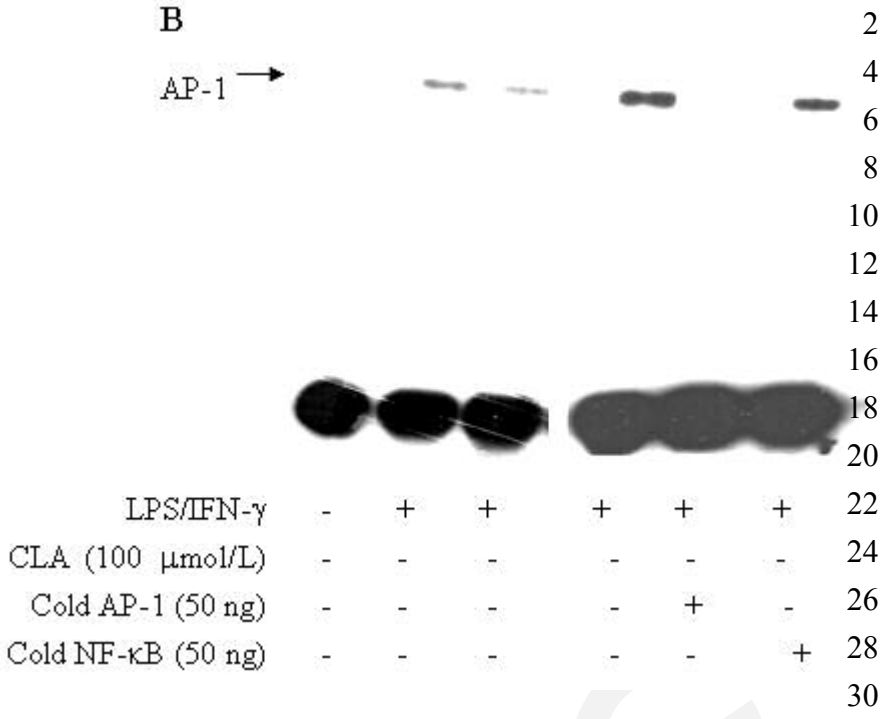


Fig. 4. Effect of CLA on LPS-IFN-γ–induced p65 protein expression in mouse mesangial cells. Cultures were preincubated with or without 100 μM CLA for 12 h and were then treated with either methanol vehicle control or a mixture of LPS (1 μg/ml) and IFN-γ (5 ng/ml) for 45 min. The nuclear fractions were used to analyze the content of p65 protein by Western blot. Relative protein levels were quantified by scanning densitometry and are expressed as a percentage of the maximal band intensity in the culture treated with LPS-IFN-γ alone. Data are the mean ± SD of at least three separate experiments, and an asterisk indicates a significant difference from treatment with LPS-IFN-γ alone ($P < 0.05$).

Fig. 5





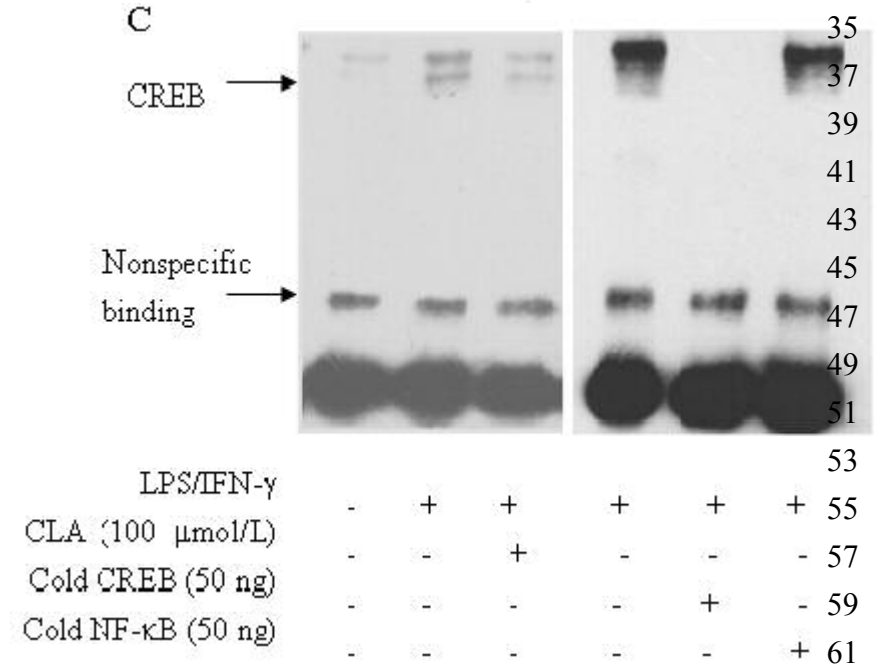


Fig. 5. Effect of CLA on LPS-IFN-γ–induced NF-κB, AP-1 and CREB nuclear protein DNA binding activity in mouse mesangial cells. Cultures were preincubated

with or without 100 μM CLA for 12 h followed by addition of either methanol vehicle control or a mixture of LPS (1 μg/ml) and IFN-γ (5 ng/ml) for 45 min (for NF-κB activity, A) and for 90 min (for AP-1 activity, B). EMSA experiment of CREB activity (C) were carried out using nuclear extract from cells treated with or without 100 μM CLA in the absence or presence of a mixture of LPS (1 μg/ml) and IFN-γ (5 ng/ml) for 12 h. EMSA experiments were carried out by using the LightShift Chemiluminescent EMSA Kit from Pierce Chemical Co. Unlabeled, double-stranded oligonucleotides (50 ng) of NF-κB, AP-1 and CREB was added for the competition assay. The unlabeled, double-stranded mutant NF-κB oligonucleotide (A) and the unlabeled, double-stranded NF-κB oligonucleotide (B and C) were added for the specificity assay. Bands were detected by using streptavidin-horseradish peroxidase and were developed by using a SuperSignal West Pico kit from Pierce Chemical Co.

CLA suppresses iNOS and TF in MES13 cells

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