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New class of linoleic acid metabolites biosynthesized by corn and rice lipoxygenases: Suppression of proinflammatory mediator expression via attenuation of MAPK- and Akt-, but not PPARγ-, dependent pathways in stimulated macrophages

Akira Murakami [a,*], Takashi Nishizawa [a], Kahori Egawa [a], Teruo Kawada [a], Yasushi Nishikawa [b], Kazuo Uenakai [b], Hajime Ohigashi [a]

[a] *Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan*
[b] *Research Center, Tamanoi Vinegar Co. Ltd., Nara, Japan*

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Abstract

In response to endogenous and exogenous stimuli, macrophages are activated to produce a cocktail of proinflammatory and anti-apoptotic mediators, thereby participating in the processes of inflammation-associated oncogenesis. Cereals, including corn and rice, have biological potentials to synthesize self-protective chemicals in order to repel the invasion of microorganisms and insects. We examined the suppressive effects of several fatty acids, including a new class of lipoxygenase metabolites of linoleic acid (LA) found in cereals, namely (±)-9-hydroxy-*trans*,*cis*-10,12-octadecadienoic acid (9-HOA from rice), (±)-13-hydroxy-10-oxo-*trans*-11-octadecenoic acid (13-HOA from corn), and (±)-10-oxo-*trans*-11-octadecen-13-olide (10-ODO from corn), on lipopolysaccharide (LPS)-induced mRNA expression of proinflammatory mediators in RAW264.7 murine macrophages. Each metabolite exhibited a suppressive activity toward nitrite production than LA, octadeca-9*Z*,11*E*-dienoic acid (a conjugated LA), and 13(*S*)-hydroxyoctadeca-9*Z*,11*E*-dienoic acid. LPS-up-regulated mRNA expression of *inducible nitric oxide synthase*, *cyclooxygenase* (*COX*)-2, *interleukin-6*, and *toll-like receptor-2*, *-4*, and *-9* was also markedly attenuated without affecting the expression levels of several constitutive genes, including *COX*-1, as detected by reverse transcription-polymerase chain reactions. In addition, Western blot and luciferase reporter assay results showed that 13-HOA suppressed the phosphorylation of mitogen-activated protein kinases (extracellular signal-regulated kinasel/2, c-Jun *N*-terminal kinasel/2, p38 mitogen-activated protein kinase), and Akt (Ser^{473}), and also attenuated degradation of inhibitor kappaB, nuclear translocation of nuclear factor kappaB (NFκB), and the transcriptional activities of NFκB and activator protein-1, both of which have essential roles in the transcription of numerous proinflammatory and oncogenic genes. In contrast, 13-HOA did not serve as a ligand for peroxisome proliferator-activated receptor-γ. Based on our findings, we propose that 13-HOA, a functionally novel LA-derivative, is a promising agent for anti-inflammatory and chemopreventive strategies with reasonable molecular mechanisms.

Keywords: Linoleic acid; Macrophage; Anti-inflammation; NFκB; AP-1; IL-6; Lipoxygenase; Toll-like receptor; iNOS; COX-2

Abbreviations: LA, linoleic acid; 9-HOA, (±)-9-hydroxy-*trans*,*cis*-10,12-octadecadienoic acid; CLA, octadeca-9*Z*,11*E*-dienoic acid; 13-HOA, (±)-13-hydroxy-10-oxo-*trans*-11-octadecenoic acid; 10-ODO, (±)-10-oxo-*trans*-11-octadecen-13-olide; LPS, lipopolysaccharide; CLA, conjugated linoleic acid; LOX, lipoxygenase; 13S-HODE, 13(*S*)-hydroxyoctadeca-9*Z*,11*E*-dienoic; iNOS, inducible nitric oxide synthase; COX, cyclooxygenase; NO, nitric oxide; PG, prostaglandin; IL, interleukin; TLR, toll-like receptor; NFκB, nuclear factor-kappaB; AP-1, activator protein-1; NSAID, nonsteroidal anti-inflammatory drugs; IFN, interferon; DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum; NO_2^-, nitrite; PBS, phosphate-buffered saline; DMSO, dimethylsulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HPLC, high performance liquid chromatography; SD, standard deviation; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF, tumor necrosis factor; TGF, transforming growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; ERK, extracellular signal-regulated kinase; JNK, c-Jun *N*-terminal kinase; MAPK, mitogen-activated protein kinase; IκB, inhibitor kappaB; HRP, horseradish peroxidase; PPAR, peroxisome proliferator-activated receptor; IR, inhibitory rate; CV, cell viability; 15d-PGJ_2, 15-deoxy-deltal2,14-PGJ_2

* Corresponding author. Fax: +81 75 753 6284.
E-mail address: cancer@kais.kyoto-u.ac.jp (A. Murakami).

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1. Introduction

Macrophages have pivotal roles in innate immunity by producing reactive oxygen and nitrogen species, cytokines, proteases, and angiogenic factors for elimination of invading bacteria, viruses, parasites, and others. Exaggerated and/or prolonged immune responses are often associated with the onset of certain diseases. In inflamed tissues, an untold number of biochemical signaling pathways are induced and/or repressed [1,2]. Of these, the production of nitric oxide (NO) and prostaglandin (PG)E_2, which are synthesized through up-regulated inducible NO synthase (iNOS) and cyclooxygenase (COX)-2, respectively, are critical steps for certain types of oncogenesis [3]. After being coupled with superoxide anion, NO is converted into peroxynitrite, which modulates biological macromolecules to form 8-nitroguanosine [4,5] and nitrotyrosine [6].

In recent years, the toll-like receptor (TLR) family has been shown to contain the key receptor components responsible for recognition of a variety of bacterial cell-wall components and CpG DNA, as well as the initiation of signal transduction pathways that subsequently lead to the production of a broad array of proinflammatory mediators [7]. Hausmann et al. presented intriguing data showing that both TLR2 and TLR4 were over-expressed in inflamed, but not normal, colonic mucosa taken from clinical specimens [8]. Conversely, exposure of renal epithelial cells to proinflammatory cytokines increased the expression of both TLR2 and TLR4 in vivo [9]. In response to proinflammatory stimuli, the above mentioned key players are known to be induced, at least in part, through the activation of a transcription factor, nuclear factor-kappaB (NFκB). In fact, endotoxin and/or proinflammatory cytokines induce *iNOS* [4], *COX-2* [4], *IL-6* [10], and *TLR* [11] genes in an NFκB-dependent manner. In addition, there is a large body of evidence showing that activator protein (AP)-l has essential roles in transcriptional activation of some of those genes [12].

It is well-established that lipoxygenase (LOX) converts arachidonic or linoleic acid (LA) to form bioactive fatty acids that have various chemical structures and physiological functions. The majority of these metabolites, together with those from the COX pathway, positively participate in the process of chronic inflammation-mediated tumorigenesis [13], whereas 13(*S*)-hydroxyoctadecandienoic acid (13S-HODE), an LA metabolized by 15-LOX-l (Fig. 1), has been reported to mediate the nonsteroidal anti-inflammatory drug (NSAID)-induced apoptotic signaling pathway, implicating that 15-LOX-l up-regulation as well as the use of 13S-HODE are novel and attractive strategies for cancer prevention [14]. Along a similar line, a group of conjugated LAs (CLAs) from animal fat sources (Fig. 1) have been shown to exert remarkable cancer preventive activities in some rodent models [15,16].

Corn germ and rice bran are known to contain another class of fatty acids, which includes (±)-9-hydroxy-*trans*,-*cis*-10,12-octadecadienoic acid (9-HOA from rice), (±)-13-hydroxy-10-oxo-*trans*-11- octadecenoic acid (13-HOA from corn), and (±)-10-oxo-*trans*-11-octadecen-13-oride (10-ODO, from corn) [17,18] (Fig. 1). They were previously characterized as cytotoxic toward several cancer cell lines [17–19], however, knowledge of their biological functions is limited. In the present study, we examined the suppressive effects of those 3 fatty acids, together with those of LA, CLA, and 13S-HODE, on lipopolysaccharide (LPS)-induced NO generation as well as on the expression of several proinflammatory genes, including *COX-2* and *iNOS*, in RAW264.7 murine macrophages. Our results

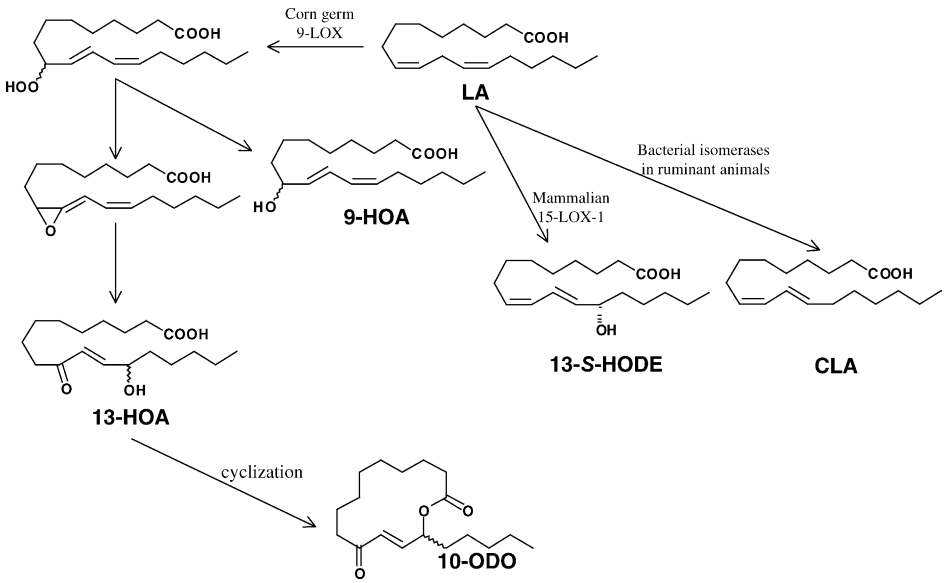

Fig. 1. Biosynthetic pathways for LA derivatives in corn germ, mammalian cells, and bacteria in ruminant animals.

showed that functionally novel cereal LOX products have a notable potential for regulating chronic inflammation-associated carcinogenesis as well as the underlying molecular mechanisms.

2. Materials and methods

2.1. Chemicals and cells

The fatty acids 9-HOA, 13-HOA, and 10-ODO were purified as previously reported [17–19]. LA and CLA (*cis*,*trans*,-9,11-octadecadienoic acid) were obtained from Calbiochem (San Diego, CA), and 13S-HODE from Sigma–Aldrich (St. Louis, MO). The purity of these fatty acids was at least 98%. Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). LPS *(E. coli* serotype 0127, B8) and interferon (IFN)-γ were purchased from Difco Labs (Detroit, MI) and Genzyme (Cambridge, MA), respectively. All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) unless specified otherwise. RAW264.7 murine macrophages and CVl monkey kidney cells were purchased from American Type Culture Collection (Manassas, VA).

2.2. Nitrite (NO_2^-) formation

RAW 264.7 cells (2×10^5) were grown to confluence in 1 mL of DMEM medium with 10% FBS on 24-well plates, then incubated in an atmosphere containing 5% CO_2 at 37 °C for 13 h. The cells were washed with phosphate-buffered saline (PBS) twice, after which the media were exchanged with FBS- and phenol-red-free media containing samples (0–100 μM) dissolved in 5 μl of dimethylsulfoxide (DMSO). After 30 min of preincubation, the cells were treated with LPS (100 ng/mL), tetrahydrobiopterin (10 mg/mL), IFN-γ (100 U/mL), and L-arginine (2 mM). After 12 or 24 h, the levels of NO_2^- and cytotoxicity were measured using Griess [20] and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, respectively. The concentrations of samples were determined to be sub-lethal ones, in which cells maintained 50% cell viability or more.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

RAW 264.7 cells (1×10^6) were grown to confluence in 5 mL of DMEM medium with 10% FBS on 6-well plates, then incubated in an atmosphere containing 5% CO_2 at 37 °C for 13 h. The cells were washed with PBS twice, after which the media were exchanged with FBS- and phenol-red-free media (2.5 mL) containing samples (0–100 μM) dissolved in 25 μl of DMSO. After 30 min of preincubation, the cells were treated with LPS (100 ng/mL), tetrahydrobiopterin (10 mg/mL), IFN-γ (100 U/mL), and L-arginine (2 mM). Following a 6-h incubation, the cells were lysed and total RNA was extracted using kits (RNeasy® mini kit and QIAshredder®, Qiagen, Valencia, CA). One microgram of total RNA was reverse transcribed using an RNA PCR kit® (Takara, Kyoto, Japan) with an oligo dT-adaptor primer, as suggested by the supplier. Then, PCR assays were performed using a thermal cycler (PTC-0100; MJ Research, Watertown, MA) for *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *iNOS*, and multiplex proinflammatory genes [*GAPDH*, *IL-6*, *tumor necrosis factor* (*TNF*)-α, *IL-1β*, *transforming growth factor* (*TGF*)-β, and *granulocyte-macrophage colony-stimulating factor* (*GM-CSF*)] using kits (Maxim Biotech, South San Francisco, CA), and for *COX-1* and *COX-2* (Ambion, Austin, TX) according to the methods shown in the manufacturer's manual. Alternatively, *TLR* genes were amplified with primers synthesized by Proligo (Boulder, CO), using 1 μL of a cDNA preparation, 45 μl of Platinum® PCR SuperMix (Invitrogen), and 2 μl of each primer (1 μM) as follows. *TLR2*: (5′-TCTgggCAgTCTTgAACATTT-3′ and 5′-AgAgTCAggTgATggATgTCg-3′), 24 cycles at 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 45 s; *TLR4*: (5′-gCAATgTCTCTggCAggTgTA-3′ and 5′-CAAgggATAA-gAACgCTgAgA-3′), 26 cycles at 95 °C for 45 s, 61 °C for 45 s, and 72 °C for 45 s; and *TLR9*: (5′-gCACAg-gAGCggTgAAggT-3′ and 5′-gCAggggTgCTCAgTg-gAG-3′), 26 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s. The PCR products were separated on 2% NuSieve® 3:1 agarose (BioWhittaker Molecular Applications, Rockland, ME) and each band was visualized using 0.01% SYBR Gold® stain (Molecular Probes, Leiden, The Netherlands). The amplified products were photographed with a digital camera and the band intensities analyzed using NIH Image software. For each target gene, we calculated the relative expression levels of mRNA in the samples as the ratio of each gene mRNA to *GAPDH* mRNA. The number of PCR cycles was optimized in such a way so that each band intensity increased proportionally as the amount of cDNA increased. Each experiment was done at least 3 times, with 1 representative picture from each shown in Figs. 3 and 4.

2.4. Western blotting

RAW 264.7 cells (2×10^6) were grown to confluence in 4 mL of DMEM medium with 10% FBS on a 60-mm dish, then incubated in an atmosphere containing 5% CO_2 at 37 °C for 13 h. The cells were washed with PBS twice, after which the media were exchanged with FBS- and phenol-red-free media (10 mL) containing 10-HOA (0 or 20 μM) dissolved in 20 μl of DMSO. After 30 min of preincubation, the cells were treated with LPS (100 ng/mL), tetrahydrobiopterin (10 mg/mL), IFN-γ (100 U/mL), and L-arginine (2 mM) for various times, as shown in the

figures. After being washed with PBS twice, the cells were separated into nuclear and cytosol fractions using a kit (Bio Vision, Research Products, Mountain View, CA). Protein concentrations were determined using a DC Protein Assay kit (Bio-Rad laboratories, Hercules, CA), with γ-globulin employed as the standard. Next, 10 or 20 μg of proteins (for the nuclear and cytosol fractions, respectively) were separated on 10% polyacrylamide gels and electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore, MA). After blocking, the membranes were incubated with rabbit anti-Pi-extracellular signal-regulated kinase (ERK)l/2, rabbit anti-ERKl/2 (Promega, Madison WI), rabbit anti-Pi-c-Jun N-terminal kinase (JNK)l/2 antibody, rabbit anti-JNKl/2 antibody, rabbit anti-Pi-p38 mitogen-activated protein kinase (MAPK) antibody, rabbit anti-p38 antibody (Cell Signaling, Beverly, MA), rabbit anti-Pi-Ser^{473} and Thr^{308} Aktl/2/3 antibodies, rabbit anti-Aktl/2/3 antibody, rabbit anti-inhibitor kappaB (IκB)-α antibody, rabbit anti-NF-κB p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and goat anti-β-actin antibody (Biochemical Technologies, Stoughton, MA) (1:1000 dilution each), and then the corresponding secondary antibodies [horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, 1:1000 dilution, Dako, Glostrup, Denmark; or HRP-conjugated anti-goat IgG, 1:1000 dilution, Dako]. The blots were developed using an ECL detection kit (Amersham Life Science, Buckinghamshire, UK). Each experiment was done at least 3 times, with 1 representative picture of each shown in Fig. 5.

2.5. Reporter assays for NFκB and AP-1 activity

The following reporter assays were done using a Mercury® Pathway Profiling System (Clontech Laboratories Inc.) with some modifications. RAW264.7 cells (3×10^5 cells/lmL) were preincubated on a 24-well plate for 12 h. Next, 625 μL of OPTI-MEM® (Invitrogen, Carlsbad, CA) and 37.5 μL of LipofectAMINE Reagent® (Invitrogen) were mixed in a tube, and 4 μg of either pNFicB- or pAP-1-luciferase vector, provided in the kit, and 4 μg of pRL-TK vector (Promega), which was served as the internal standard, were added to the 625 μL of OPTI-MEM. These were combined and allowed to stand at room temperature for 30 min, after which 5 mL of OPTI-MEM was added. After washing the cells with Hanks' buffer twice, 250 μL of transfection mixture was added to each well and the cells were incubated at 37 °C for 6 h. After washing, the cells were incubated in 1 mL of DMEM containing 10% FBS for 12 h. The cells were washed again with Hanks' buffer twice, then exposed to the vehicle (0.5% DMSO, v/v) or 20 μM of 13-HOA dissolved in DMSO in serum-free DMEM for 30 min. After stimulation of the cells with LPS (100 ng/mL) and IFN-γ (100 U/mL) for 12 h, luciferase activity in the cell lysate was determined using a Dual-Luciferase Reporter Assay Kit® (Promega).

2.6. Reporter assay for peroxisome proliferator-activated receptor (PPAR)γ ligand activity

A PPARγ ligand assay was done using a PPARγ/GAL4 chimera system and CVl cells, using a method previously reported [21]. Briefly, pM-hPPARγ, p4 × UASg-tk-luc, or pRL-CMV (internal standard) (4 μg each) was transfected into CVl cells. After 5 h, the cells were washed and incubated for an additional 19 h. The cells were then exposed to the vehicle (0.5% DMSO, v/v), 20 μM of troglitazone, 100 μM of LA, or 20 μM of 13-HOA at 37 °C for 24 h. Luciferase activity in the cell lysate was determined using a Dual-Luciferase Reporter Assay Kit (Promega).

2.7. Quantification of extracellular and intracellular 13-HOA

RAW264.7 cells (5×10^6 cells/60-mm dish) were preincubated for 13 h, then exposed to 5 ml of FBS-free DMEM containing 20 μM of 13-HOA. After 0, 30, 60, and 360 min, the medium was centrifuged to obtain the supernatant. After the resultant pellets were combined with cells on the dish, both were washed 3 times with DMEM with 10% FBS. The medium was then mixed with the supernatant and the combined fractions were extracted with an equivalent volume of ethyl acetate twice. For another experiment, 5 mL of ethyl acetate was added to the cells, then they were washed twice with DMEM with 10% FBS. Each ethyl acetate fraction thus obtained was dried with sodium sulfate and iltrated through filter paper. Filtrates from the media and cell fractions were independently evaporated in vacuo and dissolved in 50 μL of ethanol for high performance iquid chromatography (HPLC) analysis (column, YMC-Pack ODS-AQ302, YMC, Kyoto, Japan; elute, 70% methanol in H_2O; flow rate, 1.0 mL/min; detection, UV 223 nm; sample volume, 5 μL; and retention time for 13-HOA, 13 min). Quantification of 13-HOA was done using a standard curve with a proportional range (62.5 ng–5 μg). The ecovery rate of 13-HOA was 95% or more (data not shown).

2.8. Statistical analysis and inhibitory rate (IR)

Each experiment was done four times unless specified otherwise, with the data shown as he mean ± standard deviation (mean ± S.D.) values. The statistical significance of differences between groups in each assay was assessed using a Student's *t*-test (two-sided) that issumed unequal variance. The IR in each assay was calculated using the following equation: IR (%) = {1 − [(test sample data) − (blank data)] [(positive control data) − (blank data)] − 1} × 100.

3. Results

3.1. Effects on NO_2^- production

Stimulation of RAW264.7 murine macrophages with LPS for 12 h increased the NO_2^- concentration in the medium by 5.5-fold as compared to the vehicle alone (Fig. 2A). Treatment with 20 μM of LA, but not 100 μM of LA, significantly suppressed NO_2^- formation (IR = 41%), with a similar tendency observed for 13S-HODE (IR = 31% at 20 μM, no significant suppression at 100 μM). CLA inhibited NO generation by 57% at a

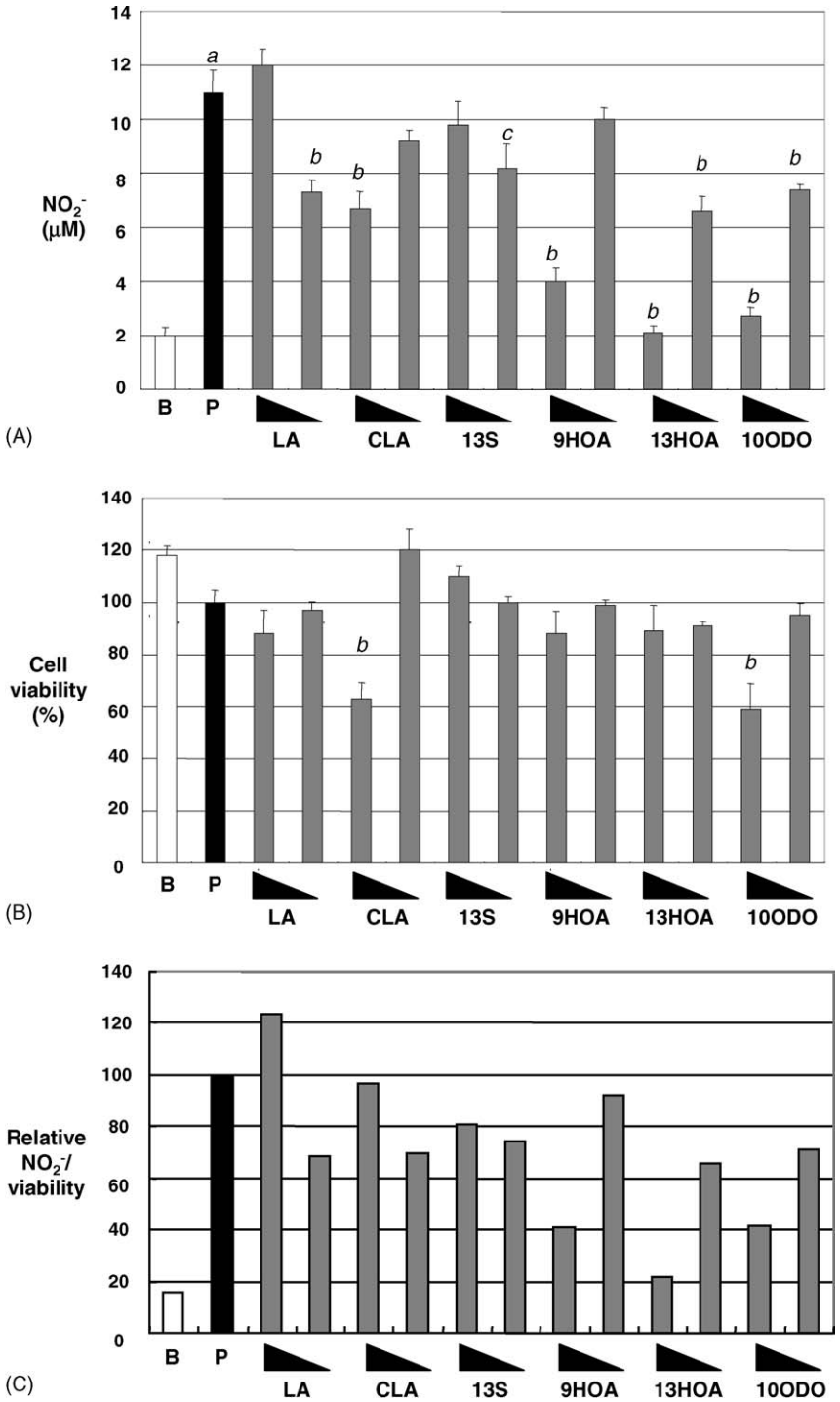

Fig. 2. Effects of LA and its derivatives on LPS-induced NO_2^- formation. RAW264.7 macrophages were treated with LPS and the vehicle (0.5% DMSO, v/v) or the test samples for 12 h. Media NO_2^- concentrations (Panel A) and cell viability (Panel B) were determined by Griess and MTT assays, respectively, as described in Section 2. B, blank; P, LPS (100 ng/mL); 13S, 13S-HODE. LA, CLA, and 13S-HODE (100 or 20 μM each); 9-HOA and 13-HOA (20 or 4 μM); and 10-ODO (4 or 0.8 μM) were tested. [a] $P < 0.001$ vs. blank, [b] $P < 0.001$ vs. LPS, [c] $P < 0.01$ vs. LPS, respectively, using Student's *t*-test. Panel C, the relative NO_2^-/cell viability values in each test. The value of positive control (vehicle + LPS) was expressed as 100.

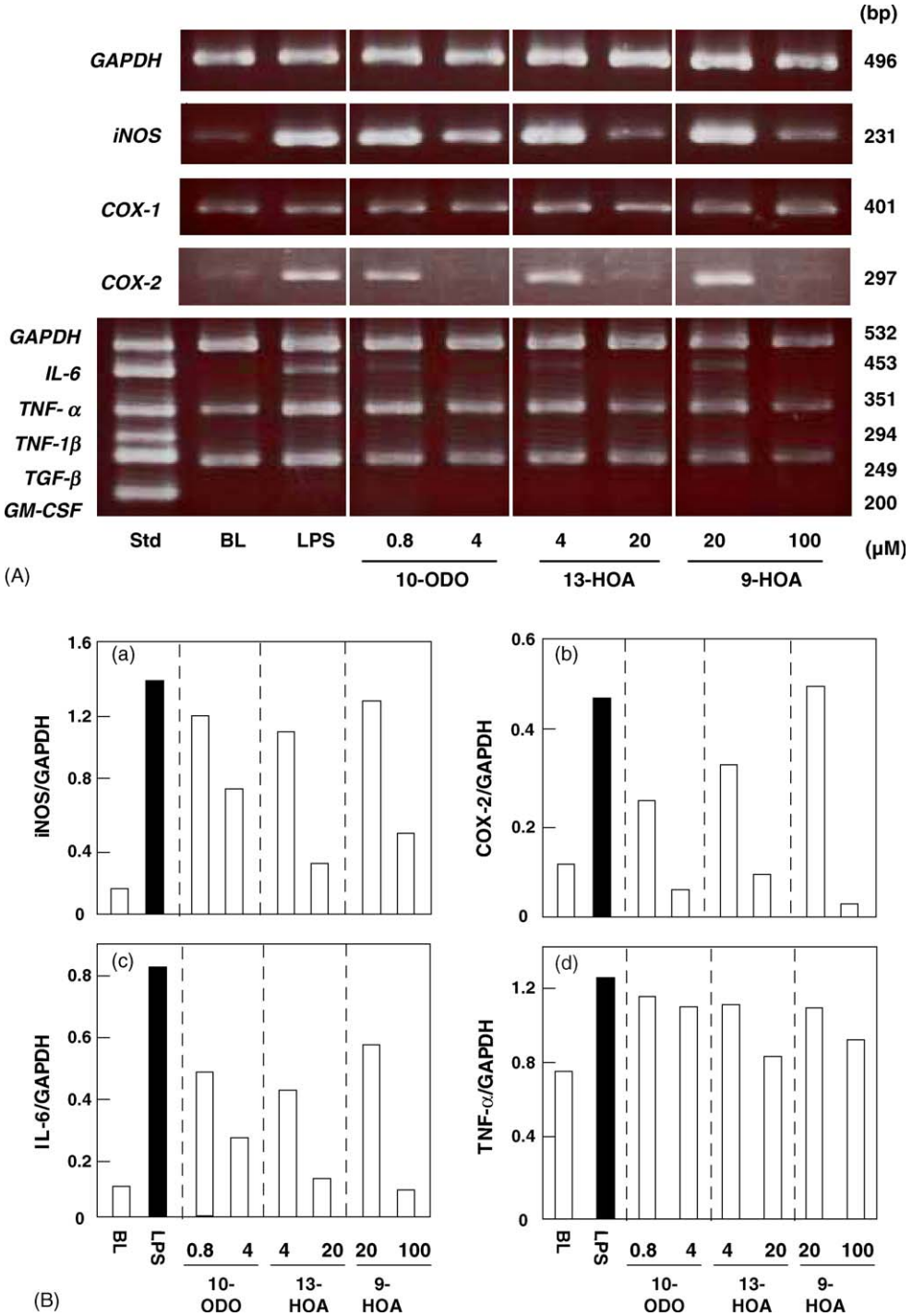

Fig. 3. Effects of 10-ODO, 13-HOA, and 9-HOA on LPS-induced proinflammatory gene mRNA expression. RAW264.7 macrophages were treated with LPS and the vehicle (0.5% DMSO, v/v) or the test samples for 6 h. RT-PCR was done with *GAPDH* transcripts as the internal control as described in Section 2. Panel A: photographs of each PCR product. Std, each standard transcript; BL, blank. The data in Panel A are expressed as bar graphs in Panel B: a, *iNOS*; b, *COX-2*; c, *IL-6*; d, *TNF*-α.

concentration of 100 μM, but not at 20 μM. 13-HOA at a concentration of 20 μM abrogated LPS-induced NO_2^- production and significantly attenuated by 63% without cytotoxicity, while 9-HOA (4 μM) failed to suppress the production. 10-ODO showed significant suppression at concentrations of 4 and 0.8 μM (IRs = 92 and 40%; CVs = 59 and 95%, respectively). We then calculated the values of NO_2^- per cell viability, and that in positive control cells treated vehicle and LPS was expressed as 100 to discern the effect of cytotoxicity on the NO_2^- generation suppressive activities. As shown in Fig. 2C, the effect of CLA at a higher concentration was suggested to be due to cytotoxicity and similar tendencey was seen for 10-ODO, but not 13-HOA. These results showed that 13-HOA was

the most selective NO generation suppressant tested, with lower cytotoxicity.

3.2. Effects on expression of proinflammatory genes

We also examined the suppressive effects of 10-ODO, 13-HOA, and 9-HOA on the LPS-induced expression of *iNOS* mRNA, as well as that of other proinflammatory genes (*COX-1*, *COX-2*, *IL-6*, *TNF-α*, *IL-1β*, *TGF-β*, and GM-CSF) and a house-keeping gene (*GAPDH*) by RT-PCR (Fig. 3A and B). *GAPDH*, *COX-1*, *TNF-α*, and *TGF-β* mRNA was expressed in a constitutive manner, and the expression levels were unchanged upon stimulation, except for the case of *TNF-α,* whose mRNA expression level was increased by 1.6-fold as compared to the vehicle alone (Fig. 3A and B, panel d). No expression of *IL-1β* or *GM-CSF* was detectable under any of the experimental conditions. Stimulation of cells for 6 h resulted in enhancement of *iNOS*, *COX-2,* and *IL-6* mRNA expression by 9.2-, 3.6-, and 8.4-fold, respectively (Fig. 3B, panels a–c). Further, 10-ODO, 13-HOA, and 9-HOA at higher concentrations (4, 20, and 100 μM, respectively) exhibited profound suppressive effects on the expression of *iNOS* (IRs = 52, 88, and 73%, respectively), *COX-2* (IR ∼100% each), *IL-6* (IRs = 78, 96, and 101%, respectively), and *TNF-α* (IRs = 31, 84, and 65%, respectively). Even at lower concentrations, each showed notable suppression toward *IL-6* expression (IR range of 35–56%), whereas LA showed no suppression of any of the proinflammatory genes examined (data not shown).

3.3. Effects on TLR expression

TLR family members are known to be expressed as pathogen sensors in certain immune cells, including macrophages, to transduce proinflammatory signals. Once stimulated, they are either up-regulated or down-regulated, depending upon the biological properties of invading agents and their isotypes. The present RT-PCR analyses revealed that *TLR2* mRNA was intensely expressed, and mRNA of both *TLR4* and *TLR9* slightly expressed, in non-treated RAW264.7 cells (Fig. 4A). Upon stimulation with LPS, the level of *TLR2* expression was decreased by 33% after 3 h and 65% after 6 h, whereas the levels of *TLR4* and *TLR9* were up-regulated by 1.9- and 2.0-fold, respectively. The expression levels of *TLR2*, *TLR4*, and *TLR9* (Fig. 3B–D, respectively) mRNA were notably decreased following treatment with 20 μM of 13-HOA (IRs = 47–50% at 3 h, 87–89% at 6 h).

3.4. Effects on MAPK and Akt phosphorylation, IkB-α degradation, and NFκB nuclear translocation

As shown in Fig. 5, simulation of RAW cells with LPS for 30 and 60 min led to a time-dependent marked increase in the amount of phosphorylated ERK2 and, to a much

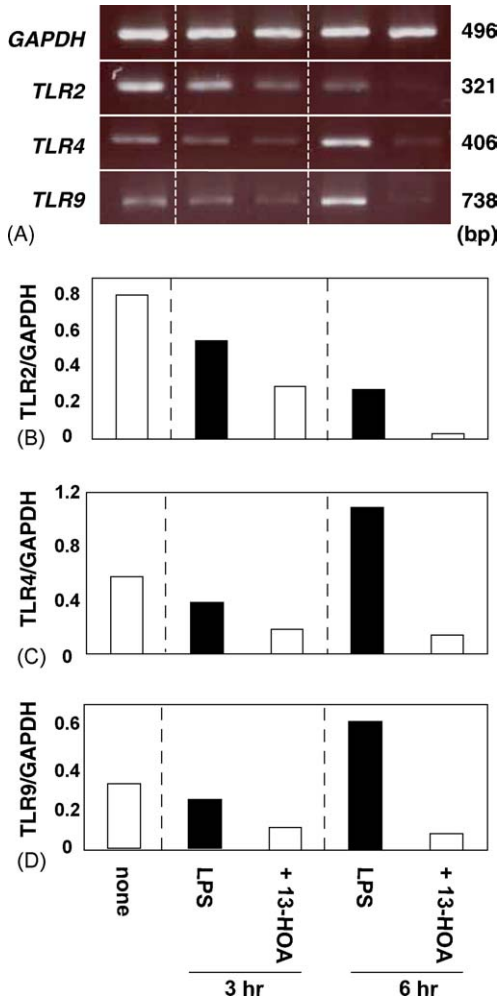

Fig. 4. Effects of 13-HOA on LPS-induced TLR mRNA expression. RAW264.7 macrophages were treated with LPS and the vehicle (0.5% DMSO, v/v) or 13-HOA (20 μM) for 3 or 6 h. RT-PCR was done with *GAPDH* transcripts as the internal control as described in Section 2. Panel A: photographs of each PCR product. The data in Panel A are expressed as bar graphs in Panel B (*TLR2*), C (*TLR4*), and D (*TLR9*).

lesser extent, ERK1. Consistent with those results, the protein levels of the corresponding inactive forms, ERK1 and 2, were decreased. In contrast, the active forms of ERK1 and 2 were not detected when the cells were pretreated with 20 μM of 13-HOA for 30 min. Further, JNK2, but not JNK1, was similarly activated by LPS, while 13-HOA caused a complete blockade. Notably, LPS transiently induced p38 MAPK phosphorylation, which peaked at 30 min, though that effect was not seen in 13-HOA-treated cells. LPS also increased Akt (protein kinase B) by phosphorylation at the Ser^{473} residue and, interestingly, pretreatment with 13-HOA dephosphorylated it from 0 to 60 min. The levels of both phosphorylated Akt at the Thr^{308} residue and its inactive form were constant. On the other hand, LPS treatment led to the degradation of IκB-α protein time-dependently until it was not detected after 60 min. The expression level of IκB-α, was unchanged following 13-HOA treatment for 0–60 min.

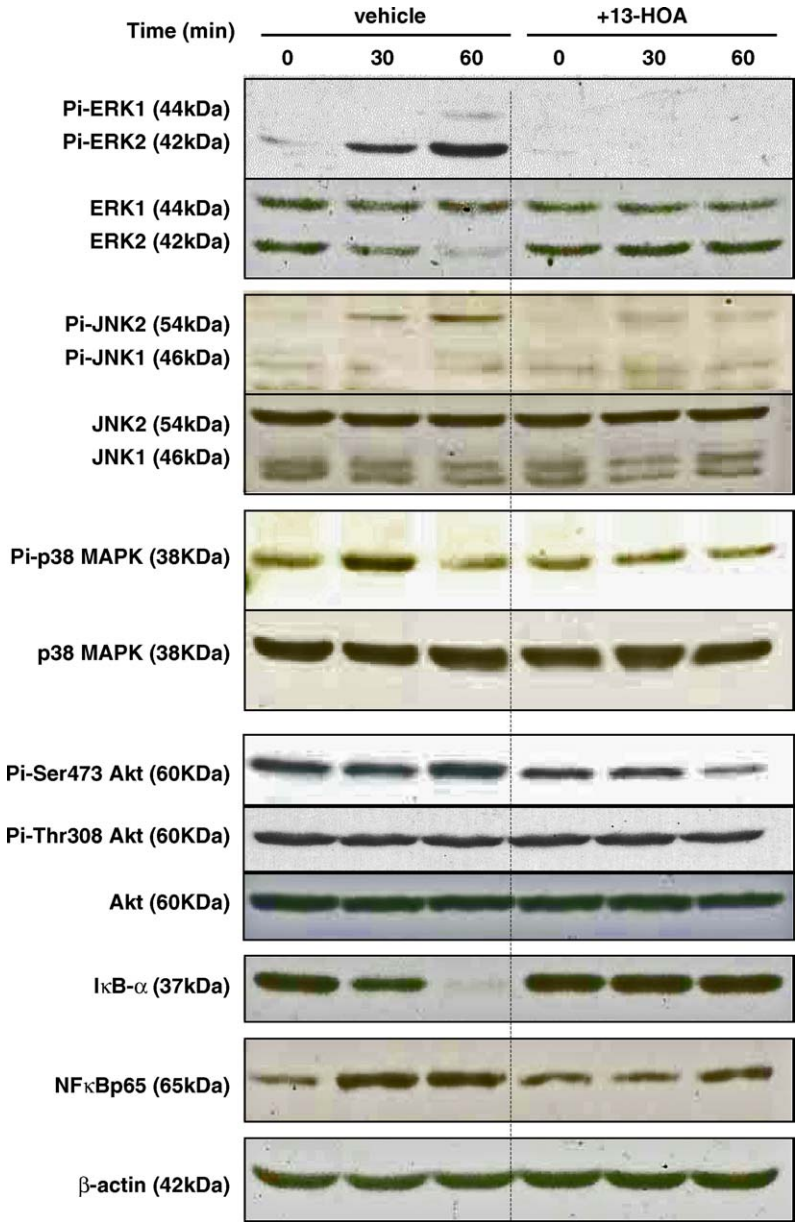

Fig. 5. Effects of 13-HOA on LPS-induced MAPK and Akt activation, degradation of IkB protein, and nuclear translocation of NFκB p65. RAW264.7 macrophages were treated with LPS and the vehicle (0.5% DMSO, v/v) or 13-HOA (20 μM) for the designated times. Western blot assays were done with β-actin as the internal control as described in Section 2. NFκB p65 protein was detected from the nuclear proteins and others from the total cell lysates.

Further, there was a remarkable increase in the amount of NFκB p65 protein in the nuclear fraction of the cell lysate 30 min after LPS stimulation, whereas 13-HOA did not allow an LPS-increased translocation of this transcription factor component.

3.5. Effects on NFκB and AP-1 transcription and PPARγ ligand activities

Induction of many, if not all, of the genes whose expression was suppressed by 13-HOA (Figs. 3 and 4) is regulated by NFκB and AP-1 transcription factors. In addition, activation of those factors is involved in the pathogenesis of some diseases including cancer, while there are number of dietary factors known to prevent carcinogenesis through attenuation of their functions [3]. As shown in Fig. 6A and B, LPS treatment for 12 h increased relative NFκB- and AP-1-luciferase reporter activities by 4.1- and 2.3-fold, respectively, and 13-HOA pretreatment suppressed them significantly. Subsequently, a PPARγ ligand assay was done using the PPARγ/GAL4 chimera system in CV1 cells [21], with troglitazone [22] used as a positive control. Treatment of CV1 cells with 20 μM of troglitazone increased reporter activity by 7.6-fold and that with 100 μM of LA increased the activity by 4.5-fold, as compared to the vehicle alone (Fig. 6C). In contrast, 13-HOA at 20 μM did not show any PPARγ ligand activity and the metabolite was cytotoxic at a

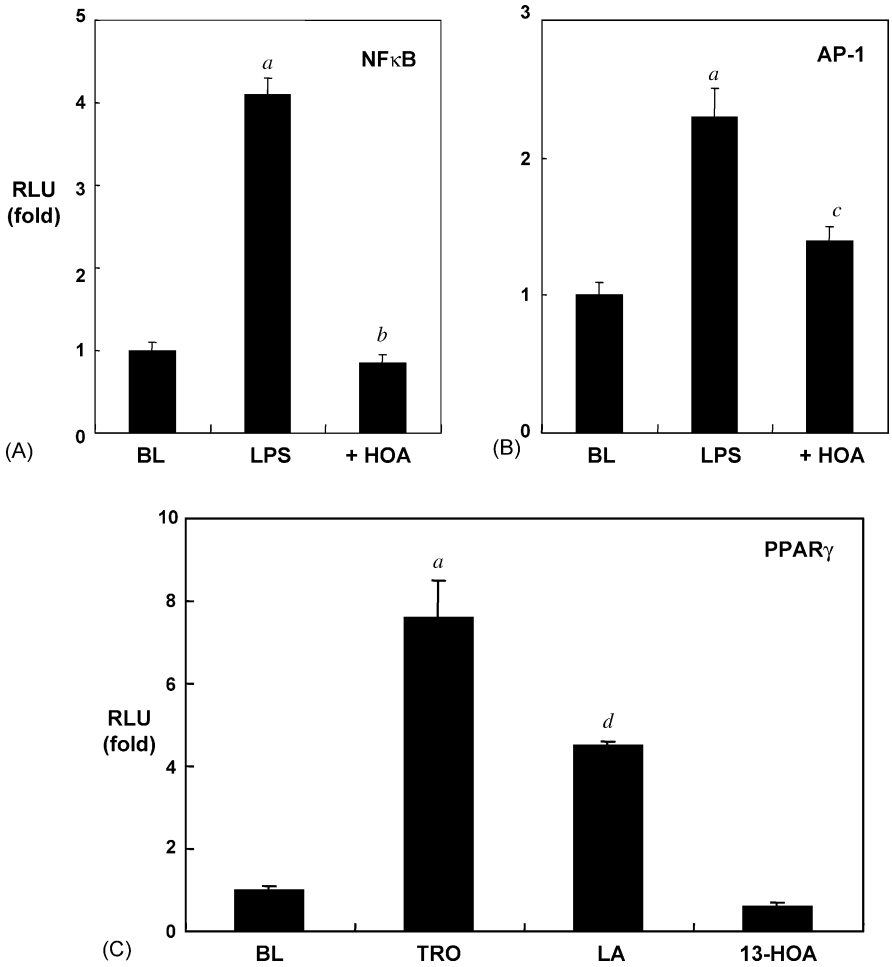

Fig. 6. Effects of 13-HOA on LPS-induced transcriptional activation of NFκB (A) and AP-1 (B), and PPARγ ligand activity (C). Panels A and B: RAW264.7 macrophages were subjected to transfection with either a pNFκB- or pAP-1-liciferase vector, with a pRL-TK vector used as the internal standard, for 6 h. After washing, the cells were exposed to LPS and the vehicle (0.5% DMSO, v/v) or 13-HOA (20 μM) for 12 h. Panel C: PPARγ ligand assays were done using a PPAR(/GAL4 chimera system with CVl cells as reported previously (26). Briefly, pM-hPPARγ, p4 × UASg-tk-luc, and pRL-CMV (internal standard) vectors were transfected into CVl cells. After 5 h, the cells were washed and incubated for 19 h. The cells were then exposed to the vehicle (0.5% DMSO, v/v), 20 μM of troglitazone, 100 μM of LA, or 20 μM of 13-HOA for 24 h. Luciferase activity in the cell lysate was determined as described in Section 2. RLU, relative luciferase activity units; BL, blank; TRO, troglitazone. $^{a}P < 0.01$ vs. blank, $^{b}P < 0.01$ vs. LPS, $^{c}P < 0.05$ vs. LPS, $^{d}V < 0.05$ vs. blank, respectively, using Student's *t*-test.

concentration of 100 μM (data not shown). In addition, 20 μM of 13-HOA did not produce any changes in the expression level of PPARγ mRNA and protein in RAW cells (data not shown).

3.6. HOA scarcely incorporated into cell as an intact structure

Based on our findings, we speculated that HOA acts extracellularly and/or upstream of the LPS-induced signaling cascade. Next, we collected media and cellular fractions from RAW264.7 cell cultures to which 20 μM of 13-HOA was added, and quantified the concentrations of 13-HOA using HPLC. As shown in Fig. 7, the concentration of 13-HOA in medium decreased in a time-dependent manner and was scarcely detectable at 6 h. Further, we did not observe significant amounts of the compound within the cells (<5%) from 30 min to 6 h.

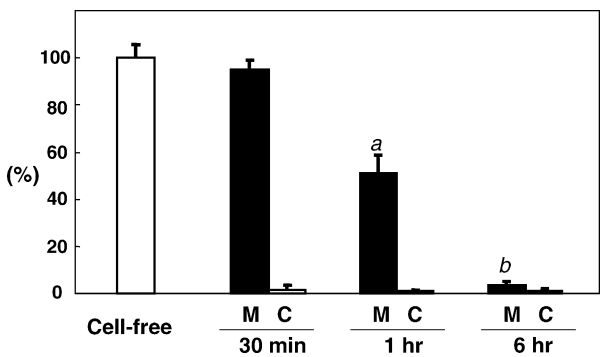

Fig. 7. Distribution of 13-HOA in a cell culture system. RAW264.7 cells were exposed to 20 μM of 13-HOA for 0, 30, 60, and 360 min. Next, the media and cells were extracted with ethyl acetate for HPLC analysis as described in Section 2. The data are expressed with the content of 13-HOA in cell-free medium considered to be 100%. The recovery rate of 13-HOA from cell-free medium was 95% or more (data not shown). M, media; C, cells. $^{a}P < 0.05$ and $^{b}P < 0.001$ vs. medium from 30-min exposure using Student's *t*-test.

4. Discussion

Chronic activation of immune cells such as macrophages is known to be highly involved in several processes of cancer development, including tumor progression and metastasis [23–27]. In the present study, 13-HOA was shown to substantially suppress LPS-induced NO generation in macrophages, which has been proposed to be associated with colon cancer development [28]. The biochemical ability of 13-HOA to highly attenuate the expression of proinflammatory genes seen in previous studies of *COX-2* [29], *iNOS* [30], *TNF*-α [31], and *IL-6* [32] knockout mice, in which tumor development was dramatically reduced, may demonstrate its cancer prevention potential. In addition, we previously reported that some dietary phytochemicals with cancer preventive potentials are able to attenuate the proinflammatory actions of immune cells in vivo [33–35]. Thus, we propose a hypothesis that 13-HOA has a notable potential for anti-inflammatory cancer prevention.

Polyunsaturated fatty acids, such as arachidonic acid and LA, are the substrates for several types of COXs and LOXs to form bioactive metabolites that enhance or suppress carcinogenic processes. In addition to the well-recognized importance of COX-2 regulation in cancer development and progression, recent studies have noted that modulation of 15-LOX-l activity as well as its product, 13S-HODE, is anticipated to prevent or reverse carcinogenesis in certain models [14]. For example, early work done by Shureiqi et al. found decreased levels of both 15-LOX-l and 13S-HODE in colon cancer and transformed colon cancer cell lines [36]. Thereafter, they and another group reported that 15-LOX-l mediated NSAIDs-induced apoptotic signaling pathways in colon [37] and gastric [38] cancer cell lines. In addition, NSAIDs were able to restore 15-LOX-l expression in esophagus cancer cells, whereas their effects on prostate cancer remain controversial [39,40]. The present study found for the first time that LOX metabolites of LA from dietary plants have remarkably higher suppressive activities toward proinflammatory genes expression as compared to LA, CLA, and 13S-HODE, again suggesting the efficacy of these functionally novel fatty acids, 13-HOA in particular, for the prevention of chronic inflammation-associated carcinogenesis.

As noted above, the production of 13S-HODE was found to be regulated by 15-LOX-l in mammalian cells (Fig. 1). CLAs, which are generally found in food sources such as beef and lamb as well as their dairy products, are believed to be formed from LA by the functions of bacterial isomerases in ruminant animals [41]. Alternatively, oral feeding of vaccenic acid led to its conversion to CLA by delta 9-desaturase in rats, thereby exhibiting cancer preventive activity in their mammary glands [42]. It has been proposed that 9-LOX plays a central role in the production of 9-HOA and 13-HOA in corn germ (Fig. 1). These bioactive lipids are derived from 9-hydroperoxy-*trans*,-*cis*-10,12-octadecadienoic acid, which is an LA metabolite by 9-LOX [43], after which 13-HOA is cyclized to form 10-ODO by unknown mechanisms.

The molecular mechanisms by which 13-HOA highly attenuates the expression of proinflammatory genes in macrophages are not fully understood. However, it is well known that certain exogenous and endogenous stimuli, including endotoxin exposure, activate the transcription factor NFκB, a master regulator for induction of numerous proinflammatory genes [44,45], including *iNOS*, *COX-2*, *IL-6,* and *TLRs* [4,11,12,46]. Thus, we hypothesized that 13-HOA is able to attenuate the expression of proinflammatory genes through a blockade of NFκB activation. Although 13-HOA abrogates the LPS-induced activation of ERK1/2, JNK1/2, and p38 MAPK, the relevance of MAPK inhibitory activities toward NFκB and AP-1 suppression, as well as its inhibition of the expression of proinflammatory genes, remain to be fully established. Nonetheless, some important observations can be cited, as follow.

Several independent groups have reported that LPS-induced NFκB activation pathways are related to the activation of protein kinase C, ERK1/2, and p38 MAPK, but not JNK1/2, in mouse macrophages [47–49]. On the contrary, 13S-HODE up-regulated MAPKs in a prostate carcinoma cell line [40], though the effects of 13-HOA on MAPK activity in the prostate have not been addressed. In parallel, AP-1 is a transcription factor shown to have a dimeric complex including Jun and Fos, as well as others, and essential roles in inflammation and tumorigenesis [50] by inducing numerous genes, including COX-2 [51]. Based on the established role of JNK1/2 in the transcriptional activation of AP-1 [17], it is reasonable to link the suppression of JNK activation by 13-HOA to that reduction in AP-1 activity. In the present study, LPS triggered the activation of JNK2, but not JNK1, in RAW264.7 macrophages and 13-HOA abolished JNK2 phosphorylation (Fig. 5). It is interesting to note that JNK2, but not JNK1, was reported to be involved in phorbol ester-induced tumor promotion in mouse skin [52], suggesting that 13-HOA is an effective anti-tumor promoter in mouse skin, which was also seen in our recent study (A. Murakami et al., in preparation).

A noticeable chemical moiety in 13-HOA is the α,β-unsaturated carbonyl group (also known as the *enone* structure), which is not present in LA, CLA, or 13S-HODE. This type of Michael reaction acceptor [53] has been shown to exert versatile biochemical and biological functions [54–60]. Further, Rungeler et al. provided intriguing data showing that sesquiterpene lactones containing this reactive moiety may directly modify cysteine residues of NFκB p65 by alkylation, thereby abrogating its DNA binding activity [61]. An endogenous PG that belongs to the above-mentioned category is 15-deoxy-deltal 2,14-prostaglandin J_2 (15d-PGJ_2) [62]. There is ample evidence showing that 15d-PGJ_2 inhibits NFκB activation [63] via a direct blockade of IκB kinase (IKK) [64], which causes

phosphorylation of IκB protein, a suppressive partner of NFκB, leading to the repression of NFκB targeting genes. Interestingly, some independent studies reported that the cyclopentanone PG directly targets ERK1/2 [65], though contrasting findings have also been presented [66]. Nevertheless, direct interactions of 13-HOA with ERK1/2, IKK, and NFκB p65 should be explored in the future. Another known target of agents that have the α,β-unsaturated carbonyl group is PPARs, which are nuclear hormone receptors whose endogenous ligands are fatty acids. Although LA was confirmed to serve as a PPARγ ligand in the present chimera system, our present data contradict the notion that 13-HOA has a similar property (Fig. 6C). On the other hand, our results for 13-HOA distribution in the present cell culture system (Fig. 7) suggest that this compound either works mainly in the extracellular compartment and immediately metabolized to be undetectable metabolite or that its intracellular, undetectable metabolite is responsible for exerting activity. Intriguingly, Bochkov et al. suggested that a phosphorylcholine interacts directly with the LPS complex for protection from endotoxin-induced tissue damage [67]. However, it must be noted that the suppressive mode of action of 13-HOA is not LPS signaling pathway-specific, because it also attenuated the expression of phorbol ester-induced proinflammatory gene expression in THP-1 human monocytic cells (Murakami et al., unpublished data). Thus, identification of the active principle remains to be accomplished in the future.

In conclusion, we found that 13-HOA, a functionally novel fatty acid derived from LA by corn and rice LOXs, markedly attenuated the expression of proinflammatory genes in LPS-stimulated macrophages via a blockade of both NFκB and AP-1, suggesting its potential for cancer prevention activities in some organs, including the colon. In fact, 13-HOA exhibited a pronounced anti-tumor promotional activity in mouse skin in a two-stage carcinogenesis model, as well as suppression of proinflammatory gene expression (Murakami et al., in preparation). While the purity and availability of CLAs and 13S-HODE is limited, 13-HOA can be prepared in large quantities by treating LA with corn LOXs, which are readily extracted from corn germ. Further investigations of its in vivo cancer prevention efficacy and mechanistic studies are warranted, and currently in progress in our laboratory.

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