Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4*

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SUMMARY

Results from our previous studies demonstrated that activation of toll-like receptor 4 (Tlr4), the lipopolysaccharide (LPS) receptor, is sufficient to induce NFκB activation and expression of inducible cyclooxygenase (COX-2) in macrophages. Saturated fatty acids (SFAs) acylated in Lipid A moiety of LPS are essential for biological activities of LPS. Thus, we determined whether these fatty acids modulate LPS-induced signaling pathways and COX-2 expression in monocyte/macrophage cells (RAW 264.7). Results show that SFAs, but not unsaturated fatty acids (UFAs), induce NFκB activation and expression of COX-2 and other inflammatory markers. This induction is inhibited by a dominant-negative Tlr4. UFAs inhibit COX-2 expression induced by SFAs, constitutively active Tlr4 or LPS. However, UFAs fail to inhibit COX-2 expression induced by activation of signaling components downstream of Tlr4. Together, these results suggest that both SFA-induced COX-2 expression and its inhibition by UFAs are mediated through a common signaling pathway derived from Tlr4. These results represent a novel mechanism by which fatty acids modulate signaling pathways and target gene expression. Furthermore, these results suggest a possibility that propensity of monocyte/macrophage activation is modulated through Tlr4 by different plasma free fatty acids, which in turn can be altered by kinds of dietary fat consumed.
INTRODUCTION

Cyclooxygenase [COX; prostaglandin endoperoxide (PGH2) synthase] catalyzes the conversion of arachidonic acid to prostaglandin endoperoxide. This is the rate-limiting step in prostaglandin (PG) and thromboxane biosynthesis. Two isoforms of COX have been cloned from various animal cells: constitutively expressed COX-1 (1-5) and mitogen-inducible COX-2 (6-11). Numerous studies have demonstrated that the levels of PGs in various tumors, or the tumor’s biosynthetic capacity of PGs, are greater when compared with normal tissues (12-16). Recently, it has been shown that the inducible form of COX is overexpressed in sites of inflammation and in many types of tumor tissues (17-20). Overexpression of COX-2 in tumor tissues occurs in both tumor cells and stromal cells including macrophages (21). What causes the overexpression of COX-2 in such pathological states is not clearly understood. COX-2 belongs to a family of immediate early response genes which do not require precedent protein synthesis for their expression (22). Therefore, elucidating the signaling pathways leading to the expression of COX-2 is a key to understanding why COX-2 is overexpressed in such pathological states, and can provide critical information for identifying potential targets of modulation by pharmacological and dietary factors.

COX-2 expression is induced by various mitogenic stimuli in different cell types (6, 9, 11, 23). The cis-acting NFκB element is present in the 5’-flanking regions of COX-2 genes of different species (24, 25). Results from our previous studies demonstrated that the activation of NFκB is required to induce maximal expression of COX-2 in the lipopolysaccharide (LPS)-stimulated macrophage cell line (26, 27). Pro-inflammatory
cytokines, such as TNFα and IL-1, also activate NFκB and induce COX-2 expression in many cell types (28, 29).

The recent finding that murine Tlr4 is the LPS receptor (30) provided a new impetus in elucidating LPS-induced signaling pathways and target gene expression. Results from our previous studies indicated that murine Tlr4 confers LPS responsiveness, and that activation of Tlr4 is sufficient to induce NFκB activation and expression of COX-2 in macrophages (27). The lipid A moiety possesses most of the biological activities of LPS (31). Lipid A of Escherichia coli and Salmonella typhimurium is a β,1-6 linked disaccharide of glucosamine, acylated with R-3-hydroxylaurate or myristate and phosphorylated at positions 1 and 4’. The 3-hydroxyl groups of these saturated fatty acids are further 3-O-acylated by lauric acid, myristic acid or palmitic acid (31). These acyl-linked saturated fatty acids are subject to hydrolysis by acyloxyacyl hydrolase; the deacylated LPS loses its endotoxic properties (32, 33). This implies that fatty acids acylated in Lipid A may play an important role in LPS-mediated signaling pathways. In light of the finding that murine Tlr4 is the LPS receptor (30), it is important to determine whether these fatty acids modulate Tlr4-mediated signaling pathways and the expression of target gene products. If they do, this will represent a new paradigm for the mechanism by which gene expression is regulated by fatty acids.

Activation of monocytes/macrophages is an important initial step in the cascades of events leading to many inflammatory diseases including endotoxemia (34). If activation of macrophages is modulated by types of fatty acids through Tlr4, it can be inferred that risk for such diseases may also be modified by different types of fatty acids.
EXPERIMENTAL PROCEDURES

Reagents—All saturated and unsaturated fatty acids were purchased from Nu-Chek (Eslyan, MN). Rumenic acid [9(Z), 11(E)-octadecadienoic acid; conjugated linoleic acid (cLA)] was purchased from Matreya (Pleasant Gap, PA). Lipopolysaccharide (LPS) was purchased from DIFCO (Detroit, Michigan). Bovine serum albumin (BSA, fatty acid free and low endotoxin, Cat.No. A8806) and human recombinant TNFα were purchased from Sigma. Polyclonal antibodies for COX-2 were prepared and characterized as described previously (21, 23). Antibodies for iNOS and IL-1α were purchased from Santa Cruz Biotech (Santa Cruz, CA). Donkey anti-rabbit immunoglobulin G (IgG) antibodies conjugated to horseradish peroxidase were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). Enhanced chemiluminescence (ECL) western blotting detection reagents were purchased from Amersham Corp (Piscataway, NJ). SuperFect Transfection Reagent was purchased from Qiagen (Valencia, CA). Luciferase Assay System and β-galactosidase Enzyme System were purchased from Promega (Madison, WI). All other reagents were purchased from Sigma unless otherwise described.

Cell culture—RAW 264.7 cells (a murine macrophage-like cell line, ATCC TIB-71) or HT-29 cells (a human colon adenocarcinoma cell line, ATCC HTB-38) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, Intergen) and 100 units/ml Penicillin and 100 µg/ml Streptomycin (GIBCO-BRL) at 37°C in a 5% CO2/air environment. Cells (2 x 10⁶) were plated in 60-mm dishes (Falcon) and cultured for an additional 18 hours to allow the
number of cells to approximately double. Cells were maintained in serum-poor (0.25% FBS) medium for another 18 hours prior to the treatment with indicated reagents.

Preparation of fatty acids-albumin complexes—All fatty acids were solubilized in ethanol. They were combined with fatty acid free and low endotoxin BSA at a molar ratio of 10:1 (fatty acid: albumin) in serum poor medium (0.25% FBS). Fatty acids-albumin complex solution was freshly prepared prior to each experiment.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting—These were performed as previously described (26, 35). Briefly, solubilized proteins were subjected to 8% SDS-PAGE for COX-2, iNOS, IL-1α and GAPDH immunoblot analyses. Following electrophoresis, the gel was transferred to a PVDF membrane and the membrane was blocked to prevent non-specific binding of antibodies in TBS-T [20 mM Tris HCl, 137 mM NaCl, 0.05% (v/v) Tween 20, pH 7.6] containing 5% non-fat dried milk (Carnation). Immunoblotting was performed using respective polyclonal antibodies followed by incubation with anti-rabbit IgG coupled to horseradish peroxidase. The membrane was exposed on an X-ray film (Kodak) using ECL western blot detection reagents (Amersham).

Plasmids—The luciferase reporter plasmids (pGL2) containing the promoter region of the murine COX-2 gene (-3201/+93 or -1017/+93) were provided by David Dewitt (Michigan State University, East Lansing, MI). To prepare the wild-type COX-2 promoter fragment, polymerase chain reaction (PCR) was performed with the primers named Kpn-COX2-For and Hind-COX2-Rev using the murine COX-2 (-1017/+93) luciferase reporter plasmid as a template. To prepare the mutant COX-2 promoter fragment containing mutated NFκB site, Kpn-COX2-Fmut and Hind-COX2-Rev were
used as primers. Each PCR fragment was inserted into the *Kpn*I and *Hind*III sites of pGL2 to generate the wild-type or mutant COX-2 (-410/+86) luciferase reporter constructs, respectively. Sequences for wild-type NFκB site, GGGATTCCC, was changed to GGCCTTCCC. All promoter sequences were confirmed by DNA sequencing. The primers used are as follows: Kpn-COX2-For, 5’-GACGGTACCGAGAGGTGAGGGATTCCC-3’; Hind-COX2-Rev, 5’-CAGAAGCTTGGTGAGCTGGCAGGATG-3’; Kpn-COX2-Fmut, 5’-GACGGTACCGAGAGGTGAGGGCCTTCCC-3’. 2xNFκB-luciferase reporter construct was a gift from Frank Mercurio (Signal Pharmaceuticals, San Diego, CA). HSP70-β-galactosidase reporter plasmid was from Robert Modlin (University of California, Los Angeles, CA). The expression plasmids for a constitutively active form of Tlr4 (ΔTlr4) and a dominant-negative mutant, ΔTlr4 (P712H) were prepared as previously described (27). The expression plasmid of the wild-type NFκB-inducing kinase (NIK), pRK-NIK(wt) was gift from Mike Rothe (Tularik, South San Francisco, CA). The dominant-negative mutant of inhibitor κB (pCMV4-IκBα(ΔN)) was provided by Dean Ballard (Vanderbilt University, Nashville, TN). The constitutively active form of MyD88 (Flag-MyD88(ΔToll)) was kindly provided by Jurg Tschopp (University of Lausanne, Switzerland) (36). The dominant-negative mutant of mouse PPARγ [pCMX-PPARγ(L466A/L467A)] was from Ira Schulman (Ligand Pharmaceuticals, San Diego, CA). All DNA constructs were prepared in large scale using EndoFree Plasmid Maxi kit (Qiagen, Chatsworth, CA) for transfection.

*Transient transfection and luciferase assay*—These were performed as described in our previous studies (27, 35). Briefly, RAW 264.7 cells were plated in 6-well plates (5
x $10^5$ cells/well) and transfected with luciferase reporter plasmids and HSP70-β-galactosidase plasmid as an internal control using SuperFect transfect reagent (Quiagen) according to the manufacturer’s instruction. Luciferase and β-galactosidase enzyme activities were determined using the Luciferase Assay System and β-galactosidase Enzyme System (Promega, Madison, WI) according to the manufacturer’s instruction. Luciferase activity was normalized by β-galactosidase activity.

Statistical analysis—Data were analyzed by paired t-test.

RESULTS

*Saturated fatty acids, but not unsaturated fatty acids, induce COX-2 expression in RAW 264.7 cells*—Saturated fatty acids induced COX-2 expression as determined by both Western blot analysis (Fig. 1A) and luciferase reporter gene assay for COX-2 (Fig. 1B). Among the saturated fatty acids (C8:0-C18:0) tested, lauric acid (C12:0) and palmitic acid (C16:0) were most potent in inducing COX-2 expression (Fig. 1C). In addition to COX-2, the expression of other inflammatory marker gene products such as iNOS and IL-1α was also induced by lauric acid in a dose-dependent manner (Fig. 1A). Unlike saturated fatty acids, all unsaturated fatty acids (C18:1n-9, C18:2n-6, C20:4n-6, C20:5n-3 and C22:6n-3) and conjugated linoleic acid (cLA) tested were unable to induce COX-2 expression in RAW 264.7 cells (Fig. 1D).

*Induction of COX-2 expression by saturated fatty acids is mediated through the activation of NFκB*—In our previous studies it was demonstrated that activation of NFκB is sufficient and required to induce maximal expression of COX-2 in LPS-
stimulated RAW 264.7 cells (27). Therefore, we determined whether saturated fatty acid-induced COX-2 expression is mediated through the activation of NFκB in RAW 264.7 cells. Lauric acid activated NFκB in a dose-dependent manner (Fig. 2A). The expression of COX-2 induced by lauric acid was inhibited by co-transfection of a dominant-negative mutant of IκBα plasmid (Fig. 2B). In addition, lauric acid-induced COX-2 expression was significantly reduced in the COX-2 promoter reporter gene containing mutated NFκB site as compared with the one containing wild-type NFκB site (Fig. 2C).

Since naturally occurring fatty acids are known to bind and activate PPARs (37-42) and some of PPAR activators induce COX-2 expression in certain cell types (35, 43), we determined whether saturated fatty acid-induced COX-2 expression is also mediated through PPAR signaling pathway. The 5′-flanking region of murine COX-2 contains PPAR response element (PPRE)-like sequences (Fig. 3A). Thus, we determined whether these sequences are required for saturated fatty acid-induced COX-2 expression. The result showed that deletion of those sequences did not affect the promoter activity of COX-2 reporter gene (Fig. 3A). Next, we determined whether a dominant-negative mutant of PPARγ (44) alters the saturated fatty acid-induced COX-2 expression. The result showed that lauric acid-induced COX-2 expression in cells co-transfected with a dominant-negative mutant of PPARγ plasmid was not altered as compared with control cells regardless of whether the COX-2 reporter gene construct contains the PPRE-like sequences or not (Fig. 3B). Together, these results suggest that saturated fatty acid-induced COX-2 expression is not directly mediated through the PPRE-like sequences in COX-2 gene.
Saturated fatty acid-induced COX-2 expression is inhibited by a dominant-negative mutant of Tlr4—Next, we attempted to identify the upstream target in the NFκB signaling pathways through which the saturated fatty acids activate NFκB and induce COX-2 expression. Activation of Tlr4 is sufficient and necessary to activate NFκB and to induce COX-2 expression in RAW264.7 cells. Because of the implication that lauric acid, myristic acid or palmitic acid acylated in the Lipid A molecule may play an important role in transmitting the LPS-mediated signal, we determined whether saturated fatty acid-induced activation of NFκB and COX-2 expression are mediated through the murine LPS receptor (Tlr4). If saturated fatty acid-induced COX-2 expression is mediated through Tlr4, co-transfection of cells with a dominant-negative mutant of Tlr4 should lead to inhibition of COX-2 expression. The results show that the dominant-negative mutant of Tlr4 [ΔTlr4(P712H)] inhibits both saturated fatty acid-induced NFκB activation and COX-2 expression (Fig. 4A and B). These results suggest that the upstream target in the signaling pathways through which saturated fatty acids mediate NFκB activation and COX-2 expression is Tlr4 or its associated molecules. However, these results do not allow us to conclude whether saturated fatty acids directly interact with Tlr4.

Unsaturated fatty acids inhibit saturated fatty acid-induced COX-2 expression, and this inhibition is mediated through suppression of NFκB—Unlike saturated fatty acids, unsaturated fatty acids were unable to induce COX-2 expression (Fig. 1D). Furthermore, they inhibited saturated fatty acid-induced NFκB activation (Fig. 5A) and
COX-2 expression (Fig. 5B). These results indicate that inhibition of saturated fatty acid-induced COX-2 expression by unsaturated fatty acids is mediated through suppression of NFκB signaling pathway. Together, these results suggest that both the induction of COX-2 by saturated fatty acids and its inhibition by unsaturated fatty acids are mediated through NFκB signaling pathway.

Unsaturated fatty acids also inhibit constitutively active Tlr4 (ΔTlr4)-induced COX-2 expression but they do not inhibit COX-2 expression induced by constitutively active MyD88 or NFκB-inducing kinase (NIK) which lies downstream of Tlr4—If saturated fatty acid-induced COX-2 expression is mediated through Tlr4, it is logical to determine whether the inhibition of saturated fatty acid-induced COX-2 expression by unsaturated fatty acids is also mediated through Tlr4. The results showed that docosahexaenoic acid (C22:6n-3) partially inhibits constitutively active Tlr4 (ΔTlr4)-induced COX-2 expression (Fig. 6A). MyD88 is the immediate downstream adaptor protein which interacts directly with the cytoplasmic domain of Tlr4. Activation of MyD88 leads to activation of NFκB and COX-2 expression in RAW 264.7 cells (27). Therefore, if the inhibition of saturated fatty acid-induced COX-2 expression by unsaturated fatty acids is mediated through Tlr4, COX-2 expression induced by the activation of signaling steps downstream of Tlr4 should not be inhibited by unsaturated fatty acids. The results indeed show that docosahexaenoic acid (C22:6n-3) is unable to inhibit COX-2 expression induced by constitutively active MyD88 or NIK (Fig. 6B and C). These results suggest that both induction of COX-2 expression by saturated fatty
acids and its inhibition by unsaturated fatty acids are mediated through Tlr4 or molecules associated with Tlr4.

*Unsaturated fatty acid also inhibits LPS-induced NFκB activation and expression of COX-2, iNOS and IL-1α*—If the inhibition of saturated fatty acid-induced COX-2 expression by unsaturated fatty acids is mediated through Tlr4 or its associated molecules, unsaturated fatty acids should also inhibit LPS-induced COX-2 expression. The results indeed show that docosahexaenoic acid (C22:6n-3) inhibits the LPS-induced expression of COX-2, iNOS, and IL-1α (Fig. 7A). Other unsaturated fatty acids tested (Fig. 1D) also inhibit LPS-induced COX-2 expression (data not shown). Inhibition of LPS-induced NFκB activation by docosahexaenoic acid (C22:6n-3) is demonstrated by inhibition of LPS-induced degradation of IκBα protein (Fig. 7B). Furthermore, docosahexaenoic acid (C22:6n-3) fails to inhibit TNFα-induced COX-2 expression in a colon tumor cell line (HT-29) (Fig. 7C) reinforcing the possibility that the inhibitory effect of unsaturated fatty acid on saturated fatty acid- or LPS-induced the expression of COX-2 is specifically mediated through Tlr4 or its associated molecules.

**DISCUSSION**

Most long-chain fatty acids are esterified in cellular lipids in mammalian cells. Therefore, the concentrations of unesterified fatty acids are believed to be low. However, fatty acids are rapidly released by the action of various phospholipase A2 and monoacylglycerol and diacylglycerol lipases in response to various extracellular stimuli. In plasma the average concentration of free fatty acid in postabsorptive state is < 0.7 mM,
and this concentration may be much higher in absorptive phase after ingestion of a fatty meal (45). Therefore, blood cells such as monocytes are constantly exposed to relatively high concentrations of free fatty acids. Fatty acids are known to regulate the expression of many genes involved in lipid metabolism (45) and modulate activity of signaling molecules such as phospholipase C and protein kinase C (46, 47). The mechanism by which fatty acids can regulate gene expression is still not well understood. However, some conceptual framework has been proposed for the possible mechanism of actions.

Fatty acids and their oxidative metabolites are known to bind and activate peroxisome proliferator-activated receptors (PPARs), the steroid-thyroid super family of nuclear receptors (37-42). Two Zn-finger motifs in the DNA binding domain of PPARs bind PPAR response elements (PPREs) located in 5’-flanking region of PPAR responsive genes. PPARs bind PPRE as a heterodimer with the retinoid X receptor (RXR). Polyunsaturated fatty acids (PUFAs) and other peroxisome proliferators induce peroxisomal \(\beta\)-oxidation and the expression of certain peroxisomal enzymes (45). Using PPAR\(\alpha\) null mice, it was demonstrated that PPAR\(\alpha\) is required for the induction of acyl-CoA oxidase by n-3 PUFAs but not for the suppression of lipogenic enzymes by n-3-PUFA (48). These results indicate that regulation of gene expression by fatty acids can be mediated through signaling pathways other than PPARs.

The inability of a dominant-negative mutant of PPAR\(\gamma\) to inhibit saturated fatty acid-induced COX-2 expression suggests that the induction was not mediated through activation of PPAR\(\gamma\). However, the possibility that the induction of COX-2 expression by saturated fatty acid is in part mediated through PPAR\(\alpha\) or PPAR\(\delta\) cannot be ruled out. Murine COX-2 gene contains PPRE-like sequences at positions [(-)2354-(-)2342] in the
5′-flanking region. Deletion of these sequences did not affect the promoter activity of COX-2 reporter gene (Fig. 3) suggesting that the PPRE-like sequences do not appear to be required for saturated fatty acid-induced COX-2 expression. However, the possibility that the saturated fatty acids in part stimulate or inhibit other PPAR-responsive gene products which in turn cause the induction of COX-2 expression cannot be ruled out.

It was shown that unsaturated fatty acids induce COX-2 expression in mammary epithelial cells (43). Whether this induction is mediated through PPARs has not been determined. However, to our surprise, saturated fatty acids, but not unsaturated fatty acids, induce COX-2 in RAW 264.7 cells (Fig. 1). Greater potency of lauric acid and palmitic acid in inducing COX-2 expression among saturated fatty acids tested (Fig. 1C) coincides with the abundance of these fatty acids in lipid A molecule (31). Lauric acid, myristic and palmitic acid are known to be major fatty acids acylated in lipid A molecule (31). The fact that deacylation of these fatty acids from LPS results in loss of endotoxic activity (32, 33) implies an important role of these fatty acids in LPS-mediated signal transmission. NFκB is one of the major downstream signaling pathways derived from activation of LPS receptor, Tlr4 in RAW 264.7 cells (27). The results demonstrating that induction of COX-2 by lauric acid is mediated through activation of NFκB (Fig. 2) and that this activation is inhibited by a dominant-negative mutant of Tlr4 (Fig. 4A), suggest that the most upstream signaling components affected by saturated fatty acids include Tlr4 or molecules associated with Tlr4. Whether saturated fatty acids can directly interact with Tlr4, or they interact with molecules associated with either extracellular or intracellular domains of Tlr4, remains to be determined.
The results presented in Fig. 4 and Fig. 6 suggest that activation of NFκB and COX-2 expression induced by saturated fatty acids and inhibition of this induction by unsaturated fatty acids are mediated through a common signaling pathway derived from Tlr4. The possibility that saturated fatty acids may act as a physiologically relevant endogenous ligand for Tlr4, and that unsaturated fatty acids interfere with saturated fatty acids in interacting with Tlr4 or molecules associated with Tlr4 remains to be determined.

While the detail mechanism by which saturated and unsaturated fatty acids interact with Tlr4 or its associated molecules is not known, the results presented in this report represent a novel mechanism by which fatty acids modulate signaling pathways and the expression of target genes. Furthermore, the results strongly imply that cellular expression of COX-2 and other inflammatory markers in monocytes and macrophages can be differentially regulated by different plasma free fatty acids which in turn can be altered by kinds of dietary fats consumed. These results further raise important questions as to whether activation of monocytes/macrophages and the propensity of endotoxemia can be modulated by types of plasma fatty acids, and whether unsaturated fatty acids can provide prophylactic efficacy against endotoxemia. Elucidating the mechanisms of the differential regulation of gene expression and activation of macrophages by types of fatty acids will help us to understand how different kinds of dietary fat modify risks of many chronic and acute inflammatory diseases.
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FOOTNOTE:

The abbreviations used are: COX-2, mitogen-inducible cyclooxygenase; LPS, lipopolysaccharide; PPAR, peroxisome proliferator-activated receptors; PPRE, peroxisome proliferator-response element; Tlr, TOLL-like receptor; MyD88, myeloid differentiation factor; NIK, NFκB-inducing kinase; NFκB, nuclear factor κB; IκBα, inhibitor κBα; IL-1α, interleukin-1 alpha; iNOS, inducible form of nitric-oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNFα, tumor necrosis factor alpha; PVDF, polyvinylidene difluoride; C8:0, octanoic acid; C10:0, decanoic acid; C12:0, lauric acid; C14:0, myristic acid; C16:0, palmitic acid; C18:0, stearic acid; C18:1n-9, oleic acid; C18:2n-6, linoleic acid; cLA, conjugated linoleic acid; C20:4n-6, arachidonic acid; C20:5n-3, eicosapentaenoic acid; C22:6n-3, docosahexaenoic acid; fatty acids are denoted by their carbon chain length:number of double bonds and n-indicates the location of the first double bond counted from the methyl end.
FIGURE LEGENDS:

Fig. 1. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of COX-2, iNOS and IL-1α. A. RAW 264.7 cells maintained in serum-poor (0.25%) medium were treated with indicated concentrations of lauric acid (C12:0) solubilized with BSA at a molar ratio of 10:1 (fatty acid:BSA). After 11 hours, cell lysates were analyzed by COX-2, iNOS, IL-1α or GAPDH immunoblot. Lane 1, cells treated in medium alone; Lane 2-5, cells treated with lauric acid in medium with BSA and lane 6, cells treated in medium with 10 µM BSA without fatty acid. B. Cells were transfected with a luciferase reporter plasmid for COX-2 promoter and HSP70-β-galactosidase reporter plasmid as an internal control and treated with various concentrations of lauric acid (C12:0) or C. 75 µM of each saturated fatty acid for 24 hours. The luciferase and β-galactosidase enzyme activities were measured as described under “Experimental Procedures”. Relative luciferase activity was determined by normalization with β-galactosidase activity. D. Cells were treated with 75 µM of each fatty acid for 11 hours. Cell lysates were analyzed by COX-2 or GAPDH immunoblot. The panels are representative data from more than three different experiments. Values are mean ± SEM (n=3). *Significantly different from the vehicle control, p<0.05. RLA, relative luciferase activity.

Fig. 2. Lauric acid (C12:0)-induced expression of COX-2 is inhibited by a dominant-negative mutant of IκBα or by mutation in the NFκB site in COX-2 promoter. A. RAW 264.7 cells were transfected with a luciferase
reporter plasmid for NFκB response element and treated with indicated concentrations of lauric acid (C12:0) for 24 hours. **B.** Cells were co-transfected with a luciferase reporter plasmid for COX-2 promoter and the expression plasmid containing a dominant-negative mutant of IκBα [IκBα(ΔN)], and then treated with 75 μM of lauric acid (C12:0) for 24 hours. **C.** Cells were transfected with a luciferase reporter plasmid for COX-2 promoter containing wild-type NFκB site or mutated NFκB site. Relative luciferase activity (RLA) was determined as described in Fig.1. The panels are representative data from more than three different experiments. Values are mean ± SEM (n=3). *Significantly different from the vehicle control (A), the control (C12:0+vector) (B), or the data obtained using COX-2 promoter with wild-type NFκB site (C), p<0.05.

**Fig. 3.** Lauric acid (C12:0)-induced COX-2 expression is not affected by deletion of PPRE-like sequences in murine COX-2 promoter or by a dominant-negative mutant of PPARγ. **A.** RAW 264.7 cells were transfected with a luciferase reporter plasmid for COX-2 promoter with or without PPRE-like sequences. **B.** Cells were co-transfected with a reporter plasmid for COX-2 promoter with or without a dominant-negative mutant of PPARγ, and then treated with lauric acid (75 μM) for 24 hours. Relative luciferase activity (RLA) was determined as described in Fig.1. The panels are representative data from more than three different experiments. Values are mean ± SEM (n=3).
Fig. 4. Lauric acid (12:0)-induced activation of NFκB and COX-2 expression are inhibited by a dominant-negative mutant of Tlr4. RAW 264.7 cells were co-transfected with a luciferase reporter plasmid for NFκB response element (A) or COX-2 promoter (B) and the expression plasmid for a dominant-negative mutant of Tlr4 [ΔTlr4(P712H)] and then treated with lauric acid (75 µM) for 24 hours. Relative luciferase activity (RLA) was determined as described in Fig.1. The panels are representative data from more than three different experiments. Values are mean ± SEM (n=3). *Significantly different from the control (C12:0+vector), p<0.05.

Fig. 5. Unsaturated fatty acids inhibit lauric acid (C12:0)-induced activation of NFκB and COX-2 expression. RAW 264.7 cells were transfected with a luciferase reporter plasmid for NFκB response element (A) or COX-2 promoter (B) and pre-treated with 5 µM of each unsaturated fatty acid for 3 hours, and then treated with lauric acid (75 µM) for additional 21 hours. Relative luciferase activity (RLA) was determined as described in Fig.1 and data are expressed as a percentage of the control (C12:0). The panels are representative data from more than three different experiments. Values are mean ± SEM (n=3). *Significantly different from the C12:0 alone, p<0.05.

Fig. 6. Docosahexaenoic acid (C22:6n-3) inhibits constitutively active Tlr4 (ΔTlr4)-induced but not constitutively active MyD88- or NIK-induced COX-2 expression. RAW 264.7 cells were co-transfected with a luciferase reporter plasmid for COX-2 promoter and the expression plasmid for a constitutively active Tlr4 (ΔTlr4) (A), a constitutively active MyD88(ΔToll)
(B), or NIK (C), and then treated with 20 µM of docosahexaenoic acid (C22:6n-3) for 11 hours. Relative luciferase activity (RLA) was determined as described in Fig.1. The panels are representative data from more than three different experiments. Values are mean ± SEM (n=3). *Significantly different from the control (ΔTlr4 without C22:6n-3), p<0.05.

Fig. 7. Docosahexaenoic acid (C22:6n-3) inhibits LPS-induced expression of COX-2, iNOS, and IL-1α and degradation of IκBα in RAW 264.7 cells, but it fails to inhibit TNFα-induced COX-2 expression in HT-29 cells. A. RAW 264.7 cells were pretreated with indicated concentrations of docosahexaenoic acid (C22:6n-3) for 3 hours and then stimulated with LPS (100 ng/ml) for 8 hours and analyzed by COX-2, iNOS, IL-1α or GAPDH immunoblot or B. for 30 min and analyzed by IκBα immunoblot. C. Colon cancer cells (HT-29) were pretreated with various concentrations of docosahexaenoic acid (C22:6n-3) for 3 hours and then treated with TNFα (20 ng/ml) for 8 hours. Cell lysates were analyzed by COX-2 and GAPDH immunoblot. The panels are representative data from more than three different experiments.
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Fig. 1

(A) COX-2 immunoblot

iNOS immunoblot

IL-1α immunoblot

GAPDH immunoblot

| C12:0 (µM) | 1 | 10 | 50 | 100 |
|-------------|---|----|----|-----|
| BSA (µM)    | 0.1 | 1 | 5 | 10 |

(B) COX-2 Immunoblot

GAPDH Immunoblot

(C) COX-2 Immunoblot

(D) COX-2 Immunoblot

Saturated fatty acids

Unsaturated fatty acids

RLA

* *
Fig. 3

(A) TGACCTTTGNCCT: consensus PPRE
(-2354) TGTGCTTTGCCAT (-2342): murine COX-2

(B) COX-2 Luc
-3201 +93

RLA

C12:0 - + - +
Mutant PPARγ - - + +
Vector + + - - + +
**Fig. 4**

(A) 2× NFκB → Luc

|          | RLA    |
|----------|--------|
| C12:0    | +      |
| ΔTlr4(P712H) | +    |
| Vector   | +      |

(B) COX-2 -3201 → +93 → Luc

|          | RLA    |
|----------|--------|
| C12:0    | +      |
| ΔTlr4(P712H) | +    |
| Vector   | +      |
**Fig. 5**

(A) 2x NFκB → Luc

| Unsaturated Fatty acids | C12:0 | + | + | + | + | + | + |
|-------------------------|-------|---|---|---|---|---|---|
| C22:6n-3               |       |   |   |   |   |   |   |
| C20:5n-3               |       |   |   |   |   |   |   |
| C20:4n-6               | *     |   |   |   |   |   |   |
| C18:2n-6               |       | *|   |   |   |   |   |
| C18:1n-9               |       |   | *|   |   |   |   |
| RLA [% of control (C12:0)] |       |   |   |   |   |   |   |

(B) COX-2 → Luc

| Unsaturated Fatty acids | C12:0 | + | + | + | + | + | + |
|-------------------------|-------|---|---|---|---|---|---|
| C22:6n-3               |       |   |   |   |   |   |   |
| C20:5n-3               |       |   |   |   |   |   |   |
| C20:4n-6               | *     |   |   |   |   |   |   |
| C18:2n-6               |       | *|   |   |   |   |   |
| C18:1n-9               |       |   | *|   |   |   |   |
| RLA [% of control (C12:0)] |       |   |   |   |   |   |   |
Fig. 6

(A) 

(RLA) 

\[ \Delta \text{Tlr4} \] 

\[ \text{Vector} \] 

\[ \text{C22:6n-3} \] 

(B) 

(RLA) 

\[ \text{MyD88(}\Delta \text{Toll)} \] 

\[ \text{Vector} \] 

\[ \text{C22:6n-3} \] 

(C) 

(RLA) 

\[ \text{NIK} \] 

\[ \text{Vector} \] 

\[ \text{C22:6n-3} \]
(A) COX-2 Immunoblot

iNOS Immunoblot

IL-1α Immunoblot

GAPDH Immunoblot

LPS
- + + + + +
C22:6n-3 (µM)
- - 5 10 20 50

(B) IκBα immunoblot

LPS
- + + + + +
C22:6n-3 (µM)
- - 1 10 50

(C) COX-2 and GAPDH Immunoblot

kd
77—
50—

← COX-2

← GAPDH

TNFα
- + + + + -
C22:6n-3 (µM)
- - 50 100 200 200