Differential modulation of Toll-like receptors by fatty acids: preferential inhibition by n-3 polyunsaturated fatty acids

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Abstract **Human subjects consuming fish oil showed a significant suppression of cyclooxygenase-2 (COX-2) expression in blood monocytes when stimulated in vitro with lipopolysaccharide (LPS), an agonist for Toll-like receptor 4 (TLR4). Results with a murine monocytic cell line (RAW 264.7) stably transfected with COX-2 promoter reporter gene also demonstrated that LPS-induced COX-2 expression was preferentially inhibited by docosahexaenoic acid (DHA, C22:6n-3) and eicosapentaenoic acid (EPA, C20:5n-3), the major n-3 polyunsaturated fatty acids (PUFAs) present in fish oil. Additionally, DHA and EPA significantly suppressed COX-2 expression induced by a synthetic lipopeptide, a TLR2 agonist. These results correlated with the preferential suppression of LPS- or lipopeptide-induced NFκB activation by DHA and EPA. The target of inhibition by DHA is TLR itself or its associated molecules, but not downstream signaling components. In contrast, COX-2 expression by TLR2 or TRL4 agonist was potentiated by lauric acid, a saturated fatty acid. These results demonstrate that inhibition of COX-2 expression by n-3 PUFAs is mediated through the modulation of TLR-mediated signaling pathways. Thus, the beneficial or detrimental effects of different types of dietary fatty acids on the risk of the development of many chonic inflammatory diseases may be in part mediated through the modulation of TLRs.**—Lee, J. Y., A. Plakidas, W. H. Lee, A. Heikkinen, P. Chanmugam, G. Bray, and D. H. Hwang. **Differential modulation of Toll-like receptors by fatty acids: preferential inhibition by n-3 polyunsaturated fatty acids.** ***J. Lipid Res.*** **2003.** 44: **479–486.**

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Toll-like receptors (TLRs) play a critical role in the detection of microbial infection and the induction of inflammatory and immune responses against conserved microbial structures, called pathogen-associated molecular patterns (PAMPs) (1). The activation of TLRs leads to the induction of nuclear factor κB (NFκB) activation and the expression of inflammatory cytokines (2, 3). Ten members of the TLR family have so far been identified in human and mouse, and these TLRs are ubiquitously expressed in human tissues (4–6). However, endogenous ligands for these TLRs have not been fully identified. Genetic and biochemical evidence demonstrated that TLR4 confers the responsiveness to lipopolysaccharide (LPS) derived from gram-negative bacteria (7–9), whereas TLR2 recognizes other bacterial cell wall components, including bacterial lipoproteins (1–3). Other agonists for TLR4 from nonmicrobial origins include heat shock protein 60, fibronectin, taxol, respiratory syncytical virus coat protein, and saturated fatty acids (10–14). Such a broad spectrum of TLR4 agonists implies the promiscuous nature of ligand specificity for this receptor. This leads to the speculation that TLRs have much broader roles than we currently understand.

Lipid A, which possesses most of the biological activities of LPS, is acylated with hydroxy saturated fatty acids. The 3-hydroxyl groups of these saturated fatty acids are further 3-*O*-acylated by saturated fatty acids. Removal of these *O*-acylated saturated fatty acids from lipid A not only results in complete loss of endotoxic activity, but also makes the lipid A act as an antagonist to the native lipid A (15, 16). Lipid A(s) containing unsaturated fatty acids are also known to be nontoxic or act as an antagonist against endotoxin (17, 18). It was also demonstrated that the deacylated bacterial lipoproteins were unable to activate TLR2 and to induce cytokine expression in monocytes (19). These results suggest that the fatty acids acylated on lipid A or bacterial lipoproteins play a critical role in ligand rec-

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Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; LPS, lipopolysaccharide; NFκB, nuclear factor κB; MyD88, myeloid differential factor 88; NIK, NFκB-inducing kinase; OA, oleic acid; Pam-CAG, palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ala-Gly-OH; PUFA, polyunsaturated fatty acid; TLR, Toll-like receptors.

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ognition and receptor activation for TLR2 and TLR4. Indeed, it was suggested that the rapid interaction of bacterial lipopeptides with plasma membrane of macrophages occurs via insertion of their acylated saturated fatty acids as determined by electron energy loss spectroscopy and freeze-fracture techniques (20, 21).

Results from our previous studies (9) demonstrated that the ligand independent activation of TLR4 is sufficient to induce the activation of NFκB and the expression of the mitogen-inducible cyclooxygenase (COX-2) in macrophages. Furthermore, saturated fatty acids induce NFκB activation and COX-2 expression, but unsaturated fatty acids inhibit both saturated fatty acid- and LPS-induced NFκB activation, and the expression of COX-2 and other inflammatory markers in a murine monocytic cell line (RAW 264.7) (14). The inhibition of LPS-induced NFκB activation and COX-2 expression by unsaturated fatty acids was mediated through the suppression of TLR4-derived signaling pathways (14). It was demonstrated that consuming n-3 polyunsaturated fatty acids (PUFAs) leads to the suppression of the production of LPS-induced proinflammatory cytokines in blood mononuclear cells in humans (22, 23). However, the mechanism is not understood. Both inducible cyclooxygenase (COX-2) and proinflammatory cytokines belong to a family of immediate early response genes. The expression of immediate early response genes does not require preceding protein synthesis (24). This suggests that the suppressed expression of LPS-induced proinflammatory cytokines by n-3 PUFAs may be mediated by modulation of LPS (TLR4 agonist)-induced signaling pathways.

Thus, we determined whether COX-2 expression is inhibited in LPS-stimulated monocytes derived from human subjects consuming fish oil, and whether this inhibition is mediated through the modulation of TLR4 signaling pathways by n-3 PUFAs. If the activation of TLRs is modulated by the types of fatty acids, then signaling pathways downstream of TLRs, target gene expression, and consequent cellular responses should also be modulated by different types of fatty acids. This modulation has profound implications for the potential role of dietary fat with varying composition of fatty acids on inflammatory and immune responses induced by the activation of TLRs that are ubiquitously expressed in human tissues.

MATERIALS AND METHODS

Reagents

Sodium salts of unsaturated and saturated fatty acids were purchased from Nu-Chek (Eslyan, MN). LPS was purchased from DIFCO (Detroit, MI). A synthetic bacterial lipoprotein [palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ala-Gly-OH (PamCAG)] was purchased from Bachem (King of Prussia, PA). All other reagents were purchased from Sigma unless otherwise described.

Plasmids

The luciferase reporter plasmid (pGL2) containing the promoter region of the murine COX-2 gene (−3.2 kb) was provided by David Dewitt (Michigan State University, East Lansing, MI). 4× NFκB-luciferase reporter construct was purchased from Clontech (Palo Alto, CA) and used for transient transfection. Heat shock protein 70 (HSP70)-β-galactosidase reporter plasmid was from Robert Modlin (University of California, Los Angeles, CA). The expression plasmids for a wild-type TLR2 and a dominant-negative mutant [TLR2(P681H)] were from C. B. Wilson (University of Washington, Seattle, WA). Constitutively active chimeric CD4-TLR4 was obtained from C. A. Janeway, Jr. (Yale University, New Haven, CT). The constitutively active form of myeloid differential factor 88 [MyD88(ΔToll)] and the dominant-negative mutant, MyD88(ΔDD), were kindly provided by Jurg Tschopp (University of Lausanne, Switzerland). The wild-type and the dominant-negative mutant of NFκB-inducing kinase (NIK) were gifts from M. Rothe (Tularik, South San Francisco, CA). All DNA constructs were prepared in large scale using Endo-Free Plasmid Maxi kit (Qiagen, Chatsworth, CA) for transfection.

Cell culture

RAW 264.7 cells (a murine monocytic cell line, ATCC TIB-71) and 293T cells (provided by Sam Lee, Beth Israel Hospital. Boston, MA) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, Intergen) and 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO-BRL) at 37°C in a 5% CO_2/air environment. RAW264.7 cells stably transfected with murine COX-2 promoter (−3.2 kb) luciferase plasmid were prepared as described below. RAW264.7 cells stably transfected with a luciferase plasmid containing 5× NFkB binding site were a gift from Jianping Ye (Pennington Biomedical Research Center, Baton Rouge, LA). Cells were plated in 6-well plates and cultured for an additional 18 h to allow the number of cells to approximately double. Cells were maintained in the serum-poor (0.25% FBS) medium for another 18 h prior to the treatment with indicated reagents.

Preparation of stably transfected cells with luciferase reporter plasmids

RAW 264.7 cells (1×10^6 cells) were plated in 100 mm dish and transfected with murine COX-2 promoter (−3.2 kb) luciferase plasmid using Superfect Transfection reagent (Qiagen) according to the manufacturer's instruction. pcDNA3/neo was cotransfected to select transfected cells using the antibiotic. After 48 h of stabilization, the new media containing Geneticin (500 μg/ml) was added and changed for appropriate time periods. Two weeks later, the colonies that survived were selected and propagated under Geneticin. After another 2 weeks of antibiotic selection, the luciferase activities were determined for each colony after treatment with LPS (100 ng/ml). The colony that showed the highest response to LPS treatment was selected.

Transient transfection and luciferase assay

These were performed as described in our previous studies (9, 14). Briefly, RAW 264.7 or 293T cells were plated in 6-well plates (5×10^5 cells/well) and cotransfected with a luciferase plasmid containing either murine COX-2 promoter (−3.2 kb) or 2× NFkB binding site and HSP70-β-galactosidase plasmid as an internal control using SuperFect transfection reagent (Quiagen, Valencia, CA) according to the manufacturer's instructions. Various expression plasmids or corresponding empty vector plasmids for signaling components were cotransfected. The total amount of transfected plasmids was equalized by supplementing with the corresponding empty vector in order to eliminate the experimental error from transfection itself. 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 instructions. Luciferase activity was normalized by β-galactosidase activity.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting

These were performed essentially the same as previously described (25, 26). For COX-2 and actin immunoblot analyses, solubilized proteins were subjected to 8% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the gel was transferred to a PVDF membrane in the transfer buffer. The membrane was blocked to prevent nonspecific binding of antibodies in TBS-T [20 mM Tris HCl, 137 mM NaCl, 0.05% (v/v) Tween 20, pH 7.6] containing 5% nonfat dried milk (NFDM, Carnation). COX-2 immunoblotting was performed using rabbit polyclonal antibody followed by incubation with anti-rabbit IgG coupled to horseradish peroxidase (1:5,000) in 5% NFDM in TBS-T. Polyclonal antibodies for COX-2 were prepared and characterized as described previously (27, 28). For actin immunoblotting, the membrane used for COX immunoblot was stripped in the stripping buffer (29) at 56°C for 1 h, reprobed with 1:10,000 dilution of mouse monoclonal anti-actin antibody (Sigma), and followed by incubation with anti-mouse IgG coupled to horseradish peroxidase (1:5,000) in 5% NFDM in TBS-T. The membrane was exposed on an X-ray film (Kodak) using ECL western blot detection reagents (Amersham). Sheep anti-mouse and donkey anti-rabbit immunoglobulin G (IgG) antibodies conjugated to horseradish peroxidase were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL).

Human studies to determine whether dietary n-3 PUFAs suppress the expression of COX-2 induced by LPS in peripheral blood monocytes

We conducted two randomized, double-blind, placebo-controlled, parallel arm studies in human subjects where the amounts of dietary n-3 and n-6 PUFAs were controlled using institutionally prepared diets with fish oil concentrate or placebo oil capsules. The study protocol was approved by the Louisiana State University Institutional Review Board, and subjects gave written informed consent. In Study I, subjects (7 to 8 per group) received 9 g of purified fish oil with varying amounts of linoleic acid (C18:2n-6) for 8 weeks, whereas in Study II, subjects (11 to 12 per group) received varying amount of fish oil (0, 6, 15g) with a constant amount of linoleic acid for 4 weeks. The control group received linoleic acid without fish oil supplementation. The studies I and II were combined to get a broader range of dose-responses to the intake of fish oil. The data for the control group in both study I and II were combined to make the control group in **Fig. 1**. The data for 9 g of fish oil intake with different amount of linoleic acid in study I were combined with the data for the group receiving 9 g of fish oil in Study II. Thus, numbers of replicates for the control and the group of 9 g of fish oil intake were approximately two times greater than those for the groups of 6 g or 15 g of fish oil intake. The combination of the data was based on the fact that the levels of linoleic acid intake did not affect the production of PGE_2. Details for the study design and provision of the diets are described elsewhere (30).

Assay for de novo synthesized COX-2 in human monocytes

Briefly, monocytes isolated from peripheral blood (31) were pretreated with aspirin to inactivate any endogenous COX. Newly expressed COX in response to LPS (100 ng/ml) stimulation was determined by measuring the production of prostaglandin E_2 (PGE_2) in the presence of arachidonic acid (AA; 30 μM, above V_{max}). COX-2, but not COX-1, is selectively expressed in LPS-stimulated macrophages (27). Therefore, COX activity as measured by PGE_2 production at these conditions reflects de novo synthesis of COX-2 (25, 27).

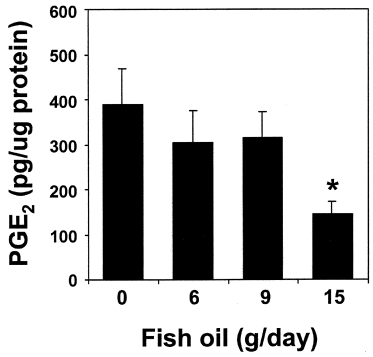

Fig. 1. Dietary intake of fish oil suppresses the expression of cyclooxygenase (COX)-2 induced by Toll-like receptor (TLR)4 agonist [lipopolysaccharide (LPS)] in human peripheral blood monocytes. Two randomized, double-blinded, placebo-controlled, parallel arm studies were conducted in healthy subjects where the amounts of dietary n-3 and n-6 polyunsaturated fatty acids (PUFAs) were controlled using institutionally prepared diets and fish oil concentrate or placebo oil capsules. Fish oil concentrate or safflower oil was used as a source of n-3 PUFAs or n-6 PUFAs (linoleic acid), respectively. In study I, subjects consumed an equal amount of fish oil (9 g/day) but varying amounts of the n-6 fatty acid (linoleic acid). In study II, subjects consumed various amounts of fish oil (0, 6, 15 g/day) with the constant amount of linoleic acid. Peripheral blood monocytes were isolated, and the production of prostaglandin E_2 (PGE_2) induced by LPS in monocytes was determined as described in Materials and Methods. The levels of PGE_2 produced in these conditions reflect the amounts of de novo synthesis of COX-2 protein. Combined data for studies I and II are presented. Values are mean ± SEM (n = 11–21). *Significantly different from the control group receiving no fish oil ($P < 0.022$).

RESULTS

Adding fish oil containing n-3 PUFAs to the diet suppressed the expression of COX-2 in human monocytes stimulated with LPS, an agonist for the Toll-like receptor 4

Here we determined whether the intake of fish oil, a major dietary source of docosahexaenoic acid (DHA, C22:6n-3) and eicosapentaenoic acid (EPA, C20:5n-3) leads to suppression of COX-2 expression in human monocytes exposed to LPS in vitro. Prostaglandin E_2 (PGE_2) production was used as a surrogate maker for COX-2 expression. The production of PGE_2 was significantly ($P < 0.022$) suppressed by 15 g of fish oil intake, but not by the lower doses of intake (Fig. 1), suggesting that COX-2 expression by LPS in human monocytes was suppressed by the fish oil diet.

It has also been demonstrated by other investigators that the suppression of the production of cytokines (IL-2, IL-1, and TNFα) in LPS-stimulated human mononuclear cells by fish oil intake occurred at the dose of 18 g/day for 6 weeks (22, 23). These results indicate that the suppression of COX-2 and cytokine expression by fish oil intake occurs at high dose levels. Since the feeding periods were relatively short (4–8 weeks), it is possible that the suppression of the expression of COX-2 and cytokines could occur at lower levels of fish oil intake if the experimental period were prolonged.

Preferential inhibition by n-3 PUFAs of LPS-induced NFκB activation and COX-2 expression in RAW 264.7 cells

To investigate the mechanism by which n-3 PUFAs inhibit LPS-induced COX-2 expression in human blood monocytes, we determined the relative potency of unsaturated fatty acids in inhibiting the TLR-induced signaling pathway and the target gene expression in murine monocytic cell line (RAW 264.7), which is stably transfected with NFκB or COX-2 promoter luciferase reporter gene. These stably transfected cell lines eliminate the necessity of transfecting plasmids for the reporter gene and the internal control. Thus, inhibitory or stimulatory effects of various fatty acids on agonist-induced TLR activation can be quantitatively determined in a high throughput mode using 96 well plates. Demonstration that LPS-induced NFκB activation and COX-2 expression in RAW 264.7 cells is mediated through TLR4 was reported previously (9). Therefore, we utilized NFκB activation and COX-2 expression as readouts for agonist-induced TLR activation and its suppression or potentiation by fatty acids in RAW 264.7 cells in these studies.

All unsaturated fatty acids tested inhibit LPS-induced NFκB activation and COX-2 expression as determined by reporter gene assays (**Fig. 2A**, **B**). Among unsaturated fatty acids, DHA and EPA are the most potent inhibitors. This finding corroborates the results from the human studies described above (Fig. 1), and demonstrates that n-3 PUFAs (DHA and EPA) as compared with n-6 PUFAs (arachidonic acid and linoleic acid) are much more potent inhibitors of TLR4 activation. In contrast, a saturated fatty acid, lauric acid (C12:0), potentiates LPS-induced NFκB activation and COX-2 expression (Fig. 2A, B). Results from previous studies showed that saturated fatty acids alone, without other agonists, can induce NFκB activation and COX-2 expression in RAW 264.7 cells (14). Immunoblot analyses also showed that LPS-induced COX-2 expression is suppressed by DHA but potentiated by the saturated fatty acid (Fig. 2C). To determine whether unsaturated fatty acids inhibit the activation of TLR4 in a reconstituted system, human embryonic kidney cells (293T) were transfected with a constitutively active form of TLR4 (CD4-TLR4) to activate TLR4-mediated signaling pathways in a ligand-independent manner (4). DHA inhibits, but C12:0 potentiates CD4-TLR4-induced NFκB activation in 293T cells (**Fig. 3A**). These results are consistent with the results demonstrating the similar pattern of modulation by fatty acids for the ligand-induced activation of TLR4 in RAW264.7 cells (Fig. 2).

We next determined whether the target of inhibition by DHA is TLR4 or its downstream signaling components. One of the common components of the immediate downstream signaling pathways of all TLRs is known to be an adaptor protein, MyD88 (1–3, 5). The activation of NFκB mediated through MyD88 is one of the major downstream signaling pathways derived from TLRs. DHA does not inhibit NFκB activation induced by the activation of downstream component (MyD88 or NIK) of TLR signaling pathways (Fig. 3B), while unsaturated fatty acids inhibit

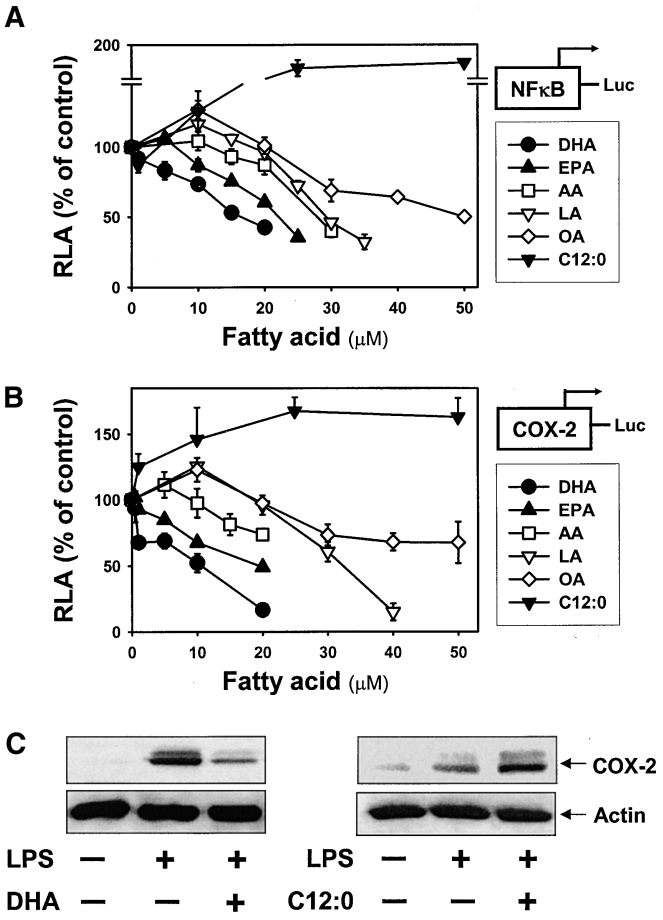

Fig. 2. Unsaturated fatty acids inhibit, but saturated fatty acid potentiates, LPS-induced nuclear factor κB (NFκB) activation and COX-2 expression in RAW 264.7 cells. Cells stably transfected with NFκB(5×) (A) or COX-2 promoter (B) reporter gene were pretreated with various concentrations of each fatty acid for 3 h. Cells were then treated with LPS (200 ng/ml). After 8 h, cell lysates were prepared and luciferase activities were determined. Data are expressed as a percentage of LPS treatment alone. Values are mean ± SEM (n = 3). RLA, relative luciferase activity. C: Cells were pretreated with docosahexaenoic acid (DHA) (20 μM) or C12:0 (75 μM) for 3 h and further stimulated with LPS (200 ng/ml for DHA; 1 ng/ml for C12:0). After 8 h, cell lysates were analyzed by COX-2 and actin immunoblotting. DHA, docosahexaenoic acid (C22:6n-3); EPA, eicosapentaenoic acid (C20:5n-3); AA, arachidonic acid (C20:4n-6); LA, linoleic acid (C18:2n-6); OA, oleic acid (C18:1n-9); C12:0, lauric acid.

NFκB activation induced by both TLR4 agonist (LPS) (Fig. 2A) and constitutively active TLR4 (CD4-TLR4) (Fig. 3A). These results suggest that the molecular target of inhibition by DHA is TLR itself or its associated molecules, but not the downstream components.

N-3 PUFAs suppress, but saturated fatty acid potentiates PamCAG-induced NFκB activation and COX-2 expression

Acylation by saturated fatty acids of bacterial lipopeptides is also required for the activation of TLR2 (19). Therefore, we determined whether unsaturated fatty acids suppress the activation of TLR2 as they do TLR4 activation. To validate our experimental model, we first demon-

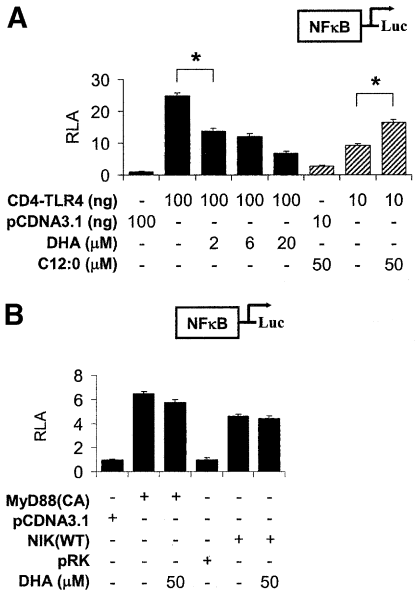

Fig. 3. Docosahexaenoic acid inhibits a constitutively active TLR4 (CD4-TLR4), but not a constitutively active myeloid differential factor 88 (MyD88) or wild-type NIK-induced NFκB activation in 293T cells. A: Cells were cotransfected with NFκB-luciferase reporter plasmid and the expression plasmid of constitutively active human TLR4 (CD4-TLR4) and treated with DHA or C12:0. B: Cells were cotransfected with NFκB-luciferase reporter plasmid and the expression plasmid of constitutively active MyD88 [MyD88(CA)] or wild-type NIK [NIK(WT)], and treated with DHA. Values are mean ± SEM (n = 3). RLA, relative luciferase activity. * Significantly different from the respective control ($P < 0.05$).

strated that PamCAG, a synthetic analog of bacterial lipopeptides that are known agonists of TLR2, activates TLR2 in a reconstituted system using 293T cells that do not express TLR2 (4, 32). PamCAG activates TLR2, as determined by NFκB activation and its inhibition by a dominant negative mutant of TLR2 or downstream signaling component (MyD88 or NIK) in TLR2-transfected 293T cells (**Fig. 4A**). The murine monocytic cell line (RAW 264.7) expresses both TLR2 and TLR4 (6). Thus, we determined whether PamCAG activates endogenous TLR2 in RAW 264.7 cells. PamCAG induces NFκB activation and expression of COX-2, and this induction was inhibited by a dominant negative mutant of TLR2 or MyD88 (Fig. 4B, C). These results demonstrate that PamCAG activates both ectopically expressed TLR2 in 293T cells and endogenous TLR2 in RAW 264.7 cells.

Similar to the results shown with TLR4 agonist (Fig. 2), n-3 PUFAs, DHA, and EPA are the most potent inhibitors among the unsaturated fatty acids tested for PamCAG-induced NFκB activation and COX-2 expression in RAW 264.7 cells (**Fig. 5A–C**). The saturated fatty acid (C12:0) potentiated PamCAG-induced NFκB activation and COX-2 expression in RAW264.7 cells. In addition, DHA inhibits, but lauric acid (C12:0) potentiates NFκB activation induced by PamCAG in TLR2-transfected 293T cells (Fig. 5D). These results demonstrate the modulatory effects of the fatty acids on the activation of both TLR2 and TLR4.

DISCUSSION

Dietary fatty acids can be divided into four major groups: saturated fatty acids (e.g., lauric, myristic, palmitic, and stearic); monounsaturated fatty acids [e.g., oleic acid (n-9)]; n-6 PUFAs (linoleic acid); and n-3 PUFAs (e.g., linolenic acid). The unsaturated fatty acids can be converted to longer chain polyunsaturated fatty acids through a series of desaturation and chain elongation steps. It is well documented that there is metabolic competition among the three groups of unsaturated fatty acids at the desaturation steps (33–35). Twenty carbon PUFAs, such as arachidonic acid (C20:4n-6) and EPA (C20:5n-3), can be enzymatically converted to eicosanoids (36–38). Revelation of diverse pathophysiological actions of eicosanoids has expanded our understanding of how fatty

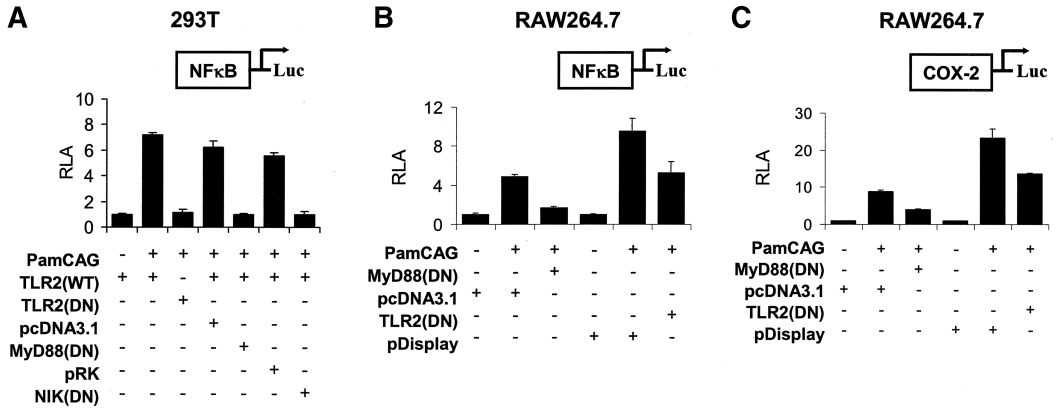

Fig. 4. Synthetic bacterial lipopeptide [palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ala-Gly-OH (PamCAG)] activates NFκB and induces COX-2 expression through TLR2-mediated signaling pathway. 293T (A) or RAW 264.7 cells (B and C) were transfected with NFκB (A and B) or COX-2 (C) luciferase reporter plasmid and cotransfected with various expression plasmids as indicated. pcDNA3.1, pRK, and pDisplay were transfected as vector controls for dominant negative (DN) mutants of MyD88, NIK, and TLR2, respectively. Twenty-four hours after the transfection, cells were stimulated with either vehicle control or PamCAG (1 μg/ml). After 18 h, luciferase activities were determined. Values are mean ± SEM (n = 3). WT, wild-type. RLA, relative luciferase activity.

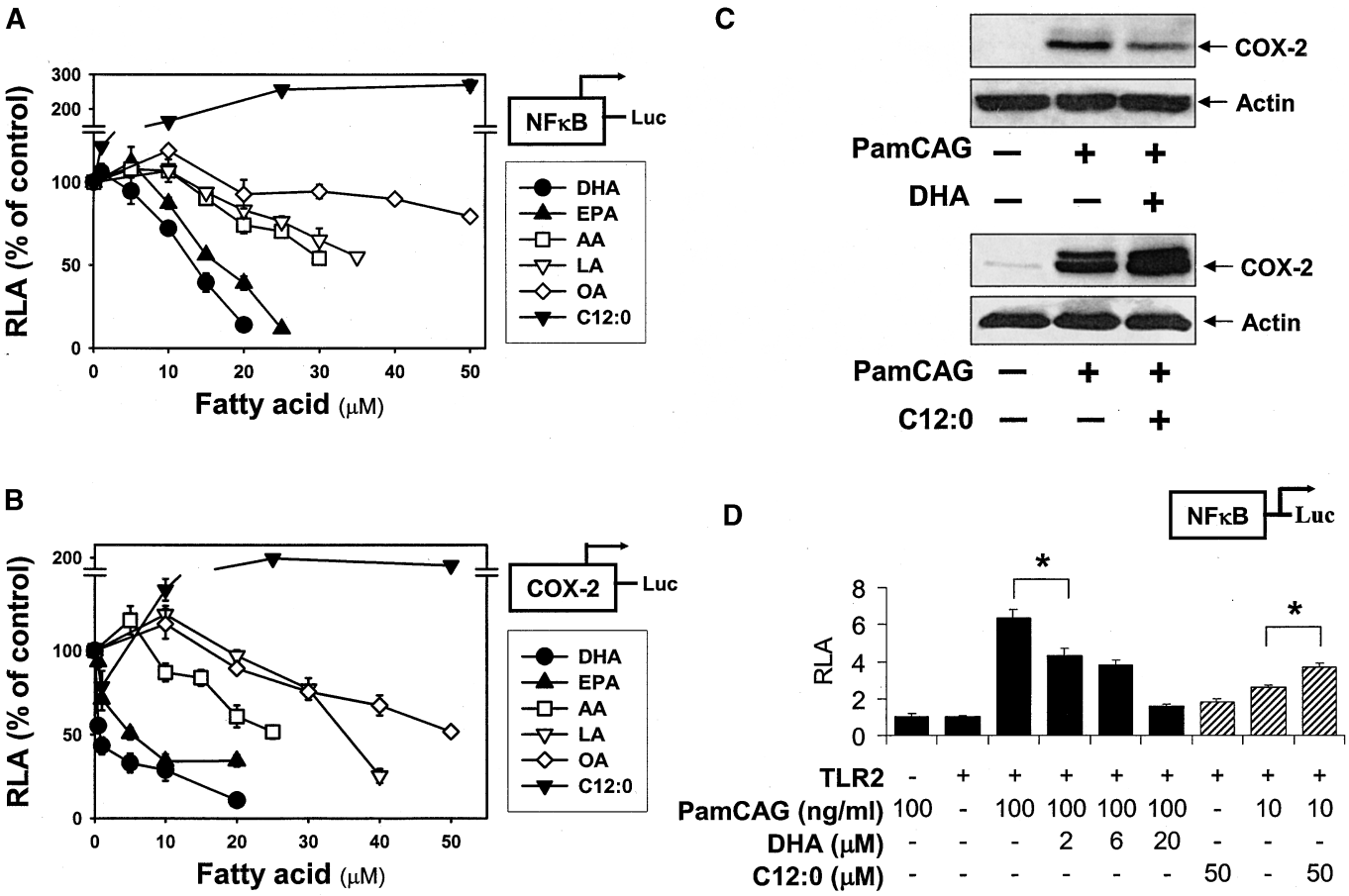

Fig. 5. Unsaturated fatty acids inhibit, but saturated fatty acid potentiates, PamCAG-induced NFκB activation and COX-2 expression. RAW 264.7 cells stably transfected with NFκB(5×) (A) or COX-2 promoter (B) reporter gene were pretreated with various concentrations of each fatty acid for 3 h. Cells were further stimulated with a synthetic bacterial lipoprotein (PamCAG, 500 ng/ml). After 8 h, luciferase activities were determined. Data are expressed as a percentage of PamCAG treatment alone. C: RAW 264.7 cells were pretreated with DHA (20 μM) or C12:0 (75 μM) for 3 h and further stimulated with PamCAG (500 ng/ml). After 8 h, cell lysates were analyzed by COX-2 and actin immunoblotting. D: 293T cells were cotransfected with NFκB-luciferase reporter plasmid and TLR2 expression plasmid, and treated with PamCAG in the presence or absence of DHA or C12:0. Values are mean ± SEM (n = 3). RLA, relative luciferase activity. * Significantly different from the respective control ($P < 0.05$).

acids modulate various cellular responses. Mounting evidence now suggests that fatty acids not only are the precursors of eicosanoids and other lipid mediators, but also can modulate signaling molecules and transcription factors (39–41). Elucidating the mechanism of this modulation could help us to understand how different types of dietary fat modify the risks of developing many chronic diseases.

DHA (C22:6n-3) and EPA are the major n-3 PUFAs present in marine lipids. Epidemiological, clinical, and biochemical studies have demonstrated beneficial effects of these n-3 PUFAs in reducing risks of cardiovascular diseases, inflammatory diseases, and cancer (42–49). However, the mechanisms by which dietary n-3 PUFAs exert such beneficial effects are not well understood. It has been demonstrated that consuming n-3 PUFAs rich in fish oil suppressed production of cytokines (IL-1, IL-2, and TNFα) in peripheral blood mononuclear cells in response to the TLR4 agonist LPS (22, 23, 50). Inhibition of IL-2 production from T-cells by the dietary intake of n-3 PUFAs has also been demonstrated in human subjects and in mice (51, 52). As both the cytokines and COX-2 belong to a family of immediate early response genes (24), we reasoned that n-3 PUFAs might also suppress the expression of COX-2 through modulation of the signaling pathways leading to its expression. Indeed, our results demonstrate that the intake of n-3 PUFAs leads to suppression of LPS-induced COX-2 expression in human monocytes.

The next question was the identity of the molecular target(s) that mediates the suppression of cytokine production or COX-2 expression by n-3 PUFAs as compared with n-6 PUFAs. To answer this question, we first examined the molecular target(s) through which PUFAs modulate signaling pathways. The results shown in Fig. 2 demonstrate that all unsaturated fatty acids inhibit LPS-induced COX-2 expression. The results presented in Fig. 3A–B, 5D suggest that the molecular target for the inhibition by DHA is TLR itself or its associated molecules, but not the components of the downstream pathways. Next, we compared the efficacy of n-3 PUFAs in modulating the molecular tar-

gets. Both LPS-induced NFκB activation and COX-2 expression were preferentially inhibited by n-3 PUFAs in RAW 264.7 cells in a similar pattern and dose range (Fig. 2). This finding corroborates the results of the clinical studies demonstrating the suppression of LPS-induced COX-2 expression in blood monocytes by high doses of fish oil (Fig. 1).

Since bacterial lipopeptides also require fatty acid acylation for the activation of TLR2 (19), we investigated whether fatty acids also modulate TLR2 signaling pathways. Similar to the results obtained with TLR4 agonist stimulation, unsaturated fatty acids inhibited but the saturated fatty acid lauric acid potentiated, TLR2 agonist-induced NFκB activation and COX-2 expression.

Together, these results represent a novel mechanism by which n-3 PUFAs inhibit the expression of COX-2 that is overexpressed in sites of inflammation and in many types of tumor tissues (28, 53–56). Furthermore, these results suggest that the anti-inflammatory effects of dietary n-3 PUFAs are mediated at least in part through the inhibition of TLR-induced signaling pathways and target gene expression. Infection and inflammation are important risks for the development of many chonic diseases (57–59). Thus, our results suggest the possibility that both the beneficial and detrimental effects of different dietary fatty acids on the risk of developing chonic inflammatory diseases may in part be mediated through the modulation of Toll-like receptors.

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