Differential Modulation of Nods Signaling Pathways by Fatty Acids in Human Colonic Epithelial HCT116 Cells*

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Nucleotide-binding oligomerization domain-containing proteins (Nods) are intracellular pattern recognition receptors recognizing conserved moieties of bacterial peptidoglycan through their leucine-rich repeats domain. The agonists for Nods activate pro-inflammatory signaling pathways, including NF-κB pathways. The results from our previous studies showed that the activation of TLR4 and TLR2, leucine-rich repeat-containing pattern recognition receptors, were differentially modulated by saturated and n-3 polyunsaturated fatty acids in macrophages and dendritic cells. Here, we show the differential modulation of NF-κB activation and interleukin-8 (IL-8) expression in colonic epithelial cells HCT116 by saturated and unsaturated fatty acids mediated through Nods proteins. Lauric acid (C12:0) dose dependently activated NF-κB and induced IL-8 expression in HCT116 cells, which express both Nod1 and Nod2, but not detectable amounts of TLR2 and TLR4. These effects of lauric acid were inhibited by dominant negative forms of Nod1 or Nod2, but not by dominant negative forms of TLR2, TLR4, and TLR5. The effects of lauric acid were also attenuated by small RNA interference targeting Nod1 or Nod2. In contrast, polyunsaturated fatty acids, especially n-3 polyunsaturated fatty acids, inhibited the activation of NF-κB and IL-8 expression induced by lauric acid or known Nods ligands in HCT116. Furthermore, lauric acid induced, but docosahexaenoic acid inhibited lauric acid- or Nod2 ligand MDP-induced, Nod2 oligomerization in HEK293T cells transfected with Nod2. Together, these results provide new insights into the role of dietary fatty acids in modulating inflammation in colon epithelial cells. The results suggest that Nods may be involved in inducing sterile inflammation, one of the key etiological conditions in the development of many chronic inflammatory diseases.

Evolution of host defense systems against microbial pathogens is the key for the survival of all multicellular organisms. Germ line-encoded transmembrane Toll-like receptors (TLRs) play a crucial role in the detection of invading microbes and the induction of immune and inflammatory responses to defend the host (1–4). In addition to TLRs, cytosolic proteins, the family of nucleotide-binding oligomerization domain-containing proteins (Nods), recognizes intracellular bacterial products and activates pro-inflammatory signaling pathways (5–8).

Currently, there are thirteen TLRs identified in mammalian cells. Each TLR is thought to respond to different types of agonists through their leucine-rich repeat domains. For examples, TLR4 recognizes lipopolysaccharide derived from Gram-negative bacteria. TLR2 forms a heterodimer with TLR1 or TLR6 to discriminate triacylated bacterial lipopeptides from diacylated mycoplasmal lipopeptides. Upon activated by agonists, the intracellular TIR domains of TLRs recruit adaptor molecules, such as MyD88 or Toll-interleukin-1 receptor domain-containing adaptor inducing interferon-β, to initiate downstream NF-κB activation and pro-inflammatory gene expression. Similarly, Nods proteins have also been demonstrated to recognize their ligands through the leucine-rich repeat domain at the COOH terminus (9, 10). The minimal peptidoglycan structure that Nod1 recognizes is a dipeptide, γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) derived mostly from Gram-negative bacteria, whereas Nod2 recognizes the minimal peptidoglycan muramyl dipeptide, MurNac-L-Ala-D-isoGln (MDP), from both Gram-positive and Gram-negative bacteria (11–14). The activation of Nod2 leads to the recruitment of a CARD domain-containing kinase, RICK/RIP2, through a CARD-CARD interaction, which then leads to the interaction with the regulatory subunit of IKK, IKKγ/NEMO, linking to the activation of NF-κB and pro-inflammatory gene expression (15, 16).

Thus, despite the apparent differences between TLRs and Nods, these two families of pattern recognition receptors both...

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2 The abbreviations used are: TLR, Toll-like receptor; Nods, family of nucleotide-binding oligomerization domain-containing proteins; NF-κB, nuclear factor-κB; iE-DAP, γ-D-glutamyl-meso-diaminopimelic acid; MDP, muramyl dipeptide MurNac-L-Ala-D-isoGln; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFAs, polyunsaturated fatty acid; RIP2, RIP-like interacting CLARP kinase; IKK, IκB kinase; IκB, inhibitor of NF-κB; DN, dominant negative; siRNA, small RNA interference; RT, reverse transcription; HA, hemagglutinin; IL-8, interleukin-8.
are thought to recognize their ligands through the leucine-rich repeats domain and activate common NF-κB pathways. Mutations in Nod2 gene have been implicated in certain inflammatory diseases. The three major Nod2 mutations (1007fs, R702W, and G908R) have been found to be associated with increased susceptibility to Crohn disease, a chronic relapsing inflammatory disease of the bowel (17).

Dietary fatty acids have been known to modulate immune responses. It has been demonstrated that the survival of mice fed with fish oil, whose major fatty acids are n-3 PUFAs, was diminished after bacterial infection as compared with mice fed with diet rich in saturated fatty acids or n-6 PUFAs (18, 19). Moreover, n-3 PUFAs have been suggested to be beneficial in ameliorating various chronic inflammatory diseases including Crohn disease because of their anti-inflammatory properties (20). However, the underlying mechanisms for the effects of these fatty acids remain largely unknown.

The results from our previous studies demonstrated that lauric acid (C12:0) activated, but docosahexaenoic acid (DHA), an n-3 PUFA, inhibited both NF-κB activation and the expression of the target gene, inducible cyclooxygenase-2, in macrophages. This was at least in part mediated through TLR4 or TLR2 dimerized with TLR6 or TLR1, for which respective bacterial agonists require acylated fatty acids, but not through other TLRs tested (21, 22). Consequently, the maturation and antigen-presenting abilities of dendritic cells (the major antigen-presenting cells), which express TLRs, were also shown to be differentially modulated by these fatty acids (23). In addition to these immune cells, intestinal epithelial cells, representing the first line of defense against invasive pathogens, play important roles in mucosal immunity against luminal pathogens through its expression of pro-inflammatory genes, secretion of inflammatory cytokines, and recruitment of inflammatory cells in response to pathogenic bacteria and their products. The dysregulation of the interaction between the intestinal epithelial cells and luminal bacteria is suggested to cause inflammatory diseases in gut, especially in susceptible individuals (24). Moreover, as major absorptive sites, intestinal epithelial cells are exposed to a relatively higher concentration of fatty acids during each meal. However, the effects of dietary fatty acids on inflammatory responses in intestinal epithelial cells have not been studied.

Here, we show the differential modulation of NF-κB activation and the target gene interleukin-8 production by saturated and unsaturated fatty acids in the human colonic epithelial cell line HCT116. These effects of fatty acids are mediated through Nod1 and Nod2, in the absence of detectable amounts of TLR2 and TLR4 expression.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Sodium salts of saturated fatty acids and unsaturated fatty acids were purchased from Nu-chek (Elysan, MN). They were dissolved in endotoxin-free water. MDP was purchased from Bachem. iE-DAP was synthesized as described previously (12). Polyclonal IkB antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). β-Actin antibody was from Sigma. All other reagents were purchased from Sigma unless otherwise indicated.

**Cell Culture**—HEK293T (a human embryonic kidney epithelial cell line, provided by Sam Lee, Beth Israel Hospital, Boston, MA) was cultured in Dulbecco’s modified Eagle’s medium containing 10% (v/v) heat-inactivated fetal bovine serum (InterGen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Human colonic epithelial cell lines HCT116 and HT29, purchased from ATCC, were cultured in McCoy’s 5A medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Human normal colonic epithelial cell line NCM460, purchased from INCELL Corp. (San Antonio, TX), was cultured in M:3:10 medium purchased from the company. Human monocytic cell line THP-1, purchased from ATCC, was cultured in RPMI medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen).

**Plasmids**—pcDNA3-HA-Nod1, pcDNA3-HA-Nod2, pcDNA3-Nod1-Δcard (DN form), and pcDNA3-Nod2L145P (DN form) were previously described (25–28). (2X)-NF-κB luciferase reporter construct was provided by Frank Mercuro (Signal Pharmaceuticals, San Diego, CA). Human IL-8 (-173bp)-Luc was from Marie Annick Buendia (Institute Pasteur, France). pRSV-β-galactosidase plasmid was from Jiongde Lee (University of California, San Diego, CA). Dominant negative forms of TLR4 (TLR4(P712H)) and TLR2 (TLR2(P681H)) were described previously (21, 22). A dominant negative form of TLR5 (TLR5(P737H)) was cloned by substituting proline at residue 737 with histidine. A dominant negative mutant of RICK/RIP2 (pcCMV-RICK/RIP2-(1–434)) was provided by Genhong Cheng (University of California, Los Angeles, CA). A dominant negative mutant of IκKB (IKKB(K44M)) was obtained from Michael Karin (University of California, San Diego). A dominant negative mutant of inhibitor κB (pCMV-4-IκBα(ΔN)) was provided by Dean Ballard (Vanderbilt University, Nashville, TN). All DNA constructs were prepared in large scale using the EndoFree Plasmid Maxi kit (Qiagen) for transfection.

**RT-PCR and Semi-quantitative Real-time RT-PCR Analysis**—Total RNA was prepared from HEK293T, HCT116, HT29, NCM460, or THP-1 cells using TRizol reagent (Invitrogen) according to the manufacturer’s instructions. Five micrograms of total RNAs was used for cDNA synthesis with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). The primers used to detect mRNA expression were as follows: human TLR2, 5'-gcc aaa gtc tgt att gat tgg-3' (forward) and 5'-ttg aag ttc tgt ccc cgg tct c-3' (reverse); TLR4, 5'-ttg atc ctt ctt ata ag-3' (forward) and 5'-gaa tga gag gca ccc ctt c-3' (reverse); TLR5, 5'-ctc aac ccc tcc aga gaa ccc c-3' (forward) and 5'-ttg gag tgt cgg cct agg ccc c-3' (reverse); TLR7, 5'-ttg gct gtg gcg cct caa ctt c-3' (forward) and 5'-aag gcc agg tta tgt tca cgc c-3' (reverse); TLR9, 5'-cag cag ctc tgt tgt cct c-3' (forward) and 5'-cag cag ctc tgt ccc ctt c-3' (reverse); Nod1, 5'-cag cat cca gat gaa cgt g-3' (reverse) and 5'-tcc aac ccc tcc aga ccc c-3' (forward); Nod2, 5'-gcc aag tgg tgt gcc ccc ctt c-3' (forward) and 5'-aga gag tgg att gta cag cct c-3' (reverse); Nod3, 5'-tcc aac ccc tcc aga gaa ccc c-3' (forward) and 5'-aag gcc agg tta tgt tca cgc c-3' (reverse); and glyceraldehyde-3-phosphate dehydrogenase, 5'-tct ccc cct ctc ctg c-3' (forward) and 5'-cga ctc ctc ccc cac ctc-3' (reverse). The following PCR program was used on iCycler (Bio-Rad): denaturation
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at 95 °C for 2 min and 40 cycles of denaturation at 95 °C for 20 s, annealing at 58 – 60 °C for 20s, and extension at 72 °C for 25 s. The resulting PCR products were resolved by 1% agarose gels. Total RNA was isolated from HCT116 cells after treatments with vehicle control, increasing doses of lauric acid or MDP for 18 h. IL-8 mRNA level was determined by semi-quantitative real-time PCR following reverse transcription with a LightCycler (Roche Molecular Biochemicals) using the QuantiTect SYBR Green PCR kit (Qiagen). The primers used for human IL-8 were 5′-gta aac atg act tcc aag cta g-3′ (forward) and 5′-gcc ctc ttc aac aat ctc c-3′ (reverse). The primers used for human β-actin (as an internal control) were 5′-gtc gtc gac aac ggc tcc gcc atg tg-3′ (forward) and 5′-cat tgt aga agg tgt ggt gcc aca t-3′ (reverse). Samples were compared using the relative crossing-point values (Cp) method. The Cp value, which is inversely proportional to the initial template copy number, was determined by the LightCycler software program provided by the manufacturer (Roche Applied Science). The -fold induction of IL-8 expression relative to vehicle control was measured three times in duplicates and calculated after adjusting for β-actin using 2ΔΔCp, where ΔΔCp = Cp β-actin - Cp IL-8, and ΔΔCp = Cp treatment - Cp control as described previously (22).

Transfections and Luciferase Assays—For transient transfections and luciferase assays, HEK293T or HCT116 were seeded 4–5 × 104 per well of 24-well plates, and co-transfected with 2xNF-κB-Luc or IL-8(-173bp)-Luc and pRSV-β-galactosidase plasmid using SuperFect transfection reagent (Qiagen) according to the manufacturer’s instructions. In Nod1 and Nod2 transfection experiments in 293T, 2 ng of pcDNA3-Nod1-HA, or 1.5 ng of pcDNA3-Nod2-HA was co-transfected with reporter gene and β-galactosidase plasmid. Various expression plasmids for dominant negative form of TLRs, Nods, or of downstream components, or their corresponding empty vectors were co-transfected as indicated in the figure legends. The total amount of plasmids was equalized by supplementing with the corresponding empty vector to eliminate the experimental errors from transfection itself. The cells were stimulated with saturated fatty acids, Nods agonist, or vehicle controls. In unsaturated fatty acid experiments, the cells were pretreated with unsaturated fatty acids for 1–2 h and co-treated further with other stimuli for 15–18 h before lysis. Luciferase and β-galactosidase enzyme activities were determined using a 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 to correct the transfection efficiency.

Immunoblotting and Co-immunoprecipitation—These experiments were performed essentially the same as previously described (21, 22, 29). For IκB degradation experiments, HCT116 cells, 4–5 × 105 per 60-mm dish, were seeded. The cells were stimulated with lauric acid, MDP, or pretreated with DHA, then co-stimulated with lauric acid or MDP as indicated in the figure legends. For Nod2 oligomerization experiments, 293T cells, 5.0 × 106 per 100-mm dish, were transfected with 2 μg of HA- and 2 μg of FLAG-tagged Nod2 for 24 h and treated as described in the figure legends. The resulting proteins co-immunoprecipitated with HA-affinity matrix (Roche Applied Science) were analyzed for the presence of FLAG-tagged Nod2 using anti-FLAG M2 antibody (Sigma). The solubilized proteins from co-immunoprecipitation or from the cell lysate from HCT116 were subjected to 7.5–10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membrane was blocked in 20 mM Tris-HCl, 137 mM NaCl, and 0.05% (v/v) Tween 20 (pH 7.6) containing 5% nonfat milk. The membrane was probed with primary antibody for 1–24 h, followed by secondary antibody coupled to horseradish peroxidase (Amersham Biosciences) for 1 h. The protein signals were detected by ECL Western blot detection reagents (Amersham Biosciences) followed by exposing to an x-ray film (Eastman Kodak Co.). To reprobe with different antibodies, the membrane was stripped in stripping buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol at 53 °C for 30 min.

Measurement of IL-8—The supernatants of HCT116 cells that were transiently transfected for reporter gene assays and/or stimulated by different stimuli were collected, and the levels of
IL-8 were determined by enzyme-linked immunosorbent assay kits (OptEIA ELISA kits, BD Pharmingen).

RNA Interference—Nod1 and Nod2 siRNA targeting 5'-AAG-AGCCTCTTTGTCTTCACC-3' of human Nod1 and 5'-AAGACATCTTTCCAGTTACTCC-3' of human Nod2 were custom synthesized; non-targeting control siRNA was purchased from Dharmacon (Lafayette, CO). Transfection of siRNA (final concentration, 200 nm) in HCT116 cells was performed using TransIT-TKO reagent (Mirus, Madison, WI) according to the manufacturer's instructions.

Data Analysis—Data were analyzed by paired t test or one-way analysis of variance with repeated measures.

**FIGURE 2.** Saturated fatty acids induced the transactivation of NF-κB and IL-8 expression in HCT116 cells. A and B, HCT116 cells were transiently transfected with 2xNF-κB-Luc or IL-8-Luc together with β-galactosidase. The cells were stimulated with iE-DAP (20 μg/ml), MDP (25 μg/ml), or increasing doses of lauric acid (C12:0, 0, 20, 50, 75, 100, and 200 μM) for 18 h. C, HCT116 cells were treated with vehicle control, increasing doses of C12:0, or MDP for 18 h. The total RNA was isolated, and the relative IL-8 mRNA levels were determined by semi-quantitative RT-PCR. D and E, HCT116 cells were transfected with IL-8-Luc and β-galactosidase. The cells were treated with saturated fatty acids with different carbon chain length (C10:0–C18:0, 100 μM) for 18 h. NF-κB and IL-8 reporter gene assays were performed (A and D), and IL-8 protein was determined from the supernatant by IL-8 enzyme-linked immunosorbent assay (B and E). RLA, relative luciferase activity. Values are mean ± S.E. (n = 3) and expressed as fold induction compared with vehicle controls (A, C, and D) or pg/ml (B and E). The data are representative of more than three independent experiments. a and b, significantly different from the corresponding vehicle controls (p < 0.05 and p < 0.01, respectively).
RESULTS

Expression and Functional Status of TLRs and Nods in Colonic Epithelial HCT116 Cells—To determine the effects of dietary fatty acids on the inflammatory responses in intestinal cells, we first analyzed the expression of TLRs and Nods and their functional status in these cells by RT-PCR and NF-κB reporter gene assays in response to synthetic ligands for these receptors. Colonic epithelial HCT116, HT29, and NCM460 cells all express TLR5, TLR7, and TLR9, but the expressions of TLR2 and TLR4 are different among these cell lines (Fig. 1A). HCT116 cells do not express detectable amounts of TLR2 and TLR4. HEK293T cells also do not express detectable amounts of TLR4 and TLR2, consistent with previous reports (11, 30). In contrast, HCT116 express both Nod1 and Nod2 mRNA. Nod2 protein expression has been reported in HCT116 (31). To confirm the expression results, we examined the functional status of these receptors by NF-κB reporter gene assays. Consistent with RT-PCR data, HCT116 cells respond only to flagellin, the TLR5 ligand, but not Pam3CAG and MALP-2, the ligands for TLR2/TLR1 and TLR2/TLR6 dimers, respectively, and lipopolysaccharide, the TLR4 ligand. This result is in agreement with the observations by others that, under normal conditions, the intestinal epithelial cells do not respond to any TLR ligands other than flagellin (32, 33). In contrast, HCT116 cells responded to iE-DAP, the minimal peptidoglycan recognized by Nod1, and MDP, the minimal peptidoglycan recognized by Nod2.

FIGURE 3. Polyunsaturated fatty acids inhibited the NF-κB activation and IL-8 expression induced by lauric acid, iE-DAP, or MDP in HCT116 cells. A–E, HCT116 cells were transfected with 2xNF-κB-Luc (A and D), or IL-8-Luc (B) and β-galactosidase. The cells were pretreated with vehicle or DHA (5 μM) (A–C), increasing doses of DHA, or other unsaturated FAs (0, 1, 5, and 20 μM) (D and E) for 2 h and then further co-stimulated with vehicle, lauric acid (C12:0, 100 μM), iE-DAP (20 μg/ml) (A–C) or MDP (25 μg/ml) (D and E) for 18 h. F, HCT116 cells were transfected with NF-κB-Luc or IL-8-Luc and β-galactosidase for 24 h. The cells were treated with vehicle, C12:0 (5 μM), DHA (5 μM), MDP (50 μM), or pretreated with C12:0 (5 μM) or DHA (5 μM) for 2 h and further co-treated with MDP for 18 h. The reporter assays were performed (A, B, D, and F), and IL-8 protein secretion was measured (C and E) as described. RLA, relative luciferase activity. Values are mean ± S.E. (n = 3) and expressed as -fold induction (A, B, and F), percentage of control (D), or pg/ml (C and E). a and b, significantly different from the corresponding controls (p < 0.05 and p < 0.01, respectively). The data are representative of more than three independent experiments.
Nod2 (Fig. 1B). Thus, HCT116 cells are chosen to be studied further for the effects of fatty acids in the absence of TLR2 and TLR4, which are known to be modulated by fatty acids (21, 22). Saturated Fatty Acids Activated, but PUFAs Inhibited, NF-κB Activation, and the Nod1 Target Gene IL-8 Expression in HCT116 Cells—Next, we examined the effects of fatty acids on inflammatory responses in HCT116 cells using NF-κB and IL-8 reporter gene assays and IL-8 protein production. Lauric acid (C12:0) dose dependently induced activation of NF-κB and the target gene IL-8 expression. The levels of activation of NF-κB and IL-8 by lauric acid (100 μM) were comparable to those by iE-DAP (20 μg/ml) or MDP (25 μg/ml) (Fig. 2A). The activation was confirmed by IL-8 protein measurement in the supernatant of the same cells (Fig. 2B). To further confirm the dose-dependent effects of lauric acid on IL-8 transactivation, we measured IL-8 mRNA levels. Lauric acid dose dependently induced IL-8 mRNA expression in these cells (Fig. 2C). Lauric acid is the most potent in inducing IL-8 as compared with other saturated fatty acids (Fig. 2, D and E). In contrast, DHA, an n-3 PUFAs, did not induce, but rather inhibited, NF-κB and IL-8 activation induced by lauric acid, iE-DAP, or MDP (Fig. 3, A–E). Moreover, we also compared the inhibitory potency among unsaturated FAs. DHA and EPA, n-3 PUFAs, are the most potent in inhibiting Nod2 ligand MDP-induced IL-8 expression as compared with arachidonic acid and linoleic acid, n-6 PUFAs and oleic acid, n-9 monounsaturated fatty acid (Fig. 3, D and E). To determine whether the preincubation of lauric acid might also interfere with the Nod2-ligand interaction, we performed the experiments by pretreating the cells with lauric acid at the concentration used for DHA in Fig. 3 (A–C), prior to MDP stimulation for 18 h. Lauric acid did not inhibit MDP-induced NF-κB and IL-8 activation, but rather potentiated MDP-induced IL-8 transactivation, although the change was not statistically significant (Fig. 3F).

Lauric Acid Induced, but DHA Inhibited MDP- or Lauric Acid-induced, IkBα Degradation in HCT116 Cells—We next determined whether the immediate upstream event, i.e. the degradation of IkBα that leads to NF-κB activation, was also affected by fatty acids. Lauric acid dose dependently induced IkBα degradation within 30 min upon stimulation (Fig. 4A, left and right panels). MDP also induced IkBα degradation, as expected (Fig. 4B). In contrast, DHA itself did not induce similar effects, but rather inhibited the basal IkBα degradation (Fig. 4C, left panel). Moreover, DHA attenuated MDP or lauric acid-induced degradation of IkBα in these cells (Fig. 4C, right panels). These results demonstrate the direct (not secondary) effects of these fatty acids on NF-κB activation leading to differential modulation of downstream target gene expression (i.e. IL-8) in HCT116 cells.

Differential Modulation of NF-κB Activation and IL-8 Expression by Lauric Acid and DHA Was Mediated through Nod2 in HCT116 Cells—The results from our previous studies suggested that the effects of saturated fatty acids can activate TLR4 and/or TLR2 dimers in macrophages (21, 22). However, HCT116 cells do not express detectable amounts of TLR4 and TLR2 and do not respond to the agonists of TLR4 and TLR2 dimers. However, HCT116 cells still responded to saturated and unsaturated fatty acids. Because HCT116 cells express both Nod1 and Nod2, we hypothesized that the effects of fatty acids might be mediated through Nod1 and/or Nod2. We first tested our hypothesis using various dominant negative mutants of TLRs and Nod2. The expression of those constructs after transfection has been verified in these cells by Western blot analysis (data not shown). Indeed, lauric acid-induced IL-8 activation was not inhibited by the dominant negative forms of TLR2, TLR4, or TLR5 but was inhibited by the dominant negative forms of Nod1 and Nod2, and of their known downstream signaling components, Rick, IKKβ, and IkBα (Fig. 5A). Transfection of the cells with both Nod1 and Nod2 dominant negative mutants resulted in more pronounced inhibition on the effects of lauric acid than either Nod1 or Nod2 dominant negative mutant alone (data not shown). The results suggest that Nod1 and Nod2 are additive in mediating lauric acid effects. To further address the roles of Nod2 in mediating lauric acid-induced IL-8 expression, we performed siRNA experiments to inhibit the endogenous Nod1 and Nod2. First, we evaluated the efficacy of those siRNA by measuring the reduction of endogenous mRNA of Nod1 and Nod2. The Nod1- or Nod2-specific oligonucleotides significantly reduced Nod1 or Nod2 mRNA levels (Fig. 5B), siRNA targeting either Nod1 or Nod2 significantly inhibited the IL-8 expression induced by lauric acid as revealed by IL-8 reporter gene activation and protein production (Fig. 5, C–F). In contrast, tumor necrosis factor-α-induced IL-8 activation was not affected by these Nod1- or Nod2-specific siRNAs, further demonstrating the specificity of these siRNAs. Therefore, these results suggest that the effects of saturated fatty acid were, at least in part, mediated through Nod1 or Nod2 in HCT116.
Modulation of Nods by Fatty Acids in HCT116 Cells

To further confirm the roles of Nods proteins in mediating the effects of fatty acids, we determined the effects of fatty acids in HEK293T cells with and without transfection of Nod1 or Nod2 expression plasmid. In non-Nod1- and -Nod2-transfected 293T cells (Fig. 6A), iE-DAP induced 1.42 ± 0.05-fold and 1.26 ± 0.08-fold, whereas MDP induced 1.56 ± 0.09-fold and 1.52 ± 0.11-fold, NF-κB and IL-8 transactivation, suggesting the presence of endogenous Nod1 and Nod2 in HEK293T cells. Nod1 or Nod2 transfection resulted in potentiation of lauric acid-induced NF-κB and IL-8 transactivation in these HEK293T cells (Fig. 6B). Nod1 or Nod2 transfection also potentiated iE-DAP or MDP-induced NF-κB and IL-8 transactivation (Fig. 6B), as compared with that of non-transfected cells. Another saturated fatty acid, capric acid (C10:0), was much less potent in inducing the activation both in the non-transfected and Nod1- or Nod2-transfected cells, consistent with the potency data in HCT116 cells (Fig. 2, D and E). In contrast, DHA not only failed to induce the activation by itself (Fig. 6A) but also significantly inhibited NF-κB and IL-8 transactivation induced by transfection of Nods alone, and by lauric acid, iE-DAP, or MDP in these Nod1- or Nod2-transfected cells (Fig. 7, A and B). To determine whether the inhibitory effects of DHA were also dependent on Nods overexpression in HEK292T cells, we tested DHA effects in Nod1- or Nod2-silenced 293T cells stably transfected with Nod1 or Nod2, respectively. The inhibitory effects of DHA were significantly attenuated when Nod1 or Nod2 was silenced in these cells (Fig. 7C; Nod1 data not shown).

The results that lauric acid is more potent in activating NF-κB and the target gene IL-8 than another saturated fatty acid, capric acid, and that polyunsaturated fatty acid DHA does not activate but rather inhibits the activation, strongly suggest that the effects of these fatty acids are specific and are not due to the overexpression of Nods protein by transfection in HEK293T cells. The activation by iE-DAP or MDP was modest compared with those reported before (13, 14). This could be due to the fact that we have added Nods ligands or fatty acids directly into the medium, not mixed with transfection reagent such as calcium phosphate, which might have facilitated the entry of the ligands into the cells (13, 14). Together, our data suggest that Nod1 or Nod2 are not only required but also sufficient to mediate the effects of fatty acids. Lauric Acid Induced, but DHA Inhibited MDP or Lauric Acid-induced, Nod2 Self-oligomerization in HEK293T Cells—To characterize the molecular target(s) of saturated and unsaturated fatty acids in modulating Nods signaling pathways, we examined the effects of fatty acids on Nod2 self-oligomerization using co-immunoprecipitation and immunoblotting of HA- and FLAG-tagged Nod2 ectopically expressed in HEK293T cells. The results show Nod2 ligand MDP-induced Nod2 self-oligomerization within 10 min upon the stimulation.
Modulation of Nods by Fatty Acids in HCT116 Cells

Our results presented here demonstrated that saturated fatty acids activated, whereas n-3 polysaturated fatty acids inhibited, NF-κB activation and IL-8 production in colonic epithelial HCT116 cells. These effects were mediated through Nod1 and Nod2, but not through TLRs. Healthy human primary colonic epithelial cells have been shown to express low levels of TLR2 and TLR4 (34). The expression of TLR2 and TLR4 is confined in crypt cells, and the expression decreases as these cells mature and move up toward the gut lumen. Thus, there is no or trace amount of TLR2 and TLR4 expression at the luminal surface (34). Therefore, human intestinal epithelial cells are generally unresponsive to defined TLR2 or TLR4 ligand (34, 33). On the other hand, both Nod1 and Nod2 have been shown to be expressed in primary colonic epithelial cells and can be up-regulated by cytokines (35–37). In HCT116 cells we used herein, protein expression, whereas n-3 polysaturated fatty acid such as DHA suppressed IL-8 protein expression in HCT116 colonic epithelial cells. Lauric acid and Nods ligands have shown greater induction at protein levels than that at transcriptional levels (Fig. 2, A and C versus Fig. 2B). IL-8 is known to be regulated both at transcriptional and post-transcriptional levels by TLR ligands and inflammatory stimulations (48, 49). Therefore, the difference of IL-8 up-regulation at transcription and protein levels by lauric acid and Nods ligands may be due to the possibility that lauric acid and Nods ligands may also regulate IL-8 expression at post-transcriptional levels such as IL-8 mRNA stability. This is an important question that needs to be addressed in the future. Together, our results have provided new insights into differential modulation of inflammatory responses by types of fatty acids in colonic epithelial cells. Whether such modulations can lead to alteration of the risk for the development of such diseases need to be determined in the future.

Our results presented here and the results from our previous studies (21, 22, 29) demonstrated that two key pattern recognition receptors, TLRs and Nods, which are involved in host defense and wound healing, are differentially modulated by saturated and n-3 polysaturated fatty acids. To our knowledge, our report is the first to show that Nod1 and Nod2 signaling can be modulated by non-microbial molecules, suggesting that Nods, like TLRs, especially TLR4 (50–52), may also participate...
in the induction of sterile inflammation (inflammation in the absence of pathogen). Both infection-induced inflammation and sterile inflammation can lead to chronic inflammation, which is now considered as one of the key etiological conditions leading to the development of many chronic diseases, including cancer. Understanding the roles of Nods in the development of these chronic diseases may hold the promise of designing more effective preventive and therapeutic dietary strategies for these diseases. Moreover, our results raised an important question as to whether chronic inflammations and thus the risk of developing the inflammatory chronic diseases in the gut can also be similarly modulated by the types of dietary fatty acids consumed.

How Nods proteins are activated by MDP- or iE-DAP-containing molecules is still poorly understood. Oligomerization of both TLRs and Nods are considered as the initial event for the activation and signaling of these receptors (15, 53, 54). Here, we show that one of the molecular targets for these fatty acids is the oligomerization of Nods. However, the detailed mechanisms by which fatty acids modulate self-oligomerization of Nods, leading to enhanced or suppressed receptor activation, remain to be determined further. Our results showed that DHA inhibited Nod oligomerization, which is one of the most upstream events of Nods activation. Once the upstream event is inhibited, then, most (if not all) downstream signaling steps should also be inhibited. Our results, showing that DHA inhibited MDP-induced IκBα degradation and NF-κB transactivation and inhibited MDP-induced ERK activation (data not shown), support such interpretation. Our results, however, do not allow us to preclude the possibility that DHA may also inhibit multiple signaling steps derived from Nods, in addition to their oligomerization. In fact, DHA also inhibited tumor necrosis factor-α-induced NF-κB transactivation in HCT116 cells (data not shown), suggesting that DHA can inhibit multiple signaling molecules.

Polyunsaturated fatty acids can be enzymatically metabolized via lipoxygenases, cyclooxygenases, or cytochrome P450. Recently, it was shown that the novel hydroxy series of DHA metabolites possesses potent anti-inflammatory effects (55). Whether the inhibitory effects of DHA on Nods activation are exerted by DHA itself or enzymatic metabolites needs to be determined in the future.

In summary, our results demonstrate differential modulation of NF-κB and the target gene IL-8 expression by saturated and n-3 polyunsaturated fatty acids in colonic epithelial HCT116 cells mediated through Nods. These results suggest that Nods
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