The Effects of Dietary Fatty Acids and Non-Steroidal Anti-Inflammatory Drugs on Cyclooxygenase-2 Expression.

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THE EFFECTS OF DIETARY FATTY ACIDS AND NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ON CYCLOOXYGENASE-2 EXPRESSION

A Dissertation

Submitted to the Graduate faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Food Science

by

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August 2000
AKNOWLEDGMENTS

The author would like to thank to her major professor, Dr. Daniel Hwang in Pennington Biomedical Research Center at Louisiana State University for his tremendous and precious support, advice, guidance, and encouragement during the entire Ph.D. program.

The author wishes to express her sincere appreciation to Dr. Ezzat Younathan, Dr. Joan M. King, Dr. Maren Hegsted and Dr. Mike Keenan for serving as her committee members.

Appreciation is also expressed to Dr. Douglas Park, the former head of the Department of Food Science and other professors at the Department of Food Science.

The author would like to express her appreciation to lab colleagues, Sang Hoon Rhee, Dr. Mary Boudreau, and Brenda N. Belton.

The author would like to thank the Korea Food and Drug Administration for giving her an opportunity to pursue this degree and the Korea Department of Education for the scholarship.

Most of all, the author deeply thanks her parents, because she could not have finished her Ph.D. degree without their support, encouragement, and love.
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ABSTRACT

Dietary n-3 fatty acids and non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to have cancer preventive and tumor regressive effects. Fatty acids and NSAIDs are substrates and inhibitors, respectively, of cyclooxygenase-2 (COX-2), which is a key enzyme in the conversion of arachidonic acids to prostaglandins. Thus, the modulating effects of different types of dietary fatty acids and NSAIDs on the expression of COX-2 are studied here. Results show that both n-3 and n-6 polyunsaturated fatty acids (PUFAs) inhibit Lipopolysaccharide (LPS)-induced COX-2 expression and NF-κB activation in macrophages. Results from an in vitro cell proliferation study indicate that growth of colon tumor cells is suppressed by n-3 PUFAs as compared with n-6 PUFAs regardless of whether COX-1 or COX-2 is expressed or not. These results suggest that signaling pathways through which PUFAs inhibit tumor cell growth and LPS-induced COX-2 expression may be different. Unlike the previous study in human colon carcinoma cells, NSAIDs, especially flufenamic acid and sulindac sulfide, upregulate the expression of COX-2 under both the presence and absence of COX-2 stimulators in a normal intestinal cell line, IEC-6 cells. The inhibitors of extracellular-signal-regulated protein kinase (ERK), and p38 MAP kinase inhibit the flufenamic acid-induced COX-2 expression, suggesting that flufenamic acid-induced COX-2 expression is at least partly mediated through activation of MAPKs in IEC-6 cells. TNFα-induced activation of NF-κB was suppressed by flufenamic acid or sulindac sulfide. However, these NSAIDs did not appear to affect TNFα-induced COX-2 expression in IEC-6 cells. This may be due to two opposing effects: one is
induction of COX-2 by NSAIDs and the other is induction of TNFα-induced COX-2 expression by NSAIDs. The results from these studies provide new insight for signaling pathways through which fatty acids and NSAIDs modulate COX-2 and other inflammatory marker gene products.
CHAPTER 1

REVIEW OF LITERATURE

1.1. Cyclooxygenase (COX)

Cyclooxygenase (COX) is the enzyme that catalyzes the conversion of arachidonic acid and O₂ to prostaglandin endoperoxide H₂ (PGH₂). COX is also known as prostaglandin H synthase. COX has two distinct enzyme activities: 1) a cyclooxygenase activity, which catalyzes the conversion of arachidonic acid to PGG₂, and 2) a peroxidase activity, which catalyzes the conversion of PGG₂ to PGH₂. PGH₂ formed by the action of COX is converted via distinct synthases to PGD₂, PGE₂, PGF₂α, PGI₂, or thromboxane A₂ (TXA₂) [see APPENDIX 1]. Non-steroidal anti-inflammatory drugs (NSAIDs) compete directly with arachidonate for binding to the cyclooxygenase site and inhibit cyclooxygenase activity but have little effect on peroxidase activity (Mizuno et al., 1982). Therefore, the cyclooxygenase and peroxidase sites are physically and functionally separate. This conclusion is based on results from many studies of protein chemistry, UV-visible spectroscopy, and x-ray crystallography about the active sites of COX (Lambeir et al., 1985; Picot et al., 1994; Smith and DeWitt, 1996).

It has been found that there are two closely related isoforms of COX, which are known as COX-1 and COX-2 (Xie et al., 1991; Kujubu et al., 1991; O’Banion et al., 1991). COX-1 is the constitutive form of the enzyme and ubiquitously expressed. It provides certain homeostatic functions, such as maintaining normal gastric mucosa and aiding in blood clotting by abetting platelet aggregation (Simon, 1996).
COX-2 is the inducible form and expressed in response to inflammatory and other physiologic stimuli, growth factors, tumor promoters, hormones, bacterial endotoxins, and cytokines (DeWitt and Meade, 1993; Evett et al., 1993; Jones et al., 1993; O'Sullivan et al., 1992). The rapid induction of COX-2 mRNA, which can be superinduced by cycloheximide, parallels the expression of c-fos leading to the classification of COX-2 as an immediate early gene (Jones et al., 1993). In fibroblasts, increased expression is due to an increased rate of COX-2 gene transcription (DeWitt and Meade, 1993). In other systems, post-transcriptional regulation contributes to the magnitude and duration of COX-2 mRNA expression (Ristimaki et al., 1996).

Although both COX-1 and COX-2 convert arachidonic acid to prostaglandins, there are many differences between the COX isoforms. One major distinction is that COX-2 is the principal isoform that participates in inflammation (Simon, 1999). Of the two isoforms, the inducible COX-2 enzyme is thought to make the more important contribution to synthesis of PGs at sites of inflammation as suggested by studies of cultured inflammatory cells (Lee et al., 1992) or of synoviocytes from rheumatoid arthritis and osteoarthritis patients (Sano et al., 1992). COX-2 expression is inhibited by glucocorticoids such as dexamethasone at the level of transcription and translation, while these agents have little effect on the expression of COX-1 (Vane and Botting, 1995; Masferrer et al., 1994). COX-1 is also not upregulated by inflammatory or other stimuli. This leads to a steady state level of COX-1 that is responsible for the production of basal levels of PGs (Crofford, 1997). Antiinflammatory cytokines, such as interleukin-10 (IL-10), can also selectively inhibit COX-2 induction (Mertz et al.,
All of this evidence suggest that COX-2 is responsible for production of prostaglandins involved in inflammation.

Another distinction involves their differential expression. The two isoforms are encoded by genes located on separate chromosomes. COX-1 is located on human chromosome 9, while COX-2 is located on human chromosome 1 (Funk et al., 1991; Jones et al., 1993). Both isoenzymes share 60% genetic homology in their coding regions (Smith and DeWitt, 1996). The gene for COX-1 is approximately 22 kilobase pairs and contains 11 exons (Kraemer et al., 1992). Typical of developmentally regulated “housekeeping” genes, the COX-1 gene lacks a TATA box and little is known about the details of the regulation of COX-1 gene expression. COX-2 is 8 kilobase pairs in length and contains 10 exons (Kujubu and Herschman, 1992). The small size of the COX-2 gene is consistent with its characterization as an immediate-early gene (Herschman, 1994). The COX-2 promoter contains a TATA box. Several relevant enhancer sequences have been identified in the COX-2 gene promoter. In bovine endothelial cells, a CEBPβ site is responsible for induction of COX-2 by lipopolysaccharides and tumor-promoting phorbol ester while the same site is responsible for tumor necrosis factor α-mediated induction of COX-2 in MC3T3-E1 cells (Inoue et al., 1995; Yamamoto et al., 1995). An E-box sequence is essential for basal and luteinizing hormone- and gonadotrophin-releasing hormone stimulated transcription, and this element binds the upstream stimulating transcription factor (Morris and Richards, 1996). A cAMP response element mediates the effect of src on COX-2 expression in fibroblasts (Xie and Herschman, 1995). Transcriptional activation of the COX-2 gene appears to be the major mechanism for increasing COX-2
expression. COX-2 expression can be induced through multiple signaling pathways involving protein kinases A and C, tyrosine kinases, phosphatase, bacterial endotoxin, and src (Evett et al., 1993; Kester et al., 1994; Smith et al., 1996).

Prostanoid production by COX-1 and COX-2 appears to be initiated through distinct signaling pathways that may rely on the activation of different phospholipases (Reddy and Herschman, 1994). In addition, products of COX-2, but not COX-1, localize to the nucleus, which suggests that COX-2 may independently signal to the nucleus (Morita et al., 1995).

Although COX-2 is usually barely detectable during normal physiologic conditions, there is also some constitutive expression of this isoform in the rat kidney and brain, human prostate and lung, bone, and female reproductive system, (Cryer and Dubois, 1998). COX-2 expression also occurs in transformed or cancerous cells. The first COX-2 cDNA was initially characterized on the basis of its elevated expression in Rous sarcoma virus transformed chicken embryo fibroblasts (Simmons et al., 1989). Constitutive overexpression of COX-2 has been detected in colon carcinomas (Eberhart et al., 1994; Kargman et al., 1995).

Within the cell, both isoforms are located on the endoplasmic reticulum and the nuclear envelope (Otto and Smith, 1994). However, the concentration of COX-2 within the nuclear envelope is approximately twice the concentration in the endoplasmic reticulum, while COX-1 is found in equivalent concentrations in both intracellular locations (Morita et al., 1995).

The two major substrates for both COX-1 and COX-2 are arachidonic acid and dihomo-γ-linolenic acid. They are also capable of catalyzing the oxygenation of
eicosapentaenoic acid (EPA), γ-linolenic acid, α-linolenic acid, and linoleic acid (Kulmacz et al., 1994; Laneuville et al., 1995). EPA is converted to PGH$_3$, whereas the 18-carbon fatty acids are converted to monohydroxy acids. There are subtle differences in substrate specificities between COX-1 and COX-2. In general, 18-carbon polyunsaturated fatty acids are more efficiently oxygenated by COX-2 than by COX-1. Docosahexaenoic acid (DHA, 22:6n-3) is a competitive inhibitor of both COX-1 and COX-2 without being a substrate for either enzyme (Meade et al., 1993).

In enzyme catalysis, COX-1 and COX-2 have similar cyclooxygenase turnover numbers (~3500 mol of arachidonate/min/mol of dimer), and the $K_m$ values for arachidonate (~5 μM) and O$_2$ (~5 μM) are about the same for both isozymes (Barnett et al., 1994). Furthermore, the key residues involved in catalysis are conserved between the isozymes, and the crystal structures of the two isozymes are essentially superimposable (Picot et al., 1994). In the overall context of catalytic mechanisms, these proteins appear to be essentially the same.

### 1.2. Prostanoids

Prostaglandins and thromboxanes, collectively known as prostanoids (PGs), are produced by biologic oxidation of arachidonic acids. PGs are important mediators of inflammation. PGs are synthesized and secreted by a wide variety of cells only when stimulated by a multitude of cell perturbations, ranging from mechanical to chemical stimuli. Once released, PGs act as autocrine or paracrine factors to regulate the functions of various differentiated cells. The ubiquitous presence of PG-metabolizing enzymes makes these mediators unstable and their actions short-lived. Due to the short
half-lives of PGs in general, the levels and activities of PG synthetic enzymes determine the bioactivity of PGs within tissues (Needleman et al., 1986; Hla et al., 1993).

In arthritis, PGs contribute to synovial inflammation by increasing local blood flow and potentiating the effects of mediators such as bradykinin and interleukin (IL)-1 that induce increases in vasopermeability (Anderson et al., 1996). PGs also may induce osteoclastic bone resorption, suggesting a mechanism through which they may contribute to joint erosion (Sano et al., 1992). In patients with inflammatory joint disease, there is an increase in synovial and cartilage COX activity (Crofford, 1998).

1.3. Lipopolysaccharide (LPS)

Macrophages secrete prostaglandins upon activation by the bacterial endotoxin LPS, due to induced transcription of the COX-2 gene and production of the COX-2 enzyme. LPS is a major cell wall component of Gram-negative bacteria. Activation by LPS constitutes the first step in a cascade of events believed to lead to the manifestation of Gram-negative sepsis, a condition that results in approximately 20,000 deaths per year in the United States (Rietschel and Brade, 1992). Macrophages and monocytes respond to LPS by inducing the expression of cytokines, cell adhesion molecules, and low molecular weight proinflammatory molecules. The activation of monocytes or macrophages by LPS requires a serum protein known as LPS-binding protein (LBP) and a glycosylphosphatidylinositol-linked cell surface glucoprotein, CD14 (Wright et al., 1990). LBP is a 60 kD serum glycoprotein that forms high-affinity stoichiometric complexes with LPS. LBP also functions as an opsonin (Rietschel and Brade, 1992). It binds to the surface of bacteria or to LPS-coated erythrocytes and mediates the adhesion of these coated particles to macrophages. Interaction of LPS-LBP complexes with
macrophages subserves not only this adhesive function but also induces the synthesis of tumor necrosis factor (TNF) by the macrophages (Wright et al., 1990). CD14 is a well-known marker for monocytes and macrophages. LPS has been shown to initiate multiple intracellular signaling events, including the activation of nuclear factor-kappa B (NF-κB), which ultimately leads to the synthesis and release of a number of proinflammatory mediators, including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-α (Yamamoto et al., 1995; Cao et al., 1996). However, CD14 is not a transmembrane protein and does not have a cytoplasmic domain. Therefore, the identity of a transmembrane protein that could relay LPS-induced signals across the cell-surface membrane remained elusive.

Recently, several Toll-like receptors (TLR) have been identified as LPS receptors and can interact with CD14 to form the LPS receptor complex (Yang et al., 1998; Yang et al., 1999). Toll is a transmembrane receptor in Drosophila involved in dorsal-ventral patterning in embryos and in the induction of an anti-fungal response. The cloning of a family of human receptors structurally related to Drosophila Toll revealed five proteins that have extracellular domains containing multiple leucine-rich repeats and cytoplasmic domains with sequence homology to the intracellular portion of the IL-1 receptor. Recent studies have suggested that TLR2 or TLR4 serves as the main mediator of responses to LPS in vitro and in vivo (Chow et al., 1999; Cario et al., 2000).

LPS treatment leads to receptor oligomerization and to subsequent recruitment of IL-1R-associated kinase (IRAK) to the signaling complex, resulting in rapid phosphorylation of p42/p44 mitogen-activated protein kinase (MAPK), p38, and c-Jun NH2-terminal kinase (JNK) in monocytic cell lines (Reimann et al., 1994; Hambleton,
1996; Han et al., 1997; Kopp et al., 1999; Yang et al., 1999). Downstream LPS signaling through TLRs rapidly leads to NF-kB activation in monocyctic cells (Zhang et al., 1999).

1.4. Mitogen-activated protein kinases (MAPKs)

Eukaryotic cells use a variety of intracellular signaling pathways to respond to changes in their external environment. Among these pathways, the MAPK cascade is a major signaling system by which cells transduce extracellular signal into intracellular responses (Waskiewicz and Cooper, 1995; Treisman, 1996). Many steps of this cascade are conserved during evolution. Thus, MAPKs might play an essential role in diverse intracellular signaling processes from yeast to mammalian cells (Nishida and Gotoh, 1993; Marshall, 1994). MAPKs were originally described as serine/threonine kinases that are activated commonly by various growth factors and tumor promoters in mammalian cultured cells (Ray and Sturgill, 1987; Hoshi et al., 1988). They are now thought to function as key molecules in signaling processes stimulated by the growth factors, cytokines, hormones and differentiating factors and also in the M phase cascade reactions downstream of maturation promoting factor (MPF) (Cobb et al., 1991; Gotoh et al., 1991; Ferrell, et al., 1991; Posada et al., 1991).

MAPKs are activated by dual threonine/tyrosine phosphorylation of residues in the activation loop catalyzed by a specific MAPK kinase (MEK). The MEK is activated by Ser/Thr protein kinase, the MAPK kinase kinase (MEKK), which phosphorylates two serine or threonine residues within a Ser-X-X-X-Ser/Thr motif. In general, the MEKK → MEK → MAPK cascade functions as a module. There are multiple MAPK modules in mammalian cells. At present, three main classes of
MAPKs pathways are recognized. They are the extracellular signal-regulated kinases (the ERKs) pathway, the c-Jun N-terminal kinases/stress-activated protein kinases (the JNK/SAPK) pathway, and the p38/HOG pathway (Davis, 1993; Davis, 1994; Cano and Mahadevan, 1995; Cobb and Goldsmith, 1995). Each pathway is a 3-kinase cascade consisting of a MAPK, which is activated by a MEK that in turn is activated by a MEKK.

The ERKs pathway consists of Raf-1, MEK-1 (or MEK-2), and ERK-1 (or ERK-2). This pathway can be activated by various extracellular stimuli including phorbol esters and growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). ERK-1 (also known as MAPK-1) and ERK-2 (also known as MAPK-2) were the first two members of the MAPK subfamily to be purified and cloned (Boulton et al., 1991; Davis, 1993). They are the best-characterized MAPKs so far in terms of the signaling pathway involved in their activation and the identification of physiological targets. They are 44- and 42-kd enzymes that display 90% amino acid identity in the protein kinase catalytic core and 83% identity overall (Cobb et al., 1994). Other closely related enzymes include ERK-3, ERK-4, and ERK-5 (Gonzalez et al., 1992; Boulton et al., 1991; Zhou et al., 1995). The activation of ERKs pathway can be initiated through activation of transmembrane receptors with intrinsic or associated protein tyrosine kinase (PTK) activity through binding of extracellular ligands to their receptors. After the association of autophosphorylated receptor and adaptor protein such as growth factor receptor-bound protein 2 (Grb2), Grb2 binds to the guanine nucleotide-exchange protein SOS which, in turn, increases the association of Ras with GTP. The GTP-bound form of Ras interacts with the protein
kinase Raf-1 (MEKK). Then, MEK-1 or MEK-2 is phosphorylated and activated by Raf-1. MEKs phosphorylate and activate the ERKs. Activated ERK translocates into the nucleus and phosphorylates other protein kinases (e.g. Rsk) and transcription factors (e.g. Elk-1, NF-IL6).

The JNK/SAPK pathway consists of MEKK-1, MEK-4, and JNKS/SAPKs. The JNK/SAPK pathway is potently and preferentially activated by cellular stresses such as heat shock, recovery from protein synthesis inhibition, ATP depletion, UV radiation, and by inflammatory cytokines such as TNFα and IL-1β (Kyriakis et al., 1994; Derijard et al., 1994). In this pathway, GTP-bound forms of the small GTP-binding proteins such as Rac or Cdc42 activate p21-activated kinase (PAK), which activate MEKK-1. Then, activation of MEK-4 by MEKK-1 leads to activation of JNK/SAPK, which include JNK-1 (p45 SAPK α/β) and JNK-2 (p54 SAPK α/β/γ) (Lin, et al., 1995; Gupta et al., 1996). The JNK/SAPK stimulates c-Jun transcriptional activity, by phosphorylation of residues Ser 63 and Ser73 in c-Jun’s activation domain (Pulverer et al., 1991; Smeal et al., 1991). JNKS also appear to be responsible for activation of transcription factor, ATF2. Phosphorylation of c-Jun and ATF2 increases their transcriptional activity, leading to enhanced c-Jun transcription followed by increased c-Jun synthesis. The newly synthesized c-Jun may combine with c-Fos or ATF2, or form homodimers, all of which can contribute to increased AP-1 activity (Karin and Hunter, 1995).

The p38/HOG pathway consists of MEKK-1, MEK-3, and p38/HOG. This pathway is activated by various extracellular stimuli including LPS, heat, chemical stress, high osmolarity, ultraviolet light, and inflammatory cytokines (Han et al., 1994; Robinson and Cobb, 1997). Waskiewicz and Cooper (1995) reported a negative role for
p38 in cell proliferation. Its activation inhibited transcription of cyclin D1. and should therefore inhibit cell cycle progression (Lavoie et al., 1996). p38 is a vertebrate homolog of the yeast HOG gene (Han et al., 1994). There are several isoforms: p38α, p38β, p38γ, and p38δ (Jiang et al., 1996; Jiang et al., 1997; Li et al., 1996). Like its homolog HOG, p38 is activated by phosphorylation on both threonine and tyrosine. Two p38 activators have been characterized: SEK1 (also the activator of JNK) and MEK3 (specific for p38) (Lin et al., 1995). The known downstream targets of p38 are a seryl/threonyl kinase, MAPK-activated protein kinase (MAPKAPK) 2, and the transcription factor ATF-2 (Rouse et al., 1994).

1.5. Nuclear factor kappa B (NF-κB)

NF-κB is a eucaryotic transcription factor that exists in virtually all cell types. It was first identified in 1986 as a nuclear factor necessary for immunoglobulin kappa light chain transcription in B cells (Sen and Baltimore, 1986). It was originally thought that NF-κB was not produced in other cells. It is now known that NF-κB preexists in the cytoplasm of most cells in an inactive form bound to the inhibitor, IκB (Baeuerle and Baltimore, 1988). Upon receipt of an appropriate signal, NF-κB is released from IκB and translocates to the nucleus where it can upregulate transcription of specific genes. Since NF-κB activity does not require new protein synthesis, the signal is transmitted quickly. Upon appropriate cellular stimulation, IκBs are specifically phosphorylated and degraded through a ubiquitin/proteasome-dependent mechanism (Ghosh et al., 1998). The kinases responsible for phosphorylating IκB are known as the IκB kinases, IKK-1 and IKK-2, and they form a large multiprotein complex that contains scaffolding proteins such as IKAP and NEMO (IKKγ) (Cohen et al., 1998;
Rothwarf et al., 1998; Yamaoka et al., 1998; May and Ghosh, 1998). The IKKs are believed to be activated through phosphorylation by a kinase belonging to the MAP kinase kinase kinase (MAPKKK) family. Those kinases include NIK (NF-κB inducing kinase) and MEKK-1 (mitogen-activated protein kinase/ERK kinase kinase-1) and they can activate NF-κB through phosphorylation and activation of the IKKs. TRAF6 is capable of binding NIK and may therefore activate NF-κB via a NIK-IKK pathway (Kopp et al., 1999). TRAF6 is a member of the TRAF family of adaptor proteins. The TRAFs (TNF-receptor associated factors) were first described as proteins that are recruited to the tumor necrosis factor (TNF) receptors during signaling (Rothe et al., 1994). There are currently six TRAF proteins known. TRAF1-5 are recruited to the TNF receptor complex and activate NF-κB via the kinase RIP (Receptor Interacting Protein), whereas TRAF6 participates in IL-1 receptor and Toll activation of NF-κB by interacting with the kinase IRAK (Cao et al., 1996; Arch et al., 1998).

1.6. Dietary fatty acids

The dietary lipids that have nutritional value are in the form of triglycerides, or neutral fat. Triglycerides are composed of two parts, glycerol and fatty acids, the fatty acids being esterified to the three hydroxyl groups of the glycerol. Fatty acids are either saturated or unsaturated. Unsaturated fatty acids contain one or more double bonds. Those which have one unsaturated bond are monounsaturated fatty acids and those which have two or more unsaturated bonds are polyunsaturated fatty acids (PUFAs). PUFAs are usually classified according to a shorthand nomenclature which designates the chain length, number of double bonds, and position of the double bond nearest to the terminal methyl group (Willis, 1987). The carbon atom of the methyl terminal end...
is referred to as the n carbon atom, as it occurs at the opposite end of the molecule to carbon-1 of the carboxyl group. The ω can be used instead of n to describe the position of the double bond nearest the methyl group. There are four families of unsaturated fatty acids: n-3, linolenic acid (LNA, C18:3n-3); n-6, linoleic acid (LA, C18:2n-6); n-7, palmitoleic acid (C16:1n-7); and n-9, oleic acid (OA, C18:1n-9) (Cave, 1991). The n-3 and n-6 polyunsaturated fatty acids are considered essential fatty acids (EFA) because they cannot be synthesized by the human body and must be obtained from the diet. They have distinctive nutritional and metabolic effects, and each has a direct precursor relationship with specific classes of eicosanoids. Thus, all eicosanoids formed in the human body are generated from polyunsaturated fatty acids that must be derived from the diet (Fischer, 1989). Oleic acid is not an essential fatty acid because animals possess desaturase and so can synthesize the n-9 fatty acids by chain elongation and desaturation.

Most human diets contain a variety of saturated fatty acids of different chain lengths. The major saturated fatty acid in the diet is palmitic acid (PA, C16:0), followed by stearic acid (SA, C18:0), myristic acid (C14:0), and lauric acid (C12:0). Short and medium chain fatty acids (C4:0-10:0), which occur mainly in dairy fat, palm kernel oils and coconut oils, also contribute to the total intake of saturated fatty acids (Bartsch et al., 1999). PA is found in all edible fats and oils and is particularly abundant in palm oil and in butter, milk, cheese and meats. SA is found predominantly in cocoa butter and in fats from cattle and sheep. Coconut and palm kernel oils, and dairy fats contain large amounts of lauric and myristic acids. The major monounsaturated fatty acid in human diets is oleic acid (OA, cis-C18:1n-9). OA is found in all edible fats and
oils and is particularly abundant in olive and rapeseed oils. The major polyunsaturated fatty acid in the diet is linoleic acid (cis,cis-C18:2n-6). LA is present in vegetable oils such as those from soybeans, corn and sunflowers (Ip, 1997). Linolenic acid (n-3) is present in dark green leafy vegetables, walnuts, and in linseed and rapeseed oil. The n-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are present in fish (Bartsch et al., 1999).

The n-3 fatty acids are derived from LNA while the n-6 fatty acids are derived from LA (Figure 1). The LA and arachidonic acid (AA) are representative of the n-6 fatty acid while LNA, EPA, and DHA represent n-3 fatty acids. AA and EPA are precursors to eicosanoids such as PG and leukotrienes. EPA and AA are known to compete for the enzymes that metabolize these PGs and leukotrienes (Culp et al., 1979). Thus, the ratio of n-3:n-6 EFA may be an important factor in the type of PG or leukotrienes synthesized.

1.7. Cancer and dietary fatty acids

Wynder and Gori (1977) and Doll and Peto (1981) reported that about 35% (range 10-70%) of all cancer mortality in the United States may be attributable to dietary factors. Epidemiological studies have demonstrated that diets particularly high in fish and other marine animals are generally associated with a reduced risk for the development of several types of cancer (Mckeown-Essen and Bright-See, 1985; Rose et al., 1986).

Colon cancer rates are lower in countries where consumption of fish oils is high, such as Japan (Lands et al., 1990). Kromann and Green (1980) reported a lowered
**OLEIC ACID**

16:0 $\rightarrow$ 18:0

$\downarrow$

18:1n-9 $\rightarrow$ 20:1n-9

$\downarrow$

18:2n-9 $\rightarrow$ 20:2n-9

$\downarrow$

20:3n-9 $\rightarrow$ 22:3n-9

**LINOLEIC ACID**

18:2n-6 $\rightarrow$ 20:2n-6

$\downarrow$

18:3n-6 $\rightarrow$ 20:3n-6 $\rightarrow$ 22:3n-6

$\downarrow$

20:4n-6 $\rightarrow$ 22:4n-6

(AA)

$\downarrow$

22:5n-6

**LINOLENIC ACID**

18:3n-3 $\rightarrow$ 20:3n-3

$\downarrow$

18:4n-3 $\rightarrow$ 20:4n-3

$\downarrow$

20:5n-3 $\rightarrow$ 22:5n-3 (DPA)

(EPA)

$\downarrow$

22:6n-3 (DHA)

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**Figure 1.1. Outline of three major groups of dietary fatty acids metabolism (Hwang, 1989).**
incidence of cancers in general among Greenland Eskimos who consume large amounts of seal and fish oil rich in the n-3 PUFAs.

Many epidemiological studies have shown that even though the overall fat consumption is high, the incidences of cancers are different depending on the primary fatty acids consumed (Hursting et al., 1990; Sasaki et al., 1993; Wynder et al., 1991). These differences in cancer incidence might come from differences in the ratio of n-6/n-3 PUFAs in the diet. Therefore, the ratio of n-6/n-3 PUFAs in the diets might be an important factor in determining cancer risk. Although an optimal ratio of n-6/n-3 fatty acids has yet to be determined, Bartsch et al. (1999) recommend a ratio of 8:1~5:1 (n-6/n-3).

Calder et al. (1998) reported that dietary fish oil suppresses human colon tumor growth in athymic mice. They found that; 1) human colon tumor growth is promoted by feeding high fat diets rich in medium chain saturated fatty acids or monounsaturated fatty acids, 2) a high fat diet, rich in long chain n-3 PUFAs does not promote colon tumor growth, 3) the effect of a high fat diet rich in n-6 PUFAs depends upon the timing of feeding: if fed before tumor cell inoculation, this diet promotes tumor growth, but if fed once tumor growth is initiated, there is no growth promoting effect, 4) therefore, n-6 PUFAs might affect initiation of colon tumor growth, but not promotion.

Karmali et al. (1987) showed that feeding menhaden oil or n-3 fatty acid-rich fish oil concentrate suppresses the growth of human prostate cancer cells after their subcutaneous injection into athymic nude mice. They suggested that this inhibitory effect was associated with a reduction in tumor prostaglandin E2.
Fay et al. (1997) reported the effects of saturated and monounsaturated fats and n-6 PUFAs and n-3 PUFAs on mammary tumor incidence. The results indicated that n-6 PUFAs have a strong and saturated fats a weaker tumor-enhancing effect, whereas the n-3 PUFAs have a small, statistically nonsignificant protective effect and monounsaturated fats had no significant effect. Willett et al. (1990) reported that there appears to be no correlation between the incidence of colon cancer and total intake of n-6 PUFAs.

Recent studies that focused on the effects of n-3 and n-6 PUFAs on tumor metastases showed conflicting results. n-3 PUFA diets either reduced or increased the number of metastases or had no effect at all (Rose and Connolly, 1993). Suzuki et al. (1997) showed the inhibitory effects of n-3 fatty acids on lung metastasis. Young and Young (1989) found that n-3 PUFAs can increase the number of lung metastasis. Griffini et al. (1998) reported that both n-3 and n-6 PUFAs promote colon cancer metastasis in rat liver without down-regulating the immune system.

Taken together, there is now substantial experimental evidence that in general n-6 fatty acids increase the risks for cancers of the breast and colon and for metastasis, whereas relatively high intakes of n-3 PUFAs and n-9 monounsaturated fatty acids reduce cancer risk by mechanisms that may involve modification of the biosynthesis of eicosanoids from n-6 PUFAs.

1.8. Fatty acids and immune response

Epidemiological study demonstrated low incidence of atherosclerosis, and inflammatory and autoimmune diseases in Greenland Eskimos who consume diets enriched in marine-derived (n-3) PUFAs (Kromann, 1980). Recent clinical studies with
fish oil supplemented diets have shown that fish oil diets might be beneficial for the prevention and treatment of atherosclerotic and atherothrombotic disorders and may improve autoimmune and inflammatory diseases (Gottschlich, 1992; Meydani and Dinarello, 1993; Meydani et al., 1993; Goodnight, 1996). Kremer et al. (1987) suggested that several diseases including atherosclerosis, insulin-resistant diabetes, and certain types of cancer such as colon cancer have been linked to the type and amount of fat we ingest. They showed that rheumatoid arthritis patients consuming 1.8 g/d of EPA had fewer clinical symptoms after 12 weeks of supplementation. Renier et al. (1993) showed that fish oil-induced reduction of IL-1β and TNF-α production in mice was associated with reduced atherosclerotic lesions. Cytokines such as IL-1 have been shown to contribute to the pathogenesis of atherosclerotic and inflammatory diseases. Therefore, an increase in n-3 PUFAs intake can be expected to affect cytokine production and biologic function (Meydani, 1996).

Meydani et al. (1991) showed that fish oil consumption significantly decreased T cell-mediated function such as IL-2 production and mitogenic response to T cell mitogen PHA in a human study. Somers et al. (1989) reported that dietary manipulation of fatty acids can alter activation of tumoricidal capacity of macrophages, possibly through the changes in eicosanoid synthesis.

Recent studies indicate that signal transduction and subsequent gene expression can be modified by n-3 fatty acids. Erickson et al. (1995) reported that dietary fatty acids might modulate signal transduction in mice macrophages. They suggested that effects of dietary fatty acids on macrophages might be a combination of several mechanisms and modulation of a more complicated set of events. Lo et al. (1999)
reported that EPA-supplemented diets decrease macrophage tumor necrosis factor gene transcription by altering NF-κB activation by reducing the p65/p50 dimers. May et al.(1993) showed that lymphocyte protein kinase C is inhibited by unsaturated fatty acids.

Moreover, EPA and DHA are incorporated into the cell membrane, where they influence membrane fluidity, receptor function, enzyme activity, and production of eicosanoids (Brouard and Pascaud, 1990).

Therefore, dietary fatty acids appear to influence the onset and progression of these various diseases by exerting an effect at two levels: (1) changes in membrane phospholipid composition; and (2) direct control of the nuclear events that govern gene transcription (Clandinin et al., 1991; Clarke and Abraham, 1992). Therefore, the beneficial as well as the detrimental effects that dietary fats exert on various diseases may involve a combination of interactive regulatory mechanisms: (1) rapid changes in gene expression and (2) a long term adaptive modulation of membrane composition, which leads to changes in hormone signaling (Clarke and Jump, 1994).

1.9. Non-steroidal anti-inflammatory drugs (NSAIDs)

By the early 1900s, the main therapeutic actions of Aspirin (and sodium salicylate itself) were known as the antipyretic, anti-inflammatory, and analgesic effects (Vane, et al., 1990). In time, several other drugs were discovered that had some or all of these effects; these drugs include antipyrine, phenacetin, acetaminophen, fenamates, indomethacin, and naproxen. Because of their similar therapeutic actions, these drugs tended to be regarded as a group and were generally known as the ‘Aspirin-like drugs’; because they were clearly distinct from the glucocorticoids, the other major group of
agents used in the treatment of inflammation, these drugs were considered the ‘non-steroidal anti-inflammatory drugs (NSAIDs)’ (Flower, 1974). Although the drugs in this group are chemically diverse, the fact that most of them possess an acidic function suggested another name that is still occasionally encountered-the ‘anti-inflammatory acid’.

Despite the diversity of their chemical structures, these drugs share to some extent the same therapeutic properties. In varying doses, they alleviate the swelling, redness, and pain of inflammation, reduce a general fever, and cure a headache. In addition, they also share, to varying degrees, many similar side effects. Depending on the dose, these drugs can cause gastric upset; a high dose can delay the birth process; and an overdose may damage the kidneys. A particularly interesting side effect, which is now known as a therapeutic action, is their antithrombotic effect.

In 1971, Vane reported that aspirin and indomethacin inhibit the biosynthesis of prostaglandins in the guinea pig lung. In 1974, [acetyl-3H] aspirin was shown to selectively acetylate a microsomal protein of molecular weight 85,000 (now known as COX-1) from a sheep and bull seminal vesicles and human platelets (Roth et al., 1975). In 1980s, numerous enzymatic studies were reported that defined the nature of the interactions of various NSAIDs with purified COX-1 (Smith and DeWitt, 1995). It was shown that only the cyclooxygenase activity of COX-1, but not the peroxidase activity of COX-1, was blocked by NSAIDs (Mizuno et al., 1982).

Soon after the discovery that COX-2 was the enzyme responsible for inflammatory responses, the effects of various NSAIDs on COX-2 were scrutinized and compared with those on COX-1 (Meade et al., 1993). On the basis of their binding
kinetics with the COXs, NSAIDs can be grouped into three classes. Class I compounds are simple, competitive inhibitors that compete reversibly with arachidonic acid for binding to the COX active site, whereas class II compounds are competitive, time-dependent, and reversible inhibitors. Class III compounds are known to covalently modify COX-1 and COX-2 and include aspirin. Acetylation of COX-1 by aspirin inactivates the COX activity, but not its peroxidase activity. Aspirin acetylation of COX-1 prevents arachidonic acid from getting contact with the active site (DeWitt et al., 1990). The active site of COX-2 is slightly larger than that of COX-1 (Lecomte, et al., 1994). This size difference is suggested by the broader fatty acid substrate specificity of COX-2 and the lower relative affinities of NSAIDs for COX-2 (Smith and DeWitt, 1995). Most NSAIDs inhibit the activity of both COX-1 and COX-2, a property that accounts for their shared therapeutic and side effects (Vane, 1971). The inhibition of COX-2 may well explain their therapeutic use as anti-inflammatory drugs, whereas the inhibition of COX-1 may explain their unwanted side effects, such as gastric damage (Vane, 1971).

Although NSAIDs act principally by inhibition of COX, thus blocking the initial step in prostanoid synthesis, they appear to possess additional pharmacological actions. Low doses of aspirin and other NSAIDs are enough for COX inhibition in vitro and in vivo, whereas higher doses are required for antirheumatic effects in vivo (Roth et al., 1983).
CHAPTER 2
THE EFFECT OF DIETARY FATTY ACIDS ON CYCLOOXYGENASE-2 EXPRESSION

2.1. Introduction

During the past two decades, epidemiological studies have shown a causal relationship between the environment and lifestyles of people and the incidence and morbidity of certain diseases, including certain types of cancers. Although it is difficult to assign the exact proportion of these diseases attributable to dietary factors alone, Wynder and Gori (1977) estimated that 35% (range 10-70%) of cancer mortality in the United States is from dietary factors. Much evidence from both epidemiological and experimental studies supports a positive association between consumption of fish and marine animals and a reduction in the risk for development of several types of cancer and some autoimmune diseases (Kremer et al., 1987; Kromann and Green, 1980; Miller, 1990; Rose, 1997).

Many epidemiological studies suggest that the intake of fish containing n-3 polyunsaturated fatty acids (PUFAs), mainly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), is associated with a reduced risk of colon cancer (Giovannucci and Goldin, 1997; Potter, 1995; Reddy, 1994). Furthermore, administration of EPA in the diet has been shown to reduce carcinogen-induced rat colon carcinogenesis (Minoura et al., 1988). Takanashi et al. (1993 and 1997) found DHA can suppress carcinogen-induced rat colon carcinoma development. Oshima et al. (1995) also showed that DHA suppresses intestinal polyp development in APC knockout mice.
n-3 PUFAs interfere with the metabolic pathway of arachidonic acid and thereby decrease prostaglandin E\textsubscript{2} levels (Corey et al., 1983). Thus, it has been postulated that the anti-cancer effect of n-3 PUFAs is due to their inhibitory effect on prostaglandin biosynthesis from arachidonic acid. However, the molecular mechanisms by which n-3 PUFAs exhibit their anti-carcinogenic effects have yet to be fully understood.

Several studies suggested that anti-inflammatory effects of dietary fatty acids might occur by modulating the macrophage intracellular signal pathways which, in turn, influence gene activation and cytokines production (Meydani and Dinarello, 1993; Lo et al., 1999). Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) is one of the major arachidonic acid metabolites involved in the modulation of inflammatory response. Prostaglandins are formed by the action of cyclooxygenase (COX). COX, also known as prostaglandin endoperoxide H synthase, is the rate-limiting enzyme which catalyzes the conversion of arachidonic acid to prostaglandin H\textsubscript{2}. There are two isoforms of COX so far identified: constitutively expressed COX-1 and mitogen-inducible COX-2. The COX-2 gene is overexpressed in several proliferative pathological conditions such as cancer and rheumatoid arthritis (Sano et al., 1995). COX-2 is induced by endotoxin and inflammation. COX-2 is responsible for increased PGE\textsubscript{2} in inflamed tissues. COX-2 gene activation is also responsible for the elevated PGE\textsubscript{2} production by LPS-stimulated macrophages (Lo et al., 1998).

It is believed that one possible mechanism for the anti-inflammatory effects of PUFAs may be the inhibition of COX-2 and prostaglandin synthesis. This is supported by a study that showed the inhibitory effect of PUFAs on cytokine production (Meydani et al., 1993). Lo et al. (1998) suggested that COX-2 gene expression by LPS-stimulated
macrophages is dependent on nuclear factor kappa B (NF-κB). NF-κB is a transcriptional activator protein and plays an important role in controlling inflammatory gene activation. NF-κBs are composed of dimers that are normally confined in the cytoplasm through their association with the inhibitory protein, IκBα. The schematic illustration of LPS signaling pathway leading to the expression of COX-2 is shown in scheme 1. A specific DNA sequence recognized by NF-κB transcription factor is present in the promoter regions of COX-2 gene.

Recent studies showed that fatty acids are ligands for peroxisome proliferator activated receptors (PPARs), suggesting that they are potential modulators for gene expression. PPARs are members of the steroid hormone receptor superfamily which regulate gene expression by binding to a recognition sequence known as a PPAR response element (PPRE). So far, three subtypes (α, β/δ and γ) have been identified. Transcriptional regulation by PPARs is achieved through PPAR-RXR (where RXR is the receptor for 9-cis retinoic acid) heterodimers. Recently, Meade et al. (1999) demonstrated that fatty acids as PPARs activators can induce COX-2 expression in the epithelial cells, and COX-2 promoter region has PPRE sequence. Staels et al. (1998) reported that PPARα activator can inhibit the expression of a variety of genes involved in the inflammatory response, including COX-2, in smooth muscle cells. They concluded that this inhibition of COX-2 induction occurs transcriptionally as a result of PPARα repression of NF-κB signaling.

It has been observed that dietary n-3 PUFAs can relieve the symptoms of rheumatoid arthritis and this is associated with decreased PGE₂ synthesis. However, the
Scheme 1. The schematic illustration of signaling pathways leading to expression of COX-2.
molecular basis on the regulation of anti-inflammatory response by dietary fatty acids is very poorly understood. Therefore, in this study, it was hypothesized that dietary fatty acids may modulate the inflammatory response by influencing the COX-2 signaling pathway. The purpose of this study is (1) to examine the effects of several dietary fatty acids on COX-2 gene expression in a macrophage cell line and, (2) to investigate the signaling pathway through which fatty acids modulate COX-2 expression in RAW 264.7 cells. Results from the present study will provide a plausible mechanism of PUFAs on the anti-inflammatory response in macrophages.

2.2. Materials and methods

2.2.1. Materials

Docosahexaenoic acid (22:6n-3; DHA), eicosapentaenoic acid (20:5n-3; EPA), arachidonic acid (20:4n-6; AA), linoleic acid (18:2n-6; LA), palmitic acid (16:0; PA) and stearic acid (18:0; SA) 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). Antibodies for iNOS, IL-1α, and IκBα were purchased from Santa Cruz Biotech (Santa Cruz, CA). Donkey anti-rabbit and goat anti-mouse 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). Phoenix amphotropic cells were
provided by Dr. G. P. Nolan (Department of Molecular Pharmacology and Microbiology and immunology, Stanford University School of Medicine, Stanford, CA). MTT (3-{4,5-Dimethylthiazol-2-yl}-2,5-diphenyltetrazoliumbromide) and bovine serum albumin were purchased from Sigma (St Louis, MO). All other reagents were purchased from Sigma unless otherwise described.

2.2.2. Cell culture

RAW 264.7 cells (a murine macrophage-like cell line, ATCC TIB-71) and HT29 (a human colon adenocarcinoma cell line, ATCC HTB-38) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, GIBCO-BRL) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, Intergen) and 100 units/ml penicillin and 100 μg/ml streptomycin (GIBCO-BRL) in a 5% CO₂/95% air humidified atmosphere at 37°C. IEC-6 cells (rat normal small intestine cell line, ATCC CRL-1592) were cultured in DMEM containing 5% FBS, 0.1 U/ml of insulin, 100 units/ml penicillin and 100 μg/ml streptomycin. Media were changed every other day.

2.2.3. Nude mice studies

Female athymic nude mice (NCr-nu/nu) were purchased from Harlen Sprague Dawley, Inc. (Indianapolis, Indiana) at three to four weeks of age and maintained in microisolator cages within a pathogen-free isolation facility. There were 8 mice in each group. Mice were housed four animals per cage. Mice were injected with 2 x 10⁶ IEC-6 cells (200 μl of cell suspension) subcutaneously using a 25 gauge needle. The experiment was terminated 21 days after injection of the cells.
2.2.4. Preparation of fatty acids-albumin complexes

All fatty acids were solubilized in ethanol. They were combined with fatty acid free albumin at a molar ratio of 10:1 (fatty acid : albumin) in serum poor medium (0.25% FBS). Fatty acids-albumin complexes solution was freshly prepared prior to each experiment. The final concentration of DHA, EPA, and AA was from 5 to 200 μM. LA, cLA, PA, and SA were tested from 1 to 200 μM. This concentration was found not to be toxic to the cells.

2.2.5. Preparation of whole cell lysates

After treatment, cell medium were removed and cells were rinsed with cold PBS twice. Then 0.5 ml of ice cold RIPA buffer [see APPENDIX 4] were added to 60 mm cell culture dishes. After 20 min incubation on ice, cells were collected by scraping the dishes with a cell scraper and transferred to 1.5 ml microcentrifuge tube. After sonification (7 strokes x 5), cell lysates were centrifuged at 10,000xg for 10 min at 4 °C. The supernatant fluid is whole cell lysates. The protein concentration was measured using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Richmond, CA).

2.2.6. SDS-PAGE and western blotting

Whole cell lysates were boiled for 5 min and electrophoretically resolved on a 8% polyacrylamide vertical slab gel [see APPENDIX 4] with an overlay of 4% polyacrylamide gel along with prestained SDS-PAGE molecular weight markers (Biorad). Electrophoretically resolved proteins were electrotransferred onto PVDF membrane in a electrophoretic transfer cell (Biorad) containing transfer buffer [see APPENDIX 4]. After transblotting the electrophoretically resolved proteins, the blots were blocked with 5% (w/v) nonfat dry milk (NFDM, Carnation) dissolved in
phosphate buffered saline-0.1 % (v/v) Tween 20 (PBS-T) for overnight at 4°C to prevent non-specific binding of antibodies. The blots were then incubated with polyclonal anti-COX-2 antibody diluted 1:5000 in PBS-T containing 5% (w/v) NFDM for 1 hour. Blots were washed in PBS-T and reincubated with antirabbit IgG coupled to horseradish peroxidase diluted 1:5000 in PBS-T containing 5% (w/v) NFDM. The blots were thoroughly washed in excess PBS-T and probed with the enhanced chemiluminescence (ECL) western blot detection system (Amersham Corp.). The blots were exposed on an X-ray film (Kodak) and the films were developed.

For GAPDH immunoblotting, membranes used for COX-2 immunoblots were stripped in the stripping buffer [see Appendix] at 54 °C for 1 hour, reprobed with 1:10,000 dilution of GAPDH antibody, and followed by incubation with anti-rabbit IgG coupled to horseradish peroxidase diluted 1:5000 in PBS-T containing 5% (w/v) NFDM. The blots were thoroughly washed in excess PBS-T and probed with the enhanced chemiluminescence (ECL) western blot detection system (Amersham Corp.). The blots were exposed on an X-ray film (Kodak) and the films were developed.

2.2.7. Antiphosphotyrosine immunoblotting

12 % of polyacrylamide gel was used for electrophoresis. Immunoblotting was carried out using 4G10 monoclonal antiphosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) diluted 1:3000 in TBS-0.1 % (v/v) Tween 20 (TBS-T) containing 5% (w/v) bovine serum albumin (BSA) followed by incubation with anti-mouse IgG coupled to horseradish peroxidase diluted 1:5000 in TBS-T.
2.2.8. Immunoprecipitation

500ug of whole cell lysates were used for COX-1 and COX-2 immunoprecipitation. Whole cell lysates were incubated with 2 µl of antibody for 3 hours at 4°C on a rotating device and reincubated for 3 hours after adding 80 µl of 20 % (v/v) protein A-sepharose beads. The pellet was collected by centrifugation at 10,000xg for 10 min at 4°C. The pellet was washed 3 times with RIPA buffer, each time repeating the centrifugation step above. After the final wash, the pellet was resuspended in 60 µl of 2x Laemmli buffer [see APPENDIX 4] and boiled at 100°C for 5 min. The denatured samples were centrifuged at 10,000xg for 5 min at 4°C and only the supernatant was used for SDS-PAGE.

2.2.9. Transfection and infection

Phoenix cells (1x10^6 cells) were plated into a 60 mm dish one day before transfection. To prepare DNA/Superfect reagent complexes, 5 µg of DNA was diluted with DMEM incomplete media in an eppendorf tube. Superfect reagent (30µl) was added to the same tube. The DNA/SuperFect reagent complexes were incubated for 10 minutes at room temperature. Then, 1 ml of complete media was added to the reaction tube. After several pipettings, the complexes were added to the phoenix cells. After 3 hours incubation, the media were removed and changed with DMEM complete media. After 24 hours post transfection, the culture media containing infectious viral particles produced by transfected phoenix cells were transferred to 15 ml culture tubes and centrifuged at 1,500 rpm for 5 minutes at room temperature. Only the supernatant was added to infect cells. Polybrene was added to increase the efficiency of infection. After
24 hours postinfection, the culture media was removed and changed with complete media.

2.2.10. Radioimmunoassay

The cell culture media were removed and collected for measuring accumulated prostaglandin E\(_2\). The cells were further incubated in the fresh medium containing arachidonic acid (30\(\mu\)m) for 10 min to determine COX activity. After 10 minutes incubation, the media were collected in sample vials. Levels of prostaglandin E\(_2\) in cell supernatants were determined by radioimmunoassay as an indication of COX enzyme activity.

2.2.11. Luciferase reporter gene assay

RAW 264.7 cells were plated in 6 well plates (5 \(\times\) 10\(^5\) cells/well) and transiently transfected with 1\(\mu\)g of reporter plasmid and 0.5 \(\mu\)g of HSP70-lacZ as an internal control by using SuperFect transfection reagent (Quiagen, Valencia, CA). After transfection, the medium was changed with complete media and further incubated for 6 hours. The cells were serum starved in the serum-poor (0.25% FBS) medium for 16 hours prior to the treatment with indicated reagents. After treatment, the cells were lysed with reporter lysis buffer. The luciferase and \(\beta\)-galactosidase enzyme activities were determined using the Luciferase Assay System and \(\beta\)-galactosidase Enzyme System (Promega, Madison, WI) according to the manufacturer’s instruction. Luciferase activity was normalized to the \(\beta\)-galactosidase activity.

2.2.12. Cell viability assay

HCT-116 cells (0.75 \(\times\) 10\(^4\) cells/well) were plated to 96 well plate. The cells were serum starved for 24 hours by replacing the culture media with McCoy’s 5A
media containing 0.25% fetal bovine serum. Then, the media was removed and fresh media containing fatty acids were added. The fatty acids were combined with fatty acid free albumin at a molar ratio of 10:1 (fatty acid : albumin) in serum poor medium. After culturing for 48 hours, 20 μl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) solution (5 μg/ml) were added to each well and incubated for another 4 hours. Insoluble formazan precipitates formed in media were solubilized with 100 μl of 10% SDS-0.01 N HCl solution. Absorbance at 595 nm was measured by using Bio-Rad plate reader.

2.2.13. Statistical analysis

Data were analyzed by paired t-test. Differences were considered statistically significant at P<0.05.

2.3. Results

2.3.1. The effects of n-3 and n-6 PUFAs on COX-2 expression in RAW 264.7 cells

Polyunsaturated fatty acids (PUFAs) such as n-3 and n-6 have diverse effects on cells. It is especially known that they can regulate gene expression in macrophages. Therefore, this study first examined the effect of n-3 and n-6 PUFAs on COX-2 gene expression in macrophage cell line, RAW 264.7 cells. Serum starved RAW 264.7 cells were exposed to indicated concentration of DHA for 11 hours. Total cell lysates were prepared and analyzed by an anti-COX-2 and GAPDH immunoblot analysis. Results showed that DHA did not induce COX-2 expression. In this study, ‘housekeeping gene’, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for COX-2 immunoblot analysis. Figure 2.1 showed that the levels of GAPDH proteins were constant at all lanes.
Figure 2.1. The effects of docosahexaenoic acid (DHA) on COX-2 expression in RAW 264.7 cells. Cells maintained in serum-poor medium were treated with DHA for 11 hours. Cell lysates were analyzed by COX-2 or GAPDH immunoblot.
Next, EPA as another n-3 PUFAs, and AA, LA, and cLA as n-6 PUFAs were also tested for their effects on COX-2 expression. All of the PUFAs that were tested did not induce COX-2 expression (Figure 2.2).

2.3.2. Suppression of LPS-induced COX-2 expression by PUFAs

Since lipopolysaccharide can induce COX-2 expression in RAW 264.7 cells, the effects of PUFAs on LPS-induced COX-2 expression were tested. Serum starved RAW 264.7 cells were pretreated with each PUFA for 3 hours, and then stimulated with LPS (0.1 μg/ml) in the medium containing each PUFA for 8 hours. All of the PUFAs that were tested inhibited LPS-induced COX-2 expression in a dose-dependent manner (Figure 2.3, 2.4, 2.5, 2.6, and 2.7). There was no difference in the magnitude of inhibition between n-3 and n-6 PUFAs. The levels of GAPDH expression were not affected in all PUFAs.

2.3.3. Suppression of other proinflammatory maker gene products, iNOS and IL-1α, by PUFAs

All PUFAs which were tested also suppressed the induction of inducible nitric oxide synthase (iNOS) and interleukin-1α (IL-1α) protein in LPS-stimulated RAW 264.7 cells (Figure 2.3, 2.4, 2.5, 2.6, and 2.7) in a dose-dependent fashion. These results indicate that all these PUFAs can suppress the expression of many other genes whose induction is involved in inflammatory reaction in cells.

2.3.4. Inhibition of LPS-induced NF-κB activation by n-3 and n-6 PUFAs

LPS can induce NF-κB activation through IκBα degradation. NF-κB is a member of transcriptional activator protein group and plays an important role in controlling inflammatory gene activation. NF-κBs are made up of dimers that are normally confined in the cytoplasm through their association with the IκBs, which mask
Figure 2.2. The effects of fatty acids on COX-2 expression in RAW 264.7 cells. Cells maintained in serum-poor medium were treated with indicated concentration of each fatty acid for 11 hours. Cell lysates were analyzed by COX-2 or GAPDH immunoblot. AA, arachidonic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; cLA, conjugated linoleic acid.
Figure 2.3. Inhibitory effect of docosahexaenoic acid (DHA) on LPS-induced expression of COX-2, IL-1, and iNOS in RAW 264.7 cells. Cells maintained in serum-poor medium were pretreated with indicated concentration of DHA for 3 hours and then stimulated with LPS (0.1 μg/ml) in the medium containing DHA for 8 hours. Cell lysates were analyzed by COX-2, IL-1α or GAPDH immunoblot.

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Figure 2.4. Inhibitory effect of eicosapentaenoic acid (EPA) on LPS-induced expression of COX-2, IL-1, and iNOS in RAW 264.7 cells. Cells maintained in serum-poor medium were pretreated with indicated concentration of EPA for 3 hours and then stimulated with LPS (0.1 μg/ml) in the medium containing EPA for 8 hours. Cell lysates were analyzed by COX-2, IL-1α or GAPDH immunoblot.
Figure 2.5. Inhibitory effect of arachidonic acid (AA) on LPS-induced expression of COX-2, IL-1, and iNOS in RAW 264.7 cells. Cells maintained in serum-poor medium were pretreated with indicated concentration of AA for 3 hours and then stimulated with LPS (0.1 μg/ml) in the medium containing AA for 8 hours. Cell lysates were analyzed by COX-2, IL-1α or GAPDH immunoblot.
Figure 2.6. Inhibitory effect of linoleic acid (LA) on LPS-induced expression of COX-2, IL-1, and iNOS in RAW 264.7 cells. Cells maintained in serum-poor medium were pretreated with indicated concentration of LA for 3 hours and then stimulated with LPS (0.1 μg/ml) in the medium containing LA for 8 hours. Cell lysates were analyzed by COX-2, IL-1α or GAPDH immunoblot.
Figure 2.7. Inhibitory effect of conjugated linoleic acid (cLA) on LPS-induced expression of COX-2, IL-1, and iNOS in RAW 264.7 cells. Cells maintained in serum-poor medium were pretreated with indicated concentration of cLA for 3 hours and then stimulated with LPS (0.1 µg/ml) in the medium containing cLA for 8 hours. Cell lysates were analyzed by COX-2, IL-1α or GAPDH immunoblot.
their nuclear localization sequence. When cells are activated by pro-inflammatory cytokines, oxidants, and LPS, the IκBα are rapidly phosphorylated and degraded to free NF-κB which migrates to the nucleus where it binds to cognate DNA binding sites and activates gene transcription. Therefore, the degradation of IκBα parallels the appearance of NF-κB in the nucleus and can be assessed by IκBα immunoblotting. DHA and LA were tested for their effects on LPS-induced NF-κB activation by IκBα immunoblotting. Both DHA and LA inhibited LPS-induced degradation of IκBα (Figure 2.8 and 2.9).

2.3.5. The effects of saturated fatty acids on COX-2 expression in RAW 264.7 cells

Since there was no difference between the effects of n-3 and n-6 PUFAs on LPS-induced COX-2 expression, saturated fatty acids were tested. Cells maintained in serum-poor medium were treated with palmitic acid (PA) or stearic acid (SA) for 11 hours under serum-poor condition. Figure 3.10A showed that palmitic acid (C16:0) induced COX-2 expression in a dose-dependent fashion without any change of GAPDH expression. However, stearic acid did not induce COX-2 expression (Figure 2.11A).

To examine the effect of saturated fatty acids on LPS-induced COX-2 expression, cells maintained in serum-poor medium were pretreated with palmitic acid or stearic acid for 3 hours, and then stimulated with LPS (0.1 μg/ml) in the medium containing each fatty acid for 8 hours under serum-poor conditions. PA inhibited LPS-induced COX-2 expression in low concentration and increased LPS-induced COX-2 expression in high concentration (Figure 2.10B). However, SA did not show any effect on LPS-induced COX-2 expression (Figure 2.11B).
Figure 2.8. Inhibition of LPS-induced degradation of IκBα by docosahexaenoic acid (DHA) in RAW 264.7 cells. Cells maintained in serum-poor medium were pretreated with indicated concentration of DHA for 3 hours, and then stimulated with LPS (0.1 μg/ml) in the medium containing DHA for 30 min. Cell lysates were analyzed by IκBα immunoblot.
**IκBα immunoblot**

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**Figure 2.9. Inhibition of LPS-induced degradation of IκBα by linoleic acid (LA) in RAW 264.7 cells.** Cells maintained in serum-poor medium were pretreated with indicated concentration of LA for 3 hours, and then stimulated with LPS (0.1 µg/ml) in the medium containing LA for 30 min. Cell lysates were analyzed by IκBα immunoblot.
Figure 2.10. Inhibitory effects of palmitic acid (PA) on LPS-induced expression of COX-2 in RAW 264.7 cells. Cells maintained in serum-poor medium were treated with PA for 3 hours and then stimulated with LPS (0.1 µg/ml) in the medium containing PA for 8 hours. Cell lysates were analyzed by COX-2 or GAPDH immunoblot.
Figure 2.11. The effects of stearic acid (SA) on LPS-induced expression of COX-2 in RAW 264.7 cells. Cells maintained in serum-poor medium were treated with SA for 3 hours and then stimulated with LPS (0.1 μg/ml) in the medium containing SA for 8 hours. Cell lysates were analyzed by COX-2 or GAPDH immunoblot.
2.3.6. The effects of DHA on COX-2 expression in HT29 cells

In order to investigate the effects of PUFAs on COX-2 expression in cancer cells, human colon adenocarcinoma cell line, HT29 cells were tested. HT29 cells maintained in serum-poor medium were treated with DHA for 11 hours under serum-poor conditions. DHA did not induce COX-2 expression (data not shown).

It is known that HT29 cells can induce COX-2 expression by TNFα, but not by LPS. Therefore, to examine the effect of DHA on TNFα-induced COX-2 expression, HT29 cells maintained in serum-poor medium were pretreated with DHA for 3 hours, and then stimulated with TNFα (20 ng/ml) in the medium containing DHA for 8 hours with either serum-poor media and 10% serum media. DHA did not inhibit TNFα-induced COX-2 expression under either condition (Figure 2.12).

2.3.7. The effects of n-3 and n-6 PUFAs on LPS-induced COX-2 promoter activity

To determine whether inhibition of LPS-induced COX-2 expression by PUFAs is transcriptionally regulated, the luciferase assay using COX-2 promoter was conducted. RAW 264.7 cells, transfected with COX-2 promoter luciferase, were treated with DHA for 11 hours. DHA did not affect the promoter activity of COX-2 (Figure 2.13). However, DHA inhibited LPS-induced transcriptional activity of COX-2 promoter in a dose dependent fashion (Figure 2.13). These results correspond with those obtained by Western blot analysis from endogenous COX-2 protein in RAW 264.7 cells (Figure 2.3). Moreover, LA showed exactly the same effects as DHA (Figure 2.14).

In an attempt to identify the signaling step which is affected by PUFAs, the effects of PUFAs on NF-kB promoter activity induced by constitutively active form of
Figure 2.12. The effects of docosahexaenoic acid (DHA) on COX-2 expression in TNFα-stimulated HT29 cells. Cells maintained in serum-poor medium were treated with DHA for 3 hours and then stimulated with TNFα (20 ng/ml) in the medium containing DHA for 8 hours. Cell lysates were analyzed by COX-2 and GAPDH immunoblot.
Docosahexaenoic acid (DHA) does not activate COX-2 promoter activity, but inhibits the induction of COX-2 promoter activity by LPS. RAW 264.7 cells were transfected with 1μg of COX-2 gene promoter luciferase and 0.5μg of β-galactosidase. Transfected cells were treated with DHA and LPS (0.1μg/ml) or DHA alone for 11 hours. Luciferase activity was measured and normalized by co-transfected β-galactosidase activity. Values represent the mean ± SEM (n=3). *, P<0.05.
Figure 2.14. Linoleic acid (LA) does not activate COX-2 promoter activity, but inhibits the induction of COX-2 promoter activity by LPS. RAW 264.7 cells were transfected with 1μg of COX-2 gene promoter luciferase and 0.5μg of β-galactosidase. Transfected cells were treated with LA and LPS (0.1μg/ml) or LA alone for 11 hours. Luciferase activity was measured and normalized by co-transfected β-galactosidase activity. Values represent the mean ± SEM (n=3). *, P<0.05.
Toll-like receptor 4 (ΔTLr4) or NF-κB-inducing kinase (NIK) were determined.

Constitutively active form of LPS receptor, ΔTLr4, or NIK can activate transcriptional activity of NF-κB reporter gene. Enhanced NF-κB promoter activity in RAW 264.7 cells transfected with ΔTLr4 was inhibited by DHA and LA (Figure 2.15). However, enhanced NF-κB promoter activity in RAW 264.7 cells transfected with NIK was not inhibited by DHA, LA, PA, or SA (Figure 2.16). These results imply that the potential target of PUFAs is upstream of NIK.

2.3.8. The effects of ligands for peroxisome proliferator-activated receptors (PPARs) on COX-2 expression in RAW 264.7 cells

Recent studies demonstrated that PUFAs are ligands for peroxisome proliferator activated receptors and can induce COX-2 expression in epithelial cells (Meade et al., 1999). The present study examined the expression of PPAR subtypes in RAW 264.7 cells. Neither of PPARα, PPARβ, or PPARγ was detected by immnoblod analysis in RAW 264.7 cells. Wy14643 and troglitazone, which are known specific ligands for PPARα and PPARγ, respectively, were used to examine which subtype of PPARs is responsible for the COX-2 expression by PUFAs in RAW 264.7 cells. As shown in Figure 2.17A and 2.18A, Wy14643 did not induce COX-2 expression, whereas troglitazone showed markedly increased COX-2 expression. In addition, LPS-induced COX-2 expression was slightly inhibited by Wy14643 (Figure 2.17B), whereas TG potentiated COX-2 expression induced by LPS (Figure 2.18B). These results indicate that the PUFAs show similar effects with PPARα on COX-2 expression and imply that the inhibiton of PUFAs on LPS-induced COX-2 expression is mediated through the activation of PPARα in RAW 264.7 cells.
Figure 2.15. Docosahexaenoic acid (DHA) and linoleic acid (LA) inhibit NF-κB reporter activity induced by constitutively active form of Toll-like receptor-4 (ΔTlr4). RAW 264.7 cells were transfected with 1 μg of NF-kB-luciferase, 0.5 μg of β-galactosidase, and 1 μg of ΔTlr4 wt (or mutant form of ΔTlr4(P712H) or pcDNA-vector). Transfected cells were treated with DHA or LA for 11 hours under serum-poor condition. Luciferase activity was measured and normalized by co-transfected β-galactosidase activity. BSA, bovine serum albumin; DHA, docosahexaenoic acid; LA, linoleic acid. Values represent the mean ± SEM (n=3).
Figure 2.16. Polyunsaturated fatty acids (PUFAs) or saturated fatty acids do not inhibit NF-κB reporter activity induced by NF-κB-inducing kinase (NIK). RAW 264.7 cells were transfected with 1 μg of NF-κB-luciferase, 0.5 μg of β-galactosidase, and 1 μg of NIK wt (or mutant form of NIK KA or pRK-vector). Transfected cells were treated with the indicated fatty acids (FA) for 11 hours under serum-poor condition. Luciferase activity was measured and normalized by co-transfected β-galactosidase activity. BSA, bovine serum albumin; DHA, docosahexaenoic acid; LA, linoleic acid; PA, palmitic acid; SA, stearic acid. Values represent the mean ± SEM (n=3).
Figure 2.17. The effects of WY14643 (Wy) on COX-2 expression in RAW 264.7 cells. Cells maintained in serum-poor medium were treated with Wy for 3 hours and then stimulated with LPS (0.1 µg/ml) in the medium containing Wy for 8 hours. Cell lysates were analyzed by COX-2 or GAPDH immunoblot.
Figure 2.18. The effects of troglitazone (TG) on COX-2 expression in RAW 264.7 cells. Cells maintained in serum-poor medium were treated with TG for 3 hours and then stimulated with LPS (0.1 μg/ml) in the medium containing TG for 8 hours. Cell lysates were analyzed by COX-2 or GAPDH immunoblot.

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2.3.9. The effects of n-3 fatty acids and n-6 fatty acids on cell proliferation in HCT116 cells overexpressing COX-1 or COX-2

Recently, our lab found that the growth of colon tumor cells xenographed into athymic nude mice was suppressed by a fish oil diet (rich n-3 PUFAs) as compared with safflower oil diet (rich in n-6 PUFAs) regardless of whether COX-1 or COX-2 was expressed or not in the cells (unpublished data). Therefore, MTT assay was conducted to investigate the effects of dietary fatty acids on cell growth using human colorectal adenocarcinoma cells (HCT-116) stably transfected with COX-1 or COX-2 cDNA [see APPENDIX 1]. The results were compared with an in vivo study. HCT-116 cells transfected with the control vector, COX-1, or COX-2 were grown for 48 hours in the media containing EPA, DHA, or LA. If cells are maintained in the media containing 10% FBS, growth factors incorporated from serum may contribute to the cell growth. Therefore, in order to eliminate any masking effect, the media containing 0.25% FBS was used for MTT assay. Both EPA and DHA inhibited cell growth in all three cells groups, whereas LA did not inhibit the cell growth (Figure 2.19). It appears that the growth inhibitory effect of DHA was greater than EPA. No significant difference between BSA control and LA was seen in all three cells groups.

2.3.10. Expression of enzymatically active COX proteins in IEC-6 cells transfected with COX cDNA

This experiment was originally conducted to study whether or not suppression of tumor cell growth by dietary fatty acids is mediated through inhibition of COX. HCT-116 cell line was selected as colon cancer cell to be transfected with COX-1 or COX-2 cDNA. IEC-6 cell line was selected as a control normal cell to be transfected with COX-1 or COX-2 cDNA. IEC-6 cells were infected with retroviral particles which
Figure 2.19. Suppression of cell proliferation by n-3 fatty acid in HCT116 cells which are expressing (A) control vector, (B) COX-1, or (C) COX-2. LA, linoleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Values are mean of six replicates.
were produced by phoenix cells transfected with COX-1 or COX-2 cDNAs. To distinguish with endogeneous COX-1 or COX-2 protein, COX-1 and COX-2 cDNA was tagged with epitope ‘flag’, which is 8 amino acids (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C) at the C-terminus of COX cDNA cloned in pLinx vector. Therefore, COX is expressed in the form of the fusion protein ‘COX-Flag’. To select the stable clones expressing enzymatically active COX-1 (Figure 2.20) or COX-2 (Figure 2.21) protein, each clone was screened under neomycin (800 µg/ml). The expression level of COX-1 or COX-2 protein in these stable clonal cells was first examined by COX-1 or COX-2 immunoprecipitation with polyclonal anti-Flag antibodies followed by immunoblotting with COX-1 or COX-2 antibodies. Figure 2.20 and 2.21 show that COX-1 or COX-2 transfected cells with no tetracycline (lane 3) expressed high levels of COX-1 and COX-2 protein. Their expression was almost completely suppressed by incubating the cells with tetracycline (lane 4). No DNA (lane 1) or control vector (lane 2) expressing cells did not express COX protein.

In addition, the enzymatic activity of COX-1 or COX-2 protein was also determined by radioimmunoassay for PGE$_2$. Figure 2.20B and 2.21B show that COX-1 or COX-2 overexpressing cells produce high levels of PGE$_2$ in a tetracycline-regulated manner. These data indicate that COX-1 or COX-2 proteins expressed in these cells are enzymatically active.

In order to investigate the possible relationship between COX overexpression and tumorigenesis, IEC-6 cells overexpressing COX-1 or COX-2 (2 x 10$^6$ cells) were injected into female athymic nude mice (NCr-nu/nu) subcutaneously. However, they did not form tumors in athymic nude mice.
Figure 2.20. Tetracycline-regulated expression of the COX-1 protein and PGE$_2$ production in normal rat intestinal epithelial cells (IEC-6). (A) Whole cell lysates from stable clonal cells were immunoprecipitated with polyclonal anti-Flag antibodies and immunoblotted with COX-1 antibodies. (B) COX-1 enzyme activity was determined by radioimmunoassay for PGE$_2$ in the same stable clonal cells used at (A).
Figure 2.21. Tetracycline-regulated expression of the COX-2 protein and PGE$_2$ production in normal rat intestinal epithelial cells (IEC-6). (A) Whole cell lysates from stable clonal cells were immunoprecipitated with polyclonal anti-Flag antibodies and immunoblotted with COX-2 antibodies. (B) COX-2 enzyme activity was determined by radioimmunoassay for PGE$_2$ in the same stable clonal cells used at (A).
2.4. Discussion

Dietary manipulation of fatty acids can reduce the severity of a number of inflammatory and immune disorders and inhibit growth of certain tumors (Kromhout et al., 1985; Meydani et al., 1993; Goodnight, 1996). Several studies suggested that the anti-inflammatory effects of dietary fatty acids might occur by modulating the macrophage intracellular signal pathways which, in turn, influence gene activation and cytokines production (Meydani and Dinarello, 1993; Lo et al., 1999). Macrophages can be stimulated to produce many kinds of cytokines such as TNFα and interferon-γ. The inhibitory effect of polyunsaturated fatty acids (PUFAs) on cytokine production has been shown in many human studies. Endres et al. (1989) reported that n-3 PUFAs suppress production of cytokines such as TNFα and IL-1 from stimulated mononuclear cells. Meydani et al. (1993) also showed that production of cytokines IL-1, TNFα, and IL-6 by mononuclear cells was significantly reduced in subjects after consumption of the low-fat, high-fish diet. However, the data from in vitro and in vivo studies are conflicting in terms of the effects of fish oil on macrophage cytokine production in response to LPS. Sato et al. (1992) reported that human peripheral monocytes incubated in EPA-rich media for 6 hours produced more TNF in response to LPS stimulation. The difference between these studies might be related to differences in the feeding duration, species variations, and the percentage of EPA and DHA in the diets.

It has been observed that dietary n-3 fatty acids can relieve the symptoms of rheumatoid arthritis and this is associated with decreased PGE2 synthesis. The molecular mechanisms on the anti-inflammatory regulation by dietary fatty acids are not well understood. Results from the present study suggest that the antiinflammatory
and immunomodulating effects of n-3 PUFAs are mediated through COX-2 independent pathways in macrophages. The important finding of the present study is that both n-3 and n-6 PUFAs inhibit LPS-induced COX-2 expression in macrophages. Results from Western blot analyses demonstrate that both n-3 and n-6 PUFAs inhibit LPS-induced COX-2 expression in RAW 264.7 cells, whereas they do not induce COX-2 expression in the absence of LPS. On the other hand, saturated fatty acids showed somewhat different effects on COX-2 expression. Palmitic acid (C16:0) induced COX-2 expression, and inhibited LPS-induced COX-2 expression at low concentrations, but it enhanced LPS-induced COX-2 expression at higher concentrations. However, stearic acid (C18:0) by itself did not induce COX expression and did not show any effect on LPS-induced COX-2 expression. Therefore, there were different effects on COX-2 expression only between PUFAs and saturated fatty acids, not between n-3 and n-6 PUFAs. There are some conflicting reports on the effects of PUFAs on COX-2 expression. Badawi et al. (1998) reported on the effect of dietary n-3 and n-6 PUFAs on the expression of COX-1 and COX-2 in rat mammary glands. They demonstrated that n-6 PUFAs upregulated COX-2 and, to some extent, COX-1 expression, but n-3 PUFAs did not affect COX expression. Singh et al. (1997) reported that corn oil (rich in n-6 PUFAs) promote colon tumorigenesis by upregulating the COX-2 expression and fish oil (rich in n-3 PUFAs) may exert its antitumor effect by inhibiting the COX-2 expression. So, they suggested differential effects of PUFAs on COX-2 expression. Recently Meade et al. (1999) however, demonstrated that both n-3 and n-6 fatty acids by themselves induce COX-2 expression in mammary epithelial cells. However, results from this study indicate that DHA does not induce COX-2 expression and does not
inhibit TNFα-induced COX-2 expression in human colon adenocarcinoma cell line, HT29 cells. These difference might be related to difference in the cell types.

Inducible nitric oxide synthase (iNOS) is the enzyme responsible for the production of nitric oxide (NO). It is widely accepted that excessive NO production in inflammation is associated with cellular injury and other pathological developments. Interleukin-1α (IL-1α) is one of the key mediators in the response to microbial invasion, immunologic reactions, inflammatory response, and tissue injury. Overproduction of IL-1 contributes to the pathogenesis of several chronic and acute inflammatory diseases including rheumatoid arthritis, cancer cachexia, atherosclerosis, and gram-negative bacteria induced infection. In this study, PUFAs were shown to inhibit not only COX-2 expression but also the expression of iNOS and IL-1α induced by LPS. These results suggest that PUFAs also inhibit the expression of other pro-inflammatory marker gene products in response to inflammatory stimuli. Consistant with the present study, the inhibition of transcription of the murine iNOS gene by DHA in Raw 264.7 cells has been reported by Khair-El-Din et al. (1996). But, they did not examine the n-6 PUFAs. Ohata et al. (1997) reported that the n-3 PUFAs (DHA, EPA, and α-linolenic acid) suppressed NO production in macrophages activated with LPS by inhibiting the induction of iNOS gene expression. On the other hand, n-6 PUFAs, n-9 PUFAs and a saturated fatty acid did not inhibit NO production.

Results from promoter-reporter assays demonstrated that both n-3 and n-6 PUFAs inhibit LPS-induced COX-2 expression at the transcriptional level and support the results obtained by Western blot analysis from endogenous COX-2 protein in RAW 264.7 cells.
Lo et al. (1998) suggested that COX-2 gene expression by LPS-stimulated macrophages is dependent on NF-κB. Lo et al. (1999) also reported that the inhibitory effect of eicosapentaenoic acid (EPA) on TNF gene transcription and protein elaboration in LPS-stimulated macrophages is mediated by altering the NF-κB activity. However, they did not examine the effects of n-6 PUFAs. Therefore, this study investigated the effects of n-3 and n-6 PUFAs on NF-κB signal transduction pathways. NF-κB is a member of transcriptional activator proteins and plays an important role in controlling inflammatory gene activation. NF-κBs are composed of dimers that are normally confined in the cytoplasm through their association with the IκBs, which mask their nuclear localization sequence. When cells are activated by pro-inflammatory cytokines, oxidants, and LPS, the IκBα are rapidly phosphorylated and degraded to free NF-κB which migrates to the nucleus where it binds to cognate DNA binding sites and activates gene transcription. Therefore, the degradation of IκB parallels the appearance of NF-κB in the nucleus. As shown in Figure 2.8 and 2.9, both DHA and LA inhibited LPS-induced NF-κB activation, suggesting an inhibitory action on NF-κB dependent other genes. Again, n-3 PUFAs and n-6 PUFAs showed the same inhibitory effect on NF-κB activation in macrophages.

In an attempt to identify the signaling step which is affected by PUFAs, the effects of PUFAs on NF-κB promoter activity induced by constitutively active form of toll-like receptor 4 (ΔTlr4) or NF-κB-inducing kinase (NIK) were determined. ΔTlr4 is the constitutively active form of the LPS receptor and activates NF-κB. NIK is a member of the MAP kinase kinase kinase (MAPKKK) family and activates NF-κB.
through phosphorylation and activation of the IKKs, which are IkB kinases (Ghosh, 1997). Enhanced NF-κB promoter activity in RAW 264.7 cells transfected with ΔTlr4 was inhibited by DHA or LA (Figure 2.15). However, enhanced NF-κB promoter activity in RAW 264.7 cells transfected with NIK was not inhibited by DHA or LA (Figure 2.16). These results suggest that the potential target of PUFAs is upstream of NIK. The molecular target of PUFAs that mediate the inhibition of the LPS-induced NF-κB activation still needs to be identified.

Recently, it was demonstrated that peroxisome proliferator-activated receptors (PPARs) are key regulators of lipid homeostasis and play a role in inflammation and cell proliferation. Recent study showed that fatty acids are ligands for PPARs (Desvergne and Wahli, 1999) and can induce COX-2 expression in epithelial cells, suggesting that they are potential modulators for gene expression (Meade et al., 1999). Staels et al. (1998) reported that PPARα activator can inhibit the expression of various genes involved in the inflammatory response, including COX-2, in smooth muscle cells. They concluded that this inhibition of COX-2 induction occurs transcriptionally as a result of repression of NF-κB signaling by PPARα. On the other hand, Rossi et al. (2000) recently reported that PPARγ activator, 15-deoxy-Δ^{12,14}-prostaglandin J₂ (15d-PGJ₂) inhibits NF-κB through PPARγ–independent mechanism. They demonstrated that 15d-PGJ₂ inhibits NF-κB by a direct covalent modification and inhibition of IkB kinase β (IKK β), which is a responsible enzyme for the activation of NF-κB. Ricote et al. (1998) suggested that PPARγ is involved in the regulation of inflammatory responses. They demonstrated that PPARγ is markedly upregulated in activated macrophages, and PPARγ ligands inhibit expression of iNOS, gelatinase B and scavenger receptor A.
genes. Although Ricote et al. (1998) reported that RAW 264.7 cells express very low levels of PPARγ mRNA, immunoblot analysis from the present study did not detect any PPAR subtypes in RAW 264.7 cells [see APPENDIX 3]. The present study also tested the effects of a known PPARα activator, Wy14643, and a known PPARγ activator, troglitazone, on COX-2 expression. Wy14643 was shown to inhibit LPS-induced COX-2 expression, whereas Wy14643 alone did not induce the COX-2 expression. On the other hand, troglitazone alone induced COX-2 expression, and troglitazone potentiated LPS-induced COX-2 expression. Therefore, PUFAs and PPARα activators show similar effects on COX-2 expression in macrophages. It needs to be determined whether the inhibitory effects of PUFAs on LPS-induced COX-2 expression are indeed mediated through the activation of PPARα in RAW 264.7 cells.

Recently it was found that the growth of colon tumor cells xenographed into athymic nude mice was suppressed by the fish oil diet (rich n-3 PUFAs) as compared with safflower oil diet (rich in n-6 PUFAs) regardless of whether COX-1 or COX-2 is expressed or not in the cells (unpublished data). As an in vitro test, MTT assay was conducted to investigate the effects of dietary fatty acids on cell proliferation using human colorectal adenocarcinoma cells (HCT116) stably transfected with COX-1 or COX-2. As shown in Figure 2.19, both EPA and DHA inhibited proliferation of all cell-lines (control vector, COX-1, and COX-2 transfected cells). However, LA did not inhibit cell proliferation. The growth inhibitory effect of DHA was greater than that of EPA. The effects of fatty acids were not different among three cell groups. Therefore, these results corroborate data obtained by in vivo experiments. If the efficacy of n-3 PUFAs to suppress tumor cell growth both in vitro and in vivo is primarily mediated
through inhibition of COX, the growth of COX overexpressing cell should differ from the growth of control vector-transfected cells. Therefore, these results indicate that the suppression of tumor growth by n-3 PUFAs is mediated through COX-independent pathways. The signaling pathways through which n-3 PUFAs suppress tumor growth still remain to be identified.

Taken together, DHA and EPA were much more potent than LA in inhibiting the cell proliferation, whereas there was no difference between n-3 and n-6 PUFAs in inhibiting LPS-induced COX-2 expression. Thus, these results suggest that the signaling pathways through which PUFAs inhibit tumor cell growth and LPS-induced COX-2 expression may be different.

Further studies are needed to resolve how n-3 PUFAs unlike n-6 PUFAs exert their anti-inflammatory effects and inhibitory effects on tumor cell growth.
CHAPTER 3
THE EFFECT OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ON CYCLOOXYGENASE-2 EXPRESSION

3.1. Introduction

Colon cancer is one of the leading causes of cancer deaths in western countries, with an estimated 94,100 new cases and 46,600 deaths in the United States in 1997 (Parker et al., 1997). Recent epidemiological studies indicate an inverse relationship between the intake of non-steroidal anti-inflammatory drugs (NSAIDs), especially aspirin, and colon cancer risk (Thun et al., 1993; Giovannucci et al., 1994). Animal studies have also demonstrated colon tumor inhibition by several NSAIDs, including aspirin, sulindac, ibuprofen, and piroxicam (Reddy et al., 1993; Rao et al., 1995). Results from clinical studies in patients with familial adenomatous polyposis indicate that administration of sulindac causes a reduction of polyps, which are precursors of colon cancer (Gardiello et al., 1993).

The major pharmacological action of NSAIDs is the inhibition of cyclooxygenase (COX). COX, also known as prostaglandin endoperoxide H synthase, is the rate-limiting enzyme for prostaglandins (PGs) production. There are at least two isoforms of COXs, which are known as COX-1 and COX-2. COX-1 is constitutively expressed in most type of cells, whereas COX-2 is mitogen-inducible.

The mechanisms by which NSAIDs inhibit colon carcinogenesis are not yet clearly understood. Possible mechanisms include induction of apoptosis in cancer cells (Piazza et al., 1997), and inhibition of COX-2, which, in turn, suppresses PG production (Rigas et al., 1993; Marnett, 1992). It has been reported that COX-2 expression is elevated in colorectal tumors (Sano et al., 1995). Oshima et al. (1996) reported that...
polyp formation in APC 716 knockout mice which were crossed with COX-2 knockout mice were significantly reduced. Xu et al., (1999) also reported that aspirin and sodium salicylate suppress COX-2 gene transcription induced by IL-1 in human endothelial cells. These results suggest that the beneficial effect of NSAIDs in reducing the risk of colon cancer is mediated through the COX-dependent mechanism.

However, recently, some NSAIDs have been demonstrated to act as peroxisome proliferators, suggesting that they may also regulate gene expression as part of their chemopreventative mechanism (Lehmann et al., 1997). In addition, Meade et al. (1999) demonstrated that some NSAIDs and PGs activate peroxisome proliferator-activated receptors (PPARs) and enhance COX-2 expression, suggesting that chemopreventive action of NSAIDs are mediated through COX-2-independent mechanism.

In order to clarify these two conflicting theories, our laboratory examined signal pathways through which NSAIDs modulate COX-2 expression in HT-29 colon cancer cell line (unpublished data). Results indicate that the NSAIDs have two opposing effects on COX-2 expression. That is, the NSAIDs inhibit TNFα-induced COX-2 expression, while NSAIDs alone can induce COX-2 expression. Furthermore, their inhibitory action on TNFα-induced COX-2 expression results partly from the suppression of NF-κB. Thus, in order to verify that two opposing effects of NSAIDs are restricted to only cancer cells, the present study examined their effects on COX-2 expression in normal intestinal cell line, IEC-6 cells. Results from the present study will help to understand the mechanisms of the action of NSAIDs in inflammatory disease and tumorigenesis.
3.2. Materials and methods

3.2.1. Materials

Flufenamic acid, indomethacin, and Tumor necrosis factor α were purchased from Sigma Chemical Company (St. Louis, MO). Sulindac sulfide was provided by Dr. G.A. Piazza (Cell pathways, Inc., Aurora, CO). PD98059 and SB203580 were purchased from Calbiochem (La Jolla, CA). Antibodies for IκBα were purchased from Santa Cruz Biotech (Santa Cruz, CA). Donkey anti-rabbit immunoglobulin G (IgG) antibodies conjugated to horseradish peroxidase was purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). Enhanced chemiluminescence (ECL) western blotting detection reagents were purchased from Amersham Corp (Piscataway, NJ). All other reagents were purchased from Sigma unless described.

3.2.2. Cell culture

IEC-6 cells (rat normal small intestine cell line, ATCC CRL-1592) was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, GIBCO-BRL) containing 5% (v/v) heat-inactivated fetal bovine serum (FBS, Intergen), 0.1 U/ml of insulin, and 100 units/ml penicillin and 100 μg/ml streptomycin (GIBCO-BRL) in a 5% CO₂/95% air humidified atmosphere at 37°C. Media were changed every other day.

3.2.3. Preparation of whole cell lysates

After treatment, cell media were removed and cells were rinsed twice with cold PBS. Then 0.5 ml of ice cold RIPA buffer [see APPENDIX 4] were added to 60 mm cell culture dishes. After 20 min incubation on ice, cells were collected by scraping the dishes with a cell scraper and transferred to 1.5 ml microcentrifuge tubes. After sonification (7 strokes x 5), cell lysates were centrifuged at 10,000xg for 10 min at
4°C. The supernatant fluid is whole cell lysates. The protein concentration was measured using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Richmond, CA).

3.2.4. SDS-PAGE and western blotting

Whole cell lysates were boiled for 5 min and electrophoretically resolved on a 8% polyacrylamide vertical slab gel [see APPENDIX 4] with an overlay of 4% polyacrylamide gel along with prestained SDS-PAGE molecular weight markers (Biorad). Electrophoretically resolved proteins were electrotransfered onto PVDF membrane in an electrophoretic transfer cell (Biorad) containing transfer buffer [see APPENDIX 4]. After transblotting the electrophoretically resolved proteins, the blots were blocked with 5% (w/v) nonfat dry milk (NFDM, Carnation) dissolved in phosphate buffered saline-0.1 % (v/v) Tween 20 (PBS-T) overnight at 4°C to prevent non-specific binding of antibodies. The blots were then incubated with polyclonal anti-COX-2 antibody diluted 1:5000 in PBS-T containing 5% (w/v) NFDM for 1 hour. Blots were washed in PBS-T and reincubated with anti-rabbit IgG coupled to horseradish peroxidase diluted 1:5000 in PBS-T containing 5% (w/v) NFDM. The blots were thoroughly washed in excess PBS-T and probed with the enhanced chemiluminescence (ECL) western blot detection system (Amersham Corp.). The blots were exposed on an X-ray film (Kodak) and the films were developed.

For GAPDH immunoblotting, membranes used for COX-2 immunoblots were stripped in the stripping buffer [see APPENDIX 4] at 54 °C for 1 hour, reprobed with 1:10,000 dilution of GAPDH antibody, and followed by incubation with anti-rabbit IgG coupled to horseradish peroxidase diluted 1:5000 in PBS-T containing 5% (w/v) NFDM. The blots were thoroughly washed in excess PBS-T and probed with the
enhanced chemiluminescence (ECL) western blot detection system (Amersham Corp.). The blots were exposed on an X-ray film (Kodak) and the films were developed.

3.3. Results

3.3.1. COX-2 expression induced by TNF\(\alpha\) is potentiated by flufenamic acid, sulindac sulfide or indomethacin in IEC-6 cells

Recently, it was found that some non-steroidal anti-inflammatory drugs (NSAIDs), such as flufenamic acid and sulindac sulfide, induce COX-2 expression and also inhibit activation of NF-\(\kappa\)B and COX-2 expression induced by TNF\(\alpha\) in a human colon adenocarcinoma cell line, HT29 cells, suggesting that NSAIDs have two opposing effects on COX-2 expression (Paik et al., unpublished data).

The present study was conducted to determine the effect of NSAIDs on COX-2 expression in normal cells. A rat normal small intestine cell line, IEC-6, was chosen. The effect of flufenamic acid on COX-2 expression in IEC-6 cells is shown in Figure 3.1. In the absence of other COX-2 inducers, flufenamic acid induced COX-2 expression in a dose-dependent fashion (Figure 3.1.A). TNF\(\alpha\) induced COX-2 expression in IEC-6 cells. Pretreatment of the cells with flufenamic acid leads to the potentiation of TNF\(\alpha\)-induced expression of COX-2 protein (Figure 3.1.B). These results suggest differential effects on mitogen-stimulated COX-2 expression in normal cells and cancer cells.

Sulindac sulfide and indomethacin show similar effects on COX-2 expression in IEC-6 cells. They also induce COX-2 expression in a dose-dependent fashion (Figure 3.2.A and 3.3.A). TNF\(\alpha\)-induced COX-2 expression is potentiated by sulindac sulfide or indomethacin in IEC-6 cells (Figure 3.2.B and 3.3.B).
Figure 3.1. COX-2 expression induced by TNFα is potentiated by flufenamic acid (Flu) in IEC-6 cells. Cells maintained in serum-poor medium were treated with Flu alone for 11 hours (A), or pretreated with Flu for 3 hours and then stimulated with TNFα (20 ng/ml) in the medium containing Flu for 8 hours (B). Cell lysates were analyzed by COX-2 or GAPDH immunoblot.
Figure 3.2. COX-2 expression induced by TNFα is potentiated by sulindac sulfide (Si) in IEC-6 cells. Cells maintained in serum-poor medium were treated with Si alone for 11 hours (A), or pretreated with Si for 3 hours and then stimulated with TNFα (20 ng/ml) in the medium containing Si for 8 hours (B). Cell lysates were analyzed by COX-2 or GAPDH immunoblot.
Figure 3.3. COX-2 expression induced by TNFα is potentiated by indomethacin (Indo) in IEC-6 cells. Cells maintained in serum-poor medium were treated with Indo alone for 11 hours (A), or pretreated with Indo for 3 hours and then stimulated with TNFα (20 ng/ml) in the medium containing Indo for 8 hours (B). Cell lysates were analyzed by COX-2 or GAPDH immunoblot.
3.3.2. Inhibition of MAP kinases by inhibitors of MAP kinases, PD98059 and SB203580, leads to the suppression of COX-2 expression induced by flufenamic acid in IEC-6 cells

PD98059 (2'-amino-3'-methoxyflavone) is a known specific inhibitor of MAP kinase kinase (MEK), which phosphorylates and activates MAP kinase. SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1 H-imidazole] is known to be a specific inhibitor of p38 MAP kinase. Using these specific MAP kinases inhibitors, the present study examined whether inhibiting MAP kinases affects the expression of COX-2 induced by flufenamic acid in IEC-6 cells.

IEC-6 cells were maintained in serum-poor medium and pretreated with one of two different concentrations of PD98059 (10 and 50 μM) or SB203580 (15 and 30 μM) for 3 hours; these concentrations were used based on a review of literatures that show their effects on MAP kinases. Cells were then further treated with a combination of flufenamic acid (400 μM) and PD98059 or SB203580 for 8 hours. Flufenamic acid-induced expression of COX-2 was partially inhibited by PD98059 at the concentration of 10 μM and completely inhibited at the concentration of 50 μM (Figure 3.4.A). Similarly, flufenamic acid-induced COX-2 expression was completely inhibited by SB203580 at the concentration of 30 μM (Figure 3.4.B). The levels of GAPDH proteins used as an internal control were constant at all lanes.

3.3.3. Flufenamic acid and sulindac sulfide inhibit TNFα-induced NF-κB activation in IEC-6 cells

In order to verify whether the induction of COX-2 by NSAIDs is mediated by NF-κB activation, the effects of flufenamic acid and sulindac sulfide on the activation of NF-κB were examined in unstimulated and TNFα-stimulated IEC-6 cells. The results from IκBα immunoblot showed that flufenamic acid and sulindac sulfide alone do not
Figure 3.4. COX-2 expression induced by flufenamic acid (Flu) is inhibited by MEK inhibitor (PD98059) or p38 inhibitor (SB203580) in IEC-6 cells. (A) Cells maintained in serum-poor medium were pretreated with PD98059 for 3 hours and then further treated with a combination of Flu (400 μM) and PD98059 for 8 hours. (B) Cells maintained in serum-poor medium were pretreated with SB203580 for 3 hours and then further treated with a combination of Flu (400 μM) and SB203580 for 8 hours. Cell lysates were analyzed by COX-2 or GAPDH immunoblot.
induce the degradation of IκBα indicating that flufenamic acid- or sulindac sulfide-induced expression of COX-2 is not mediated through the activation of NF-κB (Figure 3.5.A and B). TNFα-stimulated activation of NF-κB was suppressed by flufenamic acid or sulindac sulfide in a dose-dependent fashion (Figure 3.6.A and B). However, indomethacin did not inhibit TNFα-stimulated activation of NF-κB (Figure 3.6.C). These results indicate that inhibition of TNFα-stimulated activation of NF-κB by flufenamic acid or sulindac sulfide is not sufficient to inhibit TNFα-induced COX-2 expression in IEC-6 cells.

3.4. Discussion

The major pharmacological action of non-steroidal anti-inflammatory drugs (NSAIDs) is the inhibition of cyclooxygenase (COX). However, some studies recently reported that NSAIDs exert their anti-inflammatory effect through a COX-2 independent pathway. Meade et al., (1999) demonstrated that fatty acids, prostaglandins, and NSAIDs, compounds that are substrates, products, and inhibitors, respectively, of COX enzymatic activity, induce COX-2 expression in both mammary cells and colonic epithelial cells. They found that NSAIDs, which can activate PPARα, but not PPARγ, induce transcription of COX-2. Jiang et al. (1998) also showed that PPARγ agonists inhibit the production of inflammation cytokines in human monocyte suggesting PPARγ-dependent mechanisms in anti-inflammatory response.

Results from a previous study in our laboratory demonstrated that NSAIDs have two opposing effects on COX-2 expression in human colon cancer cell line, HT-29. One is the inhibitory effect on mitogen-induced COX-2 expression. The other is the inducing effect on COX-2 expression in the absence of stimulators. However, results
Figure 3.5. **NSAIDs alone do not induce degradation of IκBα in IEC-6 cells.** (A) Cells maintained in serum-poor medium were treated with flufenamic acid (Flu) for indicated time periods. (B) Cells maintained in serum-poor medium were treated with sulindac sulfide (Si) for indicated time periods. Cell lysates were analyzed by IκBα immunoblot.
Figure 3.6. Flufenamic acid (Flu) and sulindac sulfide (Si), but not indomethacin (Indo), inhibit TNFα-induced degradation of IκBα in IEC-6 cells. (A) Cells maintained in serum-poor medium were pretreated with flufenamic acid (Flu) for 3 hours and then stimulated with TNFα (20 ng/ml) in the presence of Flu for 8 hours. (B) Cells maintained in serum-poor medium were pretreated with sulindac sulfide (Si) for 3 hours and then stimulated with TNFα (20 ng/ml) in the presence of Si for 8 hours. (C) Cells maintained in serum-poor medium were pretreated with indomethacin (Indo) for 3 hours and then stimulated with TNFα (20 ng/ml) in the presence of Indo for 8 hours. Cell lysates were analyzed by IκBα immunoblot.
from the present study indicate that NSAIDs do not show these opposing effects on COX-2 expression in normal intestinal cell line, IEC-6. Results from Western blot analyses showed that NSAIDs alone induce COX-2 expression and they potentiate TNFα-induced COX-2 expression. All NSAIDs (flufenamic acid, sulindac sulfide, and indomethacin) which were tested here showed similar effects.

The inhibitors of extracellular-signal-regulated protein kinase (ERK), and p38 MAP kinase inhibit the flufenamic acid-induced COX-2 expression. On the other hand, the previous study in HT-29 cells showed that flufenamic acid- or sulindac sulfide-induced COX-2 expression was not affected by either the inhibitors of p38 or MEK, implying that flufenamic acid- or sulindac sulfide- induced COX-2 expression is not mediated through the activation of p38 or MEK in HT-29 cells. These results indicate that unlike the previous study in HT-29 cells, flufenamic acid-induced COX-2 expression is at least partly mediated through activation of MAPKs in IEC-6 cells.

The results from IκBα immunoblot showed that NSAIDs alone do not induce the degradation of IκBα in IEC-6 cells, indicating that flufenamic acid- or sulindac sulfide-induced COX-2 is not mediated through the activation of NF-κB. In the previous study, similar results were obtained in HT-29; flufenamic acid, sulindac sulfide and indomethacin did not induce degradation of IκBα. TNFα-stimulated activation of NF-κB was suppressed by flufenamic acid or sulindac sulfide. But, indomethacin did not inhibit TNFα-stimulated activation of NF-κB. It was already known that indomethacin is a PPARγ activator and does not inhibit NF-κB activation (Yin et al., 1998).
Similarly, TNFα-induced NF-κB activation in HT-29 cells was inhibited by flufenamic acid and sulindac sulfide, but not by indomethacin, all of which are PPARγ ligands. These results suggest that inhibition by flufenamic acid or sulindac sulfide on TNFα-induced NF-κB activation is not mediated through the activation of PPARs.

Furthermore, it was likely that NSAIDs, which do not inhibit TNFα-induced NF-κB activation, can not inhibit TNFα-induced COX-2 expression in HT-29 cells. However, results from the present study indicate that inhibition of TNFα-stimulated activation of NF-κB by flufenamic acid or sulindac sulfide is not sufficient to inhibit TNFα-induced COX-2 expression in IEC-6 cells.

The molecular target through which flufenamic acid inhibits TNF-α induced NF-κB activation still needs to be identified. Since the NF-κB pathway is involved in both the pathogenesis of the inflammatory response and in cellular growth control, this pathway is also a potential target for inhibition by some NSAIDs. Yamatomo et al. (1999) recently demonstrated that sulindac and its metabolites, sulindac sulfide and sulindac sulfone, inhibit IκB kinase β (IKKβ), the protein kinase phosphorylating IκBα. Inhibition of IKKβ prevents IκBα degradation and thus prevents NF-κB-mediated gene expression.

Taken together, results from the present study indicate that some NSAIDs show differential effects on mitogen-stimulated COX-2 expression in normal cells and cancer cells.
CHAPTER 4
SUMMARY AND CONCLUSIONS

The purpose of this study was to investigate the mechanism of PUFAs and NSAIDs on the regulation of COX-2 expression. The results demonstrate that signaling pathways through which PUFAs inhibit tumor cell growth and LPS-induced COX-2 expression may be different. Both n-3 and n-6 PUFAs inhibit LPS-induced COX-2 expression in macrophages, whereas they do not induce COX-2 expression in the absence of LPS. Both n-3 and n-6 PUFAs also inhibit LPS-induced COX-2 expression at the transcriptional level. They also showed an inhibitory effect on LPS-induced NF-κB activation, suggesting an inhibitory action on other NF-κB dependent genes. Results from the reporter gene assay indicate that the potential target of PUFAs appears to be upstream of NIK. The molecular target of PUFAs that mediate the inhibition of the LPS-induced NF-κB activation still needs to be identified. PUFAs were shown to inhibit not only COX-2 expression but also the expression of iNOS and IL-1α induced by LPS. These results suggest that PUFAs also inhibit the expression of other pro-inflammatory marker gene products in response to inflammatory stimuli. In addition, results from in vitro cell proliferation study indicate that the growth of colon tumor cells is suppressed by n-3 PUFAs as compared with n-6 PUFAs regardless of whether COX-1 or COX-2 is expressed or not in the cells.

Unlike the results from the previous study in human colon carcinoma cells, NSAIDs, especially flufenamic acid and sulindac sulfide, upregulate the expression of COX-2 under both the presence and absence of COX-2 stimulators in normal intestinal cell line, IEC-6 cells. The inhibitors of extracellular-signal-regulated protein kinase

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(ERK), and p38 MAP kinase inhibit the flufenamic acid-induced COX-2 expression, suggesting that flufenamic acid-induced COX-2 expression is at least partly mediated through activation of MAPKs in IEC-6 cells. TNFα-induced activation of NF-κB was suppressed by flufenamic acid or sulindac sulfide. However, these NSAIDs did not appear to affect TNFα-induced COX-2 expression in IEC-6 cells. This may be due to two opposing effects: one is induction of COX-2 by NSAIDs and the other is induction of TNFα-induced COX-2 expression by NSAIDs.

In conclusion, the results of these studies suggest that the anti-inflammatory and immunomodulating effects of n-3 PUFAs are mediated through not only inhibition of COX-2 enzyme activity but also suppression of COX-2 expression. Moreover, our in vivo and in vitro studies suggest that the suppression of tumor growth by dietary n-3 PUFAs is mediated through COX-independent pathways. Results from the present study indicate that some NSAIDs show differential effects on mitogen-stimulated COX-2 expression in normal cells and cancer cells.

Further studies are needed to resolve how n-3 PUFAs and NSAIDs exert their anti-inflammatory effects and inhibitory effects on tumor growth. The molecular targets of n-3 PUFAs and NSAIDs mediating the anti-inflammatory effects need to be identified. Recently, it was shown that PUFAs and NSAIDs bind and activate PPARs. Thus, it needs to be determined whether PPARs mediate anti-inflammatory and anti-neoplastic effects of n-3 PUFAs and NSAIDs.
REFERENCES


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carcinoma development by a fish oil component, docosahexaenoic acid (DHA). Carcinogenesis. 18(7):1337-1342.


APPENDIX 1

PATHWAY OF PROSTAGLANDIN BIOSYNTHESIS

Figure A1. Pathway of prostaglandin biosynthesis by COX-1 and COX-2 (Smith and DeWitt, 1996)
Figure A2. Tetracycline-regulated expression of the COX protein and PGE2 production in human colon cancer cells (HCT-116) stably transfected with COX-1-flag (A) or COX-2-flag (C). Whole cell lysates from stable clonal cells were immunoprecipitated with polyclonal anti-Flag antibodies and immunoblotted with COX antibodies (A, C). COX enzyme activity (B, D) was determined by radioimmunoassay for PGE2 in the same stable clonal cells used at (A) or (C). Lane 1, no DNA transfected cells; Lane 2, pLinx vector transfected cells; Lane 3, pLinx-COX-1or COX-2 transfected cells with no tetracyclin; Lane 4, pLinx-COX-1or COX-2 transfected cells with tetracyclin. *, P<0.05.
APPENDIX 3

PPAR SUBTYPES IMMUNOBLOTS

Figure A3. Expression of peroxisome proliferator-activated receptors (PPARs) subtypes in various cell types. Whole cell lysates from various cells were analyzed by PPARα (A), PPARβ (B), or PPARγ (C) immunoblot. Lane 1, IEC-6; Lane 2, RAW264.7; Lane 3, NIH3T3; Lane 4, HT-29; Lane 5, HCT-116; Lane 6, MCF-7; Lane 7, MCF-10A; Lane 8, 293 cells.
## APPENDIX 4

### COMPONENTS FOR BUFFERS AND GELS

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
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<tr>
<td>COX Lysis Buffer</td>
<td>50 mM Tris-Cl (pH 7.4), 1 mM sodium diethyldithiocarbamic acid (Na-DDTC), 10 mM EDTA, 1% Tween20, 1% Triton X-100, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF).</td>
</tr>
<tr>
<td>2 x Laemmli Buffer</td>
<td>125 mM Tris-Cl (pH 6.8), 20% (v/v) glycerol, 5% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 0.016% (w/v) bromophenol blue.</td>
</tr>
<tr>
<td>4 x Laemmli Buffer</td>
<td>250 mM Tris-Cl (pH 6.8), 40% (v/v) glycerol, 10% (w/v) SDS, 20% (v/v) β-mercaptoethanol, 0.032% (w/v) bromophenol blue.</td>
</tr>
<tr>
<td>PT Lysis Buffer</td>
<td>20 mM Tris-Cl (pH 8.0), 137 mM NaCl, 10% (v/v) Glycerol, 1% (v/v) Triton X-100, 1 mM Na3VO4, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin, 10 µg/ml aprotinin.</td>
</tr>
<tr>
<td>RIPA Buffer</td>
<td>50 mM Tris-Cl (pH 7.4), 1% (v/v) Nonidet P-40, 0.25% (w/v) Sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM</td>
</tr>
</tbody>
</table>
phenylmethylsulfonyl fluoride (PMSF), 1mM Na$_3$VO$_4$,  
10 µg/ml leupeptin, 10 µg/ml aprotinin.

Stripping Buffer (pH 6.5) 
2% (w/v) SDS, 50mM Tris-Cl (pH6.8), 100mM β-mercaptoethanol.

TBS-T (pH7.6) 
10mM Tris-Cl, 150mM NaCl, 0.05% (v/v) Tween 20.

TE 
10mM Tris-Cl (pH 7.6), 1mM EDTA

Transfer Buffer (pH9.9) 
10mM NaHCO$_3$, 3mM Na$_2$CO$_3$, 20% (v/v) methanol

Polyacrylamide gel 
8 % Acrylamide/Bis solution, 400 mM Tris-HCl (pH 8.8),  
10 % glycerol, 1 mM EDTA, 0.2 % SDS, 0.1 % APS

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APPENDIX 5
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Arachidonic Acid</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>ATF2</td>
<td>Activating transcription factor 2</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic AMP</td>
</tr>
<tr>
<td>COX-1</td>
<td>Cyclooxygenase-1</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CRE</td>
<td>Cyclic AMP response element</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EFA</td>
<td>Essential fatty acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial growth factor</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>ERKs</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>HOG</td>
<td>Hyperosmolarity glycerol</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IKAP</td>
<td>IKK-associated protein</td>
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<td>IkKbα</td>
<td>Inhibitory kappa B alpha</td>
</tr>
<tr>
<td>IKK</td>
<td>IkB kinase</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
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<td>IRAK</td>
<td>IL-1R-associated kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
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<td>Linoleic acid</td>
</tr>
<tr>
<td>LBP</td>
<td>LPS-Binding protein</td>
</tr>
<tr>
<td>LNA</td>
<td>Linolenic acid</td>
</tr>
<tr>
<td>LOX</td>
<td>Lipooxygenase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPKs</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MAPKK</td>
<td>MAP kinase kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>MAP kinase kinase kinase</td>
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<tr>
<td>MEK-1</td>
<td>A synonym of MAPKK-1</td>
</tr>
<tr>
<td>MEK-2</td>
<td>A synonym of MAPKK-2</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NFDM</td>
<td>Non-fat dried milk</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB-inducing kinase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>OA</td>
<td>Oleic acid</td>
</tr>
<tr>
<td>PA</td>
<td>Palmitic acid</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PGs</td>
<td>Prostaglandins</td>
</tr>
<tr>
<td>PGD&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin D&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin E&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PGF&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin F&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PGH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin H&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PGI&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin I&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKs</td>
<td>Protein kinases</td>
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<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonylfluoride</td>
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<tr>
<td>PPARs</td>
<td>Peroxisome proliferator-activated receptors</td>
</tr>
<tr>
<td>PPRE</td>
<td>PPAR response element</td>
</tr>
<tr>
<td>PT</td>
<td>Phosphotyrosine</td>
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PUFA          Polyunsaturated fatty acid
RIP           Receptor interacting protein
RSK           Ribosomal S6 protein kinase
SA            Stearic acid
SAPKs         Stress-activated protein kinases
SDS           Sodium dodecyl sulfate
TF            Transcription factor
Tlr4          Toll-like receptor 4
TNFα          Tumor necrosis factor alpha
TRAFs         TNF-receptor associated factors
TXA₂          Thromboxane A₂
VITA

The author was born in Suwon, the Republic of Korea, on Oct. 8, 1969. She graduated from Pusan National University with a bachelor of science degree in the Department of Molecular Biology in February 1991.

In March 1992, she enrolled in Graduate school to pursue a master of science degree in the Department of Molecular Biology at Pusan National University. Her research focus was the angiogenesis inhibiting agents from natural compounds.

After her graduation with the degree of a master of science in February 1993, she worked as a junior researcher in the Department of Toxicology at Korean Food and Drug Administration. While she was working, she received a scholarship to study abroad from Korean government, the Department of Education. In August, 1996, she was accepted by the graduate school of Louisiana State University and pursued her doctoral studies in the Department of Food Science. She worked as a graduate research assistant for Dr. Daniel Hwang.

The author is currently a candidate for the degree of Doctor of Philosophy in Food Science, which will be conferred in August, 2000.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Kyung-He Sohn

Major Field: Food Science

Title of Dissertation: The Effects of Dietary Fatty Acids and Non-steroidal Anti-inflammatory Drugs on Cyclooxygenase-2 Expression

Approved:

[Signatures]

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

June 30, 2000

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