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DISSERTATION

MACROPHAGE CYCLOOXYGENASE AND INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION IN RESPONSE TO COPPER DEFICIENCY MEDIATED OXIDATIVE STRESS AND CONJUGATED LINOLEIC ACID

Submitted by

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In partial fulfillment of the requirements for

the degree of Doctor of Philosophy

Colorado State University

Fort Collins, CO

Spring 2000

COLORADO STATE UNIVERSITY

March 30, 2000

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY YASUKO IWAKIRI ENTITLED MACROPHAGE CYCLOOXYGENASE AND INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION IN RESPONSE TO COPPER DEFICIENCY MEDIATED OXIDATIVE STRESS AND CONJUGATED LINOLEIC ACID BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHYLOSOPHY.

Committee on Graduate Work

Co-advisor

Advisor

Department Head

ABSTRACT OF THE DISSERTATION

MACROPHAGE CYCLOOXYGENASE AND INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION IN RESPONSE TO COPPER DEFICIENCY MEDIATED OXIDATIVE STRESS AND CONJUGATED LINOLEIC ACID

Excessive prostaglandin E_2 (PGE_2) and nitric oxide (NO), which are synthesized by inducible forms of cyclooxygenase (COX-2) and nitric oxide synthase (iNOS), respectively, play key roles in pathogenesis of cancer, atherosclerosis and arthritis. I investigated the synthesis of PGE_2 and NO in activated macrophages in response to copper deficiency-induced oxidative stress and conjugated linoleic acid (CLA).

First, I tested the hypothesis that diets marginal (CuM) and deficient (CuD) in copper would decrease Cu, Zn-superoxide dismutase (Cu, Zn-SOD) activity and increase cellular oxidative stress, which would increase PGE_2 and NO production by increasing COX-2 and iNOS expression in activated macrophages. Lipid peroxidation and intracellular superoxide levels were significantly enhanced in parallel with decreased Cu, Zn-SOD activity in both CuM and CuD groups. The CuD group, but not the CuM group, showed a significant four-fold increase in PGE_2 production in activated cells accompanied by a significant five-fold increased in COX activity and four fold increase in COX-2 mRNA levels. NO was 40 % higher in the CuD group compared to the copper adequate (CuA) group, with no significant difference in iNOS expression.

Second, I investigated effects of CLA and arachidonic acid (AA) on PGE_2 and NO synthesis in RAW264.7 macrophages. CLA significantly depressed PGE_2 and NO

production by 78% and 57% compared to control values, respectively. Northern blot analysis of COX-2 and iNOS showed significant suppressive effects of CLA. AA significantly increased PGE_2 synthesis by 62% compared to control values. AA also suppressed NO production and iNOS expression in the same manner as observed for CLA. These results suggest that beneficial effects of CLA may be related to its ability to decrease both PGE_2 and NO synthesis by suppressing transcription of COX-2 and iNOS in activated macrophages.

In conclusion, copper marginal and deficient diets increased macrophage oxidative stress. CuD up-regulates macrophage PGE_2 and NO synthesis, which may favor atherogenesis. The ability of CLA to suppress both PGE_2 and NO production in macrophages may play an important role in the mechanisms of anti-cancer and anti-atherogenic effects of CLA.

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ACKNOWLEDGMENTS

I would like to express my thanks and gratitude to a number of people who have helped and guided me in the process of PhD program at Colorado State University. First and foremost, I would like to thank my mentor and advisor, Dr. Kenneth Allen, for his encouragement, instruction, enthusiasm, and continued support throughout PhD program. I would also like to thank my co-advisor, Dr. David Sampson, for his encouragement, instruction, enthusiasm, and continued support throughout my study. I will remember both of them as dedicated scientists who set personal examples with quality laboratory work and always had the right guidance and ideas to keep the project on track and moving forward. Because of them, I had a great, enjoyable, and fruitful life at Colorado State University.

I would also like to thank the members of my graduate committee, Drs. Mary Harris and Ian Orme, who provided me not only technical support, but also good ideas and firm guidance when needed to bring this work to conclusion.

Finally, I would like to thank for the support of Teruo Utsumi, as well as my father and mother, who supported me and always encouraged me. Without their help, I would not have been able to accomplish my goal to become PhD.

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List of abbreviations

AA	**arachidonic acid**
CLA	**conjugated linoleic acid**
COX	**cyclooxygenase**
CuA	**copper adequate**
CuD	**copper deficient**
CuM	**copper marginal**
IFN-γ	**interferon-gamma**
LA	**linoleic acid**
LPS	**lipopolysaccharide**
MAPK	**mitogen-activated protein kinase**
NFkB	**nuclear factor kappa B**
NO	**nitric oxide**
NOS	**nitric oxide synthase**
PGE_2	**prostaglandin E_2**
PGI_2	**prostacyclin I_2**
TXA_2	**thromboxane A_2**

Part I

Depression of Cu, Zn-superoxide dismutase activity by copper deficient diet increases prostaglandin E_2 and nitric oxide production and up-regulates cyclooxygenase-2 expression in rat peritoneal macrophages.

Chapter 1

Abstract

Oxidative stress and inflammatory stimuli potently induce mitogenic signaling pathways that up-regulate cyclooxygenase-2 (COX-2) and inducible form of nitric oxide synthase (iNOS) gene expression, both of which are implicated in the pathogenesis of atherosclerosis and cancer. **The objective** of this study was to test the hypothesis that decreased Cu, Zn-superoxide dismutase (Cu, Zn-SOD) activity caused by copper marginal and deficient diets enhances oxidative stress and alters COX-2 and iNOS expression in macrophages. Rats were pair meal-fed diets that were adequate (CuA), marginal (CuM) or deficient (CuD) in copper (6.8, 1.6, and 0.4 μg Cu/g, respectively). After 5 wk, thioglycollate-elicited peritoneal macrophages were isolated and cultured with or without lipopolysaccharide (LPS; 100 ng/ml) or a combination of interferon-gamma (IFN-γ; 10 U/ml) and LPS (10 ng/ml) for 9 hr to stimulate macrophages. In both LPS and IFN-γ/LPS, lipid peroxidation and intracellular superoxide levels were significantly enhanced in parallel with decreased Cu, Zn-SOD activity. The CuD group, but not the CuM group, showed a significant five-fold increase ($P = 0.001$) in COX activity measured as prostaglandin E2 in LPS-stimulated cells accompanied by a significant four fold increase ($P=0.04$) in COX-2 mRNA levels. For macrophages stimulated with IFN-γ/LPS, nitric oxide was 40 % higher ($P=0.02$) in the CuD group compared to the CuA group, with no significant difference in iNOS expression. These results suggest that expression of the COX-2 gene is more responsive to oxidative stress

than is iNOS expression in stimulated macrophages. (Supported by Colo.Ag Expt.Stn.and USDA Comp.Grant)

Chapter 2

Literature Review

2.1. Copper in diets

Diets in Western countries have been reported to provide copper below or in the low range of the estimated safe and adequate daily dietary intake for all age groups (Pennington et al., 1989), although severe copper deficiency has been rarely recognized. It is often stated that some groups are at risk of developing marginal copper deficiency. The lack of accurate detection of such a condition, however, excludes critical evaluation of deficiency (Milne, 1994).

The Food and Nutrition Board estimated that the safe and adequate daily intake of dietary copper is 1.5 to 3.0 mg for adults (NRC, 1989). The middle quartiles ranged in marginal intake that is from 0.91 to 1.86 mg/day. The top five sources of copper in the U.S. diet are yeast bread, potatoes (white), tomatoes, ready-to-eat cereal, and beef (Subar et al., 1998). Data from the surveys of 849 diets indicated that only 3.2% of the diets exceed 3.0 mg/day, 61% are less than 1.5 mg/day, and approximately 30% are less than 1 mg/day (reviewed by Klevay, 1998).

Diets low in copper usually result in decreased immune functions, cardiovascular disease and osteoporosis. In human studies, diets fed close to 1 mg of copper per day (i.e. marginal intake), amounts quite frequent in the U.S., responded similarly to deficient animals with reversible, potentially harmful changes in blood pressure control (Lukaski et al., 1988), cholesterol and glucose metabolism, and electrocardiograms (Klevay, 1998).

Copper supplementation to human subjects showed beneficial effects on bone density. It is, therefore, probable that marginal copper intake frequently seen in the U.S. population may contribute at least in part to the prevalence of human diseases such as heart disease (Klevay, 1998).

2.2. Role of copper in cellular oxidation

Copper is an essential antioxidant nutrient which acts as a cofactor for Cu, Zn-superoxide dismutase (Cu, Zn-SOD) enzyme. This enzyme plays an important role in cellular defense mechanism against superoxide radicals (O_2^-) that are produced from normal metabolism (McCord and Fridvich, 1969; Linder and Goode, 1991). Removal of O_2^- is achieved through their reduction by Cu, Zn-SOD to H2O2, and the subsequent removal of H2O2 by catalase and glutathione peroxidase (Fig 1). In spite of its short life, the O_2^- has the potential to initiate intracellular damage by producing hydroxyl radicals (OH •) from its production of H2O2 and subsequent reaction with Fe^{2-} (Fenton reaction) and by serving as a major intracellular reductant of ferric (Fe^{3-}) ion (Adelman et al., 1988). While cells contain several non-enzymatic antioxidants, the activity of cytosolic Cu, Zn-SOD plays a key role in protecting against the cells from initiation of oxidative damage. In this respect, cellular oxidative damage is highly dependent upon copper adequacy in our diet.

It is known that even the copper marginal diet decreases Cu, Zn-SOD activity and increases cellular oxidative stress. In studies with rats, Cu, Zn-SOD activity is significantly associated with the amounts of copper in the diet. Furthermore, lipid peroxidation, recognized as the final stage of cellular oxidation, increases as the activity of Cu, Zn-SOD activity decreases, indicating that dietary copper is a key factor in

protecting against cellular oxidation (Nelson et al., 1992; Morin et al., 1993). When the balance of antioxidants and cellular oxidation goes in favor of cellular oxidation, cells undergo the situation called oxidative stress. Indicators of oxidative stress include the levels of lipid peroxidation and intracellular O_2^- concentration. While Babu and Failla (1990) showed that copper deficiency depresses Cu, Zn-SOD activity in macrophages and impairs macrophage functions, currently no studies have been conducted to test whether the depression of Cu, Zn-SOD activity causes oxidative stress in macrophages.

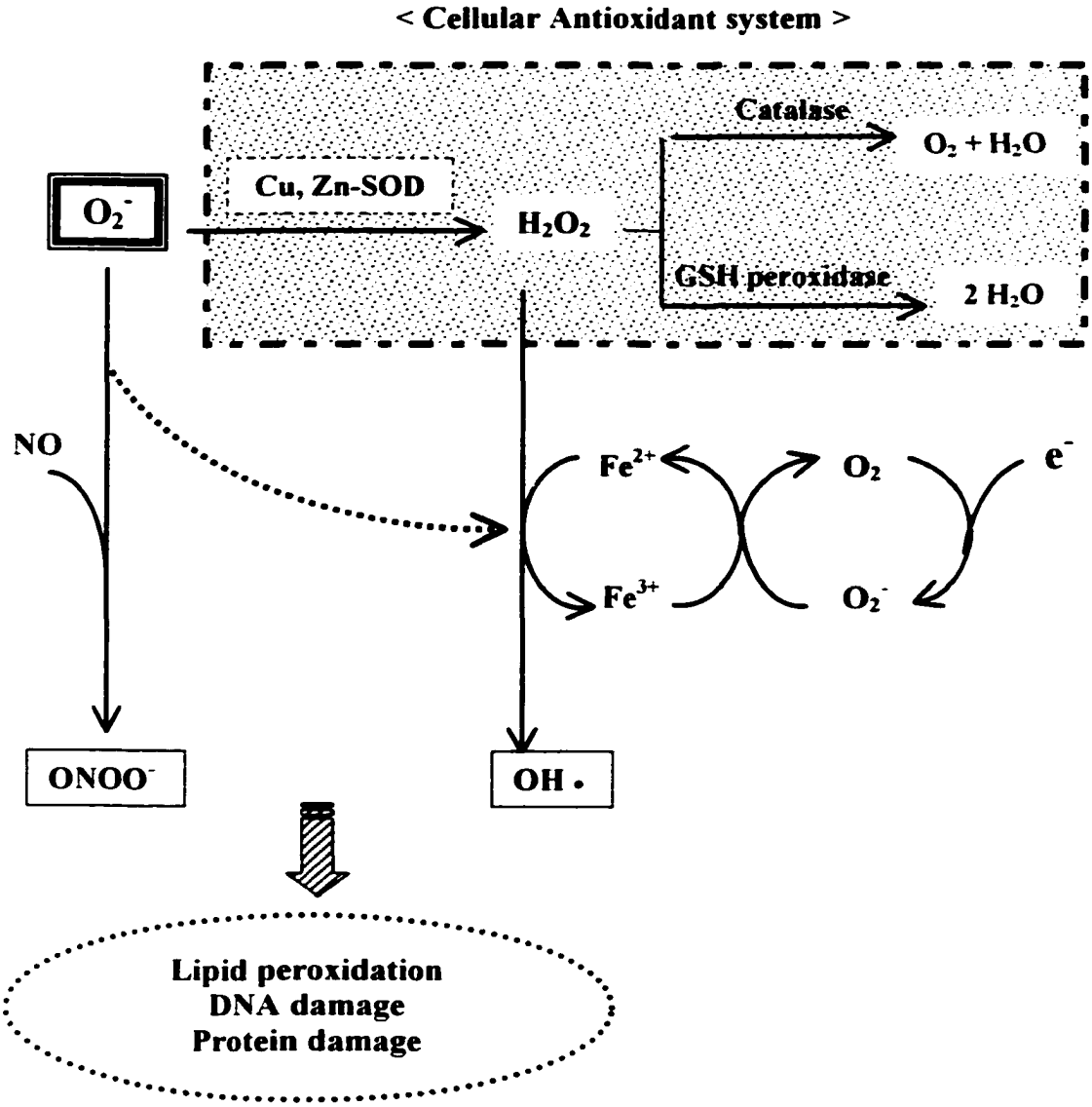

Figure 1. Role of Cu, Zn-superoxide dismutase (Cu, Zn-SOD) in cellular antioxidant system. Abbreviations are; O_2^-, superoxide radical; H_2O_2, hydrogen peroxide; GSH, glutathione; NO, nitric oxide; $ONOO^-$, peroxynitrite; OH •, hydroxyl radical. The inside of the dotted line indicates the cellular antioxidant system.

2.3. Cyclooxygenase

Cyclooxygenase (COX), also known as prostaglandin H (PGH) synthase (E.C.1.14.99.1), catalyzes a rate-limiting step of prostaglandin synthesis (Fig 2). COX is an integral membrane protein found mainly in microsomal membrane, but also in the nuclear membrane (Rollins and Smith, 1980).

Currently, two COX isoforms have been identified and are referred to as COX-1 and COX-2. The COX-1 is constitutively expressed in most cell types under basal condition and its major function is to provide prostanoid precursors for cellular homeostasis. In contrast, COX-2 is expressed in response to a wide variety of stimuli and is thought to contribute to the generation of prostanoids in certain stages of cell proliferation and differentiation at sites of inflammation (William and Dubois, 1996). In rats, COX-1 encodes 2.8 kb mRNA and COX-2 encodes 4 kb mRNA (Feng et al., 1993). Human COX-1 cDNA encodes a 599 amino acid protein, while COX-2 cDNA encodes a 604 amino acid protein (Smith et al., 1996). Although COX-1 and COX-2 catalyze the identical reaction and have similar structure with approximately 60% amino acid sequence identity, their enzyme activities and genes are regulated by two independent and quite different systems (Wu, 1996).

COX-1 and COX-2 can be expressed in the same cells, such an example includes macrophages, but are regulated in a quite different manner. The expression of the COX-2 gene accounts for the great increase and prolonged duration of prostaglandin E_2 (PGE_2) synthesis by macrophages responding to various inflammatory stimuli (DeWitt, 1991). The regulatory mechanisms of COX expression are discussed later.

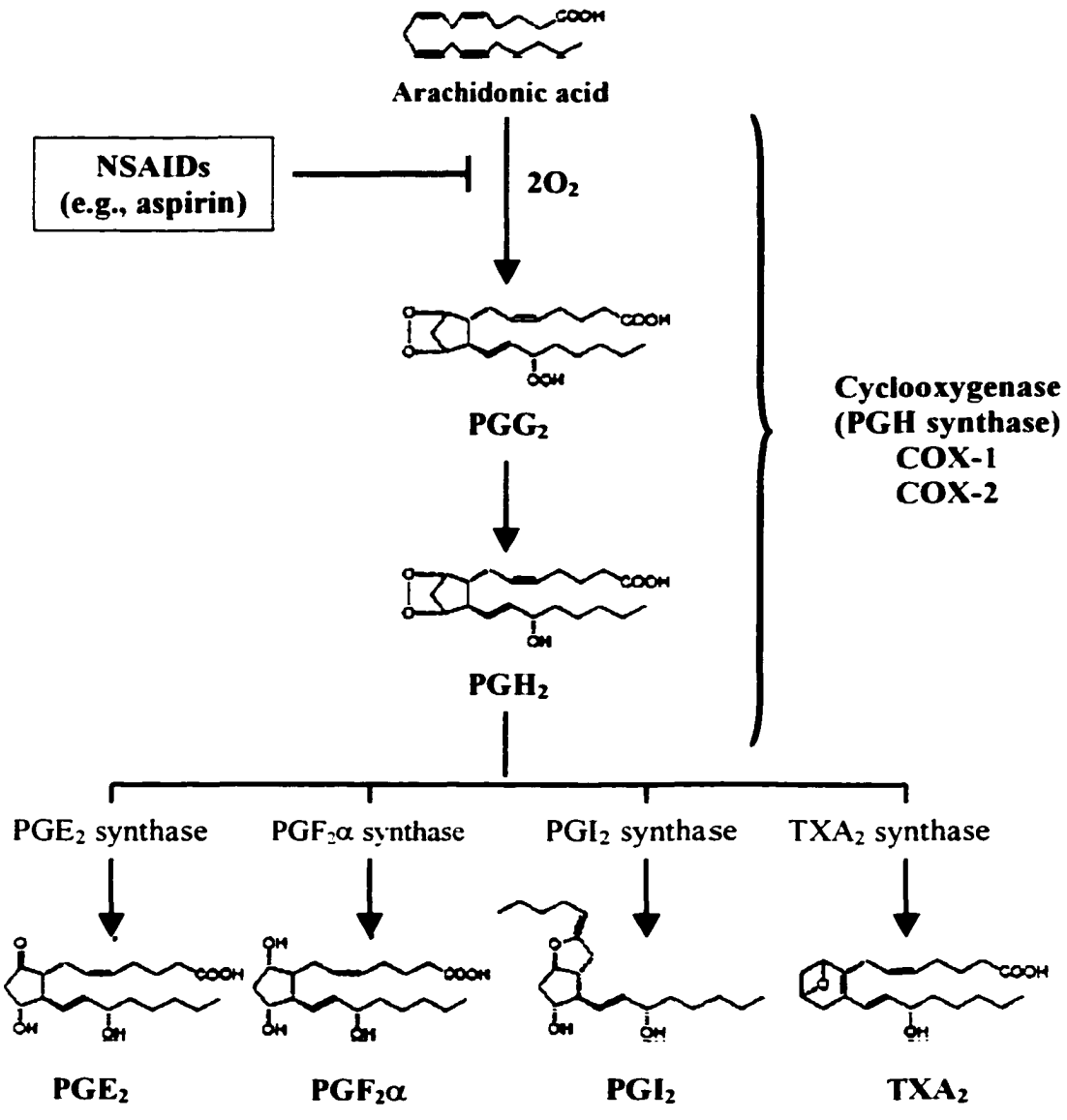

Figure 2. The biosynthetic pathway of prostanoids. Abbreviations are: NSAIDs, nonsteroidal anti-inflammatory drugs; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGI_2, prostacyclin I_2; and TXA_2, thromboxane A_2. NSAIDs, such as aspirin, block COX activity, resulting in the inhibition of prostanoid synthesis. Prostanoid synthesis is initiated by the release of arachidonic acid (AA) from the membrane phospholipids by phospholipase C (PLC) and phospholipase A_2 (PLA_2). COX catalyzes the incorporation of molecular oxygen at C11 of AA, with the formation of a cyclic endoperoxide in which an oxygen molecule bridges C9 to C11. The introduction of a second oxygen molecule at C15 yields PGG_2. Subsequently, peroxidase activity results in the formation of PGH_2, which serves as a common substrate for the synthesis of all the prostanoids such as PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2. The subsequent conversion of PGH_2 to each prostanoid is depending on the presence or absence of the respective terminal prostanoid synthases varying in cell and tissue distribution (Dubois et al., 1998).

2.4. PGE_2 in physiology and diseases

PGE_2 is a paracrine and autocrine hormone. In contrast to hormones such as cortisone or thyroxin, which have broad systemic effects despite being released from a single site in the body, PGE_2 is synthesized in a broad range of tissue types and serves as autocrine or paracrine mediators to induce signals within the immediate environment. At low concentrations, produced by COX-1, PGE_2 plays an important role in regulating homeostasis, including preserving stomach lining, respiratory, reproduction, and gastric function. In contrast, PGE_2 at high concentration, produced by COX-2, is thought to enhance inflammation and is implicated in pathophysiology of diseases. (reviewed by Dubois et al., 1998). The pathophysiological roles of PGE_2 and COX-2 are focused on the following review.

A. Pain and fever. PGE_2 in the amounts produced at sites of inflammation is believed to mediate acute pain by sensitizing pain receptors. In the dorsal root ganglion, COX-2, but not COX-1, plays an important role in the synthesis of substance P, which is released from primary afferent neurons to convey information regarding noxious stimuli (Inoue et al., 1999). The fever response is initiated by the release of pyrogen(s) from macrophages responding to diverse inflammatory stimuli, including endotoxin lipopolysaccharide (LPS), immune complexes, and cytokines. A study using a COX-2 specific inhibitor showed that COX-2 mRNA is strongly induced in the brain blood vessels during fever (Cao et al., 1995; Breder and Saper, 1996). Furthermore, light and electron microscopic immunocytochemical studies clearly indicated an enhanced COX-2 expression in brain endothelial cells in mice during LPS-induced fever (Matsumura et al., 1999). These evidence suggest that PGE_2 is an essential mediator of fever and COX-2 plays a key role in producing a large amount of PGE_2 in central nerve system.

B. Cancer. Epidemiological studies have suggested that the regular use of nonsteroidal anti-inflammatory drugs (NSAID), such as aspirin, an inhibitor of COX activity, decreases the risk of cancers, indicating an involvement of PGE_2 and COX in the pathogenesis of cancers (Smalley and Dubois, 1997). An increasing body of evidence suggests an enhanced expression of COX-2 mRNA and/or protein, but not COX-1, in human colorectal cancer (Dannenberg and Zakim, 1999; Sheehan et al., 1999; Dimberg et al., 1999; Watanabe et al., 1999), esophageal cancer (Ratnasinghe et al., 1999), hepatocellular carcinoma (Koga et al., 1999; Shiota et al., 1999), stomach cancer (Uefuji et al., 1998; Yamamoto et al., 1999; Murata et al., 1999) and lung cancer (Wolff et al., 1998). The inhibition of COX-2 expression, therefore, has been receiving considerable attention in recent years due to the notable therapeutic effects on cancers (Dubois et al., 1998).

PGE_2 affects cell proliferation, tumor growth and suppresses the immune response to malignant cells. A High level of PGE_2, therefore, could favor malignant growth (Earnest et al., 1992). A study by Watanabe et al. (1999), using an antagonist of PGE_2 receptor subtype EP1, showed a significant 60 % reduction in the number of intestinal polyps, indicating that PGE_2 contributes to colon carcinogenesis to some extent through its action at the EP1 receptor. Furthermore, a study by Sheehan et al. (1999), who studied COX-2 expression in tumor section obtained from colorectal cancer patients with different stages of cancer, showed an increase in COX-2 expression in tumor cells as indicated by COX-2 epithelial staining. In contrast, no COX-2 was observed in normal tissue. In addition, greater expression of COX-2 correlated with more advanced stages of tumor, indicating that COX-2 may play an important role in colorectal tumorigenesis and COX-2 expression in colorectal cancer may be related to survival from this cancer

(Sheehan et al., 1999). These evidence suggest that PGE_2 and COX-2 are important mediators for the development and progression of cancer.

A high level of PGE_2 is generally known as an immuno-suppressive agent produced by cancer cells and their surrounding macrophages. Several researchers have reported an enhanced PGE_2 concentration in the portal veins of patients with colorectal cancer. Okuno and researchers showed (1995) that continuous administration of PGE_2 in the portal vein suppressed the cytotoxic activity of hepatic sinusoidal lymphocytes in a dose dependent manner. Furthermore, flow cytometric analysis indicated that the numbers of large granular lymphocyte fraction, hepatic natural killer cells, and T cells were decreased in the hepatic sinusoidal lymphocyte following PGE_2 infusion. The decreased hepatic immunity was associated with an increase in the number and size of metastatic tumor nodules in the liver and promoted liver metastases, suggesting that the decreased liver-associated immunity may be related to the progression of tumor cells (Okuno et al., 1995). It is probable that the decreased immune system due to the increased production of PGE_2 by cancer cells and their surrounding macrophages may create an environment, which favors tumor growth.

C. Atherosclerosis. It is known that prostanoids are important mediators of vascular physiology and thus play critical roles in atherogenesis. PGE_2 is known to mediate inflammation by increasing vascular permeability and migration of immune cells, which appears to have a major role in the development of atheorsclerotic lesions (Ross, 1993).

COX-2 expression was examined in atherosclerotic lesions from patients with coronary disease using an antibody against COX-2. COX-2 expression was observed predominantly in macrophages/foam cells in atherosclerosis, while there was no COX-2

expression in normal arteries. COX-2 expression was also observed in smooth muscle cells and endothelial cells in those atherosclerotic lesions (Baker et al., 1999). Besides an increase in COX-2 expression, those atherosclerotic lesions had increased peroxynitrite formation, which has been shown to activate COX-2 (Landino et al., 1996), suggesting that COX-2 plays a role in atherogenesis.

2.5. Regulation of PGE_2 synthesis

Cellular PGE_2 synthesis is tightly regulated at the level of COX catalysis (Kulmacz, 1998). This catalytic regulation is particularly interesting because it appears to be different for the two isoforms. Oxidative stress, caused by increased reactive oxygen species (ROS), may play an important role in the differences in the regulation between COX-1 and COX-2. Lipid hydroperoxides produced by oxidative stress are essential cofactors for the COX catalytic activity (Smith and Lands, 1972). ROS can no longer be regarded solely as damaging species. Its appears important to characterize their contribution to the control of gene (i.e., COX-2) expression (von Knethen et al., 1999), and also their contribution to activation of COX-1 and COX-2 activity (discussed below). The regulatory mechanisms of COX activity and expression by oxidative stress are discussed in this review.

A. COX-2 expression. ROS are increasingly recognized to control signal transduction via activation of mitogen-activated protein kinase (MAPK) or as being implicated in the regulation of transcription factors such as NFkB or AP-1 (von Knethen et al., 1999). Activation of NFkB is an important component for cytokine-induced COX-2 expression (Hwang et al., 1997; D'Acquisto et al., 1997), suggesting that ROS are potent regulators of COX-2 expression.

NFkB is a heterodimer composed of two DNA-binding sites, RelA (p50) and RelB (p65), and is responsible for regulating the transcription of several genes whose products are critical for generating an appropriate immune response to insults such as infection and injury (Donald et al., 1995; Lin et al., 1995). Under physiological conditions, NFkB is held in its inactive form in the cytosol by the inhibitory protein IkB-α. Activation of cells that induce the generation of ROS causes the phosphorylation and proteolytic degradation of IkB-α (Henkel et al., 1993). Lo et al., (1998) showed that COX inhibitors prevented NFkB activation and decreased PGE_2 production by inhibiting COX-2 mRNA expression. Thus, inhibition of NFkB translocation might be beneficial in that this inhibition suppresses the synthesis of inducible enzymes, such as COX-2 and iNOS, which promote inflammation.

Superoxide radicals (O_2^-), or their products (e.g., $OH^\bullet$, $RO^\bullet$), are capable of inducing COX-2 transcription via nuclear factor kappa B (NFkB)-mediated mechanisms (Satriano and Schlondorff, 1994; Von Knethen et al., 1999). A study by Von Knethen and coworkers (1999) clearly demonstrated that an incubation of RAW264.7 macrophage cells with 2,3-dimethoxy-1,4-naphtoquinone (DMNQ), an O_2^- generator, resulted in increased p50/p65-heterodimer formation, IkB-α degradation, and stimulation of a NFkB luciferase reporter construct (which indicates increased NFkB binding to its response element). Inhibition of NFkB binding at COX-2 promoter abolished DMNQ-induced COX-2 expression, indicating that O_2^- - mediated NFkB activation enhances COX-2 expression. Furthermore, DMNQ-elicited COX-2 expression is mediated through inhibition of MAPK. It is known that LPS-induced COX-2 expression is mediated by MAPK and NFkB activation in macrophages (Hwang et al., 1997).

The regions regulating gene expression of COX-1 and COX-2 show little similarity. For example, the promoter and enhancer regions regulating COX-2 contain a wide variety of response elements (Hoff et al., 1993). Sequence analysis of the 5'-flanking region of COX-2 has shown several potential transcription regulatory sequences, including two NFkB sites, a TATA box, a C/EBP motif, two AP-2 sites, three SP1 sites, a CRE motif and an Ets-1 site (Appleby et al., 1994). This observation explains, at least in part, the inducibility of COX-2 by hormones, inflammatory factors, and cytokines.

B. COX activity. Both COX-1 and COX-2 require hydroperoxides for the enzyme to be catalytically active (Kulmacz et al., 1995; Capdevile et al., 1995). In many cells containing both isoforms, the COX-2 has been found to be catalyticaly active at the same time that the COX-1 remains latent (Kulmacz, 1998). The observation that the COX-2 is activated at hydroperoxide levels approximately 10-fold lower than those needed for COX-1 (Kulmacz et al., 1995), indicating that this difference may contribute to the distinct control of cellular prostanoid synthesis by the two isoforms. Furthermore, the COX activation by lipid hydroperoxide is distinctly faster in COX-2 than COX-1 (Lu et al., 1999).

Conversion of latent COX to catalyticaly competent enzyme is believed to involve in the formation of a key catalytic component, a free radical located on Tyr385 in COX-1 and Tyr371 in COX-2. The difference in efficiency of activation between COX-1 and COX-2 may be in the formation of the catalytically active tyrosyl radicals on Tyr385 in COX-1 and Tyr371 in COX-2 (Lu et al., 1999). There are, however, no readily apparent structural differences in the vicinity of the heme and Tyr385 (Tyr371) between COX-1 and COX-2 (Picot et al., 1994; Kurumbail et al., 1996), which gives rise to a question. It is suggested that one possible mechanism would be that the active site

tyrosyl radical in COX-2 is less readily quenched by glutathione (GSH) peroxidase than the corresponding tyrosyl radical in COX-1 (Lu et al., 1999). Most cells have a large excess of GSH peroxidase, over the peroxide generating enzymes, such as COX (Marchall et al., 1987). The increased stability of tyrosyl radical was observed in COX-2 against GSH peroxidase compared to COX-1. Further study will be needed to test this possibility that differing tyrosyl radical stability in the two COX isoforms contributes to the difference in hydroperoxide activator efficiency (Lu et al., 1999).

2.6. Nitric oxide synthase

Nitric oxide (NO) is a radical molecule which is synthesized by nitric oxide synthase (NOS) from L-arginine (Moncada and Higgs, 1993) (Fig. 3). To date three isoforms of NOS have been characterized (reviewed by Förstermann et al., 1994).

There are two constitutive isoforms, including neuronal (Bredt et al., 1990; Mayer et al., 1991) and endothelial NOS (Sessa et al., 1992; Lamas et al., 1992; Janssens et al., 1992). These constitutive isoforms are always present, thus named constitutive. eNOS has been localized to endothelium (Michel et al., 1993) and is mostly membrane bound via a myristylation site, while nNOS has been localized to the cytosol of central and peripheral neurons (Bredt et al., 1991) but is expressed also in extra-neuronal sites such as skeletal muscle, pancreas, and kidney (Nakane et al., 1993; Nathan, 1992). Small amounts of NO are generated by these isoforms and play a major role as regulator and mediator of numerous processes, including vascular tone, smooth muscle cell proliferation, and neurotransmission (Moncada and Higgs, 1995; Nathan and Xie, 1994).

The third isoform, the inducible NOS (iNOS), is present only after cells are stimulated with bacteria or bacterial endotoxin such as lipopolysaccharide (LPS) and/or

proinflammatory cytokines such as interferron-gamma (IFN-γ) (Stueher et al., 1991). Although resting unstimulated cells do not express iNOS, several cell types including macrophages, hepatocytes, vascular smooth muscle cells, glial cells, endothelial cells, chondrocytes, cardiac myocytes, pancreatic islet cells and fibroblasts have been shown to express iNOS when appropriately stimulated (reviewed by Nathan, 1992). Induction of iNOS in these cells results in the release of large quantities of NO, which exerts cytotoxic actions in mammalian tissues and anti-microbial activity toward certain pathogens. For instance, the expression of iNOS in macrophages is considered to be part of the host's defense system (Stuehr and Marletta, 1985; Nathan and Hibbs, 1991). Various aspects of iNOS expression with an emphasis on pathophysiological condition, are discussed in this review.

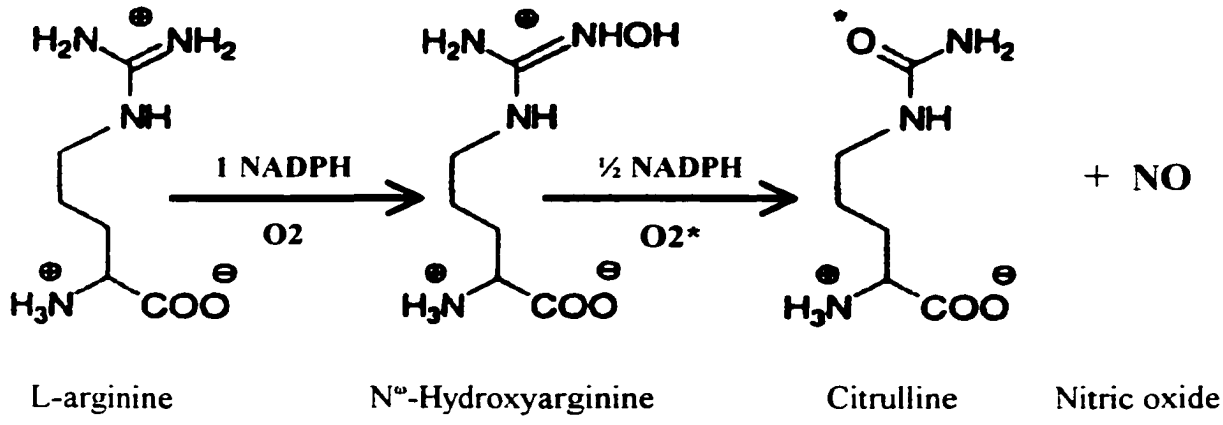

Figure 3. The L-arginine-nitric oxide (NO) biosynthetic pathway by NO synthase (NOS). Conversion of L-arginine to NO and citrulline is carried out in steps by NOS. The reaction represents a five-electron oxidation of L-arginine guanidino nitrogen. The first reaction involves a formation of N$^{\omega}$-Hydroxy-L-arginine from L-arginine by a hydroxylation reaction as an enzyme bound intermediate. In the second step, N$^{\omega}$-Hydroxy-L-arginine is converted to citrulline and NO. The biological electron donor NADPH is required for both steps in the reation. A total of 1.5 NADPH is oxidized per molecule NO formed (reviewed by Lancaster and Stuehr, 1996).

2.7. NO in physiology and diseases

In recent years, accumulated evidence indicates that enhanced NO production by iNOS may be involved in the development and progression of diseases (Jadeski and Lala, 1999). In contrast to these adverse effects on diseases, iNOS is required to fight against infectious diseases. The current findings of iNOS expression in atherosclerosis, cancers, and infectious diseases are reviewed.

A. Cancer. Although there are apparently two conflicting views, overall the vast majority of clinical and experimental evidence supports the interpretation that high levels of NO production are positively associated with tumor growth. Relative to normal tissues, increased iNOS expression has been observed in human esophagael carcinoma (Tanaka et al., 1999), human colon carcinoma (Kojima et al., 1999), and human gastric tumor (Doi et al., 1999; Goto et al., 1999). Experimental tumor model provides more direct evidence of an involvement of NO in tumorigenesis. In animal models, iNOS inhibition resulted in the reduction in angiogenesis (Ziche et al., 1997; Jadeski and Lala, 1999), suggesting that NO may enhance angiogenesis.

In contrast to these studies, some studies have shown an inverse relation between NO and tumorigenesis. When iNOS overexpressing gene was introduced into melanoma cells (Dong et al., 1994) as well as renal carcinoma cells (Juang et al., 1998), these cells lost tumorigenic and metastatic effects as a result of NO-mediated tumor apoptosis, suggesting that the production of high levels of NO by iNOS may be associated with autocytotoxicity, suppression of tumorigenicity, and abrogation of metastasis.

These opposing findings suggest a dual role for NO in tumor growth and metastasis; the susceptibility of tumor cells to NO-mediated injury may depend on levels of NO produced and the genetic makeup of the tumor cells. During the evolution of

tumors, high NO-producing cells may self-delete by apoptosis, and those making lower levels of NO, or cells capable of resisting NO-mediated injury, may have an in vivo advantage, resulting from NO-mediated stimulation of tumor cell invasiveness, tumor blood flow or tumor angiogenesis (Lala and Orucevic, 1998). Despite intensive investigations, the role of NO in carcinogenesis and tumor cell growth remains unclear and controversial (Hajri et al., 1998).

B. Atherosclerosis. A growing body of evidence showed increased expression of iNOS in atherosclerotic lesions (Depre et al., 1999; Luoma and Ylä-Herttuala, 1999; Vejlstrup et al., 1998). It is believed that macrophages play an important role in plaque stability by producing a large quantity of NO by iNOS expression (Moreno et al., 1994). It is suggested that iNOS activity in the plaque is deleterious, due to the formation of peroxynitrite ($ONOO^-$), the product of NO and superoxide (O_2^-), which enhances platelet adhesion and aggragation (Salvemini et al., 1989). The ONOO- has been shown to inhibit PGI_2 synthase (Zou et al., 1998). PGI_2 is a potent inhibitor of platelet aggregation and is a vasodilator. It blocks leukocyte adhesion to endothelial cells and inhibits monocyte activation (Wu, 1996). It is likely that elevated NO production by iNOS in atherosclerotic lesions is associated, at least to some extent, with localized oxidative damage to arterial cells and lipoproteins, which could promote atherogenesis (Leeuwenburgh et al., 1997).

Currently, however, it is not clear whether iNOS induction in atheroslerotic lesions is protective or whether it enhances lesion formation. Some suggest that NO produced by iNOS might have several anti-atherogenic effects (Darley-Usmar et al., 1995); it can favor vasodilation and inhibit cellular proliferation in the plaque (Cayatte et

al., 1994; Fukumoto et al., 1997). Further study will be needed to elucidate the roles of increased iNOS expression in atherosclerotic lesions.

C. Infectious diseases. Macrophages play an important role in eliminating infectious agents, such as *M. Tuberculosis* (Rhoades and Orme, 1997). In genetically engeneered mice, a lack of iNOS results in decreased survival rate upon infection with *L. Monocytegenes* (MacMicking et al., 1995) and *M. Tuberculosis* (MacMicking et al., 1996), suggesting that expression of iNOS is necessary for pathogen killing during infection.

2.8. Regulation of nitric oxide production

The regulation of iNOS is complex. Although a major iNOS regulation occurs at a pre-translational step such as transcription or mRNA stability, iNOS regulation also occurs at multiple levels of expression including transcription, translation, post-translational modifications and cofactor requirements. In recent years, extensive efforts have been made to elucidate the regulation of iNOS expression, in particular, via signal transduction and transcription. In contrast, relatively few studies have been performed on iNOS mRNA stability, translational effciency as well as post-translational modification of iNOS (Taylor et al., 1998).

All three NOS isoforms are structurally closely related, and among the isoforms also evolutionary well-conserved: the identity between the murine or rat and the human iNOS cDNA sequences is 80%, and between murine and rat iNOS cDNA 90%. However, the promoter regions show only 65% similarity between murine and human iNOS genes, and also a diverse responsiveness to similar stimuli (Lowenstein et al., 1993; De Vera et al., 1996), suggesting a different and a species-specific gene regulation. The

regulation of NO production by iNOS is the focus of this review.

A. Transcription. Multiple regulations of iNOS transcription could occur as is evident by the presence of various consensus sequences for binding of transcription factors in the iNOS gene (Xie et al., 1993). NFkB plays a critical role in the induction of iNOS gene by IFN-gamma and LPS. iNOS contains NFkB binding sites in both up-stream and down-stream regions. It seems that the down-stream NFkB site act as a core promoter for the iNOS promoter activity, while the up-stream NFkB site functions as an enhancer (Kim et al., 1997). In addition, there are a number of putative transcription factor binding sites, many of them probably indispensable for the gene's NFkB-dependent induction, but also may have a role in its NFkB-independent induction pathway. These include cyclic adenosine 3', 5'-monophosphate (cAMP) response elements (CRE), hypoxia response element (HRE), and activator protein 1 (AP-1) response element (Keinanen et al., 1999).

B. mRNA stability. Induction of macrophage iNOS mRNA by LPS plus IFN-gamma reflects increased iNOS gene transcription without changes in iNOS mRNA stability (Lorsbach et al., 1993). In contrast to the effects of LPS and IFN-gamma, TGF-beta suppresses macrophage iNOS expression via decreased iNOS mRNA stability and translational efficiency and by decreased stability of iNOS protein but does not alter iNOS transcription (Vodovotz et al., 1993). These effects may be cell-type specific, since TGF-beta attenuates iNOS mRNA induction in RAW264.7, but enhances induction in Swiss 3T3 cells (Gilbert and Herschman, 1993). These results suggest that expression of iNOS genes can also be regulated at the level of mRNA stability.

A recent finding suggests that tetrahydrobiopterin (BH4) plays a role in iNOS mRNA stabilization (Linscheid et al., 1998). BH4 is synthesized from GTP (54 in Morris

and Billiar, 1994). Linscheid et al. (1998) investigated roles of BH4 in iNOS expression in smooth muscle cells (SMC). SMC treated with a BH4 donor had more stable iNOS mRNA compared to control cells, whereas in SMC with a BH4 inhibitor iNOS mRNA disappeared. The loss of iNOS mRNA by the BH4 inhibitor was abolished by the treatment with a BH4 donor. These results indicate that BH4 post-transcriptionally stabilizes iNOS mRNA in SMC (Linscheid et al., 1998). Mechanism(s) by which BH4 stabilizes iNOS mRNA is not known.

C. Post-translational modifications. Post-translational modification and regulation of iNOS activity have been suggested and may have an important regulatory role in iNOS enzyme activity (Michel and Feron, 1997). The phosphorylation is an important characteristic of post-translational modification of diverse proteins, including enzymes. A study by Pan et al. (1996) showed that iNOS was phosphorylated on tyrosine residues and that this was an early event coinciding with the appearance of newly synthesized iNOS in RAW264.7 macrophages. In addition, a brief exposure of activated macrophages to a tyrosine phsophatase inhibitor significantly increased the level of iNOS tyrosine phosphorylation, suggesting that tyrosine phosphatases may be involved in the regulation of the levels of iNOS phosphorylation. The phosphatase inhibition also resulted in a rapid increase in enzyme activity, occurring within 5 min of exposure, indicating that tyrosine phosphorylation up-regulates iNOS activity. Taken together, these results demonstrated that tyrosine kinases and phosphatases are involved in the post-translational modification (covalent modification) of iNOS activity and may potentially play a role in modulating the functional activity of the enzyme in macrophages (Pan et al., 1996). It is not known whether the tyrosine phosphorylation is essential for iNOS activity.

BH4 has been well-recognized as an important factor for the iNOS post-translational modification. BH4 by binding to heme portion of the enzyme plays a critical role in promoting dimerization of iNOS, which is required for iNOS to become a catalytically active enzyme. Cells deficient in BH4 showed little iNOS activity, but acquired activity during a 60 to 120 min incubation with BH4. Furthermore, NO synthesis correlated with an increase in intracellular BH4 but no increase in iNOS protein (Tzeng et al., 1995), suggesting that BH4 is an important cofactor for iNOS catalytic activity.

The important feature in the regulation of all three isoforms is the role of calmodulin. Both eNOS and nNOS are highly regulated by the enzyme's binding to the ubiquitous Ca^{2+} regulatory protein calmodulin. A high intracellular Ca^{2+} concentration $[Ca^{2+}]$ is required for their binding to calmodulin to be fully activated. In contrast, iNOS is capable of binding to calmodulin with extremely high affinity even at low $[Ca^{2+}]$, which is the characteristic of resting cells. Thus, the intracellular activity of eNOS and nNOS may be closely modulated by transient changes in $[Ca^{2+}]$, whereas the activity of iNOS is no longer temporally regulated by intracellular Ca transient changes (reviewed by Nathan and Xie, 1994). iNOS contains calmodulin tightly bound to each subunit of the enzyme (Cho et al., 1992). It is presumed that calmodulin is incorporated into the enzyme during synthesis, resulting in permanent activation of the enzyme, which is calcium independent. Because of this, NO synthesis by iNOS is most likely limited by substrate, cofactors, protein turnover, and product inhibition (reviewed by Morris and Billiar, 1994).

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Chapter 3

Introduction

Copper is an essential antioxidant nutrient as a cofactor of Cu, Zn-superoxide dismutase (Cu, Zn-SOD) which scavenges superoxide radical (O_2^-) formed from normal oxygen metabolism (McCord and Fridvich, 1969; Linder and Goode, 1991). Insufficient copper intake, therefore, leads to the depression of the enzyme's activity (Nelson et al., 1992; Morin et al., 1993; Babu and Failla, 1990) and allows reactive oxygen species (ROS) to propagate the chain reaction of cellular oxidation, e.g., protein oxidation (Wilkins and Leake, 1994), DNA damage (Thomas et al., 1998) and lipid peroxidation of cell membranes (Nelson et al., 1992; Morin et al., 1993). Although severe copper deficiency is rarely recognized, insufficient copper intake in western diets has been reported (NRC 1989). For this reason, the majority of the US population consuming the typical US diet could be at risk of oxidative stress.

Copper intake influences prostanoid synthesis (Nelson et al., 1992; Morin et al., 1993). Prostanoids, including prostaglandin E_2 (PGE_2), thromboxane A_2 (TXA_2), and prostacyclin I_2 (PGI_2), are 20 carbon fatty acids that have a wide variety of physiological effects (Herschman, 1996). In studies with rats, both copper marginal and deficient diets altered prostanoid profiles in favor of atherogenesis: an increase in TXA_2 (a platelet aggregator and vasoconstrictor) in platelets (Morin et al., 1993) and a decrease in PGI_2 (an anti-platelet aggregator and vasodilator) in aortic ring incubations (Nelson et al., 1992). Furthermore, these changes in prostanoids were associated with Cu, Zn-SOD

depression and an increase in oxidative stress as indicated by elevated lipid hydroperoxides (Nelson et al., 1992; Morin et al., 1993). Macrophages produce PGE_2 as a primary prostanoid and play major roles in the pathogenesis of atherogenesis (Davies and MacIntyre, 1992; Ross, 1999) and tumorigenesis (Rees and Parry, 1992). Currently, however, it is not known whether copper insufficiency affects PGE_2 synthesis in macrophages.

Macrophages also produce nitric oxide (NO) for the bactericidal activity. It is suggested that excessive NO production may cause oxidative damage to neighboring cells and is implicated in the pathogenesis of diseases such as atherosclerosis (Leeuwenburgh et al., 1997) and cancer (Jadeski and Lala, 1999). High amounts of NO are produced by inducible form of nitric oxide synthase (iNOS). Currently, however, it is not known whether copper insufficiency influences NO and iNOS expression in macrophages.

Cyclooxygenase (COX), also known as prostaglandin H (PGH) synthase, catalyzes a key step in the biosynthesis of prostanoids (Rollins and Smith, 1980). To date, two isoforms of COX, COX-1 and COX-2, that have been identified. COX-1 is constitutively expressed in most cell types and is thought to serve physiological housekeeping functions. In contrast, COX-2 is induced in response to a wide variety of stimuli and is thought to contribute to the generation of prostanoids in certain stages of cell proliferation and differentiation, and at sites of inflammation (William and Dubois, 1996).

NO is derived from L-arginine by NOS, which is also found in both constitutive and inducible isoforms (Palmer et al., 1987; Ignarro et al., 1987). The latter isoform, iNOS, can be induced by the inflammatory stimuli and catalyzes the synthesis of large

amounts of NO to destroy invading bacterial, fungal, viral, or parasitic pathogens (Liew et al., 1990). Besides its beneficial roles, NO in excess can damage cells, tissues and DNA when it is inappropriately generated or overproduced by the inflammatory stimuli (Dawson, 1994; Billiar, 1995; Thomas et al., 1998).

Recent studies indicate the regulatory roles of ROS in the synthesis of PGE_2 and NO. For instance, lipid hydroperoxides, generated as a consequence of oxidative stress, are essential activators of both COX-1 and COX-2, suggesting a role of Cu, Zn-SOD in COX enzyme activation. Furthermore, ROS may act as signaling molecules through the activation of nuclear factor kappa B (NFkB). It has been shown that COX-2 and iNOS contain response elements for NFkB (Appleby et al., 1994), and thus activation of NFkB stimulates COX-2 and iNOS expression. Considering the copper regulation of oxidative stress via Cu, Zn-SOD activity, it is probable that increased oxidative stress via copper insufficient diets may enhance COX-2 and iNOS expression, then leading to an increase in PGE_2 and NO production, respectively, in macrophages. I, therefore, investigated the effects of copper insufficient (marginal and deficient) diets on cellular oxidation in macrophages, as well as on the synthesis of PGE_2 and NO in macrophages. I also tested the expression of COX-1, COX-2 and iNOS in macrophages from those rats fed copper insufficient diets.

Chapter 4

Materials and Methods

4.1. Materials.

Diet components were obtained from Dyets Inc. (Bethlehem, PA) and United States Biochemical Co. (Cleveland, OH). Thioglycollate was obtained from Fisher Scientific (Pittsburgh, PA). Ca^{2+}, Mg^{2+}, and phenol red-free Hanks' balanced salt solution (HBSS) and salmon sperm DNA solution were obtained from Life Technologies (Grand Island, NY). Heat inactivated fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), lipopolysaccharide (LPS; *Escherichia coli* serotype 011:B4), bovine serum albumin (BSA), arachidonic acid, aspirin, sodium nitrite, pyrogallol, thiobarbituric acid, 1,1,3,3-tetramethoxypropane, butylated hydroxytoluene, 2-butanol, nitroblue tetrazolium, dioxane, HOECHST33258, calf thymus DNA, formamide, L-glutamine, penicillin-streptmycin, and MOPS were obtained from Sigma Chemical Co. (St. Louis, MO). Mouse recombinant interferon-gamma (IFN-γ) was obtained from Genzyme Diagnostics (Cambridge, MA). The rat COX-1 and COX-2 (pBluescript) cDNA for Northern analysis were kindly provided from Dr. Daniel Hwang (Louisiana State University, Baton Rouge, LA). Plasmids containing human iNOS (Adams et al., 1995) cDNA and 18s rRNA (Oberbaumer, 1986 and 1992) cDNA were obtained from American Type Culture Collection. [5, 6, 8, 11, 12, 14, 15-H^3(N)]-Prostaglandin E_2 and [α-^{32}P]dCTP were obtained from DuPont NEN (Boston, MA).

4.2. Methods

A. Animals and diets. Weanling male Sprague-Dawley rats, weighing 50~60 g, were obtained from Charles River Breeding Laboratories (Wilmington, MA). Animals were housed individually in stainless steel cages at 20°C, 45% relative humidity, with a 12-h light/dark cycle. Animals were randomly assigned to one of three diet groups of 10 rats each: copper-deficient (CuD); copper-marginal (CuM) and copper-adequate (CuA).

The diet was a modification of the American Institute of Nutrition (AIN) recommendations (1977 and 1980) and contained 66.3% sucrose, 20% vitamin-free casein, 3.5% Cu- and Se-free AIN 76 mineral mix, 1% vitamin E-free AIN76A vitamin mix, 3% cellulose, 0.2% choline bitartrate, 5% vitamin E-stripped corn oil, and 1% powdered dextrose with supplemental copper. To prepare CuA and CuM diets, finely ground $CuSO_4 \cdot 5H_2O$ was dispersed in powdered dextrose prior to the addition to the other dry components. The copper concentration of each diet was determined by atomic absorption spectrophotometry of dry-ashed (550 °C, 15h) diet samples and was: CuD diet, 0.43 μg Cu/g; CuM diet, 1.60 μg Cu/g; and CuA diet, 6.77 μg Cu/g. Vitamin E was added to all diets at a final concentration of 55 IU/kg (50 mg all rac-α-tocopherol/kg) by mixing the vitamin with the vitamin E-stripped corn oil prior to adding the oil to the mixing diet components. $NaSeO_3 \cdot 2H_2O$ dissolved in 10 ml of distilled water was added to the mixing dry diet components to provide 100 μg Se/kg in all diets (Nelson et al., 1992; Morin et al., 1993).

At entry rats ate ad libitum CuD, CuM or CuA diets for 2 weeks. For the last 3 weeks rats in both CuM and CuA diet groups were individually paired with rats in the CuD diet group and an 8 h meal was offered at the beginning of the dark cycle. The amount of food eaten by rats in CuD group was measured and on the following day rats

in CuM and CuA diet groups were fed an amount equal to their paired rats in CuD diet group. Based on this pair-meal feeding protocol, food intakes were equal. The pair-meal feeding was used to equalize both food intake and the pattern of food consumption between groups. Rats were provided with distilled deionized water (<10 ngCu/ml) ad libitum.

B. Preparation of macrophages. Rat-elicited peritoneal macrophages were obtained by intraperitoneal injection of 7 ml of 7% thioglycollate in saline 4 d before the collection of cells by lavage of the peritoneal cavity with ice-cold HBSS containing 2% FBS. After centrifugation (700 x g, 10 min, 4°), cells were suspended in DMEM containing 5% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. The incubation medium contained 0.03 μg Cu/ml. Cells (~2 x 10^6) were plated to 35 mm plastic tissue culture dishes and incubated at 37 °C in an atmosphere of 95%/5% CO_2 to facilitate selective attachment of macrophages for 90 min. For assay of intracellular O_2^- concentration, cells were assayed right after the 90 min-attachment period. After 90 min, nonadherent cells were removed by rinsing the monolayer three times with warm incomplete DMEM (containing no FBS). Microscopic examination of samples treated with Wright's stain showed that more than 90 % of the adherent cells were macrophages. Cells were activated within 1 to 2 h.

C. Cell activation. Adherent cells were incubated with 100 ng/ml of LPS or without LPS for 9 h. In some experiment cells were incubated with or without a combination of IFN-γ (10 units/ml) and LPS (10 ng/ml) for 9 h. After incubation, supernatants were collected for PGE_2 measurement and for the assay of nitrites. Attached cells were either processed for RNA extraction as described below or rinsed with warm HBSS three times followed by lysing with 0.1% triton X-100 in 1x TNE buffer, and

stored at –70°C until analyzed for protein and DNA concentration, as well as for lipid peroxidation. To evaluate COX activity, after removal of supernatant, selected plates of cells were incubated in the same medium but containing 1 mg/ml BSA and 30 μM of arachidonic acid at 37 °C for 10 min as described by Fu et al. (1990). After 10 min, 42 mM aspirin solution was added to the medium to stop COX enzyme activity and PGE_2 concentration was measured in the supernatant to determine COX activity. Cells were then rinsed with HBSS three times, lysed with 0.1% TX-100 in 1x TNE buffer, and stored at –70°C for protein analysis by Bradford (Bradford 1976) Coomassie Blue dye binding protein assay (Bio-Rad, Richmond, CA).

D. Prostaglandin E_2 measurement. PGE_2 concentration in the supernatant of cells was determined by Radio Immuno assay (RIA) using [5, 6, 8, 11, 12, 14, 15-H^3(N)]-PGE_2 and a standard curve obtained using PGE_2 standard (Steinberg et al., 1982; Morin et al., 1993).

E. Nitric oxide measurement. Nitrite concentration was determined in the supernatant of cells and used as an index of NO synthesis. Nitrite was quantified colorimetrically after its reaction with Griess reagent using sodium nitrite as standard (Green et al., 1982). For the measurement of nitrite concentration in cell medium, an equal volume of Griess reagent was added to cell medium (0.5 ml), and the absorbance of the mixture was measured at 580 nm using a Beckman DU640 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA).

F. Cu, Zn-superoxide dismutase activity. The Prohaska (1983) modification of the pyrogallol autoxidation assay was used. Cell lysates were treated with 0.4 volumes of 25:15 (v/v) ethanol:chloroform, vortexed, and centrifuged at 14,000 g for 5 min to obtain two layers. Solution from the upper layer was carefully taken and used for the assay.

Ethanol:chloroform treatment eliminates contribution from Mn-dependent SOD activity without affecting the activity of Cu, Zn-SOD. Rate of pyrogallol autoxidation was then measured at 320 nm using Beckman DU640 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). One unit of Cu, Zn-SOD activity is defined as 50 % inhibition of the uninhibited rate of pyrogallol oxidation. Enzyme activity was expressed per units of protein.

G. Lipid Peroxidation. Lipid peroxidation in the cells was determined by the thiobarbituric acid (TBA) test (Yagi, 1976). Cell lysates from each rat were clarified by adding 10 % TCA, followed by centrifugation at 14,000 x g. Standard malondialdehyde solution, 1.0 μmol/l in 0.01 mol/l HCl, was freshly prepared from 1,1,3,3-tetramethoxypropane. Aliquots of malondialdehyde standard and clarified cell lysate samples were mixed with 700 μl of thiobarbituric acid reagent (two parts 27.8 mmol/l thiobarbituric acid in 0.2 mol/l HCl and one part water) and 70 μl of 22.7 mmol/l butylated hydroxytoluene in 95 % ethanol. Samples were heated in a 90 °C water bath for 60 min and extracted with 1.4 ml of 2-butanol. Butanol extracts were assayed by fluorimeter (Farrand Optical, Valhalla, NY), using excitation at 510 nm and emission at 560 nm. Data were expressed as pmol malonaldehyde equivalents per μg DNA.

H. Intracellular superoxide concentration. The nitroblue tetrazolium (NBT) test (Muller et al., 1981) was employed to evaluate intracellular O_2^- concentration with some modifications. In the presence of O_2^-, NBT dye is reduced to form a blue formazan. NBT (1 mg/ml) in HBSS was placed on cell culture dishes right after the removal of nonadherent cells, then incubated for 25 min at 37 °C. After the incubation, NBT solution was removed, 0.1 N-HCl was added to stop the reaction and cells were harvested in 1 ml HBSS by scraping the dish with a sterile rubber policeman. The cell suspension

was then centrifuged at 1,000 x g for 30 min. After discarding supernatant, cell pellets were resuspended in dioxane and incubated in a 90 °C water bath for 20 min. The suspension was centrifuged at 14,000 x g for 10 min to eliminate cell debris. The absorbance of the supernatant was measured at 580 nm using a Beckman DU640 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). Quantities of formazan were calculated using an extinction coefficient of 0.025 $nmol^{-1}.ml.cm^{-1}$ (Muller et al., 1981) and expressed as nmol formazan equivalents per μg DNA.

I. DNA concentration. DNA concentration was measured using the DNA-binding fluorochrome HOECHST33258 (Labarca and Paigen, 1980). A standard curve was constructed using calf thymus DNA. HOECHST33258 (0.2 μg/ml), prepared in 1x TNE buffer, was added to 100 μl of cell lysate samples or DNA standard solution, and assayed at an excitation wavelength of 365 nm and an emission wavelength of 460 nm by fluorometer (Farrand Optical, Valhalla, NY).

J. RNA extraction and Northern blot analysis. Cells were incubated in 35-mm dishes with appropriate stimuli as described previously. After incubation, cells were washed once with HBSS and total RNA was extracted using RNAzol B (TEL-TEST, Inc., TX), according to the manufacturer's instructions, then solubilized in FORMAzol (Molecular research center, Inc., Cincinnati, OH) and stored at –20 °C until analysis. Total RNA (18 μg) was fractionated using formaldehyde/MOPS/agarose (1.1 %) (Brown, 1993). RNA was transferred to a nitrocellulose membrane (Bio Rad, Hercules, CA) by capillary reaction, then cross-linked by UV irradiation using UV Stratalinker 2400 (Strategene, La Jolla, CA). The membrane was further baked at 80 °C for 1 h.

The sizes of cDNA probes were 1195 bp (COX-1 cDNA), 1545 bp (COX-2 cDNA), 1500 bp (iNOS cDNA), and 750 bp 18s rRNA cDNA. Probes were labeled

using Random Primers DNA Labeling System (Life Technologies, Gaithersburg, MD), according to the manufacturer's instruction. After prehybridization, membranes were hybridized at 42 °C for 24 h in 50 % formamide, 5x Denhardt, 50 mM potassium phosphate buffer, 1 %-SDS, and 100 μg/ml denatured salmon sperm DNA containing ^{32}P-dCTP labeled cDNAs. Membranes were washed twice with 5x SSC/0.1 %-SDS and twice in 0.1x SSC/0.1%-SDS at 42 °C for 10 min. Membranes were exposed to MR-2 X-Omat AR films using intensifying screens at –70 °C. The membranes were then scanned using the AMBIS Radioanalytic Imaging System (AMBIS, Inc., San Diego, CA), and hybridization signals of COX-1, COX-2, and iNOS were normalized against 18S rRNA.

K. Copper Analysis. Diets and lyophilized liver samples from each rat were ashed at 550°C for 15 h and the ash dissolved in 50 mM nitric acid. Copper content in culture medium and the ashed diet and liver samples were analyzed by flame atomic absorption spectrophotometry (model 257; Instrumentation Laboratories, Wilmington, MA).

L. Statistical analysis of data. Data were analyzed by analysis of variance (ANOVA) using SAS (SAS Institute, Cary, NC). Differences between means were separated by Fisher's protected least significant difference test. All data analyzed for differences between means by this test had overall F-values with $P<0.05$, unless otherwise stated in the legend of figures or tables. Log_{10} transformation was carried out for some data for the statistical analysis, and so stated in the figure or table legends. Values are reported as mean ± SEM.

Chapter 5

Results

5.1. Copper status and cellular oxidation.

Liver is the primary organ of copper storage. For this reason, copper status could be closely associated with copper content in liver. CuM and CuD diets significantly decreased hepatic copper concentration by 20 % ($P = 0.0001$) and 77 % ($P = 0.0001$), respectively (Table 1).

Copper is an essential cofactor of Cu, Zn-SOD activity. Thus, it was expected that Cu, Zn-SOD activity would parallel the copper content in diets. In agreement with the study by Babu and Failla (1990), Cu, Zn-SOD activity in peritoneal macrophages showed a significant 52 % ($P = 0.0001$) decrease in rats fed CuD diet, with a marginally significant 20 % ($P = 0.06$) decrease in rats fed CuM diet. In order to determine whether the depression of Cu, Zn-SOD activity influenced levels of cellular oxidation (i.e., oxidative stress), $O_2^{\cdot}$ generated inside the cells and lipid peroxidation were measured in peritoneal macrophages (Table 2). Formazan generation, an indicator of $O_2^{\cdot}$, showed a significant 91 % ($P = 0.008$) increase in macrophages from CuD rats, with a nonsignificant 44 % increase ($P = 0.15$) in CuM rats. Furthermore, lipid peroxidation in macrophages, estimated by malondialdehyde level, was significantly increased 1.7 fold ($P = 0.02$) and 3.2 fold ($P = 0.008$) from CuM and CuD rats, respectively. These data strongly suggest that the depression of macrophage Cu, Zn-SOD activity due to CuM and CuD diets significantly increased oxidative stress in macrophages.

TABLE 1

Body weight and liver copper concentrations.

	CuA	CuM	CuD
Number of animals	10	10	10
Body weight (g)	213.3 ±4.7 [a]	207.5 ± 5.9[a]	206.4 ± 3.6[a]
Liver Cu (μg/g dry)	10.1 ± 0.4[a]	8.1 ± 0.3[b]	2.3 ± 0.2[c]

Values are mean ± SEM. Values in row not sharing common letter superscripts are significantly different, $P<0.05$. Copper-adequate (CuA), -marginal (CuM) and –deficient (CuD) diet groups.

TABLE 2

Cu, Zn-SOD activity, intracellular O_2^- concentrations, and lipid peroxidation in elicited peritoneal macrophages.

	CuA	CuM	CuD
Cu, Zn-SOD (U/mg protein)[1]	34.2 ± 2.2[a]	27.5 ± 3.1[b]	16.2 ± 2.1[c]
Formazan (nmol/μg DNA)[2]	1.8 ± 0.5[a]	2.7 ± 0.7[ab]	3.6 ± 0.4[b]
MDA equivalents (pmol/μg DNA)[3]	25.1 ± 6.3[a]	42.5 ± 15.8[b]	80.6 ± 39.2[c]

Values (mean ± SEM) in row not sharing common letter superscripts are significantly different. $P<0.05$.

Diet groups: copper-adequate, CuA; -marginal, CuM; and –deficient (CuD) groups.

[1]Cu, Zn-SOD = copper dependent superoxide dismutase. 1 unit (U) = 50% inhibition of uninhibited rate of pyrogallol autoxidation, statistics on $\log_{10}$ transformed data; untransformed data shown; n=10.

[2]Formazan = an indicator of intracellular O_2^- concentration, statistics on $\log_{10}$ transformed data; untransformed data are shown; n=5.

[3]MDA = malondialdehyde, statistics on $\log_{10}$ transformed data; untransformed data are shown; n=5.

5.2. PGE_2 production and COX activity

PGE_2 production in macrophages is shown in Fig 4. Unlike unstimulated cells, a significant effect of diet was observed in PGE_2 production in cells stimulated with LPS and a combination of IFN-γ/LPS. Compared to other groups, CuD elevated PGE_2 4-fold (P=0.001) and 2-fold (P=0.002) in LPS and IFN-γ/LPS groups, respectively. PGE_2 production in CuM macrophages was not significantly different from that in CuA macrophages in both LPS and IFN-γ/LPS stimulation.

COX activity in each diet group is shown in Fig 5. COX activity requires a presence of lipid peroxides. Accordingly, it was hypothesized that increased lipid peroxidation in CuM and CuD would be associated with an increase in COX activity. CuD increased COX activity 5-fold (P=0.001), compared to CuM and CuA, which did not differ in COX activity.

In LPS-stimulated cells, PGE_2 production correlated significantly with COX activity ($r^2 = 0.96$, $P = 0.0001$). In unstimulated cells, the correlation approached statistical significance ($r^2 = 0.24$, $P = 0.06$). Results suggest that PGE_2 production is closely correlated with COX activity in LPS stimulation. Considering evidence that LPS induces COX-2 gene expression without affecting COX-1 expression, it is probable that an increase in PGE_2 production in LPS stimulation is, at least in part, due to an increase in COX-2 enzyme activity.

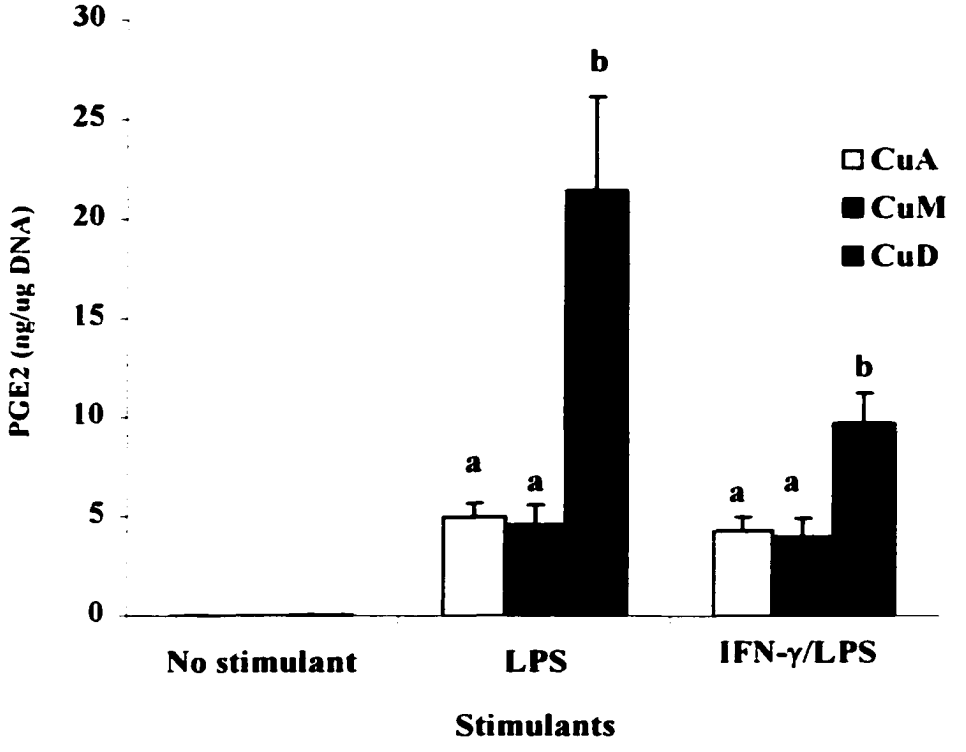

FIGURE 4. Prostaglandin E_2 (PGE_2) production in elicited peritoneal macrophages in rats fed copper-adequate (CuA), marginal (CuM), and deficient (CuD) diets. Cells from each rat were incubated in medium without stimulant, with lipopolysaccahride (LPS; 100 ng/ml), or a combination of interferon-gamma (IFN-γ; 10 U/ml) and LPS (10 ng/ml) for 9 h. Values are mean ± SEM. Each diet group had 10 rats. Bars not sharing common letters are significantly different among diet groups, $P<0.05$. Log_{10} transformed data were used for the statistical analysis. Untransformed data are used for the figure.

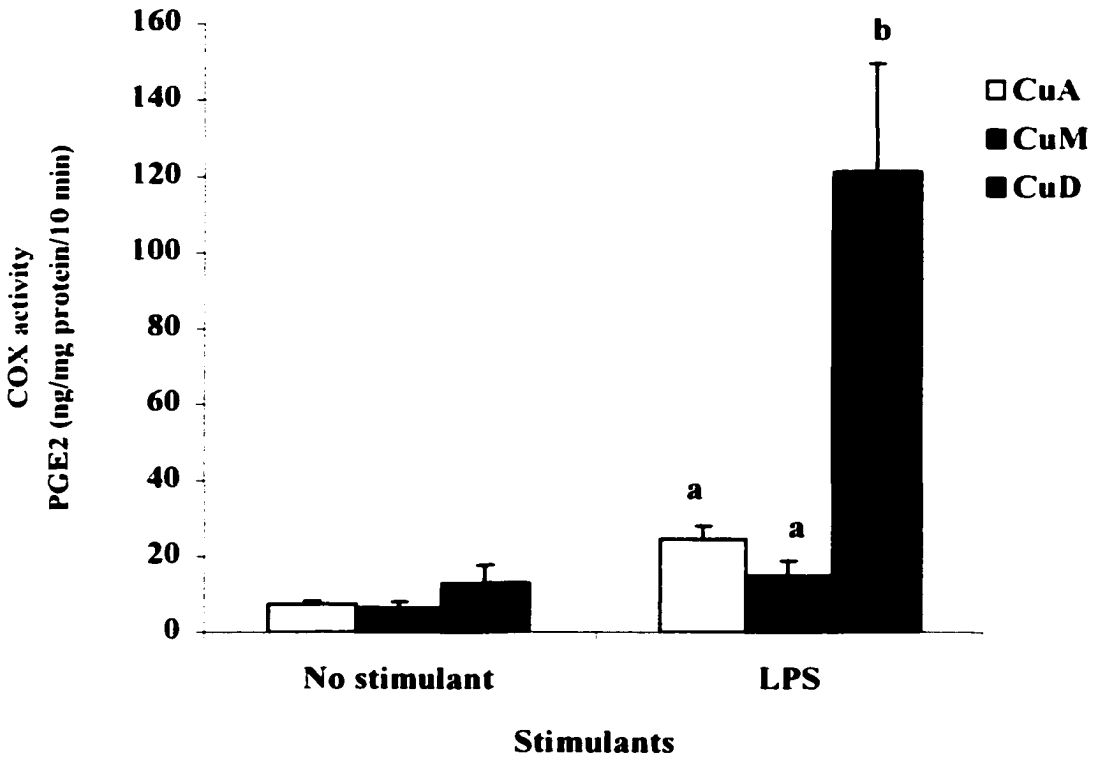

FIGURE 5. Cyclooxygenase (COX) activity in peritoneal macrophages in rats fed copper-adequate (CuA), marginal (CuM), and deficient (CuD) diets. Cells from each rat were incubated in medium with or without lipopolysaccahride (LPS; 100 ng/ml) for 9 h. After removal of supernatant, selected plates of cells were incubated in the medium containing 1 mg/ml BSA and 30 μM of arachidonic acid (AA) at 37 °C for 10 min, followed by the addition of 42 mM aspirin solution to inhibit further conversion of AA to PGE_2. PGE_2 produced in 10 min was determined in medium. Values are mean ± SEM. Each diet group had 5 rats. Bars not sharing common letters are significantly different among diet groups, $P<0.05$. Log_{10} transformed data were used for the statistical analysis. Untransformed data are used for the figure.

5.3. NO production and iNOS expression

Nitrite content, an indicator of NO production, was measured in macrophage medium from rats fed CuA, CuM and CuD diets (Fig 6). For unstimulated macrophages, there was no copper effect on nitrite production. In contrast, when cells were stimulated with LPS or IFN-γ/LPS, there was a trend toward increased nitrite production as copper content of the diet declined. For macrophages stimulated with IFN-γ/LPS, nitrite was 40 % increased in the CuD group, compared to the CuA group ($p = 0.022$), although the overall F-value was only marginally significant ($p = 0.06$). There was no significant copper effect on iNOS mRNA abundance (Fig 7).

5.4. COX-1 and COX-2 mRNA abundance

To determine whether increased PGE_2 production in cells from CuD rats was associated with increased COX-2 mRNA levels, Northern blot analyses were conducted (Fig 7). Studies have been shown that LPS induces COX-2 expression without influencing COX-1 expression. There were no significant diet effects on COX-1 mRNA abundance. In contrast, COX-2 mRNA expression was significantly increased 4-fold ($p = 0.04$) in CuD macrophages, compared to CuA and CuM. The increased COX-2 mRNA was in accord with increased PGE_2 in CuD.

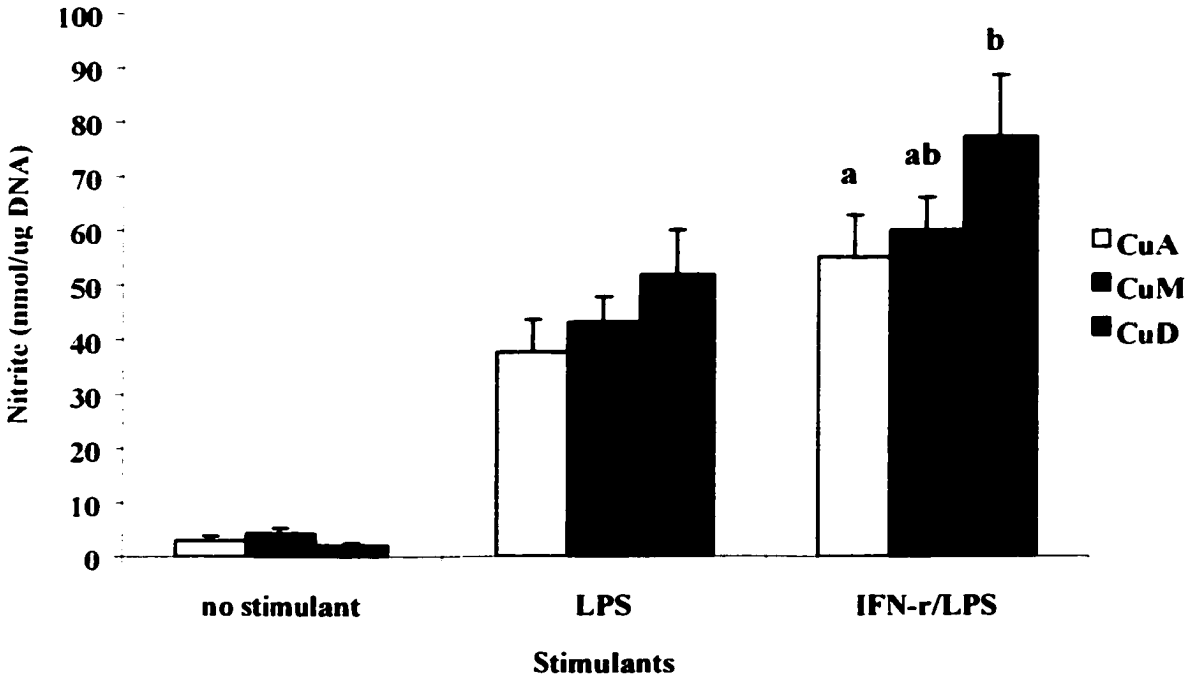

FIGURE 6. Nitrite released from elicited peritoneal macrophages in rats fed copper-adequate (CuA), marginal (CuM), and deficient (CuD) diets. Cells from each rat were incubated in medium with lipopolysaccahride (LPS; 100 ng/ml) or a combination of interferon-gamma (IFN-γ; 10 U/ml) and LPS (10 ng/ml) for 9 h. Values are mean ± SEM. Each diet group had 10 rats. For macrophages stimulated with IFN-γ/LPS, the overall F-value was only marginally significant ($P = 0.06$). Bars not sharing common letters are significantly different among diet groups, $P<0.05$. Log_{10} transformed data were used for the statistical analysis. Untransformed data are used for the figure.

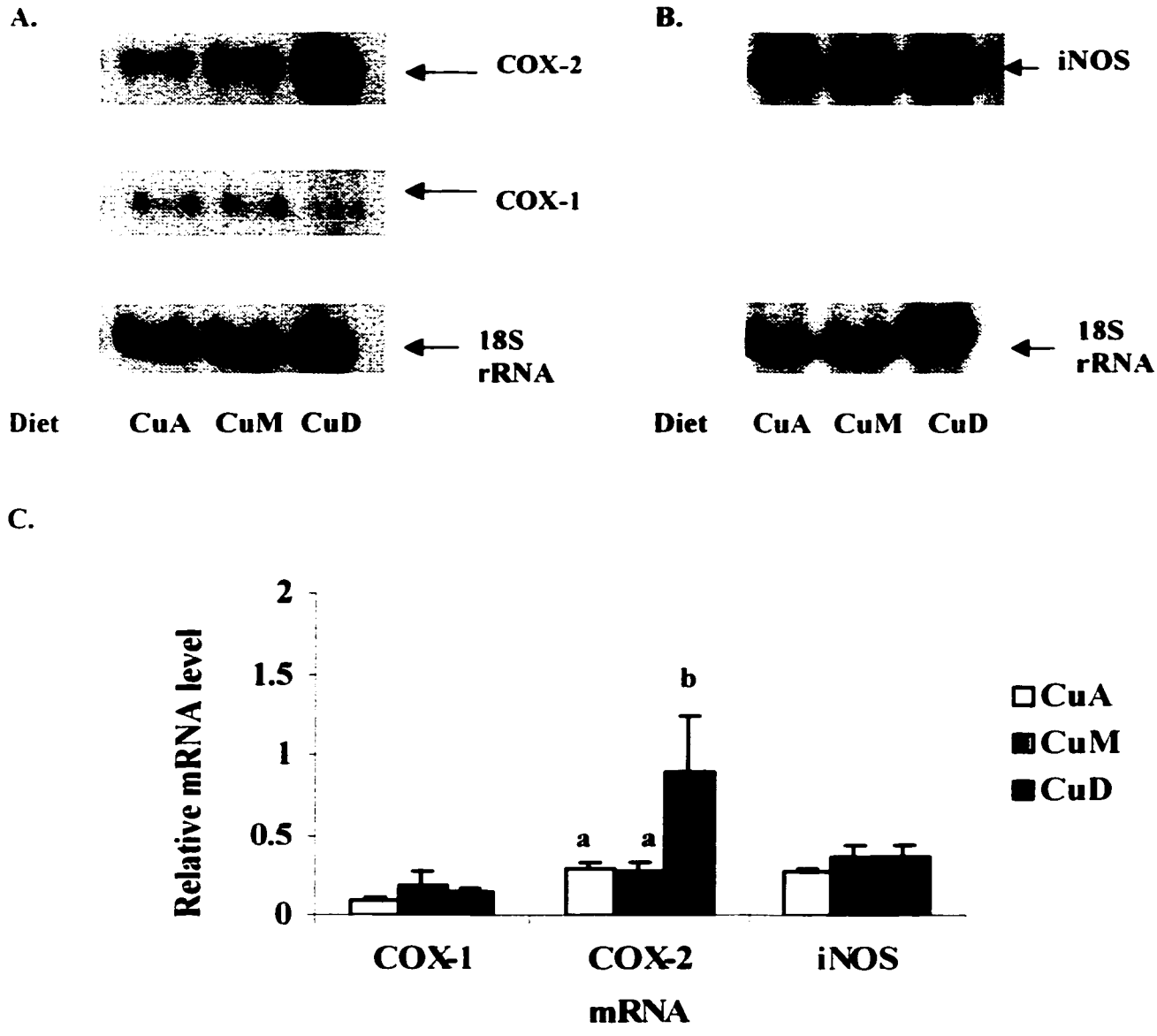

FIGURE 7. mRNA levels in elicited peritoneal macrophages in rats fed copper-adequate (CuD), -marginal (CuM), and –deficient (CuD) diets. (A) cyclooxygenase-1(COX-1), and COX-2, as well as 18S rRNA mRNA levels. Total mRNA was isolated from cells that were incubated with lipopolysaccharide (LPS; 100 ng/ml) for 9 h. (B) inducible nitric oxide synthase (iNOS) mRNA level. Total mRNA was isolated from cells incubated with a combination of interferon-gamma (IFN-γ; 10 U/ml) and LPS (10 ng/ml) for 9 h. mRNA levels were assayed by Northern blot analysis. Signals of COX-1, COX-2 and iNOS were normalized relative to those of 18S rRNA bands (C). Values in C are mean ± SEM of two to five rats from each diet group. Log_{10} transformed data were used for statistical analysis. Bars not sharing common letters are significantly different, $P<0.05$.

Chapter 6

Discussion

Copper is required for Cu, Zn-SOD catalytic activity, which removes superoxide radicals (O_2^-) produced from energy metabolism (McCord and Fridovich, 1969). The purpose of this study was to test the hypothesis that diets low in copper decrease Cu, Zn-SOD activity, which may increase oxidative stress, resulting in increased COX-2 and iNOS expression in macrophages. Both CuM and CuD diets significantly suppressed Cu, Zn-SOD activity in macrophages, which was associated with increased cellular oxidation in those cells. PGE_2 is the primary prostanoid produced by macrophages due to the predominant presence of PGE_2 synthase over other prostanoid synthase enzymes (DeWitt, 1991). CuD significantly increased synthesis of PGE_2, which was related to enhanced COX-2 mRNA and COX activity in activated macrophages. CuD increased NO production without affecting iNOS expression in activated macrophages.

Macrophages, one of the phagocytes, are widely distributed throughout the body and play an important role in immune system. Macrophage functions differ considerably depending on their developmental stages from the resting state, inflammatory state, to the activated state (reviewed in Celada and Nathan, 1994). I used inflammatory (elicited) and activated macrophage. Inflammatory macrophages can be recruited by inoculating rats with sterile irritant, thioglycollate, and are thus called elicited macrophages. Elicited macrophages, although not cytolytic themselves, readily become so when activated by bacterial endotoxin LPS or a combination of IFN-γ and LPS. These macrophages are,

then, called activated macrophages (Adams and Hamilton, 1984). Both elicited and activated macrophages provide excellent models to study effects of CuM and CuD on COX-2 and iNOS genes under the inflammatory condition. Both genes are expressed during the inflammation (DeWitt, 1991; Stueher et al., 1991).

This is the first report to show that diets low in copper cause oxidative stress in macrophages. In platelets and aorta in rats, both CuM and CuD diets also decrease Cu, Zn-SOD activity and increase cellular oxidation with an increase in lipid peroxidation (Nelson et al., 1992; Morin et al., 1993). An impaired cellular antioxidant defense is believed to cause oxidative stress with increased concentration of reactive oxygen species (ROS), including O_2^-, hydroxyl radical (OH •), and lipid hydroperoxides (LOOH), all of which are thought to cause damages to DNA, protein and membrane phospholipids (Parman et al., 1999; Oinuma et al., 1997; Morin et al., 1993; Nelson et al., 1992). It is believed that prolonged oxidative stress may accelerate the aging process and age-associated diseases, such as atherosclerosis, arthritis, and cancers. Increased cellular oxidation by low copper diets may lead to the development and progression of these diseases.

ROS are no longer regarded solely as damaging species. It appears important to characterize their contribution to the control of gene (i.e., COX-2 and iNOS) expression (von Knethen et al., 1999). A study by von Knethen et al. (1999) showed that ROS induce COX-2 expression through NFkB activation in macrophages. I showed that CuD significantly increased intracellular O_2^- concentration and enhanced COX-2 expression in activated macrophages, suggesting that O_2^- or other ROS formed as a result of the increased O_2^- by CuD may play a role in an induction of COX-2 gene in activated macrophages. COX-2 expression is induced only when macrophages are activated. LPS

activates macrophages and induces COX-2 expression through mitogen-activated protein kinase and NFkB activation in macrophages (Hwang et al., 1997). CuD affects NFkB nuclear localization (i.e., NFkB activation) (Failla and Hopkins, 1998). Taking all these observations together, I speculate that increased ROS caused by CuD may enhance NFkB activation and consequently increase COX-2 expression.

CuM, as opposed to CuD, did not enhance COX-2 expression in spite of a moderate increase in O_2^- concentration. It is suggested that moderate concentrations of ROS suppresses expression of genes, while high concentrations of ROS stimulate the induction of some genes (Morel and Barouki, 1999), indicating that ROS may control gene expression in a concentration-dependent manner. Based on this evidence, it is speculated that induction of COX-2 may require a high ROS concentration generated as a result of CuD diet, and CuM does not generate enough ROS to induce COX-2 in activated macrophages. Transcription is a central control step for COX-2 in activated macrophages (Dubois et al., 1998). ROS regulation of COX-2 transcription, therefore, may be the primary control mechanism of enhanced PGE_2 production in CuD macrophages.

It has been observed that dietary copper also influences synthesis of other prostanoids, including TXA_2 in rat platelets (Morin et al., 1993) and PGI_2 in aortic ring (Nelson et al., 1992). Both COX-1 and COX-2 require LOOH for the enzymes to be catalytically active (Kulmacz and Wang, 1995; Capdevila et al., 1995). It seems that both TXA_2 and PGI_2 are highly dependent on the cellular LOOH concentration. While LOOH activate COX enzymes, LOOH inactivate PGI_2 synthase at the same time (Weiss et al., 1979). Thus, increased LOOH via decreased copper intake inhibited PGI_2 production in rat aorta (Nelson et al., 1992), although COX enzymes may be activated by LOOH. In

contrast, TXA_2 synthesis is closely associated with an increase in LOOH concentration, which occurs as a result of decrease in copper content in diet. Platelets do not express COX-2, but express COX-1 (Patrignani et al., 1999). Thus, COX-1 is a regulatory step for TXA_2 synthesis in platelets. Studies show that COX-2 reacts with LOOH 10 times faster and has higher affinity for LOOH compared to COX-1, suggesting that LOOH may be more critical regulator for COX-1 than COX-2 (Kulmacz, 1998; Lu et al., 1999).

I also showed a trend toward increased NO production as copper content in diet decreases in activated macrophages. However, no copper effect was observed on iNOS mRNA abundance in those cells. There may be several factors associated with this outcome. First, the magnitude of difference in iNOS mRNA in CuM and CuD may be too small to be detected by Northern blot analysis. Second, the transcription of iNOS may not be as susceptible to oxidative stress as that of COX-2, although both COX-2 and iNOS are co-induced by the NFkB-mediated mechanism. Third, CuD may influence posttranscriptional and posttranslational modifications of iNOS, since these modifications are considered as important regulatory steps for iNOS activity (Michel and Feron, 1997). For instance, tetrahydrobiopterin (BH4), produced from GTP, is an important regulator for iNOS. BH4 stabilizes iNOS mRNA (Linscheid et al., 1998) and is required for catalytic activity of iNOS enzyme (Tzeng et al., 1995). It is not known whether copper influences BH4 metabolism. It is, however, suggested that copper depletion may decrease activity of soluble guanylate cyclase (GC-S), an enzyme that converts GTP to cGMP (Gerzer et al. 1981). I, thereby, speculated that diets low in copper may decrease GC-S activity, which may reduce the conversion of GTP to cGMP, causing more GTP to become available for BH4 formation, resulting in an increase in iNOS activity. Furthermore, phosphorylation and dephosphorylation of iNOS regulate iNOS catalytic

activity (Pan et al., 1996; Paul et al., 1995). Considering evidence of ROS activation of protein tyrosine kinase activity (Lowe et al., 1998), diets low in copper may activate the kinase activity by increasing ROS, resulting in an increase in iNOS activity by the phosphorylation. Further study will be needed to test whether oxidative stress imposed by CuM and CuD may play a role in the posttranscriptional and posttranslational modifications of iNOS, which may be a mechanism of increased NO in CuM and CuD.

NO produced by iNOS has bactericidal activity. NO reacts with O_2^- released from cells and forms peroxynitrite, which is a highly cytotoxic compound and thus exerts bactericidal activity. It is known that O_2^- release (i.e., extracellular concentration), which is also related to the bactericidal and candidacidal activity, is impaired by CuD (Babu and Failla, 1990). I showed increased intracellular concentration of O_2^- in CuM and CuD. Intracellular O_2^- reported in our study comes largely from energy metabolism in the microsomal compartment and perhaps mitochondria. In contrast, O_2^- concentration reported in the study by Babu and Failla (1990), is the extracellular O_2^- produced by the membrane-bound NADPH oxidase and released outside the cells (Jones, 1993). Thus, the origins of O_2^- between these two studies are different, and relevant comparisons are difficult to make.

Increased NO release in CuM and CuD may not contribute to the bactericidal activity of macrophages, since O_2^- release from macrophages is decreased in CuD, which is associated with reduced candidacidal activity (Babu and Failla, 1990). A study by Lynch et al. (1997) showed that a CuD diet causes depression of vascular Cu, Zn-SOD activity and increases vascular O_2^-. Increased NO as a result of CuM and CuD may rather react with O_2^- produced from the vascular system, and may localize oxidative damages to arterial cells and lipoproteins in the form of peroxynitrite. Several lines of

evidence suggest that peroxynitrite oxidizes LDL (Leeuwenburgh et al., 1997), enhances platelet adhesion and aggregation (Salvemini et al., 1989), and inhibits PGI2 synthase (Zou et al., 1998), all of which are associated with atherogenesis (Ross, 1993).

CuD impairs immune function (Prohaska and Lukasewycz, 1989; Failla and Hopkins, 1998). The study by Babu and Failla (1990) showed that CuD reduces respiratory burst and consequently impairs candidacidal activity in rat macrophages. It is suggested that oxidative stress may cause macrophage dysfunction (Raley and Loegering, 1999). The impaired immune function by CuD may be, at least in part, attributed to enhanced oxidative stress. Furthermore, high amounts of PGE_2 are known to suppress immune system (Okuno et al., 1995). Based on these observations, CuD may impair immune functions by enhancing oxidative stress and PGE_2 production in activated macrophages.

CuM frequently seen in the U.S. population may contribute, at least in part, to the prevalence of human diseases such as heart disease (Klevay, 1998). Copper plays an important role in the protection against atherosclerosis (Lamb et al., 1999). CuD may exacerbate inflammation and consequently may cause heart disease and cancer by increasing COX-2 expression, NO and PGE_2 production in activated macrophages. Increased COX-2 and iNOS expressions have been reported in a variety of tumors (Murata et al., 1999; Dubois et al., 1998; Jadeski and Lala, 1999) and atherosclerotic lesions (Baker et al., 1999; Landino et al., 1996; Darley-Usmar et al., 1995). The oxidative stress in our study, although it was generated a result of a nutrient deficiency, may be implicated in conditions, such as aging and diseases, where enhanced oxidative stress is believed to play an important regulatory role.

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Part II

Suppression of cyclooxygenase-2 and inducible nitric oxide synthase expression by conjugated linoleic acid in macrophages.

Chapter 1

Abstract

In response to inflammatory signals such as endotoxin, macrophages become activated to express the inducible isoforms of cyclooxygenase (COX-2) and nitric oxide synthase (iNOS), and produce excessive amount of prostaglandin E_2 (PGE_2) and nitric oxide (NO), respectively, which play key roles in cancer pathogenesis. Conjugated linoleic acid (CLA) is a potent anti-carcinogen, while arachidonic acid (AA) may be a pro-carcinogen as a substrate for PGE_2. **The objective** of this study was to investigate effects of CLA and AA on PGE_2 and NO synthesis in endotoxin-activated macrophages. RAW264.7 macrophages were incubated in medium containing no lipid (control), 30 μM AA (AA medium), or 30 μM CLA (CLA medium) for 24 h, then activated with bacterial endotoxin lipopolysaccharide (LPS; 100 ng/ml) for 9 h. CLA significantly depressed PGE_2 and NO by 78% (P=0.003) and 57% (P=0.0001) compared to control values, respectively. Northern blot analysis of COX-2 and iNOS showed significant treatment effects parallel to those seen for PGE_2 and NO. In contrast, AA significantly increased PGE_2 synthesis by 62% compared to control values (P=0.02). AA also suppressed NO production and iNOS expression in the same manner as observed for CLA. These results suggest that the anti-carcinogenic effect of CLA in endotoxin-activated macrophages may be related to its ability to decrease both PGE_2 and NO synthesis by suppressing transcription of COX-2 and iNOS. (Supported by Colo. Ag. Expt. Stn.& USDA NRI Comp.Grant)

Chapter 2

Literature Review

2.1. Conjugated linoleic acid

Conjugated linoleic acid (CLA) is a collective term used to describe positional and geometric isomers of linoleic acid (LA; 18:2, n-6) with conjugated double bonds at positions 9 and 11 or 10 and 12, and each double bond may be in the *cis* or *trans* configuration. The combination of *cis-trans* double bonds in the molecule accounts for the geometric isomers. CLA is a naturally occurring substance and found mainly in dairy products and meats (Chin et al., 1992). CLA was first identified and purified from grilled beef as an anti-mutagenic agent by Pariza (1983). Since then, the biological properties of dietary CLA has been receiving a considerable attention because of its potential anti-cancer, anti-atherogenic, and immunomodulating properties (Parodi, 1999).

2.2. Biological effects of CLA

In the following section, current literature about CLA actions in cancer, immune system, and atherosclerosis is reviewed. In addition, possible mechanisms of those CLA action are discussed.

A. Anti-carcinogenic properties. Growing evidence suggests that CLA inhibits tumor growth in a variety of tissues, including the mammary gland (Banni et al., 1999; Ip et al., 1999a; Ip et al., 1999b; Thompson et al., 1997), skin (Belury et al., 1996; Liu and Belury, 1997 and 1998), forestomach (Schutt, 1997), and colon (Liew et al., 1995; Xu

and Dashwood, 1999). Considerable effort has been made to elucidate the mechanisms of these anti-carcinogenic effects of CLA, although the mechanisms for CLA action are still largely unknown. Various studies have suggested that the ability of CLA to inhibit PGE_2 synthesis may, at least in part, play a role in the anti-cancer action of CLA (Parodi, 1999; Banni et al., 1999).

Studies with rat mammary tumor models have shown CLA as a potent anti-carcinogen (Parodi, 1999). Mammary tumorigenesis that is chemically induced occurs primarily in the rapidly proliferating epithelium at the distal end of terminal end buds (TEB). In the primary culture of rat mammary epithelial cell organoids (MEO), CLA inhibited outgrowth of MEO, which was mediated both by a reduction in DNA synthesis and a stimulation of apoptosis (Ip et al., 1999a). The study by Banni et al. (1999) showed that diet containing 0.5 and 1 % CLA had a graded and parallel reduction in TEB density. The reduction of TEB density in CLA-fed group was associated with decreased LA metabolites, including AA. In addition, the CLA dose-response effect on AA suppression corresponded closely with the CLA dose-response effect on cancer protection in the mammary gland. Therefore, it would be reasonable to expect that the biosynthesis of AA-metabolites (i.e., PGE_2) will be affected by the reduced availability of AA.

While CLA in food is predominantly (80-90%) the c9, t11-isomers in triacylglycerols, a vast majority of CLA diet studies have been done with a commercial free fatty acid preparation containing a mixture of c9,t11-, t10,c12- and c11,t13-isomers. Therefore, Ip et al. (1999b) conducted a study to test whether a high CLA butter fat has biological activities similar to those of the mixture of free fatty acid CLA isomers. Feeding butter fat CLA to rats significantly reduced mammary epithelial mass by 22%,

decreased the size of the terminal end bud population by 30%, suppressed the proliferation of terminal end bud cells by 30%, and inhibited mammary tumor yield by 53%. The magnitude of these changes in butter fat CLA were almost identical to the changes observed in the commercial mixture of CLA isomers at the level of CLA (0.8%) present in the diet. Results suggest that CLA in foods (i.e., triacylglycerol form) are also effective anti-carcinogens.

Skin (epidermal) tumors can be induced chemically by 7,12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA). Using this model, Ha et al. (1987) showed that mouse skin tumor was suppressed by topical application of CLA. Similar tumor reduction was observed with this model when CLA was provided as a 1.0% or 1.5% dietary supplement (Belury et al., 1996). Furthermore, Kavanaugh et al. (1999) showed that dietary CLA (1.5%) significantly reduced PGE_2 synthesis (two-fold) in this model. This reduction in PGE_2 was accompanied by a moderate decrease in an accumulation of c-myc mRNA (a gene commonly associated with regulating cell cycle components involved in cellular proliferation) and ornithine decarboxylase activity (a hall mark event of tumor promotion). It is known that TPA causes morphological and biological changes in skins with an increase in PGE_2 synthesis and ornithine decarboxylase activity (Verma et al., 1980). These studies clearly indicate that CLA inhibits PGE_2 and ornithine decarboxylase activity in TPA-induced skin tumor. Similarly, CLA significantly reduced ornithine decarboxylase activity in the TPA-treated murine keratinocytes (HEL-30 cells), compared to LA- and AA-supplemented groups. Furthermore, PGE_2 synthesis in CLA-supplemented cells decreased approximately 3 and 6 times, compared to LA- and AA-supplemented cells, respectively (Liu and Belury, 1998). These observations possibly

suggest that CLA inhibits tumor cell proliferation by decreasing PGE_2 synthesis in those tumor cells.

In spite of the structural similarity, LA and CLA exert opposite effects. A number of studies show that LA enhances the development and growth of tumors. In contrast, an increasing number of studies has been reported that CLA inhibits tumors. Cesano et al. (1998) studied the effects of three diets on the local growth and metastatic properties of DU-145 human prostatic carcinoma cells that were subcutaneously inoculated in mice. Animals were fed a standard diet (control) or diets supplemented with 1% LA or 1% CLA for 2 wk prior to subcutaneous inoculation of DU-145 cells and throughout the study (total 14 wk). Mice fed LA showed increased local tumor growth, while CLA-fed mice had smaller local tumors than the control diet-fed group. While 80% and 100% of mice which were fed with control and LA-supplemented diets, respectively, had metastatic spread to the lungs, only 10% of mice fed with the CLA-diet had lung metastasis. Furthermore, measurements of serum levels of human ICAM-1 (an intracellular adhesion molecule and a good indicator of tumor burden) showed significantly higher levels of this tumor marker in LA-fed mice and lower levels in CLA-fed mice as compared to the control group.

Decreased PGE_2 production by CLA has been recognized not only in those tumor cells, but also in serum (Sugano et al., 1998) and liver homogenate (Turek et al., 1998) in Sprague-Dawley rats. It seems that CLA modulation of PGE_2 depends on the cell type or organ examined. In a study of rat fed diet supplemented with either 1% CLA or LA, CLA decreased serum PGE_2 concentrations significantly, while the reduction in spleen was not significant compared to LA-fed group (Sugano et al., 1998). Furthermore, a marginally significant reduction in PGE_2 production was observed in liver from rats fed a

CLA supplemented diet (Turek et al., 1998). Currently, the effects of CLA on macrophage PGE_2 production and cyclooxygenase expression have not been studied.

B. Immuno-enhancing properties. It has been suggested that CLA enhances the immune system. Mice fed 1% CLA in diet showed increased T-cell proliferation as evaluated by the amount of [^{3}H]thymidine incorporation into DNA and interleukin-2 (IL-2) production by splenocytes (Hayek et al. 1999). A CLA diet study by Chew et al. (1997) showed that dietary CLA (0.3 and 0.9% CLA) significantly increased IL-2 production by splenic lymphocytes at wk 3 compared to control mice. These results indicate that dietary CLA is a potent modulator of the immune system, especially of lymphocyte proliferation and cytokine production.

Dietary CLA enhances macrophage phagocytosis in rats. Rats were fed 0.5% CLA diet over 4 wk and were then injected with LPS (1 mg/kg body weight) intraperitoneally. Both control and CLA-fed rats lost weight over the 24 hr period after LPS injection. The loss of weight in rats fed CLA was, however, only half of the weight loss of the control rats, suggesting that CLA not only enhance immune function by increasing macrophage phagocytosis, but was also effectively prevented the catabolic effects of immune stimulation (Cook et al., 1993). In a cell culture study, CLA addition to cell culture medium increased bactericidal activity of macrophages (Chew et al., 1997). These studies indicate that CLA are potent enhancers of immune function, at least in T-cells and macrophages.

The enhanced immune function, however, may not be associated with the anti-cancer action of CLA. Cesano et al. (1998) clearly showed that dietary CLA could inhibit prostatic tumor growth even in animals with a lack of T and B lymphocytes. This

study, however, does not exclude the possibility of macrophage involvement in anti-cancer action of CLA.

C. Anti-atherosclerosis properties. CLA have been suggested by some to possess anti-atherosclerotic properties. Studies have shown that CLA alter lipoprotein profiles (Munday, et al., 1999; Lee et al., 1994) and inhibit platelet aggregation by decreasing thromboxane A2 production (Truitt et al., 1999). Currently, however, there was no conclusive evidence to support CLA protection against atherogenesis (Munday et al., 1999). In a study with mice (Munday, et al., 1999) and rabbits (Lee et al., 1994), it was shown that CLA modify the serum lipoprotein profile in favor of anti-atherogenesis; CLA significantly reduce the LDL cholesterol to HDL cholesterol ratio and total cholesterol to HDL cholesterol ratio, and increase serum HDL-cholesterol to total cholesterol ratio. In spite of the beneficial alteration in the lipoprotein profile, the addition of CLA to the atherogenic diet increased the development of aortic fatty streaks in those mice (Munday, et al., 1999). In contrast, rabbits that were fed CLA-diet developed less atherosclerosis in aorta than those fed a semi-synthetic diet with no CLA addition (Lee et al., 1994). It is not known if the CLA action is specific to the species studied. Further study will be needed to establish CLA effects on atherogenesis.

D. Mechanisms. Mechanism(s) of CLA action still remain to be elucidated. Various studies suggested that the anti-carcinogenic action of CLA is, in part, explained by the ability of CLA to decrease the formation of PGE_2 (Parodi, 1999; Banni et al., 1999). It is also suggested that CLA may act by antioxidant mechanisms, pro-oxidant toxicity, inhibition of nucleotide and protein synthesis, reduction of cell proliferative activity, and inhibition of both DNA-adduct formation and carcinogen activation (Parodi, 1999). These speculations are derived from various changes seen with CLA

supplementation. In this review, mechanisms of CLA inhibition on PGE_2 synthesis are discussed. Based on the most current papers, at least two mechanisms could be postulated. One could be that CLA compete with LA for the desaturase and elongase enzymes, which are required for the conversion of LA to AA (speculated mechanism 1) and is shown in Fig 8. Another could be that CLA may induce a signaling pathway and affect the COX-2 expression (speculated mechanism 2) and is shown in Fig 9.

Speculated mechanism 1: CLA may compete in elongation and desaturation steps with LA that are precursors of AA, and such competition in AA synthesis may alter PGE_2 biosynthesis. Thompson and coworkers (1997) showed that 1% CLA diet significantly elevated concentrations of conjugated diene polyunsaturated fatty acids, including CLA (C18:2) 65-fold, conjugated C18:3 PUFA 6-fold, and conjugated C20:3 PUFA 14-fold in mammary tissues as compared to the control group. The presence of these conjugated dienes suggest that CLA could be desaturated further and elongated while still maintaining the conjugated diene structure, resulting in a competitive decrease in the conversion of LA to AA in CLA group (Thompson et al., 1997). Similarly, in cultured murine keratinocytes, CLA reduced AA content, while LA treatment increased AA release from cells. In association with decreased AA content, there was a marked decrease in PGE_2 production in CLA treated cells, while LA, by increasing AA content, enhanced PGE_2 production significantly (Liu and Belury, 1998). Replacement of AA by CLA and reduction of PGE_2 production varies depending of the cells, tissues, or organs used for the study, suggesting that CLA replacement of AA in phospholipid membranes might not be the sole cause of significant changes in PGE_2 production.

Speculated mechanism 2: The speculation that CLA may induce signal transduction to modulate gene expression was derived from the recent studies by Moya-

Camarena et al. (1999a and 1999b), who showed that CLA is strong ligands of peroxisome proliferator activated receptors (PPAR), in particular the PPARα isoform. PPAR is a ligand-activated transcription factor and a member of the steroid hormone receptor family. It was shown by Staels et al. (1998) that activated PPARα inhibited prostaglandin synthesis by decreasing COX-2 mRNA accumulation. Furthermore, activated PPARα was capable of inhibiting NFkB activation (Chinetti et al., 1998), which is an essential process for COX-2 expression. Based on this evidence, it was speculated that CLA may bind to PPARα and inhibit the activation of NFkB signaling pathway, preventing COX-2 expression and resulting in a reduction in PGE_2 synthesis.

Currently, three isoforms of PPAR have been recognized and are α, β (or δ) or γ. The biological importance of PPAR was discovered in 1990, ending 25 years of its classification as an orphan receptor (Issemann and Green, 1990). Since then, a growing number of studies have demonstrated the biological importance of PPAR in cardiovascular disorders and cancers (reviewed by Vamecq and Latruffe, 1999). Similar to other steroid hormone receptors, PPAR requires a ligand binding for the activation of the receptor. Upon activation, PPAR form a heterodimer with RXRα (retinoid X receptor; 9cis-retinoic acid receptor), which is required for the interaction with a peroxisome proliferator responsive element (PPRE), and induce the expression of the PPAR responsive gene. Several fatty acids bind to all three PPAR isoforms, although there is a preference of PPARα for polyunsaturated fatty acids (reviewed by Vamecq and Latruffe, 1999). CLA is able to induce accumulation of PPAR-responsive enzymes at the mRNA and protein level in mice (Belury et al., 1997), suggesting a possible involvement of CLA in PPAR activation.

PPARα negatively regulates COX-2 gene transcription and prevents COX-2 induction. A study by Staels et al. (1998) showed that activation of PPARα inhibited IL-1 induced prostaglandin production in human aortic smooth-muscle cells (SMC). This inhibition of prostaglandin synthesis was associated with decreased COX-2 mRNA by Wy14643 (a synthetic PPARα activator). These researchers also tested whether PPARα activation negatively interfered with the transcriptional activation of COX-2, using Cos-1 cells transfected with the human COX-2 promoter in the presence or absence of PPARα expressing vector. COX-2 promoter activity was significantly increased upon PMA stimulation, which was, however, significantly decreased in response to PPARα activation by Wy14643. Thus, co-transfection of human PPARα prevented PMA-mediated induction of COX-2 promoter activity, suggesting PPARα inhibition of COX-2 transcription. The inhibitory control exerted by PPARα on the COX step of AA metabolism is probably an important mechanism by which PPARα ligand (activator) might inhibit inflammation.

How could the activated PPAR prevent the induction of COX-2 expression? Several lines of evidence suggested that activated PPAR antagonizes the activation of NFkB transcription factor (Ricote et al., 1998; Brand et al., 1996; Bourcier et al., 1997; Chinetti et al., 1998), resulting in the inhibition of COX-2 expression (Staels et al., 1998). Pro-inflammatory cytokines and endotoxin are known to induce COX-2 expression by NFkB mediated signaling pathway. These observations suggest that COX-2 inhibition by PPARα activation could be mediated by the inhibition of NFkB activation.

Besides COX-2, NFkB plays an essential role in the transcriptional regulation of the inducible nitric oxide synthase (iNOS) gene (Xie et al., 1994). Macrophages express

iNOS and produce NO in response to stimulation by bacterial LPS and pro-inflammatory cytokines (Xie et al., 1992). NO accumulation in the RAW264.7 murine macrophage-like cells was inhibited by the PPARα activator WY14643, although this effect may have been mediated through the activation of PPARγ, as other activators of this PPAR isoform were also effective (Colville-Nashi et al., 1998). Currently, the effects of CLA on NO production and iNOS expression have not been studied.

Macrophages express both PPARα and PPARγ. While PPARα is expressed in undifferentiated human monocytes, PPARγ only becomes expressed in human macrophages upon differentiation. PPARα is present constitutively in the cytoplasm, whereas PPARγ is predominately localized in nucleus in differentiated human macrophages (Chinetti et al., 1998). In murine macrophages, PPARγ inhibited the activities of transcription factors of the AP-1, STAT, and NFkB families (Ricote et al., 1998). Similarly, PPARα is capable of interfering negatively with the p65/RelA subunit of the NFkB signaling pathway only in the activated macrophages (Chinetti et al., 1998). PPARα activators exert their anti-inflammatory action, at least in part, by negatively regulating NFkB transcriptional activity. P65/RelA is activated in SMC, macrophages and endothelial cells in human atheroma (Brand et al., 1996; Bourcier et al., 1997). Macrophages produce increased amounts of PGE_2 by COX-2 in inflammation. Furthermore, increased COX-2 expression has been recognized in human atheroma (Schonbeck et al., 1999) and some tumors (Sheehan et al., 1999). Thus, PPAR activation by CLA has the potential to inhibit PGE_2 production and thus may be beneficial for human health.

It is not know how PPAR inhibits NFkB activation. It is probable that: 1) the PPAR-responsive gene product may protect IkB (an inhibitory subunit of NFkB) from degradation and, therefore, the activation of NFkB may be inhibited; 2) PPAR may directly interact with NFkB in the cytosol or the nucleus; 3) PPAR may enhance cellular antioxidant defense, which then prevents the activation of NFkB by reactive oxygen species (ROS) (Scheme 2). ROS have been recognized as important activators for NFkB. Studies showed that PPARα enhances cellular antioxidant systems by up-regulating the transcription of antioxidant enzymes such as Cu, Zn-SOD and catalase, thereby reducing the ROS-induced activation of NFkB (Inoue et al., 1997; Inoue et al., 1998). Thus, increased antioxidant protection by PPAR activation could be, in part, a mechanism of NFkB inhibition. It is interesting to note that CLA increase the activity of superoxide dismutase (SOD), catalase and glutathione peroxidase in cultured MCF-7 cells and SW480 cells (O'Shea et al., 1999), suggesting a possibility of a link between CLA, PPAR and NFkB.

A growing body of evidence indicates that PPAR activation may have beneficial effects in human health. A link between CLA and PPAR has not fully established yet. Whether CLA influence NF-kappa B activation is not known. Furthermore, effects of CLA on COX-2 expression and NO, as well as iNOS expression have not studied. Based on an increasing body of evidence, it is highly possible that CLA are potent activators of PPAR, which, at least in part, could be a mechanism of anti-cancer effects of CLA.

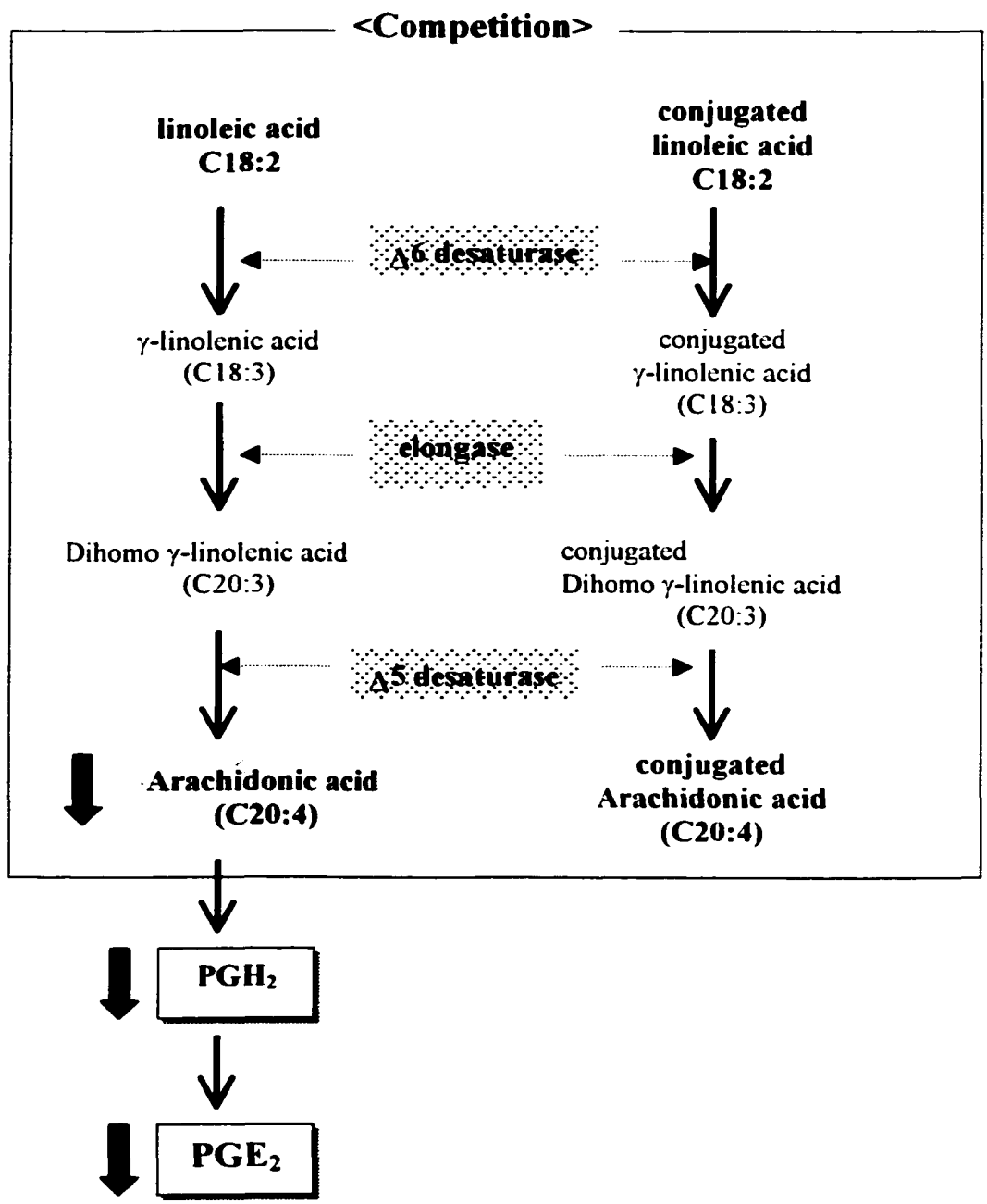

FIGURE 8. Competition for desaturase and elongase enzymes between linoleic acid (LA) and conjugated linoleic acid (LA) (speculated mechanism 1). It has been shown that CLA decreases arachidonic acid (AA) content in phospholipids. Studies have suggested that decreased AA may be due to the competition of desaturase and elongase enzymes between LA and CLA. Decreased AA may result in a reduction of prostaglandin E_2 (PGE_2) synthesis

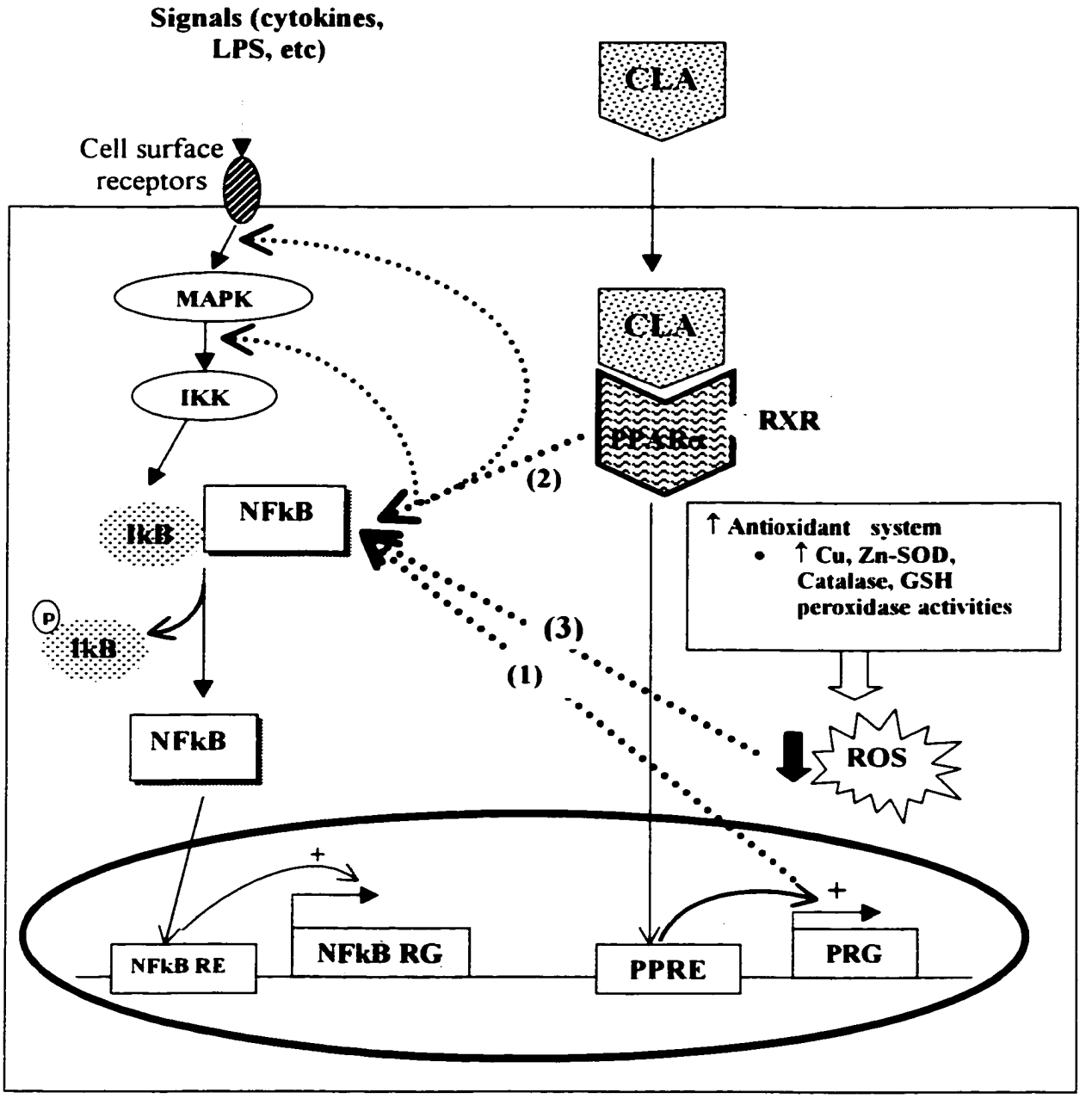

FIGURE 9. CLA induced signaling pathway which activates PPAR as ligands, inhibit NFkB activation, resulting in prevention of NFkB responsive genes such as COX-2 and iNOS (speculated mechanism 2). Abbreviations are: CLA, conjugated linoleic acid; PPARα, peroxisome proliferator activated receptor alpha; RXR, retinoid X receptor; Cu, Zn-SOD, Cu, Zn-superoxide dismutase; GSH peroxidase, glutathione peroxidase; ROS, reactive oxygen species; PPRE, PPAR response element; PRG, PPAR responsive genes; NFkB, nuclear factor kappa B; IkB, inhibitory kappa B; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; IKK, IkB kinase; NFkB RE, NFkB response element; NFkB RG, NFkB responsive genes. For the explanation of (1), (2) and (3), refer to the text.

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Chapter 3

Introduction

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid (LA; 18:2, n-6) with conjugated double bonds. CLA is a naturally occurring substance and found mainly in dairy products and meats (Chin et al., 1992). CLA was first identified and purified from grilled beef as an anti-mutagenic agent by Pariza (1983). Since then, the biological properties of dietary CLA have received considerable attention because of its anti-cancer, anti-atherogenic, and anti-diabetic effects (Parodi, 1999). CLA inhibits tumor growth in a variety of tissues, including the mammary gland (Banni et al., 1999; Ip et al., 1999a and 1999b; Thompson et al., 1997), skin (Belury et al., 1996; Liu and Belury, 1997), stomach (Schut et al., 1997), and colon (Liew et al., 1995; Xu and Dashwood, 1999).

It has been suggested that inhibition of prostaglandin E_2 (PGE_2) synthesis by CLA may account, at least in part, for its anti-cancer action (Parodi, 1999; Banni et al., 1999). A regulatory step in PGE_2 synthesis is conversion of arachidonic acid (AA) to PGH2 by cyclooxygenase (COX). Two COX isoforms have been identified, referred to as COX-1 and COX-2. COX-1 is present constitutively in most cell types and produces small quantities of PGE_2 that regulate cellular homeostasis. In contrast, COX-2 is expressed only in response to inflammatory signals such as cytokines and bacterial endotoxin lipopolysaccharide (LPS) (Williams and DuBois, 1996). COX-2 produces large amounts of PGE_2 that induce inflammation, which is implicated in pathogenesis of diseases such

as cancer. Recent studies suggest that high COX-2 expression rates are associated with cancer progression and inhibition of apoptosis (von Knethen et al., 1999). This is supported by the discovery that overexpression of COX-2 is a central event in colon carcinogenesis, and may explain why aspirin (which inhibits COX) is chemopreventive against cancer (Jones et al., 1999).

In spite of structural similarities, LA and CLA exert opposite effects in tumorigenesis. LA enhances the development and growth of tumors. In contrast, an increasing number of studies show that CLA inhibits tumor growth. Mice fed a high LA diet had increased tumor growth compared to controls, while those fed a high CLA diet showed significant reductions in tumor growth (Cesano et al., 1998). Banni et al. (1999) showed that diets containing CLA produced graded reductions in terminal end bud density (an indicator of breast cancer), associated with parallel decreases in LA metabolites, including arachidonic acid (AA).

It is well-established that CLA decreases PGE_2 production. In contrast, LA enhances PGE_2 production, because it is a precursor for AA and, therefore, the substrate for PGE_2. Macrophages play an important role in the pathogenesis of cancer and are present in all stages of tumorigenesis, producing high amounts of PGE_2 when macrophages are activated in response to pro-inflammatory stimuli, such as cytokines and LPS (Rees and Parry, 1992). Effects of CLA on COX-2 expression have not been documented in macrophages.

Nitric oxide (NO) is another factor implicated in tumorigenesis. NO is formed from L-arginine by nitric oxide synthase (NOS). Both constitutive and inducible isoforms of NOS have been identified, similar to COX. While constitutive NOS isoforms play regulatory roles in cellular homeostasis, inducible NOS (iNOS) produces high

amounts of NO in response to cytokines and LPS. Although iNOS is important in bactericidal activity of macrophages, increased NO is known to react with superoxide radicals and form peroxynitrite, which is believed to cause oxidative damage to cell membranes, protein and DNA. Furthermore, recent evidence shows that there is an increase in iNOS expression in cancers and atherosclerotic lesions, and that iNOS inhibition results in the reduction of angiogenesis (Ziche et al., 1997; Jadeski and Lala, 1999), suggesting that inhibition of iNOS may be beneficial for the treatment of cancers.

Currently, few studies have addressed effects of CLA on eicosanoid and NO biology in activated macrophages. I tested the **hypothesis** that CLA alters synthesis of PGE_2 and NO, as well as expression of COX-2 and iNOS, respectively, in activated macrophages.

Chapter 4

Materials and Methods

4.1. Materials

A. Cells and reagents. RAW264.7, a murine macrophage-like cell line, was purchased from American Type Culture Collection (ATCC; Rockville, MD). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO) (D10F medium) and maintained at 37 °C in a humidified incubator containing 5% CO_2. Bacterial lipopolysaccharide (LPS, *Escherichia coli* serotype 011:B4) was obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant mouse IFN-γ was purchased from Genzyme Diagnostics (Cambridge, MA). Ca^{2+}, Mg^{2+}, and phenol red-free Hanks' balanced salt solution (HBSS) and salmon sperm DNA solution were obtained from Life Technologies (Grand Island, NY). The rat COX-1 and COX-2 (pBluescript) cDNA for Northern blot analysis were kindly provided from Dr. Daniel Hwang (Louisiana State University, Baton Rouge, LA). Plasmids containing human iNOS (Adams et al., 1995) cDNA and 18s rRNA (Oberbaumer, 1986 and 1992) cDNA were obtained from American Type Culture Collection (ATCC). [5, 6, 8, 11, 12, 14, 15-H^3(N)]-Prostaglandin E_2 and [α-^{32}P]dCTP were obtained from Du Pont NEN (Boston, MA).

4.2. Methods

A. Lipid treatment and cell activation. Cells were recovered from freeze-back and grown in 75 cm^2 flasks until confluent. Cells were collected by rubber policeman, distributed to 35 mm plastic tissue culture dishes at ~1 x 10^6/dish and incubated at 37 °C in a humidified, 5% CO_2. Prior to the distribution, cell number was determined using a hemocytometer. After 90 min of incubation, nonadherent cells were removed by rinsing the monolayer three times with warm incomplete DMEM (containing no FBS). Adherent cells were further incubated in D10F medium (lipid medium) that had added 1 mg/ml BSA and supplemented with no added lipid, 30 μM-conjugated linoleic acid (CLA, Natural Lipid LTD, Norway), or 30 μM-arachidonic acid (AA, Sigma Chemical Co., St. Louis, MO) for 24 h (lipid treatment). There were two to three plates for each lipid treatment. After supernatant was discarded, cells were incubated in lipid medium with or without 100 ng/ml of LPS for 9 h. In some experiments cells were incubated with or without a combination of IFN-γ (10 units/ml) and LPS (10 ng/ml) for 9 h. After incubation, supernatants were collected for PGE_2 and NO measurements. Attached cells were either processed for RNA extraction, as described below, or rinsed with warm HBSS three times followed by lysing with 0.1% triton X-100 in 1x TNE buffer, and stored at –70°C until analysis for DNA concentration. Experiments were repeated three times in different time period.

B. RNA extraction and Northern blot analysis. Cells were treated in 35-mm dishes with lipid medium for 24 h, prior to the stimulation with LPS for 9 h as described above. After incubation, cells were washed once with 1 ml HBSS and total RNA was extracted using RNAzol B (TEL-TEST, Inc., TX), according to the manufacturer's instructions, then solubilized in FORMAzol (Molecular Research Center, Inc.,

Cincinnati, OH) and stored at –20 °C until analysis. Total RNA (18 μg) was fractionated using formaldehyde/MOPS/agarose (1.1 %) (Brown, 1993). RNA was transferred to a nitrocellulose membrane (Bio Rad, Hercules, CA) by capillary reaction, then cross-linked by UV irradiation using UV Stratalinker 2400 (Strategene, La Jolla, CA). The membrane was further baked at 80 °C for 1 h.

The sizes of cDNA probes were 1195 bp (COX-1 cDNA), 1545 bp (COX-2 cDNA), 1500 bp (iNOS cDNA), and 750 bp 18s rRNA cDNA. Probes were labeled using Random Primers DNA Labeling System (Life Technologies, Gaithersburg, MD), according to the manufacturer's instruction. After prehybridization, membranes were hybridized at 42 °C for 24 h in hybridization solution containing 50 % formamide, 5x Denhardt, 50 mM potassium phosphate buffer, 1 %-SDS, and 100 μg/ml denatured salmon sperm DNA containing ^{32}P-dCTP labeled cDNAs. Membranes were washed twice with 5x SSC/0.1 %-SDS and twice in 0.1x SSC/0.1%-SDS at 42 °C for 10 min. Membranes were exposed to MR-2 X-Omat AR films using intensifying screens at –70 °C. The membranes were then scanned using the AMBIS Radioanalytic Imaging System (AMBIS, Inc., San Diego, CA), and hybridization signals of COX-1, COX-2, and iNOS were normalized against 18S rRNA.

C. Prostaglandin E_2. PGE_2 concentration in the supernatant of cells was determined by Radio Immuno assay (RIA) using [5, 6, 8, 11, 12, 14, 15-H^3(N)]-PGE_2 and a standard curve obtained using PGE_2 standard (Steinberg et al., 1982; Morin et al., 1993).

D. Nitric oxide. Nitrite concentration was determined in the supernatant of cells and used as an index of NO synthesis. Nitrite was quantified colorimetrically after its reaction with Griess reagent using sodium nitrite as standard (Green et al., 1982). For the

measurement of nitrite concentration in cell medium, an equal volume of Griess reagent was added to cell medium (0.5 ml), and the absorbance of the mixture was measured at 580 nm using a Beckman DU640 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA).

E. DNA concentration. DNA concentration was measured using the DNA-binding fluorochrome HOECHST33258. Standard curve was constructed using calf thymus DNA. HOECHST33258 (0.2 μg/ml), prepared in 1x TNE buffer, was added to 100 μl of cell lysate samples or DNA standard solution, and assayed at an excitation wavelength of 365 nm and an emission wavelength of 460 nm by fluorometer (Farrand Optical, Valhalla, NY) (Labarca and Paigen, 1980).

F. Statistical analysis of data. Data were analyzed by analysis of variance using the SAS statistical program (SAS Institute, Cary, NC), followed by Fisher's protected least significant difference test. This least significant test was conduced only when overall F-value from ANOVA had p-values less than 0.05, unless otherwise stated in the legend of figures or tables. Values are reported as mean ± SEM.

Chapter 5

Results

5.1. PGE_2 production.

To determine whether CLA influences PGE_2 production in LPS-stimulated cells, RAW264.7 macrophage cells were incubated with lipid medium supplemented with no added lipid (control), 30 μM CLA, or 30 μM AA for 24 h, prior to the LPS stimulation for 9 h. PGE_2 concentration was determined in each medium and is shown in Fig. 10. Macrophages incubated with CLA medium had a significant 78 % ($P = 0.003$) decrease in PGE_2 synthesis, compared to the control medium. In contrast, macrophages in AA medium showed a significant 62 % ($P = 0.02$) increase in PGE_2 production, as expected, since AA serves as a substrate for PGE_2 synthesis. A significant 7.5 fold difference was observed in PGE_2 production between AA and CLA medium. Data suggest that CLA significantly suppress PGE_2 production in LPS-stimulated macrophages.

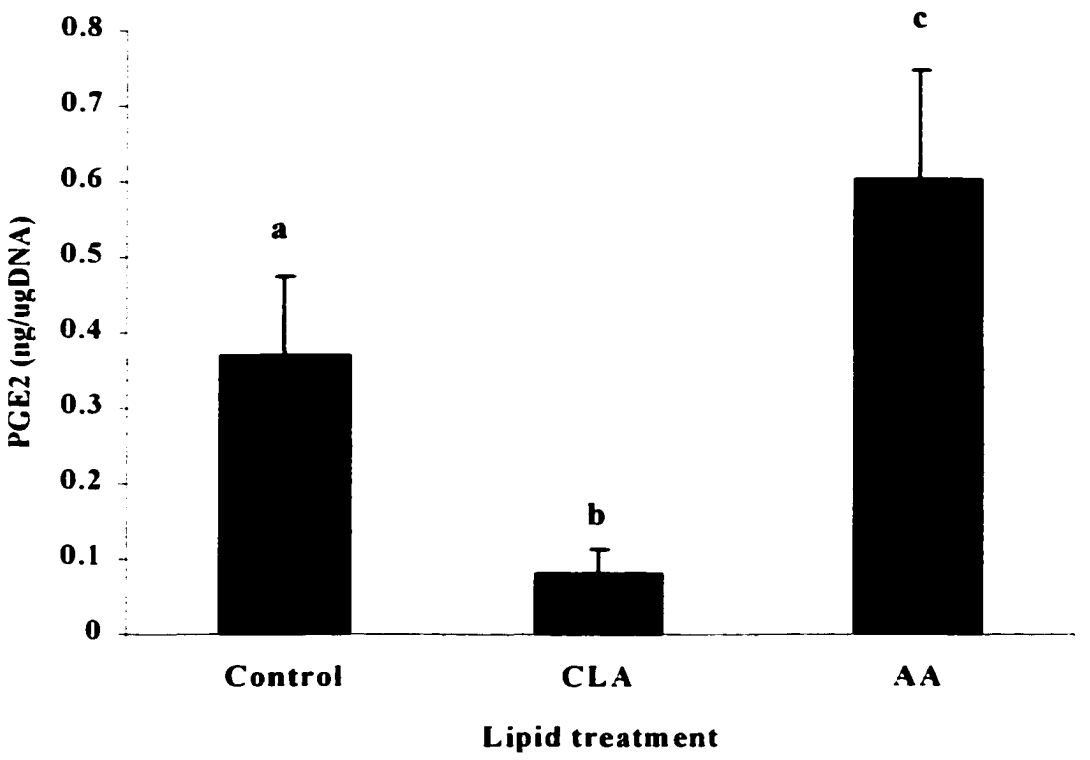


Figure 10. Prostaglandin E2 (PGE2) production in RAW264.7 macrophage-like cells incubated in medium supplemented with no added lipid (control), 30 μM conjugated linoleic acid (CLA), or 30 μM arachidonic acid (AA). Cells were incubated with control, CLA, or AA medium for 24 h, prior to the stimulation with lipopolysaccharide (100 ng/ml) for 9 h. Bars (mean ± SEM) not sharing common letters are significantly different, $P<0.05$. Values were mean of two to three plates from each of three independent experiments. Log-transformed data were used for the statistical analysis. Untransformed data are shown.

5.2. NO production

Effects of lipid treatment on NO production was determined, using nitrite concentration as an index of NO production (Fig 11). In cells with no LPS stimulation, lipid treatment did not affect NO production in cells (data not shown). In contrast, in cells stimulated with LPS, both CLA and AA significantly decreased NO production by 57 % ($P = 0.0001$) and 61 % ($P = 0.0001$), respectively. The magnitude of reduction in NO production was not significantly different between CLA and AA-cells, suggesting that NO suppression in LPS-stimulated cells is not limited to CLA, but could be a general feature of polyunsaturated fatty acids.

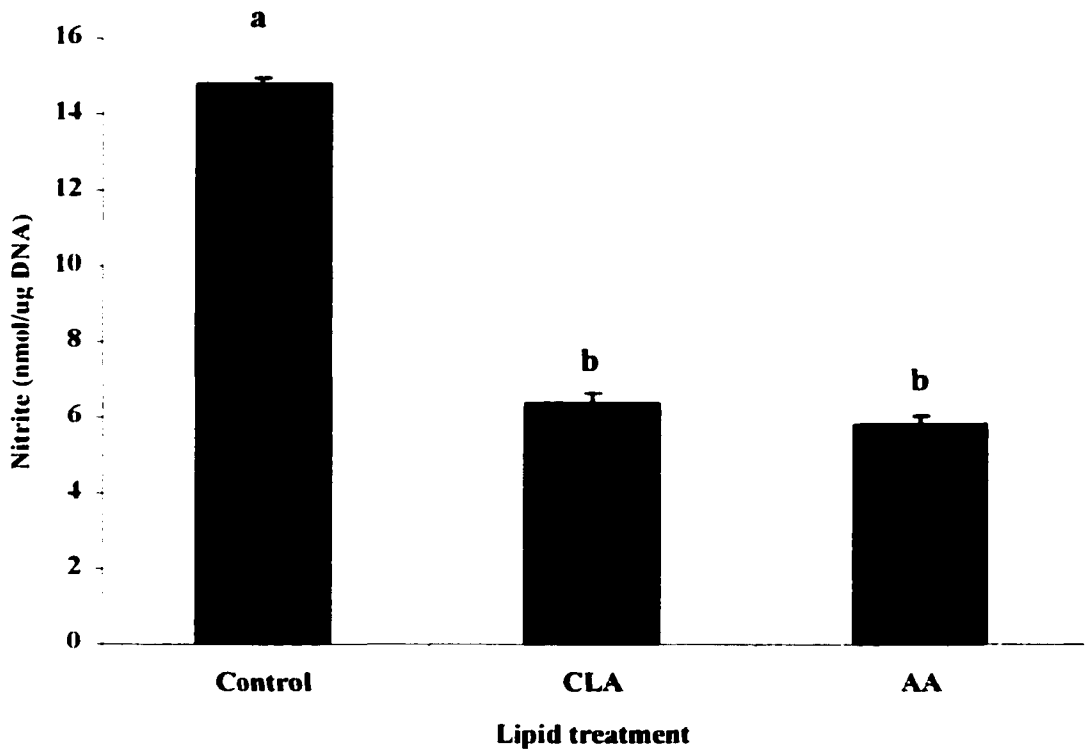


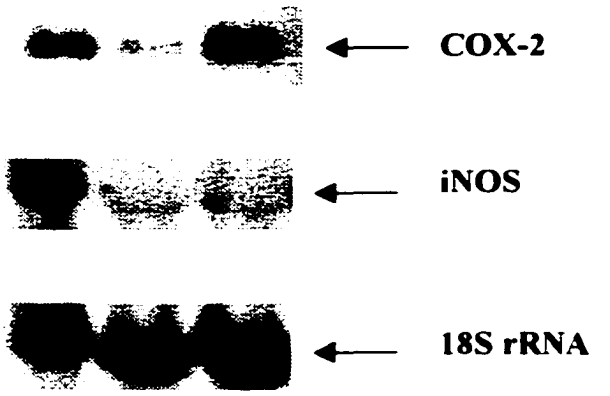
Figure 11. Nitrite production in RAW264.7 macrophage-like cells incubated in medium supplemented with no added lipid (control), 30 μM conjugated linoleic acid (CLA), or 30 μM arachidonic acid (AA). Cells were incubated with control, CLA, or AA medium for 24 h, prior to the stimulation with lipopolysaccharide (100 ng/ml) for 9 h. Bars (mean ±SEM) not sharing common letters are significantly different, $P<0.05$. Values were mean of two to three plates from each of three independent experiments.

5.3. COX-2 and iNOS mRNA levels

COX catalyzes the rate-limiting step of PGE_2 production using AA as a substrate. In order to determine whether a reduction of PGE_2 and NO syntheses in CLA-treated cells is associated with decreased mRNA abundance, Northern blot analyses were conducted (Fig 12). Unlike COX-1 that is expressed constitutively in all types of cells, COX-2 is only expressed in cytokine or endotoxin (i.e., LPS) activated cells. COX-1 mRNA was barely detectable (data not shown). A significant 33 % suppression in COX-2 mRNA levels was observed in CLA-supplemented cells compared to control cells ($P = 0.01$). There was no significant difference in COX-2 mRNA levels between control and AA-supplemented cells. Data suggest that CLA suppress PGE_2 synthesis at the level of COX-2 transcription in LPS-stimulated cells.

Like COX-2, iNOS expression is induced in some cell types such as macrophages only after exposed to cytokines and endotoxins (i.e., LSP). Significantly lower mRNA levels were observed in both CLA ($P = 0.04$) and AA-supplemented cells ($P = 0.003$) in response to LPS-simulation, compared to that of control cells. In agreement with NO data presented previously, there was no difference in iNOS mRNA levels between CLA- and AA-supplemented cells, suggesting that CLA and AA are equally suppressive toward iNOS transcription as well as NO production.

A.



B.

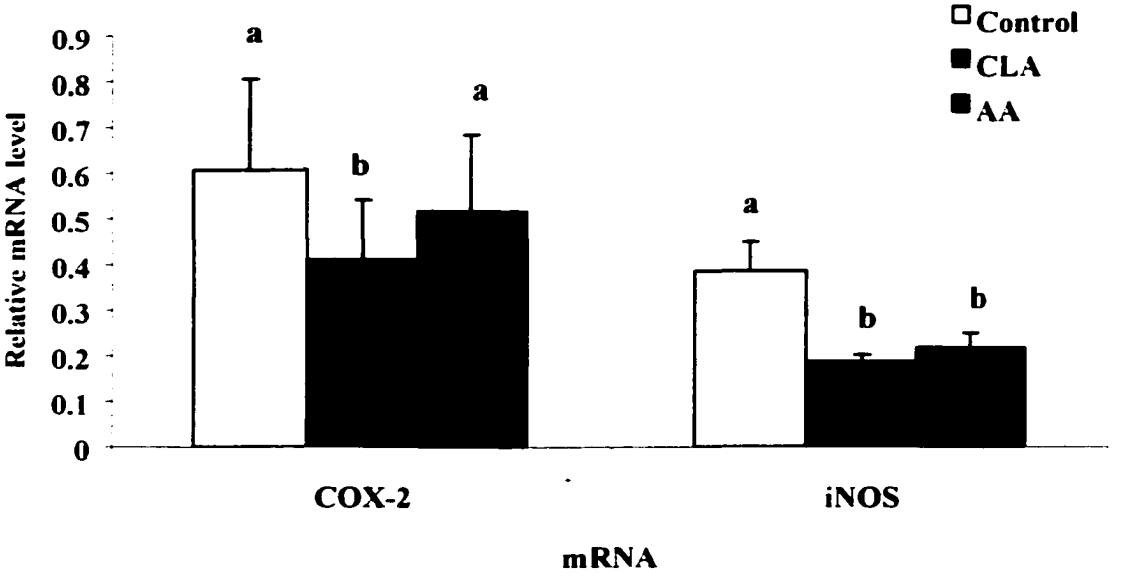


Figure 12. mRNA levels in RAW264.7 macrophage-like cells incubated medium supplemented with no added lipid (control), 30 μM conjugated linoleic acid (CLA), or 30 μM arachidonic acid (AA). (A) Cyclooxygenase-2 (COX-2) and inducible form of nitric oxide synthase (iNOS) mRNA levels are shown. Total RNA was isolated from cells that were incubated with control, CLA or AA medium for 24 h, prior to the stimulation with lipopolysaccharide (LPS; 100 ng/ml) for 9 h. mRNA levels were determined by Northern analysis. Signals of COX-2 and iNOS were normalized relative to that of 18S rRNA bands (B) mRNA abundance relative to 18S rRNA; Bars (mean ± SEM) not sharing common letters are significantly different, $P<0.05$. Values were mean of two to three plates from each of three independent experiments.

Chapter 6

Discussion

I have shown that CLA suppressed both PGE_2 and NO production in activated macrophages, which was associated with decreased COX-2 and iNOS mRNA abundance, respectively. Although it is well-established that CLA inhibits PGE_2 production, this is the first study to show that CLA inhibition of PGE_2 synthesis occurs at the level of COX-2 expression. Furthermore, this is the first study that has demonstrated effects of CLA on NO and iNOS expression in macrophages.

CLA inhibition of PGE_2 synthesis has been reported in rat serum (Sugano et al., 1998), rat liver homogenate (Turek et al., 1998), and murine keratinocytes (Liu and Belury, 1998). I showed that CLA also inhibits PGE_2 production in activated macrophages. Various studies suggest that inhibition of PGE_2 production by CLA may account for the anti-carcinogenic action of CLA (Parodi, 1999; Banni et al., 1999), since it is believed that high amounts of PGE_2 produced by COX-2 may promote tumor growth (Earnest et al., 1992). Therefore, inhibition of PGE_2 production by CLA may be beneficial for tumor suppression.

The mechanisms by which CLA suppresses PGE_2 production are still unclear. Some speculate that CLA may compete in elongation and desaturation steps with LA for the synthesis of AA, which is the precursor of PGE_2 (Fig 8). Such competition possibly results in decreased rate of LA conversion to AA, and thereby PGE_2 synthesis could be suppressed. In cultured murine keratinocytes, for instance, incubation of cells with CLA

reduces AA content in the cells, while LA increases AA. In association with decreased AA content in CLA treated cells, there is a marked decrease in PGE_2 production, while LA, by increasing AA content, enhances PGE_2 production (Liu and Belury, 1998).

The reduction of AA content in the cells by CLA may not be a sole cause of decreased PGE_2 production by CLA. CLA may affect at the level of gene expression, as indicated in our study showing that CLA suppresses mRNA abundance of COX-2 and iNOS in activated macrophages. How does CLA influence COX-2 and iNOS expression? I speculate that CLA binding to peroxisome proliferator-activated receptor alpha (PPARα) activates PPARα, which inhibits activation of the transcription factor, nuclear factor kappa B (NFkB), which in turn inhibits COX-2 and iNOS expression (Fig 9). Three lines of evidence support our speculation. Firstly, Moya-Camarena et al. (1999a and 1999b) showed that CLA is a ligand for PPARα. Secondly, PPARα activation inhibits NFkB activation (Ricote et al., 1998; Brand et al., 1996; Bourcier et al., 1997; Chinetti et al., 1998; Staels et al., 1998). Thirdly, activated NFkB up-regulates expression of both COX-2 (von Knethen et al., 1999; Hwang et al., 1997) and iNOS genes (Chen et al., 1999). Thus, NFkB inhibition by PPARα activated by CLA may account for the depressed COX-2 and iNOS expression I observed.

PPAR is a member of steroid hormone receptor superfamily. It requires ligand binding to become active. Currently, three isoforms of PPAR have been recognized, referred to as PPARα, β (or δ) or γ (Isseman and Green, 1990). Macrophages express both PPARα and PPARγ. PPARα is expressed in undifferentiated human monocytes, while PPARγ only becomes expressed in human macrophages upon differentiation. PPARα is present constitutively in the cytoplasm, whereas PPARγ is predominately

localized in nucleus in differentiated human macrophages (Chinetti et al., 1998). PPARα interferes negatively with the NFkB signaling pathway in the activated macrophages (Chinetti et al., 1998). Similarly, PPARγ inhibits the activities of transcription factors of the AP-1, STAT, and NFkB families in activated macrophages (Ricote et al., 1998). It has not been demonstrated whether CLA also binds to PPARγ. However, it is possible that PPARγ isoform, besides PPARα, may be involved in the mechanisms of CLA-inhibition of COX-2 and iNOS expression through the interaction with the NFkB pathway.

NFkB is a heterodimer composed of two DNA-binding sites, RelA (p50) and RelB (p65) and is responsible for regulating the transcription of several genes whose products are critical for generating an appropriate immune response to insults such as infection and injury (Donald et al., 1995; Lin et al., 1995). Those genes include COX-2 and iNOS. Under physiological conditions, NFkB is held in its inactive form in the cytosol by the inhibitory protein IkB-α. Upon the phosphorylation and proteolytic degradation of IkB-α, NFkB is activated and translocated into the nucleus (Henkel et al., 1993). Then, it binds to its response element to induce gene expressions of COX-2 and iNOS. LPS induces the signaling pathway which enhances IkB degradation, and thereby activates NFkB, resulting in induction of COX-2 and iNOS expression in macrophages (Chen et al., 1999). NFkB activation is a key step for COX-2 (Lo et al., 1998) and iNOS expression (Xie et al., 1994). In this regard, inhibition of NFkB activation by CLA is a potent mechanism of COX-2 and iNOS expression in LPS-activated macrophages.

Several lines of evidence support that PPARα activation affects COX-2 and iNOS expression. Colville-Nash and researchers (1998) showed that PPARα activation inhibits

NO accumulation in the RAW264.7 macrophage cells, although this effect may be mediated through the activation of PPARγ, as other activators of this PPAR isoform are also effective in NO inhibition. In addition, it has shown that macrophage activation markedly up-regulates PPARγ, which inhibites iNOS expression (Ricote et al., 1998). Staels and colleagues (1998) showed that PPARα ligands, but not PPARγ ligands, inhibited interleukin-1 induced COX-2 expression in human aortic smooth muscle cells. In contrast, PPARα activation increases COX-2 transcription in colonic epithelium by binding to PPAR response element in a region of the COX-2 promoter (Meade et al.,1999). Whether PPARα activation inhibits or stimulates COX-2 and iNOS expression may depend on the cells or tissues studied. Taking all these observations together, PPARα (possibly PPARγ also) may be involved in inhibition of COX-2 and iNOS expression in activated macrophages that I observed.

Upon ligand binding, PPAR forms a heterodimer with retinoid X receptor (RXRα, 9cis-retinoic acid receptor), which is required for the interaction with a peroxisome proliferator responsive element (PPRE), which then induces expression of the PPAR responsive gene. CLA induces accumulations of PPAR-responsive enzymes at the mRNA and protein levels in mice (Belury et al., 1997), suggesting that CLA may interact with PPAR, which may in turn induce down-stream signals to express PPAR-responsive genes. Synthetic PPAR activators exert their anti-inflammatory actions, at least in part, by negatively regulating NFkB activation (Delerive et al., 1999; Staels et al., 1999). It is not known how PPAR activation causes inhibition of NFkB activation. Testing our postulated mechanism by which CLA affects iNOS and COX-2 should prove fruitful in future research.

I also evaluated effects of AA on PGE_2 and NO, as well as COX-2 and iNOS expression, respectively. AA is the precursor of PGE_2. Thus, addition of AA to the medium is likely to increase PGE_2 production. As expected, AA addition to the medium had higher PGE_2 production, compared to control medium. The COX-2 mRNA abundance in AA group was not different from control. Thus, it seems that AA increases PGE_2 synthesis without influencing COX-2 expression, most likely by increasing COX substrate availability.

Although AA had no effect on COX-2 expression, AA suppressed iNOS expression to a similar extent as the CLA treatment. It has been reported that polyunsaturated fatty acids (PUFA) affect iNOS expression. A study by Ohata et al. (1997) showed that n-3 PUFA (e.g., α-linolenic acid, docosahexaenoic aicd, and eicosapentaenoic acid) suppress iNOS expression in activated macrophages. In contrast, no effect on iNOS expression was observed in cells incubated with n-6 PUFA (e.g.,LA) and saturated fatty acid (e.g., oleic acid). Based on these results, Ohata and researchers (1997) speculated that iNOS suppression in activated macrophages is a unique characteristics of n-3 PUFA. In agreement with their study, LA did not affect NO production in our preliminary study (data not shown). In addition, PGE_2 production was not affected by LA (data not shown), although LA is a precursor for AA (the precursor of PGE_2). I have clearly shown that AA, a n-6 PUFA, suppressed iNOS expression, indicating that some n-6 PUFA may also inhibit iNOS expression in activated macrophages. Macrophages lack the enzyme to transform LA (18:2, n-6) into AA (20:4, n-6). Thus, AA, present in macrophage phospholipids, is biosynthesized elsewhere and transported to the macrophages (Chapkin et al., 1988). These observations suggest that

LA and AA have distinct effects on iNOS expression, and AA, but not LA, may induce signaling pathway, which negatively controls iNOS expression.

It has been shown that both LA and AA can bind to PPARα (Murakami et al., 1999; Lin et al., 1999). However, it is unlikely that iNOS suppression by AA is through the interaction with PPAR as indicated by two observations. First, AA and LA shows different effects on NO synthesis, although both can bind to PPARα. AA exerts significant suppressive effects on iNOS expression in activated macrophages, while LA has no effects on iNOS expression. Second, AA exerts different effects on COX-2 and iNOS expression. While AA had no effect on COX-2 expression, AA suppressed iNOS expression in activated macrophages. If AA binds to PPAR, which in turn inhibits NFkB activation, it is likely that this AA-induced signaling event is likely to inhibit both COX-2 and iNOS expression. I speculate that AA may activate a receptor (not PPAR), which then induces down-stream signaling, in which NFkB may not be involved. As mentioned above, NFkB activation is a key step for COX-2 and iNOS expression in LPS-activated macrophages (Lo et al., 1998; Xie et al., 1994). Thus, inhibition of NFkB activation is likely to suppress expression of both COX-2 and iNOS in activated macrophages. AA, however, suppressed iNOS without affecting COX-2 expression. Thus, it is reasonable to speculate that AA may induce PPAR independent-signaling pathway, which inhibits iNOS expression with no effect on COX-2 expression. This speculation may explain why CLA and AA show different effects on COX-2 expression.

The molecular mechanisms of PUFA regulation of gene expression are not clearly elucidated. Recent evidence suggests that PUFA modulate gene transcription, mRNA stability and cellular differentiation (Sessler and Ntambi, 1998). Since PUFA activate PPAR, it has been hypothesized that PUFA are the endogenous activator of this receptor

(Gottlicher et al., 1992). It has become evident in recent studies that PPAR is not the sole mediator in PUFA-regulation of gene expression (Sessler and Ntambi, 1998). Ren and researchers (1997), using the PPARα-deficient mice, showed that induction of some hepatic enzyme gene expression by PUFA requires PPARα, while others are regulated by PUFA without the presence of PPARα. It is, therefore, suggested that there are distinct pathways for PUFA control of hepatic enzyme expression; one requires PPARα and others are independent on PPARα. Several lines of evidence also support the presence of PUFA specific receptor (not PPAR) and/or signaling pathways (Mater et al., 1999; Jump et al., 1999). Based on these observations, I speculate that CLA and AA mediate different signaling pathways for COX-2 and iNOS expression in activated macrophages. AA may mediate PPARα-independent signaling pathways, while CLA may control COX-2 and iNOS expression through PPARα.

Expression of COX-2 and iNOS has been implicated in diseases, such as cancer and atherosclerosis (Schonbeck et al., 1999; Depre et al., 1999). Recent studies have been shown that inhibition of COX-2 and iNOS expression results in positive effects on the treatment of these diseases. Macrophages are present in all stages of tumorigenesis and atherogenesis and play regulatory roles in the pathogenesis of these diseases (Rees and Parry, 1992). Macrophages express COX-2 and iNOS when macrophages are activated in response to pro-inflammatory stimuli, such as cytokines and LPS, producing high amounts of PGE_2 and NO, respectively, which are implicated in pathogenesis of these diseases. I showed that CLA inhibits both COX-2 and iNOS expression. I speculate that the anti-carcinogenic effects of CLA reported by others may be due to this ability of CLA to inhibit COX-2 and iNOS expression in macrophages.

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