MODULATION OF COX I AND COX II-MEDIATED FORMATION OF VARIOUS ARACHIDONIC ACID METABOLITES IN VITRO AND IN VIVO BY DIETARY POLYPHENOLS

By:

C2009
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M.S., Chonnam National University, 2004

B.S., Chonnam National University, 2002

Submitted to the graduate degree program in Pharmacology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Dessertation Defended: 12/03/2009

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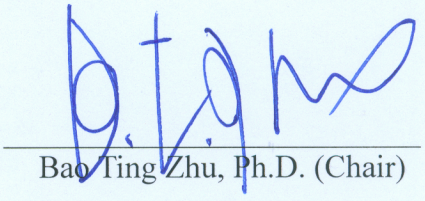

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ACKNOWLEDGEMENTS

I would like to express my gratitude to the many people who helped me complete this dissertation. First and foremost, I'd like to thank my advisor, Dr. Bao Ting Zhu, for his continued support and advice throughout my doctoral program. His hard work and guidance helped me complete this work over the last six years. I look forward to his continued guidance and mentorship throughout my professional development.

I am also grateful to the rest of my committee members, Dr. Thomas L. Pazdernik, Dr. Kenneth E. McCarson, Dr. Beth Levant, and Dr. Andrei Belousov for all of their scientific thoughts and advice, as well as for being so generous with their time whenever I needed to schedule a last-minute meeting. I would not have been able to complete any step of this project without the assistance of my committee.

I thank the faculty members of the International program and the Department of Pharmacology program at the University of Kansas Medical Center. Their efforts provided me with the fundamental knowledge to live in the United States and to complete this dissertation.

I would like to thank the Pharmacology department office staff, particularly Rosa Meagher. The office staff not only keeps the department running efficiently, but does so with the best interest of the students in mind.

Thanks also go to the previous and current members of Dr. Zhu's laboratory, especially Dr. Masayuki Fukui and Dr. Hye-Joung Choi who have provided me with unconditional guidance, and a great learning environment, and who were amazing

people to work with.

I would also like to thank my colleagues in the Department of Pharmacology, Toxicology and Therapeutics for their friendship and academic support. Special thanks go to Pan Wang, who generously provided assistance regarding computational modeling analysis as well as great friendship, and to the American Heart Association for partially funding this work by a scholarship.

I would like to express my deepest appreciation to my family who have always trusted and believed in me. I want to thank my parents, Yang-Wong Bai and Hae-Kyoung Chung, and sister, Jin Bai, who have always financially and emotionally supported me with their everlasting love that is wider than the sea and higher than the sky.

Finally, the most heartfelt thanks go to my wife, Jina Yu. She has spent countless hours with me all the time during the course of my research. Without her love and support, I would not be the person that I am, and for this reason I share this degree with her.

ABSTRACT

Cyclooxygenase (COX) is a key enzyme required for the conversion of arachidonic acid (AA) to various prostaglandins (PGs), thromboxanes (TXs), and hydroxyeicosatetraenoic acids (HETEs), by which AA exert numerous biological actions in the body such as inflammation and platelet aggregation. Therefore, the regulation of the levels of these autacoids is crucial for normal physiological functions. Experiments were performed to investigate the hypothesis that some of the bioflavonoids are naturally-occurring, physiological co-substrates and activators for COX I and II in the body, and to determine the mechanisms by which bioflavonoids modulate the catalytic activity of COXs.

To investigate the effect of bioflavonoids on COX-mediated AA metabolism, a total of 20 naturally-occurring bioflavonoids were first tested for their ability to modulate the catalytic activity of COXs *in vitro*. Some of these bioflavonoids, such as quercetin, myricetin, fisetin and morin, were found to be powerful direct stimulators of the catalytic activity of COXs *in vitro* assays, increasing the formation of PG products by up to 11-fold over the controls. Additional studies using intact cells in culture showed that some of these dietary compounds also stimulated the formation of PGE_2 (a representative PG).

Two representative dietary bioflavonoids, quercetin and myricetin, were further studied to determine if they could modulate the plasma and tissue levels of PG products in normal Sprague-Dawley rats. Both compounds strongly stimulated the formation of several representative PG products *in vivo,* in time- and dose-dependent

manners.

Computational modeling studies further revealed that bioflavonoids could bind to the peroxidase active site of COXs and directly interact with the hematin component of COXs and facilitate the electron transfer from the bioflavonoids to hematin. Biochemical analysis and site-directed mutagenesis experiments were conducted to verify these computational findings. Biochemical analysis revealed that when the cyclooxygenase activity of COXs was selectively inhibited by chemical inhibitors, myricetin could still stimulate the conversion of prostaglandin G_2 to PGE_2, catalyzed by the peroxidase activity. Using the site-directed mutagenesis assay, it was found that Q189 at the peroxidase site of COX II was essential for the binding of bioflavonoids.

Additional computational molecular modeling and structure-activity relationship studies revealed that the hydroxyl groups in the B-ring of various bioflavonoids played a crucial role in stimulating the COX's catalytic activity. Galangin, a representative bioflavonoid without a hydroxyl group in its B-ring, could function as an inhibitor of the catalytic activity of COXs both *in vitro* and *in vivo* when the enzymes were stimulated by quercetin.

In conclusion, some of the bioflavonoids, such as myricetin, quercetin, fisetin and morin, were found to have a powerful direct stimulatory effect on the COX I and II activity at physiologically-relevant doses. Based on the results obtained from these studies, it was suggested that one of the important biological functions of bioflavonoids in the human body might be to serve as the naturally-occurring co-

substrates for the COX enzymes through binding tightly into the peroxidase active site, and interacting directly with the hematin component of the COX enzymes to facilitate the electron transfer from bioflavonoids to hematin. Besides, some of the dietary compounds with no hydroxyl group on their *B-rings*, such as galangin, can function as inhibitors of COXs. These studies provide a platform for the future development of novel modulators (stimulators or inhibitors) of the human COX I and II activity.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ANOVA	analysis of variance
AP	activator protein
APC	adenomatous polyposis coli
ATCC	american type culture collection
b.w.	body weight
C/EBP	CCAAT-enhancer-binding protein
CoMFA	comparative molecular field analysis
COX	cyclooxygenase
DMEM	dulbecco's modified eagle's medium
EDTA	ethylenediaminetetraacetic acid
EFG	epidermal growth factor
EGCG	epigallocatechin gallate
EIA	enzymatic immunoassay
EPR	electron paramagnetic resonance
ERK	extracellular signal-regulated kinase
ESI	electrospray interface
FBS	fetal bovine serum
HETE	hydroxyeicosatetraenoic acid
HHT	hydroxy-5Z,8E,10E-heptadecatrienoic acid

HPETE	hydrophobic alkyl hydroperoxide
HPLC	High performance liquid chromatography
HUVEC	human umbilical vein endothelial cells
i.p.	intraperitoneal
i.v.	intravenous
IACUC	institutional animal care and use committee
IL	interleukin
IκB	inhibitor-κB
JNK	c-Jun N-terminal kinase
KUMC	university of kansas medical center
LC-MS/MS	liquid chromatography-mass spectrometry
LDL	low-density lipoproteins
LOX	lipooxygenase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MBD	membrane binding domain
MRM	multiple reaction monitoring
NFκB	nuclear factor-κB
NIH	national institutes of health
NSAID	non-steroidal anti-inflammatory drug
PCR	polymerase chain reaction
PG	prostaglandin

PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGHS	prostaglandin endoperoxidase H synthase
PGI_2	prostaglandin I_2
PMSF	phenylmethylsulfonyl fluoride
POX	peroxidase
PPHP	5-phenyl-4-pentenyl-1-hydroperoxide
PPIX	protoporphyrin IX
PVDF	polyvinylidine fluoride
QSAR	quantitative structure-activity relationship
ROS	reactive oxygen species
S.D.	standard deviation
SAPK	stress-activated protein kinase
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TNF	tumor necrosis factor
TPA	12-O-tetradecanoylphorbol 13-acetate
TX	thromboxane
TXA_2	thromboxane A_2
TXB_2	thromboxane B_2

CHAPTER ONE

GENERAL INTRODUCTION

COXs and biosynthesis of prostaglandins

The first cyclooxygenase (COX), also called prostaglandin H synthase (PGHS), was purified in 1976 and cloned in 1988 (Miyamoto et al., 1976; DeWitt and Smith, 1988). It is a key enzyme in the biosynthesis of various prostaglandins (PGs), thromboxanes (TXs) and hydroxyeicosatetraenoic acids (HETEs) from arachidonic acid (AA) (Hamberg and Samuelsson, 1967; Miyamoto et al., 1976; Marnett, 2000; Kurumbail et al., 2001). In 1991, several laboratories identified a protein with very similar COX activity that was encoded by another gene, and it was named COX II (reviewed in (Herschman, 1996)). While these two enzymes have similar catalytic properties, they have distinctly different biological functions. COX I is generally considered a constitutive enzyme and produces PGs acutely to regulate cellular responses to hormonal stimulation and to regulate vascular homeostasis. COX II, in constrast, is often considered an inducible enzyme expressed only in response to growth factors and inflammatory stimuli. Both enzymes catalyze ***a cyclooxygenase reaction*** in which AA plus two O_2 molecules are converted to prostaglandin G_2 (PGG_2), followed by ***a peroxidase reaction*** in which PGG_2 is reduced to prostaglandin H_2 (PGH_2) by two electrons. PGH_2 is then quickly transformed ***enzymatically*** or ***nonenzymically*** into various primary prostanoids including prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin D_2 (PGD_2), prostaglandin I_2 (PGI_2), and thromboxane A_2 (TXA_2) (Hamberg and Samuelsson, 1967; Miyamoto et al., 1976; Marnett, 2000; Kurumbail et al., 2001) (**Figure 1**). The resulting products then exit the cells via a carrier-mediated

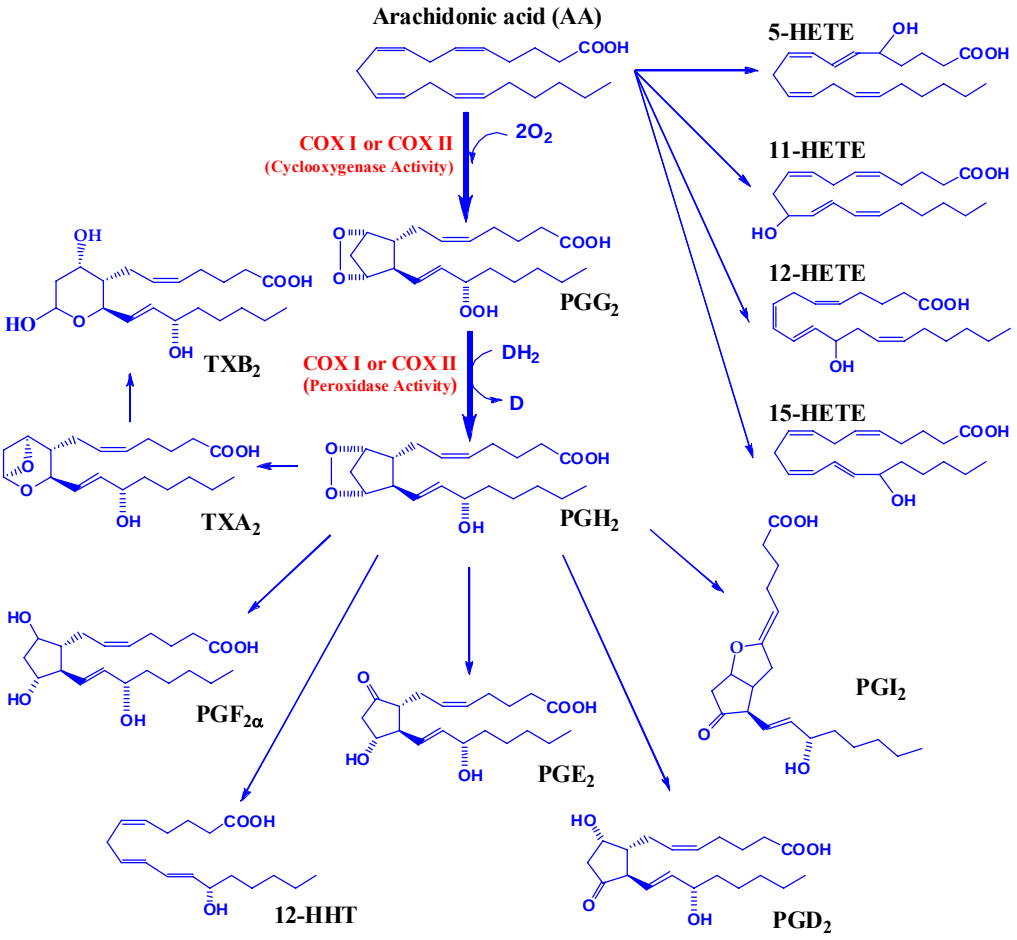


Figure 1. Metabolic pathways of AA metabolism mediated by COX I and COX II. Abbreviations used: PGH_2, prostaglandin H2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGE_2, prostaglandin E_2; PGD_2, prostaglandin D_2; 12-HHT, 12S-hydroxy-5Z,8E,10E-heptadecatrienoic acid; 5-, 11-, 12-, or 15-HETE, 5-, 11-, 12-, or 15-hydroxyeicosatetraenoic acid, respectively.

process (Chan et al., 1998) and activate G protein–linked prostanoid receptors (Murata et al., 1997; Sugimoto et al., 1998; Ushikubi et al., 1998), and in some cases, they may interact with nuclear receptors (Lim et al., 1999). The activation of specific receptors present in various target tissues or cells mediate a myriad of biological functions.

COX Structure

The primary structures of COX I and II from numerous species have been reported (Smith and Dewitt, 1996). Both isoforms have signal peptides of varying lengths. Processed mature forms of COX I and II contain 576 and 587 amino acids, respectively. There is a 60%–65% sequence identity between COX I and II from the same species and 85%–90% identity among individual isoforms from different species. The main differences between COX isoforms occur in the membrane binding domains (MBDs) (Otto and Smith, 1996; Spencer et al., 1999a). A unique difference between COX I and II is 18 amino acids inserted 6 residues in from the C terminus of COX II that are not present in COX I. The function of this insert is not established.

COXs are homodimers both functionally (Xiao et al., 1998) and structurally, but the reason that dimerization is necessary for catalysis is unknown. Each monomer consists of three structural domains: an epidermal growth factor (EGF) domain of 50 amino acids at the N terminus, a neighboring MBD of about 50 amino acids, and a large C-terminal globular catalytic domain with about 460 amino acids (**Figure 2**) (Picot et al., 1994; Kurumbail et al., 1996; Luong et al., 1996; Smith et al., 2000).

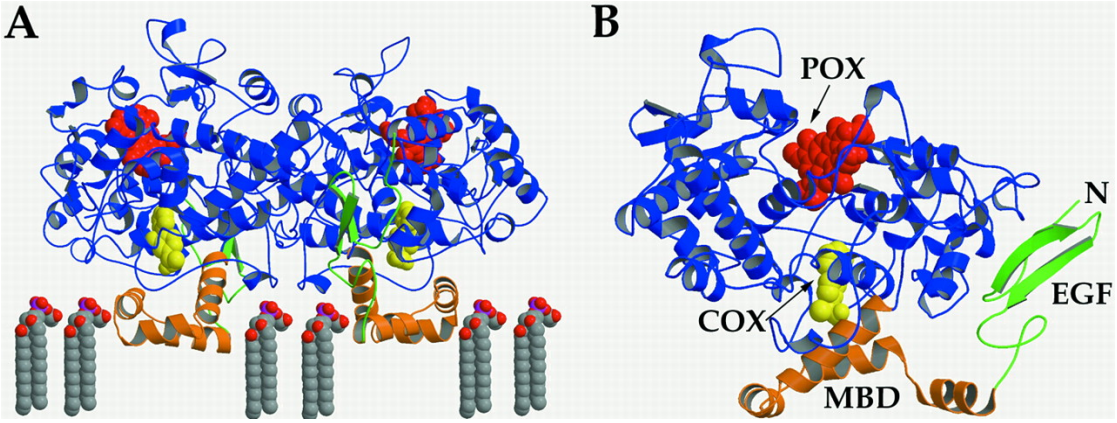


Figure 2. Structure of ovine prostaglandin endoperoxidase H synthase-1 (oPGHS-1). (*A*) Ribbon diagram of the oPGHS-1 homodimer with flurbiprofen (*yellow*) bound within the cyclooxygenase active site and the protein interdigitated into one face of the lumenal surface of the membrane bilayer. The positions of the three major folding domains are indicated: epidermal growth factor (EGF; *green*), membrane binding domain (MBD; *gold*) and globular catalytic domain (*blue*); as are the peroxidase and cyclooxygenase active sites. The heme is shown in *red*. (*B*) Ribbon drawing of the oPGHS-1 monomer with flurbiprofen bound, indicating the locations of the peroxidase (POX) and cyclooxygenase (COX) active sites and the EGF and MBD. The color scheme is the same as in (*A*) (Smith et al., 2000).

The EGF domain forms a portion of the dimer interface. The MBDs of COXs contain four short, consecutive, amphipathic α helices, the last of which, helix D, merges into the catalytic domain. Hydrophobic and aromatic residues protrude from these helices and away from the hydrophilic surface of the catalytic domain to create a hydrophobic patch that interacts with the one face of the underlying bilipid layer

(Picot et al., 1994). These helices also surround an opening through which fatty acid substrates and non-steroidal anti-inflammatory drugs (NSAIDs) are believed to enter the COX active site. The upper half of the tunnel is the COX active site and can bind fatty acid substrates and NSAIDs.

Peroxidase Kinetics

Peroxidase activities of COX I and II have been characterized by examining rates of oxidation of reducing cosubstrates such as guaiacol, 2,20-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (Landino et al., 1997), and by analyzing differential product formation from hydroperoxides that undergo one- or two-electron oxidations (Goodwin et al., 1999). The kinetic constants for compound I (oxyferryl heme radical cation) and compound II/intermediate II (oxyferryl heme) associated with heterolytic cleavage of alkyl hydroperoxides have been measured for both COX I and II. Relatively hydrophobic alkyl hydroperoxides such as 15-HPETE and 5-phenyl-4-pentenyl-1-hydroperoxide (PPHP) exhibit about 10-fold higher secondary rate constants for formation of compound I (~ 2 X 10^7 mol^{-1} s^{-1}) versus soluble peroxides such as ethylhydroperoxide and have lower apparent K_M values (~ 10 μM versus 300 μM for H_2O_2) for the peroxidase reaction as measured by rates of oxidation of reducing cosubstrates (Landino et al., 1997).

Although the second order rate constants k_1 for compound I formation with alkyl hydroperoxides are approximately the same for both isozymes (~ 2 X 10^7 mol^{-1} s^{-1}), the first-order rate constant for the conversion of compound I to compound

II/intermediate II is considerably more rapid for COX II (Lu et al., 1999). This partly accounts for the fact that for COX II, intermediate II is formed more rapidly and at lower peroxide concentrations. There is no obvious structural explanation for this property.

Cyclooxygenase Kinetics

COX kinetics are complex. In general, there are three points. The first is that the K_M values are the same for both microsomal and solubilized, purified enzymes, suggesting that the membrane-bound and purified enzyme forms behave very similarly. Interestingly, COX I and COX II have nearly identical COX turnover numbers (~3500 mol/min of arachidonate per mole of dimer) (Barnett et al., 1994; Gierse et al., 1995) and apparent K_M values for arachidonate (~5 μM). They also have similar rates of suicide inactivation ($t_{1/2}$ ~30 s). The second point is that the K_M values probably approximate the binding affinities for the substrates. This supposition is based on the observations that the *Ki* and K_M values are similar to one another for poor substrates such as eicosapentaenoic acid that behave as both inhibitors (e.g. of arachidonate oxygenation) and substrates (Kulmacz et al., 1994; Rieke et al., 1999). The third point is that the unusual negative allosteric regulation of COX I (Swinney et al., 1997; Shitashige et al., 1998; So et al., 1998; Chen et al., 1999) is not seen with COX II. This difference may be relevant to the preferential oxygenation of arachidonate by COX II in intact cells at relatively low substrate concentrations (Kulmacz, 1998; Shitashige et al., 1998). Both microsomal and purified COX I

exhibit negative allosterism at arachidonate concentrations between 50 nM and 1 μM when equivalent amounts of the two isozymes are functioning in this substrate range, the amount of product formed via COX I is less than 25% of that formed via COX II (Swinney et al., 1997); intact cells expressing both isozymes also show preferential utilization of exogenous arachidonate via COX II (Shitashige et al., 1998). This is important because this concentration of substrate is in the range likely to be available under many conditions *in vivo*. Moreover, negative allosteric regulation of COX I can explain how COX II can operate independently of COX I. Interestingly, this negative allosterism is not observed in the presence of excess hydroperoxide (which activates the peroxidase), suggesting that hydroperoxide concentrations may be key to regulating the relative activities of COX I and COX II *in vivo* (Chen et al., 1999).

Regulation of COX I Expression

Because COX I is expressed constitutively in most tissues, and expression levels of this enzyme do not vary greatly in adult animals, it has been difficult to study transcriptional regulation of the COX I gene. Nevertheless, COX I is preferentially expressed at high levels in selected cells and tissues, including endothelium, monocytes, platelets, renal collecting tubules, and seminal vesicles, indicating that it is developmentally regulated. As might be expected, expression of COX I increases in cell lines that undergo differentiation and thus mimic developmental processes (Smith and Dewitt, 1996). The promoter of the human COX I gene lacks a TATA box, has a very GC-rich region, and contained multiple

transcription start sites (Kraemer et al., 1992; Xu et al., 1997). Two elements, which contains a Sp1 binding site proximal to the transcription start sites, have been identified that contribute to constitutive expression of COX I in human umbilical vein endothelial cells (HUVEC) (Xu et al., 1997).

Regulation of COX II Expression

Examination of the cells and tissues in which COX II is expressed or can be induced and of the various factors and conditions that stimulate expression of this enzyme allows some general conclusions to be drawn about regulation of COX II. The COX II gene is particularly responsive to and most commonly elevated by growth factors and mediators of inflammation such as interleukin (IL)-1, tumor necrosis factor-α (TNFα), lipopolysaccharide (LPS), and 12-O-tetradecanoylphorbol 13-acetate (TPA); moreover, glucocorticoids and anti-inflammatory cytokines suppress COX II expression. Selective inhibitors of COX II have confirmed that this enzyme plays a critical role in inflammation, pain, and fever (DeWitt, 1999). Regulation by growth factors suggests that COX II, like immediate early genes, such as c-*fos* and c-*myc*, also plays some general role in mitogenesis, and perhaps a specialized role in wound repair. The obligatory contribution of COX II to the development of cancers of the colon further supports a role for this enzyme in control of cell growth separate from that of COX I (DuBois and Smalley, 1996; Takaku et al., 1998). Inducible or constitutive expression of COX II also occurs in specialized cell types or tissues where COX II plays specific functions in individual biological

processes. These include reproduction (Lim et al., 1997; Lim et al., 1999), immunity (Rocca et al., 1999), renal physiology (Cheng et al., 1999), neurotransmission (Breder et al., 1995), bone resorption (Pilbeam et al., 1997), and pancreatic secretion (Robertson, 1998). Thus, expression of COX II in many specialized cell types appears to be differentially sensitive to stimuli that regulate the unique physiological activities of each tissue.

Signal Transduction and Transcriptional Activation of the COX II Gene. It is difficult to identify specific signaling pathways for all of the many activators and different conditions that lead to transcriptional activation of the COX II gene. However, for the inflammatory agents, and for growth factors, a number of shared or convergent pathways are likely to regulate transcription of COX II. These include nuclear factor-κB (NFκB) and CCAAT-enhancer-binding protein (C/EBP), two common signaling pathways in inflammatory response (Ghosh et al., 1998; Poli, 1998), as well as one or all of three mitogen-activated protein kinase (MAPK) cascades: extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase /stress-activated protein kinase (JNK/SAPK), and p38/RK/Mpk2 (Su and Karin, 1996).

Regulation of COX II Expression by the NFκB Signaling Pathway. *NFκB* signaling has been implicated variously in the expression of COX II stimulated by TNFα, hypoxia, endothelin, and IL-1 in osteoblastic cells (Yamamoto et al., 1995), synoviacytes (Roshak et al., 1996; Crofford et al., 1997), epithelial cells (Crofford et al., 1997; Cheng et al., 1999), endothelial cells (Schmedtje et al., 1997), and

hepatocytes (Gallois et al., 1998). Each of these effectors, as well as LPS, can activate the NFκB signaling pathway (Zhang et al., 1997; Ghosh et al., 1998), and the COX II promoter contains two consensus sequences for the *cis*-acting regulatory sequences that are recognized by the NFκB family of transcription factors. Evidence that activation of NFκB is required for induction by these treatments, and is not simply coincidental, includes experiments that show inhibition of COX II expression by antisense oligonucleotides for the p65 protein (Crofford et al., 1997; Miller et al., 1998), by decoy oligonucleotides containing the NFκB binding motif, by expression of negative-dominant inhibitor-κB (IκB) mutants (Jobin et al., 1998), and by use of salicylate and MG-132, two inhibitors of the IκB kinase (Wu et al., 1999). Furthermore, transfection experiments with reporter plasmids have demonstrated that mutations in the NFκB *cis*-regulatory sites attenuate transcriptional activation of the COX II promoter in response to TNFα stimulation (Yamamoto et al., 1995). Together these provide convincing evidence that NFκB regulates COX II expression in response to the appropriate activators in specific cell types.

Regulation of COX I and II activity

Suicide inactivation. Earlier studies revealed an interesting phenomenon that the peroxidase and the COX activities of COX I or II are inactivated during their catalysis by mechanism-based, first-order processes (Callan et al., 1996; Smith et al., 1996; Wu et al., 1999), which is commonly referred to as "***suicide inactivation***". During this process, the peroxidase and cyclooxygenase activities of COX I or II fall

to zero within 1-2 min even in the presence of sufficient substrates. In **Figure 3**, peroxidase and COX suicide inactivation are depicted as common events involving intermediate III (Wu et al., 1999), although mechanistic details are still not resolved. For example, peroxidase inactivation is independent of the nature of the oxidizing peroxide (Wu et al., 1999), whereas COX inactivation appears to depend on the nature of the fatty acid substrate (MirAfzali et al., 2005), and thus apparently on the nature of the peroxide. Suicide inactivation originates with a reaction intermediate (Wu et al., 1999). This intermediate does not directly involve an AA-derived radical because the rate of covalent attachment of AA to COX during catalysis is 30 times slower than that of suicide inactivation (Kulmacz, 1987; Lecomte et al., 1990).

As depicted in **Figure 3**, suicide inactivation likely proceeds from intermediate II (Wu et al., 1999) and involves the formation of a tyrosyl radical other than the Tyr385 radical. Consistent with this concept are findings that protein tyrosyl radicals in COX I can be localized to tyrosines other than Tyr385 (Tsai et al., 1994; Hsi et al., 1995; Shi et al., 2000), and that an intermediate III has been detected in association with peroxidase inactivation (Tsai et al., 1994). It should be noted that the rates of both peroxidase and COX suicidal inactivation are slowed markedly by peroxidase-reducing co-substrates (Hemler and Lands, 1980; Koshkin and Dunford, 1999; Wu et al., 1999). Reducing co-substrates may bias the rate of conversion of intermediate II to compound II versus intermediate III. Suicide inactivation is an interesting chemical phenomenon, but its biological relevance is unclear.

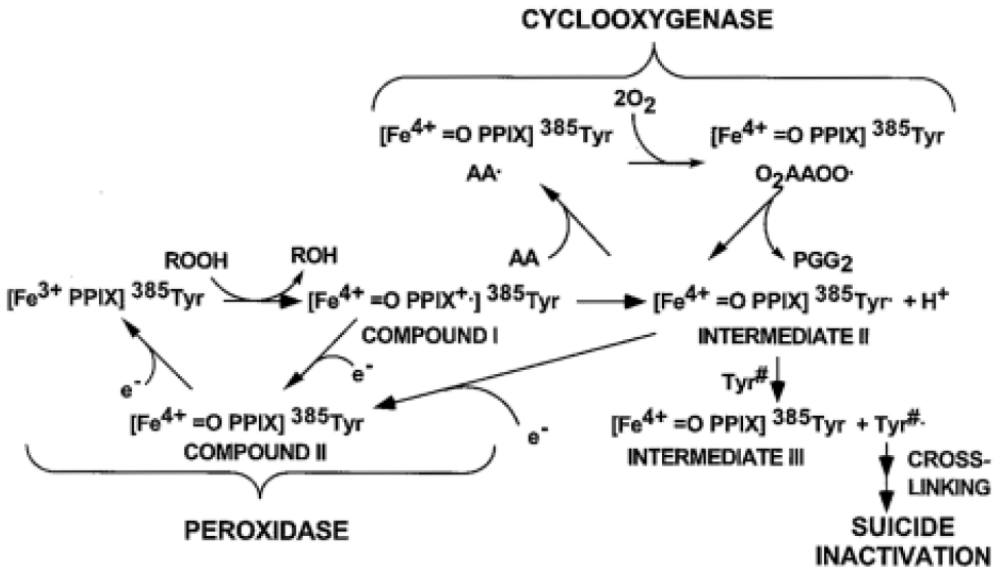


Figure 3. Cyclooxygenase and peroxidase catalysis and suicide inactivation by COXs. Fe^{3+}PPIX, ferric iron protoporphyrin IX (heme); ROOH, alkyl hydroperoxide; ROH, alcohol; AA, AA; Fe^{4+}=O PPIX, oxyferryl heme. Compound I, an oxyferryl group [Fe(IV)=O] plus a protoporphyrin IX radical cation; intermediate II, an oxyferryl group plus a neutral protoporphyrin IX plus a Tyr385 tyrosyl radical; compound II, an oxyferryl group plus a neutral protoporphyrin IX; intermediate III, a spectral intermediate of unknown structure but perhaps involving a heme group with a protein radical located on an amino acid sidechain other than Tyr385 (Smith et al., 2000).

Different biological functions of COX I and COX II

As already mentioned above, COX I is generally considered a constitutively-expressed house-keeping enzyme. Many studies have shown that the presence of the constitutive COX I activity contributes importantly to maintaining many of the normal physiological functions in a given tissue. In comparison, the COX II activity is usually undetectable in normal tissues, but its activity is often increased drastically under various pathological conditions. The common stimuli that are known to induce COX II are those associated with inflammation, and examples induce bacterial LPS and pro-inflammatory cytokines such as IL1, IL-2, and TNF-α. In comparison, the anti-inflammatory cytokines, IL-4, IL-10, and IL-13 inhibit COX II expression, and so do corticosteroids, which are powerful anti-inflammatory agents (Bakhle and Botting, 1996; Onoe et al., 1996).

COX I and II have very similar COX active site structures, catalytic mechanisms, products, and kinetics. There are, however, two structural differences between the two isozymes that have important pharmacological and biological consequences. First, the COX active site of COX II is larger and more accommodating than that of COX I. This size difference has been exploited in developing COX-2–specific NSAIDs. Second, although the gross kinetic properties (e.g. K_M and V_{MAX}) of COX I and II are nearly identical, COX I, but not COX II, exhibits negative allosterism at low arachidonate concentrations; this difference may permit COX II to compete more effectively for newly released arachidonate when the isozymes are expressed in the same cell.

NSAIDs are nonselective inhibitors of COX I and II and are widely used in the treatment of a number of medical conditions such as arthritis. NSAID interactions with the COX active sites of COX I and II have been studied extensively, and a number of crystal structures of the NSAID/COXs complexes are available (Picot et al., 1994; Kurumbail et al., 1996; Luong et al., 1996). There are, however, three general points to be made. First, there are two classes of NSAIDs: (*a*) classical (pre-1995) NSAIDs and (*b*) COX II inhibitors. All classical NSAIDs can inhibit both COX I and II but in general bind more tightly to COX I (Bors and Saran, 1987). Of course, COX II inhibitors exhibit selectivity toward COX II (DeWitt, 1999; Spencer et al., 1999a). Second, while all NSAIDs compete with arachidonate for binding to the COX active site, each NSAID exhibits one of three kinetic modes of inhibition (Smith and Dewitt, 1996; Smith et al., 1996; DeWitt, 1999; Marnett et al., 1999): (*a*) rapid, reversible binding (e.g. ibuprofen); (*b*) rapid, lower affinity reversible binding followed by time-dependent, higher affinity, slowly reversible binding (e.g. flurbiprofen), or (*c*) rapid, reversible binding followed by covalent modification (acetylation) of Ser530 (e.g. acetyl salicylic acid). The structural basis for time-dependent inhibition is not well defined and may be different for different drugs. The kinetic differences in NSAID inhibition have made simple comparisons of drug interactions with COX I versus COX II difficult, particularly *in vitro*. And finally, all COX II inhibitors cause time-dependent inhibition of COX II but not COX I.

Although inhibition of COX activity can be used to treat a number of medical conditions where COX levels are dramatically elevated, it should also be noted that

inhibition of the normal COX activity has been associated with a number of severe adverse effects, including gastrointestinal ulceration and bleeding as well as increased risk for heart attacks (Gottlieb, 2001). It has been suggested that the gastrointestinal toxicity of NSAIDs such as acetyl salicylic acid and ibuprofen is mostly related to COX I inhibition (Vane and Botting, 1998). About 1% of chronic users of NSAIDs, typically those with arthritis or other chronic inflammatory diseases, develop ulcers or other serious gastrointestinal complications each year. These ulcers result from inhibition of PG synthesis by COX I, the predominant COX isozyme in the stomach lining. Because of the widespread use of NSAIDs, these toxicities are one of the most prevalent drug-associated health risks. In comparison, the newer COX II-selective inhibitors, such as celecoxib and rofecoxib, were used for the treatment of a number of chronic inflammations, but they have been found to cause serious cardiovascular system problems in some patients. A selective COX II inhibition (following the use of celecoxib or rofecoxib) would decrease the production of vascular prostacyclin (PGI_2), thereby affecting the balance between the levels and functions of the prothrombotic and antithrombotic eicosanoids. Whereas the biological properties of PGI_2 are essentially anti-inflammatory, including vasodilation and inhibition of platelet aggregation, TXA_2 promotes platelet aggregation and vasoconstriction. An imbalance in the production of PGI_2 and TXA_2 has been suggested to be an early event in the development of thrombi in the coronary and cerebral blood vessels which will ultimately lead to myocardial infarction and stroke (Schmedtje et al., 1997). Unlike the platelet inhibition afforded by COX I inhibitors, COX II inhibitors do not

share this salutary anti-thrombotic property. In contrast, inhibition of COX II may decrease the formation of the vasodilatory and anti-aggregatory PGI_2, and thus may tip the balance in favor of prothrombotic eicosanoids (e.g., TXA_2), leading to increased cardiovascular thrombotic events (Belton et al., 2000). These clinical observations suggest that a balanced COX activity capable of producing an assortment of PGs is crucially important for maintaining the normal physiological functions.

Dietary polyphenolic compounds - Overview

Bioflavonoids have been known as plant pigments for over a century. Originally proposed to be required as vitamins, the term "vitamin P" for flavonoids was suggested, although this was later dismissed (Kuo, 1997). Bioflavonoids belong to a vast group of polyphenolic compounds that are widely distributed in all foods of plant origin. In the past half century, a great deal of research has been done to better understand the biological actions associated with bioflavonoids as well as other dietary polyphenolic compounds (Brown, 1980; Deschner et al., 1991; Ferrandiz and Alcaraz, 1991; Tzeng et al., 1991; Middleton and Kandaswami, 1992; Elangovan et al., 1994; Gil et al., 1994; Terao et al., 1994; Rump et al., 1995; Di Carlo et al., 1999). Animal and human studies have shown that dietary polyphenols have a wide range of beneficial effects, such as strong antioxidant activity, unique vascular protective actions, inhibition of cancer formation, antiviral activity, allergy-relieving activity, and others (Chassevent, 1969; MacCornack, 1977; Hertog et al., 1993;

Hollman and Katan, 1999; Yang and Landau, 2000; Higdon and Frei, 2003; Lambert and Yang, 2003; da Cunha et al., 2004; Neuhouser, 2004; Yao et al., 2004). Using bioflavonoids as an example, their well-documented health-promoting effects include improved cardiovascular health, increased capillary strength, improved structure of connective tissues and appearance of skin, a stronger immune system, better eyesight, and a reduced risk for a number of diseases (atherosclerosis, arthritis, gastrointestinal disorders and cancer) (Chassevent, 1969; MacCornack, 1977; Hertog et al., 1993; Hollman and Katan, 1999; Yang and Landau, 2000; Higdon and Frei, 2003; Lambert and Yang, 2003; da Cunha et al., 2004; Neuhouser, 2004; Yao et al., 2004). Similarly, there are also numerous health-promoting effects associated with tea polyphenols (reviewed in ref. (Chassevent, 1969; MacCornack, 1977; Hertog et al., 1993; Hollman and Katan, 1999; Yang and Landau, 2000; Higdon and Frei, 2003; Lambert and Yang, 2003; da Cunha et al., 2004; Neuhouser, 2004; Yao et al., 2004)).

Bioflavonoids in foods - Types and Sources

Several thousand polyphenol compounds have been identified in plants. These compounds could be classified into different groups base on a function of the number of phenol rings. Distinctions are thus made between the phenolic acids, flavonoids, stilbenes, and lignans. The flavonoids, which have a common structure consisting of 2 aromatic rings (A and B) that are bound together by 3 carbon atoms that form an oxygenated heterocycle (ring C), could be further divided into 6 subclasses as a function of the type of heterocycle involved: flavonols, flavones, isoflavones,

flavanones, anthocyanidins, and flavanols (Bravo, 1998). In addition, polyphenols are also associated with various carbohydrates and organic acids and with one another. Flavonols are the most ubiquitous flavonoids in foods. Flavones are much less common than flavonols in fruit and vegetables. Flavonoids are widely distributed in foods and beverages of plant origin, such as fruits, vegetables, tea, cocoa, and wine.

Dietary intake of Bioflavonoids

Only partial information is available on the quantities of polyphenols that are consumed daily throughout the world. In 1976 Kuhnau calculated that dietary flavonoid intake in the United States was about 1 g/d and consisted of the following: 16% flavonols, flavones, and flavanones; 17% anthocyanins; 20% catechins; and 45% biflavones depending on the season (Kuhnau, 1976). Hertog et al. (Hertog et al., 1997) estimated intake of flavonols and flavones in the Netherlands to be 23 mg/day, and Kuhnau estimated intake of these two subclasses to be 115 mg/day. It has been suggested that Kuhnau's estimate was inflated owing to the unreliability of analytic methods used in the 1970s (Hollman and Katan, 1999).

Absorption and Bioavailability of bioflavonoids

Knowledge of the absorption and metabolism of flavonoids is cucial to the understanding of whether these compounds or their metabolites have the potential to exert the biological activity *in vivo* that the i*n vitro* studies suggest. The polyphenols that are the most common in the human diet are not necessarily, those with the

greatest activity within the body, because either they have a lower intrinsic activity or they are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated. In addition, the metabolites that are found in blood and target organs and that result from digestive or hepatic activity may differ from the native substances in terms of biological activity. Metabolism of polyphenols occurs via a common pathway (Scalbert and Williamson, 2000). The aglycones can be absorbed from the small intestine. However, most polyphenols are present in food in the form of esters, glycosides, or polymers that cannot be absorbed in their native form. These substances must be hydrolyzed by intestinal enzymes or by the colonic microflora before they can be absorbed. When the flora is involved, the efficiency of absorption is often reduced because the flora also degrades the aglycones that it releases and produces various simple aromatic acids in the process.

Hollman et al. (Hollman and Katan, 1999) conducted a study in nine ileostomy patients to examine the extent of absorption of quercetin. Following a 12-day quercetin-free diet, subjects were randomized to the following supplemented diets over a 12-day period: fried onions (quercetin glucosides), pure quercetin rutinoside (the major quercetin glycoside found in tea), or 100 mg of pure quercetin aglycone. They found that the absorption of orally administered quercetin aglycone was approximately 24%; however, the absorption of quercetin glycosides from onions was 52%, suggesting that the glycoside moiety actually enhanced absorption.

The bioavailability studies have also shown that the concentrations of intact flavonoids in human plasma rarely exceed 1 μM when the quantities of polyphenols

ingested do not exceed those commonly ingested with our diets. These maximum concentrations are most often reached 1-2 h after ingestion (Hollman et al., 1996; Aziz et al., 1998), except for polyphenols, which are absorbed only after partial degradation by the colon microflora. With regard to rutin, the maximum concentration of quercetin in the plasma is reached 9 h after ingestion (Hollman et al., 1997). For most flavonoids absorbed in the small intestine, the plasma concentration then rapidly decreases (elimination half-life period of 1–2 h). This fast excretion is facilitated by the conjugation of the aglycone to sulfate and glucuronide groups. The elimination half-life period for quercetin is much higher (24 h) (Hollman et al., 1997).

Conjugation of Bioflavonoids and their Biological Activity

Once absorbed, polyphenols are subjected to 3 main types of conjugation: methylation, sulfation, and glucuronidation. Catechol-*O*-methyl transferase catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine to polyphenols having an *o*-diphenolic (catechol) moiety. Such a reaction is well known for quercetin, catechin, caffeic acid, and luteolin. The methylation generally occurs predominantly in the 3' position of the polyphenol, but a minor proportion of 4'-*O*-methylated product is also formed. Its activity is highest in the liver and the kidneys (Piskula and Terao, 1998). Sulfotransferases catalyze the transfer of a sulfate moiety from 3'-phosphoadenosine-5'-phosphosulfate to a hydroxyl group on various substrates (steroids, bile acids, polyphenols, etc). Neither the isoforms that are specifically involved in the conjugation of polyphenols nor the positions of sulfation for the

various polyphenols have yet been clearly identified, but sulfation clearly occurs mainly in the liver (Piskula and Terao, 1998). UDP-glucuronosyltransferases catalyze the transfer of a glucuronic acid from UDP-glucuronic acid to steroids, bile acids, polyphenols, and thousands of dietary constituents and xenobiotics. The presence of glucuronidated metabolites in the mesenteric or portal blood after perfusion of polyphenols in the small intestine of rats shows that glucuronidation of polyphenols first occurs in the enterocytes before further conjugation in the liver (Spencer et al., 1999b).

The biological activities of polyphenols have often been evaluated *in vitro* on pure enzymes, cultured cells, or isolated tissues by using polyphenol aglycones or some glycosides that are present in food. Very little is known about the biological properties of the conjugated derivatives present in plasma or tissues because of the lack of precise identification and commercial standards. However, reflection on the antioxidative activity of polyphenols suggests that the metabolism of polyphenols may have a considerable effect. Conjugation might enhance certain specific biological activities, as shown for some xenobiotics. Koga and Meydani showed that plasma metabolites of catechin have an inhibitory effect on monocyte adhesion to interleukin 1β–stimulated human aortic endothelial cells, whereas catechin and metabolites of quercetin had no effect (Koga and Meydani, 2001). In another *in vitro* study, quercetin 3-*O*-glucuronide prevented vascular smooth muscle cell hypertrophy by angiotensin II (Yoshizumi et al., 2002).

Plasma concentrations and tissue uptake

Plasma concentrations reached after polyphenol consumption vary highly according to the nature of the polyphenol and the food source. They are on the order of 0.3–0.75 μmol/L after consumption of 80–100 mg quercetin equivalent administered in the form of apples, onions, or meals rich in plant products (Manach et al., 1998). When ingested in the form of green tea (0.1–0.7 μmol/L for an intake of 90–150 mg), cocoa (0.25–0.7 μmol/L for an intake of 70–165 mg) (Rein et al., 2000), or red wine (0.09 μmol/L for an intake of 35 mg) (Donovan et al., 1999), catechin and epicatechin are as effectively absorbed as is quercetin. Isoflavones are certainly the best absorbed flavonoids: plasma concentrations of 1.4–4 μmol/L are obtained between 6 and 8 h in adults who consume relatively low quantities of soya derivatives supplying ~50 mg isoflavones (Watanabe et al., 1998).

Determination of the actual bioavailability of polyphenol metabolites in tissues may be much more important than is knowledge of their plasma concentrations. Data are still very limited, even in animals. When single doses of radiolabeled polyphenols (quercetin, epigallocatechin gallate (EGCG), quercetin 4'-glucoside, resveratrol) are given to rats or mice killed 1–6 h later, radioactivity is mainly recovered in blood and in tissues of the digestive system, such as the stomach, intestine, and liver (Vitrac et al., 2003). However, polyphenols have been detected by HPLC analysis in a wide range of tissues in mice and rats, including brain (Abd El Mohsen et al., 2002), endothelial cells (Youdim et al., 2000), heart, kidney, spleen, pancreas, prostate, uterus, ovary, mammary gland, testes, bladder, bone, and skin

(Chang et al., 2000; Coldham and Sauer, 2000).

Elimination

Metabolites of polyphenols may follow 2 pathways of excretion, via the biliary or the urinary route. Large, extensively conjugated metabolites are more likely to be eliminated in the bile, whereas small conjugates such as monosulfates are preferentially excreted in urine. In laboratory animals, the relative magnitude of urinary and biliary excretion varies from one polyphenol to another (Crespy et al., 2003). Biliary excretion seems to be a major pathway for the elimination of genistein, EGCG, and eriodictyol (Sfakianos et al., 1997). Biliary excretion of polyphenols in humans may differ greatly from that in rats because of the existence of the gall bladder in humans; however, this has never been examined. Intestinal bacteria possess ß-glucuronidases that are able to release free aglycones from conjugated metabolites secreted in bile. Aglycones can be reabsorbed, which results in enterohepatic cycling. Pharmacokinetic studies in rats have shown a second maximum plasma concentration about 7 h after genistein administration, which is consistent with enterohepatic circulation (Coldham and Sauer, 2000). A second plasma peak was also observed in some human volunteers 10–12 h after ingestion of hesperetin from orange juice or of isoflavones from soya (Manach et al., 2003).

Urinary excretion has often been determined in human studies. The total amount of metabolites excreted in urine is roughly correlated with maximum plasma concentrations. Urinary excretion of flavonols accounts for 0.3–1.4% of the ingested

dose for quercetin and its glycosides (Graefe et al., 2001) but reaches 3.6% when purified glucosides are given in hydroalcoholic solution to fasted volunteers (Olthof et al., 2000). Urinary recovery is 0.5–6% for some tea catechins (Lee et al., 1995), 2–10% for red wine catechin (Donovan et al., 2002), and up to 30% for cocoa epicatechin (Baba et al., 2000).

Biological effects of polyphenols

Diets high in fruits and vegetables are protective against a variety of diseases, particularly cardiovascular disease and some types of cancer. Antioxidants and dietary fiber are thought to be the principal nutrients responsible for these protective effects (Simonetti et al., 1997; Parr and Bolwell, 2000). Reactive oxygen species (ROSs) are formed *in vivo* during normal aerobic metabolism and can cause damage to DNA, proteins, and lipids, despite natural antioxidant defense systems. The accumulation of unrepaired damaged products may be critical to the development of cancer, atherosclerosis, diabetes, and chronic inflammation (Coward et al., 2002). Several *in vitro* studies have shown that the flavonoids, including flavonols, flavones, isoflavones, flavanols, and anthocyanidins, possess antioxidant activity.

The antioxidant capacity of phenolic compounds is determined by their structure, in particular the ease with which a hydrogen atom from an aromatic hydroxyl group can be donated to a free radical and the ability of an aromatic compound to support an unpaired electron as the result of delocalization around the M-electron system. Other important structural determinants of the antioxidant

capacity of flavonoids appear to be the 4'-OH and 3'-OH groups. The addition of hydroxyl groups to the carbon atoms ortho to the 4-C position appear to further increase antioxidant potential (Lien et al., 1999). Studies have indicated that the aglycones, including quercetin, luteolin, myricetin, and kaempferol, have greater antioxidant capacity than do the conjugate flavonoids, such as quercetin-3-glucoside, quercitrin, and rutin (Noroozi et al., 1998). Ioku et al. (Ioku et al., 1995) showed that the antioxidant activity of quercetin glycosides is lower than quercetin aglycone in an artificial membrane system, suggesting that glycosidation weakens the antioxidant activity of flavonoids. This decrease may be caused by increased blocking of the phenolic groups responsible for radical scavenging and metal chelation and possibly to a decrease in accessibility of the membranes owing to the large glycoside group. Reaction rate constants in organic media for several flavonoids exceed that of vitamin E. Suggested reasons include that flavonoids have a more extended conjugated system to support an unpaired electron, two or more reactive OH groups, and less stearic hindrance at the site of abstraction.

Cardiovascular Diseases. Many studies have focused on the beneficial cardiovascular effects of polyphenolic compounds in humans. These beneficial effects have been frequently ascribed to flavanols, a subgroup of flavonoids abundantly present in fruits and vegetables (Duffy et al., 2001). A significantly reduced incidence of coronary artery disease despite a high fat diet, little exercise, and wide-spread smoking in certain areas of France has led to the concept of the “French paradox.” This phenomenon was attributed to a higher intake of alcohol and in

particular of red wine in France (Renaud and de Lorgeril, 1992). In this context, red wine, cocoa, some chocolates, and tea received much attention, because they are particularly rich in flavanols, phytochemicals with strong antioxidant properties *in vitro* (Hammerstone et al., 2000). The benefits of red wine or flavanol-rich food have been attributed to the antioxidative activity of their polyphenolic compounds (Rice-Evans et al., 1997) and the oxidative modification of low-density lipoproteins (LDL) is a key step in the formation of an atherosclerotic lesion (Steinberg et al., 1989). Several studies have shown protective effects of flavanols on LDL by measuring their oxidative susceptibility *in vitro* (Brown et al., 1998) or *ex vivo* (Fuhrman et al., 1995). However, there is still debate about whether flavanol-rich food can decrease LDL oxidation *in vivo* (Fuhrman et al., 1995; Caccetta et al., 2000). For many years, it was accepted that this was the main mechanism by which flavanols mediate their beneficial effects. Although LDL oxidation is main mechanism for preventing cardiovascular disease, there is increasing evidence that these compounds possess additional cardioprotective functions including altering hepatic cholesterol absorption, triglyceride assembly and secretion, the processing of lipoproteins in plasma, and inflammation.

Cancer. Oxidative stress imposed by reactive oxygen species (ROS) plays a crucial role in the pathophysiology associated with neoplasia, atherosclerosis, and neurodegenerative diseases. The ROS-induced development of cancer involves malignant transformation due to altered gene expression through epigenetic mechanisms as well as DNA mutations which lead to tumor formation.

Many epidemiological studies have been conducted to investigate the effects of tea consumption on human cancer incidence. Evidence for the anticarcinogenic potential of tea polyphenols has been provided by numerous *in vitro* and experimental studies describing their action to bind directly to carcinogens, induce Phase II enzymes such as UDP-glucuronosyl transferase and inhibit heterocyclic amine formation. Molecular mechanisms, including catechin-mediated induction of apoptosis and cell cycle arrest, inhibition of transcription factors NF-κB and activator protein 1 (AP-1) and reduction of protein tyrosine kinase activity and c-jun mRNA levels have also been suggested as relevant chemopreventive pathways for tea (Mukhtar and Ahmad, 2000; Khan et al., 2006). Some epidemiological studies also support a protective role of tea against the development of cancer. Studies conducted in Asia, where green tea is consumed frequently and in large amounts, tend to show a beneficial effect on cancer prevention. Importantly, the putative chemopreventive effect of tea also varies by the specific type of cancer (Khan and Mukhtar, 2007).

Activation of COX I and II by phenol and phenolic compounds

Earlier studies showed that phenol, known as antioxidants and have been widely used as drugs or antimicrobial agents. Their peroxidatic oxidation plays an important role in chemical toxicity and chemical carcinogenesis (Eastmond et al., 1987; Schlosser et al., 1989; Eling et al., 1990). Phenols showed stimulatory effect on the catalytic activity of COX I and II *in vitro* (Smith and Lands, 1971; Markey et al., 1987; Hsuanyu and Dunford, 1992a). Because of this property, this organic chemical

has been commonly added to the reaction mixture to optimize the catalytic activity of COX I and II *in vitro*. It is thought that phenol serves as a supporting cofactor for the COX I and II-mediated metabolism of AA *in vitro*. A mechanism for COX stimulation by phenol was suggested which envisioned stimulators as radical-trapping reducing agents capable of attenuating enzyme deactivation by scavenging the destructive moiety responsible for the electron paramagnetic resonance (EPR) signal (Egan et al., 1978).

It have been also reported that some drugs have a stimulatory effect on biosynthesis of PGs by acting as a co-factor or co-substrate and that stimulant effects depends on the presence of a phenolic group (Collier et al., 1976). The self-catalyzed inactivation of COX activity can be diminished substantially by some peroxidase-reducing co-substrates (Egan et al., 1976; Hemler and Lands, 1980; Markey et al., 1987). On the other hand, some excellent reducing substrates are inhibitors of the cyclooxygenase reaction under certain conditions (Marnett and Wilcox, 1977; Panganamala et al., 1979; Egan et al., 1980). For example, nafazatrom, a thrombotic and antimetastatic agent containing phenolic group, serves as a peroxidase-reducing substrate and stimulates PGI_2 biosynthesis in ram seminal vesicle microsomes by acting as a substrate (Marnett et al., 1984).

CHAPTER TWO

STATEMENT OF PURPOSE

COX is the key enzyme required for the conversion of AA to various autacoids, including PGs, TXs, and HETEs (Hamberg and Samuelsson, 1967; Miyamoto et al., 1976; Marnett, 2000; Kurumbail et al., 2001), which exert multiple biological actions in the body (Regan, 2003; Lee et al., 2005; Sung et al., 2005). It is well known that elevated levels of these autacoids as a result of COX II induction play a crucial role in mediating inflammation. Furthermore, pharmacological inhibition of the pathologically-elevated COX II activity is an effective treatment for various inflammatory diseases (Flower and Vane, 1972). Preclinical studies have shown that inhibition of the COX I and II activity *in vivo* may inhibit the intestinal tumorigenesis in the *APC (adenomatous polyposis coli)* $^{min/+}$ mice (Ju et al., 2005). On the other hand, abnormally low levels of COX I and II activity have been associated with detrimental health consequences. Long-term inhibition of COX I and/or COX II by effective COX inhibitors has also been closely linked to a number of notorious adverse effects, including gastrointestinal ulceration and bleeding as well as increased risk for heart attacks (Gottlieb, 2001). The gastrointestinal toxicity appears to be largely related to COX I inhibition (Vane and Botting, 1998); in comparison, selective COX II inhibition (e.g., caused by the use of celicoxib or rofecoxib) would decrease the production of vascular prostacyclin (PGI_2), thereby affecting the balance between the levels and functions of the prothrombotic and antithrombotic eicosanoids (Schmedtje et al., 1997; Belton et al., 2000). These clinical observations suggest that lower basal levels of the COX I and II activity are not always better or beneficial for optimal health. On the contrary, it is believed that

balanced COX activity is required for the normal physiological functions.

Given the myriad important biological functions known to be exerted by various PG products under normal physiological conditions, it is critically important to know whether there are naturally-occurring, physiological co-substrates that are used by COX I and II by in the body to support their normal catalytic activity and functions for the biosynthesis of various PGs. Therefore, the goal of this study is to develop novel dietary approaches by using certain bioflavonoids and other polyphenols for reducing the risk of human diseases and also for improving overall human health. The specific objective of this research is to understand the modulating effects of dietary polyphenols on the biosynthesis PGs *in vitro* and *in vivo*, and also to understand the mechanisms of these effects. Therefore, the overall hypothesis of this research is that bioflavonoids and possibly other dietary polyphenols are naturally-occurring, physiological co-substrates and modulators for COX I and II in the body. These dietary chemicals jointly serve to support the normal catalytic activity of the constitutive COX enzymes in various target tissues or cells *in vivo*. This hypothesis will be systematically tested by pursuing the following four SPECIFIC AIMS:

1. To test the hypothesis that some dietary bioflavonoids may modulate the formation of PG products in vitro catalyzed by COX I and II.

The purpose of the first aim is to examine various bioflavonoids for their ability to modulate the catalytic activity of COX I and II in biochemical assays.

Earlier studies have shown that phenol, a non-physiological chemical with

antioxidant activity, has a direct stimulatory effect on the catalytic activity of COX I and II *in vitro* (Smith and Lands, 1971; Hsuanyu and Dunford, 1992a). It is generally thought that phenol functions as a supporting cofactor for the COX I and II-mediated AA metabolism *in vitro*. Notably, there are many phenolic/polyphenolic compounds abundantly present in our daily diet, such as bioflavonoids. Therefore, some dietary bioflavonoids are tested for their ability to serve as co-substrates/modulators for the COXs-mediated various PG formations *in vitro*.

Prior to analysis, an HPLC assay is developed that simultaneously detects multiple AA metabolites including PGs and escosainoides in a single HPLC run in order to identify each AA metabolites produced by COXs. A liquid chromatography-mass spectrometry (LC-MS/MS) method is used to identify each AA metabolite produced by COXs. Thereafter, a series of experiments are conducted to compare various bioflavonoids for their ability to directly modulate the catalytic activity of COX I and II in the biochemical assays. Following the biochemical study of COX activation, another set of experiments is conducted to determine the potential differences between the observed effects of COX activation by dietary polyphenols in biochemical assays and their effects using intact cells in culture to further evaluate the activation of COX-mediated AA metabolism and PG biosynthesis by dietary polyphenols.

2. To test hypothesis that some dietary bioflavonoids may modulate the formation of PG products catalyzed by COX I and II in vivo.

The purpose of the second aim is to determine the effect of dietary bioflavonoids on the circulating and tissue levels of PGs in short-term animal models. The main goal of the proposed studies described under this aim is to use short-term animal models to determine whether or not some dietary bioflavonoids selected from Aim 1 that are found to have a strong stimulatory effect on the formation of various PG products *in vitro*, would also have a similar stimulatory effect *in vivo*.

It is well known that the autacoids produced by COXs exert numerous bological actions in the body through activation of specific membrane receptors (Regan, 2003; Lee et al., 2005; Sung et al., 2005). Clinical studies demonstrated that either abnormally elevated or low levels of COX activity is associated with a number of severe pathological conditions, including inflammation and gastrointestinal ulceration (Silverstein et al., 2000; Moore et al., 2006; Blandizzi et al., 2009), and the basal levels of COX activity and ultimately the basal circulating and tissue levels of AA-derived autacoids need to be maintained within a normal range in order to exert many of their normal physiological functions. It is found that some of the dietary bioflavonoids are powerful activators of the catalytic activity of COX I and II in the *in vitro*, resulting in increased formation of various AA metabolites (Bai and Zhu, 2008). Therefore, the effect of two representative dietary compounds (quercetin and myricetin) on plasma and tissue levels of several PG products will be determined in a short-term animal model.

3. To test hypothesis that dietary bioflavonoids stimulate COX-mediated PGs formation by serving as co-substracts for COX enzyme via binding tightly to the peroxidase site, and interacting with hematin to facilitate electron transfer from bioflavonoids to hematin.

The purpose of the third aim is to determine the mechanism by which these bioflavonoids modulate COX-mediated AA metabolism, especially, how different bioflavonoids interact with COX enzymes, and how these bioflavonoids stimulate PGs production. In the previously study (Bai and Zhu, 2008), it was found that some dietary bioflavonoids are strong activators of COX I and II *in vitro* and *in vivo*. The specific mechanism of these effects, however, has not yet been established. In addition, data from the 3-D quantitative structure-activity relationship/comparative molecular field analysis (QSAR/CoMFA) study reveal that the *B*-ring of bioflavonoids played an important role for their direct activation of the catalytic activity of COX I and II. This notion is further supported by homology modeling and docking studies, which show that bioflavonoids could bind to the peroxidase active site and directly interact with hematin, thereby facilitating the electron transfer from bioflavonoids to hematin. To confirm these findings, experiments are designed to couple biochemical analysis with site-directed mutagenesis studies.

4. To test hypothesis that the hydroxyl groups on the B-ring might be key functional groups for interaction with COXs to modulate enzyme activity, either stimulation or inhibition.

The fourth aim is to verify the concept that lack the B-ring hydroxyl group of bioflavonoids may be able to function as antagonists and block the stimulatory effect of bioactive bioflavonoids such as quercetin and myricetin both *in vitro* and *in vivo*.

It is found that some of the dietary polyphenols could function as naturally-occurring co-substrates for COX I and II *in vitro*, resulting in increased catalytic activity of COXs for the formation of various AA metabolites (Bai and Zhu, 2008). This observation was also further confirmed *in vivo* by showing that administration of quercetin and myricetin (two representative dietary bioflavonoids) could strongly increase the plasma and tissue levels of several PG products in an animal model (Bai and Zhu, 2009). Molecular modeling studies have shown that bioflavonoids could bind, with high affinity, to the peroxidase site of the human COX I and II, and serve as reducing co-substrates and that the hydroxyl groups contained in the *B*-ring of various bioflavonoids play a crucial role in their stimulation of the COX catalytic activity. Based on the computational models developed, it is predicted that some of the dietary compounds that lack the *B*-ring hydroxyl groups may be able to function as antagonists that would inhibit the stimulatory effect of those bioactive bioflavonoids (serving as reducing co-substrates for the COX enzymes). Therefore, this idea will be tested using galangin, a representative bioflavonoid with no hydroxyl group in its *B*-ring, both *in vitro* and also *in vivo*.

CHAPTER THREE

MATERIALS AND METHODS

Chemicals

[^{14}C]Arachidonic acid ([^{14}C]AA, specific radioactivity of 53 Ci/mol) was purchased from PerkinElmer (Boston, USA). Indomethacin, NS-398, hematin, flavone, myricetin, quercetin, fisetin, morin, chrysin, kaempferol, baicalein, daidzein, genistein, silibinin, apigenin and LPS (from Escherichia coli, serotype 055:b5) were purchased from Sigma Co. (Missouri, USA). Chrysoeriol, isorhamnetin, diosmetin and tamarixetin were purchased from Extrasynthese (Cedex, France). COX I, COX II, 12-lipoxygenase (12-LOX), 15-LOX, PGI_2, PGG_2, TXB_2, PGE_2, 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGH_2, PGD_2, 5-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (5-HETE), 11-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (11-HETE), 12-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (12-HETE), 15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-HETE), 12-hydroxy-5Z,8E,10E-heptadecatrienoic acid (12-HHT) and the enzymatic immunoassay (EIA) kits for TXB2, PGE2, PGF2α, and PGD2 were purchased from Cayman Chemical (Ann Arbor, MI). According to the supplier, the COX I and COX II preparations used in our present study had a purity of approximately 95% and 70%, respectively, which was confirmed by our SDA-PAGE analysis (**Figure 4**). The cDNA of the human COX II was purchased from Origene Technologies (Rockville, MD). QuikChange XL site-directed mutagenesis kit and XL10-Gold competent cells were purchased from Stratagene (La Jolla, CA). QIAprep miniprep kit was obtained from Qiagen (Valencia, CA). Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Lipofectamine 2000 and goat anti-rabbit IgG conjugated to

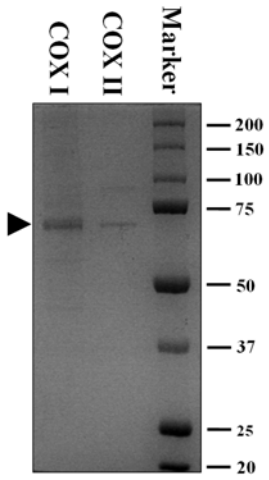


Figure 4. SDS-PAGE analysis (with Coomassie brilliant blue staining) of the relative purity of the COX I and II enzymes used in the present study. The arrow indicates the location of COX I and II protein bands.

horseradish peroxidase were purchased from Invitrogen Co. (Carlsbad, CA). Polyclonal rabbit antibody against the COX II was purchased from Cell Signaling Technology, Inc. (Danvers, MA). Cos-7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). ECL Plus kit was purchased from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ).

Assay of cyclooxygenase (COX) and lipoxygenase (LOX) activity *in vitro*

For assaying the catalytic activity of COX I and II *in vitro*, the incubation mixtures (added to an Eppendrof tube) consisted of 20 μM [^{14}C]AA (0.2 μCi) as substrate, COX I or COX II as the enzyme (0.5 or 0.97 μg/mL, respectively), 10 mM ethylenediaminetetraacetic acid (EDTA), 1 mM reduced glutathione, 1 μM hematin, and a bioflavonoid in 200 μL Tris-HCl buffer (100 mM, pH 7.4). Similarly, for

measuring 12-LOX and 15-LOX activities, the incubation mixtures included 20 μM [^{14}C]AA (0.2 μCi) as substrate, 12-LOX or 15-LOX as the enzyme (at 72 μg/mL or 75 ng/mL, respectively), and a bioflavonoid in a final volume of 200 μL Tris-HCl buffer (100 mM, pH 7.4). The bioflavonoids or other test compounds were initially dissolved in pure ethanol and then diluted with the reaction buffer. The reaction was incubated at 37°C for 5 min and terminated by adding 15 μL of 0.5 N HCl to each tube. Ethyl acetate (600 μL) was added immediately for extraction. The dried extracts were re-dissolved in acetonitrile and analyzed by HPLC for determining the metabolite compositions.

The HPLC system consisted of a Waters 2690 solvent delivery system, a Waters 484 UV-detector, and an IN/US β-RAM radioflow detector, coupled with a C18 column (Atlantis, 4.6 × 150 mm). For COX metabolites, they were eluted with a linear gradient from 93% solvent A (25% acetonitrile in water containing 0.01% acetic acid) and 7% solvent B (100% acetonitrile containing 0.01% acetic acid) to 14% A and 86% B over 27 min. The gradient was then changed to 100% A over a 3-min period at a flow rate of 1 mL/min. For LOX metabolites, they were eluted with a linear gradient from 93% A and 7% B to 32% A and 68% B over 10 min. The gradient was then changed to 100% A over a 2-min period at a flow rate of 1 mL/min. The radioactive fractions were detected using a radioflow detector, while the non-radioactive co-eluting standards were detected at 230 nm (wavelength) for HHT and various HETEs, and at 200 nm for other PG products.

Mass spectrometric analysis of various AA metabolites formed

A LC-MS/MS was used for structural identification of the AA metabolites formed. The HPLC system consisted of a Shimadzu SIL-20AC autosampler, a pair of LC-20AD pumps, a DGU-20A3 degasser, and a SCL-10AVP system controller (Shimadzu, Tokyo, Japan). The mass spectrometer was a Waters Quattro Premier triple quadrupole instrument with an ESI source (Waters, Milford, MA). The entire LC-MS/MS system is controlled by MassLynx 4.0 software. Thirty percent of the HPLC column effluent was introduced into an electrospray interface (ESI) operated in the negative ionization mode. The interface used nitrogen desolvation gas at 650 L/hour, 400°C. The instrument was operated in the multiple reaction monitoring (MRM) mode, and each standard molecule was individually tested for optimization of various parameters such as cone voltage and collision energy (various parameters are listed in **Table 1**). For optimization of the cone voltages and collision energies during the method development, a solution containing each analyte was infused into the electrospray ionization source at 10 μL/min using a syringe pump (Pump 11, Harvard Apparatus, Holliston, MA). The mass spectra for various product ions (daughter ions) were recorded using the continuum averaging mode of operation.

To do so, the metabolites were deprotonated to form their corresponding [M-H] precursor ions in the electrospray ionization source and then were detected in the negative ion mode. The MS/MS detector was used to fragment the precursor ions to form specific product ions. The fragmentation pattern of each AA metabolite

Table 1. MRM transitions and analyte-specific mass spectrometry parameters.

Compounds monitored	MRM transition		Dwell (sec)	Cone (V)	Collision energy (eV)
	Precursor ion (m/z)	Daughter ion (m/z)			
$PGF_{2\alpha}$	353	193	0.05	40	25
PGE_2	351	333	0.05	25	20
PGD_2	351	189	0.05	20	20
12-HHT	279	217	0.05	20	15
15-HETE	319	175	0.05	20	15
11-HETE	319	167	0.05	20	15
12-HETE	319	179	0.05	20	15
5-HETE	319	115	0.05	20	15

produced *in vitro* was then matched against a library of authentic standards. A complete match of the retention time of a given metabolite peak and also its mass fragmentation pattern was needed for confirmation of the identity of the metabolite formed.

Determination of the kinetic parameters (K_M, V_{MAX}, and V_{MAX}/K_M) for COX I and II

To determine the kinetic parameters (K_M and V_{MAX}) for COX II-mediated formation of $PGF_{2\alpha}$, PGE_2, PGD_2 and 12-HHT, the various concentrations of [^{14}C]AA

was used as a substrate in the absence or presence of bioflavonoid being tested. The x-axis of the Michaelis-Menten curves was the varying concentrations of [^{14}C]AA and the y-axis was the corresponding rate of formation of various AA products. The curves were analyzed using the SigmaPlot 10.0 software to determine the K_M (μM) and V_{MAX} (pmol/mg protein/min) values.

The reason that a 5-min incubation time was used is because the COX enzymes are known to be suicidal enzymes that only have a half-life of approximately 30 seconds. It would be practically difficult, if not impossible, to precisely control the time and also the temperature of the in vitro metabolic reactions that lasted shorter than 30 seconds. When the 5-min incubation time was used, it would allow us to better control the in vitro reactions and yield more reproducible data. Almost all previous studies that sought to characterize the enzyme kinetics of COX I and COX II also used the 5-min incubation time (Collier et al., 1976; Chi et al., 2001; Hong et al., 2001).

Cell culture experiments

The murine macrophage RAW264.7 cell line was obtained from ATCC (Rockville, MD), and maintained in DMEM containing L-glutamine, glucose and sodium bicarbonate. To determine the effect of myricetin (a representative dietary bioflavonoid) on the expression of COX I and II proteins in cultured RAW264.6 cells, these cells were treated with myricetin alone (0-100 μM) or in combination with LPS. The COX I and COX II protein levels were analyzed using 10% SDS-

polyacrylamide gel electrophoresis (SDS-PAGE) in a Mini-Protein system (BioRad, Hercules, CA). After electrophoresis, the protein bands on the gel were transferred onto the Polyvinylidine Fluoride (PVDF) membrane (BioRad, Hercules, CA) for Western blot analysis. The membrane was first blocked with 5% non-fat dried milk powder in Tris-HCl-buffered saline containing 0.1% Tween-20 (the blocking solution), and then it was probed with polyclonal rabbit antibodies (Upstate, Lake Placid, NY) against COX I, or the polyclonal goat antibodies (Upstate, Lake Placid, NY) against COX II. The primary antibody-antigen complexes were detected using the goat anti-rabbit IgG for COX I and the rabbit anti-goat for COX II conjugated to horseradish peroxidase (Invitrogen, Carlsbad, CA) and developed according to procedures supplied by the Amersham ECL Plus (Piscataway, NJ).

To determine the effect of bioflavonoids on the formation of PGE_2 (a representative PG) by LPS-pretreated RAW264.7 cells, the cells were first stimulated with 1 μg/mL LPS for 2 h to induce COX II expression, and then the medium was removed and replaced with 300 μL of serum-free DMEM with or without the dietary compound at concentrations of 0.01, 0.1 1, 10, and 100 μM. NS-398 (a COX II specific inhibitor, at 10 μM) and indomethacin (a non-specific inhibitor of COX I and COX II, 10 μM) were used for comparison. After additional 2-h incubation, the culture media were collected and PGE_2 levels in the culture medium were measured by using the EIA kit.

***In vivo* animal experiments**

All procedures involving the use of live animals were approved by the Institutional Animal Care and Use Committee (IACUC) of our university, and the national institutes of health (NIH) guidelines for humane treatment of animals were strictly followed. Male Sprague-Dawley rats (4 to 5-week-old, specific pathogen-free) were obtained from Harlan Laboratories (Indianapolis, IN), and maintained at the central animal facility of the University of Kansas Medical Center (KUMC). After arrival, the animals were allowed to acclimatize for one week prior to being used in the experiments. The animals were housed under constant conditions of temperature (20 ± 1°C) and a 20-h light/20-h dark cycle, and had free access to food and water. The rats were 8 weeks old at the time when they were used in the experiments.

In Experiment-I, the animals were divided into five groups (with 4 rats in each group). On the day of the experiment, myricetin and quercetin (at 0.1 and 0.3 mg/kg b.w., dissolved in 100 μL normal sterile saline containing 5% ethanol) were injected i.v. into each animal. Animals in the control group were injected i.v. 100 μL vehicle only. The blood samples were collected from the tail vein for up to 6 h. The plasma was immediately separated by centrifugation, and indomethacin was added at a 10-μM final concentration to the plasma. The concentrations of several PGs, including PGD_2, PGE_2, $PGF_{2\alpha}$ and TXB_2, were quantified using the EIA kits provided by Cayman Chemical (Ann Arbor, MI).

In Experiment-II, the animals were divided into eleven groups (with 3 rats in each group). On the day of the experiment, myricetin and quercetin (at 0.05, 0.1, 0.3,

1 and 5 mg/kg b.w., dissolved in 100 μL normal sterile saline containing 5% ethanol) were injected i.v. into each animal. Animals in the control group were injected i.v. 100 μL vehicle only. The blood samples were collected from the tail vein at 4 h after injection. The plasma was prepared and the concentrations of PGE_2 (a representative PG) were quantified as described above.

In Experiment-III, the male rats were divided into three groups (with 6 rats in each group). Myricetin and quercetin (10, and 50 mg/kg b.w., dissolved in 500 μL of 1% methyl cellulose) were given orally. The control animals were given vehicle only. The blood samples were collected for preparation of plasma, and the concentrations of PGE_2 were quantified by using the EIA kit.

In Experiment-IV, the male rats were divided into five groups (with 4 rats in each group). Myricetin and quercetin (0.1 mg/kg b.w., dissolved in 100 μL of 0.9% NaCl and 5% ethanol) were given i.v. The control animals were injected i.v. 100 μL vehicle alone. The animals were then sacrificed at 4 h after injection. The blood samples were collected for preparation of plasma, and the liver, kidney, stomach, lung and small intestine were removed and perfused through the artery with ice-cold saline, and a part of the tissue was frozen in liquid nitrogen and then kept at -80°C. The plasma levels of PGE_2 were analyzed using EIA as described above. For analysis of the tissue levels of PGE_2, tissues were thawed and then homogenized with a polytron-type homogenizer in a 100 mM Tris-HCl, pH 7.4 (5 mL/g of tissue), containing 1 mM EDTA and 10 μM indomethacin. The homogenates were centrifuged at 12000 g for

30 min at 4°C. The supernatants were then used for measurement of PGE_2 levels by using the EIA kit as described above.

In Experiment-V, the animals were divided into three groups (with 3 rats in each group). On the day of the experiment, galangin (at 1 and 5 mg/kg body weight, dissolved in 100 μL normal sterile saline containing 5% ethanol) were injected i.v. into each animal through the tail vein. Animals in the control group were injected i.v. 100 μL vehicle only. The blood samples were collected from the tail vein at 6 h after injection. The plasma was immediately separated by centrifugation, and indomethacin was added at a 10 μM concentration to inhibit the further formation of prostaglandins. The concentration of PGE_2 was quantified by using the EIA kit provided by Cayman Chemical (Ann Arbor, MI).

In Experiment-VI, the male rats were divided into four groups. Quercetin (0.1 mg/kg body weight, dissolved in 100 μL sterile saline containing 5% ethanol) and galangin (at 1 and 5 mg/kg body weight, dissolved in 100 μL sterile saline containing 5% ethanol) were given i.v. (these two chemicals were given i.v. jointly). The control animals were injected i.v. 100 μL vehicle alone. The animals were then sacrificed at 4 h after administration. The blood samples were collected for preparation of the plasma. The plasma levels of PG products were determined as described above. Liver, kidney, lung and small intestine were removed and perfused through the artery with ice-cold saline, and a part of the tissue were frozen in liquid nitrogen and then kept at □80°C. For analysis of the tissue levels of PGE_2, tissues were homogenized with polytron-type homogenizer in a 100 mM Tris-HCl, pH 7.4 (5 mL/g of tissue),

containing 1 mM EDTA and 10 μM indomethacin. The homogenates were centrifuged at 12000 g for 30 min at 4°C. The supernatants were used for measurement of various PG levels by using EIA kits (Cayman Chemical, Ann Arbor, MI) according to manufacturer's instructions.

Statistical analysis of the data

Statistical analyses were performed using SigmaPlot 10.0 (San Jose, CA). All data were expressed as mean ± S.D. Statistical difference in PG levels between different treatment groups was determined by one-way analysis of variance (ANOVA), using the using unpaired t-test, and Tukey's tests. Differences were considered statistically significant at $P < 0.05$.

Selective inhibition of the cyclooxygenase activity of COX I and II

Earlier studies showed that acetyl salicylic acid could selectively and covalently modify the cyclooxygenase active site (but not the peroxidase activity site), resulting in a selective knockout of the cyclooxygenase activity of the enzymes. This approach was adopted in the present study. COX I and II where enzymes were incubated with acetyl salicylic acid (at 0.5 and 5 mM, respectively) for 30 min at room temperature were used. For measuring the catalytic activity of untreated COX I and II, [^{14}C]arachidonic acid was used as substrate, but for measuring the activity of acetyl salicylic acid-pretreated COX I and II, PGG_2 was used as substrate. The reason to use PGG_2 as the substrate for acetyl salicylic acid-pretreated COXs was because it

was the immediate product of arachidonic acid formed by the cyclooxygenase activity of COXs, and it could still be converted to form other PGs (such as PGE_2) by the peroxidase activity of the COXs. The incubation mixtures (in Eppendrof tubes) included the following: 20 μM [^{14}C]arachidonic acid (0.2 μCi) or 10 μM PGG_2 as substrate, COX I or COX II as the enzyme (0.5 or 0.97 μg/mL, respectively), 10 mM EDTA, 1 mM reduced glutathione, 1 μM hematin, and myricetin in a final volume of 200 μL Tris-HCl buffer (100 mM, pH 7.4). Myricetin was initially dissolved in pure ethanol as stock and then further diluted with the reaction buffer. The reaction was incubated at 37°C for 5 min and terminated by adding 15 μL of 0.5 N HCl to each tube. Ethyl acetate (600 μL) was added immediately for extraction. The dried extracts were re-dissolved in acetonitrile or EIA buffer for analysis of representative PG products by HPLC or EIA kit.

***In vitro* site-directed mutagenesis study**

The cDNA of the human COX II was used as template to generate the corresponding mutant cDNAs. The *in vitro* mutagenesis was performed using a polymerase chain reaction (PCR)-based QuikChange XL site-directed mutagenesis kit according to the procedures recommended by the manufacturer. The primers used for site-directed mutagenesis were listed in **Table 2**. The reconstructed plasmids were purified from transformed XL10-Gold using the QIAprep miniprep kit. The sequences of all reconstructed plasmid DNAs were confirmed by DNA sequencing. The cos-7 cells were maintained in DMEM supplemented with 10% FBS and used to

Table 2. Primers used in the site-directed mutagenesis study. The sequences changed for each mutant developed were marked with underlines.

COX II mutants	Primers	
Q189A	**Forward**	GCATTCTTTGCCGCGCACTTCACGCATCAG
	Reverse	CTGATGCGTGAAGTGCGCGGCAAAGAATGC
H193A	**Forward**	GCCCAGCACTTCACGGCTCAGTTTTTCAAG
	Reverse	CTTGAAAAACTGAGCCGTGAAGTGCTGGGC
Q189A/H193A	**Forward**	ATTCTTTGCCGCGCACTTCACGGCTCAGTT TTTC
	Reverse	GAAAAACTGAGCCGTGAAGTGCGCGGCAAAGAAT
Q189N	**Forward**	GCATTCTTTGCCAATCACTTCACGCATCAG
	Reverse	CTGATGCGTGAAGTGATTGGCAAAGAATGC
Q189R	**Forward**	GCATTCTTTGCCCGGCACTTCACGCATCAG
	Reverse	CTGATGCGTGAAGTGCCGGGCAAAGAATGC
Q189E	**Forward**	GCATTCTTTGCCGAGCACTTCACGCATCAG
	Reverse	CTGATGCGTGAAGTGCTCGGCAAAGAATGC

express the wild-type and mutant COX II proteins. Transfection of reconstructed mutant plasmids was carried out using Lipofectamine 2000 according to the manufacturer's instructions.

Western blot analysis was used to determine the expression of the wild-type and mutant human COX II proteins. Thirty h after transfection with the plasmids, cells were collected by centrifugation and were then sonicated in ice-cold lysis buffer (50 mM Tris-HCl and 200 mM NaCl, pH 7.5). After addition of 5 mM 1,4-dithiothreitol and 1 mM phenylmethylsulfonyl fluoride (PMSF) to the crude homogenates, they were centrifuged at 10,000 × g for 10 min at 4°C. After addition of 10% glycerol, the supernatants were stored at -80°C until used as enzyme source for

assaying COX activity. The reconstructed human COX II proteins were analyzed using 10% SDS-PAGE in a Mini-Protein system (BioRad, Hercules, CA). After electrophoresis, the protein bands on the gel were transferred onto the PVDF membrane (BioRad, Hercules, CA) for Western blot analysis. The membrane was first blocked with 5% non-fat dried milk powder in Tris-buffered saline containing 0.1% Tween-20 (the blocking solution), and then it was probed with polyclonal rabbit antibodies against the COX II. The primary antibody-antigen complexes were detected using the goat anti-rabbit IgG conjugated to horseradish peroxidase and developed by using ECL Plus kit.

CHAPTER FOUR

SPECIFIC AIM 1

STRONG ACTIVATION OF CYCLOOXYGENASE I AND II CATALYTIC ACTIVITY BY DIETARY BIOFLAVONOIDS.

HYPOTHESIS

Some dietary bioflavonoids modulate the formation of PG products in vitro catalyzed by COX I and II.

RATIONALE

Past studies show that phenol, a non-physiological chemical with antioxidant activity, has a direct stimulatory effect on the catalytic activity of COX I and II *in vitro* (Smith and Lands, 1971; Hsuanyu and Dunford, 1992a) by serving as an electron donor for the reduction of PGG_2 to PGH_2 catalyzed by the hydroperoxidase activity of COX I or COX II (Smith and Lands, 1971; Hsuanyu and Dunford, 1992a). Because of this unique property, this organic chemical has been commonly added to the reaction mixture to optimize the catalytic activity of COX I and II *in vitro*. It is generally thought that phenol functions as a supporting cofactor for the COX I and II-mediated metabolism of AA *in vitro*. Notably, there are many phenolic/polyphenolic compounds that are naturally-occurring and abundantly present in the daily diet, such as the bioflavonoids. These bioflavonoids have a variety of biological functions in man. It has been reported that some of these dietary polyphenols, such as galangin and luteolin, inhibit COX-mediated AA peroxidation (Das and Das, 2007). In contrast, some have found that certain drugs or bioflavonoids have a stimulatory effect on the biosynthesis of prostaglandins by acting as a co-factor for COX, and that stimulator effect appears to the presence of a phenolic group. Nafazatrom, for example, a thrombotic and antimetastatic agent containing a phenolic group, serves as

peroxidase-reducing substrate and stimulates PGI_2 biosynthesis in ram seminal vesicle microsomes by acting as a substrate (Marnett et al., 1984). At present, however, little is known about whether there are naturally-occurring phenolic compounds that serve as physiological co-substrates/activators to support the COX I and II-mediated production of prostanoids. Therefore, I plan to test some dietary bioflavonoids (**Figure 5**) for their ability to serve as co-substrates/activators for the formation of various PG products mediated by COX I and II *in vitro*, and study the biochemical mechanisms of COX modulation by dietary bioflavonoids.

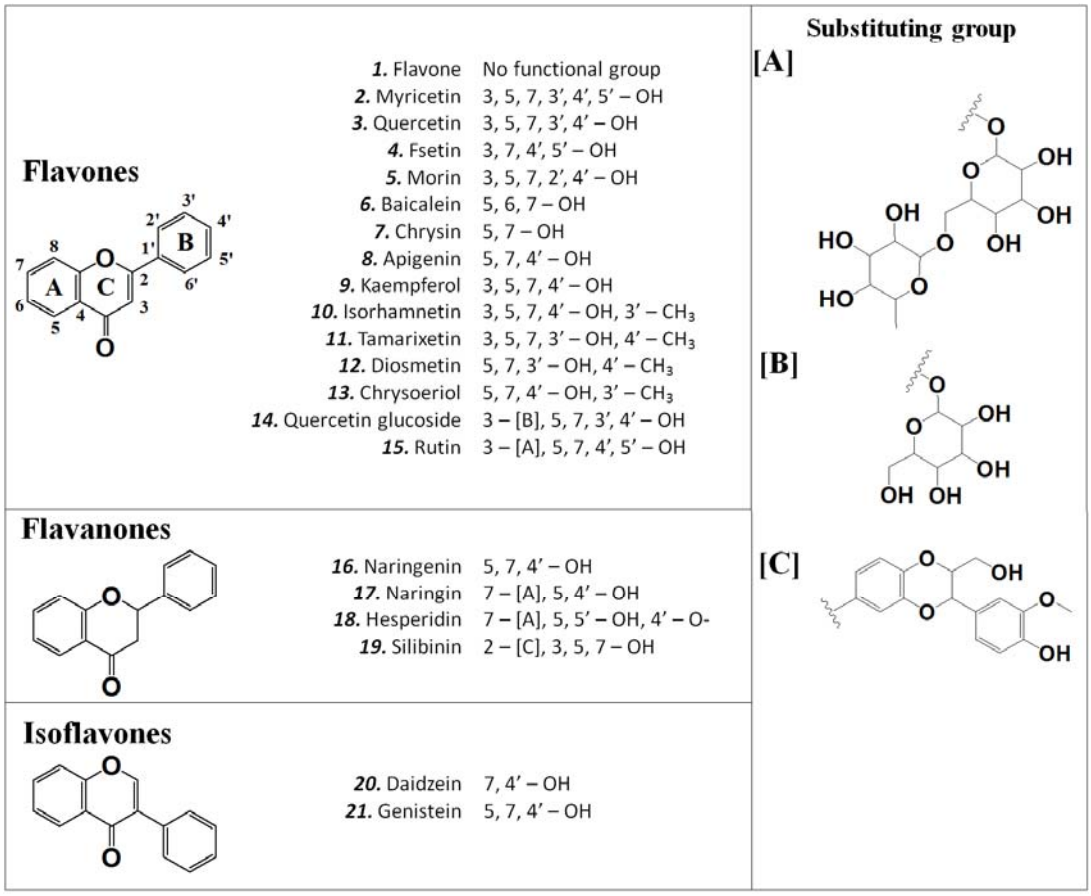


Figure 5. Chemical structures of bioflavonoids and other compounds used in this study.

RESULTS

Characterization of AA metabolites formed by COX I and II

Incubations of COX I or COX II with 20 μM [^{14}C]AA produced several radioactive AA metabolites, which included $PGF_{2\alpha}$, PGE_2, PGD_2, 12-HHT, several HETE derivatives (5-HETE, 11-HETE, 12-HETE and 15-HETE), plus a few unidentified metabolites (**Figure 6**). The overall AA metabolite profiles formed by

COX I and II were quite similar. 12-HHT was the most abundant product formed in both COX I and II-mediated reactions. PGG_2 and PGH_2, two well-known putative intermediates in AA metabolism, were not detected in these *in vitro* reactions. It is likely that these intermediates might be present transiently and then transformed to more stable PG products. TXB_2 was formed in very small quantity. This study mostly focused on the formation of four major COX products, namely, $PGF_{2\alpha}$, PGE_2, PGD_2 and 12-HHT, when evaluating the effects of bioflavonoids on AA metabolism.

The identity of various AA metabolites was first probed by comparing their HPLC retention times with those of authentic standards. Then LC-MS/MS was used to confirm the structural identity of the AA metabolites detected. To do so, the metabolites were deprotonated to form their corresponding [M-H] precursor ions in the electrospray ionization source and then were detected in the negative ion mode. The MS/MS detector was used to fragment the precursor ions to form specific product ions. The fragmentation pattern of each AA metabolite produced *in vitro* was then matched against a library of authentic standards. A complete match of the retention time of a given metabolite peak and also its mass fragmentation pattern confirmed the identity of each metabolite. This method unequivocally confirmed the identities of $PGF_{2\alpha}$, PGE_2, PGD_2, 12-HHT, 15-HETE, 11-HETE, 12-HETE and 5-HETE in these experiments.

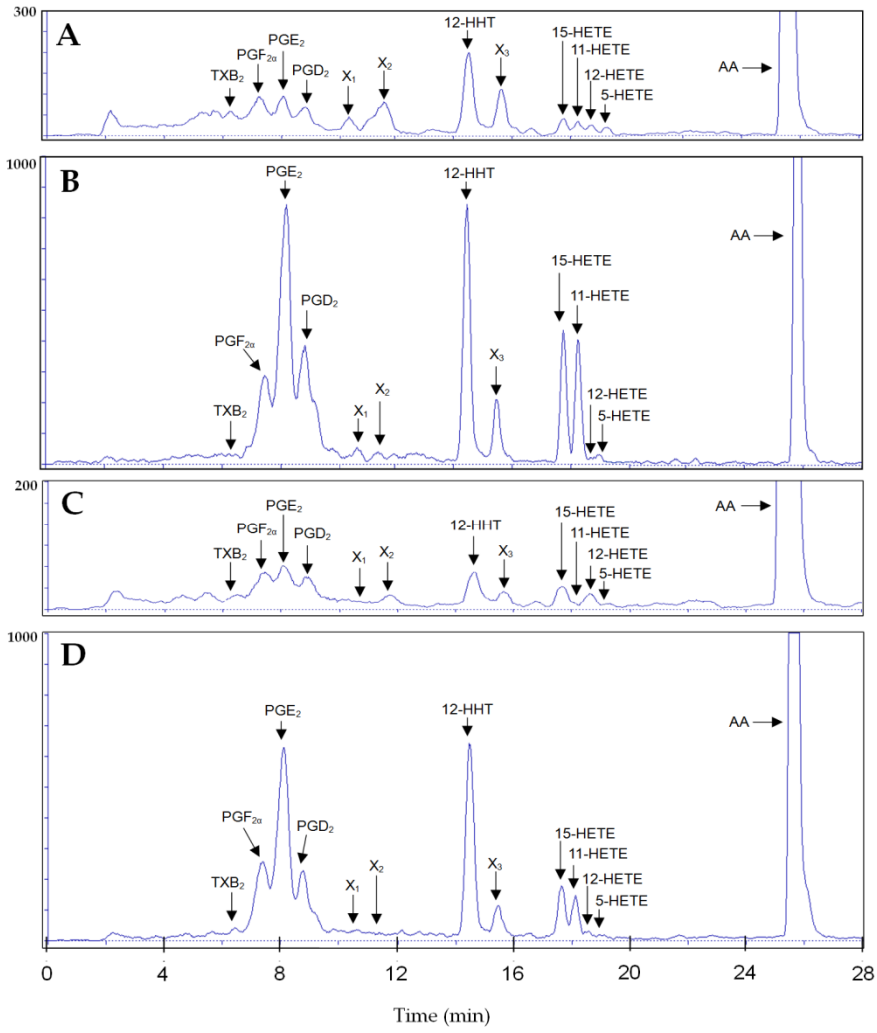


Figure 6. The HPLC chromatographs showing the separation of the [^{14}C]AA metabolites formed by COX I and II-mediated reactions. The incubation mixture (in an Eppendrof tube) consisted of 20 μM [^{14}C]AA (0.2 μCi) as substrate, COX I or COX II as the enzyme (0.5 or 0.97 μg/mL, respectively), 10 mM EDTA, 1 mM reduced glutathione, 1 μM hematin, and a stimulator chemical in 200 μL of 100 mM Tris-HCl buffer, pH 7.4. *Panels A and B*. The radioactive AA metabolites formed by COX II in the absence or presence of 100 μM myricetin as a stimulator, respectively. *Panel C and D*. The radioactive AA metabolites formed by COX I in the absence or presence of 100 μM myricetin as a stimulator, respectively. Each data point is the mean of duplicate determinations.

Stimulation of COX I and II catalytic activity *in vitro* by bioflavonoids

Optimization of AA metabolism conditions. To quantify the COX I and II-mediated metabolism of [^{14}C]AA *in vitro*, a series of assays was conducted to determine suitable concentrations of the enzymes and the substrate, as well as the incubation time for the metabolic reactions. The formation of several AA metabolites, such as $PGF_{2\alpha}$, PGE_2, PGD_2 and 12-HHT, was increased with COX I protein concentrations from 0 to 4 μg/mL and with COX II from 0 to 5 μg/mL either in the presence or absence of myricetin, a representative bioflavonoid stimulator (**Figure 7**). The K_M values for the COX II-mediated formation of $PGF_{2\alpha}$, PGE_2, PGD_2 and 12-HHT were all below 2 μM (**Table 3**). Accordingly, a saturating substrate concentration of 20 μM [^{14}C]AA was used in all *in-vitro* metabolism experiments reported here unless indicated otherwise. Notably, it was reported in several earlier studies that the COX-mediated reaction for the formation of various PG products reached a plateau very rapidly, within 1 min (Smith and Lands, 1971; Hamberg and Samuelsson, 1973; Hsuanyu and Dunford, 1992a), and our data also showed that the enzymatic reactions reached plateaus usually within 1 min of incubation. A uniform 5-min incubation time was used in all experiments presented in this study.

Effect on AA metabolite profiles. A total of some 20 naturally-occurring bioflavonoids were tested in the present study for their ability to modulate the catalytic activity of COX I and II *in vitro*, and the data are summarized in **Figure 8 and 9**. The profiles of stimulation of the COX I and II-mediated formation of AA metabolites carried for each bioflavonoids assessed. For COX II, myricetin (at 200

μM) had the highest efficacy, increasing the formation of $PGF_{2\alpha}$, PGE_2, PGD_2 and 12-HHT by 549%, 924%, 677% and 680%, respectively (**Figure 8**). Quercetin, fisetin, morin and tamarixetin also had high efficacy for stimulating the formation of some of the AA metabolites. Among the various AA metabolites formed by COX II, PGE_2 was increased with many of the bioflavonoids tested. Tamarixetin, baicalein, kaempferol, genistein, quercetin glucoside, apigenein, isorhamnetin, diosmetin, silibinin, rutin, diadzein, chrysoeriol and chrysin had moderate activity in stimulating the formation of major AA metabolites. Naringin, naringenin, hesperidin and flavone had either very weak or no appreciable effect on the COX II-mediated formation of major AA metabolites (**Figure 9**).

With COX I as the enzyme, quercetin and fisetin were the two most efficacious stimulators of PGE_2 formation (**Figure 9**). Notably, several other bioflavonoids (such as tamarixetin, genistein, chrysoeriol, diosmetin, kaempferol, apigenin, daidzein) had a preferential stimulation of the COX I-mediated formation of PGD_2 (by up to 10-fold) over other AA metabolites ($PGF_{2\alpha}$, PGE_2 and 12-HHT). Interestingly, flavone (a man-made flavonoid without any hydroxyl groups) also exerted a strong preferential efficacy in stimulating the COX I activity for the formation of PGD_2 (692%), but it only had a very weak stimulatory effect on the COX II activity. Resveratrol also inhibited COX I activity.

For comparison, the effect of phenol, the prototypical activator of the catalytic activity of COX I and II was also tested under the same conditions. The presence of phenol at 2 mM (a concentration used in many published studies) showed only a

modest stimulation of the COX I and II-mediated conversion of AA to various PG products (**Figure 8 and 9**). When phenol is compared with some of the bioflavonoids (such as quercetin and myricetin) for their ability to stimulate COX I and II-mediated formation of PGE_2, the naturally-occurring compounds had up to 20- to 30-fold higher efficacy than phenol.

Lastly, it is also of note that the degree of stimulation of COX I and II by various bioflavonoids did not show a meaningful degree of correlation, except for the formation of PGE_2 (r^2 = 0.458) and 12-HHT (r^2 = 0.299). This result suggests that although the AA metabolite profiles formed by COX I and II are very similar, their sensitivity to the stimulation by various bioflavonoids as well as the underlying mechanisms of their stimulation are likely quite different (**Figure 10**).

Concentration dependence of bioflavonoids. Using myricetin as an example, its stimulatory effect on COX I-mediated formation of various AA metabolites, such as $PGF_{2\alpha}$, PGE_2, PGD_2 and 12-HHT, was clearly concentration-dependent (**Figure 11**). The curve had a biphasic pattern: at lower concentrations (≤ 125 μM), it strongly stimulated the formation of most AA metabolites in a concentration-dependent manner and reached a plateau when 125 - 250 μM of myricetin was present. At concentrations (≥ 250 μM), myricetin started to have a concentration-dependent reduction of its stimulatory effect, and it almost returned to the basal levels (or even slightly below that) when 1000 μM of myricetin was present. The maximal stimulation of COX I-mediated formation of $PGF_{2\alpha}$, PGE_2, PGD_2, and 12-HHT by myricetin was approximately 4-, 7-, 4-, and 8-fold, respectively. Myricetin also had a

strong stimulation of the COX II-mediated formation of $PGF_{2\alpha}$, PGE_2, PGD_2 and 12-HHT (approximately 6-, 10-, 8-, and 8-fold, respectively, of the controls) (**Figure 11**). Notably, myricetin's biphasic pattern of regulation of COX II-mediated formation of $PGF_{2\alpha}$, PGE_2 and PGD_2 was somewhat less pronounced compared to COX I, with a reduced self-inhibition when it was present at higher concentrations (**Figure 11**). In addition, the concentration-dependent effects of quercetin, fisetin, and morin on the catalytic activity of COX II *in vitro*, had similar patterns as described above (**Figure 11**).

Mechanisms for the stimulation of COX II activity by bioflavonoids

Enzyme kinetics. To evaluate the enzyme kinetics for the stimulation of COX I and II activity by dietary polyphenols, several representative bioflavonoids (myricetin, quercetin, fisetin, and morin) were chosen for further studies. With a focus on analyzing the formation of $PGF_{2\alpha}$, PGE_2, PGD_2, 12-HHT, as major products. The Michaelis-Menten curves for the COX I and II-mediated formation of representative AA metabolites in the presence of myricetin is shown in **Figure 12**. All kinetic parameters (K_M, V_{MAX}, and V_{MXA}/K_M) for COX I and II-mediated formation of $PGF_{2\alpha}$, PGE_2, PGD_2, 12-HHT, 5-HETE, 11-HETE, 12-HETE and 15-HETE in the presence or absence of different bioflavonoids were calculated using curve regression analyses and were summarized in **Table 3** for comparison. The presence of a bioflavonoid greatly increased the V_{MAX} values (up to 11-fold), although the apparent the K_M value for the formation of PGE_2 was also increased (approximately 2.5-fold). In most cases,

the V_{MXA}/K_M values were significantly increased, which shows an increased catalytic efficiency for AA metabolism by COX I and II *in vitro*. It is of note that while 15-HETE was a quantitatively major metabolite formed by COX I and II, its apparent K_M values were unusually high compared to all other AA metabolites characterized (**Table 3**).

Time course of the stimulation. In the absence of any bioflavonoids, the COX II-mediated formation of PGE_2 and other AA metabolites reached a plateau within 1 min (sometimes as short as 20 to 30 seconds). However, in the presence of 100 μM myricetin, the amount of PGs formed by COX II increased continuously for nearly 5 min (**Figure 13**). When myricetin was added to the reaction mixtures after a 3-min incubation at 37°C, the formation of PGs was not further increased (**Figure 13**). This observation suggests that the presence of myricetin only slows down the self-inactivation of the COX enzyme, but it does not restore the enzyme molecules that are already inactivated.

Effect of bioflavonoids on PGE_2 formation in RAW264.7 cells

A number of experiments were conducted to determine the modulating effect of representative dietary bioflavonoids on PG formation in cultured RAW264.7 cells that were pretreated with LPS for 4 h to induce COX II expression. Western blot analysis showed that treatment of murine RAW264.7 cells with LPS increased the protein levels of COX II, but not COX I (**Figure 14**). In comparison, treatment with myricetin alone or in combination with LPS did not appreciably alter COX II protein

levels in these cells (**Figure 14**). These data showed that pretreatment of RAW264.7 cells with LPS significantly and selectively induced the levels of COX II protein but not the COX I protein.

Next the effect of these bioflavonoids on the levels of PGE_2 (a representative PG) in the culture media of LPS-pretreated RAW264.7 cells were determined by the use of EIA kit. Cells pretreated with LPS had marked increase the formation of PGE_2, and this formation was almost completely abolished when 10 μM NS-398 (a specific COX II inhibitor) or 10 μM indomethacin (a nonselective COX inhibitor) was present (**Figure 15A**). These data suggest that the PGE_2 formed by LPS-pretreated RAW264.7 cells was mainly catalyzed by the COX II enzyme that was selectively induced during LPS pretreatment. Addition of myricetin, quercetin, fisetin or morin to LPS-pretreated RAW264.7 cells exhibited a dual pattern of concentration-dependent stimulation and inhibition of PGE_2 formation (**Figure 15B**). At lower concentrations (≤1 μM), the formation of PGE_2 was stimulated in a concentration-dependent manner by the presence of these dietary compounds, and the stimulation reached a plateau when the concentration reached approximately 1 μM. However, at higher concentrations (>10 μM), these dietary compounds showed a concentration-dependent inhibition of PGE_2 formation (**Figure 15B**). In comparison, flavone (which does not have a stimulatory effect on COX II-mediated formation of PGs *in vitro*; see **Figure 8 and 9**) did not have a stimulatory effect on the formation of PGE_2 in LPS-pretreated RAW264.7 cells, instead it inhibited PGE_2 formation in a concentration-dependent manner, which is consistent with the finding from *in-vitro* enzymatic

assays.

Effect of bioflavonoids on the *in vitro* catalytic activity of LOXs

To determine whether the observed direct stimulation of COX activity by certain bioflavonoids is an enzyme-specific phenomenon, also examined their effects on LOX-mediated AA metabolism. The formation of 12-HETE by 12-LOX and the formation of 15-HETE by 15-LOX was uniformly inhibited by all bioflavonoids tested (**Figure 16**). Notably, when COX I and II were used as the enzymes, the formation of small amounts of various HETEs was also detected. Interestingly, some of the bioflavonoids stimulated the COX-mediated formation of HETEs (particularly 11-HETE and 15-HETE) in a similar way as they stimulated the COX-mediated formation of PGs (**Figure 6**).

Table 3. Kinetic parameters (K_M and V_{MAX} values) for the COX-mediated formation of $PGF_{2\alpha}$, PGE_2, PGD_2 and 12-HHT.

		COX II			COX I		
Product	Myricetin	K_M (μM)	V_{MAX} (nmol/μg/5 min)	V_{MAX} / K_M	K_M (μM)	V_{MAX} (nmol/μg/5 min)	V_{MAX} / K_M
$PGF_{2\alpha}$	0 μM	1.8	38	20.8	4.0	23	5.8
	100 μM	9.9	236	23.8	4.5	90	20
PGE_2	0 μM	1.2	41	34.2	1.9	29	15.3
	100 μM	5.5	457	83.0	2.2	160	72.7
PGD_2	0 μM	0.9	30	33.3	5.8	19	3.3
	100 μM	2.4	179	74.6	5.3	72	13.6
12-HHT	0 μM	1.0	44	43.5	1.5	26	17.3
	100 μM	2.6	375	144.0	1.8	190	105.6
5-HETE	0 μM	4.2	7	1.7	4.2	4	1
	100 μM	13	7	0.5	6.4	2	0.3
11-HETE	0 μM	9.7	14	1.4	4.6	5	1.1
	100 μM	15.4	142	9.2	4.8	35.2	7.3
12-HETE	0 μM	8.4	11	1.3	5	13	2.6
	100 μM	15.6	7	0.4	6.6	2	0.3
15-HETE	0 μM	8.2	16	2.0	261.3	222	0.8
	100 μM	35.2	168	4.8	126.1	230	1.8

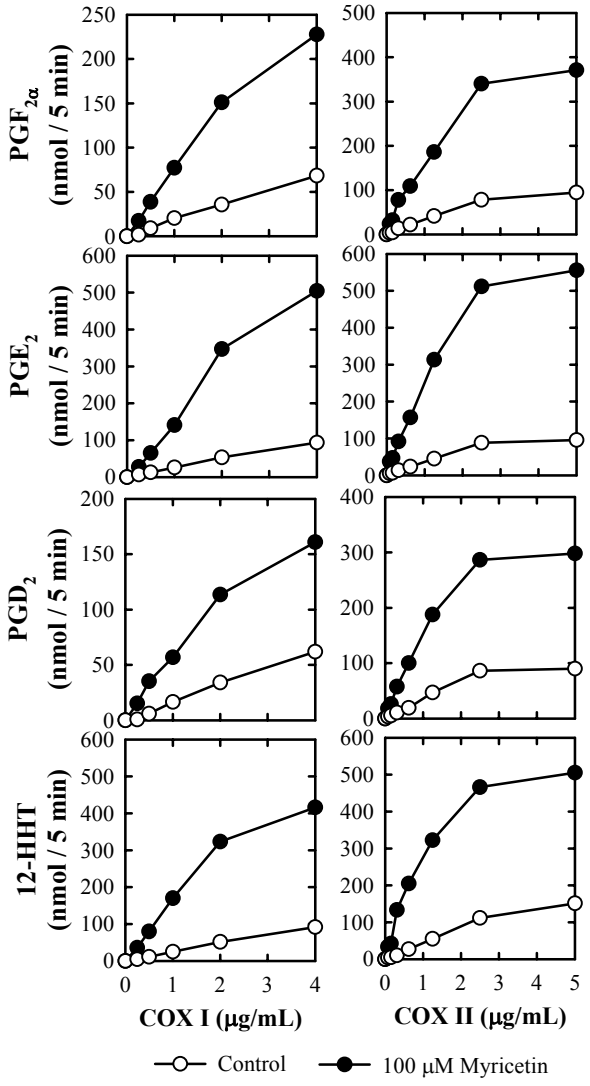


Figure 7. Effect of the COX concentrations on the formation of several quantitatively-predominant AA metabolites ($PGF_{2\alpha}$, PGE_2, PGD_2 and 12-HHT) in the absence (open circle) or presence of 100 μM myricetin (filled circle). The incubation mixtures consisted of 20 μM [^{14}C]AA (0.2 μCi) as substrate, different concentrations of COX I or COX II as indicated, 10 mM EDTA, 1 mM reduced glutathione, 1 μM hematin, and with or without 100 μM myricetin in 200 μL of 100 mM Tris-HCl buffer, pH 7.4. The incubations were carried out at 37°C for 5 min. Each data point is the mean of duplicate determinations.

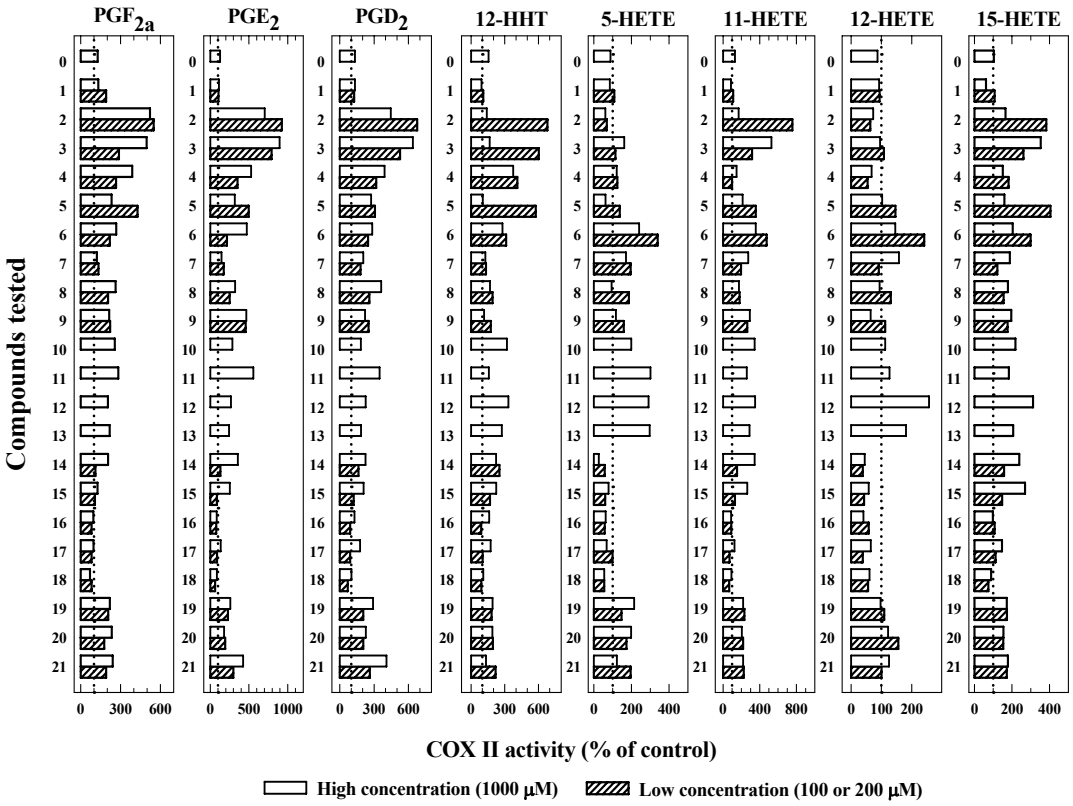


Figure 8. Effect of bioflavonoids on the catalytic activity of COX II *in vitro*. The incubation mixtures consisted of 20 μM [^{14}C]AA (0.2 μCi) as substrate, COX II (at 0.97 μg/mL) as the enzyme, a test compound, 10 mM EDTA, 1 mM reduced glutathione, and 1 μM hematin in 200 μL of 100 mM Tris-HCl buffer, pH 7.4. The incubations were carried out at 37°C for 5 min. Note that for most test compounds, the high concentration (open bar) used was 1 mM, and the low concentration (filled bar) was either 100 μM (compounds 14, 15, 16, 17 and 18) or 200 μM (compounds 1, 2, 3, 4, 5, 6, 7, 8, 9, 19, 20 and 21). For some of the test compounds, only one concentration (1 mM) was tested. Compound ***0*** is phenol. The structures of all test compounds are listed in **Figure 5**. Each data point is the mean of duplicate determinations. The dotted line denotes the control activity in the absence of any test compound.

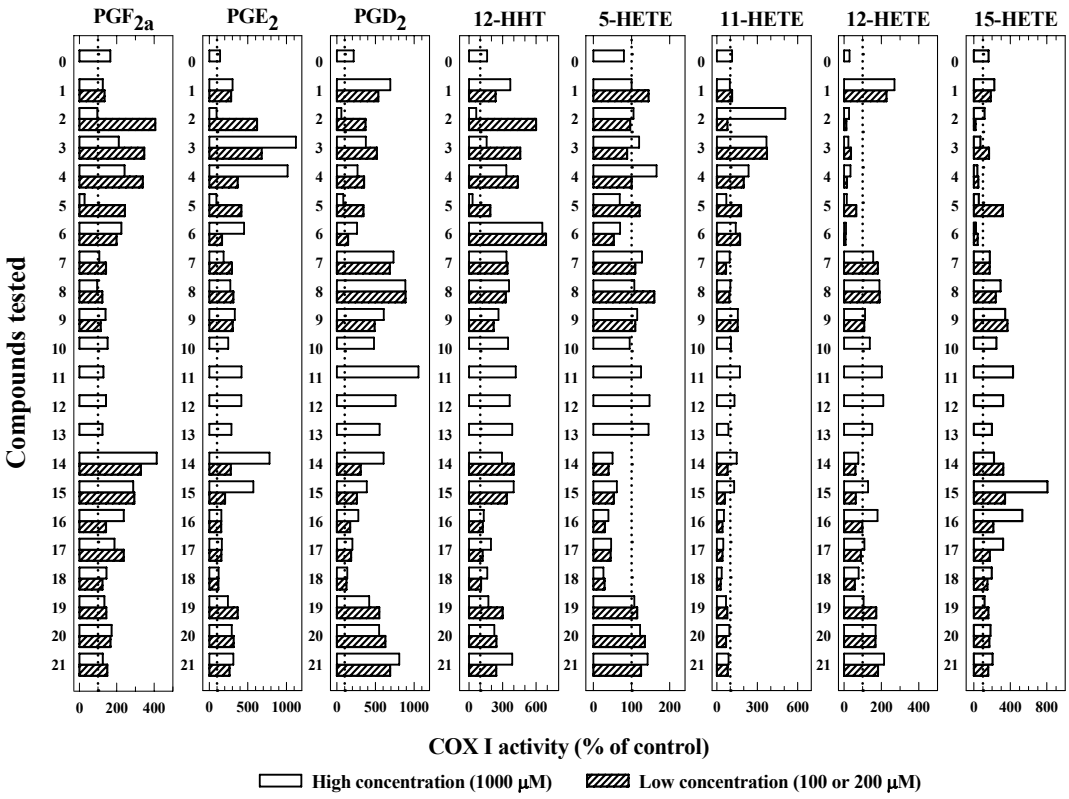


Figure 9. Effect of bioflavonoids on the catalytic activity of COX I *in vitro*. The incubation mixtures consisted of 20 μM [14C]AA (0.2 μCi) as substrate, COX I (at 0.5 μg/mL) as the enzyme, a test compound, 10 mM EDTA, 1 mM reduced glutathione, and 1 μM hematin in 200 μL of 100 mM Tris-HCl buffer, pH 7.4. The incubations were carried out at 37°C for 5 min. Note that for most test compounds, the high concentration (open bar) used was 1 mM, and the low concentration (filled bar) was either 100 μM (compounds 14, 15, 16, 17 and 18) or 200 μM (compounds 1, 2, 3, 4, 5, 6, 7, 8, 9, 19, 20 and 21). For some of the test compounds, only one concentration (1 mM) was tested. Compound 0 is phenol. The structures of all test compounds are listed in **Figure 5**. Each data point is the mean of duplicate determinations. The dotted line denotes the control activity in the absence of any test compound.

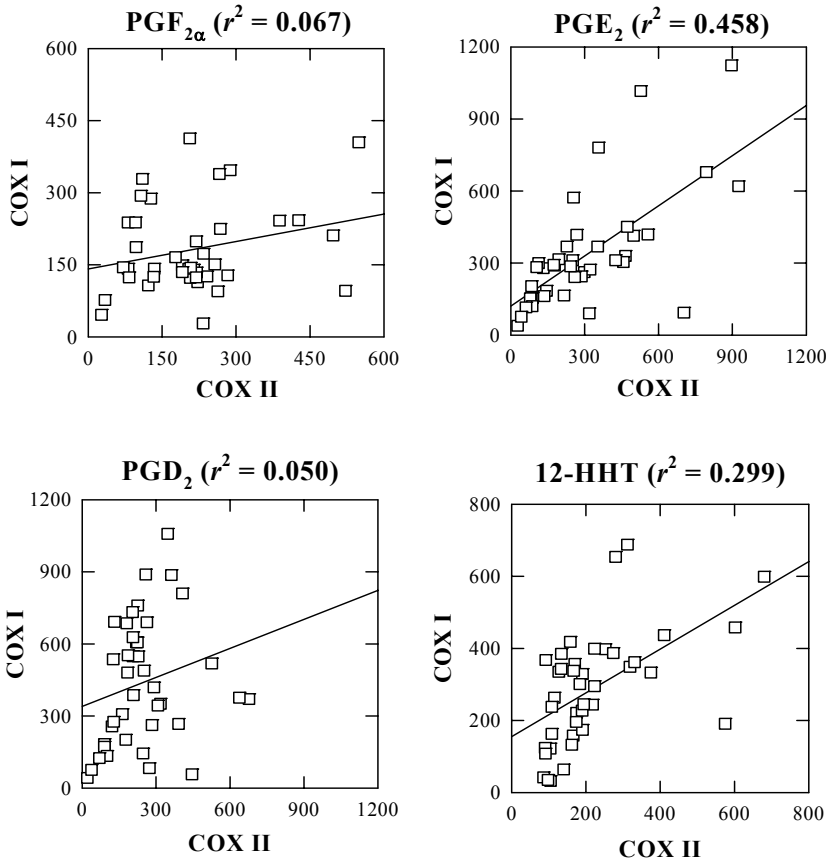


Figure 10. The relationship between the stimulatory effect of various bioflavonoids (values shown in Figure 8 and 9) on the COX I and II-mediated formation of $PGF_{2\alpha}$, PGE_2, PGD_2 and 12-HHT.

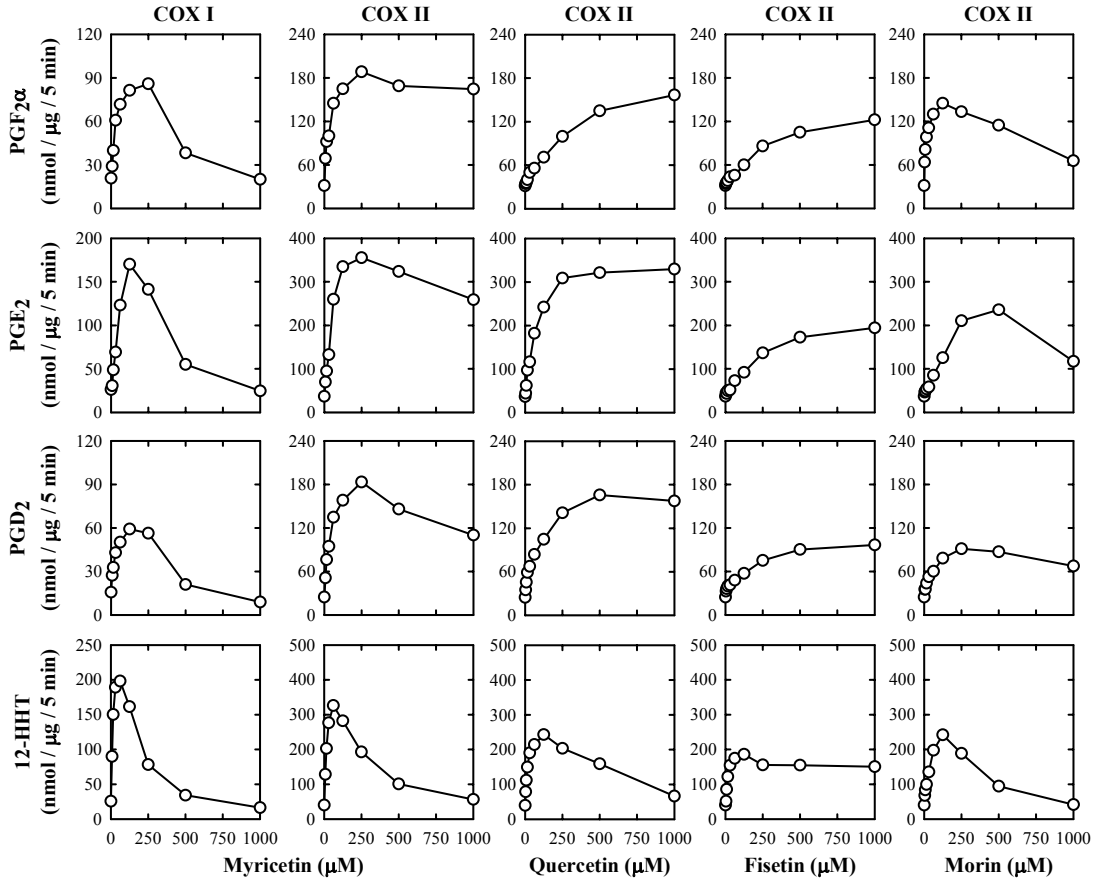


Figure 11. Effect of myricetin, quercetin, fisetin and morin on the COX activity *in vitro*. The incubation mixtures consisted of 9 different concentrations (0, 7.8, 15.6, 31.3, 62.5, 125, 250, 500 and 1000 μM) of myricetin, quercetin, fisetin or morin as the stimulator, 20 μM [^{14}C]AA (0.2 μCi) as substrate, COX I or COX II as the enzyme (0.5 or 0.97 μg/mL, respectively), 10 mM EDTA, 1 mM reduced glutathione, and 1 μM hematin in 200 μL of 100 mM Tris-HCl buffer, pH 7.4. The incubations were carried out at 37°C for 5 min. Each data point is the mean of duplicate determinations.

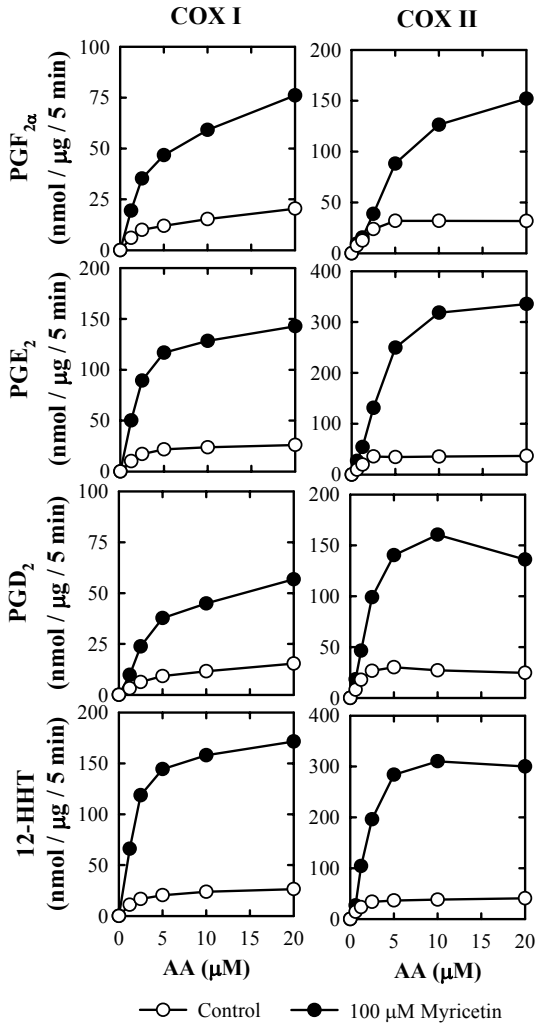


Figure 12. The dependence of AA concentrations on the formation of several quantitatively-predominant AA metabolites ($PGF_{2\alpha}$, PGE_2, PGD_2 and 12-HHT) when 100 μM myricetin of present (filled circle) or absence (open circle). The incubation mixture consisted of indicated [^{14}C]AA (0.2 μCi) as substrate, COX I or COX II as the enzyme (0.5 or 0.97 μg/mL, respectively), 10 mM EDTA, 1 mM reduced glutathione, 1 μM hematin, and with (filled circle) or without (open circle) 100 μM myricetin in 200 μL of 100 mM Tris-HCl buffer, pH 7.4. The incubations were carried out at 37°C for 5 min. Each data point is the mean of duplicate determinations. The kinetic paramters (K_M, V_{MAX} and V_{MAX} /K_M) for all AA metabolites were summarized in **Table 3**.

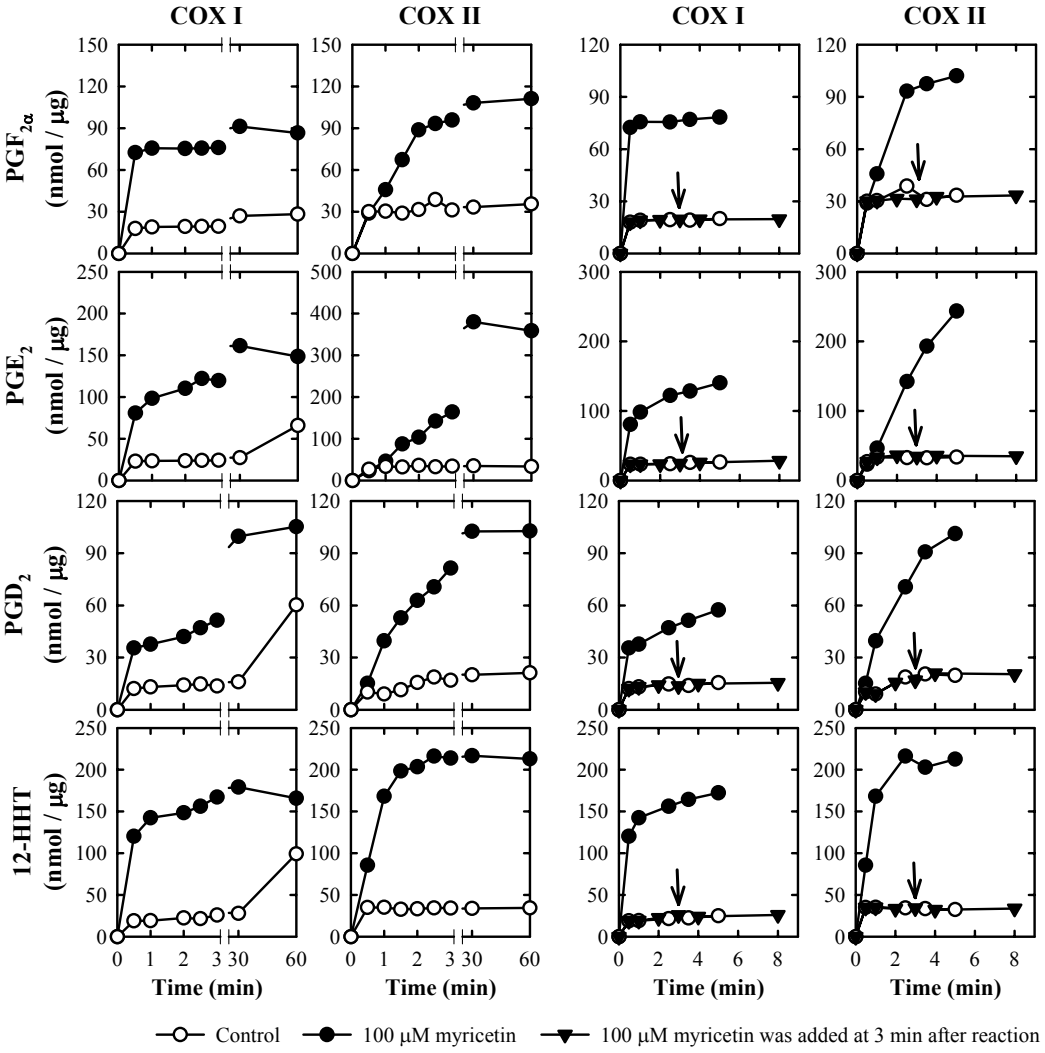


Figure 13. Effect of incubation time on the formation of several quantitatively-predominant AA metabolites ($PGF_{2\alpha}$, PGE_2, PGD_2 and 12-HHT) when 100 μM myricetin was present (filled circle) or absence (open circle). The incubation mixture consisted of 20 μM [^{14}C]AA (0.2 μCi) as substrate, COX I or COX II as the enzyme (0.5 or 0.97 μg/mL, respectively), 10 mM EDTA, 1 mM reduced glutathione, 1 μM hematin, and with or without 100 μM myricetin in 200 μL of 100 mM Tris-HCl buffer, pH 7.4. The incubations were carried out at 37°C for indicated time. Each data point is the mean of duplicate determinations. An arrow indicates the addition of myricetin at 3 min after incubation.

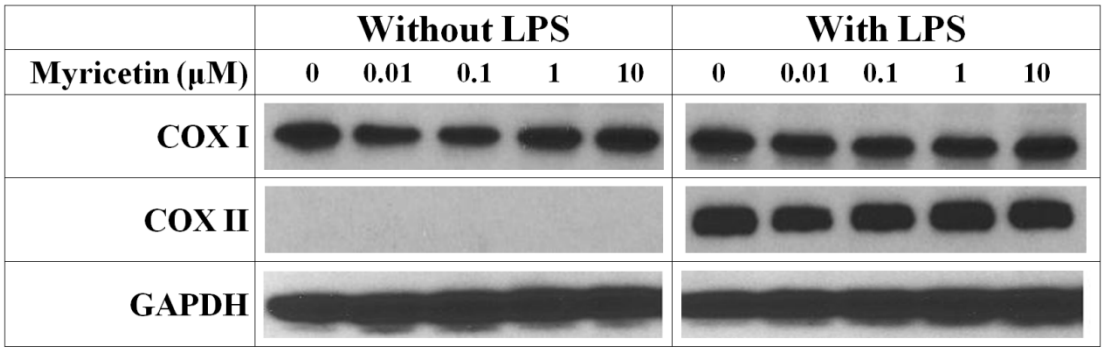


Figure 14. Effect of LPS and myricetin on COX I and COX II protein levels in RAW264.7 cells in culture. After treatment with 1 μg/mL LPS for 2 h, RAW264.7 cells were incubated with different concentrations of myricetin for an additional 2 h. Western blot analysis of cell lysates was performed with antibodies specific for COX I or COX II, coupled with a secondary antibody conjugated with horseradish peroxidase. A total of three experiments were conducted, and a representative data set from one of the experiments was shown.

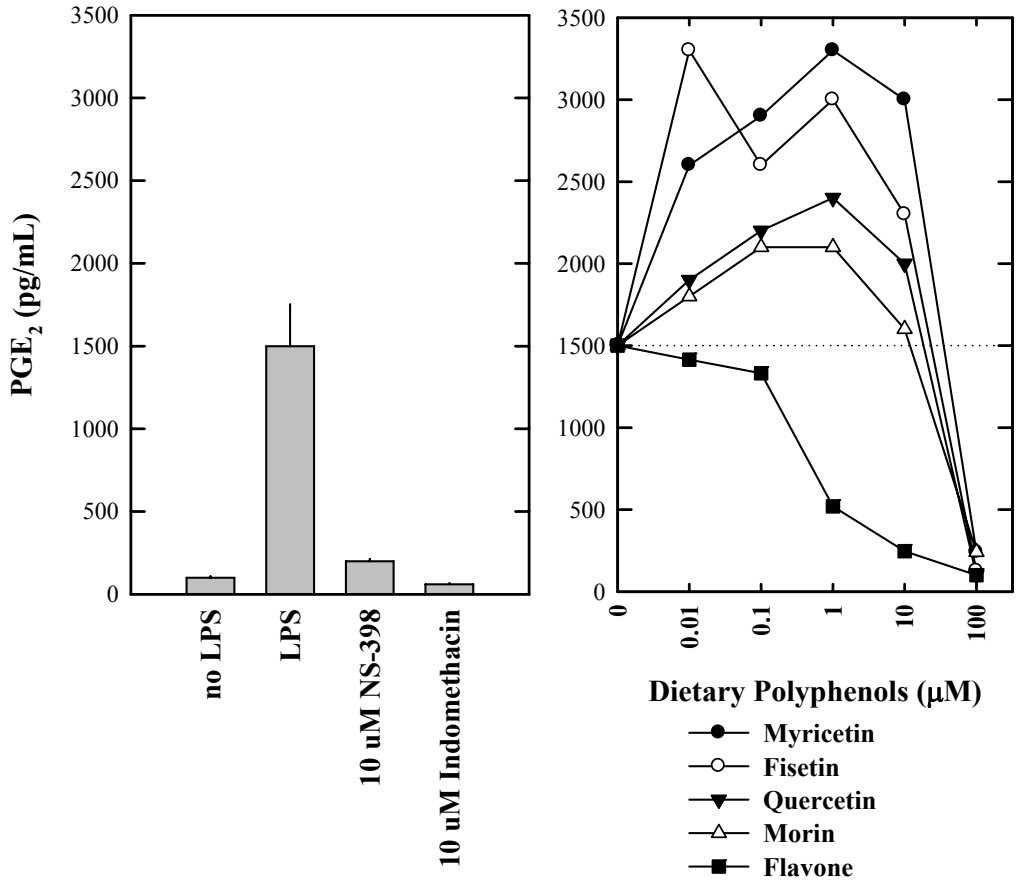


Figure 15. Effect of representative dietary bioflavonoids on the release of PGE_2 from LPS-pretreated RAW264.7 cells in culture. Cells were pretreated with 1 μg/mL LPS for 2 h to induce COX II expression, and then the culture media were removed and replaced with 300 μL serum-free medium with or with the test compound (myricetin, fisetin, quercetin or morin) for an additional 2 h. The following concentrations of the test compounds were used: 0.01, 0.1 1, 10, and 100 μM. NS-398 (a COX II specific inhibitor, at 10 μM) and indomethacin (a non-specific COX inhibitor, at 10 μM) were also tested for comparison. The levels of PGE_2 were measured using an EIA (Cayman, Ann Arbor, MI). Each point was the mean of duplicate determinations.

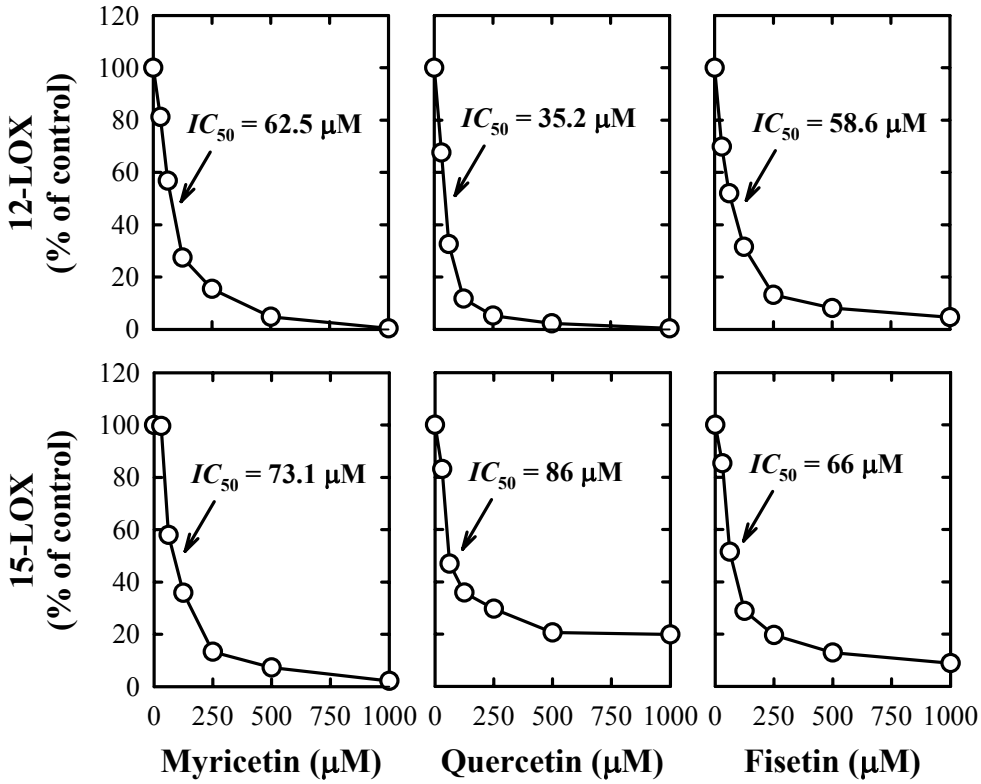


Figure 16. The inhibitory effect of myricetin, quercetin, and fisetin on the catalytic activity of 12-LOX and 15-LOX *in vitro*. The incubation mixture consisted of 7 different concentrations (0, 31.3, 62.5, 125, 250, 500, and 1000 μM) of quercetin, fisetin or morin, 20 μM [^{14}C]AA (0.2 μCi) as substrate, and 12-LOX or 15-LOX as the enzyme (72 μg/mL or 75 ng/mL, respectively) in a final volume of 200 μL of 100 mM Tris-HCl buffer, pH 7.4. The incubations were carried out at 37°C for 5 min. Each data point is the mean of duplicate determinations.

DISCUSSION

Direct stimulation of the catalytic activity of COX I and II by bioflavonoids

In 1980, Baumann et al. first reported that some of the dietary polyphenols, such as galangin and luteolin, inhibited the COX-mediated AA peroxidation (Baumann et al., 1980). Since then, several researchers have also reported that other dietary polyphenols, including many of the common bioflavonoids tested in the present study, inhibited the catalytic activity of COX I and II *in vitro* and *in vivo* (Kalkbrenner et al., 1992; Mahmoud et al., 2000). In addition, bioflavonoids have been reported to inhibit LOX activity (Welton et al., 1986). The effect of polyphenols on the catalytic activity of 5-LOX and 12-LOX has been studied quite extensively in the past in an effort to better understand their anti-inflammatory properties (Chi et al., 2001). This inhibitory effect has often been used as a mechanistic explanation for their chemopreventive effect against certain types of cancer (Ju et al., 2005).

In the present study, we have evaluated some 20 naturally-occurring bioflavonoids for their ability to modulate the catalytic activity of COX I and II *in vitro*. To our surprise, some of the common bioflavonoids, such as myricetin, quercetin and fisetin, were found to have a powerful, direct stimulatory effect (up to 11-fold increase) on the COX I and II-mediated formation of PGE_2 and other PG products. This stimulatory effect of bioflavonoids was enzyme-specific because none of them stimulated the catalytic activity of LOXs. Compared to phenol, a prototypical COX stimulator *in vitro*, the naturally-occurring bioflavonoids are up to 30 times more efficacious in stimulating the COX activity. To the best of our knowledge, these

natural compounds are the most powerful direct stimulators of the *in vitro* COX I and II catalytic activity known to date.

In addition, we have also conducted experiments using cultured cells which clearly showed that these bioflavonoids could strongly stimulate COX-mediated formation of PGE_2 (a representative PGs) in intact cells, and a strong stimulatory effect was observed at physiologically achievable concentrations (10 to 100 nM). These data provide support for the novel concept that these dietary bioflavonoids are physiologically-relevant activators of the COX I and II activity *in vivo*.

Here it should be noted that the potency of these dietary compounds in intact cells (**Figure 15**) is much higher than what was seen in the *in vitro* enzymatic assays (**Figure 8, 9 and 11**). This discrepancy may lie in the difference between the *in-vitro* reaction conditions which may not be best suited for studying the interactions of the highly-lipophilic COX enzymes with the water-soluble dietary compounds. In partial support of this explanation, we noticed that the concentration-dependent dual stimulation and inhibition was also observed in almost exactly the same pattern in both *in-vitro* biochemical assays and intact cells, although the COX enzymes in intact live cells were far more sensitive to the actions of these dietary compounds than the COX enzymes used in the *in-vitro* biochemical assays.

The exact molecular mechanism by which bioflavonoids stimulate the catalytic activity of COX I and II is not precisely understood at present. As observed in the present study and also in several other studies (Kulmacz, 1986; MacDonald et al., 1989; Hsuanyu and Dunford, 1990; Hsuanyu and Dunford, 1992b), COX I and II

rapidly undergo suicidal inactivation, which likely is mediated by chemically-reactive radical intermediates formed during enzymatic catalysis of cyclooxidation and peroxidation. Data from our present study showed that bioflavonoids mainly act to slow down the suicidal inactivation of the COX enzymes, but they cannot reactivate the inactivated enzymes.

Bioflavonoids are known to have strong radical-scavenging activity as well as antioxidant activity (Pietta, 2000). Earlier structure-activity relationship studies have shown that the presence of a 3-hydroxyl group in the heterocyclic *C*-ring, a catechol group in the *B*-ring, and a C2-C3 double bond of a bioflavonoid favored its antioxidant and free radical-scavenging activity (Middleton and Kandaswami, 1994; Cos et al., 1998; Pietta, 2000; Bors and Michel, 2002). The stimulatory effect of bioflavonoids on COX-mediated AA metabolism may be contributed, in part, by their radical-scavenging properties. It is possible that the hydroxyl groups of bioflavonoids may bind to the enzymes to act as electron donors, accelerating their peroxidative activity to convert PGG_2 to PGH_2. However, the free radical-scavenging activity of various bioflavonoids is not believed to be a major mechanism of their actions because the degree of stimulation of COX activity by bioflavonoids did not correlate with the number of hydroxyl groups present at their *B*-ring. Notably, flavone (a synthetic compound with no hydroxyl groups attached to it) also showed strong, selective activity in stimulating the formation of certain AA metabolites. This observation is rather intriguing, and it sheds light on the mechanisms of the stimulatory actions of these bioflavonoids.

Conclusion

In the present study, some 20 naturally-occurring bioflavonoids were examined for their ability to stimulate the catalytic activity of COX I and II in PG biosynthesis *in vitro*. Bioflavonoids, such as myricetin, quercetin, fisetin and morin, have a powerful direct stimulatory effect on the COX I and II activity, and are perhaps the most efficacious stimulators of the COX I and II catalytic activity known to date. Given the large amount of pre-existing information in the literature concerning the inhibitory effect of dietary polyphenols on the COX activity, these intriguing findings suggests that bioflavonoids may represent a unique class of physiological, high-efficacy stimulators of the COX I and II activity *in vivo*.

CHAPTER FIVE

SPECIFIC AIM 2

MYRICETIN AND QUERCETIN ARE NATURALLY-OCCURRING CO-SUBSTRATES OF CYCLOOXYGENASES *IN VIVO*.

HYPOTHESIS

Some dietary bioflavonoids modulate the formation of PG products in vivo catalyzed by COX I and II.

RATIONALE

COX I and II catalyze the metabolism of AA, resulting in the formation of PGs, TXs and HETEs (Hamberg and Samuelsson, 1967; Miyamoto et al., 1976; Marnett, 2000; Kurumbail et al., 2001). These autacoids exert numerous bological actions in the body through activation of specific membrane receptors (Regan, 2003; Lee et al., 2005; Sung et al., 2005). Although inhibitors of COX I and II are effectively used to treat a number of medical conditions (such as chronic inflammation) where their activity is abnormally elevated, it is also known that abnormally-low levels of COX activity are associated with a number of severe pathological conditions, including gastrointestinal ulceration and bleeding (Silverstein et al., 2000; Moore et al., 2006; Blandizzi et al., 2009) and cardiovascular diseases (low levels of PGI_2) (Gottlieb, 2001).

Therefore, it is evident that the basal levels of COX activity and, ultimately, the basal circulating and tissue levels of AA-derived autacoids need to be maintained within a normal range in order to exert many of their normal physiological functions. Thus, lower basal levels of the COX I and II activity are not always better or beneficial for optimal health. However, the physiological importance of maintaining normal levels of COX activity and PGs has not received much attention until very

recently in light of the clinical observations of severe adverse effects seen in patients chronically receiving selective COX II inhibitors such as celecoxib and rofecoxib (Bombardier et al., 2000; Silverstein et al., 2000; Gottlieb, 2001; White et al., 2002).

It was found that some of the dietary polyphenols are powerful activators of the catalytic activity of COX I and II in the *in vitro* enzyme assays as well as in cultured cells, resulting in increased formation of various AA metabolites (Bai and Zhu, 2008). Therefore, the effect of two representative dietary compounds (quercetin and myricetin) on plasma and tissue levels of several PG products will be determined using an animal model.

RESULTS

Effect of myricetin and quercetin on plasma levels of PGs

First the plasma levels of several PG products were determined in male Sprague-Dawley rats following a single *i.v.* injection of myricetin or quercetin at two very low doses: 0.1 and 0.3 mg/kg b.w. (approximately 25 or 75 μg/rat, respectively). The reason that the *i.v.* route of administration was selected was to minimize some of the confounding factors in these initial experiments that sought to determine whether or not some of the bioflavonoids identified as strong activators of the COX-mediated formation of various PG products *in vitro* (Bai and Zhu, 2008) were also activators *in vivo*. Using PGE_2 as an example [one of the major PG products detected in our *in vitro* study (Bai and Zhu, 2008)], the time dependence for the changes in its plasma levels were determined. It was found that both quercetin and myricetin at the two

very low doses, markedly increased the plasma levels of PGE_2 in a time-dependent manner (**Figure 17A and 17B**). The effect elicited by quercetin at the 0.1 and 0.3 mg/kg b.w. doses was similar, and the formation of PGE_2 reached a plateau at ≈1 h after quercetin administration. In comparison, the plasma levels of PGE_2 in animals after receiving an *i.v.* injection of 0.1 and 0.3 mg/kg b.w. of myricetin reached plateau in 6 and 2 h, respectively. It appeared that myricetin had a relatively slower onset of effect than quercetin, but the former was more efficacious than the latter for increasing the plasma levels of PGE_2. The maximal increase in PGE_2 plasma levels following administration of myricetin and quercetin was approximately 10- and 8-fold, respectively, over the basal levels in vehicle-treated animals (**Figure 17A and 17B**).

The plasma levels of three other PGs (PGD_2, $PGF_{2\alpha}$ and TXB_2) were also increased at a selected time point (4 h) post injection of quercetin or myricetin. TXB_2, a metabolite of TXA_2, was increased by approximately 5-fold in the plasma following administration of myricetin or quercetin, and it became the quantitatively-predominant arachidonic acid metabolite detected (**Figure 17E**). The basal plasma levels of PGD_2 and $PGF_{2\alpha}$ were very low in untreated animals, and treatment with myricetin or quercetin significantly increased their levels (**Figure 17C and 17D**).

The dose dependence of the stimulatory effect of quercetin and myricetin on plasma PGE_2 levels in male Sprague-Dawley rats was assessed over a wide dose range, (*i.e.*, at 0.05, 0.1, 0.3, 1 and 5 mg/kg b.w.; single *i.v.* injection). Our data showed that the stimulatory effect of these two bioflavonoids on COX-mediated

formation of PGE_2 was clearly dose-dependent and followed a unique biphasic pattern (**Figure 18**). At lower doses (< 0.3 mg/kg b.w.), these two bioflavonoids strongly stimulated the formation of PGE_2 in a dose-dependent manner. However, at higher doses (> 0.3 mg/kg b.w.), myricetin and quercetin had a lessor stimulatory effect, almost returning to the original basal levels when 5 mg/kg b.w. myricetin or quercetin was injected. The maximal increase in PGE_2 plasma levels following administration of myricetin or quercetin was seen with the 0.3 mg/kg b.w. dose (**Figure 18**). Notably, the biphasic pattern of regulation of PGE_2 plasma levels closely resembled the curve patterns for their stimulation of the formation of PGE_2 *in vitro* catalyzed by COX I or COX II or in intact cells in culture (Bai and Zhu, 2008).

Plasma levels of PGE_2 were also measured in male Sprague-Dawley rats following a single oral administration of quercetin (at 10 and 50 mg/kg b.w.). Again using PGE_2 as an example, the time dependence for the changes of PG plasma levels was assessed. Quercetin increased the plasma levels of PGE_2 in a time-dependent manner at the doses of 10 and 50 mg/kg b.w. (**Figure 19**). The formation of PGE_2 reached a plateau at approximately 6 h following administration of quercetin, and the maximal increase was approximately 3-fold (**Figure 19**).

Effect of myricetin and quercetin on tissue levels of PGs

In a separate experiment, the tissue levels of various AA metabolites (PGE_2, PGD_2, $PGF_{2\alpha}$ and TXB_2) were measured in animals that received a single *i.v.* injection of 0.1 mg/kg b.w. of myricetin or quercetin 4 h earlier (data shown in **Figure 20**). As

summarized in **Figure 20A**, the basal levels of these PG products varied for different in different tissues. For instance, while comparable levels of PGE_2 were present in rat liver, brain and lung, its level in the small intestine was unusually high, approximately 30 times higher than that in the kidney. PGD_2 had its highest level in the small intestine, followed by lung, brain, and liver. In the blood, PGE_2 and TXB_2 were the two quantitatively-predominant PGs, whereas PGD_2 and $PGF_{2\alpha}$ were present at rather low levels.

Treatment of animals with myricetin and quercetin differentially increased the levels of these PG products in different tissues. Myricetin and quercetin increased the levels of PGE_2 by ~5-fold in liver, 3-fold in kidney, and less than 2-fold in small intestine, lung and brain. Similarly, these two dietary compounds also increased the levels of PGD_2 in these tissues. For $PGF_{2\alpha}$, the major increase was observed in kidney (5-fold), followed by lung and small intestine, and the increase in liver and brain was rather small. For TXB_2, a major increase (approximately 5-fold) was observed in liver, followed by small intestine, and its increase in kidney, brain and lung was relatively small. In this experiment, the blood levels of PGs (shown in **Figure 20G**), matched the findings from the other experiments (**Figure 17**).

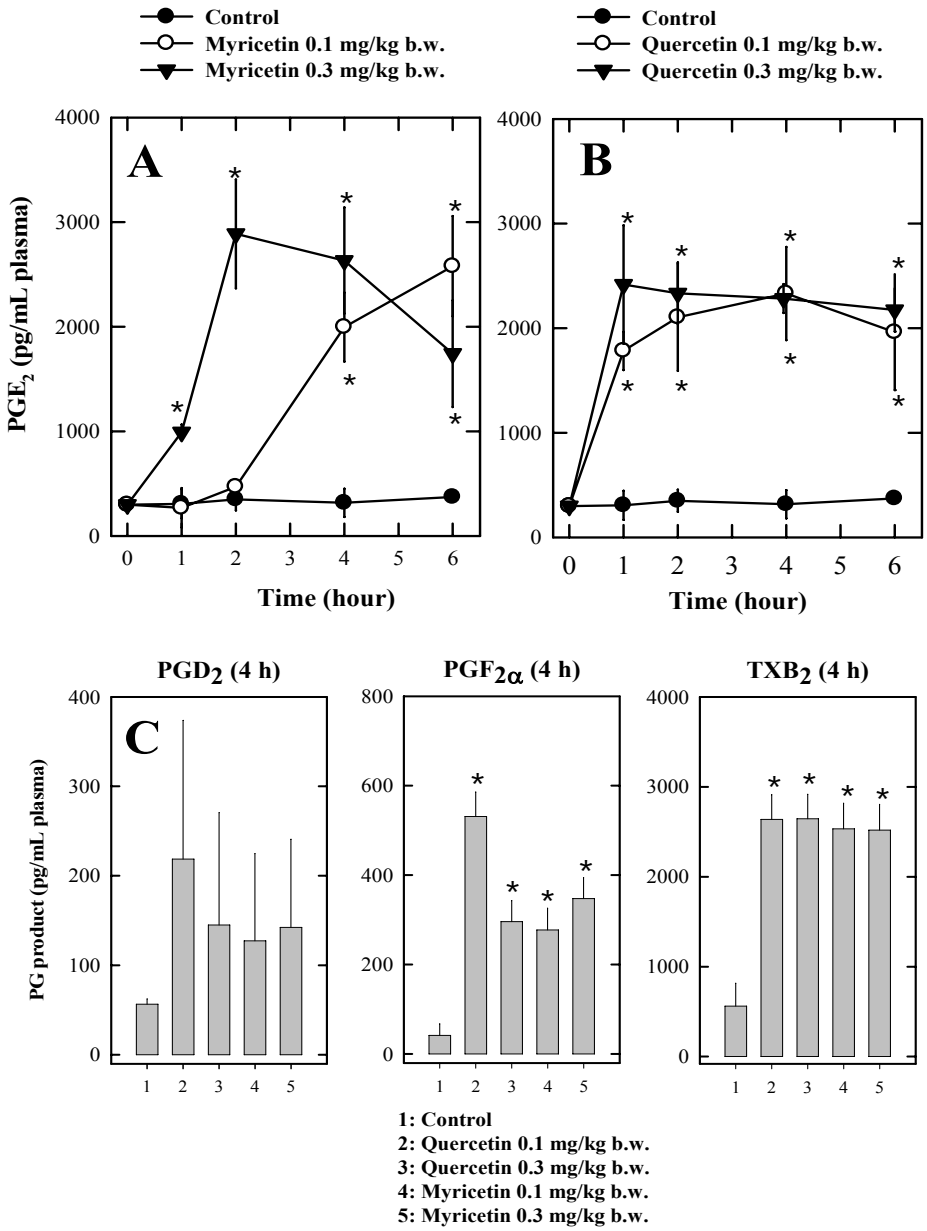


Figure 17. The plasma levels of PGE_2 in rats injected *i.v.* with 0.1 or 0.3 mg/kg b.w. of myricetin or quercetin. The blood samples were collected from the tail vein for up to 6 h. The plasma was immediately prepared and stored at −80°C for measurement of PGs (PGE_2, PGD_2, $PGF_{2\alpha}$, and TXB_2) by using EIA kits. Data represent mean ± S. D. (n = 4). * $P < 0.05$ compared to the corresponding values of the control group.

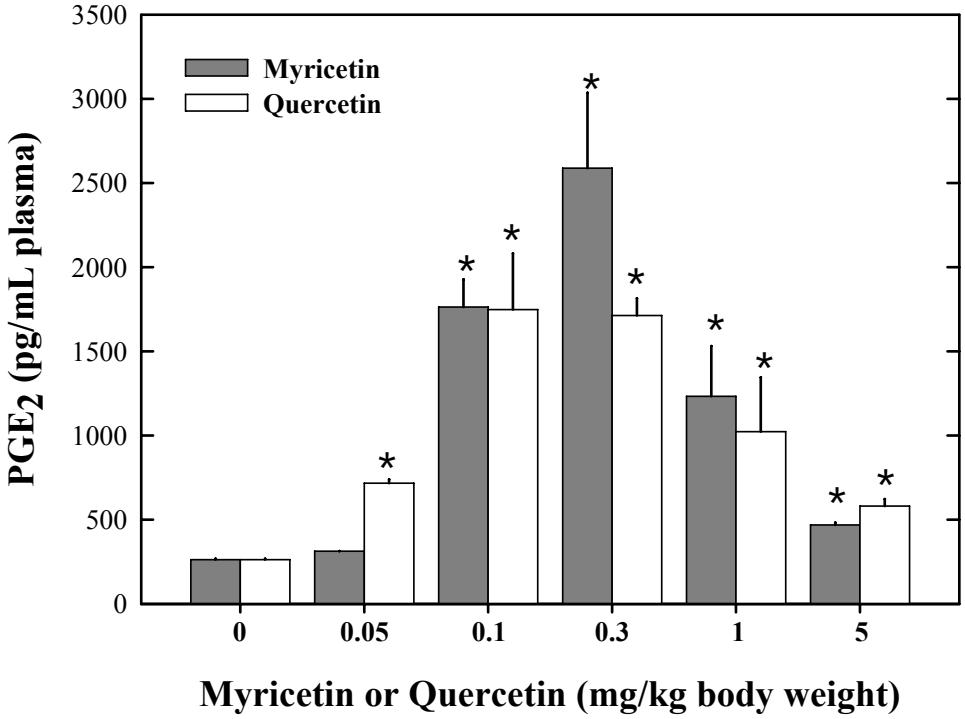


Figure 18. The dose-dependent effect of myricetin or quercetin on the levels of plasma PGE_2 in rats. Myricetin or quercetin was injected *i.v.* into each animal, and animals in the control group were injected *i.v.* with the vehicle only. The blood samples were collected for preparation of plasma from the tail vein at 4 h after injection, and the PGE_2 levels were measured by using an EIA kit. Data represent mean ± S. D. (n = 3). * $P < 0.05$ compared to the levels at 0 h.

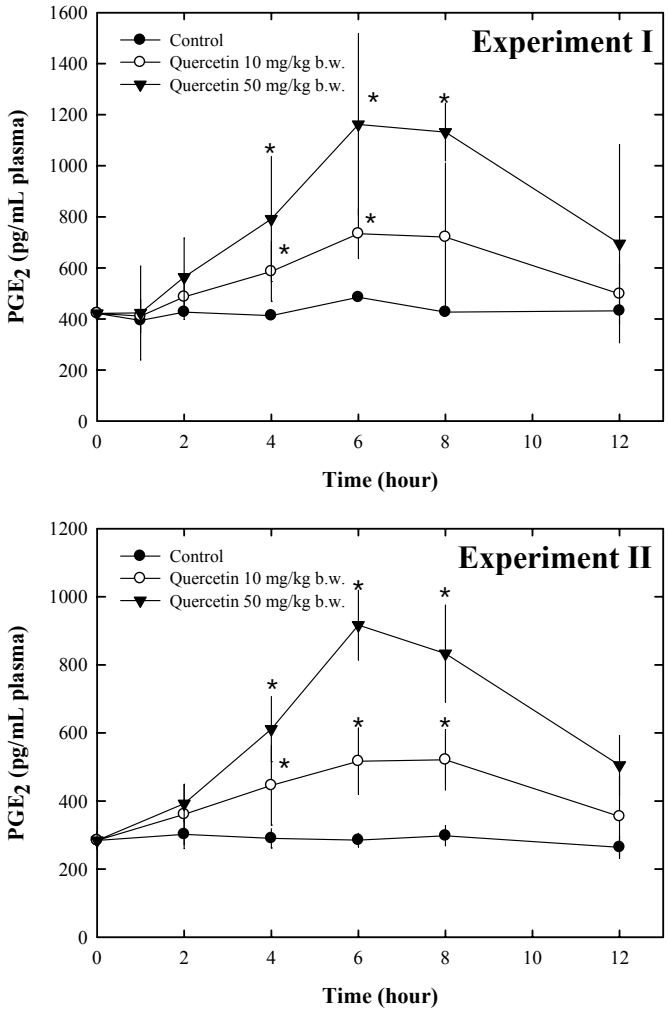


Figure 19. Effect of oral administration of quercetin on the levels of plasma PGE_2 in rats. Quercetin (10 and 50 mg/kg b.w.) was administrated orally, and animals in the control group were given the same volume of vehicle only. The blood samples were collected for preparation of plasma, and the levels of PGE_2 were measured by using an EIA kit. Data represent mean ± S. D. (For experiment *I*, $n = 3$, and for experiment *II*, $n = 6$). * $P < 0.05$ compared to the corresponding values of the control group.

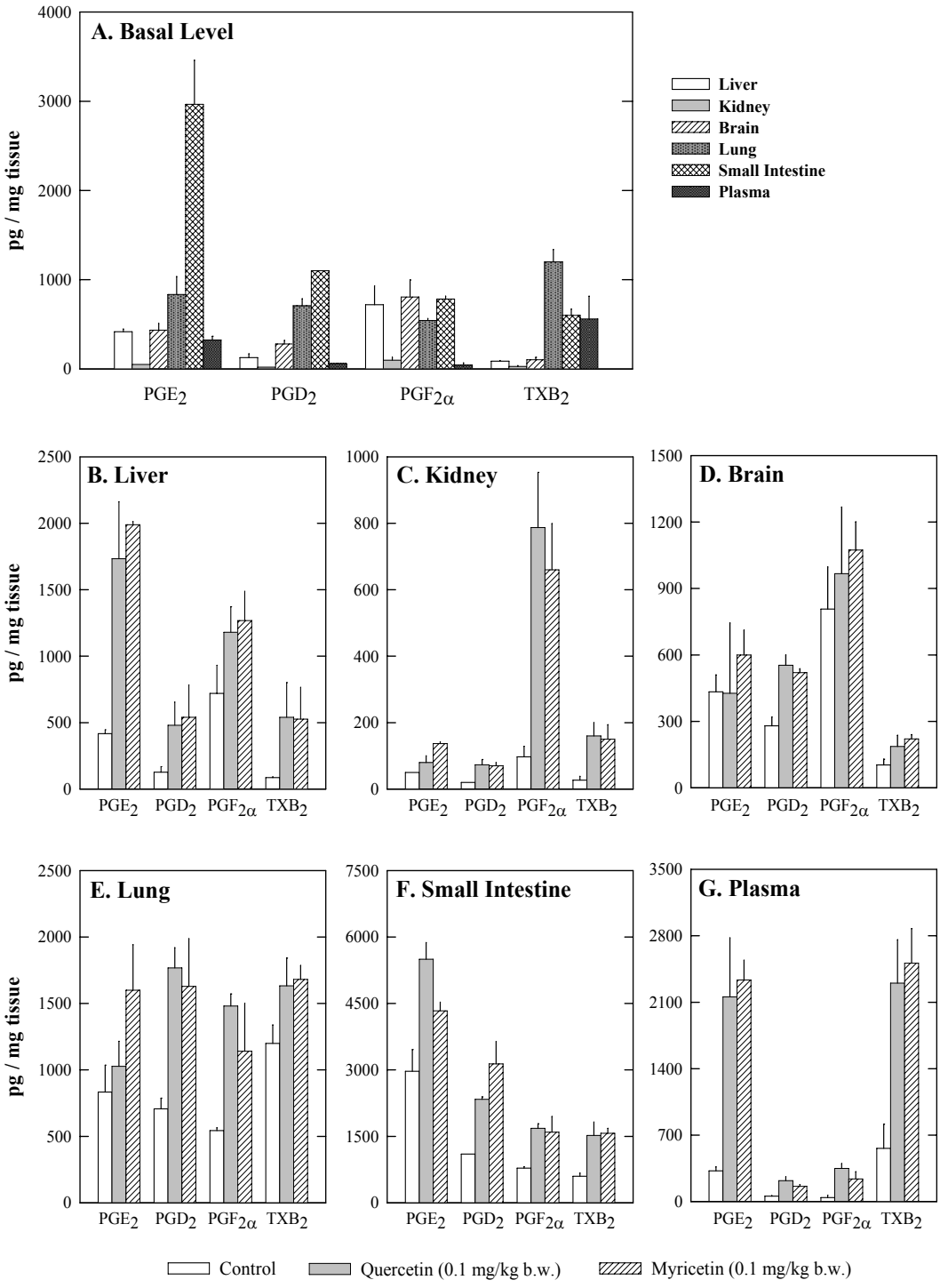

A. Basal Level
B. Liver
C. Kidney
D. Brain
E. Lung
F. Small Intestine
G. Plasma
pg / mg tissue
PGE2
PGD2
PGF2α
TXB2
Liver
Kidney
Brain
Lung
Small Intestine
Plasma
Control
Quercetin (0.1 mg/kg b.w.)
Myricetin (0.1 mg/kg b.w.)

Figure 20. The effect of quercetin and myricetin on the tissue levels of PGE_2, PGD_2, $PGF_{2\alpha}$ and TXB_2 in rats. Myricetin and quercetin (0.1 mg/kg b.w.) were injected *i.v.* into the animals, and the control animals were injected *i.v.* with 100 μL vehicle. The animals were sacrificed 4 h later. The blood samples were collected for preparation of the plasma. Liver, kidney, stomach, lung and small intestine were also removed, quickly frozen in liquid nitrogen, and then kept at −80°C. The levels of PGE_2, PGD_2, $PGF_{2\alpha}$ and TXB_2 in these tissues were measured by using the EIA kits. Data represent mean ± S. D. (n = 4).

DISCUSSION

Some dietary bioflavonoids are powerful direct stimulators of the catalytic activity of COX I and II *in vitro*, resulting in increased formation of various PGs, thromboxanes, and hydroxyeicosateraenoic acids (HETEs) from arachidonic acid (Bai and Zhu, 2008). The present study was designed to determine whether bioflavonoids at physiologically-relevant doses could exert similar stimulatory effects *in vivo*. Our results showed that the plasma and tissue levels of several PG products in male SD rats following a single *i.v.* injection of very low doses of myricetin or quercetin (*i.e.*, 0.1 and 0.3 mg/kg b.w.; approximately 25 or 75 μg/rat, respectively) were markedly increased in a time-dependent manner. In addition, when quercetin was administered orally (at 10 or 50 mg/kg b.w.), it also significantly increased the plasma levels of PGE_2 (a representative PG product measured) in male SD rats in a time dependent manner, although the effect was less efficacious compared to *i.v.* administration. The stimulatory effect of these two bioflavonoids on COX-mediated formation of PGE_2 was dose-dependent and followed a unique biphasic pattern. This biphasic pattern of PGE_2 plasma levels closely resembled the curve patterns for their stimulatory effect *in vitro* catalyzed by COXs or in cultured intact cells as reported recently (Bai and Zhu, 2008).

A number of earlier studies have reported an inhibitory effect of dietary bioflavonoids on COX-mediated PGs formations in lipopolysaccharide-induced animal model (Ruetten and Thiemermann, 1997; Kao et al., 2005; Kao et al., 2009), but little information is available concerning the effect of these dietary compounds on

COX-mediated PG formation *in vivo*. To our knowledge, this is the first report on a stimulatory effect of dietary bioflavonoids on PG formation *in vivo* at physiologically relevant doses in intact animals. The estimated combined daily dietary intake of various bioflavonoids in human is approximately one gram from food sources (Bravo, 1998; McKay and Blumberg, 2002). Generally, rodents have a much faster rate of metabolic disposition of dietary phenolic compounds than humans. However, assuming that the bioavailability of bioflavonoids in humans is as low as in rodents (at 10 mg/kg b.w.), then the estimated effective oral dose for humans should be readily attainable. Therefore, it is believed that bioflavonoids could be biologically-effective stimulators of COX I and II-mediated formation of various PG products in humans.

Bioflavonoids are known to have diverse beneficial effects in the body. In capillaries, they increase plasticity, decrease fragility, and ultimately reduce capillary bleeding (Gabor, 1972). In the blood, bioflavonoids reduce the aggregation of red blood cells and platelets (Kaneider et al., 2004). In the immune system, they act to strengthen the body's natural defense against viruses and infections, and may serve as natural anti-inflammatory agents (Paradkar et al., 2004). In addition, bioflavonoids may reduce hypertension, allergies, wound healing, and peptic ulceration (Li and Zhang, 2001; Havsteen, 2002). Thus, bioflavonoids may have the unique ability to modulate COX I and II activity and levels of prostaglandin products in circulation and tissues. This maybe the mechanistic underpinnings for some of the beneficial biological effects known to be associated with bioflavonoids. In this context, it is also

of note from our data that the levels of PG products are differentially affected in tissues by bioflavonoids.

In summary, the results of this study show that myricetin and quercetin (two representative dietary bioflavonoids) have a powerful direct stimulatory effect on the COX's catalytic activity *in vivo*. Given the large amount of pre-existing information in the literature concerning the inhibitory effect of bioflavonoids on the COX activity, our findings is rather intriguing and suggest that bioflavonoids may function as naturally-occurring, physiological co-substrates/stimulators of the COX I and II.

CHAPTER SIX

SPECIFIC AIM 3

STRUCTURAL BASIS FOR CERTAIN BIOFLAVONOIDS TO FUNCTION AS REDUCING CO-SUBSTRATES FOR CYCLOOXYGENASE I AND I

HYPOTHESIS

Dietary bioflavonoids stimulate COX-mediated formation of PG products by serving naturally-occurring cofactors for the COX enzymes via binding tightly to the peroxidase active site, and interacting directly with the hematin component of the COX enzyme to facilitate electron transfer from the bioflavonoids to hematin.

RATIONALE

Both COX I and II catalyze a cyclooxygenase reaction that converts AA to PGG_2 as well as a peroxidase reaction that reduces PGG_2 to PGH_2. These two reactions occur at distinct but functionally related catalytic sites. A branched-chain model has been proposed to explain the mechanism of these two reactions catalyzed by COX I and II (Dietz et al., 1988). Based on this model, a peroxide (such as PGG_2) is thought to initiate the peroxidase reaction by abstracting two electrons from hematin in the peroxidase active site, yielding Compound I, a protoporphyrin IX (PPIX) radical cation with an oxyferry group (Fe^{4+}=O). Next, Compound I undergoes an intramolecular reduction by Tyr385 to form Intermediate II with a neutral PPIX and a Tyr385 tyrosyl radical. Alternatively, Compound I can undergo the one-electron reduction by an exogenous electron donor, yielding Compound II with a neutral PPIX and Tyr385. Intermediate II initiates the cyclooxygenase reaction by abstracting one hydrogen atom from arachidonic acid to yield an arachidonate radical, which then reacts with two molecular O_2 to produce PGG_2.

Suicide inactivation is a well-known feature for the COX enzymes *in vitro*.

The peroxidase and cyclooxygenase activities are both inactivated usually in less than 1-2 min by a mechanism-based, first-order process even when sufficient amount of the substrates is present (Callan et al., 1996; Wu et al., 1999). Mechanistically, the Intermediate II is thought to initiate the suicidal inactivation by forming Intermediate III, which causes further unknown protein modifications resulting in enzyme inactivation (Wu et al., 1999).

In the previously study (Bai and Zhu, 2008), it was found that some of the dietary bioflavonoids were strong activators of COX I and II *in vitro* and *in vivo*. In addition, data from the 3-D quantitative structure-activity relationship/comparative molecular field analysis (QSAR/CoMFA) study revealed that the *B*-ring of bioflavonoids played an important role for their direct activation of the catalytic activity of COX I and II (**Figure 21**). This notion was further supported by homology modeling and docking studies, which showed that bioflavonoids could bind to the peroxidase active site and directly interact with hematin, thereby facilitating the electron transfer from bioflavonoids to hematin (**Figure 22**). To confirm these findings, biochemical analysis and site-directed mutagenesis experiments are conducted.

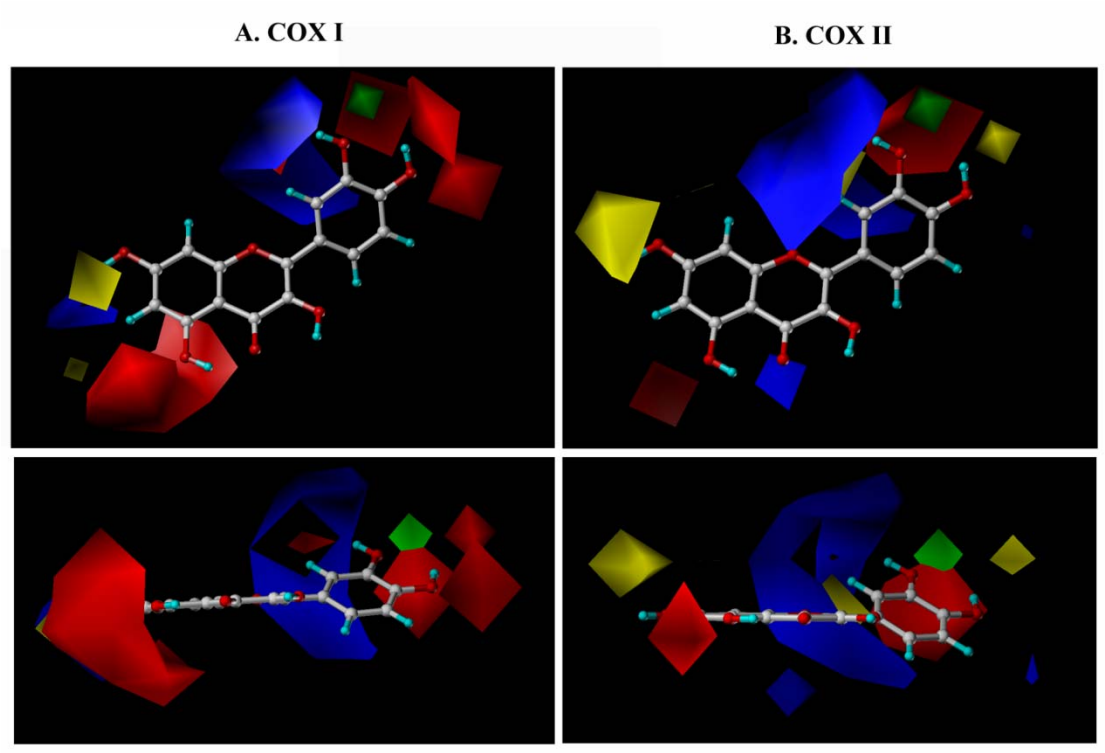


Figure 21. The color contour maps of the 3-D QSAR/CoMFA models for the COX I (A) and COX II (B). Note that quercetin was shown in the ball and stick format inside the field for demonstration. Oxygen, carbon, and hydrogen atoms are colored in red, gray, and blue, respectively. The contours of the steric maps were shown in yellow and green, and those of the electrostatic maps were shown in red and blue. Green contours indicated regions where a relatively bulkier substitution would increase induction of COX activity, whereas the yellow contours indicated areas where a bulkier substituent would decrease the COX activity. The red contours were regions where a negative-charged substitution likely would increase the COX activity whereas the blue contours showed areas where a negative-charged substitution would decrease the COX activity. Bioflavonoids with a higher ability to activate the COX enzymes were correlated with: (***i***) more bulkier substitute near green; (***ii***) less bulkier substitute near yellow; (***iii***) less negative charge near blue; and/or (***iv***) more negative charge near red. The figure in the lower panel shows the 90^{o} rotation around the *x*-axis of the figure shown in the upper panel.

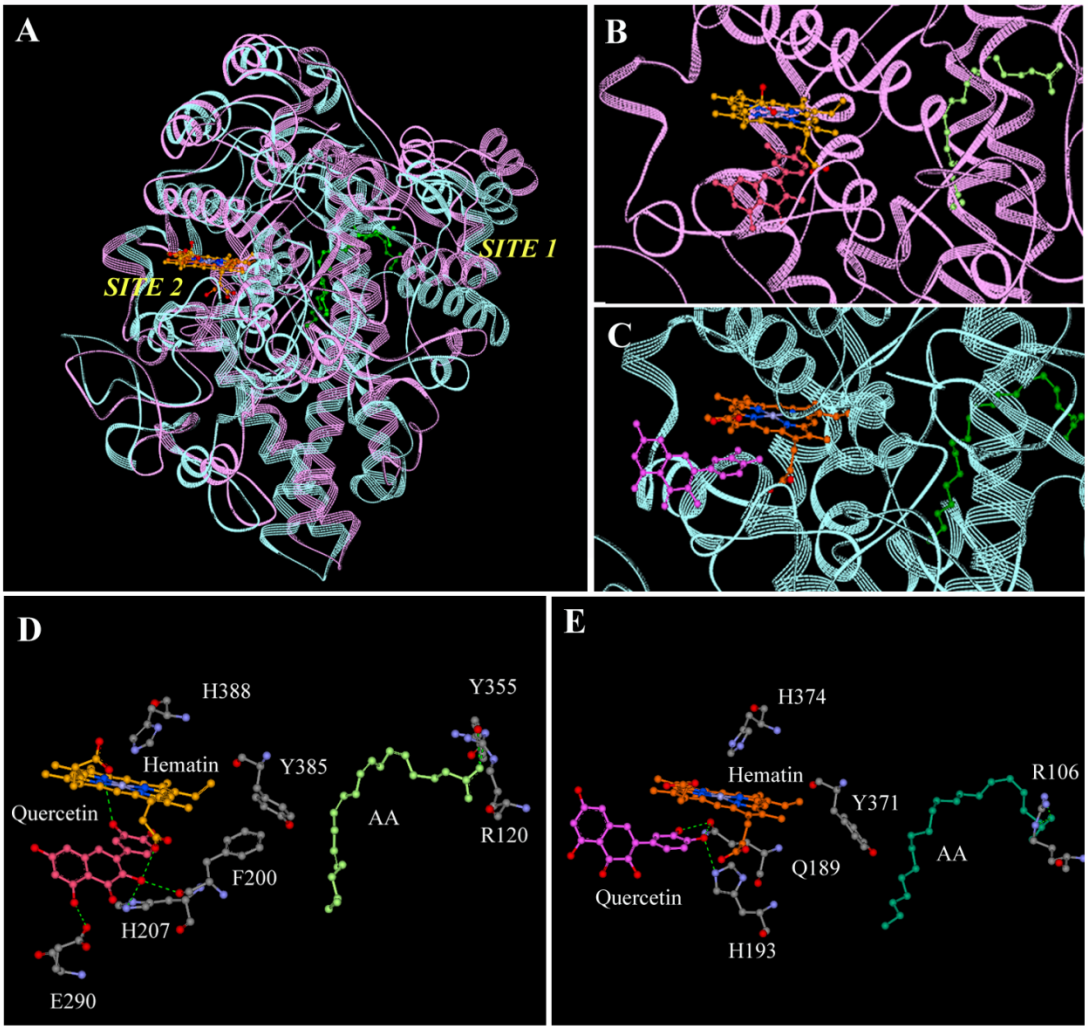

A
SITE 1
SITE 2
B
C
D
H388
Y355
Hematin
Y385
Quercetin
AA
R120
F200
H207
E290
E
H374
Hematin
R106
Y371
Q189
AA
Quercetin
H193

Figure 22. The binding site determination for COX I and COX II by molecular docking method. A. The superimposed structures of COX I and COX II in complex with hematin and the natural substrate arachidonic acid (AA). The white labels indicated the two candidate binding sites for quercetin identified by *Active-Site-Search* in *InsightII*. **B.** Docking results for quercetin in Site-2 of COX I. **C.** Docking results for quercetin in Site-2 of COX II. **D.** Enlarged view of the interaction of quercetin with hematin and key amino acid residues in the peroxidase active sites of COX I. **E.** Enlarged view of the interaction of quercetin with hematin and key amino acid residues in the peroxidase active site of COX II. The protein structure was shown with ribbons in **A**, **B** and **C**. COX I was colored in pink and COX II in purple. AA was colored in light green for COX I and dark green for COX II. Quercetin was colored in light red for COX I and magenta for COX II. Carbon atoms in hematin were colored in yellow for COX I and orange for COX II whereas nitrogen atoms were colored in blue, oxygen atoms in red, and magnesium in silver. The green dashes represented the hydrogen bonds. Hematin, AA, key amino acid residues, and quercetin were shown in the ball and stick format. For amino acid residues, oxygen atoms were shown in red, carbon atoms in gray, and nitrogen atoms in blue. Hydrogens were omitted in these molecules.

RESULTS

Biochemical analysis

To confirm that bioflavonoid stimulate COXs activity through binding to the peroxidase site but not the cyclooxygenase site, the stimulatory effect of myricetin was tested on the catalytic activity of COX I and II pre-treated with acetyl salicylic acid, which covalently acetylates and, thereby, inactivates the cyclooxygenase active site of the COX enzymes. To selectively test the effect of myricetin on peroxidase activity, PGG_2 was used as substrate to bypass the cyclooxygenation step that would convert AA to PGG_2. As shown in **Figure 24A and 24D**, pretreatment of COX I and II with acetyl salicylic acid (500 μM and 5 mM for COX I and COX II) strongly inhibited the cyclooxygenase activity when AA was used as substrate by 72% and 70%, respectively. However, when PGG_2 was used as substrate, acetyl salicylic acid-pretreated COX I and II the expected level of inhibition of COX activity was not seen.

As shown in **Figure 24B and 24E**, myricetin activated the catalytic activity of COX I and II by 5- and 8-fold, respectively, when AA was the substrate. When PGG_2 was used as substrate (as shown in **Figure 24C and 24F**), myricetin still similarly stimulated the catalytic activity of COX I and II with or without acetyl salicylic acid pre-treatment. Furthermore, the fold of stimulation was similar to what was seen with untreated COX I and II when AA was the substrate (**Figure 24B and 24E**). Altogether, these data show that myricetin activate COX I and II through interaction with the peroxidase site but not the cyclooxygenase site. Notably, at higher concentrations of myricetin (500 μM and 1000 μM), the catalytic activity of COX I

and II was inhibited to a similar degree, regardless of whether or not the enzymes were pre-treated with acetyl salicylic acid. This observation indicates that the inhibition of the COX activity by high concentrations of the bioflavonoids is due to the inhibition of peroxidase activity.

Mutagenesis studies

To further confirm results from binding models obtained from molecular docking studies showing that hydrogen bonds are formed between bioflavonoids and Q189 and H193 of COX II (**Figure 22**), site-directed mutagenesis was used to verify the functional roles of proposed two key amino acid residues. The recombinant protein expression levels were determined by western blotting (**Figure 23**). Q189A, H193A and Q189A/H193A mutant proteins of COX II (expressed in cos-7 cells) were made, and their levels of expression were confirmed using Western blot analysis (data not shown**)**. As summarized in **Table 4**, the catalytic activity of the Q189A, H193A and Q189A/H193A mutant proteins was approximately 32%, 10.2% and 0% (enzymatic activity was not detected) of the wild-type COX II activity. This observation showed that H193 plays a more important role than Q189 in catalyzing the peroxidation reaction of the substrate. As shown in **Table 4**, myricetin did not stimulate the catalytic activity of Q189A and H193A mutants, instead it inhibited their activity. Because the H193 mutant lost most of the catalytic activity, the interaction of Q189 with bioflavonoids was examined by designing additional mutant COX II proteins (*i.e.* Q189E, Q189N and Q189R). Glutamic acid (E) is similar to glutamine (Q) with the same length of side chain and similar side chain functional

group. Based on docking models, Q189E may likely be able to bind bioflavonoids in a similar way as the wild type COX II. In comparison, asparagine (N) has the same side chain functional group but with an one carbon shorter side chain, whereas arginine (R) has a positive-charged side chain compared with glutamine (Q) which has an acidic side chain. As summarized in **Table 4**, Q189E and Q189N mutants retained approximately 80% and 56%, respectively, of the catalytic activity of the wild-type COX II, whereas Q189R retained approximately 30% of the catalytic activity. As anticipated, based on the docking models, myricetin still stimulated the catalytic activity of the Q189E mutant COX II by 51.4% over the corresponding control, but did not stimulate the catalytic activity of other two mutant enzymes (Q189N and Q189E). Instead, myricetin inhibited the catalytic activity of these two mutant enzymes by 32.9% and 38.8%, respectively.

Table 4. The activity of various COX II mutants assayed in the presence or absence of myricetin.

Enzyme	Cyclooxygenase activity (formation of $PGF_{2\alpha}$ + PGE_2 + PGD_2; % of control)	
	Without myricetin	With 100 μM myricetin
Wild type	100	224.5 (↑ 124.5%)[a]
Q203A	32.0	11.5 (↓ 74.1%)
H207A	10.2	N.D. (↓ 100%)
Q203A/H207A	N.D.[b]	N.D.
Q203E	78.5	118.9 (↑ 51.4%)
Q203N	55.9	37.3 (↓ 32.9%)
Q203R	37.9	23.2 (↓ 38.8%)

a The number in parenthesis represents the % of increase or decrease over the corresponding control (in the absence of myricetin).

b N.D., not detected.

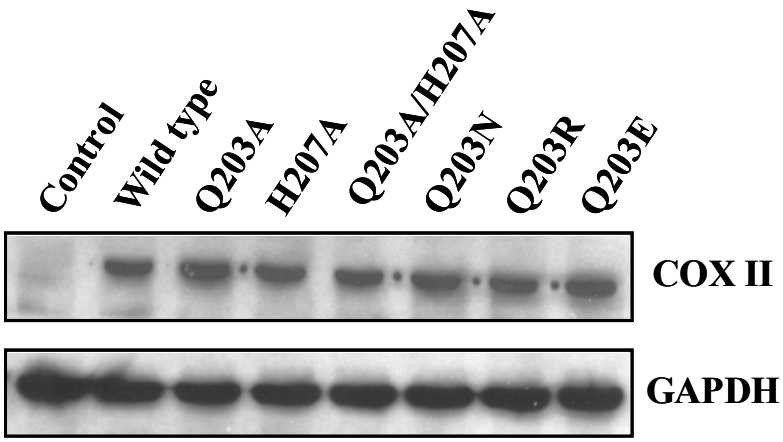


Figure 23. Recombinant COX II protein levels in transfected Cos-7 cells in culture. Western blot analysis of cell lysates was performed with antibodies specific for COX II, coupled with a secondary antibody conjugated with horseradish peroxidase. A total of three experiments were conducted, and a representative data set from one of the experiments was shown.

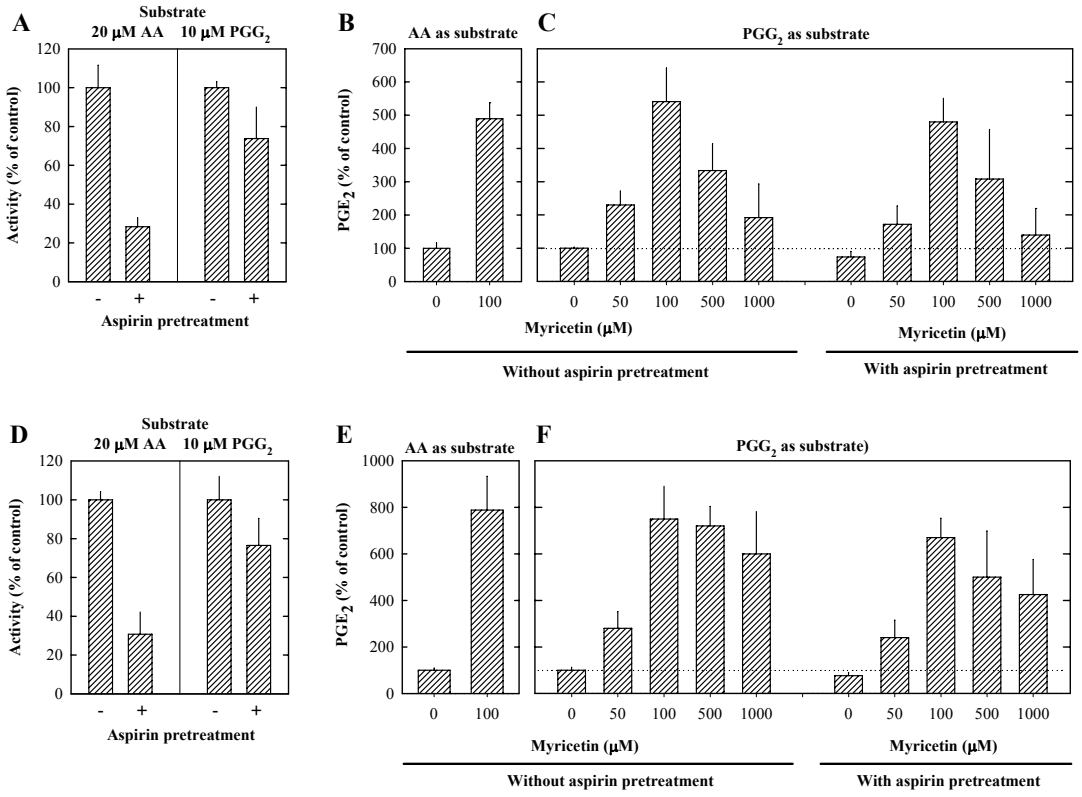


Figure 24. Myricetin stimulated the catalytic activity of COX I and II with PGG_2 as substrate. The incubation mixtures consisted of 20 μM [^{14}C]AA (0.2 μCi) or 10 μM PGG_2 as substrate, COX I or COX II as the enzyme (0.5 or 0.97 μg/mL, respectively), 10 mM EDTA, 1 mM reduced glutathione, 1 μM hematin, and myricetin in 200 μL Tris-HCl buffer (100 mM, pH 7.4). The reaction was incubated at 37°C for 5 min and terminated by adding 15 μL of 0.5 N HCl to each tube. Ethyl acetate (600 μL) was added immediately for extraction. The dried extracts were re-dissolved in acetonitrile or EIA buffer (Cayman Co. Michigan, USA) and the metabolites were analyzed using HPLC (with radioactivity detection) when [^{14}C]AA was used as substrate (American Chemical Society. Meeting (205th : 1993 : Denver Colo.) et al.) or using an EIA kit when PGG_2 was used as substrate. For acetyl salicylic acid pre-treatment, enzymes were pre-incubated with acetyl salicylic acid at 500 μM for COX I or 5 mM for COX II for 30 min at room temperature and then used as the enzyme source.

DISCUSSION

Computational molecular modeling studies revealed that the *B*-ring of bioflavonoids plays an important role for their direct activation of the catalytic activity of COX I and II. This was further supported by homology modeling and docking studies, which showed that bioflavonoids bind to the peroxidase active site, directly interacting with hematin, and thereby, facilitating the electron transfer from bioflavonoids to hematin.

Additional biochemical analyses showed that when PGG_2 was used as substrate, bioflavonoids still stimulate, to a similar degree, the catalytic activity of COX I and II with or without acetylating the active site of the enzyme with acetyl salicylic acid. Furthermore, the site-directed mutagenesis of COX II confirmed the predictions true docking models revealing that Q189 in the peroxidase active site was an important amino acid for binding interactions with bioflavonoids and subsequently the stimulation of the COX II activity. Altogether, these data provide a detailed explanation for the structural basis of certain bioflavonoids to function as high-affinity reducing co-substrates for the COX enzymes.

The structure-activity relationship for the antioxidant activity of bioflavonoids has been extensively studied (Burda and Oleszek, 2001; Bors and Michel, 2002; Silva et al., 2002). The antioxidant activity depends on the number of phenolic hydroxyl groups as well as their localization in the molecules. The presence of a catechol structure (3',4'-dihydroxyls) in the *B*-ring and a 2,3-double bond are important determinants for high antioxidant activity (Burda and Oleszek, 2001; Silva et al.,

2002). The presence of 3- and 5-dihydroxyl groups usually further enhances the antioxidant activity. The stimulation of COX activity by apigenin or naringenin, demonstrates that the 2,3-double bond is an essential structural feature for bioflavonoids to have a stimulatory effect on the COX activity, although this was not revealed by CoMFA contour maps. The similarity between the 3-D QSAR/CoMFA study and the earlier studies on the antioxidant activity of various bioflavonoids suggests that the stimulation of the COX activity by bioflavonoids is, in a large part, attributable to their antioxidant property.

The molecular docking studies implicate that bioflavonoids bind to the peroxidase sites of COX I and II, thus suggesting the possibility that bioflavonoids stimulate their catalytic activity by interacting with the peroxidase site. This was confirmed by biochemical analysis using PGG_2 as substrate to bypass the cyclooxygenase reaction. Our observation that myricetin stimulated COX activity to a similar degree with either PGG_2 or AA as substrate provided support for the notion that myricetin selectively activates the peroxidase activity. Furthermore, acetyl salicylic acid pretreatment of the COX enzymes did not affect myricetin's stimulation of their catalytic activity when PGG_2 was used as substrate, which further supports this conclusion.

These findings based on the homology modeling and molecular docking studies provided detailed molecular insights. As shown in **Figure 22**, bioflavonoids fit into the peroxidase sites tightly and form several hydrogen bonds with the hematin moiety as well as with the nearby amino acids (in the case of quercetin, E290, H207

and F200 for COX I, and H193 and Q189 for COX II). The formation of these hydrogen bonds will facilitate the electron flow from the *B*-ring of a bioflavonoid molecule to the hematin group of the enzymes. The binding energy values calculated from the docking models correlate well with the ability of the active bioflavonoids to stimulate the catalytic activity of the COX enzymes, which provides support for the docking models used in this study.

The docking model of COX II is also supported by the results from site-directed mutagenesis studies. By mutating Q189 and H193 into alanine (A), we showed that Q189 and H193 were important for COX II activity, especially the peroxidase activity, which is consistent with an earlier report (Kulmacz et al., 1994). H193 played a more important role than Q189 since the H193A mutant nearly lost all catalytic activity compared to the Q189A mutant. The observation that myricetin could not activate the Q189A, Q189N, or Q189R mutant COX II enzyme revealed that the side chain of Q189 was very important for the binding interaction with bioflavonoids and also for their stimulation of the COX II activity. This experimental observation confirms our docking results showing that Q189 forms two hydrogen bonds with bioflavonoids (**Figure 22E**). As expected, the Q189E mutant could still be stimulated by myricetin because the side chain of glutamic acid (E) is of the same length and similar chemical property as the side chain of glutamine (Q), and thus hydrogen bonds could still be formed between glutamic acid and bioflavonoids. Although about 56% activity was retained in Q189N mutant protein, myricetin could

not stimulate its activity. This indicated that the length of side chain of Q189 was crucial for binding interactions with bioflavonoids.

There were several earlier studies that investigated a number of reducing co-substrates of COXs (Van der Ouderaa et al., 1977; Hemler and Lands, 1980; Kulmacz and Lands, 1985; Hsuanyu and Dunford, 1992a; Bambai and Kulmacz, 2000). The mechanism is generally thought to be due to the reduction of the oxidized intermediates by the co-substrate (Hemler and Lands, 1980; Hsuanyu and Dunford, 1992a). As depicted in **Figure 25**, the reducing potential of bioflavonoids, like other reducing co-substrates, will help maintain the peroxidase cycle and thereby slow down the suicidal inactivation of the COX enzymes by donating one electron each to Compound I and Compound II to restore the reducing activity of the hematin, which is needed for peroxidase to convert PGG_2 to PGH_2. The oxidized quinone form of bioflavonoids is expected to have a lower binding affinity for the peroxidase site because they will lose two hydrogen bond donors (hydroxyl groups) in the original catechol structure, and some of the hydrogen bonds cannot be formed between the quinone and the peroxidase site. Accordingly, the following catalytic sequence is proposed (depicted in **Figure 25** and the events were labeled with numbers to show their sequence): It is assumed that PGG_2 has a high binding affinity for the peroxidase site of the enzyme and will tightly bind to the active site. Immediately following the catalytic conversion of PGG_2 to its product, the product will dissociate from the enzyme (due to reduced binding affinity). After that, the peroxidase site will become catalytically inactive (with an oxidized hematin), and it will be bound by a

bioflavonoid molecule (reduced form) for the reduction of the hematin to its initial state. At the same time, the bioflavonoid will be oxidized to a semiquione (as an intermediate) and finally to a quinone. The bioflavonoid quinone will then be released from the activated peroxidase site because the oxidized molecule will have reduced binding affinity for the site. In this model, it is apparent that there is a potential competition between the substrate (PGG_2) and also the co-substrate (bioflavonoid) at the peroxidase active site. When the bioflavonoid concentration becomes too high, it will increase the fraction of the active peroxidase site that is still occupied by a co-substrate, and when this occurs, it would inhibit the binding of PGG_2 to the peroxidase site and thus would reduce the catalytic activity of the enzyme for the formation of further products. This mechanistic explanation is in agreement with the data shown in **Figure 24C** and **24F** as well as a number of earlier studies showing a concentration-dependent biphasic modulation of the COX activity by co-substrates, namely, the presence of a co-substrate at low concentrations stimulated the COX activity, whereas its presence at higher concentrations inhibited the COX activity (Van der Ouderaa et al., 1977; Hemler and Lands, 1980; Kulmacz and Lands, 1985; Harvison et al., 1988; Thompson and Eling, 1989; Hsuanyu and Dunford, 1992a; Bambai and Kulmacz, 2000). Notably, some of the earlier studies suggested that the inhibition with high concentrations of the co-substrate was due to the fast reduction of Intermediate II and the loss of cyclooxygenase activity (Harvison et al., 1988). This explanation appeared to disagree with the observation (in **Figure 24C and 24F**) which showed that myricetin at high concentrations (500 and 1000 μM) inhibited the

peroxidase activity of acetyl salicylic acid-pretreated COX II (using PGG_2 as substrate) to a similar degree as their inhibition of the untreated COX II for its metabolism of AA as substrate.

In summary, biochemical analysis with acetyl salicylic acid-pretreated COX enzyme and PGG_2 as substrate provides support for the docking models. In addition, site-directed mutagenesis study of COX II confirms that Q189 in the perioxidase active site was crucial for the binding of the bioflavonoid molecule and subsequently the stimulation of the COX II activity. These findings provide the structural basis for bioflavonoids to function as naturally-occurring, high-affinity reducing co-substrates of COXs through selectively binding to the peroxidase active site, facilitating electron transfer, and ultimately, the reactivating the peroxidase's catalytic activity.

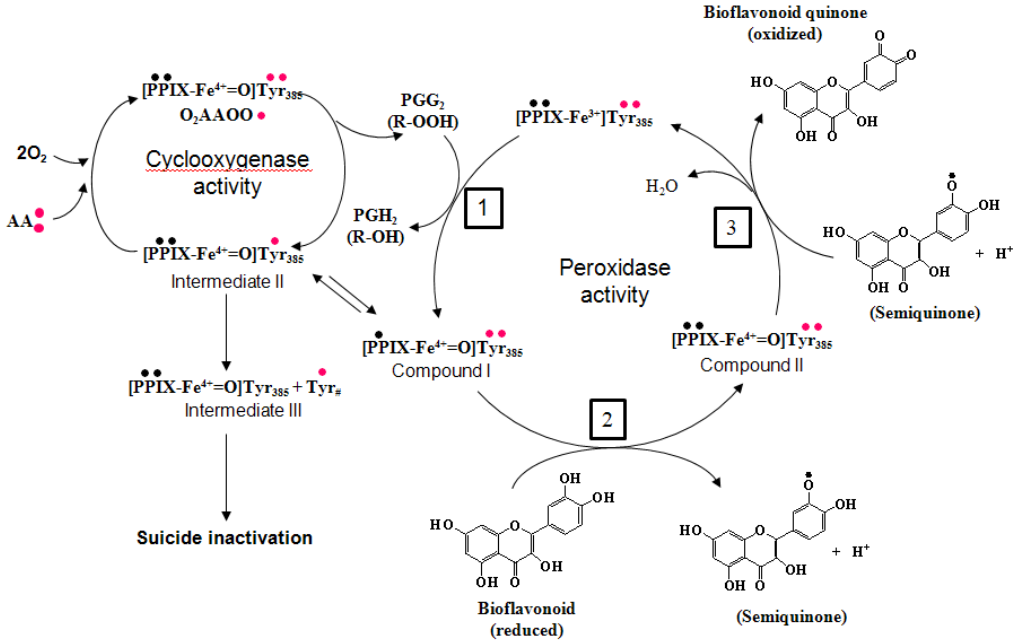


Figure 25. Schematic illustration of the catalysis and inactivation mechanism of the COXs as well as their interactions with bioflavonoids. PPIX stands for protoporphorin IX. Quercetin structure was shown as a representative bioactive bioflavonoid. Events in the peroxidase cycle were labeled with numbers to show their sequence.

CHAPTER SEVEN

SPECIFIC AIM 4

EFFECT OF GALANGIN ON CYCLOOXYGENASE-MEDIATED FORMATION OF VARIOUS PROSTAGLANDINS *IN VITRO* AND *IN VIVO*.

HYPOTHESIS

Bioflavonoids that have no hydroxyl group on B-ring might be antagonists for bioflavonoids that have stimulatory effect on COX-mediated AA metabolism.

RATIONALE

It is found that some of the dietary polyphenols could function as naturally-occurring co-substrates for COX I and II *in vitro*, resulting in increased catalytic activity of COXs for the formation of various AA metabolites (Bai and Zhu, 2008). This observation was also recently confirmed *in vivo* by showing that administration of quercetin and myricetin (two representative dietary bioflavonoids) could strongly increase the plasma and tissue levels of several PG products in an animal model. Molecular modeling studies have shown that bioflavonoids could bind, with high affinity, to the peroxidase site of the human COX I and II, and serve as reducing co-substrates and the hydroxyl groups contained in the *B*-ring of various bioflavonoids play a crucial role in their stimulation of the COX catalytic activity. Based on the computational models developed, it is predicted that some of the dietary compounds that lack the *B*-ring hydroxyl groups may be able to function as antagonists that would inhibit the stimulatory effect of those bioactive bioflavonoids (serving as reducing co-substrates for the COX enzymes). This idea will be tested using galangin, a representative bioflavonoid without any hydroxyl group in its *B*-ring (**Figure 26**), both *in vitro* and also *in vivo*.

RESULTS

Effect of galangin on COX I and II-mediated formation of various PG products *in vitro*

First, the effects of galangin on COX-mediated AA metabolism was investigated in the presence or absence of quercetin [an activator of the catalytic activity of COX I and II (Bai and Zhu, 2008)]. As shown in **Figure 27**, the presence of quercetin alone at 50 or 200 μM concentrations increased the formation of PGs and HETEs catalyzed by either COX I or COX II, as reported recently (Bai and Zhu, 2008). When galangin was also present, it reduced the formation of PGE_2 (a representative PG product measured) in a concentration-dependent manner (**Figure 27**). The profile of its inhibitory effects on the *in-vitro* formation of various PG products was slightly different with COX I and COX II. Galangin completely abrogated quercetin's effect at 100 μM for COX I-mediated PGE_2 formation, but for COX II-mediated AA metabolism, PGE_2 formation was only inhibited by approximately 50% even when the highest concentration (100 μM) of galangin was present. The formation of other PGs and HETEs had a similar pattern of inhibition by galangin as seen with PGE_2.

Interestingly, when the effects of galangin was tested alone in the absence of quercetin, it did not exert an appreciable effect on the basal levels of PG product formation catalyzed by either COX I or COX II (**Figure 27**). This observation suggests that galangin selectively antagonizes the stimulatory effect of quercetin, and this conclusion is supported by the computational modeling studies (described later).

Effect of galangin on COX I and II-mediated formation of various PG products in cultured cells

Further, experiments were conducted to determine the modulating effects of galangin on PG formation in cultured RAW264.7 cells that were pretreated with LPS for 4 h to induce COX II expression. In these experiments, only the levels of PGE_2 were measured as a representative PG product in the culture media of LPS-pretreated RAW264.7 cells by using an EIA kit. As reported (Bai and Zhu, 2008), quercetin at 100 nM stimulates the formation of PGE_2 by approximately 1.4 times in the LPS-pretreated RAW264.7 cells compared to cells in the absence of quercetin. In the presence of 100 nM quercetin, galangin diminished its stimulatory effect in a concentration-dependent manner similarly as shown in the *in-vitro* biochemical assay. The IC_{50} value of galangin for inhibiting quercetin-stimulated formation of PGE_2 was comparable to its observed effect *in vitro*, at approximately 5 μM (**Figure 28**). In the absence of quercetin, galangin also inhibited the formation of PGE_2 in a concentration-dependent manner, the IC_{50} value was approximately 6.6 μM. It is speculated that galangin inhibits the stimulatory effect of other reducing co-substrates that are present in the cell culture media or produced by the cells.

Effects of galangin on COX I and II-mediated formation of various PG products *in vivo*

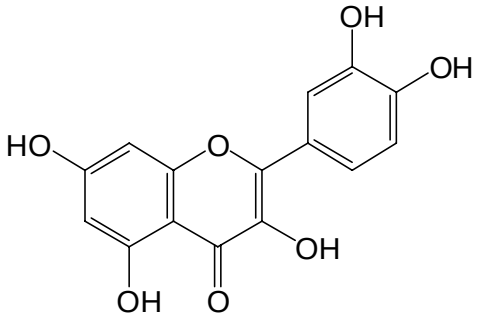
In this study, the effects of galangin on the blood levels of PGE_2 were determined in male Sprague-Dawley rats. The animals were divided into several

groups with four animals per group, and then given either a single *i.v.* injection of quercetin alone (at 0.1 mg/kg b.w.), or in combination with galangin at 1 or 5 mg/kg b.w. Administration of galangin alone did not significantly alter the blood levels of PGE_2 at either dose compared to the control animals injected with vehicle only (**Figure 29A**). Injection of quercetin alone markedly increased the plasma levels of PGE_2 by approximately 6-fold (**Figure 29B**), similar to that reported in Aim 1 to 3. This stimulatory effect of quercetin was inhibited by galangin in a dose-dependent manner. PGE_2 levels in animals treated with quercetin (0.1 mg/kg b.w.) plus galangin (5 mg/kg b.w.) were comparable to the levels seen in vehicle-treated control animals (**Figure 29B**). Blood levels of three other PGs (PGD_2, $PGF_{2\alpha}$ and TXB_2) were also increased in animals treated with quercetin. The blood levels of TXB_2, a metabolite of TXA_2, was increased by approximately 4-fold following administration of quercetin, but decreased with galangin co-administration (**Figure 29B**). The basal blood levels of PGD_2 and $PGF_{2\alpha}$ were very low in untreated animals, and treatment with quercetin significantly increased their levels. Similarly, co-treatment with galangin dose-dependently reduced the blood levels of these two PG products in these animals (**Figure 29B**).

The tissue levels of various PG products (PGE_2, PGD_2, $PGF_{2\alpha}$ and TXB_2) were also determined in these animals (the data were summarized in **Figure 30A-30D**). In the liver, PGE_2 was a major PG product among the four PG products measured, and it was increased by approximately 3-fold following quercetin administration alone (at 0.1 mg/kg b.w.). TXB_2 was increased by approximately 2-

fold compared to the control, while PGD_2 and $PGF_{2\alpha}$ were only slightly increased. Interestingly, galangin did not reduce the stimulatory effect of quercetin as it did on the blood PG levels. Other PGs also showed a similar pattern (**Figure 30A**). $PGF_{2\alpha}$ was a major PG in the kidney. Quercetin administration increased its formation by ~5-fold, and interestingly, its formation was increased even more when galangin and quercetin were co-administrated. The levels of other PGs were very low in the kidney (**Figure 30B**). In the lung, while the levels of PGE_2 and TXB_2 were not changed by either quercetin alone or quercetin and galangin, PGD_2 and $PGF_{2\alpha}$ were increased by approximately 2-fold. The increased levels of these PG products were not decreased by galangin co-treatment (**Figure 30C**). Lastly, PGE_2 was a major PG formed in small intestine. The levels of PGE_2, PGD_2, $PGF_{2\alpha}$ and TXB_2 were increased by approximately 2-fold following quercetin treatment, and its stimulatory effect was diminished by galangin in a dose-dependent manner (**Figure 30D**). Notably, the level of PGE_2 was even lower than that in the control animals when 5 mg/kg b.w. galangin was administrated together with quercetin (0.1 mg/kg b.w.).

Quercetin



Galangin

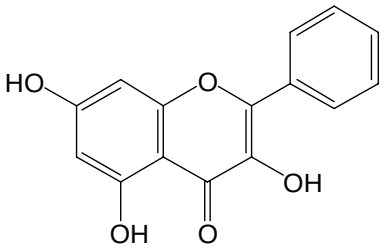


Figure 26. Chemical structures of quercetin and galangin.

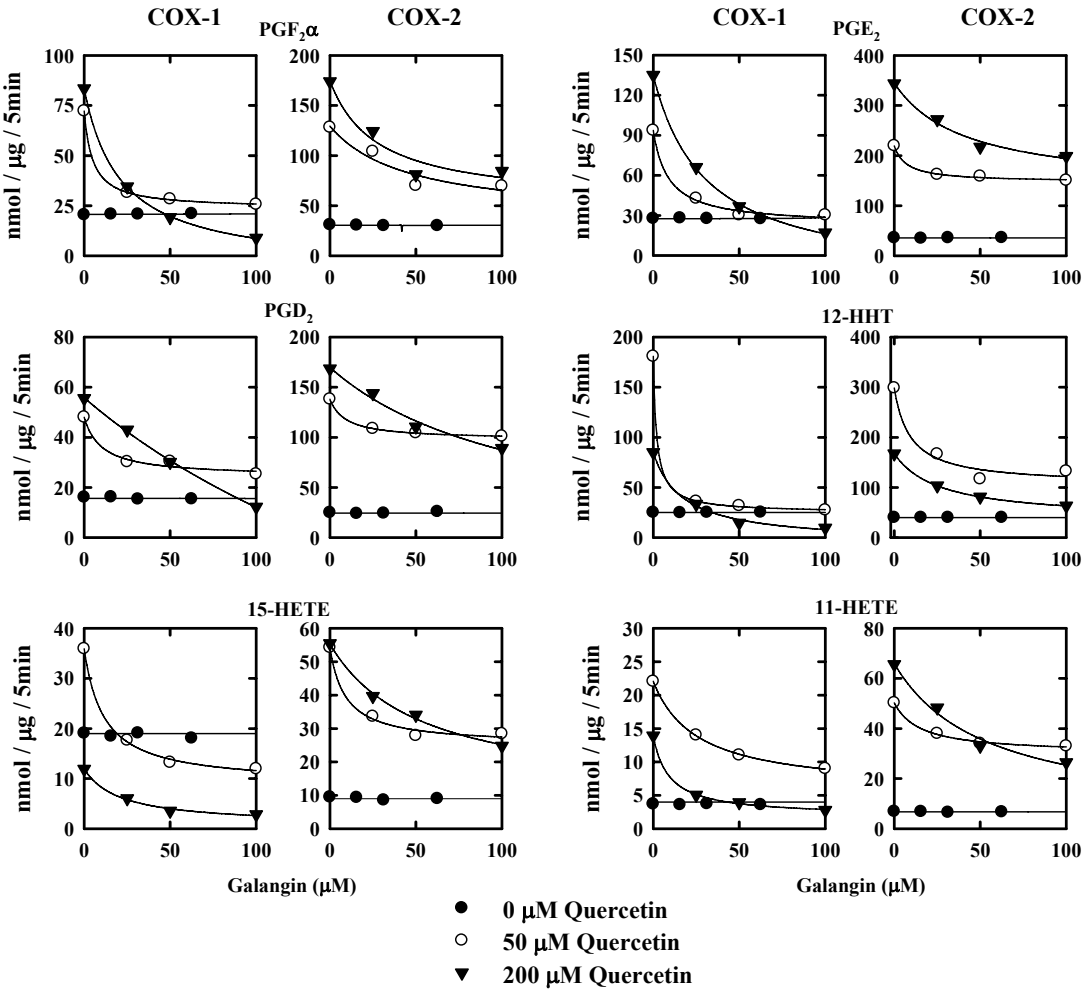


Figure 27. Effect of galangin and quercetin on the COX's catalytic activity *in vitro*. The incubation mixtures consisted of 4 different concentrations (0, 25, 50 and 100 μM) of galangin with or without 50 or 200 μM quercetin as a reducing co-substrate, 20 μM [^{14}C]AA (0.2 μCi) as substrate, COX I or COX II as the enzyme (0.5 or 0.97 μg/mL, respectively), 10 mM EDTA, 1 mM reduced glutathione, and 1 μM hematin in 200 μL of 100 mM Tris-HCl buffer, pH 7.4. The incubations were carried out at 37°C for 5 min. The levels of AA metabolites were measured using HPLC as described in the Materials and Methods section. Each point was the mean of duplicate determinations.

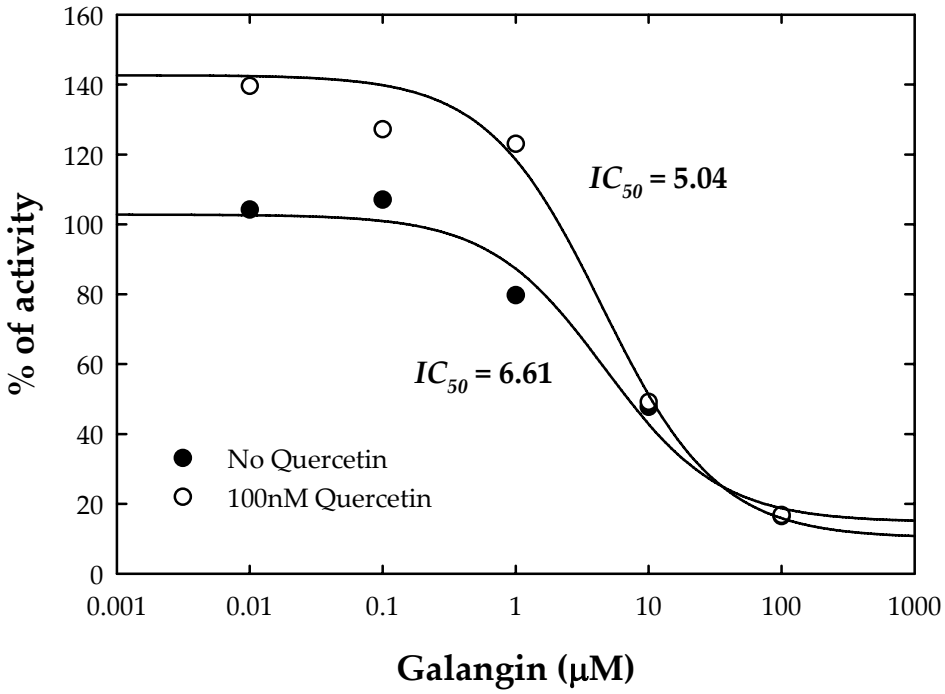


Figure 28. Effect of galangin on the release of PGE_2 from LPS-pretreated RAW264.7 cells in the absence (close circle) or presence (open circle) of 100 nM quercetin. Cells were pretreated with 1 μg/mL LPS for 2 h to induce COX II expression, and then the culture media were removed and replaced with 300 μL serum-free medium with or without quercetin for an additional 2 h. Five concentrations of galangin were tested: 0.01, 0.1 1, 10, and 100 μM. The levels of PGE_2 were measured using an EIA kit (Cayman Chemical, Ann Arbor, MI). Each point was the mean of duplicate determinations.

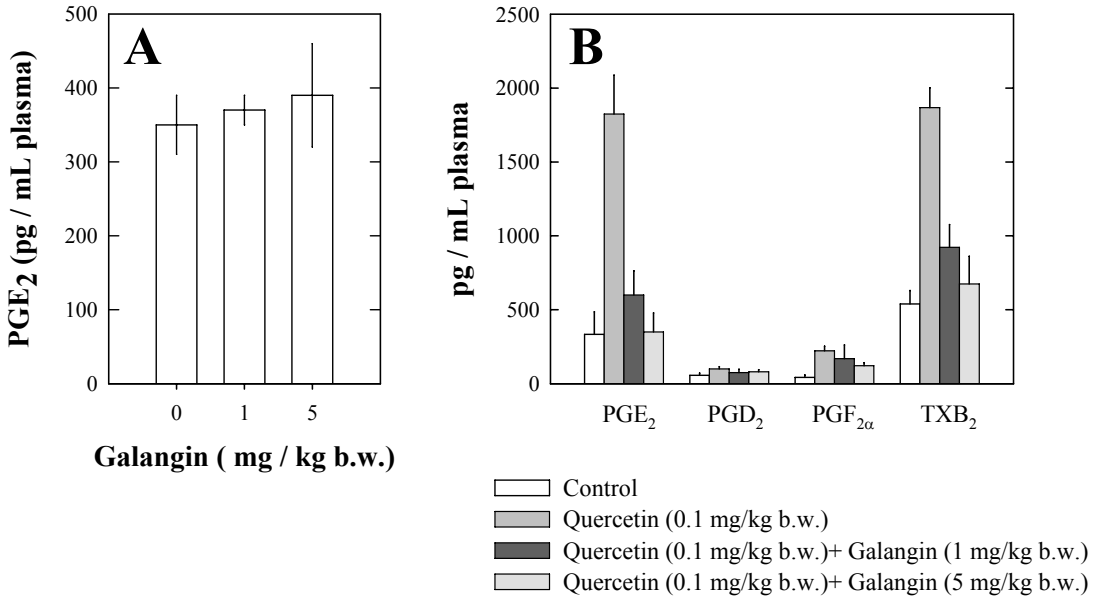


Figure 29. Plasma levels of PGs in rats injected *i.v.* with either galangin alone (at 1 or 5 mg/kg b.w.) or in combination with quercetin at 0.1 mg/kg b.w. The blood samples were collected from the tail vein at 4 h after injection. The plasma was immediately prepared and stored at -80°C for measurement of PGs (PGE_2, PGD_2, $PGF_{2\alpha}$, and TXB_2) using the EIA kits. Data represent mean ± S.D. ($N = 4$).

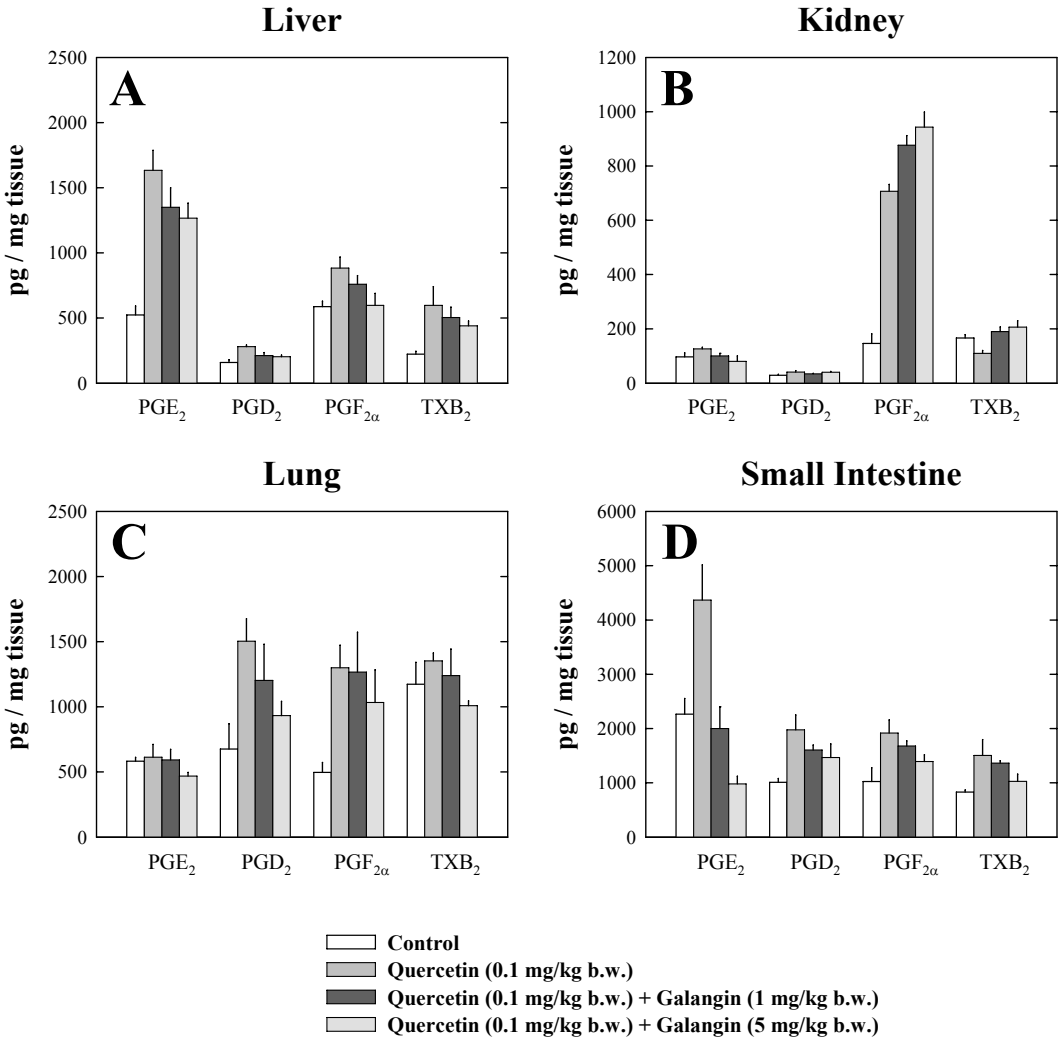


Figure 30. Effect of galangin and quercetin on the tissue levels of PGE_2, PGD_2, $PGF_{2\alpha}$ and TXB_2 in rats injected *i.v.* with either galangin alone (at 1 or 5 mg/kg b.w.) or in combination with quercetin at 0.1 mg/kg b.w. The animals were sacrificed 4 h later. Liver, kidney, lung and small intestine were removed, quickly frozen in liquid nitrogen, and then kept at -80°C. The levels of PGE_2, PGD_2, $PGF_{2\alpha}$ and TXB_2 in these tissues were measured using the EIA kits. Data represent mean ± S. D. ($N = 4$).

DISCUSSION

Galangin (3,5,7-trihydroxyflavone), a member of the flavonol-class bioflavonoids, is present in high concentrations in honey and *Alpinia officinarum*, a plant which used as a spice, and also as a traditional herbal medicine in the Orient for a number of medical conditions, such as common colds, wound swelling, and stomachache (An et al., 2008).

We have evaluated galangin, a bioflavonoid with no hydroxyl group in its *B*-ring, for its ability to modulate the catalytic activity of COX I and II stimulated by quercetin *in vitro* and *in vivo*. Interestingly, galangin itself did not have an appreciable effect on the COX mediated formation of various PG products in the *in vitro* biochemical assay and also in intact animals. However, when the COX enzymes were activated by the presence of a reducing co-substrate such as quercetin (Bai and Zhu, 2008), galangin abrogated the stimulatory effect of quercetin on COX catalytic activity *in vitro* in a concentration dependent manner. This observation was also confirmed in experiments using LPS-pretreated RAW cells (a mouse macrophage cell line). In addition, experiments with normal SD rats, demonstrated that galangin diminished the stimulatory effect of quercetin on the COX activity *in vivo*, by lowering the PGE_2 levels in plasma as well as in some of the tissues analyzed.

Based on QSAR studies, it was found that the hydroxyl groups in the *B*-ring of bioflavonoids are the key functional groups for stimulating the COX activity. This was confirmed again in the present study with galangin, which has several hydroxyl groups in its *A*- and *C*-rings but no hydroxyl groups in its *B*-ring.

In the presence of quercetin, galangin suppressed quercetin's stimulatory effect on COX I and II by competing with quercetin for binding to the peroxidase sites of the COX enzymes. This suggestion was supported by the following observations. (***i***) The docking models developed in this study showed that galangin binds to the peroxidase site in a similar way as other bioflavonoids such as quercetin, although its binding affinity was lower than quercetin. This reduced binding affinity is likely due to the lack of hydroxyl groups on its *B*-ring to form hydrogen bonds with the peroxidase's activity site. (***ii***) Recent biochemical analysis showed that the stimulation effect of quercetin on COX's catalytic activity is due to its action on peroxidase activity. (***iii***) While quercetin strongly stimulated the COX activity, galangin had no activity in stimulating COX's catalytic activity by itself. Inhibition of the COX II activity by galangin in LPS-pretreated RAW cells may be due to the presence of endogenous reducing agents in the cell culture system.

The data provids support for the concept that these dietary bioflavonoids may help to improve human health by modulating COXs activity directly at biologically achievable concentrations. It also offers insights into the development of new COX modulators either as stimulators or inhibitors.

CHAPTER EIGHT

SUMMARY OF FINDINGS AND CLINICAL IMPLICATIONS

Summary of findings

Of a total of over 20 bioflavonoids tested, some of them were found to have a powerful, direct stimulatory effect on the COX-mediated formation of PG products. Myricetin, quercetin, fisetin, morin and tamarixetin have a high efficacy for stimulating PGs formation. Their profiles for stimulating the COX-mediated AA metabolism varied depending on the compounds. These bioflavonoids also exhibited a unique biphasic concentration-dependent pattern in modulating COX-mediated AA metabolism both in test tubes and also in cultured cells. Western blotting analysis revealed that the stimulatory effect of bioflavonoids was not due to an increased COX expression.

To determine the effect of two representative dietary compounds (quercetin and myricetin) on plasma and tissue levels of several PG products *in vivo*, experiments were performed using a short-term animal model. The plasma level of PGE_2, a representative PG, was found to be markedly increased in a time- and dose-dependent manner for both quercetin and myricetin. This effect was biphasic, *i.e.*, low doses stimulated COX wherever high doses inhibited its activity. Notably, the biphasic pattern of PGE_2 plasma levels closely resembled the patterns observed for the formation of PGE_2 *in vitro*. Moreover, when quercetin was administered orally, it also significantly increased the plasma levels of PGE_2 in male SD rats in a time-dependent manner, but the effect was less pronounced than when given by *i.v.* administration. The tissue levels of various PG products in animals that received a single *i.v.* injection of myricetin or quercetin varied from tissue to tissue. According to the rodent

effective oral dose (at 10 mg/kg b.w.), it is estimated that these dose should be achievable in humans as well.

Computational molecular modeling studies and structure-activity relationship analysis revealed that the ability of bioflavonoids to activate COX I and II depends on the structural features of their *B-rings*. Using computational homology modeling as a tool, the peroxidase active site of COXs were identified as the binding site for bioflavonoids. The bioflavonoids directly interact with the hematin component of COXs and facilitate the electron transfer from bioflavonoids to hematin.

To confirm the results obtained from computational modeling studies, we determined the stimulatory effect of myricetin on the catalytic activity of COX I and II after pre-treating the enzymes with acetylsalicylic acid. Pretreatment of COX I or II with acetylsalicylic acid acetylated the cyclooxygenase active site and strongly inhibited the cyclooxygenase activity. However, when PGG_2 was used as a substrate, acetylsalicylic acid-pretreated COX I and II still retained the catalytic activity. Moreover, myricetin stimulated the catalytic activity of COX I or II when either AA or PGG_2 were used as the substrate with or without acetylsalicylic acid pretreatment. These data indicate that myricetin stimulates COX I and II through interaction with the peroxidase site but not the cyclooxygenase site.

A site-directed mutagenesis approach was used to verify the mechanisms proposed from the molecular docking studies. The study of COX II confirmed that Q189 in its perioxidase active site was crucial for the binding of the bioflavonoid molecule and subsequently the stimulation of the COX II activity. These findings

provide the structural basis for the functions of bioflavonoids as naturally-occurring, high-affinity reducing co-substrates of COXs through selectively binding to the peroxidase active site, facilitating the electron transfer, and ultimately, enhancing the peroxidase's catalytic activity.

Based computational findings, it was predicted that bioflavonoids with no hydroxyl groups in their *B-ring* would function as COX inhibitors by selectively binding to the peroxidase site of the enzymes and thereby inhibit the binding of those bioflavonoids that serve as co-substrates. To test this hypothesis, galangin was used as a representative bioflavonoid because it does not have any hydroxyl group in its *B-ring*. Galangin was found to strongly inhibit the COX activity stimulated by quercetin both *in vitro* and *in vivo* by competitively binding to the same binding site. These results verified the notion that the presence of hydroxyl groups in the *B-ring* of bioflavonoids plays a crucial role in activating the COX activity. This concept also provides a new strategy for designing novel COX inhibitors.

Physiological/pathophysiological implications

Over the past fifty years, several thousand bioflavonoids have been identified and their various pharmacological actions have been characterized (Barishaw, 1949; Smith and Lands, 1971; Deschner et al., 1991; Tzeng et al., 1991; Hsuanyu and Dunford, 1992a; Terao et al., 1994; Rump et al., 1995). Bioflavonoids are polyphenolic compounds widely distributed in human diet (such as fruits, vegetables, and wine). A number of recent studies have shown that many of these dietary

polyphenols are effectively absorbed by the digestive system in humans and are present in circulation and tissues at physiologically or pharmacologically relevant concentrations (Lee et al., 1995; Chow et al., 2001; Ross and Kasum, 2002; Chow et al., 2003; Manach et al., 2004; Manach et al., 2005).

Historically, some of the bioflavonoids were once considered to be a class of vitamin-like compounds (Barishaw, 1949), because of their unique biological property in reducing capillary fragility and permeability. Nowadays, bioflavonoids are not considered as vitamins because they have not been shown unequivocally to be essential dietary constituents required for normal physiological functions. Epidemiological studies including more than 100,000 patients have shown an inverse association between dietary bioflavonoid intake and mortality from coronary heart disease and/or risk of stroke (Hertog et al., 1993; Rimm et al., 1996). Although prospective randomized clinical trials are lacking, several studies using animal models support the potential protective effects of bioflavonoids in cardiovascular diseases (Middleton et al., 2000). Isorhamnetin and quercetin, for example, produce vasodilator effects in rat aorta, rat mesenteric arteries, rat portal vein, and porcine coronary arteries (Ibarra et al., 2002; Perez-Vizcaino et al., 2002). Mechanistically, epidemiological studies suggest that an increase intake of bioflavonoids is associated with a reduction in cardiovascular events by enhancing the endothelial functions in humans (Fisher et al., 2003). While it has been suggested that an enhancement of NO synthesis might partially contribute to their cardiovascular protective effects (Fisher et al., 2003), the exact mechanism is still not fully understood at present. Quercetin

has also been shown to have a NO-independent vasodilator effect (Duarte et al., 1993; Perez-Vizcaino et al., 2002); when given orally, it reduces blood pressure, cardiac hypertrophy, and vascular remodeling in spontaneously hypertensive (SHR) and nitric oxide (NO)-deficient rats (Duarte et al., 2001; Duarte et al., 2002). In addition, chronic use of selective COX II inhibitors (*e.g.*, rofecoxib) was associated with an elevated cardiovascular risk and mortality (Gottlieb, 2001; Weir et al., 2003). The results of our present study suggest that bioflavonoids may protect the cardiovascular diseases in an NO-independent manner by stimulating COX catalytic activity *in vivo*. If this assumption proves to be correct, then this would provide support for the original proposal of nearly half a century ago concerning bioflavonoids as vitamin-like compounds.

Because PGs produced by COXs in the stomach and intestine exert important functions in maintaining the integrity of mucosal epithelium (Silverstein et al., 2000; Moore et al., 2006; Blandizzi et al., 2009), abnormally low levels of PGs in these tissues as a result of the use of acetylsalicylic acid and other NSAIDs has also been associated with the development of peptic ulcers in humans (Bombardier et al., 2000; White et al., 2002). Studies have shown that administration of cisapride, a stimulator of the COX activity, has a cytoprotective effect on gastric mucosal lesions induced by ethanol (Motilva et al., 1996). Like cisapride, some bioflavonoids also can stimulate COX activity and thus may also be useful for the treatment of peptic ulcers. In support of this idea, an earlier study has shown that apple polyphenolic extracts could prevent damage to human gastric epithelial cells *in vitro* and to rat gastric mucosa *in*

vivo (Graziani et al., 2005).

Since some of the dietary bioflavonoids can increase the levels of PGs *in vivo* (Bai and Zhu, 2009), here it should be noted that theoretically it is also possible that if the levels of PGs are elevated too high by these dietary compounds, they may increase the risk for developing certain pathological conditions. Recent studies demonstrated that the expression of COX II is increased in various human tumors as well as in animal tumor models. Similarly, the use of COX inhibitors was effective in the prevention or treatment of many disease conditions and also cancers (Kalgutkar and Zhao, 2001). For instance, data from epidemiological and animal studies indicated that NSAIDs, which inhibit the COX activity, reduced the relative risk of colon cancer and promote tumor regression (Zha et al., 2004). Similarly, natural product curcumin, which inhibits COX II expression by blocking NF-κB activation, can function as a chemopreventive agent in colonic epithelial cells (Plummer et al., 1999). Collectively, these observations suggest that increased levels of COX II expression or PGs are associated with colon cancer. However, there is no evidence that bioflavonoids increase the risk of colon cancer. Therefore, the finding that stimulatory effect of bioflavonoids on formation of PGs have to be further studied to demonstrate if increased levels of PGs by some bioflavonoids would increase the risk of certain pathological conditions.

In conclusion, some of the bioflavonoids, such as myricetin, quercetin, fisetin and morin, were found to have a powerful direct stimulatory effect on the COX I and II activity at physiologically-relevant doses. Based on the results obtained from these

studies, it was suggested that one of the important biological functions of bioflavonoids in the human body might be to serve as the naturally-occurring co-substrates for the COX enzymes through binding tightly into the peroxidase active site, and interacting directly with the hematin component of the COX enzymes to facilitate the electron transfer from bioflavonoids to hematin. Besides, some of the dietary compounds with no hydroxyl group on their *B-rings*, such as galangin, can function as inhibitors of COXs. These studies provide a platform for the future development of novel modulators (stimulators or inhibitors) of the human COX I and II activity.

LITERATURE CITED

Abd El Mohsen MM, Kuhnle G, Rechner AR, Schroeter H, Rose S, Jenner P and Rice-Evans CA (2002) Uptake and metabolism of epicatechin and its access to the brain after oral ingestion. *Free Radic Biol Med* **33**:1693-1702.

American Chemical Society. Meeting (205th : 1993 : Denver Colo.), Kauffman GB, American Chemical Society. Division of the History of Chemistry., American Chemical Society. Division of Chemical Education. and American Chemical Society. Division of Inorganic Chemistry. (1994) *Coordination chemistry : a century of progress : developed from a symposium sponsored by the Divisions of the History of Chemistry, Chemical Education, Inc., and Inorganic Chemistry, Inc., at the 205th National Meeting of the American Chemical Society, Denver, Colorado, March 28-April 2, 1993*. American Chemical Society, Washington, DC.

An N, Zou ZM, Tian Z, Luo XZ, Yang SL and Xu LZ (2008) Diarylheptanoids from the rhizomes of Alpinia officinarum and their anticancer activity. *Fitoterapia* **79**:27-31.

Aziz AA, Edwards CA, Lean ME and Crozier A (1998) Absorption and excretion of conjugated flavonols, including quercetin-4'-O-beta-glucoside and isorhamnetin-4'-O-beta-glucoside by human volunteers after the consumption of onions. *Free Radic Res* **29**:257-269.

Baba S, Osakabe N, Yasuda A, Natsume M, Takizawa T, Nakamura T and Terao J (2000) Bioavailability of (-)-epicatechin upon intake of chocolate and cocoa in human volunteers. *Free Radic Res* **33**:635-641.

Bai HW and Zhu BT (2008) Strong activation of cyclooxygenase I and II catalytic activity by dietary bioflavonoids. *J Lipid Res* **49**:2557-2570.

Bai HW and Zhu BT (2009) Myricetin and quercetin are naturally occurring co-substrates of cyclooxygenases in vivo. *Prostaglandins Leukot Essent Fatty Acids*.

Bakhle YS and Botting RM (1996) Cyclooxygenase-2 and its regulation in inflammation. *Mediators Inflamm* **5**:305-323.

Bambai B and Kulmacz RJ (2000) Prostaglandin H synthase. Effects of peroxidase cosubstrates on cyclooxygenase velocity. *J Biol Chem* **275**:27608-27614.

Barishaw SB (1949) The use of hesperidin-C in the treatment of abnormal capillary fragility. *Exp Med Surg* **7**:358-365.

Barnett J, Chow J, Ives D, Chiou M, Mackenzie R, Osen E, Nguyen B, Tsing S, Bach C, Freire J and et al. (1994) Purification, characterization and selective inhibition of human prostaglandin G/H synthase 1 and 2 expressed in the baculovirus system. *Biochim Biophys Acta* **1209**:130-139.

Baumann J, von Bruchhausen F and Wurm G (1980) Flavonoids and related compounds as inhibition of arachidonic acid peroxidation. *Prostaglandins* **20**:627-639.

Belton O, Byrne D, Kearney D, Leahy A and Fitzgerald DJ (2000) Cyclooxygenase-1 and -2-dependent prostacyclin formation in patients with atherosclerosis. *Circulation* **102**:840-845.

Blandizzi C, Tuccori M, Colucci R, Fornai M, Antonioli L, Ghisu N and Del Tacca M (2009) Role of coxibs in the strategies for gastrointestinal protection in patients requiring chronic non-steroidal anti-inflammatory therapy. *Pharmacol Res* **59**:90-100.

Bombardier C, Laine L, Reicin A, Shapiro D, Burgos-Vargas R, Davis B, Day R, Ferraz MB, Hawkey CJ, Hochberg MC, Kvien TK and Schnitzer TJ (2000) Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group. *N Engl J Med* **343**:1520-1528, 1522 p following 1528.

Bors W and Michel C (2002) Chemistry of the antioxidant effect of polyphenols. *Ann N Y Acad Sci* **957**:57-69.

Bors W and Saran M (1987) Radical scavenging by flavonoid antioxidants. *Free Radic Res Commun* **2**:289-294.

Bravo L (1998) Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr Rev* **56**:317-333.

Breder CD, Dewitt D and Kraig RP (1995) Characterization of inducible cyclooxygenase in rat brain. *J Comp Neurol* **355**:296-315.

Brown JE, Khodr H, Hider RC and Rice-Evans CA (1998) Structural dependence of flavonoid interactions with Cu2+ ions: implications for their antioxidant properties. *Biochem J* **330 (Pt 3)**:1173-1178.

Brown JP (1980) A review of the genetic effects of naturally occurring flavonoids, anthraquinones and related compounds. *Mutat Res* **75**:243-277.

Burda S and Oleszek W (2001) Antioxidant and antiradical activities of flavonoids. *J Agric Food Chem* **49**:2774-2779.

Caccetta RA, Croft KD, Beilin LJ and Puddey IB (2000) Ingestion of red wine significantly increases plasma phenolic acid concentrations but does not acutely affect ex vivo lipoprotein oxidizability. *Am J Clin Nutr* **71**:67-74.

Callan OH, So OY and Swinney DC (1996) The kinetic factors that determine the affinity and selectivity for slow binding inhibition of human prostaglandin H synthase 1 and 2 by indomethacin and flurbiprofen. *J Biol Chem* **271**:3548-3554.

Chan BS, Satriano JA, Pucci M and Schuster VL (1998) Mechanism of prostaglandin E2 transport across the plasma membrane of HeLa cells and Xenopus oocytes expressing the prostaglandin transporter "PGT". *J Biol Chem* **273**:6689-6697.

Chang HC, Churchwell MI, Delclos KB, Newbold RR and Doerge DR (2000) Mass spectrometric determination of Genistein tissue distribution in diet-exposed Sprague-Dawley rats. *J Nutr* **130**:1963-1970.

Chassevent F (1969) [Chlorogenic acid, physiological and pharmacological activity]. *Ann Nutr Aliment* **23**:Suppl:1-14.

Chen W, Pawelek TR and Kulmacz RJ (1999) Hydroperoxide dependence and cooperative cyclooxygenase kinetics in prostaglandin H synthase-1 and -2. *J Biol Chem* **274**:20301-20306.

Cheng HF, Wang JL, Zhang MZ, Miyazaki Y, Ichikawa I, McKanna JA and Harris RC (1999) Angiotensin II attenuates renal cortical cyclooxygenase-2 expression. *J Clin Invest* **103**:953-961.

Chi YS, Jong HG, Son KH, Chang HW, Kang SS and Kim HP (2001) Effects of naturally occurring prenylated flavonoids on enzymes metabolizing arachidonic acid: cyclooxygenases and lipoxygenases. *Biochem Pharmacol* **62**:1185-1191.

Chow HH, Cai Y, Alberts DS, Hakim I, Dorr R, Shahi F, Crowell JA, Yang CS and Hara Y (2001) Phase I pharmacokinetic study of tea polyphenols following single-dose administration of epigallocatechin gallate and polyphenon E. *Cancer Epidemiol Biomarkers Prev* **10**:53-58.

Chow HH, Cai Y, Hakim IA, Crowell JA, Shahi F, Brooks CA, Dorr RT, Hara Y and Alberts DS (2003) Pharmacokinetics and safety of green tea polyphenols after multiple-dose administration of epigallocatechin gallate and polyphenon E in healthy individuals. *Clin Cancer Res* **9**:3312-3319.

Coldham NG and Sauer MJ (2000) Pharmacokinetics of [(14)C]Genistein in the rat: gender-related differences, potential mechanisms of biological action, and implications for human health. *Toxicol Appl Pharmacol* **164**:206-215.

Collier HO, McDonald-Gibson WJ and Saeed SA (1976) Stimulation of prostaglandin biosynthesis by drugs: effects in vitro of some drugs affecting gut function. *Br J Pharmacol* **58**:193-199.

Cos P, Ying L, Calomme M, Hu JP, Cimanga K, Van Poel B, Pieters L, Vlietinck AJ and Vanden Berghe D (1998) Structure-activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. *J Nat Prod* **61**:71-76.

Coward L, Barnes NC, Setchell KDR and Barnes S (2002) Genistein, daidzein, and their .beta.-glycoside conjugates: antitumor isoflavones in soybean foods from American and Asian diets. *Journal of Agricultural and Food Chemistry* **41**:1961-1967.

Crespy V, Morand C, Besson C, Cotelle N, Vezin H, Demigne C and Remesy C (2003) The splanchnic metabolism of flavonoids highly differed according to the nature of the compound. *Am J Physiol Gastrointest Liver Physiol* **284**:G980-988.

Crofford LJ, Tan B, McCarthy CJ and Hla T (1997) Involvement of nuclear factor kappa B in the regulation of cyclooxygenase-2 expression by interleukin-1 in rheumatoid synoviocytes. *Arthritis Rheum* **40**:226-236.

da Cunha FM, Duma D, Assreuy J, Buzzi FC, Niero R, Campos MM and Calixto JB (2004) Caffeic acid derivatives: in vitro and in vivo anti-inflammatory properties. *Free Radic Res* **38**:1241-1253.

Das S and Das DK (2007) Anti-inflammatory responses of resveratrol. *Inflamm Allergy Drug Targets* **6**:168-173.

Deschner EE, Ruperto J, Wong G and Newmark HL (1991) Quercetin and rutin as inhibitors of azoxymethanol-induced colonic neoplasia. *Carcinogenesis* **12**:1193-1196.

DeWitt DL (1999) Cox-2-selective inhibitors: the new super aspirins. *Mol Pharmacol* **55**:625-631.

DeWitt DL and Smith WL (1988) Primary structure of prostaglandin G/H synthase from sheep vesicular gland determined from the complementary DNA sequence. *Proc Natl Acad Sci U S A* **85**:1412-1416.

Di Carlo G, Mascolo N, Izzo AA and Capasso F (1999) Flavonoids: old and new aspects of a class of natural therapeutic drugs. *Life Sci* **65**:337-353.

Dietz R, Nastainczyk W and Ruf HH (1988) Higher oxidation states of prostaglandin H synthase. Rapid electronic spectroscopy detected two spectral intermediates during the peroxidase reaction with prostaglandin G2. *Eur J Biochem* **171**:321-328.

Donovan JL, Bell JR, Kasim-Karakas S, German JB, Walzem RL, Hansen RJ and Waterhouse AL (1999) Catechin is present as metabolites in human plasma after consumption of red wine. *J Nutr* **129**:1662-1668.

Donovan JL, Kasim-Karakas S, German JB and Waterhouse AL (2002) Urinary excretion of catechin metabolites by human subjects after red wine consumption. *Br J Nutr* **87**:31-37.

Duarte J, Jimenez R, O'Valle F, Galisteo M, Perez-Palencia R, Vargas F, Perez-Vizcaino F, Zarzuelo A and Tamargo J (2002) Protective effects of the flavonoid quercetin in chronic nitric oxide deficient rats. *J Hypertens* **20**:1843-1854.

Duarte J, Perez-Palencia R, Vargas F, Ocete MA, Perez-Vizcaino F, Zarzuelo A and Tamargo J (2001) Antihypertensive effects of the flavonoid quercetin in spontaneously hypertensive rats. *Br J Pharmacol* **133**:117-124.

Duarte J, Perez-Vizcaino F, Zarzuelo A, Jimenez J and Tamargo J (1993) Vasodilator effects of quercetin in isolated rat vascular smooth muscle. *Eur J Pharmacol* **239**:1-7.

DuBois RN and Smalley WE (1996) Cyclooxygenase, NSAIDs, and colorectal cancer. *J Gastroenterol* **31**:898-906.

Duffy SJ, Keaney JF, Jr., Holbrook M, Gokce N, Swerdloff PL, Frei B and Vita JA (2001) Short- and long-term black tea consumption reverses endothelial dysfunction in patients with coronary artery disease. *Circulation* **104**:151-156.

Eastmond DA, Smith MT and Irons RD (1987) An interaction of benzene metabolites reproduces the myelotoxicity observed with benzene exposure. *Toxicol Appl Pharmacol* **91**:85-95.

Egan RW, Gale PH, Beveridge GC, Marnett LJ and Kuehl FA, Jr. (1980) Direct and indirect involvement of radical scavengers during prostaglandin biosynthesis. *Adv Prostaglandin Thromboxane Res* **6**:153-155.

Egan RW, Gale PH, Beveridge GC, Phillips GB and Marnett LJ (1978) Radical scavenging as the mechanism for stimulation of prostaglandin cyclooxygenase and depression of inflammation by lipoic acid and sodium iodide. *Prostaglandins* **16**:861-869.

Egan RW, Paxton J and Kuehl FA, Jr. (1976) Mechanism for irreversible self-deactivation of prostaglandin synthetase. *J Biol Chem* **251**:7329-7335.

Elangovan V, Sekar N and Govindasamy S (1994) Chemopreventive potential of dietary bioflavonoids against 20-methylcholanthrene-induced tumorigenesis. *Cancer Lett* **87**:107-113.

Eling TE, Thompson DC, Foureman GL, Curtis JF and Hughes MF (1990) Prostaglandin H synthase and xenobiotic oxidation. *Annu Rev Pharmacol Toxicol* **30**:1-45.

Ferrandiz ML and Alcaraz MJ (1991) Anti-inflammatory activity and inhibition of arachidonic acid metabolism by flavonoids. *Agents Actions* **32**:283-288.

Fisher ND, Hughes M, Gerhard-Herman M and Hollenberg NK (2003) Flavanol-rich cocoa induces nitric-oxide-dependent vasodilation in healthy humans. *J Hypertens* **21**:2281-2286.

Flower RJ and Vane JR (1972) Inhibition of prostaglandin synthetase in brain explains the anti-pyretic activity of paracetamol (4-acetamidophenol). *Nature* **240**:410-411.

Fuhrman B, Lavy A and Aviram M (1995) Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation. *Am J Clin Nutr* **61**:549-554.

Gabor M (1972) Pharmacologic effects of flavonoids on blood vessels. *Angiologica* **9**:355-374.

Gallois C, Habib A, Tao J, Moulin S, Maclouf J, Mallat A and Lotersztajn S (1998) Role of NF-kappaB in the antiproliferative effect of endothelin-1 and tumor necrosis factor-alpha in human hepatic stellate cells. Involvement of cyclooxygenase-2. *J Biol Chem* **273**:23183-23190.

Ghosh S, May MJ and Kopp EB (1998) NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* **16**:225-260.

Gierse JK, Hauser SD, Creely DP, Koboldt C, Rangwala SH, Isakson PC and Seibert K (1995) Expression and selective inhibition of the constitutive and inducible forms of human cyclo-oxygenase. *Biochem J* **305 (Pt 2)**:479-484.

Gil B, Sanz MJ, Terencio MC, Ferrandiz ML, Bustos G, Paya M, Gunasegaran R and Alcaraz MJ (1994) Effects of flavonoids on Naja naja and human recombinant synovial phospholipases A2 and inflammatory responses in mice. *Life Sci* **54**:PL333-338.

Goodwin DC, Landino LM and Marnett LJ (1999) Effects of nitric oxide and nitric oxide-derived species on prostaglandin endoperoxide synthase and prostaglandin biosynthesis. *FASEB J* **13**:1121-1136.

Gottlieb S (2001) COX 2 inhibitors may increase risk of heart attack. *BMJ* **323**:471.

Graefe EU, Wittig J, Mueller S, Riethling AK, Uehleke B, Drewelow B, Pforte H, Jacobasch G, Derendorf H and Veit M (2001) Pharmacokinetics and bioavailability of quercetin glycosides in humans. *J Clin Pharmacol* **41**:492-499.

Graziani G, D'Argenio G, Tuccillo C, Loguercio C, Ritieni A, Morisco F, Del Vecchio Blanco C, Fogliano V and Romano M (2005) Apple polyphenol extracts prevent damage to human gastric epithelial cells in vitro and to rat gastric mucosa in vivo. *Gut* **54**:193-200.

Hamberg M and Samuelsson B (1967) Oxygenation of unsaturated fatty acids by the vesicular gland of sheep. *J Biol Chem* **242**:5344-5354.

Hamberg M and Samuelsson B (1973) Detection and isolation of an endoperoxide intermediate in prostaglandin biosynthesis. *Proc Natl Acad Sci U S A* **70**:899-903.

Hammerstone JF, Lazarus SA and Schmitz HH (2000) Procyanidin content and variation in some commonly consumed foods. *J Nutr* **130**:2086S-2092S.

Harvison PJ, Egan RW, Gale PH, Christian GD, Hill BS and Nelson SD (1988) Acetaminophen and analogs as cosubstrates and inhibitors of prostaglandin H synthase. *Chem Biol Interact* **64**:251-266.

Havsteen BH (2002) The biochemistry and medical significance of the flavonoids. *Pharmacol Ther* **96**:67-202.

Hemler ME and Lands WE (1980) Evidence for a peroxide-initiated free radical mechanism of prostaglandin biosynthesis. *J Biol Chem* **255**:6253-6261.

Herschman HR (1996) Prostaglandin synthase 2. *Biochim Biophys Acta* **1299**:125-140.

Hertog MG, Feskens EJ, Hollman PC, Katan MB and Kromhout D (1993) Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* **342**:1007-1011.

Hertog MG, Sweetnam PM, Fehily AM, Elwood PC and Kromhout D (1997) Antioxidant flavonols and ischemic heart disease in a Welsh population of men: the Caerphilly Study. *Am J Clin Nutr* **65**:1489-1494.

Higdon JV and Frei B (2003) Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Crit Rev Food Sci Nutr* **43**:89-143.

Hollman PC and Katan MB (1999) Dietary flavonoids: intake, health effects and bioavailability. *Food Chem Toxicol* **37**:937-942.

Hollman PC, van Trijp JM, Buysman MN, van der Gaag MS, Mengelers MJ, de Vries JH and Katan MB (1997) Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *FEBS Lett* **418**:152-156.

Hollman PC, vd Gaag M, Mengelers MJ, van Trijp JM, de Vries JH and Katan MB (1996) Absorption and disposition kinetics of the dietary antioxidant quercetin in man. *Free Radic Biol Med* **21**:703-707.

Hong J, Smith TJ, Ho CT, August DA and Yang CS (2001) Effects of purified green and black tea polyphenols on cyclooxygenase- and lipoxygenase-dependent metabolism of arachidonic acid in human colon mucosa and colon tumor tissues. *Biochem Pharmacol* **62**:1175-1183.

Hsi LC, Hoganson CW, Babcock GT, Garavito RM and Smith WL (1995) An examination of the source of the tyrosyl radical in ovine prostaglandin endoperoxide synthase-1. *Biochem Biophys Res Commun* **207**:652-660.

Hsuanyu Y and Dunford HB (1990) Reactions of prostaglandin H synthase in the presence of the stabilizing agents diethyldithiocarbamate and glycerol. *Biochem Cell Biol* **68**:965-972.

Hsuanyu Y and Dunford HB (1992a) Prostaglandin H synthase kinetics. The effect of substituted phenols on cyclooxygenase activity and the substituent effect on phenolic peroxidatic activity. *J Biol Chem* **267**:17649-17657.

Hsuanyu YC and Dunford HB (1992b) Reduction of prostaglandin H synthase compound II by phenol and hydroquinone, and the effect of indomethacin. *Arch Biochem Biophys* **292**:213-220.

Ibarra M, Perez-Vizcaino F, Cogolludo A, Duarte J, Zaragoza-Arnaez F, Lopez-Lopez JG and Tamargo J (2002) Cardiovascular effects of isorhamnetin and quercetin in isolated rat and porcine vascular smooth muscle and isolated rat atria. *Planta Med* **68**:307-310.

Ioku K, Tsushida T, Takei Y, Nakatani N and Terao J (1995) Antioxidative activity of quercetin and quercetin monoglucosides in solution and phospholipid bilayers. *Biochim Biophys Acta* **1234**:99-104.

Jobin C, Morteau O, Han DS and Balfour Sartor R (1998) Specific NF-kappaB blockade selectively inhibits tumour necrosis factor-alpha-induced COX-2 but

not constitutive COX-1 gene expression in HT-29 cells. *Immunology* **95**:537-543.

Ju J, Hong J, Zhou JN, Pan Z, Bose M, Liao J, Yang GY, Liu YY, Hou Z, Lin Y, Ma J, Shih WJ, Carothers AM and Yang CS (2005) Inhibition of intestinal tumorigenesis in Apcmin/+ mice by (-)-epigallocatechin-3-gallate, the major catechin in green tea. *Cancer Res* **65**:10623-10631.

Kalgutkar AS and Zhao Z (2001) Discovery and design of selective cyclooxygenase-2 inhibitors as non-ulcerogenic, anti-inflammatory drugs with potential utility as anti-cancer agents. *Curr Drug Targets* **2**:79-106.

Kalkbrenner F, Wurm G and von Bruchhausen F (1992) In vitro inhibition and stimulation of purified prostaglandin endoperoxide synthase by flavonoids: structure-activity relationship. *Pharmacology* **44**:1-12.

Kaneider NC, Mosheimer B, Reinisch N, Patsch JR and Wiedermann CJ (2004) Inhibition of thrombin-induced signaling by resveratrol and quercetin: effects on adenosine nucleotide metabolism in endothelial cells and platelet-neutrophil interactions. *Thromb Res* **114**:185-194.

Kao ES, Hsu JD, Wang CJ, Yang SH, Cheng SY and Lee HJ (2009) Polyphenols extracted from Hibiscus sabdariffa L. inhibited lipopolysaccharide-induced inflammation by improving antioxidative conditions and regulating cyclooxygenase-2 expression. *Biosci Biotechnol Biochem* **73**:385-390.

Kao ES, Wang CJ, Lin WL, Yin YF, Wang CP and Tseng TH (2005) Anti-inflammatory potential of flavonoid contents from dried fruit of Crataegus pinnatifida in vitro and in vivo. *J Agric Food Chem* **53**:430-436.

Khan N, Afaq F, Saleem M, Ahmad N and Mukhtar H (2006) Targeting multiple signaling pathways by green tea polyphenol (-)-epigallocatechin-3-gallate. *Cancer Res* **66**:2500-2505.

Khan N and Mukhtar H (2007) Tea polyphenols for health promotion. *Life Sci* **81**:519-533.

Koga T and Meydani M (2001) Effect of plasma metabolites of (+)-catechin and quercetin on monocyte adhesion to human aortic endothelial cells. *Am J Clin Nutr* **73**:941-948.

Koshkin V and Dunford HB (1999) Coupling of the peroxidase and cyclooxygenase reactions of prostaglandin H synthase. *Biochim Biophys Acta* **1430**:341-348.

Kraemer SA, Meade EA and DeWitt DL (1992) Prostaglandin endoperoxide synthase gene structure: identification of the transcriptional start site and 5'-flanking regulatory sequences. *Arch Biochem Biophys* **293**:391-400.

Kuhnau J (1976) The flavonoids. A class of semi-essential food components: their role in human nutrition. *World Rev Nutr Diet* **24**:117-191.

Kulmacz RJ (1986) Prostaglandin H synthase and hydroperoxides: peroxidase reaction and inactivation kinetics. *Arch Biochem Biophys* **249**:273-285.

Kulmacz RJ (1987) Attachment of substrate metabolite to prostaglandin H synthase upon reaction with arachidonic acid. *Biochem Biophys Res Commun* **148**:539-545.

Kulmacz RJ (1998) Cellular regulation of prostaglandin H synthase catalysis. *FEBS Lett* **430**:154-157.

Kulmacz RJ and Lands WE (1985) Quantitative similarities in the several actions of cyanide on prostaglandin H synthase. *Prostaglandins* **29**:175-190.

Kulmacz RJ, Pendleton RB and Lands WE (1994) Interaction between peroxidase and cyclooxygenase activities in prostaglandin-endoperoxide synthase. Interpretation of reaction kinetics. *J Biol Chem* **269**:5527-5536.

Kuo SM (1997) Dietary flavonoid and cancer prevention: evidence and potential mechanism. *Crit Rev Oncog* **8**:47-69.

Kurumbail RG, Kiefer JR and Marnett LJ (2001) Cyclooxygenase enzymes: catalysis and inhibition. *Curr Opin Struct Biol* **11**:752-760.

Kurumbail RG, Stevens AM, Gierse JK, McDonald JJ, Stegeman RA, Pak JY, Gildehaus D, Miyashiro JM, Penning TD, Seibert K, Isakson PC and Stallings WC (1996) Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. *Nature* **384**:644-648.

Lambert JD and Yang CS (2003) Cancer chemopreventive activity and bioavailability of tea and tea polyphenols. *Mutat Res* **523-524**:201-208.

Landino LM, Crews BC, Gierse JK, Hauser SD and Marnett LJ (1997) Mutational analysis of the role of the distal histidine and glutamine residues of

prostaglandin-endoperoxide synthase-2 in peroxidase catalysis, hydroperoxide reduction, and cyclooxygenase activation. *J Biol Chem* **272**:21565-21574.

Lecomte M, Lecocq R, Dumont JE and Boeynaems JM (1990) Covalent binding of arachidonic acid metabolites to human platelet proteins. Identification of prostaglandin H synthase as one of the modified substrates. *J Biol Chem* **265**:5178-5187.

Lee JL, Kim A, Kopelovich L, Bickers DR and Athar M (2005) Differential expression of E prostanoid receptors in murine and human non-melanoma skin cancer. *J Invest Dermatol* **125**:818-825.

Lee MJ, Wang ZY, Li H, Chen L, Sun Y, Gobbo S, Balentine DA and Yang CS (1995) Analysis of plasma and urinary tea polyphenols in human subjects. *Cancer Epidemiol Biomarkers Prev* **4**:393-399.

Li SQ and Zhang QH (2001) Advances in the development of functional foods from buckwheat. *Crit Rev Food Sci Nutr* **41**:451-464.

Lien EJ, Ren S, Bui HH and Wang R (1999) Quantitative structure-activity relationship analysis of phenolic antioxidants. *Free Radic Biol Med* **26**:285-294.

Lim H, Gupta RA, Ma WG, Paria BC, Moller DE, Morrow JD, DuBois RN, Trzaskos JM and Dey SK (1999) Cyclo-oxygenase-2-derived prostacyclin mediates embryo implantation in the mouse via PPARdelta. *Genes Dev* **13**:1561-1574.

Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM and Dey SK (1997) Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell* **91**:197-208.

Lu G, Tsai AL, Van Wart HE and Kulmacz RJ (1999) Comparison of the peroxidase reaction kinetics of prostaglandin H synthase-1 and -2. *J Biol Chem* **274**:16162-16167.

Luong C, Miller A, Barnett J, Chow J, Ramesha C and Browner MF (1996) Flexibility of the NSAID binding site in the structure of human cyclooxygenase-2. *Nat Struct Biol* **3**:927-933.

MacCornack FA (1977) The effects of coffee drinking on the cardiovascular system: experimental and epidemiological research. *Prev Med* **6**:104-119.

MacDonald ID, Graff G, Anderson LA and Dunford HB (1989) Optical spectra and kinetics of reactions of prostaglandin H synthase: effects of the substrates 13-hydroperoxyoctadeca-9,11-dienoic acid, arachidonic acid, N,N,N',N'-tetramethyl-p-phenylenediamine, and phenol and of the nonsteroidal anti-inflammatory drugs aspirin, indomethacin, phenylbutazone, and bromfenac. *Arch Biochem Biophys* **272**:194-202.

Mahmoud NN, Carothers AM, Grunberger D, Bilinski RT, Churchill MR, Martucci C, Newmark HL and Bertagnolli MM (2000) Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. *Carcinogenesis* **21**:921-927.

Manach C, Morand C, Crespy V, Demigne C, Texier O, Regerat F and Remesy C (1998) Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant properties. *FEBS Lett* **426**:331-336.

Manach C, Morand C, Gil-Izquierdo A, Bouteloup-Demange C and Remesy C (2003) Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two doses of orange juice. *Eur J Clin Nutr* **57**:235-242.

Manach C, Scalbert A, Morand C, Remesy C and Jimenez L (2004) Polyphenols: food sources and bioavailability. *Am J Clin Nutr* **79**:727-747.

Manach C, Williamson G, Morand C, Scalbert A and Remesy C (2005) Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr* **81**:230S-242S.

Markey CM, Alward A, Weller PE and Marnett LJ (1987) Quantitative studies of hydroperoxide reduction by prostaglandin H synthase. Reducing substrate specificity and the relationship of peroxidase to cyclooxygenase activities. *J Biol Chem* **262**:6266-6279.

Marnett LJ (2000) Cyclooxygenase mechanisms. *Curr Opin Chem Biol* **4**:545-552.

Marnett LJ, Rowlinson SW, Goodwin DC, Kalgutkar AS and Lanzo CA (1999) Arachidonic acid oxygenation by COX-1 and COX-2. Mechanisms of catalysis and inhibition. *J Biol Chem* **274**:22903-22906.

Marnett LJ, Siedlik PH, Ochs RC, Pagels WR, Das M, Honn KV, Warnock RH, Tainer BE and Eling TE (1984) Mechanism of the stimulation of

prostaglandin H synthase and prostacyclin synthase by the antithrombotic and antimetastatic agent, nafazatrom. *Mol Pharmacol* **26**:328-335.

Marnett LJ and Wilcox CL (1977) Stimulation of prostaglandin biosynthesis by lipoic acid. *Biochim Biophys Acta* **487**:222-230.

McKay DL and Blumberg JB (2002) The role of tea in human health: an update. *J Am Coll Nutr* **21**:1-13.

Middleton E, Jr. and Kandaswami C (1992) Effects of flavonoids on immune and inflammatory cell functions. *Biochem Pharmacol* **43**:1167-1179.

Middleton E, Jr. and Kandaswami C (1994) *The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer.* Chapman and Hall, London.

Middleton E, Jr., Kandaswami C and Theoharides TC (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev* **52**:673-751.

Miller C, Zhang M, He Y, Zhao J, Pelletier JP, Martel-Pelletier J and Di Battista JA (1998) Transcriptional induction of cyclooxygenase-2 gene by okadaic acid inhibition of phosphatase activity in human chondrocytes: co-stimulation of AP-1 and CRE nuclear binding proteins. *J Cell Biochem* **69**:392-413.

MirAfzali Z, Leipprandt JR, McCracken JL and DeWitt DL (2005) Fast, efficient reconstitution of the cyclooxygenases into proteoliposomes. *Arch Biochem Biophys* **443**:60-65.

Miyamoto T, Ogino N, Yamamoto S and Hayaishi O (1976) Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J Biol Chem* **251**:2629-2636.

Moore RA, Derry S, Phillips CJ and McQuay HJ (2006) Nonsteroidal anti-inflammatory drugs (NSAIDs), cyxlooxygenase-2 selective inhibitors (coxibs) and gastrointestinal harm: review of clinical trials and clinical practice. *BMC Musculoskelet Disord* **7**:79.

Motilva V, Lopez A, Martin MJ, La Casa C and Alarcon de la Lastra C (1996) Cytoprotective activity of cisapride on experimental gastric mucosal lesions induced by ethanol. Role of endogenous prostaglandins. *Prostaglandins* **52**:63-74.

Mukhtar H and Ahmad N (2000) Tea polyphenols: prevention of cancer and optimizing health. *Am J Clin Nutr* **71**:1698S-1702S; discussion 1703S-1694S.

Murata T, Ushikubi F, Matsuoka T, Hirata M, Yamasaki A, Sugimoto Y, Ichikawa A, Aze Y, Tanaka T, Yoshida N, Ueno A, Oh-ishi S and Narumiya S (1997) Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature* **388**:678-682.

Neuhouser ML (2004) Dietary flavonoids and cancer risk: evidence from human population studies. *Nutr Cancer* **50**:1-7.

Noroozi M, Angerson WJ and Lean ME (1998) Effects of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes. *Am J Clin Nutr* **67**:1210-1218.

Olthof MR, Hollman PC, Vree TB and Katan MB (2000) Bioavailabilities of quercetin-3-glucoside and quercetin-4'-glucoside do not differ in humans. *J Nutr* **130**:1200-1203.

Onoe Y, Miyaura C, Kaminakayashiki T, Nagai Y, Noguchi K, Chen QR, Seo H, Ohta H, Nozawa S, Kudo I and Suda T (1996) IL-13 and IL-4 inhibit bone resorption by suppressing cyclooxygenase-2-dependent prostaglandin synthesis in osteoblasts. *J Immunol* **156**:758-764.

Otto JC and Smith WL (1996) Photolabeling of prostaglandin endoperoxide H synthase-1 with 3-trifluoro-3-(m-[125I]iodophenyl)diazirine as a probe of membrane association and the cyclooxygenase active site. *J Biol Chem* **271**:9906-9910.

Panganamala RV, Gavino VC and Cornwell DG (1979) Effect of low and high methional concentrations on prostaglandin biosynthesis in microsomes from bovine and sheep vesicular glands. *Prostaglandins* **17**:155-162.

Paradkar PN, Blum PS, Berhow MA, Baumann H and Kuo SM (2004) Dietary isoflavones suppress endotoxin-induced inflammatory reaction in liver and intestine. *Cancer Lett* **215**:21-28.

Parr AJ and Bolwell GP (2000) Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *Journal of the Science of Food and Agriculture* **80**:985-1012.

Perez-Vizcaino F, Ibarra M, Cogolludo AL, Duarte J, Zaragoza-Arnaez F, Moreno L, Lopez-Lopez G and Tamargo J (2002) Endothelium-independent vasodilator

effects of the flavonoid quercetin and its methylated metabolites in rat conductance and resistance arteries. *J Pharmacol Exp Ther* **302**:66-72.

Picot D, Loll PJ and Garavito RM (1994) The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1. *Nature* **367**:243-249.

Pietta PG (2000) Flavonoids as antioxidants. *J Nat Prod* **63**:1035-1042.

Pilbeam CC, Fall PM, Alander CB and Raisz LG (1997) Differential effects of nonsteroidal anti-inflammatory drugs on constitutive and inducible prostaglandin G/H synthase in cultured bone cells. *J Bone Miner Res* **12**:1198-1203.

Piskula MK and Terao J (1998) Accumulation of (-)-epicatechin metabolites in rat plasma after oral administration and distribution of conjugation enzymes in rat tissues. *J Nutr* **128**:1172-1178.

Plummer SM, Holloway KA, Manson MM, Munks RJ, Kaptein A, Farrow S and Howells L (1999) Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-kappaB activation via the NIK/IKK signalling complex. *Oncogene* **18**:6013-6020.

Poli V (1998) The role of C/EBP isoforms in the control of inflammatory and native immunity functions. *J Biol Chem* **273**:29279-29282.

Regan JW (2003) EP2 and EP4 prostanoid receptor signaling. *Life Sci* **74**:143-153.

Rein D, Lotito S, Holt RR, Keen CL, Schmitz HH and Fraga CG (2000) Epicatechin in human plasma: in vivo determination and effect of chocolate consumption on plasma oxidation status. *J Nutr* **130**:2109S-2114S.

Renaud S and de Lorgeril M (1992) Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **339**:1523-1526.

Rice-Evans C, Miller N and Paganga G (1997) Antioxidant properties of phenolic compounds. **2**:152-159.

Rieke CJ, Mulichak AM, Garavito RM and Smith WL (1999) The role of arginine 120 of human prostaglandin endoperoxide H synthase-2 in the interaction with fatty acid substrates and inhibitors. *J Biol Chem* **274**:17109-17114.

Rimm EB, Katan MB, Ascherio A, Stampfer MJ and Willett WC (1996) Relation between intake of flavonoids and risk for coronary heart disease in male health professionals. *Ann Intern Med* **125**:384-389.

Robertson RP (1998) Dominance of cyclooxygenase-2 in the regulation of pancreatic islet prostaglandin synthesis. *Diabetes* **47**:1379-1383.

Rocca B, Spain LM, Pure E, Langenbach R, Patrono C and FitzGerald GA (1999) Distinct roles of prostaglandin H synthases 1 and 2 in T-cell development. *J Clin Invest* **103**:1469-1477.

Roshak AK, Jackson JR, McGough K, Chabot-Fletcher M, Mochan E and Marshall LA (1996) Manipulation of distinct NFkappaB proteins alters interleukin-1beta-induced human rheumatoid synovial fibroblast prostaglandin E2 formation. *J Biol Chem* **271**:31496-31501.

Ross JA and Kasum CM (2002) Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu Rev Nutr* **22**:19-34.

Ruetten H and Thiemermann C (1997) Effects of tyrphostins and genistein on the circulatory failure and organ dysfunction caused by endotoxin in the rat: a possible role for protein tyrosine kinase. *Br J Pharmacol* **122**:59-70.

Rump AF, Schussler M, Acar D, Cordes A, Ratke R, Theisohn M, Rosen R, Klaus W and Fricke U (1995) Effects of different inotropes with antioxidant properties on acute regional myocardial ischemia in isolated rabbit hearts. *Gen Pharmacol* **26**:603-611.

Scalbert A and Williamson G (2000) Dietary intake and bioavailability of polyphenols. *J Nutr* **130**:2073S-2085S.

Schlosser MJ, Shurina RD and Kalf GF (1989) Metabolism of phenol and hydroquinone to reactive products by macrophage peroxidase or purified prostaglandin H synthase. *Environ Health Perspect* **82**:229-237.

Schmedtje JF, Jr., Ji YS, Liu WL, DuBois RN and Runge MS (1997) Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells. *J Biol Chem* **272**:601-608.

Sfakianos J, Coward L, Kirk M and Barnes S (1997) Intestinal uptake and biliary excretion of the isoflavone genistein in rats. *J Nutr* **127**:1260-1268.

Shi W, Hoganson CW, Espe M, Bender CJ, Babcock GT, Palmer G, Kulmacz RJ and Tsai A (2000) Electron paramagnetic resonance and electron nuclear double resonance spectroscopic identification and characterization of the tyrosyl radicals in prostaglandin H synthase 1. *Biochemistry* **39**:4112-4121.

Shitashige M, Morita I and Murota S (1998) Different substrate utilization between prostaglandin endoperoxide H synthase-1 and -2 in NIH3T3 fibroblasts. *Biochim Biophys Acta* **1389**:57-66.

Silva MM, Santos MR, Caroco G, Rocha R, Justino G and Mira L (2002) Structure-antioxidant activity relationships of flavonoids: a re-examination. *Free Radic Res* **36**:1219-1227.

Silverstein FE, Faich G, Goldstein JL, Simon LS, Pincus T, Whelton A, Makuch R, Eisen G, Agrawal NM, Stenson WF, Burr AM, Zhao WW, Kent JD, Lefkowith JB, Verburg KM and Geis GS (2000) Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. *JAMA* **284**:1247-1255.

Simonetti P, Pietta P and Testolin G (1997) Polyphenol Content and Total Antioxidant Potential of Selected Italian Wines. *Journal of Agricultural and Food Chemistry* **45**:1152-1155.

Smith WL and Dewitt DL (1996) Prostaglandin endoperoxide H synthases-1 and -2. *Adv Immunol* **62**:167-215.

Smith WL, DeWitt DL and Garavito RM (2000) Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem* **69**:145-182.

Smith WL, Garavito RM and DeWitt DL (1996) Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J Biol Chem* **271**:33157-33160.

Smith WL and Lands WE (1971) Stimulation and blockade of prostaglandin biosynthesis. *J Biol Chem* **246**:6700-6702.

So OY, Scarafia LE, Mak AY, Callan OH and Swinney DC (1998) The dynamics of prostaglandin H synthases. Studies with prostaglandin h synthase 2 Y355F unmask mechanisms of time-dependent inhibition and allosteric activation. *J Biol Chem* **273**:5801-5807.

Spencer AG, Thuresson E, Otto JC, Song I, Smith T, DeWitt DL, Garavito RM and Smith WL (1999a) The membrane binding domains of prostaglandin endoperoxide H synthases 1 and 2. Peptide mapping and mutational analysis. *J Biol Chem* **274**:32936-32942.

Spencer JP, Chowrimootoo G, Choudhury R, Debnam ES, Srai SK and Rice-Evans C (1999b) The small intestine can both absorb and glucuronidate luminal flavonoids. *FEBS Lett* **458**:224-230.

Steinberg D, Parthasarathy S, Carew TE, Khoo JC and Witztum JL (1989) Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* **320**:915-924.

Su B and Karin M (1996) Mitogen-activated protein kinase cascades and regulation of gene expression. *Curr Opin Immunol* **8**:402-411.

Sugimoto Y, Segi E, Tsuboi K, Ichikawa A and Narumiya S (1998) Female reproduction in mice lacking the prostaglandin F receptor. Roles of prostaglandin and oxytocin receptors in parturition. *Adv Exp Med Biol* **449**:317-321.

Sung YM, He G and Fischer SM (2005) Lack of expression of the EP2 but not EP3 receptor for prostaglandin E2 results in suppression of skin tumor development. *Cancer Res* **65**:9304-9311.

Swinney DC, Mak AY, Barnett J and Ramesha CS (1997) Differential allosteric regulation of prostaglandin H synthase 1 and 2 by arachidonic acid. *J Biol Chem* **272**:12393-12398.

Takaku K, Oshima M, Miyoshi H, Matsui M, Seldin MF and Taketo MM (1998) Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes. *Cell* **92**:645-656.

Terao J, Piskula M and Yao Q (1994) Protective effect of epicatechin, epicatechin gallate, and quercetin on lipid peroxidation in phospholipid bilayers. *Arch Biochem Biophys* **308**:278-284.

Thompson D and Eling T (1989) Mechanism of inhibition of prostaglandin H synthase by eugenol and other phenolic peroxidase substrates. *Mol Pharmacol* **36**:809-817.

Tsai A, Hsi LC, Kulmacz RJ, Palmer G and Smith WL (1994) Characterization of the tyrosyl radicals in ovine prostaglandin H synthase-1 by isotope replacement and site-directed mutagenesis. *J Biol Chem* **269**:5085-5091.

Tzeng SH, Ko WC, Ko FN and Teng CM (1991) Inhibition of platelet aggregation by some flavonoids. *Thromb Res* **64**:91-100.

Ushikubi F, Segi E, Sugimoto Y, Murata T, Matsuoka T, Kobayashi T, Hizaki H, Tuboi K, Katsuyama M, Ichikawa A, Tanaka T, Yoshida N and Narumiya S

(1998) Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP3. *Nature* **395**:281-284.

Van der Ouderaa FJ, Buytenhek M, Nugteren DH and Van Dorp DA (1977) Purification and characterisation of prostaglandin endoperoxide synthetase from sheep vesicular glands. *Biochim Biophys Acta* **487**:315-331.

Vane JR and Botting RM (1998) Anti-inflammatory drugs and their mechanism of action. *Inflamm Res* **47 Suppl 2**:S78-87.

Vitrac X, Desmouliere A, Brouillaud B, Krisa S, Deffieux G, Barthe N, Rosenbaum J and Merillon JM (2003) Distribution of [14C]-trans-resveratrol, a cancer chemopreventive polyphenol, in mouse tissues after oral administration. *Life Sci* **72**:2219-2233.

Watanabe S, Yamaguchi M, Sobue T, Takahashi T, Miura T, Arai Y, Mazur W, Wahala K and Adlercreutz H (1998) Pharmacokinetics of soybean isoflavones in plasma, urine and feces of men after ingestion of 60 g baked soybean powder (kinako). *J Nutr* **128**:1710-1715.

Weir MR, Sperling RS, Reicin A and Gertz BJ (2003) Selective COX-2 inhibition and cardiovascular effects: a review of the rofecoxib development program. *Am Heart J* **146**:591-604.

Welton AF, Tobias LD, Fiedler-Nagy C, Anderson W, Hope W, Meyers K and Coffey JW (1986) Effect of flavonoids on arachidonic acid metabolism. *Prog Clin Biol Res* **213**:231-242.

White WB, Faich G, Whelton A, Maurath C, Ridge NJ, Verburg KM, Geis GS and Lefkowith JB (2002) Comparison of thromboembolic events in patients treated with celecoxib, a cyclooxygenase-2 specific inhibitor, versus ibuprofen or diclofenac. *Am J Cardiol* **89**:425-430.

Wu G, Wei C, Kulmacz RJ, Osawa Y and Tsai AL (1999) A mechanistic study of self-inactivation of the peroxidase activity in prostaglandin H synthase-1. *J Biol Chem* **274**:9231-9237.

Xiao G, Chen W and Kulmacz RJ (1998) Comparison of structural stabilities of prostaglandin H synthase-1 and -2. *J Biol Chem* **273**:6801-6811.

Xu XM, Tang JL, Chen X, Wang LH and Wu KK (1997) Involvement of two Sp1 elements in basal endothelial prostaglandin H synthase-1 promoter activity. *J Biol Chem* **272**:6943-6950.

Yamamoto K, Arakawa T, Ueda N and Yamamoto S (1995) Transcriptional roles of nuclear factor kappa B and nuclear factor-interleukin-6 in the tumor necrosis factor alpha-dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. *J Biol Chem* **270**:31315-31320.

Yang CS and Landau JM (2000) Effects of tea consumption on nutrition and health. *J Nutr* **130**:2409-2412.

Yao LH, Jiang YM, Shi J, Tomas-Barberan FA, Datta N, Singanusong R and Chen SS (2004) Flavonoids in food and their health benefits. *Plant Foods Hum Nutr* **59**:113-122.

Yoshizumi M, Tsuchiya K, Suzaki Y, Kirima K, Kyaw M, Moon JH, Terao J and Tamaki T (2002) Quercetin glucuronide prevents VSMC hypertrophy by angiotensin II via the inhibition of JNK and AP-1 signaling pathway. *Biochem Biophys Res Commun* **293**:1458-1465.

Youdim KA, Martin A and Joseph JA (2000) Incorporation of the elderberry anthocyanins by endothelial cells increases protection against oxidative stress. *Free Radic Biol Med* **29**:51-60.

Zha S, Yegnasubramanian V, Nelson WG, Isaacs WB and De Marzo AM (2004) Cyclooxygenases in cancer: progress and perspective. *Cancer Lett* **215**:1-20.

Zhang Y, Shaffer A, Portanova J, Seibert K and Isakson PC (1997) Inhibition of cyclooxygenase-2 rapidly reverses inflammatory hyperalgesia and prostaglandin E2 production. *J Pharmacol Exp Ther* **283**:1069-1075.