FORMATION AND METABOLISM OF 15-DEOXY-$\Delta^{12,14}$-PROSTAGLANDIN J_2 IN VIVO

By

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$^1O_2$	Singlet oxygen
8-oxo-G	7,8-dihydro-8-oxo-guanine
AA	Arachidonic acid
AAPH	2,2'-Azobis (2-amidinopropane) hydrochloride
AD	Alzheimer's disease
ALS	Amyotriphic lateral sclerosis
AP-1	Activator protein
APCI	Atmospheric pressure chemical ionization
ATM	Ataxia telangiectasia mutation
ATP	Adenosine triphosphate
BHT	Butylated hydroxytoluene
CCl_4	Carbon tetrachloride
CHO	Chinese hamster ovary
CID	Collision induced dissociation
COX	Cyclooxygenase
$cPLA_2$	Cytosolic phospholipase A_2
CRTH2	Chemoattractant receptor-homologous molecule expressed on Th2 cells
CYP	Cytochrome P450
CyPG	Cyclopentenone prostaglandin
DHA	Docosahexaenoic acid
DP	Prostaglandin D_2 receptor
EP	Prostaglandin E_2 receptor
EPA	Eicosapentaenoic acid

ERα	Estrogen receptor α
ERK	Extracellular signal-related kinase
ESI	Electrospray ionization
ETC	Electron transport chain
ETE	Epoxyeicosatrienoic acid
FLAP	5-Lipoxygenase activating protein
FP	Prostaglandin F_2 receptor
GC	Gas chromatography
GPCR	G-protein-coupled receptor
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSH	Glutathione disulfide
GST	Glutathione *S*-transferase
H_2O_2	Hydrogen peroxide
HETE	Hydroxyeicosatetraenoic acid
HIF	Hypoxia inducible factor
HLM	Human liver microsomes
HNE	4-Hydroxy-2-nonenal
$HO^{\cdot}$	Hydroxyl radical
HO-1	Hemeoxygenase 1
HOCl	Hypochlorus acid
HPETE	Hydroperoxyeicosatetraenoic acid
HPLC	High-performance liquid chromatography

Hsp70	Heat shock protein 70
HTC	Hepatoma tissue culture
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intracellular adhesion molecule 1
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
iNOS	Inducible nitric oxide synthase
iP	Isoprostane
IsoP	Isoprostane
IκB	Inhibitor of kappa B
IKK	IκB kinase
JNK	c-Jun-NH_2-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
LA	Linoleic acid
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotriene
MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
MPO	Myoloperoxidase
MRP	Multi-drug resistance associated protein
MsrA	Methionine sulfoxidereductase
MS	Mass spectrometry

MS/MS	Tandem mass spectrometry
$NaBH_4$	Sodium borohydryde
NADH	Nicotinaminde adenione dinucleotide
NADPH	Nicotinaminde adenione dinucleotide phosphate
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
Nrf2	NF-E2-related factor 2
$O_2^{\cdot -}$	Superoxide
$ONOO^-$	Peroxynitrite
oxLDL	Oxidized low density lipoprotein
PAF	Platelet-activating factor
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PDGF	Platelet-derived growth factor
PE	Phosphatidylethanolamine
PG	Prostaglandin
PI3K	Phosphoinositol 3 kinase
PPAR	Peroxisome proliferator-activated receptor
PTP1B	Protein tyrosine phosphatase 1B
PUFA	Polyunsaturated fatty acid
rAor	Recombinant alkenal/one oxidoreductase
$RO^{\cdot}$	Alkoxyl radical
$ROO^{\cdot}$	Peroxyl radical
ROS	Reactive oxygen species

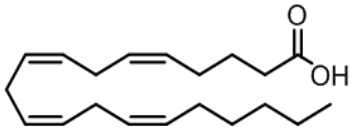
RP-HPLC	Reversed phase high-performance liquid chromatography
SIM	Selected ion monitoring
sHE	Soluble epoxide hydrolase
SOD	Superoxide dismutase
SRM	Selected reaction monitoring
STAT	Signal transducer and activator of transcription
TNFα	Tumor necrosis factor alpha
TP	Thromboxane receptor
TPP	Triphenylphosphine
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Trx	Thioredoxin
TX	Thromboxane
UGT	UDP-glucuronosyltransferases
UPLC	Ultra-performance liquid chromatography

CHAPTER I

INTRODUCTION

Generation of eicosanoids from arachidonic acid

Eicosanoids comprise a family of biologically important lipids derived from 20-carbon essential fatty acids, primarily arachidonic acid (Morrow and Roberts 2001). Arachidonic acid (5,8,11,14-eicosatetraenoic acid) (20:4) is an ω-6 polyunsaturated fatty and is the most abundant essential fatty acid precursor to eicosanoids in humans (**FIGURE 1.1**). Arachidonic acid is either derived from dietary linoleic acid (9,12-octadecadienoic acid) (18:2) or ingested as a dietary constituent, but it cannot be synthesized from other food components (Burr et al. 1930). The metabolism of linoleic acid to arachidonic acid requires the conversion of linoleic acid to γ-linolenic acid by Δ^6desaturase, followed by conversion of γ-linolenic acid to dihomo-γ-linolenic acid, and finally to arachidonic acid. Arachidonic acid is present in animal tissues esterified in the phospholipids of cell membranes or other complex lipids. Metabolism of arachidonic acid leads to the formation of a diverse array of bioactive lipid mediators, including prostaglandins (PGs), thromboxane (TX), leukotrienes (LTs), and related compounds. Other essential fatty acids that are precursors to eicosanoids include dihomo-γ-linolenic acid (8,11,14-eicosatrienoic acid) (20:3) and eicosapentaenoic acid (5,8,11,14,17-eicosapentaenoic acid, EPA) (20:5), an abundant component of fish oils. Eicosanoids are divided into various classes depending on their structural characteristics and mechanisms of formation (Morrow and Roberts 2001).



Arachidonic acid
(C20:4, ω-6)

FIGURE 1.1. Structure of arachidonic acid.

The concentration of free fatty acids is limited in most cells; thus, the biosynthesis of eicosanoids depends upon the release of arachidonic acid from cellular stores of lipids by

acylhydrolases, such as phospholipase A_2 (PLA_2) (Morrow and Roberts 2001). Cytosolic phospholipase A_2 ($cPLA_2$) plays an important role in the liberation of arachidonic acid from membrane glycerophospholipids, particularly phosphatidylcholine (PC) and phosphatidylethanolamine (PE); but other phospholipases, such as the secretory PLA_2 ($sPLA_2$) isozymes (groups IIA, IID, V, and X), are also capable releasing arachidonic acid (Morrow and Roberts 2001; Ueno et al. 2001). PLA_2 catalyzes hydrolysis of the *sn*-2 ester bond of membrane phospholipids supplying arachidonate to eicosanoid synthesizing enzymes. Eicosanoid production is induced in response to diverse stimuli, such as hormones, autocoids, and other substances (Morrow and Roberts 2001). Hormones and autocoids that have been found to stimulate biosynthesis of eicosanoids include histamine (Murayama et al. 1990), bradykinin (Grenier et al. 1981), platelet activating factor (PAF) (Glaser et al. 1990), angiotensin II (Gimbrone and Alexander 1975), interleukin-I (IL-1) (Albrightson et al. 1985; Burch et al. 1988), leukotrienes (Cramer et al. 1983), and platelet-derived growth factor (PDGF) (Habenicht et al. 1990). Other physiological stimuli include proteases such as thrombin (Weksler et al. 1978). The interaction of these agents with G-protein-coupled cell surface receptors is thought to cause the direct activation of one or more specific phoshoplipases or an increase in intracellular Ca^{2+}, which can also activate these enzymes (Smith et al. 1991; Morrow and Roberts 2001). Once released, free arachidonate is a substrate for several distinct enzyme systems, including cyclooxygenases, lipoxygenases, and cytochrome P450s, which catalyze the metabolism of arachidonate to a series of oxygenated products (Morrow and Roberts 2001).

Cyclooxygenase-derived prostaglandins

Cyclooxygenases (COXs), or prostaglandin endoperoxide H synthases (PGHSs), catalyze the committed step in the conversion of arachidonate to prostaglandins (PGs) and thromboxane

(TX) A_2. COXs are homodimeric membrane-associated glycoproteins localized to the inner leaflet of the nuclear envelope and endoplasmic reticulum bilayers (Spencer et al. 1998). The levels of PG and TX produced are determined by the expression and activity of COX enzymes (Scher et al. 2005). Two COX isoforms have been identified, a constitutive form (COX-1) and an inducible form (COX-2) (Smith et al. 1996). COX-1 was first purified from sheep seminal vesicles in 1976 (Helmer and Lands 1976) and cloned in 1988 (DeWitt and Smith 1988; Merlie et al. 1988; Yokoyama et al. 1988). This COX isoform was initially thought to be solely responsible for the production of PGs and TX. However, in 1991 Simmons and colleagues discovered the second COX isoform, COX-2, which shares 60% sequence identity with COX-1 (Xie et al. 1991). While COX-1 and COX-2 are similar in structure and catalytic activity, these two isozymes differ substantially with respect to pattern of expression and biology (Smith et al. 1996). COX-1 is expressed in most tissues and its activity is thought to serve in the production of prostanoids involved in regulation of homeostatic functions. On the other hand, COX-2 is not present in most mammalian tissues under normal conditions. Its expression is induced in response to growth factors, tumor promoters, hormones, bacterial endotoxin, and pro-inflammatory cytokines (Smith et al. 1996). COX-2 stimulated expression is inhibited by anti-inflammatory steroids, such as cortisol and dexamethasone (Kujubu and Herschman 1992; Mesferrer et al. 1994; Crofford et al. 1994). COXs are also important pharmacological targets for nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin and indomethacin, which block PG and TX synthesis through inhibition of COX (Vane 1971; Carty and Marfat 1996).

COXs are bifunctional, heme-containing enzymes which catalyze the sequential generation of PGG_2 and PGH_2 through a two-step reaction at two distinct, but interrelated catalytic sites (Smith et al. 1991). The cyclooxygenase (endoperoxide synthase) site oxygenates arachidonate to the bicyclic endoperoxide intermediate PGG_2, and the peroxidase site converts

PGG_2 to PGH_2 (Hamberg et al. 1974). Tissue specific synthases then convert PGH_2 to the parent prostanoids PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin) and TXA_2 (Morrow and Roberts 2001). Synthesis of PGE_2 from PGH_2 is catalyzed by three isomerases, cytosolic PGE synthase (cPGES), microsomal PGES-1 (mPGES-1), or mPGES-2 (Park et al. 2006). Two isomerases have been identified for the synthesis of PGD_2, hematopoietic PGD synthase (H-PGDS) and lipocalin-type PGD synthase (L-PGDS). $PGF_{2\alpha}$ is formed from the reduction of PGH_2 by PGF synthase/PGH reductase (Smith et al. 1991). Formation of PGI_2 from PGH_2 is catalyzed by PGI synthase, and TXA_2 formation is catalyzed by TXA synthase (Smith et al. 1991; Morrow and Roberts 2001). Evidence suggests that production of PGs in various cell types may involve preferential functional coupling between particular COX isoforms and PG synthases (Ueno et al. 2001; Park et al. 2006). The synthesis of prostanoids via the COX pathway is shown in **FIGURE 1.2**.

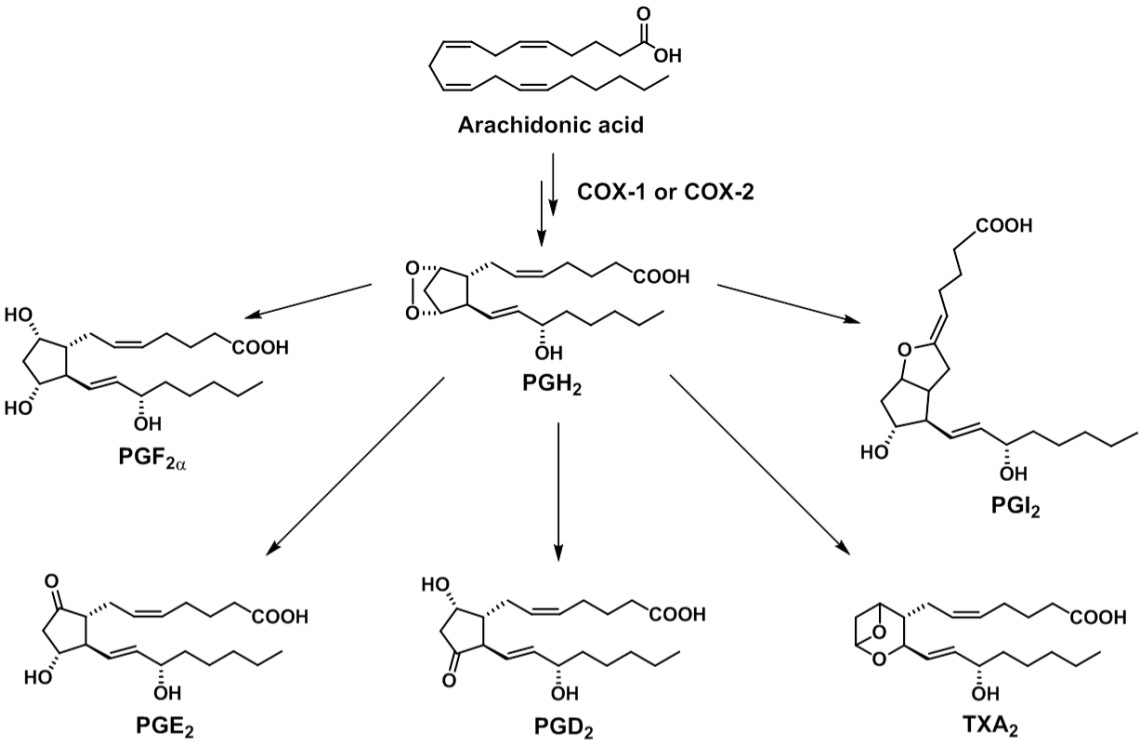


FIGURE 1.2. Prostaglandin synthesis from the enzymatic oxygenation of arachidonic acid by cyclooxygenase.

The discovery of prostaglandins is attributed to Goldblatt and von Euler, who independently reported smooth muscle-stimulating and blood pressure-lowering activity in seminal fluid and extracts and secretions from the prostate gland and seminal vesicles. Von Euler named the active substance "prostaglandin", which he characterized as a lipid-soluble acid (von Euler 1936). The first prostaglandin structure characterized was PGE_1 (Bergstrom and Samuelsson 1962), and later prostaglandins E_1, E_2, E_3, $F_{1\alpha}$, and $F_{2\alpha}$ were identified in human seminal plasma (Samuelsson 1963). Since their initial discovery, prostanoids have been detected in almost every tissue and body fluid (Morrow and Roberts 2001). Prostaglandins of the 2-series are derived from arachidonic acid, and these are the major PGs found in mammals. Dihomo-γ-linoleic acid is the precursor of 1-series PGs, and eicosapentaenoic acid (EPA) is the precursor for the 3-series PGs (Morrow and Roberts 2001).

Prostanoids (PGs and TX) are local hormones (autocoids) acting at or near their sites of synthesis to mediate a broad spectrum of biological effects. These lipid mediators elicit their effects primarily through interaction with G-protein-coupled receptors (GPCRs) expressed in the target tissue. Eight types of prostanoid GPCRs have been identified, including four subtypes of the PGE receptor (EP_1, EP_2, EP_3, and EP_4), PGD receptor (DP_1), PGF receptor (FP), PGI receptor (IP), and TXA receptor (TP) (Kobayashi and Naruyima 2002). In addition, PGD_2 activates a second GPCR termed chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2), which is a member of the chemokine receptor family (Hirai et al. 2001). Prostanoids exhibit complex and coordinated activity in the regulation of important physiological and pathophysiological processes. PGs are generally thought to be pro-inflammatory mediators. Their effects contribute to the classic signs of acute inflammation including local reddening, heat generation, and swelling, which are caused by increased blood flow and vascular permeability. In 1971, Vane proposed that NSAIDs owe their biological effects to blockade of PG formation

(Vane 1971). Recent evidence suggests that PGs may also have anti-inflammatory effects under certain conditions (Narumiya 2009). PGE_2 mediates peripheral pain signals, induction of fever, vasodilation, maintenance of renal blood flow, and suppression of intestinal inflammation. PGD_2 facilitates allergic inflammation, induction of sleep, and vasodilation. TXA_2 promotes platelet aggregation and vasoconstriction. PGI_2 inhibits platelet aggregation and mediates inflammatory swelling and vasodilation. $PGF_{2\alpha}$ causes vasoconstriction and plays a key role in the induction of labor, along with PGE_2. Further, PGE_2 and PGI_2 mediate gastrointestinal protection (Morrow and Roberts 2001; Narumiya 2009).

Prostaglandin D_2

The biological consequences of PGD_2 formation areof particular relevanceto the studies described in this dissertation. PGD_2 is produced by PGD_2 synthases in a variety of cells, including mast cells, type 2 helper T-lymphocytes (Th2 cells), macrophages, and dendritic cells (Lewis et al. 1982; Tanaka et al. 2000; Funk 2001). Hematopoeitic-PGD synthase (H-PGDS) is mainly expressed in immune and inflammatory cells, such as mast cells and Th2 cells,while lipocalin-type PGD synthase (L-PGDS) is predominately expressed in the central nervous system, male genital organs, heart, and adipose tissue (Kanaoka and Urade 2003). PGD_2 is significantly generated during IgE-dependent activation of mast cells via H-PGDS (Lewis et al. 1982). Once it is synthesized, PGD_2 acts via two GPCRs, DP_1 and CRTH2, to mediate its biological effects (Kobayashi and Naruyima 2002; Hirai et al. 2001; Nagata et al. 1999). PGD_2 plays a critical role in mediating allergic and inflammatory responses (Funk 2001). It induces bronchoconstriction, airway hyperresponsiveness, increased vascular permeability, and attraction of inflammatory cells, including Th2 cells, eosinophils, and basophils (Hirai et al. 2001). PGD_2 also promotes induction of sleep, vasodilation, and inhibition of platelet aggregation (Hayaishi and Urade 2002;

Smith et al. 1974). Pathophysiological conditions associated with overproduction of PGD_2 include systemic mastocytosis and allergic asthma (Roberts et al. 1980; Matsuoka et al. 2000).

Lipoxygenases and leukotrienes

Lipoxygenases (LOXs) are non-heme iron-containing dioxygenases that catalyze the oxygenation of fatty acids containing at least one 1-cis,4-cis-pentadiene system (two *cis* double bonds separated by a methylene carbon) (Ivanov et al. 2010). Arachidonic acid is an important substrate for mammalian LOX enzymes. LOXs convert their fatty acid substrate to a series of lipid hydroperoxides called hydroperoxyeicosatetraenoic acids (HPETEs). Several classes of LOXs have been identified, including 5-, 8-, 11-, 12-, and 15-LOX, and they are conventionally named with respect to the specific position in which they insert a hydroperoxy group in arachidonic acid (Ivanov et al. 2010). HPETEs formed by the various LOX enzymes are unstable and can be reduced to hydroxyeicosatetraenoic acids (HETEs). In addition, 12-HPETE can undergo molecular rearrangement to epoxy-hydroperoxyeicosatetraenoic acids, called hepoxilins. Leukocytes can convert 15-HPETE to trihydroxylated compounds, called lipoxins (Morrow and Roberts 2001).

5-lipoxygenase (5-LOX) is regarded as one of the most important LOX enzymes in that it initiates the biosynthesis of 4-series leukotrienes (LTs) from arachidonic acid. LTs were discovered as novel arachidonic acid metabolites generated in polymorphonuclear leukocytes (Borgeat and Samuelsson 1979). The term leukotriene (LT) was coined to describe the conjugated triene structure of these compounds and to indicate their generation from leukocytes such as human polymorphonuclear neutrophils. 5-LOX is a cytosolic protein expressed in a number of immune cells, including neutrophils, eosinphils, basophils, mast cells, monocytes, and macrophages (Murphy and Gijon 2007). Upon cell activation by inflammatory

or allergic stimuli, 5-LOX and $cPLA_2$ translocate to the nuclear membrane in a Ca^{2+}-dependent manner, and 5-LOX associates with 5-LOX activating protein (FLAP) located at the nuclear envelope. FLAP is an integral membrane protein required for LT biosynthesis (Morrow and Roberts 2001). Once $cPLA_2$ releases arachidonic acid from membrane phospholipids, FLAP is thought to transfer the arachidonic acid to 5-LOX, which catalyzes the first two steps in LT biosynthesis (Evans et al. 2008). 5-LOX first oxygenates arachidonic acid to 5*S*-HPETE and then converts this intermediate to the allylic epoxide LTA_4 (Newcomer and Gilbert 2010). LTA_4 can be transformed to LTB_4 by LTA_4 hydrolase, or it can be exported from the cell for transcellular metabolism to the cysteinyl LTs, including LTC_4, LTD_4, and LTE_4. LTC_4 synthase is a membrane-associated protein that catalyzes the conjugation of LTA_4 with glutathione to form LTC_4. Once synthesized, LTC_4 is exported out of the cell via primary ATP-dependent transport (Ishikawa 1992). LTC_4 can then be converted to LTD_4 (γ-glytamyl-cysteine conjugate) and LTE_4 (cysteine conjugate) in the extracellular space by two ectoenzymes, γ-glutamyltransferase and dipeptidase, respectively (Ishikawa 1992). The formation of LTs via the 5-LOX pathway is shown in **FIGURE 1.3**.

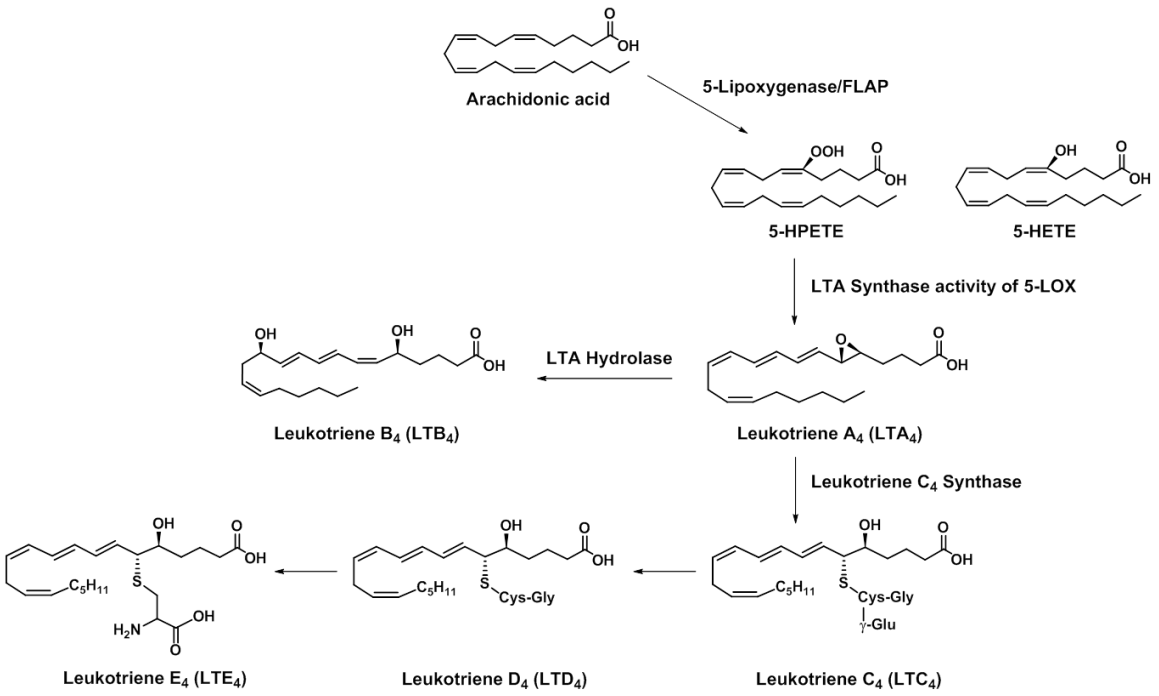


FIGURE 1.3. Leukotriene formation from the enzymatic oxygenation of arachidonic acid by 5-lipoxygenase.

Leukotrienes are key pro-inflammatory mediators in allergic inflammation and asthma (Samuelsson et al. 1987). These compounds act through specific GPCRs to produce their biological effects. LTB_4 induces neutrophil chemotaxis via activation of BLT_1 and BLT_2. The cysteinyl LTs cause bronchoconstriction, vasoconstriction, and increased vascular permeability through activation of $CysLT_1$ and $CysLT_2$. Interestingly, the active material originally known as "slow-reacting substance of anaphylaxis" (SRS-A) is now recognized to be a mixture of the cysteinyl LTs (Murphy et al. 1979). Cysteinyl LTs are involved in diseases such as local inflammation, bronchial asthma, livery injury, tissue trauma and shock (Samuelsson et al. 1987). Inhibition of LT synthesis and action is an important pharmacological target for the treatment of asthma and other respiratory diseases (Evans et al. 2008).

Cytochrome P450s and epoxyeicosatrienoic acids

Cytochrome P450 (CYP) epoxygenases catalyze the oxygenation of arachidonic acid to epoxyeicosatrienoic acids (EETs). Epoxidation of arachidonic acid at any one of its four double bonds gives rise to four regioisomers of EETs, 5,6-, 8,9-, 11,12-, and 14,15-EET (Pfister et al. 2010). EETs are formed in the endothelium of vascular beds, including the coronary and renal circulation, and they are released from endothelial cells in response to receptor activation by agonists such as acetycholine and bradykinin. EETs are regarded as important regulators of vascular tone, inflammation, and angiogenesis in the cardiovascular and renal systems (Pfister et al. 2010). These compounds induce hyperpolarization of vascular smooth muscle and vasorelaxation via activation of Ca^{2+}-activated K^{+} channels. Based on these observations, EETs have been characterized as endothelium-dependent hyperpolarizing factors (EDHFs) (Spector 2009). EETs also exhibit anti-inflammatory and cardioprotective effects in the vasculature through inhibition of adhesion molecule expression and inhibition of platelet aggregation and adhesion (Pfister et al. 2010). Further, EETs have been shown to protect ischemic myocardium and brain by promoting endothelial cell proliferation and neovascularization in ischemic tissues (Pfister et al. 2010). Metabolism of EETs by soluble epoxide hydrolase (sEH) or β-oxidation to less active metabolites limits their biological effects. Inhibition of sEH to increase the beneficial effects of EETs has emerged as a novel therapeutic strategy for the treatment of hypertension (Chiamvimonvat et al. 2007).

Prostaglandin metabolism

Prostaglandins can be metabolized by both enzymatic as well as nonenzymatic processes. The most well characterized mechanisms of PG metabolism involve their rapid transformation to predominately inactive metabolites by discrete enzyme systems *in vivo*. More

recently, it has been observed that PGE_2 and PGD_2 can also undergo spontaneous dehydrationunder various experimental conditions to form a subfamily of reactive PGs, termed cyclopentenone PGs. The physiological relevance of the latter pathway is still unclear.

Enzymatic catabolism of prostaglandins

The biological action of prostaglandins (PGs) is limited to the areas in which they are generated due to rapid metabolic inactivation. The primary mechanisms involved in PG catabolism and inactivation include dehydrogenation/oxidation reactions, double bond reduction, and β- and ω-oxidation (Morrow and Roberts 2001). β-oxidation of PGs occurs mainly in peroxisomesafter formation of the CoA ester at the C-1 carboxylic acid group, and this process is catalyzed by peroxisomal oxidases, such as acyl-CoA oxidase (Diczfalusy 1994; Schrader and Fahimi 2006). β-oxidation may also occur in the mitochondria. Sequential rounds of β-oxidation yield dinor- and tetranor-PG metabolites. In addition, ω-oxidation at the C-20 carbon of PGs produces polar dicarboxylic acids that are readily excreted into the urine (Morrow and Roberts 2001). PGE_2 and $PGF_{2\alpha}$ are rapidly metabolized in lung by 15-hydroxyprostaglandin dehydrogenase (15-OH-PGDH) and Δ^{13}reductase to yield their corresponding 15-keto-13,14-dihydro metabolites, 13,14-dihydro-15-keto-PGE_2 and 13,14-dihydro-15-keto-$PGF_{2\alpha}$, respectively (Morrow and Roberts 2001). The 15-keto-13,14-dihydro metabolitesof PGE_2 and $PGF_{2\alpha}$ are further degraded *in vivo* by β- and ω-oxidation to yield the major urinary metabolites 11α-hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid (PGE-M) and 9α,11α-dihydroxy-15-oxo-2,3,4,5-tetranor-prostane-1,20-dioic acid (PGF-M), respectively (Hamberg and Samuelsson 1969; Hamberg 1973). Metabolism of PGI_2 involves hydrolysis to 6-keto-$PGF_{1\alpha}$ followed by β-oxidation to form the urinary metabolite 2,3-dinor-6-keto-$PGF_{1\alpha}$ (Morrow and Roberts 2001). TXA_2 undergoes rapid hydrolysis to TXB_2, and subsequent β-oxidation yields the

urinary metabolite 2,3-dinor-TXB_2 (Roberts et al. 1977). PGD_2 is inactivated by different metabolic processes than those typical for other PGs. It is a poor substrate for $NAD(P)^+$-dependent 15-OH-PGDH enzymes (Lee and Levine 1975; Sun et al. 1976; Ruckrich et al. 1976). Rather, PGD_2 is preferentially metabolized by 11-ketoreductase, which catalyzes the stereo-specific reduction of PGD_2 to 9α,11β-PGF_2 (Liston and Robert 1985a). 9α,11β-PGF_2 exhibits its own spectrum of biological effects, including vasopressor activity (Liston and Robert 1985a), inhibition of platelet aggregation (Pugliese et al. 1985), and contraction of coronary and bronchial smooth muscle (Robertson et al. 1985; Seibert et al. 1987). 9α,11β-PGF_2 is further degraded *in vivo* by β- and ω-oxidation and other processes. The major urinary metabolite of PGD_2 is 9α,11β-dihydroxy-15-oxo-2,3,18,19-tetranorprost-5ene-1,20-dioic acid (PGD-M) (Liston and Robert 1985b).

Due to the extremely rapid metabolism of eicosanoids and their artifactual generation during sampling procedures, measurement of urinary metabolites has been established as the most accurate means to assess systemic eiscosanoid production (Samuelsson et al. 1975). In this regard, assessing eicosanoid formation by quantification of urinary metabolites has provided key insights into the pathophysiological processes involved in various human diseases. For example, measurement of PGD-M has had important clinical utility in the diagnosis and treatment of patients with mast cell activation disorders, such as systemic mastocytosis. Systemic mastocytosis is characterized by severe vasodilation and hemodynamic alterations that accompany episodes of systemic mast cell activation. These symptoms have been largely attributed to excessive PGD_2 release by activated mast cells (Roberts et al. 1980). Through the observation that PGD_2 mediates the severe symptoms of mastocytosis, Roberts and colleagues developed the effective treatment strategy of administering aspirin to patients with mastocytosis to inhibit PGD_2 production. In addition, measurement of PGD-M has also been

important in understanding the pharmacological actions and off-target effects of the lipid-lowering drug niacin (nicotinic acid). Niacin causes intense flushing due to marked PGD_2 release; levels of PGD-M increase 6.5 to 33-fold following niacin administration (Morrow et al. 1989; Awad et al. 1994). This bothersome side effect has limited the use of niacin as a hypolipidemic drug. However, niacin-induced flushing is significantly attenuated by pretreatment with COX inhibitors, which block production of PGD_2 (Roberts et al. 1980), as well as treatment with PGD_2 receptor antagonists to block the effects of PGD_2 release (Paolini et al. 2008).

Cyclopentenone prostaglandins

PGE_2 and PGD_2 are β-hydroxy ketones and are inherently unstable. These PGs can undergo spontaneous dehydration to generate cyclopentenone PGs of the A_2 and J_2 series (**FIGURE 1.4**). Dehydration of PGE_2 yields PGA_2 (Hamberg and Samuelsson 1966). Dehydration of PGD_2 yields J_2 series PGs, including PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15-d-PGJ_2) (Fitzpatrick and Wynalda et al. 1983). Cyclopentenone PGs (CyPGs) are characterized by the presence of an electrophilic α,β-unsaturated carbonyl moiety within their cyclopentenone ring, which renders them susceptible to nucleophilic addition reactions (Michael addition) with thiol-containing biomolecules, such as

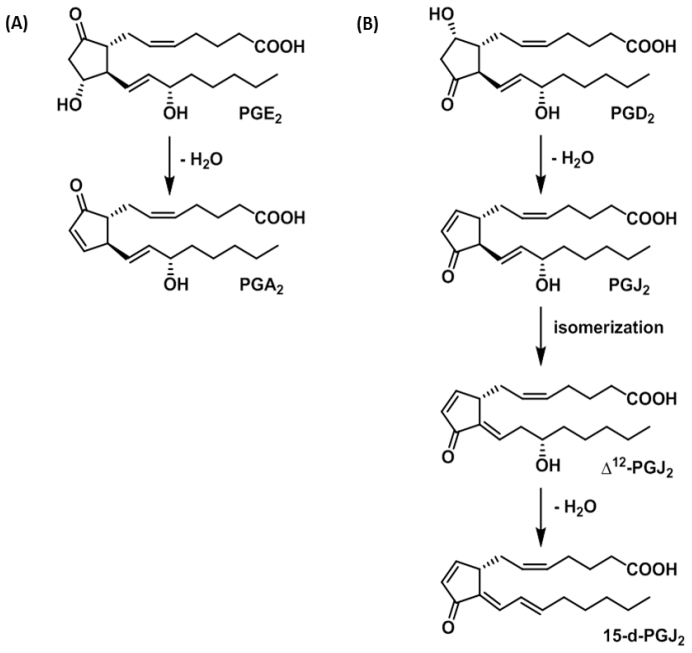


FIGURE 1.4. Formation of cyclopentenone prostaglandins. (A) Dehydration of PGE_2 yields PGA_2. (B) Dehydration of PGD_2 yields J_2 series PGs, including PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15-d-PGJ_2).

glutathione (GSH) and free cysteine residues of proteins (Straus and Glass 2001). The reactivity of the α,β-unsaturated carbonyl moiety is important to the bioactivity of CyPGs (Honn and Marnett 1985). CyPGs exhibit a unique spectrum of biological activities, such as anti-inflammatory, anti-neoplastic, and anti-viral effects, distinct from the parent PGs (Straus and Glass 2001). Unlike conventional PGs, which elicit biological responses primarily through activation of specific cell surface G-protein coupled receptors (GPCRs), CyPGs can be actively transported into cells (Naruyima et al. 1986; Naruyima et al. 1987), and they exert their biological effects through covalent modification of intracellular targets, such as transcription factors and other signaling molecules (Straus and Glass 2001).

PGA_2

PGA_2 was the first CyPG identified, discovered in seminal plasma in 1966 by Hamberg and Samuelsson (Hamberg and Samuelsson 1966). This compound was previously known as "medulin", an anti-hypertensive substance found in extracts of renal medulla (Lee et al. 1965). Shortly thereafter, Lee et al. reported the isolation of PGA_2 from rat kidney medulla indicating that PGA_2 was formed, at least in part, from dehydration of PGE_2 during the isolation procedures (Lee et al. 1967). In these studies it was found that mildly acidic or alkaline conditions were sufficient to dehydrate PGE_2 to PGA_2 (Lee et al. 1967). PGA_2 and PGA_1, the dehydration product of PGE_1, were shown to dose-dependently suppress DNA synthesis and cell proliferation in murine melanoma cells with greater potency than some known cancer chemotherapeutic agents, suggesting their chemotherapeutic potential (Honn et al. 1979). PGA_2 has also been shown to inhibit cell cycle progression, inhibit growth, and promote apoptosis in various cancer cells, such as breast cancer cells, L1210 leukemia cells, and HeLa S3 cells derived from cervical cancer (Hitomi et al. 1996; Goropse et al. 1996; Kim et al. 1993; Naruyima et al. 1987; Ohno et

al. 1986; Ohno et al. 1988). Further, PGA_2 possesses anti-viral activity (O'Brien et al. 1996). Biosynthesis of PGA_2 has been reported in cultured cells, such as including rabbit renomedullary interstitial cells, isolated rat adrenocortical cells, human leukemic Jurkat T cells, and human embryonic lung fibroblasts (Zusman and Keiser 1977; Chanderbhan et al. 1977; Aussel et al. 1987; Shindo et al. 1988). While attempts to assess *in vivo* production of PGA_2 have been largely unsuccessful, PGA_2 has been detected in human plasma and tissue extracts by various analytical methods, such as high pressure liquid chromatography (HPLC)-mass spectrometry (MS), gas chromatography (GC)/MS, and radioimmunoassay (Frolich et al. 1975; Carr et al. 1976; Green and Steffenrud 1976; Zusman et al. 1972; Jaffe et al. 1973).

PGJ_2 and Δ^{12}-PGJ_2

PGJ_2 was discovered as a dehydration product of PGD_2 in 1982 by Fukushima et al. in the course of studying the anti-tumor effects of PGD_2 in L1210 leukemia cultured cells. Fukushima et al. initially reported the potential anti-neoplastic effects of PGD_2 (Fukushima, Kato, Ueda et al. 1982), but it was later discovered that these effects were due to a cyclopentenone derivative of PGD_2, PGJ_2 (Fukushima, Kato, Ota et al. 1982). Dehydration of PGD_2 to PGJ_2 occurs spontaneously in aqueous buffer. PGJ_2 can undergo isomerization to Δ^{12}-PGJ_2 in aqueous solution containing serum albumin and in human plasma (Fitzpatrick and Wynalda 1983; Kikawa et al. 1984). Subsequent dehydration of Δ^{12}-PGJ_2 yields 15-d-PGJ_2 (Fitzpatrick and Wynalda 1983). (The discovery and biological effects of 15-d-PGJ_2 will be discussed in greater detail in the following section). PGD_2 is the major COX product in mast cells (Lewis et al. 1982), and the rapid conversion of endogenous PGD_2 to PGJ_2 and Δ^{12}-PGJ_2 has been demonstrated in murine bone marrow-derived mast cells (BMMCs) (Harberl et al. 1998). Further, Hirata et al. reported the detection of Δ^{12}-PGJ_2 in human urine using enzyme immunoassay, and production of Δ^{12}-PGJ_2

was reduced to some extent following treatment with COX inhibitors (Hirata et al. 1988). Both PGJ_2 and Δ^{12}-PGJ_2 possess anti-proliferative activity in various cell types, including vascular smooth muscle cells, murine melanoma cells, and murine fibroblasts (Fukushima et al. 1982; Kikawa et al. 1984; Mahmud et al. 1984; Narumiya and Fukushima 1985). Δ^{12}-PGJ_2 exhibits more potent anti-neoplastic effects than PGJ_2 (Narumiya and Fukushima 1985), likely due to its reactive dienone structure. Atsmon et al. demonstrated that Δ^{12}-PGJ_2 underwent conjugation with GSH in Chinese hamster ovary (CHO) and hepatoma tissue cells, and depletion of intracellular GSH enhanced the anti-proliferative activity of Δ^{12}-PGJ_2. These and other findings indicate that conjugation with GSH modulates the biological effects of CyPGs (Atsmon, Freeman et al. 1990).

Discovery of 15-d-PGJ_2

In the early 1980s, Fitzpatrick and Wynalda reported a series of investigations on the effect of albumin on the stability of prostaglandins (PGs). Albumin is an abundant plasma protein that has been previously shown to sequester fatty acids and facilitate their transport (Spector 1975). Albumins contain approximately twice the amount of basic amino acid residues typical of other protein classes, and the fatty acid binding sites of albumin are characterized by a highly alkaline microenvironment which presumably facilitates interaction with lipid anions (Fitzpatrick et al. 1981). Albumin has also been shown to bind prostaglandins. Based on this observation, Fitzpatrick and Wynalda hypothesized that albumin might expose PGs to unique environments that could affect their stability. Albumin had been previously shown to alter the inherent stability of PGI_2 (Wynalda and Fitzpatrick 1980) and promote the decomposition of PGH_2 to PGD_2 (Hamberg and Fredholm 1976). In subsequent studies Fitzpatrick and Wynalda demonstrated that albumins, derived from various vertebrate species, enhanced the

degradation of all naturally occurring primary PGs containing a β-hydroxy ketone; namely, PGs of the E and D series (Fitzpatrick et al. 1981). PGs of the E and D series had previously been shown to undergo dehydration in aqueous buffer; however, albumin was thought to accelerate the decomposition of these compounds due to the highly alkaline microenvironment in the albumin lipid binding sites, which have a local pH of 10 or greater (Fitzpatrick et al. 1981). Fitzpatrick and Wynalda suggested that this observation would have potentially important implications in the design and interpretation of experiments in PG research. For example, in experimental conditions using albumin-containing buffer, the actual concentration of PGs might be significantly reduced compared to the initial concentration administered. Further, the biological or pharmacological effects observed may be derived from the composite influence of the intact PG and its decomposition products (Fitzpatrick et al. 1981).

In 1983 Fitzpatrick and Wynalda reported the discovery that prostaglandin D_2 (PGD_2) undergoes metabolism in the presence of human serum albumin *in vitro* to form a series of cyclopentenone J_2 PGs, including PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (Fitpatrick and Wynalda 1983). Previously, PGD_2 had been shown to rapidly disappear from aqueous solutions containing vertebrate albumin (Fitpatrick and Wynalda 1981) or sera (Fukushima et al. 1982). Fitzpatrick and Wynalda proposed the following pathway for the metabolism of PGD_2 in the presence of serum albumin: PGD_2 first undergoes dehydration to form of PGJ_2, followed by isomerization to Δ^{12}-PGJ_2; finally, a second dehydration yields 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15-d-PGJ_2) (**FIGURE 1.4B**) (Fitzpatrick and Wynalda 1983). The relative amounts of PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 formed from this *in vitro* synthesis were reported to be 48:49:3, respectively (Fitzpatrick and Wynalda 1983). The C-10 proton of PGD_2 is acidic and easily extractable to promote dehydration of the C-9 hydroxyl forming PGJ_2. Further, the C-12 proton is simultaneously allylic and α to the carbonyl at C-11, thus even mildly alkaline conditions

facilitate the isomerization of the β,γ unsaturated enone (Δ^{13}) to a more stable α,β unsaturated enone (Δ^{12}) (Fitpatrick and Wynalda 1983). Albumin is thought to facilitate Δ^{12} isomerization due to the alkaline microenviroment of its lipid binding sites (Fitpatrick and Wynalda 1983). Isomerizaiton of the Δ^{13} double bond to the Δ^{12} position was thought to facilitate dehydration of the C-15 hydroxyl to form 15-d-PGJ_2. More recently, however, Shibata et al. have suggested an alternative mechanism for the formation of 15-d-PGJ_2 from PGD_2. According to their model, PGD_2 is initially converted to the dehydration products PGJ_2 and 15-d-PGD_2 in an albumin-independent manner. Subsequently, 15-d-PGJ_2 is generated via albumin-independent dehydration, and Δ^{12}-PGJ_2 is produced via albumin-dependent isomerization (Shibata et al. 2002).

Biological activity of 15-d-PGJ_2

15-d-PGJ_2 is one of the most extensively studied CyPGs. Like other CyPGs, 15-d-PGJ_2 possesses a reactive α,β-unsaturated carbonyl moiety within its cyclopentenone ring that can readily undergo Michael addition with cellular nucleophiles, such as the free sulfhydryl groups of glutathione and cysteine residues of proteins. In addition, the dienone structure of 15-d-PGJ_2 contains two reactive electrophilic carbon centers available for adduction with cellular thiols. Due to its reactivity, 15-d-PGJ_2 can modulate protein function through direct covalent modification. While the *in vitro* synthesis of 15-d-PGJ_2 was first reported by Fitzpatrick and Wynalda in 1983, considerable interest in the biological activity of 15-d-PGJ_2 was not generated until 1995, when two different studies reported that 15-d-PGJ_2 was a ligand for the orphan nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ). Since that time numerous reports have described abroad spectrum of biological effects mediated by 15-d-PGJ_2. Among its diverse cellular functions, 15-d-PGJ_2 possesses potent anti-inflammatory activity, and

it is thought play a key role in the resolution of inflammation. 15-d-PGJ_2 has also been shown to exert anti-proliferative and pro-apoptotic activity in various cancer cells and cell lines, suggesting its possible role in tumor suppression. Some of the biological effects of 15-d-PGJ_2 have been attributed to activation of PPARγ. On the other, 15-d-PGJ_2 has also been shown to possess PPARγ-independent activity through interaction with other intracellular targets, such as transcription factors and signaling molecules. Based on these observations, 15-d-PGJ_2 has emerged as a potentially important lipid mediator involved in a number of biological processes.

Chemical reactivity of 15-d-PGJ_2

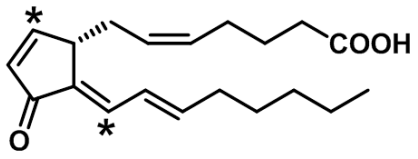


FIGURE 1.5. Structure of 15-d-PGJ_2. * Indicates positions of reactive electrophilic carbon centers.

The electrophilicity of 15-d-PGJ_2 contributes importantly to its biological activity. While 15-d-PGJ_2 functions in diverse cellular processes, its biological effects are induced primarily through covalent interaction with intracellular protein targets. The cyclopentenone ring of 15-d-PGJ_2 contains an electrophilic α,β-unsaturated carbonyl moiety that can readily react with cellular nucleophiles such as the cysteinyl thiols (R-SH) of proteins and the tripeptide glutathione (GSH) via Michael addition. In addition to the electrophilic carbon C-9, which is at the endocyclic β-position within the cyclopentenone ring, 15-d-PGJ_2 possesses a second reactive center at carbon C-13 on the lower side chain (**FIGURE 1.5**). This dienone structure is critical for the potent biological activity of 15-d-PGJ_2 (Kondo et al. 2001). Adduction of 15-d-PGJ_2 to redox active protein thiols at either of these reactive centers has been shown to contribute to its biological effects. For example, the electrophilic C-9 carbon of 15-d-PGJ_2 was shown to react with cysteine residues in IκB kinase β and the DNA binding domains of nuclear factor κB (NF-κB) to inhibit pro-inflammatory gene expression (Straus et al. 2000). The

15-d-PGJ_2 analogue 9,10-dihydro-15-d-PGJ_2 lacking the reactive α,β-unsaturated carbonyl group within the ring was not able to reproduce many of the anti-inflammatory and transactivating effects of 15-d-PGJ_2 (Sanchez-Gomez et al. 2004; Kim et al. 2007). On the other hand, the electrophilic carbon at C-13 is required for 15-d-PGJ_2-mediated activation of PPARγ (Shiraki et al. 2005). Shiraki et al. demonstrated that carbon C-13 of 15-d-PGJ_2 covalently binds a critical cysteine residue (cysteine-258) in the PPARγ ligand binding domain to cause PPARγ activation (Shiraki et al. 2005). In several studies, site-directed mutagenesis at specific cysteine residues of the target protein has been shown to abolish the effects of 15-d-PGJ_2 (Straus et al. 2000; Shiraki et al. 2005; Kim et al. 2010; Charles et al. 2011). In addition, 15-d-PGJ_2 may adduct cysteine residues of its protein targets in a selective manner. The reactivity of 15-d-PGJ_2 towards protein thiols depends on the pK_a of the thiol and the structural features of the protein, such as accessibility (Niki 2009). Deprotonation of the thiol (R-SH) to the more nucleophilic thiolate ($R\text{-}S^-$) species is favored by the Michael addition reaction (**FIGURE 1.6**) (Groeger and Freeman 2010). Proximity to basic amino acid residues such as lysine or arginine can lower the pK_a of cysteine thiols to promote formation of the thiolate (Groeger and Freeman 2010). Further, glutathione *S*-transferases (GSTs) strongly promote the conjugation of 15-d-PGJ_2 with cellular thiols (Paumi et al. 2004).

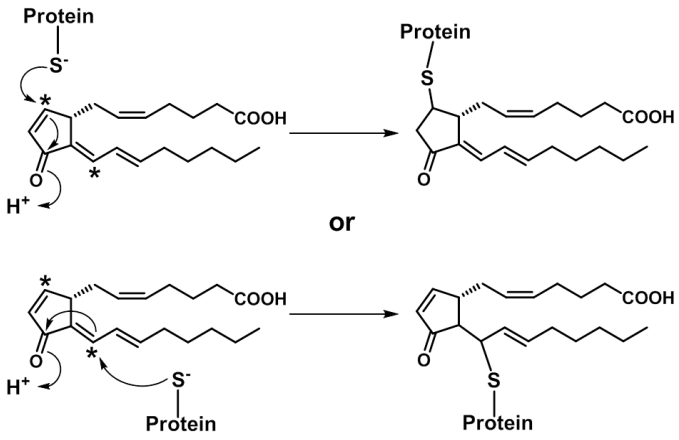


FIGURE 1.6. Michael addition reactions of 15-d-PGJ_2 with cysteinyl thiolate groups of proteins.

PPARγ-dependent effects

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily of ligand-dependent transcription factors that regulate the expression of genes involved in lipid metabolism and homeostasis, inflammation, cell proliferation, and malignancy (Scher et al. 2005). PPARs were identified as the receptors responsive to chemical and endogenous stimuli, such as fibrates or fatty acids, which lead to a significant increase in the number and size of peroxisomes and the expression of peroxisomal β-oxidation enzymes (Fahimi et al. 1982; Dussault and Forman 2000). Upon ligand binding, PPARs form heterodimeric complexes with retinoid X receptors (RXR), followed by association of the PPAR/RXR complex with peroxisome proliferator response elements (PPREs) in specific genes leading to transcription (Dussault et al. 2000). Three major subtypes of PPARs have been identified in mammals, including PPARs α, γ, and δ (Kliewer et al. 1994). PPARγ is highly expressed in adipose tissue, colon, and macrophages (Dussault et al. 2000). PPARγ is a key regulator of adipocyte differentiation, lipid storage, and glucose homeostasis, and it modulates gene expression in response to fatty acids, lipid-derived metabolites, and antidiabetic drugs, such as the thiazolidinedione class of drugs (Tontonoz et al. 1994; Lehmann et al. 1995; Saez, et al. 1998). Adipocytes mediate lipid homeostasis by storing large amounts of triglycerides during energy excess and mobilizing these lipid depots during periods of nutritional deprivation (Tontonoz et al. 1994). The differentiation of pre-adipocytes into adipocytes increases the amount of adipocytes available for lipid storage and is an important factor in controlling the level of obesity (Ghoshal et al. 2010).

15d-PGJ_2 attracted significant scientific attention and interest in 1995, when Forman and Kliewer independently reported that 15-d-PGJ_2 was an activating ligand of the orphan nuclear receptor PPARγ (Forman et al. 1995; Kliewer et al. 1995). At that time several

pharmacological agonists of PPARγ were available, such as the thiazolidinedione anti-diabetic drugs troglitazone, pioglitazone, and ciglitazone; however, no endogenous, naturally occurring ligand for PPARγ had been identified (Forman et al. 1995). In a 1995 issue of *Cell*, Forman et al. reported that 15-d-PGJ_2 activated PPARγ through direct binding and induced adiopogenesis in the NIH 3T3 cell line expressing PPARγ (NIH-PPARγ) (Forman et al. 1995). In the same issue of *Cell*, Kliewer et al. also reported that 15-d-PGJ_2 activated PPARγ as well as PPARα via direct binding and promoted differentiation of C3H10T1/2 fibroblasts to adipocytes (Kliewer et al. 1995). These findings provided the first evidence for a natural ligand of PPARγ. 15-d-PGJ_2 induced PPARγ-dependent adipogenesis at micromolar doses (EC_{50} 5-7 μM) (Forman et al. 1995; Kliewer et al. 1995); it should be noted, however, that the physiological concentrations of 15-d-PGJ_2 are unknown. Nonetheless, based on these studies, 15-d-PGJ_2 was proposed to be the physiological ligand of PPARγ and to potentially serve as a primary adipogenic signal (Forman et al. 1995; Kliewer et al. 1995).

Since these initial findings, 15-d-PGJ_2 has been shown to mediate anti-inflammatory effects in part through activation of PPARγ. Ricote et al. first demonstrated that 15-d-PGJ_2-mediated activation of PPARγ negatively regulates pro-inflammatory responses in activated macrophages (Ricote et al. 1998). PPARγ was upregulated in interferon-γ (IFN-γ)-activated macrophages, and activation of PPARγ by 15-d-PGJ_2 inhibited expression of pro-inflammatory genes, such as inducible nitric oxide synthase (iNOS), scavenger receptor A, and the matrix metalloproteinase gelatinase B in these cells (Ricote et al. 1998). 15-d-PGJ_2 also inhibited transcriptional activation of the pro-inflammatory transcription factors NF-κB, activator protein (AP)-1, and STAT (Signal Transducer and Activator of Transcription) in a PPARγ-dependent manner (Ricote et al. 1998). In a similar study, Jiang et al. showed that 15-d-PGJ_2 strongly inhibited the production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α,

interleukin (IL)-1β, and IL-6, in PMA-stimulated monocytes (Jiang et al. 1998). In addition, Fahmi et al. demonstrated that 15-d-PGJ_2 inhibited IL-1β induction of iNOS and matrix metalloproteinase 13 (MMP-13) in human chondrocytes, and it antagonized the activities of NF-κB and AP-1 in a PPARγ-dependent manner (Fahmi et al. 2001). 15-d-PGJ_2 was also shown to inhibit IL-1β-induced COX-2 expression in human synovial fibroblasts (Farrajota et al. 2005) and suppress AP-1-induced transcriptional activation of COX-2 in human epithelial cells (Subbaramaiah et al. 2001).

15-d-PGJ_2 has been shown to block cancer cell proliferation and inhibit cancer cell growth in part through activation of PPARγ. These effects are associated with induction of apoptosis or cell cycle arrest; however, the underlying mechanisms are not entirely clear (Clay et al. 2000). Shan et al. demonstrated that 15-d-PGJ_2 induced apoptosis in chondrocytes from arthritis patients in a time- and dose-dependent manner (Shan et al. 2004). 15-d-PGJ_2 has also been shown to promote apoptosis in macrophages (Chinetti et al. 1998), choriocarcinoma cells (Keelan et al. 1999), human umbilical vein endothelial cells (HUVECs) (Bishop-Bailey et al. 1999), and breast cancer cells (Clay et al. 1999; Clay et al. 2001). Further, 15-d-PGJ_2 possesses anti-neoplastic activity in various human cancer cells, including colon (Shimada et al. 2002), stomach (Sate et al. 2000), lung (Tsubouchi et al. 2000), breast (Pignatelli et al. 2005), and bone marrow (Laurora et al. 2003) cancer cells in a PPARγ-dependent manner. Moreover, Qin et al. showed that 15-d-PGJ_2-mediated activation of PPARγ inhibited breast cancer cell growth through increased proteosomal degradation of cyclin D1 and estrogen receptor a (ERα) and decreased G0-G1-->S phase progression (Qin et al. 2003). Kamagata showed that 15-d-PGJ_2 inhibited breast cancer cell growth by blocking G2-M phase progression via down-regulation of the anti-apoptotic protein cyclin B (Kamagata et al. 2007).

PPARγ-independent effects

More recently, it have become evident that 15-d-PGJ_2 mediates a number of biological effects independent of PPARγ through direct interaction with intracellular protein targets, such as transcription factors and signaling molecules. Several proteins have been identified as direct targets of 15-d-PGJ_2, including NFκB, IκB kinaseβ (IKKβ), AP-1, STAT, H-Ras, Keap1 (Kelch-like ECH-associated protein 1), p53, ATM (ataxia-telangiectasia mutated) estrogen receptor α (ERα), actin, hypoxia inducible factor (HIF), thioredoxin (Trx), and soluble epoxide hydrolase (sEH) (Straus and Glass 2001; Kim and Surh 2006; Uchida and Shibata 2008; Kim et al. 2010; Charles et al. 2011). Covalent binding of 15-d-PGJ_2 to these and other proteins has been shown to affect a diverse array of cellular processes, including inflammatory, apoptotic, proliferative, and cytoprotective responses.

Anti-inflammation

The anti-inflammatory activity of 15-d-PGJ_2 is one of its most well characterized biological properties. 15-d-PGJ_2 has been shown to exert potent anti-inflammatory activity through inhibition of inflammatory signaling pathways, most notably the NF-κB pathway. The transcription factor NF-κB is a key regulator of cellular inflammatory responses. In unstimulated cells, NF-κB exists as a heterodimeric complex, predominately p50 and p65 subunits, sequestered in the cytoplasm where it is associated with inhibitory IκB proteins (Baeuerle and Baltimore 1996; May and Ghosh 1998). However, in response to tissue injury or infection, proinflammatory cytokines, such as TNF-α and IL-1, are rapidly released, and they bind to toll-like microbial pattern recognition receptors (TLRs) leading to activation of IκB kinase (IKK) (Lawrence 2009). IKK is a complex composed of IKKα and IKKβ, and its activation results in IKKβ-mediated phosphorylation of IκBα and release of NF-κB (Baeuerle and Baltimore 1996; May and

Ghosh 1998). Once liberated, NFκB translocates to the nucleus, where it activates to the expression of pro-inflammatory genes, such as iNOS, COX-2, cytokines, chemokines, and adhesion molecules. Several groups have shown 15-d-PGJ_2 inhibits NF-κB-dependent gene transcription by targeting several proteins in the NF-κB pathway (Straus et al. 2000; Rossi et al. 2000; Castrillo et al. 2000; Cernuda-Morollon et al. 2001). 15-d-PGJ_2 inhibits IKKβ activity through covalent adduction to cysteine residue Cys179 (Straus et al. 2000; Rossi et al. 2000; Castrillo et al. 2000). 15-d-PGJ_2 also inhibits NF-κB DNA binding by covalent adduction to cysteine Cys62 in the p50 subunit and Cys38 in the p65 subunit (Straus et al. 2000; Cernuda-Morollon et al. 2001). Further, Straus et al. demonstrated that 15-d-PGJ_2 inhibited production of downstream inflammatory mediators, such as PGE_2, in macrophages (Straus et al. 2000).

In addition to inhibiting the NFκB pathway, 15-d-PGJ_2 has been shown to mediate anti-inflammatory effects by blocking the AP-1 and STAT signaling pathways. The transcription factor AP-1 (activator protein-1) exists as a heterotrimeric protein composed of members of the Jun (c-Jun, JunB, and Jun D) and Fos (c-Fos, FosB, Fra-1 and Fra-2) family (Shaulin and Karin 2002). AP-1 regulates gene transcription in response to diverse stimuli, including cytokines, growth factors, and microbial infections. Perez-Sala et al. demonstrated that 15-d-PGJ_2 directly inhibits AP-1 activity and DNA binding through covalent modification of the cysteine residue Cys269 located in the c-Jun DNA binding domain (Perez-Sala et al. 2003). Inhibition of AP-1 by 15-d-PGJ_2 is thought to reduce the expression of pro-inflammatory enzymes such as COX-2, iNOS, and matrix-metaloproteases (Kim and Surh 2006). STAT (Signal Transducer and Activator of Transcription) proteins are a family of latent cytoplasmic transcription factors, which, upon activation by extracellular stimuli such as cytokines and growth factors, translocate to the nucleus to regulate the expression of genes involved in cell growth, survival, and immune responses. Several studies have shown that 15-d-PGJ_2 inhibits cytokine-induced STAT activation

(Weber et al. 2004; Chen et al. 2003; Park et al. 2003). 15-d-PGJ_2 blocked IFNγ-induced STAT phosphorylation and DNA binding in macrophages and astrocytes as well as IL-6 and IL-10-induced phosphorylation of STAT, thus inhibiting STAT-dependent pro-inflammatory responses (Weber et al. 2004; Chen et al. 2003; Park et al. 2003; Ji et al. 2005).

Exogenous administration of 15-d-PGJ_2 has been shown to produce anti-inflammatory effects in various cell systems as well as experimental animal models. Vunta et al. showed that 15-d-PGJ_2 mediates the anti-inflammatory effects of selenium in macrophages through inhibition of IKKβ activity (Vunta et al. 2007). In addition, Cuzzocrea et al. showed that 15-d-PGJ_2 exerted potent anti-inflammatory effects *in vivo* in acute and chronic inflammation mouse models using carrageenan-induced pleurisy and collagen-induced arthritis, respectively (Cuzzocrea et al. 2000). In these studies, intraperitoneal administration of 15-d-PGJ_2 caused inhibition of pleural exudate formation, mononuclear cell infiltration, and histological injury (Cuzzocrea et al. 2000). 15-d-PGJ_2 also reduced expression of iNOS and COX-2 in the lungs of carrageenan-treated mice and in the joints of collagen-treated mice. Further, Wayman et al. reported that 15-d-PGJ_2 significantly reduced infarct size *in vivo* in rats and decreased expression of adhesion molecules ICAM-1 and P-selectin, chemokine macrophage chemotactic protein 1, and iNOS (Wayman et al. 2002). Based on these findings, 15-d-PGJ_2 has been postulated to be a key regulator of acute and chronic inflammation.

Other studies have provided evidence that 15-d-PGJ_2 is synthesized during the resolution of inflammation in primary cultured cells and *in vivo*. Using a carrageenan-induced inflammation model in rats, Gilroy et al. demonstrated that expression of COX-2 peaked at 2 h and was associated with maximal PGE_2 synthesis (Gilroy et al. 1999). This early phase was dominated by polymorphonuclear (PMN) cells. However, a second more dramatic increase in COX-2 expression at 48 h was dominated by the accumulation of macrophages and was

associated with inflammatory resolution and increased generation of 15-d-PGJ_2. Levels of 15-d-PGJ_2 were quantified by enzyme immunoassay (EIA) (Gilroy et al. 1999). These findings suggested that 15-d-PGJ_2 may be predominately produced during the resolution phase of inflammation, and synthesis of this anti-inflammatory mediator was proposed to represent as a feedback mechanism to regulate the inflammatory response (Gilroy et al. 1999). Vunta et al. also reported that lipopolysaccharide (LPS) stimulation of selenium-supplemented primary bone marrow derived macrophages (BMDMs) resulted in a time-dependent increase in 15-d-PGJ_2 generation, which was inhibited by specific COX-1 inhibitors (Vunta et al. 2007). 15-d-PGJ_2 levels were also quantified by EIA. More recently, Gilroy and colleagues have shown that 15-d-PGJ_2 is produced during zymosan-induced resolving peritonitis in mice; in this study, 15-d-PGJ_2 was detected in the inflammatory exudate by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Rajakariar et al. 2007). Based on these and previous findings, Gilroy and colleagues proposed that 15-d-PGJ_2 promotes inflammatory resolution by controlling the balance of cytokines and chemokines that regulate leukocyte influx and efflux (Rajakariar et al. 2007).

Apoptosis

The pro-apoptotic effects of 15-d-PGJ_2 have been extensively described. Apoptosis is a form of cell death requiring a well-defined chain of genetically programmed enzymatic events (Krowmer et al. 1995). 15-d-PGJ_2 has been shown to induce apoptosis through a number of different mechanisms; however, increased generation of intracellular reactive oxygen species (ROS) is thought to play a particularly important role in 15-d-PGJ_2-induced apoptosis. Overproduction of ROS can trigger mitochondrial apoptotic signaling cascades as well as lead to oxidative damage to lipids, proteins, and DNA resulting in cell death. Hortelano et al. demonstrated that 15-d-PGJ_2 promotes the resolution of inflammation by enhancing apoptosis

in activated macrophages through a p38 mitogen activated protein kinase (MAPK)-dependent increase in ROS formation (Hortelano et al. 2000). 15-d-PGJ_2-induced apoptosis in activated macrophages was shown to involve enhanced formation of superoxide anion ($O_2^{\cdot-}$) which presumably resulted in increased peroxynitrite ($OONO^-$) generation, cytochrome *c* release from the mitochondria to the cytosol, and increased protein nitration (Hortelano et al. 2000). 15-d-PGJ_2 has also been shown to induce apoptosis in human hepatic myofibroblasts (Li et al. 2001) and SH-SY57 human neuroblastoma cells (Kondo et al. 2001) through increased intracellular ROS production (Li et al. 2001). More recently, Shin et al. reported that 15-d-PGJ_2 induced apoptosis in leukemia and colorectal cancer cells in a dose-dependent manner through a series of events involving activation of NADPH oxidase, ROS generation, and ROS-mediated inactivation of the anti-apoptotic serine/threonine kinase Akt (Shin et al. 2009). In addition, 15-d-PGJ_2 was shown to activate c-Jun NH_2-terminal kinase (JNK), which initiates mitochondrial death pathways (Shin et al. 2009). Other groups have also shown that 15-d-PGJ_2 promotes apoptosis through increased intracellular ROS generation, inhibition of Akt, and activation of the JNK-mediated mitochondrial pathway (Hasegawa et al. 2007; Lee et al. 2008). Moreover, 15-d-PGJ_2 was shown to potentiate the pro-apoptotic effects of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in human leukemia cells through up-regulation of death receptor 5 (DR5) expression (Hasegawa et al. 2007).

15-d-PGJ_2 has also been shown to promote apoptosis through other mechanisms, such as activation of the p53 protein. The tumor suppressor p53 plays a central role in the regulation of cell-cycle progression, cell survival pathways, and DNA repair. p53 initiates apoptosis in cells challenged with genotoxic stress (Kim et al. 2010). Kondo et al. demonstrated that 15-d-PGJ_2 induces neuronal apoptosis in a p53-dependent manner (Kondo et al. 2002). Treatment of SH-SY5Y human neuroblastoma cells with 15-d-PGJ_2 resulted in p53 phosphorylation, nuclear

accumulation, and increased p53 DNA binding activity (Kondo et al. 2002). 15-d-PGJ_2 treatment also caused up-regulation of several genes, including heat-shock responsive genes, redox responsive genes, and p53 responsive genes. Further, Kondo et al. showed that levels of 15-d-PGJ_2 were increased in the spinal cord motor neurons of patients with sporadic amyotrophic lateral sclerosis (ALS), suggesting that 15-d-PGJ_2 may mediate neuronal cell death during chronic inflammatory processes (Kondo et al. 2002). In addition, 15-d-PGJ_2 has been shown to activate ATM (ataxia-telangiectasia mutated) through direct modification of it cysteinyl sulfhydryl groups, resulting in down-stream p53 activation and apoptosis (Kabayashi et al. 2006). Inhibition of NF-κB may also contribution to the pro-apoptotic effects of 15-d-PGJ_2. Piva et al. showed that 15-d-PGJ_2 induced apoptosis in human malignant B cells through inhibition of NF-κB activity, activation of caspases-3, -8, and -9, and down-regulation of anti-apoptotic proteins (Piva et al. 2005).

Cell growth and proliferation

There are conflicting reports regarding the effect of 15-d-PGJ_2 on cell growth, proliferation, and survival. 15-d-PGJ_2 exhibits opposing activity in various tumor cells and cell lines (Clay et al. 2001). While anti-proliferative and pro-apoptotic effects have been most frequently described, 15-d-PGJ_2 also induces proliferation and differentiation in certain cells types. The dual effects of 15-d-PGJ_2 are dependent on cell-type, concentration, and duration of exposure (Kim et al. 2010). 15-d-PGJ_2 has been shown to induce proliferation of COX-depleted human colorectal cancer cells (Chinery et al. 1999), breast cancer cells (Clay et al. 2001), mesangial cells (Rovin et al. 2002), and neural cells (Satoh et al. 1999). On the other hand, 15-d-PGJ_2 inhibited proliferation of vascular smooth muscle cells by inducing cell cycle arrest (Miwa et al. 2000). Kin et al. also showed that 15-d-PGJ_2 exerts anti-proliferative activity by inhibiting

estrogen receptor α (ERα) function via covalent modification of cysteine residues in the DNA binding domain of the receptor (Kin et al. 2007). Others have shown that 15-d-PGJ_2 induces activation of the MAPK and phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathways leading to cell survival and proliferation (Rovin et al. 2002; Wilmer et al. 2001). Further, Oliva et al. demonstrated that 15-d-PGJ_2-induced cell proliferation and MAPK activation are mediated through direct binding and activation of the H-Ras oncogene product (Oliva et al. 2003). 15-d-PGJ_2 was shown form a covalent adduct with Cys184 of H-Ras leading to activation of the Ras signaling pathway (Oliva et al. 2003). Moreover, in contrast to previous reports that 15-d-PGJ_2 induces p53 activation, Kim et al. have recently shown that treatment of breast cancer cells with 15-d-PGJ_2 lead to decreased p53 DNA binding and decreased transcriptional activity of p53 (Kim et al. 2010). 15-d-PGJ_2 was shown to covalently modify p53 via adduction to Cys277 of p53, suggesting that 15-d-PGJ_2 may contribute to carcinogenesis through functional inactivation of p53 (Kim et al. 2010). 15-d-PGJ_2 may also affect cell proliferation through direct covalent modification of c-Jun at Cys269 in the c-Jun DNA binding of the transcription factor AP-1, which is a key regulator of cell proliferation in response to mitogens (Perez-Sala et al. 2003). Moreover, Haslmayer et al. demonstrated that 15-d-PGJ_2 induced vascular endothelial growth factor (VEGF) in prostate and urinary cancer cells (Haslmayer et al. 2002). Taken together, these findings suggest that 15-d-PGJ_2 may play a potential role in tumorgenesis and cancer progression (Haslmayer et al. 2002).

Cytoprotection

15-d-PGJ_2 has been shown to exert a cytoprotective effect through activation of the Nrf2 pathway. The Nrf2-Keap1 system plays a key role in the adaptive cellular survival response to oxidative stress and xenobiotic electrophilic species (Dinkova-Kostova et al. 2005). Nrf2 (nuclear

factor erythroid 2p45 (NF-E2)-related factor) is a transcription factor that induces gene expression of antioxidant and phase II detoxification enzymes by binding the antioxidant-responsive element (ARE), also known as the electrophile-responsive element (EpRE), located in the promoter or enhancer region of many antioxidant genes (Rushmore et al. 1990). Nrf2-responsive genes include heme-oxygenase (HO-1), glutathione *S*-transferases (GSTs), γ-glutamyl cysteine ligase (γ-GCL), NAD(P)H: quione oxidoreductase (NQO1), heat shock proteins (HSPs), and glutathione peroxidase (GPx) (Lee and Surh 2005). Nrf2 activation is induced by chemical species that modify cysteine residues, including H_2O_2, ROOH lipid peroxides, peroxynitrite, oxo-aldehydes and ketones, Michael acceptors, and other electrophiles (Prestera et al. 1993). In the absence of oxidative stress signals, Nrf2 is sequestered in the cytoplasm associated with the cysteine-rich cytosolic inhibitor protein Keap1, which targets Nrf2 for ubiquitin-dependent proteosomal degradation through the Keap1-Cullin 3 complex (Itoh et al. 1999; Dinkova-Kostova et al. 2002). Keap1 acts as a sensor for electrophilic/oxidative stress. Modification of redox-sensitive cysteine residues in Keap1 by ROS or electrophiles inhibits ubiquitin conjugation of Nrf2 and destabilizes the association of Nrf2 with Keap1 resulting in release of Nrf2 (Kobayahsi et al. 2006; Gao et al. 2007). Once liberated, Nrf2 translocates to the nucleus where, in association with other transcription factors such as Maf, it binds to the ARE of antioxidant genes to activate transcription. Several studies have shown that, when administered at sublethal concentrations, 15-d-PGJ_2 activates Nrf2-dependent gene transcription via direct covalent modification of Keap1 cysteine residues (Levonen et al. 2004), resulting in induction of antioxidant proteins, including HO-1 (Zhang et al. 2004), peroxiredoxin 1 (Itoh et al. 2004), γ-GCL (Levonen et al. 2001; Chen et al. 2006), GSTs (Kawamoto et al. 2000), glutathione reductase (Levonen et al. 2001), and HSP 70 (Zhang et al. 2004). However, higher concentrations of 15-d-PGJ_2 may be cytotoxic.

Redox alteration

Several studies have shown that 15-d-PGJ_2 induces cellular redox alteration. Kondo et al. demonstrated that 15-d-PGJ_2 is a potent inducer of intracellular oxidative stress (Kondo et al. 2001). Treatment of SH-SY57 human neuroblastoma cells with 15-d-PGJ_2 (10 μM) and other CyPGs caused depletion of antioxidant defenses, such as glutathione (GSH) and GPx, decreased mitochondrial membrane potential, and induced production of protein-bound lipid peroxidation products, and accumulation of ubiquitinated proteins (Kondo et al. 2001). Pre-treatment with the antioxidant *N*-acetylcysteine significantly inhibited 15-d-PGJ_2-mediated ROS generation and cytotoxicity. Further, the 15-d-PGJ_2-induced redox alteration was not merely due to depletion of intracellular GSH; Kondo et al. proposed that 15-d-PGJ_2 may react with the selenocysteine residue of glutathione peroxidase via a Michael-type addition reaction, leading to depletion of glutathione peroxidase activity (Kondo et al. 2001). In addition, Ishii et al. showed that treatment of SH-SY57 cells with 15-d-PGJ_2 resulted in a dramatic increase in protein carbonyls, a marker frequently used to assess protein oxidation due to oxidative stress (Ishii et al. 2005). 15-d-PGJ_2 has also been shown to induce reversible *S*-oxidation of proteins via oxidative modification of cysteinyl thiols (Ishii and Uchida 2004). Using a proteomics approach to identify oxidized proteins, Ishii and Uchida showed that 15-d-PGJ_2 promoted oxidation of 26 proteins in SH-SY57 cells; the target protein identified are involved various cellular functions, such as glucolytic enzymes, redox enzymes, cytoskeletal proteins, and chaperone proteins (Ishii and Uchida 2004). Moreover, 15-d-PGJ_2 has been shown to directly modify thioredoxin (Trx), a redox sensitive protein that plays a critical role in the regulation of cellular redox status by facilitating the reduction of protein disulfide bonds via cysteine thiol-disulfide exchange (Shibata et al. 2003a). Shibata et al. demonstrated that 15-d-PGJ_2 adducted cysteine residues Cys35 and Cys69 of Trx, which resulted in inhibition of Trx function (Shibata et al. 2003a).

Protein turnover and cytoskeletal function

15-d-PGJ_2 has also been shown affect protein turnover through inhibition of proteasome activity. Shibata et al. demonstrated that 15-d-PGJ_2 acts on the ubiquitin-proteosome pathway, decreasing proteosome activity and protein turnover (Shibata et al. 2003b). Later 15-d-PGJ_2 was shown to induce oxidation of the S6 ATPase regulatory subunit of the 26 S proteasome in SH-SY57 human neuroblastoma cells, resulting in decreased S6 ATPase activity and reduced proteasomal degradation of proteins (Ishii et al. 2005). More recently, Kim et al. showed that treatment of MCF-7 human breast cancer cells with 15-d-PGJ_2 lead to accumulation of p53 protein and decreased proteasomal degradation of the protein (Kim et al. 2010). 15-d-PGJ_2 was shown to directly adduct cysteine residue Cys277 of p53, and this modification was suggested to induce a conformation change in the protein rendering p53 less susceptible to proteasomal degradation (Kim et al. 2010). In addition, Uchida and colleagues have shown that 15-d-PGJ_2causes cytoskeletal dysfunctionin SH-SY5Y cells through covalent modification of actin. 15-d-PGJ_2 was shown to bind actin through Michael adduction at Cys347 resulting in unfolding of the action structure, F-actin depolarization, and impairment of G-action polymerization (Aldini et al. 2007). These effects may play a role in the cytotoxicity of 15-d-PGJ_2 and could contribute to 15-d-PGJ_2-induced neuronal cell dysfunction (Aldini et al. 2007).

Evidence for 15-d-PGJ_2 *in vivo*

Despite significant interest in the biological activity of 15-d-PGJ_2, evidence for the biosynthesis of 15-d-PGJ_2 *in vivo* is lacking (Bell-Parikh et al. 2003; Uchida and Shibata 2008). The existence of 15-d-PGJ_2 and other cyclopentenone PGs *in vivo* has been the subject of ongoing controversy for the past three decades. It is not known whether 15-d-PGJ_2 is actually synthesized *in vivo* at physiologically relevant concentrations or whether it is merely a minor

degradation product of PGD_2 found *in vitro* (Powell 2003; Bell-Parikh et al. 2003). This is of importance because 15-d-PGJ_2 is believed to be a key lipid mediator. However, with the exception of a few studies, the biological effects of 15-d-PGJ_2 have only been observed in studies performed *in vitro* in which exogenous 15-d-PGJ_2 was administered. The lack of evidence for endogenous generationof 15-d-PGJ_2 has led some to question its physiological relevance (Powell 2003; Bell-Parikh et al. 2003). The anti-inflammatory and cytoprotective activities along with the pro-apoptotic and anti-proliferative properties of 15-d-PGJ_2 depend on cell type and concentration (Straus and Glass 2001). Additionally, pro-inflammatory effects of 15-d-PGJ_2 have been reported in several cell types, including upregulation of interleukin-8 synthesis in endothelial cells through induction of oxidative stress (Jozkowicz et al. 2008), activation of eosinophils (Monneret et al. 2002), and induction of endothelial cell apoptosis (Bishop-Bailey and Hla 1999; Levonen et al. 2001; Vosseler et al. 2003). Further, the concentration of 15-d-PGJ_2 dictates opposing biologic outcomes in several types of cancer cells and cell lines. Low concentrations of 15-d-PGJ_2 increase cellular proliferation, moderate concentrations induce cell cycle arrest and cellular differentiation, while higher concentrations induce apoptosis (Chinery et al. 1999; Clay et al. 2002). Thus, a central question regarding 15-d-PGJ_2 is what are the physiological levels of this compound in humans, and what role(s), if any, does it play in human patho/physiology? To establish a physiology role for this compound, one would have to demonstrate that it exists *in vivo* in sufficient quantities to elicit the proposed biological responses (Straus and Glass 2001).

A limited number of studies have demonstrated the detection of free 15-d-PGJ_2 in intact cells and *in vivo*. Using immunohistochemical analysis, Shibata et al. reported the presence of 15-d-PGJ_2 in the cytoplasm of macrophage foam cells located in human atherosclerotic lesions (Shibata et al. 2002). Very low levels of free 15-d-PGJ_2 have been detected in human urine by

LC/MS analysis (Bell-Parikh et al. 2003). The amounts detected were in the picomolar (pM, 10^{-12} M) range, while the concentrations of 15-d-PGJ_2 required to exert the biological activities described above are in the micromolar (μM, 10^{-6} M) range. An important critique to measuring free 15-d-PGJ_2 in biological fluids such as urine is that this compound may be generated *ex vivo* from the dehydration of PGD_2 in the urine; thus, quantification of free 15-d-PGJ_2 under these conditions likely does not reflect the actual levels of the compound produced *in vivo*. Using enzyme immunoassay Gilroy et al. quantified levels of 15-d-PGJ_2 in the inflammatory exudates from carrageenin-induced pleurisy in rats, suggesting its role in the resolution of inflammation (Gilroy et al. 1999). 15-d-PGJ_2 was also shown to be released by human articular chondrocytes and was found in joint synovial fluid obtained from patients with osteoarthritis or rheumatoid arthritis (Shan et al. 2004). Vunta et al. also demonstrated that stimulation of selenium-supplemented macrophages by LPS resulted in a time-dependent increase in 15-d-PGJ_2 formation quantified in cell lysates by enzyme immunoassay (Vunta et al. 2007). However, immunochemical procedures are of limited usefulness in defining the absolute concentrations of free 15-d-PGJ_2 in cells (Uchida and Shibata 2008). In particular, the cross reactivity of antibodies with structurally similar molecules and the reactive nature of 15-d-PGJ_2 raises questions regarding the accuracy of using an antibody-based measuring system (Rajakariar et al. 2007). Rajakariar et al. recently reported the detection of free 15-d-PGJ_2 in cell-free inflammatory exudates of mice using liquid chromatography-tandem mass spectrometry (LC-MS/MS), suggesting that 15-d-PGJ_2 may be synthesized during mammalian inflammatory processes (Rajakariar et al. 2007). Nonetheless, because this molecule can rapidly react with thiol-containing molecules *in vitro*, the measurable amount of free 15-d-PGJ_2 is likely an underrepresentation of the total amount formed *in vivo* (Brunoldi et al. 2007). Based on its reactivity, we hypothesize that 15-d-PGJ_2 is present *in vivo* primarily as a Michael conjugate,

adducted to peptide and protein thiols. Importantly, measurement of urinary metabolites has been established as the most sensitive and accurate method to assess systemic eicosanoid production *in vivo* due to their rapid biosynthesis and metabolism. Thus, studying the metabolism of 15-d-PGJ_2 *in vivo* would likely yield important insights into the quantities of this compound actually generated in biological systems.

Metabolism of cyclopentenone prostaglandins

The reactivity of CyPGs contributes importantly to their biological activity and provides a basis for understanding their metabolism. The electrophilic α,β-unsaturated carbonyl moiety within the cyclopentenone ring is a substrate for Michael addition with cellular thiols, such and GSH. Our laboratory and others have previously shown that conjugation with GSH is a major route for the metabolism of CyPGs. Gouin et al. reported that incubation of PGA_1 in neuroblastoma and glioma cells resulted in its conversion to a cysteinyl-glycine conjugate, in which the carbonyl at C-9 was reduced to an alcohol (Gouin et al. 1986). Cox et al. explored the metabolic disposition of PGJ_2 in the human colorectal cancer cell line HCA-7 (Cox et al. 2002). When PGJ_2 was added to HCA-7 cells, approximately 70% was conjugated to GSH within 2 h. Characterization of the GSH adducts by LC/MS analysis revealed two major metabolites of PGJ_2, including (1) a GSH conjugate in which the carbonyl at C-11 of PGJ_2 was reduced and (2) intact PGJ_2 conjugated to GSH (Cox et al. 2002).

In another series of studies, Atsmon et al. examined the metabolism of Δ^{12}-PGJ_2 *in vitro* and *in vivo* (Atsmon, Sweetman et al. 1990; Atsmon, Freeman et al. 1990). Incubation of Δ^{12}-PGJ_2 with excess GSH *in vitro*, resulted in its rapid conjugation with GSH, and formation the Δ^{12}-PGJ_2-GSH conjugate was enhanced by glutathione *S*-transferase (GST) (Atsmon, Sweetman et al. 1990). Conjugation of Δ^{12}-PGJ_2 with cysteine was shown to occur more rapidly than with GSH

(Atsmon, Sweetman et al. 1990). In addition, following prolonged incubation of Δ^{12}-PGJ_2 with excess GSH in the presence of GST, a small quantity of a bis conjugate of this compound was detected by mass spectrometry. H^1 nuclear magnetic resonance (H^1 NMR) analysis determined that in the mono GSH conjugate of Δ^{12}-PGJ_2, the GSH was conjugated at the C-9, suggesting that in the bis adduct, GSH was conjugated at carbon C-9 and C-13 of Δ^{12}-PGJ_2. When Δ^{12}-PGJ_2 was incubated with Chinese hamster ovary (CHO) or hepatoma tissue culture (HTC) cells, it was rapidly taken up and recovered in the cell lysate primarily as a GSH conjugate in which the carbonyl at C-11 and the Δ^{12} double bond had been reduced (Atsmon, Freeman et al. 1990). Subsequently, the formation of Δ^{12}-PGJ_2 conjugates *in vivo* was assessed by infusing radiolabeled Δ^{12}-PGJ_2 intravenously into a rat and collecting urine and bile for 4 h. Of the recovered radioactivity, 90% was excreted into the bile, and essentially all of the radioactivity in the bile was present in the form of a polar conjugate. High-performanceliquid chromatography (HPLC) analysis of the bile revealed a major radioactive peak with an elution volume characteristic for that of the mono GSH conjugate of Δ^{12}-PGJ_2 (Atsmon, Sweetman et al. 1990).

The metabolism of 15-d-PGJ_2 has also been studied in cancer cells and cell lines *in vitro*; however, little is known about the metabolic fate of 15-d-PGJ_2 *in vivo* in animals or in the human subject. Paumi et al. demonstrated that 15-d-PGJ_2 readily adducts GSH in MCF7 breast cancer cells to form the 15-d-PGJ_2-glutathione conjugate (15-d-PGJ_2-SG) (Paumi et al. 2003). Similar to it precursor Δ^{12}-PGJ_2, 15-d-PGJ_2 can potentially form bis-adducts with GSH through adduction at both the C-9 and C-13 electrophilic carbons. However, GSH conjugation preferentially occurs at carbon C-9 of 15-d-PGJ_2 (Paumi et al. 2003; Maxey et al. 2000). Interestingly, active efflux of 15-d-PGJ_2-SG by the efflux transporters multi-drug resistance associated protein (MRP)-1 and MRP3 was shown to attenuate the cytotoxic and transactivating effects of 15-d-PGJ_2 in MCF7 cells (Paumi et al. 2003). On the other hand, depletion of intracellular GSH enhanced the biological

effects of 15-d-PGJ_2 (Paumi et al. 2003). More recently, Brunoldi et al. demonstrated that 15-d-PGJ_2 is metabolized in the human hepatoma cell line HepG2 via conjugation with GSH, and three major metabolites were identified. HepG2 cells converted 15-d-PGJ_2 to a GSH conjugate in which the carbonyl at C-11 was reduced to an alcohol. Subsequently, the glutathione portion of molecule was metabolized via hydrolysis of glutamate and glycine to yield the cysteinyl-glycine- and cysteine-conjugates, respectively (**FIGURE 1.7**) (Brunoldi et al. 2007). In addition, Yu et al. demonstrated that metabolism of 15-d-PGJ_2 by recombinant alkenal/one oxidoreductase (Aor) *in vitro* resulted in reduction of the C-12, C-13 double bond to yield 12,13-dihydro-15-d-PGJ_2 (Yu et al. 2006). Taken together, these studies confirm GSH conjugation as a general pathway for the metabolic disposition of cyclopentenone eicosanoids and provide a basis for further investigations on the formation and metabolism of 15-d-PGJ_2 *in vivo*.

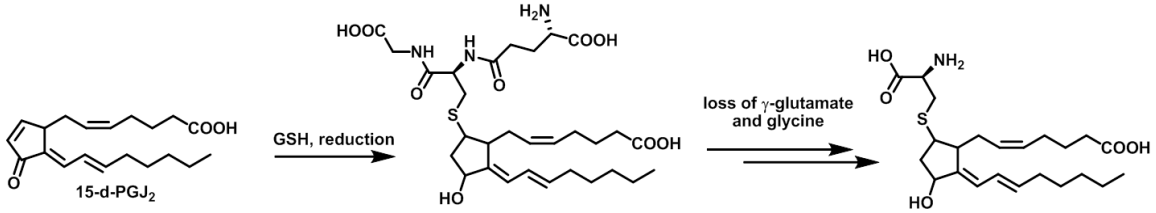


FIGURE 1.7. Metabolism of 15-d-PGJ_2 in HepG2 cells. 15-d-PGJ_2 is primarily converted to a GSH conjugate in which the carbonyl at C-11 is reduced to a hydroxyl. Subsequently, the glutathione portion of the molecule is hydrolyzed with loss of glutamate and glycine resulting in a cysteine conjugate (Brunoldi et al. 2007).

Oxidative stress

Oxidative stress is characterized by an imbalance between cellular antioxidant defenses and overproduction of free radicals, primary derived from oxygen (Montushi et al. 2004). Free radicals are reactive chemical species containing one or more unpaired electron in their outer orbitals. Reactive oxygen species (ROS) include oxygen centered free radicals. The most common ROS generated in living systems are superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), alkoxyl radicals ($RO^{\cdot}$), peroxyl radicals ($ROO^{\cdot}$), singlet oxygen ($^1O_2$), and ozone. Other reactive species formed in cells include peroxynitrite ($ONOO^-$), nitric oxide ($^{\cdot}NO$), hypochlorus acid (HOCl), and carbon centered radicals. ROS generation is induced by both endogenous and exogenous sources. Low levels of ROS are produced under normal physiological conditions, and these species are thought to mediate important biological functions. However, excessive ROS exposure can cause direct damage to important cellular macromolecules, including DNA, proteins, and lipids leading to downstream tissue injury.

Reactive oxygen species

Reactive oxygen species (ROS) are the most common free radicals generated in living systems (Montushi et al. 2004). Superoxide anion ($O_2^{\cdot-}$) is regarded as the primary ROS, and this species can lead to the formation of secondary ROS. Superoxide anion is formed enzymatically or non-enzymatically from the one-electron reduction of molecular oxygen O_2 ($O_2 + e^- \rightarrow O_2^{\cdot-}$). Hydrogen peroxide is ascribed to ROS; however, it is not a free radical because it does not contain an unpaired electron. Hydrogen peroxide is generated by cellular oxidases and from the dismutation reaction of $O_2^{\cdot-}$ ($O_2^{\cdot-} + H^+ \rightarrow HO_2^{\cdot-}$; $2HO_2^{\cdot-} \rightarrow H_2O_2 + O_2$), which is catalyzed by superoxide dismutases (SODs) (McCord and Fridovich 1969). The hydroxyl radical ($^{\cdot}OH$) is a highly reactive, toxic species that can be formed by the reaction of hydrogen peroxide with

catalytic transition metal ions, such as iron (Fe^{II}) and copper (Cu^{I}), via the Fenton reaction (Fe^{2+} $H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH$). The iron-catalyzed Haber-Weiss reaction may also occur in cells to generate ${}^{\cdot}OH$; this reaction combines the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) by $O_2^{\cdot-}$ (Fe^{3+} $O_2^{\cdot-} \rightarrow Fe^{3+} + O_2$) and a Fenton reaction, yielding the net chemical reaction: ($O_2^{\cdot-} + H_2O_2 \rightarrow OH^- +$ ${}^{\cdot}OH + O_2$) (Liochev and Fridovich 2002). In addition, hydrogen peroxide (H_2O_2) can be converted to hypochlorus acid (HOCl) by the catalytic action of myeloperoxidase (Klebanoff 1980). Alkoxyl radicals ($RO^{\cdot}$) are formed in the decomposition of hydroperoxides (ROOH) by iron and copper through the Haber-Weiss reaction. Reactive nitrogen species (RNS) are also generated in biological systems. Nitric oxide synthases (NOSs) in biological tissues catalyze the oxidation of L-arginine to nitric oxide (${}^{\cdot}NO$) (Nathan 1992). Reaction of $NO^{\cdot}$ with $O_2^{\cdot-}$ forms peroxynitrite ($ONOO^-$) ($NO^{\cdot} + O_2^{\cdot-} \rightarrow ONOO^-$), which is a potent oxidizing agent (Szabo et al. 2007).

Endogenous sources of ROS

ROS are continuously generated during aerobic metabolism through reduction-oxidation (redox) reactions in the cell. The mitochondria within a cell are a major source of endogenous ROS. The mitochondrial electron-transport chain (ETC) is the main source of energy, in the form of adenosine triphosphate (ATP), in mammalian cells and is essential for life. Movement of electrons from oxidizable organic molecules to molecular oxygen (O_2) is responsible for ATP generation by the ETC, and O_2 is the final electron acceptor in this processes yielding H_2O. The mitochondrial ETC produces small amounts of $O_2^{\cdot-}$ during normal function due to the leaking of electrons directly to oxygen. During energy transduction, an estimated 1-4% of oxygen reacting with the electron transport chain is incompletely reduced to superoxide anion by leaking electrons (Richter 1988). Complexes I and III of the ETC have been shown to be responsible for generation of $O_2^{\cdot-}$ (Muller et al. 2004; Valko et al. 2007). Under conditions of cellular stress and

ATP depletion, an excess of the superoxide radical causes release of free iron (Fe^{II}) from iron-containing molecules, such as iron-sulfur proteins within the inner mitochondrial membrane, promoting the formation of the reactive hydroxyl radical (Lenaz et al. 1999; Liochev and Fridovich 1994).

Peroxisomes are also a significant source of cellular ROS. Peroxisomes are specialized cytoplasmic organelles present in most plant and animal cells, and they are major sites of oxygen consumption. These organelles carry out important metabolic functions, including β-oxidation of long-chain and very long-chain fatty acids, degradation of uric acid, and synthesis of ether-linked lipids. Several peroxisomal oxidases produce H_2O_2 through the oxidation of various metabolites (De Duve and Baudhuin 1966; Schrader and Fahimi 2006). Peroxisomal oxidases have been shown to be responsible for approximately 35% of all H_2O_2 formed in rat liver (Boveris et al. 1972). Acyl-CoA oxidase, which is involved in the β-oxidation of fatty acids, is one such enzyme (Schrader and Fahimi 2006). During this reaction, O_2 is reduced to H_2O_2, which can be further reduced to H_2O by the enzyme catalase. The reaction of xanthine and xanthine oxidase can produce both superoxide anion and hydrogen peroxide through the one-electron and two-electron reduction of O_2, respectively, to form uric acid (Schrader and Fahimi 2006).

ROS production by phagocytic cells during oxidative burst is important in host immune defense against microbial pathogens. Oxidative or respiratory burst is a process in which activated phagocytes, such as neutrophils and macrophages, produce large, toxic quantities of ROS to kill ingested bacteria (Babior et al. 2002; DeCoursey and Ligeti 2005). Phagocytes are activated in response to microorganisms and inflammatory mediators. Upon activation, nicotine adenine dinucleotide phosphate (NADPH) oxidase (NOX) produces superoxide anion ($O_2^{\cdot-}$) within the phagosomal membrane. This superoxide-generating oxidase catalyzes the transfer of electrons from NADPH to molecular oxygen (O_2) to form $O_2^{\cdot-}$ (Lambeth 2004). In addition, the

heme-containing phagocytic enzyme myeloperoxidase (MPO) produces hypochlorus acid (HOCl) in neutrophils by the reaction of hydrogen peroxide and chloride at sites of inflammation (Klebanoff 1980; Clifford and Repine 1982). Production of ROS at sites of inflammation can cause oxidative injury to surrounding tissues.

Exogenous sources of ROS

ROS are also generated from exogenous genotoxic sources, including UV radiation, environmental toxicants, cigarette smoke, and other chemical carcinogens (Burke and Wei 2009).

Biological functions of ROS

Despite the deleterious effects of ROS overproduction, ROS have important biological functions. Low to moderate levels of ROS have been shown to play a key role in mediating signal transduction and normal physiological processes. H_2O_2 can freely diffuse across cell membranes to participate in intra- and intercellular signaling (Schrader and Fahimi 2006). H_2O_2 is a potent oxidant capable of oxidizing methionine and reactive cysteine residues of proteins. Reversible protein oxidation by H_2O_2 is thought to be an important signaling mechanism to modulate the activity of various kinases and phosphatases involved in the regulation of cell growth, proliferation, and apoptosis (Adler et al. 1999). In fact, recent reports suggest that H_2O_2 may be involved in post-translational modification of proteins in a manner that is analogous to regulatory protein phosphorylation (Lee et al. 1998). Serine/threonine kinases of the mitogen activated protein kinase (MAPK) family, including extracellular-regulated kinases (ERKs), c-Jun-NH_2-terminal kinase (JNK), and p38 MAPK are among the signaling molecules whose activity may be regulated by ROS-mediated post-translational modification (Kyriakis and Avruch 2001; Adler

et al. 1999). ROS are also thought to be involved in regulation of the phosphoinositol 3 kinase (PI3K)-Akt-p53 signaling pathway. Protein tyrosine phosphatases, such as protein tyrosine phosphatase 1B (PTP1B) (Lee et al. 1998) and the tumor suppressor PTEN (Lee et al. 2002), have been shown to be reversibly oxidized and inactivated by H_2O_2 (Meng et al. 2002). Further, the activity of transcription factors, such as activator protein (AP)-1 and nuclear factor κB (NF-κB), has been shown to be modulated by ROS (Keller et al. 1993).

Cellular antioxidant defenses

A series of antioxidant defense mechanisms have been developed to maintain redox homeostasis and protect cells against free radical-induced oxidative damage. The redox (reduction-oxidation) environment within a cell is characterized by the concentration of electrons stored in many cellular constituents, and redox homeostasis is achieved when ROS levels and antioxidant defenses are in proper balance. The primary antioxidant defenses include antioxidant enzymes and low molecular weight antioxidant molecules. Antioxidant enzymes, such as superoxide dismutase SOD), glutathione peroxidase (GPx), and catalase play a key role in the degradation of ROS. Superoxide dismutases (SODs), such as manganese SOD (MnSOD) in the mitochondrial matrix and copper-zinc SOD (CuZnSOD) catalyze the dismutation of $O_2^{\cdot-}$ to H_2O_2. Glutathione peroxidase (GPx) scavenges hydrogen peroxide with reduced glutathione (GSH) as the electron donor (Valko et al. 2004). Catalase decomposes H_2O_2 to oxygen and water. Small molecule antioxidants, such as glutathione (GSH), ascorbic acid (Vitamin C), and α-tocopherol (Vitamin E), carotenoids, and coenzyme Q, serve as free radical scavengers and/or reducing agents. In addition, the thioredoxin (Trx) system, including Trx, Trx reductase, and NADPH, is important in facilitating the enzymatic reduction of protein disulfides to regulate cellular redox status (Holmgren 1985). Because the biological status of a cell is closely related to

its redox environment, the balance between both the activities and intracellular levels of each of these antioxidants is critical for the health and survival of organisms (Schafer and Buettner 2001).

Glutathione is a major thiol antioxidant and redox buffer of the cell (Gilbert 1990). Glutathione is a tripetide, composed of γ-glutamate, cysteine, and glycine. It is synthesized in the cytosol from L-glutamate by the sequential action of γ-glutamylcysteine synthetase and glutathione synthetase. Under normal conditions, the concentration of glutathione in the cytosol is 1-11 mM, and it is the major soluble antioxidant in the cytosol, nuclei, and mitochondria (Smith et al. 1996; Valko et al. 2004). The extracellular concentrations of glutathione are 100 to 1000 times lower than the intracellular levels (Schafer and Buettner 2001). GSH is the reduced form of glutathione, and glutathione disulfide (GSSG) is the oxidized form; thus, GSSG/2GSH represents a redox couple. Oxidation of GSH to GSSH occurs in the reduction of H_2O_2 to H_2O by glutathione peroxidase (GPx), and glutathione reductase (GR) catalyzes the reduction of GSSG to GSH by NADPH. The GSH/GSSG system serves as a redox buffer by helping to regulate the reversible oxidation of the thiol groups (-SH) of protein cysteine residues to disulfides in a dynamic process termed thiol-disulfide exchange (Gilbert 1990; Di Simplicio et al. 1998; Thomas et al. 1995). The GSH/GSSG ratio is an important indicator of cellular redox status; the overall GSH/GSSG ration in a cell is usually $\geq$ 100:1 (Schafer and Buettner 2001). Under conditions of oxidative stress, the cellular GSSG content increases leading to a decreased GSH/GSSG ratio. This can result in the efflux of GSSG to restore the balance of the GSSG/2GSH redox couple within the cell (Sies and Akerboom 1984). In addition, GSH plays a key role in the detoxification of ROS as well as endogenous electrophiles and xenobiotics (Hayes and McLellan 1999). Glutathione *S*-transferases (GSTs) catalyze the conjugation of GSH with endogenous electrophiles and xenobiotics to detoxify cells from

reactive, potentially toxic species. GSH directly scavenges $^{\cdot}OH$ and $^{1}O_2$, and it reduces H_2O_2 and lipid peroxides via the catalytic action of GPx (Hayes and McLellan 1999). GSH is also able to regenerated Vitamin C and E back to their reduced form (Scholz et al. 1989).

Biological consequences of oxidative stress

Oxidative stress has become increasingly implicated in the etiology of many human diseases, including cancer, cardiovascular disease, ischemia/reperfusion injury, neurodegenerative diseases, lung disease and even the normal aging process (Halliwell and Gutteridge 1990; Southorn and Powis 1988; Ames 1983; Harman 1981; Diplock 1994). For example, Harman characterized the aging process as a decline in normal cellular function due to the accumulation of biomolecules damaged by free radicals (Harman 1981). Under conditions of oxidative stress, high levels of ROS can react non-specifically and rapidly with cellular biomolecules, including DNA, proteins, and lipids. This leads to molecular damage such as DNA mutations, protein oxidation, and lipid peroxidation.

Oxidative DNA damage

Reactive oxygen species are major contributors to spontaneous DNA damage. Oxidative DNA modifications by ROS cause damage to the structural properties of DNA, leading to formation of a variety of DNA lesions, such as base and sugar damage, DNA breaks, and DNA-protein cross-links (van Loon et al. 2010). Oxidative DNA damage is an important mechanism in cancer and aging (Ames 1988; Adelman et al. 1988). 7,8-dihydro-8-oxo-guanine (8-oxo-G) is one of the most abundant and best characterized DNA lesions caused by oxidative stress (van Loon et al. 2010). Thus, 8-oxo-G is often used as a cellular biomarker to assess the extent of oxidative stress. 8-oxo-G is formed by the introduction of an oxo group on the C-8 carbon and a

hydrogen atom to the N7 nitrogen of guanine. The steady-state level of 8-oxo-G lesions is estimated to be about 10^3 per cell/per day in normal tissues and up to 10^5 per cell/per day in cancer tissues (Klaunig and Kamendulis 2004). 8-oxo-G is highly mutagenic, and it is thought to be involved in the origin and progression of cancer. The presence of 8-oxo-G in the genome during DNA replication can cause misincorporation of adenine (A) opposite the lesion leading to C:G to A:T transversion mutations. Accumulation of such mutations can give rise to cancer. C:G to A:T transversion mutations are among the most common mutations in lung, breast, ovarian, gastric, and colorectal cancers (van Loon et al. 2010).

Protein oxidation

Excessive ROS production under settings of oxidative stress can cause to specific protein modifications leading to changes in protein activity and function. Further, oxidative modifications of proteins increase their susceptibility for proteosomal degradation (Valko et al. 2004). Protein oxidation has been implicated in many human disorders and diseases, such as atherosclerosis, ischemia/reperfusion injury, neurodegenerative diseases, and aging (Toda et al. 2010). Oxidative protein modifications include carbonylation, nitration, *S*-thiolation, and sulfoxidation. Carbonylation of proteins primarily occurs at lysine (Lys), arginine (Arg), proline (Pro), and threonine (Thr) residues leading to the formation of a-aminoadipic semialdehyde from Lys, glutamic semialdehyde for Arg and Pro, and 2-amino-3-ketobutyric acid from Thr (Aldini et al. 2007). ROS, such as $^{\cdot}OH$, and reactive lipid peroxidation products, such as the α,β-unsaturated aldehyde 4-hydroxy-2-nonenal (HNE), can cause protein carbonylation. Protein carbonylation is irreversible and irreparable and can lead to loss of protein function and subsequent cellular dysfunction. Accumulation of protein carbonyls occurs during oxidative

stress, and measurement of protein carbonyls is often used to assess protein oxidation in oxidative stress-related pathologies (Toda et al. 2010).

Protein nitration by peroxynitrite is an important mechanism of oxidative protein modification. Nitration of protein tyrosine residues involves addition of a nitrate group from peroxynitrite to the ortho position of tyrosine, leading to the formation of 3-nitrotyrosine. Protein tyrosine nitration can result in protein dysfunction and cell death and has been implicated in the pathogenesis of several diseases, including atherosclerosis (Beckman et al. 1994), inflammation (Kooy et al. 1995), and ischemia/reperfusion injury (Malinski et al. 1993). Protein tyrosine nitration has also been associated with production of insoluble protein aggregates and neuronal cell death observed in Alzheimer's disease (AD) and other age-related neurodegenerative diseases (Good et al. 1996). Measurement of 3-nitrotyrosine by immunohistochemistry and mass spectrometry is often used as a marker of oxidative protein modification (Viera et al. 1999; Abello et al. 2009). Nitrotyrosine formation has been demonstrated in atherosclerotic lesions (Beckman et al. 1994), myocardial ischemia, septic and distressed lung, inflammatory bowel disease (Beckman and Koppenol 1996), AD, Parkinson's disease, and amyotrophic lateral sclerosis (Beckman et al. 1993; Good et al. 1996).

Protein *S*-thiolation and sulfoxidation also result from ROS-induced protein oxidation. Protein *S*-thiolation occurs at cysteine residues. Oxidation of cysteine residues can lead to reversible formation of disulfides between the thiol groups (-SH) of protein cysteine residues and glutathione (*S*-glutathiolation) (Valko et al. 2004). Conjugation of glutathione to redox sensitive protein thiols generates mixed disulfides, and the formation of mixed disulfides is thought to be an early cellular response to oxidative stress. Protein *S*-glutathiolation can cause protein inactivation and has been implicated in ischemic cardiac injury (Reddy et al. 2000). Sulfoxidation of proteins occurs at methionine (Met) residues. Met is highly susceptible to

oxidative modification by ROS, such as H_2O_2. Methione sulfoxide reductase (MsrA) catalyzes the reduction of Met sulfoxides, repairing the modified protein to prevent serious cellular damage. Decreased MsrA activity and expression has been observed in AD brain and aging rats, suggesting the involvement of increased Met sulfoxidation in neurodegeneration and aging (Gabbita et al. 1999; Petropoulos et al. 2001).

Lipid peroxidation

Lipids are major targetsforfree radical attack under settings of oxidative stress (Gardner 1989). Lipid peroxidation is a key feature of oxidative stress, and excessive production of lipid peroxidation products has been implicated in the pathogenesis of a number of human diseases. Lipid peroxidation can cause damage to cellular membranes through disturbance of membrane organization and alteration of membrane integrity, fluidity, and permeability (Greenberg et al. 2008). Polyunsaturated fatty acids (PUFAs) within membrane phospholipids are especially susceptible to free radical damage. Lipids within lipoproteins also readily undergo oxidation initiated by free radicals. Free radical-mediated lipid peroxidation proceeds by a chain mechanism and involves three events: initiation, propagation, and termination. One initiating free radical can oxidize many lipid molecules through sequential, self-propagating chain reactions (Niki 2009). Radical species that can initiate the chain reaction include hydroxyl radicals, alkoxyl radicals, peroxyl radicals, and peroxynitrite, but the hydroxyl radical is the most active (Niki 2009). Catalytic metal ions such as copper (Cu^{I}) or iron (Fe^{II}) can also contribute to chain initiation (Halliwell and Gutteridge 1990). The mechanism of free radical-initiated lipid peroxidation begins withabstraction of a bisallylic hydrogen from a polyunsaturated lipid to form a carbon-centered radical which rearranges to a more stable pentadienyl radical (Porter 1986). The pentadienyl radical then combines with molecular oxygen (O_2) to generate a peroxyl

radical, which facilitates chain propagation through abstraction of a bisallylic hydrogen from a second lipid to give a conjugated diene lipid hydroperoxide and new lipid radicals (Niki 2009). This permits the continuation of another chain reaction. The distribution of lipid peroxidation products is determined by the relative contribution of competing reactions, and free radical-initiated chain reactions are terminated by antioxidants.

Biologically important PUFAs, such as linoleic acid (LA) (18:2), arachidonic acid (AA) (20:4), eicosapentaenoic acid (EPA) (20:5), and docosahexaenoic acid (DHA) (22:6), as well as cholesterol are subject to free radical-initiated lipid peroxidation, yielding a diverse array of products. Oxidation of LA and its esters proceeds by a simpler mechanism than that of more highly unsaturated fatty acids such as AA, EPA, and DHA (Niki 2009). An inner peroxyl radical of a PUFA with three or more double bonds can undergo cyclization by intramolecular rearrangement through addition of the peroxyl radical to the β,γ double bond yielding prostaglandin-like bicyclic endoperoxide products (Porter 1986; Pryor et al. 1976). Further, due to the relatively weak C–H bond of the bisallylic methylene, the oxidizability of PUFAs increases with the number of double bonds present in the fatty acid; that is, the number of bisallylic methylenes available for hydrogen abstraction. Lipid hydroperoxides are primary products of free radical-initiated peroxidation of PUFAs (Niki 2009). Oxidation of linoleates forms hydro(pero)xyoctadienoates (H(P)ODEs). The nonenzymatic free radical-catalyzed peroxidation of AA produces a series of biologically active prostaglandin-like products termed isoprostanes (IsoPs), including F_2-IsoPs (Morrow, Hill et al. 1990), E_2/D_2-IsoPs (Morrow et al. 1994), IsoTXs (Morrow et al. 1996), and cyclopentenone A_2/J_2-IsoPs (Chen et al. 1999). (The formation and biological activity of these compounds will be discussed in further detail in the following section). In addition, isoketals (isolevaglandins) are highly reactive γ-ketoaldehydes formed from the free radical-catalyzed peroxidation of AA, and these compounds exert their biological

activity by rapidly adducting to primary amines such as lysyl residues of proteins (Davies et al. 2004). Isofurans are also products of free radical-initiated peroxidation of AA that exhibit favored formation in settings of elevated oxygen tension (Fessel and Roberts 2005). Oxidation of EPA and DHA by similar mechanisms gives rise to 3-series IsoPs (Gao et al. 2006) and neuroprostanes (Roberts et al. 1998), respectively. Aldehydes such as acrolein, malondialdehyde (MDA), and 4-hydroxy-2-nonenal (HNE) are formed during lipid peroxidation as secondary or decomposition products (Esterbauer et al. 1991). HNE, the most extensively studied of these aldehyde products, is a highly reactive α,β-unsaturated aldehyde that can readily react with proteins, DNA, and phospholipids to cause deleterious effects (Esterbauer et al. 1991). Oxysterols are oxidation products of cholesterol found in oxidized low density lipoprotein (oxLDL), and these compounds have been shown to induce apoptotic cell death (Lordan et al. 2009).

Discovery of isoprostanes

In 1990, Roberts, Morrow, and colleagues reported the discovery of a series of $PGF_{2\alpha}$-like compounds generated *in vivo* in humans from the non-cyclooxygenase free radical-initiated peroxidation of arachidonic acid (Morrow, Hill et al. 1990); these novel compounds were later termed F_2-isoprostanes (F_2-IsoPs). Previously, Pryor et al. reported the formation of PG-like compounds during auto-oxidation of polyunsaturated fatty acids *in vitro* (Pryor et al. 1975). However, IsoPs were not discovered to be formed *in vivo* in humans until 1990 (Morrow, Hill et al. 1990; Morrow, Harris et al. 1990). The discovery of IsoPs was based on the identification of multiple $PGF_{2\alpha}$-like compounds in fresh plasma samples from normal human volunteers when analyzed by gas chromatography/mass spectrometry (GC/MS) (Morrow, Harris et al. 1990). Storage of plasma at -20^{o}C for several months dramatically increased the levels of these

compounds up to 50 to 100-fold (Morrow, Harris et al. 1990). In addition, base-catalyzed hydrolysis of plasma lipids also yielded significant amounts of the $PGF_{2\alpha}$-like compounds. Anti-oxidants and reducing agents suppressed the formation of these compounds (Morrow, Harris et al. 1990). Further, Morrow et al. demonstrated that treatment of rats with the hepatotoxicant carbon tetrachloride (CCl_4) resulted in a 50 to 55-fold increase in plasma F_2-IsoPs and over a 100-fold increase in liver F_2-IsoPs compared to untreated rats (Morrow, Awad, Boss et al. 1992; Morrow, Awad, Kato et al. 1992). CCl_4 is a potent inducer of lipid peroxidation due to its cytochrome P450-catalylzed conversion to the trichloromethyl radical ($CCl_3^{\cdot}$) (Recknagel 1967); this radical species catalyzes the peroxidation of lipids leading to increased IsoP generation. These studies further confirmed that F_2-IsoPs were formed from free radical-initiated lipid peroxidation (Morrow, Awad, Kato et al. 1992).

Mechanism of isoprostane formation

The mechanism for the formation of IsoPs from the free radical-catalyzed peroxidation of arachidonic acid has been well definedand is outlined in **FIGURE 1.8**. Arachidonic acid contains three bisallylic hydrogens, at positions C-7, C-10, and C-10, each of which can undergo free radical abstraction to form a carbon-centered radical which rearranges to a more stable pentadienyl radical. Addition of a molecule of oxygen (O_2) to the pentadienyl radicals generates four positional peroxyl radical isomers of arachidonic acid, which undergo 5-*exo* cyclization followed by addition of a second molecule of oxygen to the backbone of the compounds to form PGG_2-like and PGH_2-like compounds (Pryor et al. 1976; Morrow, Harris et al. 1990). These unstable endoperoxides can then be reduced to $PGF_{2\alpha}$-like compounds, termed F_2-IsoPs (Morrow, Hill et al. 1990). Based on this mechanism of formation, four regioisomers of each class of IsoPs derived from arachidonic acid are generated, each of which can theoretically be

comprised of eight racemic diastereomers, yielding a total of 64 potential F_2-IsoP isomers. Compounds are denoted as 5-, 8- 12-, or 15-series regioisomers depending on the carbon atom to which the side chain hydroxyl is attached (Taber et al. 1997). Fitzgerald and colleagues proposed an alternative nomenclature system in which the abbreviation iP is used for isoprostane, and regioisomers are denoted as III-VI based on the number of carbons between the omega carbon and the double bond (Rokach et al. 1997; Lawson et al. 1999). The four classes of IsoP regioisomers are not formed in equivalent proportions *in vivo*. The 5- and 15-series regioisomers are significantly more abundant than the 8- and 12-series compounds (Waugh et al. 1997). Yin et al. elucidated the mechanisms for this difference (Yin, Morrow et al. 2002; Yin, Havrilla et al. 2002).

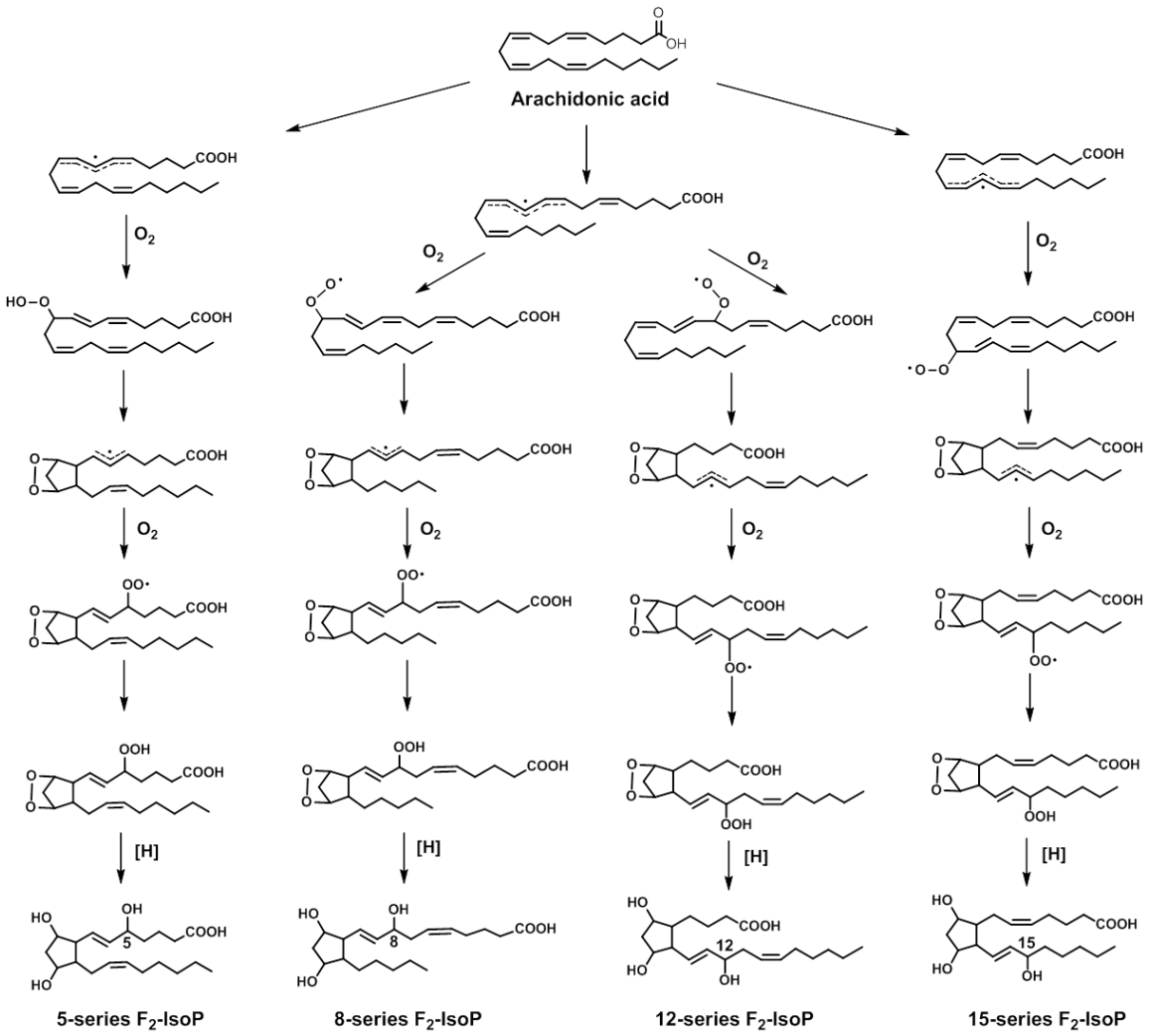


FIGURE 1.8. Mechanism of formation of F_2-IsoPs from free radical-catalyzed peroxidation of arachidonic acid. Four regioisomers are generated, each consisting of 8 racemic diastereomers. For simplicity, stereochemistry is not indicated.

Distinctions between IsoPs and COX-derived PGs

IsoPs are so named because they are isomeric to COX-derived PGs. However, IsoPs differ from PGs by important structural and biochemical distinctions. PG side chains are in the *trans*-configuration with respect to the prostane ring, whereas the majority of IsoP side chains are in the *cis*-configuration (Morrow, Hill et al. 1990). In addition, the generation of PGs via the COX pathway results in a single stereoisomer, whereas IsoPs formed via free radical-initiated lipid peroxidation consist of multiple racemic isomers. Further, unlike PGs, IsoPs are initially

formed *in situ* esterified in phospholipids and are subsequently hydrolyzed to their free form by phospholipases, such platelet-activating factor (PAF) acetylhydrolase (Morrow, Awad, Boss et al. 1992; Stafforini et al. 2006). In this regard, membrane-bound IsoPs can potentially affect the integrity and fluidity of membranes, due to their contorted 3-D structure (Morrow, Awad, Boss et al. 1992). Moreover, from a bioanalytical viewpoint, IsoPs can be quantified both in membrane-bound/esterified or free fractions.

Other products of the isoprostane pathway

After the initial discovery of F_2-IsoPs, several other classes of IsoPs and related compounds have been identified *in vivo*. Formation of these compounds is illustrated in **FIGURE 1.9**. The unstable PGH_2-like IsoP intermediates (H_2-IsoPs) can undergo rearrangement to PGE_2- and PGD_2-like compounds (E_2/D_2-IsoPs) (Morrow et al. 1994) or to TXA_2-like compounds ($IsoTxA_2$) (Morrow et al. 1996), which are hydrolyzed to TXB_2-like compounds ($IsoTxB_2$). Similar to the formation of cyclopentenone PGs, PGA_2 and PGJ_2, from dehydration of COX-derived PGE_2 and PGD_2, respectively, E_2 and D_2-IsoPs undergo dehydration *in vivo* to form reactive cyclopentenone IsoPs, A_2/J_2-IsoPs (Chen, Morrow et al. 1999). Each class of IsoPs has been shown to be abundantly produced under settings of oxidative stress *in vivo*, such as in CCl_4-treated rats. In addition, H_2-IsoPs can rearrange to form highly reactive γ-ketoaldehydes, termed isoketals (E_2/D_2-IsoKs) or isolevaglandins (Davies et al. 2004). Isofurans are also products of free radical-initiated peroxidation of arachidonic acid that are preferentially formed in settings of elevated oxygen tension (Fessel and Roberts 2005). Oxidation of EPA and DHA by similar mechanisms gives rise to 3-series IsoPs (Gao et al. 2006) and 4-series IsoP-like compounds (neuroprostanes) (Roberts et al. 1998), respectively.

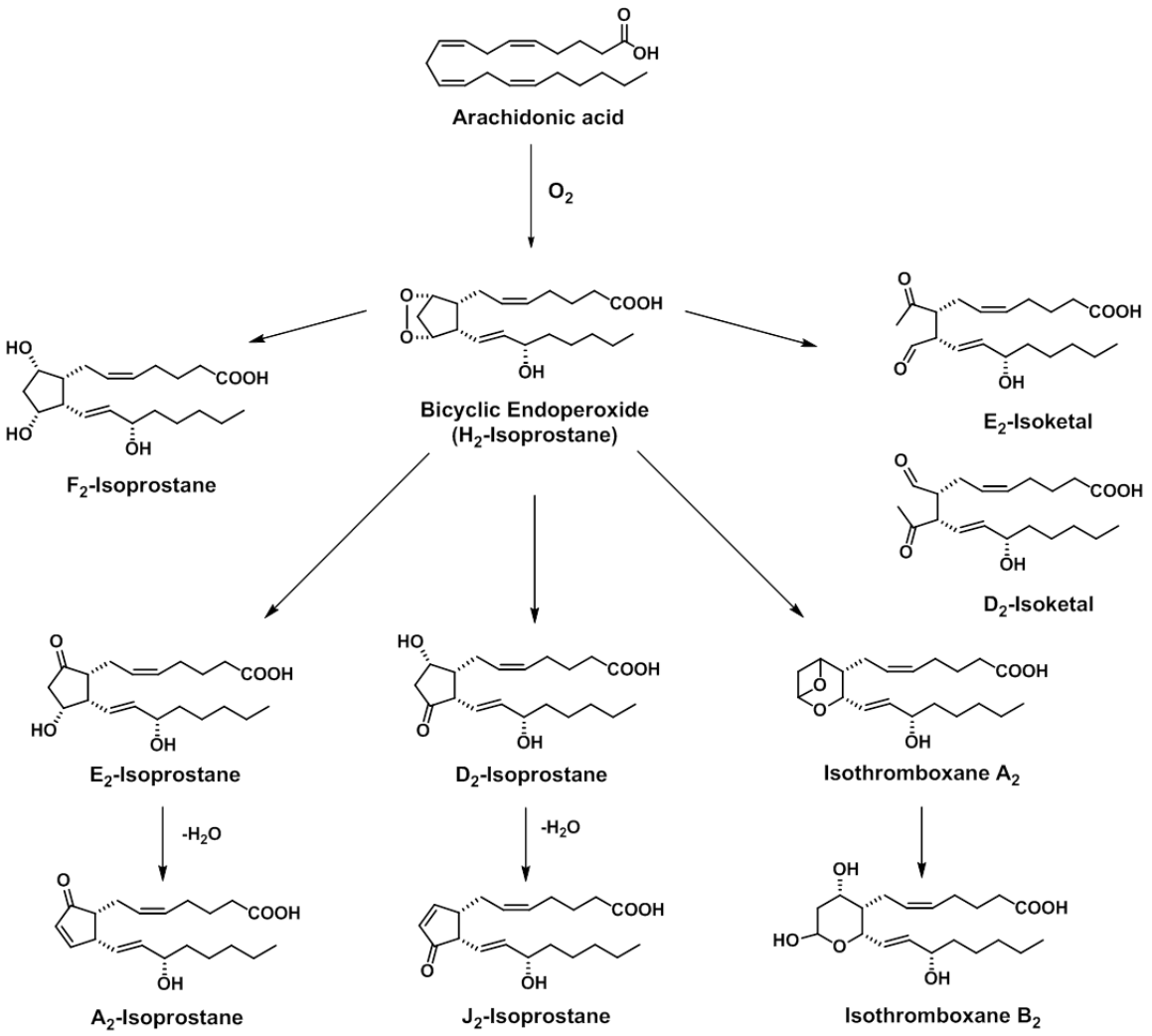


FIGURE 1.9. Structures of different classes of IsoPs and related compounds formed via IsoP endoperoxide intermediates. Multiple regioisomers are formed for each class of IsoP shown above. For simplicity, only one regioisomer is illustrated.

Isoprostanes: Biomarkers of oxidative stress

The discovery of IsoPs has provided an important tool to explore the role of oxidative stress in the pathogenesis of human disease (Montuschi et al. 2004). While various markers have been proposed to assess oxidative stress *in vivo*, measurement of F_2-IsoPs in plasma or urine has been established as the most reliable biomarker of oxidative stress status *in vivo* according to a recent multi-investigator study, termed the Biomarkers of Oxidative Stress (BOSS)

Study, sponsored by the National Institutes of Health (Kadiiska et al. 2005). IsoPs are formed in significant abundance *in vivo*, and their formation is markedly increased under settings of oxidative stress due to free radical-mediated lipid peroxidation. The advantage to measuring F_2-IsoPs over other products of lipid peroxidation is that they are chemically stable, specific products of arachidonic acid peroxidation, and they are detectable in all biological tissues and fluids under normal physiological conditions (Roberts and Morrow 2000; Morrow and Roberts 1997; Practico et al. 2001). The 15-series F_2-IsoP 15-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$) is specifically quantified in plasma and urine as well as esterified in tissue biopsies and plasma lipoproteins to provide a measure of endogenous IsoP production. In addition, quantification of the major urinary metabolite of 15-F_{2t}-IsoP, 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP, provides an accurate assessment of the systemic production of F_2-IsoPs (Montuschi et al. 2004). GC/MS is the reference analytical method for measuring F_2-IsoPs (Roberts and Morrow 2000; Milne et al. 2007), but other methods are also used such as radioimmunoassay (Wang et al. 1995). Further, due to the abundance of DHA in the central nervous system, measurement of F_4-neuroprostanes derived from DHA is used as an index of oxidative neuronal injury and has proved to be valuable in exploring the role of oxidation stress in neurodegenerative diseases (Roberts et al. 1998). F_2-IsoPs are elevated in numerous human diseases and risk factors for disease, including coronary artery disease (Vassalle et al. 2003), ischemia-reperfusion injury (Delanty et al. 1997; Reilly et al. 1997), atherosclerosis (Practico et al. 1997), renovascular disease (Minuz et al. 2002), hypercholesterolemia (Davi et al. 1997; Reilly et al. 1998), diabetes (Davi et al. 1999), obesity (Keaney et al. 2003), cigarette smoking (Morrow et al. 1995), asthma (Montuschi et al. 1999; Dworski et al. 1999), chronic obstructive pulmonary disease (Montuschi et al. 2000), Alzheimer's disease and other neurodegenerative diseases (Roberts et al. 1998; Montine et al. 2002;

Montine et al. 1999; Practico et al. 2000; Practico et al. 1998) suggesting a link or causative role of oxidative stress in disease progression (Montuschi et al. 2004).

Isoprostanes: Mediators of oxidative stress

Isoprostanes (IsoPs) are not only biomarkers of oxidative stress; recent evidence supports the contention that they are also mediators of oxidative stress (Montuschi et al. 2004). IsoPs and related compounds have been found to exert potent activity in various biological processes and may contribute to disease pathogenesis. 15-F_{2t}-IsoP is a potent vasoconstrictor in the kidney through interaction with TXA_2 receptors (Takahashi et al. 1992). This IsoP has been shown to significantly decrease glomerular filtration and renal blood flood in rats. 15-F_{2t}-IsoP also has constrictive effects in other vascular beds, such as lung (Morrow, Hill et al. 1990), heart (Kromer and Tippins 1996), and brain (Hou et al. 2000). 15-E_{2t}-IsoP is also a renal vasoconstrictor (Morrow and Roberts 1997). F_2-IsoPs and E_2-IsoPs may elicit additional biological effects through interaction with other prostanoid receptors (Montuschi et al. 2004; Milne et al. 2008).

Reactive products of the IsoP pathway, the cyclopentenone A_2/J_2 IsoPs and γ-ketoaldehyde IsoKs, mediate biological effects primarily through covalent modification of intracellular proteins. IsoKs rapidly react with lysine residues of proteins forming oxidized pyrrole adducts that can potentially lead to formation of protein-protein crosslinks (Brame et al. 1999; Davies 2004). Adduction of IsoKs with protein lysine residues can alter protein function and may contribute to the pathogenesis of diseases associated with oxidative stress (Davies 2004). The cyclopentenone A_2/J_2 IsoPs contain a reactive α,β-unsaturated carbonyl moiety within the cyclopentenone ring rendering them susceptible to Michael addition with cellular thiols, such as GSH and free cysteine residues of proteins. As such, cyclopentenone IsoPs may

exhibit biological effects similar to those induced by the cyclopentenone PGs. 15-A_{2t}-IsoP is one of the major A_2-IsoPs formed *in vivo* (Chen, Zackert et al. 1999). This compound has been shown to rapidly adduct GSH and albumin *in vitro* (Chen, Morrow et al. 1999). In addition, Levonen et al. demonstrated that 15-A_{2t}-IsoP activates the Nrf2 pathway via adduction to Keap-1 leading to induction of antioxidant response genes (Levonen et al. 2004). 15-A_2-IsoPs have been shown to suppress LPS-induced inflammatory signaling in macrophages via inhibition of the NF-κB. 15-A_2-IsoPs were also shown to reduce cytokine production in activated macrophages, suggesting that cyclopentenone IsoPs may be modulators of inflammation (Musiek et al. 2005; Milne, Musiek et al. 2005). Musiek et al. reported that 15-A_2-IsoP and 15-J_2-IsoP potently induced apoptosis in primary cortical neuronal cultures (Musiek et al. 2006). 15-J_2-IsoPs have also been shown to activate the nuclear receptor PPARγ with an EC_{50} of approximately 3 μM (Musiek et al. 2005). Moreover, similar to cyclopentenone PG 15-d-PGJ_2, 15-A_2-IsoP promoted production of VEGF, a key regulator of angiogenesis, indicating the pro-angiogenic potential of cyclopentenone IsoPs (Milne, Musiek et al. 2005). Taken together, these findings suggest that IsoPs may play an important role in mediating various physiological and pathophysiological processes under settings of oxidative stress *in vivo*.

Metabolism of cyclopentenone IsoPs

While cyclopentenone IsoPs have been readily detected *in vivo* esterified in phospholipids from rat liver following oxidant insult with CCl_4, circulating nonesterified A_2/J_2-IsoPs have not been identified in plasma and urine after CCl_4 treatment (Chen, Morrow et al. 1999; Milne, Gao et al. 2005). In contrast, E_2/D_2-IsoPs are abundant in the plasma of CCl_4-treated rats (Morrow et al. 1994). This is likely due to the marked proclivity of cyclopentenone IsoPsto rapidly react with cellular protein and peptide thiols (Chen, Morrow et al. 1999). Like

cyclopentenone PGs, the cyclopentenone IsoP 15-A_{2t}-IsoP has been shown to be primarily metabolized via conjugation with GSH *in vitro* and *in vivo* (Hubatsch et al. 2002; Milne et al. 2004; Milne, Gao et al. 2005). Hubatsch et al. demonstrated that 15-A_{2t}-IsoP is efficiently conjugated with GSH by human and rat glutathione transferase A4-4 (Hubatsch et al. 2002). Additionally, Milne et al. determined that in human cells, 15-A_{2t}-IsoP is primarily metabolized via conjugation to GSH (Milne et al. 2004). Structural characterization of adducts by LC/MS/MS revealed four major conjugates. These included the intact 15-A_{2t}-IsoP-GSH conjugate, the GSH conjugate in which the carbonyl at C-9 of 15-A_{2t}-IsoP is reduced, and the corresponding cysteine conjugates (Milne et al. 2004). In this study, formation of cysteine conjugates increased with longer cellular incubations as a consequence of hydrolysis of glutamate and glycine from GSH. Subsequently, Milne et al. explored the metabolism of 15-A_{2t}-IsoP in the rat (Milne, Gao et al. 2005). Using LC/MS/MS analysis, the major urinary metabolite of 15-A_{2t}-IsoP was determined to be the mercapturic acid sulfoxide conjugate in which the carbonyl at C-9 was reduced to an alcohol. Notably, this metabolite was found to be formed in significant quantities in the urine from rats exposed to CCl_4-induced oxidant injury (Milne, Gao et al. 2005). Further, 15-A_{2t}-IsoP was found to undergo extensive conjugation *in vivo* in humans. Upon infusion of a tracer quantity of radiolabeled 15-A_{2t}-IsoP into a normal human volunteer, the majority of the compound was excreted in the form of a polar conjugate(s) (Chen, Morrow et al. 1999). Taken together, these findings along with previous studies suggest that quantification of excreted metabolites is the best method to assess systemic production of cyclopentenone IsoPs *in vivo* under settings of oxidative stress.

Cross-talk between the COX and IsoP pathways

Emerging evidence suggests that there may be cross-talk between the IsoP pathway and the COX pathway. Recent studies by our laboratory have demonstrated that PGs can be generated *in vivo* via the IsoP pathway, independent of COX (Gao et al. 2003; Yin et al. 2007). Gao et al. reported the formation of PGE_2 and PGD_2 and their respectiveenantiomers *in vivo* through the spontaneous epimerization and subsequent hydrolysis of E_2/D_2-IsoPs, specifically 15-E_{2t}-IsoP and 15-D_{2c}-IsoP, respectively (Gao et al. 2003). Further, Yin et al. showed that $PGF_{2\alpha}$ is generated *in vivo* via the IsoP pathway, independent of the COX enzymes (Yin et al. 2007). In this study, it was found that the majorityof putative $PGF_{2\alpha}$ in human urine is derived from the nonenzymatic freeradical-initiated peroxidation of arachidonic acid, not via the COX pathway (Yin et al. 2007). Formation of PGs via the IsoP pathway may occur because small amounts of the H_2-IsoP endoperoxides containing *trans* side chains are generated *in vivo* through this mechanism (Yin et al. 2007).

At the present time, the extent to which 15-d-PGJ_2 is formed *in vivo* and mechanisms that regulate its formation are unclear. Generation of 15-d-PGJ_2 is thought to be mediated by COX-2 induction at sites of inflammation (Gilroy et al. 1999). However, given the structural similarity, it is highly likely that, similar to the dehydration of COX-derived PGD_2, D_2-IsoPs can undergo dehydration and isomerization to yield 15-d-PGJ_2 and 15-d-PGJ_2-like compounds. A proposed mechanism for formation of 15-d-PGJ_2 via the COX or free radical-catalyzed pathway is illustrated in **FIGURE 1.10**. Based on this observation, it is possible that 15-d-PGJ_2 and related compounds may be produced *in vivo* in settings of inflammation in which COX-2 activity is increased as well as under conditions of oxidative stress. This may haveimportant implicationsin diseases associated with inflammation and oxidative stress due to the potential synergistic effects of 15-d-PGJ_2 and other cyclopentenone eicosanoids.

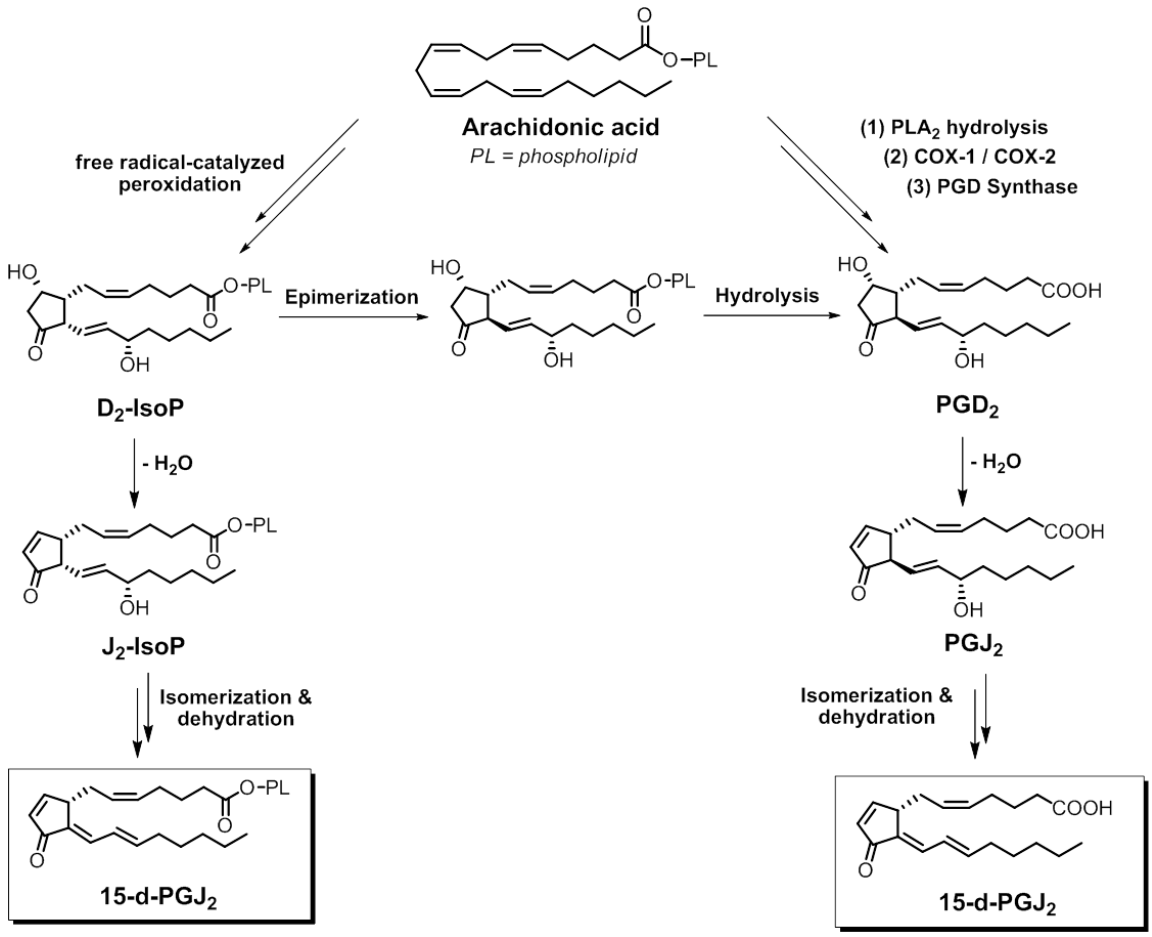


FIGURE 1.10. Proposed mechanism for the formation of 15-d-PGJ_2 via the cyclooxygenase and free radical-catalyzed pathways.

Specific aims of this research

The overall goal of this thesis research is to examine the biochemical pathways that contribute to the formation of 15-d-PGJ_2 *in vivo* and develop an analytical method to assess the systemic generation of 15-d-PGJ_2 *in vivo* in humans under normal and pathophysiological conditions. It is our hypothesis that 15-d-PGJ_2 may be generated *in vivo* via the cyclooxygenase and free radical-initiated pathways. Further, identifying the major urinary metabolite of 15-d-PGJ_2 will provide a biomarker to assess the systemic production of this compound *in vivo*. We contend that studies examining the formation and metabolism of 15-d-PGJ_2 *in vivo* will yield important insights towards understanding the biological role of this reactive cyclopentenone eicosanoid in human health and disease.

Therefore, the specific aims of this dissertation research are as follows:

1) Examine the contribution of the cyclooxygenase and the nonenzymatic free radical-catalyzed pathways to the formation of 15-d-PGJ_2 *in vivo*.
2) Determine the metabolic fate of 15-d-PGJ_2 in the rat.
3) Compare the metabolism of 15-d-PGJ_2 in rats and humans.

CHAPTER II

NONENZYMATIC FREE RADICAL-CATALYZED GENERATION OF 15-DEOXY-$\Delta^{12,14}$-PROSTAGLANDIN J_2-LIKE COMPOUNDS (DEOXY-J_2-ISOPROSTANES) *IN VIVO*

Introduction

Cyclooxygenase (COX)-1 and -2 catalyze the committed step in the formation of prostaglandins (PG) from arachidonic acid (AA) by generating the unstable bicyclic endoperoxide intermediate PGH_2 (Morrow and Roberts 2001; Marnett 2000; Marnett et al. 1999). Tissue specific synthases convert PGH_2 to the parent PGs, $PGF_{2\alpha}$, PGE_2, PGD_2, PGI_2, and thromboxane (TXA_2) (Morrow and Roberts 2001). These molecules are formed in response to various stimuli, and they exert a plethora of biological effects through interaction with cell surface receptors (Morrow and Roberts 2001).

PGE_2 and PGD_2 are unstable and readily undergo dehydration in aqueous solution to form cyclopentenone PGs, PGA_2 and PGJ_2, respectively. Dehydration of PGE_2 results in formation of PGA_2 (Hamberg and Samuelsson 1966). Dehydration of PGD_2 results in formation of PGJ_2 (Fukushima, Kato, Ota et al. 1982; Fitzpatrick and Wynalda 1983). PGJ_2 can isomerize *in vitro* in the presence of albumin to Δ^{12}-PGJ_2 and then undergo dehydration to yield 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15-d-PGJ_2) (**FIGURE 2.1**) (Fitzpatrick and Wynalda 1983). Unlike other classes of PGs, cyclopentenone PGs are characterized by the presence of an electrophilic α,β-unsaturated carbonyl moiety in the prostane ring rendering them susceptible to Michael addition with nucleophilic biomolecules, such as the free sulfhydryls of glutathione (GSH) and cysteine residues of proteins (Straus and Glass 2001). These compounds have attracted considerable attention because they exhibit a unique spectrum of biological activities, presumably through

reaction with cellular biomolecules (Straus and Glass 2001). 15-d-PGJ_2, one of the most well-defined cyclopentenone PGs, is particularly reactive because it possesses two electrophilic carbon centers that are susceptible to nucleophilic attack - one in the cyclopentenone ring (C-9), and one on the lower side chain (C-13). This compound is believed to mediate a number of cellular responses through covalent interaction with critical intracellular protein targets (Kim and Surh 2006; Uchida and Shibata 2008). 15-d-PGJ_2 is postulated as an endogenous ligand of peroxisome proliferator-activated receptor-γ (PPARγ), which plays an important role in adipocyte differentiation and anti-inflammatory processes (Forman et al. 1995; Kliewer et al. 1995). 15-d-PGJ_2 has also been shown to exhibit anti-inflammatory, anti-proliferative, and pro-apoptotic activities independent of PPARγ in studies performed *in vitro* (Straus and Glass 2001). However, the extent to which 15-d-PGJ_2 is formed *in vivo* and the mechanisms regulating its formation remain unclear.

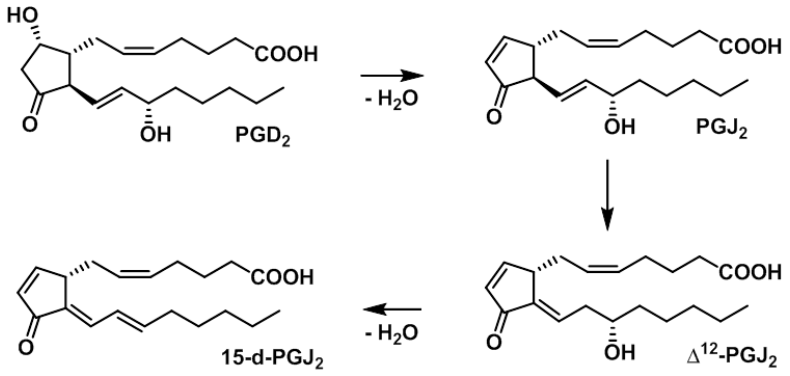


FIGURE 2.1. 15-d-PGJ_2 is formed from PGD_2 through a sequence of dehydration and isomerization reactions.

Oxidant stress has been increasingly implicated in the pathogenesis of a number of human diseases, including atherosclerosis, cancer, neurodegenerative disorders, and even the normal aging process (Halliwell and Gutteridge 1990; Southorn and Powis 1988; Ames 1983; Harman et al 1981). Lipid peroxidation is a central feature of oxidant stress. We have

previously reported the discovery of PG-like compounds, termed isoprostanes (IsoPs), which are produced *in vivo* from the free radical-induced peroxidation of arachidonic acid, independent of COX (Morrow, Hill et al. 1990). IsoPs are isomeric to PGs differing in the stereochemical relationship of the two side chains on the prostane ring. PG side chains are in the *trans*-configuration with respect to the prostane ring, whereas the majority of IsoP side chains are in the *cis*-configuration. Additionally, unlike PGs, IsoPs are initially formed *in situ* in phospholipids and then are subsequently hydrolyzed to their free form by phospholipases (Morrow, Awad, Boss et al. 1992; Stafforini et al. 2006). The mechanism for the formation of IsoPs from arachidonic acid has been well defined. Following abstraction of a bisallylic hydrogen from arachidonic acid and addition of a molecule of oxygen to form a peroxyl radical, the radical undergoes 5-*exo* cyclization, and a second molecule of oxygen adds to the backbone of the compound to form PGG_2-like compounds (Pryor et al. 1976; Morrow, Harris et al. 1990). These unstable bicycloendoperoxide intermediates can be reduced to $PGF_{2\alpha}$-like compounds termed F_2-IsoPs (Morrow, Hill et al. 1990), or undergo rearrangement to PGE_2- and PGD_2-like compounds (E_2/D_2-IsoPs) (Morrow et al. 1994) or TXA_2-like compounds (Tx-IsoPs) (Morrow et al. 1996). Based on this mechanism of formation, four regioisomers of each class of IsoPs derived from arachidonic acid are generated, each of which can theoretically be comprised of eight racemic diastereomers. Compounds are denoted as 5-, 8- 12-, or 15-series regioisomers depending on the carbon atom to which the side chain hydroxyl is attached (Taber et al. 1997).

The extent to which cyclopentenone prostanoids are generated *in vivo* has been the subject of continuing controversy for the last three decades (Hirata et al. 1988; Bell-Parikh et al. 2003; Attallah et al. 1974; Jonsson et al. 1976). We have previously reported that, analogous to the formation of PGA_2 and PGJ_2 from the dehydration of COX-generated PGE_2 and PGD_2, respectively, cyclopentenone IsoPs (A_2/J_2-IsoPs) are also formed *in vivo* from the dehydration of

E_2/D_2-IsoPs (Chen, Morrow et al. 1999). However, evidence for the formation of 15-d-PGJ_2 *in vivo* is lacking. Hirata et al. reported that the precursor of 15-d-PGJ_2, Δ^{12}-PGJ_2, can be detected in human urine, and its formation is suppressed to some extent by COX inhibitors (Hirata et al. 1988). Levels of free 15-d-PGJ_2 excreted in human urine were found to be very low (Bell-Parikh et al. 2003). Heretofore, it has been assumed that synthesis of 15-d-PGJ_2 depends upon COX generation of PGD_2. Given the structural and functional similarity, it is highly likely that, similar to the dehydration of COX-derived PGD_2, D_2-IsoPs can undergo dehydration and isomerization to yield a series of 15-d-PGJ_2-like compounds, termed deoxy-J_2-IsoPs. A proposed mechanism for formation of the four regioisomers of deoxy-J_2-IsoPs is shown in **FIGURE 2.2**.

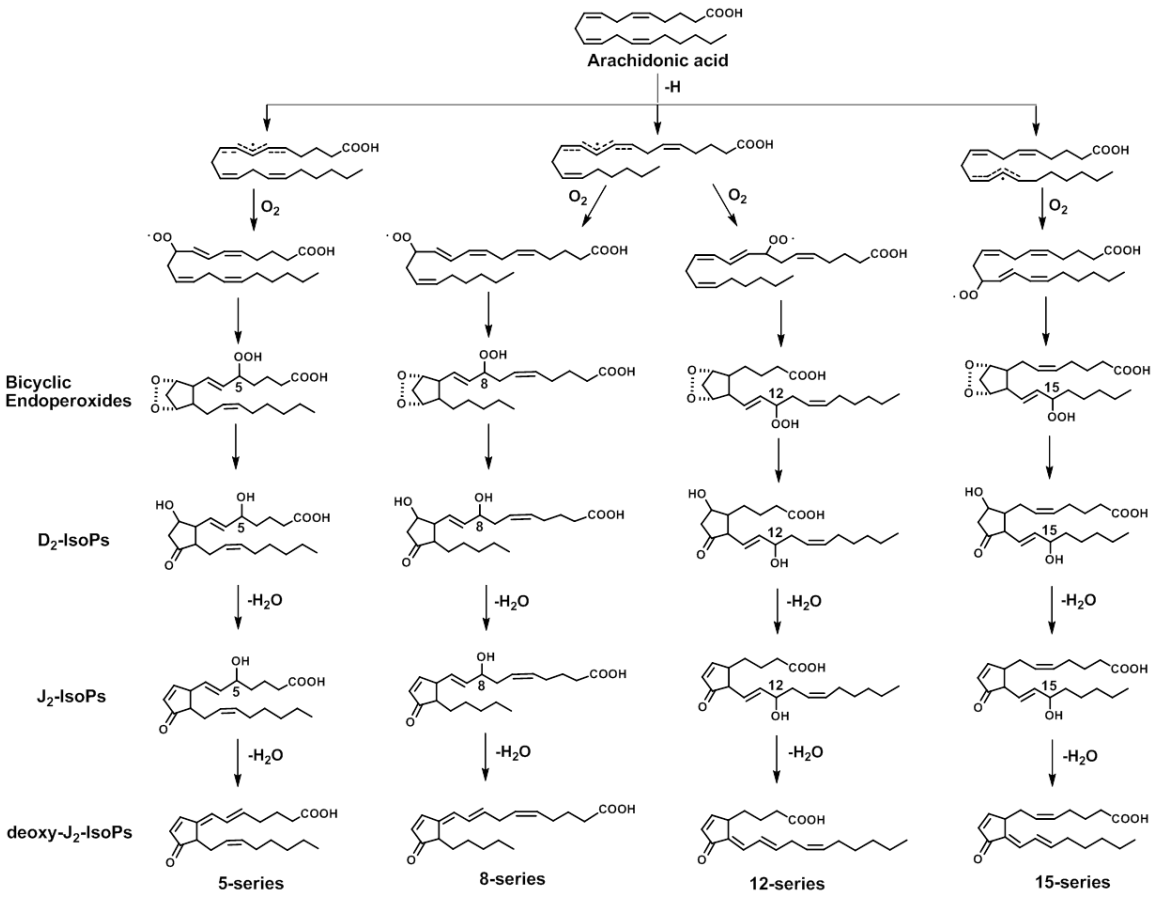


FIGURE 2.2. Proposed pathway for the formation of deoxy-J_2-IsoPs via the nonenzymatic peroxidation of arachidonic acid. Four regioisomers are generated, each consisting of racemic enantiomers. For simplicity, stereochemistry is not indicated.

It should be noted that dehydration of 15-series D_2-IsoPs followed by isomerization of the 13,14-double bond would result in loss of the chiral center at C-12, eliminating the stereochemical orientation of the lower side chain that distinguishes IsoPs from PGs. A single chiral center would be retained at C-8. Thus, a compound identical in all respects to 15-d-PGJ_2 and its corresponding enantiomer would be expected to form from dehydration and isomerization of 15-D_2-IsoP (**FIGURE 2.3**). For purposes of discussion hereafter, the PG possessing a structure identical to that generated by COX is referred to as 15-d-PGJ_2. The compound that is enantiomeric to COX-derived 15-d-PGJ_2 isreferred to as *ent*-15-d-PGJ_2. The racemic mixture is termed *rac*-15-d-PGJ_2. Dehydration of other regioisomers of D_2-IsoPs yields various regioisomers of deoxy-J_2-IsoPs (**FIGURE 2.2**).

Herein, we report that 15-d-PGJ_2 and 15-d-PGJ_2-like compounds (deoxy-J_2-IsoPs) are formed in significant abundance *in vitro* and *in vivo* via the nonenzymatic free radical-catalyzed peroxidation of arachidonic acid, independent of COX enzymes. The finding that a series of deoxy-J_2-IsoPs are generated *in vivo* through lipid peroxidation is of potential biological importance due to the predicted bioactivity of these compounds. Similar to 15-d-PGJ_2, each of the deoxy-J_2-IsoPs possess reactive electrophilic carbon centers within their prostane ring and side chains, which render them susceptible to nucleophilic addition reactions with thiol-containing biomolecules, such as GSH and cysteine residues of proteins. Because the biological effects of 15-d-PGJ_2 are often likely to be mediated by its reactivity with cellular thiols, all of the deoxy-J_2-IsoPs would be expected to exert similar biological effects *in vivo* under settings of oxidant stress.

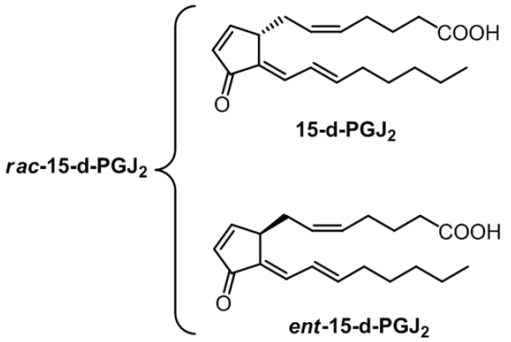


FIGURE 2.3. Structures of *rac*-15-d-PGJ_2, consisting of 15-d-PGJ_2 and its enantiomer.

Materials and Methods

Materials

Human serum albumin, carbon tetrachloride, triphenyl-phosphine (TPP), and *Apis mellifera* venom phospholipase A_2 were purchased from Sigma-Aldrich. PGD_2, 15-d-PGJ_2, and [$^2H_4$]15-d-PGJ_2 were purchased from Cayman Chemical Company (Ann Arbor, MI). Arachidonic acid was purchased from NU-Check Prep, Inc. (Elysian, MN). C_{18} Sep-Paks were purchased from Waters Corporation (Milford, MA). HPLC columns were purchased from Phenomenex (Torrance, CA). All solvents were of HPLC quality and purchased from EM Science (Gibbstown, NJ).

Oxidation of arachidonic acid *in vitro*

Five mg of arachidonic acid (AA) was dissolved in 50µl of ethanol and added immediately to 4.95 ml of phosphate-buffered saline, PBS (pH 7.4) containing 10mM AAPH (2,2"-azobis(amidinopropane)dihydrochloride), a free radical initiator. The AA oxidation reaction mixture was incubated at 37^oC for 24 h. Following oxidation, excess triphehylphosphine (TPP) was added to the oxidized AA mixture (1 mg/ml) for 5min at room temperature to reduce hydroperoxides to hydroxyls. Subsequently, the oxidized AA sample was extracted with 2 ml ethyl acetate twice, and extracts were dried under nitrogen gas. The samples were stored in 200 µl of ethanol at -80^oC until LC/MS analysis.

Isolation of 15-d-PGJ_2-like compounds (deoxy-J_2-IsoPs) from rat liver

Carbon tetrachloride (CCl_4) (1 mg/kg in 1 ml corn oil) was administered orogastrically to male Sprague-Dawley rats to induce lipid peroxidation (Morrow, Hill et al. 1990). Control animals received corn oil alone. Four hours after treatment, the animals were anesthetized with

pentobarbital (60 mg/kg) intraperitoneally and killed. The livers were removed and immediately flash-frozen in liquid nitrogen and stored at -80°C. 300-500 mg of tissue was homogenized in 5 ml of ice-cold chloroform:methanol (2:1, v/v) containing butylated hydroxytoluene, BHT (0.005%) to prevent *ex vivo* autooxidation. Esterified deoxy-J_2-IsoPs in liver phospholipids were enzymatically hydrolyzed with *A. mellifera* venom phospholipase A_2 to liberate free deoxy-J_2-IsoPs. Subsequently, free IsoPs were extracted over a C_{18} Sep-Pak cartridge that had been preconditioned by rinsing with methanol and deionized water, pH 3. The C_{18} Sep-Pak was rinsed with 10 ml each of deionized water (pH 3) and heptane, and the sample was eluted with 10 ml 1:1 ethyl acetate:heptane. The solvent was removed by evaporation under a stream of nitrogen (N_2), and samples were stored in 200 μl of ethanol at -80°C until LC/MS analysis.

Analysis of 15-d-PGJ_2-like compounds (deoxy-J_2-IsoPs) LC/MS/MS

After extraction of oxidized AA using the C_{18} Sep-Pak methodology described above, the sample was suspended in 100 μl 2:1 methanol:water, and deoxy-J_2-IsoPs were analyzed by reversed phase high-performance liquid chromatography (RP-HPLC). Online HPLC was carried out using a Surveyor MS Pump equipped with a Phenomenex Luna C-18 column (50 x 2.00 mm, 3 μm) at a flow rate of 0.3 ml/min starting with 80% Phase 1 (water/phase 2/acetic acid, 95/5/0.1) to 30% from 1 to 27.0 min, and holding at 100% Phase 2 (acetonitrile/methanol/acetic acid, 95/5/0.1) from 28.0-29.0 min, and returning to 80% Phase 1 at 30.0-32.0 min. LC/electrospray ionization (ESI)-MS/MS was carried out on a ThermoFinnigan TSQ/Quantum Ultra mass spectrometer. The ESI source was fitted with a deactivated fused silica capillary (100 μm inner diameter), and the mass spectrometer was operated in the negative ion mode. Nitrogen was used as both the sheath gas and the auxiliary gas, at 31 and 17 p.s.i., respectively. The capillary temperature was 300°C. The spray voltage was 5.0 kV, and the tube lens voltage

was 80V. In another set of experiments, deoxy-J_2-IsoPs were analyzed by normal phase HPLC/atmospheric pressure chemical ionization (APCI)-MS for separation of deoxy-J_2-IsoP regioisomers. For these experiments, deoxy-J_2-IsoPs were extracted by C_{18} Sep-Pak as described previously, and samples were resuspended in mobile phase (93:7 hexane/isopropanol/acetic acid) for normal phase HPLC. Online HPLC was carried out using a Waters Alliance HPLC equipped with a Phenomenex Luna Silica column (250 x 4.60 mm, 5 μm) at a flow rate of 1.0 ml/min with an isocratic gradient of 93:7:0.1 hexane/isopropanol/acetic acid for 20 min. LC/APCI-MS was carried out on a ThermoFinnigan TSQ/Quantum Ultra mass spectrometer operating negative ion mode with an APCI source. Tandem mass spectrometric (MS/MS) analysis employing collision-induced dissociation (CID) of the molecular ion *m/z* 315 of putative 15-d-PGJ_2 and deoxy-J_2-IsoPs was performed from 18 to 27 eV under 1.5 mTorr of argon. Spectra that are shown were obtained at 24 eV. Spectra were displayed by averaging scans across chromatographic peaks. Selected reaction monitoring (SRM) was performed according to characteristic fragmentation patterns of 15-d-PGJ_2 determined by CID. The collision energy for SRM was 24 eV. Data acquisition and analysis were performed using Xcaliber software, version 2.0.

Adduction of deoxy-J_2-IsoPs with glutathione *in vitro*

To isolate deoxy-J_2-IsoPs *in vitro*, AA was oxidized as described above and extracted with ethyl acetate. The sample was dried under a stream of N_2 and reconstituted in 100 μl ethanol. This sample was dissolved in 0.5 ml 1 N HCl and incubated for 5 hr at 37°C to facilitate acid-catalyzed dehydration of D_2/J_2-IsoPs to deoxy-J_2-IsoPs. Following the incubation, the reaction mixture was diluted to 10 ml with 50 mM aqueous ammonium acetate (pH 3.4) and extracted three times with 1.5 – 2 ml ethyl acetate. Extracts were pooled, dried under a stream of N_2, and

reconstituted in 100 μl ethanol. This sample was dissolved in 1 ml PBS (pH 6.5) and incubated in the presence of excess reduced glutathione (GSH) (~1 mg) and ~1 mg of equine liver glutathione *S*-transferase (GST) for 2 h at 37°C. A similar method was used for the incubation of chemically synthesized 15-d-PGJ_2 in the presence of GSH and GST. After the incubation, GSH adducts of deoxy-J_2-IsoPs and synthesized 15-d-PGJ_2 were purified by extraction using a C_{18} Sep-Pak cartridge preconditioned with 10 ml of acetonitrile and 50 mM aqueous ammonium acetate (pH 3.4) and eluted with 10 ml of 95% ethanol, and dried under a stream of N_2. Samples were suspended in a 3:1 mixture of Phase 1/Phase 2 (Phase 1: water/phase 2/acetic acid, 95/5/0.1; Phase 2 acetonitrile/methanol/acetic acid, 95/5/0.1), and deoxy-J_2-IsoP-GSH adducts were definitively identified by LC/ESI-MS/MS. The reversed phase-HPLC conditions described above were used. For MS/MS experiments, CID analysis of the parent ion *m/z* 622 of the 15-d-PGJ_2-GSH adduct and putative deoxy-J_2-IsoP-GSH adducts was performed from 18 to 22 eV under 1.5 mTorr of argon. Spectra that are shown were obtained at 22 eV.

Results

Formation and characterization of 15-d-PGJ_2 and 15-d-PGJ_2-like compounds *in vitro*

A representative selected ion monitoring (SIM) chromatogram obtained from LC/MS/MS analysis of chemically synthesized 15-d-PGJ_2 is shown in **FIGURE 2.4A** (upper chromatogram). When analyzed by ESI-MS in the negative ion mode, the predicted parent ion for 15-d-PGJ_2 is *m/z* 315. The peak eluting at 19.4 min was analyzed by CID and predicted fragments were obtained (**FIGURE 2.4A**, lower spectrum). These include *m/z* 271 $[M - CO_2]^-$ and *m/z* 203 (**FIGURE 2.4A**, lower spectrum). The smaller peak in the upper chromatogram is likely a

synthetic isomer of 15-d-PGJ_2 as LC/ESI-MS/MS analysis of this peak was identical to authentic 15-d-PGJ_2.

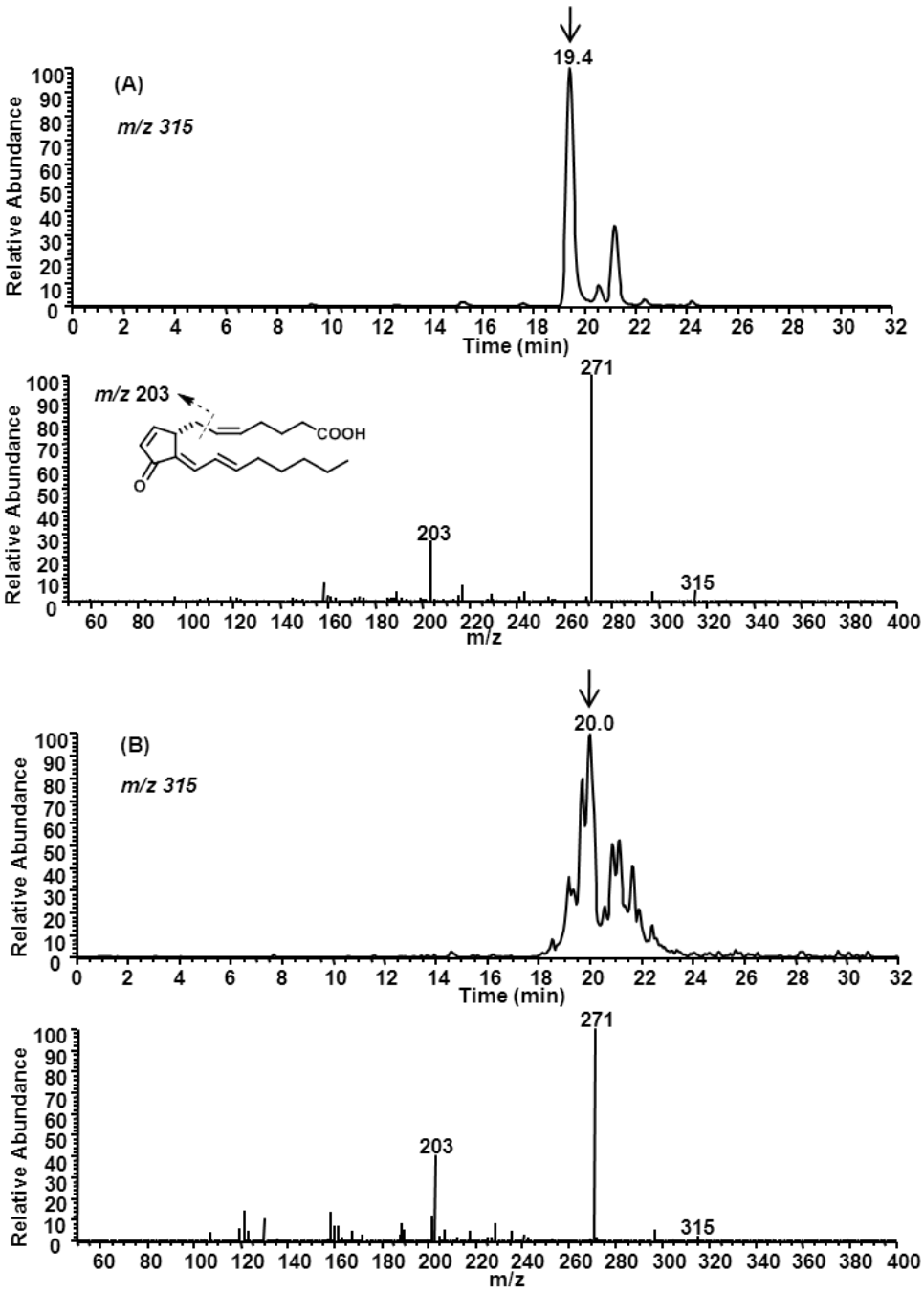


FIGURE 2.4. LC/ESI-MS/MS analysis of 15-d-PGJ_2 (A) and a mixture of 15-d-PGJ_2-like compounds (deoxy-J_2-IsoPs) obtained from the nonenzymatic oxidation of AA *in vitro* (B). A: LC/ESI-MS/MS analysis of a chemically synthesized 15-d-PGJ_2 employing SIM (upper chromatogram) and CID (lower product ion spectrum). B: LC/ESI-MS/MS analysis of 15-d-PGJ_2 -like compounds obtained from the *in vitro* oxidation of AA employing SIM for the precursor ion *m/z* 315 (upper chromatogram) and CID (lower product ion spectrum) indicative of putative 15-d-PGJ_2.

AA was then oxidized *in vitro* using the free radical initiator AAPH, and extracts were analyzed for 15-d-PGJ_2 and other 15-d-PGJ_2-like compounds (deoxy-J_2-IsoPs). When analyzed by LC/ESI-MS/MS in negative ion mode, the predicted parent ion for deoxy-J_2-IsoPs is *m/z* 315. The SIM chromatogramof this ion is shown in **FIGURE 2.4B**. As is evident, multiple chromatographic peaks are present that presumably represent different deoxy-J_2-IsoP regioisomers obtained from the nonenzymatic peroxidation of AA. The chromatographic peak eluting at 20.0 min has nearly the same retention time as authentic 15-d-PGJ_2. An authentic sample of isotopically labeled 15-d-PGJ_2 ([$^2H_4$]15-d-PGJ_2) showed the identical retention time upon co-injection (vide infra). (The analyses in **FIGURE 2.4A and 2.4B** were performed on separate days, accounting for differences in LC retention time). This peak was analyzed by CID to obtain structural information, and the CID spectrum is shown in **FIGURE 2.4B**. The observed product ions, *m/z* 271 and *m/z* 203, are consistent with fragmentation of 15-d-PGJ_2. The series of compounds representing deoxy-J_2-IsoPs elute in multiple chromatographic peaks approximately 19-22 min. CID analysis of this series of chromatographic peaks supports the contention that these are 15-d-PGJ_2-like compounds derived from different IsoP regioisomers, including compounds of the 5-, 8-, 12-, and 15-series (**FIGURE 2.5**). The composite CID spectrum obtained by summing scans over theentire series of chromatographic peaks reveals the product ion *m/z* 271 $[M - CO_2]^-$ as well as product ions resulting from specific fragmentation of each of the four deoxy-J_2-IsoPs regioisomers, including *m/z* 217 (5-series), *m/z* 95 (8-series), *m/z* 175 (12-series), and *m/z* 203 (15-series). Additionally, we utilized normal phase HPLC to obtain chromatographic separation of each of the deoxy-J_2-IsoPs regioisomers. Shown in the Figure 6 is a representative chromatogram of the different regioisomers of deoxy-J_2-IsoPs analyzed by normal phase LC/APCI-MS/MS. Again, CID analysis of the series of chromatographic peaks is consistent with fragmentation of each of the deoxy-J_2-IsoP regioisomers (**FIGURE 2.6**).

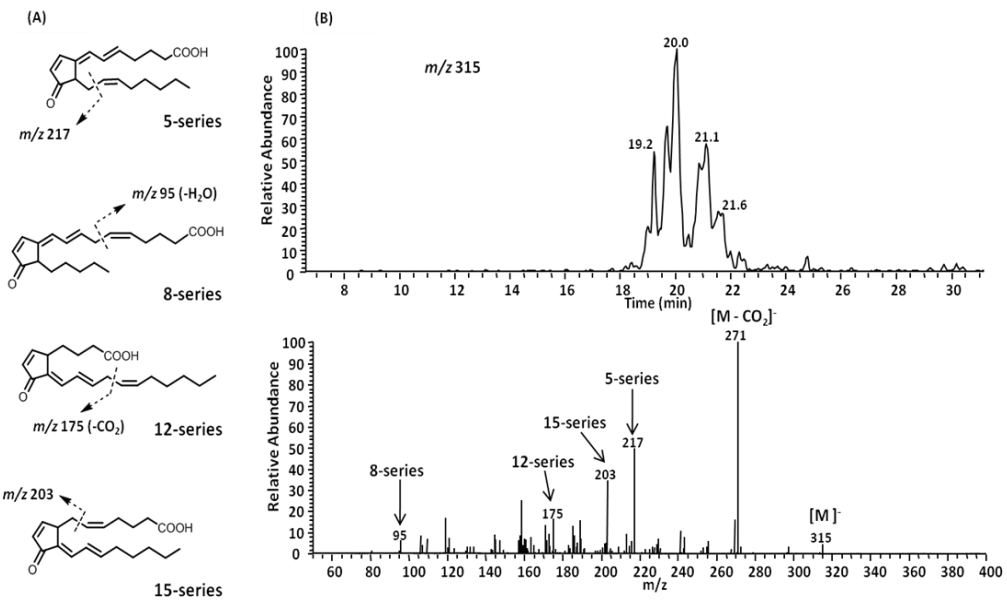


FIGURE 2.5. LC/ESI-MS/MS analysis of multiple regioisomers of deoxy-J_2-IsoPs generated from the nonenzymatic oxidation of AA *in vitro*. A: Structurally specific fragmentation of the different deoxy-J_2-IsoP regioisomers. B: SIM chromatogram of the precursor ion at *m/z* 315 (upper) and CID spectrum (lower) obtained by summing scans over the chromatographic peaks, revealing structurally specific fragmentation of the different deoxy-J_2-IsoP regioisomers.

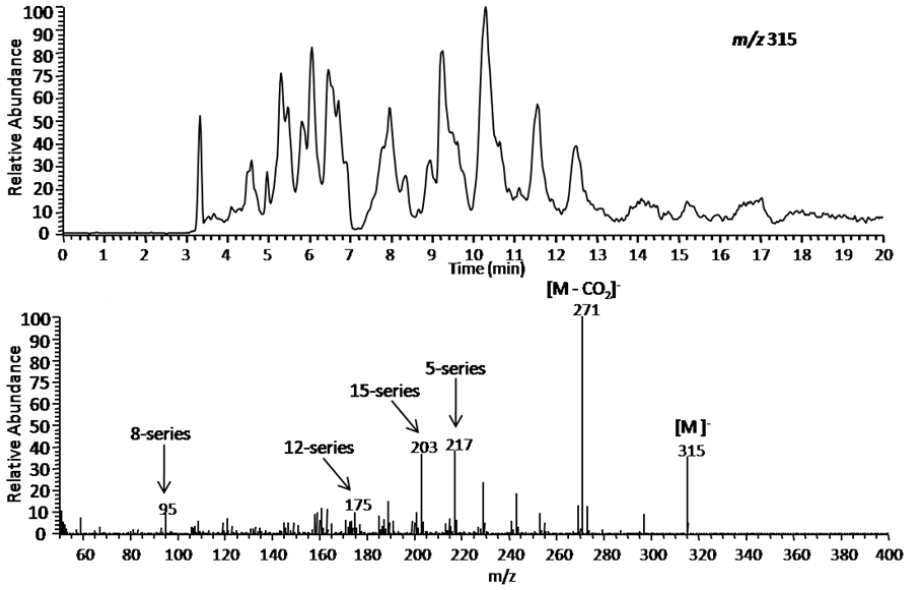


FIGURE 2.6. Normal phase LC/APCI-MS/MS analysis of multiple regioisomers of deoxy-J_2-IsoPs generated from the nonenzymatic peroxidation of AA *in vitro*. SIM chromatogram of the parent ion at *m/z* 315 (upper) and CID spectrum (lower) obtained by summing scans over the series of chromatographic peaks reveals structurally specific fragmentation of the different deoxy-J_2-IsoP regioisomers.

Due to their nonenzymatic formation, compounds generated from the free radical-catalyzed peroxidation of AA would be predicted to be racemic. Thus, 15-series deoxy-J_2-IsoPs are predicted to comprise a racemic mixture of a compound identical in all respects to COX-derived 15-d-PGJ_2 and its enantiomer, *ent*-15-d-PGJ_2. The racemic mixture is referred to as *rac*-15-d-PGJ_2 (**FIGURE 2.3**). To confirm the formation of *rac*-15-d-PGJ_2 from the nonenzymatic oxidation of AA, we utilized LC/MS/MS analysis employing SRM. The major product ion unique to *rac*-15-d-PGJ_2 is *m/z* 203; thus, we monitored the parent-to-product transition *m/z* 315 to *m/z* 203 to selectively detect *rac*-15-d-PGJ_2. [$^2H_4$]15-d-PGJ_2 (d_4-15-d-PGJ_2) was used as an internal standard for these analyses. The parent ion of d_4-15-d-PGJ_2 is *m/z* 319 due to the presence of four deuterium atoms on the upper side chain of the molecule, and CID analysis of d_4-15-d-PGJ_2 yields the major product ions *m/z* 275 $[M - CO_2]^-$ and *m/z* 203 (data not shown). Shown in **FIGURE 2.7** is a representative chromatogram for the SRM analysis of *rac*-15-d-PGJ_2 generated from the nonenzymatic oxidation of AA, and these compounds co-elute with d_4-15-d-PGJ_2. Additional support for the identification of these compounds as 15-d-PGJ_2 and *ent*-15-d-PGJ_2 was obtained through LC/UV analysis which showed that these compounds possess UV absorbancemaximum, λ_{max} = 306 nm, identical to authentic 15-d-PGJ_2 (data not shown). To assess the total amount of deoxy-J_2-IsoPs formed from the nonenzymatic oxidation of AA, LC/MS quantification was performed by measuring the relative abundance of compounds representing deoxy-J_2-IsoPs with that of the d_4-15-d-PGJ_2 internal standard. The mass spectrometer was operated in SRM mode in the negative ion mode monitoring the parent-to-product transition *m/z* 315 to *m/z* 271 for the deoxy-J_2-IsoPs and monitoring the parent-to-product transition *m/z* 319 to *m/z* 275 for d_4-15-d-PGJ_2. Deoxy-J_2-IsoPs are generated in significant abundance from the nonenzymatic oxidation of AA *in vitro*, and the levels are 16.0 $\pm$ 3.4 ng/mg AA (n=4). In additional studies, when we oxidized a phospholipid containing *sn*-2-AA

(palmitoyl-arachidonyl-phosphatidylcholine, PAPC) *in vitro*, we found that 15-d-PGJ_2 was formed on the intact phospholipid (data not shown).

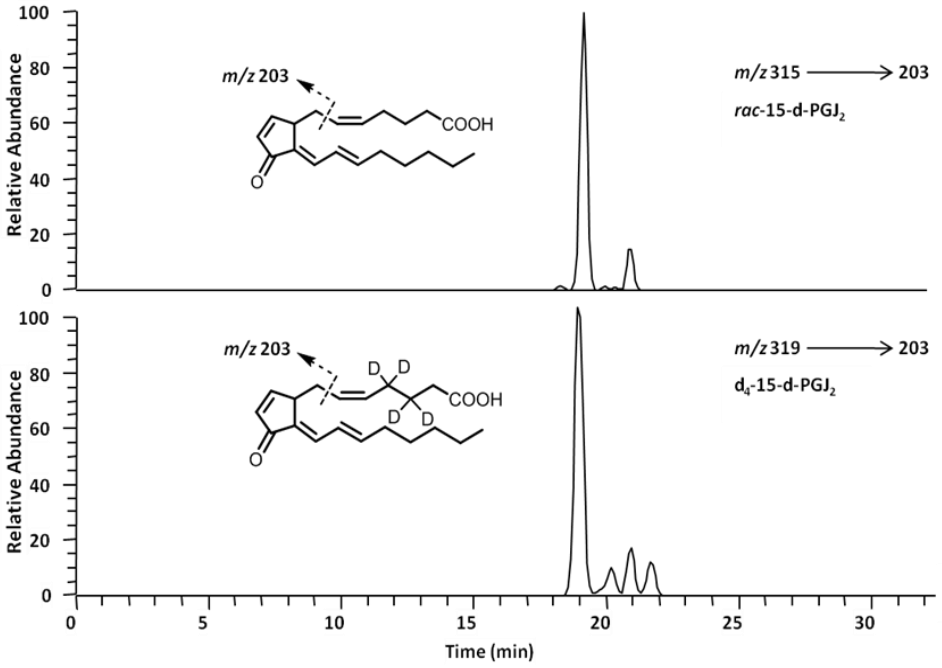


FIGURE 2.7. Analysis of putative rac-15-d-PGJ_2 *in vitro* by LC/MS/MS using SRM. LC/MS/MS analysis of *rac*-15-d-PGJ_2 (A) from the nonenzymatic oxidation of AA *in vitro* and chemically synthesized [$^2H_4$]15-d-PGJ_2 (d_4-15-d-PGJ_2) (B). The mass spectrometer was operated in negative ion mode using SRM to monitor the precursor-to-product transition of *m/z* 315–203 for *rac*-15-d-PGJ_2 (chromatogram A) and the precursor-to-product transition of *m/z* 319–203 for d_4-15-d-PGJ_2 (chromatogram B).

Adduction of deoxy-J_2-IsoPs with glutathione *in vitro*

One of our primary interests in the formation of 15-d-PGJ_2 and cyclopententone deoxy-J_2-IsoPs *in vivo* is related to the fact that these molecules contain electrophilic α,β-unsaturated carbonyl moieties which render them susceptible to Michael addiction with cellular nucleophiles. Because the biological effects of 15-d-PGJ_2 are thought to be largely mediated through its interaction with cellular thiols, all of the deoxy-J_2-IsoPs would be expected to exert similar biological effects through adduction to cellular thiols. Thus, as a further characterization of these compounds, we sought to determine whether deoxy-J_2-IsoPs would adduct glutathione

(GSH), an abundant cellular thiol, in the presence of GST. We have previously shown that 15-d-PGJ_2 and other cyclopentenone eicosanoids readily adduct GSH in the presence of GST *in vitro* (Brunoldi et al. 2007; Hubatsch et al. 2002; Milne et al. 2004; Cox et al. 2002; Atsmon, Sweetman et al. 1990). In the present study, we incubated deoxy-J_2-IsoPs generated from the nonenzymatic oxidation of AA *in vitro* with GSH in the presence of GST. Adducts were partially purified from an incubation mixture by C_{18} Sep-Pak extraction and subjected to LC/MS as described above. Putative deoxy-J_2-IsoP-GSH adducts were definitively identified by LC/ESI-MS/MS. The predicted parent ion for deoxy-J_2-IsoP-GSH adducts in negative ion mode is *m/z* 622. SIM analysis showed multiple peaks with this *m/z* eluting from 10.7 to 15.3 min, presumably representing the GSH adducts of different deoxy-J_2-IsoP regioisomers (**FIGURE 2.8A**). These adducts elute at a similar retention time as the GSH adduct of 15-d-PGJ_2. CID experiments confirmed the molecules at *m/z* 622 to be deoxy-J_2-IsoP-GSH adducts. A composite CID spectrum was obtained by summing scans over these peaks and is shown in **FIGURE 2.8B**. The parent ion M^- is present at *m/z* 622. Relevant product ions include *m/z* 306 [M – deoxy-J_2-IsoP]$^-$ (loss of the prostanoid portion of the molecule), *m/z* 272 [M – 350]$^-$ (retro-Michael fragmentation) and *m/z* 254 [272 – H_2O]$^-$. A similar CID spectrum was observed when chemically synthesized 15-d-PGJ_2 was incubated with GST and GSH (**FIGURE 2.8C**). It should be noted that the major product ions generated from CID analysis of deoxy-J_2-IsoP-GSH adducts are indistinguishable from those generated from the CID analysis of the synthetic 15-d-PGJ_2-GSH adduct. Together, these data confirm that the molecules putatively identified as cyclopentenone deoxy-J_2-IsoPs are reactive and capable of undergoing nucleophilic addition reactions with GSH.

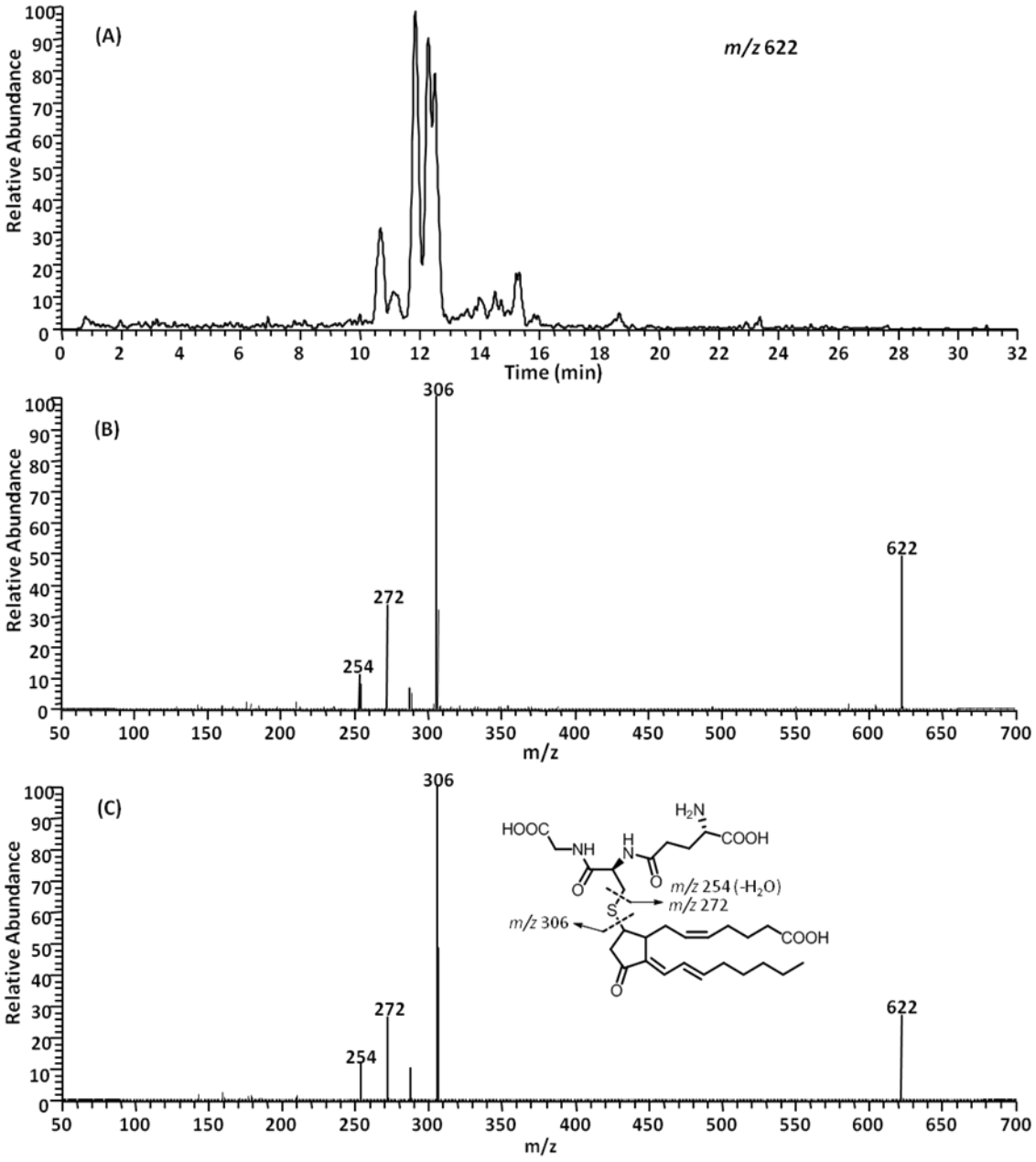


FIGURE 2.8. Analysis of GSH adducts of deoxy-J_2-IsoPs and 15-d-PGJ_2 by LC/ESI-MS/MS. (A) SIM chromatogram of putative deoxy-J_2-IsoP-GSH adducts with the precursor ion *m/z* 622. (B) Composite CID spectrum obtained by summing scans over the series of chromatographic peaks representing deoxy-J_2-IsoP-GSH adducts. (C) CID spectrum of the synthesized 15-d-PGJ_2-GSH adduct.

Isolation and quantification of deoxy-J_2-IsoPs from rat liver

After confirming the formation of *rac*-15-d-PGJ_2 and deoxy-J_2-IsoPs via the nonenzymatic oxidation of arachidonic acid *in vitro*, we then explored whether these compounds are formed *in vivo.* IsoPs are initially formed *in situ* esterified in phospholipids and hydrolyzed to their free form by platelet activating factor acetylhydrolase and possibly other phospholipases (Stafforini et al. 2006). To quantify F_2-IsoPs esterified in tissues, the tissue is initially treated with base to release F_2-IsoPs, which are then quantified. However, base treatment will induce dehydration of D_2-IsoPs to form J_2-IsoPs. Therefore, to quantify esterified deoxy-J_2-IsoPs formed in tissue, the tissue is subjected to enzymatic hydrolysis using bee venom phospholipase A_2 (PLA_2). We found that this method of hydrolysis results in <0.3% dehydration of PGD_2 to 15-d-PGJ_2. We have previously shown that treatment of rats with CCl_4 induces a dramatic increase in esterified IsoPs in the liver (Morrow et al. 1994; Chen et al. 1999). Therefore, we examined whether deoxy-J_2-IsoPs are formed *in vivo* esterified in liver phospholipids in rats that had been treated with CCl_4. Shown in **FIGURE 2.9** is a representative chromatogram from the SRM analysis of deoxy-J_2-IsoPs (**FIGURE 2.9A**), including the *rac*-15-d-PGJ_2 (**FIGURE 2.9B**), generated *in vivo* in liver phospholipids in the rat after CCl_4 treatment. Again, the entire series of deoxy-J_2-IsoPs was quantified by LC/ESI-MS employing SRM as described previously. Levels of deoxy-J_2-IsoPs *in vivo* esterified in liver phospholipids in control rats were near the limit of detection (0.55 $\pm$ 0.21 ng/g tissue, n=5) for this method. However, in animals treated with CCl_4, levels increased by ~12-fold to 6.4 $\pm$ 1.1 ng/g tissue (n=5, $p<0.0001$ compared to control). Additionally, we have identified PC containing esterified 15-d-PGJ_2 (15-d-PGJ_2-PC) generated *in vivo* in rats after oxidant insult (data not shown). These findings provide evidence that deoxy-J_2-IsoPs are produced in significant abundance *in vivo* as products of free radical-induced lipid peroxidation.

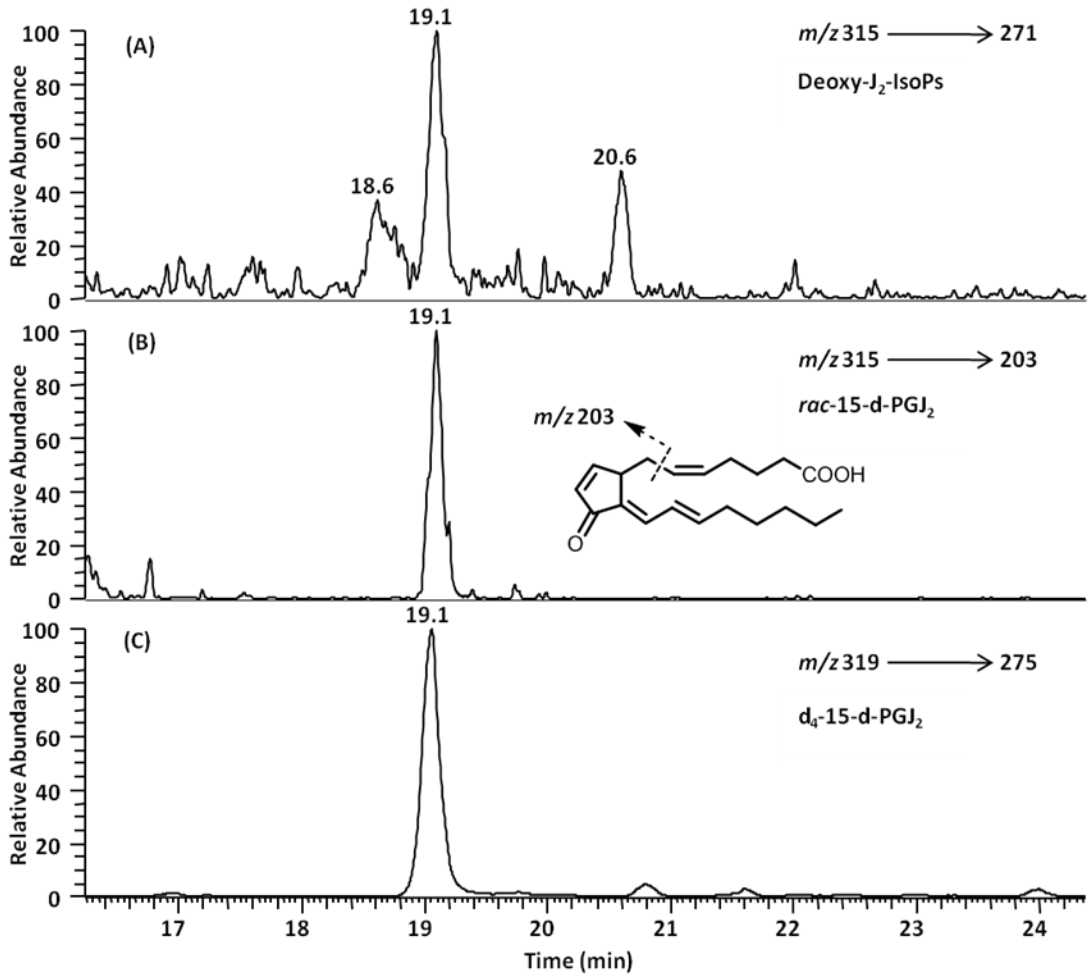


FIGURE 2.9. Analysis of putative deoxy-J_2-IsoPs *in vivo* by LC/MS/MS using SRM. LC/MS/MS analysis of (A) deoxy-J_2-IsoPs regioisomers and (B) 15-series deoxy-J_2-IsoPs (*rac*-15-d-PGJ_2) generated *in vivo* esterified in liver phospholipids in rats after treatment with CCl_4; (C) chemically synthesized [$^2H_4$]15-d-PGJ_2 (d_4-15-d-PGJ_2) was used as the internal standard. The mass spectrometer was operated in the negative ion mode with SRM for the precursor-to-product transitions of *m/z* 315–271 for all deoxy-J_2-IsoPs regioisomers (chromatogram A), *m/z* 315–203 for *rac* -15-d-PGJ_2 (chromatogram B), and *m/z* 319–275 for d_4-15-d-PGJ_2 (chromatogram C).

Discussion

We report the finding that a series of 15-d-PGJ_2-like compounds, termed deoxy-J_2-IsoPs, are formed *in vitro* and *in vivo* via the free radical-catalyzed peroxidation of arachidonic acid. Among the deoxy-J_2 products formed during this process, the 15-series deoxy-J_2-IsoPs (*rac*-15-d-PGJ_2) comprise a racemic mixture of a molecule identical in all respects to 15-d-PGJ_2 and its corresponding enantiomer. The formation of 15-d-PGJ_2 and 15-d-PGJ_2-like compounds *in vivo* is biologically relevant because 15-d-PGJ_2 is thought to be an important lipid mediator. Despite significant interest in the bioactivity of 15-d-PGJ_2, the extent to which this compound is formed *in vivo* and the mechanisms that regulate its formation have been unclear. In the present study, we have shown, for the first time, the formation of 15-d-PGJ_2 and 15-d-PGJ_2-like compounds *in vivo* via free radical-catalyzed lipid peroxidation, independent of the COX pathway. Our results suggest that a second pathway exists for the biosynthesis of 15-d-PGJ_2 *in vivo*, and these findings provide further insight into the biochemical mechanisms that can contribute to the endogenous generation of reactive lipid mediators.

15-d-PGJ_2 was originally identified as a dehydration product of COX-derived PGD_2 (Fitzpatrick and Wynalda 1983). Our initial interest in determining whether 15-d-PGJ_2 and 15-d-PGJ_2-like compounds are formed via the free radical-catalyzed pathway emerged from the observation that cyclopentenone PGA_2- and PGJ_2-like compounds (A_2/J_2-IsoPs) are generated *in vivo* in significant quantities esterified in liver phospholipids in rats following oxidant insult (Chen et al. 1999). In addition, recent studies by our lab support the contention that PGs can be generated through the IsoP pathway, independent of the COX enzymes (Gao et al. 2003; Yin et al. 2007). Gao et al. reported the formation of PGE_2 and PGD_2 and their respective enantiomers *in vitro* and *in vivo* through the nonenzymatic peroxidation of AA by epimerization of E_2/D_2-IsoPs, specifically 15-E_{2t}-IsoP and 15-D_{2c}-IsoP, respectively (Gao et al. 2003). More recently Yin

et al. reported the formation of $PGF_{2\alpha}$ *in vivo* via the IsoP pathway, and demonstrated that the vast majority of putative $PGF_{2\alpha}$ in human urine is derived from the free radical-initiated peroxidation of arachidonic acid, independent of COX (Yin et al. 2007). In the present study, we have found that J_2-IsoPs derived from D_2-IsoPs can also undergo further dehydration and isomerization *in vivo* to yield the cyclopentenone deoxy-J_2-IsoPs. Dehydration of the 15-series J_2-IsoPs yields a compound identical in all respects to COX-derived 15-d-PGJ_2 and its enantiomer.

Cyclopentenone eicosanoids as a class have received considerable attention over the past three decades due their reactivity (Straus and Glass 2001; Milne, Musiek et al. 2005). These compounds are characterized by the presence of an electrophilic α,β-unsaturated carbonyl moiety in the prostane ring. This functional group renders them susceptible to nucleophilic addition reactions with thiol-containing biomolecules, which has been attributed to their biological effects. 15-d-PGJ_2, the most extensively studied of the cyclopentenone eicosanoids, is particularly reactive due to the presence of two electrophilic carbons. The presence of two electrophilic centers in 15-d-PGJ_2 is thought to contribute to its increased reactivity and biological potency compared to other cyclopentenone eicosanoids (Brechbuhl et al. 2009). The finding that, in addition to 15-d-PGJ_2, a series of deoxy-J_2-IsoPs are formed in significant abundance *in vivo* is notable due to the fact that these compounds also possess the reactive electrophilic carbon centers that render them susceptible to reaction with cellular nucleophiles. Like other IsoPs generated via the nonenzymatic oxidation of AA, deoxy-J_2-IsoPs are formed as a series of four regioisomers, each consisting of a mixture of stereoisomers. Deoxy-J_2-IsoPs are derived from the dehydration and isomerization of the different D_2-IsoP and J_2-IsoP regioisomers, including the 5-, 8-, 12-, and 15-series. Of note, the 15-series deoxy-J_2-IsoPs comprise a racemic mixture of 15-d-PGJ_2 and its enantiomer, and are termed *rac*-15-d-PGJ_2. Consistent with the presence of the polyunsaturated carbonyl moiety in each of the deoxy-J_2-

IsoPs, we have shown that these compounds are reactive and readily undergo nucleophilic addition with GSH. Because this electrophilic reactivity is often likely to be responsible for the bioactivity of 15-d-PGJ_2, all of the deoxy-J_2-IsoPs are expected to have similar bioactivities related to covalent interaction with intracellular proteins. In this regard, the deoxy-J_2-IsoPs represent a new class of lipid peroxidation products that are predicted to have more potent bioactivity than the previously identified cyclopentenone (A_2/J_2) IsoPs due to the presence of two reactive electrophilic carbons. These compounds may be important lipid mediators involved in the pathophysiology of oxidant stress.

The extent to which 15-d-$PGJ_2$and other deoxy-J_2-IsoPs are produced *in vivo* is biologically relevant because 15-d-PGJ_2 has been shown to mediate a wide range of biological activities, including modulating inflammatory and apoptotic processes (Bishop-Bailey and Hla 1999; Levonen et al. 2001; Clay et al. 2002; Hortelano et al. 2000; Rossi et al. 2000; Straus et al. 2000; Perez-Sala et al. 2002). Unlike other PGs, which elicit their biological responses by binding specific G-protein coupled receptors (GPCRs), 15-d-PGJ_2 exerts its biological effects through interaction with key intracellular targets. Some of its activity may relate to the observation that 15-d-PGJ_2 activates the nuclear receptor PPAR-γ (Forman et al. 1995; Kliewer et al. 1995). Activation of PPAR-γ by 15-d-PGJ_2 has been recently shown to occur through covalent binding of 15-d-PGJ_2 to a critical cysteine residue in the PPARγ ligand binding pocket (Shiraki et al. 2005). Additionally, 15-d-PGJ_2 has been shown to inhibit the inflammatory response in a number of cell types, including monocytes and macrophages, by blocking NF-κB-dependent gene expression via covalent modification of a critical cysteine residue in IκB kinase and the DNA-binding domains of NF-κB subunits (Hortelano et al. 2000; Rossi et al. 2000; Straus et al. 2000; Perez-Sala et al. 2002). 15-d-PGJ_2 is also thought to be involved in the resolution of inflammation by enhancing apoptosis in activated macrophages through a p38 mitogen activated protein kinase (MAPK)-

dependent increase in reactive oxygen species formation (Hortelano et al. 2000). Further, 15-d-PGJ_2 activates the antioxidant response pathway via covalent modification of reactive cysteine residues in Keap 1, leading to Nrf2 activation and induction of antioxidant proteins, including HO-1, peroxiredoxin 1, γ-GCL, and heat shock protein 70 (Dinkova-Kostova et al. 2002; Itoh et al. 2004; Kim et al. 2004; Chen et al. 2006; Zhang et al. 2004). Finally, 15-d-PGJ_2 has been reported to inhibit proliferation of vascular smooth muscle cells by inducing cell cycle arrest (Miwa et al. 2000; Wakino et al. 2000).

The finding that 15-d-PGJ_2 and other deoxy-J_2-IsoPs are generated *in vivo* via free radical-induced lipid peroxidation has important implications for the pathophysiological role of these compounds. Generation of 15-d-PGJ_2 via the COX pathway has been associated with settings of inflammation (Shibata et al. 2002; Gilroy et al. 1999; Rajakariar et al. 2007). In the present study, we provide evidence that 15-d-PGJ_2 and 15-d-PGJ_2-like compounds, the deoxy-J_2-IsoPs, are generated *in vivo* under conditions of oxidant stress. As a result of being generated through the COX and free radical-catalyzed pathways, these compounds may have additive effects in the pathophysiology of diseases, such as atherosclerosis, in which inflammation and oxidant stress are considered to play a critical role (Berliner et al. 1997; Heineke 1998; Chisholm and Steinberg 2000). Previous studies have shown that 15-d-PGJ_2 is synthesized during mammalian inflammatory responses, and its production may represent a feedback mechanism for the resolution of inflammation (Shibata et al. 2002; Gilroy et al. 1999; Rajakariar et al. 2007). 15-d-PGJ_2 has in fact been reported to be present in significant amounts in human atherosclerotic lesions and to reside in foam cells (Shibata et al. 2002). 15-d-PGJ_2 has also been detected in the inflammatory exudates from carrageenan-induced pleurisy in rats using enzyme immunoassay (Gilroy et al. 1999), and it was detected by LC/MS in the cell-free inflammatory exudates of mice during resolving peritonitis (Rajakariar et al. 2007). Additionally, stimulation of

selenium supplemented murine macrophages with LPS was shown to result in at time-dependent increase in 15-d-PGJ_2 formation quantified in cell lysates by enzyme immunoassay (Vunta et al. 2007). This suggests that 15-d-$PGJ_2$and deoxy-J_2-IsoPs may be produced *in vivo* in settings of inflammation in which COX activity is increased as well as under conditions of oxidant stress.

Further support for the free radical-catalyzed generation of 15-d-PGJ_2 and other deoxy-J_2-IsoPs *in vivo*, independent of COX enzymes, was provided by the finding that these compounds are formed *in situ* esterified in membrane phospholipids in rat liver after induction of oxidant injury by CCl_4. Hydrolysis of deoxy-J_2-IsoPs by bee venom PLA_2 permitted detection and quantification of these compounds by LC/MS/MS. Free 15-d-PGJ_2, which would be generated through the COX pathway, was not detectable in the liver of control rats and rats treated with CCl_4. This may be due to the rapid reactivity of 15-d-PGJ_2. Previous studies have indicated that cyclopentenone eicosanoids rapidly undergo conjugation with GSH *in vitro* and *in vivo* (Brunoldi et al. 2007; Hubatsch et al. 2002; Milne et al. 2004; Cox et al. 2002; Atsmon, Sweetman et al. 1990). Brunoldi et al.reported that HepG2 cells convert 15-d-PGJ_2 to a GSH conjugate in which the carbonyl on the prostane ring is reduced to a hydroxyl, and the glutathione portion of the molecule is subsequently hydrolyzed to a cysteine conjugate (Brunoldi et al. 2007). Additionally, Milne et al. showed that the cyclopentone eicosanoid 15-A_{2t}-IsoP is primarily metabolized *in vivo* by conjugation with GSH, and the compound is excreted into the urine as the *N*-acetyl cysteine sulfoxide conjugate of 15-A_{2t}-IsoP in which the carbonyl on the prostane ring of the IsoP is reduced to a hydroxyl (Milne, Gao et al. 2005). Preliminary studies suggest that, like other cyclopentenone eicosanoids, 15-d-PGJ_2 is rapidly metabolized *in vivo* via conjugation with GSH and excreted into the urine as a metabolized GSH adduct (data not shown). Further studies are required to identify the major urinary metabolite of 15-d-$PGJ_2$to

develop a biomarker of its systemic production in humans. These studies are critical to determine to what extent 15-d-PGJ_2 is generated *in vivo* in humans and elucidate the mechanisms that regulate its production.

In summary, we have reported the formation of the cyclopentenone eicosanoids 15-d-PGJ_2 and deoxy-J_2-IsoPs *in vivo* via free radical-catalyzed lipid peroxidation, independent of COX. Deoxy-J_2-IsoPs represent a new class of reactive lipid peroxdation products that may exert unique biological actions relevant to the pathobiology of oxidant stress. This study provides the rational basis to examine the extent to which deoxy-J_2-IsoPs are generated *in vivo* in humans. Additionally, future studies aimed at exploring the biological activities of deoxy-J_2-IsoPs will likely yield insights into the pathophysiological consequences of their formation in settings of oxidant stress.

CHAPTER III

METABOLIC FATE OF 15-DEOXY-$\Delta^{12,14}$-PROSTAGLANDIN J_2 *IN VIVO*: IDENTIFICATION OF THE MAJOR URINARY METABOLITE IN THE RAT

Introduction

15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-d-PGJ_2) is a reactive cyclopentenone prostaglandin (CyPG) formed from the dehydration of prostaglandin D_2 (PGD_2). This compound is regarded as an important lipid mediator of a broad spectrum of biological effects (Fitzpatrick and Wynalda 1983; Straus and Glass 2001; Kim and Surh 2006; Uchida and Shibata 2008). Synthesis of 15-d-PGJ_2 initially depends upon the enzymatic machinery for PGD_2 generation. Cyclooxygenases (COXs) catalyze the enzymatic oxygenation of arachidonic acid to the endoperoxide intermediate PGH_2, and tissue specific PGD synthases convert PGH_2 to PGD_2. PGD_2 is the major COX product in immune cells, such as mast cells, T lymphocytes, and macrophages (Lewis et al. 1982; Funk 2001). PGD_2 is unstable and can undergo spontaneous dehydration and isomerization to yield a series of cyclopentenone J_2 PGs. Dehydration of PGD_2 yields PGJ_2, followed by isomerization to Δ^{12}-PGJ_2, and subsequent dehydration yields 15-d-PGJ_2 (Fukushima et al. 1982; Fitzpatrick and Wynalda 1983).

The reactivity of 15-d-PGJ_2 contributes importantly to its biological activity. The cyclopentenone ring of 15-d-PGJ_2 contains an electrophilic α,β-unsaturated carbonyl moiety, which is susceptible to Michael addition reactions with thiol-containing bio-molecules, such as glutathione (GSH) and cysteine residues of proteins. In addition, 15-d-PGJ_2 contains a second reactive center at carbon C-13 of its lower side chain that can adduct cellular thiols. Due to its reactivity, 15-d-PGJ_2 has been shown to modulate protein function and exert potent anti-inflammatory, anti-proliferative, and pro-apoptotic activity in a variety of cell types (Straus and

Glass 2001; Kim and Surh 2006; Uchida and Shibata 2008). 15-d-PGJ_2 was found to be a ligand for the nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ) (Forman et al. 1995; Kliewer et al. 1995), and some of its biological effects are mediated, at least in part, through activation of PPARγ. However, 15-d-PGJ_2 can also induce a number of biological responses independent of PPARγ through direct covalent modification of key intracellular protein targets (Straus and Glass 2001). In this regard, 15-d-PGJ_2 has been shown to exert potent anti-inflammatory effects by inhibiting nuclear factor κB (NF-κB)-dependent pro-inflammatory gene expression via covalent modification of a critical cysteine residue in IκB kinase β (IKKβ) and the DNA-binding domains of NF-κB subunits (Straus et al. 2000; Rossi et al. 2000; Castrillo et al. 2000; Cernuda-Morollon et al. 2001). 15-d-PGJ_2 has also been show to inhibit cell growth and promote apoptosis through a number of mechanisms, including modification of p53 (Kondo et al. 2002), inhibition of cell cycle progression (Qin et al. 2003; Kamagata et al. 2007), and induction of intracellular oxidative stress (Kondo et al. 2001; Hortelano et al. 2000).

Although 15-d-PGJ_2 has been shown to mediate a number of cellular processes in studies performed *in vitro*, there is little evidence that this compound is formed *in vivo*. Immunological approaches to measure 15-d-PGJ_2 *in vivo* have been under scrutiny due to their lack of specificity and potential for cross-reactivity. Very low levels of free 15-d-PGJ_2 have been detected in human urine, and the amounts found are likely insufficient to produce the biological effects ascribed to 15-d-PGJ_2 (Bell-Parikh et al. 2003). However, due to reactivity of 15-d-PGJ_2 with cellular proteins and peptides, it is possible that measuring free 15-d-PGJ_2 in biological fluids may lead to an underestimation of the total amount produced *in vivo.* Further, measurement of urinary metabolites by mass spectrometry has been established as the most accurate and sensitive method to assess the systemic production of prostaglandins and other

eicosanoids (Samuelsson et al. 1975). At the present time, no such methodology has been developed for the quantification of 15-d-PGJ_2.

Previous studies have shown that cyclopentenone eicosanoids are predominately metabolized via conjugation with GSH *in vitro* and *in vivo*. HCA-7 human colorectal cancer cells convert PGJ_2 to two primary metabolites: the PGJ_2-gluathione conjugate (PGJ_2-SG) and the PGJ_2-SG in which the C-11 carbonyl was reduced to an alcohol (COX et al. 2002). Incubation of Δ^{12}-PGJ_2 with Chinese hamster ovary (CHO) cells or hepatoma tissue culture (HTC) cells resulted in its conversion to a GSH conjugate in which the carbonyl at C-11 was reduced to the alcohol and the Δ^{12} double bond were reduced; subsequent metabolism yielded the cysteinyl-glycine and cysteine metabolites (Atsmon, Freeman et al. 1990). In addition, Milne et al. demonstrated that the cyclopentenone isoprostane (IsoP) 15-A_{2t}-IsoP is efficiently metabolized in the human hepatoma cell line HepG2 via GSH conjugation (Milne et al. 2004). Further, the major urinary metabolite of 15-A_{2t}-IsoP in rats was identified as a metabolized GSH adduct of the compound, the *N*-acetylcysteine sulfoxide conjugate of 15-A_{2t}-IsoP, in which the carbonyl at C-9 was reduced to an alcohol (Milne, Gao et al. 2005).

The metabolism of 15-d-PGJ_2 has also been examined in cultured cells. Incubation of 15-d-PGJ_2 in MCF7 breast cancer cells resulted in formation of the glutathione conjugate of 15-d-PGJ_2 (Paumi et al. 2003). More recently, Brunoldi et al. showed that 15-d-PGJ_2 undergoes rapid conjugation with GSH in HepG2 cells followed by reduction of the carbonyl at C-11 to the alcohol (Brunoldi et al. 2007). Subsequently, the glutathione portion of the molecule was metabolized by hydrolysis of glutamate and glycine to yield the cysteine conjugate (Brunoldi et al. 2007). In addition, Yu et al. demonstrated that recombinant alkenal/one oxidoreductase (Aor) catalyzed the reduction of the C-12,C-13 double bond of 15-d-PGJ_2 to yield 12,13-dihydro-15-d-PGJ_2 (Yu et al. 2006). Little is known about the metabolism of 15-d-PGJ_2 *in vivo*.

In the present study, we have examined the metabolite fate of 15-d-PGJ_2 in the rat to gain further insight into the metabolic disposition of this compound *in vivo*. In addition, we sought to identify the major urinary metabolite of 15-d-PGJ_2 excreted in rats that may be used as a potential biomarker to assess the endogenous production of 15-d-PGJ_2.

Materials and Methods

Materials

Chemicals and enzymes were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. 15-d-PGJ_2 and PGD_2 were purchased from Cayman Chemicals (Ann Arbor, MI). [$^3H_7$]PGD_2 was purchased from Perkin-Elemer Life Sciences, Inc. (Boston, MA). C_{18} Sep-Paks were purchased from Waters Corporation (Milford, MA). All solvents were of HPLC quality and purchased from EM Science (Gibbstown, NJ).

Preparation of [$^3H_6$]15-d-PGJ_2

[$^3H_6$]15-d-PGJ_2 was prepared as described previously (Brunoldi et al. 2007). Briefly, [$^3H_7$]PGD_2 (2 μCi) and PGD_2 (200 μg) was dissolved in 1N HCl (0.5 ml) at 37°C. After 5 h, 50 mM aqueous ammonium acetate (10 ml, pH 3.4) was added, and the resulting solution was extracted with ethyl acetate (5 x 1 ml). The organic layers were collected, and the solvent was evaporated under a stream of N_2. The resulting residue was purified by high-performance liquid chromatography (HPLC) using a Phenomenex silica column (250 mm x 4.6 mm, 5 μm) and a mobile phase of hexane isopropyl alcohol/acetic acid, 97:3:0.1 at a flow rate of 1 ml/min. 15-d-PGJ_2 was detected by UV spectroscopy (λ = 307 nm). [$^3H_6$]15-d-PGJ_2 and unlabeled 15-d-PGJ_2

were eluted together at 10 min. (The desired product co-eluted with commercially available 15-d-PGJ_2). The yield was 5-10%.

Infusion of 15-d-PGJ_2 and [$^3H_6$]15-d-PGJ_2 into a rat

Femoral vein cannulated male Sprague-Dawley rats were purchased from Charles Rivers. The procedure used for the infusion of 15-d-PGJ_2 into a rat was similar to that previously described (Milne, Gao et al. 2005). Briefly, 1 mg of 15-d-PGJ_2 combined with 1 μCi of [$^3H_6$]15-d-PGJ_2 was dissolved in 200 μl of ethanol and then suspended in 200 μl of normal sterile saline. The suspension was then infused into the femoral vein catheter of a 230-g Sprague-Dawley male rat, followed by flushing with heparinized saline (1:50) over 1 min. After the infusion, the animal was place in a metabolic cage, and urine was collected in aliquots over a 24-h period. The radiolabeled material was used to follow the excretion of metabolites, and radioactivity was assessed by scintillation counting. Approximately 62% of the total radioactivity was excreted into the urine, and 75% of the radioactivity in the urine was excreted 0 to 5 h post-infusion. These aliquots were combined together for analysis by high-performance liquid chromatography/mass spectrometry (HPLC/MS). To gain an understanding of the chemical characteristics of the metabolite(s) generated, small aliquots (50 μl) of urine were taken for extraction with organic solvents, methylene chloride and ethyl acetate.

Feces were collected separately over a 24-h period. Following collection of the final urine aliquot at 24 h, the animal was anesthetized with pentabarbitol (60 mg/kg) and sacrificed. The major organs were harvested, frozen immediately in liquid nitrogen, and then stored at -80^oC until further analysis. All of the procedures were performed in accordance with Vanderbilt University Animal Care Committee guidelines.

Preparation of organs and feces for scintillation counting

The procedure for the preparation of organs and feces for scintillation counting was performed similar to that previously described (Milne, Gao et al. 2005). For each organ harvested the total tissue was weighed, and a small portion was cut off, placed into a scintillation vial, and weighed. Soluene-350 (2ml) (Packard Biosciences B.V.), a tissue solubilizer containing 0.5 M ammonium hydroxide in toluene and methanol, was added to each vial. The samples were incubated at 50^{o}C for 18 h until the tissue samples were completely dissolved. To remove the color from the solution, 30% hydrogen peroxide (400 μl over 1 h) was added slowly to each sample. Each sample was cooled to room temperature, and 10 ml of scintillation fluid (Bio-Safe, Research Products International Corp., Mount Prospect, IL) was mixed with each sample. Samples were counted after adapting to light for 1 h. This procedure was repeated three times for each tissue (Milne, Gao et al. 2005).

For the feces, after drying under ambient air, a small portion was placed in a scintillation vial and 0.5 ml of bleach (sodium hypochlorite solution) was added. The sample was incubated at 50^{o}C for 1 h with occasional swirling. The sample was cooled, and excess chlorine gas was removed by running a stream of nitrogen gas over the sample for 5 min. Scintillation fluid (10 ml) was then mixed with the sample, and the radioactivity was counted after the sample adapted to light for 1 h (Milne, Gao et al. 2005).

Extraction and HPLC/MS analysis of 15-d-PGJ$_2$ metabolites

The procedure for the extraction and HPLC/MS analysis of 15-d-PGJ$_2$ metabolites was performed similar to that previously described (Milne et al. 2005). Briefly, aliquots of urine (0.5-2 ml) were diluted to a total volume of 10 ml with distilled deionized water then acidified to pH 3 with 1 N HCl. The samples were then loaded onto a C_{18} Sep-Pak that had been precondition by

rinsing with acetonitrile (10 ml) and 50 mM aqueous ammonium acetate, pH 3.4 (10 ml). Subsequently, the sample was rinsed with 10 ml each of ammonium acetate and heptane and eluted with 10 ml of 95% ethanol. The ethanol was removed by evaporation under a stream of nitrogen gas, and samples were stored in 100 μl of ethanol at -80^{o}C until LC/MS analysis.

The resulting sample extract was subjected to on-line LC using the ThermoFinnigan Surveryor MS Pump 2.0 equipped with a Phenomenex Luna C_{18} column (50×200 mm, 3 μm particle size) and employing a linear gradient (mobile phase A: water/mobile phase B/acetic acid (95:5:0.1, v/v/v); mobile phase B: acetonitrile/methanol/acetic acid (95:5:0.1, v/v/v)) with a flow rate of 300 μl/min. The sample was dissolved in a 3:1 mixture of mobile phase/mobile phase B for analysis, and the following gradient conditions were used: isocratic gradient of 20% phase B for 1 min followed by a linear gradient of 20 to 70% phase B for 26 min; the total run time was 32 min. A ThermoFinnigan TSQ Quantum mass spectrometer was used for sample analysis. To monitor the elution of radiolabeled metabolites, we employed a post-column splitter and approximately 75% of the sample was directed to a fraction collector. Fractions were collected every 0.5min, and the radioactivity of each fraction was determined by scintillation counting. The remaining portion of the sample was directed to the mass spectrometer for electrospray ionization (ESI)-MS analysis. The ESI source was fitted with a deactivated fused silica capillary. Nitrogen was used as both the sheath and the auxillary gas. The mass spectrometer was operated in the full scan mode monitoring *m/z* 200-1000 in the negative ion mode to identify the most abundant precursor ion *m/z* corresponding to the elution of radiolabeled metabolites. Tandem MS experiments were subsequently performed for the structural elucidation of metabolites. For this analysis, compounds were collisionally activated between the energies of -18 and -27 eV under 1.5 millitorrs of argon. Data acquisition and analysis will performed using Xcalibur software, version 2.0.

UPLC/MS analysis of 15-d-PGJ_2 metabolites in human urine

To determine if the major urinary metabolite of 15-d-PGJ_2 formed in rats was also present in humans, urine was obtained from a healthy male human volunteer 1-2 h following administration of 500 mg niacin. 10 ml of urine was acidified to pH 3 and extracted by C_{18} Sep-Pak as described above. The sample was eluted with 95% ethanol. The resulting sample extract was subjected reversed phase ultra-performance-LC/MS (RP-UPLC/MS) analysis using the ThermoFinnigan Surveryor MS Pump 2.0 equipped with an Acquity UPLC HSS T3 column (1.0 X 100 mm, 1.8 μm) and employing a linear gradient (mobile phase A_2: water/acetic acid (99.8:0.2, v/v/v); mobile phase B: acetonitrile/methanol/acetic acid (95:5:0.1, v/v/v) with a flow rate of 200 μl/min. The sample was dissolved in a 5:1 mixture of mobile phase A_2/mobile phase B for analysis, and the following gradient conditions were used: isocratic gradient of 100% mobile phase A_2 for 2 min followed by a linear gradient of 0 to 70% mobile phase B for 6 min; the total run time was 15 min. A ThermoFinnigan TSQ/Quantum Ultra mass spectrometer was used for sample analysis. The mass spectrometer was operated in the selected reaction monitoring (SRM) mode in the negative ion mode monitoring the transition of the metabolite precursor ion *m/z* 690 to the specific product ions *m/z* 514, *m/z* 175, and *m/z* 113. Data acquisition and analysis will performed using Xcalibur software, version 2.0.

Results

Infusion of 15-d-PGJ_2

15-d-PGJ_2 containing a small amount of [$^3H_6$]15-d-PGJ_2 was infused into a rat via a femoral vein catheter over the course of 1 min. The rat of was then placed in a metabolic cage, and urine was collected in aliquots up to 24 h post-infusion. 62% of the total radioactivity

infused was recovered in the urine during that time period, and 75% of the total radioactivity in the urine was excreted in the first 5 h. The remained of the radioactivity infused into the animal was recovered in the feces and tissues. The feces excreted during the 24-h period after the infusion was collected, and the major organs were harvested after the animal was sacrificed. The tissue samples and feces were solubilized as described above, and the radioactivity was measured. **FIGURE 3.1** shows the distribution of [$^3H_6$]15-d-PGJ$_2$ in the rat. After 24 h, approximately 34% of the [$^3H_6$]15-d-PGJ$_2$ infused into the rat remained in the tissues, predominately the liver, most likely bound to proteins. Approximately 3% of the [$^3H_6$]15-d-PGJ$_2$ infused was excreted in the feces.

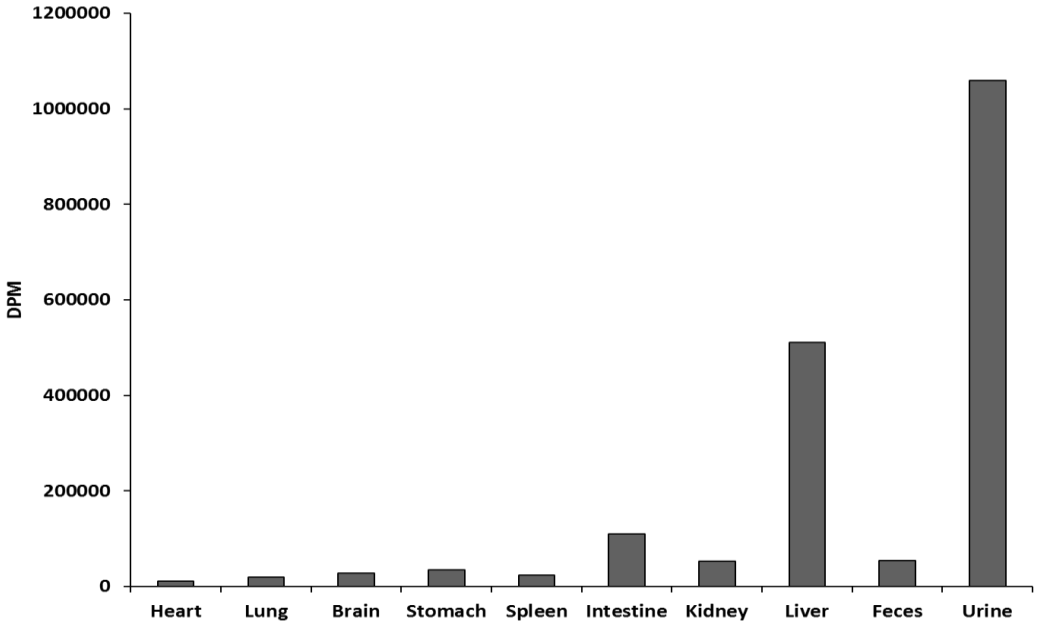


FIGURE 3.1. Distribution of [$^3H_6$]15-d-PGJ$_2$ in major organs after infusion into a rat. The values shown represent the mean radioactivity measurement in each tissue.

Extraction of 15-d-PGJ$_2$ metabolites

To gain a better understanding of the chemical characteristics of the 15-d-PGJ$_2$ urinary metabolites, small aliquots of urine were acidified to pH 3 and extracted with organic solvents. Following extraction with either methylene chloride or ethyl acetate, approximately 60% of the

radioactivity remained in the aqueous solution, suggesting the formation of a polar conjugate(s). For the purification of the conjugate(s), separate aliquots of urine (0.5-2.0 ml) were then extracted using a C_{18} Sep-Pak as previously described (Milne et al. 2004; Milne, Gao et al. 2005). 90% of the radioactivity applied to the Sep-Pak cartridge was eluted in 95% ethanol after washing with 50 mM ammonium acetate, pH 3.4, and heptane.

HPLC/MS analysis of 15-d-PGJ_2 metabolites

The eluate from the Sep-Pak was analyzed by reversed-phase HPLC/ESI-MS using the condition described in "Materials and Methods." Initially, the mass spectrometer was operated in the full scan mode monitoring *m/z* 200-1000, and analysis was performed in the negative ion mode. For these experiments, a flow-splitter was employed post-column and approximately 75% of the sample was directed to a fraction collector. Fractions were collected every 0.5 min during the course of the run to monitor the elution of radiolabeled metabolites. The RP-HPLC radiochromatogram of 15-d-PGJ_2 metabolites excreted in rat urine is shown in **FIGURE 3.2**. Metabolites are labeled according to their RP-HPLC retention time. The majority of the radioactivity eluted in a peak between 1-1.5 min. The most abundant *m/z* found in the full-scan spectrum of this fraction was *m/z* 690 in negative ion mode. Other abundant peaks were observed at 3.5-4.5 min corresponding to *m/z* 498, *m/z* 496 and at 12.5-13.5 min corresponding to *m/z* 478, *m/z* 480. To determine the structures of these metabolites, tandem MS experiments were performed utilizing collision induced dissociation (CID) analysis in negative ion mode. A summary of the urinary metabolites of 15-d-PGJ_2 identified in the rat is reported in **TABLE 3.1**. The structure elucidation for each metabolite is described below.

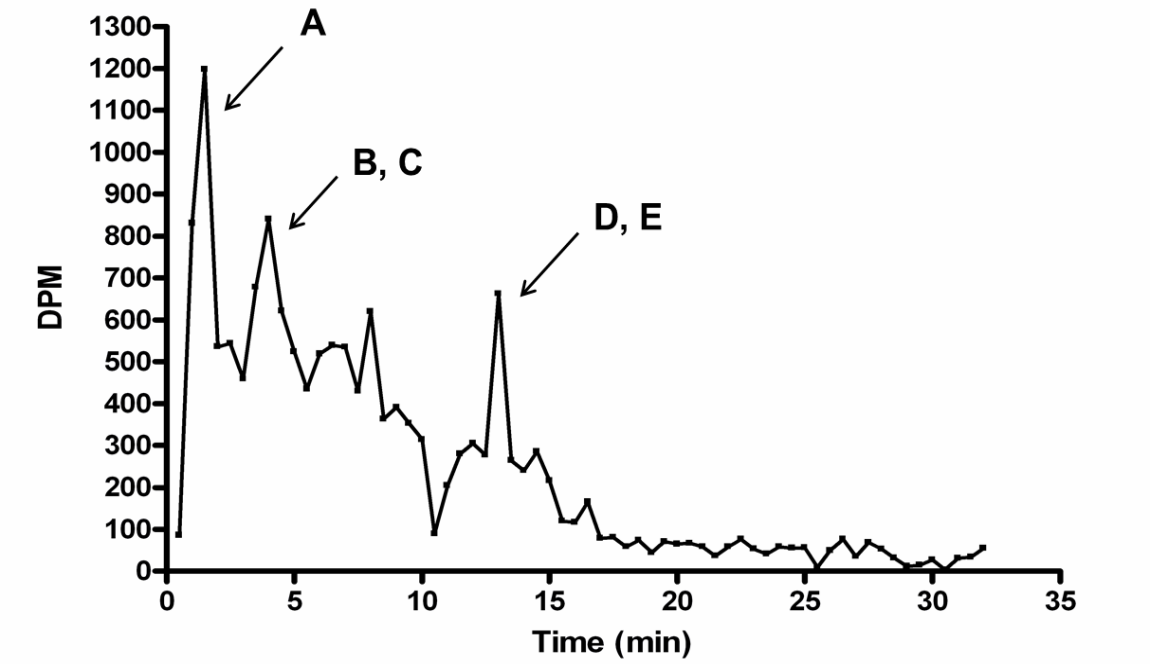


FIGURE 3.2. RP-HPLC radiochromatogram of 15-d-PGJ$_2$ metabolites excreted in rat urine. 15-d-PGJ$_2$ (1 mg) containing [^{3}H$_6$]15-d-PGJ$_2$was infused intravenously into the rat, and urine was collected over a 24-h period. Aliquots of urine containing the majority of the radioactivity excreted were extracted by C_{18} Sep-Pak, and extracts were subjected to RP-HPLC/MS. Radioactivity was monitored by collecting eluted fractions every 0.5 min and scintillation counting. Utilizing LC/MS analysis, five abundant metabolites of 15-d-PGJ$_2$ were identified. Metabolites are labeled based on their RP-HPLC retention time.

	Metabolite	[M-H]$^-$ *m/z*
A	***N*-acetylcysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ$_2$ glucuronide**	**690**
B	***N*-acetylcysteinyl-12,13-dihydro-20-hydroxy-15-d-PGJ$_2$**	**496**
C	***N*-acetylcysteinyl-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ$_2$**	**498**
D	***N*-acetylcysteinyl-15-d-PGJ$_2$**	**478**
E	***N*-acetylcysteinyl-11-hydroxy-15-d-PGJ$_2$**	**480**

TABLE 3.1. Summary of the observed metabolites of 15-d-PGJ$_2$ excreted in rat urine following intravenous administration. Each of the metabolites indicated were structurally identified and characterized by LC/MS/MS analysis, as described below.

Metabolite A (*m/z* 690), the putative *N*-acetylcysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ$_2$ glucuronide conjugate

The RP-HPLC retention time of metabolite A obtained from HPLC/MS analysis was 1.2 min with an *m/z* of 690. The mass chromatogram and CID spectrum generated from collisional activation of *m/z* 690 are shown in **FIGURE 3.3**. The major product ions observed are *m/z* 514 $[M - 176]^-$ (loss of glucuronic acid to yield the aglycone anion) and *m/z* 511 $[M - 179]^-$ (retro-Michael fragmentation). On the basis of this CID mass spectrum, metabolite A was determined to be the *N*-acetylcysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ$_2$ glucuronide conjugate. This metabolite is obtained by conjugation of 15-d-PGJ$_2$ with glutathione, followed by hydrolysis of glutamate and glycine to the cysteine conjugate, oxidation of the cysteinyl-sulfur to the sulfoxide, and *N*-acetylation of the cysteine to the *N*-acetylcysteine-sulfoxide conjugate. Additionally, the prostanoid portion of the molecule was metabolized by reduction of the carbonyl at C-11 to the hydroxyl, reduction of the C-12,C-13 double bond, and ω-oxidation of the C-20 carbon to the 20-hydroxyl. Further, conjugation of glucuronic acid with the C-11 or C-20 hydroxyl or the C-1 carboxyl yielded the glucuronide conjugate. Importantly, this metabolite was identified as the major urinary metabolite of 15-d-PGJ$_2$ in the rat.

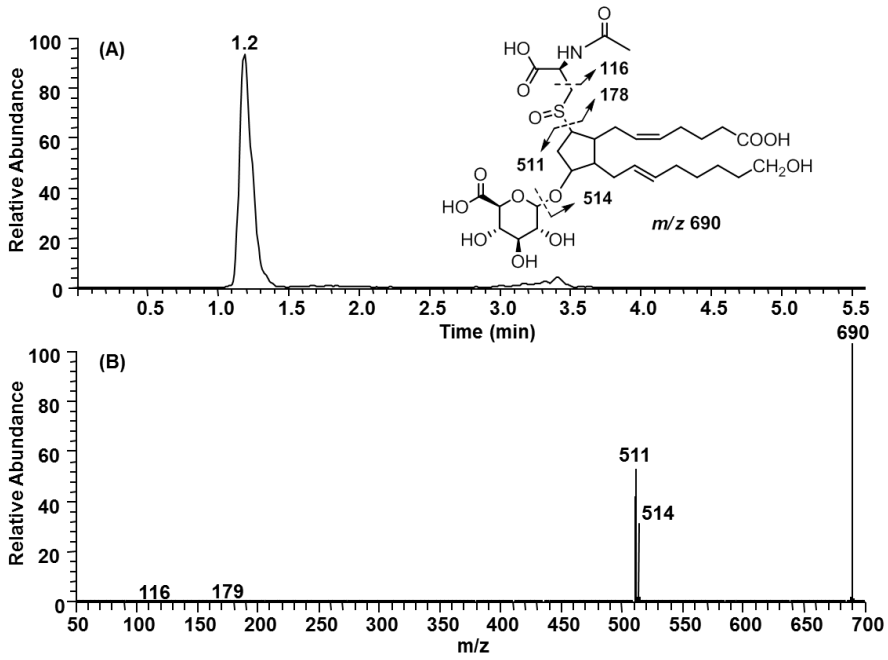


FIGURE 3.3. HPLC/MS/MS analysis of rat metabolite A (*m/z* 690), identified as the putative *N*-acetylcysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ$_2$ glucuronide conjugate. (A) The RP-HPLC retention time of metabolite A obtained from HPLC/MS analysis was 1.2 min with a precursor ion of *m/z* 690 in the negative mode. (B) The CID mass spectrum generated from collisional activation of *m/z* 690 is shown. The major product ions observed are *m/z* 514 $[M - 176]^-$ (loss of glucuronic acid to yield the aglycone anion) and *m/z* 511 $[M - 179]^-$ (retro-Michael fragmentation). The predicted structure of this metabolite is illustrated.

Metabolite B (*m/z* 496), the putative *N*-acetylcysteinyl-12,13-dihydro-20-hydroxy-15-d-PGJ$_2$

The RP-HPLC retention time of metabolite B obtained from HPLC/MS analysis was 3.7 min with an *m/z* of 496. The mass chromatogram and CID spectrum generated from collisional activation of *m/z* 496 are shown in **FIGURE 3.4**. The major product ions observed are *m/z* 367 $[M - 129]^-$ (retro-Michael fragmentation), *m/z* 315 [M – *N*-acetylcysteine – $H_2O]^-$, *m/z* 271 [315 – $CO_2]^-$, *m/z* 162 $[M - 334]^-$ (loss of the prostanoid portion of the molecule), and 128 $[M - 368]^-$ (fragmentation of the *N*-acetylcysteine, loss of the retro-Michael fragment). On the basis of this CID mass spectrum, metabolite B was determined to be the *N*-acetylcysteinyl-12,13-dihydro-20-hydroxy-15-d-PGJ$_2$. This metabolite is obtained by conjugation of 15-d-PGJ$_2$ with glutathione, followed by hydrolysis of glutamate and glycine to the cysteine conjugate, and *N*-acetylation of

the cysteine to the *N*-acetylcysteine conjugate. Additionally, the prostanoid portion of the molecule was metabolized by reduction of the C-12,C-13 double bond and ω-oxidation of the C-20 carbon to the 20-hydroxyl.

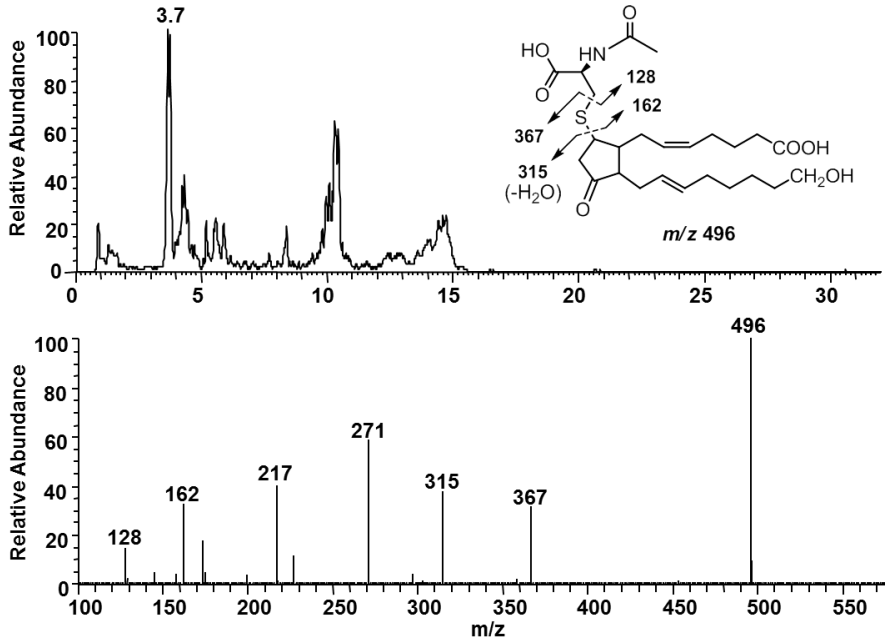


FIGURE 3.4. HPLC/MS/MS analysis of rat metabolite B (*m/z* 496), identified as the putative the putative *N*-acetylcysteinyl-12,13-dihydro-20-hydroxy-15-d-PGJ$_2$. (A) The RP-HPLC retention time of metabolite B obtained from HPLC/MS analysis was 3.7 min with a precursor ion of *m/z* 496 in the negative mode. (B) The CID mass spectrum generated from collisional activation of *m/z* 496 is shown. The major product ions observed are *m/z* 367 $[M - 129]^-$ (retro-Michael fragmentation), *m/z* 315 $[M - N\text{-acetylcysteine} - H_2O]^-$, *m/z* 271 $[315 - CO_2]^-$, *m/z* 162 $[M - 334]^-$ (loss of the prostanoid portion of the molecule), and *m/z* 128 [M – 368] (fragmentation of the *N*-acetylcysteine, loss of the retro-Michael fragment). The predicted structure of this metabolite is illustrated.

Metabolite C (*m/z* 498), the putative *N*-acetylcysteinyl-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ$_2$

The RP-HPLC retention time of metabolite C obtained from HPLC/MS analysis was 4.2 min with an *m/z* of 498. The mass chromatogram and CID spectrum generated from collisional activation of *m/z* 498 are shown in **FIGURE 3.5**. The major product ions observed are *m/z* 369 $[M - 129]^-$(retro-Michael fragmentation), *m/z* 317 $[M - N\text{-acetylcysteine} - H_2O]^-$, *m/z* 271 $[317 - H_2O - 2CH_2]^-$, *m/z* 162 $[M - 336]^-$ (loss of the prostanoid portion of the molecule), and *m/z* 128

[M – 370]$^-$ (fragmentation of the *N*-acetylcysteine, loss of the retro-Michael fragment). On the basis of this CID mass spectrum, metabolite C was determined to be the *N*-acetylcysteinyl-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ$_2$. This metabolite is obtained by conjugation of 15-d-PGJ$_2$ with glutathione, followed by hydrolysis of glutamate and glycine to the cysteine conjugate, and *N*-acetylation of the cysteine to the *N*-acetylcysteine conjugate. Additionally, the prostanoid portion of the molecule was metabolized by reduction of the carbonyl at C-11 to the hydroxyl, reduction of the C-12,C-13 double bond, and ω-oxidation of the C-20 carbon to the 20-hydroxyl.

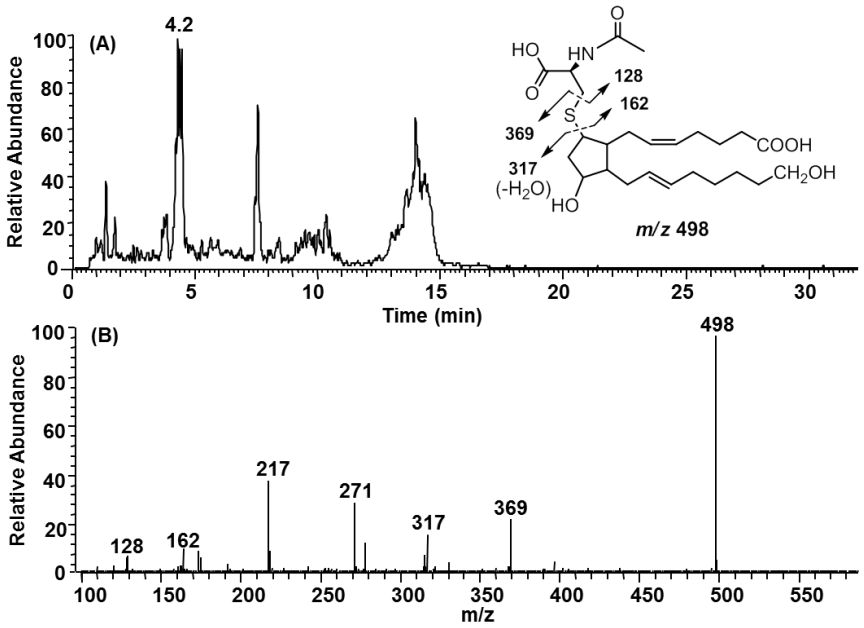


FIGURE 3.5. HPLC/MS/MS analysis of rat metabolite C (*m/z* 498), identified as the putative *N*-acetylcysteinyl-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ$_2$. (A) The RP-HPLC retention time of metabolite C obtained from HPLC/MS analysis was 4.2 min with a precursor ion of *m/z* 498 in the negative mode. (B) The CID mass spectrum generated from collisional activation of *m/z* 498 is shown. The major product ions observed are *m/z* 369 [M – 129]$^-$ (retro-Michael fragmentation), *m/z* 317 [M – *N*-acetylcysteine – H_2O]$^-$, *m/z* 271 [317 – H_2O – 2CH_2]$^-$, *m/z* 162 [M – 336]$^-$ (loss of the prostanoid portion of the molecule), and *m/z* 128 [M – 370] (fragmentation of the *N*-acetylcysteine, loss of the retro-Michael fragment). The predicted structure of this metabolite is illustrated.

Metabolite D (*m/z* 478), the putative *N*-acetylcysteinyl-15-d-PGJ$_2$

The RP-HPLC retention time of metabolite D obtained from HPLC/MS analysis was 12.8 min with an *m/z* of 478. The mass chromatogram and CID spectrum generated from collisional

activation of *m/z* 478 is shown in **FIGURE 3.6**. The major product ions observed are *m/z* 349[M – 129]$^-$ (retro-Michael fragmentation), *m/z* 315 [M – *N*-acetylcysteine]$^-$, *m/z* 162 [M – 316]$^-$ (loss of the prostanoid portion of the molecule), *m/z* 128 [M – 350]$^-$ (fragmentation of the *N*-acetylcysteine, loss of the retro-Michael fragment), and *m/z* 84 [128 – CO_2]$^-$. On the basis of this CID mass spectrum, metabolite D was determined to be *N*-acetylcysteinyl-15-d-PGJ_2. This metabolite is obtained by conjugation of 15-d-PGJ_2 with glutathione, followed by hydrolysis of glutamate and glycine to the cysteine conjugate, and *N*-acetylation of the cysteine to yield the *N*-acetylcysteine conjugate.

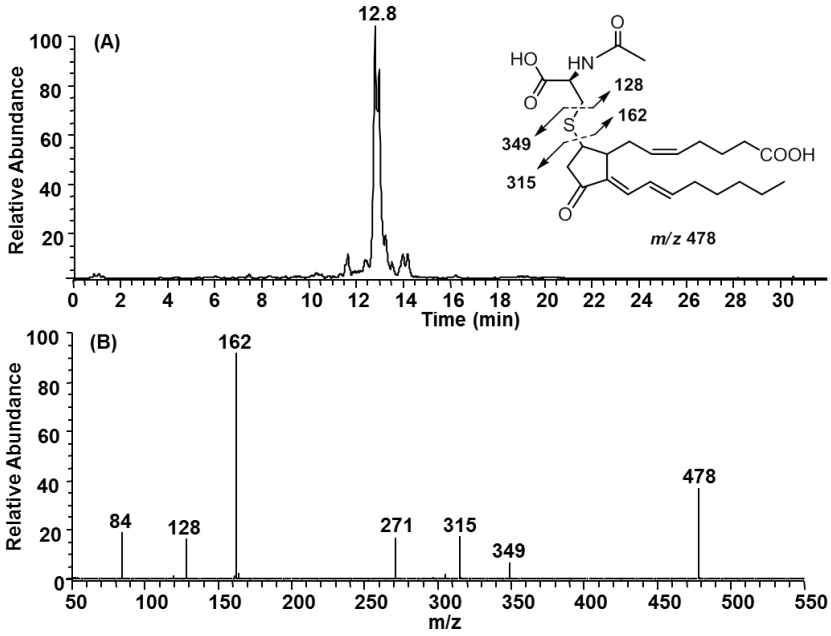


FIGURE 3.6. HPLC/MS/MS analysis of rat metabolite D (*m/z* 478), identified as the putative *N*-acetylcysteinyl-15-d-PGJ_2. (A) The RP-HPLC retention time of metabolite D obtained from HPLC/MS analysis was 12.8 min with a precursor ion of *m/z* 478 in the negative mode. (B) The CID mass spectrum generated from collisional activation of *m/z* 478 is shown. The major product ions observed are *m/z* 349[M – 129]$^-$(retro-Michael fragmentation), *m/z* 315 [M – *N*-acetylcysteine]$^-$, *m/z* 162 [M – 316]$^-$ (loss of the prostanoid portion of the molecule), *m/z* 128 [M – 350]$^-$ (fragmentation of the *N*-acetylcysteine, loss of the retro-Michael fragment), and *m/z* 84 [128 – CO_2]$^-$. The predicted structure of this metabolite is illustrated.

Metabolite E (*m/z* 480), the putative *N*-acetylcysteinyl-11-hydroxy-15-d-PGJ_2

The RP-HPLC retention time of metabolite D obtained from HPLC/MS analysis was 12.8-13.5 min with an *m/z* of 480. (The multiple peaks eluting near the same retention time likely

represent the various isomers of this metabolite). The mass chromatogram and CID spectrum generated from collisional activation of *m/z* 480 is shown in **FIGURE 3.7**. The major product ions observed are *m/z* 351 $[M - 129]^-$ (retro-Michael fragmentation), *m/z* 317[M – *N*-acetyl-cysteine]$^-$, *m/z* 299 $[317 - H_2O]^-$, *m/z* 255 $[299 - CO_2]^-$, *m/z* 162 $[M - 318]^-$ (loss of the prostanoid portion of the molecule), and *m/z* 128 $[M - 352]^-$ (fragmentation of the *N*-acetylcysteine). On the basis of this CID mass spectrum, metabolite E was determined to be *N*-acetylcysteinyl-11-hydroxy-15-d-PGJ_2. This metabolite is obtained by conjugation of 15-d-PGJ_2 with glutathione, followed by hydrolysis of glutamate and glycine to the cysteine conjugate, and *N*-acetylation of the cysteine to the *N*-acetylcysteine conjugate. Additionally, the prostanoid portion of the molecule was metabolized by reduction of the carbonyl at C-11 to the hydroxyl.

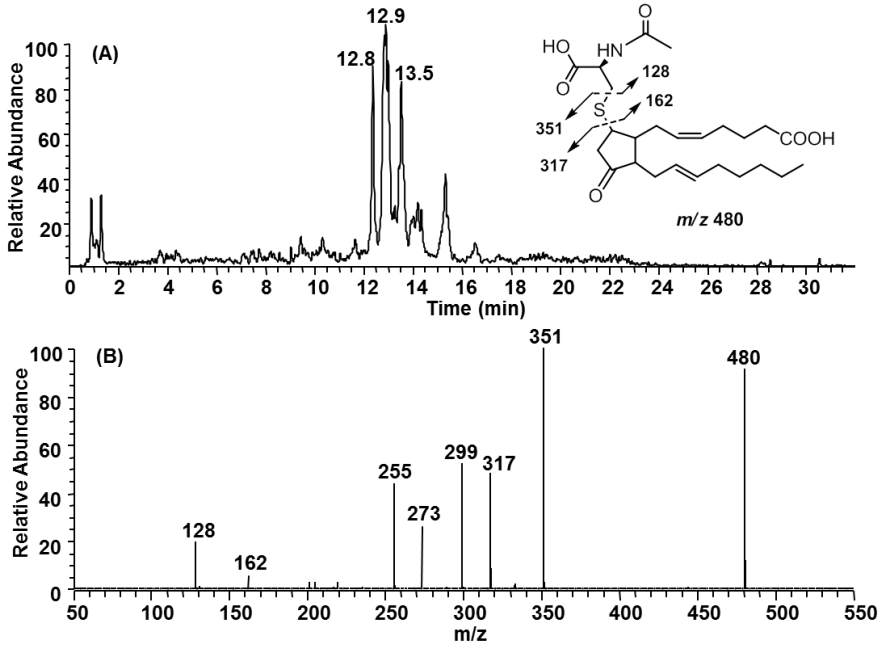


FIGURE 3.7. HPLC/MS/MS analysis of rat metabolite E (*m/z* 480), identified as the putative *N*-acetylcysteinyl-11-hydroxy-15-d-PGJ_2. (A) The RP-HPLC retention time of metabolite E obtained from HPLC/MS analysis was 12.8-13.5 min with a precursor ion of *m/z* 480 in the negative mode. (B) The CID mass spectrum generated from collisional activation of *m/z* 480 is shown. The major product ions observed are *m/z* 351 $[M - 129]^-$ (retro-Michael fragmentation), *m/z* 317 [M – *N*-acetylcysteine]$^-$, *m/z* 299 $[317 - H_2O]^-$, *m/z* 255 $[299 - CO_2]^-$, *m/z* 162 $[M - 318]^-$ (loss of the prostanoid portion of the molecule), and *m/z* 128 $[M - 352]^-$ (fragmentation of the *N*-acetylcysteine). The predicted structure of this metabolite is illustrated.

Identification of the major rat urinary metabolite of 15-d-PGJ_2 (rat metabolite A) in humans

Previously, it has been shown that PGD_2 production is markedly increased in humans after administration of the hypolipidemic drug niacin (Morrow et al. 1989; Awad et al. 1994). We hypothesized that the increased PGD_2 generated may also result in formation of 15-d-PGJ_2 *in vivo*. Metabolites of 15-d-PGJ_2 should also be generated under these conditions. Thus, to determine if the major urinary metabolite of 15-d-PGJ_2 formed in rats is also present in humans, urine was obtained from a healthy male human volunteer 1-2 h after administration of 500 mg niacin. The urine was extracted by C_{18} Sep-Pak as described in "Materials and Methods" and subjected to RP-UPLC/MS analysis. (RP-UPLC was employed to provide increased retention of the highly polar metabolite). The mass spectrometer was operated in the selected reaction monitoring (SRM) mode in the negative ion mode monitoring the transition of the metabolite precursor ion *m/z* 690 to the specific product ions *m/z* 514, *m/z* 175, and *m/z* 113. The SRM chromatogram from this analysis is shown in **FIGURE 3.8**. The precursor-to-product transition *m/z* 690–514 represents loss of glucuronic acid from the conjugate to yield the aglycone anion. In addition, the precursor-to-product transitions *m/z* 690–175 and *m/z* 690–113 represent loss of the prostanoid portion of the molecule and fragmentation of glucuronic acid, respectively. Taken together, these data support the contention that the major 15-d-PGJ_2 urinary metabolite, *N*-acetylcysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ_2 glucuronide conjugate, is formed in humans.

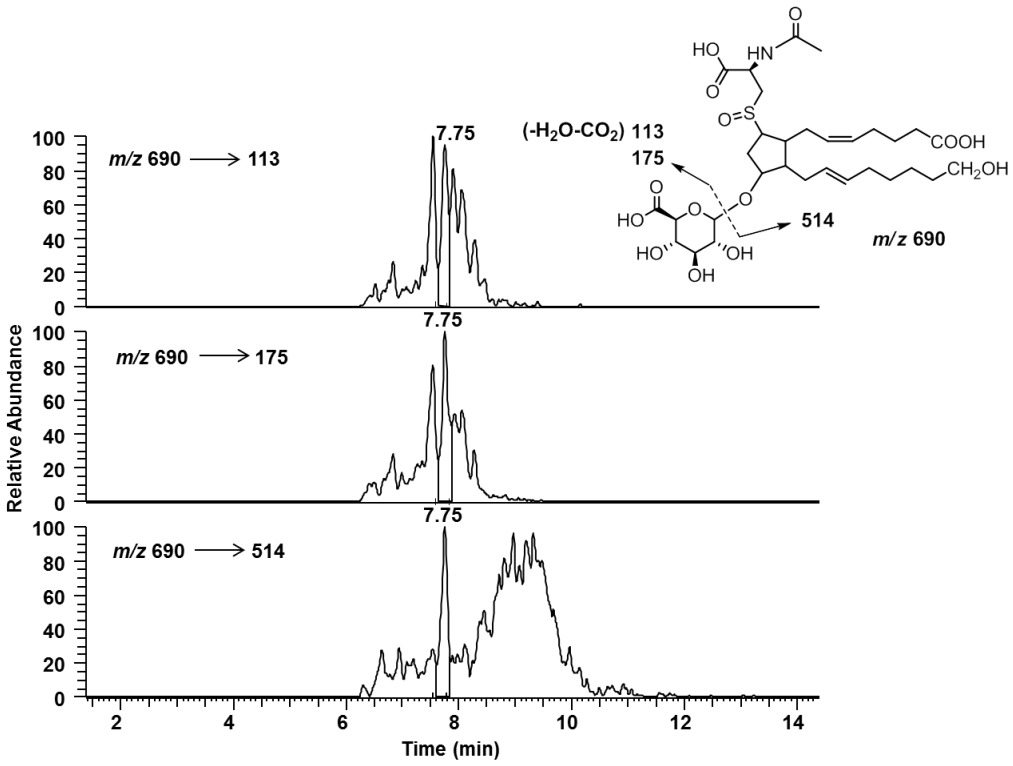


FIGURE 3.8. UPLC/MS/MS analysis of the major rat urinary metabolite of 15-d-PGJ$_2$ in human urine. The mass spectrometer was operated in SRM mode monitoring the precursor-to-product transitions *m/z* 690–113, *m/z* 690–175, and *m/z* 690–514 in negative mode. The putative 15-d-PGJ$_2$ urinary metabolite eluted at 7.75 min. The SRM transitions indicated are predicted to occur from fragmentation of the precursor ion *m/z* 690 as illustrated.

Discussion

15-d-PGJ$_2$ is a biologically active cyclopentenone PG is formed from the dehydration of COX-derived PGD$_2$ (Fitzpatrick and Wynalda 1983). We have recently made the observation that 15-d-PGJ$_2$ and a series of 15-d-PGJ$_2$-like compounds are also formed *in vivo* via the free radical-initiated peroxidation of arachidonic acid (Hardy et al. 2011). This is relevant because 15-d-PGJ$_2$ is regarded as a key mediator of various cellular processes. The electrophilicity of 15-d-PGJ$_2$ contributes importantly to its biological activity. The cyclopentenone ring of 15-d-PGJ$_2$ contains a reactive α,β-unsaturated carbonyl moiety that can undergo Michael addition with cellular nucleophiles, such as the free sulfhydryl groups of cysteine residues of proteins or GSH. 15-d-

PGJ_2 has been shown to possess potent anti-inflammatory, anti-proliferative, and pro-apoptotic activity in various cell types (Straus and Glass 2001; Kim and Surh 2006; Uchida and Shibata 2008). 15-d-PGJ_2 is postulated to be the endogenous ligand of the nuclear receptor PPARγ, and its biological effects are thought to be due in part through activation PPARγ (Forman et al. 1995; Kliewer et al. 1995). On the other hand, 15-d-PGJ_2 can also exert biological activity independent of PPARγ via direct interaction with other intracellular protein targets (Straus and Glass 2001). In this regard, 15-d-PGJ_2 has been shown to inhibit the NF-κB inflammatory signaling pathway via covalent modification of critical cysteine residues in IκB kinase β and the DNA-binding domains of NF-κB p50 and p65 subunits (Straus et al. 2000). Further, 15-d-PGJ_2 has been shown to affect a number of other cellular responses through redox alteration, modulation of MAPK and p53 activity, inhibition of protein turnover, and induction of cytoskeletal dysfunction (Uchida and Shibata 2008).

While the biological properties of 15-d-PGJ_2 have been well defined, physiological relevance of this compound has been the subject of continuing controversy. This is due to the lack of evidence that 15-d-PGJ_2 is formed *in vivo*. A limited number of studies have reported the detection of 15-d-PGJ_2 in inflammatory fluids and tissue sections *in vivo* using immunological approaches (Gilroy et al. 1999; Shibata et al. 2002); however, immunoassays are not the optimal approach for prostanoid analysis due to the potential for cross reactivity with structurally similar compounds. Further, the lack of sensitivity and specificity of the antibody used has caused these results to be questioned. Fitzgerald and colleagues reported that only trivial amounts of free 15-d-PGJ_2 were found in human urine when analyzed by LC/MS, and these levels were unaltered under various pathological conditions in which 15-d-PGJ_2 have been implicated, such as acute inflammatory responses and diabetes (Bell-Parikh et al. 2003). These finding led the authors to conclude that 15-d-PGJ_2 is not formed *in vivo* in sufficient quantities to mediate its

proposed biological activities (Bell-Parikh et al. 2003). The challenge and limitation of quantifying 15-d-PGJ_2 in free form *in vivo* is its intrinsic reactivity and its potential for artifactual generation from the dehydration of PGD_2 *ex vivo*. Like other cyclopentenone eicosanoids, 15-d-PGJ_2 readily reacts with the free sulfhydryl groups of cysteine residues of proteins and glutathione (GSH), to form Michael adducts (Chen et al. 1999; Milne et al. 2004; Paumi et al. 2003; Brunoldi et al. 2007). Moreover, measurement of urinary metabolites has been established as the most accurate and sensitive method to assess the total endogenous formation of eicosanoids (Samuelsson et al. 1975). Thus, studies on the metabolic disposition of 15-d-PGJ_2 in cells and *in vivo* are likely critical to defining the actual concentrations of 15-d-PGJ_2 generated *in vivo*.

In the present study, we have identified, for the first time, the major urinary metabolite of 15-d-PGJ_2 in the rat. The metabolite was identified as the *N*-acetylcysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ_2 glucuronide conjugate. Other abundant urinary metabolites of 15-d-PGJ_2 identified included various *N*-acetyl cysteine derivatives. Through the identification of all five metabolites observed, we have proposed a pathway for the metabolism of 15-d-PGJ_2 *in vivo* in the rat (**FIGURE 3.9**). *N*-acetyl cysteine conjugates (mercapturic acids) are common metabolites of endogenous compounds that contain α,β-unsaturated carbonyl moieties, such as the electrophilic lipid peroxidation products 15-A_{2t}-isoprostane (IsoP) and 4-hydroxy-2-nonenal (HNE) (Milne, Gao et al. 2005; Alary et al. 1995; De Zwart et al. 1996). Mercapturic acid formation begins with conjugation of the parent compound with GSH, followed by hydrolysis of glutamate and glycine from the GSH portion of the molecule to form the cysteine conjugate. Subsequent *N*-acetylation yields the mercapturic acid. This conversion occurs through a series of enzymatic reactions involving sequentially glutathione *S*-transferases, γ-glutamyltranspeptidases, cysteinyl-glycine dipeptidase, and *N*-acetyltransferases (Hinchman

and Ballatori 1994). Further, *S*-oxidation of the cysteinyl-sulfur forms the sulfoxide, and this reaction has been shown to be catalyzed by cytochrome P450s (CYPs) or flavin-containing monooxygenases (FMOs) (Werner et al. 1995; Birner et al. 1998; Krause et al. 2002; Sheffels et al. 2004).

The prostanoid portion of the 15-d-PGJ_2 mercapturic acid conjugate was further metabolized by various processes *in vivo*, including reduction of the carbonyl at C-11 to the alcohol, reduction of the Δ^{12} double bond, ω-oxidation of the C-20 carbon to the 20-hydroxyl, and conjugation of glucuronic acid. These reactions are also common in eicosanoid metabolism. Reduction of C-11 carbonyl is likely catalyzed by 11-ketoreductase, which has been shown to occur in the metabolism of PGD_2 and Δ^{12}-PGJ_2 (Liston and Robert 1985a; Atsmon, Freeman et al. 1990). Reduction of the Δ^{12} double may be catalyzes by other cellular reductases. For example, prostaglandin Δ^{13}reductase catalyzes reduction of the Δ^{13} double bond of PGE_2 and $PGF_{2\alpha}$ 15-keto derivatives (Tai et al. 2002). ω-oxidation of carbon C-20 is likely catalyzed by specific cytochrome P450s. Cytochrome P450s of the CYP4F subfamily have previously been shown to catalyze the ω-oxidation of other eicosanoids to yield 20-hydroxyl products (Wheelan et al. 1999). Finally, conjugation of glucuronic acid is catalyzed by UDP-glucuronosyltransferases (UGTs) in liver microsomes (King et al. 2000). The C-1 carboxyl of 15-d-PGJ_2 is a potential acceptor of glucuronic acid to form an acyl glucuronide. The C-11 hydroxyl and C-20 hydroxyl are also potential sites for glucuronidation. The exact position of glucuronidation could not be determined from these studies.

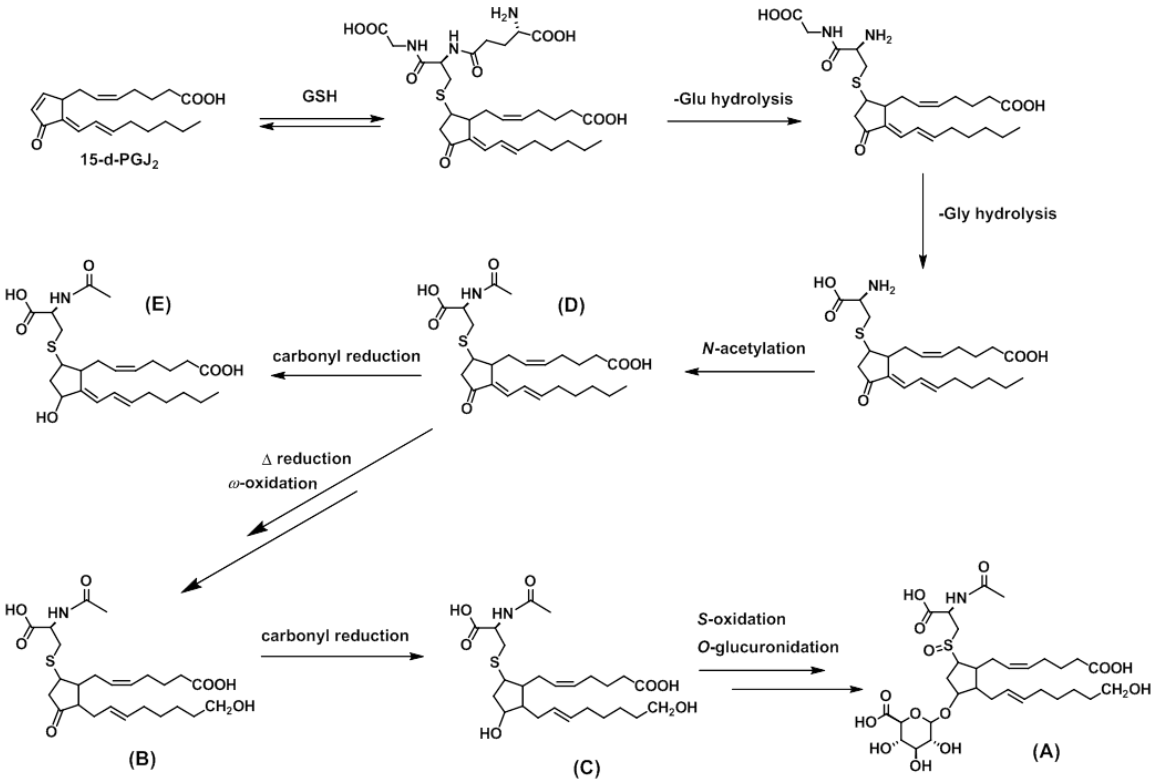


FIGURE 3.9. Proposed pathway for the metabolism of 15-d-PGJ$_2$ *in vivo* in the rat. The metabolites labeled in parentheses () were structurally identified by LC/MS/MS.

The adduction of 15-d-PGJ$_2$ to GSH and subsequent metabolism probably represents a pathway of metabolic inactivation. Conjugation of cyclopentenone eicosanoids with GSH has been shown to abolish their biological effects, most likely due to loss of the reactivity unsaturated carbonyl moiety. Although 15-d-PGJ$_2$ contains two electrophilic carbon centers at position C-9 and C-13, GSH conjugation has been shown to preferentially occur at carbon C-9 (Paumi et al. 2003; Maxey et al. 2000). Paumi et al. demonstrated that active transport of 15-d-PGJ$_2$-gluathione conjugates via the efflux transporters multi-drug resistance associated protein (MRP)-1 and MRP3 expressed in MCF-7 breast cancer cells attenuated the cytotoxic and transactivating effects of this compound (Paumi et al. 2003). On the other hand, depletion of intracellular GSH enhanced the cytotoxicity of 15-d-PGJ$_2$ (Paumi et al. 2003). Similarly, Atsmon

et al. showed that depletion of intracellular GSH augmented the anti-proliferative effects of the precursor to 15-d-PGJ_2, Δ^{12}-PGJ_2, in Chinese hamster ovary (CHO) and hepatoma tissue culture (HTC) cells (Atsmon, Freeman et al. 1990). Thus, it is likely that conjugation of 15-d-PGJ_2 with GSH is a mechanism by which cells modulate the bioactivity of this and related reactive cyclopentenone-containing compounds. In addition, reduction of the Δ^{12} double bond may also affect the biological activity of 15-d-PGJ_2 due to elimination of the electrophilic carbon at position C-13. In this regard, 15-d-PGJ_2 has been shown to activate PPARγ through covalent adduction to a critical cysteine residue in the PPARγ ligand binding domain via carbon C-13 (Shiraki et al. 2005). Further, Yu et al. showed that reduction of Δ^{12} double of 15-d-PGJ_2 by recombinant alkenal/one oxidoreductase (rAor) abolished the ability of 15-d-PGJ_2 to activate Nrf2-responsive antioxidant genes (Yu et al. 2006).

The identification of the major urinary metabolite of 15-d-PGJ_2 has important implications regarding the total amount of 15-d-PGJ_2 and related compounds *in vivo*. The extent to which 15-d-PGJ_2 is formed in humans has been the subject of continuing controversy for at least three decades, in part because previous studies have attempted to detect the unconjugated compound in animals and human body fluids (Bell-Parikh et al. 2003). In the present study, we were able to detect the major urinary metabolite of 15-d-PGJ_2 in human urine by RP-UPLC/MS analysis. These results suggest that this metabolite can potentially be used as a biomarker to assess the total endogenous production of 15-d-PGJ_2 *in vivo*.

In summary, we report that the cyclopentenone PG 15-d-PGJ_2 is rapidly metabolized in rats to the *N*-acetylcysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ_2 glucuronide conjugate. These finding provide, for the first time, the *in vivo* pathways by which this electrophilic lipid is metabolized. Further, we report the detection of the 15-d-PGJ_2 urinary metabolite in human urine. Thus, the identification of the major urinary metabolite of 15-d-PGJ_2

provides a potential biomarker that can be used to assay the *in vivo* production of 15-d-PGJ_2 and related compounds. Efforts are currently underway in our laboratory to further develop this methodology by synthesizing a heavy isotope labeled standard of this metabolite to quantify the production of 15-d-PGJ_2 in humans under normal and pathophysiological conditions. Through these studies, we hope to gain a better understanding of the physiological role of 15-d-PGJ_2 in human health and disease.

CHAPTER IV

METABOLIC TRANSFORMATIONOF 15-DEOXY-$\Delta^{12,14}$-PROSTAGLANDIN J_2 IN PRIMARY HUMAN HEPATOCYTES

Introduction

Prostaglandins (PGs) are important lipid mediators formed by the enzymatic action of cyclooxygenase (COX) on arachidonic acid (AA) (Morrow and Roberts 2001). The partent prostaglandins include $PGF_{2\alpha}$, PGE_2, PGD_2, PGI_2, and thromboxane A_2. PGE_2 and PGD_2 can undergo spontaneous dehydration in aqueous solutions to form reactive cyclopentenone PGs of the A_2 and J_2 series. Dehydration of PGE_2 yields PGA_2 (Hamberg and Samuelsson 1966), and dehydration of PGD_2 yields PGJ_2 (Fukushima, Kato, Ota et al. 1982; Fitzpatrick and Wynalda 1983). PGJ_2 can undergo isomerization to Δ^{12}-PGJ_2, and a second loss of water yields 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15-d-PGJ_2) (Fitzpatrick and Wynalda 1983). CyPGs are characterized by the presence of a chemically reactive α,β unsaturated carbonyl moiety on the cyclopentenone ring, which renders them susceptible to nucleophilic addition reactions (Michael addition) with cellular thiols, such as GSH and cysteine residues of proteins. This reactive property has been shown to contribute importantly to the bioactivity of CyPGs (Straus and Glass 2001). CyPGs exhibit a broad spectrum of biological activities unique from conventional PGs, including anti-inflammatory, anti-proliferative, and anti-neoplastic effects (Straus and Glass 2001). Unlike other PGs, which elicit biological responses primarily through binding specific cell surface G-protein coupled receptors (GPCRs), CyPGs can be actively and selectively transported into cells (Narumiyaand Fukushima 1986) where they exert their biological effects through covalent modification of intracellular protein targets, such as transcription factors and other signaling molecules. Interesting, due to their anti-inflammatory properties, these compounds have

recently been used as pharmacological tools against atherosclerosis (Gutierrez et al. 2008). CyPGs have also been investigated as potential agents in cancer therapy due to their ability to inhibit tumor cell proliferation (Kikuchi et al. 1992).

15-d-PGJ_2 is the most extensively studied CyPG and has emerged as a potentially important mediator of a number of biological effects. This moleculeoften exhibits greater biological potency compared to other CyPGs, and this property may be attributed to its high reactivity (Brechbuhlet al. 2009; Liu et al. 2010). 15-d-PGJ_2 contains two reactive electrophilic carbon centers at carbons C-9 and C-13, which can undergo Michael addition with free sulfhydryls of cysteine residues to proteins. Among its diverse biological effects, 15-d-PGJ_2 has been shown to mediate anti-inflammatory (Straus and Glass 2001; Gilroy et al. 1999; Rossi et al. 2000; Straus et al. 2000; Perez-Sala et al. 2002) and pro-apoptotic (Hortelano et al. 2000; Bishop-Baileyet al. 1999; Levonenet al. 2001; Clay et al. 2002) responses in various cell types, particularly at concentrations in the micromolar range. Based on these findings, 15-d-PGJ_2 is considered to be an important lipid mediator, which plays a critical role in the resolution of inflammation. Some of the biological activities of 15-d-PGJ_2 are related to the observation that this compound activates the nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ) (Forman et al. 1995; Kliewer et al. 1995). 15-d-PGJ_2 has also been shown to possess PPARγ-independent activity through direct adduction to intracellular proteins targets, such as NF-κB (Rossi et al. 2000; Straus et al. 2000; Perez-Sala et al. 2002), p53 (Kondo et al. 2002; Shibata et al. 2003; Kim et al. 2009), Keap1 (Dinkova-Kostovaet al. 2002; Itoh et al. 2004; Levonen et al. 2004), and mitogen activated protein kinases (MAPKs) (Hortelano et al. 2000; Shin et al. 2009).

Despite significant interest in the biological activity of 15-d-PGJ_2, evidence for its formation *in vivo* is controversial. Production of 15-d-PGJ_2 has been reported in selenium-

supplement macrophages following LPS stimulation (Vunta et al. 2007) and in rodent models of inflammation, including carrageenan-induced pleurisy in rats (Gilroy et al. 1999) and zymosan-induced resolving peritonitis in mice (Rajakariar et al. 2007). 15-d-PGJ_2 was also detected in macrophages in human atherosclerotic lesions using immunohistochemical approaches (Shibata et al. 2002). In addition, Hirata et al. reported the detection of Δ^{12}-PGJ_2, the precursor to 15-d-PGJ_2, in human urine, and its formation was suppressed to some extent by COX inhibitors (Hirata et al. 1988). On the other hand, Bell-Parikh et al. reported very low levels of 15-d-PGJ_2 (~5 pM) in human urine, and these levels are likely insufficient to exert bioactivity (Bell-Parikh et al. 2003). Additionally, levels of 15-d-PGJ_2 were unaltered in the pathophysiological settings in which 15-d-PGJ_2-mediated activation of PPAR-γ has been implicated, namely inflammation and diabetes (Bell-Parikh et al. 2003). Based on these and other findings, the physiological relevance of 15-d-PGJ_2 has been questioned (Bell-Parikh et al. 2003; Powell 2003). Further, the extent to which 15-d-PGJ_2 is formed *in vivo* in humans and the mechanisms that regulate its formation remain unclear.

Oxidant stress has been increasingly implicated in the pathogenesis of a number of human diseases, including atherosclerosis, cancer, neurodegenerative disorders, and even the normal aging process (Halliwell and Gutteridge 1990; Southorn and Powis 1988; Ames 1983; Harman et al. 1981). Free radical-initiated lipid peroxidation is a key feature of oxidant stress. Previously, Roberts, Morrow and colleagues reported the discovery of a series of PG-like compounds, termed isoprostanes (IsoPs), generated *in vivo* from the nonenymatic free radical-initiated peroxidation of arachidonic acid (Morrow, Hill et al. 1990). IsoPs are isomeric to PGs, differing in the position of their side chains in relation to the prostane ring. PG side chains are in the *trans*-configuration with respect to the prostane ring, whereas the majority of IsoPs contain side chains that are in the *cis*-configuration (Morrow, Hill et al. 1990). Additionally, unlike PGs,

IsoPs are initially formed *in situ* in phospholipids and are subsequently hydrolyzed to their free form by phospholipases (Morrow, Awad, Boss et al. 1992; Stafforini et al. 2006). Several different classes of IsoPs have been characterized, including $PGF_{2\alpha}$-like compounds, termed F_2-IsoPs (Morrow, Hill et al. 1990), PGE_2- and PGD_2-like compounds (E_2/D_2-IsoPs) (Morrow et al. 1994), TXA_2-like compounds (Tx-IsoPs) (Morrow et al. 1996), and cyclopentenone PGA_2- and PGJ_2-like compounds (A_2/J_2-IsoPs) (Chen, Morrow et al. 1999). Further, more recently, we have shown that 15-d-PGJ_2 and 15-d-PGJ_2-like compounds, termed deoxy-J_2-IsoPs, are formed *in vivo* from the dehydration of J_2-IsoPs (Hardy et al. 2011). We found that 15-d-PGJ_2 and deoxy-J_2-IsoPs are significantly generated *in vivo* esterified in membrane phospholipids in rat liver after induction of lipid peroxidation by CCl_4 (Hardy et al. 2011). The finding that 15-d-PGJ_2 is produced *in vivo* via free radical-catalyzed lipid peroxidation, independent of the COX pathway, offers new insights into the possible mechanisms that contribute to 15-d-PGJ_2 formation *in vivo* and provided the impetus for us to examine the systemic production of 15-d-PGJ_2 in humans.

Although we can readily detect 15-d-PGJ_2 and related cyclopentenone eicosanoids esterified in membrane phospholipids *in vivo* (Chen, Morrow et al. 1999; Hardy et al. 2011), quantification of free 15-d-PGJ_2 in body fluids such as urine and plasma is challenging. Only trivial amounts of 15-d-PGJ_2 have been detected in human urine (Bell Parikh et al. 2003). In these studies, production of 15-d-PGJ_2 was quantified by mass spectrometric analysis of free 15-d-PGJ_2 in urine. However, measuring free 15-d-PGJ_2 in biological fluids such as urine likely does not reflect the actual quantities of this compound generated *in vivo* for two important reasons. First, 15-d-PGJ_2 can be generated *ex vivo* from the dehydration of PGD_2 excreted in the urine. Further, the marked proclivity of 15-d-PGJ_2 to undergo Michael addition with the free sulfhydryls of GSH and cysteine residues of proteins suggests that 15-d-PGJ_2 may be rapidly metabolized *in vivo* such that it is virtually undetectable in free form. Thus, quantification of

free 15-d-PGJ_2 in the urine may lead to an underestimation of the total amount produced *in vivo.* Previous studies have shown that measurement of urinary metabolites by mass spectrometry is the most accurate and sensitive approach to assess systemic prostaglandin production (Samuelsson and Green 1974; Samuelsson et al. 1975; Roberts et al. 1981). Hence, there is a need for a reliable mass spectrometric method to quantify 15-d-PGJ_2 metabolites excreted in urine as a means to accurately estimate biological levels of this compound. In this regard, studies on the metabolism of 15-d-PGJ_2 should yield key insights into the actual quantities of 15-d-PGJ_2 generated *in vivo*.

We and others have previously shown that conjugation with GSH is a major route for the metabolism of cyclopenteone eicosanoids, including PGJ_2 (Cox et al. 2002), Δ^{12}-PGJ_2 (Atsmon, Sweetman et al. 1990; Atsmon, Freeman et al. 1990), and the cyclopentenone IsoP 15-A_{2t}-IsoP (Hubatsch et al. 2002; Milne et al. 2004; Milne, Gao et al. 2005), *in vitro* and *in vivo*. The metabolism of 15-d-PGJ_2 has also been studied in cancer cells and cell lines. Paumi et al. showed that 15-d-PGJ_2 readily adducts GSH in MCF7 breast cancer cells to form the 15-d-PGJ_2-GSH conjugate (Paumi et al. 2003). More recently, Brunoldi et al. reported that 15-d-PGJ_2 is metabolized in the human hepatoma cell line HepG2 via conjugation with GSH, and three major metabolites are produced, including the 15-d-PGJ_2-GSH conjugate in which the carbonyl at C-11 was reduced to an alcohol and the cysteinyl-glycine and cysteine derivatives (Brunoldi et al. 2007). In addition, Yu et al. demonstrated that metabolism of 15-d-PGJ_2 by recombinant alkenal/one oxidoreductase (Aor) *in vitro* resulted in reduction of the C-12, C-13 double bond to yield 12,13-dihydro-15-d-PGJ_2 (Yu et al. 2006). However, little is known about the metabolic fate of 15-d-PGJ_2 in the human subject or in primary human cells. The studies described above are limited by the fact that cancer cells and cell lines do not represent metabolically competent models. For example, HepG2 cells lack a substantial set of liver-specific functions, including low

expression of phase I and phase II enzymes involved in xenobiotic metabolism; thus, these transformed cells are not suitable for mimicking normal liver cell function *in vivo* (Guillouzo et al. 2007). Further, in addition to the metabolic transformations described above, other metabolic pathways, such as glucuronic acid conjugation and β- or ω-oxidation, may be involved in the metabolism of 15-d-PGJ_2 *in vivo*. These pathways have previously been shown to be important in eicosanoid metabolism *in vivo* (Roberts 1987; Berry et al. 2003; Yan et al. 2010).

Primary human hepatocytes represent the *in vitro* gold standard model for studying xenobiotic metabolism and are frequently used as a surrogate model for predicting metabolism *in vivo* (Guillouzo et al. 2007). These cells are capable of expressing the complete metabolic pathways occurring *in vivo*. In the present study, we have examined the metabolic transformation of 15-d-PGJ_2 in primary human hepatocytes to gain a better understanding of the potential pathways involved in the metabolic inactivation and excretion of 15-d-PGJ_2 in humans. Herein, we report the identification of several novel metabolites of 15-d-PGJ_2 formed in primary human hepatocytes. Based on our findings, we propose a pathway for the metabolism of 15-d-PGJ_2 in humans.

Materials and Methods

Materials

Chemicals and enzymes were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. *In Vitro*GRO HI Medium and Torpedo Antibiotic Mix were purchased from Celsis-In Vitro Technologies. β-glucuronidase was purchased from MP Biomedicals. 15-d-PGJ_2 and PGD_2 were purchased from Cayman Chemicals (Ann Arbor, MI). [$^3H_7$]PGD_2 was purchased from Perkin-Elemer Life Sciences, Inc. (Boston, MA). C_{18} Sep-Paks were purchased from Waters

Corporation (Milford, MA). All solvents were of HPLC quality and purchased from EM Science (Gibbstown, NJ).

Preparation of [$^3H_6$]15-d-PGJ_2

[$^3H_6$]15-d-PGJ_2 was prepared as described previously (Brunoldi et al. 2007). Briefly, [$^3H_7$]PGD_2 (2 μCi) and PGD_2 (200 μg) was dissolved in 1N HCl (0.5 ml) at 37°C. After 5 h, 50 mM aqueous ammonium acetate (10 ml, pH 3.4) was added, and the resulting solution was extracted with ethyl acetate (5 x 1 ml). The organic layers were collected, and the solvent was evaporated under a stream of N_2. The resulting residue was purified by high-performance liquid chromatography (HPLC) using a Phenomenex silica column (250 mm x 4.6 mm, 5 μm) and a mobile phase of hexane isopropyl alcohol/acetic acid, 97:3:0.1 at a flow rate of 1 ml/min. The desired product co-eluted with commercially available 15-d-PGJ_2.

Metabolism of 15-d-PGJ_2 by primary human hepatocytes

Freshly plated human hepatocytes from a male donor were purchased from Celsis-In Vitro Technologies in either a 6-well or 24-well plate. Upon arrival of the hepatocytes, the shipping media was removed from the plate, and the hepatocytes were incubated 12-24 h in *In Vitro*GRO HI Medium with Torpedo Antibiotic Mix to resume normal physiological function before the start of the experiment (in accordance with the protocol provided by Celsis-In Vitro Technologies). Hepatocytes were incubated in a 95% air/5% CO_2 humidified environment maintained at 37°C during the course of all experiments. Cells were 70-90% confluent at the time of the experiment and maintained normal hepatocyte morphology throughout the course of all experiments. To examine the metabolism of 15-d-PGJ_2 by primary human hepatoyctes, 0.25 μCi of [$^3H_6$]15-d-PGJ_2 and 23.7 μg (5 μM) of 15-d-PGJ_2 were added to 15 ml of medium as

an ethanolic solution (30 μl), the initial incubation media was removed, and 0.5 ml of the medium containing 15-d-PGJ_2 was added to each well of the 24-well plate. Aliquots of medium (0.6 ml) were taken at 0 h, 3 h, 6 h, 14 h, and 24 h. Unconjugated material was separated from conjugated material by extraction of small aliquots of medium (200 μl) with ethyl acetate (200 μl) after acidification with 1 N HCl. The extent of conjugation was determined by comparing the radioactivity of the organic phase (unconjugated) with that of the aqueous phase (conjugated).

Metabolite purification

Aliquots of cell-free medium (0.6 ml) were also taken at 0 h and 24 h from the incubation of 15-d-PGJ_2 with primary human hepatocytes for analysis of 15-d-PGJ_2 metabolites by off-line high-performance liquid chromatography (HPLC) followed by HPLC/electrospray ionization – mass spectrometry (ESI-MS). For these studies the medium was applied to a C_{18} Sep-Pak(Waters Associates, Milford, MA) preconditioned by rinsing with 10ml of acetonitrile and 10 ml of 50 mM aqueous ammonium acetate, pH 3.4. After loading the sample, the Sep-Pak was rinsed with 10 ml of 50 mM aqueous ammonium acetate (pH 3.4) and heptane. The sample was eluted with 95% ethanol (10 ml) and dried under a stream of N_2. The resulting extract was dissolved in a 3:1 mixture of phase A_1/phase B (phase A_1, 95:5:0.1 water/phase B/acetic acid; phase B, 95:5:0.1 acetonitrile/methanol/acetic acid) and subjected to reversed phase HPLC (RP-HPLC) using a Phenomenex Luna C_{18} column (50 X 2.0 mm, 3μm) with a flow of 300 μl/min. An isocratic gradient of 20% phase B for 1 min and then a gradient from 20 to 70% phase B for 26 min were used for the initial RP-HPLC/MS analysis of the samples. The total run time was 32 min. Initially the mass spectrometer was operated in the full scan mode monitoring *m/z* 200-1000 in the negative ion mode. To monitor the elution of radiolabeled metabolites, we employed a post-column splitter and approximately 75% of the sample was directed to a

fraction collector. Fractions were collected every 0.5 min, and the radioactivity of each fraction was determined by scintillation counting. The remaining portion of the sample was directed to the mass spectrometer for ESI-MS analysis to identify the predominate precursor ion *m/z* corresponding to the elution of radiolabeled metabolites.

To provide increased column retention of very polar metabolites eluting 0-2 min with the initial RP-HPLC/MS system, fractions 0-2 min were subjected to a second chromatographic purification by RP-ultra-performance liquid chromatography (UPLC)/MS. For this analysis, fractions 0-2 min were pooled, dried under N_2, and dissolved in a 5:1 mixture of mobile phase A_2/phase B (phase A_2: water/acetic acid, 99.8:0.2; phase B: acetonitrile/methanol/acetic acid, 95:5:0.1). This sample was dried under N_2 briefly to remove the organic solvent and was then subjected to RP-UPLC/MS analysis in negative ion mode employing an Acquity UPLC HSS T3 column (1.0 X 100 mm, 1.8 μm) at a flow rate of 200 μl/min. An isocratic gradient of 100% phase A_2 for 2 min and then a gradient from 0 to 70% phase B for 6 min were used for RP-UPLC/MS analysis of the pooled polar fractions. The total run time was 15 min. Fractions were collected every 1 min, and radioactivity was counted in each fraction.

Analysis of metabolites by LC/MS

Aliquots of media for MS analysis were extracted using a C_{18} Sep-Pak as described above. After extraction, the samples were resuspended in a phase A_1/phase B (3:1) and subjected to LC/MS. Online LC was carried out using a Surveryor MS Pump equipped with a Phenomenex Luna C_{18} column (50 X 2.0 mm, 3μm) utilizing the same chromatographic conditions reported above. For analysis of highly polar metabolites, fractions eluting from the initial RP-HPLC system at 0-2 min were pooled, dried under N_2, resuspended in phase A_2/phase B (5:1), and subjected RP-UPLC/MS analysis with an Acquity UPLC HSS T3 column (1.0 X 100 mm,

1.8 μm) utilizing the same chromatographic condition described above. Samples were analyzed using a ThermoFinnigan TSQ/Quantum Ultra mass spectrometer operating in the negative ion mode. The ESI source was fitted with a deactivated fused silica capillary (100 μm i.d.). Nitrogren was used as both the sheath gas and the auxillary gas, and all parameters were set in order to obtain maximum response. For MS/MS experiments, compounds were collisionally activated between energies of -18 and -27 eV under 1.5 mTorr of argon. Data acquisition and analysis were performed using Xcaliber software, version 2.0.

Hydrolysis of glucuronide conjugates of 15-d-PGJ_2

Small aliquots of media (350 – 500 ml) from the 24 h incubation 15-d-PGJ_2 with primary human hepatocytes were taken for incubation with β-glucuronidase to hydrolyze glucuronic acid from glucuronide metabolites of 15-d-PGJ_2. For this experiment, the media was acidified to pH 5 with 1 N HCl, and 10 μl of β-glucuronidase (~50 units/ml as a 65% saturated ammonium sulfate solution) was added. This sample was incubated over a time course from 0 – 24 h. Subsequently, the sample was extracted by C_{18} Sep-Pak as described above and resuspended in phase A_2/phase B (5:1) and subjected to RP-UPLC/MS analysis utilizing an Acquity UPLC HSS T3 column (1.0 X 100 mm, 1.8 μm) with an isocratic gradient of 100% phase A_2 for 2 min, a gradient of 0-70% phase B for 6 min, and a gradient of 70-100% phase B for 1 min were used for RP-UPLC/MS. The total run time was 18 min. For the analysis of 15-d-PGJ_2 metabolites and hydrolysis products RP-UPLC/MS utilizing selected reaction monitoring (SRM) for the major parent *m/z* to product *m/z* transitions of 15-d-PGJ_2 metabolites was used. The SRM transitions used in this study were obtained from the collision induced dissociation (CID) mass spectra derived from the corresponding metabolites. The SRM transitions monitored were *m/z* 632–456 and *m/z* 632–175 for metabolite B and *m/z* 648–472 and *m/z* 648–175 for metabolite C.

Compounds were collisionally activated at -22 eV under 1.5 mTorr of argon. All other MS analysis conditions and data acquisition were the same as reported above.

Synthesis of conjugates of 15-d-PGJ_2

The cysteine-sulfoxide conjugate of 15-d-PGJ_2, in which the carbonyl at the C-11 position was reduced to a hydroxyl and the Δ^{12} double bond was reduced, was prepared by first incubating 15-d-PGJ_2 (500μg) with ~1 mg of L-cysteine and ~1 mg of gluthatione *S*-transferase (GST, rat liver) in 1 ml phosphate buffered saline, pH 6.5, at 37°C for 2hr (Milne, Gao et al. 2005; Weinander et al. 1994). The reaction mixture was subsequently diluted with deionized water to a total volume of and acidified to pH 3 with 1 N HCl. The conjugate was purified by extraction using C_{18} Sep-Pak as described above. The second step of the reaction was to reduce the carbonyl at the C-11 position of the prostanoid portion of the conjugate to the alcohol and reduce the Δ^{12} double bond. For this reaction, the eluate from the C_{18} Sep-Pak extraction was dried under N_2 and resuspended in 500 μl of dry methanol. 400 ml of an aqueous sodium borohydride solution (12% by weight) was added to this solution at 0°C, and the reaction was allowed to proceed for 30 min. After 30 min, the reaction mixture was diluted to 10 ml with deionized water, acidified to pH 3 with 1 N HCl, and extracted by C_{18} Sep-Pak. In the final step of the synthesis, the cysteinyl sulfur was oxidized to the sulfoxide. To form the sulfoxide, the reduced cysteine conjugate was taken up in 500 ml methanol. 100 ml of 30% hydrogen peroxide was added to the solution at room temperature, and the reaction was allowed to proceed for 30 min. After that time, 2 ml of acetonitrile was added to the solution to act as an azetrope for the water, and the solvent was removed under N_2. Finally, the glucuronide conjugate of 11-hydroxy-12,13-dihydro-15-d-PGJ_2-cysteine-sulfoxide was prepared by incubating the cysteinyl-sulfoxide conjugate with human liver microsomes (25 mg) in the presence of UDP-

glucuronic acid. Again, the conjugate was purified by extraction using C_{18} Sep-Pak as described above. The purity of the product was confirmed by mass spectral analysis.

Results

Time course of metabolism of 15-d-PGJ_2

Initially, we examined the cellular uptake and conjugation of 15-d-PGJ_2 in primary human hepatocytes over time. For these experiments, we incubated 15-d-PGJ_2 containing a tracer amount of [$^3H_6$]15-d-PGJ_2 with freshly isolated human hepatocytes for 24 h. No exogenous GSH was added to the incubation. Aliquots of media were taken at different time intervals, and the extent to which material was present as a water soluble conjugate(s) was determined. The media was acidified to pH 3, and nonpolar compounds were separated from water soluble conjugates by extraction with ethyl acetate. It is expected that polar adducts of 15-d-PGJ_2 that form during metabolism will dissolve in water at pH < 3, while under the same conditions, free 15-d-PGJ_2 and less polar compounds will extract into ethyl acetate. The extent of conjugation was determined by comparing the radioactivity of the organic phase (unconjugated) with that of the aqueous phase (conjugated) (**FIGURE 4.1**). By 24 h over 60% of the radioactive material was present as a water soluble conjugate(s), suggesting that 15-d-PGJ_2 is predominately metabolized in primary human hepatocytes by conjugation to form polar metabolites. Of note also is that, at time 0, virtually all of the radioactivity representing unconjugated 15-d-PGJ_2 extracted into ethyl acetate. Incubation of 15-d-PGJ_2 with primary human hepatocytes for 24 h resulted in recovery of 93% of the radioactivity in the supernatant. The remaining 7% was cell-associated, presumably adducted to intracellular proteins. For purification of the conjugate(s) before LC/MS analysis, small aliquots of cell-free medium were

extracted by C_{18} Sep-Pak as described in "Materials and Methods." Approximately 90% of the radioactivity applied to the Sep-Pak cartridge was eluted in 95% ethanol after washing with 50 mM ammonium acetate, pH 3.4, and heptane.

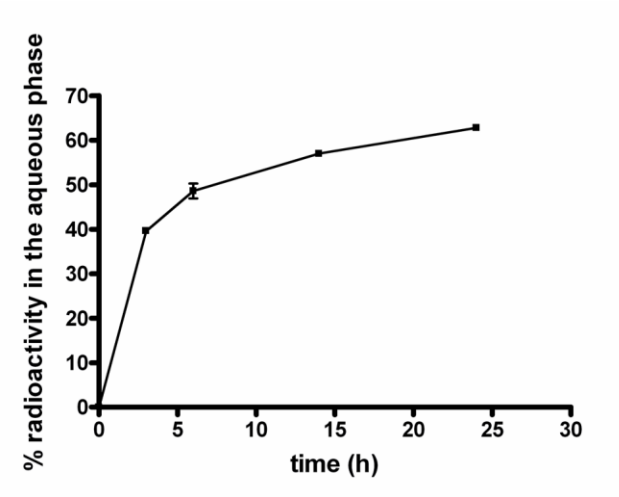


FIGURE 4.1. Time course of [$^3H_6$]15-d-PGJ$_2$ conjugation in primary human hepatocytes. Conjugation was assessed by extraction of [$^3H_6$]15-d-PGJ$_2$ from cell media into ethyl acetate at various time points (*n* = 3 experiments).

Purification and LC/MS analysis of 15-d-PGJ$_2$ metabolites

Cellular extracts were prepared and analyzed by RP-HPLC/ESI-MS as described in "Materials and Methods." The RP-HPLC radiochromatogram of metabolites formed after a 24-h incubation of 15-d-PGJ$_2$ with human hepatocytes is shown in **FIGURE 4.2A**. Twelve (12) unique metabolites were observed from this analysis. Because most of the radiolabel eluted between 0-2 min in the solvent front, it was difficult to identify these metabolites using our initial RP-HPLC system. Other abundant metabolites eluted approximately between 5-12 min, and ESI-MS analysis permitted the structural elucidation of these metabolites.

To provide increased column retention of the highly polar metabolites initially eluting between 0-2 min, fractions 0-2 min were collected and subjected to a second chromatographic purification using RP-UPLC. The RP-UPLC radiochromatogram of these highly polar metabolites is shown in **FIGURE 4.2B**. Using RP-UPLC/ESI-MS analysis, three polar metabolites, labeled A, B, and C, were identified. A summary of all of the 15-d-PGJ$_2$ metabolites that were identified from

the incubation of 15-d-PGJ_2 with primary human hepatocytes is reported in **TABLE 4.1**. Specific details are provided below. Metabolites are labeled according to their RP-HPLC retention time.

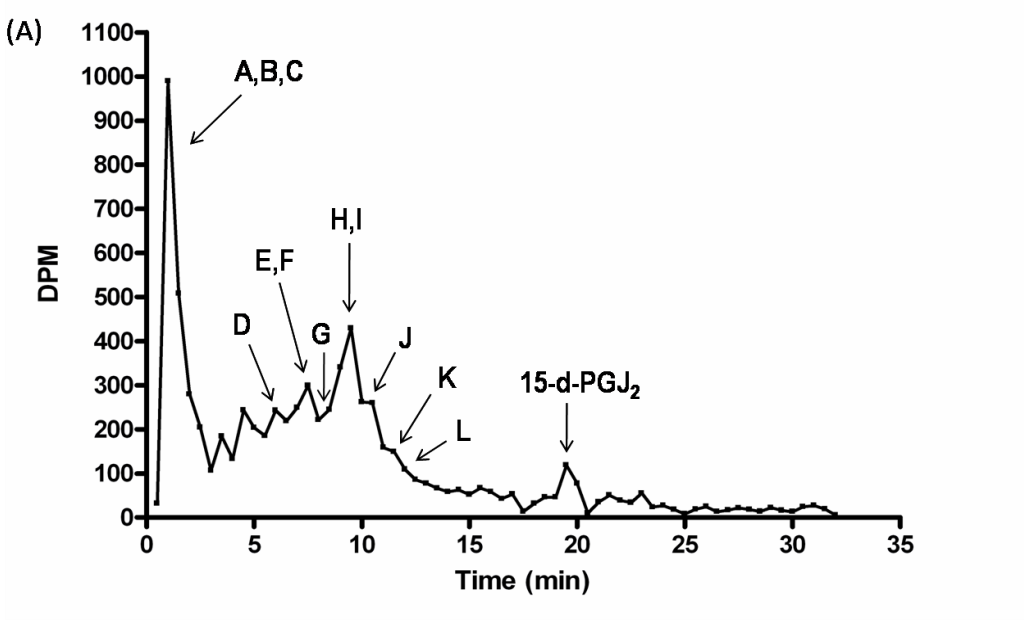


FIGURE 4.2. RP-HPLC and RP-UPLC separation of 15-d-PGJ_2 metabolites produced by primary human hepatocytes. Freshly plated human hepatocytes were incubated with 5 μM 15-d-PGJ_2 (24 μg) containing [$^3H_6$]15-d-PGJ_2 for 24 h. Aliquots of media were extracted by C_{18} Sep-Pak and extracts were subjected to RP-HPLC/MS. Radioactivity was monitored by collecting fractions eluting every 0.5 min and scintillation counting. (A) RP-HPLC radiochromatogram of 15-d-PGJ_2 metabolites. Utilizing LC/MS analysis, 12 unique metabolites of 15-d-PGJ_2 were identified. Metabolites are labeled based on their RP-HPLC retention time. (B) RP-UPLC radiochromatogram of highly polar 15-d-PGJ_2 metabolites initially eluting at 1-2 min. To provide increased column retention for identification of the highly polar metabolites eluting 1-2 min

with the initial RP-HPLC/MS system, fractions collected between 1-2 min were subjected to a second chromatographic purification by RP-ultra performance liquid chromatography (UPLC)/MS employing anAcquity UPLC HSS T3 column. Metabolites A, B, and C eluted as one peak in the radiochromatogram.

	Metabolite	$[M-H]^-$ *m/z*
A	9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ_2	472
B	9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2 glucuronide	632
C	9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ_2 glucuronide	648
D	9-cysteinyl-glycine-11-hydroxy-2,3,4,5-tetranor-15-d-PGJ_2	443
E	9-cysteinyl-glycine-sulfoxide-11-hydroxy-15-d-PGJ_2	511
F	9-cysteinyl-glycine-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2	513
G	9-cysteinyl-glycine-11-hydroxy-15-d-PGJ_2	495
H	9-cysteinyl-sulfoxide-11-hydroxy-15-d-PGJ_2	454
I	9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2	456
J	9-cysteinyl-11-hydroxy-15-d-PGJ_2	438
K	9-glutathionyl-sulfoxide-11-hydroxy-15-d-PGJ_2	640
L	9-glutathionyl-11-hydroxy-15-d-PGJ_2	624

TABLE 4.1. Summary of the observed metabolites of 15-d-PGJ_2 formed after 24-h incubation (5 μM) with primary human hepatocytes. All indicated metabolites were structurally identified and characterized by LC/MS/MS.

Metabolite A, the putative 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ_2

Metabolite A was determined to be a highly polar metabolite because it eluted in the solvent front at 0-2 min using the initial RP-HPLC system (**FIGURE 4.2A**). The RP-UPLC retention time of metabolite A obtained from the second chromatographic purification was 6.7 min with a precursor ion of *m/z* 472 (**FIGURE 4.2B, FIGURE 4.3A**). The CID mass spectrum generated from collisional activation of *m/z* 472 is shown in **FIGURE 4.3B**. The major product ions observed are *m/z* 385 $[M - 87]^-$ (retro-Michael fragmentation), *m/z* 349 $[385 - 2H_2O]^-$, *m/z* 335 [M – cysteine sulfoxide]$^-$, *m/z* 305 $[385 - CO_2 - 2H_2O]^-$, and *m/z* 136 $[M - 336]^-$ (loss of the prostanoid portion of the molecule). On the basis of this CID mass spectrum, metabolite A was identified as the

putative 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ_2. This metabolite is obtained from conjugation of 15-d-PGJ_2 with glutathione, followed by hydrolysis of glutamate and glycine to the cysteine conjugate, oxidation of cysteinyl-sulfur to the sulfoxide, and reduction of carbonyl at C-11 a hydroxyl. Additionally, the C-12,C-13 double bond was reduced, and the C-20 carbon underwent ω-oxidation to form the 20-hydroxyl.

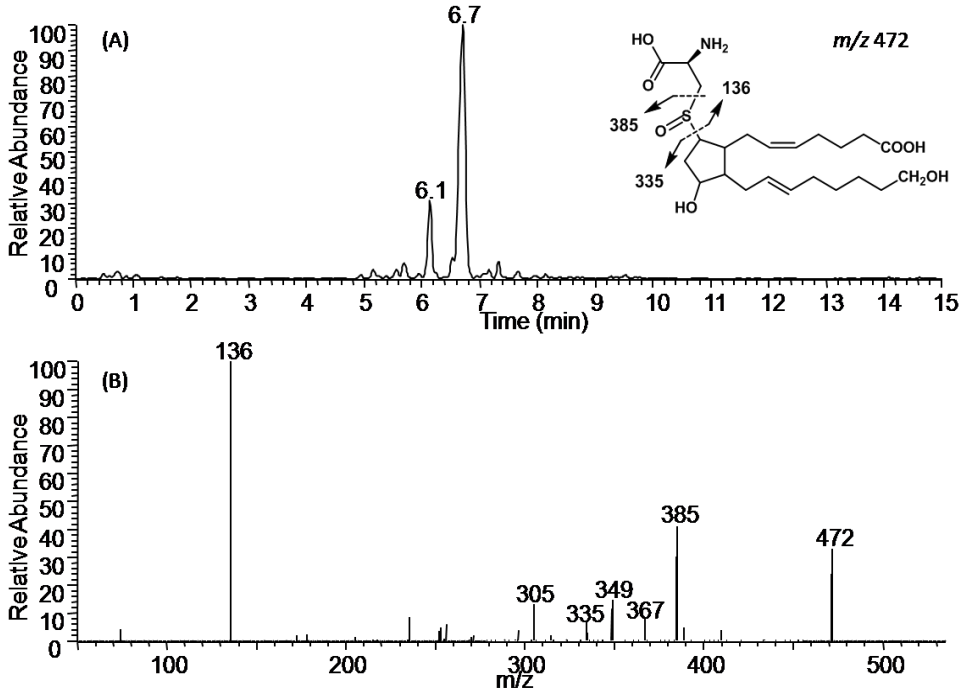


FIGURE 4.3. LC/MS/MS analysis of metabolite A (*m/z* 472), identified as 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ_2. (A) The RP-UPLC retention time of metabolite A obtained from UPLC/MS analysis was 6.7 min with an *m/z* of 472. (B) The CID mass spectrum generated from collisional activation of *m/z* 472 is shown. The major product ions observed are *m/z* 385 $[M - 87]^-$ (retro-Michael fragmentation), *m/z* 349 $[385 - 2H_2O]^-$, *m/z* 335 [M – cysteine sulfoxide]$^-$, *m/z* 305 $[385 - CO_2 - 2H_2O]^-$, and *m/z* 136 $[M - 336]^-$ (loss of the prostanoid portion of the molecule).

Metabolite B, the putative 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2 glucuronide conjugate

Metabolite B was also determined to be a highly polar metabolite because it eluted in the solvent front at 0-2 min using the initial RP-HPLC system (**FIGURE 4.2A**). The RP-UPLC retention time of metabolite B obtained from the second chromatographic purification was 6.6

min with a precursor ion of *m/z* 632 (**FIGURE 4.2B**, **FIGURE 4.4A**). The CID mass spectrum generated from collisional activation of the molecular anion *m/z* 632 is shown in **FIGURE 4.4B**. The major product ions observed are *m/z* 545 $[M - 87]^-$ (retro-Michael fragmentation), *m/z* 495 $[M - \text{cyseinly-sulfoxide}]^-$, *m/z* 456 $[M - 176]^-$ (loss of glucuronic acid to yield the aglycone anion), and *m/z* 175 [M – 457]- (decomposition of the molecular anion to the glucuronide). The exact position of the glucuronide attachment could not be determined from this analysis. On the basis of this CID mass spectrum, metabolite B was determined to be the glucuronide conjugate of 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2. This metabolite is obtained from conjugation of 15-d-PGJ_2 with glutathione, followed by hydrolysis of glutamate and glycine to the cysteine conjugate, oxidation of cysteinyl-sulfur to the sulfoxide, and reduction of carbonyl at C-11 a hydroxyl. Additionally, the C-12,C-13 double bond was reduced, and the C-11 hydroxyl or the C-1 carboxyl was conjugated with glucuronic acid.

Further confirmation of the correct identification of metabolite B as the glucuronide conjugate of 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2 was obtained through synthesis of the glucuronide conjugate by incubation of 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2 with human liver microsomes (HLMs) and UDP-glucuronic acid. For analysis of the 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2 glucuronide, we developed a UPLC/MS/MS method utilizing selected reaction monitoring (SRM) for the major parent to product transitions for metabolite B: *m/z* 632–456 and *m/z* 632–175. When the synthetic glucuronide conjugate was analyzed by UPLC/MS/MS utilizing this SRM method, it possessed the same RP-UPLC retention time as metabolite B. Additionally, treatment of the hepatocyte media (containing metabolite B, the 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2 glucuronide) with β-glucuronidase to hydrolyze the glucuronic acid and form the aglycone

metabolite resulted in disappearance of the signal for metabolite B (*m/z* 632–456 and *m/z* 632–175) from the SRM analysis (data not shown).

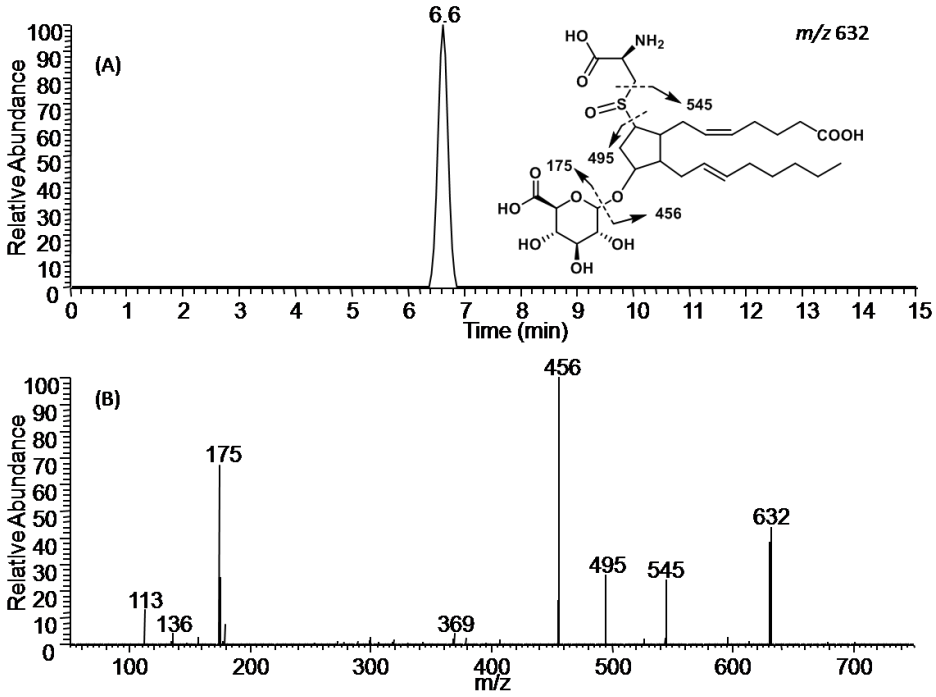


FIGURE 4.4. LC/MS/MS analysis of metabolite B (*m/z* 632), identified as 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ$_2$ glucuronide. (A) The RP-UPLC retention time of metabolite B obtained from UPLC/MS analysis was 6.6 min with an *m/z* of 632. (B) The CID mass spectrum generated from collisional activation of *m/z* 632 is shown. The major product ions observed are *m/z* 545 [M – 87]$^-$ (retro-Michael fragmentation), *m/z* 495 [M – cyseinly-sulfoxide]$^-$, *m/z* 456 [M – 176]$^-$ (loss of glucuronic acid to yield the aglycone anion), and *m/z* 175 [M – 457]- (decomposition of the molecular anion to the glucuronide). The exact position of the glucuronide attachment could not be determined from this analysis.

Metabolite C, the putative 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ$_2$ glucuronide conjugate

Similar to metabolites A and B, metabolite C was determined to be a highly polar metabolite because it eluted in the solvent front at 0-2 min using the initial RP-HPLC system (**FIGURE 4.2A**). The RP-UPLC retention time of metabolite A obtained from the second chromatographic purification was 6.5 min with a molecular anion at *m/z* 648 (**FIGURE 4.2B**, **FIGURE 4.5A**). The CID mass spectrum generated from collisional activation of the molecular

anion *m/z* 648 is shown in **FIGURE 4.5B**. The major product ions observed are *m/z* 561 $[M - 87]^-$ (retro-Michael fragmentation), *m/z* 511 [M – cyseinly-sulfoxide]$^-$, *m/z* 472 $[M - 176]^-$ (loss of glucuronic acid to yield the aglycone anion), *m/z* 175 $[M - 457]^-$ (decomposition of the molecular anion to the glucuronide), and *m/z* 136 $[M - 512]^-$ (fragmentation of cysteinyl sulfoxide from the molecular anion). The exact position of the glucuronide attachment could not be determined from this analysis. On the basis of this CID mass spectrum, metabolite C was determined to be the glucuronide conjugate of 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ_2. This metabolite is obtained by conjugation of 15-d-PGJ_2 with glutathione, followed by hydrolysis of glutamate and glycine to the cysteine conjugate, oxidation of cysteinyl-sulfur to the sulfoxide, and reduction of carbonyl at C-11 a hydroxyl. Additionally, the C-12,C-13 double bond was reduced, the C-20 carbon underwent ω-oxidation to form the 20-hydroxyl, and the C-11 or C-20 hydroxyl or the C-1 carboxyl was conjugated with glucuronic acid.

To further confirm that we had identified metabolite C correctly, we developed an UPLC/MS/MS method utilizing SRM for the major parent to product transitions for metabolite C: *m/z* 648–472 and *m/z* 648–175. Treatment of the hepatocyte media (containing metabolite C, the 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2 glucuronide) with β-glucuronidase to hydrolyze of the glucuronic acid and form the aglycone metabolite resulted in significant reduction in the signal for metabolite C (*m/z* 648–472 and *m/z* 648–175) from the SRM analysis (data not shown).

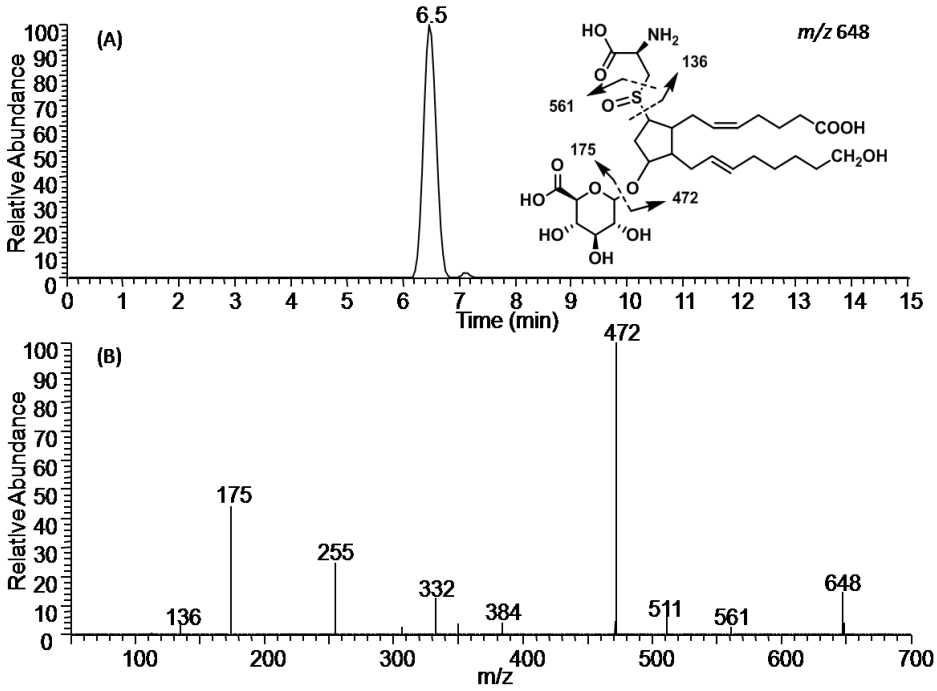


FIGURE4.5. LC/MS/MS analysis of metabolite C (*m/z* 648), identified as 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ$_2$ glucuronide. (A) The RP-UPLC retention time of metabolite C obtained from UPLC/MS analysis was 6.5 min with an *m/z* of 648. (B) The CID mass spectrum generated from collisional activation of *m/z* 648 is shown. The major product ions observed are *m/z* 561 [M – 87]$^-$ (retro-Michael fragmentation), *m/z* 511 [M – cyseinly-sulfoxide]$^-$, *m/z* 472 [M – 176]$^-$ (loss of glucuronic acid to yield the aglycone anion), *m/z* 175 [M – 457]$^-$ (decomposition of the molecular anion to the glucuronide), and m/z 136 [M – 512]$^-$ (fragmentation of cysteinyl sulfoxide from the molecular anion). The exact position of the glucuronide attachment could not be determined from this analysis.

Metabolite D, the putative 9-cysteinyl-glycine-11-hydroxy-12,13-dihydro-2,3,4,5-tetranor-15-d-PGJ$_2$

A relatively minor yet novel metabolite was observed with the RP-HPLC retention time of 5-6 min. This metabolite had a molecular anion at *m/z* 443. **FIGURE 4.6** shows the CID mass spectrum of the molecular anion *m/z* 443 generated by electrospray ionization. The major product ions observed are *m/z* 299 [M – 144]$^-$ (retro-Michael fragmentation), *m/z* 265 [M – cysteinyl-glycine]$^-$, and *m/z* 143 [M – 300]$^-$ (loss of the prostanoid portion of the molecule). On the basis of this CID mass spectrum, metabolite D was determined to be the 9-cysteinyl-glycine-11-hydroxy-12,13-dihydro-2,3,4,5-tetranor-15-d-PGJ$_2$. This metabolite is obtained from

conjugation of 15-d-PGJ_2 with glutathione, followed by hydrolysis of glutamate to the cysteinyl-glycine conjugate, reduction of the carbonyl at C-11 to a hydroxyl, and β-oxidation of the prostanoid portion of the molecule. Additionally, the C-12,C-13 double bond was reduced.

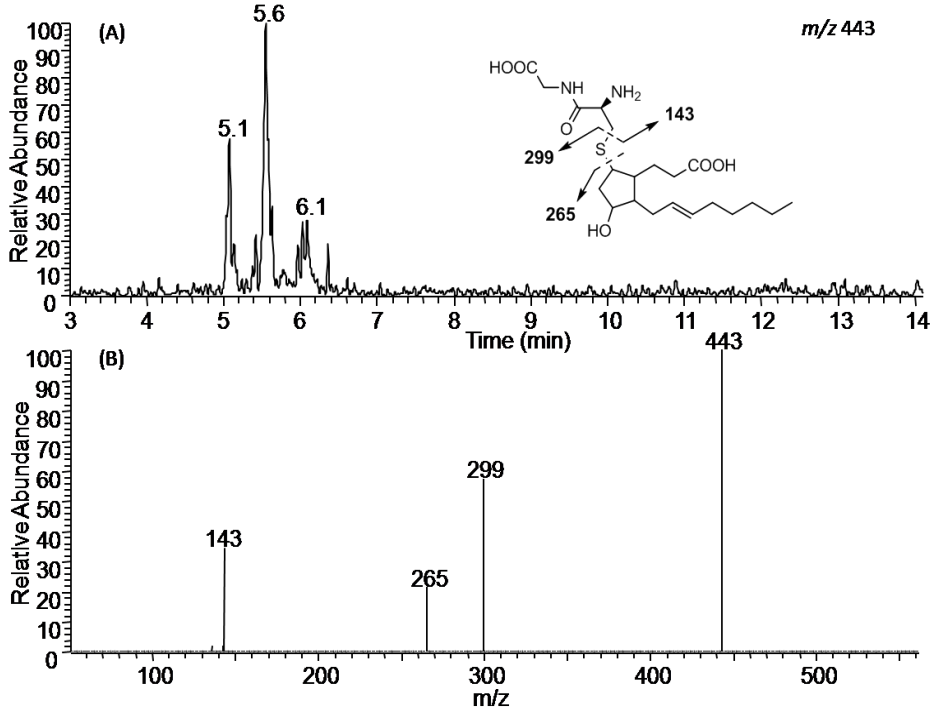


FIGURE 4.6. LC/MS/MS analysis of metabolite D (*m/z* 443), identified as 9-cysteinyl-glycine-11-hydroxy-12,13-dihydro-2,3,4,5-tetranor-15-d-PGJ_2. (A) The RP-HPLC retention time of metabolite D obtained from HPLC/MS analysis was 5-6 min with an *m/z* of 443. (B) The CID mass spectrum generated from collisional activation of *m/z* 443 is shown. The major product ions observed are *m/z* 299 $[M - 144]^-$ (retro-Michael fragmentation), *m/z* 265 [M – cysteinyl-glycine]$^-$ and *m/z* 143 $[M - 300]^-$ (loss of the prostanoid portion of the molecule).

Metabolite E, the putative 9-cysteinyl-glycine-sulfoxide-11-hydroxy-15-d-PGJ_2

The RP-HPLC retention time of metabolite E obtained from HPLC/MS analysis was 7.4-7.7 min with an *m/z* of 511. The CID mass spectrum generated from collisional activation of *m/z* 511 is shown in **FIGURE 4.7**. The major product ionsobserved are *m/z* 367 $[M - 144]^-$ (retro-Michael fragmentation), *m/z* 317 [M – cysteinyl-glycine sulfoxide], *m/z* 193 $[M - 318]^-$ (loss of the prostanoid portion of the molecule), and *m/z* 143 $[M - 368]^-$ (retro-Michael fragmentation). On the basis of this CID mass spectrum, metabolite E was determined to be the 9-cysteinyl-

glycine-sulfoxide-11-hydroxy-15-d-PGJ_2. This metabolite is obtained from conjugation of 15-d-PGJ_2 with glutathione, followed by hydrolysis of glutamate to the cysteinyl-glycine conjugate, oxidation of cysteinyl-sulfur to the sulfoxide, and reduction of the carbonyl at C-11 to a hydroxyl.

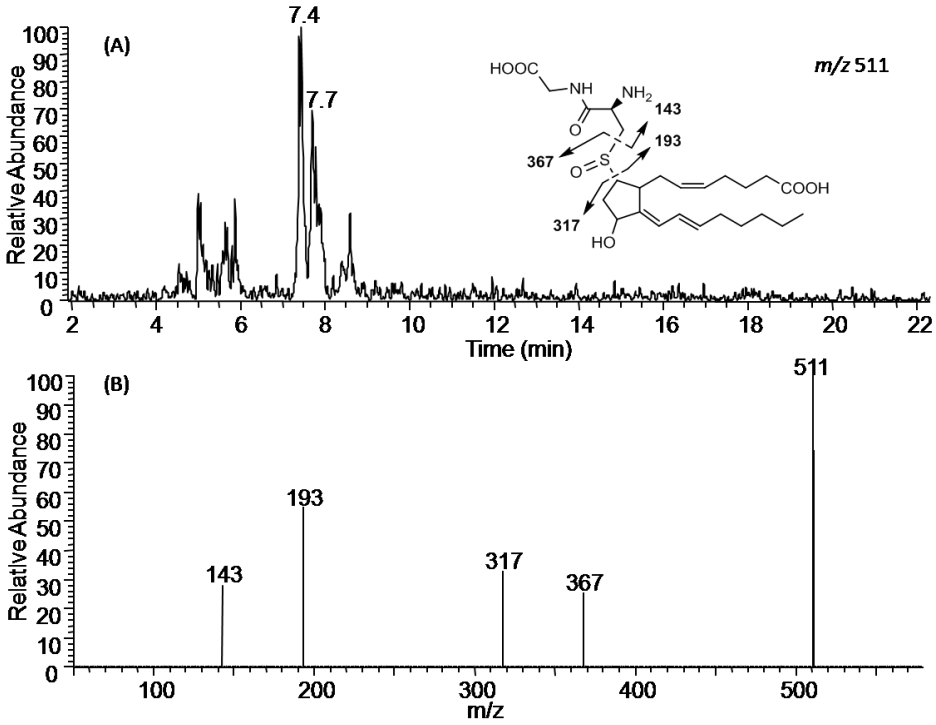


FIGURE 4.7. LC/MS/MS analysis of metabolite E (*m/z* 511), identified as 9-cysteinyl-glycine-sulfoxide-11-hydroxy-15-d-PGJ_2. (A) The RP-HPLC retention time of metabolite E obtained from HPLC/MS analysis was 7.4-7.7 min with an *m/z* of 511. (B) The CID mass spectrum generated from collisional activation of *m/z* 511 is shown. The major product ions observed are *m/z* 367 $[M - 144]^-$ (retro-Michael fragmentation), *m/z* 317 [M – cysteinyl-glycine sulfoxide], *m/z* 193 $[M - 318]^-$ (loss of the prostanoid portion of the molecule), and *m/z* 143 $[M - 368]^-$ (retro-Michael fragmentation).

Metabolite F, the putative 9-cysteinyl-glycine-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2

Metabolite F had a slightly longer RP-HPLC retention time than metabolite E (9-cysteinyl-glycine-sulfoxide-11-hydroxy-15-d-PGJ_2) and displayed a molecular anion at *m/z* 513, 2 Da higher than metabolite E. This suggested the saturation of one double bond in the conjugated diene of 9-cysteinyl-glycine-sulfoxide-11-hydroxy-15-d-PGJ_2. The CID mass spectrum generated from collisional activation of *m/z* 513 is shown in **FIGURE 4.8**. The major product ions

observed are *m/z* 369 $[M - 144]^-$ (retro-Michael fragmentation), *m/z* 333 $[369 - 2H_2O]^-$, *m/z* 193 $[M - 320]^-$ (loss of the prostanoid portion of the molecule), and *m/z* 143 $[M - 370]^-$ (retro-Michael fragmentation). These data were consistent with the identification of metabolite F as the putative 9-cysteinyl-glycine-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2. This metabolite is obtained from conjugation of 15-d-PGJ_2 with glutathione, followed by hydrolysis of glutamate to the cysteinyl-glycine conjugate, oxidation of cysteinyl-sulfur to the sulfoxide, and reduction of the carbonyl at C-11 to a hydroxyl. Additionally, the C-12,C-13 double bond was reduced.

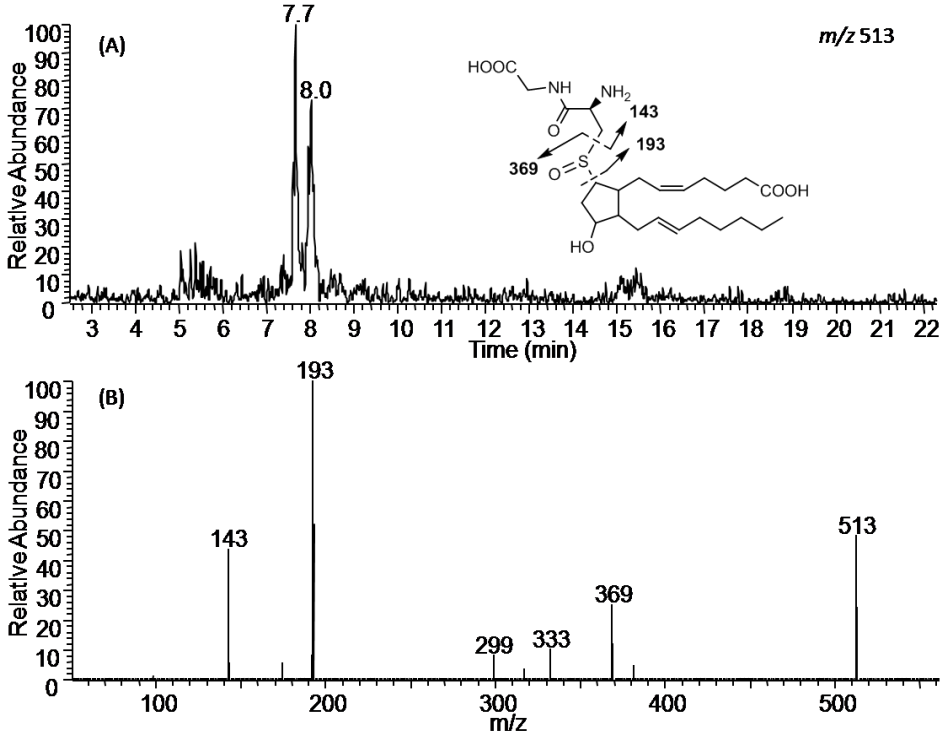


FIGURE 4.8. LC/MS/MS analysis of metabolite F (*m/z* 513), identified as 9-cysteinyl-glycine-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2. (A) The RP-HPLC retention time of metabolite F obtained from HPLC/MS analysis was 7.7-8.0 min with an *m/z* of 513. (B) The CID mass spectrum generated from collisional activation of *m/z* 513 is shown. The major product ions observed are *m/z* 369 $[M - 144]^-$ (retro-Michael fragmentation), *m/z* 333 $[369 - 2H_2O]^-$, *m/z* 193 $[M - 320]^-$ (loss of the prostanoid portion of the molecule), and *m/z* 143 $[M - 370]^-$ (retro-Michael fragmentation).

Metabolite G, the putative 9-cysteinyl-glycine-11-hydroxy-15-d-PGJ_2

The RP-HPLC retention time of metabolite G obtained from HPLC/MS analysis was 8.5 min with an *m/z* of 495. These data are consistent with the previously identified cysteinyl-glycine conjugate of 15-d-PGJ_2 in which the C-11 carbonyl was reduced to a hydroxyl (Brunoldi et al. 2007). The CID mass spectrum generated from collisional activation of *m/z* 495 is shown in **FIGURE 4.9**, and nearly identical to that of the 9-cysteinyl-glycine-11-hydroxy-15-d-PGJ_2 metabolite previously reported (Brunoldi et al. 2007). The major product ions observed are *m/z* 351 $[M - 144]^-$ (retro-Michael fragmentation), *m/z* 317 [M – cysteinyl-glycine]$^-$, *m/z* 255 $[317 - CO_2 - H_2O]^-$, and *m/z* 143 $[M - 352]^-$ (retro-Michael fragmentation). On the basis of this CID mass spectrum, metabolite G was determined to be the 9-cysteinyl-glycine-11-hydroxy-15-d-PGJ_2. This metabolite is obtained from conjugation of 15-d-PGJ_2 with glutathione, followed by hydrolysis of glutamate to the cysteinyl-glycine conjugate, and reduction of the carbonyl at C-11 to a hydroxyl.

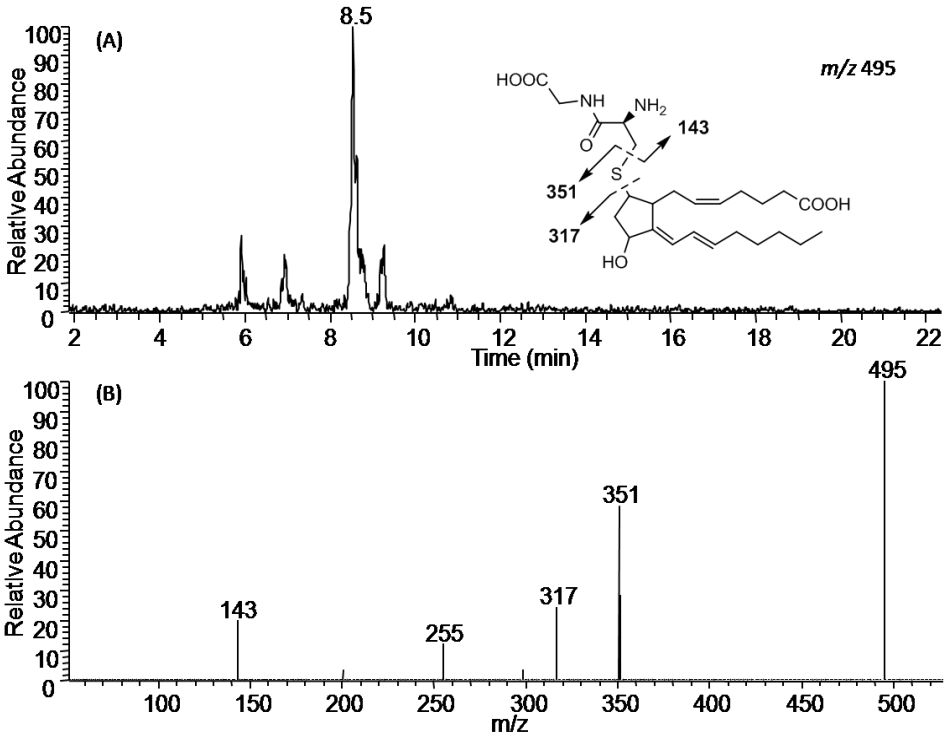


FIGURE 4.9. LC/MS/MS analysis of metabolite G (*m/z* 495), identified as 9-cysteinyl-glycine-11-hydroxy-15-d-PGJ$_2$. (A) The RP-HPLC retention time of metabolite G obtained from HPLC/MS analysis was 8.5 min with an *m/z* of 495. (B) The CID mass spectrum generated from collisional activation of *m/z* 495 is shown. The major product ions observed are *m/z* 351 $[M - 144]^-$ (retro-Michael fragmentation), *m/z* 317 $[M$ – cysteinyl-glycine$]^-$, *m/z* 255 $[317 - CO_2 - H_2O]^-$, and *m/z* 143 $[M - 352]^-$ (retro-Michael fragmentation).

Metabolite H, the putative 9-cysteinyl-sulfoxide-11-hydroxy-15-d-PGJ$_2$

Metabolite H had a RP-HPLC retention time of 9.5min with an *m/z* of 454. **FIGURE 4.10** shows the CID mass spectrum of the precursor ion *m/z* 454 generated by electrospary ionization. The major product ions observed are *m/z* 317 $[M$ – cysteine sulfoxide$]^-$, *m/z* 299 $[317 - H_2O]^-$, *m/z* 273 $[317 - CO_2]^-$, *m/z* 255 $[299 - CO_2]^-$, and *m/z* 136 $[M - 318]^-$ (loss of the prostanoid portion of the molecule). On the basis of this mass spectrum, metabolite H was determined to be the cysteine conjugate of 15-d-PGJ$_2$ in which the C-11 carbonyl was reduced to a hydroxyl and the cysteinyl sulfur was oxidized to the sulfoxide (9-cysteinyl-sulfoxide-11-hydroxy-15-d-PGJ$_2$). Further, the RP-HPLC retention time and CID spectrum of this metabolite at

m/z 454 were identical with the synthetic standard of 15-d-PGJ_2-cysteinyl-sulfoxide in which the C-11 carbonyl was reduced to a hydroxyl. This metabolite is obtained from conjugation of 15-d-PGJ_2 with L-cysteine, followed oxidation of the cysteinyl-sulfur to the sulfoxide, and reduction of the carbonyl at C-11 to a hydroxyl.

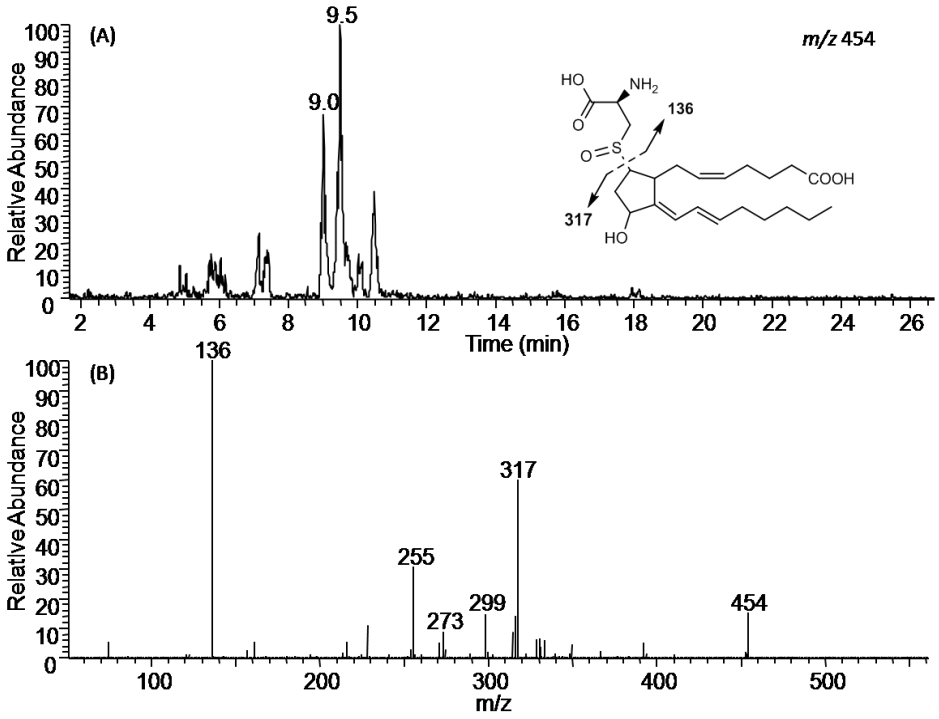


FIGURE 4.10. LC/MS/MS analysis of metabolite H (*m/z* 454), identified as 9-cysteinyl-sulfoxide-11-hydroxy-15-d-PGJ_2. (A) The RP-HPLC retention time of metabolite H obtained from HPLC/MS analysis was 9.0-9.5 min with an *m/z* of 454. (B) The CID mass spectrum generated from collisional activation of *m/z* 454 is shown. The major product ions observed are *m/z* 317 [M – cysteine sulfoxide]$^-$, *m/z* 299 [317 – H_2O]$^-$, *m/z* 273 [317 – CO_2]$^-$, *m/z* 255 [299 – CO_2]$^-$, and *m/z* 136[M – 318]$^-$ (loss of the prostanoid portion of the molecule).

Metabolite I, the putative 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2

Metabolite I had a slightly longer RP-HPLC retention time than metabolite H and displayed a molecular anion at *m/z* 456, 2 Da higher than metabolite H (9-cysteinyl-sulfoxide-11-hydroxy-15-d-PGJ_2). This suggested the saturation of one double bond in the conjugated diene of 9-cysteinyl-sulfoxide-11-hydroxy-15-d-PGJ_2. The CID mass spectrum generated from collisional activation of *m/z* 456 is shown in **FIGURE 4.11**. The major product ions observed are

m/z 369 [M – 87]$^-$ (retro-Michael fragmentation), *m/z* 351 [369 – H_2O]$^-$, *m/z* 333 [351 – H_2O]$^-$, and *m/z* 136 [M – 320]$^-$ (loss of the prostanoid portion of the molecule). On the basis of this CID mass spectrum, metabolite I was determined to be the 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2. This metabolite is obtained from conjugation of 15-d-PGJ_2 with glutathione, followed by hydrolysis of glutamate and glycine to the cysteine conjugate, oxidation of the cysteinyl-sulfur to the sulfoxide, and reduction of the carbonyl at C-11 to a hydroxyl. Additionally, the C-12,C-13 double bond was reduced.

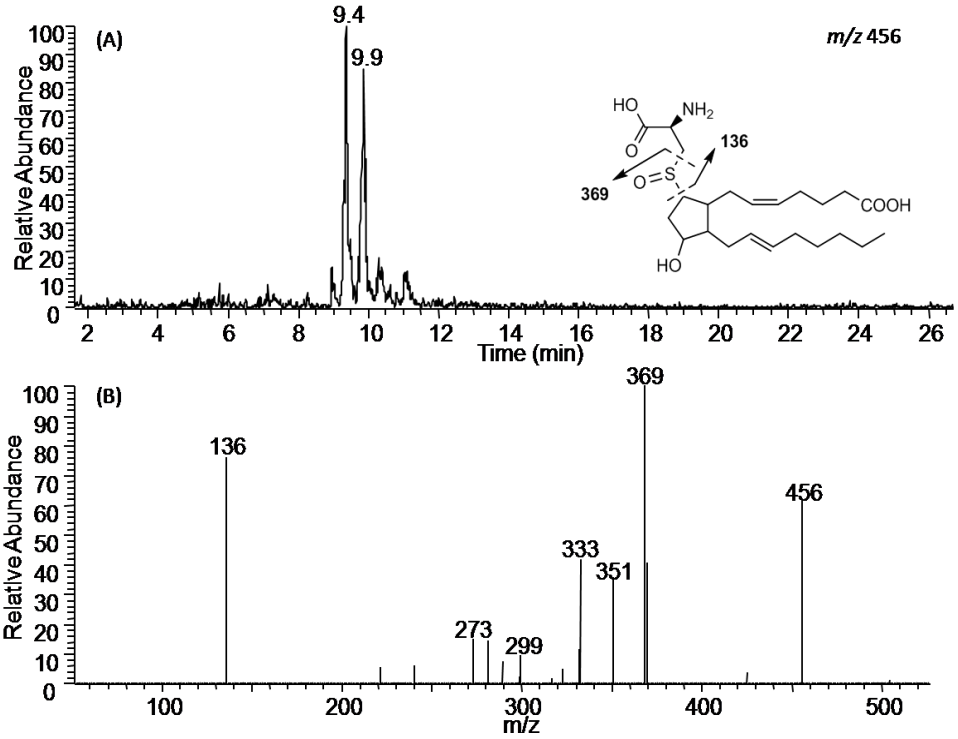


FIGURE 4.11. LC/MS/MS analysis of metabolite I (*m/z* 456), identified as 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2. (A) The RP-HPLC retention time of metabolite I obtained from HPLC/MS analysis was 9.4-9.9 min with an *m/z* of 456. (B) The CID mass spectrum generated from collisional activation of *m/z* 456 is shown. The major product ions observed are *m/z* 369 [M – 87]$^-$ (retro-Michael fragmentation), *m/z* 351 [369 – H_2O]$^-$, *m/z* 333 [351 – H_2O]$^-$, and *m/z* 136 [M – 320]$^-$(loss of the prostanoid portion of the molecule).

Metabolite J, the putative 9-cysteinyl-11-hydroxy-15-d-PGJ_2

The RP-HPLC retention time of metabolite G obtained from HPLC/MS analysis was 8.5 min with an *m/z* of 438. These data are consistent with the previously identified cysteine conjugate of 15-d-PGJ_2 in which the C-11 carbonyl was reduced to a hydroxyl (Brunoldi et al. 2007). The CID mass spectrum generated from collisional activation of *m/z* 438 is shown in **FIGURE 4.12**, and is nearly identical to that of the 9-cysteinyl-11-hydroxy-15-d-PGJ_2 metabolite previously reported (Brunoldi et al. 2007). The major product ions observed are *m/z* 351 $[M - 87]^-$ (retro-Michael fragmentation), *m/z* 317 $[M - \text{cysteine}]^-$, *m/z* 299 $[317 - H_2O]^-$, and *m/z* 120 $[M - 318]^-$ (loss of the prostanoid portion of the molecule). On the basis of this CID mass spectrum, metabolite J was identified as the 9-cysteinyl-11-hydroxy-15-d-PGJ_2. Further support for the identification of metabolite J as the 15-d-PGJ_2-cysteine conjugate in which the carbonyl is reduced to a hydroxyl came from LC/MS/MS analysis of the synthetic standard of this conjugate, which demonstrated a virtually identical RP-HPLC retention time and CID spectrum (data not shown). This metabolite is obtained from conjugation of 15-d-PGJ_2 with L-cysteine and reduction of the carbonyl at C-11 to a hydroxyl.

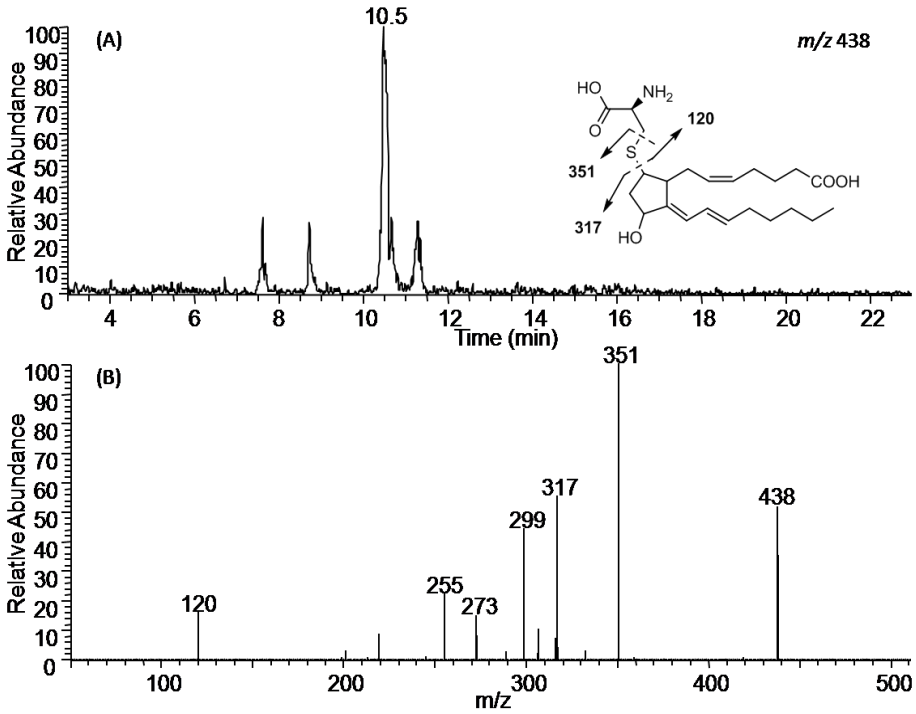


Figure 4.12. LC/MS/MS analysis of metabolite J (*m/z* 438), identified as 9-cysteinyl-11-hydroxy-15-d-PGJ$_2$. (A) The RP-HPLC retention time of metabolite J obtained from HPLC/MS analysis was 10.5 min with an *m/z* of 438. (B) The CID mass spectrum generated from collisional activation of *m/z* 438 is shown. The major product ions observed are *m/z* 351 $[M - 87]^-$ (retro-Michael fragmentation), *m/z* 317 $[M - \text{cysteine}]^-$, *m/z* 299 $[317 - H_2O]^-$, and *m/z* 120 $[M - 318]^-$ (loss of the prostanoid portion of the molecule).

Metabolite K, the putative 9-glutathionyl-sulfoxide-11-hydroxy-15-d-PGJ$_2$

Metabolite K had a RP-HPLC retention time of 11.4 min and with an *m/z* of 640. The CID mass spectrum generated from collisional activation of *m/z* 640 is shown in **FIGURE 4.13**. The major product ions observed are *m/z* 322 $[M - 318]^-$ (loss of the prostanoid portion of the molecular), m/z 304 $[322 - H_2O]^-$, and *m/z* 272 (retro-Michael fragmentation). On the basis of this CID mass spectrum, metabolite K was identified as the 9-glutathionyl-sulfoxide-11-hydroxy-15-d-PGJ$_2$. Further, the RP-HPLC retention time and CID spectrum of metabolite K at *m/z* 640 were identical with the synthetic standard of 9-glutathionyl-sulfoxide-11-hydroxy-15-d-PGJ$_2$. This metabolite is obtained from conjugation of 15-d-PGJ$_2$ with glutathione, followed by

oxidation of the cysteinyl-sulfur to the sulfoxide, and reduction of the carbonyl at C-11 to a hydroxyl.

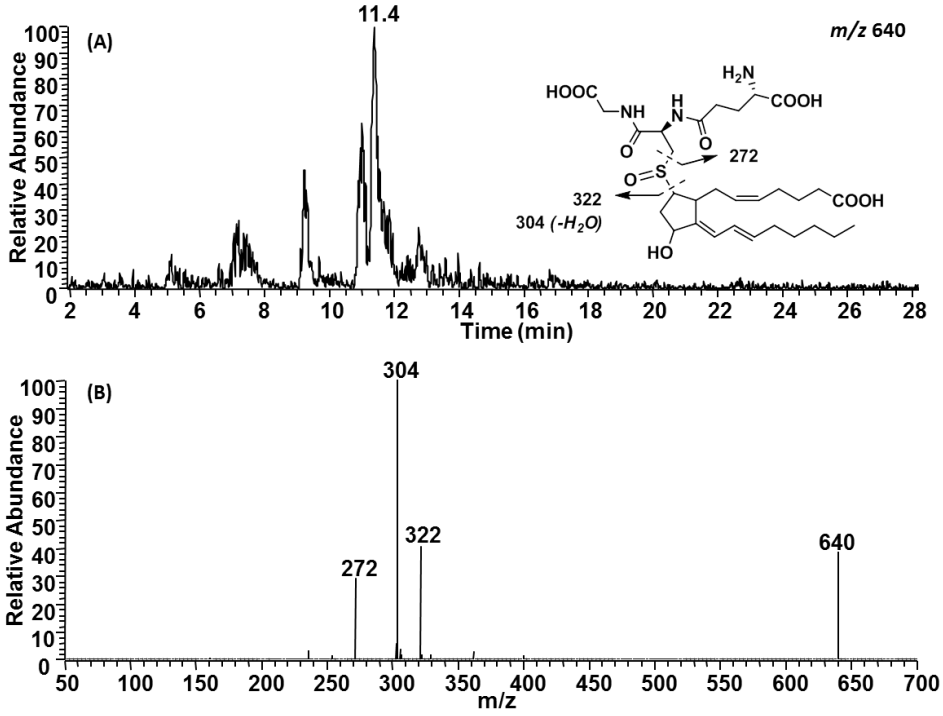


Figure 4.13. LC/MS/MS analysis of metabolite K (*m/z* 640), identified as 9-glutathionyl-sulfoxide-11-hydroxy-15-d-PGJ$_2$. (A) The RP-HPLC retention time of metabolite K obtained from HPLC/MS analysis was 11.4 min with an *m/z* of 640. (B) The CID mass spectrum generated from collisional activation of *m/z* 640 is shown. The major product ions observed are *m/z* 322 [M – 318]$^-$ (loss of the prostanoid portion of the molecular), *m/z* 304 [322 – H_2O]$^-$, and *m/z* 272 (retro-Michael fragmentation).

Metabolite L, the putative 9-glutathionyl-11-hydroxy-15-d-PGJ$_2$

In addition, the previously identified glutathione conjugate of 15-d-PGJ$_2$ in which the C-11 carbonyl was reduced to a hydroxyl was also identified in this study; however, this metabolite was present at relatively low abundance compared to the other metabolites observed. The RP-HPLC retention time at 12.7 min and molecular anion at *m/z* 624 were identical to the glutathione conjugate of 15-d-PGJ$_2$ in which the C-11 carbonyl was reduced to a hydroxyl, 9-glutathionyl-11-hydroxy-15-d-PGJ$_2$ (data not shown). The CID mass spectrum of the metabolite

9-glutathionyl-11-hydroxy-15-d-PGJ_2 has been reported previously (Brunoldi et al. 2007) and contains the same fragment ions observed in the CID spectrum of the metabolite with the molecular ion *m/z* 624 identified in the present study (data not shown). The major product ions observed were *m/z* 606 [M – H_2O]-, *m/z* 495 [M – 129]- (loss of glutamic acid from GSH), *m/z* 351 [M – 273]$^-$ (retro-Michael fragmentation), *m/z* 272 [M – 352]$^-$ (retro-Michael fragmentation), *m/z* 254 [272 – H_2O]$^-$, and *m/z* 210 [254 – CO_2]$^-$ (data not shown).

Discussion

In this study, we have examined the metabolic transformation of 15-d-PGJ_2 by primary human hepatocytes to identify pathways that may be involved in the metabolism of 15-d-PGJ_2 *in vivo* in humans. Additionally, we sought to identify metabolite(s) of 15-d-PGJ_2 formed in primary human hepatocytes that may be used as a potential biomarker to assess the *in vivo* production of 15-d-PGJ_2 in humans. Primary human hepatocytes are considered the *in vitro* model of choice for predicting xenobiotic metabolism *in vivo* in humans as these cells are capable of expressing the complete metabolic pathways occurring *in vivo* in human liver. Thus, primary human hepatocytes are frequently used as surrogate models for studying xenobiotic metabolism *in vivo* in humans.

Biosynthesis of the reactive cyclopentenone prostaglandin 15-d-PGJ_2 is biologically relevant because this molecule is thought to be an important lipid mediator. 15-d-PGJ_2, a dehydration of product of PGD_2, exhibits a broad spectrum of biological activities unique from other PGs. This molecule possesses two electrophilic α,β-unsaturated carbonyl moieties, which render it susceptible to Michael addition with cellular thiols, such as GSH and cysteine resides of proteins. The biological effects of 15-d-PGJ_2 are attributed to its reactivity with key protein targets, such as transcription factors and other signaling molecules. In studies performed *in*

vitro, 15-d-PGJ_2 has been shown to activate peroxisome proliferator-activated receptor-γ (PPARγ) (Forman et al. 1995; Kliewer et al. 1995) and exerts potent anti-inflammatory, anti-proliferative, and pro-apoptotic activity through PPARγ-dependent and independent mechanisms. However, the extent to which 15-d-PGJ_2 is formed *in vivo* is unclear. Moreover, the physiological relevance of 15-d-PGJ_2 has been the subject of continuing controversy due to an inability to assess its biosynthesis *in vivo*. This may be due in part to fact that previous studies have attempted to measure free 15-d-PGJ_2 in biological fluids. Trivial amounts of free 15-d-PGJ_2 have been reported in human urine, and the amounts detected are regarded as insufficient to yield biological effects (Bell-Parikh et al. 2003). Because of its reactivity, however, measurement of free 15-d-PGJ_2 in biological fluids may lead to an underestimation of the total amount produced *in vivo.* Thus, it is important to develop a specific biomarker for the endogenous production of 15-d-PGJ_2.

Our results in the present study indicate that multiple processes are involved in the metabolism of 15-d-PGJ_2 by primary human hepatocytes. These pathways include conjugation of 15-d-PGJ_2 with GSH and reduction of the Δ^{12} double bond, which have been described previously (Brunoldi et al. 2007; Yu et al. 2006). In addition, we have identified new pathways for the metabolic transformation of 15-d-PGJ_2 which lead to the formation of several novel metabolites. Electrospray ionization tandem mass spectrometry (ESI/MS/MS) provided critical information to suggest structural assignment of these metabolites. This information, in combination with reverse phase HPLC or UPLC retention time, was used to identify twelve (12) unique metabolites of 15-d-PGJ_2 formed by primary human hepatocytes after *in vitro* incubation (**TABLE 4.1**). The four (4) most abundant metabolites identified were 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2, 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ_2, and their corresponding glucuronide conjugates (**FIGURE 4.2A**). Through the

identification of all twelve (12) metabolites, we have proposed a pathway for the metabolism of 15-d-PGJ_2 in primary human hepatocytes (**FIGURE 4.14**).

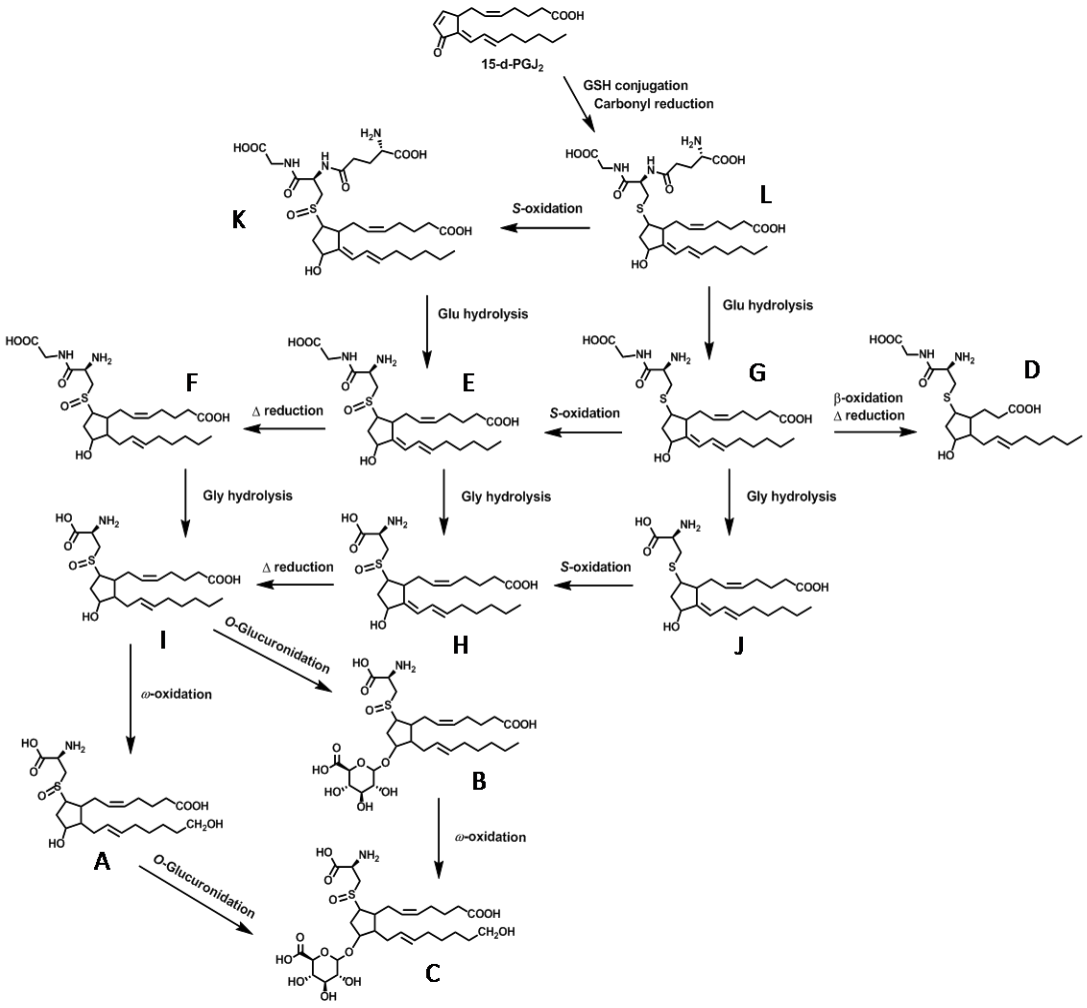


Figure 4.14. Summary of possible metabolic pathways involved in the observed metabolites of 15-d-PGJ_2 formed by primary human hepatocytes. All indicated metabolites were structurally identified and characterized by LC/MS/MS. The letters A-L designate metabolites in **TABLE 4.1.**

The first step in the metabolism of 15-d-PGJ_2 is likely conjugation with GSH via Michael addition. The formation of GSH adducts of cyclopentenone-containing molecules is strongly promoted by glutathione *S*-transferases (GSTs) (Brunoldi et al. 2007), but may also occur nonenzymatically (Meister and Anderson 1983). Conjugation likely occurs rapidly due to millimolar concentrations of intracellular GSH (Meister and Anderson 1983). 15-d-PGJ_2 contains

two electrophilic carbon centers, carbons C-9 and C-13, which are subject to nucleophilic attack by GSH. However, previous studies have shown that GSH preferentially conjugates 15-d-PGJ_2 at the C-9 carbon (Paumi et al. 2003). Conjugation of GSH is likely followed by reduction of the carbonyl at C-11 on the eicosanoid portion of the molecule to the hydroxyl. If the carbonyl at C-11 was reduced before conjugation with GSH, the molecule would lack the α,β-unsaturated ketone group which is key for adduction with GSH. Moreover, reduction of the C-11 carbonyl may represent an important step in the irreversible degradation of 15-d-PGJ_2 as it prevents retro-Michael addition of the GSH conjugate (Brunoldi et al. 2007). This reaction is likely catalyzed by NADPH-dependent 11-ketoreductase found in human liver (Atsmon, Freeman et al. 1990; Seibertet al. 1987).

Alternatively, reduction of the Δ^{12} double bond of 15-d-PGJ_2 may occur before the carbonyl at C-11 is reduced to the hydroxyl. Reduction of the Δ^{12} double bond eliminates the electrophilic carbon center on the lower side chain of 15-d-PGJ_2. Maintaining the ketone group at carbon C-11 may facilitate the catalytic mechanism of the double bond reductase. A similar pathway has been shown to be important in the metabolic inactivation of other eicosanoids (Atsmon, Freeman et al. 1990; Wheelan et al. 1999; Hori et al. 2004). Reduction of the Δ^{13} double bond of 15-ketoprostaglandins and a variety of α,β-unsaturated ketone derivatives is catalyzed by the NADH/NADPH-dependent Δ^{13}-ketoprostaglandin reductase (13-PGR) (Tai et al. 2002). Based on structure-function studies of 13-PGR, the catalytic mechanism of 13-PGR-mediated reduction involves formation of an α,β-conjugated enolate intermediate in equilibrium with the Δ^{13} conjugated double bond of 15-ketoprostaglandins (Tai et al. 2002). A similar enzymatic mechanism may occur for the reduction of the Δ^{12} double bond of 15-d-PGJ_2 to yield a series of 12,13-dihydro metabolites. Nonetheless, our data are consistent with the

formation of GSH-conjugates of 15-d-PGJ_2, in which the carbonyl on the eicosanoid portion has been reduced to the hydroxyl, independent of reduction of the Δ^{12} double bond to form 12,13-dihydro metabolites. Subsequently, hydrolysis of the GSH portion of the molecule yields the cysteinyl-glycine and cysteine conjugates. These reactions require the sequential enzymatic actions of γ-glutamyl transpeptidase and dipeptidase, respectively (Meister and Anderson 1983). Oxidation of the cysteinyl sulfur to the sulfoxide is likely catalyzed by a cytochrome P450s or flavin-containing monooxygenases (Werner et al. 1995; Altuntaset al. 2004; Sheffels et al. 2004).

Two abundant metabolites observed following the 24-h incubation of 15-d-PGJ_2 with human hepatocytes were the glucuronide conjugates of 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2 (metabolite B) and 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ_2 (metabolite C). These metabolites represent the first identification of 15-d-PGJ_2 glucuronide conjugates observed during *in vitro* incubations. Glucuronic acid conjugation is catalyzed by UDP-glucuronosyltransferases (UGTs) in human liver microsomes. The C-1 carboxyl of 15-d-PGJ_2 is a potential acceptor of glucuronic acid to form an acyl glucuronide. Additionally, the C-11 hydroxyl and C-20 hydroxyl (of the latter metabolite) are potential sites for glucuronidation. The exact position of glucuronidation could not be determined from these studies. Formation of the 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ_2 involves ω-oxidation of the C-20. This reaction is likely catalyzed by a specific cytochrome P450. Cytochrome P450s of the CYP4F superfamily have previously been shown to catalyze the ω-oxidation of other eicosanoids to yield 20-hydroxyl products (Wheelan et al. 1999). An alternative pathway for 15-d-PGJ_2 metabolism identified in this study occurs via β-oxidation from the carboxyl terminus. Prostaglandins have been shown to be largely metabolized by β-oxidation in peroxisomes after formation of the CoA ester at the C-1 carboxylic acid group in the endoplasmic reticulum (Diczfalusy 1994). β-oxidation can also take place in the mitochondria.

This pathway produces the chain shortened product 9-cysteinyl-glycine-11-hydroxy-12,13-dihydro-2,3,4,5-tetranor-15-d-PGJ_2.

The metabolic pathways identified in this study likely represent routes by which 15-d-PGJ_2 is inactivated. Conjugation of 15-d-PGJ_2 with GSH at the carbon C-9 results in loss of the α,β-unsaturated carbonyl moiety within the cyclopentenone ring. Loss of this reactive moiety has been previously shown to abolish the biological activity of other cyclopenteone eicosanoids. Atsmon et al. showed that the cyclopentone prostaglandin Δ^{12}-PGJ_2 loses it cytotoxic effects in HTC and CHO cells after conjugation with GSH (Atsmon, Freeman et al. 1990). Additionally, Musiek et al. reported that adduction of the cyclopentenone IsoP 15-A_{2t}-IsoP with GSH resulted in loss of its biological effects in macrophages (Musiek et al. 2005). GSH conjugate efflux transporters, such as multidrug resistance protein 1 (MRP1) and MRP3, may be important in transporting 15-d-PGJ_2-GSH conjugates out of cells, thus, further attenuating the biological effects of 15-d-PGJ_2 (Paumi et al. 2003). Due to its dienone structure, 15-d-PGJ_2 possesses an additional electrophilic center at carbon C-13, which is capable of adducting cellular thiols via Michael addition. Indeed, the C-13 reactive center has been shown to be responsible for some of the 15-d-PGJ_2-mediated biological effects, including activation of PPARγ and Nrf2 signaling. Shiraki et al. showed that 15-d-PGJ_2 activates PPARγ by covalently binding a critical cysteine residue in the ligand binding domain of PPARγ via carbon C-13 (Shiraki et al. 2005). The 15-d-PGJ_2 analogue, 9,10-dihydro-15-d-PGJ_2, which lacks the reactive center within the cyclopentenone ring but retains the electrophilic carbon C-13, has equal potency to 15-d-PGJ_2 in activating PPARγ. More recently, Yu et al. demonstrated that the reactivity of carbon C-13 is responsible for the ability of 15-d-PGJ_2 to activate Nrf2-mediated induction of cytoprotective genes. Metabolism of 15-d-PGJ_2 by rAor resulted in reduction of the C-12,C-13 double bond and abolished the ability of 15-d-PGJ_2 to modulate Nrf2 signaling (Yu et al. 2006). Taken together,

these findings suggest that conjugation with GSH and reduction of the Δ^{12} double bond are mechanisms by which cells eliminate the reactive electrophilic centers of 15-d-PGJ_2.

The findings of the present study are consistent with previous studies regarding the metabolism of CyPGs. In addition to being important in their bioactivity, the reactive property of cyclopentenone eicoanoids provides a basis for understanding their metabolism *in vitro* and *in vivo*. Previous studies by our lab and others have shown that conjugation with GSH is a major route for the metabolism of cyclopentenone prostanoids and other lipid electrophiles. We have shown that 15-A_{2t}-IsoP, one of the abundant cyclopentenone IsoPs formed *in vivo*, is efficiently conjugated with GSH *in vitro* in the presence of human and rat glutathione *S*-transferase (GST) A4-4 (Hubatsch et al. 2002) and in HepG2 cells (Milne et al. 2004). Moreover, the major urinary metabolite of 15-A_{2t}-IsoP was determined to be a metabolized GSH adduct of the compound, the *N*-acetylcysteine sulfoxide conjugate of 15-A_{2t}-IsoP, in which the carbonyl at C-9 was reduced to an alcohol (Milne, Gao et al. 2005). The metabolism of J_2-series cyclopentone PGs, including PGJ_2, Δ^{12}-PGJ_2, and 15-d-PGJ_2 has also been examined. Cox et al. explored the metabolic transformation of PGJ_2 in the human colorectal cancer cell line HCA-7. When PGJ_2 was incubated with HCA-7 cells for 12 h, the two major metabolites identified were the PGJ_2-GSH conjugate and the PGJ_2-GSH conjugate in which the C-11 carbonyl was reduced to an alcohol (Cox et al. 2002). Atsmon et al. explored the metabolism of Δ^{12}-PGJ_2 *in vitro* and *in vivo* in a series of studies. Incubation of Δ^{12}-PGJ_2 with excess GSH *in vitro* resulted in its rapid conjugation with GSH, and formation the Δ^{12}-PGJ_2-GSH conjugate was enhanced in the presence of GST (Atsmon, Sweetman et al. 1990). When Δ^{12}-PGJ_2 was incubated with Chinese hamster ovary (CHO) cells or hepatoma tissue culture (HTC) cells, it was primarily metabolized to a GSH conjugate in which the carbonyl at C-11 was reduced to the hydroxyl and the Δ^{12} double bond were reduced (Atsmon, Freeman et al. 1990). Subsequent metabolism of this GSH conjugate

yielded the cysteinyl-glycine and cysteine metabolites (Atsmon, Freeman et al. 1990). To examine the metabolic fate of Δ^{12}-PGJ_2 *in vivo*, radiolabeled Δ^{12}-PGJ_2 was infused intravenously into a rat, and urine and bile were collected for 4 h. The vast majority of the radioactivity excreted was recovered in the bile, and the primary biliary metabolite was determined to be the Δ^{12}-PGJ_2-GSH conjugate (Atsmon, Sweetman et al. 1990). Further, the metabolism of 15-d-PGJ_2 has been examined *in vitro* and in intact cells. 15-d-PGJ_2 was shown to readily conjugate GSH *in vitro* and in MCF7 breast cancer cells (Paumi et al. 2003). Notably, in this study, Paumi et al. reported the detection of the glutathione conjugate of 15-d-PGJ_2 (15-d-PGH_2-SG) in MCF7 cells without exogenous administration of 15-d-PGJ_2 (Paumi et al. 2003), suggesting that 15-d-PGJ_2 was endogenously formed in these cells. Brunoldi et al. also reported that human HepG2 cells primarily convert 15-d-PGJ_2 to a GSH conjugate in which the carbonyl at C-11 is reduced to a hydroxyl. Subsequently, the glutathione portion of the molecule is hydrolyzed to yield the cysteine conjugate (Brunoldi et al. 2007).

In addition to metabolic transformations leading to inactivation, our results indicate that 15-d-PGJ_2 undergoes further polar modifications that likely promote its excretion from cells. Two of the most abundant metabolites of 15-d-PGJ_2 (metabolites B and C) were glucuronide conjugates. These metabolites represent the first identification of glucuronide conjugates of a CyPG observed during *in vitro* incubations. Glucuronic acid conjugation is a common means that the intact organism can enhance the excretion of lipophilic compounds. Many endogenous compounds and xenobiotics are metabolized by conjugation with glucuronic acid to yield water-soluable derivatives readily excreted into the urine. Some eicosanoids, such as F_2-isoprostanes and leukotriene B_4, are significantly excreted in human urine as glucuronide conjugates (Berry et al. 2003; Yan et al. 2010). In addition, metabolite D and Metabolites A and C represent the first identification of 15-d-PGJ_2 metabolites as products of β- and ω-oxidation, respectively. β- and

ω-oxidation are common transformations involved in the metabolism of many eicosanoids *in vivo* (Morrow and Roberts 2001; Roberts 1987). These findings suggest that similar pathways may play an important role in the elimination of 15-d-PGJ_2 *in vivo*.

The identification of several novel metabolites of 15-d-PGJ_2 formed by primary human hepatocytes yields important insights into the potential pathways involved in the metabolism and excretion of 15-d-PGJ_2 in humans and provides a basis to develop a specific biomarker for the biosynthesis of 15-d-PGJ_2 *in vivo*. The most abundant metabolites observed from the 24-h incubation of 15-d-PGJ_2 with primary humans hepatocytes were 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2 (metabolite I), 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ_2 (metabolite A), and their corresponding glucuronide conjugates (metabolites B and C, respectively). These results suggest that, similar to other eicosanoids, 15-d-PGJ_2 undergoes extensive metabolism *in vivo* such that little, if any, escapes into the circulation or appears in the urine as an intact molecule (Berry et al. 2003). In this regard, previous attempts to quantify free 15-d-PGJ_2 in biological fluids may have resulted in an underestimation of the actual quantities formed *in vivo*. Importantly, previous studies have shown that measurement of urinary metabolites by mass spectrometry is the most accurate and sensitive method to assess systemic eicosanoid production. One or more of the metabolites identified in the present may be utilized as a potential biomarker for the production of 15-d-PGJ_2 *in vivo*. However, additional studies are required to determine which of the observed metabolite(s) best reflects the systemic production of 15-d-PGJ_2 in humans. In addition to the liver, extrahepatic tissues, such as the kidney, may be involved in the metabolism of 15-d-PGJ_2 *in vivo*. Previous studies have shown that cysteine *S*-conjugates are converted to the corresponding *N*-acetylcysteine *S*-conjugates (mercapturic acids) by liver microsomal *N*-acetyl-transferases (Meister and Anderson 1983). Formation of mercapturic acid conjugates is thought

to involve interorgan transport between the liver and kidney, and the final mercapturic acid is excreted in the urine (Inoue et al. 1982). On the other hand, recent studies have shown that formation of cysteine *S*-conjugates and subsequent mercapturic acids can also occur as an intrahepatic process (Josch et al. 1998; Hinchman and Ballatori 1994).

In addition to the studies described herein, we have recently undertaken studies to examine the metabolic fate of 15-d-PGJ_2 *in vivo* in rats. Intravenous administration of [$^3H_6$]15-d-PGJ_2 into a rat resulted in its rapid metabolism and excretion of radiolabeled metabolites in the urine within 0-6 h after the infusion. The remainder of the radioactivity was accumulated primarily in the liver (refer to chapter III of this dissertation). 15-d-PGJ_2 was present in the urine predominately in the form of a polar conjugate(s) that was not extractable by organic solvents. Using LC/ESI-MS/MS analysis, the major urinary metabolite of 15-d-PGJ_2 observed in the rat was determined to be the glucuronide conjugate of *N*-acetylcysteine-sulfoxide-11-hydroxy-12,13,-dihydro-20-hydroxy-15-d-PGJ_2 polar conjugates (refer to chapter III of this dissertation). This metabolite is in fact the mercapturic acid (*N*-acetylcysteine) derivative of the glucuronide conjugate of 9-cysteinyl-sulfoxide-11-hydroxy-12,13,-dihydro-20-hydroxy-15-d-PGJ_2, an abundant metabolite observed in primary human hepatocytes. It is likely that we only observed the cysteine conjugate in human hepaptocytes and not its corresponding mercapturic acid derivative because mercapturic acid biosynthesis is thought to involve the liver and kidney. As discussed above, cysteine conjugates are readily converted to mercapturic acid conjugates by *N*-acetylation before being excreted in urine. Indeed, studies examining the metabolism of 15-d-PGJ_2 in the whole animal *in vivo* permitted identification of the mercapturic acid metabolite of 15-d-PGJ_2. Further, in recent preliminary studies we have detected this metabolite in human urine using UPLC/MS/MS analysis (our unpublished data). Importantly, these finding suggest that this metabolite may be a useful biomarker to assess biosynthesis of 15-d-PGJ_2 *in vivo* in

humans. Studies are currently underway to develop UPLC/MS/MS assay for the quantification of this metabolite in humans under normal and pathophysiological conditions.

Developing a biomarker to assess 15-d-PGJ_2 production *in vivo* will provide important insights into the physiological and pathophysiological mechanisms that contribute to the endogenous formation of this reactive lipid mediator. 15-d-PGJ_2 was originally identified as a product of PGD_2 dehydration *in vitro*, and its formation has been shown to occur in an albumin-dependent and –independent manner (Fitzpatrick and Wynalda 1983; Shibata et al. 2002). Generation of 15-d-PGJ_2 is thought to be localized at sites of inflammation, in which COX-2 is induced (Gilroy et al. 1999; Rajakariar et al. 2007; Shibata et al. 2002). Indeed, it has been postulated that because PG production is significantly increased under pathological conditions, the intracellular concentrations of 15-d-PGJ_2 and other CyPGs may reach biologically relevant levels at sites of inflammation (Gutierrez et al. 2008; Offenbacker et al. 1986). Bell-Parikh et al. demonstrated that COX-2 is the dominant source of 15-d-PGJ_2 biosynthesis in humans; however, induction of acute inflammation by administration of LPS, which results in expression of COX-2, showed no alteration in levels of free 15-d-PGJ_2 excreted in urine compared to controls levels (Bell-Parikh et al. 2003). On the other hand, using immunochemical detection approaches, Shibata et al. demonstrated that 15-d-PGJ_2 is produced by activated macrophages *in vitro* and is present in foamy macrophages within a human atherosclerotic lesion (Shibata et al. 2002). In addition, production of 15-d-PGJ_2 has been reported in selenium-supplement macrophages following LPS stimulation (Vunta et al. 2007) and in rodent models of inflammation, including carrageenan-induced pleurisy in rats (Gilroy et al. 1999) and zymosan-induced resolving peritonitis in mice (Rajakariar et al. 2007).

Although the cyclooxygenases are primarily responsible for the generation of PGs, studies in our laboratory and others have shown that biologically active prostanoids are also

generated in abundance *in vivo* via nonenyzmatic free radical-initiated lipid peroxidation, independent of COX. Morrow et al. first reported the discovery of a series of PG-like compounds, termed isoprostanes (IsoPs), which are generated in abundance *in vivo* via the nonenyzmatic free radical-initiated peroxidation of arachidonic acid (Morrow, Hill et al. 1990). The generation of PGs via COX results in a single stereoisomer, whereas IsoPs formed via the free radical-initiated pathway consist of multiple racemic isomers. Moreover, formation of IsoPs significantly increases under settings of oxidant stress *in vivo* (Morrow, Hill et al. 1990). Recent studies in our laboratory support the contention that PGs can be generated *in vivo* independent of the COX enzymes, via free radical-initiated lipid peroxidation. Gao et al. reported the formation of PGE_2 and PGD_2 and their respective enantiomers *in vitro* and *in vivo* through the nonenzymatic peroxidation of AA by epimerization of E_2/D_2-IsoPs, specifically 15-E_{2t}-IsoP and 15-D_{2c}-IsoP, respectively (Gao et al. 2003). More recently Yin et al. reported the formation of $PGF_{2\alpha}$ *in vivo* via the IsoP pathway, and demonstrated that the vast majority of putative $PGF_{2\alpha}$ in human urine is derived from the free radical-initiated peroxidation of arachidonic acid, independent of COX (Yin et al. 2007). Further, more recently, we have discovered that 15-d-PGJ_2 and multiple isomers of 15-d-PGJ_2-like compounds (deoxy-J_2-IsoPs) are also formed *in vivo* via free radical-catalyzed lipid peroxidation, independent of COX. We found that 15-d-PGJ_2 and deoxy-J_2-IsoPs were significantly generated esterified in phospholipids in rat liver after induction of oxidant stress by CCl_4 (Hardy et al. 2011). Taken together, this suggests that 15-d-PGJ_2 and deoxy-J_2-IsoPs may be produced *in vivo* in settings of inflammation in which COX activity is increased as well as under conditions of oxidant stress. Further studies are necessary to establish the relative contributions of the COX and free radical-catalyzed pathways to biosynthesis of 15-d-PGJ_2 and related compounds *in vivo*.

In summary, we report the identification of several novel metabolites of 15-d-PGJ_2 formed by primary human hepatocytes. The most abundant metabolites of 15-d-PGJ_2 observed were 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-15-d-PGJ_2 (metabolite I), 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ_2 (metabolite A), and their corresponding glucuronide conjugates (metabolites B and C, respectively). Formation of the metabolites identified involves conjugation of 15-d-PGJ_2 with GSH, subsequent hydrolysis of the GSH portion of the molecule to the cysteine conjugate, and oxidation of the cysteinyl sulfur to the sulfoxide. Further, the prostanoid portion of the molecule was metabolized by reduction of the Δ^{12} double bond, reduction of the C-11 carbonyl to the hydroxyl, β- and ω-oxidation, and glucuronidation. These findings yield important insights into the potential pathways occurring in vivo for the metabolism and excretion of 15-d-PGJ_2 in humans and provide the basis to develop a specific biomarker for the biosynthesis of 15-d-PGJ_2 *in vivo*. In addition, we have recently undertaken studies to examine the metabolic fate of 15-d-PGJ_2 *in vivo* in rats. The major urinary metabolite of 15-d-PGJ_2 identified in the rat is the *N*-acetylcysteine-sulfoxide-11-hydroxy-12,13,-dihydro-20-hydroxy-15-d-PGJ_2 glucuronide conjugate, the mercapturic acid derivative of Metabolite C observed in the present study. Of particular interest is the finding that we can detect this metabolite in human urine using UPLC/MS/MS analysis (refer to chapter III of this dissertation). Importantly, this urinary metabolite may be utilized as a biomarker to assess the systemic production of 15-d-PGJ_2. The findings described herein provide the basis to develop a mass spectrometric method to quantify 15-d-PGJ_2 metabolites excreted in urine. This assay should be a valuable tool to accurately estimate biological levels of 15-d-PGJ_2. Studies are currently underway to further develop this methodology and assess the biosynthesis of 15-d-PGJ_2 in humans under normal and pathophysiological conditions. Determining the extent to which 15-d-PGJ_2 is formed *in vivo* and the defining mechanisms that regulate its formation will

contribute importantly to our understanding of the potential role of this lipid mediator in human health and disease.

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-d-PGJ_2) is a reactive cyclopentenone PG generated from the dehydration of cyclooxygenase (COX)-derived PGD_2, and this compound has emerged as a putative mediator of numerous biological effects. The chemical reactivity of 15-d-PGJ_2 contributes importantly to its biological activity. 15-d-PGJ_2 possesses an electrophilic α,β-unsaturated carbonyl moiety within its cyclopentenone ring that can readily adduct cellular nucleophiles, such as the free sulfhydryl groups of glutathione and cysteine residues of proteins, via nucleophilic addition reactions (Michael addition). In addition, 15-d-PGJ_2 contains a second reactive carbon center on its lower side chain that can undergo Michael with cellular thiols. Due to its reactivity, 15-d-PGJ_2 can modulate protein function and exert potent biological activity in various cell types through interaction with intracellular protein targets. Significant scientific interest in 15-d-PGJ_2 was sparked by the finding that it is an activating ligand of the nuclear receptor PPARγ. Since that time, 15-d-PGJ_2 has been shown to induce a number of cellular responses through PPARγ-dependent and -independent mechanisms. Among its diverse functions, 15-d-PGJ_2 is regarded as a key anti-inflammatory mediator involved in the resolution of inflammation. This molecule has also been shown to exert potent anti-proliferative and pro-apoptotic activity in various cancer cell lines. Although the biological properties of 15-d-PGJ_2 have been well characterized *in vitro*, the extent to which 15-d-PGJ_2 is formed *in vivo* and mechanisms that regulate its formation are unknown. In view of its potential biological significance, it is critical to provide evidence that 15-d-PGJ_2 is synthesized *in vivo* and determine what are the concentrates of this compound generated at its biological targets.

Our research group has extensively studied the free radical-initiated peroxidation of arachidonic acid *in vivo* and has identified a series of PG-like compounds, termed isoprostanes (IsoPs) formed via this pathway. These studies have provided key insights into the pathobiology of oxidative stress, which has become increasingly implicated in a number of human diseases. Several of the compounds formed via the IsoP pathway are biologically active, and thus, they likely mediate various physiological and pathophysiological processes. In addition, recent studies by our laboratory have demonstrated that biologically active PGs and their corresponding entantiomers can be generated *in vivo* via the IsoP pathway, independent of COX. We hypothesized that 15-d-PGJ_2 may also be formed via the free radical-initiated pathway. The overall goal of this thesis research was to examine the biochemical pathways that contribute to the formation of 15-d-PGJ_2 *in vivo* and develop an analytical approach to assess the systemic generation of 15-d-PGJ_2 *in vivo* in humans under normal and pathophysiological conditions. Our overall hypothesis was that 15-d-PGJ_2 may be generated *in vivo* via the COX and free radical-initiated pathways.

Nonenzymatic free radical-catalyzed generation of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2-like compounds (deoxy-J_2-isoprostanes) *in vivo*: summary and future directions

Our research group has previously reported that biologically active cyclopentenone prostaglandin A_2-like and prostaglandin J_2-like compounds, termed A_2/J_2-IsoPs, are generated *in vivo* via the free radical-catalyzed peroxidation of arachidonic acid. Based on these observations, we questioned whether 15-d-PGJ_2-like compounds are formed from the oxidation of arachidonic acid *in vivo*. Herein in Chapter II, we reported the formation of reactive cyclopentenone 15-d-PGJ_2-like compounds, termed deoxy-J_2-IsoPs, generated in abundance *in vitro* and *in vivo* from arachidonic acid peroxidation. Biochemical approaches coupled with liquid chromatography/mass spectrometry (LC/MS) were used to structurally characterize these

compounds as deoxy-J_2-IsoPs. We found that these molecules were significantly formed from the oxidation of arachidonic acid *in vitro*, and levels of these compounds markedly increased following acid-catalyzed dehydration *in vitro*. We also detected these compounds esterified in the liver of control rats at basal levels of 0.55 $\pm$ 0.21 ng/g tissue, and quantities increased 12-fold to 6.4 $\pm$ 1.1 ng/g tissue *in vivo* in settings of oxidative stress. In addition, we showed that, similar to 15-d-PGJ_2, deoxy-J_2-IsoPs rapidly adducted glutathione (GSH) in the presence of glutathione *S*-transferase *in vitro*, and we identified GSH conjugates of these compounds by LC/MS. These studies have, for the first time, definitively characterized novel, reactive deoxy-J_2-IsoPs forming in abundance from the nonenzymatic free radical-catalyzed peroxidation of arachidonic acid *in vivo*.

Our proposed mechanism of deoxy-J_2-IsoP formation has been based on that of other IsoPs formed from the free radical-initiated peroxidation of arachidonic acid. According to our hypothesis, these compounds are formed from the spontaneous dehydration and isomerization of D_2- and J_2-IsoPs. We have previously shown that D_2- and J_2-IsoPs are formed in abundance *in vivo*, and there formation is significantly increased under settings of oxidative stress. Deoxy-J_2-IsoPs of the 5-, 8-, 12-, and 15-series were identified, and these are presumably formed from dehydration of the various D_2- and J_2-IsoP regioisomers. Each would theoretically comprise racemic enantiomers. Notably, we found that a compound identical in all respects to COX-derived 15-d-PGJ_2 and its corresponding enantiomer were generated through this mechanism, demonstrating, for the first time, the formation of 15-d-PGJ_2 *in vivo* via the free radical-initiated pathway, independent of COX.

The identification of 15-d-PGJ_2 and other reactive deoxy-J_2-IsoPs formed *in vivo* from lipid peroxidation has important implications regarding the potential role of these compounds in the pathobiology of diseases associated with oxidative stress. The biosynthesis of 15-d-PGJ_2 via

the COX pathway *in vivo* is likely limited to sites in which PGD_2 is produced, namely tissues expressing PGD synthases. On the other, free radical-induced lipid peroxidation occurs throughout the body in virtually all tissues. Thus, it is possible that the generation of 15-d-PGJ_2 and deoxy-J_2-IsoPs via the free radical-mediated pathway may occur more broadly in various tissues under settings of oxidative stress. Future investigations aimed at characterizing the biological activity of these compounds should yield further insight into the patho/physiological consequences of their formation *in vivo*. Important scientific considerations include: what are the biological effects of deoxy-J_2-IsoPs; how do esterified deoxy-J_2-IsoPs affect membrane structure and function; can these compounds covalently modify membrane-associated proteins; do these compounds have synergistic effects with COX-derived 15-d-PGJ_2 under settings of inflammation and oxidative stress?

Metabolic fate of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 *in vivo* – identification of the major urinary metabolite in the rat: summary and future directions

Given the reactive polyunsaturated carbonyl moiety of 15-d-PGJ_2 and related compounds, these compounds cannot be readily measured *in vivo* in plasma or urine. We hypothesize that they undergo rapid adduction with the thiol groups of cellular proteins or GSH, yielding them virtually undetectable in free form. This analytical challenge has hindered scientific progress in understanding the physiological relevance of 15-d-PGJ_2 and other cyclopentenone eicosanoids. Our research group has previously shown that conjugation with GSH is the primary route for the metabolism of cyclopentenone eicosanoids, including PGJ_2, Δ^{12}-PGJ_2, and 15-A_{2t}-IsoP. Studies in Chapter III of this dissertation determined the metabolic fate of 15-d-PGJ_2 *in vivo* in the rat and characterized the urinary metabolites of 15-d-PGJ_2 by LC/MS. We found that 15-d-PGJ_2 undergoes adduction to GSH and subsequent metabolism *in vivo* to yield the major urinary metabolite the *N*-acetylcysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-

hydroxy-15-d-PGJ_2 glucuronide conjugate. We also identified several other *N*-acetyl cysteine (mercapturic acid) conjugates of 15-d-PGJ_2 excreted in rat urine. Based on these findings, we proposed a route for the metabolism of 15-d-PGJ_2 *in vivo* via the mercapturic acid biosynthesis pathway. In addition, utilizing ultra-performance LC/MS (UPLC/MS) analysis, we obtained preliminary evidence that the major urinary metabolite of 15-d-PGJ_2 is formed *in vivo* in humans. Importantly, this finding provides, for the first time, a potential biomarker to assess the endogenous production of 15-d-PGJ_2 and 15-d-PGJ_2-like compounds *in vivo*. To further develop this methodology, a heavy isotope labeled standard of the 15-d-PGJ_2 urinary metabolite will be synthesized to quantify this metabolite in human urine using UPLC/MS analysis. Future studies will utilize this approach to establish normal levels of 15-d-PGJ_2 and 15-d-PGJ_2-like compounds *in vivo* in humans and determine the relative extent to which the COX and free radical-initiated pathways contribute to the systemic generation of 15-d-PGJ_2 under various pathophysiological conditions associated with PGD_2 overproduction and oxidative stress. These studies should yield important insights into the role of 15-d-PGJ_2 and related compounds in human health and disease.

Metabolic transformation of 15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2 in primary human hepatocytes: summary and future directions

Because the metabolic fate of eicosanoids can vary between rodents and humans, we sought to gain further insight into the potential pathways involved in the metabolism of 15-d-PGJ_2 in humans. To this end, we undertook studies to examine the metabolic transformation of 15-d-PGJ_2 in primary human hepatocytes as a surrogate model for predicting metabolism *in vivo*. In Chapter IV of this dissertation, we reported the identification of twelve metabolites of 15-d-PGJ_2 generated from the 24-h incubation of this compound with freshly isolated human hepatocytes. The four most abundant metabolites were 9-cysteinyl-sulfoxide-11-hydroxy-12,13-

dihydro-15-d-PGJ_2, 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ_2, and their corresponding glucuronide conjugates. Based on these findings, we proposed a pathway for the metabolism of 15-d-PGJ_2 in primary human cells, which involves conjugation with GSH and subsequent metabolism via β- and ω-oxidation, glucuronic acid conjugation, and other processes. It is possible that these conjugates may be converted to mercapturic acid metabolites prior to urinary excretion, or they may be targeted for direct biliary excretion. It should be noted that the structural insights gained from identification of metabolites in primary human hepatocytes were instrumental in determining the major urinary metabolite of 15-d-PGJ_2 described in Chapter III; the major urinary metabolite in rats is the mercapturic acid derivative of the 9-cysteinyl-sulfoxide-11-hydroxy-12,13-dihydro-20-hydroxy-15-d-PGJ_2 glucuronide conjugate formed in primary human hepatocytes. This metabolite was also detected in human urine by UPLC/MS. Taken together, these findings support the contention that similar pathways may contribute to the metabolism of 15-d-PGJ_2 in rodents and humans.

Summary

The overall goal of this thesis research was to examine the biochemical pathways that contribute to the formation of 15-d-PGJ_2 *in vivo* and develop an analytical approach to assess the systemic generation of 15-d-PGJ_2 *in vivo* in humans under normal and pathophysiological conditions. We hypothesized that 15-d-PGJ_2 may be generated *in vivo* via the COX and free radical-initiated pathways. Through the studies described herein, we found that, in addition to the COX pathway, 15-d-PGJ_2 and 15-d-PGJ_2-like compounds (deoxy-J_2-IsoPs) are significantly generated *in vivo* via free radical-initiated lipid peroxidation. This finding provided the impetus for us to examine the systemic generation of these compounds *in vivo* in humans. Due to the reactivity of these molecules, we hypothesized that they undergo rapid adduction to protein

and peptide thiols. Thus, we determined the metabolic fate of 15-d-PGJ_2 in primary human cells and *in vivo* in the rat. Four abundant metabolites were identified in primary human hepatocytes. The major urinary metabolite of 15-d-PGJ_2 was identified in the rat, and our results have provided evidence that this metabolite is formed in humans. The identification of this metabolite provides a potential method to quantify, for the first time, the production of 15-d-PGJ_2 and related compounds *in vivo* in humans. Taken together, these studies yield new insight into the biochemical mechanisms that contribute to the endogenous generation of reactive lipid mediators. Further, our findings provide a potential biomarker that may be used to assess the formation of 15-d-PGJ_2 and 15-d-PGJ_2-like compounds in human disease.

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