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CHAPTER 13

The eicosanoids: cyclooxygenase, lipoxygenase, and epoxygenase pathways

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1. Introduction

1.1. Terminology, structures, and nomenclature

The term 'eicosanoids' is used to denote a group of oxygenated, twenty carbon fatty acids (Fig. 1) [1]. The major precursor of these compounds is arachidonic acid (all *cis* 5,8,11,14-eicosatetraenoic acid), and the pathways leading to the eicosanoids are known collectively as the 'arachidonate cascade'. There are three major pathways within the cascade, including the cyclooxygenase, lipoxygenase, and epoxygenase pathways. In each case, these pathways are named after the enzyme(s) that catalyzes the first committed step. The prostanoids, which include the prostaglandins and thromboxanes, are formed via the cyclooxygenase pathway. The first part of our discussion will focus on the prostanoids. Later in this chapter, we will describe the lipoxygenase and epoxygenase pathways.

The structures and biosynthetic interrelationships of the most important prostanoids are shown in Fig. 2 [1]. PG is the abbreviation for prostaglandin, and TX is the abbreviation for thromboxane. Naturally occurring prostaglandins contain a cyclopentane ring, a *trans* double bond between C-13 and C-14, and an hydroxyl group at C-15. The letters following the abbreviation PG indicate the nature and location of the oxygen-containing substituents present in the cyclopentane ring. Letters are also used to label thromboxane derivatives (e.g., TXA and TXB). The numerical subscripts indicate the number of carbon–carbon double bonds in the side chains emanating from the cyclopentane ring (e.g., PGE_1 vs. PGE_2). In general, those prostanoids with the '2' subscript are derived from arachidonate; the '1' series prostanoids are formed from 8,11,14-eicosatetraenoate, and the '3' series compounds are derived from 5,8,11,14,17-eicosapentaenoate. Greek subscripts are used to denote the orientation of ring hydroxyl groups (e.g., PGF_α).

Prostanoids formed by the action of cyclooxygenases have their aliphatic side chains emanating from C-8 and C-12 of the cyclopentane ring in the orientations shown in Fig. 2. Prostanoids known as isoprostanes have their aliphatic groups in various other orientations [2]. Isoprostanes are formed from arachidonic acid by nonenzymatic autooxidation, and, somewhat surprisingly, isoprostanes and their metabolites are found in greater quantities in urine than metabolites of prostanoids formed enzymatically via

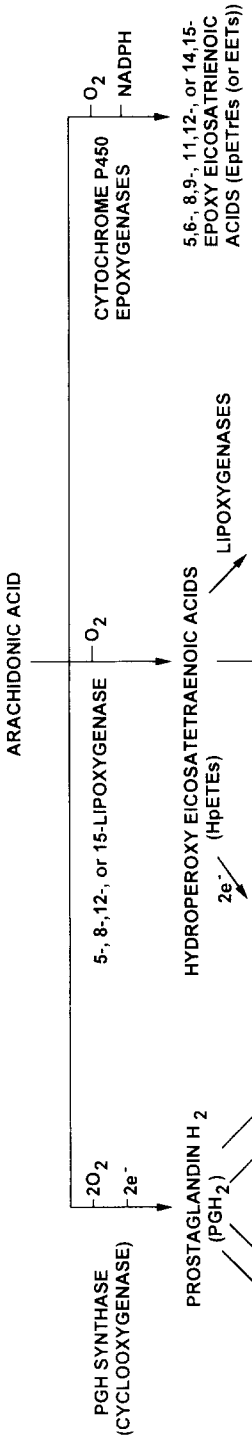

Fig. 1. Cyclooxygenase, lipoxygenase, and epoxygenase pathways leading to the formation of eicosanoids from arachidonic acid.

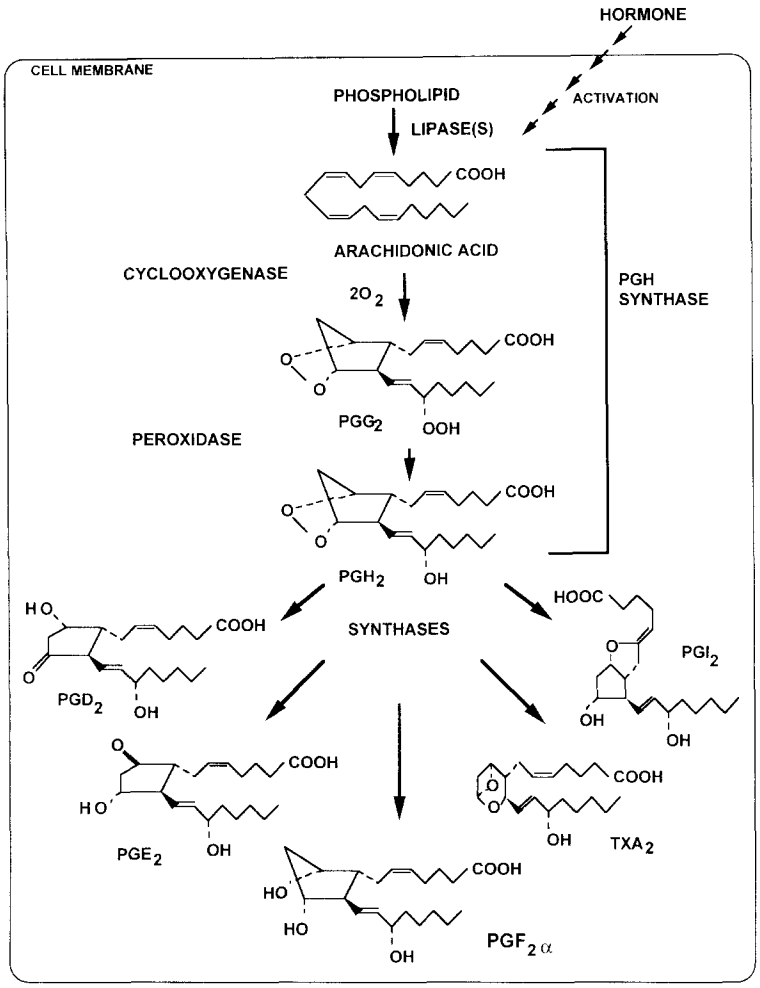

Fig. 2. Structures and biosynthetic relationships among the most common prostanoids.

the cyclooxygenase. Particularly in pathological conditions which support autooxidation (e.g., CCl_4 toxicity), isoprostanes are produced in abundance [2].

1.2. Prostanoid chemistry

Prostaglandins are soluble in lipid solvents below pH 3.0 and are typically extracted from acidified aqueous solutions with ether, chloroform/methanol, or ethyl acetate. PGE, PGF, and PGD derivatives are relatively stable in aqueous solution at pH 4–9; above pH 10, both PGE and PGD are subject to dehydration. PGI_2, which is also known as prostacyclin, contains a vinyl ether group that is very sensitive to acid-catalyzed hydrolysis; PGI_2 is unstable below pH 8.0. The stable hydrolysis product

of PGI_2 is 6-keto-$PGF_{1\alpha}$. PGI_2 formation is usually monitored by measuring 6-keto-$PGF_{1\alpha}$ formation. TXA_2, which contains an oxane–oxetane grouping in place of the cyclopentane ring, is hydrolyzed rapidly ($t_{1/2} = 30$ s at 37°C in neutral aqueous solution) to TXB_2; TXA_2 formation is assayed by quantifying TXB_2. Prostaglandin derivatives are commonly quantified with immunoassays or by mass spectrometry using deuterium-labeled internal standards.

2. *Prostanoid biosynthesis*

Eicosanoids are not stored by cells, but rather are synthesized and released rapidly (5–60 s) in response to extracellular hormonal stimuli. The pathway for stimulus-induced prostanoid formation as it might occur in a model cell is illustrated in Fig. 2 [1]. Prostanoid formation occurs in three stages: (a) mobilization of free arachidonic acid (or 2-arachidonyl-glycerol (2-AG); see below) from membrane phospholipids; (b) conversion of arachidonate (or 2-AG) to the prostaglandin endoperoxide PGH_2 (or 2-PGH_2-glycerol); and (c) cell-specific conversion of PGH_2 (or 2-PGH_2-glycerol) to one of the major prostanoids.

2.1. Mobilization of arachidonate

Prostaglandin synthesis is initiated by the interaction of various hormones (e.g., bradykinin, angiotensin II, thrombin) with their cognate cell surface receptors (Figs. 2 and 3) which, in turn, causes the activation of one or more cellular lipases. Although in principle, there are a variety of lipases and phospholipases that could participate in this arachidonate mobilizing phase, the high molecular weight cytosolic phospholipase A_2 (PLA_2) and certain of the nonpancreatic, secretory PLA_2s appear to be the relevant lipases (Chapter 11). The current consensus regarding the roles of PLA_2s in prostanoid synthesis is that typically cytosolic PLA_2 is involved directly in mobilizing arachidonic acid for the constitutive, prostaglandin endoperoxide H synthase-1 (PGHS-1) whereas cytosolic PLA_2 is indirectly, and secretory PLA_2 is directly, involved in mobilizing arachidonate for the inducible PGHS-2 (Fig. 3); [3–5]. This is discussed in more detail below in describing the functions of the PGHS isoforms.

2.2. Cytosolic and secreted phospholipase A_2s

Cytosolic PLA_2 is found in the cytosol of resting cells, but as illustrated in Fig. 3, hormone-induced mobilization of intracellular Ca^{2+} leads to the translocation of cytosolic PLA_2 to the ER and nuclear envelope. There, cytosolic PLA_2 cleaves arachidonate from the *sn*2 position of phospholipids on the cytosolic surface of the membranes. The arachidonate then traverses the membrane where it acts as a substrate for PGHSs which are located on the luminal surfaces of the ER and the associated inner and outer membranes of the nuclear envelope [1]. The activity of cytosolic PLA_2 is also augmented by phosphorylation by a variety of kinases [3]. The translocation of cytosolic PLA_2 involves the binding of Ca^{2+} to an N-terminal CalB domain and then the binding of the

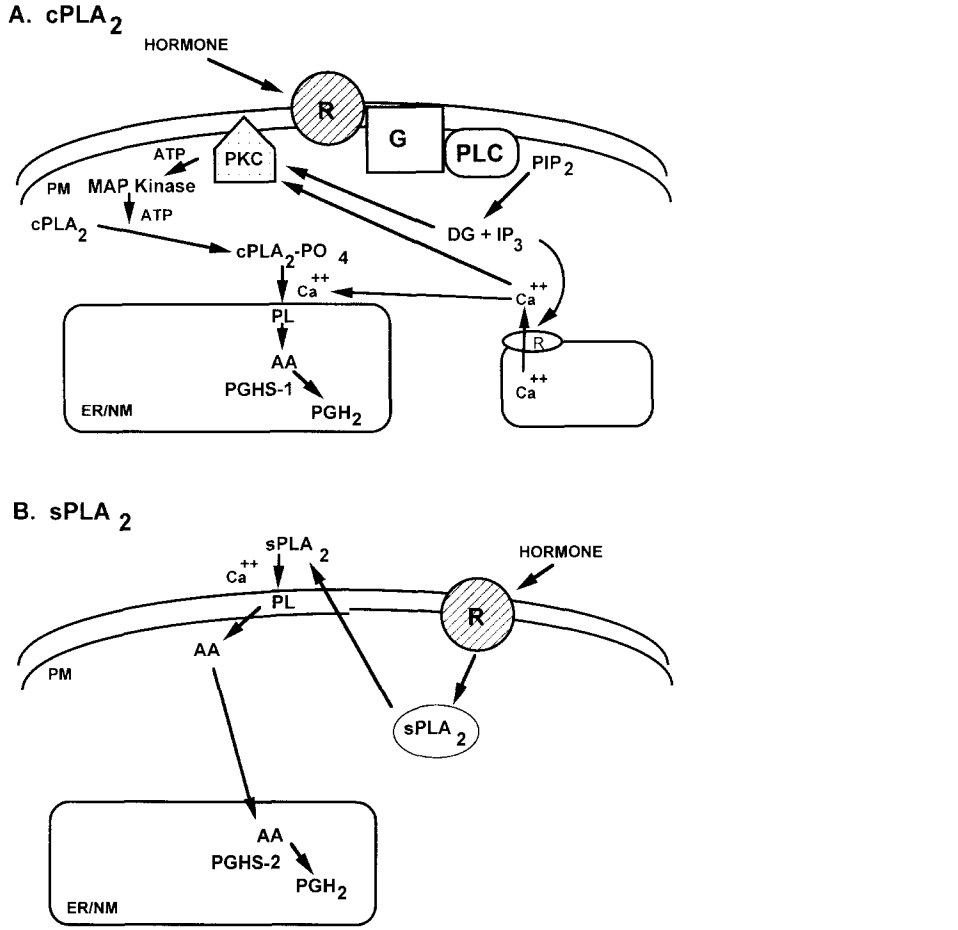

Fig. 3. Activation of cytosolic (c) PLA_2 (panel A) and Type II nonpancreatic secretory (s) PLA_2 (panel B) involved in mobilizing arachidonic acid from phospholipid. Abbreviations include: PL, phospholipid; ER/NM, endoplasmic reticulum/nuclear membrane; PM, plasma membrane; AA, arachidonic acid; PGHS, prostaglandin endoperoxide H synthase; R, receptor; G, G protein, PLC, phospholipase C; PIP_2, phosphatidylinositol-4,5-*bis*-phosphate; PKC, protein kinase C. The activation of secretory PLA_2 requires participation of cytosolic PLA_2 but the mechanism has not yet been determined.

Ca^{2+}/CalB domain to intracellular membranes [6]; Ca^{2+} is involved in translocation of the enzyme but not in the catalytic mechanism of cytosolic PLA_2 (see Dessen et al., 1999).

Stimulus-dependent arachidonate mobilization by secretory PLA_2s depends on the ability of these enzymes to be released from cells and to rebind to the cell surface through heparin sulfate containing proteoglycans (Fig. 3). Once bound, these enzymes appear to act on phosphatidylcholine present on the extracellular face of the plasma membrane [7]. Secretory PLA_2 is shown in Fig. 3 in a vesicle that moves to the surface of the cell in response to an agonist, is secreted into the medium and then binds to

a proteoglycan on the cell surface. Presumably, free arachidonate released through the actions of secretory PLA_2 can enter the cell and make its way to the ER, where it is acted upon by PGHSs. There are many different nonpancreatic secretory PLA_2s but to date, only secretory PLA_2s IIA and V have been shown to be involved in releasing arachidonate for prostanoid synthesis [7].

The crystal structure of secretory PLA_2 IIA from human synovial fluid has been determined. Secretory PLA_2s require high concentrations of Ca^{2+} (~1 mM) such as those found extracellularly for maximal activity. Ca^{2+} is involved in phospholipid substrate binding and catalysis by secretory PLA_2s. Unlike cytosolic PLA_2, secretory PLA_2 shows no specificity toward either the phospholipid head group or the acyl group at the *sn*2 position. The levels of secretory PLA_2s are regulated transcriptionally in response to cell activation.

Cytosolic PLA_2 is directly involved in the immediate arachidonate release that occurs when a cell is challenged with a circulating hormone or protease. For example, thrombin acting through its cell surface receptor activates cytosolic PLA_2 in platelet cells to cause arachidonate release that results in TXA_2 formation. This entire process occurs in seconds. secretory PLA_2, on the other hand, plays a prominent role in 'late-phase' prostaglandin formation which occurs 2–3 h after cells have been exposed to a mediator of inflammation (e.g., endotoxin or interleukin-1) or a growth factor such as platelet-derived growth factor.

2.3. Mobilization of 2-arachidonyl-glycerol (2-AG)

In 2000, Kozak et al. made the insightful discovery that 2-AG is an efficient substrate for PGHS-2 but not PGHS-1 [8]. PGHS-2 converts 2-AG to 2-PGH_2-glycerol, and this intermediate is converted to the 2-prostanyl-glycerol derivatives but not 2-thromboxane-glycerol. At the time of this writing, it is not clear under what conditions or to what degree alternative substrates such as 2-AG are used in vivo to form products like the 2-prostanyl-glycerol derivatives. However, one can imagine that these products have a unique set of biological roles that distinguish them from the classical prostaglandins derived from arachidonic acid itself. Although the pathway leading to the formation of 2-AG itself has not been defined in the context of prostanoid metabolism, 2-AG could be formed from phosphatidylcholine through the sequential actions of phospholipase C and acylglycerol lipase [8].

2.4. Prostaglandin endoperoxide H_2 (PGH_2) formation

Once arachidonate is released, it can be acted upon by PGHS [9]. There are two PGHS isozymes, PGHS-1 and PGHS-2. PGHS-1 appears to use fatty acids such as arachidonate exclusively as substrates. In contrast, PGHS-2 utilizes both fatty acids and 2-AG about equally well [8]. The PGHSs exhibit two different but complementary enzymatic activities (Fig. 2): (a) a cyclooxygenase (*bis*-oxygenase) which catalyzes the formation of PGG_2 (or 2-PGG_2-glycerol) from arachidonate (or 2-AG) and two molecules of O_2; and (b) a peroxidase which facilitates the two-electron reduction of the 15-hydroperoxyl group of PGG_2 (or 2-PGG_2-glycerol) to PGH_2 (or 2-PGH_2-glycerol)

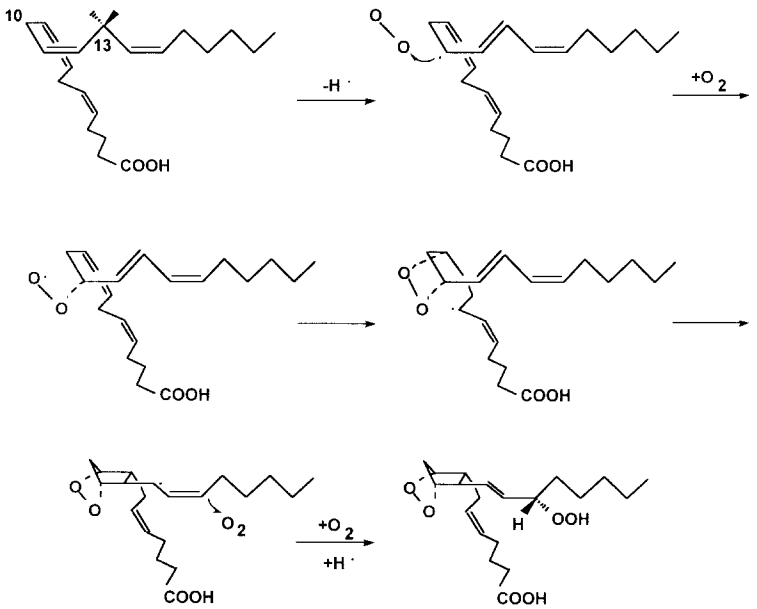

Fig. 4. Mechanism for the cyclooxygenase reaction showing the conversion of arachidonic acid and two molecules of oxygen to PGG_2.

(Fig. 2). The oxygenase and peroxidase activities occur at distinct but interactive sites within the protein.

The initial step in the cyclooxygenase reaction is the stereospecific removal of the 13-pro-*S* hydrogen from arachidonate. As depicted in Fig. 4 [9], an arachidonate molecule becomes oriented in the cyclooxygenase active site with a kink in the carbon chain at C-9. Abstraction of the 13-pro-*S* hydrogen and subsequent isomerization leads to a carbon-centered radical at C-11 and attack of molecular oxygen at C-11 from the side opposite that of hydrogen abstraction. The resulting 11-hydroperoxyl radical adds to the double bond at C-9, leading to intramolecular rearrangement and formation of another carbon-centered radical at C-15. This radical then reacts with another molecule of oxygen. The 15-hydroperoxyl group of PGG_2 can undergo a two-electron reduction to an alcohol yielding PGH_2 in a reaction catalyzed by the peroxidase activity of PGHSs.

2.5. PGHS active site

Depicted in Fig. 5 is a model of the cyclooxygenase and peroxidase active sites of ovine PGHS-1 [1]. The cyclooxygenase is an unusual activity that exhibits a requirement for hydroperoxide and undergoes a suicide inactivation [1]. The reason for the hydroperoxide activating requirement is that in order for the cyclooxygenase to function, a hydroperoxide must oxidize the heme prosthetic group located at the peroxidase active site to an oxo-ferryl heme radical cation. This oxidized heme intermediate abstracts an electron from Tyr385. Finally, the resulting Tyr385 tyrosyl

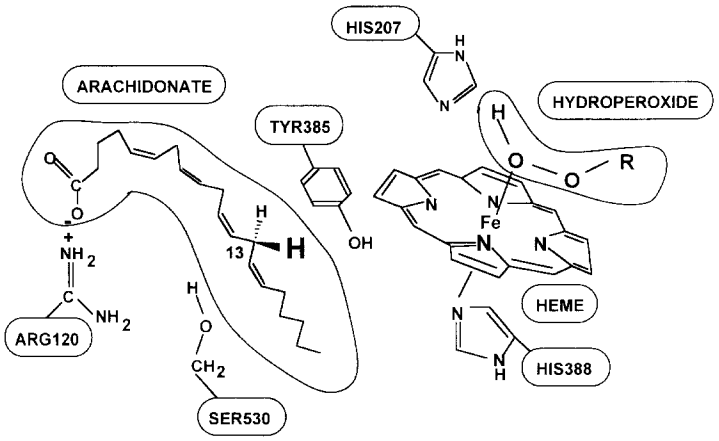

Fig. 5. Model of the cyclooxygenase and peroxidase active sites of the ovine PGHS-1.

radical abstracts the 13-pro-*S* hydrogen from arachidonate, initiating the cyclooxygenase reaction. Once the cyclooxygenase reaction begins, newly formed PGG_2 can serve as the source of the activating hydroperoxide; prior to PGG_2 formation, ambient cellular hydroperoxides apparently serve to initiate heme oxidation and cyclooxygenase catalysis. Ser530, the site of acetylation of PGHS-1 by aspirin (Section 2.7), is shown within the cyclooxygenase active site in Fig. 5. Also shown is Arg120. The guanidino group of this residue serves as the counterion for the carboxylate group of arachidonate.

2.6. Physico-chemical properties of PGHSs

PGHS-1 was first purified from ovine vesicular gland, and most biochemical studies have been performed using this protein. PGHS-1 is associated with the luminal surfaces of the ER and the inner and outer membranes of the nuclear envelope. Detergent-solubilized ovine PGHS-1 is a dimer with a subunit molecular mass of 72 kDa. The reason for the existence of the dimer is unknown, but separation of the enzyme into monomers eliminates enzyme activity. The protein is *N*-glycosylated and is a hemoprotein containing one protoporphyrin IX per monomer [1]. The sequences of cDNA clones for PGHS-1 from many mammals indicate that initially the protein has a signal peptide of 24–26 amino acids, that is cleaved to yield a mature protein of 574 amino acids.

PGHS-2 was discovered in 1991 as an immediate early gene product in phorbol ester-activated murine 3T3 cells and in *v-src*-transformed chicken fibroblasts [1,10]. PGHS-1 and PGHS-2 from the same species have amino acid sequences that are 60% identical. The major sequence differences are in the signal peptides and the membrane binding domains (residues 70–120 of PGHS-1); in addition, PGHS-2 contains a unique 18 amino acid insert near its carboxyl terminus. The role of this 18 amino acid cassette in PGHS-2 is unknown.

The crystal structures of PGHS-1 and PGHS-2 have been determined. As noted earlier, two subunits of the enzymes form homodimers. Each monomer contains three

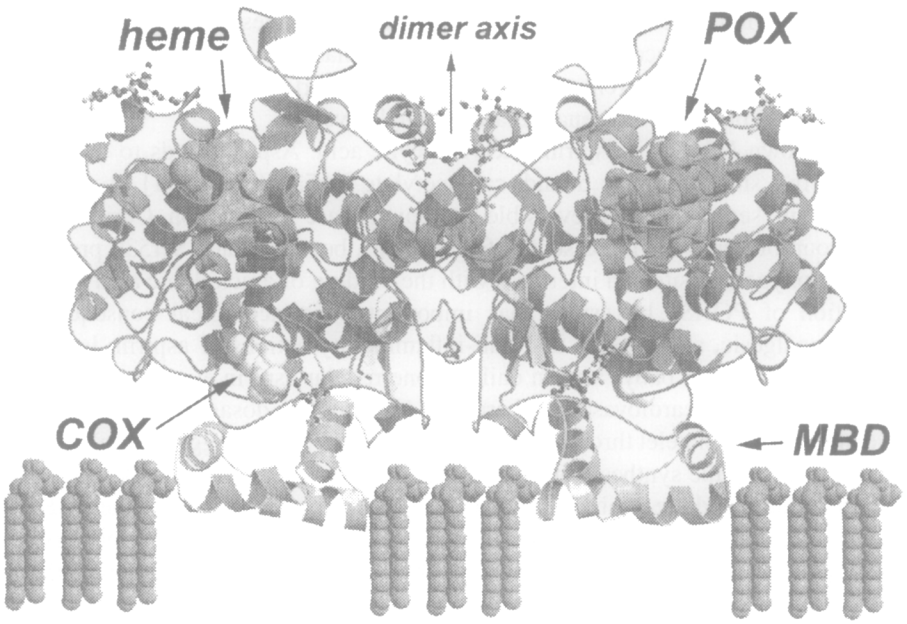

Fig. 6. Ribbon diagram of the structure of ovine PGHS-1 homodimer interdigitated via its membrane binding domain (MBD) into the luminal surface of the endoplasmic reticulum. The arrow denotes the dimer interface. Abbreviations include: POX, peroxidase; COX, cyclooxygenase.

sequential folding domains: an N-terminal epidermal growth factor-like domain of about 50 amino acids, an adjoining region containing about 70 amino acids that serves as the membrane binding domain, and a C-terminal globular catalytic domain (Fig. 6).

PGHS-1 and PGHS-2 are integral membrane proteins. However, their interactions with membranes do not involve typical transmembrane helices. Instead, analysis of the crystal structures and membrane domain labeling studies have established that PGHSs interact *monotopically* with only one surface of the membrane bilayer [1]. The interaction involves four short amphipathic α-helices present in the membrane binding domain noted above. The side chains of hydrophobic residues located on one surface of these helices interdigitate into and anchor PGHSs to the luminal surface of the ER and the inner and outer membranes of the nuclear envelope (Fig. 6).

2.7. PGHSs and nonsteroidal anti-inflammatory drugs

Prostaglandin synthesis can be inhibited by both nonsteroidal anti-inflammatory drugs (NSAIDs) and anti-inflammatory steroids. Both PGHS isozymes are pharmacological targets of common NSAIDs (e.g., aspirin, ibuprofen, naproxen). However, only prostaglandin synthesis mediated by PGHS-2 is inhibited by anti-inflammatory steroids, which block the synthesis of PGHS-2, at least in part, at the level of transcription [1]. Additionally, PGHS-2 is inhibited by 'COX-2 inhibitors' including rofecoxib and celecoxib. These drugs belong to a special class of NSAIDs specific for this isoform

and are often referred to as COX-2 drugs. The selectivity of these latter drugs depends on subtle structural differences between the cyclooxygenase active sites of PGHS-1 and PGHS-2 [1]. These same differences account, at least in part, for the ability of PGHS-2 but not PGHS-1 to bind and oxygenate 2-arachidonyl-glycerol.

The best known NSAID is aspirin, acetylsalicylic acid. Aspirin binds to the cyclooxygenase active site, and, once bound, can acetylate Ser530 (Fig. 5) [1]. Acetylation of this active site serine causes irreversible cyclooxygenase inactivation. Curiously, the hydroxyl group of Ser530 is not essential for catalysis, but acetylated Ser530 protrudes into the cyclooxygenase site and interferes with the binding of arachidonic acid [1].

Acetylation of PGHSs by aspirin has important pharmacological consequences. Besides the analgesic, anti-pyretic, and anti-inflammatory actions of aspirin, low-dose aspirin treatment — one 'baby' aspirin daily or one regular aspirin every three days — is a useful anti-platelet cardiovascular therapy [11]. This low-dosage regimen leads to selective inhibition of platelet thromboxane formation (and platelet aggregation) without appreciably affecting the synthesis of other prostanoids in other cells. Circulating blood platelets lack nuclei and are unable to synthesize new protein. Exposure of the PGHS-1 of platelets to circulating aspirin causes irreversible inactivation of the platelet enzyme. Of course, PGHS-1 (and PGHS-2) inactivation also occurs in other cell types, but cell types other than platelets can resynthesize PGHSs relatively quickly. For new PGHS-1 activity to appear in platelets, new platelets must be formed. Because the replacement time for platelets is five to ten days, it takes time for the circulating platelet pool to regain its original complement of active PGHS-1.

There are many NSAIDs other than aspirin [1,12]. In fact, this is one of the largest niches in the pharmaceutical market, currently accounting for about five billion dollars in annual sales. Like aspirin, other NSAIDs act by inhibiting the cyclooxygenase activity of PGHS [1,11,12]. However, unlike aspirin, most of these drugs cause reversible enzyme inhibition simply by competing with arachidonate for binding. A well-known example of a reversible nonsteroidal anti-inflammatory drug is ibuprofen. All currently available NSAIDs inhibit both PGHS-1 and PGHS-2 [1,12]. However, inhibition of PGHS-2 appears to be primarily responsible for both the anti-inflammatory and analgesic actions of NSAIDs [11]. Dual inhibition of PGHS-1 and PGHS-2 with common NSAIDs causes unwanted ulcerogenic side-effects [1,5,12]. Indeed, the newly developed COX-2 drugs rofecoxib and celecoxib exhibit the anti-inflammatory and analgesic actions of classical NSAIDs but have lesser side-effects, particularly gastro-intestinal side-effects [11].

Recent attention has been focussed on COX-2 inhibitors as prophylactic agents in the prevention of colon cancer. About 85% of tumors of the colon express elevated levels of PGHS-2 and classical NSAIDs and COX-2 inhibitors reduce mortality due to colon cancer (Takuku et al., 1998; Dubois, 2001).

2.8. Regulation of PGHS-1 and PGHS-2 gene expression

PGHS-1 and PGHS-2 are encoded by separate genes [1]. Apart from the first two exons, the intron/exon arrangements are similar. However, the PGHS-2 gene (~8 kb) is considerably smaller than the PGHS-1 gene (~22 kb). The PGHS-1 gene is on human chromosome 9, while the PGHS-2 gene is located on human chromosome 1.

The expressions of the PGHS-1 and PGHS-2 genes are regulated in quite different ways. PGHS-1 is expressed more or less constitutively in almost all tissues, whereas PGHS-2 is absent from cells unless induced in response to cytokines, tumor promoters, or growth factors [1]. Cells use PGHS-1 to produce prostaglandins needed to regulate 'housekeeping activities' typically involving rapid responses to circulating hormones (Fig. 2). PGHS-2 produces prostanoids which function during specific stages of cell differentiation or replication; it is not yet clear what these latter functions are nor whether they involve products formed from arachidonate or 2-AG or both. There is some indirect evidence suggesting that at least some of the products formed via PGHS-2 operate at the level of the nucleus through peroxisomal proliferator-activated receptors to modulate transcription of specific genes (Chapters 10 and 16) [1,5].

Relatively little is known concerning the regulation of expression of PGHS-1, although the enzyme must be under developmental control. The regulation of expression of PGHS-2 continues to be an area of intensive investigation. Much of what is known about PGHS-2 comes from studies with cultured fibroblasts, endothelial cells and macrophages [1]. Typically, PGHS-2 is induced rapidly (1–3 h) and dramatically (20- to 80-fold). Platelet-derived growth factor, phorbol ester, and interleukin-1β induce PGHS-2 expression in fibroblasts and endothelial cells. Bacterial lipolysaccharide, interleukin-1β, and tumor necrosis factor a stimulate PGHS-2 in monocytes and macrophages. While only a limited number of tissues and cell types have been examined, it is likely that PGHS-2 can be induced in almost any cell or tissue with the appropriate stimuli. Importantly, as noted earlier, PGHS-2 expression, but not PGHS-1 expression, can be completely inhibited by anti-inflammatory glucocorticoids such as dexamethasone [1,12].

The promoters of the two PGHS genes are indicative of their mode of regulation. PGHS-1 has a TATA-less promoter, a feature common to housekeeping genes. Reporter plasmids constructed with the 5′-upstream region of the PGHS-1 gene have failed to show any significant inducible transcription from this promoter, supporting the concept that regulation of PGHS-1 occurs only developmentally. The PGHS-2 promoter, on the other hand, contains a TATA box, and experiments with reporter plasmids containing the PGHS-2 promoter and upstream 5′-flanking sequence have demonstrated that PGHS-2 is highly regulatable. Transcriptional activation of the PGHS-2 gene appears to be one important mechanism for increasing PGHS-2 expression. Transcription of PGHS-2 can be controlled by multiple signaling pathways including the cAMP pathway, the protein kinase C pathway (phorbol esters), viral transformation (*src*), and other pleiotropic pathways such as those activated by growth factors, endotoxin, and inflammatory cytokines. These latter agents (e.g., platelet-derived growth factor, lipopolysaccharide, interleukin-1β, tumor necrosis factor α) likely share convergent pathways involving nuclear factor κB (NFκB) and the CAAT enhancer binding protein (C/EBP), two transcription factors common to inflammatory responses, and one or more of the established mitogen activated protein kinase cascades: ERK1/2, JNK/SAPK, and p38/RK/Mpk2.

The nucleotide sequence of the PGHS-2 gene promoter is known but identification of *cis*-elements responsible for the regulation of this gene is incomplete. Regulatory elements in the 5′-flanking regions of the PGHS-2 gene that are known to regulate

transcription are the overlapping E-box and CRE-1 sequence most proximal to the TATA box, a C/EBP-2 sequence, a NFκB binding site and a downstream CRE-2. In fibroblasts and endothelial cells, the most critical of the regulatory sequences is the CRE-1. This type of response element typically is activated by hetero- and homo-dimers of the c-Fos, c-Jun and ATF families of bZIP proteins (AP-1) and the cAMP regulatory binding protein (CREB). Activation of c-Jun is required for transcriptional activation of the PGHS-2 promoter in response to growth factors. It is not known if c-Jun forms homodimers, or interacts with other bZIP proteins.

In macrophages bacterial endotoxin stimulates PGHS-2 expression through cooperative activation via the CRE-1, C/EBP-2, NFκB and CRE-2 regulatory elements (Wingerd, 2002). Tumor necrosis factor α-stimulated expression is dependent on a NFκB site and the C/EBP-1 site. The C/EBPβ and C/EBPδ transcription factors are commonly involved in the regulation of inflammatory responses, and C/EBP regulatory elements are frequently found in promoters of so called acute phase genes. This family of transcription factors is activated by most of the inflammatory stimuli which induce PGHS-2 expression. C/EBPs bind to PGHS-2 promoters and function in conjunction with USF-1, NFκB, and c-Jun *cis*-regulatory proteins to activate transcription.

2.9. PGH_2 metabolism

Although all the major prostanoids are depicted in Fig. 2 as being formed by a single cell, prostanoid synthesis appears to be cell-specific [1]. For example, platelets form mainly TXA_2, endothelial cells form PGI_2 as their major prostanoid, and PGE_2 is the major prostanoid produced by renal collecting tubule cells. The syntheses of PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2, and TXA_2 from PGH_2 are catalyzed by PGE synthase, PGD synthase, PGF_α synthase, PGI synthase, and TXA synthase, respectively [1]. Formation of $PGF_{2\alpha}$ involves a two-electron reduction of PGH_2, and a PGF_α synthase utilizing NADPH can catalyze this reaction. All other prostanoids are formed via isomerization reactions involving no net change in oxidation state from PGH_2.

PGI synthase and TXA synthase are hemoproteins with molecular weights of 50–55,000. Both of these proteins are cytochrome P-450s. Both enzymes, like PGHSs, undergo suicide inactivation during catalysis. TXA synthase is found in abundance in platelets and lung. PGI synthase is localized to endothelial cells, as well as both vascular and nonvascular smooth muscle [13]. Both TXA and PGI synthases are found on the cytoplasmic face of the ER. PGH_2 formed in the lumen of the ER via PGHSs diffuses across the membrane and is converted to a prostanoid end product on the cytoplasmic side of the membrane.

At least four different proteins have been shown to have PGE synthase activity including a cytosolic PGE synthase, an inducible, membrane-associated PGE synthase and two cytosolic glutathione-*S*-transferase isozymes [13]. Cytosolic PGE synthase appears to be more tightly coupled to PGHS-1. Microsomal PGE synthase has been reported to be more tightly coupled to PGHS-2 and interestingly, its expression like that of PGHS-2 is inhibited by anti-inflammatory steroids such as dexamethasone. All PGE synthases require reduced glutathione as a cofactor. Glutathione facilitates cleavage of the endoperoxide group and formation of the 9-keto group [14]. PGF_α synthase

activity has been partially purified from lung. Structurally, the enzyme is a member of the aldose reductase family of proteins. Glutathione-dependent and -independent PGD synthases have been isolated. The glutathione-dependent forms also exhibit glutathione-*S*-transferase activity. A glutathione-independent form of PGD synthase has been purified from brain [13].

3. Prostanoid catabolism and mechanisms of action

3.1. Prostanoid catabolism

Once a prostanoid is formed on the cytoplasmic surface of the ER, it diffuses to the cell membrane and exits the cell probably via carrier-mediated transport [14]. Prostanoids are local hormones that act very near their sites of synthesis. Unlike typical circulating hormones that are released from one major endocrine site, prostanoids are synthesized and released by virtually all organs. In addition, all prostanoids are inactivated rapidly in the circulation. The initial step of inactivation of PGE_2 is oxidation to a 15-keto compound in a reaction catalyzed by a family of 15-hydroxyprostaglandin dehydrogenases. Further catabolism involves reduction of the double bond between C-13 and C-14, ω-oxidation, and β-oxidation.

3.2. Physiological actions of prostanoids

Prostanoids act both in an autocrine fashion on the parent cell and in a paracrine fashion on neighboring cells [15,16]. Typically, the role of a prostanoid is to coordinate the responses of the parent cells and neighboring cells to the biosynthetic stimulus, a circulating hormone. The actions of prostanoids are mediated by G-protein-linked prostanoid receptors of the seven *trans* membrane domain receptor superfamily [16].

Those examples which have been studied in the most detail are the renal collecting tubule-thick limb interactions involving PGE_2 synthesized by the collecting tubule and the platelet–vessel wall interactions involving PGI_2 and TXA_2 [14]. For example, in the case of platelets TXA_2 is synthesized by platelets when they bind to subendothelial collagen that is exposed by microinjury to the vascular endothelium. Newly synthesized TXA_2 promotes subsequent adherence and aggregation of circulating platelets to the subendothelium. In addition, TXA_2 produced by platelets causes constriction of vascular smooth muscle. The net effect is to coordinate the actions of platelets and the vasculature in response to deendothelialization of arterial vessels. Thus, prostanoids can be viewed as local hormones which coordinate the effects of circulating hormones and other agents (e.g., collagen) that activate their synthesis.

3.3. Prostanoid receptors

The identification and characterization of prostanoid receptors has occurred during the past ten years [16]. These results coupled with studies of PGHS and prostanoid receptor knockout mice [5,15] have been critical in beginning to rationalize earlier results of

studies on the physiological and pharmacological actions of prostanoids that had been somewhat confusing and difficult to interpret because prostaglandins were found to cause such a wide variety of seemingly paradoxical effects.

The seminal step in understanding the structures of prostanoid receptors and their coupling to second messenger systems resulted from the cloning of receptors for each of the prostanoids by Narumiya and others [16]. Prostanoid receptor cloning began with the TXA/PGH receptor known as the TP receptor. cDNA encoding this receptor was cloned using oligonucleotide probes designed from protein sequence data obtained from the TP receptor purified from platelets. The results confirmed biochemical predictions that the TP receptor was a seven-membrane spanning domain receptor of the rhodopsin family. Subsequent cloning of other receptors was performed by homology screening using receptor cDNA fragments as cross-hybridization probes. All of these prostanoid receptors are of the G-protein-linked receptor family.

It is now clear that there are pharmacologically distinct receptors for each of the known prostanoids. In the case of PGE_2, four different prostaglandin E (EP) receptors have been identified and designated as EP1, EP2, EP3, and EP4 receptors. Based on studies with selective agonists for each of the EP receptors and their effects on second messenger production, it appears that EP1 is coupled through G_q to the activation of phospholipase C, EP2 and EP4 are coupled via G_s to the stimulation of adenylate cyclase and EP3 receptors are coupled via G_i to the inhibition of adenylate cyclase [16].

In order to determine the physiological roles of various prostanoid receptors a number of knockout mice have been developed [15,16]. These studies indicate that prostacyclin receptors are involved in at least some types of pain responses, EP3 receptors are involved in the development of fever, PGE receptors are important in allergy, EP2 and EP4 function in bone resorption and EP1 receptors are involved in chemically induced colon cancer. The availability of cloned prostanoid receptors provides a rationale and the appropriate technology to search for receptor agonists and antagonists that might provide some specificity beyond the currently available cyclooxygenase inhibitors which prevent broadly the synthesis of all prostaglandins.

The realization that the gene for PGHS-2 is an immediate early gene associated with cell replication and differentiation suggests that prostanoids synthesized via PGHS-2 may have nuclear effects. As noted above there have been several reports indicating that prostanoid derivatives can activate some isoforms of peroxisomal proliferator activated receptors (PPARs). There is evidence that PGI_2 can be involved in PPARδ-mediated responses such as decidualization and apoptosis [5] (S.K. Dey, 2000).

4. Leukotrienes and lipoxygenase products

4.1. Introduction and overview

Leukotrienes are produced by the action of 5-lipoxygenase (5-LO) which carries out the insertion of a diatomic oxygen at carbon atom-5 of arachidonic acid yielding 5(*S*)-hydroperoxy eicosatetraenoic acid (5-HpETE). A subsequent dehydration reaction catalyzed by the same enzyme, 5-LO, results in formation of leukotriene A_4 (LTA_4),

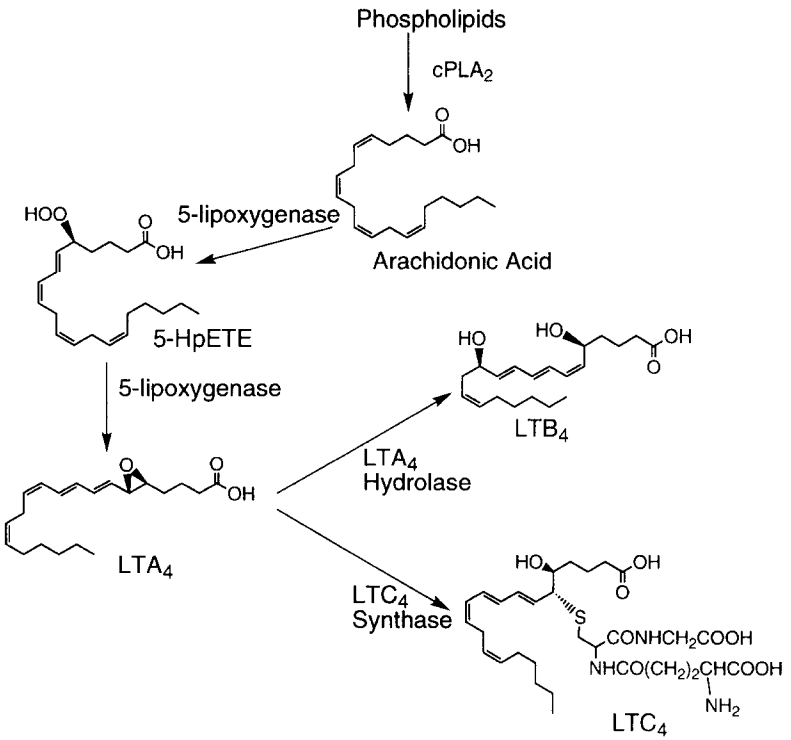

Fig. 7. Biochemical pathway of the metabolism of arachidonic acid into the biologically active leukotrienes. Arachidonic acid released from phospholipase by cytosolic (c) PLA_2 is metabolized by 5-lipoxygenase to 5-hydroperoxyeicosatetraenoic acid (5-HpETE) and leukotriene A_4 (LTA_4) which is then enzymatically converted into leukotriene B_4 (LTB_4) or conjugated by glutathione to yield leukotriene C_4 (LTC_4).

the chemically reactive precursor of biologically active leukotrienes. As was the case with prostanoid biosynthesis, leukotriene biosynthesis depends upon the availability of arachidonic acid as a free carboxylic acid as the 5-LO substrate, which typically requires the action of cytosolic phospholipase A_2 to release arachidonic acid from membrane phospholipids. Also, leukotrienes are not stored in cells, but synthesis and release from cells are rapid events following cellular activation. Interest in the leukotriene family of arachidonate metabolites arises from the potent biological activities of two products derived from LTA_4, that being leukotriene B_4 (LTB_4) and leukotriene C_4 (LTC_4) (Fig. 7). LTB_4 is a very potent chemotactic and chemokinetic agent for the human polymorphonuclear leukocyte, while LTC_4 powerfully constricts specific smooth muscle such as bronchial smooth muscle, and mediates leakage of vascular fluid in the process of edema [17]. The name leukotriene was conceived to capture two unique attributes of these molecules. The first attribute relates to those white blood cells derived from the bone marrow that have the capacity to synthesize this class of eicosanoid, for example the polymorphonuclear *leukocyte*. The last part of the name refers to the unique chemical structure, a conjugated *triene*, retained within these eicosanoids.

There are numerous other biochemical products of arachidonate metabolism formed

by lipoxygenase enzymes other than 5-LO. Other monooxygenases expressed in mammalian cells include 12-lipoxygenase, 15-lipoxygenase and much less frequently 8-lipoxygenase. These enzymes are named in accordance with the carbon atom position of arachidonate initially oxygenated even though other polyunsaturated fatty acids can be substrates. In addition, arachidonic acid can be oxidized by specific isozymes of cytochrome P-450, leading to a family of epoxyeicosatrienoic acids (EETs). Methyl terminus oxidized arachidonate as well as lipoxygenase-like monohydroxy eicosatetraenoic acid (HETE) products are also formed. In general, much more is known about the biochemical role of prostaglandins and leukotrienes as mediators of biochemical events; little is known concerning the exact role played by the other lipoxygenase products or cytochrome P-450 products. In accord with the body of information available, various pharmacological tools are available to inhibit 5-LO as well as specific leukotriene receptors.

4.2. Leukotriene biosynthesis

The arachidonate 5-lipoxygenase (5-LO, EC 1.13.11.34) is a metalloenzyme with bound iron coordinated by four histidine residues. This 77,852 Da protein (human 5-LO) catalyzes the addition of molecular oxygen to the 1,4-*cis*-pentadienyl structural moiety closest to the carboxyl group of arachidonic acid to yield a conjugated diene hydroperoxide, typical for all lipoxygenase reactions. Some details of the mechanism of 5-LO are known in that 5-LO removes the pro-*S* hydrogen atom from carbon-7 of arachidonic acid, leading to reduction of Fe(III) to Fe(II) likely in a radical type mechanism [18]. Molecular oxygen then adds to carbon-5 to yield the hydroperoxy radical. The hydroperoxy radical abstracts a hydrogen atom to yield 5(*S*)-HpETE (5(*S*)-hydroperoxy-6,8,11,14-(*E*,*Z*,*Z*,*Z*)-eicosatetraenoic acid). A second enzymatic activity of 5-LO, sometimes termed LTA_4 synthase activity, catalyzes the stereospecific removal of the pro-*R* hydrogen atom at carbon-10 of the 5(*S*)-HpETE through a second redox cycle followed by a sigmatropic shift of the electrons to form the conjugated triene epoxide and loss of hydroxide (Fig. 8). One unique feature of the iron redox cycle is that these reactions involve one electron rather than two electron transfers, typical of most peroxidase enzymes, in what has been called a pseudoperoxidase reaction [19]. The product of this reaction, 5(*S*),6(*S*)-oxido-7,9,11,14-(*E*,*E*,*Z*,*Z*)-eicosatetraenoic acid,

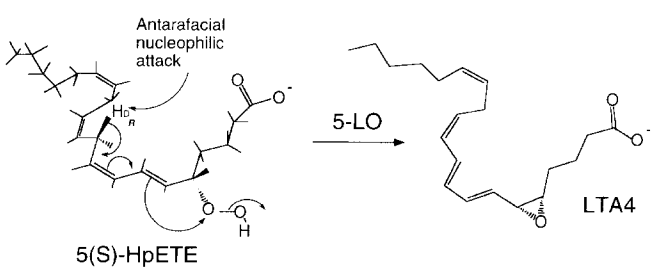

Fig. 8. Detailed mechanism of the 5-lipoxygenase reaction where the pro-*R* hydrogen from carbon-10 in 5-HpETE is removed by 5-lipoxygenase followed by sigmatropic rearrangement of electrons to form LTA_4.

is LTA_4, a conjugated triene epoxide that is highly unstable. LTA_4 undergoes rapid hydrolysis in water with a half-life of less than 10 s at pH 7.4 and also can react with proteins as well as DNA. Nonetheless within cells, LTA_4 is stabilized by binding to proteins that remove water from the immediate environment of the epoxide structure. The nonenzymatic hydrolysis products of LTA_4 include several biologically inactive and enantiomeric 5,12- and 5,6-diHETEs. However, the hydrolysis of LTA_4 catalyzed by LTA_4 hydrolase [20] produces the biologically active LTB_4, 5(*S*),12(*R*)-dihydroxy-6,8,10,14-(*Z*,*E*,*E*,*Z*)-eicosatetraenoic acid. A second pathway for LTA_4 metabolism is prominent in cells expressing the enzyme LTC_4 synthase [21] which catalyzes the addition of glutathione to carbon-6 of the triene epoxide yielding 5(*S*),6(*R*)-*S*-glutathionyl-7,9,11,14-(*E*,*E*,*Z*,*Z*)-eicosatetraenoic acid (LTC_4). LTC_4 synthase has been found to be localized on the nuclear membrane and is a unique glutathione (*S*) transferase. The formation of either LTC_4 or LTB_4 is controlled by the expression of either LTA_4 hydrolase or LTC_4 synthase by specific cell types. The human neutrophil, for example, expresses LTA_4 hydrolase and produces LTB_4 while the mast cell and eosinophil produce LTC_4, since they express LTC_4 synthase. Interestingly, cells have been found which do not have 5-LO, but do express either LTA_4 hydrolase (e.g., erythrocytes and lymphocytes) or LTC_4 synthase (e.g., platelets and endothelial cells). Studies have found that cells in fact cooperate in the production of biologically active leukotrienes through a process termed transcellular biosynthesis (J. Maclouf, 1989) where a cell such as the neutrophil or mast cell generates LTA_4 which is then released from the cell and is then taken up by either a platelet to make LTC_4 or red blood cell to make LTB_4. In spite of the chemical reactivity of LTA_4, this process is known to be highly efficient and approximately 60 to 70% of the LTA_4 produced by the activated neutrophil can be released to another cell for transcellular biosynthesis of leukotrienes (A. Sala, 1996).

4.3. Enzymes involved in leukotriene biosynthesis

4.3.1. 5-Lipoxygenase

The human 5-LO gene is unusually large and is present on chromosome 10. This gene covers more than 80 kb of DNA and has 14 exons that encode a 673 amino acid protein (without the initiator methionine residue) [22]. 5-Lipoxygenase has been purified from human, pig, rat, and guinea pig leukocytes, all with close to 90% homology. It is interesting to note that the purified or recombinant enzyme requires several cofactors for activity that include Ca^{2+}, ATP, fatty acid hydroperoxides, and phosphatidylcholine in addition to the arachidonic acid and molecular oxygen substrates [17,19]. Purified 5-LO was found to catalyze the initial oxidation of arachidonic acid to yield 5-HpETE as well as the second enzymatic reaction to convert 5-HpETE into LTA_4. Recombinant human 5-LO has been expressed in osteosarcoma cells, cos-M6 cells, baculovirus-infected SF9 insect cells, yeast, and *Escherichia coli* [19].

Low concentrations of calcium ion (1–2 μM) are required for maximal activity of purified 5-LO, but the major role of calcium appears to be that of increasing lipophilicity of 5-LO in order to promote membrane association. ATP has a stimulatory effect on 5-LO at 20 nM and lipid hydroperoxides are important to initiate the 5-LO catalytic

cycle since they readily form Fe(III) within 5-LO by the pseudoperoxidase mechanism. Microsomal membranes as well as phosphatidylcholine vesicles can stimulate purified 5-LO activity since 5-LO performs the oxidation of arachidonic acid at the interface between the membrane and cytosol in a manner similar to that of cytosolic PLA_2. Calcium ions increase the association of 5-LO with phosphatidylcholine vesicles that likely recapitulates events within the cell where 5-LO becomes associated with the nuclear membrane [23] and to which arachidonic acid is presented by a second gene product which has been termed 5-LO activating protein (FLAP) [19]. In cells such as the neutrophil and mast cell where 5-LO is found in the cytosol, 5-LO is only catalytically active when bound to a membrane, typically the nuclear membrane. In fact, in some cells 5-LO is found to be constitutively associated with the nuclear membrane, likely a result of a process of cellular activation while 5-LO is found in the alveolar macrophage within the nucleus itself [23].

4.3.2. 5-Lipoxygenase activating protein (FLAP)

FLAP was found during the course of development of the drug MK-886 by workers at Merck-Frosst in Montreal [19]. They found this drug bound to a novel protein that was essential for the production of leukotrienes in stimulated, intact cells and hence, the name 'five lipoxygenase activating protein'. FLAP is a unique 161 amino acid containing protein (18,157 Da). While the role of FLAP is not entirely clear, experiments using a ^{125}I-labeled photoaffinity analog of arachidonic acid suggested that FLAP functions as a substrate transfer protein and in this manner stimulates 5-LO catalyzed formation of leukotrienes. Recently, it has been found that LTC_4 synthase has 31% amino acid identity to FLAP with a highly conserved region possibly involved in arachidonate binding for both proteins [19].

4.3.3. LTA_4 hydrolase

LTA_4 hydrolase catalyzes the stereochemical addition of water to form the neutrophil chemotactic factor LTB_4. LTA_4 hydrolase contains 610 amino acids (excluding the first methionine) with a molecular weight of 69,399 Da. LTA_4 hydrolase contains one zinc atom per enzyme molecule and this metal ion is essential for the catalytic activity [20]. LTA_4 hydrolase is also a member of a family of zinc metalloproteases and exhibits some protease activity. The finding of this activity led to the discovery of several drugs such as bestatin and captopril that are inhibitors of this enzyme. LTA_4 hydrolase is found in many cells including those which do not contain 5-LO and it is felt that these cells play an important role in transcellular biosynthesis of LTB_4 through cell–cell cooperation. LTA_4 is thought to be localized in the cytosol of the cell and is the only protein in the leukotriene biosynthetic cascade that is not found on the nuclear membrane following cellular activation. Therefore, in order to efficiently metabolize the chemically reactive LTA_4, either the LTA_4 hydrolase must come in close contact with the nuclear membrane during LTA_4 biosynthesis or a carrier protein must present LTA_4 to LTA_4 hydrolase in the cytosol. LTA_4 hydrolase is known to be efficiently suicide inactivated by LTA_4 when the electrophilic epoxide becomes covalently bound to the enzyme presumably within the active site (F.A. Fitzpatrick, 1990). A specific tyrosine residue has been found to be modified by LTA_4 and this residue has been implicated as the potential proton donor

in the suggested mechanism of LTA_4 hydration. The 3-dimensional structure of LTA_4 hydrolase has now been determined by X-ray crystallography [20].

4.3.4. LTC_4 synthase

The conjugation of the tripeptide glutathione (γ-glutamyl-cysteinyl glycine) to the triene epoxide LTA_4 is carried out by LTC_4 synthase (EC 2.5.1.37). This enzyme is found localized on the nuclear envelope of cells and has little homology to soluble glutathione (*S*) transferases. LTC_4 synthase does have some primary amino acid sequence homology to the recently described microsomal glutathione (*S*) transferases and FLAP [21]. LTC_4 synthase has a restricted distribution and is found predominantly in mast cells, macrophages, eosinophils, and monocytes. However, human platelets and endothelial cells have been found to express LTC_4 synthase. Purified recombinant LTC_4 synthase will conjugate glutathione to both LTA_4 and LTA_4 methyl ester with a K_m of approximately 2–4 μM for the free acid and 7–10 μM for the methyl ester. The K_m for glutathione is approximately 2 mM. The drug MK-886 was found to inhibit LTC_4 synthase with an IC_{50} of approximately 2–3 μM [19]. The gene for LTC_4 synthase is located on human chromosome 5, distal to that of cytokine, growth factor, and receptor genes relevant to the TH2 phenotype (T.D. Bigby, 2000).

4.4. Regulation of leukotriene biosynthesis

The biosynthesis of leukotrienes within cells is highly regulated and depends not only on the availability of arachidonic acid and molecular oxygen, but also on the subcellular location of 5-LO. Resting neutrophils synthesize little if any leukotrienes; however, following the elevation of intracellular calcium either through a physiological event such as phagocytosis or by pharmacological manipulation with the calcium ionophore A23187, the neutrophil produces a substantial amount of 5-LO products including leukotrienes from either endogenous or exogenous arachidonic acid. The need for an increase in intracellular calcium ion concentrations is a distinguishing feature of 5-LO that differentiates this enzyme from other lipoxygenases and cyclooxygenase.

A major determinant of 5-LO activity is the translocation of 5-LO to the nuclear membrane (Fig. 9). It has even been possible to demonstrate that the site of localization of 5-LO is at the inner nuclear membrane. The Ca^{2+}-dependent translocation event is thought to bring 5-LO to the same region where FLAP and cytosolic PLA_2 translocate. Regulation of leukotriene biosynthesis is thus a process of assembly of the leukotriene biosynthetic machine at a nuclear envelope site. It is this site where arachidonic acid is released from nuclear membrane phospholipids, then converted to LTA_4 and ultimately conjugated with glutathione by nuclear membrane LTC_4 synthase. The mechanism by which 5-LO is trafficked to the nuclear membrane is as yet undefined, but the discovery of this site of leukotriene biosynthesis was unexpected and suggests novel intracellular actions of leukotrienes or of 5-LO itself within the immediate nuclear environment [23].

The activity of 5-LO is also regulated by a suicide inactivation mechanism where LTA_4 rapidly inactivates 5-LO, likely through a covalent modification mechanism [18]. Continued synthesis of leukotrienes then requires synthesis of new 5-LO. The production of 5-LO is known to be regulated at the level of gene transcription as well

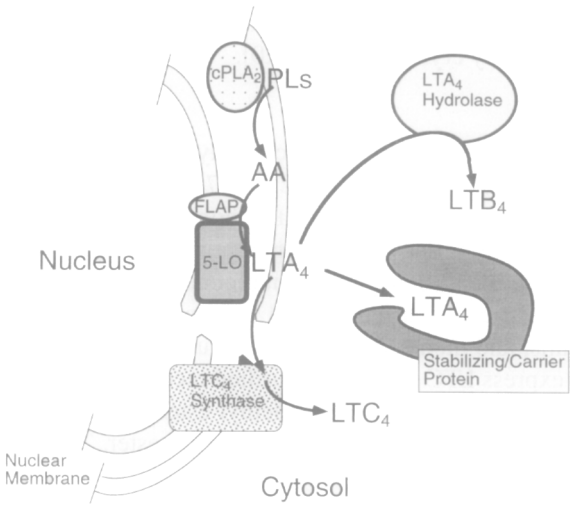

Fig. 9. Proposed model for the location of biosynthetic events occurring during leukotriene biosynthesis at the nuclear membrane of cells. Arachidonic acid is released from membrane glycerophospholipids (PLs) by translocated and nuclear membrane associated cytosolic (c) PLA_2 and is then presented to 5-lipoxygenase (5-LO) by way of FLAP. LTA_4 is either converted by nuclear membrane associated LTC_4 synthase into LTC_4 or carried to LTA_4 hydrolase possibly by a fatty acid binding protein which can stabilize the chemically reactive LTA_4 that is available for transcellular biosynthesis.

as mRNA translation in addition to the translocation mechanism [22,23]. The 5-LO gene promoter contains several consensus-binding sites for known transcription factors including Sp1 and EGR-1. In a region located 212 to 88 base pairs upstream from the translation start site of the human 5-LO gene, a highly rich G + C region is found that contains the consensus sequence or SP1 and EGR-1 transcription factors. Furthermore, fairly common variations have been found in human populations in which deletions of one or two of the Sp1 binding motifs were observed [22]. This genetic polymorphism could have substantial effects on the induction of 5-LO transcription.

Various biochemical mechanisms can also alter leukotriene biosynthesis within cells. Because of the complexity of 5-LO activation and the requirement of the Ca^{2+}-dependent translocation event, modification of the signal transduction pathways are known to alter leukotriene biosynthesis. For example, elevation of cellular levels of cyclic AMP have been known for some time to inhibit leukotriene biosynthesis even when synthesis is stimulated by the powerful calcium ionophore A23187. Adenosine and A2A receptor agonists are known to inhibit production of leukotrienes in human neutrophils, most likely through an enhanced production of cyclic AMP (P. Borgeat, 1999). The product in neutrophil leukotriene biosynthesis, namely LTB_4, can also inhibit the synthesis of leukotrienes when initiated during the course of phagocytosis. These effects can be observed at 1–3 nM, are mediated through the LTB_4 receptor, and likely represent a feedback-like inhibition of leukotriene biosynthesis through inactivation of 5-LO as well as cytosolic PLA_2 (J. Fiedler, 1998). Pharmacological agents have been developed to inhibit leukotriene production through direct action on 5-LO. The drug

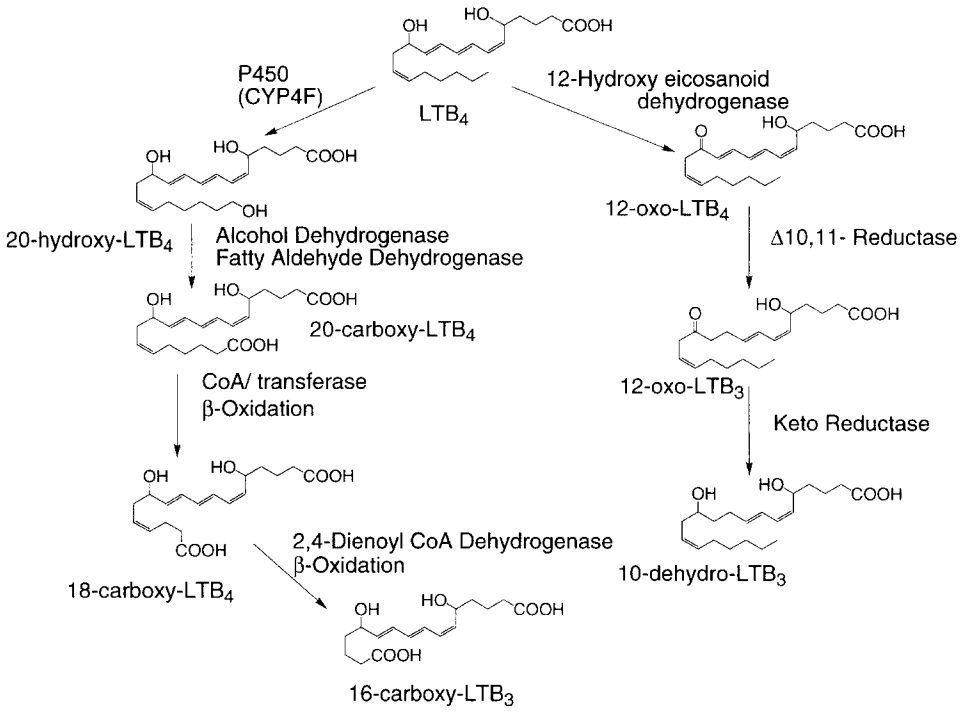

Fig. 10. Common metabolic transformations of LTB_4 either by the cytochrome P-450 (CYP4F-family members) and ω-oxidation followed by β-oxidation or by the 12-hydroxyeicosanoid dehydrogenase pathway which leads to reduction of the $\Delta^{10,11}$ double bond.

zileuton likely causes the reduction of activated 5-LO Fe(III) to the inactive 5-LO Fe(II) or prevents the oxidation of Fe(II) by lipid hydroperoxides, the pseudoperoxidase step, by serving as a competitive substrate [19]. Inhibitors of FLAP have been discussed above and include MK-886 and the related agent BAY x1005. Because of its close similarity to the FLAP protein, LTC_4 synthase can be inhibited by MK-886 albeit at higher concentrations.

4.5. Metabolism of leukotrienes

The conversion of leukotrienes into alternative structural entities is an important feature of inactivation of these potent biologically active eicosanoids. Metabolism of leukotrienes is rapid and the exact pathway depends upon whether the substrate is LTB_4 or LTC_4. LTB_4 is rapidly metabolized through both oxidative and reductive pathways (Fig. 10) [24]. The most prominent pathway present in the human neutrophil (CYP4F3) as well as hepatocyte (CYP4F2) involves specific and unique cytochrome P-450s of the CYP4F family. cDNAs encoding sixteen different proteins have now been cloned and expressed in several animal species [25] and each of these enzymes efficiently converts LTB_4 into 20-hydroxy-LTB_4. 20-Hydroxy-LTB_4 has some biological activity since it is a competitive agonist for the LTB_4 receptor. In the human neutrophil, 20-hydroxy-LTB_4

is further metabolized into 20-carboxy-LTB_4 by CYP4F3. In the hepatocyte and other tissues, 20-hydroxy-LTB_4 is further metabolized by alcohol dehydrogenase to form 20-oxo-LTB_4 then by fatty aldehyde dehydrogenase to form 20-carboxy-LTB_4 both of which reactions require NAD^+ (Fig. 10).

A unique reductive pathway has been observed to be highly expressed in cells such as keratinocytes, endothelial cells and kidney cells. This pathway arises from an initial oxidation of the 12-hydroxy group to a 12-oxo moiety followed by reduction of the conjugated dienone and double bond $\Delta^{10,11}$. The products of the 12-hydroxy eicosanoid dehydrogenase pathway have been found to be devoid of biological activity and in certain cells represent the major pathway of inactivation.

A secondary metabolic pathway is β-oxidation which was found to predominate from the 20-carboxy terminus of 20-carboxy-LTB_4 [14]. These events take place both within the peroxisome as well as the mitochondria of the hepatocyte. β-oxidation can also occur from the C-1 carboxyl moiety of LTB_4 which eventually results in the loss of the C-5 hydroxyl group. The importance of the each oxidation processes in LTB_4 metabolism is seen in human subjects with various genetic abnormalities. Deficiencies in peroxisomal metabolism (Zellweger disease) leads to a reduction in β-oxidation and in these individuals LTB_4 and 20-carboxy-LTB_4 can be measured as urinary excretion products (E. Mayatepek, 1999). Individuals with a deficiency in fatty aldehyde dehydrogenase termed Sjogren–Larsson syndrome were found to excrete measurable levels of LTB_4 and 20-hydroxy-LTB_4 (M.A. Willemsen, 2001). None of these compounds can be measured in the urine of normal individuals even when exogenous LTB_4 is administered.

The metabolism of LTC_4 results in activation as well as inactivation of biological activity of the sulfidopeptide leukotrienes. Initial peptide cleavage reactions including γ-glutamyl transpeptidase and various dipeptidases lead to the production of LTD_4 and LTE_4, both of which are biologically active metabolites (Fig. 11). Sulfidopeptide leukotrienes can also be metabolized specifically at the sulfur atom through oxidation reactions initiated by reactive oxygen species. More specific metabolic processing of the sulfidopeptide leukotrienes include ω-oxidation by cytochrome P-450 followed by β-oxidation from the ω-terminus resulting in a series of chain-shortened products [24]. Formation of 20-carboxy-LTE_4 results in complete inactivation of the biological activities of this molecule due to poor receptor recognition. Acetylation of the terminal amino group in LTE_4 and formation of *N*-acetyl-LTE_4 is an abundant metabolite in rodent tissue. In man, some LTE_4 is excreted in urine and has been used to reflect whole body production of sulfidopeptide leukotriene in vivo.

4.6. Biological activities of leukotrienes

LTB_4 is thought to play an important role in the inflammatory process by way of its chemotactic and chemokinetic effects on the human polymorphonuclear leukocyte. LTB_4 induces the adherence of neutrophils to vascular endothelial cells and enhances the migration of neutrophils (diapedesis) into extravascular tissues. The biological activity of LTB_4 is mediated through two specific G-protein-coupled receptors termed BLT_1 and BLT_2 [26]. BLT_1 (human receptor 37,591 Da) is almost exclusively expressed in human polymorphonuclear leukocytes and to a much lesser extent on macrophages

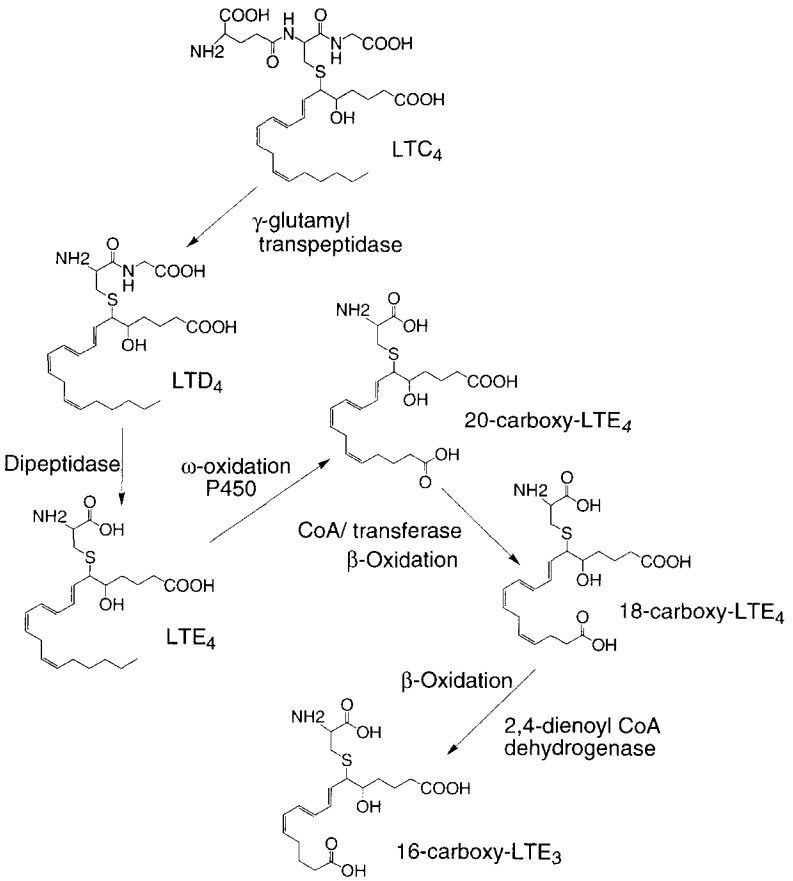

Fig. 11. Common metabolic transformations of LTC_4 to the biologically active sulfidopeptide leukotrienes, LTD_4 and LTE_4. Subsequent ω-oxidation of LTE_4 by cytochrome P-450 leads to the formation of 20-carboxy-LTE_4 which can undergo β-oxidation after formation of the CoA ester into a series of chain-shortened cysteinyl leukotriene metabolites.

and in tissues such as thymus and secretory PLeen. The human BLT gene is located on chromosome 14. The chemotactic effect of LTB_4 was shown to be mediated through the BLT_1 and BLT_2 receptors. Several specific agents have been developed by pharmaceutical companies to inhibit the LTB_4 receptor; however, none has been fully developed to be used in humans.

LTC_4 and the peptide cleavage products LTD_4 and LTE_4 have been identified as mediators causing bronchial smooth muscle contraction in asthma. These sulfidopeptide leukotrienes also increase vascular leakage leading to edema. The discovery of leukotrienes was, in fact, a result of the search for the chemical structure of the biologically active principle called 'slow reacting substance of anaphylaxis' (R.C. Murphy, 1979). Two receptors for the cysteinyl leukotriene have recently been characterized and termed $CysLT_1$ and $CysLT_2$. The $CysLT_1$ receptor was found to be a G-protein-coupled

receptor with seven *trans* membrane regions [27]. The $CysLT_1$ receptor has limited distribution in tissues with the most prominent being in smooth muscle of the lung and small intestine and both LTD_4 and LTC_4 activate this receptor. Several drugs are now available for inhibition of the $CysLT_1$ receptor in human subjects. These are montelukast (2–5 nM), pranlukast (4–7 nM), and zafirlukast (2–3 nM). Interestingly, the gene encoding the human $CysLT_1$ receptor is located on the X chromosome [27].

4.7. Other lipoxygenase pathways

Numerous lipoxygenases occur within the plant and animal kingdoms and these enzymes have in common several aspects. First, iron is an essential component of the catalytic activity of these enzymes and is held in place through histidine residues rather than by heme. Furthermore, these enzymes catalyze the insertion of molecular oxygen into polyunsaturated fatty acids, predominantly linoleic and arachidonic acids, with the initial formation of lipid hydroperoxides. In general, the overall biological activities of the lipoxygenase products are incompletely known and the significance of 12- and 15-lipoxygenase in man remains undefined.

4.7.1. 12-Lipoxygenase

Two different enzymes termed 12-lipoxygenase (12-LO) catalyze the formation of 12-hydroperoxyeicosatetraenoic acid from arachidonic acid which is subsequently reduced to 12-hydroxyeicosatetraenoic acid (Fig. 12). The human platelet expresses one 12-LO

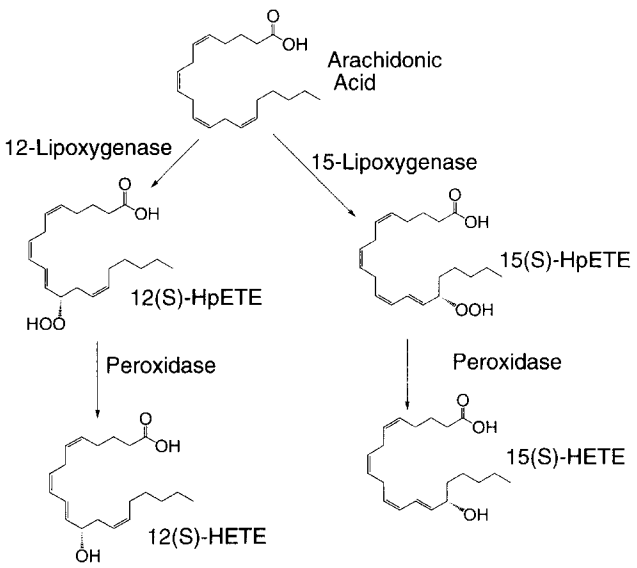

Fig. 12. Metabolism of arachidonic acid by 12- and 15-lipoxygenase pathways with corresponding stereospecific formation of hydroperoxyeicosatetraenoic acids (HpETE). Subsequent reduction of these hydroperoxides leads to the corresponding HETE at either carbon-12 or -15 which are thought to mediate biological activities of these enzymatic pathways.

type (EC 1.13.11.31) the cDNA of which has been cloned, sequenced, and found to encode a 662 amino acid protein with a molecular weight of 75,535 Da. A second 12-LO is observed in other mammalian systems including the mouse and rat and has been termed the leukocyte-type 12-LO. This latter lipoxygenase is very similar in many respects to a 15-lipoxygenase (15-LO) in terms of its substrate specificity and capability of forming both 12-HpETE and 15-HpETE from arachidonic acid. The human platelet 12-LO has approximately 65% identity in primary structure to that of 15-LO from human reticulocytes. In addition, there are other lipoxygenases less well studied including an epidermal lipoxygenase from newly differentiated keratinocytes and the lipoxygenase that oxygenates arachidonic acid at position C-15 and C-8. Both 12/15-LO types are suicide inactivated, but the leukocyte 12-LO undergoes autoinactivation at a much higher rate. Several lines of evidence suggest that the 12-LO pathway of arachidonate metabolism plays an important role in regulating cell survival and apoptosis (A.R. Brash, 1999).

4.7.2. 15-Lipoxygenase

The oxidation of arachidonic acid at carbon-15 is catalyzed by 15-LO, a soluble 661 amino acid containing protein with a molecular weight of 74,673 Da. Many cells express this enzyme which also efficiently oxidizes linoleic acid to 13-hydroperoxyoctadecadienoic acid and lesser extent 9-hydroperoxyoctadecadienoic acid because of broad substrate specificity as well as arachidonate to both 12-HpETE and 15-HpETE [28]. One distinguishing feature of this lipoxygenase is that it can oxidize arachidonic acid esterified to membrane phospholipids, thus forming esterified 15-HpETE. Expression of 15-LO is enhanced by several interleukins, suggesting a role of this enzyme in events such as atherosclerosis.

The X-ray crystal structure of mammalian 15-LO has revealed two domains, a catalytic domain and a β-barrel domain [29]. The β-barrel domain may be involved in the binding of this enzyme to phospholipid membranes, the source of either arachidonate or phospholipids in the oxidation process. The catalytic domain, which contains the histidine-coordinated Fe(III), holds the arachidonic acid assisted by an ionic bond between R403 and the ionized carboxyl group of arachidonic acid. The methyl terminus of arachidonic acid is thus placed deep within a hydrophobic binding pocket in an arrangement that is likely similar for other lipoxygenases.

Mammalian 15-LO is involved in the production of more complicated eicosanoids including the biologically active lipoxins [26]. Lipoxins are formed by the sequential reaction of both 15-LO and 5-LO acting on precursor arachidonate. For example, LTA_4 (the 5-LO product) can be converted to a lipoxin by action of 15-LO.

There are a host of biological activities initiated by these 15-LO dependent eicosanoids [28]. The unique activity of 15-LO in oxidizing intact phospholipids has been featured in several hypotheses linking the oxidation of phospholipids in atherosclerotic lesions to important role of these lipoxygenases. The recent availability of strains of mice which have a targeted deficiency in 5-, 12-, and 15-lipoxygenases (C. Funk, 2000) provides a powerful tool to ask specific questions concerning the role these lipoxygenases play in host defense reactions, cellular function, and perhaps disease processes.

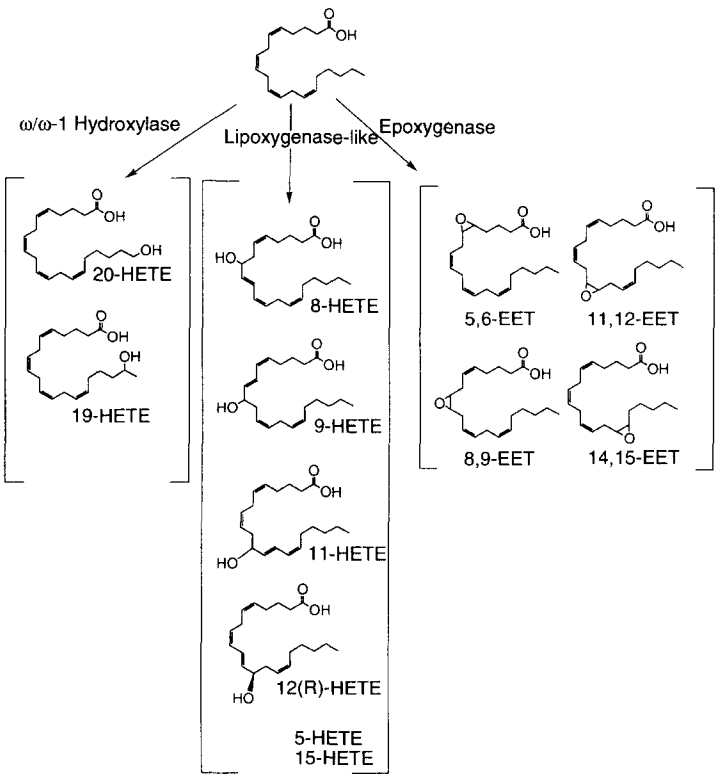

Fig. 13. Metabolism of arachidonic acid by cytochrome P-450 enzymes and the formation of three structurally distinct metabolite families. Omega-oxidation leads to a family of ω to ω-4 products of which 20-HETE (ω-oxidation) and 19-HETE (ω-1 oxidation) are indicated. The lipoxygenase-like mechanism of cytochrome P-450 metabolism leads to the formation of six different conjugated dienols, for which the structures of four are indicated. One unique biologically active lipoxygenase-like P-450 metabolite is 12(*R*)-HETE. The epoxygenase pathway leads to the formation of four regioisomeric epoxyeicosatrienoic acid (EETs) all of which are biologically active.

5. *Cytochrome P-450s and epoxygenase pathways*

Arachidonic acid can be metabolized to a series of products characterized by the introduction of a single oxygen atom from molecular oxygen and formation of three different types of initial products catalyzed by various cytochrome P-450 mixed function oxidases (Fig. 13) [30,31]. The three classes of products include a series of hydroxyeicosatetraenoic acids (HETEs) formed by an allylic oxidation mechanism resulting in a family of conjugated dienes isomeric to the reduced products of a lipoxygenase reaction. P-450 metabolites formed by this mechanism have been characterized as 5-, 8-, 9-, 11-, 12-, and 15-HETE, some of which are epimeric to the lipoxygenase catalyzed products, e.g., 12(*R*)-HETE. A second class of reactions involves oxidation of the terminal alkyl chain region of arachidonic acid with placement of a hydroxyl group between the terminal carbon atom (ω) through ω-4 position with formation of a family of ω-oxidized

monohydroxyeicosatetraenoic acids. Insertion of oxygen into the carbon–carbon bond results in the formation of a family of regioisomeric *cis* epoxyeicosatetraenoic acids (EETs) from which the general pathway has been named, the epoxygenase pathway. These regioisomers include 14,15-, 11,12-, 8,9-, and 5,6-EETs which can be formed either as an *R,S*, or the *S,R* enantiomer (Fig. 13).

5.1. Epoxygenase P-450 isozymes

With the availability of recombinant P-450 isozymes, it has been possible to identify specific isozymes that can metabolize arachidonic acid. EET biosynthesis can be accomplished by CYP1A, CYP2B, CYP2C, CYP2D, CYP2G, CYP2J, CYP2N, and CYP4A subfamilies [31]. For each of these, unique EET regioisomers are formed. For example CYP2C8 produces 14(*R*),15(*S*)-EET and 11(*R*),12(*S*)-EET with optical purities of 86% and 81%, respectively. However, it is likely that more than a single P-450 contributes to EET biosynthesis within a specific cell or tissue and thus the individual arachidonate epoxygenase metabolite may depend upon expression of specific P-450 isoforms. It is thought that the majority of EET biosynthesis in human and rat kidney is a result of CYP2C expression in these tissues. However, the induction of specific P-450s can greatly alter the production of specific epoxygenase products.

5.2. Occurrence of EETs

Various EETs have been measured in tissues as well as physiological fluids such as urine (G. FitzGerald, 1990). Biologically active lipids originally defined as an endothelium-derived hyperpolarizing factor and an inhibitor of Na^+/K^+ATPase found in the thick ascending loop of Henley cells were structurally characterized as 11(*R*),12(*S*)-EET and 20-hydroxyeicosatetraenoic acid, both derived from cytochrome P-450 mediated metabolism of arachidonic acid [30]. Interestingly, the EETs can readily form CoA esters and participate in reacylation of lysophospholipids which results in the reincorporation of these oxidized metabolites of arachidonic acid into phospholipid membranes, a biochemical feature not observed for prostaglandins, thromboxanes or leukotrienes. For example, human platelets have been found to contain 14,15-EET esterified within membrane phospholipids (Y. Zhu, 1995). It is felt that the majority of EETs produced within cells become reesterified to cellular glycerophospholipids.

5.3. Metabolism of EETs

A number of metabolic pathways operate on the primary epoxygenase metabolites of arachidonic acid. Some of the more abundant pathways include CoA-dependent reesterification as mentioned above as well as β-oxidation chain-shortening. A unique pathway involves epoxide hydrolase, a cytosolic enzyme that hydrates EETs to the corresponding vicinal dihydroxyeicosatrienoic acids [30]. There is also a microsomal epoxide hydrolase that can metabolize EETs, but at a somewhat lower rate. The soluble epoxide hydrolase does have substrate specificity, both in terms of the stereochemistry of the EET as well as its position in the arachidonic acid chain. As expected, there is

nonenzymatic hydration of these epoxides especially under acidic conditions that can be accelerated during the isolation of these arachidonate metabolites. Therefore, it is sometimes difficult to distinguish between nonenzymatic and enzymatic hydration of EETs. The 5,6-EET is a poor substrate for cytosolic as well as microsomal epoxide hydrolase; however, it has been observed to be an efficient substrate for PGH synthase, leading to the formation of 5,6-epoxy-PGH_1. This reactive intermediate can subsequently be transformed into corresponding 5,6-epoxy-prostaglandins of the E, F, and I series or into an epoxy thromboxane analog. All of the EETs can also be substrates for lipoxygenases which would introduce molecular oxygen at any 1,4-*cis*-pentadienyl position not interrupted by the epoxide ring. The EETs can also be conjugated with reduced glutathione catalyzed by glutathione (*S*) transferases. Studies of the metabolism of EETs by rat or mouse liver microsomal P-450 revealed the formation of a series of diepoxyeicosadienoic acids as well as monohydroxyepoxyeicosatrienoic acids. Interestingly, the diepoxides were found to be further transformed into tetrahydrofurandiols mediated by intermediate diol epoxides formed by soluble epoxide hydrolase. The characterization of specific products of EET and PGH synthase metabolism of arachidonic acid have led to the observation of 5,6-epoxy-PGE_1 as a renal vasodilator with similar potency to that of PGE_2 and the metabolism of 8,9-EET by PGH synthase leading to 11-hydroxy-8,9-epoxyeicosatrienoic acid which is a mitogen for rat glomerular mesangial cells.

5.4. Biological actions of EETs

Metabolites of arachidonic acid derived from the epoxygenase P-450 pathway have been studied extensively in terms of their pharmacological properties. Potent effects have been observed in modulating various ion channels, membrane bound transport proteins, mitogenesis, PPARα agonists, and activators of tyrosine kinase cascades [30]. EETs likely play an important role in mediating Na^+/K^+-ATPase and inhibiting the hydroosmotic effect of arginine vasopressin in the kidney. A picture has emerged for an important role of EETs in regulating renal vascular tone and fluid/electrolyte transport placing the EETs in the pathogenesis of hypertension.

6. Future directions

There is currently a reasonable understanding of the structures of PGHS-1 and -2, but many of the relationships between structure and function remain to be identified. For example, it is known that the peroxidase activities of PGHSs preferentially utilize alkyl hydroperoxides such as PGG_2 versus hydrogen peroxide; the basis for this specificity is not evident from simple observation of the structures. It will also be important to characterize further the membrane binding domain of PGHSs in the context of the interaction of these domains with specific membrane lipids and the role of this domain in governing substrate entry into the cyclooxygenase site and product exit from this site.

Our understanding of the different biological roles of PGHS-1 and PGHS-2 is only beginning to emerge. The functions of these two isozymes in apoptosis, particularly as it relates to the development of a variety of cancers, angiogenesis, respiration,

inflammation, pain and reproduction need further exploration. Of key importance is understanding the reason for the existence of the two PGHS isozymes and how coupling occurs between these enzymes, upstream lipases, and downstream synthases and receptors.

Considerable challenges remain in understanding the detailed biochemistry involved in the synthesis and release of biologically active leukotrienes. Little is known how these highly lipophilic molecules are released from cells; but even more curious is how the chemically reactive intermediate leukotriene A_4, made on the perinuclear membrane, can find its way into a neighboring cell in the process of transcellular biosynthesis. The mechanism of 5-lipoxygenase is still poorly understood; however, the detailed structure of 5-lipoxygenase (X-ray structure) may likely reveal important facets relevant to translocation events of 5-lipoxygenase as well as mechanism of suicide inactivation. Such information would be of great value in designing specific drugs as novel inhibitors of 5-lipoxygenase, a pharmacological approach highly successful for cyclooxygenase. Last, but not least, little is known concerning a potential intracellular role for leukotrienes. The currently known biological actions of leukotrienes all involve cell membrane G-protein-linked receptors, yet an understanding of why biosynthesis of these lipophilic molecules occurs deep within the cell remains a mystery.

Abbreviations

2-AG	2-arachidonyl-glycerol
BLT (1 or 2)	leukotriene B_4 receptor (subclass 1 or 2)
cysLT (1 or 2)	cysteinyl leukotriene receptor (subclass 1 or 2) for which leukotriene C_4, D_4, and E_4 are agonists
CYP4F(x)	cytochrome P-450 isozyme that carries out ω-oxidation of leukotriene B_4 (subclass x)
EET	epoxyeicosatetraenoic acid
EP	prostaglandin E receptor
FLAP	5-lipoxygenase activating protein
HETE	hydroxyeicosatetraenoic acid
HpETE	hydroperoxyeicosatetraenoic acid
LO	lipoxygenase
LT	leukotriene (followed by letter to designate structural type)
NSAID	nonsteroidal anti-inflammatory drug
PG	prostaglandin (followed by letter to designate structural type)
PGHS	prostaglandin endoperoxide H synthase
PLA_2	phospholipase A_2

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