The organization and consequences of eicosanoid signaling

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Organization of leukotriene and prostaglandin synthesis

As described in the introduction to this Perspective series (1), signaling by arachidonic acid represents a paradigm for the use of oxygen in the transmission of information. At the same time, arachidonic acid signaling can also contribute to the propagation of cellular damage. This duality is typified by a signaling cascade that (a) prevents the activation of 5-lipoxygenase (5-LO) in resting cells and (b) results in the formation and release of leukotrienes (LTs), which requires the sequential activation and interaction of at least eight different proteins. In fact, all lipoxygenases require membrane translocation to exert activity. In the case of the formation of COX products, particularly prostaglandin E_2 (PGE_2) and PGD_2, humans have evolved two sets of biosynthetic enzymes that differ not only in their cell- and tissue-specific localization, but also in their subcellular localization and requirement for reduced glutathione, a cellular defense against oxidative damage. This review will focus on three aspects of arachidonic acid biology. First, the compartmentalization and organization of eicosanoid synthesis, specifically LTs and PGs, will be discussed. This will illustrate the elaborate mechanisms that keep unwanted lipoxygenation at arm's length and also show that the enzymes such as glutathione-*S*-transferases, epoxide hydrolases, and carrier proteins that are commonly thought of as biosynthetic also belong to families that are generally considered to play a role in detoxification. Second, the potential cellular oxidative damage that is produced as a by-product of the use of oxygen and lipid substrates is examined. Finally, mechanisms that are used to amplify signaling diversity from a core of LTs and PGs are discussed.

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Nonstandard abbreviations used: 5-lipoxygenase (5-LO); leukotriene (LT); cytoplasmic phospholipase A_2 ($cPLA_2$); 5(S)-hydroxy,6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE); 5-lipoxygenase–activating protein (FLAP); multidrug-resistance protein 1 (MRP1); reactive oxygen species (ROS); membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG); glutathione-*S*-transferase 2 (GST2); microsomal prostaglandin E_2 synthase–1 (mPGES-1); epoxyeicosatrienoic acid (EET).

The role of leukotrienes C_4 and D_4 in disease

LTs are the products of the 5-LO pathway of arachidonic acid metabolism (Figure 1). The initial interest in LTs followed largely from their association with the pathogenesis of asthma (2). LTC_4, identified as the parent molecule of the sulfidopeptide LTs, is generated from eosinophils and mast cells in large amounts, and also from monocytes and macrophages (2–7). However, it is not formed by polymorphonuclear leukocytes (2–5). When released from cells, LTC_4 is converted to LTD_4 (2–7), and both exert their biological effects via G protein–coupled receptors (8, 9). LTD_4 and LTC_4 cause the constriction of smooth muscle, and the clinical correlate is bronchial smooth muscle constriction in asthma (2, 10, 11). The role of LTD_4 as a major contributor to asthmatic bronchospasm has been firmly established, and aerosolized LTD_4 and LTC_4 cause bronchospasm when taken by inhaler (10, 11). The metabolic product of LTD_4, 5(S)-hydroxy,6(R)-cysteinyl-7,9,11-*trans*,14-*cis*-eicosatetraenoic acid, (LTE_4), has been found at high levels in the serum and urine of patients with asthma and allergic rhinitis (12). When cold-induced bronchoconstriction, allergen-induced asthma, and exercise-induced asthma were analyzed, antagonists of the 5-LO pathway were able to alleviate bronchospasm (13–17). Pretreatment with the LTD_4 receptor antagonist MK-0476 has been shown to block the bronchoconstrictive response to all forms of challenge and alleviate ongoing brochoconstriction (16). The use of LTD_4 receptor and 5-LO antagonists has also blocked the severity and occurrence of asthmatic attacks in clinical trials, and these agents are particularly effective in chronic mild asthma, for which they are recommended for clinical use (18–23). LTs are also considered to be central to the pathogenesis of allergic rhinitis, in which cells expressing the cysteinyl LT1 receptor are highly expressed in $CD45^+$ nasal leukocytes, and recent studies have shown that cysteinyl LTs regulate the trafficking of T cells in vivo (24, 25). Subsequently, LTD_4 receptor antagonists have proved to constitute an effective therapeutic modality for this disorder. Knockout animals that lack LTC_4 synthase have markedly attenuated vascular responses that are dependent on IgE-mediated LTC_4 release (26).

As described above, LTD_4 and LTC_4 have been shown to function via both high- and low-affinity G protein–coupled receptors (8, 9, 27, 28), and these receptors are present on human eosinophils and monocytes,

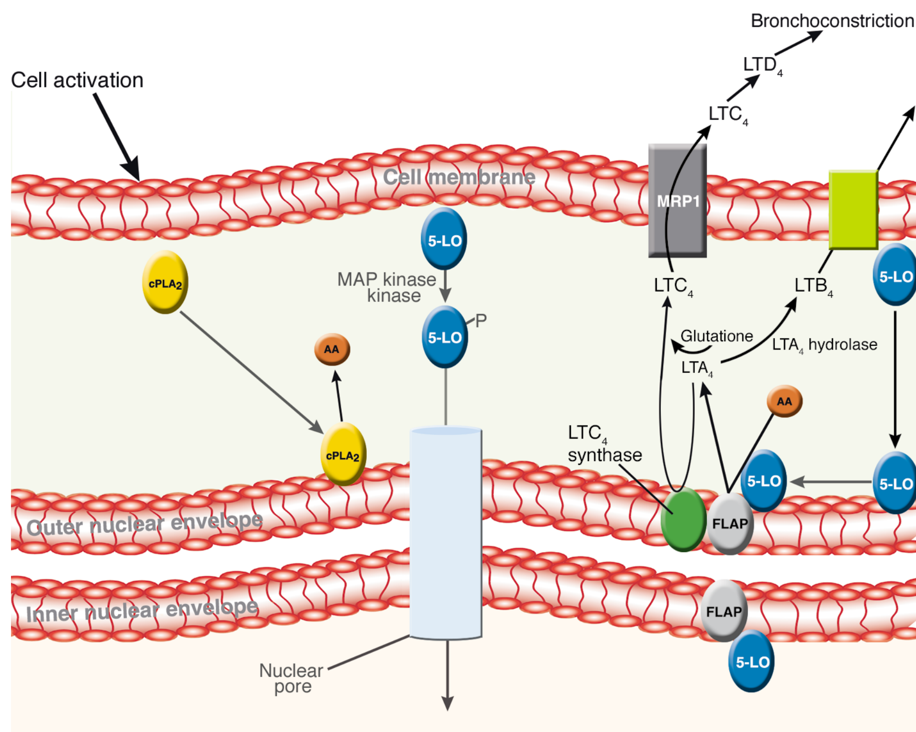

Figure 1
LT biosynthesis and assembly. Upon cellular activation of a mast cell or macrophage by IgE-antigen complexes or other stimuli, a cascade of cell activation events results in LT biosynthesis. A concomitant rise in free calcium induces translocation of $cPLA_2$ to intracellular membranes, where it releases arachidonic acid. In parallel, 5-LO is phosphorylated by MAP kinase kinase and traffics through the nuclear pore to the nucleus (possibly in association with NF-κB) or directly to the outer nuclear envelope. 5-LO then associates with the nuclear membrane, and possibly with FLAP. FLAP facilitates arachidonic acid presentation to 5-LO and subsequent conversion of arachidonic acid to LTA_4. LTA_4 interacts with LTA_4 hydrolase to form LTB_4, or with LTC_4 synthase to form LTC_4. The synthesis of LTs A_4, B_4, and C_4 probably takes place within the lumen of, or in close proximity to, the nuclear membranes. However, for clarity they are shown here throughout the cytosol. FLAP is present on both the inner and the outer nuclear envelope, but LTC_4 synthase is exclusively expressed on the outer nuclear membrane and ER.

where they function in chemotaxis and cell activation. Thus, insight into the cellular mechanisms that regulate the enzymatic interactions during LT biosynthesis represents a critical step in our understanding of the formation and activity of these molecules in both health and disease.

Cellular and molecular biology of LT biosynthesis

The formation of LTs is initiated when eosinophils, mast cells, polymorphonuclear leukocytes, or monocytes are activated to release arachidonic acid. This occurs after the translocation of the 86-kDa calcium-dependent cytoplasmic phospholipase A_2 ($cPLA_2$) to the nuclear envelope, ER, or Golgi apparatus (Figure 1) (29). 5-LO is also translocated to the nuclear envelope (30–32) and acts on arachidonic acid in sequential steps to generate 5(S)-hydroxy,6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE) and then 5(S),6(R)-oxo-7,9,11-*trans*-14-*cis*-eicosatetraenoic acid, (LTA_4) (33). Within cells, these enzymatic steps require the expression of the 17-kDa nuclear envelope protein 5-LO–activating protein (FLAP). FLAP is critical to cellular 5-LO activity and to its membrane interactions, but not to 5-LO translocation (34, 35). It has been postulated that FLAP presents arachidonic acid to 5-LO (36) but may also restrict the diffusion of arachidonic acid through cellular membranes. FLAP may also "dock" 5-LO to its membrane target after translocation. LTA_4 is then converted to LTB_4 by the action of the enzyme LTA_4 hydrolase (37) (Figure 1). Once formed, LTB_4 is exported from cells by an active process (38) and exerts its effects on other phagocytic cells via high- or low-affinity G protein–coupled receptors (39, 40). Alternatively, in eosinophils, monocytes, and mast cells, LTA_4 is conjugated with reduced glutathione to form LTC_4. This reaction is catalyzed by the enzyme LTC_4 synthase, a 17-kDa protein also located in the ER and outer nuclear membrane (41–44). LTC_4 is exported from cells by the multidrug-resistance protein 1 (MRP1) and then metabolized by LTD_4, which plays a subsequent role in the induction of bronchoconstriction and edema (45–47). MRP1 knockout animals are characterized by intracellular retention of LTC_4 and significant asthmatic responses to allergen challenge (48), and dendritic cells from these animals are deficient in trafficking under certain circumstances (49). As described above, the fact that the enzymes commonly

thought of as biosynthetic belong to families that are generally considered to play a role in detoxification raises the intriguing possibility that LT signaling may have evolved as an adaptive response to oxidative stress.

LT formation is regulated by compartmentalization
The generation of LTs and PGs is under a complex set of controls. As exemplified in mast cells, the synthesis of LTs and PGs is initiated by $cPLA_2$, which is specific for phospholipids that contain arachidonic acid in the SN2 position (50). The enzyme is calcium-dependent and is translocated to the nuclear membrane in rat basophilic leukemia cells stimulated with IgE or calcium ionophore A23187 (29). This translocation is required for cellular activity and is mediated by a phospholipid-binding domain (51). Phosphorylation by MAPK at S505 can augment activity three- to fourfold but is not required for membrane association (52–55). The central role of $cPLA_2$ in LT production has been confirmed by studies using peritoneal macrophages prepared from $cPLA_2$ knockout mice (56). These cells produce no LTs in response to cell activation with calcium ionophore A23187. The enzyme is also found to localize within the cytosol of resting cells in this system. However, in other systems, detailed studies have shown that $cPLA_2$ traffics to the Golgi apparatus (57). This implies that arachidonic acid can be rapidly moved to the ER and nuclear envelope, where it can subsequently interact with FLAP and 5-LO.

5-LO is phosphorylated concomitantly with translocation to the nucleus and nuclear envelope (58–60). Phosphorylation via MAPK-activated protein kinase-2 is critical in controlling targeting and activation of this enzyme (61). A second critical aspect of 5-LO control may be its interaction with the p65 subunit of NF-κB (62). Whether 5-LO activation can modify the activity of NF-κB remains to be determined, though this has been suggested to occur via 5-LO–mediated generation of reactive oxygen species (ROS) (63). 5-LO has a functional C2-like domain, which is important in mediating its membrane interactions, specifically with phosphatidyl choline (64). Recent studies have also shown that LTA_4 hydrolase is translocated to the nucleus upon stimulation of rat basophilic leukemia cells (65), but not in human peripheral blood leukocytes. This would place the site of LTB_4 synthesis within the nucleus of rat basophilic leukemia cells, a site where LTB_4 has been suggested to function as a ligand for PPARα (66).

FLAP, LTC_4 synthase, and LTC_4 generation: the compartmentalization of LTC_4 and LTB_4 biosynthesis
Both sides of the nuclear envelope are potential sites of LT formation, and the interactions between the enzymes mediating LT biosynthesis at the membrane interface ultimately determine the fate of LTA_4 and the regulation of LTC_4 synthesis (30–32, 34–36, 67–71). As described above, the two proteins that regulate the synthesis of LTC_4 in bone marrow–derived cells are FLAP and LTC_4 synthase. These proteins are members of the superfamily known as membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG). This family is composed of six members that are central to the synthesis of LTs and PGs. MAPEG proteins are 17-kDa integral membrane proteins with three transmembrane domains and high structural identity. Three MAPEG proteins, FLAP, LTC_4 synthase, and microsomal glutathione-*S*-transferase 2 (GST2) (72), control the synthesis of LTC_4 and LTB_4. A fourth member of the MAPEG family, inducible microsomal prostaglandin E_2 synthase (mPGES-1), regulates the formation of PGE_2 in inflammatory settings (73–76). Very little is known about the regulation of these MAPEG proteins, but it is becoming increasingly clear that their membrane interactions may play a critical role in their regulation.

Figure 2 illustrates the topological relationships of the membrane domains of LTC_4 synthase as recently determined by our group (44), in addition to the likely orientation of FLAP and the topology of both COX1 and COX2 (77). Within transmembrane domains 1 and 3, LTC_4 synthase and FLAP share 52% identity between amino acids 41 and 97 of FLAP and between amino acids 45 and 101 of LTC_4 synthase (32–36, 42). LTC_4 synthase and microsomal GST2 share an even higher identity within this region. FLAP, characterized by three hydrophobic regions, has been localized to the nuclear envelope in polymorphonuclear leukocytes and monocytes, and the lipid-binding moiety is contained within the first hydrophilic loop (36). Immunoelectron microscopy of FLAP, using antibody to the first hydrophilic loop, has shown that FLAP is preferentially localized to the inner nuclear membrane, with the lipid-binding site oriented to the lumen (32).

LTC_4 synthase has recently been found to localize to the ER and outer nuclear membrane (44). However, in contrast to FLAP, it is excluded from the inner nuclear membrane. Similar to FLAP, LTC_4 has its active site localized to the ER lumen.

These results, combined with the observation that LTA_4 hydrolase translocates to the nucleus, indicate that in certain settings, the synthesis of LTB_4 and LTC_4 is compartmentalized. The molecular mechanism by which LTC_4 synthase and FLAP are segregated between the inner and outer nuclear membrane is one basis for

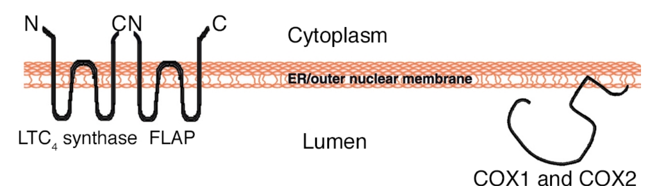

Figure 2
Membrane topology of proteins involved in eicosanoid synthesis. The active sites of LTC_4 synthase, COX1, COX2, and the lipid-binding domain of FLAP are all oriented to the lumen of the ER and the nuclear envelope.

the compartmentalized synthesis of LTB_4 and LTC_4, but the molecular basis for the compartmentalization of the latter two proteins remains unknown.

Recent studies of PGE_2 biosynthesis emphasize that an understanding of the membrane interactions of MAPEG proteins is central to an understanding of their biological role and suggest that regulated protein interactions may be important to their biological activity. PGES2 is coinduced and functionally coupled with COX2 in inflammatory settings, whereas cytosolic PGE_2 synthase 1 is coupled to COX1 (73–77). How COX2 and mPGES-1 interact is not known, but three possible mechanisms have been proposed (73–77). First, direct passage of PGH_2 between COX2 and mPGES-1 may occur, indicating that specific interactions between proteins of eicosanoid biosynthesis are critical to the efficient formation of PGE_2. Alternatively, the association of COX2 and mPGES-1 may simply be kinetically favored. Finally, differential membrane compartmentalization has also been postulated to play a role.

Because the formation of LTC_4 is controlled by two members of the MAPEG family (FLAP and LTC_4 synthase), a critical question in understanding the intracellular biology of LT formation should be raised: Do the biosynthetic enzymes of LT formation interact with each other? Two possible models can be proposed. In one, a multiprotein complex is formed that mediates the efficient transfer of LTA_4 to LTC_4 synthase. This would have the advantage of preventing oxidative damage secondary to the formation of LTA_4 adducts. In a second model, all the proteins are independent, and LTA_4 passes between them and is captured by individual molecules. Evidence supports the existence of at least some protein interactions. As described above, 5-LO is targeted to the nuclear envelope. This interaction is partly dependent on FLAP, and cells that lack FLAP or are treated with the FLAP inhibitor MK-886 do not synthesize LTs and have deficient targeting of 5-LO to nuclear membranes (34, 78). Direct interactions between 5-LO and FLAP have been postulated, but not demonstrated; however, this may be secondary to the technology employed. An ability of FLAP and LTC_4 synthase to form heterodimers would imply that interactions between MAPEG proteins are critical in controlling the formation of LTs. The situation is made more complex by the fact that both COXs and LTC_4 synthase are known to form homodimers (77).

An additional important protein interaction occurs between PKC and LTC_4 synthase. Pretreatment of cells with the activator of PKC, phorbol myristate acetate, inhibits subsequent LTC_4 formation by up to 70% (79–82), suggesting another potential interaction on the outer nuclear envelope. This interaction has not been directly demonstrated, and the specific isoform of PKC that interacts with LTC_4 synthase are unknown. Whether LTC_4 synthase is phosphorylated during cell activation has not been demonstrated. Two intriguing possibilities are that phosphorylation disrupts protein interactions and/or that it causes membrane redistribution of LTC_4 synthase.

Consequences of eicosanoid signaling

Why have cells evolved such complex mechanisms to control the initiation of signaling by 5-LO, other lipoxygenases, and COXs? Increasing evidence indicates that the initiation of lipid signaling comes at a potential cost to cells, particularly with respect to the generation of ROS and lipid hydroperoxides. In several model systems, the 5-LO enzyme contributes to the generation of ROS that activate NF-κB (62). Secondly, LTA_4 has the potential to form adducts with DNA bases, suggesting that it may potentially serve as a modulator of transcription or as a mutagen (83). In the case of terminally differentiated myeloid cells, the adduction of LTA_4 may function as a modulator of gene expression similar to methylation. More dire consequences may occur in conditions of chronic inflammation. Recent studies have shown that transcellular LTA_4 conversion of LTA_4 to LTB_4 occurs in in vivo models of inflammation (84). This finding suggests that LTA_4, generated by phagocytes, has the potential to form adducts in tissue DNA. In addition, the initial product of 5-LO, 5-HPETE, is a reactive hydroperoxide. The potential for the products of lipoxygenase reactions to generate cellular damage is typified by 15-LO, which has been implicated in the pathogenesis of atherosclerosis secondarily to its ability to utilize esterified phospholipids as a substrate (85, 86).

A second series of studies has suggested a mechanism by which the generation of PGs and electrophilic lipid hydroperoxides might couple inflammation and cancer but may also downregulate inflammation by interacting with NF-κB (87–95). Cyclopentanone PGs are generated late in the inflammatory process. PGs of the J and A series can inactivate wild-type p53 tumor-suppressor protein. This is based on the chemical reactivity of molecules that contain α,β-unsaturated ketones. The J series of PGs are more potent antiproliferative molecules than the A series and are more stable, and last longer. They are derived from PGD_2, and their addition to cells results in the inactivation of wild-type tumor suppressor p53. It is becoming clear that these molecules have the ability to modify multiple redox-sensitive transcription factors. The addition of PGJ_2 to cells triggers a series of events that are dependent on the generation of ROS and that can be prevented by the addition of the radical quencher *N*-acetyl-L-cysteine. These effects suggest that cyclopentanone PGs, 15-deoxy-$\Delta^{12,14}$-PGJ_2 in particular, are either a source of markedly increased ROS generation or modulators of ROS sensitivity. Recently, a mechanism has been proposed that integrates these observations. As a consequence of its unique chemical reactivity properties, PGA_1 and a PGA analog can react with and covalently modify selenium-containing enzymes and proteins, including thioredoxin reductase. This impairs the reduction of redox-sensitive proteins by thioredoxin, indicating that electrophilic PGs and lipids can function as amplifiers of oxidative stress. This would include the activation of apoptosis signal–related kinase-1, which would be released during the oxidation of thioredoxin. Δ12-PGJ_2 can also inactivate ubiquitin isopeptidase

activity of the proteasome pathway. A series PGs antagonize p53-dependent apoptosis but not cell-cycle arrest. These effects are consistent with inhibition of thioredoxin reductase. Moos et al. (87, 90) have suggested that inhibition of thioredoxin reductase–thioredoxin cycling would prevent the assembly of p53 into a transcriptionally competent form, blocking apoptosis. Inhibition of thioredoxin reductase–thioredoxin cycling would disrupt ribonucleotide reductase activity, resulting in cell-cycle arrest during G1, because ribonucleotide reductase is the rate-limiting enzyme in DNA synthesis.

The generation of lipid hydroperoxides within or near the cell nucleus must confer a benefit. One possibility is that the generation of PGs and LTs on the nuclear envelope is important for the signaling or regulation of transcription. For example, LTB_4 has been suggested as an endogenous ligand for PPARα (66), though the significance of this observation is unclear. An intriguing, though untested, possibility is that the generation of PGs and LTs on the nuclear envelope could provide a mechanism for altering nuclear redox tone as a result of the "controlled" lipoxygenase or COX reactions. This would regulate a series of redox-sensitive transcriptional events. The physical association of 5-LO with other proteins suggests a direct role in transcription. The role of redox signaling and oxygen-dependent transcription in regulating the inflammatory response has recently been supported by the observation that hypoxia-inducible factor 1α is required for an inflammatory response by myeloid cells. The potential for 5-LO to generate sufficient ROS to affect NF-κB (62) provides a direct and feasible link between 5-LO–generated redox changes and transcription.

The topology of the biosynthetic enzymes involved in PG and LT synthesis may also be an adaption to the generation of lipid hydroperoxides. The active sites of COX1 and COX2 (96), FLAP (39), and LTC_4 synthase (42) (as shown in Figure 2) are all oriented toward the ER lumen, a location with high glutathione levels. Whether mPGES-1 has the same topology as these other MAPEG enzymes is unknown, but it would not be surprising if they were similarly oriented.

Signaling by LTs and PGs

Although signaling that is mediated by a combination of oxygen and lipid interaction has potentially disastrous consequences for cells, it provides one great advantage: diversity. Whereas a large portion of the genome is devoted to coding for kinases and phosphatases, the multiple signaling pathways of arachidonic acid are governed by only three classes of enzymes that initially add oxygen to the substrate. These are (a) COXs, which initiate the synthesis of PGs; (b) lipoxygenases such as 5-LO, which initiate the synthesis of LTs, 12-, 15-, and 8-LO; and (c) cytochrome P450s, which catalyze the formation of epoxyeicosatrienoic acids (EETs) or the formation of 20-hydroxyeicosatetraenoic acid. These enzymes generate products of differing biological activity by inserting oxygen at different positions in arachidonic acid. The use of enzymes of different classes to initiate oxygenation has a distinct advantage in that, because of their different mechanisms, the initial oxygenations result in molecules of different properties, limiting the next series of oxygenations, isomerizations, or reductions that can be performed. Thus, cytochrome P450 epoxygenases can generate 5,6-EET, but the biology of this molecule is completely distinct from that of LTA_4, another 5,6-epoxide. Similarly, the products of COX, but not of lipoxygenases, can be converted to thromboxane and prostacyclin in subsequent reactions.

The second mechanism that generates functional diversity within any individual class of enzymes is regiospecificity for the substrate. The 5-, 12-, and 15-LOs each generate products with different potential biological function. In addition, 12- and 15-LO can utilize esterified fatty acids as substrates. This positional specificity and the ability to utilize esterified phospholipids as substrates provide a basis for the proposed role of 15-LO in the early phases of atherosclerosis with the generation of reactive lipid aldehydes and free radicals (85, 86).

It was initially presumed that the presence of two COX genes was a unique property of the COX enzyme class, but it now appears to be more the rule than the exception in eicosanoid biochemistry. As described above, there are two PGE_2 synthase enzymes. There are also two enzymes that catalyze the synthesis of PGD_2 (one in hematopoietic cells and one in the brain), two 15-LOs, two 12-LOs, and multiple cytochrome P450 epoxygenases. Each of these variants may function differently by being coupled to different stimuli in different tissues, or by being differentially compartmentalized within cells. The advantage of this is that different pools of eicosanoids can potentially be coupled to different functions (for examples see refs. 73–76, 96). In addition, the recent discovery of a G protein–coupled receptor for arachidonic acid indicates that even the lack of enzymatic oxygen can import biological information mediated by arachidonic acid (97).

As recently described by Narumiya and Fitzgerald (98), this theme is echoed by PG receptors. Eight types and subtypes of membrane prostanoid receptors are conserved in mammals from mice to humans: the PGD receptor, DP; four subtypes of the PGE receptor, EP1, EP2, EP3, and EP4; the PGF receptor, FP; the PGI receptor, IP; and the thromboxane A receptor, TP. They have different cell- and tissue-specific functions, determined by selective coupling to G proteins and by the expression of splicing isoforms. Their role as inhibitory versus constrictive is determined by coupling and splicing. The theme of multiple receptors has been expanded to include the LT receptors, whose different functions have been found to be mediated by two LTB_4 receptors, two cysteinyl LT receptors, and two PGD_2 receptors. Thus, the temporal, cell-specific, and intracellular distribution of biosynthetic enzymes

controls the generation of eicosanoids within inflammation, whereas the cellular distribution, temporal induction, and coupling of their G protein–coupled receptors can diversify responses and control whether the responses are proinflammatory. G_s, G_i, or G_q proteins can couple signaling by a single molecule to multiple responses in different tissues or cells, and in a given cell.

In summary, the combination of 20 carbons and four unsaturated double bonds has proved to be one of the most flexible molecular combinations yet described and has provided the stimulus for over 50 years of intense investigation. As more roles and interactions for arachidonic acid products are identified, it is clear that this molecule will remain an integral component of biomedical research for at least another 50 years.

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