Regulated formation of eicosanoids

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Between 1965 and the mid-1980s, investigators laid a durable foundation for understanding the regulated formation and metabolic disposition of eicosanoids. First, they sought, and found in arachidonic acid, the biosynthetic precursor for the prostaglandins. Second, they identified phospholipids as the cellular compartment that harbored arachidonic acid, and they identified phospholipases as the enzymes essential for its liberation and for the ensuing biosynthesis of prostaglandins (1). Third, they deduced the existence of transient intermediates in the prostanoid biosynthetic pathway, leading to the eventual discovery of the prostaglandin endoperoxides, PGG_2 and PGH_2 (2, 3). Fourth, they established that the enzyme PGH synthase, or cyclooxygenase (COX), was a molecular target of great medical significance in reproductive, cardiovascular, and inflammatory disorders (4). Fifth, they established that rapid, comprehensive pulmonary metabolism limited the steady-state levels and duration of action of prostanoids, implying that they acted as autocoid lipid mediators, not hormones. Finally, they identified new prostanoids (5, 6) and their biosynthetic enzymes, as well as new lipoxygenase enzymatic pathways and their leukotriene products (7, 8), as medically significant molecules.

Over this period, the most prominent themes in eicosanoid research were the identification of novel eicosanoid mediators, the determination of their molecular structures, and the establishment of their pharmacological activities. In a typical study of the time, investigators exposed tissues or cells to 50–100 μM of exogenous arachidonic acid, a concentration five- to tenfold greater than the K_m for COX or lipoxygenases ($K_m \approx 10$ μM) (see Brash, this Perspective series, ref. 9). The contemporary model for eicosanoid biosynthesis, circa 1985, asserted that liberation of free arachidonic acid by phospholipase was the rate-limiting step in biosynthesis. Accordingly, providing saturating amounts of exogenous arachidonic acid ought to reveal all biosynthetic products and pathways.

By 1985 or thereabouts, the quest for novel eicosanoid mediators of medical significance had reached the point of diminishing returns. Simultaneously, more and more investigators sought to understand the role of eicosanoid biosynthesis in disease processes. Accordingly, investigators shifted from exogenous arachidonic acid and Ca^{2+} ionophore as preferred tools and embraced natural ligands, relevant to disease, to initiate receptor-coupled activation of eicosanoid formation.

By the late 1980s, two lines of experimental inquiry began to provoke a reconsideration and refinement of the accepted model of eicosanoid biosynthesis. First, kinetic and quantitative aspects of eicosanoid biosynthesis initiated by growth factors or cytokines on mitotically competent cells suggested that it was an oversimplification to regard availability of arachidonic acid as the sole, rate-limiting step in cellular eicosanoid biosynthesis. Second, the discovery of the 5-lipoxygenase activating protein (FLAP) in 1990 proved unambiguously that some eicosanoids (leukotrienes) originated under conditions that were not rate-limited by arachidonic acid availability. The discovery of novel regulatory processes, particularly Ca^{2++}-dependent redistribution of 5-lipoxygenase (5-LO) and the interaction between 5-LO and FLAP (10), offered a new framework on which to reconstruct the earlier model of eicosanoid biosynthesis.

Here, we comment on several mechanisms through which cells attain more subtle control of eicosanoid biosynthesis than would be possible by simply limiting the availability of arachidonic acid. We first discuss the coordinated action of specific phospholipases, the enzymes that generate this substrate, with specific COXs and PGH isomerase enzymes. We then consider the restricted expression of eicosanoid biosynthetic enzymes and, finally, turn to the "suicide" inactivation of these biosynthetic enzymes, a general mechanism that may help terminate their proinflammatory function.

Functional coupling of phospholipases, COXs, and PGH isomerases

In 1989 Lin et al. (11) provided an accurate glimpse of two regulatory processes that have assumed great importance during the past decade. First, they showed that PDGF, a typical growth factor, caused a sustained increase in PGE_2 biosynthesis by NIH 3T3 cells, commencing within 10 minutes and reaching a maximum after 2 hours. Second, they showed that cycloheximide inhibited more than 90% of the PGE_2 synthesis initiated by PDGF, suggesting that de novo protein syn-

thesis, presumably de novo synthesis of the COX enzyme, contributed to the process. However, additional experiments in which NIH 3T3 cells were exposed to exogenous arachidonic acid, instead of PDGF, showed that these cells had ample basal capacity for COX-catalyzed formation of PGE_2. Furthermore, the steady-state level of COX mRNA in NIH 3T3 cells rose, but this rise followed, rather than preceded, the increase in PGE_2 synthesis. Finally, COX protein levels did not rise appreciably following PDGF stimulation, even when the corresponding mRNA levels were elevated.

These data prompted two questions. First, why would cells rely on de novo synthesis of COX enzyme if they already had sufficient COX enzymatic capacity to convert any available arachidonic acid into PGE_2? Second, if de novo synthesis of COX enzyme did account for increased PGE_2 formation in cells incubated with PGDF, why were the temporal and stoichiometric relationships between mRNA accumulation, protein accumulation, and PGE_2 formation so distorted? Herschman and colleagues' discovery (12) of the COX-2 isoenzyme (see Smith and Langenbach, this Perspective series, ref. 13) would eventually clarify the quantitative and temporal distortions observed by Lin et al. (11). However, absent this discovery, Lin et al. proposed that coupling of the PDGF receptor to phospholipase and ultimately to COX relied on de novo synthesis of an unidentified protein. In other words, the PDGF-mediated increase in cellular PGE_2 synthesis depends on de novo synthesis of proteins that coordinate the availability of arachidonic acid to its metabolism by COX during growth factor receptor occupancy. This hypothesis remains attractive and active today. For instance, Murakami et al. (14) have proposed the existence of a hypothetical accessory protein that integrates phospholipase–COX-2 interactions to explain how COX-2 expression enables $cPLA_2$ activation. Given the renewed interest in functional coupling among the enzymes of the arachidonic acid cascade (15), it will be interesting to see if as-yet unidentified coupling proteins promote interactions among component receptors and enzymes.

During the past year the concept of coupling among the phospholipase-COX-PGH isomerase enzymes has gained particular experimental support and clarity from the investigations by Kudo and colleagues (14, 16, 17). By expressing any of several forms of phospholipase A2 (the Ca^{2+}-dependent cytosolic phospholipase $cPLA_2$, the secretory phospholipase $sPLA_2$; or the Ca^{2+}-independent phospholipase $iPLA_2$) in combination with ectopically expressed COX-1 or COX-2, these authors have established a hierarchy of functional interactions among the enzymes.

First, under what might be termed basal conditions (Figure 1, red text and arrows), the Ca^{2+}-independent $iPLA_2$ is the dominant phospholipase involved in the liberation of arachidonic acid and related polyunsaturated fatty acids from membrane phospholipids. $iPLA_2$ serves primarily in cell membrane remodeling and does not induce eicosanoid biosynthesis because, under basal conditions, the rate of arachidonic release via $iPLA_2$ is less than or equal to the rate of its reincorporation into cell membranes; thus there is negligible accumulation

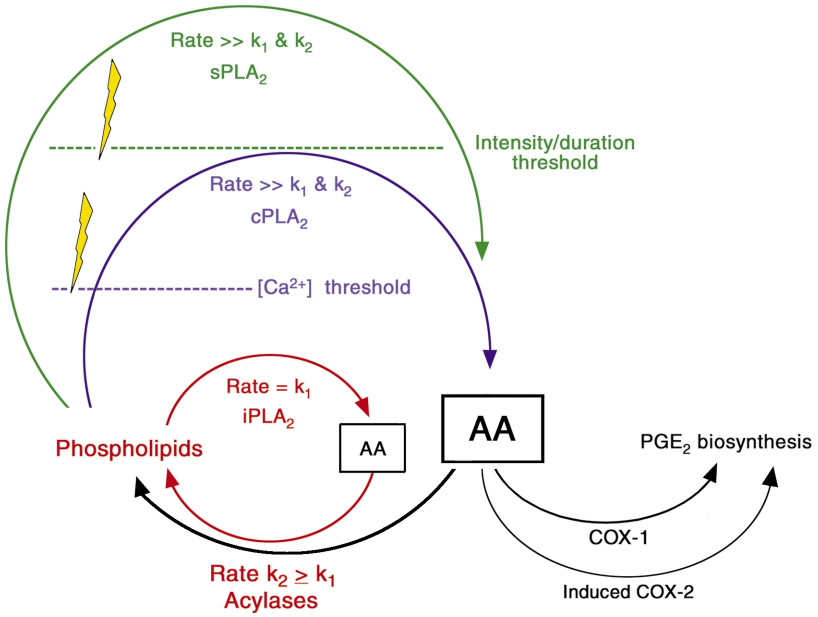

Figure 1
Stages of eicosanoid biosynthesis. Under basal conditions, release and metabolism of arachidonic acid (AA) are balanced, leaving little AA available as a substrate for COXs, so little prostaglandin E_2 is formed. With the breaching (lightning bolts) of two successive metabolic thresholds, two additional phospholipases are activated, permitting the accumulation of AA and the formation of its downstream metabolites. Prior to stimulation, the cycle shown in red predominates, wherein membrane phospholipids are hydrolyzed by the phospholipase A_2 isoform $iPLA_2$, yielding low quantities of AA that are recycled by the action of acylases to reform membrane phospholipid. However, following growth factor receptor activation, Ca^{2+} levels rise and the inducible phospholipase $cPLA_2$ (shown in blue) begins to generate sufficient AA that COX-1 and, later, COX-2 become active. COX-1 activity is coupled to a cytosolic form of the PGH-PGE isomerase, whereas COX-2 is coupled to a membrane-bound form of the enzyme. Following prolonged receptor occupancy and activation, yet another phospholipase, $sPLA_2$ (shown in green), is induced. This enzyme is secreted and can therefore activate prostaglandin synthesis in neighboring cells by a paracrine mechanism.

of free arachidonic acid substrate for conversion by either COX isoenzyme. Effectively, the acylase enzymes competitively inhibit the COX isoenzymes.

Second, following receptor activation, intracellular Ca^{2+} levels rise abruptly, exceeding a Ca^{2+} concentration threshold. At this point, the Ca^{2+}-dependent $cPLA_2$ becomes the dominant phospholipase involved in liberation of arachidonic acid from membrane phospholipids (Figure 1, blue text and arrows). During receptor activation, the rate of arachidonic acid release by $cPLA_2$ exceeds the rate of its reincorporation into cell membranes; thus there is sufficient accumulation of free arachidonic acid substrate for metabolism by either of the COX isoenzymes or both together. The model implies that inaugural formation of PGE_2 involves preferential coupling between $cPLA_2$ and COX-1 and a cytosolic PGH-PGE isomerase, all of which are expressed constitutively. As time passes and conditions favor the induction of COX-2 and a membrane-bound PGH-PGE isomerase, terminal formation of PGE_2 involves coupling between $cPLA_2$ and these latter two enzymes.

Finally, when exposure to receptor ligands is enduring and intense, the inducible, secreted $sPLA_2$ isoenzyme begins to participate, creating an amplification loop to align arachidonic acid availability with the sustained capacity for biosynthesis by inducible COX-2 and inducible PGH-PGE isomerase-2. This paracrine amplification loop helps release eicosanoid formation from its focal origins, allowing it to spread to surrounding cells (Figure 1, green text and arrows).

We draw attention to two further observations by Kudo and colleagues (14, 16, 17). First, COX-1 and COX-2 have rather similar K_m for arachidonic acid, yet it is COX-2 that metabolizes the bulk of the available arachidonic acid when the substrate is present at low concentrations (see Smith and Langenbach, this Perspective series, ref. 13). Studies of COX enzyme kinetics by Kulmacz and colleagues (18) point to cellular levels of hydrogen peroxide, which may contribute to COX activation, as a basis for this phenomenon, but it is an unusual trait that warrants investigation. Second, the occurrence of the PGH-PGE isomerase enzymes within discrete cellular compartments, cytosol and membrane, corresponds with but does not necessarily explain their preferential coupling to COX-1 and COX-2. A compartmentalization mechanism seems flawed, since both COX isoenzymes are located on membranes. In seeking an explanation, one is drawn, again, to hypothetical proteins (11, 12) that might couple the separate steps depicted in Figure 1.

Restricted expression of oxygenase and isomerase enzymes

Restricted enzyme expression provides a second mechanism for regulating eicosanoid biosynthesis. For example, restricted expression of thromboxane (Tx) synthase in platelets and restricted expression of PGI_2 synthase in endothelial cells account for the medical benefits of low-dose aspirin in thromboocclusive disorders (19). The distribution also accounts for the prothrombotic properties of platelets and the antithrombotic properties of the vascular endothelium (20, 21). Restricted expression of 5-LO is also critical for the so-called transcellular biosynthesis of leukotrienes, in which an eicosanoid precursor generated by granulocytes or other cells can be enzymatically activated by platelets or endothelial cells (22–24). Likewise, the inducible expression of COX-2 during inflammation provides the rationale for using COX-2–selective inhibitors in treating rheumatological disorders and helps explain the general observation that COX-1 participates in physiological processes whereas COX-2 acts in disease.

Emerging evidence suggests that restricted expression of COX-2 will be as important in oncology and developmental biology as it has been in understanding inflammation. For instance, nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit COX-1 and COX-2 with comparable efficacy have been associated with cleft palate during embryogenesis in experimental animals and in humans (25). FDA-approved COX-2–selective inhibitors presumably have no greater liability than do nonselective inhibitors. As

Table 1
Disease-restricted expression of COX-2 in oncology

Organ system	Observation	Incidence
Colon carcinoma (27)	COX-2 mRNA at site of pathology greater than at noninvolved site	5/5 subjects
Colon adenocarcinoma (28)	COX-2 protein at site of pathology greater than at noninvolved site	5/7 subjects
Breast tumor (28)	COX-2 protein at site of pathology greater than at noninvolved site	11/20 subjects
Lung tumor (28)	COX-2 protein at site of pathology greater than at noninvolved site	18/20 subjects
Gastric tumor (29)	COX-2 protein at site of pathology greater than at noninvolved site	73/104 subjects
Head and neck squamous cell carcinoma (HNSCC) (30)	Mean COX-2 mRNA in HNSCC 150-fold greater than in normal volunteers	
Pancreatic cancer (31)	Mean COX-2 mRNA in pancreatic cancer 60-fold greater than in adjacent noninvolved tissue. COX-2 protein at site of pathology greater than at noninvolved site	9/10 subjects
Colon carcinoma (32)	COX-2 levels greater than in tumors with larger sizes and deeper invasion	

their use grows it will be interesting to see whether they have reduced liability. In a different developmental context, recent experiments show an obligate role for COX isoenzymes in T cell maturation in the fetal thymus. Rocca et al. (26) propose a minimal model suggesting that PGE_2 formation by COX-1 converts $CD4^-/CD8^-$ thymocytes into $CD4^+/CD8^+$ and that PGE_2 formation by COX-2 subsequently converts $CD4^+/CD8^+$ into the $CD4^+$ lineage. This investigation provides a glimpse of the type of experimental design, involving both genetic and pharmacological experiments, that seems necessary to investigate the role of eicosanoid enzymes in developmental biology.

Disease-restricted expression of COX-2 is not limited to inflammation. Several types of neoplastic and preneoplastic tissue overexpress COX-2, compared with adjacent noninvolved tissue, with incidences ranging from about 50–90% in studies involving a minimum of ten subjects (27–32) (Table 1). Is this induction a cause or an effect of tumor progression? Investigations with the $Min^{+/-}$ mouse model of intestinal polyposis suggest that COX-2 expression favors progression from a preneoplastic to a neoplastic condition. Chulada et al. (33) report that homologous disruption of the *COX-2* gene diminishes polyp formation in $Min^{+/-}$ mice by about 80%. Investigations with these mice also suggest that the COX-1 isoenzyme participates in tumorigenesis. Episodically, investigators have reported that stromal tissue adjacent to tumor harbors elevated levels of the COX-1 isoenzyme, not COX-2 (34). Such reports reinforce the notion that disease-restricted expression of COX-2 is associated directly with the disease at the cellular level, but they also indicate that altered eicosanoid biosynthesis occurring as an adaptation to the diseased state of neighboring cells can be mediated by COX-1.

The high expression of COX-2 in neoplastic tissue derives, in part, from the accumulation of somatic mutations that persistently activate the ras pathway or various receptor tyrosine kinase pathways. Most tumors should exhibit this trait, but to explain the minority of cases without COX-2 expression, Toyota et al. (35) examined CpG island methylation in *COX-2* and found that methylation of CpG islands near exon 1 silenced COX-2 expression in 12 of 92 (13%) sporadic colorectal cancers and 7 of 50 (14%) colorectal adenomas. This observation has important implications. First, adenomas with a methylation-silenced *COX-2* gene may have a diminished response to treatment with COX-2–specific inhibitors or a better prognosis, depending on what other genetic defects are harbored by the tumor. Second, and more speculatively, a similar mechanism may apply to other oxygenase genes that are induced downstream of tumorigenic signaling pathways. Consistent with a number of other reports, Gao et al. (36) have shown that 12-lipoxygenase is expressed in a disease-restricted fashion in prostate cancer. Among 122 subjects examined, 12-lipoxygenase expression levels were elevated in about 50%, and 12-lipoxygenase elevation correlated with stage. It would be interesting to determine whether CpG methylation silences transcription of 12-lipoxygenase or other lipoxygenase enzymes in tumors that fail to show this inductive response.

"Suicide inactivation" of eicosanoid biosynthetic enzymes

The regulated formation of eicosanoids, as depicted in Figure 1, implies that cells have appreciable ability to amplify the rate and amount of eicosanoid biosynthesis. Once these processes reach maximal velocity, how can the cell return to its basal state? No doubt, part of the answer involves silencing the expression of $sPLA_2$, COX-2, and the other biosynthetic enzymes, but a phenomenon termed "suicide inactivation," or mechanism-based autoinactivation, could also impose a vital, but overlooked, limit on eicosanoid biosynthesis. This process occurs for COX (37), Tx synthase (38), PGI synthase (39), leukotriene A_4 hydrolase (40), and the 5-, 12-, and 15-lipoxygenases (41, 42).

Suicide inactivation is often regarded as a special tactic for developing irreversible pharmacological inhibitors. It is seldom regarded as a regulatory process. However, many substrate-product pairs in eicosanoid biosynthesis contain at least one electrophile, which might be expected to interact with and irreversibly modify the enzyme involved. In this context, it seems less surprising that these enzymes are subject to two alternative fates. One fate is a normal catalytic cycle, yielding product and regenerating enzyme. A second fate is a catalytic cycle leading to irreversible inactivation of the enzyme instead of product formation.

Little is known about the intracellular degradation of COX-2 protein, or other suicide-inactivated proteins. For instance, how is autoinactivated COX recognized and targeted for degradation? Is this different from degradation of active COX? Does cellular accumulation of suicide-inactivated enzyme account, in part, for any poor correlation between protein expression and product formation (e.g., COX-2 protein versus PGE_2)? If protein degradation processes fail to degrade suicide-inactivated enzymes, are there any cellular consequences? The implications of suicide inactivation have not yet been fully appreciated, or fully integrated, into models of eicosanoid biosynthesis. Future efforts to correlate the amount of cellular enzymes with the amount of product will need to take into account the catalytic history of the enzyme.

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