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Signalling networks in focus

Genetic insights into the *in vivo* functions of prostaglandin signaling

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ABSTRACT

Prostaglandins (PGs) are lipid signals that are produced at their sites of action by cyclooxygenase (COX) enzymes, the targets of non-steroidal anti-inflammatory drugs (NSAIDs), and PG-type specific synthases. Active PGs serve as ligands for G protein-coupled receptors (GPCRs). The functions of PGs have largely been elucidated using pharmacologic, expression-based (synthesis and signaling components), and genetic studies. In this review, we discuss the *in vivo* roles of PGs in cancer, development, and reproduction that have been characterized using genetic knockout/knockdown and overexpression approaches in mice, zebrafish, and invertebrate model systems, and how pharmacologic inhibition of PG synthesis affects cardiovascular health/disease and cancer incidence and progression.

Signalling networks facts

- PGs are autocrine and paracrine lipid signals produced by COX enzymes and PG-type specific synthases.
- There are four major PGs (PGE_2, PGD_2, PGI_2, $PGF_{2\alpha}$) and thromboxane (TXA_2).
- PGs bind to PG-type specific GPCRs to activate Gα-signaling cascades.
- Genetic studies on PG synthesis and signaling pathways have revealed the roles of PGs in many physiological processes, including embryonic development, hematopoiesis, reproduction, and cancer.
- Misregulation of PG signaling is associated with numerous diseases, including cardiovascular diseases and cancer.

1. Introduction

Prostaglandins (PGs) are autocrine and paracrine lipid signals that mediate many processes including fever, sleep, pain, inflammation, allergy, immunity, bone metabolism, adipogenesis, cancer, and cardiovascular functions (Kobayashi and Narumiya, 2002). Much of our understanding about PGs arose from pharmacologic studies using non-steroidal anti-inflammatory drugs (NSAIDs) to differentially inhibit the two cyclooxygenase enzymes (COX1 and COX2) responsible for PG production, and more recently, PG G-protein coupled receptor (GPCR) agonists and antagonists. Because the specificity of such reagents can be unclear, it is necessary to complement pharmacological experiments with genetic analyses. Indeed, genetic knockout and overexpression studies have further elucidated the roles of specific PGs (PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2) and TXA_2, identified new functions, and revealed evolutionarily conserved actions. Here we will (i) summarize the *in vivo* functions of PGs as revealed by genetic studies, (ii) overview the PG synthesis and signaling cascades, and (iii) discuss therapeutic approaches to target PG pathways.

2. *In vivo* functions of PG signaling

2.1. Insights into cancer from mouse models

PGs are widely implicated in cancer, including those of the colon, lung, prostate, cervical, ovarian, breast, and pancreas. Initial pharmacologic studies suggested COX2, the inducible enzyme, was responsible for generating PGs mediating cancer development and progression. Somewhat surprisingly, genetic studies in mouse models of cancer, particular gastrointestinal (Oshima and Oshima, 2012) and skin (Rundhaug et al., 2011), have implicated both COX1 and COX2.

Knockout of either COX in the *Min* model or COX2 in the *Apc*Δ*716* model of sporadic intestinal tumorigenesis reduces polyp formation. This is due to the loss of PGE_2 signaling *via* EP2 but not EP1 or EP3 receptors (Oshima and Oshima, 2012). In the AOM model, mPGES-1 knockout reduces, while overexpression increases tumor number and size. Furthermore, knockout of

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15-PGDH doubles PGE_2 levels and increases tumor formation in the *Min* model (Myung et al., 2006). Conversely, in the colitis-associated model of intestinal tumorigenesis (AOM/dextran sodium sulfate), knockout of either COX1 or COX2 did not effect tumor development (Ishikawa and Herschman, 2010), and COX2 plays a protective role against colitis (Ishikawa et al., 2011). Thus, PGs mediate both gastrointestinal homeostasis, and cancer. This finding complicates the use of NSAIDs as a preventative therapy against colon cancer.

PGs also play critical roles in non-melanoma skin cancers (NMSCs) (Rundhaug et al., 2011). In both two-stage chemical and UV-induced models, knockout of COX2 protects against, while overexpression increases tumorigenesis. The role of COX1 in skin cancer is less clear, as one study implicates it while others do not (Rundhaug et al., 2011).

Multiple PGE_2 pathways contribute to NMSC. In a chemical model, EP1 overexpression results in Ca^{2+} mobilization, activation of PLC/PKC, and elevated COX2 expression, resulting in increased tumor multiplicity and incidence, and the percentage of squamous cell carcinomas (Surh et al., 2011). A similar phenotype is observed with EP4 and EP2 overexpression, while EP2 knockout has the opposite phenotype (Rundhaug et al., 2011). Conversely, in the UV model EP2 knockout results in reduced incidence; however, tumors that form are larger, less differentiated, and more aggressive due to increased metalloprotease and decreased E-cadherin expression (Brouxhon et al., 2007). Therefore, like gastrointestinal cancers, the roles of PGE_2 in skin cancer are context dependent. In order to appropriately therapeutically target PGE_2, further studies are needed to fully delineate the signaling cascades through which PGE_2 mediates specific cellular outcomes.

While genetic studies clearly implicate PGE_2-dependent signaling in cancer, expression and/or lipid analyses of human patient samples reveal other PGs are likely involved. Thus, it will be important to explore the roles of each PG and their signaling cascades (see Table 1) in mouse models of cancer using both genetic and biochemical approaches.

2.2. Zebrafish studies reveal developmental roles of PGs

The roles of PGs in mammalian embryonic development are unclear; this may be due to the passage of PGs from mother to embryo obscuring development roles. This is not true in zebrafish where embryogenesis occurs outside the mother. Strong knockdown of COX1 results in a developmental delay and at high doses causes gastrulation defects and lethality; knockdown of COX2 does not cause similar effects (Grosser et al., 2002). A mild reduction of COX1 results in nephric duct and vascular defects (Cha et al., 2005). Similarly, mouse COX2 is required for kidney development and function (Morham et al., 1995), and cardiovascular remodeling at birth (Loftin et al., 2001).

PGs also regulate zebrafish hematopoiesis. COX1 mediates the formation of the hematopoietic niche, while COX2 regulates stem cell self-renewal and proliferation (North et al., 2007). Knockdown of two PGE_2 receptors (*ptger21* and *ptger41*) results in similar defects. The role of PGs in hematopoiesis is likely conserved as COX2 knockout mice exhibit reduced hematocrit levels and delayed recovery after bone marrow injury (Lorenz et al., 1999). Furthermore, both inhibitor (North et al., 2007) and EP4 knockout studies (Hoggatt et al., 2013) implicate PGE_2 in regulating mouse hematopoietic stem cells.

Expression and pharmacologic studies suggest PGs also regulate organogenesis, follicle development, ovulation, and immunity in zebrafish. Additionally, zebrafish has recently become a model for studying the role of inflammatory cells in cancer initiation. PGE_2 produced downstream of COX2 in innate immune cells signals to transformed cells to mediate overgrowth *via* EP1 (Feng et al., 2012). Thus, genetic studies in zebrafish hold the promise of elucidating the developmental, but also pathological roles of PGs.

2.3. Invertebrate insights into the functions of PGs

PGs regulate many processes in invertebrates and have been extensively studied in insects, where they regulate egg development, ovulation, ion transport, and immunity (Stanley and Kim, 2011). However, the study of PGs in genetically tractable invertebrate models has been limited due to the lack of clearly conserved PG synthesis machinery. *Caenorhabditis elegans* does not have a COX homolog and treatment with NSAIDs have had no discernable effects. However, lipid biochemistry in combination with studies using exogenous $PGF_{2\alpha}$ strongly indicates $PGF_{2\alpha}$-like lipids regulate sperm migration and fertilization (Hoang et al., 2013). PGs also regulate female fertility in *Drosophila*, as both treatment with COX inhibitors or genetic loss of the COX-like enzyme Pxt results in multiple defects during follicle development, including aberrant actin cytoskeletal remodeling. These defects are rescued by exogenous $PGF_{2\alpha}$ (Tootle and Spradling, 2008). Subsequent studies have identified the actin bundling protein Fascin as a new effector of PGs (Groen et al., 2012). It will be interesting to determine if the role of PGs in regulating Fascin is conserved in other systems, particularly since both play critical roles in cancer. Thus, genetic invertebrate model systems can provide novel insights into PG signaling.

Notably, PGs regulate female reproduction in every organism examined thus far. Indeed, mice deficient for COX1 fail to undergo parturition due to loss of $PGF_{2\alpha}$ (Gross et al., 1998), while mice deficient for COX2 are female sterile due to defects in ovulation, fertilization, implantation, and decidualization due to loss of PGE_2 (Lim et al., 1997). Furthermore, in women, NSAIDs cause reversible infertility, perhaps due to defects during oocyte maturation and/or ovulation (Pall et al., 2001).

Table 1
PG synthesis and signaling components.

PG	Synthase	GPCR	Common signaling		Alternative signaling
PGD_2	H-PGDS	DP	Gs	↑cAMP ↑$[Ca^{2+}]$	
	L-PGDS	CRTH2	Gi	↓cAMP	↑$[Ca^{2+}]$, PLC, PI3K, MAPK
PGE_2	mPGES-1	EP1	?	↑$[Ca^{2+}]$	
	mPGES-2	EP2	Gs	↑cAMP	EGFR, β-catenin
	cPGES	EP3	Gi	↓cAMP	Gq: ↑IP3/DAG Gs: ↑cAMP
		EP4	Gs	↑cAMP	PI3K, ERK1/2, β-catenin
$PGF_{2\alpha}$	Human: AKR1B1 Murine: AKR1B3, AKR1B7	FP	Gq	↑IP3/DAG	Rho, EGFR, β-catenin
PGI_2	PGIS	IP	Gs	↑cAMP	Gq: ↑IP3/DAG Gi: ↓cAMP
TXA_2	TXAS	TP	Gq	↑IP3/DAG/$[Ca^{2+}]$	Gs, Gi, Gh, G12, ↑cAMP, ↓cAMP

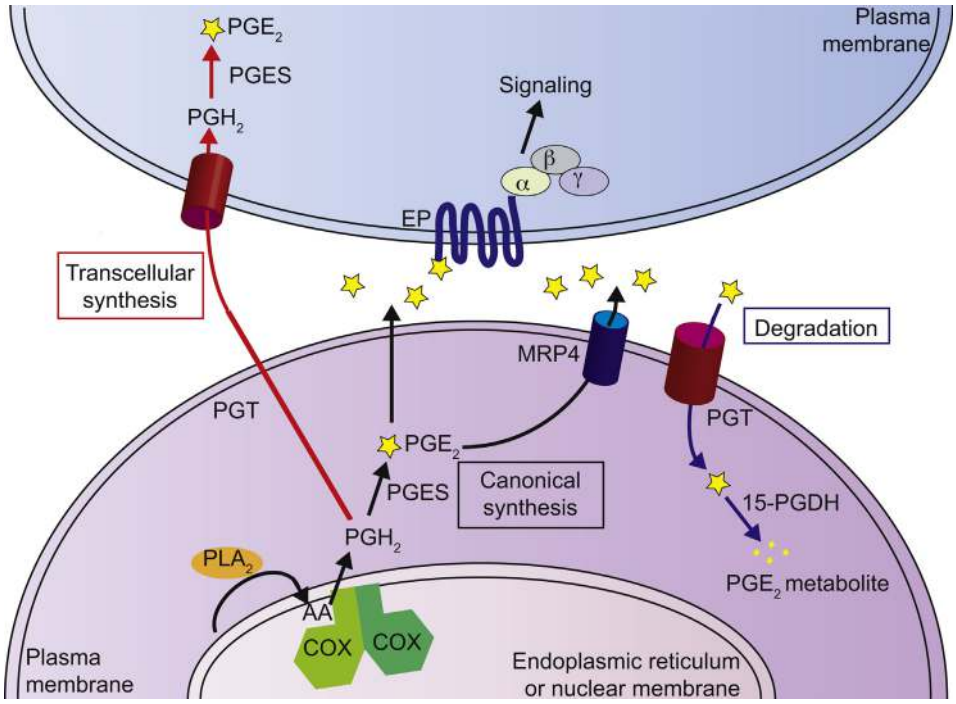

Fig. 1. PGs are synthesized at their sites of action. Phospholipases (PLA_2) cleave membrane phospholipids to release arachidonic acid (AA). AA is converted into PGH_2 by the COX dimer, which is found on the inside of the endoplasmic reticulum or nuclear membranes. PGH_2 can then be used for canonical (black lines) or transcellular synthesis (red lines). In transcellular synthesis, PGH_2 leaves the cell and is taken up *via* PGT into an adjacent cell where synthases convert it into an active PG, such as PGE_2. In canonical synthesis (black lines), PGH_2 is processed within the original cell into an active PG, illustrated as PGE_2. PGE_2 can diffuse or be transported by MRP4 out of the cell, where it activates its GPCR on either the synthesizing cell (not shown) or an adjacent cell to initiate a signaling cascade. Excess PGE_2 is taken back up into the synthesizing cell *via* PGT and is broken down by 15-PGDH (blue lines).

3. PG synthesis and signaling cascades and key molecules

PGs are produced at their sites of action by COX enzymes (a.k.a. PTGS or PGHS). Vertebrates have two COX enzymes – COX1 and COX2 – that differ in the size of their substrate-binding pocket, which allows them to be differentially targeted by pharmacologic inhibitors. NSAIDs including aspirin and ibuprofen inhibit both enzymes at similar concentrations; while COX2 inhibitors (COX-IBs) including celecoxib (Celebrex) and rofecoxib (Vioxx) inhibit COX2 at significantly lower concentrations than for COX1. Generally, COX1 mediates the homeostatic functions of PGs, while COX2 is inducible; however, there are exceptions to this rule (Zidar et al., 2009). Thus, it is important to genetically determine the roles of both COX enzymes in any given process.

COX enzymes are endoplasmic reticulum or nuclear membrane bound, and function as homodimers with one catalytic and one regulatory subunit (Yuan et al., 2009). Phospholipases release arachidonic acid (AA) from membrane lipids into the catalytic site (Fig. 1). Through heme-dependent peroxidase and cyclooxygenase activities, COX enzymes produce the PG intermediate PGH_2. Specific synthases (Table 1) convert PGH_2 into active PGs (PGE_2, $PGF_{2\alpha}$, PGD_2 and PGI_2) or TXA_2. Additionally, PGD_2 can be non-enzymatically metabolized into 15d-PGJ_2; however, the *in vivo* relevance of this PG is unclear. While both COX and PG synthases generally act within a cell to produce PGs, PGs can also be generated through transcellular synthesis (Fig. 1, red lines), with COX and synthase activities residing in separate cells (Sala et al., 2010).

Active PGs diffuse or are transported, by multidrug resistance proteins, out of the cell to signal *via* G protein-coupled receptors (GPCRs; Table 1 and Fig. 1, black lines) (Hirata et al., 1991). While each GPCR is referred to as a receptor for a specific PG (i.e. EP1-4 are PGE_2 receptors), other PGs (albeit at higher concentrations) can also activate these receptors (Kiriyama et al., 1997). This finding complicates the interpretation of receptor knockout and inhibitor studies. Each GPCR initiates one or more Gα-dependent signaling cascades (Table 1). PGs can also activate MAPK signaling by either transactivation of receptor tyrosine kinases through their GPCRs or independently of their receptors, and may serve as ligands for PPAR nuclear hormone receptors. Excess extracellular PGs can be taken back up into the synthesizing cell *via* PGTs and inactivated by 15-hydroxyprostaglandin dehydrogenase (15-PGDH; Fig. 1, blue lines).

While the PG synthesis and signaling pathways are well established, it is difficult to determine the complement and levels of PGs a particular cell makes. This is because PGs signal at picomolar to nanomolar ranges and have extremely short half-lives in aqueous solutions (30 seconds to a few minutes). Additionally, there are limited methods for detecting PGs. One such method is enzyme immunoassays (EIAs), which can be used to quantify an individual active PG. Another method is liquid chromatography coupled to mass spectrometry, which simultaneously quantifies multiple PGs and related eicosanoids. Fully elucidating the actions of all of the PGs acting in a given context will require biochemical analysis of the PGs present to complement expression, pharmacologic, and genetic studies.

4. Associated pathologies and therapeutic implications

Aberrant PG signaling is implicated in pathologies such as asthma, arthritis, infertility, and neurodegeneration (Miller, 2006). Here we will discuss the roles of PGs in cardiovascular diseases and cancers.

PGs regulate vasoconstriction/dilation, platelet aggregation, and atherosclerosis. Thus, misregulation of PGs results in both heart attack and stroke (Miller, 2006). Low dose aspirin treatment reduces the risk of such diseases. However, high doses of

NSAIDs and COXIBs are associated with cardiovascular complications, including hypertension, myocardial infarction and stroke; these effects may be due to altered PGI_2 levels.

Upregulation of COX2 and increased PG levels are associated with cancer development and metastasis (Ulrich et al., 2006). Low dose aspirin treatment reduces the incidence of colorectal cancer, and perhaps other cancers including esophageal, gastric, prostate, ovarian, breast, and lung. Furthermore, NSAIDs and COXIBs are utilized as chemotherapeutic adjuvants. Unfortunately, their utility is limited by gastrointestinal toxicity and cardiovascular complications. Additionally, the effectiveness of these drugs depends upon genetic background. COX2 polymorphisms (V511A and −765G>C) reduce the risk of colorectal and non-small cell lung cancer, but abrogate the beneficial effects of NSAIDs. Intriguingly, a COX1 polymorphism (P17L) is associated with a three-fold increase in the risk of colorectal adenomas (Ulrich et al., 2006). Thus, the outcomes of NSAID and COXIB treatment are likely to vary across individuals.

In summary, PGs are conserved lipid signaling molecules that mediate a wide array of biological functions. While their synthesis and signaling pathways are well established and can be grossly inhibited by NSAIDs and COXIBs, the events further downstream remain largely unknown. Thus, there is a critical need to define the molecular outcomes of specific PG signaling events. These newly identified mechanisms of PG action will provide novel targets for the development of therapies to more specifically target PG-dependent events.

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