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Cyclooxygenase-1 signaling is required for vascular tube formation during development

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Abstract

Prostaglandin endoperoxide synthases (PTGS), commonly referred to as cyclooxygenases (COX-1 and COX-2), catalyze the key step in the synthesis of biologically active prostaglandins (PGs), the conversion of arachidonic acid (AA) into prostaglandin H2 (PGH2). Although COX and prostaglandins have been implicated in a wide variety of physiologic processes, an evaluation of the role of prostaglandins in early mammalian development has been difficult due to the maternal contribution of prostaglandins from the uterus: COX null mouse embryos develop normally during embryogenesis. Here, we verify that inhibition of COX-1 results in zebrafish gastrulation arrest and shows that COX-1 expression becomes restricted to the posterior mesoderm during somitogenesis and to posterior mesoderm organs at pharyngula stage. Inhibition of COX-1 signaling after gastrulation results in defective vascular tube formation and shortened intersomitic vessels in the posterior body region. These defects are rescued completely by PGE_2 treatment or, to a lesser extent, by $PGF_{2\alpha}$, but not by other prostaglandins, such as PGI_2, TxB_2, or PGD_2. Functional knockdown of COX-1 using antisense morpholino oligonucleotide translation interference also results in posterior vessel defect in addition to enlarged posterior nephric duct, phenocopying the defects caused by inhibition of COX-1 activity. Together, we provide the first evidence that COX-1 signaling is required for development of posterior mesoderm organs, specifically in the vascular tube formation and posterior nephric duct development.

Keywords: Cyclooxygenase; Prostaglandin; Prostaglandin E_2 (PGE_2); Zebrafish; Tubulogenesis; Vascular tube; Angiogenesis; Kidney; Nephric duct; Posterior mesoderm

Introduction

Cyclooxygenases (COXs), prostaglandin (PG) endoperoxide G/H synthases (PTGS), catalyze the initial step in the conversion of arachidonic acid to prostaglandins (PGs) (Supplementary Fig. 1). Although COX-1 and COX-2 are encoded on separate chromosomes, they share similar structural and kinetic properties and show distinct expression and regulation. COX-1 is thought to serve "housekeeping" functions as a constitutive enzyme, while COX-2 is highly inducible by diverse stimuli including cytokine, mitogen, growth factor, and tumor promoters. PGs are important in a wide variety of normal physiologic and pathologic processes (Smith et al., 2000).

Prostaglandin biosynthetic pathway is initiated by conversion of arachidonic acid into PGH_2 by the enzymatic action of cyclooxygenases. COX enzymes are inserted in the ER and nuclear membrane with the substrate-binding pocket precisely orientated to take up released arachidonic acid (Smith et al., 2000). The crystal structures of COX-1 and COX-2 are remarkably similar, with one notable amino acid

Abbreviations: PG, prostaglandin; COX, prostaglandin endoperoxide synthase; Indo, indomethacin; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; WT, wild-type.

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difference that leads to larger "side-pocket" for substrate access in COX-2. PGH_2 is subsequently converted to one of several structurally related prostaglandins, including PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2 (TxA_2), by the activity of specific PG synthases. The coupling of PGH_2 synthesis to metabolism by downstream enzymes is intricately orchestrated in a cell-specific fashion. Thromboxane synthase is found in platelets and macrophages, prostacyclin synthase is found in endothelial cells, and PGF synthase in uterus, and two types of PGD synthase are found in brain and mast cells. Two PGE synthases, microsomal PGE synthase (mPGES), a member of the MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism) family, and cytosolic PGE synthase (cPGES), are responsible for PGE_2 synthesis (Jakobsson et al., 1999).

Understanding the developmental role of cyclooxygenases and the downstream signaling molecules did not reveal much insight into embryonic role for prostaglandins. COX-1 homozygous mutant mice did not reveal much pathology, which was surprising given its "housekeeping" function. COX-$2^{-/-}$ mice exhibit multiple defects in reproduction that include ovulation, fertilization, implantation, and decidualization, but did not exhibit overt abnormalities during embryonic development (Langenbach et al., 1995; Loftin et al., 2002; Morham et al., 1995). Subsequently, EP receptors and various prostaglandin synthases were knocked out in mice, but none of the mutants revealed phenotype during embryonic development (Kobayashi and Narumiya, 2002). From these studies, it was hypothesized that either fetal or maternal production of prostaglandins allows normal development of embryos in the uterus.

Recently, both COX isoforms were isolated in zebrafish and shown to be genetically and functionally equivalent to their mammalian orthologs (Grosser et al., 2002). In this study, knockdown of COX-1, using antisense oligonucleotides morpholino, resulted in gastrulation arrest, while knockdown of COX-2 failed to produce any phenotype. Zebrafish is an attractive model for many reasons (Amatruda et al., 2002). Each mating gives rise to hundreds of embryos for study; embryogenesis can be visualized due to the transparency of the developing embryos; molecular markers are available for virtually all the tissues; and most importantly for prostaglandin biology, embryogenesis occurs outside of mother's body. Therefore, we sought to explore the specific role of COX-1 during zebrafish embryogenesis after the gastrulation period.

Materials and methods

Zebrafish maintenance, embryo generation, and staging

AB* and TL WT zebrafish strains were maintained as described (Solnica-Krezel et al., 1996). Embryos were obtained from natural spawnings and staged according to morphology as described (Kimmel et al., 1995).

In situ hybridization and antibody staining

Antisense probes for zebrafish *cox1* and *cox2* were synthesized as described (Grosser et al., 2002). Antisense RNA probes were synthesized from cDNAs encoding *fli1* (Krauss et al., 1993), *flk-1*, and *pax2.1* (Krauss et al., 1991). Whole mount in situ hybridization was performed according to Marlow et al. (2002). All images were captured with Nikon Coolpix 4500. Each in situ experiment was done at least twice, and approximately 20 embryos were used in each experiment. The primary antibody was a monoclonal mouse anti-vinculin and the secondary antibody was a CY2 anti-mouse IgG (Jackson Immuno). Images were acquired using the Zeiss LSM 510 laser scanning inverted microscope.

Morpholino design and RNA injections

Morpholino design and RNA injections were performed at the one-cell stage as described (Marlow et al., 2002). Morpholino antisense oligonucleotide was designed to the predicted start codon of *cox1* (underline indicates the predicted start codon): *cox1-MO* (5′-TCAGCAAAAAGTTACACTCTCTCAT-3′) as described (Grosser et al., 2002). Mismatch morpholino against *cox1* was also designed to the predicted start codon of *cox1* (underline indicated the predicted start codon and italic letters indicates mismatched resides): (5′-TCAcCAtAAAcTTACACTgTCTgAT-3′). Both morpholinos were obtained from Gene Tools, LLC (Philomath, OR). Zebrafish *cox1* sense-capped RNA was synthesized using mMessage Machine (Ambion, Austin, TX) after template linearization. For phenotype rescue and phenocopy experiments, 40–100 pg *cox1* RNA and 4–10 ng *cox1-MO* were used per embryo.

NSAID pharmacology

To inhibit COX activity, embryos were treated with indomethacin, a non-selective cyclooxygenase inhibitor. Embryos were incubated overnight at 28°C in 24-well dishes. Indomethacin (Cayman), celecoxib (Pfizer), and NS-398 (Cayman) were incubated in embryo medium (EM) with 1% DMSO. Treatment of embryos at 2–8 cell stage with 50 μM indomethacin results in gastrulation arrest phenotype. We treated embryos with 40 μM indomethacin at tailbud stage or early somitogenesis stage to observe posterior vessel defect. Celecoxib was used in dose ranging from 1 μM to 150 μM and NS-398 was used at dose ranging from 1 μM to 150 μM. Both were treated starting at tailbud stage.

Prostaglandin assays

Endogenous levels of prostanoids were measured. Embryos were isolated at 80% epiboly stage and 24 hpf. The PG content (PGE_2, $PGF_{2\alpha}$, PGD_2, thromboxane B_2