Role of Microsomal Prostaglandin E Synthase-1 (mPGES1)-Derived PGE₂ in Patency of the Ductus Arteriosus in the Mouse

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ABSTRACT: Prostaglandin E₂ (PGE₂) plays a key role in the ductus arteriosus, prenatally by maintaining patency and postnatally by promoting tissue remodeling for closure. Here, by using near-term mouse fetuses with (wild-type, WT) and without microsomal PGE synthase-1 (mPGES1^{-/-}), we have examined the importance of this enzyme for PGE₂ formation and function. mPGES1^{-/-} ductus, unlike WT ductus, contracted little, or not all, to indomethacin *in vitro*. Coincidentally, as evident from responses to N^G-nitro-L-arginine methyl ester and zinc photoporphyrin, the mutant showed no significant enhancement of nitric oxide (NO)- and carbon monoxide (CO)-based relaxation. mPGES1 suppression differs, therefore, from cyclooxygenase (COX) suppression, whether genetically or pharmacologically induced, where NO is markedly up-regulated. *In vivo*, the ductus was patent, albeit occasionally with a narrowed lumen, in all mPGES1^{-/-} fetuses. Conversely, postnatal closure progressed regularly in mPGES1^{-/-} animals thanks to residual PGE₂ originating via mPGES2. We conclude that mPGES1 is critical for PGE₂ formation in the ductus but its loss does not entail compensatory up-regulation of other relaxing mechanisms. Accordingly, an mPGES1 inhibitor stands out as a prospective better tool, compared with the currently used COX inhibitors, for the management of premature infants with persistent ductus. (*Pediatr Res* 64: 523–527, 2008)

Prostaglandin (PG) E₂ is viewed as the prime agent for prenatal patency of the ductus arteriosus (1). Conversely, PGI₂ is without a role, notwithstanding its occurrence in the vessel and its known importance for vasoregulation elsewhere (2,3). Ductal PGE₂ is formed primarily through the cyclooxygenase-2 (COX2)/microsomal PGE synthase-1 (mPGES1) complex, while little of the compound originates from the COX1/mPGES1 complex and none at all from cytosolic PGES (cPGES). No information is available on microsomal PGE synthase-2 (mPGES2), but evidence from other vascular districts points to its minor function (4). Several findings support this arrangement in the ductus, namely, immunocytochemical data showing a predominant colocalization of COX2 with mPGES1 (5); the increase in COX2 and mPGES1 expression occurring selectively through development (6); the constrictor action of COX2 inhibitors on the ductus, being

close or even equal in magnitude to that of a dual COX1/COX2 inhibitor (7–9); the greater impairment of PGE₂-based relaxation upon COX2 than COX1 deletion (5) along with the marked curtailment of PGE₂ formation after inhibition of either COX2 or mPGES1 (6,7). Consistent with the preferential operation of the COX2/mPGES1 route for PGE₂ synthesis is also evidence from adult blood vessels (10) and the notion that mPGES1 is catalytically most active among the PGES (11).

Despite these facts, previous attempts to prove the actual importance of the COX2/mPGES1 pathway for ductus patency have failed. As shown by others and ourselves (5,12,13), COX2 removal does not result in loss of patency, likely thanks to compensation from COX1-derived PGE₂ and nonPG agents such as nitric oxide (NO) and carbon monoxide (CO). Indeed, COX2 removal, even if limited to a single allele, is followed by selective up-regulation of NO (5,14). An additional complication in addressing this issue is that PGE₂ not only sustains patency of the ductus *in utero*, but also promotes its closure after birth (9,15,16). Hence, COX2/mPGES1 function needs to be considered pre and postnatally.

Our objective here was 2-fold: first, to assess the importance of mPGES1 in ensuring normal patency and closure of the ductus, and second, to ascertain whether mPGES1 removal mimics COX2 removal in up-regulating NO. CO was examined comparatively to verify the specificity of any such effect. An answer to these questions is important insofar as it may provide a better insight into the functional organization of the PGE₂ system and the role of PGE₂ vis-à-vis NO and CO in ductus relaxation. In addition, any advance in this area may lead to better agents, in comparison to the currently used COX inhibitors (17), for the management of the prematurely born infant with patent ductus (PDA).

METHODS

Animals. Experiments were carried out in 129×C57BL/6 mice with mPGES1^{-/-} genotype (18) (litter size, 2–9; mean, 4), and wild-type (WT) C57BL/6 mice (litter size, 1–12; mean, 5) served as a control. In either case, genotype was confirmed in tail specimens by polymerase chain reaction (PCR). Animals were housed in temperature- and humidity-controlled quar-

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Abbreviations: COX, cyclooxygenase; L-NAME, N^G-nitro-L-arginine methyl ester; mPGES1, microsomal PGE synthase-1; mPGES2, microsomal PGE synthase-2; ONO-11113, 9, 11-epithio-11, 12-methano-thromboxane A₂; PDA, patent ductus arteriosus; PG, prostaglandin; ZnPP, zinc photoporphyrin

ters with constant 12:12-h light-dark cycles and were given food and water *ad libitum*. Surgical procedures and experimental protocols were approved by the Animal Care Committee of the Ministry of Health.

In vitro studies. Term fetal mice, both WT and gene-deleted (gestational age, 19 d), were delivered by cesarean section under halothane anesthesia and were killed by cervical dislocation. Body weight varied between 0.9 and 1.4 g, and only one fetus was used in each experiment. The procedure for dissection of the ductus arteriosus, normalization of internal circumference and mechanical record has been described previously (19). In brief, the animal was secured with its left side up in a dissection chamber containing ice-cold Krebs solution gassed with 5% CO₂ in N₂. Through a thoracotomy, the ductus was exposed, separated from the adjoining large blood vessels and then suspended onto 25- μ m tungsten wires (Cooner wire, Chatsworth, CA) inside an organ bath. The fluid of the bath was gassed with a mixture containing 2.5% O₂ to mimic the fetal condition, and the same gas mixture was flushed through a hood covering the bath. Preparations were then equilibrated (about 60 min at 37°C) with a minimal stretch being applied (all values in mN mm⁻¹) (WT, 0.09 ± 0.01 ($n = 14$); mPGES1^{-/-}, 0.08 ± 0.008 ($n = 16$)) and the attendant internal circumference (C₀) with the related resting dimension (Table 1) served as a reference for choosing the appropriate load. Afterward, tension was applied to attain an operating circumference (*i.e.* C₅₀) coinciding with the condition *in vivo* (WT, 0.45 ± 0.007 ($n = 14$); mPGES1^{-/-}, 0.45 ± 0.009 ($n = 16$); values in mN mm⁻¹), and the actual experiment was started after a second, 60- to 150-min period of equilibration.

The study consisted of a single protocol with two test procedures, addressing respectively the effect of N^G-nitro-L-arginine methyl ester (L-NAME, 100 μ M; $n = 13$) and zinc protoporphyrin (ZnPP, 10 μ M; $n = 8$) on the basal tone. Indomethacin (2.8 μ M; $n = 9$) was tested comparatively to verify the degree of loss of PGE₂ function upon mPGES1 deletion. In all cases, treatment lasted 60 min or as long as required to achieve a maximal effect, and the contraction to the thromboxane (TX) A₂ analogue (0.1 μ M) provided a reference. Only one test procedure was used with each vessel.

In vivo studies. Experiments were carried out in fetuses (gestational age, 19 d) or newborns (3 h after birth) depending on the protocol. In the former case, animals (WT and mPGES1-deleted) were delivered by cesarean section under halothane anesthesia, whereas in the latter they were used at the stated interval after vaginal delivery. No obvious difference in the length of gestation was noted between WT and mutant, and time zero (*i.e.* the time at which the delivery was completed) was assessed for each animal in the litter. Throughout the survival period, newborns were kept with the mother. All animals were killed by cervical dislocation.

Changes in ductus caliber through the transition from the pre to the postnatal condition were assessed by fixing the vessel *in situ* with the whole-body freezing technique (19,20). Briefly, animals were placed with their right side up in a Petri dish and were covered with an embedding medium (Tissue-Tek optimum cutting temperature compound; Sakura Finetek, Torrance, CA). The dish with the animal was then wrapped with aluminum foil and immersed in liquid N₂. Once frozen, specimens were stored at -20°C for further work-up. To prepare a block of tissue with the ductus, the carcass was freed of its embedding in the frozen state. With a razor blade, soft tissues covering the dorsum were removed making certain that the exposed surface would be parallel to the underlying descending aorta, hence perpendicular to the ductus. Several, transversal sections (5 μ m thick), progressing from this cut through the whole length of the vessel, were obtained on a Zeiss freezing microtome (model HM500 OM, Welldorf, Germany) and were stained with

1% methylene blue. Care was taken to collect a complete series of ductus sections. Afterward, each section was photographed with a charge-coupled device solid-state camera (COHU, San Diego, CA) and lumen area of the ductus was measured with a MCID-M4 program (Brock University, St. Catharines, Canada). Both maximal and minimal values of this area were retrieved from each series for computation.

Total RNA preparation and quantitative RT-PCR. mPGES2 expression was measured to assess any change resulting from mPGES1 deletion. For this purpose, ductuses were collected from WT and mPGES1 null fetuses and were pooled in three separate groups for each genotype (35–41 specimens/group). RNA was then isolated as described earlier (5), and its yield was measured spectrophotometrically. DNase I (Roche, Indianapolis, IN)—treated total RNA (2.5 μ g) was reverse transcribed with 1 U of Thermoscript RT (Invitrogen, Carlsbad, CA) in the presence of random hexanucleotide primers according to the manufacturer's instructions. QRT-PCR reactions (40 cycles) were performed on an ABI Prism 7700 instrument (Applied Biosystems, Foster City, CA) using the TaqMan Universal PCR Master Mix (Applied Biosystems). Primer sequences for mPGES2 and cyclophilin B (internal standard) were obtained from an online library (Applied Biosystems). Gene expression was quantified in triplicate for each group, using the comparative cycle-threshold method and cyclophilin for normalization.

Solutions and drugs. The Krebs medium had the following composition (mM): NaCl 118, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1, MgSO₄ 0.9, dextrose 11.1, and NaHCO₃ 25. Depending on the stage of the experiment (see above), the solution was bubbled with gas mixtures containing either no O₂ or 2.5% O₂ plus 5% CO₂ and balance N₂. Po₂ was measured with a Chiron gas analyzer (mod. 248, Halstead, UK) and was 0.95 ± 0.0009 and 2.14 ± 0.02 kPa (pH, 7.41 ± 0.003) when gas mixtures had 0 and 2.5% O₂, respectively.

The following compounds were used: the NO synthase inhibitor, L-NAME (Sigma Chemical Co., St. Louis, MO); the heme oxygenase (HO) inhibitor, ZnPP (Porphyrin Products, Canforth, UK); the dual COX1/COX2 inhibitor, indomethacin (Sigma Chemical Co.); the TXA₂ analogue 9, 11-epithio-11, 12-methano-TXA₂ (ONO-11113, courtesy of ONO Pharmaceutical, Osaka, Japan). Concentrations of the inhibitors were derived from the literature with the aim of combining efficacy with selectivity. In particular, at the stated concentrations, ZnPP exerts a specific effect on the HO enzyme complex in the ductus (21).

ONO-11113 was dissolved in distilled ethanol (5 mg ml⁻¹), and aliquots (stored at -70°C) were diluted with Tris buffer (pH 7.4). Indomethacin was also dissolved in ethanol (10 mg ml⁻¹) before preparation of the final solution in the Krebs medium. Likewise, ZnPP was first prepared as a stock solution in 0.1 M NaOH (1 mM) on the day of the experiment. Ethanol in the fluid bathing isolated ductus preparations did not exceed 0.01% (indomethacin) or 0.001% (ONO-11113). Solutions of ZnPP were protected from light.

Concentrations of compounds are given in molar units and refer to their final value in the bath. Vehicle alone had no effect on vessel tone.

Analysis of data. Baseline contractile tension, which varied with the preparation (see "Results"), refers to the net active tension (*i.e.* total tension minus applied tension) developed by the preparation before any treatment. Contractile responses were measured by the rise in tension over baseline and were expressed in absolute values.

Data are presented as means $\pm$ SEM. Comparisons were made using a *t* test or analysis of variance (ANOVA), followed by the Bonferroni test. Differences are considered significant for $p < 0.05$.

RESULTS

mRNA analysis. The mPGES2 transcript was barely detectable in the WT ductus (reading at 36–38 cycles), indicating a minor contribution of this enzyme to PGE₂ synthesis. Its level of expression remained marginal upon mPGES1 deletion and, consequently, no importance can be attached to any apparent variation, either upward ($n = 2$) or downward ($n = 1$), relative to the WT value.

In vitro studies. The isolated ductus arteriosus was slightly shorter in mPGES1^{-/-} than WT and, in one case, it also showed the signs of a prior constriction *in utero* (Table 1). With either genotype, preparations developed a variable degree of tension during equilibration (WT, 0.26 ± 0.06 ; mPGES1^{-/-} 0.18 ± 0.04 , all values in mN mm⁻¹), which, most of the times, was maintained throughout the experiment. In addition, the baseline was often interrupted by transient

Table 1. Resting dimension of isolated ductus arteriosus from WT and mPGES1^{-/-} term fetal mice

Genotype	Internal diameter	Vessel length*		Wall thickness
		Short side	Long side	
WT ($n = 14$)	132 ± 1	568 ± 19	581 ± 19	20 ± 0.5
mPGES1 ^{-/-} ($n = 16$)†	132 ± 2	$513 \pm 11‡$	$524 \pm 11‡$	20 ± 0.5

All values (μ m) expressed as mean $\pm$ SEM.

* Vessel length of the ductus arteriosus is uneven because of its insertion into aorta at an angle. Short side was taken for normalisation of internal circumference and calculation of tension output (see "Methods").

† One of the fetuses presented a clearly constricted ductus (internal diameter: 107; length, 496 and 488, respectively, for long and short side; wall thickness, 22; all values in μ m) on dissection. Otherwise, it behaved normally through the experiment and was included in the computation.

‡ $p = 0.01$ vs WT (ANOVA with Bonferroni *post hoc* test).

In vivo studies. The ductus was patent in all fetuses, with no significant difference in lumen area between WT (max area: $139674 \pm 12415 \mu\text{m}^2$; min area: $103312 \pm 10910 \mu\text{m}^2$) and mPGES1 $^{-/-}$ (max area: $124821 \pm 14358 \mu\text{m}^2$; min area: $92174 \pm 10012 \mu\text{m}^2$) (Fig. 2A). However, two mutants departed from the group for their smaller vessel (Fig. 2A), which is indicative of a preexisting constriction *in utero*. Postnatally, both WT and mPGES1 $^{-/-}$ animals closed their duct rapidly (Fig. 2B), with narrowing of the lumen being uniform over the entire length or more prominent in the centro-pulmonary portion.

How is, then, patency of the ductus maintained in the face of a marked curtailment of the PGE₂ mechanism? Compensation by other intramural prostanoids, in particular PGI₂ being up-regulated by mPGES1 deletion (10,22,23), is unlikely. PGI₂ is far less potent than PGE₂ in dilating the vessel (1) and, moreover, its involvement in tone regulation is ruled out by findings in animals lacking the receptor (2). More plausibly, other local agents, specifically NO and CO, come into play even though their function is not overtly enhanced as a result of mPGES1 suppression. Furthermore, a relaxant influence may derive from blood-borne PGE₂ being formed elsewhere, particularly in the placenta, through alternative PGE synthases (24,25). Conversely, no contribution is expected from the endothelium—derived hyperpolarizing factor (EDHF) because its action unfolds only in the absence of NO and CO (14). Whatever its mechanism(s), the intervening compensation is expectedly limited and, in fact, appears inadequate in the few mPGES1^{-/-} fetuses showing a constricted ductus.

The mPGES1^{-/-} fetus differs from fetuses lacking COX1 or COX2 whose duct presents a marked up-regulation of NO (5). Not fortuitously, the latter mutants have always an open ductus without any sign of constriction (5). The question, then, can be raised on why is the NO system unevenly affected by mPGES1 *versus* COX deletion, with the lesser activation occurring somewhat paradoxically in a situation (*i.e.* the mPGES1 mutation) where a compensation for PGE₂ loss would be more acutely needed. No firm explanation can be provided with the available data. However, by considering the results in mutants along with work documenting an enhanced NO function upon indomethacin treatment (26), one may conclude that the up-regulatory drive is linked specifically to interference with COX. Extending this reasoning further, one could then assume that COX suppression is coupled with a rebound of parallel pathways in arachidonic acid metabolism (lipoxygenase, monooxygenase) wherefrom a stimulatory signal is generated for the NO system and, particularly for eNOS (*i.e.* the main synthetic enzyme; see Refs. 5,14). Among the many potential candidates for this messenger role, products from 12S-lipoxygenase are most promising in view of the prominence of this pathway in the ductus (27). 12-hydroxytetraenoic acid (12-HETE), for example, could stimulate eNOS not only through its transcription factors SP1 and AP2 acting on specific recognition sites in the promoter (28; see <http://www.genomatix.de>), but also through changes in phosphorylating kinases and free Ca²⁺ favoring catalytic activity (29,30).

Contrary to animals lacking both COXs or the prime PGE₂ receptor, EP₄, the mPGES1 mutant closed its duct normally after birth. The former mutants present instead a persistent duct, which is incompatible with extra-uterine life (13,15). These findings raise important questions, both conceptual and methodological. Persistency of the ductus upon complete removal of PGE₂ may be linked to two conditions: NO up-regulation expectedly occurring with the double COX knockout but not with the EP₄ knockout, and interference with the remodeling process being shared by both mutants. Ductus closure requires, in fact, PGE₂ for its smooth progression (9,15,16). No such events take place in the mPGES1^{-/-} ductus where, conversely, NO function is practically normal and some PGE₂ may still be produced via the COX/mPGES2 pathway (31), both locally (this study) and systemically (24,25). Hence, this mutant not only affords a better insight into the operation of the ductus, but also represents a useful model for the study of PGE₂ function without the limitations of the double COX and EP₄ mutants.

The present study has significant implications for the clinic inasmuch as it may lead to a better tool for the management of premature infants with PDA. The currently used COX inhibitors carry, in fact, a high incidence of failures due largely, in our view, to the rebound activation of nonPG relaxing mechanisms, NO in particular (26). No such complication is envisaged with an mPGES1 inhibitor. In addition, this inhibitor would be more selective and, specifically, would preserve the synthesis of PGI₂ in vascular districts critically dependent on this compound for their normal function. Significant is also the fact that an mPGES1 inhibitor may be particularly useful with PDA complicated by an infectious state (see Ref. 18) and may

also preserve some PGE₂ synthesis for the orderly progression of the remodeling process within the closing ductus.

In conclusion, we have demonstrated that mPGES1 is critical for PGE₂ synthesis in the ductus. mPGES1 deletion, unlike COX inhibition or deletion, is not associated with a major compensation from NO. Based on these findings, an mPGES1 inhibitor stands out as a prospective new tool, superior to the currently used COX inhibitors, for the management of the premature infant with PDA.

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