Developmental Changes in Prostaglandin E\textsubscript{2} Receptor Subtypes in Porcine Ductus Arteriosus

Possible Contribution in Altered Responsiveness to Prostaglandin E\textsubscript{2}

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Background—Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) is important in ductus arteriosus (DA) patency, but the types of functional PGE\textsubscript{2} receptors (EP) in the developing DA are not known. We postulated that age-dependent alterations in EP and/or their subtypes may possibly contribute to the reduced responsiveness of the newborn DA to PGE\textsubscript{2}.

Methods and Results—We determined PGE\textsubscript{2} receptor subtypes by competition binding and immunoblot studies on the DA of fetal (\textasciitilde\textasciitilde75\% and 90\% gestation) and newborn (<45 minutes old) pigs. We studied the effects of EP receptor stimulation on cAMP signaling in vitro and on term newborn (<3 hours old) DA patency in vivo. Fetal pig DA expressed EP\textsubscript{2}, EP\textsubscript{3}, and EP\textsubscript{4} receptors equivalently, but not EP\textsubscript{1}. In neonatal DA, EP\textsubscript{1}, EP\textsubscript{3}, and EP\textsubscript{4} were undetectable, whereas EP\textsubscript{2} density was similar in fetus and newborn. Prostaglandin-induced changes in cAMP mirrored binding data. 16,16-Dimethyl PGE\textsubscript{2} and 11-deoxy PGE\textsubscript{1} (EP\textsubscript{2}/EP\textsubscript{3}/EP\textsubscript{4} agonist) produced more cAMP in fetus than newborn, but butaprost (selective EP\textsubscript{2} agonist) caused similar cAMP increases in both; EP\textsubscript{3} and EP\textsubscript{4} ligands (M&B28767 and AH23848B, respectively) affected cAMP production only in fetus. After birth, administration of butaprost alone was as effective as 11-deoxy PGE\textsubscript{1} and 16,16-dimethyl PGE\textsubscript{2} in dilating DA in vivo.

Conclusions—The data reveal fewer PGE\textsubscript{2} receptors in the DA of the newborn than in that of the fetus; this may contribute to the decreased responsiveness of the DA to PGE\textsubscript{2} in newborn. Because EP\textsubscript{2} receptors seem to mediate the effects of PGE\textsubscript{2} on the newborn DA, one may propose that a selective EP\textsubscript{2} agonist may be preferred as a pharmacological agent to maintain DA patency in infants with certain congenital heart diseases. (Circulation. 1999;100:1751-1756.)

Key Words: ductus arteriosus ■ receptors ■ prostaglandins ■ pediatrics

Prostaglandins play a major role in maintaining patency of the fetal ductus arteriosus (DA).\textsuperscript{1,2} The marked sensitivity of the DA to prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) suggests that this is the most important prostanoid regulating vessel patency.\textsuperscript{3} PGE\textsubscript{2} exerts its effects through a diverse group of receptors, classified as EP\textsubscript{1}, EP\textsubscript{2}, EP\textsubscript{3}, and EP\textsubscript{4}.\textsuperscript{4} Activation of EP\textsubscript{1} increases inositol 1,4,5-triphosphate (IP\textsubscript{3}) formation and elicits vasoconstriction,\textsuperscript{5} stimulation of EP\textsubscript{2} and EP\textsubscript{4} increases cAMP and leads to vasodilation,\textsuperscript{4,6} and stimulation of EP\textsubscript{3} decreases cAMP and opposes vasodilation.\textsuperscript{4}

At present, the relative types and importance of EP receptors in the DA are uncertain. Pharmacological evidence suggests that the EP\textsubscript{4} receptor is the predominant PGE\textsubscript{2} receptor in the fetal rabbit DA.\textsuperscript{7} Conversely, genetic disruption of the EP\textsubscript{4} receptor does not diminish DA patency in the fetal and neonatal mouse.\textsuperscript{8} However, the type of EP receptors expressed in higher species, and especially in the newborn, is not known. This is of particular relevance because responsiveness of the DA to PGE\textsubscript{2} in the newborn is significantly less than that in the fetus.\textsuperscript{9,10} Because PGE\textsubscript{2} acts on several distinct receptor subtypes, each coupled to different second messengers, we hypothesized that differences in the relative density and/or proportion of EP receptors could explain, at least in part, the differences in the responsiveness of the fetal and neonatal DA to PGE\textsubscript{2}.\textsuperscript{9,10}

We therefore studied the expression of EP receptor subtypes and EP receptor signaling factors in the DA of fetal and newborn pigs. We also assessed the role of these receptors in the newborn in vivo. Our findings reveal that the DA of the fetal pig expresses negligible amounts of the EP\textsubscript{1} receptor but does express the other 3 EP subtypes (EP\textsubscript{2}, EP\textsubscript{3}, EP\textsubscript{4}) in equivalent proportions. In the DA of the newborn pig, the density of PGE\textsubscript{2} receptors is significantly reduced compared with that in the fetus because of complete loss of EP\textsubscript{3} and EP\textsubscript{4}.

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receptors; however, the number of EP2 receptors remains unchanged, such that EP2 seems to mediate all PGE2-dependent relaxation in the newborn DA.

Methods

Animals

DAs were removed from fetal pigs (78 to 90 and 100 to 105 days of gestation [term, 114 days]) and term newborn piglets (1.25 to 1.7 kg, killed within 45 minutes of vaginal birth with pentobarbital [120 mg/kg intracardiac] under halothane anesthesia). Tissues were immediately rinsed in ice-cold Krebs buffer (pH 7.4) of the following composition (mmol/L): NaCl 120, KCl 4.5, CaCl2 2.5, MgSO4 1.0, NaHCO3 27, KH2PO4 1.0, and glucose 10 and then frozen in liquid N2; we have shown that freezing does not affect PGE2 binding.13 A group of newborn piglets (<3 hours old) was used to study the effects of prostaglandin analogues on DA diameter in vivo.

EP Receptor Characterization

Tissues were prepared for prostaglandin binding as described.11–13 Aliquots of DA homogenate (200 μg protein) were incubated at 37°C for 30 minutes with various concentrations of [3H]PGE2 (Amersham) in the presence or absence of 25 μmol/L 16,16-dimethyl PGE2. The reaction was terminated with ice-cold 5 mmol/L Tris-HCl buffer (pH 7.4), the homogenate was filtered through Whatman GF/C glass filter disks, and the radioactivity was counted in Tris-HCl buffer (pH 7.4), the homogenate was filtered through Whatman GF/C glass filter disks, and the radioactivity was counted.

Data were analyzed by Student’s t test and by 2-way ANOVA factoring for time and treatment; means tests were compared by the Tukey-Kramer method. Statistical significance was set at P<0.05. Data are expressed as mean±SEM.

Immunoblotting of EP Receptors

Western blotting of EP1, EP3, and EP2 receptors was conducted on DA membranes prepared as described13 and on cell lysates from human embryonic kidney (HEK) 293 cells (Invitrogen), which overexpress each of these receptors14 and hence were used as positive controls. After immunoblotting with EP1, EP3, or EP2-specific polyclonal rabbit antibodies17 (1:1000; EP2 antibodies are not available), immunoreactive bands were visualized by chemiluminescence (Amersham) according to the manufacturer’s instructions.

cAMP Assay

The effects of PGE2 analogues on cAMP were determined12,13 Briefly, DA homogenates (100 μg protein) were incubated in an assay mixture (100 μL) containing 10 mmol/L Tris-HCl buffer (pH 8.0), 1 mmol/L ATP, 7.5 mmol/L MgCl2, 15 mmol/L creatine phosphate, 185 μmol/L creatine phosphokinase, 200 μmol/L aspirin, 0.5 mmol/L EGTA, 0.5 mmol/L 3-isobutyl-1-methylxanthine, 1 mmol/L dithiothreitol, 1 mmol/L benzamidine, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 100 μg/mL soybean trypsin inhibitor in the presence or absence of test agents at 37°C for 10 minutes. The reaction was terminated with 200 μL acidic ethanol. After centrifugation, cAMP was measured by radiomunnoassay (Diagnostic Products).

Surgical Preparation and Echocardiography

Newborn pigs (between 1.5 and 2 hours after delivery; 1.3 to 1.7 kg) were prepared to test the effects of specific EP receptor agonists on DA patency; in the piglet, the DA closes completely by 4 to 6 hours of age.15 We were not able to perform in vivo studies on fetal pigs because facilities were not available to operate on sows. The newborn piglets were anesthetized with halothane (2%) for 15 minutes during tracheostomy and catheterization of the umbilical vein and artery, and anesthesia was discontinued after surgery. Animals were ventilated with air with a Harvard small-animal respirator, maintained on α-chloralose (50 mg/kg bolus followed by 10 mg · kg⁻¹ · h⁻¹ infusion), and paralyzed with pancuronium (0.1 mg/kg); body temperature was maintained at 38°C. The surgical procedure was completed within 15 minutes, and piglets were allowed to stabilize for an additional 30 minutes.

Echocardiographic measurements were performed with an Acuson 128 XP/10C real-time ultrasound imaging system using 7.5- and 5-MHz transducers duplexed with a range-gated Doppler as previously described16,20; Doppler signals were filtered by 100-Hz high-pass filter. The DA was visualized through a left second intercostal parasternal approach. Measurements of the DA diameter were repeated 3 times. The smallest diameter of the DA lumen was monitored until it reached 0.6 to 0.8 mm in diameter. Experimental drugs were then injected, and the smallest diameter was measured every minute for the next 10 to 25 minutes.

Animals (3 to 4 per treatment) were randomly assigned to receive 10-minute infusions of saline (1 mL, controls) or 0.083 μg · kg⁻¹ · min⁻¹ of EP receptor agonists; the doses used have been shown previously to be effective in vivo.21 The effect of EP receptor agonists on DA patency was also tested in animals pretreated with indomethacin (3 mg/kg IV for 5 minutes), once the DA diameter reached 0.6 to 0.9 mm (within 20 minutes).

Statistical Analysis

Data were analyzed by Student’s t test and by 2-way ANOVA factoring for time and treatment; means tests were compared by the Tukey-Kramer method. Statistical significance was set at P<0.05. Data are expressed as mean±SEM.

Results

PGE2 Receptors in Fetal and Newborn DA

[3H]PGE2 bound specifically to fetal and newborn DA membranes. Maximum binding of [3H]PGE2 to the fetal DA was similar at both gestational ages studied (78 to 90 days: 34.9±2.5 fmol/mg protein, n=3; 100 to 105 days: 35.1±3.1 fmol/mg protein, n=3). Maximum specific binding of [3H]PGE2 was 3-fold greater in fetal than newborn DA (Figure 1A and 1B and Table 1); the Kd did not differ between the fetus and newborn.

In fetal DA membrane preparations (Figure 1C), butaprost (EP2 agonist), M&B28,767 (EP3 agonist), and AH23848B (EP1 antagonist) caused an equivalent displacement of [3H]PGE2 (Figure 1C and 1E and Table 2); 11-deoxy PGE1 (EP2/EP3/EP4 agonist) displaced virtually all [3H]PGE2, but the EP1 antagonist AH6809 was ineffective (Figure 1C and 1E and Table 2). IC50 values of specific polyclonal rabbit antibodies17 (1:1000; EP2 antibodies are not available), immunoreactive bands were visualized by chemiluminescence (Amersham) according to the manufacturer’s instructions.

In contrast to fetal DA, [3H]PGE2 bound to newborn DA membranes was no longer displaced by M&B28,767, sulprostone (EP3 agonist), and AH23848B (EP1 antagonist) displaced virtually all [3H]PGE2, but the EP1 antagonist AH6809 was ineffective (Figure 1C and 1E and Table 2). In contrast to fetal DA, [3H]PGE2 bound to newborn DA membranes was no longer displaced by M&B28,767, sulprostone, or AH23848B (Figure 1D). Butaprost and 11-deoxy PGE1 were the only PGE1 analogues (along with 16,16-dimethyl PGE1) capable of fully displacing [3H]PGE2 from the newborn DA (Figure 1D and Table 2). IC50 values of ligands tested were comparable to those reported in other porcine tissues.11,12 Results indicate the presence of EP2, EP3, and EP1 in fetal DA.

In the immediate postnatal newborn, EP3 and EP1 are absent, without significant change in the number of EP2 receptors (Figure 1E); this is reflected by the reduced [3H]PGE2 binding in the newborn DA (Figure 1A and 1B and Table 1).
Expression of EP₁, EP₃, and EP₄ Immunoreactive Protein in DA of Fetal and Newborn Pig
The DA of the fetal pig, but not of the newborn, expressed EP₁ (55-kDa band) and EP₄ (63-kDa band) immunoreactive protein consistent with binding data (Figure 2); EP₂ antibodies are unavailable.

PGE₂ Analogue–Induced Production of cAMP
PGE₂ receptor–coupled changes in cAMP generation were also consistent with the binding data. 16,16-Dimethyl PGE₂ and 11-deoxy PGE₁ (EP₁/EP₃/EP₄ agonist) produced a comparable dose-dependent increase in cAMP synthesis, which was greater in fetus than in newborn (Table 3).

In fetal DA, butaprost (EP₂ agonist) stimulated cAMP production in a concentration-dependent manner. The EP₃ agonist M&B28,767 and EP₁/EP₃ agonist sulprostone had no effect on cAMP formation; this suggests that EP₃ is coupled to inhibition of cAMP formation. Because no selective EP₃ agonist is currently available, we estimated the effects attributed to EP₃, as we previously reported, by subtracting the increase in cAMP produced by the combination of 16,16-dimethyl PGE₂ and EP₄ antagonist AH23848B (which would stimulate all of the EP receptors except EP₄) from the increase in cAMP produced by 16,16-dimethyl PGE₂ alone (which stimulates all EP receptors). In the fetal DA, AH23848B decreased the cAMP production induced by 16,16-dimethyl PGE₂ but not that stimulated by butaprost (Table 3).

### TABLE 1. Maximum Binding and Affinity Constant of [³H]PGE₂ on DA Membranes From Fetal and Newborn Pigs

<table>
<thead>
<tr>
<th>Variables</th>
<th>Fetus</th>
<th>Newborn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bₘₐₓ, fmol/mg protein</td>
<td>34.9±2.5</td>
<td>12.3±1.6*</td>
</tr>
<tr>
<td>Kᵦ, nmol/L</td>
<td>10.8±3.0</td>
<td>6.1±2.3</td>
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</table>

Values are the mean±SEM of 3 or 4 experiments, each performed in duplicate. Because Bₘₐₓ in fetus 78 to 90 and 100 to 105 days of gestation was similar, fetal tissue from both ages were combined for all tables.

*P<0.01 vs corresponding value for fetus.
In the newborn DA, butaprost increased cAMP generation to values comparable to those in the fetus (Table 3); M&B28,767, sulprostone, and AH23848B had no effect on cAMP production.

### Effects of EP Receptor Agonists on DA Diameter In Vivo

We examined the effects of EP receptor agonists (17-phenyl-trinor PGE$_2$ [EP$_1$]; butaprost [EP$_2$]; M&B28,767 [EP$_3$]; sulprostone [EP$_2$/EP$_3$]; and 11-deoxy PGE$_1$ [EP$_2$/EP$_3$/EP$_4$]) on patency of the newborn DA in vivo (Figure 3). 16,16-Dimethyl PGE$_2$ dilated both the untreated, spontaneously closing (Figure 3A) and the indomethacin-constricted DA (Figure 3B). Butaprost and 11-deoxy PGE$_1$ dilated the constricting DA as seen with 16,16-dimethyl PGE$_2$ (Figure 3); cessation of infusion of these PGE$_2$ analogues resulted in constriction of the DA. In contrast, 17-phenyl-trinor PGE$_2$, M&B28,767, and sulprostone did not affect DA diameter; 2- to 3-fold higher infusion rates of the EP$_1$ and EP$_3$ agonists also had no effects (data not shown). Hence, the vasodilatory effects of PGE$_2$ in the newly born seem to be accounted for by action on EP$_2$.

### Discussion

Previous pharmacological studies have suggested that EP$_4$ is the dominant (relaxant) PGE$_2$ receptor in the fetal rabbit DA. In the mouse, however, disruption of the EP$_4$ gene did not affect ductal patency, suggesting possible species differences. We therefore set out to characterize the EP receptors in the DA of the fetus and newborn of a higher species, namely the pig.

Using binding and displacement, immunoblot, and stimulation of second-messenger cAMP, our studies revealed that the number and types of PGE$_2$ receptors differ between fetal and immediate postnatal newborn DA. There was a 3-fold higher density of PGE$_2$ receptors and a greater PGE$_2$-induced increase in cAMP in the fetus than in the newborn (Table 3); a rise in cAMP is usually associated with vasorelaxation. We identified EP$_3$, EP$_4$, and EP$_3$ receptors in the fetal pig DA; EP$_1$ receptor was undetectable (Figures 1 and 2). In the newborn DA, we observed a decrease in PGE$_2$ binding due to a loss of EP$_3$ and EP$_4$ receptors (Figures 1C through 1E and 2); the number of EP$_3$ receptors was essentially the same in the fetus and newborn. As a result, the EP$_2$ receptor appeared to mediate the vasorelaxant effects of PGE$_2$ on the full-term neonatal DA (Figure 3).

The mechanism(s) responsible for the birth-related decrease in PGE$_2$ receptors in the DA are currently unknown. We have previously observed that the loss of DA responsiveness to PGE$_2$ is directly related to the degree of postnatal DA constriction. However, a role for hypoxia to explain the selective loss of EP$_1$ and EP$_4$ is unlikely. Our data on [H]PGE$_2$ binding, immunoreactivity, and cAMP generation in newborn were obtained on DA of animals <45 minutes after birth. Although a 50% ductal constriction occurs over this time period, the partial pressure of oxygen also rises markedly immediately after birth; as a result, the DA tunica media may not develop significant hypoxia during this time.

### Table 2. Competitive Inhibition by Different Agents of [H]PGE$_2$ Binding to DA Membranes From Fetal and Newborn Pigs

<table>
<thead>
<tr>
<th>Competing Agents</th>
<th>% Inhibition of Binding</th>
<th>IC$_{50}$, nmol/L</th>
<th>% Inhibition of Binding</th>
<th>IC$_{50}$, nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>16,16-Dimethyl PGE$_2$</td>
<td>100±0</td>
<td>31±4</td>
<td>100±0</td>
<td>28.5±2.5</td>
</tr>
<tr>
<td>AH8809</td>
<td>&lt;1</td>
<td>ND</td>
<td>&lt;1</td>
<td>ND</td>
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<tr>
<td>11-Deoxy PGE$_1$</td>
<td>94±6</td>
<td>30±5.0</td>
<td>96±4</td>
<td>38±5.5</td>
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<td>Butaprost</td>
<td>32±2*</td>
<td>157±12</td>
<td>98±1</td>
<td>187±9.6</td>
</tr>
<tr>
<td>M&amp;B28,767</td>
<td>42±3</td>
<td>260±13</td>
<td>1&lt;1</td>
<td>ND</td>
</tr>
<tr>
<td>Sulprostone</td>
<td>43±4</td>
<td>128±13</td>
<td>1&lt;1</td>
<td>ND</td>
</tr>
<tr>
<td>AH23848B</td>
<td>32±2</td>
<td>15050±323</td>
<td>1&lt;1</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are mean±SEM of 3 to 5 experiments, each performed in duplicate. Concentration of [H]PGE$_2$ was 8 nmol/L. ND indicates not determined (inhibition of binding was too low for accurate calculation of IC$_{50}$). *P<0.01 vs corresponding value in newborn.

In the newborn DA, butaprost increased cAMP generation to values comparable to those in the fetus (Table 3); M&B28,767, sulprostone, and AH23848B had no effect on cAMP production.

**Figure 2.** Immunoblot of EP$_1$, EP$_3$, and EP$_4$ receptor proteins (arrows) in DA of fetal (F) and newborn (N) pigs and in cell lysates from HEK 293 cells overexpressing EP$_1$, EP$_3$, and EP$_4$ receptors separately (positive controls). Molecular weight protein markers (kDa) are on right.
butaprost (1 μmol/L), 11-deoxy PGE1 (0.1 μmol/L), 16,16-dimethyl PGE2 (0.1 μmol/L), AH23848B (1 μmol/L), sulprostone (1 μmol/L), and forskolin (0.1 μmol/L). The net maximum stimulated production of cAMP was corrected for basal (unstimulated) synthesis of cAMP, which was 4.1 ± 0.7 pmol · mg protein⁻¹ · min⁻¹ for newborn and 2.9 ± 0.6 pmol · mg protein⁻¹ · min⁻¹ for fetus.

*P < 0.05 vs values in fetus; †P < 0.05 vs corresponding values with 16,16-dimethyl PGE2 (1 μmol/L); ‡P < 0.05 vs corresponding values with 16,16-dimethyl PGE2 (0.1 μmol/L); §P < 0.05 vs corresponding values with forskolin alone.

### Figure 3. Effects of PGE2 receptor agonists on DA diameter in newborn pigs.

A. DA diameter in newborn pigs after infusion of saline (○), 16,16-dimethyl PGE2 (■), EP2/EP3 agonist AH23848B (▲), EP1 agonist 17-phenyl-trinor PGE2 (□), EP2 agonist butaprost (●), EP1 agonist M&B28,767 (▲), or EP1 agonist sulprostone (△); diameter of aorta at isthmus was 5.8 ± 0.9 mm. Effects of agents were studied when DA closed spontaneously to reach a diameter of 0.6 to 0.8 mm (at ~2.5 to 3 hours old). Shaded area along abscissa corresponds to period of PGE2 analogue infusion (0.083 μg · kg⁻¹ · min⁻¹ · IV). B. DA diameter of newborn pigs (1.5 hours old) first treated with indomethacin (INDO, 3 mg/kg IV) and subsequently infused with saline (○), 16,16-dimethyl PGE2 (■), 17-phenyl-trinor PGE2 (□), butaprost (●), or M&B28,767 (▲). Shaded area along abscissa corresponds to period of PGE2 analogue infusion (0.083 μg · kg⁻¹ · min⁻¹ · IV). Arrow points to time of administration of indomethacin. Effects of PGE2 analogues were determined on DA when it reached a diameter similar to that in A. Ductal diameter was measured by echocardiogram as described in Methods section. Values are mean ± SEM of 3 to 4 experiments for each agent. *P < 0.05 vs corresponding values for saline, 17-phenyl-trinor PGE2, M&B28,767, and sulprostone (ANOVA and Tukey-Kramer method).

focused on ontogenic changes in PGE2 receptors in the DA. Other mechanisms are also likely to participate in this age-dependent altered responsiveness of the DA to PGE2. These include developmental changes in the rates of prostaglandin production, uptake, and degradation as well as in prostaglandin-coupled signal transduction and relaxant mechanisms, and the role of increasing oxygen tension on ductal...
function, which has been debated, these various aspects need to be examined separately.

In summary, the present study demonstrates a developmental alteration of EP receptors in the DA. However, changes in EP receptor profile in DA of the prematurely born newborn remain to be determined. The present study also provides a physiological basis for the potential use of more selective EP receptor ligands to control DA patency and potentially diminish the side effects associated with nonselective PGE therapy. For example, one could suggest specific therapies for maintaining ductal patency in infants with congenital heart disease, such as selective EP2 agonists, and possibly reduce PGE-mediated fever due to EP2 stimulation. In the future, selective EP receptor antagonists targeted toward inflammation, fever, and pain in the pregnant subject will also need to be evaluated for their effects on the fetal DA.

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References

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