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Distribution of Prostacyclin Synthase, 6-Keto-Prostaglandin $F_{1\alpha}$, and 15-Hydroxy-Prostaglandin Dehydrogenase in the Normal and Persistent Ductus Arteriosus of the Dog

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The presence of prostacyclin synthase (PGI_2 synthase), 6-keto-prostaglandin $F_{1\alpha}$ (6k-$PGF_{1\alpha}$), and the stable hydrolysis product of prostacyclin (PGI_2), prostaglandin E_2 (PGE_2), as well as the activity of 15-hydroxy-prostaglandin dehydrogenase (PGDH) were studied in the aorta, pulmonary artery, the normal ductus arteriosus (DA), and persistent DA (PDA) of the dog using histochemical and immunohistochemical techniques. The normal DA is characterized by the development of intimal thickening, a process that does not occur in the persistent DA. Distribution of PGI_2 synthase was identical in the aorta, pulmonary artery, and persistent DA. In these vessels endothelial cells contained higher levels of PGI_2 synthase as compared with medial smooth muscle cells. In the normal DA, levels of PGI_2 synthase were clearly higher in smooth muscle cells at the sites of intimal thickening than at other sites. Distribution of 6-keto-$PGF_{1\alpha}$ resembled the localization of PGI_2 synthase. Presence of PGE_2 and activity of PGDH could not be demonstrated. The results demonstrated existence of a clear relationship between ductal morphology and the presence of PGI_2 synthase. This finding suggests a more important role for PGI_2 in regulating ductal patency than has heretofore been appreciated. It was assumed that the role of PGI_2 in regulating ductal patency is, at birth, at least overruled by the constrictive effect of the cytochrome P450 mono-oxygenase mechanism. It is still possible to attribute a role to PGI_2 in the regulation of cushion formation. Once smooth muscle cell activity has been enhanced by the presence of a glycosaminoglycan rich environment, increase in PGI_2 may produce a concurrent inhibition of smooth muscle cell growth. (Am J Pathol 1989, 135:881–887)

Patency of the ductus arteriosus (DA) is regulated by both prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2).[1,2] In the DA, PGE_2 is a stronger vasodilator than PGI_2, although the latter appears to be the main product of prostaglandin metabolism in this vessel.[1-7] A fall in locally synthesized and/or systematically produced PGE_2 induces constriction after birth.[1,2] A local decrease of PGE_2 concentration could be due to an increase in activity of 15-hydroxy-prostaglandin dehydrogenase (PGDH), which degrades PGE_2 into the vaso-inactive compound 15-keto-PGE_2. The activity of PGDH in the DA has not been studied, however. There is also increasing evidence for the involvement of a cytochrome P-450-mediated mechanism in ductal constriction.[8] Activation of this system would convert arachidonic acid into a compound with vasoconstrictive properties, at the expense of PGE_2 and PGI_2 synthesis.[2,8]

The closure mechanism is diminished or absent in the persistent DA (PDA). Although the PDA, like the DA, constricts after exposure to indomethacin and may show some initial contraction after birth,[9,10] it remains patent. Synthesis of PGE_2, PGI_2, or both, therefore, is likely to be different in the PDA compared with the normal DA. The difference between DA and PDA is also reflected in an altered morphology of the vessel wall.[11,12] Maturation of the normal DA is characterized by development of intimal cushions,[10,11] which are absent in the PDA, indicating that their presence is a prerequisite for ductal closure. A possi-

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Table 1. *Material Used in This Study: Canine DA*

N	Age	Strain	Morphology
1	6 Hours	Beagle	Normal
2	6 Hours	Beagle	Normal
3	1 Day	Beagle	Normal
4	1 Day	Beagle	Normal
5	1 Day	Beagle	Normal
6	2 Days	Beagle	Normal
7	5 Days	Poodle	Mixed type
8	15 Days	Poodle	Mixed type

ble relationship between intimal cushion formation and prostaglandin metabolism can therefore not be excluded. To determine whether there truly is a relationship between intimal cushion formation and prostaglandin metabolism, the normal DA and PDA of the dog were studied. Histochemical and immunohistochemical techniques were used to determine the distribution of PGI_2 synthase, 6-keto-prostaglandin $F_{1\alpha}$ (6 keto $PGF_{1\alpha}$: the stable hydrolysis product of PGI_2), PGE_2, and the activity of PGDH.

Material and Methods

DA were obtained from littermates of a strain of beagles, known to have a high incidence of a normal closing DA,[13] and a strain of poodles with a high frequency of a genetically persistent DA[14] (Table 1). The beagle strain is maintained at the Department of Obstetrics, the University of Utrecht, The Netherlands. The poodle strain is maintained at the University of Veterinary Medicine, Philadelphia, PA, according to guidelines set forth in the Guide to the Care and Use of Laboratory Animals.[15]

Normal DA are characterized by the development of intimal thickening (Figure 1), a process that starts with separation of the endothelial cells from the internal elastic lamina by a widened subendothelial region (SR). This is followed by migration of medial smooth muscle cells and invagination of the endothelial cells. Finally, the SR is filled with both smooth muscle cells and endothelial cells. This process always starts at the pulmonary end of the DA and proceeds towards the aortic end. Different stages of intimal thickening therefore can be observed along the length of the DA.[12]

In this study a series of subsequent frozen sections of canine DA and PDA were used (Table 1). In all experiments sections taken from the aortic connection towards the pulmonary end were examined. Alternate sections were stained for the various components studied. For each DA the aorta was used as a control. DA derived from the poodle strain can be divided into three different categories[12]: PDA, mixed type DA, and a DA that is morphologically indistinct from a DA derived from the beagle strain. PDA is characterized by the absence of intimal thickening, whereas mixed type DA has characteristics of both normal DA and PDA, showing normal DA morphology and intimal thickening at only one side, whereas a part of the vessel wall has an abnormal morphology and lacks intimal thickening. The term side is used to indicate a restricted part of the vessel wall. It does not refer to the aortic or pulmonary end of the DA.

Dogs were anesthetized with ether and perfused through the left ventricle with physiologic saline containing 10 IU/ml heparin (Organon Teknika, Oss, The Netherlands) and 0.0001% $NaNO_2$. DA with adjacent aorta (AO) and pulmonary artery (PA) was removed, rinsed in phosphate-buffered saline (PBS), pH 7.2, containing 6.8% sucrose, submerged in Tissue tek (Miles laboratories, Naperville, IL) and quickly frozen in chilled isopentane (−196 C). Sections measuring 6 μm were cut in a cryostat at −12 C and stored at −70 C. Sections of cases 1, 2, and 5 (Table 1) were first fixed in cold acetone for 10 minutes at −12 C before being stored.

Immunohistochemical Detection of Prostacyclin Synthase, 6-Keto-$PGF_{1\alpha}$ and PGE_2

For detection of PGI_2 synthase, sections were fixed in cold acetone (−20 C) for 10 minutes, air-dried for 1 hour at 20 C, and incubated overnight with monoclonal anti-PGI_2 synthase (isn-1).[16] For detection of PGE_2 and 6-keto-$PGF_{1\alpha}$, sections were air-dried for 1 hour at 20 C and incubated overnight with rabbit anti-PGE_2 (1:10 to 1:20, Sigma P3038, Sigma Chemical Co., St. Louis, MO) or rabbit anti-6-keto-$PGF_{1\alpha}$ (1:20 to 1:80, Sigma P1791). The freeze-dried anti-6k-$PGF_{1\alpha}$ and anti-PGE_2 were reconstituted in 500 μl aqua destillate and stored at −70 C until use. Anti-

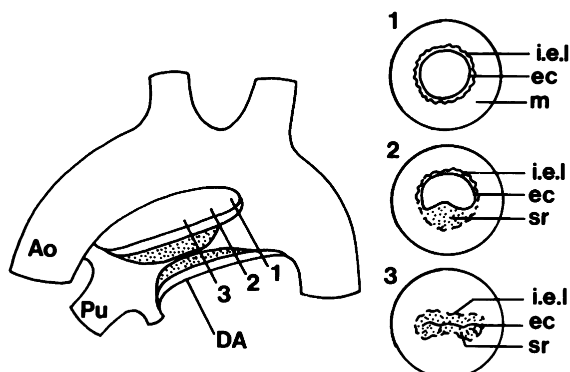

Figure 1. *Schematic structure of the ductus arteriosus of a 1-day-old beagle. 1) The aortic end still lacks intimal thickening. 2) Intimal thickening starts at the "bottom" of the DA; later stages of this process can be observed toward the pulmonary end. 3) Closure is observed from approximately the middle of the DA to the pulmonary end. ec, endothelial cells; i.e.l, internal elastic lamina; sr, subendothelial region; m, media.*

6k-$PGF_{1\alpha}$ and anti-PGE_2 were diluted in PBS, pH 7.2, containing 0.1% BSA (Sigma) and 0.05% Tween 20 (Sigma). Anti-PGI_2 synthase was used undiluted (cell culture medium).[16] Bound antibodies were detected using either TRITC-conjugated rabbit anti-mouse antibodies (DAKO, Glostrup, Denmark), FITC-conjugated goat anti-mouse antibodies (Nordic, Tilburg, the Netherlands), or FITC-conjugated swine anti-rabbit antibodies (DAKO). To increase the sensibility of detection of PGE_2 the double PAP technique was also used. In this case bound anti-PGE_2 antibodies were detected using incubation with swine anti-rabbit antibodies (DAKO) for 60 minutes (20 C) followed by incubation with PAP/R (DAKO) for 60 minutes (20 C). This procedure was repeated once and sections were stained for 7 minutes at room temperature using diaminobenzidine (0.4 mg/ml, Sigma) dissolved in 0.1 M Tris-maleate buffer (pH 7.6) containing 0.02% H_2O_2. Sections were counterstained with hematoxylin, dehydrated in graded ethanol, and mounted in Entellan (Merck, Darmstadt, FRD).

Controls

Frozen sections of human placenta measuring 6 μm were used as positive controls.[17] For negative controls, sections were incubated overnight with either PBS or the cell culture medium used for PGI_2 synthase production (see above).

Histochemical Detection of 15-Hydroxy-Prostaglandin Dehydrogenase (PGDH)

Activity of PGDH was demonstrated using a modification of the technique described by Nissen et al.[18] Air-dried or acetone-fixed frozen sections (6 μm) were incubated for 120 minutes at 37 C in a medium (50 μl/section) containing 1 mM NAD (Sigma), 5 mM Nitro-Blue Tetrazolium salt (Sigma), 4 mM Sodiumazide (Sigma), and 2.8 mM (1 mg/ml) PGE_2 (Upjohn, Kalamazoo, MI) dissolved in 0.033 M phosphatebuffer with and without 17% "hot water soluble" polyvinylalcohol (PVA, Sigma). After incubation sections were rinsed in warm tap water, air dried, and mounted in glycerine-jelly.

Controls

Frozen sections (6 μm) of human placenta known to be rich in PGDH were used as control. Negative controls were obtained by omitting PGE_2 as a substrate.

Results

Prostacyclin Synthase

Beagle DA

Six DA were studied with special attention to the different stages of intimal thickening that can be observed in a single specimen. The process of intimal thickening always starts at the pulmonary end and proceeds towards the junction with the aorta. A series of consecutive sections therefore show the process of development of intimal thickening when the DA is followed in the opposite direction. Intimal thickening was not observed at the aortic end of the DA. No difference in morphology between the DA and aorta could be observed in this region. More distal from the aortic end the process started at the bottom of the DA (Figure 1, level 2) with detachment of the endothelial cells. More advanced stages of this process were observed toward the pulmonary end. All DA appeared to be closed from approximately the middle of the DA to the pulmonary end (Figure 1).

Distribution of PGI_2 synthase was similar in the aorta (Figure 2a) and the aortic end of the DA (Figure 2b). PGI_2 synthase was present in both endothelial and smooth muscle cells but most of the fluorescence was observed in the endothelial cells. In all DA studied, this pattern changed at points where intimal thickening had developed. Fluorescence of smooth muscle cells was clearly higher at sides showing detachment of endothelial cells compared with sides where detachment had not yet occurred (Figure 2b,c). The increase in PGI_2 synthase concentrations in smooth muscle cells followed the course of intimal thickening and spread over the entire ductal wall toward the pulmonary end. Sections at the level of the bifurcation of DA and pulmonary artery clearly demonstrated the higher level of PGI_2 synthase in ductal tissue (Figure 3a).

Poodle DA

Both DA studied showed the morphology of a mixed type DA. They were still open at the ages of five and fifteen days. Both DA were characterized by the presence of a side with normal DA structure showing intimal thickening towards the pulmonary end and a side lacking intimal thickening. The morphology of the abnormal side strongly resembled the elastic structure of the aortic wall. The distribution of PGI_2 synthase in the abnormal side was similar to that in the aorta; most fluorescence for PGI_2 synthase was noted in the endothelial cells (Figure 2d). Increased fluorescence in smooth muscle cells, however, was observed at the side showing normal DA morphology. At this side the intensity of fluorescence in smooth muscle cells

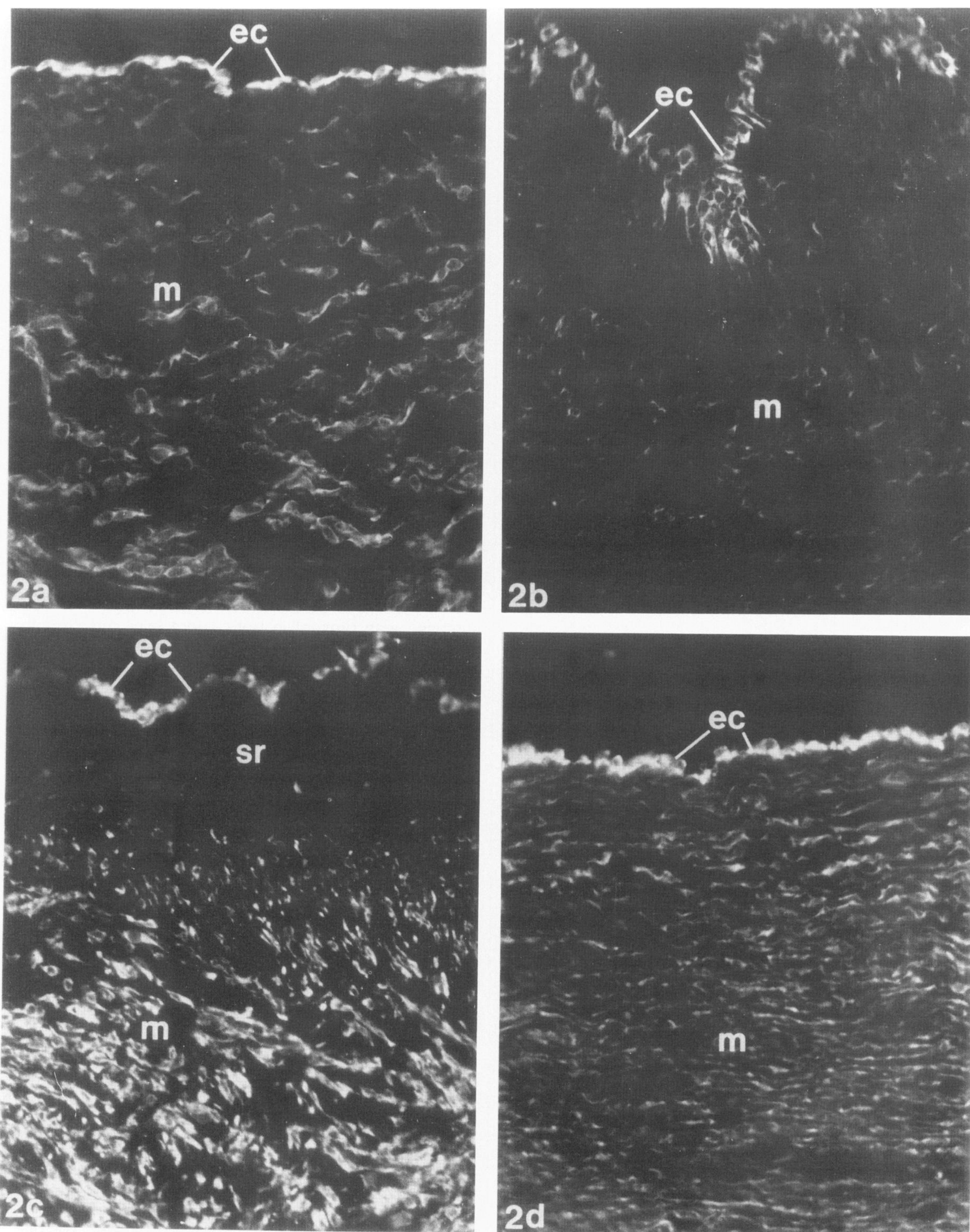

Figure 2. *Distribution of PGI_2 synthase in the aorta, DA, and PDA before and after detachment of the endothelial cells.* **a**: *Aorta. Endothelial cells (ec) stain strongly for the presence of PGI_2 synthase. Less fluorescence is present in the media (m) (original magnification ×272).* **b**: *Undetached endothelial cells (ec) in the DA at the aortic end. EC stain strongly for the presence of PGI_2 synthase. Less fluorescence is present in the media (m) (original magnification ×272).* **c**: *Detached endothelial cells (ec) in the DA. Strong fluorescence for PGI_2 synthase is present in both the EC and the smooth muscle cells. m, media; sr, sub-endothelial region (original magnification ×272).* **d**: *Abnormal side in a PDA. Endothelial cells (ec) stain strongly for PGI_2 synthase. Less fluorescence is observed in the media (m) (×272).*

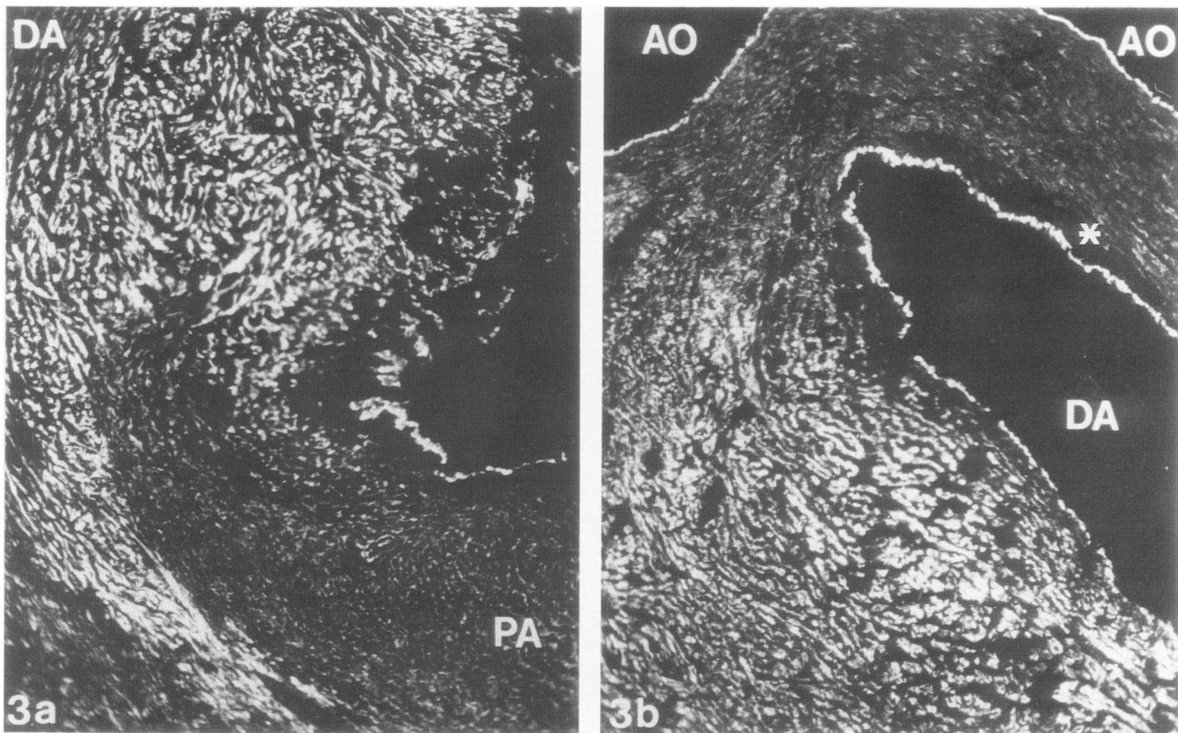

Figure 3. *Transverse section showing distribution of PGI_2 synthase.* **a**: *Normal DA. Ductal tissue (DA) stains more strongly for PGI_2 synthase than the pulmonary artery (PA) (original magnification ×150).* **b**: *Mixed type DA. The normal side stains more strongly for PGI_2 synthase than the side lacking intimal thickening (asterisk). Ao, aorta (original magnification ×88).*

appeared to be as strong as that observed in the endothelial cells.

6-Keto-Prostaglandin $F_{1\alpha}$

The distribution of 6-keto-$PGF_{1\alpha}$ resembled the distribution of PGI_2 synthase in the aorta, DA, and PDA, as well as the pulmonary artery, but the intensity of fluorescence appeared to be much lower. Stronger fluorescence was observed at those regions that also showed higher levels of PGI_2 synthase. In the aorta, the pulmonary artery, and at sides without intimal thickening in the normal DA and the mixed type DA, most fluorescence was noted in the endothelial cells. Parts of the DA and mixed type DA with intimal thickening showed more fluorescence in medial smooth muscle cells than was seen at points without intimal thickening.

Prostaglandin E_2

Presence of prostaglandin E_2 was shown in sections of the placenta, but it could not be demonstrated in the aorta, DA, or pulmonary artery.

15-Hydroxy-Prostaglandin Dehydrogenase

Activity of PGDH was clearly demonstrated in frozen sections of the placenta. Intensity of staining in sections of DA, mixed type DA, aorta, and pulmonary artery did not exceed the level of background staining in the control sections. Addition of PVA in the incubation media, to prevent diffusion of PGDH out of the section, did not alter these findings.

Discussion

This study demonstrated a clear relationship between the presence of PGI_2 synthase and the structure of the DA. Previous work[10] showed that the development of intimal thickening in the DA is a prerequisite for its closure after birth. The present study showed that formation of intimal cushions is accompanied by an increase in PGI_2 synthase concentrations in medial smooth muscle cells. This increase is not observed at the sides that lack intimal thickening in the mixed type DA. Levels of PGI_2 synthesis were not measured quantitatively in this study. Fluorescence for 6-keto $PGF_{1\alpha}$ was rather weak; nevertheless, slightly more fluorescence was noted at those sides that also showed increased levels of PGI_2 synthase, indicating that

at least some of the PGI_2 synthase is functionally active, a finding that is consistent with data in the literature showing that the DA primarily produces PGI_2.[3–5] The low intensity of staining for 6-keto-$PGF_{1\alpha}$, despite the presence of high levels of PGI_2 synthase, is not well understood, but several mechanisms may be involved. It may be that the detection method used is not sensitive enough or loss of 6-keto-$PGF_{1\alpha}$ during incubation may be involved. Moreover, it is possible that activation of the cytochrome-P450 mono-oxygenase system[8] decreases the availability of arachidonic acid for PGE_2 and PGI_2 synthesis, thereby influencing the level of 6-keto-$PGF_{1\alpha}$.

That PGE_2 synthesis is low or absent in the DA[1–5] explains why we were unable to demonstrate the presence of PGE_2 immunohistochemically. The absence of PGDH activity suggests that increased degradation of PGE_2 in the DA after birth does not play a role in the process of constriction. Activity of PGDH in the DA, aorta, and pulmonary artery was demonstrated biochemically, but evidently its activity in the DA, aorta, and pulmonary artery is at too low a level to be demonstrated histochemically. The results indicated a more crucial role for PGI_2 in maturation of the DA than has been appreciated before.

The DA is known as a vessel with a high sensitivity for PGE_2 compared with other blood vessels.[1] Various studies showed that ductal patency is primarily regulated by PGE_2.[6,7,19] Like PGE_2, PGI_2 is believed to be involved in regulating the patency of the DA before birth.[1,2,6,7] Considering the increase in PGI_2 synthase, a synergistic function of PGI_2 and PGE_2 could be hypothesized. This hypothesis if true would imply that the sensitivity of the DA to PGE_2[1] is enhanced by the presence of high levels of PGI_2. If this is the case, it would mean that PGI_2 has a more important role in regulating ductal patency and does not simply have a relaxing effect in addition to PGE_2.

That the process of intimal thickening in the DA is accompanied by an increase in PGI_2 synthase suggests that PGI_2 or one of its metabolites is involved in this process. Perhaps the increase in PGI_2 production is somehow linked to a stimulation of glycosaminoglycan synthesis, which is an important event in the process of intimal thickening, and thus to smooth muscle cell migration in the DA.[20] Involvement of prostaglandins in stimulation of glycosaminoglycan synthesis has been suggested as occurring in other cell types.[21] A stimulatory role of PGI_2 in intimal cushion formation, however, would appear to contradict data in the literature showing a protective role of PGI_2[22] on structural vascular changes.

It is tempting to attribute a role to PGI_2 in the regulation of intimal cushion formation. Once smooth muscle cell activity has been enhanced by the presence of a glycosaminoglycan-rich environment, an increase in PGI_2 may produce a concurrent inhibition of smooth muscle cell growth. This could explain the increase in PGI_2 at the time of intimal cushion formation. This study provides no clues as to what factor may be responsible for triggering the smooth muscle cells to increase their level of PGI_2 synthase.

According to the response-to-injury hypothesis[23] intimal thickening is initiated by activation of the endothelial cells. It would thus be interesting to know whether the endothelial cells synthesize a factor that, directly or indirectly, enhances the level of PGI_2 synthase in ductal smooth muscle cells.

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