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Expression of prostanoid receptors in human ductus arteriosus

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1 Prostaglandins play a major role in maintaining ductal patency *in utero*. Ductal tone is regulated by both locally released and circulating vasodilatory prostaglandins. In infants with ductus arteriosus-dependent congenital heart disease, ductal patency is maintained by intravenous administration of prostaglandin (PG) E_1. Little information is available regarding the expression of prostaglandin receptors in man.

2 By means of RT–PCR and immunohistochemistry we studied the expression of the PGI_2 receptor (IP), the four different PGE_2 receptors (EP1, EP2, EP3 and EP4), and the receptors for thromboxane (Tx) A_2 (TP), PGD_2 (DP) and $PGF_{2\alpha}$ (FP) in the ductus arteriosus of three newborn infants with ductus arteriosus-dependent congenital heart disease and intravenous infusion of PGE_1 and of one 8 month old child with a patent ductus arteriosus.

3 The EP3, EP4, FP, IP and TP receptor were markedly expressed at the mRNA and protein level, whereas the EP2 receptor was weakly expressed and the EP1 receptor was detected in two out of four tissue specimens only. The DP receptor was not detected in any of the samples. The most pronounced expression, which was located in the media of the ductus arteriosus, was observed for the EP4 and TP receptors followed by IP and FP receptor protein.

4 These data indicate that ductal patency during the infusion of PGE_1 in infants with ductus arteriosus-dependent congenital heart disease might be mediated by the EP4 and IP receptor. The data further suggest that a heterogeneous population of prostanoid receptors may contribute to the regulation of ductus arteriosus tone in humans.

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Abbreviations: DA, ductus arteriosus; PG, prostaglandin; Tx, thromboxane

Introduction

The ductus arteriosus (DA) is a foetal shunt blood vessel that extends between the pulmonary artery and the aorta. This vessel plays a crucial role in normal and abnormal cardiovascular physiology of the foetus and newborn (Smith, 1998). *In utero*, patency of the DA allows blood to flow from right ventricle to aorta. The DA constricts within 24–48 h after birth and later undergoes permanent closure through structural remodelling (Clyman *et al*., 2002; Drayton & Skidmore, 1987; Gittenberger-de Groot *et al*., 1985). Failure of postnatal DA closure may result in pulmonary oedema and severe respiratory distress in preterm infants. The survival of infants with certain cardiac malformations with ductus arteriosus-dependent pulmonary or systemic circulation (e.g. pulmonary atresia, hypoplastic left heart) depends on the patency of the DA which is maintained by the intravenous infusion of prostaglandin (PG) E_1 (Freed *et al*., 1981). Patency of the vessel in the foetus is an active process, which comprises the inhibition of procontractile mechanisms by vasodilators. Several dilator systems, including prostaglandins, carbon monoxide and nitric oxide, are involved in maintaining ductal patency in the foetus (Smith, 1998). Prostaglandins, however, play the most important role (Coceani & Olley, 1973; Smith, 1998). The vasodilatory prostaglandins prostaglandin (PG) E_2 and PGI_2 relax the DA, but PGI_2 is less active than PGE_2 (Clyman *et al*., 1978; Coceani *et al*., 1978) and PGE_2 is considered to be the most important endogenous prostaglandin involved in the regulation of DA patency. PGI_2 has also a role in pre- and postnatal ductal dilation (Seyberth *et al*., 1984; Smith *et al*., 1994). Whether the potentially constrictory-acting prostanoids thromboxane (TX) A_2 and $PGF_{2\alpha}$ also contribute to vessel tone is still uncertain (Clyman *et al*., 1978; Coceani *et al*., 1978; Smith & McGrath, 1995; Starling & Elliott, 1974).

Prostanoids act on their target cells *via* distinct G protein coupled receptors (Narumiya *et al*., 1999). For each prostanoid a specific receptor gene exists (i.e., IP for PGI_2, DP for PGD_2, FP for $PGF_{2\alpha}$), except for PGE_2, which acts on four different receptors, EP1, EP2, EP3 and EP4. So far, the expression of PGE_2 receptors in DA tissue has been studied in animals (Bhattacharya *et al*., 1999; Bouayad *et al*., 2001a, b; Nguyen *et al*., 1997; Segi *et al*., 1998; Smith *et al*., 2001). Expression of EP2, EP3 and EP4 has been observed in the foetal pig and lamb DA, whereas in the newborn animal DA only EP2 receptor expression was detected (Bhattacharya *et al*., 1999; Bouayad *et al*., 2001a, b). In the present study, we describe the mRNA and protein expression of the E, F, T and I prostanoid receptors in the human ductus arteriosus.

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Methods

Tissue collection and preparation

DA tissue was obtained during cardiovascular surgery from three newborn infants with ductus arteriosus-dependent congenital heart disease and from one 8 month old child with a patent DA. The newborn infants were treated with intravenous PGE_1 in order to maintain ductal patency until cardiac surgery. Only infants who were from 3–28 days old and responded to PGE_1 administration were included in the study. Responsiveness was evaluated by serial colour Doppler echocardiography and was defined as reopening or dilation of a DA that was closed or constricted prior to the administration of PGE_1 and stable ductal diameter and flow during the administration of PGE_1 for at least 3 days. After excision, the DA tissues were immediately snap frozen in liquid nitrogen and stored at −80°C until cryosectioning or RNA isolation. The study was carried out following a protocol approved by the local ethical committee.

Reverse transcriptase (RT)-PCR

Total RNA was isolated from the DA samples using the guanidinium thiocyanate method with acidic phenol (Chomczynski & Sacchi, 1987). After annealing to oligo(dT), 1 μg RNA was reverse transcribed to cDNA by SuperScript II reverse transcriptase (GIBCO). The primer design for PCR amplification was based on the human nucleotide sequences of the prostanoid receptors (Table 1). The reactions were cycled 40 times in a cycle profile of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C after a 5 min denaturating step at 95°C. Amplification products were analysed by 2% agarose gel electrophoresis and ethidium bromide staining. Size determination and dideoxy sequencing verified the identity of the amplified fragments. PCR amplification of RT reactions without reverse transcriptase revealed no PCR product, thereby excluding amplification of genomic DNA. Samples were assayed at various dilutions to ensure proportionality in the yield of PCR products. A fragment of β-actin was amplified as internal control. Abundance of receptor mRNA was expressed relative to β-actin mRNA.

Immunohistochemistry

Slices (5 μM) of human DA were obtained by cryosectioning, thereafter air dried and stored at −80°C. Immunohistochemical staining was performed according to the method described by us (Morath *et al*., 1999). Slides were kept at room temperature for 20 min, incubated in acetone at 4°C for 10 min, air dried at room temperature and incubated with 10% goat serum/phosphate buffered saline (PBS) for 30 min. Rabbit polyclonal antibodies directed against EP1, EP2, EP3, EP4, FP and IP receptor have been recently described by us (Kömhoff *et al*., 1998; Morath *et al*., 1999; Schlotzer-Schrehardt *et al*., 2002). A rabbit polyclonal antibody against human thromboxane receptor was gained by immunization with peptide ^{270}PPAMSPAGQLSRTTE^{284}, located at the C-terminus, coupled on a lysine matrix (using the multiple antigene peptide-system according to (Tam, 1988)). Incubation of DA sections with the preimmune sera gave no staining signals (data not shown). Immunohistochemistry was not performed for the DP receptor, due to the lack of a specific antibody. Overnight incubation with anti-prostanoid receptor antibodies (the following dilutions in PBS/10% goat serum were used: anti-EP1 1:250, anti-EP2 1:40, anti-EP3 1:40, anti-EP4 1:250, anti-FP 1:100, and anti-TP 1:100) was followed by 30 min incubation at 37°C with monoclonal mouse anti-rabbit antibody (1:70), polyclonal rabbit anti-mouse antibody (1:70), and alkaline phosphatase anti-alkaline phosphatase complex (1:70, Dako, Copenhagen, Denmark). Following further PBS washes the colour reaction with naphthol-AS-BI-phosphate/New Fuchsin was performed by the addition of substrate solution according to the protocol of the supplier (Dako, Copenhagen, Denmark). Washing stopped the colour reaction and the slides were counterstained with Mayer's hemalaun solution for 1 min and finally mounted in Mowiol. Staining was independently scored by two blinded observers according to no staining, −; weak staining, +; moderate staining, ++; strong staining, +++. Sometimes only a scattered pattern was observed which was indicated by (+).

Results

RT–PCR studies revealed the expression of all prostanoid receptors except DP, albeit with different expression levels (Figure 1 and 2). Strong mRNA expression was observed for the prostanoid receptors IP and EP4, known to mediate vasodilation. Based on the relative expression to β-actin mRNA, the potentially constrictory-acting prostanoid receptors TP and FP were expressed to a similar extent. Further, mRNA expression of the EP3 receptor was detected, whereas only weak amplification signals of the EP1 receptor and the EP2 receptor were observed. mRNA expression of the DP

Table 1 Primer design for PCR amplification of prostanoid receptors and β-actin

Product	*Accession number*	*Forward*	*Reverse*	*Size (bp)*
EP1	L22647	aggcactgcttgctggcctctt	tggcccaccatctccac	295
EP2	L25124	tgctggactatgggcagtacgtcc	aaggtgatggtcatgatagccagg	304
EP3	X83857	actggtatgcgagccacatga	cataagctgaatggccgtctc	368
EP4	L28175	tccgcatgcaccgccagttcat	tcggatggcctgcaaatctgg	317
FP	L24470	tggaaatggtaatccagctcctgg	catgcactccacagcattgactgg	246
IP	D25418	ttcgtgcaggaacctcacctacg	tccagctgcgcgtagaggta	384
TP	D38081	tgggagccccgcagatgaggtctct	accacgatggtaccggtcaccagca	369
DP	U31332	gctttatccagatggtccacgagg	ccttaaatgctccatagtaagcgc	327
β-actin	X00351	actcaccattggcaatgagcg	ctagaagcatttgcggtggac	400

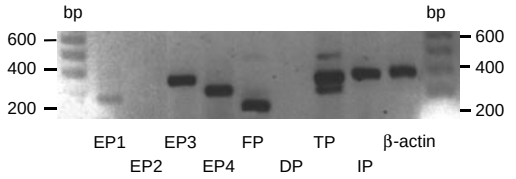

Figure 1 Expression of prostanoid receptor mRNA in human DA. RNA was isolated from excised tissue, reverse-transcribed by oligo(dT) and PCR amplification for all tissue samples was repeated three times. A fragment of β-actin was amplified as internal control. A representative expression pattern of one tissue sample is shown, base pair markers (bp) are indicated on both sides.

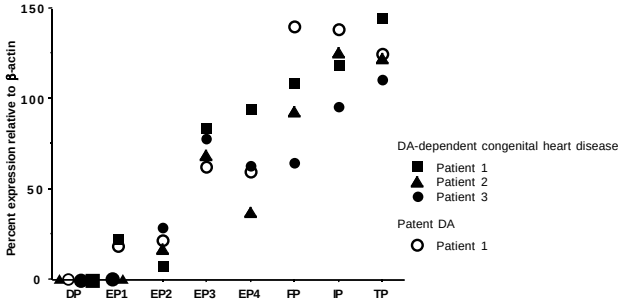

Figure 2 Expression of the different prostanoid receptors relative to the expression of β-actin. RNA was isolated from excised tissue, reverse-transcribed by oligo(dT) and PCR amplification was performed for the indicated prostanoid receptors. Intensity of DNA bands is presented as percentage of amplification of β-actin fragment.

Table 2 Localization of prostanoid receptor proteins in human ductus arteriosus

Receptor type	*Smooth muscle cells*	*Endothelial cells*	*Stromal cells*
EP1	+	–	–
EP2	+	–	(+)
EP3	+	+++	(+)
EP4	+++	(+)	(+)
IP	++	(+)	–
TP	+++	–	–
FP	++	–	(+)

No staining, –; weak staining, +; moderate staining, ++; strong staining, +++; scattered staining (+).

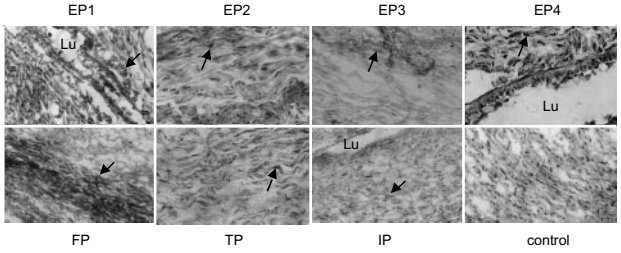

Figure 3 Immunohistochemical localization of prostanoid receptors in human DA. Cryosections were prepared and incubated with anti prostanoid receptor antibodies. Antibody binding was visualized by APAAP technique (for all except EP4 receptor) or ABC technique (for EP4 receptor). The target receptors of the used antibodies are indicated below each figure. Control represents staining without primary antibody. A representative experiment staining is shown, exemplarily stained cells are indicated by arrows. Light microscope, $\times 200$. Lu, lumen of ductus arteriosus.

receptor could not be detected in any of the samples. Using specific antibodies, we observed a similar expression of the different prostanoid receptors (Table 2). The strongest signal in smooth muscle cells of the DA was observed using anti-FP and anti-TP receptor antibodies (Figure 3). Using the different EP receptor antibodies, we observed a strong signal with the anti-EP4 receptor antibody in smooth muscle cells of DA (Table 2). Antibody reactivity towards EP3 receptor was mainly observed in endothelial cells and scattered in stromal cells. Occasionally weak staining of EP3 receptor was detected in smooth muscle cells. Weak positive immunolabelling was observed for EP1 and EP2 receptor. The pattern of prostanoid receptor expression was not obviously different between newborn infant and child DA (Figure 2).

Discussion

We found a consistently strong expression of the EP4 and the IP receptor in newborn infant and child DA. The EP4 receptor is probably the most important prostanoid receptor that regulates ductal tone in the perinatal period. Binding analysis with specific analogues in foetal rabbits indicates that EP4 mediates the PGE_2-induced dilation of the DA (Smith *et al*., 1994), which is crucial with respect to the regulation of ductal tone. *In situ* hybridization analysis demonstrated a strong expression of EP4 mRNA in both foetal and neonatal mouse DA (Nguyen *et al*., 1997; Segi *et al*., 1998). In EP4 deficient mice, the DA is patent and failed to close after birth and in these genetically modifed mice administration of indomethacin failed to close the DA (Nguyen *et al*., 1997; Segi *et al*., 1998). It is suggested that a compensatory mechanism is active in the absence of EP4 receptor which maintains DA patency also after birth. Noteworthy, in contrast to COX-1-deficient mice closure of the DA and survival of COX-2-deficient mice were compromised (Loftin *et al*., 2001). The observation that mice lacking a functional gene for 15-hydroxy-prostaglandin dehydrogenase die with a patent DA after birth indicates that sensing the postnatal drop in PGE_2 formation is an important step towards closure of DA (Coggins *et al*., 2002). The function of the IP receptor with respect to the regulation of ductal tone is less clear. In IP deficient mice, patency of the DA after birth does not occur (Murata *et al*., 1997). Studies with selective IP agonists, however, indicate that the IP receptor is present in the DA of foetal rabbits and may have a role in the dilation of the DA (Smith *et al*., 1994). Although PGI_2 is less active than PGE_2 it induces dilation of the DA (Clyman *et al*., 1978; Coceani *et al*., 1978). In the present investigation, we did not perform functional studies *ex vivo*. However, in the infants studied, ductal patency was maintained by intravenous administration of PGE_1, which has the same affinity as PGE_2 for the EP4 receptor and approximately one third of the affinity of PGI_2 for the IP receptor (Narumiya *et al*., 1999). Thus our data indicate that the EP4 and the IP receptor are present in human DA and probably either of them or both receptors are functionally active and contribute to the dilator effect of PGE_1 administration in infants.

Binding studies, RT–PCR assays and immunoblot studies demonstrated the expression of the EP3 receptor in foetal rabbit, lamb and porcine DA (Bhattacharya *et al*., 1999; Bouayad *et al*., 2001b; Smith & McGrath, 1995; 2001). Functional studies with EP3 receptor agonists and antagonists point to a contractile effect of PGE_2 that is mediated by the EP3 receptor in foetal rabbit DA (Smith & McGrath, 1995). It has been suggested that this contractile effect is of particular importance after birth because increasing oxygen tension potentiates the response of the DA to vasoconstrictors (Smith, 1998). In contrast, in foetal lamb DA EP3 receptor stimulation caused DA relaxation which was dependent on the stimulation of the ATP-sensitive K^+ channel and was not modified by removal of luminal endothelium (Bouayad *et al*., 2001b), indicating that the EP3 receptor is localized on the smooth muscle cells. In the present investigation, distinct EP3 receptor protein expression was detected on endothelial cells suggesting a different indirect mechanism, which couples the EP3 receptor to smooth muscle cells. Whether the effect of EP3 receptor stimulation is contraction or relaxation remains uncertain. If EP3-dependent stimulation of contractile mechanisms occurs, these are probably of minor importance because the infusion of PGE_1, which has a high affinity to the EP3 receptor (Narumiya *et al*., 1999), resulted in DA patency in the infants studied.

Studies on the presence and function of the potentially contractile TP receptor in animal DA have been rarely performed. In foetal lambs, TxA_2 is not active on the DA (Coceani *et al*., 1978) and is not formed by DA tissue (Pace-Asciak & Rangaraj, 1978). More recent investigations with selective agonists and antagonists, however, demonstrated the presence of functionally active TP receptors on foetal rabbit DA (Smith & McGrath, 1995). $PGF_{2\alpha}$ has a weak contractile effect on bovine DA but is inactive on lamb DA (Clyman *et al*., 1978). To our knowledge, the FP receptor has not been studied so far. Despite the strong expression of the TP and FP receptor, we assume that neither TxA_2 nor $PGF_{2\alpha}$ contribute substantially to DA contraction, because non-selective inhibition of prostanoid formation by indomethacin results in closure of the DA (Smith, 1998). However, other ligands that are produced independently from the cyclooxygenase could trigger the receptor. 8-epi-$PGF_{2\alpha}$, a TP receptor ligand with strong vasoconstrictive properties is formed by radical-triggered mechanisms and may accumulate under high oxygen tension (Roberts & Morrow, 1997). In foetal rats paraquat, a strong radical-producing agent which is known to increase 8-epi-$PGF_{2\alpha}$, causes constriction of the DA (Shirai *et al*., 1995). In neonates, a major factor that stimulates contraction of the DA, is increasing oxygen tension (Smith, 1998). Thus, increasing formation of 8-epi-$PGF_{2\alpha}$ with increasing oxygen tension after birth might contribute to active DA contraction by binding to the TP receptor. The hypothesis, that prostanoids may play a role in active constriction of DA is supported by data from Loftin *et al*. (2001) demonstrating that mice lacking both COX isoforms die within minutes after birth. Normally COX-2 is expressed in smooth muscle cells of the DA. Therefore it appears plausible that a COX-dependent product effects early processes of DA closure.

In our study, the DP receptor was absent and only a very weak expression of the EP1 receptor was observed. In line with our results is the observation that the rabbit DA lacks DP and EP2 receptors (Smith *et al*., 1994). PGD_2 is not formed by lamb DA tissue (Pace-Asciak *et al*., 1978).

The developmental regulation of EP receptor expression has been studied in the foetal and neonatal lamb and pig DA in animals without cardiac malformation (Bhattacharya *et al*., 1999; Bouayad *et al*., 2001a, b; Smith *et al*., 2001). Binding studies and RT–PCR assays identified the EP2, EP3 and EP4 receptor in foetal DA, whereas in neonatal DA only the EP2 receptor was detected (Bhattacharya *et al*., 1999; Bouayad *et al*., 2001a, b). The present investigation studies the prostanoid receptor expression in DA tissue of newborn infants with ductus arteriosus-dependent cardiac malformation. The results of strong mRNA and protein expression of EP3 and EP4 but only weak expression of EP2 differ from the results obtained in healthy animals. The reason for this difference is unclear, but several possibilities can be considered. The developmental expression of EP receptors may be species specific. More likely, however, is a modification of the receptor population and density either by the underlying cardiac malformation and altered haemodynamics or by the administration of PGE_1. Because the receptor expression was not obviously different between newborn infant and child DA we assume that administration of PGE_1 in the infants did not substantially affect the receptor expression. This assumption, however, is limited by the small number of patients studied. Based on studies in newborn animals without cardiac malformation, it has been proposed that a selective EP2 agonist may be more appropriate than the nonselective PGE_1 for the treatment of infants with ductus arteriosus-dependent congenital heart disease (Bhattacharya *et al*., 1999; Bouayad *et al*., 2001b). Our results in newborn infants with cardiac malformation, however, do not support this suggestion. Instead, selective agonists of the EP4 or IP receptor may be better candidates.

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