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ORIGINAL PAPER

Developmental changes in the effects of prostaglandin E_2 in the chicken ductus arteriosus

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Abstract Prostaglandin E_2 (PGE_2) is the major vasodilator prostanoid of the mammalian ductus arteriosus (DA). In the present study we analyzed the response of isolated DA rings from 15-, 19- and 21-day-old chicken embryos to PGE_2 and other vascular smooth muscle relaxing agents acting through the cyclic AMP signaling pathway. PGE_2 exhibited a relaxant response in the 15-day DA, but not in the 19- and 21-day DA. Moreover, high concentrations of PGE_2 ($\geq$3 μM in 15-day and $\geq$1 μM in 19-day and 21-day DA) induced contraction of the chicken DA. The presence of the TP receptor antagonist SQ29,548, unmasked a relaxant effect of PGE_2 in the 19- and 21-day DA and increased the relaxation induced by PGE_2 in the 15-day DA. The presence of the EP receptor antagonist AH6809 abolished PGE_2-mediated relaxation. The relaxant responses induced by PGE_2 and the β-adrenoceptor agonist isoproterenol, but not those elicited by the adenylate cyclase activator forskolin or the phosphodiesterase 3 inhibitor milrinone, decreased with maturation. High oxygen concentrations (95%) decreased the relaxation to PGE_2. The relaxing potency and efficacy of isoproterenol and milrinone were higher in the pulmonary than in the aortic side of the DA, whereas no regional differences were found in the response to PGE_2. We conclude that, in contrast to the mammalian situation, PGE_2 is a weak relaxant agent of the chicken DA and, with advancing incubation, it even stimulates TP vasoconstrictive receptors.

Keywords Ductus arteriosus · Chicken embryo · Prostaglandins · Adenylate cyclase · cAMP

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Introduction

The ductus arteriosus (DA), a fetal arterial connection between the pulmonary artery and the descending aorta, is indispensable for fetal life. Considerable evidence supports a major role for the cyclooxygenase (COX) product prostaglandin (PG) E_2 in maintaining mammalian fetal patency and for the rapid decline in PGE_2 levels as trigger of DA closure after birth (see Clyman 2006; Smith 1998 for reviews).

In blood vessels, PGE_2 acts on four different prostanoid receptors: EP_1, EP_2, EP_3 and EP_4. Both EP_2- and EP_4-receptor subtypes mediate PGE_2 relaxation through a cyclic AMP-dependent mechanism (Narumiya and FitzGerald 2001). EP_1 is a constrictive receptor and EP_3 induces a decline in cAMP, inhibiting smooth muscle relaxation (Narumiya and FitzGerald 2001). The main receptor responsible for PGE_2-induced DA relaxation varies among mammalian species and was reported as EP_4 in rabbits (Smith 1998; Smith et al. 1994; Smith and McGrath 1995), mice (Nguyen et al. 1997), rats (Kajino et al. 2004), lambs (Waleh et al. 2004), baboon (Waleh et al. 2004), and humans (Leonhardt et al. 2003), and as EP_2 in pigs (Bhattacharya et al. 1999) and lambs (Bouayad et al. 2001). The cAMP-inhibiting EP_3 receptor is also present in the rabbit and piglet DA, modulating the dilating effect of PGE_2

(Bhattacharya et al. 1999; Smith 1998; Smith et al. 1994; Smith and McGrath 1995). In contrast, stimulation of EP_3 receptor in the lamb DA produces not contraction but relaxation via a cAMP-independent pathway (Bouayad et al. 2001).

Significant progress in our understanding of the ductal effects of PGE_2, as well as other aspects of DA physiology and pathophysiology, has been achieved with the use of mammalian models (Sutendra and Michelakis 2007). However, these models are technically complex and experimental manipulations affect both the mother and the fetus. Therefore, there is a need for additional models, addressing these limitations (Sutendra and Michelakis 2007). Recently, we analyzed the ontogeny of the chicken DA response to O_2 and other vasoconstrictors (Agren et al. 2007), as well as the relaxation induced by agonists acting through the nitric oxide (NO)-soluble guanylate cyclase (sGC)-cyclic GMP (cGMP) pathway (Agren et al. 2008). We found that the effects of vasoactive mediators on chicken DA tone are developmentally regulated with loss of responsiveness to vasodilators and increase of responsiveness to vasoconstrictors with advancing incubation age.

Most of the physiological functions that prostanoids exhibit in mammals seem to be present in avian and other non-mammalian vertebrates (Rowley 1991). Prostanoids are produced in chicken blood vessels and participate in the regulation of vascular tone (Chand and Eyre 1976; Claeys et al. 1981; Wideman et al. 1999). However, we have observed that in the chicken DA COX inhibitors did not produce significant contractile effects (Agren et al. 2007) and did not affect endothelium-dependent relaxation (Agren et al. 2008), suggesting that locally produced PGs are not important mediators in the control of chicken ductal tone. Accordingly, Dzialowski and Greyner (2008) demonstrated that COX inhibition did not produce any change in the basal tension of DA of another avian species, the emu. However, they also observed that the emu DA was relaxed by exogenous PGE_2 (Dzialowski and Greyner 2008). This indicates that PGE_2, the most potent vasodilator in the regulation of mammalian DA tone, is also active in the avian DA. In the present study we hypothesized that the chicken DA is responsive to PGE_2 and that its responsiveness is developmentally regulated. Therefore, we analyzed, at different ages, the ex vivo response of chicken DA to PGE_2 and other vascular smooth muscle relaxing agents acting through the cAMP signaling pathway.

Material and methods

Embryos incubation and vessel isolation

Experiments were performed in accordance with Dutch law for animal experimentation. Fertilized eggs of White Leghorn chickens were incubated at 37.8°C, 45% humidity and rotated once per hour (Incubator model 25HS, Masalles Comercial, Spain). Embryos incubated for 15, 19 and 21 days of the 21-day incubation period were studied. The majority of the experiments were performed in 19-day (non-internally pipped) embryos, while for the study of developmental changes all three ages were compared (see below). On the experimental day the embryos were killed by decapitation and the right DA was carefully dissected free and severed distal to the takeoff of the right pulmonary artery and proximal to the insertion into the dorsal aorta. The average in situ length (in mm) of the right DA was 3.48 (SD 0.27), 4.71 (SD 0.40), and 5.58 (SD 0.40) at embryonic day 15, 19, and 21, respectively, (Agren et al. 2007), whereas the maximal length allowed in the myograph was 2 mm. Therefore, a similar length of ductal tissue was removed from the pulmonary and aortic ends of the vessel to obtain a segment of the central part of the DA with a length of approximately 2 mm. The right DA was selected because its shorter length allowed having a higher proportion of the vessel represented in the myograph.

Recording of arterial reactivity

Two stainless steel wires (diameter 40 μm) were inserted into the lumen of the DA, which was mounted as a ring segment between an isometric force transducer and a displacement device in a myograph (Danish Myo Technology A/S model 610 M, Aarhus, Denmark). The myograph organ bath (5 mL vol) was filled with Krebs–Ringer bicarbonate (KRB) buffer maintained at 39°C and continuously aerated with one of the following gas mixtures: 0% O_2/95% N_2/5% CO_2 (pO_2 = 2.6–3.3 kPa), 5% O_2/90%N_2/5% CO_2 (pO_2 = 6.8–7.2 kPa), or 95% O_2/5% CO_2 (pO_2 = 72–76 kPa). The final pH was 7.38–7.42 and pCO_2 was 4.6–5.6 kPa in all solutions. Each DA was stretched to its individual optimal lumen diameter, i.e., the diameter at which it developed the strongest contractile response to 62.5 mM K^+, using a diameter-tension protocol as previously described (Agren et al. 2007; Villamor et al. 2002). During the first phase of stabilization and determination of optimal diameter, DA rings were maintained in KRB buffer with a pO_2 of ~3 kPa. Afterward, oxygen concentration was switched to 5% (pO_2 ~7 kPa), unless the effect of O_2 on the relaxant responses was evaluated. When two or more agonists were studied in the same arterial preparation, the vessels were repeatedly washed and allowed to equilibrate for at least 30 min. If the tone did not recover to resting level or 62.5 mM K^+ failed to induce an adequate level of contraction, the vessels were discarded for further experiments.

The effects of PGE_2 (0.1 nM–10 μM), the direct activator of adenylate cyclase forskolin (0.1–10 μM), the β-adrenoceptor

agonist isoproterenol (1 nM–10 μM) and the phosphodiesterase 3 (PDE3) inhibitor milrinone (0.1 μM–0.1 mM) were evaluated in DA rings contracted with K^+ (62.5 mM), or the thromboxane (Tx)A_2-mimetic (selective TP-receptor agonist) U46619 (0.1 μM). When stable contractions were obtained, agonists were added cumulatively to the bath until a maximal response was achieved. In some experiments, the relaxation evoked by PGE_2 was studied in the presence of the TP-receptor antagonist SQ29,548 (10 μM), or the non-selective EP receptor antagonist AH6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid; 10 μM) and the relaxations evoked by isoproterenol and forskolin were studied in the presence of the non-selective COX inhibitor indomethacin (10 μM), the COX-1 inhibitor valeryl salicylate (0.5 mM), or the COX-2 inhibitor nimesulide (0.1 μM).

In order to analyze developmental changes on relaxation, the responses to PGE_2, forskolin, isoproterenol, and milrinone were studied in the central part of DA from 15-, 19-, and 21-day-old embryos. The 21-day embryos were externally pipped for about 1-3 h. In another set of experiments, aimed to evaluate the modulatory role of O_2 in DA relaxation, the responses to PGE_2, were studied under three different conditions of pO_2: ~3, 7, and 74 kPa. In these experiments, optimal diameter was determined at 3 kPa. Thereafter the O_2 concentration was switched to the appropriate level and the vessels were allowed to equilibrate for at least 30 min before the concentration- response curve for the agonists was obtained. Finally and in order to analyze the differences along the DA in the responses to PGE_2, forskolin, isoproterenol, milrinone and U46619, we performed a third set of experiments in which the pulmonary and the aortic parts of the vessel were compared in 19-day embryos. The responses to U46619 and milrinone were also analyzed in the pulmonary and the aortic parts of the DA from 15-day embryos. The boundary between pulmonary and aortic segments was determined during the dissection based on the marked differences of diameter observed along the chicken DA (see Agren et al. 2007).

Data analysis

Results are shown as mean (SD) of measurements in n embryos. For clarity, results are shown in the figures as mean ± SE. Contractions are expressed in terms of active wall tension (N/m), calculated as the force divided by twice the length of the segment, while the relaxant responses are expressed as the percentage of reduction of the contraction induced by K^+, or U46619. Sensitivity (expressed as $pD_2 = -\log EC_{50}$) and maximal relaxation (E_{max}) to agonists was determined for each artery by fitting individual concentration–response data to a non-linear sigmoidal regression curve and interpolating (Graphpad Prism version 2.01; GraphPad Software Inc, San Diego, CA, USA). When a maximal response was not achieved with a given agonist, the regression curve could not be calculated and the responses for each concentration of the agonist were used for comparison. Differences between mean values were assessed by one-way ANOVA followed by Bonferroni post hoc *t* test. Non-paired *t* tests were used if only two groups were compared. Differences were considered significant at a $P < 0.05$. All analyses were performed using a commercially available statistics package (GraphPad InStat version 3.00; GraphPad Software Inc, San Diego, CA, USA).

Drugs and solutions

PGE_2, U46619, SQ29,548, and AH6809 were obtained from Cayman Chemical (Ann Arbor, MI, USA). Valeryl salicylate, nimesulide, and milrinone were obtained from Alexis Biochemicals (Lausen, Switzerland). All the other drugs were obtained from Sigma Chemical Co (St Louis, MO, USA). PGE_2, U46619, and AH6809 were dissolved in DMSO and SQ29,548 indomethacin, valeryl salicylate, nimesulide and milrinone in ethanol to prepare adequate stock solutions and further dilutions were made in KRB. The final bath concentration of DMSO and ethanol did not exceed 0.1%, which we have found elsewhere to have little or no effect on mechanical activity (Agren et al. 2007). All the other drugs were dissolved initially in distilled deionised water.

Results

Potassium-induced contraction

Isolated DA obtained from 15-, 19- and 21-day chicken embryos responded to depolarizing high-K^+ (62.5 mM) solution with a tonic contraction and this response significantly increased with age (15-day: 0.21 N/m, SD 0.05, $n = 30$, $P < 0.001$ from both 19 and 21-day; 19-day: 0.61 N/m, SD 0.19, $n = 69$; 21-day: 0.99 N/m, SD 0.37, $n = 21$, $P < 0.001$ from 19-day old.). The diameter at which a maximal response to 62.5 mM KCl was obtained increased between day 15 (787 μm, SD 89, $n = 30$) and 19 (830 μm, SD 121, $n = 69$, $P < 0.001$ vs.15-day), but decreased at day 21 (483 μm, SD 227, $n = 21$, $P < 0.001$ vs.15- and 19-day).

Effects of PGE_2 in chicken DA

In the first set of experiments we tested the relaxant effects induced by PGE_2 in chicken DA stimulated with KCl 62.5 mM. We found that PGE_2 exhibited a relaxant response in 15-day DA (Figs. 1a, 2a; Table 1), but no significant relaxation was observed in 19- and 21-day DA

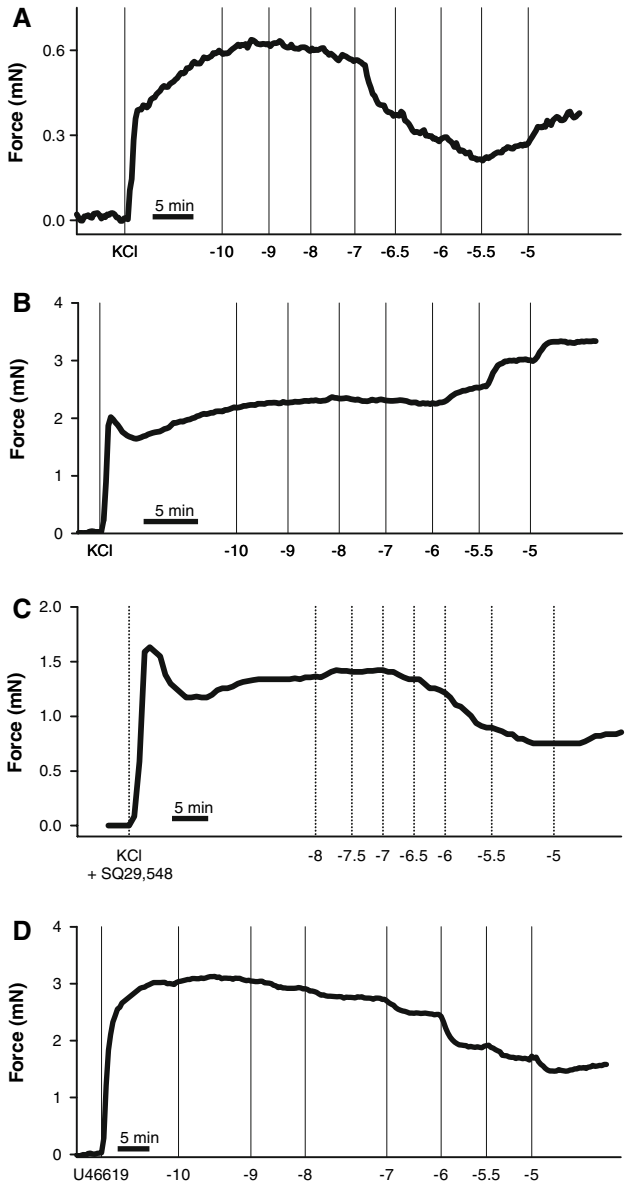

Fig. 1 Typical tracings illustrating the vascular effects of prostaglandin E_2 (PGE_2) in chicken DA. During contraction induced by K^+ (62.5 mM), PGE_2 induced a concentration-dependent relaxation of the 15-day DA (**a**) but a contraction of the 19-day DA (**b**). In the presence of the TP receptor antagonist SQ 29,548 (10 μM, **c**) or when the vessel was contracted with the TP receptor agonist U46619 (0.1 μM, **d**), a relaxant effect of PGE_2 was observed in the 19-day DA

(Figs. 1b, 2a; Table 1). Moreover, high concentrations of PGE_2 (≥3 μM in 15-day and ≥1 μM in 19- and 21-day DA) induced contraction of the chicken DA (Figs. 1a, b, 2a). The presence of the TP receptor antagonist SQ29,548, unmasked a relaxant effect of PGE_2 in the 19- (Figs. 1c, 2b; Table 1) and 21-day DA (Fig. 2b; Table 1) and increased the relaxation induced by PGE_2 in the 15-day DA (Fig. 2b; Table 1). Under these conditions (i.e., presence of SQ29,548), the maximal relaxant response induced by PGE_2 was reduced in term (externally pipped 21-day) versus preterm (non-internally pipped 15-day and 19-day) chicken DA, while the sensitivity to PGE_2 was not affected by age (Table 1). When the 19-day DA was contracted with the TP receptor agonist U46619 (0.1 μM), PGE_2 induced a concentration-dependent relaxation with a threshold concentration of 1 nM (Figs. 1d, 2d) and without achieving the maximal effect with the highest concentration tested (10 μM). The presence of the EP receptor antagonist AH6809 reduced the contraction induced by U46619 from 0.8 N/m (SD 0.11, $n = 6$) to 0.58 N/m (SD 0.12, $n = 6$, $P < 0.01$ vs. control) and abolished PGE_2-mediated relaxation of U46619-contracted DA (Fig. 2d). As shown in Fig. 2c, bubbling the organ chamber with 95% O_2 (pO_2 ~74 kPa) produced a marked reduction of the relaxation evoked by PGE_2 in 19-day DA (contracted with 62.5 mM K^+ and in the presence of SQ29,548).

Relaxant effects of isoproterenol, forskolin, and milrinone in chicken DA

In K^+-stimulated DA, the addition of the β-adrenoceptor agonist isoproterenol, the direct activator of adenylate cyclase forskolin, or the PDE3 inhibitor milrinone caused a concentration-dependent relaxation (Fig. 3a, c, e; Table 1). As expected the β-adrenergic receptor blocker propanolol drastically reduced the relaxation response induced by isoproterenol (Fig. 3a). Similar to PGE_2, the relaxation induced by isoproterenol was markedly diminished in 21-day versus 15- or 19-day chicken DA (Fig. 3a). In contrast, the relaxant effect of forskolin did not change with age (Fig. 3c; Table 1) and milrinone was less potent (but similarly efficacious) in relaxing the 15-day than the 19-, or 21-day chicken DA (Fig. 3e; Table 1). Finally, as shown in Fig. 3b and d, the relaxation evoked by isoproterenol and forskolin were not significantly affected by the presence of the COX inhibitors indomethacin, valeryl salicylate, or nimesulide, compared to control vessels (incubated with vehicle, ethanol 0.1%).

Heterogeneity of relaxant responses along the DA

In the last set of experiments the responses to the drugs studied above were compared in the pulmonary versus the aortic side of the DA. The contractions induced by KCl were not significantly different when the pulmonary and the aortic side of the DA from the 19-day (pulmonary: 0.72 N/m, SD 0.16, $n = 14$; aortic: 0.67 N/m, SD 0.2, $n = 14$) and 15-day embryos (pulmonary: 0.21 N/m, SD 0.03, $n = 6$; aortic: 0.24 N/m, SD 0.05, $n = 6$) were compared. Similarly, the optimal diameters were not significantly different between the pulmonary and the aortic side of the 19-day DA (pulmonary: 889 μm, SD 88, $n = 14$; aortic: 936 μm, SD 87, $n = 14$) and 15-day DA (pulmonary: 742 μm, SD

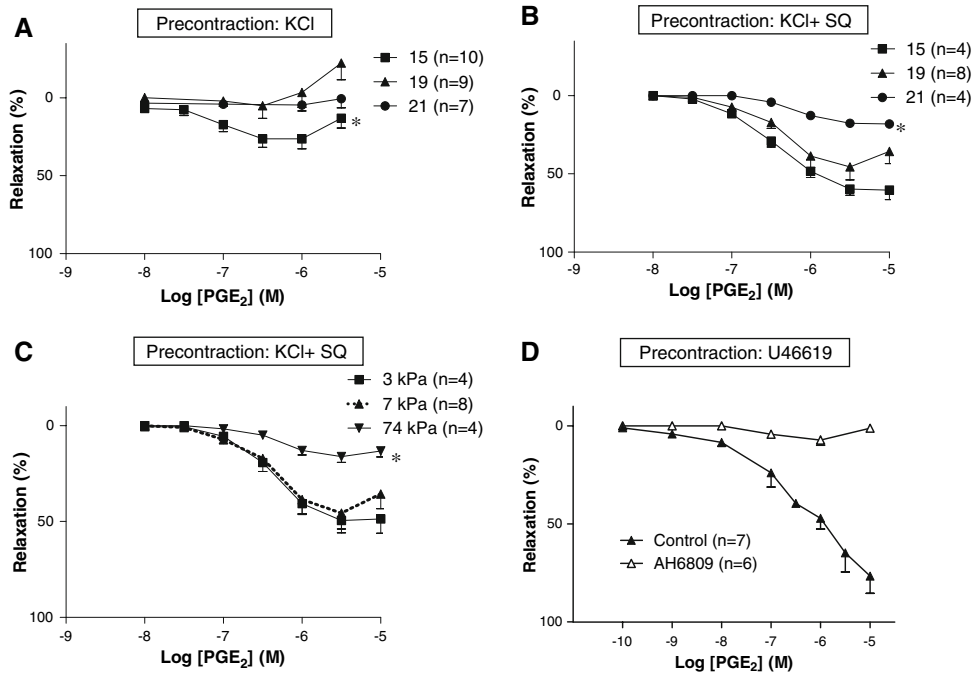

Fig. 2 Concentration-dependent relaxant effects of prostaglandin E_2 (PGE_2,) in chicken DA during contraction induced by K^+ (62.5 mM, **a**), K^+ in the presence of the TP receptor antagonist SQ 29,548 (**b** and **c**), or the TP receptor agonist U46619 (0.1 μM, **d**). The organ chambers were areated with 5% O_2/90%N_2/5% CO_2 (**a**, **b**, **d**). The effect of different oxygen concentrations on the relaxations induced by PGE_2 in the 19-day DA is depicted in **c**, in which the DA rings were aerated with 0% O_2/95% N_2/5% CO_2, or 95% O_2/5% CO_2. For clarity, the curve to PGE_2 in 5% O_2 from panel B is repeated in **c** (*dashed line*). The effects of the EP receptor antagonist AH6809 (10 μM) on PGE_2-induced relaxation is depicted **d** (19-day DA). * $P < 0.05$ for difference from the other ages (**a**, **b**) and for difference from other oxygen concentrations (**c**)

Table 1 Concentration-response parameters for relaxant substances in chicken ductus arteriosus

	15-day-old			19-day-old			21-day-old, e.p.		
	E_{max}	pD_2	*n*	E_{max}	pD_2	*n*	E_{max}	pD_2	*n*
PGE_2	29.79 (20.1)	7.15 (0.43)	10	5.06 (24.0)	–	9	4.7 (8.1)	–	7
PGE_2 (SQ)	64.14 (11.5)	6.45 (0.21)	4	46.89 (24.5)	6.36 (0.06)	8	18.27 (3.7)*	6.19 (0.15)	4
Isoproterenol	120.7 (25.2)	6.63 (0.31)†	8	107.3 (23.8)	7.03 (0.46)	29	64.0 (15.3)*	6.99 (0.26)	12
Forskolin	97.2 (9.5)	5.59 (0.09)	8	124.6 (37.5)	5.58 (0.28)	7	111.7 (21.3)	5.44 (0.05)	8
Milrinone	75.12 (10.1)	5.4 (0.14)‡	6	86.31 (9.8)	6.0 (0.15)	8	86.16 (19.4)	6.06 (0.18)	8

Values are means (SD); *n* = no. of embryos (arteries), e.p. externally pipped embryos; $pD_2 = -\log EC_{50}$; E_{max}, maximal relaxant effect. The pD_2 was not calculated when a non-significant relaxation was observed

* $P < 0.05$ from both 15 and 19-day old; † $P < 0.05$ from 19-day old; ‡ $P < 0.05$ for 19 and 21-day old (5% O_2 and precontraction with 62.5 mM KCl)

38, $n = 6$; aortic: 792 μm, SD 49, $n = 6$). As shown in Fig. 4a, the relaxation induced by PGE_2 (vessels were contracted with 62.5 mM K^+ in the presence of SQ29,548) was similar in both sides of the DA. In contrast, the relaxing potency and efficacy of isoproterenol (Fig. 4b) was significantly higher ($P < 0.05$) in the pulmonary (pD_2 7.71, SD 0.26, E_{max} 117.95%, SD 8.3, $n = 6$) than in the aortic side (pD_2 7.37, SD 0.15; E_{max} 72.77%, SD 3.1, $n = 6$). Forskolin (Fig. 4c) also induced a higher relaxation in the pulmonary than in the aortic side of the 19-day DA, but significant differences were only observed with the highest concentration tested (10 μM). Similarly to isoproterenol, the relaxing potency and efficacy of milrinone (Fig. 4d) was significantly ($P < 0.05$) higher in the pulmonary (pD_2 5.96, SD 0.16, E_{max} 80.2%, SD 9.8, $n = 6$) than in the aortic side (pD_2 5.0, SD 0.16; E_{max} 59.2%, SD 14, $n = 4$). The effects of milrinone were also studied in the 15-day DA and a similar significantly higher relaxation was observed in the pulmonary (pD_2 5.4, SD 0.18, E_{max} 77.1%, SD 9.4, $n = 6$) than in the aortic side (pD_2 4.9, SD 0.17, E_{max} 47.2%, SD 11, $n = 6$). Finally, as shown in Fig. 4e, the contractile potency and efficacy of U46619 was also significantly higher

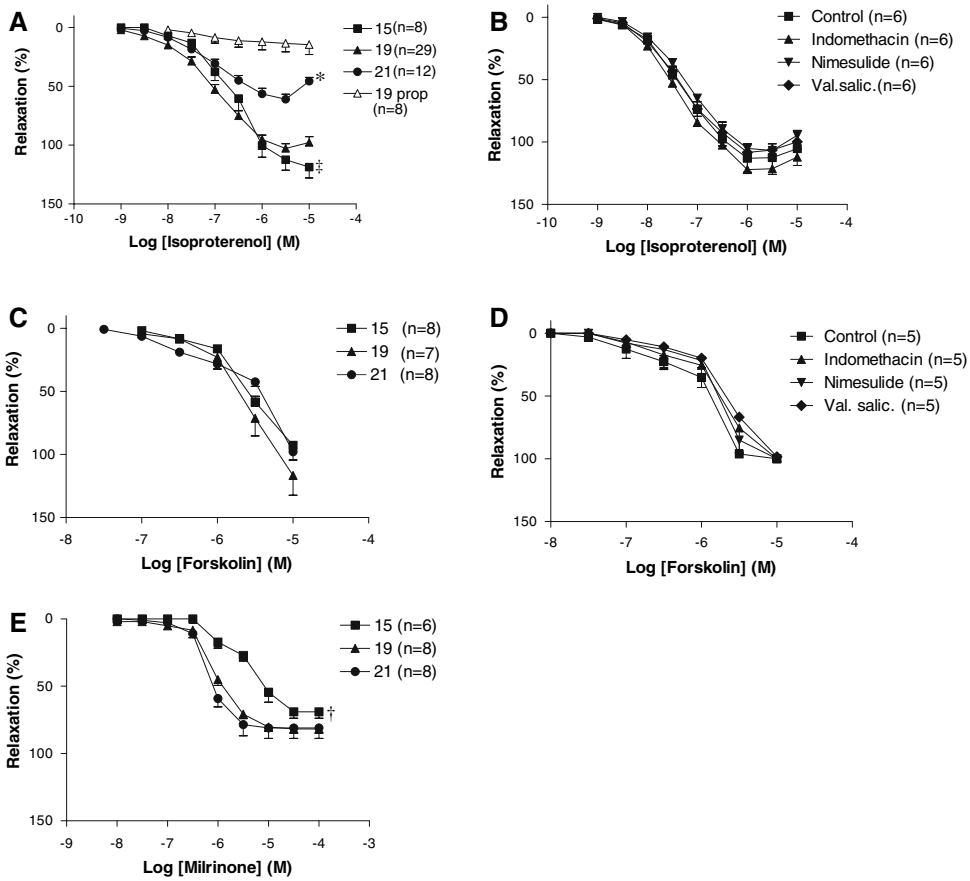

Fig. 3 Concentration-dependent relaxant effects of isoproterenol (**a**, **b**), forskolin (**c**, **d**) and milrinone (**e**) in 15, 19 and 21-day DA rings aerated with 5% O_2/90%N_2/5% CO_2. The effects of the β-adrenergic receptor blocker propanolol (prop) on isoproterenol-evoked relaxation are shown in **a**. **b**, **d** Depict the effects of the nonselective COX inhibitor indomethacin (10 μM), the COX-1 inhibitor valeryl salicylate (Val. salic., 0.5 mM), or the COX-2 inhibitor nimesulide (0.1 μM) on the relaxations induced by isoproterenol (**b**) and forskolin (**d**) in 19-day DA. Potassium (62.5 mM) was used as precontractile agent. * $P < 0.05$ for difference in E_{max} from the other ages. ‡ $P < 0.05$ for difference in E_{max} from 19 day. † $P < 0.05$ for difference in pD_2 from the other ages

($P < 0.05$) in the pulmonary than in the aortic side of the 19-day (pulmonary: pD_2 6.52, SD 0.15, E_{max} 1.58 N/m, SD 0.61, $n = 6$; aortic: pD_2 6.1, SD 0.13, E_{max} 0.34 N/m, SD 0.18, $n = 6$) and the 15-day (pulmonary: pD_2 6.84, SD 0.185, E_{max} 0.33 N/m, SD 0.24, $n = 6$; aortic: pD_2 5.3, SD 0.16, E_{max} 0.08 N/m, SD 0.06, $n = 6$) chicken DA.

Discussion

PGE_2 plays a major role in prenatal patency and postnatal closure of the mammalian DA, namely during fetal life by exerting a potent relaxant effect (Clyman 2006; Smith 1998), possibly in conjunction with nitric oxide (NO, Coceani et al. 1994; Momma and Toyono 1999; Seidner et al. 2001; Takizawa et al. 2000), and after birth by abruptly withdrawing its action. We have previously shown that the transition to ex ovo life is accompanied by dramatic changes in chicken DA reactivity (Agren et al. 2007, 2008). In the present paper, we have characterized, for the first time, the effects of PGE_2 and other drugs acting through the adenylate cyclase/cAMP pathway on chicken ductal tone. Our main findings are: (1) PGE_2 induced an EP receptor-mediated relaxation of the chicken DA that was partially or wholly masked by a simultaneous contractile effect mediated through TP receptors; (2) the relaxant responses induced by PGE_2 and the β-adrenoceptor agonist isoproterenol, but not those elicited by the adenylate cyclase activator forskolin or the PDE3 inhibitor milrinone, decreased with maturation, (3) relaxations to PGE_2 are decreased with increasing oxygen concentrations and (4) the effects of PGE_2 were similar in the pulmonary and aortic sides of the DA, whereas isoproterenol and milrinone caused a more efficient relaxation in the pulmonary side of the chicken DA.

As mentioned elsewhere, COX inhibitors did not induce significant changes in the basal tension of the chicken (Agren et al. 2007) or the emu (Dzialowski and Greyner 2008) DA and did not alter the vasoactive effects of other agonists in the chicken DA (Agren et al. 2007, 2008), suggesting that locally produced PGs are not important mediators in the control of ductal tone in these avian species. However, Dzialowski and Greyner (2008) observed that exogenous PGE_2 relaxed the oxygen-constricted emu DA with a threshold concentration of 0.1–1 μM and a full relaxation at 10 μM, the highest concentration tested. In the present paper, we demonstrate that exogenous PGE_2 is indeed a DA vasodilator in the chicken but its relaxant response is masked by its ability to activate TP receptors. We found that TP receptor occupancy either by an agonist

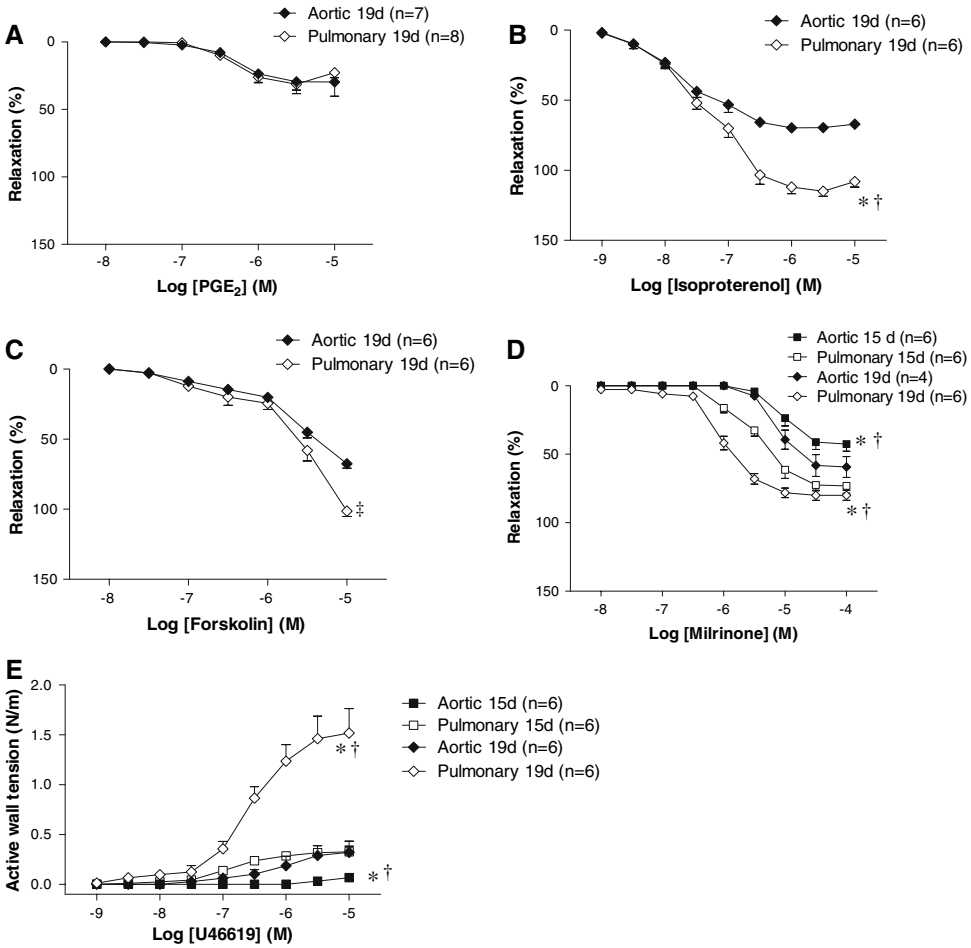

Fig. 4 Heterogeneity of relaxant and contractile responses along chicken ductus arteriosus (DA). Response of segments from the pulmonary and aortic side of 19-day chicken DA to PGE_2 (**a**), isoproterenol (**b**), and forskolin (**c**), milrinone (**d**), and U46619 (**e**). The responses to milrinone and U46619 in the pulmonary and aortic side of 15-day chicken DA are also shown. Relaxations were studied during contraction induced by K^+ (62.5 mM). The TP receptor antagonist SQ 29,548 (10 μM) was present in the experiments involving PGE_2. The vessels were aerated with 5% O_2/90%N_2/5% CO_2 (pO_2 ~7 kPa). * $P < 0.05$ for difference in E_{max} aortic versus pulmonary side. ‡ $P < 0.05$ for difference in the response to a given concentration. † $P < 0.05$ for difference in pD_2 aortic versus pulmonary side

(U46619) or an antagonist (SQ29,548) results in the appearance or the increase of the relaxant response to PGE_2. This is consistent with a stimulatory action of PGE_2 on TP receptors. The PGE_2 promiscuous interaction with TP receptors, especially at concentrations higher than 1 μM, has also been observed in several vascular tissues including human umbilical arteries (Boersma et al. 1999) and veins (Daray et al. 2003), human uterine arteries (Baxter et al. 1995) and aorta of the spontaneously hypertensive rat (Tang et al. 2008). An alternative explanation for the effects of SQ29,548 on PGE2-induced relaxation might be the blockade of a baseline TxA2- or isoprostane-induced tone (Gonzalez-Luis et al. 2005), allowing greater relaxatory actions of PGE on its dilatory receptors. However, this possibility is unlikely, since SQ29,548 have no effect on DA mechanical activity, suggesting the lack of basal release of a TP receptor stimulator.

PGE_2-induced relaxation of the chicken DA was abolished by AH6809 suggesting the involvement of EP receptors. However, AH6809 is a non selective EP receptor antagonist, which may also block DP and TP receptors (Tang et al. 2008). Accordingly, we observed that AH6809 impaired the contraction induced by U46619. Future functional experiments with more selective EP antagonists, as well as investigations addressing the expression and binding activity of prostanoid receptors in the chicken DA are, therefore, warranted. Nevertheless, we observed that the relaxant potency and efficacy of PGE_2 in the chicken DA, even after blocking its effect on the TP receptor, was very low. Dzialowski and Greyner (2008) also described a low relaxant potency of PGE_2 in the emu DA. In contrast, PGE_1 and PGE_2 in the picomolar and low nanomolar range relaxed the rat, rabbit, pig and lamb DA (Bhattacharya et al. 1999; Clyman et al. 1983, 1980; Smith 1998; Waleh et al. 2004), while the only mammalian species studied where PGs have not been shown to exert a significant DA relaxation is the guinea pig (Bodach et al. 1980; Smith 1998). With our present results we can only speculate about the functional consequences of the low relaxant potency of PGE2 in the chicken DA. In the mammalian fetus, circulating concentrations of PGE2 appear to be of placental origin (Clyman 2006). To the best of our knowledge, there is no study in the literature examining prostanoids levels in avian embryos or the role of the choriallantoic membrane, the analogous of mammalian placenta, in the production of prostaglandins. These issues remain to be investigated.

In mammals, the immature fetal DA is more sensitive than the late-gestation fetal DA to the relaxing effects of PGE_2 (Bhattacharya et al. 1999; Clyman et al. 1983, 1980; Waleh et al. 2004). Herein, we have found that chicken DA is more sensitive to PGE_2 in preterm than in term. The activation of TP receptors by PGE_2 is, at least partially, responsible for these changes with age. Thus, in the absence of TP antagonist a relaxant response is observed in the 15-day DA, whereas no relaxation, but contraction, is found in the 19-day DA. However, in the presence of the TP antagonist the relaxant responses to PGE_2 are similar in 15 and 19 days. These results indicate that the contribution of TP receptor activation in the response to PGE_2 increases during maturation. Accordingly, we have previously demonstrated a developmental increase in the sensitivity of chicken DA to the specific TP agonist U46619 (Agren et al. 2007). In addition, from 19- to 21-day there is an additional reduction in the relaxant responses to PGE_2 when SQ29,548 is present (i.e., independent of TP receptor activation). Similar to PGE_2, the β-adrenoceptor agonist isoproterenol induced less relaxation in 21-day when compared with 15- or 19-day DA. PGE_2 and isoproterenol induced relaxation, at least partially, through the adenylate cyclase/cAMP pathway. Therefore, our results suggest a developmental decrease in the relaxant efficacy of this pathway during the transition to ex ovo life. Nevertheless, direct activation of adenylate cyclase with forskolin induced a similar relaxation at all stages of incubation. Thus, the developmental differences in the responses to PGE_2 and isoproterenol from 19- to 21-day seem to be due to changes in the coupling between their receptors and adenylate cyclase. Accordingly, Waleh et al. (2004) found that the EP receptor agonists PGE_2 (nonselective), butaprost (EP_2 selective) and M&B 28767 (EP_3 selective) produced a greater increase in cAMP production in the immature than in the mature lamb DA. However, there were no differences between the immature and mature ductus in the relaxation or cAMP production induced by forskolin (Waleh et al. 2004). In the emu, the other avian DA that has been studied, Dzialowski and Greyner found similar PGE_2-relaxations in the day 45 and day 49 DA (total incubation in the emu: 50 days) (Dzialowski and Greyner 2008). Unfortunately, they did not analyze the response to PGE_2 of the DA of less mature emu ductus.

The role of β-adrenoceptors in the control of mammalian DA tone also varies among species (Smith 1998). Infusion of the β-adrenoceptor antagonist propanolol had no effect on ductal tone in the in vivo lamb (Friedman et al. 1983). Furthermore, in the presence of the α-adrenoceptor antagonist phenoxybenzamine, norepinephrine was able to relax lamb DA preparations, but the effects were modest even at the highest doses (Bodach et al. 1980). In the newborn rat, propanolol paradoxically delayed DA closure (Arishima et al. 1995), but it has been suggested that this is secondary to the effects of β-adrenergic blockade on the perinatal cardiovascular homeostasis and not due to a direct effect on ductal tone (Smith 1998). On the other hand, in the guinea pig DA, phenoxybenzamine converts the contractile effect of norepinephrine to a relaxation and isoproterenol causes a marked relaxation (Bodach et al. 1980). These results together with the lack of a prostaglandin-mediated relaxation could point to a special role for β-adrenoceptors in prenatal patency in the guinea pig. Herein, we have observed a similar pattern in the chicken DA. Previously, we demonstrated that the nonselective adrenergic receptor agonist norepinephrine and the α_1-adrenegic receptor agonist phenylephrine induced a developmentally increased contraction of the chicken DA. Secretion of catecholamines plays an important role in several of the adaptations that characterize the transition of the chicken to ex ovo life (Mulder et al. 2000; Wittmann and Prechtl 1991). The augmentation of the α-adrenergic-mediated contraction and the concomitant reduction of the β-adrenergic-mediated relaxation suggest an active participation of catecholamines in the closure of the chicken DA.

The cyclic nucleotides cAMP and cGMP are considered the main intracellular secondary messengers involved in smooth muscle relaxation (Omori and Kotera 2007; Rybalkin et al. 2003). These molecules are degraded by a family of enzymes known as phosphodiesterases (PDEs). There are now known to be 11 different PDE families expressed in mammalian tissues. Depending on the species, the major PDEs present in vascular smooth muscle are PDE1, PDE3 and PDE5 (Moreno et al. 2004; Omori and Kotera 2007; Rybalkin et al. 2003). PDE3 s are termed cGMP-inhibited cAMP PDEs, whereas PDE5 specifically hydrolyzes cGMP (Moreno et al. 2004; Omori and Kotera 2007; Rybalkin et al. 2003). We have demonstrated that the chicken DA is relaxed by the PDE5 inhibitor sildenafil (Agren et al. 2008) and by the PDE3 inhibitor milrinone (present work). When clinically used in premature infants, it appeared that PDE3 inhibitors inhibited closure of the DA (Toyoshima et al. 2006). Toyoshima et al. (2006) demonstrated that the PDE3 inhibitors milrinone and amrinone exhibited dose-dependent DA-dilating effects in rat fetuses and pups and were more effective in the preterm than in the near-term period. Liu et al. (2008) observed, in the ovine DA, a developmental increase in the activity and expression of PDE1, 3, 4, and 5 and reported that the mature ductus required higher concentrations of PDE1, PDE3, and PDE4 inhibitors to inhibit its tension to the same extent as in the immature ductus. In contrast, we have observed a reduced relaxant effect of milrinone in the 15-day chicken DA when compared with the 19- or 21-day DA. This reduced response to milrinone in the 15-day DA was homogeneously present when segments of the central part, the pulmonary end or the

aortic end of the DA were analyzed (see below). Our results might suggest that an increase in PDE3-mediated cAMP hydrolysis is involved in the age-related decrease of PGE_2- and isoproterenol-induced relaxation observed in the chicken DA. However, if that was the case, the relaxation induced by forskolin should also have exhibited a reduction with maturation.

Another observation of the present work was that hyperoxia markedly impaired PGE_2-induced relaxation. Previously, we reported that high oxygen concentrations decreased the endothelium- dependent and -independent relaxation induced by, respectively, acetylcholine and the nitric oxide donor, sodium nitroprusside and increased the contraction mediated by adrenergic agonists (Agren et al. 2007, 2008). Accordingly, it has been demonstrated that increasing oxygen tension from fetal to neonatal levels desensitizes the rabbit DA to a range of vasodilators, including PGE_2 (Smith and McGrath 1991, 1993). Thus the postnatal increase in O_2 is not only one of the main triggers for constriction of the DA but also has a profound modulatory effect on other vasoactive systems favoring the action of vasoconstrictors and decreasing the action of vasodilators (Smith 1998).

The chicken DA presents morphological and functional heterogeneity along its path between the pulmonary artery and the aorta (Agren et al. 2007; Belanger et al. 2008; Bergwerff et al. 1999, 1996). Thus, the pulmonary side shows the structure of a muscular artery and responds to O_2 with contraction, whereas the aortic part shows the morphology of an elastic artery and relaxes in response to O_2 (Agren et al. 2007; Belanger et al. 2008; Bergwerff et al. 1999, 1996). In addition, acetylcholine, SNP and the NO-independent stimulator of sGC BAY 41-2272 induced larger relaxations in the aortic side of the vessel (Agren et al. 2008). In the present work, we observed that isoproterenol, forskolin, and milrinone evoked significantly larger relaxations in the pulmonary than in the aortic side of the chicken DA. This might indicate that the pulmonary side of the vessel is more sensitive to the vasodilators acting through cAMP, whereas the aortic side would be more sensitive to cGMP-mediated relaxation. However, and surprisingly, the relaxation evoked by PGE_2 was similar in both regions of the DA. We can only speculate about the nature of this finding. As the contraction induced by the TP receptor agonist U46619 was significantly higher in the pulmonary side, the masking effect of PGE_2-induced TP stimulation might be smaller in the aortic than in the pulmonary side. However, the experiments were performed in the presence of TP antagonism. Another speculation might be that cAMP-mediated mechanisms are less involved in the relaxation evoked by PGE_2 in the aortic side. In fact, it has been demonstrated that PGE_2 dilates the late gestation fetal lamb DA through pathways that involve either cAMP (via EP_2 and EP_4 receptors) or ATP-dependent potassium channels (via EP3, Bouayad et al. 2001). However, in the experiments that compared the pulmonary and the aortic side of the chicken DA, the vessels were contracted with a high potassium solution which precluded the relaxation induced by potassium channels activation.

The heterogeneity of the chicken DA makes the vessel particularly attractive for the study of its developmental vascular biology but also poses a methodological problem. As we mentioned elsewhere, the maximal length of the rings allowed in the myographs that we used is 2 mm, whereas the length of the chicken DA is ~4–6 mm. Although the pulmonary and the aortic side of the ductus can be clearly differentiated during the dissection (Agren et al. 2007), when mounting the central portion of the DA it is difficult to control for the exact amount of tissue of each part that is finally present in the myograph. In addition, putative developmental changes in the proportion between the two parts of the DA, might influence developmental changes in the responsiveness to the different agonists. This limitation of our experimental setting was particularly relevant when the response to milrinone was analyzed. We observed that milrinone-induced relaxation increased with development but also was higher in the pulmonary than in the aortic side of the DA. Therefore the reduced relaxation in the 15-day DA could be related to the presence of higher amounts of tissue from the aortic side of the vessel. We controlled for this possibility by analyzing the response to milrinone in the pulmonary and aortic sides of the 15-day DA and we observed that the developmental increase was present in both sides of the vessel.

In summary, with the present and our previous studies (Agren et al. 2007, 2008), we have completed an extensive characterization of the pharmacology of the chicken DA and demonstrated its sensitivity to a wide range of vasoactive agonists including oxygen, prostanoids, potassium channels blockers, NO, catecholamines, endothelin-1, adenylate cyclase activators, guanylate cyclase activators and PDE inhibitors. As occurs in the mammalian, the multiplicity of these vasoactive systems seems at odds with the relatively simple physiological role of the DA (Smith 1998). The main vasoconstrictor of the mammalian DA, the postnatal increase in oxygen tension, appears to play also a relevant role in the closure of the chicken DA (Agren et al. 2007; Belanger et al. 2008), whereas the main vasodilator of the mammalian DA, PGE_2, is a weak relaxant agent of the chicken DA which even stimulates vasoconstrictive receptors. As the mammalian DA, the chicken DA undergoes a process of maturation to prepare the task of postnatal closure. Due to the above similarities and taking into account the differences, the chicken embryo appears as an extremely interesting model for translational developmental vascular biology (Sutendra and Michelakis 2007).

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References

Agren P, Cogolludo AL, Kessels CG, Perez-Vizcaino F, De Mey JG, Blanco CE, Villamor E (2007) Ontogeny of chicken ductus arteriosus response to oxygen and vasoconstrictors. Am J Physiol Regul Integr Comp Physiol 292:R485–R496

Agren P, van der Sterren S, Cogolludo AL, Frazziano G, de Mey JG, Blanco CE, Villamor E (2008) Developmental changes in endothelium-dependent relaxation of the chicken ductus arteriosus. J Physiol Pharmacol 59:55–76

Arishima K, Takizawa T, Oda T, Yamamoto M, Togashi H, Somiya H, Eguchi Y (1995) Propranolol inhibits the spontaneous closure of the ductus arteriosus in newborn rats. J Vet Med Sci 57:943–944

Baxter GS, Clayton JK, Coleman RA, Marshall K, Sangha R, Senior J (1995) Characterization of the prostanoid receptors mediating constriction and relaxation of human isolated uterine artery. Br J Pharmacol 116:1692–1696

Belanger C, Copeland J, Muirhead D, Heinz D, Dzialowski EM (2008) Morphological changes in the chicken ductus arteriosi during closure at hatching. Anat Rec (Hoboken)

Bergwerff M, DeRuiter MC, Gittenberger-de Groot AC (1999) Comparative anatomy and ontogeny of the ductus arteriosus, a vascular outsider. Anat Embryol (Berl) 200:559–571

Bergwerff M, DeRuiter MC, Poelmann RE, Gittenberger-de Groot AC (1996) Onset of elastogenesis and downregulation of smooth muscle actin as distinguishing phenomena in artery differentiation in the chick embryo. Anat Embryol (Berl) 194:545–557

Bhattacharya M, Asselin P, Hardy P, Guerguerian AM, Shichi H, Hou X, Varma DR, Bouayad A, Fouron JC, Clyman RI, Chemtob S (1999) Developmental changes in prostaglandin E(2) receptor subtypes in porcine ductus arteriosus. Possible contribution in altered responsiveness to prostaglandin E(2). Circulation 100:1751–1756

Bodach E, Coceani F, Dumbrille A, Okpako DT, Olley PM (1980) The response of the isolated ductus arteriosus to transmural stimulation and drugs. Br J Pharmacol 71:419–427

Boersma JI, Janzen KM, Oliveira L, Crankshaw DJ (1999) Characterization of excitatory prostanoid receptors in the human umbilical artery in vitro. Br J Pharmacol 128:1505–1512

Bouayad A, Kajino H, Waleh N, Fouron JC, Andelfinger G, Varma DR, Skoll A, Vazquez A, Gobeil F Jr, Clyman RI, Chemtob S (2001) Characterization of PGE2 receptors in fetal and newborn lamb ductus arteriosus. Am J Physiol Heart Circ Physiol 280:H2342–H2349

Chand N, Eyre P (1976) Effects of prostaglandins E1, E2 and F2 alpha and polyphloretin phosphate of domestic fowl. Arch Int Pharmacodyn Ther 221:261–265

Claeys M, Wechsung E, Herman AG, Nugteren DH (1981) Prostaglandin E2 is the prevalent metabolite of arachidonic acid formed by aortic tissue of the chicken. Arch Int Pharmacodyn Ther 249:312–315

Clyman RI (2006) Mechanisms regulating the ductus arteriosus. Biol Neonate 89:330–335

Clyman RI, Mauray F, Roman C, Heymann MA, Payne B (1983) Effect of gestational age on ductus arteriosus response to circulating prostaglandin E2. J Pediatr 102:907–911

Clyman RI, Mauray F, Rudolph AM, Heymann MA (1980) Age-dependent sensitivity of the lamb ductus arteriosus to indomethacin and prostaglandins. J Pediatr 96:94–98

Coceani F, Kelsey L, Seidlitz E (1994) Occurrence of endothelium-derived relaxing factor–nitric oxide in the lamb ductus arteriosus. Can J Physiol Pharmacol 72:82–88

Daray FM, Minvielle AI, Puppo S, Rothlin RP (2003) Pharmacological characterization of prostanoid receptors mediating vasoconstriction in human umbilical vein. Br J Pharmacol 139:1409–1416

Dzialowski EM, Greyner H (2008) Maturation of the contractile response of the Emu ductus arteriosus. J Comp Physiol 178:401–412

Friedman WF, Printz MP, Kirkpatrick SE, Hoskins EJ (1983) The vasoactivity of the fetal lamb ductus arteriosus studied in utero. Pediatr Res 17:331–337

Gonzalez-Luis G, Perez-Vizcaino F, Garcia-Munoz F, de Mey JG, Blanco CE, Villamor E (2005) Age-related differences in vasoconstrictor responses to isoprostanes in piglet pulmonary and mesenteric vascular smooth muscle. Pediatr Res 57:845–852

Kajino H, Taniguchi T, Fujieda K, Ushikubi F, Muramatsu I (2004) An EP4 receptor agonist prevents indomethacin-induced closure of rat ductus arteriosus in vivo. Pediatr Res 56:586–590

Leonhardt A, Glaser A, Wegmann M, Schranz D, Seyberth H, Nusing R (2003) Expression of prostanoid receptors in human ductus arteriosus. Br J Pharmacol 138:655–659

Liu H, Manganiello V, Waleh N, Clyman RI (2008) Expression, activity and function of phosphodiesterases in the mature and immature ductus arteriosus. Pediatric research

Momma K, Toyono M (1999) The role of nitric oxide in dilating the fetal ductus arteriosus in rats. Pediatr Res 46:311–315

Moreno L, Losada B, Cogolludo A, Lodi F, Lugnier C, Villamor E, Moro M, Tamargo J, Perez-Vizcaino F (2004) Postnatal maturation of phosphodiesterase 5 (PDE5) in piglet pulmonary arteries: activity, expression, effects of PDE5 inhibitors, and role of the nitric oxide/cyclic GMP pathway. Pediatr Res 56:563–570

Mulder AL, Golde JM, Goor AA, Giussani DA, Blanco CE (2000) Developmental changes in plasma catecholamine concentrations during normoxia and acute hypoxia in the chick embryo. J Physiol 527(Pt 3):593–599

Narumiya S, FitzGerald GA (2001) Genetic and pharmacological analysis of prostanoid receptor function. J Clin Invest 108:25–30

Nguyen M, Camenisch T, Snouwaert JN, Hicks E, Coffman TM, Anderson PA, Malouf NN, Koller BH (1997) The prostaglandin receptor EP4 triggers remodelling of the cardiovascular system at birth. Nature 390:78–81

Omori K, Kotera J (2007) Overview of PDEs and their regulation. Circ Res 100:309–327

Rowley A (1991) Eicosanoids: aspects of their structure, function, and evolution. In: Warr G, Cohen N (eds) The phylogenesis of immune functions. CRC Press, Boca Ration, pp 269–294

Rybalkin SD, Yan C, Bornfeldt KE, Beavo JA (2003) Cyclic GMP phosphodiesterases and regulation of smooth muscle function. Circ Res 93:280–291

Seidner SR, Chen YQ, Oprysko PR, Mauray F, Tse MM, Lin E, Koch C, Clyman RI (2001) Combined prostaglandin and nitric oxide inhibition produces anatomic remodeling and closure of the ductus arteriosus in the premature newborn baboon. Pediatr Res 50:365–373

Smith GC (1998) The pharmacology of the ductus arteriosus. Pharmacol Rev 50:35–58

Smith GC, Coleman RA, McGrath JC (1994) Characterization of dilator prostanoid receptors in the fetal rabbit ductus arteriosus. J Pharmacol Exp Ther 271:390–396

Smith GC, McGrath JC (1991) Prostaglandin E2 and fetal oxygen tension synergistically inhibit response of isolated fetal rabbit ductus arteriosus to norepinephrine. J Cardiovasc Pharmacol 17:861–866

Smith GC, McGrath JC (1993) Characterisation of the effect of oxygen tension on response of fetal rabbit ductus arteriosus to vasodilators. Cardiovasc Res 27:2205–2211

Smith GC, McGrath JC (1995) Contractile effects of prostanoids on fetal rabbit ductus arteriosus. J Cardiovasc Pharmacol 25:113–118

Sutendra G, Michelakis ED (2007) The chicken embryo as a model for ductus arteriosus developmental biology: cracking into new territory. Am J Physiol Regul Integr Comp Physiol 292:R481–R484

Takizawa T, Kihara T, Kamata A, Yamamoto M, Arishima K (2000) Role of nitric oxide in regulating the ductus arteriosus caliber in fetal rats. J Vet Med Sci 62:707–710

Tang EH, Jensen BL, Skott O, Leung GP, Feletou M, Man RY, Vanhoutte PM (2008) The role of prostaglandin E and thromboxane-prostanoid receptors in the response to prostaglandin E2 in the aorta of Wistar Kyoto rats and spontaneously hypertensive rats. Cardiovasc Res 78:130–138

Toyoshima K, Momma K, Imamura S, Nakanishi T (2006) In vivo dilatation of the fetal and postnatal ductus arteriosus by inhibition of phosphodiesterase 3 in rats. Biol Neonate 89:251–256

Villamor E, Ruijtenbeek K, Pulgar V, De Mey JG, Blanco CE (2002) Vascular reactivity in intrapulmonary arteries of chicken embryos during transition to ex ovo life. Am J Physiol Regul Integr Comp Physiol 282:R917–R927

Waleh N, Kajino H, Marrache AM, Ginzinger D, Roman C, Seidner SR, Moss TJ, Fouron JC, Vazquez-Tello A, Chemtob S, Clyman RI (2004) Prostaglandin E2–mediated relaxation of the ductus arteriosus: effects of gestational age on g protein-coupled receptor expression, signaling, and vasomotor control. Circulation 110:2326–2332

Wideman RF Jr, Maynard P, Bottje WG (1999) Thromboxane mimics the pulmonary but not systemic vascular responses to bolus HCl injections in broiler chickens. Poult Sci 78:714–721

Wittmann J, Prechtl J (1991) Respiratory function of catecholamines during the late period of avian development. Respir Physiol 83:375–386