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Developmental changes in endothelium-dependent vasodilation and the influence of superoxide anions in perinatal rabbit pulmonary arteries

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1 ACh-induced vasodilation was investigated in pulmonary arteries from 8 and 2 day pre-term foetal, neonatal (0–12 h and 4 day old) and adult rabbits. The effects of superoxide anion generation [with hypoxanthine (HX, 0.1 mM)/xanthine oxidase (XO, 15 mu ml^{-1})], endogenous superoxide dismutase (SOD) inhibition [with the Cu-Zn SOD inhibitor triethylenetetramine (TETA, 1 mM)], endogenous superoxide anion scavenging [by superoxide dismutase (SOD, 50 u ml^{-1})] and inhibition of endothelial nitric oxide synthase (eNOS) [with, N^{ω}-nitro-L-arginine methylester (L-NAME, 0.1 mM)], on basal and ACh-induced NO activity were studied by examining phenylephrine-induced contraction and ACh-induced vasodilation respectively.

2 L-NAME and endothelium removal abolished all ACh-induced vasodilation and 1 μM sodium nitroprusside fully dilated all vessels. ACh-induced vasodilation was absent in the 8 day pre-term foetus and 0–12 h neonate but present at all other ages. L-NAME itself contracted 2 day pre-term foetal vessels. At 0–12 h, SOD, but not the phosphodiesterase 5 inhibitor zaprinast (1 μM), uncovered ACh-induced vasodilation. At this age SOD reduced phenylephrine-induced contraction which was not influenced by TETA, L-NAME or HX/XO, and L-NAME itself did not cause contraction. This suggests both ACh-induced and basal NO activity are compromise in these vessels by endogenous superoxide anion production and deficiencies in endogenous SOD activity.

3 In 4 day vessels, but not adult vessels, L-NAME, TETA and HX/XO augmented contractions to phenylephrine, and L-NAME itself induced vasoconstriction, suggesting that basal NO and SOD activities were present by 4 days but were not evident in the adult. ACh-induced NO activity, and the influence of endogenous SOD on this, were present in the adult (and 4 day) vessels as superoxide generation with HX/XO significantly reduced ACh-induced vasodilation and this effect was inhibited by SOD and augmented by TETA.

4 Increased oxygen tensions >500 mmHg attenuated ACh-induced vasodilation in the foetal but not neonatal rabbits. Raising the oxygen tension from $\sim$20 to $\sim$120 mmHg revealed ACh-induced vasodilation in the 8 day pre-term vessels.

5 In summary, superoxide anion accumulation combined with deficiencies in SOD activity may transiently compromise basal and ACh-induced NO activity at birth. Experimental oxygen tensions markedly influence ACh-induced vasodilation in foetal rabbit pulmonary arteries.

Keywords: Nitric oxide; superoxide anion; vasodilation; pulmonary arteries; foetal; neonate

Introduction

The pulmonary circulation undergoes a rapid transition at birth in order to adapt to extra uterine life and allow normal gas exchange to occur. This is characterized by an increase in pulmonary arterial blood flow and concomitant sharp decline in pulmonary vascular resistance (Fineman *et al*., 1995). Endothelium-dependent vasodilation has been demonstrated in pulmonary arteries from the foetus, newborn and adult of several species (Davidson & Eldemerdash, 1990; Steinhorn *et al*., 1993; Shaul *et al*., 1993). ACh-induced vasodilation can be due to the release of the endothelium-derived relaxing factor nitric oxide (NO) synthesized by the action of endothelial NO synthase (eNOS) (Furchgott & Zawadki, 1980; Palmer *et al*., 1987). Hence, NO may modulate pulmonary vascular tone in the developing pulmonary circulation. The free radical superoxide anion has been shown to interact with NO preventing its vasodilator activity and producing the cytotoxic oxidant peroxynitrite (Rubanyi & Vanhoutte, 1986; Beckman *et al*., 1990). Endogenous superoxide dismutase (SOD) is the major mammalian antioxidant enzymes and catalyzes the dismutation of superoxide anion to hydrogen peroxide (Abrahamsson *et al*., 1992; Marin & Rodrigez-Martinez, 1995). Little, however, is known about the influence of superoxide anions and SOD activity on pulmonary arterial reactivity in the perinatal period.

In this study we investigated ACh-induced vasodilation in rabbit pulmonary conduit arteries from 8 day pre-term foetus, 2 day pre-term foetus, 0–12 h, 4-day and adult rabbits. We investigated the effect of inhibiting eNOS, superoxide anion generation, exogenous SOD and inhibition of SOD on basal and ACh-induced NO activity in these vessels by examining their effects on phenylephrine-induced contraction and ACh-induced vasodilation respectively.

NO induces vasodilation by the activation of soluble guanylate cyclase and subsequent rise in guanosine 3′,5′-cyclic monophosphate (cyclic GMP) which is metabolized by phosphodiesterase 5 (PDE 5, Sunahara *et al*., 1996). PDE 5 activity has been shown to be present in rat foetal lung tissue but to have increased markedly in neonatal (1 day old) rat lungs then to decrease again by adulthood (Sanchez *et al*., 1998). We therefore examined the effects of the PDE 5 inhibitor, zaprinast, on ACh-induced vasodilation in 0–12 h vessels.

Before birth, foetal pulmonary arteries are in a hypoxic environment but at birth they are exposed to $\sim$120 mmHg oxygen. Many previous studies have, however, studied

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perinatal pulmonary arteries bubbled with 95% oxygen (Steinhorn *et al*., 1993; Liu *et al*., 1992; Shaul *et al*., 1993). Therefore, we compared ACh-induced vasodilation in foetal vessels bubbled with 95, 16 and 3% oxygen as well as in 4 day vessels bubbled with 16 and 95% oxygen.

Methods

First branch pulmonary arteries from 8 day pre-term foetal (400 μ i.d.), 2 day pre-term foetal (1–2 mm i.d.), 0–12 h (1–2 mm i.d.), 4 day (1.5–2.5 mm i.d.), and adult (2–4 mm i.d.) New Zealand White rabbits were studied. All vessel rings, except those from the 8 day pre-term rabbits, were set up in 5 ml organ baths. Vessels were set up under tensions at which maximum responses to 50 mM KCl were obtained (1.5 g for adult vessels and 0.8–1 g for all other vessels). Those from the 8 day pre-term rabbits were set up on a Mulvaney-Halpern wire myograph. Tension was applied to give an equivalent transmural pressure of $\sim$16 mmHg. This is the pressure existing *in vivo* in neonatal and adult rabbits and the minimum tension at which contractions were observed in the foetal vessels. All vessels were bathed in Krebs-bicarbonate solution (pH 7.4). To mimic *in vivo* gas tensions different gas mixtures were initially used to bubble the Krebs solution to give the following gas tensions: adults and neonates $pO_2 \sim 120$ mmHg, $pCO_2 \sim 35$–40 mmHg (16% O_2/5% CO_2); foetus $pO_2 \sim 18$–23 mmHg, $pCO_2 \sim 35$–40 mmHg (3% O_2/5% CO_2). Subsequently, some foetal vessels were bubbled with either 16 or 95% O_2 ($pO_2 > 500$ mmHg) and some 4 day vessels were also bubbled with 95% O_2. Gas tensions were measured using an oxygen electrode (Strathkelvin Instruments, U.K.) and verified using a blood gas analyser (Corning 166). Rings were equilibrated for 1 h and then each ring was exposed to 50 mM KCl for 5 min KCl caused an immediate and well sustained contraction.

Endothelium-dependent vasodilation

In all age groups except the 8 day pre-term foetus, the pulmonary arterial rings were pre-contracted with a concentration of phenylephrine (0.1–0.3 μM) which produced a contraction 70% of maximum. Once a stable plateau had been reached, cumulative concentration-dependent-response curves (CCRCs) to ACh (1 nM–10 μM) were constructed in half log increments, each increment added when response to previous addition had plateaued (about 2 min). The 8 day pre-term foetal rings were pre-contracted with 30 nM endothelin-1 (ET-1) (pre-determined in preliminary experiments), as phenylephrine did not produce an appreciable contraction at this age. Subsequently, control studies were carried out by precontracting vessels of all ages with ET-1 to demonstrate that this did not affect the results of the study (data not shown).

In the 0–12 h rabbit vessels, CCRCs to ACh were repeated after 20 min pre-treatment with superoxide dismutase (SOD, 50 u ml^{-1}) whilst in others the experiment was repeated in the presence of zaprinast (1 μM, 40 min pre-treatment). In other 0–12 h arteries CCRCs to phenylephrine (1 nM–10 μM) were obtained in the presence and absence of 50 u ml^{-1} SOD (20 min pre-treatment).

Influence of superoxide anions on basal and ACh-induced NO activity

Basal activity of NO was assessed indirectly both by the addition of 100 μM L-NAME (30 min pre-treatment) to quiescent vessels and by the endothelium-dependent attenuation of phenylephrine-induced contraction (Mian & Martin, 1995). Two day pre-term, 0–12 h, 4 day and adult vessels were pre-contracted with 0.1–0.3 μM PE. Some vessels were pre-treated with the Cu-Zn SOD inhibitor triethylenetetramine (TETA, 1 mM, 45 min pre-treatment followed by wash-out). The effect of superoxide anion generation and/or SOD was studied by applying hypoxanthine (HX, 0.1 mM)/xanthine oxidase (XO, 15 mu ml^{-1}) and/or SOD (50 u ml^{-1}) once the phenylephrine-induced contraction had stabilized.

The influence of HX/XO and/or SOD on ACh-induced NO activity were assessed by examining their effects on ACh-induced (0.3 μM) relaxation in phenylephrine-precontracted (0.1–0.3 μM) vessels. Some of these experiments were repeated after pre-treatment with the Cu-Zn SOD inhibitor triethylenetetramine (TETA, 1 mM, 45 min pre-treatment followed by wash-out). The effect of sodium nitroprusside (1 μM) was also studied on all pre-contracted vessels. Most experiments were repeated in endothelium-denuded vessels. The endothelium was removed by gentle rubbing of the intimal surface. All experiments involving HX/XO and or TETA were performed in the presence of catalase (1800 u ml^{-1}) to prevent hydrogen peroxide accumulation.

Drugs and solutions

The following drugs were used: Acetylcholine chloride, phenylephrine hydrochloride, catalase (bovine liver), N^{ω}-nitro-L-arginine methylester (L-NAME), hypoxanthine, xanthine oxidase (from buttermilk) superoxide dismutase (from bovine erythrocytes) triethylenetetramine (TETA) and zaprinast were all purchased from SIGMA (Poole, Dorset, U.K.); endothelin-1 (ET-1) was purchased from Thistle Peptides, Glasgow, Scotland. The composition of the Krebs solution was as follows (mM): NaCl(118.4), KCl(4.7), $NaHCO_3$(24.8), $MgSO_4$(0.6), KH_2PO_4(1.2), $CaCl_2$(2.5) and Glucose (11.1). Acetylcholine, phenylephrine, TETA and L-NAME were dissolved in distilled water whilst catalase, xanthine oxidase and SOD were dissolved in 0.9% saline. Dilutions of zaprinast (stock in DMSO) and hypoxanthine (stock in 0.4% sodium hydroxide) were made up in distilled water.

Data analysis

pEC_{50} values were calculated from individual CCRCs using computer-aided interpolation of responses from each vessel. These were expressed as the $-\log$ M concentration. Graphical results are expressed as a percentage of the reference response to 50 mM KCl or as a percentage of agonist-induced tone where appropriate. Statistical analysis was made by one-way analysis of variance (ANOVA) followed by an appropriate *post hoc* test (Tukeys) for multiple comparisons: $P < 0.05$ was considered statistically significant. Data throughout are expressed as means $\pm$ s.e. mean and n = the number of animals studied.

Results

Endothelium-dependent vasodilation

Figure 1 illustrates the effect of ACh on pulmonary arteries pre-contracted with phenylephrine (except for the 8 day pre-term vessels which were pre-contracted with ET-1). pEC_{50} values are summarized in Table 1. Pulmonary arteries from the

8 day pre-term foetus and 0–12 h neonate did not vasodilate to ACh. ACh caused vasodilation in all other vessels with the following order of potency: adult > 4 day = 2 day pre-term (Table 1). Maximum vasodilation was in the following order (% preconstriction): 4 day (105.4 ± 3.0%) = 2 day pre-term (86.4 ± 4.2%) > adult (67.4 ± 6.6%, $P < 0.01$ *c.f.* 4 day). Levels of pre-constriction were not significantly different between groups being (% of the response to 50 mM KCl): 107 ± 8% (8 day pre-term), 103 ± 11% (2 day pre-term), 104 ± 9% (0–12 h), 112 ± 11% (4 day) and 83 ± 12% (adult). Vasodilation in all vessels was abolished by removal of the endothelium. Sodium nitroprusside (1 μM) caused 100% vasodilation in arteries from all age groups ($n = 3–4$).

Effect of changing oxygen tension on ACh-induced vasodilation

No vasodilation to ACh was observed in the 8 day pre-term foetal vessels bubbled with 3% O_2/5% CO_2, whilst vasodilation was observed in those bubbled with 16% O_2/5% CO_2 (Figure 2A). A further increase in oxygen tension by bubbling with 95% O_2/5% CO_2 virtually abolished the response to ACh (Figure 2A). Levels of pre-constriction were not significantly different between compared groups being (% of the response to 50 mM KCl): 118 ± 27% (8 day pre-term, 16%), 98 ± 14% (8 day pre-term, 95%), 114 ± 22% (2 day pre-term, 16%), 98 ± 14% (2 day pre-term, 95%), 93 ± 5% (4 day, 95%) and 76.5 ± 7% (adult, 95%).

In the 2 day pre-term foetal vessels, bubbling with 16% O_2/5% CO_2 did not have any significant effect on the ACh-induced vasodilation when compared to control (3% oxygen, Figure 2B). Vasodilation was significantly attenuated in those vessels bubbled with 95% O_2/5% CO_2 (maximum vasodilation, $P < 0.001$ *c.f.* 3%, Figure 2B). By 4 days, vasodilation to ACh was not significantly affected by bubbling with 95% O_2/5% CO_2 (Figure 2C).

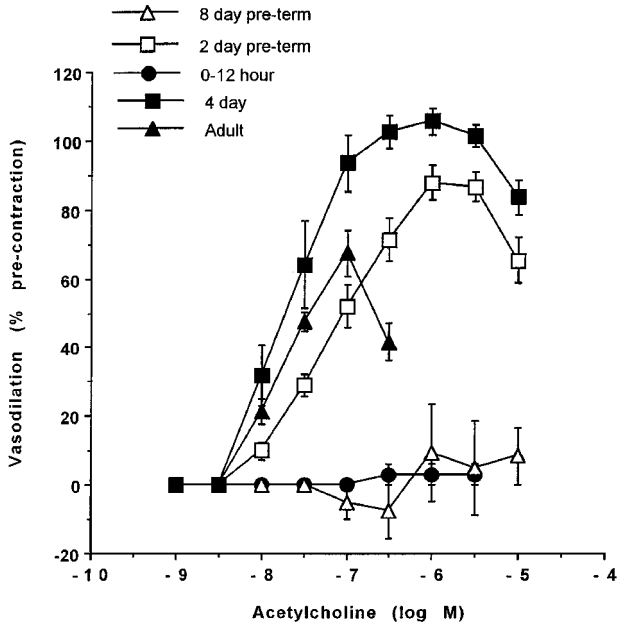

Figure 1 ACh-induced vasodilation in endothelium-intact, pulmonary artery rings pre-contracted with phenylephrine (unless otherwise stated) from 8 day pre-term foetal ($n = 4$, precontracted with endothelin-1), 2 day pre-term foetal ($n = 6$), 0–12 h ($n = 12$), 4 day ($n = 7$) and adult ($n = 8$) rabbits. Foetal vessels were bubbled with 3% oxygen, neonatal and adult vessels with 16% oxygen. Vasodilation is expressed as % reversal of pre-constriction. Each point represents mean ± s.e.mean.

Effect of L-NAME

Sustained contractions to L-NAME (100 μM) developed over 3–4 min in vessels from both foetal and 4 day rabbits. Little response, however was observed in the 0–12 h and adult vessels (Figure 3). L-NAME inhibited ACh-induced vasodilation at all ages (data not shown).

Effect of superoxide dismutase and zaprinast on 0–12 h vessels

Zaprinast (1 μM) did not reveal a response to ACh in the 0–12 h rabbit pulmonary conduit arteries (Figure 4A). It had no effect on vascular tone itself. Pre-treatment with SOD (50 u ml^{-1}) however, restored ACh-induced vasodilation. The maximum vasodilation in the presence of SOD (84.2 ± 11.2%, $n = 6$) was not significantly different from that observed in adult vessels (67.4 ± 6.6%, $n = 8$) although the potency of ACh was less (Figure 4A, Table 1). As maximum vasodilation induced by ACh was significantly less in the adult than at earlier ages, the effect of SOD was examined in these vessels also. Pre-incubation with SOD did not, however, significantly change either the potency of ACh ($pEC_{50} = 7.74 \pm 0.2$, $n = 6$) or the maximum vasodilation (Figure 4A).

0–12 h rabbit vessels contracted to phenylephrine in a concentration-dependent fashion ($pEC_{50} = 6.7 \pm 0.2$, $n = 6$; Figure 4B). Pre-incubating the intact vessels with SOD resulted in a significant attenuation of the response to phenylephrine without any significant change in potency ($pEC_{50} = 6.3 \pm 2.0$, $n = 6$) whilst responses to phenylephrine were unaffected by SOD in endothelium-denuded rings ($pEC_{50} = 6.9 \pm 0.2$, $n = 5$, Figure 4B).

Table 1 pEC_{50} values for acetylcholine-induced vasodilation in foetal (8 day pre-term and 2 day pre-term), neonatal (0–12 h post-natal and 4 days post-natal) and adult rabbit pulmonary conduit arteries: influence of experimental oxygen tensions

Percentage of oxygen used to aerate the vessels	*3% O_2*	n	*16% O_2*	n	*95% O_2*	n
8 day pre-term	no response	6	6.96 ± 0.15†††	6	no response	5
2 day pre-term	7.19 ± 0.06***	6	7.40 ± 0.10	6	6.46 ± 0.20†††	6
0–12 h			no response	12		
0–12 h + SOD			6.38 ± 0.16	6		
4 day			7.38 ± 0.20	7	7.20 ± 0.10	6
Adult			7.73 ± 0.04	5	7.58 ± 0.10	5

n, number of animals; + SOD, in the presence of 50 u ml^{-1} superoxide dismutase. Data are shown as means ± s.e.mean. Statistical analysis was by ANOVA, vs adult ***$P < 0.001$; vs 3% O_2 †††$P < 0.001$.

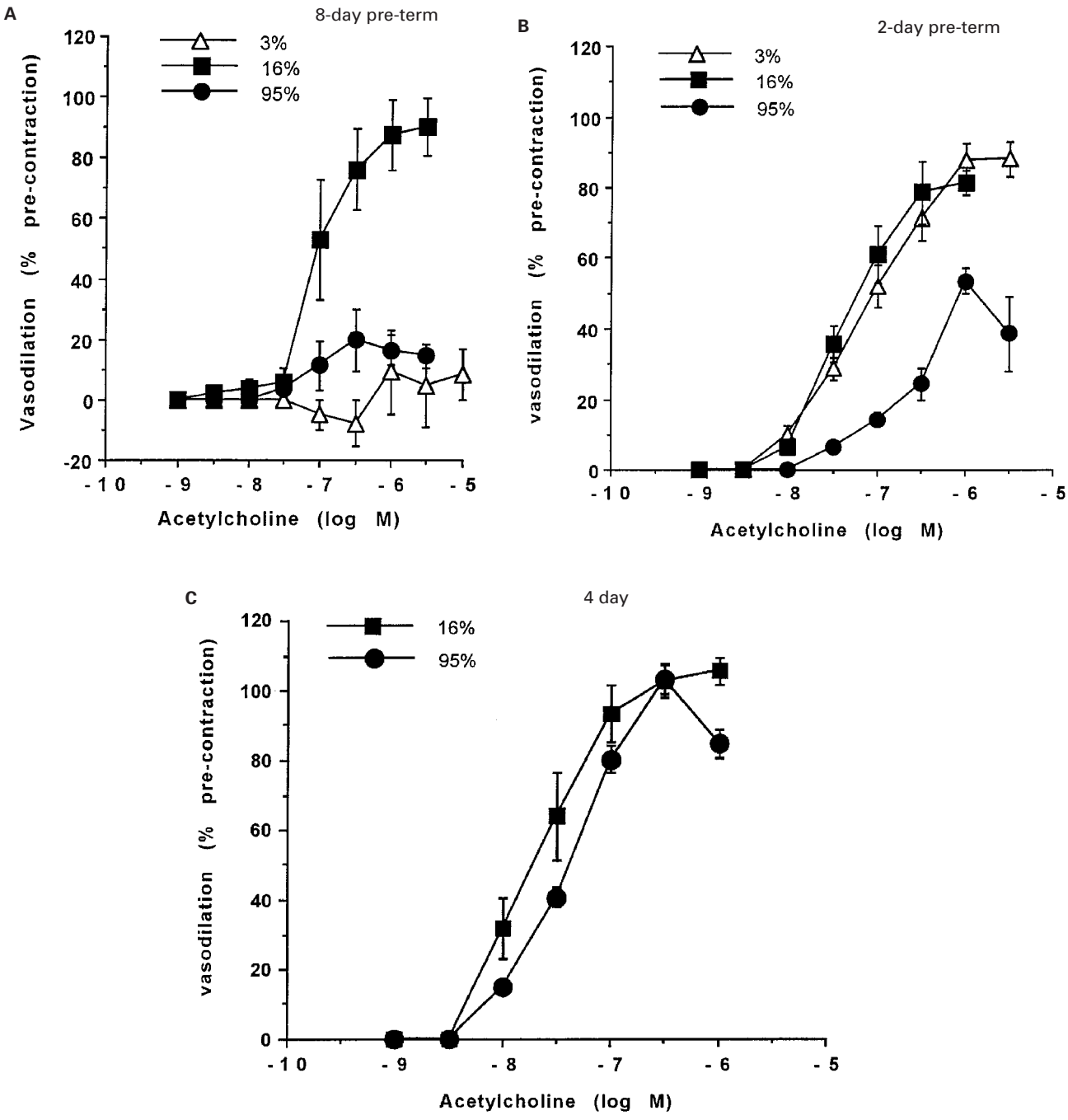

Figure 2 The effect of oxygen concentration on ACh-induced vasodilation in rabbit pulmonary conduit arteries. (A) 8 day pre-term foetal rabbits ($n=6$, pre-contracted with endothelin-1). (B) 2 day pre-term foetal rabbits ($n=6$, pre-contracted with phenylephrine). (C) 4 day old rabbits ($n=6$, pre-contracted with phenylephrine). Vasodilation is expressed as % reversal of pre-constriction. Each point represents mean ± s.e.mean.

Effect of superoxide anion generation on basal NO activity

In 2 day pre-term vessels, hypoxanthine (HX) (0.1 mM/ xanthine oxidase (XO) (15 mu ml^{-1}) with or without SOD had no effect on phenylephrine-induced contraction (Figure 5A). L-NAME significantly potentiated the phenylephrine-induced contraction in these vessels whilst neither TETA (1 mM) nor SOD itself had any significant effect (Figure 5A).

In 0–12 h vessels, neither HX/XO, L-NAME nor TETA affected phenylephrine-induced contraction whilst this was significantly reduced by SOD (Figure 5B). This attenuation was absent in the presence of L-NAME (data not shown).

In the 4 day old vessels, HX/XO significantly increased phenylephrine-induced tone and this effect was reversed in the presence of SOD (Figure 5C). L-NAME potentiated the phenylephrine-induced contraction as did administration of TETA whilst SOD alone had no effect (Figure 5C).

In the adult rabbit arteries, HX/XO (with or without SOD), L-NAME, TETA and SOD did not significantly affect the phenylephrine-induced contraction (Figure 5D).

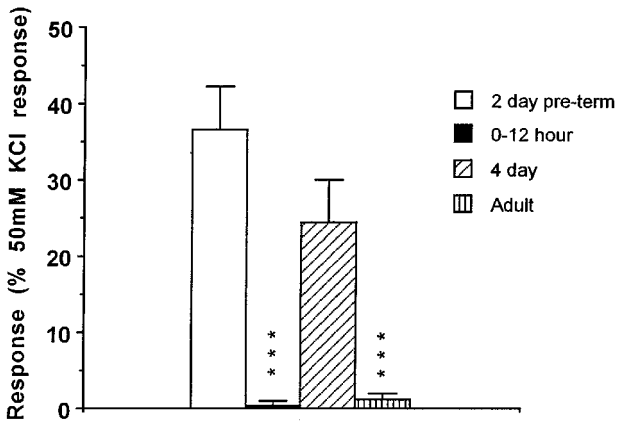

Figure 3 The effect of L-NAME (100 μM) on basal tone in endothelium intact, pulmonary conduit artery rings from (left to right) 2 day pre-term foetal ($n=4$), 0–12 h ($n=6$), 4 day ($n=6$), and adult ($n=4$) rabbits. Response expressed as % of response to 50 mM KCl. Statistical analysis by one-way ANOVA $***P<0.001$ compared with 2 day pre-term foetal response. Each point represents mean ± s.e.mean.

Effect of superoxide anion generation on ACh-induced NO activity

In 2 day pre-term vessels, HX/XO significantly inhibited ACh-induced vasodilation and this effect was reversed by SOD (Figure 6A). Pre-treatment with TETA to inhibit endogenous Cu-Zn SOD, did not increase the ability of HX/XO to inhibit ACh-induced vasodilation.

In 4 day vessels and adult vessels, HX/XO significantly inhibited ACh-induced vasodilation and this effect was reversed by SOD. Pre-treatment with TETA increased the ability of HX/XO to inhibit ACh-induced vasodilation (Figure 6B and C).

Discussion

Endothelium-dependent vasodilation: changes with development

Our results show that ACh-induced vasodilation changes markedly with developmental age, in rabbit conduit pulmonary arteries. ACh-induced vasodilation was not apparent in the 8 day pre-term foetal vessels (bubbled with 3% O_2) but by 2 days pre-term vasodilation was substantial. ACh-induced vasodilation was absent in the 0–12 h vessels but was restored by 4 days and still evident in adults.

The loss of ACh-induced vasodilation in pulmonary arteries immediately after birth is in agreement with the work of others in the pig and sheep (Liu *et al*., 1992; Steinhorn *et al*., 1993). This could reflect changes in expression of eNOS, ACh-induced prostaglandin release, changes in muscarinic receptor number or decreased sensitivity of the tissue to NO at birth (Liu *et al*., 1992; Steinhorn *et al*., 1993). Our results show that all vessels were sensitive to NO as they were fully dilated by 1 μM sodium nitroprusside. Deficiencies in endothelium-dependent vasodilation at birth are unlikely to be due to a lack of eNOS as several groups have shown high levels of immunoreactivity for eNOS in foetal and neonatal pulmonary arteries (Halbower *et al*., 1994; Hislop *et al*., 1995). We show that ACh-induced vasodilation was present in the late term foetus and at 4 days which suggests that NO activity is transiently compromised immediately after birth.

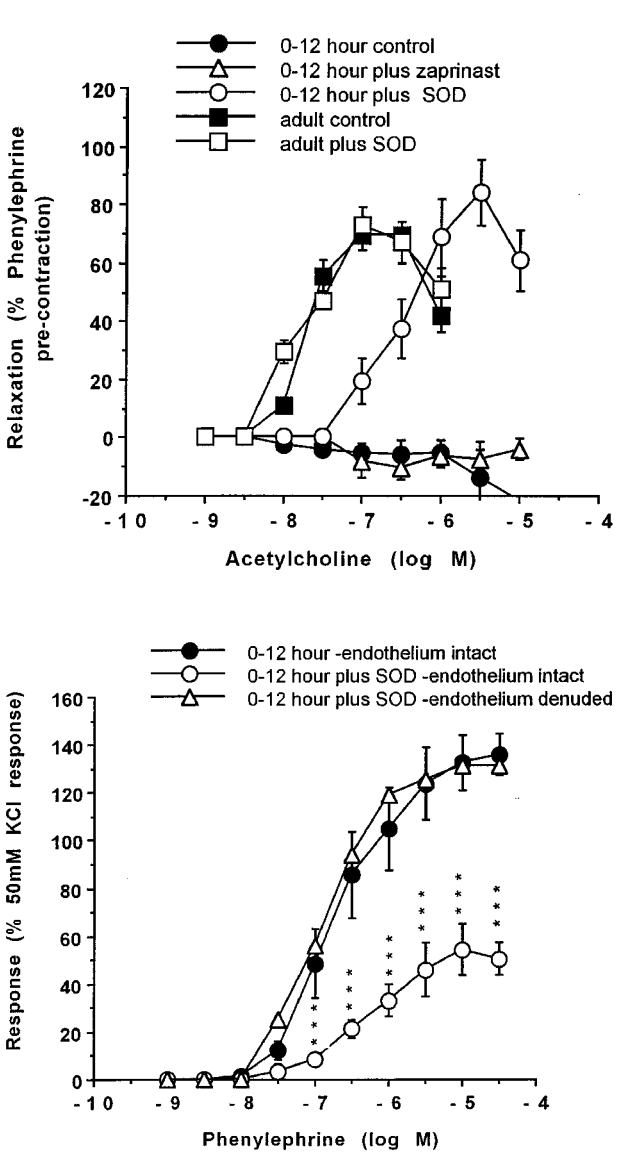

Figure 4 (A) Effect of superoxide dismutase (50 u ml^{-1}) and zaprinast (1 μM) on ACh-induced vasodilation in pulmonary artery rings, precontracted with phenylephrine, from 0–12 h rabbits, and superoxide dismutase on ACh-induced vasodilation in adult vessels. Controls: $n=12$, 0–12 h; $n=5$, adult; in the presence of SOD: $n=6$, 0–12 h and adult; in the presence of zaprinast : $n=6$. Vasodilation is expressed as % reversal of pre-constriction. Each point represents mean ± s.e.mean. (B) Effect of superoxide dismutase (50 u ml^{-1}) on phenylephrine induced contractions in endothelium intact ($n=6$) and endothelium-denuded, ($n=5$) 0–12 h rabbit pulmonary conduit arteries. Each point represents mean ± s.e.mean. Statistical analysis by one-way ANOVA $***P<0.001$ compared with control vessels.

Cyclic GMP is hydrolyzed by PDE5 in the adult rat pulmonary arterial circulation (MacLean *et al*., 1997), cyclic GMP-specific PDE5 gene expression is present in the perinatal lung (Kinsella *et al*., 1995; Ziegler *et al*., 1995) and PDE5 activity is increased in 1 day old rat lung (Sanchez *et al*., 1998). Increased PDE5 activity could, therefore, prevent cyclic GMP accumulation which would compromise NO dependent vasodilation. In this study, however, zaprinast, a PDE5 inhibitor, did not restore ACh-induced vasodilation in the 0–12 h vessels ruling out this possibility. Zaprinast did not cause a change in vascular tone confirming that there was no influence of basal cyclic GMP on cyclic vascular tone in these vessels.

Influence of superoxide dismutase

Superoxide anions interact with NO, rendering NO inactive and reducing its ability to vasodilate (Rubanyi & Vanhoutte, 1986; Gryglewski *et al*., 1986). Here we show that the superoxide anion scavenger SOD revealed an ACh-induced vasodilation in the 0–12 h arteries which was of the same magnitude as the vasodilation observed in the adult vessels. Hence, an excess of superoxide anion production at birth may compromise NO activity. The arteries from the late foetus developed sustained contractions in the presence of L-NAME highlighting a significant contribution of endogenous NO to basal tone in these vessels. 0–12 h arteries did not contract to L-NAME, and the observation that L-NAME did constrict 4 day vessels, again suggests that basal NO was compromised in the 0–12 h arteries.

Influence of superoxide anions and SOD on basal NO activity

We investigated further if endogenous superoxide anions were compromising basal NO activity in the 0–12 h vessels by examining the effect of SOD on phenylephrine-induced contraction. SOD attenuated phenylephrine-induced contraction in these vessels, suggesting that there was indeed basal release of superoxide anions compromising NO activity. The relaxation of these vessels by SOD was inhibited by L-NAME and endothelium removal, suggesting that SOD removed endothelium-derived superoxide anions which were, in turn destroying basally produced NO. There was no effect of HX/XO, L-NAME, or TETA on the phenylephrine-induced contraction in the 0–12 h vessels suggesting the absence of basally produced NO and SOD. Hence, these vessels were not protected against the destruction of NO by the production of

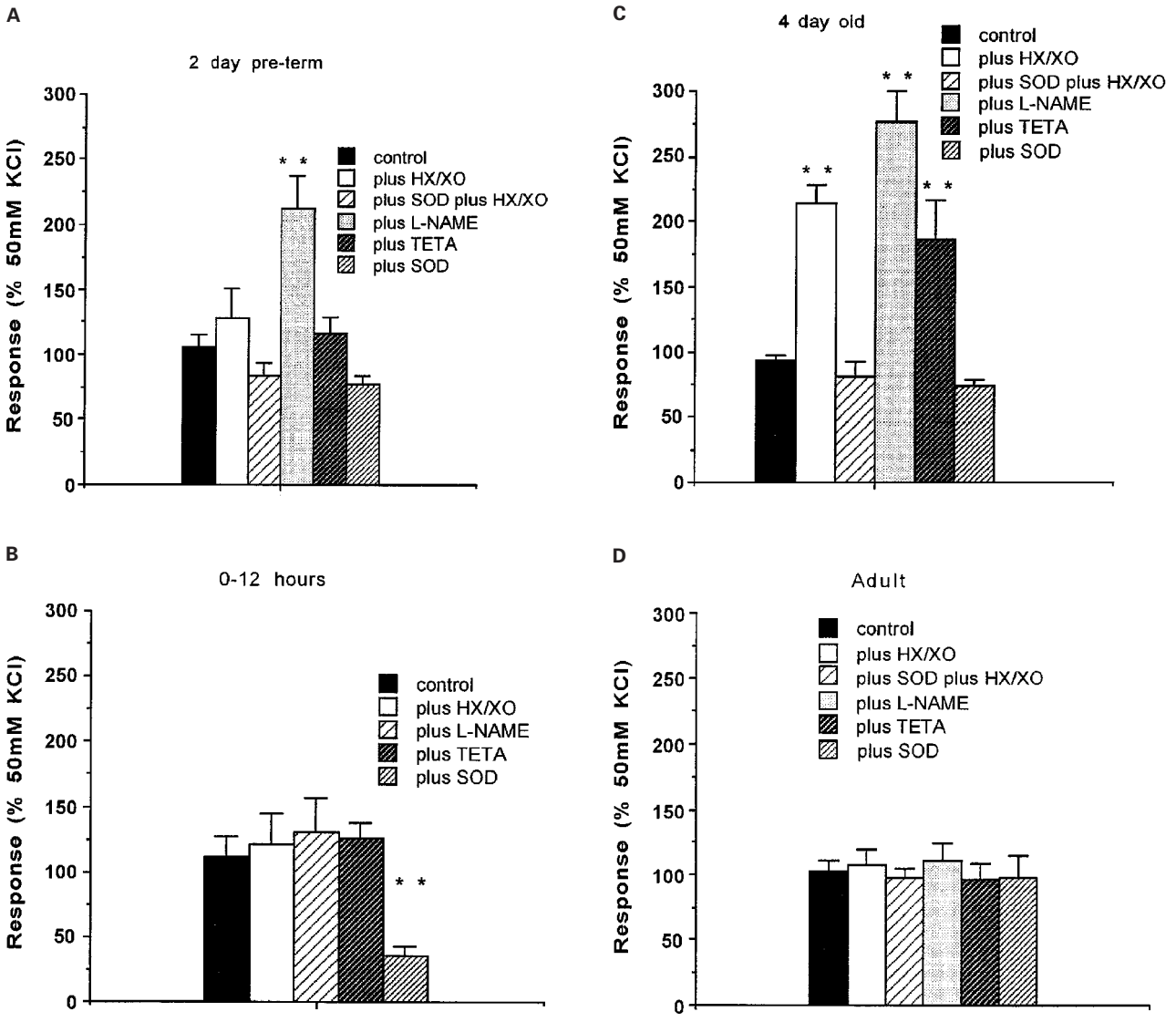

Figure 5 Effect of superoxide anion generation on phenylephrine-induced tone in (A) 2 day pre-term foetal, (B) 0–12 h; (C) 4 day, and (D) adult rabbit pulmonary conduit arteries. From left to right: control; plus hypoxanthine (HX) (0.1 mM)/xanthine oxidase (XO) (15 mu ml^{-1}); plus SOD (50 u ml^{-1}) plus HX (0.1 mM)/XO (15 mu ml^{-1}); plus L-NAME (0.1 mM) ($n=6$); plus 1 mM triethylenetetramine (TETA); plus SOD (50 u ml^{-1}) ($n=6$). All groups, $n=6$. Responses are expressed as % of the response to 50 mM KCl. Statistical analysis by one-way ANOVA $^{**}P<0.01$ compared with control phenylephrine response. Each point represents mean ± s.e.mean.

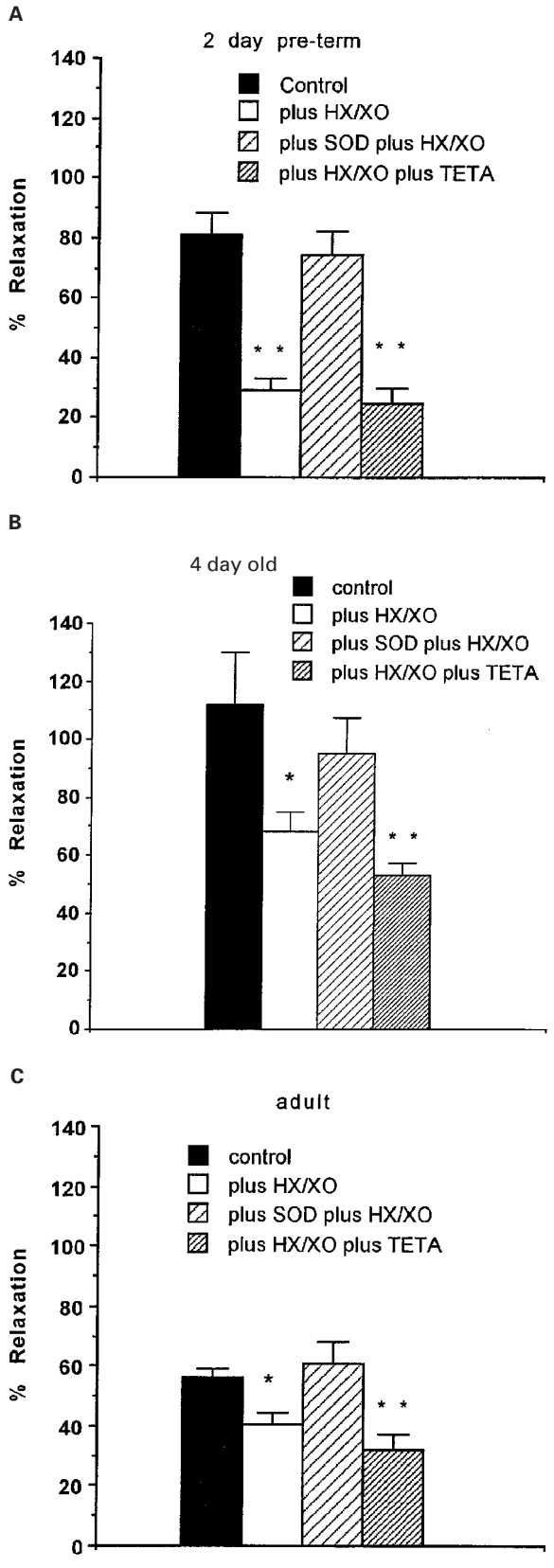

Figure 6 Effect of superoxide anion generation on ACh-induced vasodilation in phenylephrine pre-contracted pulmonary arteries from 2 day pre-term foetal (A), 4 day old (B), and adult (C) rabbit endothelium-intact pulmonary conduit arteries. From left to right, Control; plus hypoxanthine (HX)(0.1 mM)/xanthine oxidase (XO) (15 mu ml^{-1}); plus SOD (50 u ml^{-1}) plus HX (0.1 mM)/XO (15 mu ml^{-1}); plus HX (0.1 mM)/XO (15 mu ml^{-1}) plus 1 mM triethylenetetramine (TETA). All groups $n=6$. Statistical analysis by one-way ANOVA; $*P<0.05$, $**P<0.01$ compared with control phenylephrine response. Vasodilation is expressed as % relaxation of pre-constriction. Each point represents mean ± s.e.mean.

superoxide anions. This would contribute to the failure of these vessels to vasodilate to ACh.

In the 2 day pre-term vessels, superoxide generation by HX/XO did not affect the phenylephrine-induced contraction. This was surprising as phenylephrine-induced contraction was potentiated by L-NAME, clearly demonstrating basal NO activity. The copper chelating compound TETA has been shown to be an inhibitor of Cu-Zn SOD (De Man *et al.*, 1996). It is unlikely that these foetal vessels contain a rich source of endogenous SOD which would protect NO from destruction by superoxide anions as TETA had no effect on phenylephrine-induced tone. Furthermore, research has shown that baseline levels of antioxidant enzymes are significantly reduced in foetal, compared to newborn rat lungs (Chen *et al.*, 1994). In the foetal-placental vasculature, the interaction between NO and superoxide anion has been shown to generate a vasodilator, thought to be peroxynitrite (Holcberg *et al.*, 1995). One possibility therefore, could be that, in these foetal vessels, exogenous superoxide anion, generated from HX/XO, interacts with the basally released NO forming peroxynitrite or another vasodilator substance which counteracts any vasoconstrictor effects of HX/XO. SOD itself had no effect on phenylephrine-induced tone in the foetal vessels, suggesting that there was no basal production of superoxide anions.

A very different profile of basal NO/SOD activities was observed by 4 days. Here, superoxide generation with HX/XO resulted in increased phenylephrine-induced contraction. Both SOD and L-NAME pre-treatment prevented these effects of HX/XO, suggesting the effect was due to the production of superoxide anions destroying basal NO. Basal NO production is clearly present as L-NAME markedly potentiated the phenylephrine-induced contraction and caused contraction on its own. TETA caused an increase in phenylephrine-induced contraction suggesting that endogenous SOD activity is present in these vessels, protecting them from endogenous superoxide anion production. SOD itself did not influence phenylephrine-induced contraction, suggesting that the elevated basal production of superoxide anions evident in the 0–12 h vessels no longer exist by 4 days. There must be further maturation in this system between 4 days and adulthood, as in the adult vessels, there was no effect of HX/XO (with or without SOD), L-NAME or SOD itself on responses to PE. This suggests that there is no basal production of NO in these vessels and therefore no longer any evidence of the influence of endogenous SOD activity on basal NO activity.

Influence of superoxide anion activity on ACh-induced NO production

In the 2 day pre-term vessels, HX/XO markedly reduced ACh-induced vasodilation, and this effect was inhibited by SOD. Hence, the ACh-induced NO activity was sensitive to destruction by superoxide anions. Inhibition of SOD with TETA did not, however, enhance the effect of HX/XO suggesting that endogenous Cu-Zn SOD is not present in these vessels. Similar results were obtained in the 4 day and adult vessels except that there was evidence of a protective effect of endogenous SOD by 4 days in that TETA enhanced the ability of HX/XO to reduce the effects of ACh-induced NO release. The magnitude of ACh-induced vasodilation was reduced in adults compared with at earlier age points. This was not due to superoxide accumulation as exogenous SOD had no effect on ACh-induced vasodilation in adult vessels.

Pulmonary hypertension in the neonate

In some newborn babies, the normal decline in pulmonary vascular resistance and increased pulmonary blood flow does not occur resulting in the development of persistent pulmonary hypertension of the newborn (PPHN). This occurs in more than 1 per 1000 live births and results in high morbidity and mortality with a high percentage of deaths occurring before 3–4 days of age (Hageman *et al*., 1984; Steinhorn *et al*., 1995). Our study indicates that pulmonary arteries in the rabbit are particularly unprotected against vasoconstrictors immediately after birth due to the absence of the protective effects of endogenous SOD against both basal and insult-induced superoxide production. These protective mechanisms are, however, in place by 4 days. If this situation exists in human neonates it may explain why there is a high mortality to PPHN before 3–4 days of age. Absence of the protective actions of NO immediately after birth would render the immediate postnatal pulmonary circulation sensitive to the effects of circulation vasoconstrictors such as ET-1. Indeed, plasma ET-1 levels are high at birth and further elevated in PPHN (Rosenberg *et al*., 1993; Malamitsi-Puchner *et al*., 1993; Endo *et al*., 1996). In addition, we have recently shown that 0–24 h rabbit pulmonary resistance arteries are extremely sensitive to ET-1 and noradrenaline (Docherty & MacLean, 1998) and that 5-HT-induced vasodilation is also absent in these vessels (Morecroft & MacLean, 1998). Inhaled NO itself has been shown to be effective in reducing pulmonary arterial pressure in PPHN (Kinsella *et al*., 1993). The results of our study invite the possibility that manipulation of the superoxide dismutase/superoxide anion system, to protect endothelium-dependent vasodilation, may be a useful therapeutic strategy in the treatment of PPHN.

Effect of oxygen tension

A great variety of oxygenation conditions have been used in previous studies of sheep and pig perinatal pulmonary arteries (e.g. Steinhorn *et al*., 1993; Abman *et al*., 1991; Liu *et al*., 1992; Shaul *et al*., 1992; 1993). In the present study, no ACh-induced vasodilation was observed in the 8 day pre-term vessels when the vessels were bubbled with 3% oxygen whilst there was ACh-induced vasodilation when the oxygen was raised to 16%. This is in keeping with the results of Shaul *et al*. (1992) who demonstrated that a reduction in oxygen tension inhibits ACh-induced vasodilation and cyclic GMP accumulation in the sheep foetal pulmonary artery. Physiologically, this would be advantageous as it would serve to maintain vasoconstriction of the foetal pulmonary circulation *in utero*. ACh-mediated vasodilation was present in vessels from 2 day pre-term foetus at 3% oxygen and this was not affected by raising the oxygen to 16%. This reflects the rapid changes that occur in the pulmonary artery during late gestation to optimize the capacity for NO production at the time of birth (Shaul, 1997). This includes an upregulation of eNOS expression which may be influenced both by oxygen and oestrogen (Shaul, 1997). 95% O_2 virtually abolished ACh-induced vasodilation in the 8 day pre-term foetal vessels in keeping with observations that extreme hyperoxia can destroy NO by the generation of superoxide anions (Rubanyi & Vanhoutte, 1986; Gryglewski *et al*., 1986). This effect of 95% O_2 was reduced by 2 days pre-term and by 4 days, ACh-induced relaxation was not influenced by raising oxygen from 16–95%. This may reflect the developmental changes in the activity of endogenous SOD or other antioxidant enzymes.

In conclusion, at low oxygen tensions equivalent to those *in utero*, ACh-induced vasodilation of the rabbit conduit pulmonary artery is absent 8 days pre-term but present by 2 days pre-term. Activity of basal and ACh-induced NO is, however, compromised in the 0–12 h neonate, possibly due to accumulation of superoxide anions and deficiencies in SOD activity. These deficiencies are restored by 4 days. Experimental oxygen tension markedly influence ACh-induced vasodilation in rabbit foetal pulmonary arteries.

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