

Role of NO in recovery from neonatal hypoxic pulmonary hypertension

R M R Tulloh, A A Hislop, S G Haworth

Abstract

Background—The management of sick newborn infants who have sustained a hypoxic insult is a common clinical problem but relatively little is known about the recovery process. The aim of this study was to investigate this process in newborn piglets.

Methods—Thirty five newborn piglets were exposed to chronic hypobaric hypoxia for three days, either from birth, three or 14 days of age, and were allowed to recover for one, three, or six days. Control animals of relevant age were also studied. The heart weight ratio and pulmonary arterial muscularity were measured. Endothelial dependent and independent relaxation of the isolated intrapulmonary conduit arteries was determined in classical organ chamber studies, together with measurement of basal and stimulated cGMP accumulation.

Results—After six days of recovery the hypoxia induced right ventricular hypertrophy and pulmonary arterial medial hypertrophy had decreased in all animals but values were still abnormal in the two younger age groups. Relaxation was still impaired during the first three days of recovery in all groups, had normalised by six days in the two youngest groups, but relaxation (both endothelium dependent and independent) remained impaired in older animals. In these older animals basal nitric oxide (NO) production and basal and stimulated cGMP accumulation was normal.

Conclusions—The recovery of the smooth muscle cells lags behind that of the endothelial cells. A normal stimulated increase in cGMP with reduced relaxation suggests an altered threshold for cGMP effected relaxation. These findings help to explain why some hypoxic infants require protracted NO therapy.

(Thorax 1999;54:796–804)

Keywords: nitric oxide; porcine pulmonary hypertension; endothelium; smooth muscle cells

Pulmonary hypertension in the newborn infant remains a significant clinical problem with a mortality of 20–50%.¹ Hypoxia is a frequent cause of persistent pulmonary hypertension, usually associated with parenchymal lung disease such as the respiratory distress syndrome, meconium aspiration or congenital abnormalities of lung development such as

pulmonary hypoplasia. Of those who survive, a few develop cor pulmonale. Other infants appear to do well, but if they do die in infancy, histological examination of the lungs reveals pulmonary arterial medial hypertrophy, indicating a persistent increase in pulmonary arterial pressure.² These findings indicate that apparent clinical recovery from hypoxia in infancy does not necessarily imply a return to normality.

Neonatal pulmonary hypertension is treated with inhaled nitric oxide (NO) with varying degrees of success. We assume that we supplement an hypoxia induced deficiency of NO but experimental studies have demonstrated, in addition, attenuated endothelium independent relaxation in clinically hypoxic newborn piglets.³ Once the hypoxic insult has been removed, we do not understand the recovery process and it is this which will determine the response to treatment. In this study we have therefore studied endothelium dependent and independent relaxation in the intrapulmonary arteries of neonatal piglets which had been exposed to chronic hypobaric hypoxia for three days and then allowed to recover in room air.

In the intrapulmonary arteries of the healthy newborn piglet endothelium dependent and independent relaxation is immature at birth.³ Conduit pulmonary arteries fail to relax to acetylcholine until three days of age. Endothelium independent relaxation to NO is present at birth, although relatively poor. Both endothelium dependent and independent relaxation improve gradually during the first two weeks of life. We have previously shown that exposing piglets to chronic hypoxia from birth prevented the normal postnatal development of endothelium dependent relaxation, while hypoxia inhibited or attenuated the normal newly established response in animals first exposed after the third day of life. Chronic hypoxia also attenuated endothelium independent relaxation. We hypothesised that, on recovery, like the response to hypoxia itself, the speed and extent of recovery would depend on the age at which the animal sustained the hypoxic insult. Animals were therefore exposed to hypoxia at different ages during the first three weeks of life before being allowed to recover in room air. Endothelium dependent relaxation to acetylcholine and endothelium independent relaxation to NO was studied in isolated intrapulmonary arteries using classical organ chamber pharmacology and the basal and stimulated accumulation of guanosine 3',5'-cyclic monophosphate (cGMP) was measured to find out whether any persistent impairment of relaxation might be attributed to failure to accumu-

Vascular Biology & Pharmacology Unit,
Institute of Child Health, London
WC1N 1EH, UK
R M R Tulloh
A A Hislop
S G Haworth

Correspondence to:
Professor S G Haworth.

Received 26 November 1998
Returned to authors
2 March 1999
Revised version received
19 April 1999
Accepted for publication
8 June 1999

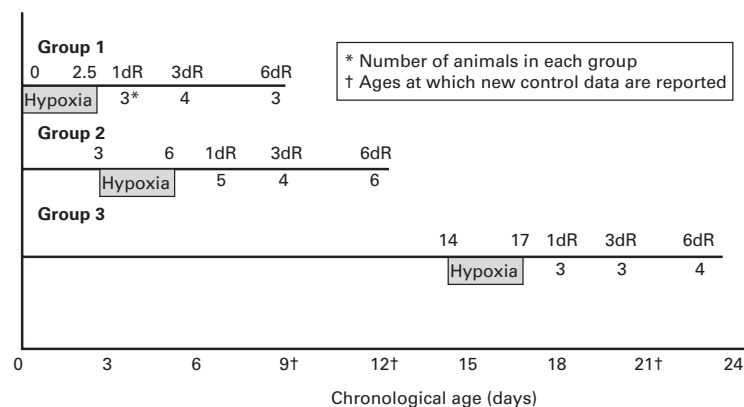


Figure 1 Flow chart of experimental protocol. dR = days of recovery.

late sufficient cGMP in the arterial smooth muscle cells. We also hypothesised that the fall in pulmonary arterial pressure would be reflected in a decrease in right ventricular weight and pulmonary arterial muscularity. This we assessed post mortem using quantitative morphometric techniques.

Methods

EXPERIMENTAL DESIGN

Piglets were delivered from 10 pregnant Large White sows which farrowed normally at term. One group of piglets was exposed to hypobaric hypoxia at 50.8 kPa from birth until 2.5 days of age, a second group was exposed to hypoxia from three to six days of age, and a third group was exposed to hypoxia from 14 to 17 days.³ In the hypobaric chamber the internal temperature (25°C) and light were controlled, the air pressure was maintained at 50.8 kPa, and the fraction of carbon dioxide did not rise above 0.4% as assessed by mass spectrometry of the exhaust gases. Animals were sacrificed by means of a lethal injection of sodium pentobarbitone (100 mg/kg). Details of this model have been described by Hislop *et al.*⁴

Some animals were killed immediately after removal from the hypobaric chamber following each of the periods of exposure (n = 14). The pharmacological findings in the pulmonary arteries of these animals and of the 19 age matched controls have been reported previously.³ For the purposes of the present study 35 other animals were allowed to recover in room air and were sacrificed one, three, or six days later (n = 3–6 for each recovery period). Additional control animals of appropriate ages (nine, 12, and 21 days) for the recovery periods were also studied (n = 12; fig 1). Thus, findings are reported on a total of 80 animals, 33 of which have previously been published but are included here as baseline data.³

The work carried out comprised structural studies on the heart and lungs to assess the severity of pulmonary hypertension, pharmacological organ bath experiments on isolated intrapulmonary arteries, and the biochemical determination of basal and stimulated cyclic GMP generation.

STRUCTURAL STUDIES

Assessment of the severity of pulmonary hypertension

After sacrifice, in each animal the heart was fixed for at least one week in 10% buffered formal saline. The left ventricle plus septum (LV+S) and the right ventricle (RV) were weighed to give the heart weight ratio [(LV+S)/RV].⁵ Lung tissue was also fixed in 10% buffered formal saline, processed into paraffin wax, and sections were cut at 4 µm and stained with either haematoxylin and eosin or Miller's elastic van Gieson stain for light microscopic measurement of wall thickness. Pulmonary arterial medial wall thickness was measured in vessels less than 100 µm in diameter and expressed as a percentage of the external diameter.^{3,6}

PHARMACOLOGICAL STUDIES

Tissue preparation for pharmacology studies

The heart and lungs were removed en bloc and transferred to pre-gassed Krebs-Ringer bicarbonate solution at room temperature. The middle portion of the axial intrapulmonary arteries from the lower lobes accompanying the small bronchi was dissected free from the surrounding lung parenchyma and cut into rings (2.5–3 mm length), taking care not to touch the luminal surface. Care was taken to obtain the rings from the equivalent portion of the pulmonary artery at each age. The external diameter ranged from 2 mm in the neonate to 3 mm at 23 days.

Organ bath experiments

Pulmonary artery rings were studied in pairs with and without endothelium. Each ring was mounted in a 5 ml organ bath between two wires, one fixed and the other attached by surgical silk to a Grass FT03 transducer. The output was to a Grass model 7 polygraph at a paper speed of 5 mm/min and sensitivity setting of 0.5 g/cm, and also to an on-line computerised analysis system (MacLab/8, AD Instruments) at a sampling frequency of 2 Hz. The bathing solutions were gassed with 95% O₂ and 5% CO₂ at a constant temperature of 37°C. Indomethacin (10 µM) was added for all experiments and this did not change the basal tone. All rings were first exposed to potassium chloride (KCl) in order to ensure their viability, and were then exposed to prostaglandin F_{2α} (PGF_{2α}).

Each intrapulmonary artery ring was pre-contracted with its own EC₅₀ of PGF_{2α} before being exposed to cumulative log molar increments of either acetylcholine (ACh) (10 nM to 0.1 mM), NO (3 nM to 30 µM), sodium nitroprusside (SNP) (1 nM to 0.1 mM), or to a single dose of the phosphodiesterase inhibitor M+B 22948 (Zaprinast) (10 µM)⁷ or L-nitro monomethyl arginine acetate (L-NMMA), a NOS inhibitor (30 µM).⁸ The response to each substance was expressed as a percentage of PGF_{2α} induced tension. Using pulmonary arteries from both lungs, four different agonists were studied in each animal. Time dependent controls were also studied. The effect of each agonist was determined in a minimum of three

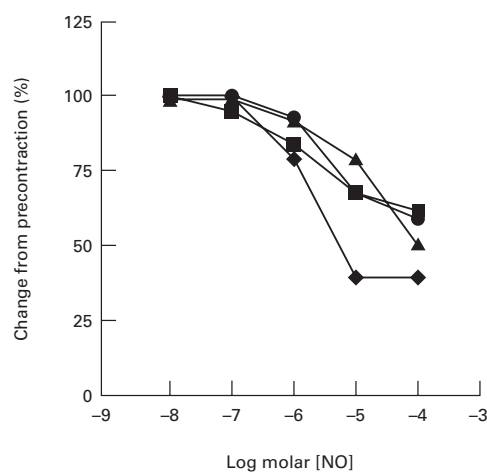


Figure 2 Cumulative response curves to NO in four pulmonary artery rings from four different animals killed after three days of recovery in room air after hypoxic exposure from birth to 2.5 days.

rings (each from a different animal) at each recovery time point following each period of hypoxic exposure. At the end of each experiment all the rings were exposed to papaverine (0.1 mM) to abolish any residual tone. This end point was taken as 100% relaxation, against which other relaxant responses were judged. Figure 2 illustrates a cumulative dose response curve to NO from four individual rings from different animals (birth to 2.5 days of hypoxia + three days of recovery) to show the variability between cases.

The rings were then fixed in 2.5% glutaraldehyde. 1 μ m sections embedded in epoxy resin were stained with toluidine blue and examined histologically to confirm the presence or absence of endothelium.

BASAL AND STIMULATED ACCUMULATION OF GUANOSINE 3',5'-CYCLIC MONOPHOSPHATE (cGMP)

Determinations were carried out using radioimmunoassay in weighed rings with endothelium from segments of intrapulmonary arteries adjacent to those used in the organ bath studies. The same bathing solutions, including indomethacin, were used for the cGMP determinations as for the pharmacology studies. Basal cGMP accumulation and cGMP accumulation in response to ACh, SNP, Zaprinast, and NO were determined. For the response to ACh and NO, preliminary studies showed that the peak response in cGMP accumulation occurred after 30 seconds exposure and at doses of 10 μ M and 3 μ M, respectively. The peak response in cGMP accumulation to SNP (0.1 mM) occurred at five minutes and to Zaprinast (10 μ M) at 30 minutes.³ Incubation was terminated by transferring the tissue to liquid nitrogen. The frozen tissue was thawed in 6% trichloroacetic acid and homogenised. The extracted tissues were centrifuged at 2000g for 15 minutes at 4°C. The supernatant was washed four times with five volumes of water saturated ether and then freeze dried overnight. The cGMP content was measured in each sample by radioimmunoassay using a modifica-

tion of the method of Brooker *et al.*⁹ This consisted of adjusting to pH 6.2 with sodium acetate buffer and acetylating by the addition of triethylamine and acetic anhydride. Bovine serum antibody was added followed by cGMP, 2'-O-succinyl 3-[¹²⁵I] iodotyrosine methylester. Anti-cGMP Amerlex-M rabbit antibody was added, vortexed, and incubated at room temperature for 10 minutes. Free and antibody bound cGMP were then separated by magnetic separation. The radioactivity present in each sample was determined by counting for 60 seconds in a gamma scintillation counter and compared with a standard curve. The results were expressed as fmol cGMP/mg wet tissue.

DRUGS AND SOLUTIONS

The Krebs bicarbonate Ringer solution had the composition (in mM) of NaCl 118.3, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, NaHCO₃ 25, glucose 11.1. The following drugs were used: ACh, indomethacin, PGF_{2 α} , SNP, A23187, L-NMMA (Sigma), Zaprinast (gift of Rhone-Poulenc Rorer), and NO (BDH). The cGMP radioimmunoassay kits were obtained from Amersham International (UK). Indomethacin was dissolved in 10 μ M sodium carbonate, the PGF_{2 α} and Zaprinast were dissolved in ethanol, and the A23187 was dissolved in dimethylsulphoxide (DMSO). The solvents did not exceed a concentration of more than one part in 1000 in the organ bath and, for both ethanol and DMSO, solvent controls were performed. All other drugs were dissolved in distilled water. Drug concentrations are expressed as final molar concentrations in the organ bath. The drugs and solutions were made fresh daily except for PGF_{2 α} , A23187, Zaprinast, and L-NMMA which were frozen as aliquots of 10 mM solutions at -20°C.

Nitric oxide preparation

NO was obtained by transferring the gas from a cylinder to a helium flushed glass container. Appropriate volumes (100–10 000 μ l) were transferred to glass flasks containing 100 ml distilled water which had been bubbled with helium for two hours. NO concentrations of 3 nM to 30 μ M were obtained in the organ bath by transferring the appropriate quantities and allowing for a solubility constant of 4.6 ml NO in 100 ml water. The solution was allowed to stand for two hours to allow for equilibration.¹⁰

DATA ANALYSIS

The findings in the animals recovering from hypoxia were compared with those in the previously reported hypoxic animals and in normal age matched controls from the present and previous studies.³ Data from the concentration response curves from animals recovering from hypoxia were compared with those from age matched control or hypoxic animals by an analysis of variance (ANOVA). Heart weight ratio, mean percentage arterial medial thickness, maximum response values, and cGMP accumulation values were compared by ANOVA and, where appropriate, Student's *t* test was used with Bonferroni correction. Statistical significance was taken at *p*<0.05.

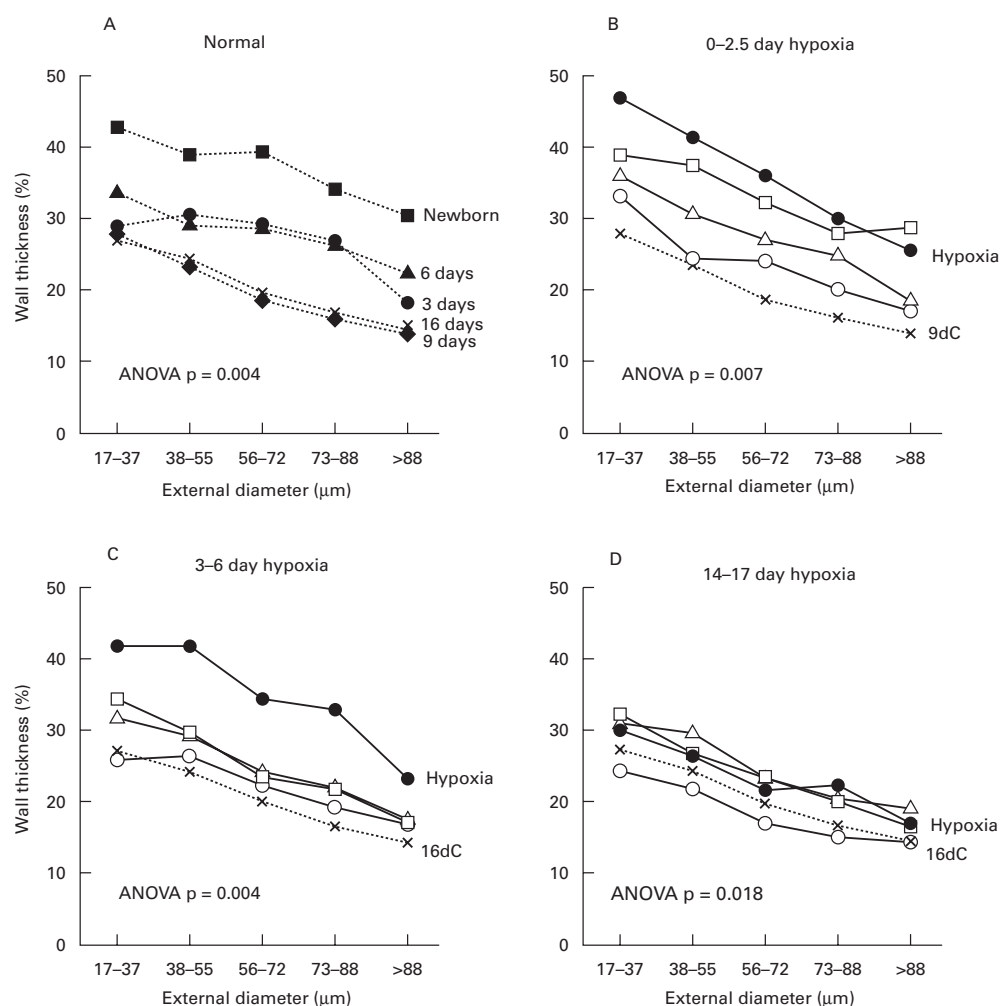


Figure 3 Percentage arterial medial thickness in vessels less than 100 μm external diameter in (A) normal animals and in animals recovering from exposure to hypoxia from (B) birth to 2.5 days, (C) 3–6 days, and (D) 14–17 days. Standard errors were less than 5% of the mean. \square = one day of recovery; \triangle = three days of recovery; \circ = six days of recovery. dC = day control.

Results

STRUCTURAL STUDIES

Assessment of right ventricular hypertrophy

Normal piglets show an increase in the heart weight ratio ((LV+S)/RV) during the first three weeks of life which is significantly reduced after exposure to hypoxia.³ During the recovery period the heart weight ratio increased. In those exposed to hypoxia from 14 to 17 days of age it increased from 2.32 to 2.65 after six days recovery and was not significantly different from the normal value at three weeks of age. In the youngest two groups the ratio had increased after six days of recovery from 1.38 to 2.04 ($p < 0.05$) in those exposed from birth, and from 1.74 to 2.43 in those exposed from three to six days of age, but these ratios were still significantly less than in age matched normal animals (2.77 (9 day olds) and 2.8 (12 day olds)), respectively ($p < 0.05$ for both comparisons).

Assessment of pulmonary arterial medial hypertrophy

We have previously described the structure of arteries less than 100 μm diameter after hypoxic exposure and have shown that the medial wall thickness became significantly greater than in

age matched normals.³ In the two youngest age groups the muscle extended into smaller arteries than normal.¹¹ On recovery from hypoxia the pattern of change was different in the three age groups (fig 3). After hypoxic exposure from birth to 2.5 days (fig 3B) the mean percentage arterial medial thickness decreased gradually, mirroring the normal decrease in wall thickness with age, but after six days of recovery the medial thickness was still greater than normal for age (9 days) ($p < 0.05$). In animals exposed to hypoxia from three to six days of age (fig 3C) there was a marked decrease in wall thickness after one day recovery ($p < 0.05$) with little further change during the next two days, followed by a further reduction to a value within normal limits for 12 day old animals by the sixth day of recovery. In animals exposed to hypoxia from 14 to 17 days of age (fig 3D) the wall thickness remained increased during the first three days of recovery, and then decreased so that the vessel walls were thinner than normal for age after six days of recovery ($p < 0.05$).

PHARMACOLOGICAL STUDIES

Examination of the toluidine blue stained sections by light microscopy confirmed that the

endothelium was present in all intact preparations and absent from those in which the endothelium had been removed.

Pharmacological studies were carried out on vessel rings from animals killed either one, three, or six days after return to normoxia. Arteries from normal animals of the same age as the experimental animals at the end of the recovery period were also studied. The results were thus compared with those previously reported in control and hypoxic animals and with those of the newly studied control animals.

Response to L-NMMA

Pulmonary artery rings with endothelium taken from normal animals show a significant enhancement of the $\text{PGF}_{2\alpha}$ induced contraction when L-NMMA is added during the first week of life, but the ability of L-NMMA to increase the $\text{PGF}_{2\alpha}$ induced contraction was inhibited after hypoxic exposure, suggesting a reduction in basal NO production.³ On recovery, the enhancement of the contractile response by L-NMMA after one and three days had not improved and was similar to that at the end of hypoxic exposure, irrespective of the age at the time of exposure. After six days of recovery, however, the enhancement of the contractile response was significantly greater than at the end of hypoxic exposure in the two younger age groups, increasing from a mean (SE) of 34 (13)% to 94 (23)% in those exposed from birth and from 35 (7)% to 122 (20)% in those exposed from three to six days ($p < 0.05$ for each age group). This response was normal for age in animals that had been exposed to hypoxia from birth (90 (10)% in nine day controls) and excessive in those exposed from three days of age where the normal value was 90 (11)% ($p < 0.05$). In the 14–17 day hypoxic group after six days of recovery basal NO release was similar to normal. Rings without endothelium showed no response to L-NMMA.

Response to ACh

Pulmonary arteries with intact endothelium taken from normal animals at birth contract in response to acetylcholine and do not relax until 2.5 days of age.^{3, 8} Rings from animals exposed to hypoxia from birth to 2.5 days still show a contractile response and, in animals exposed from three to six days and from 14 to 17 days of age the relaxant response is inhibited (fig 4).^{3, 8} After hypoxic exposure from birth, ACh stimulated relaxation was still absent after one day of recovery (at age 3.5 days), but the contractile response present at the end of hypoxic exposure was abolished (fig 4A). Relaxation occurred after three days of recovery but was less than in normal six day old animals ($p < 0.05$). Relaxation was normal for age (nine days) after six days of recovery. The pattern of recovery was different after hypoxic exposure from three to six days of age, where the relaxant response was normal after one day of recovery and remained so (fig 4B). After hypoxic exposure from 14 to 17 days of age, relaxation had improved significantly after three days ($p < 0.05$) but there was no further improve-

ment by six days of recovery, making the maximal extent of relaxation significantly less than in normal animals aged 21 days ($p < 0.05$) (fig 4C). Rings without endothelium showed no relaxation response in any group.

Response to A23187

Pulmonary arteries taken from normal animals show a significant increase in the relaxant response to A23187 during the first week of life, and hypoxic exposure inhibits the response, irrespective of the age at the time of exposure.³ After hypoxic exposure from birth the relaxant response to A23187 did not improve until after six days when it was significantly greater than at the end of hypoxic exposure ($p < 0.05$) and normal for age. After hypoxic exposure from either 3–6 days or 14–17 days of age, relaxation was still significantly less than in age matched normal animals after six days recovery ($p < 0.05$ for each). In rings without endothelium there was no relaxant response in any group.

Response to nitric oxide

Pulmonary arteries, whether with or without endothelium, taken from normal animals show a significant increase in the relaxant response to NO during the first two weeks of life and hypoxia attenuates the relaxant response at all ages.³ After hypoxic exposure from birth, maximal relaxation did not improve until after six days of recovery when relaxation was greater than at the end of hypoxic exposure ($p < 0.05$) and was normal for age (nine days) (fig 4D). After hypoxic exposure from three to six days of age the relaxant response after six days of recovery was significantly greater than at the end of hypoxic exposure ($p < 0.05$), but still less than in age matched 12 day old normal animals (fig 4E; $p < 0.05$). After hypoxic exposure from 14 to 17 days of age the relaxant response was still reduced after six days of recovery and the maximum relaxation was less than half that seen in age matched normal animals (fig 4F; $p < 0.05$). Rings without endothelium were not significantly different in their response from those with endothelium.

Response to SNP

Pulmonary arteries, both with and without endothelium, taken from control animals relax in response to SNP at birth and the response was not affected by exposure to hypoxia.³ The response remained normal during recovery, irrespective of the age at which the animals were exposed to hypoxia (data not shown).

Response to Zaprinast

Pulmonary arteries taken from normal animals show a significant increase in the relaxant response to Zaprinast during the first week of life and the response is attenuated in rings from animals exposed to hypoxia at all ages.³ On recovery, after exposure to hypoxia from birth to 2.5 and 3–6 days, the relaxant response as a percentage of $\text{PGF}_{2\alpha}$ induced precontraction after three days was similar to that in age matched normal animals and greater than the response at the end of the hypoxic period. The

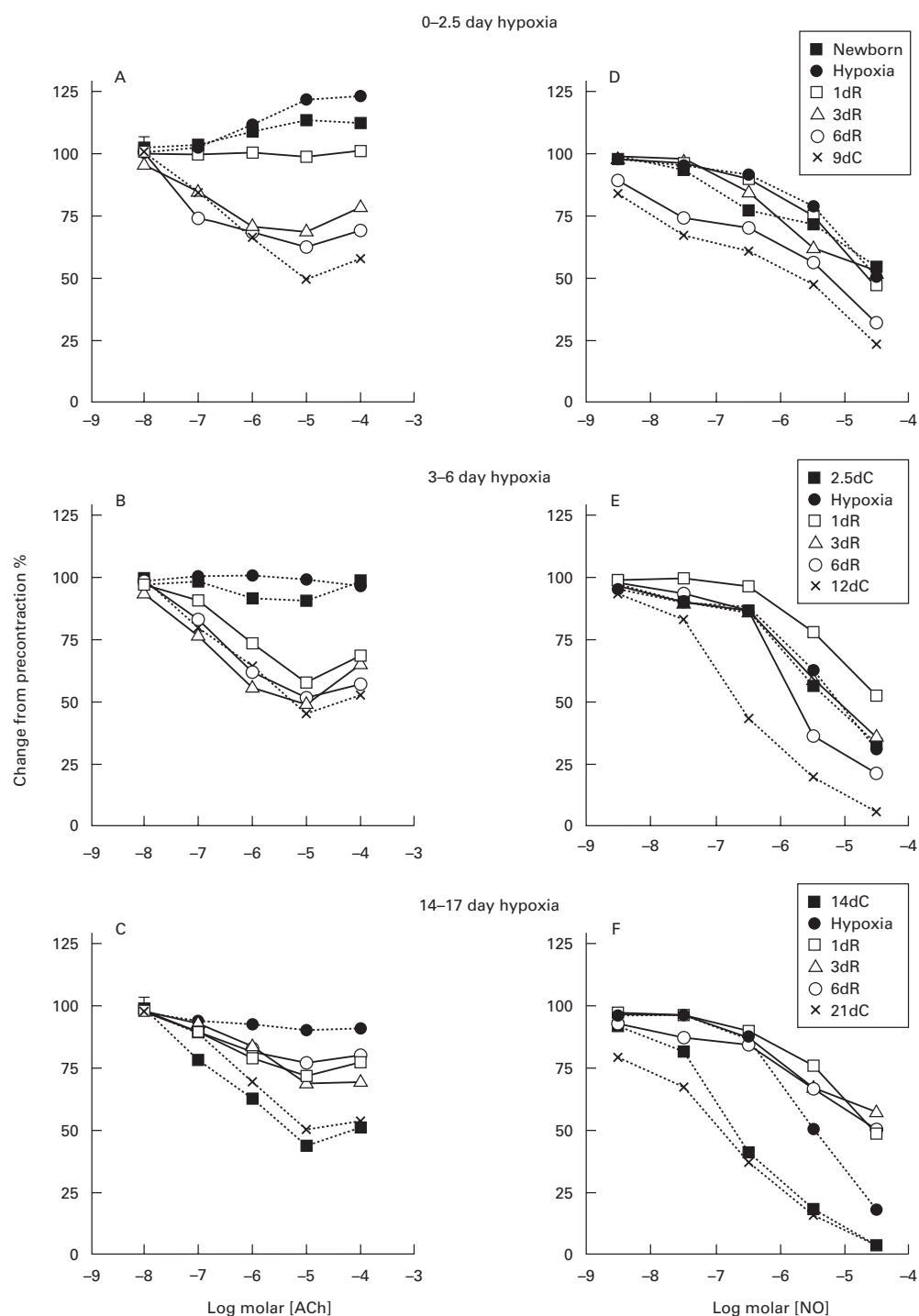


Figure 4 Mean cumulative concentration-response curves to acetylcholine (A–C) and nitric oxide (D–F) in pulmonary artery rings following precontraction with prostaglandin $F_{2\alpha}$ during recovery after hypoxic exposure from birth to 2.5 days, 3–6 days, and 14–17 days. Mean of 3–6 for each group. dR = days of recovery; dC = day control.

maximum improvement in relaxation showed a change in tone of 35 (4.5)% and 53 (9.5)% versus 7(2.4)% and 30 (4.8)%, respectively, after hypoxic exposure ($p < 0.05$ for each). After six days the relaxant response was significantly greater than normal for age, whether the animals were exposed from birth (70 (8)% versus 48 (3.1)% at nine days) or from three days of age (70 (5)% versus 54 (5)% at 12 days; $p < 0.05$ for both comparisons). After hypoxic exposure from 14 to 17 days of age the relaxant response was similar to normal after three days of recovery (46

(11)% versus 54 (5)% at 21 days). There was no difference between the response of arteries with and without endothelium.

BASAL AND STIMULATED ACCUMULATION OF CGMP

In pulmonary arteries taken from normal animals basal cGMP accumulation was greater at birth than at three days of age, after which it remained unchanged and was not altered by hypoxic exposure.³ After one day of recovery basal cGMP accumulation increased in animals

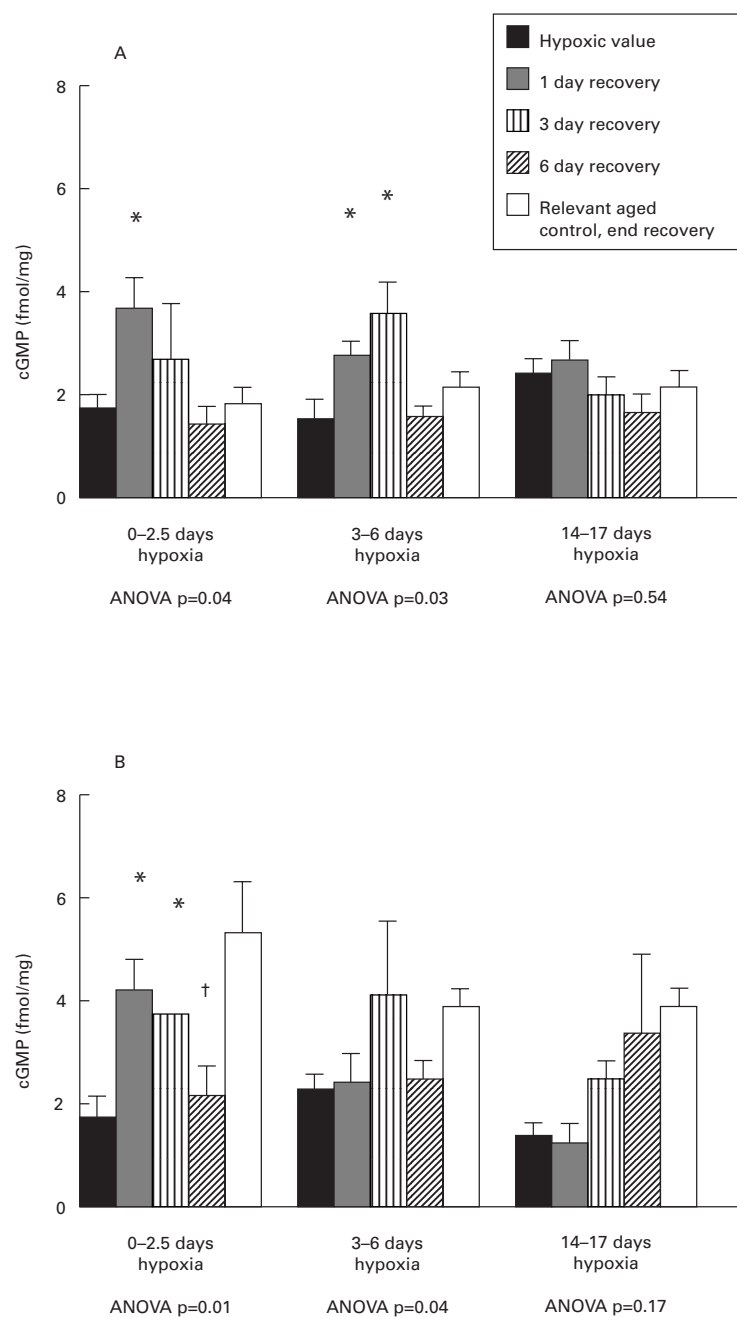


Figure 5 cGMP accumulation (A) under basal conditions and (B) in response to acetylcholine during recovery after hypoxic exposure from birth to 2.5 days, 3-6 days, and 14-17 days. Mean of 3-6 for each group. * $p < 0.05$ in comparison with hypoxic value; † $p < 0.05$ in comparison with normal.

exposed to hypoxia from birth and from three days ($p < 0.05$) and had returned to normal for age (nine and 12 days, respectively) after six days of recovery (fig 5A). In animals exposed from 14 to 17 days basal accumulation remained normal throughout the recovery period.

Chronic hypoxia inhibited the normal cGMP accumulation in response to ACh in animals exposed from three or 14 days of age. ACh has no stimulatory effect on either normal animals at 2.5 days or on hypoxic animals exposed from birth and killed at 2.5 days of age.³ During recovery, following hypoxic exposure from birth, ACh stimulated cGMP accumulation appeared greater than at the end

of hypoxic exposure after one and three days of recovery ($p < 0.05$), but the response was less than in normal age matched controls after six days of recovery (fig 5B). After exposure to hypoxia from three to six days of age ACh failed to increase cGMP accumulation over basal levels during the recovery period. After hypoxic exposure at 14 to 17 days stimulated cGMP increased progressively to become normal after six days of recovery. During hypoxic exposure³ and during the recovery period the accumulation of cGMP in response to NO, Zaprinast, and SNP was not different from that seen in the normal animals, irrespective of the age at initial exposure (data not shown).

Discussion

The aim of this study was to assess recovery in an animal model of neonatal pulmonary hypertension, particularly the ability to respond to NO. The studies were designed, in so far as it was possible, to mimic the situation in infants recovering from a hypoxic insult in the neonatal unit. The piglets exposed to hypoxia from birth were ill and cyanosed, continuing to shunt from right to left through persistent fetal channels.³ Recovery from chronic hypoxia has previously been studied in adult animals, usually rats; the animals were less ill and longer periods of hypoxia could be tolerated. Age was not a factor in these adult studies. In our developmental study, however, the morphological and pharmacological response to recovery depended upon the age at which the animals were first exposed to hypoxia. Also, improvement in morphological and functional abnormalities did not always occur in parallel.

Exposure to chronic hypobaric hypoxia from birth did not prevent the pulmonary vasculature from adapting to extrauterine life once the hypoxic insult was removed, and after six days of recovery both endothelium dependent and independent relaxation was normal. At this time, however, both the heart weight ratio and the pulmonary arterial medial hypertrophy, although improved, were not normal. By contrast, in animals first exposed to hypoxia later (from 14 to 17 days) when adaptation to extrauterine life is largely complete, relaxation was still impaired after six days of recovery although the heart weight ratio and pulmonary arterial medial hypertrophy had become normal. Following hypoxic exposure from three to six days of age, the recovery pattern was intermediate between those of younger and older animals.

In those animals kept hypoxic from birth, the arterial wall thickness remained similar to that seen in fetal life and, on recovery, wall thickness decreased over a similar time course to that seen during the first days of life in normal animals and therefore was similar to six day old but not nine day old animals. In animals made hypoxic from three to six days, the wall thickness had returned to the fetal level during hypoxic exposure but became almost normal for age after one day of recovery, indicating that these newborn arteries can undergo rapid structural remodelling. In the younger animals exposed either from birth or from three days of

age the regression of right ventricular hypertrophy lagged behind normalisation of the pulmonary arterial muscularity and the heart weight ratio was still abnormal after six days. Adult rats which had been exposed to chronic hypoxia showed a rapid early reduction in pulmonary artery pressure and right ventricular hypertrophy, followed by a slow phase of recovery,¹² and pulmonary arterial medial hypertrophy continued to regress for up to 20 weeks.¹²⁻¹⁵ A similar response was seen in cattle recovering from hypoxic exposure.¹⁶ In both the normal and hypoxic newborn lung the most dramatic structural remodelling occurs in small resistance arteries which we therefore studied during recovery. The extent to which the morphological changes in these vessels relate to the pharmacological findings in the conduit arteries is not known. However, in a previous study on lungs from chronically hypoxic newborn piglets in which the pulmonary arteries were injected with barium sulphate, the larger vessels also showed an increase in muscle thickness.¹⁷ It is these larger vessels that were used for the functional studies in the present work.

There is little information on the pharmacology of the pulmonary vasculature during recovery from chronic hypoxia,^{18, 19} particularly in the newborn period. In piglets exposed to hypoxia from birth the endothelium dependent relaxation which had been depressed during hypoxic exposure was present after three days of recovery and normal after six days in room air. The release of NO, as judged by the effect of L-NMMA on precontracted vessels, was also depressed by hypoxia³ and remained depressed after three days of recovery but had returned to normal after six days of recovery. In the same animal model earlier immunohistochemical studies had shown depression of endothelial NO synthase during hypoxic exposure, with restoration of normal expression after six days of recovery.⁴ Thus, those animals exposed from birth appear to recapitulate the normal adaptation of endothelium dependent relaxation on return to a normoxic environment.^{3, 20} Recovery of endothelial function also occurred rapidly following exposure from three to six days of life. The response to ACh became similar to normal after only one day of recovery, baseline accumulation of cGMP increased significantly after one day, and basal release of NO, as judged by the contractile response to L-NMMA, became greater than normal after six days of recovery. Chronic hypoxia appeared to have a more prolonged effect on endothelium dependent relaxation in animals exposed from 14 to 17 days, in which it was still impaired after six days of recovery. However, the studies using L-NMMA and the stimulation of cGMP by ACh suggested that NO was being produced by the endothelial cells during this time, and the earlier immunohistochemical studies had demonstrated the presence of NOS.⁴ However, ACh failed to elicit normal relaxation despite stimulating cGMP accumulation and the response to exogenous NO was also impaired. Thus, in the oldest group there was evidence to suggest persistent smooth

muscle cell dysfunction after six days of recovery. The relaxant response to Zaprinast, although normal, was less than in the younger animals. These findings might be explained by there being a transient block in the relaxant pathway distal to cGMP accumulation, possibly resulting from the excessive depolarisation of the smooth muscle membrane which occurs during hypoxic exposure.^{21, 22} This proposition might also help to explain why, in animals exposed to hypoxia from birth, the basal and ACh stimulated cGMP accumulation was normal after one day of recovery and yet both endothelium dependent and independent relaxation was still impaired. The smooth muscle cells are known to be depolarised at birth in normal piglets.²² The findings suggest that, following hypoxic exposure, the sensitivity for cGMP mediated relaxation is decreased.

In the present study the NO pathway was markedly affected by chronic hypoxic exposure in all the immature animals. The NO pathway is also vulnerable in the hypoxic adult rat. Maruyama and Maruyama¹⁹ found that the relaxant response to isoproterenol in adult rats which had been exposed to hypoxia for 10 days was normal after three days of recovery but the response to SNP was not normal after 28 days of recovery from hypoxia and the response to ACh was still impaired after 56 days. These findings appeared to be associated with a cyclo-oxygenase dependent production of a vasoconstrictor substance. In the present study the relaxant response to SNP was present at birth, was not impaired during hypoxic exposure, and remained normal on recovery. This suggests that, in these studies, SNP may be able to cause relaxation by a recognised alternative mechanism, possibly the sodium-potassium ATPase pump.²¹

In conclusion, irrespective of the age at the onset of hypoxic exposure, relaxation was impaired during the first few days of recovery. The abnormalities persisted for longer in the animals allowed to adapt normally to extrauterine life for two weeks before exposure to hypoxia and there appeared to be persistent smooth muscle cell dysfunction. By two weeks of age the rapid postnatal changes in pulmonary arterial smooth muscle cell phenotype are complete, apparently making it more difficult to reverse the abnormalities caused by hypoxic exposure.^{23, 24}

Our findings help to explain why it can be difficult to manage newborn infants recovering from a hypoxic insult. The emphasis during treatment is to improve relaxation, supplementing endogenous NO with the inhaled gas, with or without addition of a phosphodiesterase inhibitor to increase cGMP accumulation. The results of this study show a heightened threshold for cGMP mediated relaxation, indicating smooth muscle cell dysfunction. Clinically, therefore, we should use the most appropriate management strategy, allowing time for endothelial and smooth muscle cell function to recover. A greater understanding of the mechanisms involved in the recovery process ought to indicate more specific therapeutic targets.

The authors wish to thank the British Heart Foundation for their continued support. Dr R Tulloh was a British Heart Foundation Junior Research Fellow.

- 1 Spitzer AR, Davis J, Clarke WT, *et al.* Pulmonary hypertension and persistent fetal circulation in the newborn. *Clin Perinatol* 1988;15:389-410.
- 2 Hislop AA, Haworth SG. Pulmonary vascular damage in the development of cor pulmonale following hyaline membrane disease. *Paediatr Pulmonol* 1990;9:152-61.
- 3 Tulloh RMR, Hislop AA, Boels PJ, *et al.* Chronic hypoxia inhibits postnatal maturation of porcine intrapulmonary artery relaxation. *Am J Physiol* 1997;272:H2436-45.
- 4 Hislop AA, Springall DR, Oliveira H, *et al.* Endothelial nitric oxide synthase in hypoxic newborn porcine pulmonary vessels. *Arch Dis Child* 1997;76:F16-22.
- 5 Fulton RM, Hutchinson EC, Jones AM. Ventricular weight in cardiac hypertrophy. *Br Heart J* 1952;14:413-20.
- 6 Hislop A, Reid L. New pathological findings in emphysema of childhood. 1. Polyalveolar lobe with emphysema. *Thorax* 1970;25:682-90.
- 7 Griffiths TM, Edwards DH, Lewis MJ, *et al.* Evidence that cyclic guanine monophosphate (cGMP) mediates endothelium-dependent relaxation. *Eur J Pharmacol* 1985;112:195-202.
- 8 Liu SF, Hislop AA, Haworth SG, *et al.* Developmental changes in endothelium-dependent pulmonary vasodilatation in pigs. *Br J Pharmacol* 1992;106:324-30.
- 9 Brooker G, Harper JF, Terasaki WL, *et al.* Radioimmunoassay of cyclic AMP and cyclic GMP. *Adv Cyclic Nucleotide Res* 1979;10:1-33.
- 10 Young CL. *Oxides of nitrogen*. Solubility Data Series. 1991; 260-4.
- 11 Haworth SG, Hislop AA. Adaptation of the pulmonary circulation to extra-uterine life in the pig and its relevance to the human infant. *Cardiovasc Res* 1981;15:108-19.
- 12 Fried R, Reid LM. Early recovery from hypoxic pulmonary hypertension: a structural and functional study. *J Appl Physiol* 1984;57:1247-53.
- 13 Hislop A, Reid L. Changes in the pulmonary artery of the rat during recovery from hypoxia-induced pulmonary hypertension. *Br J Exp Pathol* 1977;58:653-62.
- 14 Abraham AK, Kay JM, Cole RB, *et al.* Haemodynamic and pathological study of the effect of chronic hypoxia and subsequent recovery of the heart and pulmonary vasculature of the rat. *Cardiovasc Res* 1971;5:95-103.
- 15 Herget J, Suggett AJ, Leach E, *et al.* Resolution of pulmonary hypertension and other features induced by chronic hypoxia in rats during complete and intermittent normoxia. *Thorax* 1978;33:468-73.
- 16 Weir EK, Tucker A, Reeves JT, *et al.* The genetic factor influencing pulmonary hypertension in cattle at high altitude. *Cardiovasc Res* 1974;8:745-9.
- 17 Haworth SG, Hislop AA. Effect of hypoxia on adaptation of the pulmonary circulation to extra-uterine life in the pig. *Cardiovasc Res* 1982;16:293-303.
- 18 Packer CS, Bennie RE, Powell DR, *et al.* Neonatal hypoxia: long term effects on pulmonary arterial muscle. *Respir Physiol* 1994;96:213-29.
- 19 Maruyama J, Maruyama K. Impaired nitric oxide-dependent responses and their recovery in hypertensive pulmonary arteries of rats. *Am J Physiol* 1994;266:H2476-88.
- 20 Hislop AA, Springall DR, Buttery LDK, *et al.* Abundance of endothelial nitric oxide synthase in newborn intrapulmonary arteries. *Arch Dis Child* 1995;12:F17-21.
- 21 Rapoport RM, Schwartz K, Murad F. Effects of Na⁺, K⁺ pump inhibitors and membrane depolarizing agents on acetylcholine-induced endothelium-dependent relaxation and cyclic GMP accumulation in rat aorta. *Eur J Pharmacol* 1985;110:203-9.
- 22 Evans AM, Osipenko ON, Haworth SG, *et al.* Resting potentials and potassium currents during the development of pulmonary artery smooth muscle cells. *Am J Physiol* 1998;275:H887-99.
- 23 Haworth SG, Hall SM, Chew M, *et al.* Thinning of fetal pulmonary arterial wall and postnatal remodelling: ultrastructural studies on the respiratory unit arteries of the pig. *Virchow's Arch Pathol A* 1987;411:161-71.
- 24 Hall SM, Haworth SG. Conducting pulmonary arteries: structural adaptation to extrauterine life. *Cardiovascular Res* 1987;21:208-16.