Reversal of pulmonary vascular remodelling following hypoxic exposure: no effect of infusion of atrial natriuretic factor and neutral endopeptidase inhibitor

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Objective: The aim was to investigate whether infusion of either atrial natriuretic factor (ANF, 800 $ng \cdot h^{-1} \cdot rat^{-1}$) or a specific inhibitor of neutral endopeptidase 24.11 (NEI, UK 73,967, 5.4 $mg \cdot kg^{-1} \cdot d^{-1}$) can influence the reversal of the pulmonary vascular remodelling produced by exposure to hypoxia. **Methods:** Male Wistar rats were kept in a normobaric hypoxic chamber ($Fio_2 = 10\%$) for 7 d. Chronically hypoxic rats were then treated with intravenous infusion of vehicle, ANF, or NEI by osmotic minipumps. Measurements of pulmonary artery pressure, systemic blood pressure, heart rate, right ventricular hypertrophy, microhaematocrit, and pulmonary vascular remodelling (percentage of thick walled peripheral vessels) were made in all the rats at different time points. **Results:** The changes in packed cell volume, right ventricular hypertrophy, and pulmonary hypertension induced by a 7 d hypoxic exposure diminished gradually and returned to normal at different time points during the 24 d recovery period. In contrast, vascular hypertrophy in peripheral pulmonary arteries was present after 24 d. There were no significant differences in pulmonary arterial pressure, packed cell volume, right ventricular hypertrophy and vascular remodelling between ANF, NEI, and vehicle treatment groups at either day 8 or day 15. **Conclusions:** ANF and NEI treatment had no effect on the reversal of pulmonary hypertension, right ventricular hypertrophy, and vascular remodelling, in contrast to the beneficial actions of ANF and NEI during the development of pulmonary vascular remodelling.
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Chronic hypoxia causes an increase in pulmonary arterial pressure associated with right ventricular hypertrophy, polycythaemia, and muscularisation of small pulmonary arteries.[1-6] The time course of the development of these hypoxia induced changes has been well defined. Right ventricular hypertrophy, increased arterial wall thickness, and peripheral extension of vascular smooth muscle occur after only three days of exposure to chronic hypoxia.[6] Pulmonary hypertension, pulmonary vascular remodelling, and right ventricular hypertrophy are about 70% developed after seven days of exposure in a normobaric chamber at an Fio_2 of 10%.[1] Fewer data are available about the reversal of the changes caused by hypoxia, although several studies have indicated that the reversal phase is either incomplete or, if ultimately complete, much slower.[7-9]

Whereas various drugs have been assessed during development of pulmonary hypertension,[10] few studies have measured their effects during reversal. Increased synthesis and release of atrial natriuretic factor (ANF), a natriuretic, diuretic, and vasorelaxant peptide, occurs during development of pulmonary hypertension in both patients and experimental models.[11-16] Previous studies have shown that increases in plasma ANF produced by chronic infusion of either physiological doses of synthetic ANF[17,18] or of a specific inhibitor of neutral endopeptidase 24.11 (NEI, candoxatrilat, UK 73,967, Pfizer) can attenuate the development of pulmonary hypertension and vascular remodelling.[19] ANF also displays an antiproliferative and antihypertrophic effect on vascular smooth muscle cells in vitro, when stimulated by platelet derived growth factor-B (PDGF-B).[20,21] In the present study we examined the time course of the reversal of the changes caused by seven days of hypoxia, and the effects of infusion of ANF and NEI during the reversal phase of hypoxia induced pulmonary hypertension in rats.

Methods

These investigations were performed in accordance with *Guidance on the operation of the Animals (Scientific Procedures) Act 1986*, published by HMSO, London.

Animals and environmental chamber

Specified pathogen-free albino male Wistar rats weight range 200 to 250 g were used throughout. Chronically hypoxic rats were placed in a normobaric hypoxic environmental chamber for 7 d, where the fractional inspired oxygen concentration was maintained at 10%, with excess humidity and carbon dioxide removed by means of scrub filters.[22] Gas was sampled periodically and analysed by mass spectrometer, and $Fico_2$ was shown to be less than 0.04% at all times. Relative humidity was always less than ambient and temperature was constantly maintained within 1°C of air temperature. Normoxic control rats were placed in the same room and exposed to the same light dark cycle. Food and standard laboratory chow were given to all animals ad libitum.

Experimental design

Experiment 1 – Six experimental groups ($n = 4$ all groups except normoxic control group $n = 7$) were designated as follows: normoxic control (C1); 7 d hypoxia (H1); 7 d hypoxia with recovery in air for 4 d (HR4), 8 d (HR8), 15 d (HR15), and 24 d (HR24).

Experiment 2 – Eight experimental groups ($n = 8$ in all groups) were designated as follows: normoxic control (C2); 7 d hypoxic (H2); 8 d

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and 15 d recovery in air treated with vehicle (0.9% NaCl, HR8S, HR15S), ANF (800 $ng \cdot h^{-1} \cdot rat^{-1}$, HR8ANF, HR15ANF),[18] or NEI (5.4 $mg \cdot kg^{-1} \cdot d^{-1}$, HR8NEI, HR15NEI)[19] by osmotic minipumps.

Implantation of osmotic minipumps
Pentobarbitone sodium (6 $mg \cdot 100\ g^{-1}$ body weight) was used for anaesthesia intraperitoneally both during implantation of osmotic minipumps and for measurement of pulmonary artery pressure and systemic blood pressure. Synthetic ANF (rat 3-28, Bachem, CA, USA) and the neutral endopeptidase inhibitor UK-73,967 (NEI, Candoxatrilat, Pfizer) were dissolved in saline (0.9% NaCl). Osmotic minipumps filled with vehicle (saline, 0.9% NaCl), ANF, and NEI were connected to the jugular vein by a polyethylene catheter (PE-60), tunnelled subcutaneously, and connected to the osmotic minipumps which were also positioned subcutaneously. A washout period of 1 d was allowed at the end of the 7 d and 14 d period of drug delivery to allow haemodynamic measurement to be made.

Haemodynamic measurements
The pulmonary artery was cannulated via the right jugular vein without an introducer using a precurved catheter, using the technique of Po and Wenli.[23] The left carotid artery was cannulated with a heparinised polyethylene cannula and simultaneous recordings of systemic blood pressure and pulmonary artery pressure in normoxia were made with a three channel thermal array recorder.

Right ventricular hypertrophy and packed cell volume
The heart was removed en bloc, the right ventricle was dissected free from the left ventricle and septum, and the right ventricle (RV) and left ventricle and septum (LV) were weighed separately using a chemical balance. Right ventricular hypertrophy was expressed as right ventricular weight/left ventricular weight (RV/LV). Blood obtained from the carotid artery cannula was placed in heparinised microtubes which were spun in a microhaematocrit centrifuge and packed cell volumes were read with a microhaematocrit reader.

Pulmonary vascular remodelling
The trachea was cannulated and the lungs were insufflated and fixed with 10% buffered formol-saline. A block 3 mm in thickness was taken by complete transverse medial to lateral section of the left lung below the hilum. Sections 3 μm thick were stained with elastic Van Gieson and coded slides examined systematically using the ×40 magnification objective, using a previously described method.[24] All vessels with a definite elastic coat adjacent to alveoli and alveolar ducts (25-55 μm diameter) were counted and the proportion of these having a double elastic lamina for between 35-100% of the circumference, indicating a muscular media, were counted. The proportion of vessels with a double elastic lamina (designated thick walled peripheral lung vessels, %TWPV) was then expressed as a percentage of all vessels examined.

Statistical analysis
Results are expressed as mean(SEM). Statistical analysis between treated and untreated groups and between different time points during induction of hypoxia and recovery from hypoxia was performed with an analysis of variance and Student's *t* test. Significance was assumed when $p < 0.05$.

Results

EXPERIMENT 1. RESOLUTION OF PULMONARY HYPERTENSION

Pulmonary artery pressure, systemic blood pressure, and heart rate

Chronic hypoxia increased mean pulmonary artery pressure from 15.8(SEM 0.8) to 26.2(0.6) mm Hg ($p < 0.001$) (fig 1). After removal from the hypoxic chamber, pulmonary artery pressure decreased gradually following 4 and 8 d of recovery in room air and returned to normal range at days 15 and 24 [HR15, 18.1(0.3) mm Hg; HR24, 17.0(0.8) mm Hg; $p > 0.05$

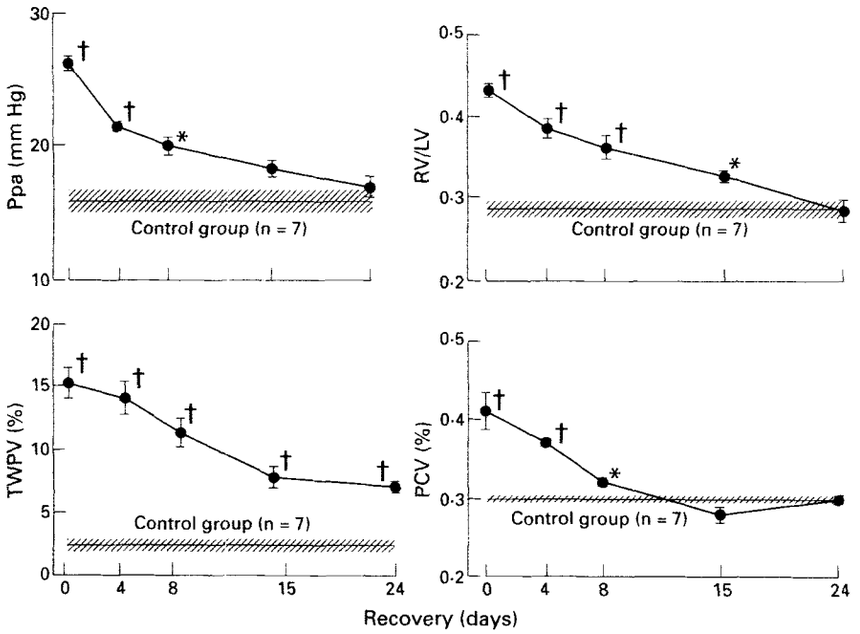

Figure 1 *Results of the recovery of pulmonary artery pressure (Ppa), right ventricular hypertrophy (RV/LV), packed cell volume (PCV), and pulmonary vascular remodelling (TWPV) after 7 days of chronic hypoxic exposure at day 4, day 8, day 15, and day 24 (n = 4 in all groups except normoxic control n = 7). Values are means, bars = SEM.*
**p < 0.01, †p < 0.001 v normoxic control group.*

Systemic systolic and diastolic blood pressure (SBP, DBP) and heart rate of the rats were not significantly changed during chronic hypoxic exposure or 24 d recovery in room air. Values are means(SEM).

	Normoxic control	*Hypoxia 7 days*	*Recovery*			
			4 days	*8days*	*15 days*	*24 days*
Number	7	4	4	4	4	4
Heart rate ($beats \cdot min^{-1}$)	398(5)	395(10)	395(4)	405(18)	408(9)	412(5)
SBP (mm Hg)	127(5)	128(10)	126(7)	126(6)	125(7)	130(6)
DBP (mm Hg)	102(3)	103(7)	107(7)	106(6)	104(7)	105(6)

v C1]. Systemic blood pressure and heart rate were not significantly changed during chronic hypoxic exposure or 24 d of recovery in room air (table).

Right ventricular hypertrophy and packed cell volume
Chronic hypoxia produced right ventricular hypertrophy [fig 1. RV/LV: C1, 0.27(0.01); H1, 0.43(0.01); $p < 0.001$] and significantly increased the packed cell volume [fig 1. C1, 50(1); H1, 61(2). The RV/LV ratio decreased gradually after 4, 8, and 15 d of recovery in air and was within the normal range after 24 d [HR24, 0.28(0.01)]. Normal packed cell volume values were found after both 15 and 24 d recovery [HR15, 48(1); HR24, 50(1)].

Pulmonary vascular remodelling
Chronic hypoxia produced extension of smooth muscle of the small pulmonary arteries toward the periphery of the lung as reflected in an increase percentage of TWPV in the 7 d hypoxic group compared to controls [fig 1. C1, 2.8(0.5)%; H1, 15.3(1.3)%; $p < 0.001$]. The percentage of TWPV decreased gradually during the 4, 8, 15, and 24 d of recovery in air, but evidence of pulmonary vascular remodelling still remained at day 24 [HR24, 7.3(0.4)%; $p < 0.001$ *v* C1].

EXPERIMENT 2. EFFECT OF ANF AND NEI ON RESOLUTION OF PULMONARY HYPERTENSION

Pulmonary artery pressure, systemic blood pressure, and heart rate
As with the first experimental group, chronic hypoxia increased mean pulmonary artery pressure significantly from 16.6(0.3) to 25.4(0.8) mm Hg ($p < 0.001$) (fig 2A). After removal from the hypoxic chamber, the pulmonary artery pressure of the vehicle treated group decreased gradually [HR8S, 19.1(0.4) mm Hg] and returned to normal range at day 15 [HR15S, 17.5(0.5) mm Hg; $p > 0.05$ *v* C2]. The pulmonary arterial pressures of ANF or NEI treated groups were similar to those of vehicle treated group at both day 8 and day 15 [HR8ANF, 19.0(0.6) mm Hg; HR8NEI, 19.8(0.7) mm Hg; HR15ANF, 16.0(0.6) mm Hg; HR15NEI, 16.5(0.7) mm Hg; fig 2A]. Systemic blood pressure and heart rate were not significantly altered by chronic hypoxic exposure or vehicle, ANF, and NEI treatment (fig 2B).

Packed cell volume measurements
Chronic hypoxia produced significant increases in packed cell volume [C2, 48(1)%; H1, 62(1)%; $p < 0.001$]. The packed cell volume of the vehicle treated group and ANF and NEI treated groups decreased gradually after removal from hypoxic chamber and returned to normal at day 15[HR15S, 47(1)%, HR15ANF, 47(1)%, HR15NEI, 47(1)%].

Right ventricular hypertrophy
Hypoxia produced right ventricular hypertrophy similarly to experiment 1[RV/LV: C2, 0.28(0.01); H2, 0.48(0.02); $p < 0.001$; fig 2C]. The RV/LV ratio of the vehicle treated group decreased gradually but it was still significantly higher than the normoxic control at day 15[HR15S, 0.32(0.01); $p < 0.001$ *v* C2]. ANF and NEI infusion for 7 or 14 d had no significant effect on RV/LV as shown in fig 2C.

Pulmonary vascular remodelling
Chronic hypoxia produced highly significant increases in the percentage of TWPV [C2, 2.3(0.5); H2, 13.8(1.2); $p < 0.001$]. Although the percentage of TWPV of the vehicle group decreased gradually during recovery in room air for 15 d, evidence of pulmonary vascular remodelling still remained at day 15[HR15S, 6.2(0.7); $p < 0.001$ *v* C2]. Treatment with ANF or NEI infusion did not result in any significant change in the percentage of TWPV (fig 2D).

Discussion

Chronic hypoxia produces structural changes in the pulmonary circulation very rapidly. Increases in the muscle of pulmonary arteries occur after only three days of hypoxic exposure and all features of pulmonary hypertension are established by 14 days, with no further increase in

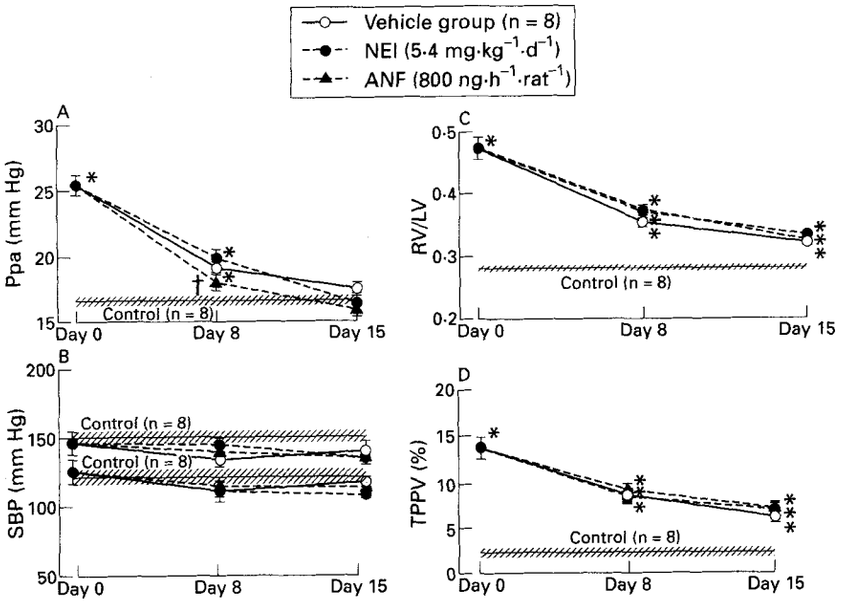

Figure 2 *Effects of chronic infusion of ANF and NEI on the reversal of pulmonary artery pressure (Ppa) (A), systemic blood pressure (SBP) (B), right ventricular hypertrophy (RV/LV) (C), and pulmonary vascular remodelling (TWPV) (D) at day 8 and day 15 following 7 days of chronic hypoxia exposure. Values are means, bars = SEM.*
**$p < 0.001$, †$p < 0.01$ v normoxic control group.*

muscularity up to 28 days of exposure.[6] In the present experiment after rats were exposed to hypoxia for seven days, the development of vessels with double elastic lamina confirmed the extension of muscle from the small arteries toward the periphery of the lung.

Withdrawal of the hypoxic stimulus leads to regression of the pulmonary hypertension of chronic hypoxia. The pulmonary changes induced by seven days of chronic hypoxia resolved at different rates on return to normoxia. Polycythaemia regressed within 15 days; pulmonary hypertension resolved from 15 to 24 days; right ventricular hypertrophy returned to normal by day 24 but some remodelling of pulmonary vessels still remained. Thus the features of pulmonary hypertension induced by hypoxia are reversible, although the structural changes do not regress as rapidly as they develop. Similar results were also noted previously in rats with much longer hypoxia exposure. Rabinovitch *et al*[25] exposed rats to half atmospheric pressure for one month and then allowed them to recover in room air for up to three months. They observed only partial regression of pulmonary hypertension and vascular remodelling. Herget and colleagues[7] reported that, after exposing rats to three weeks of hypoxia, pulmonary arterial pressure returned to baseline between six and 20 weeks. Right ventricular hypertrophy regressed a little more quickly, but the muscularisation of small pulmonary vessels was still apparent after 20 weeks. In an earlier study, Hislop and Reid[6] exposed rats to hypoxia for two weeks; there was a decrease in right ventricular hypertrophy and pulmonary vessel muscularisation after two weeks in room air with a further improvement at one month. These results have a parallel in human lung diseases. In humans, after descent from chronic high altitude exposure to sea level, pulmonary hypertension takes several years to regress.[26] In the clinical situation, long term oxygen therapy only slightly reduces the rate of progression of pulmonary hypertension. Reversal of the features of pulmonary hypertension is a slow process compared to their rate of development during hypoxia exposure, which suggests that the factors involved in reversal are different from those involved in the development of the pulmonary hypertension. Furthermore, the temporal dissociation between the recovery from right ventricular hypertrophy and that of pulmonary arteriolar thickening indicates that the mechanisms controlling reversal of these two structural changes may be different.

In the rat model of chronic hypoxia, continuous infusion of synthetic ANF exerts an antiproliferative action on the vascular smooth muscle during pulmonary remodelling.[17 18] UK 73,967 results in raised endogenous ANF and attenuation of remodelling and cardiac hypertrophy.[19] In vitro experiments demonstrated antiproliferative and antihypertrophic effects of ANF on vascular smooth muscle cells which are independent of any action on vascular tone. ANF inhibited proliferation of cultured vascular smooth muscle cells stimulated by platelet derived growth factor (PDGF) in a dose dependent manner, effects that are mimicked by 8-bromo-cyclic-GMP.[21] Further studies have shown that ANF exerted potent inhibitory effects not only on DNA replication and cell division in smooth muscle cells, but also on RNA and protein synthesis that may be mediators of the increase in vascular mass.[20] Rubin *et al*[27] provided evidence that there is increased expression of receptors for PDGF in various vascular proliferative lesions and Kourembanas *et al*[28] have shown that oxygen tension regulates the expression of PDGF B chain gene in human endothelial cells. Another candidate gene for pulmonary vascular remodelling is endothelin-1 the expression of which in cultured endothelial cells[29] and in rat lung[30] is induced by hypoxia.

In the present experiments, continuous infusion of NEI and ANF had no effect on the reversal of hypoxia induced pulmonary hypertension, right ventricular hypertrophy, and pulmonary vascular remodelling. These data suggest that factors involved in the reversal are different from those involved in the development of pulmonary remodelling. In the non-working isolated perfused heart, hypoxia stimulates ANF release.[31] In the lung, increased secretion and release of ANF may limit the progression of pulmonary hypertension by partially inhibiting the vascular hypertrophy induced by hypoxia. When rats are withdrawn from a hypoxic environment, the atrial and ventricular wall stress may be relieved, and the stimulus for ANF release may be removed. In other words, the recovery process may be independent of ANF synthesis and secretion. Hence ANF or NEI infusion has no effect. Alternatively, ANF may inhibit the expression of vascular growth factors which are induced during exposure to hypoxia. If these factors are switched off on the return to a normoxic environment, to permit reversal of the remodelled circulation, elevation of ANF levels would be without effect.

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Key terms: atrial natriuretic factor; neutral endopeptidase 24.11; hypoxia; pulmonary vascular remodelling.

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