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Alterations in pulmonary vascular function in rats exposed to intermittent hypoxia

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Abstract

Vasoactive agents were examined in arteries from control rats and rats exposed to intermittent hypoxia (10% oxygen; 8 h/day) for 3, 5 or 20 days. Hypoxic rats developed right ventricular hypertrophy after 5 days, but became pulmonary hypertensive (elevated right ventricular systolic pressure; RVSP) only after 20 days. In pulmonary arteries (main and intralobar), responses to acetylcholine and ionomycin (endothelium-dependent vasodilators) were reduced after 20 and 5 days of intermittent hypoxia, whereas contractions to 5-hydroxytryptamine (5-HT) were enhanced (potency increase >10-fold) after 20, 5 and 3 days. Contractions to endothelin-1 and a thromboxane-mimetic, but not Ca^{2+}, were also increased. No changes in vascular function occurred in aorta. Since changes in pulmonary vascular function preceded the increase in RVSP they do not result from, but may contribute to, the development of hypoxia-induced pulmonary hypertension. If similar changes occur in humans, they may be important in conditions characterised by intermittent, as opposed to continuous, hypoxia.
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1. Introduction

Exposure of rats to chronic continuous hypoxia results in the development of pulmonary hypertension and right ventricular hypertrophy. There are two important pathological features that contribute to the increase in pulmonary artery pressure, viz., alterations in pulmonary vascular structure (pulmonary vascular remodelling) and changes in pulmonary vascular function (Rabinovitch et al., 1979; Davies and Reid, 1991; Jeffery and Wanstall, 2001). The changes in pulmonary vascular function, most of which favour abnormal vasoconstriction, occur in both the endothelium and the vascular smooth muscle (Park et al., 1977; Maruyama and Maruyama, 1994; MacLean et al., 1996; Lal et al., 1999; Jeffery and Wanstall, 2001). These functional changes appear to be mainly independent of the changes in blood vessel structure (Jeffery and Wanstall, 1999).

There are some clinical or life-style situations in which hypoxia is intermittent rather than continuous, e.g. repeated short ascents into high altitude or exacerbation of hypoxia in patients with chronic obstructive lung disease during an acute respiratory infection. Previous studies have shown that exposure of rats to intermittent hypoxia can, like continuous hypoxia, cause pulmonary hypertension, right ventricular hypertrophy and pulmonary vascular remodelling (Widimsky et al., 1980; Stanbrook et al., 1984; Michael et al., 1986). However, it is not known whether intermittent hypoxia also leads to changes in pulmonary vascular function and the objective of this study was to investigate this possibility.

Hence, reactivity to various vasoactive agents has been assessed in isolated pulmonary arteries from rats exposed, for varying periods of time, to intermittent hypoxia. Endothelial and smooth muscle functions have been investigated. Parallel experiments were carried out on isolated aorta to determine whether any functional changes observed in the pulmonary blood vessels extended to the systemic circulation. In previous studies, exposure of rats to 8% to 10.5% oxygen for between 4 and 12 h per day has been shown to cause pulmonary hypertension, right ventricular hypertrophy and pulmonary vascular remodelling (Widimsky et al., 1980; Stanbrook et al., 1984; Michael et al., 1986). Based on this information, the intermittent hypoxic regime chosen for the present study was 10% oxygen for 8 h per day. In

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addition to assessing vascular function, the development of pulmonary hypertension has been monitored.

2. Materials and methods

2.1. Treatment of rats

This study conforms to the Code of Practice for Animal Experiments issued by the National Health and Medical Research Council of Australia and was approved by the University of Queensland Animal Experimentation Ethics Committee.

Male Wistar rats were housed intermittently in a chamber containing 10% oxygen (hypoxic rats). The hypoxic chamber was the same as that previously used in studies in which rats were exposed to continuous hypoxia (Wanstall et al., 1992). The intermittent regime consisted of 8 h of hypoxia per day, i.e. 8 h in the hypoxic chamber followed by 16 h in room air (21% oxygen). Rats were exposed to these conditions for a total of 3, 5 or 20 days. All hypoxic rats were in room air for the 16 h immediately preceding each experiment. Control rats were housed continuously in room air. The age of the rats was selected so that all rats were within the same age range (7–9 weeks) at the end of the treatment period. The weights of the rats in each group on the day of the experiment were (g): control 308 ± 5.5, $n = 57$; hypoxic, 3 days 289 ± 6.9, $n = 5$; hypoxic, 5 days 303 ± 6.2, $n = 34$; hypoxic, 20 days 315 ± 8.8, $n = 14$.

2.2. Haemodynamic measurements

On the day of the experiment, rats were anaesthetised with sodium pentobarbitone (90 mg/kg i.p.). The trachea was cannulated and the rat was artificially ventilated with room air (Ugo Basile Rodent Ventilator; 60 strokes/min; 1.5 ml/stroke; Ugo Basile, Comerio-Varese, Italy). The thorax was opened and a heparin-filled blunt-ended hypodermic needle connected to a Bentley Trantec pressure transducer (Model 60-800; American Edwards Laboratories, Santa Ana, CA, USA) was inserted into the right ventricle to record right ventricular pressure. In some preliminary experiments (in both normoxic and hypoxic rats), pulmonary artery pressure was recorded as well, by carefully advancing the needle into the main pulmonary artery. In these experiments, there was a significant positive correlation between right ventricular systolic pressure (RVSP) and pulmonary artery systolic pressure (slope of regression line 1.09 ± 0.12; $r^2 = 0.75$; $P < 0.001$; number of data points 29; range of RVSP values 11–29 mm Hg). On the basis of these results, RVSP, rather than pulmonary artery pressure, was measured to detect pulmonary hypertension. This avoided any risk of damage to the endothelium of the main pulmonary artery that might conceivably have occurred as a result of the procedure for measuring pulmonary artery pressure.

A blood sample was taken for measurement of haematocrit. One or more of the following tissues were removed: main pulmonary artery, descending thoracic aorta, lungs (for obtaining intralobar pulmonary arteries). The heart was then dissected out, divided into right ventricle (RV) and left ventricle + septum ([LV + S]), blotted and weighed. The ratios: RV/[LV + S], RV/body weight and [LV + S]/body weight were determined. Increases in RVSP, haematocrit and RV/[LV + S] were taken as indicators of pulmonary hypertension, polycythemia and right ventricular hypertrophy, respectively. The ratios RV/body weight and [LV + S]/body weight were used to ascertain that any increase in RV/[LV + S] reflected an increase in right ventricular weight rather than a decrease in left ventricular weight.

2.3. Isolated blood vessel preparations

Single-ring preparations (3 mm in length) of main pulmonary artery or descending thoracic aorta (endothelium intact) were set up in vertical organ baths containing physiological salt solution (PSS) at 37 °C, bubbled with 95% O_2/5% CO_2. The composition of the PSS was (mM): NaCl 118, KCl 5.9, $CaCl_2$ 1.5, $MgSO_4$ 0.72, $NaHCO_3$ 25, glucose 11.7, Na_2EDTA 0.024. Force in the circular muscle was recorded isometrically via a Statham Universal Transducer (UC3 + UL5, Statham Instruments, Oxnard, CA, USA) or a Grass FT03 force displacement transducer (Grass Instruments, Quincy, MA, USA), attached to a micrometer (Mitutoyo, Tokyo, Japan). Resting forces for the preparations were 10 mN (1.7 mN/mm; Doggrell et al., 1999).

Table 1
Haemodynamic variables; effects of exposure of rats to intermittent hypoxia

No. of hypoxic exposures[a]	n	RVSP (mm Hg)	RV/[LV + S] (mg/mg)	RV/body wt (mg/g)	[LV + S]/body wt (mg/g)	Haematocrit (%)
0 (control)	53	19 ± 0.6	0.22 ± 0.01	0.50 ± 0.01	2.33 ± 0.01	42 ± 0.6
3	5	17 ± 2.0	0.23 ± 0.01	0.57 ± 0.04	2.50 ± 0.05[b]	46 ± 3.2
5	30	20 ± 0.7	0.25 ± 0.01[c]	0.63 ± 0.02[c]	2.50 ± 0.04[c]	52 ± 0.9[c]
20	10	24 ± 1.8[c]	0.31 ± 0.01[c]	0.70 ± 0.03[c]	2.26 ± 0.06	56 ± 1.7[c]

Values are means ± S.E.M. from n rats.
RVSP = right ventricular systolic pressure. RV = right ventricle. [LV + S]=[left ventricle + septum].
[a] Each hypoxic exposure (in 10% oxygen) was for 8 h/day.
[b] $0.05 > P > 0.01$ when compared with corresponding value in control rats (one-way ANOVA and Dunnett's post test).
[c] $0.01 > P > 0.001$ when compared with corresponding value in control rats (one-way ANOVA and Dunnett's post test).

Intralobar pulmonary arteries (i.d. 450–660 μm) were dissected from the left lung lobe (second or third lateral branch of the main intralobar pulmonary artery). Ring preparations (length 0.95–2.00 mm) with the endothelium intact were mounted on 40 μm diameter stainless steel wires in a small vessel myograph (Mulvany-Halpern type; Model 400A; JP Trading, Aarhus, Denmark). The tissue chamber contained PSS at 37 °C, bubbled with 95% O_2/5% CO_2. The preparations were individually normalised to resting tensions that corresponded to a transmural pressure of 15 mm Hg (Doggrell et al., 1999); the mean resting tension was 0.51 ± 0.03 mN/mm; $n = 11$. Active force was recorded isometrically via the transducer incorporated in the myograph. Changes in force were recorded via the Myo-interface, on a chart recorder.

2.4. Experimental protocols

Preparations were allowed to equilibrate in PSS for 1 h. They were then contracted submaximally with phenylephrine (0.1 μM; main pulmonary artery and aorta) or U46619 (thromboxane-mimetic; 0.3 μM; intralobar pulmonary artery). U46619 was used instead of phenylephrine on intralobar pulmonary arteries because in the latter vessels responses to phenylephrine are minimal. Once the contraction had stabilised, cumulative concentrations (three-fold increments) of acetylcholine (0.01–10 μM) or ionomycin (calcium ionophore, 1 nM–1 μM) were added to the bath to obtain a concentration–response (relaxation) curve. Once the maximum relaxant response had been achieved, preparations were washed with PSS and then incubated for 30 min with N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM) and indomethacin (3 μM). These two drugs remained in the bath throughout the rest of the experiment and were present to prevent any interference from endothelium-derived nitric oxide (NO) or prostanoids when determining smooth muscle contractile responses. A reference contraction to K^+-depolarising PSS (in which 80 mM NaCl was replaced with 80 mM KCl) was then obtained. The preparations were washed with PSS to restore baseline force before obtaining cumulative concentration–response (contraction) curves to one or more of the following contractile agents: 5-hydroxytryptamine (5-HT; 1 nM–300 μM); 5-carboxamidotryptamine (5-CT; 1 nM–100 μM); U46619 (1 nM–1 μM); endothelin-1 (0.1 nM–0.3 μM); Ca^{2+} (20 μM–10 mM). The Ca^{2+} concentration–response curve was obtained in the presence of Ca^{2+}-free PSS containing 80 mM K^+, after a 30-min period of washing in Ca^{2+}-free PSS (Ca^{2+}-free PSS changed every 10 min). When endothelin-1 was one of the agents tested it was always the final agent examined because it does not readily wash off the tissue.

2.5. Expression of data

Responses to relaxant agents were expressed as "% reversal" of the submaximal contraction to phenylephrine or U46619. The submaximal contractions to phenylephrine and U46619 and also the reference contractions to K^+ were expressed as increases in tension (mN/mm, where mm represents twice the length of the preparation). All other contractile responses were expressed as a percentage of the reference contraction to K^+. The potencies of relaxant and contractile agents were calculated as the negative logarithm of the EC_{50} (negative log EC_{50}) where EC_{50} is the concentration producing 50% of the maximum response to the particular agent. EC_{50} values were interpolated from plots of response versus log molar concentration.

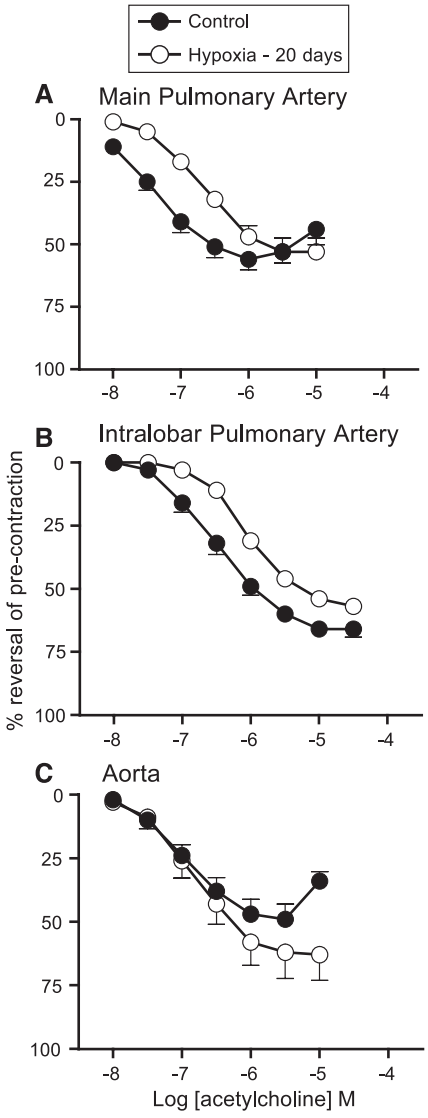

Fig. 1. Acetylcholine. Mean concentration–response (relaxation) curves on (A) main pulmonary artery, (B) intralobar pulmonary artery and (C) aorta from control normoxic rats (closed symbols) and rats exposed to intermittent hypoxia (8 h per day; open symbols) for 20 days. Responses are expressed as "percentage reversal" of contractions induced by phenylephrine (0.1 μM; main pulmonary artery and aorta) or U46619 (0.3 μM; intralobar pulmonary artery) and are shown as mean values with S.E.M. indicated by vertical lines except when smaller than the size of the symbols. Numbers of rats and potency values are shown in Table 2. There were no differences in maximum relaxation between the two groups of rats.

Table 2
Acetylcholine potency (negative log EC_{50}): effects of exposure of rats to intermittent hypoxia

No. of hypoxic exposures[a]	Acetylcholine negative log EC_{50}		
	Main pulmonary artery	Intralobar pulmonary artery	Aorta
0 (control)	7.36 ± 0.06 (17)	6.47 ± 0.13 (5)	6.96 ± 0.07 (6)
5	6.88 ± 0.12 (12)[b]	6.49 ± 0.10 (4)	7.24 ± 0.07 (4)
20	6.73 ± 0.12 (6)[b]	6.06 ± 0.06 (4)[c]	6.83 ± 0.11 (6)

Values are means ± S.E.M. Numbers of rats in parentheses.
Preparations were pre-contracted with phenylephrine (0.1 μM; main pulmonary artery, aorta) or U46619 (0.3 μM; intralobar pulmonary artery).
[a] Each hypoxic exposure (in 10% oxygen) was for 8 h/day.
[b] $0.01>P>0.001$ when compared with corresponding value in control rats (one-way ANOVA and Dunnett's post test).
[c] $0.05>P>0.01$ when compared with corresponding value in control rats (one-way ANOVA and Dunnett's post test).

2.6. Drugs and solutions

Acetylcholine chloride (Sigma, St. Louis, MO, USA); 5-carboxamidotryptamine maleate (5-CT; Sigma); 9, 11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619; Upjohn, Kalamazoo, MI, USA); endothelin-1 (Auspep, Melbourne, Victoria, Australia); 5-hydroxytryptamine creatinine sulphate (5-HT; Sigma); indomethacin (Sigma); ionomycin (Biomol, Plymouth Meeting, PA, USA); N^G-nitro-L-arginine methyl ester (L-NAME; Sigma); pentobarbitone sodium (Nembutal; Boehringer Ingelheim, Ingelheim, Germany); phenylephrine hydrochloride (Sigma).

Solutions of drugs were prepared as follows: acetylcholine (10 mM), 5-CT (10 mM), endothelin-1 (10 μM), 5-HT (10 mM) and L-NAME (10 mM) in deionised water; indomethacin (10 mM), ionomycin (2 mM) and U46619 (10 mM) in absolute ethanol; phenylephrine (10 mM) in 0.01 M HCl. All dilutions were prepared in PSS and discarded at the end of the experiment.

2.7. Statistical analyses

Mean values were calculated for data from *n* rats and are cited with their standard errors (S.E.M.). Mean values were compared by either Student's *t*-test (when comparing two values) or one-way analysis of variance (ANOVA) followed by Dunnett's post test (when comparing three or more values).

Table 3
K^+ (80 mM) contraction: effects of exposure of rats to intermittent hypoxia

No. of hypoxic exposures[a]	K^+ contraction (mN/mm)		
	Main pulmonary artery	Intralobar pulmonary artery	Aorta
0 (control)	4.25 ± 0.14 (28)	1.72 ± 0.27 (6)	3.94 ± 0.34 (10)
5	4.10 ± 0.22 (14)	1.55 ± 0.12 (4)	4.30 ± 0.36 (8)
20	4.85 ± 0.40 (10)	1.43 ± 0.10 (4)	4.45 ± 0.31 (10)

Values are means ± S.E.M. Numbers of rats in parentheses.
L-NAME (100 μM) and indomethacin (3 μM) were present.
There were no significant differences between values in either group of hypoxic rats and corresponding values in control rats.
[a] Each hypoxic exposure (in 10% oxygen) was for 8 h/day.

3. Results

3.1. Haemodynamic variables

Rats subjected to intermittent hypoxia for 20 days had (i) pulmonary hypertension, evidenced by a significant increase in RVSP, when compared with the value in control rats, and

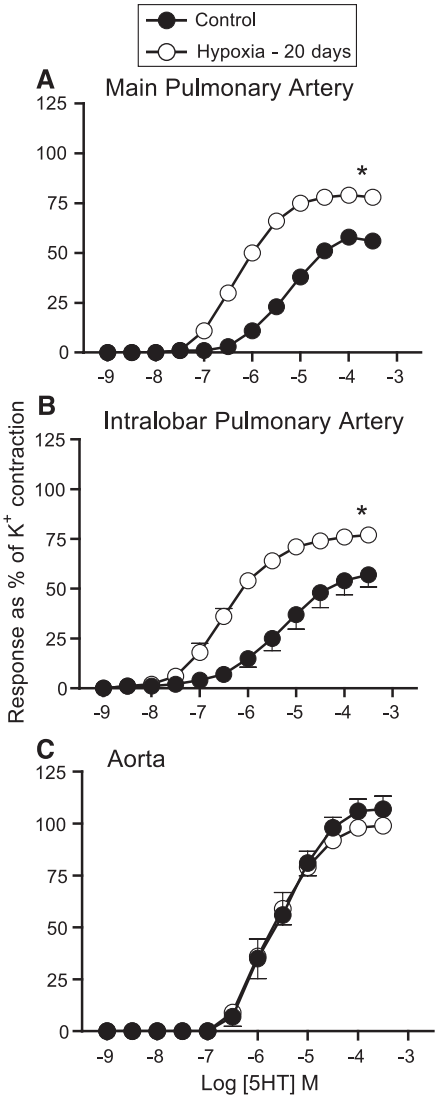

Fig. 2. 5-HT. Mean concentration–response (contraction) curves on (A) main pulmonary artery, (B) intralobar pulmonary artery and (C) aorta from control normoxic rats (closed symbols) and rats exposed to intermittent hypoxia (8 h per day; open symbols) for 20 days. Responses are expressed as a percentage of the reference contraction to 80 mM K^+ and are shown as mean values with S.E.M. indicated by vertical lines except when smaller than the size of the symbols. Numbers of rats and potency values are shown in Table 4. *Significant difference ($P<0.05$) between maximum responses in preparations from hypoxic and control rats (one-way ANOVA and Dunnett's post test).

Table 4
5-HT potency (negative log EC_{50}): effects of exposure of rats to intermittent hypoxia

No. of hypoxic exposures[a]	5-HT negative log EC_{50}		
	Main pulmonary artery	Intralobar pulmonary artery	Aorta
0 (control)	5.26 ± 0.09 (14)	5.17 ± 0.20 (6)	5.67 ± 0.18 (6)
5	6.38 ± 0.20 (7)[b]	5.84 ± 0.20 (4)[c]	6.17 ± 0.18 (4)
20	6.25 ± 0.04 (6)[b]	6.46 ± 0.11 (4)[b]	5.80 ± 0.15 (6)

Values are means ± S.E.M. Numbers of rats in parentheses.
L-NAME (100 μM) and indomethacin (3 μM) were present throughout.
[a] Each hypoxic exposure (in 10% oxygen) was for 8 h/day.
[b] $0.01 > P > 0.001$ when compared with corresponding value in control rats (one-way ANOVA and Dunnett's post test).
[c] $0.05 > P > 0.01$ when compared with corresponding value in control rats (one-way ANOVA and Dunnett's post test).

(ii) polycythemia, evidenced by an increase in haematocrit (Table 1). They also had right ventricular hypertrophy because there were significant increases in the ratios RV/[LV + S] and RV/body weight without any decrease in [LV + S]/body weight (Table 1). Rats subjected to 5 days of intermittent hypoxia had right ventricular hypertrophy and polycythemia but no pulmonary hypertension. After 3 days of intermittent hypoxia, none of these pathological features was present (Table 1).

3.2. Endothelium-dependent vasodilators

Responses to acetylcholine in pulmonary arteries (main and intralobar) from rats exposed to intermittent hypoxia for 20 days were reduced when compared with responses in corresponding control arteries (Fig. 1A,B). The reduction in responses in pulmonary arteries was seen as a reduction in potency (negative log EC_{50}) (Table 2) without any change in maximum response (Fig. 1). In main pulmonary artery, but

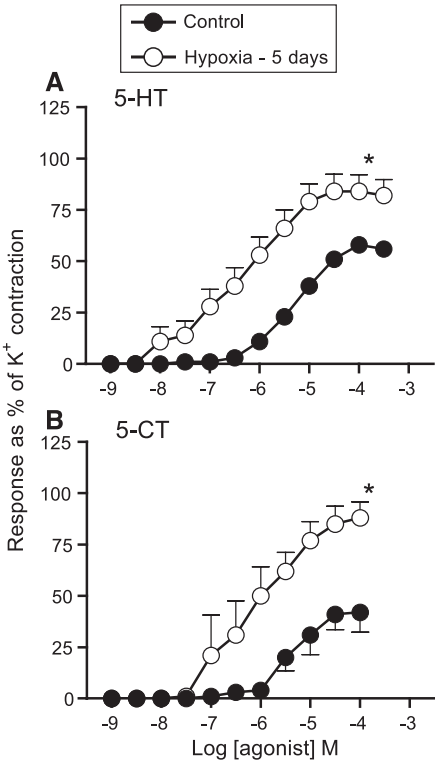

Fig. 3. 5-HT and 5-CT. Mean concentration–response (contraction) curves on main pulmonary artery from control normoxic rats (closed symbols) and rats exposed to intermittent hypoxia (8 h per day; open symbols) for 5 days. Responses are expressed as a percentage of the reference contraction to 80 mM K^+ and are shown as mean values with S.E.M. indicated by vertical lines except when smaller than the size of the symbols. Numbers of rats and potency values are shown in Table 4 (5-HT) and Section 3.4 of the text (5-CT). *Significant difference ($P < 0.05$) between maximum responses in preparations from hypoxic and control rats (one-way ANOVA and Dunnett's post test).

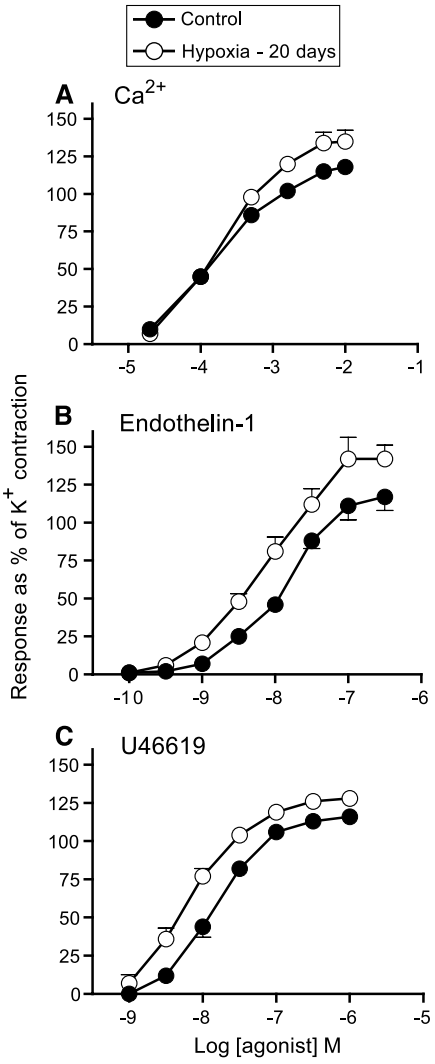

Fig. 4. Ca^{2+}, endothelin-1 and U46619 (thromboxane-mimetic). Mean concentration–response (contraction) curves on main pulmonary artery from control normoxic rats (closed symbols) and rats exposed to intermittent hypoxia (8 h per day; open symbols) for 20 days. Responses are expressed as a percentage of the reference contraction to 80 mM K^+ and are shown as mean values with S.E.M. indicated by vertical lines except when smaller than the size of the symbols. Numbers of rats and potency values are shown in Table 5. There were no significant differences in maximum contractions between the two groups of rats.

not intralobar pulmonary artery, a comparable reduction in the potency of acetylcholine was also seen in rats exposed to hypoxia for 5 days (Table 2); however, there was no change after 3 days of hypoxia (negative log EC_{50}: 7.18 ± 0.12, $n = 5$; $P > 0.05$ when compared with control value for main pulmonary artery shown in Table 2; one-way ANOVA and Dunnett's post test). In aorta, responses to acetylcholine were not different in the two groups of rats (Fig. 1C; Table 2).

A second endothelium-dependent vasodilator, ionomycin, was studied in main pulmonary artery. Like acetylcholine, the potency of ionomycin was lower in preparations from hypoxic rats (5 and 20 days) than in vessels from control rats (negative log EC_{50}; hypoxia 5 days 7.90 ± 0.11, $n = 6$; hypoxia 20 days 7.85 ± 0.01, $n = 4$; both $0.05 > P > 0.01$ when compared with control value, 8.20 ± 0.07, $n = 11$; one-way ANOVA and Dunnett's post test).

Note that the magnitudes of the pre-contractions in these experiments were not significantly different in pulmonary arteries from control and hypoxic rats. Values were: main pulmonary artery, phenylephrine, mN/mm: control 1.82 ± 0.07, $n = 28$; hypoxia 5 days 2.08 ± 0.15, $n = 18$; hypoxia 20 days 2.28 ± 0.34, $n = 10$ ($P > 0.05$; one-way ANOVA); intralobar pulmonary artery, U46619, mN/mm: control 0.84 ± 0.10, $n = 5$; hypoxia 5 days 1.0 ± 0.10, $n = 4$; hypoxia 20 days 1.2 ± 0.11, $n = 4$ ($P > 0.05$; one-way ANOVA).

3.3. Contractions to K^+

The reference contractions to K^+ (80 mM) in main and intralobar pulmonary arteries and aorta are shown in Table 3. For each of the vessel types, K^+ contractions in preparations from rats exposed to hypoxia for 5 or 20 days were not significantly different from those obtained in control preparations (Table 3).

3.4. Contractions to 5-HT and 5-CT

Contractile responses to 5-HT were markedly greater in pulmonary arteries (main and intralobar) from rats exposed to hypoxia for 20 days than in corresponding arteries from control rats (Fig. 2A,B). The increases in responses were seen as increases in both potency (≥ 10-fold; Table 4) and maximum response (Fig. 2). Increases in potency and maxima were also seen after 5 days of hypoxia (Table 4; Fig. 3A). Additional experiments were carried out in main pulmonary artery from rats exposed to hypoxia for 3 days and even after this short time there was still an increase in potency (negative log EC_{50}: 6.03 ± 0.16; $n = 5$; $0.01 > P > 0.001$ when compared with corresponding control value in Table 4; one-way ANOVA and Dunnett's post test). In contrast, exposure of rats to hypoxia had no effect on responses to 5-HT in aorta (Fig. 2C; Table 4).

In an additional series of experiments in main pulmonary artery from rats exposed to hypoxia for 5 days, the 5-HT_1 receptor-selective agonist, 5-CT, was examined. As with 5-HT, responses were increased when compared with those in arteries from control rats, with an increase in both maximum response (Fig. 3B) and potency (negative log EC_{50}; hypoxic rats 6.29 ± 0.39; $n = 3$; control rats 5.33 ± 0.18, $n = 4$).

3.5. Contractions to Ca^{2+}, endothelin-1 and thromboxane-mimetic (U46619)

To investigate whether the sensitisation to 5-HT described above was selective for this particular contractile agent, three other contractile agents were studied in arteries from rats exposed to intermittent hypoxia for 20 days (Fig. 4). In main pulmonary artery, the potencies of endothelin-1 and U46619 were greater in preparations from hypoxic rats than in control preparations, but the sensitisation (2-fold increase in negative log EC_{50}; Table 5) was much less pronounced than the 10-fold increase described above for 5-HT (Table 4). In contrast, the potency of Ca^{2+} was the same in preparations from hypoxic and control rats (Table 5). There were no significant changes in maximum response for any of the agents (Fig. 4). In aorta, exposure of rats to hypoxia had no effect on the potency of any of the three contractile agents studied (Table 5).

4. Discussion

This study represents the first systematic investigation of pulmonary vascular function in rats exposed to a regime of

Table 5
Ca^{2+}, endothelin-1 and U46619 (thromboxane-mimetic) potency (negative log EC_{50}): effects of exposure of rats to intermittent hypoxia

No. of hypoxic exposures[a]	Negative log EC_{50}					
	Main pulmonary artery			Aorta		
	Ca^{2+}	Endothelin-1	U46619	Ca^{2+}	Endothelin-1	U46619
0 (control)	3.77 ± 0.02 (4)	7.86 ± 0.04 (4)	7.87 ± 0.06 (10)	3.87 ± 0.17 (4)	8.31 ± 0.13 (4)	8.11 ± 0.20 (4)
20	3.72 ± 0.06 (4)	8.09 ± 0.06 (4)[b]	8.17 ± 0.06 (6)[c]	3.93 ± 0.08 (4)	8.31 ± 0.16 (4)	8.35 ± 0.08 (6)

Values are means ± S.E.M. with numbers of rats in parentheses.
Ca^{2+} concentration–response curves were carried out in Ca^{2+}-free, K^+ (80 mM)-depolarising PSS. L-NAME (100 μM) and indomethacin (3 μM) were present throughout.
[a] Each hypoxic exposure (in 10% oxygen) was for 8 h/day.
[b] $0.05 > P > 0.01$ when compared with corresponding value in control rats (Student's *t*-test).
[c] $0.01 > P > 0.001$ when compared with corresponding value in control rats (Student's *t*-test).

intermittent hypoxia. The results have shown that intermittent hypoxia causes marked changes in the functional properties of pulmonary arteries, in both the endothelium and vascular smooth muscle. Some, but not all, of the functional changes are reminiscent of alterations in pulmonary vascular function seen in models of continuous hypoxia. Also, in contrast to the findings reported in continuous hypoxia, the functional changes could be detected well before the development of sustained pulmonary hypertension.

The hypoxic regime used (10% oxygen for 8 h per day) did eventually lead to sustained pulmonary hypertension (seen in this study as an increase in RVSP measured 16 h after the rats had been returned to a normoxic environment). However, this occurred only after the largest number of exposures studied (viz. 20 exposures); it was not seen after 3 or 5 exposures. Direct comparison of these data with those from other studies in rats is difficult because of the wide range of conditions used, i.e. variations in the numbers of hours of hypoxia per day, the severity of the hypoxia and/or the total number of hypoxic exposures. Pulmonary hypertension has previously been reported after 20–24 hypoxic exposures but in each of these studies either (i) the duration of each hypoxic exposure was greater than in the present study (Stanbrook et al., 1984; 12 h per day), (ii) the oxygen level was lower (7–8%; Widimsky et al., 1980; Pelouch et al., 1997) or (iii) there was concurrent hypercapnia (Nattie et al., 1978). In one study, pulmonary hypertension was reported after fewer than 20 hypoxic exposures (viz. 14 exposures) but each exposure was of longer duration, i.e. 15 h/day (Lai et al., 1995). Importantly, in the present study sustained pulmonary hypertension did not occur after three or five hypoxic exposures, even though alterations in pulmonary vascular function were first seen at these early time-points.

Right ventricular hypertrophy and polycythemia have been reported in most of the previous studies on intermittent hypoxia (Nattie et al., 1978; Michael et al., 1986; Ostadal and Widimsky, 1990; Lai et al., 1995; Ostman-Smith, 1995) and were also seen in the present study. Interestingly, as few as five hypoxic exposures led to right ventricular hypertrophy, despite the absence of sustained pulmonary hypertension at this time. Right ventricular hypertrophy is frequently regarded as providing evidence for the presence of pulmonary hypertension in animal models of the disease. The current finding that right ventricular hypertrophy preceded pulmonary hypertension indicates that this practice is not totally reliable. This view is supported by Ostadal and Widimsky (1990) who likewise noted that right ventricular hypertrophy could occur in the absence of sustained pulmonary hypertension, viz. 24 hypoxic exposures of 4 h per day in rats caused right ventricular hypertrophy but no change in RVSP. In addition, right ventricular hypertrophy in the absence of elevations in RVSP has been reported in a study on the effects of hypoxia in vascular endothelial growth factor-B knockout mice (Wanstall et al., 2002). A speculative explanation for these findings is that elevations in pulmonary artery pressure occurring only during the time the animals are in the hypoxic chamber (reflecting acute hypoxic pulmonary vasoconstriction rather than sustained pulmonary hypertension) are sufficient to stimulate right ventricular hypertrophy. Alternatively, hypoxia may conceivably have a direct effect on right ventricular growth. Marked polycythemia was seen after 5 days of intermittent hypoxia, before pulmonary hypertension was seen. It has been suggested that polycythemia makes a significant contribution to the increase in pulmonary artery pressure in hypoxic rats (Fried et al., 1983). Our data show that an increase in haematocrit alone is insufficient to cause an increase in pressure.

The primary purpose of the study was the assessment of pulmonary vascular function (endothelial and smooth muscle) following intermittent hypoxia, since this has not previously been investigated. Two endothelium-dependent vasodilators, acetylcholine and ionomycin, were examined on isolated pulmonary arteries, and responses were found to be reduced after 20 days of intermittent hypoxia in both main and intralobar pulmonary arteries. On main pulmonary artery, a reduction in endothelium-dependent relaxation was also seen after as little as 5 days of intermittent hypoxia, but in intralobar pulmonary artery endothelial function was not impaired at this early time-point. The reason for this discrepancy between large and small pulmonary arteries is not known, but in a study on the effects of continuous hypoxia on pulmonary vascular function the influence of hypoxia on various vasoactive agents was not always identical in pulmonary arteries of different sizes/locations (Jeffery and Wanstall, 2001). The finding that responses were reduced for the receptor-independent Ca^{2+} ionophore, ionomycin as well as the receptor-linked dilator, acetylcholine, points to generalised impairment of the endothelial NO/relaxation pathway rather than a specific effect on the muscarinic receptor pathway.

In view of the dysfunction in the endothelial NO pathway seen in the hypoxic rats, it was important that smooth muscle responses to the various vasoconstrictor agents were obtained in preparations where any influence of this pathway was blocked. Bearing this in mind, the vasoconstrictor agents (including the reference contractile agent, K^+) were all examined in the presence of the NO synthase inhibitor, L-NAME. Under these conditions, the magnitude of the reference contraction to K^+ and the potency of Ca^{2+} were unchanged in arteries from hypoxic rats. This indicated that neither the overall contractile ability of the smooth muscle nor the sensitivity of the contractile proteins to Ca^{2+} was affected by exposing rats to intermittent hypoxia. In contrast, clear increases in reactivity to three spasmogens acting via specific receptors, viz. 5-HT, endothelin-1 and a thromboxane-mimetic, were seen in pulmonary arteries from rats exposed to 20 days of intermittent hypoxia. The most dramatic change in sensitivity seen after 20 days of hypoxia was for 5-HT where potency was increased by more than one order of magnitude in pulmonary arteries of both sizes

studied. For this reason, 5-HT was also studied after fewer hypoxic exposures. To our surprise, an increase in the potency of 5-HT was seen after as little as 3 days of intermittent hypoxia, long before pulmonary hypertension developed (present study), before the appearance of endothelial dysfunction (present study) and also before there is any evidence of pulmonary vascular structural remodelling (Thomas and Wanstall, unpublished observation).

It was not the purpose of this study to investigate possible mechanisms for the changes in 5-HT potency, but there are several points that are worthy of comment. First, an increase in potency was also seen for the $5HT_1$ receptor agonist, 5-CT. This finding suggests a possible increase in $5\text{-}HT_1$ receptor-mediated contractions, as has been reported to occur following chronic continuous hypoxia (MacLean et al., 1996). Second, as discussed above, the experimental conditions used (i.e. L-NAME present) meant that any changes in contractile function could not be attributed to alterations in the modulating effect of endothelium-derived NO. Finally, because the increase in 5-HT potency preceded the development of pulmonary hypertension, the change in potency cannot be a consequence of elevated pulmonary artery pressure. On the contrary, an increase in 5-HT-induced pulmonary vasoconstriction is evidently a forerunner to the development of pulmonary hypertension and is likely to exacerbate the rise in pulmonary artery pressure caused later by pulmonary vascular remodelling (Widimsky et al., 1980; Michael et al., 1986).

Some of the changes in pulmonary vascular reactivity, viz. the decrease in acetylcholine responses and increases in 5-HT and U46619 responses, are qualitatively the same as reported in rats exposed to chronic continuous hypoxia (Maruyama and Maruyama, 1994; MacLean et al., 1996; Lal et al., 1999; Jeffery and Wanstall, 2001). However, this does not apply to all of the findings. For example, in the present study responses to endothelin-1 were increased following intermittent hypoxia but in studies with continuous hypoxia responses to this vasoconstrictor peptide were reduced (Bialecki et al., 1998; Lal et al., 1999). The reason for this discrepancy is not known but Bialecki et al. (1998) attributed the reduction in endothelin-1 responses to the increase in plasma endothelin levels seen in rats exposed to continuous hypoxia. It is not known whether intermittent hypoxia causes changes in plasma endothelin levels. Whatever the reason for the difference, it is clear that one cannot necessarily extrapolate findings from continuous hypoxia to intermittent hypoxia, or vice versa. It should also be noted that it is the use of the intermittent hypoxic regime, where pulmonary hypertension is comparatively slow to develop, that has enabled us to obtain clear evidence that hypoxia-induced changes in pulmonary vascular function precede the development of pulmonary hypertension. Continuous hypoxia produces pulmonary hypertension much more rapidly, i.e. pulmonary artery pressure is elevated after as little as 3 days of continuous hypoxia (Rabinovitch et al., 1979; Wanstall et al., 1992). Therefore, in studies using continuous hypoxia, it is much more difficult to separate, on a temporal basis, vascular functional changes from the rise in pulmonary artery pressure.

None of the changes in vascular function that were seen in pulmonary blood vessels occurred in aorta. In this systemic vessel, endothelium-dependent relaxation and smooth muscle contraction were the same whether preparations were from control or hypoxic rats, suggesting that altered vascular function caused by intermittent hypoxia is probably confined to the pulmonary circulation. This is also true of changes in pulmonary vascular reactivity associated with continuous hypoxia (Wanstall and O'Donnell, 1992; Bialecki et al., 1998) and with other models of pulmonary hypertension (Wanstall and O'Donnell, 1990, 1992).

In conclusion, exposure of rats to intermittent hypoxia results in impaired endothelium-dependent vasodilatation and enhanced smooth muscle responsiveness to specific vasoconstrictors, especially 5-HT, in pulmonary arteries but not aorta. Alone, these changes in pulmonary vascular function were not sufficient to cause pulmonary hypertension but, by their nature, they could exacerbate increases in pulmonary vascular resistance caused later by vascular remodelling, and hence contribute to the development of pulmonary hypertension. Because the functional changes appear to be confined to the pulmonary circulation, they would not affect systemic vascular resistance. The changes in endothelial and smooth muscle function were seen after remarkably mild hypoxic insults, viz. as little as 5 days of intermittent hypoxia caused impairment of responses to endothelium-dependent vasodilators and a mere 3 days of intermittent hypoxia caused enhancement of vasoconstrictor responses to 5-HT. Hence, it is quite conceivable that pulmonary vascular reactivity could be altered in clinical or lifestyle situations characterised by short periods of intermittent hypoxia, such as repeated excursions into high altitude or acute respiratory infections in patients with chronic obstructive lung disease.

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