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European Journal of Pharmacology 416 (2001) 123–131

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Pulmonary vascular remodelling in hypoxic rats: effects of amlodipine, alone and with perindopril

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Received 23 November 2000; received in revised form 9 February 2001; accepted 16 February 2001

Abstract

This study investigated whether pulmonary vascular remodelling in hypoxic pulmonary hypertensive rats (10% oxygen; 4 weeks) could be prevented by treatment, during hypoxia, with amlodipine (10 mg/kg/day, p.o.), either alone or in combination with the angiotensin converting enzyme inhibitor, perindopril (30 mg/kg/day, p.o.). Medial thickening of pulmonary arteries (30-500 μm o.d.) was attenuated by amlodipine whereas it was totally prevented by the combination treatment (amlodipine plus perindopril); neomuscularisation of small alveolar arteries (assessed from critical closing pressure in isolated perfused lungs) was not affected. Pulmonary vascular resistance (isolated perfused lungs) was reduced by both treatment regimes but only combination treatment reduced right ventricular hypertrophy. Thus, amlodipine has anti-remodelling properties in pulmonary hypertensive rats. The finding that combining amlodipine with another anti-remodelling drug produced effects on vascular structure that were additive raises the question of whether combination therapy with two different anti-remodelling drugs may be of value in the treatment of patients with hypoxic (and possibly other forms of) pulmonary hypertension.

Keywords: Pulmonary hypertension; Pulmonary vascular remodelling; Ca^{2+} channel antagonist; Angiotensin converting enzyme inhibitor

1. Introduction

Pulmonary vascular remodelling (altered vessel structure) is one of the key pathological features contributing to the rise in pulmonary artery pressure in pulmonary hypertension. The changes in pulmonary vascular structure include hypertrophy/hyperplasia of the media and neomuscularisation of normally non-muscular arteries in the alveolar region (Reid, 1979). Current drug therapy for pulmonary hypertension is limited to vasodilators, which oppose the other key pathological feature of this disease, i.e. abnormal pulmonary vasoconstriction (Reeves et al., 1986). Present research efforts are directed toward identifying drugs that inhibit pulmonary vascular remodelling. We have recently shown that treatment of rats with the angiotensin converting enzyme inhibitor, perindopril, attenuated a number of the changes in pulmonary vascular structure associated with the development of hypoxic pulmonary hypertension (Jeffery and Wanstall, 1999). These findings supported the view that angiotensin II, acting as a growth factor, is involved in the remodelling process (Morrell et al., 1995; Nong et al., 1996). However, it is unlikely that angiotensin II is the only factor involved. This is supported by our study on perindopril, in which it was shown that 30 mg/kg/day was a maximally effective dose of this drug but, even at this dose, remodelling was not totally prevented (Jeffery and Wanstall, 1999).

The aim of the present study was to determine whether changes in pulmonary vascular structure in hypoxic pulmonary hypertensive rats could be totally prevented either by an anti-remodelling drug with a different mechanism of action or, alternatively, by treatment with a combination of two different drugs. There is evidence that Ca^{2+} channel antagonists, apart from their well-known vasodilator properties, also may inhibit vascular remodelling. The mechanism involved appears to be inhibition of the action of various growth factors. For example, in in vitro experiments in rat and human vascular smooth muscle cells, DNA synthesis and smooth muscle cell proliferation in response to a variety of growth factors, e.g. platelet derived growth factor, epidermal growth factor (e.g. Block et al., 1989; Kataoka et al., 1997; Stepien et al., 1997), was

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)00855-X

prevented by Ca^{2+} channel antagonists. Therefore, we chose to examine the dihydropyridine Ca^{2+} channel antagonist, amlodipine, in this study.

A Ca^{2+} channel antagonist of the dihydropyridine type was chosen because drugs in this group are vascular selective and are therefore unlikely to cause depressant effects on the heart (i.e. negative inotropy and chronotropy). Amlodipine was the particular dihydropyridine selected for three reasons. Firstly, it is more potent than most other dihydropyridines including nifedipine, nitrendipine and felodipine (Burges et al., 1989; Fleckenstein et al., 1989). Secondly, and more importantly, it has a slow onset and offset of action coupled with a long plasma half-life and therefore has the advantage of requiring only once daily administration (Stopher et al., 1988; Burges et al., 1989). Thirdly, amlodipine has not previously been examined in hypoxic pulmonary hypertensive rats.

Amlodipine was examined initially alone and subsequently in combination with the angiotensin converting enzyme inhibitor used in our previous study, i.e. perindopril (Jeffery and Wanstall, 1999). These two classes of drugs have been examined together in models of systemic hypertension (e.g. Scalbert et al., 1990; Mervaala et al., 1997) but there are no studies in which this combination of drugs has been studied on the changes in pulmonary vascular structure in pulmonary hypertension.

2. Materials and methods

2.1. Rats

Male Wistar rats, aged 8–9 weeks on the day of the experiment, were used. Some of the rats were housed in hypoxic chambers (10% O_2) for 4 weeks before the experiment (Jeffery and Wanstall, 1998). Normoxic rats were housed in room air (21% O_2). During their 4-week exposure to hypoxia or normoxia, rats were treated once a day, by oral gavage, with either (i) water (no drug), (ii) amlodipine besylate (10 mg/kg) or (iii) amlodipine besylate (10 mg/kg) plus perindopril (30 mg/kg). Groups of rats were weight-matched at the commencement of treatment (Table 1). Rats exposed to hypoxia gained less weight than their normoxic counterparts over the 4-week period, but neither of the drug treatments had any effect on weight gain (Table 1). This investigation conforms to the *Code of Practice for Animal Experiments* issued by the National Health and Medical Research Council of Australia.

2.2. Haemodynamic and heart weight measurements

On the day of the experiment, rats were anaesthetized with pentobarbitone (70 mg/kg i.p.) and were given an i.p. injection of heparin (250 IU). The trachea was cannulated and the lungs were artificially ventilated (60 strokes/min; Ugo Basile Rodent ventilator, Comerio-Varese, Italy). Systemic artery pressure was determined via a cannula inserted into the right carotid artery. Pulmonary artery pressure was determined by inserting a heparin-filled blunt-ended hypodermic needle (23 gauge) into the main pulmonary artery via the right ventricle immediately after opening the thorax. Pressures were recorded via a Bentley Trantec pressure transducer (Ugo Basile,) connected to a Gemini chart recorder (Ugo Basile). Pressures of hypoxic rats were recorded between 20 and 60 min after rats had been removed from the hypoxic chamber. Hence, measurements of pulmonary artery pressure were not confounded by any acute hypoxic pulmonary vasoconstriction and, therefore, any increase in pulmonary artery pressure represented sustained pulmonary hypertension. Mean pulmonary artery pressure was taken as diastolic pressure + 1/3 (systolic pressure − diastolic pressure). Heart rate was determined from the pulmonary artery pressure record.

Hearts were removed and were divided into right ventricle (RV) and left ventricle plus septum (LV + S), blotted and weighed. Ratios of RV/body weight and (LV + S)/body weight were calculated.

Table 1
Initial and final body weights and weight gain of normoxic and hypoxic rats: effect of treatment of rats with amlodipine or a combination of amlodipine plus perindopril
Data are mean ± S.E.M.

Treatment	n[a]	Initial body weight (g)		Final body weight (g)		Weight gain (g)	
		Normoxic	Hypoxic[b]	Normoxic	Hypoxic[b]	Normoxic	Hypoxic[b]
No drug	8	137 ± 13.6	151 ± 18.0	272 ± 24.0	228 ± 20.9	135 ± 10.5	77 ± 3.8[c]
Amlodipine[d]	12	134 ± 4.8	147 ± 9.3	305 ± 5.0	225 ± 12.2[c]	169 ± 6.0	75 ± 3.9[c]
Amlodipine + Perindopril[e]	8	128 ± 5.8	149 ± 10.3	268 ± 9.9	243 ± 13.2	140 ± 8.5	95 ± 7.4[c]

[a] n = number of rats.
[b] Rats exposed to 10% oxygen for 4 weeks.
[c] Significantly less than corresponding values for normoxic rats ($P < 0.05$).
[d] Rats treated with 10 mg/kg/day amlodipine by oral gavage for 4 weeks.
[e] Rats treated with 10 mg/kg/day amlodipine plus 30 mg/kg/day perindopril by oral gavage for 4 weeks.

2.3. Morphological measurements in histological sections of lungs

The pulmonary artery and trachea were cannulated, the left atrium was cut and the lung was perfused via the pulmonary artery cannula with normal saline (0.9% w/v NaCl in deionised water) via the pulmonary artery cannula under a head of pressure that approximated the in vivo pulmonary artery pressure of the rats (20 or 40 cm H_2O in normoxic and hypoxic rats, respectively). The left atrium and pulmonary artery were then ligated to maintain the pressure within the pulmonary circulation. The lungs were subsequently fixed by perfusing them via the trachea with 10% v/v formalin at a pressure of 25 cm H_2O for approximately 1 min (Meyrick and Reid, 1980). The trachea was tied off and the lungs and heart were removed en bloc and immersed in 10% formalin for 1 week. After fixation, longitudinal sections (3 μm thick) were taken from the left lobe and transverse sections were taken from the right lobe. The sections were stained with Miller's stain for elastin and Van Gieson's stain for smooth muscle. The sections from the left lobe provided images of arteries 101–500 μm o.d. and the sections from the right lobe provided images of arteries 30–100 μm o.d.

Images of individual pulmonary arteries (4–11 arteries per section) were captured using a video camera (Video 7, 8 bit 765×512 pixel CCD), mounted on a light microscope (Olympus BH5), linked to a computer (Macintosh Centris 650, with scion LG3 frame grabber). The sections were examined at $\times 40$, 100 or 400 magnification. Measurements of vessel diameter and medial thickness were obtained from these images using a computer programme (NIH image adapted for PC by Scion). Arterial diameter was defined as the distance between two diametrically opposed external elastic laminae; two measurements were made: (i) along the line corresponding to the longest distance and (ii) along the line perpendicular to it, and these two values were averaged to give "mean arterial diameter". Medial thickness was defined and measured as the distance between internal and external elastic laminae; four measurements were made, one in each of the four quadrants defined by the perpendicular lines, and were then averaged to give "mean medial thickness". Percent medial thickness was calculated as $(2 \times \text{mean medial thickness}) \times 100 \div \text{mean arterial diameter}$ (Jeffery and Wanstall, 1999).

2.4. Isolated perfused lungs

Rats were exsanguinated via the vena cava. Cannulae were inserted into the pulmonary artery (via the right ventricle) and left atrium (via the left ventricle). The lungs were carefully removed and set up in a humidified chamber (37°C). Lungs were ventilated via the previously inserted tracheal cannula (see above) with normoxic gas (20% O_2, 5% CO_2, balance N_2) using an Ugo Basile Rodent ventilator (60 strokes/min; inspiratory pressure 9 cm H_2O; end expiratory pressure 2.5 cm H_2O). Lungs were perfused via the pulmonary artery cannula initially at a constant flow rate (8 ml/min; Desaga peristaltic pump, Heidelberg, Germany) with a physiological salt solution (PSS) of the following composition (in mM); NaCl 119; KCl 4.7; $MgSO_4$ 1.17; $CaCl_2$ 3.2; KH_2PO_4 1.18; $NaHCO_3$ 22.6; glucose 5.5; sucrose 50. The perfusate also contained 4% w/v bovine serum albumin, 3 μM indomethacin and 2.5 nM angiotensin II (McMurtry, 1984). Angiotensin II was included because in salt-perfused, as opposed to blood-perfused, lungs from rats the presence of a vasoconstrictor agent is required to facilitate the production of hypoxic vasoconstrictor responses; the choice of vasoconstrictor is unimportant (McMurtry, 1984). Angiotensin II was used at a concentration that, in itself, did not cause constriction (i.e. a just sub-threshold concentration) because, in previous studies, we have found this to be adequate (Crilley et al., 1998; Jeffery and Wanstall, 1999). Indomethacin (cyclooxygenase inhibitor) was present since inhibition of prostaglandin production can enhance and stabilise hypoxic pulmonary vasoconstriction in rat lungs (McMurtry et al., 1976, 1978). The effluent perfusate was collected via the atrial cannula and was not recirculated. Changes in perfusion pressure were measured via a side arm located in the perfusion line close to the pulmonary artery cannula and connected to a Bentley Trantec transducer.

The lung preparations were allowed to equilibrate for 20 min at a flow rate of 8 ml/min. The flow rate was then increased to 20, 16 or 12 ml/min. The highest flow rate was used in lungs from normoxic rats; the two lower flow rates were used in lungs from hypoxic rats, drug-treated and untreated, respectively, and were selected so that perfusion pressures of 35 mm Hg were not exceeded. This avoided the development of oedema. The flow rate was then reduced in a stepwise manner (2 ml/min decrements) down to a rate of 4 ml/min in order to obtain a passive pressure-flow relationship. The flow rate was then returned to 8 ml/min and successive hypoxic pulmonary vasoconstrictor responses were obtained until the response was reproducible. These constrictor responses were induced by ventilating the lungs with a hypoxic gas mixture (2% O_2, 5% CO_2, balance N_2) rather than normoxic gas for ≥ 4 min. Successive hypoxic challenges were separated by 4 min of ventilation with normoxic gas. Hypoxic pulmonary vasoconstrictor responses were measured at equilibrium and were expressed in mm Hg.

From these pressure-flow plots obtained in individual lungs the *Y*-intercept, which was determined by extrapolation, and the slope were obtained. The *Y*-intercept represents the critical closing pressure, i.e. the minimum pressure that is required to keep the pulmonary vessels of the alveolar region open (Wach et al., 1987). An increase in critical closing pressure indicates extension of smooth muscle into the normally non-muscular arteries of the

alveolar region (referred to as neomuscularisation). The slope represents the pulmonary vascular resistance of the perfused lung (Wach et al., 1987). The mean pressure-flow plots were generated by linear regression using the mean of the pressures obtained at each flow rate in a particular group of rats.

In some experiments, after the pressure flow plots and hypoxic pulmonary vasoconstrictor responses had been determined, responses to the calcium channel activator, Bay K8644 (1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-pyridine-3-carboxylic acid methyl ester; administered by infusion at a rate of 0.01 ml/min to achieve a final concentration of 1 μM in the perfusate; Razel infusion pump, Model A-99.z, Stanford, CN, USA) were obtained. In lungs from normoxic rats (but not hypoxic rats), it was necessary to include an additional 6 mM KCl in the perfusate in order to obtain reliable constrictor responses to Bay K8644 (Su et al., 1984; Wanstall and O'Donnell, 1989).

2.5. Drugs

Amlodipine besylate (gift from Pfizer, Sandwich, UK); angiotensin II (Sigma, St Louis, USA); Bay K8644 (1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-pyridine-3-carboxylic acid methyl ester; Sigma); bovine serum albumin (Sigma); heparin (Fisons, Sydney, Australia); indomethacin (Sigma); pentobarbitone sodium (Rhone Merieux, Brisbane, Australia); perindopril (gift from Servier Laboratories, Melbourne, Australia).

Solutions of drugs were prepared as follows; angiotensin II (1 mM) in 0.01 M HCl; amlodipine besylate (5 mg/ml) and perindopril (15 mg/ml) in deionised water. Bay K8644 (10 mM) and indomethacin (10 mM), in absolute ethanol. Dilutions, when required, were made in PSS.

2.6. Data analysis

Mean values were calculated from data obtained in preparations from a number (n) of different animals and are quoted together with their S.E.M. All values compared statistically were first shown to be normally distributed using the Kolmogorov–Smirnov test. Mean values were then analysed by two-way analysis of variance (ANOVA) with normoxic and hypoxic rats as one factor and treatment regime (i.e. no drug, amlodipine and amlodipine plus perindopril) as the other factor. If there was a significant effect of hypoxic exposure, a Student's t-test (unpaired values) was carried out in order to compare normoxic and hypoxic rats. If there was a significant effect of drug treatment, a Student's t-test or one-way ANOVA with a Newman–Keul's post hoc test was carried out separately for normoxic rats and hypoxic rats. If there was a significant interaction between the two factors, both of the above post tests were performed regardless of whether the two-way ANOVA indicated a significant effect of hypoxia or drug treatment. $P < 0.05$ was taken as indicating statistical significance. The statistics programmes that were used were Prism (version 3.0) and Statistica.

3. Results

3.1. Effects of chronic amlodipine treatment of rats on vasoconstrictor responses in isolated perfused lungs

Data were obtained to verify that the selected dose of amlodipine (10 mg/kg/day) was effective in inhibiting two types of constrictor response known to involve the opening of voltage gated L-type Ca^{2+} channels, i.e. responses to the Ca^{2+} channel activator, Bay K8644 (1 μM) and to acute alveolar hypoxia (2% O_2) (Weir and Archer, 1995). These responses were measured in isolated perfused lungs. Vasoconstrictor responses to both Bay K8644 and acute alveolar hypoxia were significantly reduced in lungs from rats treated with amlodipine for 4 weeks when compared with responses obtained in untreated rats (Table 2). This was seen in both normoxic and hypoxic rats. Therefore, chronic in vivo administration of the selected dose of

Table 2
Constrictor responses to Bay K8644 and acute alveolar hypoxia in isolated perfused lungs from normoxic and hypoxic rats: effects of treatment of rats with amlodipine
Data are mean ± SEM. Numbers of rats are in parentheses.

	Vasoconstrictor responses (mm Hg)			
	Bay K8644 (1 μM)		Acute alveolar hypoxia (2% O_2)	
	Normoxic	Hypoxic[a]	Normoxic	Hypoxic[a]
No drug	7.5 ± 1.9 (5)	17, 12 (2)[b]	3.4 ± 0.5 (6)	11.6 ± 2.2[c] (8)
Amlodipine[d]	2.8 ± 0.6[e] (5)	6.0 ± 1.1[c] (4)	1.8 ± 0.1[e] (4)	4.8 ± 1.3[e] (6)

[a] Rats exposed to 10 oxygen for 4 weeks.
[b] In all except two of the rats in this group, Bay K8644 caused such a large vasoconstriction that the lungs became oedematous and the response could not be quantified.
[c] Significantly greater than corresponding values in normoxic rats ($P < 0.05$).
[d] Rats treated with 10 mg/kg/day amlodipine by oral gavage for 4 weeks.
[e] Significantly less than corresponding values in untreated (no drug) rats ($P < 0.05$).

amlodipine was adequate to inhibit the opening of L-type Ca^{2+} channels in the pulmonary vasculature.

3.2. Pulmonary vascular structure

In rats exposed to hypoxia, there were changes in structure throughout the pulmonary vascular tree, as follows. In pulmonary arteries, 101–500 μm and 30–100 μm o.d (examined in histological sections of lungs) values of % medial thickness were approximately double those seen in normoxic rats (Fig. 1). In isolated perfused lungs, there was an increase in the critical closing pressure (Fig. 2; *Y*-intercept of passive pressure-flow plots, normoxia

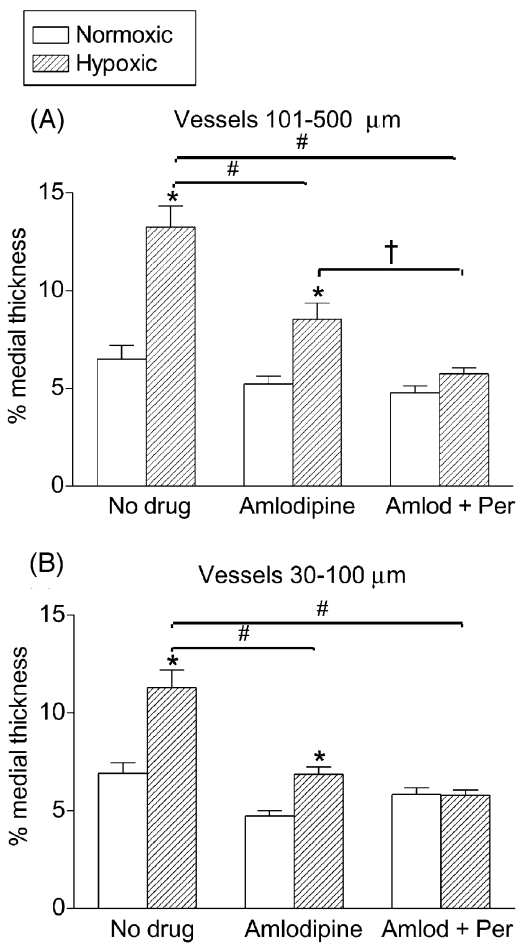

Fig. 1. Medial thickness (expressed as a percent of vessel diameter) of pulmonary arteries (A) 101–500 μm o.d. and (B) 30–100 μm o.d from rats exposed to normoxia or hypoxia, either given no drug ($n = 4$) or treated with amlodipine (10 mg/kg/day; $n = 4$) or a combination of amlodipine plus perindopril (10 and 30 mg/kg/day, respectively; Amlod + Per; $n = 4$). Values are means with S.E.M. shown by vertical lines. Each group of data represents 21–40 vessels. * Significantly greater than corresponding values for normoxic rats ($P < 0.05$). #Significant difference between values for drug-treated (amlodipine or amlodipine plus perindopril) and untreated (no drug) hypoxic rats ($P < 0.05$). †Significant difference between values for amlodipine-treated hypoxic rats and amlodipine plus perindopril-treated hypoxic rats ($P < 0.05$).

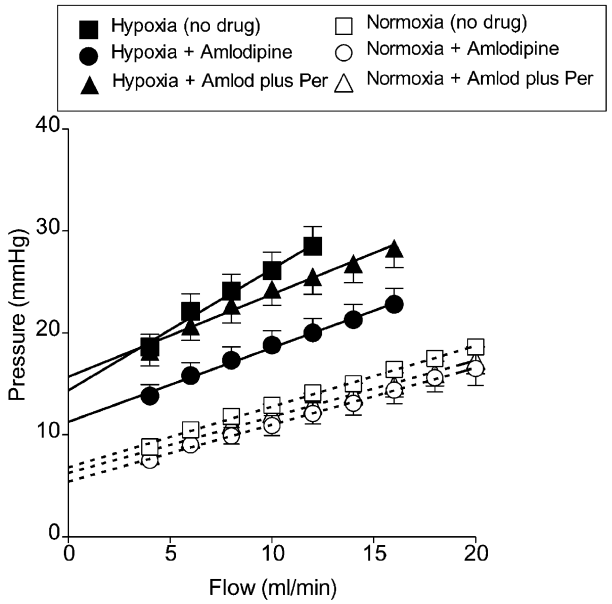

Fig. 2. Pressure flow plots obtained in isolated perfused lungs from normoxic and hypoxic rats either given no drug ($n = 4$) or treated with amlodipine (10 mg/kg/day; $n = 6–7$) or amlodipine plus perindopril (10 and 30 mg/kg/day, respectively; Amlod plus Per; $n = 3–4$). Points are means with S.E.M. shown by vertical lines, except when smaller than the symbols.

6.8 ± 0.91 mm Hg, $n = 4$; hypoxia, 14.4 ± 1.06 mm Hg, $n = 4$; $P < 0.05$) indicating an extension of smooth muscle into the normally non-muscular arteries in the alveolar region.

Treatment of hypoxic rats with amlodipine led to significant reductions in medial thickness of vessels 101–500 and 30–100 μm but the values were still significantly greater than corresponding values in normoxic rats (Fig. 1). When rats were treated with a combination of amlodipine plus perindopril, the reductions in medial thickness were more pronounced and, consequently, in both sizes of vessels, there was no longer any difference between normoxic and hypoxic rats (Fig. 1).

In isolated perfused lungs from rats treated with amlodipine or a combination of amlodipine plus perindopril, there was no significant reduction in critical closing pressure (11.4 ± 1.01 mm Hg, $n = 6$ and 15.6 ± 1.29 mm Hg, $n = 3$, respectively; $P > 0.05$ when compared with the value in untreated hypoxic rats cited above).

3.3. Pulmonary vascular resistance and pulmonary artery pressure

Pulmonary vascular resistance (determined from the slope of pressure-flow plots) and mean pulmonary artery pressure (measured in vivo) were significantly increased in hypoxic rats when compared with corresponding values obtained in normoxic rats (Table 3).

Table 3
Values for pulmonary vascular resistance (PVR) and mean pulmonary artery pressure (PAP) in normoxic and hypoxic rats: effects of treatment of rats with amlodipine or a combination of amlodipine plus perindopril
Data are mean ± S.E.M. Number of rats in parentheses.

	PVR (mm Hg/ml/min) (in isolated perfused lungs)		PAP (mm Hg) (in vivo)	
	Normoxic	Hypoxic[a]	Normoxic	Hypoxic[a]
No drug	0.60 ± 0.09 (4)	1.19 ± 0.08[b] (4)	12 ± 1.0 (6)	29 ± 1.7[b] (8)
Amlodipine[c]	0.55 ± 0.06 (7)	0.72 ± 0.05[d] (6)	17 ± 0.5[g] (12)	34 ± 0.8[b,d] (10)
Amlodipine + Perindopril[e]	0.55 ± 0.03 (4)	0.81 ± 0.04[b,d] (3)	17 ± 0.8[d] (8)	29 ± 1.8[b,f] (8)

[a] Rats exposed to 10% oxygen for 4 weeks.
[b] Significantly greater than corresponding values for normoxic rats ($P < 0.05$).
[c] Rats treated with 10 mg/kg/day amlodipine by oral gavage for 4 weeks.
[d] Significantly different from corresponding values for untreated (no drug) rats ($P < 0.05$).
[e] Rats treated with 10 mg/kg/day amlodipine plus 30 mg/kg/day perindopril by oral gavage for 4 weeks.
[f] Significantly less than corresponding value for amlodipine-treated hypoxic rats ($P < 0.05$).

In normoxic rats, treatment with amlodipine had no effect on pulmonary vascular resistance. However, treatment of hypoxic rats with amlodipine alone or in combination with perindopril caused a significant reduction in pulmonary vascular resistance (Table 3). Despite this reduction in pulmonary vascular resistance, there was a 15% increase in pulmonary artery pressure when rats were treated with amlodipine, but this increase was no longer apparent when rats were treated with amlodipine plus perindopril (Table 3). A significant increase in pulmonary artery pressure was seen in normoxic rats also, whether treated with amlodipine alone or in combination with perindopril. Since pulmonary vascular resistance was reduced in amlodipine treated hypoxic rats (Table 3), the increase in pulmonary artery pressure must reflect an increase in cardiac output, and this was presumably due to an increase in stroke volume since heart rate (beats/min) did not differ in any of the groups of rats (normoxic: no drug 377 ± 14.2, $n = 8$, amlodipine 403 ± 11.4, $n = 12$, amlodipine plus perindopril 360 ± 11.3, $n = 8$; hypoxic: no drug 364 ± 13.2, $n = 8$, amlodipine 357 ± 7.5, $n = 11$, amlodipine plus perindopril 349 ± 9.7, $n = 6$; $P > 0.05$).

3.4. Heart weights

In rats exposed to hypoxia, there was right ventricular hypertrophy as evidenced by a marked increase in RV/body weight (Table 4). Although there was a significant increase in (LV + S)/body weight, the increase was much smaller than the increase in RV/body weight (1.2-fold compared with 3-fold) and, unlike the increase in RV/body weight, it could be accounted for by the 1.1- to 1.3-fold decrease in body weight seen in each group of hypoxic rats compared with the corresponding group of normoxic rats (Table 1).

Treatment of hypoxic rats with amlodipine had no effect on right ventricular hypertrophy, i.e. RV/body weight was not reduced (Table 4). However, treatment with a combination of amlodipine plus perindopril did reduce right ventricular hypertrophy because there was a

Table 4
Ratios of RV/body weight and (LV + S)/body weight in normoxic and hypoxic rats: effect of treatment of rats with amlodipine or a combination of amlodipine plus perindopril
Data are mean ± S.E.M.

Treatment	n[a]	RV/body weight (mg/g)		(LV + S)/body weight (mg/g)	
		Normoxic	Hypoxic[b]	Normoxic	Hypoxic[b]
No drug	8	0.59 ± 0.02	1.65 ± 0.06[c]	2.46 ± 0.05	2.96 ± 0.06[c]
Amlodipine[d]	12	0.65 ± 0.02	1.69 ± 0.09[c]	2.41 ± 0.04	3.12 ± 0.11[c]
Amlodipine + Perindopril[e]	8	0.63 ± 0.03	1.36 ± 0.05[c,f,g]	1.91 ± 0.07[f,g]	2.35 ± 0.09[c,f,g]

[a] n = number of rats.
[b] Rats exposed to 10% oxygen for 4 weeks.
[c] Significantly greater than corresponding values for normoxic rats ($P < 0.05$).
[d] Rats treated with 10 mg/kg/day amlodipine by oral gavage for 4 weeks.
[e] Rats treated with 10 mg/kg/day amlodipine plus 30 mg/kg/day perindopril by oral gavage for 4 weeks.
[f] Significantly less than corresponding values for untreated (no drug) rats ($P < 0.05$).
[g] Significantly less than corresponding values for amlodipine-treated hypoxic rats ($P < 0.05$).

significant reduction in RV/body weight (Table 4). In this group, unlike the group treated with amlodipine alone, it was noted that there was also a marked reduction in (LV + S)/body weight. However, this reduction was seen in both normoxic and hypoxic rats (Table 4) and very likely reflects the marked reduction (34–38%; $P < 0.05$) in systemic artery pressure seen in rats treated with amlodipine plus perindopril (mm Hg; normoxic: no drug 80 ± 3.6, $n = 6$, amlodipine 82 ± 4.1, $n = 11$, amlodipine + perindopril 56 ± 7.5, $n = 8$; hypoxic: no drug 98 ± 2.6, $n = 8$, amlodipine 83 ± 2.6, $n = 11$, amlodipine + perindopril 60 ± 4.9).

4. Discussion

In this study, two different effects of chronic in vivo treatment of rats with amlodipine have been demonstrated in the pulmonary vasculature. Firstly, in isolated perfused lungs from both normoxic and hypoxic rats, there was inhibition of two vasoconstrictor responses known to involve Ca^{2+} entry via L-type channels, namely, responses to Bay K8644 (Ca^{2+} channel activator) and acute alveolar hypoxia (Weir and Archer, 1995). Secondly, in hypoxic pulmonary hypertensive rats there was attenuation of pulmonary vascular remodelling, i.e. medial thickening of pulmonary arteries 30–100 and 101–500 μm o.d. was reduced (though not totally prevented). Total prevention of medial thickening in these vessels was, however, achieved when amlodipine was administered in combination with the angiotensin converting enzyme inhibitor, perindopril. The inhibitory effects on remodelling were accompanied by reductions in pulmonary vascular resistance, as measured in isolated perfused lungs but, despite this, pulmonary artery pressure was not reduced. The possible reasons for this are discussed later.

Attenuation of pulmonary vascular remodelling by amlodipine was confined to vessels > 30 μm (measured in histological sections) and the reduction in medial thickening in these arteries was comparable in magnitude to data previously obtained with the angiotensin converting enzyme inhibitor, perindopril (Jeffery and Wanstall, 1999). In the very small arteries of the alveolar region, neomuscularisation (determined from critical closing pressure in perfused lungs) was not, however, altered by amlodipine and this was in contrast to perindopril (Jeffery and Wanstall, 1999). The finding that amlodipine attenuated medial thickening is in agreement with data from the two other studies with dihydropyridine Ca^{2+} channel antagonists (nifedipine and nitrendipine) in hypoxic pulmonary hypertensive rats (Michael et al., 1986; Stanbrook et al., 1984).

Total prevention, as opposed to partial attenuation, of the development of medial thickening was achieved when hypoxic pulmonary hypertensive rats were treated with a combination of amlodipine with perindopril. There are at least two possible reasons for the additive effect of these two drugs. It may reflect the fact that perindopril and amlodipine inhibit remodelling via different mechanisms, i.e. perindopril inhibits the production of a growth factor whereas amlodipine reduces growth factor action; in other words, the two drugs inhibit events that occur sequentially. Alternatively, the Ca^{2+} channel antagonist may not be effective against every growth factor involved in pulmonary vascular remodelling, and angiotensin II may be one of the factors that it does not inhibit.

The precise mechanism whereby Ca^{2+} channel antagonists inhibit the action of growth factors remains to be determined. Although the mitogenic action of some growth factors is known to involve the influx of extracellular Ca^{2+} (Mogami and Kojima, 1993; Roe et al., 1989), it is not known whether the anti-mitogenic properties of Ca^{2+} channel antagonists are dependent on their action on L-type Ca^{2+} channels or not (Ahmed et al., 1998; Stepien et al., 1997). Possible mechanisms independent of L-type Ca^{2+} channels include inhibition of (i) intracellular release of Ca^{2+} by a direct action on phosphoinositide turnover (Block et al., 1989; Roe et al., 1989), (ii) protein kinase C (Kataoka et al., 1997), (iii) the expression of early growth responses genes (e.g. c-myc, c-fos; Stepien et al., 1997) and (iv) mRNA expression of growth factors (e.g. transforming growth factor β; Kim et al., 1995).

Amlodipine, both alone and in combination with perindopril, reduced pulmonary vascular resistance in hypoxic rats as assessed in perfused lungs. This probably reflects, at least in part, the attenuation of medial thickening seen in vessels examined histologically since pulmonary vascular resistance is governed by vessels that occur proximal to the small resistance vessels in the alveolar region (Bee and Wach, 1984; Emery et al., 1981). The vasodilator action of amlodipine may also make a contribution by inhibiting resting levels of tone in the vessels, especially since perindopril (a non-vasodilator) alone had remarkably little effect on pulmonary vascular resistance (Jeffery and Wanstall, 1999). Despite the total prevention of medial thickening with the combination of the two drugs, values of pulmonary vascular resistance, though markedly reduced, were not completely restored to values seen in normoxic rats. The slope of the pressure-flow plots may have a component that reflects pulmonary vascular compliance, in addition to pulmonary vascular resistance (Bee and Wach, 1984). Therefore, we cannot exclude the possibility that the drugs may lack effect on the compliance of the vessels.

In spite of the beneficial effects of amlodipine treatment on vascular structure and pulmonary vascular resistance, there was not an associated reduction in pulmonary artery pressure. On the contrary, there was a small increase in pressure. This was also seen in normoxic rats treated with amlodipine and must reflect an increase in cardiac output secondary to systemic arterial dilation. This is supported by studies with other dihydropyridines in which direct

measurements of cardiac output have been made (e.g. Stanbrook et al., 1984). Since heart rate was not changed, any increase in cardiac output is presumably due to an increase in stroke volume. In contrast to our findings in hypoxic rats, in a different model of pulmonary hypertension (the monocrotaline-treated rat) amlodipine treatment caused a reduction in right ventricular systolic pressure, a measure that parallels pulmonary artery systolic pressure (Takahashi et al., 1996). The reason for this discrepancy is not known but it may reflect the fact that, without amlodipine treatment, pulmonary artery pressure was much higher in the monocrotaline-treated rats (Takahashi et al., 1996) than in the hypoxic rats (present study).

The 15% increase in pulmonary artery pressure induced by amlodipine treatment was in contrast to an 18% reduction in pulmonary artery pressure seen in perindopril-treated hypoxic rats (Jeffery and Wanstall, 1999). When both drugs were administered together, these two opposing effects evidently cancelled each other out, because pulmonary artery pressure was neither increased nor decreased.

Amlodipine treatment alone did not reduce right ventricular hypertrophy. This is in contrast to other studies, in both hypoxic and monocrotaline-induced pulmonary hypertension, in which Ca^{2+} channel antagonists have been shown to attenuate the development of right ventricular hypertrophy (Michael et al., 1986; Stanbrook et al., 1984; Takahashi et al., 1996). The persistence of right ventricular hypertrophy in the present study may be related to the observed increase in pulmonary artery pressure. When rats were treated with amlodipine together with perindopril, right ventricular hypertrophy was reduced even though pulmonary artery pressure remained elevated. This supports the idea that angiotensin converting enzyme inhibitors have a direct anti-growth effect on the heart (Morrell et al., 1997). In the only other study where a Ca^{2+} channel antagonist and an angiotensin converting enzyme inhibitor have been studied in combination in pulmonary hypertensive rats, verapamil plus captopril did not have any greater effect on pulmonary artery pressure and right ventricular hypertrophy than either drug alone (Kentera et al., 1984). Note that the study of Kentera et al. (1984) did not investigate pulmonary vascular structure.

In summary, this is the first study of chronic amlodipine treatment in vivo in hypoxic pulmonary hypertensive rats. It is also the first study in which the effects of a combination of a Ca^{2+} channel antagonist and an angiotensin converting enzyme inhibitor have been examined on pulmonary vascular structure. The data showed that amlodipine alone has beneficial effects on pulmonary vascular remodelling and this is translated into a beneficial effect on pulmonary vascular resistance. The fact that this did not result in a reduction in pulmonary artery pressure highlights the disadvantage of any drug therapy that causes marked systemic arterial dilation. The experiments using a combination of amlodipine with perindopril have shown that total prevention of medial thickening of small pulmonary arteries is achievable. The finding that combining amlodipine with another anti-remodelling drug produced effects on vascular structure that were additive raises the question of whether combination therapy with two anti-remodelling drugs with different mechanisms of action may be of value in the treatment of pulmonary hypertensive patients. It is conceivable that by using two drugs one could use lower doses of one or both of the drugs and still see a beneficial effect; if so, this might circumvent the problem of systemic arterial dilation. Ca^{2+} channel antagonists have been successfully used in humans with pulmonary hypertension and it has been assumed that their value lies in their vasodilator properties. However, it is common to use higher doses of these drugs than are used in other types of cardiovascular disease. This fact, together with the findings from the present study, raises the possibility that their benefit may be due in part to an anti-remodelling effect.

Acknowledgements

This work was supported by the National Health and Medical Research Council of Australia (JCW) and the National Heart Foundation of Australia (TKJ) and this financial assistance is gratefully acknowledged. The authors thank Pfizer for the generous provision of amlodipine and Servier Laboratories (Australia) for their generous provision of perindopril. The technical assistance of Paul Addison is also acknowledged.

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