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Neutral endopeptidase (NEP) inhibition in rats with established pulmonary hypertension secondary to chronic hypoxia

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1 Atrial natriuretic peptide (ANP) causes vasorelaxation in the pulmonary vasculature. ANP levels are elevated in conditions characterized by pulmonary hypertension and it has been hypothesized that ANP may be autoregulatory in the pulmonary circulation.

2 One route of ANP metabolism *in vivo* is by the action of the enzyme neutral endopeptidase (NEP). We have studied the effects of the NEP inhibitor, SCH 42495, in rats with established pulmonary hypertension secondary to chronic hypoxia.

3 Rats ($n = 32$) were divided into 4 groups. Normoxic controls were kept in air for 10 days (NC10) and all other animals were placed in a normobaric hypoxic chamber (Fi O_2 10%). Chronic hypoxic controls were studied at 10 days (CHC10). After 10 days hypoxia the two remaining groups received oral treatment for a further 10 days, consisting of either SCH 42495 (30 mg kg^{-1}, twice daily CHT20) or methyl cellulose vehicle (0.4%, twice daily, CHV20).

4 Animals were anaesthetized and blood collected for measurement of plasma ANP. Hearts were dissected and ventricles weighed and the histology of the pulmonary vasculature examined.

5 CHC10 rats had significant right ventricular hypertrophy (0.53 ± 0.08) and pulmonary vascular remodelling ($29.0 \pm 0.01\%$) and had gained significantly less body weight (33.2 ± 5.5 g) than NC10 rats (0.31 ± 0.04, $10.9 \pm 0.01\%$, and 59.2 ± 11.9 g respectively). CHC10 rats had significantly elevated plasma ANP levels (58.4 ± 9.9 pM) compared with NC10 rats (23.9 ± 32 pM). Treatment with SCH 42495 caused a significant reduction in pulmonary vascular remodelling ($25.0 \pm 0.01\%$) and right ventricular hypertrophy (0.52 ± 0.09) in CHT20 rats compared with CHV20 controls ($33.0 \pm 0.02\%$ and 0.61 ± 0.09 respectively). Pulmonary vascular remodelling was also significantly lower in CHT20 rats than CHC10 animals.

6 Thus, short term inhibition of NEP causes regression of established pulmonary vascular remodelling and may be a useful therapeutic strategy in pulmonary hypertension.

Keywords: Atrial natriuretic peptide; neutral endopeptidase inhibitor; chronic hypoxic pulmonary hypertension; remodelling

Introduction

Atrial natriuretic peptide (ANP) is a 28 amino acid hormone of cardiac origin which exhibits vasoactive, diuretic and natriuretic properties (Adams, 1987). ANP is released in response to an increase in pulmonary artery pressure because of right atrial and ventricular distension. In disease states characterized by an increased pulmonary artery pressure, such as primary pulmonary hypertension (Morice *et al.*, 1990), chronic obstructive airways disease (COAD) (Winter *et al.*, 1989), acute respiratory distress syndrome (Eison *et al.*, 1988), and chronic thromboembolism (Noll *et al.*, 1990), ANP synthesis and secretion is increased and plasma levels are elevated.

ANP is a potent vasodilator in the pulmonary vasculature. Jansen *et al.* (1987) showed that ANP was more potent in isolated pulmonary, as opposed to renal, vessels of the pig. We have demonstrated that ANP causes a dose-dependent relaxation in preconstricted isolated perfused lungs (Stewart *et al.*, 1991b), and isolated pulmonary resistance vessels (Rogers *et al.*, 1992) in the rat and that relaxation appears to be particularly potent against hypoxic vasoconstriction (Rogers *et al.*, 1992).

Chronic pulmonary hypertension causes remodelling of the pulmonary vasculature, with neomuscularization of pulmonary vessels towards the periphery of the lung. Infusion of ANP with osmotic minipumps has been shown to reduce the cardio-pulmonary remodelling associated with the development of hypoxic pulmonary hypertension in rats (Zhao *et al.*, 1991), as has chronic elevation of ANP in mice that have been genetically engineered to over-express ANP (Klinger *et al.*, 1993a). However, the therapeutic potential of ANP is limited by rapid *in vivo* metabolism with a plasma half life of ANP of less than 3 min in the rat (Hollister *et al.*, 1989). The metabolic action of the enzyme neutral endopeptidase 24.11 (NEP) and clearance receptor binding are the two identified mechanisms of ANP clearance. Because of this dual pathway of clearance, attempts to elevate plasma ANP solely by inhibiting NEP have met with limited success. However, NEP inhibition does prolong the half life of ANP, elevate plasma ANP levels, and cause diuresis on volume loading in rats (Watkins *et al.*, 1993), normal human volunteers (Danielwicz *et al.*, 1989) and patients in chronic heart failure (Northridge *et al.*, 1989).

We and others have previously found that NEP inhibition caused a significant reduction in the cardio-pulmonary remodelling associated with the development of pulmonary hypertension in rats exposed to chronic hypoxia (Winter *et al.*, 1991; Stewart *et al.*, 1992; Klinger *et al.*, 1993b; Thompson *et al.*, 1994). Unfortunately, these models are of limited clinical relevance in that they study the development of pulmonary hypertension. Of more interest is the effect of NEP inhibition on established pulmonary hypertension. In the present study therefore, we have investigated the effects of the NEP inhibitor SCH 42495 on rats with established cardio-pulmonary remodelling secondary to chronic hypoxia.

Methods

Litters of male Wistar rats (Royal Hallamshire Hospital) were obtained at 21 days of age and allowed to acclimatize to

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the laboratory for 5 days. Animals were maintained in 12 h/12 h dark/light cycle with free access to standard rat chow and water. The litters were divided into four groups, two of 6 rats and two of 10 rats. One group of 6 animals were placed in the normobaric, hypoxic chamber at an Fi O_2 of 10% for 10 days (chronic hypoxic controls; CHC10), while the other group of 6 rats were kept in the same room but exposed to air for 10 days (normoxic controls; NC10).

The two remaining groups of 10 rats were placed in the normobaric, hypoxic chamber at an Fi O_2 of 10% for 20 days. After 10 days, one group (chronic hypoxic drug-treated; CHT20) received SCH 42495 (30 mg kg^{-1}, 1 to 2 ml) while the other group (chronic hypoxic vehicle-treated; CHV 20) received aqueous methyl cellulose vehicle (0.4%, 1 to 2 ml) at 12 hourly intervals by oral gavage. All animals were weighed daily prior to the morning dose. Treatment resulted in rats in the hypoxic chamber being exposed to room air for approximately 30 min twice daily. Groups of rats were anaesthetized with pentobarbitone (60 mg kg^{-1}, i.p.) and blood samples were collected in a heparinized syringe from the main pulmonary artery via the right ventricle. Blood was immediately transferred to chilled EDTA tubes, centrifuged (3000 r.p.m., 15 min), and plasma stored at − 40°C for subsequent radioimmunoassay of ANP (Stewart et al., 1991a) (intra-assay coefficient of variation, 16.3%; inter-assay coefficient of variation, 14.6%; assay sensitivity, 8.9 pM; and limit of detection, 2.0 pM).

The heart was carefully removed from the animal and the atria cut away and discarded. The right ventricle (RV) was dissected from the left ventricle plus septum (LV + S) and post-mortem blood removed by blotting on a piece of tissue paper. The ventricles were weighed on a chemical balance (Ohaus Analytical Plus) and the ratio of RV to LV + S determined. The trachea was cannulated and lungs inflated and fixed with 10% buffered formol saline (pH 7.4) at a pressure of 20 cm water. Blocks (3 mm) were cut from the left lung just below the hilum and vacuum embedded in paraffin wax; 5 μm sections were cut and stained with Miller's solution. Slides were examined under an optical microscope (× 40 magnification) without prior knowledge of their identity. All vessels less than 50 μm diameter with an elastic coat, situated adjacent to an alveolar duct or alveoli, were counted. The proportion of these vessels with a double elastic lamina (DEL) around at least 50% of the vessels' internal wall were calculated, as previously described (Leach et al., 1977).

Results were expressed as mean ± standard error of the mean (s.e.mean). Groups were compared by Student's two-tailed unpaired t test.

Results

Body weight

After 10 days exposure to a normobaric, hypoxic environment, the CHC10 animals had gained significantly ($P<0.001$) less weight (g) than the NC10 controls (33.2 ± 5.5 and 59.2 ± 11.9 respectively). There was no significant difference in change in body weight between the CHV20 group, which received vehicle for a further 10 days in the hypoxic environment, and the CHT20 group, which received SCH 42495 for a further 10 days in the hypoxic environment (84.4 × 10.2 and 87.6 ± 13.1 respectively). Results are shown graphically in Figure 1.

RV/[LV + S]

The CHC10 rats had developed significant ($P<0.001$) right ventricular hypertrophy when compared with NC10 controls (0.53 ± 0.08 and 0.31 ± 0.04 respectively) after 10 days in the hypoxic chamber. Treatment with SCH 42495 for 10 days arrested any further development of ventricular hypertrophy, the ventricular ratio for the CHT20 animals was similar to the ratio of the CHC10 animals (0.52 ± 0.09 and 0.53 ± 0.08 respectively). Those animals receiving vehicle for 10 days (CHV20) demonstrated continued progression of right ventricular hypertrophy, the ratio being significantly greater ($P<0.05$) than the CHC10 animals (0.61 ± 0.09 and 0.53 ± 0.08 respectively). Results are shown graphically in Figure 2.

Pulmonary vascular remodelling

Ten days exposure to normobaric hypoxia resulted in significant remodelling of the pulmonary vasculature (Figure 3). The proportion of vessels < 50 μm that had developed a DEL in CHC10 animals was significantly greater ($P<0.005$) than the NC10 rats (29.0 ± 0.01% and 10.9 ± 0.01% respectively). Treatment with SCH 42495 caused an attenuation in the development of DEL. Not only was the degree of pulmonary vascular remodelling in the CHT20 rats less than that in the CHV20 animals (25.0 ± 0.01% and 33.0 ± 0.02% respectively, $P<0.05$), but it was also significantly less ($P<0.05$) than the degree of pulmonary vascular remodelling observed after 10 days exposure to hypoxia in the CHC10 rats (29.0 ± 0.01%). Results are shown graphically in Figure 4.

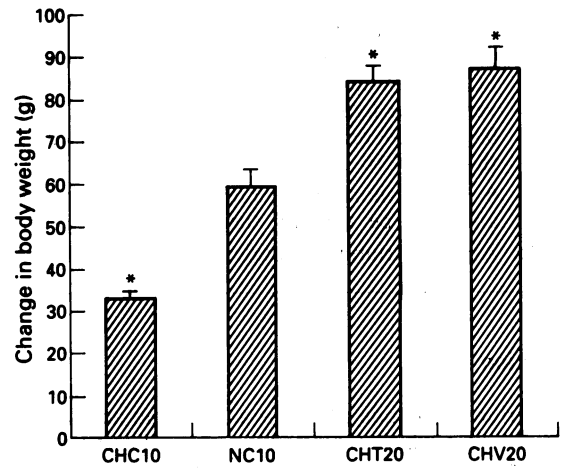

Figure 1 Change in body weight (g) in untreated, control rats exposed to chronic hypoxia for 10 days (CHC10) and normoxia for 10 days (NC10), and in rats treated with SCH 42495 (CHT20) or vehicle (CHV20) during a further 10 days in the chronic hypoxic environment. *$P<0.001$ compared with NC10. Results are as mean ± s.e.mean.

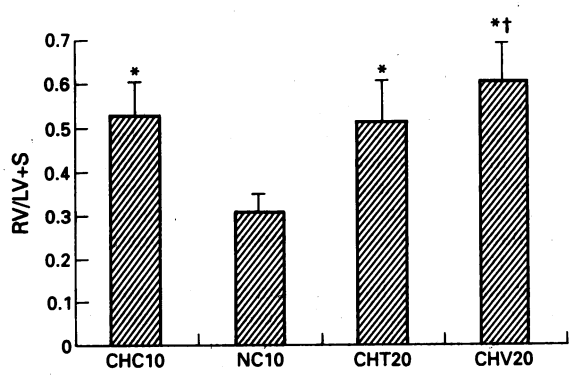

Figure 2 Ventricular ratios in untreated, control rats exposed to chronic hypoxia for 10 days (CHC10) and normoxia for 10 days (NC10), and in rats treated with SCH 42495 (CHT20) or vehicle (CHV20) during a further 10 days in the chronic hypoxic environment. *$P<0.001$ compared with NC10. †$P<0.05$ compared with CHC10. Results are as mean ± s.e.mean.

ANP levels

Plasma ANP levels (pM) were significantly ($P<0.05$) elevated after 10 days in CHC10 rats (58.4 ± 9.9) compared with NC10 controls (23.9 ± 3.2). Treatment with SCH 42495 did not result in any further elevation of plasma ANP levels in CHT20 rats (23.9 ± 5.7). Indeed, treatment with SCH 42495 caused a significant decrease in plasma ANP levels towards levels observed in the NC10 rats. Vehicle-treated animals (CHV20) continued to demonstrate significantly elevated plasma ANP levels (47.4 ± 11.2 pM). Results are shown graphically in Figure 5.

Discussion

Chronic alveolar hypoxia, whether it is normobaric or hypobaric, is a well-documented model of man at high altitude and of hypoxic lung disease. Rats exposed to low inspired oxygen tensions develop pulmonary hypertension (Rabinovitch *et al.*, 1979) which is associated with pulmonary vascular remodelling, right ventricular hypertrophy, and an increase in haematocrit secondary to the renal response to hypoxia (Abraham *et al.*, 1971; Leach *et al.*, 1977). The development of pulmonary hypertension is closely correlated with the morphological changes seen in the cardio-pulmonary system, particularly the pulmonary vascular remodelling (Rabinovitch *et al.*, 1979).

In the pulmonary vascular bed remodelling is characterized by a thickening of the vessel wall predominantly by hyperplasia of smooth muscle. These changes occur primarily in the small, non-muscularized, precapillary pulmonary arteries and result in the apparent duplication of the elastic laminae (Rabinovitch *et al.*, 1979). The structural changes in the pulmonary vascular tree caused by chronic exposure to hypoxia are largely reversible on return to air. In the rat, pulmonary vascular remodelling begins after about 2 days exposure and is complete after about 10 days (Hunter *et al.*, 1974).

In this study we examined pulmonary vessels less than 50 μm internal diameter following exposure to normobaric hypoxia using the development of a double elastic lamina as a marker for pulmonary vascular remodelling. Hypoxia caused a threefold increase in the proportion of small vessels

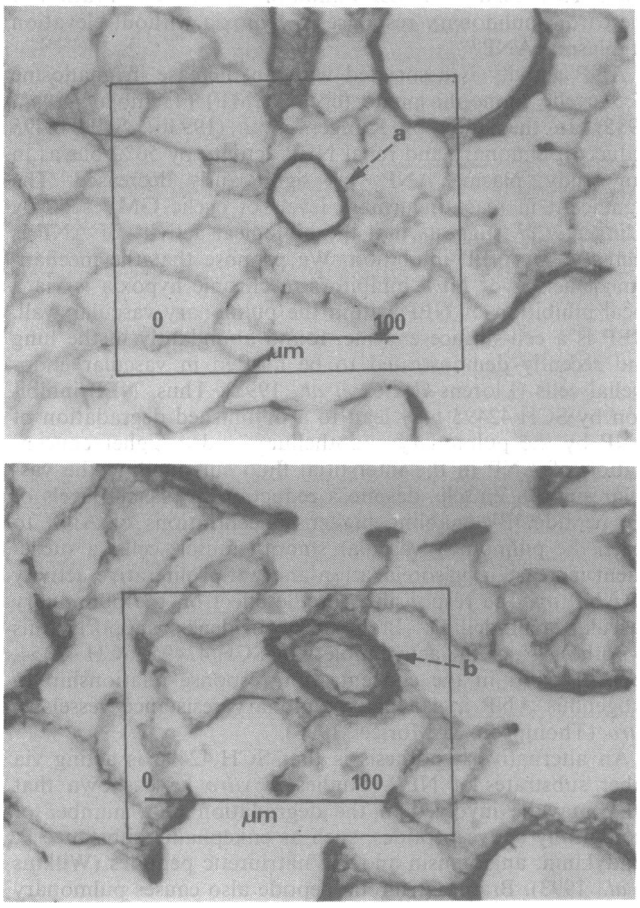

Figure 3 The light microscopical appearance of (a) a normal pulmonary vessel (< 50 μm external diameter) from a littermate kept in room air and (b) a remodelled rat pulmonary vessel (< 50 μm external diameter) after 10 days exposure to chronic, normobaric hypoxia. Sections were stained with Miller's solution and viewed under a magnification of × 40. Treatment (SCH 42495, 30 mg kg^{-1}) or rats with established pulmonary vascular remodelling for a further 10 days (CHT20) resulted in significant ($P<0.05$) fewer pulmonary vessels which had developed a DEL around at least 50% of the internal circumference compared with vehicle-treated, chronic hypoxic rats (CHV20).

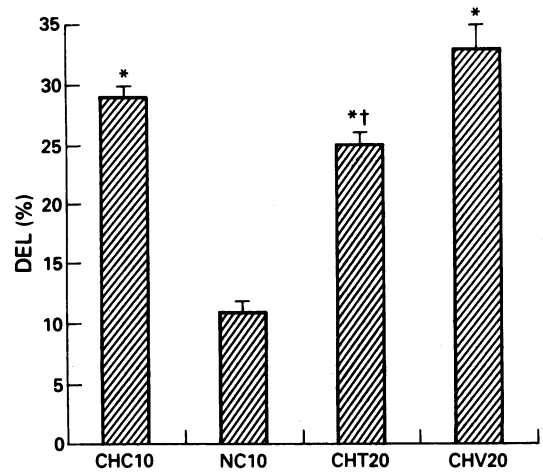

Figure 4 Proportion of vessels (< 50 μm internal diameter) which had developed a double elastic lamina (DEL, %) around at least 50% on the internal circumference in the four groups of rats, were compared. Groups were untreated, control rats exposed to chronic hypoxia for 10 days (CHC10) and normoxia for 10 days (NC10), and in rats treated with SCH 42495 (CHT20) or vehicle (CHV20) during a further 10 days in the chronic hypoxic environment. *$P<0.005$ compared with NC10. †$P<0.05$ compared with CHC10 and CHV20. Results are as mean ± s.e.mean.

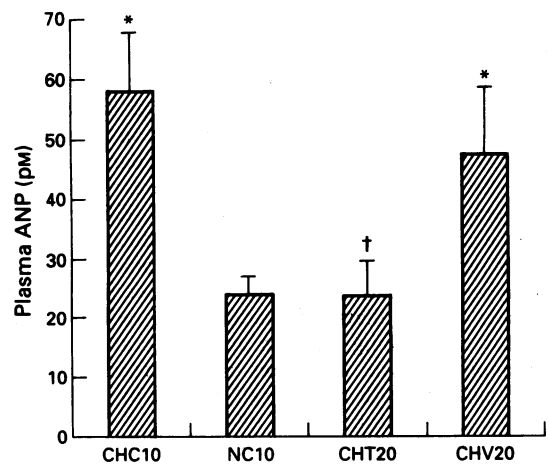

Figure 5 Plasma ANP levels (pM) in untreated, control rats exposed to chronic hypoxia for 10 days (CHC10) and normoxia for 10 days (NC10), and in rats treated with SCH 42495 (CHT20) or vehicle (CHV20) during a further 10 days in the chronic hypoxic environment. *$P<0.05$ compared with NC10. †$P<0.05$ compared with CHC10 and CHV20. Results are as mean ± s.e.mean.

exhibiting a double elastic lamina, an effect which was established after 10 days. Animals also demonstrated significant right ventricular hypertrophy, the ratio of RV/LV + S increasing by two thirds after 10 days hypoxia. Unlike the changes of pulmonary vascular remodelling, right ventricular hypertrophy progressed with continued hypoxic exposure, the RV/LV + S ratio doubling after 20 days exposure.

On return to normoxia, the pathophysiological changes which develop during chronic hypoxia in this rat model resolve at different rates. Right ventricular hypertrophy takes approximately 12 weeks to resolve whereas pulmonary vascular remodelling is still evident after 20 weeks and never fully resolves (Hunter *et al.*, 1974; Leach *et al.*, 1977). In man, descent from high altitude to sea level leads to similar resolution of cardio-pulmonary remodelling, but with a much slower time course (Heath & Williams, 1981).

In patients with COAD, pulmonary vascular resistance is elevated (Matthay *et al.*, 1990), due in part to remodelling of the small pulmonary arteries and arterioles (Singer *et al.*, 1987). The only treatment available to date which has altered mortality in COAD is long-term oxygen therapy, which has been suggested to act by inhibiting the further development of pulmonary vascular remodelling secondary to hypoxia. Thus, treatment that causes a reduction in the degree of vascular remodelling, as opposed to simple pulmonary vasodilatation, may be preferable in diseases characterized by hypoxic pulmonary hypertension, since studies investigating the use of vasodilators in COAD have so far failed to demonstrate any effect on prognosis (Howard, 1989).

In animal models, the majority of studies looking at pulmonary vascular remodelling have examined the effect of therapy on the development of remodelling during exposure to a stimulus (usually hypoxia). Whilst this is a good screening test for potential activity, in the clinical situation the patient presents with established pulmonary hypertension and ideally therapy should reverse established pulmonary vascular remodelling.

It has been hypothesized that ANP may be involved in a homeostatic feedback loop to protect the pulmonary vasculature from pulmonary hypertension. In support of this, plasma levels of ANP have been shown to be elevated in response to increased pulmonary artery pressure both in animal models (McKenzie *et al.*, 1986; Jin *et al.*, 1990) and in man (Winter *et al.*, 1989; Morice *et al.*, 1990). The vasodilatation caused by ANP is particularly potent in the pulmonary circulation (Jansen *et al.*, 1987) with hypoxic vasoconstriction being preferentially inhibited (Rogers *et al.*, 1992). In a recent study we have shown that an acute infusion of ANP in patients with COAD and pulmonary hypertension caused specific pulmonary vasodilatation without adversely effecting systemic haemodynamics or ventilation perfusion matching (Rogers *et al.*, 1994).

The effect of ANP on pulmonary vascular remodelling in response to chronic hypoxia has been studied by continuous infusion of exogenous ANP and a reduction of DEL has been demonstrated (McKenzie *et al.*, 1986; Zhao *et al.*, 1991). Similarly, transgenic mice that overexpress ANP have reduced remodelling in chronic hypoxia (Klinger *et al.*, 1993a). The mechanism by which ANP reduces the cardio-pulmonary remodelling associated with chronic hypoxic pulmonary hypertension is undefined. It is most likely to be a combination of vasodilatation leading to a reduction in the stimulus for remodelling, and an antiproliferative action on vascular smooth muscle, as studies have demonstrated inhibition of vascular smooth muscle cell growth by ANP *in vitro* (Abell *et al.*, 1989; Itoh *et al.*, 1990).

ANP is rapidly cleared from the circulation by clearance receptor binding and the metabolic action of the membrane-bound enzyme, neutral endopeptidase (NEP) EC 24.11. ANP therefore has a relative short half life *in vivo* (Hollister *et al.*, 1989), and has little therapeutic potential. Attempts to prolong the bioavailability of ANP *in vivo* have included ANP clearance receptor blockade, and inhibition of NEP. Studies using NEP inhibitors *in vivo* have demonstrated an increase in the half-life and natriuretic effect of exogenous ANP (Lafferty *et al.*, 1989; Sybertz *et al.*, 1990a). This is a specific effect since ANP antisera blocks the activity of NEP inhibitors (Shepperson *et al.*, 1991). We have previously demonstrated an attenuation in the development of pulmonary hypertension secondary to chronic hypoxia in rats by the NEP inhibitor, SCH 42495 (Thompson *et al.*, 1994), although no significant elevation in plasma ANP levels were observed. Similar results have also been reported with the less potent (Watkins *et al.*, 1993) compound, SCH 34826 (Stewart *et al.*, 1992), and the structurally unrelated drug, Candoxatrilat (Winter *et al.*, 1991). However, in this latter study, Winter *et al.* observed a significant elevation of plasma ANP levels in rats treated with Candoxatrilat during seven days exposure to hypoxia and proposed that the mechanism by which NEP inhibition reduced pulmonary vascular remodelling was by elevation of plasma ANP levels.

In the present study, NEP inhibition with SCH 42495 led to significant reduction in the progression of pulmonary vascular remodelling, which was clearly not attributable to elevations in plasma ANP levels, as we observed a significant fall in plasma ANP with drug treatment. Similarly, Klinger *et al.* (1993b) showed that rats treated with the less potent NEP inhibitor, SCH 34826 (90 mg kg^{-1}; twice daily) during 3 weeks of chronic hypoxia had a significantly lower degree of right ventricular hypertrophy, and less pulmonary vascular remodelling, despite a significant reduction in plasma ANP levels. How then does NEP inhibition lead to modification of the cardio-pulmonary response to hypoxia without elevation of plasma ANP?

ANP activity is associated with an increase in guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Tremblay *et al.*, 1985). In the study by Klinger *et al.* (1993b), SCH 42495 reduced pulmonary and renal NEP activity by 50% but as in our study, plasma ANP was significantly decreased. The significant increase in urinary levels of cyclic GMP seen by Klinger *et al.* suggests that the biological activity of ANP is increased by NEP inhibition. We propose that the mechanism of action of NEP inhibitors in chronic hypoxia is via a local inhibition of NEP within the pulmonary vascular wall. NEP is a cell-surface enzyme, found abundantly in the lung and recently demonstrated to be located in vascular endothelial cells (Llorens-Cortez *et al.*, 1992). Thus, NEP inhibition by SCH 42495 may lead to a diminished degradation of ANP by the pulmonary endothelium, and a higher concentration of ANP in the interstitial fluid surrounding the vascular smooth muscle, despite a reduction in plasma levels of the peptide. By enabling higher concentrations of ANP to reach the pulmonary vascular smooth muscle cells, a subsequent increase in vasorelaxation and antiproliferative activity of ANP may be responsible for the effect on the pulmonary vascular remodelling. Preliminary evidence supports this hypothesis since the active moiety of SCH 42495, SCH 42354, causes a shift in the concentration-response relationship to exogenous ANP in isolated pulmonary resistance vessels *in vitro* (Thompson & Morice, 1993).

An alternative hypothesis is that SCH 42495 is acting via other substrates of NEP. Studies *in vitro* have shown that NEP may be involved in the degradation of a number of biologically active peptides, such as enkephalin, substance P, bradykinin, angiotensin and the natriuretic peptides (Wilkins *et al.*, 1993). Brain natriuretic peptide also causes pulmonary vasodilatation (Rogers *et al.*, 1993), and C-type natriuretic peptide has vasorelaxant and antiproliferative actions (Furuya *et al.*, 1991; Stingo *et al.*, 1992).

SCH 42495 appears to be relatively specific to NEP, with little activity against other peptidases. Watkins *et al.* (1993) demonstrated that the *in vivo* hypotensive response to SCH 42495 (3 and 30 mg kg^{-1}), seen in volume expanded rats, was not affected by intravenous administration of two substrates of ACE, bradykinin or angiotensin II. Similarly, Sybertz *et al.* (1990b) showed that, whilst the antihypertensive response

to SCH 42495 in DOCA-salt hypertensive rats was abruptly reversed by ANP antibodies, intravenous administration of a bradykinin antagonist had no effect.

Treatment with the NEP inhibitor, SCH 42495, partially reversed the proportion of DEL and attenuated the further development of right ventricular hypertrophy, in rats in which pulmonary vasculature had already been remodelled by exposure to 10 days hypoxia. To date only two studies have demonstrated a reduction in pulmonary vascular remodelling in established pulmonary hypertension, both using calcium channel blockade (Stanbrook *et al.*, 1984; Michael *et al.*, 1986). In man, calcium channel blockade is used in the treatment of primary pulmonary hypertension and may affect prognosis (Rich *et al.*, 1992). In pulmonary hypertension secondary to COAD, calcium channel blockade has proven less successful (Howard, 1989). Thus, the demonstration that inhibitors of NEP cause a reduction in established pulmonary vascular remodelling may have important implications for the treatment of this disease.

In summary, we have demonstrated that treatment with the NEP inhibitor, SCH 42495, reduced the established cardio-pulmonary changes associated with pulmonary hypertension secondary to chronic hypoxia in rats. However, this occurred despite a reduction in plasma ANP levels suggesting that elevated tissue, rather than plasma, levels of the peptide are involved in the action of this NEP inhibitor.

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