Atrial natriuretic peptide levels in plasma and in cardiac tissues after chronic hypoxia in rats

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SUMMARY

1. Atrial natriuretic peptide (ANP) levels were measured in cardiac tissues and in plasma from adult rats exposed to chronic alveolar hypoxia for periods of 2 h, 24 h and 7 days. Levels were also measured in rats that were maintained in hypoxia for 7 days and then returned to air for 24 h.

2. Plasma ANP was not altered at 2 h but was significantly increased at both 24 h and at 7 days. Plasma ANP in animals exposed to hypoxia for 7 days was normal 24 h after returning to air breathing, despite the persistence of indices of pulmonary hypertension.

3. No significant right atrial hypertrophy was observed under these conditions of chronic hypoxia. A reduction in right atrial ANP content was found at 24 h and was accompanied by a decrease in the number of electron-dense granules per right atrial muscle cell. After exposure to hypoxia for 7 days, right atrial ANP and granule number was not different from control, and no alteration was found in right atrial ANP level after removal from the hypoxic environment.

4. No significant right ventricular hypertrophy was produced by exposure to hypoxia for 2 or 24 h. In the former group ventricular ANP had decreased significantly compared with control. Right ventricular hypertrophy was found in both the hypoxic groups after exposure for 7 days, when selective increases in right ventricular ANP content were found.

5. These findings are consistent with the hypothesis that ANP release occurs on exposure to chronic hypoxia and is independent of the associated cardiac hypertrophy and pulmonary vascular remodelling. The findings may have relevance to the natriuresis and reported changes in the renin-angiotensin-aldosterone axis under hypoxic conditions.

Key words: anoxia, atrial natriuretic peptide, myocardium.

Abbreviations: ANP, atrial natriuretic peptide; FiO₂, fractional inspired oxygen; RA/LVW, right atrial weight/left ventricular weight; RV/LVW, right ventricular weight/left ventricular weight.

INTRODUCTION

Mammalian atrial muscle cells possess numerous electron-dense granules containing the 28 amino acid atrial natriuretic peptide (ANP) stored as a 126 amino acid prohormone [1]. Release occurs in response to atrial distension [2] and can be modified by volume loading [3] and by alteration of venous return [4] or, more directly, by induced disorders of cardiac rhythm [5]. Smaller quantities of ANP immunoreactivity are found in ventricular tissue, but the role of this and other extra-atrial sites may have in secretion are not understood. In addition to described natriuretic and vasorelaxant effects, ANP has important regulatory actions on the renin-angiotensin-aldosterone system that are seen at physiological concentrations of the peptide [6]. Thus ANP inhibits the stimulation by angiotensin II, adrenocorticotrophic hormone and potassium of aldosterone secretion by rat adrenal glomerulosa cells [7]. An inhibitory effect of ANP in vivo has also been documented on the action of endogenously released angiotensin II-stimulated aldosterone secretion [8].

Natriuresis and diuresis are conspicuous features of exposure to chronic hypoxia in man, and individual differences have been considered to be an important factor in the development of altitude sickness [9]. Milledge et al. [10] found increased levels of plasma renin activity in humans at altitude and have shown reduced aldosterone responses for a given increment in plasma renin activity. These findings have been subsequently confirmed by other investigators [11]. The suggested mechanism was a
decreased level of plasma angiotensin-converting enzyme activity, but this possibility has not been supported by further experiments [12].

Thus, a body of evidence now exists to suggest a change in salt and water homeostasis and an altered inter-relationship between the components of the renin-angiotensin-aldosterone axis in hypoxia. We therefore reasoned that if right heart pressures were raised as a result of hypoxic pulmonary artery vasoconstriction, then ANP release might occur and explain the observed changes in aldosterone secretion. We have therefore investigated this possibility by simultaneous measurement of ANP immunoreactivity in plasma and cardiac tissue (prepared from both pre-hypertrophied and hypertrophied tissue) during 7 days exposure to chronic hypoxia and recovery. A further aim was to study the appearance of right atrial electron-dense granules during hypoxic exposure.

MATERIALS AND METHODS

Animals

Male Wistar rats in the weight range 250–400 g were used for these experiments. They were maintained in a flexible film hypoxic chamber [fractional inspired oxygen $FIO_2 = 10\%$] with carbon dioxide and excess humidity removed by means of filters [13]. The fractional oxygen concentration was continuously monitored by a polarographic oxygen analyser. Gas was sampled periodically for analysis by mass spectrometer and showed $FIO_2$ to be within 0.5% of the prescribed level and $FCO_2$ to be less than 0.2% at all times. Pellet diet and water were given to all groups ad libitum. Normoxic control animals were kept in the same room and exposed to the same light–dark cycle.

Experimental design

Experiment 1. Animals were removed after exposure to hypoxia for 2 or 24 h and were compared with controls in normoxia (10 animals in the three groups).

Experiment 2. Animals were maintained in the hypoxic environment for 7 days and compared with their normoxic control group. In this study an additional group was removed after 7 days in the hypoxic chamber and allowed to recover in normoxic conditions for a further 24 h (10 animals in the three groups).

Measurements and collection of samples

Tissue and plasma were collected from the six groups at the same time of day to obviate any effect of diurnal variation [14]. Particular care was taken to avoid stress during the handling of the animals. A short time (<10 min) after removal from their respective environments blood was obtained by decapitation. This method was used for collection of blood because anaesthetic agents reportedly have a large effect on plasma levels of ANP [15]. Plasma was removed, samples frozen rapidly and stored at −20°C until assay. The heart and lungs were removed en bloc, and the heart was separated. The right ventricle was opened at the apex and together with the right atrium was dissected free. The right atrium was then separated. The left ventricle and septum were removed and left atrium and aortic root were removed. The right ventricle, right atrium and left ventricle were lightly blotted and then weighed using a chemical balance. Right ventricular and right atrial hypertrophy was expressed as right ventricular weight/total ventricular weight (RV/LVW) and right atrial weight/left ventricular weight (RA/LVW), respectively. This method has been used because left ventricular weight relative to both log (body weight) does not alter under conditions of hypoxia, whereas right ventricular weight relative to both log (body weight) and left ventricular weight is increased, reflecting selective hypertrophy of this chamber [16]. As in previous experiments the septum was included with the left ventricle, since functionally it forms part of it [17].

After the right atrium and right ventricle were divided, the parts to be used for radioimmunoassay of ANP content were weighed and then extracted in boiling 0.5 mol/l acetic acid for 10 min. A ratio of 1:10 wet weight of tissue/volume of 0.5 mol/l acetic acid was employed, thus a volume of 0.5–1.0 ml was used depending on the tissue weight. This is a well-validated method of extraction that has been used for several different peptides [18] and has been used previously for ANP [19]. Tissue extracted was stored at −20°C until later assay.

Portions of fresh right atrial tissue were immediately cut into 1 mm cubes, fixed in 3% (v/v) glutaraldehyde (2 h) and post fixed in 1% (w/v) osmium tetroxide (1 h). Blocks were processed by standard procedures and embedded in epoxy resin. Ultrathin sections were cut with glass knives, stained by uranyl acetate/lead citrate (0.12%, w/v), and examined in an AEI 801 electron microscope.

Radioimmunoassay of ANP

Plasma ANP immunoreactivity was measured by direct assay of plasma [20]. Serial plasma dilution curves were found to be parallel with the synthetic α-ANP standard. The plasma sample volume was 100 μl, assayed in duplicate as previously. The assay detection limit in rat plasma is 5 fmol/ml. Tissue ANP reactivity was measured by the addition of 10 μl of the acid extract, directly (for ventricles) or after 1:1000 or 1:10 000 dilution (for the atria) to the assay tubes. Samples from each experiment were assayed at the same time as their normoxic controls, to overcome any difficulties in interpretation as a result of known interassay variability. Moreover, since measurement of tissue ANP requires an initial extraction process, we considered it especially important not to use historical controls. Tissue ANP levels were expressed per g wet weight.

Atrial granule morphometry

Semi-quantitative estimation of the atrial muscle electron-dense granules was undertaken. Comparison was
made between the 24 h hypoxic and normoxic groups (experiment 1), and between 7 day hypoxic (experiment 2) and normoxic animals. The number of granules was counted in three cells per animal, where abundant cytoplasm surrounded each pole of the nucleus. Assessment was made by the same observer from electron micrographs at the same magnification (×8000). Four animals were assessed in both the hypoxic groups together with four normoxic control animals.

Data analysis
Values for cardiac hypertrophy, tissue and plasma ANP were compared with an analysis of variance. Comparison between the granule count in the groups was made with Student’s t-test. Significance was assumed if \( P < 0.05 \).

RESULTS
Cardiac hypertrophy
Individual results for cardiac hypertrophy are given in Figs. 1(a) (experiment 1) and 1(b) (experiment 2). No significant right atrial or right ventricular hypertrophy was apparent up to 24 h: RA/LVW (mean ± SEM) was 0.072 ± 0.004 in the control group, which was not significantly different from the values of 0.068 ± 0.005 and 0.077 ± 0.006 at 2 and 24 h hypoxia, respectively, as shown in Fig. 1(a). The corresponding values for RV/LVW were 0.214 ± 0.009 in the control group, and 0.237 ± 0.008 and 0.239 ± 0.009 for exposure to hypoxia for 2 and 24 h, respectively (both non-significant).

No significant change in RA/LVW was seen in either group after 7 days hypoxia: RA/LVW was 0.075 ± 0.006 in control animals, 0.088 ± 0.008 in 7 day hypoxia and 0.096 ± 0.007 in hypoxia and recovery as shown in Fig. 1(b). After 7 days significant right ventricular hypertrophy was seen (RV/LVW hypoxic 0.320 ± 0.012; normoxic 0.224 ± 0.012, \( P < 0.001 \)) that was also present in the group returned to air breathing for a further 24 h (RV/LVW 0.316 ± 0.015, \( P < 0.001, n = 10 \)).

Plasma ANP
Plasma ANP was increased after 24 h hypoxia (24 ± 1 pmol/l) compared with control (19 ± 1 pmol/l, \( P < 0.05, n = 10 \)), but not after 2 h hypoxia (21 ± 1 pmol/l) as shown in Fig. 2. After 7 days hypoxia a 60% increase in plasma ANP was found (hypoxic 43 ± 3 pmol/l, control 27 ± 2 pmol/l, \( P < 0.01 \)). Plasma ANP was normal in the hypoxic group returned to air breathing for 24 h (30 ± 2 pmol/l) and was not significantly different from control animals (Fig. 3).

Ventricular ANP
In the pre-hypertrophied ventricle a significant depletion of ventricular ANP was evident after 2 h hypoxic exposure (16 ± 3 pmol/g wet tissue compared with 62 ± 15 pmol/g wet tissue in controls, \( P < 0.01, n = 10 \)). Right ventricular ANP levels were 70 ± 17 pmol/g after 24 h, not different from controls (Fig. 2). In experiment 2, the levels of ANP in the hypertrophied right ventricular tissue were 30 ± 5 and 27 ± 4 pmol/g, after 7 days and 7 days with 24 h recovery, respectively, both significantly
greater than in the control group (6 ± 1 pmol/g), as shown in Fig. 3 (P < 0.001 in both cases).

**Atrial ANP**

Individual results for atrial ANP levels are given in Fig. 2 (experiment 1) and Fig. 3 (experiment 2). Control right atrial ANP was 56 ± 5 nmol/g wet tissue, not different from the value of 60 ± 6 nmol/g wet tissue after 2 h hypoxic exposure. After 24 h exposure right atrial ANP level was 35 ± 3 nmol/g wet tissue, significantly reduced compared with control (P < 0.01, n = 10). Atrial ANP content did not differ in the three groups in experiment 2 (normoxic control 131 ± 11 nmol/g tissue, hypoxic 138 ± 18 nmol/g, hypoxic with recovery 120 ± 14 nmol/g).
Atrial electron-dense granules

Examination was made at 24 h and after 7 days. At 24 h there was a decrease in the number of granules per cell, with 110 ± 13 granules per cell in control group compared with 50 ± 9 in the hypoxic group (P<0.01) [Figs. 4 and 5]. After 7 days the number of granules per cell was 99 ± 13 per cell, not significantly different from control.

DISCUSSION

The use of this model enabled us to examine changes in tissue ANP and granule morphology, in tandem with plasma levels, during various periods of hypoxic exposure and recovery.

The increases in RV/LVW indicate that the hypoxic stimulus was sufficient to produce right ventricular hypertrophy after exposure of 1 week, but not after 24 h. These findings are concordant with information about the early time course at this level of hypoxia in rats, where detectable pulmonary hypertension, pulmonary vascular remodelling, significant increases in packed cell volume and right ventricular hypertrophy are seen as early as 4 days [21]. Limited information is available about whether there are also changes in the relative weight of the right atrium with prolonged hypoxia, but we did not find significant changes in RA/LVW, the chosen index of right atrial hypertrophy, suggesting that this is not a feature of early exposure.

The results show clearly that plasma ANP is increased during the initial 7 days exposure to chronic hypoxia, from as early as 24 h. The concomitant reduction in atrial levels suggests that the initial increase in plasma ANP was derived from an atrial source. This is further supported by the reduction in electron-dense granules in right atrial tissue at 24 h. This contrasts with the findings at 7 days when elevated plasma ANP levels were seen but with normal right atrial ANP levels and atrial granule number, which we interpret as showing increased synthesis. The finding that after 24 h removal from the hypoxic conditions plasma ANP had returned to normal, without detectable accumulation of ANP in the right atrium, also shows that ANP synthesis adjusts rapidly after removal from a provocative stimulus.

In the study we tried to examine whether ANP release was the result of the hypoxia itself, or of associated cardiac and pulmonary vascular remodelling. Experiment 2 was designed to dissociate these two possible stimuli through the inclusion of a group returned to air breathing after hypoxic exposure. Although pulmonary vascular remodelling takes as little as 4 days to develop in these conditions, it persists for a surprisingly long period after removal; this disparity between rapid development and slow deviation has been well described previously [22]. Although not measured directly, previous work has indicated that the group allowed to recover would have had raised pulmonary artery pressures as a result of pulmonary vascular remodelling. Since RV/LVW is known to be closely correlated with pulmonary artery pressure in this model [23]. Thus, if raised pulmonary artery pressure were directly or indirectly the main signal for ANP release, then increased levels would be predicted in the group allowed to recover, but this was not found. It is not possible to accurately measure atrial pressure in conscious animals within the chamber, but removal from the hypoxic environment may have been associated with a reduction in right atrial pressure and may have been the mechanism involved (through relief of hypoxic pulmonary artery vasoconstriction and right ventricular pressure overload). The observation that plasma ANP was normal after 24 h air breathing could also be explained by a capability of the system to reset to higher pressure levels, and thus sensing pressure change rather than absolute levels.

The finding of a 74% reduction in the ventricular ANP content after 2 h hypoxia shows that the ventricle is capable of responding quickly to pressure overload. That this large decrease in right ventricular immunoreactivity after 2 h was not accompanied by an increased plasma level suggests that ANP derived from the non-hypertrophied ventricle does not contribute to any appreciable extent to plasma levels, a finding that may reflect the 1000-fold smaller concentration of ANP immunoreactivity in the ventricles compared with the atria. However, depletion of ventricular ANP immunoreactivity has been seen in patients with heart failure, raising the possibility that this and perhaps other extra-atrial sources may have a role in certain pathophysiological conditions [24]. The considerable increase in right ventricular ANP immunoreactivity in hypertrophy found after 7 days hypoxia is unexplained on the basis of present knowledge; furthermore, because ANP levels were expressed per g wet weight of tissue, the selective increase seen in the hypertrophied right ventricle implies an even greater increase in total ANP immunoreactivity at this site. Cardiac tissue ANP will be determined by the balance between synthesis and release, and the change in ventricular content seen in

![Fig. 4. Number of electron-dense granules/muscle cell in right atrial tissue from controls, 2 h and 7 days hypoxic groups. The number of granules was counted in three cells per animal, where abundant cytoplasm surrounded each pole of the nucleus (see the Methods section). n = 4 animals in each group. Statistical significance: * P<0.01.](image-url)
Fig. 5. Electron micrographs (original magnification ×8000) of representative right atrial muscle cells in normoxic tissue (a) and 24 h hypoxic tissue (b).

Experiment 2 raises the possibility that this balance has in some way been reset under conditions of hypertrophy. These studies, however, do not allow one to comment directly upon the synthesis of ANP. It will be of particular interest to employ quantitative hybridization methods in situ to obtain, more directly, information about ANP synthesis under conditions of hypoxia and selective ventricular hypertrophy.

These results showing a substantial increase in plasma ANP in hypoxia accord with a previous paper describing increases in rats after 21 days exposure to chronic hypoxia [25]. Our finding that plasma ANP is increased during the first 7 days of exposure to hypoxia, is in keeping with a physiological role for the peptide in the natriuresis and changes in the renin-angiotensin-aldosterone system, since both occur during the initial few days
exposure [9, 10]. The physiological importance of these findings was outside the scope of the present enquiry but will be an important area for further study, as will their applicability to man. Changes in ANP, the renin–angiotensin–aldosterone cascade system and the carotid body require further study, but there is now evidence to suggest that these operate together to effect the changes in water and sodium balance in hypoxic conditions.

REFERENCES

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