Expressions of adrenomedullin mRNA and protein in rats with hypobaric hypoxia-induced pulmonary hypertension

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A high-altitude hypoxic environment (HHE) is known to cause pulmonary hypertension in both humans and animals (5, 18, 20, 21, 23, 24, 30). This hypoxic hypoxia-induced pulmonary hypertension has been shown to be accompanied by structural remodeling of the heart and pulmonary arteries (18, 20, 21–23, 24, 30). Over the last decade or so, there have been many studies dealing with the changes in cardiovascular hormones that occur in people with high altitude disease (3, 15) and in animals exposed to a hypoxic environment (1, 2) or chronic hypoxia (6, 9, 21, 23, 28, 33, 37). However, few reports have dealt with alterations in adrenomedullin (AM) in response to high altitude disease (3, 15) and in animals exposed to hypobaric hypoxia (40). AM, which was originally isolated from human pheochromocytoma, plays an important role as a potent hypotensive peptide, serving to promote vasodilation, diuresis, and natriuresis and to inhibit aldosterone production in normal animals (12, 13). AM peptide and mRNA are reportedly expressed in the adrenal gland, heart, kidney, lung, and endothelium in humans and rats (13, 31), and several investigators have found plasma AM levels to be increased in patients with pulmonary hypertension (25, 26, 35). Furthermore, tissue levels of AM peptide and mRNA have been shown to be increased in the heart, kidney, and lung in monocrotaline-induced pulmonary hypertension (MIPH) (38). These findings suggest that AM may play a role in modulation of pulmonary hypertension (MIPH) (38). These findings suggest that AM may play a role in modulating pulmonary hypertension (MIPH) (38). These findings suggest that AM may play a role in modulating pulmonary hypertension (MIPH) (38).

MATERIALS AND METHODS

We used essentially the same methods as in previous reports (20, 21, 23, 24). Although we intended several experiments to involve 4–12 wk of exposure to HHE, many rats scheduled for 6–12 wk of exposure became weak and emaciated, or even died, some time after 4 wk of exposure (22). Therefore, in the present study, we elected to use a maximum of 21 days of exposure to HHE. Briefly, a total of 150 adult male Wistar rats, ~8 wk old and weighing 190–210 g, were divided into seven groups. Groups of 17 or 18 rats each were housed in a 5.6 m × 3.0 m × 3.0-m mechanical chamber (Hitachi) and exposed to an HHE equivalent to 5,500 m in altitude (380 mmHg for 3 wk). We measured PAP as well as the expression of AM mRNA and the concentration of AM peptide in plasma and a variety of organs (by in situ hybridization and immunohistochemical methods) to help us examine the possible causes of the morphological and physiological changes occurring during HHE exposure.
in the accessory room. The oxygen content in the main room was maintained constant at a fraction of inspired oxygen of 0.105 to simulate 5,500 m in altitude. The age of the rats when exposure to HHE was initiated was such that all were ~11 wk old at the termination of the experiment. The chamber was kept at an ambient temperature of 23 ± 1°C and a relative humidity of 60–70%, with light exposure daily from 0600 to 1800 h. All rats were given commercial chow and tap water ad libitum. After 0.5, 1, 3, 5, 7, 14, or 21 days of exposure to HHE, rats were killed by decapitation. An additional 17 rats kept in the ground level environment served as controls. This experimental study was carried out in accordance with the recommendations in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Committee on the Ethics of Animal Experiments of the National Defense Medical College.

Measurement of Pulmonary Arterial Pressure

At 0.5, 1, 3, 7, 14, or 21 days of exposure to HHE, six rats in each group were subjected to measurement of mean PAP and mean systemic arterial pressure (SAP) under anesthesia produced by an intramuscular injection of ketamine hydrochloride (60 mg/kg body wt), as previously described (20, 21, 23, 24). To this end, a polyethylene catheter [inner diameter (ID) 0.28 mm and outer diameter (OD) 0.61 mm for PAP; ID 1.0 mm and OD 1.5 mm for SAP] was inserted into the right jugular vein or left jugular artery as appropriate. With the pressure being monitored all the time, the catheter was advanced to either the pulmonary artery through the right ventricle (RV) of the heart or to the ascending aorta. Mean PAP and mean SAP were then recorded.

Blood Sampling and Autopsy

Before an autopsy, the rats were decapitated and blood samples were taken (from rats not subjected to arterial pressure measurement) for the determination of hematocrit and AM. The blood samples were placed in chilled tubes containing EDTA (2 mg/ml) and aprotinin (1 million IU/ml; Peptide Institute; Osaka, Japan) and then centrifuged. The plasma was frozen immediately and stored at −80°C until assay. At autopsy, the heart was removed and dissected on ice into bilateral atrial and ventricular tissues. The apical one-half of the ventricular tissue was used as the ventricular sample to prevent atrial contamination. Tissues were weighed, frozen in liquid nitrogen and stored at −80°C until use. The cardiac tissues so obtained were subjected to RNA and peptide extractions. The ratio of the left ventricle plus septum (LV + S) over RV (LV + S/RV) was calculated for the assessment of RV hypertrophy. All the parts of the various organs isolated from each of 11 rats were frozen in liquid nitrogen and stored at −80°C until used for RT-PCR and ELISA. Tissues (including cardiac tissues) isolated from five rats were fixed separately in OCT compound, 10% formalin, or 4% paraformaldehyde solution for in situ hybridization and immunohistochemistry.

Total RNA Extraction and Semiquantitative RT-PCR

In all 21 organs or tissues isolated from 3 control rats, as well as in all 9 tissues isolated from 5 rats in each of the groups exposed to HHE, total RNA was isolated using acid guanidinium isothiocyanate-phe- nol-chloroform extraction and ethanol precipitation (20, 23). RT-PCR was performed using an amplification reagent kit (TaqMan EZRT-PCR kit, Applied Biosystems; Alameda, CA) with rat AM or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, as previously reported (20). AM and GAPDH primers were synthesized by an automated DNA synthesizer. The sense and antisense sequences of the AM primers were 5′-AGA ATG GGA ATA AGT GGG CGC T-3′ and 5′-ATG CGG CCG TCC TTG TCT TG TCT G-3′, respectively, whereas that of the TaqMan probe was 5′-CAC AGC CCA CAT TCG AGT CAA ACG CTA C-3′. The cDNA amplification product was predicted to be a 301-bp fragment from position 248 to 548 in the cDNA of rat AM. Primers were also synthesized to amplify the cDNA encoding GAPDH, a constitutively expressed gene, as a control. For GAPDH, the primers and TaqMan probe used were 5′-CCT CAC CAT GAT GGA AGA GCC-3′ (sense), 5′-GCG ATG GAC TGT GGT CAT GAG-3′ (antisense), and 5′-CCT GGC CAA GGT CAT CCA TGA CAA CTT T-3′ (TaqMan probe), giving rise to a 237-bp PCR product corresponding to bases 368–605 in the cDNA base sequence of rat GAPDH. Both TaqMan probes were labeled at the 5′ end with the reporter dye molecule 6-carboxyfluorescein and at the 3′ end with the quencher dye 6-carboxytetramethylrhodamine. The reaction master mix was prepared according to the manufacturer’s protocol to give final concentrations of 1× reaction buffer, 300 μM dATP, 300 μM dCTP, 300 μM dGTP, 600 μM dUTP, 3 mM Mg(OAc)₂, 0.1 U/μl rTth DNA polymerase, 0.01 U/μl AmplErase UNG, 200 nM AM primers, and 100 nM TaqMan probe. To perform PCR, the RT reaction was incubated at 60°C for 30 min, followed by incubation at 95°C for 5 min to deactivate AmplErase UNG. PCR was performed using 50 or 40 amplification cycles (for AM mRNA and GAPDH mRNA, respectively) at 95°C for 20 s and at 68 or 60°C for 1 min using an ABI PRISM 7700 Sequence detector (Applied Biosystems). Total RNA extracts from the adrenal gland and liver were used as control templates for AM mRNA and GAPDH mRNA, respectively. PCR products were separated by electrophoresis in a 3% agarose gel and stained with ethidium bromide.

In Situ Hybridization

In situ hybridization was performed essentially as previously described (20, 21, 23). Briefly, deparaffinized 4% paraformaldehyde-fixed sections were treated with 0.2 N HCl for 20 min, then incubated in 2× SSC for 10 min at 37°C, and finally incubated in 5 μg/ml proteinase K for 10 min at 37°C. Sections were subsequently postfixed in 4% paraformaldehyde for 5 min and then incubated for 10 min in 0.1 mol/l tritionanolime buffer (pH 8.0) containing 0.25% (vol/vol) acetic anhydride to prevent nonspecific binding due to oxidation of the tissue. The rat AM cDNA probe used was a 301-bp fragment (obtained from positions 248 to 548 in the cDNA of rat AM) subcloned into the EcoRI site of a pGM-Easy Vector (Promega). Antisense probes and the corresponding sense probes were labeled with digoxigenin using SP6 and T7 polymerases, respectively, by means of an RNA labeling kit (Boehringer Mannheim). Hybridization was carried out overnight at 42°C in 50% (vol/vol) deionized formamide, 5× Denhardt’s solution, 5% (vol/vol) dextran sulfate, 2× SSC, 0.3 mg/ml salmon sperm DNA, 5 mM EDTA, and 0.01 μg/ml digoxigenin-labeled probes. After a final stringency wash was performed at 55°C for 20 min, hybridization was detected immunologically.

Extraction of Tissues and Plasma and Determination of AM Protein

Tissue samples from six rats in each group were boiled for 10 min in 10 volumes of 0.1 M acetic acid containing 0.1% Triton X-100 to avoid intrinsic proteinolyis and then homogenized using a polytron homogenizer (Kinematica; Lucerne, Switzerland). The homogenate was centrifuged at 15,000 g for 30 min at 4°C. The supernatant and a plasma sample from six rats in each group were then applied to a Sep-Pak C18 cartridge (Waters; Milford, MS) that had been preequilibrated with 0.5 M acetic acid, and adsorbed materials were eluted using 2.0 ml of 60% acetonitrile in 0.1% trifluoroacetic acid. The eluates were then evaporated in a vacuum to dryness and stored at −80°C until the concentration of AM protein was measured using ELISA (Phoenix Pharmaceuticals; Belmont CA). The minimum detection limit for the AM protein concentration was 1.0 ng/ml.

Immunohistochemistry

The indirect immunoperoxidase method was applied to 4-μm-thick frozen sections (20). Primary rabbit polyclonal antibodies against AM protein (Peninsula Laboratories; Belmont, CA) diluted at 1:200 and

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horseradish peroxidase-labeled secondary antibody against rabbit immunoglobulins (Chemicon International; Temecula, CA) diluted at 1:250 were used. For the negative control, the incubation step with the primary antibody was omitted.

**Statistical Analysis**

The results are expressed as means ± SE. Fisher’s protected least-significant difference test or Scheffé’s test was applied to the data when significant $F$ ratios were obtained in ANOVA. Differences were considered significant at $P < 0.05$.

**RESULTS**

**Normal Rats**

*Expression of AM mRNA and protein.* Cell-specific expressions of AM mRNA and protein were observed in the adrenal gland, kidney, stomach, salivary gland, pancreas, and intestine. In the adrenal gland, such signals were observed in medullary cells (Figs. 1a and 2a). In the kidney, AM mRNA and protein were expressed strongly in the epithelial cells of the proximal and distal tubules, but they were barely detectable in the mesangial cells or endothelial cells of the glomeruli or in transitional cells (Figs. 1b and 2b). In the stomach, epithelial cells in the lower portion of the glands showed focally positive reactions for both AM mRNA and protein (Figs. 1c and 2c). In the salivary gland, AM mRNA and protein were each evident in the ductal epithelial cells (Fig. 1d and 2d). In the remaining tissues, AM mRNA and protein were each present in pancreatic islet cells (Figs. 1e and 2e) and focally and weakly present in epithelial cells in the intestine. However, AM mRNA and protein were barely or only weakly detectable in myocytes (Figs. 1f and 2f). No expressions of AM mRNA and protein were seen in the spleen, lung, brain, esophagus, testis, skin, skeletal muscle, pituitary gland, or thyroid gland. Hybridization with an AM mRNA sense probe revealed weak levels of background grains in all preparations.

*Semiquantitative RT-PCR for AM mRNA and level of AM protein.* By RT-PCR, AM mRNA was detected in all 21 organs or tissues examined from control rats (Fig. 3A). We then performed semiquantitative RT-PCR on these tissues [for 18 tissues, samples were examined for each of 3 rats separately, but for 3 tissues (pituitary gland, thyroid gland, and salivary gland) samples from 3 rats were combined to give 1 sample/tissue because of the small size of the individual samples]. Relatively high levels of AM mRNA were detected in the

![Fig. 1.](image_url)
adrenal gland and lung, intermediate levels in the right atrium (RV), kidney, stomach, large intestine, spleen, testis, skin and pituitary gland, and low levels in the LV, RV, left atrium (LA), brain, liver, aorta, esophagus, small intestine, skeletal muscle, thyroid gland, and salivary gland (Fig. 3). By ELISA, AM protein was detected in the plasma and in all nine tissues or organs examined (from 6 rats in the control group): these were RV, LV, LA, and RA of the heart, adrenal gland, brain, lung, kidney, and liver (Fig. 4). Relatively high levels of AM protein were detected in the adrenal gland (1.6 ± 0.11 ng/mg protein) and lung (0.67 ± 0.07 ng/mg protein), whereas low levels were detected in both atria (RA, 0.25 ± 0.11 ng/mg protein; LA, 0.20 ± 0.02 ng/mg protein), both ventricles (RV, 0.19 ± 0.06 ng/mg protein; LV, 0.19 ± 0.04 ng/mg protein), brain (0.16 ± 0.02 ng/mg protein), kidney (0.05 ± 0.01 ng/mg protein), and liver (0.11 ± 0.12 ng/mg protein). The plasma level of AM protein was 0.20 ± 0.01 ng/ml.

Rats Exposed to HHE

Growth characterization and hemodynamic study. The body weight of rats exposed to HHE decreased from 335 ± 6.3 g (control rats) to 280 ± 5.2 g at 21 days of exposure (Table 1). At all time points at or after 3 days of exposure, the body weight of HHE rats was significantly less than that of the control rats (P < 0.05). The hematocrit of HHE rats was significantly higher than that of control rats at all time points at or after 0.5 days of exposure to HHE (P < 0.05). The mean PAP of HHE rats was significantly higher than that of control rats at 14 and 21 days of exposure to HHE (P < 0.05), whereas mean SAP was not changed by exposure to HHE. Among the cardioventricular measurements, the ratios of LV+S/RV and RV over body weight were significantly lower than control after 0.5 or more days and 5 or more days, respectively, of exposure to HHE (P < 0.05 and P < 0.05, respectively), indicating RV hypertrophy.

In situ hybridization and immunohistochemistry. The RV tissues of control rats showed barely detectable signals for AM mRNA and protein, but staining for both AM mRNA and protein was more intense in the middle to late phase of HHE exposure (Figs. 1, f and g, and 2, f and g). However, RVs displayed some regional variations in staining intensity and in the proportion of cells with a positive cytoplasm. No changes in the staining for AM mRNA or protein were seen...
in the other organs or tissues throughout the period of exposure to HHE.

Expressions of AM mRNA and protein in rat tissues. Semi-quantitative RT-PCR for AM mRNA was performed on the adrenal gland, heart, lung, brain, kidney, and liver (Fig. 5). In the heart, the AM mRNA level in the RV was significantly increased at 0.5 and 1 days of exposure and also at 14 and 21 days of exposure to HHE. AM mRNA levels were increased at day 14 of exposure to HHE in the LA and at 14 and 21 days in the RA. AM mRNA levels in the lung and brain were also increased at 0.5 or 0.5–5 days of exposure to HHE, respectively, but they returned to normal thereafter. In contrast, in the adrenal gland, AM mRNA showed significant decreases at 0.5 and 1 days of exposure to HHE. No changes in AM mRNA levels were seen in the LV, kidney, or liver.

The plasma level of AM protein was significantly increased at 21 days of exposure to HHE by comparison with that of control rats (P < 0.05; Fig. 4). In the RV and RA from 5 to 14 days, and in the LA after 14 or more days of exposure to HHE, AM protein levels were significantly higher than in control rats (P < 0.05). AM protein levels were significantly higher than in control rats at 3 days in the lung and at 0.5–5 days in the brain, but they returned to normal thereafter. In contrast, the AM protein level in the adrenal gland was significantly lower than in control rats after 14 or more days of exposure to HHE (P < 0.05).

DISCUSSION

Although there have been two studies dealing with alterations in AM in an experimental model of pulmonary hypertension (induced by monocrotaline) (32, 38), no report has focused specifically on the quantitative and qualitative changes in AM in rats with pulmonary hypertension induced by hypobaric hypoxia. We maintained rats under hypobaric hypoxic conditions for up to 21 days. We demonstrated an elevation in PAP and RV hypertrophy and an increase in the circulating AM concentration after exposure to HHE. In the heart, we observed overexpression of AM mRNA in RV tissues and RA tissues at 14 and 21 days of exposure to HHE (using semi-quantitative RT-PCR analysis), and we also observed increases in the amounts of AM protein in the plasma, RV tissues, and both RA and LA tissues in the middle to late period of exposure (using ELISA). Conceivably, these changes could play an important role in modulating the pulmonary hypertension induced by HHE, although we have no direct evidence for such a causal link. In contrast, although both AM mRNA and protein levels were increased in the brain and lung at an early stage of the exposure to HHE, they had returned to control levels after 7 days of exposure. Possibly, the elevated AM levels in the brain and lung may reflect a protective mechanism against acute hypoxia and a mechanism serving to improve gas exchange, respectively. In each tissue, this would involve dilatation of local vessels early on in the period of hypobaric hypoxia. If this is so, AM may prove to be a sensitive biochemical marker for acute hypoxia and hypoxia-induced pulmonary hypertension.

The plasma level of AM protein is increased in a variety of diseases, including cardiovascular, respiratory, hepatic, and renal disorders (4, 10). In cardiovascular diseases, plasma AM is significantly increased in patients with hypertension, congestive heart failure, acute myocardial infarction, pulmonary hypertension, and so on (4, 10, 14, 25, 26, 35). However, circulating levels of AM were found not to be changed by either acute or chronic salt loading in normotensive subjects and patients with essential hypertension (8). In hypertensive animal models, the increase in the plasma AM level partici-
Fig. 4. Concentrations of AM protein in the adrenal gland, plasma, LV, RV, RA, and RA of the heart, lung, brain, kidney, and liver in rats exposed to a HHE. *P < 0.05 vs. value at time 0.

Table 1. Body weight, PAP<sub>m</sub>, SAP<sub>m</sub>, hematocrit, and cardioventricular measurements in rats exposed to a hypobaric hypoxic environment

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<th>Hypobaric Period, days</th>
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<td>0</td>
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<tr>
<td><strong>Body weight, g</strong></td>
<td></td>
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<tr>
<td>10–11</td>
<td>335±6</td>
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<tr>
<td><strong>PAP&lt;sub&gt;m&lt;/sub&gt;, mmHg</strong></td>
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<tr>
<td>6</td>
<td>14.7±1.0</td>
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<td><strong>SAP&lt;sub&gt;m&lt;/sub&gt;, mmHg</strong></td>
<td></td>
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<tr>
<td>4–6</td>
<td>120.4±11.6</td>
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<tr>
<td><strong>Hematocrit, %</strong></td>
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<tr>
<td>6</td>
<td>42.2±0.1</td>
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<tr>
<td><strong>Cardioventricular measurements</strong></td>
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<tr>
<td><strong>RV/body weight, %</strong></td>
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<tr>
<td>10–11</td>
<td>0.055±0.005</td>
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<tr>
<td><strong>LV+S/RV</strong></td>
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<td>10–11</td>
<td>3.8±0.06</td>
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Values are means ± SE; n = no. of rats. PAP<sub>m</sub>, mean pulmonary arterial pressure; SAP<sub>m</sub>, mean systemic arterial pressure; RV, right ventricle; LV+S, left ventricle plus septum. Fisher’s protected least-significant difference test or Scheffe’s test was applied to the data when significant F ratios were obtained in an analysis of variance. *P < 0.05 vs. the value at time 0.
pates in a mechanism to counteract the raised blood pressure (11, 19). Moreover, increased levels of locally synthesized AM and systemic plasma AM are considered to compensate for, or prevent, damage to target tissues, such as the kidney, heart, and vasculature (19). In the present study, we demonstrated an increase in the plasma AM level at 21 days of exposure to HHE. Although such an elevation in plasma AM might prevent damage in target tissues, as in systemic hypertension, we could not find a local elevation in AM protein levels in the lung, kidney, liver, or brain tissues in the late period of exposure to HHE. The reason for these unchanged levels of AM protein in the lung and other tissues is unclear. However, our findings are consistent with the observation by Zhao et al. (40) that in the lungs of rats exposed to normobaric hypoxia (10% oxygen) for 7 days, endogenous immunoreactive AM concentrations and AM mRNA levels were unchanged (compared with control rats).

With the use of experimental models of chronic heart failure, a number of investigations have revealed that AM mRNA and protein are present in the heart and that AM protein may be released from the heart in association with hemodynamic changes, including atrial stretch and increases in ventricular wall stress, mean PAP, and/or pulmonary capillary wedge pressure (11, 14, 19). Thus the authors indicated that the

![Fig. 5. Semiquantitative RT-PCR of AM mRNA in the adrenal gland, LV, RV, LA, and RA of the heart, lung, brain, kidney, and liver in rats exposed to a HHE. To derive the data, the actual AM mRNA/GAPDH mRNA value obtained for a given tissue at 0 days in one individual rat was expressed as 1.0 and all other values for that tissue were expressed in a relative manner. *P < 0.05 vs. value at time 0.](http://ajpheart.physiology.org/doi/10.1152/ajpheart.00110.2004)
elevation of PAP may play a causal role in the increased secretion of AM, although it is unknown whether it is the elevated pressure in the pulmonary circulation per se that directly stimulates the secretion of AM. In the present study, we demonstrated an increase in the plasma AM level concomitant with a severe increase in mean PAP. These findings therefore support the above idea.

Although AM has been shown to have diuretic and natriuretic activities and to be secreted predominantly by the adrenal gland, lung, and digestive tissues in normal states, vascular tissues are considered to be the main source of circulating AM (12, 13, 31, 34). However, the mechanism responsible for the increase in circulating AM protein is well not understood. In experimental pulmonary hypertension, recent studies demonstrated that the AM level in the RV increased with increases in RV systolic pressure and RV weight after the injection of monacrotaline (32, 38). Hofbauer et al. (7) demonstrated that AM mRNA and protein levels in plasma and all organs examined (brain, lung, heart, kidney, and liver) increased in response to a 6-h exposure to normobaric hypoxia (10% oxygen), functional anemia (0.1% CO), or cobalt chloride (6 mg/kg), each of which would cause hypoxemia. They suggested that the increase in AM mRNA in these organs may have led to an increased synthesis and release of AM protein, resulting in the elevation in circulating AM. In pulmonary hypertension in patients with mitral stenosis, however, Nishikimi et al. (25) showed no increase in plasma AM between the femoral vein and pulmonary artery. This result suggests that secretion of AM from the heart may not contribute greatly to the circulating AM concentration in mitral stenosis. Thus the sites responsible for producing the circulating AM have not yet been identified.

Among the changes in AM levels seen in a number of organs in our study, we could discern two patterns in response to exposure to HHE for 21 days: one involved elevations in AM levels in the brain and lung at an early stage of HHE exposure, and the other involved elevations in AM levels in the RV, RA, and LA in the middle to late stage of exposure. Because the former (viz. that involving changes in the brain and lung) was not associated with an increase in the circulating AM level, AM may act in a local paracrine and/or autocrine fashion within the brain and lung so as to protect against acute hypoxia and improve gas exchange, respectively, at an early stage in HHE exposure. In normal rat and human brains, AM mRNA and protein have been found only at low levels (13, 31). Wang et al. (36) demonstrated that AM mRNA expression was increased in the ischemic cerebral cortex at an early stage (from 3 to 15 days) in an experimental focal stroke model of middle cerebral artery occlusion in rats. They suggested that an upregulation of AM in the ischemic cortex may dilate cerebral vessels as an acute response to ischemia. Furthermore, Oie et al. (27), who examined myocardial infarction in rats, demonstrated that AM levels in the ischemic area of the heart were increased temporarily, with the peak at 7 days after the infarction. Thus these findings are in line with the above idea that AM may play a role as an autocrine/paracrine factor in the early period after the start of hypoxia or ischemia. In contrast, the later increase in plasma AM protein suggests that it may be available to act in another fashion in the whole body, perhaps including an action preventing or limiting lung damage. The elevation in plasma AM protein in the late stage of the present exposure to hypobaric hypoxia (after its increase in the RV) may suggest that one of the main sources of plasma AM protein is synthesis by and secretion from the RV. Our findings are similar to those of Yoshihara et al. (38) and Shimokubo et al. (32), who noted increases in AM mRNA and protein in the hypertrophied RV after 3 wk in rats treated with monocrotaline (to induce experimental pulmonary hypertension). However, we had no way of examining whether RV tissues alone were responsible for the increase we observed in the circulating AM level.

There have been several reports indicating that an attenuation of pulmonary hypertension occurs after AM infusions (39). Despite the increased concentration of AM protein and overexpression of AM mRNA in the RV during HHE in our experiment, pulmonary hypertension developed rapidly and was most pronounced at 21 days of HHE, perhaps suggesting that the levels of AM protein were insufficient to produce a major moderating effect under our conditions (or that the pulmonary hypertension would have been even more pronounced in the absence of AM).

In the present study, we found a significant decrease in body weight in HHE rats after 3 or more days of exposure. Furthermore, the levels of AM protein in the brain and plasma were increased at 0.5–5 days and 21 days of HHE exposure, respectively. Recent studies have demonstrated that amylin-related peptides such as AM, calcitonin, and calcitonin gene-related peptide inhibit food intake and gastric emptying in rats when administered systemically or into the brain (16, 29). It would be interesting to examine a group of pair-fed rats maintained in a hypobaric normoxic chamber identical to that used to produce a HHE, thus enabling us to test whether factors other than hypoxia might have been responsible for the changes in AM levels reported here. Unfortunately, serious equipment failure has so far prevented us from doing this. At present, our suspicion is that the increase in AM in the brain and plasma of HHE rats may contribute to the sustained loss in body weight seen here.

In our RT-PCR study, AM mRNA was present in multiple organs in normal rats. The adrenal gland and lung exhibited high levels of AM mRNA by semiquantitative RT-PCR. These AM mRNA findings are consistent with the Northern blotting results in normal rats obtained by Sakata et al. (31). In our in situ hybridization experiment, however, no signal was detected in the lung. In contrast, AM mRNA was detected in the salivary gland by in situ hybridization, whereas it was at a low level by semiquantitative RT-PCR. The reason for these discrepancies is unclear. One possibility is that the sensitivities of the AM mRNA probe and primers may be different and/or that the AM mRNA may be localized to foci within the organs. In the heart, the levels of AM mRNA and protein were quite low in control rats (by semiquantitative RT-PCR, in situ hybridization, and immunohistochemistry) in our study. During exposure to HHE, however, the staining obtained for AM mRNA by in situ hybridization and for AM protein by immunohistochemistry became strong in the RV. These findings were supported by the changes in AM (both mRNA and protein) in the RV that were detected in the heart by semiquantitative RT-PCR and ELISA.

In conclusion, we have demonstrated that during 21 days of exposure to HHE, increases both in the synthesis of AM in the RV and in plasma AM protein occur alongside the increase in

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PAP and the development of RV hypertrophy. These changes could conceivably play an important role in modulating pulmonary hypertension during HHE. However, we did not try to determine whether increased AM expression is the cause of the pulmonary hypertension seen during HHE. Recently, McLatchie et al. (17) demonstrated that the calcitonin receptor-like receptor (CRLR) can function either as a calcitonin gene-related peptide receptor or as an AM receptor. Furthermore, CRLR/receptor activity-modifying protein 2 (CRLR/RAMP2) and CRLR/RAMP3 cause the receptor at the cell surface to function as an AM receptor (17). Further studies will be needed to identify which, if any, effects of HHE are exerted via the AM receptors located within the cardiovascular system.

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