Repeated inhalation of adrenomedullin ameliorates pulmonary hypertension and survival in monocrotaline rats

Noritoshi Nagaya,1 Hiroyuki Okumura,2 Masaaki Uematsu,1 Wataru Shimizu,1 Fumiaki Ono,1 Mikiyasu Shirai,1 Hidezo Mori,4 Kunio Miyatake,1 and Kenji Kangawa2

1Department of Internal Medicine, National Cardiovascular Center, Osaka 565-8565; 2Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka 565-8565; 3Cardiovascular Division, Kansai Rosai Hospital, Hyogo 660-0064, Japan; and 4Department of Cardiac Physiology, National Cardiovascular Center Research Institute, Osaka, Japan 565-8565

Submitted 1 July 2002; accepted in final form 31 December 2002

Nagaya, Noritoshi, Hiroyuki Okumura, Masaaki Uematsu, Wataru Shimizu, Fumiaki Ono, Mikiyasu Shirai, Hidezo Mori, Kunio Miyatake, and Kenji Kangawa. Repeated inhalation of adrenomedullin ameliorates pulmonary hypertension and survival in monocrotaline rats. Am J Physiol Heart Circ Physiol 285: H2125–H2131, 2003; 10.1152/ajpheart.00548.2002.—Adrenomedullin (AM) is a potent vasodilator peptide. We investigated whether inhalation of aerosolized AM ameliorates monocrotaline (MCT)-induced pulmonary hypertension in rats. Male Wistar rats given MCT (MCT rats) were assigned to receive repeated inhalation of AM (n = 8) or 0.9% saline (n = 8). AM (5 μg/kg) or saline was inhaled as an aerosol using an ultrasonic nebulizer for 30 min four times a day. After 3 wk of inhalation therapy, mean pulmonary arterial pressure and total pulmonary resistance were markedly lower in rats treated with AM than in those given saline (mean pulmonary arterial pressure: 22 ± 2 vs. 35 ± 1 mmHg (−37%); total pulmonary resistance: 0.048 ± 0.004 vs. 0.104 ± 0.066 mmHg·ml⁻¹·kg⁻¹·min⁻¹ (−54%), both P < 0.01). Neither systemic arterial pressure nor heart rate was altered. Inhalation of AM significantly attenuated the increase in medial wall thickness of peripheral pulmonary arteries in MCT rats. Kaplan-Meier survival curves demonstrated that MCT rats treated with aerosolized AM had a significantly higher survival rate than those given saline (70% vs. 10% 6-wk survival, log-rank test, P < 0.01). In conclusion, repeated inhalation of AM inhibited MCT-induced pulmonary hypertension without systemic hypotension and thereby improved survival in MCT rats.

ADRENOMEDULLIN (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma (13). Immunoreactive AM has subsequently been detected in plasma and a variety of tissues, including blood vessels and the lungs (9, 27). It has been reported that there are abundant binding sites for AM in the lungs (24). We (11, 30) have shown that the plasma AM level increases in proportion to the severity of pulmonary hypertension and that circulating AM is partially metabolized in the lungs. Interestingly, AM has been shown to inhibit the migration and proliferation of vascular smooth muscle cells (8, 12). These findings suggest that AM plays an important role in the regulation of pulmonary vascular tone and vascular remodeling.

In fact, experimental studies (5, 14, 22) have demonstrated that intralobar arterial infusion of AM induces pulmonary vasodilation in rats and cats. In humans, we have shown that short-term intravenous infusion of AM significantly decreases pulmonary vascular resistance in patients with congestive heart failure (19) or primary pulmonary hypertension (PPH) (18). Unfortunately, however, intravenously administered AM also decreases systemic arterial pressure in such patients because of its nonselective vasodilation in pulmonary and systemic vascular beds.

Recently, inhaled prostacyclin and its analog, iloprost, have been shown to cause pulmonary vasodilation without systemic hypotension in patients with PPH (7, 28, 29). In addition, the inhalant application of vasodilators does not induce negative side effects on gas exchange, because ventilation-matched deposition of the drugs in the alveoli causes pulmonary vasodilation matched to ventilated areas (28). In clinical settings, inhalation therapy may be more simple, noninvasive, and relatively comfortable than continuous intravenous infusion therapy. These findings raise the possibility that intratracheal delivery of aerosolized AM may have beneficial effects in patients with pre-capillary pulmonary hypertension.

Thus the purpose of the present study was to investigate whether inhalation of AM ameliorates monocrotaline (MCT)-induced pulmonary hypertension and thereby improves survival in MCT-treated rats.

METHODS

Animals. Male Wistar rats weighing 80 to 100 g were used in this study. The rats were given a subcutaneous injection of 60 mg/kg MCT (MCT rats) and assigned to receive a single inhalation of AM (n = 5) or 0.9% saline (n = 5) or repeated inhalation of AM (n = 8) or 0.9% saline (n = 8). Sham rats not
given a MCT injection also received repeated inhalation of AM (n = 8) or 0.9% saline (n = 8). An additional 20 rats were studied to evaluate the effects of inhaled AM on survival in MCT rats. Finally, rats that had developed pulmonary hypertension 3 wk after the MCT injection received repeated inhalation of AM (n = 8) or 0.9% saline (n = 8). All protocols were performed in accordance with guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute (Osaka, Japan).

Preparation of AM. Recombinant human AM was obtained from Shionogi (Osaka, Japan). The homogeneity of AM was confirmed by reverse-phase HPLC and amino acid analysis. AM was dissolved in 0.9% saline, and the solution was stored as 20-ml volumes containing 200 μg AM/tube at −80°C until the time of preparation for administration.

Inhalation of AM. We used an unrestrained, whole body aerosol exposure system. Each rat was placed in a plastic cage for aerosol delivery. AM or saline was aerosolized using an ultrasonic nebulizer (Sonicator 305, Atom) connected to six cages. The 20 ml solution containing 200 μg AM was delivered as an aerosol into the six cages at a constant flow rate (0.6 ml solution/min) for 30 min. Inhalation of fluorescent isothiocyanate-dextran demonstrated that a single inhalation of AM delivered 0.5 μg AM to the lungs in each rat (5 μg/kg body wt).

To assess the acute effect of inhaled AM, hemodynamic studies were carried out at 3 wk after the MCT injection. Hemodynamics were measured at 15-min intervals before, during, and after a single inhalation of AM or saline. Blood was obtained from the carotid artery at the same time points for measurement of plasma AM.

To assess the chronic effect of inhaled AM, 30-min inhalation of AM (5 μg/kg body wt) or saline was repeated four times a day for 3 wk after the MCT injection. Finally, to investigate the effects of inhaled AM on developed pulmonary hypertension, aerosolized AM or saline was given for 1 wk to rats that had developed pulmonary hypertension 3 wk after the MCT injection. After completion of the inhalation therapy, hemodynamic studies were performed. Blood was then drawn from the carotid artery for measurement of plasma hormone levels. Finally, cardiac arrest was induced by the injection of 2 mmol KCl through the catheter. The ventricles and lungs were excised, dissected free, and weighed. The measurement of right ventricular weight excluded the interventricular septum. The ratio of right ventricular weight to body weight and the ratio of left ventricular weight to body weight were calculated as indexes of ventricular hypertrophy.

Hemodynamic measurements. Rats were anesthetized with intraperitoneal pentobarbital (30 mg/kg) and placed on a heating pad to maintain body temperature at 37–38°C throughout the study. A polyethylene catheter (PE-10) was inserted into the right femoral artery to measure heart rate and mean arterial pressure. An umbilical vessel catheter was inserted through the right jugular vein into the pulmonary artery for the measurement of right ventricular pressure and pulmonary arterial pressure. These hemodynamic variables were measured using a pressure transducer (model P23ID, Gould) connected to a polygraph and recorded with a thermal recorder (7756B system, Hewlett-Packard). A thermocapillary probe was advanced into the ascending aorta via the right carotid artery and connected to a cardiac output computer (Cardiotherm-500, Columbus Instruments). Cardiac output was measured in triplicate by the thermodilution method. Total pulmonary resistance was calculated by dividing the mean pulmonary arterial pressure by the cardiac output.

Morphometric analysis of pulmonary arteries. Paraffin sections 4 μm in thickness were obtained from the middle region of the right lung and stained with hematoxylin and eosin for examination by light microscopy. Analysis of the medial wall thickness of the pulmonary arteries was performed as described previously (23). In brief, the external diameter and the medial wall thickness were measured in 20 muscular arteries (ranging in external diameter from 25 to 50 and from 51 to 100 μm) per lung section. For each artery, the medial wall thickness was expressed as follows: percent wall thickness = [(medial thickness × 2)/external diameter] × 100. A lung section was obtained from individual rats for comparison among the four groups (n = 5 each).

Hormonal analysis. The plasma AM level was measured by an immunoradiometric assay using a specific kit (Shionogi) (22). For the assessment of right ventricular function (17, 21), the plasma atrial natriuretic peptide (ANP) level was measured using an enzyme immunoassay kit (ANF Rat EIA kit; Peninsula, CA).

Survival analysis. To evaluate the effects of inhaled AM on survival in MCT rats, 20 rats received repeated inhalation of AM (n = 10) or saline (n = 10) four times a day from the date of the MCT injection until death. Survival was estimated from the date of the MCT injection to the death of the rat or 6 wk after the injection.

Statistical analysis. All data are expressed as means ± SE unless otherwise indicated. Comparisons of parameters among three groups were made by one-way ANOVA, followed by Scheffe’s multiple-comparison test. Comparisons of the time course of parameters between two groups were made by two-way ANOVA for repeated measures, followed by Scheffe’s multiple-comparison test. Survival curves according to the presence or absence of AM inhalation were derived using the Kaplan-Meier method and compared using a log-rank test. A P value < 0.05 was considered statistically significant.

RESULTS

Acute effect of single inhalation of AM. Acute hemodynamic studies were carried out at 3 wk after the MCT injection. AM inhalation slightly increased the circulating level of human AM (from 0 to 3.6 ± 1.0 fmol/ml, P < 0.05). A 30-min inhalation of AM slightly but significantly decreased the mean pulmonary arterial pressure in MCT rats (from 32 ± 2 to 29 ± 2 mmHg, P < 0.05; Fig. 1) without a significant decrease in mean arterial pressure (from 113 ± 5 to 111 ± 4 mmHg, P = not significant). AM inhalation markedly increased cardiac output by 42% (from 405 ± 22 to 575 ± 34 ml/min·1.0 kg–1, P < 0.05) at the end of inhalation. Thus AM resulted in a 36% decrease in total pulmonary resistance (from 0.081 ± 0.006 to 0.052 ± 0.004 mmHg·ml·min·1·kg–1, P < 0.05). The ratio of total pulmonary resistance to systemic vascular resistance was significantly decreased at the end of inhalation (from 0.29 ± 0.01 to 0.26 ± 0.01, P < 0.05). Interestingly, these hemodynamic effects of AM lasted at least 60 min after the end of inhalation. Inhalation of saline did not alter any hemodynamic or hormonal parameter.

Chronic effect of repeated inhalation of AM. The physiological profiles of the four experimental groups are summarized in Table 1. Body weight was significantly lower in both MCT groups than in sham rats.
Right ventricular weight was significantly lower in MCT rats receiving repeated inhalation of AM than in those given aerosolized saline. There was no significant difference in left ventricular weight among the four groups.

Three weeks after the MCT injection, pulmonary hypertension developed compared with findings in sham rats, but the rise in mean pulmonary arterial pressure was markedly attenuated in MCT rats treated with repeated inhalation of AM (by 37%) compared with that in MCT rats given aerosolized saline (22 ± 2 vs. 35 ± 1 mmHg, P < 0.05; Fig. 2). Cardiac output was significantly higher in MCT rats treated with AM (by 30%) compared with that in MCT rats given saline (444 ± 18 vs. 342 ± 18 ml·min⁻¹·kg⁻¹, P < 0.05). Therefore, total pulmonary resistance was markedly lower in MCT rats treated with AM (by 54%) compared with that in MCT rats given saline (0.048 ± 0.004 vs. 0.104 ± 0.006 mmHg·ml⁻¹·min⁻¹·kg⁻¹, P < 0.05). Similarly, the increase in right ventricular systolic pressure was significantly attenuated by AM inhalation (Table 1). In contrast, neither mean arterial pressure nor heart rate differed among the four groups. The ratio of total pulmonary resistance to systemic vascular resistance was markedly lower in MCT rats treated with aerosolized AM (by 44%) compared with that in MCT rats given aerosolized saline (0.19 ± 0.01 vs. 0.34 ± 0.01, P < 0.05). Inhalation of AM did not significantly alter any hemodynamic parameters in sham rats.

Representative photomicrographs of pulmonary arteries showed that hypertrophy of the pulmonary vessel wall was inhibited in MCT rats treated with AM compared with that in MCT rats given saline (Fig. 3). Quantitative analysis of peripheral pulmonary arteries demonstrated that the percent wall thickness of pulmonary arteries was significantly lower in MCT rats treated with aerosolized AM than in those given aerosolized saline (20 ± 1% vs. 28 ± 1% in vasculature with an external diameter of 25–50 μm and 21 ± 1% vs. 27 ± 1% in vasculature with an external diameter of 51–100 μm, both P < 0.05; Fig. 3). Inhalation of AM did not significantly alter vascular morphology in sham rats.

**Effect of AM inhalation on long-term prognosis in MCT rats.** Kaplan-Meier survival curves demonstrated that MCT rats treated with aerosolized AM had a significantly higher survival rate than those given saline (70% vs. 10% in 6-wk survival, log-rank test, P < 0.01; Fig. 4). No definite adverse effects were detected after repeated inhalation of AM.

**Effect of AM inhalation on developed pulmonary hypertension.** AM or saline was inhaled by rats that had developed pulmonary hypertension 3 wk after the MCT injection. Mean pulmonary arterial pressure was significantly lower in MCT rats treated with AM (by 14%) compared with that in rats given saline (32 ± 1 vs. 37 ± 1 mmHg, P < 0.05). Cardiac output was also higher in MCT rats treated with AM (by 15%) compared with that in rats given saline (360 ± 11 vs. 313 ± 14 ml·min⁻¹·kg⁻¹, P < 0.05). Therefore, total pulmonary resistance was significantly lower in MCT rats treated with AM (by 24%) compared with that in rats given saline (0.091 ± 0.005 vs. 0.119 ± 0.008 mmHg·ml⁻¹·min⁻¹·kg⁻¹, P < 0.05).

**DISCUSSION**

In the present study, we demonstrated that 1) a single inhalation of AM using an ultrasonic nebulizer induced relatively long-lasting pulmonary vasodilation without systemic hypotension, 2) repeated inhalation
of AM ameliorated MCT-induced pulmonary hypertension and attenuated the development of pulmonary vascular remodeling, and 3) inhalation of AM improved survival in MCT rats without definite adverse effects.

PPH is a rare but life-threatening disease characterized by progressive pulmonary hypertension, ultimately producing right ventricular failure and death (25). Although intravenous administration of prostacyclin has become recognized as a therapeutic breakthrough (1, 6, 16, 26), some patients with PPH are refractory to this treatment. Thus a new therapeutic strategy for the treatment of PPH is desirable.

AM is one of the most potent endogenous vasodilators in the pulmonary vascular bed (5, 13, 14, 22). The vasodilating effect is mediated by a cAMP-dependent and/or nitric oxide-dependent mechanism (10, 20). Recently, we (19) have shown that intravenous administration of AM markedly decreases pulmonary vascular resistance in patients with PPH. Nevertheless, systematically administered AM decreases systemic arterial pressure, which may be harmful in treating patients with PPH. In the present study, inhalation of AM markedly decreased total pulmonary resistance, whereas it did not significantly decrease mean arterial pressure. The ratio of total pulmonary resistance to systemic vascular resistance was significantly reduced by AM inhalation. These results suggest that this novel route of AM administration causes relatively selective pulmonary vasodilation. Expectedly, inhalation of AM markedly increased the cardiac index in MCT rats, consistent with our previous results from intravenous delivery (18). Considering the strong vasodilator activity of AM in the pulmonary vasculature, the significant decrease in cardiac afterload may be responsible for the increased cardiac index with AM. Interestingly, the hemodynamic effects of AM lasted at least 60 min after a single inhalation of AM. Although a single inhalation of AM delivered 0.5 μg into the lungs in each rat, it induced only a slight increase in the plasma AM level (3.6 ± 1.0 fmol/ml). These results raise the possibility that inhaled AM is retained in lung tissue for a while and acts transepithelially on the pulmonary vasculature. Thus inhalation of AM may cause potent, long-lasting pulmonary vasodilator activity in MCT rats.

Table 1. Physiological profiles of the four experimental groups

<table>
<thead>
<tr>
<th></th>
<th>Sham-Saline</th>
<th>Sham-AM</th>
<th>MCT-Saline</th>
<th>MCT-AM</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>150 ± 3</td>
<td>154 ± 3</td>
<td>132 ± 2†</td>
<td>146 ± 4‡</td>
</tr>
<tr>
<td>RV/body wt, g/kg</td>
<td>0.59 ± 0.02</td>
<td>0.58 ± 0.01</td>
<td>0.92 ± 0.06§</td>
<td>0.66 ± 0.02†</td>
</tr>
<tr>
<td>LV/body wt, g/kg</td>
<td>2.32 ± 0.04</td>
<td>2.27 ± 0.05</td>
<td>2.48 ± 0.05</td>
<td>2.33 ± 0.05</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>409 ± 15</td>
<td>428 ± 20</td>
<td>424 ± 15</td>
<td>413 ± 14</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td>120 ± 3</td>
<td>117 ± 3</td>
<td>104 ± 3§</td>
<td>115 ± 3‡</td>
</tr>
<tr>
<td>RV systolic pressure, mmHg</td>
<td>35 ± 1</td>
<td>34 ± 1</td>
<td>67 ± 2*</td>
<td>45 ± 3†‡</td>
</tr>
<tr>
<td>Right atrial pressure, mmHg</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
<td>7.2 ± 1*</td>
<td>2 ± 1‡</td>
</tr>
<tr>
<td>Plasma ANP level, pg/ml</td>
<td>275 ± 40</td>
<td>238 ± 29</td>
<td>694 ± 61*</td>
<td>346 ± 44†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of rats. Sham-saline, sham rats given aerosolized saline; sham-AM, sham rats given aerosolized AM; MCT-saline, rats treated with monocrotaline (MCT) and given aerosolized saline; MCT-AM, rats treated with MCT and given aerosolized AM; RV, right ventricular LV, left ventricular; ANP, atrial natriuretic peptide. *P < 0.05 vs. sham-saline; †P < 0.05 vs. MCT-saline.

Fig. 2. Chronic effects of AM inhalation on mean pulmonary arterial pressure (A), cardiac output (B), and total pulmonary resistance (C). Sham-saline, sham rats given aerosolized AM; MCT-saline, MCT rats given aerosolized saline; sham-AM, sham rats given aerosolized AM; MCT-AM, MCT rats given aerosolized AM. Data are means ± SE. *P < 0.05 vs. sham-saline; †P < 0.05 vs. MCT-saline rats.
The present study also demonstrated that repeated inhalation of AM four times a day for 3 wk markedly decreased mean pulmonary arterial pressure and total pulmonary resistance in MCT rats without systemic hypotension. The potent, long-lasting pulmonary vasodilator effect of inhaled AM may contribute to the strong inhibition of the development of pulmonary hypertension. In addition, considering intermittent delivery of AM to the lungs, the chronic effects of inhaled AM appear to go beyond acute pulmonary vasodilation. In the present study, inhalation of AM inhibited an increase in the medial wall thickness of peripheral pulmonary arteries of MCT rats. Earlier studies (8, 12) have shown that AM inhibits the migration and proliferation of vascular smooth muscle cells. Given the known potent vasoprotective effects of AM, such as vasodilation and inhibition of smooth muscle cell migration and proliferation, it is interesting to speculate that AM trapped in the bronchial epithelium or alveoli leaks to the pulmonary arteries to maintain pulmonary vascular integrity in MCT rats. Inhalation of AM also decreased plasma ANP, a potential marker for right ventricular dysfunction (17, 21). It is possible that the decreased pulmonary vascular resistance by AM may ameliorate increased wall stress in the right ventricle and improve right ventricular dysfunction in MCT rats.

Importantly, Kaplan-Meier analysis demonstrated that the 6-wk survival rate for MCT rats treated with aerosolized AM was significantly high (70%) compared with those given saline (10%). Thus treatment with aerosolized AM may be an alternative approach for severe pulmonary hypertension that is refractory to conventional therapy.

In the pulmonary circulation, the AM receptor acts not only as a functional receptor but also as a clearance receptor, the expression of which is stimulated by basal AM itself (3). Thus exogenously administered AM may have differing effects depending on the basal levels of AM.

Champion et al. (2) showed that intratracheal gene transfer of prepro-calcitonin gene-related peptide (CGRP) to the lung attenuates chronic hypoxia-induced pulmonary hypertension in mice. The gene for AM belongs to the CGRP family, and the receptors for CGRP and AM bind both peptides (15). In addition, the AM receptor is expressed at high levels in the pulmonary vascular endothelium, and there is an interaction of CGRP and AM with the receptor in the pulmonary endothelium (4). Thus it is not surprising that AM attenuates pulmonary hypertension in a similar manner as CGRP. In fact, we (31) have previously reported a beneficial effect of AM in a rat model of pulmonary hypertension. In our previous study, however, AM was administered subcutaneously. In contrast, in the present study, AM was inhaled to amerilolate pulmonary hypertension, which may have a pharmacological...
and clinical implication of the treatment for this disorder.

In conclusion, repeated inhalation of AM inhibited MCT-induced pulmonary hypertension without systemic hypotension and thereby improved survival in MCT rats. Thus long-term treatment with aerosolized AM may be a new therapeutic strategy for the treatment of pulmonary hypertension.

We thank Yumi Takara for technical assistance.

DISCLOSURES

This work was supported by grants from the Japan Cardiovascular Research Foundation, Kanae Foundation for Life and Sociomedical Science, Research on Health Sciences Focusing on Drug Innovation, Research Grant for Cardiovascular Disease 12C-2 from the Ministry of Health, Labour and Welfare, and the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan.

REFERENCES


