Effects of Adrenomedullin Inhalation on Hemodynamics and Exercise Capacity in Patients With Idiopathic Pulmonary Arterial Hypertension

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Background—Adrenomedullin (AM) is a potent pulmonary vasodilator peptide. However, whether intratracheal delivery of aerosolized AM has beneficial effects in patients with idiopathic pulmonary arterial hypertension remains unknown. Accordingly, we investigated the effects of AM inhalation on pulmonary hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension.

Methods and Results—Acute hemodynamic responses to inhalation of aerosolized AM (10 μg/kg body wt) were examined in 11 patients with idiopathic pulmonary arterial hypertension during cardiac catheterization. Cardiopulmonary exercise testing was performed immediately after inhalation of aerosolized AM or placebo. The work rate was increased by 15 W/min until the symptom-limited maximum, with breath-by-breath gas analysis. Inhalation of AM produced a 13% decrease in mean pulmonary arterial pressure (54 ± 3 to 47 ± 3 mm Hg, $P<0.05$) and a 22% decrease in pulmonary vascular resistance (12.6 ± 1.5 to 9.8 ± 1.3 Wood units, $P<0.05$). However, neither systemic arterial pressure nor heart rate was altered. Inhalation of AM significantly increased peak oxygen consumption during exercise (peak $\dot{V}O_2$, 14.6 ± 0.6 to 15.7 ± 0.6 mL $\cdot$ kg^{-1} $\cdot$ min^{-1}, $P<0.05$) and the ratio of change in oxygen uptake to that in work rate ($\Delta\dot{V}O_2/\Delta W$ ratio, 6.3 ± 0.4 to 7.0 ± 0.5 mL $\cdot$ min^{-1} $\cdot$ W^{-1}, $P<0.05$). These parameters remained unchanged during placebo inhalation.

Conclusions—Inhalation of AM may have beneficial effects on pulmonary hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension. (***Circulation*. 2004;109:351-356.**)

Key Words: peptides ■ hypertension, pulmonary ■ respiration ■ exercise ■ hemodynamics

Idiopathic pulmonary arterial hypertension is a rare but life-threatening disease characterized by progressive pulmonary hypertension, ultimately producing right heart failure and death.[1,2] Although a variety of vasodilators have been proposed as potential therapy for this disease over the past 30 years,[3-7] some patients ultimately require heart-lung or lung transplantation.[8,9] Thus, a novel therapeutic strategy is desirable.

Adrenomedullin (AM) is a potent, long-lasting vasodilator peptide that was originally isolated from human pheochromocytoma.[10] Immunoreactive AM has subsequently been detected in plasma and a variety of tissues, including blood vessels and lungs.[11,12] It has been reported that there are abundant binding sites for AM in the lungs.[13] We 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.[14,15] Interestingly, AM has been shown to inhibit the migration and proliferation of vascular smooth muscle cells.[16,17] These findings suggest that AM plays an important role in the regulation of pulmonary vascular tone and vascular remodeling. In fact, we have shown that short-term intravenous infusion of AM significantly decreases pulmonary vascular resistance in patients with congestive heart failure[18] or pulmonary arterial hypertension.[19] Unfortunately, however, intravenously administered AM induced systemic hypotension in such patients because of nonselective vasodilation in the pulmonary and systemic vascular beds.

More recently, inhalation of aerosolized prostacyclin and its analogue iloprost has been shown to cause pulmonary vasodilation without systemic hypotension in patients with idiopathic pulmonary arterial hypertension.[20,21] In addition, inhalant application of vasodilators does not impair gas exchange because the ventilation-matched deposition of drug

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TABLE 1. Baseline Characteristics of Patients With Idiopathic Pulmonary Arterial Hypertension

Demographics	
Age, y	39±3
Male/female, n	2/9
NYHA functional class, n	
III	10
IV	1
Baseline hemodynamics	
MPAP, mm Hg	54±3
CI, $L \cdot min^{-1} \cdot m^{-2}$	2.4±0.1
PVR, Wood units	12.6±1.5
RAP, mm Hg	7±1
PCWP, mm Hg	7±1
Pulmonary function	
Sa_{O_2}, %	94±3
Sv_{O_2}, %	63±4
FVC, % predicted	86±4
FEV_1, % predicted	75±1
6-Minute walk test, m	355±35
Medication use, n	
Anticoagulant agents	10
Diuretics	9
Digitalis	7
Oral prostacyclin analogue	6
Calcium antagonists	2

NYHA indicates New York Heart Association; MPAP, mean pulmonary arterial pressure; CI, cardiac index; PVR, pulmonary vascular resistance; RAP, mean right atrial pressure; PCWP, pulmonary capillary wedge pressure; Sa_{O_2}, arterial oxygen pressure; Sv_{O_2}, mixed venous oxygen saturation; FVC, forced vital capacity; and FEV_1, forced expiratory volume in 1 second. Data are mean±SEM.

in the alveoli causes pulmonary vasodilation matched to ventilated areas.[20] In clinical settings, inhalation therapy may be more simple, noninvasive, and comfortable than continuous intravenous infusion therapy. Thus, the purpose of the present study was to investigate the effects of AM inhalation on hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension.

Methods

Study Subjects

Eleven patients with idiopathic pulmonary arterial hypertension (9 women and 2 men; age, 39±3 years) were included in this study. Idiopathic pulmonary arterial hypertension was defined as pulmonary hypertension unexplained by any secondary cause, on the basis of the criteria of the National Institutes of Health registry.[1] Ten patients were classified as New York Heart Association (NYHA) functional class III and 1 as class IV (Table 1). Two of the 11 patients (18%) were acute responders who showed a significant decrease in mean pulmonary arterial pressure of ≥20% with a decrease in mean pulmonary arterial pressure to <35 mm Hg and no change or an increase in cardiac index during short-term infusion of epoprostenol. Long-term medication, including anticoagulant agents, digitalis, and diuretics, was kept constant. Vasodilator agents, such as oral prostacyclin analogue and calcium antagonists, were stopped ≥12 hours before the study procedure was begun. The ethics committee of the National Cardiovascular Center approved the study, and all patients gave written informed consent.

Preparation of Human AM

Human AM was dissolved in saline with 4% D-mannitol and sterilized by passage through a 0.22-μm filter (Millipore Co). At the time of dispensing, randomly selected vials were submitted for sterility and pyrogen testing. The chemical nature and content of the human AM in vials were verified by high-performance liquid chromatography and radioimmunoassay. All vials were stored frozen at −80°C from the time of dispensing until the time of preparation for administration.

Hemodynamic Studies

Acute hemodynamic responses to AM inhalation were assessed in all patients while they were in a stable condition during hospitalization. Hemodynamic variables, including pulmonary arterial pressure, right atrial pressure, pulmonary capillary wedge pressure, and cardiac output (in triplicate), were determined with a thermodilution catheter (TOO21H-7.5F, Baxter Co).[22] A 22-gauge cannula was inserted into a radial artery for hemodynamic measurements and blood sampling. After an equilibration period of 30 minutes, baseline hemodynamics were measured. Then, AM (10 μg/kg body wt) was inhaled as an aerosol with a jet nebulizer (Porta-Nebu, MEDIC-AID) for 15 minutes, which resulted in a cumulative dose of 400 to 600 μg AM. Hemodynamic parameters were measured at 15-minute intervals starting 15 minutes before AM inhalation until 60 minutes after inhalation. Blood samples for AM measurement were taken at 15-minute intervals from 15 minutes before inhalation until 60 minutes after the end of inhalation.

Cardiopulmonary Exercise Testing

The effects of AM inhalation on exercise capacity were examined in 10 of 11 patients; 1 patient with NYHA class IV underwent the 6-minute walk test according to decision of attending physicians. Cardiopulmonary exercise testing was performed immediately after inhalation of aerosolized AM (10 μg/kg body wt) or saline in a double-blind, randomized, crossover design. This study was performed on 2 separate days, 1 week apart. The first cardiopulmonary exercise testing was performed within 10 days after the cardiac catheterization. The patients performed exercise seated on a cycle ergometer. They first pedaled at 55 rpm without any added load for 1 minute. The work rate was then increased by 15 W/min up to the symptom-limited maximum. Breath-by-breath gas analysis was performed with an AE280 (Minato Medical Science) connected to a personal computer running analyzing software.[23] The ratio of change in oxygen uptake to that in work rate ($\Delta\dot{V}O_2/\Delta W$ ratio) was calculated as the slope of oxygen consumption per unit workload from 1 minute after the start of load addition until 85% maximal $\dot{V}O_2$. Exercise capacity was evaluated by peak oxygen consumption (peak $\dot{V}O_2$), which was defined as the value of averaged data during the final 15 seconds of exercise. Ventilatory efficiency during exercise was represented by the $\dot{V}E$-$\dot{V}CO_2$ slope, which was determined as the linear regression slope of $\dot{V}E$ and $\dot{V}CO_2$ from the start of exercise until the RC point (the time until which ventilation is stimulated by CO_2 output and end-tidal CO_2 tension begins to decrease).

Measurement of Plasma AM, cAMP, and cGMP

Blood samples were immediately transferred into chilled glass tubes containing disodium EDTA (1 mg/mL) and aprotinin (500 U/mL) and centrifuged immediately at 4°C, and the plasma was frozen and stored at −80°C until assayed. Plasma AM level was measured by a specific immunoradiometric assay kit (Shionogi Pharmaceutical Co Ltd).[24] Plasma cAMP and cGMP were determined with radioimmunoassay kits (cAMP assay kit, cGMP assay kit, Yamasa Shoyu).[18]

Statistical Analysis

All data were expressed as mean±SEM unless otherwise indicated. Changes in hemodynamic and hormonal parameters by AM inhalation were analyzed by 1-way ANOVA for repeated measures,

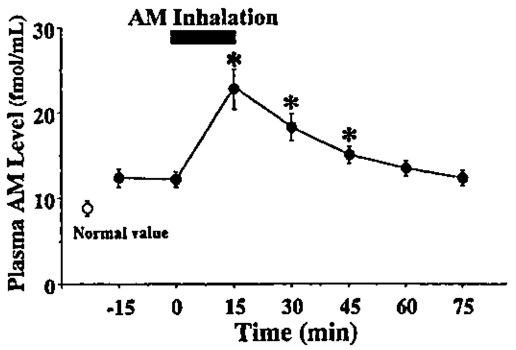

Figure 1. Changes in plasma AM level by inhalation of aerosolized AM in patients with idiopathic pulmonary arterial hypertension. Normal value indicates plasma AM level derived from 15 age-matched healthy subjects. Data are mean±SEM. *$P<0.05$ vs value at time 0.

followed by Newman-Keuls test. Comparisons of exercise parameters between the 2 groups were analyzed with paired Student's t test. A probability value of $P<0.05$ was considered statistically significant.

Results

All patients tolerated this study protocol. One patient developed a headache, and another patient had mild arterial hypoxemia during AM inhalation. None of them experienced other adverse effects, such as systemic hypotension, infection, or arrhythmia.

Plasma AM Level After Inhalation

Baseline plasma AM level in patients with idiopathic pulmonary arterial hypertension was significantly higher than the normal value, which was determined from pooled data of 15 age-matched healthy subjects (11.9±0.8 versus 9.3±0.1 fmol/mL, $P<0.05$). Inhalation of AM significantly increased the plasma AM level to 22.9±2.1 fmol/mL immediately after inhalation (Figure 1). The half-life of plasma AM after inhalation was approximately 20 minutes, and the elevation of AM lasted for >45 minutes. Plasma cAMP level increased significantly 30 minutes after the initiation of AM inhalation (10.8±0.7 to 12.0±0.6 pmol/mL, $P<0.05$), although plasma cGMP level was not significantly altered (6.5±1.0 to 6.8±1.0 pmol/mL, P=NS).

Hemodynamic Effects of AM Inhalation

Inhalation of AM significantly decreased mean pulmonary arterial pressure in patients with idiopathic pulmonary arterial hypertension (54±3 to 47±3 mm Hg, $P<0.05$) without a significant decrease in mean arterial pressure (85±4 to 83±4 mm Hg, P=NS) (Figure 2). AM inhalation slightly but significantly increased cardiac index by 12% (2.4±0.1 to 2.7±0.2 $L \cdot min^{-1} \cdot m^{-2}$, $P<0.05$). Thus, AM inhalation resulted in a 22% decrease in pulmonary vascular resistance (12.6±1.5 to 9.8±1.3 Wood units, $P<0.05$) (Figure 3). Inhaled AM did not significantly alter systemic vascular resistance. The ratio of pulmonary vascular resistance to systemic vascular resistance was decreased significantly at the end of inhalation (0.63±0.08 to 0.55±0.07, $P<0.05$). These hemodynamic effects of AM lasted for >45 minutes.

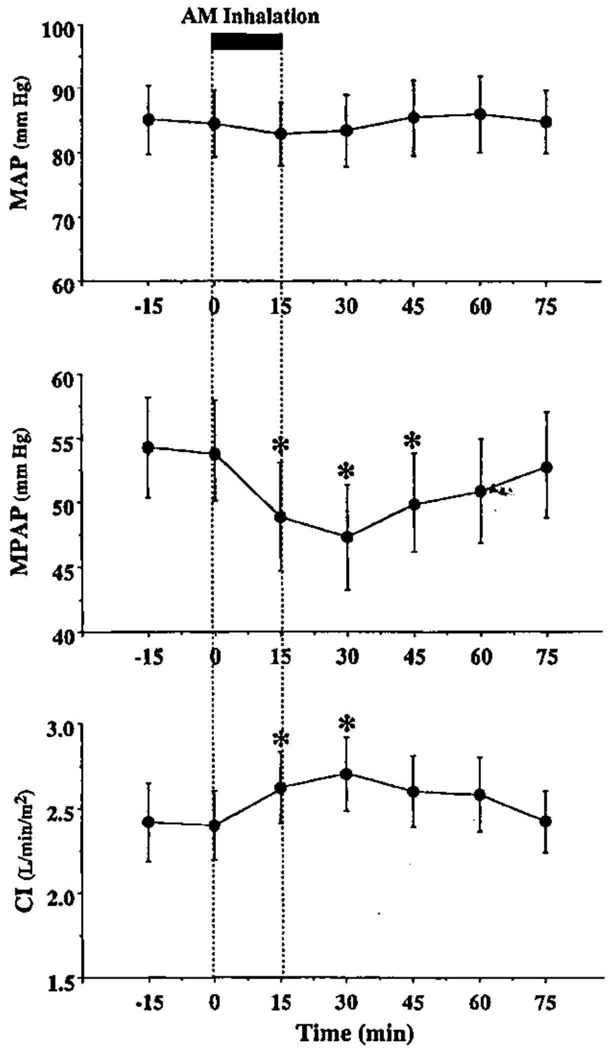

Figure 2. Changes in mean arterial pressure (MAP), mean pulmonary arterial pressure (MPAP), and cardiac index (CI) by inhalation of aerosolized AM in patients with idiopathic pulmonary arterial hypertension. Data are mean±SEM. *$P<0.05$ vs value at time 0.

No significant change in heart rate, pulmonary capillary wedge pressure, or right atrial pressure was observed. There was no significant change in arterial oxygen saturation (94±3% to 93±3%).

Effects of AM Inhalation on Exercise Capacity and Ventilatory Efficiency

As the limiting symptom at the end of exercise, 6 patients reported muscle weakness and 4 reported dyspnea. There was no difference in these symptoms when exercise testing was performed with or without inhalation of AM. Inhalation of AM altered neither heart rate nor blood pressure either at rest or at peak exercise (Table 2). Inhalation of AM significantly increased peak workload (86±5 to 93±6 W, $P<0.05$) (Table 2). AM also significantly increased peak $\dot{V}O_2$ (14.6±0.6 to 15.7±0.6 $mL \cdot kg^{-1} \cdot min^{-1}$, $P<0.05$) (Figure 4). Inhalation of AM significantly increased $\Delta\dot{V}O_2/\Delta W$ ratio (6.3±0.4 to

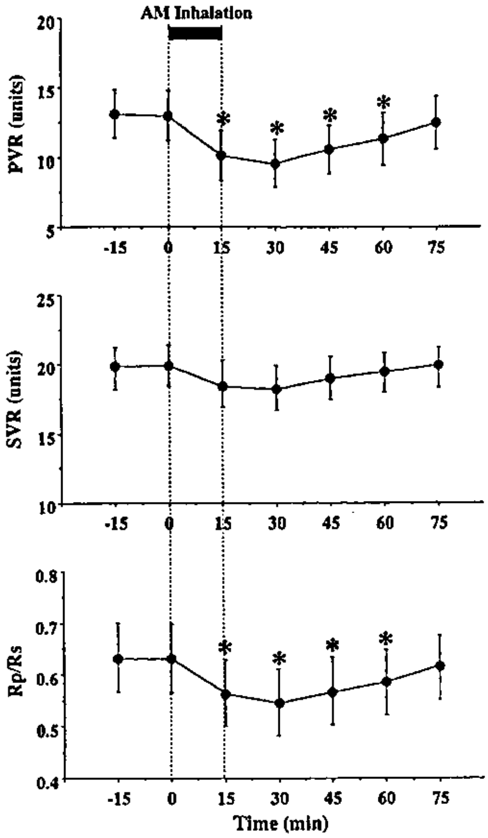

Figure 3. Changes in pulmonary vascular resistance (PVR), systemic vascular resistance (SVR), and ratio of pulmonary vascular resistance to systemic vascular resistance (Rp/Rs) by inhalation of aerosolized AM in patients with idiopathic pulmonary arterial hypertension. Data are mean±SEM. *$P<0.05$ vs value at time 0.

7.0±0.5 mL · min^{-1} · W^{-1}, $P<0.05$). AM did not significantly alter the $\dot{V}_E$-$\dot{V}CO_2$ slope (Table 2). No significant changes in arterial oxygen saturation were observed either at rest or at peak exercise. In 1 patient with NYHA class IV who did not undergo cardiopulmonary exercise testing, the distance walked in 6 minutes increased from 150 to 180 m by inhalation of AM.

Discussion

In the present study, we demonstrated that inhalation of AM improved hemodynamics with pulmonary selectivity and exercise capacity in patients with idiopathic pulmonary arterial hypertension.

AM is one of the most potent endogenous vasodilators in the pulmonary vascular bed.[25–27] The vasodilatory effect is mediated by cAMP-dependent and nitric oxide–dependent mechanisms.[28,29] Endogenous AM production is enhanced in a variety of cardiovascular diseases through a compensatory mechanism.[14,30] Nonetheless, additional supplementation of AM has beneficial effects in these diseases.[18,19] These results suggest that endogenous AM level is not sufficient to improve deteriorated conditions despite the increased AM production. Interestingly, Champion et al[31] have shown that intratracheal gene transfer of calcitonin gene–related peptide, a member of the same peptide family as AM, to bronchial

TABLE 2. Changes in Exercise Parameters by Inhalation of AM or Placebo

Variables	Placebo	AM	*P*
Peak workload, W	86±5	93±6	<0.05
HR, bpm			
Rest	75±5	75±3	NS
Peak	144±6	148±6	NS
MAP, mm Hg			
Rest	85±3	87±5	NS
Peak	108±5	110±6	NS
Peak Borg score (D/L)	17/18	18/18	NS
Peak $\dot{V}O_2$, mL · kg^{-1} · min^{-1}	14.6±0.6	15.7±0.6	<0.05
$\Delta\dot{V}O_2/\Delta W$ ratio, mL · min^{-1} · W^{-1}	6.3±0.4	7.0±0.5	<0.05
$\dot{V}_E$-$\dot{V}CO_2$ slope	37±2	36±2	NS
SaO_2, %			
Rest	97±1	97±1	NS
Peak	95±1	95±1	NS

HR indicates heart rate; MAP, mean arterial pressure; Peak Borg score (D/L), Borg score at peak exercise (dyspnea/leg fatigue); Peak $\dot{V}O_2$, peak oxygen consumption; $\Delta\dot{V}O_2/\Delta W$ ratio, $\dot{V}O_2$ increase per unit workload; $\dot{V}_E$-$\dot{V}CO_2$ slope, slope of regression line of relation between $\dot{V}_E$ and $\dot{V}CO_2$; and SaO_2, arterial oxygen saturation. Data are mean±SEM.

epithelial cells attenuates chronic hypoxia-induced pulmonary hypertension in the mouse. These results raise the possibility that intratracheal delivery of a vasodilator peptide may be sufficient to alter pulmonary vascular function. In fact, in the present study, inhalation of AM significantly decreased pulmonary vascular resistance, whereas it did not alter systemic arterial pressure or systemic vascular resistance. The ratio of pulmonary vascular resistance to systemic vascular resistance was reduced significantly by AM inhalation. These results suggest that inhaled AM improves hemodynamics with pulmonary selectivity. This is consistent with earlier findings that inhaled prostacyclin or its analogue iloprost acts transepithelially with pulmonary selectivity and improves pulmonary hypertension.[20,21] Inhalation of AM slightly but significantly increased cardiac index in patients with idiopathic pulmonary arterial hypertension. Considering the strong vasodilator activity of AM in the pulmonary vasculature, the significant decrease in cardiac afterload may be responsible for increased cardiac index with

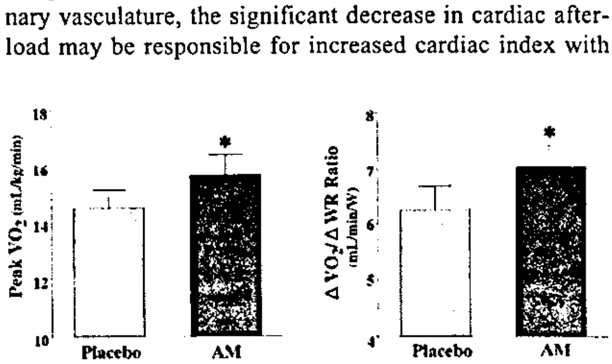

Figure 4. Changes in peak oxygen consumption (peak $\dot{V}O_2$) and ratio of change in oxygen uptake to that in work rate ($\Delta\dot{V}O_2/\Delta W$ ratio) by inhalation of aerosolized AM or placebo in patients with idiopathic pulmonary arterial hypertension. Data are mean±SEM. *$P<0.05$ vs placebo.

AM. Interestingly, the hemodynamic effects of inhaled AM lasted for >45 minutes. A previous study demonstrated that intravenous injection of AM produces a long-lasting vasodilator response because of its long half-life (≈15 minutes).[32] The half-life of plasma AM after inhalation was longer (20 minutes). Thus, inhalation of AM may cause relatively long-lasting pulmonary vasodilator activity in patients with idiopathic pulmonary arterial hypertension. In the present study, plasma cAMP level increased after AM inhalation, suggesting that the hemodynamic effects of AM may be mediated by activation of cAMP.

Earlier studies have shown that peak $\dot{V}O_2$ during exercise is markedly lower in patients with idiopathic pulmonary arterial hypertension than in healthy subjects.[33,34] Peak $\dot{V}O_2$ is determined primarily by the maximal cardiac output during exercise and the potential for O_2 extraction by the exercising muscle.[35] Thus, the decreased peak $\dot{V}O_2$ may reflect insufficient oxygen delivery to the body during exercise, at least in part because of an inadequate increase in cardiac output under conditions of severe pulmonary hypertension. In the present study, inhalation of AM significantly increased peak $\dot{V}O_2$ in patients with pulmonary hypertension. AM also increased the $\Delta\dot{V}O_2/\Delta W$ ratio, which indicates oxygen transport per unit workload to the exercising legs. These results suggest that inhalation of AM improves exercise capacity in patients with idiopathic pulmonary arterial hypertension. It is possible that an increase in cardiac output during exercise may contribute to increases in peak $\dot{V}O_2$ and the $\Delta\dot{V}O_2/\Delta W$ ratio.

The major limitation of this pilot trial relates to the lack of a randomized, placebo-controlled group in acute hemodynamic studies, which was as result not only of invasive assessment of hemodynamics but also of the limited number of patients available. Nevertheless, cardiopulmonary exercise testing was performed in a double-blind, randomized, crossover design. Thus, it is unlikely that the hemodynamic effects of inhaled AM are attributable to the placebo effect.

Inhalation therapy may be more simple, noninvasive, and comfortable than continuous intravenous infusion therapy. An experimental study demonstrated that repeated inhalation of AM (for 30 minutes, 4 times a day) inhibited monocrotaline-induced pulmonary hypertension and markedly improved survival in rats.[36] Recently, pulmonary delivery of a dry-powder insulin has been shown to improve glycemic control without adverse pulmonary effects.[37] Although further studies are necessary to maximize the efficiency and reproducibility of pulmonary AM delivery, combining AM inhalation therapy with other modalities that have a different mode of action may have beneficial effects in patients with idiopathic pulmonary arterial hypertension.

Conclusions

These preliminary results suggest that inhalation of AM may have beneficial effects on pulmonary hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension.

Acknowledgments

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Adrenomedullin Infusion Attenuates Myocardial Ischemia/Reperfusion Injury Through the Phosphatidylinositol 3-Kinase/Akt-Dependent Pathway

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Background—Infusion of adrenomedullin (AM) has beneficial hemodynamic effects in patients with heart failure. However, the effect of AM on myocardial ischemia/reperfusion remains unknown.

Methods and Results—Male Sprague-Dawley rats were exposed to a 30-minute period of ischemia induced by ligation of the left coronary artery. They were randomized to receive AM, AM plus wortmannin (a phosphatidylinositol 3-kinase [PI3K] inhibitor), or saline for 60 minutes after coronary ligation. Hemodynamics and infarct size were examined 24 hours after reperfusion. Myocardial apoptosis was also examined 6 hours after reperfusion. The effect of AM on Akt phosphorylation in cardiac tissues was examined by Western blotting. Intravenous administration of AM significantly reduced myocardial infarct size (28±4% to 16±1%, $P<0.01$), left ventricular end-diastolic pressure (19±2 to 8±2 mm Hg, $P<0.05$), and myocardial apoptotic death (19±2% to 9±4%, $P<0.05$). Western blot analysis showed that AM infusion accelerated Akt phosphorylation in cardiac tissues and that pretreatment with wortmannin significantly attenuated AM-induced Akt phosphorylation. Moreover, pretreatment with wortmannin abolished the beneficial effects of AM: a reduction of infarct size, a decrease in left ventricular end-diastolic pressure, and inhibition of myocardial apoptosis after ischemia/reperfusion.

Conclusions—Short-term infusion of AM significantly attenuated myocardial ischemia/reperfusion injury. These cardioprotective effects are attributed mainly to antiapoptotic effects of AM via a PI3K/Akt-dependent pathway. (***Circulation*. 2004;109:242-248.**)

Key Words: peptides ■ reperfusion ■ apoptosis ■ myocardial infarction ■ hemodynamics

Coronary revascularization has been established as the most effective treatment for coronary artery disease. However, reperfusion can elicit a number of adverse reactions that may limit its beneficial actions. Although it has been attempted to reduce ischemia/reperfusion injury in many basic or clinical studies, few agents are clinically available for ischemia/reperfusion injury.

Adrenomedullin (AM) is a potent vasodilatory peptide that was originally isolated from human pheochromocytoma.[1] We have shown that AM peptide and mRNA are distributed in the heart[2,3] and that plasma and cardiac AM markedly increase after acute myocardial infarction.[4,5] AM has been shown to be a possible endogenous suppressor of myocyte hypertrophy[6] and fibroblast proliferation.[7] In addition, intravenous infusion of AM has beneficial hemodynamic effects in patients with heart failure.[8] These findings suggest that AM induces cardioprotective effects not only as a circulating factor but also as a paracrine and/or autocrine factor.

Recently, AM has been shown to activate the Akt pathway in vascular endothelial cells.[9] Interestingly, the Akt activation has been reported to lead to the prevention of myocardial injury after transient ischemia in vivo through antiapoptotic effects.[10] However, whether AM, a potent Akt activator, attenuates myocardial ischemia/reperfusion injury remains unknown.

Thus, the purposes of this study were (1) to investigate whether short-term infusion of AM reduces myocardial infarct size, inhibits myocyte apoptosis, and thereby improves cardiac function after ischemia/reperfusion and (2) to determine whether the underlying mechanisms are associated with

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the phosphatidylinositol 3-kinase (PI3K)/Akt-dependent pathway.

Methods

Reperfusion Model

We used male Sprague-Dawley rats (Japan SLC Inc, Hamamatsu, Japan) weighing 180 to 220 g. Ligation of the left coronary artery was performed as described previously.[11] In brief, under anesthesia with pentobarbital sodium (30 mg/kg) and artificial ventilation, the heart was exposed via left thoracotomy, and the left coronary artery was ligated 2 to 3 mm from its origin between the pulmonary artery conus and the left atrium with a 6-0 Prolene suture. The heart was subjected to regional ischemia for 30 minutes, followed by coronary reperfusion through release of the tie. After ligation of the left coronary artery, AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$), AM plus wortmannin (16 $\mu g/kg$ intravenous injection 15 minutes before AM infusion; a PI3K inhibitor),[12] or placebo (0.9% saline) was administered for 60 minutes through a catheter inserted into the left jugular vein. Sham-operated rats only underwent left thoracotomy. The chest wall was then closed, and the animal was allowed to recover. This protocol resulted in the creation of 4 groups: sham-operated rats (sham group, n=12), placebo-treated rats with ischemia/reperfusion (I/R-placebo group, n=19), AM-treated rats with ischemia/reperfusion (I/R-AM group, n=19) and AM plus wortmannin–treated rats with ischemia/reperfusion (I/R-Wo+AM group, n=15).

All animal experiments were conducted in accordance with the principles and procedures outlined in the *National Cardiovascular Center Guide for the Care and Use of Laboratory Animals*, which adheres strictly to the National Institutes of Health animal experimental guidelines, with the approval of the National Cardiovascular Center Animal Experimental Committee.

Hemodynamic Studies

We performed hemodynamic measurements 24 hours after ischemia/reperfusion. A 1.5F micromanometer-tipped catheter was advanced into the left ventricle through the right carotid artery, and a polyethylene catheter (PE-50) was advanced into the right ventricle through the right jugular vein to measure right ventricular pressure. Heart rate was also monitored with an ECG.

Measurement of Plasma AM Level

Blood samples were obtained from the right carotid artery during 0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$ AM infusion. Plasma AM level was measured by immunoradiometric assay, as described previously.[8,11]

Assessment of Infarct Size

After hemodynamic measurements, the heart was removed and perfused with a Langendorff apparatus for 10 minutes to wash out the blood and then fixed with 10% neutral buffered formalin. The heart was sliced transversely from the apex to the atrioventricular groove in 2.5-mm thicknesses and weighed separately. Within 24 hours after fixation, each section was embedded in paraffin. Serial 5-μm myocardial sections were cut with microtome and mounted on siliconized slides. After Masson trichrome staining, infarct size of each slice was analyzed by microscopy. Myocardial coagulation necrosis could be distinguished from viable myocardium as a definite alteration of staining, and then the infarct area was outlined and measured by planimetry. Infarct weight was determined with the following equation: % infarct area × weight of each slice, as described previously.[13] Finally, we determined percent infarct size as total infarct weight divided by total left ventricular (LV) weight.

TUNEL Staining

Hearts were isolated from each group (n=5) 6 hours after reperfusion for the terminal dUTP nick-end labeling (TUNEL) assay. After the blood and the fixation were washed out, the heart was also sliced transversely in 2.5-mm thicknesses. Paraffin-embedded, 5-μm-thick myocardial sections were used as described previously.[14] In brief, after deparaffinization and enzyme-mediated antigen retrieval, TUNEL staining was performed with a commercially available kit (Apop Tag Plus, Intergen). Samples were incubated with monoclonal anti-desmin antibody (Sigma) followed by tetramethylrhodamine isothiocyanate-conjugated rabbit anti-mouse antibody (DAKO). Counterstaining was performed with propidium iodide. Finally, these slides were mounted with Vector Shield (Vector Laboratories) containing an antifade reagent. We measured the number of TUNEL-positive nuclei in myocytes by means of confocal microscopy (Olympus, Fluoview 500). Quantitative analysis was performed on 60 high-power fields (magnification ×600) with at least 10 randomly selected fields used per section. We counted the number of cardiomyocytes at least $>10^4$ cells per heart.

DNA Ladder Assay

We used 10 additional rats for the DNA ladder assay (sham group, n=2; I/R-placebo group, n=4; I/R-AM group, n=4). Rats were killed, and the heart was excised 24 hours after ischemia/reperfusion. Immediately before heart isolation, 1% Evans blue was infused slowly into the left ventricle to delineate the risk area after coronary religation. Then, 40 mg of myocardium in the posterolateral border zone between the nonrisk area and the risk area was resected. Each specimen was frozen in liquid nitrogen and stored at −80°C until DNA extraction. DNA extraction and electrophoresis were performed with a commercially available kit (Apoptosis Ladder Detection Kit, WAKO).

Immunohistochemical Analysis

To assess localization of calcitonin receptor-like receptor (CRLR), a receptor for AM, in cardiac tissues, we performed immunohistochemical analysis using rabbit anti-rat CRLR antibody (Zymed). Localization of Akt phosphorylation was examined with rabbit anti-rat phospho-Akt antibody (Cell Signaling).

Western Blot Analysis

To identify Akt phosphorylation in myocardial tissues after AM infusion, Western blotting was performed with a commercially available kit (PhosphoPlus Akt [Ser 473] antibody kit, Cell Signaling). Myocardial tissues were obtained from rats treated with intravenous AM (0.01, 0.05, and 0.25 $\mu g \cdot kg^{-1} \cdot min^{-1}$), AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$) plus wortmannin (16 $\mu g/kg$ intravenous injection 15 minutes before AM infusion), or saline for 60 minutes during ischemia/reperfusion. These samples were homogenized on ice in a 0.1% Tween 20 homogenization buffer with a protease inhibitor (Complete, Roche). After centrifugation for 20 minutes at 4°C, the clear supernatant was used for Western blot analysis. Protein concentration was measured by Bradford's method (Bio-Rad). Fifty micrograms of each protein extract were transferred in sample buffer, loaded on 7.5% SDS-polyacrylamide gel, and blotted onto nitrocellulose membrane (Bio-Rad) with a wet blotting system. After being blocked for 60 minutes, the membranes were incubated with primary antibodies in blocking buffer (1:500) at 4°C overnight. Antibodies were used at the manufacturer's recommended dilution (Cell Signaling). The membranes were incubated with secondary antibodies, which were conjugated with horseradish peroxidase (Cell Signaling), at a final dilution of 1:2000. Signals were detected with LumiGLO chemiluminescence reagents (Cell Signaling).

Statistical Analysis

All data are expressed as mean±SEM unless otherwise indicated. Comparisons of parameters among the 3 or 4 groups were made by 1-way ANOVA for repeated measures, followed by Scheffé test. A probability value <0.05 was considered to indicate statistical significance.

Results

Reduction of Myocardial Infarct Size After AM Infusion

Moderate to large infarcts were observed in Masson trichrome–stained myocardial sections 24 hours after ische-

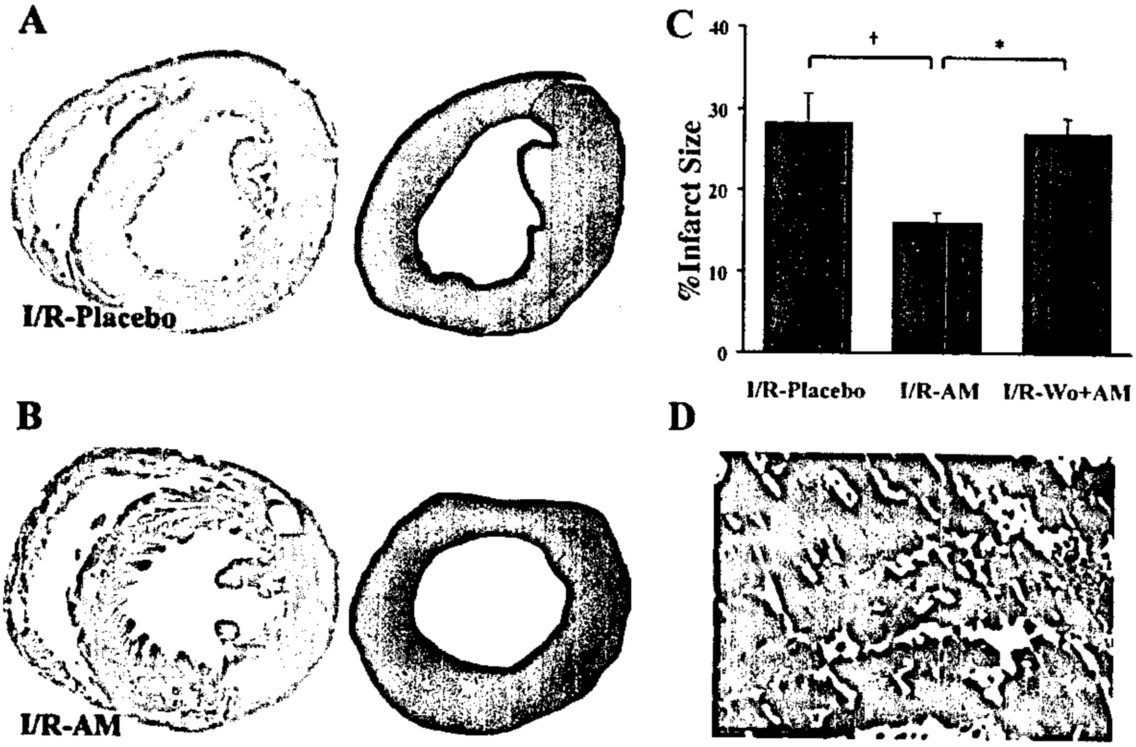

Figure 1. Effect of AM on myocardial infarct size 24 hours after ischemia/reperfusion. A and B, Photomicrographs show representative myocardial sections stained with Masson trichrome in I/R-placebo (A) and I/R-AM groups (B). Light red area indicates coagulation necrosis (right). C, Quantitative analysis demonstrated that AM infusion decreased infarct size after ischemia/reperfusion. However, pretreatment with wortmannin attenuated effect of AM. D, Typical reperfusion injury was observed in all groups on high-power field. Bar=100 μm. Data are mean±SEM. *$P<0.05$, †$P<0.01$.

mia/reperfusion (Figures 1A and 1B). Quantitative analysis revealed that 60-minute infusion of AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$) significantly reduced myocardial infarct size compared with placebo infusion (16±1 versus 28±4%, $P<0.01$; Figure 1C). Infusion of AM markedly increased plasma AM level (from 10±2 fmol/mL at baseline to 96±13 fmol/mL at 60 minutes), which suggests that the plasma AM level was pharmacologically high. Pretreatment with wortmannin reversed the reducing effects of AM on myocardial infarct size (from 16±1% to 27±2%, $P<0.05$ versus I/R-AM group; Figure 1D). Although typical reperfusion injury, including contraction bands, hemorrhage, myocardial cell coagulation, and inflammatory cell infiltration, was observed after ischemia/reperfusion (Figure 1D), there were no histological differences among the 3 groups.

Hemodynamic Effects of AM

Twenty-four hours after ischemia/reperfusion, LV end-diastolic pressure (LVEDP) showed a marked elevation in the I/R-placebo group (19±2 mm Hg); the elevation was significantly attenuated in the I/R-AM group (8±2 mm Hg, $P<0.05$; Figure 2A). Pretreatment with wortmannin attenuated the reducing effects of AM on LVEDP (from 8±2 to 17±2 mm Hg, $P<0.05$ versus I/R-AM group; Figure 2A) 24 hours after ischemia/reperfusion. LV dP/dt_{max} tended to be higher in the I/R-AM group than in the I/R-placebo group (5285±285 versus 4524±247 mm Hg/s), and LV dP/dt_{min} tended to be lower in the I/R-AM group than in the I/R-placebo group (−4700±303 versus −3695±165 mm Hg/s; Figure 2B). Furthermore, pretreatment with wortmannin reversed the effects of AM on LV dP/dt_{max} and LV dP/dt_{min} after ischemia/reperfusion (5285±285 to 4570±239 mm Hg/s, −4700±303 to −3843±227 mm Hg/s, respectively; Figure 2B). These results suggest that AM infusion improved LV systolic and diastolic function after ischemia/reperfusion through the PI3K pathway. Interestingly, heart rate was significantly higher in the I/R-placebo and I/R-AM groups than in the sham group (Table). Although mean aortic pressure was significantly lower in the I/R-placebo group than in the sham group, a significant decrease in mean aortic pressure was not observed in the I/R-AM group. Right ventricular systolic pressure was significantly lower in the I/R-AM group than in the I/R-placebo group.

Antiapoptotic Effect of AM in Cardiomyocytes

Representative photomicrographs showed that TUNEL-positive myocytes were more frequently observed in the I/R-placebo group than in the sham group. However, TUNEL-positive myocytes were less frequently observed in the I/R-AM group than in the I/R-placebo group (Figure 3). Although a typical DNA ladder indicating fragmented DNA in cardiomyocytes was also observed in the I/R-placebo group, it was attenuated in the I/R-AM group (Figure 4). Quantitative analyses demonstrated that the number of TUNEL-positive cardiomyocytes was significantly smaller in the I/R-AM group than in the I/R-placebo group (9±4%

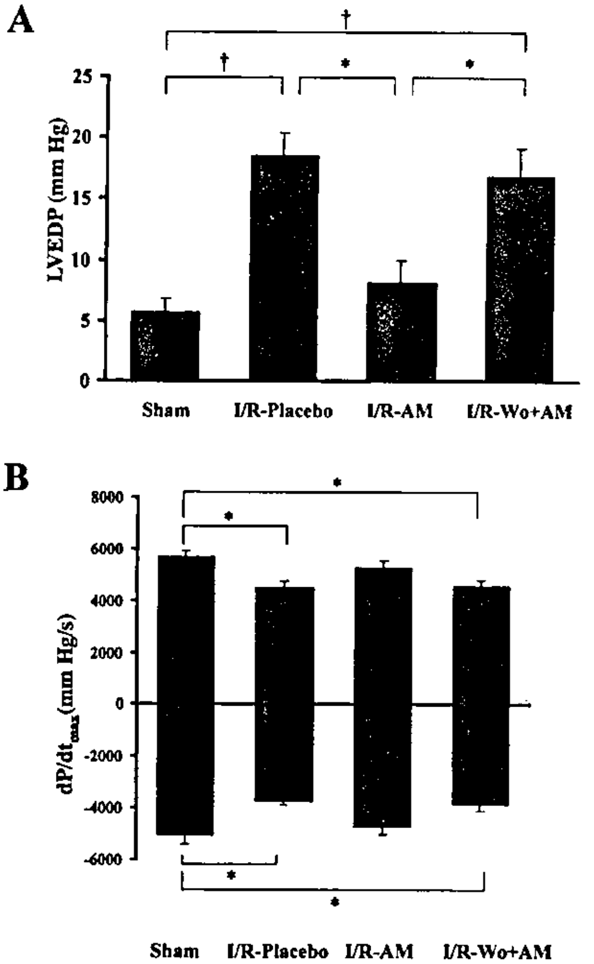

Figure 2. Effects of AM on LVEDP (A) and LV dP/dt (B) 24 hours after ischemia/reperfusion. AM infusion significantly inhibited increase in LVEDP compared with placebo infusion. AM infusion also improved LV dP/dt 24 hours after ischemia/reperfusion. Pretreatment with wortmannin attenuated effects of AM on LVEDP and LV dP/dt. Data are mean±SEM. *$P<0.05$; †$P<0.01$.

versus 19±2%, $P<0.05$; Figure 5). Furthermore, pretreatment with wortmannin abolished the AM-induced antiapoptotic effect in cardiomyocytes (from 9±4% to 20±1%, $P<0.05$; Figure 5). These results suggest that AM exerted antiapoptotic effects through the PI3K-dependent signal.

Summary of Hemodynamic Studies

	Sham (n=5)	I/R-Placebo (n=8)	I/R-AM (n=8)	I/R-Wo+AM (n=10)
Body weight, g	184±10	184±9	183±7	195±6
Heart rate, bpm	450±10	501±5*	494±9*	488±4
MAP, mm Hg	120±3	97±3*	105±4	99±7*
RAP, mm Hg	3±1	5±2	4±1	3±1
RVSP, mm Hg	32±1	47±1†	43±2†‡	48±2†

MAP indicates mean aortic pressure; RAP, right atrial pressure; and RVSP, right ventricular systolic pressure. Data are mean±SEM.

*$P<0.05$ vs sham group.

†$P<0.01$ vs Sham group.

‡$P<0.01$ vs I/R-placebo group.

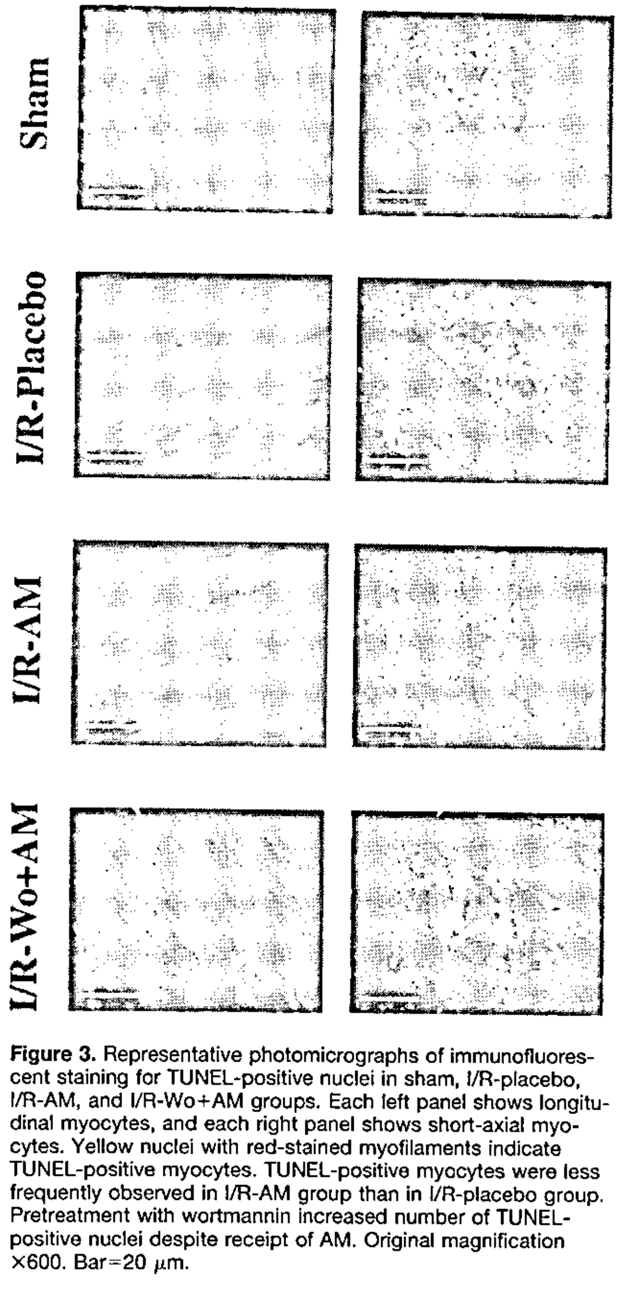

Figure 3. Representative photomicrographs of immunofluorescent staining for TUNEL-positive nuclei in sham, I/R-placebo, I/R-AM, and I/R-Wo+AM groups. Each left panel shows longitudinal myocytes, and each right panel shows short-axial myocytes. Yellow nuclei with red-stained myofilaments indicate TUNEL-positive myocytes. TUNEL-positive myocytes were less frequently observed in I/R-AM group than in I/R-placebo group. Pretreatment with wortmannin increased number of TUNEL-positive nuclei despite receipt of AM. Original magnification ×600. Bar=20 μm.

Akt Phosphorylation Induced by AM Infusion in Cardiac Tissue

Immunohistochemical analysis revealed that CRLR, a receptor for AM, was localized in cardiomyocytes and vascular endothelial cells (Figure 6). After 60-minute infusion of AM, Akt phosphorylation was detected in the nuclei of cardiomyocytes and vascular endothelial cells (Figures 7A and 7B). Western blot analyses also revealed that AM at 0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$ significantly phosphorylated Akt in cardiac tissue that was exposed to ischemia/reperfusion (Figure 7C). The effect of AM on Akt was inhibited by pretreatment with wortmannin. These results suggest that AM acts directly on myocardium and induces cardioprotective effects through the activation of PI3K/Akt-pathway.

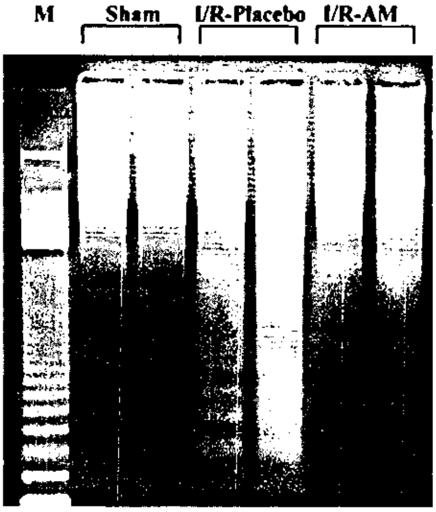

Figure 4. DNA ladder in sham, I/R-placebo, and I/R-AM groups. Although typical DNA ladder indicating fragmented DNA in cardiomyocytes was observed in I/R-placebo group, it was attenuated in I/R-AM group. M indicates molecular marker.

Discussion

In the present study, we demonstrated that short-term infusion of AM during the early phase of ischemia/reperfusion significantly reduced myocardial infarct size and inhibited myocyte apoptosis, and AM significantly decreased LVEDP and tended to improve LV dP/dt_{max} and dP/dt_{min}. We also demonstrated that AM enhanced Akt phosphorylation in cardiac tissue and that pretreatment with a PI3K inhibitor attenuated AM-induced cardioprotective effects against ischemia/reperfusion and inhibited AM-induced Akt phosphorylation.

Intravenous infusion of AM has beneficial hemodynamic and renal effects in patients with heart failure.[8] However, whether AM has direct cardioprotective effects in vivo remains unclear. In the present study, we demonstrated that short-term infusion of AM during the early phase of ischemia/reperfusion markedly reduced myocardial infarct size. Cardiomyocyte apoptosis is one of the major contributors to the development of myocardial infarcts,[15,16] which is related to the pathogenesis of heart failure. Thus, we examined whether AM has antiapoptotic effects in cardiomyocytes. Interestingly, short-term infusion of AM significantly reduced myocyte apoptosis after ischemia/reperfusion. This is the first study to demonstrate antiapoptotic effects of AM against myocardial ischemia/reperfusion injury, although AM has been shown to have antiapoptotic effects in vascular endothelial cells.[17,18] Given that cardiomyocyte apoptosis rather than necrosis contributes to myocyte death after ischemia/reperfusion, the antiapoptotic effects of AM may result in the reduced infarct size after ischemia/reperfusion.

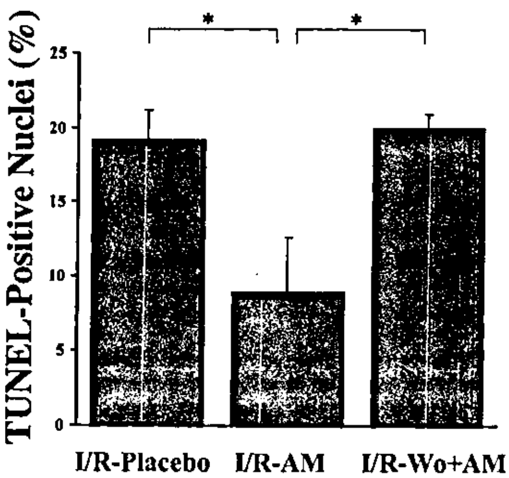

Figure 5. Quantitative analysis of TUNEL-positive nuclei in myocytes. Number of TUNEL-positive myocytes was lower in I/R-AM group than in I/R-placebo group. However, number of TUNEL-positive myocytes in I/R-Wo+AM group was as large as in I/R-placebo group. Data are mean±SEM. *$P<0.05$.

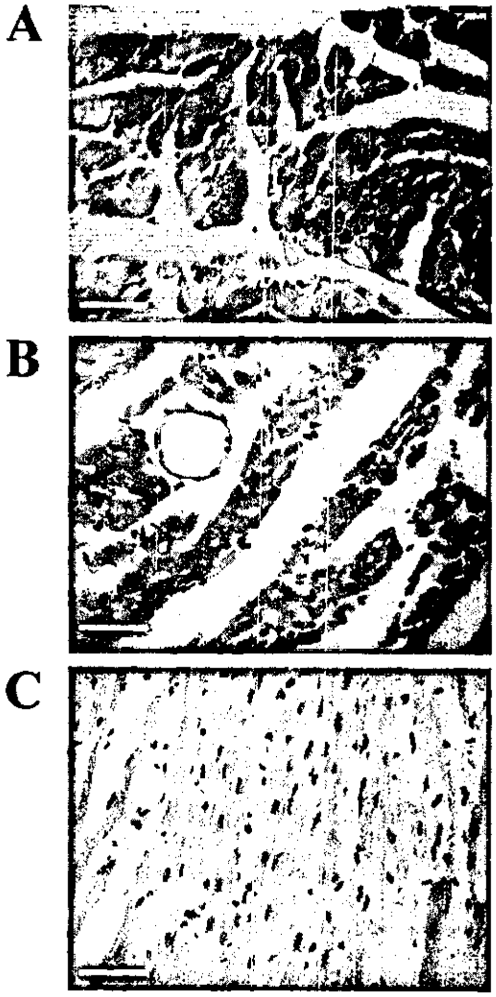

Figure 6. Immunohistochemistry for CRLR in rat cardiac tissue. Representative photomicrographs revealed that CRLR was localized in cardiomyocytes (A) and vascular endothelial cells (B). Negative control study (using mouse IgG) showed no positive staining in cardiac tissue (C). Original magnification ×400. Bar=20 μm.

In the present study, 60-minute infusion of AM improved cardiac function after ischemia/reperfusion, as indicated by a significant decrease in LVEDP and a tendency for an increase in LV dP/dt_{max} and a decrease in LV dP/dt_{min}. Previous studies have shown that the susceptibility to cardiac dysfunction

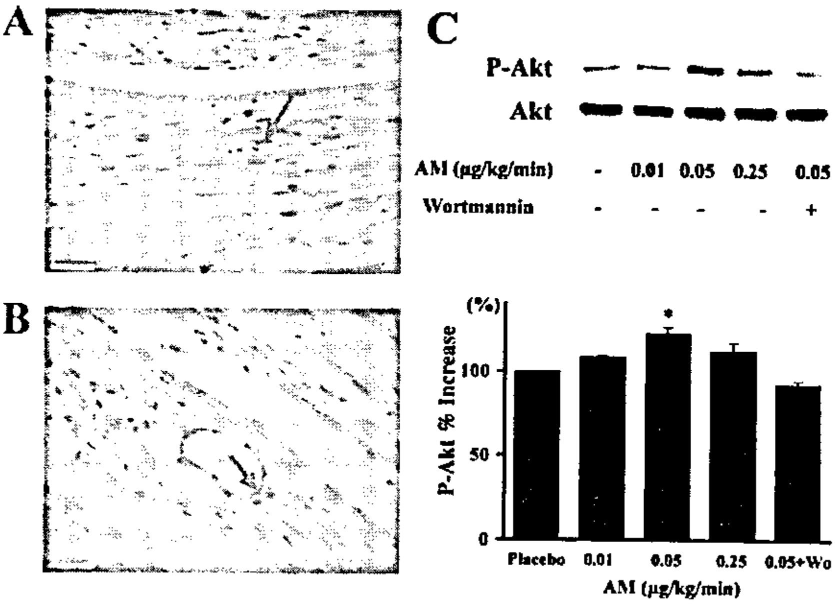

Figure 7. A and B, Immunohistochemistry for Akt phosphorylation in rat cardiac tissue. Infusion of AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$) phosphorylated Akt predominantly in nuclei of cardiomyocytes (A, B) and vascular endothelial cells (B). Arrow indicates nuclei of cardiomyocytes with positive staining for P-Akt antibody. Arrowhead indicates nuclei of endothelium with positive staining for P-Akt antibody. Original magnification ×400. Bar=20 μm. C, Western blot analysis of AM-induced Akt phosphorylation in cardiac tissues. Infusion of AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$) activated Akt in myocardial tissues exposed to ischemia/reperfusion. Pretreatment with wortmannin significantly inhibited AM-induced Akt phosphorylation. P-Akt indicates phosphorylated Akt; Wo, wortmannin. Data are mean±SEM. *$P<0.05$ vs placebo.

depends on the degree of myocyte apoptosis within 24 hours after ischemia/reperfusion.[19] Thus, the early prevention of myocyte apoptosis and the resultant reduced infarct size by AM may contribute to the hemodynamic improvement after ischemia/reperfusion. AM infusion reduced right ventricular systolic pressure, which may be attributable not only to the potent vasodilatory effects of AM but also to improvement in cardiac function.

Recently, Akt activation has been shown to reduce myocyte apoptosis and thereby prevent myocardial injury after transient ischemia.[10] Akt is the downstream effector molecule for signal transduction initiated by cardioprotective hormones such as insulin-like growth factor I.[20] Thus, Akt is considered to be a powerful survival signal in myocytes.[21] More recently, AM has been shown to activate the PI3K/Akt-pathway in vascular endothelial cells.[9] However, localization of AM-specific receptors in cardiac tissue had been unknown. The present study demonstrated that CRLR was present in rat cardiomyocytes and vascular endothelial cells and that AM infusion accelerated Akt phosphorylation in nuclei of cardiomyocytes and vascular endothelial cells. Furthermore, Western blot analyses demonstrated that AM 0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$ significantly increased phosphorylated Akt in cardiac tissue compared with placebo treatment and that pretreatment with wortmannin significantly inhibited Akt phosphorylation. Interestingly, pretreatment with wortmannin attenuated the AM-induced beneficial effects, such as reduction of infarct size, hemodynamic improvements, and inhibition of apoptosis. These findings suggest that AM infusion directly induces cardioprotective effects through the PI3K/Akt-dependent pathway.

In the present study, plasma AM level during infusion was much higher than baseline plasma level in rats, plasma level in normal human subjects (≈10 fmol/mL),[8] and plasma level in patients with acute myocardial infarction (≈14 fmol/mL).[22] These findings suggest that exogenously administered AM functions at pharmacological levels.

Preclinical studies have demonstrated that a variety of antioxidative or antiapoptotic agents reduce myocardial infarct size after ischemia/reperfusion.[23,24] However, few agents are clinically available for patients with coronary artery disease. In contrast, the safety and hemodynamic benefits of short-term treatment with intravenous AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$) have been demonstrated in patients with heart failure[8] and patients with myocardial infarction.[25] Given the results of the present study, a prospective, randomized, placebo-controlled clinical trial should be planned.

Conclusions

Short-term infusion of AM significantly attenuated myocardial ischemia/reperfusion injury. These cardioprotective effects were attributed mainly to the antiapoptotic effects of AM via a PI3K/Akt-dependent pathway.

Acknowledgments

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Adrenomedullin Enhances Angiogenic Potency of Bone Marrow Transplantation in a Rat Model of Hindlimb Ischemia

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Background—Previous studies have shown that adrenomedullin (AM) inhibits vascular endothelial cell apoptosis and induces angiogenesis. We investigated whether AM enhances bone marrow cell–induced angiogenesis.

Methods and Results—Immediately after hindlimb ischemia was created, rats were randomized to receive AM infusion plus bone marrow–derived mononuclear cell (MNC) transplantation (AM+MNC group), AM infusion alone (AM group), MNC transplantation alone (MNC group), or vehicle infusion (control group). The laser Doppler perfusion index was significantly higher in the AM and MNC groups than in the control group (0.74±0.11 and 0.69±0.07 versus 0.59±0.07, respectively, $P<0.01$), which suggests the angiogenic potency of AM and MNC. Importantly, improvement in blood perfusion was marked in the AM+MNC group (0.84±0.08). Capillary density was highest in the AM+MNC group, followed by the AM and MNC groups. In vitro, AM inhibited MNC apoptosis, promoted MNC adhesiveness to a human umbilical vein endothelial cell monolayer, and increased the number of MNC-derived endothelial progenitor cells. In vivo, AM administration not only enhanced the differentiation of MNC into endothelial cells but also produced mature vessels that included smooth muscle cells.

Conclusions—A combination of AM infusion and MNC transplantation caused significantly greater improvement in hindlimb ischemia than MNC transplantation alone. This effect may be mediated in part by the angiogenic potency of AM itself and the beneficial effects of AM on the survival, adhesion, and differentiation of transplanted MNCs. **(*Circulation*. 2005;111:356-362.)**

Key Words: peptides ■ angiogenesis ■ peripheral vascular disease

Peripheral vascular disease is a crucial health issue that affects an estimated 27 million people.[1] Despite recent advances in medical intervention, the symptoms of some patients with critical limb ischemia fail to be controlled. Bone marrow–derived mononuclear cells (MNCs) include a variety of stem and progenitor cells, such as endothelial progenitor cells (EPCs), and contribute to pathological neovascularization.[2] MNC transplantation induces therapeutic angiogenesis in ischemic limb[3,4]; however, some patients fail to respond to this cell therapy. Thus, a novel therapeutic strategy to enhance the angiogenic property of MNCs is desirable.

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma.[5] Previous studies have reported that abnormalities of vascular structure are present in homozygous AM knockout mice.[6,7] A recent study has demonstrated that blood flow recovery in ischemic limb and tumor angiogenesis are substantially impaired in heterozygous AM knockout mice.[8] Furthermore, AM has been shown to inhibit vascular endothelial cell apoptosis and induce angiogenesis through the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway.[9,10] These results suggest that AM is indispensable for modulating angiogenesis and vasculogenesis. When these findings are taken together, combination therapy with MNC transplantation and AM infusion may have additional or synergetic effects on therapeutic angiogenesis for the treatment of severe peripheral vascular disease. Thus, the purposes of the present study were (1) to investigate whether local infusion of AM enhances the angiogenic potency of MNC transplantation in a rat model of hindlimb ischemia and (2) to investigate the effects of AM on the survival, adhesion, and differentiation of transplanted MNCs.

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Methods

Animal Model of Hindlimb Ischemia

Male Lewis rats (weight 250 to 275 g; Japan SLC Inc, Hamamatsu, Japan) were used in the present study. The left common iliac artery of each rat was resected under anesthesia with pentobarbital sodium (50 mg/kg). The distal portion of the saphenous artery and all side branches and veins were dissected free and excised. The right hindlimb was kept intact and used as the nonischemic limb. Transplantation of bone marrow–derived MNCs and infusion of AM were performed in 40 rats immediately after hindlimb ischemia was created. This protocol resulted in the creation of 4 groups: (1) AM infusion plus MNC transplantation (AM+MNC group, n=10), (2) AM infusion plus PBS injection (AM group, n=10), (3) vehicle infusion plus MNC transplantation (MNC group, n=10), and (4) vehicle infusion plus PBS injection (control group, n=10). The Animal Care Committee of the National Cardiovascular Center approved this experimental protocol.

MNC Transplantation and AM Infusion

Bone marrow was harvested from the femur and tibia in other male Lewis rats, and MNCs were isolated by Ficoll density gradient centrifugation (Lymphoprep, Nycomed). MNCs (5×10^6 cells per animal) or PBS was injected into the ischemic thigh muscle with a 26-gauge needle at 5 different points. Human recombinant AM (0.01 $\mu g \cdot kg^{-1} \cdot min^{-1}$) or vehicle was administered for 7 days with a mini-osmotic pump (ALZET, Palo Alto) implanted in the left inguinal region.

Assessment of Blood Perfusion

To measure serial blood flow for 3 weeks, we used a laser Doppler perfusion image (LDPI) analyzer (Moor Instrument). After blood flow was scanned twice, the average flow values of the ischemic and nonischemic limbs were calculated by computer-assisted quantification. The LDPI index was determined as the ratio of ischemic to nonischemic hindlimb blood perfusion.[11]

Histological Assessment

Three weeks after MNC transplantation and/or AM infusion, 4 pieces of ischemic tissue from the adductor and semimembranous muscles were obtained and snap-frozen in liquid nitrogen. Frozen tissue sections were stained with alkaline phosphatase by an indoxyl tetrazolium method to detect capillary endothelial cells.[3,11] Five fields were randomly selected to count the number of capillaries. The capillary number adjusted per muscle fiber was used to compare the differences in capillary density among the 4 groups.[3]

Monitoring of Transplanted MNCs in Ischemic Hindlimb Muscle

To examine differentiation of transplanted MNCs, 5×10^6 MNCs labeled with red fluorescent dye (PKH26-GL, Sigma Chemical Co) were transplanted into the ischemic thigh muscle in rats with (n=3) and without (n=3) AM infusion. Three weeks after transplantation, frozen tissue sections from ischemic muscle were incubated with anti-von Willebrand factor antibody (vWF, DAKO), anti-CD31 antibody (BD Pharmingen), and anti-α-smooth muscle actin antibody (α-SMA, DAKO), followed by incubation with Alexa Fluor 633 IgG antibody (Molecular Probes) and FITC-conjugated IgG antibody (BD Pharmingen), respectively. Five high-power fields (40×) of each section were randomly selected to count the number of transplanted MNCs, vWF-positive cells, and α-SMA–positive cells.

In Situ Detection of MNC Apoptosis

PKH26-labeled MNCs (5×10^6 cells per animal) were transplanted into the ischemic muscle in rats with (n=2) and without (n=2) AM infusion. Twenty-four hours after transplantation, apoptosis of transplanted MNCs in ischemic tissue was evaluated by terminal dUTP nick-end labeling (TUNEL) assay (ApopTag Fluorescein kit, Serological Corporation), as reported previously.[12]

In Vitro Apoptosis Assay

The antiapoptotic effect of AM on MNCs was evaluated by TUNEL assay. Human MNCs, isolated from peripheral blood, were plated on 12-well plates (1×10^6 cells per well) and cultured in serum-free medium for 24 hours with control buffer, AM, or AM plus wortmannin, a PI3K inhibitor (50 nmol/L). TUNEL for detection of apoptotic nuclei was performed according to the manufacturer's instructions. MNCs were then mounted in medium that contained 4',6-diamidino-2-phenylindole (DAPI). Randomly selected microscopic fields (n=10) were evaluated to calculate the ratio of TUNEL-positive cells to total cells.

Adhesion Assay

We evaluated whether AM enhances MNC adhesiveness according to a previously reported method.[13] In brief, human umbilical vein endothelial cells (HUVECs) were cultured to confluence on 6-well plates with or without pretreatment with tumor necrosis factor-α (1 ng/mL). In the absence or presence of AM (10^{-7} mol/L), 1×10^6 MNCs labeled with PKH26 were incubated on an HUVEC monolayer for 24 hours. Nonadherent MNCs were removed, and the number of PKH26-positive cells in each well was counted.

Cell ELISA

Expression of adhesion molecules in HUVECs was measured by cell ELISA, as reported previously.[14] In brief, confluent HUVECs on 96-well plates were treated with AM (10^{-7} mol/L) or control buffer for 4 hours. HUVECs were then incubated with monoclonal mouse antibodies against intercellular adhesion molecule-1 (ICAM-1, R&D Systems) and vascular adhesion molecule-1 (VCAM-1, R&D Systems). A protein detector ELISA kit (KPL) was used to detect bound monoclonal antibodies.

EPC Culture Assay

Culture of EPCs was performed as described previously.[11,15,16] In brief, 2×10^6 MNCs were plated in Medium-199 supplemented with 20% FCS, heparin, and antibiotics on fibronectin-coated 6-well plates. AM (10^{-7} mol/L), human recombinant vascular endothelial growth factor (VEGF; 20 ng/mL), or control buffer was added to each plate. After 7 days of culture, nonadherent cells were removed, and adherent cells were incubated with acetylated LDL labeled with DiI (DiI-acLDL, Biomedical Technologies) and FITC-labeled lectin from ulex europaeus (Sigma). Double-positive cells for DiI-acLDL and FITC-labeled lectin were identified as EPCs.[16] Randomly selected microscopic fields (n=10) were evaluated to count the number of EPCs.

Fluorescence-Activated Cell Sorting Analysis

Fluorescence-activated cell sorting was performed to identify characteristics of adherent cells after 7 days of culture.[16] Cells were incubated for 30 minutes at 4°C with anti-human CD31 antibodies (clone L133.1, Becton Dickinson), anti-human KDR antibodies (clone KDR-1, Sigma), and anti-human VE-cadherin antibodies (clone BV6, Chemicon). Isotype-identical antibodies served as controls. Fluorescence-activated cell sorting analyses were performed with a FACSCalibur flow cytometer and Cell Quest software (BD Biosciences).

Real-Time Polymerase Chain Reaction

Expression of calcitonin receptor-like receptor (CRLR), a receptor for AM, was examined by real-time polymerase chain reaction (PCR). Total RNA was extracted from MNCs, EPCs, and HUVECs with an RNA extraction kit (RNeasy Mini Kit, Qiagen) and converted to cDNA by reverse transcription. Real-time PCR was performed with SYBR green dye (QuantiTect SYBR Green PCR kit, Qiagen) and a Prism 7700 sequence detection system (Applied Biosystems). The PCR primers for CRLR were as follows: sense primer 5'-CATTCAACAAGCAGAAGGCG-3' and antisense primer 5'-AGCCATCCATCCCAGGTTC-3'. For GAPDH, the primers were as follows: sense primer 5'-CAATGCCTCCTGCACCACCAA-3' and antisense primer 5'-GAGGCAGGGATGATGTTCTGGA-3'. Levels of CRLR mRNA were normalized to that of

GAPDH mRNA. PCR-amplified products were also electrophoresed on 2% agarose gels to confirm that single bands were amplified.

In Vitro Matrigel Assay

HUVECs (1×10^5 cells) were seeded onto 24-well plates coated with Matrigel (Becton Dickinson) in the presence of the combination of control buffer, AM (10^{-7} mol/L), VEGF (10 ng/mL), or neutralizing antibodies against KDR (2 μg/mL, R&D Systems). After incubation for 18 hours, tube formation area was measured as described previously.[17] The control was defined as 100% tube formation, and the percent increase was calculated for each sample.

Measurements of Cytokines

A total of 1×10^6 MNCs or HUVECs were plated in serum-free medium with or without AM (10^{-7} mol/L) on 12-well plates. After 24-hour incubation, the conditioned medium was collected, and VEGF, basic fibroblast growth factor, and hepatocyte growth factor were measured with enzyme immunoassay kits (R&D Systems).

Migration Assay

Migration assay of smooth muscle cells (SMCs) was performed with Transwell (Coster) 24-well plates composed of a collagen-coated membrane with 8-μm pores. Human aortic SMCs, preincubated with serum-free medium for 24 hours to maintain quiescence, were seeded on the upper chamber at a concentration of 1×10^6 cells/mL. Serum-free medium containing control buffer, AM (10^{-7} mol/L), or AM plus wortmannin (50 nmol/L) was placed in the lower chamber. After incubation for 12 hours, the number of migrated cells was counted in the randomly selected fields (n=5).

Statistical Analysis

All values are expressed as mean±SEM. Student's unpaired *t* test was used to compare differences between 2 groups. Comparisons of parameters among 3 or 4 groups were made by 1-way ANOVA, followed by Scheffé multiple comparison test. Comparisons of the time course of the LDPI index were made by 2-way ANOVA for repeated measures, followed by Scheffé multiple comparison tests. A probability value <0.05 was considered statistically significant.

Results

Blood Perfusion and Capillary Density

Blood perfusion of the ischemic hindlimb increased modestly but gradually in the AM and MNC groups after treatment (Figure 1A). Interestingly, blood perfusion in the AM+MNC group markedly improved within 2 weeks after treatment and showed further improvement thereafter. The LDPI index was significantly higher in the AM, MNC, and AM+MNC groups than in the control group 3 weeks after surgery (Figure 1B). Importantly, the LDPI index was highest in the AM+MNC group among the 4 groups.

Alkaline phosphatase staining of ischemic muscle showed significant augmentation of neovascularization in the AM, MNC, and AM+MNC groups (Figure 2A). The capillary/muscle fiber ratio of ischemic muscle was highest in the AM+MNC group, followed by the MNC group, AM group, and control group (Figure 2B).

Differentiation of Transplanted MNCs

Three weeks after MNC transplantation, PKH26-labeled MNCs were frequently observed in the AM+MNC group, and these transplanted cells were positive for vWF (Figure 3A). Most of these cells were also stained by CD31 (data not shown). The number of PKH26/vWF double-positive cells was significantly higher in the AM+MNC group than in the MNC group (Figure 3B). Although PKH26/α-SMA double-positive cells were not detected in ischemic muscle of each group, newly formed vascular structures in the AM+MNC group included α-SMA–positive cells (Figure 3C). The number of α-SMA–positive cells in the MNC-derived vascular structures was significantly higher in the AM+MNC group than in the MNC group (Figure 3D).

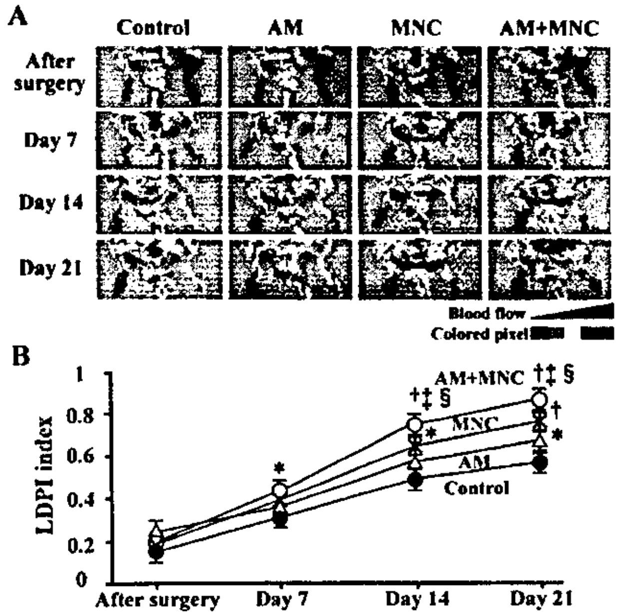

Figure 1. A, Representative examples of serial laser Doppler perfusion images. Blood perfusion of ischemic hindlimb increased notably in AM+MNC group (red to yellow). B, Quantitative analysis of hindlimb blood perfusion with LDPI index, ratio of ischemic to nonischemic hindlimb blood perfusion. Data are mean±SEM. *P<0.05 and †P<0.01 vs control; ‡P<0.01 vs AM; §P<0.05 vs MNC.

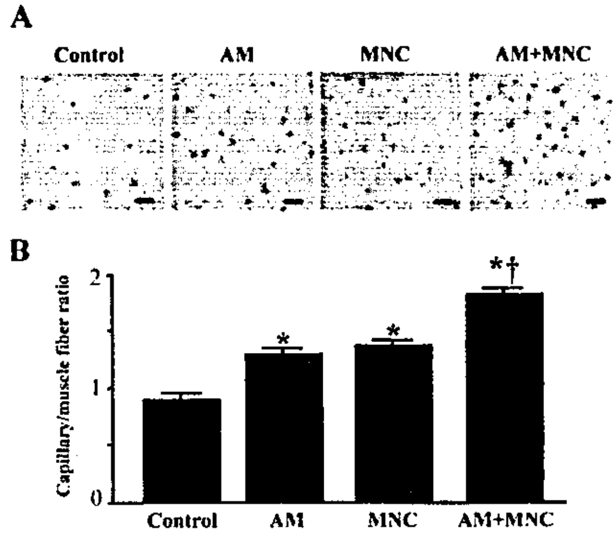

Figure 2. A, Representative photographs of alkaline phosphatase staining in ischemic hindlimb muscles. Capillary density in AM+MNC group was markedly higher than that in other groups. B, Quantitative analysis of capillary density in ischemic hindlimb muscles. Data are mean±SEM. *P<0.01 vs control; †P<0.01 vs AM and MNC. Scale bars: 50 μm.

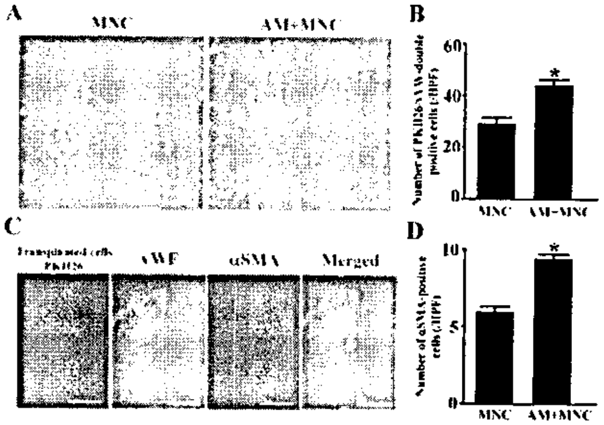

Figure 3. In vivo differentiation of transplanted MNCs. A, Representative photographs of MNC-derived vascular structures in MNC and AM+MNC groups. Red fluorescence (PKH26)–labeled MNCs were transplanted into ischemic thigh muscle. PKH26 (red)/vWF (blue) double-positive cells (pink, arrows) were frequently observed in AM+MNC group. B, Number of PKH26/vWF double-positive cells (MNC-derived endothelial cells) was significantly higher in AM+MNC group than in MNC group. C, Representative photographs of newly formed mature vessels in AM+MNC group. MNC-derived vascular structures often included α-SMA–positive cells (green). D, Number of α-SMA–positive cells in MNC-derived vessels was significantly higher in AM+MNC group than in MNC group. Data are mean±SEM. *$P<0.01$ vs MNC. Bars: 50 μm. HPF indicates high-power field.

Antiapoptotic Effect of AM on MNCs

In vitro, serum starvation induced MNC apoptosis, as indicated by detection of TUNEL-positive cells (Figure 4A). When incubated in the presence of AM, the percentage of TUNEL-positive cells markedly decreased in a dose-dependent manner (Figure 4B). However, pretreatment with wortmannin, a PI3K inhibitor, diminished the antiapoptotic effect of AM. Similarly, in vivo, local administration of AM decreased TUNEL-positive MNC 24 hours after transplantation (data not shown).

Effect of AM on MNC Adhesiveness

The number of adherent MNCs on an HUVEC monolayer increased significantly in the presence of AM (10^{-7} mol/L) compared with control (Figures 5A and 5B). With pretreatment using tumor necrosis factor-α, AM also enhanced the adhesiveness of MNCs to HUVECs. AM significantly enhanced expression of ICAM-1 and VCAM-1 in HUVECs (Figure 5C).

Effect of AM on EPC Expansion

After 7-day culture of human MNCs, spindle-shaped or cobblestone-like adherent cells were observed (Figure 6A). Most of the adherent cells were double stained with DiI-acLDL and FITC-labeled lectin. These adherent cells expressed endothelial cell–specific markers: KDR, VE cadherin, and CD31 (Figure 6B). Thus, we identified the major population of the adherent cells as EPCs. Culture of MNCs with AM significantly increased the number of EPCs (Figure 6C). The effect of AM was equivalent to that of VEGF. Real-time PCR revealed that MNCs, EPCs, and HUVECs expressed mRNA of CRLR (Figure 6D). Expression of CRLR mRNA was highest in HUVECs, followed by EPCs and MNCs.

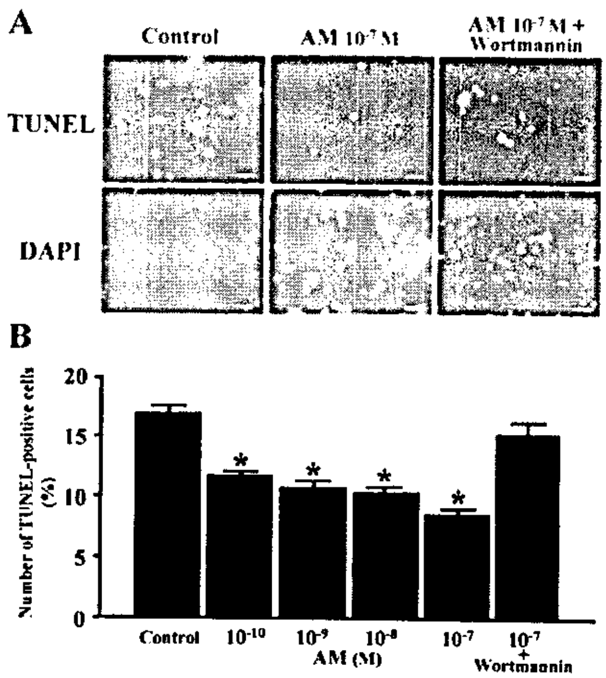

Figure 4. Apoptosis assay. A, Apoptosis of MNC was detected by TUNEL assay (green). Nuclei of MNC were stained with DAPI (blue). AM inhibited MNC apoptosis in serum-free medium. B, Quantitative analysis. AM decreased percentage of TUNEL-positive cells in dose-dependent manner. Pretreatment with wortmannin, a PI3K inhibitor, diminished antiapoptotic effect of AM. Data are mean±SEM. *$P<0.01$ vs control. Bars: 50 μm.

Effects of AM on Tube Formation and SMC Migration

Like VEGF, AM induced tube formation in HUVECs in vitro (Figure 7A). Blocking antibodies against KDR significantly inhibited VEGF-induced tube formation, whereas they did not suppress AM-induced tube formation (Figure 7B). AM did not significantly alter VEGF, basic fibroblast growth factor, or hepatocyte growth factor levels in conditioned medium of cultured MNCs or HUVECs (data not shown). AM significantly increased the number of migrated SMCs compared with control (Figures 7C and 7D). Pretreatment with wortmannin diminished the effect of AM on SMC migration.

Discussion

In the present study, we demonstrated in vivo that AM infusion or MNC transplantation alone induced angiogenesis in a rat model of hindlimb ischemia, the combination of AM infusion and MNC transplantation enhanced MNC-induced angiogenesis, and AM increased the number of MNC-derived vWF-positive cells and generated α-SMA–positive vascular structures. We also demonstrated in vitro that AM inhibited serum starvation–induced MNC apoptosis, promoted MNC adhesiveness to an HUVEC monolayer, increased the number of MNC-derived EPCs, and stimulated SMC migration.

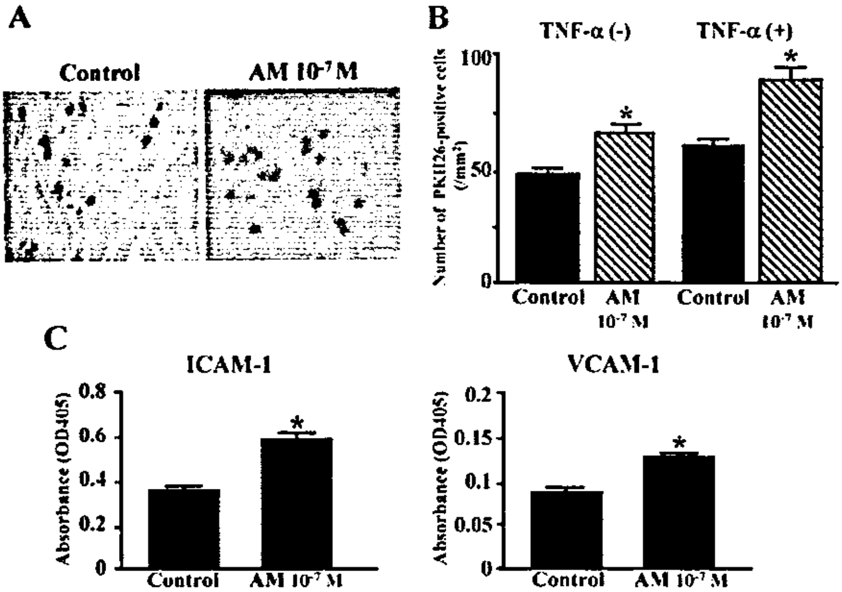

Figure 5. A and B, Adhesion assay. Representative photographs of red fluorescence–labeled MNC adhesion to HUVEC monolayer with and without AM (A). Quantitative analysis of MNC adhesion (B). Bars: 50 μm. C, Surface expression of ICAM-1 and VCAM-1 in HUVECs with or without AM. Data are mean±SEM. TNF indicates tumor necrosis factor. *$P<0.01$ vs control.

MNC transplantation causes therapeutic angiogenesis by supplying EPCs and multiple angiogenic cytokines such as VEGF.[3,4] The present study showed that local infusion of AM significantly increased blood perfusion and capillary density in ischemic hindlimb muscle. Furthermore, a combination of AM infusion and MNC transplantation significantly increased blood perfusion and capillary density of the ischemic hindlimb compared with MNC transplantation alone. AM has been shown to induce angiogenesis in vitro and in vivo through the PI3K/Akt pathway.[10,18] In the present study, AM-induced tube formation was not blocked by neutralizing antibodies against KDR. In addition, AM did not enhance VEGF secretion from MNCs and HUVECs. Thus, beneficial effects of combination therapy

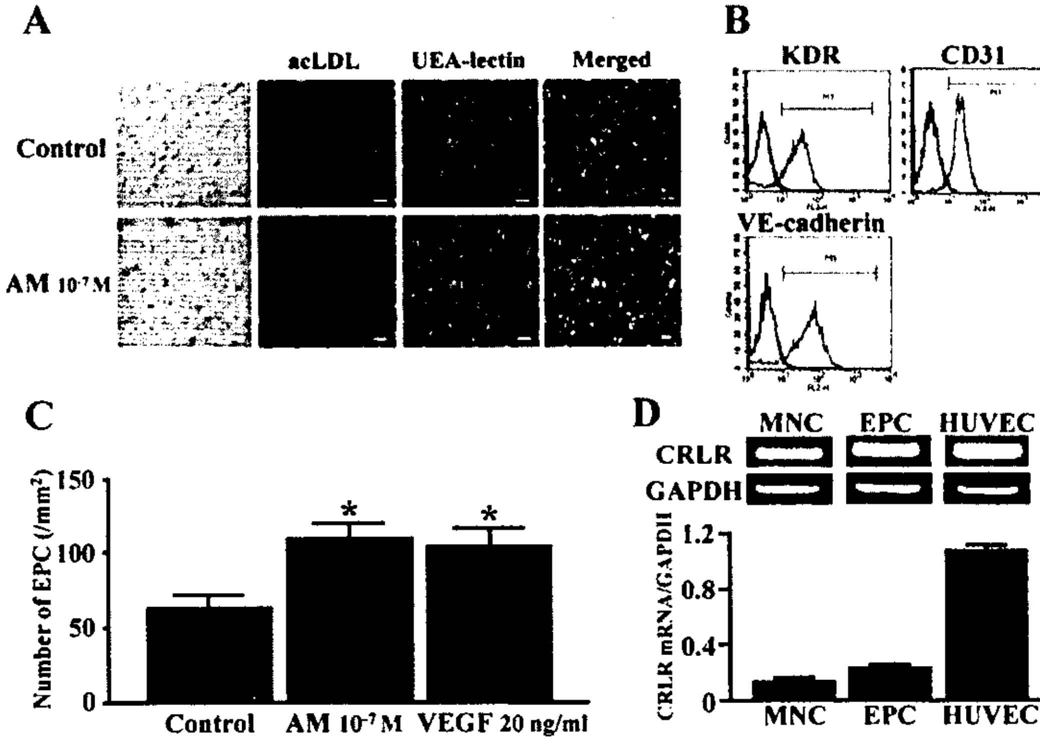

Figure 6. A through C, EPC culture assay. Cultured adherent cells took up DiI-acLDL (red) and FITC-labeled lectin (green) in same fields (A). Fluorescence-activated cell sorting analyses revealed that most adherent cells expressed KDR, VE cadherin, and CD31 (B). Culture of MNCs with AM significantly increased number of EPCs. Effect of AM was equivalent to that of VEGF (C). Data are mean±SEM. *$P<0.01$ vs control. Bars: 50 μm. D, Quantitative analysis of AM receptor (CRLR) mRNA expression in MNCs, EPCs, and HUVECs. UEA indicates ulex europaeus.

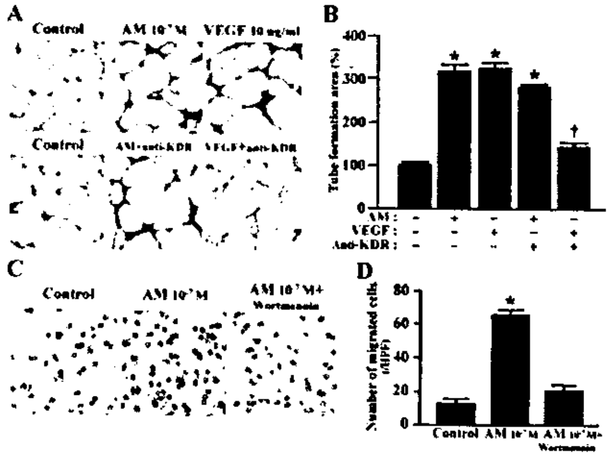

Figure 7. A and B, Matrigel assay. Representative photographs of tube formation (A). Quantitative analysis of tube formation area (B). Data are mean±SEM. *$P<0.01$ vs control; †$P<0.01$ vs VEGF. Bars: 20 μm. C and D, Migration assay. Representative photographs of migrated SMCs (C). Quantitative analyses of SMC migration (D). Data are mean±SEM. *$P<0.01$ vs control. Bars: 50 μm.

with AM and MNCs may be attributable in part to the angiogenic properties of AM itself.

An earlier study has shown that transplanted MNCs disappear from ischemic muscle 7 days after transplantation.[19] We demonstrated that apoptosis of MNCs occurred in ischemic muscle 24 hours after MNC transplantation. These results raise the possibility that the angiogenic potency of MNC transplantation is attenuated by MNC apoptosis. In the present study, AM inhibited apoptosis of MNCs in vitro and in vivo, and the antiapoptotic effect of AM was suppressed by wortmannin, a PI3K inhibitor. These findings suggest that AM prolongs MNC survival through the PI3K/Akt pathway and thereby enhances neovascularization in ischemic tissue.

In the present study, AM promoted adhesiveness of MNCs to an HUVEC monolayer. AM significantly enhanced expression of ICAM-1 and VCAM-1 in HUVECs, both of which facilitate adhesion of MNCs to endothelial cells.[20] These findings suggest that AM increases MNC adhesiveness to endothelial cells via activation of adhesion molecules. A recent study has shown that MNC adhesiveness to endothelial cells is indispensable for MNC differentiation into endothelial lineage.[21] Thus, it is possible that AM infusion enhances the angiogenic potency of MNCs at least in part through promotion of adhesion of MNC to host vascular endothelial cells.

VEGF has been shown to increase the number of EPCs in vitro and in vivo, resulting in angiogenesis and vasculogenesis.[13,22] The present study showed that MNCs and EPCs expressed CRLR, a receptor of AM. In vitro, AM increased the number of MNC-derived EPCs that expressed VE cadherin, KDR, and CD31. The effect of AM on EPC expansion was equivalent to that of VEGF. In vivo, AM infusion increased the number of MNC-derived vWF-positive cells, although incorporation of these cells in the capillaries may be due in part to incorporation of hematopoietic cells. These findings suggest that AM may accelerate MNC differentiation into endothelial lineage.

SMC is essential for the generation of functional and mature blood vessels.[23] We demonstrated in vivo that local infusion of AM increased the number of α-SMA–positive cells (SMCs) in MNC-derived vascular structures. In vitro, AM enhanced SMC migration, which was inhibited by wortmannin, a PI3K inhibitor. Recent studies using homozygous AM knockout mice have suggested that AM is indispensable for vascular morphogenesis.[6,7] When these findings are taken together, it is possible that AM contributes to vessel maturation through enhancement of SMC migration via the PI3K/Akt-dependent pathway.

Currently, a new therapeutic approach to augment the efficacy of MNC transplantation is awaited for the treatment of severe peripheral vascular disease. The present study demonstrated that local infusion of AM enhanced the angiogenic potency of MNC transplantation. In the present study, AM inhibited MNC apoptosis and increased the total number of engrafted cells in ischemic tissue, although this study did not show the effect of AM on specific cell populations of MNCs. In addition, AM promoted cell proliferation, migration, and differentiation. We have already demonstrated the safety of AM infusion in patients with congestive heart failure.[24] Thus, combination therapy with AM infusion and MNC transplantation may be a novel and promising therapeutic strategy for the treatment of severe peripheral vascular disease.

Conclusions

A combination of AM infusion and MNC transplantation caused significantly greater improvement in hindlimb ischemia than MNC transplantation alone. This effect may be mediated in part by the angiogenic potency of AM itself and the beneficial effects of AM on the survival, adhesion, and differentiation of transplanted MNCs.

Acknowledgments

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