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H. 知的財産権の出願・登録状況

「霊長類動物胚性幹細胞から血管系細胞への分化方法」米国特許出願（平成16年2月27日）
「内皮細胞分化増殖方法」特願2004-25631号

厚生労働科学研究費補助金（基礎研究成果の臨床応用推進研究事業）
分担研究報告書

アドレノメデユリンによる肺高血圧治療

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アドレノメデユリン(AM)は血管平滑筋に直接作用して、また血管内皮に働き NO を介して強力な肺血管拡張作用を発揮する。しかし静脈内投与では、体血圧の低下を伴うこと、また持続点滴が必要であるという欠点が存在する。我々は、これらの欠点を補うべく、AM の吸入療法を開発した。この投与法は、体血圧に影響を与えずに肺血管抵抗を低下させた。また運動耐容能を有意に改善させた。この吸入システムにはジェットネブライザーを用いており、取り扱いが容易であり自宅での繰り返し投与が可能である。

A. 研究目的

近年、基礎的研究からAMは強力な肺血管拡張作用が明らかとなった。これまでに我々は AM の静脈内投与が肺血管抵抗を低下させることを報告してきた。しかし静脈内投与では、体血圧の低下を伴うという欠点がある。そこで今回、原発性肺高血圧症(PPH)に代表される肺動脈性肺高血圧症に対するAMの気道内吸入治療効果を検討した。

B. 研究方法

吸入投与では PPH11 例(肺動脈平均圧 54±3 mmHg)を対象とし、全例に AM 吸入治療を行った。投与方法は、ジェットネブライザーを用いてAM(10μg/kg)のエアロゾルを 15 分間吸入させた。AM 吸入前より右心カテーテルを留置し、経時的に血行動態を測定した。また血中の AM 濃度の変化をラジノイムノアッセイにて測定した。最後に心肺運動負荷試験を行うことが可能であった 10 例に対して、AM 吸入の運動耐容能改善効果を検討した。AM または生理食塩水の吸入を無作為化二重盲検試験で行った。

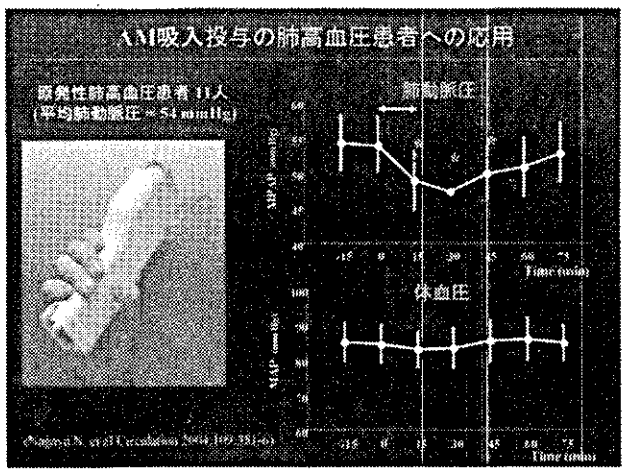
C. 研究結果

AM 吸入時に 1 人が頭痛を訴えた。また他の1人が軽度の低酸素状態となった。しかし重篤な副作用は出現しなかった。また不整脈、低血圧は認められなかった。

AM 吸入により血漿 AM 濃度は有意に上昇し(11.9±0.8 から 22.9±2.1 fmol/mL)、AM 濃度の上昇は 45 分以上持続した。このとき血漿 cAMP の有意な上昇を認めたが、血漿 cGMP 濃度の上昇はなかった。

ジェットネブライザーによるAM吸入は体血圧に影響を与えることなく(平均動脈圧 85±4 から 83±4 mmHg, p=NS)、平均肺動脈を有意に低下させた(54±3 から 47±3 mmHg, p<0.05)。心係数は有意に増加し(+12%)、肺血管抵抗は有意に低下した(-22%)。これらの効果は 45 分以上持続した。AM 吸入は酸素濃度に影響を与えなかった(SaO2: 94±3 から 93±3 %)。

AM 吸入後に心肺運動負荷試験を行った結果、AM 吸入はコントロールと比較して肺高血圧患者の最大酸素消費量 peakVO2 を有意に増加させた(15.7±0.6 vs. 14.6±0.6, p<0.05)。また AM 吸入はΔVO2/ΔWR ratio を有意に増加させた。これらの結果より、AM は肺高血圧患者の運動耐容能を改善させることが明らかとなった。



D. 考察

これまでの動物実験から、AMは強力な肺血管拡張作用を有することが明らかとなってきた2)。またAMは血管保護作用(血管内皮細胞のアポトーシス抑制、平滑筋細胞の遊走や増殖の抑制)を持つことが報告されている 5)。肺高血圧患者では内因性AMの産生が亢進することが知られているが、今回我々はさらに体外よりAMを補充することで、肺高血圧改善効果が得られることを証明した。またAM投与により血漿cAMP濃度が上昇した。血管内皮細胞、血管平滑筋細胞にはAMの特異的受容体が多数存在する。AMは血管平滑筋細胞の受容体に直接作用し、アデニル酸シクラーゼを活性化し細胞内 cAMP を増加させることで血管拡張に働く。一方、AM は血管内皮細胞の受容体に結合し一酸化窒素(NO)合成を促進させる。こうして AM は血管平滑筋に直接作用して、また血管内皮に働き NO を介して血管拡張をきたすと考えられる。AMの強力な血管拡張作用は、このような二つの経路が重なり発揮されると考えられる。

AM の投与方法の検討では、静脈内投与は肺血管抵抗を低下させるも体血圧の低下を伴うこと、また持続点滴が必要であるという欠点が存在する。我々は、これらの欠点を補うべく、AM の吸入療法を開発した。この投与法は、体血圧に影響を与えずに肺血管抵抗を低下させた。また吸入システムにはジェットネブライザーを用いており、取り扱いが容易であり自宅での繰り返し投与が可能である。また我々は、1日4回のAM吸入投与が肺高血圧ラットの生命予後を改善させることを証明した。これらの結果は、AM 吸入の肺高血圧治療法としての可能性を示唆するものである。今後は症例を重ね、肺高血圧に対する慢性治療効果を検討していく必要がある。

E. 結論

AM 静脈内投与では、体血圧の低下を伴うこと、また持続点滴が必要であるという欠点が存在する。我々は、これらの欠点を補うべく、ジェットネブライザーを用いた AM の吸入療法を開発した。この投与法は、体血圧に影響を与えずに肺血管抵抗を低下させ、運動耐容能を改善させた。安全性と治療効果はさらに症例を増やして検討していく必要があると考えられた。

F. 健康危険情報

なし

G. 研究発表

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1. 特許取得
特願2005-036419
特願2005-062951
2. 実用新案登録
なし
3. その他
なし

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

Hiroyuki Okumura, MD; Noritoshi Nagaya, MD; Takefumi Itoh, MD; Ichiro Okano, PhD; Jun Hino, PhD; Kenji Mori, PhD; Yoshitane Tsukamoto, MD; Hatsue Ishibashi-Ueda, MD; Senri Miwa, MD; Keiichi Tambara, MD; Shinya Toyokuni, MD; Chikao Yutani, MD; Kenji Kangawa, PhD

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 μg/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 μg/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-

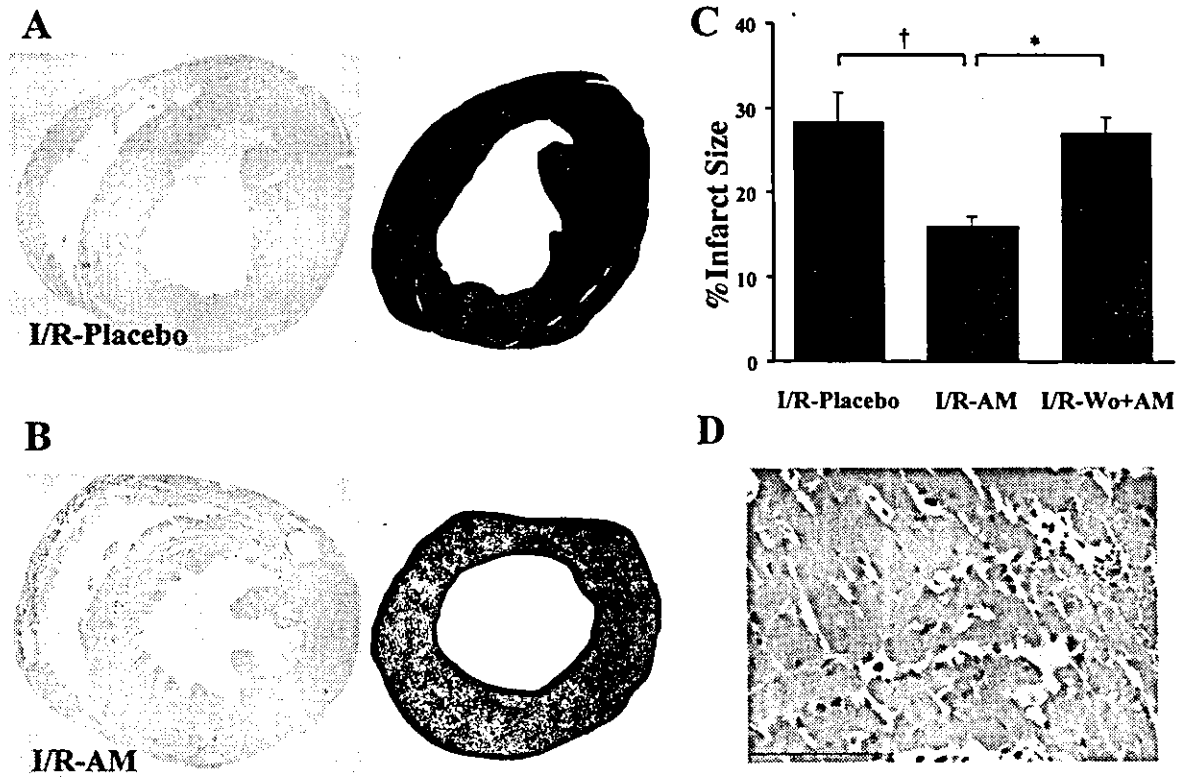

Figure 1. Effect of AM on myocardial infarct size 24 hours after ischemia/reperfusion. A and B, Photomicrographs show representative myocardial sections stained with Masson trichrome in I/R-placebo (A) and I/R-AM groups (B). Light red area indicates coagulation necrosis (right). C, Quantitative analysis 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%

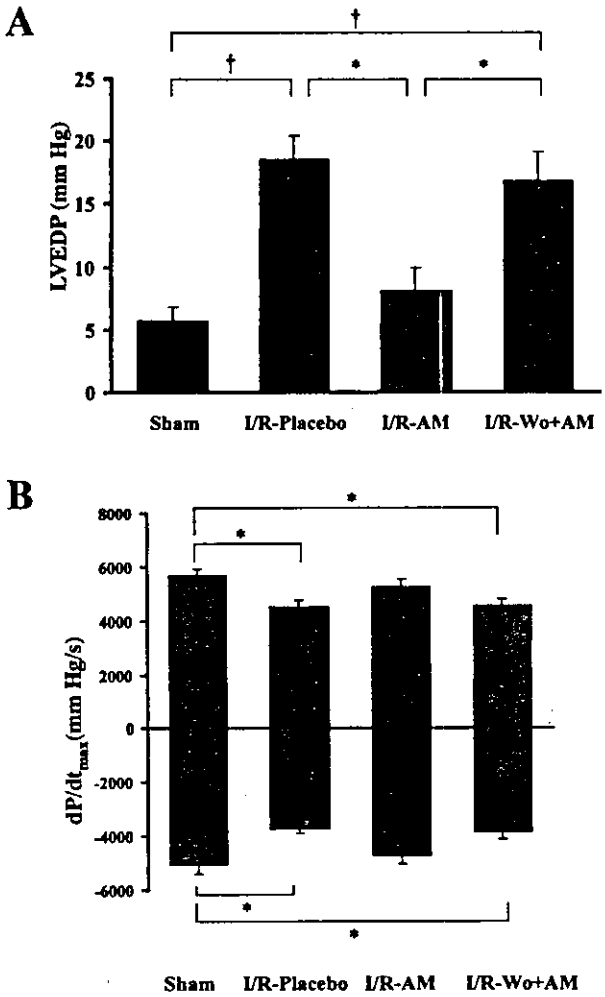

Figure 2. Effects of AM on LVEDP (A) and LV dP/dt (B) 24 hours after ischemia/reperfusion. AM infusion significantly inhibited increase in LVEDP compared with placebo infusion. AM infusion also improved LV dP/dt 24 hours after ischemia/reperfusion. Pretreatment with wortmannin attenuated effects of AM 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.

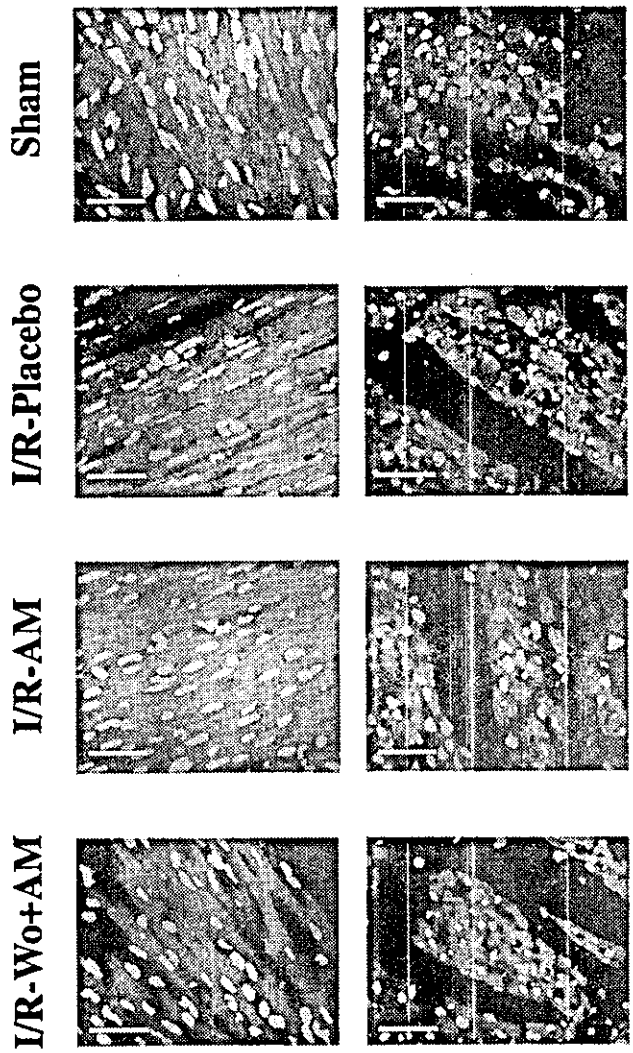

Figure 3. Representative photomicrographs of immunofluorescent staining for TUNEL-positive nuclei in sham, I/R-placebo, I/R-AM, and I/R-Wo+AM groups. Each left panel shows longitudinal myocytes, and each right panel shows short-axial myocytes. Yellow nuclei with red-stained myofilaments indicate TUNEL-positive 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.

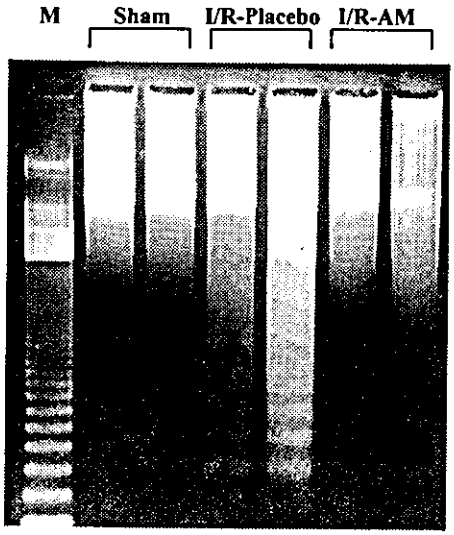

Figure 4. DNA ladder in sham, I/R-placebo, and I/R-AM groups. Although typical DNA ladder indicating fragmented DNA in cardiomyocytes was observed in I/R-placebo group, it was attenuated in I/R-AM group. M indicates molecular marker.

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.

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

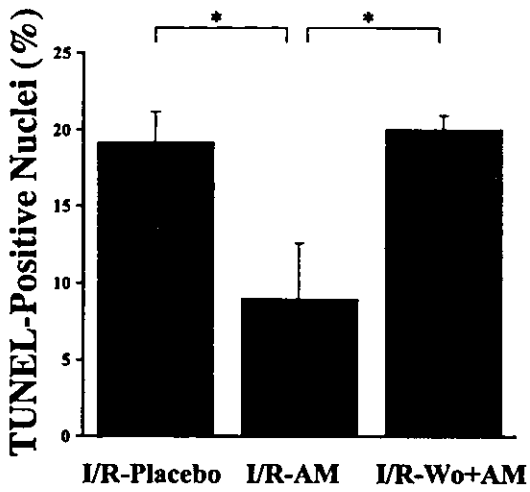

Figure 5. Quantitative analysis of TUNEL-positive nuclei in myocytes. Number of TUNEL-positive myocytes was lower in I/R-AM group than in I/R-placebo group. However, number of TUNEL-positive myocytes in I/R-Wo+AM group was as large as in I/R-placebo group. Data are mean±SEM. *$P<0.05$.

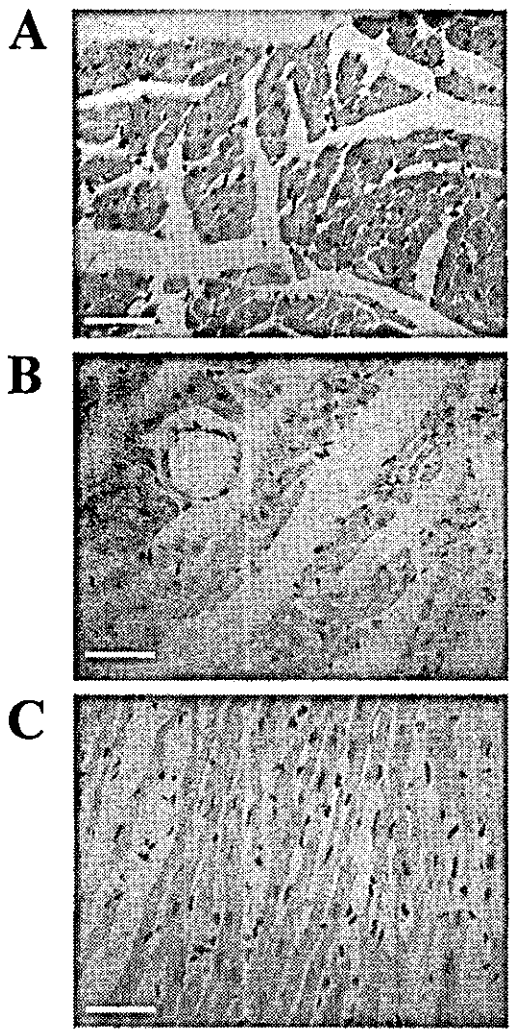

Figure 6. Immunohistochemistry for CRLR in rat cardiac tissue. Representative photomicrographs revealed that CRLR was localized in cardiomyocytes (A) and vascular endothelial cells (B). Negative control study (using mouse IgG) showed no positive staining in cardiac tissue (C). Original magnification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

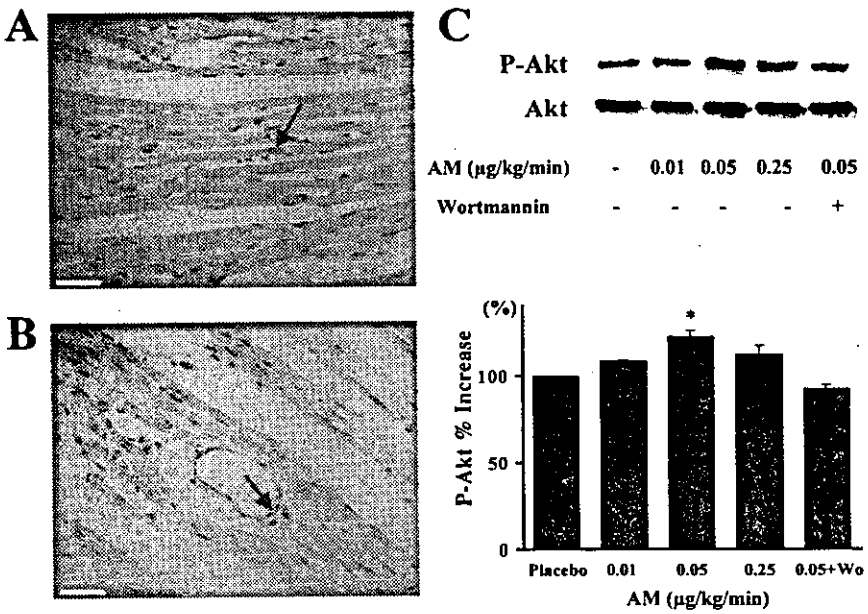

Figure 7. A and B, Immunohistochemistry for Akt phosphorylation in rat cardiac tissue. Infusion of AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$) phosphorylated Akt predominantly in nuclei of cardiomyocytes (A, B) and vascular endothelial cells (B). Arrow indicates nuclei of cardiomyocytes with positive 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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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 · kg^{-1} · 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 · min^{-1} · 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	
Sao_2, %	94±3
Svo_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; Sao_2, arterial oxygen pressure; Svo_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,

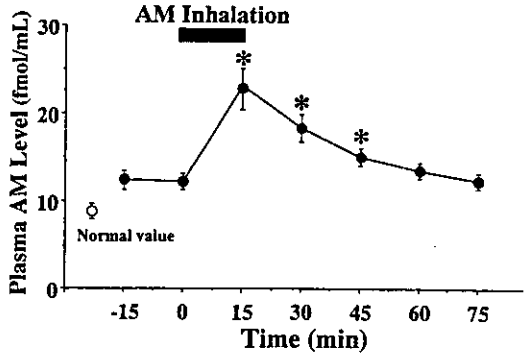

Figure 1. Changes in plasma AM level by inhalation of aerosolized AM in patients with idiopathic pulmonary arterial hypertension. Normal value indicates plasma AM level derived from 15 age-matched healthy subjects. Data are mean±SEM. *P<0.05 vs value at time 0.

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 · min^{-1} · 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.

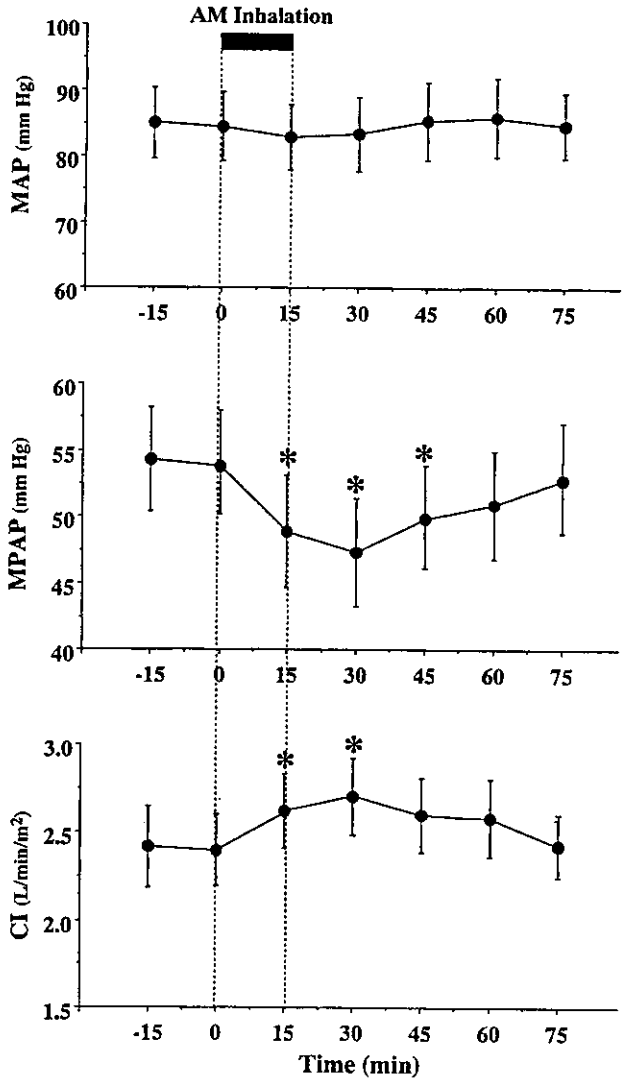

Figure 2. Changes in mean arterial pressure (MAP), mean pulmonary arterial pressure (MPAP), and cardiac index (CI) by inhalation of aerosolized AM in patients with idiopathic pulmonary arterial hypertension. Data are mean±SEM. *P<0.05 vs value at time 0.

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 · kg^{-1} · 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