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

アドレノメデュリンを用いた循環器疾患の画期的治療法の開発

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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）
総合研究報告書

アドレノメデュリンを用いた循環器疾患の画期的治療法の開発

主任研究者　宮武邦夫　大阪南医療センター　病院長

研究要旨

アドレノメデュリン（AM）による心筋および脳の保護効果を病態モデルや遺伝子改変動物を用いて明らかにした。また、急性心筋梗塞患者に対する臨床試験を行い、安全性と有効性を確認した。特にAM投与量を変えて検討した結果、低容量では血圧の著明な低下をきたすことなく12時間投与できることが分った。以上の結果より、AMは虚血性心疾患を含む難治性循環器疾患に対する我が国独自の画期的治療薬となる可能性が示唆された。

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A. 研究目的

医療技術が進歩した現在においても虚血性心疾患、脳虚血疾患は常に死因の上位を占め、また高額な医療費の原因にもなっている。アドレノメデュリン（AM）は1993年、研究分担者の寒川らが発見した内因性循環調節ペプチドである。近年、AMにはさまざまな心血管保護効果があることが報告された。我々は3年間の研究期間の前半には動物実験主体に研究を行い、後半には急性心筋梗塞や脳梗塞に対するAMの治療薬としての可能性を検討するために、実際に急性心筋梗塞患者や脳梗塞患者にAMを投与して安全性を検討した。また、探索的研究として、AMの血管再生医療における有用性について、基礎的検討を行った。本研究の目的は、AMによる心筋および脳の保護効果を病態モデルや遺伝子改変動物を用いて明らかにし、また急性心筋梗塞、心不全、脳梗塞、肺高血圧に対してAMを治療薬として臨床応用し、AM投与による新

たな循環器治療法の開発を行なうことであった。

B. 研究方法

まず動物実験により、AMによる虚血心筋保護作用とメカニズムを検討した。ラット心筋梗塞、高血圧性心肥大、急性心筋炎の各モデルを作製し、AMの投与を行った。また、脳虚血治療効果を検証する動物モデルとしてAM単独過剰発現トランスジェニックマウスを開発し、脳虚血に対するAMの効果を検討した。

動物実験の後に、急性心筋梗塞症患者や脳梗塞患者に対してパイロット臨床試験を行った。初回急性心筋梗塞症患者を対象にAMを虚血再灌流時に投与し、AMによる虚血心筋保護効果と投与の安全性を検討した。低容量と高容量を12時間投与し、心筋保護効果と安全性を検討した。

脳梗塞に対する臨床試験としては、軽症の脳梗塞既往者を対象に、AMの27時間持続投与を行い、脳血流、代謝を含む血行動態、各種液性因子の変化を検討した。その他、原発性肺高血圧症患者を対象とし、ネブライザーを用いたAM吸入投与の安全性と有効性(肺高血圧軽減効果)を検討した。また、原発性アルドステロン症による高血圧患者にAMを投与し、アルドステロン抑制効果を検討した。

(倫理面への配慮)

動物操作にあたっては、各施設の動物実験指針に従って行う。臨床応用は国立循環器病センターの倫理委員会承認のもとで行う。

C. 研究結果

アドレノメデュリンによる虚血心筋保護作用の検討:

ラットを用いて短時間のAM投与による虚血再灌流障害の抑制効果とそのメカニズムを検討した。急性心筋梗塞再灌流時にAMを1時間投与したところ心筋細胞のアポトーシスが抑制され、また梗塞サイズが約半分に縮小した(図1)。このAMの抗アポトーシス効果はPI3K/Aktを介することが示された。

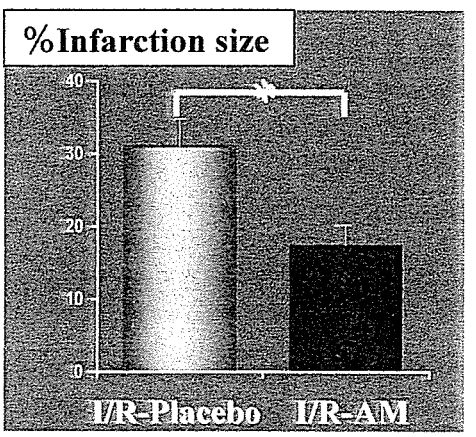
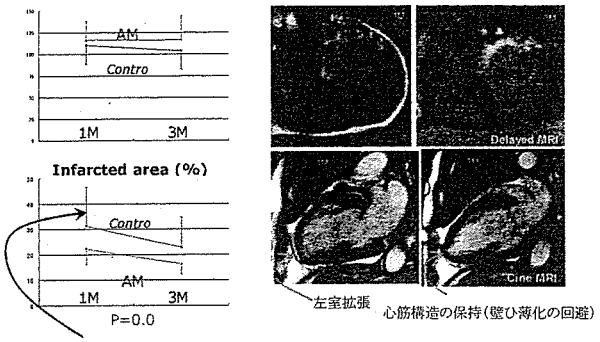


図1. アドレノメデュリンによる
梗塞サイズの縮小が認められた

動物実験の結果を踏まえ、国立循環器病センター倫理委員会承認のもと臨床試験を行った。12例の急性心筋梗塞患者にAMを投与し、静脈内投与の安全性を確認した。先行の急性心筋梗塞6例に対しては再灌流療法に先行してアドレノメデュリン(0.025 μ g/kg/min)の静脈内投与を開始以後12時間継続した。血圧が低下

する症例があったため、次の5例は 0.025 μg/kg/min で3時間投与した後に半量の 0.0125 μg/kg/min で 9 時間投与した。

図2. MRI を用いた梗塞サイズ、LVmass



これにより過度の低血圧を生じることはなくなった。ただし、11例目の症例が急性心筋梗塞に伴う左室自由壁破裂のために死亡した。安全評価委員会において審議したが、AM 投与との明らかな因果関係は認められないと結論された。
核磁気共鳴装置(MRI)による解析では梗塞サイズの縮小が認められた(図2)。

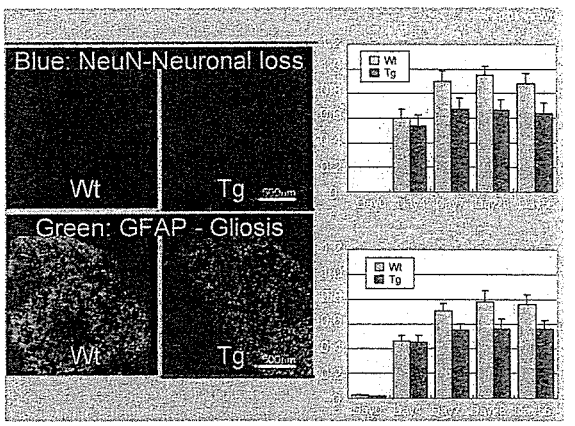
アドレノメデュリンによる心不全治療効果の検討:

ラットにアンジオテンシンIIを投与し高血圧性心不全のモデルを作製した。高血圧に伴い、左心室内の冠動脈周囲の線維芽細胞数は増加したが、14 日間の AM の同時皮下投与により、収縮期血圧に非依存的にそれらは有意に減少した。左室内 TGF-β1 と type 1 collagen 遺伝子発現は減少した。また、ラット急性心筋炎モデルにおいて心筋炎急性期に AM を持続投与すると心筋組織の炎症細胞浸潤・浮腫等が抑制され、心機能が改善した。以上より AM の高血圧性心血管リモデリング効果、心筋炎抑制効果が明らかとなった。

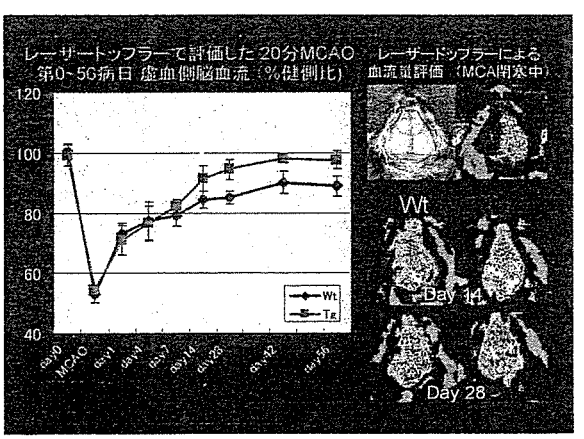
アドレノメデュリンによる脳虚血保護作用の検討:

AM の慢性投与による脳虚血治療効果を検証する動物モデルとして AM 単独過剰発現トランスジェニックマウスを開発。同マウスにおいて中大脳動脈20分閉塞脳梗塞モデルを作成し、梗塞域、グリオーシス、アポトーシス、白血球浸潤、血管再生を評価し、脳虚血に対する AM の効果を検討した。その結果、AM が血管再生促

AMTg マウスの脳梗塞域の減少効果



AMTg マウスの脳梗塞域の血流改善効果



進作用、内皮前駆細胞動員促進作用、炎症細胞浸潤抑制作用、ニューロンのアポトーシス抑制と再生促進作用を有していることを証明した。

軽症の脳梗塞既往者にAMの27時間持続投与を行ったところ、AM 投与により収縮期血圧は約 30 mmHg 低下し、心拍数は有意に上昇した。しかし副作用はみられず、投与中止 15 時間後には基礎値に復帰した。また、血圧低下にもかかわらず、脳血流への影響は軽微であり、脳血管障害患者への臨床応用の安全性が示された。

アドレノメデユリンによる血管再生効果の検討:

In vitro においてヒトES細胞由来血管前駆細胞や脂肪由来幹細胞に AM＋VEGF を添加すると、これらの細胞は内皮系への分化した。こうして得た血管前駆細胞は強力な血管再生能を in vitro のみでなく、in vivo で示した。また、AM-Tg マウス下肢虚血モデルに血管前駆細胞移植を施行し、AM の細胞移植増強効果を検討した。AM は血管前駆細胞による血管再生効果を増強することを確認した。

アドレノメデユリンによる肺血管保護作用の検討:

AM は強力な肺血管拡張作用を有し肺高血圧治療に有効と考えられるが、静脈内投与では体血圧の低下を伴うこと、持続点滴が必要等の欠点がある。そこで、原発性肺高血圧症患者11人を対象とし、ジェットネブライザーを用いた AM の吸入による治療効果を検討した。AM を15分間行い血行動態、血中 AM 濃度、心肺運動負荷試験を施行し AM の安全性と有効性(肺高血圧軽減効果と運動耐容能改善効果)を検討した。その結果AMが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ることを証明した。ジェットネブライザーを用いた吸入システムは取り扱いが容易であり患者自身による自宅での繰り返し投与が可能である。

D. 考察

当初、計画していた動物実験はすべてが完了し、AM の心血管脳保護作用とそのメカニズムが明らかとなった。特に、AMがアポトーシス抑制作用、血管再生促進作用、内皮前駆細胞動員促進作用、炎症細胞浸潤抑制作用、ニューロンのアポトーシス抑制と再生促進作用を有しているが、心血管脳保護に働いている可能性が示唆された。

臨床試験は急性心筋梗塞症に対してパイロット臨床試験を完了するにとどまったが、低容量と高容量の比較試験を行い、低容量の投与がより安全であることが示された。医療技術が進歩した現在においても虚血性心疾患、脳虚血疾患は常に死因の上位を占め、また高額な医療費の原因にもなっている。AM はこれらの疾患の治療に有効である可能性が示された。本

ペプチドは日本で発見されたこともあり、虚血性心疾患、脳虚血疾患に対する創薬が行える可能性がある。

E. 結論

AM は虚血性心疾患を含む難治性循環器疾患に対する我が国独自の画期的治療薬となる可能性が示唆された。

F. 研究発表

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G．知的所有権の取得状況

1．特許取得

特願 2005-036419
特願 2005-062951
特願 2005-117588

「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
「非細菌性の炎症性疾患の予防又は治療剤」
特願 2005-111889（出願日：平成 17 年 4 月 8 日）
「心筋障害の予防又は治療剤」
特願 2005-114786（出願日：平成 17 年 4 月 12 日）
「アドレノメデュリン前駆体 C 端末ペプチドの濃度の上昇を指標として循環器疾患又は炎症性疾患を診断する方法」
特願 2006-148348（出願日：2006 年 5 月 29 日）

2．実用新案登録
なし

3．その他
なし

研究成果の刊行に関する一覧表（16年度）

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Miyashita K, Itoh H, Arai H, Suganami T, Sawada N, Fukunaga Y, Sone M, Yamahara K, Yurugi-Kobayashi T, Park K, Oyamada N, Sawada N, Taura D, Tsujimoto H, Chao TH, Tamura N, Mukoyama M, Nakao K.	The neuroprotective and vasculo-neuro-regenerative roles of adrenomedullin in ischemic brain and its therapeutic potential.	Endocrinology	147	1642-53	2006
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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-

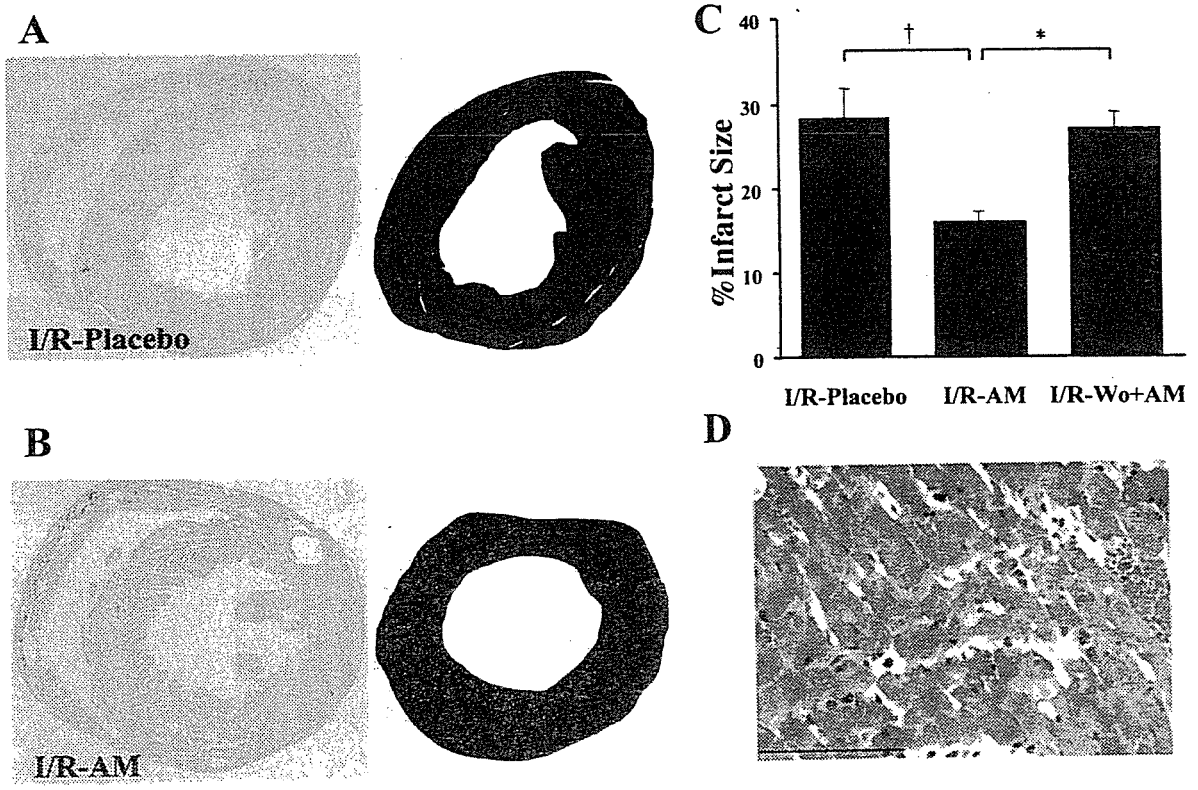

Figure 1. Effect of AM on myocardial infarct size 24 hours after ischemia/reperfusion. A and B, Photomicrographs show representative myocardial sections stained with Masson trichrome in I/R-placebo (A) and I/R-AM groups (B). Light red area indicates coagulation necrosis (right). C, Quantitative analysis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

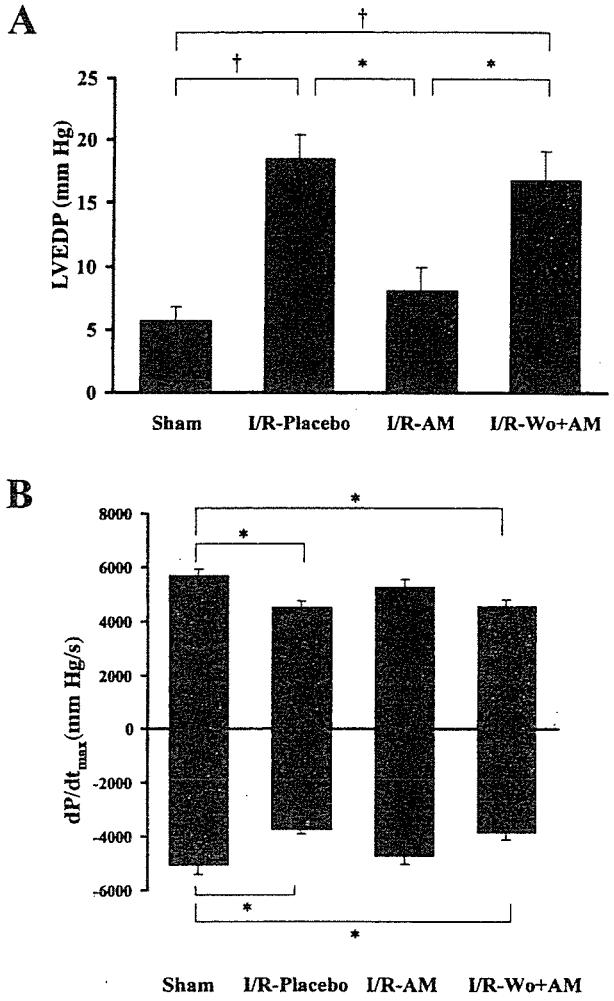

Figure 2. Effects of AM on LVEDP (A) and LV dP/dt (B) 24 hours after ischemia/reperfusion. AM infusion significantly inhibited increase in LVEDP compared with placebo infusion. AM infusion also improved LV dP/dt 24 hours after ischemia/reperfusion. Pretreatment with wortmannin attenuated effects of AM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

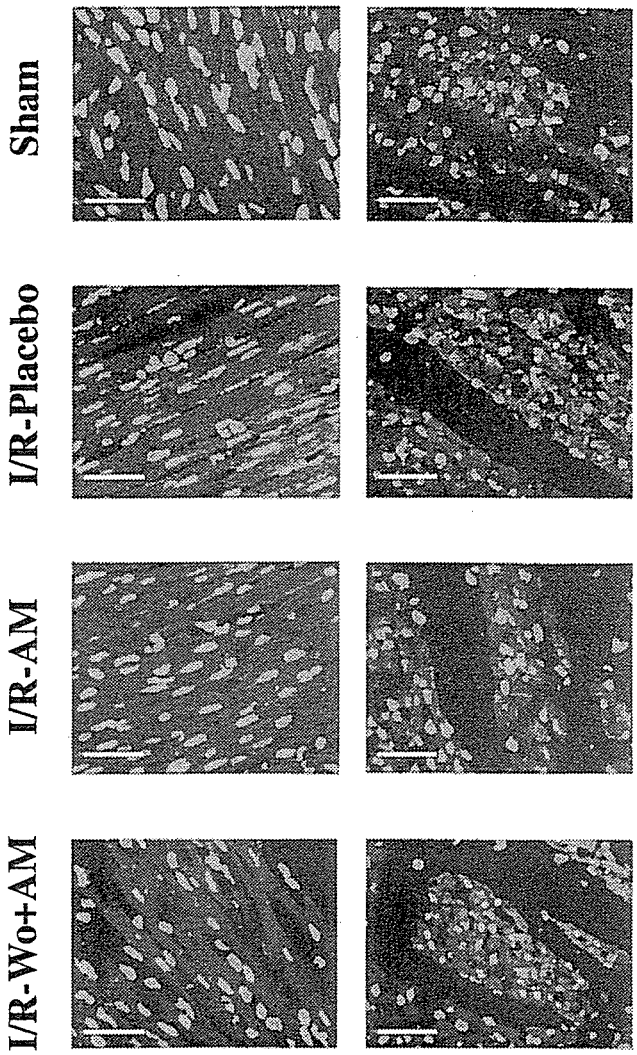

Figure 3. Representative photomicrographs of immunofluorescent staining for TUNEL-positive nuclei in sham, I/R-placebo, I/R-AM, and I/R-Wo+AM groups. Each left panel shows longitudinal myocytes, and each right panel shows short-axial myocytes. Yellow nuclei with red-stained myofilaments indicate TUNEL-positive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