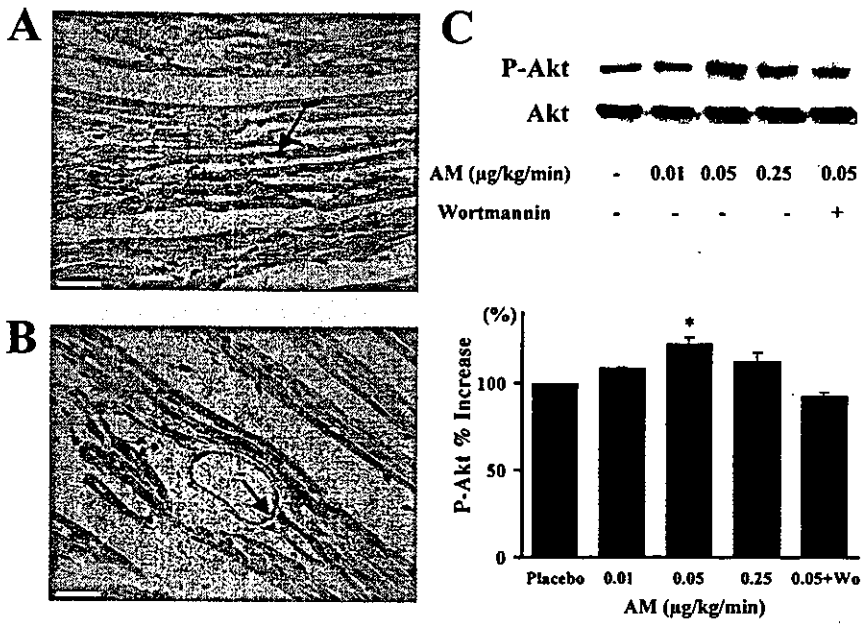

Figure 7. A and B, Immunohistochemistry for Akt phosphorylation in rat cardiac tissue. Infusion of AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$) phosphorylated Akt predominantly in nuclei of cardiomyocytes (A, B) and vascular endothelial cells (B). Arrow indicates nuclei of cardiomyocytes with positive 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 Ghrelin Administration on Left Ventricular Function, Exercise Capacity, and Muscle Wasting in Patients With Chronic Heart Failure

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Background—Ghrelin is a novel growth hormone–releasing peptide that also induces vasodilation, inhibits sympathetic nerve activity, and stimulates feeding through growth hormone–independent mechanisms. We investigated the effects of ghrelin on left ventricular (LV) function, exercise capacity, and muscle wasting in patients with chronic heart failure (CHF).

Methods and Results—Human synthetic ghrelin (2 μg/kg twice a day) was intravenously administered to 10 patients with CHF for 3 weeks. Echocardiography, cardiopulmonary exercise testing, dual x-ray absorptiometry, and blood sampling were performed before and after ghrelin therapy. A single administration of ghrelin elicited a marked increase in serum GH (25-fold). Three-week administration of ghrelin resulted in a significant decrease in plasma norepinephrine (1132±188 to 655±134 pg/mL; $P<0.001$). Ghrelin increased LV ejection fraction (27±2% to 31±2%; $P<0.05$) in association with an increase in LV mass and a decrease in LV end-systolic volume. Treatment with ghrelin increased peak workload and peak oxygen consumption during exercise. Ghrelin improved muscle wasting, as indicated by increases in muscle strength and lean body mass. These parameters remained unchanged in 8 patients with CHF who did not receive ghrelin therapy.

Conclusions—These preliminary results suggest that repeated administration of ghrelin improves LV function, exercise capacity, and muscle wasting in patients with CHF. (***Circulation.*** **2004;110:3674-3679.)**

Key Words: growth substances ■ heart failure ■ hormones ■ nutrition

Left ventricular (LV) remodeling (dilatation and wall thinning) and cardiac cachexia (body weight loss and muscle wasting) often are observed in patients with end-stage chronic heart failure (CHF).[1,2] Growth hormone (GH) and its mediator, insulinlike growth factor-1 (IGF-1), are anabolic hormones that are essential for skeletal and myocardial growth and metabolic homeostasis.[3,4] Earlier studies have shown that GH supplementation may have beneficial effects on LV myocardial structure and function in some patients with CHF,[5] although the importance of GH resistance[6] and neutral results of randomized trials also have been reported.[7,8]

Ghrelin is a novel GH-releasing peptide that was isolated from the stomach and has been identified as an endogenous ligand for the growth hormone secretagogue receptor.[9] Therefore, we believed that administration of ghrelin may induce beneficial changes in LV function and energy metabolism in patients with CHF via a GH-dependent mechanism. On the other hand, growth hormone secretagogue receptor mRNA is detected not only in the hypothalamus and pituitary but also in the heart and blood vessels,[10] implying direct cardiovascular effects of ghrelin. Wiley and Davenport[11] have demonstrated that ghrelin is an endothelium-independent vasodilator in isolated human arteries. We have shown that intravenous administration of ghrelin decreases systemic vascular resistance and increases cardiac output in patients with CHF.[12] Furthermore, ghrelin induces a positive energy balance by stimulating food intake[13,14] and adiposity[15] through GH-independent mechanisms. These findings raise the possibility that ghrelin administration may have beneficial effects in cachectic patients with CHF. In fact, we recently have demonstrated that treatment with ghrelin improves not only LV function but also cardiac cachexia in rats with CHF.[16] In humans, however, the potential effects of ghrelin as a therapeutic agent for CHF remain unknown.

Thus, the purposes of this study were as follows: (1) to investigate whether repeated administration of ghrelin im-

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TABLE 1. Patient Characteristics

	Control Group (n=8)	Ghrelin Group (n=10)
Age, y	74±2	75±2
Sex, M/F	6/2	7/3
Body mass index, kg/m²	19.0±1.1	19.0±0.9
Cause of CHF, n		
Dilated cardiomyopathy	4	4
Ischemic cardiomyopathy	1	3
Hypertensive heart disease	2	1
Valvular heart disease	1	2
NYHA functional class, n		
III	8	9
IV	0	1
LVEF, %	28±2	27±2
Presence of cardiac cachexia, n	6	8
Medication use, n		
Digoxin	6	9
ACE inhibitors	7	9
A II blockers	2	2
β-Blockers	6	7
Diuretics	7	10

LVEF indicates LV ejection fraction; A II, angiotensin II. Data are mean±SEM.

proves LV myocardial structure and function in patients with CHF, (2) to examine whether ghrelin improves exercise capacity in such patients, and (3) to examine whether ghrelin induces anabolic effects in patients with CHF.

Methods

Study Subjects

Eighteen patients with CHF (13 men, 5 women; mean age, 75 years; range, 63 to 80 years) were included in this study. Inclusion criteria were as follows: (1) LV ejection fraction <35% as assessed by cardiac catheterization, (2) a stable clinical condition, and (3) clinical evidence of heart failure despite conventional therapy. Exclusion criteria were the presence of any of the following: (1) chronic renal impairment (serum creatinine level ≥2.0 mg/dL), (2) significant liver dysfunction, (3) evidence of malignant diseases, (4) active infection, (5) hematologic abnormalities, or (6) systolic blood pressure <90 mm Hg. Ten patients with CHF (ghrelin group) received repeated administrations of ghrelin. Although this study was neither randomized nor placebo controlled, 8 patients with CHF who did not receive ghrelin (control group) were studied to exclude time-course effects during hospitalization. Patients in the ghrelin group were admitted only for the study. Those in the control group had been in hospital for diagnostic examination and stayed for 3 weeks for the study. There was no significant difference in demographic, clinical, or hemodynamic data at baseline between the ghrelin and control groups (Table 1). Eight patients in the ghrelin group and 6 patients in the control group were defined as exhibiting cardiac cachexia, as reported previously.[17] The weight loss in cachectic patients amounted to 6.4±0.4 kg or 11.8±0.7% loss of previous body weight during 14±2 months. The ethics committee of the National Cardiovascular Center approved the study, and all patients gave written informed consent.

Preparation of Human Ghrelin

Human synthetic ghrelin was obtained from the Peptide Institute Inc. This peptide is not commercially available. Ghrelin was dissolved in distilled water with 4% D-mannitol and sterilized by passage through a 0.22-μm filter (Millipore Co). Ghrelin was stored in 2-mL volumes, each containing 200 μg ghrelin. The chemical nature and content of the human ghrelin 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.

Study Protocol

This study was performed while patients were in a stable clinical condition during hospitalization. Ghrelin (2 μg/kg, 10 mL solution) was administered intravenously over 30 minutes at a constant rate. The infusion was repeated twice a day (before breakfast and before dinner) for 3 weeks. Study patients in both groups remained hospitalized for 3 weeks. Echocardiography, cardiopulmonary exercise testing, dual x-ray absorptiometry, hand-grip test, and blood sampling were performed at baseline and after 3 weeks of treatment with ghrelin (ghrelin group) or without ghrelin (control group). Long-term medication, including digitalis, diuretics, ACE inhibitors, and β-blockers, was kept constant during this study protocol.

Echocardiographic Studies

Echocardiography was performed by an investigator blinded to treatment allocation. Two-dimensional targeted M-mode tracings were obtained at the level of the papillary muscles with an echocardiographic system equipped with a 3.5-MHz sector scan probe (SONOS 2000, Hewlett Packard). LV wall thickness, dimensions, and fractional shortening were measured according to the recommendations of the American Society of Echocardiology from at least 3 consecutive cardiac cycles. LV end-diastolic volume, end-systolic volume, and ejection fraction were calculated with a modified version of Simpson's method.[18]

Cardiopulmonary Exercise Testing

Cardiopulmonary exercise testing was performed in all patients except 1, who underwent a 6-minute walk test as recommended by attending physicians. The patients exercised seated on a cycle ergometer. The work rate was then increased by 15 W/min up to their symptom-limited maximum. Breath-by-breath gas analysis was performed with an AE280 (Minato Medical Science).[19] Exercise capacity was evaluated by peak oxygen consumption (peak $\dot{V}O$). Ventilatory efficiency during exercise was represented by the $\dot{V}E$–$\dot{V}CO_2$ slope.[19]

Food Intake and Body Mass Analyses

Food intake for 3 consecutive days was assessed before ghrelin administration and during the last week of ghrelin therapy. Food intake was semiquantitatively assessed by a calorie count based on a 10-point scale method (0=null intake, 10=full intake or 1800 kcal), which was averaged for 3 days. Dual x-ray absorptiometry (DPX-L, Lunar Radiation) was repeated in all patients to examine changes in lean body mass, fat mass, and bone mineral content. Hand-grip strength was determined with a dynamometer.

Blood Sampling and Assay

Blood samples were taken from the antecubital vein the morning after an overnight fast. Serum GH and IGF-1 were measured by immunoradiometric assay (Ab Bead HGH Eiken, Eiken Chemical Co, Ltd, sensitivity=0.1 ng/mL; Somatomedin CII Bayer, Bayer Medical Ltd, sensitivity=0.3 ng/mL). Plasma norepinephrine and epinephrine were measured by high-performance liquid chromatography (HLC8030, Tosoh Co, sensitivity=6 pg/mL). Serum cortisol and insulin were measured by enzyme immunoassay (AIA-PACK CORT, sensitivity=0.2 μg/dL; AIA-PACK IRI, sensitivity=2.0 μU/mL, Tosoh Co). Serum tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) were measured by enzyme immunoassay (Quantikine HS, R&D Systems Inc, sensitivity=0.18 pg/mL; TFB kit, TFB Co, Ltd, sensitivity=0.3 pg/mL). Plasma renin and aldosterone were measured with radioimmunoassay kits (RENIN RIABEAD, sensitivity=0.1 ng/mL; ALDOSTERONE RIAKIT II, sensitivity=2.0

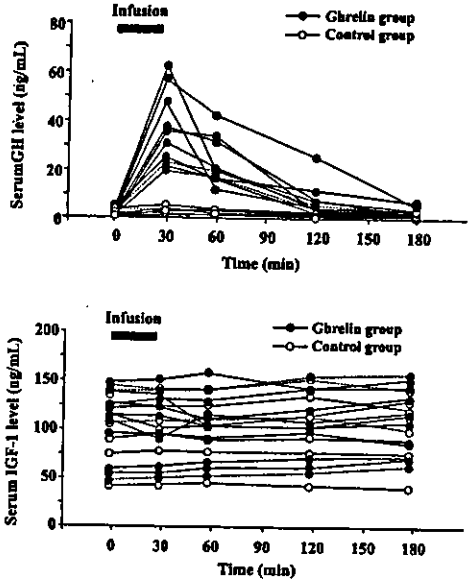

Figure 1. Changes in serum GH and IGF-1 after single administration of ghrelin. Solid line indicates cachectic patients; dotted line, noncachectic patients.

ng/dL, DAINABOT Co). Plasma brain natriuretic peptide (BNP) was measured by immunoradiometric assay (SHIONORIA BNP, sensitivity=4.0 pg/mL).

Statistical Analysis

Numerical values are expressed as mean±SEM. Comparisons of parameters between the 2 groups were made by unpaired Student's *t* test. Comparisons of the time course of serum GH and IGF-1 between the 2 groups were made by 2-way ANOVA for repeated measures, followed by the Newman-Keuls test. Comparisons of changes in parameters during the 3-week follow-up between the 2 groups were also made by 2-way ANOVA for repeated measures, followed by the Newman-Keuls test. A value of $P<0.05$ was considered significant.

Results

Administration of ghrelin transiently caused stomach rumbles in 6 patients and a slight feeling of being warm and sleepy in 4 subjects. Two patients felt slightly thirsty during ghrelin infusion. Other than these minor complaints, all subjects tolerated 3-week administration of ghrelin without incident. After 3-week administration of ghrelin, NYHA functional class improved in 4 patients and was unchanged in 6 patients. No change in NYHA functional class was observed in patients who did not receive ghrelin.

Effects of Ghrelin on Somatotropic Function

A single administration of ghrelin markedly increased serum GH level (baseline, 1.4±0.4; peak, 35.0±5.0 ng/mL; $P<0.001$; Figure 1). This elevation lasted >60 minutes after the start of ghrelin infusion. Serum IGF-1 level tended to increase 3 hours after the start of ghrelin infusion (101±12 to 110±12 ng/mL; $P=0.08$). Three-week administration of ghrelin tended to increase basal serum IGF-1 level (99±13 to 116±13 ng/mL; $P=0.07$). There was no significant difference in basal serum GH level between before and after 3 weeks of ghrelin therapy (2.0±0.8 to 1.2±0.3 ng/mL; P=NS).

Effects of Ghrelin on Food Intake, Body Weight, and Lean Body Mass

Administration of ghrelin significantly increased food intake (Figure 2). Three-week administration of ghrelin tended to increase body weight (49.6±2.7 to 50.4±2.7 kg; $P=0.09$). No development of edema was observed during ghrelin therapy. Dual x-ray absorptiometry demonstrated that treatment with ghrelin significantly increased lean body mass in patients with CHF (38.3±2.1 to 39.1±2.1 kg; $P<0.05$). Ghrelin did not significantly alter bone mineral content (2243±191 to 2265±189 g; P=NS) or fat mass (8877±1353 to 8748±1311 g; P=NS). Hand-grip strength was increased significantly by ghrelin therapy (20.5±1.7 to 22.7±2.0 kg; $P<0.01$). All of these parameters remained unchanged in patients who did not receive ghrelin.

Effects of Ghrelin on Cardiac Structure and Function

Neither heart rate nor blood pressure was significantly changed by 3-week administration of ghrelin (Table 2). Ghrelin increased LV ejection fraction (27±2% to 31±2%; $P<0.05$) in association with a decrease in LV end-systolic volume and an increase in LV mass (Figure 3), although ghrelin did not significantly alter LV end-diastolic volume. All of these parameters remained unchanged in patients who did not receive ghrelin.

Effects of Ghrelin on Exercise Capacity and Ventilatory Efficiency

Three-week administration of ghrelin significantly increased peak workload and peak $\dot{V}o$ during exercise (739±127 to 801±126 mL/min; $P<0.05$; Figure 4). Treatment with ghrelin did not significantly alter the $\dot{V}_E$–$\dot{V}co_2$ slope. In 1 patient

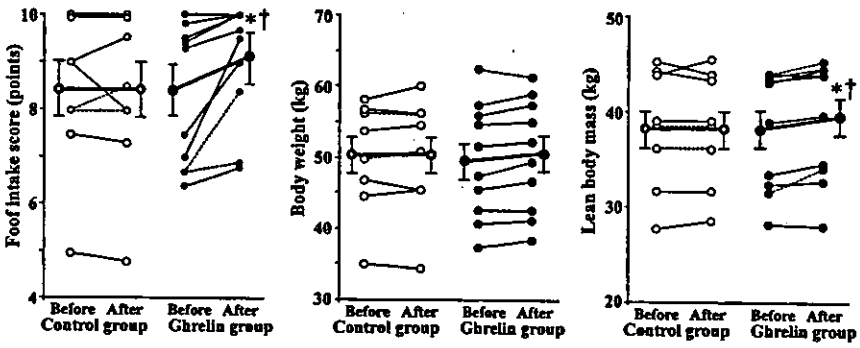

Figure 2. Food intake, body weight, and lean body mass before and after 3-week administration of ghrelin. Food intake was described semiquantitatively with 10-point scale method (0=null intake, 10=full intake). Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients. *$P<0.05$ vs before; †$P<0.05$ vs respective control group.

TABLE 2. Physiological and Echocardiographic Measurements

	Control Group	Ghrelin Group
Heart rate, bpm		
Before	77±3	78±3
After	76±3	74±3
Mean arterial pressure, mm Hg		
Before	79±4	81±2
After	80±3	78±3
LVDd, mm		
Before	65.6±3.2	66.6±2.5
After	64.4±3.7	63.7±3.3
LVDs, mm		
Before	55.1±3.0	56.9 ± 2.9
After	53.9±3.6	52.8 ± 3.4*
FS, %		
Before	16.1±1.2	14.8±1.7
After	16.0±1.3	17.3±2.3
AWT diastole, mm		
Before	10.0±0.8	9.5±1.0
After	10.1±0.9	10.0±1.0*
PWT diastole, mm		
Before	9.2±0.4	9.3±0.6
After	9.4±0.4	9.9±0.5*†

LVDd indicates LV end-diastolic dimension; LVDs, LV end-systolic dimension; FS, fractional shortening; AWT, anterior wall thickness; and PWT, posterior wall thickness. Data are mean±SEM.

*$P<0.05$ vs before; †$P<0.05$ vs respective control group.

who did not undergo cardiopulmonary exercise testing, the distance walked in 6 minutes increased from 300 m to 410 m with ghrelin treatment. Exercise parameters remained unchanged without ghrelin.

Effects of Ghrelin on Sympathetic Nerve Activity

Three-week administration of ghrelin significantly decreased plasma norepinephrine and epinephrine (Figure 5). Treatment with ghrelin significantly decreased plasma BNP level (Table 3). Ghrelin did not significantly alter circulating glucose, insulin, cortisol, TNF-α, or IL-6. Neither plasma renin activity nor plasma aldosterone level was changed significantly. All of these parameters remained unchanged in patients who did not receive ghrelin.

Discussion

Ghrelin is a novel GH-releasing peptide that acts through a mechanism independent of that of hypothalamic GH-releasing hormone.[9] The GH-releasing effect of ghrelin has been shown to be more potent than that of GH-releasing hormone.[20] In fact, in the present study, ghrelin infusion elicited potent GH release in patients with CHF. Three-week administration of ghrelin increased LV ejection fraction in association with an increase in LV mass, which is consistent with findings from a previous experimental study in rats.[16] Plasma BNP level, a marker for LV function and wall stress, was decreased by ghrelin therapy. GH and its mediator, IGF-1, have been shown to enhance physiological compensatory hypertrophy in rats with CHF, resulting in a decrease in LV wall stress, leading to improvement in cardiac function.[21] Thus, ghrelin may also improve cardiac function partly through GH-dependent mechanisms. On the other hand, Baldanzi et al[22] have shown that ghrelin inhibits apoptosis of cardiomyocytes and endothelial cells through activation of extracellular signal-regulated kinase-1/2 and Akt serine kinases. Furthermore, stimulation of GHS-R by hexarelin has been shown to prevent cardiac damage after ischemia-reperfusion in hypophysectomized rats.[23] When these results are considered together, improvement in cardiac function by ghrelin therapy may be related to direct effects of ghrelin on myocardium. Importantly, ghrelin significantly decreased plasma norepinephrine levels in the present study. It is possible that improvement in cardiac function may lead to attenuation of sympathetic nerve activity. Interestingly, a recent study has demonstrated that ghrelin acts directly on the central nerve system to decrease sympathetic nerve activity.[24] Thus, inhibitory effects of ghrelin on sympathetic nerve activity may contribute to a decrease in plasma norepinephrine, which may have beneficial effects on cardiac performance in patients with CHF.

In the present study, 3-week administration of ghrelin improved exercise capacity in patients with CHF, as indicated by an increase in peak workload and peak $\dot{V}o$. A decrease in peak $\dot{V}o$ in patients with CHF is attributable not only to an inadequate increase in cardiac output during exercise, which is a central effect, but also to muscle wasting, a peripheral effect. Recently, we have shown that infusion of ghrelin increases cardiac output in patients with CHF.[12] In the present study, ghrelin increased lean body mass and muscle strength. These results suggest that ghrelin may improve exercise capacity through both central and peripheral effects.

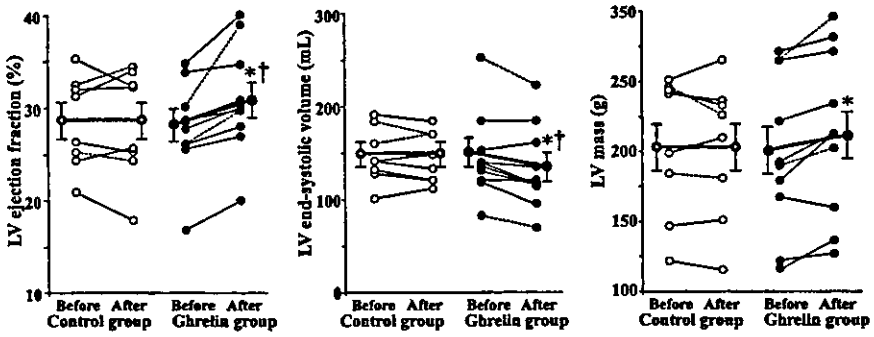

Figure 3. LV geometry and function before and after ghrelin therapy. Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients. *$P<0.05$ vs before; †$P<0.05$ vs respective control group.

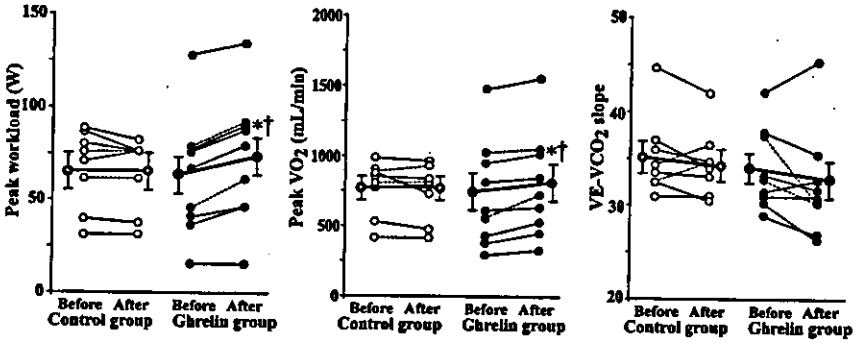

Figure 4. Exercise capacity and ventilatory efficiency before and after ghrelin therapy. Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients. *$P<0.05$ vs before; †$P<0.05$ vs respective control group.

Cardiac cachexia, a catabolic state characterized by weight loss and muscle wasting, occurs frequently in patients with end-stage CHF[25] and is a strong independent risk factor for mortality in such patients.[26] Recently, we have shown that plasma ghrelin level is increased in cachectic patients with CHF as a compensatory mechanism in response to anabolic-catabolic imbalance.[17] In the present study, 3-week administration of ghrelin tended to increase body weight and significantly increased lean body mass and muscle strength. These results suggest that treatment with ghrelin improves muscle wasting in patients with CHF. These effects may be mediated, at least in part, by GH/IGF-1, which is considered essential for skeletal muscle. Earlier studies have shown that ghrelin induces orexigenic effects via activation of neuropeptide Y neurons in the hypothalamic arcuate nucleus.[13,14] In the present study, intravenous administration of ghrelin increased food intake in patients with CHF, which may contribute to anabolic effects of ghrelin. Tschop et al[15] have shown that administration of ghrelin induces adiposity through a GH-independent mechanism. In the present study, however, ghrelin did not significantly increase fat mass. This difference may be explained by the high dose of ghrelin (>2000-fold) used by Tschop et al. Ghrelin itself decreases fat utilization and increases fat, whereas GH decreases fat tissue and increases lean tissue. Thus, in the present study, ghrelin-induced GH may have attenuated an increase in fat and enhanced an increase in lean tissue.

The major limitation of this pilot trial relates to the lack of a randomized, placebo-controlled group. Patients in the control group were not treated identically because a placebo infusion was not performed. Nonetheless, this study was performed while patients were in a stable clinical condition during hospitalization. In addition, 8 patients in the control group were studied to exclude time-course effects during hospitalization. On the basis of the results of this study, a double-blind, randomized, and placebo-controlled study should be conducted. Second, this clinical study did not clarify mechanisms of increased LV ejection fraction by ghrelin therapy. Further studies are necessary to examine which mechanism predominantly contributes to improvement in LV ejection fraction.

Except for a few minor complications, long-term treatment with ghrelin was tolerated well in patients with CHF. Although a preliminary study documented the beneficial effects

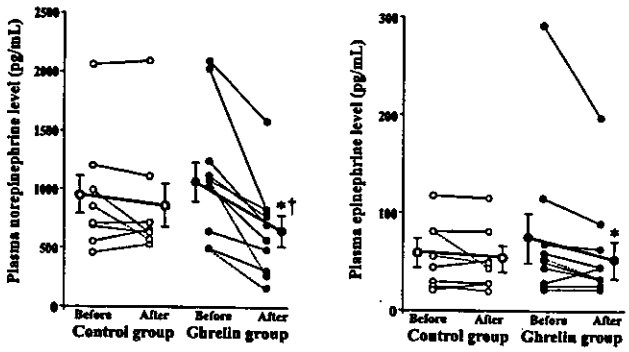

Figure 5. Plasma levels of norepinephrine and epinephrine before and after ghrelin therapy. Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients. *$P<0.05$ vs before; †$P<0.05$ vs respective control group.

TABLE 3. Hormone Analysis in Patients With CHF

	Control Group	Ghrelin Group
BNP, pg/mL		
Before	180±53	238±59
After	181±62	190±60*
Fasting glucose, mg/dL		
Before	105±5	101±4
After	102±6	102±7
Insulin, μU/mL		
Before	6.0±1.4	3.9±0.7
After	6.8±2.0	5.5±1.2
Cortisol, μg/dL		
Before	15.5±1.9	17.9±1.6
After	14.5±2.6	17.2±1.5
TNF-α, pg/mL		
Before	5.3±0.9	5.7±0.8
After	5.4±0.9	5.6±0.8
IL-6, pg/mL		
Before	3.2±0.5	3.8±0.7
After	3.4±0.5	3.6±0.7
Renin, $ng \cdot mL^{-1} \cdot h^{-1}$		
Before	9.3±4.6	7.3±3.0
After	10.1±4.1	6.9±3.7
Aldosterone, ng/dL		
Before	11.6±4.1	15.0±4.7
After	12.7±4.1	11.9±4.2

Data are mean±SEM.
*$P<0.05$ vs before.

of GH,[5] controlled studies in humans have been predominantly negative.[7,8] Nevertheless, ghrelin has been shown to have GH-independent effects, stimulating vasodilation,[10–12] reversing cachexia,[13–15] and inhibiting sympathetic nerve activity[24] and myocyte apoptosis.[22] Thus, ghrelin may have additional therapeutic potential compared with GH supplementation. Ghrelin improved cardiac function and exercise capacity in not only cachectic CHF patients but also noncachectic ones. Nevertheless, the best candidates may be cachectic CHF patients because ghrelin stimulates feeding and improves muscle wasting.

Conclusions

These preliminary results suggest that repeated administration of ghrelin improves LV structure and function, exercise capacity, and muscle wasting in patients with CHF. Thus, administration of ghrelin may be a new therapeutic approach for the treatment of CHF.

Acknowledgments

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Optimum Collection and Storage Conditions for Ghrelin Measurements: Octanoyl Modification of Ghrelin Is Rapidly Hydrolyzed to Desacyl Ghrelin in Blood Samples, *Hiroshi Hosoda,[1,3] Kentaro Doi,[1] Noritoshi Nagaya,[2] Hiroyuki Okumura,[2] Eiichiro Nakagawa,[2] Mitsunobu Enomoto,[2] Fumiaki Ono,[2] and Kenji Kangawa[1,3]** ([1] Department of Biochemistry, National Cardiovascular Center Research Institute, and [2] Department of Internal Medicine, National Cardiovascular Center, Osaka 565-8565, Japan; [3] Translational Research Center, Kyoto University Hospital, Kyoto 606-8507, Japan; * address correspondence to this author at: Department of Biochemistry, National Cardiovascular Center Research Institute, National Cardiovascular Center, Osaka 565-8565, Japan; fax 81-6-6835-5402, e-mail kangawa@ri.ncvc.go.jp)

Ghrelin is an acylated peptide with growth-hormone-releasing activity *(1)*. It was first isolated from rat and human stomach during the search for an endogenous ligand to the "orphan" G-protein-coupled receptor, growth hormone secretagogue receptor *(2)*. The peptide contains 28 amino acids, and n-octanoylation of the Ser-3 hydroxyl group is necessary for biological activity. Most studies have focused on the somatotropic and orexigenic roles of ghrelin; therefore, little is known about the kinetics of this peptide. Because the ester bond is both chemically and enzymatically unstable, elimination of the octanoyl modification of ghrelin can occur during storage, handling, and/or dissolution in culture medium *(3)*. Because of increased interest in ghrelin measurements, a standardized method of sample collection is required.

In the present study, which focused on the active form of ghrelin, we investigated the effects of anticoagulants and storage conditions on ghrelin stability. To distinguish the active form of ghrelin, we established two ghrelin-specific RIAs; N-RIA recognizes the N-terminal, octanoyl-modified portion of the peptide, whereas C-RIA recognizes the C-terminal portion. Thus, the value determined by N-RIA specifically measures active ghrelin, whereas the value determined by C-RIA gives the total ghrelin immunoreactivity, including both active and desacyl ghrelin *(4–6)*. The minimum detectable quantities in the N- and C-RIAs were 5.0 and 50 pmol/L, respectively. The respective intra- and interassay CV were 3% and 6% for the N-RIA and 6% and 9% for the C-RIA (n = 8 assays). Data are reported as the mean (SD). Comparisons of the time course of ghrelin concentrations between subgroups were made by two-way ANOVA for repeated measures, followed by the Scheffé test. $P < 0.05$ was considered statistically significant.

All blood samples were taken from three healthy male volunteers who gave written informed consent. Blood was taken from the forearm vein and immediately divided into tubes for serum and plasma preparation using (*a*) disodium EDTA (1 g/L) with aprotinin (500 000 kIU/L), (*b*) disodium EDTA alone, (*c*) heparin sodium, or (*d*) no anticoagulant. Synthetic human ghrelin was added to each blood sample at a final concentration of 40 μg/L; each sample was then sequentially divided into two aliquots for incubation at either 4 or 37 °C. After incubation for 0, 30, and 60 min, blood samples were centrifuged, diluted 1:200 in RIA buffer, and subjected to ghrelin-specific RIAs. A comparison of the effects of different anticoagulants on the detected ghrelin concentrations is shown in Table 1A. Although the serum and three different plasma samples tested gave comparable results for total ghrelin by C-RIA, the N-RIA gave ghrelin concentrations that were significantly decreased at 37 °C. When the ghrelin was measured by N-RIA, serum samples were highly affected by such treatment; samples stored for 60 min at 37 °C lost ~35% of the ghrelin compared with the basal values at 0 min ($P < 0.05$). The ghrelin concentrations in samples containing heparin as an anticoagulant were also significantly decreased ($P < 0.05$). When EDTA–aprotinin was used as the anticoagulant for plasma treatment, the decreases in ghrelin stability were smaller than for other procedures. Storage at 4 °C also improved ghrelin stability.

To explore optimum storage conditions, we examined the effect of plasma pH on ghrelin stability. The EDTA–aprotinin-treated plasma (n = 3) was divided into five samples; the pH was then adjusted to 3, 4, 5, 6, or 7.4 with 1 mol/L HCl. Synthetic human ghrelin was then added to each sample aliquot at a final concentration of 75 μg/L. Each of the five plasma aliquots was then subdivided into two, with one stored at 4 °C and the other stored at 37 °C. The effects of acidification on ghrelin stability in plasma are summarized in Table 1B. When stored at 37 °C, ghrelin concentrations measured by N-RIA gradually decreased at all pH values tested. However, ghrelin was most stable in highly acidified plasma samples (pH 3–4). At pH 3–5 and a storage temperature of 4 °C, the stability of ghrelin in plasma did not change significantly over a 6-h period. By C-RIA, ghrelin concentrations remained stable across the different pH and storage temperature conditions.

We then evaluated the effects of repeated freezing and thawing on the stability of ghrelin. EDTA–aprotinin-treated plasma samples were divided into two pH groups; one was acidified to pH 4, whereas the other was not acidified (pH 7.4). After the addition of synthetic human ghrelin (75 μg/L), we subjected the samples to four freeze–thaw cycles. Repeated freezing and thawing also influenced ghrelin stability (Table 1C). As in the N-RIA, ghrelin concentrations in untreated plasma samples decreased significantly with each successive freeze–thaw cycle, whereas the ghrelin remained relatively stable after acidification. Ghrelin concentrations by C-RIA were unchanged despite repeated freeze–thaw treatments in both acidified and untreated plasma samples.

As well as differences in assay methodologies, differences in sample handling, such as the method of storage, effects of anticoagulants, or previous freezing and thawing of the samples, could influence the reported values *(7–10)*. Instability of peptides and proteins can be divided into two forms: chemical and physical instability *(11, 12)*.

Table 1. Effect of anticoagulants and storage conditions on ghrelin stability.[a]

A. Ghrelin measurements in serum and different plasma samples

		Mean (SD) percentage of baseline					
		C-RIA			N-RIA		
		0 min	30 min	60 min	0 min	30 min	60 min
EDTA–aprotinin	37°C	100.0 (6.2)	101.0 (4.4)	102.9 (10.4)	100.0 (9.1)	102.6 (5.1)	89.6 (1.8)
	4°C		100.4 (4.0)	101.1 (4.5)		97.4 (1.3)	99.9 (12.3)
EDTA	37°C	100.0 (7.4)	98.5 (5.4)	98.4 (3.8)	100.0 (3.7)	83.6 (9.9)	85.3 (3.3)
	4°C		98.2 (2.6)	98.6 (7.6)		96.5 (1.8)	88.3 (6.5)
Heparin	37°C	100.0 (10.0)	104.3 (6.6)	91.1 (13.1)	100.0 (5.0)	88.1 (2.3)	77.4 (2.2)[b]
	4°C		104.4 (6.2)	102.1 (12.2)		92.5 (0.8)	86.9 (4.3)
Serum	37°C	100.0 (9.9)	100.4 (10.3)	98.2 (11.8)	100.0 (10.9)	87.5 (1.3)	65.1 (6.9)[b]
	4°C		96.2 (7.1)	98.4 (8.7)		96.5 (0.8)	94.6 (7.4)

B. Effects of storage pH, duration, and temperature on ghrelin stability

			Mean (SD) percentage of baseline					
		pH	0 h	1 h	2 h	3 h	4 h	6 h
C-RIA	RT	7.5	100.0 (6.6)	95.7 (5.0)	95.5 (6.3)		98.5 (5.4)	102.1 (6.5)
		6	100.0 (5.2)	96.0 (5.3)	91.3 (8.2)		96.6 (6.1)	97.9 (4.6)
		5	100.0 (6.1)	101.9 (8.2)	99.0 (6.0)		100.3 (8.4)	104.1 (5.2)
		4	100.0 (9.2)	96.0 (6.4)	98.0 (3.5)		100.2 (2.9)	97.1 (3.4)
		3	100.0 (2.2)	96.1 (4.9)	95.6 (4.7)		95.2 (2.7)	89.8 (3.9)
	4°C	7.5	100.0 (2.0)			100.9 (8.3)		99.3 (3.3)
		6	100.0 (3.6)			95.1 (4.1)		98.1 (1.8)
		5	100.0 (4.0)			101.2 (7.8)		105.9 (5.1)
		4	100.0 (7.1)			99.1 (3.3)		99.1 (4.1)
		3	100.0 (4.2)			99.4 (1.7)		101.3 (5.0)
N-RIA	RT	7.5	100.0 (5.0)	81.8 (1.0)[c]	72.0 (3.1)[d]		50.6 (2.9)[d]	37.5 (2.6)[d]
		6	100.0 (8.8)	93.3 (5.7)	78.8 (2.7)[c]		54.7 (1.2)[d]	38.8 (2.9)[d]
		5	100.0 (12.8)	92.8 (4.1)	86.4 (2.2)		74.1 (3.8)[b]	59.2 (7.6)[c]
		4	100.0 (12.5)	94.5 (7.4)	90.8 (5.2)		78.8 (3.1)[b]	74.0 (9.8)[c]
		3	100.0 (6.0)	98.4 (1.9)	96.9 (0.9)		82.2 (2.4)[b]	76.4 (6.1)[c]
	4°C	7.5	100.0 (6.9)			66.5 (4.4)		40.4 (3.5)[b]
		6	100.0 (6.1)			96.3 (0.2)		83.0 (1.2)[b]
		5	100.0 (5.5)			103.2 (2.0)		87.7 (11.7)
		4	100.0 (0.8)			102.2 (0.4)		99.6 (5.7)
		3	100.0 (12.8)			105.8 (0.3)		103.5 (1.6)

C. Effects of repeated freeze–thaw cycles on plasma ghrelin stability

		Cycles				
		1	2	3	4	5
C-RIA	HCl (−)	100.0 (8.8)	94.7 (7.2)	92.8 (6.6)	95.5 (5.1)	91.2 (7.3)
	HCl (+)	100.0 (5.2)	96.0 (5.3)	91.3 (8.2)	96.6 (6.1)	97.9 (4.6)
N-RIA	HCl (−)	100.0 (4.0)	89.8 (2.7)[b]	59.9 (6.3)[d]	28.1 (5.2)[d]	14.5 (3.3)[d]
	HCl (+)	100.0 (4.3)	94.1 (4.2)	94.4 (5.7)	95.4 (4.2)	93.8 (7.0)

[a] Results are for triplicate measurements. Values measured at 0 min, 0 h, or zero cycles are the baseline values.
[b–d] Compared with baseline: [b] $P < 0.05$; [c] $P < 0.01$; [d] $P < 0.001$.

The chemical degradation of peptides is influenced by the pH of the aqueous solution; human parathyroid hormone and luteinizing-hormone-releasing hormone derivatives are examples *(13–15)*. We demonstrated that in whole blood and plasma, ghrelin is unstable. The degradation of octanoylated ghrelin was shown to be attributable to hydrolysis to desacyl ghrelin (see Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue6/). Acidification is a simple, reliable procedure that protected against degradation of the acylated modification and dramatically improved stability at pH 4. On the other hand, the stability of the octanoyl modification of ghrelin was markedly decreased in strongly acidic (below pH 2), neutral, and alkaline solutions (data not shown).

We evaluated the effectiveness of measuring active ghrelin compared with total ghrelin in response to oral glucose tolerance tests (OGTTs). Four healthy male volunteers (age range, 28–35 years; body mass index, 21.5–23.7 kg/m^2) were examined on 2 separate days (100 g of glucose administered on 1 day, and 50 g of glucose administered on the other day) at least 2 weeks apart in a randomized, crossover study. After the volunteers fasted overnight, 50 or 100 g of glucose was administered orally between 0930–1000. Blood samples were obtained at 0, 1, 2, 3, and 4 h after glucose ingestion. To each plasma sample was added 1 mol/L HCl (10% of plasma volume), which acidified the sample to pH ~4; samples were then treated with Sep-Pak C_{18} cartridges for ghrelin RIAs. After glucose ingestion, the mean plasma ghrelin concentrations as determined by N-RIA and C-RIA decreased to a nadir at 1 h (Fig. 1). At this point, 60.3% and 73.0% of the basal concentration was detected by the N-RIA and C-RIA, respectively, after the 100-g OGTT, and 64.2% and 78.7% of the basal concentration was detected after the 50-g OGTT. Plasma ghrelin values increased thereafter, although plasma ghrelin concentrations measured by the C-RIA were significantly lower for up to 2 h after the 100-g glucose load. The N-RIA for ghrelin could detect differences in the changes in ghrelin concentrations between the 50-g and 100-g OGTTs at 3 h. The ghrelin values observed with the C-RIA exhibited changes similar those in the N-RIA, but the changes were small and delayed. These effects may be attributable to the differential rates of metabolic turnover for octanoylated and desacylated ghrelin in circulating blood (see Fig. 2 in the online Date Supplement).

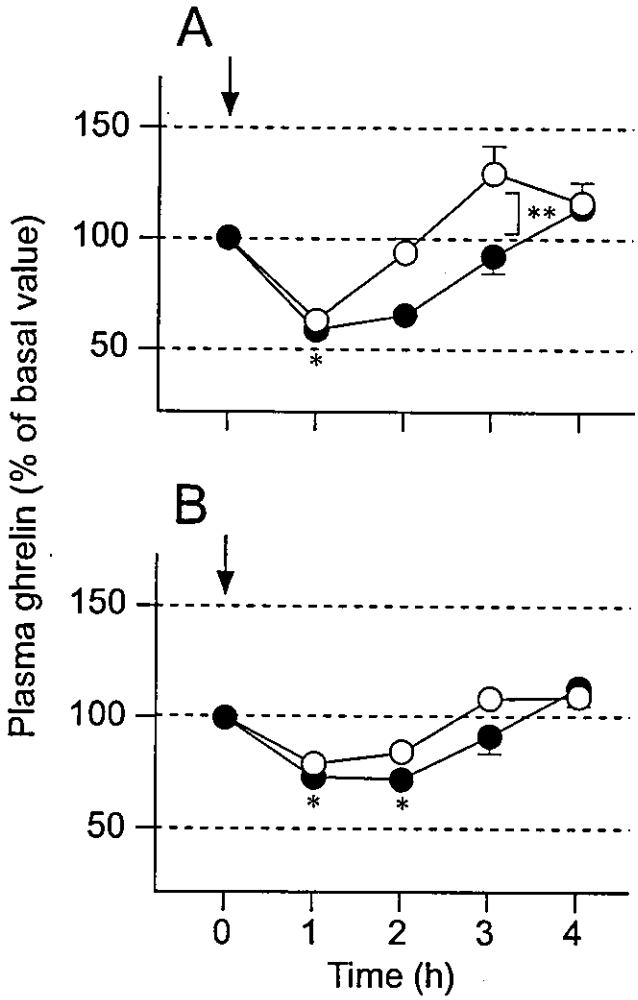

Fig. 1. Plasma ghrelin response to 50-g (○) and 100-g (●) OGTTs in four healthy individuals.

Plasma ghrelin concentrations assayed by N-RIA (*A*) and C-RIA (*B*) are given as the mean (SD; *error bars*) percentage change from basal values. *, $P < 0.05$ compared with basal values; **, $P < 0.05$ for difference in plasma ghrelin between 50-g and 100-g glucose loads.

The results for the plasma ghrelin response to the OGTTs show that measuring the concentration of active ghrelin is useful for studying plasma ghrelin changes over short time periods. Plasma concentrations of active ghrelin changed more rapidly and dynamically than those of total ghrelin immunoreactivity. Fasting led to markedly increased plasma ghrelin values as measured by N-RIA, and the values decreased in a clearer dose-dependent manner in rats after glucose injection compared with those measured by C-RIA *(16)*. The proportion of active ghrelin in plasma was 2–5% of total ghrelin in rodents. In this study, the quantity of active ghrelin was ~10% of the total ghrelin in human plasma (data not shown). These findings imply that inactive desacyl ghrelin circulates in the bloodstream at much higher concentrations than active ghrelin. Similar to previous studies in which ghrelin concentrations were measured by C-RIA *(17)*, desacyl ghrelin is relatively stable, and its stability is not altered by different storage conditions. An analogous situation has been reported for the activity of pancreatic beta cells, which secrete insulin and C-peptide in a 1:1 molar ratio. However, the half-life of C-peptide is much longer than that of insulin, leaving more C-peptide available in the circulation for quantification *(18, 19)*. Measurement of C-peptide provides an assessment of β-cell secretory activity. Similarly, desacyl ghrelin concentrations may serve as an indicator of ghrelin secretory function *(20)*.

To acquire accurate data on ghrelin concentrations, this study recommends a standard procedure for the collection of blood samples: (*a*) the collection of blood samples with EDTA–aprotinin is preferred; (*b*) blood samples should be chilled and centrifuged as soon as possible, at least within 30 min after collection; and (*c*) because acidification is the best method for the preservation of plasma ghrelin, 1 mol/L HCl (10% of sample volume) can be added to the plasma sample for adjustment to pH 4.

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Substitution of 3′-Phosphate Cap with a Carbon-Based Blocker Reduces the Possibility of Fluorescence Resonance Energy Transfer Probe Failure in Real-Time PCR Assays, *Kendall W. Cradic,[1] Jason E. Wells,[2] Lindsay Allen,[2] Kent E. Kruckeberg,[1] Ravinder J. Singh,[1] and Stefan K.G. Grebe[1,3]** ([1] Departments of Laboratory Medicine and Pathology and [3] Medicine, Mayo Clinic, Rochester, MN; [2] Idaho Technology Inc., Salt Lake City, UT; * address correspondence to this author at: Endocrine Laboratory, Hilton 730C, Mayo Clinic, 200 1st St. SW, Rochester, MN 55905; fax 507-284-9758, e-mail grebs@mayo.edu)

During the last decade, research and clinical use of real-time PCR applications has continued to grow in importance *(1)*. Many laboratories that use real-time PCR with fluorescent probes experience an unexplained loss of probe fluorescence at some stage, in particular with pairs of fluorescence resonance energy transfer (FRET) probes. Photobleaching is often assumed to be the cause. Structural integrity of the oligonucleotides is also a major factor, and its loss has been shown to correlate with repeated freeze–thaw cycles *(2)*. Laboratories guard against these two problems by aliquoting probes and protecting them from light. Despite these precautions, inexplicable FRET probe failures are still observed. In one recent such case, we were able to determine an additional mechanism for FRET probe failure: loss of the phosphate cap from the 3′ end of a probe. To our knowledge, this has not been described previously. Our studies revealed that this may be a common and important problem, intrinsic to 3′-phosphate-blocking chemistry. We also found that alternative terminating groups may be a preferable option to 3′-phosphate blocking.

A 3-nmol/L synthesis-scale LightCycler™ hybridization probe set was purchased from Idaho Technology Inc. Biochem in April 2003. As is common practice, the manufacturer produced a large-scale synthesis and, after shipping our order, archived the remainder for a possible future reorder. The first half of the batch (α-probe set) was sent immediately, whereas the second half (β-probe set) was stored lyophilized for 6 months at −20 °C and then shipped with our next order.

Oligonucleotides from the first shipment were used in PCR reactions in a LightCycler with satisfactory results. PCR conditions were as follows: 1× LightCycler FastStart DNA Master Hybridization Probe Mix (Roche Diagnostics), $MgCl_2$ (final concentration, 3.5 mM), 0.5 μM each of the forward (5′-GGCCTTTCTGAAGCAAG-3′) and reverse (5′-GACGATTTCTTATTTCACAGCTCC-3′) primers, 0.2 μM each of the donor (5′-GGACGCAGAGGGGATGG-FITC-3′, where FITC is fluorescein isothiocyanate) and acceptor (LCRed640-GTGTATGGGACCCGCCAG-phosphate) probes, and 2 μL of cDNA template mixture. The final reaction volume was 10 μL. The reaction started with an initial melting step at 95 °C for 10 min followed by 45 cycles of 95 °C for 2 s, 57 °C for 10 s, and 72 °C for 5 s.

Loss of fluorescence activity was first observed when we received the β-probe set. PCR reactions were carried out under the same conditions, but the amplification

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,

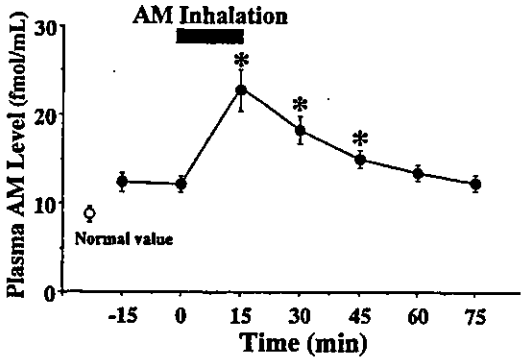

Figure 1. Changes in plasma AM level by inhalation of aerosolized AM in patients with idiopathic pulmonary arterial hypertension. Normal value indicates plasma AM level derived from 15 age-matched healthy subjects. Data are mean±SEM. *$P<0.05$ vs value at time 0.

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.

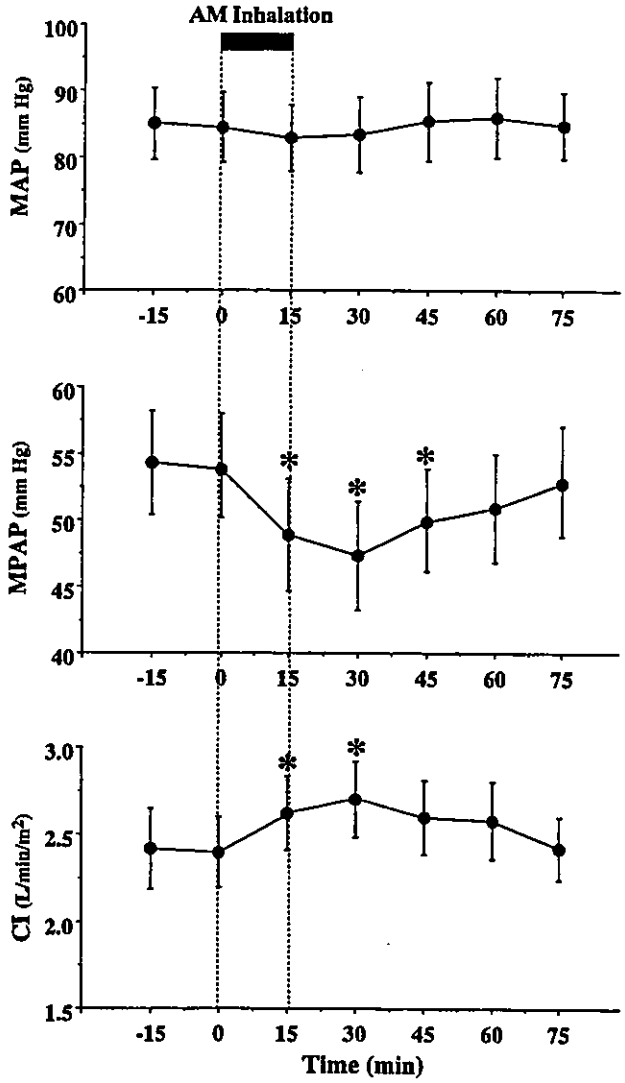

Figure 2. Changes in mean arterial pressure (MAP), mean pulmonary arterial pressure (MPAP), and cardiac index (CI) by inhalation of aerosolized AM in patients with idiopathic pulmonary arterial hypertension. Data are mean±SEM. *$P<0.05$ vs value at time 0.

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

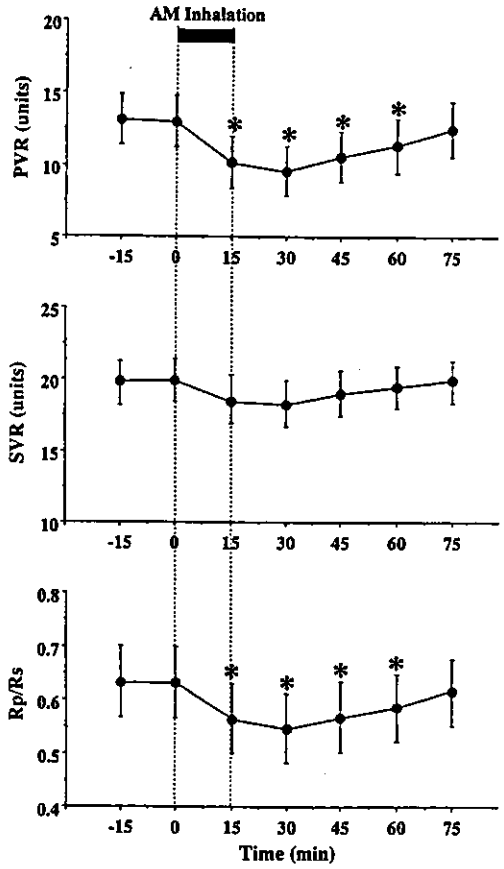

Figure 3. Changes in pulmonary vascular resistance (PVR), systemic vascular resistance (SVR), and ratio of pulmonary vascular resistance to systemic vascular resistance (Rp/Rs) by inhalation of aerosolized AM in patients with idiopathic pulmonary arterial hypertension. Data are mean±SEM. *$P<0.05$ vs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

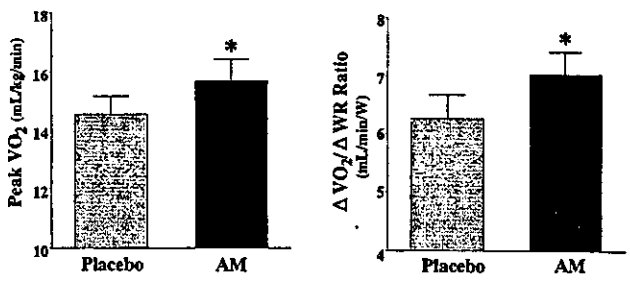

Figure 4. Changes in peak oxygen consumption (peak $\dot{V}_{O_2}$) and ratio of change in oxygen uptake to that in work rate ($\Delta\dot{V}_{O_2}/\Delta$W ratio) by inhalation of aerosolized AM or placebo in patients with idiopathic pulmonary arterial hypertension. Data are mean±SEM. *$P<0.05$ vs placebo.

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 Gene Transfer Induces Therapeutic Angiogenesis in a Rabbit Model of Chronic Hind Limb Ischemia

Benefits of a Novel Nonviral Vector, Gelatin

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Background—Earlier studies have shown that adrenomedullin (AM), a potent vasodilator peptide, has a variety of cardiovascular effects. However, whether AM has angiogenic potential remains unknown. This study investigated whether AM gene transfer induces therapeutic angiogenesis in chronic hind limb ischemia.

Methods and Results—Ischemia was induced in the hind limb of 21 Japanese White rabbits. Positively charged biodegradable gelatin was used to produce ionically linked DNA-gelatin complexes that could delay DNA degradation. Human AM DNA (naked AM group), AM DNA-gelatin complex (AM-gelatin group), or gelatin alone (control group) was injected into the ischemic thigh muscles. Four weeks after gene transfer, significant improvements in collateral formation and hind limb perfusion were observed in the naked AM group and AM-gelatin group compared with the control group (calf blood pressure ratio: 0.60±0.02, 0.72±0.03, 0.42±0.06, respectively). Interestingly, hind limb perfusion and capillary density of ischemic muscles were highest in the AM-gelatin group, which revealed the highest content of AM in the muscles among the three groups. As a result, necrosis of lower hind limb and thigh muscles was minimal in the AM-gelatin group.

Conclusions—AM gene transfer induced therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia. Furthermore, the use of biodegradable gelatin as a nonviral vector augmented AM expression and thereby enhanced the therapeutic effects of AM gene transfer. Thus, gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of peripheral vascular diseases. (***Circulation.*** **2004;109:526-531.)**

Key Words: peripheral vascular disease ■ angiogenesis ■ gene therapy ■ ischemia

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma.[1] AM and its receptor are expressed mainly in vascular endothelial cells and vascular smooth muscle cells.[2–4] AM not only induces vasorelaxation but also regulates growth and death of these vascular cells.[5–10] These findings suggest that AM plays an important role in maintaining vascular homeostasis in an autocrine and/or paracrine manner.

A recent study has shown that vascular abnormalities are present in homozygous AM knockout mice, suggesting that AM is indispensable for vascular morphogenesis.[11–13] More recently, AM has been shown to activate the PI3K/Akt-dependent pathway in vascular endothelial cells, which is considered to regulate multiple critical steps in angiogenesis, including endothelial cell survival, proliferation, migration, and capillary-like structure formation.[7,14] These results raise the possibility that AM plays a role in modulating vasculogenesis and angiogenesis. However, whether AM induces therapeutic angiogenesis remains unknown.

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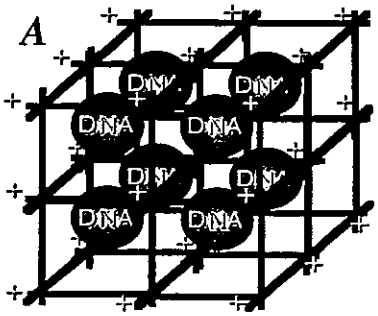

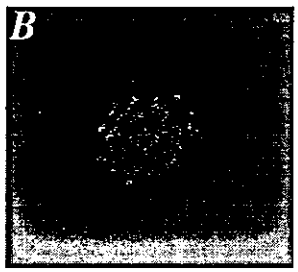

Figure 1. A, Schema of DNA-gelatin complex. Biodegradable gelatin can hold negatively charged plasmid DNA in its positively charged lattice structure. B, RITC-labeled AM DNA particles were incorporated into gelatin.

We prepared biodegradable gelatin that could hold negatively charged protein or plasmid DNA in its positively charged lattice structure.[15,16] Biodegradable gelatin has been widely used as a carrier of protein because of its capacity to delay protein degradation.[15] Similarly, ionically linked DNA-gelatin complexes can delay gene degradation.[16] These findings raise the possibility that gelatin may serve as a nonviral vector for gene therapy.

Thus, the purposes of this study were (1) to investigate whether AM gene transfer induces therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia and (2) to examine whether the use of biodegradable gelatin as a vector augments AM expression and thereby enhances the therapeutic effects of AM gene transfer.

Methods

Animal Model

All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute. Twenty-one male Japanese White rabbits (body weight, 2.9±0.1 kg; Japan Animal Co, Osaka, Japan) were used for physiological and morphological assessment. In addition, 30 rabbits were used for radioimmunoassay, immunohistochemical examination, and Western blot analysis. After anesthetization with pentobarbital sodium (30 to 35 mg/kg), a longitudinal incision was made in the left thigh, extending inferiorly from the inguinal ligament to a point just proximal to the patella. Hind limb ischemia was induced by ligation of the distal left external iliac artery and complete resection of the left femoral artery, as described previously.[17]

Construction of Plasmid DNA

To construct the expression vector for human AM, the *EcoRI/XhoI* fragment of the full-length human AM cDNA was ligated into the EcoRI/XhoI fragment of the pcDNA1.1-CMV expression plasmid (Invitrogen). To verify that the pcDNA1.1-CMV vector encoding AM cDNA produces a biologically active AM protein, the expression vector was transfected into 293 cells, and AM activity in the transfected cells was measured by high-performance liquid chromatography and radioimmunoassay. The pcDNA1.1-CMV vector encoding β-galactosidase (LacZ) cDNA was used as a control DNA.

Preparation of AM DNA-Gelatin Complex

Biodegradable gelatin was prepared from pig skin. The gelatin was characterized by a spheroid shape with a diameter of approximately 30 μm, water content of 95%, and an isoelectric point (pI) of 9 after swelling in water.[15,16] Gelatin can hold negatively charged protein or plasmid DNA in its positively charged lattice structure (Figure 1A). Dried gelatin (4 mg, pI 9) was added to human AM DNA solution (500 μg/100 μL in phosphate-buffered saline, pH 7.4). After mixture of DNA and gelatin, DNA-gelatin complexes were incubated at 37°C for 2 hours.

To visualize incorporation of DNA into gelatin, AM plasmid DNA was labeled with rhodamine B isothiocyanate (RITC), as reported previously.[16] In brief, the coupling reaction of RITC to plasmid DNA was carried out by mixing the two substances in 0.2 mol/L sodium carbonate-buffered solution (pH 9.7), followed by gel filtration with a PD 10 column (Amersham-Pharmacia). RITC-labeled AM DNA was incorporated into positively charged gelatin (Figure 1B).

Study Protocol

Ten days after the induction of hind limb ischemia (day 10), AM DNA (naked AM group, n=7), AM DNA-gelatin complex (AM-gelatin group, n=7), or gelatin alone (control group, n=7) was administered intramuscularly into 3 different sites in the ischemic adductor muscle and 2 different sites in the semimembranous muscle. In addition, Lac Z DNA-gelatin complex served as a control DNA (Lac Z-gelatin group, n=5). The amount of plasmid was 500 μg (1 mL) and that of gelatin was 4 mg. Morphological and angiographic analyses and measurements of calf blood pressure and laser Doppler flow were performed 4 weeks after gene transfer (day 38). After completion of these measurements, the adductor, semimembranous, and gastrocnemius muscles were weighed in each hind limb.[18] The muscle weight ratio was calculated for each muscle as follows: muscle weight ratio=muscle weight in ischemic hind limb/muscle weight in nonischemic hind limb. Specimens of the adductor muscle of the ischemic hind limb were obtained for histological examination.

Measurement of Calf Blood Pressure

Calf blood pressure was measured on days 10 and 38 in both hind limbs with a Doppler flowmeter (Hayashi Denki Co, Ltd) and a 25-mm-wide cuff. The pulse of the posterior tibial artery was identified with the use of a Doppler probe, and the systolic blood pressure in both hind limbs was determined by standard techniques. The calf blood pressure ratio was defined for each rabbit as the ratio of systolic pressure of the ischemic hind limb to that of the normal hind limb.[17]

Laser Doppler Blood Perfusion Analysis

Blood flow of the ischemic hind limb was measured with the use of a laser Doppler blood perfusion image system (moorLDI, Moor Instruments) on day 38.

Angiographic Analysis

Development of collateral arteries was evaluated by angiography on days 0 and 38. A 4F catheter was placed in the left internal iliac artery through the common carotid artery, and 3 mL contrast medium (Iopamiron 300, SCHERING) was injected with an automated angiography injector at a rate of 2.5 mL/s. Quantitative angiographic analysis of collateral vessel development in the ischemic hind limb was performed with the use of a 5-mm^2 grid overlay, as described previously.[17] The angiographic score was calculated for each film as the ratio of grid intersections crossed by opacified arteries divided by the total number of grid intersections in the ischemic medial thigh. The angiographic score was determined by 2 blinded observers.