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

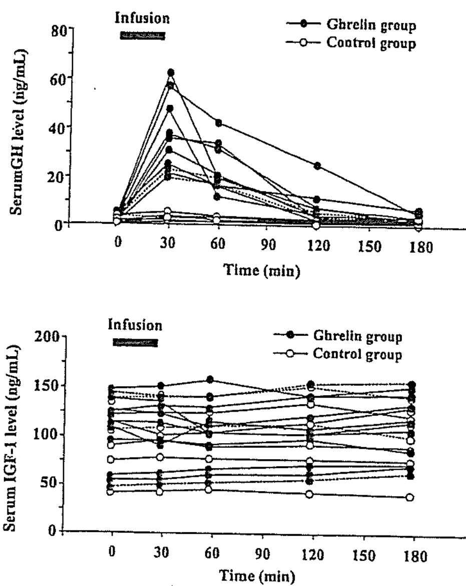

Figure 1. Changes in serum GH and IGF-1 after single administration of ghrelin. Solid line indicates cachectic patients; dotted line, noncachectic patients.

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

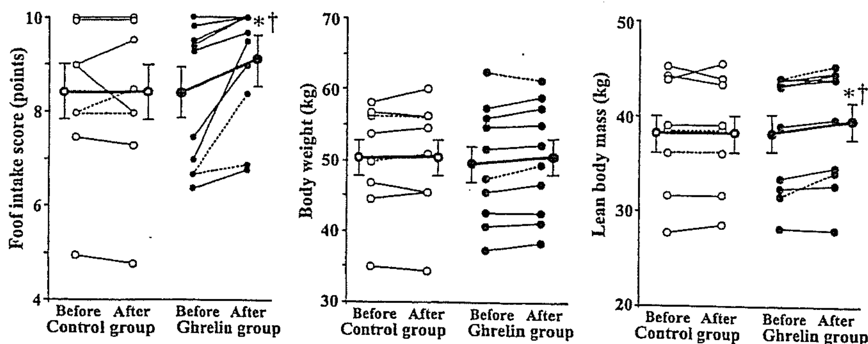

Figure 2. Food intake, body weight, and lean body mass before and after 3-week administration of ghrelin. Food intake was described semiquantitatively with 10-point scale method (0=null intake, 10=full intake). Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients.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

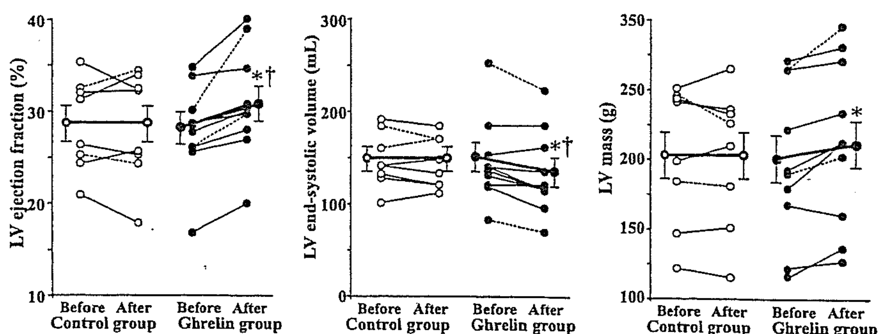

Figure 3. LV geometry and function before and after ghrelin therapy. Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients. *$P<0.05$ vs before; †$P<0.05$ vs respective control group.

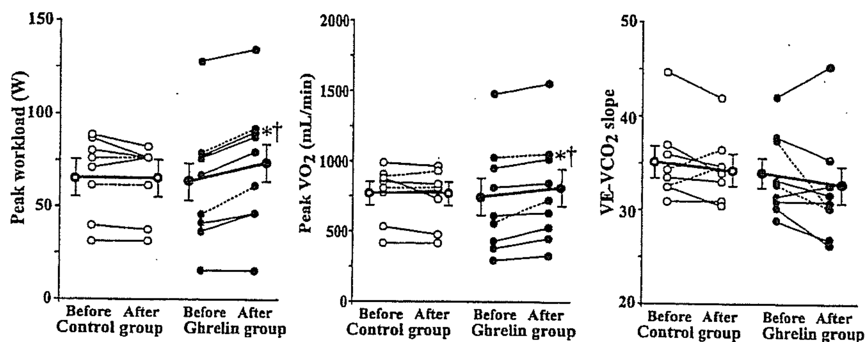

Figure 4. Exercise capacity and ventilatory efficiency before and after ghrelin therapy. Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients. $^*P<0.05$ vs before; $^\dagger P<0.05$ vs respective control group.

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

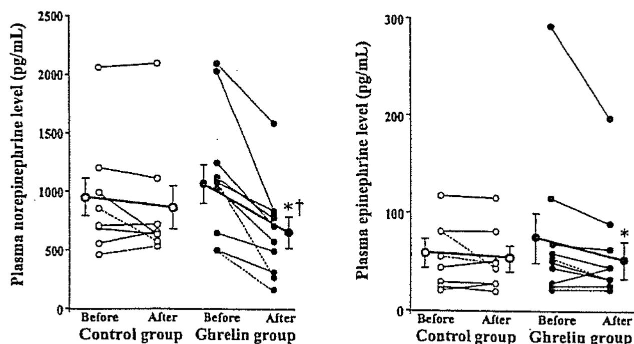

Figure 5. Plasma levels of norepinephrine and epinephrine before and after ghrelin therapy. Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients. $^*P<0.05$ vs before; $^\dagger P<0.05$ vs respective control group.

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 · mL⁻¹ · 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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SCIENTIFIC LETTER

Green tea reverses endothelial dysfunction in healthy smokers

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Green tea is popular in Asia, including Japan, although it is rarely consumed by western people. It contains large amounts of catechins which are potent free radical scavengers and therefore has an antioxidant effect.[1] Oxidative stress participates in the pathogenesis of many cardiovascular diseases. Recently, black tea consumption has been shown to reverse endothelial dysfunction in patients with coronary artery disease through its antioxidant effects.[2] However, the number of catechins in non-fermented tea (green tea) is higher than that in fermented tea (black tea).[1] These findings raise the possibility that green tea acts as a potent antioxidant.

Smoking is a well recognised risk factor for cardiovascular diseases. Free radicals in cigarette smoke are responsible for endothelial dysfunction, leading to atherosclerosis.[3] Earlier studies have shown that the flavonoid components of red wine and purple grape juice, which are powerful antioxidants, reverse endothelial dysfunction and reduce cardiovascular risk. However, any beneficial effect of green tea on vascular function in smokers remains unknown. Accordingly, the purpose of this study was to investigate whether green tea consumption reverses endothelial dysfunction in association with improvement of oxidative stress in healthy smokers.

METHODS

We studied 20 healthy male smokers (mean (SEM) age 33 (1) years, body mass index 22.9 (0.9) kg/m^2, smoking 15.5 (2.1) packs/year). They were randomised to consume green tea or hot water in a crossover design. All subjects gave written informed consent and the study was approved by the ethics committee of the National Cardiovascular Center. Subjects were studied on two separate days, at least one week apart, at 8:30 in the morning after they had fasted for at least 12 hours. Subjects did not smoke on the morning of the study. At baseline and two hours after consumption of 400 ml green tea or hot water, we measured the response of forearm blood flow (FBF) to reactive hyperaemia, an index of endothelium dependent vasodilatation, and to sublingual administration of glyceryl trinitrate (GTN), an index of endothelium independent vasodilatation. To induce reactive hyperaemia, FBF was occluded by inflating a cuff around the left upper arm to a pressure of 280 mm ?Hg for five minutes. After release of the ischaemic cuff occlusion, FBF was measured for three minutes. After FBF returned to the baseline in a 15 minute recovery period, 0.3 mg GTN was administered sublingually and FBF was measured for five minutes. Seven healthy volunteers (mean age 32 (2) years), who had not smoked for more than one year, served as age matched non-smoking controls.

FBF was measured using a mercury filled Silastic strain-gauge plethysmograph (EC-5R, DE Hokanson Inc, Washington, USA), as described previously.[1] FBF was expressed in ml/min/100 ml of forearm tissue volume. Blood and urine samples were repeatedly obtained for measurement of plasma catechin and urine 8-iso-prostaglandin-F2α (8-iso-PGF2α), an index of oxidative stress.[4] Numerical values were expressed as mean (SEM). Comparisons of the time course of parameters between the two groups were made by two way analysis of variance (ANOVA) for repeated measures, followed by Scheffe's multiple comparison test.

RESULTS

Green tea contained very large amounts of catechins (173.1 mg/dl), including epigallocatechin gallate (61.8 mg/dl), which have potent antioxidant effects. The content of epigallocatechin gallate in green tea was 10-fold higher than that in black tea. Unlike black tea, green tea also contained ascorbic acid (3.0 mg/dl). Plasma epigallocatechin gallate concentration was notably increased two hours after a single consumption of green tea (3 (1) pmol/ml to 694 (81) pmol/ml, $p < 0.001$). Green tea consumption significantly decreased the urinary concentration of 8-iso-PGF2α, an index of oxidative stress (541 (188) pg/mg creatinine to 396 (136) pg/mg creatinine, $p < 0.05$). Green tea had no significant effect on systolic and diastolic blood pressure, heart rate, fasting plasma lipid, or glucose concentration. These parameters remained unchanged after hot water consumption.

During reactive hyperaemia in healthy smokers, FBF was significantly less than that in age matched non-smoking controls (maximum FBF, 16.9 (1.1) ml/min/100 ml tissue *v* 29.3 (2.1) ml/min/100 ml tissue, $p < 0.01$), suggesting the presence of endothelial dysfunction in healthy smokers. Time course curves of FBF during reactive hyperaemia were similar before consumption of green tea and hot water. When the results of subjects randomised to receive green tea first and hot water first were combined, green tea consumption significantly increased FBF during reactive hyperaemia (maximum FBF, 16.9 (1.1) ml/min/100 ml tissue to 21.3 (1.3) ml/min/100 ml tissue, $p < 0.001$, fig 1).

These results suggest that green tea consumption improved endothelium dependent vasodilatation in smokers. In contrast, green tea had no effect on the increase in FBF after sublingual administration of GTN, an index of endothelium independent vasodilatation (maximum FBF, 16.9 (1.1) ml/min/100 ml tissue to 21.3 (1.3) ml/min/100 ml tissue, p = NS). These parameters remained unchanged after hot water consumption.

DISCUSSION

The present study demonstrated that, compared with black tea, green tea contained extremely large amounts of catechins including epigallocatechin gallate. We also showed that, unlike black tea, green tea contained ascorbic acid, which also has antioxidant effects. These results suggest that green tea may act as a potent antioxidant.

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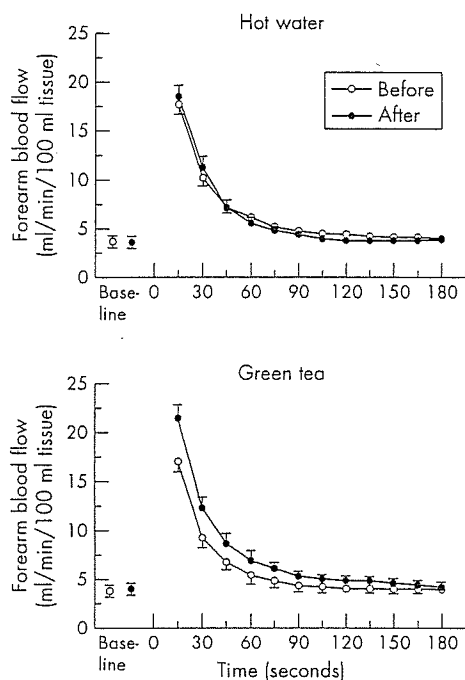

Figure 1 Forearm blood flow (FBF) at rest and during reactive hyperaemia before (open circles) and after (solid circles) consumption of hot water (upper panel) or green tea (lower panel). Green tea consumption augmented FBF during reactive hyperaemia ($p < 0.001$), whereas hot water consumption did not significantly alter it.

Cigarette smoke has been reported to contain nicotine and large amounts of free radicals, such as superoxide anion and hydroxyradicals.[3] Free radicals in cigarette smoke may not only degrade nitric oxide released from the endothelium, but also produce highly reactive intermediates, resulting in endothelial injury. In the present study, a single consumption of green tea notably increased plasma epigallocatechin gallate concentration and significantly decreased urinary 8-iso-PGF2α, a specific and stable product of lipid peroxidation, in healthy smokers. Another study has shown that green tea decreases oxidative DNA damage (measured through 8-OHdG (8-hydroxy-2'-deoxyguanosine) in smokers.[5] These results suggest that green tea consumption attenuates oxidative stress in healthy smokers, at least in part, through the antioxidant effects of catechins. Ascorbic acid included in green tea may also play a role in the regulation of oxidative stress. As expected, endothelium dependent vasodilatation was impaired in healthy smokers, consistent with many previous studies.

In the present study, a single consumption of green tea significantly increased FBF during reactive hyperaemia in smokers. In contrast, green tea had no effect on FBF at rest or GTN induced vasodilatation. These results suggest that green tea consumption reverses endothelial dysfunction in healthy smokers, possibly through its antioxidant effect. Earlier studies have demonstrated that endothelial dysfunction is important in the pathogenesis and clinical manifestation of cardiovascular disease. Thus, green tea consumption may be beneficial for the prevention and treatment of atherosclerotic vascular disease. Further prospective, randomised studies of green tea consumption are necessary to examine whether green tea consumption reduces the risk of cardiovascular events and mortality.

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

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

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

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

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

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

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

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

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

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

Methods

Reperfusion Model

We used male Sprague-Dawley rats (Japan SLC Inc, Hamamatsu, Japan) weighing 180 to 220 g. Ligation of the left coronary artery was performed as described previously.[11] In brief, under anesthesia with pentobarbital sodium (30 mg/kg) and artificial ventilation, the heart was exposed via left thoracotomy, and the left coronary artery was ligated 2 to 3 mm from its origin between the pulmonary artery conus and the left atrium with a 6-0 Prolene suture. The heart was subjected to regional ischemia for 30 minutes, followed by coronary reperfusion through release of the tie. After ligation of the left coronary artery, AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$), AM plus wortmannin (16 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-

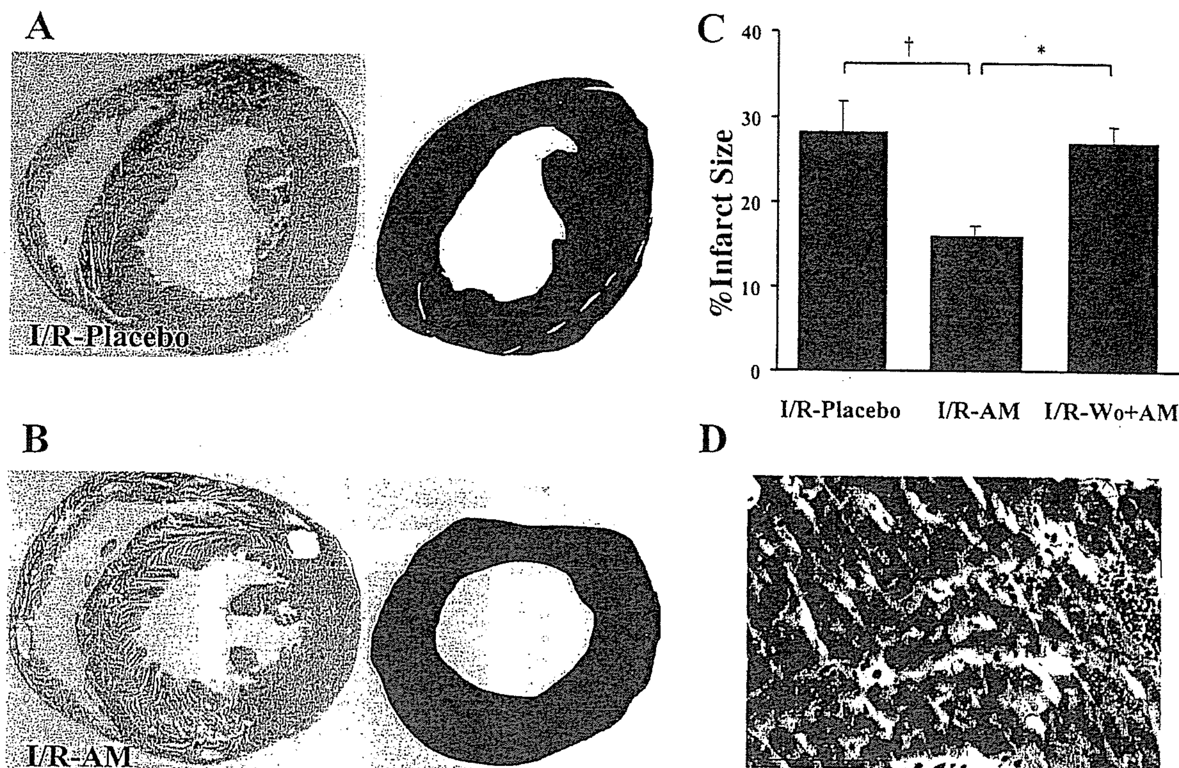

Figure 1. Effect of AM on myocardial infarct size 24 hours after ischemia/reperfusion. A and B, Photomicrographs show representative myocardial sections stained with Masson trichrome in I/R-placebo (A) and I/R-AM groups (B). Light red area indicates coagulation necrosis (right). C, Quantitative analysis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

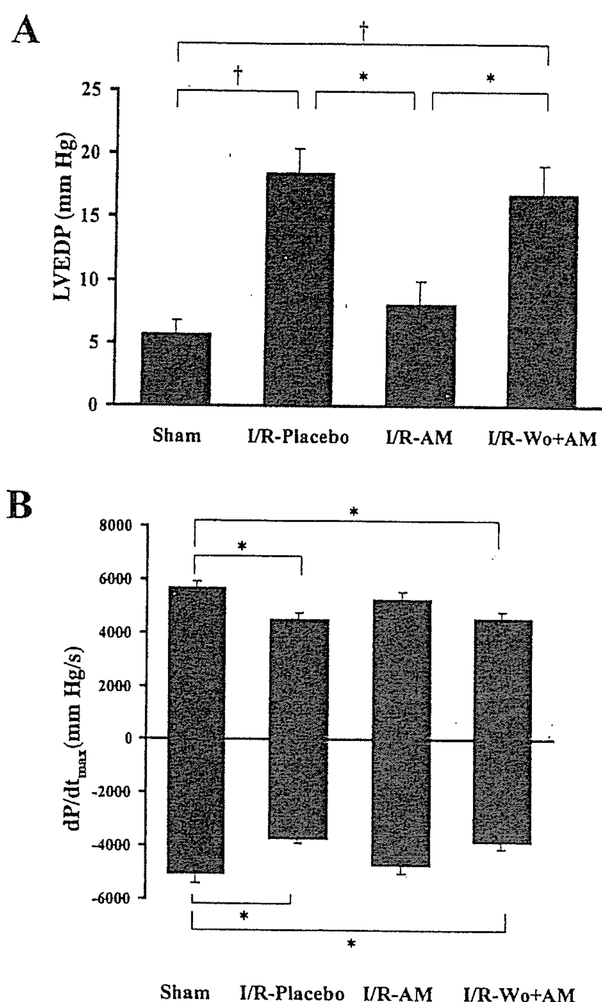

Figure 2. Effects of AM on LVEDP (A) and LV dP/dt (B) 24 hours after ischemia/reperfusion. AM infusion significantly inhibited increase in LVEDP compared with placebo infusion. AM infusion also improved LV dP/dt 24 hours after ischemia/reperfusion. Pretreatment with wortmannin attenuated effects of AM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

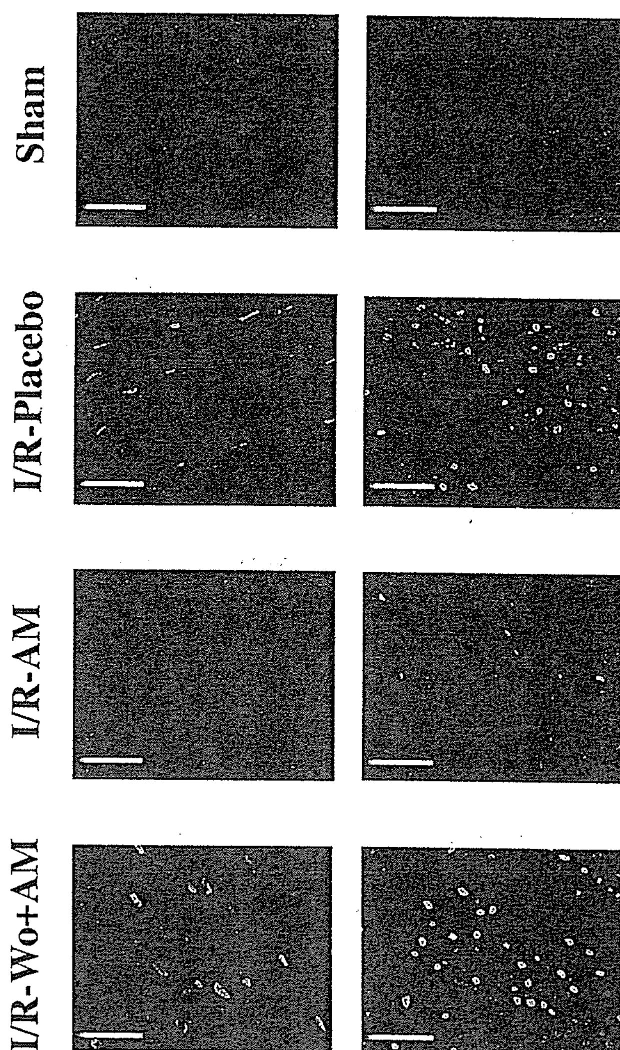

Figure 3. Representative photomicrographs of immunofluorescent staining for TUNEL-positive nuclei in sham, I/R-placebo, I/R-AM, and I/R-Wo+AM groups. Each left panel shows longitudinal myocytes, and each right panel shows short-axial myocytes. Yellow nuclei with red-stained myofilaments indicate TUNEL-positive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

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.

Akt Phosphorylation Induced by AM Infusion in Cardiac Tissue

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

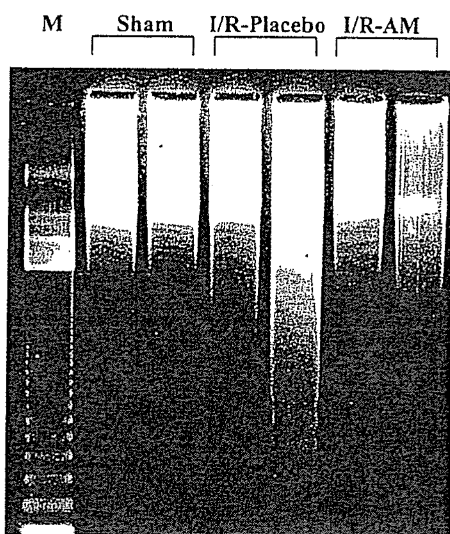

Figure 4. DNA ladder in sham, I/R-placebo, and I/R-AM groups. Although typical DNA ladder indicating fragmented DNA in cardiomyocytes was observed in I/R-placebo group, it was attenuated in I/R-AM group. M indicates molecular marker.

Discussion

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

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

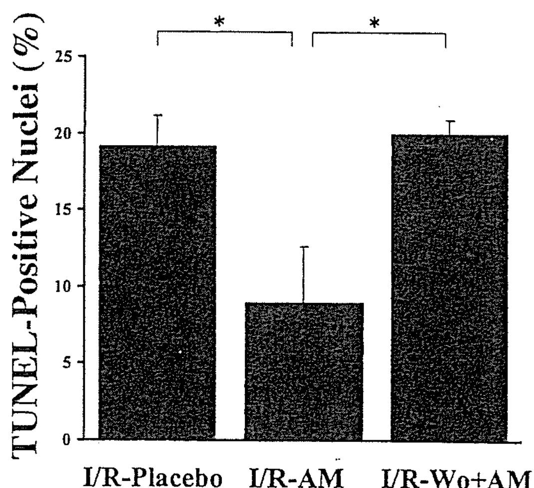

Figure 5. Quantitative analysis of TUNEL-positive nuclei in myocytes. Number of TUNEL-positive myocytes was lower in I/R-AM group than in I/R-placebo group. However, number of TUNEL-positive myocytes in I/R-Wo+AM group was as large as in I/R-placebo group. Data are mean±SEM. $^{*}P<0.05$.

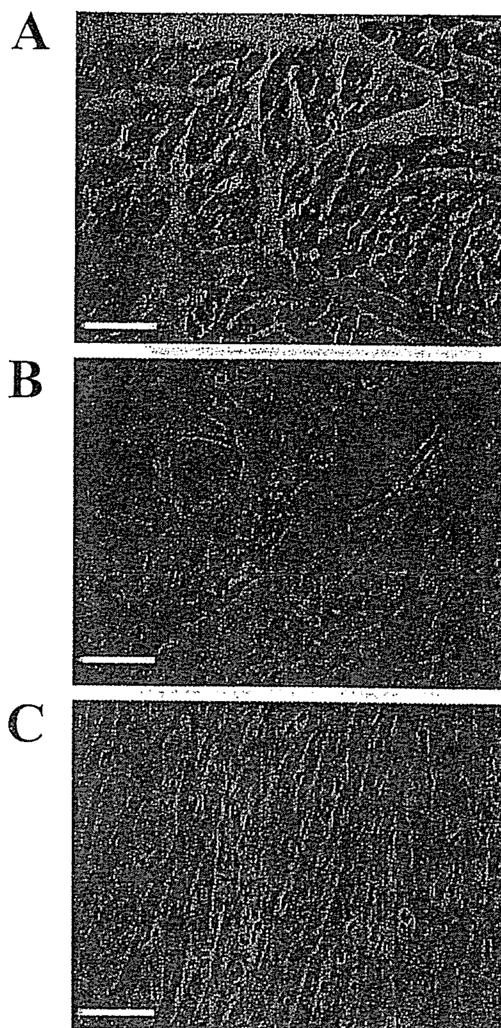

Figure 6. Immunohistochemistry for CRLR in rat cardiac tissue. Representative photomicrographs revealed that CRLR was localized in cardiomyocytes (A) and vascular endothelial cells (B). Negative control study (using mouse IgG) showed no positive staining in cardiac tissue (C). Original magnification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

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

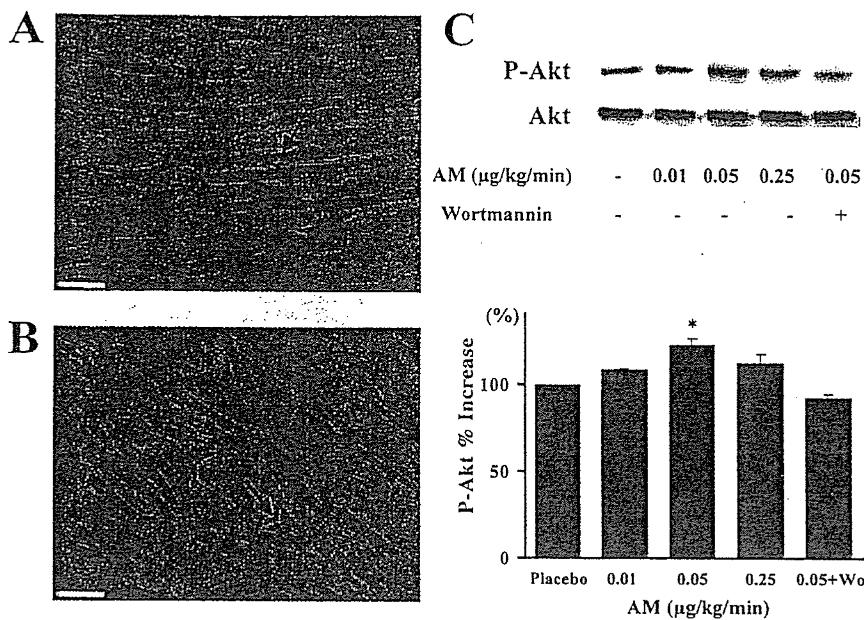

Figure 7. A and B, Immunohistochemistry for Akt phosphorylation in rat cardiac tissue. Infusion of AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$) phosphorylated Akt predominantly in nuclei of cardiomyocytes (A, B) and vascular endothelial cells (B). Arrow indicates nuclei of cardiomyocytes with positive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

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

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

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

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

Conclusions

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

Acknowledgments

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Diagnostic value of epinephrine test for genotyping LQT1, LQT2, and LQT3 forms of congenital long QT syndrome

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OBJECTIVES The aim of this study was to test the hypothesis that epinephrine test may have diagnostic value for genotyping LQT1, LQT2, and LQT3 forms of congenital long QT syndrome (LQTS).

BACKGROUND A differential response of dynamic QT interval to epinephrine infusion between LQT1, LQT2, and LQT3 syndromes has been reported, indicating the potential diagnostic value of the epinephrine test for genotyping the three forms.

METHODS The responses of 12-lead ECG parameters to epinephrine were retrospectively examined in 15 LQT1, 10 LQT2, 8 LQT3, and 10 healthy volunteers to select the best ECG criteria for separating the four groups. The epinephrine test then was prospectively conducted in 42 probands clinically affected with LQTS, their 67 family members, and 10 new volunteers. The best criteria were applied in a blinded fashion to prospectively separate a different group of 31 LQT1, 23 LQT2, 6 LQT3, and 30 Control patients (10 genotype-negative LQT1, 10 genotype-negative LQT2 family members, and 10 volunteers).

RESULTS The sensitivity (penetrance) by ECG diagnostic criteria was lower in LQT1 (68%) than in LQT2 (83%) or LQT3 (83%) before epinephrine and was improved with steady-state epinephrine in LQT1 (87%) and LQT2 (91%) but not in LQT3 (83%), without the expense of specificity (100%). The sensitivity and specificity to differentiate LQT1 from LQT2 were 97% and 96%, those from LQT3 were 97% and 100%, and those from Control were 97% and 100%, respectively, when Δ mean corrected Q-Tend $\geq$35ms at steady state was used. The sensitivity and specificity to differentiate LQT2 from LQT3 or Control were 100% and 100%, respectively, when Δ mean corrected Q-Tend $\geq$80ms at peak was used.

CONCLUSIONS Epinephrine infusion is a powerful test to predict the genotype of LQT1, LQT2, and LQT3 syndromes as well as to improve the clinical diagnosis of genotype-positive patients, especially those with LQT1 syndrome.

KEYWORDS Arrhythmia; Diagnosis; Long QT syndrome; Catecholamines; Genes

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Table 1 Clinical characteristics of LQT1, LQT2, LQT3, and control groups in prospective study

	LQT1 (n = 31)	LQT2 (n = 23)	LQT3 (n = 6)	Control (n = 30)
Age [yr (range)]	21 ± 14 (4–55)	27 ± 16 (6–61)	21 ± 16 (7–43)	29 ± 15 (6–64)
Age <15 yr	16/31 (52%)	7/23 (30%)	3/6 (50%)	5/30 (17%)
Female sex	17/31 (55%)	16/23 (70%)	3/6 (50%)	16/30 (53%)
Baseline heart rate (beats/min)	67 ± 9	66 ± 12	60 ± 10	72 ± 13
Peak heart rate with Epi (beats/min)	99 ± 14	96 ± 16	95 ± 10	99 ± 13
Steady-state heart rate with Epi (beats/min)	85 ± 12	76 ± 14	70 ± 12	79 ± 13
Baseline QTc interval (ms)	470 ± 41†	503 ± 33*	506 ± 41*	408 ± 19
Syncope or aborted cardiac arrest	14/31 (45%)	12/23 (52%)	2/6 (33%)	(0%)
Beta-blockers	(0%)	(0%)	(0%)	(0%)

Values are given as mean ± SD where indicated. Epi = epinephrine; QTc = corrected QT.
*P < 0.05 vs LQT1 and control.
†P < 0.05 vs control.

The congenital long QT syndrome (LQTS) is a hereditary disorder caused by mutations in genes of the potassium and sodium channels or membrane adapter located on chromosomes 3, 4, 7, 11, 17, and 21.[1–4] Among the LQT1, LQT2, and LQT3 forms, which account for approximately two thirds of genotyped patients, cardiac events are more often associated with sympathetic stimulation (physical or emotional stress) in LQT1 than in either LQT2 or LQT3 syndrome.[5–8] Concordant with the influence of sympathetic stimulation, beta-blockers are the most effective in LQT1 syndrome.[9,10] Therefore, genotyping of LQTS is of major importance because it would be helpful in managing and treating patients more effectively.[11] Preliminary studies by our and other groups have demonstrated the differential response of dynamic QT interval to epinephrine infusion between LQT1, LQT2, and LQT3 syndromes,[12,13] indicating the potential diagnostic value of the epinephrine test for genotyping the three forms. The present study was designed to test this hypothesis.

Methods

Study design and population

First, we retrospectively analyzed the response of ECG parameters to epinephrine infusion in 15 LQT1 patients (5 families), 10 LQT2 patients (5 families), 8 LQT3 patients (2 families), and 10 healthy volunteers (Control), some of whom were included in our previous study.[12] The best ECG criteria separating LQT1, LQT2, LQT3, and Control patients were selected. Then, we prospectively conducted an epinephrine test in 42 probands who were clinically diagnosed as having congenital LQTS, their 67 family members, and 10 new healthy volunteers. The best ECG criteria with the epinephrine test derived from the retrospective study were applied in a blinded fashion to differentiate LQT1, LQT2, LQT3, and Control groups in a total of 119 subjects. Molecular screening, which was performed after the epinephrine test, identified 31 genotype-positive LQT1 patients (12 families), 23 genotype-positive LQT2 patients (12 families), 6 genotype-positive LQT3 patients (3 families), 10 genotype-negative LQT1 patients (9 families), and 10 genotype-negative LQT2 patients (4 families). The study population of the prospective study included the 31 LQT1, 23 LQT2, and 6 LQT3 patients. The data from the 10 genotype-negative LQT1 patients, 10 genotype-negative LQT2 patients, and 10 healthy volunteers were pooled and referred to as Control group, because there were no significant differences in the clinical and ECG characteristics among the three groups. In the remaining 29 patients including 15 probands (15 families), no responsible mutations were identified in any LQTS genes. There were no significant differences among LQT1, LQT2, LQT3, and Control groups with regard to age, percentage of age <15 years old, gender, baseline heart rate, and peak and steady-state heart rate with epinephrine in the prospective study (Table 1). Percentage of syncope or aborted cardiac arrest was no different among LQT1, LQT2, and LQT3 groups (Table 1). The baseline corrected QT intervals in LQT2 and LQT3 groups were significantly longer than that in the LQT1 group; those in the LQT1, LQT2, and LQT3 groups were all significantly longer than that in the Control group (Table 1). Genotyping of LQTS was reviewed and approved by our Ethical Review Committee, and written informed consent was obtained from all patients or their parents when the patients were younger than 20 years. All epinephrine tests were conducted in the National Cardiovascular Center as part of a clinical

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evaluation of LQTS patients. We previously reported that the oral beta-blocker propranolol (0.5–2 mg/kg) completely suppressed the effects of epinephrine on repolarization parameters[14]; therefore, no subjects took beta-blockers at the time of the epinephrine test in either the retrospective or prospective study. Among a total of 93 genotyped LQTS patients in the retrospective and prospective studies, 85 patients were transferred to our hospital for initial evaluation of LQTS without any medications including beta-blockers, and the epinephrine test could be conducted in the absence of beta-blockers. Appropriate therapies, including beta-blockers, were started after the evaluation of LQTS. In the remaining 8 patients (3 LQT1 and 5 LQT2), beta-blockers were withheld during the evaluation of LQTS, including the epinephrine test, and then reinstated.

Clinical diagnosis

LQTS-affected individuals were diagnosed based on the ECG criteria of Keating et al,[15] including a corrected QT ≥470 ms in asymptomatic individuals and a corrected QT >440 ms for males and >460 ms for females associated with ≥1 of the following: (1) stress-related syncope, (2) documented torsades de pointes, or (3) family history of early sudden cardiac death. The LQTS score was calculated using the diagnostic criteria of Schwartz et al.[16]

Recording of standard 12-lead ECG

A standard 12-lead ECG was recorded using an FDX6521 (Fukuda Denshi Co., Tokyo, Japan) with the patient in the supine position. These ECG data were digitized using analog-to-digital converters at a sampling rate of 1,000 samples per second per channel.

Measurements

Measurement of the ECG parameters was performed against five averaged QRS complexes by an off-line computer with an analysis program developed by our institution. Q-Tend was defined as the interval between QRS onset and the point at which an isoelectric line intersected a tangential line drawn at the minimum dV/dt point of a positive T wave or at the maximum dV/dt point of a negative T wave. When a bifurcated or secondary T wave (pathologic U wave) appeared, it was included as part of the measurement of the Q-Tend, but a normal U wave, which was apparently separated from a T wave, was not included. Q-Tpeak was defined as the interval between QRS onset and the peak of the positive T wave or the nadir of the negative T wave. When the T wave had a biphasic or a notched configuration, the peak of the T wave was defined as that of dominant T deflection. Q-Tend, Q-Tpeak, and Tpeak-end (Q-Tend − Q-Tpeak) as an index of transmural dispersion of repolarization were measured automatically from all 12-lead ECGs, corrected by Bazett's method, and averaged among all 12 leads. Data of corrected Q-Tend, Q-Tpeak, and Tpeak-end, which were measured simply from lead V_5, also were evaluated. As an index of spatial dispersion of repolarization, dispersion of the corrected Q-Tend was defined as the interval between the maximum and the minimum of the corrected Q-Tend among the 12 leads.

Epinephrine administration

A bolus injection of epinephrine (0.1 μg/kg) was immediately followed by continuous infusion (0.1 μg/kg/min). The 12-lead ECG was continuously recorded during sinus rhythm under baseline conditions and usually for 5 minutes under epinephrine infusion. The effect of epinephrine on both RR and QT intervals usually reached steady-state conditions 2 to 3 minutes after the start of epinephrine infusion. Epinephrine infusion for >5 minutes was avoided, and ECG monitoring was continued for another 5 minutes after epinephrine infusion to detect the possible occurrence of torsades de pointes. The ECG data as a representative of the peak epinephrine effect were collected 1 to 2 minutes after the start of epinephrine infusion when the RR interval was the shortest, whereas the data as a representative of the steady-state epinephrine effect were collected 3 to 5 minutes after the start of epinephrine infusion.

Statistical analysis

Data are expressed as mean ± SD, except for those shown in Figure 3, which are expressed as mean ± SEM. Repeated-measures two-way ANOVA followed by the Scheffé test was used to compare measurements made before and after epinephrine infusion and to compare differences between groups (STATISTICA, 98 Edition). Repeated-measures one-way ANOVA followed by the Scheffé test was used to compare changes (Δ) in the measurements with epinephrine between groups. Differences in frequencies were analyzed by Chi-square test. A two-sided $P < .05$ was considered statistically significant.

Results

Retrospective study

Best ECG criteria to differentiate LQT1, LQT2, LQT3, and Control groups

The retrospective study as well as our previous study[12] suggested the differential response of the mean corrected Q-Tend interval to epinephrine test among LQT1, LQT2, and LQT3 groups. The mean corrected Q-Tend intervals were more prominently prolonged at peak epinephrine effect in LQT1 and LQT2 groups than in either the LQT3 or the Control group. On the other hand, they remained pro-

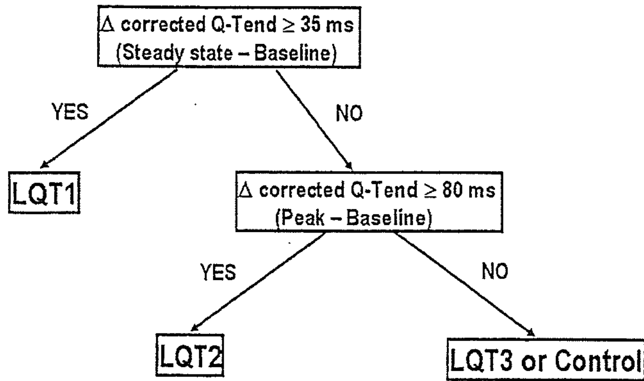

Figure 1 Flow chart for predicting genotype with the epinephrine test.

longed at steady-state epinephrine effect only in the LQT1 group but not in the other three groups.

Figure 1 illustrates a flow chart for predicting LQT1, LQT2, LQT3, and Control patients with the epinephrine test derived from the retrospective study. If the Δ mean corrected Q-Tend was ≥35 ms at steady-state epinephrine effect, the patient was expected to be affected with LQT1 syndrome. If not, and the Δ mean corrected Q-Tend was ≥80 ms at peak epinephrine effect, the patient was expected to be linked to LQT2 syndrome. If not, the patient was expected to be an LQT3 or Control patient.

Prospective study

Differential responses of ECG parameters to epinephrine infusion

Figure 2 illustrates ECG lead V_4 under baseline conditions and at peak and steady-state epinephrine effects in representative LQT1, LQT2, LQT3, and Control patients.

Figure 3 shows composite data of the ECG parameters under baseline conditions and at peak and steady-state epinephrine effects in the four groups of the prospective study. Under baseline conditions, the mean corrected Q-Tend and Q-Tpeak were significantly longer in the LQT1, LQT2, and LQT3 groups than in the Control group; both were significantly longer in the LQT2 and LQT3 groups than in LQT1 group (Figure 3A and 3B). The mean corrected Tpeak-end was significantly greater in the LQT2 group than in the LQT3 or Control group (Figure 3C). The dispersion of corrected Q-Tend was significantly larger in the LQT1 and LQT2 groups than in the Control group (Figure 3D). The mean corrected Q-Tend and Q-Tpeak were dramatically prolonged at peak epinephrine effect (470 ± 41 to 596 ± 56 ms, 385 ± 34 to 480 ± 53 ms; $P < .05$, respectively) and remained prolonged at steady state (549 ± 55 ms, 438 ± 50 ms; $P < .05$ vs baseline, respectively) in the LQT1 group (Figure 3A and 3B, closed circles). The mean corrected Tpeak-end also was markedly increased at peak epinephrine effect (85 ± 11 to 115 ± 19 ms; $P < .05$), and remained increased at steady state (111 ± 17 ms; $P < .05$ vs baseline) as a result of a greater prolongation in the mean corrected

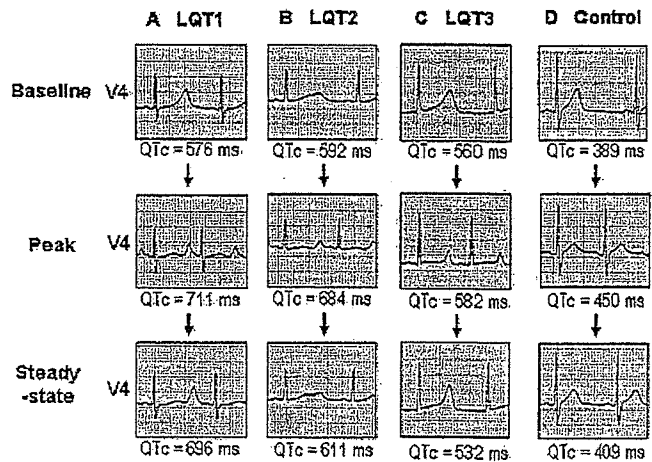

Figure 2 ECG lead V_4 under baseline conditions and at peak and steady-state epinephrine effects in LQT1 (**A**), LQT2 (**B**), LQT3 (**C**), and Control (**D**) patients. The mean corrected Q-Tend was prominently prolonged from 576 to 711 ms at peak epinephrine effect and remained prolonged at steady state (696 ms) in the LQT1 patient. In the LQT2 patient, the mean corrected Q-Tend also was dramatically prolonged from 592 to 684 ms at peak but returned to the baseline level at steady-state (611 ms). It was much less prolonged (LQT3: 560 to 582 ms, Control: 389 to 450 ms) at peak in the LQT3 and Control patients than in either the LQT1 or LQT2 patient and was shortened to the baseline level at steady state (532, 409 ms).

Q-Tend than in the mean corrected Q-Tpeak at both peak and steady-state conditions (Figure 3C, closed circles). The mean corrected Q-Tend and Q-Tpeak also were dramati-

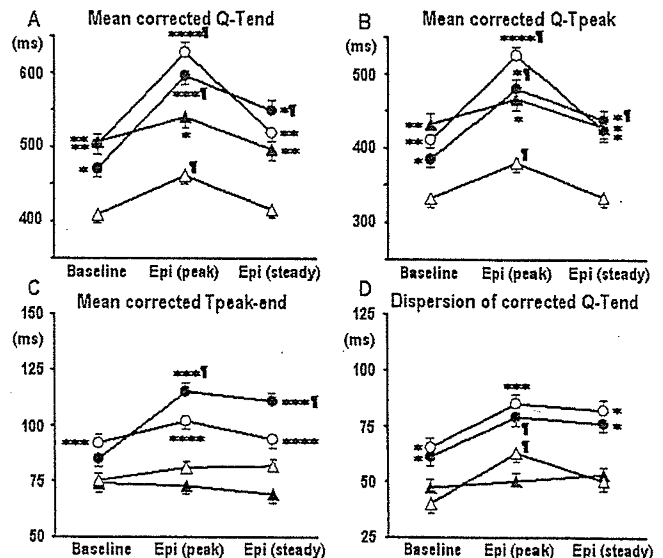

Figure 3 Composite data of the mean corrected Q-Tend (**A**), Q-Tpeak (**B**), Tpeak-end (**C**), and dispersion of corrected Q-Tend (**D**) under baseline conditions and at peak and steady-state epinephrine effects in LQT1 *(closed circle)*, LQT2 *(open circle)*, LQT3 *(closed triangle)*, and Control *(open triangle)* groups of the prospective study. *$P < .05$ vs Control; **$P < .05$ vs LQT1 and Control; ***$P < .05$ vs LQT3 and Control; ****$P < .05$ vs LQT1, LQT3, and Control; ¶$P < .05$ vs baseline.

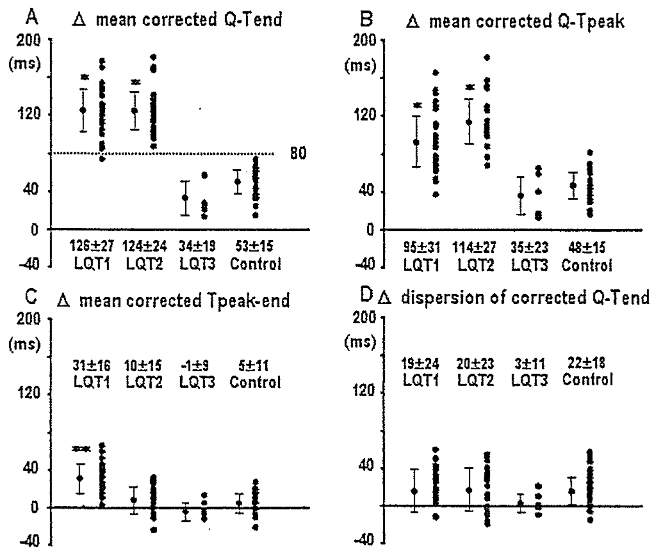

Figure 4 Composite data of changes (Δ) in the mean corrected Q-Tend (**A**), Q-Tpeak (**B**), Tpeak-end (**C**), and dispersion of corrected Q-Tend (**D**) between baseline conditions and peak epinephrine effects in LQT1, LQT2, LQT3, and Control groups of the prospective study. *$P < .05$ vs LQT3 and Control; **$P < .05$ vs LQT2, LQT3 and Control.

cally prolonged at peak epinephrine effect (503 ± 33 to 627 ± 30 ms, 411 ± 26 to 525 ± 32 ms; $P < .05$, respectively) in the LQT2 group but returned to baseline levels at steady state (518 ± 38 ms, 424 ± 36 ms; P = NS vs baseline, respectively; Figure 3A and 3B, open circles). The mean corrected Tpeak-end was unchanged with epinephrine (92 ± 23 to 102 ± 18 to 94 ± 19 ms) in the LQT2 group (Figure 3C, open circles). The mean corrected Q-Tend and Q-Tpeak were much less prolonged at peak epinephrine effect (LQT3: 506 ± 41 to 540 ± 28 ms; P = NS, 432 ± 40 to 467 ± 26 ms; P = NS, Control: 408 ± 19 to 461 ± 19 ms, 332 ± 17 to 380 ± 23 ms; $P < .05$, respectively) in the LQT3 and Control groups than in the LQT1 or LQT2 group and were shortened to the baseline levels at steady state (LQT3: 496 ± 37 ms, 427 ± 30 ms; Control: 415 ± 18 ms, 333 ± 19 ms; P = NS vs baseline, respectively) (Figure 3A and 3B, closed triangles and open triangles). The mean corrected Tpeak-end was unchanged with epinephrine (LQT3: 74 ± 7 to 73 ± 4 to 69 ± 10 ms; Control: 75 ± 8 to 81 ± 13 to 82 ± 11 ms) in the LQT3 and Control groups (Figure 3C, closed triangles and open triangles). The dispersion of corrected Q-Tend was increased at peak epinephrine effect in the LQT1 and Control groups (LQT1: 61 ± 21 ms, 79 ± 27 ms; Control: 40 ± 14 ms, 63 ± 19 ms; $P < .05$, respectively).

Figure 4 illustrates the changes (Δ) in the ECG parameters between baseline conditions and peak epinephrine effects in the four groups of the prospective study. Both the Δ mean corrected Q-Tend and Q-Tpeak were no different between the LQT1 and LQT2 groups, but they were significantly greater than those in the LQT3 and Control groups ($P < .05$; Figure 4A and 4B). No significant differences were observed in the Δ mean corrected Q-Tend and Q-Tpeak between the LQT3 and Control groups. The Δ mean corrected Tpeak-end was significantly greater in the LQT1 group than in the other three groups ($P < .05$; Figure 4C). No significant differences were observed in the Δ dispersion of corrected Q-Tend among the four groups (Figure 4D). As suggested by the retrospective study, the Δ mean corrected Q-Tend ≥80 ms at peak epinephrine effect could most effectively differentiate the LQT1 and LQT2 groups from the LQT3 or Control group (Figure 4A).

Figure 5 illustrates Δ in the ECG parameters between baseline conditions and steady-state epinephrine effects in the four groups of the prospective study. The Δ mean corrected Q-Tend, Q-Tpeak, and Tpeak-end were significantly greater in LQT1 than in the other three groups ($P < .05$; Figure 5A–5C). The Δ mean corrected Q-Tend was significantly larger in the LQT2 than in LQT3 group ($P < .05$; Figure 5A). There were no significant differences in the Δ dispersion of corrected Q-Tend among the four groups (Figure 5D). As suggested by the retrospective study, the Δ mean corrected Q-Tend ≥35 ms at steady-state epinephrine effect could most effectively differentiate the LQT1 group from the other three groups (Figure 5A).

Improvement of clinical diagnosis with epinephrine test

The sensitivity (i.e., penetrance) and specificity for identifying genotype-positive LQT1, LQT2, and LQT3 patients by the ECG diagnostic criteria before and after steady-state epinephrine effects were evaluated in the prospective study.

The sensitivity for identifying genotype-positive LQT1 patients among the LQT1 and Control groups was low under baseline conditions; 68% (21/31) using the ECG

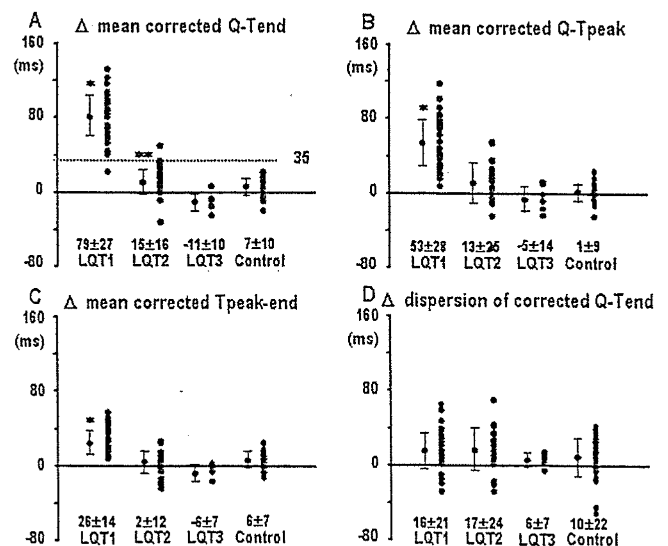

Figure 5 Composite data of changes (Δ) in the mean corrected Q-Tend (**A**), Q-Tpeak (**B**), Tpeak-end (**C**), and dispersion of corrected Q-Tend (**D**) between baseline conditions and steady-state epinephrine effects in LQT1, LQT2, LQT3 and Control groups of the prospective study. *$P < .05$ vs LQT2, LQT3 and Control; **$P < .05$ vs LQT3.

Table 2 Prediction of genotype with the epinephrine test in prospective study

	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Accuracy
LQT1 vs LQT2	97%	96%	97%	96%	96%
Δ Mean corrected Q-Tend ≥35 ms (Steady state-Baseline)	(90%)	(83%)	(88%)	(86%)	(87%)
LQT1 vs LQT3	97%	100%	100%	86%	97%
Δ Mean corrected Q-Tend ≥35 ms (Steady state-Baseline)	(90%)	(100%)	(100%)	(67%)	(92%)
LQT1 vs control	97%	100%	100%	97%	98%
Δ Mean corrected Q-Tend ≥35 ms (Steady state-Baseline)	(90%)	(97%)	(97%)	(91%)	(93%)
LQT2 vs LQT3	100%	100%	100%	100%	100%
Δ Mean corrected Q-Tend ≥80 ms (Peak-Baseline)	(91%)	(100%)	(100%)	(75%)	(93%)
LQT2 vs control	100%	100%	100%	100%	100%
Δ Mean corrected Q-Tend ≥80 ms (Peak-Baseline)	(91%)	(90%)	(88%)	(93%)	(91%)

Percentages in parentheses indicate those calculated by data measured simply from ECG lead V_5. Δ Increase with epinephrine.

diagnostic criteria, 68% (21/31) when an LQTS score ≥4 was used, and 74% (23/31) when a score ≥2 was used. The specificity was 100% (30/30) regardless of the criteria. The sensitivity was substantially improved by measurement of the mean corrected Q-Tend at steady-state epinephrine effect without the expense of specificity (100% [30/30]); 87% (27/31), 81% (25/31), and 90% (28/31), respectively.

The sensitivity for identifying genotype-positive LQT2 patients among the LQT2 and Control groups was relatively high under baseline conditions; 83% (19/23), 83% (19/23), and 96% (22/23), respectively. The sensitivity was further improved at steady-state epinephrine effect to 91% (21/23), 91% (21/23), and 96% (22/23), respectively, without the expense of specificity (100% [30/30]).

The sensitivity for identifying genotype-positive LQT3 patients among the LQT3 and Control groups under baseline conditions was 83% (5/6), 50% (3/6), and 100% (6/6), respectively, which was unchanged at steady-state epinephrine effect by any of the three criteria.

Prediction of genotype with epinephrine test

Table 2 illustrates the predictive values with the epinephrine test for genotyping in the prospective study. The Δ mean corrected Q-Tend ≥35 ms at steady-state epinephrine effect could differentiate LQT1 from the LQT2, LQT3, or Control group with predictive accuracy ≥90%. The Δ mean corrected Q-Tend ≥80 ms at peak epinephrine effect could differentiate LQT2 from LQT3 or Control group with predictive accuracy of 100%. Even if we calculated the predictive values by the Δ corrected Q-Tend, which was measured simply from ECG lead V_5, the predictive accuracy still was high (≥80%).

At molecular screening, the responsible mutations could be identified in the first targeted gene suspected by the epinephrine test in all of the 12 LQT1, 12 LQT2, and 3 LQT3 families of the prospective study.

Response to epinephrine test in genotype-unknown patients

Figure 6 illustrates Δ mean corrected Q-Tend at peak (Figure 6A) and steady-state (Figure 6B) epinephrine effects in the 29 patients (15 probands and 14 family members) of the prospective study in whom the responsible mutations could not be identified in any LQTS genes. Among the 15 probands, the response to the epinephrine test was LQT1 pattern in 11 probands and LQT2 pattern in 4 probands. Among the 14 family members, the response was LQT1 pattern in 3 members, LQT2 pattern in 3 members, and LQT3 or Control pattern in 8 members. Even though these 29 patients without causative mutations were included in the analysis for genotype prediction, the positive predictive values were 67% (30/31+14) for LQT1 syndrome and 73% (22/23+7) for LQT2 syndrome, respectively.

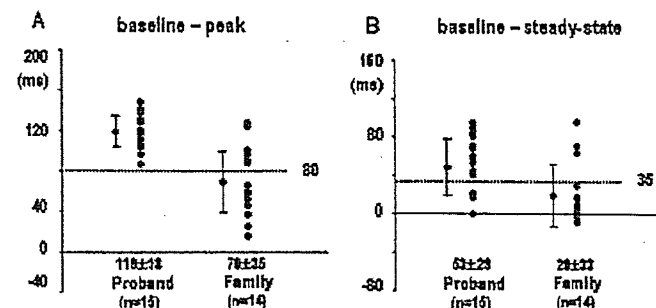

Figure 6 Composite data of changes (Δ) in the mean corrected Q-Tend between baseline conditions and peak epinephrine effects (A) and between baseline conditions and steady-state epinephrine effects (B) in the 29 patients (15 probands and 14 family members) of the prospective study in whom the responsible mutations could not be identified in any LQTS genes.