KATP channels [62] after activation of the bradykinin B_2 receptor [61–63].

While bradykinin is considered to be an endogenous trigger of preconditioning, as mentioned above, activation of the δ-opioid receptor by opioid peptides has also been investigated as a potent exogenous trigger of preconditioning in isolated human cardiac tissue [64]. Many reports have suggested that KATP channels are the downstream mechanism of cardioprotection, but the relative contribution of either sarcolemmal [65] or mitochondrial [62] KATP channels to the triggering phase is still controversial.

4.5. Other emerging factors

In addition, some of the cell membrane ion exchangers are also possible candidates for the mediation of cardioprotection due to preconditioning such as the Na^+/H^+ exchanger (NHE) [66], Na^+/Ca^{2+} exchanger (NCX), and Na^+/K^+ ATPase [67]. Because these channels contribute directly to ischemia/reperfusion injury, they have been studied as components of the cardioprotection afforded by ischemic preconditioning. However, the latest studies have positioned them as pharmacological therapeutic targets separately from their effect on preconditioning.

One of the emerging concepts is the involvement of the mitochondrial transition pore (MTP) which interacts directly with the activated PKC-ε isoform and inhibits calcium-induced pore opening to decrease mitochondrial permeability [68] or decrease mitochondrial release of cytochrome *c* in response to various stresses. On the other hand, recent studies have proposed a role of Bcl-2, an antiapoptotic protein that is also induced by ischemic preconditioning, in ischemic metabolic changes of the mitochondria through association with components of the respiratory chain.

Furthermore, Lochner et al. [69] reported that transient β-adrenoceptor stimulation mimics preconditioning independently of PKC, while we have found that activation of protein kinase A and subsequent transient p38MAPK activation confers a similar cardioprotective effect in dogs [70] (Fig. 5). The molecular mechanism of this PKA-induced transient activation of p38MAPK, which may help to explain how p38MAPK is transiently activated, may be that PKA phosphorylates the catalytic site of protein tyrosine phosphatase and inhibits dephosphorylation of p38MAPK, which leads to physiological augmentation of p38MAPK activity independently of PKC [71,72]. In addition, we observed in an in vivo dog model that Rho and Rho-kinase play an important role downstream of PKA during sustained ischemia to confer cardioprotection of ischemic preconditioning independent of PKC (published in abstract of Ref. [113]). These findings might also be useful with regard to clinical application because widely used drugs can elicit this effect without a negative inotropic effect even in acute heart failure. Taken together with the cardioprotection induced by α_1-adrenoceptor stimulation that we described previously, it seems possible that both the α_1-PKC pathway and the β-PKA pathway may "independently but synergistically" mediate catecholamine-induced preconditioning in response

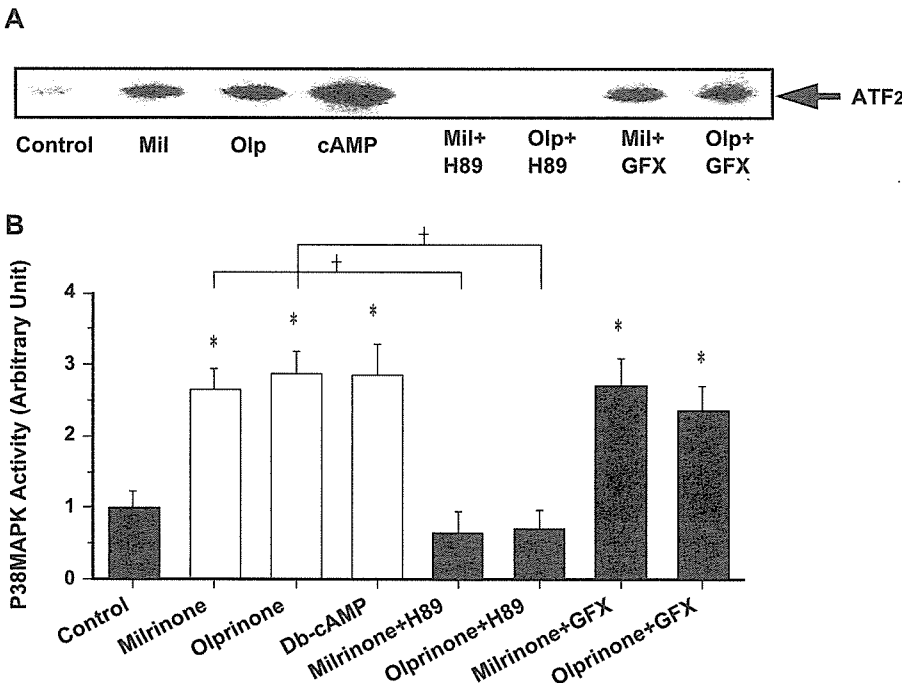

Fig. 5. p38MAPK activity in an anesthetized dog model (estimated by the phosphorylation of its substrate). Myocardial samples were obtained after 30 min of drug infusion. (A) Representative immunoblot specimens and (B) changes of the mean value (mean ± S.E.M.). Transient stimulation by cyclic AMP caused activation of p38MAPK which was blocked by a PKA inhibitor but not by a PKC inhibitor, suggesting that PKA can activate p38MAPK independent of PKC. Bars show the mean values ($n=4$). Mil: milrinone; Olp: olprinone; GFX: GF109203X. $^{\dagger}P<0.05$; $^{*}P<0.05$ vs. control.

to various stimuli, including brief periods of ischemia, which may help to provide novel insights into the development of strategies against ischemic injury.

5. Cellular mechanisms of late ischemic preconditioning

The late phase of preconditioning, which was initially reported as the "second window of preconditioning" [11,12] in 1993, occurs about 24 h (the actual time varies among species and experimental models) after transient preconditioning and lasts for much longer than the early phase.

As described in the Introduction, the late phase of ischemic preconditioning has essentially different signaling mechanisms from the early phase (classical ischemic preconditioning); that is, late phase preconditioning involves processes that require a longer time to occur such as modulation of the genes for channel proteins, receptor proteins, enzymes, molecular chaperon proteins, or immune factors.

Recent studies have revealed that these two types of preconditioning share some triggering mechanisms, while certain mediators and effectors are reported to be specific for the late phase of preconditioning. Here, we will discuss various mediators or effectors involved in late preconditioning.

5.1. Adenosine and PKC: booster of the triggering mechanism

In 1994 and 1995, Baxter et al. reported that either the adenosine receptor [73] or PKC [74] was involved in triggering the late phase of preconditioning, as is the case for the early phase. Many subsequent in vitro studies have suggested that a specific subtype of PKC, especially a Ca^{2+}-independent subtype (PKC-ε) [24], may be a critical mediator of late preconditioning in contrast with the involvement of multiple PKC subtypes (α/δ/ε) [19–24] in the early phase. Experiments using PKC-ε overexpressing mice have shown that PKC-ε increases cardioprotection against ischemia/reperfusion injury [75] with remaining whole PKC activity. Although an accumulating number of reports support this concept, there are some issues that need to be discussed further because continuous activation of PKC generally leads to myocardial hypertrophy [76,77] or apoptotic myocyte death. Furthermore, sustained ischemia with or without ischemic preconditioning decreases the total level of PKC activity. Furthermore, PKC activation during ischemia is reported to be harmful [78], and specific actions of PKC-ε are reported to be blunted by potent inhibitors of Ca^{2+}-sensitive PKC such as GF109203X.

The adenosine-triggered cardioprotection that appears after 24 to 72 h is reported to have some unique characteristics [13]. Adenosine-triggered cardioprotection involves both adenosine A_1 and A_3 receptors, and it only reduces the infarct size rather than having an influence on stunning or arrhythmias. Initial transient activation of adenosine A_1 receptors causes activation of subcellular signaling pathways. such as p38MAPK or HSP27 [79], and increases the synthesis of manganese superoxide dismutase (Mn–SOD) [80], but the role of A_3 receptors is yet to be established.

5.2. Nitric oxide (NO): multiple roles in preconditioning

Nitric oxide (NO) is an endogenous vessel relaxant that was initially identified as endothelium-derived relaxation factor (EDRF). Its physiological role in the cardiovascular system is quite similar to that of adenosine especially its actions related to adenosine A_1 receptor activation [81], such as a negative inotropic effect, vasodilator effect, and inhibition of platelet aggregation or cytokine production, with all of these actions being mediated through cyclic GMP as a messenger [82]. Furthermore, some authors have suggested that an increase of NO prior to sustained ischemia plays a part in either ischemic or pharmacological preconditioning [83], indicating that NO could act as either a trigger or an effector of preconditioning which also resembles the role of adenosine. However, these two factors are reported to be independently and differentially involved in the triggering of preconditioning [13,84]. Furthermore, in contrast with the established role of NO in the triggering of late phase cardioprotection, whether NO can trigger the early phase of cardioprotection remains controversial. This is because a negative role of endogenous NO in early preconditioning has been reported [85] which implies a different position in the mechanism of preconditioning from that of adenosine. NO is reported to share downstream pathways with various other triggers [such as acetylcholine (Ach), bradykinin, opioids, and phenylephrine), involving PKC, KATP channels, and the generation of reactive oxygen species (ROS) in the triggering phase [62], but its other downstream effects are unknown. Dawn and Bolli [82] reported that PKC activates NF-κB after several hours which leads to the increased expression of inducible NO synthase (iNOS) after 4 to 8 h.

Although we have mentioned a direct cardioprotective effect of NO, it also forms peroxynitrite ($ONOO^-$) and promptly loses its bioactivity as a stimulator of cyclic GMP in the presence of high levels of free radicals. It remains unclear whether peroxynitrite is beneficial or harmful to the myocardium because it has been reported to damage vascular endothelial cells [86], whereas another study showed that formation of $ONOO^-$ is required for NO to act as a trigger of preconditioning [87]. In addition, some studies using mice with gene targeting have indicated that endothelial NOS (eNOS) is responsible for cardioprotection [88]. It could probably be concluded that the overall effect of NO on the cardiovascular system is beneficial, although the results might vary among species and experimental models, and may also depend on other circumstances such as the presence of free radicals.

5.3. Products of the arachidonic acid cascade: emerging mediators

The most recent studies on the downstream pathways have addressed cardioprotection mediated by the induction of enzymes in the arachidonic acid cascade {12-lipoxygenase (12-LO) [89] and cyclooxygenase-2 (COX-2) [90]}. 12-LO is located downstream of the opioid receptors, and its cardioprotective effect is linked with an increase of its product (12-HETE). iNOS and its product NO may activate COX-2 in the late phase of cardioprotection [91], but the contribution of its products is unknown. These findings are interesting with respect to possible links with further downstream internal mediators that are believed to be the final effectors of preconditioning, as well as the concept that the immune response may be a direct effector of the cardioprotection afforded by preconditioning, and are also encouraging with regard to clinical application. Further

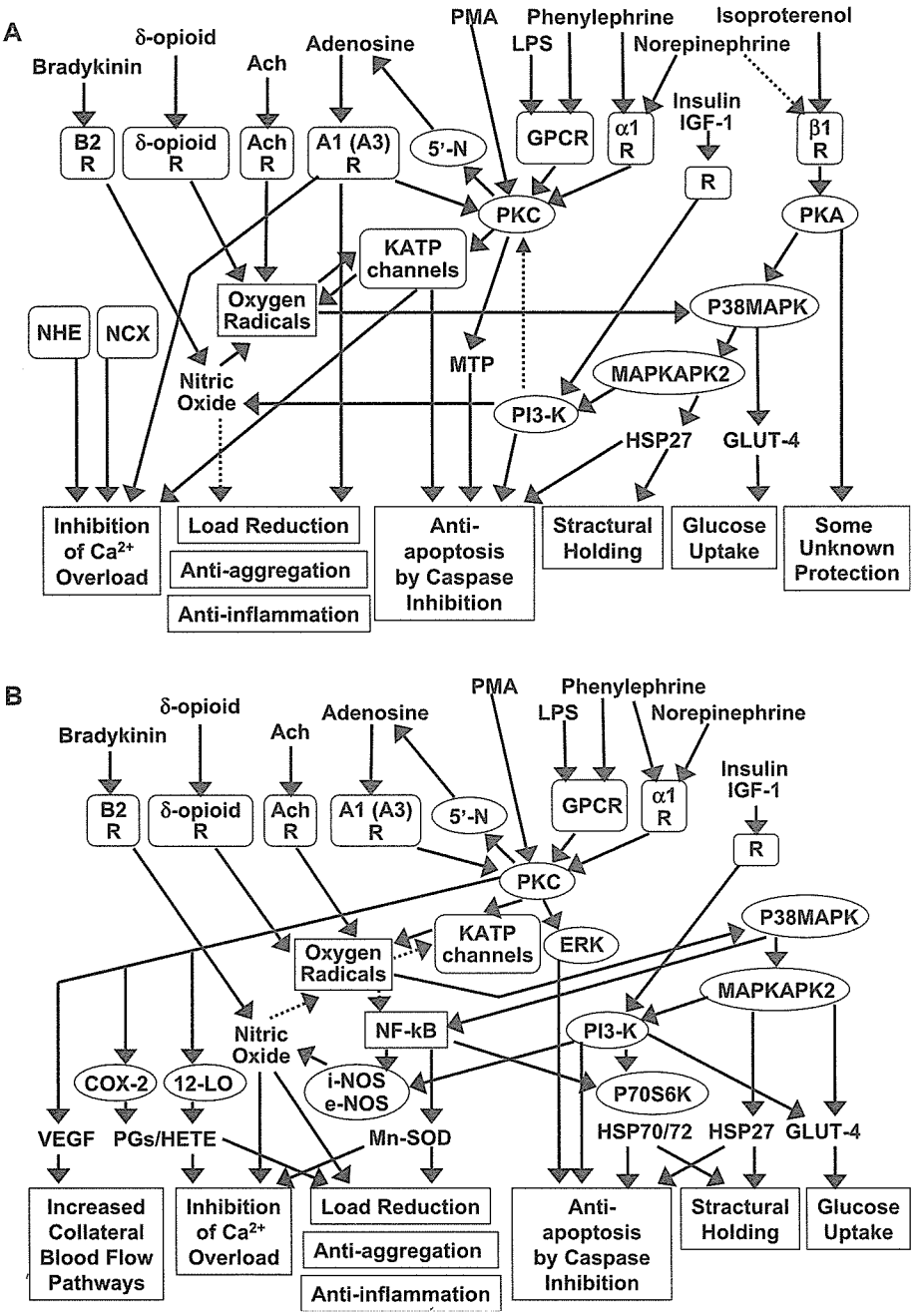

Fig. 6. Diagrams showing the mechanisms of early phase (A) and late phase (B) cardioprotection. Each trigger causing ischemic and pharmacological preconditioning is listed at the top, and the major effects of early phase cardioprotection are indicated at the bottom. Dashed arrow indicates the mechanism of premature or controversial issue.

investigations are warranted because many clinical reports have established strong relationships between reduction of the local/systemic inflammatory response and a better prognosis for patients with various kinds of heart disease including IHD and/or cardiac failure.

5.4. Heat shock proteins and superoxide dismutase (SOD)

It has been reported that the expression of HSP70/72, a high molecular weight heat shock protein, increases to reach a peak at 24 to 48 h after preconditioning ischemia [94]. HSP 70/72 acts as a molecular chaperone, but its cardioprotective effect may be related to other factors induced by HSPs as was described for HSP27. Induction of these HSPs during preconditioning is blunted by inhibition of PKC [92], suggesting that PKC also mediates cardioprotection through the generation of HSPs.

On the other hand, ischemic preconditioning also activates Mn–SOD, and the peak level is seen at 24 h after preconditioning [93]. Because increased expression of Mn–SOD has been detected in a model of HSP72 overexpression [94] and neutralizing antibodies against either IL-1β or TNF-α can abolish the induction of Mn–SOD by intermittent heat stress [95], these factors may also be sequentially involved in ischemic or heat stress-induced preconditioning.

However, some studies [96] have indicated that exogenous Mn–SOD does not protect the myocardium against ischemic damage or reperfusion injury. This suggests that free radicals generated inside or outside cardiac myocytes may play different roles during the process of ischemia and reperfusion injury. Moreover, ischemic preconditioning might largely contribute to cardioprotection by reduction of free radical formation within cells, although further investigations are needed.

5.5. Growth factors: contribution of neovascularization

Although in vivo studies, including ours, indicate that early cardioprotection afforded by preconditioning is not related to changes of regional blood flow, some reports have suggested that an increase in collateral blood flow per se contributes to late cardioprotection [97]. It has also been found that this increase of collateral flow is accompanied by increased regional expression of vascular endothelial growth factor (VEGF) [98], while the increase of collateral flow and VEGF are both blunted by adenosine receptor blockade [99]. The main feature of the cardioprotection afforded by VEGF is thought to be neovascularization in the infarct area [99].

Finally, our current understanding of the intricate mechanisms involving triggers, mediators, and effectors of preconditioning, as well as the factors that subsequently regulate gene expression or protein synthesis and their major cardioprotective effects are outlined in Fig. 6 (Fig. 6A for early phase and Fig. 6B for late phase, respectively).

6. Translational clinical strategies based on cardioprotection by preconditioning

Recent clinical investigations have confirmed that prodromal angina (transient angina attack(s) prior to myocardial infarction) limits the size of infarcts and their lethal consequences (i.e., ventricular fibrillation and acute heart failure), thus improving the prognosis of patients with acute myocardial infarction [10,100]. Although we cannot directly apply "preconditioning ischemia" to patients with heart disease because it needs to be done prior to an acute cardiac event, there have been many attempts to transform the cardioprotective effect of ischemic preconditioning into useful therapeutic strategies for various kinds of heart disease, e.g., by using potent final effectors to treat acute disease or by inducing preconditioninglike effects that can prevent (or ameliorate) chronic disease. Here, we summarize some of these attempts and the encouraging results that have been obtained.

6.1. Acute pharmacological cardioprotection against ischemia/reperfusion injury

Currently, there are at least two strategies that have been tried. One method involves the treatment of cardiac injuries, and the other is primary prevention by continuous promotion of cardioprotective factors. Adjunctive therapy following PTCA or PTCR is a typical example of the former strategy. Among the many candidates suggested by experimental studies, there are two agents that have also been shown to ameliorate reperfusion injury in the clinical setting: adenosine (or its parent compound ATP) and the KATP channel opener nicorandil.

6.1.1. Adenosine and its parent compounds

In the AMISTAD trial [101], patients with acute anterior myocardial infarction who underwent continuous intravenous infusion of adenosine together with PTCR had smaller infarcts and better functional recovery than those without adenosine infusion. However, this study had certain fundamental problems because angiography was not used to evaluate the infarct in the acute phase and obvious improvement of cardiac function was only seen in the patients with anterior infarction. Therefore, our group is now conducting a clinical trial (the COAT study) to investigate whether intracoronary administration of ATP for a few minutes after successful recanalization by PTCA can reduce the infarct size and improve functional recovery. We are also performing a large-scale multicenter clinical trial (the J-WIND trial of the National Cardiovascular Center involving 54 institutions in Japan) to test the acute effect of either nicorandil or calperitide (recombinant human ANP) on

reperfusion injury after successful PTCA, and over 700 patients have been enrolled.

6.1.2. KATP channel openers

Marked reduction of regional myocardial perfusion and severe impairment of contraction (hypokinesis) of the heart frequently occur after reperfusion despite successful recanalization of a major coronary artery, and this "no-reflow" phenomenon causes prolonged cardiac dysfunction. Some experimental [102] and clinical trials using either intravenous [103] or intracoronary [104] administration have shown that the no-reflow phenomenon can be prevented after successful PTCA, together with a reduced infarct size and better recovery from hypokinesis, by continuous infusion of a KATP channel opener (nicorandil) just after the procedure. The pathophysiological mechanism of the no-reflow phenomenon and the manner in which nicorandil improves it remain controversial, although some authors have proposed that the no-reflow phenomenon is based on vasospasm [104] or microembolization [105]. Furthermore, the component of the KATP channel (mitochondrial or sarcolemmal) involved in this action of nicorandil is also controversial because many reports have confirmed that nicorandil has a stronger effect (10- to 100-fold) on the mitochondrial KATP channel than on the sarcolemmal KATP channel in vitro [106], whereas we observed that nicorandil was much less selective for the mitochondrial KATP channel in an in vivo dog model [41], and we found that a specific sarcolemmal KATP channel blocker (HMR1098) could completely abolish the effect of nicorandil on the no-reflow phenomenon (unpublished data). Interestingly, the recent IONA study [107] has shown that long-term oral administration of nicorandil to patients with moderate/severe stable angina reduces the risk of cardiac events and improves their prognosis.

Despite the development of some successful interventions derived from cardioprotection by preconditioning, it is also true that these methods are still less effective than ischemic preconditioning itself. To establish safe and more potent strategies that are applicable in the clinical setting, further investigations will be needed.

6.2. Extended concept of cardioprotection based on preconditioning

6.2.1. Cardioprotection in patients with nonischemic heart disease

Recent findings have indicated that continuous exposure of the heart to mediators of ischemia and reperfusion injury, such as signaling via GPCR, Ca^{2+} overload, free radicals, or cytokines, leads to inadaptive hypertrophy or contractile dysfunction. Ischemic preconditioning can attenuate the effect of these deleterious stimuli, and thus may also help to prevent both cardiac hypertrophy and heart failure. Furthermore, it could be suggested that brief and repeated stimulation of the signals leading to hypertrophy or heart failure might protect the nonischemic heart against further progression of the underlying disease.

Some studies along these lines have already been performed. For example, intermittent pharmacological treatment has been tried using β-adrenergic stimulators [108]. All of the studies done so far have used the triggering mechanism of preconditioning, and the duration of stimulation has been limited because continuous stimulation fails to protect the heart [109] as is the case with ischemic preconditioning.

In contrast, we have attempted to continuously modulate one of the potent effectors of preconditioning. In a pilot study by our group [110], an inhibitor of adenosine reuptake (dipyridamole) was continuously administered to 26 patients with stable moderate heart failure in addition to conservative therapy, resulting in better cardiac function and improved exercise capacity after 6 months. We are now performing a multicenter trial (the ROAD trial) on a larger number of subjects. In this context, methods of genetic modulation (including gene transfer) might also be applicable to activate the cardioprotective effectors of ischemic preconditioning.

6.2.2. Cardioprotective gene therapy

Some frontier attempts to promote gene therapy have also been reported. By stimulation of critical mediators or potent effectors in various experimental studies, a cardioprotective effect has been successfully obtained through genetic modulation of PKC-ε [75], HSPs [94], SODs [111], and NO synthases [88,91]. The problem is how to identify the target clinical population for these therapies before signs or symptoms develop in order to decrease cardiac damage. An eventual answer might be provided by individual genetic analysis and tailored therapy, but these strategies might initially be applied to a restricted population of patients at high risk or with prior attacks. However, patients with any obvious signs of cardiac disorders, such as ischemia, arrhythmia, or heart failure, should be encouraged to undergo treatment by these strategies in the near future.

7. Conclusion

Currently, there is an increasing need for "evidence-based medicine" (EBM) as the foundation of therapy to meet current requirements for security, efficacy, and cost-effectiveness. However, it is quite difficult for most of the procedures that have been well supported in experimental studies or pilot clinical studies to accumulate sufficient evidence for certification as standard therapies according to EBM. Furthermore, statistical analysis clearly indicates that some of the most powerful therapeutic strategies that are presently supported by strong clinical evidence, such as use of statins (HMG-CoA reductase inhibitors) for cholesterol-lowering therapy or angiotensin-converting enzyme inhibitors for cardiovascular disease, may only benefit a small percentage of all patients. On the other hand, a strong

cardioprotective effect of ischemic preconditioning has been demonstrated by experimental and clinical studies without exception. This supports the safety and efficacy of cardioprotection by ischemic preconditioning which has been intensively investigated for over 15 years since its detection. We continue to hope that the ultimate essence of cardioprotection will be found within the treasure box named "ischemic preconditioning" in the near future.

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Cardiovascular Drugs and Therapy 18 189–195 2004

CLINICAL PHARMACOLOGY AND DRUG STUDIES

Roles of Systemic Nitric Oxide Metabolites for Human Coronary Circulation

Yoji Kato[1], Yoshiyuki Kijima[1], Masafumi Kitakaze[2], Tetsuo Minamino[3], Yasuhiko Matsu-ura[1], Kazuhiko Hashimura[2], Takenori Yasuda[1], Tetsuya Ueda[1], Isao Mori[1], and Masatsugu Hori[3]

[1]*The Cardiovascular Division, Ishinkai Yao General Hospital, Osaka 581-0036, Japan;* [2]*Cardiovascular Division of Medicine, National Cardiovascular Center, Osaka 565-8565, Japan;* [3]*Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan*

Summary. **Several previous studies have suggested decreased bioactivity of nitric oxide (NO) in coronary artery diseases using NO synthase inhibitors. Nitrite is delivered as bioactive NO in the forearm circulation. However, the role(s) of NO metabolites in the systemic and coronary circulation are still unknown. The aim of this study was to investigate the role(s) of systemic NO metabolites for human coronary circulation in patients with and without coronary spastic angina (CSA). Twenty-nine patients with chest symptoms were enrolled to perform the acetylcholine (Ach) provocative test. Blood was sampled from the aorta at baseline, and from the great cardiac vein at baseline and after Ach to measure plasma levels of nitrate and nitrite (NOx). The epicardial left anterior descending artery was examined by quantitative angiography. The patients were divided into the two groups according to the Ach provocative test. In the non-CSA group, the NOx uptake across the coronary circulation correlated with the endothelium-dependent vasoresponse to Ach ($r = -0.61$, $p < 0.05$) and NOx levels of the aorta also correlated ($r = -0.72$, $p < 0.005$), which suggested the compensatory increase of systemic NOx levels for impaired endothelial function. In the CSA group, the NOx uptake across the coronary circulation did not correlate with the vasoresponse to Ach ($r = 0.29$, $p = 0.28$). However, NOx levels of the aorta correlated with vasosensitivity to Ach ($r = 0.61$, $p < 0.005$). The higher systemic NOx levels correlated well with the vasodilator responsiveness to Ach. These results suggest that systemic NOx is delivered into the coronary circulation as bioactive NO to preserve endothelial function in the non-CSA patients, and to attenuate Ach-induced vasoconstriction in the CSA patients. There is a possibility that systemic NOx plays a complementary role on impaired coronary vasoregulation.**

Key Words. **nitrite/nitrate, acetylcholine, coronary vasospasm**

Introduction

Nitric oxide (NO) has been widely accepted as an endothelium-derived relaxing factor, which is produced from L-arginine through the catalytic reaction by nitric oxide synthase [1–3]. Several previous studies have provided evidence of NO activity in the human coronary circulation using a NO synthase inhibitor [4,5]. Furthermore, it has been demonstrated that the gradients of nitric oxide metabolites are frequently negative in the human coronary circulation, being indicative of uptake or consumption within the microcirculation. However, the physiological and pathophysiological significance of the negative gradient have not been elucidated. Recently, Gladwin et al. suggested that the circulating nitrite is bioactive and provides a delivery gradient of intravascular NO [6]. To investigate the role(s) of systemic NO metabolites in the human coronary circulation, we measured NO metabolites of systemic and coronary circulation in patients with and without coronary spastic angina (CSA).

Methods

Study patients

Twenty nine patients (16 males and 13 females, mean age 55 ± 12 years) who complained of chest symptoms were enrolled (Table 1). They did not have any history of myocardial infarction, acute heart failure or cardiogenic shock. The patients with renal dysfunction, whose creatinine clearance was less than 50 ml/min according to Cockcroft formula [7], were excluded. Their echocardiograms did not show either left ventricular dysfunction (ejection fraction <50%) or valvular disease. Their coronary arteries were angiographically normal or of minimal change.

Address for correspondence: Yoji Kato, MD, KATO Cardiovascular Clinic 10-10, Dejima-3, Sakai, Osaka 590-0834, Japan. Tel.: 81-722-41-0016; Fax: 81-722-41-0033; E-mail: kato-cl@ka2.so-net.ne.jp

Table 1. Patients characteristics

	Non-CSA group	CSA group	*p*-value
Gender (M/F)	7/6	9/7	ns
Age	53 ± 10	56 ± 14	ns
Total cholesterol	200 ± 25	205 ± 39	ns
Hypertension	3/10	3/13	ns
Diabetes	1/12	3/13	ns
Smoking	5/8	4/12	ns

Acetylcholine provocative test

To diagnose CSA, the patients were subjected to the acetylcholine (Ach) provocative test [8]. All antianginal agents except sublingual nitroglycerin were suspended at least for 48 hours prior to the catheterization. After the angiography and blood sampling for the nitrite and nitrate (NOx) measurements at baseline, 25 μg of acetylcholine (Ach 25 μg) dissolved in 10 ml saline were injected at a rate of 1ml/sec into the left coronary artery. A temporary pacing catheter was inserted into a right ventricle to prevent transient bradycardia caused by Ach. Coronary angiograms were recorded 1 min after the injection of Ach 25 μg to evaluate the vasoresponse to Ach. At the time the ECG showed no ischemic change even in the spasm group (Fig. 1). We regarded the vasoresponse to Ach 25 μg as the endothelium-dependent vasoresponse to Ach in the non-CSA group, and as vasosensitivity to Ach in the CSA group. If the spasm did not develop, 50 and 100 μg Ach was injected for the purpose of diagnosing CSA. Twelve-lead electrocardiography was continuously recorded during the Ach provocative test. When an ischemic electrocardiographic change was detected, 2–4 mg of isosorbide dinitrate was injected into the coronary artery to release coronary spasm (Fig. 1). The definition of the ischemic electrocardiographic change consisted of: (1) ST-segment elevation or depression more than 1 mm; or (2) sustained T wave inversion in any of precordial leads.

Blood sampling and nitric oxide measurement

According to the conventional Seldinger's technique, a 6-Fr Judkins catheter was positioned at the orifice of the left coronary artery. A 6-Fr coronary sinus catheter (Goodman, Japan) was positioned at the great cardiac vein (GCV). Two minutes after the baseline angiography, Ach 25 μg was injected into a left coronary artery. Two ml of blood was sampled from the aorta and the GCV at baseline simultaneously and from the GCV at 40 seconds after the intracoronary injection of Ach 25 μg (Fig. 1). The plasma level of NO was evaluated by measurement of NO*x*, stable end products of NO [9], using the Griess reagent as described previously [10]. NO*x* uptake across coronary circulation was defined as follows:

NOx uptake across coronary circulation at baseline:
NO*x* of the aorta at baseline—NO*x* of the GCV at baseline

NOx uptake across coronary circulation at Ach 25 μg:
NO*x* of the aorta at baseline—NO*x* of the GCV at Ach 25 μg

Coronary artery diameter measurement

Coronary angiograms were recorded with a cineangiographic system (Toshiba, Japan). The epicardial coronary diameter was evaluated using a computer-assisted

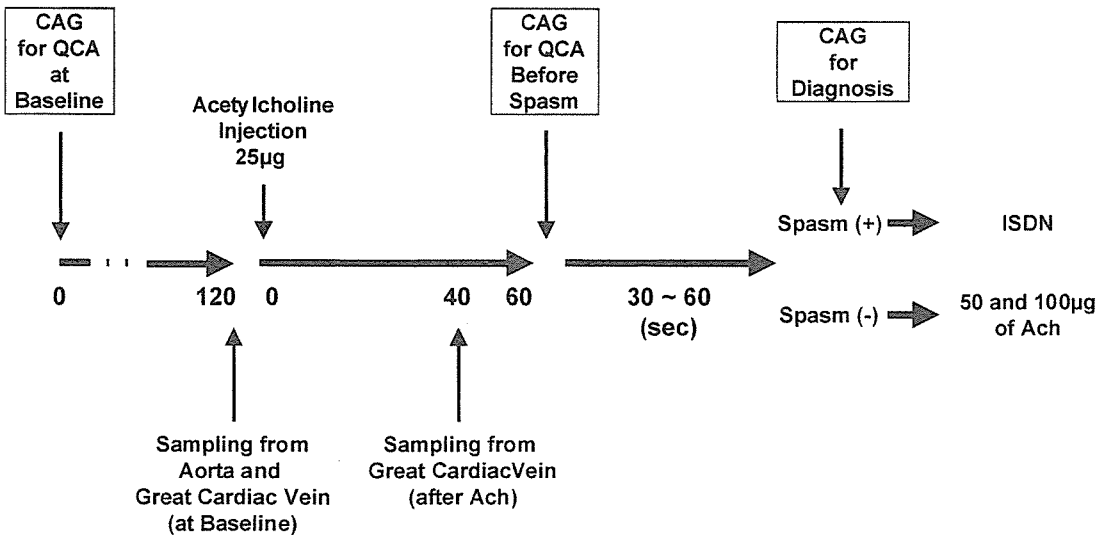

Fig. 1. The protocol of this study is shown. Mean epicardial diameters were measured 60 seconds after 25 μg of acetylcholine before developing spasm. CAG: coronary angiography, QCA: quantitative coronary angiography, Ach: acetylcholine, ISDN: isosorbide dinitrate.

program, QCA-CMS (MEDIS, Netherlands) in all patients. An appropriate view that permitted clear visualization of the left anterior descending artery was selected. The view angle, the distance from the x-ray focus to the object, and the distance from the object to the image intensifier were maintained constant during the study. An end-diastolic frame of the arteriogram was selected. The coronary diameters were measured by an examiner who did not know clinical characteristics of the patients. The 6F Judkins catheter was used for calibrating the arterial diameter.

To evaluate percent change of epicardial coronary artery after Ach 25 μg, we measured a mean coronary diameter throughout proximal and middle segments of a left anterior descending artery. The analyzed segments spanned from the origin of the left anterior descending artery to the bifurcation of the second diagonal branch (segment length 47.1 ± 7.2 mm). Cineangiograms were performed at the baseline and at 60 seconds after Ach 25 μg. The vasoresponse to Ach 25 μg was calculated as follows: Vasoresponse to Ach 25 μg(%) = $(D_{Ach} - D_{Base})/D_{Base} \times 100$, where D_{Base} and D_{Ach} represented a mean epicardial diameter at the baseline and at 60 seconds after the injection of Ach 25 μg, respectively.

The protocol was approved by the Institutional Ethical Committee at Ishinkai Yao General Hospital. Written informed consent was obtained from each patient.

Statistical analysis

All data in the table were shown as mean ± SD. Statistical comparison within a group was performed by paired t-test and between the groups by unpaired t-test. Correlation between NOx levels and the vasoresponse to Ach were analyzed by simple regression test. A probability less than 0.05 was considered to be statistically significant.

Results

Classification of patients and acetylcholine provocative test

The characteristics of the patients enrolled in this study are summarized in Table 1. The patients were divided into two groups according to the results of the Ach provocative test and documented history of spontaneous chest symptom with ischemic ECG changes. In the non-CSA group, patients did not have an evidence of ischemic electrocardiogram during the 25, 50 and 100 μg doses of the Ach provocative test. In the CSA group, the 13 patients showed a positive Ach provocative test, of whom 5 patients showed ischemic ST-T changes after Ach 25 μg (cases 14–18), 4 patients did so after 50 μg of Ach (cases 19–22), 4 patients did so after 100 μg of Ach (cases 23–26), and 2 patients did so after completion of the Ach provocative test (cases 28 and 29). The one patient showed no ischemic ST-T change, but had presented with a positive Ach provocative test two years prior (case 27). Three of the CSA group also showed vasospasm in the left circumflex artery. The cases with right coronary spasm, which could not be relieved spontaneously, were excluded from this study. In the non-CSA group, 25 and 50 μg of Ach was also subsequently injected into a right coronary artery, without developing significant vasospasm. There were no statistically significant differences between the two groups in gender, age, total cholesterol level, hypertension, diabetes mellitus and smoking (Table 1).

Vasoresponse to acetylcholine in each group

In the non-CSA group, the endothelium-dependent vasoresponse to Ach 25 μg was −7.0 ± 9.6% (Table 2). In the CSA group, vasosensitivity to Ach 25 μg before spasm was −14.9 ± 15.3% (Table 2). Among these patients, the vasosensitivity to Ach 25 μg was −32.4 ± 9.1% (cases 14–18; spasm developed at Ach 25 μg), −6.1 ± 1.5% (cases 19–22; spasm developed at Ach 50 μg), −7.9 ± 8.9% (cases 23–26; spasm developed at Ach 100 μg) and −6.6 ± 5.3% (cases 27–29: Spasm developed at not this provocative test but the other situation).

NOx and vasoresponse to Ach in the non-CSA group

In the non-CSA group, NOx levels of the aorta at baseline, NOx of the GCV at baseline, and NOx of the GCV after Ach 25 μg were 8.9 ± 3.7, 8.0 ± 3.0 and 8.1 ± 1.9 μmol/L, respectively (Table 2). NOx uptake across the coronary circulation at baseline and that after Ach 25 μg were −0.9 ± 2.1 and −0.8 ± 2.4 μmol/L, respectively. However, NOx uptake across the coronary circulation correlated negatively with the endothelium-dependent vasoresponse to Ach 25 μg ($r = -0.61$, $p < 0.05$, Fig. 2A). Moreover, NOx levels of the GCV and NOx uptake across the coronary circulation tended to increase in patients with the dilative response to Ach (case 1–4, $p = 0.08$ by 2-tailed t-test). Unexpectedly, NOx levels of aorta also correlated negatively with the vasoresponse to Ach 25 μg ($r = -0.72$, $p < 0.005$, Fig. 2B). The greater NOx uptake and NOx levels of the aorta correlated well with impairment of the endothelial function.

NOx and vasoresponse to Ach in the CSA Group

In the CSA group, NOx levels of the aorta at baseline, those of the GCV at baseline, and those of the GCV after Ach 25 μg were 10.8 ± 5.2, 10.7 ± 5.9, 11.5 ± 5.7 μmol/L, respectively (Table 2). NOx uptake across the coronary circulation at baseline and that after Ach 25 μg were 0.1 ± 1.6 μmol/L and −0.7 ± 2.3 μmol/L, respectively (Table 2). NOx uptake across the coronary circulation at baseline did not correlate with the vasosensitivity to Ach ($r = 0.29$, $p = 0.28$, Fig. 3A). However, NOx levels of aorta at baseline correlated positively with

Table 2. Vasoresponses and NOx levels to acetylcholine 25 μg in the Non-CSA and CSA groups

Case	Age	Gender	*Vasoresponse to Ach 25 (%)	NOx level (μmol/L)					Ischemic change of ECG was documented at
					GCV		NOx uptake		
				Aorta	Baseline	Ach 25	Baseline	Ach 25	
Non-CSA group									
1	51	F	5.8	4.8	4.2	5.7	0.6	−0.9	–
2	57	M	3.5	4.4	5.6	7.3	−1.2	−2.9	–
3	68	F	0.0	7.1	6.7	7.1	0.4	0.0	–
4	40	M	0.0	6.5	5.3	5.6	1.2	0.9	–
5	55	F	−0.4	7.5	8.9	8.8	−1.4	−1.3	–
6	56	M	−1.5	7.5	7.0	5.7	0.5	1.8	–
7	40	F	−2.5	11.3	5.0	9.9	6.3	1.4	–
8	62	F	−11.1	12.1	10.3	11.0	1.8	1.0	–
9	48	M	−11.4	7.7	8.6	7.6	−0.9	0.1	–
10	50	F	−13.2	8.6	7.5	7.4	1.2	1.2	–
11	37	M	−13.8	11.5	10.5	10.1	1.0	1.4	–
12	55	M	−23.0	8.5	9.4	8.7	−0.9	−0.2	–
13	67	M	−23.4	18.2	15.2	10.9	3.0	7.3	–
M	53		−7.0	8.9	8.0	8.1	0.9	0.8	
SD	10		9.6	3.7	3.0	1.9	2.1	2.4	
CSA group									
14	33	M	−47.5	5.5	5.9	4.9	−0.3	0.7	Ach 25
15	50	M	−33.5	8.1	7.5	10.6	0.5	−2.5	Ach 25
16	66	M	−29.6	9.1	9.1	8.9	0.0	0.3	Ach 25
17	46	M	−26.2	8.1	7.3	6.4	0.8	1.7	Ach 25
18	75	M	−25.0	7.7	6.8	7.8	0.9	−0.1	Ach 25
19	70	F	−21.6	6.1	5.8	5.6	0.3	0.5	Ach 50
20	69	F	−14.9	15.8	12.8	14.7	3.0	1.2	Ach 50
21	77	F	3.1	12.2	11.0	12.2	1.2	0.0	Ach 50
22	38	F	9.0	23.9	28.0	24.1	−4.1	−0.2	Ach 50
23	38	M	−21.1	10.5	8.5	10.4	2.0	0.1	Ach 100
24	45	F	−5.1	6.4	5.8	6.5	0.6	−0.1	Ach 100
25	72	F	−4.1	9.6	11.7	17.7	−2.1	−8.1	Ach 100
26	54	M	−1.4	14.0	14.9	15.6	−0.9	−1.6	Ach 100
27	48	M	−12.7	11.0	11.0	10.8	0.1	0.3	Other situation
28	62	F	−4.4	5.8	6.4	6.9	−0.6	−1.1	Other situation
29	56	M	−2.8	18.9	18.8	21.3	0.1	−2.3	Other situation
M	56		−14.9	10.8	10.7	11.5	0.1	−0.7	
SD	14		15.3	5.2	5.9	5.7	1.6	2.3	

*Vasoresponse to acetylcholine (%) was defined as $(D_{Ach} - D_{Base})/D_{Base} \times 100$, where D_{Base} and D_{Ach} represent the mean epicardial diameters under the baseline condition and at 1 min after the intracoronary injection of 25 μg acetylcholine, respectively. The mean epicardial diameters were determined by the computer-based quantitative coronary angiography as described in the Methods. NOx levels were measured by Griess reagent. GCV: great cardiac vein, VA difference: veno-arterial difference, NOx: nitrite and nitrate, Ach: acetylcholine.

the vasosensitivity to Ach ($r = 0.61$, $p < 0.05$, Fig. 3B). The higher systemic NOx levels of the aorta were associated with a greater dilative vasoresponse to Ach.

No differences of NOx levels between the two groups

There were no differences in NOx levels of the aorta at baseline, of the GCV at baseline, or after Ach 25 μg. NOx uptake across the coronary circulation at baseline and that after Ach 25 μg between the non-CSA group and the CSA group was not significantly different (Table 2).

Discussion

In this study we found that (1) NOx uptake across the coronary circulation after Ach and systemic NOx levels correlated with the endothelium-dependent vasoresponse in the non-CSA group; (2) the systemic NOx

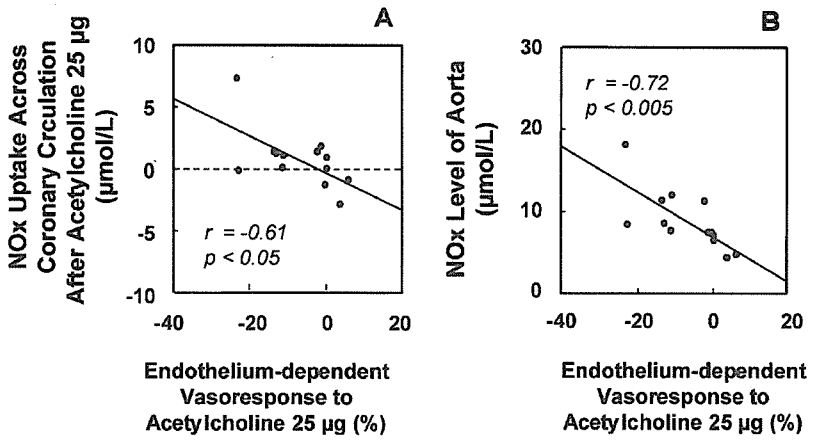

Fig. 2. *NOx uptake across the coronary circulation after acetylcholine correlated negatively with the acetylcholine-induced vasoresponse (Panel A, $r = -0.61$, $p < 0.05$). NOx level of aorta correlated negatively with the endothelium-dependent vasoresponse (Panel B, $r = -0.72$, $p < 0.005$). *Vasoresponse to acetylcholine (%) was defined as $(D_{Ach} - D_{Base})/D_{Base} \times 100$, where D_{Base} and D_{Ach} represent the mean epicardial diameters under the baseline condition and at 1 min after the intracoronary injection of acetylcholine 25 μg, respectively.*

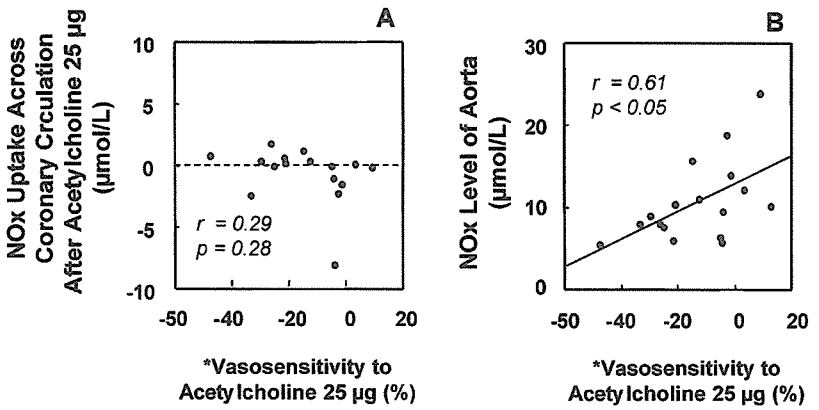

Fig. 3. *NOx uptake across the coronary circulation after acetylcholine did not correlate with vasosensitivity to acetylcholine (Panel A, $r = 0.29$, $p = 0.28$). NOx level of aorta positively correlated with vasosensitivity to acetylcholine (Panel B, $r = 0.61$, $p < 0.05$), *Vasoresponse to acetylcholine (%) was defined as in the Figure 2.*

levels correlated with vasosensitivity to Ach, however NOx uptake after Ach did not correlate in the spasm group; (3) there were no differences in NOx uptake across the coronary circulation and systemic NOx levels between the two groups.

We suggest that NOx uptake across the coronary circulation contributes to the changes in coronary vascular tone, which are modified by Ach in the non-CSA patients, but not in the CSA patients. Furthermore, the coronary vascular tone due to Ach in the CSA group is mainly attributable to the aortic levels of NOx that perfuse the spastic coronary artery. Interestingly, NOx levels of the aorta in the non-CSA group correlated inversely with coronary vascular tone, which may indicate the importance of NOx uptake across the coronary circulation in the non-CSA patients.

NOx uptake across the coronary circulation after Ach and systemic NOx in the non-CSA group

To confirm the peak NOx levels after Ach in this protocol, we performed serial NOx measurements following the injection of Ach. The study demonstrated that the NOx levels in the GCV peaked at 40 seconds. Our study and the previous report [11] found that Ach maximally increased coronary blood flow at 40 to 50 seconds. And the NOx levels in the GCV also demonstrated so rapid responsiveness to Ach, and NOx uptake after Ach across the coronary circulation correlated with the endothelium-dependent vasoresponse. These findings suggest that NOx acts as an endothelium-derived relaxing factor, as previously reported by Furchgott and Oemar [1,12]. However, why did the higher systemic

NOx levels correlate with a greater constrictor response? Nitric oxide metabolites may be elevated as oxidative stress related to endothelial dysfunction, as reported previously [13,14]. However, Gladwin and Cannon III have reported that arterial levels of nitrite are significantly higher than venous levels, suggesting delivery or metabolism within human forearm circulation, and that inhibition of NO synthase increases nitrite uptake across forearm circulation after exercise [6]. In the present study, endothelial dysfunction increased NOx uptake across the coronary circulation after Ach injection (Fig. 2A). Taken together, we speculate that complementary elevated systemic NOx is delivered into the diseased coronary circulation to preserve endothelial function as a bioactive NO.

Systemic NOx in the CSA group

NOx uptake across the coronary circulation did not correlate with the coronary vasoresponse in the CSA group. Why didn't the NOx uptake correlate with the vasoresponse to Ach? We believe that, in this setting, these vasoresponses were the net result of a combination of the endothelium-dependent vasoresponse and hypersensitivity of vascular smooth muscle to Ach [25]. However, systemic NOx levels correlated well with vasoresponsiveness in the patients with CSA. These considerations suggest that systemic NOx may be complementary to the coronary circulation, compensating for decreased coronary NO production in patients with CSA. There are several reports to support these findings; (1) some long acting Ca^{2+}-antagonists, which have been frequently used for coronary spastic angina [15], enhance nitric oxide production and increase nitrite and nitrate level [16–19]; (2) exercise induces NOx release [20] and spasm does not usually occur during exercise; (3) smoking decreases NO activity [21] and is a major trigger to induce spasm; (4) estradiol suppresses hyperventilation-induced attack in postmenopausal women with variant angina [22] and increases NOx levels [23]; (5) It is well known that, above all, coronary spasm is relieved by nitrovasodilators upon conversion into bio-active NO [24]. Systemic NOx may play a complementary role on coronary spasm, and thus enhancement of systemic NOx appears to be a goal to treat CSA.

NOx role in the spasm group

The precise mechanisms of coronary spasm have not yet been established. Hypersensitivity of smooth muscle cells [25] and abnormality of NO production [5] have been suggested. Previous investigations in which the authors used nitric oxide synthase inhibitors, have raised a controversy as to whether nitric oxide activity is deficient or preserved in spastic coronary arteries [4,5]. However, in the present study there were no differences in systemic NOx levels and NOx uptake across the coronary circulation between the two groups, which suggests that nitric oxide is just a modulator for coronary spasm.

NOx from ischemic cardiocyte in the CSA group?

In our study, NOx sampling was performed prior to the ECG change, and at this point coronary blood flow had not yet peaked by Doppler examination. There was no evidence of NOx production from ischemic cardiocytes.

Study limitation

Firstly, the changes in NOx by Ach were very subtle in this study, which is likely dependent upon the characteristics of the patients, including higher age [26] or the Japanese population [27], and upon the study methods. We measured the total amount of nitrite and nitrate, as a whole (i.e., not independently). Further examination by direct measurement of nitrite should be performed in this protocol. Nevertheless, by the current method we performed the Ach provocative test with a high concentration of Ach, since slow injection of Ach as in the previous report [28] may not demonstrate the subtle changes of NOx in response to Ach.

In conclusion, these findings suggest that systemic NO metabolites are delivered into the coronary circulation to preserve endothelial function in non-CSA patients, and to attenuate Ach-induced vasoconstriction in CSA patients. Circulating NO metabolites appear to play a complementary role on diseased human coronary circulation.

Acknowledgment

We are grateful to Mr. Ichiro Miyamoto, Koji Matsuno, and Jo Sogyuku (Ishinkai Yao General Hospital) for their co-operation in blood sampling. We also thank Ms. Tomi Fukushima, Akiko Ogai, and Junko Yamada for their technical assistance in NOx measurements. This study was supported by Grants-in-aid for Scientific Research (No. 10557068 and 10670649) from the Ministry of Education, Science, and Culture, Japan, and in part by grants from the Smoking Research Foundation in Japan.

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Effects of Ghrelin Administration on Left Ventricular Function, Exercise Capacity, and Muscle Wasting in Patients With Chronic Heart Failure

Noritoshi Nagaya, MD; Junji Moriya, MD; Yoshio Yasumura, MD; Masaaki Uematsu, MD; Fumiaki Ono, MD; Wataru Shimizu, MD; Kazuyuki Ueno, PhD; Masafumi Kitakaze, MD; Kunio Miyatake, MD; Kenji Kangawa, PhD

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-

Received April 30, 2004; revision received July 6, 2004; accepted August 23, 2004.

From the Department of Internal Medicine, National Cardiovascular Center, Osaka (N.N., J.M., Y.Y., F.O., W.S., M.K., K.M.); Cardiovascular Division, Kansai Rosai Hospital, Hyogo (M.U.); and Departments of Pharmacy (K.U.) and Biochemistry (K.K.), National Cardiovascular Center Research Institute, Osaka, Japan.

Reprint requests to Noritoshi Nagaya, MD, Department of Internal Medicine, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. E-mail nagayann@hsp.ncvc.go.jp

***Circulation* is available at http://www.circulationaha.org** **DOI: 10.1161/01.CIR.0000149746.62908.BB**

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/m^2	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

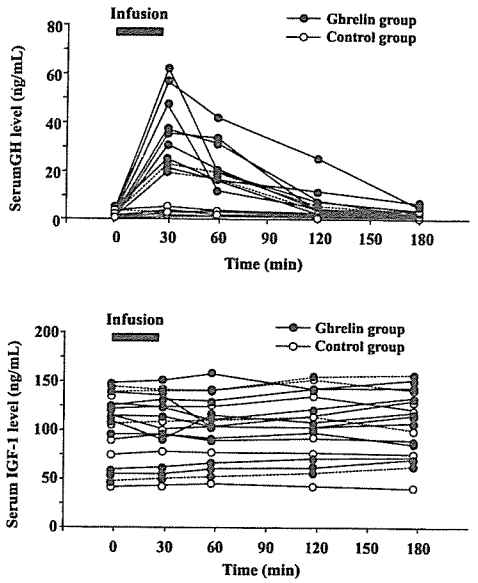

Figure 1. Changes in serum GH and IGF-1 after single administration of ghrelin. Solid line indicates cachectic patients; dotted line, noncachectic patients.

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

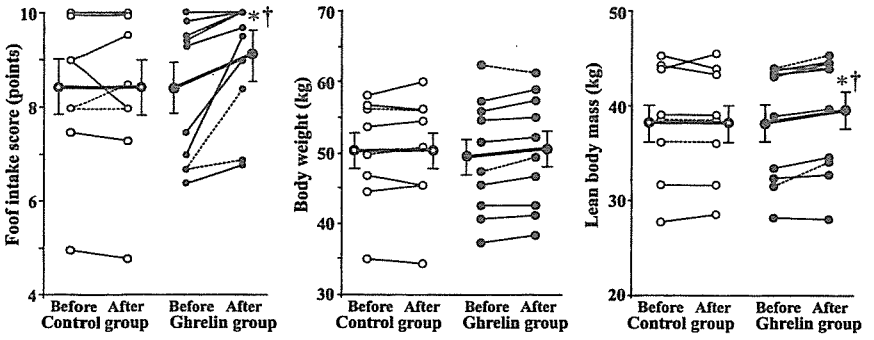

Figure 2. Food intake, body weight, and lean body mass before and after 3-week administration of ghrelin. Food intake was described semiquantitatively with 10-point scale method (0=null intake, 10=full intake). Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients.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

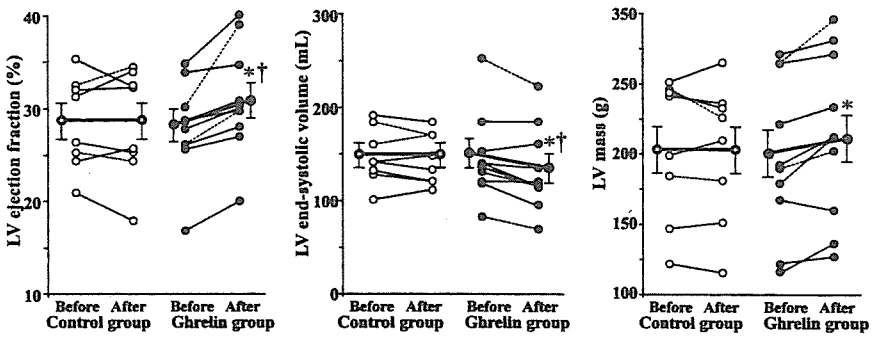

Figure 3. LV geometry and function before and after ghrelin therapy. Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients. *$P<0.05$ vs before; †$P<0.05$ vs respective control group.

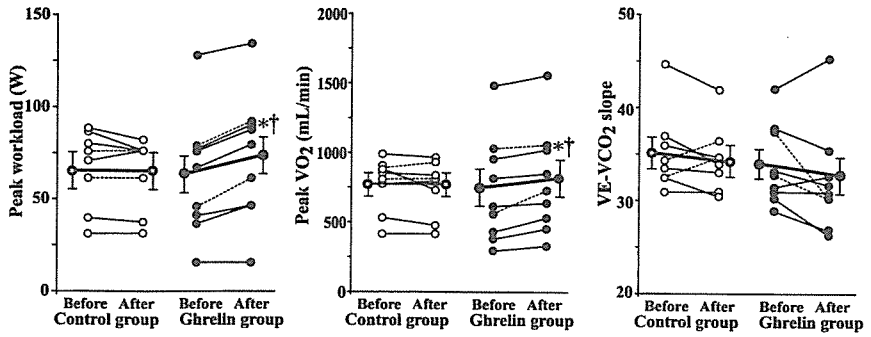

Figure 4. Exercise capacity and ventilatory efficiency before and after ghrelin therapy. Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients. *$P<0.05$ vs before; †$P<0.05$ vs respective control group.

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

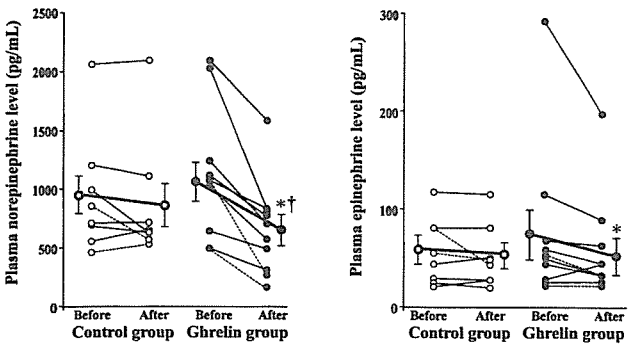

Figure 5. Plasma levels of norepinephrine and epinephrine before and after ghrelin therapy. Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients. *$P<0.05$ vs before; †$P<0.05$ vs respective control group.

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.