

Recently, adiponectin was reported to have a cardioprotective effect against ischemia-reperfusion injury (11) and hemodynamic stress (12,13) in mice. Interestingly, it has been reported that the level of N-terminal pro-brain natriuretic peptide shows a positive correlation with the plasma adiponectin concentration in patients with chronic heart failure (14).

Given these experimental and clinical observations, we hypothesized that natriuretic peptides might increase adiponectin production in patients with heart failure to protect the cardiovascular system. Accordingly, in the present study, we investigated whether natriuretic peptides could directly increase adiponectin production by these adipocytes (and the cellular mechanisms involved) and confirmed this effect on adiponectin in the clinical setting.

## Methods

**Agents.** Both human ANP and BNP were purchased from Sigma-Aldrich (St. Louis, Missouri). HS142-1, a functional guanylyl cyclase-A type receptor antagonist, was provided by Kyowa Hakko Kogyo Co., Ltd. (Mishima, Japan). A cGMP analog (8-pCPT-cGMP) and a selective cGMP-dependent protein kinase G (PKG) inhibitor ( $R_p$ -8-Br-PET-cGMP-S) were obtained from Biolog Life Science Institute (Bremen, Germany). An antibody directed against mouse adiponectin (MAB3608) was purchased from Chemicon International, Inc.

**Primary culture and in vitro study of human adipocytes.** Subcutaneous adipocytes derived from the adipose tissue of 6 women were obtained commercially together with culture medium from Zen-Bio, Inc. (Research Triangle Park, North Carolina). The donors were nonsmokers with a mean body mass index of 27.0 kg/m<sup>2</sup> (range 25.9 to 29.1 kg/m<sup>2</sup>) and an average age of 47 years (range 29 to 63 years). Cells were maintained in adipocytes maintenance medium (i.e., AM-1) containing Dulbecco's modified Eagle medium/Ham's F-12 (1:1, v/v), 3% fetal calf serum, 15 mmol/l HEPES (pH 7.4), biotin, pantothenate, human insulin, 1  $\mu$ mol/l dexamethasone, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 0.25  $\mu$ g/ml amphotericin B at 37°C in a humidified atmosphere of 95% air/5% CO<sub>2</sub>. The medium was changed every 2 days. Primary cultures of the adipocytes were used to examine the effects of natriuretic peptides (ANP or BNP) on the expression of adiponectin.

Before these experiments, the cells were plated in adipocyte basal medium (i.e., BM-1) containing Dulbecco's modified Eagle medium/Ham's F-12 (1:1, volume/volume), 15 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), biotin, and pantothenate for 24 h. Then the indicated concentrations of either natriuretic peptide (from 10<sup>-11</sup> to 10<sup>-9</sup> mol/l) were added to the BM-1 medium. After 24 h of incubation, the medium was harvested for Western blotting to measure the secretion of adiponectin, and the cells were also harvested for ribonucleic acid (RNA) analysis. The effect of each natriuretic peptide on adiponectin messenger ribonucleic acid (mRNA) levels

was determined by quantitative real-time polymerase chain reaction (PCR).

**Measurement of adiponectin.** In patients with CHF, the plasma adiponectin concentration was measured by the use of an ELISA kit (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) according to the manufacturer's protocol. Adiponectin secretion by primary cultured human adipocytes was assessed by Western blotting of the culture medium, as previously described (15), and the immunoreactive bands were quantified by densitometry (Molecular Dynamics, Sunnyvale, California).

**Reverse transcriptional-PCR.** Total RNA was extracted from adipocytes derived from human white fat with the use of RNA-Bee-RNA Isolation Reagent (Tel-Test, Inc., Gainesville, Florida). Then, 200 ng of total RNA was reversed transcribed and amplified by the use of an Omniscript RT kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The forward primers for type A guanylyl cyclase receptor (GC-A) and natriuretic peptide receptor (NPR)-C were 5'-CCAGTTCCAAGTCTTTGCCAA-GACAGCA and 5'-GGAAGACATCGTGCGCAATA, respectively, and the reverse primers for GC-A and NPR-C were 5'-CATTGTGTAGAAACAGCATGCCCTTGA-CGA and 5'-TGCTCCGGATGGTGTCACT, respectively. As a positive control, we used the samples of human cardiac tissue under the protocol approved by the institutional review board of the National Cardiovascular Center (No. 14-18) (16).

**Quantitative real-time PCR analysis.** Quantitative real-time PCR was performed as described previously (17). Oligonucleotide primers and TaqMan probes for human adiponectin and glyceraldehyde 3-phosphate dehydrogenase were purchased from Applied Biosystems (Foster City, California).

**Subjects and design of the clinical study.** We prospectively studied 30 consecutive CHF patients who were admitted to the emergency department of the National Cardiovascular Center between April and July 2006. The exclusion criteria were as follows: age >80 years, cardiogenic shock or hypotension (systolic blood pressure <100 mm Hg), and renal failure (serum creatinine >2.0 mg/dl). This study was approved by the Committee on Human Investigation of the National Cardiovascular Center, and all patients who participated gave informed consent. The 30 patients were randomized to 2 groups, a human atrial natriuretic peptide (hANP) group consisting of 15 patients who received administration of hANP and a control group consisting of 15 patients who were administered saline. In the hANP group, from immediately after the diagnosis of

### Abbreviations and Acronyms

ANP	= atrial natriuretic peptide
BNP	= brain natriuretic peptide
CHF	= congestive heart failure
GC-A	= type A guanylyl cyclase receptor
hANP	= human atrial natriuretic peptide
NPR	= natriuretic peptide receptor
PKG	= protein kinase G

acute exacerbation of CHF, hANP (0.025  $\mu\text{g}/\text{kg}/\text{min}$ ) was infused intravenously for 3 days. The study protocol did not restrict or specify any other diagnostic or therapeutic strategies. Blood for measuring the plasma adiponectin level was sampled before and 1 and 7 days after finishing the administration of hANP or saline (days 1, 4, and 10, respectively) (Fig. 3A).

**Statistical analysis.** For analysis of differences between the various treatments of adipocytes, analysis of variance was performed, followed by the appropriate post-hoc test. The differences in adiponectin levels between days 1 and 4 in each group were tested with a paired *t* test. The changes in adiponectin levels from day 1 to 4 between ANP group and saline group was tested with an unpaired *t* test. Results are expressed as the mean  $\pm$  SEM, and *p* values of  $<0.05$  were considered significant.

## Results

**Effect of natriuretic peptides on the expression and secretion of adiponectin by primary cultured human adipocytes.** First, we checked the expression of GC-A and NPR-C mRNA by using reverse transcriptional-PCR. As shown in Figure 1A, both GC-A and NPR-C mRNA was detectable in primary cultured human adipocytes. To investigate the effects of natriuretic peptides on the regulation of adiponectin production in adipocytes, we incubated primary cultured human adipocytes with recombinant ANP. When ANP was used at a concentration of  $10^{-10}$  mol/l (pathological plasma concentration), it increased adiponectin mRNA expression after 6 h of incubation and reached a maximum after 12 h (Fig. 1B). Next, we incubated human adipocytes with ANP at the concentration of from  $10^{-11}$  mol/l (normal plasma concentration) to  $10^{-9}$  mol/l (pharmacological plasma concentrations) and demonstrated enhanced adiponectin mRNA expression and adiponectin secretion into the medium in a dose-dependent manner, whereas these changes were completely inhibited by pretreatment with HS142-1 (Figs. 1C and 1D). Incubation of adipocytes with BNP also increased the expression of adiponectin mRNA in a dose-dependent manner and this effect was completely blocked by pretreatment with HS142-1 (Figs. 1E and 1F).

**Involvement of cGMP/PKG signaling in natriuretic peptide-induced synthesis of adiponectin.** Because both ANP and BNP exert their biological effects by promoting cGMP production, to investigate the role of the GC-A/cGMP/PKG signaling pathway in adiponectin production, we measured the changes of cGMP in ANP-treated primary cultured human adipocytes. We found that incubation with ANP increased the cGMP level and that this effect was blunted by co-treatment with HS142-1 (data not shown). Next, we treated human adipocytes with the cGMP analog 8-pCPT-cGMP and the PKG inhibitor ( $R_p$ )-8-Br-PET-cGMP-S. The activation of PKG by 8-pCPT-cGMP (50  $\mu\text{mol}/\text{l}$  for 12 h) produced an increase of adiponectin

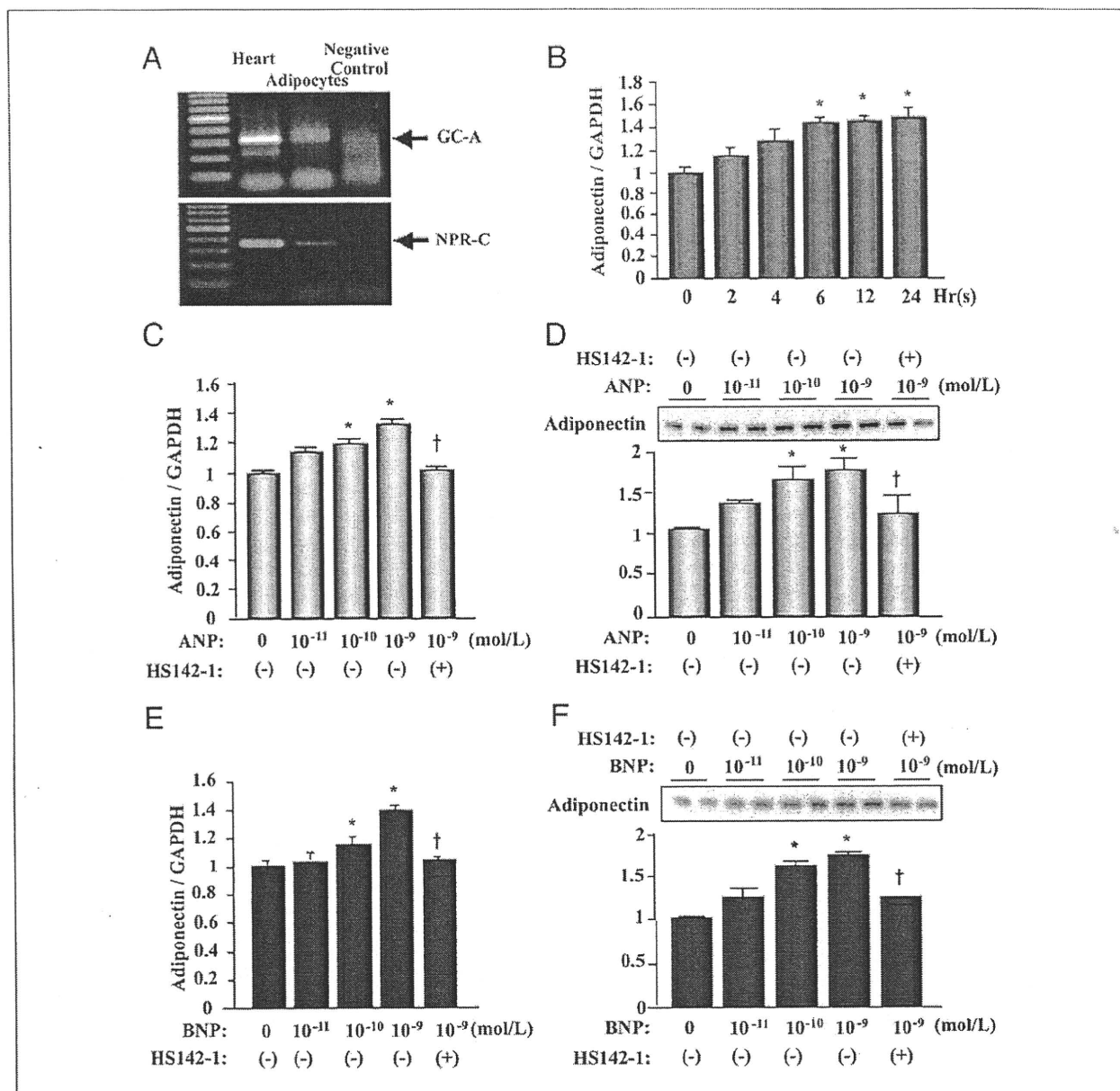
mRNA expression similar to that observed after incubation with ANP. The effect of ANP on adiponectin mRNA expression was abolished in the presence of ( $R_p$ )-8-Br-PET-cGMP-S (100 nmol/l) (Fig. 2A). Consistent with these findings, adiponectin secretion into the culture medium also was increased by stimulation of the cGMP/PKG-dependent pathway (Fig. 2B). These results suggested that natriuretic peptides promote adiponectin synthesis via the GC-A/cGMP/PKG-dependent pathway.

**Increase of plasma adiponectin levels in CHF patients treated with hANP.** To confirm the effect of natriuretic peptides on the production of adiponectin, we conducted the clinical study. Thirty consecutive patients who met the inclusion criteria were enrolled in this clinical study. Fifteen patients were randomized to the ANP group, and 15 were assigned to the saline group. Baseline variables and treatments of the 2 groups are shown in Table 1. There were no differences in baseline clinical characteristics, hemodynamics, biochemical data, or medications. There was also no significant difference in the baseline plasma level of adiponectin between the 2 groups. As shown in Figure 3B, the plasma level of adiponectin did not change throughout the study in the saline group. On the other hand, the plasma adiponectin level at 1 day after finishing the administration of hANP (day 4) was significantly increased compared with the baseline value (day 1) in the ANP group, and it returned to baseline by 7 days after the completion of hANP infusion (day 10). These results suggested that hANP infusion led to an increase of the plasma adiponectin level in patients with CHF.

## Discussion

In the present study, we demonstrated a novel effect of natriuretic peptides (ANP and BNP) on the production of adiponectin by adipocytes in both experimental and clinical studies. First, we clearly demonstrated that pathophysiological and pharmacological concentrations of either ANP or BNP increased adiponectin synthesis by primary cultured human adipocytes. Second, we showed that administration of recombinant ANP increased the plasma adiponectin level in patients with CHF.

ANP and BNP play an important role in the regulation of cardiovascular homeostasis. Their actions are primarily mediated via GC-A, which is expressed in various tissues and organs, including the kidneys, blood vessels, adrenal glands, and heart (18). Consistent with a previous report (19), we demonstrated that GC-A and NPR-C are expressed by human adipocytes. In the present study, we demonstrated a novel effect of both ANP and BNP on primary cultured human adipocytes, which was that pathophysiological or pharmacological concentrations of both peptides augmented adiponectin production by human adipocytes, with this effect being inhibited by treatment with HS142-1. Furthermore, we demonstrated that natriuretic peptides augment the production of adiponectin via a cGMP-dependent



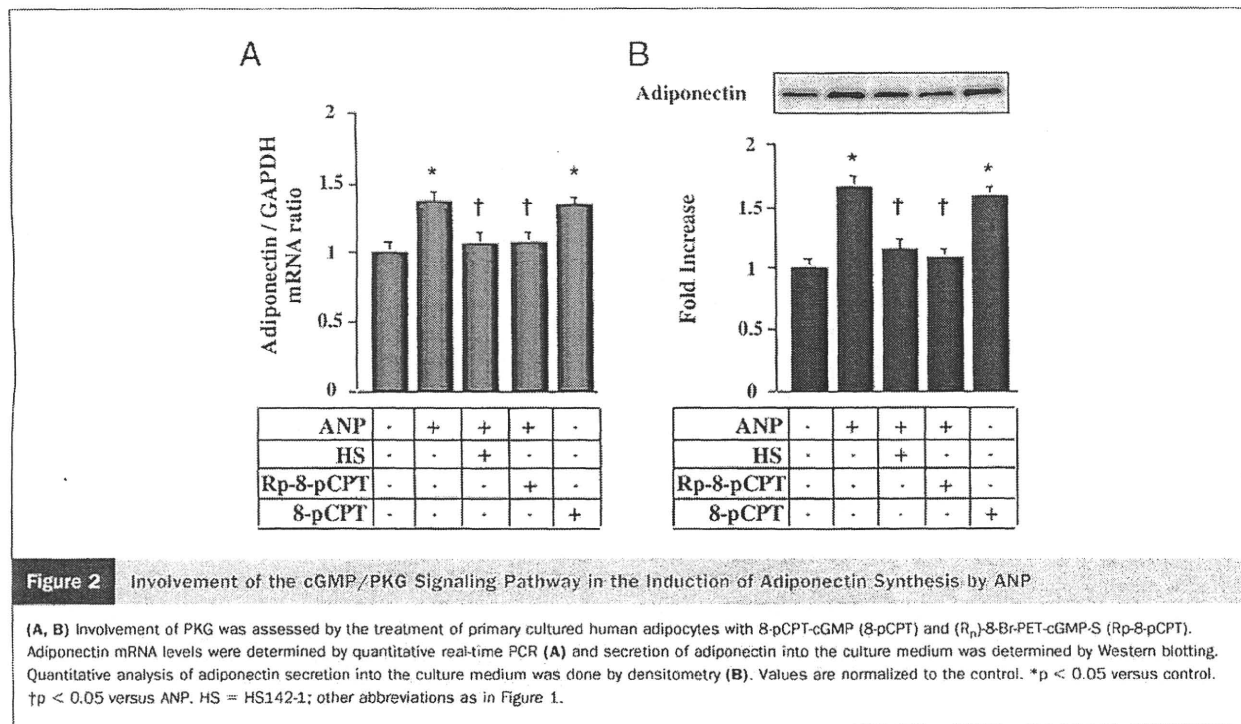
**Figure 1.** Effect of Natriuretic Peptides on the Expression and Secretion of Adiponectin by Primary Human Adipocytes

(A) Expression of GC-A receptors (top) and NPR-C (bottom) mRNA by primary cultured human adipocytes. Reverse-transcription PCR revealed expression of both GC-A receptors and NPR-C by human adipocytes. (B) Effect of ANP ( $10^{-10}$  mol/l) on the expression of adiponectin mRNA as determined by quantitative real-time PCR. (C) Dose-dependent effect of ANP on adiponectin mRNA expression, as determined by quantitative real-time PCR. Human adipocytes were treated with the indicated concentrations of ANP for 24 h. (D) Dose-dependent effect of ANP on adiponectin secretion into the culture medium. (Top) A representative Western blot of adiponectin. (Bottom) Quantitative analysis of adiponectin by densitometry. Values are normalized to the control. \* $p < 0.05$  versus control, † $p < 0.05$  versus ANP  $10^{-9}$  mol/l. (E) Dose-dependent effect of BNP on adiponectin mRNA expression, as determined by quantitative real-time PCR. (F) Dose-dependent effect of BNP on adiponectin secretion into the culture medium as determined by Western blotting. (Top) Representative Western blot of adiponectin. (Bottom) Quantitative analysis of adiponectin by densitometry. Values are normalized to the control. \* $p < 0.05$  versus control, † $p < 0.05$  versus BNP  $10^{-9}$  mol/l. ANP = atrial natriuretic peptide; BNP = brain natriuretic peptide; GC-A = type A guanylyl cyclase receptor; mRNA = messenger ribonucleic acid; NPR-C = natriuretic peptide receptor C; PCR = polymerase chain reaction.

pathway. These findings are important evidence that ANP and BNP regulate adiponectin production by human adipocytes.

Intravenous infusion of nesiritide (recombinant human BNP) has been reported to have beneficial hemodynamic

effects in patients with CHF (4,5). The use of ANP also has been reported to have beneficial effects in patients with acute myocardial infarction (20,21). These beneficial effects have been attributed to the cardiovascular-protective actions of natriuretic peptides, including diuresis, natriuresis, vaso-



dilation, and reduction of activity of the sympathetic nervous system and the renin-angiotensin-aldosterone system (3-5). In the present study, we administered recombinant ANP to patients with CHF and observed the changes of plasma adiponectin. The plasma adiponectin level of the ANP group was increased at 1 day after the finish of ANP administration compared with that in the control group, and then returned to baseline by 7 days after the completion of administration in patients with CHF.

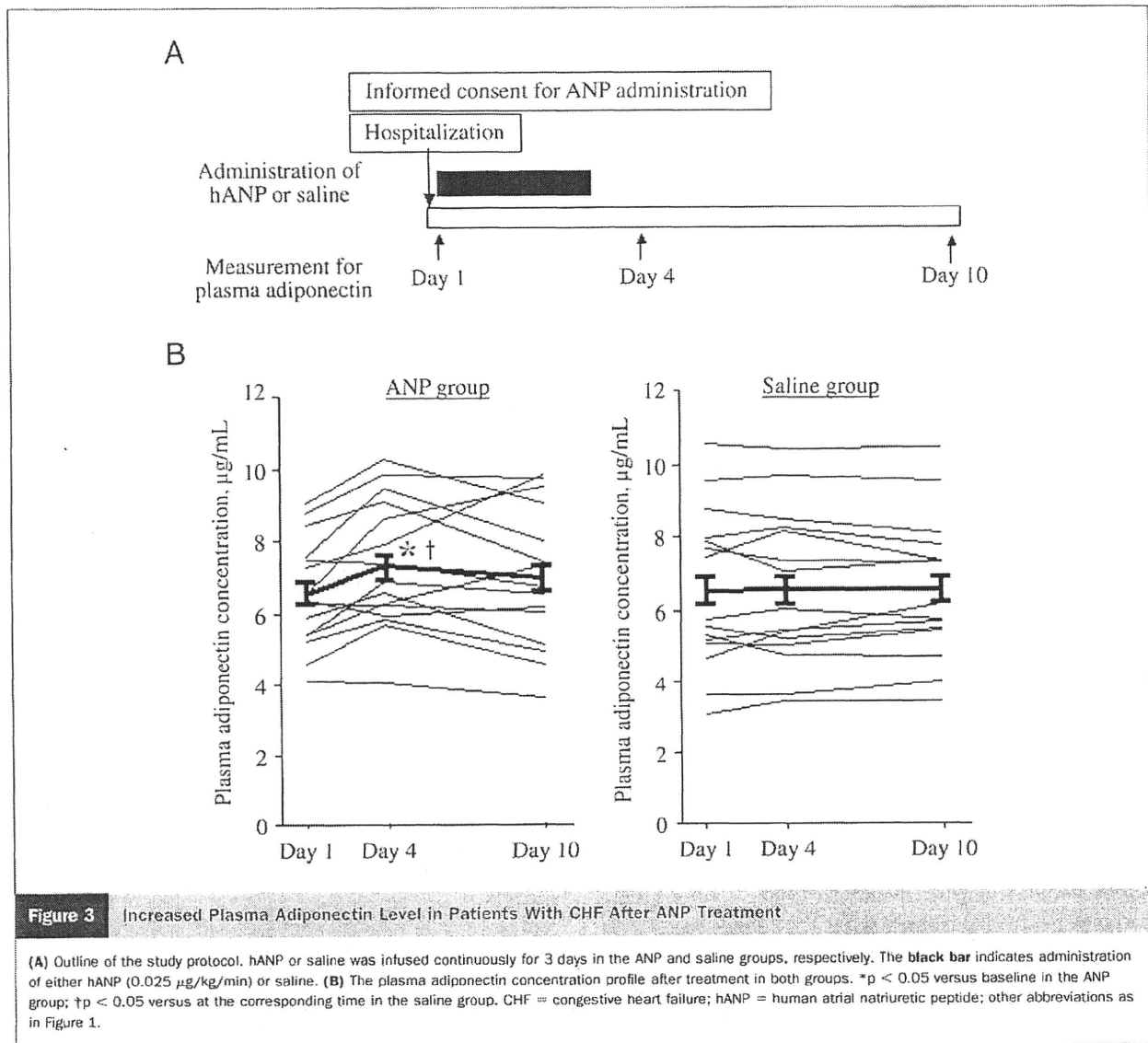
Importantly, Moro et al. (22) showed that ANP did not affect the secretion of adiponectin in human abdominal

adipose tissue from overweight women. This result may appear contradict ours, but we believe that is not the case. First, the concentration of ANP they used ( $10^{-6}$  mol/l) in the experiment of cultured adipocytes was greater than our concentration. Second, our data that recombinant ANP increased the plasma adiponectin levels were drawn from patients with heart failure, whereas the data of Moro et al. (22) were from cultured fat tissues of overweight women who underwent plastic surgery. However, they also demonstrated the potential stimulatory effect of ANP on adiponectin production from human adipose tissue in the presence of

**Table 1** Clinical Characteristics of the 2 Groups

	hANP Group (n = 15)	Saline Group (n = 15)	p Value
Age (yrs)	60 ± 19	59 ± 19	NS
Sex (male/female)	9/6	10/5	NS
Heart rate (beats/min)	62 ± 11	66 ± 7	NS
Body mass index (kg/m <sup>2</sup> )	21.4 ± 1.1	21.1 ± 1.7	NS
Systolic blood pressure (mm Hg)	116 ± 9	113 ± 9	NS
Diastolic blood pressure (mm Hg)	76 ± 12	74 ± 6	NS
NYHA functional class (II/III)	14/1	10/5	NS
LVEF by echocardiography (%)	32 ± 2	31 ± 8	NS
Plasma BNP (pg/ml)	506 ± 39	537 ± 33	NS
Other medications n (%)			
Loop diuretics	9 (60)	10 (67)	NS
Spironolactone	5 (33)	8 (53)	NS
ACEI or ARB	12 (80)	11 (80)	NS
Beta-blockers	13 (86)	12 (80)	NS

ACEI = angiotensin-converting enzyme inhibitors; ARB = angiotensin II receptor blockers; BNP = brain natriuretic peptide; hANP = human atrial natriuretic peptide; LVEF = left ventricular ejection fraction; NS = not significant; NYHA = New York Heart Association.



hormone-sensitive lipase inhibitor, which inhibits the formation of lipolysis-derived byproducts by ANP-induced lipolysis (22).

Recently, Yu et al. (23) demonstrated the increased ANP-induced lipolysis rates in large adipocytes compared with small adipocytes. Thus, the difference of adipocyte size between patients with CHF and obesity might contribute to the different pattern of adiponectin secretion. Finally, catecholamines also are involved in the control of lipolysis in humans (24). Thus, the prolonged exposure of high plasma level of catecholamines or the treatment with beta-adrenergic receptor blockers in patients with CHF also might affect the distinct pattern of adiponectin secretion from adipocytes. Although precise mechanisms are unknown, the human adipocytes could secrete adiponectin when the certain stress was loaded. However, it remains possible that factors such as tumor necrosis factor-alpha (25)

and alpha-adrenergic stimulation (26), both of which are increased in patients with CHF, may influence the expression of adiponectin or that adiponectin levels are affected by medical treatment, so further investigations are needed.

It is not clear whether ANP augments the plasma adiponectin levels in healthy subjects because of the ethical problems. However, we have reported that the plasma adiponectin level increased along with an increase of plasma BNP levels in 1,538 healthy subjects (27). These results suggest that an increase of natriuretic peptides augments the plasma adiponectin levels and exerts a cardioprotective effect in clinical settings.

Under normal conditions the adult heart utilizes predominantly fatty acids to derive the majority of its energy (28). However, metabolic remodeling such as a marked shift in substrate preference away from fatty acids toward glucose is observed in hypertrophic and failing hearts and the decrease

in fatty acid oxidation is not fully compensated for by an increase in glucose oxidation (29). Thus, the failing heart suffers from chronic energy starvation (30). Insulin resistance also is common in patients with heart failure (31). Adiponectin improves both glucose metabolism and insulin resistance via the AMPK signaling pathway (32). Therefore, we believe that the administration of recombinant natriuretic peptide has beneficial effects on cardiac energy metabolism via adiponectin in patients with CHF.

Interestingly, the plasma adiponectin level was reported to be decreased in patients with risk factors for heart failure (9,33-35) and increased along with BNP after the onset of heart failure (14). Although approximately 10% increase in adiponectin levels in the ANP group seems relatively small, this would not be the case because there was about a 20% reduction in plasma adiponectin levels in patients with coronary artery disease compared with those in control subjects (35), which leads us to believe that the 10% increase in adiponectin is important from the viewpoint of pathophysiology of heart diseases. Therefore, we hypothesized that ANP and/or BNP regulates the plasma level of adiponectin in patients with CHF and conducted this study.

## Conclusions

We demonstrated that natriuretic peptides increase the production of adiponectin by human adipocytes, as well as in patients with CHF. These findings may help to shed more light on the pathophysiology of heart failure.

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**Key Words:** adiponectin ■ natriuretic peptides ■ heart failure ■ adipose tissue.

# Metformin Prevents Progression of Heart Failure in Dogs

## Role of AMP-Activated Protein Kinase

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**Background**—Some studies have shown that metformin activates AMP-activated protein kinase (AMPK) and has a potent cardioprotective effect against ischemia/reperfusion injury. Because AMPK also is activated in animal models of heart failure, we investigated whether metformin decreases cardiomyocyte apoptosis and attenuates the progression of heart failure in dogs.

**Methods and Results**—Treatment with metformin (10  $\mu\text{mol/L}$ ) protected cultured cardiomyocytes from cell death during exposure to  $\text{H}_2\text{O}_2$  (50  $\mu\text{mol/L}$ ) via AMPK activation, as shown by the MTT assay, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining, and flow cytometry. Continuous rapid ventricular pacing (230 bpm for 4 weeks) caused typical heart failure in dogs. Both left ventricular fractional shortening and left ventricular end-diastolic pressure were significantly improved in dogs treated with oral metformin at 100  $\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$  ( $n=8$ ) ( $18.6 \pm 1.8\%$  and  $11.8 \pm 1.1$  mm Hg, respectively) compared with dogs receiving vehicle ( $n=8$ ) ( $9.6 \pm 0.7\%$  and  $22 \pm 0.9$  mm Hg, respectively). Metformin also promoted phosphorylation of both AMPK and endothelial nitric oxide synthase, increased plasma nitric oxide levels, and improved insulin resistance. As a result of these effects, metformin decreased apoptosis and improved cardiac function in failing canine hearts. Interestingly, another AMPK activator (AICAR) had effects equivalent to those of metformin, suggesting the primary role of AMPK activation in reducing apoptosis and preventing heart failure.

**Conclusions**—Metformin attenuated oxidative stress-induced cardiomyocyte apoptosis and prevented the progression of heart failure in dogs, along with activation of AMPK. Therefore, metformin may be a potential new therapy for heart failure. (*Circulation*. 2009;119:2568-2577.)

**Key Words:** AMP-activated protein kinase ■ heart failure ■ metformin ■ nitric oxide

Metformin is widely used as an antidiabetic drug with an insulin-sensitizing effect. A large-scale clinical trial (the UK Prospective Diabetes Study [UKPDS] 34) has shown that metformin therapy decreased the risk of cardiovascular death and the incidence of myocardial infarction associated with diabetes mellitus,<sup>1</sup> suggesting that this drug may be useful for patients who have both cardiovascular disease and diabetes mellitus. Eurich and colleagues<sup>2</sup> recently reported the results of a meta-analysis showing that metformin was the only antidiabetic agent to reduce all-cause mortality without causing any harm in patients who had heart failure and diabetes mellitus. These results suggest that a tight link exists between cardiovascular disease and diabetes mellitus and that metformin has a cardioprotective effect. Metformin is known

to activate AMP-activated protein kinase (AMPK),<sup>3-5</sup> which is expressed in various tissues, including the myocardium, and plays a central role in the regulation of energy metabolism under stress conditions.<sup>6</sup> AMPK is activated by ischemia/reperfusion,<sup>7-9</sup> as well as in hearts with pressure overload hypertrophy<sup>10</sup> and subsequent heart failure.<sup>11,12</sup> In addition, Russell et al<sup>9</sup> have demonstrated that isolated hearts of AMPK-deleted mice show increased apoptosis and dysfunction after ischemia/reperfusion. Activation of AMPK by adiponectin also has been reported to protect cardiomyocytes against apoptosis and to attenuate myocardial ischemia/reperfusion injury in mice.<sup>8</sup> Furthermore, metformin has been reported to increase the production of nitric oxide (NO),<sup>13-15</sup> which is known to have various beneficial cardiovascular

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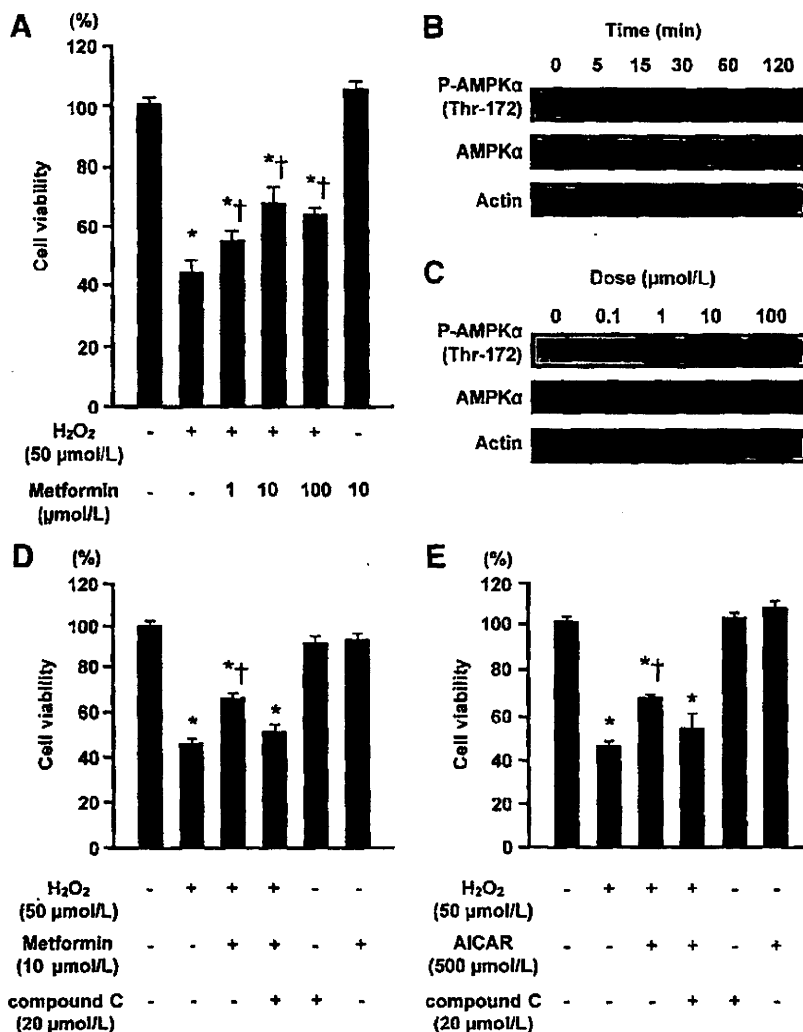
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**Figure 1.** Effect of metformin on oxidative stress-induced cell death via AMPK activation in cultured rat cardiomyocytes. **A**, Cardiomyocyte viability after treatment with metformin (1, 10, or 100 μmol/L) and exposure to H<sub>2</sub>O<sub>2</sub> (50 μmol/L). **B**, Time (0, 5, 15, 30, 60, 120 minutes)-dependent changes in AMPK phosphorylation in cardiomyocytes after treatment with metformin (10 μmol/L). **C**, Dose-dependent changes in AMPK phosphorylation in cardiomyocytes after treatment with metformin (0.1, 1, 10, or 100 μmol/L). **D**, Effect of an AMPK inhibitor (compound C; 20 μmol/L) on cardiomyocyte viability after treatment with metformin (10 μmol/L). **E**, Effect of an AMPK activator (AICAR; 500 μmol/L) on cardiomyocyte viability after treatment with metformin (10 μmol/L). Values are mean±SEM. P-AMPKα indicates phosphorylation of AMPKα. \*P<0.05 vs no treatment; †P<0.05 vs H<sub>2</sub>O<sub>2</sub> (50 μmol/L) treatment.

effects<sup>16</sup> and may alleviate mechanical or neurohormonal stress on the heart.

**Clinical Perspective on p 2577**

These findings led us to hypothesize that activation of AMPK by metformin may exert a cardioprotective effect under stress conditions. Accordingly, metformin might be a potential new treatment for cardiac failure because it activates AMPK and increases NO production. Therefore, we investigated the influence of metformin on apoptosis, an important feature of heart failure, using cultured neonatal cardiomyocytes exposed to H<sub>2</sub>O<sub>2</sub> and the effect of metformin on the progression of pacing-induced heart failure in dogs, along with activation of AMPK.

**Methods**

Experimental procedures are described in the online-only Data Supplement.

**Statistical Analysis**

Results are expressed as mean±SEM. Comparison of changes between groups over time was performed by 2-way repeated-measures ANOVA. Other data were compared between groups by

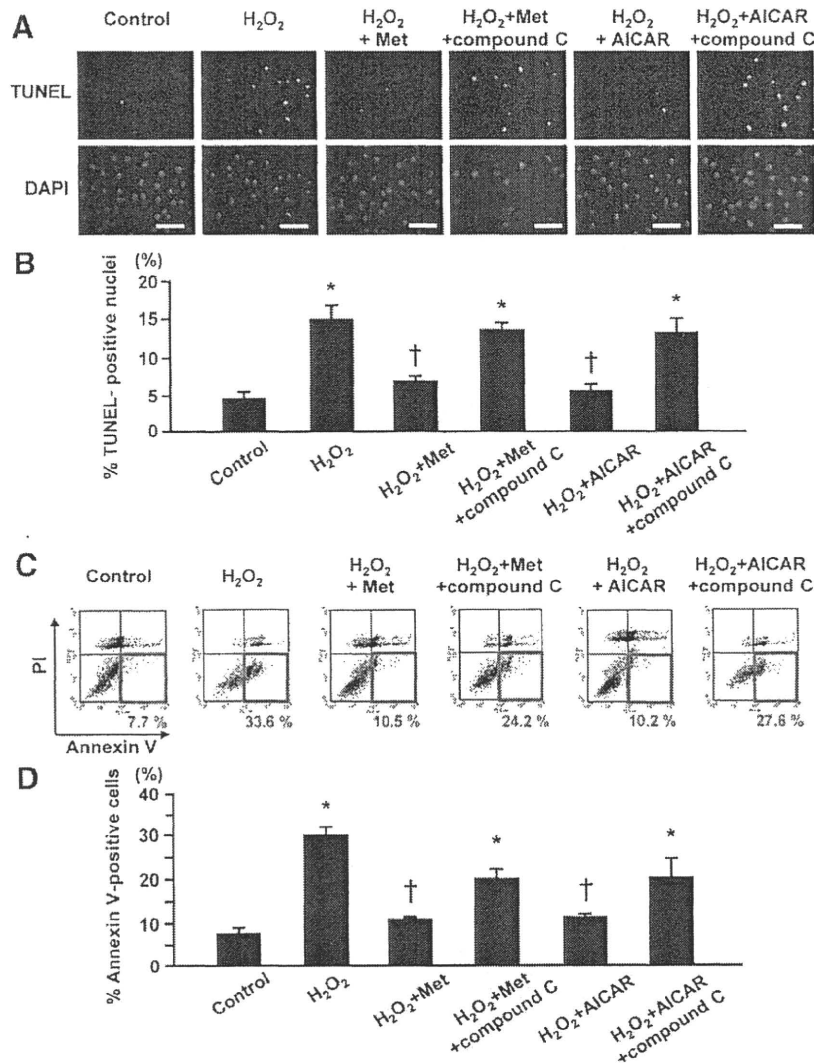
1-way fractional ANOVA. The Tukey-Kramer test was used to correct for multiple comparisons. In all analyses, values of P<0.05 were considered to indicate statistical significance.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Metformin Attenuates Oxidative Stress-Induced Cell Death and Apoptosis in Cultured Cardiomyocytes via AMPK Activation**

Cell viability was decreased in the presence of H<sub>2</sub>O<sub>2</sub>, as shown by the MTT assay, but this change was blunted by treatment with metformin in a dose-dependent manner (Figure 1A). Treatment with metformin (10 μmol/L) stimulated phosphorylation of AMPK in cultured cardiomyocytes in a time- and dose-dependent manner (Figure 1B and 1C). The effect of metformin on cell viability was blunted by cotreatment with compound C, an AMPK inhibitor (20 μmol/L) (Figure 1D). 5-Amino-4-imidazole-1-β-D-carboxamide ribofuranoside (AICAR; another AMPK activator) had an effect similar to metformin on cardiomyocyte viability after exposure to H<sub>2</sub>O<sub>2</sub> (Figure 1E). These results suggested that



**Figure 2.** Effect of metformin on oxidative stress-induced apoptosis via AMPK activation in cultured rat cardiomyocytes. Representative (A) and quantitative (B) data on cardiomyocyte apoptosis obtained by TUNEL staining (n=3 in each experiment). Representative (C) and quantitative (D) data on cardiomyocyte apoptosis obtained by flow cytometry (n=3 in each experiment). Values are mean±SEM. PI indicates propidine iodide. \*P<0.05 vs control; †P<0.05 vs H<sub>2</sub>O<sub>2</sub> (50 μmol/L) treatment.

activation of AMPK protected cardiomyocytes against damage caused by H<sub>2</sub>O<sub>2</sub>.

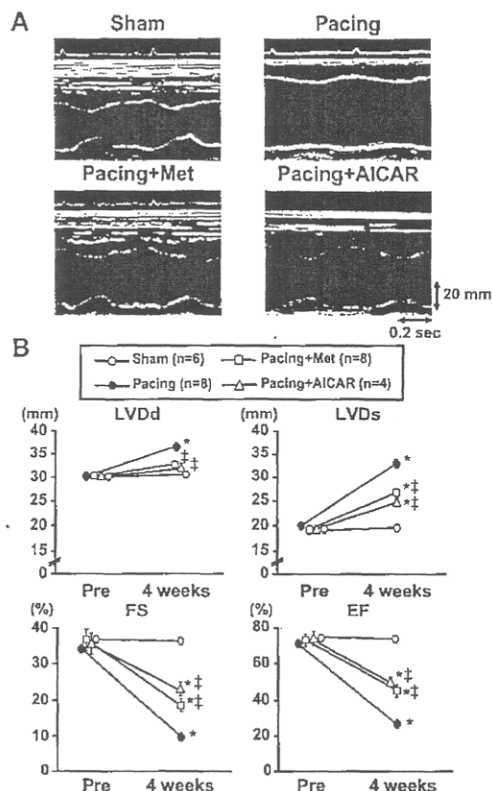
H<sub>2</sub>O<sub>2</sub> also increased cardiomyocyte apoptosis, as shown by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining and flow cytometry (annexin V-positive and propidine iodide-negative cells) (Figure 2A through 2D). Metformin pretreatment significantly reduced the extent of cardiomyocyte apoptosis compared with that in untreated control cells (Figure 2A through 2D). Treatment with compound C inhibited the effects of metformin and AICAR (which was similar to that of metformin) on apoptosis in cardiomyocytes exposed to H<sub>2</sub>O<sub>2</sub> (Figure 2A through 2D). These results suggested that the activation of AMPK by metformin could prevent apoptosis of cardiomyocytes induced by H<sub>2</sub>O<sub>2</sub>.

### Effect of Metformin on Cardiac Function in Dogs With Pacing-Induced Heart Failure

#### Cardiac Physiological and Pathophysiological Parameters

Four weeks after the rapid right ventricular (RV) pacing, left ventricular (LV) end-diastolic dimension, LV end-systolic

dimension, LV fractional shortening, and LV ejection fraction of the pacing group showed significant deterioration compared with the sham group (Figure 3A and 3B). Treatment with metformin significantly reduced both LV dimensions and increased both LV fractional shortening and LV ejection fraction compared with the pacing group (Figure 3A and 3B). Before RV pacing, both mean aortic pressure and heart rate were similar in all groups, and these parameters did not change throughout the study (Table). Four weeks after the RV pacing, pulmonary capillary wedge pressure, mean pulmonary artery pressure, and LV end-diastolic pressure were all significantly higher in the pacing group compared with the sham group (Figure 4A and 4B). Metformin treatment significantly reduced pulmonary capillary wedge pressure, mean pulmonary artery pressure, and LV end-diastolic pressure compared with the pacing group (Figure 4A and 4B). Furthermore, cardiac output was decreased and systemic vascular resistance was increased in the pacing group compared with the sham group, whereas metformin increased cardiac output and decreased systemic vascular resistance compared with the levels in the pacing group (the Table).



**Figure 3.** Effect of metformin on echocardiographic parameters. **A**, Representative M-mode echocardiograms obtained 4 weeks after sham surgery or after RV pacing. **B**, Echocardiographic parameters before and after sham surgery or after RV pacing in the sham group ( $n=6$ ), pacing group ( $n=8$ ), pacing plus metformin group ( $n=8$ ), and pacing plus AICAR group ( $n=4$ ). Values are mean  $\pm$  SEM. LVDd indicates LV end-diastolic dimension; LVDs, LV end-systolic dimension; LVFS, LV fractional shortening; and LVEF, LV ejection fraction. \* $P<0.01$  vs sham group; † $P<0.01$  vs pacing group.

Importantly, the percentage of TUNEL-positive cells to total cells in LV myocardium in the pacing group increased compared with that in the sham group, which was blunted by treatment with either metformin or AICAR (Figure 5A through 5E).

Consistent with previous data,<sup>17</sup> no significant differences were found in body weight, the ratio of LV plus septal weight to body weight, and the ratio of RV weight to body weight among all groups (the Table).

To explore established markers of cardiac failure, we analyzed LV myocardial expression of the atrial natriuretic peptide and brain natriuretic peptide genes, which showed an increase in the pacing group, whereas metformin significantly suppressed this increase (Figure 6A and 6B). Metformin also significantly reduced the levels of angiotensin II and norepinephrine compared with the pacing group (the Table).

Pedometer counts were significantly reduced in the pacing group compared with the sham group, suggesting that heart failure led to reduced physical activities (the Table). Metformin increased the pedometer count compared with that in the pacing group. No differences in body fat were found among all groups (the Table).

### Cardiac Molecular Parameters

To assess the molecular basis of the improvement in cardiac performance achieved by metformin administration for 4 weeks, we examined the collagen volume fraction in LV myocardium after staining with Masson's trichrome stain. Metformin reduced the collagen volume fraction compared with the pacing group (Figure 6C and 6D). To further investigate the mechanism of this antifibrotic effect of metformin, we examined the level of transforming growth factor- $\beta 1$  (TGF- $\beta 1$ ) mRNA associated with fibrosis in canine LV myocardium 4 weeks after pacing. Metformin suppressed the increase in TGF- $\beta 1$  mRNA expression (Figure 6E).

AMPK was phosphorylated in the pacing group, and its phosphorylation was significantly enhanced by administration of metformin (Figure 7A and 7B). Phosphorylation was used as an index of enzymatic activity because AMPK is activated by phosphorylation.<sup>18</sup> This increase in AMPK phosphorylation was accompanied by augmented phosphorylation of acetyl-CoA carboxylase (ACC; a downstream target of AMPK) at Ser-79 (Figure 7A and 7C). Endothelial NO synthase (eNOS) also showed an increase in phosphorylation at Ser-1177 with metformin treatment (Figure 7A and 7D). Furthermore, metformin significantly upregulated eNOS mRNA expression and increased  $\Delta$ NO (the difference between the plasma NO level before and after 4 weeks of RV pacing) compared with the pacing group (Figure 8A and 8B).

To investigate the level of insulin signaling in the heart, we examined the phosphorylation of Akt in the left ventricles in all groups. Significant increases were found in phosphorylation of Akt at Ser-473 in the pacing group compared with the sham group, and such increases were blunted by either metformin or AICAR treatment (Figure 8C and 8D).

### Plasma and Cardiac Metabolic Parameters

To investigate whether activation of AMPK by metformin influenced metabolic parameters in the periphery or the heart, we assessed glucose and lipid metabolism after 4 weeks of pacing. Plasma free fatty acids tended to increase in the pacing group compared with the sham group, although no statistically significant difference was found. Fasting plasma levels of both glucose and lactate were similar among all groups (the Table). Both the fasting plasma insulin level and the homeostasis model assessment–insulin resistance value were significantly increased in the pacing group, whereas metformin reduced both parameters until they were similar to those of the sham group (the Table).

In the heart, both glucose extraction and the arterial–coronary sinus difference were increased in the pacing group compared with the sham group (the Table). In the pacing group, the free fatty acids extraction was not increased, but the arterial–coronary sinus difference tended to increase compared with the sham group (the Table). Lactate extraction and the arterial–coronary sinus difference were similar among all groups (the Table).

### AICAR Mimics the Effect of Metformin in This Canine Pacing Model

To further confirm that activation of AMPK contributed to inhibition of the progression of heart failure, we administered

**Table. Characteristics of the Dogs at 4 Weeks**

	Sham Group (n=6)	Pacing Group (n=8)	Pacing+Metformin Group (n=8)	Pacing+AICAR Group (n=4)
<b>Organ weight</b>				
Body weight, kg	9.5±0.2	9.4±0.2	9.7±0.1	9.6±0.3
LV+septal weight, g	42±0.6	47.3±1.2	43.6±0.9	44.8±1.3
LV+septal weight/body weight ratio, g/kg	4.4±0.1	5.0±0.1	4.5±0.1	4.7±0.2
RV weight, g	14.7±0.5	15.6±0.6	15.0±1.2	14.7±1.0
RV weight/body weight ratio, g/kg	1.5±0.1	1.7±0.1	1.5±0.1	1.5±0.1
<b>Hemodynamic parameters</b>				
Mean aortic pressure, mm Hg	105±5	109±2	100±2	97±3.3
Heart rate, bpm	118±5	136±4	128±5	126±3.6
Cardiac output, L/min	2.6±0.1	1.6±0.1*	2.2±0.3†	2.2±0.3†
Systemic vascular resistance, dynes·s·cm <sup>-5</sup>	3317±189	4769±235*	3775±334†	3763±237†
<b>Plasma metabolic parameters</b>				
Fasting glucose, mmol/L	5.3±0.3	5.3±0.1	5.3±0.1	5.3±0.2
Fasting insulin, μU/mL	14.2±3.3	67.6±13.7*	18.9±7.3†	24.4±10.5†
HOMA-IR	3.4±0.1	15.8±0.1*	4.4±0.1†	5.8±0.1†
Free fatty acids, μmol/L	305±67	716±68	554±101	595±69
Lactate, mmol/L	1.4±0.2	1.5±0.2	1.5±0.1	1.4±0.1
<b>Cardiac metabolic substrates</b>				
<b>Glucose</b>				
Arterial, mmol/L	5.8±0.1	6.4±0.2	6.6±0.1	6.6±0.4
Arterial–coronary sinus difference, mmol/L	0.6±0.1	1.6±0.3*	0.9±0.1	1.1±0.3
Extraction rate, %	10.5±1.2	28.6±4.7*	13.3±1.8	17.7±4.7
<b>Free fatty acids</b>				
Arterial, mmol/L	213.5±44.9	532.3±98.5*	312.8±56.6	294.5±22.8
Arterial–coronary sinus difference, mmol/L	90.4±13.2	153.7±20.6	99.0±9.1	103.2±20.6
Extraction rate, %	47.5±9.2	29.9±2.8	33.9±5.1	36.9±8.6
<b>Lactate</b>				
Arterial, mmol/L	1.8±0.1	1.9±0.3	2.3±0.7	1.8±0.8
Arterial–coronary sinus difference, mmol/L	1.2±0.3	1.0±0.2	1.3±0.5	1.1±0.4
Extraction rate, %	62.6±16.0	48.2±3.8	55.0±12.2	61.8±6.9
<b>Plasma neurohormone levels</b>				
Norepinephrine, pg/mL	34.9±13.0	195.9±21.3*	59.2±11.2†	79.3±8.9†
Angiotensin II, pg/mL	34.7±15.0	153.6±24.3*	78.1±14.8†	73.4±11.8†
<b>Body fat and activity</b>				
Body fat, %	13.7±1.2	18.7±2.9	16±1.2	14.3±0.8
Pedometer count	88 783±2899	64 541±2530*	78 423±3292†	77 716±1472†

HOMA-IR indicates homeostasis model assessment–insulin resistance. Values are mean±SEM.

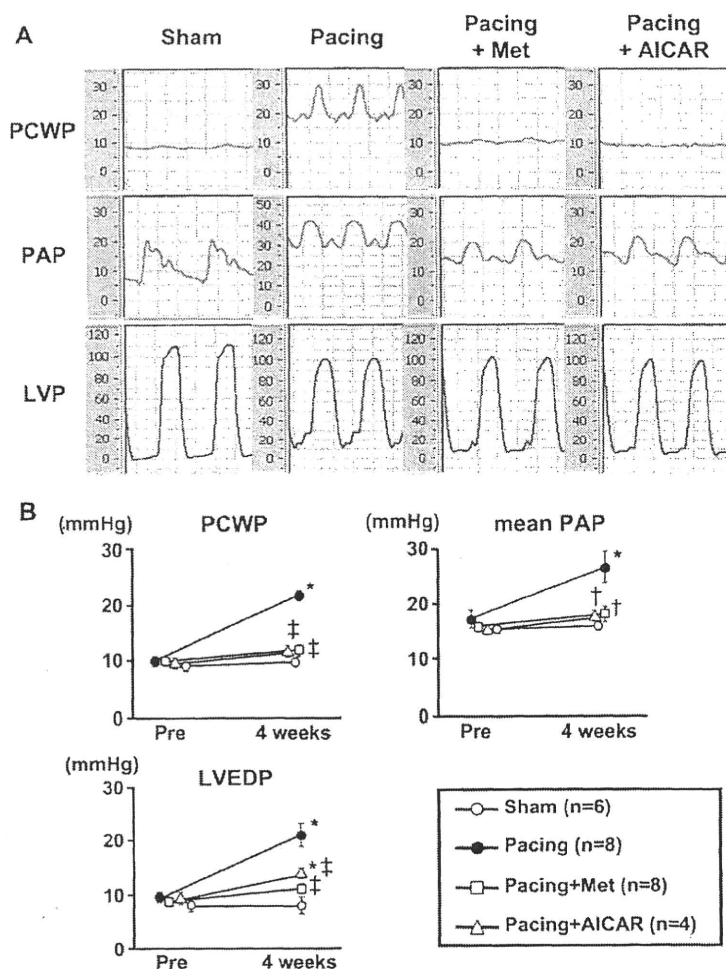
\**P*<0.05 vs the sham group; †*P*<0.05 vs the pacing group.

another AMPK activator (AICAR at a dose of 5 mg/kg SC every other day) to dogs. As expected, AICAR reproduced the effects of metformin in this canine pacing model (Figures 3 through 8).

### Discussion

To the best of our knowledge, this is the first study to demonstrate clearly that long-term (not short-term) oral administration of metformin, which is used as an antidiabetic agent worldwide, inhibits cardiac remodeling and prevents the progression of heart failure in dogs, along with increases in AMPK activation and NO production. Of course, we and

others have previously shown that in rodent either AMPK activation or NO production attenuates myocardial ischemia/reperfusion injury in the ischemic model<sup>7–9</sup> and prevents cardiac remodeling in the pressure overload model.<sup>11,12,19,20</sup> However, it has been unclear whether AMPK or NO can modulate cardiac remodeling and inhibit the progression of heart failure in a canine model with another pathogenic mechanism that is not an ischemic or a pressure overload heart failure model. Therefore, we used a rapid pacing-induced heart failure dog model, which is considered to be similar to human dilated cardiomyopathy<sup>21,22</sup> and can be superimposed on translational study for human heart failure.



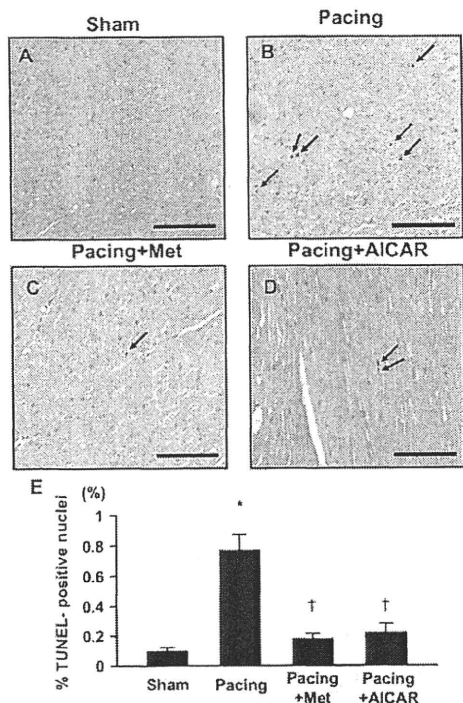
**Figure 4.** Effect of metformin on hemodynamic parameters. **A**, Representative graphs of hemodynamic parameters obtained at 4 weeks. **B**, Hemodynamic parameters before and after the 4-week study period in the sham ( $n=6$ ), pacing ( $n=8$ ), pacing plus metformin ( $n=8$ ), and pacing plus AICAR ( $n=4$ ) groups. Values are mean  $\pm$  SEM. PAP indicates pulmonary artery pressure; PCWP, pulmonary capillary wedge pressure; and LVEDP, LV end-diastolic pressure. \* $P < 0.05$  vs sham group; † $P < 0.05$  vs pacing group; ‡ $P < 0.01$  vs pacing group.

Furthermore, we provide sufficient insight because dogs can be monitored more precisely for hemodynamic data than rodents.

#### Possible Cardioprotective Mechanism of Metformin Mediated via AMPK

Metformin has previously been shown to reduce high fat-induced apoptosis,<sup>23</sup> and AMPK has been reported to protect against hypoxic apoptosis in cardiomyocytes through attenuation of endoplasmic reticulum stress.<sup>24</sup> Consistent with these previous reports, we confirmed that metformin could ameliorate oxidative stress-induced apoptosis in cardiomyocytes. This effect was blunted by compound C, an AMPK inhibitor, suggesting that activation of AMPK was responsible for the inhibition of cardiomyocyte apoptosis. Furthermore, using a dog model, we demonstrated that metformin ameliorated the progression of heart failure induced by rapid RV pacing and decreased apoptosis in the LV myocardium, as indicated by TUNEL staining. Interestingly, AICAR, another AMPK activator, had effects almost identical to those of metformin, supporting that the activation of AMPK contributed to the observed cardioprotective effect. Indeed, AICAR also has been reported to reduce myocardial ischemia/reperfusion injury in humans and animals.<sup>25,26</sup> What processes following AMPK activation are involved in cardioprotection?

The first possibility is enhancement of NO production. Recchia et al<sup>27</sup> reported that basal cardiac NO release is decreased in dogs with heart failure induced by rapid pacing. We found that the difference in plasma NO levels between baseline and 4 weeks of RV pacing was significantly increased by metformin treatment compared with the pacing group. Metformin has been shown to phosphorylate AMPK at Thr-172 in cardiomyocytes and murine hearts,<sup>4,5</sup> whereas AMPK is known to phosphorylate eNOS at Ser-1177 in rat hearts,<sup>28</sup> resulting in an increase in NO production. Indeed, a recent report has indicated that short-term metformin treatment protects against myocardial infarction via AMPK-eNOS-mediated signaling in mice.<sup>7</sup> Other studies have suggested involvement of the AMPK-eNOS pathway in the response of endothelial cells to shear stress,<sup>29</sup> metformin,<sup>30</sup> and statins.<sup>31</sup> Consistent with these reports, we found that either metformin or AICAR promoted the phosphorylation of eNOS at Ser-1177 and increased both mRNA and protein levels of eNOS, possibly leading to increased plasma NO levels and reduced systemic vascular resistance. Although the precise mechanism of the effects of phosphorylation of AMPK by either metformin or AICAR on eNOS protein expression is not clear, these findings suggest that metformin or AICAR increased NO production, which improves endothelial

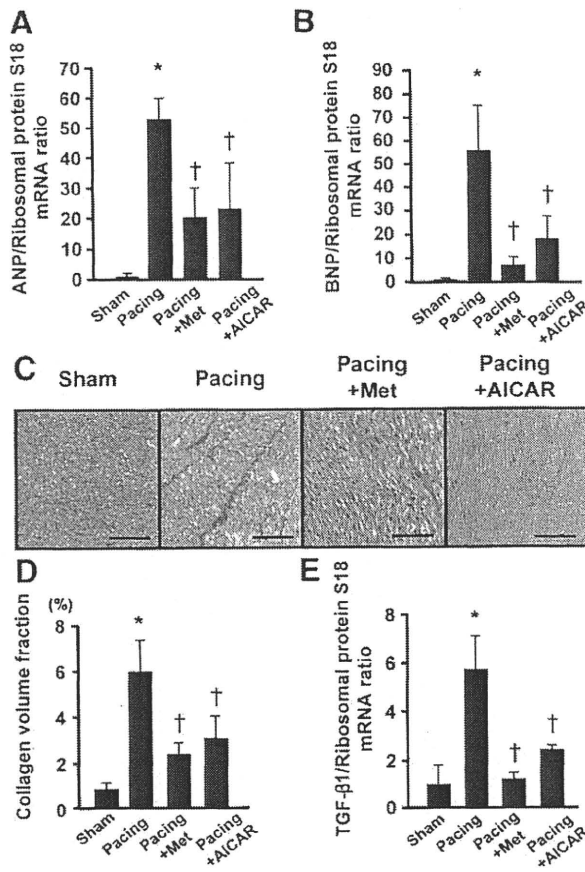


**Figure 5.** TUNEL staining of canine hearts at 4 weeks. Representative examples of TUNEL-stained hearts from sham (A), pacing (B), pacing plus metformin (C), and pacing plus AICAR (D) groups. Arrows indicate TUNEL-positive nuclei (brown). Scale bar=100  $\mu$ m. E, Quantitative data on the percentage of TUNEL-positive nuclei to total cell nuclei. \* $P$ <0.05 vs sham group; † $P$ <0.05 vs pacing group.

function. NO is believed to have various cardioprotective effects.<sup>16</sup> Therefore, enhancement of NO production by metformin via activation of AMPK may have contributed to alleviating the progression of heart failure induced by rapid RV pacing.

The second possibility is related to the improvement in insulin resistance. It is known that insulin resistance is associated with the progression of chronic heart failure, whereas chronic heart failure may provoke insulin resistance by increasing sympathetic activity, activating the renin-angiotensin system, or both.<sup>32,33</sup> We found that rapid RV pacing for 4 weeks induced heart failure and that metformin treatment improved insulin resistance (estimated by homeostasis model assessment–insulin resistance) compared with the pacing group, suggesting that the beneficial effect of metformin on heart failure mediated via AMPK may have been due in part to an improvement in insulin resistance.

The third possibility is the metabolic effects of AMPK activation. Both metformin and AICAR are reported to increase glucose extraction in heart,<sup>34,35</sup> which may decrease the severity of the failing hearts. However, we found a 2- to 3-fold increase in myocardial glucose extraction of pacing dogs, and metformin returned glucose extraction to the value of the sham group. Numerous studies have shown a switch from free fatty acids to glucose as the primary energy substrate in humans and animals with advanced heart failure,<sup>27,36–38</sup> suggesting that the reduction in glucose extraction by the improvement in heart failure by AMPK activation is



**Figure 6.** Natriuretic peptide expression, cardiac collagen volume fraction, and TGF- $\beta$ 1 expression. A, B, and E, Quantitative real-time reverse-transcriptase polymerase chain reaction analysis of myocardial atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and TGF- $\beta$ 1 expression, respectively. The mRNA values were corrected for the ribosomal protein S18 mRNA level. The sham group was arbitrarily assigned a value of 1.0. Results are mean $\pm$ SEM. Representative results from 3 independent experiments are shown. \* $P$ <0.05 vs sham group; † $P$ <0.05 vs pacing group. C, Representative histological appearance of LV myocardium stained with Masson's trichrome stain (light blue). Scale bar=100  $\mu$ m. D, Collagen volume fraction in the LV myocardium. Values are mean $\pm$ SEM. \* $P$ <0.05 vs sham group; † $P$ <0.05 vs pacing group.

likely to be greater than the induction of glucose extraction by direct activation of AMPK. The possibility exists that AMPK-induced glucose extraction triggers the improvement in heart failure, followed by the restoration of metabolic switch. On the other hand, we found that the net free fatty acids extraction of the pacing group tended to increase despite no statistical significance, which is consistent with the report by Paolisso et al<sup>39</sup> that myocardial free fatty acids extraction increased in patients with congestive heart failure<sup>39</sup> but is contrary to the reports of the metabolic switch.<sup>27,36–38</sup> The metabolic switch may differ in relatively acute or chronic heart failure and by the severity of heart failure.

The increased phosphorylation of Akt in the pacing group was attenuated in either the pacing plus metformin or the pacing plus AICAR group, suggesting that the levels of activation of insulin signaling decreased in either the

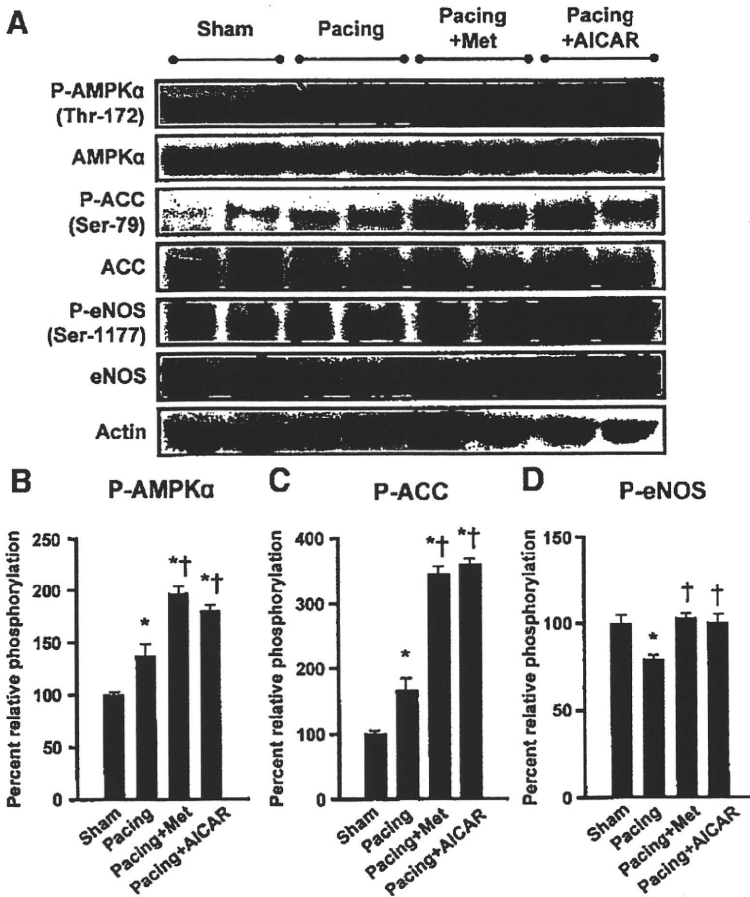


Figure 7. Phosphorylation of AMPK $\alpha$ , ACC, and eNOS in canine hearts after 4 weeks of treatment with or without metformin and AICAR. A, Representative immunoblots of phospho-AMPK $\alpha$ , ACC, and eNOS. B through D, Percentage relative phosphorylation of AMPK $\alpha$ , ACC, and eNOS, respectively. Values are mean  $\pm$  SEM. Representative results from 3 independent experiments are shown. \* $P$ <0.05 vs sham group; † $P$ <0.05 vs pacing group.

metformin- or AICAR-treated group. Considering that glucose extraction was decreased in the pacing plus metformin and pacing plus AICAR groups and that AMPK was phosphorylated by either metformin or AICAR, which may increase in glucose extraction in the heart, the present data may be contradictory, but they are not contradictory when we consider the changes in phosphorylated Akt. The reason is that in this pacing-induced canine heart failure model, glucose extraction in the heart was influenced predominantly by insulin resistance, accompanied by the severity of heart failure, rather than AMPK phosphorylation, although further investigation on this issue is needed.

The fourth possibility is the antifibrotic effect of metformin. Several studies have indicated that AMPK activation inhibits protein synthesis through effects on both the eEF-2 and mTOR pathways.<sup>40,41</sup> We demonstrated that no significant difference in ventricular mass existed at autopsy among the groups. This dog pacing model has been reported to preserve wall thickness without hypertrophy or a consistent increase in heart weight, unlike the pressure overload model.<sup>42</sup> We found that metformin attenuated fibrosis and reduced the TGF- $\beta$ 1 mRNA level after 4 weeks of RV pacing compared with the pacing group. Metformin also improved representative markers of heart failure, including LV end-diastolic pressure, brain natriuretic peptide, angiotensin II, and norepinephrine. Although a number of factors may have

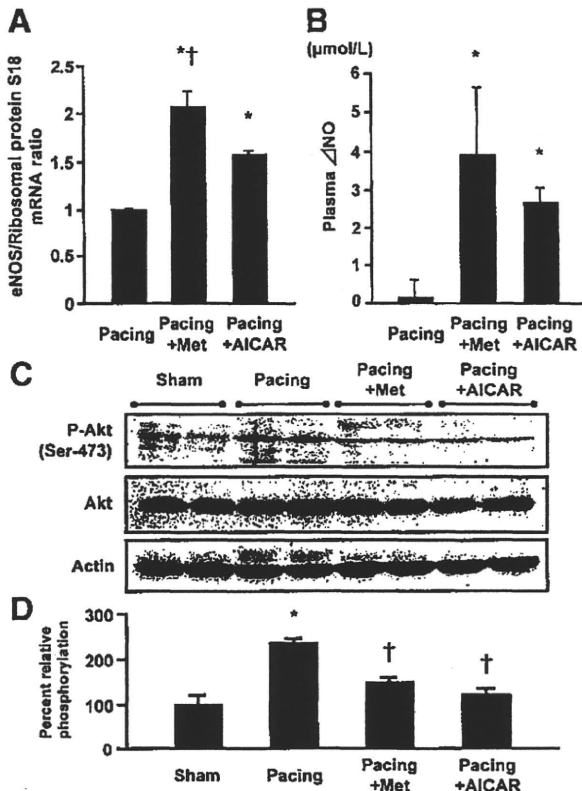
contributed to the antifibrotic effect of metformin, our data suggest that inhibition of TGF- $\beta$ 1 by metformin has at least some role, resulting in the prevention of heart failure.

Taken together, these data suggest that metformin has a direct cardioprotective effect, has effects on the improvements of peripheral vascular system and insulin resistance, and inhibits fibrosis. All these actions might contribute to the improvement in the pathophysiology of heart failure, although we could not identify the exact role of each factor. It remains to be determined whether these results were a cause or consequence of improved cardiac function, especially in systemic effects of both insulin resistance and systemic vascular resistance.

### Study Limitations

We found that the extent of phosphorylation of eNOS decreased despite the increase in the phosphorylated Akt in the pacing-induced failing canine hearts, which may be contradictory to previous reports that the phosphorylation of Akt leads to eNOS phosphorylation.<sup>43,44</sup> Because the signal transduction to modulate eNOS is unclear in the failing myocardium and the pathophysiological role and importance of Akt also are unclear, this discrepancy should be clarified in future studies.<sup>45</sup>

We need to consider the dose of metformin used in the present study, which was at least 3-fold higher than that used clinically. Nevertheless, adverse effects such as hypoglycemia and lactic acidosis were not detected during the experiment.



**Figure 8.** Effect of metformin on eNOS mRNA expression and plasma ΔNO levels, and phosphorylation of Akt in canine hearts. **A**, Quantitative real-time reverse-transcriptase polymerase chain reaction for eNOS mRNA. The mRNA levels were normalized to ribosomal protein S18 mRNA, and the pacing group was arbitrarily assigned a value of 1.0. **B**, Plasma ΔNO level after 4 weeks of RV pacing with or without metformin and AICAR administration. Values are mean±SEM. Representative results from 3 independent experiments are shown. \**P*<0.05 vs pacing group; †*P*<0.05 vs pacing plus AICAR group. **C**, Representative immunoblots of phospho-Akt. **D**, Percent relative phosphorylation of Akt. Values are mean±SEM. Representative results from 3 independent experiments are shown. \**P*<0.05 vs sham group; †*P*<0.05 vs pacing group.

**Conclusions**

We demonstrated that metformin prevents the progression of pacing-induced heart failure in dogs, along with the activation of AMPK. Metformin may offer a novel treatment strategy for heart failure.

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**Disclosures**

None.

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### CLINICAL PERSPECTIVE

Metformin is widely used as an antidiabetic drug with an insulin-sensitizing effect. A large-scale clinical trial (the UK Prospective Diabetes Study [UKPDS] 34) has shown that metformin therapy decreased the risk of cardiovascular death and the incidence of myocardial infarction associated with diabetes mellitus; metformin reduced the hemoglobin A<sub>1c</sub> levels in treated patients to the same extent as in the other patients treated with conventional therapies. These results suggest that metformin might exert cardioprotective effects beyond its glucose-lowering action such as either activation of AMP-activated protein kinase (AMPK) or elevation of nitric oxide. Metformin is known to activate AMPK, which mediates potent cardioprotection against ischemia/reperfusion injury. AMPK also is activated in experimental failing myocardium, suggesting that activation of AMPK is beneficial for the pathophysiology of heart failure. The present study demonstrated that long-term oral administration of metformin prevents the progression of heart failure as indicated by hemodynamic and echocardiographic parameters. Metformin also promoted phosphorylation of both AMPK and endothelial nitric oxide synthase, increased plasma nitric oxide levels, and improved insulin resistance. As a result of these effects, metformin decreased apoptosis and improved cardiac function in failing canine hearts. Interestingly, another AMPK activator (AICAR) had effects equivalent to those of metformin, suggesting the primary role of AMPK activation in reducing apoptosis and preventing heart failure. Drugs that activate AMPK, especially metformin, may provide a novel strategy for the treatment of heart failure in clinical settings.



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## Identification of genes related to heart failure using global gene expression profiling of human failing myocardium

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### ABSTRACT

Although various management methods have been developed for heart failure, it is necessary to investigate the diagnostic or therapeutic targets of heart failure. Accordingly, we have developed different approaches for managing heart failure by using conventional microarray analyses. We analyzed gene expression profiles of myocardial samples from 12 patients with heart failure and constructed datasets of heart failure-associated genes using clinical parameters such as pulmonary artery pressure (PAP) and ejection fraction (EF). From these 12 genes, we selected four genes with high expression levels in the heart, and examined their novelty by performing a literature-based search. In addition, we included four G-protein-coupled receptor (GPCR)-encoding genes, three enzyme-encoding genes, and one ion-channel protein-encoding gene to identify a drug target for heart failure using *in silico* microarray database. After the *in vitro* functional screening using adenovirus transfections of 12 genes into rat cardiomyocytes, we generated gene-targeting mice of five candidate genes, namely, *MYLK3*, *GPR37L1*, *GPR35*, *MMP23*, and *NBC1*. The results revealed that systolic blood pressure differed significantly between *GPR35*-KO and *GPR35*-WT mice as well as between *GPR37L1*-Tg and *GPR37L1*-KO mice. Further, the heart weight/body weight ratio between *MYLK3*-Tg and *MYLK3*-WT mice and between *GPR37L1*-Tg and *GPR37L1*-KO mice differed significantly. Hence, microarray analysis combined with clinical parameters can be an effective method to identify novel therapeutic targets for the prevention or management of heart failure.

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### Introduction

Heart failure is a multi-factorial condition with increasing prevalence worldwide; further, a significant increase has been observed in the mortality rate and economic impact associated with this condition. In the last 20 years, substantial development of treatment for heart failure, including angiotensin-converting-enzyme inhibitors [1] and beta-blockers [2,3], has greatly improved the

prognosis of the patients with heart failure. However, despite these rapid advancements in the management of heart failure, effective treatment of end-stage heart failure without providing ventricular assistance or heart transplantation is still difficult. Investigation of new and unexplored targets for the prevention or treatment of heart failure is warranted. Global gene expression analysis using microarray technique has been used in the last decade to identify biomarkers or drug targets for heart failure [4–10]. Several gene expression signatures of heart failure have been identified by analyzing independent microarray datasets [11,12]. However, most of these analyses did not consider the severity of heart failure. Because the severity of heart failure may quantitatively reflect the expression levels of genes such as the natriuretic

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peptide-encoding gene, expression analysis combined with the severity of heart failure could be an appropriate method to identify heart failure-related genes. However, microarray analysis of genes expressed in failing myocardium while considering the severity of heart failure has not yet been reported.

Hence, we investigated the genes whose expression level correlated with clinical parameters such as pulmonary artery pressure (PAP), left ventricular ejection fraction (EF), and brain natriuretic peptide (BNP) mRNA level. Using this approach, we identified cardiac myosin light chain kinase as a novel heart failure-related gene [13]. Here, we describe newly identified several genes whose expression correlated with clinical parameters and additional genes encoding G-protein-coupled receptor genes (GPCRs), other enzymes and ion-channel proteins, and performed the functional analysis of these heart failure-related genes. This novel strategy involving the use of clinical parameters might find potential applications for the identification of disease-associated genes that could not be detected using conventional microarray techniques.

## Materials and methods

**Patient characteristics.** We recruited 12 patients (11 males and 1 female) with heart failure and obtained written informed consent from them. The patients were diagnosed with severe chronic heart failure due to various cardiac diseases such as dilated cardiomyopathy and myocardial infarction [13]. The average age of patients was  $55 \pm 13$  years. The plasma level of BNP, which is the best marker for the severity of heart failure, ranged from 80 to 2710 pg/ml. The mean PAP measured using a Swan-Ganz catheter 1–4 weeks before the operation varied between 16 and 59 mmHg. The average of EF determined by echocardiography on the day before the operation was  $32.5\% \pm 12.4\%$ .

**Microarray analysis and subsequent in silico functional analysis.** RNA was extracted from myocardium samples of 12 heart failure patients who had undergone either Batista or Dor surgeries. RNA samples of non-failing hearts were purchased from Biochain, Inc. Complementary RNA (cRNA) was prepared from RNA samples and hybridized to HG-U95 Affymetrix GeneChip (Affymetrix, US). The expression data were analyzed using Microarray Analysis Suite version 5.0 software. Among all the genes detected on the microarray, we selected the genes whose expression was significantly different in the failing and non-failing myocardial samples ( $p < 0.005$ ). From these genes, we selected genes whose expression was correlated with PAP, EF, and BNP mRNA level, with 0.7 being the cutoff value of the correlation coefficient. The values of PAP, EF, and BNP mRNA level used for the correlation analysis were normalized to their median during the measurements. Subsequently, the functional analysis of datasets was performed using Ingenuity Pathway Analysis (Ingenuity® Systems; www.ingenuity.com), and the biological functions most significant to the dataset were identified.

**Cell culture.** Cardiomyocytes were harvested before the experiments from 2- to 3-day-old neonatal rats and cultured as described in previous studies [14]. Briefly, primary cardiomyocytes isolated from neonatal rats were grown in Dulbecco's modified Eagle medium/F12 (Gibco) supplemented with 10% fetal calf serum for 72 h, and then cultured in a serum-free condition for 24 h.

**Adenovirus generation and transfection.** Adenovirus constructs encoding the genes of interest were generated using the ViraPower Adenoviral Expression System (Invitrogen, US) according to the manufacturer's method. Adenovirus vectors were transfected to cultured cardiomyocytes for 12 h according to the published protocol.

**In vitro functional analysis of genes.** Cultured rat cardiomyocytes were infected by adenovirus vectors. After 24 h, hypertrophic

reaction, cell viability, and cellular morphology were assessed. Hypertrophic reaction was determined by estimating the incorporation of [<sup>3</sup>H]phenylalanine. In brief, [<sup>3</sup>H]phenylalanine was added to the culture medium at the final concentration of 0.1  $\mu$ Ci/ml, and the cells were incubated for an additional 24 h. Then, the incorporation of [<sup>3</sup>H]phenylalanine was determined by counting the radioactivity of each sample with a liquid scintillation counter. The viability of cardiomyocytes was evaluated by the Alamar blue assay according to the manufacturer's method. The morphology of cardiomyocytes was evaluated 24 h after adenovirus transfections.

**Generation of transgenic and knockout mice.** To generate transgenic mice, open reading frame of each gene, namely, *Mylk3*, *Gpr3711*, or *Nbc1* was amplified from mouse cDNA by PCR, with Sal I site linker on each end, and cloned into Sal I site of alpha-MHC clone 26 vector. Then the DNAs used in the microinjections were released from the vector by digestion with NotI and were microinjected into fertilized eggs of mouse. Founder mice were identified by PCR analysis with appropriate primers. To develop *Gpr3711* knockout mice, the targeting vector was assembled to replace the exon 1 and 2 by neomycin selection cassette resulting in the absence of *Gpr3711* protein. W9.5 ES cells were electroporated with linearized targeting vector. ES cell clones with successful homologous recombination was determined by the PCR and subsequent direct sequence. From these targeted ES cells, the chimera mice were bred to C57 BL/6 females to generate F1 and F2 offsprings were obtained. The *Gpr3711* null mice were determined by PCR genotyping of F2 offsprings. The knockout mice of *Gpr35* and *Mmp23* (the mouse ortholog of MMP23B) were purchased from Deltagen, Inc. (California, US).

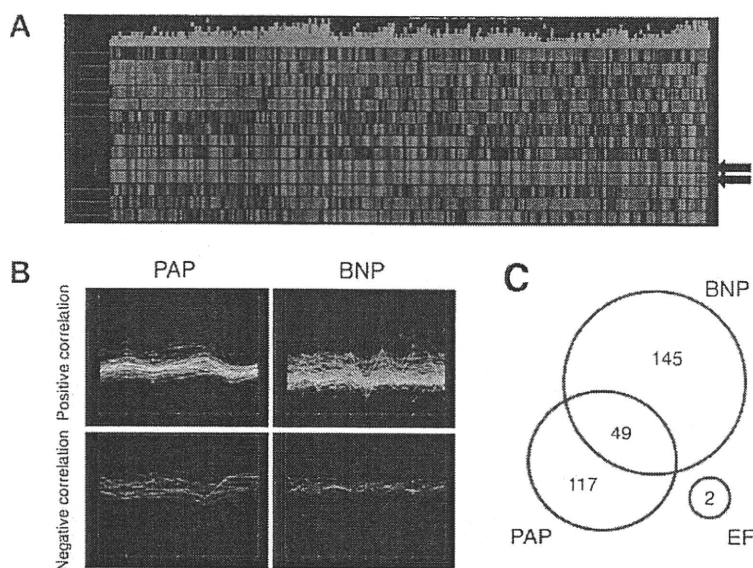
**Invasive blood pressure measurement.** The phenotype of the gene-targeted mice was examined. Before sacrificing the mice, their hemodynamic parameters were evaluated. The mice were anesthetized and ventilated, and a Millar catheter was inserted via right carotid artery. The left-ventricular systolic and end-diastolic pressures were measured. Then, the mice were sacrificed and the weight of the whole body and heart was determined.

**Statistical analysis.** Unpaired Student's t-test was used for comparing the two groups. Results are expressed as means  $\pm$  SEM, and  $p$  value less than 0.05 was considered statistically significant.

## Results

### Identification of heart failure-related genes by expression analysis using clinical parameters

We performed microarray analysis of the genes expressed in failing myocardium obtained from 12 patients with heart failure and the genes expressed in non-failing myocardium from two normal objects whose characteristics were reported in the previous study [13]. Although all patients were diagnosed with chronic heart failure, the plasma BNP level, which is an index of the severity of heart failure, ranged from 80 to 2710 pg/ml, suggesting that the severity of heart failure varied extensively among the patients. This marked difference in the severity of heart failure reflects the fact that the gene expression patterns in the 12 patients were not uniform, as shown in Fig. 1A. Thus, we analyzed gene expression profiles of failing myocardium using clinical parameters representing the severity of heart failure. We identified 166 and 194 genes whose expressions were correlated with PAP and BNP mRNA level, respectively (Fig. 1B and Supplementary Tables S1, S2). Among these, 49 genes correlated with both PAP and BNP mRNA level (Fig. 1C). The expression of only two genes, namely, *FMO2* and *LMAN1L*, correlated with the EF. We investigated the functional categories of these genes by performing Ingenuity Pathway Analysis. The number of genes in each group, functional categories, and



**Fig. 1.** The gene expression profile of human failing or non-failing myocardium. Gene expression levels of myocardial samples from 12 patients with severe heart failure and from two normals were analyzed using microarray. (A) Heat maps showing the genes with differential expression between the 12 failing myocardial samples and the two non-failing myocardial samples. Red color indicates upregulated gene expression. Green color indicates downregulated gene expression. Arrows indicate non-failing samples. (B) Expression profile of positively or negatively correlated genes to pulmonary artery pressure (PAP) or brain natriuretic peptide (BNP) mRNA level ( $r > 0.7$ ). (C) Venn diagram of genes correlated with PAP, BNP, and ejection fraction.

**Table 1**  
Datasets of genes whose expressions were correlated to clinical parameters.

	PAP	EF	BNP mRNA level
<i>Positive correlation</i>			
Number	124	1	175
Function	Cardiovascular system development and function Cell death	–	Cardiovascular system development and function Cell cycle
Representative genes	<i>ARNT, MYOCD, SMARCA4, BGN, CFLAR, EEF2, MTPN</i>	<i>LMAN1L</i>	<i>BTG1, NPPA, NPPB, SERPINF1, CKS1B, DDR2, FCGR2B, FN1</i>
<i>Negative correlation</i>			
Number	42	1	19
Function	Skeletal and muscular system development and function Cellular assembly and organization	–	Skeletal and muscular system development and function Cellular assembly and organization
Representative genes	<i>PIK3R1, PRKARIA, SLMAP, C19ORF20, RAB9A, SYNGAP1, TTN</i>	<i>FMO2</i>	<i>ACTC1, RBBP4, TTN</i>

The function of gene sets was analyzed by Ingenuity Pathway Analysis.  
PAP, pulmonary artery pressure; EF, ejection fraction; BNP, brain natriuretic peptide.

representative genes are shown in Table 1. Interestingly, both gene sets correlated positively with PAP and BNP mRNA level were most associated with the same functional category of “cardiovascular system development and function”, although the included genes were different. Similarly, the gene sets correlated negatively with both PAP and BNP mRNA level had most association with common functional categories of “skeletal and muscular system development and function” and “cellular assembly and organization”.

#### Selection of 12 genes for *in vitro* screening

Among the genes selected using clinical parameters, we selected those genes that showed high expression levels in the heart by performing microarray analysis. On the basis of their novelty determined by a literature-based search, we selected four genes for further investigation (Table 2). Concurrently, to identify possible drug targets, we included four orphan GPCRs and four additional genes (three enzyme-encoding genes and one ion-channel protein-encoding gene) in the further analysis. The *RHOQ* and

*STK38* genes were selected based on their correlation with BNP mRNA level and PAP, respectively. *GPR161* and *NBC1* were selected owing to their high expression level in the heart. *GPR37L1*, *GPR35*, *F2RL2*, and *MMP23B* were selected because of their high expression level in the heart, and their association with the cardiac diseases-related genes listed in the database was determined by *in silico* analysis.

#### Functional analysis of genes on the basis of adenovirus-mediated overexpression of proteins in neonatal rat cardiomyocytes

To determine which of the selected genes were associated with the physiological functions of the heart, we first generated adenovirus vectors for each gene listed in Table 2 and transfected these vectors into neonatal rat cardiomyocytes. Next, we evaluated the hypertrophic reaction, viability, and morphology of the transfected cardiomyocytes. Among the 12 selected genes, three adenovirus-mediated genes decreased the incorporation of [<sup>3</sup>H]phenylalanine in neonatal rat cardiomyocytes (Table 2); the expression of one