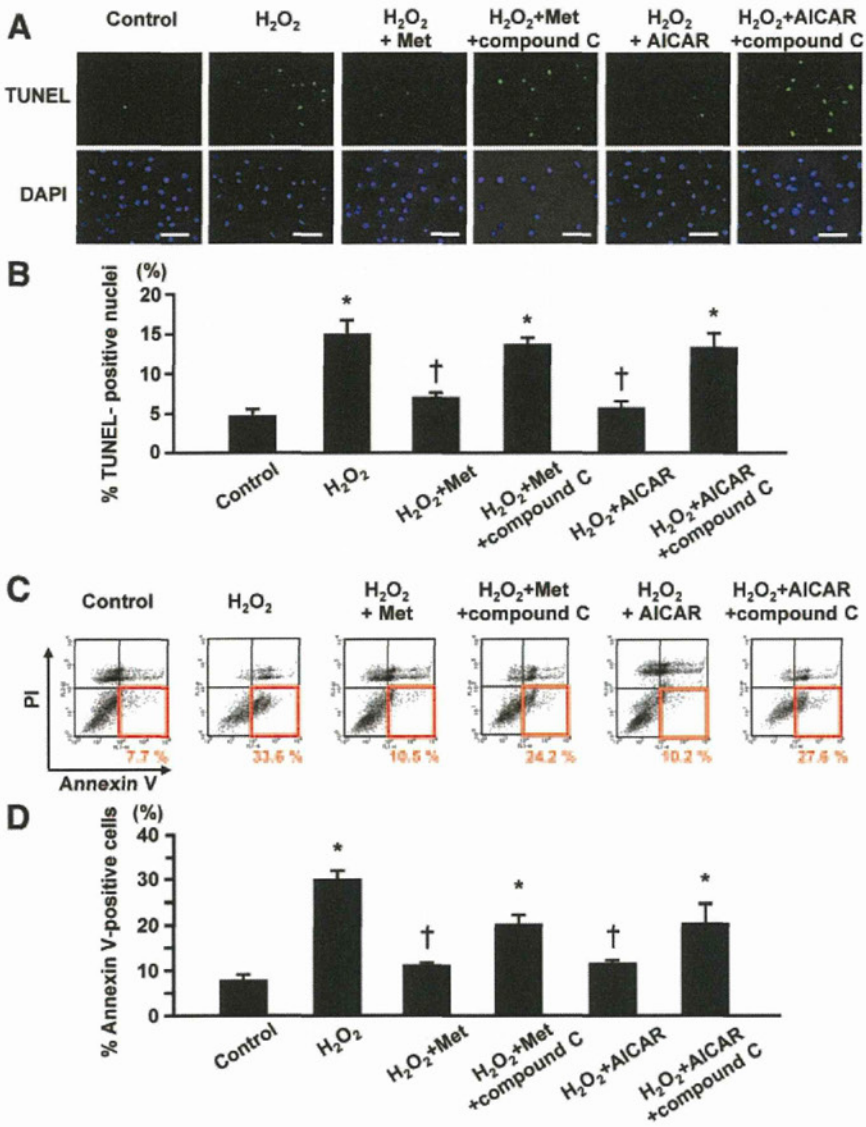


**Figure 2.** Effect of metformin on oxidative stress–induced apoptosis via AMPK activation in cultured rat cardiomyocytes. Representative (A) and quantitative (B) data on cardiomyocyte apoptosis obtained by TUNEL staining (n=3 in each experiment). Representative (C) and quantitative (D) data on cardiomyocyte 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 $H_2O_2$ (50 μmol/L) treatment.

activation of AMPK protected cardiomyocytes against damage caused by $H_2O_2$.

$H_2O_2$ 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 $H_2O_2$ (Figure 2A through 2D). These results suggested that the activation of AMPK by metformin could prevent apoptosis of cardiomyocytes induced by $H_2O_2$.

## 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).

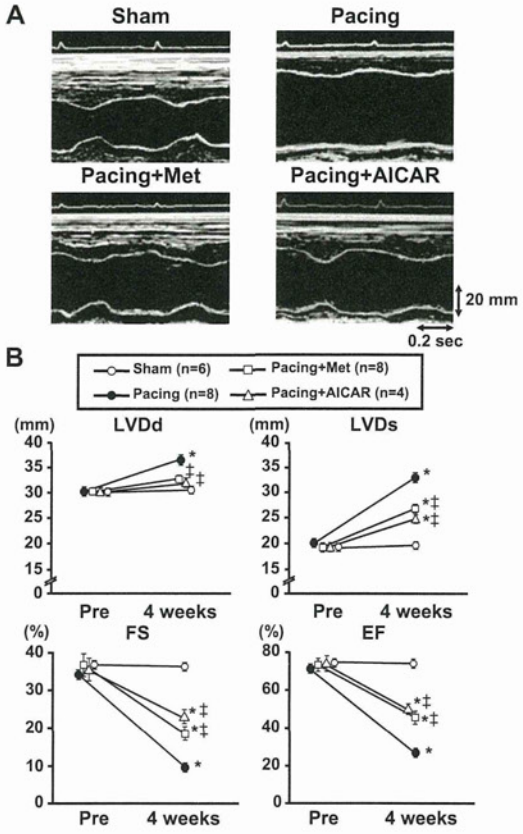


**Figure 3.** Effect of metformin on echocardiographic parameters. A, Representative M-mode echocardiograms obtained 4 weeks after sham surgery or after RV pacing. B, Echocardiographic parameters before and after sham surgery or after RV pacing in the sham group (n=6), pacing group (n=8), pacing plus metformin group (n=8), and pacing plus AICAR group (n=4). Values are mean±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,[17] 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.[18] 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) |
|---|---|---|---|---|
| Organ weight | | | | |
| 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 |
| Hemodynamic parameters | | | | |
| 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 · $cm^{-5}$ | 3317±189 | 4769±235* | 3775±334† | 3763±237† |
| Plasma metabolic parameters | | | | |
| 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 |
| Cardiac metabolic substrates | | | | |
| Glucose | | | | |
| 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 |
| Free fatty acids | | | | |
| 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 |
| Lactate | | | | |
| 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 |
| Plasma neurohormone levels | | | | |
| 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† |
| Body fat and activity | | | | |
| 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[7–9] and prevents cardiac remodeling in the pressure overload model.[11,12,19,20] 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[21,22] and can be superimposed on translational study for human heart failure.

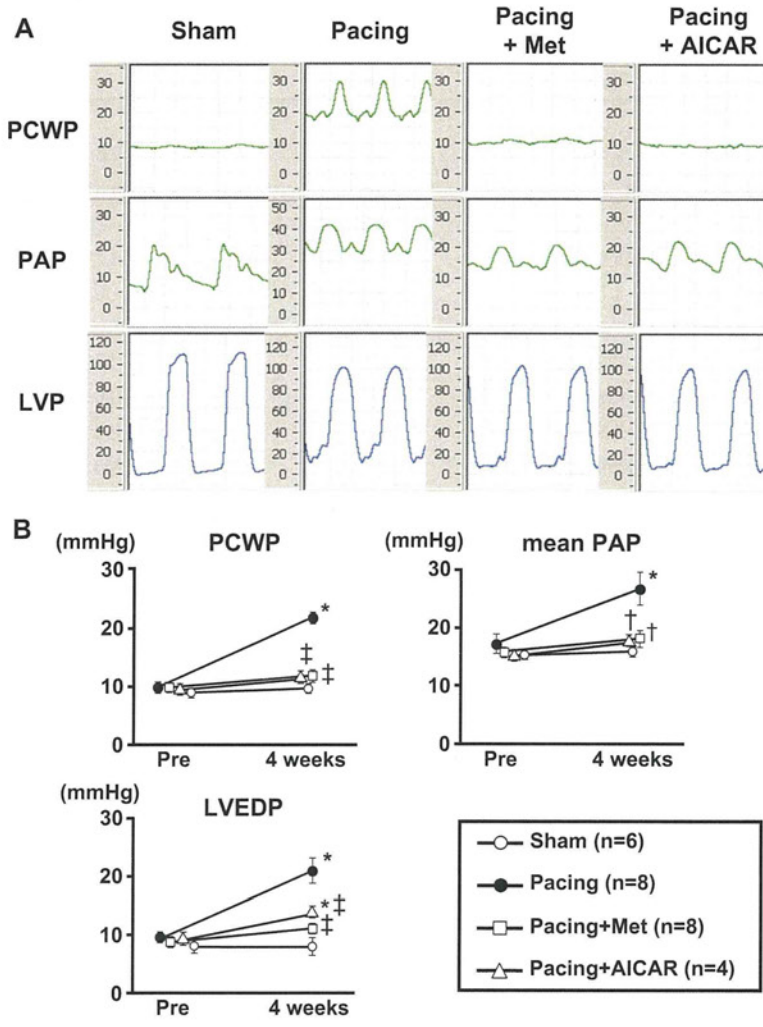


**Figure 4.** Effect of metformin on hemodynamic parameters. A, Representative graphs of hemodynamic parameters obtained at 4 weeks. B, Hemodynamic parameters before and after the 4-week study period in the sham (n=6), pacing (n=8), pacing plus metformin (n=8), and pacing plus AICAR (n=4) groups. Values are mean±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,[23] and AMPK has been reported to protect against hypoxic apoptosis in cardiomyocytes through attenuation of endoplasmic reticulum stress.[24] 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.[25,26] What processes following AMPK activation are involved in cardioprotection?

The first possibility is enhancement of NO production. Recchia et al[27] 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,[4,5] whereas AMPK is known to phosphorylate eNOS at Ser-1177 in rat hearts,[28] 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.[7] Other studies have suggested involvement of the AMPK-eNOS pathway in the response of endothelial cells to shear stress,[29] metformin,[30] and statins.[31] 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

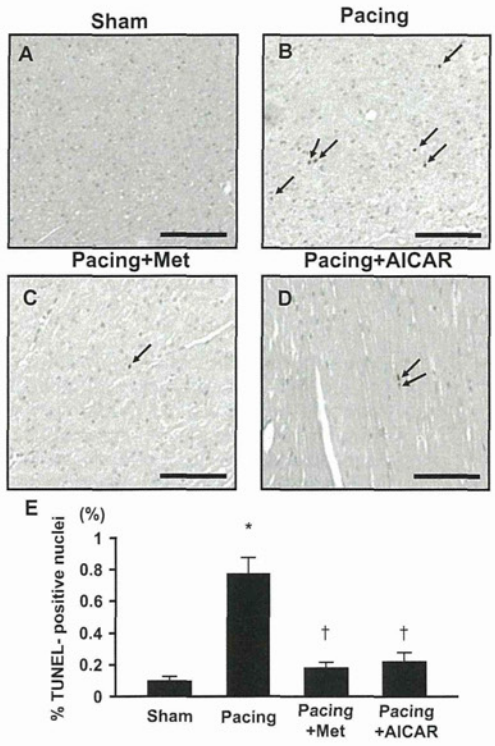


**Figure 5.** TUNEL staining of canine hearts at 4 weeks. Representative examples of TUNEL-stained hearts from sham (A), pacing (B), pacing plus metformin (C), and pacing plus AICAR (D) groups. Arrows indicate TUNEL-positive nuclei (brown). Scale bar=100 $\mu$m. E, Quantitative data on the percentage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

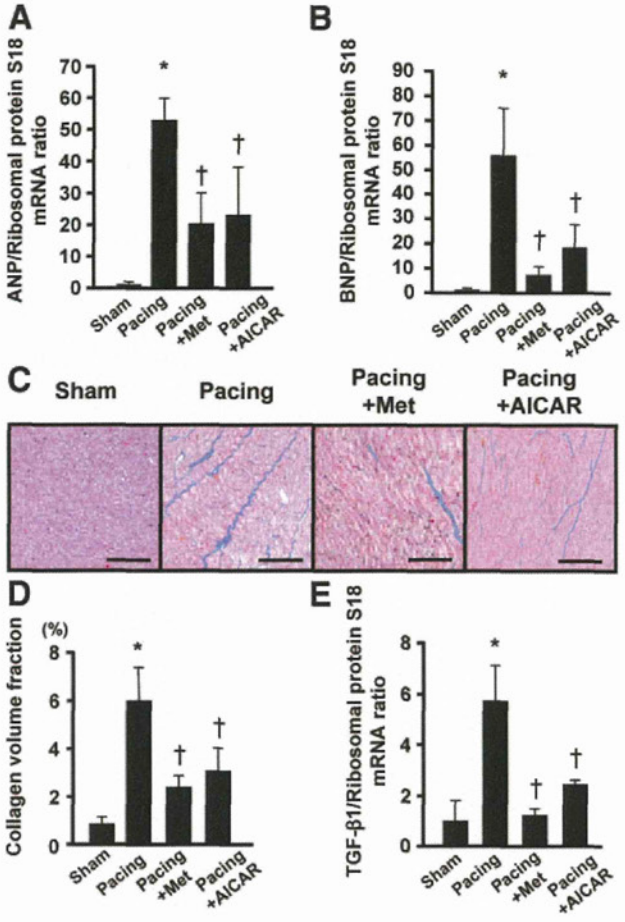


**Figure 6.** Natriuretic peptide expression, cardiac collagen volume fraction, and TGF-$\beta$1 expression. A, B, and E, Quantitative real-time reverse-transcriptase polymerase chain reaction analysis of myocardial atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and TGF-$\beta$1 expression, respectively. The mRNA values were corrected for the ribosomal protein S18 mRNA level. The sham group was arbitrarily assigned a value of 1.0. Results are mean±SEM. Representative results from 3 independent experiments are shown. *$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±SEM. *$P$<0.05 vs sham group; †$P$<0.05 vs pacing group.

function. NO is believed to have various cardioprotective effects.[16] 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.[32,33] 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,[34,35] 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,[27,36–38] suggesting that the reduction in glucose extraction by the improvement in heart failure by AMPK activation is 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[39] that myocardial free fatty acids extraction increased in patients with congestive heart failure[39] but is contrary to the reports of the metabolic switch.[27,36–38] 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

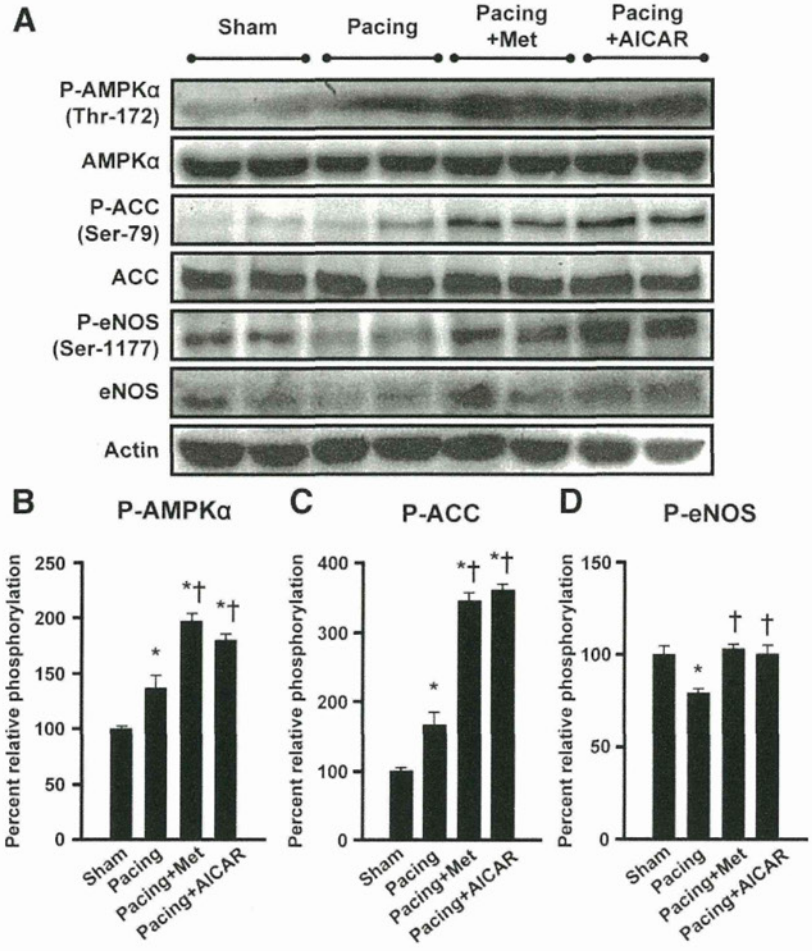


**Figure 7.** Phosphorylation of AMPK$\alpha$, ACC, and eNOS in canine hearts after 4 weeks of treatment with or without metformin and AICAR. A, Representative immunoblots of phospho-AMPK$\alpha$, ACC, and eNOS. B through D, Percentage relative phosphorylation of AMPK$\alpha$, ACC, and eNOS, respectively. Values are mean±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.[40,41] 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.[42] 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.[43,44] 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.[45]

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.

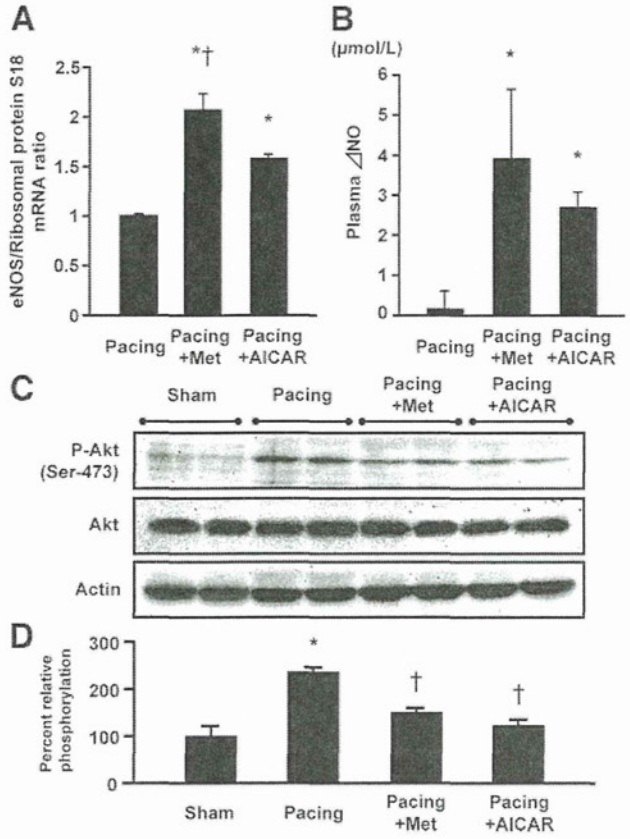


**Figure 8.** Effect of metformin on eNOS mRNA expression and plasma ΔNO levels, and phosphorylation of Akt in canine hearts. A, Quantitative real-time reverse-transcriptase polymerase chain reaction for eNOS mRNA. The mRNA levels were normalized to ribosomal protein S18 mRNA, and the pacing group was 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 $A_{1c}$ 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.

# Supplemental Methods

The animal experiments were approved by the National Cardiovascular Center Research Committee and were performed according to institutional guidelines.

**Experimental Protocols**

**1) Effects of Metformin on Cardiomyocyte Viability and Apoptosis After Exposure to $H_2O_2$**

To investigate whether metformin has a cardioprotective effect against damage due to $H_2O_2$ in vitro, we assessed cell viability and apoptosis in cultured cardiomyocytes using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and both the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining plus flow cytometry, respectively. The cells were cultured in serum-free media for 24 hours and then incubated in the presence of 50 μmol/L $H_2O_2$ for 24 hours. Cardiomyocytes were pretreated with either metformin (1 to 100 μmol/L) or 5-amino-4-imidazole-1-β-D-carboxamide ribofuranoside (AICAR; an AMPK activator) (500 μmol/L) for 60 minutes before the addition of $H_2O_2$. Other cells were preincubated with an AMPK inhibitor, compound-C (20 μmol/L) for 6 hours before the addition of either metformin or AICAR. Then cell viability and apoptosis were analyzed.

**2) Effects of Metformin on Cardiac Performance in Dogs With Pacing-Induced Heart Failure**

After pacemaker implantation, the dogs were randomly assigned to 3 groups as follows: 1) a group that received a normal diet and drinking water (Pacing group, n=8), 2) a group that received metformin orally at a dose of 100 mg/kg/day (Pacing+Met group, n=8), and 3) a group received AICAR

subcutaneously every other day at 5 mg/kg (Pacing+AICAR group, n=4). We also performed a sham operation in another 6 dogs (Sham group, n=6). The dose of metformin (100 mg/kg/day) was selected because our preliminary study showed that this was the maximum dose that did not induce hypoglycemia (data not shown). The dose of AICAR (5 mg/kg subcutaneously on alternate days) was selected because we preliminarily confirmed that phosphorylation of AMPK was elevated at least 48 hours after subcutaneous injection of AICAR, by reference to previous report in rats, due to the lack of any data for dogs (Supplemental Figures).[1] Echocardiography was performed and hemodynamic parameters were measured before and after 4 weeks of right ventricular (RV) pacing. After assessment of these parameters, each heart was excised and divided into three parts for immunoblotting, quantitative reverse-transcriptase polymerase chain reaction (PCR), and histological examination.

**Materials**

1, 1-Dimethylbiguanide hydrochloride (metformin hydrochloride) was a kind gift from Nippon Shinyaku Co. Ltd. (Kyoto, Japan), while AICAR (an AMPK activator) and compound-C (an AMPK inhibitor) were purchased from Calbiochem (California, USA). Antibodies directed against endothelial nitric oxide synthase (eNOS) were obtained from Affinity BioReagents (Colorado, USA). Other antibodies were purchased from Cell Signaling Technology (Massachusetts, USA).

**Cell Culture**

Primary cultures of cardiomyocytes were prepared from ventricles of 1-day-old Wistar rats, as described previously.[2] In brief, cardiomyocytes were plated at a density of $5\times10^5$ cells/mL on

collagen-coated culture dishes and incubated in standard medium (DMEM with 10% FBS) for 72 hours, after which incubation was continued under serum-free conditions for 48 hours.

**Cell Viability Assay (MTT Assay)**

Cell viability was analyzed by a nonradioactive cell proliferation assay using MTT, as described previously with minor modifications[3].

**Assessment of Cardiomyocyte Apoptosis**

To investigate the influence of metformin on cardiomyocyte viability, TUNEL assay was performed as reported previously.[3] Apoptosis was also quantified by flow cytometry (FACScan; Becton, Dickinson and Company, New Jersey, USA) after cells were stained with annexin V and propidine iodide (PI) according to the manufacturer's instructions (Annexin V-FITC Apoptosis Detection Kit; Sigma, Saint Louis, USA).

**Canine Pacing Model**

Beagle dogs (Oriental Yeast Co. Ltd, Tokyo, Japan) weighing 8 to 10 kg were sedated with intravenous sodium pentobarbital at a dose of 25 mg/kg. After intubation with a cuffed endotracheal tube, anesthesia was maintained with 0.5 % to 1% isoflurane and an equal mixture of air and oxygen. Ventilation was provided with a tidal volume of 22 mL/kg at a rate of 15 times per minute. A bipolar pacing lead (Model BT-45P, Star Medical Inc., Tokyo, Japan) was advanced under fluoroscopic guidance through the right jugular vein to the RV apex and was connected to a programmable pacemaker (VOO mode; Model SIP-501, Star Medical Inc., Tokyo, Japan) that was implanted in a

subcutaneous pocket in the neck. The success of this procedure was confirmed by electrocardiography. Cefazolin sodium (1 g) was given intravenously after surgery, and the dogs were allowed to recover for a few hours. Then heart failure was induced by rapid RV pacing at a rate of 230 beats per minute for 4 weeks, as reported previously.[4]

**Echocardiography**

Transthoracic echocardiography was performed by using an echocardiographic system equipped with a 4-MHz phased-array transducer (SONOS 5500, PHILIPS, Eindhoven, the Netherlands) in conscious dogs before pacemaker implantation and 30 minutes after the cessation of right ventricular (RV) pacing at 4 weeks. A two-dimensional short-axis view of the left ventricle was obtained at the level of the papillary muscles. All measurements were made by two observers, who were blinded with respect to the source of the tracings.

**Hemodynamic Studies**

Both left ventricular end-diastolic pressure (LVEDP) and mean aortic pressure were measured by pressure transducers using a 5 Fr pig tail catheter (Terumo Co. Ltd., Tokyo, Japan) that was inserted into the left ventricle from the left femoral artery. The mean pulmonary artery pressure (PAP) and the pulmonary capillary wedge pressure (PCWP) were measured using a 7 Fr Swan-Ganz catheter (American Edwards Laboratories, California, USA). Cardiac output (CO) was determined at least three times by the thermodilution technique. Systemic vascular resistance (SVR) was calculated as follows: (mean aortic pressure-right atrial pressure) × 80/CO.

**Histological examination**

The collagen volume fraction was examined in sections of the left ventricular (LV) free wall, after excluding vessels, artifacts, minor scars, and incomplete tissue. Specimens were stained with Masson's trichrome stain to evaluate the extent of interstitial fibrosis, as described previously.[5] The area of stained tissue was calculated as a percentage of the total area within a field by using Scion image software (Beta 4.0.2).

**Quantitative Reverse-Transcriptase PCR**

The quantitative reverse-transcriptase PCR was performed as described previously.[6] Total RNA was extracted from LV myocardium with RNA-Bee-RNA Isolation Reagent (Tel-Test, Texas, USA). Then 1,000 ng of total RNA was reverse transcribed and amplified with an Omniscript RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Oligonucleotide primers and TaqMan probes for canine atrial natriuretic peptide (ANP) (Cf 02705687_g1), canine transforming growth factor-β1 (TGF-β1) (Cf 02741608_m1), and canine ribosomal protein S18 (Cf 02681523_g1) were purchased from Applied Biosystems (California, USA). Both Taqman probe and primer designs were optimized to enhance stability on the basis of the known sequences of canine brain natriuretic peptide (BNP)[7] and canine endothelial NO synthase (eNOS).[8] We used the following probes, sense primers, and antisense primers: 5'-FAM-CAGTTGGCCCTGGAA-MGB-3', 5'-GAAGGACGCAGTTTCAGAGCTG -3' and 5'-AAAGCACCCTGACTTGTGCATC-3' for canine BNP; and

5'-FAM-CCTGGAGGATGTGGC-MGB-3', 5'-AACCTGTGTGACCCTCATCGAT-3' and 5'-TCACTTTGGCCAGCTGGTAACT-3' for canine eNOS, respectively.

**Immunoblotting**

Immunoblotting was performed as described previously.[9] A Bio-Rad ChemiDoc XRS system (Bio-Rad Laboratories, Inc., California, USA) was used for chemiluminescence imaging and immunoreactive bands were quantified with Bio-Rad Quantity One 1-D analysis software (Bio-Rad Laboratories, Inc., California, USA).

**Measurement of Nitric Oxide End-Products**

The plasma level of nitric oxide (NO) metabolic end-products (nitrite + nitrate) was measured by the Griess method, as reported previously.[10] Subsequently, ΔNO was defined as the difference between the plasma NO level before and after 4 weeks of RV pacing.

**Metabolic Parameters**

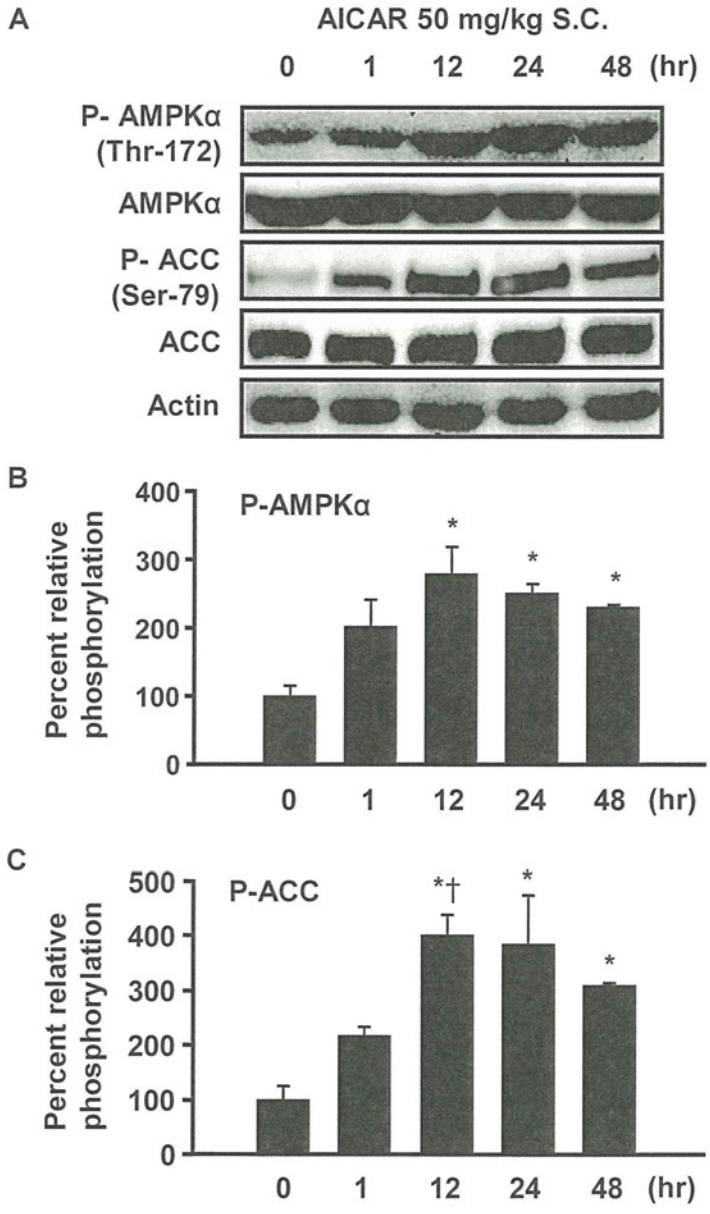
All dogs were fed a standard diet with a fixed carbohydrate and fat content (DS-A, Oriental Yeast Co. Ltd, Tokyo, Japan). After fasting for 14 hours, metabolic parameters such as the plasma levels of glucose, lactate, free fatty acids (FFA), and insulin were measured with a quick-auto-neo-GLU-HK (Shino-Test Corporation, Tokyo, Japan.), Determiner LA (KYOWA MEDEX Co., Ltd., Tokyo, Japan.), NEFA-SS Eiken, Eiken Chemical Co., Ltd., Tokyo, Japan.), and YK060 Insulin ELISA Kit (Yanaihara Institute Inc. Shizuoka, Japan), respectively. Insulin resistance was assessed from the fasting insulin and glucose levels by the homeostasis model assessment-insulin resistance (HOMA-IR) method, i.e.,

HOMA-IR is [fasting glucose (mmol/L) × fasting insulin (μU/mL)] / 22.5.[11] The levels of norepinephrine and angiotensin II were measured by using a CA test TOSOH (Tosoh Corporation, Tokyo, Japan.) and a NEX-105 (125I)-Tyr4-Angiotensin II test (PerkinElmer Inc., Massachusetts, USA.), respectively. Myocardial substrate extraction was calculated as described previously.[12]

**Measurement of Body Fat and Activity in Dogs**

To examine the effects of metformin on body fat and physical activity in this dog model of pacing-induced heart failure, we measured body fat with a dog body fat counter (IBF-D02, Kao Corporation, Tokyo, Japan) and evaluated physical activity by using a pedometer (SE-MG10, SATO KEIRYOUKI MFG. Co., Ltd., Tokyo, Japan) attached to each dog's collar.

## Supplemental Figures




**Changes in the phosphorylation of AMPKα and ACC in canine hearts after subcutaneous administration of AICAR. A)** Representative immunoblots of phospho-AMPKα and ACC. **B)** and **C)** The percent relative phosphorylation of AMPKα and ACC, respectively. Values are the mean±SEM. **P*<0.05 vs. no treatment; †*P*<0.05 vs. one hour after subcutaneous administration of AICAR. Representative results from 3 independent experiments are shown.

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# In vivo direct monitoring of vagal acetylcholine release to the sinoatrial node

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ABSTRACT

To directly monitor vagal acetylcholine (ACh) release into the sinoatrial node, which regulates heart rate, we implanted a microdialysis probe in the right atrium near the sinoatrial node and in the right ventricle of anesthetized rabbits, and perfused with Ringer's solution containing eserine. (1) Electrical stimulation of right or left cervical vagal nerve decreased atrial rate and increased dialysate ACh concentration in the right atrium in a frequency-dependent manner. Compared to left vagal stimulation, right vagal nerve stimulation decreased atrial rate to a greater extent at all frequencies, and increased dialysate ACh concentration to a greater extent at 10 and 20 Hz. However, dialysate ACh concentration in the right atrium correlated well with atrial rate independent of whether electrical stimulation was applied to the right or left vagal nerve (atrial rate $= 304 - 131 \times \log[\mathrm{ACh}]$, $R^2 = 0.77$). (2) Right or left vagal nerve stimulation at 20 Hz decreased atrial rate and increased dialysate ACh concentrations in both the right atrium (right, $17.9 \pm 4.0$ nM; left, $7.9 \pm 1.4$ nM) and right ventricle (right, $0.9 \pm 0.3$ nM; left, $1.0 \pm 0.4$ nM). However, atrial dialysate ACh concentrations were significantly higher than ventricular concentrations, while ventricular dialysate ACh concentrations were not significantly different between right and left vagal nerve stimulation. (3) The response of ACh release to right and left vagal nerve stimulation was abolished by intravenous administration of a ganglionic blocker, hexamethonium bromide. In conclusion, ACh concentration in dialysate from the right atrium, sampled by microdialysis, is a good marker of ACh release from postganglionic vagal nerves to the sinoatrial node.


## 1. Introduction

Parasympathetic nerves play an important role in the regulation of heart rate under physiological conditions. To better understand the parasympathetic control of heart rate, it is important to quantitatively assess the efferent cardiac vagal nerve activity. Several methods have been used to assess this activity. Efferent cardiac vagal nerve electrical activity has been measured directly at the preganglionic site in several studies (Jewett, 1964; Kunze, 1972). We have developed a microdialysis technique which is used with high-performance liquid chromatography (HPLC) to monitor in vivo endogenous acetylcholine (ACh) release in the heart (Akiyama et al., 1994). Using this technique, we were able to monitor endogenous ACh release into the ventricular myocardium (Akiyama et al., 1994; Kawada et al., 2001). This technique permits the estimation of relative changes in postganglionic efferent cardiac vagal nerve activity in the ventricle.

However, vagal innervation is known to be heterogeneous in the heart. Kilbinger and Löffelholz (1976) reported that the ACh content of the ventricle was 41% and 19% of the atrial content in chicken and rabbit, respectively. Brown (1976) reported that ACh concentration was higher in the atrium than the ventricle, and that ACh content was higher in the right than the left portions in both the atrium and ventricle of the cat. Thus, to better understand the parasympathetic control of heart rate, which is the sinus rate under physiological conditions, we need information about the activities of postganglionic vagal nerves innervating the sinoatrial (SA) node.

In this study, we developed a dialysis probe using shorter dialysis fiber, which was suitable for implantation into the atrium. Using this dialysis probe, we tried to monitor myocardial interstitial ACh levels in the right atrium, especially near the SA node. Furthermore, we investigated whether the myocardial interstitial ACh levels reflect relative changes in activity of postganglionic vagal nerves innervating the SA node.

## 2. Materials and methods

### 2.1. Surgical preparation

Animal care was provided in accordance with the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences*

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