might attenuate vagal neurotransmission, contributing to the aggravation of disease states.

Effects of ANG II on the transfer function from sympathetic stimulation to HR. Although ANG II attenuated the dynamic gain of the transfer function from sympathetic stimulation to HR without affecting the natural frequency, damping ratio, or pure delay, the attenuating effect was not definitive because the effect was not significant on the steady-state response in the calculated step response (Fig. 4 and Table 4). There are conflicting reports about the effects of ANG II on sympathetic control of the heart. Starke (33) reported that ANG II (1 ng/ml) potentiated NE release in response to postganglionic sympathetic nerve stimulation in isolated rabbit hearts, whereas no effect on spontaneous or tyramine-induced NE output was observed. Farrell et al. (10) demonstrated that administration of ANG II (100 μM at 1 ml/min for 10 min; ~35–42 $\mu g \cdot kg^{-1}$) into right atrial ganglionated plexus neurons via a branch of the right coronary artery caused the release of catecholamine into the myocardial interstitial fluid of anesthetized dogs, suggesting that ANG II affects intrinsic cardiac neurons. In that study, the effect of ANG II on the catecholamine release induced by cardiac sympathetic nerve stimulation was not investigated. On the other hand, Lameris et al. (19) demonstrated that administration of ANG II (0.5 $ng \cdot kg^{-1} \cdot min^{-1}$ or 30 $ng \cdot kg^{-1} \cdot h^{-1}$) into the left anterior descending coronary artery of anesthetized pigs did not yield spontaneous NE release or enhance the NE release induced by cardiac sympathetic nerve stimulation. Cardiac ganglia derived from different species can demonstrate differences in phenotype for ANG II receptors, and this may impact on the resultant neurohumoral interactions. Dendorfer et al. (7) demonstrated that ANG II (0.3 to 1 μg/kg bolus) increased renal sympathetic nerve activity during ganglionic blockade in pithed rats, suggesting direct ganglionic excitation by ANG II. In the present study, because we stimulated the postganglionic cardiac sympathetic nerve, possible direct ganglionic excitation by ANG II might not have affected the dynamic sympathetic control of HR. In addition, postganglionic CSNA did not change significantly in our experimental conditions (Tables 1 and 3), indicating that the 10 $\mu g \cdot kg^{-1} \cdot h^{-1}$ dose of intravenous ANG II was not high enough to produce direct ganglionic excitation.

In isolated rabbit hearts, Peach et al. (30) demonstrated that ANG II (0.2 ng/ml) inhibited NE uptake. Starke (33) reported a higher dose of ANG II (10 μg/ml) to inhibit NE uptake. In a previous study from our laboratory, blockade of neuronal NE uptake using desipramine attenuated the dynamic gain, decreased the natural frequency, and increased the pure delay of the transfer function from sympathetic stimulation to HR (28). In the present study, however, neither the natural frequency nor the pure delay was changed by ANG II, suggesting that NE uptake was not inhibited. In an in vivo study using canine hearts, Lokhandwala et al. (22) demonstrated that ANG II (100 and 200 $ng \cdot kg^{-1} \cdot min^{-1}$ or 6 and 12 $\mu g \cdot kg^{-1} \cdot min^{-1}$ iv) did not affect the positive chronotropic effects of either postganglionic cardiac sympathetic nerve stimulation or intravenous NE infusion. In that study, ANG II enhanced the positive chronotropic effects of sympathetic nerve stimulation but not of intravenous NE infusion after blocking neuronal NE uptake with desipramine. The authors' interpretation of the results was that ANG II facilitated NE release in response to sympathetic nerve stimulation, whereas any effects of ANG II might be masked in animals with functioning neuronal NE uptake mechanisms (22). To make matters more complex, Lameris et al. (19) did not observe enhanced NE release during cardiac sympathetic stimulation in porcine hearts even after neuronal NE uptake was blocked with desipramine. Thus it appears that differences in species, ANG II doses, and experimental settings (in vivo vs. isolated hearts, intravenous vs. intracoronary administration, with or without the contribution of sympathetic ganglia) critically affected the experimental results. Therefore, we believe that assessing the relative effects of ANG II on the vagal and sympathetic systems is important to understand the pathophysiological roles of ANG II in the autonomic regulation of HR.

Limitations. Our results should be interpreted in the context of various experimental limitations. First, we obtained data from anesthetized animals. If the data had been obtained under conscious conditions, the results might have been different. Because we disabled the arterial baroreflexes and cut the autonomic efferent pathways, however, the anesthetics should not have markedly affected our results. Second, because we stimulated the postganglionic cardiac sympathetic nerve, the possible effects of ANG II on the sympathetic ganglia were not assessed. Further studies that stimulate the preganglionic cardiac sympathetic nerve with various doses of ANG II are required to determine the effects of ANG II on the cardiac sympathetic ganglionic transmission. Finally, ANG II may affect the autonomic regulation of HR chronically. Further studies focused on the effects of chronically elevated ANG II levels on the autonomic regulation of HR are required to elucidate the pathophysiological significance of elevated ANG II levels.

In conclusion, continuous intravenous administration of ANG II at a dose that did not induce direct cardiac sympathetic ganglionic excitation significantly attenuated the dynamic gain of the transfer function from vagal stimulation to HR. The attenuation of the transfer gain was observed uniformly in the frequency range under study, suggesting that ANG II can attenuate the HF component of HRV even when vagal outflow from the central nervous system remains unchanged. In addition, the same dose of ANG II did not markedly affect the dynamic gain of the transfer function from postganglionic sympathetic stimulation to HR. Although there remains a room for arguments relating to the different site of stimulation (preganglionic for vagal vs. postganglionic for sympathetic), possible disproportional suppression of the dynamic vagal and sympathetic regulation of HR likely results in a relative dominance of sympathetic control in the presence of ANG II. Because many neurohumoral elements remodel or adapt during the evolution of cardiac pathology (18), we cannot directly extrapolate the results of acute neurohumoral interactions observed in the present study to the chronic pathological situations. If we do so, however, the reduction of the HF component of HRV in patients with cardiovascular diseases, such as myocardial infarction and heart failure (34), may be partly explained by the peripheral effects of ANG II on the dynamic autonomic regulation of HR.

APPENDIX A

Meaning of a step response calculated from a transfer function. We calculated a step response from a transfer function relating to the vagal or sympathetic HR control. The calculated step response is useful for time-domain interpretation of the low-pass filter characteristics described by the frequency-domain transfer function but does not necessarily conform to an experimentally estimated step response because of the following reasons. The transfer function identifies the

linear input-output relationship of a given system around a mean input signal (5 Hz for vagal and 2.5 Hz for sympathetic stimulation in the present study). The step response is then calculated for a unit change in the input signal. If we perform a kind of experiment where we change the stimulation frequency from 4.5 to 5.5 Hz for the vagal system and from 2 to 3 Hz for the sympathetic system, the resultant step response is most likely close to the calculated step response. The ordinary experimental step response is, however, estimated by a step input in which the stimulation is completely turned off before the stimulation starts. The calculated step response and the ordinary experimental step response can conform only when the system is purely linear. Whenever nonlinearities exist such as threshold and saturation commonly observed in biological systems, the two step responses disagree. Conversely, information gained by the ordinary experimental step response has a limited ability to estimate the dynamic HR response unless the system is purely linear.

Once vagal or sympathetic transfer function is identified, an impulse response of the system is obtained by an inverse Fourier transform of the transfer function. We can estimate the dynamic HR response from a convolution of a input signal and the impulse response. Figure 5 represents typical data of measured HR and calculated HR based on the transfer function. Figure 5*A* is a continuation of the time series obtained under the control condition depicted in Fig. 1*A*. Figure 5*B* shows a scatter plot of measured HR versus calculated HR during dynamic vagal stimulation. The solid line indicates a linear regression line ($r^2 = 0.94$). Figure 5*C* is a continuation of the time series obtained under the control condition depicted in Fig. 3*A*. Figure 5*D* shows the scatter plot of measured HR versus calculated HR during dynamic sympathetic stimulation. The solid line indicates a linear regression line. Although a slight convex nonlinearity is noted between the measured HR and calculated HR, squared correlation coefficient is high ($r^2 = 0.89$). These results indicate that the transfer function can represent the dynamic HR response reasonably well.

APPENDIX B

Binary white noise versus Gaussian white noise. In a previous study from our laboratory (29), we reported a corner frequency of ~0.1 Hz for a transfer function from vagal stimulation to HR, which was distinctly different from the result of the present study. Possible explanation for the discrepancy is the difference in the input variance (or power) of vagal stimulation. In the previous study, we used a Gaussian white noise (GWN) with a mean stimulation frequency of 5 Hz and a SD of 2 Hz so that the input signal covered at most 98.8% (means ± 2.5 SD) of the Gaussian distribution when the actual stimulation frequency was limited between 0 and 10 Hz. The variance of the GWN signal is 4 Hz^2. In contrast, the 0–10 Hz binary white noise used in the present study has a variance of 25 Hz^2. Hence, the binary white noise has a merit of increasing the input variance over the GWN when the stimulation frequency is limited between 0 and 10 Hz. Increasing the

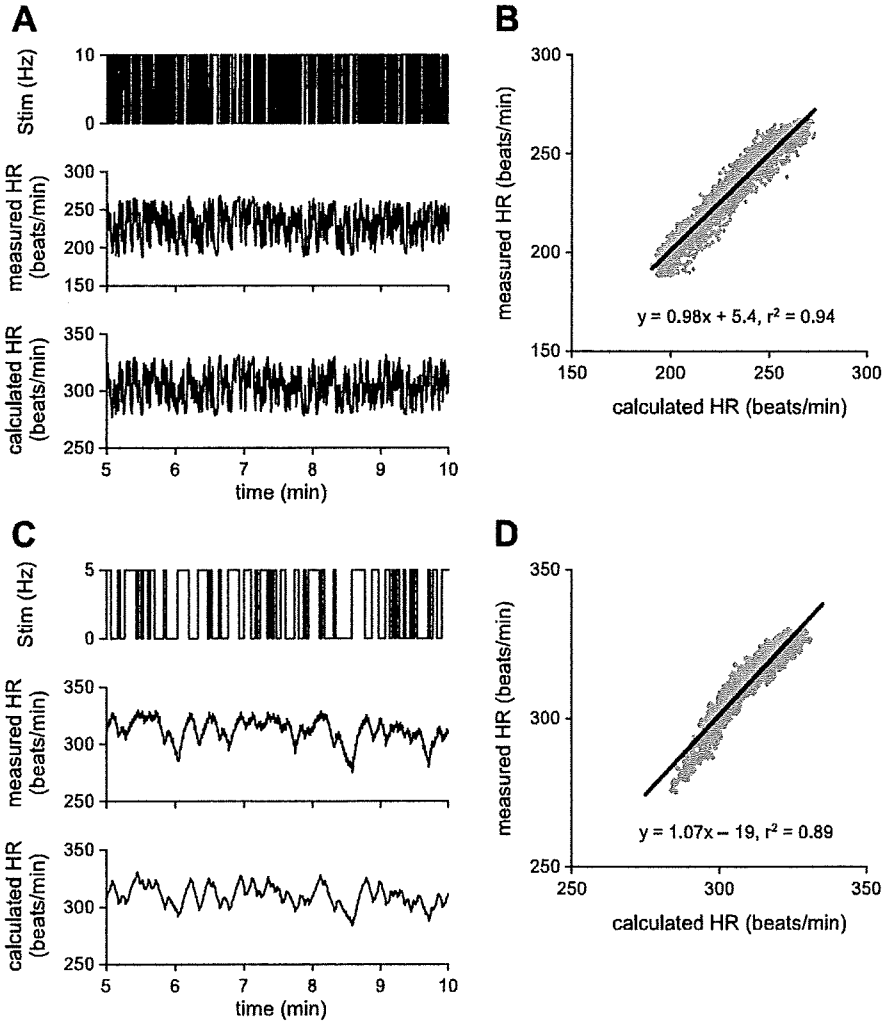

Fig. 5. *A*: data showing vagal stimulation (Stim), measured HR, and calculated HR based on the identified vagal transfer function of this animal. Time axis indicates the minutes after the initiation of random vagal stimulation (continuation of Fig. 1*A*). *B*: scatter plot between measured and calculated HR values. A solid line indicates a liner regression line. *C*: data showing sympathetic stimulation (Stim), measured HR, and calculated HR based on the identified sympathetic transfer function of this animal. Time axis indicates the minutes after the initiation of random sympathetic stimulation (continuation of Fig. 3*A*). *D*: scatter plot between measured and calculated HR values. A solid line indicates a linear regression line.

input variance is effective to increase the signal-to-noise ratio in the output signal and to improve the estimation of the transfer function.

In an earlier study on the transfer function analysis, Berger et al. (3) demonstrated that the roll-off of the vagal transfer function was gentle (i.e., the corner frequency was high) at high mean stimulatory rates and became more abrupt (i.e., the corner frequency was lower) with lower mean stimulatory rates. Although they attributed the difference in the roll-off characteristics to the difference in mean stimulatory rates, because they set the variance of input signal at ~1/4 of the mean stimulatory rates, which of the mean stimulatory rates or the input variance contributed to the determination of corner frequency seems inconclusive. Because there was no significant difference in the corner frequency between the vagal transfer functions estimated by GWNs of 5 ± 2 Hz and 10 ± 2 Hz (means ± SD) in a previous study from our laboratory (29), we speculate that the difference in the input variance rather than the mean stimulation frequency might have caused the different values of the corner frequency between the previous and the present results. This speculation requires further verification in future.

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REFERENCES

1. **Akselrod S, Gordon D, Ubel FA, Shannon DC, Berger AC, Cohen RJ.** Power spectrum analysis of heart rate fluctuation: a quantitative probe of beat-to-beat cardiovascular control. *Science* 213: 220–222, 1981.
2. **Andrews PL, Dutia MB, Harris PJ.** Angiotensin II does not inhibit vagally-induced bradycardia or gastric contractions in the anaesthetized ferret. *Br J Pharmacol* 82: 833–837, 1984.
3. **Berger RD, Saul JP, Cohen RJ.** Transfer function analysis of autonomic regulation. I. Canine atrial rate response. *Am J Physiol Heart Circ Physiol* 256: H142–H152, 1989.
4. **Brigham EO.** FFT transform applications. In: *The Fast Fourier Transform and Its Applications*. Englewood Cliffs, NJ: Prentice-Hall, 1988, p. 167–203.
5. **Brooks VL.** Chronic infusion of angiotensin II resets baroreflex control of heart rate by an arterial pressure-independent mechanism. *Hypertension* 26: 420–424, 1995.
6. **Castillo-Hernandez JR, Rubio-Gayosso I, Sada-Ovalle I, Garcia-Vazquez A, Ceballos G, Rubio R.** Intracoronary angiotensin II causes inotropic and vascular effects via different paracrine mechanisms. *Vascul Pharmacol* 41: 147–158, 2004.
7. **Dendorfer A, Thornagel A, Raasch W, Grisk O, Tempel K, Dominiak P.** Angiotensin II induces catecholamine release by direct ganglionic excitation. *Hypertension* 40: 348–354, 2002.
8. **DiBona GF.** Physiology in perspective: the wisdom of the body. Neural control of the kidney. *Am J Physiol Regul Integr Comp Physiol* 289: R633–R641, 2005.
9. **Diz DI, Averill DB.** Angiotensin II/autonomic interactions. In: *Primer on the Autonomic Nervous System*, edited by Robertson D, Biaggioni I, Burnstock G, and Low PA. San Diego: Elsevier Academic Press, 2004, p. 168–171.
10. **Farrell DM, Wei CC, Tallaj J, Ardell JL, Armour JA, Hageman GR, Bradley WE, Dell'Italia LJ.** Angiotensin II modulates catecholamine release into interstitial fluid of canine myocardium in vivo. *Am J Physiol Heart Circ Physiol* 281: H813–H822, 2001.
11. **Glantz SA.** *Primer of Biostatistics* (5th ed.). New York: McGraw-Hill, 2002.
12. **Jackson EK.** Autonomic control of the kidney. In: *Primer on the Autonomic Nervous System*, edited by Robertson D, Biaggioni I, Burnstock G, and Low PA. San Diego: Elsevier Academic Press, 2004, p. 157–161.
13. **Kashihara K, Takahashi Y, Chatani K, Kawada T, Zheng C, Li M, Sugimachi M, Sunagawa K.** Intravenous angiotensin II does not affect dynamic baroreflex characteristics of the neural or peripheral arc. *Jpn J Physiol* 53: 135–143, 2003.
14. **Kawada T, Ikeda Y, Sugimachi M, Shishido T, Kawaguchi O, Yamazaki T, Alexander J Jr, Sunagawa K.** Bidirectional augmentation of heart rate regulation by autonomic nervous system in rabbits. *Am J Physiol Heart Circ Physiol* 271: H288–H295, 1996.
15. **Kawada T, Miyamoto T, Miyoshi Y, Yamaguchi S, Tanabe Y, Kamiya A, Shishido T, Sugimachi M.** Sympathetic neural regulation of heart rate is robust against high plasma catecholamine. *J Physiol Sci* 56: 235–245, 2006.
16. **Kawada T, Uemura K, Kashihara K, Jin Y, Li M, Zheng C, Sugimachi M, Sunagawa K.** Uniformity in dynamic baroreflex regulation of left and right cardiac sympathetic nerve activities. *Am J Physiol Regul Integr Comp Physiol* 284: R1506–R1512, 2003.
17. **Kawada T, Yamazaki T, Akiyama T, Li M, Zheng C, Shishido T, Mori H, Sugimachi M.** Angiotensin II attenuates myocardial interstitial acetylcholine release in response to vagal stimulation. *Am J Physiol Heart Circ Physiol* 293: H2516–H2522, 2007.
18. **Khan MH, Sinoway LI.** Congestive heart failure. In: *Primer on the Autonomic Nervous System*, edited by Robertson D, Biaggioni I, Burnstock G, and Low PA. San Diego: Elsevier Academic Press, 2004, p. 247–248.
19. **Lameris TW, de Zeeuw S, Duncker DJ, Alberts G, Boomsma F, Verdouw PD, van den Meiracker AH.** Exogenous angiotensin II does not facilitate norepinephrine release in the heart. *Hypertension* 40: 491–497, 2002.
20. **Levy MN.** Sympathetic-parasympathetic interactions in the heart. *Circ Res* 29: 437–445, 1971.
21. **Li M, Zheng C, Sato T, Kawada T, Sugimachi M, Sunagawa K.** Vagal nerve stimulation markedly improves long-term survival after chronic heart failure in rats. *Circulation* 109: 120–124, 2004.
22. **Lokhandwala MF, Amelang E, Buckley JP.** Facilitation of cardiac sympathetic function by angiotensin II: role of presynaptic angiotensin receptors. *Eur J Pharmacol* 52: 405–409, 1978.
23. **Marmarelis PZ, Marmarelis VZ.** The white noise method in system identification. In: *Analysis of Physiological Systems*. New York: Plenum, 1978, p. 131–221.
24. **Miyamoto T, Kawada T, Takaki H, Inagaki M, Yanagiya Y, Jin Y, Sugimachi M, Sunagawa K.** High plasma norepinephrine attenuates the dynamic heart rate response to vagal stimulation. *Am J Physiol Heart Circ Physiol* 284: H2412–H2418, 2003.
25. **Miyamoto T, Kawada T, Yanagiya Y, Inagaki M, Takaki H, Sugimachi M, Sunagawa K.** Cardiac sympathetic nerve stimulation does not attenuate dynamic vagal control of heart rate via α-adrenergic mechanism. *Am J Physiol Heart Circ Physiol* 287: H860–H865, 2004.
26. **Mizuno M, Kamiya A, Kawada T, Miyamoto T, Shimizu S, Sugimachi M.** Muscarinic potassium channels augment dynamic and static heart rate responses to vagal stimulation. *Am J Physiol Heart Circ Physiol* 293: H1564–H1570, 2007.
27. **Nakahara T, Kawada T, Sugimachi M, Miyano H, Sato T, Shishido T, Yoshimura R, Miyashita H, Inagaki M, Alexander J Jr, Sunagawa K.** Accumulation of cAMP augments dynamic vagal control of heart rate. *Am J Physiol Heart Circ Physiol* 275: H562–H567, 1998.
28. **Nakahara T, Kawada T, Sugimachi M, Miyano H, Sato T, Shishido T, Yoshimura R, Miyashita H, Inagaki M, Alexander J Jr, Sunagawa K.** Neuronal uptake affects dynamic characteristics of heart rate response to sympathetic stimulation. *Am J Physiol Regul Integr Comp Physiol* 277: R140–R146, 1999.
29. **Nakahara T, Kawada T, Sugimachi M, Miyano H, Sato T, Shishido T, Yoshimura R, Miyashita H, Sunagawa K.** Cholinesterase affects dynamic transduction properties from vagal stimulation to heart rate. *Am J Physiol Regul Integr Comp Physiol* 275: R541–R547, 1998.
30. **Peach MJ, Bumpus FM, Khairallah PA.** Inhibition of norepinephrine uptake in hearts by angiotensin II and analogs. *J Pharmacol Exp Ther* 167: 291–299, 1969.
31. **Potter EK.** Angiotensin inhibits action of vagus nerve at the heart. *Br J Pharmacol* 75: 9–11, 1982.
32. **Reid IA, Chou L.** Analysis of the action of angiotensin II on the baroreflex control of heart rate in conscious rabbits. *Endocrinology* 126: 2749–2756, 1990.
33. **Starke K.** Action of angiotensin on uptake, release and metabolism of ^{14}C-noradrenaline by isolated rabbit hearts. *Eur J Pharmacol* 14: 112–123, 1971.
34. **Task Force of the European Society of Cardiology, the North American Society of Pacing and Electrophysiology.** Heart rate variability: standards of measurement, physiological interpretation and clinical use. *Circulation* 93: 1043–1065, 1996.
35. **Zimmerman BG, Sybertz EJ, Wong PC.** Interaction between sympathetic and renin-angiotensin system. *J Hypertens* 2: 581–587, 1984.

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 μmol/L) protected cultured cardiomyocytes from cell death during exposure to H_2O_2 (50 μmol/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 $mg \cdot kg^{-1} \cdot d^{-1}$ (n=8) (18.6±1.8% and 11.8±1.1 mm Hg, respectively) compared with dogs receiving vehicle (n=8) (9.6±0.7% and 22±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,[1] suggesting that this drug may be useful for patients who have both cardiovascular disease and diabetes mellitus. Eurich and colleagues[2] 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),[3–5] which is expressed in various tissues, including the myocardium, and plays a central role in the regulation of energy metabolism under stress conditions.[6] AMPK is activated by ischemia/reperfusion,[7–9] as well as in hearts with pressure overload hypertrophy[10] and subsequent heart failure.[11,12] In addition, Russell et al[9] 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.[8] Furthermore, metformin has been reported to increase the production of nitric oxide (NO),[13–15] which is known to have various beneficial cardiovascular

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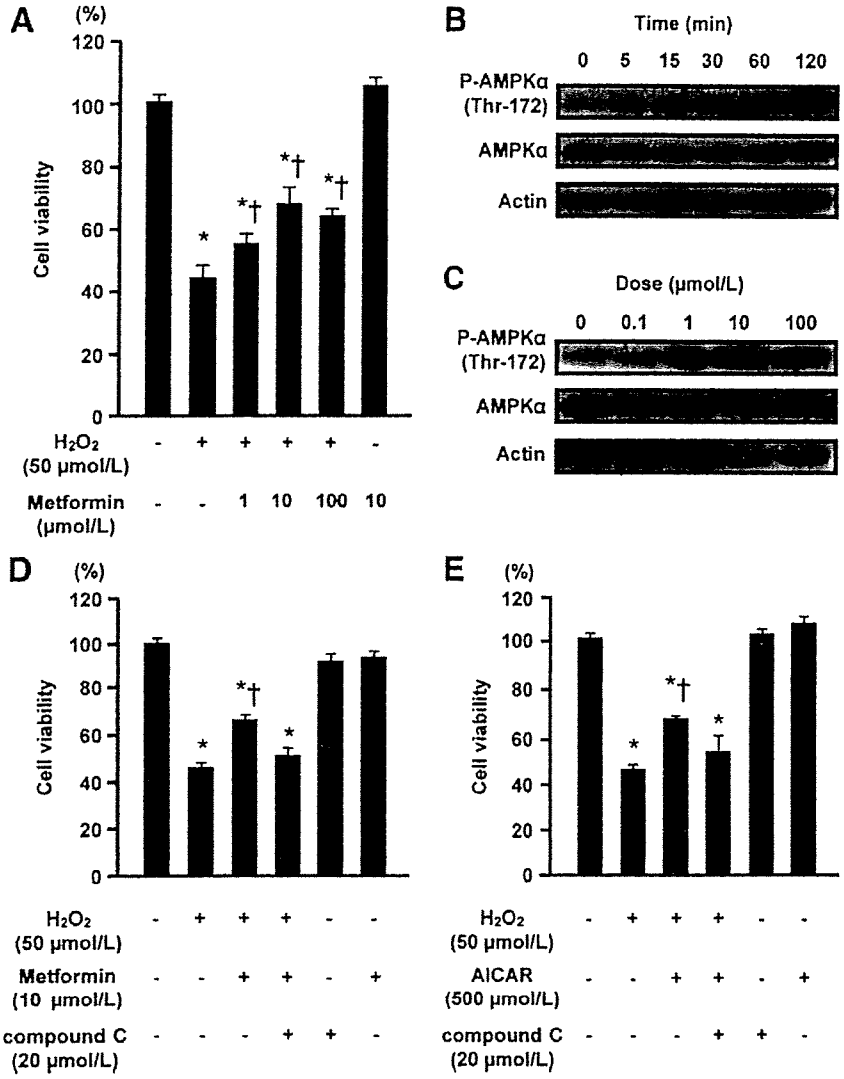

Figure 1. Effect of metformin on oxidative stress–induced cell death via AMPK activation in cultured rat cardiomyocytes. A, Cardiomyocyte viability after treatment with metformin (1, 10, or 100 μmol/L) and exposure to H_2O_2 (50 μmol/L). B, Time (0, 5, 15, 30, 60, 120 minutes) -dependent changes in AMPK 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_2O_2 (50 μmol/L) treatment.

effects[16] 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_2O_2 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_2O_2, 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_2O_2 (Figure 1E). These results suggested that

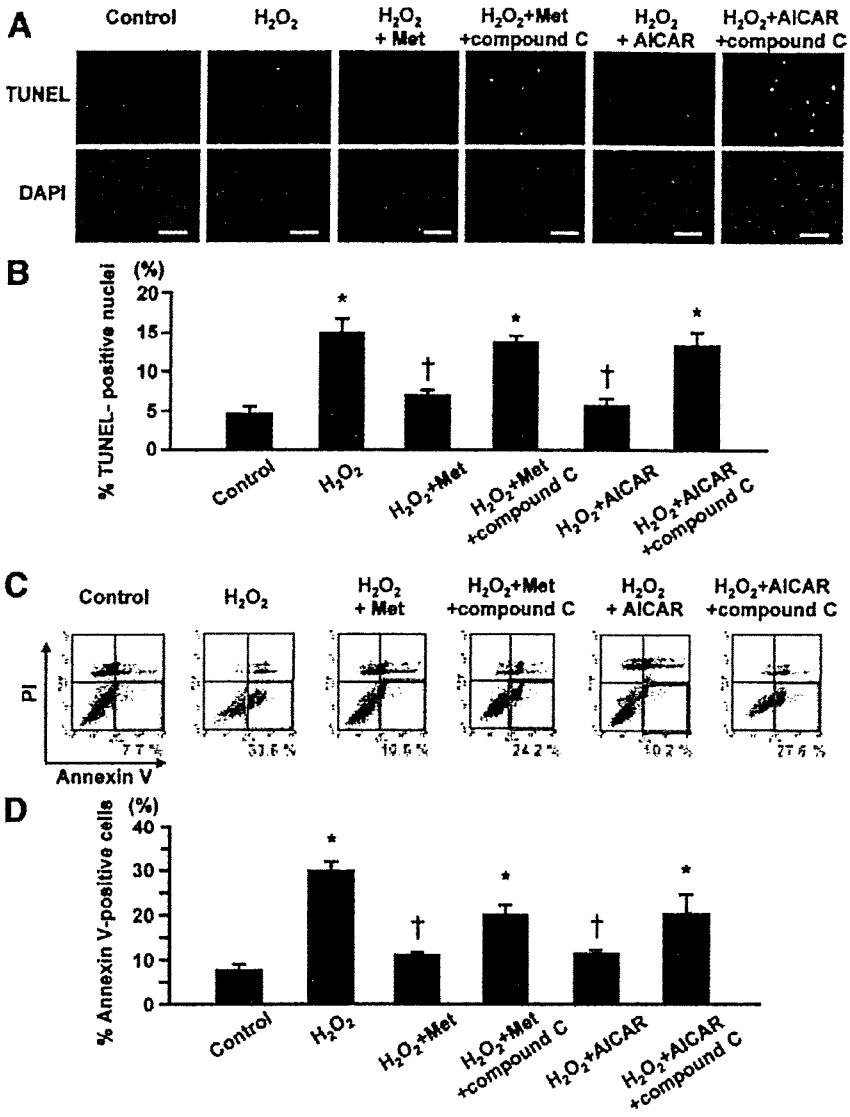

Figure 2. Effect of metformin on oxidative stress–induced apoptosis via AMPK activation in cultured rat cardiomyocytes. Representative (A) and quantitative (B) data on cardiomyocyte apoptosis obtained by TUNEL staining (n=3 in each experiment). Representative (C) and quantitative (D) data on cardiomyocyte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

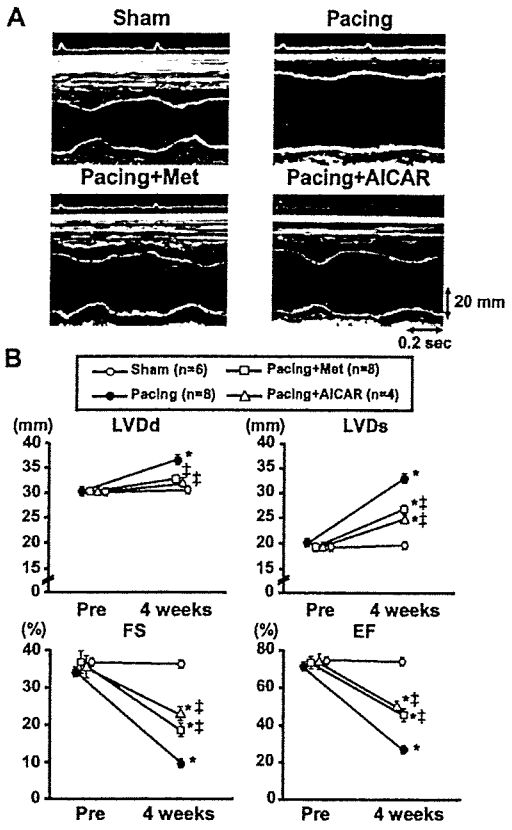

Figure 3. Effect of metformin on echocardiographic parameters. A, Representative M-mode echocardiograms obtained 4 weeks after sham surgery or after RV pacing. B, Echocardiographic parameters before and after sham surgery or after RV pacing in the sham group (n=6), pacing group (n=8), pacing plus metformin 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-β1 (TGF-β1) mRNA associated with fibrosis in canine LV myocardium 4 weeks after pacing. Metformin suppressed the increase in TGF-β1 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 ΔNO (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.

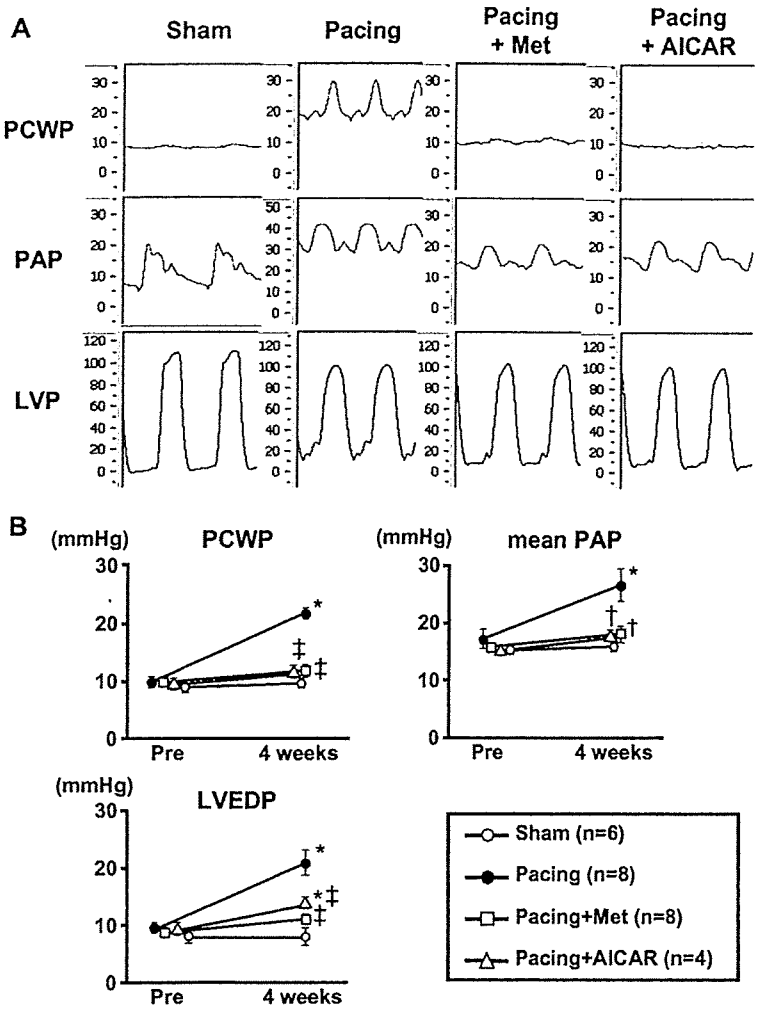

Figure 4. Effect of metformin on hemodynamic parameters. A, Representative graphs of hemodynamic parameters obtained at 4 weeks. B, Hemodynamic parameters before and after the 4-week study period in the sham (n=6), pacing (n=8), pacing plus metformin (n=8), and pacing plus AICAR (n=4) groups. Values 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

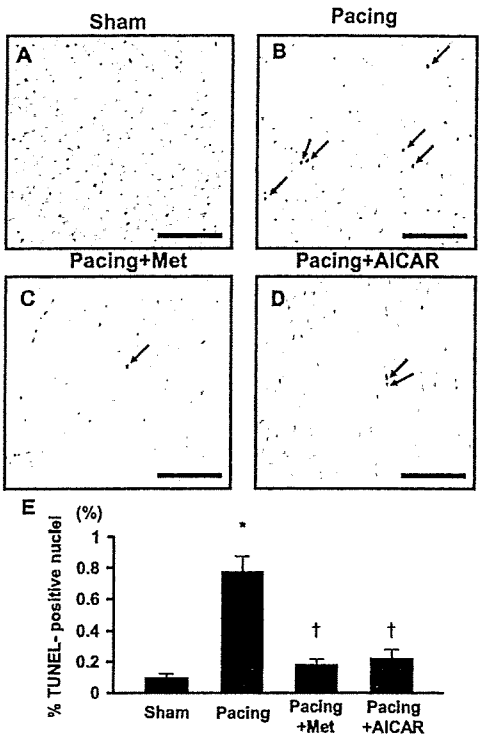

Figure 5. TUNEL staining of canine hearts at 4 weeks. Representative examples of TUNEL-stained hearts from sham (A), pacing (B), pacing plus metformin (C), and pacing plus AICAR (D) groups. Arrows indicate TUNEL-positive nuclei (brown). Scale bar=100 μm. E, Quantitative data on the percentage of TUNEL-positive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

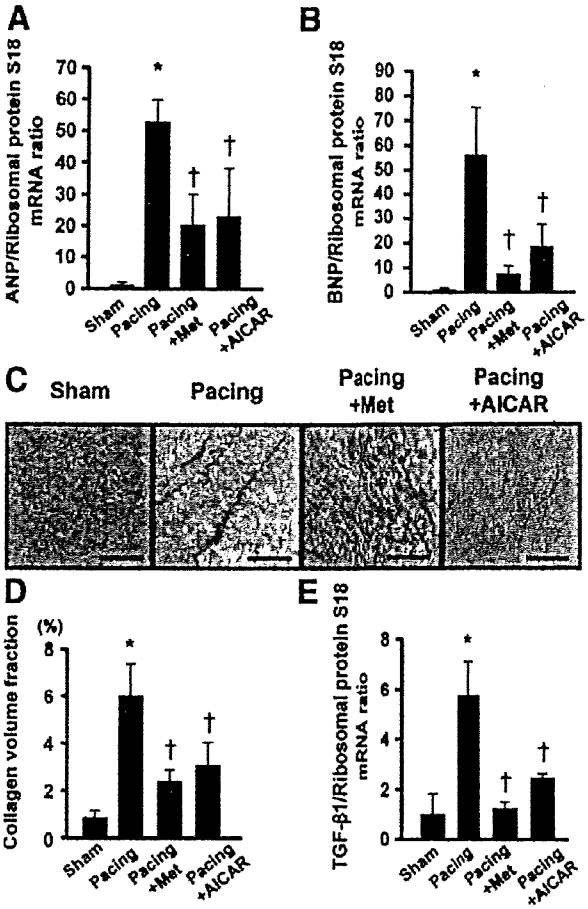

Figure 6. Natriuretic peptide expression, cardiac collagen volume fraction, and TGF-β1 expression. A, B, and E, Quantitative real-time reverse-transcriptase polymerase chain reaction analysis of myocardial atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and TGF-β1 expression, respectively. 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 μm. 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

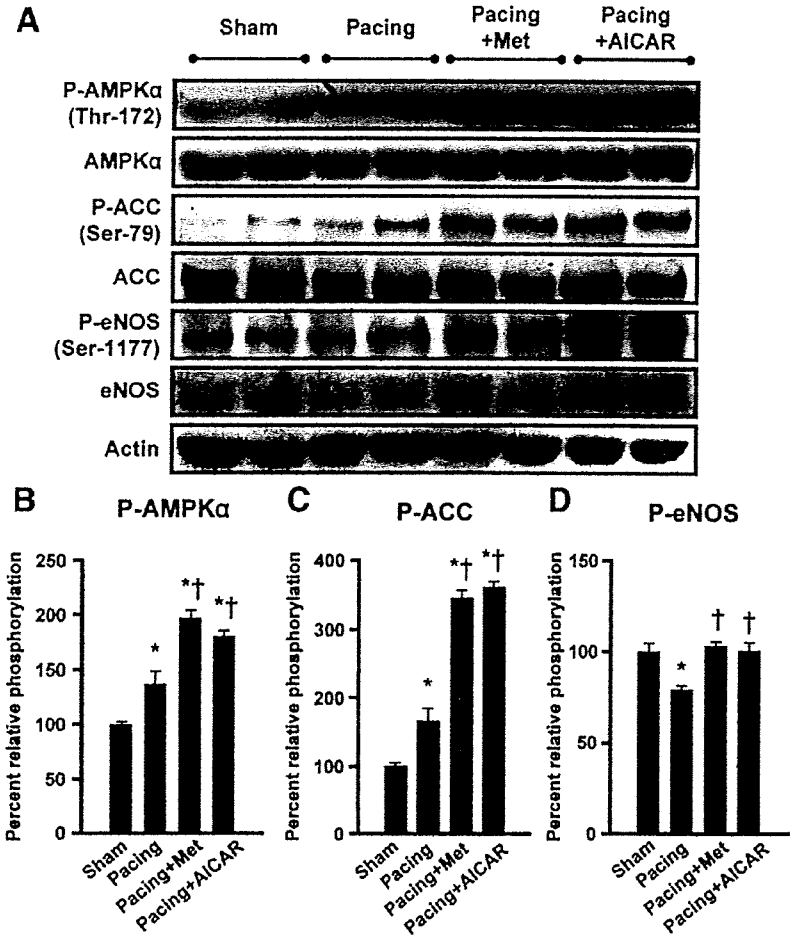

Figure 7. Phosphorylation of AMPKα, ACC, and eNOS in canine hearts after 4 weeks of treatment with or without metformin and AICAR. A, Representative immunoblots of phospho-AMPKα, ACC, and eNOS. B through D, Percentage relative phosphorylation of AMPKα, ACC, and eNOS, respectively. Values are mean±SEM. 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-β1 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-β1 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.

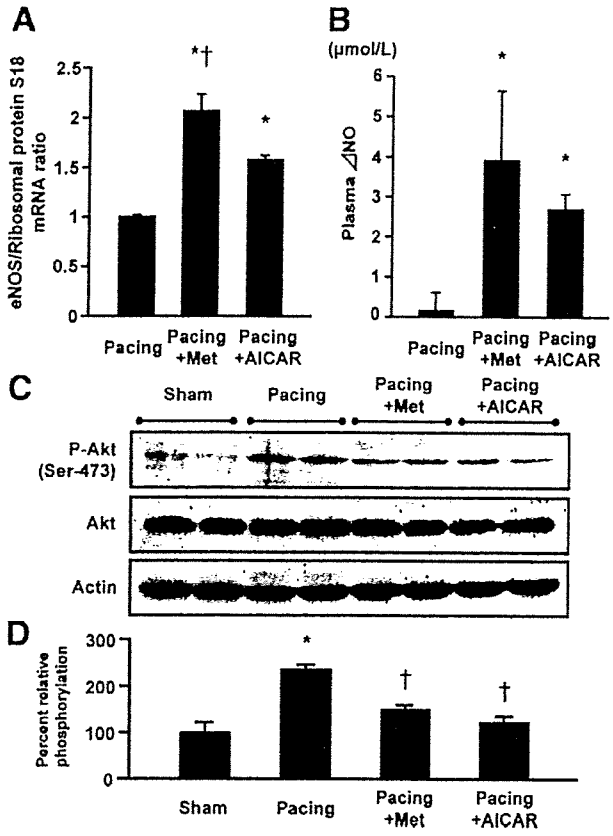

Figure 8. Effect of metformin on eNOS mRNA expression and plasma ΔNO levels, and phosphorylation of Akt in canine hearts. A, Quantitative real-time reverse-transcriptase polymerase chain reaction for eNOS mRNA. The mRNA levels were normalized to ribosomal protein S18 mRNA, and the pacing group was arbitrarily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

References

1. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34): UK Prospective Diabetes Study (UKPDS) Group. *Lancet.* 1998;352: 854–865.
2. Eurich DT, McAlister FA, Blackburn DF, Majumdar SR, Tsuyuki RT, Varney J, Johnson JA. Benefits and harms of antidiabetic agents in patients with diabetes and heart failure: systematic review. *BMJ.* 2007; 335:497.
3. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest.* 2001;108:1167–1174.
4. Chan AY, Soltys CL, Young ME, Proud CG, Dyck JR. Activation of AMP-activated protein kinase inhibits protein synthesis associated with hypertrophy in the cardiac myocyte. *J Biol Chem.* 2004;279: 32771–32779.
5. Zou MH, Kirkpatrick SS, Davis BJ, Nelson JS, Wiles WG 4th, Schlattner U, Neumann D, Brownlee M, Freeman MB, Goldman MH. Activation of the AMP-activated protein kinase by the anti-diabetic drug metformin in vivo: role of mitochondrial reactive nitrogen species. *J Biol Chem.* 2004; 279:43940–43951.
6. Hardie DG. AMP-activated protein kinase: the guardian of cardiac energy status. *J Clin Invest.* 2004;114:465–468.
7. Calvert JW, Gundewar S, Jha S, Greer JJ, Bestermann WH, Tian R, Lefer DJ. Acute metformin therapy confers cardioprotection against myocardial infarction via AMPK-eNOS-mediated signaling. *Diabetes.* 2008;57: 696–705.
8. Shibata R, Sato K, Pimentel DR, Takemura Y, Kihara S, Ohashi K, Funahashi T, Ouchi N, Walsh K. Adiponectin protects against myocardial ischemia-reperfusion injury through AMPK- and COX-2-dependent mechanisms. *Nat Med.* 2005;11:1096–1103.
9. Russell RR 3rd, Li J, Coven DL, Pypaert M, Zechner C, Palmeri M, Giordano FJ, Mu J, Birnbaum MJ, Young LH. AMP-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis, and injury. *J Clin Invest.* 2004;114: 495–503.
10. Tian R, Musi N, D'Agostino J, Hirshman MF, Goodyear LJ. Increased adenosine monophosphate-activated protein kinase activity in rat hearts with pressure-overload hypertrophy. *Circulation.* 2001;104:1664–1669.
11. Shibata R, Ouchi N, Ito M, Kihara S, Shiojima I, Pimentel DR, Kumada M, Sato K, Schiekofer S, Ohashi K, Funahashi T, Colucci WS, Walsh K. Adiponectin-mediated modulation of hypertrophic signals in the heart. *Nat Med.* 2004;10:1384–1389.
12. Liao Y, Takashima S, Maeda N, Ouchi N, Komamura K, Shimomura I, Hori M, Matsuzawa Y, Funahashi T, Kitakaze M. Exacerbation of heart failure in adiponectin-deficient mice due to impaired regulation of AMPK and glucose metabolism. *Cardiovasc Res.* 2005;67:705–713.
13. Bhalla RC, Toth KF, Tan E, Bhatty RA, Mathias E, Sharma RV. Vascular effects of metformin: possible mechanisms for its antihypertensive action in the spontaneously hypertensive rat. *Am J Hypertens.* 1996;9:570–576.
14. Marfella R, Acampora R, Verrazzo G, Ziccardi P, De Rosa N, Giunta R, Giugliano D. Metformin improves hemodynamic and rheological responses to L-arginine in NIDDM patients. *Diabetes Care.* 1996;19: 934–939.
15. Katakam PV, Ujhelyi MR, Hoenig M, Miller AW. Metformin improves vascular function in insulin-resistant rats. *Hypertension.* 2000;35: 108–112.
16. Rakhit RD, Marber MS. Nitric oxide: an emerging role in cardioprotection? *Heart.* 2001;86:368–372.
17. Komamura K, Shannon RP, Pasipoularides A, Ihara T, Lader AS, Patrick TA, Bishop SP, Vatner SF. Alterations in left ventricular diastolic function in conscious dogs with pacing-induced heart failure. *J Clin Invest.* 1992;89:1825–1838.
18. Lei B, Matsuo K, Labinskyy V, Sharma N, Chandler MP, Ahn A, Hintze TH, Stanley WC, Recchia FA. Exogenous nitric oxide reduces glucose transporters translocation and lactate production in ischemic myocardium in vivo. *Proc Natl Acad Sci U S A.* 2005;102:6966–6971.
19. Li HL, Yin R, Chen D, Liu D, Wang D, Yang Q, Dong YG. Long-term activation of adenosine monophosphate-activated protein kinase

attenuates pressure-overload-induced cardiac hypertrophy. *J Cell Biochem.* 2007;100:1086–1099.
20. Liao Y, Asakura M, Takashima S, Ogai A, Asano Y, Shintani Y, Minamino T, Asanuma H, Sanada S, Kim J, Kitamura S, Tomoike H, Hori M, Kitakaze M. Celiprolol, a vasodilatory beta-blocker, inhibits pressure overload-induced cardiac hypertrophy and prevents the transition to heart failure via nitric oxide-dependent mechanisms in mice. *Circulation.* 2004;110:692–699.
21. Elsner D, Riegger GA. Characteristics and clinical relevance of animal models of heart failure. *Curr Opin Cardiol.* 1995;10:253–259.
22. Lee WL, Chen JW, Ting CT, Ishiwata T, Lin SJ, Korc M, Wang PH. Insulin-like growth factor I improves cardiovascular function and suppresses apoptosis of cardiomyocytes in dilated cardiomyopathy. *Endocrinology.* 1999;140:4831–4840.
23. An D, Kewalramani G, Chan JK, Qi D, Ghosh S, Pulinilkunnil T, Abrahani A, Innis SM, Rodrigues B. Metformin influences cardiomyocyte cell death by pathways that are dependent and independent of caspase-3. *Diabetologia.* 2006;49:2174–2184.
24. Terai K, Hiramoto Y, Masaki M, Sugiyama S, Kuroda T, Hori M, Kawase I, Hirota H. AMP-activated protein kinase protects cardiomyocytes against hypoxic injury through attenuation of endoplasmic reticulum stress. *Mol Cell Biol.* 2005;25:9554–9575.
25. Mangano DT. Effects of acadesine on myocardial infarction, stroke, and death following surgery: a meta-analysis of the 5 international randomized trials: the Multicenter Study of Perioperative Ischemia (McSPI) Research Group. *JAMA.* 1997;277:325–332.
26. Kitakaze M, Takashima S, Minamino T, Node K, Shinozaki Y, Mori H, Kuzuya T, Hori M. Improvement by 5-amino-4-imidazole carboxamide riboside of the contractile dysfunction that follows brief periods of ischemia through increases in ecto-5-nucleotidase activity and adenosine release in canine hearts. *Jpn Circ J.* 1999;63:542–553.
27. Recchia FA, McConnell PI, Bernstein RD, Vogel TR, Xu X, Hintze TH. Reduced nitric oxide production and altered myocardial metabolism during the decompensation of pacing-induced heart failure in the conscious dog. *Circ Res.* 1998;83:969–979.
28. Chen ZP, Mitchelhill KI, Michell BJ, Stapleton D, Rodriguez-Crespo I, Witters LA, Power DA, Ortiz de Montellano PR, Kemp BE. AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Lett.* 1999;443:285–289.
29. Zhang Y, Lee TS, Kolb EM, Sun K, Lu X, Sladek FM, Kassab GS, Garland T Jr, Shyy JY. AMP-activated protein kinase is involved in endothelial NO synthase activation in response to shear stress. *Arterioscler Thromb Vasc Biol.* 2006;26:1281–1287.
30. Davis BJ, Xie Z, Viollet B, Zou MH. Activation of the AMP-activated kinase by antidiabetes drug metformin stimulates nitric oxide synthesis in vivo by promoting the association of heat shock protein 90 and endothelial nitric oxide synthase. *Diabetes.* 2006;55:496–505.
31. Sun W, Lee TS, Zhu M, Gu C, Wang Y, Zhu Y, Shyy JY. Statins activate AMP-activated protein kinase in vitro and in vivo. *Circulation.* 2006; 114:2655–2662.
32. Swan JW, Anker SD, Walton C, Godsland IF, Clark AL, Leyva F, Stevenson JC, Coats AJ. Insulin resistance in chronic heart failure: relation to severity and etiology of heart failure. *J Am Coll Cardiol.* 1997;30:527–532.
33. Nielson C, Lange T. Blood glucose and heart failure in nondiabetic patients. *Diabetes Care.* 2005;28:607–611.
34. Fischer Y, Thomas J, Rosen P, Kammermeier H. Action of metformin on glucose transport and glucose transporter GLUT1 and GLUT4 in heart muscle cells from healthy and diabetic rats. *Endocrinology.* 1995;136: 412–420.
35. Russell RR 3rd, Bergeron R, Shulman GI, Young LH. Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. *Am J Physiol.* 1999;277:H643–H649.
36. Davila-Roman VG, Vedala G, Herrero P, de las Fuentes L, Rogers JG, Kelly DP, Gropler RJ. Altered myocardial fatty acid and glucose metabolism in idiopathic dilated cardiomyopathy. *J Am Coll Cardiol.* 2002;40: 271–277.
37. Sack MN, Rader TA, Park S, Bastin J, McCune SA, Kelly DP. Fatty acid oxidation enzyme gene expression is downregulated in the failing heart. *Circulation.* 1996;94:2837–2842.
38. Osorio JC, Stanley WC, Linke A, Castellari M, Diep QN, Panchal AR, Hintze TH, Lopaschuk GD, Recchia FA. Impaired myocardial fatty acid oxidation and reduced protein expression of retinoid X receptor-alpha in pacing-induced heart failure. *Circulation.* 2002;106:606–612.
39. Paolisso G, Gambardella A, Galzerano D, D'Amore A, Rubino P, Verza M, Teasuro P, Varricchio M, D'Onofrio F. Total-body and myocardial substrate oxidation in congestive heart failure. *Metabolism.* 1994;43: 174–179.
40. Horman S, Beauloye C, Vertommen D, Vanoverschelde JL, Hue L, Rider MH. Myocardial ischemia and increased heart work modulate the phosphorylation state of eukaryotic elongation factor-2. *J Biol Chem.* 2003; 278:41970–41976.
41. Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. *Cell.* 2003;115:577–590.
42. Shinbane JS, Wood MA, Jensen DN, Ellenbogen KA, Fitzpatrick AP, Scheinman MM. Tachycardia-induced cardiomyopathy: a review of animal models and clinical studies. *J Am Coll Cardiol.* 1997;29: 709–715.
43. Montagnani M, Chen H, Barr VA, Quon MJ. Insulin-stimulated activation of eNOS is independent of Ca^{2+} but requires phosphorylation by Akt at Ser(1179). *J Biol Chem.* 2001;276:30392–30398.
44. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature.* 1999;399: 597–601.
45. Shiojima I, Walsh K. Regulation of cardiac growth and coronary angiogenesis by the Akt/PKB signaling pathway. *Genes Dev.* 2006;20: 3347–3365.

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.

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Metformin Prevents Progression of Heart Failure in Dogs: Role of AMP-Activated Protein Kinase

Hideyuki Sasaki, Hiroshi Asanuma, Masashi Fujita, Hiroyuki Takahama, Masakatsu Wakeno, Shin Ito, Akiko Ogai, Masanori Asakura, Jiyoong Kim, Tetsuo Minamino, Seiji Takashima, Shoji Sanada, Masaru Sugimachi, Kazuo Komamura, Naoki Mochizuki and Masafumi Kitakaze

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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 5×10^5 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