

Table 2 Echocardiographic features

Variable	
EDV _{2D} (ml)	104.4 ± 32
EDV _{3D} (ml)	84.7 ± 23
dP/dt (mm Hg s ⁻¹)	625 ± 124
dP/dt/EDV _{2D} (mm Hg s ml ⁻¹)	6.6 ± 2.8
dP/dt/EDV _{3D} (mm Hg s ml ⁻¹)	7.8 ± 1.9
Tricuspid regurgitation grade	2.3 ± 1.0
(RV-RA) pressure gradient (mm Hg)	78.3 ± 37

Abbreviations: EDV_{2D}, end-diastolic volume measured by two-dimensional echocardiography; EDV_{3D}, end-diastolic volume measured by three-dimensional echocardiography; RV-RA: right ventriculo-atrial.

Table 3 Correlation coefficient

	CI	mRAP	SvO ₂	BNP	6 mWD
EDV _{2D}	NS	0.70**	NS	0.58*	NS
EDV _{3D}	-0.69**	0.65*	-0.70**	NS	NS
dP/dt	NS	NS	NS	NS	NS
dP/dt/EDV _{2D}	NS	NS	NS	NS	NS
dP/dt/EDV _{3D}	0.60*	-0.55*	NS	-0.61*	NS

Abbreviations: 6 mWD, 6-min walk distance; CI, cardiac index; EDV_{2D}, end-diastolic volume measured by two-dimensional echocardiography; EDV_{3D}, end-diastolic volume measured by three-dimensional echocardiography; mRAP, mean right atrial pressure; SvO₂, mixed venous oxygen saturation.

P* < 0.05, *P* < 0.01.

ranged from 1.4 to 2.7 l/min. The distance of 6-min walk was shorter in patients with class III, compared with those with NYHA class II (370 ± 28 vs 491 ± 21 m, *P* < 0.01). CI was smaller and PVR and BNP were larger in patients with NYHA class III, but the difference was not significant. Echocardiographic measurements are shown in Table 2. EDV measured by 2D echocardiography was significantly larger than that measured by 3D echocardiography (*P* < 0.05).

Relationship between echocardiographic parameters and hemodynamics

The correlation coefficients between echocardiographic measurements and hemodynamics, both plasma BNP levels and 6 mWD are listed in Table 3. EDV_{3D} showed better correlations with the hemodynamics compared with EDV_{2D}. EDV_{2D} had significant correlations with mean RAP and plasma BNP levels, but not with CI and SvO₂. RV dP/dt and dP/dt/EDV_{2D} were not correlated with any of those measurements. However, RV dP/dt/EDV_{3D} showed a good correlation with the invasive and the non-invasive parameters of RV function (Table 3).

Reproducibility

The interobserver variability was 25 ± 13% of the measured EDV_{2D}, ranging between 5 and 55% between patients, and 11 ± 8% of the measured EDV_{3D}, ranging between 3 and 23% (*P* < 0.05 between techniques). The intraobserver variability was 21 ± 8% of the measured EDV_{2D}, ranging between 11 and 41% between patients, and 9 ± 5% of the measured EDV_{3D}, ranging between 3 and 19% between patients (*P* < 0.05 between techniques).

DISCUSSION

In this study, we found 3D-echocardiography-derived RV EDV and dP/dt/EDV showed a good correlation with indices of the cardiac function compared with those determined by 2D echocardiography.

Assessment of severity and the prognosis of PPH patients with conventional echocardiographic indices

Earlier investigators showed the clinical significance of 2D echocardiographic parameters in patients with PPH. Increasing severity of pericardial effusion was found to be associated with prognosis of PPH.¹⁰ Another study showed pericardial effusion and indexed RA were independently associated with the mortality.¹¹ However, these parameters simply reflect the end stage of RV failure. It is important to assess the disease status and the effect of the therapy in the management of PPH. Therefore, quantitative analysis of RV function is necessary in the clinical setting. Our findings shed light on the possibility of determining the severity of PPH non-invasively.

Index of RV systolic function

There have been several methods for assessing RV function. The conductance catheter is a frequently used instrument to assess RV function.^{12,13} Conductance-derived pressure-volume data are preload-independent measurements of RV contractile function. However, conductance catheterization is invasive and not cost-effective. Therefore, they are predominantly used as a research tool for assessment of ventricular function. Attempt has been made to measure RV function by echocardiography, but the simple M-mode or 2D echocardiographic indices cannot be reliably applied for assessing the RV function because of RV complex geometry.¹⁴ Other non-invasive methods, such as MRI, can be used accurately to measure end-diastolic and end-systolic volumes and calculate the ejection fraction,^{15,16} but these methods are time consuming and cannot be performed at the bedside.

Left-ventricular dP/dt has been well established as an index of contractility and is known to correlate well with the prognosis of left-ventricular dysfunction.¹⁷ There have been few studies on Doppler-derived RV dP/dt. Anconina *et al.*¹⁸ measured the mean rate of right ventriculo-atrial pressure gradient and showed good correlation with invasively determined maximal dP/dt. We have previously reported that non-invasive measurement of RV peak-positive dP/dt by echocardiography correlated well with that derived by catheterization irrespective of the level of right atrial pressure.¹⁹ However, dP/dt is dependent on preload,⁵ and may overestimate the contractility in RV dilatation accompanied by PPH.

Measuring 3D-echocardiography-derived RV dP/dt/EDV

In this study, we analyzed RV dP/dt divided by RV EDV obtained by 3D echocardiography. As RV EDV has been shown to be a marker of preload,^{20,21} RV dP/dt divided by RV EDV should be a marker of preload-independent RV function. We have shown that RV dP/dt/EDV_{3D} correlated well with both invasively and non-invasively derived RV function indices.

Although there are many parameters that relate to the severity and prognosis of PPH, no single parameter can be the gold standard. We used invasive measurements as the next best standard and investigated the correlations. The correlation coefficients were not so high, but we believe that RV dP/dt/EDV_{3D} was still clinically useful because no other parameters were good enough to assess the severity of the disease.

Study limitations

Measuring RV volume by 3D echocardiography is challenging in the severely enlarged RV, especially in patients in the end stage. In our study, we could not measure RV volume in 4 out of 20 patients by 3D echocardiography, because the RV size exceeded the scan volume. It is a major limitation of this study. The latest version of the 3D

ultrasound equipment provides us the wider scan area, which may increase the clinical applicability.

dP/dt is also affected by afterload. However, in patients with PPH, pulmonary artery pressure is elevated, and the timing of the opening of pulmonary valve is delayed. Therefore, the influence of afterload can be neglected.

Medical therapy also could have affected the result. However, the number of patients with oral medication or with intravenous epoprostenol infusion was too small to investigate the effect.

In this study, all patients had echocardiography and 6-mWD test within 1 week of catheterization. In addition to the scarcity of PPH itself, this strict criterion was also partly the reason why the number of the patients was small.

Our study population consisted of rather severe PPH patients. It is a limitation of this study that we did not have patients with mild-to-moderate PPH among our study subjects. We believe that our method is applicable even to those with milder disease. It is warranted to conduct larger trials to extend these findings to patients with a different stage of disease.

Clinical implications

By using our index, we could estimate the catheterization-derived RV function in patients with PPH non-invasively. It is useful in a setting where the patient condition is unstable and the catheter examination is extremely dangerous. It is also useful when a new drug is administered and the catheter-derived index is needed for repeated assessment of the drug effect.

CONCLUSION

In patients with PPH, 3D-echocardiography-determined RV EDV and $dP/dt/EDV$ correlated well with prognostic markers of PPH, including CI, mean right atrial pressure and BNP. 3D-echocardiography-determined RV $dP/dt/EDV$ seems to be a potential marker of disease severity, and may be useful in assessing therapeutic effects and the prognosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

- 1 Runo JR, Loyd JE. Primary pulmonary hypertension. *Lancet* 2003; **361**: 1533–1544.
- 2 Sandoval J, Bauerle O, Palomar A, Gomez A, Martinez-Guerra ML, Beltran M, Guerrero ML. Survival in primary pulmonary hypertension. Validation of a prognostic equation. *Circulation* 1994; **89**: 1733–1744.
- 3 D'Alonzo GE, Barst RJ, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, Fishman AP, Goldring RM, Groves BM, Kernis JT, Levy PS, Pietra GG, Reid LM, Reeves JT, Rich S,

- Veim CE, Williams GW, Wu M. Survival in patients with primary pulmonary hypertension. Results from a national prospective registry. *Ann Intern Med* 1991; **115**: 343–349.
- 4 Rich S, Dantzker DR, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, Fishman AP, Goldring RM, Groves BM, Koerner SK, Levy PC, Reid LM, Veim CE, Williams GW. Primary pulmonary hypertension. A national prospective study. *Ann Intern Med* 1987; **107**: 216–223.
- 5 Imanishi T, Nakatani S, Yamada S, Nakanishi N, Beppu S, Nagata S, Miyatake K. Validation of continuous wave Doppler-determined right ventricular peak positive and negative dP/dt : effect of right atrial pressure on measurement. *J Am Coll Cardiol* 1994; **23**: 1638–1643.
- 6 Little WC. The left ventricular dP/dt_{max} -end-diastolic volume relation in closed-chest dogs. *Circ Res* 1985; **56**: 808–815.
- 7 Linker DT, Moritz WE, Pearlman AS. A new three-dimensional echocardiographic method of right ventricular volume measurement: *in vitro* validation. *J Am Coll Cardiol* 1986; **8**: 101–106.
- 8 Shiota T, Jones M, Chikada M, Fleishman CE, Castellucci JB, Cotter B, DeMaria AN, von Ramm OT, Kisslo J, Ryan T, Sahn DJ. Real-time three-dimensional echocardiography for determining right ventricular stroke volume in an animal model of chronic right ventricular volume overload. *Circulation* 1998; **97**: 1897–1900.
- 9 Dorosz JL, Bolson EL, Weiss MS, Sheehan FH. Three-dimensional visual guidance improves the accuracy of calculating right ventricular volume with two-dimensional echocardiography. *J Am Soc Echocardiogr* 2003; **16**: 675–681.
- 10 Eysmann SB, Palevsky HI, Reichel N, Hackney K, Douglas PS. Two-dimensional and Doppler-echocardiographic and cardiac catheterization correlates of survival in primary pulmonary hypertension. *Circulation* 1989; **80**: 353–360.
- 11 Raymond RJ, Hinderliter AL, Willis PW, Ralph D, Caldwell EJ, Williams W, Ettinger NA, Hill NS, Sumner WR, de Boisblanc B, Schwartz T, Koch G, Clayton LM, Jobsis MM, Crow JW, Long W. Echocardiographic predictors of adverse outcomes in primary pulmonary hypertension. *J Am Coll Cardiol* 2002; **39**: 1214–1219.
- 12 Segers P, Leather HA, Verdonck P, Sun YY, Wouters PF. Preload-adjusted maximal power of right ventricle: contribution of end-systolic P-V relation intercept. *Am J Physiol Heart Circ Physiol* 2002; **283**: H1681–H1687.
- 13 Brown KA, Ditchey RV. Human right ventricular end-systolic pressure-volume relation defined by maximal elastance. *Circulation* 1988; **78**: 81–91.
- 14 Levine RA, Gibson TC, Aretz T, Gillam LD, Guyer DE, King ME, Weyman AE. Echocardiographic measurement of right ventricular volume. *Circulation* 1984; **69**: 497–505.
- 15 Helbing WA, Bosch HG, Maliepaard C, Rebergen SA, van der Geest RJ, Hansen B, Ottenkamp J, Reiber JH, de Roos A. Comparison of echocardiographic methods with magnetic resonance imaging for assessment of right ventricular function in children. *Am J Cardiol* 1995; **76**: 589–594.
- 16 Vogel M, Gutberlet M, Dittrich S, Hosten N, Lange PE. Comparison of transthoracic three-dimensional echocardiography with magnetic resonance imaging in the assessment of right ventricular volume and mass. *Heart* 1997; **78**: 127–130.
- 17 Koliass TJ, Aaronson KD, Armstrong WF. Doppler-derived dP/dt and $-dP/dt$ predict survival in congestive heart failure. *J Am Coll Cardiol* 2000; **36**: 1594–1599.
- 18 Anconina J, Danchin N, Selton-Suty C, Isaaz K, Juilliere Y, Buffet P, Edel F, Cherrier F. Noninvasive estimation of right ventricular dP/dt in patients with tricuspid valve regurgitation. *Am J Cardiol* 1993; **71**: 1495–1497.
- 19 Kanzaki H, Nakatani S, Kawada T, Yamagishi M, Sunagawa K, Miyatake K. Right ventricular $dP/dt/P(max)$, not $dP/dt(max)$, noninvasively derived from tricuspid regurgitation velocity is a useful index of right ventricular contractility. *J Am Soc Echocardiogr* 2002; **15**: 136–142.
- 20 Siniscalchi A, Pavesi M, Piraccini E, De Pietri L, Braglia V, Di Benedetto F, Lauro A, Spedicato S, Dante A, Pinna AD, Faenza S. Right ventricular end-diastolic volume index as a predictor of preload status in patients with low right ventricular ejection fraction during orthotopic liver transplantation. *Transplant Proc* 2005; **37**: 2541–2543.
- 21 Durham R, Neunaber K, Vogler G, Shapiro M, Mazuski J. Right ventricular end-diastolic volume as a measure of preload. *J Trauma* 1995; **39**: 218–223 (discussion 223–4).

Metformin Prevents Progression of Heart Failure in Dogs

Role of AMP-Activated Protein Kinase

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

Methods and Results—Treatment with metformin (10 $\mu\text{mol/L}$) protected cultured cardiomyocytes from cell death during exposure to H_2O_2 (50 $\mu\text{mol/L}$) via AMPK activation, as shown by the MTT assay, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining, and flow cytometry. Continuous rapid ventricular pacing (230 bpm for 4 weeks) caused typical heart failure in dogs. Both left ventricular fractional shortening and left ventricular end-diastolic pressure were significantly improved in dogs treated with oral metformin at 100 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ($n=8$) ($18.6 \pm 1.8\%$ and 11.8 ± 1.1 mm Hg, respectively) compared with dogs receiving vehicle ($n=8$) ($9.6 \pm 0.7\%$ 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 colleagues² 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 hypertrophy¹⁰ and subsequent heart failure.^{11,12} In addition, Russell et al⁹ 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

Received August 23, 2007; accepted February 24, 2009.

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The online-only Data Supplement is available with this article at <http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.108.798561/DC1>.

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Circulation is available at <http://circ.ahajournals.org>

DOI: 10.1161/CIRCULATIONAHA.108.798561

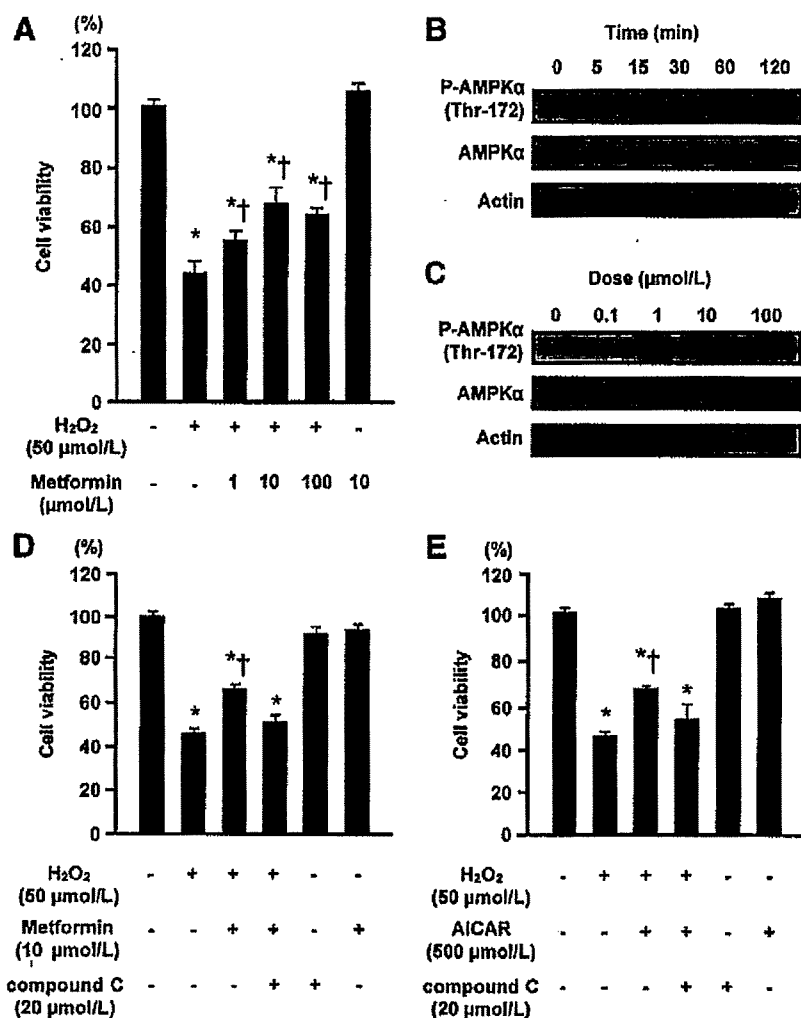


Figure 1. Effect of metformin on oxidative stress-induced cell death via AMPK activation in cultured rat cardiomyocytes. **A**, Cardiomyocyte viability after treatment with metformin (1, 10, or 100 μmol/L) and exposure to H₂O₂ (50 μmol/L). **B**, Time (0, 5, 15, 30, 60, 120 minutes)-dependent changes 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 H₂O₂ (50 μmol/L) treatment.

effects¹⁶ and may alleviate mechanical or neurohormonal stress on the heart.

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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 H₂O₂ 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 H₂O₂, 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 H₂O₂ (Figure 1E). These results suggested that

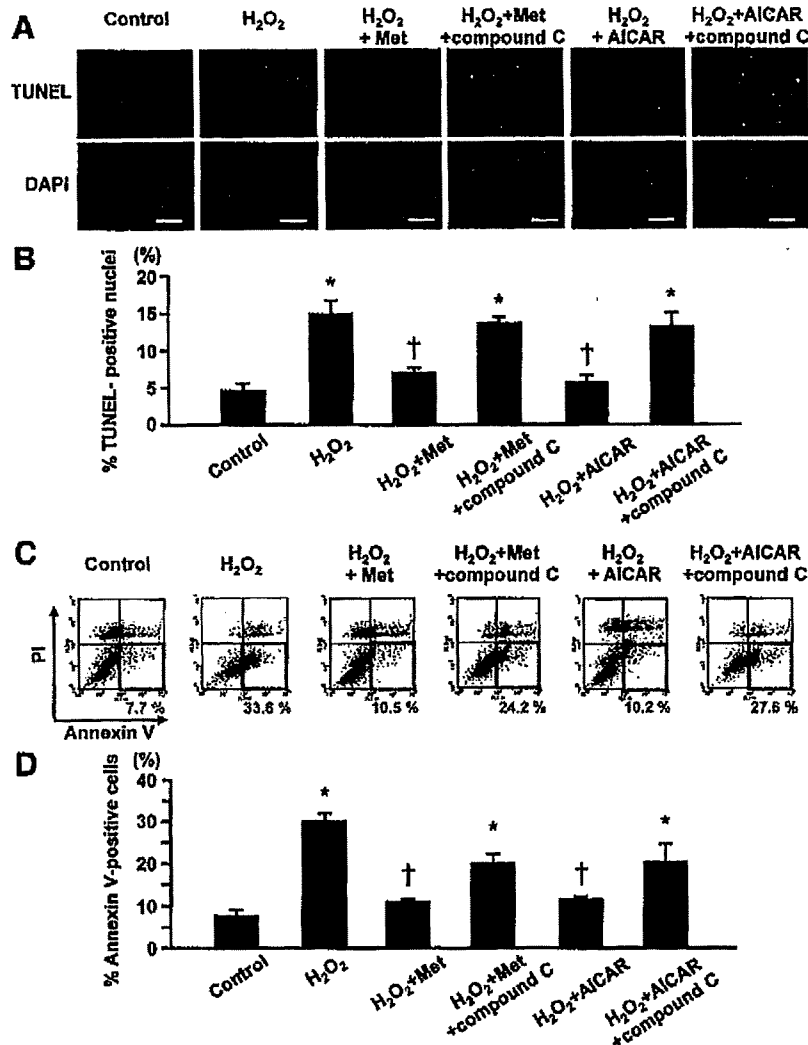


Figure 2. Effect of metformin on oxidative stress-induced apoptosis via AMPK activation in cultured rat cardiomyocytes. Representative (A) and quantitative (B) data on cardiomyocyte apoptosis obtained by TUNEL staining (n=3 in each experiment). Representative (C) and quantitative (D) data on cardiomyocyte apoptosis obtained by flow cytometry (n=3 in each experiment). Values are mean±SEM. PI indicates propidium iodide. *P<0.05 vs control; †P<0.05 vs H₂O₂ (50 μmol/L) treatment.

activation of AMPK protected cardiomyocytes against damage caused by H₂O₂.

H₂O₂ 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 propidium 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 H₂O₂ (Figure 2A through 2D). These results suggested that the activation of AMPK by metformin could prevent apoptosis of cardiomyocytes induced by H₂O₂.

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

Cardiac Physiological and Pathophysiological Parameters
Four weeks after the rapid right ventricular (RV) pacing, left ventricular (LV) end-diastolic dimension, LV end-systolic

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

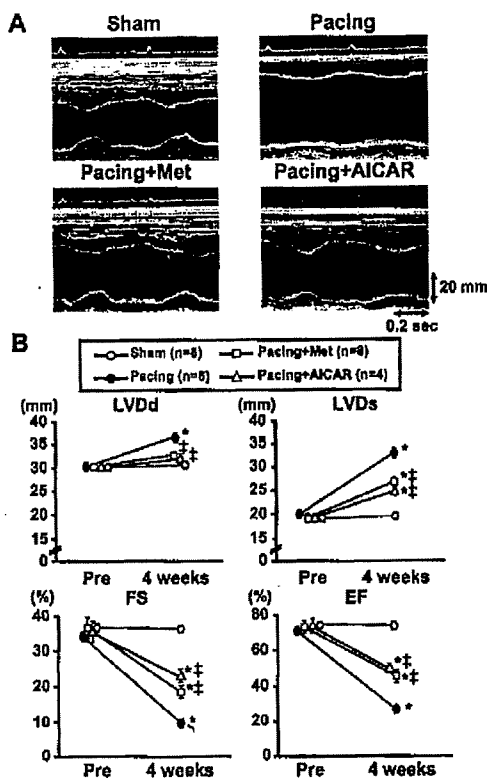


Figure 3. Effect of metformin on echocardiographic parameters. **A**, Representative M-mode echocardiograms obtained 4 weeks after sham surgery or after RV pacing. **B**, Echocardiographic parameters before and after sham surgery or after RV pacing in the sham group ($n=6$), pacing group ($n=8$), pacing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

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

Consistent with previous data,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 Δ 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	1.8±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±87	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.

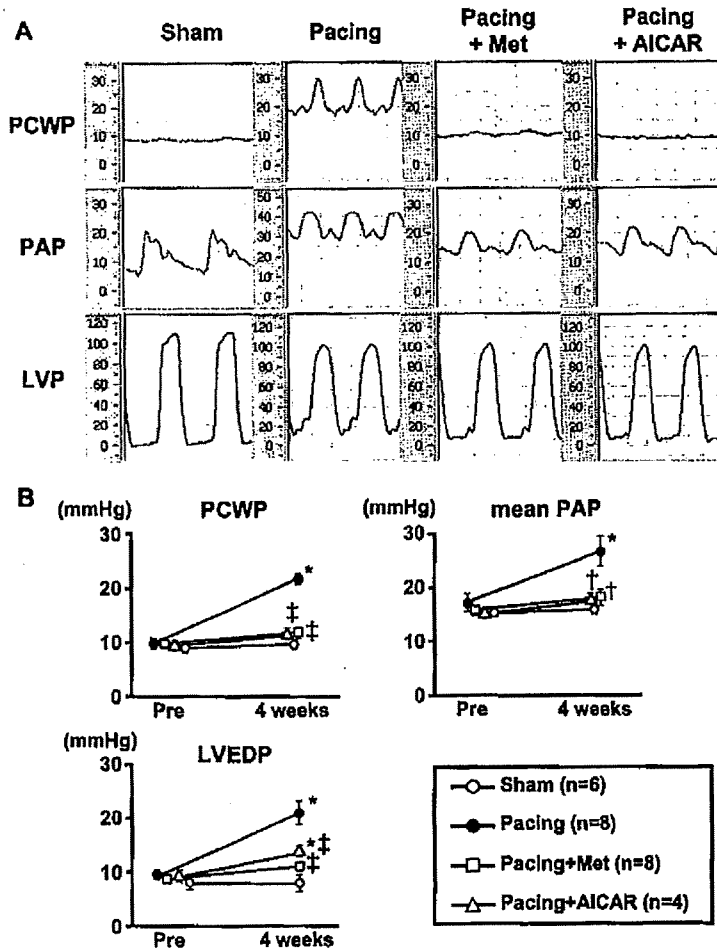


Figure 4. Effect of metformin on hemodynamic parameters. A, Representative graphs of hemodynamic parameters obtained at 4 weeks. B, Hemodynamic parameters before and after the 4-week study period in the sham (n=6), pacing (n=8), pacing plus metformin (n=8), and pacing plus AICAR (n=4) groups. Values 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 al²⁷ 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

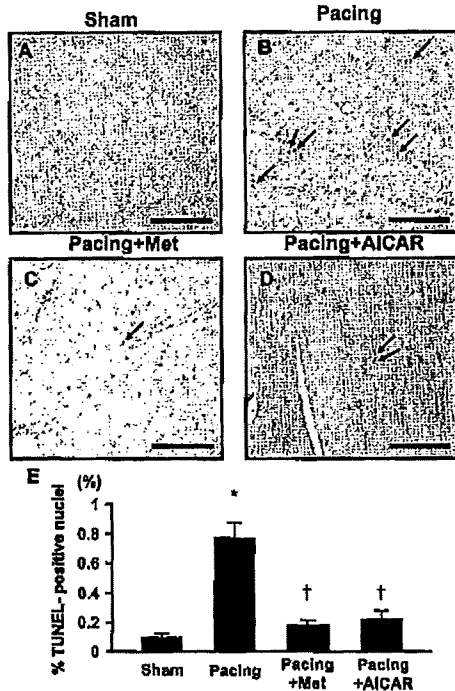


Figure 5. TUNEL staining of canine hearts at 4 weeks. Representative examples of TUNEL-stained hearts from sham (A), pacing (B), pacing plus metformin (C), and pacing plus AICAR (D) groups. Arrows indicate TUNEL-positive nuclei (brown). Scale bar=100 μ m. E, Quantitative data on the percentage of TUNEL-positive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

function. NO is believed to have various cardioprotective effects.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

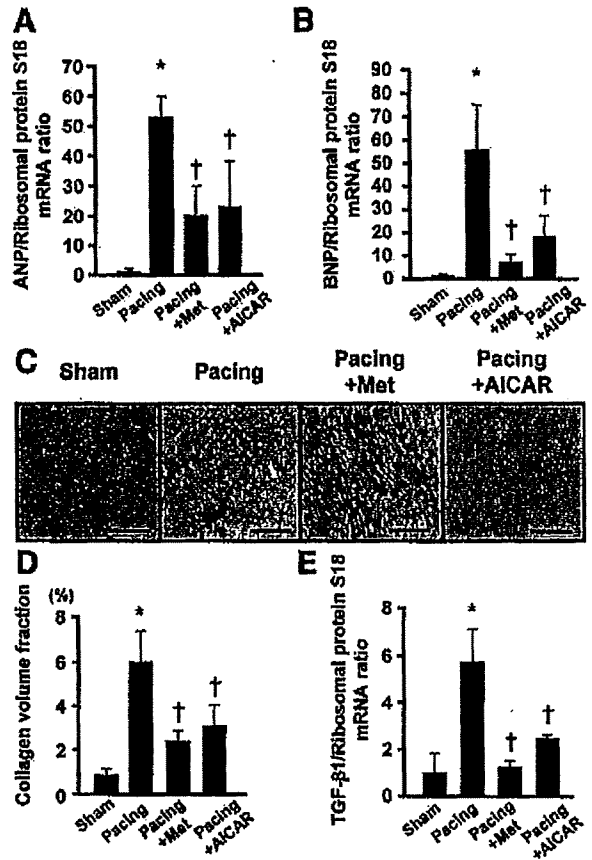


Figure 6. Natriuretic peptide expression, cardiac collagen volume fraction, and TGF- β 1 expression. A, B, and E, Quantitative real-time reverse-transcriptase polymerase chain reaction analysis of myocardial atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and TGF- β 1 expression, respectively.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

likely to be greater than the induction of glucose extraction by direct activation of AMPK. The possibility exists that AMPK-induced glucose extraction triggers the improvement in heart failure, followed by the restoration of metabolic switch. On the other hand, we found that the net free fatty acids extraction of the pacing group tended to increase despite no statistical significance, which is consistent with the report by Paolisso et al³⁹ that myocardial free fatty acids extraction increased in patients with congestive heart failure³⁹ 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

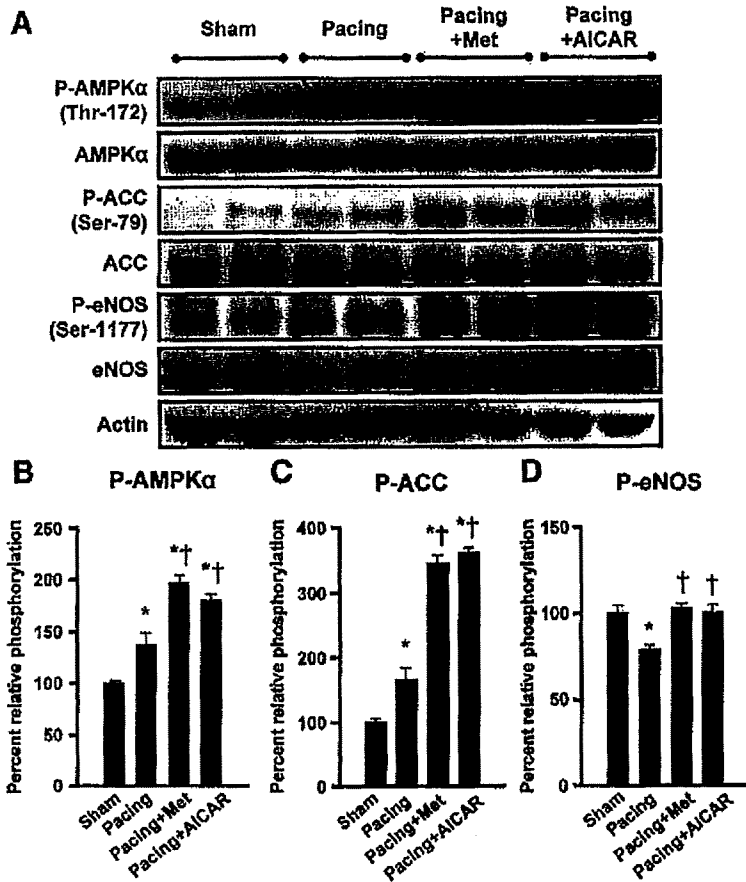


Figure 7. Phosphorylation of AMPK α , ACC, and eNOS in canine hearts after 4 weeks of treatment with or without metformin and AICAR. A, Representative immunoblots of phospho-AMPK α , ACC, and eNOS. B through D, Percentage relative phosphorylation of AMPK α , ACC, and eNOS, respectively. Values are mean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

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

The fourth possibility is the antifibrotic effect of metformin. Several studies have indicated that AMPK activation inhibits protein synthesis through effects on both the eEF-2 and mTOR pathways.^{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- β 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- β 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.

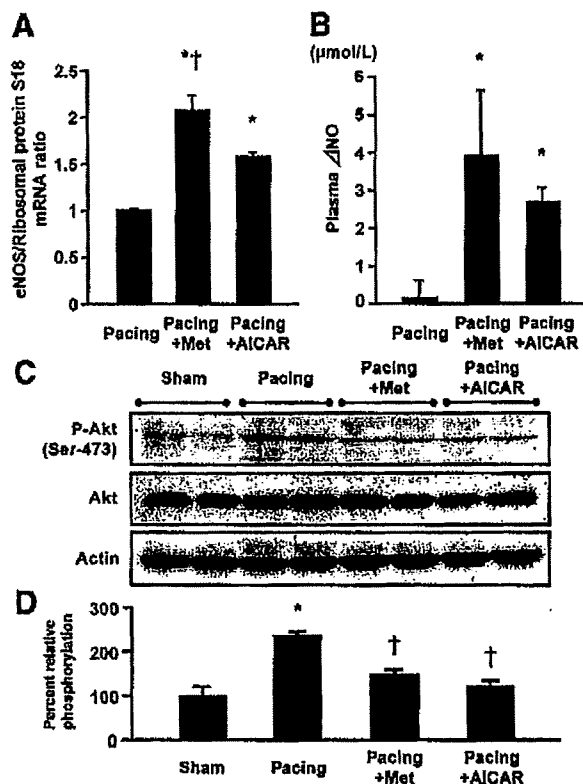


Figure 8. Effect of metformin on eNOS mRNA expression and plasma Δ NO levels, and phosphorylation of Akt in canine hearts. **A**, Quantitative real-time reverse-transcriptase polymerase chain reaction for eNOS mRNA. The mRNA levels were normalized to ribosomal protein S18 mRNA, and the pacing group was arbitrarily assigned a value of 1.0. **B**, Plasma Δ NO level after 4 weeks of RV pacing with or without metformin and AICAR administration. Values are mean \pm 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 \pm 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.

Acknowledgments

We thank Yoko Horiguchi for her technical assistance; Dr Masafumi Myoishi for his assistance with TUNEL staining; Dr Hai Ying Fu for her assistance with flow cytometry; Dr Hatsue-Ishibashi-Ueda for her assistance with fluorescence microscopy; Dr Kyoko Shioya for her assistance with animal care; Tsunehisa Nakao (Nippon Shinyaku Co Ltd) for providing information about metformin; and the Evidence Finders' Club for their encouragement of this study.

Sources of Funding

This work was supported by grants in aid from the Ministry of Health, Labor, and Welfare—Japan and the Ministry of Education, Culture, Sports, Science and Technology—Japan and grants from the Japan Heart Foundation and the Japan Cardiovascular Research Foundation.

Disclosures

None.

References

- 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.
- Eurich DT, McAllister 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.
- 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.
- 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.
- 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.
- Hardie DG. AMP-activated protein kinase: the guardian of cardiac energy status. *J Clin Invest*. 2004;114:465–468.
- Calvert JW, Gundewar S, Jha S, Greer JJ, Bestermann WH, Tian R, Lefler DJ. Acute metformin therapy confers cardioprotection against myocardial infarction via AMPK-eNOS-mediated signaling. *Diabetes*. 2008;57:696–705.
- 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.
- 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.
- 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.
- Shibata R, Ouchi N, Ito M, Kihara S, Shiojima J, Pimentel DR, Kumada M, Sato K, Schiekfer S, Ohashi K, Funahashi T, Colucci WS, Walsh K. Adiponectin-mediated modulation of hypertrophic signals in the heart. *Nat Med*. 2004;10:1384–1389.
- 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.
- Bhalla RC, Toth KF, Tan E, Bhaty 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.
- 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.
- Katakam PV, Ujhelyi MR, Hoenig M, Miller AW. Metformin improves vascular function in insulin-resistant rats. *Hypertension*. 2000;35:108–112.
- Rakhit RD, Marber MS. Nitric oxide: an emerging role in cardioprotection? *Heart*. 2001;86:368–372.
- 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.
- 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.
- 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, Kore 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, Pulimkunnil T, Abrahami 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, Mitchellhill 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, Violette B, Zou MH. Activation of the AMP-activated kinase by antidiabetic 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, Goddard 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, Loposchuk 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, Teasoro P, Varricchio M, D'Onofrio F. Total-body and myocardial substrate oxidation in congestive heart failure. *Metabolism*. 1994;43:174-179.
 40. Horman S, Brauloye 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, Scholman 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 Ca²⁺ 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.

PRE-CLINICAL RESEARCH

Prolonged Targeting of Ischemic/ Reperfused Myocardium by Liposomal Adenosine Augments Cardioprotection in Rats

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Objectives	The purpose of this study was to investigate whether liposomal adenosine has stronger cardioprotective effects and fewer side effects than free adenosine.
Background	Liposomes are nanoparticles that can deliver various agents to target tissues and delay degradation of these agents. Liposomes coated with polyethylene glycol (PEG) prolong the residence time of drugs in the blood. Although adenosine reduces the myocardial infarct (MI) size in clinical trials, it also causes hypotension and bradycardia.
Methods	We prepared PEGylated liposomal adenosine (mean diameter 134 ± 21 nm) by the hydration method. In rats, we evaluated the myocardial accumulation of liposomes and MI size at 3 h after 30 min of ischemia followed by reperfusion.
Results	The electron microscopy and ex vivo bioluminescence imaging showed the specific accumulation of liposomes in ischemic/reperfused myocardium. Investigation of radioisotope-labeled adenosine encapsulated in PEGylated liposomes revealed a prolonged blood residence time. An intravenous infusion of PEGylated liposomal adenosine ($450 \mu\text{g}/\text{kg}/\text{min}$) had a weaker effect on blood pressure and heart rate than the corresponding dose of free adenosine. An intravenous infusion of PEGylated liposomal adenosine ($450 \mu\text{g}/\text{kg}/\text{min}$) for 10 min from 5 min before the onset of reperfusion significantly reduced MI size ($29.5 \pm 6.5\%$) compared with an infusion of saline ($53.2 \pm 3.5\%$, $p < 0.05$). The antagonist of adenosine A_1 , A_{2a} , A_{2b} , or A_3 subtype receptor blocked cardioprotection observed in the PEGylated liposomal adenosine-treated group.
Conclusions	An infusion as PEGylated liposomes augmented the cardioprotective effects of adenosine against ischemia/reperfusion injury and reduced its unfavorable hemodynamic effects. Liposomes are promising for developing new treatments for acute MI. (J Am Coll Cardiol 2009;53:709–17) © 2009 by the American College of Cardiology Foundation

Liposomes are now widely used for drug delivery in cancer treatment to target specific organs actively or passively and to prevent the degradation of chemotherapy agents (1). However, the application of liposomes for cardiovascular diseases is still limited. In ischemic/reperfused myocardium,

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cellular permeability is enhanced and vascular endothelial integrity is disrupted (2,3), suggesting that nanoparticles

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Manuscript received September 4, 2008; revised manuscript received October 21, 2008, accepted November 3, 2008.

Abbreviations and Acronyms

8-SPT = 8-(*p*-sulfophenyl)
theophylline

EM = electron microscopy

MI = myocardial infarction

PEG = polyethylene glycol

RI = radioisotope

TTC = triphenyltetrazolium
chloride

such as liposomes may be a promising drug delivery system for targeting damaged myocardium with cardioprotective agents. Additionally, coating liposomes with polyethylene glycol (PEG) prolongs their residence time in the circulation (1). Because enhanced microvascular permeability persists for at least 48 h after the occurrence of myocardial infarction (MI) (2), drugs delivered in PEGylated li-

posomes should be able to display their maximum beneficial effects on myocardial damage after MI.

Adenosine has multiple physiological functions that are mediated via the adenosine A₁, A_{2a}, A_{2b}, and A₃ receptors (4,5). Although large-scale clinical trials suggested the potential value of adenosine therapy for patients with acute MI (6,7), this agent has an extremely short half-life (1 to 2 s) and causes hypotension and bradycardia because of vasodilatory and negative chronotropic effects (4). Because a high dose of adenosine is required to exert cardioprotective effects, it is difficult to use clinically because of the associated hemodynamic consequences. Therefore, we hypothesized that adenosine encapsulated in PEGylated liposomes would cause less hemodynamic disturbance and might also specifically accumulate in ischemic/reperfused myocardium, leading to augmented cardioprotective effects. To test this hypothesis, we created PEGylated liposomal adenosine by the hydration method and investigated: 1) whether liposomal adenosine accumulated in ischemic/reperfused myocardium and prolonged blood residence time; 2) whether liposomal adenosine caused less severe hypotension and bradycardia than free adenosine; and 3) which adenosine receptor subtype was involved in mediating the cardioprotective effects of liposomal adenosine against ischemia/reperfusion injury.

Methods

Materials. The materials for preparing PEGylated liposomes, including hydrogenated soy phosphatidyl choline (HSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*n*-[methoxy (polyethylene glycol)-2000] (DSPE-PEG2000), and cholesterol were obtained from Nissei Oil Co., Ltd. (Tokyo, Japan) and Wako Pure Chemical Co., Ltd. (Osaka, Japan). [³H]-adenosine was purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Other materials were obtained from Sigma (St. Louis, Missouri), including 8-(*p*-sulfophenyl)theophylline (8-SPT; a nonselective adenosine receptor antagonist), 1,3-diethyl-8-phenylxanthine (DPCPX; a selective adenosine A₁ receptor antagonist), 5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH58261; a selective adenosine A_{2a} receptor antagonist), 8-[4-[[[(4-cyanophenyl)carbamoylethyl]oxy]phenyl]-1,3-di-(*n*-propyl)xanthine (MRS1754; a selective

adenosine A_{2b} receptor antagonist), and 5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate (MRS1523, a selective adenosine A₃ receptor antagonist).

Animals. Male Wistar rats (9 weeks old and weighing 250 to 310 g, Japan Animals, Osaka, Japan) were used. The animal experiments were approved by the National Cardiovascular Center Research Committee and were performed according to institutional guidelines.

Preparation of PEGylated liposomes. The PEGylated liposomes were prepared by the hydration method. Briefly, adenosine was added to the lipid solution. After mixture of lipid and adenosine, DSPE-PEG2000 was added and incubated. The final composition of PEGylated liposomes was HSPC:cholesterol:DSPE-PEG2000 = 6.0:4.0:0.3 (molar ratio). After ultracentrifugation several times, the pellet of liposomal adenosine was resuspended in sodium lactate at each required concentration for use in the experimental protocols. Some samples of final liposomal adenosine were disrupted by dilution with 50% methanol (1.5 ml per 30- μ l of liposomes). After 10 min of ultracentrifugation, the concentration of adenosine in the supernatant was measured by high-performance liquid chromatography.

To prepare fluorescent-labeled liposomes, 0.5 mol% tetramethylrhodamine isothiocyanate (rhodamine) was added to the lipid mixture. To prepare radioisotope (RI)-labeled adenosine encapsulated in liposomes, [³H]-radiolabeled adenosine (Daiichi Pure Chemicals, Tokyo, Japan) was diluted with free adenosine and was encapsulated in liposomes as described above.

Characterization of PEGylated liposomal adenosine. The characterization of the liposomes was performed by the dynamic scatter analysis (Zetasizer Nano ZS, Malvern, Worcestershire, United Kingdom). The analyses were performed 10 times per sample, and results represented analyses of 4 independent experiments.

Experimental protocols. PROTOCOL 1: EFFECTS OF PEGYLATED LIPOSOMAL ADENOSINE ON HEMODYNAMICS IN RATS. Rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). Catheters were advanced into a femoral artery and vein for the measurement of systemic blood pressure and infusion of drugs, respectively. Both blood pressure and heart rate were monitored continuously during the study using a Power Lab (AD Instruments, Castle Hill, Australia). After hemodynamics became stable, we intravenously administered empty PEGylated liposomes (*n* = 8), free adenosine (*n* = 8), or PEGylated liposomal adenosine (*n* = 8) for 10 min. Either PEGylated liposomal or free adenosine was infused at an initial dose of 225 μ g/kg/min (0.1 ml/min) for 10 min. After a 5-min interval, either PEGylated liposomal adenosine or free adenosine was infused at 450 μ g/kg/min (0.1 ml/min) for 10 min. In the same manner, PEGylated liposomal adenosine or free adenosine was then infused at 900 μ g/kg/min (0.1 ml/min).

PROTOCOL 2: EFFECTS OF PEGYLATED LIPOSOMAL ADENOSINE ON INFARCT SIZE IN RATS. The MI was induced by transient ligation of the left coronary artery as described previously (8). In the first series of experiments, to examine the dose-dependent effects of liposomal adenosine on MI size, PEGylated liposomal adenosine was infused intravenously at 50, 150, or 450 $\mu\text{g}/\text{kg}/\text{min}$ for a 10-min period starting from 5 min before the onset of reperfusion. In the second series of experiments, to determine the adenosine receptor subtype involved in cardioprotective effects by the liposomal adenosine, the antagonist of adenosine subtype receptor was intravenously injected as a bolus followed by the infusion of liposomal adenosine for 10 min. The MI size was evaluated at 3 h after the start of reperfusion. The doses of adenosine receptor subtype antagonists were determined according to the previous reports (9–11).

Measurement of infarct size. At 3 h after the onset of reperfusion, the area at risk and the infarcted area were determined by Evans blue and triphenyltetrazolium chloride (TTC) staining, respectively, as previously described (8). Infarct size was calculated as $[\text{infarcted area}/\text{area at risk}] \times 100(\%)$ in a blind manner. The area at risk was composed of border (TTC staining) and infarcted (TTC nonstaining) areas.

Electron microscopy (EM). Myocardial samples for EM were obtained from the central and peripheral areas in ischemic/reperfused myocardium, which roughly corresponded to the infarcted and border areas, respectively, after the left coronary artery was occluded for 30 min of ischemia followed by 3 h of reperfusion. Samples were prepared as previously reported (12). Liposomes, whose major membrane component is unsaturated phospholipids, were visualized as homogenous dark dots with a diameter of 100 to 150 nm (13).

Accumulation of fluorescent-labeled PEGylated liposomes in ischemic/reperfused myocardium. Unlabeled or fluorescent-labeled PEGylated liposomes were infused intravenously at a dose of 0.1 ml/min as liposomal adenosine was infused in protocol 2. At 3 h after reperfusion, hearts were quickly removed and cut into 4 sections parallel to the axis from base to apex. Then *ex vivo* bioluminescence imaging was performed with an Olympus OV 100 imaging system (Olympus, Tokyo, Japan) and signals were quantified using WASABI quantitative software (Hamamatsu Photonics K.K., Shizuoka, Japan). Fluorescent intensity in the region of interest was measured as previously reported (14). Control intensity indicated the fluorescent intensity in the nonischemic area of the individual rat.

Time-course changes of free and PEGylated liposomal RI-labeled adenosine in plasma and myocardium. Free or PEGylated liposomal [^3H]-adenosine (83 kBq per rat) was infused intravenously at a dose of 0.1 ml/min as liposomal adenosine was infused in protocol 2. At the time indicated, rat hearts were harvested for counting of radioactivity (LSC-3100, Aloka Co., Tokyo, Japan). Results are expressed as a percentage of the injected dose per 1 ml of blood or 1 g of wet tissue weight.

Statistical analysis. The parameters of the liposomes were expressed as the average \pm SD, whereas other data were expressed as the average \pm SEM. Comparison of time-course changes in hemodynamic parameters between groups was performed by 2-way repeated-measures analysis of variance (ANOVA) followed by a post-hoc Bonferroni test. For comparison of RI activity between groups, statistical analysis was done with the Mann-Whitney *U* test. To address the differences in infarct size among groups, we performed a nonparametric (Kruskal-Wallis) test followed by evaluation with the Mann-Whitney *U* test. Resulting *p* values were corrected according to the Bonferroni method. To compare parameters of liposomes, an unpaired *t* test was performed. In all analyses, *p* < 0.05 was considered to indicate statistical significance.

Results

Characterization of liposomes by dynamic light scatter analysis. The dynamic light scatter analysis showed no significant difference in mean diameter, polydispersity index, or zeta-potential distribution between empty and adenosine-loaded PEGylated liposomes (Table 1).

Liposomes in ischemic/reperfused myocardium. The EM revealed the intact vascular endothelial cells and cardiomyocytes in the nonischemic myocardium (Figs. 1A and 1B). There were no homogenous dark dots indicating liposomes in the nonischemic myocardium of rats that received either saline (Fig. 1A) or liposomes (Fig. 1B). In the border area, many homogenous dark dots indicating liposomes were accumulated in rats that received liposomes, but not saline (Figs. 1C and 1D). In this area, significant structural damage was not observed in endothelium, but slight swelling of mitochondria was often observed. In the infarcted area, numerous liposomes were detected in rats that received liposomes, but not saline (Figs. 1E and 1F). In this area, the disrupted endothelial integrity and marked swelling of mitochondria were often observed.

Table 1 Characterization of Liposomes by Dynamic Light Scatter Analysis

	Mean Diameter (nm)	Polydispersity Index	Zeta Potential (mV)
PEGylated liposomes (empty liposomes)	126 \pm 12	0.035 \pm 0.003	-1.7 \pm 0.4
PEGylated liposomal adenosine	134 \pm 21	0.094 \pm 0.002	-2.3 \pm 1.1

Results represented analysis of 4 independent experiments. Values are expressed as mean \pm SD.
PEG = polyethylene glycol.

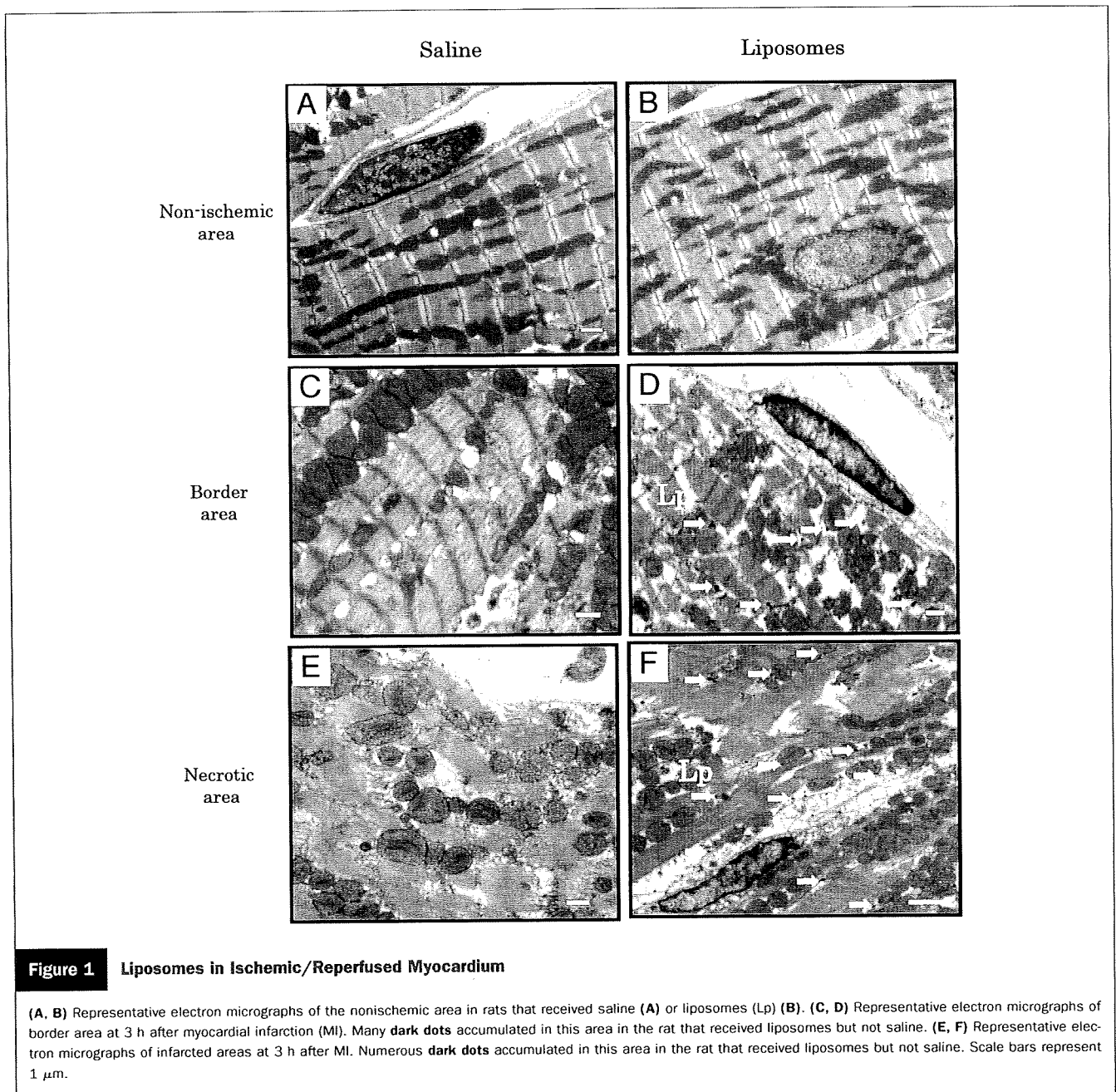


Figure 1 Liposomes in Ischemic/Reperfused Myocardium

(A, B) Representative electron micrographs of the nonischemic area in rats that received saline (A) or liposomes (Lp) (B). (C, D) Representative electron micrographs of border area at 3 h after myocardial infarction (MI). Many dark dots accumulated in this area in the rat that received liposomes but not saline. (E, F) Representative electron micrographs of infarcted areas at 3 h after MI. Numerous dark dots accumulated in this area in the rat that received liposomes but not saline. Scale bars represent 1 μm .

Fluorescent-labeled PEGylated liposomes in ischemic/reperfused myocardium. Quantitative analysis by bioluminescence *ex vivo* bioluminescence imaging revealed that the target to control fluorescent intensity ratio was higher in the border (noninfarcted area at risk) as well as infarcted areas compared with a nonischemic one, suggesting that fluorescent-labeled liposomes were accumulated in the border as well as infarcted areas. Since there was no high-intensity area when unlabeled liposomes were infused, it was suggested that this was not a nonspecific phenomenon to MI by the *ex vivo* bioluminescence imaging system (Fig. 2). The Evans blue staining was unrelated to the fluorescence intensity (data not shown).

Plasma radioactivity of RI-labeled adenosine was markedly higher in the PEGylated liposomal adenosine group at 10 min and 3 h after the intravenous infusion than in the free adenosine group (Fig. 3A). Encapsulation within PEGylated liposomes also augmented the accumulation of adenosine in ischemic/reperfused myocardium compared with that of free adenosine (Fig. 3B).

Hemodynamic effects of PEGylated liposomal adenosine. Baseline hemodynamic parameters did not differ among the groups. An intravenous infusion of free adenosine at doses of 225, 450, and 900 $\mu\text{g}/\text{kg}/\text{min}$ decreased the mean blood pressure by 14.8%, 25.4%, and 33.7%, respectively, compared with the effect of empty PEGylated lipo-

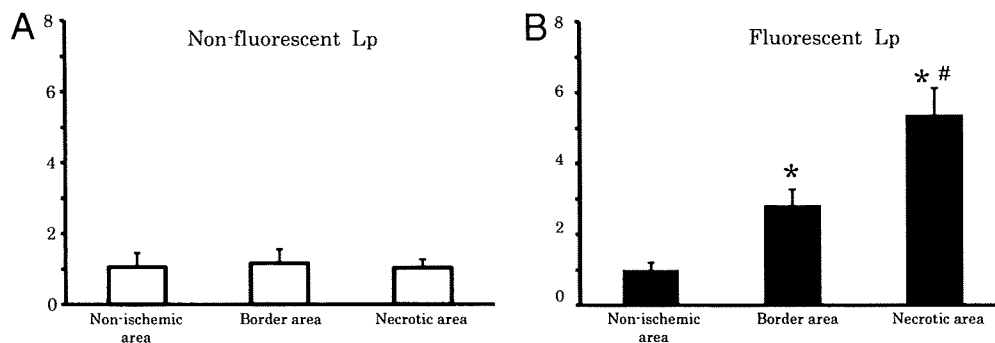


Figure 2 Detection of Fluorescence-Labeled PEGylated Liposomes in Ischemic/Reperfused Myocardium

Quantitative analysis of target-to-control fluorescent intensity ratio for each area in rats (n = 3 each group) that received nonfluorescent (A) or fluorescent (B) liposomes. The values of bioluminescence signals in the border and infarcted areas were expressed as the fold to that of the each nonischemic area. Values are expressed as the mean \pm SEM (error bars). *p < 0.05 versus nonischemic areas. #p < 0.05 versus border areas.

somes. In contrast, the intravenous infusion of PEGylated liposomal adenosine at a dose of either 225 or 450 $\mu\text{g}/\text{kg}/\text{min}$ did not significantly alter mean blood pressure (Fig. 4). Changes of the heart rate after infusion of PEGylated liposomal adenosine or free adenosine were similar to those observed for mean blood pressure (Fig. 4).

Effects of PEGylated liposomal adenosine on MI size. Baseline hemodynamic parameters were similar among all of the groups (Table 2). Intravenous infusion of free adenosine for 10 min reduced both the blood pressure and the heart rate, although these parameters returned to baseline within 5 min of ceasing infusion (Table 2). In contrast, hemodynamic parameters of the other groups were not altered (Table 2). The area at risk in the control group ($61 \pm 3\%$) did not differ compared with those of other groups that received liposomal adenosine. Intravenous infusion of PEGylated liposo-

mal adenosine caused a dose-dependent decrease of MI size compared with that in the control group, whereas intravenous infusion of empty PEGylated liposomes or free adenosine did not (Fig. 5B).

The bolus injection of adenosine receptor antagonist did not alter the hemodynamic parameters (Table 3). The area at risk in the liposomal adenosine group ($58 \pm 3\%$) did not differ compared with those of other groups that received adenosine receptor antagonist. Infusion of 8-SPT, a non-specific adenosine receptor antagonist, blunted the cardioprotective effect of liposomal adenosine (Fig. 6B). Furthermore, the infusion of the adenosine A_1 , A_{2a} , A_{2b} , or A_3 receptor antagonist also blunted cardioprotective effects of liposomal adenosine (Fig. 6B). Infusion of 8-SPT alone did not significantly affect myocardial infarct size compared with the control ($52 \pm 5\%$, n = 4).

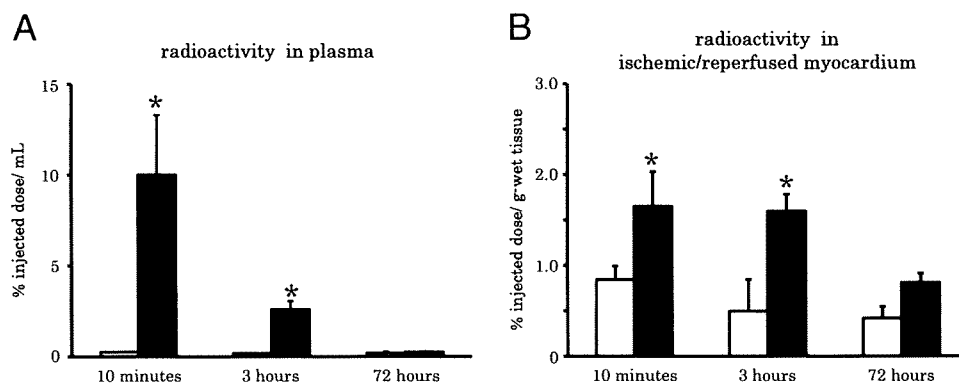


Figure 3 Radioisotope-Labeled Adenosine in Plasma and Ischemic/Reperfused Myocardium

(A) Changes in plasma radioactivity after infusion of radioisotope-labeled adenosine. Solid and open bars indicate the PEGylated liposomal adenosine and free adenosine groups, respectively (n = 4 each). In the PEGylated liposomal adenosine group, plasma radioactivity was markedly higher than in the free adenosine group. (B) Changes in radioactivity in ischemic/reperfused myocardium. Solid and open bars indicate the PEGylated liposomal adenosine and free adenosine groups, respectively (n = 4 each). In the PEGylated liposomal adenosine group, myocardial radioactivity was markedly higher than in the free adenosine group. Values are expressed as the mean \pm SEM (error bars). *p < 0.05 versus the free adenosine group at the corresponding time.

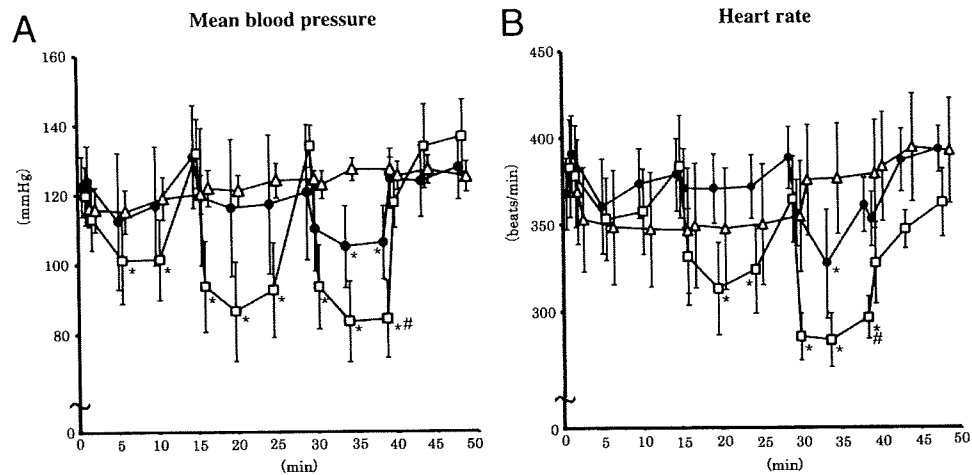


Figure 4 Hemodynamic Effects of PEGylated Liposomal Adenosine

Changes in the mean blood pressure (A) and heart rate (B) after intravenous infusion of various doses of empty PEGylated liposomes (triangles), PEGylated liposomal adenosine (circles), or free adenosine (squares) ($n = 8$ each). Values are expressed as the mean \pm SEM. * $p < 0.05$ versus baseline at the corresponding group. # $p < 0.05$ versus PEGylated liposomes.

Discussion

In the present study, EM, bioluminescence ex vivo imaging, and fluorescent analysis revealed the accumulation of liposomes in the border (noninfarcted areas at risk) as well as infarcted ones, but not nonischemic myocardium, at 3 h after MI. These findings suggested that liposomes could specifically accumulate in ischemic/reperfused myocardium. Interestingly, EM revealed the existence of liposomes at sites where endothelial integrity was still morphologically maintained. Endothelial dysfunction such as enhanced permeability is induced by ischemic insult without morphological endothelial disruption (3,15). Enhanced permeability might lead to the accumulation of liposomes in the border as well as infarcted area, which will

contribute to salvage the ischemic/reperfused myocardium. However, further investigation will be needed to determine the precise mechanism by which liposomes accumulate in ischemic/reperfused myocardium.

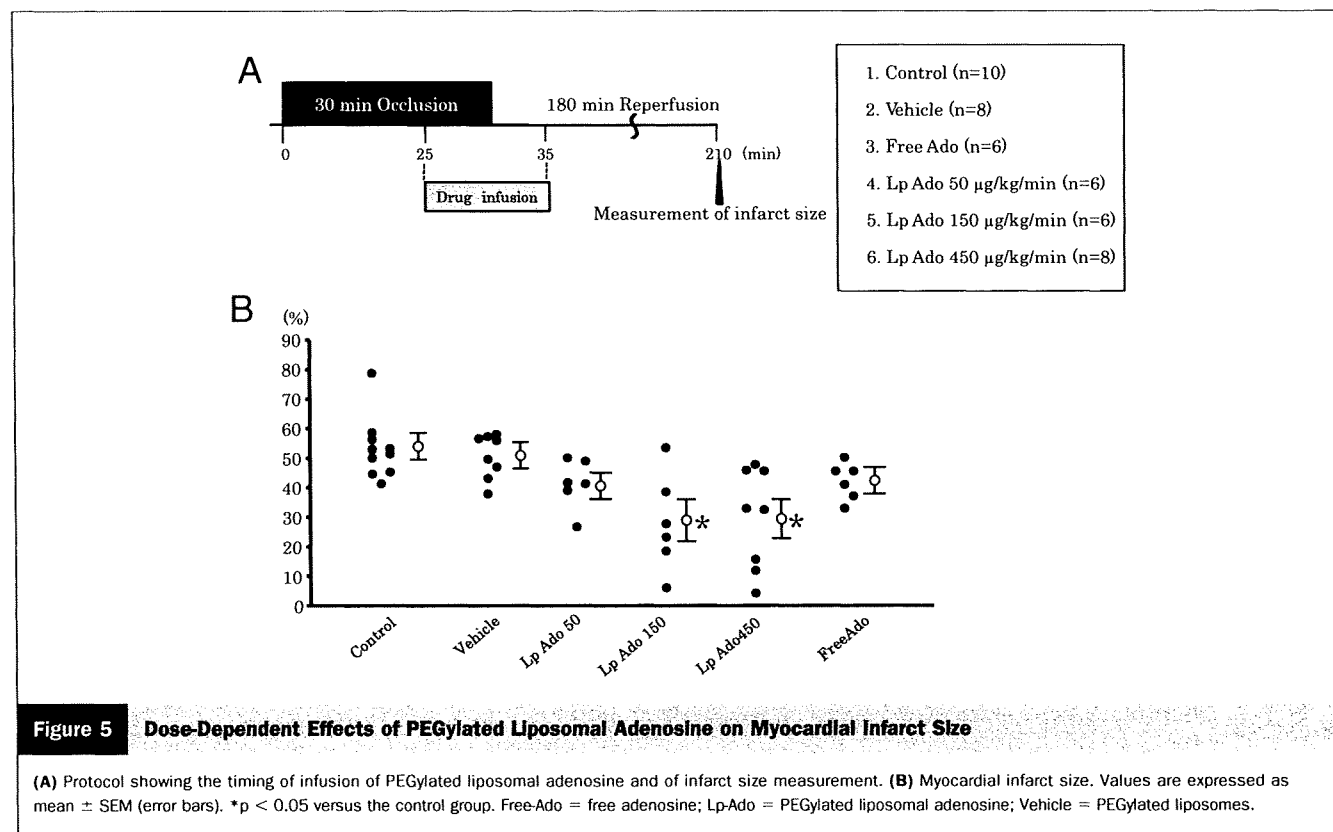
Analysis using RI-labeled adenosine encapsulated in liposomes revealed that plasma radioactivity was markedly higher in the PEGylated liposomal adenosine group compared with the free adenosine group. This indicates that encapsulation of adenosine by PEGylated liposomes considerably prolonged its residence time in the circulation and delayed its degradation. Consistent with the histological data, RI-labeled adenosine also showed preferential accumulation in ischemic/reperfused myocardium.

Table 2 Effects of Liposomal Adenosine on Hemodynamic Parameters

	Baseline	Ischemia				Reperfusion	
		0 min	15 min	25 min	30 min	5 min	10 min
Mean blood pressure (mm Hg)							
Saline	122 \pm 5	102 \pm 10	108 \pm 7	107 \pm 9	108 \pm 7	105 \pm 9	104 \pm 9
Vehicle	127 \pm 4	109 \pm 8	108 \pm 7	111 \pm 9	111 \pm 5	105 \pm 5	103 \pm 5
Free-Ado	124 \pm 8	115 \pm 8	111 \pm 5	109 \pm 4	66 \pm 4*	62 \pm 4*	112 \pm 6
Lp-Ado 50 μ g/kg/min	121 \pm 5	106 \pm 6	105 \pm 6	110 \pm 10	102 \pm 6	101 \pm 6	104 \pm 4
Lp-Ado 150 μ g/kg/min	122 \pm 3	107 \pm 6	107 \pm 6	109 \pm 11	105 \pm 6	100 \pm 6	103 \pm 4
Lp-Ado 450 μ g/kg/min	124 \pm 3	104 \pm 6	105 \pm 6	107 \pm 5	102 \pm 6	99 \pm 6	104 \pm 4
Heart rate (beats/min)							
Saline	363 \pm 22	366 \pm 19	369 \pm 14	413 \pm 22	372 \pm 12	372 \pm 16	371 \pm 14
Vehicle	363 \pm 32	363 \pm 6	383 \pm 6	396 \pm 25	367 \pm 6	374 \pm 7	372 \pm 7
Free-Ado	360 \pm 18	361 \pm 17	384 \pm 13	379 \pm 18	305 \pm 11*	293 \pm 13*	356 \pm 14
Lp-Ado 50 μ g/kg/min	378 \pm 19	386 \pm 21	366 \pm 12	376 \pm 12	367 \pm 19	369 \pm 9	377 \pm 17
Lp-Ado 150 μ g/kg/min	388 \pm 27	376 \pm 20	371 \pm 14	377 \pm 13	378 \pm 16	373 \pm 16	369 \pm 17
Lp-Ado 450 μ g/kg/min	368 \pm 17	376 \pm 21	361 \pm 13	386 \pm 15	368 \pm 15	363 \pm 6	367 \pm 7

Values are expressed as mean \pm SEM. * $p < 0.05$ versus baseline.

Free-Ado = free adenosine; Lp-Ado = PEGylated liposomal adenosine; PEG = polyethylene glycol; vehicle = PEGylated liposomes.



Furthermore, this study showed that PEGylated liposomal adenosine had a weaker effect on the blood pressure and heart rate than free adenosine. Thus, encapsulating adenosine in PEGylated liposomes attenuated its vasodilatory and negative chronotropic effects, presumably by reducing the amount of circulating free adenosine. However, the changes of hemodynamic parameters in this in vivo model suggested that significant release of adenosine from PEGylated liposomes would still occur if a large dose of liposomal adenosine (e.g., 900 $\mu\text{g}/\text{kg}/\text{min}$) were administered. Thus, further investi-

gation of the in vivo pharmacodynamics of PEGylated liposomal adenosine is needed.

An intravenous infusion of PEGylated liposomal adenosine at the maximum dose that did not disturb hemodynamic parameters for 10 min before reperfusion reduced MI size in a dose-dependent manner, and this improvement was blocked by 8-SPT, a nonselective adenosine receptor antagonist. These findings suggest that adenosine released from liposomes acts via an adenosine receptor-dependent pathway. One possible mechanism by which PEGylated lipo-

Table 3 Effects of Adenosine Receptor Antagonist on Hemodynamic Parameters

	Baseline	Ischemia				Reperfusion	
		0 min	15 min	25 min	30 min	5 min	10 min
Mean blood pressure (mm Hg)							
Lp-Ado + 8SPT	120 \pm 6	113 \pm 4	112 \pm 6	112 \pm 5	107 \pm 6	102 \pm 8	109 \pm 7
Lp-Ado + DPCPX	130 \pm 6	105 \pm 4	121 \pm 4	100 \pm 10	122 \pm 6	120 \pm 6	111 \pm 4
Lp-Ado + SCH58261	132 \pm 2	98 \pm 12	99 \pm 8	110 \pm 8	118 \pm 10	113 \pm 10	109 \pm 6
Lp-Ado + MRS1754	130 \pm 3	95 \pm 12	106 \pm 8	105 \pm 10	100 \pm 10	96 \pm 10	99 \pm 7
Lp-Ado + MRS1523	130 \pm 2	109 \pm 8	104 \pm 8	105 \pm 9	100 \pm 9	101 \pm 10	104 \pm 6
Heart rate (beats/min)							
Lp-Ado + 8SPT	404 \pm 17	385 \pm 10	374 \pm 8	396 \pm 8	389 \pm 9	383 \pm 8	385 \pm 9
Lp-Ado + DPCPX	396 \pm 24	380 \pm 11	399 \pm 9	398 \pm 12	385 \pm 9	382 \pm 9	380 \pm 7
Lp-Ado + SCH58261	393 \pm 14	399 \pm 15	381 \pm 9	395 \pm 15	376 \pm 9	373 \pm 9	385 \pm 7
Lp-Ado + MRS1754	398 \pm 14	392 \pm 11	401 \pm 9	379 \pm 15	378 \pm 9	374 \pm 9	377 \pm 7
Lp-Ado + MRS1523	396 \pm 9	390 \pm 11	390 \pm 11	392 \pm 10	373 \pm 9	391 \pm 7	388 \pm 11

Values were expressed as mean \pm SEM. * $p < 0.05$ versus baseline.

Lp-Ado = PEGylated liposomal adenosine; PEG = polyethylene glycol; Vehicle = PEGylated liposomes.

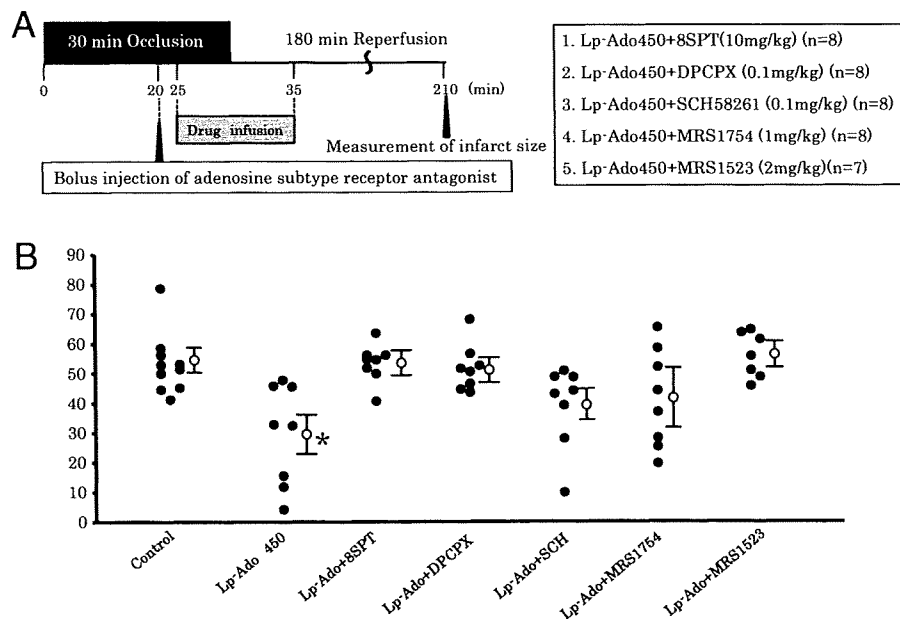


Figure 6 Effects of Adenosine Receptor Antagonists on Myocardial Infarct Size

(A) Protocol showing the timing of infusion of PEGylated liposomal adenosine and bolus injection adenosine receptor antagonists. (B) Myocardial infarct size. Values are expressed as the mean \pm SEM (error bars). * $p < 0.05$ versus the control group. The abbreviations for adenosine antagonists were described in the text. Abbreviations as in Figure 5.

some could augment cardioprotective effects of liposomal adenosine with minimum effects on hemodynamic parameters is the enhanced accumulation of PEGylated liposomal adenosine in ischemic/reperfused myocardium, which could augment various beneficial actions such as preventing calcium overload in the myocardium (5). The prolonged persistence of PEGylated liposomal adenosine would also increase its beneficial effect on ischemic/reperfused myocardium. Although continuous high-dose, long-term infusion of free adenosine was reported to reduce infarct size in rats (16), the present study did not confirm such a cardioprotective effect, probably because the total dose of free adenosine that we used was not high enough.

We found that myocardial infarct size in the group that received PEGylated liposomal adenosine with the antagonist of adenosine A_1 , A_{2a} , A_{2b} , or A_3 subtype receptor was no different from the control group, indicating that every adenosine subtype receptor could possibly play a role in mediating cardioprotection by liposomal adenosine and that it was difficult to identify one particular subtype in the present study. Numerous studies reported that A_1 , A_{2a} , A_{2b} , and A_3 receptors have been involved in cardioprotection against ischemia/reperfusion injury, and it remains controversial which adenosine subtype receptor is most responsible for cardioprotection (17–20). Furthermore, because the adenosine receptor antagonists used in the present study had some nonspecific effects, future investigation will be needed to examine the precise role of each adenosine receptor subtype using genetically engineered mice.

Because liposomal adenosine infused during reperfusion could reduce MI size, this agent could be a candidate for the adjunctive therapy of patients with acute MI. Importantly, adenosine is currently used for the diagnosis of ischemic heart disease and PEGylated liposomes are used to deliver anticancer agents (21). Thus, it should not be difficult to introduce PEGylated liposomal adenosine into clinical practice. Finally, PEGylated liposomes may provide a useful drug delivery system for targeting ischemic/reperfused myocardium with other agents.

Acknowledgments

The authors thank Akiko Ogai and Yoko Nakano for their excellent technical assistance; Motohide Takahama, Hiroyuki Hao, and Hatsue Ishibashi-Ueda for advice about the electron microscopy figure; and Syunichi Kuroda and Takashi Matsuzaki for assistance with bioluminescence imaging.

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REFERENCES

- Papahadjopoulos D, Allen TM, Gabizon A, et al. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc Natl Acad Sci U S A* 1991;24:11460–4.