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Control of plasma glucose with alpha-glucosidase inhibitor attenuates oxidative stress and slows the progression of heart failure in mice

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Abstract

Objective: It has been suggested that reduction in glucose levels contributes to the prolongation of life span of rodents in conjunction with restricted food intake, and hyperglycemia has been confirmed as a risk factor for cardiovascular disease (CVD), raising the possibility that better glycemic control could slow the progression of CVD. This study was designed to determine whether impaired glucose tolerance develops during the progression of cardiac hypertrophy and heart failure, and whether tight glycemic control could reduce the severity of heart failure.
Methods: In male C57BL/6 mice, transverse aortic constriction (TAC) was employed to create cardiac hypertrophy and heart failure. The involvement of NADPH in TAC mice and cardiac myocytes in the neonatal rat was investigated.
Results: The random-fed plasma glucose concentration was higher in TAC mice, and it was reduced to about 100 mg/dL by voglibose (an alpha-glycosidase inhibitor). Four weeks after TAC, both the heart weight/body weight ratio and the lung weight/body weight ratio were lower in the voglibose group than in the TAC group. Echocardiographic and invasive hemodynamic examination showed improvement of left ventricular function in voglibose-treated mice. Voglibose treatment decreased the myocardial expression of an NADPH oxidase subunit ($p47^{phox}$). Glucose dose-dependently increased both neonatal rat myocyte protein synthesis and the expression of $p47^{phox}$ protein, while apocynin (an NADPH oxidase inhibitor) blocked the enhancement of protein synthesis by high glucose.
Conclusion: Improvement of glycemic control through voglibose therapy inhibited cardiac remodeling by decreasing myocardial oxidative stress in mice with cardiac pressure overload.

Keywords: Glucose metabolism; Myocardial hypertrophy; Oxidative stress; Heart failure

1. Introduction

Hyperglycemia or impaired glucose tolerance (IGT) is a common feature of both acute myocardial infarction [1] and chronic heart failure (CHF) [2,3]. IGT can either be the cause or the result of CHF [4]. Patients with type 2 diabetes have a high propensity to develop CHF [5], and IGT is believed to be an independent risk factor for cardiovascular events [6–8]. Hyperglycemia or IGT can accelerate the progression of CHF [1,9]. On the other hand, increasing evidence supports a reciprocal relationship between CHF and IGT showing CHF patients are susceptible to developing IGT or diabetes [2,3]. These findings suggest the important impact of glycemic levels on the progression of CHF. Because CHF may cause hyperglycemia or IGT via increased sympathetic activity [10] or promotion of the renin–angiotensin system [11], it could be hypothesized that improved glycemic control might ameliorate CHF.

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In addition to dietary restrictions, two other approaches are usually employed to control blood glucose levels, namely, increasing glucose utilization and decreasing glucose absorption. Stimulation of carbohydrate oxidation has been shown to have a favorable impact on cardiac function [12,13]. A recent clinical study has also suggested that improvement of glycemic control in patients with IGT by administration of an alpha-glycosidase inhibitor, acarbose, was associated with a reduced risk of cardiovascular disease [14]. In addition, prior retrospective clinical investigations from our laboratory revealed that another alpha-glycosidase inhibitor (voglibose) was also beneficial in the treatment of CHF [15]. It has been established that IGT or hyperglycemia leads to oxidative stress [16,17], which in turn accelerates cardiac remodeling [18,19], further supporting the concept that better glycemic control might slow the progression of cardiac hypertrophy and cardiac failure.

In the present study, we investigated whether IGT develops in mice with left ventricular pressure overload, and explored the beneficial effect of voglibose on cardiac remodeling as well as the possible underlying mechanism.

2. Methods

2.1. Transverse aortic constriction (TAC) model and experimental protocols

All procedures were performed in accordance with our institutional guidelines for animal research conforming to NIH Guidelines. Male C57BL/6 mice (7–8 weeks old, wt 20–25 g) were anesthetized with a mixture of xylazine (5 mg/kg) and ketamine (100 mg/kg) via intraperitoneal injection. TAC was performed to create pressure overload-induced cardiac hypertrophy and heart failure, as described previously [20,21].

Seventy-one mice were included in this study. We treated the mice with water (TAC group, $n=25$, Sham group: $n=21$) or the alpha-glycosidase inhibitor voglibose (supplied gratis by Takeda Pharmaceutical Co. Ltd.) at a daily oral dose of 10 mg/kg (in tap water, TAC+Voglibose group: $n=19$; Sham+Voglibose: $n=6$). The dose of voglibose was set according to the results of a previous study [22]. Mice were fed ad libitum and given free access to water. There were no differences among all the experimental groups with regard to age and body weight before surgery. On the 3rd day following TAC, two mice from the TAC group and the TAC+Voglibose group were used to measure the trans-stenosis pressure gradient to confirm whether the LV pressure overload was similar between the two groups. Mice were euthanized at 4 weeks after TAC for morphometric and molecular analyses. Cell surface area, myocardial and perivascular fibrosis were quantified using 4 hearts from each group, as described previously [21,23].

2.2. Invasive measurement of hemodynamics

To determine the pressure gradient on the third day after TAC, two mice each from the TAC and TAC+Voglibose groups were randomly selected and anesthetized, as mentioned above, and an endotracheal tube was inserted and connected to a volume-cycled rodent ventilator as described elsewhere [20]. Ventilation was necessary to avoid respiratory arrest due to ligation of both carotid arteries. A 1.4 F Millar pressure catheter (Millar Instruments) was inserted into each of the left and right carotid arteries, and the blood pressures were measured simultaneously with a data acquisition and analysis system (PowerLab, AD Instruments). Left ventricular (LV) hemodynamics were evaluated at 4 weeks after TAC. A Millar catheter was inserted via the right carotid artery and carefully introduced into the LV, after which the heart rate, systolic pressure (LVSP), end-diastolic pressure (LVEDP), the maximal slope during the upstroke or downstroke of the pressure wave (max dP/dt and min dP/dt), max dP/dt divided by the pressure at the time of max dP/dt (contractility index) and the exponential time constant of relaxation (Tau) were analyzed using an application program Blood Pressure Module.

2.3. Echocardiography

Transthoracic echocardiography was performed with a Sonos 4500 and a 15–6 L MHz transducer (Philips, the Netherlands). Mice were immobilized without anesthesia. Two-dimensional short-axis views of the LV were obtained for guided M-mode measurement of the LV posterior wall thickness (LVPW), LV end-diastolic diameter (LVEDd), and LV end-systolic diameter (LVESd). LV fractional shortening (FS) was calculated as follows: $\mathrm{LVFS}=(\mathrm{LVEDd}-\mathrm{LVESd})/\mathrm{LVEDd}\times 100$. LV volume was calculated using the Teichholz formula: $V=7D^3/(2.4+D)$, where V=volume and D=the echocardiographically measured internal dimension [24]. Accordingly, LV end-diastolic volume $(\mathrm{LVEDV})=7(\mathrm{LVEDd})^3/(2.4+\mathrm{LVEDd})$, LV end-systolic volume $(\mathrm{LVESV})=7(\mathrm{LVESd})^3/(2.4+\mathrm{LVESd})$, stroke volume $(\mathrm{SV})=\mathrm{LVEDV}-\mathrm{LVESV}$, LV ejection fraction (LVEF %)$=\mathrm{SV/LVEDV}\times 100$. LV mass was calculated according to cube assumptions and modified with Teichholz formula: LV mass $(\mathrm{mg})=1.0557[7(\mathrm{LVEDd}+\mathrm{LVPWd}+\mathrm{VSTd})^3/(2.4+\mathrm{LVEDd}+\mathrm{LVPWd}+\mathrm{VSTd})-\mathrm{LVEDV}]$, where 1.055 is the gravity of myocardium, VSTd is diastolic ventricular septal thickness.

2.4. Measurement of glucose and insulin and free fatty acid (FFA)

The plasma concentrations of glucose and insulin were determined under fasting conditions, as described previously [25]. Random plasma glucose levels were also measured in each group. Insulin resistance was determined

by homeostasis model assessment: HOMA-IR=(fasting plasma glucose [mg/dL] × fasting serum insulin [ng/mL])/22.5. An intraperitoneal glucose tolerance test (IPGTT) was performed at 4 weeks. IPGTT is extensively used in the study of glucose metabolism in rodent animals [26,27]. After fasting for 14 h, glucose (2 g/kg) was injected intraperitoneally and the plasma glucose level was measured at baseline and at 30, 60, and 120 min. Serum nonesterified (free) fatty acid concentrations were measured by spectrophotometric enzymatic assay (Wako Chemicals).

2.5. RNA preparation and analysis

Total RNA of homogenized mouse whole heart or cultured neonatal rat cardiac myocytes was prepared using RNA-Bee isolation reagent (Tel-Test, Inc.) according to the protocol of the manufacturer. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to generate cDNA templates from extracted RNA. cDNA template (1 μg) was then used for subsequent PCR amplification with primers targeting the genes of atrial natriuretic factor (ANF), collagen IV (procollagen IV alpha) and collagen I. PCR products were loaded onto a 2.0% agarose gel and electrophoresed at 100 V for 45 min. Gels were stained with ethidium bromide and quantified using Scion Image software. β-Actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control.

2.6. Western blot analysis

Membrane proteins were prepared from whole heart tissue homogenate or cultured cardiac myocytes, as described elsewhere [28]. Then immunoblotting was performed to detect the nicotinamide adenine dinucleotide 3-phosphate (NADPH) oxidase subunits $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $gp91^{phox}$ (Santa Cruz Biotechnology), and beta-actin was used as a loading control. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham) and quantified by densitometry with Scion Image software.

2.7. Validation of glucose effects on oxidant stress and cellular hypertrophy in an in vitro model of neonatal rat cardiomyocyte culture

Ventricular myocytes were isolated from neonatal rats (2 to 3 days old) and cultured as described previously [29]. In brief, myocytes were incubated in Dulbecco's Modified Eagle's Medium containing 100 mg/dL glucose supplemented with 10% fetal calf serum for 72 h and then grown under serum-free conditions for 48 h. Finally, the myocytes were exposed to 100, 450, or 900 mg/dL glucose with or without the addition of 10^{-4} mol/L apocynin (an inhibitor of superoxide production by NADPH oxidase; Sigma-Aldrich) for 24 h and then harvested for analysis of protein synthesis based on ^{3}H-leucine incorporation [21] and for determination of the expression of NADPH oxidase subunits proteins.

2.8. Statistical analysis

The unpaired Student's *t*-test was used for comparisons between two groups, and one-way ANOVA with post hoc analysis by the Tukey–Kramer exact probability test was used for multiple comparisons. Skewed data were log-transformed before parameter testing was performed. Results were expressed as the mean±S.E.M. and $P<0.05$ was considered statistically significant.

3. Results

3.1. Levels of plasma glucose and serum insulin and FFA

As shown in Fig. 1A, random plasma glucose levels were increased in TAC mice at 3 weeks after surgery, while the administration of voglibose (10 mg/kg/day) returned the plasma glucose level to about 100 mg/dL. Fasting glucose before sacrifice was also higher in the TAC group than in the sham group (Fig. 1B). An increase of serum insulin levels relative to those in sham mice was noted in TAC mice, and this was not changed by voglibose treatment (Fig. 1C). Insulin resistance index HOMA-IR was significantly increased in TAC mice (Fig. 1D). The IPGTT showed significantly lower peak glucose levels in sham and voglibose-treated mice (Fig. 1E), suggesting an improvement of glucose tolerance by voglibose. These findings indicate the development of IGT with postprandial hyperglycemia in TAC mice. On the other hand, the serum FFA level was significantly lower in TAC mice than in the sham group, while no difference was found between TAC and voglibose-treated TAC mice (Fig. 1F).

3.2. Amelioration of cardiac hypertrophy by voglibose

The pressure gradient was about 50 mm Hg on the 3rd day after TAC, indicating that LV pressure overload was similar in the TAC and voglibose groups, which is also supported by the evidence that LVSP was similar between the two groups at 4 weeks after TAC (Table 1). There was no significant difference in body weight at 4 weeks (21.24±0.32 g vs. 21.94±0.46 g in the TAC and TAC+Voglibose groups, respectively). Both the heart weight-to-body weight ratio (HW/BW mg/g) and the cross-sectional surface area of cardiomyocytes were significantly smaller in voglibose-treated TAC mice (Fig. 2A, B, E and F), but there was no significant difference in cardiac fibrosis indexed histologically by Azan staining (myocardial fibrosis: 19.7±4% vs. 17.4±2%; perivascular

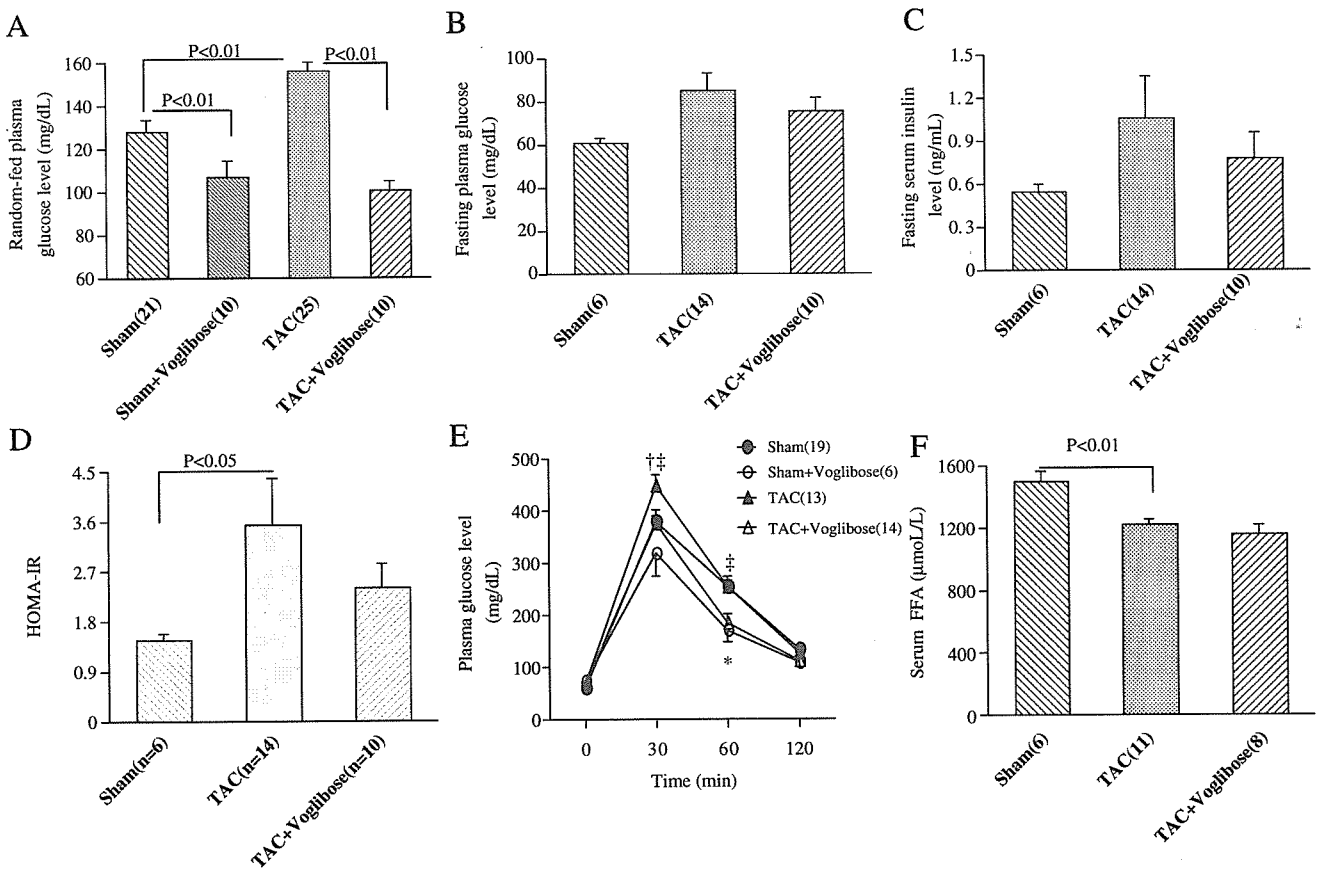

Fig. 1. Glucose and fatty acid metabolism in hypertrophic and failing hearts. (A) Random plasma glucose concentrations at 3 weeks after transverse aortic constriction (TAC) or a sham operation. (B) Fasting plasma glucose concentration at 4 weeks. (C) Fasting serum insulin concentration at 4 weeks. (D) The insulin resistance index HOMA-IR at 4 weeks. A *t*-test was performed after log-transformation. (E) Intraperitoneal glucose tolerance test. $^{\dagger}P<0.05$ vs. sham, $^{\ddagger}P<0.05$ vs. voglibose-treated mice, $^{*}P<0.05$ vs. sham. (F) Serum free fatty acid at 4 weeks (FFA).

fibrosis: 81±12% vs. 73±9%) and expression of the markers of fibrosis, collagen I and IV genes (Fig. 2C and D) between the TAC and TAC+Voglibose groups.

3.3. Improvement of LV hemodynamics by voglibose

It has been reported that high concentrations of glucose impair cardiomyocyte contractility [28]. Since the present study showed that LV pressure overload could induce moderate postprandial hyperglycemia (Fig. 1A), we postulated that inhibition of postprandial hyperglycemia by voglibose might improve cardiac function. As expected, both max d*P*/d*t* and contractility index were higher in the voglibose-treated mice compared with the untreated TAC mice (Fig. 3A and Table 1). LVSP was slightly higher, while both LVEDP and Tau were

Table 1
Left ventricular hemodynamics at 4 weeks after TAC or sham operation

Group	HR (beats/min)	LVSP (mm Hg)	LVEDP (mm Hg)	Max d*P*/d*t* (mm Hg/s)	Min d*P*/d*t* (mm Hg/s)	Contractility index	Tau (ms)
Sham	236±25	86±3.0*	8.3±1.6*	3074±344	2695±270	77.3±5.5*	19.2±1.3†
Sham+V	243±37	87±11*	8.9±1.4*	2892±269	2601±195	75.0±9.7*	19.2±1.2††
TAC	188±20	164±5.4	24.5±1.4	3167±106	2995±161	43.6±2.1	24.8±1.3
TAC+V	170±10	169±13.1	16.3±2.4†	3776±328	3904±369††	54.6±4.7††	18.7±1.6†

TAC, transverse aortic constriction; HR, heart rate; LVSP, maximum left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; Max d*P*/d*t*, the steepest slope during the upstroke of the pressure curve; Min d*P*/d*t*, the steepest slope during the downstroke of the pressure curve; Contractility index: Max d*P*/d*t* divided by the pressure at the time of Max d*P*/d*t*; Tau, the exponential time constant of relaxation; Sham+V, sham+voglibose 10 mg/kg/day; TAC+V, TAC+voglibose 10 mg/kg/day. The number of mice in groups of Sham, Sham+V, TAC and TAC+V was 10, 6, 11 and 9, respectively. Data are mean±S.E.M.

* $P<0.001$, compared with TAC.

† $P<0.01$, compared with TAC.

†† $P<0.05$, compared with TAC.

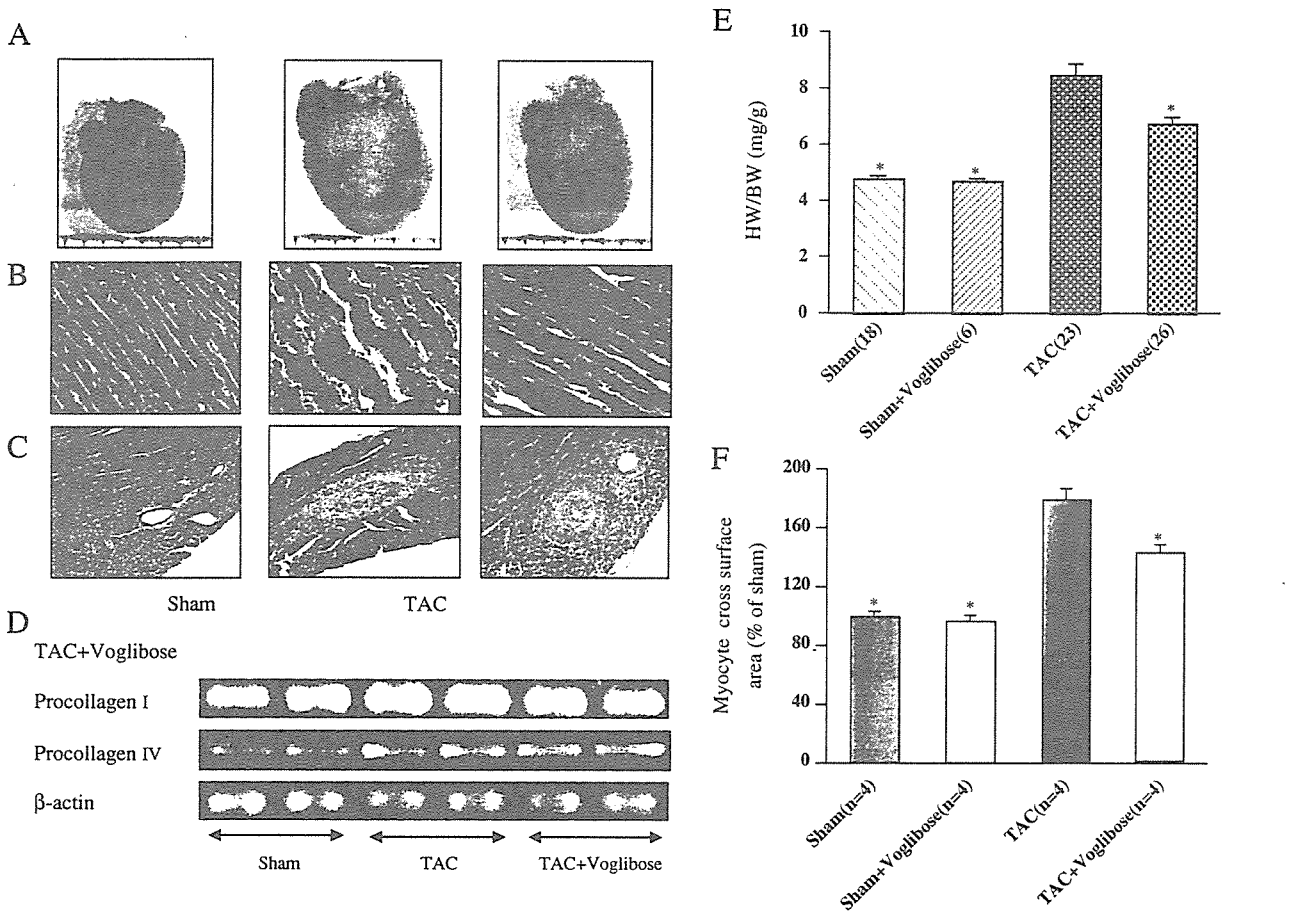

Fig. 2. Results of cardiac hypertrophy and fibrosis at 4 weeks. (A) Representative images of the whole heart. (B) Long-axis view of cardiac myocytes (HE stain, ×200). (C) Cardiac fibrosis with Azan staining (×100). (D) Expression of collagen I and collagen IV genes detected by RT-PCR with β-actin as internal control. The heart weight-to-body weight ratio (HW/BW) (E) and cardiac myocyte cross-sectional area (F) were decreased in voglibose-treated mice. *$P<0.01$ vs. TAC.

significantly lower and min dP/dt was significantly higher in voglibose-treated TAC mice (Table 1), and no significant change was found in voglibose-treated sham mice, suggesting that voglibose improves both systolic and diastolic function in TAC mice (Table 1). These results indicate that voglibose treatment delayed the progression of cardiac dysfunction due to pressure overload.

3.4. Pulmonary and echocardiographic findings

Four weeks after TAC, the lung weight-to-body weight ratio (LW/BW) was markedly decreased in voglibose-treated mice (Fig. 3C), suggesting improvement of cardiac function. In agreement with this finding and the data on LV hemodynamics, echocardiography showed a marked increase in LVFS and LVEF in voglibose-treated TAC mice, indicating an improvement in systolic function (Fig. 3B, Table 2). Both LV dimensions and posterior wall thickness were also decreased in TAC mice by voglibose treatment (Table 2), indicating the inhibition of cardiac remodeling. No significant change was found in voglibose-treated sham mice.

3.5. High glucose enhances protein synthesis by cardiac myocytes: role of oxidative stress in vitro

The most commonly used in vitro and in vivo models for myocardial hypertrophy studies are primary culture of neonatal rat cardiac myocytes and the mouse TAC model, respectively [30]. Since mouse cardiac myocyte culture is not an established method for experimental investigation of hypertrophy, we used cultured neonatal rat cardiac myocytes to examine the effect of high glucose levels on protein synthesis to test whether hyperglycemia causes cardiac myocyte hypertrophy. As shown in Fig. 4A, glucose dose-dependently increased ^{3}H-leucine incorporation by cultured cardiac myocytes. Expression of the marker of pathological hypertrophy ANF was upregulated in high glucose-medium cultured cells (Fig. 4B).

To investigate the mechanism underlying glucose-induced myocyte hypertrophy, we examined NADPH

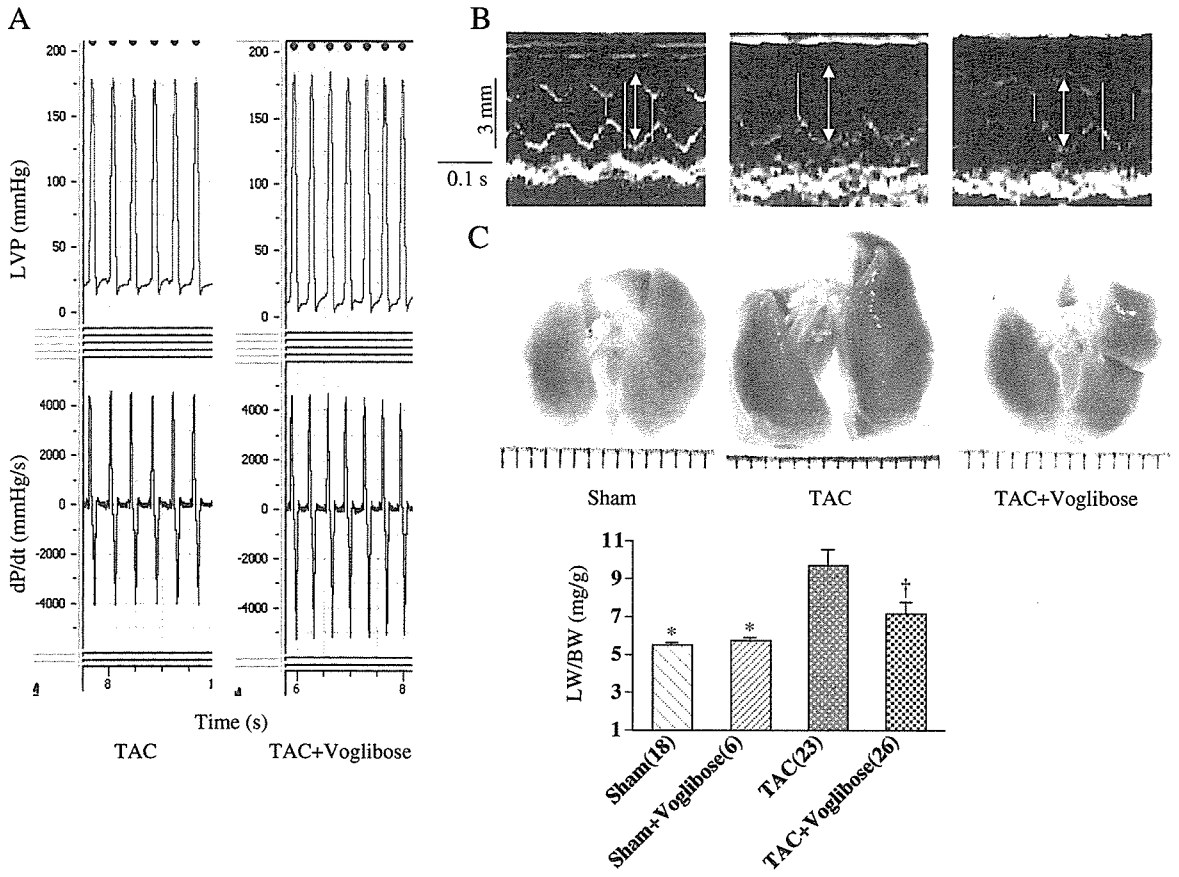

Fig. 3. Improvement in heart function by voglibose treatment. (A) Representative graphs of left ventricular pressure and its rate of change (dP/dt) showed a lower diastolic pressure and higher minimum dP/dt. (B) Representative M-mode echocardiographic images. (C) Pulmonary congestion was ameliorated by voglibose treatment at 4 weeks after TAC and the lung weight-to-body weight ratio (LW/BW) was significantly lower in voglibose-treated mice than in the untreated TAC mice. *P<0.01, †P<0.05 vs. TAC.

oxidase expression because this enzyme is a potential source of reactive oxygen species (ROS) and makes an important contribution to cardiac hypertrophy and heart failure [19,31,32]. Membrane expression of NADPH subunit proteins ($p22^{phox}$, $p47^{phox}$, $p67^{phox}$) and gp 91 was determined by western blotting. Neonatal rat myocyte expression of $p47^{phox}$ was upregulated by culture in high glucose medium (Fig. 5A) and was

Table 2
Echocardiographic findings at 4 weeks after TAC or sham operation

Parameters	Sham (n=16)	Sham+Voglibose (n=6)	TAC (n=21)	TAC+Voglibose (n=17)
LVEDd (mm)	2.93±0.05*	2.99±0.06††	3.46±0.12	3.09±0.12††
LVPWd (mm)	0.66±0.01*	0.61±0.02*	0.81±0.03	0.76±0.02††
LVESd (mm)	1.27±0.05*	1.35±0.05†	2.23±0.16	1.70±0.17††
LVFS (%)	56.7±1.3†	55.0±1.4†	37.0±2.8	46.5±3.3††
LVEDV (μL)	33.2±1.3*	34.9±1.7††	51.0±4.4	39.1±4.0††
LVESV (μL)	4.1±0.4*	4.7±0.4††	20.0±3.4	10.9±2.6††
SV (μL)	29.1±1.1	30.3±1.4	31.3±1.3	28.2±1.7
LVEF (%)	88±1*	87±1†	66±3	77±4††
LV mass (mg)	47.3±1.3*	44.6±1.6*	73.7±2.7	59.7±2.3*

TAC, transverse aortic constriction; LVEDd, left ventricular end-diastolic dimension; LVPWd, left ventricular diastolic posterior wall thickness; LVESd, left ventricular end-systolic dimension; LVFS: left ventricular fractional shortening; LVEDV, left ventricular end-diastolic volume; LVESV: left ventricular end-systolic volume; SV, stroke volume; LVEF, left ventricular ejection fraction; Sham+V, sham+voglibose 10 mg/kg/day; TAC+V, TAC+voglibose 10 mg/kg/day. Data are mean±S.E.M.

* P<0.001, compared with TAC.

†† P<0.05, compared with TAC.

† P<0.01, compared with TAC.

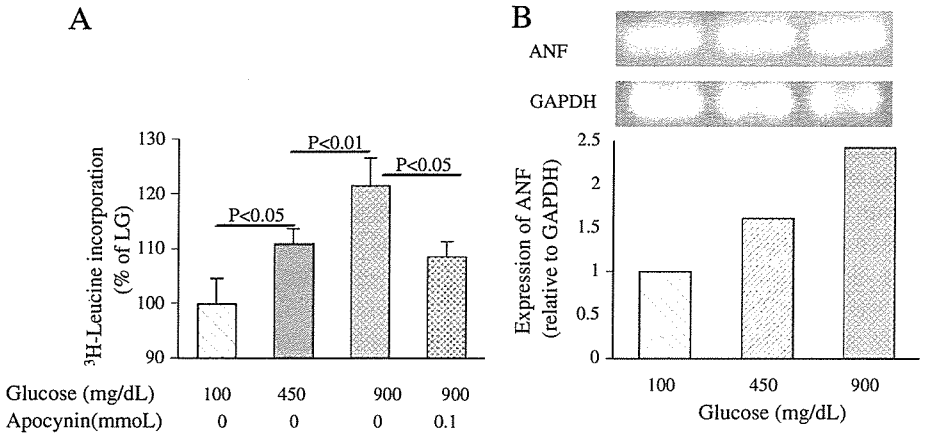

Fig. 4. High glucose culture enhances protein synthesis by neonatal rat cardiac myocytes. (A) Glucose caused a concentration-dependent increase of protein synthesis in cardiac myocytes, which was blocked by an NADPH oxidase inhibitor (apocynin). Each experiment was repeated at least 3 times. (B) A representative result of atrial natriuretic factor (ANF) detected by RT-PCR using GAPDH as an internal control. ANF gene was upregulated in high-glucose culture medium.

also upregulated in the hearts of TAC mice at 4 weeks (Fig. 5B). In contrast, the other 3 NADPH subunits were unchanged (data not shown). Treatment with voglibose caused a decrease in p47^{phox} expression ($P<0.05$ vs. TAC mice, Fig. 5B).

To confirm that the increase in p47^{phox} contributed to the glucose-induced increase in protein synthesis by cardiac myocytes, we used an inhibitor of NADPH oxidase (apocynin) to block protein synthesis. As shown in Fig. 4, co-treatment with 10^{-4} mol/L apocynin and high glucose caused a decrease in ^{3}H-leucine incorporation by cardiac myocytes.

4. Discussion

In this study, we demonstrated that glucose intolerance was induced in mice with cardiac hypertrophy and heart failure due to pressure overload, and that oral treatment with voglibose effectively controlled postprandial hyperglycemia, thereby ameliorating cardiac hypertrophy and slowing progression to heart failure. We further showed that culture with high concentrations of glucose led to enhancement of oxidative stress-mediated protein synthesis by cardiac myocytes, and voglibose inhibited myocardial NADPH oxidase expression via improved control of blood glucose levels.

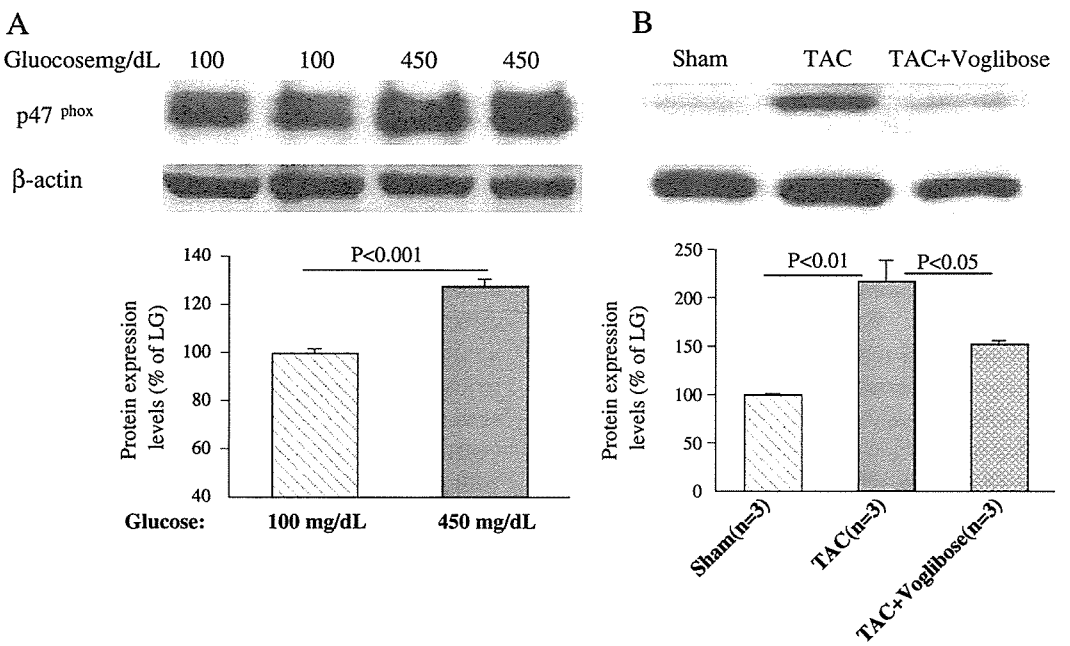

Fig. 5. Western blotting of NADPH oxidase subunits. (A) High glucose culture increased the expression of p47^{phox} protein in neonatal rat myocytes. LG: low glucose (100 mg/dL). (B) Voglibose decreased myocardial expression of p47^{phox} protein in TAC mice.

We speculate that sympathetic activation might have contributed to hyperglycemia associated with CHF. Consistent with previous reports [33–35], we have noted enhancement of sympathetic activity (demonstrated by an increase in plasma catecholamines) in mice with cardiac hypertrophy and cardiac failure [21]. Supportively, alpha$_1$-adrenergic blockade with prazosin [36] has been demonstrated to increase insulin sensitivity and improve glucose metabolism. Moreover, downregulation of glycolytic enzyme encoding genes was observed in pressure-overload-induced hypertrophy in spontaneously hypertensive rats, and a sympathetic inhibitory treatment with carvedilol attenuates the downregulation of glucose metabolic gene expression [37] and increases glucose utilization in hypertension patients [38]. It is also known that the renin–angiotensin system (RAS) plays an important role in the metabolic syndrome, including hyperglycemia and IGT [11], and that inhibition of RAS activity by either angiotensin-converting enzyme inhibitors [39] or angiotensin type 1 receptor blockers [40] is effective both for treating cardiovascular disease and for preventing the onset of diabetes. Evidence from clinical studies also supports our finding that IGT is associated with heart failure. Tenenbaum et al. reported that there was a significantly higher risk of diabetes in patients with advanced heart failure [2]. In 308 non-ischemic heart disease patients with CHF, we have also found a 75% incidence of IGT and diabetes (unpublished data).

The failing heart is postulated to suffer from chronic energy starvation despite an excess of substrates. Therefore, we targeted the excessive supply of glucose by pharmacological intervention in an attempt to improve cardiac function. A very recently published retrospective study by Kosiborod et al. firmly supports the hypothesis that excess glucose is detrimental. They reported that hyperglycemia was common in patients with acute myocardial infarction and the risk of death was higher in hyperglycemic patients without recognized diabetes than in those with diabetes [1]. Furthermore, it was reported that hyperglycemia exacerbates LV remodeling and heart failure in rats after myocardial infarction [9]. Considering these findings, it is plausible that better control of postprandial hyperglycemia by voglibose treatment could ameliorate cardiac hypertrophy and slow the progression to heart failure. We investigated the effect of voglibose on lipid metabolism and did not find any change in serum FFA concentrations, consistent with previous studies showing alpha-glycosidase inhibitors had no effect on FFA metabolism (review [41]). Except for a reduction in triglycerides, voglibose was reported not to change other lipid profiles such as total cholesterol, low-density lipoprotein cholesterol and FFA levels [42]. In this study, we noted a significantly lower serum FFA level in TAC mice, which could be a result of an increase in fatty acid oxidation. Because fatty acid oxidation requires a greater rate of oxygen consumption than does glucose oxidation for a given rate of ATP synthesis [43], an increase of fatty acid oxidation is likely to increase the consumption of cardiac energy and consequently lead to the depression of contractile performance. This is also supported by the evidence that inhibition of FFA oxidation attenuates the severity of heart failure [33,44,45]. On the other hand, an increased plasma FFA concentration [33] and down regulated fatty acid oxidation were reported to appear in advanced stage CHF [46,47], while moderate heart failure does not decrease fatty acid oxidation [48].

Substantial evidence supports an important role of oxidative stress due to an excess of reactive oxygen species (ROS) in the pathophysiology of cardiac hypertrophy and failure [18,19,49]. NADPH oxidase is a major source of ROS. In this study, we confirmed that high glucose contributed to enhancement of oxidative stress in both cultured cardiac myocytes and mouse hearts. The level of $p47^{phox}$ protein, a subunit of NADPH oxidase, was increased in the hearts of mice with cardiac failure and in cardiac myocytes cultured with high-glucose medium, in agreement with the reported results of clinical and experimental studies [19,28,31,50]. Accordingly, inhibition of $p47^{phox}$ via a decrease in the plasma glucose level could be a mechanism by which voglibose slows the progression of heart failure, as it was shown that high glucose led to a moderate increase in protein synthesis by cardiac myocytes and co-treatment with an NADPH oxidase inhibitor abrogated this effect. Among the NADPH oxidase subunits, $p47^{phox}$ is consistently reported to be upregulated by CHF in both animals and humans [19,28,31]. Pharmacological interventions targeting the inhibition of oxidative stress have been frequently shown to be effective in inhibiting cardiac remodeling [18,49,51]. However, we should note that anti-oxidant stress is not the sole mechanism. The beneficial impact of glycerol control on mechanical efficiency and energy conservation has also been reported to contribute to improved heart function [43,52,53]. In addition, although our data implicate that voglibose improves heart failure independent of the inhibition of cardiac fibrosis, further investigation to obtain firm evidence on the molecular makers of fibrosis from the protein level would be helpful to clarify this issue.

Although the change of glucose concentration in the in vitro model of neonatal rat cardiomyocyte culture was different from that observed in our in vivo model of TAC induced myocardial hypertrophy in mice, the impact on oxidant stress was similar. It has been reported that a long-term decrease in plasma glucose levels by 15 mg/dL in rats contributes to the prolongation of life span in conjunction with caloric restriction via attenuation of oxidant stress [54].

Metabolic modulators, as a novel form of therapy for cardiac hypertrophy and failure, could serve as an effective adjunct to traditional regimens. There is mounting evidence that stimulation of glucose utilization can improve cardiac function in heart failure [12,13]. Our results suggest that a target glucose level of $\leq$110 mg/dL is beneficial, in agreement with the position statement released by the

American College of Endocrinology [55]. The present study provides the firm evidence that reduction in the postprandial blood glucose level can slow the progression of cardiac hypertrophy and heart failure, which may suggest a novel therapy for addition to the current pharmacological regimens. Alpha-glucosidase inhibitors are devoid of any direct negative hemodynamic or inotropic effects, and no significant side effects such as hypoglycemia were observed in this study, which is another advantage when treating CHF. With this in mind, we are now conducting prospective clinical trials to test the effect of voglibose in patients with CHF or myocardial infarction, and the preliminary results have been encouraging. It seems reasonable that improved glycemic control in combination with approaches to increase energy utilization would be an effective alternative for the treatment of heart failure.

Acknowledgements

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PRECLINICAL STUDY

Erythropoietin Enhances Neovascularization of Ischemic Myocardium and Improves Left Ventricular Dysfunction After Myocardial Infarction in Dogs

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OBJECTIVES We investigated the effects of erythropoietin (EPO) on neovascularization and cardiac function after myocardial infarction (MI).

BACKGROUND Erythropoietin exerts antiapoptotic effects and mobilizes endothelial progenitor cells (EPCs).

METHODS We intravenously administered EPO (1,000 IU/kg) immediately [EPO(0) group], 6 h [EPO(6h) group], or 1 week [EPO(1wk) group] after the permanent ligation of the coronary artery in dogs. Control animals received saline immediately after the ligation.

RESULTS The infarct size 6 h after MI was significantly smaller in the EPO(0) group than in the control group (61.5 ± 6.0% vs. 22.9 ± 2.2%). One week after MI, the circulating CD34-positive mononuclear cell numbers in both the EPO(0) and the EPO(6h) groups were significantly higher than in the control group. In the ischemic region, the capillary density and myocardial blood flow 4 weeks after MI was significantly higher in both the EPO(0) and the EPO(6h) groups than in the control group. Four weeks after MI, left ventricular (LV) ejection fraction in the EPO(6h) (48.6 ± 1.9%) group was significantly higher than that in either the control (41.9 ± 0.9%) or the EPO(1wk) (42.6 ± 1.2%) group but significantly lower than that in the EPO(0) group (56.1 ± 2.3%). The LV end-diastolic pressure 4 weeks after MI in both the EPO(0) and the EPO(6h) groups was significantly lower than either the control or the EPO(1wk) group. Hematologic parameters did not differ among the groups.

CONCLUSIONS In addition to its acute infarct size-limiting effect, EPO enhances neovascularization, likely via EPC mobilization, and improves cardiac dysfunction in the chronic phase, although it has time-window limitations. (J Am Coll Cardiol 2006;48:176–84) © 2006 by the American College of Cardiology Foundation

Erythropoietin (EPO) is a cytokine that promotes proliferation and differentiation of erythroid precursor cells (1) and is widely used for the treatment of anemia in patients with chronic renal failure (2). Erythropoietin can also exert antiapoptotic and radical scavenger effects on nonerythroid cells (3,4). Indeed, we and others showed that an administration of EPO before or shortly after the onset of ischemia

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reduced myocardial infarct size and improved cardiac function in acute phases (5–8). Another interesting nonerythroid function of EPO is the promotion of endothelial progenitor cell (EPC) mobilization in animals and humans (9–11), which may enhance neovascularization of ischemic areas (12,13). We hypothesized that EPO increases blood supply to ischemic regions through promoting neovascularization and improves cardiac dysfunction after ischemic insult. Thus, the goal of this study was to characterize the effects of EPO on neovascularization and cardiac function after myocardial infarction (MI) in the chronic phase.

METHODS

All procedures were performed in conformity with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, 1996 revision) and were approved by the Osaka University Committee for Laboratory Animal Use.

Instrumentation. Forty-seven beagle dogs (Kitayama Labes, Yoshiki Farm Gifu, Japan), weighing 8 to 12 kg were used in these experiments. After an intravenous injection of sodium pentobarbital (15 mg/kg), the dogs were intubated and ventilated. General anesthesia was maintained with 0.5% to 2.0% inhaled isoflurane. After baseline echocardiography and hemodynamic assessment, minimal thoracot-

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Abbreviations and Acronyms

ABP	= arterial mean blood pressure
Dil-ac-LDL	= 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated low density lipoprotein
EPC	= endothelial progenitor cell
EPO	= erythropoietin
HR	= heart rate
LAD	= left anterior descending coronary artery
LCX	= left circumflex coronary artery
LV	= left ventricle/ventricular
LVEDD	= left ventricular end-diastolic dimension
LVEDP	= left ventricular end-diastolic pressure
MBF	= myocardial blood flow
MI	= myocardial infarction
MNC	= mononuclear cell
UEA-I	= *Ulex europaeus* agglutinin I
VEGF	= vascular endothelial growth factor

omy was performed, and then the left anterior descending coronary artery (LAD) was ligated just distal to the first diagonal branch. To ensure that all animals included in the data analysis were exposed to a similar extent of ischemia, animals with excessive myocardial collateral blood flow (>15 ml/100 g/min) were excluded from study as previously described (14).

Experimental protocols. ACUTE EFFECTS OF EPO ON MYOCARDIAL INFARCT SIZE. Either a single dose of EPO (1,000 IU/kg; 5 ml) (n = 6) or the same volume of saline (n = 6) was administered intravenously immediately after the LAD ligation. Regional myocardial blood flow (MBF), area at risk, and infarct size at 6 h after the LAD ligation were determined as described previously (Fig. 1) (14). Recombinant human EPO was provided by Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan). Recombinant human EPO is effective for correcting anemia in the beagle dog (15).

EFFECTS OF IMMEDIATE OR DELAYED TREATMENT WITH EPO ON NEOVASCULARIZATION AND CARDIAC FUNCTION. A single dose of EPO (1,000 IU/kg; 5 ml) was administered intravenously immediately [EPO(0) group, n = 8], 6 h [EPO(6h) group, n = 8], or 1 week [EPO(1wk) group, n = 7] after the LAD ligation. Control animals received the same volume of saline (control group, n = 8) immediately after the LAD ligation.

Hematologic parameters. Blood was sampled from a peripheral vein under pentobarbital (15 mg/kg) anesthesia at the time points indicated in Figure 2. Hematologic parameters, including hematocrit, white blood cell count, and platelet count, were measured.

Cytokine measurements. Plasma levels of vascular endothelial growth factor (VEGF) were measured by enzyme-linked immunosorbent assay (R & D Systems, Minneapolis, Minnesota). The detection limit of the assays was 9 pg/ml. The reliability of this assay in dogs has already been reported previously (16).

Quantification of CD34-positive mononuclear cells. The circulating CD34-positive mononuclear cells (CD34+MNCs) were quantified at the time points indicated in Figure 2. In brief, peripheral white blood cells were stained with a phycoerythrin-conjugated anticanine CD34 monoclonal antibody (BD Pharmingen, San Diego, California). Samples were then subjected to a two-dimensional side-scatter-fluorescence dot plot analysis (FACScan, Becton-Dickinson, Tokyo, Japan). After appropriate gating of

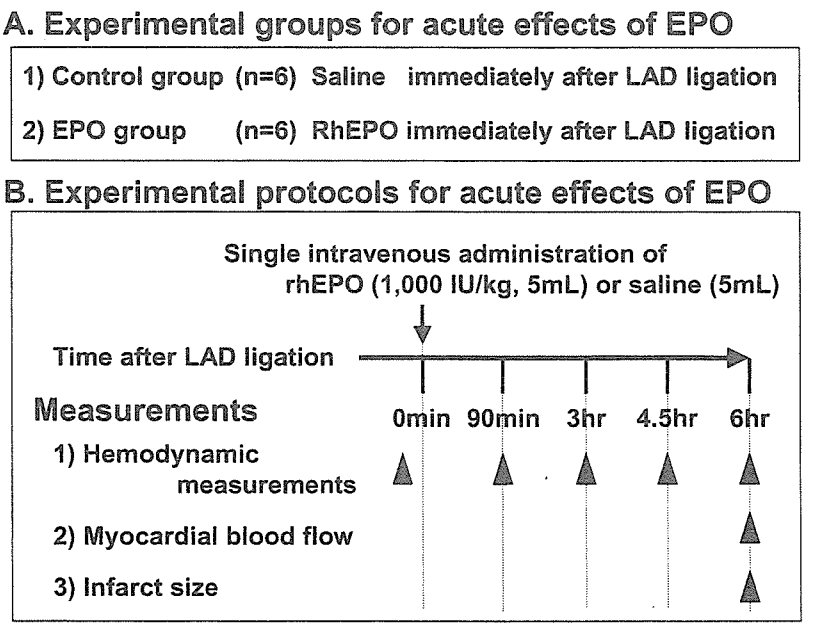

Figure 1. Experimental protocols to investigate acute effects of erythropoietin (EPO) on myocardial infarct size. LAD = left anterior descending coronary artery; RhEPO = recombinant human erythropoietin.

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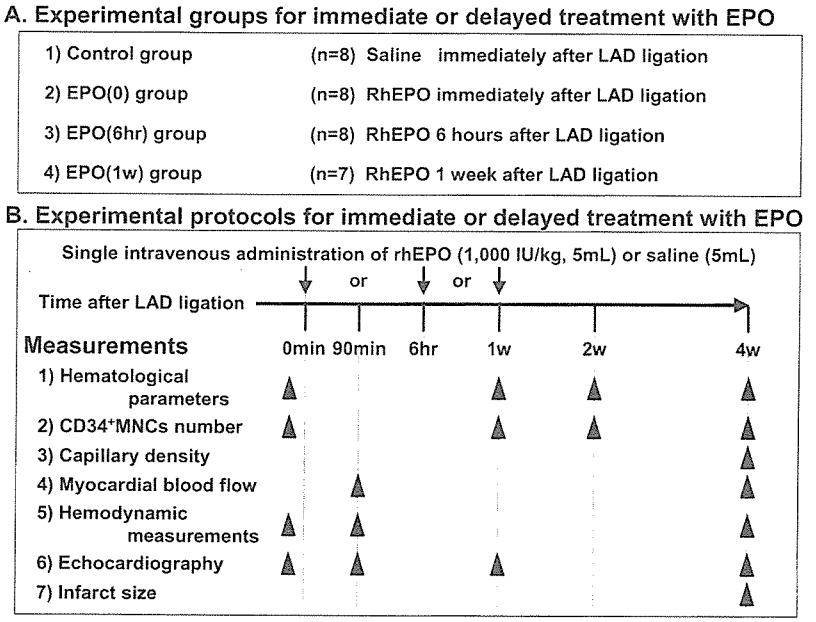

Figure 2. Experimental protocols to investigate effects of immediate or delayed treatment with erythropoietin (EPO) on neovascularization and cardiac function. CD34+MNC = CD34-positive mononuclear cell; other abbreviations as in Figure 1.

MNCs, the number of CD34+MNCs with low cytoplasmic granularity (low sideward scatter) was quantified and expressed as the number of cells per 1-μl blood sample.

In vitro MNC culture assay. Circulating MNCs were isolated from blood (10 ml) of dogs at baseline and 1 week after MI in the control and EPO(0) groups (n = 4 each) by Ficoll density-gradient centrifugation. After MNCs (107 per well) were plated in Medium 199 (Gibco, Grand Island, New York) supplemented with 20% fetal calf serum and antibiotics on human fibronectin-coated six-well dishes. After 7 days in culture, adherent cells were stained for the uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (DiI-ac-LDL) (Biomedical Technologies, Stoughton, Massachusetts) and the binding of fluorescein isothiocyanate-labeled *Ulex europaeus* agglutinin I (UEA-I) (Vector Laboratories, Peterborough, England). Double-staining cells were quantified by examining five random microscopic fields (×200 power) (10,11).

Histologic assessments. Four weeks after MI, myocardial tissue was sampled from both ischemic (LAD) and non-ischemic (left circumflex coronary artery [LCX]) regions in each group. The tissues in the ischemic region were identified as the edge of the region showing necrosis. These samples were then fixed in 10% buffered formalin, embedded in paraffin, and serially sectioned in the frontal plane at 5-μm thickness. Endothelial cells were immunohistologically stained using rabbit antihuman von Willebrand factor antibody (Dako, Kyoto, Japan) and the Envision+/HRP Kit (Dako) (17). The peroxidase was visualized by incubation with 3,3'-diaminobenzidine, followed by incubation with a DAB-enhancing solution (Dako). We counted the numbers of capillaries and cardiomyocytes in 20 random high-power fields (×400 power), and then calculated the average capillary density and capillary-to-myocyte ratio (18).

Measurements of regional MBF. Regional MBF was determined as described previously (19). Nonradioactive microspheres (Sekisui Plastic Co., Tokyo, Japan) made of inert plastic were labeled with bromine or niobium. Microspheres were administered at 90 min and 4 weeks after MI. The MBF in the LAD region was calculated according to the following formula: time flow = (tissue count) × (reference flow)/(reference count), and was expressed in ml/g wet weight/min.

Hemodynamic measurements. Hemodynamic parameters, such as arterial mean blood pressure (ABP), heart rate (HR), and left ventricular end-diastolic pressure (LVEDP), were measured at the time points indicated in Figure 2. A 5-F sidearm sheath (Radifocus, Terumo, Tokyo, Japan) was placed in the right femoral artery for hemodynamic measurements. A 4-F pigtail catheter (Outlook, Terumo) was placed in the LV for measurement of LVEDP and was connected to a pressure transducer (model DX-200, Nihon Kohden, Tokyo, Japan). The ABP and HR were monitored via the 5-F sidearm sheath.

Echocardiography. Cardiac function was assessed by echocardiography (Sonos 5500, S4-probe, 2-4 MHz, Philips, Bothell, Washington) at the time points indicated in Figure 2. Short-axis views were recorded at the level of midpapillary muscles, and two-dimensional and M-mode views were recorded at the same level. Measurements of left ventricular end-diastolic dimension (LVEDD) and LV ejection fraction were obtained from M-mode views. All measurements were made by one observer, who was blinded with respect to the identity of the tracings.

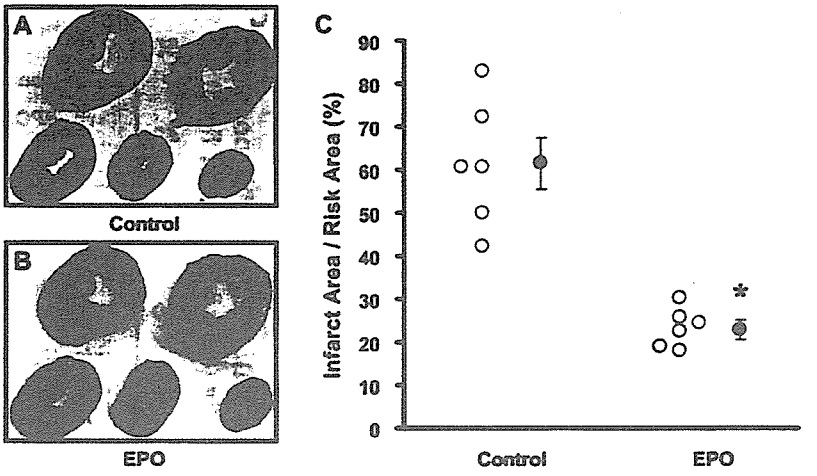

Figure 3. Representative left ventricular cross sections at 6 h after myocardial infarction (MI) in dogs with **(B)** and without **(A)** erythropoietin (EPO) treatment. **(C)** Infarct size at 6 h after MI. *p < 0.05 vs. the control group. **Open circles** = infarct size in each animal.

Infarct size 4 weeks after MI. Myocardial infarct area was determined at the end of the protocol by triphenyltetrazolium chloride staining as described previously (14). Infarct size was expressed as a percentage of the total LV area.

Statistical analysis. Results are expressed as the mean ± standard error of the mean. Comparisons of the time course of the change between groups were performed using two-way repeated measures analysis of variance. Comparisons of other data between groups were performed using one-way factional analysis of variance. If statistical significance was found for a group, a time effect, or a group-by-time interaction, further comparisons were made with paired *t* tests between all possible pairs of four groups at individual time points. The Bonferroni-Holm procedure was used for correction of multiple comparisons. A p value <0.05 was considered to represent statistical significance (20).

RESULTS

Exclusion. Four dogs [acute effects protocol; control: 1, EPO: 0, delayed treatment effects protocol; control: 1, EPO(0): 1, EPO(6h): 0, EPO(1wk): 1] were excluded from analysis because of excessive regional MBF (>15 ml/100 g/min). Thus, 12 and 31 dogs in acute and delayed EPO treatment protocols, respectively, were included.

Acute effects of EPO on infarct size. Myocardial infarct size was significantly smaller in animals receiving EPO compared with those that received saline, but there was no significant difference in regional MBF (9.0 ± 1.0 ml/100 g/min vs. 8.5 ± 1.2 ml/100 g/min) or area at risk (42.9 ± 2.3% vs. 42.3 ± 0.9%) when comparing the two groups (Fig. 3).

Effects of EPO on hematologic parameters. The average change in hematologic parameters was not different when comparing the three EPO-treated groups and the control group over the 4-week experimental protocol (Table 1).

Plasma VEGF levels. Table 2 shows the time course of changes in plasma VEGF level after MI. The plasma VEGF level was significantly and comparably elevated in both control and EPO(0) groups, peaking on 6 h after MI, and returned to baseline at 1 week after MI.

Circulating CD34+MNCs and in vitro cultured MNCs. Figure 4A shows the time course of changes in circulating CD34+MNC number in the different groups. One week after MI, the number of circulating CD34+MNCs increased in all groups. Furthermore, the number of circulating CD34+MNCs at 1 week after MI was higher in the EPO(0) and EPO(6h) groups than in either control or EPO(1wk) group. Two weeks after MI, the number of CD34+MNCs in the control group returned to the baseline. By contrast, the number of CD34+MNCs in the EPO(0) and EPO(6h) groups also decreased but still remained higher than those in either the control or

Table 1. Time Course of Changes in Hematologic Parameters

Parameters	Baseline	1 Week	2 Weeks	4 Weeks
Hematocrit (%)				
Control	52.9 ± 1.7	47.0 ± 1.6	48.9 ± 2.3	53.1 ± 1.8
EPO(0)	52.4 ± 1.1	48.2 ± 1.2	47.9 ± 1.4	53.4 ± 0.7
EPO(6h)	51.5 ± 1.6	49.3 ± 1.6	51.4 ± 1.1	51.3 ± 2.3
EPO(1wk)	48.9 ± 1.0	46.4 ± 1.1	49.4 ± 0.5	50.1 ± 1.0
WBC ($10^3/\mu l$)				
Control	13.8 ± 0.4	15.4 ± 1.4	15.3 ± 0.9	13.5 ± 0.9
EPO(0)	12.6 ± 0.6	14.0 ± 1.1	14.4 ± 0.3	12.3 ± 1.4
EPO(6h)	12.6 ± 0.8	15.6 ± 1.1	13.9 ± 1.0	12.0 ± 0.8
EPO(1wk)	13.1 ± 0.8	14.8 ± 1.2	13.3 ± 0.4	12.9 ± 0.8
Platelet ($10^4/mm^3$)				
Control	27.3 ± 2.0	26.5 ± 1.9	28.4 ± 1.2	26.2 ± 2.0
EPO(0)	28.5 ± 2.0	26.8 ± 4.3	27.0 ± 3.4	28.2 ± 1.8
EPO(6h)	26.9 ± 0.9	27.0 ± 1.4	26.1 ± 1.8	26.1 ± 1.5

Data are presented as mean ± SEM (n = 7 to 8).
EPO = erythropoietin; WBC = white blood cell.

Table 2. Time Course of Changes in Plasma VEGF Levels

Groups	n	Baseline	6 Hours	1 Week	2 Weeks
VEGF (pg/ml)					
Control	4	<9.0	22.5 ± 3.3*	<9.0	<9.0
EPO(0)	4	<9.0	21.6 ± 5.0*	<9.0	<9.0

Data are presented as mean ± SEM. *p < 0.05 vs. baseline.
EPO = erythropoietin; VEGF = vascular endothelial growth factor.

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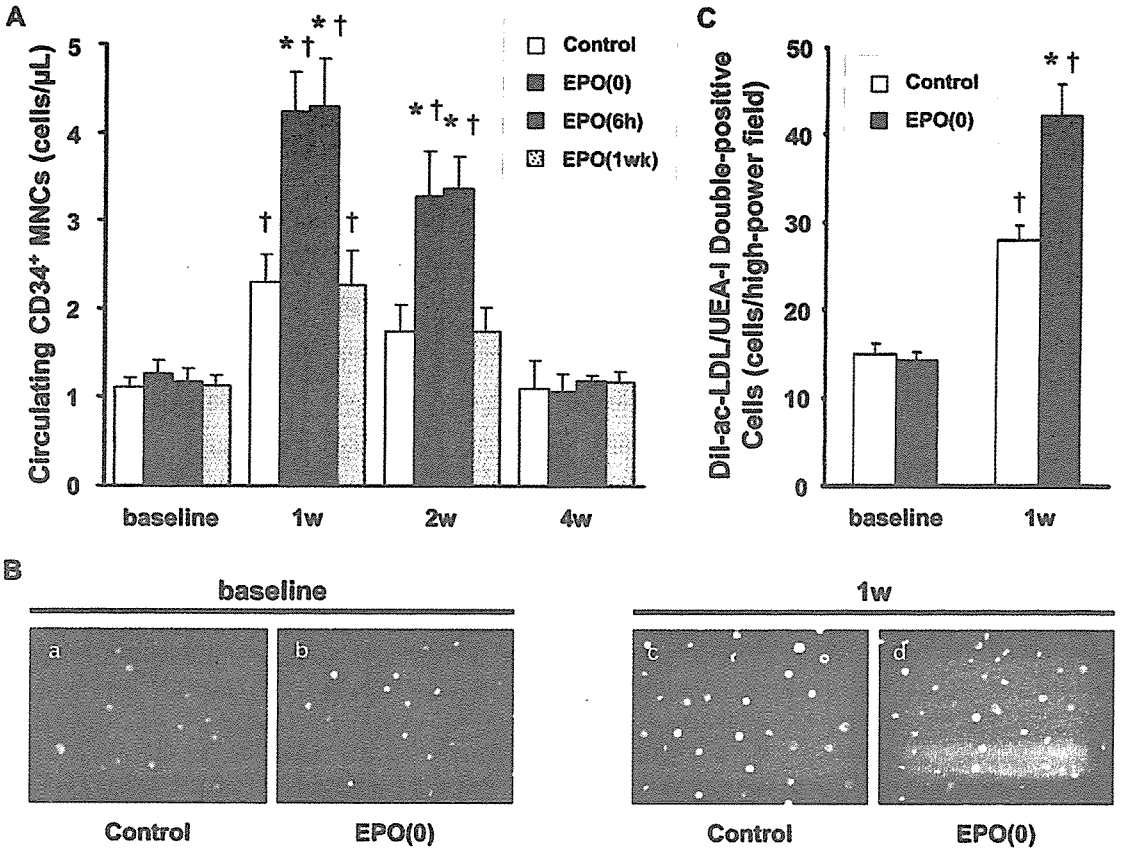

Figure 4. **(A)** Time course of changes in circulating CD34+MNC count after left anterior descending coronary artery (LAD) ligation in different experimental groups. **(B)** Representative images of double-stained cultured cells (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated low density lipoprotein [Dil-ac-LDL] and *Ulex europaeus* agglutinin I [UEA-I]) at baseline **(a, b)** and 1 week after LAD ligation **(c, d)** from dogs with and without erythropoietin (EPO) treatment immediately after LAD ligation. **(C)** Quantitative analysis of endothelial progenitor cell culture assay. *$p < 0.05$ vs. the control group. †$p < 0.05$ vs. baseline.

EPO(1wk) group. Furthermore, the administration of EPO 1 week after the LAD ligation did not affect the number of CD34+MNCs at any given time point.

In the culture assay of MNCs, the number of Dil-ac-LDL/UEA-I double-positive cells obtained from blood 1 week after MI increased compared with that at baseline in both control and EPO(0) groups. Importantly, the double-positive cell number obtained from blood 1 week after MI in the EPO(0) group was significantly higher than in the control group (Figs. 4B and 4C).

Capillary density and regional MBF. Figure 5A shows the representative immunohistologic findings in the non-ischemic (panels a to d) and ischemic (panels e to h) regions at 4 weeks after MI. In the nonischemic region, there was no difference in the capillary density and capillary-to-myocyte ratio when comparing groups. In the ischemic region, the capillary-to-myocyte ratio as well as capillary density was significantly higher in the EPO(0) and EPO(6h) groups, but not in the EPO(1wk) group, than in the control group (Figs. 5B to 5C).

Figure 6 shows the changes in regional MBF in the ischemic regions in different experimental groups. There was no significant difference in MBF at 90 min when comparing experimental groups. At 4 weeks after MI, MBF was more increased in the EPO(0) and EPO(6h) groups, but not in the EPO(1wk) group, than in the control group.

Effects of immediate or delayed EPO treatment on cardiac function and infarct size. Throughout the experimental protocols, there was no difference in either ABP or HR when comparing the groups (Table 3).

Figure 7 shows the time course of changes in LVEF (panel A), LVEDD (panel B), and LVEDP (panel C) in different experimental groups. There were no significant differences in baseline LVEF, LVEDD, and LVEDP when comparing the groups.

Ninety minutes, 1 week, and 4 weeks after MI, LVEF was higher in the EPO(0) group than in the other groups. Ninety minutes and 1 week after MI, there was no difference in LVEF when comparing the EPO(6h) group and the control group. When comparing the time points of 1 week and 4 weeks after MI, LVEF decreased in the control and the EPO(1wk) groups but not in the EPO(6h) group. One and 4 weeks after MI, LVEDD was lower in the EPO(0) group than in the other groups. When comparing the time points of 1 week and 4 weeks after MI, LVEDD increased in the control and EPO(1wk) groups but not in the EPO(6h) group. Ninety minutes after MI, LVEDP was lower in the

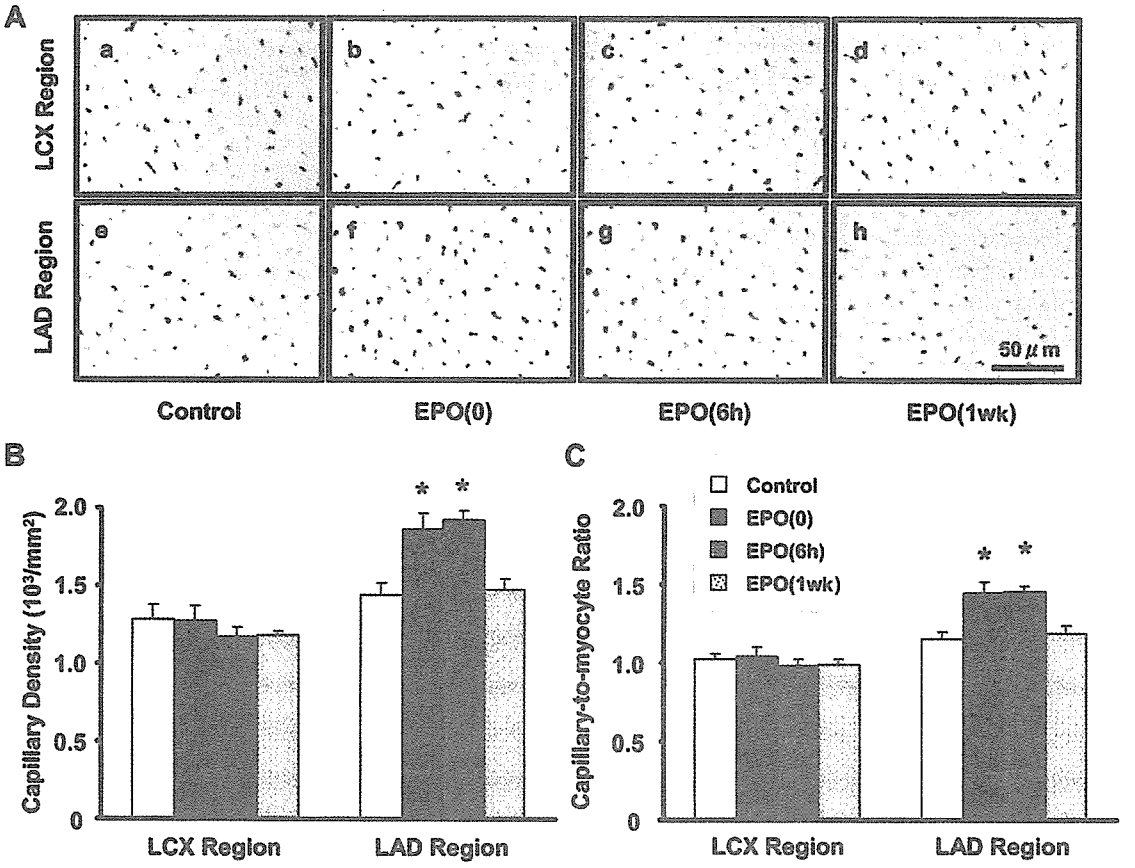

Figure 5. **(A)** Representative immunohistologic staining with an antibody against von Willebrand factor in nonischemic (left circumflex coronary artery [LCX]) **(a, b, c, d)** and ischemic (left anterior descending coronary artery [LAD]) **(e, f, g, h)** regions in different experimental groups. Capillary density **(B)** and capillary-to-myocyte ratio **(C)** of nonischemic (LCX) and ischemic (LAD) regions in different experimental groups. *$p < 0.05$ versus the control group. Abbreviations as in Figure 1.

EPO(0) group than in the other groups. Four weeks after MI, LVEDP was lower in the EPO(0) and EPO(6h) groups than in either the control or the EPO(1wk) group.

Myocardial infarct size 4 weeks after MI was smaller in the EPO(0) group than in the control group, although EPO treatment, initiated 6 h and 1 week after MI, did not reduce infarct size (Fig. 7D).

DISCUSSION

The present study showed that EPO administered 6 h after LAD ligation increased circulating CD34+MNCs, capillary density, MBF in the ischemic region, and prevented the worsening of cardiac function without reducing infarct size. The EPO enhances neovascularization, likely via EPC mobilization, and improves cardiac dysfunction in the chronic phase, although EPO has time-window limitations.

We showed that the EPO treatment immediately after the LAD ligation reduced infarct size, which is consistent with observations of previous reports (5–8). Because the infarct size-limiting effects of EPO appear rapidly, the nonerythroid effects of EPO, such as antiapoptosis and radical scavenging (4–8), may contribute to the reduction of infarct size.

Recent reports have shown that circulating CD34+MNC count correlated with EPC number in MNCs culture assay, and both increased at 1 to 2 weeks after EPO administration in animals and humans (9–11). In the culture assay, the number of Dil-ac-LDL/UEA-I double-positive cells obtained from blood at baseline did not differ between the two groups. The number of double-positive cells obtained from blood at 1 week after MI significantly increased compared with that at baseline in the control and EPO(0) groups. Further, the double-positive cell number obtained from blood in the EPO(0) group was higher than in the control group. These findings suggest that EPO augments increases in the number of MNCs that can differentiate into Dil-ac-LDL/UEA-I double-positive cells, an indicator of endothelial cells. Increases in the number of both CD34-positive cells and Dil-ac-LDL/UEA-I double-positive cells strongly suggest that EPO promotes EPC mobilization. The number of CD34+MNCs increased 1 week after MI in the canine model, which is consistent with observations from studies of patients with acute MI (21,22). Furthermore, the number of CD34+MNCs was higher in the EPO(0) and EPO(6h) groups than in the control group. This finding suggests that a single dose of EPO was effective in increasing the number of circulating EPCs after MI. Interestingly,

EPO administered 1 week after MI failed to produce the identical effect, suggesting that EPO has a time window for promotion of EPC mobilization. We found that plasma VEGF levels were elevated, peaking at 6 h after MI and returned to the baseline 1 week after MI. The EPO did not affect plasma VEGF levels. Because both VEGF and EPO are known to promote EPC mobilization in experimental conditions and are independent predictors for the number of circulating EPCs in patients with coronary heart disease (9–11,23), they may additionally or synergistically contribute to EPC mobilization. Thus, it is likely that EPO alone, at least at the dose used in the present study, might not be enough to promote CD34+MNC mobilization 1 week after MI when VEGF returns to the baseline. Although we only investigated the low dose of EPO to consider the clinical implication, it is possible that high doses of EPO would show the different results. Further investigations are needed to clarify the mechanism of EPO-stimulated EPC mobilization.

The present study also showed that EPO increased capillary-to-myocyte ratio corrected for LV hypertrophy as well as capillary density in the EPO(0) and EPO(6h) groups, suggesting that EPO promotes the neovascularization in the ischemic region. Investigators have also reported that EPO enhances neovascularization in the ischemic region in the hind-limb occlusion model (9). As suggested in the present study, EPO may enhance neovascularization via EPC mobilization. Indeed, bone marrow-derived EPCs incorporate into foci of neovascularization at the border zone of MI (12,13), and administration of ex vivo-expanded EPCs resulted in increased myocardial neovascularization (24,25). In a rat stroke model, Wang et al. (26) showed that EPO treatment, initiated 24 h after MI, enhances angiogenesis. In addition, van der Meer et al. (27) showed that capillary density was increased in the rat post-MI model even when EPO was administered 3 weeks after MI. In contrast, we showed that EPO administered 1 week after MI failed to increase capillary density. The possible explanation for this discrepancy is attributable to the different doses of EPO used. In the studies by Wang et al. (26) (5,000 IU/kg for 7 days) and van der Meer et al. (27) (8,000 IU/kg every 3 weeks), relatively high doses of EPO were administered. In contrast, in the present study, a relatively low dose (1,000 IU/kg) of EPO was administered with a single injection, and the reason for this dose in the present study is for the possible translation of our results to clinical settings more easily (6), because 8,000 or 5,000 IU/kg EPO may cause side effects. On the other hand, we noticed that a higher dose of EPO would increase capillary density and improve the cardiac function even by the late administration of EPO for clinical use.

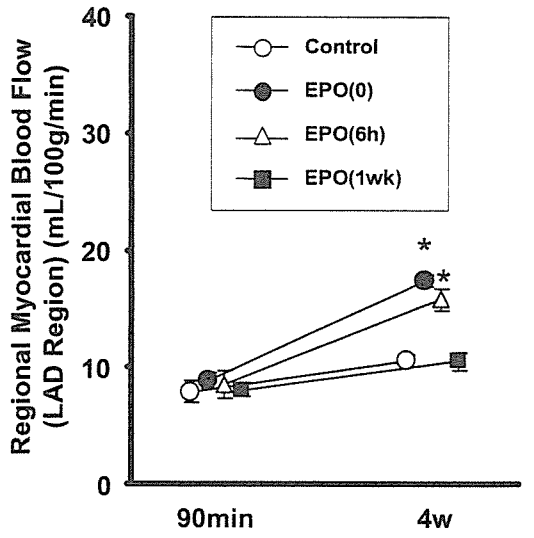

Figure 6. Regional myocardial blood flow in the ischemic (left anterior descending coronary artery [LAD]) region 90 min and 4 weeks after myocardial infarction in different experimental groups. *$p < 0.05$ versus the control group. EPO = erythropoietin.

Table 3. Time Course of Changes in Hemodynamic Parameters

Parameters	Baseline	90 Min	4 Weeks
ABP (mm Hg)			
Control	99 ± 3	101 ± 3	103 ± 2
EPO(0)	102 ± 3	99 ± 3	102 ± 2
EPO(6h)	101 ± 1	98 ± 2	101 ± 1
EPO(1wk)	102 ± 2	102 ± 3	103 ± 2
HR (per min)			
Control	131 ± 6	135 ± 6	129 ± 6
EPO(0)	128 ± 6	131 ± 3	131 ± 5
EPO(6h)	130 ± 7	135 ± 7	126 ± 6
EPO(1wk)	128 ± 6	128 ± 3	126 ± 6

Data are presented as mean ± SEM (n = 7 to 8).
ABP = arterial mean blood pressure; EPO = erythropoietin; HR = heart rate.

In the present study, MBF in the ischemic region was increased in both the EPO(0) and the EPO(6h) groups. Because neovascularization was also enhanced in these groups, increased MBF may occur secondary to the enhanced neovascularization.

The present study also showed that an administration of EPO immediately after the LAD ligation improved cardiac function at 90 min after MI, likely because of infarct size reduction, and subsequently prevented the development of cardiac dysfunction in the chronic phase. Because the previous reports showed that myocardial necrosis progresses within 6 h after the onset of MI (28,29), EPO was administered at time points of 6 h and later after LAD ligation to determine whether its activity is directed toward the acute phase of MI or the chronic phase of cardiac dysfunction. One week after MI, LVEF, LVEDD, or LVEDP was similar among the EPO(6h), EPO(1wk), and control groups. However, EPO administered 6 h, but not 1 week, after the LAD ligation improved cardiac dysfunction 4 weeks after MI when compared with the control group. Because we did not find any difference in infarct size at 4 weeks after MI between the EPO(6h) and the EPO(1wk) groups, the improvement of cardiac function in the EPO(6h) group was not attributable to the reduction of infarct size, but to the increased blood flow to the ischemic regions.

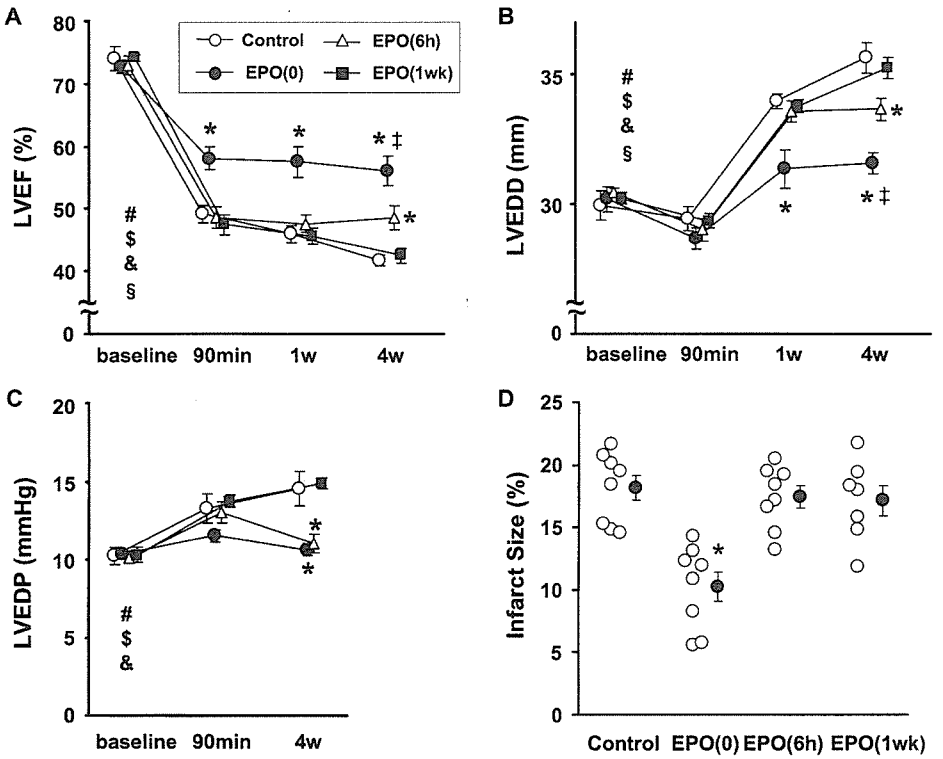

Figure 7. The time course of changes in left ventricular ejection fraction (LVEF) **(A)**, left ventricular end-diastolic dimension (LVEDD) **(B)**, and left ventricular end-diastolic pressure (LVEDP) **(C)** in different experimental groups. Statistically significant ($p < 0.05$) group-by-time interactions (analysis of variance for repeated measurements) are indicated by the following: # = all groups; $ = control × EPO(0) group; & = control × EPO(6h) group; § = EPO(0) × EPO(6h) group. **(D)** Infarct size at 4 weeks after myocardial infarction in different experimental groups. **Open circles** = infarct size in each animal. *$p < 0.05$ versus the control group. EPO = erythropoietin.

In conclusion, in addition to its acute effect on infarct size reduction, EPO may exert chronic cardioprotective effects through neovascularization and may be a useful adjunct for the treatment of patients with myocardial infarction.

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