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Endogenous erythropoietin system in non-hematopoietic lineage cells plays a protective role in myocardial ischemia/reperfusion

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Abstract

Objective: Recent studies suggested that erythropoietin (Epo) receptors (EpoR) are expressed not only in the hematopoietic lineage cells but also in the heart and that the administration of recombinant human Epo elicits protective effects in myocardial ischemia and reperfusion (I/R). We tested our hypothesis that endogenous Epo signals mediated by EpoR expressed in the non-hematopoietic lineage cells play a protective role against myocardial I/R injury.
Methods: Transgene-rescued EpoR null mutant mice (RES), which express EpoR exclusively in the hematopoietic lineage cells, were subjected to 30 min left coronary artery occlusion followed by reperfusion.
Results: Hematocrit, heart rate, blood pressure, heart weight, and echocardiographic parameters were comparable between wild-type mice (WT) and RES under the baseline condition. After 24 h of reperfusion, the infarct size in RES with I/R (RES/MI) was larger than that in WT/MI. Caspase-3 activity and number of TUNEL-positive cardiomyocytes in the ischemic area were increased in RES/MI compared with WT/MI. The extents of p38 and JNK phosphorylations in the ischemic area were significantly increased in WT/MI, but not in RES/MI as compared with corresponding sham-operated mice. Plasma Epo concentration in RES/MI did not differ from that in sham-operated RES, while that in WT/MI was peaked at 24 h post I/R. Additionally, left ventricular (LV) end-diastolic diameter was increased and LV fractional shortening tended to be reduced in the RES/MI compared with WT/MI at 21 days after I/R.
Conclusions: These results suggest that the endogenous Epo–EpoR system in the non-hematopoietic lineage cells plays an important protective role against myocardial I/R injury.
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Keywords: Apoptosis; Cytokines; Ischemia; Remodeling; MAP kinase

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1. Introduction

Erythropoietin (Epo), which is produced in the adult kidney and fetal liver in mammals, promotes the proliferation and differentiation of erythroid progenitor cells by preventing apoptosis [1]. In this process, the Epo–Epo receptor (EpoR) signal is mediated by JAK-STAT, MAPK, and PI3K-Akt [2–4]. Recently, Epo mRNA or protein has been shown to be expressed not only in the kidney but also in the testis, liver, central nervous system and the female reproductive organs [5]. Furthermore, it has been reported that EpoR are expressed not only in hematopoietic cells but also in other non-hematopoietic cells [6–8] including cardiomyocytes [9–11]. The role of Epo in non-hematopoietic cells include the promotion of endothelial

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cell proliferation [6] and the amelioration of neuronal recovery from injury [12,13]. More recent studies have demonstrated that the administration of recombinant human Epo (rhEpo) elicits cardioprotective effects in myocardial ischemia and reperfusion (I/R) [10,11,14–16]. Furthermore, we have recently demonstrated that a high serum level of endogenous Epo can predict a smaller infarct size in patients with acute myocardial infarction subjected to successful primary percutaneous coronary intervention [17], suggesting that endogenous Epo–EpoR signals play a protective role against I/R injury in humans. However, it is unclear whether the beneficial effects of Epo in myocardial I/R can be attributed to Epo–EpoR signaling in non-hematopoietic cells. In the present study, we tested our hypothesis that endogenous Epo signals mediated by EpoR expressed in the non-hematopoietic lineage cells play a protective role against myocardial I/R injury using transgene-rescued EpoR null mutant mice (RES), which express EpoR exclusively in the hematopoietic lineage cells [18].

2. Materials and methods

The present study conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Ethics Committee on Animal Experiments of Tohoku University School of Medicine.

2.1. Animals

EpoR null mouse embryos die approximately on embryonic day 13 due to severe anemia caused by the failure of erythropoiesis in the fetal liver [19]. Therefore, we analyzed transgene-rescued EpoR null mutant mice (RES). These mice possess the transgene that drives the EpoR mRNA expression only in hematopoietic cells with the activity of the hematopoietic regulatory domain of GATA-1 gene. RES develop normally and are fertile despite the lack of EpoR expression in non-hematopoietic cells. The detailed procedures to generate the RES have been described previously [8,18]. We employed the RES line that expresses approximately 40% of the wild-type EpoR level in the erythroid cells. A total of 139 RES and 115 wild type mice (WT) were tested in the present study.

2.2. Measurements of hematocrit, plasma Epo concentration, and blood pressure

Immediately after thoracotomy and before harvesting the heart, blood samples were collected under anesthesia from the right ventricular cavity using a 26-gauge needle to determine the hematocrit (a hematocrit centrifuge) and plasma Epo concentration (a radioimmunoassay kit, Nippon DPC Corporation, Chiba, Japan). Systolic blood pressure and heart rate under baseline conditions were measured using a non-invasive computerized tail-cuff system (MK-2000, Muromachi Kikai, Tokyo, Japan).

2.3. Mouse model of myocardial I/R injury

Left coronary artery occlusion and reperfusion were performed by a modification of the procedure previously described [20]. Male mice (8–12-week-old, 24–30 g) were anesthetized with an intraperitoneal injection of pentobarbital sodium (0.04 mg/g body weight) and were ventilated using an endotracheal tube and a ventilator. After left thoracotomy, the pericardium was opened, and the proximal portion of the left coronary artery was ligated using an 8-0 nylon thread. Myocardial ischemia was confirmed by the discoloration of the heart and typical ECG changes. After 30 min occlusion, the left coronary artery was reperfused by loosening the ligature, and the thorax was closed. In sham-operated mice (SHAM), the pericardium was opened, but the coronary artery was not ligated.

2.4. Assessment of infarct size at 24 h after I/R

The infarct size was assessed using triphenyltetrazolium chloride (TTC) staining. After the 24-h reperfusion, mice were anesthetized and ventilated. After re-thoracotomy, the left coronary artery was ligated again using the thread that had been loosened after the 30-min ischemia. Evans blue dye (0.2 mL of the 2.0% solution) was infused into the inferior vena cava to determine the non-ischemic area. The heart was then excised, washed in PBS, and sliced into 1-mm-thick cross sections. After the ex-vivo incubation in 1.0% TTC solution for 30 min at 37 °C, the cross sectional images of the left ventricle (LV) were stored as digital images. The sizes of the infarct area, risk area, and the total LV area were determined using computer-assisted planimetry to calculate the area at risk/total LV area (% area at risk) and infarct size/area at risk (% infarct size) ratios [20].

2.5. TUNEL and caspase-3 activity assays

After 6 or 24 h of reperfusion, the hearts were excised, fixed with formalin, and embedded in paraffin. We then prepared 1.5-μm-thick sections taken perpendicular to the long axis of the LV from the mid-ventricular level. TUNEL assay was performed using an apoptosis detection kit (ApopTag, CHEMICON international, Inc., Temecula, CA) followed by methyl green counterstaining. The number of TUNEL-positive cells was counted in 9 random fields in each heart at a magnification of $\times 400$, which were selected from the ischemic area.

Caspase-3 activity was determined using a caspase-3 colorimetric activity assay kit (CHEMICON international, Inc., Temecula, CA) by means of the detection of

chromophore *p*-nitoroaniline cleaved from labeled substrate Ac-DEVD-*p*-nitroaniline. After 3 h of reperfusion, the hearts were excised and divided into the ischemic and non-ischemic areas. The fresh myocardium was homogenized in the lysis buffer. Aliquots of the protein lysates were then reacted with an equal volume of 3 mg/mL Ac-DEVD-*p*-nitroaniline for 2 h at 37 °C. Caspase-3 activity was assessed in a microtiter plate reader at 405 nm and was calculated using the calibration curve obtained with the known concentrations of *p*-nitroaniline [21].

2.6. Western blotting

The LV isolated following 1-h reperfusion was divided into the ischemic and non-ischemic areas, homogenized with lysis buffer containing 250 mmol/L sucrose, 0.1 mol/L Tris–HCl (pH8.0), 1 mmol/L EDTA, 3 mmol/L DTT, 1% Triton X, protease inhibitor cocktail (1:100) (Sigma, Saint Louis, Missouri) and phosphatase inhibitor cocktail (1:100) (Sigma), and centrifuged at 1000×*g* for 10 min at 4 °C. The supernatants were collected, and 50 μg of protein derived from both ischemic and non-ischemic tissues were subjected to 7.5% or 10% SDS-PAGE immunoblotting using antibodies for STAT1, phospho-STAT1 (Tyr701), STAT3, phospho-STAT3 (Tyr705), phospho-STAT5 (Tyr694), Akt, phospho-Akt (Ser473), p38MAPK, phospho-p38MAPK (Thr180/Tyr182), phospho-JNK (Thr183/Tyr185) (Cell Signaling Technology, Inc. Beverly, MA), STAT5, and JNK (SantaCruz). The immunoreactive bands were detected by an ECL or ECL advance kit (Amersham).

2.7. Echocardiography, histological examination, and survival

Transthoracic echocardiographic study was performed using an echocardiographic system equipped with a 12-MHz phased-array transducer (Aplio, Toshiba, Otawara, Japan). Mice were adequately acclimated, and the measurements were performed in the conscious state. LV end-diastolic dimension (LVDd) and LV fractional shortening (LVFS) were measured using M-mode echocardiography, which was assisted by a two-dimensional LV short axis image at the mid-papillary level [22].

The hearts were sectioned into three 1-mm-thick slices taken perpendicular to the long axis of the LV. The slices were fixed in neutral buffered 10% formalin, and embedded in paraffin. The sections (1.5-μm-thick) were cut from each slice and stained with hematoxylin–eosin, Masson-trichrom or Sirius-red. The infarct size at 21 days after I/R was measured in two sections from each heart, and the value was expressed as the ratio of Masson-trichrome stained area to total LV area [23].

We included 36 WT/MI and 23 RES/MI to determine whether the survival rate at 6 weeks after I/R differs between the two groups.

2.8. Statistical analysis

Data are expressed as mean±S.E.M. Differences between the groups were evaluated using unpaired Student's *t*-test or one-way ANOVA followed by Bonferroni correction (StatView version 4.5, SAS Institute, San Francisco, CA). Survival was assessed by the standard Kaplan–Meier analysis with log-rank test. A value of $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Characteristics of animals at basal condition

Characteristics of WT and RES at the age of 8–12 weeks are shown in Table 1. There were no significant differences between WT and RES with regard to body weight, heart rate, systolic blood pressure, or heart weight/body weight ratio under basal condition. Although hematocrit was similar in the two groups, plasma concentration of Epo was significantly increased in the RES as compared with WT. LVDd and LVFS determined by echocardiography were comparable between the 2 strains. The microscopic characteristics of RES were also comparable to those of the WT with regard to the morphology of cardiomyocytes (hematoxylin–eosin) and interstitial fibrosis (Masson-trichrome) (Fig. 1A).

3.2. Increased infarct size after I/R

Representative TTC staining after 24 h of I/R in both the WT (WT/MI) and RES (RES/MI) is shown in Fig. 1B. The % area at risk (area at risk/total LV area×100) was comparable between WT/MI ($n=12$) and RES/MI ($n=11$) (62±3 vs. 68±2%, NS). The % infarct size (infarct size/area at risk×100) in RES/MI was significantly larger than that in WT/MI (54±3 vs. 41±3%, $P<0.01$) (Fig. 1C).

3.3. Accelerated apoptosis of cardiomyocytes after I/R

The number of TUNEL-positive cardiomyocytes was significantly increased in RES/MI as compared with WT/MI both after 6 h (31±2 vs. 24±2%, $n=6$ each, $P<0.05$) and

Table 1
Characteristics of RES at basal condition

	WT (*n*)	RES (*n*)
Body weight (g)	26.3±0.6 (15)	25.8±0.6 (15)
Heart rate (bpm)	657±24 (15)	634±30 (15)
Systolic blood pressure (mm Hg)	110±3 (15)	106±3 (15)
Heart weight/body weight (mg/g)	4.6±0.1 (10)	4.6±0.1 (10)
Hematocrit (%)	43.6±2.2 (10)	44.0±1.5 (10)
Plasma Epo concentration (mIU/mL)	20.5±2.5 (10)	40.0±5.4 (10)[a]
LV end diastolic diameter (mm)	3.2±0.1 (15)	3.2±0.1 (15)
LV fractional shortening (%)	50.4±1.4 (15)	48.8±1.3 (15)

LV, left ventricular. Values are mean±S.E.M.
[a] $P<0.01$ vs. WT.

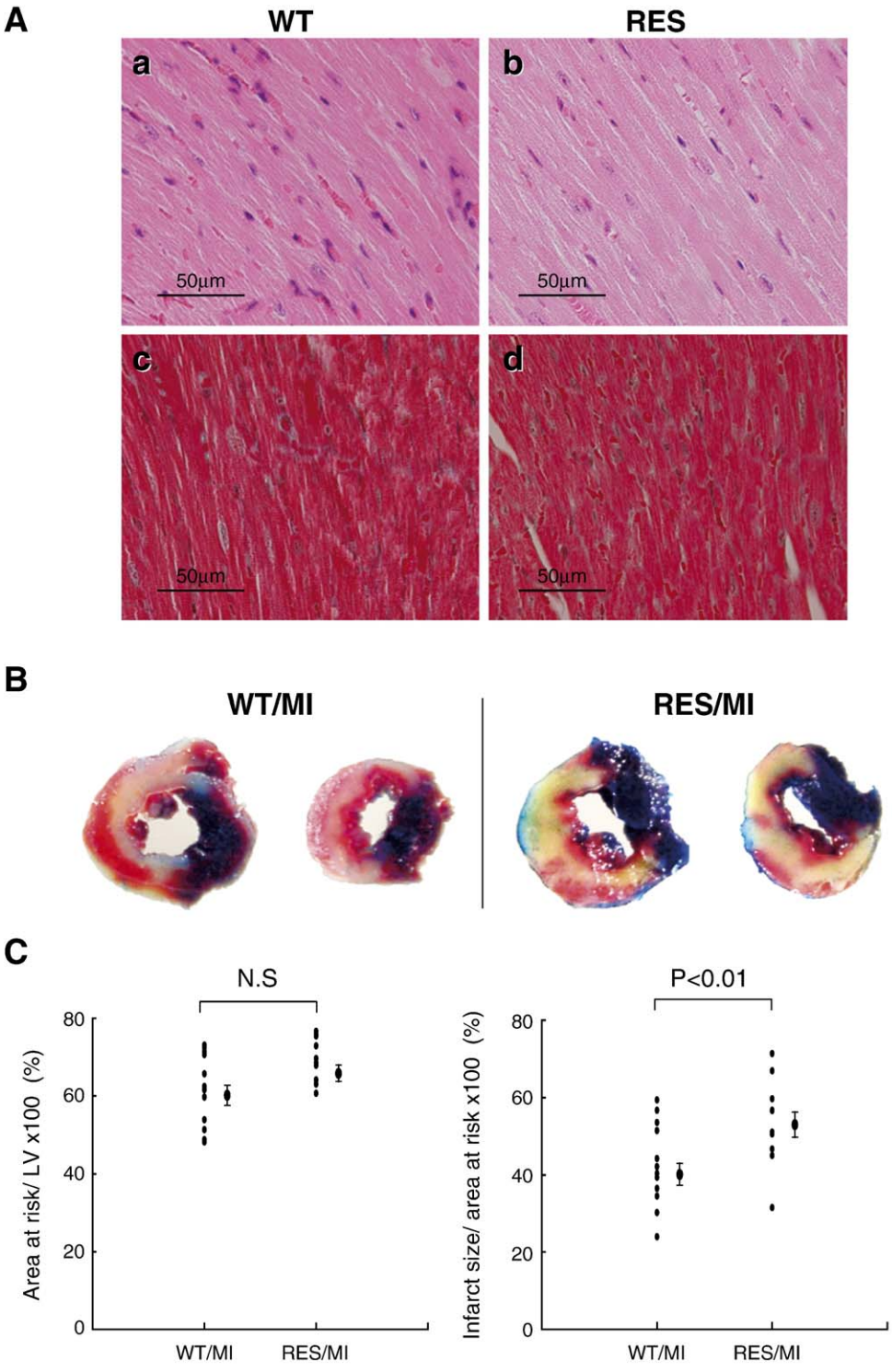

Fig. 1. A: Histological micrographs of the LV myocardium stained with hematoxylin–eosin (a, b) and Masson-trichrome (c, d) in the baseline condition in WT (a, c) and RES (b, d). These micrographs of RES show the normal morphology of cardiomyocytes (hematoxylin–eosin) with very little interstitial fibrosis (Masson-trichrome). B: Infarct size 24 h after I/R determined by TTC staining. Representative photographs of heart sections from WT/MI and RES/MI. The blue stain shows the non-ischemic area, the red stain represents viable myocardium in the ischemic area, and the infarcted tissue remains unstained (white). C: Result of infarct size 24 h after I/R. The area at risk/total LV area ratio and the infarct size/area at risk in WT/MI ($n=12$) and RES/MI ($n=11$) are shown. Mean±S.E.M.

24 h (42±4 vs. 26±2%, $n=4$ each, $P<0.005$) of reperfusion (Fig. 2A–C). The difference in the percentage of TUNEL positive cells between 6 and 24 h after I/R was statistically significant in RES/MI ($P<0.05$), but not in WT/MI. The activities of caspase-3 after the 3-h reperfusion in the ischemic areas of both WT/MI and RES/MI (128±4 and

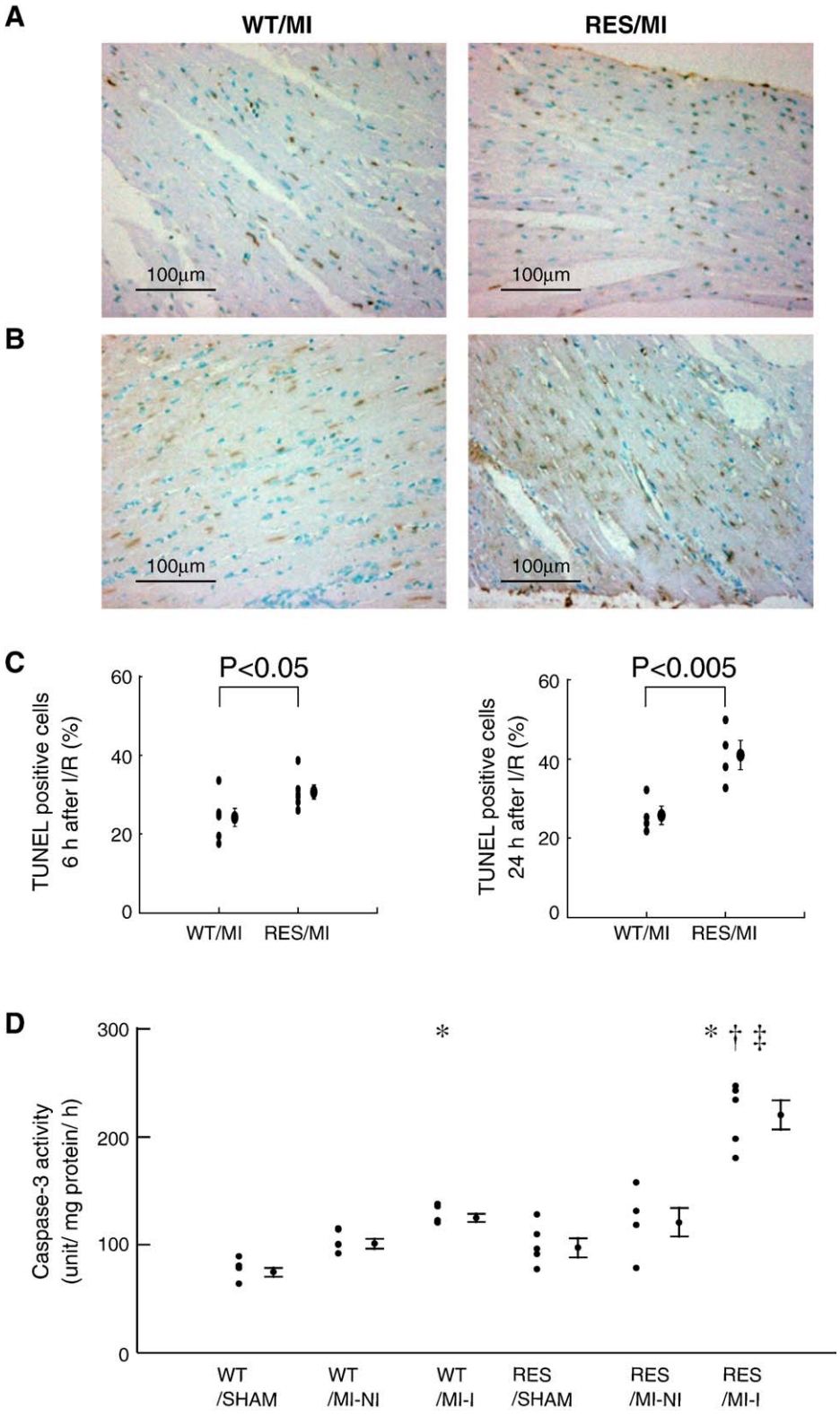

Fig. 2. A, B and C: Representative TUNEL staining in ischemic myocardium from WT/MI and RES/MI 6 h (A) and 24 h (B) after I/R. Brown staining indicates TUNEL positive nuclei. Numbers of TUNEL positive cardiomyocytes in the ischemic area in WT/MI and RES/MI at 6 h ($n=6$ for each group) and 24 h ($n=4$ for each group) after I/R are shown (C). Mean±S.E.M. D: Caspase-3 activity in the LV myocardial tissue 3 h after I/R. NI and I represent non-ischemic area and ischemic area, respectively. *$P<0.05$ vs. SHAM, $^{\dagger}P<0.05$ vs. RES/MI-NI, and $^{\ddagger}P<0.05$ vs. WT/MI-I. Mean±S.E.M. $N=5$ for each group.

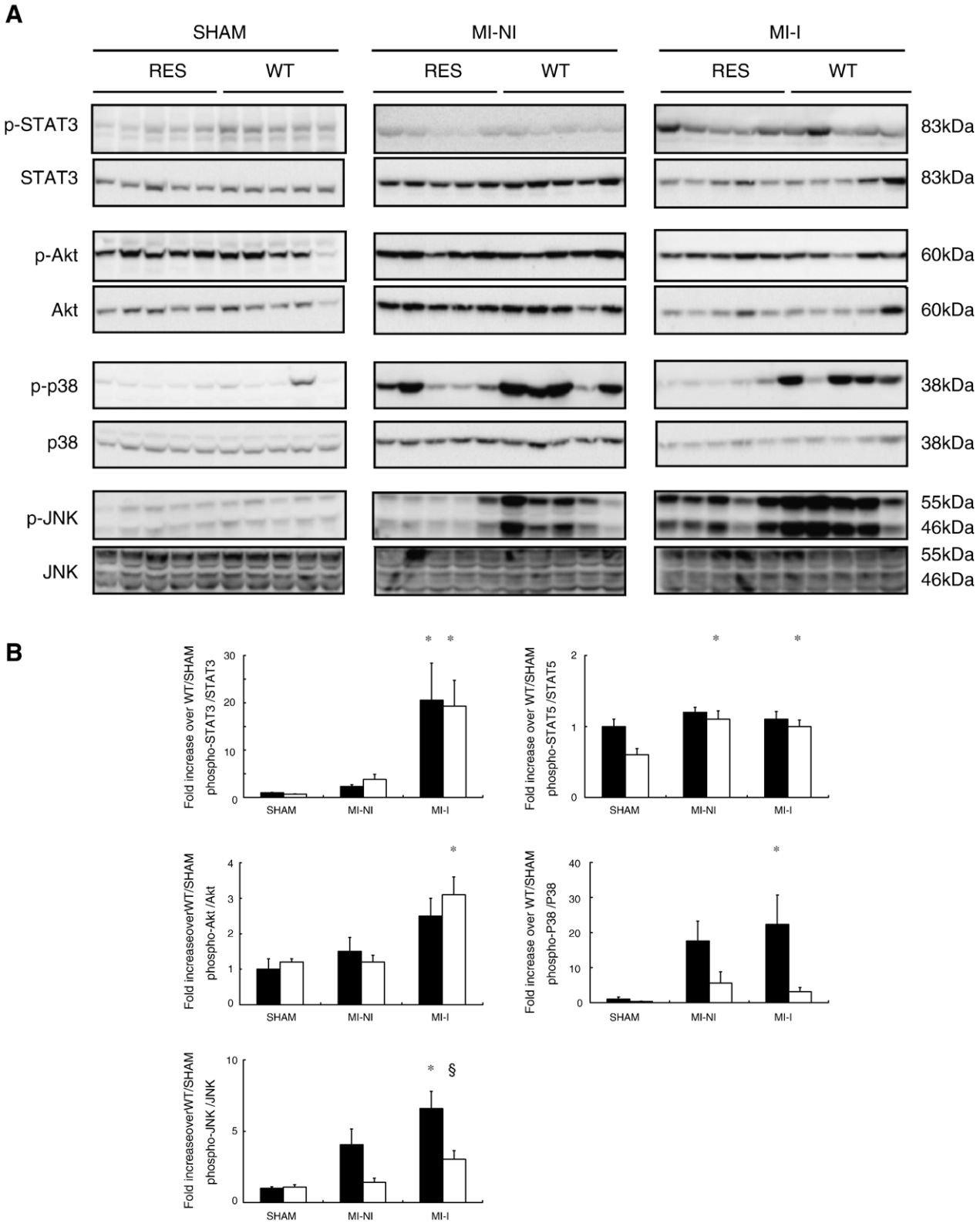

Fig. 3. A: Western blots of LV myocardial tissue from WT/MI, RES/MI and their corresponding SHAMs ($n=5$ for each group). NI and I represent the non-ischemic area and ischemic area, respectively. B: Results of densitometric assessment. The graphs show a fold increase over WT/SHAM. The densitometric data were corrected by those of WT/SHAM which were obtained from the same membrane. Black bars and white bars represent WT and RES, respectively ($n=5$ for each group). Mean±S.E.M. *$P<0.05$ vs. SHAM, $^{\S}P<0.05$ vs. WT.

221±13 unit/mg protein/h, respectively) were significantly increased compared with their corresponding SHAMs (79±4 and 101±9 unit/mg protein/h, respectively, $n=5$ each for 4 groups, all $P<0.05$, Fig. 2D). Furthermore, the caspase-3 activity in the ischemic area of RES/MI was significantly higher than that in the non-ischemic area of RES/MI and that in the ischemic area of WT/MI ($P<0.05$ for each comparison).

3.4. Affected Epo–EpoR signaling pathway

In both WT/MI and RES/MI, the ratio of phospho-STAT3/STAT3 was significantly increased in the ischemic area as compared with the corresponding LV myocardium of SHAM. The ratio of phospho-Akt/Akt of RES/MI was significantly increased, and that of WT/MI also tended to be increased compared with the corresponding LV myocardium of SHAM. However, there was no significant difference in the ratios of phospho-STAT3/STAT3 and phospho-Akt/Akt of the ischemic area between WT/MI and RES/MI (Fig. 3A and B). The ratio of phospho-STAT5/STAT5 tended to be lower in RES/SHAM than in WT/SHAM. Although the ratio did not change after I/R in both the ischemic and non-ischemic areas of WT/MI, it was significantly increased in both areas of RES/MI to the levels similar to those of WT/MI. The expression level of STAT1 was relatively low, and there was no significant difference in the ratio of phospho-STAT1/STAT1 among the regions or between the WT/MI and RES/MI (data not shown). Although the ratio of phospho-p38/p38 was significantly increased in the ischemic area of WT/MI compared with WT/SHAM, that of RES/MI did not change significantly in the ischemic area compared with RES/SHAM. The ratio of phospho-JNK/JNK of WT/MI was significantly increased in the ischemic area compared with WT/SHAM. The ratio of phospho-JNK/JNK in the ischemic area of RES/MI did not change significantly compared with that of RES/SHAM.

3.5. Response of plasma Epo concentration to myocardial I/R

At the basal condition, the plasma Epo concentration of RES was significantly higher than that of WT (Table 1 and Fig. 4). Following I/R, the plasma Epo concentration in WT/MI was significantly increased, peaked at 24 h, and returned to the baseline level at 48 h after I/R (Fig. 4). In contrast, the time course of the plasma Epo concentration in RES/MI did not differ from that in RES/SHAM.

3.6. Effects of Epo-R deficiency on LV remodeling after I/R

The LV cavity was remarkably enlarged and the wall was globally thin in RES/MI compared with WT/MI at 21 days after I/R, suggesting that the LV remodeling after myocardial I/R was deteriorated in RES/MI as compared with WT/MI (Fig. 5A a–d). The extent of myocardial fibrosis in the non-ischemic remote area determined by Sirius-red staining

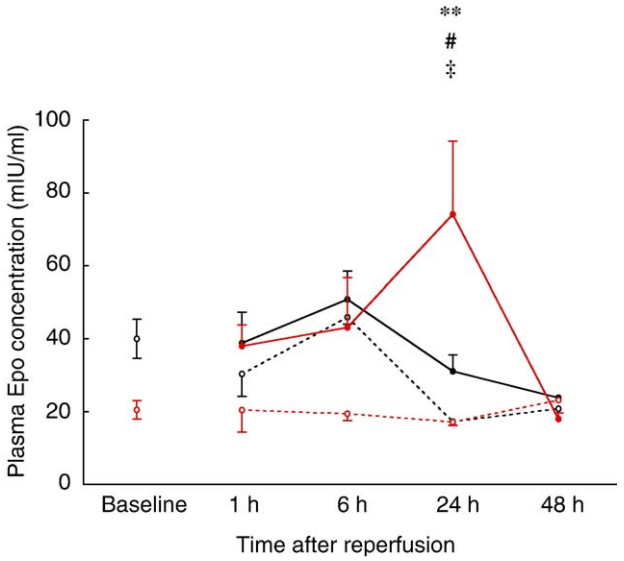

Fig. 4. The time course of plasma Epo concentration. Blood samples at baseline condition were collected from 10 WT and 10 RES (Table 1). The samples were also collected at 1, 6, 24, or 48 h after I/R from 16 WT/SHAM (red open circles, $n=4$ for each time point after I/R), 16 RES/SHAM (black open circles, $n=4$ for each time point), 24 WT/MI (red closed circles, $n=6$ for each time point), and 24 RES/MI (black closed circles, $n=6$ for each time point). $^{\#}P<0.05$ vs. WT/SHAM, $^{**}P<0.05$ vs. RES/MI, and $^{\ddagger}P<0.05$ vs. WT/MI at baseline and 48 h after reperfusion. Mean±S.E.M.

was comparable between WT/MI and RES/MI (Fig. 5A e and f). Table 2 shows the basic characteristics of the mice at 21 days after I/R. Body weight, heart rate, heart weight/body weight ratio, and hematocrit were comparable among the 4 groups. The infarct size at 21 days after I/R as defined by the ratio of Masson-trichrome stained area to the total LV area was significantly increased in RES/MI compared with WT/MI. As shown in Fig. 6, LVDd was significantly increased and LVFS was significantly reduced in the mice 21 days after I/R compared with the corresponding SHAMs. Furthermore, LVDd was significantly enlarged (Fig. 6B) and LVFS tended to be reduced in the RES/MI compared with the WT/MI (Fig. 6C). Although the difference was not statistically significant, the survival rate at 6 weeks after I/R tended to be lower by 37% in RES/MI than in WT/MI (26% vs. 41%) (Fig. 5B).

4. Discussion

The novel finding of the present study was that in transgene-rescued EpoR null mutant mice, RES, which express EpoR exclusively in the hematopoietic lineage cells, the infarct size after I/R was increased and LV remodeling was deteriorated as compared with WT. The increased infarct size in RES may be explained, at least in part, by accelerated apoptosis. The present results indicate that endogenous Epo–EpoR system in the non-hematopoietic lineage cells play an important protective role against I/R injury in vivo.

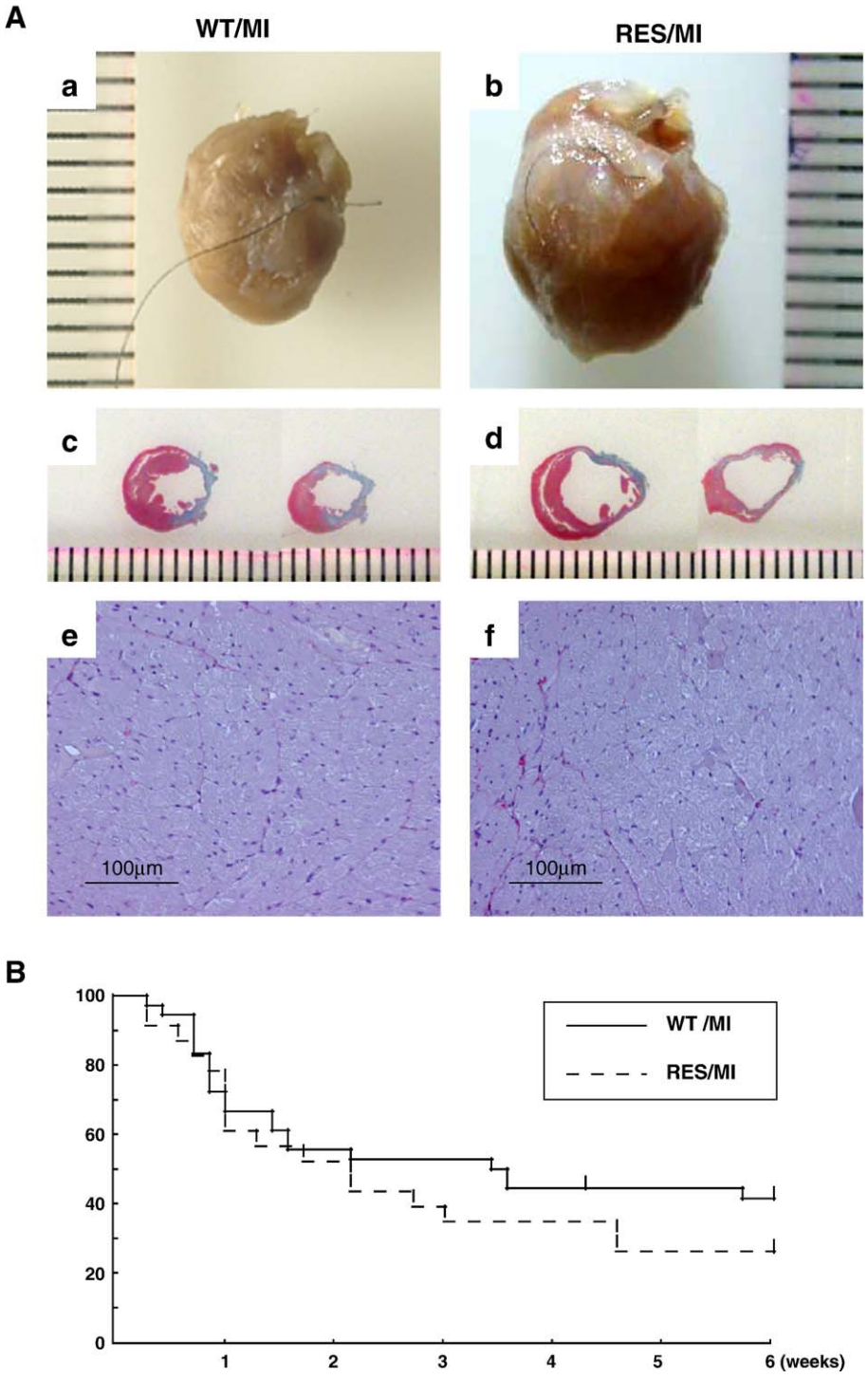

Fig. 5. A: Representative photographs of hearts (a, b), Masson-trichrome stained sections of hearts (c, d), and micrographs of Sirius-red staining (e, f) from WT/MI (a, c and e) and RES/MI (b, d and f) 21 days after I/R are shown. B: Kaplan–Meier survival curve during the 6-week observation period after I/R. The survival rate at 6 weeks after I/R tended to be lower in RES/MI than in WT/MI (26% vs. 41%).

4.1. Cardioprotective effect of endogenous Epo–EpoR system

The present results regarding the caspase-3 activity and number of apoptotic cells in the I/R region suggest that the increased infarct size in the RES/MI can be explained, at least in part, by accelerated apoptosis. It is well known that Epo inhibits apoptosis of erythroid progenitor cells in process of the maturation of erythroid cells [24]. The present results indicate that endogenous Epo–EpoR system in the LV myocardium also has anti-apoptotic effects against myocardial I/R injury. The

Table 2
Characteristics of mice at 21 days after ischemia and reperfusion

	WT/SHAM ($n=5$)	RES/SHAM ($n=5$)	WT/MI ($n=8$)	RES/MI ($n=8$)
Body weight (g)	25.6±0.9	26.2±0.2	24.6±0.7	25.2±0.6
Heart rate (bpm)	678±20	668±8	683±20	665±30
Heart weight (mg)	126±8	125±2	116±7	147±11
Heart weight/body weight (mg/g)	5.0±0.3	4.8±0.1	4.8±0.3	5.6±0.3
Infarct size (scar/LV) (%)			18.7±2.7	32.9±4.2[a]
Hematocrit (%)[b]	45.9±2.5	47.5±1.8	44.9±1.3	47.5±2.0

Values are mean±S.E.M.
[a] $P<0.01$ vs. WT.
[b] Hematocrit could not be measured in one RES/SHAM and one WT/MI due to technical problems during blood sampling.

difference in the percentage of TUNEL positive cells between 6 and 24 h after I/R was statistically significant in RES/MI, but not in WT/MI (Fig. 2A–C). These data suggest that not only cardiomyocyte death determined in the early phase of I/R but also its progression is modulated, at least in part, by Epo–EpoR signaling in non-hematopoietic lineage cells.

In the process of proliferation and differentiation of erythroid progenitor cells, the Epo–EpoR signals mediated by JAK-STAT, PI3K-Akt, and MAPK play a crucial role in the prevention of apoptosis in these cells [2–4]. Several investigators reported that the phosphorylation of STAT3, Akt, and p38 in ischemic myocardium was accelerated after administration of rhEpo [16,25–28], although the results were inconsistent among different experimental models. Both JNK and p38 are also activated in myocardial I/R [29,30]. However, it is still controversial whether the activation of signaling from these proteins is beneficial in terms of the protection against I/R injury [30–32]. Kaiser et al. [33] reported that both genetic inhibition and activation of JNK protects the myocardium from I/R induced cell death. Furthermore, Kaiser et al. [34] demonstrated that genetic inhibition of p38 in the myocardium provide protection from I/R injury while Martindale et al. [35] showed that activation of constitutive p38 was also associated with protection against I/R injury. In the present study, the phosphorylation of p38 and JNK in the region subjected to I/R, which was remarkably activated in WT/MI, did not change in RES/MI. The results are in line with those of Rafiee et al. [26,27] who reported that the administration of rhEpo increased the phosphorylation of p38 and protect myocardium against I/R injury in isolated buffer-perfused Langendorf rabbit heart preparations, and suggest that the increased EpoR-mediated phosphorylation of p38 and JNK after myocardial I/R mediates anti-apoptotic signals elicited by endogenous Epo–EpoR system in non-hematopoietic cells.

The phosphorylation of STAT3 and Akt in the I/R region was similarly increased in WT/MI and RES/MI. Although the phosphorylation of these molecules has been shown to elicit cytoprotective and anti-apoptotic effects [36,37], the difference in caspase-3 activity and subsequent apoptosis between WT/MI and RES/MI cannot be attributed to the difference in the phosphorylation of STAT3 and Akt (Fig. 3). The increased phosphorylation of STAT3 and Akt in the I/R region of both WT/MI and RES/MI might be explained by other cytokines such as interleukin-6 as the circulating level and myocardial expression of the cytokine have been reported to be elevated after myocardial infarction [38]. The different profiles of the extent of STAT5 phosphorylation observed between WT and RES (Fig. 3B) might be attributed to the difference in the activity of endogenous Epo–EpoR signaling in the basal condition between the 2 strains.

Accelerated ischemia-induced neovascularization might be involved in the mechanisms of beneficial effects of Epo–EpoR signaling on LV remodeling after I/R. Heeschen et al. reported that the administration of high dose rhEpo stimulated the mobilization of endothelial progenitor cells from the bone marrow and increased neovascularization at 2 weeks after the induction of hind-limb ischemia [39]. Furthermore, van der Meer et al. [40] demonstrated that Epo treatment starting 3 weeks after the induction of MI improved cardiac function presumably by accelerated neovascularization in a rat model of post-MI heart failure. Therefore, it is possible that the lack of Epo–EpoR system in the non-hematopoietic cells deteriorates neovascularization in the LV myocardium in RES/MI. Indeed, we recently reported that endogenous Epo–EpoR system plays an important role in the mobilization of endothelial progenitor cells and their recruitment to the pulmonary artery, and prevents the development of pulmonary hypertension due to systemic hypoxia by using RES [41].

4.2. Plasma Epo concentration

High plasma Epo concentration in RES under basal condition with a hematocrit level comparable to that of WT suggests that some non-hematopoietic cells regulate the production and/or metabolic turnover of Epo through Epo–EpoR interaction. The time courses of endogenous plasma Epo after I/R were also different between the WT/MI and RES/MI. Suzuki et al. previously reported that the anemia induced by bleeding increases the plasma Epo concentration in RES [18]. Furthermore, our preliminary study demonstrated an increase in the plasma Epo level under systemic hypoxic condition in RES (unpublished observations). These results suggest that the mechanism underlying the

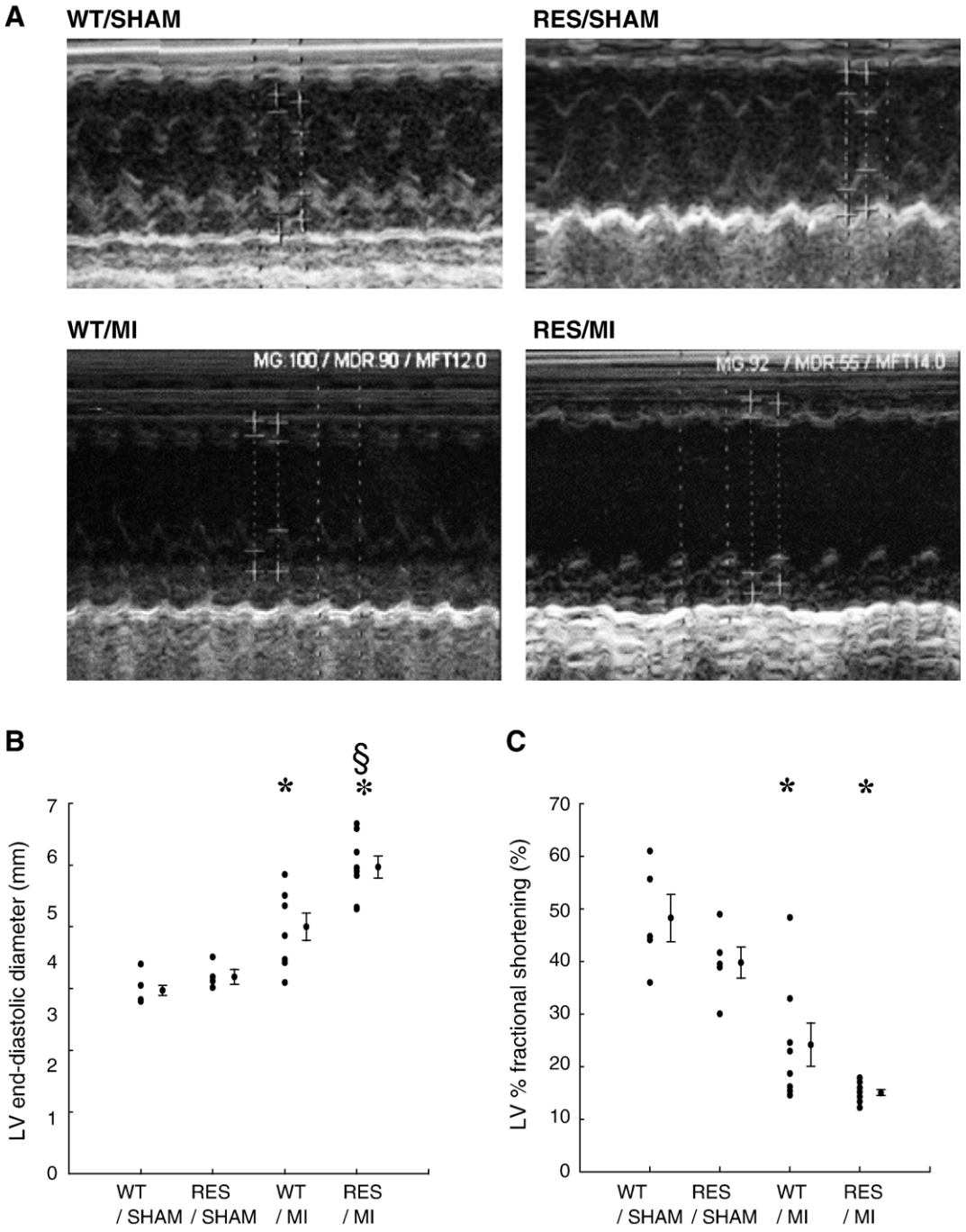

Fig. 6. Representative M-mode traces (A), LVDd (B), and LV %FS (C) in WT/SHAM ($n=5$), WT/MI ($n=8$), RES/SHAM ($n=5$), and RES/MI ($n=8$) 21 days after I/R are shown. *$P<0.05$ vs. SHAM, $^{\S}P<0.05$ vs. WT. Mean±S.E.M.

increased production of plasma Epo during myocardial I/R may differ from that induced by anemia or systemic hypoxia. Although the precise mechanism of the increased plasma Epo level in WT/MI after myocardial I/R is unclear, the present results suggest the existence of some protective mechanism that increases the endogenous plasma Epo level in vivo. The lack of increase in the plasma Epo level in RES/MI suggests that the protective mechanism is regulated by the endogenous Epo–EpoR system in the non-hematopoietic lineage cells.

4.3. Limitations of the study

Several limitations should be mentioned for the present study. First, despite the deterioration in ventricular remodeling after myocardial I/R in RES/MI, no significant difference was observed in the survival rate at 6 weeks after I/R between WT/MI and RES/MI. This discrepancy might be attributed to the relatively short observation period after I/R in the present study. Second, infarct size is one of the most important determinants of LV remodeling after

myocardial infarction [42]. As the infarct size was significantly increased in RES/MI compared with WT/MI, we were not able to determine whether the deteriorated LV remodeling in RES/MI can be attributed to the lack of some beneficial effects of endogenous Epo–EpoR system in the non-hematopoietic lineage cells on the development of LV remodeling or only to the increased infarct size. Finally, the percentages of apoptotic cells in the ischemic area of WT/MI were 24% and 26% at 6 and 24 h after I/R, respectively, and were relatively high as compared with the previously reported numbers of 13% [43] and 21% [44] at 3 and 24 h after I/R, respectively. We do not know how many TUNEL-positive cardiomyocytes in the ischemic area actually represented apoptotic cells in the present study because we did not perform an electron microscopic study [45]. We therefore cannot exclude the possibility that a significant number of these TUNEL-positive cells were actually necrotic cardiomyocytes. We however believe that accelerated apoptotic cell death contributed to the larger infarct size in RES/MI than in WT/MI because the caspase-3 activity in the ischemic area was significantly increased in RES/MI compared with WT/MI (Fig. 2D).

5. Conclusion

A deficiency of endogenous Epo–EpoR system in the non-hematopoietic lineage cells may deteriorate cardiomyocyte survival after I/R and subsequent LV remodeling. The endogenous Epo–EpoR system in the non-hematopoietic lineage cells may play an important protective role against I/R injury, at least in part, by preventing apoptosis.

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