Chronic Treatment With an Erythropoietin Receptor Ligand Prevents Chronic Kidney Disease–Induced Enlargement of Myocardial Infarct Size

Keitaro Nishizawa, Toshiyuki Yano, Masaya Tanno, Takayuki Miki, Atsushi Kuno, Toshiyuki Tobisawa, Makoto Ogasawara, Shingo Muratushiki, Kouhei Ohno, Satoko Ishikawa, Tetsuji Miura

Abstract—Chronic kidney disease (CKD) is known to increase myocardial infarct size after ischemia/reperfusion. However, a strategy to prevent the CKD-induced myocardial susceptibility to ischemia/reperfusion injury has not been developed. Here, we examined whether erythropoietin β pegol, a continuous erythropoietin receptor activator (CERA), normalizes myocardial susceptibility to ischemia/reperfusion injury by its effects on protective signaling and metabolomes in CKD. CKD was induced by 5/6 nephrectomy in rats (subtotal nephrectomy, SNx), whereas sham-operated rats served controls (Sham). Infarct size as percentage of area at risk after 20-minutes coronary occlusion/2-hour reperfusion was larger in SNx than in Sham: 60.0±4.0% versus 43.9±2.2%. Administration of CERA (0.6 μg/kg SC every 7 days) for 4 weeks reduced infarct size in SNx (infarct size as percentage of area at risk=36.9±3.9%), although a protective effect was not detected for the acute injection of CERA. Immunoblot analyses revealed that myocardial phospho-Akt-Ser473 levels under baseline conditions and on reperfusion were lower in SNx than in Sham, and CERA restored the Akt phosphorylation on reperfusion. Metabolomic analyses showed that glucose 6-phosphate and glucose 1-phosphate were reduced and malate:aspartate ratio was 1.6-fold higher in SNx than in Sham, suggesting disturbed flux of malate–aspartate shuttle by CKD. The CERA improved the malate:aspartate ratio in SNx to the control level. In H9c2 cells, mitochondrial Akt phosphorylation by insulin-like growth factor-1 was attenuated by malate–aspartate shuttle inhibition. In conclusion, the results suggest that a CERA prevents CKD-induced susceptibility of the myocardium to ischemia/reperfusion injury by restoration of Akt-mediated signaling possibly via normalized malate–aspartate shuttle flux. (Hypertension. 2016;68:697-706. DOI: 10.1161/HYPERTENSIONAHA.116.07480.) • Online Data Supplement

Key Words: chronic kidney disease ■ erythropoietin ■ metabolome ■ myocardial infarction ■ signal transduction

Myocardial infarct size is a major determinant of clinical outcome after acute myocardial infarction. Reduced renal function tightly correlates with adverse outcomes in patients with acute myocardial infarction,1,2 and chronic kidney disease (CKD) has been shown to enlarge infarct size in animal models of acute myocardial infarction.3-4 However, the mechanism by which CKD enlarges myocardial infarct size has not been clarified. Cardiomyocytes are equipped with endogenous protective mechanisms that are regulated by intracellular signal pathways. Some of the protective signal pathways are activated at the time of reperfusion after ischemia, and they are called RISK (reperfusion injury salvage kinase) pathways.5 We recently found that insufficient activation of Akt-mediated signaling underlies aggravation of reperfusion injury, leading to enlarged infarct size.6 In addition to modified signal transduction, alteration in metabolism is possibly involved in CKD-induced increase in myocardial sensitivity to ischemia/reperfusion (I/R) injury. Insulin resistance is a metabolic hallmark of uremia,7,8 and Tamaki et al8 showed that CKD induces substantial changes in metabolites and the number of mitochondria in skeletal muscle. However, to our knowledge, the effect of CKD on cardiac metabolomes has not been reported, and its relationship to modification of the cytoprotective signal pathway also remains unclear.

Erythropoietin is produced in the adult kidney and plays a major role in promotion of erythropoiesis. However, erythropoietin protein is also expressed in nonrenal tissues, including the liver and central nervous system.9 Erythropoietin receptors are also expressed not only in hematopoietic cells but also in other types of cells, such as endothelial cells, neurons, and cardiomyocytes.10-12 A series of studies using a mutant mouse that expresses the erythropoietin receptor exclusively in hematopoietic cells (EpoR−/− rescued) showed that deletion of the erythropoietin receptor aggravates I/R injury and

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pressure overload–induced heart failure and blunts angio genesis in response to ischemia. Thus, the outcomes of reduction of erythropoietin by CKD is unlikely to be limited to anemia (renal anemia) and possibly includes cardiovascular dysfunctions.

We hypothesized that chronic activation of the erythropoietin receptor normalizes myocardial susceptibility to I/R injury in CKD by its effects on protective signaling and metabolomes. The rationale for this hypothesis is 3-fold. First, homometric and heteromeric erythropoietin receptors are present in cardiomyocytes and the receptors are coupled with signaling pathways including a pathway that induces mitochondrial translocation of Akt, which plays a major role in protection of cells from necrosis. Second, activation of endogenous erythropoietin signaling is involved in cell repair and recovery from damage in muscle cells. Third, insulin resistance in skeletal muscle has been shown to be alleviated by activation of the heteromeric erythropoietin receptor. To test the hypothesis, we used an established model of CKD, 5/6 nephrectomized rat, and epoetin β pegol, a continuous erythropoietin receptor activator (CERA). Cardiovascular effects of CKD are replicated in the rat 5/6 nephrectomy model of CKD, and we examined the myocardium 5 weeks after the first nephrectomy, when mild hypertension and mild proteinuria are known to be induced without cardiac hypertrophy, cardiac fibrosis, or vascular calcification. Epoetin β pegol was selected as a CERA because this CERA has much longer half-life than that of a conventional erythropoiesis-stimulating agent (ESA) and persistently corrects anemia. We examined effects of CKD and CERA on activation of cytoprotective signaling on reperfusion and on metabolomes in rat hearts and relationship between CKD-induced metabolomic changes and impaired response of Akt signaling by using H9c2 cells.

Methods
This study was conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals published by National Research Council of the National Academies, USA (2011) and was approved by the Animal Use Committee of Sapporo Medical University. Detailed Methods section is in the online-only Data Supplement.

Statistical Analyses
Data are presented as means±SEM. Differences between treatment groups were tested by 1- or 2-way ANOVA, and the Tukey post hoc test was used to test for multiple comparisons when ANOVA indicated significant differences. The difference was considered significant if the P value was <0.05. The relationship between serum creatinine concentration and infarct size was analyzed by the use of simple linear regression analysis. All of the above statistical analyses were performed using EZR software (Jichi Medical University, Saitama, Japan). Hemodynamic data were analyzed by 2-way repeated measures ANOVA using SigmaStat software.

Results
Chronic Erythropoietin Receptor Activation Prevents Infarct Size Enlargement in CKD
First, as shown in Figure 1A, rats were assigned to a subtotal nephrectomy group (SNx) or to a Sham group (Sham). SNx underwent 5/6 nephrectomy, and Sham received only sham operation. The effect of CERA administration (every 7 days for 4 weeks) on infarct size after 20-minute ischemia/2-hour reperfusion in Sham and SNx was examined. The effect of CKD on blood pressure in the present model was assessed in separate groups of rats by a catheter indwelled in the carotid artery. Mean blood pressure under conscious state was significantly higher by ≈15 mm Hg in SNx than in Sham (Table S1). However, histological analyses showed no discernible cardiomyocyte hypertrophy or interstitial fibrosis in the myocardium of SNx compared with those in Sham regardless of CERA treatment (Figure S1). Vehicle-treated SNx showed lower hemoglobin level (14.2±0.2 g/dL versus 17.5±0.2 g/dL) and higher serum creatinine level (0.83±0.05 g/dL versus 0.34±0.02 mg/dL) than those in the vehicle-treated Sham (Figure 1B and 1C; Table S2), confirming successful induction of CKD. In SNx, the hemoglobin level was preserved by treatment with the CERA (16.4±0.7 g/dL) without change in creatinine level (0.79±0.07 mg/dL), although the CERA had no effect on hemoglobin and creatinine levels in Sham. In infarct size experiments, heart rates, and blood pressures under anesthesia were comparable in Sham and SNx regardless of treatment with the CERA (Table S3).

The time courses of heart rates and blood pressures during the infarct size experiment were comparable in Sham and SNx with or without CERA treatment (Tables S3). Serum erythropoietin in SNx before ischemia was elevated in SNx treated with the CERA compared with that in vehicle-treated SNx (12.2±3.3 mIU/mL versus 0.3±0.2 mIU/mL). Infarct and area at risk were visualized by tetrazolium staining and by infusing fluorescent particles into the aorta after coronary reocclusion ex vivo, respectively (Detailed Methods section is in the online-only Data Supplement). Sizes of area at risk were similar in all the treatment groups (Table S4), and infarct size as a percentage of area at risk was significantly larger in the SNx group than in the Sham group when treated with the vehicle (60.0±4.0% versus 43.9±2.2%) as shown in Figure 1D. The CERA significantly reduced infarct size in SNx (36.9±3.9%), although the effect of the CERA in Sham was modest (31.8±4.6%) with no statistical significance.

In the second protocol (Figure 2A), we assessed the effect of single injection of the CERA 1 day before I/R as shown. Serum erythropoietin levels were elevated by a single injection of the CERA in Sham (66.5±13.5 mIU/mL) and SNx (79.1±14.2 mIU/mL) compared with those in the vehicle-treated Sham (0.5±0.2 mIU/mL) and SNx (0.9±0.3 mIU/mL), respectively (Table S5), whereas heart rates and blood pressures under anesthesia were similar in the 4 groups (Table S6). However, infarct size was not reduced by a single injection of the CERA in either Sham or SNx (Figure 2B; Table S7).

A significant correlation between infarct size and hemoglobin level (Figure 1E) indicates the possibility that reduction in infant size by the CERA in the SNx is attributable to improvement of anemia. Thus, in the third protocol (Figure 2C), we examined whether elimination of CERA-induced improvement of anemia inhibits protection afforded by CERA against I/R injury in the SNx. The rats were divided into a bloodletting group and a nonbloodletting group after 5/6 nephrectomy, and both groups received the CERA at a dose of 0.6 μg/kg every 7 days for 4 weeks. In the bloodletting group, 3 mL/kg of bloodletting with supplementation of equal volume of saline via the tail vein was performed at each time of CERA injection.
The hemoglobin level was significantly lower (13.7±0.6 g/dL) in the bloodletting group than in the CERA-treated SNx (17.4±0.5 g/dL) without significant change in serum creatinine or blood urea nitrogen level (Table S8). Blood pressures and heart rates before ischemia were similar in both of the groups (Table S9). Infarct size as percentage of area at risk tended to be larger in the bloodletting group (51.5±5.6%) than in the non-bloodletting group (41.5±3.6%), but the difference did not reach statistical significance (Figure 2D; Table S10).

Reperfusion-Induced Activation of Akt Was Restored by the CERA in SNx

We examined how cytoprotective signal pathways were modified during the development of CKD. Levels of erythropoietin receptor mRNA and protein in the myocardium were similar among the 4 treatment groups (Figures S2 and S3). Protein levels of janus kinase-2, Akt, 70kDa ribosomal S6 kinase (p70s6K), glycogen synthase kinase 3β (GSK3β), mechanistic target of rapamycin complex 2 subunits (mTOR, Rictor, Sin1, and Deptor), PH domain and leucine-rich repeat protein phosphatases-1, and phosphatase and tensin homolog deleted from chromosome 10 in the myocardium were comparable among the treatment groups at baseline (Figure 3A). Phosphorylation of janus kinase-2 was similarly observed in Sham and SNx with or without CERA administration. However, the level of phospho-Akt-Ser473 was lower in the vehicle-treated SNx than in the vehicle-treated Sham (Figure 3A and 3B). The reduction in phosphorylation of Akt-Ser473 in SNx was associated with attenuated phosphorylation of p70s6K and GSK3β, downstream substrates of Akt (Figure 3A and 3B). The CKD-induced reduction in baseline Akt phosphorylation was not improved by CERA administration.

We next examined the effects of CKD and CERA on Akt signaling on reperfusion, because reperfusion-induced
activation of Akt signaling plays a major role in protection against reperfusion injury. As was the case with the baseline condition, the level of phospho-Akt-Ser473 in the myocardium after 5 minutes of reperfusion was lower in the vehicle-treated SNx than in the vehicle-treated Sham (Figure 4A and 4B). The CERA significantly enhanced Akt-Ser473 phosphorylation in SNx, leading to increased Akt activation as shown by increases in phosphorylation of p70s6K and GSK3β in the
CERA-treated SNx. Janus kinase-2 phosphorylation levels on reperfusion were comparable among the 4 treatment groups.

In contrast to chronic treatment with the CERA (Figure 4), a single injection of the CERA a day before I/R did not significantly change phosphorylation of janus kinase-2, Akt, p70s6K, or GSK3β on reperfusion in Sham or SNx (Figure S4).

Metabolomic Analysis of the Myocardium Showed Disturbance of MAS by CKD and Its Restoration by the CERA

Changes in myocardial metabolomes caused by CKD and CERA were examined using comprehensive tissue metabolome analyses. Of 116 metabolites measurable by capillary electrophoresis time-of-flight mass spectrometry and by positive and negative modes of capillary electrophoresis tandem mass spectrometry, 100 were detected in myocardial samples. In principal component (PC) analysis (Figure 5A), the first component (PC1), which accounted for 33.9% of the total variation, clearly separated SNx from Sham. In a factor loading plot for PC1, the top metabolites that contribute more to the total variance indicating SNx were reduced glucose 6-phosphate (G6P), glucose 1-phosphate (G1P), and fructose 6-phosphate, indicating reduction of glycolytic flux in SNx. This finding is consistent with previous findings that renal failure induces insulin resistance in muscle and consistent with our results indicating reduction of Akt activity in SNx (Figure 3). However, the top metabolites that contribute more to the total variance indicating Sham were GTP and ATP (Figure 5B). We also focused on the fourth component (PC4), which accounted for 7.2% of the total variation, because CERA-treated SNx rats were clearly separated from vehicle-treated SNx rats by this component analysis (Figure 5A). A factor loading plot for PC4 showed that the top metabolites indicating rats treated with the CERA and those with the vehicle were aspartate and malate, respectively (Figure 5C). The ratio of malate to aspartate was 1.6-fold higher in SNx than in Sham (Table S11), suggesting that CKD disturbed flux of malate–aspartate shuttle (MAS), presumably in the mitochondria. Treatment with the CERA did not affect the levels of G6P and G1P in SNx but improved the malate:aspartate ratio to a level similar to that in Sham (Table S11). Protein levels of enzymes that catalyze reactions involved in MAS (GOT2, MDH2, and oxoglutarate carrier) and activities of GOT2 and MDH2 were comparable among the groups (data not shown).

Hemoglobin level was positively correlated with aspartate, G1P and G6P levels in the myocardium ($r=0.57$, $r=0.678$, and $r=0.714$; $P<0.05$) and tended to be negatively correlated with malate:aspartate ratio ($r=-0.44$, $P=0.05$). However, there was no correlation between serum creatinine level and malate:aspartate ratio ($r=0.12$, $P=0.60$).

Inhibition of MAS Flux Disturbed Mitochondrial Akt Signaling

To explore a mechanistic link between suppression of Akt activation at the time of reperfusion and inhibition of MAS in the myocardium in SNx, we examined the effect of MAS...
inhibition on Akt activation in response to its upstream stimulus. In H9c2 cells, aminooxyacetate, an MAS inhibitor, alone did not affect the level of baseline phosphorylation of Akt in both mitochondria and cytosol fractions. However, aminooxyacetate abolished Akt phosphorylation induced by insulin-like growth factor-1 in both fractions (Figure 6). These findings support the notion that inhibition of MAS by CKD contributes to impaired Akt activation at the time of reperfusion.

**Discussion**

The mechanism by which CKD increases myocardial infarct size has not been fully clarified, and a strategy to prevent the adverse effect of CKD has not been investigated. In this study, phosphorylation of Akt and GSK3β on reperfusion was significantly attenuated in the SNx group (Figure 4), indicating that the cytoprotective signal at the time of reperfusion is attenuated by CKD. A crucial role of Akt-Ser473 phosphorylation in infarct size limitation is supported by close association of Akt-Ser473 phosphorylation on reperfusion with protection afforded by ischemia preconditioning and postconditioning5,17 and by our recent findings that an mechanistic target of rapamycin complex inhibitor, Ku-0063794, injected before reperfusion inhibited Akt-Ser473 and increased myocardial infarct size in non-CKD rats.6 Furthermore, continuous erythropoietin receptor activation by the CERA for 4 weeks improved phosphorylation of Akt-Ser473 on reperfusion in the SNx group (Figure 4), and the restored Akt signaling on reperfusion was associated with the elimination of infarct size enlargement by SNx (Figure 1D). In contrast to chronic treatment with the CERA, single-dose administration of the CERA before ischemia did not reduce myocardial infarct size in SNx (Figure 2B) and did not enhance phosphorylation of Akt, p70s6K or GSK3β on reperfusion (Figure S4). The failure of a single injection of CERA at a dose of 0.6 μg/kg to enhance Akt phosphorylation on reperfusion is not surprising because the dose was much smaller than doses of ESAs that have been shown to limit infarct size by activation of PI3K-Akt-GSK3β signaling (eg, 5000 IU/kg of epoetin-β).14–16 Taken together, the findings indicate that chronic treatment with a CERA prevents CKD-induced infarct size enlargement by restoration of Akt-Ser473-mediated signaling on reperfusion.

Chronic treatment with the CERA prevented not only infarct size enlargement but also anemia in SNx. A significant correlation between hemoglobin level and infarct size (Figure 1E) indicates the possibility that prevention of renal anemia by the CERA contributed to the prevention of infarct size enlargement in SNx. Thus, we examined the effect of chronic anemia, induced by bloodletting at each time of CERA injection, on infarct size limitation in SNx treated with the CERA. The difference in infarct sizes between CERA-treated SNx rats with and without chronic bloodletting was statistically insignificant (Figure 2D). Although we cannot exclude a type II error, infarct size in the group treated with both the CERA and chronic bloodletting (51.5±5.6%) was still smaller than infarct size in the untreated SNx group (60.0±4.0%). Taken together, the results shown in Figures 1C and 1D and 2D indicate that prevention of CKD-induced infarct enlargement by chronic CERA treatment cannot be explained simply by improvement of anemia.

Metabolomic analyses in this study revealed a significant impact of CKD on myocardial metabolomes (Figure 5; Table S11), which were associated with increased susceptibility to I/R injury. A factor loading plot for PC1 showed that the top 2 metabolites characterizing CKD were reduced G6P and G1P. The findings suggest that glycolysis via glycogen phosphorylase and glycolysis via hexokinase are impaired in SNx,

![Figure 4](http://hyper.ahajournals.org/) Effects of chronic kidney disease and continuous erythropoietin receptor activator (CERA) on reperfusion-induced Akt activation. Representative blots (A) and summary data (B) of immunoblotting for signal molecules on reperfusion in myocardial samples from Sham and subtotal nephrectomy (SNx) with or without CERA administration. Myocardial samples were taken from areas at risk subjected to 20-min ischemia/5-min reperfusion; n=7 to 9 in each group. a.u. indicates arbitrary units. *P<0.05 vs Sham+vehicle. †P<0.05 vs SNx+vehicle.
although we did not directly confirm change in activities of those enzymes. The effect of the CERA was also distinguished from that of the vehicle by PC4 analysis. The top metabolite indicating rats treated with CERA was aspartate, whereas malate ranked top to predict the likelihood of untreated rats. Without the CERA, SNx rats showed a trend of increase in malate and significant decrease in aspartate, with elevation of malate:aspartate ratio. Elevation of malate:aspartate ratio is most likely to be attributable to disturbance of MAS, probably at malate dehydrogenase and aspartate transaminase in the mitochondria. MAS constitutes the primary metabolic pathway for transfer of NADH, a reducing equivalent from the cytosol into the mitochondria for oxidation. Besides, MAS shares intermediates, that is, oxaloacetate, malate, and α-ketoglutarate, with the tricarboxylic acid cycle. Thus, the flux of MAS is tightly linked to the flux of electron transport chain and tricarboxylic acid cycle and its dysfunction is likely to be detrimental to susceptibility to I/R injury. Interestingly, chronic treatment with the CERA normalized levels of malate, aspartate, and malate:aspartate ratio in CKD rats, although reduced G6P and G1P levels were unchanged. Hence, dysfunctional MAS rather than modified glucose utilization may contribute to the CKD-induced increase in myocardial susceptibility to I/R injury. The dysfunction of MAS might be related to anemia but not to the accumulation of uremic toxins because malate:aspartate ratio tended to correlate with hemoglobin level ($r=-0.44$, $P=0.05$) but not with serum creatinine level.
In conclusion, CKD has a significant impact on myocardial
CERA treatment (Figure 3). Thus, we postulated that modification
of MAS, which is suggested by reduced malate:aspartate
ratio, contributed to the blunted response of Akt to reperfu-
sion in CKD, and we examined this possibility by the use of
H9c2 cells. As shown in Figure 6, insulin-like growth fac-
TOR-1–induced phosphorylation of Akt in the mitochondria was
suppressed by an inhibitor of MAS, aminooxyacetate, in H9c2
cardiomyoblasts. Because activated Akt in mitochondria plays
a major role in suppression of opening of the mitochondrial perme-
ability transition pore,27 the results shown in Figure 6 sup-
port the notion that CKD-induced inhibition of MAS is casually
related to blunted response of Akt-Ser473 to its upstream sig-
als, leading to increased I/R injury by CKD. A contribution of
retrograde signaling, in which metabolic disturbance precedes
impaired activation of prosurvival kinases, has been observed in
some previous studies28–31; mitochondrial uncoupling and sub-
sequent reactive oxygen species overproduction reduce activa-
tion of the Akt–GSK3β axis, although a defect of mitochondrial
respiration induces compensated upregulation of Akt activity.32
Nevertheless, the relationship between amelioration of the
CKD-induced metabolic disturbance by a CERA and restora-
tion of Akt signaling in the heart warrants further investigation.

Epoetin β pegol, known as a CERA, is currently used for
the treatment of anemia in patients with CKD. The CERA
is an ESA modified by integration of methoxypolyethylene
glycol into the molecule and has a long half-life with slow
clearance33 and low-binding affinity to erythropoietin recep-
tors.34 Thus, it is expected to activate intracellular signals
downstream of erythropoietin receptors more persistently
and, thus, to more efficiently compensate deficiency of endog-
ous erythropoietin than does a conventional ESA in CKD.
In fact, a CERA prevented endothelial dysfunction in femoral
arteries of rats with 5/6 nephrectomy-induced CKD through
reduction of Nox4 expression and eNOS phosphorylation.35
Furthermore, albumin excretion and podocyte loss in advanced
diabetic nephropathy in the db/db mouse were ameliorated by
treatment with a CERA, via decrease in p27 expression and
increase in NRP1 expression.36 This study provides another
example that chronic treatment with CERA repairs defects in
important signal transductions in CKD.

This study showed a novel beneficial effect of chronic
CERA treatment in a model of CKD, that is, prevention of
CKD-induced infarct size enlargement. However, clinical
studies to date have not demonstrated that treatment with ESA
significantly reduces cardiovascular events in patients with
CKD,37 although protective effects of ESA on the heart and
blood vessels have been shown by several preclinical stud-
ies.13,35,36 The reason for the discrepant findings in clinical and
preclinical studies is not clear, but a plausible explanation
is detrimental effects of ESA on the cardiovascular system,
such as elevation of blood pressure and possible promotion of
thrombosis in patients with CKD.38,39 Whether the net benefit
of an ESA on the cardiovascular event can be maximized by
adjusting the dose of ESA alone or whether it requires a com-
bination of ESA with an agent for preventing adverse effects
of ESA is an important question that remains to be answered.

In conclusion, CKD has a significant impact on myocardial
metabolomes and impairs the Akt-mediated endogenous pro-
tective mechanism. Chronic treatment with a CERA prevents
increase in myocardial susceptibility to I/R injury in CKD by restoration of Akt-mediated signaling on reperfusion. The recovery of Akt response might be mediated by restoration of MAS.

Perspectives

CKD is known to significantly increase cardiovascular events and the mortality rate after acute myocardial infarction. However, strategies to reduce such risks by CKD has not been developed. CKD-induced enlargement of myocardial infarct size was not preventable by blood pressure lowering or blockade of sympathetic nerve activities in an earlier study. This study uncovered that infarct size enlargement by CKD is induced by impaired activation of Akt on reperfusion and metabolomic derangements but not by renal anemia alone. Furthermore, suppressed MAS was found to link with blunted response of Akt in the myocardium to upstream signals. We found that chronic activation of the erythropoietin receptor prevents CKD-induced impairments of Akt activation and MAS in the myocardium in addition to anemia. The findings uncover significant impacts of CKD on both cytoprotective signaling and metabolome in the myocardium and suggest that signal-regulating metabolomes may be a new target of therapy for cardioprotection.

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Disclosures

None.

References


What Is New?
- Chronic treatment with epoetin β pegol prevented increase in myocardial susceptibility to ischemia/reperfusion injury in a rat model of chronic kidney disease (CKD).
- The protection was closely associated with restoration of Akt-mediated protective signaling on reperfusion and normalization of metabolomics derangements in the myocardium.
- Infarct size enlargement by CKD is not attributable to renal anemia.

What Is Relevant?
- CKD is known to increase myocardial infarct size after ischemia/reperfusion. However, a strategy to prevent the CKD-induced myocardial susceptibility to ischemia/reperfusion injury has not been developed. The results suggest that chronic erythropoietin therapy has beneficial effects not only on renal anemia but also on myocardial susceptibility to infarction.
- This study demonstrated significant untoward modification of myocardial metabolomes by CKD, which is potentially treatable by pharmacological agents.

Summary
We showed that chronic activation of erythropoietin receptors prevents CKD-induced increase in myocardial susceptibility to ischemia/reperfusion injury by repair of cytoprotective signaling and metabolomic derangements.
Chronic Treatment With an Erythropoietin Receptor Ligand Prevents Chronic Kidney Disease–Induced Enlargement of Myocardial Infarct Size
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CHRONIC TREATMENT WITH AN EPO RECEPTOR LIGAND PREVENTS CKD-INDUCED ENLARGEMENT OF MYOCARDIAL INFARCT SIZE.

*Department of Cardiovascular, Renal and Metabolic Medicine, †Department of Pharmacology, Sapporo Medical University School of Medicine, Sapporo, Japan.

Address for correspondence
Tetsuji Miura, MD, PhD
Department of Cardiovascular, Renal, and Metabolic Medicine
Sapporo Medical University School of Medicine
South-1, West-16, Chuo-ku
Sapporo 060-8543
Japan
Phone: +81-11-611-2111, ext. 3225
Fax: +81-11-644-7958
Email: miura@sapmed.ac.jp

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Detailed Methods
This study was conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals published by National Research Council of the National Academies, USA (2011) and was approved by the Animal Use Committee of Sapporo Medical University.

Preparation of the subtotal nephrectomy model of chronic kidney disease (CKD) and pharmacological treatments
Male Sprague-Dawley (SD) rats (8 weeks) were prepared for the induction of CKD by the subtotal nephrectomy (5/6 nephrectomy) as described previously 1,2. Rats were anesthetized with isoflurane inhalation and assigned to a subtotal nephrectomy group (SNx) or to a Sham group (Sham). In the SNx, the right kidney was decapsulated and removed, and 7 days later, approximately two-thirds of the left kidney was resected as shown in Figure 1A. Rats in the Sham received only decapsulation of kidneys. Infarct size experiments and experiments for tissue sampling were performed 5 weeks after the second operation.

Treatment protocols: In the first protocol, subcutaneous administration of the CERA at a dose of 0.6 μg/kg or its vehicle every 7 days was commenced at one week after the second operation. This dose of CERA was selected as a dose of the CERA to modestly increase hemoglobin level without marked change in blood pressure based on results...
of earlier studies in which effects of different doses of a CERA (0.6, 1.25 and 5 μg/kg) and different intervals of its administration (i.e., 1, 2 and 4 weeks) on erythropoiesis in rats were examined. In the second protocol (Figure 2A), rats in Sham and SNx received a single injection of the CERA or its vehicle 24 hr before infarct size experiments. In the third protocol (Figure 2C), rats after the 5/6 nephrectomy were divided into a bloodletting group and a non-bloodletting group, and both groups received the CERA at a dose of 0.6 μg/kg every 7 days for 4 weeks. In the bloodletting group, approximately 3 ml/kg of blood was drawn from the tail vein and the same amount of saline was infused into the vein at each time of CERA injection.

Using separate groups of Sham and SNx rats, blood pressures and heart rates were measured in a conscious state. A catheter (PE50) filled with heparinized saline was indwelled in the right carotid artery under isoflurane anesthesia. Twenty-four hours after recovery from the anesthesia and surgery, the rats were placed in strainers and arterial blood pressure was determined by connecting a pressure transducer (SCK-590, Nihon-Kohden, Japan) to the arterial catheter.

Infarct size experiment in situ
Infarct size experiments were performed as in our previous studies with slight modifications. In brief, rats were anesthetized with pentobarbital sodium (80 mg/kg, intraperitoneal injection) and ventilated with a Harvard Model 683 respirator (Harvard Apparatus, South Natick, NA). The chest was opened via a left thoracotomy, and a coronary snare was prepared around the left main coronary artery. A saline-filled catheter was inserted into a carotid artery for monitoring for blood pressure. The catheter placed in the carotid artery was connected into a Nihon-Kohden SCK-590 pressure transducer. An electrocardiogram was recorded by precordial bipolar electrodes. Rectal temperature was adjusted within 37.5°C-38.5°C by using a heating lamp when necessary.

After 30 minutes of stabilization, rats underwent 20 minutes of left coronary artery occlusion and 2 hours of reperfusion. Myocardial ischemia and reperfusion were confirmed by the appearance of regional cyanosis and ST segment elevation on an electrocardiogram and visible hyperemia on the ventricular surface, respectively. After 2 hours of reperfusion, 200 U of heparin was intravenously administered and the hearts were quickly excised. The excised hearts were mounted onto a Langendorff apparatus and perfused with saline to wash out blood, and then the coronary artery was re-oocluded. A saline suspension of fluorescent polymer microspheres (Duke Scientific, Palo Alto, CA) was infused into the aorta to negatively mark the area at risk, and the heart were frozen at -20°C.

Frozen hearts were sliced into 1.5-mm-thick sections and stained with 1% triphenyltetrazolium chloride in phosphate buffer (pH 7.4). Areas of infarct and those of region at risk were determined by using Image J software (National Institutes of Health). Their volumes were obtained by multiplying each area by 1.5 mm, i.e., the thickness of the heart slice.

Since infarct size larger than 20% of the ventricle is associated with significant reduction in left ejection fraction and ventricular remodeling in patients with acute myocardial infarction, we investigated whether infarct size enlargement by CKD reaches that size of infarct. Based on the results of pilot experiments, we assumed that the mean size of area at risk is 0.3 mm³ (corresponding to 30% of the ventricle) and that the mean value of infarct size as a percentage of area at risk (%IS/AR) after 20-min ischemia/reperfusion is 45±9 (SD)% in untreated control rats. In this model, %IS/AR of 67% corresponds to infarct size of 20% of the ventricle. To detect 22% change in %IS/AR from the control value, seven rats in each group give statistical power of 0.73. Based on the assumption, we used 7-8 animals for each treatment group in infarct size experiments in this study.
Tissue sampling for biochemical analyses
Anesthesia and surgical preparation of rats, including placement of a coronary snare, were the same as those in infarct size experiments. Hearts were excised after 30-min stabilization or at 5 min after reperfusion following 20-min coronary occlusion, soaked in ice-cold saline, quickly mounted onto a Langendorff apparatus, and perfused with ice-cold saline to wash out blood. The coronary artery was re-occluded and Evans blue dye was infused into the aorta to negatively mark the area at risk, from where myocardial tissue was quickly sampled and frozen in liquid nitrogen. The frozen tissues were stored at -80°C until use for immunoblotting and/or determination of mRNA levels.

Immunoblotting
Frozen heart samples were homogenized in ice-cold buffer (Celllytic™ MT Cell Lysis Reagent) including 0.5 mmol/L Na3VO4, a protease inhibitor cocktail (Complete mini, Roche Molecular Biochemicals, Mannheim, Germany), and 1 mmol/l phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 13,000 g for 15 min to obtain the supernatant. Protein concentration of the supernatant was determined using the Bradford assay. Equal amounts of proteins were electrophoresed on 7.5% or 12.5% polyacrylamide gels and then blotted onto PVDF membranes (Millipore, Bedford, MA). After blocking had been performed with a TBS-T buffer containing 5% nonfat dry milk or 5% BSA, the blots were incubated with antibodies that recognize the following: phospho-Akt (Ser473) and total Akt, phospho-GSK3β (Ser9) and total GSK3β, phospho-p70s6K (Thr389) and total p70s6K, phospho-JAK2 and total JAK2, PTEN, mTOR, Rictor, Sin1, Deptor, (Cell Signaling Technology, Beverly, MA); PHLPP-1 (Bethyl Laboratories, Montgomery, TX); vinculin (Sigma Aldrich, St Louis, MO); erythropoietin receptor (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoblotted proteins were visualized by using an Immobilon Western detection kit (Millipore, Billerica, MA).

Metabolome analysis
Comprehensive analyses of metabolites of the ventricular myocardium were performed according to the methods developed by Soga et al.8,9 The ventricular myocardial samples were taken 5 weeks after the second operation as shown in Figure 1A. Approximately 50 mg of frozen tissue was plunged into 1,500 μL of 50% acetonitrile/Milli-Q water containing internal standards (H3304-1002, Human Metabolome Technologies, Inc., Tsuruoka, Japan) at 0°C in order to inactivate enzymes. The tissue was homogenized thrice at 1,500 rpm for 120 sec using a tissue homogenizer (Microsmash MS100R, Tomy Digital Biology Co., Ltd., Tokyo, Japan) and then the homogenate was centrifuged at 2,300 ×g for 5 min at 4°C. Subsequently, 800 μL of the upper aqueous layer was centrifugally filtered through a Millipore 5-kDa cutoff filter at 9,100 ×g for 120 min at 4°C to remove proteins. The filtrate was centrifugally concentrated and re-suspended in 50 μL of Milli-Q water for subsequent analysis. Metabolome measurements were carried out through a facility service at Human Metabolome Technologies, Inc., Tsuruoka, Japan. Cationic compounds were measured in the positive mode of capillary electrophoresis-time-of-flight mass spectrometry (CE-TOFMS) and anionic compounds were measured in the positive and negative modes of capillary electrophoresis tandem mass spectrometry (CE-MS/MS)8,9. Peaks detected by CE-TOFMS and CE-MS/MS were extracted using automatic integration software (MasterHands, Keio University, Tsuruoka, Japan and MassHunter Quantitative Analysis B.04.00, Agilent Technologies, Santa Clara, CA, USA, respectively) in order to obtain peak information including m/z, migration time (MT), and peak area. The peaks were annotated with putative metabolites from the Human Metabolome Technologies metabolite database based on their MTs in CE and m/z values determined by TOFMS. The tolerance ranges for the peak annotation were
configured at ±0.5 min for MT and ±10 ppm for m/z. In addition, concentrations of metabolites were calculated by normalizing the peak area of each metabolite with respect to the area of the internal standard and by using standard curves, which were obtained by three-point calibrations. Principal component analysis to cluster samples based on variance was performed.

**mRNA quantification**

Total RNA was isolated from myocardial tissues by using an RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA) and mRNA levels were determined as previously reported. First-strand sDNA was synthesized using a SuperScript VILO™ cDNA synthesis Kit (Life Technologies). DNA amplification was performed in ABI PRISM7500 (Life Technologies) by using Taqman Universal PCR Master Mix (Applied Biosystems, Inc) and Taqman gene expression assays for rat erythropoietin receptor (Rn00566533_m1) and β-actin (Rn00667869_m1, Applied Biosystems, Inc). All assays were performed in duplicate and by the standard curve method using serial cDNA dilution.

**Serum erythropoietin level**

Blood was sampled at the time of cannulation of the carotid artery during the surgical preparation, and serum erythropoietin levels were determined by using an ELISA kit (Access EPO, Beckman Coulter, Fullerton, CA).

**Cell culture and isolation of mitochondria**

H9c2 cells were obtained from ATCC (American Type Culture Collection). Cells were cultured in Dulbecco’s Modified Eagle Medium (4.5 g/L glucose) supplemented with 10% fetal bovine serum and antibiotics. Treatment with insulin-like growth factor-1 (IGF1, 50 nM) for 15 min was used for activating Akt. To examine the effects of malate-aspartate shuttle inhibition on mitochondrial Akt phosphorylation, H9c2 cells were incubated with aminooxyacetate (5 mM) or a vehicle for 45 min before addition of IGF1 to the medium, and the treatment was continued until the end of the experiment. Mitochondrial and cytosolic fractions of H9c2 cells were prepared by using a mitochondrial isolation kit (Pierce Biotechnology, Rockford, IL), and the fractions were used for immunoblotting.

**Statistical analyses**

Data are presented as means ± SEM. Differences between treatment groups were tested by 1-way or 2-way analysis of variance (ANOVA), and the Tukey post hoc test was used to test for multiple comparisons when ANOVA indicated significant differences. The difference was considered significant if the P value was <0.05. The relationship between serum creatinine concentration and infarct size was analyzed by the use of simple linear regression analysis. All of the above statistical analyses were performed using EZR software (Jichi Medical University, Saitama, Japan). Hemodynamic data were analyzed by 2-way repeated measures ANOVA using SigmaStat software.

**References**


Supplemental Table S1. Baseline profiles (Blood pressure study)

<table>
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<tr>
<th>Group</th>
<th>n</th>
<th>SBP (mmHg)</th>
<th>DBP (mmHg)</th>
<th>MBP (mmHg)</th>
<th>HR (bpm)</th>
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<tbody>
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<td>Sham</td>
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<td></td>
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<tr>
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<td>5</td>
<td>136± 4</td>
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<td>110± 5</td>
<td>361± 8</td>
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<td>CERA</td>
<td>4</td>
<td>134± 2</td>
<td>96± 1</td>
<td>109± 1</td>
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<td></td>
</tr>
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<td>109± 2</td>
<td>125± 1*</td>
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</tr>
<tr>
<td>CERA</td>
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<td>158± 2*</td>
<td>111± 4</td>
<td>127± 3*</td>
<td>365± 6</td>
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</table>

Data are mean±SEM.  * P<0.05 vs. Sham+Vehicle.
SBP= systolic blood pressure, DBP= diastolic blood pressure, MBP= mean blood pressure, HR= heart rate, CERA= chronic erythropoietin receptor activator
<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>HW (g)</th>
<th>BW (g)</th>
<th>Cr (mg/dl)</th>
<th>BUN (mg/dl)</th>
<th>Hb (g/dl)</th>
<th>EPO (mIU/ml)</th>
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</thead>
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<tr>
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<td>1.7±0.1</td>
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<td>17.5±0.2</td>
<td>0.2±0.1</td>
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<td>7.4±3.0</td>
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<td>Vehicle</td>
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<td>1.8±0.1</td>
<td>453±12</td>
<td>0.83±0.05*</td>
<td>47.9±3.0*</td>
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Data are mean±SEM. * P<0.05 vs. Sham+Vehicle. † P<0.05 vs. SNx+Vehicle.
HW= heart weight, BW= body weight, Cr= creatinine, BUN= blood urea nitrogen, Hb= hemoglobin, EPO= erythropoietin.
CERA= chronic erythropoietin receptor activator.
### Supplemental Table S3. Hemodynamic parameters (Protocol 1)

<table>
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<tr>
<th>Group</th>
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<th>Baseline</th>
<th>Ischemia</th>
<th>Reperfusion</th>
</tr>
</thead>
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<td><strong>HR (bpm)</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sham</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>7</td>
<td>418±14</td>
<td>390±15</td>
<td>363±13*</td>
</tr>
<tr>
<td>CERA</td>
<td>8</td>
<td>431±11</td>
<td>408±13</td>
<td>366±7*</td>
</tr>
<tr>
<td><strong>SNx</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>8</td>
<td>419±16</td>
<td>415±11</td>
<td>371±4*</td>
</tr>
<tr>
<td>CERA</td>
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<td>437±14</td>
<td>420±8</td>
<td>397±18*</td>
</tr>
</tbody>
</table>

| **MBP (mmHg)** | | | | |
| **Sham** | | | | |
| Vehicle | 7  | 89±4      | 93±6      | 92±5        |
| CERA    | 8  | 87±5      | 86±4      | 90±4        |
| **SNx** | | | | |
| Vehicle | 8  | 80±3      | 77±3      | 84±3        |
| CERA    | 7  | 86±3      | 87±5      | 91±5        |

Data are mean±SEM. *P<0.05 vs. Baseline.
HR= heart rate, MBP= mean blood pressure, Ischemia= 20 min after ischemia, Reperfusion= 120 min after reperfusion.
### Supplemental Table S4. Infarct size data (Protocol 1)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>AR (mm³)</th>
<th>IS (mm³)</th>
<th>IS/AR (%)</th>
</tr>
</thead>
<tbody>
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<td><strong>Sham</strong></td>
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</tr>
<tr>
<td>Vehicle</td>
<td>7</td>
<td>0.31±0.03</td>
<td>0.14±0.01</td>
<td>43.9±2.2</td>
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<tr>
<td>CERA</td>
<td>8</td>
<td>0.32±0.04</td>
<td>0.10±0.02</td>
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</tr>
<tr>
<td><strong>SNx</strong></td>
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<tr>
<td>Vehicle</td>
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<td>0.35±0.04</td>
<td>0.22±0.03</td>
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<td>CERA</td>
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<td>0.31±0.02</td>
<td>0.12±0.02</td>
<td>36.9±3.9†</td>
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</table>

Data are mean±SEM. * P<0.05 vs. Sham+Vehicle. † P<0.05 vs. SNx+Vehicle. AR= area at risk, IS= infarct size, IS/AR=infarct size as % of area at risk, CERA= chronic erythropoietin receptor activator.
Supplemental Table S5. Baseline profiles (Protocol 2)

<table>
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<th>Group</th>
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<th>BW (g)</th>
<th>Cr (mg/dl)</th>
<th>BUN (mg/dl)</th>
<th>Hb (g/dl)</th>
<th>EPO (mIU/ml)</th>
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<tr>
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<tr>
<td>Vehicle</td>
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<td>1.7±0.1</td>
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<tr>
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<td>1.7±0.1</td>
<td>464±7</td>
<td>0.89±0.08*</td>
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<tr>
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<td>79.1±14.2†</td>
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</table>

Data are mean±SEM. * P<0.05 vs. Sham + Vehicle. † P<0.05 vs. SNx + Vehicle.
HW= heart weight, BW= body weight, Cr= creatinine, BUN= blood urea nitrogen, Hb= hemoglobin, EPO= erythropoietin, CERA= chronic erythropoietin receptor activator.
**Supplemental Table S6. Hemodynamic parameters (Protocol 2)**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Baseline</th>
<th>Ischemia</th>
<th>Reperfusion</th>
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</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
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</tr>
<tr>
<td>Vehicle</td>
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<td>99± 5</td>
<td>94± 4</td>
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<tr>
<td>Vehicle</td>
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<td>78± 7†</td>
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</table>

Data are mean±SEM. *P<0.05 vs. Baseline. †P<0.05 vs. Sham Vehicle. ‡P<0.05 vs. Sham CERA. HR= heart rate, MBP= mean blood pressure, Ischemia= 20 min after ischemia, Reperfusion= 120 min after reperfusion.
# Supplemental Table S7. Infarct size data (Protocol 2)

<table>
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<tr>
<th>Group</th>
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<th>AR (mm$^3$)</th>
<th>IS (mm$^3$)</th>
<th>IS/AR (%)</th>
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<td></td>
</tr>
<tr>
<td>Vehicle</td>
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<td>0.31±0.02</td>
<td>0.12±0.01</td>
<td>37.6±2.2</td>
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<td>CERA</td>
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<td>45.8±3.9</td>
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</tr>
<tr>
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Data are mean±SEM. * P<0.05 vs. Sham+Vehicle.
AR= area at risk, IS= infarct size, IS/AR=infarct size as % of area at risk, CERA= chronic erythropoietin receptor activator.
Supplemental Table S8. Baseline profiles (Protocol 3)

<table>
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<tr>
<th>Group</th>
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<th>HW (g)</th>
<th>BW (g)</th>
<th>Cr (mg/dl)</th>
<th>BUN (mg/dl)</th>
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Data are mean±SEM. * P<0.05 vs. Bloodletting (-).

HW= heart weight, BW= body weight, Cr= creatinine, BUN= blood urea nitrogen, Hb= hemoglobin, CERA= chronic erythropoietin receptor activator.
Supplemental Table S9. Hemodynamic parameters (Protocol 3)

<table>
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<tr>
<td>Bloodletting</td>
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<tr>
<td><strong>MBP (mmHg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bloodletting</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-)</td>
<td>7</td>
<td>86±3</td>
<td>87±6</td>
<td>87±4</td>
</tr>
<tr>
<td>(+)</td>
<td>7</td>
<td>78±2</td>
<td>86±3</td>
<td>83±3</td>
</tr>
</tbody>
</table>

Data are mean±SEM. *P<0.05 vs. Baseline.
HR= heart rate, MBP= mean blood pressure,
Ischemia= 20 min after ischemia, Reperfusion= 120 min after reperfusion.
Supplemental Table S10. Infarct size data (Protocol 3)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>AR (mm³)</th>
<th>IS (mm³)</th>
<th>IS/AR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bloodletting</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-)</td>
<td>7</td>
<td>0.34±0.08</td>
<td>0.14±0.01</td>
<td>41.5±3.6</td>
</tr>
<tr>
<td>(+)</td>
<td>7</td>
<td>0.38±0.01</td>
<td>0.19±0.02</td>
<td>51.5±5.6</td>
</tr>
</tbody>
</table>

Data are mean±SEM. * P<0.05 vs. Bloodletting (-). AR= area at risk, IS= infarct size, IS/AR=infarct size as % of area at risk.
Supplemental Table S11. Concentration of metabolites in the myocardium

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Sham Vehicle</th>
<th>Sham CERA</th>
<th>SNx Vehicle</th>
<th>SNx CERA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose 6-phosphate</td>
<td>1021±257.5</td>
<td>1281±164.2</td>
<td>322±79.3</td>
<td>421±35.1</td>
</tr>
<tr>
<td>Glucose 1-phosphate</td>
<td>83±24.6</td>
<td>107±20.3</td>
<td>18±5.4</td>
<td>23±3.3</td>
</tr>
<tr>
<td>Fructose 6-phosphate</td>
<td>161±49.1</td>
<td>244±50.8</td>
<td>57±12.0</td>
<td>70±4.9</td>
</tr>
<tr>
<td>Fructose 1,6-diphosphate</td>
<td>145±36.4</td>
<td>115±43.6</td>
<td>100±24.6</td>
<td>100±12.2</td>
</tr>
<tr>
<td>Lactate</td>
<td>3039±585.1</td>
<td>2522±339.7</td>
<td>1986±240.7</td>
<td>1981±272.9</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>37.9±5.0</td>
<td>46.7±4.2</td>
<td>24.3±4.4</td>
<td>34.2±5.6</td>
</tr>
<tr>
<td>Lactate/Pyruvate</td>
<td>78.5±9.4</td>
<td>54.7±6.9</td>
<td>88.1±11.8</td>
<td>60.3±5.3</td>
</tr>
<tr>
<td>Malate</td>
<td>223±28.6</td>
<td>226±41.8</td>
<td>260±51.4</td>
<td>170±12.1</td>
</tr>
<tr>
<td>Aspartate</td>
<td>583±29.0</td>
<td>711±140.6</td>
<td>397±36.2*</td>
<td>632±62.6†</td>
</tr>
<tr>
<td>Malate/Aspartate</td>
<td>0.39±0.06</td>
<td>0.37±0.12</td>
<td>0.63±0.09</td>
<td>0.28±0.04†</td>
</tr>
<tr>
<td>Glutamate</td>
<td>6209±153.1</td>
<td>6407±207.6</td>
<td>6855±276.2</td>
<td>6854±237.3</td>
</tr>
<tr>
<td>NADH</td>
<td>17.4±2.2</td>
<td>16.6±2.5</td>
<td>22.8±1.3</td>
<td>16.9±1.0†</td>
</tr>
<tr>
<td>NAD*</td>
<td>873.2±41.9</td>
<td>830.4±62.0</td>
<td>920.6±32.3</td>
<td>951.8±49.5</td>
</tr>
<tr>
<td>NADH/NAD*</td>
<td>0.020±0.0025</td>
<td>0.020±0.0018</td>
<td>0.025±0.0013</td>
<td>0.018±0.0014†</td>
</tr>
</tbody>
</table>

Data are mean±SEM. Concentrations of all metabolites are expressed as nmol/g.
N = 5 in each group. *p<0.05 vs. Sham+vehicle. † p<0.05 vs. SNx+vehicle.
Supplemental Figure S1. Histological findings in the myocardium. Representative images of hematoxylin staining (A, B, C, D) and Azan staining (E, F, G, H) are shown. (A, E) Sham+Vehicle; (B, F) Sham+CERA; (C, G) SNx+Vehicle; (D, H) SNx+CERA. CERA = continuous erythropoietin receptor activator. Four animals in each treatment groups were examined and showed similar images.
Supplemental Figure S2. mRNA levels of the erythropoietin receptor in the myocardium. CERA = continuous erythropoietin receptor activator. a.u. = arbitrary unit. N=5 in each group.
Supplemental Figure S3. Protein levels of the erythropoietin receptor in the myocardium. EPO-R = erythropoietin receptor. CERA = continuous erythropoietin receptor activator. a.u. = arbitrary unit. N = 5 in each group.
Supplemental Figure S4. Effects of a single injection of CERA on reperfusion-induced activation of kinases. Representative blots (A) and summary data (B) of immunoblotting for signal molecules upon reperfusion in myocardial samples from Sham and SNx with or without a single injection of CERA. Myocardial samples were taken from areas at risk subjected to 20-min ischemia/5-min reperfusion. N = 4~5 in each group. a.u. = arbitrary units. *p<0.05 vs. Sham+vehicle