Hypertensive Hypertrophied Myocardium Is Vulnerable to Infarction and Refractory to Erythropoietin-Induced Protection

Toshiyuki Yano, Takayuki Miki, Masaya Tanno, Atsushi Kuno, Takahito Itoh, Akifumi Takada, Tatsuya Sato, Hidemichi Kouzu, Kazuaki Shimamoto, Tetsuji Miura

Abstract—The objective of this study was to examine the hypothesis that hypertensive hypertrophy is vulnerable to infarction and defective in cytoprotective mechanisms by modification of intracellular signaling and mitochondrial proteins. Myocardial infarction was induced by 20-minute coronary occlusion/reperfusion in spontaneously hypertensive stroke-prone rats (SHR-SPs) and their controls (Wistar-Kyoto rats [WKYs]). Infarct size expressed as a percentage of area-at-risk was larger by 29% in SHR-SPs than in WKYs. Pretreatment with erythropoietin (EPO) significantly limited infarct size in WKYs but not in SHR-SPs. Ca\(^{2+}\) retention capacity after reperfusion and limited infarct size in SHR-SPs to levels in WKYs. EPO induced phosphorylation of Akt, extracellular signal-related kinase, and glycogen synthase kinase-\(3\beta\) in the myocardium in both WKYs and SHR-SPs. EPO enhanced interaction of phospho-glycogen synthase kinase-\(3\beta\) and adenine nucleotide translocase on reperfusion in WKYs, although such an effect of EPO was not detected in SHR-SPs. The results suggest that enhanced opening of mitochondrial permeability transition pores by reactive oxygen species and modification of the signal downstream of phospho-glycogen synthase kinase-3\(\beta\) in the mitochondria underlie the increased vulnerability to infarction and the lack of anti-infarct tolerance by EPO, respectively, in hypertensive hypertrophied hearts.

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Key Words: signal transduction □ mitochondria □ permeability transition pore □ myocardial infarction

Myocardial infarct size after ischemia/reperfusion can be reduced by a number of interventions, including ischemic preconditioning (IPC), in animal hearts and human hearts. However, recent animal studies have shown that some comorbidities, such as postinfarct heart failure and type 2 diabetes mellitus, modify and/or disturb signaling mechanisms of cardiomyocyte protection, resulting in resistance to cardioprotective agents.\(^1\)\(^-\)\(^3\) Left ventricular hypertrophy (LVH) has been known for some time to increase mortality after acute myocardial infarction.\(^4\) However, effects of hypertensive hypertrophy on myocardial vulnerability to ischemia/reperfusion-induced necrosis and on myocardial responses to protective agents remain unclear.

In the present study, we aimed to examine the hypothesis that hypertensive ventricular hypertrophy increases myocardial vulnerability to necrosis and impairs cytoprotective signaling. The rationale for this hypothesis is 2-fold. First, regulation of the mitochondrial permeability transition pore (mPTP), opening of which triggers cell necrosis, is known to be modified by mechanical stress on the myocardium.\(^5\)\(^-\)\(^6\) Second, disruption or impairment of prosurvival signaling has been observed in the myocardium under mechanical stress.\(^1\)\(^,\)\(^2\)

As possible modifications induced by hypertensive hypertrophy, we focused on changes in phosphorylation of glycogen synthase kinase-3\(\beta\) (GSK-3\(\beta\)),\(^7\)\(^-\)\(^9\) a kinase on which multiple prosurvival signal pathways converge for inhibition of the mPTP.\(^10\) As a model of hypertensive LVH, we mainly used spontaneous hypertensive stroke-prone rats (SHR-SPs).

Materials and Methods
Details of methods are described in the Expanded Methods section in the online Data Supplement (please see http://hyper.ahajournals.org).

Animals
Male SHR-SPs and their controls (Wistar-Kyoto/Izm rats [WKYs]) at ages between 12 and 16 weeks were used in this study. In a part of the infarct size experiments, Sprague-Dawley rats were used to induce LVH by transverse aortic constriction (TAC).
Infarct Size Experiments
Myocardial infarction was induced by 20-minute coronary occlusion/2-hour reperfusion in vivo, and infarct size was expressed as a percentage of area at risk (%IS/AR).

Immunoblot Experiments
Hearts were isolated and perfused with buffer as reported previously,2,3 and ventricular tissues were sampled before and after ischemia/reperfusion. In this series of experiments, 25 minutes, instead of 20 minutes, was selected as the ischemia duration, because %IS/AR after 20-minute regional ischemia in vivo has been shown to be similar to that after 25-minute global ischemia in our isolated heart preparation.9,11

Ca2+ Retention Capacity
In isolated mitochondria, the amount of Ca2+ that can be taken up in response to repetitive Ca2+ loading without opening of the mPTP was determined as Ca2+ retention capacity (CRC). We used methods reported by Tissier et al12 with slight modification.

Statistics
All of the data are presented as mean±SEM. Differences between treatment groups were tested by 1-way or 2-way ANOVA, and the Student Newman-Keuls 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.

Results
Hypertension and LVH in SHR-SPs
Mean blood pressure measured in a conscious state using the tail-cuff method was higher in SHR-SPs than in age-matched WKYs (158.9±9.7 versus 96.7±3.6 mm Hg), as was heart rate (366±4 versus 306±12 beats per min). The ratio of heart weight:body weight (Table S1, available in the online Data Supplement) and posterior wall thickness of the left ventricle assessed by echocardiography (2.15±0.05 versus 1.59±0.02 mm) were significantly larger in SHR-SPs than in WKYs, indicating LVH.

Infarct Size and Its Response to Cardioprotective Ligands in Hypertensive Hypertrophied Hearts
In all of the protocols of infarct size experiments, mortality rates after coronary occlusion (8.3% to 9.5%) did not significantly differ between the study groups.

In the first protocol, effects of pretreatment with erythropoietin (EPO); effects of a δ-opioid receptor agonist (D-Ala2,D-Leu5)-enkephalin acetate (DADLE), which is a Jak2-activating ligand like EPO; and effects of IPC on infarct size in WKYs and SHR-SPs were determined. Pretreatments did not significantly affect time courses of heart rates and blood pressures (Table S2). Risk area sizes were comparable in WKYs and SHR-SPs with or without treatment (Table S1). As shown in Figure 1, EPO reduced %IS/AR in WKYs. Infarct size in the SHR-SP control group was larger by 29% than that in the WKY control group, and EPO failed to limit infarct size in SHR-SPs. No protection was detected for pretreatment with a 3-fold higher dose of EPO (ie, 15 000 U/kg) in post hoc experiments (%IS/AR: 65.1±8.5%; n=3). Administration of DADLE before ischemia reduced infarct size in WKYs but not in SHR-SPs. In contrast, IPC afforded significant infarct size limitation in both SHR-SPs and WKYs.

In the second protocol, we assessed the effects of inhibition of reactive oxygen species (ROS) by N-(2-mercaptopropionyl)-glycine (MPG) on infarct size in SHR-SPs. MPG reduced mean blood pressure by ~25 mm Hg but not heart rate (Table S3). MPG significantly reduced %IS/AR in SHR-SPs, but no additive reduction of infarct size was achieved by MPG plus EPO (Figure 2 and Table S4).

To confirm that association of loss of myocardial response to EPO with hypertensive LVH, we used rats with TAC in the third protocol. Twenty-eight days after TAC, the ratio of heart weight:body weight (4.89±0.02 mm) were larger than in the sham-operated group but not in the TAC group (Figure S1).

Receptor-Mediated Phosphorylation of Prosurvival Kinases
Under baseline conditions, levels of total and phosphorylated forms of Akt, extracellular signal-related kinase-1 and -2 (ERK1/2), and GSK-3β were similar in SHR-SPs and WKYs (data not shown). Activation of the EPO receptor induced phosphorylation of myocardial Akt, ERK1/2, and GSK-3β similarly in both WKYs and SHR-SPs (Figure 3A and 3B).

Interaction of Phospho-GSK-3β With ANT on Reperfusion
Protein levels of regulatory subunits of the mPTP, adenine nucleotide translocase (ANT), voltage-dependent anion channel and cyclophilin D (CypD) in the myocardium under baseline conditions were comparable between WKYs and
SHR-SPs (Figure S2). ANT coimmunoprecipitated with phospho-GSK-3β on reperfusion was increased by pretreatment with EPO in WKYs but not in SHR-SPs (Figure 3C). This augmented carbonylation of mitochondrial proteins in SHR-SPs was attenuated by IPC but not by EPO (Figure 4C).

Effects of Ischemia/Reperfusion on Threshold for Opening of the mPTP
CRC, an index of threshold for mPTP opening, under baseline conditions tended to be lower in SHR-SPs than in WKYs, although the difference did not reach statistical significance (Figure 5). Twenty-five-minute ischemia/10-minute reperfusion reduced CRC, and CRC after ischemia/reperfusion was significantly lower in SHR-SPs than in WKYs (Figure 5B). Treatment with MPG attenuated the reduction of CRC after reperfusion in SHR-SPs to a level comparable with CRC in WKYs (Figure 5C).

Discussion
Enhanced Vulnerability of Hypertensive Hypertrophied Myocardium to Infarction
Earlier studies have shown that some types of LVH increase myocardial necrosis during ischemia/reperfusion. However, the mechanism underlying the vulnerability to infarction has been poorly understood. In a model of volume overload–induced LVH, CRC of mitochondria was significantly reduced, which was associated with increase in both expression of CypD and its binding to mitochondrial membranes. The change in CypD expression appears to be causally related to reduced CRC, because CypD binding to the mPTP increases sensitivity of this channel to Ca$^{2+}$, a major stimulus of mPTP...
In the present study, infarct size after ischemia/reperfusion was larger by \( \approx 30\% \) in hypertrophied hearts of SHR-SPs than in hearts of WKYs (Figure 1). Neither CypD expression nor CRC of mitochondria in SHR-SPs was different from those in WKYs under baseline conditions, but reduction of CRC after ischemia/reperfusion was augmented in SHR-SPs (Figure 5). Interestingly, MPG eliminated the change in mitochondrial and also reduced infarct size to a level comparable to the size in untreated WKY controls. These findings suggest that enhanced production of ROS lowers the threshold for mPTP opening, leading to increase in myocardial necrosis after ischemia/reperfusion in SHR-SPs.

Earlier observations support the contribution of ROS to CypD-mPTP interaction during ischemia/reperfusion in the myocardium. McStay et al. examined the effects of different thiols reagents and an oxidative protocol on thiol residues in ANT, a regulatory subunit of the mPTP, and on mPTP opening in mitochondria. Their results suggest that cross-linking between Cys160 and Cys257 in ANT increases CypD binding to this molecule. In the present study, carbonylation of mitochondrial proteins <43 kDa, which include ANT, inorganic phosphate carrier, and other mPTP subunits, after ischemia/reperfusion was at a significantly higher level in SHR-SPs than in WKYs (Figure 4). This finding is consistent with the results obtained by McStay et al. and also with an observation by Kalenikova et al. that the ROS level assessed by tissue 2,3-dihydroxybenzoic acid after 30-minute ischemia/reperfusion was 1.8-fold higher in SHR-SP hearts than in WKY controls.

The finding that SHR-SPs had larger infarcts than those in WKYs was consistent with results of an earlier study using SHR rats by Dai et al. In contrast, infarct size did not differ between rats with TAC and sham-operated controls, although TAC induced LVH, the extent of which was similar to LVH in SHR-SPs. The reason for the difference between SHR-SPs and rats with TAC remains unclear, although different features between the 2 models of LVH (e.g., duration of pressure overload) are possibly involved.

### Loss of Myocardial Response to EPO in Hypertensive Hypertrophied Myocardium

The present study showed for the first time that pressure-overload LVH impairs myocardial response to activation of receptors that trigger cardioprotective signals. Loss of myocardial response in SHR-SPs was not specific to EPO but was observed also to an agonist of the \( \delta \)-opioid receptor, a G protein–coupled receptor that also activates Jak2 (Figure 1). Studies using healthy animals have demonstrated that Jak2-phosphatidylinositol 3-kinase-Akt-GSK-3\( \beta \) signaling plays a major role in infarct size limitation by EPO receptor activation. Interestingly, Akt, ERK, and GSK-3\( \beta \) phosphorylated by EPO receptor activation before ischemia are dephosphorylated during sustained ischemia to levels comparable to those in untreated controls, but they are rephosphorylated to significantly higher levels after reperfusion than those in controls. The importance of enhanced phosphorylation of Akt, ERK, and GSK-3\( \beta \) at the time of reperfusion has been indicated in protection afforded by IPC and EPO, and GSK-3\( \beta \) phosphorylated by Akt, ERK, and protein kinase C-\( \epsilon \) has been shown to inhibit the mPTP opening. In SHR-SPs, EPO induced phosphorylation of Akt, ERK, and GSK-3\( \beta \) as in WKYs, but interaction of phospho-GSK-3\( \beta \) with ANT at the time of reperfusion was not increased by EPO (Figure 3A). ANT plays a crucial role in transport of ATP generated by mitochondria for energy-consuming processes in the cytosol and also in regulation of the threshold for opening of the mPTP. Although the functional outcome of phospho-GSK-3\( \beta \)-ANT interaction
remains unclear, the present findings indicate that signaling downstream of mitochondrial GSK-3β phosphorylation at the time of reperfusion is modified in SHR-SPs.

Signaling defects in the translocation of phospho-GSK-3β to mitochondria14 or in the mechanism of phosphorylation of GSK-3β preexisting in mitochondria18 might be involved in the failure for EPO to increase phospho-GSK-3β-ANT interaction. Another possibility is ROS-induced GSK-3β dephosphorylation on reperfusion.21,22 Nevertheless, regulation of intracellular translocation and interaction of GSK-3β with proteins within mitochondria need to be further investigated.

Different Responses to EPO and IPC in Hypertensive Hypertrophied Myocardium

In contrast to EPO, IPC could limit infarct size in SHR-SPs. The most plausible explanation for the difference is that IPC activates multiple classes of receptors, leading to activation of redundant prosurvival signal pathways.18,23 Repetition of IPC augments activation of redundant signal pathways so that blockade of a single signaling pathway in IPC does not abrogate protection because of compensation by other signal pathways. For example, blockade of bradykinin B2 receptor or inhibition of protein kinase C abolishes infarct size limitation by IPC with a single cycle of ischemia/reperfusion but does not affect protection afforded by IPC with multiple cycles of ischemia/reperfusion.18,23 IPC, but not EPO, suppressed levels of mitochondrial protein oxidation, which could have modified the threshold for opening of mPTPs in SHR-SPs (Figure 4). These findings suggest that some of the prosurvival signals provoked by IPC could bypass signaling defects downstream of the EPO receptor in SHR-SPs and suppress opening of the mPTP, leading to cytoprotection.

Perspectives

Results of the present study indicate that hypertensive ventricular remodeling not only increases vulnerability to infarction but also induces insensitivity to EPO and possibly other cardioprotective agents targeting mPTPs. Dysfunction of cardioprotective mechanisms in hypertensive patients and its impact on clinical outcomes warrant further investigation.

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Disclosures

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References


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Short title: Lack of EPO-induced protection in SHR-SP

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Expanded Methods

Infarct Size Experiments

Surgical preparation

Surgical preparation was performed as in our previous studies using rats. In brief, rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and ventilated with a rodent respirator (model 683, South Natick, MA) using room air and supplemented oxygen. Rectal temperature in each rat was maintained within 37.5°C ~38.5°C by the use of a heating lamp when necessary. The hearts were exposed via left thoracotomy, and a coronary snares was placed around the left coronary artery by using a 5-0 silk thread. Saline-filled catheters were placed in the jugular vein and the carotid artery for drug infusion and monitoring arterial blood pressure by the use of an SCK-590 pressure transducer (Nihon-Kohden, Tokyo, Japan), respectively. Precordial bipolar electrodes were placed for recording an electrocardiogram.

Protocols of infarct size experiments

Myocardial infarction was induced by 20-min coronary occlusion and 2-hr 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. In protocol 1, male spontaneous hypertensive stroke-prone rats (SHR-SP) and their controls (Wistar-Kyoto/Izm rats [WKY]) at ages between 12~16 weeks old were assigned to one of four treatments: no pretreatment (control), erythropoietin (EPO), [D-Ala², D-Leu⁵]-enkephalin acetate (DADLE) or ischemic preconditioning (IPC), as shown in Figure 1. EPO (human recombinant EPO, Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) and DADLE were administered intravenously at doses of 5,000 U/kg and 1 mg/kg, respectively, at 15 min before coronary occlusion. IPC was performed with two cycles of 5-min ischemia/5-min reperfusion. Since results of immunoblotting experiments suggested possible contribution of reactive oxygen species (ROS) to change in myocardial response to protection afforded by EPO, we examined the effects of a ROS scavenger, N-(2-mercaptopropionyl)-glycine (MPG), on infarct size with or without EPO pretreatment in protocol 2. In this protocol, MPG (20 mg/kg) was administered at 3 min before coronary artery occlusion and also at 3 min before reperfusion. Finally, to demonstrate whether inability in EPO-induced cardioprotection against myocardial infarction is a common phenomenon in the pressure-overloaded myocardium, the effect of EPO on infarct size was examined in Sprague-Dawley rats (11~12 weeks old) that received transverse aortic constriction (TAC) 28 days before coronary occlusion/reperfusion.

TAC

Induction of TAC was performed by the method of Kobayashi et al. with slight modifications. Under pentobarbital anesthesia and mechanical ventilation (as described in Surgical preparation), left thoracotomy was performed in Sprague-Dawley rats. To induce TAC, an 18-gauge needle was placed alongside the aortic arch and both the needle and the aorta were tied together by using a 3-0 silk thread between the first and second branches of the aortic arch. The needle was then removed, yielding an internal diameter of 0.8 mm. Sham operated rats served as control. At 28 days after the operation, cardiac dimensions and function were assessed by echocardiography and rats were used for infarct size experiments.

Infarct size measurement
After 2 hours of reperfusion, 200 U of heparin was administered intravenously and the heart was quickly excised. The excised heart was mounted onto a Langendorff apparatus and perfused with saline to wash out blood, and then the coronary artery was reoccluded. 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. Areas of infarct and those of region at risk were determined by SigmaScan (SPSS, Chicago, IL). Their volumes were obtained by multiplying each area by 1.5 mm, i.e., the thickness of the heart slice.

Immunoblotting Experiments
Isolated heart preparation
An isolated perfused heart preparation was selected for immunoblotting and immunoprecipitation experiments since concentrations of pharmacological agents can be strictly controlled. Rats were anesthetized as per the infarct size protocol and hearts were quickly excised and perfused at a pressure of 75 mmHg with non-circulating Krebs-Henseleit buffer (NaCl 118.5, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, NaHCO3 24.8, CaCl2 2.5 and glucose 10 mmol/L). The buffer was gassed with 95% O2/5% CO2, and the temperature of the perfusate was maintained at 38°C. A fluid-filled latex balloon with a PE-50 polyethylene tube was inserted into the left ventricle and was connected to an SCK-590 transducer. Heart preparations were stabilized for 30 min before entry to the tissue sampling protocol.

Protocols of tissue sampling
In protocol 1, biopsy samples (0.2~0.3 g) were taken from the left ventricle under the baseline condition and at 15 min after EPO infusion (10 U/ml). Tissues were snap-frozen in liquid nitrogen and stored at -80°C until biochemical analysis. In protocol 2, hearts were assigned to one of four pretreatments before 25-min global ischemia: no treatment (controls), IPC with two cycles of 5-min ischemia/5-min reperfusion, infusion of EPO (10 U/ml) or infusion of MPG (1 mM). EPO was infused for 15 min commencing at 15 min before global ischemia. Infusion of MPG was commenced at 10 min before global ischemia and continued until 5 min after reperfusion. At 5 min after reperfusion following 25-min global ischemia, ventricular tissues were sampled for immunoprecipitation and immunoblotting. In this series of experiments, 25 min, instead of 20 min, was selected as the ischemia duration because infarct size as a percentage of area at risk (%IS/AR) after 20-min regional ischemia in vivo has been shown to be similar to that after 25-min global ischemia in our isolated heart preparation. In protocol 3, rats were assigned into no pretreatment, EPO infusion or IPC before 25-min global ischemia and tissues were sampled for determination of mitochondrial protein carbonylation.

Immunoblotting and immunoprecipitation
To obtain total homogenate, frozen heart samples were homogenized in ice-cold buffer containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na2EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4, 1 µg/ml leupeptin, 50 µg/ml phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail (Complete mini, Roche Molecular Biochemicals, Mannheim, Germany). The homogenate was centrifuged at 13,000 g for 15 min to obtain the supernatant. Protein concentration was determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA).

Equal amounts of proteins were electrophoresed on 10% 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 phosphorylated Akt (Ser473) and total Akt, phosphorylated ERK1/2 (Thr202/Tyr204) and total ERK1/2, phosphorylated GSK-3β (Ser9) and total GSK-3β (Cell Signaling Technology, Beverly, MA), and β-actin (Sigma-Aldrich, St Louis, MO). Immunoblotted proteins were visualized by using an Immobilon Western detection kit (Millipore, Billerica, MA) and quantified by a lumino-image analyzer LAS-2000mini (Fujifilm, Tokyo, Japan).

To immunoprecipitate adenine nucleotide translocase (ANT) or phospho-GSK-3β, mitochondria-enriched fractions were prepared by the method used in our previous study with slight modifications. Ventricular tissues were homogenized using a Polytron PT-3100 (Kinematica; Littau, Switzerland) in ice-cold MSE buffer A containing (in mmol/L) 225 mannitol, 75 sucrose, 1 EGTA, 20 HEPES-KOH pH 7.4, and a protease inhibitor cocktail. The homogenates was centrifuged at 1,000 g for 10 min, and then the supernatant was centrifuged at 10,000 g for 30 min. The 10,000 g mitochondria-enriched pellet was solubilized in ice-cold IP buffer containing 20 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 5 mmol/L NaNH3, 1 mmol/L EGTA, 1% Triton X-100, 0.5% NP-40, 50 mmol/L Na3VO4, 1 mmol/L PMSF and a protease inhibitor cocktail. For immunoprecipitation, 500 µg of protein was pre-incubated with 50 µl of protein G magnetic beads (New England Biolabs, Ipswich, MA) for 1 hr. After the beads had been discarded, the supernatant was incubated with mouse monoclonal rat ANT antibody (Calbiochem, San Diego, CA) or phospho-Ser9-GSK-3β antibody (Cell Signaling) for 1 hr and the mixture was then incubated with 50 µl of fresh beads for 1 hr. A magnetic field was applied to this IP mixture, and the supernatant was removed. The beads were washed 2 times using 500 µl of IP buffer, re-suspended in 30 µl of SDS sample loading buffer (125 mmol/L Tris-HCl [pH 7.5], 4.3% SDS, 30% glycerol, 10% β-mercaptoethanol, 0.01% bromophenol blue), and incubated at 70 °C for 5 min. Finally, 20 µl of the supernatant was taken after applying a magnetic field to the mixture and was used for immunoblotting.

Determination of mitochondrial protein carbonylation

In this series of experiments, mitochondrial fractions were prepared in accordance with the method by Clarke et al. with slight modifications. In brief, the tissue was quickly minced with scissors in isolation buffer (300 mmol/L sucrose, 2 mmol/L EGTA, 10 mmol/L Tris-HCl, 5 mmol/L EDTA, 1 mmol/L PMSF, pH 7.4) and homogenized by a Potter-type homogenizer. The homogenate was centrifuged at 2,000 g for 2 min, and the supernatant was recentrifuged at 10,000 g for 5 min to obtain the final mitochondrial pellet. The pellet was solubilized in anti-oxidant buffer (25 mmol/L Tris-HCl, 0.15 mmol/L NaCl, 2% CHAPS, 50 mmol/L DTT, 1 mmol/L PMSF and a protease inhibitor cocktail (pH 7.4). Carbonylated proteins in mitochondrial fractions were determined by using an OxyBlot™ protein oxidation detection kit (Chemicon International Inc., Temecula, CA). Two aliquots of each mitochondrial protein were denatured by adding 12% SDS, and one aliquot was derivatized with dinitrophenylhydrazine (DNPH) and the other aliquot (negative control) was incubated with derivatization control solution for 15 min. Samples were electrophoresed on 10% polyacrylamide gels and then blotted onto PVDF membranes. The blots were incubated with anti-DNP antibodies (1:150). Equal protein loading was confirmed by Coumassie blue-stained polyacrylamide gels.

Ca2+ Retention Capacity Experiments

Isolation of mitochondria

Ventricular tissues were sampled from isolated perfused hearts under the baseline conditions.
condition or at 10 min after reperfusion following 25-min global ischemia. In a group of hearts, infusion of MPG (1 mM) was commenced at 10 min before ischemia and continued until tissue sampling (i.e., at 10 min after reperfusion). Isolated mitochondria were prepared from tissue samples as previously reported with slight modification. In brief, ventricular tissues were immediately placed in ice-cold MSE buffer A containing (in mmol/L) 225 mannitol, 75 sucrose, 1 EGTA, and 20 HEPES-KOH (pH 7.4) and then finely minced with scissors. The minced samples were homogenized for 5 s using a Polytron PT-MR 3100 (Kinematica; Littau, Switzerland). The homogenates were centrifuged at 1,000 g for 3 min, and then the supernatant was centrifuged at 10,000 g for 10 min. The mitochondrial pellet was suspended in MSE buffer B containing (in mmol/L) 225 mannitol, 75 sucrose, 0.1 EGTA, and 20 HEPES-KOH (pH 7.4). Protein concentration was determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA).

Determination of Ca^{2+} retention capacity

Ca^{2+} retention capacity was fluorometrically analyzed under an energized condition according to methods by Tissier et al. and Gomez et al. with slight modification. Extramitochondrial Ca^{2+} in a suspension of mitochondria was determined by 1 μM Calcium Green-5N hexapotassium salt (Invitrogen, Carlsbad, CA) and a Perkin-Elmer LS 50 B spectrofluorimeter. Cuvette temperature was maintained at 25°C, and excitation and emission wavelengths of 506 and 532 nm, respectively, were used to monitor free Ca^{2+} levels. Isolated mitochondria (0.25 mg/ml) were incubated in Calcium Green-added respiration buffer containing (in mmol/L) 2 KH_{2}PO_{4}, 250 sucrose, 5 sodium succinate, 1 ATP, 0.125 ADP, and 10 HEPES-KOH (pH 7.5) for 90 sec, and then pulsing with 10 μM CaCl_{2} was performed under gentle stirring at intervals of 60 sec. Although Ca^{2+} is taken up by energized mitochondria, accumulated Ca^{2+} is released from the mitochondrial matrix when mPTPs open.

Animals and Chemicals

SHR-SP/Izm and WKY/Izm were obtained from Japan SLC (Tokyo, Japan). Recombinant human EPO was kindly provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). DADLE and MPG were purchased from Sigma-Aldrich (St Louis, MO, USA).
References


Table S1. Summary of heart weight and infarct size data in Protocol 1

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<th>Group</th>
<th>n</th>
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<th>IS, cm³</th>
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<td>0.28±0.02</td>
<td>0.13±0.01</td>
<td>44.2±2.8*</td>
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<tr>
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<td>1.51±0.03</td>
<td>4.07±0.09</td>
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<td>0.12±0.03</td>
<td>40.5±5.9*</td>
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<tr>
<td>IPC</td>
<td>5</td>
<td>1.28±0.07</td>
<td>3.73±0.28</td>
<td>0.30±0.03</td>
<td>0.04±0.02*</td>
<td>12.1±5.7*</td>
</tr>
<tr>
<td><strong>SHR-SP</strong></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>1.42±0.08</td>
<td>5.27±0.16*</td>
<td>0.33±0.02</td>
<td>0.24±0.02*</td>
<td>71.2±3.1*</td>
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<tr>
<td>EPO</td>
<td>5</td>
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<td>0.31±0.02</td>
<td>0.21±0.02*</td>
<td>67.4±4.7*</td>
</tr>
<tr>
<td>DADLE</td>
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<td>1.61±0.04</td>
<td>5.32±0.09*</td>
<td>0.32±0.03</td>
<td>0.22±0.02*</td>
<td>67.5±4.9*</td>
</tr>
<tr>
<td>IPC</td>
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<td>1.44±0.12</td>
<td>5.32±0.18*</td>
<td>0.30±0.02</td>
<td>0.06±0.01*†</td>
<td>20.8±1.6*†</td>
</tr>
</tbody>
</table>

* P<0.05 vs. WKY control,
†P<0.05 vs. SHR-SP control

HW = heart weight, BW = body weight, AR = area at risk, IS = infarct size, %IS/AR = IS as % of AR.
Table S2. Hemodynamic parameters in Protocol 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Baseline</th>
<th>Treatment</th>
<th>Ischemia</th>
<th>Reperfusion</th>
</tr>
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<tbody>
<tr>
<td><strong>WKY</strong></td>
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<tr>
<td>Control</td>
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<td>355±12</td>
<td>337±8</td>
<td>325±9*</td>
<td>317±9*</td>
</tr>
<tr>
<td>EPO</td>
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<td>332±10*</td>
<td>323±7*</td>
<td>320±12*</td>
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<tr>
<td>DADLE</td>
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<td>364±12</td>
<td>351±11</td>
<td>342±11</td>
</tr>
<tr>
<td>IPC</td>
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<td>353±10</td>
<td>365±8</td>
<td>343±16</td>
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<tr>
<td>MBP (mmHg)</td>
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</tr>
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</tr>
<tr>
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<td>86±3</td>
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<td>88±4</td>
<td>82±4</td>
<td>81±6</td>
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<tr>
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<td>80±7</td>
<td>88±5</td>
<td>79±8</td>
<td>88±10</td>
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<td><strong>SHR-SP</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
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<td>412±9</td>
<td>405±8</td>
<td>404±9</td>
<td>375±5*</td>
</tr>
<tr>
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<td>398±11</td>
<td>384±3</td>
<td>357±6*</td>
</tr>
<tr>
<td>DADLE</td>
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<td>435±10</td>
<td>413±13*</td>
<td>397±15</td>
<td>341±17*</td>
</tr>
<tr>
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<td>402±14</td>
<td>395±13</td>
<td>381±10*</td>
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<tr>
<td>MBP (mmHg)</td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>Control</td>
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<td>125±11</td>
<td>115±9</td>
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<td>EPO</td>
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<td>115±14</td>
<td>119±11</td>
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<tr>
<td>DADLE</td>
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<td>129±9</td>
<td>121±11</td>
<td>105±7</td>
<td>91±13</td>
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<tr>
<td>IPC</td>
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<td>122±12</td>
<td>127±11</td>
<td>115±14</td>
<td>130±16</td>
</tr>
</tbody>
</table>

Data are mean±SEM. * P<0.05 Baseline

HR= heart rate, MBP= mean blood pressure,
Treatment= 1 min before ischemia, Ischemia= 20 min after ischemia,
Reperfusion= 120 min after reperfusion
Table S3.  Hemodynamic parameters in Protocol 2.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Baseline</th>
<th>Treatment</th>
<th>Ischemia</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR-SP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>409± 7</td>
<td>402± 6</td>
<td>395± 9</td>
<td>368± 8</td>
</tr>
<tr>
<td>MPG</td>
<td>8</td>
<td>391± 9</td>
<td>381± 8</td>
<td>376± 4</td>
<td>361±11</td>
</tr>
<tr>
<td>MPG+EPO</td>
<td>5</td>
<td>405± 18</td>
<td>386± 21</td>
<td>391±16</td>
<td>367± 15</td>
</tr>
<tr>
<td>MBP (mmHg)</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>130± 9</td>
<td>128± 9</td>
<td>115± 6</td>
<td>127± 5</td>
</tr>
<tr>
<td>MPG</td>
<td>8</td>
<td>119± 9</td>
<td>92± 8</td>
<td>88± 6</td>
<td>106± 6</td>
</tr>
<tr>
<td>MPG+EPO</td>
<td>5</td>
<td>124±10</td>
<td>97±11</td>
<td>89± 8</td>
<td>106±14</td>
</tr>
</tbody>
</table>

Data are mean±SEM.
HR= heart rate, MBP= mean blood pressure,
Treatment= 1 min before ischemia,
Ischemia= 20 min after ischemia,
Reperfusion= 120 min after reperfusion
Table S4. Summary of heart weight and infarct size data in Protocol 2.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>HW, g</th>
<th>HW/BW, g/kg</th>
<th>AR, cm³</th>
<th>IS, cm³</th>
<th>%IS/AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR-SP</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>1.51±0.08</td>
<td>5.39±0.14</td>
<td>0.31±0.02</td>
<td>0.21±0.02</td>
<td>68.6±3.3</td>
</tr>
<tr>
<td>MPG</td>
<td>8</td>
<td>1.54±0.03</td>
<td>5.60±0.10</td>
<td>0.31±0.02</td>
<td>0.16±0.02</td>
<td>50.8±5.6*</td>
</tr>
<tr>
<td>MPG+EPO</td>
<td>5</td>
<td>1.73±0.04</td>
<td>5.65±0.11</td>
<td>0.33±0.04</td>
<td>0.18±0.04</td>
<td>52.1±7.0*</td>
</tr>
</tbody>
</table>

* P<0.05 vs. SHR-SP control

HW = heart weight, BW = body weight, AR = area at risk, IS = infarct size, %IS/AR = IS as % of AR
Figure S1. Effects of EPO on infarct size in hearts with TAC-induced ventricular hypertrophy.
EPO was administered intravenously at a dose of 5,000 U/kg at 15 min before coronary occlusion. Infarct size is expressed as a percentage of the area at risk. *P<0.05 vs. Sham Control. N = 4~5 in each group.
Figure S2. Expression of major mPTP subunit proteins in WKY and SHR-SP. Prohibitin, a marker of mitochondria fraction, was used as a loading control. Representative immunoblots (A) and group mean data (B) are presented. Level of each protein was normalized by prohibitin level in the same blot. IB = immunoblotting. a.u. = arbitrary unit. N = 7 in each group.