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

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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 of mitochondria, an index of the threshold for opening of the mitochondrial permeability transition pore, on reperfusion was reduced in SHR-SPs compared with that in WKYs. Suppression of reactive oxygen species by N-(2-mercaptopropionyl)-glycine increased Ca\(^{2+}\) retention capacity after reperfusion and limited infarct size in SHR-SPs compared with that in WKYs. EPO induced phosphorylation of Akt, extracellular signal-related kinase, and glycogen synthase kinase-3β in the myocardium in both WKYs and SHR-SPs. EPO enhanced interaction of phospho-glycogen synthase kinase-3β 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-3β in the mitochondria underlie the increased vulnerability to infarction and the lack of anti-infarct tolerance by EPO, respectively, in hypertensive hypertrophied hearts. (Hypertension. 2011;57:110-115.) ● Online Data Supplement

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-3β (GSK-3β).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, 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.

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 al 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±24 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)-enkephaline 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.

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.
weights, we separately determined levels of carbonylation in proteins below and above 43 kDa using a molecular weight marker in the blot (Figure 4). The level of carbonylation in proteins <43 kDa was significantly higher in SHR-SPs than in WKYs, whereas an intergroup difference was not detected for carbonylation of larger mitochondrial proteins (Figure 4B). 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.\textsuperscript{13,14} 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.\textsuperscript{6} 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\textsuperscript{2+}, a major stimulus of mPTP

![Figure 2: Effects of MPG on infarct size in SHR-SPs. A, Experimental protocol. Solid arrow indicates injection of EPO; open arrow, injection of MPG (20 mg/kg). B, Infarct size expressed as a percentage of the area at risk. *P<0.05 vs control. N=5 to 8 in each group.](#)

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).

**Carbonylation of Mitochondrial Proteins After Ischemia/Reperfusion**

Because major subunit proteins of the mPTP (eg, ANT and inorganic phosphate carrier) have relatively small molecular weights, we separately determined levels of carbonylation in proteins below and above 43 kDa using a molecular weight marker in the blot (Figure 4). The level of carbonylation in proteins <43 kDa was significantly higher in SHR-SPs than in WKYs, whereas an intergroup difference was not detected for carbonylation of larger mitochondrial proteins (Figure 4B). This augmented carbonylation of mitochondrial proteins in SHR-SPs was attenuated by IPC but not by EPO (Figure 4C).

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![Figure 3: Phosphorylations of prosurvival kinases and interaction of phospho-GSK-3β with ANT by EPO receptor stimulation. A, Representative immunoblots. B, Levels of phosphorylated forms of kinases were normalized by total kinase levels, and their changes after EPO infusion are expressed as percentages of baseline level. Base indicates baseline conditions; EPO, after treatment with EPO; solid bar, ERK1; stippled bar, ERK2. N=4 to 7 in each group. C, Protein levels of ANT coimmunoprecipitated with phospho-GSK-3β. Level of ANT in each sample was normalized by p-GSK-3β level determined by rebloking. *P<0.05 vs WKY control. a.u. indicates arbitrary unit (mean ANT/p-GSK-3β level in the WKY control group was defined as 1 U). N=5 to 6 in each group.](#)
opening. 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 mitochondria 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 Cyp-D-mPTP interaction during ischemia/reperfusion in the myocardium. \(^{15,16}\) McStay et al. \(^{15}\) examined the effects of different thiol 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 \(<43 \text{kDa}\), 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. \(^{15}\) and also with an observation by Kalenikova et al. \(^{16}\) 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 by Dai et al. \(^{14}\) 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 (eg, 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. \(^{3,9,11,17}\) 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. \(^{17}\) 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\(^{18}\) and EPO, \(^{3,9}\) and GSK-3\( \beta \) phosphorylated by Akt, ERK, and protein kinase C-\( \varepsilon \) has been shown to inhibit the mPTP opening. \(^{8}\) 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 \(^{19}\) and also in regulation of the threshold for opening of the mPTP. \(^{10,19}\) 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 mitochondria or in the mechanism of phosphorylation of GSK-3β preexisting in mitochondria 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. 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. 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. 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**


