Renalase is a novel target gene of hypoxia-inducible factor-1 in protection against cardiac ischaemia–reperfusion injury

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Aims
Renalase, an enzyme that can metabolize catecholamine, was recently reported to attenuate the ischaemia/reperfusion (I/R)-induced cardiac injury. This work was undertaken to investigate the functions and regulation mechanisms of renalase in protection against cardiac I/R injury.

Methods and results
An elevated level of renalase was found in C57BL/6 mice challenged with I/R injury. Then, we generated a mouse model with cardiac administration of cholesterol-conjugated renalase siRNA followed by I/R operation. The mice treated with renalase siRNA exhibited increased infarction size and decreased cardiac function compared with the scramble siRNA group. Subsequently, we identified four potential hypoxia-inducible factor-1 alpha (HIF-1α)-binding motifs in the promoter of renalase through bioinformatics approaches. Dual-luciferase reporter assay, electrophoretic mobility shift assay, chromatin immunoprecipitation assay, and western blot were conducted and demonstrated that renalase was a novel target gene of HIF-1α. Furthermore, administration of renalase reduced the infarct area and rescued the deterioration of cardiac function in myocardial HIF-1α knockdown mice subjected to I/R injury. In addition, the levels of norepinephrine in serum as well as nicotinamide adenine dinucleotide (NAD+) and ATP in myocardium were determined, which implied that cardiac protection of renalase against I/R may be related, at least in part, to its metabolism of catecholamine and regulation of energy.

Conclusion
These findings have revealed renalase as a novel target gene of HIF-1α in protection against myocardial I/R injury, which provided a basis for therapeutic strategies for enhancing cardiomyocyte survival in patients associated with ischaemic heart diseases.

Keywords
Renalase • Hypoxia-inducible factor-1 alpha • Ischaemia/reperfusion injury • Transcriptional regulation

1. Introduction
Ischaemic heart disease, a leading cause of death throughout the world, is the most common consequence of coronary artery disease.1 It has been established that restoration of blood flow within occluded coronary artery by mechanical or pharmacological intervention is an efficient way to limit infarct size and improve the clinical outcomes of myocardial infarction.2 However, reperfusion after ischaemia itself causes additional death of cardiomyocytes and increases infarct size in a process known as ischaemia/reperfusion (I/R) injury. Thus, it is urgent to investigate the underlying mechanisms and potential therapeutic strategies for I/R-induced myocardial damage. Interestingly, recent evidence has
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implicated the potential roles of renalase, a ubiquitous flavin adenine dinucleotide-containing amino oxidase, in I/R-triggered cardiac injury. Since first identified in 2005, renalase is reported to be synthesized not only in the kidney but also in cardiomyocytes, liver, and adipose tissues. It has slight amino acid similarities with monoamine oxidase A and B (MAO-A and -B), and is characterized by distinct function specificity. MAO-A and MAO-B are anchored to the outer mitochondrial membrane and metabolize intracellular catecholamines, whereas renalase is secreted into the blood and metabolizes circulating catecholamines. Renalase exerts a protective effect in response to mortality risk that appears to be correlated with increased oxidative stress and heightened sympathetic tone, including ischemic acute kidney injury, heart failure, stroke, and heart transplantation. The elevated renalase level under stress conditions suggests that it may be involved in the cardioprotection against hypoxia-related cardiac diseases, but the underlying molecular mechanisms remain largely unknown.

A major transcription factor involved in the molecular effects triggered by hypoxia is hypoxia-inducible factor-1 (HIF-1α), of which the biological activity is mainly determined by the HIF-1α subunit. Under hypoxic condition, HIF-1α translocates from cytoplasm to nucleus, dimerizes with HIF-1β, and binds to hypoxia response elements (HREs) containing the consensus sequence 5′-RCGTG-3′ to activate transcription of target genes. These target genes, such as VEGF, glycolysis enzymes, erythropoietin, and pyruvate dehydrogenase kinases, can promote cellular survival in low-oxygen conditions. Meanwhile, it is still valuable to identify novel target genes of HIF-1α in hypoxic status. In the present study, the functions and regulations of renalase were explored in myocardial I/R injury. We demonstrated, for the first time, that renalase was a hypoxia-responsive gene in cardiomyocytes and correlated with HIF-1α expression. Renalase attenuated cardiac function loss and reduced infarction size in myocardial HIF-1α knockdown mice suffering from I/R challenge. Moreover, we found that HIF-1α could directly bind to the promoter of renalase to facilitate its transcription. These findings provide evidence that renalase serves as a novel target gene of HIF-1α in mediating protection against myocardial I/R injury.

2. Methods

2.1 Ethics statement

All animal experiments were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th edition, 2011) and approved by the Ethics Committee of Tongji Medical College, the Huazhong University of Science and Technology, China.

2.2 In vivo myocardial I/R model

Male C57BL/6 mice, aged 6–8 weeks, were purchased from Beijing University (Beijing, China) and maintained on a chow diet in a 12-h light/12-h dark environment at 25°C at the Tongji Medical School Animal Care Facility. Surgical induction of myocardial I/R was performed as previously described. Briefly, mice were anaesthetized by an intraperitoneal injection with pentobarbital sodium (50 mg/kg, P3761, Sigma), orally intubated, connected to a rodent ventilator, and placed in the supine position. A left thoracotomy was performed. Left anterior descending (LAD) coronary artery was visualized and ligated using a 6-0 silk suture around fine PE-10 tubing with a slip knot. Mice were subjected to 30 min of LAD ischaemia followed by varying periods of reperfusion, respectively. In the sham group, a suture was passed under the LAD but not tied. After experiments, mice were euthanized with pentobarbital sodium (200 mg/kg) by an intraperitoneal injection, and the ischaemic-reperfused tissue was isolated and used for further assays. Before sacrifice, echocardiographic analysis was performed.

2.3 In vivo delivery of siRNA and renalase recombinant protein

In vivo siRNA, delivery was performed as previously described. Mice were anaesthetized with pentobarbital sodium (50 mg/kg) by an intraperitoneal injection, orally intubated, and connected to a rodent ventilator. The cholesterol-conjugated-specific siRNAs for renalse or HIF-1α (Ribobio Co., Guangzhou, China) were intramuscularly injected into the left ventricle (LV) in approximately five sites before LAD occlusion. This occlusion established infarction within the injected zone (left ventricular front wall proximal to the apex). After 12 h of siRNA injection, mice were subjected to I/R operation. Western blotting and real-time RT-PCR were performed at 12 h after operation, and echocardiography was detected at 24 h. Scramble siRNA served as a negative control.

Renalase recombinant protein (0.5 mg/kg, H00055328-P01, Abnova, Walnut, CA, USA) was injected 15 min before or after I/R operation, respectively, through the tail vein. Twice injections were performed to increase the amount of blood protein level and to prolong its action. Saline was injected as a negative control.

The methods for infarct size, haemodynamic, and echocardiographic analyses were described in Supplementary material online, Materials and Methods. Levels of nicotinamide adenine dinucleotide (NAD) and ATP in myocardium as well as the activity of norepinephrine and renalase in serum were also detected according to the manufacturer’s instructions.

![Figure 1](image-url) Enhanced renalase expression in myocardial I/R injury. The mRNA (A) and protein (B) levels of renalase and serum renalase activity (C) at 0, 6, 12, or 24 h after myocardial I/R injury in C57BL/6 mice (n = 6). *P < 0.05 vs. I/R 0 h group.
Human cardiomyocytes (HCMs, Cat. No. 6200) were purchased from ScienCell Research Laboratories (San Diego, CA, USA). HCM cells were cultured in commercial Cardiac Myocyte media in poly-lysine-coated plates (no. 6201, ScienCell Research Laboratories), according to the manufacturer’s recommendation. HCM cells were characterized by IF or flow cytometry with antibody α-actinin, and the purity of HCM cells used in our study was over 96% (see Supplementary material online, Figure S1A). A cardiac muscle cell line HL-1 cells, derived from the AT-1 mouse and atrial cardiomyocyte tumour lineage, were cultured in Claycomb medium (JRH Biosciences, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 2 mM L-glutamine (Gibco, USA), and 1% antibiotics (penicillin and streptomycin). The cells were incubated in a humidified condition with 5% CO₂ at 37°C.

**Figure 2** Protective roles of renalase in myocardial I/R injury. Representative images of LV slices from C57BL/6 mice in different groups (n = 6) (Ai). Quantification of myocardial infarct size/area at risk (MI/AAR) and AAR/LV (Aii and iii, respectively). Data are expressed as the mean ± SD, *P < 0.05 vs. I/R + vehicle group; #P < 0.05 vs. I/R + scramble siRNA group. Representative M-mode images of LV from C57BL/6 mice in different groups (n = 6). (Bi) Quantification of EF and FS (Bii and iii, respectively). Data are expressed as the mean ± SD, *P < 0.05 vs. sham group; #P < 0.05 vs. I/R + vehicle group; &P < 0.05 vs. I/R + scramble siRNA group.
Hypoxia/reoxygenation (H/R)-induced cardiomyocyte injury was performed as described previously.19 Cells were cultured in regular media or D-Hanks solution in a modular incubator chamber (Forma Scientific) with 1% O2, 5% CO2, and 94% N2 for 4, 8, 12, or 24 h. In addition, after suffering from hypoxia for 12 h, cells were exposed to atmosphere of 21% O2, 5% CO2, and 74% N2, and cultured with complete medium for another 2, 6, 12, or 24 h.

Methods for cell culture, dual-luciferase reporter assay, western blot, quantitative real-time RT-PCR, chromat immunoprecipitation (ChiP) assay, electrophoretic mobility shift assay (EMSA) was included in Supplementary material online, Materials and Methods.

2.5 Statistical analysis
Data representative of at least three independent experiments were expressed as the mean ± SD. Differences were evaluated by unpaired Student’s t-test between two groups or one-way analysis of variance (ANOVA) for multiple comparison, followed by a post hoc Student–Newmann–Keuls test when necessary. All analyses were performed with SPSS 13.0 (SPSS, Chicago, IL, USA), and statistical significance was set at P < 0.05.

3. Results
3.1 Renalase was elevated during the myocardial I/R injury process
Previous studies have implied that renalase is closely related to ischaemic conditions such as stroke, heart transplantation, or acute kidney injury.3–7 To investigate the roles of renalase in myocardial I/R injury, we examined the expression of renalase in the myocardium. The results showed that renalase expression peaked at 12 h after I/R injury in both mRNA and protein levels (Figure 1A and B). The activity of renalase in serum was also investigated at different reperfusion time points following the I/R episode. As depicted in Figure 1C, renalase activity significantly elevated at 6 h, and peaked at 12 h after I/R injury, and decreased thereafter.

3.2 Protective roles of renalase in myocardial I/R injury
To further elucidate the significance of renalase elevation in myocardial I/R injury, we performed the in vivo knockdown experiments with cholesterol-conjugated siRNAs. The expression level of renalase was determined by western blot (see Supplementary material online, Figure S1B). Renalase was markedly reduced in the cholesterol-conjugated renalase siRNA treatment group. However, there was no significant difference in renalase protein expression between the renalase supplied group and vehicle group (see Supplementary material online, Figure S1B). Thus, we speculated that renalase might concentrate in blood circulation by tail vein injection, but not accumulate in the myocardium. In addition, we analysed the expression of renalase in different tissues, including heart, liver, spleen, lung, and kidney. We observed a significant reduction of renalase expression in hearts of mice treated with renalase siRNA. However, when compared with the scramble siRNA group, no significant difference was found in other tissues (see Supplementary material online, Figure S1C).

Thereafter, the siRNA specific for renalase was used to knockdown myocardial renalase expression prior to operation. This led to a significantly higher ratio of myocardial infarct size/area at risk (MI/AAR), when compared with that in the scramble siRNA-treated group (Figure 2Ai and ii). Meanwhile, the ratio of AAR to LV area was similar in each group (Figure 2Aiii), indicating a reproducible ligation performed at the same level of left anterior coronary artery. Moreover, echocardiography was performed to determine the effects of renalase knockdown in cardiac function. Ejection fraction (EF) and fraction shortening (FS) were both remarkably reduced in the renalase knockdown group at 24 h after I/R surgery, compared with the scramble siRNA treatment group (Figure 2B). Importantly, administration of renalase recombinant protein by the tail vein injection restored the MI/AAR ratio and cardiac function as revealed by EF and FS (Figure 2B). Data of heart rate (HR) and left ventricular end-systolic and end-diastolic diameters and volumes were shown in Supplementary material online, Table S1.

In the mice I/R model, HR (bpm) and mean arterial pressure (MAP, mmHg) were recorded by means of cardiac catheterization at different...
time points, and the rate pressure product (RPP), an cardiac contractile index, was calculated (Table 1). Compared with the sham-operating group, mice suffering from I/R injury exhibited significantly lower RPP levels after 1 h of reperfusion. However, administration of renalase recombinant protein restored the RPP values (Table 1), indicating that renalase ameliorated the deterioration of cardiac function in I/R injury. Additionally, when renalase was knocked down prior to operation, serum level of norepinephrine increased, whereas myocardial NAD$^+$

**Figure 3** HIF-1α facilitated the transcription of renalase in hypoxic condition. Luciferase reporter constructs driven by the indicated renalase promoter fragments (0.1 μg) were transfected into HCM cells. Cells were cotransfected with HIF-1α expression plasmid (0.1 μg) or treated with CoCl$_2$ (200 μmol/L; 24 h), respectively. HRE1–4: hypoxia response elements 1–4, putative HIF-1α-binding sites in computer-assisted analysis; TSS: transcription start site (A). EMSA indicated the binding of HCM nuclear extracts to biotin-labelled HRE2 consensus in vitro. Cells were treated with CoCl$_2$ (50, 100, or 200 μmol/L; 24 h) (Bii), transfected with HIF-1α expression plasmid (Biii), or HIF-1α-specific siRNA (Biii), respectively. ChIP assay revealed the affinity of HIF-1α on renalase promoter in vivo, as shown by 2% agarose gel electrophoresis (Cii) and quantitative real-time PCR (Ciii). Cells were treated with CoCl$_2$ (200 μmol/L; 24 h) and transfected with HIF-1α expression plasmid or HIF-1α-specific siRNA, respectively. Data are representative of three independent experiments and expressed as the mean ± SD, *P < 0.05, **P < 0.01 vs. control group; #P < 0.05 vs. empty vector group; &P < 0.05 vs. scramble siRNA group.
and ATP levels declined. Treatment with renalase recombinant protein suppressed the up-regulation of norepinephrine and ameliorated the decreases in NAD$^+$ and ATP induced by I/R operation (see Supplementary material online, Figure S2). These results indicated that the protective roles of renalase in myocardial I/R injury may be related to the levels of norepinephrine as well as NAD$^+$ and ATP.

3.3 HIF-1α facilitated the renalase expression at transcriptional levels

To explore the mechanisms underlying the renalase elevation in myocardial I/R injury, we performed in silico analysis of the promoter sequence of renalase. Four putative HRE sequences (CGTG) were found and denoted as HRE1, HRE2, HRE3, and HRE4 (Figure 3A). Since it has been well established that HIF-1α is a key transcription factor that binds to HRE to mediate gene expression in I/R-induced cardiac injury,$^{11,20}$ it is rational to suspect that HIF-1α might regulate the renalase expression.

The 2000 bp renalase promoter-luciferase reporter construct and its truncations were further established and transfected into cultured HCM cells. The putative functions of these elements were further explored by HIF-1α overexpression or CoCl$_2$ (200 μmol/L, 24 h). HIF-1α overexpression and CoCl$_2$ treatment significantly induced luciferase activity of the −1948 bp renalase gene promoter, indicating that renalase was a putative HIF-1α target gene (Figure 3A). HIF-1α-dependent renalase promoter activation was maintained upon 5’ deletion to −1782 bp (Figure 3A). Further nucleotide deletion to −1395 bp resulted in a significant and incomplete reduction of renalase promoter activation by HIF-1α (Figure 3A). Finally, deletion of −356 bp completely abolished the HIF-1α-induced activation of renalase promoter (Figure 3A). Similar results were obtained in cells incubated with CoCl$_2$. In line with these results, a mutation of HRE2 in renalase promoter significantly reduced the luciferase activity induced by HIF-1α overexpression as well as transactivation (Figure 3A). These results illustrated that 5’ to −1430 bp elements were involved in HIF-1α-dependent renalase promoter activation, with the HRE2 element mediating the majority of this response.

Additionally, to explore the influence of endogenous HIF-1α on renalase expression, the HCM cells were transfected with the renalase promoter reporter and exposed to hypoxia for 12 h in regular media or D-Hanks solution. In consistence with our previous results, the luciferase activities of renalase were also prominently increased under hypoxic conditions, and HRE2 played a crucial role in mediating these effects (see Supplementary material online, Figure S3). These results suggested an essential role of HIF-1α in transactivation of renalase.

To determine whether HIF-1α physically binds to the promoter of renalase, EMSA was performed in HCM cells by the use of biotin-labelled oligonucleotide probe containing HRE2 element in vitro. As shown in Figure 3Bi, HIF-1α agonist CoCl$_2$ facilitated the formation of HIF-1α–DNA complex. Forced overexpression of HIF-1α also increased its DNA-binding activity (Figure 3Bi). In contrast, knockdown of HIF-1α by specific siRNA decreased the formation of HIF-1α–DNA complex (Figure 3Bii). These results demonstrated that HIF-1α specifically binds to the renalase HRE2 site within renalase promoter in vitro.

ChIP was also performed in HCM cells to investigate the in vivo occupancy of renalase promoter by HIF-1α. In accordance with the EMSA results, the region spanning from −1552 to −1366 bp of renalase

**Figure 4** HIF-1α-induced renalase expression in HCM cells. The protein (A) and mRNA (B) levels of renalase in HCM cells treated with CoCl$_2$ (50, 100, or 200 μmol/L; 24 h). The protein (C) and mRNA (D) levels of renalase in HCM cells transfected with HIF-1α plasmid or specific siRNA, respectively. Data are representative of three independent experiments and are expressed as the mean ± SD. *P < 0.05 vs. control group; #P < 0.05 vs. scramble siRNA group; &P < 0.05 vs. empty vector group.
promoter was immunoprecipitated by the anti-HIF-1α antibody. This effect was enhanced by CoCl2 treatment or HIF-1α overexpression, and attenuated by knockdown of HIF-1α (Figure 3C). Taken together, these results illustrated that HIF-1α could directly bind to the HRE2 site within the renalase promoter to induce renalase transcription.

3.4 Activation of HIF-1α-induced renalase expression

HCM cells were incubated with different concentrations of HIF-1α agonist CoCl2. Results showed that CoCl2 significantly increased both mRNA and protein levels of renalase (Figure 4A and B). In addition, HCM cells were transfected with HIF-1α-expressing plasmid or HIF-1α siRNA, respectively. In consistence with our speculation, overexpression of HIF-1α significantly increased the protein and mRNA levels of renalase, whereas knockdown of HIF-1α attenuated the renalase levels in HCM cells (Figure 4C and D).

As numerous studies have demonstrated the accumulation and activation of HIF-1α in cells exposed to hypoxic conditions, it is rational to speculate that renalase could be induced in response to hypoxia. To clarify this speculation, HCM cells incubated in regular media were subjected to hypoxia for 4, 8, 12, or 24 h. Western blot was conducted and results showed that hypoxia induced a significant increase in expression of HIF-1α and renalase, as well as VEGFα and glucose transporter 1 (GLUT1), which have been verified as HIF-1α-dependent genes (Figure 5A i). In the cellular H/R model, cells were subjected to hypoxia for 12 h, and then followed by 2, 6, 12, or 24 h of reoxygenation. We observed that renalase expression peaked at 2 h after reoxygenation in regular media and decreased thereafter (Figure 4E ii). VEGFα served as a positive control (Figure 5A ii). Moreover, we performed similar experiments in HCM cells incubated with D-Hanks solutions. Results showed that HIF-1α protein was induced by hypoxia in a shorter period in D-Hanks solution and a delayed decrease was found in 6 h of reoxygenation (Figure 4F), implying that D-Hanks solution were more stressful. In consistence, high expression level of renalase was found after 6 h of reoxygenation in D-Hanks media. Similar results were obtained in HL-1 cell lines (see Supplementary material online, Figure S4A and B).

Furthermore, to explore the effects of HIF-1α knockdown on renalase expression under hypoxic status, knockdown of HIF-1α was performed prior to hypoxia in HCM cells. Hypoxia-induced up-regulation of renalase was attenuated when HIF-1α was knocked down in regular media or in D-Hanks solution, (see Supplementary material online, Figure S4C). Similar results could be obtained in HL-1 cell line (see

![Figure 5](image-url) - HIF-1α-induced renalase expression in H/R injury. (A and B) The protein expression of renalase, HIF-1α, VEGFα, and GLUT1 in HCM cells incubated in regular media subjected to hypoxia for 0, 4, 8, 12, or 24 h (A i) or reoxygenation for 2, 6, 12, or 24 h following 12 h of hypoxia (A ii). Similar experiments were repeated in D-Hanks solution (B). Data are representative of five independent experiments and are expressed as the mean ± SD, *P < 0.05 vs. control group; **P < 0.01 vs. control group.
Supplementary material online, Figure S4D). These results demonstrated that activation of HIF-1α induced renalase expression.

### 3.5 Renalase rescued the HIF-1α knockdown-induced deterioration of myocardial I/R injury in vivo

To further explore the roles of renalase in HIF-1α-mediated protection against myocardial I/R injury, we performed the rescue experiments via administration of renalase recombinant protein. When myocardial HIF-1α expression was knocked down via the cholesterol-conjugated siRNA prior to surgery, the ratio of MI/AAR was significantly higher compared with the scramble siRNA group (Figure 6Ai and ii). Administration of renalse recombinant protein to the myocardial HIF-1α knockdown mice by the tail vein injection resulted in a lower ratio of MI/AAR compared with the vehicle treatment group (Figure 6Ai and ii). The ratio of AAR to LV area was similar in each group (Figure 6Aiii), indicating that ligation was reproducibly performed at the same level of left anterior coronary artery.

Figure 6: Protective roles of renalse in HIF-1α knockdown myocardial I/R injury mice. Representative images of LV slices from mice in different groups (n = 6) (Ai). Quantification of MI/AAR (Ai) and AAR/LV (Aiii). Data are expressed as the mean ± SD, *P < 0.05 vs. I/R group; #P < 0.05 vs. I/R + HIF-1α siRNA + vehicle group. Representative M-mode images of LV from mice in different groups (n = 6) (Bi). Quantification of EF (Bi) and fraction FS (Biii). Data are expressed as the mean ± SD, *P < 0.05 vs. sham group; #P < 0.05 vs. I/R + scramble siRNA group; &P < 0.05 vs. I/R + HIF-1α siRNA + vehicle group.

Renalse is a target gene of HIF-1α.
Moreover, echocardiography was performed to determine the effect of HIF-1α and renalase in cardiac function after I/R injury. Data of heart rate and LV end-systolic and end-diastolic diameters and volumes were shown in Supplementary material online. Table S2. EF and FS were both remarkably reduced in the HIF-1α knockout group at 24 h after I/R compared with the scramble siRNA treatment group (Figure 6B). However, administration of renalase recombinant protein resulted in a remarkable amelioration of cardiac function, as shown by an increase of EF and FS compared with the vehicle group (Figure 6B). To meet this end, western blot and real-time RT-PCR revealed that knockdown of HIF-1α in heart significantly reduced the elevation of renalase induced by myocardial I/R injury, in which HIF-1α target genes including VEGFα and GLUT1 served as positive controls (Figure 7A and B). All these results indicated that knockdown of HIF-1α decreased the toleration for I/R challenge, which was rescued by renalase.

4. Discussion
Increasing evidence has implicated the potential roles of renalase in ischaemic heart disease, whereas we are at the very beginning to solve several problems. In the current study, we focused on the functions and regulation mechanisms of renalase in this process. Elevated myocardial renalase levels and enhanced renalase activity in serum were found in the I/R mouse model. Knockdown of renalase by siRNA in vivo caused poor toleration for cardiac ischaemia and developed serious myocardial necrosis, whereas treatment with recombinant renalase resulted in better cardiac phenotypes. Moreover, we demonstrated for the first time that renalase was a novel target gene of HIF-1α in protection against I/R injury.

Human renalase gene locates at chromosome 10 at q23.33 and encodes a conserved protein consisting 342 amino acids throughout evolution. Renalase was reported to efficiently degrade dopamine, followed by epinephrine, and norepinephrine. Previous studies have showed that infusion of renalase in rats causes a decrease in cardiac contractility, heart rate, and blood pressure, and prevents a compensatory increase in peripheral vascular tone. In our study, the norepinephrine content increased in the I/R group treated with renalase siRNA, and decreased when renalase was supplied via a tail vein. Therefore, it is likely that the cardioprotective effect of renalase is mediated, at least in part, by its ability to metabolize excess norepinephrine. Moreover, data showed that there is no difference of renalase expression between the vehicle group and renalase recombinant group. It implies that the effect of renalase on cardiac damage may rely on increased local norepinephrine levels in renalase siRNA-treated mice, but a whole body norepinephrine decline in recombinant protein-treated mice. In addition, the levels of NAD+/NADH and ATP, which were critically important in energy metabolism, electron transfer reactions, Ca2+ homeostasis, and thus cell survival, were also decreased when renalase was knocked down and increased following the recombinant renalase protein supply. This result was consistent with Wu’s study in a Langendorff-perfused isolated mouse heart model. Taken together, these data revealed that catecholamine, NAD+/NADH, and ATP were involved in renalase’s protection against ischaemia, while the underlying mechanisms need further investigation.

Reperfusion after ischaemia is associated with increased oxidative stress as the heart reverts to aerobic respiration and thereby generates toxic levels of reactive oxygen species (ROS), which subsequently activates HIF-1α. Tremendous amounts of research have been conducted to address the roles of HIF-1α in infarcted hearts. It has been revealed that HIF-1α overexpression induced by recombinant adenovirus ameliorated the I/R-induced injury, whereas studies regarding the I/R injury in the heterozygous HIF-1α knockout mice demonstrated no cardiac function loss, which may be due to the incompletely abolished HIF-1α expression in HIF-1α+/− mice. HIF-1α regulates the transcription of extensive repertoire of genes, including many involved in angiogenesis, glycolysis, apoptosis, and control of ROS in response to I/R injury. The transactivation of these genes, such as VEGFα, GLUT1, and inducible nitric oxide synthase (iNOS), contribute to the survival of ischaemic myocardium. Here, we illustrated a new mechanism that HIF-1α was involved in I/R injury through induction of renalase gene expression.

Interestingly, the decline of HIF-1α after reoxygenation in this study has taken longer (~2 h) when compared with previous studies (normally few minutes). In our study, cells were subjected to reoxygenation following 12 h of hypoxia in 1% O2, compared with 4 h of hypoxia in 0.5% O2 in Appelhoff et al.’s study. More time needed for decline of HIF-1α after reoxygenation in our study may be due to a longer time of hypoxia.

In conclusion, to our best knowledge, this is the first report to demonstrate that the protective roles of HIF-1α in I/R are mediated, at least in part, by increasing the transcription of renalase. These findings
have revealed renalase as a novel target gene of HIF-1α in protection against myocardial I/R injury, which provided a basis for therapeutic strategies for enhancing cardiomyocyte survival in patients associated with ischaemic heart diseases.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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**References**