Up-regulation of hypoxia-inducible factor-1α enhanced the cardioprotection effects of ischemic postconditioning in hyperlipidemic rats

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Hyperlipidemia is an independent risk factor in the development of ischemic heart disease, which can increase myocardial susceptibility to ischemia/reperfusion (I/R) injury. Ischemic postconditioning (PostC) has been demonstrated as a novel strategy to harness nature’s protection against myocardial I/R injury in normal conditions. However, the effect of PostC on hyperlipidemic animals remains elusive. It has been shown in our previous study that PostC reduces the myocardial I/R injury, and hypoxia-inducible factor-1α (HIF-1α) may play an important role in the cardioprotective mechanisms of PostC on normal rats. Here, we tested the hypothesis that the cardioprotection of PostC on hyperlipidemic rats is associated with the up-regulated HIF-1α expression. Male Wistar rats were fed with a high-fat diet for 8 weeks, and then randomly divided into five groups: sham, I/R, dimethyloxalylglycine (DMOG) + I/R, PostC, and DMOG + PostC group. The detrimental indices induced by I/R injury included infarct size, plasma creatine kinase (CK) activity and caspase-3 activity. The results showed that PostC could reduce the infarct size, when compared with the I/R group, which was consistent with the significant lower levels of plasma CK activity and caspase-3 activity. The results showed that PostC could reduce the infarct size, when compared with the I/R group, which was consistent with the significant lower levels of plasma CK activity and caspase-3 activity. The results showed that PostC could reduce the infarct size, when compared with the I/R group, which was consistent with the significant lower levels of plasma CK activity and caspase-3 activity. The results showed that PostC could reduce the infarct size, when compared with the I/R group, which was consistent with the significant lower levels of plasma CK activity and caspase-3 activity.

Keywords hyperlipidemia; ischemic postconditioning; hypoxia-inducible factor-1; DMOG
as well as necrosis and then lead to cardiac dysfunction. Hypoxia-inducible factor-1α (HIF-1α) is a transcriptional activator that regulates gene expression of various stress proteins involved in physiological and pathological adaptive responses to hypoxia [18,19]. It has been reported that HIF-1α is one of the first response elements to I/R injury at the molecular level [19]. Accordingly, HIF-1α may play a pivotal role in the endogenous protective mechanism against ischemia [20–22]. It has been reported that the expression of HIF-1α is significantly increased after PostC procedure [20,21]. However, it is still not clear whether this occurs in hyperlipidemic condition, and whether the up-regulation of HIF-1α is responsible for PostC-elicited cardioprotection. Here, we tested the hypothesis that cardioprotection with PostC in hyperlipidemic rats is associated with up-regulated HIF-1α expression.

Materials and Methods

Animals and diet
The experimental procedures were approved by the Institutional Animal Care and Use Committee of Shanxi Medical University (Shanxi, China). Sixty male Wistar rats weighing (110 ± 10 g) were included in the study. The rats were fed with a high-fat diet for 8 weeks [7]. At the very beginning of the experiment and the end of the 8 weeks of the high-fat diet feeding, blood samples were taken from the rats’ vena caudalis for the determination of plasma levels of cholesterol (TC) and triglycerides (TG).

Surgical preparation of animals
The rats were anesthetized by intraperitoneal injection of urthane (1 g/kg), and were mechanically ventilated with room air by using a small animal respirator through endotracheal intubation. After the chest was opened and the heart exposed, a silk suture was passed under the left anterior descending coronary artery (LAD) just below the left atrial appendage. The suture was threaded through a small plastic tube to create a snare, which was tightened or released to produce ischemia or reperfusion.

Experimental procedure
After 8 weeks of high-fat diet feeding, the rats were assigned randomly to five groups (n = 12): sham, rats were given a high-fat diet for 8 weeks (hyperlipidemic rats) and underwent the same surgical procedures except that the suture passed under LAD without being tightened; I/R, hyperlipidemic rats were subjected to 30 min of LAD occlusion followed by 180 min of reperfusion; dimethyloxalylglycine (DMOG) + I/R, animals were treated with DMOG (a HIF-1α prolyl hydroxylase inhibitor) at 40 mg/kg for 24 h before ischemia; PostC, at the onset of reperfusion, three cycles of 10 s reperfusion, and 10 s ischemia were given preceding the 180 min of reperfusion to the hyperlipidemic rats; DMOG + PostC, the hyperlipidemic rats were received DMOG at 40 mg/kg for 24 h before PostC [21].

Measurement of infarct size
At the end of reperfusion, LAD was re-ligated at its original site. Evans blue dye was then injected into the aortic root from femoral vein to stain the normally perfused region blue and outline the AAR. The atria and right ventricle were excised and discarded, and the left ventricle was cut into transverse slices. In each slice, the AAR was then separated from the non-ischemic zone and incubated in 1% triphenyltetrazolium chloride solution at 37°C for 15 min to differentiate necrotic (pale) from non-necrotic (red) area at risk [7,21]. The infarct size was measured and expressed as the percentage of AAR (infarction/AAR).

Plasma creatine kinase activity
Arterial blood was collected at the end of reperfusion for creatine kinase (CK) quantitation to confirm morphologic injury (necrosis). Samples were centrifuged at 3000 rpm for 10 min. The plasma supernatants were analyzed spectrophotometrically (SoftMax Pro Software; Molecular Devices, Sunnyvale, USA) for CK activity according to manufacturer’s instruction.

Determine of caspase-3 activity
The activity of caspase-3 was evaluated by using a colorimetric activity assay kit (BIOMOL, Houston, USA) according to manufacturer’s instruction. In brief, myocardial tissue from the area at risk was lysed with ice-cold buffer provided in the kit. The supernatant was incubated with caspase-3 substrate Ac-DEVD-pNA. A spectrophotometer (Molecular Devices) was used to measure the absorbance at 405 nm and the protein concentration in supernatant was determined. Data were expressed as the fold of the sham group.

Western blot assay for HIF-1α
Western blot assay for HIF-1α was carried out as described previously [7,21]. In brief, myocardial tissue samples (100 mg) from the area at risk were lysed with ice-cold buffer. The tissue lysate was centrifuged, mixed with loaded buffer, and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The samples were then transferred onto polyvinylidene difluoride membranes and subsequently exposed to rabbit polyclonal anti-rat HIF-1α antibody (1 : 200; Santa Cruz Biotechnology, Santa Cruz, USA). Bound antibody was detected by horseradish peroxidase-conjugated goat anti-rabbit IgG (1 : 1000 polyclonal; Santa Cruz Biotechnology). Specific antibody binding was detected by
using electrochemiluminescence. The densities of the scanned protein bands were measured by using image analysis software and data were expressed as percentage change of loading control.

Measurement of HIF-1α mRNA level by real-time polymerase chain reaction
The mRNA level of HIF-1α was measured by using real-time quantitative polymerase chain reaction (PCR) as described previously [7, 21]. Briefly, the primer sequences were as follows: sense 5'-ACTGAGATGATCTTG-3' and antisense 5'-CTACAGCTTCACCAAG-3' (GenBank accession No. NM_024359). The mRNA level of the gene of interest was normalized to β-actin (sense 5'-GGCTACAGCTTCACCAAG-3' and antisense 5'-TCAGGAGGGAATGCCTTG-3', GenBank accession No. NM_031144) and expressed as fold increase over control.

Statistical analysis
Data were expressed as means ± SEM and analyzed by analysis of variance. P < 0.05 was considered statistically significant.

Results
The levels of plasma lipid are increased
Eight weeks of high-fat feeding resulted in a dramatic increase in plasma TC, TG, and low-density lipoprotein levels (Table 1).

PostC significantly reduces infarct size induced by I/R
The AAR was comparable among all groups (Fig. 1A), indicating that a comparable degree of ischemia was induced among these groups. Infarct size induced by I/R in the hyperlipidemic rats was significantly increased, when compared with the sham group (38.36% ± 1.04% vs. 4.15% ± 1.16%, P < 0.05, Fig. 1B). After application of PostC, infarct size was significantly reduced relative to the I/R group (28.43% ± 1.66% vs. 38.36% ± 1.04%, P < 0.05, Fig. 1B).

Table 1 The blood fat levels of rats (mmol/l)

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>TC</th>
<th>TG</th>
<th>LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Weeks</td>
<td>60</td>
<td>2.17 ± 0.32</td>
<td>0.86 ± 0.23</td>
<td>1.33 ± 0.27</td>
</tr>
<tr>
<td>8 Weeks</td>
<td>60</td>
<td>3.23 ± 0.29**</td>
<td>1.65 ± 0.36**</td>
<td>2.32 ± 0.21**</td>
</tr>
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TC, total cholesterol; TG, triglyceride; LDL, low-density lipoprotein.
After fed with high diet for 8 weeks. The blood levels of TC, TG, and LDL showed a comparable degree in all groups before baseline level.
**P < 0.01 vs. 0 weeks.

PostC diminishes plasma CK activity
Consistent with the increase of infarct size, I/R increased CK activity in the hyperlipidemic group. However, PostC intervention significantly reduced CK activity (0.48 ± 0.11 vs. 0.68 ± 0.15 U/ml, P < 0.05, Fig. 1C).

PostC reduces myocardial caspase-3 activity
Apoptosis is an important phenomenon in the myocardial I/R injury and caspase-3 is identified as a key protein in the final pathway of cell apoptosis. Therefore, we detected caspase-3 activity to further illustrate the myocardial injury induced by I/R. As shown in Fig. 1D, the activity of caspase-3 was significantly increased in the I/R group, but reduced in the PostC group.

PostC increases myocardial tissue HIF-1α protein level but does not influence the level of mRNA
As shown in Fig. 2, in hyperlipidemic rats, the low HIF-1α protein level was detected in the sham group. I/R dramatically increased HIF-1α protein level, while PostC further enhanced the increase of HIF-1α protein expression induced by I/R. However, there were no significant differences in HIF-1α mRNA level among all groups, suggesting that the changes in HIF-1α expression were regulated at the post-translational level but not at the transcriptional level.

A linear relationship between HIF-1α level and infarct size
To demonstrate whether the up-regulation of HIF-1α level correlates to the infarct-sparing effect of PostC, a linear correlation analysis between infarct size and HIF-1α protein level was performed based on method reported previously [21]. As shown in Fig. 3, there was a linear inverse relationship between infarct size and HIF-1α protein level. Associated with the increase in HIF-1α protein level, the infarct size was accordingly decreased (r = −0.82, P < 0.01). This result suggested a role of HIF-1α up-regulation in PostC-elicted cardioprotection.

DMOG up-regulates HIF-1α protein level
It has been previously reported that DMOG, a pan-hydroxylase inhibitor, targets HIF-1α through suppressing the ubiquitination and proteasomal degradation to stabilize and activate HIF-α. We selected DMOG to demonstrate whether PostC-enhanced HIF-1α can be further modulated. As shown in Fig. 4, DMOG pre-treatment plus PostC further enhanced HIF-1α protein level than that plus I/R or PostC alone. However, DMOG pre-treatment before I/R or PostC did not show any effects on HIF-1α mRNA level.

DMOG enhances the cardioprotective effects of PostC
DMOG pre-treatment before I/R or PostC alone significantly reduced infarct size to a compatible level. As shown in Fig. 5,
Figure 1. Area at risk, infarct size, plasma CK, and caspase-3 activity after I/R and PostC in hyperlipidemic rats. After application of PostC, infarct size was significantly reduced relative to the I/R group (A, B). PostC intervention significantly reduced CK (C) and caspase-3 (D) activity relative to the I/R. *$P < 0.05$ vs. sham, $^#P < 0.05$ vs. I/R.

Figure 2. Expression of HIF-1α protein and mRNA after I/R and PostC. (A) The western blot image for HIF-1α, and equal loading of samples was verified by staining with β-actin-specific antibody. (B) No change in HIF-1α mRNA level was detected among all groups. *$P < 0.05$ vs. sham, $^#P < 0.05$ vs. I/R.
a reduction in infarct size by PostC was further enhanced when DMOG was given 24 h before PostC, averaging a 30% reduction compared with that in the PostC group alone. This result was consistent with a significant reduction in CK activity in these groups. Furthermore, DMOG pre-treatment plus PostC also significantly reduced caspase-3 activity.

**Discussion**

Hyperlipidemia is regarded as an independent risk factor of ischemic heart disease. It has been reported that hyperlipidemia increased the myocardial susceptibility to I/R injury [7,8,23], which was confirmed in the present study by using hyperlipidemic rat models induced by a 8-week feeding with high-fat diet. PostC has been demonstrated as a novel strategy to harness nature’s protection against myocardial I/R injury [9]. The cardioprotection with PostC has been well demonstrated from different laboratories in different species including human [9,24–29]. It has been reported that the effectiveness of PostC may be influenced in some disease states such as hypertension, diabetes, and hyperlipidemia. However, in our previous study, it has been found that hyperlipidemia does not prevent the cardioprotection by PostC against I/R injury [7].

The mechanisms responsible for the cardioprotection induced by PostC have been associated with a few of cellular and subcellular adaptive responses to I/R injury, including activation of survival kinases, inhibition of mitochondria permeability transition pore opening, and so on [30–32]. It has been observed in our previous study for the first time that HIF-1 may play an important role in the protective mechanisms of PostC in normal rat models [21].
HIF-1 is a principal transcription factor composed of a HIF-1α functional subunit and HIF-1β constitutional subunit, and has been reported to play a crucial role in the maintenance of oxygen homeostasis [33]. HIF-1β subunit is maintained at a constant level regardless of oxygen availability, but the expression and function of HIF-1α are regulated by cellular oxygen concentrations [34]. Under normoxic conditions, HIF-1α proteins are rapidly degraded by prolyl 4-hydroxylases (PHDs) in the presence of oxygen. Under hypoxic conditions, these enzymes fail to hydroxylate HIF-1α, which then results in the translocation of HIF-1α to nucleus to involve in hypoxic adaptation by regulating the expression of target genes.

In the present study, HIF-1α expression was maintained at a very low level in hyperlipidemic rats. Although I/R significantly increased HIF-1α expression, its level was markedly elevated by PostC. These findings were all consistent with those in normal rats [15]. In order to further identify the role of HIF-1α in PostC-elicited cardioprotection, we selected DMOG, a nonspecific inhibitor of PHDs, to demonstrate the effects of PostC by stabilizing HIF-1α protein. Results showed that DMOG pre-treatment before PostC further enhanced HIF-1α expression, when compared with PostC intervention alone. Furthermore, consistent with increased expression of HIF-1α, the injury induced by I/R was inhibited. A linear correlation between HIF-1α protein level and infarct size indicated a role of HIF-1α in the regulation of myocardial damage. These results indicated that the cardioprotection of PostC may be mediated by the up-regulation of HIF-1α. However, the pathway of HIF-1α-mediated PostC cardioprotective effects is currently unknown. More studies are needed to further to demonstrate whether some downstream target genes are activated after the up-regulation of HIF-1α protein by PostC.

To determine whether the change in HIF-1α protein expression in the PostC group is regulated at a transcriptional level, steady-state mRNA levels of HIF-1α were analyzed by a real-time PCR assay. In our experiments, no changes in the expression of HIF-1α mRNA were observed, suggesting that protective modulation of HIF-1α gene expression mainly happens at protein level. It has been demonstrated that the stability and activity of HIF-1α protein are chiefly controlled by post-translational events [35]. DMOG pre-treatment before PostC did not alter the level of HIF-1α mRNA, but significantly increased HIF-1α protein levels and reduced the myocardial injury.

In conclusion, our findings showed that PostC attenuated the myocardial I/R injury and up-regulated HIF-1α expression in hyperlipidemic rats, providing a new insight into protective mechanisms of PostC after ischemia and reperfusion under hyperlipidemic condition.

**Funding**

This work was supported by grants from the National Nature Scientific Foundation of China (No. 81100150 and No. 81170144) and the Nature Scientific Foundation of Shanxi Province (2012011040-1).
References