Original Article

Heme oxygenase-1 (HO-1) alleviates vascular restenosis after balloon injury in a rabbit carotid artery model

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Abstract: Percutaneous coronary intervention (PCI) is used commonly for coronary artery disease (CAD); however, restenosis is a proliferative response and frequent sequela to this treatment. Although the introduction of drug-eluting stents has convincingly reduced the incidence of vascular restenosis, restenosis remains a problem. The present study was designed to investigate the effects of the heme oxygenase-1 (HO-1) on restenosis formation after balloon injury in a rabbit carotid artery model. We found that involvement of the HO-1 in defensive restenosis formation was independent of the levels of blood lipid. Activation of HO-1 induced by chlorhematin treatment alleviated vascular restenosis after balloon injury in a rabbit carotid artery model, whereas inhibition of HO-1 by zinc protoporphyrin treatment exacerbated restenosis formation. Furthermore, overexpression of HO-1 inhibited nuclear factor kappa B subunit 1 (NF-κB) activity and decreased tumor necrosis factor-alpha (TNF-α) and endothelin 1 (ET-1) expression. In conclusion, our study provides preliminary data suggesting that HO-1 alleviates vascular restenosis after balloon injury in a rabbit carotid artery model by inhibiting NF-κB, TNF-α and ET-1 expression, indicating induction of HO-1 activation may be a feasible therapeutic target for treating vessels resistant to restenosis.

Keywords: HO-1, restenosis, balloon injury, activation

Introduction

Percutaneous coronary intervention (PCI) is a minimally invasive, safe, and effective treatment for atheroproliferative diseases, especially for acute coronary syndrome [1, 2]. PCI has become the coronary revascularization procedure most frequently performed worldwide. However, this procedure is plagued by a high incidence of restenosis, which is responsible for 30-50% of long-term failure [3, 4]. Although the introduction of drug-eluting stents has convincingly reduced the incidence of restenosis, vascular restenosis remains a problem. Dysfunction of the vascular endothelium induced by mechanical stimuli, tissue inflammation and proliferation of vascular smooth muscle cells (VSMCs) are essential for vascular restenosis [5, 6]. The pathobiology of restenosis comprises a complicated interaction among many vasoactive factors and inflammatory mediators. Prevention of restenosis and improved prognosis will require improved endothelial function, reduced inflammation and inhibited VSMC proliferation.

Heme oxygenase (HO) catalyzes the rate-limiting step in the oxidative degradation of cellular heme that liberates carbon monoxide (CO), iron and biliverdin. The protective effects of HO on the vascular system appear to be mediated in large part by the actions of its three metabolic byproducts through multiple distinct mechanisms, including antioxidant stress, inhibition of inflammatory responses, reduction of cellular proliferation, anti-apoptosis effects, anti-thrombogenic effects, normal angiostasis maintenance, cell signal transduction pathway regulation and more [7]. HO-1 is a stress-responsive effector that is highly induced by many agents, including some cytokines, endotoxin, stress, heat shock, ultraviolet radiation, ischemia-reperfusion, heavy metals, nitric oxide (NO) and heme [8]. In addition to its induced expression in various physiological and pathophysiological states in the vascular system, HO-1 plays an
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In the present study, we evaluated the efficacy of activation and inactivation of HO-1 on restenosis formation after balloon injury in a rabbit carotid artery model. We found that involvement of HO-1 in defensive restenosis formation was independent of the levels of blood lipid. Activation of HO-1 effectively suppressed vascular restenosis after balloon injury by inhibiting nuclear factor kappa B subunit 1 (NF-κB) activity and decreasing tumor necrosis factor-alpha (TNF-α) and endothelin 1 (ET-1) expression. On the contrary, inactivation of HO-1 exacerbated restenosis formation.

Materials and methods

Animals

Fifty-two adult New Zealand white rabbits (2.5~3.0 kg) purchased from the Animal Experiment Center of Zhejiang Chinese Medicine University). All animals were maintained on a 12/12-hour light/dark cycle at an ambient temperature of 20~25°C and 55~65% humidity. Food and water were provided ad libitum. All animals were treated according to the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources for the National Research Council. The study was approved by The Ethics Committee of the Affiliated Hospital of Guizhou Medical University (Guiyang, China).

Rabbit carotid artery model

The rabbits were randomly divided in four groups (n=13): sham group (S), operation group (O), operation + chlorhematin treated group (OC) and operation + zinc protoporphyrin treated group (OZ). All rabbits were received 1.5% cholesterol food for 5 weeks to produce the early-stage lesions of atherosclerosis. The OC and OZ groups were treated with chlorhematin (15 mg/kg/d, i.p., Sigma, CA, USA) and zinc protoporphyrin (45 µmol/kg/d, i.p., Sigma, CA, USA), respectively, whereas the S group was treated with the same dose of a vehicle (0.1% DMSO in saline per day, i.p. Sigma, CA, USA). After 2 weeks, a balloon-catheter injury to the right common carotid artery of the rabbits of O, OC and OZ groups was performed as described previously [12]. Briefly, a 2.5 French Fogarty balloon catheter (Baxter) was inserted through the external carotid artery, inflated with air to 6 standard atmospheres, and passed 3 times along the length (4 to 5 cm) of the isolated segment.

Blood collection and biochemical test

Fasted rabbits (12 h) were anesthetized with a mixture of ketamine (25 mg/kg) and xylazine (10 mg/kg) by intravenous injection. Blood from the ear vein was withdrawn into a heparinized syringe and centrifuged at 3,000 rpm for 20 min at 4°C. Serum triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) concentrations were determined enzymatically by using a kit (KHB, Shanghai, China). Optical density was measured using a spectrophotometer (BioPharma, UK) at 546 nm and 500 nm. Oxidized LDL (ox-LDL) was analyzed using a double-antibody sandwich method kit (Robio, Shanghai, China).

Tissue collection and immunohistochemical (IHC) analysis

Rabbits were anesthetized as previously described. The common carotid arteries were ligated at the third proximal to the carotid bifurcation. The distal part was divided in eight 5-mm segments and stored in liquid nitrogen. The injury segments of the carotid arteries were excised and cut into 10 fragments, which were then fixed overnight in 4% paraformaldehyde (Sigma, CA, USA) at 4°C and embedded in paraffin. The fragments were cut to 10 sections (4 µm per section). Hematoxylin and eosin (HE) staining was performed for analysis of neointimal hyperplasia. Neointimal and medial areas were measured in 2~9 individual sections (taken from the middle portion of the segments) by delineating the external elastic lamina, internal elastic lamina and endothelium. Neointima/media ratios were used to compare neointima formation among the various groups.

Immunohistochemical (IHC) detection of HO-1 or ET-1 in the 4-µm-thick vessel sections was performed using a 1:1, 000 dilution of polyclonal anti-rabbit HO-1 or ET-1 antibodies (Santa Cruz, CA, USA) overnight at 4°C. After the paraffin was removed from serial sections with xylene, sections were dehydrated with
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Enzyme-linked immunosorbent assay (ELISA)

Carotid arteries were homogenized in ice-cold saline. Then, the homogenate was centrifuged at 13,000 g at 4°C for 60 min. The levels of NF-κB and TNF-α were measured with the ELISA kit (Active Motif Ltd. USA, CA, USA) according to the manufacturer’s protocols.

Reverse transcription-PCR (RT-PCR)

The total mRNA from carotid artery segment was extracted using Trizol reagent (Roche, USA) and quantified using A160/280 absorption. The first-strand cDNA was synthesized with Oligo(dT)$_{12-18}$ primer (Takara, Japan). The RT-PCR assay was performed with a EmeraldAmp PCR Master Mix (Takara, Japan) according to the instruction manuals. A 118-bp fragment of rabbit HO-1 was amplified using the forward primer, 5'-GGAAGTAGAGCGG-GCGTAG-3'; a 543-bp fragment was also amplified for rabbit ET-1 using the forward primer, 5'-AAGATCCAGCCAGCATGGAGACC-3', and reverse primer, 5'-CGTGGCTCTGCTCTCCTGATG-3'. β-actin, which served as an internal control, was amplified for the 543-bp fragment using the forward primer 5'-CCCATCTACGAG-GGCTACGC-3' and reverse primer 5'-CAGGAAGGA-GGGCTGAACA-3'. PCR products were separated on 2% agarose gel. Gel images were captured using a ChemiImager™ 5500 Gel Image System (Alpha Innotech, CA, USA). PCR reactions for HO-1 consisted of 32 cycles of amplification with the following conditions: 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, followed by a terminal 7 min elongation at 72°C. PCR reactions for ET-1 consisted of 30 cycles of amplification with the following conditions: 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, followed by a terminal 7 min elongation at 72°C.

Western blot analysis

The carotid artery segment samples were homogenized in liquid nitrogen and then diluted in RIPA Lysis Buffer (Biocolor BioScience & Technology Company, Shanghai, China) containing a phosphatase inhibitor cocktail (Roche, USA) and a 5% complete protease inhibitor cocktail (Roche, USA). The tissue homogenate was centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatant containing the protein lysate was collected. The protein concentrations were subsequently determined by a smart spectrophotometer (Bio-RAD, USA). Immunoblotting detection of HO-1 and ET-1 was performed as follows: 200 μg samples were separately resolved on 10% SDS-PAGE under reducing and denaturing conditions and transferred to a nitrocellulose membrane. The blots were blocked overnight at 4°C in Tris-buffered saline containing 0.1% Tween (TBST) and 2% BSA. Subsequently, the blots were incubated for 60 min in a shaker at 37°C with goat anti-HO-1 (diluted 1:500; Santa Cruz, CA, USA), goat anti-ET-1 (diluted 1:500; Santa Cruz, CA, USA) and sheep anti-β-actin (diluted 1:500; Santa Cruz, CA, USA), and then rinsed with TBST (10 min each, 3 times total) in a shaker at 37°C. The blots were incubated for 45 min in a shaker at room temperature with a 1:2,000 dilution of HRP-conjugated donkey anti-goat or sheep IgG.
The protein bands were detected by adding a chemiluminescent substrate and analyzed using a digital image processing system (Tanon GI52010, China). The relative intensity of each band was determined and normalized to the intensity of β-ACTIN.

**Statistical analysis**

All experiments were repeated at least three times. Statistical analysis was detected by using SPSS17.0. The data are presented as the mean ± SD, and compared by Student’s t-test or one-way multiple-range ANOVA with subsequent t-tests employing a Bonferroni a-correction for multiple comparisons. *P*<0.05 was considered significant.

**Results**

**Chlorhematin treatment induced HO-1 over-expression in rabbit carotid artery model**

Firstly, we detected the expression of HO-1 at mRNA and protein levels by employing RT-PCR, IHC and western blotting methods. RT-PCR
analysis showed that the carotid artery HO-1 mRNA levels in the OC group was significantly higher than in the S, O and OZ groups (Figure 1, \( P<0.01 \)), whereas that in the OZ group was significantly lower than in the S, O and OC group (Figure 1, \( P<0.01 \)). In addition, there were no differences between the S group and the O group (Figure 1).

The carotid artery IHC assay showed that HO-1 was mainly expressed in the endothelial cells, neointimal cells, foam cells and smooth muscle cells of the carotid artery (Figure 2A). HO-1 expression in the OC group was significantly higher than in the other three groups (Figure 2A, \( P<0.01 \)), while HO-1 expression in OZ group was significantly lower than in the other three groups (Figure 2A, \( P<0.01 \)). In addition, there was no significant difference in HO-1 expression between O and S groups (Figure 2A). As shown in Figure 2B, similar results were obtained by further analysis of HO-1 expression by Western blot. These results suggest that chlorhematin treatment induces HO-1 overexpression after balloon injury in a rabbit carotid artery model, whereas zinc protoporphyrin treatment inhibits HO-1 expression.

**HO-1 over-expression had no effect on serum lipid levels of rabbit carotid artery model**

To determine whether HO-1 expression affected serum lipid levels, we measured levels of serum TC, TG, LDL-C, HDL-C, and ox-LDL in rabbit carotid artery model. The results were shown in Table 1. There were no significant differences of serum TC, TG, LDL-C, HDL-C and ox-LDL levels in the S, O, OC and OZ groups.

**HO-1 over-expression alleviated restenosis formation in a rabbit carotid artery model**

As shown in Figure 3, the intimal hyperplasia involved primarily neo-VSMCs, foam cells and ECM in O group. In this context, endothelial cell density decreased and proliferating VSMCs migrated across the inner elastic lamina layer, which was disrupted. The tube lumen became severely stenotic, leading eventually to occlusion. The OC group exhibited intact endothelium, fewer VSMCs, foam cells, lipid deposition, fiber matrix and elastic fibers, as well as increases in vessel lumen diameter (Figure 3), while the severity of carotid artery pathology in the OZ group was similar to that in the O group (Figure 3).

Quantitative analysis was measured as follows: for intimal area, 0.586 ± 0.090 (S group), 0.634 ± 0.096 (O group), 0.386 ± 0.076 (OC group) and 0.775 ± 0.096 (OZ group). For intimal thickness, 431.23 ± 46.08 (S group), 442.17 ± 59.14 (O group), 281.47 ± 21.10 (OC group) and 698.71 ± 58.37 (OZ group); for neointima/media area, 1.334 ± 0.108 (S group), 1.381 ± 0.180 (O group), 0.862 ± 0.164 (OC group) and 1.843 ± 0.212 (OZ group); for neointima/media thickness, 3.880 ± 0.549 (S group), 3.988 ± 0.520 (O group), 2.491 ± 0.173 (OC group) and 6.172 ± 0.522 (OZ group); for stenosis ratios, 53.5 ± 5.62% (S group), 54.2 ± 6.38% (O group), 38.8 ± 2.43% (OC group) and 78.5 ± 6.10% (OZ group). Among these groups, the intimal area, the intimal thickness, the neointima/media area ratios, the neointima/media thickness ratios and stenosis ratio in the OZ group were significantly higher than in the S, O and OC groups (Table 2, \( P<0.01 \)), whereas those in the OC group were significantly lower than in the other three groups (Table 2, \( P<0.01 \)).
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These data suggest that up-regulation of HO-1 induced by chlorhemin treatment alleviates restenosis formation after balloon injury in a rabbit carotid artery model, whereas downregulation of HO-1 evoked by zinc protoporphyrin facilitates restenosis formation.

**HO-1 over-expression decreased NF-kB activity and inhibited TNF-α expression in a rabbit carotid artery model**

NF-kB activity and TNF-α expression were measured by ELISA assay. The results showed that NF-kB activity and TNF-α expression in the OZ group were significantly higher than in the S, O and OC (Table 3, P<0.01), whereas that in the OC group were significantly lower than in the S, O and OZ groups (Table 3, P<0.01). In addition, there were no significant differences in NF-kB and TNF-α expression between the S and the O groups (Table 3). These data suggest HO-1 over-expression decreases NF-kB activity and inhibited TNF-α expression, whereas zinc protoporphyrin treatment promotes NF-kB activity and TNF-α expression.

**Discussion**

Percutaneous coronary intervention (PCI) is a prevalent and feasible approach to the reconstruction of blood supply for treatment of coronary ischemia diseases [1, 2]. However, the high incidence rate of restenosis after the operation is a key factor in prediction of the prognosis and the long-term follow-up results of patients [13-15]. There is increasing evidence that HO-1 plays an important role in coronary disease [11, 16]. In the present study, we evaluated the efficacy of activation (induced by chlorhemin) or inactivation (induced by zinc protoporphyrin) of HO-1 in inhibiting neointimal hyperplasia, thrombosis and restenosis in balloon-injury carotid arteries of high-cholesterol rabbits, a typical model of coronary disease. We found that involvement of HO-1 in defensive restenosis formation was indepen-

### Table 2. Effects of HO-1 expression on restenosis formation in a rabbit carotid artery model

<table>
<thead>
<tr>
<th>Groups</th>
<th>Intimal area (mm²)</th>
<th>Intimal thickness (µm)</th>
<th>Neointimal/media area</th>
<th>Neointima/media thickness</th>
<th>Stenosis ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>0.586 ± 0.090</td>
<td>431.23 ± 46.08</td>
<td>1.334 ± 0.108</td>
<td>3.880 ± 0.549</td>
<td>53.5 ± 5.62%</td>
</tr>
<tr>
<td>O</td>
<td>0.634 ± 0.096</td>
<td>442.17 ± 59.14</td>
<td>1.381 ± 0.180</td>
<td>3.988 ± 0.520</td>
<td>54.2 ± 6.38%</td>
</tr>
<tr>
<td>OC</td>
<td>0.386 ± 0.076**</td>
<td>281.47 ± 21.10**</td>
<td>0.862 ± 0.164**</td>
<td>2.491 ± 0.173**</td>
<td>38.8 ± 2.43%**</td>
</tr>
<tr>
<td>OZ</td>
<td>0.775 ± 0.096**</td>
<td>698.71 ± 58.37**</td>
<td>1.843 ± 0.212**</td>
<td>6.172 ± 0.522**</td>
<td>78.5 ± 6.10%**</td>
</tr>
</tbody>
</table>

**P<0.01 vs S or O group.

Figure 4. HO-1 overexpression suppressed ET-1 expression. RT-PCR analysis of ET-1 mRNA levels in the carotid artery of S, O, OC and OZ groups. Relative quantitative analysis of ET-1 expression is shown. ET-1: endothelin 1. **P<0.01 vs S or O group.
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We found that activation of HO-1 effectively alleviated the degree of restenosis and that inhibition of the HO-1 exacerbated restenosis. In addition, we showed that HO-1 overexpression inhibited NF-κB activity and decreased TNF-α and endothelin 1 (ET-1) expression. In addition to its well-defined role in heme catabolism and erythrocyte turnover, HO-1 also plays an important role in various physiological and pathophysiological states associated with cellular stress [17, 18]. HO-1 is a stress responsive effector that is highly induced by many agents, including its own substrate heme and NO [19]. Therefore, we first quantified HO-1 expression in the carotid artery of experimental rabbits after treatment with chlorhematin or zinc protoporphyrin. Interesting, we found HO-1 expression was significantly up-regulated in the chlorhematin treated group, whereas that in the zinc protoporphyrin treated group was significantly downregulated.

Several lines of evidence suggest that the multiple roles of HO-1 in the vascular system, including vascular tone regulation, anti-smooth muscle cell proliferation, anti-endothelial apoptosis, and angiogenesis [9-11]. The protective effects of HO-1 on the vascular system appear to be mediated through multiple distinct mechanisms, including antioxidant stress, inhibition of inflammatory responses, reduction of cell proliferation, anti-apoptosis, anti-thrombogenic effects, maintenance of normal angiogenesis, and regulation of cell signal trans-

Table 3. Effects of HO-1 expression on NF-κB activity and TNF-α expression

<table>
<thead>
<tr>
<th>Groups</th>
<th>S</th>
<th>O</th>
<th>OC</th>
<th>OZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK-κB (ng/ml)</td>
<td>1.75 ± 0.14</td>
<td>1.79 ± 0.13</td>
<td>1.43 ± 0.11**</td>
<td>2.09 ± 0.13**</td>
</tr>
<tr>
<td>TNF-α (ng/L)</td>
<td>226.22 ± 15.80</td>
<td>232.35 ± 19.01</td>
<td>190.31 ± 18.03**</td>
<td>271.69 ± 21.30**</td>
</tr>
</tbody>
</table>

NF-κB: nuclear factor kappa B subunit 1; TNF-α: tumor necrosis factor-alpha. **P<0.01 vs S or O group.

Figure 5. ET-1 expression was down-regulated in the carotid artery after treatment with chlorhematin. A. Immunohistochemical (IHC) analysis of ET-1 proteins levels in the carotid artery of S, O, OC and OZ groups. B. Western blotting was performed to detect ET-1 expression in the four groups. Relative quantitative analysis of ET-1 expression was showed. **P<0.01 vs S or O group.
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Considering that restenosis is a form of intimal hyperplasia, we further investigated the changes in intimal area, intimal thickness, neointima/media area ratio, neointima/media thickness ratio and stenosis ratio. We found that up-regulation of HO-1 induced by chlorhematin treatment inhibited restenosis formation in a rabbit carotid artery model, whereas downregulation of HO-1 evoked by zinc protoporphyrin can facilitate restenosis formation.

There is accumulating evidence that some cytokines such as TNF-α, ET-1 and IL-1β, as well as nuclear transcriptional factor NF-κB, contribute to the progression of arteriosclerosis and vascular stenosis, indicating that inhibition of those elements could prevent and inhibit these diseases [20-23]. HO-1 has also been shown to protect the cell by modulating NF-κB and other injury-causing agents [24]. In addition, HO-1 has also been shown to decrease the expression of IL-1, TNF-α, ET-1, platelet-derived growth factor (PDGF)-B and macrophage inflammatory protein (MIP)-1B, and to increase anti-inflammatory IL-10-induced expression in endothelial cells [25, 26]. Moreover, HO-1 directly or indirectly interferes with the cell division cycle by regulating specific transcription factors involved in the cell cycle and serving as an inhibitor of VSMC proliferation and migration [27, 28]. ET-1-mediated increases in secretion contribute to abnormal vascular remodeling and atherosclerosis [29, 30]. These investigations revealed that the HO-1 exerts anti-inflammatory, anti-apoptotic and anti-atherosclerotic effects. Accordingly, the present study shows that activation of the HO-1 decreased NF-κB, TNF-α and ET-1 protein levels in vivo, suggesting that HO-1 exhibits anti-inflammatory effects by inhibiting NF-κB activity, as well as TNF-α and ET-1 secretion, which alleviates local inflammatory injury to the carotid artery and inhibit restenosis formation.

In conclusion, the present study demonstrates that activation of HO-1 alleviates restenosis formation after balloon injury in a rabbit carotid artery model by inhibiting NF-κB activity, as well as TNF-α and ET-1 expression, while inhibition of the HO-1 exacerbates restenosis formation. Considering the thrombosis and restenosis associated with multiple mechanisms, our findings suggest that induction of HO-1 activity provides a way to make vessels resistant to thrombosis and restenosis.

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Disclosure of conflict of interest

None.

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