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Pre-emptive hypoxia-regulated HO-1 gene therapy improves post-ischaemic limb perfusion and tissue regeneration in mice

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Aims Haem oxygenase-1 (HO-1) is a haem-degrading enzyme that generates carbon monoxide, bilirubin, and iron ions. Through these compounds, HO-1 mitigates cellular injury by exerting antioxidant, anti-apoptotic, and anti-inflammatory effects. Here, we examined the influence of HO-1 deficiency and transient hypoxia/ischaemia-induced HO-1 overexpression on post-injury hindlimb recovery.

Methods and results Mice lacking functional HO-1 (HO-$1^{-/-}$) showed reduced reparative neovascularization in ischaemic skeletal muscles, impaired blood flow (BF) recovery, and increased muscle cell death compared with their wild-type littermates. Human microvascular endothelial cells (HMEC-1) transfected with plasmid vector (pHRE-HO-1) carrying human HO-1 driven by three hypoxia response elements (HREs) and cultured in 0.5% oxygen demonstrated markedly increased expression of HO-1. Such upregulated HO-1 levels were effective in conferring protection against H_2O_2-induced cell death and in promoting the proangiogenic phenotype of HMEC-1 cells. More importantly, when delivered *in vivo*, pHRE-HO-1 significantly improved the post-ischaemic foot BF in mice subjected to femoral artery ligation. These effects were associated with reduced levels of pro-inflammatory cytokines (IL-6 and CXCL1) and lower numbers of transferase-mediated dUTP nick-end labelling-positive cells. Moreover, HO-1 delivered into mouse skeletal muscles seems to influence the regenerative potential of myocytes as it significantly changed the expression of transcriptional (Pax7, MyoD, myogenin) and post-transcriptional (miR-146a, miR-206) regulators of skeletal muscle regeneration.

Conclusion Our results suggest the therapeutic potential of HO-1 for prevention of adverse effects in critical limb ischaemia.

Keywords Angiogenesis • Gene therapy • HO-1 • MicroRNA • Satellite cells

1. Introduction

Interruption of blood supply results in ischaemic injury which can rapidly damage any tissue. Critical limb ischaemia, usually caused by atherosclerotic plaques blocking the blood vessels, is a common and devastating manifestation of peripheral arterial disease. It is associated with a very high morbidity and mortality risk.[1] In many cases, the use of bypass surgery or balloon angioplasty is limited, necessitating amputation. For these reasons, the development of new therapies continues to be an area of substantial scientific and clinical interest.

Haem oxygenase-1 (HO-1) degrades haem to carbon monoxide (CO), biliverdin/bilirubin, and ferrous iron. These products, either directly or indirectly, exert anti-oxidative, anti-apoptotic, and anti-inflammatory effects.[2] Proangiogenic properties of HO-1 appear to

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be mediated in large part by CO through the stimulation of soluble guanylate cyclase in endothelial cells (ECs).[3] Moreover, our latest data demonstrate that HO-1 may be an important regulator of myogenic regulatory factors (MRFs) and microRNAs (miRNAs) involved in myoblast differentiation.[4] All of this makes HO-1 a promising candidate for gene therapy. Importantly, however, uncontrolled overexpression of HO-1 can cause serious detrimental side effects. The release of high amounts of reactive iron under conditions of strongly enhanced HO activity and a high supply of haem may potentially be harmful to the cells.[5] Therefore, creating a beneficial threshold of this protein by its controlled expression seems to be crucial for obtaining the therapeutic effects. This objective can be achieved with hypoxia-regulated gene carriers.

Such an approach involves the naturally occurring mechanism, an interaction of hypoxia-inducible factor-1 (HIF-1) with hypoxia response element (HRE) what results in upregulation of the transgene in pathologic hypoxic/ischaemic conditions. HIF-1 is a basic helix–loop–helix heterodimeric transcription factor that consists of oxygen-sensitive subunit HIF-1α or, in tissues such as the heart, lungs, kidney and endothelium, HIF-2α and oxygen insensitive, constitutively expressed subunit HIF-1β. It initiates the expression of several hypoxia responsive genes, like erythropoietin, vascular endothelial growth factor, or mouse HO-1.[6] The main advantage of using vectors containing HRE sequence is the induction of transgene expression only upon a drop in oxygen tension. Therefore, the production of therapeutic agent is limited to the ischaemic region where its presence might be beneficial. Additionally, when the tissue heals, it becomes re-oxygenated and the transgene expression goes down.

Previously, hypoxia-regulated HO-1 gene transfer reduced cellular damage during myocardial ischaemia–reperfusion (I/R) injury.[7,8] Moreover, Tongers *et al.*[9] demonstrated a significantly blunted blood flow (BF) recovery after femoral artery ligation (FAL) in mice treated with the HO inhibitor tin protoporphyrin-IX. However, as the protoporphyrin inhibitors of HO activity may exert many unspecific effects, the genetic models are required to prove the role of HO-1 in revascularization. Hence, to the best of our knowledge, in this study we tested for the first time the importance of HO-1 in post-ischaemic neovascularization and BF recovery in HO-1-deficient mice. Moreover, we generated and successfully used a plasmid harbouring human HO-1 driven by three HRE sequences. We demonstrated anti-apoptotic, anti-inflammatory, and proangiogenic action of HO-1, revealing additionally the new mechanisms of its beneficial effect on muscle regeneration.

2. Methods

Detailed procedures and protocols are provided in the Supplementary material online.

2.1 Reagents

Cell culture reagents, MCDB 131, and foetal bovine serum (FBS) were from PAA (Lodz, Poland). Oligo(dT) primers, dNTPs, MMLV reverse transcriptase, and lactate dehydrogenase (LDH) assay were obtained from Promega (Gdansk, Poland). NCode miRNA First-Strand cDNA Synthesis Kit was from Invitrogen. Propidium iodide (PI), mouse CXCL1-, mouse IL-6-, and mouse TNFα-recognizing ELISA kits were procured from R&D Systems (Warszawa, Poland). HMVEC-L Nucleofector Kit was purchased from Lonza (Warszawa, Poland). Ketamine hydrochloride (Bioketan) and xylazine hydrochloride (Sedazin) were from Biowet (Pulawy, Poland). All other reagents and chemicals, unless otherwise stated, were purchased from Sigma (Poznan, Poland).

2.2 Cell culture

Human microvascular endothelial cells (HMEC-1) were kindly provided by Dr Francis Candal (Center for Disease Control and Prevention, Atlanta, GA, USA) and cultured in the MCDB 131 medium containing 10% heat-inactivated FBS, L-glutamine (2 mM), epidermal growth factor (10 ng/mL) and hydrocortisone (1 μg/mL), penicillin (100 U/mL), and streptomycin (10 μg/mL) at standard conditions: 37°C, 5% CO_2, and humidified atmosphere. For induction of HIF-1, the cells were placed in hypoxic chamber (Billups Rothenberg), flushed with a low-oxygen gas mixture (containing 0.5% O_2, 5% CO_2, 94.5% N_2) for 20 min. After flushing, gas ports were tightly sealed and chambers were placed in the incubator at 37°C. For evaluation of cell survival, HMEC-1 cells were stimulated with 500 μM hydrogen peroxide (H_2O_2) in the presence of 2% FBS.

2.3 Plasmids

The following plasmids were used in this study: pCMV-EGFP and pCMV-HO-1—in *in vitro* experiments; pHRE-minCMV (pHRE-empty) and pHRE-minCMV-HO-1 (pHRE-HO-1)—in *in vitro* and *in vivo* experiments. cDNA for human HO-1 was obtained from cDNA library of human keratinocytes (HaCaT) and subcloned into two vectors either downstream of the cytomegalovirus (CMV) constitutive promoter (pCMV-HO-1) or downstream of three copies of HRE (5′-GACGTG-3′) and the minimal CMV promoter (pHRE-HO-1). This sequence enabled hypoxia-regulated gene expression.

2.4 Nucleofection of HMEC-1 cells

Cells were transfected under serum-free conditions with 2 μg of plasmid DNA using the Nucleofector (Amaxa) and HMVEC-L Nucleofector Kit according to vendor's protocol. The transfection efficiency was determined 24 h later with Nikon Eclipse TS 1000 Fluorescence Microscope by counting the cells expressing enhanced green fluorescent protein (EGFP).

2.5 Western blotting

Protein extracts from transfected HMEC-1 cells and fragments of gastrocnemius muscles were prepared and analysed as previously described[4] using antibodies and the conditions described in Supplementary material online.

2.6 Analysis of cell death

Cell death after appropriate H_2O_2 treatment was analysed with (i) LDH assay in the conditioned media according to vendor's protocol and (ii) red propidium iodide (PI) fluorescence.

2.7 Analysis of cell migration

Migratory properties of transfected HMEC-1 cells were analysed in (i) scratch assay (cells were pre-treated with 5 mM hydroxyurea in order to induce growth arrest) and (ii) Boyden chamber assay (transwell plates with 8 μm pores coated with 20 μg/mL fibronectin mixed with 0.5% gelatin in 1:1 ratio) according to the protocols described previously in Grochot-Przeczek *et al.*[10] and Jazwa *et al.*,[11] respectively. After 12 h of culture, scratches and transwell membranes were stained with haematoxylin and eosin (H&E), photographed with Nikon Eclipse TS 1000 Microscope at ×200 magnification, and analysed with the Image J software.

2.8 Murine hindlimb ischaemia and gene delivery

All animal procedures were in accordance with the Directive 2010/63/EU of the European Parliament and carried out under a license from the Ethical Committee of the Jagiellonian University. Twelve-week-old male and female HO-$1^{-/-}$ and HO-$1^{+/+}$ mice of C57BL/6×FVB background (courtesy of Dr Anupam Agarwal, Birmingham, USA) and 12-week-old male C57/BL6 mice were used in the experiments. All animals were

maintained under controlled environmental conditions (12 h light/dark cycle at ~23°C), and provided with standard laboratory food and water *ad libitum*. Unilateral FAL was performed by double ligation of the superficial left femoral artery under either 2% isoflurane (Aerrane, Baxter) gas anaesthesia (C57BL/6×FVB mice) or mixture of ketamine hydrochloride (90 mg/kg) and xylazine hydrochloride (20 mg/kg) injected intraperitoneally (C57BL/6 mice). The limb withdrawal response to toe pinch was monitored to ensure the adequacy of anaesthesia. About 10–15 min before vessel ligation, pHRE-empty or pHRE-HO-1 was delivered into the left gastrocnemius muscle of each C57BL/6 mouse by three injections (total dose 10 μg in 30 μL). Control animals were left untreated. BF was measured in anaesthetized animals up to 30 min after the surgery and then at day 7 and day 14 using Laser Doppler Perfusion Imager (LDPI) System (PIM II, Perimed). The ischaemic-to-non-ischaemic foot BF ratio was calculated as an index of BF recovery. The readings of individual measurements per mouse were averaged for each time point. Animals were euthanized via an anaesthetic overdose (200 mg/kg of ketamine mixed with 40 mg/kg of xylazine delivered by intraperitoneal injection) at day 1 (to study the gene expression) and day 14 (to evaluate the gene expression and vessel density) after surgery and gene transfer.

2.9 Histological analyses

Skeletal muscle sections were stained with H&E for overall morphology. Microvessel density was evaluated in isolectin B_4 (dilution 1:100, Vector Laboratories) and CD31 (1:100, BD Biosciences) stained sections of 14 days post-ischaemic and intact mouse adductor skeletal muscles. Then, respectively, streptavidin–fluorochrome-conjugated antibodies (Streptavidin Alexa Fluor 546) and rhodamine-conjugated anti-rat antibodies (1:250, BD Biosciences) were used to visualize endothelial cells in the muscle tissue. Apoptosis was determined in the gastrocnemius muscle cryosections (6 μm) 1 day after gene transfer and/or FAL by transferase-mediated dUTP nick-end labelling (TUNEL) staining (*in situ* cell death detection kit Fluorescein, Roche, Indianapolis, IN, USA) according to vendor's protocol. Immunofluorescent staining against Pax-7 was performed in the gastrocnemius muscle cryosections (6 μm) 14 days after FAL and HO-1 gene transfer with the use of mouse anti-Pax7 antibody (1:200; DSHB, University of Iowa) followed by secondary anti-mouse antibody Alexa Fluor 488 (1:250; Molecular Probes). The counting was performed with the use of a fluorescent microscope (Nikon) at ×400 magnification.

2.10 Total RNA isolation

At 1 and 14 days post-gene transfer and/or FAL, fragments of gastrocnemius muscles were snap-frozen in liquid nitrogen and stored at −80°C. Total RNA including small RNA fraction was isolated as previously described[4] by lysis in 1 mL of Qiazol Total RNA Isolation Reagent using Tissue Lyzer (Qiagen).

2.11 RT–PCR evaluation of gene expression and miRNA levels

RT–PCR was performed as previously described.[4] cDNA template was synthesized using NCode miRNA First-Strand cDNA Synthesis Kit following the manufacturer protocol. Quantitative PCR (qPCR) was performed using StepOne Plus Real-Time PCR (Applied Biosystems) in a mixture containing SYBR Green PCR Master Mix (SYBR Green qPCR Kit), 50 ng of cDNA and specific primers described in Supplementary Material online. Relative quantification of gene expression was calculated based on the comparative C_T (threshold cycle value) method ($\Delta C_T = C_{T\ \text{gene of interest}} - C_{T\ \text{housekeeping gene}}$). Comparison of gene expression in different samples was performed based on the differences in ΔC_T of individual samples ($\Delta\Delta C_T$).

2.12 ELISA

The content of mouse CXCL1, IL-6, and TNFα in gastrocnemius muscle homogenates was evaluated according to vendor's protocol. After evaluation of total protein content with Bicinchoninic Acid Protein Assay Kit, the amount of mouse CXCL1, IL-6, and TNFα was expressed in pg/mg protein.

2.13 Statistical analysis

Results are expressed as mean ± SEM unless otherwise stated. One-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test or unpaired Student's *t*-test was used to evaluate the statistical significance between investigated groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1 BF recovery and neovascularization after hindlimb ischaemia is impaired in HO-1-deficient mice

Immediately after the surgery (day 0), LDPI analysis demonstrated similar reductions of blood perfusion in HO-$1^{+/+}$ and HO-$1^{-/-}$ mice (*Figure 1A* and *B*). Two weeks after the surgery, BF in ischaemic hindlimbs of HO-$1^{+/+}$ animals had almost returned to normal (*Figure 1A* and *B*), whereas it was significantly impaired in HO-1-deficient mice (*Figure 1A* and *B*). The number of capillaries was markedly increased in the 14 days post-ischaemic hindlimb adductor muscles of HO-$1^{+/+}$ animals when compared with intact muscles from untreated controls (*Figure 1C* and *D*). Conversely, abolished angiogenic response to hindlimb ischaemia was observed in mice lacking HO-1 (*Figure 1C* and *D*). Already 1 day post-ischaemia, murine HO-1 mRNA levels detected in skeletal muscles of HO-$1^{+/+}$ animals were upregulated more than four times and we still detected some induction at day 14 (*Figure 1E*). Accordingly, HO-$1^{-/-}$ mice submitted to the same treatment did not exhibit neither basal nor ischaemia-induced HO-1 expression (*Figure 1E*). Moreover, in the 1 day post-ischaemic skeletal muscle cross-sections of HO-$1^{-/-}$ mice, we detected significantly higher numbers of TUNEL-positive cells when compared with HO-$1^{+/+}$ animals indicating an increased cell death and muscle damage in the absence of HO-1 (*Figure 1F*).

3.2 Beneficial effects of HO-1 overexpressed in normoxic endothelial cells

To evaluate whether activation of HO-1 in ischaemic conditions may have any functional consequences, we overexpressed HO-1 in HMEC-1 cells growing *in vitro* and compared their angiogenic properties and survival under stressful conditions with control cells expressing EGFP. HMEC-1 cells were chosen because in human cells, particularly endothelial, in contrary to rodent cells the expression of HO-1 is not induced and sometimes even downregulated under hypoxic conditions.[12,13] HMEC-1 cells were transfected by nucleofection with plasmids encoding EGFP (pCMV-EGFP) and human HO-1 (pCMV-HO-1). The transfection efficiency varied between 50 and 70% (Supplementary material online, *Figure S1A*). Accordingly, western blot analysis performed 24 h after nucleofection revealed a potent overexpression of human HO-1 in cells transfected with pCMV-HO-1 (*Figure 2A*). Then, HMEC-1 cells overexpressing HO-1 and EGFP were subjected to H_2O_2-induced damage mimicking reperfusion injury. Incubation of control pCMV-EGFP-transfected HMEC-1 cells with graded concentrations of H_2O_2 for 24 h provoked a dose-dependent decrease in the proportion of surviving cells (data not shown). After 24 h of treatment with 500 μM H_2O_2, the level of

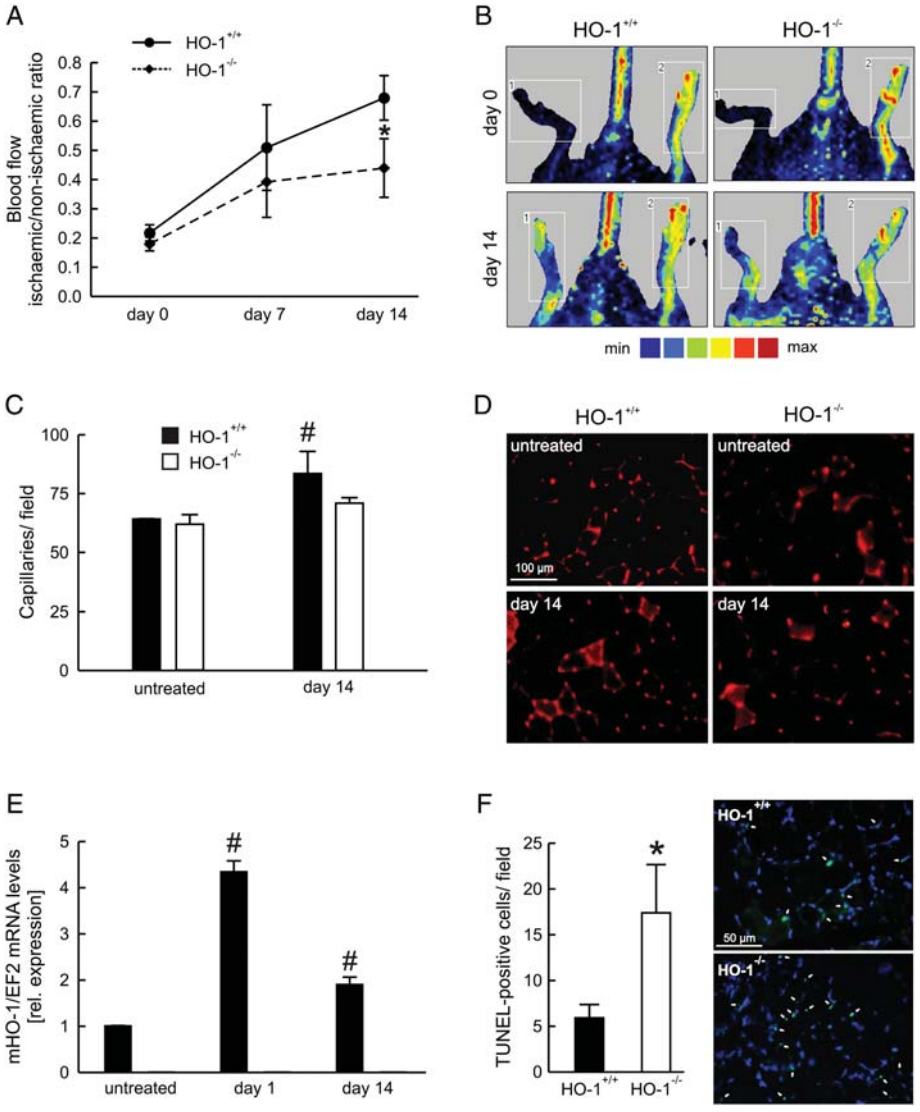

Figure 1 Post-ischaemic hindlimb perfusion and muscle vascularity is impaired in HO-$1^{-/-}$ mice. (*A*) Graph illustrates the time course of the recovery of BF to the ischaemic foot measured by Laser Doppler Flowmetry. (*B*) Images show typical Laser Doppler pictures of superficial BF in lower limbs. Squares include the area of interest (the feet) in which average perfusion was computed by the software (1, ischaemic leg; 2, non-ischaemic leg). Colour scale from blue to brown indicates progressive increases in BF. (*C*) Graph illustrates total capillary density in HO-$1^{+/+}$ (black bars) and HO-$1^{-/-}$ (white bars) mice quantified 14 days after FAL and in untreated subjects. (*D*) Representative pictures from the immunofluorescent staining detecting biotinylated isolectin B4 bound to endothelial cells. Magnification $\times 200$. (*E*) qPCR for endogenous mouse HO-1 (mHO-1) normalized to EF2. (*F*) Bar graphs show the numbers of TUNEL-positive apoptotic cells in the gastrocnemius muscle 1 day after FAL. Insets show representative merged images of the muscle cross-sections stained for TUNEL (green) and nuclei (blue). Double positive cells are indicated by arrows. Magnification $\times 400$. Values are means $\pm$ SEM of 4–6 animals per group and per time point. $^{*}P < 0.05$ vs. HO-$1^{+/+}$ at the appropriate time point. $^{\#}P < 0.05$ vs. untreated HO-$1^{+/+}$.

LDH released by necrotic cells increased by about 50% and this H_2O_2 concentration was employed in all experiments investigating the pro-survival effects of HO-1. The level of LDH released to the cell culture media by pCMV-HO-1-transfected HMEC-1 cells treated with H_2O_2 was significantly lower when compared with the pCMV-EGFP-transfected cells subjected to the same H_2O_2 treatment (*Figure 2B*). Similar effect has been observed when cell death was assessed with PI staining (*Figure 2C*). Moreover, overexpression of HO-1 with pCMV-HO-1 vector markedly enhanced the migratory potential of HMEC-1 cells evaluated in two different tests—scratch assay (*Figure 2D*) and Boyden chamber assay (*Figure 2E* and Supplementary material online, *Figure S1B*).

3.3 Beneficial effects of HO-1 overexpressed in hypoxic endothelial cells

Next, HMEC-1 cells were transfected by nucleofection with pHRE-HO-1 or pHRE-empty vector. Western blot analysis revealed potent overexpression of HO-1 in cells transfected with pHRE-HO-1 and kept in hypoxia (*Figure 3A*, right panel) comparable

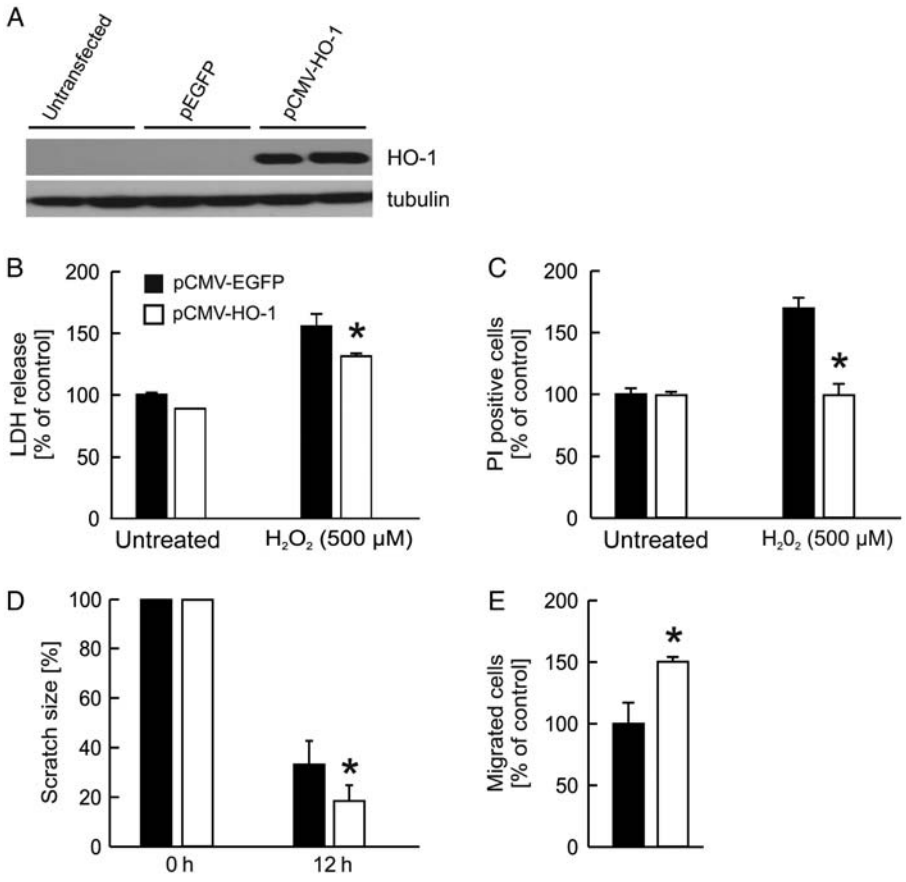

Figure 2 Beneficial effects of constitutive HO-1 overexpression on H_2O_2-induced mortality and migratory properties of HMEC-1 cells. (*A*) Western blot demonstrating the overexpression of HO-1 in HMEC-1 cells transfected with pCMV-HO-1. HMEC-1 cells transfected with pCMV-EGFP (black bars) or pCMV-HO-1 (white bars) were treated with 500 μM H_2O_2 for 24 h. Mortality was determined using (*B*) LDH assay and (*C*) PI staining of cell nuclei. Migration of pCMV-EGFP- or pCMV-HO-1-transfected HMEC-1 cells was analysed in (*D*) scratch closure assay and (*E*) Boyden chamber assay. Each bar represents mean ± SD of representative experiment performed three times in duplicates. $^*P < 0.05$ vs. pCMV-EGFP under appropriate treatment.

with the one obtained after pCMV-HO-1 transfection (*Figure 2A*). LDH released to the cell culture media by pHRE-HO-1-transfected HMEC-1 cells treated with 500 μM H_2O_2 in hypoxic conditions was mildly but significantly lower when compared with the pHRE-empty vector-transfected cells subjected to the same treatment (*Figure 3B*, right panel). Moreover, pHRE-HO-1 transfection resulted in acceleration of cell migration in hypoxic conditions (*Figure 3C*, right panel).

3.4 Administration of pHRE-HO-1 improves BF and reduces the incidence of necrosis in ischaemic hindlimbs

On the basis of *in vitro* results, our next aim was to investigate whether local overexpression of HO-1 will exert cytoprotective and proangiogenic effects *in vivo*. Therefore, shortly before induction of unilateral hindlimb ischaemia, we performed intramuscular injection of pHRE-HO-1 construct encoding human HO-1 under the control of three HRE sequences into the gastrocnemius muscle below the ligation site. Control animals were injected with pHRE-empty vector. The presence of human HO-1 mRNA in mouse gastrocnemius muscle homogenates of pHRE-HO-1-injected animals was confirmed by quantitative RT–PCR (*Figure 4A*) and at the protein level (Supplementary material online, *Figure S2A*). Because available antibodies did not allow to distinguish endogenous murine HO-1 from human HO-1, the western blot analysis was done on samples collected from HO-$1^{-/-}$ mice subjected to FAL and pHRE-HO-1 gene transfer (Supplementary material online, *Figure S2A*). In wild-type mice, hindlimb ischaemia induced endogenous HO-1 expression 1 day after the insult (*Figure 4A*). However, transfer of pHRE-HO-1 resulted in much stronger expression of HO-1 in ischaemic muscles (*Figure 4A*). Accordingly, overexpression of HO-1 resulted in a much better recovery of BF in ischaemic hindlimbs at day 14 after the surgery and HO-1 gene transfer than in control animals treated with pHRE-empty (*Figure 4B* and Supplementary material online, *Figure S2B*). Moreover, this effect was associated with decreased number of necrotic toes (1.5 ± 0.5 vs. 2.5 ± 0.9 in pHRE-empty-treated mice). Neovascularization in the adductor muscles 14 days after insult was significantly increased in the hindlimbs of mice treated with pHRE-HO-1, but not in pHRE-empty-injected mice, when compared with untreated animals (*Figure 4C*).

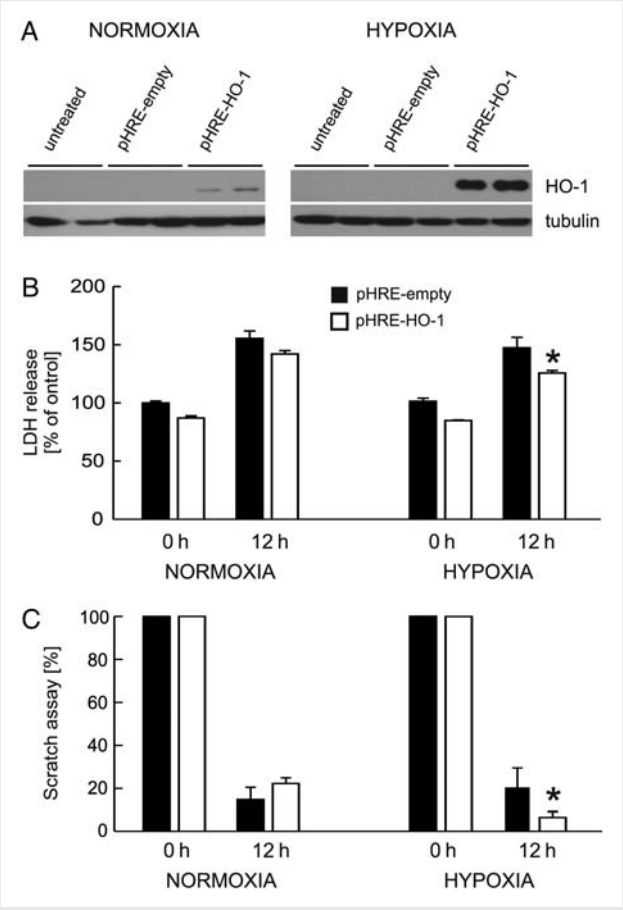

Figure 3 Beneficial effects of hypoxia-induced HO-1 overexpression on H_2O_2-mediated mortality and migratory properties of HMEC-1 cells. HMEC-1 cells transfected with pHRE-empty (black bars) or pHRE-HO-1 (white bars) were cultured either in normoxic (21% O_2) or hypoxic (0.5% O_2) conditions. (*A*) Western blot demonstrating the overexpression of HO-1 in HMEC-1 cells transfected with pHRE-HO-1 and kept in hypoxia (right panel). (*B*) LDH detected in the conditioned media from HMEC-1 cells treated with 500 μM H_2O_2 for 12 h. (*C*) Scratch closure assay was performed on confluent monolayers of transfected HMEC-1 cells and the scratch closure rate was determined by comparing the denuded area immediately and 12 h later. Each bar represents mean ± SD of representative experiment performed three times in duplicates. *$P < 0.05$ vs. pHRE-empty at 12 h in hypoxia.

3.5 Administration of pHRE-HO-1 diminishes inflammatory response and apoptosis in ischaemic skeletal muscles

Hindlimb ischaemia significantly increased production of pro-inflammatory cytokines—murine functional homolog of human IL-8 (CXCL1) and IL-6—already 1 day post-surgery (*Figure 5A* and *B*, respectively). Importantly, injection of pHRE-HO-1 shortly before FAL lowered the levels of both cytokines at day 1, although only the inhibition of CXCL1 but not IL-6 (*Figure 5A* and *B*) nor TNFα (data not shown) was statistically significant. Accordingly, the general morphology analysis of the H&E stained transverse sections of the 1 day post-ischaemic gastrocnemius muscle (Supplementary material online, *Figure S3*) revealed smaller inflammatory cell infiltrates in mice treated with pHRE-HO-1 when compared with pHRE-empty-injected individuals (Supplementary material online, *Figure S3C* vs. *B*). Importantly, HO-1 gene decreased also the number of apoptotic TUNEL-positive cells already at day 1 post-surgery (*Figure 5C* and Supplementary material online, *Figure S2C*), providing evidence that local, transient HO-1 overexpression improves skeletal muscle cell survival under ischaemic conditions.

3.6 Overexpression of HO-1 in skeletal muscles modulates levels of molecules involved in skeletal muscle regeneration

Postnatal growth and regeneration of skeletal muscle mainly depend on adult muscle stem cells, named satellite cells. Activation of satellite cells is governed by MRFs which appear to be regulated by HO-1.[4] Accordingly, we observed about four-fold upregulation of MyoD mRNA (*Figure 6A*) and also an increase in this protein levels (Supplementary material online, *Figure S4A* and *B*), and about two-fold upregulation of myogenin mRNA (*Figure 6B*) 1 day after ischaemia in gastrocnemius muscles of animals treated with pHRE-empty. Slightly lower levels of MyoD mRNA (*Figure 6A*, middle bars) and protein (Supplementary material online, *Figure S4B*, middle bars) were detected in mice overexpressing HO-1 at day 1 after FAL and were accompanied by significant upregulation of Pax7 protein when compared with 1 day post-ischaemic pHRE-empty-treated individuals (Supplementary material online, *Figure S4A*). We have also detected a significant decrease in myogenin mRNA levels (*Figure 6B*, middle bars) in mice overexpressing HO-1 at 1 day after ischaemia. In contrast, after BF reconstitution at day 14 resulting in deactivation of human HO-1 expression from the pHRE-HO-1 construct, much higher mRNA levels of myogenin were detected in muscles injected with pHRE-HO-1 than treated with pHRE-empty vector (*Figure 6B*, right bars). Of note, no effect of transient HO-1 overexpression on other myocyte differentiation-related genes (Mef2c, myocardin, and desmin) has been found (data not shown).

MRFs control also the generation of myomirs, a set of conserved miRNAs specific for skeletal muscles.[14,15] Temporal upregulation of myomirs negatively regulates the target genes, and is necessary for proper muscle development.[15] In the present study, we have observed a significant increase in miR-206 (*Figure 6C*) in skeletal muscles injected with pHRE-HO-1. Reversely, the expression of miR-146a which may inhibit myoblast differentiation[16] was downregulated in pHRE-HO-1-transfected muscles (*Figure 6D*). In contrary, we did not observe any significant differences between both groups of animals in miR-1, miR-133a, and miR-133b levels (data not shown). Additionally, we detected significantly lower levels of both Pax3 (*Figure 6E*) and Pax7 (*Figure 6F*, Supplementary material online, *Figure S4C*) 14 days after the surgery in mice injected with pHRE-HO-1 in comparison with pHRE-empty-treated controls.

Finally, the analysis of H&E-stained paraffin sections of 14 days post-ischaemic skeletal muscles revealed that, in contrast to pHRE-empty-treated individuals, mice subjected to pHRE-HO-1 gene transfer formed many centrally nucleated fibres arising from muscle satellite cells (Supplementary material online, *Figure S3E* and *F*).

4. Discussion

The salient finding of the present study is that hypoxia-regulated overexpression of HO-1 accelerates the recovery of the ischaemic limbs,

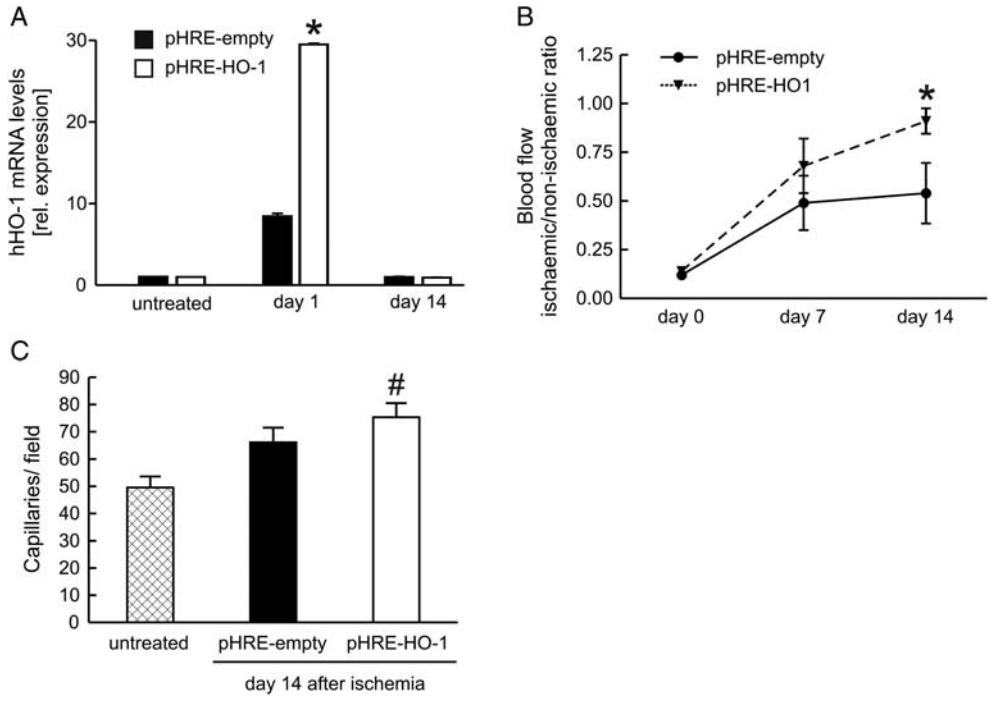

Figure 4 Post-ischaemic BF is improved in mouse hindlimbs overexpressing HO-1. (*A*) Human HO-1 was significantly upregulated in gastrocnemius muscles injected with pHRE-HO-1 1 day after FAL. (*B*) Graph illustrates the time course of the recovery of BF to the ischaemic foot measured by Laser Doppler Flowmetry. (*C*) Graph shows number of CD31-positive capillaries in the adductor muscles 14 days after FAL and gene transfer. Values are means ± SEM of four animals per group and per time point. $^{*}P < 0.05$ vs. pHRE-empty at day 14. $^{\#}P < 0.05$ vs. untreated.

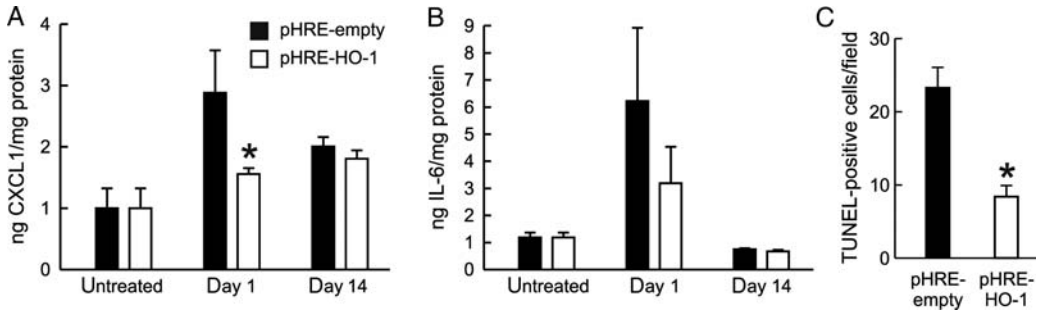

Figure 5 HO-1 modulates inflammation and apoptosis in ischaemic skeletal muscles. ELISA for (*A*) CXCL1 chemokine—mouse homolog of human IL-8 and (*B*) mouse IL-6. (*C*) TUNEL-positive apoptotic cells in the gastrocnemius muscle 1 day after FAL and gene transfer. Values are means ± SEM of four animals per group and per time point. $^{*}P < 0.05$ vs. pHRE-empty at the appropriate time point.

the process potentially dependent on increased revascularization, decreased inflammation, and improved muscle regeneration in animals treated with HO-1 harbouring plasmid.

HO-1 is an inducible, cytoprotective enzyme that catabolizes haem to free iron, CO, and biliverdin, which is converted to bilirubin. Pharmacological inhibition of HO was previously shown to interfere with the establishment of an SDF-1 gradient between the ischaemic hindlimb and bone marrow, and the mobilization and recruitment of putative EPCs defined as Sca-1^{+}/Kdr^{+} cells.[9] In the present study, we have shown the diminished post-ischaemic reparative neovascularization, increased muscle cell death, and impaired post-ischaemic BF recovery in mice lacking the HO-1 gene.

It was previously shown that constitutive overexpression of HO-1 in mouse[17] or rat[18] ischaemic limbs improves the post-ischaemic BF and promotes tissue survival. Unfortunately, however, iron ions, although being capable of inducing protective ferritin,[19] can also accelerate tissue injury via production of potentially harmful-free hydroxyl radicals.[5] Therefore, creating a beneficial threshold of HO-1 by its controlled expression seems to be crucial for conferring the therapeutic effects, especially in terms of long-term protection.

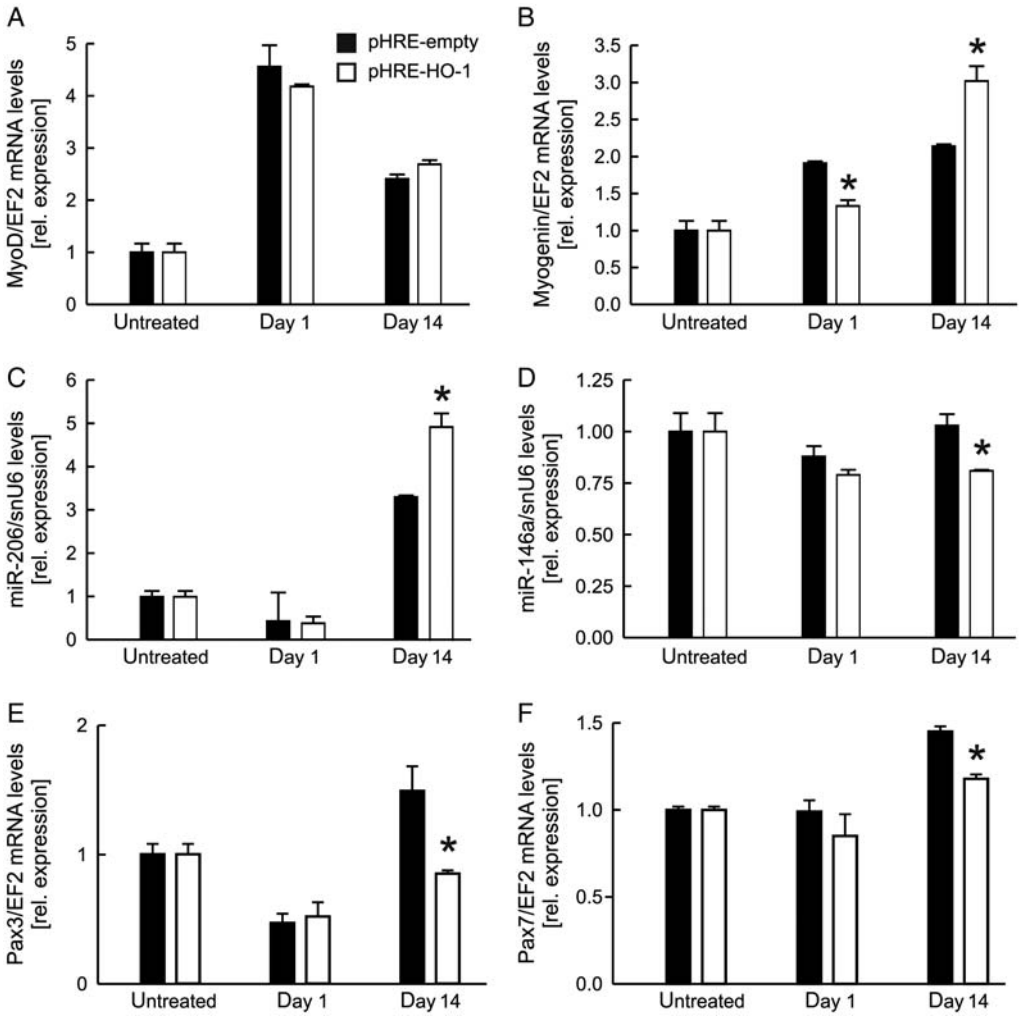

Figure 6 HO-1 modulates levels of molecules involved in post-injury myocyte regeneration. qPCR for (*A*) MyoD, (*B*) myogenin, (*C*) miR-206, (*D*) miR-146a, (*E*) Pax3, and (*F*) Pax7. In (*A*), (*B*), (*E*), and (*F*) EF2 served as a constitutive control gene, in (*C*) and (*D*) snU6 served as a constitutive control. Values are means ± SEM of four animals per group and per time point. $^{*}P < 0.05$ vs. pHRE-empty at the appropriate time point.

The concept of hypoxia-regulated HO-1 gene transfer was previously investigated as a suitable strategy for the treatment of cardiovascular diseases by reducing cellular damage during I/R injury and preserving heart function.[7,20] Here, we show that such strategy can be effective also in the treatment of acute limb ischaemia and, importantly, indicate for the other mechanisms of HO-1 effect, namely enhanced revascularization and improved muscle regeneration. In the present study, we delivered pHRE-HO-1 and the control pHRE-empty vector into skeletal muscles by local injections. We have detected human HO-1 mRNA already 1 day after surgical induction of ischaemia in mice, whereas 2 weeks later the transgene expression disappeared upon restoration of circulation. Therefore, incorporation of hypoxia responsive elements in our pHRE-HO-1 construct provided a useful on–off physiological switch, which rendered relatively fast transcription of the therapeutic gene dependent on the hypoxic stimulus triggered by ischaemia.

Reactive oxygen species (ROS) play an important role in the pathophysiology of ischaemic disease.[7,8,21] Sudden occlusion of the blood vessel often leads to I/R injury. It refers to paradoxical damage of the tissue caused when blood supply returns to the tissue after a period of ischaemia. It has been shown that HO-1 overexpression may have a sparing effect on the ischaemic myocardium by reducing (I/R)-induced injury and preventing the sequence of events leading to cell loss and subsequent ventricular dysfunction.[22] In the present study, we have observed markedly better survival of HO-1 overexpressing ECs exposed to the high doses of H_2O_2. However, we did not observe any significant differences between analysed groups of mice neither in tissue ROS levels detected by dichlorodihydrofluorescein nor in DNA oxidation evaluated by 8-hydroxy-2′-deoxyguanosine fluorescent staining (data not shown). These may suggest that, unlike the I/R model, in which the tissue undergoes repeated brief ischaemic episodes, the acute ischaemia is associated with a more extensive injury that is not completely dependent on ROS generation. Widespread activity of HO-1 and its products may hence provide better protection in such conditions.

In the present study, we have shown that HO-1 gene transfer was able to lower the pro-inflammatory IL-6 production by post-ischaemic murine skeletal muscles. Similar effects of HO-1 were previously

observed in patients subjected to bypass surgery.[23] The relationship between HO-1 and IL-8 raises more controversy, because HO-1-dependent[24] as well as HO-1-independent[13,25] changes in IL-8 production were noted. In the present study, we have evaluated the levels of chemokine CXCL1, which is a murine functional homologue of human IL-8. CXCL1 was significantly lower upon HO-1 gene delivery. It has been shown that pro-inflammatory markers correlate with peripheral arterial disease progression and risk of amputation.[26] Thus, the possibility of modulating the inflammatory response at the site of injury with HO-1 may be beneficial.

It has also been shown that HO-1 might provide an anti-inflammatory action *in vivo* through the downmodulation of cell adhesion molecules accompanied by inhibition of leucocyte infiltration[27] and via promotion of non-inflammatory angiogenesis facilitating tissue repair.[28] Importantly, our study shows another, new mechanism of improved tissue repair by intramuscular HO-1 gene transfer. Activation of HO-1 was previously reported to induce osteoblast,[29] bone marrow mesenchymal stem cell,[30] and monocyte[31] differentiation, whereas HO-1 haploinsufficiency led to disrupted erythroblast differentiation.[32] Satellite cells, the muscle progenitors, preserve the regenerative capacity of adult skeletal muscle. In response to muscle injury, satellite cells become activated, proliferate, and fuse to either existing muscle fibres or fuse together to form new muscle fibres.[33] Here, we showed that MyoD, as reported elsewhere,[34] was strongly upregulated shortly (1 day) after the surgery and its expression decreased 2 weeks later during the regeneration phase. Moreover, in the present study, we have observed a slight decrease in MyoD and more evident decrease in myogenin mRNA levels 1 day after pHRE-HO-1 delivery and hypoxia/ischaemia-mediated activation of human HO-1 expression. On the contrary, when HO-1 expression in pHRE-HO-1-transfected murine hindlimbs has been switched-off at day 14, the level of myogenin was significantly increased in comparison with muscles treated with control pHRE-empty vector. Accordingly, Pax7 protein levels were higher in mice overexpressing HO-1 at 1 day after FAL when compared with empty vector-treated individuals. We propose that in this way, HO-1 may be implicated in the early phases of myogenic differentiation and participate in the generation of a pool of reserve cells which are closely related to satellite cells responsible for adult muscle regeneration. Indeed, our recent study indicates that stable, long-term expression of HO-1 may inhibit differentiation of myoblasts while increasing their proliferation.[4] Hence, regulated expression of HO-1 in ischaemic limbs may exert better effect than constitutive one.

In the present study, we have also evaluated the levels of muscle-specific miRNAs. It is known that their generation can be modulated by MRFs,[15,35] and as our recent data indicate also by HO-1.[4] We have observed significant increase in miR-206, which promotes differentiation,[36] and decrease in miR-146a, known to inhibit myoblast differentiation[16] in skeletal muscles injected with pHRE-HO-1 when compared with the control group 14 days post-treatment. Again, switching-off the HO-1 expression after the period when it stimulates myoblast proliferation may be beneficial and stimulate myoblast differentiation.

Summing up, our study suggests that the effect of HO-1 on muscle regeneration is multifactorial. First, it can be related to the known cytoprotective action of HO-1, as reflected by decrease in apoptosis of the muscle fibres in animals overexpressing HO-1 and attenuation of inflammatory response in the injured tissue; second, the stimulation of revascularization through the improvement of endothelial cell viability, migration, and proliferation, resulting in increased formation of capillaries; third, by the influence on myoblasts proliferation and differentiation. It may be supposed that temporal expression of HO-1 initially promotes myoblasts proliferation, as suggested by increase in Pax7 expression and decrease in MyoD and myogenin expression at early phase of regeneration in muscle overexpressing HO-1. Then switching off HO-1 expression may accelerate muscle differentiation by downregulating the expression of Pax7 as well as miR-146a, while stimulating the expression of differentiation drivers miR-206 and myogenin.

In conclusion, our results demonstrate that HO-1 may play an important role in the protection of ischaemic tissue by decreasing inflammation and apoptosis, and promoting neovascularization. Moreover, for the first time, we have shown that HO-1 may be an important new player in the muscular regeneration by promoting renewal of the satellite cell compartment. The pHRE-HO-1 vector provided a useful on–off physiological switch, which rendered relatively fast transcription of the HO-1 gene in hypoxic/ischaemic conditions and can be further investigated as a tool to provide cytoprotection in ischaemic cardiovascular diseases.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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