Haeme oxygenase promotes progenitor cell mobilization, neovascularization, and functional recovery after critical hindlimb ischaemia in mice

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Aims Neovascularization is an important element of long-term functional recovery during chronic ischaemia. We postulated that haeme oxygenase (HO) is required for progenitor cell recruitment, neovascularization, and blood flow recovery after critical hindlimb ischaemia (HLI).

Methods and results The femoral artery was ligated in FVB/N mice proximal to its superficial and deep branches. Blood flow in the ischaemic hindlimb was determined by laser Doppler perfusion imaging. Capillary density was measured by isoelectin staining, and mobilization of Sca-1⁺/Kdr⁺ progenitor cells by FACS analysis. Progenitor cell recruitment to the ischaemic hindlimb was assessed after Tie2-lacZ transgenic bone marrow transplantation. Blood flow recovery after femoral artery ligation was significantly blunted in mice treated with the HO inhibitor tin protoporphyrin-IX (25 mg/kg i.p., every other day). HO-inhibited mice developed more pronounced limb necrosis, associated with impaired hindlimb motor function. Capillary density in the ischaemic hindlimb and mobilization of Sca-1⁺/Kdr⁺ progenitor cells were significantly reduced after HO inhibition. After transplantation of Tie2-lacZ transgenic bone marrow cells into lethally irradiated wild-type mice, fewer LacZ⁺ cells were detected in the ischaemic hindlimb muscle of HO-inhibited mice. Mechanistically, HO inhibition prevented the establishment of a stromal cell-derived factor-1 gradient for progenitor cell mobilization between the ischaemic hindlimb and bone marrow.

Conclusion HO-1 are required for progenitor cell recruitment, neovascularization, and functional recovery after HLI.

KEYWORDS
Angiogenesis; Microcirculation; Regional blood flow

1. Introduction
Neovascularization is an adaptive response to relieve ischaemia in tissues with compromised arterial blood supply. Neovascularization in the adult was originally thought to result largely from angiogenesis, i.e. the sprouting of pre-existing, fully differentiated endothelial cells. More recent studies have shown that circulating endothelial progenitor cells (EPCs) contribute to blood flow recovery after ischaemia by homing to sites of neovascularization, by differentiating into endothelial cells in situ, and/or by contributing to the release of vasogenic substances in the underperfused tissue; these processes have been referred to as (post-natal) vasculogenesis. The bone marrow constitutes an important source of circulating EPCs. Mobilization of EPCs from the bone marrow niche is significantly enhanced following peripheral or myocardial ischaemia, and is triggered by the local release of cytokines, such as vascular endothelial growth factor (VEGF), and chemokines, such as stromal cell-derived factor-1 (SDF-1). Establishment of a concentration gradient of SDF-1 between ischaemic tissues and the bone marrow is thought to play a major role in the recruitment of EPCs to sites of neovascularization.

Haeme oxygenase (HO) catalyses the rate-limiting step in the degradation of haeme to ferrous iron, carbon monoxide, and biliverdin, which is subsequently converted to bilirubin by biliverdin-reductase. There are two distinct isoforms of HO. HO-1 is expressed at low basal levels in most tissues,
including endothelial cells and haematopoietic stem cells, and is rapidly induced by a broad spectrum of stressful stimuli, including tissue ischaemia. Conversely, HO-2 is constitutively expressed at low levels in most tissues, including the vasculature. During recent years, interest in HO has shifted from its metabolic function to its more general role as a signaling mediator in various disease states associated with cellular stress and inflammation. It has been recognized that HO, in particular the inducible HO-1 isoform, promotes protective effects in the cardiovascular system. HO has been shown to inhibit atherosclerotic plaque formation, to decrease intimal hyperplasia following vascular trauma, to protect from myocardial ischaemia-reperfusion injury, and to inhibit cardiac hypertrophy in animal models. HO also appears to play an important role during angiogenesis, for example, by mediating VEGF-induced endothelial cell proliferation.

However, the role of HO during neovascularization after critical hindlimb ischaemia (HLI) has never been investigated. Moreover, the potential contribution of HO to postnatal vasculogenesis has not been assessed. In the present study, we therefore explored whether HO is required for EPC recruitment, neovascularization, and blood flow recovery after critical HLI.

2. Methods

2.1 Hindlimb ischaemia model

Ten- to 12-week-old male FVB/N mice (Charles-River) were subjected to permanent femoral artery ligation. Anesthesia was induced by i.p. injection of ketamine (2 mg/kg body weight) and xylazine (13 mg/kg body weight), and maintained by isoflurane (2%) ventilation. To induce HLI, the right femoral artery was ligated proximal to the deep femoral artery including all superficial and deep branches. Blood flow recovery in this animal model of chronic ischaemia depends on arteriogenesis and ischaemia-driven angiogenesis and vasculogenesis. Immediately after the procedure and during follow-up examinations on days 3 and 7, mice were placed on 37°C heated pads, and limb perfusion was measured by laser Doppler imaging (PIM II, Perimed, Sweden). Tissue perfusion of the distal part of the hindlimb (below the knee) was expressed as the number of capillary fields (20 magnification) from three cross-sections were evaluated, and capillary density was expressed as the number of capillary profiles per myocyte. As an additional readout for capillarization, we performed CD31 immunofluorescence staining using an antibody from BD Biosciences. Skin infiltration by leucocytes (CD45) and monocytes (CD68) was assessed by immunofluorescence staining using antibodies from BD Biosciences and Serotec, respectively. After bone marrow cell transplantation, β-galactosidase-expressing cells were identified by X-gal staining. Slides were counter-stained with H&E, and LacZ+ cells per mm² were quantified in three cross-sections per animal.

2.4 Reverse transcription real-time polymerase chain reaction

Three days after femoral artery ligation, total RNA was prepared from the gastrocnemius muscle using the TRIzol reagent (Invitrogen). After reverse transcription, SDF-1 mRNA and 18S rRNA were amplified using murine primer sets from SuperArray Bioscience followed by the manufacturer’s instructions.

2.5 Fluorescence-activated cell sorting

Circulating Sca-1+ /Kdr+ progenitor cells were quantified by FACS analysis using fluorescein isothiocyanate and phycoerythrin-labelled antibodies from BD Pharmingen, and a flow cytometer (FACSCalibur) and CellQuest software from BD Biosciences.

2.6 Immunoblotting

Standard immunoblotting techniques were employed using primary antibodies against α-actinin and HO-1 (both from Sigma). Protein concentrations were determined by the Bradford assay, and equal amounts of protein (30 μg) were loaded per lane. Membranes were developed with horseradish peroxidase-coupled secondary antibodies and an enhanced chemiluminescence kit (both from Amersham).

2.7 Stromal cell-derived factor-1 enzyme-linked immunosorbent assay

SDF-1 concentrations in serum and bone marrow were determined by ELISA (R&D Systems). Bone marrow was obtained from the femurs and tibias, as described. The bones were flushed with 500 μL of phosphate-buffered saline. After centrifugation, the
supernatant was frozen at −80°C. SDF-1 concentrations in bone marrow supernatants were normalized for protein content as measured by the Bradford assay.

2.8 Statistical analyses
Data are presented as mean ± SEM. Differences between groups were analysed by one-way ANOVA followed by Student–Newman–Keuls post hoc test. A two-tailed P-value of <0.05 was considered statistically significant.

3 Results

3.1 Haeme oxygenase inhibition impairs blood flow recovery after hindlimb ischaemia

As shown by immunoblotting, HO-1 protein abundance increased in the gastrocnemius muscle 12 and 24 h after femoral artery ligation (Figure 1A). Seven days after femoral artery ligation, the majority of saline-treated animals displayed no apparent hindlimb necrosis, only two out of 12 mice (17%) developed toe necrosis (Figure 1B). HO inhibition resulted in more extensive tissue damage in the ischaemic hindlimb: out of 13 SnPP-treated animals, seven animals (54%) developed toe necrosis, one animal (8%) developed necrosis of the entire foot (Figure 1B); this was associated with a more severe impairment of active foot movement (Figure 1C). SnPP did not induce hindlimb necrosis (Figure 1B) and did not affect hindlimb function (Figure 1C) in non-operated control mice. Immediately after femoral artery ligation, laser Doppler perfusion imaging demonstrated similar reductions in blood flow in saline- and SnPP-treated animals (Figure 1D and E). Seven days after the procedure, blood flow in the ischaemic hindlimb had almost returned to normal in saline-treated animals (91 ± 4% of non-ischaemic leg) (Figure 1D).

Figure 1 Haeme oxygenase inhibition impairs blood flow recovery and limb function after ischaemia. Mice underwent right femoral artery ligation and were treated with saline hindlimb ischaemia or SnPP; non-operated mice were not treated (no OP) or treated with SnPP (no OP/SnPP). Twelve and 24 h after femoral artery ligation, Haeme oxygenase-1 protein expression was determined by immunoblotting in the ischaemic (isch) and non-ischaemic (n-isch) gastrocnemius muscles from operated mice, and in muscle from non-operated animals (control); data from four animals are shown on the left, data from one animal at 12 h, one animal at 24 h, and one control animal are shown on the right (in each case, protein was isolated from 100–200 mg pieces of hindlimb muscle, and 30 μg of protein were loaded per lane) (A). The severity of hindlimb necrosis was assessed by a scoring system on day 7 (n = 9–13 animals per group) (B). Ischaemic hindlimb motor function was evaluated on day 7 by the active foot movement score (n = 14–15 animals/group) (C). Blood flow recovery was assessed by laser Doppler imaging immediately after femoral artery ligation (day 0) and on postoperative days 3 and 7 (n = 9–13 animals per group and time point). Perfusion is expressed as the ratio of the ischaemic to the non-ischaemic hindlimb (D). Typical laser Doppler perfusion images are shown in (E). *P < 0.05 vs. no OP; **P < 0.01, ***P < 0.001 vs. hindlimb ischaemia.
Conversely, blood flow recovery was delayed in SnPP-treated animals, and significantly reduced 3 and 7 days after femoral artery ligation (Figure 1D and E). SnPP did not affect hindlimb blood flow in non-operated mice (not shown). At day 7, no differences in arteriogenesis in the ischaemic hindlimb were observed by contrast angiography in five SnPP-treated animals as compared to five saline-treated animals (data not shown).

### 3.2 Reduced neovascularization after hindlimb ischaemia in haeme oxygenase-inhibited mice

Seven days after femoral artery ligation, capillary density was significantly increased in the gastrocnemius muscle in saline-treated animals (Figure 2A and B). Treatment with SnPP did not affect capillary density in non-operated mice, but abolished the angiogenic response to HLI (Figure 2A and B). Haeme oxygenase inhibition attenuates neovascularization after hindlimb ischaemia gastrocnemius muscle tissue was harvested 7 days after femoral artery ligation. Cryosections were stained by the endothelial marker biotinylated isoelectin B4 (yellow), 4,6-diamidine-2-phenylindoldihydrochloride (blue) and wheat germ agglutinin (red) for quantification of capillary-like structures and myocytes. Representative cryosections demonstrating fewer capillary profiles in haeme oxygenase-inhibited (HLI/SnPP) when compared with saline-treated animals (hindlimb ischaemia) are shown; non-operated mice were not treated (no OP) or treated with SnPP (no OP/SnPP) (A). Data from n = 7–10 animals per group are summarized in the bar graph; capillary density is expressed as the number of capillary profiles per myocytes (B). *P < 0.05 vs. no OP; **P < 0.01 vs. hindlimb ischaemia.

Haeme oxygenase inhibition decreases progenitor cell mobilization and reduces bone marrow cell recruitment to the ischaemic region after hindlimb ischaemia circulating progenitor cells expressing Sca-1 and Kdr were quantified by FACS analysis 7 days after femoral artery ligation. Exemplary plots are shown in (A). The X- and Y-axes of the FACS plots represent phycoerythrin/Kdr and fluorescein isothiocyanate/Sca1, respectively. The gates were chosen based on isotype control stainings. Data from n = 7–11 animals per group are summarized in (B). Tie2-lacZ-transgenic bone marrow cells were transplanted into lethally irradiated wild-type mice. Four weeks after bone marrow reconstitution, hindlimb ischaemia was induced, and mice were treated with saline (hindlimb ischaemia) or SnPP; transplanted but non-operated mice were either not treated (no OP) or treated with SnPP (no OP/SnPP). Gastrocnemius muscle tissue was harvested 7 days after hindlimb ischaemia. Cross-sections were stained by X-gal to identify lacZ⁺ cells and counter-stained by haematoxylin–eosin. Representative sections with fewer lacZ⁺ cells following hindlimb ischaemia and haeme oxygenase inhibition are shown in (C). Results from n = 8–14 animals per group are summarized in (D). ***P < 0.001 vs. no OP; *P < 0.05, **P < 0.01 vs. hindlimb ischaemia.

**Figure 2** Haeme oxygenase inhibition attenuates neovascularization after hindlimb ischaemia gastrocnemius muscle tissue was harvested 7 days after femoral artery ligation. Cryosections were stained by the endothelial marker biotinylated isoelectin B4 (yellow), 4,6-diamidine-2-phenylindoldihydrochloride (blue) and wheat germ agglutinin (red) for quantification of capillary-like structures and myocytes. Representative cryosections demonstrating fewer capillary profiles in haeme oxygenase-inhibited (HLI/SnPP) when compared with saline-treated animals (hindlimb ischaemia) are shown; non-operated mice were not treated (no OP) or treated with SnPP (no OP/SnPP) (A). Data from n = 7–10 animals per group are summarized in the bar graph; capillary density is expressed as the number of capillary profiles per myocytes (B). *P < 0.05 vs. no OP; **P < 0.01 vs. hindlimb ischaemia.

**Figure 3** Haeme oxygenase inhibition decreases progenitor cell mobilization and reduces bone marrow cell recruitment to the ischaemic region after hindlimb ischaemia circulating progenitor cells expressing Sca-1 and Kdr were quantified by FACS analysis 7 days after femoral artery ligation. Exemplary plots are shown in (A). The X- and Y-axes of the FACS plots represent phycoerythrin/Kdr and fluorescein isothiocyanate/Sca1, respectively. The gates were chosen based on isotype control stainings. Data from n = 7–11 animals per group are summarized in (B). Tie2-lacZ-transgenic bone marrow cells were transplanted into lethally irradiated wild-type mice. Four weeks after bone marrow reconstitution, hindlimb ischaemia was induced, and mice were treated with saline (hindlimb ischaemia) or SnPP; transplanted but non-operated mice were either not treated (no OP) or treated with SnPP (no OP/SnPP). Gastrocnemius muscle tissue was harvested 7 days after hindlimb ischaemia. Cross-sections were stained by X-gal to identify lacZ⁺ cells and counter-stained by haematoxylin–eosin. Representative sections with fewer lacZ⁺ cells following hindlimb ischaemia and haeme oxygenase inhibition are shown in (C). Results from n = 8–14 animals per group are summarized in (D). ***P < 0.001 vs. no OP; *P < 0.05, **P < 0.01 vs. hindlimb ischaemia.
and B). Very similar results were obtained when using CD31 immunostaining instead of isolectin staining (data not shown). To explore whether SnPP interferes with the EPC-dependent process of vasculogenesis, we determined the number of circulating Sca-1<sup>+</sup>/Kdr<sup>+</sup> cells by FACS analysis. HLI led to a significant increase in circulating Sca-1<sup>+</sup>/Kdr<sup>+</sup> cells in saline-treated animals (Figure 3A and B). SnPP had no influence on the number of circulating Sca-1<sup>+</sup>/Kdr<sup>+</sup> cells in non-operated mice, but strongly reduced the mobilization of Sca-1<sup>+</sup>/Kdr<sup>+</sup> cells following HLI (Figure 3A and B). Recruitment of bone marrow-derived cells to the ischaemic hindlimb was assessed after transplantation of Tie2-lacZ-transgenic bone marrow cells into wild-type recipient mice. Only very few lacZ<sup>+</sup> cells were present in gastrocnemius muscle in non-operated mice, regardless of SnPP-treatment. Seven days after femoral artery ligation, however, a massive increase in lacZ<sup>+</sup> cells was observed in saline-treated animals (Figure 3C and D). SnPP significantly reduced lacZ<sup>+</sup> cell recruitment to the ischaemic gastrocnemius muscle (Figure 3C and D).

### 3.3 Reduced stromal cell-derived factor-1 gradient between plasma and bone marrow in haeme oxygenase-1 inhibited mice

Next, we explored how HO may promote progenitor cell recruitment after HLI. As shown by real-time PCR, expression levels of SDF-1 mRNA relative to 18S rRNA were significantly elevated in the ischaemic gastrocnemius muscle in saline-treated animals (Figure 4A). At the same time, SDF-1 protein levels increased in the peripheral blood (Figure 4B) and decreased in the bone marrow (Figure 4C), thereby establishing a significant SDF-1 gradient between blood and bone marrow (Figure 4D). SnPP did not have an effect on basal SDF-1 levels in non-operated mice. After femoral artery ligation, however, SnPP significantly blunted the decrease in expression of SDF-1 in bone marrow (Figure 4C) and the increase in expression of SDF-1 in the ischaemic hindlimb muscles (Figure 4A) and in the peripheral circulation (Figure 4B). As a result, the SDF-1 gradient between blood and bone marrow was significantly reduced (Figure 4D).

### 4. Discussion

Neovascularization is a complex biological response that is important for limb salvage after critical ischaemia. Data from the present study show, for the first time, that HO inhibition interferes with blood flow recovery after HLI. Specifically, HO inhibition interferes with the establishment of an SDF-1 gradient between the ischaemic hindlimb and bone marrow and the mobilization and recruitment of putative EPCs. These data add to the growing body of evidence implicating HO in the regulation of neovascularization and point towards an important role of HO in vasculogenesis.

**Mobilization of Sca-1<sup>+</sup>/Kdr<sup>+</sup> (endothelial progenitor) cells after HLI was blunted in HO-inhibited mice. Moreover, the number of bone marrow-derived, Tie2-expressing cells in the ischaemic hindlimb was reduced following HO inhibition. Previous studies have shown that bone marrow-derived cells expressing a reporter gene under the control of the Tie2 promoter at sites of neovascularization represent a mixed population, consisting of mature endothelial cells and monocytes with paracrine and pro-angiogenic capabilities, suggesting:**

![Figure 4](image-url)
that reduced bone marrow cell recruitment to the ischaemic hindlimb may have contributed to the impairment of blood flow recovery in HO-inhibited mice. Indeed, it has been reported that blockade of progenitor cell recruitment is sufficient to reduce neovascularization in animal models. 28–30

Mechanistically, our study shows that HO is required for establishment of the SDF-1 gradient between the ischaemic hindlimb and bone marrow, which is essential for EPC trafficking and neovascularization. 7–9,30,31 HO inhibition interfered, both, with the increase in expression of SDF-1 in the ischaemic hindlimb and circulating blood and the decrease in expression of SDF-1 in the bone marrow compartment. Induction of SDF-1 and other chemokines, such as interleukin-8 (IL-8), in ischaemic tissues is regulated on a transcriptional level via hypoxia-inducible factor (HIF)-1α. 32 Notably, HO-1 has been implicated in the HIF-1α-mediated induction of IL-8 after myocardial ischaemia. 32

It has been observed in a retinal ischaemia model that HO-1 is required for the migratory capacity of murine EPCs. 33 It is conceivable therefore, that HO inhibition not only interfered with EPC mobilization, but also with EPC migration in our study. Importantly, not all check points controlling angiogenesis and EPC mobilization appear to be dependent on HO; in fact, HO inhibition did not interfere with the induction of VEGF protein expression in the ischaemic hindlimb, or Ser1177-phosphorylation (activation) of endothelial NO synthase in the bone marrow in our animal model (data not shown).

Although our findings highlight an important role of HO upstream from SDF-1, a recent study indicates that HO can also act downstream from SDF-1. 34 As shown in that report, SDF-1-induced angiogenesis in fully differentiated endothelial cells in vitro and Matrigel plugs in vivo critically depends on HO-1. 33 Similarly, HO-1 plays an important role during VEGF-induced non-inflammatory angiogenesis. 16 Although the molecular mechanisms are not fully understood, all haeme metabolites appear to contribute to the pro-angiogenic potential of HO-1. 15–18 Considering the significant impairment in capillarization and blood flow recovery in HO-inhibited mice in our study, it is likely that SnPP not only inhibited vasculogenesis but also angiogenesis after HLI. For example, treatment with SnPP may have inhibited angiogenesis downstream from VEGF, which was significantly induced in the ischaemic hindlimb in our animal model, regardless of SnPP treatment. Notably, SnPP treatment did not appear to affect arteriogenesis in our animal model.

We used an established pharmacological approach to inhibit HO in our study. 11 This design allowed us to inhibit HO precisely at the time of ischaemic injury, and to avoid the anaemia and chronic inflammation observed in HO-1 gene-targeted mice. 34 Indeed, by using CD45 and CD68 immunohistochemistry, we did not find evidence for enhanced inflammation in the hindlimb skin of mice treated for 7 days with SnPP (data not shown). This is important, as a pro-inflammatory effect of SnPP may have induced hyperaemia thereby interfering with our laser Doppler blood flow measurements which are known to reflect mostly skin perfusion. Using a pharmacological strategy, however, we were not able to discriminate between HO-1 and HO-2 mediated effects, although the significant increase in expression of HO-1 in the ischaemic hindlimb suggests an important role of HO-1 in neovascularization after HLI. Also, off-target effects of a pharmacological agent like SnPP cannot be excluded.

In conclusion, our study identifies HO as a mediator of EPC recruitment and ischaemia-driven neovascularization, and adds to the growing body of evidence showing that HO is important for maintaining vascular homeostasis. Further research is needed to elucidate whether modification of the HO system could open new therapeutic avenues for patients with cardiovascular disease. 25

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