

# Heme oxygenase-1 in neovascularisation: A diabetic perspective

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## Summary

Neovascularisation is crucial both for physiological processes, like development, wound healing, tissue regeneration, hair growth or menstrual cycle, and for pathological states, such as tumour progression, retinopathy and psoriasis. Blood vessel formation is orchestrated by numerous pro-angiogenic and anti-angiogenic factors, acting together to keep tight rein on this complicated, desirable but also dangerous process. One of the proteins important for neovascularisation is heme

oxygenase-1 (HO-1), an enzyme degrading heme. This review focuses on the role of HO-1 in angiogenesis and vasculogenesis, having a closer look at the significance of this system in diabetes.

## Keywords

Heme oxygenase-1, neovascularisation, diabetes, wound healing, endothelial progenitor cells

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## Heme oxygenase-1 and products of its activity

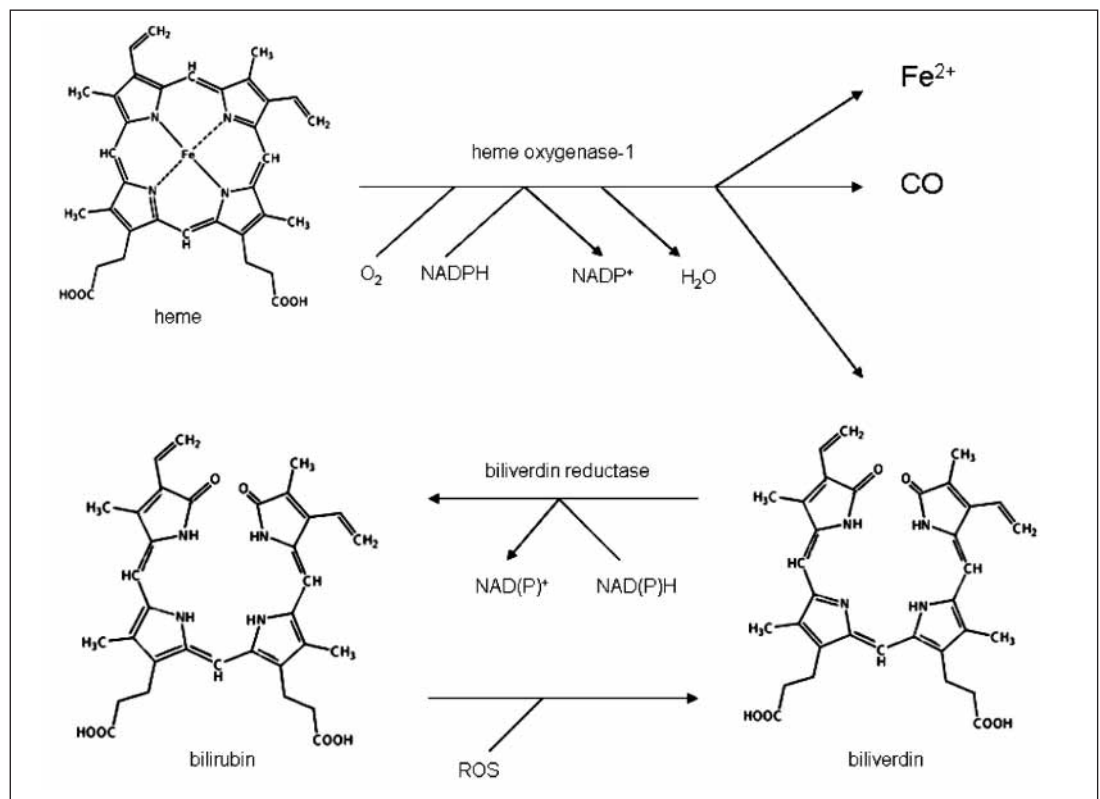
Heme, ferrous protoporphyrin IX, is a prosthetic group of numerous proteins, such as haemoglobin, myoglobin, cytochromes, catalase, peroxidase, nitric oxide synthase, guanyl cyclase and tryptophan pyrrolase (1). Free heme is a prooxidative compound, which induces generation of free radicals and lipid peroxidation (2). It is also a source of free iron, that contributes to free radical generation in Fenton's reaction (3). Moreover, heme molecule has lipophilic properties and can destroy cell membrane, mitochondria, cytoskeleton and nucleus (4). Therefore it must be efficiently removed from the cellular microenvironment. An enzyme responsible for this process is heme oxygenase (HO). There have been two isoforms of HO described so far: an inducible HO-1 and a constitutively expressed HO-2 (5)]. A third isoform HO-3, which is in fact a pseudogene derived from HO-2 transcript, has been found only in rats (6). HO degrades heme to equimolar quantities of carbon monoxide (CO), iron ion and biliverdin (7). Biliverdin subsequently undergoes conversion to bilirubin by biliverdin reductase (BVR), the second enzyme of heme catabolism pathway (► Fig. 1) (8).

HO-1 is a microsomal protein, however it was also demonstrated to be localised in mitochondria (9), caveoli (10) and in nucleus, where it can activate transcription factors important for proper response to oxidative stress (11). It is ubiquitously expressed in mammalian tissues and its expression is highly induced by various stimuli, including heme, ultraviolet (UV) light, lipopoly-

saccharide (LPS) and other oxidative factors (12). The products of HO-1 activity exert several biological effects in regulation of cellular metabolism and homeostasis, as well as play a role in pathological processes (13, 14). They have cytoprotective, proangiogenic and anti-apoptotic properties and are the second, after glutathione, most important part of anti-oxidative protection system (4, 15).

CO has similar features to nitric oxide (NO). It is a signaling molecule, which regulates vascular functions: induces guanyl cyclase activity leading to blood vessel relaxation (16, 17), and inhibits platelets aggregation (18). CO influences also gene expression (15, 19). Moreover, it exerts anti-apoptotic and cytoprotective effects, preventing apoptosis in several cell types, including endothelial cells (20), vascular smooth muscle cells (21), fibroblasts (22), osteoblasts (23), and pancreatic  $\beta$ -cells (24). Furthermore, CO protects lungs against hyperoxic injury (25). Carbon monoxide plays an important role also in angiogenesis: it stimulates proliferation of endothelial cells (26, 27), inhibits their apoptosis (20) and induces vascular endothelial growth factor (VEGF) expression in vascular smooth muscle cells, macrophages and microvascular endothelium (28–30).

Free ferrous ion, the second product of HO-1 activity, is toxic to the cells; therefore after release from heme it is quickly transported to bone marrow, where it is utilised in erythropoiesis. There are several proteins binding free iron. One of them is ferritin, a primary intracellular iron-storage protein, exerting anti-oxidative properties (31), whose expression is induced by  $Fe^{2+}$  (32). Moreover, HO-1 directly takes part in utilisation of ferrous ions via activation of  $Fe^{2+}$ -ATP pump (4, 33, 34).



**Figure 1: Heme catabolism pathway.** Heme molecule is degraded by heme oxygenase-1 (HO-1) to three compounds: iron ion, carbon monoxide and biliverdin, which is subsequently reduced to bilirubin by biliverdin reductase (BVR). Bilirubin scavenges reactive oxygen species (ROS) and is oxidised to biliverdin.

Finally, biliverdin and bilirubin possess strong anti-oxidant features. They scavenge free radicals and prevent lipid peroxidation (35–36). Bilirubin reacts with hydroxyl radical, singlet oxygen and superoxide anion (37, 38). However, recently published paper has suggested that the role of BVR-mediated redox cycle, as a cellular antioxidant defense mechanism, is overestimated (39).

## HO-1 knockout mice and HO-1 deficiency in humans

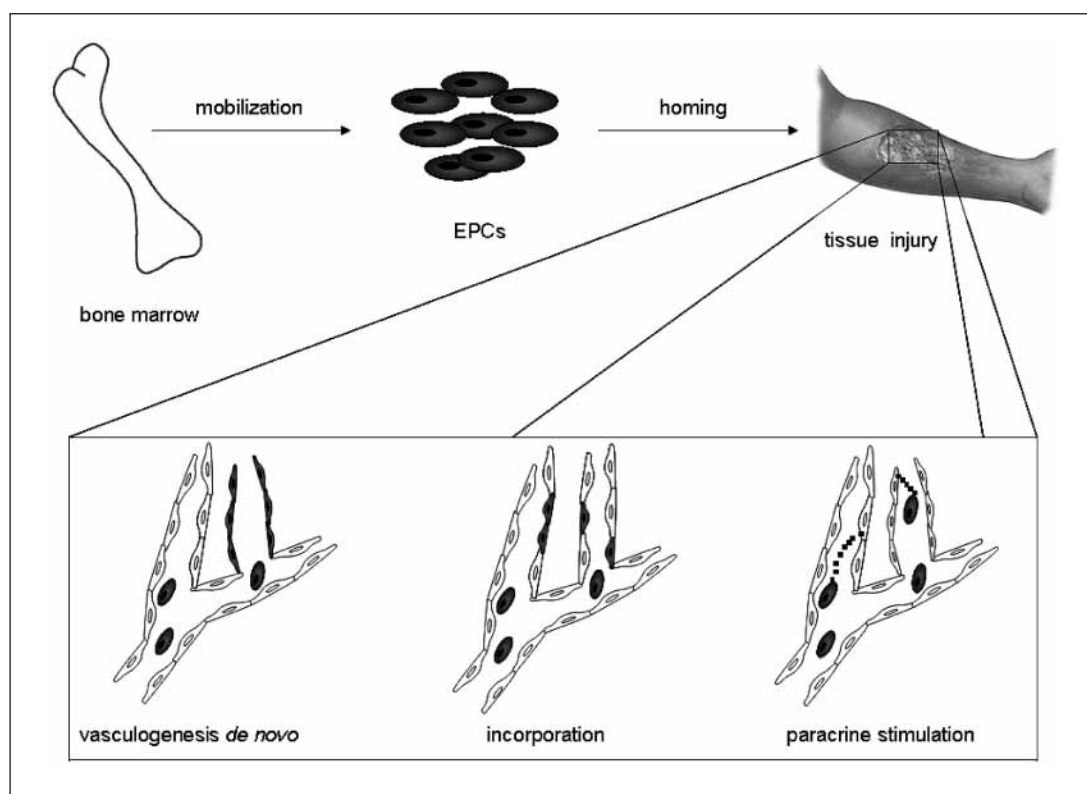
The consequences of HO-1 deficiency are severe. HO-1 knockout mice have lower body weight and suffer from anaemia with very low level of iron and reduced number of erythrocytes in blood. In these animals iron is stored in tissues, especially in kidneys and livers, what leads to intensification of oxidative processes, chronic inflammation and cell damage (40). The cells isolated from HO-1 deficient mice demonstrate lower resistance to oxidative stress induced by hemin, hydrogen peroxide and cadmium chloride (41).

There has been only one case of human two-allele HO-1 deficiency described so far. The symptoms were similar and even more pronounced than those observed in HO-1 knockout mice. The boy suffered from anaemia, erythrocyte fragmentation and intravascular haemolysis, low level of bilirubin, as well as severe and chronic impairment of endothelium. In liver and kidney the accumulations of iron were observed, and the cells were very vulnerable to hemin-induced damage (42).

## HO-1 in angiogenesis

There are numerous processes directly dependent on tissue neovascularisation. One of them is skin wound healing. Neovascularisation can be achieved in two different ways: via vasculogenesis and angiogenesis. Vasculogenesis includes capillary formation either from angioblasts (stem cells) or from endothelial progenitor cells (EPC) in response to mediators such as basic fibroblast growth factor (bFGF) or VEGF (43). Angiogenesis is a process of blood vessel formation from the pre-existing ones, based on migration and proliferation of mature, totally differentiated endothelial cells, which are stimulated by various factors, among which VEGF is doubtless the most important. This process occurs both in embryogenesis as well as postnatally. Angiogenesis contributes to many disorders, where excessive (cancer, rheumatoid arthritis, psoriasis) or impaired vascularisation (coronary artery and peripheral arterial diseases, ulcers) causes disease development or treatment failure.

Previously, postnatal vascularisation was thought to occur exclusively due to angiogenesis. However, recent investigations have shown EPC to be involved in blood vessels formation during repair processes. EPC are mobilised mainly from the bone marrow by mediators released in ischaemic tissue-like VEGF, stromal cell-derived factor-1 (SDF-1), angiopoietin-1, or FGFs – and home to disrupted or ischaemic tissue (44, 45). In this way, EPC may stimulate new blood vessel formation *de novo*. It is also possible, that EPC are incorporated into existing vessel structures. So far the significance of this phenomenon has not been investigated sufficiently. It is



**Figure 2: Involvement of endothelial progenitor cells (EPC) in blood vessel formation.** EPC are mobilised from bone marrow into peripheral blood in response to tissue injury. They home to injured area and participate in blood vessel formation via three possible ways. EPC can either form blood vessels de novo or incorporate into existing vasculature or paracrine stimulate mature endothelial cells.

commonly accepted, based on labelled EPC incorporation into vessel wall, that progenitor cells participate in formation of new capillaries, but the assessments of the scale of this process range from 0–80% (44, 46–48). EPC may play also a role in neovascularisation due to paracrine *in situ* stimulation of endothelial cells in existing capillaries, since they produce growth factors, such as VEGF, bFGF, HGF (hepatocyte growth factor), IL-8 (interleukin-8), PDGF-BB (platelet derived growth factor-BB), MCP-1 (macrophage chemoattractant protein-1), and MIP-1 (macrophage inflammatory protein-1) (49–52) (► Fig. 2).

There is a growing number of papers demonstrating the significance of HO-1 in angiogenesis (reviewed in [12, 53]). Overexpression of HO-1 leads to the stimulation of VEGF synthesis in various cell types and in animals (26, 28, 54–56). Furthermore, HO-1 mediates VEGF synthesis driven by oxidative stress (54). Activation of HO-1 in endothelial cells is also crucial for pro-angiogenic response to VEGF, since pharmacological inhibition of HO-1 or knockout of HO-1 gene impair proliferation, migration and angiogenic potency of VEGF-stimulated endothelial cells (26, 54). The involvement of HO-1 in VEGF synthesis was confirmed by inhibition of VEGF expression by attenuation of HO-1 mRNA expression with specific siRNA (57). Hitherto, results of *in vivo* experiments support those observations, showing that increased expression of HO-1 leads to the induction of angiogenesis. Such effect has been observed in rat femoral muscle after injection of adenoviral vectors with HO-1 encoding sequence (58). Enhancement of angiogenesis in cancer and rheumatoid arthritis also depends on HO-1 overexpression (59–63). Noteworthy, HO-1 was also

shown to regulate the expression of anti-angiogenic factors such as soluble endoglin (sEng) and soluble VEGFR-1. These inhibitors of angiogenesis were upregulated in case of HO-1 deficiency (64).

SDF-1 is the second, very important pro-angiogenic mediator, whose activity is dependent on heme oxygenase-1. Injection of viral vectors with HO-1 leads to increase in SDF-1 expression (55, 65). On the other hand, SDF-1 upregulates HO-1 in endothelial cells (66) and HO-1 derived CO is required for the phosphorylation of vasodilator-stimulated phosphoprotein (VASP), a cytoskeletal-associated protein involved in cell migration (67, 68), necessary for the formation of blood vessels. siRNA studies showed HO-1 to be crucial for SDF-1 induced sprouting in ring aortic assay and tube formation assay *in vitro* (66); the significance of HO-1 was also confirmed in SDF-1 induced capillary sprouting from HO-1 deficient rings (66). Moreover, HO-1 was shown to mediate the function of EPC in a model of retinal ischaemia and critical hind limb ischaemia (66, 69).

However, it must be kept in mind that the role of HO-1 in angiogenesis depends on the underlying conditions, since for example the inflammation-induced blood vessel formation is attenuated, whereas VEGF-driven non-inflammatory angiogenesis is facilitated by HO-1 (70).

Since many data show an important role of HO-1 in neovascularisation one can suppose that decreased HO-1 activity may contribute to the impaired revascularisation processes in ischaemic or damaged tissues. In such a case, patients might benefit from overexpression of HO-1 in the injured organ, achieved by gene transfer or combined gene and cell therapy.

## Impaired wound healing in diabetes

Impairment of endothelial function and reduced tissue vascularisation is one of the major reasons of delayed wound healing in diabetic patients (71, 72). Considerable attention was paid to the expression of growth factors associated with angiogenesis in diabetic wounds. It was demonstrated that induction of VEGF after injury in db/db (diabetic and hyperglycaemic) mice was reduced, compared to the wild-type animals (73, 74). The defect in VEGF production may be, at least in part, caused by lipid peroxidation, as administration of an inhibition of lipid peroxidation normalised VEGF level in the wound, improved angiogenesis, re-epithelialisation and extracellular matrix structure in diabetic mice (75). Moreover, it was shown, that topical VEGF is able to improve wound healing in db/db mice, as it increased re-epithelialisation, cell proliferation, expression of PDGF and bFGF, and even recruited bone marrow-derived EPC to form new vessels in the wound (76). Galiano et al. demonstrated that EPC mobilisation and stimulation of neovascularisation by VEGF is a major factor which accelerates wound healing in db/db mice (76), although the other proangiogenic factors, such as bFGF, also play an important role (77).

Some studies indicate that impaired diabetic wound healing is the result of prolonged inflammation (78). It was demonstrated that sustained inflammatory response and induction of chemokines accompany wound repair in db/db mice. *In vitro* studies revealed that fibroblasts isolated from db/db mice are characterized by impaired migration and response to hypoxia. The production of VEGF was reduced, whereas MMP-9 activity was greater than in wild-type fibroblasts, which may lead to the prolongation of inflammatory state.

Another hypothesis, which does not exclude the previous ones, is that the defects in healing of diabetic wounds result from increased apoptosis accompanied by decreased cell proliferation (79). Significance of neovascularisation, inflammation and apop-

tosis may suggest a potential involvement of HO-1, a proangiogenic, anti-inflammatory and anti-apoptotic enzyme in regulation of wound healing.

## HO-1 and diabetes

The effect of diabetic conditions on HO-1 expression and activity has been investigated in various models *in vitro* and *in vivo* and the results of those experiments are inconsistent (► Table 1). However, even if regulation of HO-1 expression in diabetes is still uncertain, doubtlessly hyperglycaemia leads to endothelial dysfunction, impaired cell replication and increased apoptosis (80–82) and these effects are reversed by overexpression of anti-oxidative enzymes, such as HO-1 (83, 84).

The most commonly used experimental model for investigating the HO-1 expression in diabetic animals are the rats injected intravenously with streptozotocin (STZ). Upregulation of both HO-1 expression and activity is considered to play a protective role against the development of diabetic complications. Accordingly, in glomeruli of diabetic rats the significant increase in HO-1 mRNA levels was observed, whereas expression HO-2 remained unchanged. It was proposed that induction of HO-1 results from excessive oxidative stress and may attenuate the symptoms of diabetic nephropathy, however the report did not provide a conclusive evidence supporting this hypothesis (85). Similarly, HO-1 mRNA level was up-regulated in the retina of STZ-induced diabetic rats. Surprisingly, also the retinal HO-2 expression was enhanced (86). In accordance with these results, HO mRNA, protein (both isozymes) and activity was up-regulated in the heart of diabetic rats (87). In this case, increased HO activity was associated with the presence of stainable iron in cardiomyocytes and SnPPIX, an HO activity inhibitor treatment, prevented diabetes-induced oxidative stress, as well as iron accumulation in the cells. This suggests a pro-

**Table 1: HO-1 expression and activity in diabetes.**

Animal models			
Model	Tissue	HO-1 expression/activity	Ref.
STZ-injected rats	glomeruli	increase in HO-1 expression	[85]
	retina	increase in HO-1 expression	[86]
	heart	increase in HO-1 expression and activity	[87]
	aorta	decrease in HO-1 activity, no change in HO-1 expression	[90]
	endothelial cells	decrease in HO-1 activity	[90]
ob/ob mice	pancreatic $\beta$ -cells	increase in HO-1 expression	[88]
db/db mice	pancreatic $\beta$ -cells	increase in HO-1 expression	[89]
	skin	weaker HO-1 expression in hemin-injected skin	[74]
	skin injury	delayed and weaker HO-1 induction	[74]
	fibroblasts	decreased HO-1 expression	[91]
Human research			
Diabetic patients	Tissue	HO-1 expression/activity	Ref.
	retinal pigment epithelium	decrease in HO-1 expression	[92]
	peripheral blood mononuclear cells	decrease in HO-1 expression	[98]

oxidant activity of HO in the heart in diabetes. Moreover, treating the non-diabetic animals with hemin (an HO-1 inducer) led to augmented HO protein levels and to development of abnormalities similar to those in diabetic rats (87). Up-regulated expression of HO-1 was also found in pancreatic  $\beta$ -cells of ob/ob mice (88) and db/db mice (89).

Different results were demonstrated for aorta of STZ-treated rats, where an inhibition of HO activity was observed, despite no changes in HO-1 protein expression (90). In another study, a decrease in HO activity in the early stages of diabetes was shown in endothelial cells (90), also in fibroblasts isolated from db/db mice (91). Moreover, hemin-induced HO-1 expression was weaker in skin of db/db mice in comparison to wild-type (74). Finally, post-injury HO-1 induction was demonstrated to be delayed and weaker in diabetic mice in comparison to healthy animals (74).

In contrast to the most of data obtained from STZ-induced diabetes in animals, analyses performed in humans show downregulation of HO-1 expression in diabetic patients. For example, in human retinal pigment epithelium from diabetic donors decreased expression of HO-1 mRNA was demonstrated and suggested to augment the vulnerability of neuroretina to diabetic injury (92). Interesting suggestions can be also driven from analysis of HO-1 promoter gene polymorphism in diabetes. Human HO-1 gene promoter contains a fragment of (GT) $n$  microsatellite DNA, ranging from 11 to 40 (93). Longer (GT) $n$  sequences in this region have been associated with attenuated HO-1 transcriptional activ-

ity (94–96) and were demonstrated to be associated with susceptibility to coronary artery disease in type 2 diabetic patients (95, 97). Moreover, recently published paper has demonstrated that HO-1 expression level was significantly reduced in patients with type 2 diabetes carrying the longer (GT) $n$  allelic variants compared with persons with the shorter (more active) ones in Chinese population (98). Taken together, it seems that HO-1 expression is rather downregulated in diabetic patients at least in some tissues. This can exert detrimental effects, for example on the vascular function.

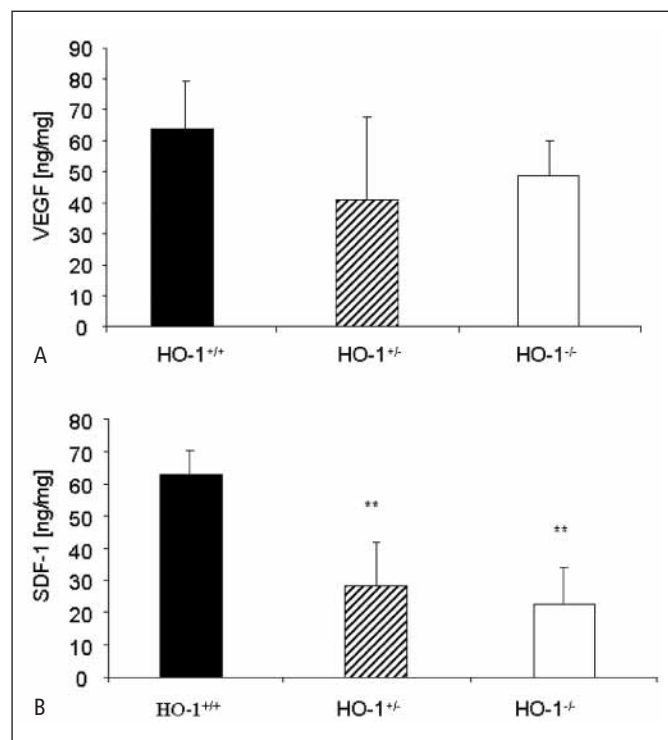
## Neovascularisation in diabetes: Significance of HO-1

Non-healing, chronic or reappearing wounds in diabetic patients are a severe consequence of vascular dysfunction (99). They may lead to development of ulcers and necrosis and finally to necessity of amputation (100). Therefore, gene or cell therapy targeted to the endothelium can be considered as a therapeutic approach to ameliorate diabetic complications. Since HO-1 exerts beneficial effects on survival, inflammatory response and angiogenic potential of endothelial cells, it represents a potential candidate for such a treatment, at least in terms of vascular dysfunction.

It was demonstrated that growth factors' production, including VEGF, is impaired by high glucose level (57, 101), and HO-1 induction reverses this disadvantageous effect (57). HO-1 is also considered to attenuate hyperglycemia-induced damage to endothelium. Endothelial cell sloughing in STZ-induced diabetic rats, caused by excessive ROS production, is reduced by retroviral-mediated HO-1 overexpression (102). Moreover, HO-1 was also shown to ameliorate glucose-induced apoptosis of human microvessel endothelial cells (103). However, it was also reported that HO-1 induction under hyperglycaemic conditions can lead to oxidative DNA and protein damage in HUVECs (104).

Carbon monoxide seems to be the product of HO-1 activity, which is directly responsible for the improvement of diabetic endothelial function. Increased generation of HO-1-derived CO was demonstrated to provide vascular protection in type 1 diabetes, decreasing endothelial cell fragmentation, reducing expression of ICAM-1, VCAM-1 and caspase 3 activity (105). Moreover, CO up-regulated the expression of adiponectin, adipose tissue-derived factor, which exert anti-inflammatory and anti-oxidative effects (105–107). This protein has a vascular protective properties in terms of endothelial cell function in diabetic and non-diabetic patients with the metabolic syndrome (108–110).

The beneficial results of HO-1 overexpression in diabetic complications have been recently demonstrated in a murine wound healing model (74). Skin regeneration in HO-1<sup>-/-</sup> mice was delayed in comparison to wild-type animals and production of important proangiogenic factors in injured skin was weaker in HO-1 deficient animals (► Fig. 3). On the other hand, wound healing was improved in transgenic HO-1 overexpressing individuals. These effects were associated with decreased wound vascularity and increased number of blood vessels in HO-1 deficient and transgenic



**Figure 3: Expression of VEGF and SDF-1 in wounded skin of mice three days post-injury.** VEGF (A) and SDF-1 (B) expression was measured by ELISA in skin wound lysates of HO-1<sup>+/+</sup>, HO-1<sup>+/-</sup> and HO-1<sup>-/-</sup> mice. \*\*  $p < 0.01$  vs. HO-1<sup>+/+</sup>.

HO-1 overexpressing mice, respectively. Noteworthy, HO-1 induction upon injury in db/db mice, in which wound healing was strongly impaired, was delayed and weaker in comparison to wild-type animals. At the same time, the formation of blood vessels was decreased. Finally, adenoviral vector-mediated HO-1 gene transfer accelerated wound healing in diabetic mice and this effect was associated with increased angiogenesis within the wounds (74). These observations indicate that activation of HO-1 facilitates wound healing both in normoglycaemic and diabetic mice, and the effect is associated with augmented angiogenesis.

A growing number of reports indicate that HO-1 overexpression might be regarded as a potential therapeutic approach in treatment of diabetic disorders. Viral HO-1 gene transfer is one of the possibilities to achieve this goal. Recently, considerable attention has been paid to cell therapy of diseases linked to vascular dysfunction, and possibly the most beneficial effects might be brought by combined cell and gene transfer strategies, with EPC as potential therapeutic agents. However, impairment of EPC functions in diabetic patients must be taken into account, when considering autologous cell therapy. Therefore, inquiry into the role of cytoprotective and provasculogenic genes, such as HO-1, may be useful for establishing the strategy aimed at improving the EPC regenerative potency.

Not only impaired function but also decreased numbers of EPC have been observed in type 1 and type 2 diabetic patients (111, 112). Abnormalities in EPC mobilisation, homing and re-endothelialisation in diabetes were identified as well (reviewed in [113]). Noteworthy, these alterations are regarded to be the cause of vascular dysfunction-linked complications in diabetes, such as delayed wound healing (113, 114). One of the most important proteins responsible for EPC mobilisation is SDF-1. In hyperglycaemic animals, the expression of SDF-1 is decreased in injured tissue. Local application of recombinant SDF-1 accelerated wound healing, due to induction of EPC mobilisation (115). Deshane et al. demonstrated that the number of blood vessels formed during skin tissue regeneration is increased upon administration of SDF-1 into the wound, and this effect is dependent on HO-1 (66). Again, HO-1

was demonstrated as a crucial factor necessary for promigratory and proangiogenic activities of SDF-1, the effect dependent possibly on CO production, cGMP elevation and PKG-mediated VASP phosphorylation (66). Consequently, EPC function and mobilisation are also regarded to be influenced by HO-1 (66, 116). First papers confirming the significance of HO-1 in diabetic EPC activity have already been published (117, 118).

In summary, it seems that HO-1 induction plays a permissive role in wound healing, and that efficacy of this pathway may be ameliorated in diabetic patients. *In vitro* experiments and studies performed in animal models confirm that HO-1 overexpression can facilitate vascularisation and healing of the wounds. HO-1 can be also used to improve EPC survival and angiogenic potential in cell therapies of ischaemic tissues. This strategy appears to be especially attractive for the potential clinical applications.

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### Abbreviations

aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; CO, carbon monoxide; eNOS, endothelial nitric oxide synthase; EPCs, endothelial progenitor cells; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; HO-1, heme oxygenase-1; HUVEC, human umbilical vein endothelial cells; ICAM-1, intracellular cell adhesion molecule; IL-8, interleukin 8; iNOS, inducible nitric oxide synthase; MCP-1, macrophage chemoattractant protein-1; MIP-1, macrophage inflammatory protein-1; MMP-9, metalloproteinase-9; PDGF-BB, platelet-derived growth factor-BB; PlGF, placental growth factor; SDF-1, stromal cell-derived factor-1; STZ, streptozotocin; TGF $\beta$ , tumour growth factor  $\beta$ ; VASP, vasodilator-stimulated phosphoprotein; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; VEGFR-1, vascular endothelial growth factor receptor 1; VEGFR-2, vascular endothelial growth factor receptor 2.

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