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Cardioprotection by hypoxia-inducible factor 1 alpha transfection in skeletal muscle is dependent on haem oxygenase activity in mice

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Aims The present study investigates whether the cardioprotection achieved by gene delivery of hypoxia-inducible factor-1α (HIF-1α) depends on the downstream factor haem oxygenase (HMOX)-1.

Methods and results Immortalized cardiomyocytes (HL-1 cells) were transfected with HIF-1α or HMOX-1 and injured with hydrogen peroxide (H_2O_2), and death was evaluated by trypan blue staining. Quadriceps muscles of mice were treated with DNA for HIF-1α and HMOX-1, or sham-treated and electroporated, and 3 days later, hearts were isolated and subjected to global ischaemia and reperfusion. Some HIF-1α- and sham-treated mice received the HMOX blocker zinc deuteroporphyrin 2,4-*bis*-glycol (ZnBG) ($n = 6$–8 in each group). HL-1 cells were stimulated with bilirubin or the carbon monoxide donor CORM-2 before injury with H_2O_2. HL-1 cells which were transfected with HIF-1α or HMOX-1 had an increased survival to H_2O_2-induced injury compared with empty vector ($n = 10$–12 per group; $P < 0.01$ for both). When HMOX-1-luciferase reporter mice were treated with HIF-1α in the quadriceps muscle, increased luciferase activity was found locally, but nowhere else. Mice pre-treated with HIF-1α or HMOX-1 had a reduced infarct size, improved post-ischaemic function, and increased serum bilirubin ($P < 0.05$). ZnBG inhibited all these effects afforded by HIF-1α. Stimulation of HL-1 cells with bilirubin and CORM-2 reduced cell death evoked by H_2O_2 ($P < 0.05$ for both, $n = 11$–15 in each group).

Conclusion HIF-1α and HMOX-1 provided protection against H_2O_2-induced damage in HL-1 cells. Remote gene delivery of HIF-1α afforded cardioprotective effects. These were dependent on HMOX activity, as an HMOX blocker abolished the effects, and they were mimicked by pre-treatment with HMOX-1. Downstream to HMOX-1, bilirubin as well as carbon monoxide may be organ effectors.

1. Introduction

Revascularization is essential to salvage ischaemic myocardium. However, some patients with ischaemic heart disease are not suitable for conventional revascularization procedures, and need other options to salvage myocytes. Genes encoding for cardioprotective factors could potentially diminish damage caused by ischaemia and reperfusion. Depending on the way and route of delivery, delivered genes can be expressed for a varied time-span. This opens a perspective of genetic cardioprotection, i.e. for patients undergoing elective coronary artery bypass grafting or balloon dilatation, where particularly those with pre-intervention low ejection fraction may benefit from extra protection. Also, patients with unstable angina waiting for revascularization procedures may benefit from pre-intervention gene therapy. Gene delivery directly to the myocardium is as yet difficult to perform without invasive procedures. The current paper is concerned with the possibility of using remote, genetic cardioprotection.

Hypoxia-inducible factor 1 alpha (HIF-1α) is a heterodimeric subunit of the transcription factor HIF-1, whose target genes cause adaptive responses to hypoxia/ischaemia. The HIF-1 complex regulates genes involved in angiogenesis, vascular tone, oxygen transport, glycolysis, iron metabolism, cell survival and proliferation.[1] HIF-1α is cardioprotective: injection of naked DNA encoding for HIF-1α directly into the left ventricle induced angiogenesis and reduced infarct size in a rat model of *in vivo* myocardial infarction with permanent occlusion.[2] Mice with cardiac overexpression of HIF-1α subjected to *in vivo* myocardial

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infarction developed reduced infarctions and attenuated progression of cardiac dysfunction 4 weeks after infarct induction.[3] We have previously delivered DNA encoding for HIF-1α into murine quadriceps muscle to protect hearts, demonstrating that HIF-1α-regulated genes are produced in skeletal muscle but in no other place in the organism.[4]

Some target genes of HIF-1 may directly exercise cardioprotective actions. Platelet-derived growth factor-B (PDGF-B) is known to phosphorylate and activate cardioprotective chaperons such as αB-crystallin and heat shock protein 27 in cardiomyocytes.[5] Locally administered PDGF-B decreased the extent of myocardial infarction in rats.[6] Adrenomedullin (ADM) has antiapoptotic effects in the myocardium and protects against ischaemia–reperfusion injury.[7] Insulin-like-growth factor-2 (IGF-2) reduces apoptosis and promotes cardiomyocyte survival.[8] Furthermore, IGF-2 delays myocardial infarction in experimental coronary artery occlusion.[9] Cardiac overexpression of haem oxygenase (HMOX)-1 protects against ischaemia–reperfusion injury,[10] and mice lacking one allele for HMOX-1 are more susceptible to ischaemia–reperfusion injury.[11] HMOX-1 catabolizes haem into free iron, biliverdin, which is rapidly converted to bilirubin, and carbon monoxide (CO).

We selected the HIF-1α target genes PDGF-B, IGF-2, ADM, and HMOX-1 along with HIF-1α itself for studies of cardioprotection. For an initial screening, DNA encoding for the factors was transfected into HL-1 cells subsequently subjected to H_2O_2-induced damage. Only HMOX-1 reduced cell death comparable with HIF-1α, and was chosen for further animal experiments. DNA encoding for HIF-1α or HMOX-1 was delivered to the skeletal muscle, and uptake was enhanced by electroporation. Pharmacological blocking of HMOX activity was performed to evaluate whether HMOX-1 could be a downstream factor to cardioprotection evoked by HIF-1α delivery. The protective effects of bilirubin and a CO-donor were studied in cell culture.

2. Methods

2.1 Cloning

The pCEP4/HIF-1α construct deriving from human HIF-1α cDNA sequence was purchased from ATCC, Johns Hopkins Special Collection.[12] pEGFP-N1 encoding for enhanced green fluorescent protein (EGFP) was purchased from Clontech. PDGF-B, ADM, IGF-2, and HMOX-1 were cloned from murine cDNA into pCR-Blunt II-TOPO vector, and subsequently into pcDNA3.1+ vector (Invitrogen) along 5′ *Eco*RI and 3′ *Xba*I. In all constructs, a Kozak translation initiation sequence (−6 to +3) was used,[13] and inserts were under the control of the cytomegalovirus promoter to yield efficient expression of the inserts.

2.2 Culture and transfection of HL-1 cardiomyocytes

HL-1-immortalized cardiomyocytes were a gift from Dr William Claycomb (Louisiana State University, New Orleans, LA, USA). Cells were seeded on gelatine/fibronectin-coated six-well plates at a density of 5×10^5 cells/well and cultured as described in Supplementary material online and elsewhere.[14] HL-1 cardiomyocytes were transiently transfected with 1.6 μg of vectors carrying either HIF-1α, PDGF-B, ADM, IGF-2, HMOX-1, EGFP or empty vector using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The myocytes, 44 h after transfection, were subjected to 300 μM H_2O_2 for 4 h. Cell viability was determined using trypan blue (0.1% final concentration) exclusion assay and a total of 400 cells were evaluated under the microscope by a blinded observer. Owing to variations in cell death induced by H_2O_2 (13–62% of all cells), results were related to H_2O_2-treated, empty vector-transfected cells in every single experiment, thus setting H_2O_2-treated cells to 1 and the rest of the samples relative to it. A pilot study indicated that relative death of cells transfected with PDGF before H_2O_2 stimulation was 82 ± 17% of death achieved by empty vector before H_2O_2 stimulation. For ADM, it was 87 ± 21%, and for IGF-2, 99 ± 14% (P = n.s. for all; $n = 6$ for each group). As HIF-1α and HMOX-1 (n = 10–12 per group in final experiments) reduced cell death more profoundly, the subsequent study focused on these two factors. Efficient transfection of HL-1 cells was first assessed by EGFP, then by real-time polymerase chain reaction (PCR) (n = 4–5/group/factor), *in situ* hybridization (n = 4/group/factor), and immunohistochemistry (n = 6/group for HIF-1α and HMOX-1).

2.3 Pharmacological stimulation of HL-1 cells

To test whether bilirubin downstream to HMOX-1 was a possible candidate for protecting heart cells, HL-1 cells were treated with bilirubin (Sigma Aldrich) at concentrations of 500, 250, and 100 nM 2 h before stimulation with H_2O_2 as described earlier. To reduce cell death, 250 nM bilirubin was selected as an optimal concentration. Altogether, 15 stimulations were performed and compared with H_2O_2 alone. To test possible protective effects of CO, the selective CO-donor tricarbonyldichlororuthenium (II) dimer[15] (CORM-2, Sigma Aldrich) was dissolved in DMSO, and initially 400, 200, 100, 50, 25, and 10 nM were added to HL-1 cells for 2 h before stimulation with H_2O_2. This was not protective against H_2O_2-induced injury. Then, 30 min incubation of 10 nM CORM-2 was attempted. When a beneficial effect was found, experiments were increased to include a total of 11 individual stimulations compared with H_2O_2 in DMSO, and control stimulations with CORM-2 were compared with DMSO only (n = 6 of each).

2.4 RNA extraction, cDNA synthesis

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN) with in-column DNase treatment (QIAGEN). The quantity of RNA was measured with NanoDrop 1000 and RNA integrity estimated with Bioanalyzer 2100. One microgram of RNA was reverse transcribed using random hexamers for priming (3 min at 70°C) followed by the first strand cDNA synthesis protocol with Superscript III (Invitrogen) and RNasin (Promega) enzymes (10 min at 25°C, 50 min at 42°C, and 4 min at 94°C).

2.5 Real-time polymerase chain reaction

Oligos were designed with Primer Express Software 3.0 (Applied Biosystems), and oligo specificity was dry-tested against the Refseq database using BLAST. For details on primer sequences and setting of the PCR reaction, see Supplementary material online. 18S rRNA was used as endogenous control. PCR reactions took place in 96-well plates using SYBR Green detection. Gene expression relative to 18S rRNA was calculated as described in Supplementary material online.

2.6 RNA *in situ* hybridization

A PCR-nested primer approach was used for the generation of DNA templates for run-off transcription of cRNA probes (Ambion Technical Bulletin 154). Briefly, after a two-step PCR, digoxygenin-11-UTP-labelled antisense and sense cRNA probes were synthesized with T7 or SP6 polymerases (Roche Diagnostics) according to the manufacturer's instructions. Primer sequences for HIF-1α, PDGF-B, ADM, IGF-2, and HMOX-1 are available upon request. HL-1 cells grown on coverslips were fixed, rinsed, and dehydrated. For details on how the coverslips were treated, see Supplementary material online. Hybridized probes were detected

with a nucleic acid detection kit (Roche Diagnostics) using alkaline phosphatase-labelled digoxigenin antibody and nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate as substrate according to the manufacturer's instructions. Colour was developed in a dark humid chamber at room temperature overnight.

2.7 Immunocyto/histochemistry

HL-1 cells were fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Cells were blocked with 3% BSA in PBS at room temperature for 30 min. Sections were incubated with the primary antibody (rabbit anti-HIF-1α 1:200, Affinity Bioreagents; mouse anti-HMOX-1 1:200, Abcam) in 1% BSA in PBS overnight at 4°C, followed by incubation with the secondary antibody (goat anti-rabbit, goat anti-mouse Alexa Fluor 488 antibodies, respectively; Invitrogen) in 1:400 in 1% BSA/PBS. Negative controls for immunocyto- and histochemistry were done by incubation with the secondary antibody alone.

Muscles were embedded in OCT compound (TissueTek) and 12 μm sections were fixed with 4% paraformaldehyde and pre-incubated with 0.1% Triton X-100 and 3% BSA in PBS at 4°C. Incubation with the primary antibody (HIF-1α, 1:50, mouse monoclonal BD Biosciences) was performed overnight at 4°C, and the secondary antibody (AlexaFluor488; Invitrogen; 1:400) in room temperature. Sections were treated with Hoechst 33342 for nuclear staining.

2.8 Immunoblot

Proteins were extracted from skeletal muscle at the time of heart isolation (see what follows). Additional mice were collected for isolation of heart and spleen proteins, as well as for the collection of serum at the same time point ($n = 7$ of HIF-1α, HMOX-1, and sham-treated each). Protein concentration was determined by the BCA assay (Pierce) and samples were prepared with Laemmli buffer as described previously.[16] Then, 30 μg protein/lane was separated on denaturing polyacrylamide gel and blotted to a nitrocellulose membrane (Amersham). Equal loading and transfer efficacy were evaluated by Ponceau staining, and membranes were blocked with 3% BSA. Membranes were incubated with mouse anti-HMOX-1 antibody (1:250, Abcam) over night, thereafter with rat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:1000, Dako) and developed using the ECL kit (Amersham). Optical density was measured with ImageJ software (NIH) and related to the optical density of Ponceau-staining used as loading control.

2.9 Animals

Male C57BL/6 mice (25–30 g) were used in Langendorff experiments ($n = 6$–8/group), as explained in what follows. HMOX-1-luciferase reporter mice on FVB background were used for the localization of HMOX-derived temporospatial signal generation in intact animals ($n = 8$/group).[17] Reporter mice were injected with DNA encoding for HIF-1α or sham-treated with saline (see Gene delivery). An amount of 150 mg/kg of D-luciferin (Biosynth, Staad, Switzerland) dissolved in PBS was injected intraperitoneally in isofluran-anaesthetized mice 7–8 min before mice were exposed for 1 min. Images taken of the treated muscle and the heart with an IVIS-100 CCD camera (Xenogen/Caliper) were analysed with Living Image 2.5 software (Igor Pro, WaveMetrics). The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health, and was approved by the local Ethics Committee for animal research.

2.10 Gene delivery

Animals were anaesthetized with Equithesin (35 mg pentobarbital and 153 mg chloral hydrate per kilogram of animal) before gene delivery. The right hindlimb was shaved, and 15 μg of either pCEP4/HIF-1α or pcDNA3.1/HMOX-1 was injected into the right quadriceps muscle in a total volume of 50 μL saline. Shams were treated with 50 μL saline. To enhance nuclear uptake, electroporation was performed as described in Supplementary material online and elsewhere.[18,19] *In vivo* transfection efficiency for HIF-1α ($n = 7$–8) and HMOX-1 ($n = 6$–8) was evaluated with real-time PCR and immunohistochemistry/immunoblot. After completion of the series, a supplementary series was performed to evaluate the effect of empty vector injected in the same amount as vector with HMOX-1, and compared with saline injection ($n = 7$ in each group).

2.11 Preparation of haem oxygenase blocker zinc deuteroporphyrin 2,4 *bis*-glycol and assessment of haem oxygenase activity

Zinc deuteroporphyrin 2,4-*bis*-glycol (ZnBG) was purchased from Porphyrin Products and re-dissolved in 50 mmol/L Na_2CO_3[20] to a concentration of 1.4 mg/mL. The blocker solution was sterile-filtered and protected from light to avoid photodegradation. An amount of 30 mg/kg of ZnBG solution was administered to mice intraperitoneally to block HMOX activity (the dose was selected according to the literature)[21] every 24 h starting at the time of gene- or sham delivery until animals were sacrificed for isolated heart perfusion.

HMOX activity was estimated from total serum bilirubin level from all mice subjected to Langendorff perfusion with a Modular Analytics SWA System (Roche). Additionally, bilirubin was measured in the serum of five unstimulated mice to record the basal level.

2.12 Isolated heart perfusion

Three days after pre-treatment, mice were re-anaesthetized with pentobarbital (60 mg/kg), and hearts were isolated and Langendorff-perfused ($n = 6$–8/group) as described in detail elsewhere.[16] Constant pressure perfusion (55 cm H_2O) with Krebs–Henseleit buffer was used. A balloon was inserted into the left ventricle for the determination of heart rate, systolic (LVSP) and end-diastolic (LVEDP) pressures, and developed pressure (LVDP = LVSP − LVEDP) was calculated. Coronary flow was measured with a flowmeter (Transonic Systems). Data were continuously collected into a computer program (PharmLab, Astra Zeneca). After 20 min of stabilization, 40 min global ischaemia was induced by clamping the inflow tubing, followed by 60 min of reperfusion. LVEDP was set to 5–6 mmHg pre-ischaemically in all groups. Animals with LVDP >60 mmHg, coronary flow 1–4.5 mL/min, and heart rate >200 b.p.m. at the end of stabilization were included. At the end of isolated heart perfusion, the whole hearts were sectioned and stained with 1% triphenyl-tetrazolium chloride solution at 37°C for 20 min and then fixed in 4% paraformaldehyde for 1 h. Digital images were taken of all sections from both sides by scanning (Epson Perfection V700), and infarct size was measured using Photoshop CS2 software (Adobe) by a blinded person. Infarct areas (whole heart minus cavities) were averaged into one value and used for statistics.

2.13 Statistics

The non-parametric Mann–Whitney *U* test was used to compare infarct sizes, death of HL-1 cells, and expression data between groups, where a non-Gaussian distribution was assumed. ANOVA for repeated measurements was applied to evaluate haemodynamic changes in Langendorff experiments and luciferase activity. Values are presented either as individual data + mean (cell death, infarct size) or mean ± SD (expression, luciferase, and haemodynamic data). Differences were considered significant when $P < 0.05$, and a tendency was noted when P was 0.05–0.08.

3. Results

3.1 Transfection of cells

HL-1 cardiomyocytes were transfected with vectors carrying either HIF-1α, ADM, IGF-2, PDGF-B, HMOX-1, EGFP or empty vector. EGFP indicated that ∼60% of cells were transfected (data not shown). Forty-four hours after transfection with these factors, real-time PCR showed a fold-increase relative to empty vector-transfected cells, which was 304 ± 115 for HIF-1α, 45 ± 18 for ADM, 150 ± 68 for IGF-2, 6899 ± 2220 for PDGF-B, and 51 ± 36 for HMOX-1. *In situ* hybridization similarly indicated successful transfection of cells (*Figure 1*), as did immunohistochemistry (*Figure 2A*). *In situ* hybridization with sense probes showed low background (data not shown).

3.2 Reduced cell death after transfection with HIF-1α or HMOX-1

To see whether transfection of HL-1 cells reduced cell death determined by trypan blue staining, transfected cells were subjected to H_2O_2-induced cell damage mimicking reperfusion injury (*Figure 2B*). Empty vector (pcDNA3.1)-transfected cells had a relative cell death rate of 0.99 ± 0.14, and this was reduced by HIF-1α-transfection and HMOX-1-transfection ($P < 0.01$ for both). Transfection with PDGF-B, IGF-2, and ADM did not reduce H_2O_2-induced cell death (see Methods).

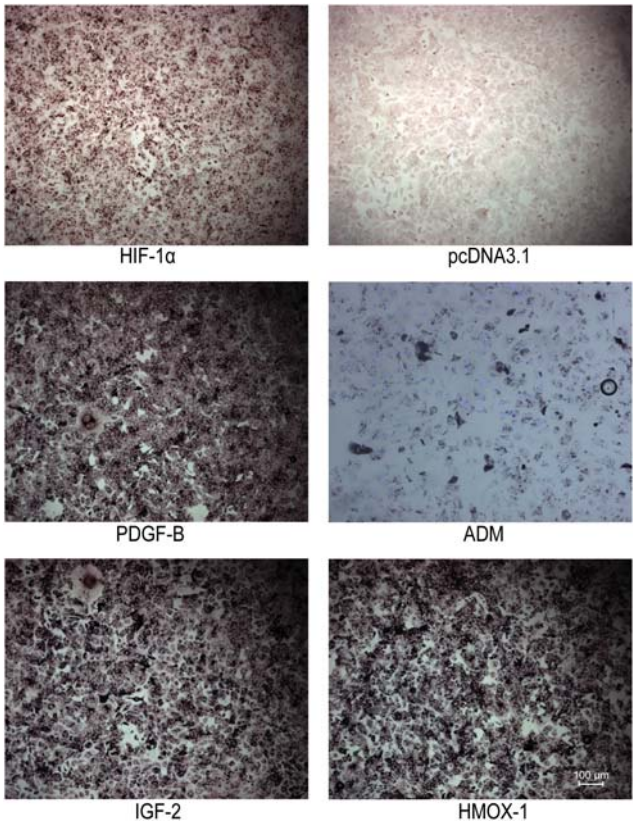

Figure 1 *In situ* hybridization of HL-1 cardiomyocytes after transfection with hypoxia-inducible factor 1 alpha (HIF-1α), platelet-derived growth factor-B (PDGF-B), adrenomedullin (ADM), insulin-like growth factor-2 (IGF-2), and haem oxygenase-1 (HMOX-1). Representative results of hybridization with antisense probe and detection with a nucleic acid detection kit are shown. When the antisense probe to HIF-1α was hybridized to the empty vector (pcDNA3.1), no RNA binding was seen. Original magnification: ×100.

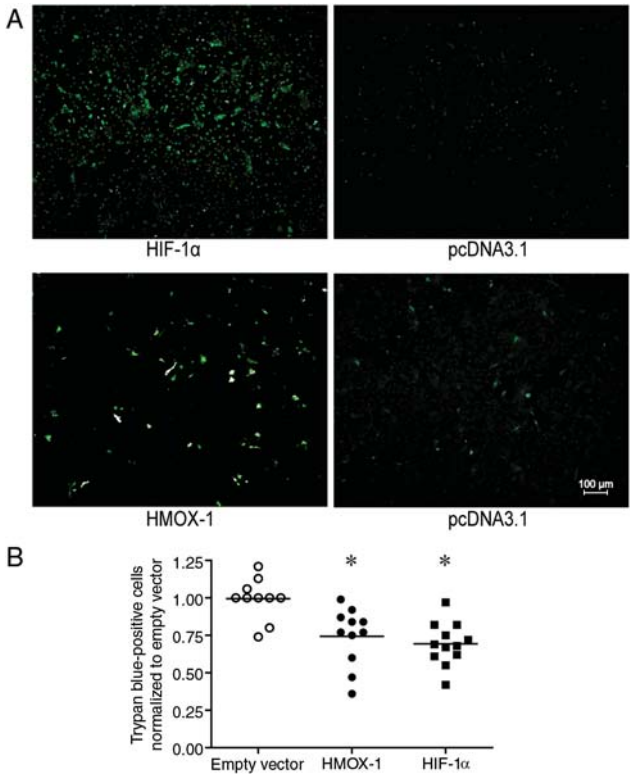

Figure 2 (*A*) Representative immunocytochemistry of HL-1 cardiomyocytes transfected with hypoxia-inducible factor-1 alpha (HIF-1α), haem oxygenase-1 (HMOX-1), or empty vector (pcDNA3.1). Detection with HIF-1α or HMOX-1 antibody and Alexa488-labelled secondary antibody. Original magnification: ×100. (*B*) HL-1 cells transfected with hypoxia-inducible factor-1 alpha (HIF-1α), haem oxygenase-1 (HMOX-1), or empty vector (pcDNA3.1) were subjected to 300 μM H_2O_2 for 4 h. Cell death, as evaluated by trypan blue staining, in single experiments, was normalized to H_2O_2-treated pcDNA3.1-transfected cells. Asterisk denotes $P < 0.05$ compared with empty vector. Individual values + mean of 10–12 experiments are shown.

3.3 Expression of HIF-1α or HMOX-1 in the skeletal muscle

DNA encoding for HIF-1α or HMOX-1 was delivered to the quadriceps muscle. There was no mortality due to gene delivery. Real-time PCR ascertained an increased HIF-1α mRNA expression after HIF-1α-delivery (*Figure 3A*). When HMOX-1 was delivered, mRNA for HMOX-1 increased locally 1 day later, and was still higher 3 days later compared with shams ($P < 0.05$; *Figure 3B*). Immunohistochemistry showed increased HIF-1α protein expression after HIF-1α transfection co-localized with the nuclear dye Hoechst 33342, which was not seen in sham-pre-treated muscles (*Figure 3C*). An increased HMOX-1 protein expression in the HMOX-1-pre-treated muscle was confirmed by immunoblot (*Figure 3D*). There was no increased expression of HMOX-1 protein in hearts, spleens, or serum after HIF- or HMOX-1 treatment (results not shown).

To investigate whether HIF-1α gene delivery influenced HMOX-1 expression, real-time PCR was performed. HMOX-1 mRNA increased 1 day after the delivery of HIF-1α in the treated muscle, and was still somewhat higher than in the sham-treated muscle 3 days later ($P < 0.05$; *Figure 4A*). Immunoblots of the same HIF-1α-treated muscles showed

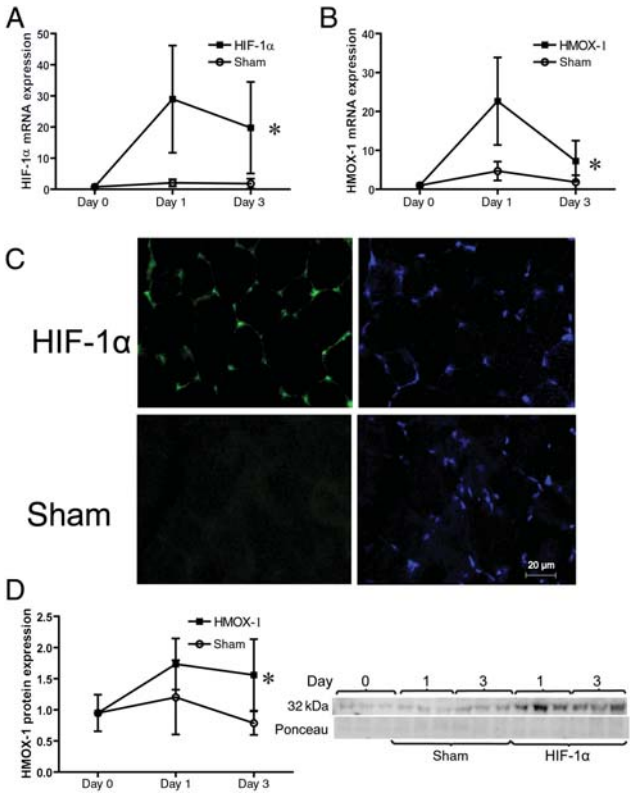

Figure 3 Expression of hypoxia-inducible factor-1 alpha (HIF-1α) and haem oxygenase-1 (HMOX-1) after delivery of DNA to quadriceps muscles, followed by electroporation. (*A*) HIF-1α mRNA expression after HIF-1α pre-treatment. (*B*) HMOX-1 mRNA expression after HMOX-1 pre-treatment. (*C*) HIF-1α protein expression after HIF-1α pre-treatment. (*D*) HMOX-1 protein expression after HMOX-1 pre-treatment. Note that in panel *C*, HIF-1α protein (green) after HIF-1α pre-treatment co-localizes with the nuclear dye Hoechst (blue), while HIF-1α protein is not expressed in sham-treated muscle (original magnification: ×200). In panel *D*, quantification of HMOX-1 protein (left), and representative immunoblot with Ponceau loading (below, right) are presented. Asterisk denotes $P < 0.05$. Mean ± SD of $n = 6$–8 per group is shown.

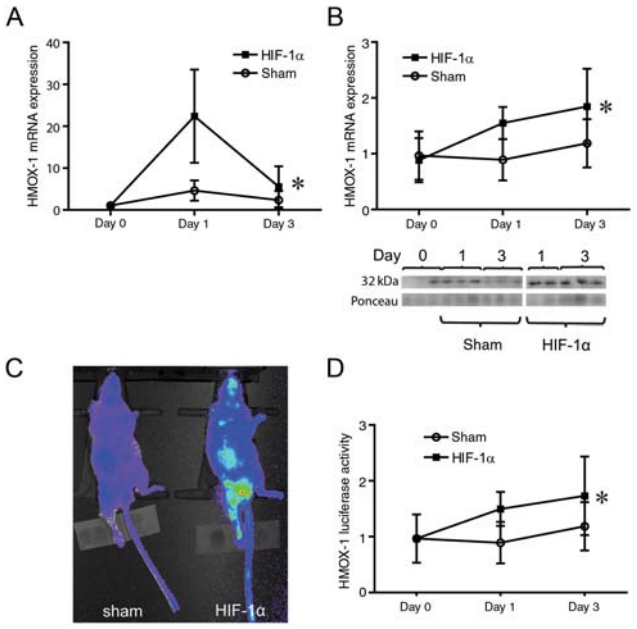

Figure 4 Haem oxygenase-1 (HMOX-1) expression after delivery of DNA encoding for hypoxia-inducible factor-1 alpha (HIF-1α) in quadriceps muscles. (*A*) HMOX-1 mRNA expression was evaluated by real-time PCR. (*B*) HMOX-1 protein expression, where the upper panel shows quantification of immunoblots and the lower panel shows a representative immunoblot (above) with protein loading (below) by Ponceau staining. (*C*) Representative image of luciferase activity in HMOX-1-luciferase reporter mice after delivery of saline (sham) or DNA encoding for HIF-1α. (*D*) Quantification of all reporter experiments. Asterisk denotes $P < 0.05$. Mean ± SD of $n = 6$–8 per group is shown.

that HMOX-1 protein was higher also 3 days after transfection ($P < 0.05$) than in sham-treated muscles (*Figure 4B*).

To explore how HIF-1α remote gene delivery can affect *in vivo* HMOX-1 expression in the intact mouse, we used an HMOX-1 luciferase reporter mouse. After gene delivery of HIF-1α, there was an increased luciferase activity for 3 days in the HIF-1α-treated muscle ($P < 0.05$) compared with the sham (*Figure 4C* and *D*). There was no increase of luciferase activity in the heart region after HIF-1α gene delivery to the quadriceps muscle (data not shown).

3.4 Improved cardiac function in mice transfected with HIF-1α or HMOX-1

To see whether HIF-1α or HMOX-1 pre-treatment of skeletal muscle was cardioprotective, hearts were isolated, perfused, and subjected to 40 min of global ischaemia followed by 60 min of reperfusion 3 days later. LVEDP increased during reperfusion in sham animals (*Figure 5A*), and this increase was significantly attenuated by either HIF-1α or HMOX-1 pre-treatment ($P < 0.05$). The HMOX blocker ZnBG given *in vivo* daily abolished the HIF-1α-induced protection of LVEDP ($P < 0.05$). When the HMOX blocker was given to sham-pre-treated mice, LVEDP was not different from sham only ($P =$ n.s.). When the effect of sham plus ZnBG was compared with HIF-1α plus ZnBG, LVEDP was not different ($P =$ n.s.). LVEDP was lower in the hearts of HIF-1α-pre-treated animals than in those of HMOX-1-pre-treated animals ($P < 0.05$). In contrast, blocking HMOX-1 with ZnBG in HIF-1α-pre-treated animals resulted in higher LVEDP than in animals that received HMOX-1 pre-treatment ($P < 0.05$; *Figure 5A*).

LVDP was reduced during reperfusion of sham-pre-treated hearts (*Figure 5B*), which was attenuated by HIF-1α pre-treatment ($P < 0.01$). HMOX-1 pre-treatment tended to attenuate the depression of LVDP ($P = 0.07$). The HMOX blocker ZnBG given in conjunction with HIF-1α pre-treatment abolished the HIF-1α-induced attenuation of LVDP depression ($P < 0.05$). ZnBG in sham- or HIF-1α-pre-treated animals did not change LVDP compared with animals that got sham pre-treatment alone ($P =$ n.s.). Further, blocking HMOX in sham- or HIF-1α-pre-treated animals resulted in similarly decreased LVDP ($P =$ n.s.). Finally, hearts of HIF-1α-treated animals had higher LVDP than those of HMOX-1-pre-treated animals ($P < 0.05$). Blocking HMOX activity by ZnBG in HIF-1α-pre-treated animals resulted in a similar LVDP as HMOX-1 pre-treatment (*Figure 5B*).

LVSP, heart rate, and coronary flow were not significantly different between groups (not shown).

3.5 Reduced infarct size in mice transfected with HIF-1α or HMOX-1

Hearts were stained with TTC to evaluate infarct size (*Figure 5C*). Hearts of sham-pre-treated animals had an infarct size of 36 ± 11%, and this was reduced by pre-treatment with either HIF-1α or HMOX-1 ($P < 0.05$ for both). The HMOX blocker ZnBG tended to abolish the

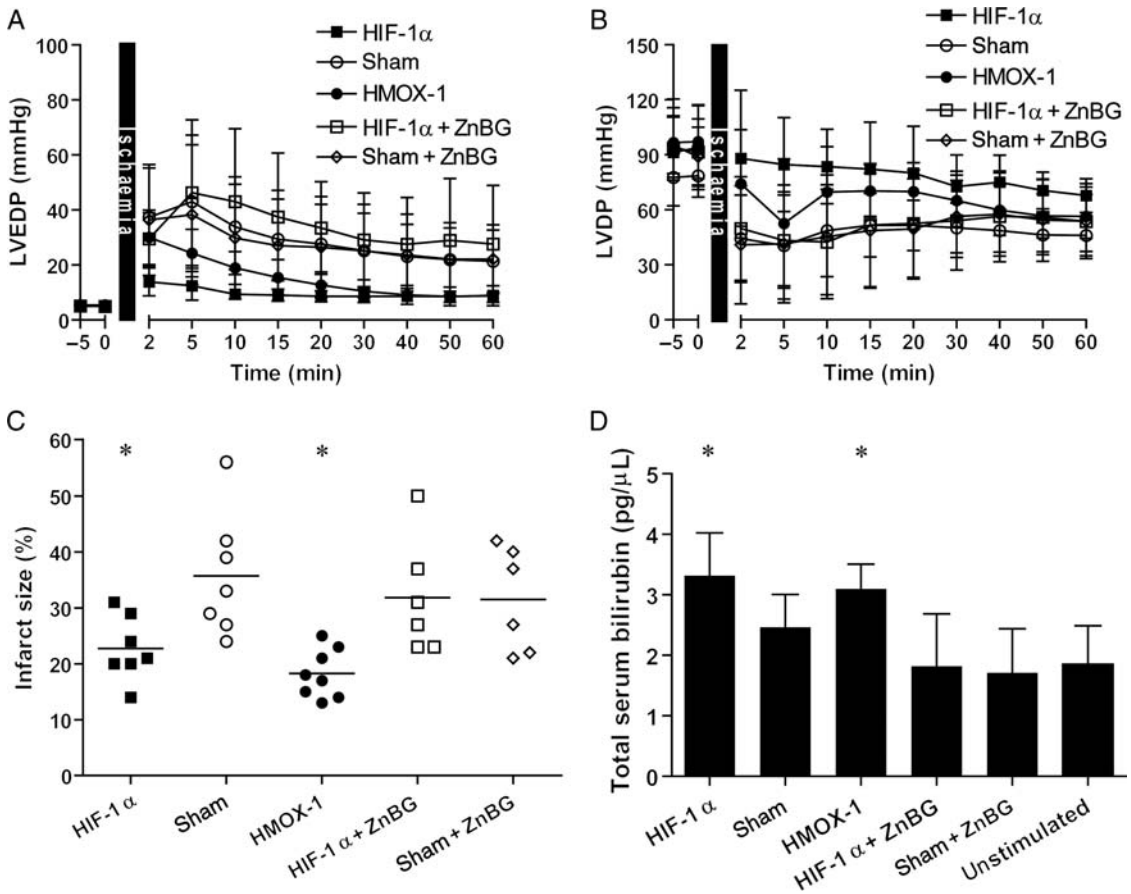

Figure 5 Mouse hearts were isolated and perfused with induced global ischaemia and reperfusion 3 days after *in vivo* treatment. The effects on (*A*) left ventricular end-diastolic pressure (LVEDP), (*B*) left ventricular developed pressure (LVDP), and (*C*) infarct size are shown. (*D*) Serum was collected at the time of heart isolation and analysed for bilirubin after delivering DNA encoding for hypoxia-inducible factor-1 alpha (HIF-1α)-, haem oxygenase-1 (HMOX-1) or sham pre-treatment, and HIF-1α- and sham-pre-treated mice with the haem oxygenase blocker zinc deuteroporphyrin 2,4-*bis*-glycol (ZnBG). For multiple comparisons and *P*-values, see Results. Mean ± SD of $n = 6$–8 per group is shown.

infarct-sparing effect of HIF-1α when compared with HIF-1α-pre-treatment alone ($P = 0.07$). Blocking HMOX activity in sham- (32 ± 9%) or HIF-1α-pre-treated animals did not change the infarct size compared with animals that got sham treatment alone ($P =$ n.s.). Further, ZnBG in sham- or HIF-1α-pre-treated animals resulted in similar infarct size ($P =$ n.s.). When the effect of HIF-1α pre-treatment was compared with that of HMOX-1, infarct size was similar ($P =$ n.s.). However, ZnBG in HIF-1α-pre-treated animals resulted in larger infarct size compared with HMOX-1 pre-treatment alone ($P < 0.05$; *Figure 5C*).

3.6 The effect of the vector

To verify that the empty vector *per se* did not have any effect, a subgroup of mice were treated with empty vector and compared with mice treated with the same vector containing HMOX-1 or saline before ischaemia and reperfusion. Infarct size was 47 ± 8% in saline-treated hearts. In hearts treated with empty vector, it was 49 ± 8%, and this was reduced to 25 ± 5% by HMOX-1 ($P < 0.0001$).

3.7 Increased haem oxygenase activity after delivery of HIF-1α and HMOX-1

HMOX activity was evaluated as total serum bilirubin concentration (*Figure 5D*). HMOX-1 increased bilirubin compared with sham ($P < 0.05$), and HIF-1α tended to do so ($P = 0.054$). Blocking HMOX activity in HIF-1α-pre-treated animals decreased bilirubin level compared with HIF-1α pre-treatment alone ($P < 0.01$) and tended to decrease compared with sham pre-treatment ($P = 0.07$), but was not different from sham-pre-treated animals with ZnBG ($P =$ n.s.). Sham treatment did not increase bilirubin compared with untreated mice (*Figure 5D*). ZnBG given in conjunction with sham pre-treatment reduced bilirubin compared with sham pre-treatment alone ($P < 0.05$). Bilirubin levels were not different between HIF-1α- and HMOX-1-pre-treated animals. When HIF-1α-treated animals got ZnBG, bilirubin was lowered compared with HMOX-1-pre-treated animals ($P < 0.01$; *Figure 5D*). Sham treatment did not significantly alter bilirubin compared with unstimulated animals (*Figure 5D*).

3.8 Treatment of HL-1 cells with bilirubin or CORM-2

Pre-treating HL-1 cells with bilirubin prior to stimulation with H_2O_2 reduced cell death ($P < 0.0001$) (*Figure 6*). Bilirubin itself did not influence the survival of HL-1 cells. The carbon monoxide donor CORM-2 used as pre-treatment likewise protected against H_2O_2-induced cell death ($P < 0.02$). CORM-2 in itself led to increased death of unstimulated

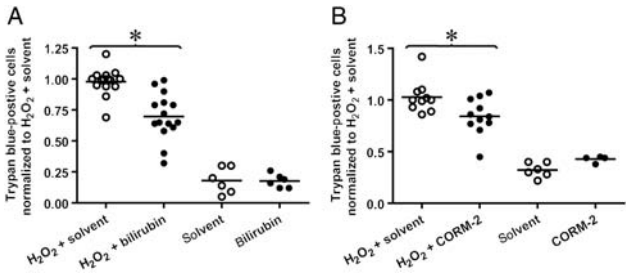

Figure 6 (*A*) HL-1 cells were pre-treated with bilirubin prior to stimulation with 300 μM H_2O_2 for 4 h. Cell death, as evaluated by trypan blue staining, in single experiments was normalized to H_2O_2-treated cells. Bilirubin alone did not induce cell death. (*B*) HL-1 cells were stimulated with the carbon monoxide donor CORM dissolved in DMSO prior to stimulation with H_2O_2 as in *A*, with control experiments using either CORM-2 alone or its solvent without H_2O_2. Individual values + mean of 6–15 experiments are shown. Asterisk denotes $P < 0.05$.

cells compared with its solvent DMSO alone ($P < 0.04$) (*Figure 6*).

4. Discussion

We hypothesized that local delivery of HIF-1α led to the activation of downstream genes, whose protein products would be secreted into the circulation and act on the heart. Specifically, we hypothesized that the downstream target HMOX-1 upregulated in the skeletal muscle would locally catabolize haem, thus increasing serum and coronary content of the antioxidant bilirubin. Gene delivery of HIF-1α to the skeletal muscle gives a local and lasting expression of HIF-1α in the treated muscle, but in no other organ.[4] The main findings of the present study are summarized as follows. Transfection with HIF-1α and HMOX-1, but not with PDGF-B, ADM, or IGF-2, increased the survival of HL-1 cells subjected to H_2O_2. When mice were pre-treated with DNA encoding for HIF-1α or HMOX-1 in the skeletal muscle and their hearts were isolated and perfused 3 days later, preservation of left ventricular function and reduction of infarct size were found. HIF-1α pre-treatment increased HMOX-1 expression locally. Pharmacological blockade of HMOX activity inhibited HIF-1α-induced cardioprotection. Serum bilirubin was increased by HIF-1α or HMOX-1 and reduced by the HMOX blocker, supporting a role of HMOX-1 for cardioprotection. In further support, the products of HMOX-1 activity, CO and bilirubin, protected HL-1 cells against induced death by H_2O_2. The novelty of the present study is using remote gene delivery of HIF-1α and HMOX-1 to protect the heart, and that cardioprotection afforded by HIF-1α is dependent on HMOX activity.

We chose gene delivery to the skeletal muscle according to the hypothesis that genes encoding for autocrine/paracrine factors act indirectly through their locally produced and secreted products. The skeletal muscle has the ability to act as an endocrine organ.[18,19] We have found previously that the delivery of HIF-1α to the skeletal muscle resulted in a local, detectable expression from 1–8 weeks later.[4] However, we have not previously been able to determine which possible downstream target(s) convey myocardial protection. One week after HIF-1α gene delivery, a number of HIF-1α target genes were upregulated in the treated muscle.[4] These genes included PDGF-B, ADM, IGF-2, and HMOX-1. Having found only HMOX-1 in addition to HIF-1α cardioprotective in our cell-based screening, we focused on these factors for further studies.

HMOX-1 is an inducible, nearly ubiquitous cytoprotective enzyme that catabolizes haem to free iron, CO, and biliverdin, which is endogenously converted to bilirubin.[10,17,20] HMOX-1 may in itself have antioxidant properties.[22] In the present study, no increased HMOX-1 expression was found in the heart, serum, or any other investigated organ. It is possible that bilirubin and/or CO formed in the skeletal muscle were secreted into the blood stream, and acted on the heart. We measured serum bilirubin as an endpoint of HMOX activity. Bilirubin increased after treatment with both HIF-1α and HMOX-1 and decreased in the presence of ZnBG, where the protective effect against induced ischaemia was no longer present. We cannot exclude the possibility that ZnBG had other effects than inhibiting HMOX-1 in the present study. Bilirubin is a potent antioxidant.[10] When HL-1 cells were stimulated with bilirubin prior to injury with H_2O_2, improved survival was observed, further indicating that bilirubin downstream to HMOX-1 could evoke cardioprotection. This is in accordance with an analogue study by Ockaili *et al.*,[23] who gave a prolyl hydroxylase inhibitor known to induce the activation of HIF-1 systemically prior to *in vivo* myocardial infarction in rabbits. The protection afforded was mediated by HMOX-1 and bilirubin. In other studies, HMOX-1-derived bilirubin ameliorates post-ischaemic myocardial dysfunction and delivered to the heart, HMOX-1 has been used as cardioprotective prevention.[24,25] The other downstream target of HMOX-1 activity, CO, is a vasodilator with antiapoptotic and anti-inflammatory properties.[24] Further, CO is protective against cardiac ischaemia–reperfusion injury *in vivo*.[26] In the present study, we could not measure serum CO for practical reasons. When HL-1 cells were stimulated with CORM-2 before injury with H_2O_2, increased cell survival was observed. Thus, CO is also a possible endpoint of induced cardioprotection in the present study.

HL-1 cells are described as a hybrid between embryonic and adult myocytes rather than an intermediate stage of myocyte maturation.[27] Their ultrastructure resembles immature mitotic mouse atrial cardiomyocytes *in situ* with nascent myofibril- and glycogen-filled cytoplasm, but they express the adult isoform of myosin heavy chain (α-MHC), α-cardiac actin, and connexin43. Cells in culture for numerous passages can still spontaneously contract.[27] We established a model of reperfusion injury through subjecting them to H_2O_2. This model was used to screen for cardioprotective factors downstream to HIF-1α. As HL-1 cells differ from adult cardiomyocytes, and H_2O_2 treatment mimics only one feature of reperfusion injury, we may have missed a possible cardioprotection by the other investigated factors.

When HIF-1α and HMOX-1 were delivered to the quadriceps muscle of mice 3 days prior to heart isolation with induced ischaemia and reperfusion, a reduced infarct size and improved function were found. The haemodynamic protection afforded by HMOX-1 pre-treatment was less robust than that of HIF-1α, as LVDP in HMOX-1-treated animals was lower than in HIF-1α-treated. The slightly less robust cardioprotection afforded by HMOX-1 suggests that there may be other HIF-1α-regulated genes which are important.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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