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RESEARCH PAPER

Haem arginate infusion stimulates haem oxygenase-1 expression in healthy subjects

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BACKGROUND AND PURPOSE

Haem oxygenase 1 (HO-1) is an inducible protein that plays a major protective role in conditions such as ischaemia-reperfusion injury and inflammation. In this study, we have investigated the role of haem arginate (HA) in human male subjects in the modulation of HO-1 expression and its correlation with the GT length polymorphism (GT_n) in the promoter of the HO-1 gene.

EXPERIMENTAL APPROACH

In a dose-escalation, randomized, placebo-controlled trial, seven healthy male subjects with a homozygous short (S/S) and eight with a long (L/L) GT_n genotype received intravenous HA. HO-1 protein expression and mRNA levels in peripheral blood monocytes, bilirubin, haptoglobin, haemopexin and haem levels were analysed over a 48 h observation period.

KEY RESULTS

We found that the baseline mRNA levels of HO-1 were higher in L/L subjects, while protein levels were higher in S/S subjects. HA induced a dose-dependent increase in the baseline corrected area under the curve values of HO-1 mRNA and protein over 48 h. The response of HO-1 mRNA was more pronounced in L/L subjects but the protein level was similar across the groups.

CONCLUSIONS AND IMPLICATION

HA is an effective inducer of HO-1 in humans irrespective of the GT_n genotype. The potential therapeutic application of HA needs to be evaluated in clinical trials.

Abbreviations

AUC, area under the curve; BW, body weight; CO, carbon monoxide; GEE, generalised estimating equations; HA, haem arginate; HO, haem oxygenase; IRI, ischaemia-reperfusion injury; L/L, homozygous for the long GT repeat length polymorphism in the promoter of the HO-1 gene; PBMCs, perhipheral blood mononuclear cells; S/S, homozygous for the short GT repeat length polymorphism in the promoter of the HO-1 gene.

Introduction

Haem oxygenase (HO) is the rate-limiting enzyme that catalyzes the degradation of haem b (Fe-protoporphyrin-IX) into carbon monoxide (CO), biliverdin (which is rapidly converted to bilirubin) and free iron (Fe^{2+}, which stimulates the induction of the iron-binding compound ferritin). In humans, two genetically distinct isoenzymes of HO have been characterized: a constitutively expressed form (HO-2) and an inducible form (HO-1) (Ryter *et al.*, 2006). HO-1 is a member of the heat-shock protein family (HSP 32) that is expressed in several organs (e.g. spleen and liver) and cell types, including

endothelial, epithelial, mononuclear and smooth muscle cells (Otterbein *et al.*, 2003). HO-1 is induced by a variety of stimuli, including oxidative stress. Up-regulation of HO-1 is mostly dependent on transcriptional modulation of the HO-1 gene. HO-1 has emerged as a major 'protective' gene, that is, a gene that when expressed restores homeostasis in many situations by its anti-inflammatory, anti-apoptotic and anti-proliferative actions (Otterbein *et al.*, 2003). The suggested use of HO-1 as a therapeutic target is based on these attributes. The cytoprotective action of HO-1 is mediated in part by its three products, CO, ferritin and biliverdin/bilirubin, which are partly overlapping in their mechanisms and effects.

The GT length polymorphism (GT_n) dinucleotide repeat polymorphism is specific for humans and has been identified in the proximal promoter region of the HO-1 gene (Lavrovsky *et al.*, 1994). This GT_n repeat is highly polymorphic and modulates gene transcription by means of oxidative challenge (Yamada *et al.*, 2000). It has been shown *in vitro* that a longer GT_n repeat corresponds to lower transcriptional activity of the HO-1 promoter (Chen *et al.*, 2002; Hirai *et al.*, 2003; Rueda *et al.*, 2007) and is associated with a susceptibility to various diseases (Exner *et al.*, 2004), such as coronary artery disease (Chen *et al.*, 2002; 2008), aortic aneurysm (Schillinger *et al.*, 2002), emphysema (Yamada *et al.*, 2000), pneumonia (Yasuda *et al.*, 2006), asthma (Islam *et al.*, 2008), idiopathic recurrent miscarriage (Denschlag *et al.*, 2004), rheumatoid arthritis (Rueda *et al.*, 2007) and cerebral malaria (Takeda *et al.*, 2005). The GT_n repeats show a bimodal distribution, with peaks at 22–23 and 27–30 repeats depending on the cohort. Alleles are usually classified into two subgroups based on the number of GT_n repeats: the shorter component (<25 or <27 repeats), which is designated as 'class S', and the upper component (≥25 or ≥27), also known as 'class L'.

It has been shown that haem acts as an inducer of HO-1 in human cells, including macrophages, *in vitro* (Yoshida *et al.*, 1988; Salahudeen *et al.*, 2001; Nakaso *et al.*, 2003; Devadas and Dhawan, 2006; Jazwa *et al.*, 2006). The molecular steps and signal transduction pathways underlying HO-1 up-regulation in general, and by haem in particular, remain largely undefined. A recent study in humans showed that the administration of 3 mg kg^{-1} hemin (Panhematin) increased the plasma level of HO-1 (Bharucha *et al.*, 2010). However, no studies have been done so far in humans to investigate the responsiveness of HO-1 to haem arginate (HA) and the influence of the human specific GT_n polymorphism on HO-1 induction. HA is a ferriporphyrin with less vasculotoxic and thrombotic side effects than other hemin preparations (Balla *et al.*, 2000), and it is an approved treatment (in Europe and South Africa) for acute porphyric attacks (Mustajoki and Nordmann, 1993). In animal studies, HA has protective effects against oxidative stress, which are probably mediated by HO-1 induction (Kubulus *et al.*, 2005; 2008; Maeshima *et al.*, 2005; Sasaki *et al.*, 2006; Jadhav and Ndisang, 2009). No human studies with HA have been done thus far in this field. Currently, a study is investigating HA in non-ST-elevating myocardial infarction (clinicaltrials.gov registration number: NCT00483587, http://clinicaltrials.gov/ct2/show/NCT00682370?term=HEMAHS&rank=1).

Hence, the aim of this study was to demonstrate the ability of intravenous HA to induce HO-1 in humans *in vivo* and to investigate the role of the GT_n polymorphism.

Methods

Subjects

Following approval of the study protocol by the Ethics Commission of the Medical University of Vienna and after written informed consent was obtained, 132 healthy white European male subjects were screened for the GT_n in the promoter region of the HO-1 gene. Eight subjects who were homozygous for the long GT_n and seven subjects who were homozygous for the short GT_n (for classification, see next) were exposed to haem infusion in a clinical study. These subjects were aged between 22 and 43 years, weighed between 61 and 91 kg (see Table 1), and were non-smokers and drug free. Each participant passed a screening examination that included medical history, a physical examination, vital sign measurement, a 12-lead electrocardiogram, laboratory tests, drug screening, and a urine test strip between 3 and 14 days before the first drug infusion. Studies were performed after an overnight fast in rooms with an ambient temperature of 22°C. The trial protocol is registered at the European Clinical Trials database (EudraCT no: 2007-003790-11) and ClinicalTrials.gov (no: NCT00682370).

Study protocol

In an open-label, stratified, three-period, placebo-controlled dose-escalation study, subjects were randomised to one of four dosage sequences (Figure 1). The dose-escalation design was chosen for the participants' safety. Three dosage sequences contained one placebo dose, and one sequence contained only active doses. Each dosage sequence consisted of three investigation periods that included three days (0, 1 and 2). On trial day 0, the study drug was

administered followed by a 6 h in-hospital stay. Blood samples were performed before and 30, 60, 180, 240 and 360 min, and 24 and 48 h after the end of the study drug administration. Washout between investigational periods was at least 10 days. Adverse events were recorded throughout the study.

Study drugs (HA and placebo)

Haem arginate was selected because it is a well-tolerated and a stable haem compound, which contains human haemin (ferriporphyrin with the iron atom in the ferric state), and because it is commercially available and labelled for human use (NORMOSANG®, Orphan Europe, Paris, France). HA was administered at increasing doses of 0.3, 1.0 and 3.0 mg kg^{-1} body weight (BW) in investigational periods 1, 2 and 3 respectively. The drug was diluted to 100 mL with 0.9% sodium chloride solution and administered i.v. over 15 min with a post-treatment rinsing infusion for 15 min with 0.9% sodium chloride solution. The dosing was based on the maximal approved dose of 3 mg kg^{-1} BW in the treatment of acute episodes of certain types of porphyria. As a placebo, infusions of 100 mL 0.9% sodium chloride solution were used.

Table 1

Demographic data and baseline levels of bilirubin, haem, haem binding proteins, HO-1 mRNA and protein across treatment periods (GT_n genotype cut-off level = 27)

	S/S	L/L
GT_n repeats	23.5 ± 1.0	31.5 ± 3.1
age (years)	30.7 ± 6.0	25.3 ± 3.7
weight (kg)	74.8 ± 8.1	75.1 ± 12.3
body mass index (kg m^{-2})	22.7 ± 1.5	22.8 ± 2.2
direct bilirubin (mg L^{-1})*	1.2 ± 0.3#	2.2 ± 0.8‡
indirect bilirubin (mg L^{-1})*	4.9 ± 1.4#	9.6 ± 4.4‡
haem (μM)	23.4 ± 4.2#	266 ± 6.5‡
haptoglobin (mg L^{-1})	975 ± 435#	766 ± 283‡
haemopexin (mg L^{-1})†	653 ± 66#	725 ± 85‡
HO-1 mRNA (ΔCT)*	2.76 ± 0.88#	3.38 ± 0.50‡
HO-1 protein (HO-1/β-actin)*	0.16 ± 0.14#	0.06 ± 0.05‡

Data are presented as means ± SD, and statistical differences are indicated (*$P < 0.01$, †$P = 0.014$; test: generalized estimating equations)
Baseline values from all investigational periods: #19 measurements from seven subjects; ‡23 measurements from eight subjects.
GT_n, GT length polymorphism; HO, haem oxygenase.

Adverse events

The adverse event profile was evaluated according to known and published side effects (e.g. injection site pain, phlebitis at injection site and elevated serum ferritin concentration) by means of standardized questions, observation, vital signs, ECG and ferritin measurements on each study day.

HO-1 genotype assessment

HO-1 promoter polymorphism analysis was carried out as previously described (Denschlag *et al.*, 2004). Genomic DNA was isolated from peripheral blood mononuclear cells (PBMCs) using commercially available kits, according to the vendors' protocols. The 5′-flanking region of the HO-1 gene containing a GT_n repeat was amplified by the PCR using a fluorescent-labelled sense primer (5′-FAM-AGA GCC TGC AGC TTC TCA GA-3′) and an unlabelled antisense primer (5′-ACA AAG TCT GGC CAT AGG AC-3′). The sizes of the PCR products were analysed

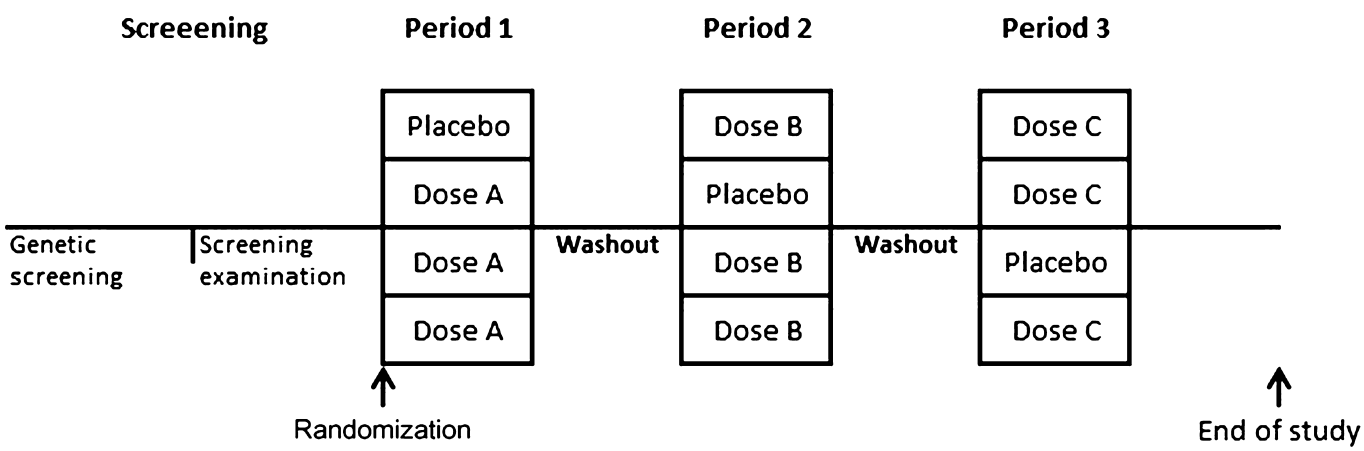

Figure 1

Study design: randomization to one of the four dosage sequences with three periods (1–3) consisting of either placebo or treatment doses A, B or C, comprising 0.3, 1.0 and 3.0 mg kg^{-1} BW haem arginate respectively.

using an internal size-standard (GeneScan ROX 350 size standard, Applied Biosystems, Foster City, CA, USA), on a laser-based ABI Prism®3100 automated DNA capillary sequencer (Applied Biosystems). The fragment length determination and GT-repeat length attribution were completed semi-automatically using ABI Prism Software (Gene Scan Analysis Version 3.7 and Genotyper Software Version 3.7, both from Applied Biosystems). Allelic repeats were divided into two subclasses using the two different previously described classification systems: short repeats, with <27 GT_n, were designated as allele class S (short), and longer repeats with ≥27 GT_n as allele class L (long) (Kaneda *et al.*, 2002; Chen *et al.*, 2008). Alternatively, a length of 25 repeats was used as a cut-off (Endler *et al.*, 2004; Exner *et al.*, 2004; Schillinger *et al.*, 2004) in *post hoc* analysis.

HO-1 protein and mRNA levels in PBMCs

HO-1 levels were analysed in PBMCs because these cells are considered effectors of oxidative stress-mediated injury and influenced by HO-1 expression. PBMCs were isolated from EDTA-blood in Ficoll-Plaque® (Amersham BioSciences, Buckinghamshire, UK) prefilled tubes (Leusosep®, Greiner bio-one, Frickenhausen, Austria). Random samples of differential blood counts of the PBMCs have shown the following subpopulations: approximately 63% lymphocytes (42% CD3+ lymphocytes), 18% monocytes (17% CD14+ monocytes), and 7% granulocytes. Cell pellets for the HO-1 mRNA analysis were treated with lysis buffer (Buffer RLT, Qiagen Sciences, MD, USA) and immediately frozen on liquid nitrogen. Lysates and cell pellets were stored at –80°C until analysis. First-strand cDNAs were synthesized from approximately 1 μg of total RNA by the use of MLV reverse transcriptase and random hexamer primers according to the manufacturer's instructions (RT-PCR Core Kit; Takara Bio, Otsu, Shiga, Japan). For quantitative real-time PCR analyses, sense and antisense primers (Invitrogen, Paisley, Scotland, UK) and fluorogenic probes (Eurogentec, Herstal, Belgium) for HO-1 and the Abelson gene (ABL) were used as previously described (Beillard *et al.*, 2003). ABL was chosen as a reference gene because it was previously shown that it is one of the most consistently expressed housekeeping genes in haematopoietic cells (Beillard *et al.*, 2003). The ABI PRISM 7700 (Applied Biosystems) was used for PCR. Results are expressed as the target/reference ratio. The difference between the HO-1 mRNA levels of HA and those of vehicle-treated cells was considered to reflect the capacity of cells to upregulate HO-1 mRNA and is expressed as ΔHO-1 mRNA.

For Western blot analyses, the dry cell pellets were transferred from –80°C to dry ice, and the cells were immediately lysed with a lysis buffer (5× extraction reagent diluted with water) containing protease inhibitors. After centrifugation to remove cell debris (18 000× *g* for 15 min at 4°C), the resulting supernatant was used for measurements using a bicinchoninic acid protein assay kit using a BSA protein standard (Thermo Scientific, Waltham, MA, USA). Then, 30 μL of a solution containing SDS loading buffer and 2-mercaptoethanol was combined with 50 μg of the sample protein and lysis buffer, bringing the volume up to 100 μL. Samples were heated at 93°C for 5 min and loaded onto a 4–12% gradient SDS/polyacrylamide gel (Invitrogen) for protein separation. The gel was run at 120 V for 1.5–2 h and then transferred (20 V for 1.5 h) to a polyvinylidene fluoride-Western blot membrane (GE Healthcare, Amersham, Buckinghamshire, UK) using the semi-dry method. The membranes were blocked in 5% milk in tris-buffered saline containing 0.05 % Tween 20 (TBST; Bio-Rad, Hercules, CA, USA) for 1 h, washed three times for 5 min with TBST, incubated with the primary mouse monoclonal anti-human HO-1 antibody (Clone OSA110, Assay Designs, Ann Arbor, MI, USA) overnight at 4°C, and washed and incubated with the second antibody (anti-mouse Ig horseradish peroxidase linked, Amersham; 1:20.000) for 1 h. The membranes were incubated for 5 min in the substrate solution (ECL Plus Western Blotting Detection System; Amersham Pharmacia Biotech, Piscataway, NJ, USA) and imaged using clear X-ray films (Thermo Scientific). The antibody staining and development procedure was repeated again using a mouse monoclonal anti-human-β-actin antibody (Abcam, Cambridge, MA, USA) for normalization of the results.

Haem, haemopexin and haptoglobin levels

The plasma haem concentration was measured in 96-well plates at 405 nm using a colorimetric haem assay kit (DIHM-250, BioAssay Systems, CA, USA). This assay reduces all haemin to ferrous haem and measures free and protein bound haem to give 'total' haem. Serum haemopexin and haptoglobin were measured by immunonephelometry (BN II System, Siemens Health Care Diagnostics, Eschborn, Germany) at the central laboratory facility of the General Hospital of Vienna.

Statistical analysis

Mean and SD were used to describe variables of interest. Pharmacokinetic and pharmacodynamic data (AUC, C_{max} and t_{max}) were calculated using the validated software Kinetica™ (Version 4.4, Innaphase Corporation, Philadelphia, PA, USA).

Differences between baseline characteristics were assessed by use of Student's *t*-test.

Differences in pharmacokinetic and pharmacodynamic parameters were assessed by generalized estimating equations models (Zeger and Liang, 1986), with identity as the link function. An AR(1) working correlation matrix for repeated observations within one patient was assumed. Numbers of observations were different between groups due to the inability to recruit the eighth subject for the S/S group and a few subjects that did not complete all three periods. Missing observations were regarded as missing at random. To test a possible interaction (effect modification) between genetics and treatment, the product of the predictors was added to the model term. For the statistical analyses, SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA) was used.

All *P* values are results of two-sided tests, and *P* values <0.05 were considered statistically significant. In this exploratory study, no adjustment for multiple testing was performed.

Results

HO-1 genotype characteristics

A total of 132 subjects were screened for the GT length polymorphism in the HO-1 promoter region. The GT_n repeats spread between 21 and 37, with 23 and 30 being the most common alleles (Figure 2A). Depending on the cut-off used (27 or 25 repeats), the prevalence of the genotypes for homozygous S/S carriers was 9.1% or 7.6%, for L/L 40.2 or 45.5.%, and for heterozygous S/L 50.8% or 47.0%, respectively (see Figure 2B). With the 25 cut-off level, only five of the recruited S/S subjects were included in the data analysis. Only data for the 27 cut-off level are presented in the following analysis because statistical analysis yielded results similar to those of the 25 cut-off.

Baseline characteristics

Eight subjects with the L/L and seven subjects carrying the S/S genotype participated in the drug infusion studies. The demographic data for these subjects are summarized in Table 1. Notably, mean bilirubin (total and sub-fractions) and haemopexin baseline levels before the investigational periods were lower in the S/S group compared with the L/L group (see Table 1). The detailed numbers of subjects available for a particular investigational period are listed in Table 2.

On the screening day, there was no difference in the biochemical parameters between the two genetic groups except in the mean triglyceride levels (S/S vs. L/L: 150.3 ± 53.7 vs. 74.1 ± 24.1; $P < 0.01$), which might be due to differences in their fasting states (S/S vs. L/L: 1 of 7 vs. 5 of 8; ns).

Effect of HA infusion

The plasma haem and haemopexin concentrations versus time curves after a single HA administration are shown in Figure 3. The dose-dependent increase in the area under the curve (AUC) over 48 h, calculated as a change from the individual baseline level (ΔAUC48) of haem ($P < 0.001$), was paralleled by a decrease in the ΔAUC48 of haemopexin ($P < 0.001$). No genetic effect was observed regarding HO-1

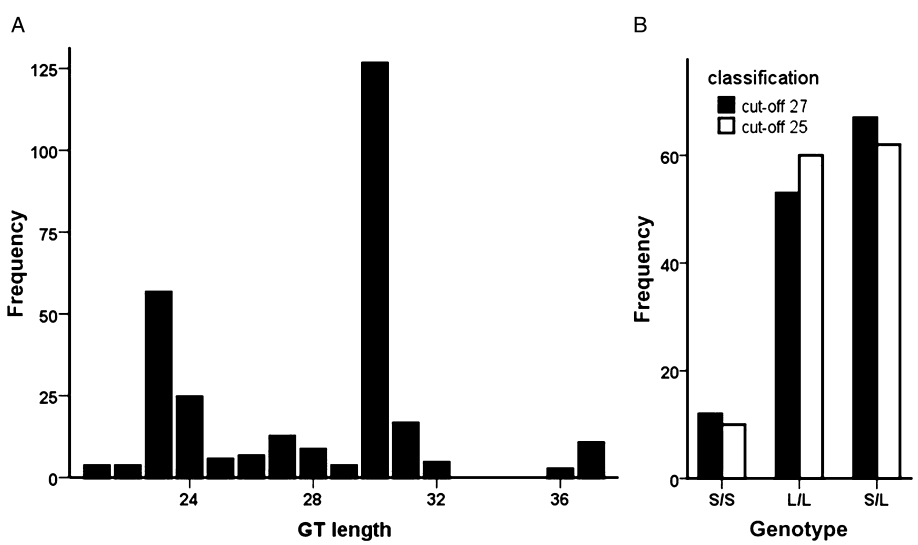

Figure 2

GT_n genotype. (A) Allele distribution of the haem oxygenase gene from 132 subjects. The GT length is shown on the x-axis and allele frequency on the y-axis. (B) Prevalence of the GT length genotypes for the two classification cut-offs, 25 and 27 (S: < 25 or 27; L: ≥ 25 or 27).

Table 2

Pharmacodynamic parameters for HO-1 mRNA and protein levels after a single treatment with different doses of HA

	Treatment dose	Number of subjects S/S	L/L	C_{max} S/S	L/L	t_{max} [h] S/S	L/L	ΔAUC_{48}* S/S	L/L
HO-1 mRNA	Placebo	5	6	–	–	–	–	−2.0 ± 14.9	−6.5 ± 7.8
	0.3 mg kg^{-1}	5	5	3.3 ± 0.58	4.1 ± 0.50	8.3 ± 8.5	4.4 ± 0.9	0.35 ± 13.8	11.4 ± 3.5
	1.0 mg kg^{-1}	4	6	3.6 ± 0.82	4.7 ± 1.00	4.3 ± 1.2	11.0 ± 9.7	14.9 ± 12.6	24.9 ± 13.0
	3.0 mg kg^{-1}	4	6	4.2 ± 1.2	5.1 ± 1.2	19.4 ± 9.0	20.7 ± 8.2	17.8 ± 11.9	47.6 ± 15.6
HO-1 protein	Placebo	5	6	–	–	–	–	−2.1 ± 8.4	−0.44 ± 5.4
	0.3 mg kg^{-1}	5	5	0.24 ± 0.095	0.19 ± 0.10	16.2 ± 19.8	15.6 ± 11.2	1.7 ± 4.1	3.2 ± 3.8
	1.0 mg kg^{-1}	5	6	0.39 ± 0.097	0.25 ± 0.074	11.6 ± 11.5	29.4 ± 21.4	1.6 ± 7.9	5.3 ± 3.0
	3.0 mg kg^{-1}	4	5	0.48 ± 0.17	0.31 ± 0.15	35.7 ± 13.5	20.2 ± 18.9	8.4 ± 10.5	8.2 ± 5.4

Data are presented as means ± SD, and statistical differences are reported in the text; C_{max}: maximal concentration (mRNA [ΔCT]; protein [(HO-1/β-actin]); t_{max} time to maximal concentration; ΔAUC_{48}: area under the concentration versus time curve over 48 h as change from the individual baseline level; *mRNA [ΔCT × h], protein [(HO-1/β-actin) × h].

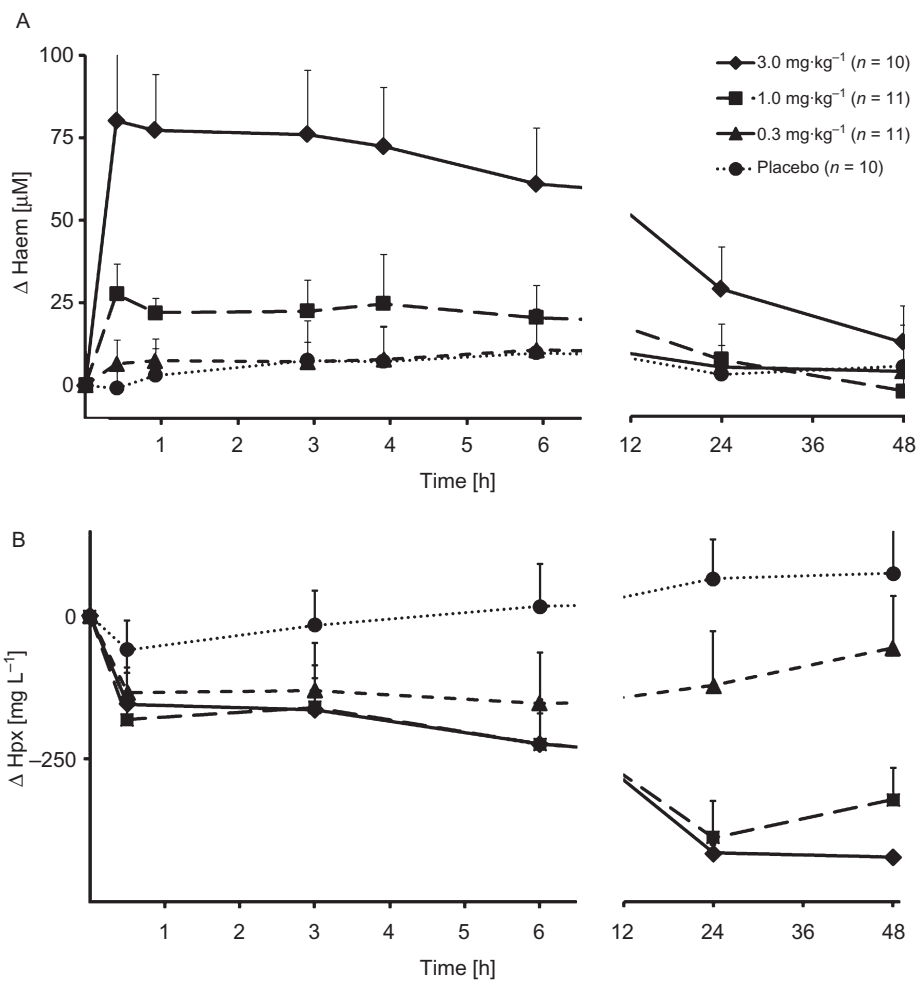

Figure 3

Plasma concentrations of total haem and haemopexin after a single infusion of haem arginate (HA). HA was infused at the indicated doses. Haem (A) and haemopexin (Hpx) (B) are plotted as a change from the individual baseline with bars showing SD. Plots summarize both genotypes (L/L and S/S). Numbers of subjects per group are indicated.

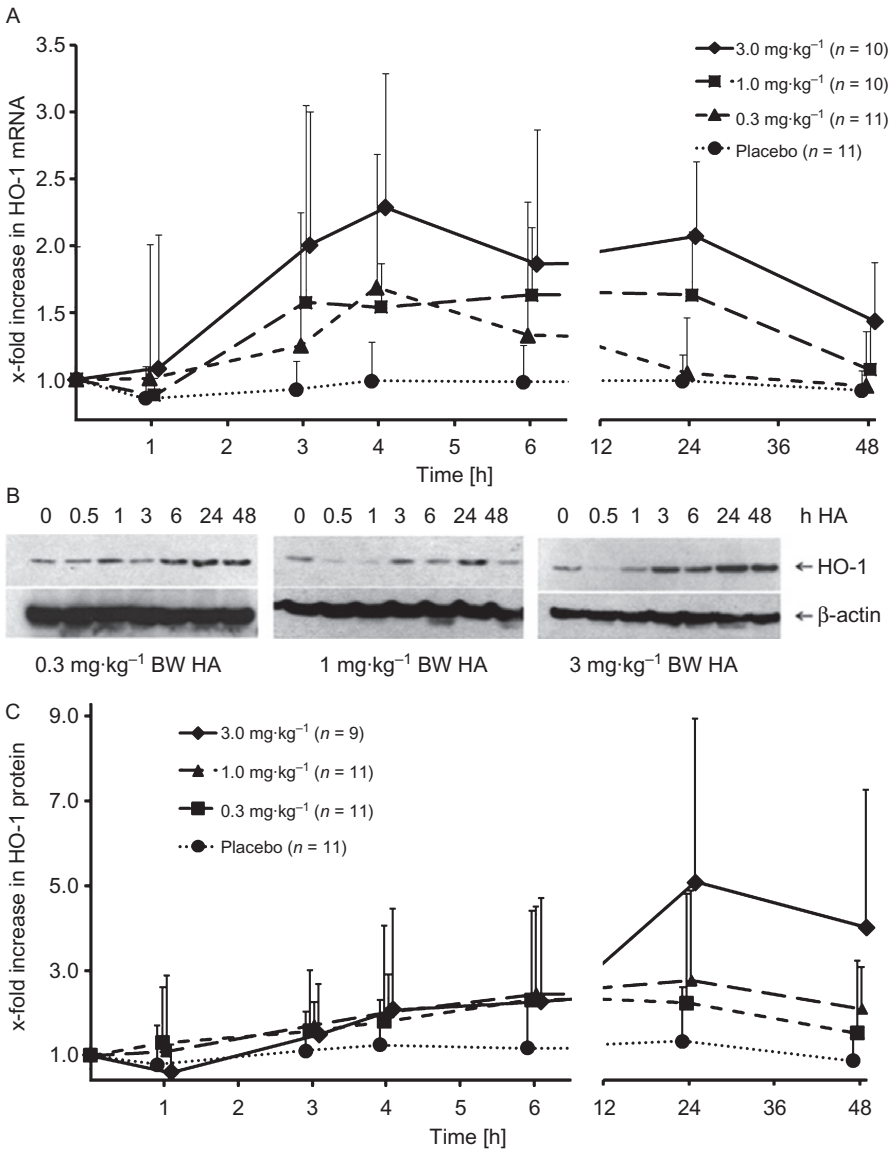

Figure 4

Haem arginate (HA) infusion induces haem oxygenase (HO-1) expression in peripheral blood mononuclear cell *in vivo*. HO-1 mRNA (A) and protein (C) expression after treatment with different doses of HA; bars show SD. (B) Representative HO-1 Western blots for the three treatment doses. β-Actin was used as a loading control. Plots summarize both genotypes (L/L and S/S). Numbers of subjects per group are indicated. BW, body weight.

GT-repeat length for haem or haemopexin ($P = 0.389$ and $P = 0.397$ respectively). The ΔAUC48 of haptoglobin did not change after HA infusion.

Interestingly, baseline levels of HO-1 mRNA were lower and protein concentrations were higher in genotype S/S compared with L/L (Table 1). HA infusion resulted in a dose-dependent increase in the ΔAUC48 of protein ($P = 0.016$). No effect of the HO-1 GT_n genotype on protein levels could be shown ($P = 0.252$) (for details, see Table 2 and Figures 4 and 5).

HA infusion resulted in a dose-dependent increase in the ΔAUC48 of HO-1 mRNA, but this effect depended strongly on the genetic group ($P < 0.001$ for the treatment-genetics interaction). As can be seen from Table 2 and Figure 5A, there is a much steeper dose-effect in the L/L group than the S/S group. Note that even the weaker dose effect of the S/S group is significant ($P < 0.001$ from subgroup analysis). The HO-1 induction curves for the individual subjects are presented in Figure S1.

The maximal concentration (C_{max}) was also dose-dependent ($P < 0.001$ for mRNA and protein), with a higher C_{max} for higher doses (Table 2). C_{max} in the S/S group was lower for mRNA ($P < 0.001$) and higher for protein ($P < 0.001$) levels compared with the L/L group. No specific differences were observed for the time to maximal concentration (t_{max}).

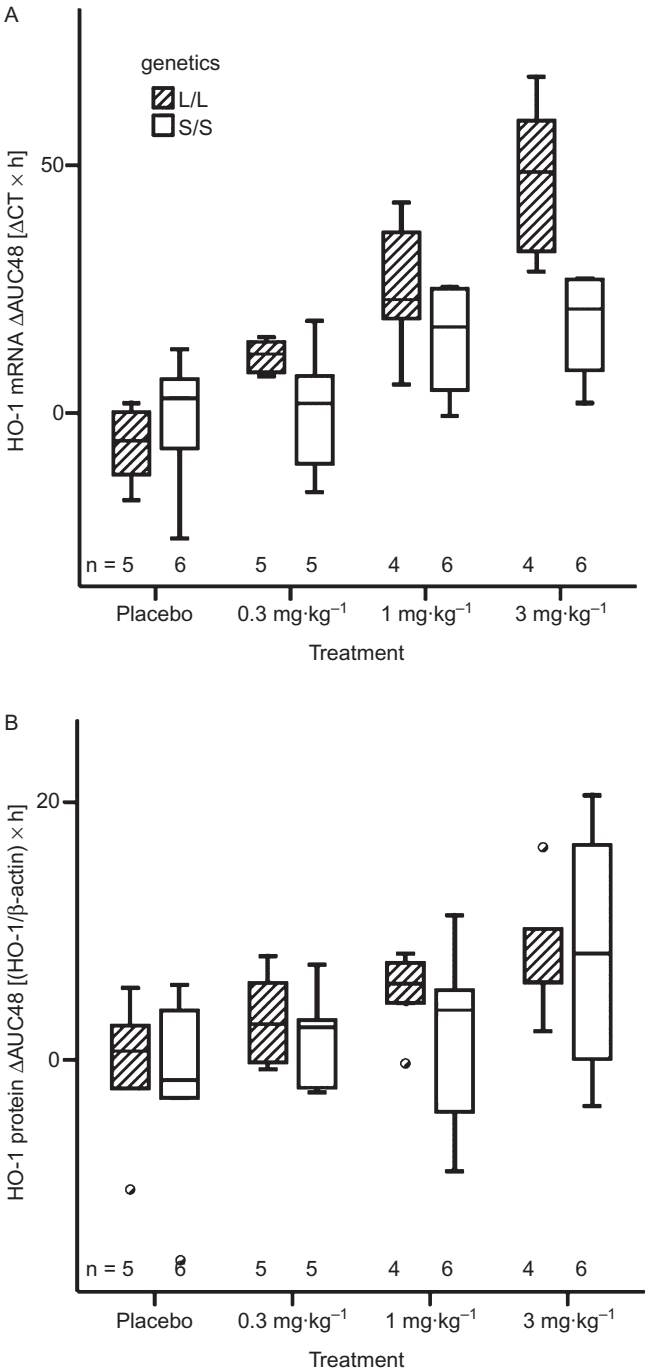

Figure 5

Area under the curve (AUC) over 48 h showing changes from individual baseline levels (ΔAUC48) for haem oxygenase (HO-1) mRNA and protein levels. Box and whisker plots of the ΔAUC48 for the HO-1 mRNA (A) and of the ODs of the protein levels normalized to β-actin (B) in peripheral blood mononuclear cells for the L/L and S/S genotypes. Numbers of subjects per group are indicated.

Adverse effects

In total, 21 adverse events were documented. All were mild or moderate in intensity and resolved without sequelae. Injection site pain and headache were the most common reports (7 and 8, respectively) and were regarded as drug related. Injection site pain was found to be dose-related but headache was not. Common cold/flu-like symptoms (rhinitis, sore throat, cough, sweating, arthralgia) and gastrointestinal symptoms (diarrhoea, nausea, abdominal pain) were regarded as not drug related.

Discussion

Induction of HO-1 expression has been shown to be highly effective in various animal models of the ischaemia-reperfusion injury (IRI) (Tsuchihashi *et al.*, 2004; Ryter *et al.*, 2006; Peterson *et al.*, 2009). However, similar studies have not been done in in humans. In the present work, we showed that HO-1 mRNA and protein levels can be augmented in PBMCs by a single i.v. dose of HA infused into healthy subjects.

HO-1 expression was measured in PBMCs because these cells are regarded as important mediators of inflammation in many vascular diseases and prime targets of the cytoprotective actions of HO-1 (Bilban *et al.*, 2008). The total amount, as well as the time to reach the maximal HO-1 mRNA and protein levels (t_{max}), was increased with higher HA dosing (Table 2).

When considering the HO-1 genotype, the induction (ΔAUC48) of HO-1 mRNA was higher in the L/L group (Figures 4A and 5A). This finding does not accord with other published data (Yamada *et al.*, 2000; Chen *et al.*, 2002; Kronke *et al.*, 2007). Several explanations for the apparent difference are proposed.

- Published data regarding functional analyses were based on *in vitro* luciferase reporter assays using truncated fragments of the HO-1 promoter or by assessment of HO-1 mRNA levels in specific cell cultures (Yamada *et al.*, 2000; Chen *et al.*, 2002; Hirai *et al.*, 2003; Rueda *et al.*, 2007). However, a promoter assay *in vitro* does not necessarily recapitulate the gene expression *in vivo*. Of note, a recent study demonstrated that a longer GT_n dinucleotide repeat did not inhibit HO-1 promoter activity (Zhang *et al.*, 2006). Additional regulatory sequences located upstream of the transcription initiation site, as well as in the intronic region of the HO-1 gene, are required for haem-induced HO-1 mRNA expression.
- Regulation of promoter activity by the GT_n repeat length may differ in cell types and in response to different stimuli (Yamada *et al.*, 2000; Rueda *et al.*, 2007). We cannot exclude the possibility that the proportion of HO-1 expressing cells varied across

PBMCs tested in S/S and L/L groups. Further studies are needed to determine whether different PBMC subpopulations respond differently to HA.

With regard to the HO-1 protein level, by measuring t_{max} or ΔAUC48, we did not observe significant differences in expression between S/S and L/L carriers; only C_{max} was higher in S/S carriers (Figure 4C and Table 2). Thus, the number of GT_n repeats does not seem to affect HA-induced HO-1 protein expression *in vivo* to a major degree under the conditions tested.

In several reports where HO-1 protein levels have been associated with clinical outcomes, HO-1 protein expression was measured after the onset of disease, thus reflecting induced rather than basal HO-1 expression. We noted higher basal (i.e. inducer-independent) HO-1 protein levels in S/S subjects (Table 2). In a murine model of liver IRI, basal rather than induced HO-1 protein levels were predictive of the antioxidant cytoprotection conferred by HO-1 (Tsuchihashi *et al.*, 2006). Such regulation is likely to be mediated, at least in part, by transcription factors distinct from those used by HA. Further studies are needed to determine whether higher basal HO-1 protein levels are also protective in humans.

Higher basal HO-1 mRNA expression in L/L subjects may also be explained, in part, by the action of a GATA2-SP1 transcription factor module flanking the GT_n repeat sequence. This module efficiently operates when the respective binding sites (i.e. GATA2 and SP1) are between 150 and 156 bases apart, as shown for basal eNOS expression in bovine aortic endothelial cells (Zhang *et al.*, 1995). In the human HO-1 5′ flanking sequence, this module exists only when the number of GT repeats is >27 or <32, which is true for most L/L carriers. Whether or not this module contributes to basal and/or induced human HO-1 gene activity needs to be verified experimentally.

In this study, data analyses were performed for two commonly used cut-offs (≤25 or ≤27) for the S/S classification. Applying the 25 cut-off resulted in the exclusion of two S/S subjects. However, similar statistical results were obtained irrespective of the cut-off level. The prevalence of the S/S genotype in the cohort under study is in agreement with published data from Austria (Funk *et al.*, 2004) and Germany (Hausmann *et al.*, 2008; Lublinghoff *et al.*, 2009), whereas the prevalence of the S/S genotype seems to be more frequent in Asian populations (17–23 %) (Chen *et al.*, 2002; 2008; Kaneda *et al.*, 2002). Previous reports described higher serum bilirubin levels in a large sample of S/S carriers (Endler *et al.*, 2004; Immenschuh *et al.*, 2007; Chen *et al.*, 2008). Differences in dietary conditions could have affected serum bilirubin concentrations in the subjects under study (Ishihara *et al.*, 2001; Zucker *et al.*, 2004).

HA infusion rapidly increased haem plasma concentrations (up to approximately 120 μM with 3 mg kg^{-1}), which is required for transcriptional induction of HO-1 in PBMC (Balla *et al.*, 2000) and is well above the haem levels used to stimulate cells *in vitro* (5–100 μM) (Kawamura *et al.*, 2005; Lang *et al.*, 2005; Devadas and Dhawan, 2006). However, *in vivo* haem is rapidly complexed by the high-affinity (K_d < 1 pmol L^{-1}) 'free haem' scavenger haemopexin (Delanghe and Langlois, 2001), which is quickly depleted, followed by 'free haem' being bound by the low-affinity albumin. Therefore, *in vivo* and *in vitro* haem levels are not comparable because measurement of the 'total' haem cannot differentiate between free unbound and complexed haem. The haem–haemopexin complex has nearly a sixfold lower activity regarding HO-1 induction in monocytes *in vitro* than free haem (Hvidberg *et al.*, 2005).

Consistently, HA infusion decreased circulating haemopexin levels. Interestingly, haem as well as haemopexin levels were found to decrease after a plateau phase of about 4–6 h. The haem–haemopexin complex is degraded via a LRP/CD91-mediated endocytosis pathway, mainly in macrophages and hepatocytes (Hvidberg *et al.*, 2005). As shown previously (Tokola *et al.*, 1986; Volin *et al.*, 1988; Kumar and Bandyopadhyay, 2005), a single infusion of 3 mg kg^{-1} HA-sustained induced lower levels of haemopexin for more than 48 h (Figure 3B), which ultimately may lead to a longer half-life of HA. Although plasma haem concentrations peaked above 100 μM, no signs of haem toxicity (e.g. haemolysis) were observed, as indicated by unchanged haptoglobin levels.

During the preparation of this manuscript, Bharucha *et al.* (2010) reported induction of plasma HO-1 protein by haemin (3 mg kg^{-1} i.v.) in humans, in addition to an increase in HO-1 enzymatic activity in PBMC. However, a potential HO-1 GT_n genotype effect was not investigated and plasma HO-1 protein might not be a good measure for induced HO-1 in healthy individuals. In our study, we report HO-1 mRNA and protein levels in PBMCs, representing important mediators of inflammation in many vascular diseases and prime targets of the cytoprotective actions of HO-1 (Bilban *et al.*, 2008). Nonetheless, the findings of our study are in good agreement with the HO-1 activity levels in PBMCs following haemin infusion.

In conclusion, this study demonstrates that a single dose of HA induces HO-1 mRNA and protein expression in PBMCs *in vivo*. The genetic

background of the GT_n length polymorphisms in the HO-1 promoter (S/S or L/L) does not have clinically relevant influence on this HO-1 induction. Further clinical studies addressing potentially beneficial effects of HA-induced HO-1 expression will be able to evaluate whether HA can be used therapeutically in IRI.

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Conflict of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Individual plots of HO-1 expression in PBMCs. Relative increase of HO-1 mRNA and protein expression after treatment with different doses of HA or placebo is plotted for all individuals (eight subjects with the L/L and seven subjects with the S/S genotype).

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