Role of Heme Oxygenase-1 in Human Endothelial Cells
Lesson From the Promoter Allelic Variants

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Objective—Heme oxygenase-1 (HO-1) is an antioxidative, antiinflammatory, and cytoprotective enzyme that is induced in response to cellular stress. The HO-1 promoter contains a (GT)n microsatellite DNA, and the number of GT repeats can influence the occurrence of cardiovascular diseases. We elucidated the effect of this polymorphism on endothelial cells isolated from newborns of different genotypes.

Methods and Results—On the basis of HO-1 expression, we classified the HO-1 promoter alleles into 3 groups: short (S) (most active, GT ≤23), medium (moderately active, GT =24 to 28), and long (least active, GT ≥29). The presence of the S allele led to higher basal HO-1 expression and stronger induction in response to cobalt protoporphyrin, prostaglandin-J2, hydrogen peroxide, and lipopolysaccharide. Cells carrying the S allele survived better under oxidative stress, a fact associated with the lower concentration of oxidized glutathione and more favorable oxidative status, as determined by measurement of the ratio of glutathione to oxidized glutathione. Moreover, they proliferated more efficiently in response to vascular endothelial growth factor A, although the vascular endothelial growth factor–induced migration and sprouting of capillaries were not influenced. Finally, the presence of the S allele was associated with lower production of some proinflammatory mediators, such as interleukin-1β, interleukin-6, and soluble intercellular adhesion molecule-1.

Conclusion—The (GT)n promoter polymorphism significantly modulates a cytoprotective, proangiogenic, and antiinflammatory function of HO-1 in human endothelium. (Arterioscler Thromb Vasc Biol. 2010;30:1634-1641.)

Key Words: angiogenesis ■ antioxidants ■ cytokines ■ endothelium ■ growth factors

Heme oxygenase-1 (HO-1) is an enzyme degrading heme to iron ions, carbon monoxide (CO), and biliverdin; the latter is subsequently converted to bilirubin by biliverdin reductase. Products of HO-1 activity perform important physiological functions in the vascular system, which, together with the removal of toxic heme, are ultimately linked to the protection of endothelium.

Accordingly, endothelial cells isolated from HO-1 knockout mice are more sensitive to oxidized lipid–induced injury and more susceptible to H2O2-induced cell death than those isolated from wild type individuals. Also, in the sole known human case of HO-1 deficiency, endothelial cell damages were prominent because of the heme-mediated oxidation of low-density lipoprotein and lack of adaptive responses. It seems, however, that a much more important and common phenomenon in the human population is variability in HO-1 expression levels, resulting from allelic variants of the HO-1 promoter.

The 5′-flanking region of the human HO-1 gene contains a fragment of (GT)n microsatellite DNA. A number of dinucleotide repeats, ranging from 11 to 42, can modulate the level of transcription. For example, reporter assays demonstrated that the HO-1 promoter constructs harboring (GT)29 or (GT)38 sequences were less active than those with (GT)11, (GT)16 or (GT)20. However, such experiments were performed only with fragments of the HO-1 promoter, whereas some regulatory sequences are located far upstream of the transcription initiation site, as well as in the introns of HO-1 gene. Until now, only 2 reports have been published in which the lymphoblastoid cells or mononuclear blood cells possessing short alleles displayed a higher level of HO-1 than cells carrying long alleles.

Importantly, though, although large scale analysis did not confirm a meaningful effect of HO-1 promoter polymorphism on coronary artery disease or myocardial infarction, there are many clinical data indicating its influence on cardiovascular complications, at least in some groups of patients. Thus, the presence of longer, less active alleles was associated with an increased risk of arteriovenous fistula failure in people...
subjected to hemodialysis,\textsuperscript{12} higher incidence of coronary artery disease in type 2 diabetic or hemodialyzed patients,\textsuperscript{7,10,12,13} elevated rate of restenosis after balloon angioplasty,\textsuperscript{14} or more frequent aortic aneurysms\textsuperscript{15} and cerebrovascular events.\textsuperscript{16} Moreover, among patients with peripheral artery disease, those carrying longer HO-1 alleles had higher rates of myocardial infarction, percutaneous coronary interventions, and coronary bypass operations.\textsuperscript{17}

Some reports have suggested that polymorphism of the HO-1 promoter may also influence the outcomes of stenting, as the presence of more active alleles correlates with reduced adverse cardiac events.\textsuperscript{18,19} These observations have not been confirmed by larger-scale studies.\textsuperscript{4,20} Nevertheless, patients carrying the short alleles had less pronounced serum lipid peroxidation\textsuperscript{7,4} and a milder postintervention inflammatory response.\textsuperscript{4}

Thus, the number of GT repeats seems to influence the progression of some cardiovascular diseases, especially those associated with oxidative stress, inflammation, and endothelial dysfunction. Our aim was to elucidate whether (GT)n promoter allelic variants can actually modulate the expression and biological effects of HO-1 in primary endothelial cells.

### Materials and Methods

For a more detailed description of methods, please see the supplemental text, available online at http://atvb.ahajournals.org.

#### Cell Culture

Experiments were carried out using human umbilical vein endothelial cells (HUVEC) freshly isolated from healthy, anonymous newborns. Collecting the umbilical cords was conducted according to the guidelines of the ethical commissions on human research of the authors’ hospitals in Vienna and Lodz.

#### Genotyping of HO-1 Promoter

Determination of the number of GT repeats in the HO-1 promoter was performed as described earlier.\textsuperscript{18}

#### Analysis of mRNA Expression

Analysis of mRNA was performed using quantitative RT-PCR.

#### Measurement of the HO-1 Protein

Concentration of the HO-1 protein in cell lysates was determined using enzyme-linked immunosorbent assay (ELISA) according to the vendor’s protocol.

#### Measurement of Inflammatory Mediators

Concentrations of interleukin (IL)-1\(\beta\), IL-6, IL-8, tumor necrosis factor-\(\alpha\), and soluble E-selectin were determined using ELISA.

#### Measurement of Glutathione

Each HUVEC batch was cultured in 2 25-cm\(^2\) flasks. Cells in one flask remained intact, but cells in the other flask were exposed to \(\text{H}_2\text{O}_2\) (100 \(\mu\text{mol/L}\)) for 16 hours. Reverse-phase high-performance liquid chromatography was used to quantify glutathione (GSH) in the cell lysates according to the procedure described elsewhere,\textsuperscript{21} with a slight modification.

#### 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide Reduction Assay

Cells were cultured in 96-well plates (100 \(\mu\text{L}\) medium per well) and exposed to \(\text{H}_2\text{O}_2\) (100 to 800 \(\mu\text{mol/L}\)) for 3 to 24 hours. Then, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (final concentration of 0.5 mg/mL) was added for 2 hours. Reaction was stopped by adding 50 \(\mu\text{L}\) of lysis buffer (20% SDS, 50% dimethylformamide). Absorbance was read at a wavelength of 570 nm.

#### Annexin-V Staining

Cells were cultured in 96-well plates and exposed to \(\text{H}_2\text{O}_2\) (100 \(\mu\text{mol/L}\)) for 24 hours. Annexin-V staining was performed using a TiterTACS 96-well apoptosis detection kit, according to the manufacturer’s instructions.

#### Cell Migration Assay

For each HUVEC batch, the spontaneous and vascular endothelial growth factor (VEGF)-A\textsubscript{165}–induced migration was analyzed using modified Boyden chambers.

#### Capillary Sprouting

The measurements of capillary sprouting were performed according to the procedure described by Korff and Augustin.\textsuperscript{22} HUVEC spheroids were embedded in collagen gels and remained intact or were stimulated with VEGF-A\textsubscript{165} (30 ng/mL). The sprouting of capillary-like structures was quantified 24 hours later by measuring the length of the sprouts that had grown out of each spheroid.

#### Cell Proliferation Assay

Cells were seeded in 96-well plates (5000 per well) in a medium devoid of endothelial cell growth supplement but supplemented with 10% FCS, and they remained intact or were stimulated with VEGF-A\textsubscript{165} (30 ng/mL). After a 42-hour incubation period, the 5-bromodeoxyuridine solution (1 \(\mu\text{mol/L}\)) was added for 6 hours, and then proliferation was measured using colorimetric ELISA, according to the vendor’s protocol.

#### Statistical Analysis

All data are shown as the mean±SEM. One-way ANOVA followed by a Tukey posteriori test (for comparison of multiple samples) or Student t test (for comparison of 2 samples) was used to analyze the statistical differences.

#### Results

Distribution of (GT)n alleles in the population studied and the response of HUVEC to the stimuli are described in the supplemental text and shown in Supplemental Figures I through III.

#### Effect of HO-1 Promoter Allelic Variants on HO-1 Expression

We compared the expression of HO-1 mRNA and protein in HUVEC batches classified according to the number of GT repeats in the shorter allele of the HO-1 promoter. Under control conditions, the HO-1 mRNA level was the highest in cells possessing the short (S) (22 or 23 repeats) allele (Figure 1). Basal expressions in medium (M) (24 to 28 repeats) and long (L) (29 to 37 repeats) carriers were lower by \(\approx 40\%\) (\(P<0.05\)) and \(\approx 60\%\) (\(P<0.001\)), respectively. Measurements of HO-1 protein concentrations in cell lysates generally confirmed the results of quantitative RT-PCR, although differences between groups were smaller, reaching statistical significance only when S and L alleles were compared (Figure 1). This was possibly caused by weaker sensitivity of ELISA.

Comparison of the results obtained for individual HUVEC batches showed that there was a significant positive correlation between the expression of HO-1 measured at the mRNA and protein levels \((r=0.63, P=0.002)\).

Exposure to \(\text{H}_2\text{O}_2\), cobalt protoporphyrin (CoPP) and 15-Deoxy-\(\Delta^{12,14}\)-prostaglandin-J\(_2\) (15d-PGJ\(_2\)) led to much
stronger upregulation of HO-1 mRNA in carriers of S alleles than in cells possessing M or L types (Figure 1). Analysis of protein showed the same tendency, but with statistically significant differences apparent only between the S and L groups. The influence of HO-1 promoter polymorphism was also observed in cells incubated with lipopolysaccharide (LPS). Here, the meaningful differences were noted only at the mRNA level between the S and L carriers. A similar but not statistically significant trend was visible in HUVEC treated with interferon γ (IFNγ) (200 ng/mL), and hemin (10 μmol/L). Some cells were cultured for 6 hours under hypoxic conditions (2% O2, 5% CO2). EF2 was used as a constitutive gene in ΔCt analysis. *P<0.05, **P<0.01, ***P<0.001 in comparison with cells carrying S allele.

Importantly, gas chromatography measurements of CO concentrations performed in media harvested from different HUVEC batches, suggest that differences in HO-1 expression are reflected in HO-1 activity (Supplemental Figure VI). Namely, untreated cells carrying the S alleles released more CO to the culture media than those with L alleles. A similar tendency was observed after the stimulation of cells with H2O2, whereas the most significant differences were found in cells stimulated with 15d-PGJ2 (P=0.050, 0.067, and 0.004 for untreated, H2O2-stimulated, and 15-PGJ2-stimulated cells, respectively; N=10 for the L group and N=17 for the S group).

**Effect of HO-1 Promoter Allelic Variants on Inflammatory Response**

Release of IL-1β, IL-6, and soluble intercellular adhesion molecule-1 was significantly lower in the control HUVEC...
carrying short (GT)n fragments than in their counterparts with longer HO-1 alleles (Figure 2). The same tendency was observed in cells treated with LPS, but here only differences in IL-1β production were statistically significant. On the other hand, the HO-1 promoter polymorphism did not seem to affect basal production of soluble E-selectin and tumor necrosis factor-α, although there was some tendency for a more pronounced response to LPS in cells from the M and L groups. Finally, it did not modulate expression of IL-8 (Figure 2).

Effect of HO-1 Promoter Allelic Variants on the Oxidative Status of Endothelial Cells

To assess the antioxidative efficacy of HO-1, we measured the concentration of total GSH, as well as GSH and oxidized glutathione (GSSG), in cells cultured under control conditions or exposed for 16 hours to H₂O₂ (100 μmol/L). The levels of total GSH in the control cells were similar in the S, M, and L groups (Figure 3A) and were slightly increased in the M and L carriers treated with H₂O₂ (to 2.03±0.65 and 2.48±0.39 mmol/mg, respectively). Concentrations of reduced GSH (Figure 3B) were also comparable in the HUVEC of all genotypes (P>0.5 and P>0.4 for control and H₂O₂-treated cells, respectively). In contrast, we observed the strong effect of HO-1 promoter polymorphism on the level of GSSG, which was the highest in the L group (Figure 3C). As a consequence, oxidative status measured as a GSH:GSSG ratio was much more favorable in cells with the S allele than in carriers of M or L variants (12.62±3.63 versus 4.11±1.19 or 3.55±0.82 in control cells, P<0.05) (Figure 3D). Unexpectedly, we did not find significant differences between untreated and H₂O₂-treated cells. Possibly, to observe the response to H₂O₂ we should perform analyses at earlier time points.

The effect of H₂O₂ on expression of other cytoprotective genes is described in the supplemental text and shown in Supplemental Figure VII.

Effect of HO-1 Promoter Allelic Variants on Viability of Endothelial Cells

The oxidative status of cells was reflected by the survival of HUVEC exposed to H₂O₂ (100 to 800 μmol/L, 3 to 24 hours). Results of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reduction assay demonstrated that endothelial cells carrying the S allele are much more resistant to oxidative stress than those with the less active HO-1 promoter (Figure 4A through 4D). Staining for annexin-V performed after 24 hours of incubation with H₂O₂ (100 μmol/L) confirmed the highest rate of apoptosis in endothelial cells with L HO-1 allele and thus with the lowest activity of HO-1 promoter (Figure 4E).

Effect of HO-1 Promoter Allelic Variants on Angiogenic Potential

Finally, we assessed the response of HUVEC to stimulation with VEGF-A, that being the crucial proangiogenic factor. The 5-bromodeoxyuridine incorporation assay showed that proliferation after 48 hours of incubation with VEGF-A (30 ng/mL) was much more pronounced in cells carrying the S allele than in those with the M or L allele (Figure 5A). The fold of induction was, respectively, 3.38±0.34, 1.98±0.41, and 1.53±0.23 (P<0.001). A similar relationship was demonstrated in which cells from SS and LL genotypes were compared (Supplemental Figure VIIIA).

In contrast, the migration of cells in response to VEGF-A was not significantly influenced by HO-1 promoter polymorphism, as estimated by using Boyden chamber assay (Figure 5B). This observation was confirmed by measuring the sprouts growing out of endothelial spheroids embedded in collagen gel, a process relying primarily on cell motility (Figure 5C). Again, a similar lack of influence was demonstrated when the HUVEC of SS and LL cells were compared (Supplemental Figure VIIIB).
Discussion

We have demonstrated that allelic variants of (GT)n repeats in human HO-1 promoter can modulate the level of gene transcription in primary endothelial cells, cultured in basal conditions or treated with H$_2$O$_2$, CoPP, 15d-PGJ$_2$, and LPS. In contrast, these variants do not significantly affect HO-1 expression in cells exposed to interferon γ, hemin, and hypoxia. This may suggest that activities of general transcription factors and transcription initiation complex are not affected by the length of the (GT)n repeat sequence.

One might notice that the Nrf2 transcription factor is the common mediator involved in HO-1 induction on treatment with CoPP,$^{23}$ 15d-PGJ$_2$, H$_2$O$_2$, and LPS. Nrf2 was shown to recruit BRG1 (Brahma-related gene-1) protein to the proximal, GT-repeat-containing fragment of the HO-1 promoter, which thereby may interact with the distal enhancer motifs.$^{26,27}$ Our data might suggest that long microsatellite fragments reduce such transactivation. This speculation, however, needs further experimental verification.

Many articles have described the significant effects of (GT)n polymorphism in the HO-1 promoter on cerebrovascular and cardiac disorders, the outcomes of transplantations, and progression of some cancers.$^{7,10,12–16,18,19}$ Strikingly, in all the earlier articles, the criteria for classification of HO-1 promoter alleles as short” or “long” were chosen arbitrarily. Thus, the maximal, threshold number of GT repeats in alleles regarded as short varied widely: 24,11,16–18 25,19 26,13 27,11 29,30 30,12,14 32,7 or 36 repeats.$^{15}$ In some analyses, the alleles were divided into 3 groups: short, medium, and long. In these cases, the cutoffs were also different, being located at 24 and 30 repeats,$^{6,30}$ 25 and 31,$^{31}$ 26 and 33$^{9,29}$ or 29 and 38.$^{4}$

Figure 3. Concentration of total GSH (A), GSH (B), GSSG (C), and the GSH:GSSG ratio (D) measured by high-performance liquid chromatography in HUVEC carrying S, M, or L alleles of HO-1 promoter, cultured for 16 hours with or without H$_2$O$_2$. *P<0.05, **P<0.01 in comparison with cells carrying S allele.

Figure 4. Viability of HUVEC carrying S, M, or L alleles of HO-1 promoter cultured with or without H$_2$O$_2$ for 3 hours (A), 6 hours (B), 12 hours (C), or 24 hours (D), measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. Shown is the percentage of annexin-V-positive, apoptotic cells (E) in HUVEC cultured with or without H$_2$O$_2$ (100 μmol/L). *P<0.05, **P<0.01, ***P<0.001 in comparison with cells carrying the S allele.
overexpression and HO-1 knockout or transgenic mice. The models were used, including cells with enforced HO-1
increase in the free iron pool. 

may be detrimental to the cells, probably because of an oxidation of heme and an associated generation of biologically active products. It has been postulated that too high an activity of HO-1, especially in the case of prolonged induction, could result in a lower concentration of IL-6. Our data fully confirm the antiinflammatory potential of the HO-1/biliverdin reductase pathway. A similar relationship was observed in transgenic mice after cardiovascular damage induced by angiotensin-II, in which enforced cardiac overexpression of HO-1 protected cells from decrease in the GSH:GSSG ratio.

Interestingly, experiments performed in a pheochromocytoma cell line exposed to the CO-releasing molecule indicate that CO may increase the concentration of GSH by Nrf2-dependent upregulation of the catalytic subunit of glutamate-cysteine ligase, which represents the rate-limiting enzyme in GSH synthesis. However, it seems that this mechanism does not play a role in our experimental setting. Instead, we suppose that higher expression of HO-1 in endothelial cells carrying the S allelic variant of the HO-1 promoter was associated with prevention of GSH oxidation, leading to a decreased requirement as well as decreased synthesis of GSH. This resulted in a better GSH:GSSG ratio and lower concentration of GSH in the cells.

It should also be remembered that treatment of HUVEC with H2O2 induces many other genes, some of them directly involved in H2O2 inactivation, such as catalase, Thrx, and ThrxR. Thus, the protective effects of HO-1 can be supported by the activity of additional antioxidative pathways, although induction of HO-1 seems to be one of the strongest responses to the oxidative stress.

Interestingly, HO-1 upregulation on treatment with 15d-PGJ2, CoPP, or H2O2 is accompanied by an augmented expression of ferritin (see supplemental text). An association between HO-1 induction and synthesis of ferritin has been already described. It has also been suggested that type 2 diabetes patients carrying short (GT)n repeats in HO-1 promoter may have a higher serum ferritin concentration. We demonstrated that ferritin upregulation in endothelial cells correlates with HO-1 inducibility and is significantly augmented in carriers of S alleles. This may provide protection from a possible increase in the free iron pool.

HO-1 is commonly regarded as an antiinflammatory enzyme. Its importance is elegantly illustrated in HO-1 knockout mice, in which HO-1 deficiency leads to increased production of proinflammatory cytokines. Also, in patients subjected to bypass surgery, a higher activity of HO-1 resulted in a lower concentration of IL-6. Our data fully confirm the antiinflammatory potential of HO-1 and show that even relatively small differences in HO-1 expression in cells carrying distinct promoter alleles are enough to modulate generation of IL-1β, IL-6, and soluble intercellular adhesion molecule-1. Similar trends were observed for tumor necrosis factor-α and soluble E-selectin.

We have demonstrated for the first time the real effect of different numbers of GT repeats on basal and induced HO-1 expression (see supplemental text). The reasonable threshold values for allele classification are 23 repeats (for S, the most active alleles) and 28 repeats (for moderately active alleles). L alleles with microsatellite DNA fragments carrying 29 or more GTs are the least active. We chose a threshold of 23 for the S allele, similar to the most common cutoff (24) chosen in other studies. Because alleles with 24 GTs are relatively rare, our study generally confirms that the arbitrary classification used in most articles reflects the actual effect of (GT)n on HO-1 expression. Furthermore, we showed that the most important factor is the number of repeats in the shorter allele. The length of the (GT)n fragment in the second allele of the HO-1 promoter is negligible (Supplemental Figure IVB).

One of the most studied functions of HO-1 is cytoprotection, resulting from both the removal of prooxidant heme and generation of biologically active products. It has been postulated, however, that too high an activity of HO-1, especially when not associated with sufficient expression of ferritin, may be detrimental to the cells, probably because of an increase in the free iron pool.

To investigate the HO-1-mediated cytoprotection, various models were used, including cells with enforced HO-1 overexpression and HO-1 knockout or transgenic mice. The question that was still unresolved, however, was whether relatively subtle changes in HO-1 expression resulting from promoter allelic variants can exert a biologically significant impact. Our data show that variation of HO-1 inducibility observed in the human population influences the sensitivity of primary endothelial cells to oxidative stress and that induction of HO-1 within the physiological range does play a protective and antiapoptotic role. Moreover, cells carrying the more active HO-1 variant display a more appropriate GSH:GSSG ratio, which may reflect the antioxidative potential of the HO-1/biliverdin reductase pathway. A similar relationship was observed in transgenic mice after cardiovascular damage induced by angiotensin-II, in which enforced cardiac overexpression of HO-1 protected cells from decrease in the GSH:GSSG ratio.

Figure 5. Proliferation measured using the 5-bromodeoxyuridine incorporation assay (A), migration determined using Boyden chambers (B), and sprouting of capillaries from endothelial spheroids (C) of HUVEC carrying S, M, or L alleles of HO-1 promoter, cultured with or without VEGF-A. **P <0.01, ***P <0.001, in comparison with cells carrying S allele; #P <0.05, ##P <0.01, ###P <0.001 in comparison with control, unstimulated cells. AU indicates arbitrary units.
The relationship between HO-1 and IL-8 raises more controversy. Some data suggest that activation of HO-1 by CoPP and CoCl₂ leads to a decrease in production of IL-8. In contrast, we demonstrated that upregulation of IL-8 in response to 15d-PGJ₂, CoPP, and CoCl₂ was HO-1-independent. Measurements of concentrations of IL-8 released from HUVEC of different HO-1 genotypes provided evidence that generation of IL-8 was not influenced by HO-1 expression.

Finally, we analyzed the effect of HO-1 promoter allelic variants on the angiogenic potential of HUVEC. The link of HO-1 and angiogenesis was first indicated by augmented endothelial proliferation resulting from HO-1 overexpression. Accordingly, inhibition of HO-1 disrupted the response of endothelial cells to growth factors. This defect was also seen in vivo, as the neovascularization of wounds was impaired in HO-1 knockout mice. Current results demonstrate the importance of small changes in HO-1 expression for the endothelial mitogenic response to VEGF and may suggest the weaker angiogenic potential of endothelium in patients with less active variants of HO-1.

In contrast, HO-1 promoter polymorphism did not influence VEGF-induced migration of HUVEC. This was unexpected, as the tin protoporphyrin, HO-1 inhibitor, markedly decreased endothelial cell motility. Moreover, recent experiments have shown the essential role of HO-1-derived CO in stromal cell-derived factor-1-induced migration of endothelial progenitors, as in the cells isolated from HO-1/−/− mice, the migration was potently attenuated.

Therefore, we suppose that some activity of HO-1 is necessary for cell migration, but after crossing a threshold value, further upregulation of HO-1 is not so important. This suggestion is supported by our unpublished observation that migration measured by scratch assay, although strongly reduced in HO-1/−/− endothelial progenitors, is very similar in endothelial progenitor cells isolated from HO-1/−/+ and HO-1/−/− mice (data not shown). Thus, analysis of HUVEC indicates that possibly, the impairment of migration is not a significant factor inhibiting the angiogenic potential of endothelial cells in patients with a less active HO-1 promoter.

In summary, (GT)n allelic variants of the HO-1 promoter directly modulate the level of HO-1 expression in human primary endothelial cells. HUVEC batches carrying the shorter, more active HO-1 alleles survive better under oxidative stress, proliferate more effectively in response to VEGF-A, and produce lower levels of proinflammatory mediators. Thus, the lesson from HO-1 promoter polymorphism confirms the cytoprotective, promitogenic, and antiinflammatory role of HO-1 in endothelium and indicates that the efficacy of this enzyme can vary significantly in the human population.

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**Disclosures**

None.

**References**


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Correction

In the article “Role of Heme Oxygenase-1 in Human Endothelial Cells: Lesson From the Promoter Allelic Variants” by Taha et al, which appeared in the August 2010 issue of the journal *Arterioscler Thromb Vasc Biol. 2010;30:1634–1641; DOI: 10.1161/ATVBAHA.110.207316*, the last sentence of the Sources of Funding should have appeared as: The Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University is a beneficiary of the structural funds from the European Union (grant No: POIG.02.01.00-12-064/08, 01.02-00-109/99 and 02.02.00-00-014/08).

The online version has been corrected.

The authors regret the error.

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Supplement Material

Materials and Methods

Cell culture. Umbilical cords were collected in hospitals in Vienna (Austria) and Lodz (Poland) using collagenase digestion. Cells were cultured to the first passage and then frozen.

After collecting all HUVEC batches, cells were thawed and their culturing continued in order to perform set experiments on cells at the same passages: 2nd passage – genotyping and measurements of glutathione concentration; 3rd passage – analyses of HO-1, BvR, eNOS and ferritin expressions, and measuring the cytokine productions; and 4th passage – assays for cell viability, proliferation, migration, and formation of capillaries. Cell were cultured in a M199 medium supplemented with FCS (20%), ECGS (30 µg/mL), glutamine (2 mmol/L), HEPES (18 mmol/L), penicillin (100 u/mL) and streptomycin (100 µg/mL), in standard conditions (37°C, 5% CO₂).

Genotyping of HO-1 promoter. Genomic DNA was isolated from HUVEC using commercially available kits, according to the vendors' protocols. The 5’-flanking region of the HO-1 gene containing a poly (GT)n repeat, was amplified by the polymerase chain reaction (PCR) using a fluorescent-labeled sense primer (5’- FAM-AGA GCC TGC AGC TTC TCA GA-3’) and an unlabeled antisense primer (5’-ACA AA G TCT GGC CAT AGG AC-3’). The sizes of PCR products were analyzed using an internal size-standard (GeneScan ROX 350 size standard, Applied Biosystems, Foster City, CA), on a laser-based ABI Prism®3100 automated DNA capillary sequencer (Applied Biosystems, Foster City, CA). Fragment length determination and GT-repeat length attribution was completed semi automatically using ABI Prism Software (Gene Scan Analysis Version 3.7 and Genotyper Software Version 3.7, both Applied Biosystems, Foster City, CA).

Induction of HO-1 expression. Each HUVEC batch was seeded to two sets of 6-well plates. Cells were cultured to confluence, and remained intact or were stimulated for 6 h with
HO-1 activators. Additionally, some cells were incubated for 6 h in hypoxia (2% O₂, 5% CO₂), created using a Modular Incubator Chamber (Billups-Rothenberg Inc.). Then, cells in one set of plates were subjected to isolation of total RNA, while in the second to isolation of total protein. Therefore, we were able to measure the basal and induced HO-1 expressions simultaneously, at mRNA and protein level in each cell batch.

**Real-time RT-PCR.** RNA was extracted from cultured cells using a modified acid guanidinium thiocyanate-phenol-chloroform extraction method, along with a Total RNA Extraction Kit, according to the vendor's instruction. Then, 2 µg of isolated RNA were used for reverse transcription with MMLV and oligo(dT) primers. The obtained cDNA was diluted in nuclease-free water and stored at -20°C.

Quantitative real time PCR reaction was performed on 50 ng of cDNA using SYBR Green Master Mix, according to the manufacturer's recommendation, using a Rotor-Gene 3000 machine (Corbett Life Science). We used the following primers: EF2-F: 5' GCG GTC AGC ACA ATG GCA TA3', EF2-R: 5' GAC ATC ACC AAG GGT GTG CAG 3' (length of product: 218 bp), HO-1-F: 5' GTG GAA MCG GTT YAC RTA GYG C 3', HO-1-R: 5' CTT TCA GAA GGG YCA GGT GWC C 3' (length of product: 250 bp), ferritin-F: 5' CTT CGA CCC TGA GCC CTT TG 3', ferritin-R: 5' CAG GTT GAT G CG CTT GA 3' (length of product: 157 bp), BvR-F: 5' CAG AGC CCG AGA GGA AGT TT 3', BvR-R: 5' ACA TGC TCC TCG TGC AAG AC 3' (length of product: 369 bp), eNOS-F: 5' CGG TGA TGG CGA AGC GAG TG 3', eNOS-R: 5' CGA GCC CGA ACA CAC AGA ACC 3' (length of product: 423 bp), GPx-F: 5' TGG GGC ATT CTC TTC TCC CAC 3', GPx-R: 5' CAT GCC CTT TTC ATC CTT CTC 3' (length of product: 150 bp), catalase-F: 5' TGA CAT GGT CTG GGA CTT CGT G 3', catalase-R: 5' TTG ATG CCC TGG TCG GTC TT 3' (length of product: 192 bp), Thrx-F: 5' GAC AAG CCC TGC AAG ACT CTC G 3', Thrx-R: 5' TTC TCC CGC AGA GCT ACT CG 3' (length of product: 139 bp), ThrxR-F: 5' TAG AGC ACA TGG CAT CTC
ATG GC 3’, ThrxR-R: 5’ CAG ACT TCT GGT GTC TGG GAC TC 3’ (length of product: 182 bp), where W – A or T; Y – C or T; M – A or C; R – A or G.

For each reaction, the following temperatures and times were applied: 95°C, 10 minutes (initial denaturation), 40 cycles of 95°C, 30 s (denaturation), 58°C, 60 s (annealing), 72°C, 45 s (elongation), followed by 72°C, 5 minutes (final elongation). Data analysis was undertaken using the ΔCt method, with EF2 used as a reference gene.

**Measurement of HO-1 protein.** Cells were rinsed twice with PBS, scrapped, transferred to pre-chilled tubes and centrifuged (8,000 g, 4°C, 10 minutes). Pellets were re-suspended in 100 µl of HO-1 Extraction Reagent containing protease inhibitors (0.1 mmol/L of PMSF, 1 µg/mL of leupeptin and 1 µg/mL of aprotinin), incubated in ice for 30 minutes, vortexed and then centrifuged again (21,000 g, 4°C, 10 minutes). Clear supernatants were collected and stored at -80°C. Total protein concentration was measured with a BCA kit, following the vendor's protocol. HO-1 ELISA was performed on 50 µg of total proteins.

**Measurements of carbon monoxide.** Culture media harvested from 9 HUVEC batches (260 µL) were added to CO-free, septum-sealed vials containing 40 µL of 30% sulfosalicylic acid. CO released into the vial headspace was quantified using gas chromatography as described elsewhere.

**Measurement of inflammatory mediators.** Each batch of HUVEC was cultured to confluence in a 24 well plate. Then, the culture medium in each well was replaced with a fresh 1 mL culture and some cells were stimulated with LPS (100 ng/mL). After 24 h, concentrations of cytokines were determined using ELISAs, according to the vendor's instructions.

**Measurement of glutathione.** Cells were rinsed twice with PBS, scrapped, transferred to pre-chilled tubes and centrifuged (300 g, 5 minutes, 4°C). Then, 500 µL of distilled water were added and cells were lysed by three freeze-thaw cycles in liquid nitrogen.
Next, 100 µL of cell lysate was deproteinated with 400 µL of 6% MPA. After centrifugation (8,000 g, 5 minutes, 4ºC) the supernatant was neutralized with 0.2 M NaOH.

GSH derivation was performed by adding equal volumes of a neutralized sample and 0.5% OPA solution in 0.1 M sodium-borate buffer (pH 9.4), followed by neutralization by 5-fold dilution with 0.5 M sodium phosphate (pH 7.0). Next, the GSH-OPA adducts were separated on a Supercosil LC-18 column (Supelco) with Shimadzu LC-10AD VP pump and SIL-10AD VP auto injector, at the rate flow of 1 mL/minute. The fluorescence was monitored at the excitation and emission wavelength of 340 nm and 425 nm, respectively. Then, samples were eluted with 30% gradient of acetonitrile in 0.05 M sodium acetate (pH 6.2). Shimadzu CLASS-VP software was used for peak integration. Quantification of GSH was prepared on the basis of the standard curve, with linearity ranging from 0 to 50 pmol. Finally, total glutathione (GSHt) was measured after GSSG reduction with 0.05 M DDT before deproteination and the GSSG concentration was obtained by subtraction of GSH from GSHt.

It should be stressed that the measurements were performed using the highly sensitive, fluorimetric method, and cell lysates were prepared in the absence of thiol scavengers, which can potentially lead to overestimation of GSSG. Thus, although the differences between the groups in our experiment are clear, the absolute values of GSH and GSSG concentrations can be directly compared only with the results obtained using the same protocol.

Cell migration assay. For each HUVEC batch the spontaneous and VEF165-induced migration was analyzed, using a modified Boyden chambers (diameter of pores 8 µm) coated with vitronectin. Cells (150,000 per well) were seeded on the transwell insert in 300 µL of medium devoid of FBS and ECGS but supplemented with 5% BSA. To the lower part, 500 µL of the same medium with or without VEGF-A165 (30 ng/mL) were added. Migration was assessed 24 h later according to the vendor's instruction. In short, cells on the lower surface of inserts were fixed and stained with crystal violet (CV) solution. Quantification of migration
was performed by measurement of absorbance after methanol extraction of CV at the wavelength of 570 nm.

Results and Discussion

Distribution of (GT)n alleles. We analyzed the DNA of HUVEC isolated from 99 healthy newborns delivered in Vienna (Austria) and in Lodz (Poland). The frequencies of HO-1 alleles were similar in both places, and so all data were pooled. The number of GT repeats ranged from 22 to 37 (Fig. I), and the frequency distribution was trimodal, with peaks at 23 (frequency 0.106), 30 (frequency 0.268) and 36 (frequency 0.04).

Quantitative RT-PCR analysis suggests that the level of HO-1 mRNA expression is influenced by allele containing the shorter GT sequence (Fig. II). Taking into consideration cells cultured in control conditions or stimulated with CoPP (10 µmol/L), H₂O₂ (100 µmol/L), and 15d-PGJ₂ (10 µmol/L), we decided to divide the alleles into three groups, according to the transcription activity: S (short, 22-23 repeats, the most active), M (median, 24-28 repeats, of moderate activity), and L (long, 29-37 repeats, the least active). The frequencies of the S, M, and L allele groups in analyzed DNA samples were 0.192, 0.207, and 0.601 (Fig. I) respectively, whereas the frequencies of genotypes (Fig. III) were 0.050 (S/S), 0.098 (S/M), 0.204 (S/L), 0.031 (M/M), 0.245 (M/L), and 0.377 (L/L). Experiments were performed on 47 HUVEC cell batches, classified according to the number of GT repeats in the shorter allele, as S (N=22), M (N=12), and L (N=13) groups.

Induction of HO-1 expression. HUVEC carrying the S, M or L alleles were cultured in a complete medium and stimulated for 6 h with hemin (10 µmol/L), CoPP, H₂O₂, 15d-PGJ₂, IFNγ (200 ng/mL), and LPS (100 ng/mL), or exposed to hypoxia (2% O₂). Control cells were incubated without any stimulation. All activators significantly increased the HO-1 mRNA expression (Fig. IVA). The most powerful one was 15d-PGJ₂, followed by CoPP and H₂O₂.
The effects of LPS, INFγ, hemin, and hypoxia were statistically significant, but moderate. The low level of response to hemin may seem unexpected, especially if the same treatment, also in our hands, induced much higher response in different cell types\textsuperscript{2,3}. In fact, in the presented study hemin induced statistically significant increase in HO-1 expression (1.65, 1.70 and 1.54 fold in the S, M, and L carriers respectively), but it was very low, when compared to the effect of 15d-PGJ\(_2\). Possibly it is a consequence of applying the serum-reach media (20% FCS) in our experiments and relatively low concentration of hemin used for stimulation (10 \(\mu\)mol/L). Hemin can be sequestered by hemopexin, and by serum albumin\textsuperscript{4}. It has been demonstrated that the added serum may reduce the amount of hemin available for cell stimulation, and for stimulation of cells in serum-reach media high concentrations of hemin were used (200 \(\mu\)mol/L)\textsuperscript{5}.

Effect of hypoxia on HO-1 expression is species- and the cell type-dependent. In some human cells the Bach1 acts as a hypoxia inducible regulator that represses the transcription of the HO-1 gene. Although the hypoxia responsive element (HRE)-like sequences were found in the promoter regions of human \textit{Hmox-1}, it is still not clear if they correspond to typical HRE. Data concerning effects of hypoxia on HO-1 are contradictory\textsuperscript{6}. It was reported that effect of hypoxia can be even inhibitory in human cells (e.g. in HUVEC, A549 human lung cancer cells, human astrocytes and coronary artery endothelial cells), but there are also results showing induction of human HO-1 in hypoxic conditions (e.g. in retinal pigment epithelial cell line, dermal fibroblasts, and keratinocytes)\textsuperscript{6}. Finally, low oxygen tension does not affect the expression of HO-1 in explants of normal human chorionic villi from term placentas and does not change the protein level of HO-1 in HMEC-1\textsuperscript{6}. In our hands, incubation of endothelial cells under hypoxic conditions for 6 h slightly, although statistically significantly, increased HO-1 mRNA expression, that was not reflected by increase in protein level.
Prolongation of hypoxia to 24 h led to reduced HO-1 expression both at mRNA and protein levels\(^7\).

To verify whether the HO-1 expression is dependent only on the shorter allele we compared the cells of S/S, S/M, and S/L genotype. As shown in Fig. IVB, we did not find any statistically significant differences between the S carriers, regardless of the number of GT repeats in the second allele.

We also demonstrated that ferritin, which can scavenge the iron ions released by HO-1, was upregulated on treatment with CoPP and 15d-PGJ\(_2\), and slightly decreased in hypoxia (Fig. IVC). Interestingly, the level of ferritin upregulation was affected by differences in HO-1 expression between HUVEC carrying S, M and L alleles. Namely, in the cells cultured in control conditions or stimulated with H\(_2\)O\(_2\), CoPP, 15d-PGJ\(_2\), and LPS, the lowest expression of ferritin mRNA was detected in the L group (Fig. IVC). In contrast, no differences were found in HUVEC treated with IFN\(_\gamma\), hemin, or incubated in hypoxia. A similar analysis of BvR or eNOS mRNAs did not demonstrate any association with HO-1 promoter polymorphism (data not shown).

Finally, we checked whether HO-1 promoter polymorphism influences only the absolute levels of HO-1 or also the fold-change in response to stimulations. As shown in Fig. V, even though the length of (GT)n repeats affects both baseline and post-treatment levels, it also influences the fold-change, although this effect is weaker and reaches statistical significance only in the case of stimulation with H\(_2\)O\(_2\) (18.0 \pm 4.16, 17.6 \pm 2.64 and 4.2 \pm 2.27 fold in S, M and L carriers, respectively) and 15d-PGJ\(_2\) (143.2 \pm 37.34, 89.7 \pm 11.86, 44.6 \pm 18.76 fold in S, M, and L carriers, respectively).

**Effect of H\(_2\)O\(_2\) on expression of cytoprotective genes.** To check the induction of antioxidative genes we incubated HUVEC cells with H\(_2\)O\(_2\). The use of exogenous added H\(_2\)O\(_2\) will not wholly mimic the physiological situation. However, it can be considered as a model
of response to oxidative stress. H$_2$O$_2$ is generated endogenously, mostly by NADPH oxidases or leaks from the mitochondrial electron transport chain, and is a part of normal cell signalling. Exogenous H$_2$O$_2$, produced for example by phagocytes, can be used in signalling to other cells, which is relevant in inflammation.

In cells treated with H$_2$O$_2$ we checked the expression of thioredoxin (Thrx), thioredoxin reductase (ThrxR), catalase, and glutathione peroxidase (GP). As shown in Fig. VII, the exposure of HUVEC carrying the S allelic variant of HO-1 promoter to H$_2$O$_2$ (100 µmol/L, 6 h), may upregulate the expression of Thrx and catalase in a statistically significant manner and show a tendency for an increase in ThrxR. Similar effects were also observed in cells of the M and L groups (data not shown). The folds of induction of Thrx, ThrxR, and GP are, however, much lower than that for HO-1 (13.6 ± 1.3 in S, 19.4 ± 2.8 in M, and 7.8 ± 2.0 in L cells). Thus, the protective effects of HO-1 can be also supported by the activity of other antioxidative pathways, but the induction of HO-1 seems to be one of the most pronounced responses to the oxidative stress.

**Summary.** Taken together, we have demonstrated the protective effect of the S alleles in endothelial cells, which can explain, at least in part, the better outcome of patients with the S alleles suffering from cardiovascular diseases. However, analysis of the allele frequency shows that in the population studied, the carriers of S alleles are less frequent (fraction 0.35) than non-carriers (fraction 0.65). Very similar frequencies were described earlier. The preponderance of L alleles may suggest that a highly active HO-1 pathway, although beneficial in elderly individuals, may have some unfavourable influences in the earlier phases of the lifecycle, thereby being subject to natural selection and Darwinian evolution. Accordingly, the S allele was significantly more frequent in women suffering idiopathic recurrent miscarriages, and its presence was associated with an increased risk of prolonged newborn jaundice and hyperbilirubinemia. Moreover, short (GT)n alleles may represent a
genetic risk factor for cerebral malaria\textsuperscript{14,15}. Finally, animal models suggest that a high level of HO-1 activity promotes plasmodium infection\textsuperscript{16} and facilitates the \textit{Mycobacterium tuberculosis} dormant infection, thereby enabling a bacterium to spread across a population\textsuperscript{17,18}.

\textbf{References}


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Fig. I. Frequency distribution of the numbers of (GT)n repeats in the population studied. S: ≤23 repeats, M: 24-28 repeats, L: ≥29 GT repeats.
Fig. II. Expression of HO-1 mRNA measured using qRT-PCR in HUVEC cells carrying different HO-1 promoter alleles, cultured in control conditions or stimulated for 6 h with CoPP (10 µmol/L), H₂O₂ (100 µmol/L) and 15d-PGJ₂ (10 µmol/L). Sh – cells classified according to the number of GT repeats in the shorter allele; lo – cells classified according to the number of GT repeats in the longer allele; sh+lo – cells classified according to sum of the number of GT repeats in the shorter and longer alleles. Some alleles were very rare in the population analysed and therefore, several bars represent a single measurement.
Fig. III. Frequency distribution of the HO-1 promoter genotypes in the population studied. S: \( \leq 23 \) repeats, M: 24-28 repeats, L: \( \geq 29 \) GT repeats.
Fig. IV. A – Expression of HO-1 mRNA in HUVEC carrying S, M, or L alleles of HO-1 promoter stimulated for 6 h with H\textsubscript{2}O\textsubscript{2}, CoPP, 15d-PGJ\textsubscript{2}, LPS, IFN\textgreek{g}, hemin, and hypoxia. B – Expression of HO-1 mRNA in HUVEC of S/S, S/M, or S/L genotype of HO-1 promoter. Both in control and stimulated cells there were no statistically significant differences between S/S, S/M, and S/L groups. C – Expression of ferritin mRNA in HUVEC carrying S, M, or L alleles of HO-1 promoter. EF2 was used as a constitutive gene in ∆Ct analysis of qRT-PCR. * P<0.05 in comparison with the S carriers, # - P<0.05 in comparison with the untreated, control cells of a respective genotype.
Fig. V. Expression of HO-1 mRNA in HUVEC carrying S, M, or L alleles of HO-1 promoter, stimulated for 6 h with H$_2$O$_2$, CoPP, 15d-PGJ$_2$, LPS, IFN$\gamma$, hemin, and hypoxia, presented as a fold of induction of each cell batch, where untreated cells served as a control. EF2 was used as a constitutive gene in $\Delta$Ct analysis of qRT-PCR. * P<0.05 in comparison with the S carriers, and # - P<0.05 in comparison with the untreated, control cells of a respective genotype.
Fig. VI. Concentration of CO measured using gas chromatography, in culture media harvested from HUVEC carrying the S or L alleles of HO-1 promoter, incubated in control conditions or stimulated for 6 h with H$_2$O$_2$ or 15d-PGJ$_2$. * P<0.05, **P<0.01 in comparison with S carriers; and # - P<0.05 in comparison with the untreated, control cells of a respective genotype.
Fig. VII. Expression of HO-1, Thrx, ThrXR, catalase (Cat), and GPx in HUVEC carrying the S allele of the HO-1 promoter, cultured in control conditions or stimulated for 6 h with H$_2$O$_2$ (100 µmol/L). EF2 was used as a constitutive gene in ΔCt analysis of qRT-PCR. Data are shown as a fold of induction in comparison to the control, unstimulated cells. * P<0.05, ***P<0.001 in comparison with the control.
Fig. VIII. VEGF-induced proliferation measured by BrdU incorporation assay (A) and VEGF-induced migration measured in the modified Boyden chambers (B) of HUVEC of SS and LL genotypes. Data are shown as a percentage of control, non-stimulated cells. * P<0.05 in comparison with the control.
Correction

In the article “Role of Heme Oxygenase-1 in Human Endothelial Cells: Lesson From the Promoter Allelic Variants” by Taha et al, which appeared in the August 2010 issue of the journal (Arterioscler Thromb Vasc Biol. 2010:30:1634–1641; DOI: 10.1161/ATVBAHA.110.207316), the last sentence of the Sources of Funding should have appeared as: The Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University is a beneficiary of the structural funds from the European Union (grant No: POIG.02.01.00-12-064/08, 01.02-00-109/99 and 02.02.00-00-014/08).

The online version has been corrected.

The authors regret the error.