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: 2^{nd} passage – genotyping and measurements of glutathione concentration; 3^{rd} passage – analyses of HO-1, BvR, eNOS and ferritin expressions, and measuring the cytokine productions; and 4^{th} 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 (37ºC, 5% CO_2).

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 AAG 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% O_2, 5% CO_2), 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 GCG 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 CTG 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: 95ºC, 10 minutes (initial denaturation), 40 cycles of 95ºC, 30 s (denaturation), 58ºC, 60 s (annealing), 72ºC, 45 s (elongation), followed by 72ºC, 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[1].

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 VEF_{165}-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-A_{165} (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 wave length 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), H_2O_2 (100 μmol/L), and 15d-PGJ_2 (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, H_2O_2, 15d-PGJ_2, IFNγ (200 ng/mL), and LPS (100 ng/mL), or exposed to hypoxia (2% O_2). Control cells were incubated without any stimulation. All activators significantly increased the HO-1 mRNA expression (Fig. IVA). The most powerful one was 15d-PGJ_2, followed by CoPP and H_2O_2.

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[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 μmol/L). Hemin can be sequestered by hemopexin, and by serum albumin[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 μmol/L)[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 *Hmox-1*, it is still not clear if they correspond to typical HRE. Data concerning effects of hypoxia on HO-1 are contradictory[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)[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[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_2O_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 IFNγ, 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_2O_2 (18.0 ± 4.16, 17.6 ± 2.64 and 4.2 ± 2.27 fold in S, M and L carriers, respectively) and 15d-PGJ_2 (143.2 ± 37.34, 89.7 ± 11.86, 44.6 ± 18.76 fold in S, M, and L carriers, respectively).

Effect of H_2O_2 on expression of cytoprotective genes. To check the induction of antioxidative genes we incubated HUVEC cells with H_2O_2. The use of exogenous added H_2O_2 will not wholly mimic the physiological situation. However, it can be considered as a model

of response to oxidative stress[8]. H_2O_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_2O_2, produced for example by phagocytes, can be used in signalling to other cells, which is relevant in inflammation[9].

In cells treated with H_2O_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_2O_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[10,11,12]. 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[12], and its presence was associated with an increased risk of prolonged newborn jaundice and hyperbilirubinemia[13]. Moreover, short (GT)n alleles may represent a

genetic risk factor for cerebral malaria[14,15]. Finally, animal models suggest that a high level of HO-1 activity promotes plasmodium infection[16] and facilitates the *Mycobacterium tuberculosis* dormant infection, thereby enabling a bacterium to spread across a population[17,18.]

References

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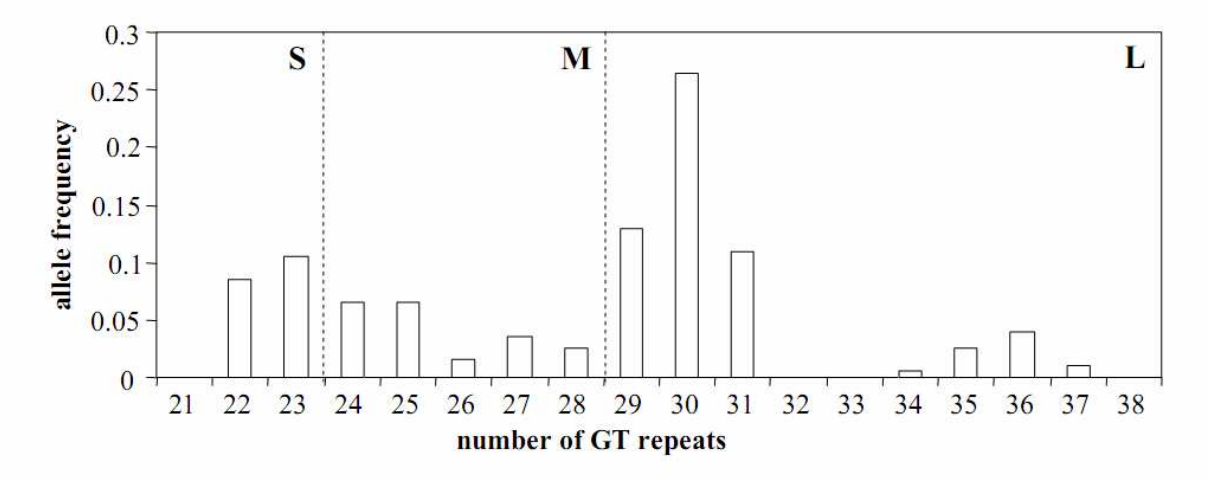

Fig. I. Frequency distribution of the numbers of (GT)n repeats in the population studied. S: ≤23 repeats, M: 24-28 repeats, L: ≥29 GT repeats.

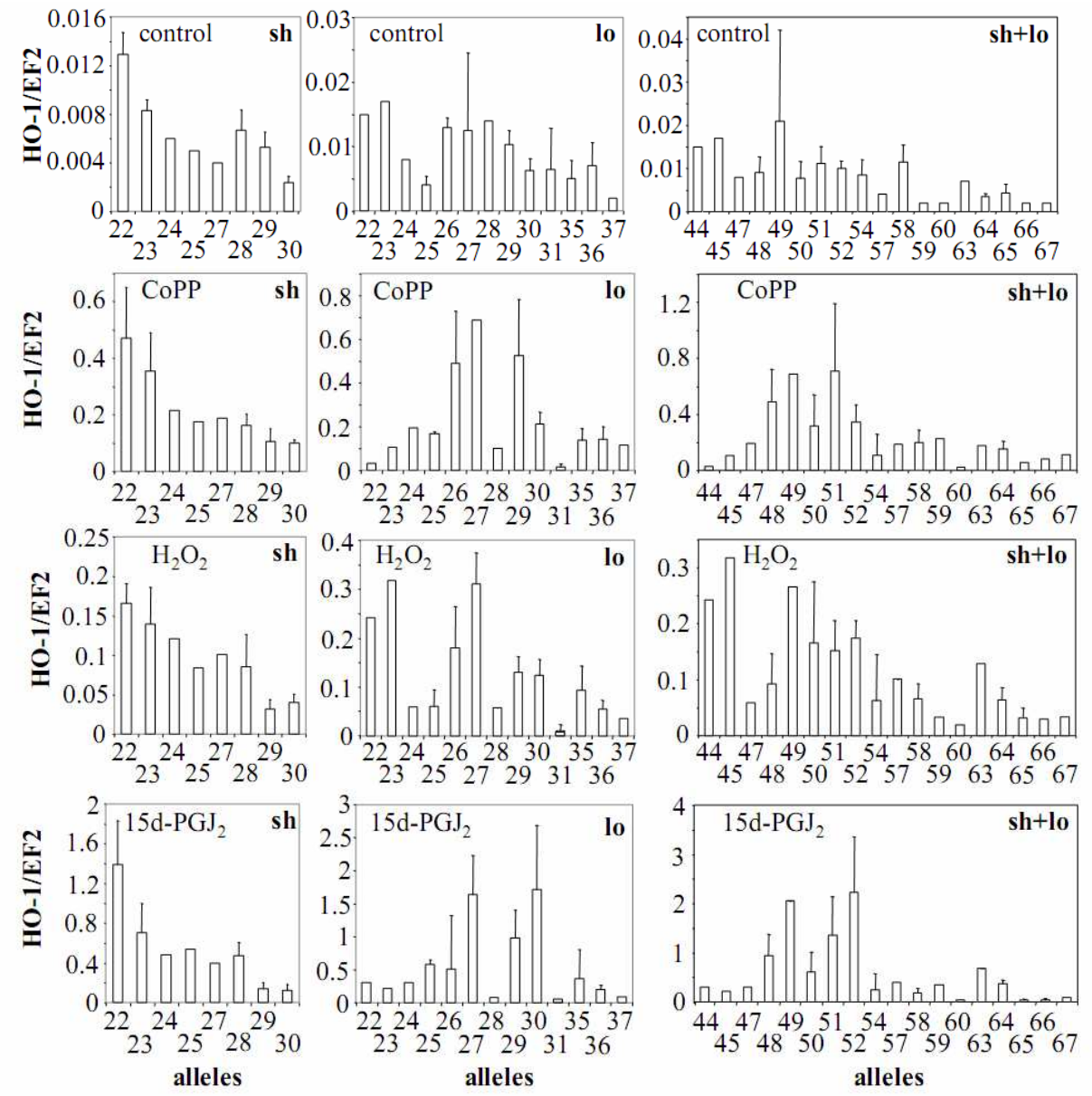

Fig. II. Expression of HO-1 mRNA measured using qRT-PCR in HUVEC cells carrying different HO-1 promoter alleles, cultured in control conditions or stimulated for 6 h with CoPP (10 μmol/L), H_2O_2 (100 μmol/L) and 15d-PGJ_2 (10 μmol/L). **Sh** – cells classified according to the number of GT repeats in 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.

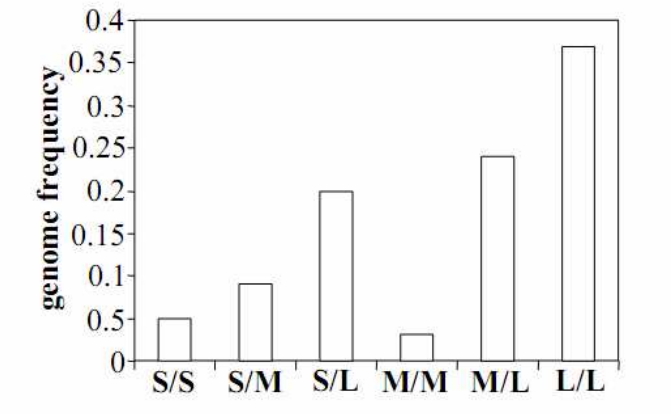

Fig. III. Frequency distribution of the HO-1 promoter genotypes in the population studied. S: ≤23 repeats, M: 24-28 repeats, L: ≥29 GT repeats.

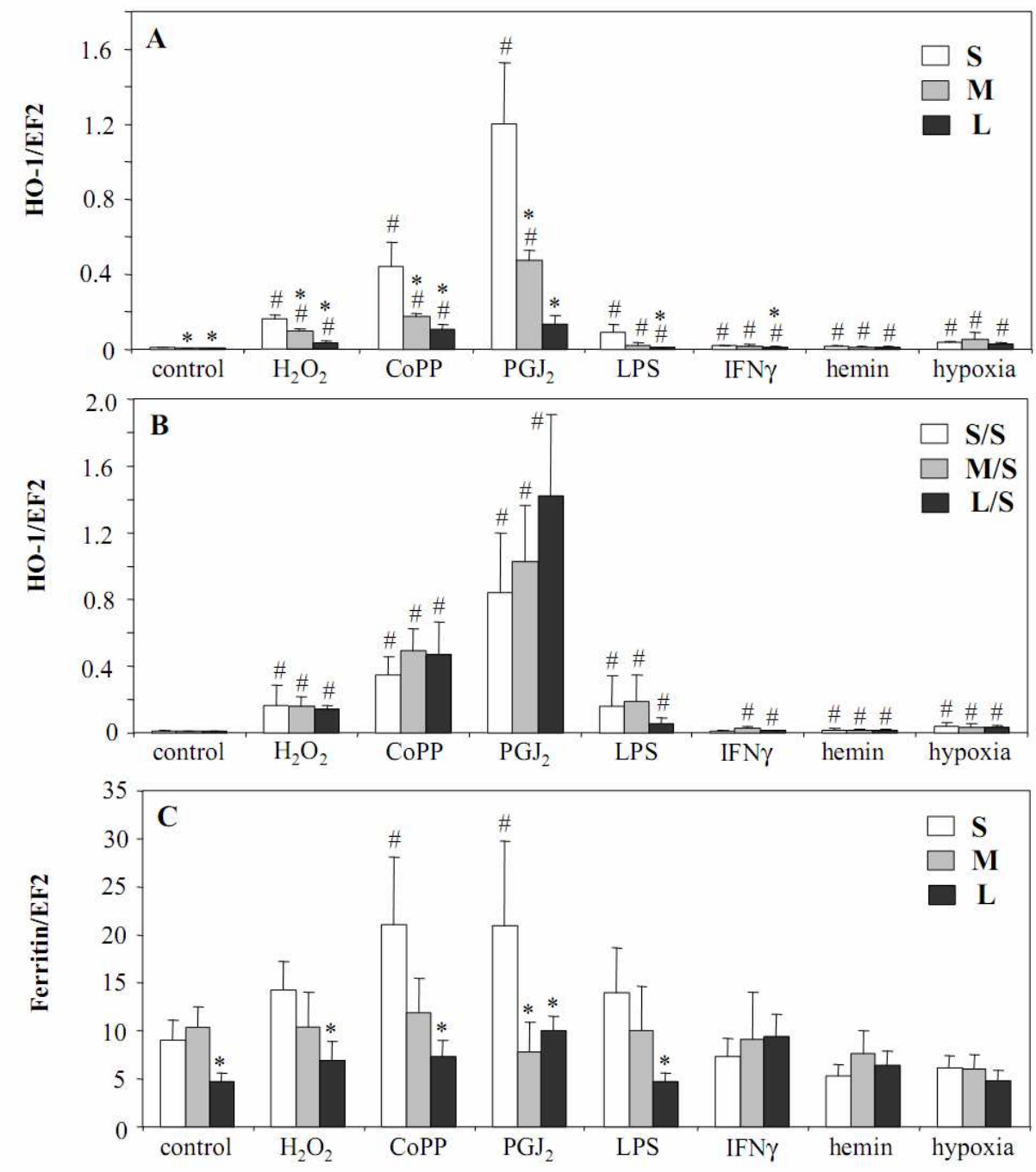

Fig. IV. A – Expression of HO-1 mRNA in HUVEC carrying S, M, or L alleles of HO-1 promoter stimulated for 6 h with H_2O_2, CoPP, 15d-PGJ_2, LPS, IFNγ, hemin, and hypoxia. **B –** Expression of HO-1 mRNA in HUVEC of S/S, S/M, or S/L genotype of HO-1 promoter. Both in control and stimulated cells there 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 ΔCt 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.

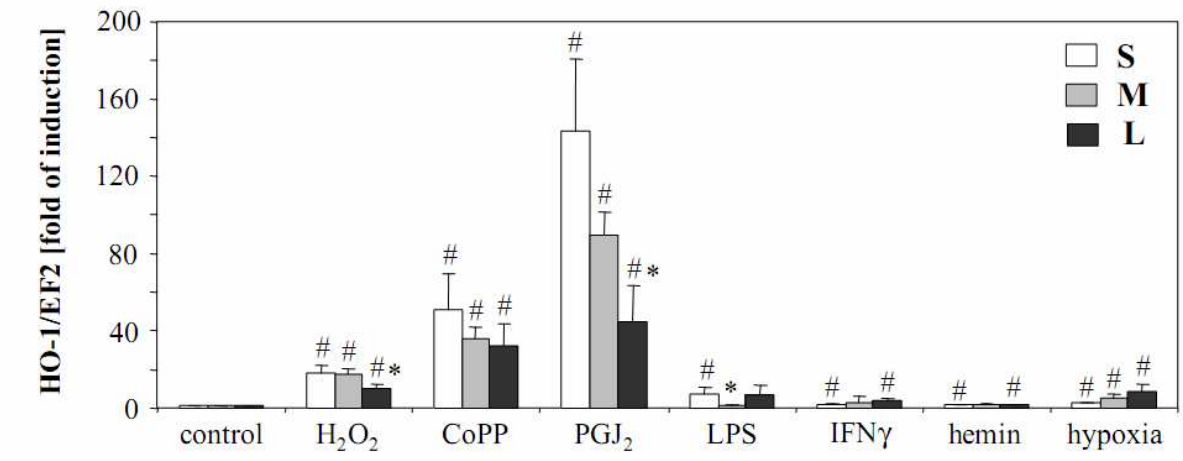

Fig. V. Expression of HO-1 mRNA in HUVEC carrying S, M, or L alleles of HO-1 promoter, stimulated for 6 h with H_2O_2, CoPP, 15d-PGJ_2, LPS, IFNγ, hemin, and hypoxia, presented as a fold of induction of each cell batch, where untreated cells served as a control. EF2 was used as a constitutive gene in ΔCt 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.

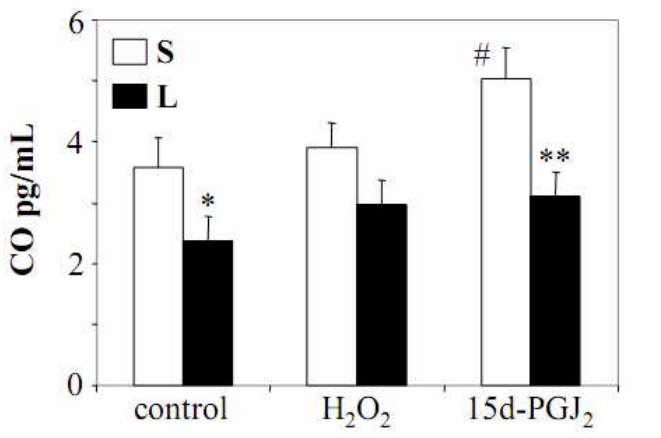

Fig. VI. Concentration of CO measured using gas chromatography, in culture media harvested from HUVEC carrying the S or L alleles of HO-1 promoter, incubated in control conditions or stimulated for 6 h with H_2O_2 or 15d-PGJ_2. * $P<0.05$, **$P<0.01$ in comparison with S carriers; and # - $P<0.05$ in comparison with the untreated, control cells of a respective genotype.

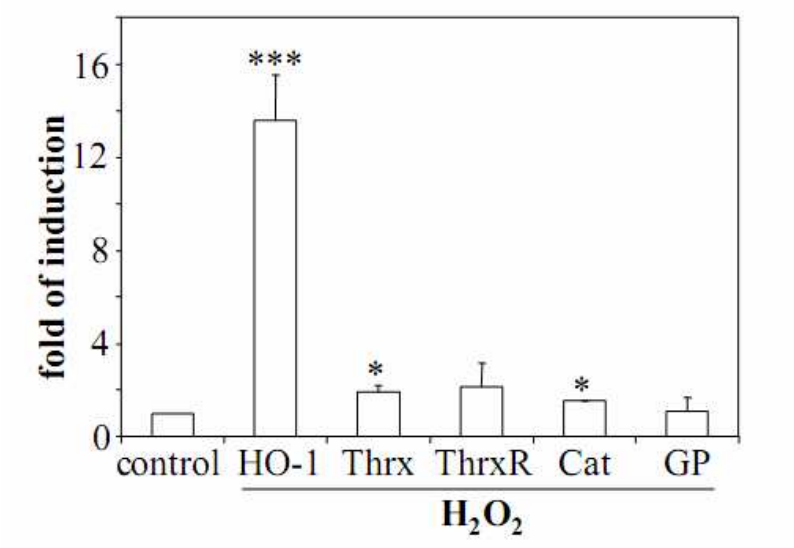

Fig. VII. Expression of HO-1, Thrx, ThrxR, catalase (Cat), and GPx in HUVEC carrying the S allele of the HO-1 promoter, cultured in control conditions or stimulated for 6 h with H_2O_2 (100 µmol/L). EF2 was used as a constitutive gene in ΔCt analysis of qRT-PCR. Data are shown as a fold of induction in comparison to the control, unstimulated cells. * $P<0.05$, ***$P<0.001$ in comparison with the control.

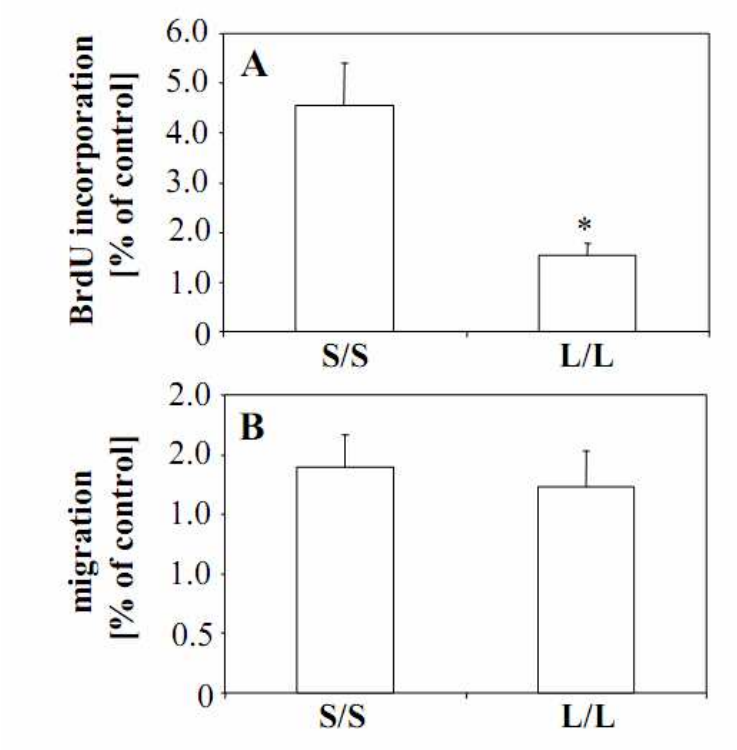

Fig. VIII. VEGF-induced proliferation measured by BrdU incorporation assay **(A)** and VEGF-induced migration measured in the modified Boyden chambers **(B)** of HUVEC of SS and LL genotypes. Data are shown as a percentage of control, non-stimulated cells. * $P<0.05$ in comparison with the control.