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# Original Research Communication

# HIF-1 Induction Attenuates Nrf2-Dependent IL-8 Expression in Human Endothelial Cells

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## Abstract

Through hypoxia-inducible factor 1 (HIF-1), hypoxia regulates the expression of numerous genes and is a potent inducer of angiogenesis. However, interleukin-8 (IL-8), an important angiogenic mediator, has been reported to be downregulated by HIF-1, although the mechanisms have not been elucidated. HIF-1 was induced in human endothelial cells by hypoxia and dimethyloxaloylglycine (DMOG). Interestingly, both hypoxia and DMOG attenuated IL-8 expression, and a similar effect has been obtained by adenoviral overexpression of the stable form of HIF-1α. Heme oxygenase-1 (HO-1) expression was also downregulated by HIF-1 induction. This suggests similar mechanisms of regulation of IL-8 and HO-1, indicating the involvement of Nrf2, a transcription factor previously linked to hypoxia-mediated inhibition of HO-1. Indeed, HIF-1-mediated downregulation of both IL-8 and HO-1 was associated with both lowered Nrf2 expression and induction of Bach1, a repressor of Nrf2 transcriptional activity. Accordingly, overexpression of Nrf2 reversed the inhibitory effect of HIF-1 on IL-8 and HO-1 expression. However, neither overexpression of HO-1 nor HO-1 inhibition affected IL-8 synthesis. The data indicate that HIF-1-dependent inhibition of IL-8 expression is caused by downregulation of Nrf2. However, expression of IL-8 is independent of HO-1. Cross-talk between HIF-1 and Nrf2 may influence the outcome of anti-angiogenic therapies aimed at targeting HIF-1. *Antioxid. Redox Signal.* 11, 1501–1517.

## Introduction

The hypoxia-dependent regulation of genes involved in angiogenesis, glucose metabolism, maturation of red blood cells, and cell proliferation occurs at the transcriptional level and is to a large extent mediated by hypoxia-inducible factor 1 (HIF-1), a transcription factor which consists of α and β subunits (32). In contrast to the HIF-1β subunit, HIF-1α is degraded at normal oxygen concentration due to the hydroxylation of HIF-1α prolines 402 and 564, mediated by specific oxygen-, iron- and 2-oxoglutarate-dependent proline hydroxylases (PHDs) (31). HIF-1 cooperates with a functional p300 and/or CBP homologous transcriptional coactivators, binding to the hypoxia responsive element (HRE) present in the promoter region of many genes (6). HIF-1α hydroxylation is abolished in hypoxia and also diminished by treatment with hypoxia mimics, including 2-oxoglutarate analogues, such as N-oxaloylglycine (NOG) or its cell-permeable precursor dimethyloxaloylglycine (DMOG) (9).

It is well understood that hypoxia arising in various pathological conditions is a potent inducer of angiogenesis. Accordingly, the expression of the main angiogenic mediator, vascular endothelial growth factor (VEGF) is strongly enhanced under reduced oxygen tension (17). The expression of interleukin-8 (IL-8), a potent chemoattractant for neutrophils and T lymphocytes, controller of leukocyte survival (7), and mediator of angiogenesis (34), cancer growth, and metastasis (33), is regulated by various tumor microenvironmental

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factors including hypoxia (12). Low oxygen tension also influences the expression of the stress inducible protein, heme oxygenase-1 (HO-1), which degrades heme to carbon monoxide, iron, and biliverdin (27). However, the effect of hypoxia on HO-1 expression is cell-type dependent. Recent studies (14, 24) demonstrated that at least in some human cells, particularly in endothelium, HO-1 is not induced or may be even attenuated by hypoxia.

HO-1 and IL-8 regulation by their multiple inducers is tightly controlled at the transcriptional level. NF-E2-related factor 2 (Nrf2), a member of the basic leucine zipper (bZIP) transcription factors family, is a potent activator of antioxidant response element (ARE)-mediated gene expression, and is involved both in IL-8 (37) and HO-1 (4) regulation. Interestingly, HO-1 dependent (26, 29) or independent (11, 18) regulation of IL-8 expression has recently been reported. Thus, cobalt protoporphyrin (CoPPIX), a potent HO-1 inducer has been shown to enhance IL-8 expression both in an HO-1-dependent (29) and independent (18) manner. Finally, HIF-1 induction by very high cobalt chloride ($CoCl_2$) concentrations enhanced IL-8 synthesis (13) although the unspecific influence of such high doses of $CoCl_2$ on HIF-1 was suggested by us in a comment to that paper (2). On the other hand, Ockaili *et al*. (26) claimed that induction of HO-1 by hypoxia attenuated TNF-induced IL-8 expression in human endothelial cells. To investigate the mechanisms of regulation of IL-8 by HIF-1 and to elucidate the aforementioned discrepancies, we decided to test the effect of HIF-1 induction on IL-8 and HO-1 expression in human endothelial cells. We provide here strong evidence that, in human endothelial cells, attenuation of IL-8 synthesis due to HIF-1 activation occurs in an HO-1 independent manner. Moreover, our results indicate that blocking of Nrf2 signaling pathway is responsible for HIF-1-mediated inhibition of IL-8 expression.

## Materials and Methods

### *Reagents*

Dimethyloxaloylglycine (DMOG), N-oxaloylglycine (NOG), and chetomin were obtained from Alexis Biochemicals (Warszawa, Poland). Tin protoporphyrin-IX (SnPPIX) was purchased from Porphyrin Products (Carnforth, Lancashire, England). Prostaglandin-J2 (PGJ2) was obtained from Biomol (Warszawa, Poland). The ELISA kits for human IL-8 and VEGF were procured from R&D Systems Europe (Warszawa, Poland) and the ELISA kit for human HO-1 was from Stressgen (Warszawa, Poland). TransAM assays for Nrf2 and HIF-1 activation were purchased from Active Motif (Rixensart, Belgium). The cell proliferation BrdU ELISA kit was bought from Roche (Warszawa, Poland). Endothelial cell growth supplement (ECGS) was obtained from Upstate Biotechnology (Warszawa, Poland). Oligo(dT) primers, dNTPs, MMLV reverse transcriptase, and Luciferase Activity Assay were obtained from Promega (Gdansk, Poland). The Great Escape SEAP Chemiluminescent Detection kit was from Clontech BD Biosciences (Chorzow, Poland). SuperFect Transfection Reagent was procured from Qiagen (Wroclaw, Poland). M199 medium and fetal bovine serum (FBS) were from PAA (Lodz, Poland). Primary antibodies: rabbit polyclonal anti-Nrf2, rabbit polyclonal anti-HIF1$\alpha$, goat polyclonal anti-Bach1 and goat polyclonal anti-HO-1 were from Santa Cruz Biotechnology (Lodz, Poland). Mouse monoclonal anti-$\alpha$-tubulin primary antibody was from Calbiochem (Warszawa, Poland). Lipofectamine2000 and oligofectamine were obtained from InVitrogen (Warszawa, Poland), and scrambled, HIF-1 and HO-1 siRNA from Dharmacon (Gdansk, Poland). Adeno-X Adenoviral Expression System and Adeno-X Rapid Titer ELISA kit were purchased from Clontech. Unless stated otherwise, other reagents were from Sigma-Aldrich (Poznan, Poland).

### *Cell culture and incubation experiments*

Human microvascular endothelial cells (HMEC-1) and human umbilical vein endothelial cells (HUVEC) were cultured as described previously (21). In brief, HMEC-1 cells were cultured in a MCDB 131 medium containing 10% FBS, L-glutamine (2 m*M*), EGF (10 ng/ml), hydrocortisone (1 $\mu$g/ml), and antibiotics. HUVEC cells were isolated from umbilical veins by collagenase digestion and cultured in M199 medium supplemented with 10% FBS, ECGS, heparin, L-glutamine (2 m*M*), hydrocortisone (1 $\mu$g/ml), and antibiotics. Mouse NIH3T3 fibroblasts were purchased from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium supplemented with 25 m*M* glucose, 10% FBS, and antibiotics. Cells were cultured in an incubator containing 5% $CO_2$, at 37°C and 95% humidity.

For induction of HIF-1, cells were cultured in hypoxia (1% $O_2$, 5% $CO_2$, 94% $N_2$) or treated with DMOG (250–1,000 $\mu M$) or NOG (500–1,000 $\mu M$) for various time points. For inhibition of HO activity, SnPPIX (10 $\mu M$) was applied 1 h before stimulation. Solvents (0.1 *M* NaOH for SnPPIX, DMSO for DMOG and NOG) at appropriate concentration were included in controls. Moreover, transduction with an adenoviral vector containing AdHIF1$\alpha$ cDNA was performed and after 48 h the appropriate tests were conducted.

In experiments with PGJ2 treatment, cells were pretreated for 1 h with DMOG and then costimulated with PGJ2 and DMOG for the next 24 h. Similarly, when chetomin and DMOG were used together, chetomin was added to the cells 30 min prior to DMOG administration.

The effect of DMOG (250–1,000 $\mu M$) on endothelial cell viability and proliferation has been determined by nonradioactive cytotoxic lactate dehydrogenase (LDH) assay (Promega) or cell proliferation BrdU ELISA kit (Roche), respectively, according to manufacturer's instructions.

### *Transfection with reporter plasmids*

HMEC-1 cells growing to 70% confluence in 24-well plates were transfected as described previously (18) with a construct containing the HRE fragment of the VEGF promoter (kindly provided by Dr. Hideo Kimura, Chiba, Japan) or with a construct containing the antioxidant response element (ARE) sequences driving the expression of luciferase (a kind gift from J. A. Johnson, University of Wisconsin, School of Pharmacy, Madison, WI). The plasmid containing the full-length promoter of the IL-8 gene driving luciferase expression was kindly supplied by Dr. Rainer de Martin (Vienna, Austria). The pAP-1-SEAP and pNF$\kappa$B-SEAP vectors, containing the AP-1 and NF$\kappa$B binding regions connected to the secreted alkaline phosphatase (SEAP) reporter gene were purchased from Clontech. The pCMV-lacZ plasmid containing the $\beta$-galactosidase gene driven by the CMV promoter was obtained from Promega and was co-transfected to cells together

with one of the above described reporter vectors. The activity of the luciferase or SEAP reporter genes was determined in cell lysates or cell culture media, respectively. Determination of luciferase enzyme activity was done according to manufacturer's protocol (Promega) using a plate reader luminometer. Chemiluminescent SEAP assay was performed according to the vendor's protocol (Clontech) with a modification, as described previously (1). The activity of luciferase or SEAP has been normalized to $\beta$-galactosidase activity or to protein content in the sample. Both normalizations, when assessed in the same sample, gave essentially the same results.

### *Western blot analysis and ELISA assays*

The total cellular protein was isolated and Western blots were performed as described previously (18). IL-8 and VEGF concentrations were determined in conditioned media. HO-1 protein was detected in cell lysates.

### *Transfection of cells with small interfering RNA*

The cells were transfected with 50 n*M* of chemically synthesized siRNA targeting human HO-1 mRNA or human HIF-1$\alpha$ mRNA, similarly to previous work (10). Scrambled siRNA served as a control. Oligofectamine or lipofectamine 2000 were used as a vehicle.

### *Transduction of the cells with adenoviral vectors*

Adenoviral vector containing rat HO-1 cDNA (AdHO-1) was a kind gift from Dr. Gisa Tiegs (Erlangen, Germany). The pAdNrf2 was obtained as described previously (16). A control vector harboring green fluorescent protein (GFP) cDNA (AdGFP) was produced using the Adeno-X system. Vectors were propagated in HEK 293 cells and then titered by detection of hexon protein with Adeno-X rapid titer ELISA kit, according to the vendor's protocol. The pAdHIF-1$\alpha$ was generated as described ((30); Pajusola *et al*., unpublished).

### *Reverse transcription—polymerase chain reaction*

Quantitative RT-PCR was performed in a Rotor Gene RG-3000 (Corbett Research) in a mixture containing SYBR Green PCR Master Mix (SYBR Green qPCR Kit, Finnzymes), specific primers and 50 ng of cDNA in a total volume of 15 $\mu$l. EF2 (elongation factor 2) was used as a housekeeping gene. Primers and cycling conditions are shown in Table 1.

### *Preparation of nuclear extracts and electrophoretic mobility-shift assay (EMSA)*

HMEC-1 cells were lysed by incubation in ice-cold low-salt buffer (10 m*M* HEPES, pH 7.9, 10 m*M* NaCl, 1.5 m*M* MgCl2, 0.1 m*M* NaVO4, 1 m*M* EGTA, 0.2% Nonidet P-40 and 1 m*M* PMSF). The nuclei were separated by centrifugation, washed with lysis buffer, resuspended in high-salt buffer (10 m*M* HEPES, pH 7.9, 420 m*M* NaCl, 0.1 m*M* NaVO4, 1 m*M* EDTA, 1 m*M* EGTA, 2 m*M* DTT, 20% glycerol) and extracted with shaking on ice for 30 min.

The following double-stranded oligonucleotides were used for DNA electrophoretic mobility shift assay: NF$\kappa$B (5′-AG CTTCAGAGACTTTCCGAGAGG-3′), and HIF-1 (5′-TCTG TACGTCACCACACTCACCTC-3′). Oligonucleotides were end-labeled with [$^{32}$P]ATP using Klenow polymerase and assay was performed according to standard procedure.

### *HIF-1 and Nrf2 activation assay (TransAM assay)*

HIF-1 and Nrf2 activation was assayed using ELISA-based transactivation TransAM kits (Active Motif) following the manufacturer's protocol. The HIF-1 TransAM kit contains a 96-well plate with immobilized oligonucleotides encoding an HRE consensus site (5′-TACGTGCT-3′) from the erythropoietin gene, whereas in the Nrf2 TransAM assay immobilized oligonucleotides encoding an ARE consensus binding site (5′-GTCACAGTGACTCAGCAGAATCTG-3′) are present. The active form of HIF-1 or Nrf2 contained in the cell extract specifically binds to the appropriate oligonucleotides. The primary antibody used to detect HIF-1 or Nrf2 recognizes an epitope on HIF-1 or Nrf2 when the proteins are activated and bound to its target DNA. A horseradish peroxidase (HRP)-conjugated secondary antibody provides a sensitive colorimetric readout that is quantified by a spectrophotometer at 450 nm with a reference wavelength of 655 nm. The positive control ($CoCl_2$-treated COS-7 nuclear extract for HIF-1 and Nrf2-transfected COS-7 nuclear extract for Nrf2) provided with the kits was used to assess assay specificity. A wild type or mutated HRE sequence was added to prove the specificity of interactions.

### *Statistical analysis*

All experiments were performed in duplicates and were repeated at least three times. All data are presented as

Table 1. PCR Primers and Conditions

| *Gene* | *Sequence* | *Annealing T* |
|---|---|---|
| IL-8 | F: CTCTCTTGGCAGCCTTCCTGA<br>R: CCCTCTGCACCCAGTTTTCCTT | 60°C |
| HO-1 | F: GTGGAGMCGCTTYACRTAGYGC<br>R: CTTTCAGAAGGGYCAGGTGWCC | 60°C |
| HIF-1 | F: TGCTTGGTGCTGATTTGTGA<br>R: GGTCAGATGATCAGAGTCCA | 60°C |
| VEGF | F: CTGGTCTTGGGTGCATTG<br>R: CACCGCCTCGGCTTGTCACAT | 58°C |
| eNOS | F: GTG ATG GCG AAG CGA GTG AA<br>R: CCG AGC CCG AAC ACA CAG AA | 58°C |
| EF2 | F: TCAGCACACTGGCATAGAGGC<br>R: GACATCACCAAGGGTGTGCAG | 60°C |

means ± standard deviations (SD) and analyzed by analysis of variance (ANOVA), followed by a Bonferoni post-hoc test for multiple comparisons, or with Student's *t*-test for two-group comparisons. Differences were accepted as statistically significant at $p < 0.05$.

## Results

### *DMOG acts similarly to hypoxia in human microvascular endothelial cells*

DMOG inhibits prolyl hydroxylases and activates HIF-1 (9). In our hands, this compound enhanced HRE activity to an almost similar level as hypoxia (Fig. 1A) and potently increased HIF-1 binding after 3 h of incubation as shown by EMSA assay (Fig. 1B). Specific activation of HIF-1 was evidenced in TransAM HIF-1 assay by blocking the HIF-1 induction by DMOG with the wild type but not the mutated version of HRE sequence motif (Fig. 1C). HIF-1α protein levels were augmented after DMOG (Fig. 1D) or hypoxia (Fig. 1E) treatment in HMEC-1 cells. Concomitantly, the expression of VEGF, a gene known to be HIF-1 inducible. was strongly induced (8.9 ± 1.1 fold of induction after 1 m*M* DMOG, $n = 3$), what indicates the correct function of our model for HIF-1 induction. Moreover, VEGF synthesis was induced to a smaller extent by another 2-oxoglutarate analogue, NOG (only 2.29 ± 0.46 fold of induction after 1 m*M* NOG, $n = 3$), than by cell-permeable DMOG. Finally, the decrease in endothelial nitric oxide synthase (eNOS) expression was also observed after DMOG treatment, similarly to the effect observed in hypoxia (Fig. 1F), confirming the previous observations of the effect of hypoxia on eNOS expression (20). Overall, these data indicate that treatment with DMOG can be considered as the equivalent of hypoxia at least with regard to regulation of major targets (*i.e.*, HIF-1 and VEGF). DMOG in the range of applied concentrations did affect neither cell viability nor proliferation (data not shown).

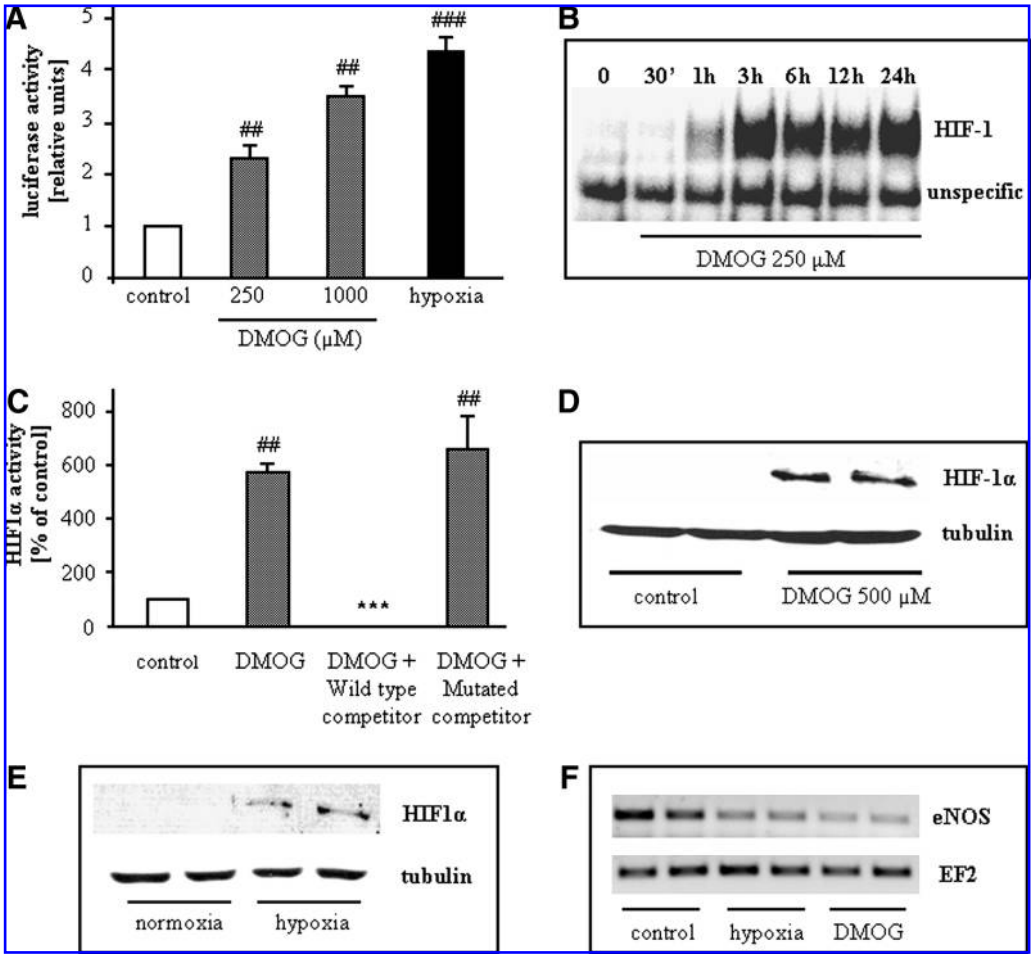


**FIG. 1. DMOG activates HIF-1 similarly as hypoxia.** Cells were cultured in the presence of 250–1,000 μ*M* DMOG or in hypoxic conditions (1% $O_2$). 24 h after transfection with the reporter plasmid HRE-luc luciferase activity was measured (**A**). HIF-1 DNA binding is shown by representative EMSAs using nuclear extracts of untreated and DMOG-treated HMEC-1 cells after different time periods (**B**). Specific activation of HIF-1 by 500 μ*M* DMOG has been demonstrated in TransAM ELISA assay (**C**). Induction of HIF-1 protein by DMOG (**D**) and hypoxia (**E**) was evaluated by Western blot analysis. The decrease in eNOS (**F**) expression was observed after hypoxia and DMOG treatment. Mean of three (**A**) and two (**C**) independent experiments, ##$p < 0.01$ *vs*. control; ###$p < 0.001$ *vs*. control, ***$p < 0.05$ *vs*. DMOG. Representative EMSA (**B**), Western blot (**D**, **E**) and RT-PCR (**F**) results.

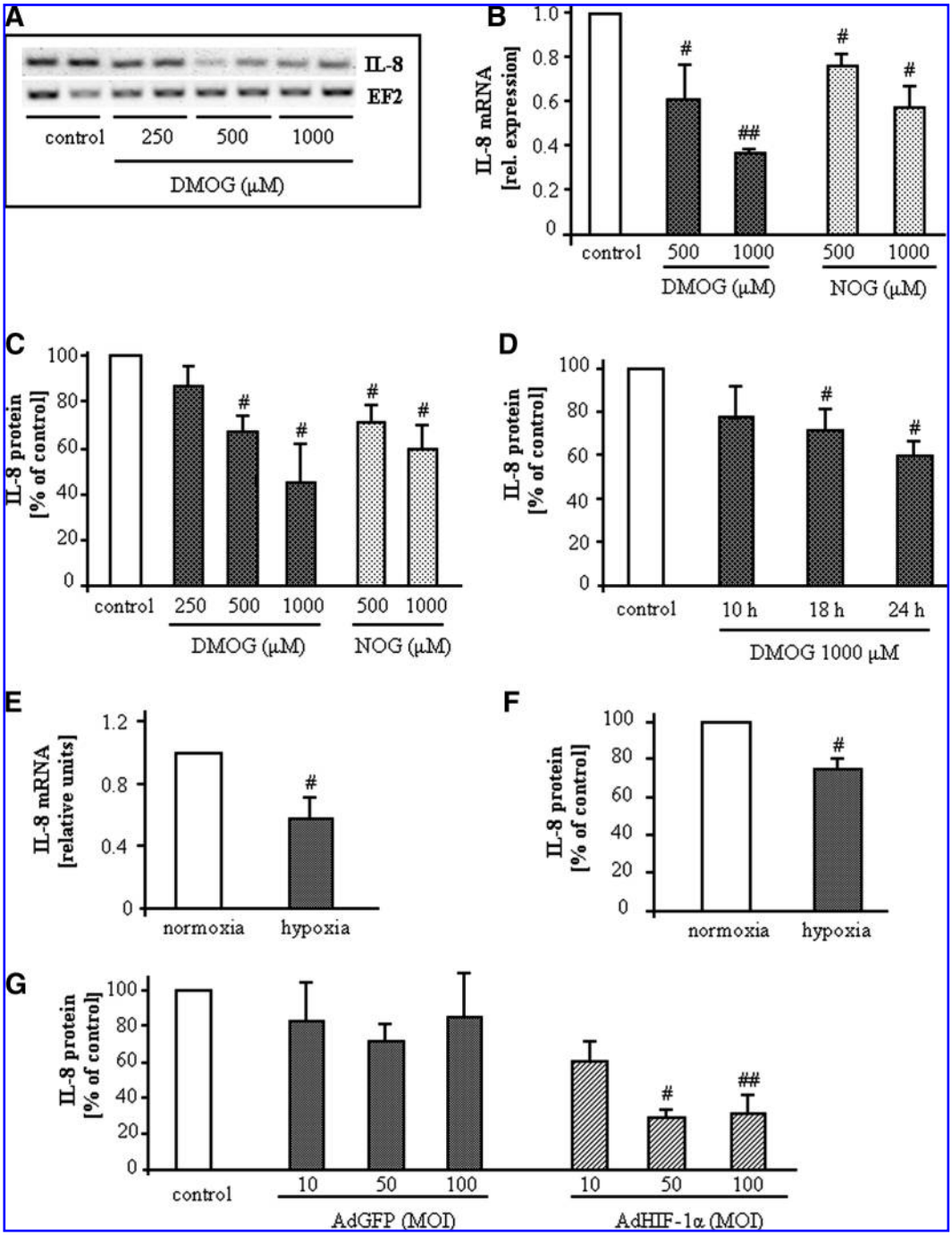


**FIG. 2. HIF-1 activation inhibits IL-8 synthesis in human microvascular endothelial cells.** Cells were cultured in the presence of 250–1,000 $\mu M$ DMOG or 500–1,000 $\mu M$ NOG for 24 h. HIF-1 activation decreased IL-8 mRNA expression as shown by qualitative (**A**) and quantitative (**B**) RT-PCR. Moreover, IL-8 production after DMOG and NOG treatment was attenuated, as determined by ELISA (**C**). Inhibition of IL-8 synthesis is observed already after 18 h incubation with 1 m$M$ DMOG (**D**). IL-8 mRNA (**E**) and protein (**F**) synthesis was also inhibited by hypoxia and in cells overexpressing HIF-1$\alpha$ (**G**). Representative RT-PCR result (**A**). Mean of three independent experiments, #$p < 0.05$ *vs.* control (**B**, **C**, **D**, **E**); *vs.* normoxia (**F**); *vs.* relevant AdGFP control (**G**), ##$p < 0.01$ *vs.* control (**B**); *vs.* relevant AdGFP control (**G**).

### *HIF-1 attenuates IL-8 expression in human endothelial cells*

Interestingly, in contrast to VEGF, treatment of HMEC-1 with DMOG (250–1,000 $\mu M$) attenuated the expression of IL-8 mRNA (Fig. 2A). Real-time PCR has shown the inhibitory effect of both HIF-1 inducers (DMOG and NOG) on IL-8 expression (Fig. 2B). Concomitantly, IL-8 protein secretion was significantly inhibited by both DMOG and NOG in a concentration- (Fig. 2C) and time-dependent (Fig. 2D) manner. Hypoxia also decreased IL-8 mRNA (Fig. 2E) and protein (Fig. 2F) levels, indicating similar mechanisms of hypoxia

and DMOG action on IL-8. Moreover, adenoviral transduction of the stable form of HIF-1α downregulated IL-8 synthesis in a concentration dependent manner (Fig. 2G), while it upregulated VEGF synthesis ($1.86 \pm 0.12$, $2.73 \pm 0.19$, $2.69 \pm 0.17$-fold induction in cells transduced with 10, 50, or 100 MOI of AdHIF-1α compared to a proper AdGFP control, respectively, $n = 3$).

### *HIF-1α siRNA reverses DMOG-induced inhibition of IL-8 synthesis*

Downregulation of IL-8 by HIF-1 overexpression indicates the involvement of this transcription factor in the effect of DMOG. However, to further confirm the supposition that the downregulation of IL-8 expression by DMOG depends on HIF-1 activation, we used siRNA targeting HIF-1α. 50 n*M* of siRNA against HIF-1α strongly diminished expression of this transcription factor (Fig. 3A). Basal level of IL-8 were not affected by HIF-1α siRNA, but eradication of HIF-1 reversed the inhibitory effect of DMOG on IL-8 (Fig. 3B). Conversely, upregulation of VEGF synthesis by DMOG was diminished after HIF-1α siRNA treatment (Fig. 3C).

### *HO-1 protein is downregulated by HIF-1 induction in human cells but upregulated in murine fibroblasts*

Recently, Ockaili *et al*. (26) suggested that, in the same HMEC-1 cell line, DMOG downregulated IL-8 synthesis through induction of HO-1. In contrast, in our hands, DMOG decreased the expression of HO-1. This effect was observed both at the mRNA (Fig. 4A and B) and protein level (Fig. 4C–E), in a concentration- (Fig. 4C and D) and time-dependent (Fig. 4E) manner. Importantly, the mRNA level for the constitutive form of the enzyme, HO-2, was not affected by DMOG (Fig. 4A). Inhibitory effect of HIF-1 induction on HO-1 expression was also observed in HMEC-1 cells treated with

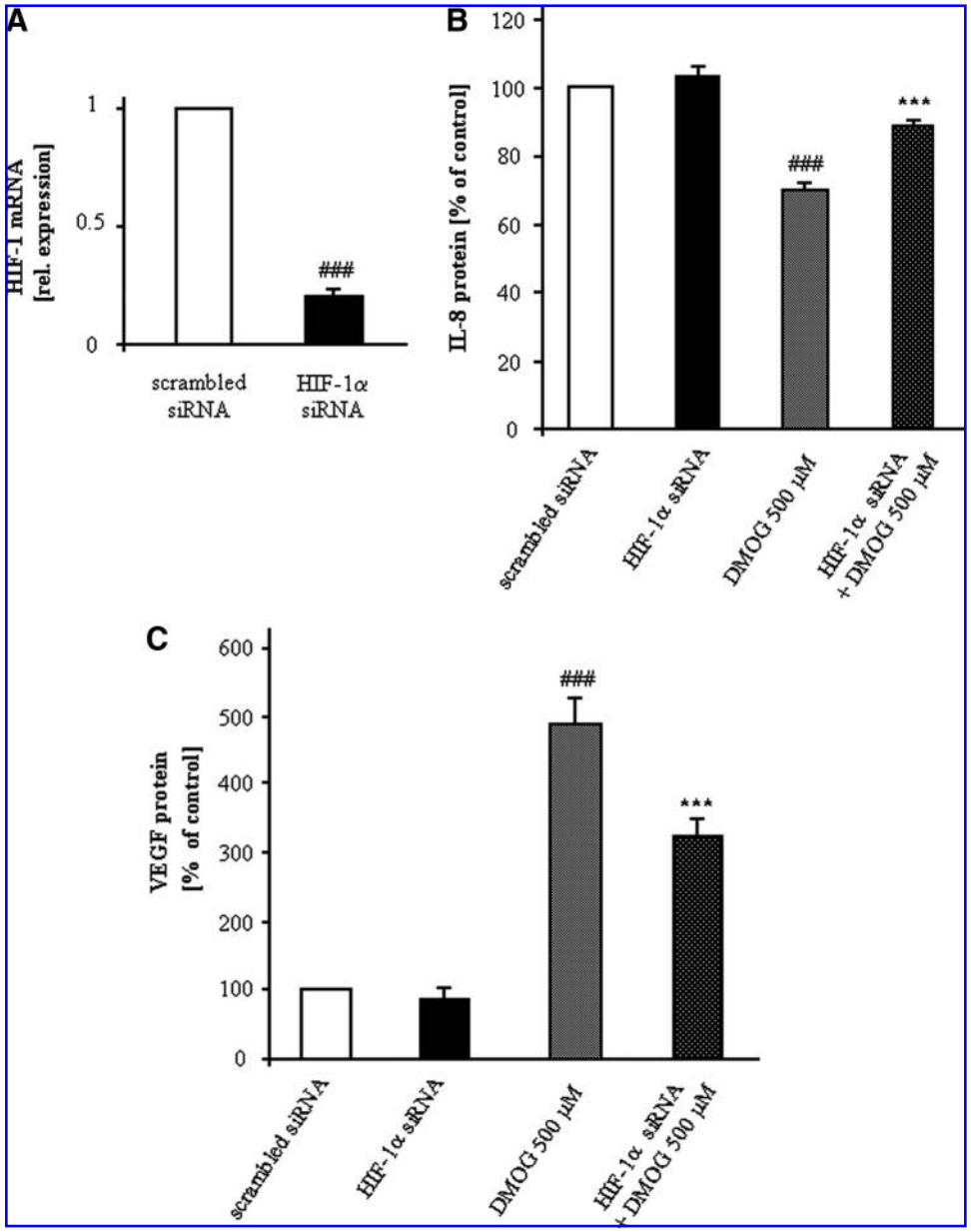


**FIG. 3. Inhibition of HIF-1α with siRNA reverses DMOG effects on IL-8 and VEGF synthesis.** (**A–C**) HIF-1α siRNA (50 n*M*) abolishes HIF-1 mRNA expression (**A**), reverses the inhibitory effect of DMOG on IL-8 synthesis (**B**), and attenuates HIF-1-induced VEGF synthesis (**C**). Control cells were transfected with scrambled siRNA. Mean of three independent experiments, ###$p <$ 0.001 *vs.* control cells transfected with scrambled siRNA, ***$p < 0.001$ *vs.* cells treated with DMOG.

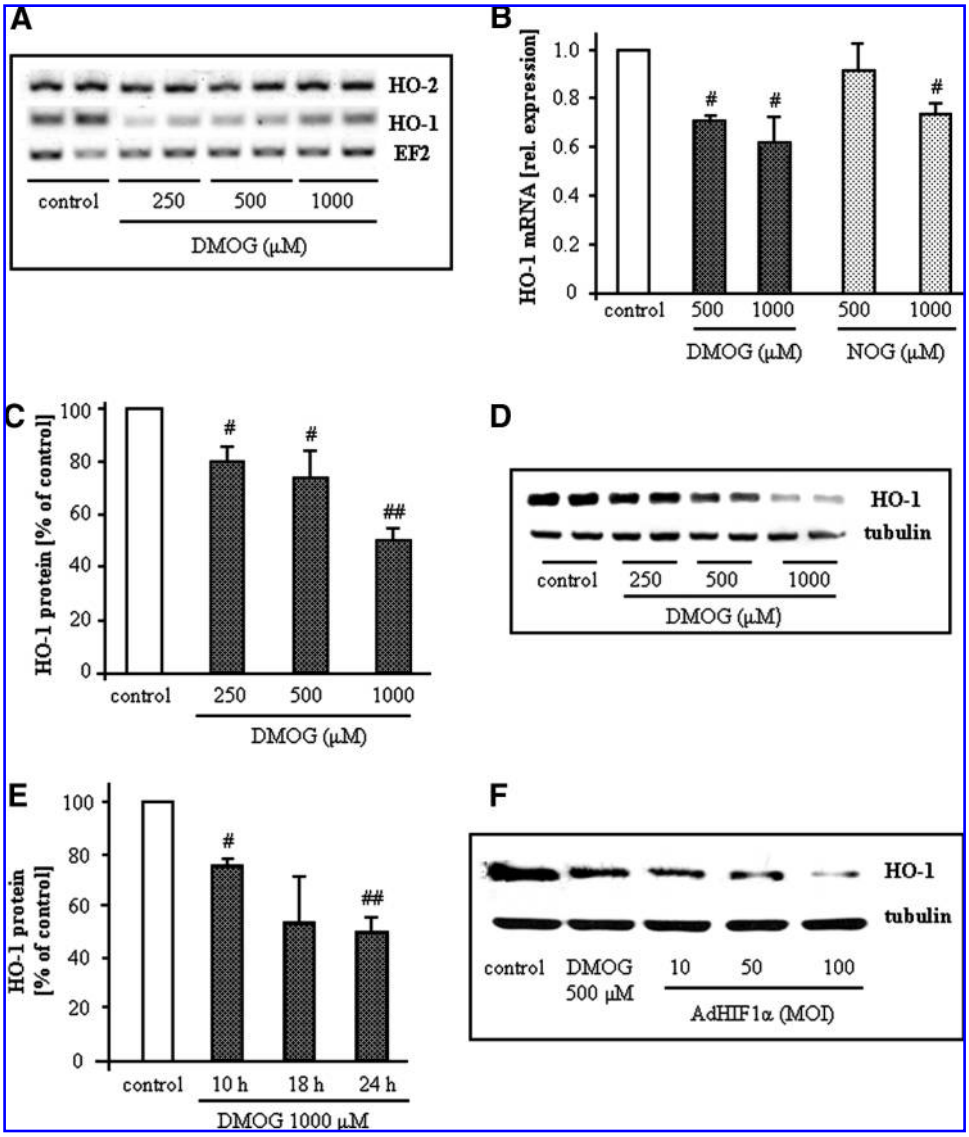


**FIG. 4. Activation of HIF-1 attenuates HO-1 expression in human endothelial cells.** HMEC-1 cells were cultured in the presence of 250–1,000 $\mu M$ DMOG or 500–1,000 $\mu M$ NOG for 24 h. DMOG concentration-dependently decreased HO-1 expression and protein synthesis, as shown by qualitative (**A**) and quantitative (**B**) RT-PCR, ELISA (**C**), and Western blot analysis (**D**). The effect of DMOG is time dependent (**E**). Overexpression of HIF-1α also downregulated HO-1 expression (**F**). Also, NOG, another analogue of 2-oxoglutarate, inhibits HO-1 expression (**B**). Expression of constitutive HO-2 did not change (**A**). Mean of three independent experiments, $\#p < 0.05$ *vs.* control; $\#\#p < 0.01$ *vs.* control. Representative of RT-PCR (**A**) and Western blot (**D**, **F**) results are shown.

NOG (1 m*M*) (Fig. 4B). Finally, overexpression of the stable form of HIF-1α downregulated HO-1 expression in HMEC-1 cells (Fig. 4F).

To exclude the cell-type specificity of the above described effects, we investigated the expression of IL-8 and HO-1 in the HUVEC primary cell line. Similarly as in HMEC-1, IL-8 mRNA (Fig. 5A) and protein (Fig. 5B) expression was downregulated by DMOG treatment in these endothelial primary cells. In addition, HO-1 mRNA (Fig. 5C) and protein (Fig. 5D) synthesis was decreased by DMOG. This indicates that the observed attenuation of IL-8 expression is not restricted only to HMEC-1 cells, and inhibition of IL-8 and HO-1 expression by HIF-1 induction occurs in human endothelial cells from different vascular beds.

Interestingly, in murine NIH3T3 fibroblasts, DMOG treatment induced HO-1 expression (Fig. 5E and F). Therefore, as regulation of HO-1 expression by HIF-1 inducers is different in at least some human cells and the observed inhibitory effect in endothelial cells may be more relevant to human pathophysiology, we concentrated on human endothelial cells only. As HMEC-1 is an immortalized cell line and only small differences between passages can be observed, we decided to use these cells for most of the experiments.

### *Neither inhibition of HO-1 expression nor HO-1 overexpression affect IL-8 synthesis*

As shown above, HO-1 is not induced in HMEC-1 by prolyl hydroxylase inhibitors in contrast to a previous report (26). Nevertheless, to check if inhibition of HO-1 would affect IL-8 production, we treated cells with tin protoporphyrin (SnPPIX), a commonly used inhibitor of HO enzymatic activity. Additionally, as protoporphyrins may exert HO-independent effects (5), we also introduced to our study siRNA against HO-1 mRNA.

The results obtained after HO-1 siRNA and SnPPIX treatment were similar. Inhibition of HO-1 with specific siRNA (to $36\% \pm 4.7\%$ of the level of basal expression, $n = 3$) did not influence basal synthesis of IL-8 (Fig. 6A). Moreover, DMOG-inhibited production of this chemokine was also not affected (Fig. 6A). Similarly, pretreatment of HMEC-1 cells with

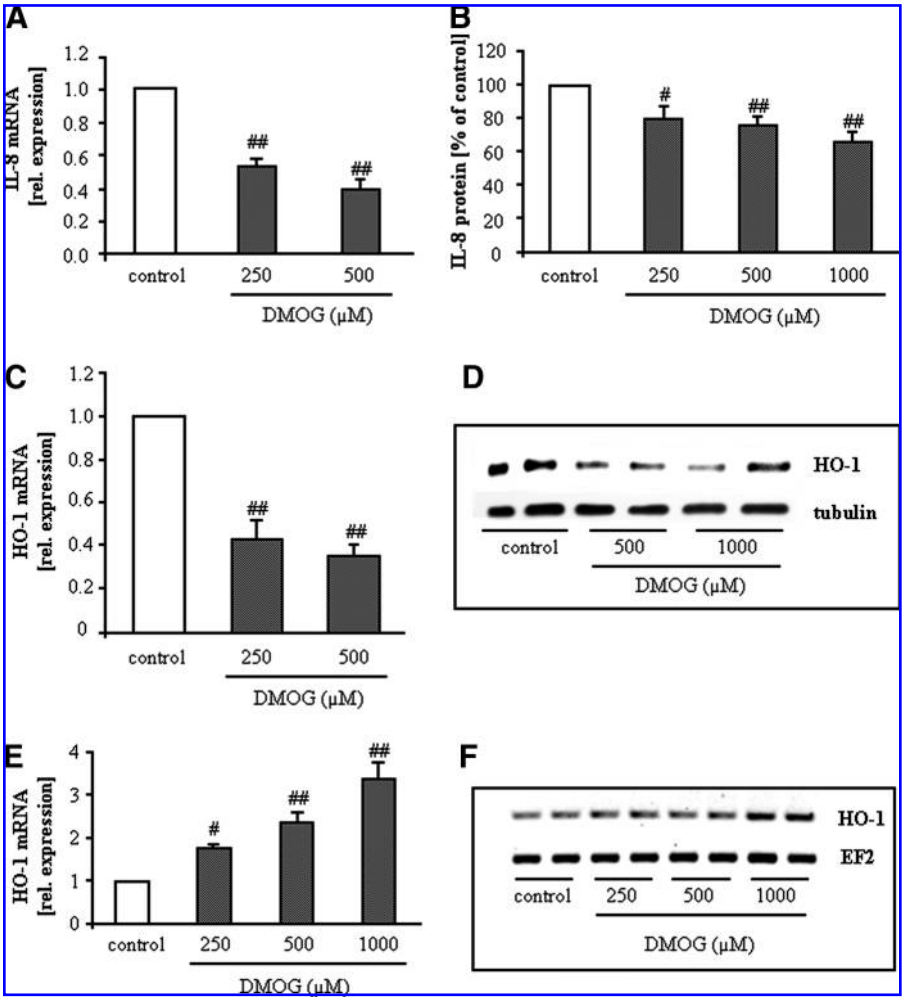


**FIG. 5. DMOG decreases IL-8 and HO-1 expression in HUVEC while upregulates in murine NIH3T3 fibroblasts.** HUVEC and NIH3T3 cells were cultured in the presence of 250–1,000 $\mu M$ DMOG for 24 h. IL-8 mRNA (**A**) and protein (**B**) expression was diminished in HUVEC cells. Similarly, DMOG exerts an inhibitory effect on HO-1 mRNA (**C**) and protein (**D**) levels in these endothelial cells. In contrast, in murine NIH3T3 cells, upregulation of HO-1 expression has been observed as shown by real-time RT-PCR (**E**) and qualitative RT-PCR (**F**). Mean of three independent experiments, $\#p < 0.05$ *vs.* control; $\#\#p < 0.01$ *vs.* control. Representative of Western blot (**D**) and RT-PCR (**F**) results are shown.

SnPPIX (10 $\mu M$) influenced neither the basal nor DMOG-downregulated production of IL-8 (Fig. 6B).

To investigate the effect of HO-1 overexpression on IL-8 production, cells were transduced with adenoviral vectors containing HO-1 cDNA (AdHO-1, 50 MOI). Control cells were transduced with the same dose of AdGFP. Transduction efficacy and overexpression of HO-1 were confirmed by microscopic analysis (50–60% GFP-positive cells, not shown), by real-time RT-PCR (17.94 ± 2.19-fold of HO-1 induction) and by Western blot (Fig. 6C). Strong induction of HO-1 affected IL-8 protein synthesis neither 24 nor 48 h after transduction with AdHO-1 (Fig. 6D). Thus, the data obtained from HO-1 overexpression confirm that HO-1 is not responsible for downregulation of IL-8 expression.

Overall, the data conclusively show that HO-1 is not involved in the regulation of IL-8 in HMEC-1 cells and that the inhibitory effect of DMOG on IL-8 synthesis cannot be due to induction of HO-1.

### *Attenuation of IL-8 expression by HIF-1 activation is accompanied by a decrease in IL-8 promoter and NFκB and AP-1 transcription factor activity*

AP-1 and NFκB are important transcriptional regulators of IL-8 expression (33). Therefore, to assess their role in HIF-1-dependent regulation of IL-8, cells were transfected with reporter plasmids (IL-8-luc, AP-1-SEAP, and NFκB-SEAP) and treated with DMOG. The results demonstrated that the effect of HIF-1 induction on IL-8 expression is exerted at transcriptional level, as inhibition of IL-8 promoter activity to 36% ± 4.2% after 1 m$M$ DMOG was observed (Fig. 7A). Moreover, AP-1 (Fig. 7B) and NFκB (Fig. 7C) transcription factor activities were also significantly lowered in cells exposed to DMOG, as evidenced also by EMSA for NFκB (not shown).

### *HIF-1 induction attenuates Nrf2 expression and activity and induces the Bach1 repressor*

As both IL-8 and HO-1 are regulated by Nrf2 (4, 37), we wondered if, in addition to AP-1 and NFκB factors, Nrf2 transcription factor is also involved in HIF-1-dependent regulation of gene expression. First, we checked the effect of HIF-1 induction on cells transfected with a plasmid containing ARE sequences to which Nrf2 binds. DMOG was found to decrease Nrf2-driven luciferase expression (Fig. 7D). Treatment with DMOG may also decrease Nrf2 mRNA (Fig. 7E) and protein (Fig. 7F) levels. Similarly, overexpression of HIF-1α downregulated Nrf2 protein levels (Fig. 7G). However, in HMEC-1 cells, DMOG did not affect Nrf2 localization, as we did not observe nuclear translocation of Nrf2 (data not shown).

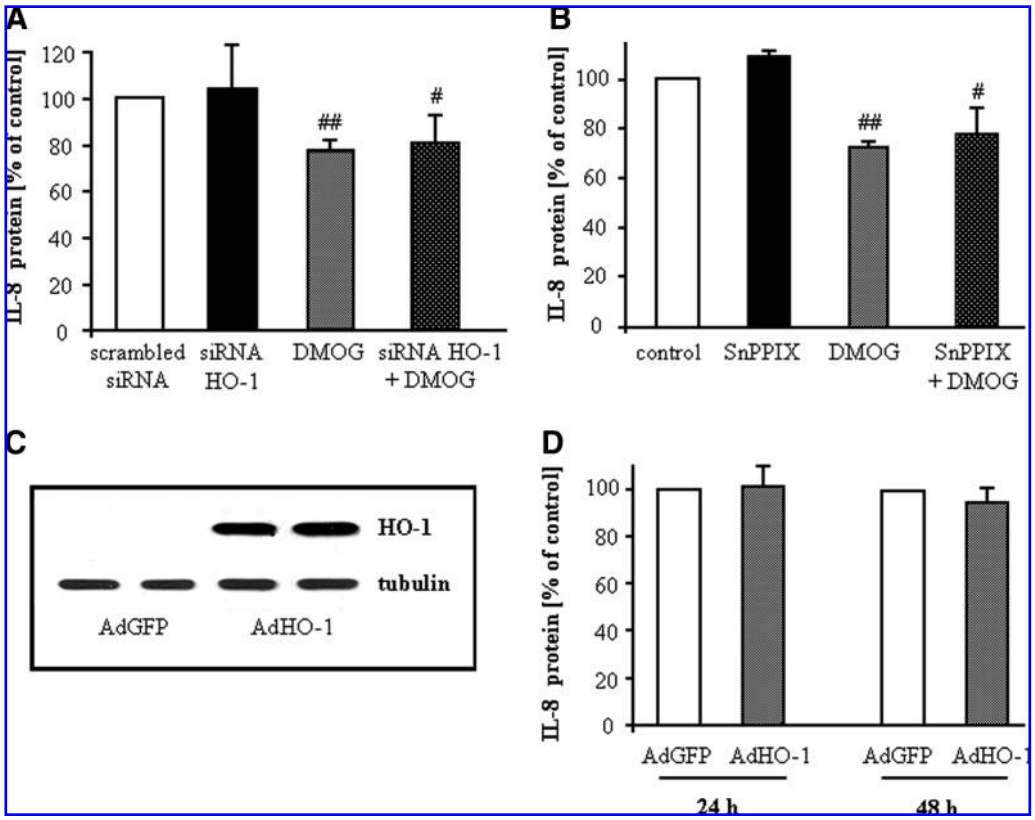


**FIG. 6. Effect of HO-1 inhibition/overexpression on IL-8 production in HMEC-1.** Inhibition of HO-1 with 50 n*M* siRNA (**A**) and SnPPIX (10 $\mu M$) (**B**) did not revert the inhibitory effect of 500 $\mu M$ DMOG on IL-8 synthesis. Control cells were transfected with scrambled siRNA (**A**). Moreover, overexpression of HO-1 with 50 MOI of AdHO-1 (**C**) did not augment IL-8 production (**D**). Mean of three independent experiments, #$p < 0.05$ *vs.* control cells transfected with scrambled siRNA (**A**) *vs.* control (**B**); ##$p < 0.01$ *vs.* control cells transfected with scrambled siRNA (**A**) *vs.* control (**B**).

Bach1 is a known repressor of Nrf2 activity, preventing the induction of Nrf2-dependent genes by blocking ARE activation (28). Importantly, in cells exposed to hypoxia (Fig. 8A), treated with DMOG (Fig. 8B and C) or transduced with Ad-HIF-1α (Fig. 8D), the expression of Bach1 was upregulated.

As demonstrated above, hypoxia, DMOG, or HIF-1α overexpression also downregulated HO-1 synthesis. As the expression of this gene is mediated by Nrf2, we checked whether induction of HIF-1 can affect Nrf2-dependent HO-1 expression. We used PGJ2, a very potent inducer of HO-1 which acts through Nrf2 activation (4). Indeed, DMOG pretreatment attenuated HO-1 expression enhanced by PGJ2, as evidenced both at the mRNA and protein level (Fig. 8E and F), again indicating the inhibitory effect of HIF-1 induction on HO-1 expression.

### *Nrf2 enhances both IL-8 transcription and mRNA stability and overexpression of Nrf2 restores IL-8 production attenuated by DMOG*

To further evaluate if the effect of HIF-1 induction on IL-8 synthesis is mediated by Nrf2, we used adenoviral vectors encoding Nrf2. After transduction with 50 MOI AdNrf2, high Nrf2 expression at the mRNA (Fig. 9A) and protein level (Fig. 9B) was observed. Interestingly, in contrast to nontransduced cells or cells transduced with control vectors (AdGFP), DMOG stimulation resulted in additional upregulation of Nrf2 expression in Nrf2 overexpressing cells (Fig. 9A and B).

A previous study demonstrated the involvement of Nrf2 in the regulation of IL-8 expression at the post-transcriptional level, whereas the activation of the IL-8 promoter was negligible (37). In contrast, in our hands, in HMEC-1 cells, adenoviral overexpression of Nrf2 enhanced both IL-8 transcription (Fig. 9C) and mRNA stability (Fig. 9D).

Next, we checked if overexpression of Nrf2 could reverse effects of DMOG on IL-8 and HO-1 generation. After AdNrf2 transduction, strong upregulation of IL-8 (Fig. 10A and B) and HO-1 (Fig. 10C and D) mRNA and protein synthesis was observed. Interestingly, although nontransduced cells or cells transduced with control vectors (AdGFP) show inhibition of IL-8 expression after DMOG treatment, concomitant treatment with this HIF-1 inducer and AdNrf2 transduction resulted in higher augmentation of IL-8 expression than obtained after Nrf2 upregulation alone (Fig. 10A and B). A similar pattern of combined DMOG and Nrf2 action was observed when HO-1 expression was evaluated (Fig. 10C and D).

### *Chetomin reverses the effect of DMOG by induction of Nrf2*

Chetomin is an agent destabilizing HIF-1α cooperation with p300/CBP (15). Therefore, we decided to apply this

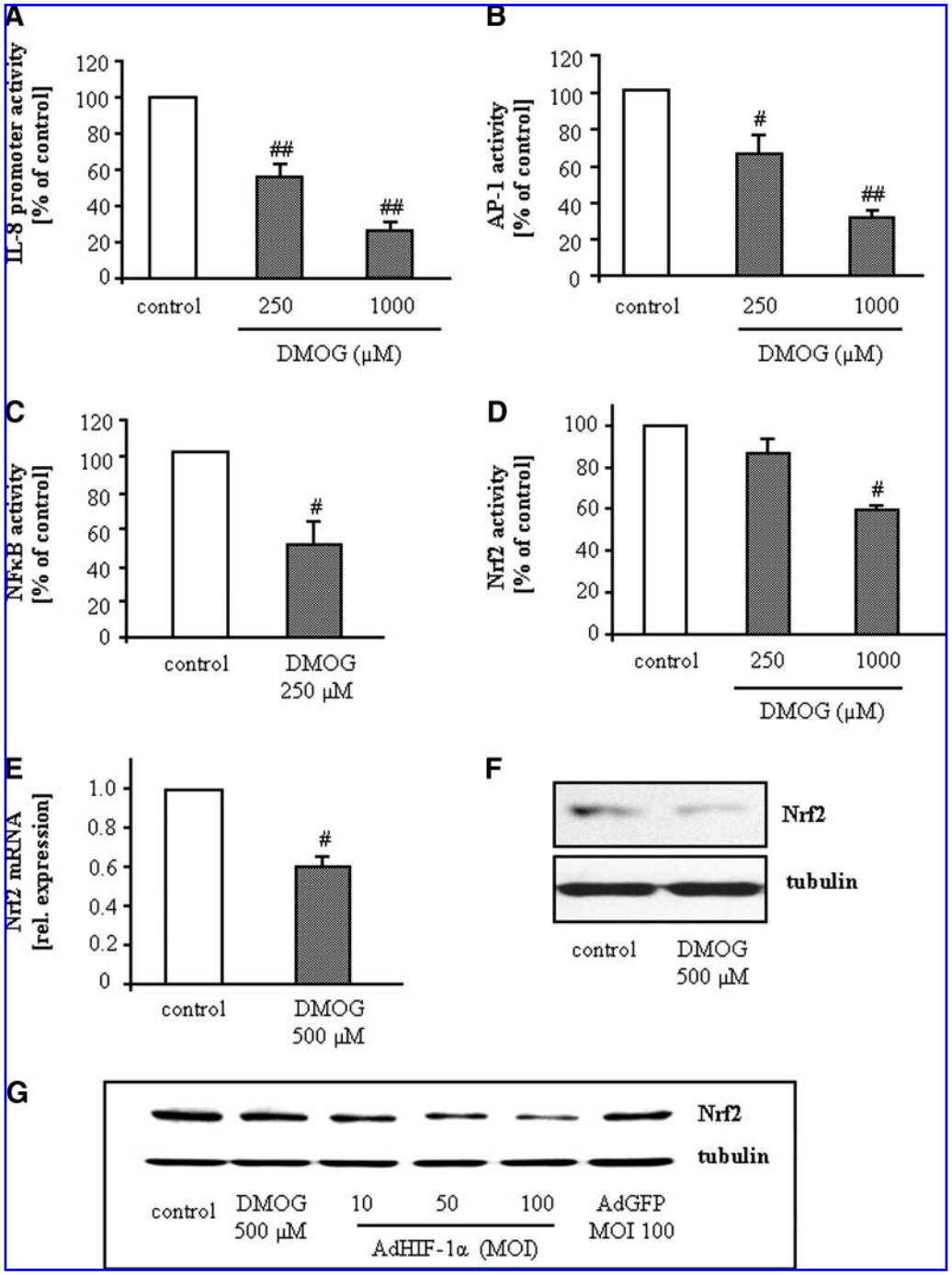


**FIG. 7. Effect of DMOG and HIF-1 overexpression on activity and expression of transcription factors.** DMOG decreased IL-8 promoter activity (**A**). This effect can be mediated by inhibition of various transcription factors, as AP-1 (**B**), NFκB (**C**), and Nrf2 (**D**) activities were downregulated, as evidenced in reporter gene assays. Additionally, DMOG treatment decreased Nrf2 mRNA (**E**) and protein (**F**) expression. The effect of DMOG is mimicked by HIF-1α overexpression (**G**). Gene transfer of a reporter gene (GFP) did not affect Nrf2 expression. Mean of three experiments (**A**–**E**), #$p < 0.05$ *vs.* control, ##$p < 0.01$ *vs.* control. Representative out of three (**F**) and two (**G**) Western blot results.

compound to check if it can restore the production of IL-8 attenuated by DMOG treatment. Indeed, chetomin reversed DMOG-mediated down-regulation of Nrf2 activity (Fig. 11A). Accordingly, chetomin reversed downregulation of IL-8 expression caused by DMOG (Fig. 11B) or hypoxia (Fig. 11C). It also induced HO-1 expression (Fig. 11D). The ability of chetomin to inhibit the binding of p300 to HIF-1 was corroborated by downregulation of DMOG-induced VEGF synthesis (Fig. 11E). However, the effect of this compound on IL-8 expression appears to be related to induction of Nrf2. Indeed, chetomin upregulated in a concentration-dependent manner Nrf2 activity, as evidenced by reporter gene assays (Fig. 11A) and the TransAM assay for Nrf2 (Fig. 11F). Importantly, in contrast to its effect on Nrf2, chetomin alone downregulated

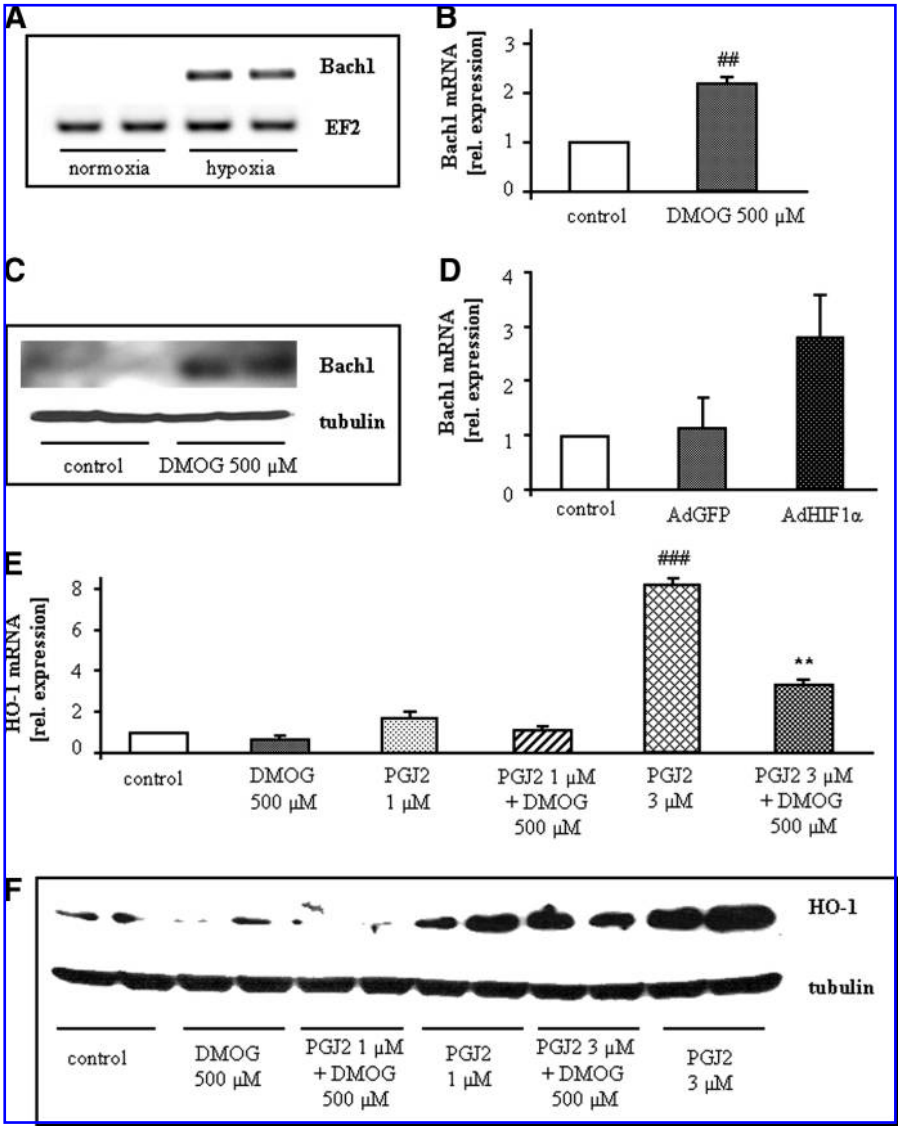


**FIG. 8. HIF-1 induction induces expression of Bach1 repressor and attenuates Nrf2-dependent HO-1 induction.** Hypoxia (**A**), DMOG (**B**, **C**) and overexpression of HIF-1α with 100 MOI AdHIF-1α (**D**) induce the expression of Bach1, a repressor of Nrf2 activity. Treatment with DMOG also attenuated the expression of HO-1 induced by PGJ2, a potent activator of the Nrf2 transcription factor, as evidenced at the mRNA (**E**) and protein (**F**) level. Mean of three (**B**) and two (**D**, **E**) independent real-time experiments performed in duplicates. ##$p < 0.05$ *vs.* control, ###$p < 0.01$ *vs.* control; **$p < 0.01$ *vs.* cells treated with 3 $\mu M$ PGJ2. Representative result from RT-PCR (**A**) and Western blot (**C**, **F**) analysis.

AP-1 and NFκB activity and further aggravated DMOG-induced inhibition of AP-1 and NFκB transcription factor activity (Fig. 12A and B). Taken together, these data demonstrate the dependence of IL-8 expression on Nrf2 transcription factor.

## Discussion

The salient finding of our study is the demonstration that activation of HIF-1 in endothelial cells decreases the production of IL-8 through inhibition of Nrf2 activity but in an HO-1 independent way. These data provide a strong mechanistic explanation demonstrating that regulation of this chemokine in endothelial cells by HIF-1 is different than another pro-angiogenic mediator, VEGF.

Induction of HIF-1 occurs when oxygen level decreases (36). However, as HIF-1α stabilization relies on oxygen, iron, and 2-oxoglutarate-dependent PHDs, it can be stabilized also by 2-oxoglutarate analogues, such as DMOG and NOG used in the present study. Here we have shown that activation of HIF-1 by hypoxia and DMOG are comparable and expression of VEGF is strongly upregulated by both hypoxia and DMOG. On the other hand, hypoxia was reported by others (22) and by our group (20) to decrease eNOS in human endothelial cells. Similarly, in the present study, DMOG has potently inhibited the eNOS mRNA level. These results suggest that DMOG can be used instead of hypoxia for HIF-1 activation.

Accordingly, to prove the role of HIF-1 we used RNA interference strategy and gene transfer of the stable form of HIF-1α. DMOG induced HIF-1α expression was down-regulated by siRNA, which resulted in restoration of IL-8 production. Moreover, adenoviral overexpression of HIF-1α significantly diminished IL-8 synthesis. Overall, the data in this study indicate that activation of HIF-1, while upregulating VEGF, decreases IL-8 synthesis in HMEC-1 cells.

Previously, in different cell types, DMOG has been reported to influence chemokines (*e.g.*, IL-8 and its ortholog in mice: KC) and HO-1 expression. Natarajan *et al.* (25) observed that HIF-1 induction increases HO-1 expression in mouse cardiomyocytes. Additionally, they also reported significant attenuation of TNF-induced KC expression. Similarly, Ockaili *et al.* (26) also observed that activation of HIF-1 attenuates IL-8

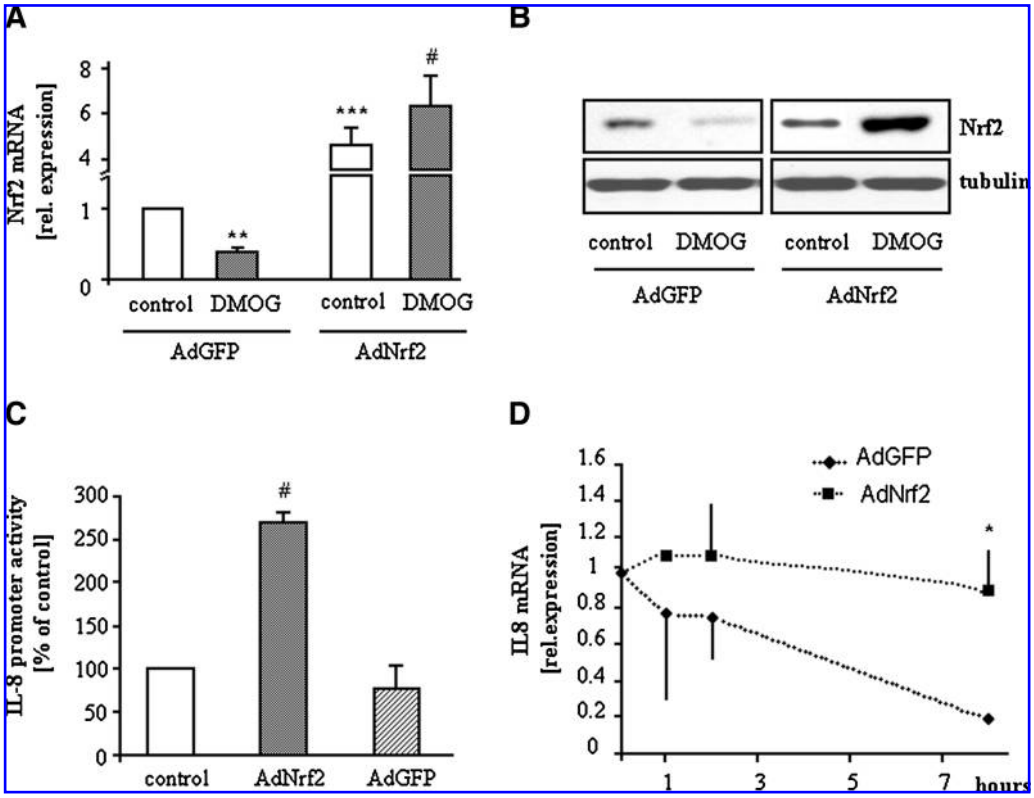


**FIG. 9. Overexpression of Nrf2 activates IL-8 promoter and enhances IL-8 mRNA stability.** After stimulation with 500 $\mu M$ DMOG real-time PCR (**A**) and western blot (**B**) for Nrf2 have been performed. DMOG decreased Nrf2 expression and protein synthesis in cells transduced with a control vector (AdGFP, 50 MOI). In contrast, after transduction with 50 MOI of AdNrf2, an increase in Nrf2 expression after DMOG delivery was observed (**A**, **B**). Overexpression of Nrf2 increased IL-8 promoter activity (**C**) and enhanced IL-8 mRNA stability (**D**), as evidenced by reporter gene assay (**C**) and real-time RT-PCR (**D**), respectively. Mean of three independent experiments performed in duplicates (**A**), two experiments done in triplicates (**C**), and one experiment in duplicates (**D**), #$p < 0.05$ *vs.* control in each group, *$p < 0.05$ *vs.* control in AdGFP group, **$p < 0.01$ *vs.* control in AdGFP group. Representative results from two Western blot analyses (**B**).

production in HMEC-1 cells but, in contrast to the present study, an induction of HO-1 expression after DMOG treatment was noted. Additionally, the authors suggested that increased HO-1 expression is responsible for the inhibition of IL-8 synthesis (26).

In the present study, we also observed downregulation of IL-8 production both after DMOG and another inhibitor of prolyl hydroxylases, NOG, in HMEC-1 cells. However, the induction of HO-1 by HIF-1 activation observed in previous studies (25, 26) stands in contrast to our results. In fact, as demonstrated by qualitative and quantitative RT-PCR, Western blot, and ELISA, the expression of HO-1 is concentration- and time-dependently attenuated by DMOG. Such an effect is also exerted by hypoxia (14) or HIF-1$\alpha$ overexpression (this study).

The discrepancies between our work and that of Natarajan *et al*. (25) can be easily explained. It is well known that the regulation of HO-1 expression by hypoxia is different in humans and rodents. This is due to action of Bach1, a hypoxia-induced repressor of Nrf2 and HO-1 in human cells that does not occur in murine cells (14, 24). Indeed, our data indicate that the expression of Bach1 increases after HIF-1 induction, after hypoxia, DMOG treatment or HIF-1$\alpha$ overexpression in HMEC-1 cells. Thus, results from mouse cardiomyocytes (25) and human microvascular endothelial cells (this study) can be different. Indeed, we also observed that in murine NIH3T3 fibroblasts DMOG enhances HO-1 expression. Thus, in hypoxic rodent cells, induction of HO-1 can occur.

However, Ockaili *et al*. (26) also showed that DMOG induced HO-1 expression in HMEC-1, the same cell line that we used. We do not have a reasonable explanation for such completely different results, as similar concentrations of DMOG were used in both studies. We can presume, however, that although cells were kept in the same type of medium, slight differences in culture conditions can somehow influence the pattern of gene expression.

Importantly, results concerning the effect of prolyl hydroxylases inhibitors on IL-8 and HO-1 expression from this paper are in agreement with our previous observations on the effect of hypoxia. Indeed, HMEC-1 cells cultured in 1% oxygen express less HO-1 (20) and produce less IL-8 (19) than cells growing in atmospheric conditions. Additionally, both hypoxia and DMOG attenuate IL-8 synthesis in HUVEC primary cells, suggesting that such a regulation is not only reserved to HMEC-1 cells.

Although Ockaili *et al.* (26) demonstrated that HO-1 induction attenuates the expression of IL-8, they did not investigate the effect of inhibitors of HO-1 activity. Therefore, in our experiments we used both a chemical inhibitor of HO activity, SnPPIX, and more specific siRNA targeting HO-1 to

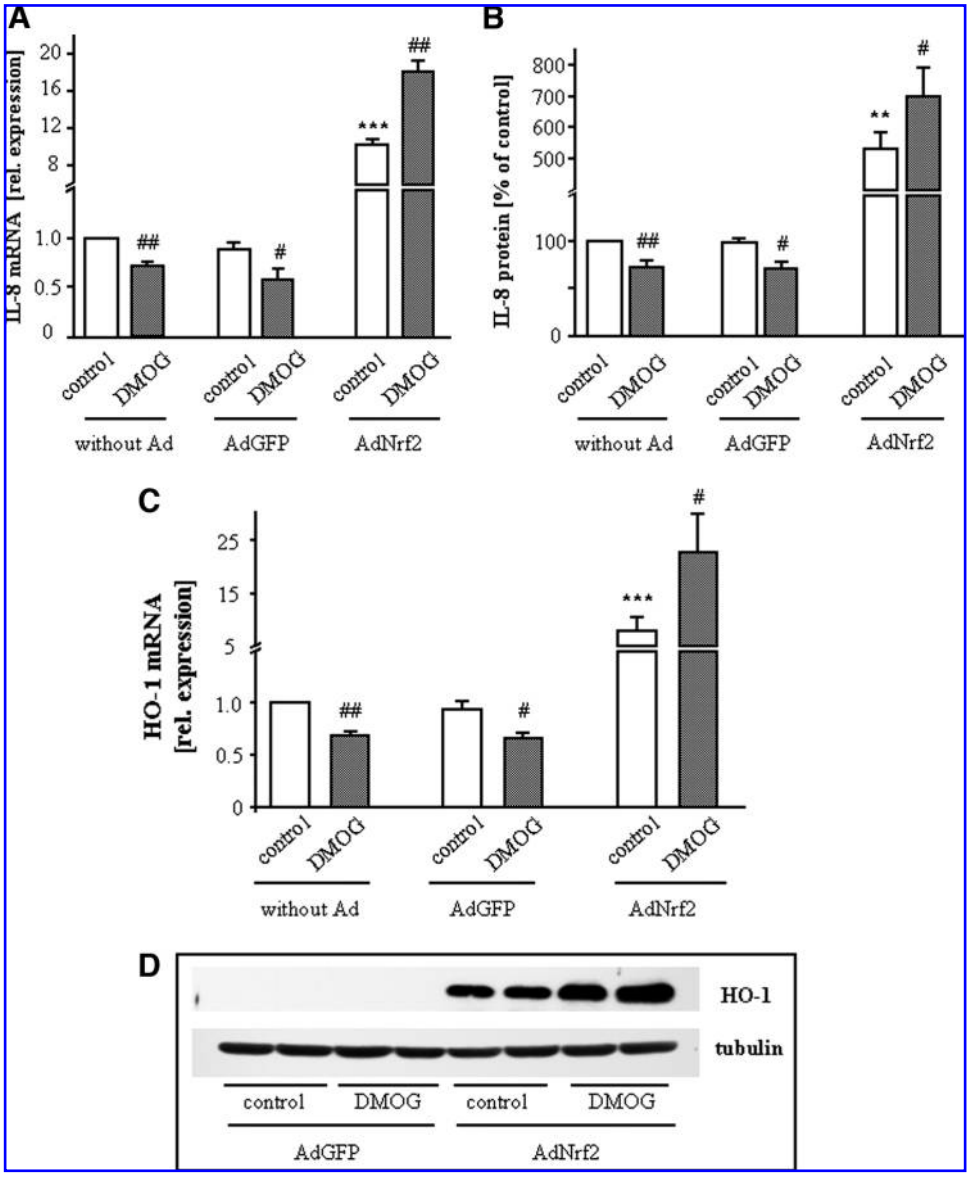


**FIG. 10. HIF-1-mediated inhibition of IL-8 and HO-1 expression is dependent on Nrf2 attenuation.** Cells were transduced with an adenoviral vector encoding GFP (AdGFP) or Nrf2 (AdNrf2) at 50 MOI or were not transduced (without Ad) and 24 h later treated with 500 $\mu M$ DMOG. After 24 h RNA was isolated and IL-8 (**A**) and HO-1 (**C**) mRNA was assessed by real-time RT-PCR. Cell-free supernatants were collected and assayed for IL-8 protein synthesis using ELISA (**B**). The protein level of HO-1 was evaluated by Western blot analysis. Because of the very strong induction of HO-1 expression, basal expression of HO-1 is not visible in the AdGFP transduced group (**D**). Overexpression of Nrf2 reversed the inhibitory effect of DMOG on IL-8 and HO-1 synthesis, indicating for the role of Nrf2 in HIF-1 mediated downregulation of IL-8. Mean of three independent experiments, #$p < 0.05$ *vs.* control in each group, ##$p < 0.01$ *vs.* control in non-transduced cells, ***$p < 0.01$ *vs.* control in non-transduced cells and AdGFP group. Representative results from Western blot analysis (**D**).

investigate the role of HO-1 in IL-8 expression. Our results clearly demonstrated that inhibition of HO-1 activity did affect neither the basal IL-8 production nor this downregulated by DMOG. Accordingly, transduction of HMEC-1 cells with an adenoviral vector containing HO-1 cDNA did not influence IL-8 synthesis. Thus, we can conclude that HO-1 is not involved in IL-8 regulation, at least in HMEC-1 cells in conditions described in the present study.

Of note, our other recent studies (11, 18) also corroborate that induction of HO-1 expression by HO-1 inducers does not affect the synthesis of IL-8. We have shown that cobalt protoporphyrin (CoPPIX), which is a potent inducer of HO-1, enhanced IL-8 expression in an HO-1-independent manner (18). Also, PGJ2, a potent inducer of HO-1, enhances IL-8 expression independently of HO-1 (11).

Nevertheless, attenuation of IL-8 expression by HIF-1 activation stands in contrast with previously published studies demonstrating the enhancement of IL-8 in hypoxia in endothelial (12) or human ovarian carcinoma (35) cells. At the moment we do not have a clear explanation for those difference and we can only suppose that the stimulatory effect of hypoxia on IL-8 described by some investigators (12, 35) may be caused by variations in hypoxic conditions, resulting in the activation of various transcription factors involved in the regulation of IL-8 expression. Further studies should also elucidate the role and extent of influence of HIF-2 on IL-8 regulation. It cannot be excluded that tumor cells can respond differently than normal cells and it might be possible that IL-8 is upregulated by hypoxia in some tumor cells (35). However, an article published by Mizukami *et al.* (23) clearly demonstrated that HIF-1 attenuates IL-8 production in hypoxia in various human tumor cell lines, what additionally support the presumptions that the inhibitory effect of HIF-1 on IL-8 may be a general phenomenon.

Importantly, knocking down of HIF-1 expression by HIF-1α siRNA reversed the inhibitory effect of DMOG on IL-8 expression, while HIF-1α overexpression decreased IL-8 synthesis. Reversely, DMOG and HIF-1α overexpression induced

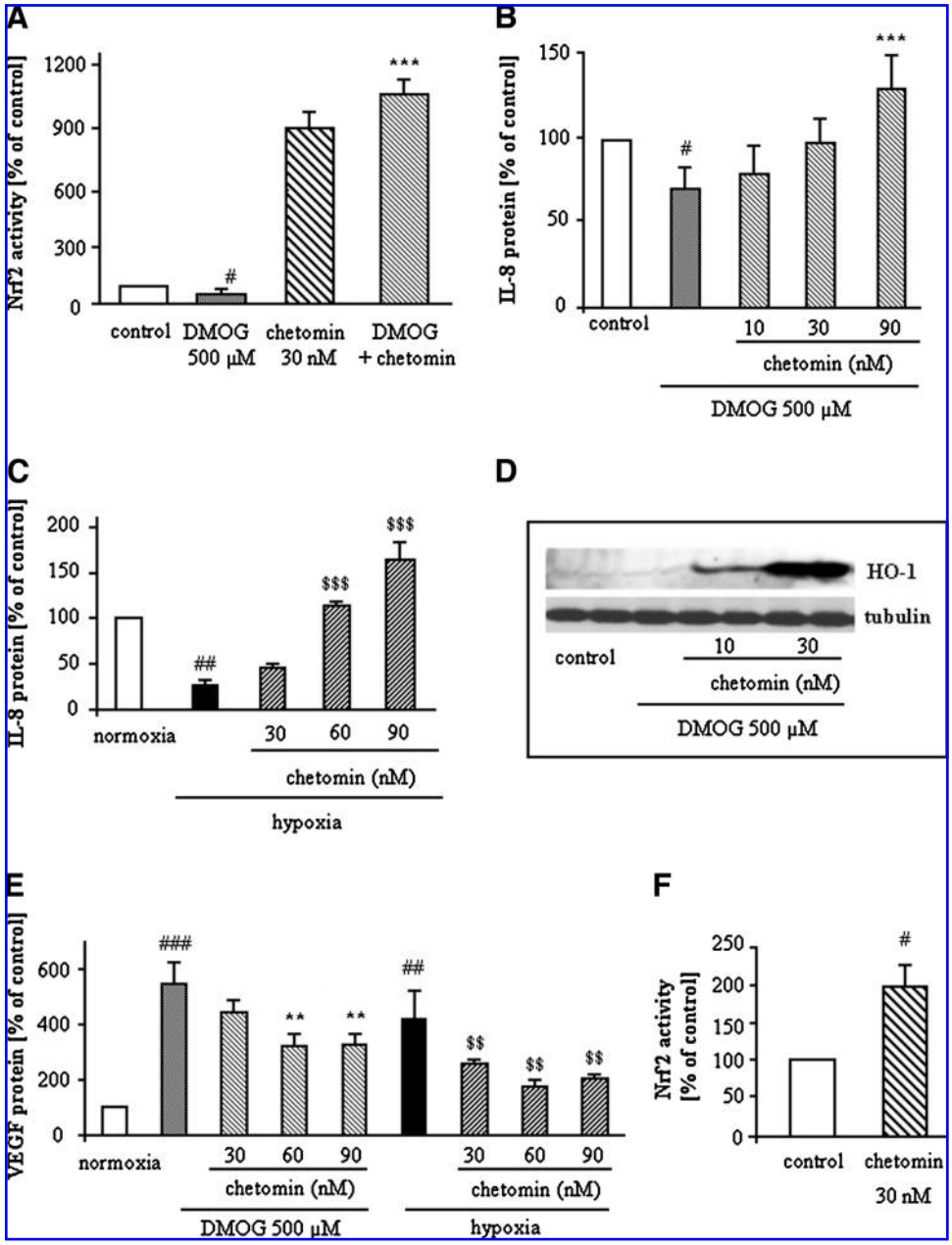


**FIG. 11. Chetomin, a blocker of p300 binding to HIF-1, activates Nrf2.** 24 h after transfection with the ARE-luc reporter plasmid (**A**), cells were stimulated with DMOG (500 $\mu M$) and 30 n$M$ chetomin and then luciferase activity was measured. Chetomin reverted the inhibitory effect of DMOG on Nrf2 activity, as it itself potently activated Nrf2 (**A**). Accordingly, chetomin reversed the inhibitory effect of DMOG (**B**) and hypoxia (**C**) on IL-8. Chetomin also strongly induced HO-1 expression (**D**). Inhibition of HIF-1 activity by chetomin resulted in downregulation of VEGF synthesis induced by DMOG or hypoxia (**E**). Activation of Nrf2 by chetomin has been confirmed in TransAM ELISA assay (**F**). Mean of three (**A**, **B**, **C**, **E** ) and representative out of two (**F**) independent experiments, $\#p < 0.05$ *vs.* control, $\#\#p < 0.01$ *vs.* normoxia (**C**, **E**); *vs.* control (**F**), $\#\#\#p < 0.001$ *vs.* normoxia, $^{**}p < 0.01$ *vs.* cells treated with DMOG, $^{***}p < 0.001$ *vs.* cells treated with DMOG, $\$\$p < 0.01$ *vs.* hypoxia, $\$\$\$p < 0.001$ *vs.* hypoxia. Representative results from Western blot analysis (**D**).

VEGF while siRNA against HIF-1$\alpha$ mRNA diminished VEGF synthesis induced by DMOG. This led us to the conclusion that DMOG acts specifically by activating HIF-1. Consequently, it can be suggested that stabilization of HIF1$\alpha$ is at least partially responsible for the observed hypoxic downregulation of IL-8.

In this study, we additionally observed that AP-1 and NF$\kappa$B activity was also downregulated by DMOG treatment. However, their effect on IL-8 expression under investigated conditions appears probably to be less important than Nrf2. This is evidenced by experiments, in which chetomin, a compound known to inhibit p300 binding to HIF-1$\alpha$ (15), was used to revert the effect of DMOG. Although such an activity of chetomin has been evidenced in downregulation of VEGF synthesis in hypoxia or after DMOG treatment, the main effect of chetomin on IL-8 synthesis seems to be related to induction of Nrf2. Importantly, although the activity of NF$\kappa$B or AP-1 was further attenuated by chetomin, as evidenced by reporter gene assay, this compound strongly induced IL-8 and HO-1 synthesis, indicating for the crucial role of Nrf2. Noteworthy, overexpression of Nrf2 using an adenoviral vector resulted in a reversal of DMOG's effect on both HO-1 and IL-8 synthesis. Thus, Nrf2 appears to be crucial for regulation of IL-8 expression.

The study also reveals the mechanism of HIF-1-dependent inhibition of Nrf2 activity and IL-8 or HO-1 expression. In all cases, when HIF-1 was induced (*i.e.*, in hypoxia), after DMOG treatment or in cells overexpressing a stable form of HIF-1$\alpha$, the induction of Bach1, a repressor of Nrf2 activity, was observed. Bach1 binds to ARE, similarly to Nrf2, but does not possess a transcriptional activation domain, hence it inhibits gene expression by blocking Nrf2 access (8).

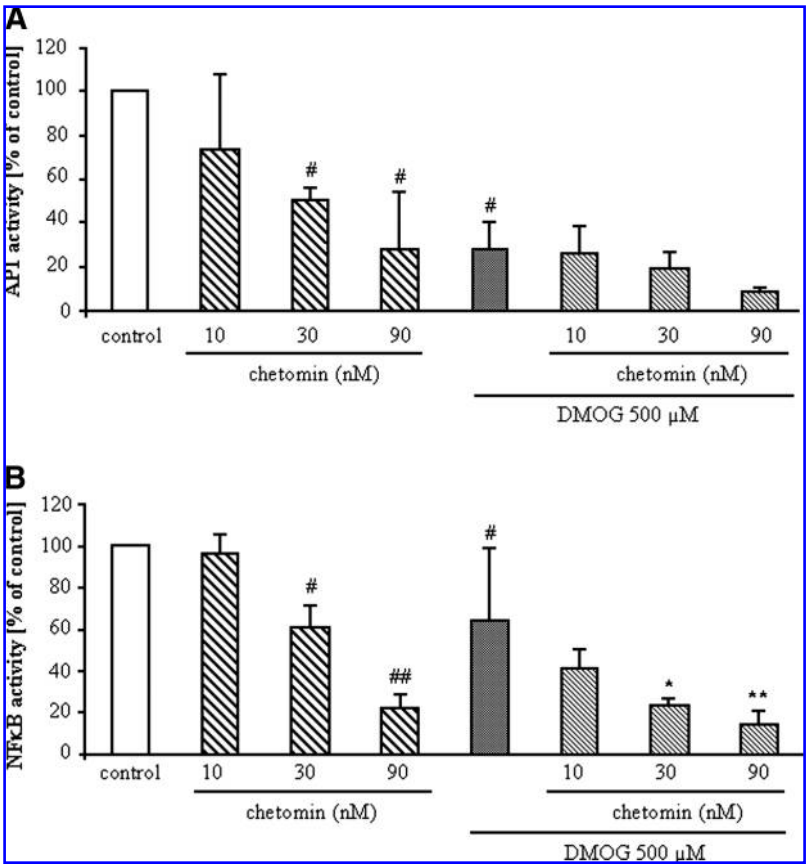


**FIG. 12. Chetomin inhibits activity of AP-1 and NFκB transcription factors and aggravates the inhibitory effect induced by DMOG.** 24 h after transfection with AP-1-SEAP (**A**) and NFκB-SEAP (**B**) reporter plasmids cells were stimulated with DMOG (500 $\mu M$) and chetomin at different concentrations and then alkaline phosphatase activity was measured. Mean of two independent experiments, #$p < 0.05$ *vs.* control, ##$p < 0.01$ *vs.* control, *$p < 0.05$ *vs.* cells treated with DMOG, **$p < 0.01$ *vs.* cells treated with DMOG.

Additionally, decreased expression of Nrf2 in cells in which HIF-1 is activated may add to the inhibitory effect observed. A similar mechanism may also operate in conditions in which IL-8 expression is induced by other Nrf2 activators (25).

Data presented here may have potential implications in clinics. Induction of angiogenesis is desirable in treating cardiovascular, cerebral, and peripheral ischemia, whereas strategies based on inhibition of pro-angiogenic factors expression are used mostly in anticancer therapies. As we and others (3, 23) have shown, inhibition of HIF-1 can lead to increase in IL-8 production. Consequently, anti-angiogenic therapy based on the inhibition of the HIF-1 pathway may decrease the expression of some genes (*e.g.*, VEGF). However it may lead to an increase in IL-8 and probably some other genes' expression, which can take over the function of angiogenic mediators in the absence of VEGF.

In summary, our data indicate that IL-8 expression is downregulated by the activation of HIF-1 in contrast to the effect exerted on VEGF, another potent angiogenic mediator. This effect on IL-8 is, however, independent of HO-1. The mechanism described here may have relevance for the effectiveness of anti-angiogenic therapies.

## Acknowledgments

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## Abbreviations

ARE, antioxidant response element; AdGFP, adenoviral vectors containing GFP cDNA; AdHIF-1α, adenoviral vectors containing HIF-1 α cDNA; AdHO-1, adenoviral vectors containing HO-1 cDNA; AdNrf2, adenoviral vectors containing Nrf2 cDNA; CoPPIX, cobalt protoporphyrin; DMOG, dimethyloxaloylglycine; eNOS, endothelial nitric oxide synthase; GFP, green fluorescent protein; HIF-1, hypoxia-inducible factor 1; HMEC-1, human microvascular endothelial cells; HO-1, heme oxygenase-1; HRE, hypoxia responsive element; HUVEC, human umbilical vein endothelial cells; IL-8, interleukin 8; NOG, N-oxalylglycine; Nrf2, NF-E2-related factor 2; PGJ2, prostaglandin J2; PHDs, proline hydroxylases; siRNA, small interfering RNA; SnPPIX, tin protopoporphyrin; VEGF, vascular endothelial growth factor.

## Author Disclosure Statement

No competing financial interests exist.

## References


1. Boesch–Saadatmandi C, Loboda A, Jozkowicz A, Huebbe P, Blank R, Wolffram S, Dulak J, and Rimbach G. Effect of ochratoxin A on redox-regulated transcription factors, antioxidant enzymes and glutathione-S-transferase in cultured kidney tubulus cells. *Food Chem Toxicol* 46: 2665–2671, 2008.
2. Dulak J, Loboda A, Jazwa A, and Jozkowicz A. Comment on "A novel role of hypoxia-inducible factor in cobalt chloride- and hypoxia-mediated expression of IL-8 chemokine in human endothelial cells". *J Immunol* 178: 4707; author reply 4707–4708, 2007.
3. Forooghian F and Das B. Anti-angiogenic effects of ribonucleic acid interference targeting vascular endothelial growth factor and hypoxia-inducible factor-1alpha. *Am J Ophthalmol* 144: 761–768, 2007.
4. Gong P, Stewart D, Hu B, Li N, Cook J, Nel A, and Alam J. Activation of the mouse heme oxygenase-1 gene by 15-deoxy-delta(12,14)-prostaglandin J(2) is mediated by the stress response elements and transcription factor Nrf2. *Antioxid Redox Signal* 4: 249–257, 2002.
5. Grundemar L and Ny L. Pitfalls using metalloporphyrins in carbon monoxide research. *Trends Pharmacol Sci* 18: 193–195, 1997.
6. Gu J, Milligan J, and Huang LE. Molecular mechanism of hypoxia-inducible factor 1alpha–p300 interaction. A leucine-rich interface regulated by a single cysteine. *J Biol Chem* 276: 3550–3554, 2001.
7. Harada A, Sekido N, Akahoshi T, Wada T, Mukaida N, and Matsushima K. Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J Leukoc Biol* 56: 559–564, 1994.
8. Igarashi K, Hoshino H, Muto A, Suwabe N, Nishikawa S, Nakauchi H, and Yamamoto M. Multivalent DNA binding complex generated by small Maf and Bach1 as a possible biochemical basis for beta-globin locus control region complex. *J Biol Chem* 273: 11783–11790, 1998.
9. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, and Ratcliffe PJ. Targeting of HIF-alpha to the von Hippel–Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science* 292: 468–472, 2001.
10. Jazwa A, Loboda A, Golda S, Cisowski J, Szelag M, Zagorska A, Sroczynska P, Drukala J, Jozkowicz A, and Dulak J. Effect of heme and heme oxygenase-1 on vascular endothelial growth factor synthesis and angiogenic potency of human keratinocytes. *Free Radic Biol Med* 40: 1250–1263, 2006.
11. Jozkowicz A, Dulak J, Prager M, Nanobashvili J, Nigisch A, Winter B, Weigel G, and Huk I. Prostaglandin-J2 induces synthesis of interleukin-8 by endothelial cells in a PPAR-gamma-independent manner. *Prostaglandins Other Lipid Mediat* 66: 165–177, 2001.
12. Karakurum M, Shreeniwas R, Chen J, Pinsky D, Yan SD, Anderson M, Sunouchi K, Major J, Hamilton T, Kuwabara K, and *et al.* Hypoxic induction of interleukin-8 gene expression in human endothelial cells. *J Clin Invest* 93: 1564–1570, 1994.
13. Kim KS, Rajagopal V, Gonsalves C, Johnson C, and Kalra VK. A novel role of hypoxia-inducible factor in cobalt chloride- and hypoxia-mediated expression of IL-8 chemokine in human endothelial cells. *J Immunol* 177: 7211–7224, 2006.
14. Kitamuro T, Takahashi K, Ogawa K, Udono-Fujimori R, Takeda K, Furuyama K, Nakayama M, Sun J, Fujita H, Hida W, Hattori T, Shirato K, Igarashi K, and Shibahara S. Bach1 functions as a hypoxia-inducible repressor for the heme oxygenase-1 gene in human cells. *J Biol Chem* 278: 9125–9133, 2003.
15. Kung AL, Zabludoff SD, France DS, Freedman SJ, Tanner EA, Vieira A, Cornell-Kennon S, Lee J, Wang B, Wang J, Memmert K, Naegeli HU, Petersen F, Eck MJ, Bair KW, Wood AW, and Livingston DM. Small molecule blockade of transcriptional coactivation of the hypoxia-inducible factor pathway. *Cancer Cell* 6: 33–43, 2004.
16. Levonen AL, Inkala M, Heikura T, Jauhiainen S, Jyrkkanen HK, Kansanen E, Maatta K, Romppanen E, Turunen P, Rutanen J, and Yla–Herttuala S. Nrf2 gene transfer induces antioxidant enzymes and suppresses smooth muscle cell growth in vitro and reduces oxidative stress in rabbit aorta in vivo. *Arterioscler Thromb Vasc Biol* 27: 741–747, 2007.
17. Levy AP, Levy NS, Wegner S, and Goldberg MA. Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. *J Biol Chem* 270: 13333–13340, 1995.
18. Loboda A, Jazwa A, Wegiel B, Jozkowicz A, and Dulak J. Heme oxygenase-1-dependent and -independent regulation of angiogenic genes expression: effect of cobalt protoporphyrin and cobalt chloride on VEGF and IL-8 synthesis in human microvascular endothelial cells. *Cell Mol Biol* 51: 347–355, 2005.
19. Loboda A, Jazwa A, Jozkowicz A, Molema G, and Dulak J. Angiogenic transcriptome of human microvascular endothelial cells: Effect of hypoxia, modulation by atorvastatin. *Vascul Pharmacol* 44: 206–214, 2006.
20. Loboda A, Jazwa A, Jozkowicz A, Dorosz J, Balla J, Molema G, and Dulak J. Atorvastatin prevents hypoxia-induced inhibition of endothelial nitric oxide synthase expression but does not affect heme oxygenase-1 in human microvascular endothelial cells. *Atherosclerosis* 187: 26–30, 2006.
21. Loboda A, Cisowski J, Zarebski A, Jazwa A, Riviera Nunez D, Kypriotakis Z, Heinrich M, and Dulak J. Effects of plant extracts on angiogenic activities of endothelial cells and keratinocytes. *J Physiol Pharmacol* 56 Suppl 1: 125–137, 2005.
22. McQuillan LP, Leung GK, Marsden PA, Kostyk SK, and Kourembanas S. Hypoxia inhibits expression of eNOS via transcriptional and posttranscriptional mechanisms. *Am J Physiol* 267: H1921–1927, 1994.
23. Mizukami Y, Jo WS, Duerr EM, Gala M, Li J, Zhang X, Zimmer MA, Iliopoulos O, Zukerberg LR, Kohgo Y, Lynch MP, Rueda BR, and Chung DC. Induction of interleukin-8 preserves the angiogenic response in HIF-1alpha-deficient colon cancer cells. *Nat Med* 11: 992–997, 2005.
24. Nakayama M, Takahashi K, Kitamuro T, Yasumoto K, Katayose D, Shirato K, Fujii–Kuriyama Y, and Shibahara S. Repression of heme oxygenase-1 by hypoxia in vascular endothelial cells. *Biochem Biophys Res Commun* 271: 665–671, 2000.
25. Natarajan R, Salloum FN, Fisher BJ, Ownby ED, Kukreja RC, and Fowler AA. Activation of hypoxia inducible factor-1 via prolyl-4 hydoxylase-2 gene silencing attenuates acute inflammatory responses in post-ischemic myocardium. *Am J Physiol Heart Circ Physiol* 293: H1571–1580, 2007.
26. Ockaili R, Natarajan R, Salloum F, Fisher BJ, Jones D, Fowler AA, 3rd, and Kukreja RC. HIF-1 activation attenuates post-ischemic myocardial injury: Role for heme oxygenase-1 in modulating microvascular chemokine generation. *Am J Physiol Heart Circ Physiol* 289: H542–548, 2005.
27. Otterbein LE, Soares MP, Yamashita K, and Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol* 24: 449–455, 2003.

28. Oyake T, Itoh K, Motohashi H, Hayashi N, Hoshino H, Nishizawa M, Yamamoto M, and Igarashi K. Bach proteins belong to a novel family of BTB-basic leucine zipper transcription factors that interact with MafK and regulate transcription through the NF-E2 site. *Mol Cell Biol* 16: 6083–6095, 1996.
29. Pae HO, Oh GS, Choi BM, Kim YM, and Chung HT. A molecular cascade showing nitric oxide-heme oxygenase-1-vascular endothelial growth factor-interleukin-8 sequence in human endothelial cells. *Endocrinology* 146: 2229–2238, 2005.
30. Pajusola K, Kunnapuu J, Vuorikoski S, Soronen J, Andre H, Pereira T, Korpisalo P, Yla-Herttuala S, Poellinger L, and Alitalo K. Stabilized HIF-1alpha is superior to VEGF for angiogenesis in skeletal muscle via adeno-associated virus gene transfer. *FASEB J* 19: 1365–1367, 2005.
31. Schofield CJ and Ratcliffe PJ. Signalling hypoxia by HIF hydroxylases. *Biochem Biophys Res Commun* 338: 617–626, 2005.
32. Semenza GL. Hydroxylation of HIF-1: Oxygen sensing at the molecular level. *Physiology* 19: 176–182, 2004.
33. Xie K. Interleukin-8 and human cancer biology. *Cytokine Growth Factor Rev* 12: 375–391, 2001.
34. Yoshida A, Yoshida S, Khalil AK, Ishibashi T, and Inomata H. Role of NF-kappaB-mediated interleukin-8 expression in intraocular neovascularization. *Invest Ophthalmol Vis Sci* 39: 1097–1106, 1998.
35. Yoshino O, Osuga Y, Koga K, Hirota Y, Yano T, Tsutsumi O, Fujimoto A, Kugu K, Momoeda M, Fujiwara T, and Taketani Y. Upregulation of interleukin-8 by hypoxia in human ovaries. *Am J Reprod Immunol* 50: 286–290, 2003.
36. Zagorska A and Dulak J. HIF-1: The knowns and unknowns of hypoxia sensing. *Acta Biochim Pol* 51: 563–585, 2004.
37. Zhang X, Chen X, Song H, Chen HZ, and Rovin BH. Activation of the Nrf2/antioxidant response pathway increases IL-8 expression. *Eur J Immunol* 35: 3258–3267, 2005.

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1. S Famulla, A Horrighs, A Cramer, H Sell, J Eckel. 2012. Hypoxia reduces the response of human adipocytes towards TNF# resulting in reduced NF-#B signaling and MCP-1 secretion. *International Journal of Obesity* **36**:7, 986-992. [CrossRef]
2. Agnieszka Loboda, Alicja Jozkowicz, Jozef Dulak. 2012. HIF-1 versus HIF-2 — Is one more important than the other?. *Vascular Pharmacology* **56**:5-6, 245-251. [CrossRef]
3. M. N. Valcarcel-Ares, T. Gautam, J. P. Warrington, L. Bailey-Downs, D. Sosnowska, R. de Cabo, G. Losonczy, W. E. Sonntag, Z. Ungvari, A. Csiszar. 2012. Disruption of Nrf2 Signaling Impairs Angiogenic Capacity of Endothelial Cells: Implications for Microvascular Aging. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* . [CrossRef]
4. Jun-Lae Cho, Munif Allanson, Vivienne E. Reeve. 2012. Hypoxia inducible factor-1# contributes to UV radiation-induced inflammation, epidermal hyperplasia and immunosuppression in mice. *Photochemical & Photobiological Sciences* . [CrossRef]
5. Thomas Haarmann-Stemmann, Josef AbelInfluence of HIF1# and NRF2 Signaling on AHR-Mediated Gene Expression, Toxicity, and Biological Functions 109-126. [CrossRef]
6. H. Agis, G. Watzek, R. Gruber. 2011. Prolyl hydroxylase inhibitors increase the production of vascular endothelial growth factor by periodontal fibroblasts. *Journal of Periodontal Research* no-no. [CrossRef]
7. Urszula Florczyk, Szymon Czauderna, Anna Stachurska, Magdalena Tertil, Witold Nowak, Magdalena Kozakowska, Lorenz Poellinger, Alicja Jozkowicz, Agnieszka Loboda, Jozef Dulak. 2011. Opposite effects of HIF-1# and HIF-2# on the regulation of IL-8 expression in endothelial cells. *Free Radical Biology and Medicine* . [CrossRef]
8. Kyril Turpaev, Mikhail Ermolenko, Thierry Cresteil, Jean Claude Drapier. 2011. Benzylidenemalononitrile compounds as activators of cell resistance to oxidative stress and modulators of multiple signaling pathways. A structure–activity relationship study. *Biochemical Pharmacology* . [CrossRef]
9. Peter Dorfmuller, Marie-Camille Chaumais, Maria Giannakouli, Ingrid Durand-Gasselin, Nicolas Raymond, Elie Fadel, Olaf Mercier, Frederic Charlotte, David Montani, Gerald Simonneau, Marc Humbert, Frederic Perros. 2011. Increased oxidative stress and severe arterial remodeling induced by permanent high-flow challenge in experimental pulmonary hypertension. *Respiratory Research* **12**:1, 119. [CrossRef]
10. Sang Hun Lee, Han Na Suh, Yu Jin Lee, Bit Na Seo, Jeong Won Ha, Ho Jae Han. 2011. Midkine prevented hypoxic injury of mouse embryonic stem cells through activation of Akt and HIF-1# via low-density lipoprotein receptor-related protein-1. *Journal of Cellular Physiology* n/a-n/a. [CrossRef]
11. Agnieszka Loboda, Alicja Jozkowicz, Jozef Dulak. 2010. HIF-1 and HIF-2 transcription factors — Similar but not identical. *Molecules and Cells* **29**:5, 435-442. [CrossRef]
12. Agnieszka Loboda, Anna Stachurska, Jerzy Dorosz, Marek Zurawski, Joanna Wegrzyn, Magdalena Kozakowska, Alicja Jozkowicz, Jozef Dulak. 2009. HIF-1 attenuates Ref-1 expression in endothelial cells: Reversal by siRNA and inhibition of geranylgeranylation. *Vascular Pharmacology* **51**:2-3, 133-139. [CrossRef]