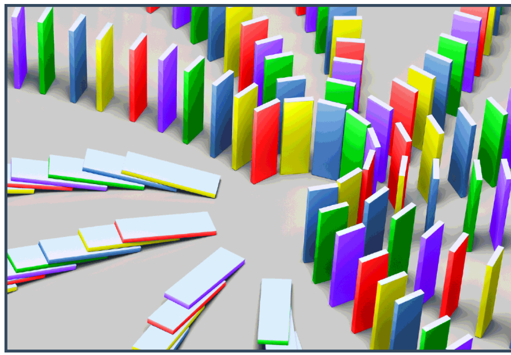
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Heme Oxygenase-1 Modulates the Expression of Adhesion Molecules Associated with Endothelial Cell Activation[1]

Miguel P. Soares,[2]*† Mark P. Seldon,† Isabel Pombo Gregoire,† Tatiana Vassilevskaia,† Pascal O. Berberat,* Jia Yu,* Tung-Yu Tsui,[3]* and Fritz H. Bach*

Heme oxygenase-1 (HO-1) cleaves the porphyrin ring of heme into carbon monoxide, Fe^{2+}, and biliverdin, which is then converted into bilirubin. Heme-derived Fe^{2+} induces the expression of the iron-sequestering protein ferritin and activates the ATPase Fe^{2+}-secreting pump, which decrease intracellular free Fe^{2+} content. Based on the antioxidant effect of bilirubin and that of decreased free cellular Fe^{2+}, we questioned whether HO-1 would modulate the expression of proinflammatory genes associated with endothelial cell (EC) activation. We tested this hypothesis specifically for the genes *E-selectin* (*CD62*), *ICAM-1* (*CD54*), and *VCAM-1* (*CD106*). We found that HO-1 overexpression in EC inhibited TNF-α-mediated E-selectin and VCAM-1, but not ICAM-1 expression, as tested at the RNA and protein level. Heme-driven HO-1 expression had similar effects to those of overexpressed HO-1. In addition, HO-1 inhibited the activation of NF-κB, a transcription factor required for TNF-α-mediated up-regulation of these genes in EC. Bilirubin and/or Fe^{2+} chelation mimicked the effects of HO-1, whereas biliverdin or carbon monoxide did not. In conclusion, HO-1 inhibits the expression of proinflammatory genes associated with EC activation via a mechanism that is associated with the inhibition of NF-κB activation. This effect of HO-1 is mediated by bilirubin and/or by a decrease of free intracellular Fe^{2+} but probably not by biliverdin or carbon monoxide. *The Journal of Immunology,* **2004, 172: 3553–3563.**

Endothelial cells (EC)[4] play a critical role in coordinating leukocyte trafficking to specific tissues that regulate their activation, differentiation, and survival (1, 2). When confronted by an inflammatory stimulus, e.g., bacterial LPS (3), TNF-α (4), or IL-1αβ (5), EC modify their phenotype and become activated (reviewed in Ref. 6), promoting adhesion, activation, and transmigration of circulating leukocytes (reviewed in Refs. 1 and 7). This is accomplished primarily via the up-regulation of a series of proinflammatory genes that promote leukocyte adhesion activation and transmigration. These include the adhesion molecules E-selectin (CD62E) (8), P-selectin (CD62P) (9), ICAM-1 (CD54) (3), and VCAM-1 (CD106) (10). The expression of these genes is regulated primarily at the transcriptional level through a mechanism that requires the activation of the transcription factor NF-κB (11, 12).

To avoid unfettered leukocyte activation and transmigration in a manner that would cause tissue injury, the expression of proinflammatory genes associated with EC activation must be strictly controlled (13). One of the mechanisms by which this occurs relies on the expression by activated EC of anti-inflammatory genes. We refer to these genes as protective genes based on their dual ability to inhibit the expression of proinflammatory genes associated with EC activation and to protect EC from undergoing apoptosis (13). We have hypothesized that the stress-responsive gene heme oxygenase-1 (HO-1) (hmox-1/hsp-32) acts in such a manner.

We have previously shown that HO-1 protects EC from undergoing apoptosis (14). We now tested whether HO-1 would also inhibit the expression of proinflammatory genes associated with EC activation. Such an effect would be consistent with the potent anti-inflammatory and cytoprotective effects attributed to the expression of HO-1 in an increased number of experimental models of acute and/or chronic inflammation in vivo.

Under inflammatory conditions, HO-1 becomes the rate-limiting enzyme in the catabolism of heme into biliverdin, free Fe^{2+}, and CO (15) (reviewed in Ref. 16). Biliverdin is subsequently catabolized into bilirubin by biliverdin reductase (17) (reviewed in Ref. 18). Free Fe^{2+} up-regulates the expression of ferritin, an Fe^{2+}-sequestering protein (19, 20) and activates an ATPase iron pump which also decreases the level of intracellular Fe^{2+} (21, 22). Expression of HO-1 has potent anti-inflammatory (23) and antithrombotic (24) effects in monocyte/macrophages as well as antiapoptotic effects in EC (25). These are exerted, not only via heme degradation, but also through the generation of bilirubin (26), CO (23–25), and ferritin (19), as well as the activation of ferroportin (21).

We demonstrate in this study that expression of HO-1 in cultured EC inhibits the ability of proinflammatory cytokines such as TNF-α or IL-1β to induce the expression of adhesion molecules associated with EC activation. This effect can be mimicked by

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[4] Abbreviations used in this paper: EC, endothelial cell; HO-1, heme oxygenase-1; BAEC, bovine aortic EC; PAEC, porcine aortic EC; DFO, desferoxamine mesylate; SIH, salicylaldehyde isonicotinoyl hydrazone; RT, room temperature; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; β-gal, β-galactosidase.

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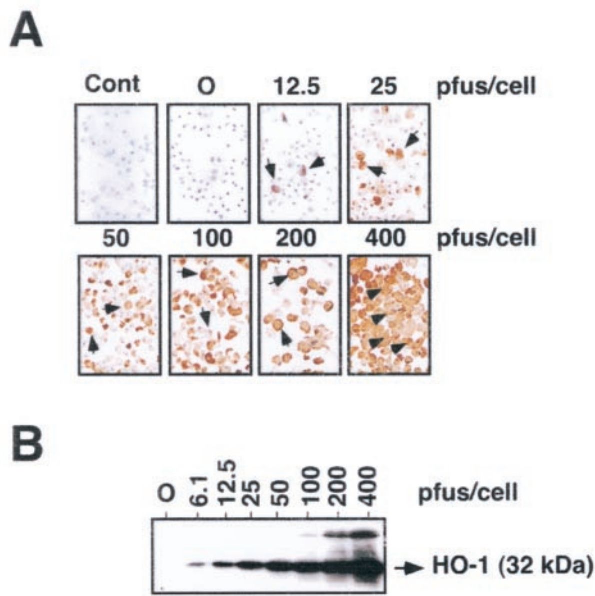

FIGURE 1. Expression of HO-1 recombinant adenovirus in cultured EC. *A*, PAEC were transduced with increasing PFU of the HO-1 recombinant adenovirus, as described in *Materials and Methods*. Control EC (Cont) were transduced with 400 PFU of β-gal recombinant adenovirus. HO-1 protein was detected 24–48 h after transduction, as described in *Materials and Methods*. Black arrows indicate positive staining. *B*, PAEC were transduced as in *A*, and HO-1 was detected by Western blot, as described in *Materials and Methods*.

bilirubin or depletion of free Fe^{2+}. The likely mechanism by which these effects occur involves the inhibition of NF-κB activation, a transcription factor strictly required for the transcription of these proinflammatory genes.

Materials and Methods

Cell culture

Primary bovine aortic EC (BAEC) and porcine aortic EC (PAEC) were isolated and cultured as described before (11). Alternatively, BAEC were purchased from Cell Systems Corporation (Kirkland, WA) and cultured in MCDB-131 (Life Technologies, Paisley, U.K.) supplemented with 10% heat-inactivated FCS (Life Technologies), 10 mM L-glutamine (Life Technologies), 50 U/ml penicillin (Life Technologies), and 50 μg/ml streptomycin (Life Technologies). When required, fungizone (2.5 μg/ml) was added (Life Technologies). Pooled HUVEC (Clonetics, Zurich, Switzerland) were cultured in EBM-2 medium (Clonetics), supplemented with 1% heparin, 0.04% hydrocortisone, 50 μg/ml gentamicin sulfate/amphotericin, 0.1 ng/ml ascorbic acid, 0.5% human fibroblast growth factor B, 0.1% human recombinant endothelial growth factor (vascular), 0.1% human recombinant epidermal growth factor, and 0.1% insulin-like growth factor (Clonetics).

Reagents

Human rTNF-α, IL-1β (R&D Systems, Minneapolis, MN), or bacterial LPS (*Escherichia coli* serotype 0127:B8; Sigma-Aldrich, St. Louis, MO) were used to induce the expression of proinflammatory genes in cultured EC (6–8 h for protein expression; 2 h for mRNA expression). Desferoxamine mesylate (DFO; 50–500 μM; Sigma-Aldrich) was used to chelate iron (incubation for 16 h before exposure to TNF-α). DFO was prepared at a stock solution of 100 mM in sterile distilled water and kept frozen at −20°C until used. Alternatively, iron chelation was achieved using salicylaldehyde isonicotinoyl hydrazone (SIH; 100 μM) (27) (a kind gift from Dr. P. Ponka (McGill University, Montreal, Quebec, Canada)). SIH was prepared and kept at 4°C until used, as described elsewhere (27). Hemin (Frontier Scientific, Logan, UT) was dissolved in 0.2 N NaOH, neutralized to pH of 7.4 using 1 N HCl, and stored at −20°C until used. Hemin was used to induce HO-1 expression in cultured EC as described by Balla et al. (19). Briefly, cultured EC were washed once in HBSS containing Ca^{2+} and Mg^{2+} (Life Technologies) and exposed (1 h) to hemin (5, 10, or 25 μM) in HBSS. EC were then cultured overnight in EBM-2 and supplemented as described above. The HO-1 inhibitor tin protoporphyrin IX (Frontier Scientific) was prepared and used in a similar manner. Biliverdin hydrochloride (ICN Biomedicals, Aurora, OH) was diluted in 25% DMSO at a stock solution of 2.5 mM and kept frozen until used. Bilirubin (Frontier Scientific) was dissolved in 0.2 N NaOH, neutralized to pH 7.4 using 1 N HCl, and used fresh.

Recombinant adenoviruses

The recombinant β-galactosidase (β-gal) adenovirus was a kind gift from Dr. R. Gerard (University of Texas Southwest Medical Center, Dallas, TX). The recombinant adenovirus expressing the rat HO-1 cDNA has been described elsewhere (28). Recombinant adenoviruses were produced in human embryonic kidney 293 cells (American Type Culture Collection, Manassas, VA), extracted, and purified through two cesium chloride gradient ultracentrifugations, and their titer was determined by limiting dilution in human embryonic kidney 293 cells as described before (12). Confluent HUVEC, BAEC, and PAEC were infected with a multiplicity of infection of 100, 200, and 400 PFU/cell, respectively, as described elsewhere (11).

Flow cytometry

Transduced or nontransduced confluent HUVEC in six-well plates were treated with recombinant human TNF-α (10 ng/ml; 6 h; R&D Systems) and harvested by trypsin digestion (100 μl/well; 0.05% in PBS). Cells were collected and washed in PBS (pH 7.2; 5% FCS and 0.01% NaN_3; 4°C) and incubated (1×10^2 cells in 100 μl; 30 min; 4°C) with mouse anti-human E-selectin (clone BBIG-E6; 13D5), ICAM-1 (clone BBIG-1; 11C81), or VCAM-1 (clone BBIG-V1; 4B2) mAbs (R&D Systems), 2 μg/ml in PBS, 5% FBS, and 0.1% NaN_3. Mouse anti-human TNF-R1 (clone 16803) and TNF-R2 (clone TR75-89) mAbs (R&D Systems) were used at a concentration of 20 μg/ml (60 min; 4°C). After washing in PBS, 5% FBS, and 0.01% NaN_3 (300 × *g*; 5 min), cells were stained (30 min; 4°C) with an FITC-labeled polyclonal goat anti-mouse IgG Ab (Sigma-Aldrich). Fluorescent labeling was evaluated using a FACSort equipped with CellQuest software (BD Biosciences, Palo Alto, CA). Specific labeling was compared with nonspecific staining using FITC-labeled isotype-matched control

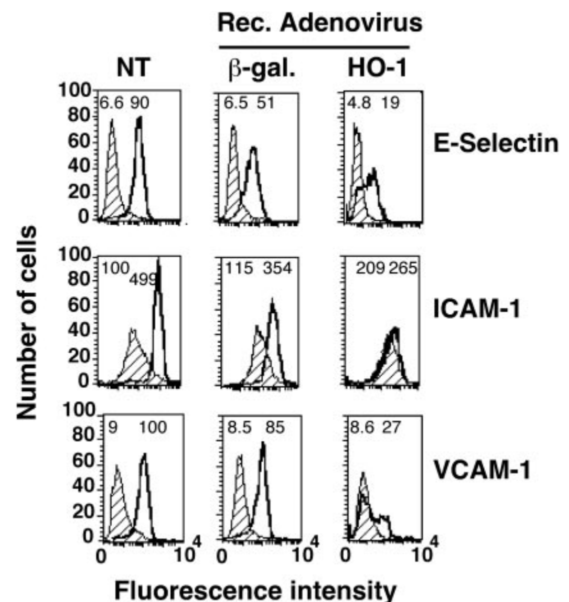

FIGURE 2. HO-1 inhibits TNF-α-mediated E-selectin and VCAM-1 expression at the surface of EC. HUVEC were either not transduced (NT) or transduced with β-gal or HO-1 recombinant adenoviruses and exposed 24 h after to human rTNF-α (10 ng/ml; 6 h). Expression of cell surface E-selectin, ICAM-1, and VCAM-1 was analyzed by flow cytometry, as described in *Materials and Methods*. Hatched histograms indicate EC not exposed to TNF-α, and linear histograms indicate EC exposed to TNF-α. Numbers in each panel indicate the mean fluorescent intensity for each specific staining. Results shown are representative of three independent experiments.

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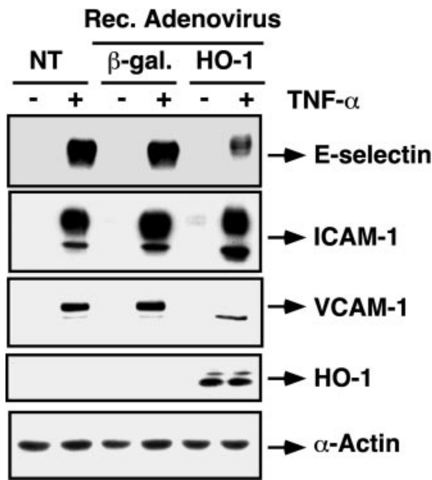

FIGURE 3. HO-1 inhibits TNF-α-mediated E-selectin and VCAM-1 protein expression. HUVEC were not transduced (NT), or transduced with β-gal or HO-1 recombinant adenoviruses and exposed (+) or not (−) to human rTNF-α (10 ng/ml; for 6 h). Expression of E-selectin, ICAM-1, VCAM-1, HO-1, and α-actin was detected by Western blot, as described in *Materials and Methods*. Results shown are representative of three independent experiments.

mAb. Modulation of the expression of the different molecules analyzed was assessed, comparing the expression on TNF-α-treated vs untreated cells.

Cellular ELISA

HUVEC were cultured in 0.2% bovine gelatin (Life Technologies)-coated 96-well culture plates (Life Technologies) in culture medium, as described above. One or 2 days postconfluence, cells were stimulated with human rTNF-α (10 ng/ml; 6 h), washed twice in PBS/0.05% Tween 20 (Sigma-Aldrich), and fixed in 0.01% glutaraldehyde (Sigma-Aldrich) in PBS/0.05% Tween 20 (30 min; 4°C). Nonspecific binding sites were saturated with 5% skimmed milk (Primor, Lisbon, Portugal) in PBS/0.05% Tween 20 (60 min; room temperature (RT)). Cells were washed twice in PBS/0.05% Tween 20 and incubated (1–2 h; 37°C) in the presence of anti-E-selectin (R&D Systems), ICAM-1 (R&D Systems), or VCAM-1 (R&D Systems) mAbs (2 μg/ml; 1–2 h; RT) in PBS/5% skimmed milk/0.05% Tween 20 (Sigma-Aldrich). In some experiments, VCAM-1 was detected using a goat anti-human VCAM-1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were washed twice in PBS/0.05% Tween 20, and primary Abs were detected using HRP-labeled goat anti-mouse Fab′ (0.1 μg/ml; 1 h; RT) (Pierce, Rockford, IL). HRP was revealed using orthophenyldiamine (Sigma-Aldrich) and H_2O_2 (0.03%) in citrate buffer (pH 4.9). Absorbance was measured at $\lambda = 490$ nm. The relative level of expression of the different molecules detected was expressed as OD units ($\lambda = 490$).

Cell extracts and Western blot analysis

Cell extracts were prepared and subjected to electrophoresis as described elsewhere (11). HO-1 was detected using a rabbit anti-human HO-1 polyclonal Ab (StressGen Biotechnologies, Victoria, BC, Canada). For the detection of E-selectin, ICAM-1, and VCAM-1, protein extracts were not subjected to 2-ME reduction. E-selectin was detected using the mAb h4/18 (kind gift from M. Gimbrone (Brigham and Women's Hospital, Boston, MA)). ICAM-1 and VCAM-1 were detected using rabbit anti-human ICAM-1 and VCAM-1 Abs (Santa Cruz Biotechnology), respectively. β-Tubulin was detected using anti-human β-tubulin mAb (Boehringer Mannheim, Indianapolis, IN). Total and activated/phosphorylated forms of extracellular signal-regulated kinases (ERK-1 and -2), c-Jun N-terminal kinases (JNK-1, -2, and 3), and p38 mitogen-activated protein kinases (MAPK) were detected using rabbit polyclonal Abs directed against the total or phosphorylated forms of these MAPK, according to the manufacturer's suggestions (Cell Signaling, Beverly, MA). Primary Abs were detected using HRP-conjugated donkey anti-rabbit or goat anti-mouse or rabbit IgG secondary Abs (Pierce). Peroxidase activity was visualized using the ECL assay (Amersham, Arlington Heights, IL), according to the manufacturer's instructions and stored in the form of photoradiographs (Biomax MS; Eastman Kodak, Rochester, NY). When indicated, membranes were stripped (62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-ME; 30 min; 50°C). Phosphorylated ERK, JNK, and p38 were normalized to the total amount of total ERK, JNK, and p38, detected in the same membrane.

Cytotoxicity assays

HUVEC were exposed to hemin, as described above (19), collected by trypsin digestion (Invitrogen, San Diego, CA) and resuspended in PBS, 3% FCS, and 10 μg/ml propidium iodide (15 min; RT) (Sigma-Aldrich). Cells were analyzed for viability by flow cytometry (1×10^4 cells/sample) using a FACScan cytometer (BD Biosciences) equipped with CellQuest software (BD Biosciences). For positive controls, HUVEC were exposed to 5% Triton X-100 (Sigma-Aldrich) following trypsinization.

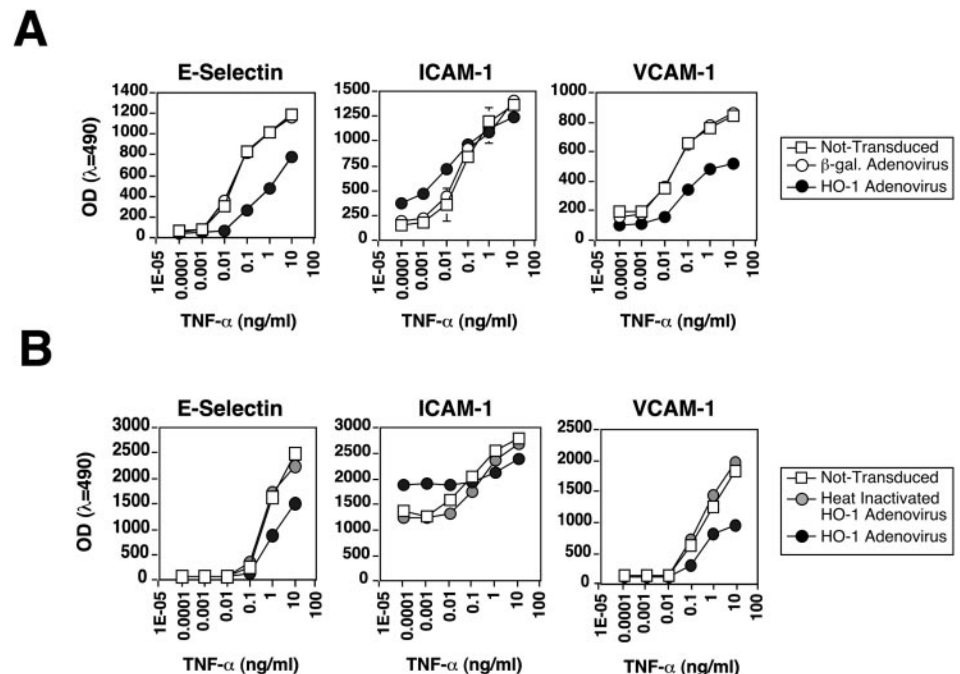

FIGURE 4. Dose-response effect of HO-1 on the level of expression of E-selectin, ICAM-1, and VCAM-1 proteins. Results shown are the mean ± SD of three independent wells. *A*, HUVEC were either not transduced (□), or transduced with β-gal (○) or HO-1 (●) recombinant adenoviruses and exposed to increasing concentrations of human rTNF-α (6 h). Expression of E-selectin, ICAM-1, and VCAM-1 was detected by ELISA, as described in *Materials and Methods*. The experiment shown is representative of three independent experiments. *B*, HUVEC were not transduced (□), or transduced with HO-1 (●) or heat-inactivated HO-1 (◍) recombinant adenovirus and exposed to increasing concentrations of human rTNF-α (6 h).

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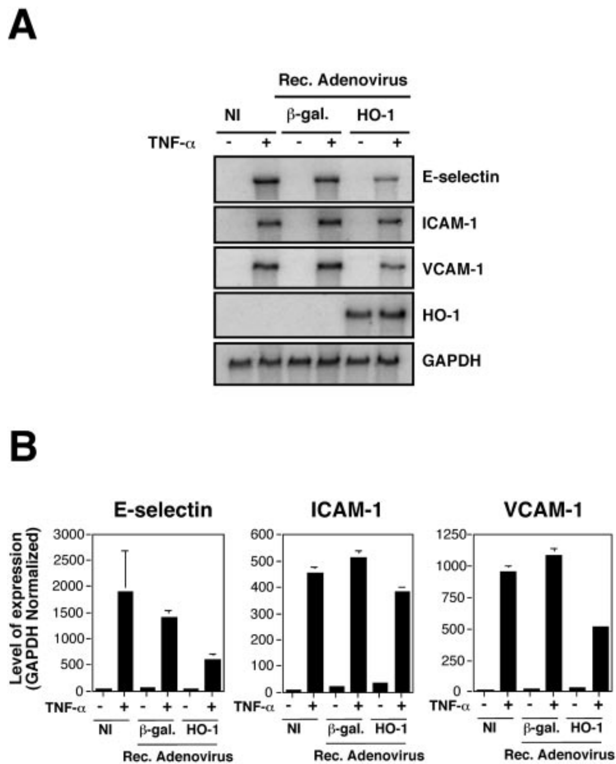

FIGURE 5. Effect of HO-1 on the expression of E-selectin, ICAM-1, and VCAM-1 mRNAs. *A*, Not transduced (NT), β-gal-transduced, or HO-1 recombinant adenovirus-transduced HUVEC were exposed (+) or not (−) to human rTNF-α (10 ng/ml; for 6 h). Expression of E-selectin, ICAM-1, VCAM-1, HO-1, and GAPDH mRNAs was detected by Northern blot, as described in *Materials and Methods*. Results shown are representative of three independent experiments. *B*, The level of RNA encoding each protein was quantified by phosphor imager analyses and normalized to the level of expression of GAPDH. Results from two independent experiments were plotted and are shown as mean ± SD.

Fluorescent microscopy

HUVEC were cultured on gelatinized glass slides (PerkinElmer, Wellesley, MA), fixed in 100% acetone (Sigma-Aldrich), and stained with an anti-HO-1 polyclonal Ab (2 μg/ml; SPA-896; StressGen Biotechnologies). Primary Ab was detected using Alexa Fluor 594-labeled goat anti-rabbit Ab (Molecular Probes, Eugene, OR). Fluorescent labeling was detected (λ_{ex} = 590 nm; λ_{em} = 617 nm) using a DMR2 microscope (Leica, Deerfield, IL) equipped with Metamorph3 software (Universal Imaging, Downingtown, PA).

Northern blot

HUVEC were treated with TNF-α (2 h), and RNA was extracted using TRIzol, according to the manufacturer's suggestions (Life Technologies). Total RNA was separated on a 1.3% agarose formaldehyde gel, transferred overnight to Hybond-N nylon membranes (Amersham), and analyzed by specific hybridization to radiolabeled cDNA probes for human E-selectin (kind gift from Dr. T. Collins (Children's Hospital, Boston, MA)), ICAM-1 (kind gift from Dr. T. Collins), VCAM-1 (kind gift from Dr. T. Collins), and rat HO-1 as described before (11). All probes were labeled with [α-^{32}P]dATP (Amersham) using a random primer labeling kit (Stratagene, La Jolla, CA). Membranes were probed for GAPDH to control for equal RNA loading. Digital images were obtained using a PhosphorImager (Storm Imager; Molecular Dynamics, Sunnyvale, CA). Intensity of [α-^{32}P]dATP labeling was quantified using ImageQuant software (Amersham).

CO exposure

Briefly, CO at a concentration of 1% (10,000 ppm) in compressed air plus 5% CO_2 was mixed with balanced air (21% O_2 plus 5% CO_2) in a stainless-steel mixing cylinder before entering the exposure chamber. CO concentrations were controlled by varying the flow rates of CO in a mixing cylinder before delivery to the chamber. Because the flow rate is primarily determined by the O_2 flow, only the CO flow was changed to deliver the final concentration to the exposure chamber. A CO analyzer (Interscan, Chatsworth, CA) was used to measure CO levels in the chamber. Cells were exposed to CO 16 h before stimulation with TNF-α and continuously thereafter.

Transient transfections and reporter assays

BAEC were seeded at 200 × 10^3 cells per well in six-well plates and transiently transfected 24 h after using Lipofectamine 2000 (Life Technologies). Briefly, Lipofectamine 2000 (2 μl) was incubated (20 min; RT) with purified plasmid DNA (3 μg) in MCDB-131 (200 μl). Cells were washed in MCDB-131, and BAEC were exposed to DNA/Lipofectamine (2 h) in a final volume of 1 ml of MCDB-131. DNA/Lipofectamine was removed, and cells were cultured for an additional 48 h in MCDB-131, 10% heat-inactivated FCS, 10 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Life Technologies). Cells were exposed to human rTNF-α (10 ng/ml; 8 h), and cell extracts were obtained using the Promega (Madison, WI) cell culture lysis reagent extraction kit, according to the manufacturer's instructions. Cell extracts were assayed for β-gal (Galacto-Light; Applied Biosystems, Tropix, Bedford, MA) and luciferase activity (luciferase assay system; Promega), according to the manufacturer's instructions. Luciferase and β-gal activities were measured using a Micro-Lumat Plus luminometer (LB96V; Berthold, Bad Wildbad, Germany). Luciferase activity was normalized for β-gal as follows: luciferase activity/β-gal activity × 100. Normalized luciferase activity is shown in arbitrary units.

Expression plasmids

The PSV-β-gal cDNA was driven by the SV40 early promoter and enhancer (E1081; Promega). The rat HO-1 cDNA was driven by the CMV enhancer/promoter (pcDNA3/HO-1) (29). The NF-κB luciferase reporter has been described elsewhere (30).

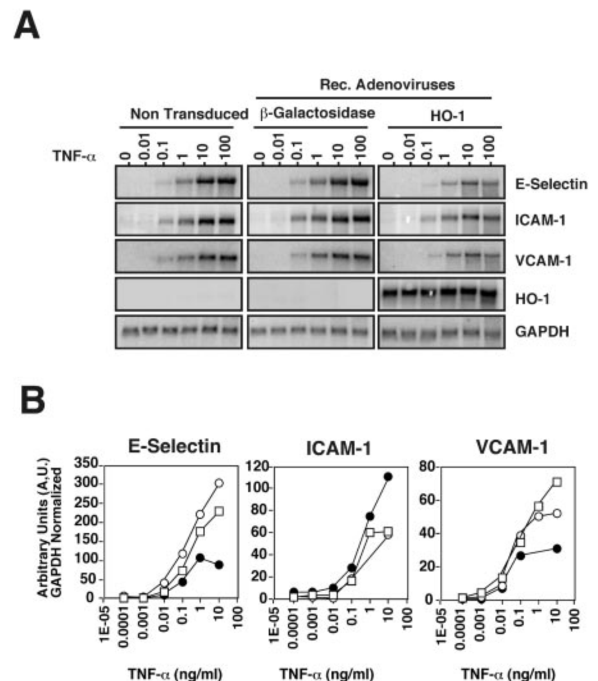

FIGURE 6. Dose-response analyses of the effect of HO-1 on E-selectin, ICAM-1, and VCAM-1 mRNA expression. *A*, Nontransduced, β-gal-transduced, or HO-1 recombinant adenovirus-transduced HUVEC were exposed to increasing concentrations of human rTNF-α (2 h), as described in *Materials and Methods*. Expression of E-selectin, ICAM-1, VCAM-1, HO-1, and GAPDH mRNAs was detected by Northern blot, as described in *Materials and Methods*. *B*, The level of RNA was quantified by phosphor imager analyses and normalized to the level of expression of the housekeeping gene *GAPDH*, as described in *Materials and Methods*. Not transduced (○), β-gal-transduced (□), or HO-1 (●) recombinant adenovirus-transduced HUVEC are shown.

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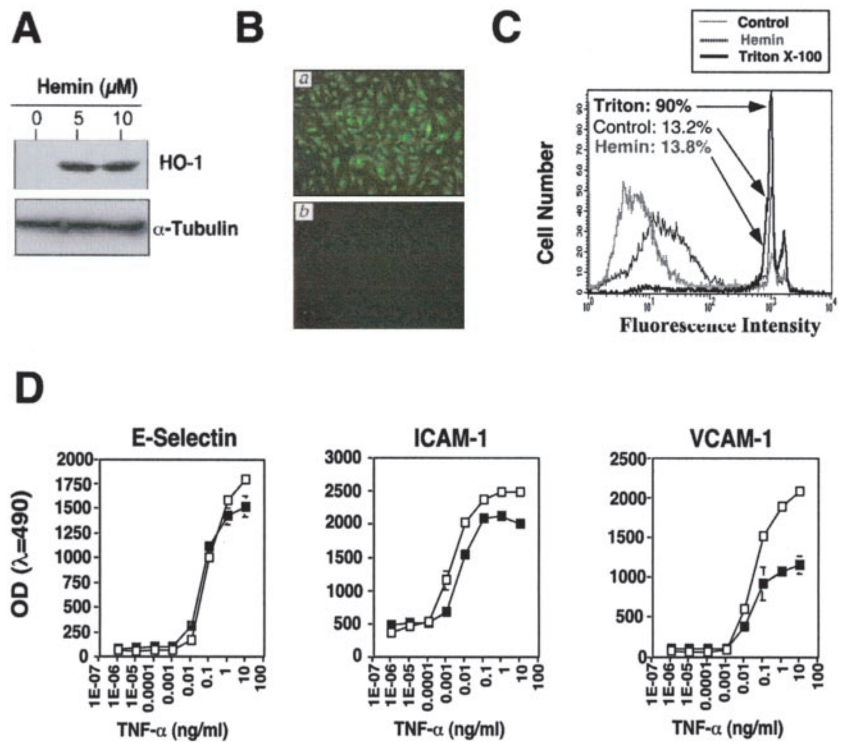

FIGURE 7. Endogenous HO-1 expression modulates the level E-selectin, ICAM-1, and VCAM-1 expression. *A*, BAEC were not treated (□) or exposed to hemin, and HO-1 as well as α-tubulin expression was detected by Western blot, as described in *Materials and Methods*. *B*, HUVEC were either treated with hemin (10 μM) (*a*) or not treated (*b*) as described in *A*. HO-1 expression was detected by fluorescent microscopy, as described in *Materials and Methods*. *C*, HUVEC were either treated with hemin (10 μM) (Hemin; gray histogram) or not treated (Control; thin histogram) as described in *A*. Cyotoxicity was detected by propidium iodide staining, as described in *Materials and Methods*. Positive control was conducted using 5% Triton X-100, as described in *Materials and Methods*. *D*, HUVEC were either not treated (□) or treated with hemin (25 μM; 16 h; ●) and then exposed to increasing concentrations of human rTNF-α, as described in *Materials and Methods*. Expression of E-selectin, ICAM-1, and VCAM-1 was analyzed by ELISA, as described in *Materials and Methods*. Results shown are the mean ± SD of three independent wells and are representative of three independent experiments.

Results

Recombinant adenovirus-mediated HO-1 overexpression in EC inhibits E-selectin and VCAM-1, but not ICAM-1 expression

To assess the effect of HO-1 overexpression on the up-regulation of proinflammatory genes associated with EC activation, we have used a HO-1 recombinant adenovirus, allowing to achieve overexpression of HO-1 in virtually 100% of cultured EC. Maximal transduction efficiency was observed between 100 and 400 PFU of recombinant adenovirus per cell, i.e., 100 PFU for HUVEC, 200 PFU for BAEC (data not shown), or 400 PFU for PAEC (Fig. 1*A*). Expression of HO-1 was confirmed by Western blot 24 h after transduction (Fig. 1*B*). Expression of β-gal recombinant adenovirus did not induce HO-1 expression (Fig. 1).

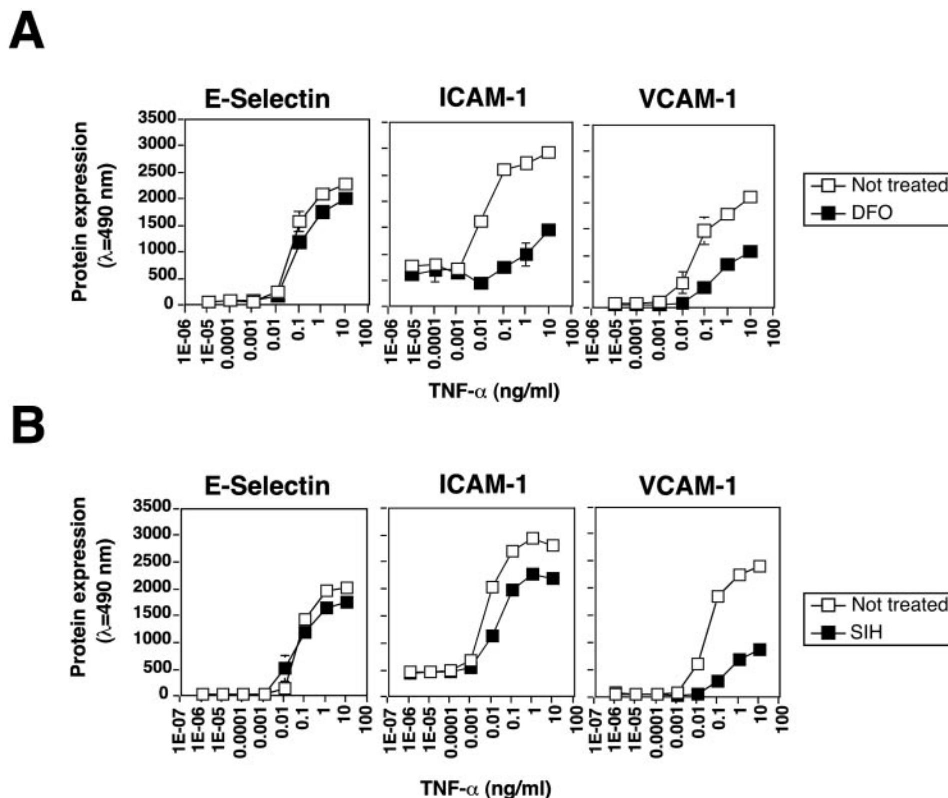

FIGURE 8. Effect of iron chelation on E-selectin, ICAM-1, and VCAM-1 protein expression. *A*, HUVEC were either not treated (□) or treated with the iron chelator DFO (500 μM; 16 h) (■) before being exposed to increasing concentrations of human rTNF-α (6 h). Expression of E-selectin, ICAM-1, and VCAM-1 was detected by ELISA, as described in *Materials and Methods*. Results shown are the mean ± SD of three independent wells. The experiment shown is representative of three independent experiments. *B*, HUVEC were cultured as in *A* and were either not treated (□) or treated with the iron chelator SIH (100 μM; 16 h) (■). The rest of the procedure was conducted as in *A*. Results shown are the mean ± SD of three independent wells.

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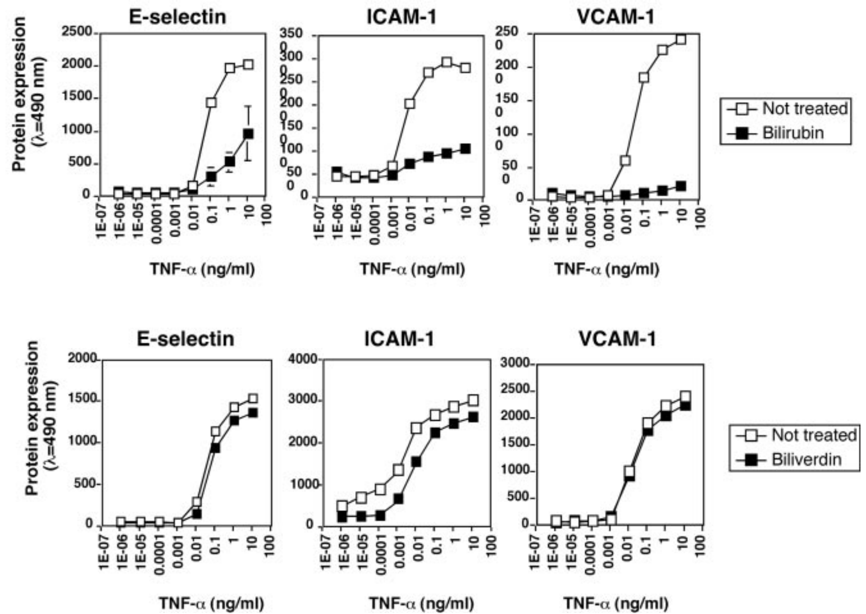

FIGURE 9. Effect of bilirubin and biliverdin E-selectin, ICAM-1, and VCAM-1 protein expression. *A*, HUVEC were either not treated (□) or treated with bilirubin (5 μM; 16 h) (squlf]) before being exposed to increasing concentrations of human rTNF-α (6 h). Expression of E-selectin, ICAM-1, and VCAM-1 was detected by ELISA, as described in *Materials and Methods*. Results shown are the mean ± SD of three independent wells. The experiment shown is representative of at least three independent experiments. *B*, HUVEC were cultured as in *A* and were either not treated (□) or treated with biliverdin (5 μM; 16 h) (■). The rest of the procedure was conducted as in *A*. Results shown are the mean ± SD of three independent wells.

Nontransduced EC expressed undetectable levels E-selectin as well as VCAM-1 at the cell surface, as assessed by flow cytometry (Fig. 2). Expression was significantly increased upon TNF-α stimulation in nontransduced or β-gal recombinant adenovirus-transduced EC (Fig. 2). VCAM-1 expression was significantly inhibited in EC transduced with a HO-1 recombinant adenovirus, compared with nontransduced or β-gal-transduced EC (Fig. 2). Similar results were observed when the expression of these proteins was detected by Western blot (Fig. 3).

Nontransduced or β-gal-transduced EC expressed basal levels of ICAM-1 at the cell surface, which were significantly increased when EC were exposed to TNF-α (Fig. 2). ICAM-1 expression was significantly increased in HO-1, but not in β-gal recombinant adenovirus-transduced EC compared with nontransduced controls (Fig. 2). TNF-α did not further increase ICAM-1 expression in HO-1-transduced EC (Fig. 2). Similar results were observed when ICAM-1 expression was detected by Western blot except that the basal level of ICAM-1 expression was somehow not detectable by Western blot (Fig. 3).

In nontransduced or β-gal-transduced EC, the level of expression of these adhesion molecules increased in a dose-dependent manner when the cells were exposed to TNF-α, i.e., the higher the TNF-α concentration, the higher the level of expression (Fig. 4). HO-1-transduced EC required a 10- to 100-fold higher concentration of TNF-α to induce a similar level of E-selectin or VCAM-1 expression, compared with nontransduced or β-gal-transduced EC (Fig. 4*A*). Heat inactivation of the HO-1 recombinant adenovirus blunted its inhibitory effect (Fig. 4*B*), excluding the possibility that the inhibitory effect of this recombinant adenovirus was related to contamination by endotoxin.

Consistent with the above data examining protein expression, EC transduced with the HO-1 recombinant adenovirus also showed significant inhibition of TNF-α-mediated E-selectin and VCAM-1 mRNA expression, compared with nontransduced or β-gal-transduced EC (Fig. 5). Expression of HO-1 had no significant effect on ICAM-1 mRNA expression (Fig. 5). EC that overexpressed HO-1 required 10–100× higher concentrations of TNF-α to achieve a similar level of E-selectin and VCAM-1

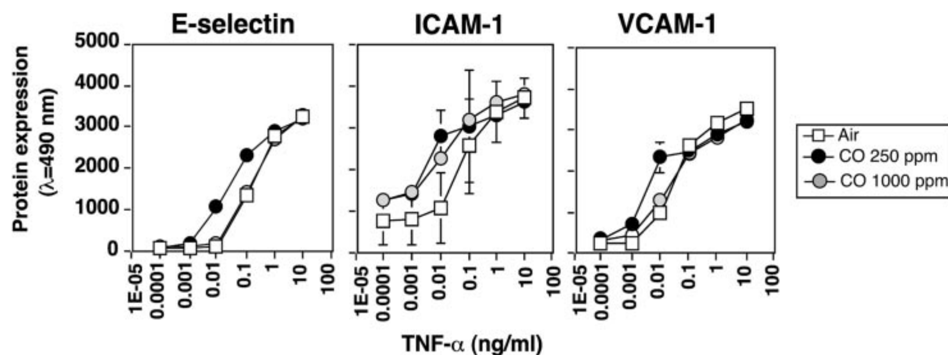

FIGURE 10. Effect of CO on E-selectin, ICAM-1, and VCAM-1 protein expression. HUVEC were exposed to air plus 5% CO_2 (□), air plus 5% CO_2 plus 250 ppm CO (●), or air plus 5% CO_2 plus 1000 ppm CO (◍) (16 h) before exposure to increasing concentrations of human rTNF-α (6 h). Expression of E-selectin, ICAM-1, and VCAM-1 was detected by ELISA, as described in *Materials and Methods*. Results shown are the mean ± SD of three independent wells. The experiment shown is representative of at least three independent experiments.

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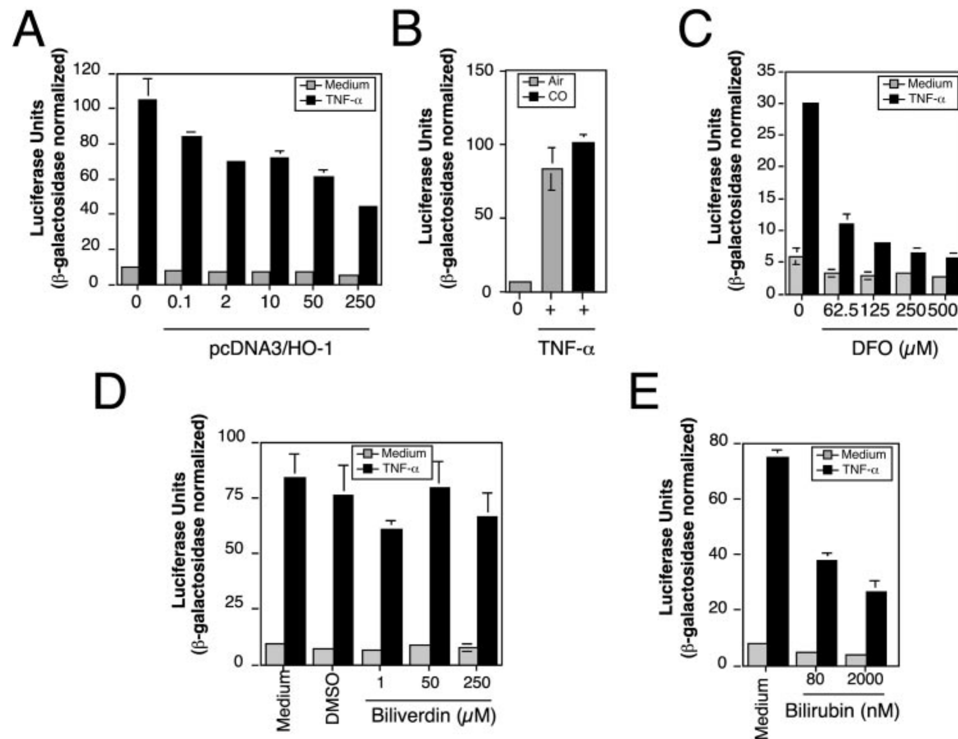

FIGURE 11. Effect of HO-1 and the end products of its action on heme on NF-κB activation. BAEC were transiently transfected with PSV-β-gal and NF-κB-luciferase reporters, as described in *Materials and Methods*. When indicated, EC were exposed to TNF-α (10 ng/ml; 8 h) 48 h after transfection. The data illustrated represent the mean ± SD of luciferase units normalized for the expression of β-gal, in three independent readings from one of five representative experiments. *A*, BAEC were transiently cotransfected with increasing amounts of a HO-1 cDNA expression plasmid (pcDNA3/HO-1) (nanograms per 300 × 10^3 cells). Cells were either not treated (▨) or exposed to TNF-α (■). *B*, BAEC were exposed to air (air; 5% CO_2) (▨) or CO (air; 5% CO_2; 10,000 ppm CO) (■) 32 h after transfection. *C*, BAEC were exposed to increasing concentrations of the iron chelator DFO 32 h after transfection. Cells were either not treated (▨) or exposed to TNF-α (■). *D*, BAEC were exposed to increasing concentrations of biliverdin 32 h after transfection. Cells were either not treated (▨) or exposed to TNF-α (■). *E*, BAEC were exposed to increasing concentrations of bilirubin 32 h after transfection. Cells were either not treated (▨) or exposed to TNF-α (■).

mRNA expression, compared with nontransduced or β-gal-transduced EC (Fig. 6).

Heme-induced HO-1 expression inhibits TNF-α-mediated up-regulation of VCAM-1

Exposure of EC to hemin (5 or 10 μM) induced high levels of HO-1 expression, as detected by Western blot or immunocytochemistry (Fig. 7, *A* and *B*) (19). HO-1 expression was noticed in the cytoplasm as well as in the nuclei, as revealed by immunocytochemistry (Fig. 7*B*). Exposure of EC to hemin (5–10 μM) did not result in detectable cytoxicity (Fig. 7*C*), a result consistent with those of others (19). TNF-α-mediated expression of VCAM-1 was significantly inhibited in EC exposed to hemin, compared with control-treated EC (Fig. 7*D*). Expression of E-selectin and ICAM-1 were not inhibited to the same extent of VCAM-1 (Fig. 7*D*). Similar results were obtained in EC exposed to cobalt protoporphyrin IX, a synthetic protoporphyrin that also induces the expression of HO-1 (data not shown). Tin protoporphyrin IX, a synthetic protoporphyrin that blocks HO-1 activity, did not inhibit VCAM-1 expression (data not shown).

Iron chelation inhibits E-selectin, ICAM-1, and VCAM-1 expression

Expression of HO-1 results in the up-regulation of ferritin (19) (data not shown) and is coupled to the activation of an ATPase iron pump (21, 22). Given that ferritin and an ATPase iron pump act to reduce the level of intracellular Fe^{2+}, we asked whether reduction of the reactive pool of cellular Fe^{2+} would mimic the effect of HO-1. Iron chelation by DFO or the permeable iron chelator SIH significantly inhibited E-selectin as well as VCAM-1 expression in a similar manner to HO-1 (Fig. 8). DFO and, to a lesser extent, SIH also inhibited ICAM-1 expression (Fig. 8).

Bilirubin inhibits E-selectin, ICAM-1, and VCAM-1 expression

We asked whether biliverdin and/or bilirubin could account for the ability of HO-1 to modulate the expression of E-selectin, and/or VCAM-1 in EC. Bilirubin inhibited the expression of E-selectin and VCAM-1 in a manner that was similar to that of HO-1 (Fig. 9*A*). Bilirubin also inhibited the expression of ICAM-1 (Fig. 9*A*). When used at a similar concentration, biliverdin did not modulate the expression of E-selectin, VCAM-1, or ICAM-1 (Fig. 9*B*).

CO does not modulate the expression of E-selectin, ICAM-1, or VCAM-1

Because of the potent anti-inflammatory effects of CO in monocyte macrophages (23), we asked whether the ability of HO-1 to modulate the expression of the proinflammatory genes *E-selectin* and *VCAM-1* in EC was also due to CO. The data illustrated in Fig. 10 suggest that this is not the case. Pre-exposure of EC to exogenous CO (100 or 250 ppm; 16 h) had no significant effect in terms of modulating TNF-α-driven E-selectin, ICAM-1, or VCAM-1 expression in EC (Fig. 10). Similar results were obtained using 1,000 or 10,000 ppm CO (data not shown). We cannot rule out that a different dose or dosing schedule of CO would lead to inhibition.

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HO-1 inhibits the activation of the transcription factor NF-κB

We asked by which mechanism HO-1 modulated TNF-α-driven E-selectin and VCAM-1 expression in EC. One possibility would be that HO-1 modulates the level of expression of TNFRs at the EC surface, thus inhibiting the ability of TNF-α to trigger E-selectin or VCAM expression. However, we found that the level of expression of TNFR-1, the predominant TNFR in EC, was not inhibited in EC transduced with an HO-1 recombinant adenovirus, compared with β-gal-transduced or nontransduced EC (Fig. 11*A*). We then asked whether HO-1 would also inhibit the ability of IL-1β to up-regulate the expression of E-selectin or VCAM. We found that HO-1 inhibited IL-1β-mediated VCAM-1 and, to a lesser extent, E-selectin expression (Fig. 11*B*).

Given that both TNF-α and IL-1β induce the expression of proinflammatory genes associated with EC activation through a mechanism that is dependent on the activation of the transcription factor NF-κB (11, 30, 31), we tested whether HO-1 inhibited NF-κB activity in EC. We monitored NF-κB transcription activity in EC transiently transfected with an NF-κB luciferase reporter. In EC exposed to TNF-α, NF-κB activity increased by 8- to 12-fold, compared with controls not exposed to TNF-α (Fig. 12). TNF-α did not modulate the transcription activity of SV40-driven β-gal or luciferase reporters, used to monitor variations in transfection efficiency in this assay (data not shown). Overexpression of HO-1 inhibited TNF-α-driven NF-κB luciferase activity by 50–75%, compared with control EC (Fig. 12*A*). This effect was dose dependent, in that increasing levels of HO-1 led to a corresponding decrease in NF-κB activation (Fig. 12*A*). HO-1 did not modulate the transcription activity of SV40-driven β-gal or luciferase reporters (data not shown).

We then asked whether the ability of HO-1 to inhibit NF-κB transcription activity was linked to its ability to generate CO, biliverdin, or bilirubin, and/or the reduction of intracellular Fe^{2+} levels. Both iron chelation by DFO or exogenous bilirubin inhibited TNF-α-mediated NF-κB transcription activity (Fig. 12, *C* and *D*). Neither exogenous CO nor biliverdin modulated NF-κB activation (Fig. 12, *B* and *E*).

HO-1 promotes the activation of the p38 MAPK signal transduction pathway

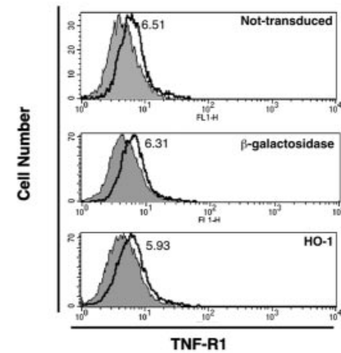
Expression of E-selectin, ICAM-1, and VCAM-1 can be modulated by the activation of MAPK signal transduction pathways. Because we found that the expression of HO-1 can modulate the p38 MAPK signal transduction pathway (23, 25), we asked whether this would occur in EC that overexpress HO-1. Expression of HO-1 increased TNF-α-mediated p38 activation, but not the activation of JNK or ERK (Fig. 13). Whether activation of p38 MAPK plays a role in the inhibition of E-selectin or VCAM-1 expression by HO-1 remains to be established.

Discussion

HO-1 expression plays a central role in the regulation of inflammatory reactions, as illustrated by the increasing number of reports showing that induction of HO-1 expression down-modulates inflammatory responses in a variety of experimental systems (reviewed in Ref. 32). Perhaps more important to support this notion is the observation that HO-1 genetic deficiency is characterized by a severe inflammatory syndrome (33, 34) that can be lethal in humans (35, 36).

The molecular basis of the anti-inflammatory action of HO-1 remains to be fully elucidated, but so far, CO seems to be responsible for most of the anti-inflammatory effects attributed to HO-1. For example, expression of HO-1 in monocyte/macrophages

A



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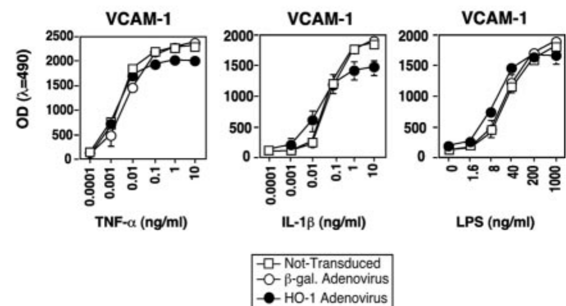

FIGURE 12. The effect of HO-1 on the expression of adhesion molecules associated with EC activation is not specific to the signal transduction pathway triggered by TNF-α. *A*, HUVEC were either not transduced (NT), or transduced with β-gal or HO-1 recombinant adenoviruses. After 24 h, expression of cell surface TNFR-1 was analyzed by flow cytometry, as described in *Materials and Methods*. Expression of TNFR-2 was not detectable by this method (data not shown). Gray histograms indicate stainings with isotype-matched control Ab, and open histograms represent stainings using an anti-TNFR-1 mAb. Numbers in each panel indicate the mean fluorescent intensity for each specific TNFR-1 staining. Results shown are representative of three independent experiments. *B*, HUVEC were either not transduced (□), or transduced with β-gal (○) or HO-1 (●) recombinant adenoviruses and exposed to increasing concentrations of human rTNF-α or IL-1β (6 h). VCAM-1 expression was detected by ELISA, as described in *Materials and Methods*. The experiment shown is representative of three independent experiments.

down-regulates the expression of proinflammatory cytokines such as TNF-α and up-regulates the expression of the anti-inflammatory cytokine IL-10 (23). TNF-α is a key mediator in the initiation of certain inflammatory reactions (37), whereas IL-10 is anti-inflammatory. CO mimics HO-1 in this respect, suggesting that it mediates its anti-inflammatory effect (23). More recently, the anti-inflammatory effect of IL-10 itself has been shown to act via the up-regulation of HO-1 and the generation of CO (38). CO also acts in an antithrombotic manner by promoting vasodilation via its action on smooth muscle cells (39), inhibiting platelet activation (40, 41) and suppressing the plasminogen activator inhibitor type 1 (serpin-1) expression, a prothrombotic molecule, in monocyte/macrophages (24). These actions of CO are likely to contribute to the anti-inflammatory effect of HO-1.

HO-1 has cytoprotective/antiapoptotic effects in a variety of cell types, including in EC (19, 25, 42). The physiological raison d'être of this may relate to the fact that free heme generated during inflammatory reactions primes EC to oxidative stress-mediated cytotoxicity (19). Therefore, expression of HO-1 would act to protect EC from the cytotoxic effects of high levels of free heme (42, 43).

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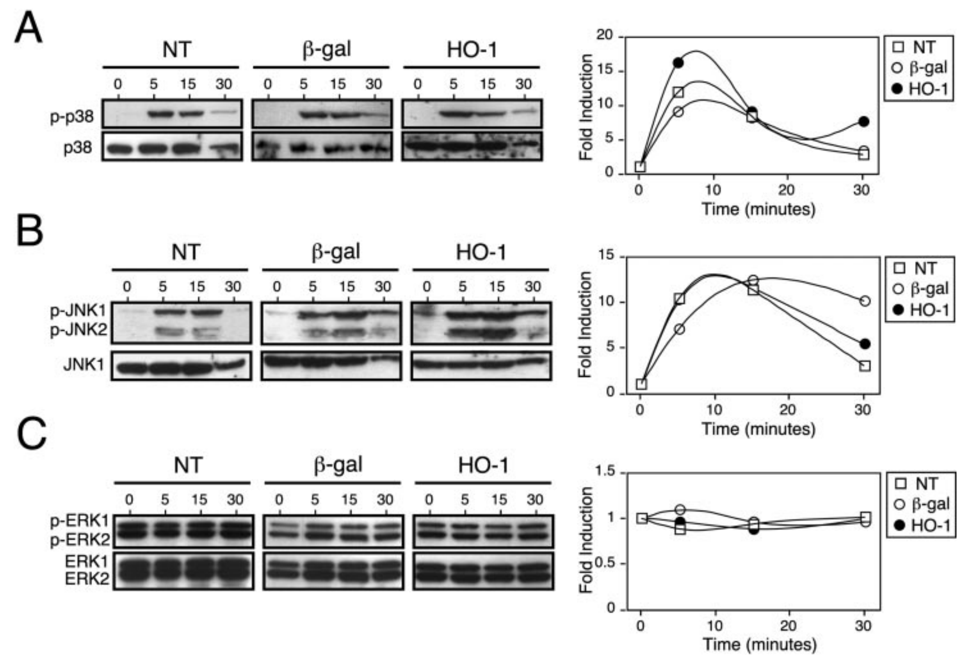

FIGURE 13. Expression of HO-1 promotes the activation of the p38 MAPK signal transduction pathway. HUVEC were either not transduced (NT), or transduced with β-gal or HO-1 recombinant adenoviruses and exposed 24 h after to human rTNF-α (10 ng/ml; 0, 5, 15, and 30 min). Activation of the p38 (*A*), JNK (*B*), and ERK (*C*) MAPK signal transduction pathways was analyzed by Western blot, using Abs specific to the phosphorylated and total forms of these kinases, as described in *Materials and Methods*. Signal obtained using Abs against the phosphorylated kinases was normalized to the one obtained using corresponding Abs recognizing the corresponding kinases and plotted graphically. Activation of each MAPK is represented as fold induction compared with nonstimulated EC (time 0).

We have shown that the cytoprotective/antiapoptotic action of HO-1 is mediated via CO (14, 25). Given that EC apoptosis can exacerbate inflammatory reactions (44), the antiapoptotic action of CO may also contribute to its anti-inflammatory effect (43).

In the present study, we asked whether HO-1 would have additional functions that could contribute to its anti-inflammatory effect. We found that overexpression of HO-1 in EC significantly inhibits the ability of TNF-α to induce the expression of proinflammatory adhesion molecules associated with EC activation, i.e., E-selectin and VCAM-1 (Figs. 2–7). This effect was also observed when the expression of HO-1 was induced by heme (Fig. 7), suggesting that it is physiologically relevant. However, contrary to HO-1 overexpression, induction of HO-1 by heme did not result in the inhibition of E-selectin expression (Fig. 7). Whether this is due to a qualitative and/or a quantitative difference between overexpressing HO-1 or inducing HO-1 expression by heme, which occurs in the context of a pro-oxidant response, remains to be established.

Contrary to the anti-inflammatory effect of HO-1 in monocyte/macrophages (23), its ability to inhibit the expression of proinflammatory adhesion molecules in EC is probably not mediated by CO. This is suggested by the observation that exogenous CO does not mimic the effect of HO-1 in terms of inhibiting E-selectin or VCAM-1 expression (Fig. 10). Instead, we found that bilirubin (Fig. 9) and/or Fe^{2+} chelation (Fig. 8) can mimic this effect of HO-1. This suggests that the ability of HO-1 to inhibit the expression of these adhesion molecules is mediated by bilirubin and/or by the decrease of intracellular Fe^{2+} levels associated with HO-1 activity but not by CO.

Our present finding that HO-1 inhibits the expression of E-selectin and VCAM-1 is in keeping with those of others, suggesting that induction of HO-1 by cobalt protoporphyrin IX also inhibits the expression of these genes in vivo (45, 46). However, in those studies, it was not clear whether HO-1 acted directly on EC or whether this effect was indirect, i.e., mediated via the anti-inflammatory effect of HO-1 on monocyte/macrophages, thereby decreasing the stimulus, e.g., TNF-α, leading to the expression of these adhesion molecules in EC. We now demonstrate that, independently of its anti-inflammatory effects in monocyte/macrophages, HO-1 acts directly in EC to inhibit the expression of these adhesion molecules (Figs. 1–7). Moreover, we found that HO-1 decreased the E-selectin and VCAM-1 expression at the mRNA level (Figs. 5 and 6) without interfering with the mRNA half-life (data not shown). This suggests that HO-1 inhibits the accumulation of these mRNAs by interfering with their rate of transcription. This notion is further supported by the observation that HO-1 inhibits the activity of the transcription factor NF-κB (Fig. 12), which is strictly required for the expression of E-selectin and VCAM-1 in EC (11, 47).

While the mechanism by which HO-1 inhibits NF-κB activation remains to be elucidated, we can only speculate how this may occur. TNF-α-mediated NF-κB activation is thought to involve an oxidative step (31, 47), required for triggering the phosphorylation/degradation of the natural NF-κB cytoplasmic inhibitor IκBα (reviewed in Ref. 48) by the IκB kinase signalosome (49). At first, our data would be consistent with the notion that, by reducing the levels of free radicals in the cell, the antioxidant bilirubin (50) and/or the reduction of the levels of intracellular Fe^{2+} could account for this effect of HO-1 (Fig. 12). However, we have recently found that overexpression of HO-1 in EC does not inhibit IκBα phosphorylation/degradation or nuclear translocation of the NF-κB heterodimer p65/RelA (our unpublished data). This suggests that HO-1 inhibits NF-κB activity downstream of these events. We have previously demonstrated that this can occur at the level of p65/RelA phosphorylation (51) and have preliminary evidence to suggest that HO-1 may act in a similar manner (our unpublished data).

Inhibition of NF-κB transcription activity by HO-1 was only partial, i.e., 50–75% inhibition vs control EC (Fig. 12). This effect is consistent with the extent of inhibition of E-selectin and VCAM-1 at both the protein (Figs. 2–4) and mRNA (Figs. 5 and 6) levels. Although speculative, this may have important implications for the overall biological function of HO-1, because we have recently shown that, to prevent EC apoptosis, HO-1 requires that NF-κB activation must be maintained above a certain threshold level (29). This level of NF-κB activity seems to be required to sustain the expression of a subset of NF-κB-dependent antiapoptotic genes that interact functionally with HO-1-derived CO to prevent EC apoptosis (29). Thus, if HO-1 would suppress NF-κB

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activity below this threshold level, the antiapoptotic action of HO-1 would be disabled, which would most probably have detrimental effects (44). We propose that HO-1 has reached a functional compromise in that it blocks NF-κB-mediated transcription activity in a manner that inhibits the expression of a subset of NF-κB dependent genes, e.g., *E-selectin* and *VCAM-1*, but allows the expression of other NF-κB-dependent genes, e.g., *ICAM-1*, including those required to support its antiapoptotic action (29). Whether this selective effect is purely due to the extent of NF-κB inhibition or whether there are additional mechanisms that could account for it is not clear.

In contrast to HO-1, bilirubin and to some extent Fe^{2+} chelation also inhibit the expression of ICAM-1 (Figs. 8 and 9). It seems unlikely that this difference is related to a distinct mechanism of action of bilirubin compared with HO-1. Rather, the concentration of bilirubin or the extent of Fe^{2+} depletion generated by the action of HO-1 may not be as great as that permitted by the exogenous administration of bilirubin or DFO.

In conclusion, we have demonstrated that HO-1 can inhibit the expression of a subset of proinflammatory genes associated with EC activation. This anti-inflammatory effect involves the inhibition of NF-κB activity, a transcription factor strictly required for the expression of most proinflammatory genes associated with EC activation. Contrary to other anti-inflammatory effects attributed to HO-1, its ability to modulate the expression of NF-κB-dependent genes in EC seems to be mediated by bilirubin and/or by removal of intracellular Fe^{2+}, but probably does involve the generation of CO.

Acknowledgments

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