Heme Oxygenase-1 Inhibits the Expression of Adhesion Molecules Associated with Endothelial Cell Activation via Inhibition of NF-κB RelA Phosphorylation at Serine 276

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Heme Oxygenase-1 Inhibits the Expression of Adhesion Molecules Associated with Endothelial Cell Activation via Inhibition of NF-kB RelA Phosphorylation at Serine 276

Mark P. Seldon,* Gabriela Silva,† Nadja Pejanovic,* Rasmus Larsen,* Isabel Pombo Gregoire,* Josina Filipe,* Josef Anrather,† and Miguel P. Soares‡*†

Heme oxygenase-1 (HO-1; encoded by the Hmox1 gene) catalyzes the degradation of free heme into biliverdin, via a reaction that releases iron (Fe) and carbon monoxide. We report that HO-1 down-regulates the proinflammatory phenotype associated with endothelial cell (EC) activation by reducing intracellular nonprotein-bound Fe (labile Fe). EC isolated from Hmox1−/− mice have higher levels of intracellular labile Fe and reactive oxygen species (ROS) as compared with EC isolated from Hmox1+/+ mice. Basal and TNF-induced expression of VCAM-1, ICAM-1, and E-selectin were increased in Hmox1−/− vs Hmox1+/+ EC, an effect reversed by Fe chelation using deferoxamine mesylate (DFO). Fe chelation inhibits TNF-driven transcription of Vcam-1, Icam-1, and E-selectin, as assessed using luciferase reporter assays. This effect is associated with inhibition of the transcription factor NF-κB via a mechanism that is not associated with the inhibition of IκB phosphorylation/degradation or NF-κB (i.e., RelA) nuclear translocation, although it affects very modestly NF-κB binding to DNA κB consensus sequences in the Vcam-1 and E-selectin promoters. HO-1 inhibits NF-κB (i.e., RelA) phosphorylation at Ser276, a phosphoacceptor that is critical to sustain TNF-driven NF-κB activity in EC. This effect was mimicked by Fe chelation as well as by antioxidants (N-acetylcysteine). In conclusion, we demonstrate a novel mechanism via which HO-1 down-modulates the proinflammatory phenotype of activated EC, i.e., the inhibition of RelA phosphorylation at Ser276. The Journal of Immunology, 2007, 179: 7840–7851.

Under homeostasis, endothelial cells (EC) regulate blood flow and orchestrate leukocyte trafficking throughout tissues via the expression of genes that regulate vasoconstriction and thrombosis and inhibit leukocyte adhesion (reviewed in Refs. 1 and 2). Under inflammatory conditions, however, EC undergo profound phenotypic modifications, becoming highly vasoconstrictive, prothrombotic, and proadhesive, a phenomenon referred to as EC activation (reviewed in Ref. 1). These phenotypic modifications rely on the down-regulation of vasodilatory, antiinflammatory, and antiadhesive genes as well as on the induction of several immediate early responsive vasoconstrictive, prothrombotic, and proadhesive genes (2). Induction of these proinflammatory genes occurs through a mechanism that requires the activation of the transcription factor NF-κB (3, 4).

The NF-κB family of transcription factors is composed of five members sharing a consensus Rel homology domain (RHD), i.e., p50, p52, cRel, RelB, and RelA (5), the latter of which is probably the predominant active form in EC (6). NF-κB proteins form homodimers and heterodimers that can bind κB DNA motifs in the promoter regions of “NF-κB-dependent genes” (7). In quiescent EC, NF-κB dimers are maintained mostly in the cytoplasm by virtue of their interaction with inhibitors of NF-κB (IκB) molecules that occlude the nuclear localization signals (8, 9) in their RHD. However, when exposed to proinflammatory agonists such as TNF, IκBs are phosphorylated, polyubiquitinated, and degraded via the 26S proteasome pathway (10) so that nuclear localization signal exposure on NF-κB dimers allows for nuclear translocation and binding to DNA κB motifs (reviewed in Ref. 9).

There are additional mechanisms that modulate NF-κB transcriptional activity (reviewed in Ref. 11). These include, but are not restricted to, phosphorylation of Ser205 (12), Ser276 (13–15), Ser281 (12), and Ser311 (16) in the N-terminal domain of RelA. Modulation of these phosphoacceptors is under the control of several kinases, including protein kinase A (PKA) (14), mitogen- and stress-activated kinase-1 (MSK1) (15), and protein kinase C (PKC) (13) (16). The phosphorylation status of RelA controls not only its transcriptional activity but also specificity for different

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4 Abbreviations used in this paper: EC, endothelial cell; BAEC, bovine aortic EC; CM-H2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorofluorescein diacetate; acetyl ester; DAPI, 4′,6-diamidino-2-phenylindole; DFO, deferoxamine mesylate; Fe, iron; HB/ Ser, N-(2-hydroxybenzyl)-L-serine; HEK-293, human epithelial kidney 293 (cell); H-ferritin, heavy chain ferritin; HO-1, heme oxygenase 1; HPRT, hypoxanthine-guanine phosphoribosyltransferase; luc, luciferase; MEC, mouse endothelial cell; MEF, mouse embryonic fibroblast; NAC, N-acetyl-l-cysteine; PKA, protein kinase A; RHD, Rel homology domain; ROS, reactive oxygen species; SIH, salicylaldehyde isonicotinoylhydrazone; TAD, transactivation domain; tet2, tetracycline operon; TET, tet2 DNA binding domain; VP16, viron protein 16.

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subsets of target genes (12). How these phosphoacceptors modulate RelA activity is not clear but presumably involves the interaction of RelA with coactivators such as the CREB-binding protein (CBP)/p300 (14, 17).

Although essential to elicit inflammatory responses, the expression of proinflammatory genes associated with EC activation must be tightly regulated so as to prevent unfettered inflammation. One of the mechanisms via which this occurs relies on the expression of “protective genes” (18). These have a dual role in that they regulate not only NF-κB activity but in addition protect EC from undergoing apoptosis (18). We have previously demonstrated that the stress responsive enzyme heme oxygenase-1 (HO-1, encoded by the Hmox1 gene) acts in such a manner (19, 20).

When exposed to proinflammatory agonists, EC up-regulate the expression of HO-1 via a mechanism that probably requires the loss of its constitutive transcriptional repression by the complex tramtrack and bric a brac (BTB and cap’ and collar (CNC) homologue 1 (BACH-1) (21, 22). Once BACH-1 is released from partaking in the generation of reactive oxygen species (ROS) via the Fenton reaction (25), a highly deleterious effect (26–28). Free Fe also induces the expression/activity of an ATPase Fexflux pump that decreases cellular Fe content (29).

Biliverdin, another end product of heme catabolism by HO-1 (23), is converted into the antioxidant bilirubin (30) by biliverdin reductase (31). Although bilirubin can inhibit NF-κB activation in EC (20), the relatively low level of biliverdin reductase expressed in EC makes it unlikely that this antioxidant would act as an autocrine manner to regulate NF-κB activity. Therefore, we reasoned that HO-1 might control NF-κB activation in EC via its ability to down-modulate the levels of intracellular labile Fe. We provide evidence that the reduction of EC labile Fe content associated with HO-1 expression inhibits specifically the phosphorylation of RelA at Ser276 and probably at Ser205, thus reducing NF-κB transcriptional activity. This mechanism underlies the inhibition of proinflammatory gene expression associated with the expression of HO-1 in EC and should contribute in a critical manner to the resolution of inflammatory reactions such as afforded by HO-1 expression.

Materials and Methods

Mice

BALB/c Hmox1+/− mice, generated by S.-F. Yet of the Pulmonary and Critical Care Division, Brigham and Women’s Hospital (32), were maintained at the Instituto Gulbenkian de Ciência (Oeiras, Portugal) under specific pathogen-free conditions in accordance with guidelines from the Animal Use and Institutional Ethical Committee of this institute. Genotypes were verified by genomic PCR using the following primers: Hmox1 (5’-GGTGACAGAAAGGCTAAG-3’ and 5’-CTGTAATCCACCTCACC AAC-3’) and neomycin (5’-TCTGAGGTCGTTCTTGAG-3’ and 5’-ACAGAAGTGCACGATGTCG-3’).

Cell culture

Murine endothelial cells (MEC) were isolated as described (33). Briefly, three hearts were mechanically minced, digested (2.2U/ml collagenase A at 37°C for 30 min; Sigma-Aldrich), washed, and labeled with rat anti-mouse PECAM-1 Ab (CD31; BD Pharmingen) plus goat anti-rat IgG microbeads. Labeled EC were purified in magnetic separation columns as per the manufacturer’s instructions (Miltenyi Biotec). EC phenotype was assessed by cobblestone morphology and confirmed by flow cytometry (CD31/CD105 expression). MEC were passaged 1/3 and used between passages 6 and 12. Bovine aortic endothelial cells (BAEC) were obtained as previously described (19) or purchased from Vec Technologies or Cell Systems and cultured on gelatinized plates in complete medium (MCDB-131; 20% FCS, 20 mM l-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin; Invitrogen Life Technologies). BAEC were passaged 1/3 and used between passages 6 and 10. Immortalized human epithelial kidney 293 (HEK-293) cells were obtained from American Type Culture Collection and cultured as described for BAEC. Pooled HUVEC (Clonetics) were cultured in EBM-2 medium supplemented according to the provider’s instructions (Clonetics). HUVEC were passaged 1/3 and used between passages 6 and 6. Mouse embryonic fibroblasts (MEF) from Hmox1−/− mice were provided by Dr. A. Beg from Columbia University, New York, NY (34). MEF were cultured in DMEM (Mediatech) supplemented with FCS (10%), penicillin (50 U/ml), and streptomycin (50 μg/ml) (Atlanta Biologicals). RelA−/− MEF were stably transfected with human RelA recombinant retroviruses essentially as described (12).

Immunoprecipitation

Cells were lysed in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl2, 5 mM KCl, 1% Triton X-100, and a Roche Protease Inhibitor Cocktail (complete EDTA-free, one tablet per 10 ml of lysis solution). Soluble fractions were incubated with a protein G-Sepharose bead/antibody complex in borate-buffered solution with 1% Triton X-100 (for 4 h at 4°C). RelA was immunoprecipitated with anti-mouse mAb (2 μg; catalog no. SC-8008, Santa Cruz Biotechnology). Immunoprecipitated proteins were washed in lysis buffer, resuspended in 2× sample buffer, heated to 100°C for 5 min, resolved by SDS-PAGE, and subjected to Western blotting.

Western blots

Cells were lysed in Laemmli sample buffer and proteins were resolved by electrophoresis on 8–10% SDS-polyacrylamide gels (35), transferred to polyvinylidine fluoride membrane (Bio-Rad), and blotted with Abs to HO-1 (catalog no. SPA-895 or SPA-896, Stressgen), RelA (catalog no. SC-372, Santa Cruz Biotechnology), phospho-Ser536-RelA (catalog no. 3031, Cell Signaling), phospho-Ser276-RelA (catalog no. 3037, Cell Signaling), IκBα (catalog no. SC-371, Santa Cruz Biotechnology), phospho-Ser32, Ser36, IκBα (catalog no. 9246, Cell Signaling), c-Myc (clone 9E10; American Type Culture Collection), or α-tubulin (clone DM 1A; catalog no. T-9026, Sigma-Aldrich). Primary Abs were detected using HRP-conjugated secondary Abs (Pierce). Labeling was visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to BioMax film (Kodak).

Flow cytometry

Confluent EC (~4 × 105) were washed in PBS and detached with 0.125% trypsin/0.05% EDTA (at 37°C for 5 min; Invitrogen Life Technologies). Digestion was stopped with complete MCDB-131 medium and cells were pelleted by centrifugation (200 × g at 4°C for 3 min), resuspended in 150 μl of FACS medium (PBS, 3% FCS, and 0.01% sodium azide), transferred into round-bottom 96-well plates (TPP) and pelleted (200 × g at 4°C for 3 min). Supernatant was removed and cells were incubated in 25–50 μl of FACS medium with primary Ab (5 μg/ml for 30 min at 4°C against endoglin/CD105 (catalog no.550546, BD Biosciences), PECAM/CD31 (catalog no.553370, BD Biosciences), or VCAM-1/CD106 (catalog no.553331, BD Biosciences). Cells were washed in FACS medium (3 × 200 μl at 200 × g and 4°C for 3 min) and primary Abs were detected using FITC-labeled secondary Abs (catalog no. F-6258, Sigma-Aldrich) in FACS medium (25 μl for 30 min at 4°C). Cells were stained with propidium iodide (0.5 μg/ml) to assess cellular viability. Fluorescence was measured by flow cytometry on a FACSCalibur device (BD Biosciences).

Cellular Fe assay

Labile and nonlabile Fe were measured essentially as described previously (36) using Ferene-S (37). Briefly, confluent EC (1 × 106) were harvested by trypsinization, pelleted (200 × g at 4°C for 3 min), washed three times with Fe-depleted PBS (1% Chexel-100 for 12 h) (Bio-Rad), and digested (0.5 ml of 25% perchloric acid at 4°C for 30 min). Supernatants (containing labile Fe) were collected after centrifugation (10,000 × g at 4°C for 15 min), pellets were dissolved (0.1 ml of 10 M nitric acid at 60°C for 16 h), pH was neutralized (10 M NaOH), cellular debris was pelleted (10000 × g at 4°C for 15 min), and supernatants were collected as the nonlabile
cellular Fe fraction. Fe in both fractions was converted to the ferrous (Fe^{2+}) form using sodium ascorbate (0.25 M, 0.1 ml) plus an equal volume of ammonium acetate (40%). Ferene-S (600 μM; BioVectra) was added and OD was measured at A_{594} nm. Fe levels were normalized to total protein content as determined by Bradford assay. Fe fractions are expressed as moles of Fe per milligram of cellular protein. Assays were performed at least in triplicate for each condition.

Quantitative real-time PCR
cDNA was obtained from total RNA as described (38). The relative number of mRNA transcripts was assessed using LightCycler real-time quantitative PCR (Roche) using FastStart DNA SYBR Green I mix (Roche). The expression ratio was calculated using an A_2^{ΔΔct} method (relative number) (39) or quantified as an absolute value using standard cDNA plasmids encoding the same sequences as the ones amplified by PCR. Results were normalized to hypoxanthine-guanine-phosphoribosyltransferase (HPRT) (ber) (39) or quantified as an absolute value using standard cDNA plasmids.

Reporters and expression constructs
Rattus norvegicus Hmox1 cDNA was expressed in pcDNA3 (Invitrogen Life Technologies) (41). N-terminal Myc-tagged RelA, p50, and cRel expression vectors have been described in Refs. 13 and 42, respectively. The NF-κB luciferase (luc) reporter (κB-luc) consists of three NF-κB binding sites, i.e., NF-E-selectin, κB-2, and κB-3, derived from the porcine E-selectin promoter (42). Another NF-κB luciferase reporter construct, i.e., VCAM-1.2-κB-luc, ICAM-1-κB-luc, and E-selectin-κB-luc have been described elsewhere (12). Briefly, the minimal SV40 promoter in the pGL3-luciferase vector (Promega) was replaced with a minimal 5'-GATCTGGG TACCCGCAGTTCCCCAGGTTCATGC-3' and the synthetic oligonucleotides coding for the κB consensus sequence derived from the Vcam-1, Icam-1, or E-selectin promoter were cloned up-stream of this minimal promoter so that they differ only in their decameric κB consensus sequences. The prototypical sequence used was 5'-GCTCκB decamer-CTGAGCTCCT-κB-deamer-CTCAGCT-3', in which the decameric κB consensus were GGGATTTCGG for VCAM-1.2-κB-luc, TCCA AATTC for ICAM-1-κB-luc, and GGGGATTTC for E-selectin-κB-luc (12). The human VCA M1 (1716–119) luciferase reporter was kindly provided by J. S. Pober (Deaconess Medical Center, Boston, MA) (44). The porcine E-selectin promoter were cloned upstream of this minimal promoter so that they differ only in their decameric κB consensus sequences. The prototypical sequence used was 5'-GCTCκB decamer-CTGAGCTCCT-κB-deamer-CTCAGCT-3', in which the decameric κB consensus were GGGATTTCGG for VCAM-1.2-κB-luc, TCCA AATTC for ICAM-1-κB-luc, and GGGGATTTC for E-selectin-κB-luc (12). Protein concentration was determined by Bradford assay. EMSA

Transient transfection and luciferase assays
BAEC, HUVEC, and HEK-293 cells were transfected at 60–70% confluence using the Lipofectamine 2000 reagent according to the manufacturer’s instructions (Invitrogen Life Science Technologies). In BAEC and HEK-293, 3 μg of total DNA was used per well of a 6-well plate with 2 μl of Lipofectamine 2000. For HUVEC, 1.5–2.5 μg of DNA was used with 1 μl of Lipofectamine 2000. The total amount of DNA was kept constant using pcDNA3 (Promega). Briefly, cells were washed and placed in serum and antibiotic-free medium followed by the addition of the DNA/Lipofectamine and replaced thereafter (2.5 h) with medium containing sera. Transfection efficiency was determined by either flow cytometry or fluorescence microscopy in cells cotransfected with pCAGGS-AFP. Transfection efficiency was typically 50% for BAEC, >95% for HEK-293, and 5–20% for HUVEC.

Luciferase assays were performed using a single (firefly) luciferase asay system (Promega) according to manufacturers instructions with a β-galactosidase control expression vector (pSV-β-galactosidase) as the control for transfection and transfection efficiency. β-Galactosidase expression was detected using Galacto-Light Plus β-Galactosidase Reporter Gene Assay System (Applied Biosystems). Alternatively, a Dual Luciferase Assay System (Promega) was used with a Renilla control expression vector (pRL-SV40). Assays were performed in triplicate and luciferase values were normalized to either β-galactosidase or Renilla luciferase.

Cellular ELISA
Two days postconfluence, HUVEC were exposed to human recombinant TNF at the indicated concentrations and times. The procedure has been described in detail elsewhere (20).

Reagents
Human recombinant TNF (R & D Systems) was resuspended in PBS plus 0.1% BSA and added directly to culture medium. Deferoxamine mesylate (DFO) (Sigma-Aldrich) was dissolved in culture medium at 30 and 250 μM. N-(2-hydroxybenzyl)-l-serine (HBSer; 0.5–5 mM) (48) was a gift from R. Tyrell, Department of Pharmacology, University of Bath, Bath, U.K. (49). Salicylaldehyde isonicotinoylhydrazide (SIH; 0.5–100 μM) (gift from Dr. P. Ponka, McGill University, Montreal, Quebec, Canada) was prepared as described (50). N-acetyl-l-cysteine (Sigma-Aldrich) was dissolved in culture medium, neutralized to pH 7.5 with 0.2–1 M NaOH, and added to culture medium (0.5 and 25 mM). 5-(and)-6-Chloromethyl-2',7'-dichlorofluorescein diacetate, acetyl ester (CM-H2DCFDA, Molecular Probes) was dissolved in DMSO (Sigma-Aldrich) and added to culture medium (3 μM).

ROS detection
Cellular ROS content was assayed by flow cytometry using CM-H2DCFDA. Two days postconfluence, MEC were transfected with 0.25–20% for HUVEC.

EMSA
Nuclear extracts were obtained from confluent BAEC as described (13, 42). Protein concentration was determined by Bradford assay. EMSA was performed essentially as previously described (13, 42). Briefly, double-stranded oligonucleotides were generated with the following sequences: 5'-AGTTGAGGACCTCCCCAGGACC3' (VCA M1-1); 5'-AGTT GAGGAGTTCCCCAAGCCG3' (VCA M1-2); 5'-AGTTGAGGATGGA A ATTC GGAGGACTCCAGGC3' (ICAM-1); 5'-AGTTGAGGAGGATGGA TGGAGGACCTCCCCAGGACC3' (E-selectin); and 5'-AGTT GAGGAGGACTCCAGGC3' (embryonic stem cells).
(Ig-κB). Oligonucleotides were labeled by phosphorylation with [γ-32P]ATP. EMSA was visualized in the form of autoradiographs indicating DNA/protein binding.

Statistical analysis
Statistical significance was assessed using an unpaired two-tailed t test assuming unequal variances as specified in Results according to experimental design, except for data in Fig. 1e where a one-sample, one-tailed z test was used.

Results
Reduction of EC labile Fe associated with HO-1 expression down-modulates VCAM-1 expression
HO-1 deficient mice (51) and humans (52) have deregulated Fe metabolism and widespread EC activation/injury, suggesting that HO-1 expression is essential in the regulation of Fe homeostasis (Ref. 51 and reviewed in Ref. 53) and in preventing unfettered EC activation. Because a causal link between deregulated Fe metabolism and unfettered EC activation has not been established, we asked whether deregulated Fe metabolism would promote EC activation. Primary EC were isolated from Hmox1+/+ and Hmox1–/– mice and their phenotype was confirmed by flow cytometry, i.e., expression of endoglin/CD105 (Fig. 1a). Genotype was confirmed by PCR (Fig. 1b) and Western blotting (Fig. 1c). Labile Fe content increased by 23% in Hmox1–/– vs Hmox1+/+ EC (Fig. 1d), while nonlabile Fe content was similar whether or not HO-1 was expressed (Fig. 1d), suggesting that HO-1 regulates specifically the labile Fe content of EC.

We then asked whether increased labile Fe content resulting from Hmox1 deletion would modulate the expression of adhesion molecules in EC. Expression of Vcam-1 mRNA, an adhesion molecule associated with EC activation (reviewed in Refs. 1 and 2), was increased by 3- to 4-fold in the liver of Hmox1–/– vs Hmox1+/+ mice as quantified by quantitative real-time PCR (Fig. 1e). To ascertain that HO-1 expression in EC accounted for this effect, Vcam-1 mRNA expression was quantified specifically in EC isolated from Hmox1–/– or Hmox1+/+ mice. The basal level of Vcam-1 mRNA expression increased by 4- to 5-fold in EC from Hmox1–/– vs Hmox1+/+ mice (Fig. 1f). Expression of VCAM-1 protein was also significantly increased in Hmox1–/– vs Hmox1+/+ EC (Fig. 1g). This effect was probably due to higher levels of labile Fe in Hmox1–/– EC (Fig. 1d), as Fe chelation by DFO reduced VCAM-1 expression in Hmox1–/– EC to levels similar to those of Hmox1+/+ EC (Fig. 1g). Taken together, these observations suggest that HO-1 expression in EC might be a physiologic regulator of basal VCAM-1 expression, an effect that probably relies on the control of labile Fe.

Down-regulation of labile Fe associated with the expression of HO-1 inhibits TNF-driven up-regulation of adhesion molecules in EC
We have previously shown that when overexpressed in EC, HO-1 inhibits the expression of adhesion molecules associated with EC activation, including VCAM-1, E-selectin, and ICAM-1 (20). We now asked to what extent this effect is associated with decreased labile Fe content (Fig. 1d). Transient HO-1 overexpression in EC reduced labile Fe content by 17% as compared with control EC (Fig. 2a). A similar effect was observed when nontransfected EC were treated with the Fe chelator DFO, i.e., an 18% reduction in labile Fe content as compared with untreated EC (Fig. 2a). TNF, a well-established proinflammatory agonist, induced a transient increase of 12–20% in EC labile Fe content (Fig. 2b), an effect in keeping with that reported in other cell types (54). HO-1 overexpression (Fig. 2c) or Fe chelation by DFO (Fig. 2d) reduced by 30–40% the ability of TNF to up-regulate VCAM-1 expression in EC. The inhibitory effect of Fe chelation was dose-dependent in that higher DFO concentrations enhanced its effect (Fig. 2e). To exclude the possibility that DFO would function independently of its Fe-chelating activity, experiments were performed using HBSer, a “milder” Fe chelator (49). HBSer mimicked the effect of

![Figure 1](https://example.com/fig1.png)
HO-1 inhibits TNF-driven expression of VCAM-1, E-selectin, and ICAM-1 via reduction of labile Fe content. a, Labile Fe was quantified in BAEC transiently transfected with control or HO-1 expression vectors as well as in nontransfected BAEC treated or not treated (NT) with DFO (250 μM; 16 h). Mean values ± SD (nine independent samples). b, Labile Fe was quantified in confluent BAEC not-treated (0) or treated with TNF (50 ng/ml). Mean values ± SD from triplicate samples (one of three representative experiments) are shown. c, Nontransduced (NT), β-galactosidase (β-gal), and HO-1 recombinant adenovirus-transduced HUVEC (24 h) were stimulated with TNF (6 h). VCAM-1 was detected by cellular ELISA. d, HUVEC treated with DFO (250 μM; 16 h) and stimulated with increasing concentrations of TNF (6 h). VCAM-1 was detected by cellular ELISA. e, HUVEC were treated with increasing concentrations of DFO (16 h) and stimulated with TNF (10 ng/ml). VCAM-1 expression was assessed as in c and d. Results shown in c–e are mean values ± SD from triplicate samples in one of three independent experiments. f, HUVEC were not treated (NT) or treated with the Fe chelator HBSer (5 mM; 16 h) and not further treated (Cont) or exposed to TNF (0.1 ng/ml; 8 h). VCAM-1 expression was detected as in c–e. g, Mean values ± SD (triplicate samples in one of four independent experiments). h, i, Confluent MEC from Hmox1+/− or Hmox1+/+ mice were treated with DFO (250 μM; 16 h) and TNF-α (50 ng/ml; 3 h) when indicated (+). Vcam-1 (g), E-selectin (h), and Icam-1 (i) mRNA were quantified by quantitative real-time PCR. Values are normalized to Hprt mRNA and shown as the mean number of molecules ± SD (n = 3 independent wells in one representative experiment). *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, not significant (p > 0.05).

DFO, but only when TNF concentrations were < 1 ng/ml, i.e., a 45% reduction in VCAM-1 expression, as compared with control EC (Fig. 2f). Similar results were obtained using SHI, a cell-permeable Fe chelator (50) (data not shown). These data suggest that despite their relative differences in efficiency, it is the Fe chelation activity of these compounds, i.e., DFO, HBSer, and SHI, that affords their effects.

To ascertain whether, when expressed under physiological conditions, HO-1 controls the induction of VCAM-1 expression in response to TNF, Vcam-1 mRNA expression was quantified by quantitative real-time PCR after the exposure of Hmox1−/− or Hmox1+/+ EC to TNF. Accumulation of Vcam-1 mRNA was ∼5-fold higher in Hmox1−/− vs Hmox1+/+ EC (Fig. 2g). Higher Vcam-1 mRNA expression in Hmox1−/− EC was ablated by DFO, suggesting that it is the higher labile Fe content in Hmox1−/− EC that accounts for this effect (Fig. 2g).

We then asked whether, when expressed under physiological conditions, HO-1 would control the expression of other TNF-responsive adhesion molecules in EC. Up-regulation of E-selectin (Fig. 2h) and Icam-1 (Fig. 2i) mRNA in response to TNF was increased by ∼3- and ∼11-fold in Hmox1−/− vs Hmox1+/+ EC, respectively. Higher E-selectin (Fig. 2h) and Icam-1 (Fig. 2i) mRNA expression in Hmox1−/− EC was ablated by DFO, suggesting again that it is the higher labile Fe of Hmox1−/− EC that accounts for this effect. Taken together, these data suggest that, when expressed under physiological conditions, HO-1 reduces the expression of adhesion molecules associated with EC activation, most probably via down-modulation of labile Fe.

Fe chelation inhibits the transcription of genes encoding adhesion molecules in EC

We tested whether down-modulation of labile Fe inhibits Vcam-1, E-selectin, or Icam-1 transcription. EC were transiently transfected with a VCAM-1-luc reporter and treated or not treated with DFO. Induction of VCAM-1 reporter activity by TNF was reduced by 77–100% in EC treated with DFO vs control EC (Fig. 3a). This effect was dose dependent in that increasing DFO concentrations...
Reduction of EC labile Fe content inhibits NF-κB activity

Induction of Vcam-1, E-selectin, and Icam-1 transcription by TNF is strictly dependent on activation of the transcription factor NF-κB in EC (3, 4, 19, 55). Therefore, we reasoned that the reduction of labile Fe might inhibit NF-κB activation (Fig. 3). EC were transiently transfected with a synthetic NF-κB-luc reporter derived from the porcine E-selectin promoter (43) plus or minus an HO-1 expression vector. TNF-driven NF-κB reporter activity was reduced by 42–57% in EC transfected with HO-1 as compared with control EC (Fig. 4a). The inhibitory effect of HO-1 was dose dependent; i.e., higher HO-1 expression resulted in lower NF-κB reporter activity (Fig. 4a). This effect was observed over a broad range of TNF concentrations (1–10 ng/ml) (Fig. 4b). Given that HO-1 overexpression reduces the labile Fe content of EC (Fig. 2a), we asked whether Fe chelation would mimic the inhibitory effect of HO-1. DFO inhibited TNF-driven NF-κB reporter activity by 63–78% as compared with control EC not treated with DFO (Fig. 4c). This effect was dose dependent; i.e., higher DFO concentrations resulting in lower NF-κB reporter activity (Fig. 4c), and this was observed over a broad range of TNF concentrations (1–10 ng/ml) (Fig. 4d). These data suggest that HO-1 inhibits NF-κB activation via a mechanism probably involving the reduction of labile Fe content in EC.

Reduction of labile Fe in EC does not affect IkBα phosphorylation/degradation or RelA nuclear translocation

When exposed to DFO under the same experimental conditions shown to inhibit NF-κB activation (Fig. 4, c and d), TNF-driven IkBα phosphorylation and/or IkBα degradation was not affected as compared with control EC (Fig. 5a). DFO also did not alter de novo IkBα synthesis after TNF stimulation as compared with control EC (Fig. 5a). In addition, DFO failed to modulate the levels of RelA protein expression or RelA phosphorylation at the C-terminal region, i.e., Ser536, as compared with control EC (Fig. 5a). RelA nuclear translocation in response to TNF was also not affected by DFO as compared with control EC (Fig. 5b). Total nuclear RelA was also not significantly modulated by DFO as assessed by Western blot analysis (Fig. 5c) (56).
Fe chelation interferes modestly with NF-κB binding to specific κB DNA binding motifs in the Vcam-1, E-selectin, and Icam-1 promoters.

Fe chelation interferes modestly with NF-κB binding to specific κB DNA binding motifs in the Vcam-1, E-selectin, and Icam-1 promoters.

Given that Fe chelation failed to modulate IκBα phosphorylation/degradation (Fig. 5a) and/or RelA nuclear translocation (Fig. 5, b and c), we reasoned that inhibition of NF-κB activity (Fig. 4) might occur “downstream” of nuclear translocation. Binding of NF-κB to κB DNA binding motifs derived from the Vcam-1, E-selectin, and Icam-1 promoters was compared by EMSA, using nuclear extracts from EC pre-exposed or not pre-exposed to DFO (16 h) and stimulated or not stimulated thereafter with TNF (60 min). DFO failed to modulate NF-κB binding to a “standard” κB binding motif derived from the Ig promoter (IκB) as compared with control EC (Fig. 5d). In a similar manner, DFO failed to modulate the very modest binding of NF-κB to the −167-bp Vcam-1 κB binding motif (VCAM1.1). There was a slight but reproducible inhibition of NF-κB binding to the −152-bp Vcam-1 κB binding motif (VCAM1.2) in EC treated with DFO, as compared with control EC (Fig. 5d). DFO reduced very slightly NF-κB binding to the −99bp E-selectin κB binding motif while not affecting NF-κB binding to the −1390-bp Icam-1 κB DNA binding motif (Fig. 5d) as compared with control EC. In all cases, the characteristic pattern of NF-κB/DNA binding was observed, namely the appearance of “specific bands” upon TNF stimulation that were competed out using 20-fold excess unlabeled κB probe, corresponding to the probe used in each EMSA, respectively (Fig. 5d).

Fe chelation inhibits RelA transcriptional activity

Because the inhibition of NF-κB activation by HO-1 or Fe chelation (Fig. 4) was not associated with the inhibition of NF-κB nuclear translocation (Fig. 5, b and c), and only to a modest extent with DNA binding (Fig. 5d), we tested whether the inhibitory effect of HO-1 acted directly on RelA, presumably the main NF-κB transactivator in EC (6). EC from Hmox1+/+ and Hmox1−/− mice were transiently transfected with increasing amounts of RelA plus an NF-κB luciferase reporter derived from the porcine E-selectin promoter (43). RelA activity was 2-3-fold higher in EC from Hmox1−/− mice as compared with EC from Hmox1+/+ mice (Fig. 6a). This suggests that, when expressed under physiologic conditions, HO-1 inhibits RelA activity.

We then asked whether Fe chelation would inhibit RelA activity. Expression of the same NF-κB luciferase reporter was increased by ~40-fold when EC were cotransfected with RelA, RelA plus p50, or RelA plus cRel expression vectors as compared with control EC that did not overexpress these NF-κB family members (Fig. 6b). Fe chelation by DFO inhibited RelA-, RelA plus p50-, and RelA plus cRel-driven reporter transactivation by ~45–57% as compared with control EC (Fig. 6b). Fe chelation did not alter RelA expression as assessed by Western blot analysis.
using an anti-Myc-tagged Ab recognizing specifically overexpressed RelA (Fig. 6c). Inhibition of RelA activity was also observed in HEK 293 cells transiently cotransfected with RelA and the same NF-κB luciferase reporter (Fig. 6d), illustrating that this effect is not restricted to EC.

We then asked whether Fe chelation would inhibit endogenous Vcam-1, E-selectin, and Icam-1 transcription when driven specifically by RelA. For this purpose, RelA −/− MEF were transfected or not transfected with a recombinant RelA retrovirus, allowing for the assessment of RelA-driven Vcam-1, E-selectin, and Icam-1 mRNA transcription by quantitative real-time PCR. RelA −/− MEF reconstituted with RelA expressed Vcam-1, E-selectin, and Icam-1 mRNA transcripts that were ~150-, ~2500-, and ~30,000-fold more abundant to those detected in RelA −/− MEF, respectively (Fig. 6e). DFO inhibited by 69, 58, and 70% Vcam-1, E-selectin, and Icam-1 mRNA expression driven by RelA, respectively (Fig. 6e). This observation provides direct evidence that Fe chelation inhibits RelA-mediated transcription of endogenous Vcam-1, E-selectin, and Icam-1.

As for DFO, SIH, a cell-permeable Fe chelator (50), also inhibited RelA activity as assessed in EC transiently cotransfected with RelA plus NF-κB luciferase reporter (Fig. 6f). This suggests that the inhibitory effect of these molecules is due to their Fe-chelating activity. We reasoned that the reduction of EC labile Fe content might down-regulate RelA activity by disabling labile Fe from promoting the generation of ROS through the Fenton reaction. If this is the case, then HO-1 expression should inhibit ROS accumulation in EC. Intracellular ROS content was ~2-fold higher in EC isolated from Hmox1−/− vs Hmox1+/- mice (Fig. 6g), suggesting that when expressed under physiologic conditions, HO-1 limits the accumulation of ROS in EC. Fe chelation by DFO reduced intracellular ROS in Hmox1−/− EC to levels similar to those of Hmox1+/- EC (Fig. 6g). This suggests that the higher levels of intracellular ROS in Hmox1−/− EC is due to the higher levels of intracellular labile Fe in these cells (Fig. 1d). Similar results were observed using another Fe chelator, i.e., SIH (data not shown). TNF failed to increase ROS levels in EC as compared with untreated EC (Fig. 6g).

If HO-1 inhibits RelA activity by disabling labile Fe from promoting the generation of ROS, then an antioxidant such as N-acetylcysteine (NAC) should mimic the inhibitory effects of HO-1 and/or that of Fe chelation. In keeping with this notion, NAC inhibited by ~50% RelA activity as assessed in EC transiently cotransfected with RelA plus NF-κB luciferase reporter (Fig. 6h).

**Fe chelation targets the N-terminal domain of RelA**

We used a series of chimeric DNA constructs encoding the N- or C-terminal domains from RelA that allowed us to test whether Fe chelation inhibits RelA activity via a mechanism that targets its N- and/or C-terminal domains (13). The chimeric constructs were transiently cotransfected in EC with a NF-κB luciferase reporter derived from the porcine E-selectin promoter (43). DFO inhibited...
Fe chelation modulates RelA transcriptional activity via a mechanism that targets the N-terminal domain of RelA. a, BAEC were transiently transfected with an NF-κB luciferase reporter derived from the porcine E-selectin promoter (κB-luc) (43) plus an expression vector encoding the full-length RelA or the N-terminal domain of RelA (aa 2–320) fused to the VP16 TAD (RelA(2–320)/VP16). EC were not further treated (NT) or treated with DFO (250 μM; 16 h). Shown is mean luciferase normalized to β-galactosidase units ± SD (triplicate sample in one of three independent experiments). b, BAEC were transiently cotransfected and treated as in a and overexpressed RelA and RelA(2–320)/VP16 were detected by Western blotting using an anti-c-Myc Ab recognizing the c-Myc tag in the N terminus of these constructs. c, BAEC were transiently transfected with tet-luc reporter alone (control) or cotransfected with tet-luc plus TET/VP16 or a construct encoding a TET DNA binding domain fused to the RelA TAD (TET/RelA(268–551)). EC were treated as in a. d, BAEC were transiently transfected with NF-κB luciferase reporter (κB-luc) alone (Control) or cotransfected with κB-luc (same as in a) plus an expression vector encoding the TET DNA binding domain fused to the full-length RelA (TET/RelA). EC were treated as in a and b. e, BAEC were transiently transfected with tet-luc reporter (tet-luc) alone (control) or cotransfected with tet-luc plus TET/RelA and treated as in a–c. Data illustrated in a–e are mean luciferase normalized to β-galactosidase units ± SD (triplicate sample in one of three independent experiments). f, BAEC were transiently transfected and treated as in e and TET/RelA was detected by Western blotting using an anti-RelA Ab. *p < 0.05.

We hypothesized that Fe chelation might target one or several phosphoacceptors in the N-terminal domain of RelA, an effect that would be consistent with the observed inhibition of RelA activity (reviewed in Ref. 11) (12, 13). We tested this hypothesis specifically for the phosphoacceptors Ser276, Ser205, Ser276, and Ser311. EC were transiently cotransfected with wild-type RelA or with RelA S205A, S276A, S281A, and S311A point mutants plus an NF-κB luciferase reporter derived from the porcine E-selectin promoter (43). DFO inhibited by 53 and 43% wild-type RelA and S311A RelA-driven transcription, respectively, as compared with control EC not treated with DFO (Fig. 8a). This suggests that Fe chelation does not act via Ser311 to suppress RelA activity. Because the transcriptional activity of the RelA S205A, S276A, and S281A point mutants is abolished using this NF-κB reporter (Fig. 8a) (12), we made use of synthetic NF-κB reporters consisting of two tandem decameric κB sites derived from the human Vcam-1 (VCAM-1-1.2-κB-luc), E-selectin (E-selectin-κB-luc), or Icam-1 (ICAM-1-1.2-κB-luc) promoters (12).

Fe chelation inhibited by 38% the transcription of VCAM-1-1.2-κB-luc driven by wild-type RelA. It also inhibited by 52 and 32% transcription driven by the RelA S281A or S311A point mutants, respectively (Fig. 8b), while it failed to inhibit RelA S205A- or S276A-driven transcription (Fig. 8b). Similar results were obtained using the ICAM-1-κB-luc reporter; i.e., Fe chelation inhibited by 53% transcriptional activity by wild-type RelA but failed to inhibit transcription by RelA S205A or S276A point mutants (Fig. 8c). Fe chelation inhibited by 38% the transcription of ICAM-1-κB-luc by the S311A RelA mutant while RelA S281A activity was abolished using this reporter (Fig. 8c). Similarly, Fe chelation inhibited by 25% the transcriptional activity of the E-selectin-κB-luc reporter by wild-type RelA (Fig. 8d), consistent with our previous observation that HO-1 and/or Fe chelation are less efficient in blocking E-selectin as compared with VCAM-1 expression (20). Again, Fe chelation failed to suppress S205A or S276A RelA-driven E-selectin-κB-luc reporter transcription (Fig. 8d). Taken together, these data suggest that Fe chelation inhibits RelA activity.
Several mechanisms have been proposed as to how HO-1 modulates cellular labile Fe content, including through the up-regulation of H-ferritin (24) and/or the activation of ATPase Fe efflux pumps (29). Given that free Fe can induce the expression of H-ferritin, one would predict that increased labile Fe content in Hmox1−/− EC would lead to higher H-ferritin expression (24). However, H-ferritin expression was very significantly decreased in Hmox1−/− versus Hmox1+/+ EC (data not shown). One possible explanation for this apparently contradictory observation would be that the accumulation of ROS in Hmox1−/− (Fig. 6g) might promote H-ferritin degradation (57), an effect that could account for an overall decrease in H-ferritin expression and thus for the increased labile Fe content observed in Hmox1−/− EC (Fig. 1d).

EC from Hmox1−/− mice expressed higher basal levels of VCAM-1 as compared with EC from Hmox1+/+ mice (Fig. 1, e–g). This suggests that HO-1 is a physiologic regulator of VCAM-1 expression in EC. Increased VCAM-1 expression in Hmox1−/− EC was reversed by Fe chelation (Fig. 1g), suggesting that there is a functional link between the ability of HO-1 to down-regulate labile Fe and to inhibit VCAM-1 expression.

HO-1 overexpression in EC decreases labile Fe content to the same extent as Fe chelation by DFO (Fig. 2a), an effect that probably explains the ability of HO-1 to inhibit the up-regulation of VCAM-1 expression in response to proinflammatory agonists such as TNF. That labile Fe may be involved in the regulation of proinflammatory gene expression in response to TNF is suggested by the observation that TNF triggers a transient rise of labile Fe content in EC (Fig. 2b). When labile Fe is decreased, either by overexpressing HO-1 or by chelating Fe, the ability of TNF to induce the expression of VCAM-1 is inhibited (Fig. 2, c–f), a finding in keeping with our previous results (20). The physiologic relevance of these findings is supported by the observation that Hmox1 deletion increases TNF-driven VCAM-1 expression, an effect reversed when labile Fe is chelated by DFO (Fig. 2g). Similar effects were observed for other adhesion molecules such as E-selectin (Fig. 2h) and ICAM-1 (Fig. 2i), suggesting that the inhibitory effects of HO-1/Fe chelation can be extended to other proinflammatory genes associated with EC activation.

The observation that Fe chelation inhibits TNF-driven Vcam-1 (Fig. 3, a and b), E-selectin (Fig. 3, c and d), and Icam-1 (Fig. 3, e and f) transcription suggests that NF-κB, a transcription factor required for TNF-driven transcription of these genes in EC (3, 20, 55), might be the target of Fe chelation (20). Although our data suggest that this is the case (Fig. 4, a–d), we found that inhibition of NF-κB does not occur at the level of IkBα phosphorylation/degradation (Fig. 5a), C-terminal RelA phosphorylation, i.e., Ser536 (Fig. 5a), or RelA nuclear translocation (Fig. 5b), and most probably not via the inhibition of NF-κB binding to κB DNA binding motifs in the promoter of Vcam-1, E-selectin, or Icam-1 (Fig. 5d). Fe chelation inhibits RelA transcriptional activity via a mechanism that targets specifically its N-terminal domain (Fig. 7, a and b).

Because N-terminal phosphorylation is required to support RelA transactivating activity (12, 13), we tested whether Fe chelation inhibited RelA activity via a mechanism dependent on serine phosphoacceptors in the N-terminal domain of RelA. Fe chelation failed to inhibit the transcriptional activity of RelA S205A or S276A point mutants as assessed by using synthetic NF-κB luciferase reporters containing κB DNA motifs from the human Vcam-1 (Fig. 8b), Icam-1 (Fig. 8c), or E-selectin (Fig. 8d) promoters. This led to the hypothesis that HO-1 and/or Fe chelation might interfere with Ser276 phosphorylation. We found that Fe chelation inhibits RelA Ser276 phosphorylation (Fig. 8, e and f) in

**FIGURE 8.** Fe chelation modulates RelA transcriptional activity via Ser205 and Ser276. BAEC were transiently cotransfected with RelA or RelA S205A, S276A, S281A, or S311A point mutants plus an NF-κB luciferase reporter derived from the porcine E-selectin (κB-luc) (43) (a), human VCAM-1 (VCAM-1.2-κB-luc) (12) (b), human ICAM-1 (ICAM-1-κB-luc) (12) (c), or human E-selectin (E-selectin-κB-luc) (12) (d) promoters. EC were either not further treated (NT) or treated with DFO (250 μM; 24–48 h). The data illustrated in a–d represent mean luciferase normalized to β-galactosidase units ± SD (triplicate in one of at least two independent experiments for each promoter used). e, f, Confluent MEF (e) or MEC (f) were either left untreated (−) or treated (+) with DFO (250 μM; 16 h) and not further treated (−) or treated (+) with TNF (50 ng/ml; 15 min). RelA was immunoprecipitated (IP) and total RelA and RelA Ser276 phosphorylation were detected by Western blotting (WB). Immunoprecipitation with irrelevant Ig was performed as a negative control. *p < 0.05.

**Discussion**

Given that HO-1 overexpression reduces cellular Fe content (29) we asked whether HO-1 would act in a similar manner when expressed under physiologic conditions in EC. Our data suggest that this is the case, because labile Fe content was significantly higher in EC isolated from Hmox1−/− mice as compared with those isolated from Hmox1+/+ mice (Fig. 1d). Based on the above, we suggest that HO-1 might act as a physiologic regulator of EC labile Fe content, a notion in keeping with the deposition of labile Fe in the vasculature of Hmox1-deficient mice (51) as well as that associated with the one reported case of HMOX1 deficiency in humans (52).

via a mechanism that targets specifically RelA Ser205 and Ser276 but not Ser281 or Ser311.

We then asked whether Fe chelation modulates RelA phosphorylation at Ser276, an effect that would justify its inhibitory effect (Fig. 8, a–d) (12, 13). As described previously (13), RelA Ser276 is constitutively phosphorylated in resting MEF (Fig. 8e) as well as in MEC (Fig. 8f). Exposure to TNF failed to increase RelA Ser276 phosphorylation in MEF (Fig. 8e) but did so in MEC (Fig. 8f). Fe chelation by DFO decreased Ser276 phosphorylation by 50–60% in resting as well as in TNF-stimulated MEF (Fig. 8e). Fe chelation fully suppressed TNF-driven Ser276 phosphorylation in MEC (Fig. 8f) while not affecting basal RelA Ser276 phosphorylation (Fig. 8g). Taken together, these observations suggest that down-modulation of labile Fe inhibits RelA Ser276 phosphorylation, thus explaining its ability to suppress RelA activity (12, 13).
a manner that should account for its inhibitory effect over RelA activity (12, 13). Whether Fe chelation modulates RelA Ser205 phosphorylation could not be established, as reagents are not available to monitor this phosphoacceptor.

A likely scenario to explain the inhibitory effects observed would be that labile Fe availability, which promotes the generation of ROS (Fig. 6g) via the Fenton reaction (reviewed in Ref. 53), might regulate the expression and/or activity of kinase(s) and/or phosphatase(s) that target RelA at Ser276 and/or Ser205 (Fig. 8, a–d) and thus regulate RelA activity (17). The notion that labile Fe targets RelA activity via ROS is supported by the observation that the antioxidant NAC, a radical scavenger and glutathione precursor, suppressed RelA activity in a manner that mimicked Fe chelation (Fig. 6h). This inhibitory effect is in keeping with our previous observation that bilirubin, another potent antioxidant (30), also inhibits NF-κB activation in EC (20). Whether bilirubin targets RelA in a similar manner to Fe chelation, i.e., inhibiting RelA Ser276 phosphorylation, remains to be established but is likely to be the case.

Putative candidate kinases modulated by ROS and targeting RelA activity in EC include PKA (58, 59), mitogen- and stress-activated kinase-1 (MSK1) (15), and protein kinase C ζ (PKCζ) (12, 13). Among these, PKA, which has been shown to target RelA Ser276, is probably the most likely candidate because Fe chelation can regulate its expression/activity (60). Whether the ability of HO-1 and/or Fe chelation to inhibit RelA activity acts via PKA remains to be established.

We suggest, that the putative kinase(s) and/or phosphatase(s) that target RelA Ser276 interfere with the mechanism via which the RHD promotes transcriptional activity upon binding to κB DNA motifs. This is supported by the observation that Fe chelation inhibits the transcriptional activity of a RelA chimeric protein (TET/RelA) only when this protein binds DNA via the RHD (i.e., binding to κB DNA recognition motifs in a NF-κB reporter; Fig. 7d) but not when the same protein binds DNA via its TET domain (i.e., binding to tet” recognition motifs in a Tet reporter) (Fig. 7e). It is possible that such kinase(s) and/or phosphatase(s) might target RHD after binding to κB DNA recognition motifs. This hypothesis remains, however, to be tested.

The mechanism via which HO-1 controls NF-κB activation in EC provides clues as to its protective effect in the context of vascular inflammation (61–63). We suggest that down-regulation of EC labile Fe content may inhibit specifically the expression of proinflammatory genes associated with EC activation, e.g., adhesion molecules, while sparing that of anti-inflammatory genes (42). Such an effect is consistent with targeting of RelA Ser276, a phosphoacceptor that controls RelA cis-acting element specificity (12). That is, by down modulating Ser276 RelA phosphorylation, HO-1 and/or Fe chelation might direct this NF-κB family member to transcribe specific subsets of genes (12). It is likely that this would occur via modulation of RelA interaction with coactivators required to support the transcription of specific gene subsets (17). Such a mechanism would explain how HO-1 has reached a “functional compromise” in which it inhibits specifically the transcription of proinflammatory genes but probably not that of anti-inflammatory genes required to support its cytoprotective effects, i.e., A1 and c-IAP2 (42). These two biologic effects, i.e., anti-inflammatory and cytoprotective, are accomplished by different end products of heme degradation, i.e., labile Fe controls NF-κB while CO is cytoprotective without interfering with NF-κB (20).

In conclusion, the effects reported hereby provide clues as to our understanding of the protective effect of HO-1 in countering the pathogenesis of inflammatory diseases, including autoimmune neuroinflammation (63) as well as neuroinflammation emanating from unfettered responses to microbial challenge, e.g., cerebral malaria (62). Our present data support the notion that these protective effects are mediated, at least partially, via the ability of HO-1 to modulate EC labile Fe content, an effect that regulates NF-κB activity in a manner that is anti-inflammatory and cytotoxic.

Finally, these data might also help in providing a mechanism for the palliative effects of Fe chelation therapies.

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