British Journal of Pharmacology (2009), 157, 769–780

0007-1188/09
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RESEARCH PAPER

The carbon monoxide releasing molecule (CORM-3) inhibits expression of vascular cell adhesion molecule-1 and E-selectin independently of haem oxygenase-1 expression

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Background and purpose: Although carbon monoxide (CO) can modulate inflammatory processes, the influence of CO on adhesion molecules is less clear. This might be due to the limited amount of CO generated by haem degradation. We therefore tested the ability of a CO releasing molecule (CORM-3), used in supra-physiological concentrations, to modulate the expression of vascular cell adhesion molecule (VCAM)-1 and E-selectin on endothelial cells and the mechanism(s) involved.
Experimental approach: Human umbilical vein endothelial cells (HUVECs) were stimulated with tumour necrosis factor (TNF)-α in the presence or absence of CORM-3. The influence of CORM-3 on VCAM-1 and E-selectin expression and the nuclear factor (NF)-κB pathway was assessed by flow cytometry, Western blotting and electrophoretic mobility shift assay.
Key results: CORM-3 inhibited the expression of VCAM-1 and E-selectin on TNF-α-stimulated HUVEC. VCAM-1 expression was also inhibited when CORM-3 was added 24 h after TNF-α stimulation or when TNF-α was removed. This was paralleled by deactivation of NF-κB and a reduction in VCAM-1 mRNA. Although TNF-α removal was more effective in this regard, VCAM-1 protein was down-regulated more rapidly when CORM-3 was added. CORM-3 induced haem oxygenase-1 (HO-1) in a dose- and time-dependent manner, mediated by the transcription factor, Nrf2. CORM-3 was still able to down-regulate VCAM-1 expression in HUVEC transfected with siRNA for HO-1 or Nrf2.
Conclusions and implications: Down-regulation of VCAM and E-selectin expression induced by CORM-3 was independent of HO-1 up-regulation and was predominantly due to inhibition of sustained NF-κB activation.
British Journal of Pharmacology (2009) **157,** 769–780; doi:10.1111/j.1476-5381.2009.00215.x; published online 5 May 2009

Keywords: endothelial cells, adhesion molecules, inflammation, carbon monoxide

Abbreviations: CORM, carbon monoxide releasing molecule; CORM-3, tricarbonylchloro(glycinato)ruthenium(II); eNOS, endothelial nitric oxide synthase; HO-1, haem oxygenase-1; HUVEC, human umbilical vein endothelial cell; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-κB; ODQ, 1H-[1, 2, 4]oxadiazolo[4, 3-alpha]quinoxalin-1-one; SNP, sodium nitroprusside; TNF, tumour necrosis factor; TEMED, N,N,N′,N′-Tetramethylethylenediamine; VCAM-1, vascular cell adhesion molecule-1

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Received 26 November 2008; revised 13 January 2009; accepted 15 January 2009

Introduction

Leukocyte extravasation occurring at the onset of inflammation is a highly regulated process characterized by bidirectional communication between endothelial cells and

leukocytes. Upon exposure of endothelial cells to inflammatory mediators, adhesion molecules and chemokines are rapidly up-regulated, which in turn facilitate leukocyte migration (Pober and Cotran, 1990; Cines *et al*., 1998; Golias *et al*., 2007; Rao *et al*., 2007). In addition, ligand binding to CXC chemokine receptor-1 (CXCR1) and CXCR2 stimulates neutrophils to release a number of factors including proteases, cytokines, chemokines and other chemoattractants that amplify inflammation and extend duration of the latter (Jaeschke and Hasegawa, 2006). Hence, understanding the mechanisms controlling down-regulation of pro-inflammatory cytokines and adhesion molecules is now being widely recognized as a prerequisite for the identification of novel drug targets for inflammatory disease (Bach *et al*., 1997; Van Assche *et al*., 2006; Golias *et al*., 2007).

In addition to its unambiguous role in oxidant-induced injury (Ryter and Tyrrell, 2000; Grosser *et al*., 2004; Erdmann *et al*., 2005), the haem oxygenase (HO) system is also clearly involved in the control of inflammatory processes (Willis *et al*., 1996; Otterbein *et al*., 2000; Soares *et al*., 2004; Seldon *et al*., 2007). The HO system comprises several isoenzymes (Maines *et al*., 1986; Maines, 1997), of which the inducible HO-1 isoenzyme appears to be particularly important as an anti-inflammatory mediator (Yachie *et al*., 1999; Otterbein *et al*., 2000; Soares *et al*., 2004; Seldon *et al*., 2007). HOs are the rate-limiting enzymes in degradation of haem into carbon monoxide (CO), Fe^{2+} and biliverdin, the latter being subsequently converted to bilirubin (Tenhunen *et al*., 1968; Kutty and Maines, 1981).

Although a number of studies have postulated putative mechanisms by which HO-1 exerts its anti-inflammatory effect, there is still debate about these mechanisms. While Soares *et al*. (2004) have shown that HO-1 down-regulates vascular cell adhesion molecule-1 (VCAM-1) and E-selectin expression via bilirubin and iron chelation with no apparent involvement of CO; Otterbein *et al*. (2000) and Sethi *et al*. (2002) clearly demonstrate the anti-inflammatory potential of CO in macrophages and monocytes as well as in endothelial cells. The salutary effect of CO has also been shown for organ transplantation and ischaemia–reperfusion injury (Neto *et al*., 2004; Nakao *et al*., 2005).

Recently, a new class of molecules, termed CO releasing molecules (CORM), has been described (Motterlini *et al*., 2002), composed of transition metal carbonyls. They are capable of liberating CO under appropriate conditions. In particular, CORM-3 [tricarbonylchloro(glycinato)ruthenium(II)] and CORM-A1 (sodium boranocarbonate), which both are fully water-soluble, rapidly liberate CO when dissolved in physiological solutions. These molecules might therefore be of therapeutic interest for their ability to modulate ongoing inflammatory reactions by delivering CO in a controllable fashion (Alcaraz *et al*., 2008). In addition, these molecules have been widely used to increase our understanding of the biological function of CO (Foresti *et al*., 2004; Motterlini, 2007).

In the present study, we investigated if high concentrations of CORM-3, releasing CO in excess of endogenously produced CO, were able to down-regulate the expression of adhesion molecules and if such effects were mediated by mechanisms similar to those reported for HO-1.

Methods

Cell culture

The use of human umbilical cords for this study was approved by the institutional ethics committee. Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cords, as described previously (Yard *et al*., 2004). Human lung microvascular endothelial cells were purchased from CellSystems (Remagen, Germany) at passage 4. The cells were grown in basal endothelial medium supplemented with 10% fetal bovine serum (FBS) and essential growth factors until they formed a confluent monolayer. Cells were stimulated with 50 $ng \cdot mL^{-1}$ of tumour necrosis factor (TNF)-α in the presence or absence of different concentrations of CORM-3. Unstimulated cells served as control.

Synthesis of CORM-3

Tricarbonylchloro(glycinato)ruthenium(II) ($[Ru(CO)_3Cl(glycinate)]$) was synthesized starting from a commercially available compound, tricarbonyldichlororuthenium(II) dimer ($[Ru(CO)_3Cl_2]_2$) as previously described (Clark *et al*., 2003). Briefly, $[Ru(CO)_3Cl_2]_2$ (0.5 g) and glycine (0.151 g) were placed under nitrogen in a round-bottomed flask. Methanol (291 mL) and sodium ethoxide (0.132 g) were added, and the reaction was allowed to continue under stirring for 18 h at room temperature. The solvent was then removed under pressure and the yellow residue redissolved in tetrahydrofuran. The yellow solution was evaporated down to give a pale yellow solid (yield 92–96%) and was stored in closed vials at –20°C. For each experiment, CORM-3 was dissolved freshly in phosphate-buffered saline (PBS, pH 7.4).

FACS analysis

Human umbilical vein endothelial cells were cultured in medium supplemented with 50 $ng \cdot mL^{-1}$ TNF-α in the presence or absence of 1 $mmol \cdot L^{-1}$ CORM-3. This concentration was chosen on the basis of concentration–response studies and was not toxic to HUVECs. In some experiments the cells were stimulated with TNF-α in the presence of different concentrations (10–500 $\mu mol \cdot L^{-1}$) of sodium nitroprusside (SNP). Also in some experiments the guanylate cyclase inhibitor 1H-[1, 2, 4]oxadiazolo[4, 3-alpha]quinoxalin-1-one (ODQ) was used to assess the role of cGMP in CORM-3-mediated effects. The concentration of ODQ was chosen based on previous publications (Foresti *et al*., 2004). FACS analysis was performed with 2×10^6 cells using the following FITC-conjugated monoclonal antibodies: anti-human intercellular adhesion molecule-1 (BBIG-I1), anti-human VCAM-1 (BBIG-V3) and anti-human E-selectin (BBIG-E5). Antibodies were added for 40 min at 4°C followed by extensive washing with PBS. FACS analysis was performed on a FACScalibur equipped with the CELLQuest software. The data were analysed by Windows Multiple Document Interface (WinMDI) software (Version 2.8).

Western blot analysis

Human umbilical vein endothelial cells were stimulated for different time periods with 50 $ng \cdot mL^{-1}$ TNF-α in the presence

or absence of CORM (1 mmol·L^{-1}). Hereafter, the cells were harvested and lysed in 50 µL lysis buffer containing 10 mmol·L^{-1} Tris-base, 150 mmol·L^{-1} NaCl, 5 mmol·L^{-1} EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 1 µmol·L^{-1} dithiothreitol (DTT) and proteinase inhibitor cocktail. In some experiments, nuclear proteins were isolated as previously described (Anrather *et al.*, 1999; Brouard *et al.*, 2002). Protein concentrations were assessed by using Coommassie reagents. Sodium dodecyl sulphate (SDS)-PAGE and Western blotting were performed essentially similar as described (Yard *et al.*, 2004). Briefly, samples were resolved on 10% SDS-PAGE and transferred onto PVDF filters by semi-dry blotting. The filters were incubated with 5% non-fat dry milk powder in PBS for 1 h at room temperature to block unspecific background staining and hereafter incubated overnight at 4°C with specific polyclonal antibodies, depending on the experiment that was performed. The following antibodies were used: polyclonal rabbit anti-HO-1 antibody, polyclonal rabbit anti-Nrf2 antibody, polyclonal rabbit anti-inhibitor of κB (IκB)α antibody, polyclonal goat anti-VCAM-1 antibody. After extensive washing in PBS/Tween/5% non-fat dry milk powder, the filters were incubated for 60 min with appropriate horseradish peroxidase-conjugated polyclonal IgG, followed by three times washing in PBS/Tween. Visualization of immunoreactive bands was performed by chemiluminescence reagent according to the manufacturer's instructions. The filters were re-probed with monoclonal anti-histone H1 antibody or monoclonal anti-GAPDH antibody to demonstrate equal loading.

Electrophoretic mobility shift assay

Nuclear extracts were obtained from HUVECs as described above. Protein concentrations were determined by Bradford assay. Electrophoretic mobility shift assay was performed essentially as previously described (Anrather *et al.*, 1999; Brouard *et al.*, 2002). Briefly, nuclear factor-κB (NF-κB) consensus oligonucleotides were labelled to a specific activity > 5×10^7 cpm·µg^{-1} DNA. Ten micrograms of nuclear extracts were added to 1 ng of labelled oligonucleotide in a total volume of 20 µL containing 10 mmol·L^{-1} HEPES (pH 7.5), 0.5 mmol·L^{-1} EDTA, 70 mmol·L^{-1} KCl, 2 mmol·L^{-1} DTT, 2% glycerol, 0.025% NP-40, 4% Ficoll, 0.1 mol·L^{-1} PMSF, 1 mg·mL^{-1} bovine serum albumin and 0.1 µg·µL^{-1} poly di/dc. In each experiment, specificity of binding was demonstrated by adding cold consensus or mutated NF-κB oligonucleotides to the nuclear extracts. In addition, supershifts were performed by adding anti-p65 and p55 antibodies to the samples. DNA–protein complexes were separated on 5% non-denaturating polyacrylamide gels and electrophoresis in low ionic strength buffer and visualized by autoradiography.

RNA isolation, reverse-transcriptase PCR, quantitative TaqMan PCR

Total RNA was isolated from confluent endothelial cell monolayers by using Trizol®-Reagent. Thereafter, DNase treatment was carried out, using RNase free DNase I (Ambion, Darmstadt, Germany). RNA concentration and quality were assessed by RNA 6000 Nano assays on a Bioanalyzer 2100 system (Agilent, Boeblingen, Germany). A total of 500 ng of total RNA was reverse-transcribed into cDNA using the 1st Strand cDNA Synthesis Kit. cDNA was diluted in 20 µL DEPC-treated water and stored at –20°C until use.

For reverse-transcriptase PCR 1 µL of cDNA was amplified in a 50 µL reaction mix containing 10 mmol·L^{-1} dNTPs, 50 pmol·L^{-1} of each primer, 2.5 units Taq polymerase and 1.5 mmol·L^{-1} $MgCl_2$. The primers used were as follows: HO-1 forward: 5′- GCT CAA CAT CCA GCT CTT TGA GG-3′ and reverse: 5′- GAC AAA GTT CAT GGC CCT GGG A-3′; VCAM forward: 5′- CGA TCA CAG TCA AGT GTT CAG TTG-3′ and reserve: 5′- GCA ATT CTT TTA CAG CCT GCC T-3′; GAPDH forward: 5′- GTC TTC ACC ACC ATG GAG AA-3′ and reserve: 5′- ATC CAC AGT CTT CTG GGT GG-3′. The cycling conditions used for various primers were as follows: 4 min of denaturation at 94°C, followed by 28 (VCAM-1) or 25 (HO-1 and GAPDH) cycles of amplification, each consisting of denaturation for 30 s at 94°C, annealing for 30 s at 59°C (VCAM-1), 62°C (HO-1) or 55°C (GAPDH) and extension for 45 s at 72°C. After the last amplification a final extension for 10 min at 72°C was performed for each reaction. PCR products were analysed on a 1% agarose gel containing ethidium bromide.

Quantitative real-time RT-PCR was performed on the ABI-Prism 7700 sequence detection system with the TaqMan universal PCR master mix No AmpErase UNG (part no. 4324018). Taqman probes for VCAM-1 (part No. HS00174239_m1) and β-actin (part No. HS99999903_m1). Samples were run under the following conditions: initial denaturation for 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The levels of gene expression in each sample were determined with the comparative cycle threshold method. PCR efficiency was assessed from the slopes of the standard curves and was found to be between 90% and 100%. Linearity of the assay could be demonstrated by serial dilution of all standards and cDNA. All samples were normalized for an equal expression of β-actin. Each experiment was repeated three times with similar results.

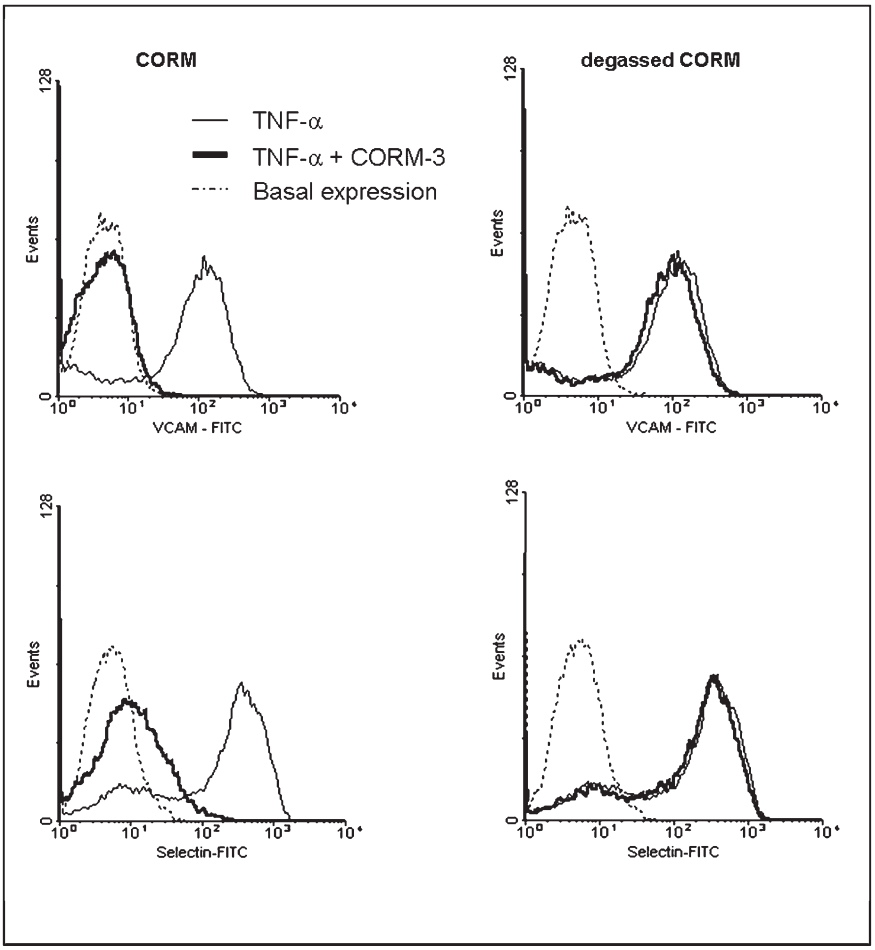
Reporter assays

Human umbilical vein endothelial cells were transfected with a reporter construct containing 3 hypoxia-responsive elements (3HRE.luc) (Viemann *et al.*, 2007) or a construct containing an antioxidant-responsive element from the HO-1 promoter (HO-ARE.luc) (Banning and Brigelius-Flohe, 2005). All transfections were performed by using DEAE-dextran as described previously (Goebeler *et al.*, 1999), and in each case the ubiquitin-dependent Renilla luciferase reporter was co-transfected. Two days after transfection, cells were stimulated for 24 h with CORM-3. Luciferase activity was measured by using the Dual-Glo Luciferase Assay System. All experiments were performed in triplicate and ARE- or HRE-dependent luciferase activities were normalized for luciferase activity generated by the Renilla luciferase control reporter. The results are expressed as fold increase compared with unstimulated controls.

Cell transfection with siRNA

Human umbilical vein endothelial cells were seeded in 12 well plates at a density of 0.5–2 × 10^5 cells, 1 day before transfection

A


CORM
degassed CORM
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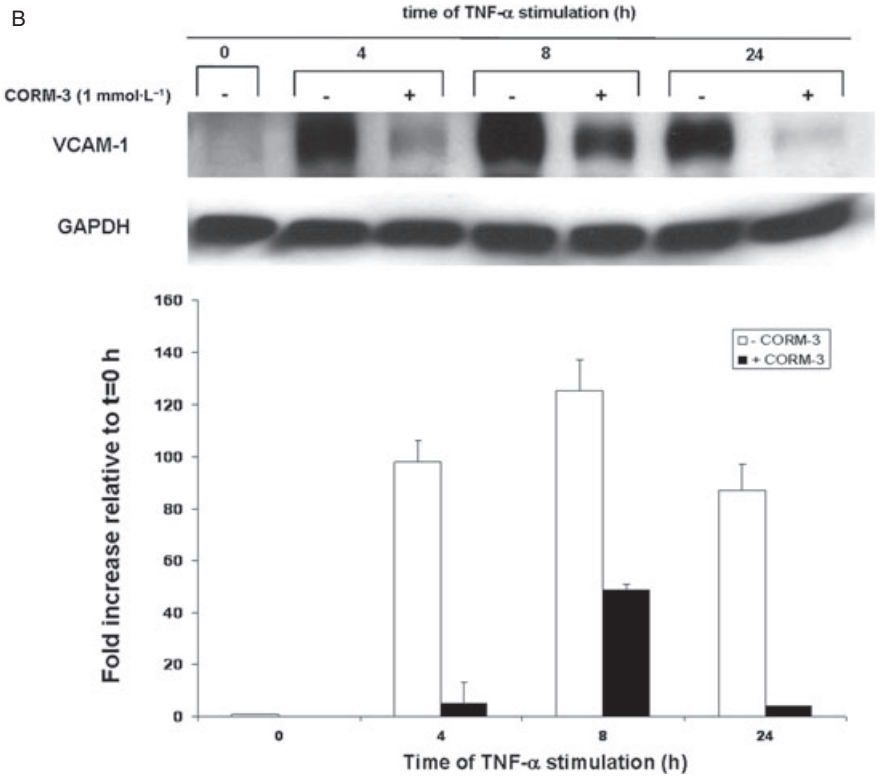
B


time of TNF-α stimulation (h)
0
4
8
24
CORM-3 (1 mmol·L⁻¹)
VCAM-1
GAPDH
Fold increase relative to t=0 h
- CORM-3
+ CORM-3
Time of TNF-α stimulation (h)

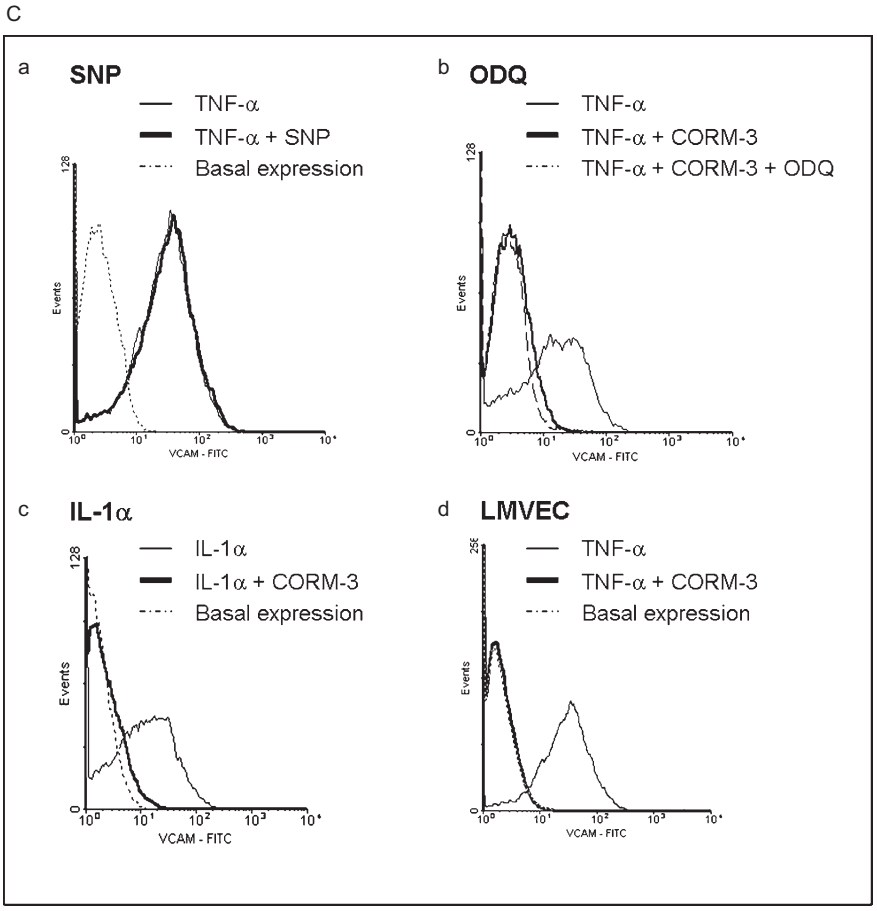


Figure 1 Modulation of TNF-α-induced expression of adhesion molecules by CORM-3. (A) HUVEC were stimulated for 24 h with TNF-α (50 ng·mL^{-1}) in the absence or presence of CORM-3 (1 mmol·L^{-1}) and surface expression of VCAM-1 and E-selectin determined by flow cytometry. The basal expression of adhesion molecules is also shown. Expression of VCAM-1 and E-selectin was inhibited in cells exposed to CORM-3 (left panel). Addition of degassed CORM-3 solution at the same concentration did not affect expression of these two adhesion molecules (right panel). Flow cytometry profiles shown are from one experiment and are representative of eight independent preparations of HUVECs. (B) Time-dependent modulation of VCAM-1 expression by CORM-3. HUVEC were stimulated for different time periods with TNF-α (50 ng·mL^{-1}) in the absence (–) or presence (+) of CORM-3 (1 mmol·L^{-1}). Cell lysates were prepared and subjected to Western blotting. HUVEC cultured in growth medium were used as control. Membranes were incubated with anti-VCAM-1 antibody and then reprobed with anti-GAPDH antibody to control for equal loading (upper panel). Summary data (mean ± SD) from four independent experiments is shown in the lower panel. (C) In the upper left panel (a), HUVEC were stimulated with TNF-α in the presence or absence of SNP (500 μmol·L^{-1}). Unstimulated HUVEC were included in each experiment. In (b), HUVEC were stimulated with TNF-α (50 ng·mL^{-1}), TNF-α and CORM-3 (1 mmol·L^{-1}), or HUVEC were pretreated for 1 h with ODQ (10 μmol·L^{-1}) before TNF-α and CORM-3 were added. The lower panels show the influence of COM-3 on VCAM-1 expression in HUVEC stimulated with IL-1α (c) and on VCAM-1 expression in LMVEC stimulated with TNF-α (d). The results of a single experiment are shown, representative of three independent experiments performed. CORM, carbon monoxide releasing molecule; CORM-3, tricarbonylchloro(glycinato)ruthenium(II); HUVEC, human umbilical vein endothelial cell; LMVEC, lung microvascular endothelial cell; ODQ, 1H-[1, 2, 4]oxadiazolo[4, 3-alpha]quinoxalin-1-one; SNP, sodium nitroprusside; TNF, tumour necrosis factor; VCAM-1, vascular cell adhesion molecule-1.

with HO-1 siRNA, Nrf2 siRNA or control siRNA. Transfection was performed according to the manufacturer's instructions. Briefly, cells were incubated for 6 h in transfection medium supplemented with siRNA and transfection reagent. Hereafter, endothelial cell culture medium containing 20% FBS was added without removing the transfection solution, and the cells were allowed to grow for an additional 24 h. For each experiment the efficacy of siRNA was demonstrated by disappearance of the specific band in Western blot analysis.

Statistical analysis

Data are presented as mean ± SD for the indicated number of separate experiments. All analyses were based on more than three separate experiments. Differences between groups were determined by Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

Materials

Reagents were obtained from the sources as indicated: endothelial cell culture medium (Promocell, Heidelberg, Germany), PBS (GIBCO, Invitrogen, NY, USA), FBS Gold (PAA laboratories GmbH, Pasching, Austria), trypsin/EDTA solution, DMSO, Tween 20, DEAE-Dextran, chloroquine, HEPES, Triton X-100, DTT, sodium deoxycholate, Tris-base, EDTA, ammonium persulphate, SDS, N,N,N′,N′-tetramethylethylenediamine, TEMED, tricarbonyldichlororuthenium(II) dimer, glycine,

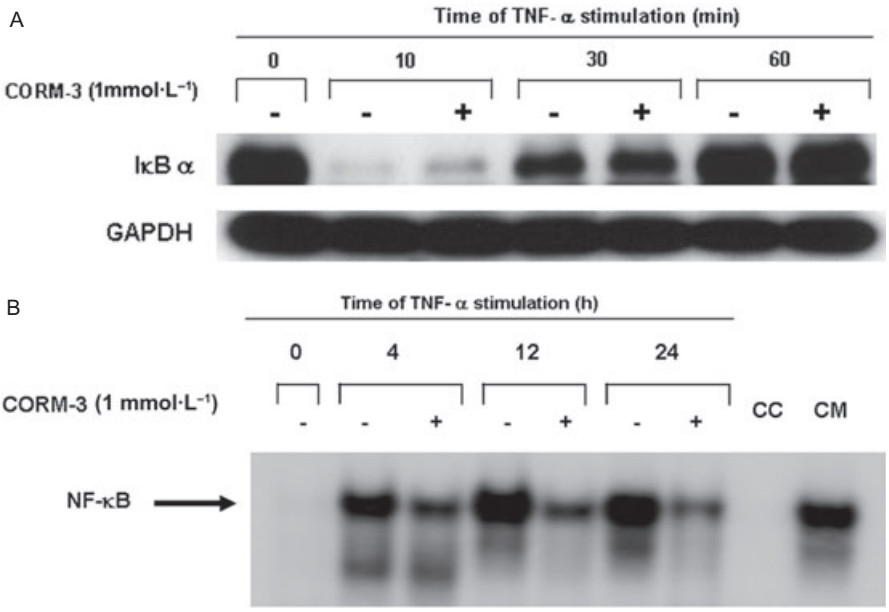
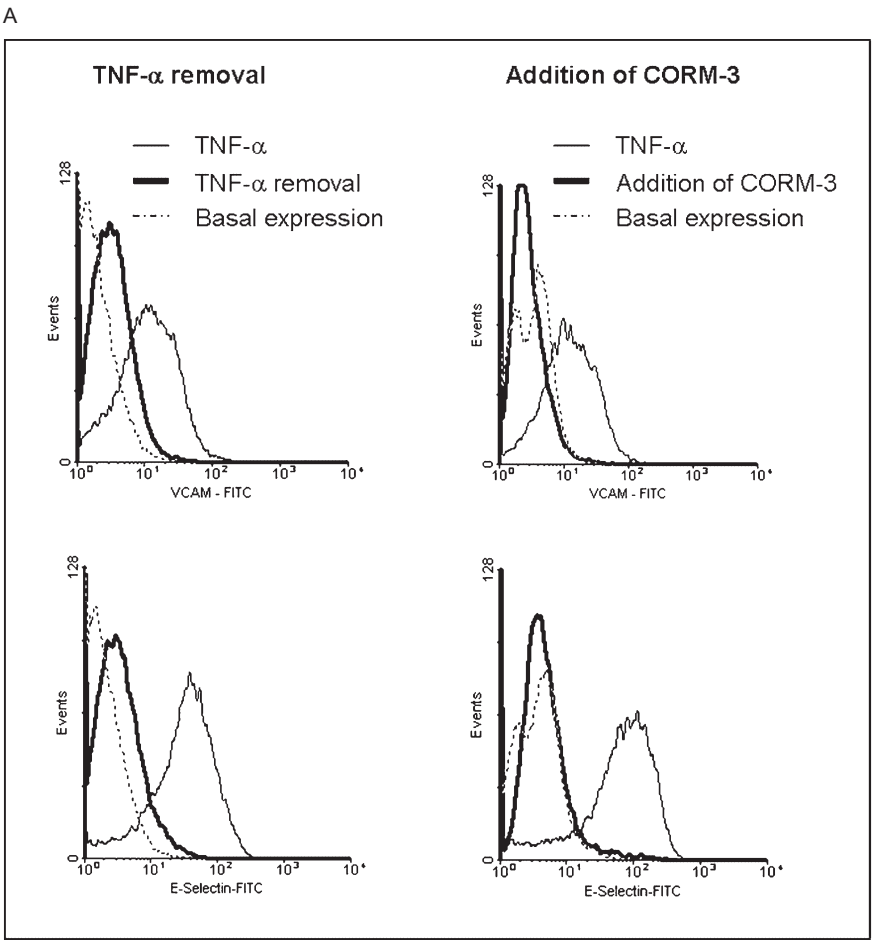


Figure 2 Influence of CORM-3 on TNF-α-mediated NF-κB activation. (A) HUVEC were stimulated for 10, 30 or 60 min with TNF-α (50 ng·mL^{-1}) in the absence (–) or presence (+) of CORM-3 (1 mmol·L^{-1}). Endothelial cells left in culture medium served as control ($t = 0$). Whole cell lysates were prepared and analysed by Western blotting for IκBα (and GAPDH to demonstrate equal sample loading; lower blots). (B) Influence of CORM-3 on NF-κB activation. HUVEC were stimulated as in (A), nuclear extracts were prepared at 0, 4, 12 and 24 h of TNF-α stimulation and used for electrophoretic mobility shift assays as described in *Methods*. Specificity of the bands was determined by addition of a 100-fold of unlabelled consensus (CC: cold consensus) or unlabelled mutant (CM: cold mutant) oligonucleotides to the samples. In (A) and (B), the results of a single experiment are shown, representative of at least four different experiments performed. CORM-3, tricarbonylchloro(glycinato)ruthenium(II); HUVEC, human umbilical vein endothelial cell; NF-κB, nuclear factor-κB; TNF, tumour necrosis factor.



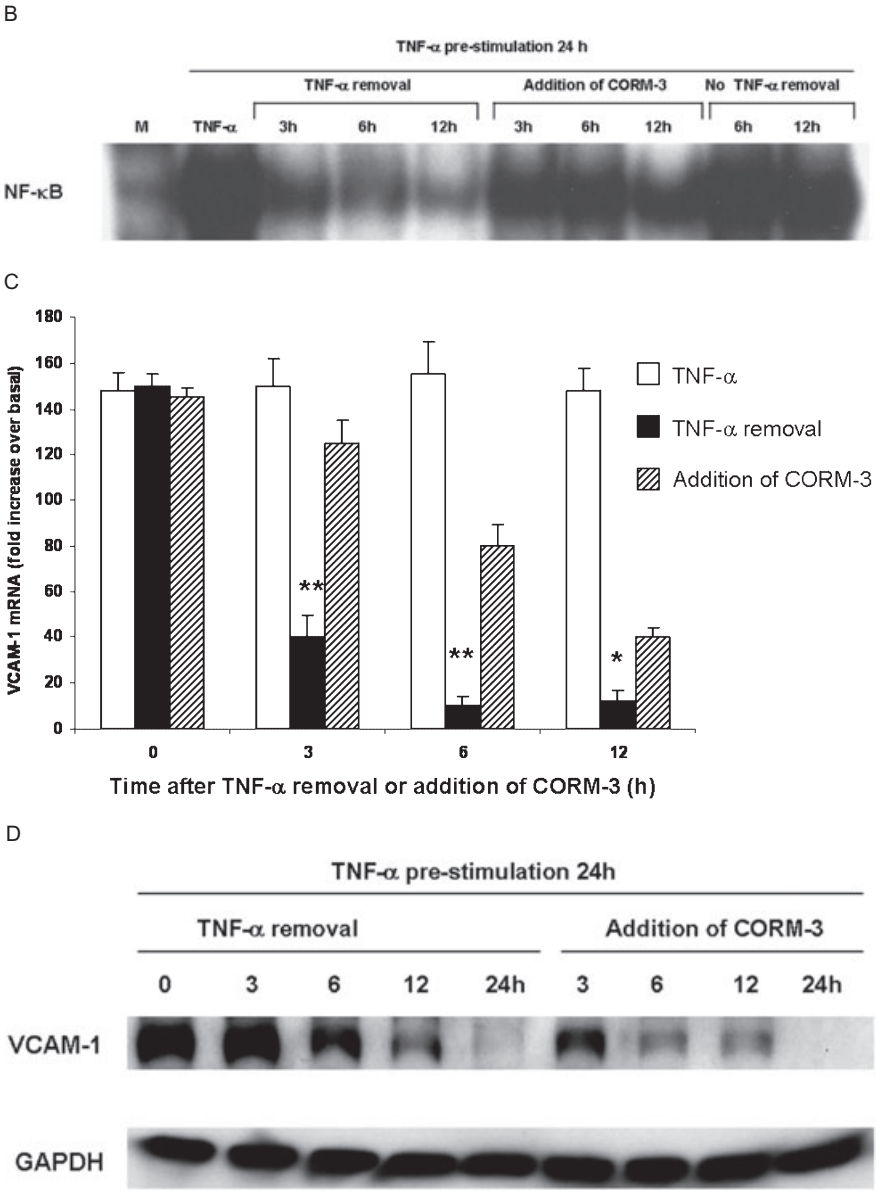


Figure 3 Influence of CORM-3 or TNF-α removal on (A) surface expression of adhesion molecules, (B) NF-κB activation, (C) expression of VCAM-1 mRNA and (D) expression of VCAM-1 protein. HUVEC were stimulated for 24 h with TNF-α (50 ng·mL^{-1}) and then the cells were washed to remove TNF-α, or CORM-3 was added in the continued presence of TNF-α. Cells that were kept in the presence of TNF-α alone for similar time periods served as control. (A) Surface expression of VCAM-1 and E-selectin after TNF-α stimulation, after addition of CORM-3 or removal of TNF-α. Basal expression of these adhesion molecules is also shown. (B) Nuclear extracts were prepared for electrophoretic mobility shift assays at 3, 6 and 12 h after TNF-α removal or after addition of CORM-3. Nuclear extracts were also prepared directly after 24 h of TNF-α stimulation (TNF-α) or from cells that were kept in the presence of TNF-α but not exposed to CORM-3 (no TNF-α removal). Cells that were kept in culture medium (M) were included to demonstrate TNF-α-mediated NF-κB activation. (C) Steady-state VCAM-1 mRNA expression. HUVEC were stimulated with TNF-α for 24 h. Then cells were kept in the presence of TNF-α or washed, or CORM-3 was added to the cells. Subsequently, the cells were cultured for various time periods and total RNA was isolated. Results are expressed as fold increase compared with basal VCAM-1 mRNA expression. Significant differences were found between TNF-α removal and CORM-3 addition at $T = 3$ h, $T = 6$ h (**$P < 0.01$) and $T = 12$ h (*$P < 0.05$; $n = 6$). (D) HUVECs were treated as in (C). At various time periods after TNF-α removal or CORM-3 addition, cell extracts were prepared. VCAM-1 protein was analysed by Western blotting. In (A), (B) and (D), the results of a single experiment are shown, representative of at least three independent experiments performed. CORM-3, tricarbonylchloro(glycinato)ruthenium(II); HUVEC, human umbilical vein endothelial cell; NF-κB, nuclear factor-κB; TNF, tumour necrosis factor; VCAM-1, vascular cell adhesion molecule-1.

sodium ethoxide, human recombinant TNF-α, human recombinant IL-1β, SNP, ODQ (Sigma, St. Louis, MO, USA), bovine serum albumin (SERVA, Heidelberg, Germany), protease inhibitor cocktail, first strand cDNA synthesis Kit (Roche Diagnostic, Mannheim, Germany), NF-κB consensus oligonucleotides, Dual-Glo Luciferase Assay System (Promega, Mannheim, Germany), Coomassie protein assay reagent (Pierce, Rockford, IL, USA), Trizol (Invitrogen, Carlsbad, CA, USA), chloroform, isopropanol, tetrahydrofuran, β-mercaptoethanol (Merck, Darmstadt, Germany). Primers

and all reagents were purchased for TaqMan PCR (ABI, Darmstadt, Germany). All antibodies for flow cytometric analysis were purchased from R&D System (Minneapolis, MN, USA) and all FACS reagents from Becton Dickinson (Heidelberg, Germany). All antibodies used for Western blotting, including horseradish peroxidase conjugates, antibodies for supershifts as well as siRNAs were purchased from Santa Cruz Biotechnology (Heidelberg, Germany) with exception of anti-HO-1 antibody (Stressgen, Victoria, Canada), anti-GAPDH and anti-eNOS (endothelial nitric oxide synthase) antibodies (Abcam Cambridge, UK). Chemiluminescence reagent was purchased from PerkinElmer LAS Inc. (Boston, MA, USA).

Results

Inhibition of adhesion molecules by CORM-3

CORM-3 inhibited TNF-α-mediated induction of VCAM-1 and E-selectin in all cell lines tested ($n = 8$) (Figure 1A, left panel). Inhibition of adhesion molecule expression was mediated by the release of CO as a degassed solution of CORM-3 was ineffective (Figure 1A, right panel). To exclude the possibility that loss of adhesion molecule expression was due to proteolytic cleavage from the cell membrane, Western blot analysis with whole cell lysates was performed. As demonstrated for VCAM-1, induction by TNF-α was significantly attenuated by CORM-3 and was completely absent when endothelial cells were stimulated for 24 h in the presence of CORM-3 (Figure 1B). CORM-3 did not induce the expression of iNOS (inducible nitric oxide synthase) nor was the expression of eNOS influenced by CORM-3 (data not shown). To formally show that down-regulation of VCAM-1 was not mediated by NO, the influence of the NO donor SNP on TNF-α-mediated VCAM-1 expression was tested. SNP used in a wide range of concentrations (10–1000 $\mu mol \cdot L^{-1}$) did not influence the expression of VCAM-1 on TNF-α-stimulated HUVEC (Figure 1C a). Down-regulation of VCAM-1 was also not mediated via cGMP as inhibition of guanylate cyclase by ODQ did not alter the effect of CORM-3 (Figure 1C b). We also assessed whether down-regulation of VCAM-1 by CORM-3 also occurred when HUVEC were stimulated with IL-1β and whether CORM-3 was also effective on lung microvascular endothelial cells. As shown in Figure 1C, CORM-3 also inhibited the expression of VCAM-1 in IL-1α-stimulated HUVEC (Figure 1C c) and was also effective when microvascular endothelial cells were used (Figure 1C d).

CORM-3 acts through the NF-κB pathway

As up-regulation of adhesion molecules depends on activation of NF-κB, we next assessed whether CORM-3 interferes with this process. Within 1 h of TNF-α stimulation, NF-κB-binding activity was detected in nuclear extracts of endothelial cells. However, neither at this time point nor after 2 h of TNF-α stimulation, did CORM-3 significantly affect NF-κB-binding activity (data not shown). Moreover, there was no effect of CORM-3 on the degradation of IκBα (Figure 2A). In contrast to these early time points, NF-κB-binding activity was significantly reduced at later times (4–24 h) after TNF-α stimulation in CORM-3-treated cells (Figure 2B).

The presence of TNF-α was required to maintain VCAM-1 and E-selectin expression on endothelial cells, as an almost complete down-regulation of these adhesion molecules occurred within 24 h after TNF-α removal. Interestingly, when CORM-3 was added after 24 h to the TNF-α-containing culture medium, this also resulted in a complete loss of VCAM-1 and E-selectin expression (Figure 3A). Hence, CORM-3 is able to down-regulate VCAM-1 and E-selectin even in the continued presence of TNF-α.

Tumour necrosis factor-α removal resulted in a rapid decrease in nuclear NF-κB-binding activity, while this was clearly much slower when CORM-3 was added (Figure 3B). In line with these observations, steady-state VCAM-1 mRNA expression decreased much faster when TNF-α was removed compared with addition of CORM-3 (Figure 3C). Nevertheless, in time–response experiments we repeatedly observed that disappearance of VCAM-1 protein occurred much faster upon addition of CORM-3 than after TNF-α removal (Figure 3D). CORM-3 did not influence stabilization of VCAM-1 mRNA, as steady-state VCAM-1 mRNA decreased to a similar extent in actinomycin D-treated HUVEC both in the absence or presence of CORM-3 (data not shown).

HO-1 is induced by CORM-3 in an Nrf2-dependent fashion

CORM-3 induced the expression of HO-1 in a time- and dose-dependent manner. While HO-1 was already slightly up-regulated at a concentration of 100 $\mu mol \cdot L^{-1}$, this was much more pronounced when 1 $mmol \cdot L^{-1}$ was used (data not shown). At the latter concentration, up-regulation of HO-1 protein was apparent as soon as 3 h after addition of CORM-3 (Figure 4A, upper panel). PCR analysis revealed that up-regulation of HO-1 mRNA occurred at concentrations similar to those inducing HO-1 protein (Figure 4A, lower panel).

Because these data suggested that CORM-3-mediated HO-1 expression was transcriptionally regulated, we next investigated the involvement of three transcription factors (NF-κB, hypoxia induced factor (HIF)-1α and Nrf2), shown to influence HO-1 transcription. Neither NF-κB activation nor HIF-1α accumulation occurred after CORM treatment (data not shown). In contrast, nuclear translocation of Nrf2 was clearly evident 1 h after CORM-3 treatment (Figure 4B). CORM-3 did not induce the expression of a HRE-dependent luciferase reporter construct, while the expression of luciferase was significantly increased by CORM-3 in cells transfected with an ARE-dependent reporter (Figure 4C).

CORM-3-induced HO-1 expression does not contribute to inhibition of VCAM-1 expression

Because CORM-3 induces HO-1 expression, an HO-1-mediated rather than a direct CO effect on VCAM-1 expression could not be excluded. To address this issue, we first stimulated endothelial cells with 1 $mmol \cdot L^{-1}$ of CORM-3 to induce HO-1 followed by a challenge with TNF-α. Despite an increase in HO-1 expression, induction of VCAM-1 by TNF-α was not influenced in these cells (data not shown). To formally exclude a role for HO-1 in the down-regulation of VCAM-1 expression, we used appropriate siRNAs to silence HO-1 or Nrf2 expression and the expression of both HO-1 and

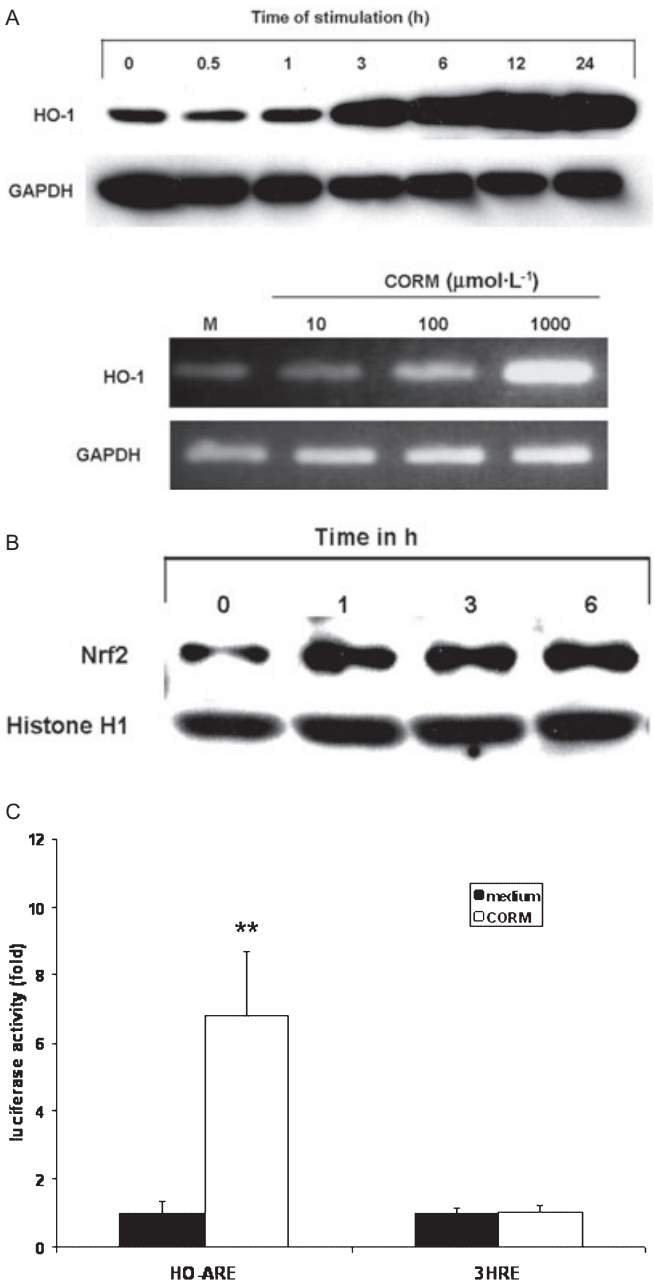


Figure 4 Induction of HO-1 by CORM-3. (A) HUVECs were incubated for different times CORM-3 (1 mmol·L^{-1}). Cell lysates were analysed by Western blot for HO-1 and GAPDH expression (upper panel). To demonstrate that HO-1 expression was regulated at the transcriptional level, total RNA was isolated from HUVEC incubated with different concentrations of CORM-3. RT-PCR for HO-1 and GAPDH was subsequently performed (lower panel). (B) Nuclear translocation of Nrf2 was assessed by Western blot analysis of nuclear protein isolated from HUVEC that were stimulated for different times with CORM-3 (1 mmol·L^{-1}). (C) HUVECs were transfected with reporter constructs containing either the ARE consensus sequence of the HO-1 promoter (HO-ARE) or with a reporter construct containing 3HRE. In each case co-transfection was performed with the ubiquitin-dependent Renilla luciferase reporter. Luciferase activities were normalized for luciferase activity generated by the Renilla luciferase control reporter. The results are expressed as fold increase compared with unstimulated controls. A significant effect was found only for the HO-ARE reporter, $^{**}P < 0.01$ untreated versus CORM-3 treated ($n = 5$). In (A) and (B) the results of a single experiment are shown, representative of at least four independent experiments performed. 3HRE, 3 hypoxia-responsive elements; ARE, antioxidant-responsive element; CORM, carbon monoxide releasing molecule; CORM-3, tricarbonylchloro(glycinato)ruthenium(II); HO-1, haem oxygenase-1; HUVEC, human umbilical vein endothelial cell.

Nrf2 was almost completely prevented (Figure 5A). Nevertheless, neither TNF-α-induced VCAM-1 expression, nor its inhibition by CORM-3 was significantly changed in HO-1- and Nrf2-depleted endothelial cells respectively (Figure 5B upper panel). However as expected from the critical role of Nrf2 in HO-1 induction, treatment of cells with siRNA for Nrf2 abolished the up-regulation of HO-1 by CORM-3 (Figure 5B, lower panel).

Discussion

Based on current evidence, the HO-1 pathway is of undisputable importance for the control of inflammation. Overexpression of HO-1 modulates pro-inflammatory processes in a number of cells including macrophages and endothelial cells (Otterbein *et al.*, 2000; Soares *et al.*, 2004; Chung *et al.*, 2005; Sacerdoti *et al.*, 2005), yet, the mechanisms by which HO-1 exerts its salutary effects are not completely delineated. In particular there is controversy over the ability of CO to down-regulate the expression of adhesion molecules on endothelial cells (Soares *et al.*, 2004; Seldon *et al.*, 2007; Urquhart *et al.*, 2007). In the present study, we have shown that CORM-3, used at 1 mmol·L^{-1}, consistently inhibited the up-regulation of VCAM-1 and E-selectin after TNF-α stimulation. In addition, we demonstrated that CORM-3 did not inhibit the initial activation of NF-κB by TNF-α, but CORM-3 did inhibit the sustained activation of this pathway. Finally we report that although CORM-3 induced HO-1 in an Nrf2-dependent fashion, neither HO-1 nor Nrf2 contributed to the inhibitory effect of CORM-3 on VCAM-1 and E-selectin expression.

CORM-3 modulates inflammatory processes in a variety of *in vivo* and *in vitro* models (Urquhart *et al.*, 2007; Ferrándiz *et al.*, 2008; Masini *et al.*, 2008). In most of these models, CORM-3 was used at substantially lower concentrations than we have used in the present study. This concentration was chosen on the basis of concentration–response experiments. While 100 µmol·L^{-1} of CORM-3 was ineffective, concentrations above 0.5 mmol·L^{-1} effectively decreased TNF-α-mediated VCAM-1 and E-selectin expression. No cell toxicity was observed for the concentrations that were tested as demonstrated by Trypan blue exclusion. Both in the studies from Urquhart *et al.* (2007) and Soares *et al.* (2004), CO, either given as CORM-3 or as gas, was not able to inhibit VCAM-1 and E-selectin expression on TNF-α-stimulated endothelial cells. This is likely to be due to the lower concentrations that were used in these studies. It therefore appears that CO only inhibits VCAM-1 and E-selectin expression when used at supra-physiological concentrations. While CORM-3 at 1 mmol·L^{-1} results in the release of 1 mmol·L^{-1} CO, a concentration far in excess of that endogenously generated, this

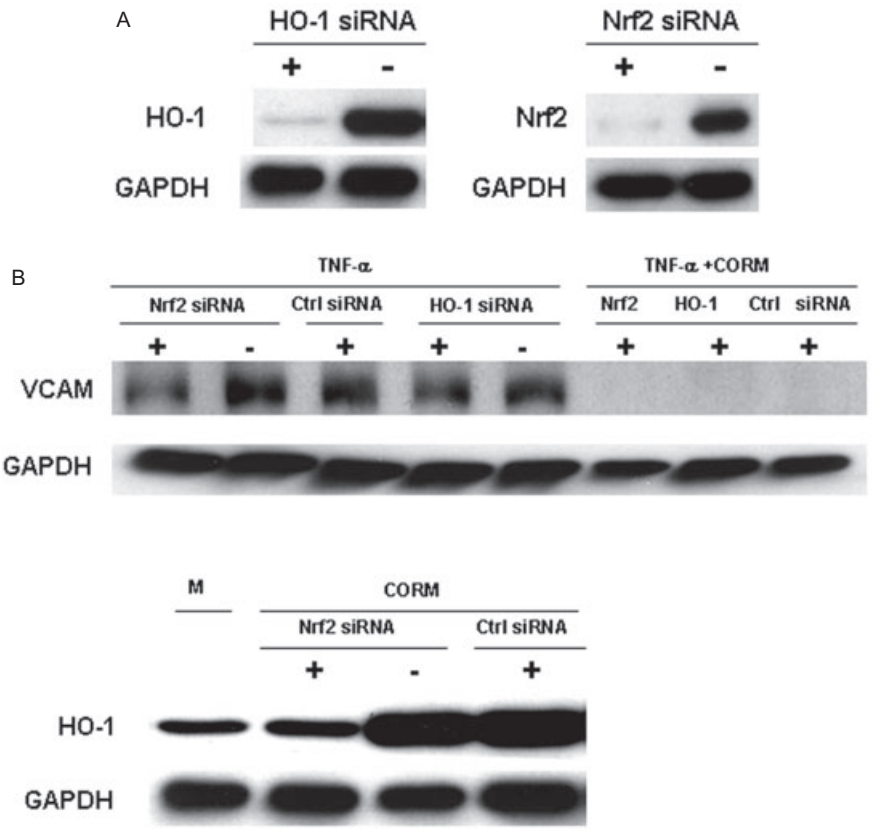


Figure 5 Induction of HO-1 is not involved in CORM-3-mediated down-regulation of VCAM-1 expression. (A) HUVECs were transfected with Nrf2, HO-1 or control siRNA (Ctrl siRNA). Two days after transfection, the expression of HO-1 and Nrf2 was assessed by Western blot analysis. (B) Transfected cells were subsequently stimulated for 24 h with TNF-α (50 ng·mL^{-1}) or TNF-α plus CORM-3 (1 mmol·L^{-1}). The expression of VCAM-1 (upper panel) and HO-1 (lower panel) was assessed by Western blotting. The results of a single experiment are shown, representative of at least three independent experiments performed. CORM, carbon monoxide releasing molecule; CORM-3, tricarbonylchloro(glycinato) ruthenium(II); HO-1, haem oxygenase-1; TNF, tumour necrosis factor; VCAM-1, vascular cell adhesion molecule-1.

finding also suggests that under physiological conditions CO generated by the HO-1 system might not be able to inhibit the expression of these adhesion molecules. Nevertheless, our findings could be of therapeutic relevance in situations of unwanted inflammation that are not sufficiently controlled by HO-1.

Interestingly, the mechanism by which CORM-3 inhibits VCAM-1 and E-selectin expression is almost similar to that described for HO-1 over-expression. Recent studies have shown that bilirubin and/or Fe^{2+} chelation, both of which are increased as a consequence of HO-1 activity, play an essential role in the HO-1-mediated down-regulation of adhesion molecules (Soares *et al.*, 2004; Seldon *et al.*, 2007). Bearing in mind that CO has a high affinity for Fe^{2+} and acts as ligand to complete the coordination shell of this ion, it is likely that CO–Fe complexes will form (Piantadosi, 2002; Watts *et al.*, 2003). Hence, if Fe^{2+} chelation can inhibit TNF-α-mediated expression of adhesion molecules, not surprisingly, similar effects can be expected by the addition of CORM-3.

Our study also suggests that inhibition of VCAM-1 and E-selectin expression by CORM-3 might, to some extent, be independent of its effect on the NF-κB pathway. This is emphasized by the finding that TNF-α removal switched off the NF-κB pathway much faster than addition of CORM-3 did, although the levels of VCAM-1 protein fell much more rapidly after CORM-3 treatment. Hence CORM-3 not only regulates VCAM-1 at the transcriptional level, but also possibly acts on VCAM-1 translation or protein turnover. To our knowledge, this has not been described for HO-1-mediated down-regulation of adhesion molecules.

Given that over-expression of HO-1 inhibits VCAM-1 expression (Soares *et al.*, 2004) and that CORM-3 at 100 μmol·L^{-1} was already able to up-regulate HO-1, it was surprising that these low concentrations of CORM-3 were not able to modulate VCAM-1 or E-selectin expression. This argues against the assumption that up-regulation of HO-1 per se exerts anti-inflammatory properties. Indeed, Foresti *et al.* (2001) showed in a hypoxia model using cardiomyocytes that over-expression of HO-1 was only protective if sufficient haemin was added during the hypoxic phase. It would therefore seem that substrate availability is an important factor that influences the anti-inflammatory effect of HO-1. Because CO can stabilize haem-containing proteins, as was recently demonstrated for cytochrome P450 (Nakao *et al.*, 2008), limited substrate availability and/or insufficient up-regulation of HO-1 at low CORM-3 concentrations may explain our findings. HO-1 up-regulation was more pronounced when 1 mmol·L^{-1} of CORM-3 was used, but even under these conditions HO-1 did not play a significant role in the inhibition of VCAM-1 and E-selectin expression. This is emphasized by

the finding that CORM-3 at this high concentration was still an effective inhibitor of adhesion molecule expression when induction of HO-1 was blocked by the relevant siRNA.

In conclusion, our study demonstrates that supra-physiological CORM-3 concentrations can down-regulate VCAM-1 and E-selectin expression, even in the continued presence of TNF-α. Although HO-1 was induced by CORM-3, it did not influence the expression of adhesion molecules. CORM-3 inhibited sustained NF-κB activation leading to down-regulation of VCAM-1 and E-selectin. In addition to its effects on the NF-κB pathway, CORM-3 also inhibited the expression of VCAM-1 either via translational regulation or regulation in protein turnover. How CORM-3 regulates translation or protein turnover is at present unclear.

Acknowledgement

This study was supported by a grant of the German Research Foundation (DFG, European Graduate College, GRK880).

Conflict of interest

None.

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