

The Contact Allergen Nickel Triggers a Unique Inflammatory and Proangiogenic Gene Expression Pattern via Activation of NF- κ B and Hypoxia-Inducible Factor-1 α ¹

Dorothee Viemann,* Marc Schmidt,[†] Klaus Tenbrock,* Sybille Schmid,[†] Verena Müller,[†] Kerstin Klimmek,* Stephan Ludwig,[‡] Johannes Roth,* and Matthias Goebeler^{2†}

Nickel compounds are prime inducers of contact allergy reactions in humans. To identify the signal transduction pathways mediating the cellular responses to nickel and to elucidate their hierarchy, we performed Affymetrix gene profiling using human primary endothelial cells, which strongly respond to nickel stimulation. Overall, we found 258 significantly modulated transcripts, comprising 140 up-regulated and 118 down-regulated genes. The bulk of those genes were identified as targets of two distinct signaling cascades, the IKK2/NF- κ B pathway and a proangiogenic pathway mediated by HIF-1 α , which accumulates upon exposure to nickel. Using dominant-interfering mutants and retroviral RNA interference technology, we demonstrate that both pathways act independently to regulate expression of nonoverlapping gene pools. NF- κ B activation mediates most of the proinflammatory responses to nickel. Nickel-dependent HIF-1 α activation primarily modulates expression of genes involved in proliferation, survival, metabolism, and signaling, albeit the induction of some proinflammatory nickel-response genes, most prominently IL-6, which we identified as novel bona fide HIF-1 α target in this study, is also critically dependent on this pathway. Furthermore, we provide evidence that transactivation of both transcription factors partially depends on p38 MAPK activation that contributes to the intensity of at least some target genes. Taken together, our data provide mechanistic insight into the complex network of nickel-induced cellular events and identify IKK2/NF- κ B and HIF-1 α as important pathways involved in processes such as delivery of “second signals” in contact hypersensitivity reactions to nickel. *The Journal of Immunology*, 2007, 178: 3198–3207.

Endothelial cells (ECs),³ located between blood and tissue compartments are important components of the innate immune system. Their activation by endogenous or environmental factors is a key event in the initiation of inflammatory responses. A strong activator of endothelium is the transition metal nickel that is widely distributed in a multitude of products. Contact with nickel is potentially hazardous for health: nickel compounds not only act as carcinogens in humans and animals (1) but also represent potent allergens (haptens). In industrialized countries, nickel is the most frequent cause for contact hypersensitivity re-

actions (2) with an estimated rate of 39% of young women being sensitized (3). Nickel is furthermore of great relevance in the context of biocompatibility of cardiovascular stents and orthopedic and dental biomedical alloys (4–6). Nickel ions exert proinflammatory and irritant properties, which, in addition to their sensitizing capacity, provide a “second signal” in hypersensitivity reactions (7).

Studying primary ECs, we earlier demonstrated that divalent nickel ions are capable of directly inducing endothelial adhesion molecules such as ICAM-1, VCAM-1, and E-selectin (8). Further analysis revealed that transcription of these genes is mediated via the IKK2/I κ B α /NF- κ B signal transduction pathway, which is activated in response to soluble nickel compounds but not to other divalent cations (9, 10). NF- κ B activation is initiated by IKK2-mediated phosphorylation and subsequent proteasomal degradation of I κ B proteins, which allows nuclear translocation and binding of the transcription factor to specific DNA motifs in the promoter region of target genes (11). The effects of nickel thus resemble those induced by proinflammatory cytokines such as TNF- α or IL-1 β that activate NF- κ B and MAPK pathways in ECs (10, 12).

In an attempt to better understand the molecular effects of nickel, we used Affymetrix U133A gene chip arrays to study gene expression profiles of primary human ECs upon exposure to nickel. Unlike other proinflammatory stimuli such as TNF- α , which exert their effect on endothelial gene expression almost exclusively via activation of the IKK2/NF- κ B pathway (13), nickel modulated endothelial gene expression via stimulation of at least two separate signaling cascades, the IKK2/NF- κ B pathway and the hypoxia-inducible transcription factor-1 α (HIF-1 α) pathway. Both pathways are partially dependent on the p38 MAPK pathway and

*Institute of Experimental Dermatology, Interdisciplinary Center of Clinical Research, and Department of Pediatrics, University of Münster, Münster, Germany;

[†]Department of Dermatology, University Medical Center Mannheim, University of Heidelberg, Mannheim, Germany; and [‡]Institute of Molecular Virology, University of Münster, Münster, Germany

Received for publication July 18, 2006. Accepted for publication December 16, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work is supported by the Deutsche Forschungsgemeinschaft Grants Go 811/1-5 (to M.G.), SFB293, A16 (to J.R.), and A17 (to S.L.) and Interdisciplinary Clinical Research Centre of the University of Münster Grant F62/005/06 (to D.V.).

² Address correspondence and reprint requests to Dr. Matthias Goebeler, Department of Dermatology, University Hospital Medical Center, University of Heidelberg, Theodor-Kutzer-Ufer 1-3, D-68135 Mannheim, Germany. E-mail address: matthias.goebeler@haut.ma.uni-heidelberg.de

³ Abbreviations used in this paper: EC, endothelial cell; ChIP, chromatin immunoprecipitation; dn, dominant negative; dnHIF-1 α , dn mutant of HIF-1 α ; HIF-1 α , hypoxia-inducible transcription factor-1 α ; HRE, hypoxia-responsive element; IKK2kd, kinase-dead IKK2; qRT-PCR, quantitative real-time RT-PCR; PCA, principal component analysis; shRNA, small hairpin RNA; VEGF, vascular endothelial growth factor.

regulate functionally distinct, nonredundant pools of genes that in combination account for the proinflammatory properties of nickel.

Materials and Methods

Cell Culture

Primary HUVECs were obtained from Cambrex and cultured as described previously (13). Cells were stimulated with 1.5 mM NiCl₂·6H₂O (Merck; subsequently referred to as Ni²⁺), 2 ng/ml TNF-α (R&D Systems), or medium as control in the absence or presence of SB202190 (Calbiochem).

Plasmids

The pCFG5-IEGZ retroviral vector containing kinase-dead IKK2 (IKK2kd), coupling its expression to a GFP-zeocin resistance fusion gene via an internal ribosomal entry site, has been described earlier (14). A dominant-negative (dn) mutant of human HIF-1 α (aa 1–311) was provided by Dr. D. Richard (Centre de Recherche de L'Hôtel-Dieu de Quebec, Quebec, Canada) (15) and cloned into pCFG5-IEGZ; a dn mutant of MKK6 (MKK6(Ala)) was supplied by Dr. R. J. Davies (Howard Hughes Medical School, University of Massachusetts, Worcester, MA) and inserted into pBABE.puro for retroviral infections. For RNA interference studies, a 64-mer dsDNA oligo (Sigma-Genosys) containing a specific 19-mer sequence complementary to human HIF-1 α (sequences as published by Ref. 16) was ligated into the pRETRO-SUPER (pRS) retroviral vector (17) that enables stable expression of small hairpin RNA (shRNA) for degradation of an mRNA of interest.

DNA microarray and statistical data analysis

In four independent experiments, total RNA from medium-stimulated controls or Ni^{2+} -treated HUVECs was isolated and subsequently processed for microarray hybridization using Affymetrix Human Genome 133A Gene Chip arrays according to the manufacturer's instructions (Affymetrix). Microarray data were analyzed using MicroArray Suite Software 5.0 (Affymetrix) and further studied applying the Expressionist Suite software package (GeneData), which allows identification of genes that are significantly regulated in multiple independent experiments (13). Only genes with a fold change of >2.5 or ≤ 2.5 and a $p \leq 0.05$ (t test) were considered. "On/off"-regulated genes were evaluated as described previously (13). To discriminate between background variance and on/off regulations at high intensity levels, on/off events were categorized in grades of expression above background level (+/+ +/+ +/+ +/+ +/+), which was calculated at a $p \leq 0.05$. Principal component analysis (PCA) was applied to mathematically reduce the dimensionality of the entire spectrum of gene expression values of a microarray experiment to three components (18).

Retroviral infections and retrovirus-mediated expression of shRNA

For stable expression of IKK2kd, dn mutant of HIF-1 α (dnHIF-1 α), and dnMKK6, retroviral infection of HUVECs was performed as described previously (14). Briefly, for each retrovirus, stable amphotrophic Φ NX producer cell lines (Orbigen) were established and culture supernatant containing the viral particles, and 5 μ g/ml polybrene was added to HUVECs in two consecutive rounds. After selection using an appropriate antibiotic, stably transduced HUVECs were incubated overnight in growth medium without selection antibiotic and processed for further analysis. For pCFG5-IEGZ-derived retroviruses, infection efficiency was routinely monitored the day before stimulation by measuring GFP positivity by flow cytometry and found to be in the range of >85%. For RNA interference studies, HUVECs were infected with retroviral expression vectors encoding either shRNA against HIF-1 α (pRS-HIF-1 α), scrambled control shRNA (pRS-scrambled), or an empty pRS retrovirus, respectively. Seventy-two hours after infection, positively infected cells were selected for puromycin resistance conferred by the pRS retrovirus backbone with 2 μ g/ml puromycin for 24 h, and cells were further incubated for an additional 18 h in puromycin-free medium before stimulation in the respective experiments. Knockdown efficiencies in the selected cell population were routinely monitored by Western blot.

Quantitative real-time RT-PCR (qRT-PCR)

qRT-PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen) (19), and data were acquired with the ABI PRISM 7900 (Applied Biosystems). Primers were designed using Primer Express software package (Applied Biosystems) and purchased from MWG Biotech. The following primers were used: forward, 5'-GATTGCGACGAGCTGTTC AG-3', and reverse, 5'-CAGTTCACCAAAATGGCGG-3' for angiotensin-

like 4; forward, 5'-TTCATTGCACCATGTGTGGC-3', and reverse, 5'-CTG GACCTCTGTGTGCAATCTTC-3', for asparagine synthetase; forward, 5'-C AAGTGTTTCCAAGGTGTGAGTAC-3', and reverse, 5'-GATGTGGAT AGCAGCTGTCCAAGTAG-3', for c-IAP-2; forward, 5'-TCCTTCCATCTC TGCTGCTCTC-3', and reverse, 5'-TCAAAAGGTGCTGGTGGAGG-3', for Bcl2-interacting protein; forward, 5'-TCGCCTCCAGCATGAAAGTC-3', and reverse, 5'-TTGCATCTGGTGAGCGAG-3', for CCL2 (MCP-1); forward, 5'-TTCTGGAATGGAATTGGACATAGC-3', and reverse, 5'-ACCC TCCATGATGTGCAAGTG-3', for CCL20 (MIP-3 α); forward, 5'-CAGAG CACTTGGCAGGTCATG-3', and reverse, 5'-TCGAGGTTGGAATCTCTC TTCTTG-3', for cyclin G2; forward, 5'-TATGAAGAGCATGACAAGGCC TG-3', and reverse, 5'-AAAGACATTTCTTGACCTTCTCCAGC-3', for M-CSF; forward, 5'-CATCCAAAGTGTAACGTAAGTC-3', and reverse, 5'-TTCCGCCCATTTCTTGAGTGT-3', for CXCL1 (Gro- α); forward, 5'-ACATCCAAAGTGTAAGGTGAAGTC-3', and reverse, 5'-AAGCTTT CTGCCATTTTGAGT-3', for CXCL2 (Gro- β); forward, 5'-AGCGTAT CATTGACACTTCTCG-3', and reverse, 5'-TCCCTTTCCAGCTGCTCCCT AG-3', for CXCL3 (Gro- γ); forward, 5'-GATCCAGAAGCCCCTTTTCTA AAG-3', and reverse, 5'-AGAGACCTCCAGAAAACCTTCTCTGC-3', for CXCL5 (ENA78); forward, 5'-CGATTGGTAACTGCAGGTTGTC-3', and reverse, 5'-TCCGGGTTCAGACAAACTTG-3', for CXCL6 (GCP2); forward, 5'-ACCACCGGAAGGAACCATTC-3', and reverse, 5'-TTCACACA GAGCTGCAGAAATCA-3', for CXCL8 (IL-8); forward, 5'-ACATACAGT GCAGCAAGATGAC-3', and reverse, 5'-GATGATTGCGCGTTTGCC-3', for CX3CL1 (fractalkine); forward, 5'-GGTTCGCACACCCATCAAG-3', and reverse, 5'-GAAGCGGTCCCAAAGGCTAG-3', for HIF-1-responsive RTP801; forward, 5'-AGGTGGTCTCTCTGACTTCAACA-3', and reverse, 5'-AGCTTGACCAAGTGTCTGTTGAG-3', for GAPDH; forward, 5'-GCCCTTACAGCATCTCAAGTTC-3', and reverse, 5'-AAAGTGGTCATGG CCGTGTC-3', for hemoxygenase 1; forward, 5'-ACCTCCCCACCCACAT ACATTT-3', and reverse, 5'-GGCATAGCTTGGGCATATTCC-3', for ICAM-1; forward, 5'-AGAGGCATGGCAGAAACAAC-3', and reverse, 5'-AGGCAAGTCTCTCATTTGAATCC-3', for IL-6; forward, 5'-CAAGA AGGGTTTTTGTGACTGAATC-3', and reverse, 5'-TCCTTGTTTTGCTCC AACACTAATC-3', for IMAGE:4711494; forward, 5'-CACCCCGATATG GTGGACTTC-3', and reverse, 5'-CATTTTTCCTACTGCCATGG-3', for lymphotoxin- β ; forward, 5'-GGGTGCTGGTGGTAGAATG-3', and reverse, 5'-AGCATAAAGCGTTTTGCGGTACTC-3', for Cox2; forward, 5'-A CATGGAATTCGAACCCAAACA-3', and reverse, 5'-GGCTGACCAAGAC GGTGTGATC-3', for VCAM-1; and forward, 5'-TGGAAGAAGCAGCCC ATGAC-3', and reverse, 5'-TTTTAGCTGCACCCCAAGG-3', for vascular endothelial growth factor (VEGF)-A. Gene expression was normalized to the endogenous housekeeping control gene GAPDH. The relative expression of respective genes was calculated using the comparative threshold cycle method (20). The significance of differences between experimental groups of independent experiments was analyzed by the Mann-Whitney *U* test.

Chromatin immunoprecipitation (ChIP)

After stimulation, HUVECs were formalin-fixed, washed, lysed, and sonicated. DNA-protein complexes were immunoprecipitated with an anti-HIF-1 α mAb (clone 54; BD Biosciences), extracted by protein A/G-Sepharose beads (Santa Cruz Biotechnology), washed, and digested with proteinase K. After reversing the cross-link between DNA and protein, DNA was extracted and amplified with primers flanking the RTP801 promoter including the putative HIF-1 α binding sites from -413 to -634 (forward, 5'-GCTTTCTGGGGCTCAATG-3', and reverse, 5'-GTCCACGCTCGCACCTC-3') or the IL-6 promoter from -9804 to -10028 (forward 5'-GGCGGGTCTCGAATGTTATGC-3', and reverse, 5'-GGTTTGTCCTCCAGTCTC-3'). PCR products were run on a 1.5% agarose gel and quantified using Alpha Ease FC software (Bio-Rad).

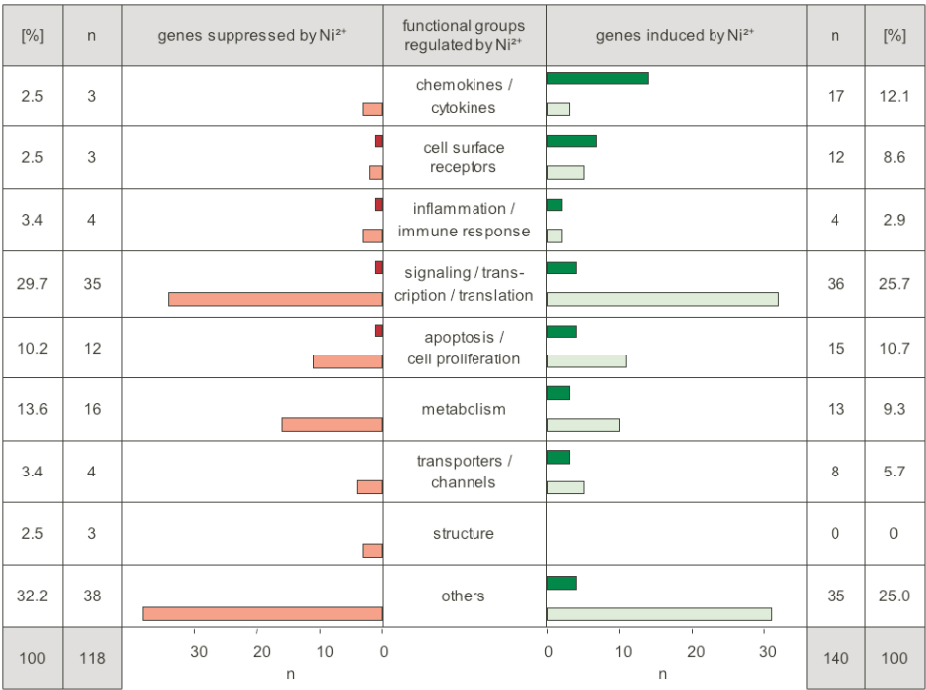
Transient transfection and promoter-reporter gene analysis

HUVECs were transiently transfected according to a DEAE-dextran protocol (12) with 6κB.luc (provided by Dr. B. Baumann, University of Ulm, Ulm, Germany) or 3HRE.luc reporter constructs (gift from Dr. S. L. McKnight, University of Texas Southwestern Medical Center, Dallas, TX) and ubiquitin-dependent *Renilla* luciferase. After stimulation, luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega). NF-κB- or HRE-dependent luciferase activities were normalized to the luminescence generated by the *Renilla* luciferase control reporter and expressed as fold stimulation compared with nonstimulated controls.

Western blot analysis

ECs were lysed in Lämmli buffer, protein lysates were separated by SDS-PAGE and blotted onto polyvinylidene difluoride or nitrocellulose membranes. Proteins were detected using mouse mAbs against HIF-1 α and IL-8 (BD Biosciences). Expression of dnMKK6 was confirmed using an

FIGURE 1. Assignment of Ni²⁺-regulated genes to functional groups. Genes that are induced (green bars) or repressed (red bars) in primary ECs, at least 2.5-fold or switched on or switched off upon exposure to 1.5 mM Ni²⁺, are assigned to functional groups. Absolute numbers and relative distribution of regulated genes are indicated. Ni²⁺-regulated genes that have been identified to be equally regulated by TNF-α in parallel experiments are shown as dark bars, and genes that are disparately regulated are bright bars.



anti-flag Ab (Sigma-Aldrich) and equal loading controlled by labeling with a rabbit antiserum against Erk2 (Santa Cruz Biotechnology). Membranes were stained with peroxidase-coupled second stage Abs and bands visualized by ECL.

Flow cytometry

Flow cytometry for endothelial surface molecules and intracellular detection of chemokines and cytokines was performed as described previously (10). Successful retroviral infection of cells was monitored by detection of GFP; only successfully infected, i.e., GFP-positive, cells were considered for analysis (14).

Immunofluorescence

HUVECs exposed to medium or Ni²⁺ were fixed with 3.7% formaldehyde, permeabilized with Triton X-100, and stained with anti-HIF-1α followed by a FITC-coupled second stage Ab. Cell nuclei were visualized by 4',6'-diamidino-2-phenylindole staining. Exposure settings were equal in all conditions.

ELISA

For determination of IL-6 and IL-8 in the culture supernatant, commercially available ELISAs were used according to the manufacturers' instructions (R&D Systems, BD Biosciences).

Results

Ni²⁺ induces expression of multiple genes in primary ECs

To determine the global gene expression profile induced by nickel, we treated exponentially growing HUVECs for 5 h with 1.5 mM NiCl₂ (subsequently referred to as Ni²⁺) and subjected RNA extractions from those samples to microarray hybridization. To ensure a high statistical significance, material from four individual experiments were analyzed independently. Only genes with a fold change of at least 2.5 at a significance level of *p* < 0.05 (*t* test) or a significant on/off-switch were considered as Ni²⁺ regulated. We identified 140 genes that were induced or up-regulated by Ni²⁺, whereas 118 were down-regulated or switched off. Almost 50% of the inducible genes could be assigned to the three functional groups: “signaling/transcription/translation,” “cytokines/chemokines,” or “apoptosis/cell proliferation,” respectively (Fig. 1). Among the down-regulated or switched-off genes, those annotated

to the categories chemokines/cytokines and cell surface receptors were considerably underrepresented (Fig. 1). The complete and detailed list of Ni²⁺-regulated genes is provided as supplemental data (Tables S1 and S2).⁴ To validate microarray data by independent methods, we additionally performed qRT-PCR (for 22 selected genes; i.e., ~10% of the Ni²⁺-regulated transcriptome; Fig. 2 and data not shown) and flow cytometry (for five selected proteins; data not shown). For >95% of these genes, regulation could be confirmed. Overall, control experiments showed a high degree of congruence with the microarray data, confirming the reliability and validity of our statistical evaluation.

A considerable number of Ni²⁺-inducible genes are regulated independently of IKK2/NF-κB activation

Our previous work revealed that some of the signaling and gene expression events induced by Ni²⁺ resembled those of proinflammatory cytokines such as TNF-α (8, 10). Therefore, we compared the Ni²⁺-induced endothelial gene profile with the gene expression pattern elicited by TNF-α using identical experimental conditions. We realized that 45 genes were equally regulated by both stimuli (Fig. 1). Since expression of almost all of the TNF-α-induced genes in ECs depends on signaling via IKK2/NF-κB (13), we also assumed a central regulatory role of this pathway for Ni²⁺. To assess this point, we selected 15 genes coregulated by both stimuli, e.g., ICAM-1, VCAM-1, CXCL8, c-IAP-2, IMAGE 4711, and exemplarily analyzed expression in response to Ni²⁺ in cells that are incapable of IKK2-mediated NF-κB activation due to stable expression of kinase-inactive IKK2. Using qRT-PCR and/or flow cytometry, we demonstrated IKK2/NF-κB-dependent regulation by Ni²⁺ in all of these cases (Fig. 2A and data not shown).

The overlap of the Ni²⁺-induced gene expression profile with the TNF-α-regulated NF-κB-dependent transcriptome prompted us to perform a discriminatory analysis to evaluate the degree of diversity. Thus, we performed PCA (18) for gene expression with

⁴ The online version of this article contains supplemental material.

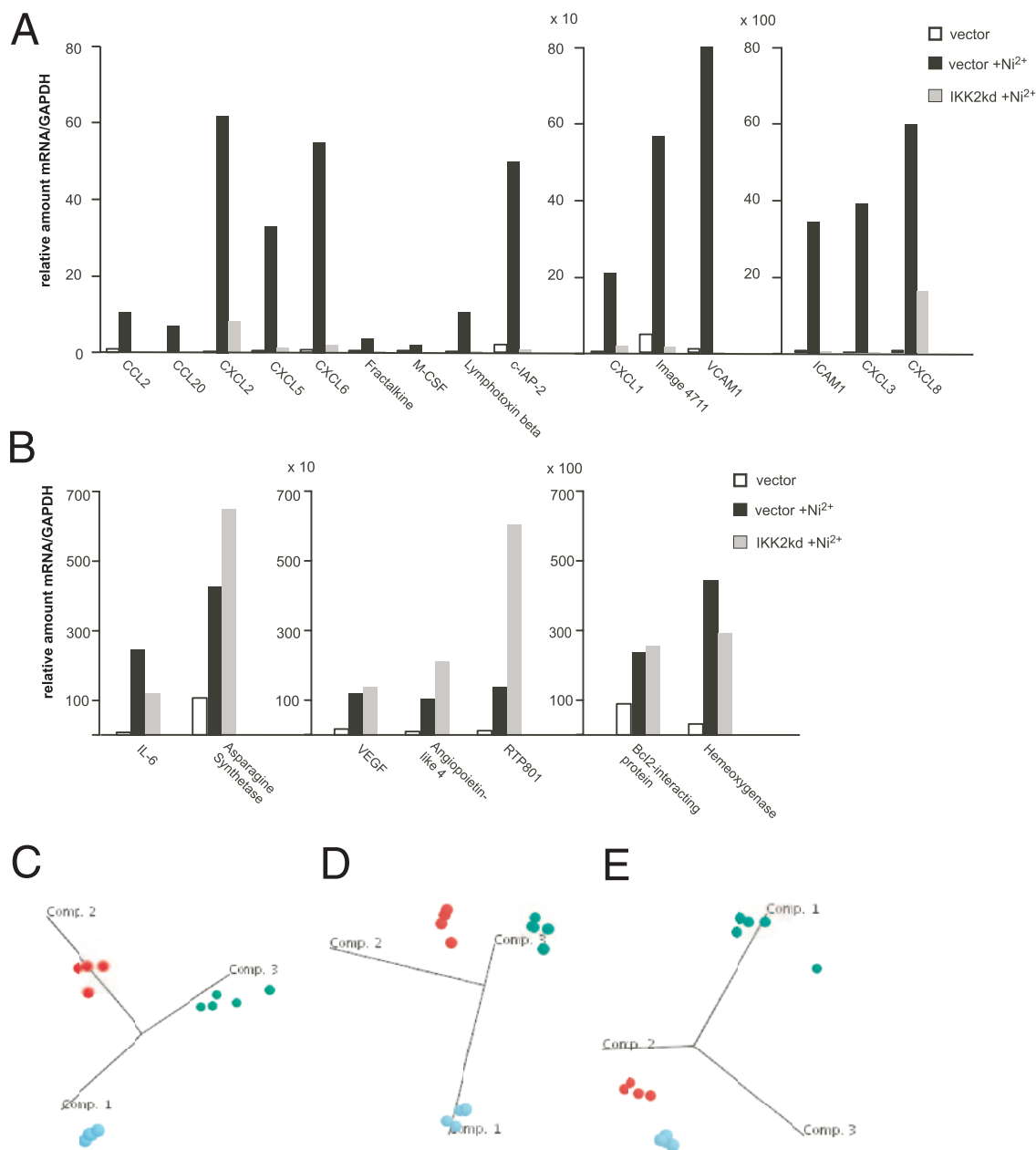


FIGURE 2. IKK2/NF- κ B-dependent and -independent regulation of gene expression in primary ECs by Ni²⁺. To identify genes that are regulated by Ni²⁺ independently of IKK2/NF- κ B signaling, HUVECs were infected with a control retrovirus or a retrovirus allowing stable expression of IKK2kd. Cells were then exposed to 1.5 mM Ni²⁺ or medium as control and further processed for qRT-PCR. mRNA levels of respective genes were normalized to the housekeeping gene GAPDH and relative expression calculated as outlined in *Materials and Methods*. The data shown represent mean values of three independent experiments each performed in duplicate. Ni²⁺-regulated genes that were significantly inhibited by the IKK2/NF- κ B pathway ($p \leq 0.005$ (Mann-Whitney- U test)) are shown in *A*, those that showed no significant inhibition by IKK2kd in *B*. □, Mock-infected HUVECs exposed to medium; ■, Mock-infected HUVEC exposed to Ni²⁺; ▨, HUVECs expressing IKK2kd exposed to Ni²⁺. The data shown in *A* and *B* are presented as mean values obtained from three independent experiments. *C–E*, PCA comparing untreated control cells (blue), Ni²⁺-exposed (green), and TNF- α -stimulated HUVECs (red). The entire dimension of gene expression values was mathematically transformed and reduced to three components and is presented as scatter plots in a three-dimensional space. PCA was based on all genes regulated by either TNF- α or Ni²⁺ (*C*), on TNF- α -regulated NF- κ B-dependent genes (*D*), or on genes solely regulated by Ni²⁺ but not TNF- α (*E*). These data illustrate that Ni²⁺ and TNF- α induce a partially overlapping fraction of genes that depends on NF- κ B (*D*) while another group of Ni²⁺-induced genes is apparently regulated independently of NF- κ B (*E*).

Ni²⁺- and TNF- α -stimulated HUVECs or untreated controls, respectively. Based on all genes that are regulated, i.e., the differential profiles of TNF- α and Ni²⁺, PCA revealed that the individual experiments of each treatment group were excellently reproducible. Importantly, the gene profiles of control cells, TNF- α -treated and Ni²⁺-exposed HUVECs, were clearly separated (Fig. 2*C*). The partial overlap between TNF- α - and Ni²⁺-regulated

profiles became obvious when PCA was restricted to NF- κ B-dependent genes. Under such conditions, TNF- α - and Ni²⁺-regulated profiles were in close vicinity but clearly separated from untreated controls (Fig. 2*D*), indicating that both stimuli induced expression of an almost identical group of genes via activation of NF- κ B. However, when PCA was applied to only those Ni²⁺-regulated genes that were not regulated by TNF- α , still a distinct cloud of genes remained that

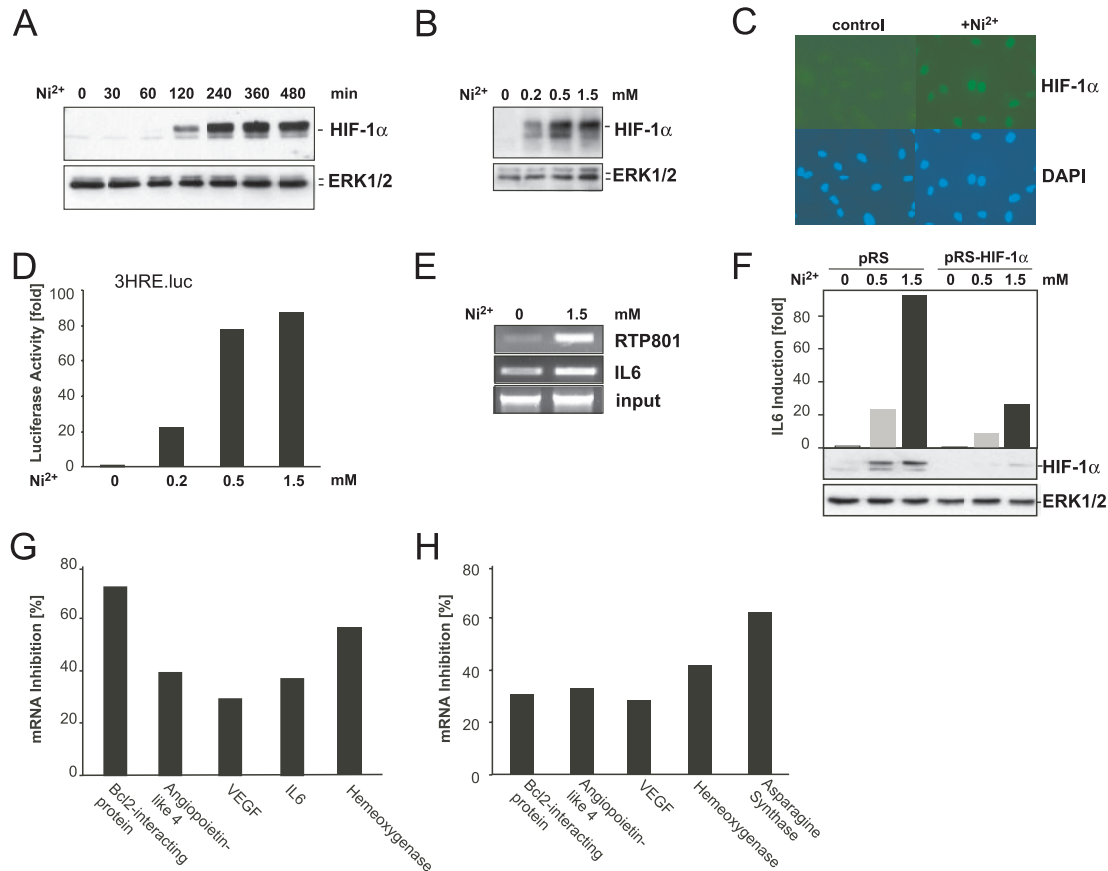


FIGURE 3. Ni²⁺ induces accumulation and DNA binding of HIF-1α and HIF-1α-dependent gene expression in primary ECs. Ni²⁺ leads to accumulation of HIF-1α protein in a time-dependent (A) and concentration-dependent manner (B). HUVECs were exposed to 1.5 mM Ni²⁺ for the time intervals indicated (A) or for 240 min to various concentrations of Ni²⁺ (B), and lysates were subsequently studied by Western blot analysis. C, HUVECs were exposed to 1.5 mM Ni²⁺ for 240 min, and accumulation and nuclear translocation of HIF-1α were visualized by immunofluorescence microscopy using an anti-HIF-1α Ab. 4',6'-Diamidino-2-phenylindole (DAPI) staining indicates nuclear localization. Exposure settings were equal in all conditions. D, Promoter reporter gene assay showing Ni²⁺-induced HRE-dependent gene expression in ECs. HUVECs transiently transfected with a HRE-dependent luciferase construct (3HRE.luc) were exposed to 0.2, 0.5, or 1.5 mM Ni²⁺ or medium as control for 16 h. Luciferase activities generated by the 3HRE.luc construct were normalized to the luminescence generated by an ubiquitin-dependent *Renilla* luciferase control reporter gene. Data from one of three independent experiments are shown. E, ChIP demonstrating that Ni²⁺ induces binding of HIF-1α to putative binding sites of the RTP801 and IL-6 promoters. HUVECs were exposed to 1.5 mM Ni²⁺ for 240 min or left untreated as indicated. F, To study the impact of HIF-1α on Ni²⁺-mediated gene expression, HIF-1α induction was suppressed by retrovirus-mediated expression of shRNA against HIF-1α. Effective HIF-1α knockdown was confirmed by Western blot analysis after exposure to Ni²⁺ for 4 h as indicated. In the same experiment, Ni²⁺-induced expression of IL-6 protein is reduced after shRNA-mediated depletion of HIF-1α as determined by ELISA. Data are presented as fold stimulation of IL-6 secretion after exposure to 0, 0.5, or 1.5 mM Ni²⁺ for 8 h as indicated and are representative for one of two independent experiments. G and H, HUVECs stably expressing shRNA against HIF-1α (G) or dnHIF-1α (H) or infected with respective control retroviruses were exposed to 1.5 mM Ni²⁺ or medium for 5 h. Thereafter, mRNA was obtained and expression of selected genes determined by qRT-PCR. Data are presented as percentage of inhibition of Ni²⁺-induced mRNA expression of HUVECs expressing shRNA against HIF-1α (G) or dnHIF-1α (H) as compared with HUVECs infected with control retroviruses. Data are representative for one of two independent experiments.

was clearly separated from the medium-stimulated control (Fig. 2E). Indeed, >100 genes were exclusively regulated by Ni²⁺ but not by TNF-α, suggesting a regulation independently of the IKK2/NF-κB-signaling pathway (Fig. 1, Supplementary Tables S1 and S2). This group of genes included molecules such as VEGF, hemeoxygenase, asparagine synthetase, Bcl2-interacting protein, angiopoietin like-4, IL-6, and others. For all of seven randomly selected genes that we identified as differentially regulated by Ni²⁺ and TNF-α in our profiling experiments, we could confirm an IKK2/NF-κB-independent control by Ni²⁺ in qRT-PCR experiments (Fig. 2B).

Ni²⁺ induces accumulation of HIF-1α in primary ECs and stimulates its DNA-binding and transactivation capacity on HIF-1α-dependent promoters in vivo

We noticed that some of the genes differentially regulated by Ni²⁺ and TNF-α are prominent targets of HIF-1α, a transcription factor

involved in hypoxia-induced gene expression. These include genes such as VEGF, HIF-1-responsive RTP801, adrenomedullin, adenylyl kinase, or cyclin G2. Thus, we analyzed the effect of Ni²⁺ on HIF-1α both on mRNA and protein level. While Ni²⁺ had no effect on HIF-1α transcript levels, as evident by the lack of HIF-1α mRNA induction by Ni²⁺ in both Affymetrix gene chip analysis and additional qRT-PCR experiments (data not shown), exposure of ECs to Ni²⁺ resulted in a dose-dependent accumulation and nuclear translocation of HIF-1α protein, which became apparent after 120 min (Fig. 3, A–C). Furthermore, Ni²⁺ induced transactivation of a HIF-1α-dependent HRE.luc promoter construct in ECs (Fig. 3D). To study whether Ni²⁺-induced HIF-1α can bind to the promoter of selected target genes in live ECs, we performed ChIP analysis. We chose HIF-1-responsive RTP801, a known target gene of HIF-1α (21), and IL-6, which contains a putative HIF-1α binding site but is currently not established as a primary

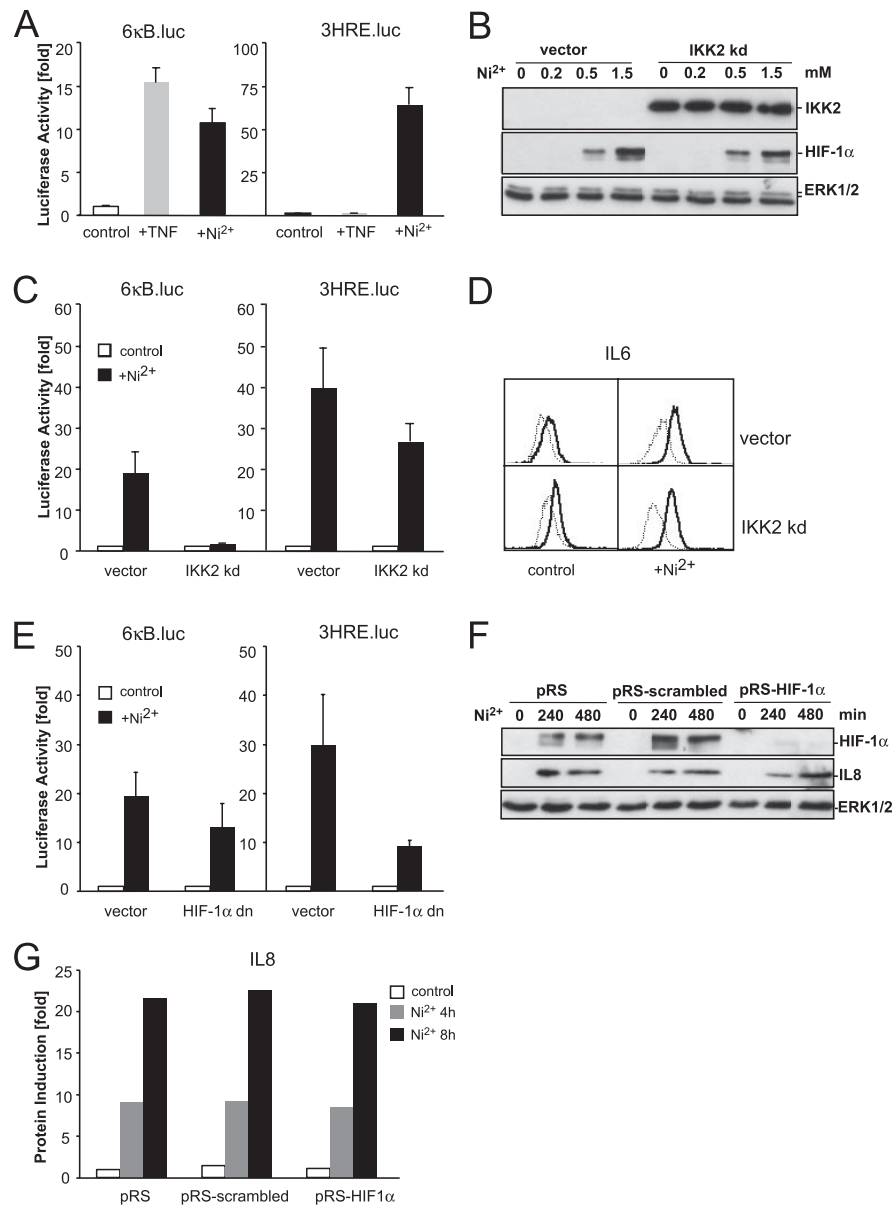


FIGURE 4. Ni^{2+} -mediated accumulation of HIF-1 α and HIF-1 α -dependent gene expression occur independently of IKK2/NF- κ B. **A**, Ni^{2+} simultaneously induces NF- κ B- and HRE-dependent gene expression in ECs. HUVECs were transiently transfected with 6 κ B.luc or 3HRE.luc reporter gene constructs and subsequently exposed to medium, 1.5 mM Ni^{2+} , or 2 ng/ml TNF- α for 16 h. Luciferase activity is expressed as fold induction as described in *Materials and Methods*. Data are derived from three to six independent experiments and presented as mean $\pm$ SEM. **B**, HUVECs were retrovirally infected to stably express a dn mutant of IKK2, i.e., IKK2kd, or a control vector and subsequently exposed to 0, 0.2, 0.5, or 1.5 mM Ni^{2+} . Thereafter, HIF-1 α protein levels were determined by Western blot analysis, and equal loading of lanes was confirmed by staining for ERK2. One of six independent experiments is shown. **C**, HUVECs stably expressing IKK2kd or infected with an empty control retrovirus were transiently transfected with 6 κ B.luc or 3HRE.luc reporter gene constructs. Thereafter, cells were exposed to 1.5 mM Ni^{2+} or medium as indicated. Fold induction was determined from data obtained from four to six independent experiments and are presented as mean $\pm$ SEM. **D**, IL-6, a putative HIF-1 α -dependent gene, is up-regulated by Ni^{2+} in a NF- κ B-independent manner. HUVECs stably expressing IKK2kd or infected with an empty control retrovirus were exposed to 1.5 mM Ni^{2+} or medium as control for 16 h and subsequently processed for intracellular flow cytometry. Bold profiles show staining for IL-6, and thin profiles depict IgG isotype controls. One of two independent experiments is shown. **E**, HUVECs stably expressing dnHIF-1 α or infected with an empty control retrovirus were transiently transfected with 6 κ B.luc or 3HRE.luc reporter gene constructs. Cells were then exposed to medium as control or 1.5 mM Ni^{2+} for 16 h. Thereafter, luciferase activities were determined and expressed as fold stimulation. Data are derived from four independent experiments and are shown as mean $\pm$ SEM. **F** and **G**, HIF-1 α -dependent signaling is not involved in regulation of the Ni^{2+} -induced NF- κ B-dependent target gene IL-8. HIF-1 α was knocked down in HUVECs by retrovirus-mediated RNA interference, and Ni^{2+} -induced IL-8 synthesis was subsequently studied by Western blot analysis (**F**) and ELISA (**G**). HIF-1 α knockdown levels were determined by Western blot analysis (**F**, upper lanes). One of two independent experiments is shown.

HIF-1 α -regulated gene. Indeed, Ni^{2+} enhanced the recruitment of HIF-1 α to both promoters significantly (Fig. 3E).

We next studied whether Ni^{2+} -mediated gene expression is indeed dependent on HIF-1 α . The pRS retroviral vector system was used to knock down HIF-1 α by RNA interference (Fig. 3F). In

HIF-1 α -depleted cells, Ni^{2+} -induced IL-6 expression was largely blocked as shown by ELISA (Fig. 3F) and Western blot analysis (data not shown). qRT-PCR revealed a decreased endothelial expression of several Ni^{2+} -inducible genes, including IL-6, VEGF, angiopoietin-like 4, and Bcl2-interacting protein, upon expression

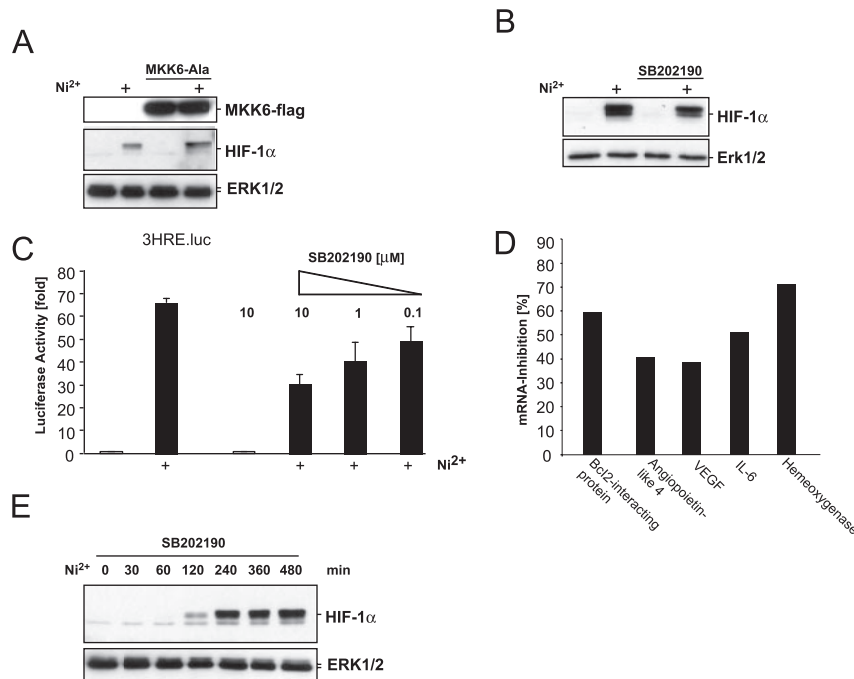


FIGURE 5. Ni^{2+} -activated p38 MAPK does not interfere with HIF-1 α accumulation but modulates HIF-1 α -dependent transactivation and gene expression. **A**, HUVECs stably expressing a dn mutant of MKK6 (MKK6-Ala), the upstream kinase of p38, or mock-infected with a retrovirus were exposed to Ni^{2+} and probed for HIF-1 α and Erk2 as loading control by Western blot analysis. Expression of the tagged dnMKK6 transgene was controlled by monitoring flag expression. HIF-1 α expression was not affected. **B**, HUVECs were preincubated with 10 μM SB202190, a pharmacological p38 inhibitor, and subsequently stimulated with Ni^{2+} for 240 min. HIF-1 α expression was analyzed by Western blot thereafter. **C**, HUVECs transiently transfected with HIF-1 α -dependent 3HRE.luc reporter gene constructs were pretreated with 0.1–10 μM SB202190 and exposed to Ni^{2+} . Fold induction of luciferase activities obtained from three independent experiments is shown as mean $\pm$ SEM. Each concentration of SB202190 significantly inhibited Ni^{2+} -induced 3HRE.luc activity (Wilcoxon test, $p \leq 0.05$, $n = 5$). **D**, HUVECs were preincubated with 10 μM SB202190 and subsequently exposed to 1.5 mM Ni^{2+} for 5 h. Thereafter, mRNA was obtained and expression of selected genes determined by qRT-PCR. Data are presented as percentage of inhibition of Ni^{2+} -induced mRNA expression of HUVECs preincubated with SB202190 as compared with HUVECs not treated with the inhibitor. Data are presented as average values from two independent experiments. **E**, Inhibition of p38 MAPK does not alter kinetics of Ni^{2+} -mediated HIF-1 α stabilization. ECs were pretreated with 10 μM SB202190 and exposed to Ni^{2+} for the time intervals indicated and subsequently probed by Western blot analysis.

of shRNA against HIF-1 α (Fig. 3G). Essentially similar results were obtained with a dnHIF-1 α stably expressed in HUVECs (Fig. 3H). These data clearly demonstrate that Ni^{2+} modulates several of its response genes via HIF-1 α .

Ni^{2+} activates HIF-1 α and IKK2/NF- κB independently from each other

Since Ni^{2+} simultaneously activates both NF- κB - and HIF-1 α -dependent gene transcription (Fig. 4A), we wondered whether the activation state of the IKK2/NF- κB signaling pathway might interfere with HIF-1 α -dependent endothelial gene regulation. To address this point, we studied Ni^{2+} -induced HIF-1 α protein expression in ECs stably expressing IKK2kd in comparison to control ECs with stable integration of empty retrovirus. As shown in Fig. 4B, Ni^{2+} -induced HIF-1 α accumulation was largely unaffected by IKK2kd expression. Consistently, Ni^{2+} -stimulated transactivation of a HRE-responsive luciferase construct was comparable under both conditions (Fig. 4C). Furthermore, IL-6, which we identified as direct target of HIF-1 α (Fig. 3, E–G), was still induced by Ni^{2+} in the absence of active IKK2, indicating that HIF-1 α -mediated gene expression does not depend on NF- κB activity (Fig. 4D).

We next studied NF- κB -dependent gene expression in HUVECs in which HIF-1 α has been blocked by stable expression of dn HIF-1 α . In these cells, Ni^{2+} -induced transactivation of a κB -dependent promoter was largely unaffected while 3HRE-dependent luciferase activity was clearly repressed (Fig. 4E). In agreement, depletion of HIF-1 α by stable expression of shRNA against HIF-

1 α , which significantly interferes with expression of established HIF-1 α target genes such as VEGF (compare Fig. 3G), is still capable of inducing expression of the NF- κB -dependent gene IL-8 (Fig. 4, F and G). Thus, Ni^{2+} triggers activation of both pathways largely independently of each other.

Ni^{2+} -induced activation of the MKK6/p38 MAPK pathway does not interfere with HIF-1 α protein accumulation but modulates HIF-1 α -dependent transactivation

We previously reported that Ni^{2+} , like TNF- α , is capable of activating the stress-activated MAPK p38 (10). To analyze whether HIF-1 α protein accumulation is dependent on p38 activation, HIF-1 α expression was studied in HUVECs stably expressing dnMKK6, the upstream activator of p38. While dnMKK6 completely abrogated Ni^{2+} -induced phosphorylation of p38 (data not shown), it did not alter protein levels of HIF-1 α (Fig. 5A). Consistently, the pharmacological p38 inhibitor SB202190 did not significantly affect Ni^{2+} -mediated expression of HIF-1 α protein (Fig. 5B). Moreover, the kinetics of Ni^{2+} -induced HIF-1 α accumulation was not perturbed upon p38 inhibition (Figs. 3A and 5E). However, blockade of p38 partially inhibited Ni^{2+} -induced transactivation of a 3HRE-dependent promoter in luciferase reporter assays (Fig. 5C). Similar results were obtained with cells stably expressing dnMKK6 (data not shown). Thus, p38 does not affect the capacity of Ni^{2+} to induce HIF-1 α protein but regulates HIF-1 α -dependent gene transcription at the level of transactivation.

To confirm that p38 indeed can influence the induction level of HIF-1 α target genes, we further tested the effect of p38 inhibition on Ni²⁺-dependent expression of five different Ni²⁺-response genes that we previously showed to be dependent on HIF-1 α (Fig. 3*G*). Fig. 5*D* illustrates that indeed for all those HIF-1 α response genes pharmacological blockade of p38 resulted in a partial inhibition of Ni²⁺-induced mRNA expression.

In conclusion, our observations indicate that Ni²⁺ induces gene expression in ECs by virtually two independent signal transduction pathways that both may be modulated by the activation status p38 MAPK.

Discussion

To investigate the complex gene response patterns induced by the proinflammatory and allergy-causing metal ion Ni²⁺ and to elucidate the responsible regulatory intracellular signaling pathways, we performed DNA microarray analysis. Primary human ECs were chosen as target cells since, as lining cells between different compartments, they critically determine the recruitment of leukocytes during the course of inflammatory reactions. They strongly respond to Ni²⁺ in vitro as well as in vivo, e.g., by expressing endothelial adhesion molecules and chemokines (8, 10, 22). Importantly, Ni²⁺ exerts its effects on ECs directly rather than acting indirectly via autocrine release of proinflammatory cytokines such as TNF- α or IL-1 since inhibition of the latter by neutralizing Abs, soluble receptors, or receptor antagonists shows no effect at all (9) (data not shown). In total, we have identified 140 endothelial genes that were significantly up-regulated or switched on upon Ni²⁺ treatment and 118 genes that were down-regulated or switched off. By strict adherence to a rigid statistical evaluation of our microarray data, we were able to confirm our results by independent experimental approaches on gene and protein level in >95% of all examined cases, underscoring the reliability of our data sets. In an earlier study, we have investigated the transcriptome of TNF- α -stimulated ECs and found that virtually all genes were regulated in a NF- κ B-dependent manner (13). Since these data were obtained in parallel and conducted under identical experimental conditions in ECs from the same source and applying the same rigid statistics, it was possible to directly compare the two data sets. From these comparisons of Ni²⁺-mediated gene expression patterns with those elicited by TNF- α , two important conclusion can be drawn: first, similar to TNF- α , Ni²⁺ induces a broad panel of NF- κ B-dependent genes in ECs. Second, the spectrum of Ni²⁺-regulated genes by far exceeds that observed after TNF- α stimulation, indicating that at least one additional pathway is involved in mediating the signals generated by Ni²⁺. While genes regulated by NF- κ B primarily reflect a proinflammatory response pattern resulting in the expression of chemokines, cytokines, cell surface receptors, and inflammation/immune response genes, those attributed to the second identified pathway were primarily associated with angiogenesis, cellular homeostasis, cell cycle control, apoptosis, and cell proliferation. In almost all cases, a dependency or independency of selected Ni²⁺ response genes from NF- κ B signaling, as predicted by comparison with the TNF- α -regulated transcriptome, could be confirmed by qRT-PCR.

Among the group of NF- κ B-independent transcripts, a considerable number represents genes that are known to be regulated by hypoxia via the transcription factor HIF-1 α (see supplemental Tables S1 and S2). Co²⁺, a transition metal chemically related to Ni²⁺, has occasionally been used as an agent mimicking hypoxia and activates the transcription factor HIF- α . Three closely related forms of HIF- α have been identified (23); in HUVECs, HIF-1 α is the predominant one (24, 25). As shown here, HIF-1 α accumulates upon stimulation with Ni²⁺ in a time- and concentration-depen-

dent manner, translocates to the nucleus, binds to hypoxia-responsive elements (HREs) in the promoters of target genes, and induces transcription. However, recent evidence indicates that only a limited number of hypoxia-inducible genes is similarly regulated by transition metals (26), indicating that data obtained from hypoxia studies cannot merely be extended to other potential activators of HIF-1 α . In earlier studies, Ni²⁺-regulated gene expression has been analyzed in the context of carcinogenesis using different cell lines such as mouse embryonic fibroblasts or hepatocellular carcinoma cells (Hep3B) but not ECs, which lead to contradictory results. However, differences in experimental settings and lack of strict statistical criteria in these earlier studies do not allow direct comparison of expression patterns (26–28).

At least four potential mechanisms could mediate accumulation and activation of HIF-1 α by Ni²⁺: first, Ni²⁺ blocks activity of prolyl hydroxylase domain enzymes, which are required for hydroxylation and subsequent von Hippel-Lindau protein-mediated degradation of HIF-1 α (23, 29). Second, transition metals may directly bind to HIF-1 α at carboxylate residues of its oxygen-dependent degradation domain, thereby preventing interaction with von Hippel-Lindau protein (30). Third, Ni²⁺ may interact with an asparaginyl hydroxylase named FIH that prevents recruitment of the coactivator p300/CBP via hydroxylation of an asparaginyl residue in the transactivation domain of HIF-1 α (23). Such a mechanism may be effective independently of changes in HIF-1 α stability even at lower concentrations of Ni²⁺ (31). Finally, Ni²⁺ depletes intracellular ascorbate, which is essential for proper function of prolyl hydroxylase domains and FIH (32). Experiments are currently underway to elucidate, which of the above mechanisms is the most relevant one for Ni²⁺-dependent HIF-1 α activation.

Different lines of evidence suggest a cross-talk between HIF-1 α and NF- κ B signaling. In some cell types, TNF- α stimulates accumulation (33) and DNA binding of HIF-1 α under hypoxic conditions (34) via NF- κ B-dependent pathways. In view of these findings, it was important to analyze whether expression of HIF-1 α is dependent on the activation state of NF- κ B in our experimental system. Stable expression of IKK2kd, which completely blocks agonist-induced activation of NF- κ B (14), only slightly reduced Ni²⁺-regulated HIF-1 α levels and HRE-dependent transcription (Fig. 4). Moreover, the potent NF- κ B activator TNF- α neither led to accumulation of HIF-1 α (data not shown) nor induced HRE-dependent gene expression in HUVECs (Fig. 4*A*). In line with these observations, stable expression of constitutively active IKK2, which results in NF- κ B activation, did not affect HIF-1 α expression (data not shown), indicating that Ni²⁺-induced NF- κ B activation is not a prerequisite for accumulation of HIF-1 α . In neutrophils, hypoxic induction of NF- κ B requires the presence of HIF-1 α (35), whereas in 293T and Jurkat cells, neither overexpression of HIF-1 α nor of HIF-2 α affected NF- κ B activity (36). Our data are consistent with the latter observations since neither depletion of HIF-1 α by retrovirus-mediated expression of shRNA against HIF-1 α nor stable expression of dnHIF-1 α had major effects on NF- κ B-dependent gene expression.

A third pathway activated by Ni²⁺, which potentially could modulate Ni²⁺-mediated HIF-1 α signaling, is the MKK6/p38 MAPK pathway (10). In HUVECs, neither pharmacological p38 inhibition nor a blockade of the pathway by expression of a dn mutant of MKK6, the upstream kinase of p38, affected Ni²⁺-mediated accumulation of HIF-1 α protein (Fig. 5). However, in both cases, we observed an impaired transactivation of a HIF-1 α -dependent promoter construct. Accordingly, blockade of p38 partially repressed transcript induction of five HIF-1 α -target genes. This observation does not necessarily mean that all HIF-1 α -dependent genes are under control of p38 MAPK. When analyzing

the TNF α -induced gene expression profile of HUVECs, which is almost entirely dependent on IKK2/NF- κ B signaling, we noticed that pharmacological inhibition of p38 partially blocked TNF α -induced expression of 6 κ B.luc but inhibited only 13 of 58 NF- κ B-dependent genes when studied at the mRNA level by Affymetrix gene chip analysis (13). A similar scenario may be conceived with respect to modulation of HIF-1 α -dependent genes by p38. It is currently not clear how p38 targets HIF-1 α -mediated transactivation, but it may well be that, in analogy to our earlier findings regarding NF- κ B-dependent gene expression (10), the kinase may modulate the activity of transcriptional coactivators such as CBP/p300, which are essential for both HIF-1 α - and NF- κ B-dependent transcription (37).

ECs of both macrovascular (HUVECs) and microvascular origin (HMEC-1 and human dermal microvascular endothelial cells; data not shown) respond to Ni²⁺ with activation of NF- κ B- and HIF-1 α -dependent signaling pathways; while there may be differences in individual gene expression patterns between both EC types (38), the basic molecular mechanisms of response to Ni²⁺ appear still comparable. Ni²⁺-regulated genes can be grouped into different functional categories. A substantial portion of genes belongs to the groups chemokines/cytokines, 'cell surface receptors' and 'inflammation/immune response' and is preferentially associated with the NF- κ B signaling pathway. The expression of some of these genes has been confirmed earlier in the context of Ni²⁺-induced inflammation such as contact hypersensitivity reactions (22). Another major fraction of genes falls to the categories signaling/transcription/translation, apoptosis/cell proliferation, "metabolism," and "others" and can be annotated as genes related to cell turnover. Most of those were regulated independently of the NF- κ B-pathway. For several of these genes, we confirmed that HIF-1 α induction mediates their responsiveness to Ni²⁺. Interestingly, HIF-1 α activation was also found to be crucial for Ni²⁺-dependent expression of the pleiotropic cytokine IL-6, which we identified as a novel HIF-1 α -dependent gene in this study. This indicates that the HIF-1 α pathway also importantly contributes to the proinflammatory potential of Ni²⁺. Thus far, only a limited number of the HIF-1 α -dependent genes have been studied in the context of vascular biology, mostly for their role in angiogenesis. Particularly, the role of these molecules during inflammatory reactions is only poorly defined. However, there is evidence that, for example, VEGF besides its proangiogenic activity can also promote Ni²⁺-associated inflammation by increasing the permeability of capillaries (39). Likewise, hemeoxygenase-1 has been reported to inhibit proinflammatory immune responses while preserving the production of the anti-inflammatory cytokine IL-10 (40, 41). Thus, activation of hemeoxygenase-1 by Ni²⁺ could provide a feedback mechanism that limits the extent of Ni²⁺-induced inflammation. Even if the exact function of individual Ni²⁺-induced genes during inflammatory processes remains to be elucidated, our data clearly indicate that the net result of Ni²⁺ stimulation is a combination of inflammatory and proangiogenic gene expression patterns resulting from simultaneous activation of two different signal transduction pathways. This bifurcated signaling response may provide the molecular basis for the broad spectrum of Ni²⁺-associated diseases in humans, including allergic reactions, incompatibility of Ni²⁺-containing biomaterial, and carcinogenesis, and may also be the basis for the action of other contact allergens. At any rate, our data underscore that gene profiling is a suitable method to decipher the relevant pathways involved in reactions to environmental agents and open the door for new approaches to therapeutically intervene with such diseases.

Acknowledgments

We thank Nicole Endres and Ulla Nordhues for excellent technical assistance.

Disclosures

The authors have no financial conflict of interest.

References

- Kasprzak, K. S., F. W. Sunderman, and K. Salnikow. 2003. Nickel carcinogenesis. *Mutat. Res.* 533: 67–97.
- Krob, H. A., A. B. Fleischer, Jr., R. D'Agostino, Jr., C. L. Haverstock, and S. Feldman. 2004. Prevalence and relevance of contact dermatitis allergens: a meta-analysis of 15 years of published T.R.U.E test data. *J. Am. Acad. Dermatol.* 51: 349–353.
- Mattila, L., M. Kilpelainen, E. O. Terho, M. Koskenvuo, H. Helenius, and K. Kalimo. 2001. Prevalence of nickel allergy among Finnish University students in 1995. *Contact Dermatitis* 44: 218–223.
- Köster, R., D. Vieluf, M. Kiehn, M. Sommerauer, J. Kähler, S. Baldus, T. Meinertz, and C. W. Hamm. 2000. Nickel and molybdenum contact allergies in patients with coronary in-stent restenosis. *Lancet* 356: 1895–1897.
- Black, J. 1988. Does corrosion matter? *J. Bone Joint Surg. Br.* 70: 517–520.
- Wataha, J. C., N. L. O'Dell, B. B. Singh, M. Ghazi, G. M. Whitford, and P. E. Lockwood. 2001. Relating nickel-induced tissue inflammation to nickel release in vivo. *J. Biomed. Mater. Res.* 58: 537–544.
- Grabbe, S., and T. Schwarz. 1998. Immunoregulatory mechanisms involved in elicitation of allergic contact hypersensitivity. *Immunol. Today* 19: 37–44.
- Goebeler, M., G. Meinardus Hager, J. Roth, S. Goerd, and C. Sorg. 1993. Nickel chloride and cobalt chloride, two common contact sensitizers, directly induce expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule (ELAM-1) by endothelial cells. *J. Invest. Dermatol.* 100: 759–765.
- Goebeler, M., J. Roth, E. B. Bröcker, C. Sorg, and K. Schulze-Osthoff. 1995. Activation of nuclear factor κ B and gene expression in human endothelial cells by the common haptens nickel and cobalt. *J. Immunol.* 155: 2459–2467.
- Goebeler, M., R. Gillitzer, K. Kilian, K. Utzel, E. B. Bröcker, U. R. Rapp, and S. Ludwig. 2001. Multiple signaling pathways regulate NF- κ B-dependent transcription of the monocyte chemoattractant protein-1 (MCP-1) gene in primary endothelial cells. *Blood* 97: 46–55.
- Karin, M., Y. Yamamoto, and Q. M. Wang. 2004. The IKK NF- κ B system: a treasure trove for drug development. *Nat. Rev. Drug Discov.* 3: 17–26.
- Goebeler, M., K. Kilian, R. Gillitzer, M. Kunz, T. Yoshimura, E. B. Bröcker, U. R. Rapp, and S. Ludwig. 1999. The MKK6/p38 stress kinase cascade is critical for tumor necrosis factor α -induced expression of monocyte-chemoattractant protein-1 in endothelial cells. *Blood* 93: 857–865.
- Viemann, D., M. Goebeler, S. Schmid, K. Klimmek, C. Sorg, S. Ludwig, and J. Roth. 2004. Transcriptional profiling of IKK2/NF- κ B- and p38 MAP kinase-dependent gene expression in TNF- α -stimulated primary human endothelial cells. *Blood* 103: 3365–3373.
- Denk, A., M. Goebeler, S. Schmid, I. Berberich, O. Ritz, D. Lindemann, S. Ludwig, and T. Wirth. 2001. Activation of NF- κ B via the I κ B kinase complex is both essential and sufficient for proinflammatory gene expression in primary endothelial cells. *J. Biol. Chem.* 276: 28451–28458.
- Richard, D. E., E. Berra, and J. Pouyssegur. 2000. Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1 α in vascular smooth muscle cells. *J. Biol. Chem.* 275: 26765–26771.
- Mizukami, Y., J. Li, X. Zhang, M. A. Zimmer, O. Iliopoulos, and D. C. Chung. 2004. Hypoxia-inducible factor-1-independent regulation of vascular endothelial growth factor by hypoxia in colon cancer. *Cancer Res.* 64: 1765–1772.
- Brummelkamp, T. R., R. Bernards, and R. Agami. 2002. Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell* 2: 243–247.
- Alter, O., P. O. Brown, and D. Botstein. 2000. Singular value decomposition for genome-wide expression data processing and modeling. *Proc. Natl. Acad. Sci. USA* 97: 10101–10106.
- Rajeevan, M. S., D. G. Ranamukhaarachchi, S. D. Vernon, and E. R. Unger. 2001. Use of real-time quantitative PCR to validate the results of cDNA array and differential display PCR technologies. *Methods* 25: 443–451.
- Liu, W., and D. A. Saint. 2002. A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. *Anal. Biochem.* 302: 52–59.
- Shoshani, T., A. Faerman, I. Mett, E. Zelin, T. Tenne, S. Gorodin, Y. Moshel, S. Elbaz, A. Budanov, A. Chajut, et al. 2002. Identification of a novel hypoxia-inducible factor 1-responsive gene, RTP801, involved in apoptosis. *Mol. Cell. Biol.* 22: 2283–2293.
- Goebeler, M., A. Trautmann, A. Voss, E. B. Bröcker, A. Toksoy, and R. Gillitzer. 2001. Differential and sequential expression of multiple chemokines during elicitation of allergic contact hypersensitivity. *Am. J. Pathol.* 158: 431–440.
- Schofield, C. J., and P. J. Ratcliffe. 2004. Oxygen sensing by HIF hydroxylases. *Nat. Rev. Mol. Cell Biol.* 5: 343–354.
- Sowter, H. M., R. Raval, J. Moore, P. J. Ratcliffe, and A. L. Harris. 2003. Predominant role of hypoxia-inducible transcription factor (Hif)-1 α versus Hif-2 α in regulation of the transcriptional response to hypoxia. *Cancer Res.* 63: 6130–6134.
- Nilsson, I., M. Shibuya, and S. Wennström. 2004. Different activation of vascular genes by hypoxia in primary endothelial cells. *Exp. Cell Res.* 299: 476–485.

26. Vengellur, A., J. M. Phillips, J. B. Hogenesch, and J. J. LaPres. 2005. Gene expression profiling of hypoxia signaling in human hepatocellular carcinoma cells. *Physiol. Genomics* 22: 308–318.
27. Salnikow, K., T. Davidson, Q. Zhang, L. C. Chen, W. Su, and M. Costa. 2003. The involvement of hypoxia-inducible transcription factor-1-dependent pathway in nickel carcinogenesis. *Cancer Res.* 63: 3524–3530.
28. Huang, Y., G. Davidson, J. Li, Y. Yan, F. Chen, M. Costa, L. C. Chen, and C. Huang. 2002. Activation of nuclear factor κ B and not activator protein-1 in cellular response to nickel compounds. *Environ. Health Perspect.* 110(Suppl. 5): 835–839.
29. Pugh, C. W., and P. J. Ratcliffe. 2003. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat. Med.* 9: 677–684.
30. Yuan, Y., G. Hilliard, T. Ferguson, and D. E. Millhorn. 2003. Cobalt inhibits the interaction between hypoxia-inducible factor α and von Hippel-Lindau protein by direct binding to hypoxia-inducible factor α . *J. Biol. Chem.* 278: 15911–15916.
31. Hirsilä, M., P. Koivunen, L. Xu, T. Seeley, K. I. Kivirikko, and J. Myllyharju. 2005. Effect of desferrioxamine and metals on the hydroxylases of the oxygen sensing pathway. *FASEB J.* 19: 1308–1310.
32. Salnikow, K., S. P. Donald, R. K. Bruick, A. Zhitkovich, J. M. Phang, and K. S. Kasprzak. 2004. Depletion of intracellular ascorbate by the carcinogenic metals nickel and cobalt results in the induction of hypoxic stress. *J. Biol. Chem.* 279: 40337–40344.
33. Zhou, J., T. Schmid, and B. Brüne. 2003. Tumor necrosis factor α causes accumulation of a ubiquitinated form of hypoxia inducible factor-1 α through a nuclear factor κ B-dependent pathway. *Mol. Biol. Cell* 14: 2216–2225.
34. Hellwig-Bürgel, T., K. Rutkowski, E. Metzen, J. Fandrey, and W. Jelkmann. 1999. Interleukin-1 β and tumor necrosis factor α stimulate DNA binding of hypoxia-inducible factor-1. *Blood* 94: 1561–1567.
35. Walmsley, S. R., C. Print, N. Farahi, C. Peyssonnaud, R. S. Johnson, T. Cramer, A. Sobolewski, A. M. Condliffe, A. S. Cowburn, N. Johnson, and E. R. Chilvers. 2005. Hypoxia-induced neutrophil survival is mediated by HIF-1 α -dependent NF- κ B activity. *J. Exp. Med.* 201: 105–115.
36. Bracken, C. P., M. L. Whitelaw, and D. J. Peet. 2005. Activity of hypoxia-inducible factor 2 α is regulated by association with NF- κ B essential modulator. *J. Biol. Chem.* 280: 14240–14251.
37. Kasper, L. H., and P. K. Brindle. 2006. Mammalian gene expression program resiliency: the roles of multiple coactivator mechanisms in hypoxia-responsive transcription. *Cell Cycle* 5: 142–146.
38. Viemann, D., M. Goebeler, S. Schmid, U. Nordhues, K. Klimmek, C. Sorg, and J. Roth. 2006. TNF induces distinct gene expression programs in microvascular and macrovascular human endothelial cells. *J. Leukocyte Biol.* 80: 174–185.
39. Brown, L. F., S. M. Olbricht, B. Berse, R. W. Jackman, G. Matsueda, K. A. Tognazzi, E. J. Manseau, H. F. Dvorak, and L. Van de Water. 1995. Overexpression of vascular permeability factor (VPF/VEGF) and its endothelial cell receptors in delayed hypersensitivity skin reactions. *J. Immunol.* 154: 2801–2807.
40. Lee, T. S., and L. Y. Chau. 2002. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. *Nat. Med.* 8: 240–246.
41. Chauveau, C., S. Remy, P. J. Royer, M. Hill, S. Tanguy-Royer, F. X. Hubert, L. Tesson, R. Brion, G. Beriou, M. Gregoire, et al. 2005. Heme oxygenase-1 expression inhibits dendritic cell maturation and proinflammatory function but conserves IL-10 expression. *Blood* 106: 1694–1702.