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A Novel Role of Hypoxia-Inducible Factor in Cobalt Chloride- and Hypoxia-Mediated Expression of IL-8 Chemokine in Human Endothelial Cells[1,2]

Kyoung S. Kim,[3]* Vikram Rajagopal,[3]* Caryn Gonsalves,* Cage Johnson,† and Vijay K. Kalra[4]*

Tissue hypoxemia is common in several pathological diseases, including vaso-occlusion in sickle cell disease and myocardial infarction. One finds increased presence of leukocytes during lung injury and at sites of inflammation in vascular endothelium. In this study, we used human pulmonary microvascular endothelial cells and human dermal microvascular endothelial immortalized cell line to delineate the cellular signaling mechanism of hypoxia- and $CoCl_2$ (a mimetic of hypoxia)-induced IL-8 expression, and the latter's role in chemotaxis of polmorphonuclear neutrophils. We show that hypoxia- and $CoCl_2$-induced IL-8 mRNA and protein expression involved activation of PI3K/Akt and p38 MAPK, but not MEK kinase. Analysis of some transcription factors associated with IL-8 promoter revealed that hypoxia and $CoCl_2$ increased DNA-binding activity of hypoxia-inducible factor-1α (HIF-1α), NF-κB, and AP-1. In addition, we show that hypoxia- and $CoCl_2$-induced IL-8 expression requires activation of HIF as demonstrated by the following: 1) EMSA; 2) transfection studies with IL-8 promoter reporter constructs with mutation in HIF-1α binding site; 3) attenuation of IL-8 expression by both HIF-1α small interfering RNA and R59949; 4) augmentation of IL-8 expression by either transfection with HIF-prolyl hydroxylase-2 small interfering RNA or overexpression of HIF-1α; and 5) chromatin immunoprecipitation analysis. Moreover, conditioned medium from hypoxia-treated endothelial cells augmented chemotaxis of neutrophils, due to release of IL-8. These data indicate that hypoxia-induced signaling in vascular endothelium for transcriptional activation of IL-8 involves PI3K/Akt, p38 MAPK, and HIF-1α. Pharmacological agents, which inhibit HIF-1α, may possibly ameliorate inflammation associated with hypoxia in pathological diseases. ***The Journal of Immunology,*** **2006, 177: 7211–7224.**

Endothelial cells that line the lumen of blood vessels are exposed to environments of low oxygen tension in various pathological states, such as myocardial infarction, cerebral infarction, and sickle cell disease (SCD).[5] In SCD, during low oxygen tension in the vasculature, hemoglobin S undergoes polymerization, leading to sickled shape and altered deformability of sickle RBC, with increased adherence of sickle RBC to vascular endothelium, causing sluggish flow of blood in the venular bed (1). In addition, platelets, polymorphonuclear neutrophils (PMN), and monocytes adhere to vascular endothelium in SCD, resulting in occlusion of blood vessels, characterized clinically by recurrent episodes of painful crises (1–4). The adherence of these blood cells to vascular endothelium occurs as a result of activation of endothelium (5), which expresses adhesion molecules, such as VCAM-1, ICAM-1, and P-selectin (6–9). Tissue hypoxia is common in patients with SCD (10–12), particularly in venules. The activation of endothelium can occur in response to diverse inflammatory stimuli such as infections, proinflammatory cytokines (TNF-α and IL-1β), oxidative stress including hypoxia, and vasoactive molecules such as vascular endothelial growth factor (VEGF), placenta growth factor, NO, and ET-1 (5, 8, 11, 13, 14). There is substantial evidence that there is an increased adhesion of leukocytes (PMN and monocytes) to the vascular endothelium, followed by diapedesis of PMN/monocytes through the blood vessel wall into alveolar space in SCD patients with acute chest syndrome, the major cause of morbidity and mortality (15). The adhesion of leukocytes to and diapedesis through the blood vessel has been thought to be one of the major factors contributing to tissue injury following in vivo hypoxia in SCD (16). Exposure of transgenic sickle mice, which show mild sickle cell syndrome, to hypoxia, followed by reoxygenation led to increased bronchoalveolar total leukocyte and neutrophil counts, release of inflammatory cytochemokines (17), tissue factor expression (18), and concomitant lung injury. However, relatively less is known how hypoxia activates vascular endothelium to allow PMNs and monocytes to adhere and undergo transmigration. The activation of endothelium and transmigration of leukocytes could occur as a result of inflammatory cytokines and chemokines elaborated from pulmonary endothelium in response to oxidative

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[5] Abbreviations used in this paper: SCD, sickle cell disease; ChIP, chromatin immunoprecipitation; CM, conditioned medium; Dn, dominant negative; EBM, endothelial basal medium; Epo, erythropoietin; HBEC, human brain endothelial cell; HDMVEC, human dermal microvascular endothelial cell; HIF, hypoxia-inducible factor; HPAEC, human pulmonary aortic endothelial cell; HPMVEC, human pulmonary microvascular endothelial cell; HRE, hypoxia response element; PHD, proline hydroxylase; PMN, polymorphonuclear neutrophil; RPA, RNase protection assay; siRNA, small interfering RNA; VHL, Von Hippel Lindau; wt, wild type; sc, scrambled.

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stress, including hypoxia. Thus, studies were undertaken to elucidate the molecular mechanism of expression of cytochemokines in human pulmonary microvascular endothelial cells (HPMVEC) in response to hypoxia.

Extensive studies have been conducted on the oxygen-dependent induction of different genes, including those encoding for erythropoietin (Epo), VEGF, and glycolytic enzymes (19, 20). Earlier studies of Bunn and his coworkers (21, 22) suggested that mammalian oxygen sensor for the regulation of Epo gene could be a heme protein. Further studies revealed that both Co^{2+} and Ni^{2+} mimic the effect of hypoxia by inducing Epo gene expression (21), indicating that Fe^{2+} in heme protein was replaced by the nonoxygen-binding cations. However, in some studies, the effect of hypoxia has been shown not to parallel the effect mediated by Co^{2+} (23), indicating that the oxygen sensor may be different heme protein (20).

The best-characterized response to hypoxia is the induction of hypoxia-inducible factor (HIF)-1, which has been shown to govern the oxygen-dependent induction of Epo, VEGF, and glycolytic enzymes (19, 20). The HIF are $\alpha\beta$ heterodimers, in which in the β-subunit it is constitutively expressed in contrast to the α-subunit, which is tightly regulated by oxygen (19, 20). We first determined whether hypoxia and cobalt chloride increased the gene expression of inflammatory cytochemokines in HPMVEC and human dermal microvascular endothelial cell line (HDMVEC). Second, we elucidated whether HIF played a role in the regulation of these cytochemokines.

We show that cobalt chloride and hypoxia (1% oxygen) increase the expression of MCP-1 and IL-8, among several cytochemokines examined, in HPMVEC and in HDMVEC. Moreover, the hypoxia- or cobalt chloride-induced expression of IL-8 was abrogated by small interfering RNA (siRNA) for HIF-1α, indicating the role of HIF-1α in the regulation of IL-8 expression. Previously (24), it has been shown that dimethyl oxallyl glycine, a prolyl hydroxylase inhibitor, reduced TNF-α-induced IL-8 secretion in endothelial cells, indicating the role of HIF-1α in IL-8 expression. In this study, we demonstrate for the first time, to our knowledge, the role of HIF-1α in the regulation of IL-8 chemokine expression, in response to hypoxia and cobalt chloride in human endothelial cells.

Materials and Methods

Cell culture and reagents

HPMVEC and human pulmonary aortic endothelial cells (HPAEC), primary cell cultures at passage two, were obtained from Cambrex. These were grown in endothelial basal medium (EBM-2MV; obtained from Cambrex) on 1% gelatin-coated tissue culture flasks or dishes according to manufacturer's instructions. These cells displayed endothelial phenotype and were positive for PECAM-1 (CD31) and von Willebrand factor. Both HPMVEC and HPAEC primary cells in culture were used up to passage 6–7, without undue effect on morphology or experimental protocol used in this study. Immortalized HDMVEC were obtained through the courtesy of E. Abes and T. Lawley of Emory University (Atlanta, GA), and these cell lines are referred to as HDMVEC. The latter were grown in the same medium as HPMVEC. Total leukocytes (PMN and monocytes) were isolated from blood collected in EDTA tubes. Ten volumes of blood were mixed with 1 vol of dextran-500 in PBS, and contents were kept for 45 min at room temperature. Then buffy layer on top of RBC was aspirated (25). To the buffy coat, PBS was added and centrifuged at 3000 $\times$ *g*. Cells were washed with PBS three times to remove platelets. This leukocyte-rich fraction was used for chemotaxis assay.

LY294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one), R59949, and PD98059 (2′-amino-3′-methoxyflavone) were obtained from Calbiochem. SB203580 and SP600125 were obtained from Tocris. HIF-1α mAb was obtained from BD Biosciences. Secondary Abs conjugated to HRP were purchased from Santa Cruz Biotechnology. All other reagents, unless otherwise specified, were purchased from Sigma-Aldrich.

RNase protection assay (RPA)

Endothelial cells were treated with cobalt chloride or exposed to hypoxia (1% oxygen in Forma tissue culture incubator with attached oxygen sensor) for various time periods, and total RNA was isolated with TRIzol reagent (Invitrogen Life Technologies). RPA were performed on total RNA extracted from endothelial cells using custom-made multiprobe templates H-1 comprised of TNF-α, IL-1β, RANTES, MIP-1β, MCP-1, IL-8, and the housekeeping genes L-32 and GAPDH (BD Biosciences). Briefly, RNA probes were uniformly labeled with [α-^{32}P]UTP using T7 RNA polymerase according to manufacturer's protocol. Five micrograms of total RNA was hybridized with ^{32}P-labeled probes (8×10^5 cpm) for 12–16 h at 56°C. The contents were treated with RNase mixture (BD Pharmingen), followed by extraction with phenol-chloroform. Protected RNA duplexes were resolved on a 6% denaturing polyacrylamide-sequencing gel and exposed to x-ray film for 24 h.

Quantification of chemokines

Endothelial cells (0.5×10^6 cells/ml) were incubated in serum-free basal medium in the presence or absence of $CoCl_2$ (0.5–1 mM). At indicated periods, supernatant was collected and stored at −80°C until further use. The amounts of secreted chemokines (MCP-1 and IL-8) were assayed using specific Duo-Set, ELISA development systems (R&D Systems), according to the manufacturer's instructions. The total protein was estimated in the cells by the Bradford method.

Transfection with siRNA

The forward and reverse HIF-α RNA strands (UGUGAGUUCGCAUCU UGAUTT) and (AUCAAGAUGCGAACUCACATT); forward and reverse scrambled (sc)HIF-α RNA strands (UACACCGUUAGCAGACAC CTT) and (GGUGUCUGCUAACGGUGUATT), as previously described (26); and proline hydroxylase (PHD)-2 siRNA (sense, AAGGACAUC CGAGGCGAUAAG) and (antisense, CUUAUCGCCUCGGAUGUC CUU), as previously described (25) were synthesized and purified at Microchemical Core Facility of University of Southern California Norris Cancer Center. The sense and antisense oligonucleotides were annealed at 95°C for 1 h, as described (26). HDMVEC ($3–5 \times 10^5$ cells) were transfected with HIF-1α siRNA (4 μg) using Lipofectamine 2000, according to the manufacturer's protocol (Invitrogen Life Technologies).

Western blot analysis

Endothelial cells were incubated in RPMI 1640 containing 2% FBS for 18 h. Medium was aspirated and replaced with fresh serum-free medium. After 3 h, they were either treated with $CoCl_2$ or exposed to hypoxia. Where indicated, cells (5×10^6 cells) were incubated with pharmacological inhibitors for 30 min before $CoCl_2$ or hypoxia treatment. At the end of the treatment, medium was aspirated and cells were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris (pH 8.0), 1 mM EGTA, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 10 μg/ml PMSF, and 1 μl/ml protease inhibitor mixture). The lysate was centrifuged at 10,000 $\times$ *g* for 20 min at 4°C. An aliquot (10 μl) of supernatant was subjected to electrophoresis on a 7.5% SDS-PAGE, followed by transfer to a nitrocellulose membrane (Bio-Rad). Blots were probed with 1/500 dilutions of HIF-1α-specific mAbs (BD Biosciences). HRP-conjugated secondary Abs were used to develop the membrane. The protein bands were detected with SuperSignal chemiluminescent substrate (Pierce Biotechnology). Blots were stripped and reprobed using a 1/1000 dilution of Abs against actin to monitor protein loading.

Preparation of nuclear extracts

Nuclear extracts were prepared from endothelial cells, according to the modified procedure of Dignam et al. (27). Briefly, 5×10^6 cells were washed with cold PBS. Cells were resuspended in 400 μl of cell lysis buffer (10 mM HEPES (pH 7.9), 100 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF, 0.5% Nonidet P-40, and 1 μl/ml protease inhibitor mixture) and allowed to swell on ice for 30 min, followed by vigorous vortex mixing for 5–10 s. The homogenate was centrifuged in a microfuge at 10,000 $\times$ *g* for 30 s. Supernatant was discarded and the nuclear pellet was resuspended in 50 μl of nuclear extraction buffer (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 420 mM NaCl, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol, 0.5 mM PMSF, and 1 μl/ml protease inhibitor mixture). The tube was mixed intermittently for 60 min. The nuclear extract was obtained by centrifuging at 10,000 $\times$ *g* for 10 min at 4°C.

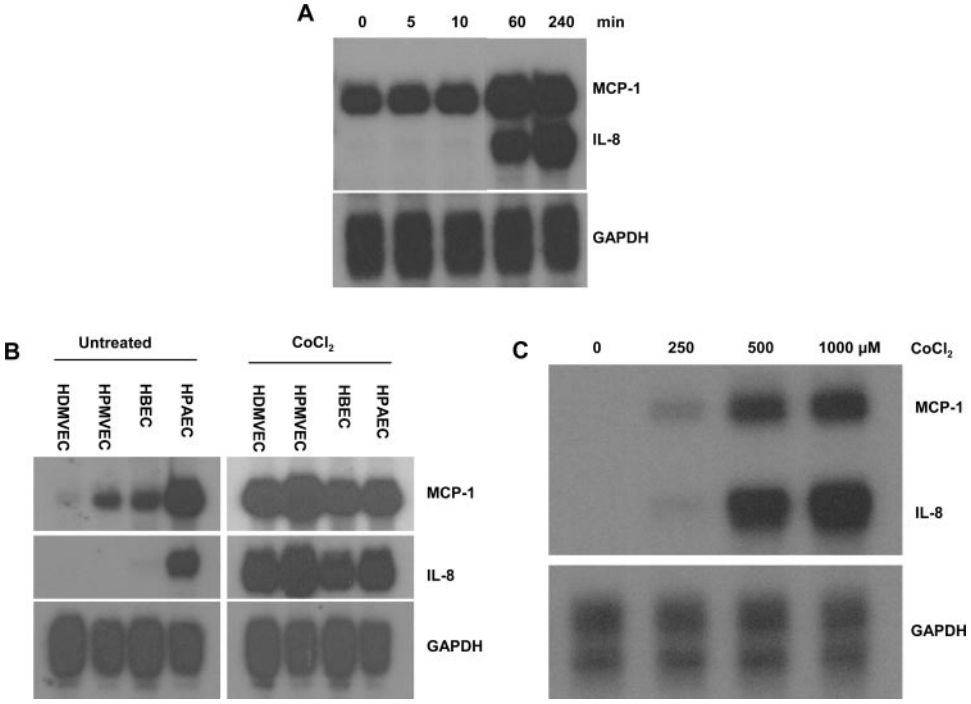

FIGURE 1. Effect of $CoCl_2$ on MCP-1 and IL-8 mRNA expression in human microvascular endothelial cells from different vascular beds. *A*, HPMVEC were treated with $CoCl_2$ (1 mM) for 1 h. RNA (5 μg) was subjected to RPA analysis, as described in *Materials and Methods*. GAPDH signals show equal loading of each RNA sample. *B*, Human endothelial cells of different vascular beds, namely dermal (HDMVEC), pulmonary microvascular (HPMVEC), brain (HBEC), and pulmonary aortic (HPAEC), were treated with $CoCl_2$ (1 mM) for 1 h, followed by RPA analysis. *C*, HDMVEC-immortalized cells were treated with $CoCl_2$ (0–1000 μM) for 1 h, and RNA from these were subjected to RPA analysis.

EMSA for transcription factors NF-κB, AP-1, and HIF-α

The oligonucleotide probes were as follows: AP-1, 5′-CGC TTG ATG ACT CAG CCG GAA-3′ and 3′-GCG AAC TAC TGA GTC GGC CTT-5′; NF-κB, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ and 3′-TCA ACT CCC CTG AAA GGG TCC G-5′; and HIF-α, 5′-TCT GTA CGT GAC CAC ACT CAC CTC-3′ and 3′-AGA CAT GCA CTG GTG TGA GTG GAG-5′, which were obtained from Santa Cruz Biotechnology. The putative probes for HIF-1α in IL-8 promoter 5′-TTGCAAATCGTG GAATTTCCT-3′ and 3′-AACGTTTAGCACCTTAAAGGA-5′ were synthesized by Microchemical Core Facility of Norris Cancer Center at University of Southern California. Probes were 5′ end labeled with 100 μCi of [γ-^{32}P]ATP using T4-polynucleotide kinase. The labeled, single-stranded sense oligonucleotide probe was mixed with labeled antisense probe, and incubated at 65°C for 5 min, followed by annealing at room temperature for 15 min. The DNA-binding reaction mixture contained nuclear proteins (2–4 μg), ^{32}P-labeled double-stranded oligonucleotide probe (~50,000 cpm), and 2 μg of poly(dI-dC). To demonstrate specificity of DNA-protein interaction, 50-fold excess of unlabeled double-stranded oligonucleotide probe was added. The DNA-protein complex was then subjected to electrophoresis in a 4% nondenaturing polyacrylamide gel. The gel was vacuum dried and exposed to x-ray film.

Transfection of HDMVEC with dominant-negative (Dn) Akt, Dn PI3K, and PTEN

HDMVECs were transfected with a plasmid expressing a Dn mutant of Akt and a Dn mutant of PI3K (Dn-Δp85) provided by D. Ann (University of Southern California School of Pharmacy, Los Angeles, CA), as previously described for THP-1 cells (14). PTEN plasmid was provided by D. Johnson (University of Southern California, Los Angeles, CA). A total of 4 μg of plasmid in 250 μl of serum-free EBM-2 medium and 10 μl of Lipofectamine 2000 (Invitrogen Life Technologies) in 250 μl of serum-free EBM-2 was mixed, and contents were incubated at room temperature for 20 min. The liposome-DNA complexes (500 μl) were added to the endothelial cells (0.5×10^6 cells/ml) in 1.5 ml of EBM-2 medium in 60-mm petri dish and incubated at 37°C for 36 h, as described (28). The medium was removed, and cells were gently washed with serum-free medium. Then 2 ml of serum-free EBM-2 medium was added to cells and incubated for 4 h, which was followed by either treatment for 24 h with $CoCl_2$ to determine the release of IL-8 in the supernatant or treatment for 1 h with $CoCl_2$ for extraction of RNA for RPA analysis.

Human IL-8 promoter-luciferase constructs and luciferase assay

The full-length IL-8 luciferase construct containing 1521 bp (nt −1481 to +40) of the promoter region of IL-8 gene, and IL-8 reporter constructs containing mutation (indicated by capital letters) in the NF-κB sequence (cgtTAaCtttcctct) were provided by C. Pothoulakis and A. Keates (Harvard Medical School, Boston, MA), as described (29). IL-8 reporter constructs containing mutations in HIF binding site (cAtg), mutations in only NF-κB binding site (cgtgAaCtttcctct) referred to as NF-κB mut, and mutation in both HIF and NF-κB site (cAtgAaCtttcctct) were generated. The substitution mutants were based on the human IL-8 promoter sequence (GenBank accession no. D14283). The cloned sequences were confirmed by DNA sequence analysis using primers specific for pGL3 basic (29). HDMVEC were grown to 50–60% confluence in six-well plates, washed in EBM-2, and incubated in 1 ml of serum-free medium with IL-8 reporter construct (~ 2 μg) and β-galactoside plasmid (~2 μg of DNA) for 3 h, followed by addition of 1 ml of complete medium, and incubated for 24 h. Cells were washed with EBM-2 medium and incubated further in 2 ml of serum-free medium for 3 h, and then cells were treated with $CoCl_2$ for 1 h. Cells were harvested and analyzed for luciferase activity, using a luminometer (Berthold Technologies; Lumat LB 9501), for the light emitted during the initial 10 s of the reaction. β-galactosidase activity was assayed by colorimetric assay using *o*-nitrophenyl-β-D-galactoside as a substrate. The data are normalized for β-galactosidase activity and expressed as relative luciferase unit.

Chromatin immunoprecipitation (ChIP) assay

HDMVEC (10×10^6 cells) were serum starved for 6 h, followed by treatment with $CoCl_2$ for indicated time period. ChIP analysis was performed, as described (30). Briefly, after stimulation of cells with $CoCl_2$, cells were washed with PBS and then cross-linked with 1% formaldehyde at room temperature for 10 min. Cells were lysed and sonicated, and supernatants were then recovered by centrifugation of lysate at 12,000 rpm for 10 min at 4°C. The supernatant was diluted 4-fold in dilution buffer (20 mM Tris-HCl (pH 8.1), 1% Triton X-100, 2 mM EDTA, and 150 mM NaCl), followed by the addition of 2 μg of sheared salmon-sperm DNA, 2.5 μg of preimmune serum, and 20 μl of protein A-Sepharose (50% slurry). The contents were kept at 4°C for 2 h. The precleared supernatant was immunoprecipitated by adding Ab (2 μg/ml) to HIF-1α, 2 μg of sheared salmon-sperm DNA, and 20 μl of protein A-Sepharose (50% slurry), followed by incubation at 4°C for 12–16 h. After several washings, the protein was digested with proteinase K (10 μg/ml) for 1 h. The cross-linking between DNA and protein was reversed by incubating the immunoprecipitate at 65°C overnight. DNA was extracted using phenol-chloroform, followed by ethanol precipitation and air drying, and finally the pellet was dissolved in 50 μl of TE buffer (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA). A total of 5 μl of DNA sample was subjected to PCR amplification using primers (5′-GATTGGCTGGCTTATCTTC-3′, forward primer, and (5′-CATCAC CCTACTAGAGAAC-3′, reverse primer) corresponding to the promoter region of IL-8 (from −21 to −420 respective to the transcription start site).

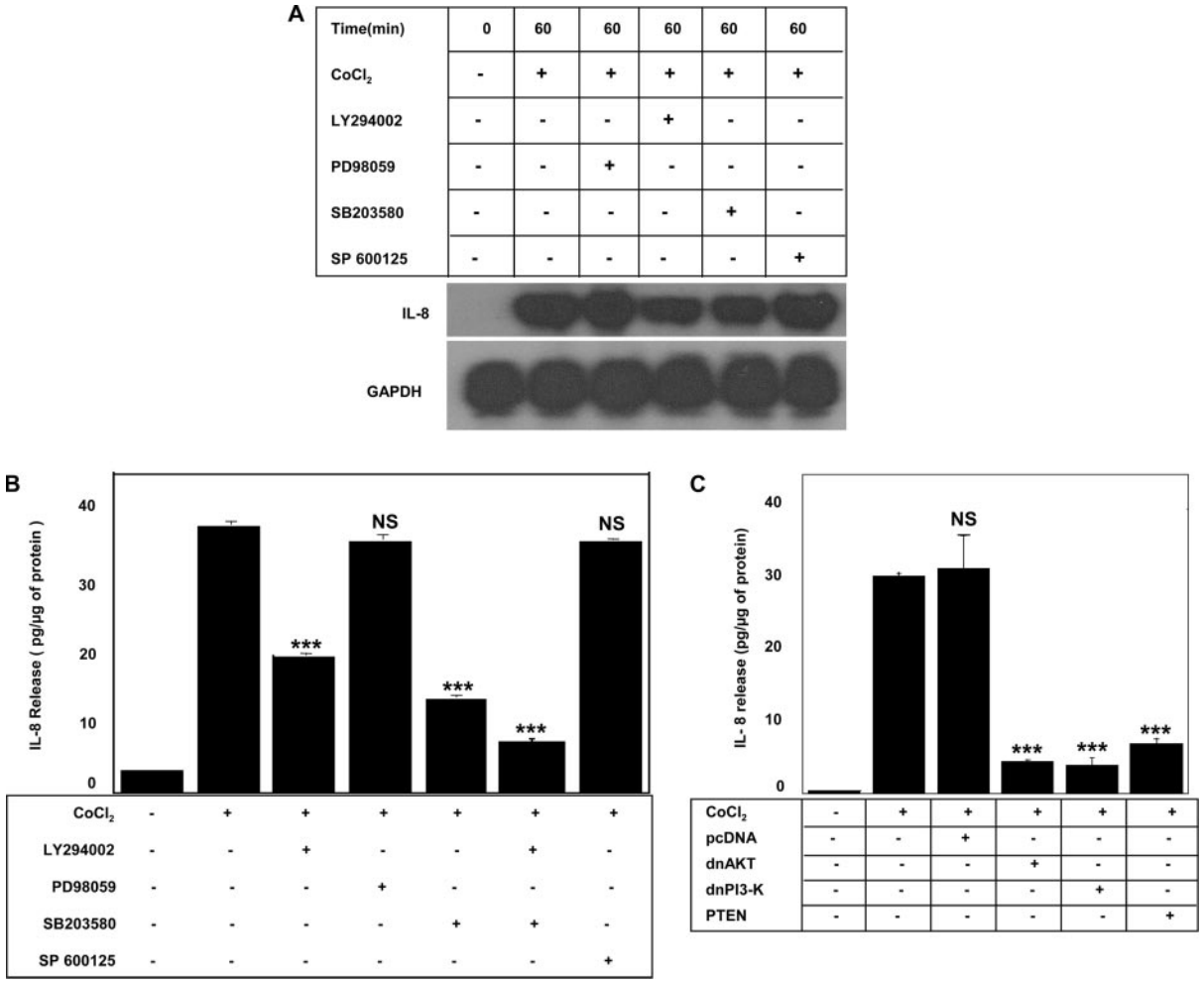

FIGURE 2. Effect of protein kinase inhibitors and transfection with Dn constructs on IL-8 expression. *A*, HDMVEC were preincubated for 30 min with LY294002 (15 μm), PD98059 (10 μM), SB203580 (1 μM), and SP600125 (100 nM), followed by treatment with $CoCl_2$ (0.5 mM) for 1 h. RNA samples were subjected to RPA analysis, as described in Fig. 1. *B*, HPMVEC were preincubated for 30 min with indicated inhibitors, followed by treatment with $CoCl_2$ (0.5 mM) for 24 h. Cell-free supernatant was collected, and 100 μl from the total 1-ml supernatant was used for determining levels of IL-8 by ELISA; $n = 3$. *C*, HDMVEC (5×10^5) were transfected with 4 μg of DNA of Dn Akt, Dn PI3K, and PTEN. Following 36-h transfection, cells were stimulated with $CoCl_2$ (0.5 mM) for 24 h. Cell-free supernatant was collected, and 100 μl from the total 2-ml supernatant was used for determining levels of IL-8 by ELISA. Data are expressed as the mean $\pm$ SD of $n = 3$. ***, $p < 0.001$. $CoCl_2$ treated vs $CoCl_2$ treated in the presence of inhibitors or Dn constructs.

Chemotaxis assay

Chemotaxis was assayed in 96-well plates (NeuroProbe) with Transwell inserts of 5-μm pore size. Briefly, leukocyte fractions resuspended in serum-free RPMI 1640 (5×10^5 cells/50 μl) were added to the upper chamber of the Boyden chamber. Chemotaxis medium (30 μl of serum-free RPMI 1640) containing indicated amounts of chemokines or conditioned medium (CM) elaborated from HDMVEC under various treatment conditions was placed in the bottom compartment. After 2 h of incubation at 37°C in a 5% CO_2 incubator, cells were scraped from the upper chamber, and then inserts were washed with PBS (100 μl) to remove nonmigrated cells. This was followed by addition of PBS containing 2 mM EDTA (30 μl) to the upper chamber and incubation at 4°C for 15 min. Cells that had migrated into the lower compartment of the Boyden chamber were counted by taking an aliquot and counting in a hemocytometer. Where indicated, the effect of neutralizing Ab against chemokines was determined by adding Ab to the chemoattractant protein in the lower compartment of the chemotaxis chamber. Each sample was tested in triplicate.

Statistical analysis

Statistical analysis of the responses obtained from control and $CoCl_2$-treated endothelial cells was conducted by one-way ANOVA using Instat 2 software program (GraphPad). Student's *t* test was used for multiple comparisons. Values of $p < 0.05$ were considered as significant.

Results

Effect of cobalt chloride on the expression of cytokines and chemokines in HPMVEC

Studies have shown that there are increased levels of IL-1β, TNF-α (31, 32), and IL-8 (33) in sera from patients with SCD. Moreover, monocytes are in activated state, because of increased expression of IL-1β and TNF-α. Our recent studies (13, 14) show that monocytes isolated from normal individuals become activated in response to placenta growth factor, resulting in increased mRNA and protein expression of some cytokines (TNF-α, IL-1β) and chemokines (MCP-1, IL-8, and MIP-1β), whereas the expression of these same cytochemokines was significantly higher in mononuclear cells from SCD at steady state. We determined whether hypoxia augmented the expression of cytochemokines in vascular endothelial cells. Treatment of HPMVEC with cobalt chloride (1 mM), a mimetic of hypoxia, resulted in 2-fold increase in mRNA expression of MCP-1 and a several fold increase in IL-8 expression at 60 and 240 min, as determined by RPA (Fig. 1*A*). The maximal expression of these chemokines occurred at 60 min.

We examined the dose (125–1000 μM)-dependent effect of cobalt chloride on the expression of both MCP-1 and IL-8 in HPMVEC. The effect was optimal at 0.5 mM $CoCl_2$ (data not shown).

Effect of $CoCl_2$ on the expression of MCP-1 and IL-8 by endothelial cells derived from different vascular beds

To determine whether the effect of $CoCl_2$ was specific for HPMVEC, we studied HPAEC, human brain endothelial cells (HBEC), and an immortalized cell line of HDMVEC. As shown in Fig. 1*B*, HPMVEC and HDMVEC (immortalized endothelial cell line) showed robust increase in the expression of MCP-1 and IL-8 mRNA above basal expression, in response to $CoCl_2$ (1 mM). HBEC displayed a modest increase in MCP-1, although an increase in IL-8 expression was significantly above the basal level. However, in HPAEC there were higher endogenous levels of MCP-1 and IL-8, which increased only modestly in response to $CoCl_2$. As shown in Fig. 1*C*, 0.5–1 mM $CoCl_2$ was optimal for the mRNA expression of MCP-1 and IL-8 in HDMVEC. The viability of cells, as measured by trypan blue exclusion assay, remained unchanged with either 0.5 or 1 mM cobalt chloride during the treatment period of 24 h. Thus, we used these concentrations of $CoCl_2$ for experiments described in this work.

Effect of pharmacological inhibitors on $CoCl_2$-induced IL-8 expression in HDMVEC

Because $CoCl_2$ treatment resulted in an increased gene expression of MCP-1 and IL-8, we determined the effect of pharmacologic inhibitors, which have been shown previously to be specific for specific protein kinases in the cell signaling pathways (34). We focused our studies on the expression of IL-8. As shown in Fig. 2*A*, pretreatment of HDMVEC with LY294002, a specific inhibitor of PI3K, reduced by ~50% the $CoCl_2$-induced mRNA expression of IL-8 (Fig. 2*A*, *lane 4*). PD98059, a specific inhibitor of MEK (MAPKK or MEK), did not reduce $CoCl_2$-induced mRNA expression of IL-8 (Fig. 2*A*, *lane 3*). However, SB203580, a selective p38MAPK inhibitor, reduced IL-8 expression by ~50% (Fig. 2*B*, *lane 5*). However, SP600125, a JNK kinase inhibitor, did not affect the mRNA expression of IL-8 (Fig. 2*A*, *lane 6*).

We next examined whether induction of IL-8 mRNA levels by $CoCl_2$ translated into increased IL-8 protein expression. As shown in Fig. 2*B*, $CoCl_2$ caused substantial increase in IL-8 protein levels in HPMVEC, which was absent under basal conditions. Both PD98059 and SP60025 (JNK kinase inhibitor) did not affect $CoCl_2$-induced release of IL-8, whereas LY294002 (a PI3K inhibitor) reduced by >50% the release of IL-8. SB203580 reduced by ~70% the $CoCl_2$-induced release of IL-8 (data not shown). We determined whether same pharmacological kinase inhibitors affected IL-8 protein expression in HDMVEC. In HDMVEC, $CoCl_2$-induced IL-8 release into the supernatant was reduced in response to LY294002 and SB203580 by ~50 and ~65%, respectively. However, the addition of both LY294002 and SB203580 inhibitors reduced IL-8 release by ~80%. These results indicate that the effect may be additive. Nonetheless, PD98059 (a MAPK inhibitor) and SP60025 (JNK kinase inhibitor) did not inhibit IL-8 release (Fig. 2*B*). These results indicate that cobalt chloride-mediated IL-8 expression in both HDMVEC and HPMVEC required activation of PI3K and p38MAPK, but did not involve MAPK and JNK kinase. Because HPMVEC are primary cultures and can be grown up to 6–7 passages, we used HDMVEC (immortalized human endothelial cell line) for ease of culture and transfection, to delineate the cell signaling pathways.

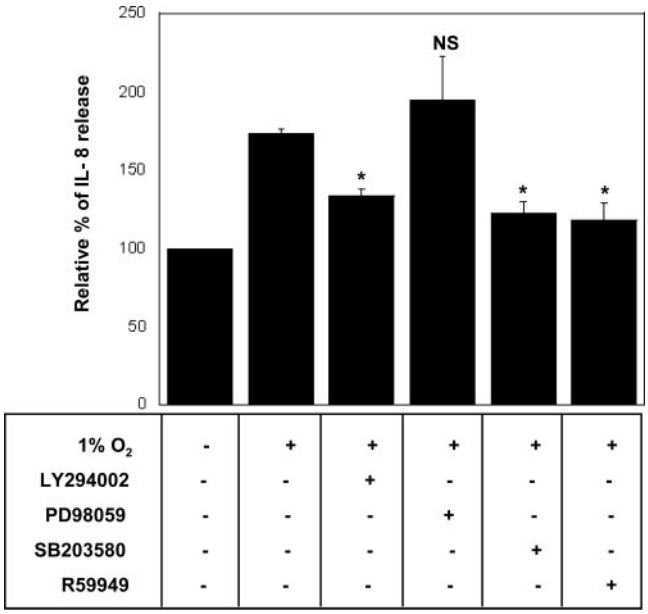

FIGURE 3. HDMVEC were preincubated for 30 min with LY294002 (15 μm), PD98059 (10 μM), SB203580 (1 μM), and R59959 (30 μM). Cells were exposed to 1% O_2 (hypoxia) for 6 h. Cell-free supernatant was collected, and 100 μl from the total 2-ml supernatant was used for determining levels of IL-8 by ELISA. Data are expressed as percentage of IL-8 released relative to control as 100%. At 6-h time period, the amount of IL-8 released in response to hypoxia treatment was 1.12 pg/μg protein vs 1.9 pg/μg protein with $CoCl_2$. Data are means ± SD of $n = 3$. *, $p < 0.05$. Hypoxia vs hypoxia treatment in the presence of inhibitors.

Effect of expression of Dn PI3K and Akt, and overexpression of PTEN on $CoCl_2$-mediated expression of IL-8 in HDMVEC

Our studies show that LY294002 reduced $CoCl_2$-mediated mRNA expression and protein secretion of IL-8, indicating the role of PI3K/Akt in the signaling pathway. To unequivocally establish that the effect of pharmacologic inhibitor of PI3K was specific, we overexpressed a Dn Akt and a Dn PI3K p85 subunit (Dn-Δp85) in HDMVEC. As shown in Fig. 2*C*, expression of Dn Akt and Dn PI3K p85subunit reduced IL-8 protein release by ~85%, in response to $CoCl_2$. As shown in Fig. 2*C*, transfection of HDMVEC with PTEN, a key regulatory component of PI3K-Akt signaling cascade (35), followed by treatment with cobalt chloride for 24 h, resulted in ~75% reduction in IL-8 release from HDMVEC.

Effect of hypoxia and HIF-1α antagonists on the release of IL-8 from HDMVEC

Because we observed that $CoCl_2$-induced IL-8 protein expression was regulated by PI3K and p38 MAPK, we determined whether similar effects were observed under hypoxic conditions. As shown in Fig. 3, exposure of HDMVEC to hypoxia (1% oxygen) for 24 h resulted in ~1.6-fold increase in IL-8, which was inhibited ~50 and ~75% by LY294002 and SB203580, respectively, as also observed in $CoCl_2$-treated cells. PD98059 was ineffective in inhibiting hypoxia-induced IL-8 release as was observed in $CoCl_2$-treated HDMVEC. However, pretreatment of HDMVEC with R59949, a diacyl glycerol kinase inhibitor and a putative activator of HIF prolyl hydroxylase, reduced by ~75% of hypoxia-induced IL-8 release by HDMVEC (Fig. 3).

HIF-1α siRNA attenuate $CoCl_2$-mediated IL-8 expression in HDMVEC

Analysis of the IL-8 promoter region (GenBank accession no. M28130) shows (Fig. 4) consensus binding sites for HIF-1α, i.e.,

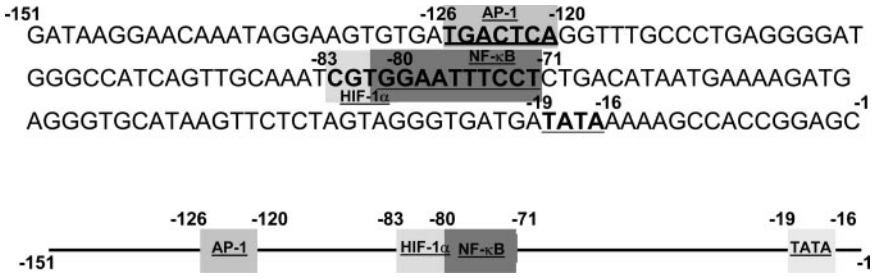

FIGURE 4. Schematics of IL-8 promoter region showing HIF-1α, NF-κB, and AP-1 DNA binding sites.

hypoxia response element (HRE; CGTG), which was confirmed by performing EMSA analysis using probes corresponding to HIF-α binding sites in IL-8 promoter regions (data shown below; Fig. 5). We hypothesized that $CoCl_2$-mediated increased IL-8 mRNA expression could be regulated through HIF-1α transcription factor. Thus, interference of HIF-α production by siRNA for HIF-1α could possibly interfere with $CoCl_2$-mediated HIF-1α DNA-binding activity and concomitant inflammatory chemokine gene expression. As shown (Fig. 6*A*, *lane 4*), treatment of HIF-1α siRNA-transfected HDMVEC with $CoCl_2$ resulted in >90% inhibition of IL-8 mRNA expression. However, there was no significant effect in IL-8 gene expression (Fig. 6*A*, *lane 3*) by $CoCl_2$ in HDMVEC transfected with scHIF-1α RNA (scHIF-1α siRNA). The effect was specific for HIF-1α siRNA, as transfection with Egr-1 siRNA did not reduce IL-8 mRNA expression (Fig. 6*A*, *lane 5*), as was observed with scHIF-1α siRNA (Fig. 6*A*, *lane 3*). The results in Fig. 6*A* show that HDMVEC transfected with HIF-1α siRNA show complete reduction in HIF-1α RNA (Fig. 6*A*, *lane 4*) compared with cells transfected with scHIF-1α siRNA (Fig. 6*A*, *lane 3*), Egr-1 siRNA (Fig. 6*A*, *lane 5*), and nontransfected cells treated with $CoCl_2$ (Fig. 6*A*, *lane 2*). $CoCl_2$-induced release of IL-8 was reduced by ~60% in HDMVEC transfected with siRNA for HIF-1α (Fig. 6*C*, *lane 4*). The effect of scHIF-1α (Fig. 6*C*, *lane 3*) and siRNA for Egr-1 (Fig. 6*C*, *lane 5*) was not significant on the $CoCl_2$-induced release of IL-8. Transfection with HIF-2α siRNA did not reduce HIF-1α mRNA as well as expression of IL-8 (Fig. 6*B*, *lane 4*). These results indicate that cobalt chloride-mediated expression of IL-8 requires activation of HIF-1α.

Effect of $CoCl_2$ on the activation of NF-κB, AP-1, and HIF-1α as determined by EMSA

Next, we wanted to determine whether $CoCl_2$ promoted an increase in the DNA binding of HIF-1α, as HIF-1α siRNA attenuated $CoCl_2$-mediated IL-8 expression. As shown in Fig. 5*A*, $CoCl_2$ caused an increased HIF-1α binding to DNA in the nuclear extracts, using bona fide HIF-1α probe (wild-type (wt) HIF-1α

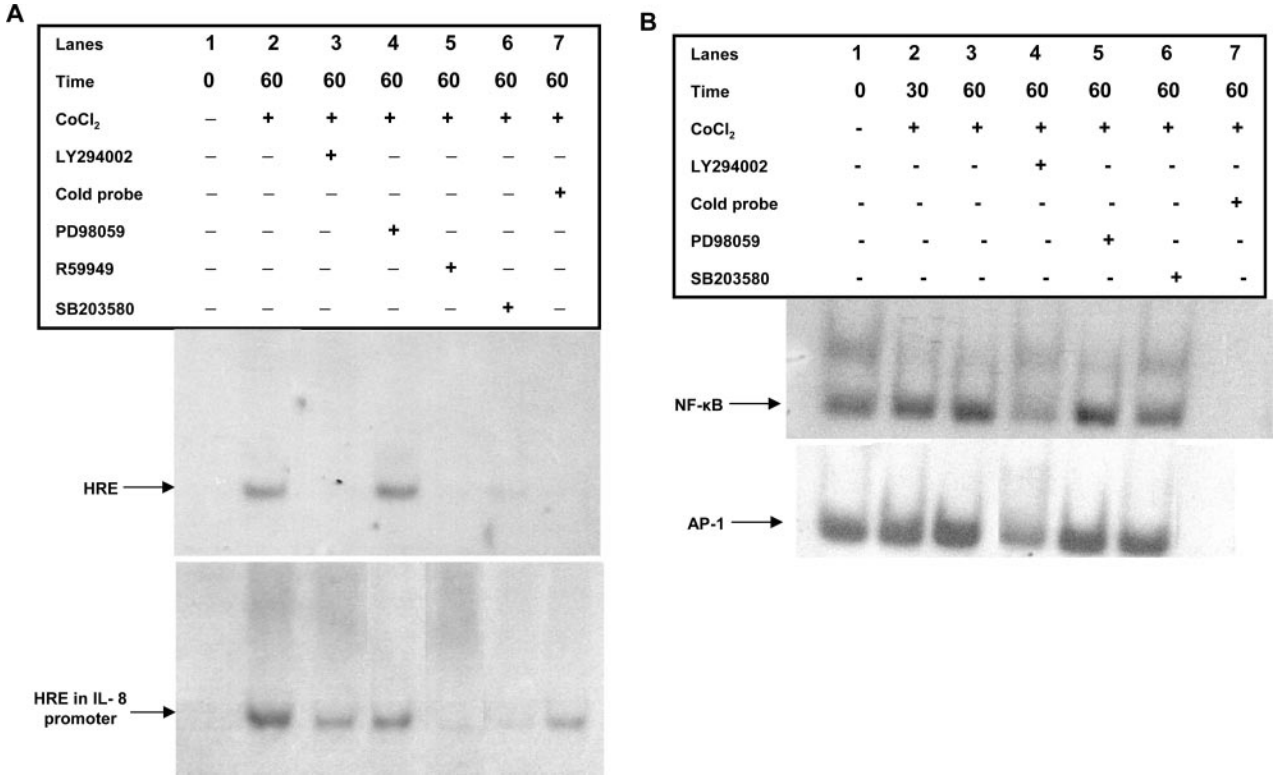

FIGURE 5. Effect of $CoCl_2$ treatment on HIF-1α-, NF-κB-, and AP-1-binding activities in nuclear extracts of HDMVEC by gel shift assay. *A*, HDMVEC were pretreated for 30 min with inhibitors LY294002 (15 μm), PD98059 (10 μM), SB203580 (1 μM), and R59959 (30 μM). The cells were then treated with $CoCl_2$ (0.5 mM) for 1 h. Nuclear extracts were prepared and incubated with ^{32}P-labeled oligonucleotide probes for HRE (bona fide) or HRE in IL-8 promoter. Where indicated, excess cold probe was added to nuclear extracts to show competition. *B*, HDMVEC treated with $CoCl_2$ (0.5 μM) for different time intervals (0, 30, and 60 min) in the presence and absence of inhibitors. Nuclear extracts from these samples were subjected to EMSA for NF-κB and AP-1 DNA-binding activity. Data are representative of two independent experiments.

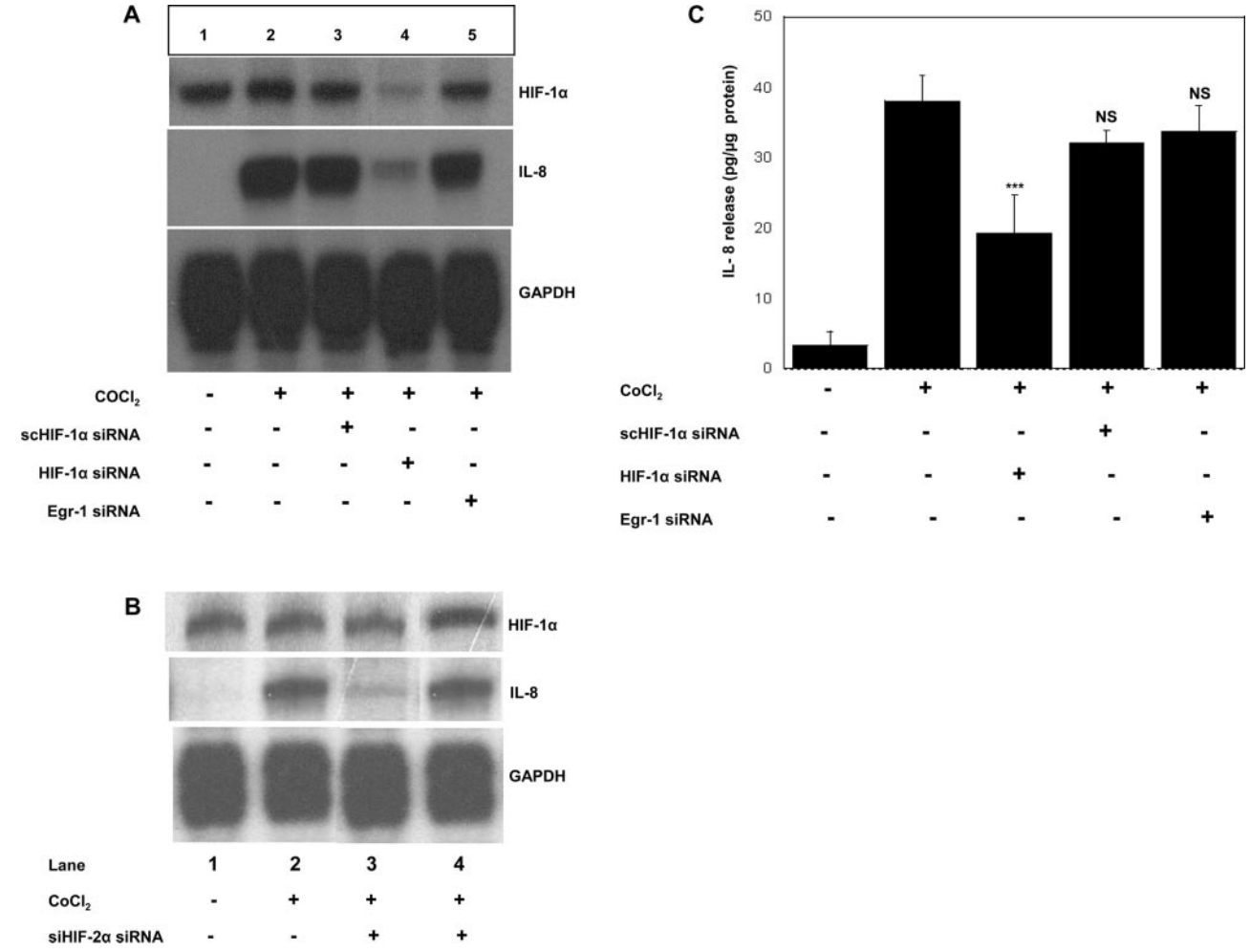

FIGURE 6. Transfection with HIF-1α siRNA suppresses $CoCl_2$-mediated IL-8 mRNA and protein expression in HDMVEC. *A* and *B*, HDMVEC (5×10^5 cells) were transfected with HIF-1α siRNA, scHIF-1α siRNA, Egr-1 siRNA, and siHIF-2α siRNA (100 pmol). After 36 h of transfection, the cells were treated with $CoCl_2$ (0.5 mM) for 1 h. The RNA samples were analyzed by RPA. *C*, Cell-free supernatants were collected from HDMVEC (5×10^5 cells), which were transfected with HIF-1α siRNA, scHIF-1α siRNA, and Egr-1 siRNA (100 pmol), followed by treatment with $CoCl_2$ for 24 h. One hundred microliters from the total 2-ml supernatant was used for determining levels of IL-8 by ELISA. Data are expressed as the means $\pm$ SD of $n = 3$. ***, $p < 0.001$; $CoCl_2$ treated vs $CoCl_2$ treatment in siRNA-transfected cells.

probe). The binding was optimal at 60 min (Fig. 5*A*, *lanes 2*). As shown in Fig. 5*A* (*lane 3*), LY294002 reduced by ~75% HIF-1α DNA binding in $CoCl_2$-treated HDMVEC. As expected PD98059, which did not inhibit $CoCl_2$-mediated increase in IL-8, also had no effect on HIF-1α DNA-binding activity (Fig. 5*A*, *lane 4*). Both R59949 (Fig. 5*A*, *lane 5*) and SB203580 (Fig. 5*A*, *lane 6*) completely reduced $CoCl_2$-mediated HIF-1α DNA-binding activity. Excess cold probe abrogated HIF-1α DNA-binding activity (Fig. 5*A*, *lane 7*). These results indicate the presence of HIF-1α binding region in the IL-8 promoter.

A search for potential HIF-1α transcription factor binding sites was performed in the human IL-8 promoter region (GenBank accession no. M28130). The data show that human IL-8 promoter contains CGTG at positions -83 to -80 (Fig. 4). On the basis of these observations, we hypothesized that IL-8 promoter with the CGTG motif could be a DNA binding site for HIF-1α. We used oligonucleotides upper strand, 5′-TTGCAAATCGTGGAATT TCCT, as the putative HIF-1α consensus sequence in IL-8 promoter (-91 to -71) for EMSA analysis.

As shown in Fig. 5*A*, *lower panel* (*lane 2*), $CoCl_2$ caused an optimal increase in HIF-1α-binding activity after 60-min treatment using HRE of IL-8 promoter sequence as an oligonucleotide probe. Moreover, excess cold HIF-1α probe reduced by >90% the HIF-1α-DNA bands in EMSA (Fig. 5*A*, *lane 7*). As expected, LY294002 (Fig. 5*B*, *lane 3*) and R59949 (Fig. 5*B*, *lane 5*) reduced $CoCl_2$-induced HIF-1α binding, whereas PD98059 had no effect (Fig. 5*B*, *lane 4*). SB203580 reduced HIF-1α binding by ~50% (Fig. 5*B*, *lane 6*).

Because IL-8 promoter contains a consensus binding site for the transcriptional factor NF-κB and AP-1 (36) and is also indicated in schematics (Fig. 4), we determined whether these transcription factor activities were augmented by $CoCl_2$. As shown in Fig. 5*B*, $CoCl_2$ caused a time-dependent increase in NF-κB-binding activity (Fig. 5*B*, *lanes 2* and *3*) in nuclear extracts prepared from $CoCl_2$-treated HDMVEC, which was inhibited by LY294002 (Fig. 5*B*, *lane 4*) and SB203580 (*lane 6*), and unaffected by PD98059 (Fig. 5*B*, *lane 5*). As shown, $CoCl_2$ caused a time-dependent increase in AP-1-binding activity (Fig. 5*B*, *lower panel lanes 2* and *3*), which was inhibited by LY294002 (Fig. 5*B*, *lane 4*) and modestly by SB203580 (Fig. 5*B*, *lane 6*), but not by PD98059 (Fig. 5*B*, *lane 5*). These results showed that $CoCl_2$ activated HIF-1α, NF-κB, and AP-1 in HDMVEC, which are inhibited by inhibitors of PI3K and p38MAPK, but not by MAPK inhibitor.

Hypoxia causes increased HIF-1α DNA-binding activity

Because $CoCl_2$ caused increased HIF-1α DNA-binding activity in HDMVEC, we determined whether hypoxia caused a similar effect as $CoCl_2$. As shown in Fig. 7*A*, hypoxia (1%) caused a time-dependent (1–6 h) increase in HIF-1α DNA binding in nuclear extracts, with optimal binding occurring at 4 h (*lane 4*). The HIF-1α DNA binding was reduced ~90% by LY294002 (Fig. 7*A*, *lane 6*), R59949 (Fig. 7*A*, *lane 7*), and SB203580 (Fig. 7*A*, *lane 8*). The specificity of HIF-1α binding was shown by competition of HIF-1α binding by either excess cold wt HIF-1α probe (*lane 9*) or putative IL-8 promoter HIF-1α cold probe (*lane 10*). Next, we examined whether the putative HIF-1α binding site in IL-8 promoter behaved in similar way as the bona fide (CGTG) sequence within HIF-1α. As shown in Fig. 7*B*, there was a time-dependent increase in HIF-1α binding (Fig. 7*B*, *lane 4*) at 4 h, which was >90% inhibited by LY294002 (*lane 8*) and ~50% inhibited by R59949 (*lane 9*). SB203580 reduced by ~90% HIF-1α binding (*lane 10*). Both excess cold probes for wt HIF-1α (*lane 6*) and putative HIF-1α in IL-8 promoter (*lane 7*) completely abrogated HIF-1α binding, showing specificity of the band for HIF-1α in EMSA. Moreover, with an Ab to HIF-1α, one sees supershift in HIF-DNA complex (Fig. 7*C*, *lane 4*). These studies show that both hypoxia- and $CoCl_2$-induced HIF-1α-DNA-binding activity are attenuated by LY294002, R59949, and SB203580.

$CoCl_2$ augments the HIF-1α protein expression in HDMVEC

As shown in Fig. 8, $CoCl_2$ treatment of HDMVEC exhibited a time-dependent increase in HIF-1α protein expression in the cell extract, which was maximal between 2 and 4 h. Both LY294002 (*lane 6*) and R59949 (*lane 7*) reduced >80% HIF-1α protein expression. SB203580 also reduced HIF-1α protein expression by ~80% (*lane 5*), whereas PD98059 showed ~25% reduction (*lane 8*). However, R59949, an activator of HIF-prolyl hydroxylase (37), induced complete degradation of HIF-1α in response to $CoCl_2$ in HDMVEC. These results indicated that $CoCl_2$ increases HIF-1α protein expression, which is inhibited by LY294002, SB203580, and R59949.

Effect of overexpression of HIF-1α and HIF-1α prolyl hydroxylase siRNA in HDMVEC on IL-8 expression

Because our studies indicated that HIF-1α possibly could be regulating the expression of IL-8, we examined the effect of overexpression of HIF-1α in HDMVEC. As shown in Fig. 9*A* (*lane 3*),

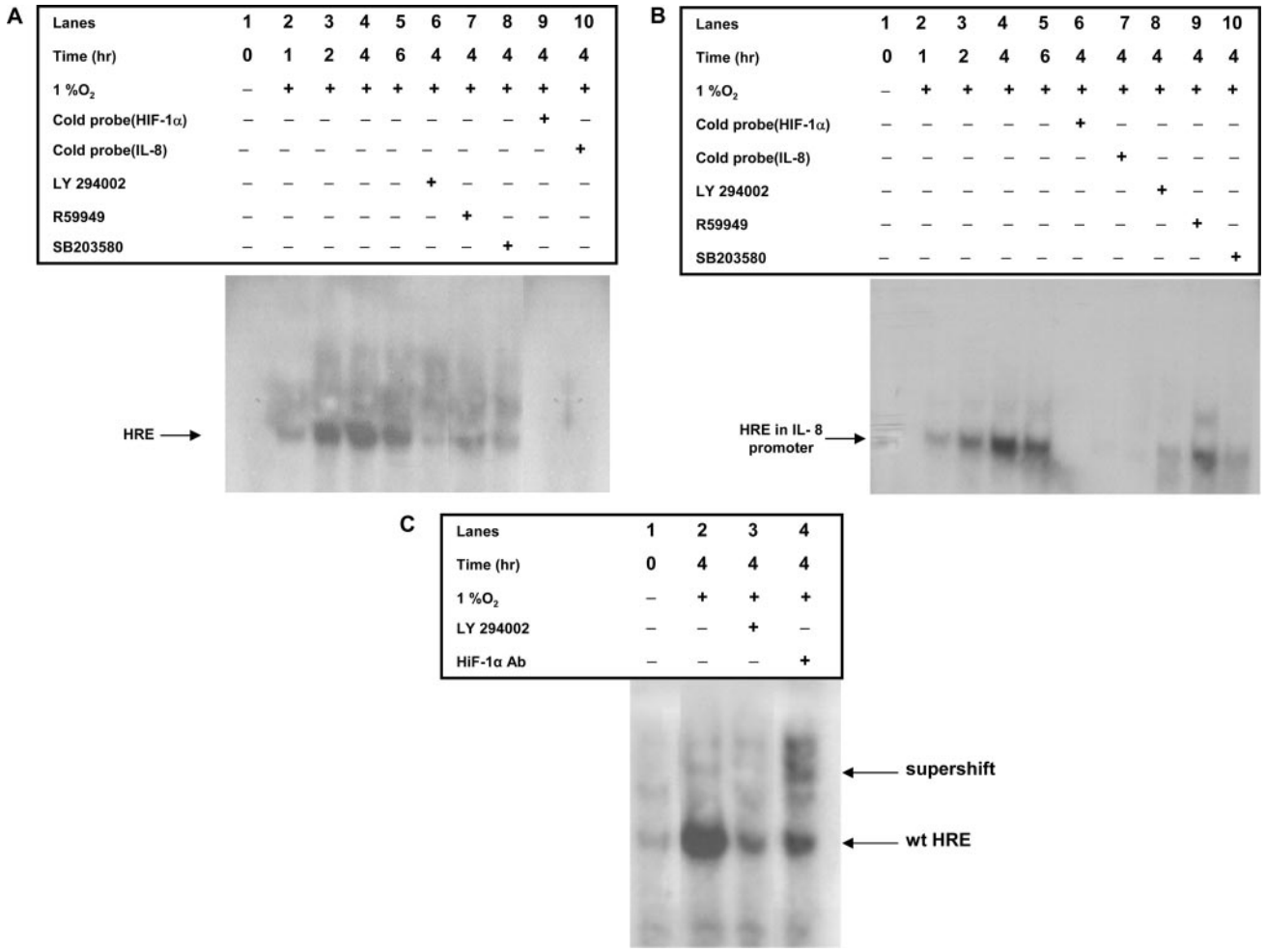

FIGURE 7. Effect of hypoxia (1% O_2) on HIF-1α-binding activities in nuclear extracts of HDMVEC by gel shift assay. *A*, HDMVEC were subjected to hypoxia (1% O_2) in the presence of various inhibitors, as used in Fig. 5, for different time intervals (1, 2, 4, and 6 h). Nuclear extracts were prepared and incubated with ^{32}P-labeled oligonucleotide probe corresponding to HRE (bona fide). *B*, Nuclear extracts were incubated with ^{32}P-labeled oligonucleotide probe for HRE in IL-8 promoter. Where indicated, excess cold probe for bona fide HIF-1α (wt) and corresponding to HRE in IL-8 promoter was added to nuclear extracts before incubation with the labeled probe. *C*, Nuclear extracts prepared from hypoxia-treated cells were preincubated with an Ab to HIF-1α (2 μg), followed by incubation with the radiolabeled probe bona fide HIF-1α (wt).

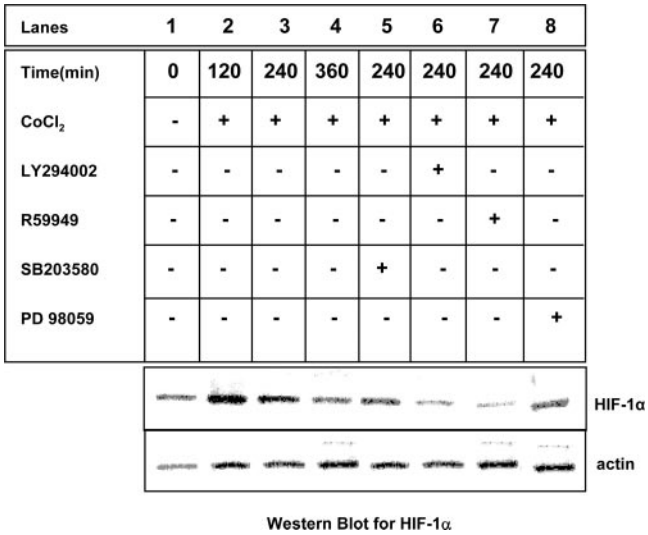

FIGURE 8. Effects of pharmacological inhibitors on $CoCl_2$-mediated HIF-1α protein expression in HDMVEC. HDMVEC were preincubated with LY294002 (15 μm), PD98059 (10 μM), SB203580 (1 μM), and R59959 (30 μM) for 30 min. Cells were then treated with $CoCl_2$ (0.5 mM). Cell lysates were subjected to 7.5% SDS-PAGE, followed by Western blotting with anti-HIF-1α Ab (*upper panel*). The same blot was stripped and reprobed with an Ab to actin to normalize any loading differences. Data are representative of two independent experiments.

overexpression of HIF-1α (Fig. 9*A*, *lanes 3* and *4*) led to an increase in IL-8 mRNA expression, which was augmented in response to $CoCl_2$ (*lane 4*). However, transfection with overexpressing HIF-1β plasmid followed by $CoCl_2$ treatment did not alter IL-8 expression (Fig. 9*A*, *lane 5*). To further substantiate the role of HIF-1α in the regulation of IL-8 transcription in response to $CoCl_2$, HDMVEC were transfected with siRNA for PHD-1 and 2 (PHD-1 and PHD-2). As shown in Fig. 9*C*, transfection of HDMVEC with PHD-2 siRNA (*lane 6*) resulted in a 2- to 3-fold increase of IL-8 mRNA expression compared with cells that were either transfected with scPHD-2 siRNA (*lane 8*) or PHD-1 siRNA (*lane 4*), in response to $CoCl_2$. It is pertinent to note that transfection with HIF-1α siRNA (Fig. 9*B*, *lane 3*) reduced HIF-1α protein levels, whereas transfection with PHD-2 siRNA (Fig. 9*B*, *lane 6*) resulted in increase in HIF-1α protein levels following hypoxic treatment. These data suggested that PHD-2 affects the hydroxylation of HIF-1α for it to undergo degradation under normoxic condition and is rescued by PHD-2 siRNA.

$CoCl_2$ induces IL-8 promoter activity in HDMVEC

To determine whether $CoCl_2$ induces IL-8 mRNA expression at the transcriptional level, HDMVEC were transfected with the full-length IL-8 luciferase construct containing 1521 bp (nt +40 to −1481) of the promoter region of IL-8 gene. As shown in Fig. 10, transiently transfected endothelial cells were treated with $CoCl_2$. There was ~8-fold increase in luciferase activity compared with untreated cells. Single nucleotide replacement (CATG instead of CGTG at −80 to −83 of IL-8 promoter (Fig. 4)) in the putative HIF-1α binding site abrogated >50% response to $CoCl_2$ (Fig. 10*B*). Similarly, mutation in the HIF-1α and NF-κB binding site of IL-8 luc promoter resulted in >80% reduction in luciferase activity in response to $CoCl_2$ (Fig. 10*B*). Furthermore, mutation in NF-κB region alone in IL-8 luc resulted in >85% reduction in IL-8 promoter activity (Fig. 10*B*). These results indicate that NF-κB is presumably involved in coactivation of HIF-1α in the expression of IL-8. Moreover, PI3K inhibitor (LY294002) and putative HIF-1α activator (R59949) reduced by ~50% of IL-8 promoter-associated luciferase activity (Fig. 10). However, MAPK inhibitor PD98059 did not significantly affect IL-8 luc activity. These data indicate roles of PI3K in HIF-1α-mediated transcription of IL-8.

ChIP assay

To determine whether HIF-1α binds to the IL-8 promoter in native chromatin of endothelial cells, we performed ChIP assays. HDMVEC pretreated with $CoCl_2$ for 1 h were subjected to ChIP analysis. Chromatin samples were immunoprecipitated with Ab to HIF-1α, Ab to SP-1, and normal rabbit IgG (control). DNA recovered from the Ab-bound fractions, and DNA from input chromatin (before immunoprecipitation) was analyzed by semiquantitative PCR using primers corresponding to the promoter region of IL-8 (from −21 to −420), relative to the transcription start site. As shown in Fig. 11, HDMVEC treated with $CoCl_2$ for 1 h exhibited increased amplification of PCR products, corresponding to the expected length of 400 bp. LY294002 reduced by ~90% the in vivo HIF-1α chromatin-binding activity, whereas PD98059 did not affect HIF-1α chromatin-binding activity in HDMVEC treated with $CoCl_2$. Fig. 11*B*, *middle panel*, shows amplification of input DNA before immunoprecipitation with HIF-1α. There is no change in the amplification of the input DNA in all the samples. However, immunoprecipitation with Ab to SP-1 showed equal amplification of PCR products (400 bp) (Fig. 11*B*, *lower panel*) in the untreated and CoCl2-treated HDMVEC, indicating that basal IL-8 promoter is also regulated by SP-1. As expected, immunoprecipitation of chromatin samples with rabbit IgG (control) did not show any amplification of expected products (400 bp) (Fig. 11*B*, *lower panel*). These data show that $CoCl_2$ stimulates the binding of HIF-1α to IL-8 promoter region in vivo.

Chemotactic response of human PBMC to CM elaborated from HDMVEC transiently transfected with HIF-1α siRNA followed by hypoxia

Because exposure of HDMVEC to hypoxia and $CoCl_2$ causes release of IL-8, we determined whether CM elaborated from these cells could augment chemotaxis of leukocytes (monocytes and neutrophils). As shown in Fig. 12*A*, we first determined the transmigration of leukocytes toward chemotactic gradient of IL-8 (20 ng/ml) as a control. There was ~8-fold increase in chemotaxis of leukocyte fraction in response to IL-8 in the lower compartment of chemotaxis chamber (Fig. 12*A*, *lane 2*), which was inhibited ~50% by Ab to IL-8 (*lane 3*). The CM from untreated HDMVEC in the lower compartment of the Nucleopore chamber increased the transmigration of leukocytes as enumerated by counting both monocytes and neutrophils (Fig. 12*A*, *lane 4*). However, CM from hypoxia (1%)-treated cells almost doubled the transmigration of leukocytes (*lane 5*), which was inhibited by ~50% with Ab to IL-8 (*lane 6*), but not with nonspecific IL-1α Ab (*lane 7*). Moreover, CM from HDMVEC pretreated with LY294002 (*lane 8*) and R59949 (*lane 9*), followed by exposure to hypoxia, showed >50% reduced chemotaxis of leukocytes as compared with hypoxia-treated cells (*lane 5*). It is pertinent to note that addition of exogenous LY294002 or R59949 to the hypoxia-elaborated medium did not alter chemotaxis of leukocyte subfractions or PMN (data not shown). Moreover, CM elaborated from HDMVEC transiently transfected with HIF-1α siRNA (*lane 10*) showed >50% reduction in chemotaxis of leukocytes. In contrast, transfection with scHIF-1α siRNA (*lane 11*) did not affect hypoxia-induced chemotaxis of leukocytes. Next, we determined whether neutrophils present in leukocyte fraction underwent chemotaxis (Fig. 12*B*). In

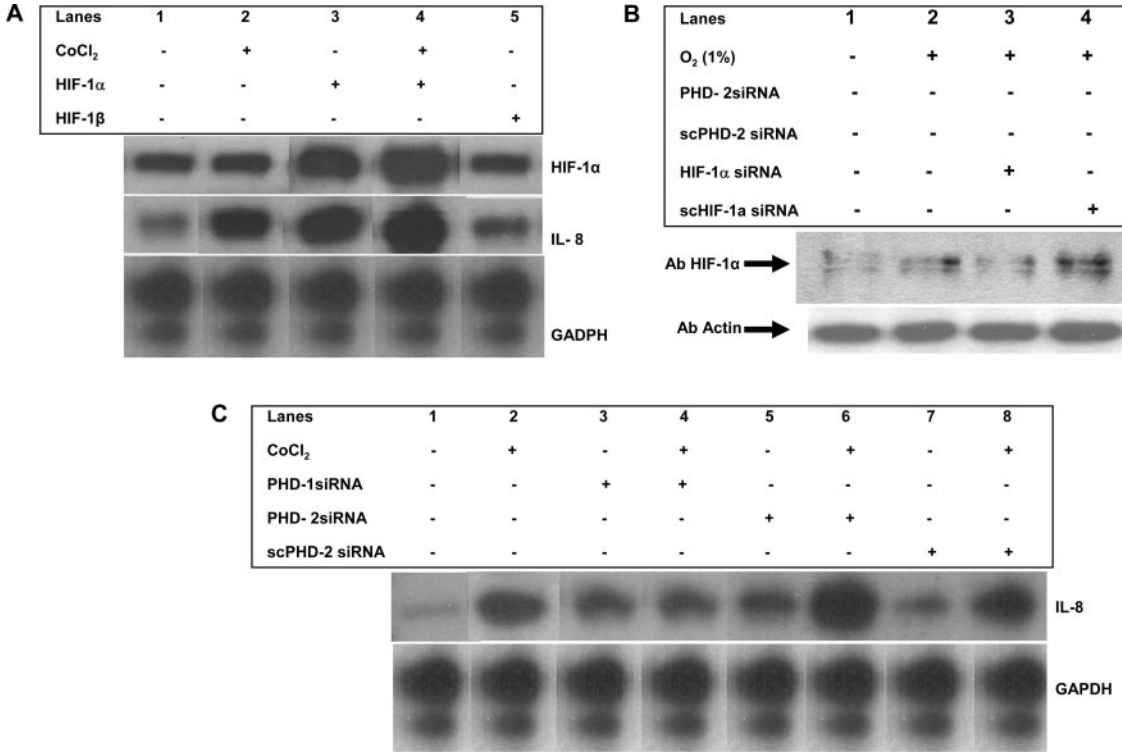

FIGURE 9. Effect of expression of HIF-1α plasmid and HIF-1α PHD siRNA on IL-8 mRNA. *A*, HDMVEC (5×10^5) were transfected with either HIF-1α or HIF-1β (4 μg of plasmid DNA). After 36 h posttransfection, the cells were treated with $CoCl_2$ (0.5 mM) for 1 h. The RNA samples were analyzed by RPA analysis. *B*, HDMVEC (5×10^5) were transfected with PHD-1 siRNA, PHD-2 siRNA, and scPHD-2 siRNA (50 pmol). After 36 h of transfection, the cells were stimulated with O_2 (1%) for 4 h (*B*) or treated with $CoCl_2$ for 1 h (*C*). The RNA was then analyzed by RPA, as described earlier. Data are representative of three independent experiments.

Fig. 12, the neutrophils present in the leukocyte fraction were enumerated by hemacytometer. As shown in Fig. 12*B*, neutrophils present in leukocyte fraction transmigrated in response to rIL-8 (*lane 2*), which were inhibited by Ab to IL-8 (*lane 3*). CM from hypoxia-treated HDMVEC caused ~2-fold increase in transmigration of neutrophils (Fig. 12*B*, *lane 5*), which was inhibited by Ab to IL-8 (*lane 6*). CM from HDMVEC treated with $CoCl_2$ in the presence of LY294002 (*lane 8*) and R59949 (*lane 9*) exhibited ~80 and ~50% and reduced transmigration of neutrophils, respectively. As expected, CM from HIF-1α siRNA-transfected cells (*lane 10*), but not scHIF-siRNA (*lane 11*), in response to hypoxia, showed ~60% reduction in chemotaxis compared with CM from hypoxia-treated HDMVEC (*lane 5*). We observed that application of IL-8 or CM from hypoxia-treated HDMVEC on both sides of Nucleopore chamber filter, at equivalent concentrations, gave the same net results, as observed with background control, indicating that migration of leukocytes in response to these chemoattractants was due to chemotaxis and not by chemokinesis (data not shown). These results show that inhibition of HIF-1α expression in HDMVEC reduces IL-8 protein expression and concomitantly reduced chemotaxis of leukocytes or neutrophils.

Discussion

Endothelial cells in the vasculature are activated in response to a variety of stimuli, such as endotoxins generated during Gram-negative bacterial infection, proinflammatory molecules, and oxidant stress generated during low oxygen tension. The activation of endothelium leads to expression of adhesion molecules, which promote adhesion of PMN and monocytes, followed by their transmigration either in response to chemokines (MCP-1 and IL-8) or diapedesis through cell-cell junctions. Sequestration of PMN within the lung is characteristic of acute and chronic lung injury, including acute chest syndrome in SCD patients. Most of the previous studies have focused on human alveolar macrophage as a primary source of this chemotactic activity (38) and proinflammatory cytokine-mediated expression of IL-8 in endothelial cells (39). In this study, we examined the molecular mechanism by which hypoxia up-regulates the expression of CXC chemokine IL-8 in human vascular endothelial cells. IL-8 has been implicated in the activation and chemotaxis of neutrophils, transendothelial migration of neutrophils, modulation of chemotaxis of T lymphocytes, and contraction of airway smooth muscle cells (40).

We show that both $CoCl_2$ and hypoxia (1% O_2) increased the expression of MCP-1 and IL-8 among several cytochemokines (TNF-1α, IL-1β, and MIP-1β) examined by RPA in HPMVEC, human aortic endothelial cells, HBEC, and HDMVEC. The expression of IL-8 in HPMVEC and HDMVEC was induced similarly in response to $CoCl_2$ and hypoxia. Moreover, pharmacological protein kinase inhibitors behaved similarly in both of these cells; thus, we used HDMVEC for ease of culture and transfection, where necessary. Because hypoxia has been shown to increase gene expression of plasminogen activator inhibitor-1, Epo, VEGF, and glycolytic enzymes via binding of transcription factor HIF-1α to HRE consensus sequence (CGTG) in their gene promoter regions (19, 20, 41), we examined the presence of such sequences in the IL-8 promoter (GenBank accession no. M28130). As shown in Fig. 4, IL-8 promoter has a single CGTG element (−80 to −83 bp) relative to the transcriptional start site; thus, we speculated that HIF-1α transcription factor may have a role in IL-8 gene expression, in response to hypoxia. The IL-8 promoter is found between –1481 and +44 bp of the transcriptional cap site of IL-8 gene and contains multiple potential regulatory transcription factor binding

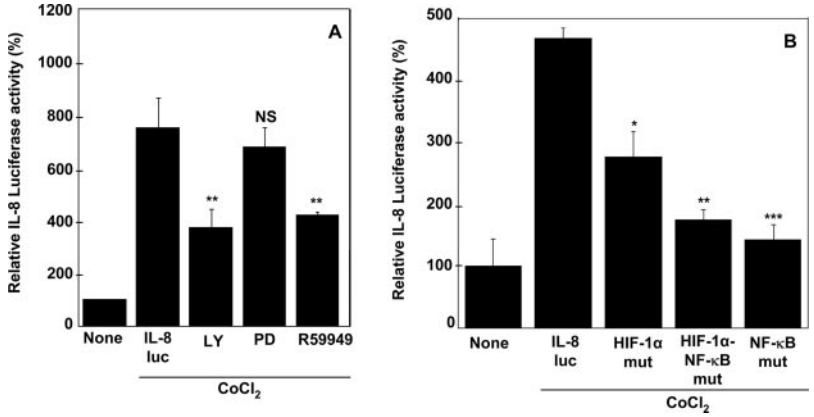

FIGURE 10. IL-8 promoter analysis by transfection with IL-8 promoter coupled to luciferase reporter. *A*, HDMVEC was transfected with IL-8 luc (wt) construct along with β-galactosidase construct. After 36 h posttransfection, cells were washed with serum-free medium. Then the cells were preincubated with LY294002 (15 μm), PD98059 (10 μM), SB203580 (1 μM), and R59959 (30 μM) for 30 min. Cells were then treated with $CoCl_2$ (0.5 mM). *B*, IL-8 promoter constructs (IL-8 luc (wt), IL-8 luc with mutation in HRE, IL-8 luc with mutation in HRE and NF-κB, and IL-8 luc with mutation in only NF-κB region) were cotransfected along with β-galactosidase constructs in HDMVEC. After 36 h posttransfection, cells were washed with serum-free medium and treated with $CoCl_2$ (0.5 mM) for 1 h. Cells were pelleted and washed once with PBS. Cell lysates were prepared, and luciferase and β-galactosidase activities were measured. Results are expressed as percentage of luciferase activity normalized with β-galactosidase activity relative to untreated cells taken as 100%. **, $p < 0.05$; $CoCl_2$ treated vs $CoCl_2$ treated in the presence of inhibitors or IL-8 promoter mutant constructs. Data are expressed as means ± SD of $n = 3$.

sites, including NF-κB, AP-1, NF-IL-6, and C/EBP (42). The minimal promoter region of −130 bp has essential NF-κB, AP-1, and NF-IL-6 sites, which is sufficient for physiological regulation of IL-8 in response to TNF-1α, IL-1β, and endotoxin (43). To determine whether the putative HRE of the IL-8 promoter played a role in hypoxia-induced transcription, we transfected HDMVEC with HIF-1α siRNA. We show that transfection with HIF-1α siRNA, but not scHIF-1α siRNA, attenuated $CoCl_2$-induced IL-8 mRNA and protein expression and also reduced mRNA levels of HIF-1α (Fig. 6). Moreover, transfection with Egr-1 siRNA did not reduce IL-8 expression, indicating the specificity of HIF-1α siRNA in reducing IL-8 expression by $CoCl_2$ (a mimetic of hypoxia).

Next, we examined the $CoCl_2$- and hypoxia-mediated cell signaling mechanism leading to increased expression of IL-8 mRNA and protein. We show that $CoCl_2$-mediated IL-8 mRNA and expression in HPMVEC are inhibited by LY294002 (a PI3K

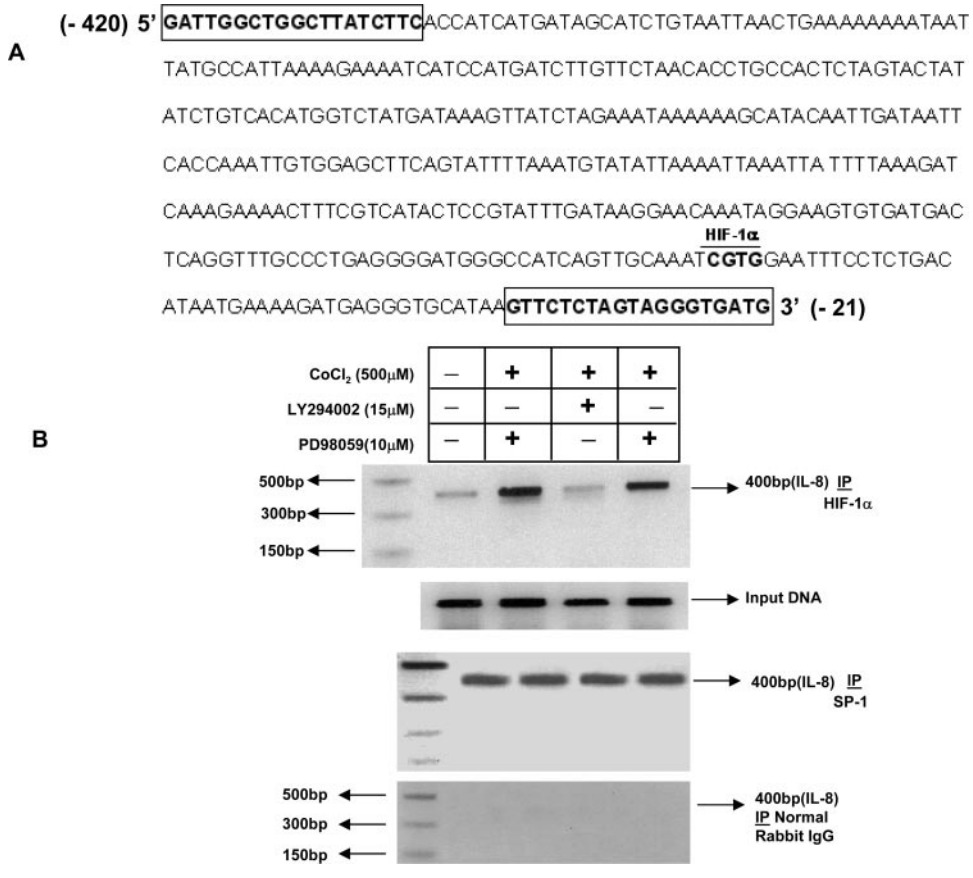

FIGURE 11. $CoCl_2$ induced HIF-1α binding to native chromatin of HDMVEC, as demonstrated by ChIP assay. *A*, Schematic of promoter region of IL-8 (−83 to −80) showing a putative HIF-1α-binding element. *B*, HDMVEC were treated with $CoCl_2$ (0.5 mM) for 1 h, in the absence or presence of pharmacological inhibitors LY294002 (15 μM) and PD98059 (10 μM). Soluble chromatin was isolated and immunoprecipitated with HIF-1α Ab, SP-1 Ab, or normal rabbit IgG (control). The immunoprecipitated DNA was PCR amplified with the use of IL-8 primers (shown in boxes; *A*). The *lower panel* is amplification of input DNA before immunoprecipitation. Data are representative of two independent experiments.

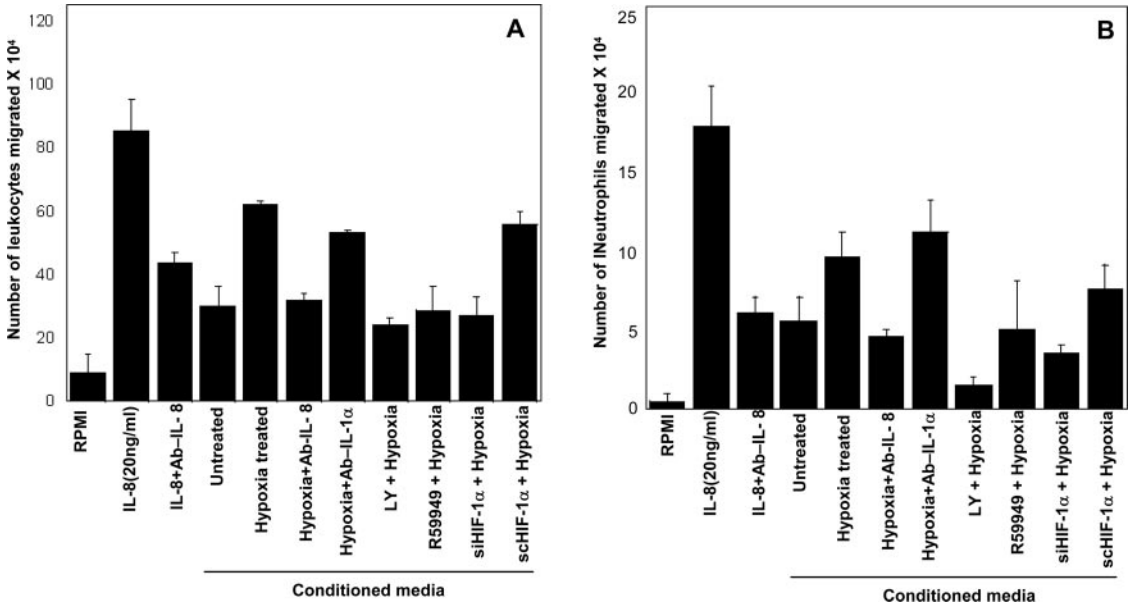

FIGURE 12. Hypoxia-induced chemokine released promotes chemotaxis of leukocytes and neutrophils. Twenty microliters of leukocytes (1.5×10^5 cells) from fresh human peripheral blood was added to the top compartment of the Boyden chamber, whereas the bottom chamber contained 28 μl of RPMI 1640, IL-8 (20 ng/ml), or CM elaborated from HDMVEC exposed to hypoxia (1% O_2) for 8 h. HDMVEC were pretreated with inhibitors or transfected with siRNA, followed by exposure to hypoxia (1% O_2) for 8 h. CM from these cells was used in the lower compartment of the chamber, where indicated. Where indicated, the Ab to IL-8 or IL-1α (1 μg/ml) were added to the lower compartment of the Boyden chamber. Cells that transmigrated across 5-μm pore filters were stained with trypan blue and differentially counted by hemocytometer. *A*, Number of leukocytes. *B*, Neutrophils. Each bar, means ± SD ($n = 3$).

inhibitor) and SB203580 (a p38 MAPK inhibitor). However, both PD98059 (inhibitors of MEK kinase) and SP600125 (a specific inhibitor of JNK) did not affect IL-8 expression. Because pharmacological inhibitors may have nonspecific effects, we transfected HDMVEC with Dn constructs (Dn Akt and Dn PI3K p85 subunit). We observed complete inhibition of IL-8 protein expression in response to $CoCl_2$, indicating the role of PI3K/Akt in hypoxia-induced IL-8 expression. This is further supported by the finding that transfection of HDMVEC with PTEN, a key regulatory component of PI3K-Akt signaling cascade (35), followed by treatment with cobalt chloride for 24 h resulted in ~75% reduction in IL-8 release from HDMVEC. Moreover, LY294002 and SB203580, but not PD98059, were effective in inhibiting hypoxia (1% O_2)-induced IL-8 protein expression in HDMVEC. Taken together, these results indicate that $CoCl_2$- and hypoxia-mediated signaling for IL-8 expression involves activation of PI3K/Akt and p38 MAPK, but not MEK kinase.

To delineate whether HIF-1α transcription factor is involved in hypoxia- or $CoCl_2$-mediated IL-8 expression in these endothelial cells, we show that R59949 inhibits hypoxia-induced IL-8 protein expression. R59949 is a diacylglycerol kinase inhibitor and an activator of HIF prolyl hydroxylase (37), and as a consequence increases degradation of HIF-1α protein by a mechanism involving Von Hippel Lindau (VHL) protein. VHL protein is a component of E3 ubiquitin ligase complex that causes ubiquitinization and proteosome-dependent degradation of HIF-1α subunits. VHL protein interaction occurs with the hydroxylated form of HIF-1α to promote its degradation. However, the hydroxylation of specific proline residues in HIF-1α is catalyzed by a group of mammalian PHD, PHD1, PHD2, and PHD3 (44). However, PHD2 is thought to be most important in regulating the levels of HIF-1α protein in normoxia. Thus, we transfected HDMVEC with siRNA for PHD1 and siRNA for PHD2 to determine whether inhibition of hydroxylation by these HIF-1α PHD will augment the expression of IL-8. We show that PHD2 siRNA augments the mRNA expression of IL-8 under normoxia, and much more in $CoCl_2$-treated HDMVEC (Fig. 9). scPHD2 siRNA had no significant effect on IL-8 expression over and above that observed with $CoCl_2$ alone. However, PHD1 siRNA modestly increased IL-8 expression under normoxia, but no further increase occurred in response to $CoCl_2$. These studies indicate that PHD2-mediated hydroxylation of HIF-1α in the presence of oxygen attenuated IL-8 mRNA expression. Concomitantly, inhibition of PHD2-mediated hydroxylation of HIF-1α augments IL-8 expression. Additionally, we observed that overexpression of HIF-1α, but not HIF-1β, augmented IL-8 mRNA expression under normoxic condition, which was further increased in response to $CoCl_2$. Taken together, these studies provide further credence that IL-8 expression in response to $CoCl_2$ or hypoxia is regulated through HIF-1α in HDMVEC and HPMVEC.

We next determined whether HRE in IL-8 promoter increased DNA-binding activity of HIF-1α. We showed by EMSA that there is increased binding of nuclear extracts prepared from $CoCl_2$-treated HDMVEC to bona fide HIF-1α oligonucleotide probe (wt) as well as to putative HIF-1α-binding oligonucleotide in IL-8 promoter (Fig. 5*A*, *lower panel*). Moreover, the binding of HIF-1α to oligonucleotide was specific as it was completely reduced by cold, excess wt HIF-1α probe or putative IL-8 HIF-1α probe. Furthermore, in nuclear extracts from HDMVEC pretreated with either LY294002 (PI3K inhibitor) or SB203580 (p38 MAPK inhibitor), followed by $CoCl_2$ treatment showed reduced HIF-1α DNA binding. However, PD98059, which did not reduce $CoCl_2$-induced IL-8 expression, also did not affect $CoCl_2$-induced HIF-1α binding to DNA. Furthermore, R59949-treated HDMVEC nuclear extracts showed >75% reduced binding in both $CoCl_2$- and hypoxia-treated HDMVEC, showing that HIF-1α binds to HRE present in IL-8 promoter. It is pertinent to note that $CoCl_2$ augments NF-κB and AP-1 activity in HDMVEC, as these responsive elements are present in IL-8 and other genes. To further support that HRE present in IL-8 promoter played a role in hypoxia-induced IL-8 expression, HDMVEC were transfected with a reporter construct

containing 1521 bp (nt −1481 to +40) of the promoter region of human IL-8 gene (29) and IL-8 reporter containing a mutation in HIF sites, i.e., CATG in place of CGTG at −80 to −83 of IL-8 promoter relative to transcription start site. We showed that single nucleotide mutation in HRE element of IL-8 promoter leads to attenuation of luciferase activity, indicating the HRE in IL-8 promoter is important for hypoxia-induced IL-8 activity. It is pertinent to note that mutation of NF-κB region in IL-8 promoter also led to reduced IL-8 luciferase activity, indicating the importance of NF-κB in regulating the IL-8 activity. The region of NF-κB in IL-8 promoter overlaps the HRE of HIF-1α binding (Fig. 4). It has been shown that nickel disulfide (Ni_3S_2), a constituent of environmental inhalation, increases IL-8 gene expression via activation of AP-1 independently of HIF-1α (42). In this study, we demonstrate by ChIP analysis that HIF-1α binds in vivo to the IL-8 promoter and interaction with this transcription factor increases after $CoCl_2$ treatment. Moreover, pharmacological inhibitors (LY-294002), which attenuated $CoCl_2$-induced IL-8 expression, also reduced HIF-1α binding to IL-8 promoter in the ChIP assay. However, the mechanisms by which metal ions such as cobalt and nickel, as well as desferroxamine, stabilize HIF-1α remain unknown (44).

Because IL-8 mediates chemotaxis of PMN, we hypothesized that CM elaborated from $CoCl_2$-treated HDMVEC would promote chemotaxis of human PMN, and conversely, CM from HIF-1α siRNA-transfected HDMVEC should exhibit reduced chemotaxis. As expected, CM from either $CoCl_2$-treated HDMVEC or hypoxia-exposed HDMVEC exhibited substantial increase in chemotaxis of neutrophils. Moreover, CM from HDMVEC transfected with siRNA, but not scHIF-1α siRNA, showed reduced chemotaxis of neutrophils. Similar results were obtained in CM from HDMVEC pretreated with R59949. These studies showed that hypoxia-stimulated IL-8 protein secretion from HDMVEC, possibly regulated by HIF-1α transcription factor, plays a role in the chemotaxis of leukocytes and PMN.

In conclusion, this is the first report, to our knowledge, showing that hypoxia- or $CoCl_2$ (a mimetic of hypoxia)-mediated increase in IL-8 gene expression in HPMVEC and HDMVEC involves activation through HIF-1α. This is supported by our finding of the following: 1) a functional HIF-1α binding site (CGTG) in the promoter region of IL-8; 2) attenuation of IL-8 expression by both siRNA for HIF-1α and R59949; 3) an increase in IL-8 expression by both siRNA for prolyl hydoroxylase-2 (PHD-2 siRNA) and overexpression of HIF-1α; and 4) ChIP showing that HIF-1α binds in vivo to the IL-8 promoter. We speculate that tissue hypoxia, which occurs in vaso-occlusion in SCD, infarction, and stroke, up-regulates the expression of chemokines, i.e., IL-8 in vascular endothelium, which may facilitate the migratory response of PMN to chemokines elaborated from activated endothelium as well as from activated monocytes/macrophages. Our studies thus provide one more avenue, among several approaches, to ameliorate inflammation and injury to vascular endothelium in pathological states such as vaso-occlusion in SCD, using pharmacological inhibitors that inhibit or degrade HIF-1α.

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Disclosures

The authors have no financial conflict of interest.

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