Difference in proangiogenic potential of systemic and pulmonary endothelium: role of CXCR2

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Submitted 4 October 2004; accepted in final form 11 February 2005

Moldobaeva, Aigul, and Elizabeth M. Wagner. Difference in proangiogenic potential of systemic and pulmonary endothelium: role of CXCR2. Am J Physiol Lung Cell Mol Physiol 288: L1117–L1123, 2005.—The systemic vasculature in and surrounding the lung is proangiogenic, whereas the pulmonary vasculature rarely participates in neovascularization. We studied the effects of the proangiogenic ELR+CXC chemokine MIP-2 (macrophage inflammatory protein-2) on endothelial cell proliferation and chemotaxis. Mouse aortic, pulmonary arterial, and lung microvascular endothelial cells were isolated and subcultured. Proliferation ([3H]thymidine uptake) and migration (Transwell chemotaxis) were evaluated in each cell type at baseline and upon exposure to MIP-2 (1–100 ng/ml) without and with exposure to hypoxia (24 h)-reoxygenation. Baseline proliferation did not vary among cell types, and all cells showed increased proliferation after MIP-2. Aortic cell chemotaxis increased markedly upon exposure to MIP-2; however, neither pulmonary artery nor lung microvascular endothelial cells responded to this chemokine. Assessment of CXCR2, the G protein-coupled receptor through which MIP-2 signals, displayed no baseline difference in mRNA, protein, or cell surface expression among cell types. Exposure to hypoxia increased expression of CXCR2 of aortic endothelial cells only. Additionally, aortic cells, compared with pulmonary cells, showed significantly greater protein and activity of cathepsin S, a proteolytic enzyme important for cell motility. Thus the combined effects of increased cathepsin S activity, providing increased motility and enhanced CXCR2 expression after hypoxia, both contribute to the proangiogenic phenotype of systemic arterial endothelial cells.

angiogenesis; cathepsin S; hypoxia-reoxygenation; macrophage inflammatory protein-2

NEOVASCULARIZATION OF THE LUNG after chronic pulmonary embolism (5), interstitial pulmonary fibrosis (30), and bacterial infection (29) requires systemic vascular angiogenesis. New blood vessels invade the lung parenchyma from systemic bronchial arteries and/or intercostal arteries (5). Surprisingly, the pulmonary vasculature does not participate in the process and appears to be resistant to proliferation and neovascularization (19). This heterogeneity in endothelial cell responsiveness raises the question of why pulmonary arterial endothelium lacks proangiogenic potential in these pathological situations. A possible mechanism may be related to the prevailing ambient oxygen level. In systemic organs, vascular obstruction and ischemia result in systemic endothelial cell hypoxia. In general, tissue hypoxia increases the angiogenic potential of systemic arterial endothelial cells by inducing the upregulation of angiogenic factors and downregulation of antiangiogenic factors. However, an opposite situation exists for pulmonary arterial endothelium within the ventilated lung during pulmonary ischemia where normoxic or hyperoxic conditions prevail. In vitro assays of endothelial cell proliferation and migration have been performed exposing cells to a variety of proangiogenic factors, yet little attention has been given to examining the anatomically relevant endothelial cell subtype or appropriate ambient oxygen tension. There is increasing evidence for endothelial cell heterogeneity (7, 26), yet responsiveness to changes in ambient oxygen tensions likely has additional phenotypic affects on vital aspects of neovascularization such as endothelial cell proliferation and migration.

In previous studies, we showed that obstruction of the left pulmonary artery in mice induced the formation of a new vasculature that developed from intercostal arteries that crossed the pleural space and invaded the lung (20). These studies confirmed a process that has been identified in humans after chronic pulmonary thromboembolism as well as in models in several other species (3, 6, 11, 32) and provided an experimental model in which to study the progression of lung angiogenesis. Our subsequent studies established the importance of the Glu-Leu-Arg (ELR+) CXC chemokines in this angiogenesis model (25). These results provide further support for work by Arenal and colleagues (2), who have shown that in several human disease states and animal models, the process of increased systemic vascularization of the lung involves these chemokines. The ELR+CXC chemokines [IL-8; growth-related oncogene (GRO)-α, -β, -γ; ENA-78 (78-amino acid epithelial cell-derived neutrophil activator); GCP-2 (granulocyte chemotactic protein-2); NAP-2 (neutrophil-activating peptide-2)] have been implicated in the regulation of endothelial cell function including proliferation, migration, and tube formation (13, 14, 17, 27). The specific effects of ELR+CXC chemokines on their target cells are mediated through binding CXCR1 and CXCR2 (1). Expression of CXCR2 on human endothelial cells has been confirmed in several studies (21) (22). In murine tissue, the ELR+CXC ligands [macrophage inflammatory protein-2 (MIP-2), keratinocyte-derived chemokine, lipopolysaccharide inducible CXC chemokine] bind exclusively through CXCR2 (9). The effects of hypoxia on CXCR2 expression have not been determined. Thus we undertook the following series of experiments to test the hypothesis that the difference in proangiogenic potential of systemic endothelial cells relative to pulmonary endothelial cells is related to CXCR2 expression and influenced by ambient oxygen levels. We developed techniques to isolate and study primary cultures of mouse aortic, pulmonary artery, and lung microvascular endothelial cells. Specific experiments were performed exposing cells to a variety of proangiogenic factors, yet little attention has been given to examining the anatomically relevant endothelial cell subtype or appropriate ambient oxygen tension. There is increasing evidence for endothelial cell heterogeneity (7, 26), yet responsiveness to changes in ambient oxygen tensions likely has additional phenotypic affects on vital aspects of neovascularization such as endothelial cell proliferation and migration.

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designed to 1) confirm the presence of CXCR2 on mouse endothelial cells, 2) determine whether the difference in proangiogenic potential (proliferation and chemotaxis) of mouse systemic arterial endothelial cells and mouse pulmonary endothelial cells is determined by CXCR2 expression, 3) determine the effects of ambient oxygen conditions on CXCR2 expression, and 4) compare the expression of cathepsin S, a proteolytic enzyme important for endothelial cell migration and invasion (24), in arterial endothelial cells and pulmonary artery endothelial cells. Our results confirm that, relative to pulmonary artery endothelium, systemic arterial endothelial cells demonstrate increased responsiveness to a CXCR2 ligand, increased CXCR2 expression after hypoxia-reoxygenation, and increased cathepsin S activity. Furthermore, these results provide a mechanism for the lack of proangiogenic potential of pulmonary artery endothelium during inflammatory conditions that promote neovascularization.

METHODS

Pulmonary artery and aortic endothelial cells. Procedures using animals were approved by the Johns Hopkins University Animal Care and Use Committee. The pulmonary artery and aorta were dissected from C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME). The vessels were opened longitudinally and cut into two or three pieces. These pieces were placed with the intimal side down on Matrigel-coated, 35-mm tissue culture dishes in culture medium (2 ml of DMEM with 20% fetal calf serum (FCS), 150 mg/ml endothelial cell growth supplement, 100 mg/ml penicillin/streptomycin, 0.25 mg/ml amphotericin B, and 0.1 mM MEM with nonessential amino acids). After 4–6 days, the pieces of vessels were removed and the endothelial cells that had migrated were treated with trypsin and replated to 0.2% gelatin-coated T-25 flasks. When confluent, endothelial cells were again transferred to T-75 flasks and subcultured. To confirm endothelial phenotypes, cells were immunostained for platelet endothelial cell adhesion molecule, von Willebrand’s factor, and modified lipoprotein uptake (Dil-ac-LDL). Only cells with positive staining were used for subsequent experiments. All experiments were performed using endothelial cells isolated from seven mice and used between passages 2 and 10.

Lung microvascular endothelial cells. Mouse lung parenchyma was removed from a peripheral region devoid of large airways, rinsed with DMEM, minced, and digested in 1 ml of collagenase (1 mg/ml; Sigma, St. Louis, MO) at 37°C for 20 min with occasional agitation. The cellular digest was filtered through sterile mesh and centrifuged (400 g for 7 min). The cell pellet was resuspended (1 ml of complete medium) and plated on 0.2% gelatin-coated 24-well plates. After ~5–7 days, areas of cells exhibiting cobblestone morphology were selected, treated with trypsin, and replated to 0.2% gelatin-coated 24-well plates. When confluent, cells were transferred to T-25 flasks and subcultured.

Proliferation assay. Cellular DNA synthesis was assessed by [3H]thymidine uptake. Endothelial cells were maintained in medium with 5% FCS for 48 h, treated with trypsin, and seeded on 0.2% gelatin-coated 24-well plates (4 x 10^4 cells per well) in DMEM with 5% FCS and 5 μCi/ml [3H]thymidine. After 24 h, cells were washed and fixed (10 min on ice with 5% trichloroacetic acid), the DNA was isolated in a beta-counter (Beckmann LS6000SC; Beckman Instruments, Fullerton, CA) and quantified in a beta-counter (Beckmann LS6000SC; Beckman Instruments, Fullerton, CA). The pieces of vessels were removed and the endothelial cells that had migrated were treated with trypsin and replated to 0.2% gelatin-coated T-25 flasks. When confluent, endothelial cells were again transferred to T-75 flasks and subcultured. To confirm endothelial phenotypes, cells were immunostained for platelet endothelial cell adhesion molecule, von Willebrand’s factor, and modified lipoprotein uptake (Dil-ac-LDL). Only cells with positive staining were used for subsequent experiments. All experiments were performed using endothelial cells isolated from seven mice and used between passages 2 and 10.

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Chemotaxis assay. Polycarbonate filters (5-μm pore size; Corning Costar, Cambridge, MA) were coated with 0.2% gelatin. Endothelial cells were detached with 2 mM EDTA/PBS, washed, and resuspended in DMEM with 0.1% BSA fatty acid free. 100 x 10^3 cells were added to the upper chamber and incubated (37°C, 1 h). After 1 h, medium in bottom chambers was replaced with DMEM with 0.1% BSA containing MIP-2 [1–100 ng/ml (27)] and incubated for 2 h. After 2 h, nonmigrated cells were removed with cotton tips, and migrated cells were fixed and stained using Diff-Quik stain set (Dade Behring, Newark, DE). Membranes were mounted on slides, and the average number of migrated cells was counted under a microscope (~20 objective) six fields across each membrane.

Hypoxia exposure. Endothelial cells were loaded into a modular incubator chamber and flooded with a hypoxic gas mixture (4% O2, 5% CO2, and balance N2) for 24 h. Thereafter, cells were incubated in 95% air and 5% CO2 for 2 days before the experiment.

CXCR2 RNA isolation and RT-PCR. Total RNA was isolated from confluent endothelial cell cultures by a single-step guanidium thiocyanate/phenol-chloroform extraction using TRIZol reagent (Invitrogen, Carlsbad, CA). Using 100 ng of cDNA as a template, we performed quantification by an ABI Prism 5700 Sequence Detector (Applied Biosystems, Foster City, CA) using the TaqMan 5′ nuclelease activity from the TaqMan Universal PCR Master Mix, fluorescent probes (Applied Biosystems), and oligonucleotide primers (Invitrogen). The mRNA expression levels of all samples were normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from the same sample. The primer sequence used for CXCR2 was: forward primer (5′ GTG CCG CTG CTC ATG ATG 3′), reverse primer (5′ AAG GAC GAC AGC GAA GAT GAC 3′), and TaqMan probe for GAPDH (5′ TGC TAC GGG TTC ACA CTG CGC AC 3′).

CXCR2 expression by flow cytometry. Endothelial cells were washed and detached with 2 mM EDTA/PBS and resuspended in PBS with 0.2% BSA. After being washed, cells were incubated with primary antibodies (anti-CXCR2 polyclonal antibodies, 1:10), washed, incubated with Alexa 488 anti-goat antibody (Molecular Probes, Eugene, OR), and analyzed. Mouse isotype IgG served as a negative control. Mean channel fluorescence for each experiment was normalized to normoxia controls.

CXCR2 and cathepsin S protein. Endothelial cells were treated with lysis buffer (1% SDS, 1 mM Na3VO4, 5 mM NaF, and 5 μg/ml of leupeptin, aprotinin, and peptatin) on ice. Lysates were boiled for 5 min, and total protein concentration was determined by bicinchoninic acid assay ( Pierce, Rockford, IL). Protein (20 μg) was separated by SDS-PAGE, blotted on nitrocellulose, and blocked for 1 h at room temperature in 3% BSA/3% nonfat dry milk in Tris-buffered saline (50 mM Tris-HCl, pH 7.4) containing 0.1% Tween 20. After being washed in TBS-T, blots were incubated (1 h) with polyclonal rabbit anti-human antibodies to CXCR2 (Abcam, Cambridge, UK) or polyclonal goat anti-rat antibodies to cathepsin S (Santa Cruz Biotechnology, Santa Cruz, CA). Immunodetection was performed using horseradish peroxidase-conjugated anti-rabbit or anti-rat antibodies (Sigma) and enhanced chemiluminescence (Amersham Pharmacia Biotech, Arlington Heights, IL).

Cathepsin S activity. Specific catalytic activity of cathepsin S was determined fluorometrically by the hydrolysis of the synthetic substrate Z-VAL-VAL-Arg-MCA (Peptide Institute, Minoh, Osaka, Japan) according to Ref. 28 with the following modifications. After a 1-h incubation period with MIP-2 (10 ng/ml), cells were washed (3 x 3) with cold PBS, suspended in extraction buffer (0.01% Triton X-100 in 1 M potassium phosphate buffer containing 1 mM EDTA, pH 7.5), and sonicated on ice. After centrifugation at 4°C (30 min at 14,000 rpm), the supernatant was removed for the cathepsin S activity assay. Supernatant (25 μl) was added to the buffer-activator solution (50 μl; 1 M potassium phosphate buffer, 5 mM EDTA, pH 7.5, and 5 mM DTT) and incubated (45 min, 40°C). After cooling, substrate (50 μl of 12.5 μM) was added to the mixture, and the increase in fluorescence measured (excitation 360 nm, emission 460 nm) after 30 min at 40°C. The calibration solution was prepared using 7-hydroxy-4-methylcoumarin (Molecular Probes). Cathepsin S activity was calculated as nmol of 7-hydroxy-4-methylcoumarin produced·mg protein−1·min−1. Cathepsin inhibitor II (10 μM; Z-FG-NHO-BzME; Calbiochem, San...
HYPOXIA INCREASES CXCR2 EXPRESSION IN SYSTEMIC ARTERIAL ENDOTHELIAL CELLS

Diego, CA), an agent known to cause nonspecific cathepsin inhibition (4), was used to interfere with MIP-2-induced chemotaxis. Statistics. Paired comparisons before and after treatment within a given endothelial cell type were evaluated with the Wilcoxon signed-rank test for paired comparisons. Unpaired comparisons were evaluated by the Mann-Whitney test. Comparisons across all cell types were made using the Kruskal-Wallis ANOVA, followed by Dunnett’s multiple-comparison test. The n noted in RESULTS refers to the number of times the experiment was repeated on different cells, usually from different animals. Data are presented as means ± SE.

RESULTS

Endothelial cell proliferation. Basal proliferation did not differ among the three endothelial cell subtypes. However, each cell type showed a vigorous response to MIP-2 treatment. Although the specific concentration of MIP-2 (1–100 ng/ml) that resulted in the maximum increase in proliferation varied both within and among cell types, the average maximum proliferation was similar across cell types (252–258% of control, n = 5–6 experiments/cell type). Furthermore, the 24-h exposure to the hypoxic gas mixture did not alter basal proliferation evaluated 48 h after the exposure, nor did it alter the average maximum increase in proliferation observed after MIP-2 treatment in any cell type (213–277% of control).

Endothelial cell chemotaxis. Basal chemotaxis showed no difference across the three endothelial cell subtypes (n = 6 experiments/cell type, P = 0.0798). However, the response to MIP-2 demonstrated clear differences among cell types (Fig. 1, n = 7 experiments/cell type−1·concentration−1; P = 0.0004). Mouse aortic endothelial cells demonstrated a vigorous, dose-dependent, and significantly greater chemotaxis to MIP-2 than pulmonary endothelial cells (P < 0.01). Neither mouse pulmonary artery endothelial cells nor lung microvascular endothelial cells showed significant chemotaxis to the range of MIP-2 studied (1–100 ng/ml, P > 0.05). Each endothelial subtype demonstrated a trend toward increased chemotaxis after exposure to hypoxia alone (aortic endothelial cells: P = 0.059). The average effects of MIP-2 (1 ng/ml), hypoxia, and the combined effects of hypoxia and MIP-2 (1 ng/ml) are presented in Fig. 2, with individual experiments normalized to control responses observed during normoxia. Aortic cell responses to treatment were significantly greater than chemotaxis observed during basal conditions (P = 0.0098). However, neither pulmonary artery endothelial cells (P = 0.661) nor lung microvascular cells (P = 0.8990) displayed differences from basal chemotaxis. In aortic endothelial cells, average responses to hypoxia and MIP-2 (1 ng/ml) individually appeared approximately additive to the experimental condition of MIP-2 after hypoxia. However, due to inherent variability this result did not reach statistical significance (P = 0.6277). When comparing chemotaxis in aortic cells exposed to both hypoxia and MIP-2 with the sum of the individual effect of hypoxia plus the individual effect of MIP-2, we found the changes to be similar (P = 0.85). This analysis of individual experiments suggests that the responses to MIP-2 and hypoxia were additive.

CXCR2 expression. To confirm the presence of CXCR2 on the three endothelial cell subtypes, we measured mRNA and CXCR2 protein and cell surface expression by fluorescence-activated cell sorting analysis. As shown in Fig. 3, basal expression of CXCR2 mRNA normalized to GAPDH was equivalent among cell types (P = 0.9062, n = 5–7 experiments/cell type). After exposure to hypoxia followed by reoxygenation, there was a significant increase in the CXCR2 expression in aortic endothelial cells only (P = 0.0180). A representative Western blot of total CXCR2 protein in endothelial cell lysates using polyclonal anti-CXCR2 antibody demonstrated a specific band (55 kDa) corresponding with CXCR2 in cell lysates from all three populations of endothelial cells (Fig. 4). Basal protein levels of CXCR2 were equivalent among cell types (P = 0.6703, n = 3 experiments/group). Only aortic endothelial cells consistently in each replicate experiment showed an increase in CXCR2 protein after hypoxiareoxygen-
Results from the assessment of cell surface receptors using polyclonal anti-CXCR2 are shown in Fig. 5 (n = 6–7 experiments/group). Mean fluorescence from anti-CXCR2 binding did not vary among cell types during basal, normoxic conditions (P = 0.9062). However, the expression of CXCR2 receptors of aortic endothelial cells only was significantly enhanced after hypoxia-reoxygenation (P = 0.0180).

*Cathepsin S activity.* Based on pilot microarray data (not shown) and the work of others (24), we studied protein expression and activity of the lysosomal, proteolytic enzyme cathepsin S in aortic and pulmonary artery endothelial cells. These two endothelial cell subtypes were selected because they were expected to represent homogeneous populations, whereas lung microvascular cells likely represent a more heterogeneous population. Figure 6A shows a representative Western blot displaying distinct bands representative of cathepsin S at 28 kDa. Quantification of pixel intensity normalized to β-actin demonstrated a small but significant difference between the level of cathepsin S protein of aortic endothelial cells compared with pulmonary artery endothelial cells after MIP-2 exposure (Fig. 6B; P = 0.0079, n = 5 experiments/group). As shown in Fig. 7, overall cathepsin S activity in aortic cells was significantly greater than in pulmonary artery endothelial cells (P = 0.0036). Specifically, MIP-2 significantly increased cathepsin S activity in aortic cells relative to pulmonary artery endothelial cells (P = 0.0087, n = 6 experiments/cell type). The combined effects of hypoxia-reoxygenation and MIP-2 further enhanced cathepsin S activity in aortic cells (P < 0.05, n = 3 experiments/cell type). Additional experiments in aortic endothelial cells (Fig. 8, n = 4 experiments) demonstrated the effects of cathepsin II inhibitor on MIP-2-induced chemotaxis. Chemotaxis was completely blocked by this cathepsin S inhibitor (P = 0.02).

**DISCUSSION**

Increased attention has focused recently on the heterogeneity of endothelial cells and the unique attributes of endothelial
CXCR1 and CXCR2 have been shown to account for all ELR of the proangiogenic ELR receptor expression. In mice, MIP-2 is one of the most potent cells with regard to CXC chemokine responsiveness and CXC the differences in systemic arterial and pulmonary endothelial the reported series of experiments, we focused on determining ad-
ditionally, the importance of CXC chemokines in promoting thery endothelium in defining a proangiogenic phenotype. Ad-
distinguish systemic arterial endothelium from pulmonary ar-
ners to proliferate and to invade ischemic lung tissues has been observed. In this study, we sought to determine factors that distinguish systemic arterial endothelium from pulmonary ar-	ery endothelium in defining a proangiogenic phenotype. Ad-
ditionally, the importance of CXC chemokines in promoting systemic neovascularization in the lung has been reported for several different angiogenesis models (2, 12, 25). Therefore, in the reported series of experiments, we focused on determining the differences in systemic arterial and pulmonary endothelial cells with regard to CXC chemokine responsiveness and CXC receptor expression. In mice, MIP-2 is one of the most potent of the proangiogenic ELR+ CXC chemokine ligands, which binds to the G protein-coupled receptor CXCR2. Although mice have been shown to lack CXCR4, in other species, CXCR1 and CXCR2 have been shown to account for all ELR+ CXC chemokine signaling (9). Because chemokines typically elicitation activation and migration of their target cells in vivo, we compared proliferation and chemotaxis of three endothelial cell subtypes in vitro. We found that endothelial cell chemotaxis was markedly enhanced in systemic arterial endothelial cells compared with either pulmonary artery or lung microvascular endothelial cells in response to MIP-2. This response was observed at all doses of MIP-2 studied (Fig. 1). Strieter and colleagues (27) showed a somewhat greater chemotactic re-
sponse of human adrenal gland capillary endothelial cells to the human analog of MIP-2, GRO-β,γ averaging 3.5- to 4.5-fold increase at a chemokine concentration of 10 ng/ml. These observations further support the overall heterogeneity of endothelial cell responsiveness. Interestingly, however, in the present study, equivalent levels of CXCR2 expression among the three endothelial cell subtypes was confirmed by measurement of CXCR2 mRNA, protein, and cell surface receptor activity (Figs. 3–5). Thus enhanced chemotaxis of systemic arterial endothelial cells in the presence of MIP-2 could not be attributed to increased receptor expression. Furthermore, al-
though systemic arterial endothelial cell chemotaxis differed markedly in response to MIP-2, basal chemotaxis did not differ, and MIP-2 was equally effective at increasing endothelial cell proliferation among all three endothelial cell subtypes. Overall these results suggest that the proangiogenic influence of MIP-2 on aortic endothelial cells is related to cell motility.

Endothelial cell migration is central to the overall process of angiogenesis and requires the proteolytic modification of extracellular matrix. Many studies have focused on the impor-
tance of the matrix metalloproteases and serine proteases in permitting endothelial cells to bore into surrounding intersti-
tium and form new vessels (31). Several recent studies have focused on the lysosomal cysteine proteases, or cathepsins, in supporting a similar role in tumorigenesis (8, 10, 15). A recent study specifically demonstrated a role for cathepsin S in mediating angiogenesis (24). Cathepsin S is capable of degrading extracellular matrix proteins such as laminin, collagens, elas-
tin, and chondroitin sulphate proteoglycan. Shi and colleagues (24) showed that inflammatory mediators (TNF-α, IFN-γ, IL-1β) and growth factors (VEGF, bFGF) upregulate cathepsin S expression in endothelial cells. Additionally, endothelial cells recovered from cathepsin S−/− mice showed impaired invasion in Matrigel and collagen gel assays. These properties of inducibility and the capacity to degrade extracellular matrix are consistent with the requirements of endothelial cells during neovascularization. An initial preliminary screening experiment using microarray analysis suggested cathepsin expression might differ among endothelial cell subtypes. We focused our experiments on primary cultures of the two most homogeneous endothelial cell populations, those of systemic aortic endothelial cells and pulmonary arterial endothelial cells, to determine whether the marked differences in chemotaxis to MIP-2 might be related to genotypic differences in cathepsin S. MIP-2 caused a significant increase in cathepsin S protein expression in aortic endothelial cells relative to pulmonary artery endothelium. More impressive, however, was the increase in cathepsin S activity in aortic relative to pulmonary artery endothelial cells after MIP-2 treatment (Fig. 7). These results are consistent with the hypothesis that cathepsin S activity modulates chemotaxis in aortic but not pulmonary artery endothelial cells. By use of a nonspecific cathepsin inhibitor, chemotaxis induced by MIP-2 was largely prevented. This result lends additional support for the overall importance of cathepsins for systemic endothelial cell migration.

We also studied the effects of hypoxia-reoxygenation on proliferation and chemotaxis in the three endothelial cell sub-
types. Tissue hypoxia is an important stimulus and regulator for new vessel growth in most systemic organs. Hypoxia can increase the angiogenic potential of endothelial cells by pro-
ducing an oxidative stress. Brief exposure to hypoxia (30–60 min) followed by reoxygenation significantly accelerated (threefold) the rate of tubular morphogenesis (16). Addition-
ally, Maulik and Das (18) showed that human coronary endo-

![Graph](http://ajplung.physiology.org/)

**Fig. 8.** Average effects of cathepsin inhibitor on MIP-2-induced chemotaxis of ao endothelial cells (n = 4 experiments). Chemotaxis was completely blocked by this cathepsin S inhibitor (*P = 0.02).
thelial cells plated on Matrigel after hypoxia-reoxygenation showed increased tubular morphogenesis. Thus we questioned whether a change in the ambient oxygen conditions might alter the responsiveness of the endothelial cells studied. Specifically, since pulmonary artery endothelium is normally exposed to mixed venous PO$_2$, we questioned whether the in vitro culture conditions routinely used predispose these cells to artificial responses. Our choice of a 24-h exposure to hypoxia followed by 48 h of reoxygenation was based on experimental constraints and preliminary studies. Different results might be obtained if cells were exposed to a shorter/longer time course of hypoxia-reoxygenation or lesser/greater level of hypoxia. However, we selected this PO$_2$ based on the normal environment of pulmonary artery endothelial cells hoping to mimic in vivo oxygen conditions. We did not see significantly different responses in pulmonary endothelial cells after hypoxia-reoxygenation compared with the normoxia controls. The 24-h exposure may have been insufficient to cause lasting changes in these cells. A persistent hypoxic growth environment may be required to fully restore in vivo attributes to pulmonary artery endothelial cells. Conversely, the effects of hypoxia on systemic arterial endothelial cells more accurately mimic in vivo systemic ischemia. Our results showed that CXCR2 expression was sensitive to hypoxia-reoxygenation only in aortic endothelial cells (Figs. 3–5). CXCR2 mRNA, protein, and cell surface expression were significantly increased after hypoxia-reoxygenation. These results could account for an enhanced responsiveness of systemic arterial endothelial cells to ELR+ CXC chemokines after an exposure to hypoxia. Furthermore, our results demonstrated that the effects of MIP-2 and hypoxia-reoxygenation appeared to be additive. Previous work by Schioppa and colleagues (23) showed the upregulation of another chemokine receptor CXCR4 in human umbilical vein endothelial cells after exposure to hypoxia (1% O$_2$, 24 h) and increased chemotaxis to the chemokine ligand stromal-derived factor 1 (CXCL12). Overall, these results demonstrate selective induction of chemokine receptors exposed to low oxygen tensions and the importance of ambient oxygen tensions in assessing angiogenic properties of endothelial cells.

In summary, we have shown that systemic arterial endothelial cells are more responsive to the ELR+ C-X-C chemokine MIP-2 than pulmonary endothelial cells. Under normoxic control conditions, the increased migration observed in arterial endothelial cells could not be accounted for by differences in chemokine receptor levels. However, the increased responsiveness is due, in part, by enhanced cathepsin S activity in arterial endothelial cells. Additionally, hypoxia-reoxygenation causes an increase in CXCR3 expression in aortic endothelial cells that contributes to an enhanced proangiogenic phenotype.

REFERENCES


