Supporting Information

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SI Methods

Cell Culture and Exposure. HLMVECs and HCAECs were cultured in endothelial cell basal medium (EBM-2) supplemented with VEGF, human FGF, human EGF, hydrocortisone, ascorbic acid, insulin-like growth factor-1, GA-1000 (gentamicin/amphotericin-B), and 5% FBS per the supplier's protocol (Lonza). Murine brain microvascular endothelial cells (MB114) and SV-40-transformed mouse endothelial cells (SVECs) were cultured in DMEM supplemented with 10% FBS, penicillin, and streptomycin.

RNA Isolation and Northern Blot Analysis. Twenty-four hours after transduction or treatment, cells were washed twice with HBSS and harvested in guanidine isothiocyanate (GITC). RNA was purified by using the CsCl method, as described previously (1). A total of 15 μg of total RNA was resolved on 1% formaldehyde-agarose gels and transferred to nylon membranes. Probes used for Northern blotting were derived from human A_{2A} cDNA and human A_{2B} cDNA, kindly provided by Marlene Jacobson. The VEGF cDNA was obtained from the Harvard Institute of Proteomics (Boston). The cDNA probes were labeled with [^{32}P]α-dCTP (ICN) by random priming and were hybridized with the membrane for 18 h at 42 °C. Membranes were then washed and autoradiographed. For loading controls, membranes were stripped of radioactive probe in a 2% glyceraldehyde solution at 80 °C and rehybridized with an end-labeled 28S rRNA oligonucleotide (Ambion). The intensity of the radiolabeled bands was measured by using a PhosphorImager running ImageQuant software (Molecular Dynamics).

Thymidine Incorporation Assay. Proliferation of HLMVECs was measured by using [^{3}H]thymidine incorporation. About 20,000 cells were plated in each well of a 24-well plate in endothelial cell complete medium. After 24 h, cells were washed once with HBSS and were serum starved in EBM-2 medium containing 1% FBS, hydrocortisone, ascorbic acid, and GA-1000. After 24 h, cells were incubated with [^{3}H]thymidine (1 μCi per well) in the presence or absence of the adenosine A_{2A} receptor agonist, CGS-21680, at varying concentrations for an additional 24 h. Subsequently, cells were washed twice with ice-cold PBS, precipitated with 0.1 M perchloric acid, and solubilized with 0.01 M NaOH containing 0.1% SDS before scintillation counting. Similarly, proliferation was also measured in cells transduced with Ad.A_{2A} or Ad.LacZ at a multiplicity of infection of 10 pfu per cell.

Angiogenesis Assay. Angiogenesis in HLMVECs was assessed by using the Matrigel tube-formation assay. Growth factor-reduced Matrigel matrix was coated onto 24-well plates and allowed to solidify at 37 °C for 30 min. HLMVECs were then trypsinized and plated onto the Matrigel in the absence of growth factors or serum and were incubated at 37 °C in a CO_2 incubator. The A_{2A} receptor agonist, CGS-21680, or the diluent control was included both in the Matrigel matrix and the overlying medium. Four hours after plating of cells, 3 randomly chosen fields from each well were photographed. Branch points were counted and plotted.

Angiogenic Migration Assay. HLMVECs were either untransduced, transduced with Ad.A_{2A}, or transduced with Ad.LacZ at a multiplicity of infection of 10 pfu per cell. Twenty-four hours after transduction, cells were split, and 100,000 cells were plated on a fibronectin-coated insert in EBM-2 medium containing 0.1% FBS, hydrocortisone, ascorbic acid, and GA-1000. Before plating cells, inserts were coated with 50 μg/mL fibronectin solution in PBS by adding 0.3 mL of the solution to the lower side of the insert and were kept at 4 °C for 24 h. Just before adding cells, the inserts were washed twice with PBS to remove unbound fibronectin. Cells were incubated in a humidified cell culture incubator with 5% CO_2, balance air, for an additional 24 h, after which they were washed twice with PBS, followed by fixation with 95% EtOH. The inserts were then stained with crystal violet and washed with water to remove unincorporated dye. Stained cells on the apical side of the insert were removed by using a swab. The membrane was cut along the edges and scanned for photography. A minimum of 8 frames per membrane were collected, and cells in each frame were counted. The mean number of cells per frame was plotted.

Promoter Luciferase Assay. The A_{2A} receptor promoter constructs, cloned in pSSG-luciferase reporter vectors (a variant of pGL4.11 vector from Promega), were obtained from SwitchGear Genomics. Promoter activity of the A_{2A} receptor gene was assessed by using a luciferase reporter construct, R5. HLMVECs were transfected with the A_{2A} reporter vectors or the empty control (pGL4.11), together with mutHIF-2α construct by using the DharmaFect Duo transfection reagent (Dharmacon). A CMV-β-gal plasmid cotransfection was also used to control for transfection efficiency. Forty hours after transfection, cells were harvested and lysed by using the reporter lysis buffer (Promega). β-Gal assays were performed with a commercially available kit (Stratagene). Luciferase activities were determined with equal amounts of protein by using a commercially available luciferase assay system (PharMingen) and a Monolight 3010 luminometer (Analytical Luminescence Laboratory). The relative luciferase units were normalized to the internal β-gal control values and plotted.

ChIP. ChIP assays were performed on HLMVECs by using standard protocol. About 45 million cells in 100-mm plates were exposed to air (21% O_2) or hypoxia (1% O_2) for 6 h. After hypoxic exposure, cells were washed with PBS and cross-linked in a solution of 10% formaldehyde with gentle shaking for 20 min. The cross-linking was stopped by the addition of glycine to a final concentration of 0.125 M. Cells were then washed with cold PBS, scraped, and pelleted. The pellet was resuspended in lysis buffer (50 mM Tris·HCl, pH 8.1, containing 1% SDS, 5 mM EDTA, and Calbiochem protease inhibitor mixture) for 10 min, after which the samples were sonicated 5 times for 15 sec each by using a Branson Sonicator. After clearing the lysate, a part of the soluble chromatin was diluted 5-fold in PBS and reverse cross-linked at 65 °C overnight for use as an input control. The remaining soluble chromatin was diluted 10-fold with the dilution buffer (20 mM Tris, pH 8.1; 2 mM EDTA; and 1% Triton X-100) and precleared with Protein G beads (GE Healthcare Life Sciences). The samples were incubated at 4 °C overnight with either a control antibody or rabbit polyclonal antibody against HIF-2α (Novus Biologicals). The chromatin-immunoprecipitated DNA was PCR amplified by using specific primers for A_{2A} receptor (forward: 5′-CAGGTTGCCAGTCCTGCTCCATC; and reverse: ACCTGCCTGGGGACAAGAGGTC-3′) and PGK-1 (forward: 5′-GTTCGCAGCGTCACCCGGATCTTCG-3′; and reverse: 5′-AGGCTTGCAGAATGCGGAACACC-3′. The following conditions were used for PCR amplification of PGK-1: 1 cycle of 95 °C for 3 min; 33 cycles of 95 °C for 30 sec, 65 °C for 30 sec, and 72 °C for 20 sec; and 1 cycle of 72 °C for 5 min; and for A_{2A} receptor: 1 cycle of 95 °C for 3 min; 31 cycles of 95 °C for 30 sec, 62 °C for 30 sec, and 72 °C for 20 sec; and 1 cycle of 72 °C 5 min.

1. Riddle SR, et al. (2000) Hypoxia induces hexokinase II gene expression in human lung cell line A549. *Am J Physiol Lung Cell Mol Physiol* 278:L407–L416.

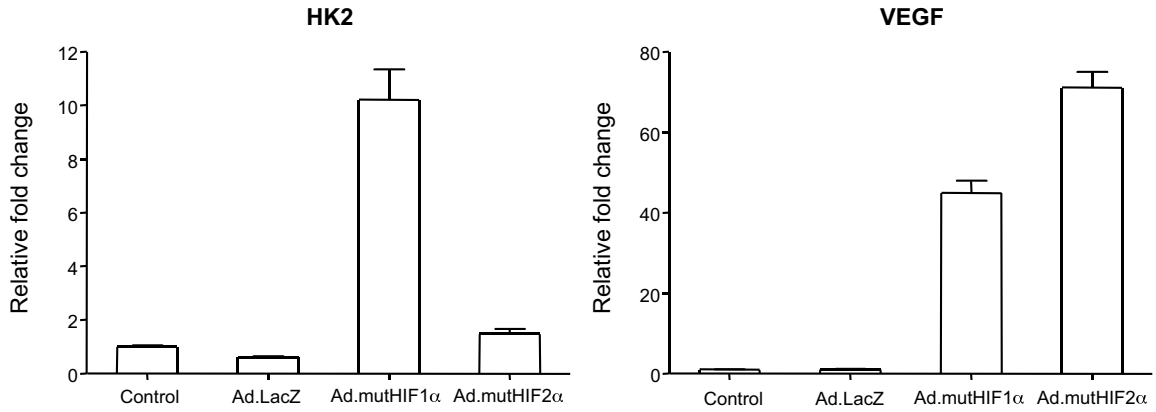

Fig. S1. Effect of HIFs on the expression of HK2 and VEGFA. Endothelial cells (HLMVECs) were transduced with Ad.LacZ, Ad.HIF-1α, or Ad.HIF-2α at a multiplicity of infection of 15 pfu per cell for 24 h. Total RNA was isolated, and 1 μg was reverse transcribed by using random primed hexamers. Real-time PCR was carried out by using a total of 25 ng of reverse-transcribed cDNA along with specific primers and probes for HK2, VEGFA, and 18S rRNA. Data were normalized per 18S.

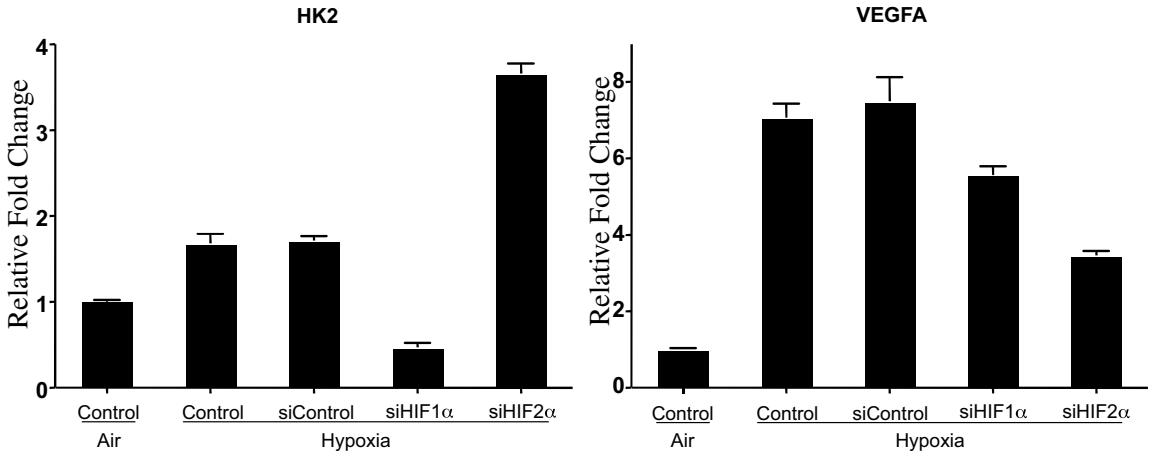

Fig. S2. Effect of siRNA knockdown of HIFs on the expression of HK2 and VEGFA under hypoxia. Endothelial cells (HLMVECs) were transfected with siRNA against HIF-1α, HIF-2α, or the control siRNA. Twenty-four hours after transfection, cells were exposed to hypoxia (0% O_2) for an additional 24 h. Total RNA was isolated, and 1 μg was reverse transcribed by using random primed hexamers. Real-time PCR was carried out by using a total of 25 ng of reverse-transcribed cDNA along with specific primers and probes for HK2, VEGFA, and 18S rRNA. Data were normalized per 18S.

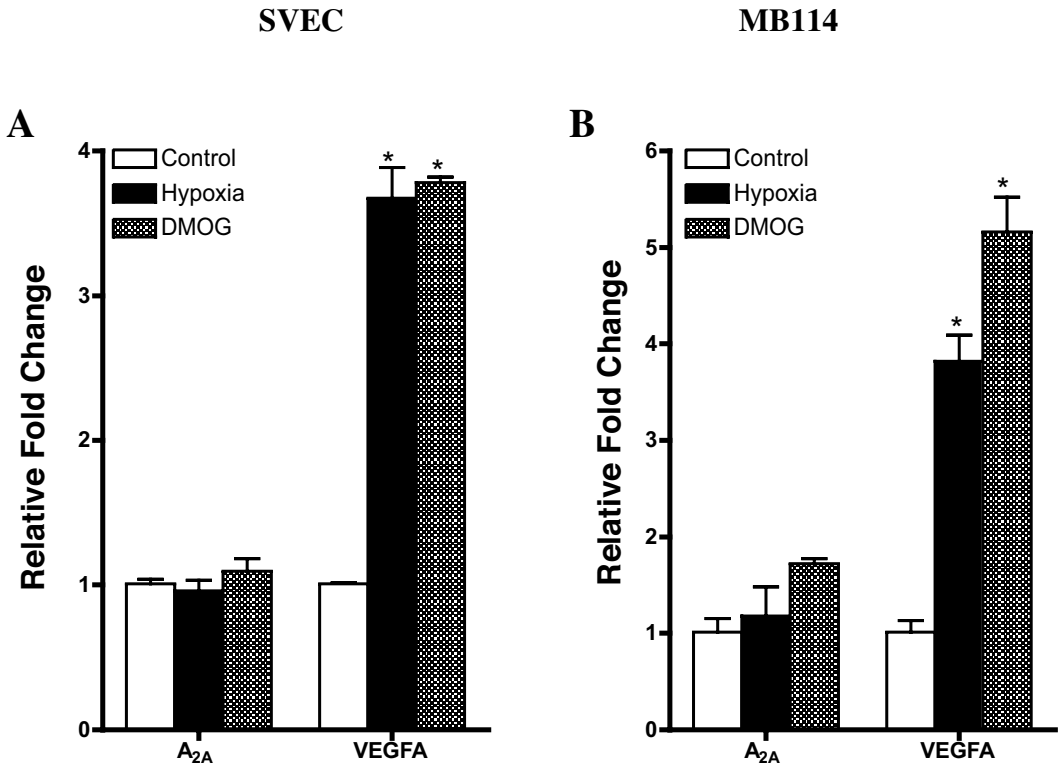

Fig. S3. Effect of hypoxia and DMOG, an inhibitor of HIF prolyl-4-hydroxylases, which causes stabilization of both HIF-1α and HIF-2α, on the levels of adenosine A_{2A} receptor steady-state mRNA levels in mouse-derived endothelial cells. Endothelial cells, SVECs (*A*) and MB114 cells (*B*), were exposed to air (21% O_2), hypoxia (0% O_2), or 1 mM DMOG for 24 h. Total RNA was isolated, and 1 μg was reverse transcribed by using random primed hexamers. Real-time PCR was carried out by using a total of 25 ng of reverse-transcribed cDNA along with specific primers and probes for adenosine A_{2A} receptor, VEGFA, and 18S rRNA. Data were normalized per 18S.