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Role of Adenosine Receptors in the Regulation of Angiogenic Factors and Neovascularization in Hypoxia

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ABSTRACT

Because hypoxia increases extracellular adenosine levels and stimulates angiogenesis, we evaluated the relative roles of reduced oxygen concentrations and adenosine receptor activation in the production of angiogenic factors. In vitro, we analyzed the effects of hypoxia and adenosine on the secretion of angiogenic factors from human microvascular endothelial cells (HMEC-1). To study the effects of hypoxia alone, we scavenged adenosine from the hypoxic medium with adenosine deaminase, and we used the stable adenosine analog 5′-*N*-ethylcarboxamidoadenosine (NECA) to study the effects of stimulation of adenosine receptors. In the absence of adenosine, hypoxia stimulated vascular endothelial growth factor (VEGF) but not interleukin-8 (IL-8) secretion from HMEC-1. In contrast, NECA stimulated both VEGF and IL-8 secretion. VEGF secretion was increased 1.9 ± 0.04-fold with NECA (10 μM) and 1.7 ± 0.1-fold with hypoxia (5% O_2) but 3.8 ± 0.1-fold when these two stimuli were combined. Thus, adenosine receptors act in a cooperative fashion with hypoxia to stimulate VEGF and induce IL-8 secretion not stimulated by hypoxia alone. In vivo, antagonism of adenosine receptors with caffeine abrogated VEGF up-regulation induced by local injection of NECA into the mouse hind limb and produced a 46% reduction of neovascularization in a mouse ischemic hind limb model. Our study suggests that adenosine actions are not redundant but rather are complementary to the direct effects of hypoxia. Stimulation of adenosine receptors not only contributes to the overall effect of hypoxia but also has additional actions in the regulation of angiogenic factors. Thus, adenosine receptors represent a potential therapeutic target for regulation of neovascularization.

Hypoxia elicits a number of compensatory homeostatic mechanisms aimed to restore oxygen supply. Acutely, it induces local vasodilation, resulting in reactive hyperemia. Chronically, hypoxia promotes growth of new blood vessels in a process known as angiogenesis. Angiogenesis is regulated by a delicate balance of multiple pro- and antiangiogenic factors. Hypoxia promotes angiogenesis by up-regulating the expression of several angiogenic factors including vascular endothelial growth factor (VEGF). It is commonly accepted that VEGF production is regulated by the oxygen-sensitive hypoxia-inducible factor-1 (HIF-1). Accumulating evidence, however, suggests that hypoxia-induced secretion of angiogenic factors involves diverse mechanisms. Several studies implicate interleukin-8 (IL-8) as contributing to hypoxia-induced angiogenesis through mechanisms independent of HIF-1 (Xie, 2001; Mizukami et al., 2005). It is possible therefore, that other alternative oxygen-sensitive mechanisms could be involved in the overall effect of hypoxia. Adenosine is known to be released in hypoxic tissues and could be an ideal candidate to contribute to hypoxia-induced angiogenesis.

Adenosine is an intermediate product of adenine nucleotide metabolism. It is generated as ATP is catabolized when energy demands increase or oxygen supply decreases at sites of tissue stress, injury, and local hypoxia. This results in increased adenosine concentrations in the interstitium, where it exerts its actions via binding to extracellular G protein-coupled adenosine receptors (Fredholm et al., 2001a). We have shown that stimulation of adenosine A_{2B} receptors up-regulates the angiogenic factors VEGF and IL-8 in human endothelial cells under normoxic conditions (Grant et al., 1999; Feoktistov et al., 2002). Studies in other cell culture models conducted under normoxic conditions have also indi-

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ABBREVIATIONS: VEGF, vascular endothelial growth factor; HIF-1, hypoxia-inducible factor-1; IL-8, interleukin-8; HMEC-1, human microvascular endothelial cells; NECA, 5′-*N*-ethylcarboxamidoadenosine; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine hydrochloride; DMSO, dimethyl sulfoxide; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pairs; PBS, phosphate-buffered saline.

cated that adenosine up-regulates proangiogenic and down-regulates antiangiogenic factors (for review, see Adair, 2005). Remarkably, chronic infusion of adenosine induced neovascularization in the skeletal muscle and the heart (Ziada et al., 1984). It is possible therefore, that the elevation of adenosine levels observed in ischemic tissues (Hagberg et al., 1987; Fredholm et al., 2001a) contributes to the overall effect of hypoxia on angiogenesis. If this is true, then adenosine receptors could become a target for pharmacological modulation of hypoxia-induced angiogenesis. However, the role of adenosine receptors in the regulation of angiogenic factors under hypoxic conditions remains unclear. Adenosine has been suggested to play a less important role in the regulation of VEGF expression under hypoxic than under normoxic conditions (Gu et al., 1999), and the regulation of IL-8 by adenosine has not been studied during hypoxia. It is also unclear whether hypoxia-induced angiogenesis could be reduced by inhibition of adenosine receptors in vivo.

In the current work, we tested the hypothesis that adenosine actions complement those of hypoxia in the regulation of angiogenesis. The results suggest that adenosine receptors up-regulate VEGF and IL-8 not only under normoxic but also under hypoxic conditions. Stimulation of adenosine receptors results in secretion of additional angiogenic factors (IL-8) not induced by hypoxia per se and in greater VEGF production. We further demonstrate that hypoxia-induced angiogenesis can be reduced by blocking adenosine receptors in vivo.

Materials and Methods

Cell Culture and Treatment Conditions

HMEC-1, developed by Dr. Edwin Ades and Fransisco J. Candal of the Centers for Disease Control and Prevention and Dr. Thomas Lawley of Emory University (Ades et al., 1992), were obtained from Centers for Disease Control and Prevention/National Center for Infectious Diseases (Atlanta, GA). Cells were maintained in M-199 medium supplemented with 15% (v/v) fetal bovine serum, 1× antibiotic-antimycotic mixture (Invitrogen, Carlsbad, CA), and 0.3 μg/ml bovine hypothalamus endothelial mitogen (Biomedical Technologies, Stoughton, MA) under a humidified atmosphere of air-CO_2 (19:1) at 37°C.

Confluent monolayer cultures were exposed to hypoxia in an incubation chamber (Billups-Rothenberg, Del Mar, CA) equipped with an oxygen meter. Growth media were replaced by serum-free media preconditioned overnight at corresponding oxygen concentrations in a 37°C cell culture incubator. Hypoxic conditions were created by flushing with a 5% CO_2-95% N_2 gas mixture until oxygen inside the chamber reached desired concentrations. The hypoxic chamber was then sealed and placed in a 37°C cell culture incubator for the indicated time.

Chemicals

5′-*N*-Ethylcarboxamidoadenosine (NECA) and *erythro*-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA) were purchased from Sigma-RBI (St. Louis, MO). [U-$^{13}C_{10}$-U-$^{15}N_5$]Adenosine was obtained from Cambridge Isotope Laboratories (Andover, MA). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (St. Louis, MO) and was used as a solvent for NECA and EHNA stock solutions. Final DMSO concentrations in all assays were 0.01%, and this concentration was used as a vehicle control.

Real-Time Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from HMEC-1 and mouse skeletal muscle tissue using RNeasy Mini and RNeasy Fibrous Tissue kits (QIAGEN, Valencia, CA), respectively. Real-time RT-PCR was performed on an ABI PRISM 7900HT Sequence Detection System (PE Applied Biosystems, Foster City, CA). Published sequences were used for generation of specific primer pairs for VEGF, IL-8, and β-actin (Schulz et al., 2003; Garcia-Velasco et al., 2004). For IL-8, the forward primer was 5′-TGCCAAGGAGTGCTAAAG-3′ and the reverse primer was 5′-TCCACAACCCTCTGCAC-3′ (197-bp fragment). For human β-actin the forward primer was 5′-CGCCCCAGGCACCAGGGC-3′, and the reverse primer was 5′-GGCTGGGGTGTTGAAGGT-3′ (285-bp fragment). The human VEGF forward primer was 5′-GGGCAGAATCATCACGAAGTG-3′, and the reverse primer was 5′-ATTGGATGGCAGTAGCTGCG-3′ (65-bp fragment). The murine VEGF forward primer was 5′-CTGCACCCACGACAGAAG-3′, and the reverse primer was 5′-ACACAGGACGGCTTGAAGAT-3′ (154-bp fragment). For murine β-actin, the forward primer was 5′-AGTGTGACGTTGACATCCGTA-3′ and the reverse primer was 5′-GCCAGAGCAGTAATCTCCTTCT-3′ (112-bp fragment). RT-PCR reactions using 1 μg of DNase-treated total RNA were performed under conditions recommended by the manufacturer. A dissociation curve was generated at the end of the polymerase chain reaction cycle to verify that a single product was amplified. A standard curve for each amplicon was obtained using serial dilutions of total RNA. The results from triplicate polymerase chain reactions for a given gene at each time point were used to determine mRNA quantity relative to the corresponding standard curve. The relative mRNA quantity for a given gene measured from a single reverse transcription reaction was divided by the value obtained for β-actin to correct for fluctuations in input RNA levels and varying efficiencies of reverse transcription reactions.

Determination of IL-8 and VEGF Levels in Conditioned Media

Cells were incubated in serum-free media containing 1 U/ml adenosine deaminase (Calbiochem, San Diego, CA) under conditions and in the presence of reagents described under *Results*. After incubations, culture media were collected by centrifugation at 12,000*g* for 1 min at 4°C. IL-8 and VEGF concentrations were measured using enzyme-linked immunosorbent kits (R&D Systems, Minneapolis, MN).

Measurement of Adenosine Concentrations in HMEC-1 Conditioned Media

We used a sensitive and specific ion-pair liquid chromatographic electrospray ionization tandem mass spectrometry assay to measure adenosine concentrations as described previously (Gamboa et al., 2003). HMEC-1 were placed in hypoxic chambers and incubated in serum-free DMEM containing 5 μM EHNA under normoxic or hypoxic conditions for 1 h. Cell culture media were collected by centrifugation at 12,000*g* for 1 min at 4°C, diluted 10-fold with water containing 10 nM [U-$^{13}C_{10}$-U-$^{15}N_5$]adenosine as an internal standard and precleared by filtration through Amicon Ultrafree-MS centrifugal filters (Millipore Corporation, Bedford, MA). High-performance liquid chromatography separations were performed on a 5-μm C_{18} column using pentadecafluorooctanoic acid as an ion-pairing reagent. Mass spectrometric analysis was performed on a Finnigan MAT TSQ-7000 triple-quadruple mass spectrometer (Thermo Electron Corporation, San Jose, CA) using positive-ion electrospray ionization. Selected reaction monitoring was used to measure adenosine (*m/z* from 268 to 136) and the internal standard (from 282 to 145). The calibration range was 23 to 1150 fmol on column (10–500 nM adenosine in original samples). The chromatographic peak at the lower limit of quantitation (10 nM; 25 fmol on column) gave a signal/noise ratio of 25. The inter-run coefficient of variability was 8.1%.

Animal Procedures

All studies were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by

the United States National Institutes of Health (NIH publication 85-23, revised 1996).

Caffeine Treatment. Male 3-month-old C57BL/6J mice (15 animals/group) were used. To block adenosine receptors in mice, caffeine was administered orally according to a published protocol (Kuzmin et al., 2000). Caffeine (Sigma-Aldrich) was dissolved in tap water at a concentration of 0.3 g/l. The mice were allowed to drink either tap water (control group) or caffeinated water. On the 3rd day both groups of animals (10 mice/group) underwent femoral artery ligation to create unilateral hind limb ischemia as described below. In a separate set of experiments, caffeine-treated and control mice (5 animals/group) were used to evaluate the effect of 3-day caffeine consumption on NECA-induced VEGF up-regulation in the hind limb. The mice with surgically induced hind limb ischemia continued to receive caffeine or vehicle treatment until they were sacrificed on day 21 postsurgery.

Stimulation of Adenosine Receptors and Sample Preparation for Analysis of VEGF mRNA in Hind Limb Muscle Tissue. Mice were anesthetized with i.p. ketamine (100 mg/kg) and xylazine (10 mg/kg). Hair from the skin covering the muscular belly of the gastrocnemius muscle group was removed from both hind limbs with a depilatory agent (Nair; Carter-Wallace, New York, NY). NECA (10 μM) or its vehicle control (0.01% DMSO) solutions were prepared in saline and sterile-filtered through 0.22-μm syringe filters. NECA solution (30 μl) was injected into the left hind limb, and an equal volume of its vehicle was injected into the right hind limb of each animal. To ensure accuracy of i.m. drug delivery, we followed a technique based on a previously published study of anatomical distribution of i.m. injected India Ink into hind limbs of C57BL mice (Fargas et al., 2003). The site of puncture was at the confluence between a superficial vein and an imaginary vertical line that crosses the Achilles tendon. Injections were performed using a disposable tuberculin syringe with a 29-gauge needle, which was introduced at a maximal depth of 2 mm, perpendicular to the longitudinal axis of the leg. One hour after injections, animals were sacrificed by cervical dislocation while remaining unconscious under anesthesia. Samples of muscle tissue (60–80 mg) were excised at the sites of injection and stored in an RNA stabilization solution (RNAlater, Ambion, Austin, TX) at 4°C before processing for real-time RT-PCR analysis of VEGF mRNA expression.

Unilateral Hind Limb Ischemia Model. Induction of ischemia in mouse hind limbs was performed according to published methods (Couffinhal et al., 1998). Mice were anesthetized with 2 to 3% isoflurane (in 100% O_2) and placed on a Deltaphase isothermal pad (Braintree Scientific, Braintree, MA) to maintain body temperature at 36 to 37°C. Hair in the middle portion of left hind limb region was removed with a depilatory agent, and the skin was prepared with antiseptic (Betadine followed by alcohol). As soon as the animal demonstrated absence of a withdrawal response to nociceptive stimulation of a hind paw, a 1-cm incision in the skin overlaying the middle portion of the leg was made. After the proximal end of the femoral artery was ligated with a suture (7-0 silk), the distal portion of the saphenous artery was ligated, and the artery and all side branches were dissected free; then the femoral artery and attached side branches were excised. The overlaying skin was then closed using a surgical stapler. Animals were placed in individual cages on a heated isothermal pad until fully recovered from anesthesia.

Quantification of Angiogenesis. The capillary/muscle fiber ratio was used as an index of angiogenesis and was evaluated in microscopic sections of muscle tissue taken from the ischemic and nonischemic limbs according to published methods (Couffinhal et al., 1998). The mice were euthanized on day 21 postsurgery, and both hind limbs were removed. The excised limbs were fixed overnight in 4% formaldehyde, and the muscles below the knee were excised from both ischemic and nonischemic limbs. The distal portions of muscles (5 mm) were cross-sectioned and then embedded in paraffin. Each block was cut transversely 7-μm-thick in sequential order. Immunoperoxidase staining of endothelial cells was performed using rabbit anti-mouse von Willebrand factor (Sigma-Aldrich) as a primary antibody to visualize capillaries. After deparaffinization, rehydration, and antigen retrieval, the sections on microscopic slides were incubated in 3% hydrogen peroxide to block endogenous peroxidase activity. To prevent nonspecific antibody binding, the sections were preincubated for 15 min in PBS containing 10% Power Block (BioGenex, San Ramon, CA) and 0.4% Triton X-100. Sections were then incubated with rabbit anti-mouse von Willebrand factor diluted 1:200 in PBS containing 10% Power Block and 0.4% Triton X-100 overnight at 4°C. Slides were washed with 0.4% Triton X-100 in PBS and incubated with biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) diluted 1:250 in PBS containing 10% Power Block and 0.4% Triton X-100 for 20 min at room temperature. Washing was repeated with 0.4% Triton X-100 in PBS, and sections were incubated with a streptavidin-horseradish peroxidase complex reagent (Vector Laboratories, Burlingame, CA) for another 30 min. After washing with 0.4% Triton X-100 in PBS, the slides were incubated with DAB (3,3′-diaminobenzidine tetrahydrochloride) Peroxidase Substrate Solution (Sigma-Aldrich) for 30 s and then counterstained with hematoxylin (Sigma-Aldrich). Capillaries and muscle fibers were counted under a microscope by an investigator blinded to the treatment received. A total of 12 random fields (100×) from different sections were counted for each limb. To ensure that capillary densities were not overestimated because of skeletal muscle atrophy observed in this model (Chalothorn et al., 2005), the capillary/muscle fiber ratio for each field was determined.

Results

Effects of Stimulation of Adenosine Receptors and Hypoxia on VEGF and IL-8 mRNA. To selectively explore the role of hypoxia and adenosine receptors without the confounding influence of endogenous adenosine release, experiments were conducted in the presence of 1 U/ml adenosine deaminase. We initially examined how adenosine and hypoxia interact in regulation of VEGF and IL-8 mRNA expression levels in HMEC-1. In these cells, stimulation of A_{2B} adenosine receptors results in up-regulation of VEGF and IL-8 under normoxic conditions (Feoktistov et al., 2002). From our previous studies, we selected a concentration of the stable adenosine analog NECA (10 μM) producing a near maximal effect. As seen in Fig. 1A, NECA stimulated VEGF mRNA expression by 2.6 ± 0.1-fold after a 1-h incubation in normoxia, but this stimulation declined later to only a 1.3 ± 0.1-fold increase over the basal level at 12 h. In contrast, hypoxia alone (5% O_2) induced a steady increase of VEGF mRNA, by 1.2 ± 0.1-fold at 1 h and by 6.2 ± 0.2-fold at 6 h. The combination of hypoxia and NECA resulted in greater increases of VEGF mRNA. Stimulation of adenosine receptors accounted for virtually all of the combined increase in VEGF mRNA in response to hypoxia and NECA at 1 h but comprised only 10 ± 2% of the total response after a 12-h stimulation.

NECA also induced a rapid, but transient, elevation of IL-8 mRNA, with levels increasing by 8.6 ± 0.5-fold at 1 h of incubation in normoxia and returning to near basal levels at 12 h (Fig. 1B). Hypoxia alone did not elevate IL-8 mRNA at 1 or 3 h and had only modest effects at later time points. The effects of NECA during hypoxia were essentially the same as during normoxia; NECA rapidly increased IL-8 mRNA levels by 8.3 ± 0.2-fold at 1 h and had no effect on IL-8 mRNA at later time points (6 and 12 h).

Effects of Stimulation of Adenosine Receptors and Hypoxia on VEGF and IL-8 Secretion. Changes in mRNA

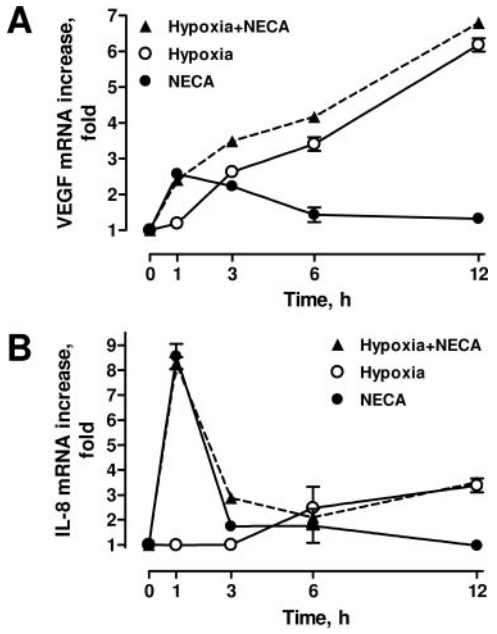

Fig. 1. Time course of the effects of hypoxia and the stable adenosine agonist NECA on mRNA levels of VEGF (A) and IL-8 (B). Cells were incubated under normoxia in the presence of 10 μM NECA (NECA) or under an atmosphere containing 5% O_2 in the absence (Hypoxia) or in the presence of 10 μM NECA (Hypoxia+NECA). The data are presented as an increase over basal mRNA levels. Values are expressed as means ± S.E.M. of three experiments.

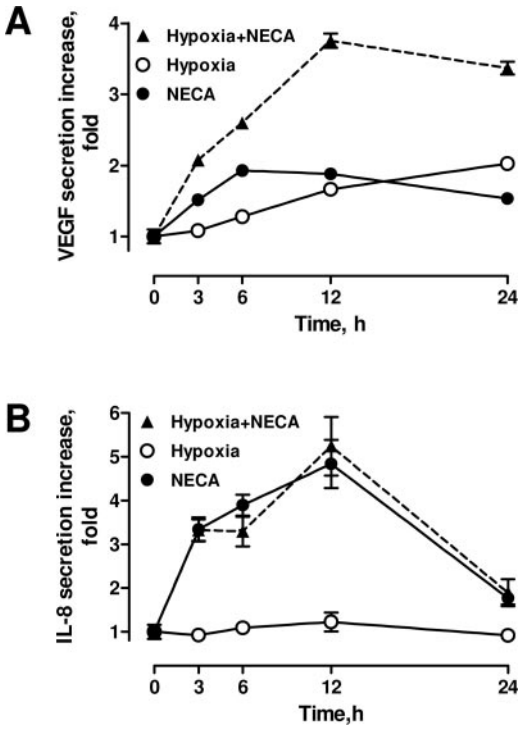

Fig. 2. Time course of the effects of hypoxia and the stable adenosine agonist NECA on VEGF (A) and IL-8 (B) secretion. Cells were incubated under normoxia in the presence of 10 μM NECA (NECA) or under an atmosphere containing 5% O_2 in the absence (Hypoxia) or in the presence of 10 μM NECA (Hypoxia+NECA). The data are presented as an increase over basal secretion measured in the absence of NECA under normoxia at each time point. Values are expressed as means ± S.E.M. of three experiments.

levels are not always expected to result in corresponding changes in protein levels because of potential post-transcriptional regulation. In addition, the release of angiogenic factors can be differentially regulated by diverse signals. Therefore, we thought it was important to determine how adenosine and hypoxia interact in regulation of VEGF and IL-8 secretion. Under normoxic conditions and in the absence of NECA, there was a time-related tonic basal secretion of VEGF and IL-8, resulting in media concentrations of 114 ± 7 and 366 ± 17 pg/ml, respectively, at 24 h. Figure 2 shows the effects of 10 μM NECA and hypoxia (5% O_2) on VEGF and IL-8 secretion presented as an increase over basal secretion at each time point. As expected, we observed a lag time between increases in mRNA and protein levels. Stimulation of VEGF secretion by hypoxia gradually increased over a 24-h period (Fig. 2A). NECA also induced VEGF secretion under normoxic conditions, but with a time course different from that of hypoxia; stimulation of VEGF secretion by NECA was substantially higher at early time points and reached a maximum by 6 h of incubation. The effects of NECA were even greater under hypoxic conditions, with the maximal increase in VEGF secretion at 12 h. VEGF secretion was increased by 1.9 ± 0.04-fold in the presence of NECA alone, by 1.7 ± 0.1-fold in hypoxia alone, but by 3.8 ± 0.1-fold when these two stimuli were combined. Thus, the combined effects of hypoxia and NECA were more prominent on VEGF protein secretion than on mRNA levels compared with the separate effects of hypoxia and NECA. Although it is tempting to speculate that these discrepancies may be related to post-transcriptional regulation, the exact mechanism is unclear and requires further investigation.

Hypoxia alone did not stimulate IL-8 production in HMEC-1 over all periods of observation (Fig. 2B). In contrast, 10 μM NECA increased IL-8 secretion to similar levels under normoxic and hypoxic conditions, with maximal stimulation observed by 12 h. At this time point, NECA increased IL-8 secretion by 4.8 ± 0.5-fold in normoxia and by 5.2 ± 0.6-fold in hypoxia.

We then questioned whether an increase in the severity of hypoxia would affect the ability of adenosine receptors to stimulate VEGF and IL-8 secretion. HMEC-1 were incubated for 6 h at varying concentrations of oxygen or anoxia. Hypoxia alone stimulated VEGF secretion with an estimated concentration of oxygen producing 50% of the maximal effect (EC_{50}) of 1.9% (Fig. 3A). NECA increased VEGF production by 3.5 ± 0.4-fold under normoxic conditions (21% O_2), by 4.5 ± 0.3, 3.8 ± 0.1, and 2.9 ± 0.1-fold under 10, 5, and 2% O_2 hypoxic conditions, respectively, and by 2.3 ± 0.2-fold in anoxia. Thus, stimulation of adenosine receptors even in anoxia produced a significant increase in VEGF secretion compared with the effect of oxygen depletion per se.

Hypoxia had no significant effect on IL-8 production at any O_2 concentrations tested (Fig. 3B). Only anoxia induced a slight 22 ± 5% increase in IL-8 secretion compared with normoxia. In contrast, NECA was effective in stimulating IL-8 release during normoxia or hypoxia. There was only a small decrease in its efficacy with increasing hypoxia; NECA stimulated IL-8 secretion by 3.5 ± 0.2-fold in normoxia, by 3.2 ± 0.1, 3.0 ± 0.3, and 2.9 ± 0.2-fold at 10, 5, and 2% O_2, respectively, and by 2.4 ± 0.2-fold in anoxia. We concluded that stimulation of VEGF and IL-8 secretion via adenosine receptors was preserved at any degree of hypoxia.

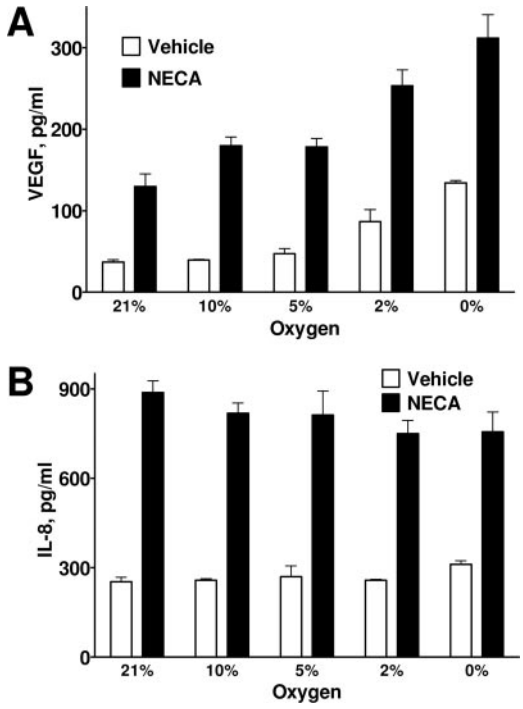

Fig. 3. Effect of hypoxia and the stable adenosine analog NECA on VEGF (A) and IL-8 (B) secretion from HMEC-1. Cells were incubated under reduced oxygen conditions for 6 h in the absence (Vehicle) or in the presence of 10 μM NECA (NECA). Values are expressed as means ± S.E.M. of three experiments.

Effect of Hypoxia on Adenosine Release from HMEC-1. Stimulation of proangiogenic factors by NECA under hypoxic conditions observed in our cell culture model implies that adenosine receptors may contribute to angiogenesis induced by hypoxia when adenosine levels increase. This contention could be tested in cell culture by using antagonists of adenosine receptors, but only if hypoxia-induced changes in adenosine concentrations in medium would approximate reported changes in interstitial adenosine levels in ischemic tissues. However, we found that omission of exogenously added adenosine deaminase from cell culture media and even inhibition of endogenous adenosine deaminase with 5 μM EHNA did not significantly increase the effect of hypoxia on VEGF release from HMEC-1. For example, incubation of HMEC-1 in the presence of 1 U/ml adenosine deaminase for 6 h under hypoxic (5% O_2) conditions resulted in VEGF concentrations of 43.5 ± 2.5 pg/ml (n = 3) in cell culture medium compared with VEGF concentrations of 22.1 ± 1.5 pg/ml (n = 3) produced in normoxia. In the absence of exogenously added adenosine deaminase and in the presence of 5 μM EHNA, concentrations of VEGF in medium reached only 48.9 ± 0.5 pg/ml (n = 3) in hypoxia and 21.1 ± 1.4 pg/ml (n = 3) in normoxia. These results strongly suggest that concentrations of adenosine in cell culture media do not reach levels high enough to stimulate adenosine receptors in our cell model. To determine whether this suggestion is correct, we measured adenosine concentrations in media collected from cells exposed for 1 h to varying concentrations of oxygen or anoxia. The incubation medium contained no serum but included 5 μM EHNA to prevent potential adenosine degradation by endogenous extracellular adenosine deaminase. A decrease in oxygen concentrations induced an exponential increase in adenosine concentrations in cell medium from 24 ± 1 nM in normoxia to 47 ± 4 nM in anoxia with an estimated EC_{50} value of 2.4% (Fig. 4). The concentration of adenosine reached in cell medium during hypoxia was much lower than that required to stimulate adenosine receptors; the K_d values for all adenosine receptor subtypes to adenosine are in the range of 0.2 to 23 μM (Fredholm et al., 2001b). This rendered our model not suitable for the use of adenosine antagonists to study the contribution of adenosine receptors to the overall effect of hypoxia.

Effect of Inhibition of Adenosine Receptors on Angiogenesis in Vivo in Hind Limb Ischemia Model. In vivo, the interstitial concentration of adenosine can increase from basal levels on the order of 100 nM (Ontyd and Schrader, 1984) to as high as 40 μM (Hagberg et al., 1987) during hypoxic conditions. These adenosine concentrations are sufficient to stimulate all adenosine receptor subtypes. Therefore, we decided next to evaluate the contribution of adenosine receptors to the regulation of neovascularization in hypoxic conditions in vivo. We chose caffeine to block adenosine receptors because it has been used previously for this purpose in mice, and its pharmacokinetics is well known (Kuzmin et al., 2000). In particular, it has been reported that chronic oral consumption of caffeine (0.3 g/l in tap water) by mice results in plasma and tissue concentrations of caffeine and its active metabolites (paraxanthine, theophylline, and theobromine) sufficient to block all but A_3 adenosine receptor subtypes (Fredholm et al., 1999; Kuzmin et al., 2000). Therefore, we sought first to confirm that caffeine administration would either reduce or abrogate adenosine-dependent upregulation of angiogenic factors in the mouse hind limb. For this purpose, we compared changes in VEGF mRNA levels resulting from stimulation of adenosine receptors in hind limbs of caffeine-treated and control mice. Each mouse received intramuscular injections with 30 μl of 10 μM NECA into one hind limb and an equal volume of vehicle into the contralateral hind limb. We reasoned that even though NECA could spill over into the systemic circulation and eventually reach the contralateral limb, the difference in local tissue concentrations of NECA would be large enough to result in greater VEGF mRNA levels in the limb injected with NECA compared with the limb injected with vehicle. As seen in Fig. 5, VEGF mRNA levels were significantly increased to 2.9 ± 0.2% β-actin in NECA-injected hind limbs in the control group of animals compared with 1.9 ± 0.2% β-actin in vehicle-injected limbs (mean ± S.E.M., $p < 0.05$, n = 5, t test). In contrast, this NECA-dependent effect was almost completely abrogated in caffeine-treated animals.

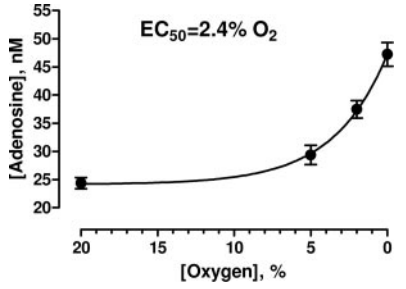

Fig. 4. Effect of hypoxia on release of adenosine from HMEC-1. Cells were incubated under reduced oxygen conditions for 1 h. Means ± S.E.M. of three experiments are shown.

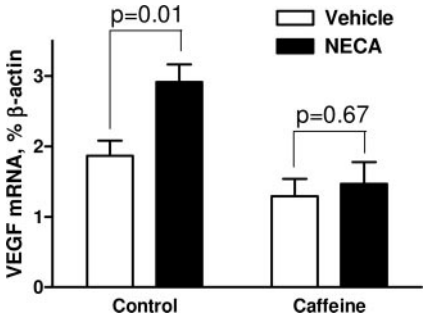

Fig. 5. Effect of caffeine on VEGF up-regulation by NECA in the mouse hind limb. Two groups of animals were allowed to drink either 0.3 g/l caffeine solution (Caffeine) or tap water (Control) for 3 days. Each mouse then received i.m. injections with 30 μl of 10 μM NECA into one hind limb and an equal volume of vehicle into the contralateral hind limb. Tissue mRNA levels were measured in muscle samples excised from NECA-injected (■) and vehicle-injected (□) limbs 1 h later. Data are expressed as means ± S.E.M. of five animals.

Two-way repeated measurement analysis of variance, performed to account for pairing of legs and followed by Bonferroni post-tests, showed that there was a significant effect of NECA in increasing VEGF mRNA levels ($p < 0.01$) and that caffeine significantly blunted the effect of NECA ($p < 0.01$ for the interaction between factors). Thus, our results confirmed that the chosen protocol of chronic caffeine administration would allow blocking of adenosine-dependent processes relevant to angiogenesis in the mouse hind limb.

Unilateral femoral artery ligation is an established model to study ischemia-induced angiogenesis in the mouse hind limb (Couffinhal et al., 1998). Twenty-one days after surgery, capillary density in the ischemic limbs of control mice was increased to 40.9 ± 4.8 compared with 19.9 ± 1.9 capillaries/field in the nonischemic limbs (mean ± S.E.M., $p < 0.001$, $n = 10$, t test). Representative micrographs in Fig. 6A illustrate the difference in capillary density in nonischemic and ischemic limbs of the same mouse. Femoral artery ligation also reduces muscle fiber size as a consequence of ischemia and/or limited limb use (Chalothorn et al., 2005). The resulting increase in number of fibers per field can confound the interpretation of changes in capillary density vis-à-vis angiogenesis. Therefore, we used the ratio of capillary number to muscle fiber number to evaluate ischemia-induced angiogenesis. As seen in Fig. 6B, caffeine administration reduced angiogenesis in the ischemic limbs from 1.75 ± 0.12 to 1.33 ± 0.11 capillaries/myofiber (mean ± S.E.M., $p < 0.05$, $n = 10$, t test). In contrast, caffeine had no effect on the capillary/fiber ratio in the nonischemic hind limbs. These data were also analyzed using two-way repeated measurement analysis of variance to account for pairing of legs followed by Bonferroni post-tests. This analysis showed a significant difference in the ischemic hind limb capillary/fiber ratio between mice treated with caffeine and control animals ($p < 0.01$). Furthermore, this analysis also indicated a significant interaction between ischemia and caffeine treatment ($p < 0.05$).

Discussion

Adenosine is considered a retaliatory autacoid released by ischemia with the goal of restoring oxygen supply. Whereas most of the research in this area has focused on the acute vasodilatory actions of adenosine, its potential longer-term role in hypoxia-induced formation of new blood vessels re-

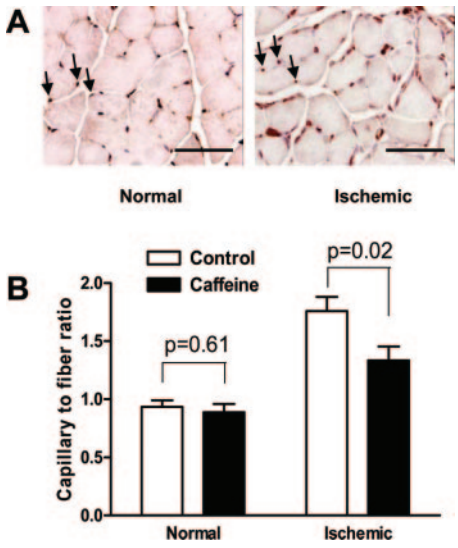

Fig. 6. Ischemia-induced angiogenesis in the mouse hind limb. A, immunohistochemical identification of vascular endothelial cells. Skeletal muscle sections were harvested from normal and ischemic hind limbs 21 days after surgery. Representative micrographs showing higher capillary density of ischemic muscle compared with normal controls are presented. As an example of von Willebrand factor immunostaining, three capillaries in each image are indicated by arrows. Scale bars, 50 μm. B, effect of chronic blockade of adenosine receptors by caffeine on ischemia-induced angiogenesis in the mouse hind limb. The ratio of capillaries to myofibers was determined in ischemic and normal limbs of control (□) and caffeine-treated mice (■) 21 days after unilateral femoral artery ligation. Data are expressed as means ± S.E.M. of 10 animals.

mains unclear. Accumulating evidence suggests that hypoxia-induced secretion of angiogenic factors involves diverse mechanisms. HIF-1-dependent stimulation of VEGF is arguably the best-understood mechanism. This process involves the loss of binding of oxygen to hydroxylases that control HIF-1 transcriptional activity and stability of its α-subunit (Semenza, 2004). However, this mechanism alone cannot explain all of the proangiogenic effects of hypoxia.

We analyzed the effects of hypoxia and adenosine, separately and in combination, on the secretion of two angiogenic factors, VEGF and IL-8, from HMEC-1. To study the effects of hypoxia alone, we scavenged adenosine from the hypoxic medium with adenosine deaminase, and we used the stable adenosine analog NECA to study the effects of stimulation of adenosine receptors. We found that hypoxia alone stimulated VEGF but not IL-8 secretion. In contrast, adenosine receptor activation stimulated both.

Differences between the actions of NECA and hypoxia were evident at the mRNA level. NECA induced a relatively fast increase in VEGF mRNA, with a maximum observed at 1 h, at a time when there was virtually no effect of hypoxia alone. The increase in VEGF mRNA by NECA, however, was transient and declined at later time points, when stimulation by hypoxia became evident. These data agree with previous studies examining the contribution of adenosine to hypoxic regulation of VEGF expression in vascular smooth muscle cells. It has been shown that adenosine increases VEGF mRNA expression in cultured rat aortic smooth muscle cells when the HIF-1 dependent pathway was stimulated for 4 h with $CoCl_2$ to mimic hypoxia (Pueyo et al., 1998). In contrast, adenosine was found to play a minor role in the regulation of VEGF mRNA expression in cultured dog myocardial smooth muscle cells exposed to 1% oxygen for 18 h (Gu et al., 1999).

This apparent contradiction could be explained by our observation that up-regulation of hypoxia-induced VEGF mRNA via stimulation of adenosine receptors occurs early during hypoxic stimulation and declines thereafter.

As expected, time courses for NECA- and hypoxia-induced VEGF protein secretion lagged behind the corresponding changes in mRNA. NECA increased VEGF secretion with a maximum observed at 6 h, whereas stimulation by hypoxia, significant only at later time points, continued to rise during the 24-h observation period. The effects of NECA were much greater under hypoxic conditions, with the maximal increase in VEGF secretion seen at 12 h. VEGF secretion was increased by 88 ± 4% in the presence of NECA alone, by 66 ± 5% in hypoxia alone, but by 275 ± 10% when these two stimuli were combined. Of interest, this synergism between NECA and hypoxia was observed at the protein but not at the mRNA level, suggesting potential amplification of translational efficiency and/or post-translational actions exerted by NECA in hypoxic cells. However, the exact mechanism of this phenomenon is unclear and requires further investigation. Stimulation of adenosine receptors considerably increased VEGF release from HMEC-1 in addition to that induced by hypoxia at all concentrations of O_2 tested including anoxia. Thus, our data indicate that adenosine and hypoxia cooperate in up-regulation of VEGF.

In contrast to VEGF, we found no evidence of a positive cooperation between adenosine and hypoxia in the regulation of IL-8 production. Several studies have shown that IL-8 is predominantly expressed in necrotic/hypoxic areas of solid tumors, suggesting that hypoxia may contribute to the overexpression of IL-8 that then plays an important and specific role in promoting angiogenesis (for review, see Xie, 2001). Our results show that hypoxia alone has virtually no effect on IL-8 secretion in HMEC-1 and even attenuated the stimulation of IL-8 secretion induced by NECA. These data agree with recent evidence indicating an inhibitory action of HIF-1 on IL-8 expression in HMEC-1 and in the rabbit postischemic myocardium (Ockaili et al., 2005). Likewise, knockdown of HIF-1α reduced VEGF but increased IL-8 levels in human DLD-1 colon cancer xenografts (Mizukami et al., 2005). Despite the putative opposing effects of hypoxia on IL-8 secretion, possibly involving HIF-1-dependent mechanisms, NECA stimulated IL-8 secretion even in anoxia. Because extracellular adenosine concentrations are increased at sites of tissue hypoxia, necrosis, and injury (Fredholm et al., 2001a), we speculate that adenosine may contribute to the regulation of IL-8 secretion from cells that otherwise are not responsive to hypoxia in these pathophysiological situations.

However, it should be noted that the physiological relevance of data obtained from in vitro studies should be interpreted with caution. First, we used the term "normoxia" to refer to the standard experimental conditions (21% O_2) used in vitro. Oxygen concentrations are considerably lower in vivo, depending on the location of cells within a tissue, or, in the case of endothelial cells, on the vascular bed. Second, we used HMEC-1 as an in vitro model because of their well-characterized ability to produce several angiogenic factors in response to adenosine (Feoktistov et al., 2002). However, there is currently no consensus about the physiological relevance of endothelial cells as a source of angiogenic factors. Cells located outside the vasculature are thought to release greater amounts of angiogenic factors, establishing a gradient that recruits new blood vessel formation toward the hypoxic focus. Finally, in vitro studies do not provide a suitable model to answer the question of whether endogenous adenosine contributes to the overall angiogenic effect of hypoxia. Stimulation of "proangiogenic" A_{2B} adenosine receptors requires adenosine concentrations in the low micromolar range (Fredholm et al., 2001b). However, the concentration of adenosine reached in our in vitro model during hypoxia was relatively modest compared with micromolar concentrations reported in hypoxic tissues in vivo (Fredholm et al., 2001a). This discrepancy can be explained by the limited number of cells present in a monolayer culture and dilution of adenosine released into a relatively large volume of medium. It is also possible that the capacity to release adenosine is greater in vivo.

Therefore, we thought it was important to evaluate the contribution of adenosine receptors to the regulation of neovascularization in hypoxic conditions in vivo using a mouse model of ischemia-induced angiogenesis. This was achieved by blocking adenosine receptors with caffeine in mice. Administration of caffeine almost completely abrogated the up-regulation of VEGF expression induced by direct injection of NECA into the mouse hind limb, indicating its effectiveness in antagonizing adenosine receptor-mediated angiogenic effects. Furthermore, inhibition of adenosine receptors substantially reduced (by 46%) the development of new capillaries stimulated by ischemia in this model, indicating a significant role of adenosine receptors in the angiogenic response induced by hypoxia in vivo. Although we cannot rule out the possibility that the systemic effects of caffeine could contribute to this outcome, adenosine antagonism would be expected to be more effective in the ischemic limb, where adenosine production is increased.

We would like to emphasize the fact that the proangiogenic effect of adenosine is not limited to the up-regulation of VEGF and IL-8 only. This is a complex process apparently involving regulation of several pro- and antiangiogenic factors via different adenosine receptor subtypes. We have previously demonstrated that stimulation of A_{2B} adenosine receptors results in up-regulation of several proangiogenic factors in endothelial, tumor, and mast cells (Grant et al., 1999; Feoktistov et al., 2002, 2003; Zeng et al., 2003). In particular, we have shown that HMEC-1, the cells used in the current study, functionally express A_{2B} adenosine receptors that mediate VEGF and IL-8 up-regulation (Feoktistov et al., 2002). Other adenosine receptor subtypes have also been implicated in angiogenesis. A_3 adenosine receptors were shown to induce secretion of angiopoetin-2 in tumor and mast cells, thereby potentiating the effects of VEGF (Feoktistov et al., 2003; Merighi et al., 2005). Of interest, stimulation of A_{2A} receptors down-regulated an antiangiogenic factor, thrombospondin-1, in endothelial cells, indicating another mechanism by which adenosine can regulate angiogenesis (Desai et al., 2005). It remains to be determined whether inhibition of a single adenosine receptor subtype would be sufficient to significantly reduce hypoxia-induced angiogenesis in vivo, or it could be achieved only when several adenosine subtypes are blocked as has been demonstrated in this study using caffeine, a nonselective adenosine receptor antagonist. This will require future studies using a combination of selective antagonists and genetically engineered mice lacking specific adenosine receptor subtypes.

In summary, the results of our study suggest that adenosine receptors represent a potential therapeutic target for regulation of angiogenesis. Adenosine receptors up-regulate angiogenic factors not only under normoxic but also under hypoxic conditions. Our study demonstrated that adenosine actions are not redundant but rather are complementary to the direct effects of hypoxia. Adenosine cooperates with hypoxia to stimulate VEGF secretion and induces secretion of additional angiogenic factors, e.g., IL-8, not stimulated by hypoxia alone. These factors, secreted in response to combined stimulation by hypoxia and adenosine, may then promote neovascularization and ultimately adequate tissue oxygenation.

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