

Synergistic Up-Regulation of Vascular Endothelial Growth Factor Expression in Murine Macrophages by Adenosine A_{2A} Receptor Agonists and Endotoxin

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Under normoxic conditions, macrophages from C57BL mice produce low levels of vascular endothelial growth factor (VEGF). Hypoxia stimulates VEGF expression by ~500%; interferon- γ (IFN- γ) with endotoxin [lipopolysaccharide (LPS)] also stimulates VEGF expression by ~50 to 150% in an inducible nitric oxide synthase (iNOS)-dependent manner. Treatment of normoxic macrophages with 5'-N-ethyl-carboxamido-adenosine (NECA), a nonselective adenosine A₂ receptor agonist, or with 2-[p-(2-carboxyethyl)-phenylethyl amino]-5'-N-ethyl-carboxamido-adenosine (CGS21680), a specific adenosine A_{2A} receptor agonist, modestly increases VEGF expression, whereas 2-chloro-N⁶-cyclopentyl adenosine (CCPA), an adenosine A₁ agonist, does not. Treatment with LPS (0 to 1000 ng/ml), or with IFN- γ (0 to 300 U/ml), does not affect VEGF expression. In the presence of LPS (EC₅₀ < 10 ng/ml), but not of IFN- γ , both NECA and CGS21680 synergistically up-regulate VEGF expression by as much as 10-fold. This VEGF is biologically active *in vivo* in the rat corneal bioassay of angiogenesis. Inhibitors of iNOS do not affect this synergistic induction of VEGF, and macrophages from iNOS^{-/-} mice produce similar levels of VEGF as wild-type mice, indicating that NO does not play a role in this induction. Under hypoxic conditions, VEGF expression is slightly increased by adenosine receptor agonists but adenosine A₂ or A₁ receptor antagonists 3,7-dimethyl-1-propargyl xanthine (DMPX), ZM241385, and 8-cyclopentyl-1,3-dipropylxanthine (DCPCX) do not modulate VEGF expression. VEGF expression is also not reduced in hypoxic macrophages from A₃^{-/-} and A_{2A}^{-/-}

mice. Thus, VEGF expression by hypoxic macrophages does not seem to depend on endogenously released or exogenous adenosine. VEGF expression is strongly up-regulated by LPS/NECA in macrophages from A₃^{-/-} but not A_{2A}^{-/-} mice, confirming the role of adenosine A_{2A} receptors in this pathway. LPS with NECA strongly up-regulates VEGF expression by macrophages from C₃H/HeN mice (with intact Tlr4 receptors), but not by macrophages from C₃H/HeJ mice (with mutated, functionally inactive Tlr4 receptors), implicating signaling through the Tlr4 pathway in this synergistic up-regulation. Finally, Western blot analysis of adenosine A_{2A} receptor expression indicated that the synergistic interaction of LPS with A_{2A} receptor agonists does not involve up-regulation of A_{2A} receptors by LPS. These results indicate that in murine macrophages there is a novel pathway regulating VEGF production, that involves the synergistic interaction of adenosine A_{2A} receptor agonists through A_{2A} receptors with LPS through the Tlr4 pathway, resulting in the strong up-regulation of VEGF expression by macrophages in a hypoxia- and NO-independent manner. (*Am J Pathol* 2002, 160:2231-2244)

During wound healing macrophages play a key role in the induction of new blood vessel formation by producing the angiogenic growth factor vascular endothelial cell growth factor (VEGF).¹⁻⁴ Although resting macrophages produce low levels of VEGF under normoxic conditions, hypoxia strongly stimulates VEGF production by macrophages.^{1,5} Moreover, when normoxic macrophages are stimulated with interferon (IFN)- γ and lipopolysaccharide (LPS), they also up-regulate their expression of VEGF.³ This up-regulation depends, in part, on the production of nitric oxide (NO) by the inducible NO synthase (iNOS) in activated macrophages.

In response to a variety of stimuli, including hypoxia, many different tissues and cell types release the purine

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nucleoside adenosine.^{6–8} Adenosine, acting at both internal sites and external receptors, modulates a variety of macrophage functions. Acting at an intracellular site adenosine affects the production of NO.⁹ Via occupancy of cell surface G-protein-coupled receptors adenosine modulates a variety of cellular functions, including phagocytosis; antigen presentation; target cell killing; production of IL-6, IL-10, and IL-12; and expression of MHC class II molecules.^{10–15} Four different adenosine receptors, A₁, A_{2A}, A_{2B}, and A₃ receptors, have been characterized at the molecular level.^{16–21} Ligand of adenosine receptors regulates cellular function via cAMP-dependent and -independent pathways.^{22,23} A number of different highly receptor-specific agonists and antagonists of adenosine receptors have been developed that either mimic or block the effects of adenosine.^{24–26} Adenosine mediates the anti-inflammatory effects of a number of currently available and experimental agents in the treatment of immune-mediated diseases such as rheumatoid arthritis and models of endotoxin shock, nephritis, and uveitis.^{27–30} More recently adenosine A_{2A} receptor-specific agonists have been used to diminish inflammation in animal models of inflammation.^{31–33}

Adenosine also plays a role in the promotion of angiogenesis. Adenosine applied to the chick chorioallantoic membrane induces growth of new microvascular blood vessels.³⁴ Regulation of expression of the angiogenic growth factor VEGF via adenosine receptors has been demonstrated in several cell types, including endothelial cells, smooth muscle cells, and the human U937 cell line.^{35–41} As macrophages play a key role in inducing angiogenesis, we have studied the role of adenosine receptors in mediating the production of VEGF by primary macrophages. Our observations indicate that agonists of adenosine A_{2A} receptors stimulate increased expression of VEGF by macrophages. Up-regulation of VEGF production by adenosine A_{2A} receptor ligation is synergistically enhanced by exposure of macrophages to low levels of endotoxin (LPS). Adenosine A_{2A} receptor/LPS-stimulated up-regulation of VEGF expression is at least as strong as that induced by hypoxia alone, and considerably stronger than that induced by IFN- γ with LPS. The synergistic up-regulation of VEGF expression is absent in macrophages from mice that lack the adenosine A_{2A} but not the A₃ receptor, confirming the specificity of this response. Similarly, the response is absent in macrophages from C₃H/HeJ mice that lack functional Tlr4 receptors because of a mutation in their cytoplasmic domain,⁴² indicating a critical role for the Tlr4 receptor in the signaling pathway.

Materials and Methods

Reagents

5'-N-ethylcarboxamidoadenosine (NECA), 2-[p-(2-carboxylethyl)-phenylethyl amino]-5'-N-ethyl-carboxamidoadenosine (CGS 21680), 2-chloro-N⁶-cyclopentyl adenosine (CCPA), 3,7-dimethyl-1-propargyl xanthine (DMPX),

8-cyclopentyl-1,3-dipropylxanthine (DCPCX), 8-sulphophenyltheophylline (8-SPT), U1026, SB203580, KT5720, recombinant murine interferon- γ (IFN- γ), LPS (*Escherichia coli* serotype 055:B5), aminoguanidine, and N⁹-nitro-L-arginine-methyl-ester (L-NAME) were purchased from Sigma (St. Louis, MO). CMI (4-cyano-3-methyl-isoquinoline) was purchased from Calbiochem (San Diego, CA). ZM241385 was purchased from Tocris-Cookson (Bristol, UK). Polyclonal antibodies to adenosine A_{2A} receptors were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Protease inhibitor cocktail for use with mammalian cell and tissue extracts, containing 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin-A, E-64, bestatin, leupeptin, and aprotinin was purchased from Sigma. Hydron was purchased from Interferon Sciences (New Brunswick, NJ).

Animals

C57BL and C₃H/HeJ mice (female, 7 to 12 weeks) were purchased from Jackson Laboratories (Bar Harbor, ME), C₃H/HeN mice were purchased from Charles River (Wilmington, MA). Mice with a targeted disruption of the iNOS gene (iNOS^{-/-} mice) were derived from an original homozygous breeding pair, and were kindly provided by Drs. John MacMicking and Dr. Carl Nathan (Cornell University Medical College, Ithaca, NY), and Dr. John Mudgett (Merck Research Laboratories, West Point, PA). These mice were derived from C57BL/6J \times 129Sv/Ev lines originally generated at Merck, and were housed in the University of Medicine and Dentistry of New Jersey (UMDNJ) animal facility. Mice with a targeted disruption of the gene for the adenosine A_{2A} and A₃ receptor have been described in detail elsewhere.^{43,44} The mice used in these experiments were derived from four original heterozygous breeding pairs for each mouse. Adenosine A_{2A} receptor knockout mice (A_{2A}^{-/-}) and their wild-type controls (A_{2A}^{+/+}) were bred on a C57BL/6 \times 129SvEv-STEEL background; adenosine A₃ receptor knockout mice (A₃^{-/-}) and their wild-type controls were also bred on a C57BL background. These mice were housed in the New York University (NYU) animal facility. Mice described as wild type were specific for the related knockout mice. All mice were fed regular mouse chow and given access to drinking water *ad libitum*. All procedures described below were reviewed and approved by the Institutional Animal Care and Use Committee of NYU Medical Center and the UMDNJ and performed under the supervision of the facility veterinary staff.

Polymerase Chain Reaction (PCR) Confirmation of Mouse Genotype

DNA was extracted from the tips of mouse tails using a standard protocol. Briefly, tail tips were lysed in 500 μ l of lysis buffer (100 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 8.0, 10 mmol/L ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulfate, 400 μ g/ml proteinase K) overnight at 55°C. To the lysed tips 300 μ l of a saturated solution of NaCl was added and, after 10 minutes on ice,

tubes were centrifuged ($16,000 \times g$, 4°C for 10 minutes). Genomic DNA present in the supernatant was precipitated by addition of $800 \mu\text{l}$ of isopropanol. Precipitates were washed once with 70% ethanol, vacuum dried, and resuspended in $30 \mu\text{l}$ of 10 mM Tris-1 mM EDTA (TE) buffer, pH 8.0. Using a method originally worked out by the laboratory of Dr. M. Sitkovsky (personal communication) the genomic DNA was then subjected to PCR using the following primers 5'-AGCCAGGGGTTACATCTGTG-3' (upstream) and 5'-TACAGACAGCCTCGACATGTG-3' (downstream), which detect a 163-bp band for the wild-type A_{2A} allele; and 5'-AGACAATCGGCTGCTCTGAT-3' (upstream) and 5'-CAAGCTCTTCAGCAATATCACG-3' (downstream), which detect a 618-bp band for the mutated A_{2A} allele; 5'-ACTTCTGGGCAGAAAGTCTGACAAGA-3' (upstream) and 5'-TTCGTCAACCCTGTACTGACTGT-3' (downstream), which detect a 570-bp band for the wild-type A_3 allele and 5'-ACTTCTGGGCAAGTCTGACAAGA-3' (upstream) and 5'-AGATCTATAGATCTCTCGTGGGATC-3' (downstream), which detect a 260-bp band for the mutated A_3 allele. To perform the PCR, $0.3 \mu\text{g}$ of genomic DNA was used in $30 \mu\text{l}$ of final reaction. The PCR was performed in a GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer, Branchburg, NJ) under the following conditions: 95°C for 2 minutes followed by 40 cycles (94°C for 1 minute, 55°C for 20 seconds, and 72°C for 1 minute) and a final extension of 72°C for 10 minutes.

Preparation and Treatment of Peritoneal Macrophages

Mice were injected intraperitoneally with 2.5 ml of sterile Brewer's thioglycolate broth (3% w/v) (Difco Laboratories, Detroit, MI). Four days later, the mice were sacrificed and peritoneal exudate cells were harvested using phosphate-buffered saline (PBS). Cells were centrifuged at $300 \times g$ for 5 minutes at 4°C , washed twice with serum-free medium (RPMI), and resuspended in RPMI containing 10% fetal calf serum and $50 \mu\text{g/ml}$ of gentamicin (RPMI-10% fetal calf serum). Cells were seeded into Falcon multiwell six-well tissue culture plates (2×10^6 cells/well; Becton-Dickinson Labware, Franklin Lakes, NJ) in 2 ml of medium. Dishes were then incubated at 37°C in a humidified incubator in 95% air/5% CO_2 overnight to allow the cells to adhere. Nonadherent cells were removed by washing with serum-free RPMI, and the cells were refed with RPMI containing 1% fetal calf serum (RPMI-1% fetal calf serum).

To test the effects of hypoxia, macrophages were incubated in 95% $\text{N}_2/4\% \text{CO}_2/1\% \text{O}_2$ at 37°C . To test the effects of IFN- γ and LPS, macrophages were treated with IFN- γ alone (0 to 300 U/ml), LPS alone (0 to 1000 ng/ml), or a combination of IFN- γ (100 U/ml) and LPS (100 ng/ml), and incubated under normoxic conditions (95% air/5% CO_2). The various test compounds (adenosine agonists and antagonists) were added to the macrophages within 30 minutes of the addition of IFN- γ and/or LPS. iNOS inhibitors were added before the addition of IFN- γ and/or LPS. Kinase inhibitors were added 2 hours

before addition of IFN- γ and/or LPS. Conditioned media were harvested 24 hours after the initiation of treatments. Within each experiment, each test condition was assayed in duplicate. Each experiment was performed at least three times. Results of a typical experiment are shown in each figure.

Assay of VEGF Protein Levels by Enzyme-Linked Immunosorbent Assay (ELISA)

VEGF protein in conditioned media was assayed using a sandwich ELISA kit (Quantikine M; R&D Systems, Minneapolis, MN), following the manufacturer's protocol. This assay detects murine VEGF in the range of 3 to 500 pg/ml. Samples with VEGF concentrations greater than this range were diluted with RPMI and re-assayed. All samples were assayed in duplicate. Results are presented as means \pm SD.

Assay of Nitrite Production

To determine the production of NO by the macrophages under various conditions tested, the conditioned media were analyzed for nitrite using the Griess reaction, as described previously.¹ All samples were assayed in duplicate. Results are presented as means \pm SD.

Assay of Macrophage Viability by the MTT Assay

To assess macrophage viability after incubation under the various test conditions, media were removed from each well and replaced with 1 ml of methyl thiazole tetrazolium (MTT) assay medium (RPMI-1640 without serum and phenol red, containing $50 \mu\text{g/ml}$ MTT). The plates were then incubated at 37°C for 2 hours. Medium was then removed and the cells were washed (two times) with PBS. One ml of cold 95% ethanol was then added, and the plates were incubated at 4°C for 5 minutes to allow the blue formazan product to dissolve. An aliquot ($100 \mu\text{l}$) of the solution from each well was then transferred to a 96-well plate, and absorption read at 560 nm with a 690-nm reference wavelength.

Isolation of Total Cellular RNA

Total cellular RNA was isolated from macrophages as described previously.¹ Medium was removed from the cells, and TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) was added directly to the culture dishes. The cell lysate was passed several times through a 21-gauge syringe needle. Samples were stored at room temperature for 5 minutes, 0.2 ml of chloroform was then added per ml of lysis reagent, and the mixture was vortexed for 15 seconds and then incubated at room temperature for 10 minutes. The resultant mixture was centrifuged at $12,000 \times g$ for 15 minutes at 4°C . The aqueous (upper) phase was transferred to a fresh microfuge tube, and RNA was precipitated by adding 0.5 ml of isopropa-

nol per 1 ml of TRI reagent used for the original extraction. Samples were incubated at room temperature for 5 minutes and then centrifuged at $12,000 \times g$ for 10 minutes at 4°C . The RNA pellets were washed with 75% ethanol, air-dried for 5 minutes, and dissolved in RNase-free water.

Quantitation of VEGF mRNA Steady-State Levels by Ribonuclease Protection Assay (RPA)

VEGF mRNA levels were determined by means of a RPA. A 360-bp fragment of the VEGF-coding sequence, between exon 1 and exon 4 (present in all isoforms of VEGF) was amplified by PCR, and subcloned into the PCRScript vector (Stratagene, La Jolla, CA) in an anti-sense orientation. The plasmid was linearized with *EcoRI*, and *in vitro*-transcribed with T3 RNA polymerase to obtain anti-sense RNA. The probe was labeled with $\alpha^{32}\text{P}$ -UTP (specific activity, 3000 Ci/mmol; NEN Life Science Products, Boston, MA). Specific activity of the labeled probe was 7×10^8 cpm/ μg . The probe was diluted to a concentration of 10^5 cpm/ml, and 1 ml was used for each sample in the RPA. The RPA procedure followed the manufacturer's protocol (Ambion, Austin, TX). Twenty μg of total RNA were hybridized with the labeled probe at 68°C overnight. RNaseA/T1 cocktail was diluted 1:25 in RNase digestion buffer at 65°C for 30 minutes. Samples were then run on a 6% polyacrylamide denaturing gel (GelMix-6; Life Technologies, Inc., Rockville, MD) in $1 \times$ TBE buffer at 250 V for 1.5 hours. The gel was then dried, exposed in a PhosphorImager cassette and analyzed using a PhosphorImage Analyzer (Molecular Dynamics, Sunnyvale, CA). VEGF mRNA levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (G3PDH), as a control housekeeping gene.

RT-PCR Analysis of VEGF mRNA Isoforms

RT-PCR analysis of VEGF mRNA isoforms was performed as described previously.¹ Oligonucleotide primers in exons 3 and 8 were used to amplify the regions of the three differentially spliced VEGF mRNA isoforms formed from the VEGF gene. These VEGF mRNA isoforms are derived from a gene containing eight exons. The largest, VEGF-1, is formed using all eight exons. VEGF-2 lacks exon 7, and VEGF-3 lacks exons 6 and 7. Using PCR primers in exons 3 and 8, the three different isoforms of VEGF generate PCR amplification products of different sizes, and because they amplify from the same primers, the ratio of intensities of the three bands gives an estimate of the relative abundance of the three differentially spliced mRNA isoforms.

Analysis of Adenosine A_{2A} Receptor Expression by Western Blotting

Western blot analyses were performed on cell membrane preparations after 24 hours of treatment of macrophages. Crude membrane proteins were isolated after sonication

of cells as described previously.⁴⁵ Briefly, cells were washed (two times) with phosphate-buffered saline (PBS) at 4°C and suspended in 50 mmol/L of Tris/HCl, pH 7.4, containing 1% protease inhibitor cocktail (Sigma) and 1 mmol/L of ethylenediaminetetraacetic acid. The cells were sonicated (four times) on ice for 10 seconds each. The extract was then cleared by centrifugation at $300 \times g$ for 10 minutes at 4°C . The supernatant containing crude membranes was centrifuged at $20,000 \times g$ for 20 minutes at 4°C in polycarbonate tubes. The membrane pellet was resuspended in 50 μl of RIPA buffer containing 1% protease inhibitor cocktail, and stored at -20°C until analysis. Proteins (18 μg /lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electrophoretically transferred to nitrocellulose membranes. Nonspecific antibody binding to the membrane was blocked with 3% nonfat milk in PBS containing 0.3% TWEEN-20 (PBS-T). The membranes were then incubated overnight at 4°C in primary antibody (goat polyclonal antibody against adenosine A_{2A} receptors). After several washes with PBS-T, the membranes were incubated for 1 hour at room temperature in alkaline phosphatase-conjugated secondary antibody (donkey anti-goat IgG). After several washes, the blots were exposed to a fluorescent-enhanced chemifluorescence substrate (Santa Cruz), and scanned using a FluorImage Analyzer (Molecular Dynamics, Sunnyvale, CA). Band densities were then directly quantitated using ImageQuant software (Molecular Dynamics).

Assay of Angiogenic Activity

Angiogenic activity of conditioned media from macrophage cultures was determined using the rat corneal bioassay of angiogenesis, as described previously.¹ Briefly, conditioned media were concentrated (20 times) using Amicon centrifugal spin filters (3-kd cutoff) (Amicon, Beverly, MA), and diafiltered. Aliquots of concentrated media were combined with an equal volume of slow-release Hydron (12% w/v in ethanol) and 10-ml droplets were allowed to dry and form pellets. These pellets were implanted aseptically in pockets within rat corneal stromas. Angiogenic responses were assessed 7 days after implantation. Four corneal implants were assessed per test sample, and the responses graded as previously described.¹ To determine the contribution of VEGF to the angiogenic activity, an affinity-purified neutralizing polyclonal antibody to VEGF (gift of Dr. Napoleone Ferrara, Genentech, Inc., South San Francisco, CA) was used, as previously described.¹

Statistics

Data are presented as means \pm SD. Statistical analysis of results was performed using the unpaired Student's *t*-test. Differences with a *P* value of <0.05 were considered significant.

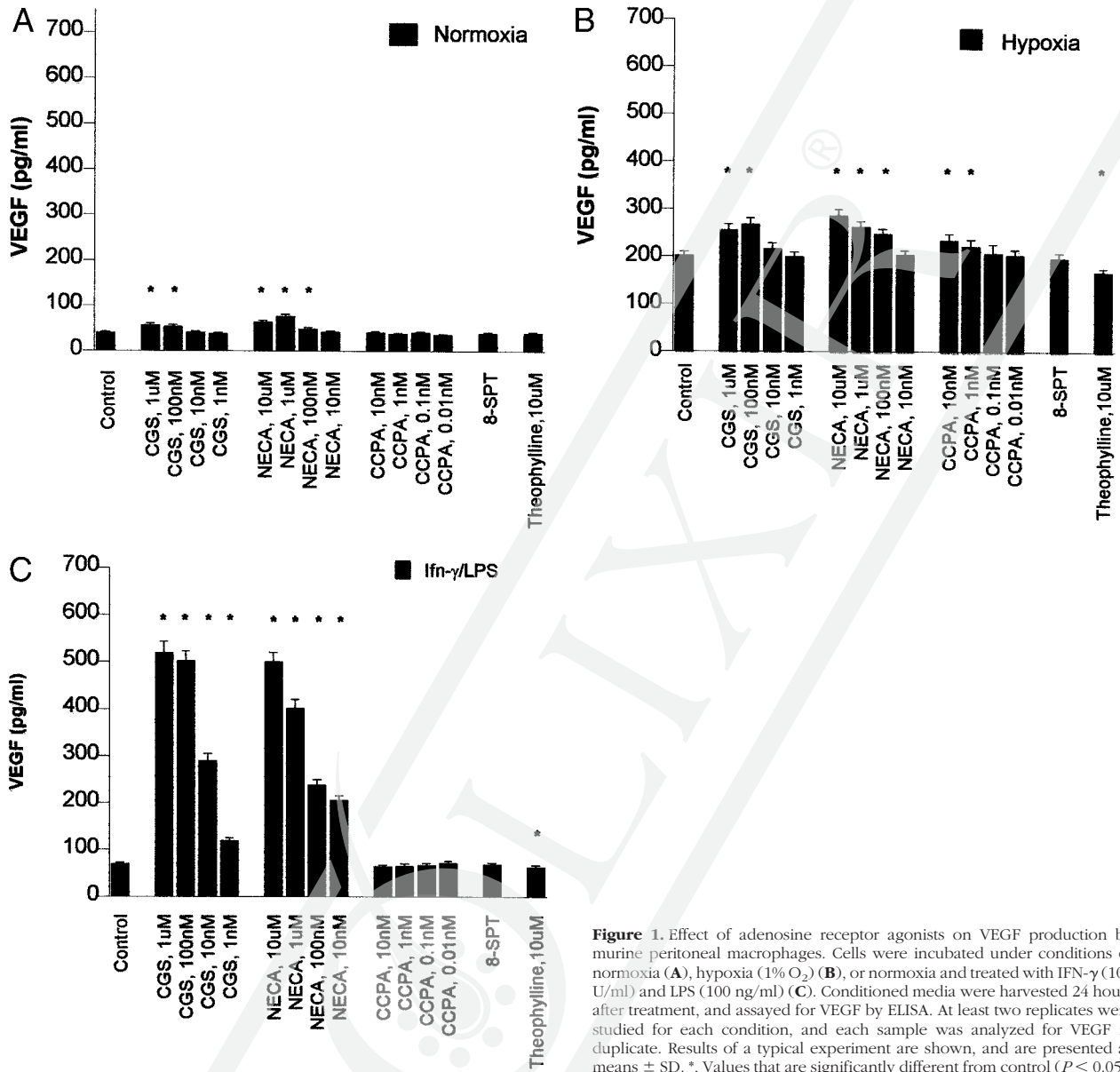


Figure 1. Effect of adenosine receptor agonists on VEGF production by murine peritoneal macrophages. Cells were incubated under conditions of normoxia (A), hypoxia (1% O₂) (B), or normoxia and treated with IFN- γ (100 U/ml) and LPS (100 ng/ml) (C). Conditioned media were harvested 24 hours after treatment, and assayed for VEGF by ELISA. At least two replicates were studied for each condition, and each sample was analyzed for VEGF in duplicate. Results of a typical experiment are shown, and are presented as means \pm SD. *, Values that are significantly different from control ($P < 0.05$).

Results

Effects of Adenosine Receptor Agonists on VEGF Production by Murine Peritoneal Macrophages

We examined the effects of a nonselective and an A_{2A}-selective adenosine receptor agonist on VEGF production by murine peritoneal macrophages under various conditions *in vitro*. Macrophages were incubated with agonists, either under normoxic conditions (95% air/5% CO₂), under hypoxic conditions (94% N₂/5% CO₂/1% O₂), or under normoxic conditions after activation with either IFN- γ alone (0 to 300 U/ml), LPS alone (0 to 1000 ng/ml), or a combination of IFN- γ (100 U/ml) and LPS (100 ng/ml).

Normoxia

Under normoxic conditions, macrophages produced low levels of VEGF. The nonselective adenosine receptor agonist NECA and the adenosine A_{2A}-specific agonist CGS21680 induced minor increases in VEGF production in a dose-dependent manner, with a maximal increase to 50 to 80% above baseline (Figure 1A). The potency of the agonists used was consistent with that expected for an adenosine A_{2A} receptor-mediated phenomenon; the EC₅₀ for NECA was <100 nmol/L, and for CGS21680 the EC₅₀ was <10 nmol/L, and CCPA, a selective adenosine A₁ receptor agonist, had no effect on VEGF production. 8-SPT and theophylline, poorly selective adenosine receptor antagonists, also had little effect on VEGF production (Figure 1A).

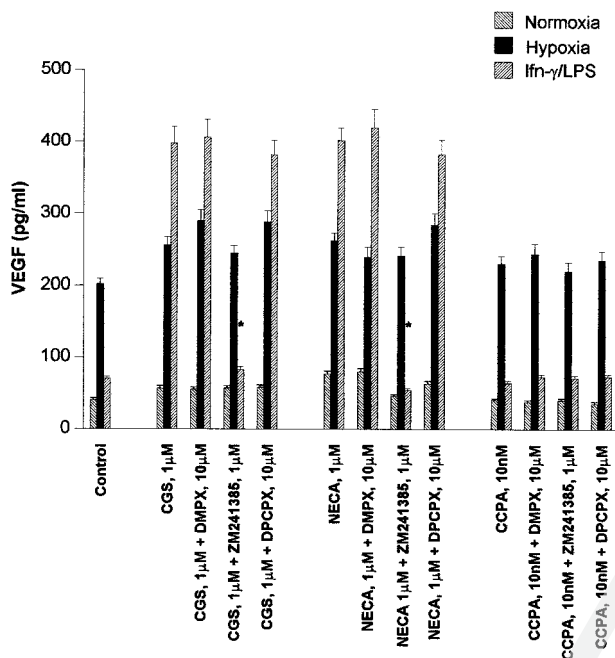


Figure 2. Effect of adenosine receptor antagonists on VEGF production by macrophages. Cells were incubated under normoxic or hypoxic conditions, and under normoxic conditions in the presence of IFN- γ and LPS. Macrophages were treated with CGS21680, NECA, or CCPA, and the adenosine receptor antagonists DMPX, ZM241384, or DPCPX were added to the cultures at the indicated concentrations. Conditioned media were harvested 24 hours after addition of test compounds, and assayed for VEGF by ELISA. At least two replicates were studied for each condition, and each sample was analyzed for VEGF in duplicate. Results of a typical experiment are shown, and are presented as means \pm SD. *, ZM241385 significantly reduced the synergistic stimulatory effects of CGS and NECA with IFN- γ and LPS on VEGF production ($P < 0.001$). DMPX and DPCPX did not significantly reduce VEGF production.

Hypoxia

Under hypoxic conditions, VEGF production increased to \sim 500% of that produced by normoxic macrophages (Figure 1B). CGS21680 and NECA further increased the production of VEGF under hypoxic conditions although the increase was modest (\sim 25% above baseline, Figure 1B). The adenosine A_2 antagonist DMPX, the A_{2A} -specific antagonist ZM241384, and the A_1 antagonist DPCPX did not significantly affect VEGF production induced by hypoxia (Figure 2), a finding that is not consistent with a role for endogenously-generated adenosine in the regulation of VEGF expression by hypoxic macrophages.

IFN- γ and LPS

Neither IFN- γ (100 μ M) nor LPS (100 ng/ml) alone stimulated increased VEGF production. However, treatment with IFN- γ in combination with LPS (IFN- γ /LPS) stimulated VEGF production by macrophages to 50 to 150% of control, as we have previously reported.¹ The effect of IFN- γ /LPS on VEGF production is considerably weaker than that of hypoxia (500% greater than normoxic cells). Surprisingly, treatment of IFN- γ /LPS-activated macrophages with adenosine analogs profoundly increased their production of VEGF (Figure 1C), increasing VEGF production by an additional 500 to 600% (Figure 1C).

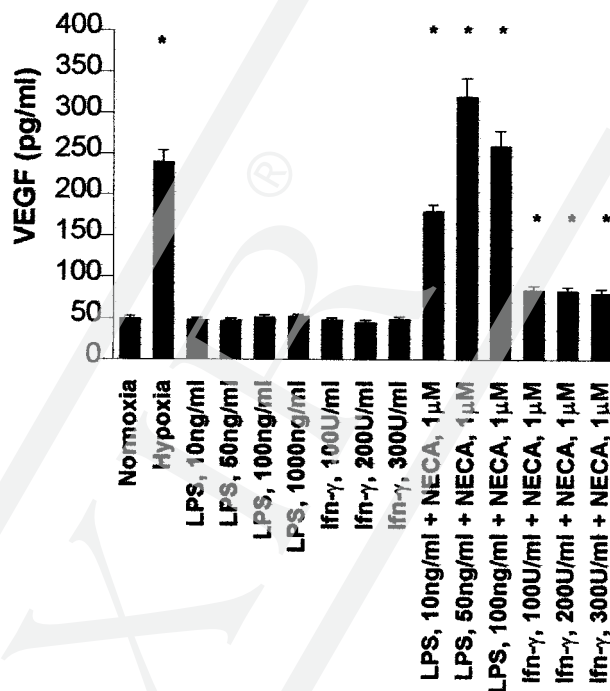


Figure 3. Effects of LPS and IFN- γ on VEGF production by murine peritoneal macrophages. Cells were incubated with the indicated concentrations of either LPS alone or IFN- γ alone, in the presence or absence of NECA (1 μ M). Media were harvested after 24 hours and assayed for VEGF by ELISA. At least two replicates were studied for each condition, and each sample was analyzed for VEGF in duplicate. Results of a typical experiment are shown and are presented as means \pm SD. *, Values that are significantly different from control ($P < 0.05$).

DPCPX, an A_1 receptor antagonist, did not antagonize the action of CGS21680 or NECA in stimulating VEGF production (Figure 2). DMPX, a nonspecific A_2 receptor antagonist had little effect, but ZM241385, a selective A_{2A} antagonist, strongly inhibited the effects of LPS/NECA and LPS/CGS on VEGF production. Alloxazine, a selective A_{2B} antagonist, had no effect on VEGF production.

The effects of adenosine receptor occupancy on macrophage VEGF production were similar in the presence of either LPS alone or LPS plus IFN- γ , but not in the presence of IFN- γ alone (Figure 3). Treatment with IFN- γ alone (0 to 300 U/ml) did not increase VEGF expression and adenosine agonists did not increase VEGF production in the presence of IFN- γ . In contrast, treatment with LPS alone (0 to 1000 ng/ml) had little effect on VEGF expression; however treatment with LPS plus either NECA or CGS21680 strongly increased VEGF expression. This effect was apparent at LPS concentrations as low as 10 ng/ml, and was maximal at 50 to 100 ng/ml ($EC_{50} < 10$ ng/ml) (Figure 3).

Role of NO in the Up-Regulation of VEGF by LPS and Adenosine A_{2A} Agonists

Effect of iNOS Inhibitors on VEGF Production

To determine whether up-regulation of VEGF by the combination of LPS and adenosine A_{2A} agonists depended on expression of NO by iNOS, macrophages

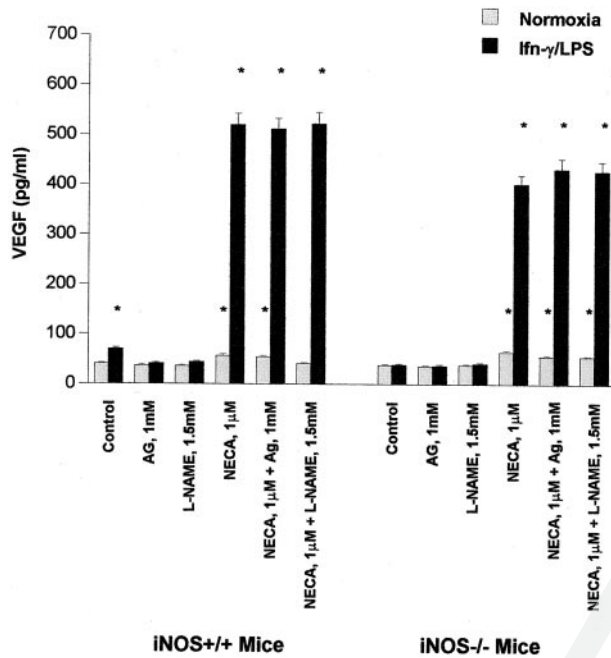


Figure 4. Effects of NO on VEGF production by murine peritoneal macrophages. Macrophages were prepared from either normal (iNOS^{+/+}) mice or mice with a targeted disruption of the iNOS gene (iNOS^{-/-}). Macrophages were incubated under normoxic conditions either with or without IFN-γ and LPS, and stimulated with CGS21680 (1 μmol/L). The iNOS inhibitors AG or L-NAME were added to the cultures at the indicated concentrations. Media were harvested after 24 hours, and assayed for VEGF by ELISA. At least two replicates were studied for each condition, and each sample was analyzed for VEGF in duplicate. Results of a typical experiment are shown and are presented as means ± SD. *, Values that are significantly different from control ($P < 0.05$).

from wild-type (iNOS^{+/+}) mice were incubated with LPS (100 ng/ml) plus NECA or CGS21680, in the presence of either L-NAME (1.5 mmol/L) or aminoguanidine (1 mmol/L), agents that strongly inhibit NO production. L-NAME and aminoguanidine reduced VEGF production by IFN-γ/LPS-treated macrophages by at least 80%. However, no effect of these inhibitors was observed on the production of VEGF by LPS/NECA-treated macrophages (Figure 4).

VEGF Production by Macrophages from iNOS^{-/-} Mice

Macrophages from iNOS^{-/-} mice did not show significant up-regulation of VEGF production after treatment with IFN-γ/LPS, in contrast to wild-type (iNOS^{+/+}) mice, which showed an ~80% increase (Figure 4). On the other hand, macrophages from iNOS^{-/-} mice showed strong up-regulation of VEGF expression after NECA/LPS treatment, comparable to that observed in iNOS^{+/+} macrophages. These results clearly indicate that while the IFN-γ/LPS-induced up-regulation of VEGF expression is NO-dependent, the LPS/adenosine A_{2A} receptor agonist-mediated up-regulation of VEGF expression is independent of iNOS and NO production.

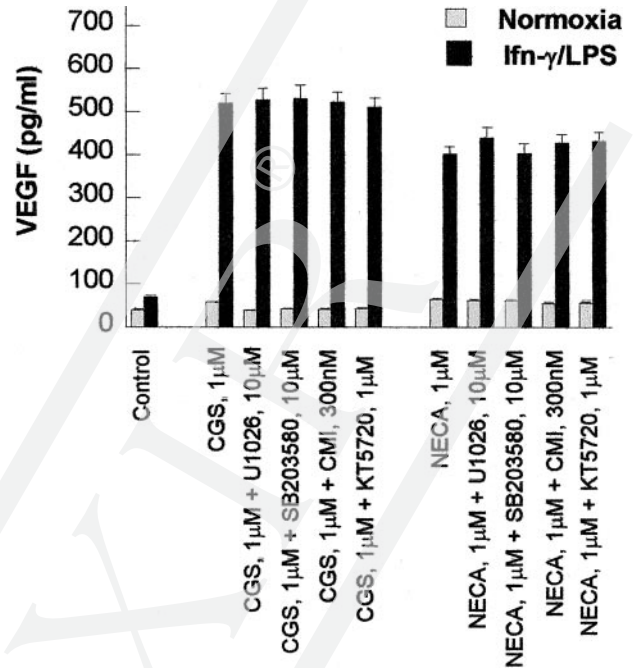


Figure 5. Effects of specific kinase inhibitors on VEGF production by murine peritoneal macrophages. Macrophages were incubated under normoxic conditions either in the presence or absence of IFN-γ and LPS, and stimulated with either CGS21680 (1 μmol/L) or NECA (1 μmol/L). The cells were treated with U1026 (a MAP-ERK kinase inhibitor), SB203580 (a p38 MAP kinase inhibitor), CMI, or KT5720 (protein kinase A inhibitors), as indicated. Media were harvested after 24 hours and assayed for VEGF by ELISA. At least two replicates were studied for each condition, and each sample was analyzed for VEGF in duplicate. Results of a typical experiment are shown, and are presented as means ± SD. The U1026-, SB203580-, CMI-, and KT5720-treated cells did not show a significant change in VEGF production in relation to the IFN-γ- and LPS-treated cells in either the CGS- or NECA-treated groups.

Effect of Specific Kinase Inhibitors on VEGF Production

Previous results have indicated that adenosine A_{2A} receptor occupancy modulates cellular function via either adenylate cyclase/protein kinase A-dependent pathways or stimulation of MAP kinase activation. To determine the signal transduction pathways involved in the LPS/A_{2A} agonist-mediated pathway VEGF up-regulation, U1026 (a MAP ERK kinase inhibitor), SB203580 (a p38 MAP kinase inhibitor), CMI, and KT5720 (protein kinase A inhibitors) were tested. The inhibitors were added to the macrophages 2 hours before stimulation with LPS and either NECA or CGS21680. None of these inhibitors significantly affected the production of VEGF by the macrophages (Figure 5). Inhibitors alone, in the absence of LPS and/or A_{2A} agonists, did not modulate VEGF production in the nonstimulated cells. These results indicate that other signal transduction pathways, yet to be determined, must be involved in the up-regulation of VEGF expression in this system.

VEGF Production By Macrophages from Adenosine Receptor-Deficient Mice

The pharmacological studies with adenosine receptor agonists presented above are most consistent with the

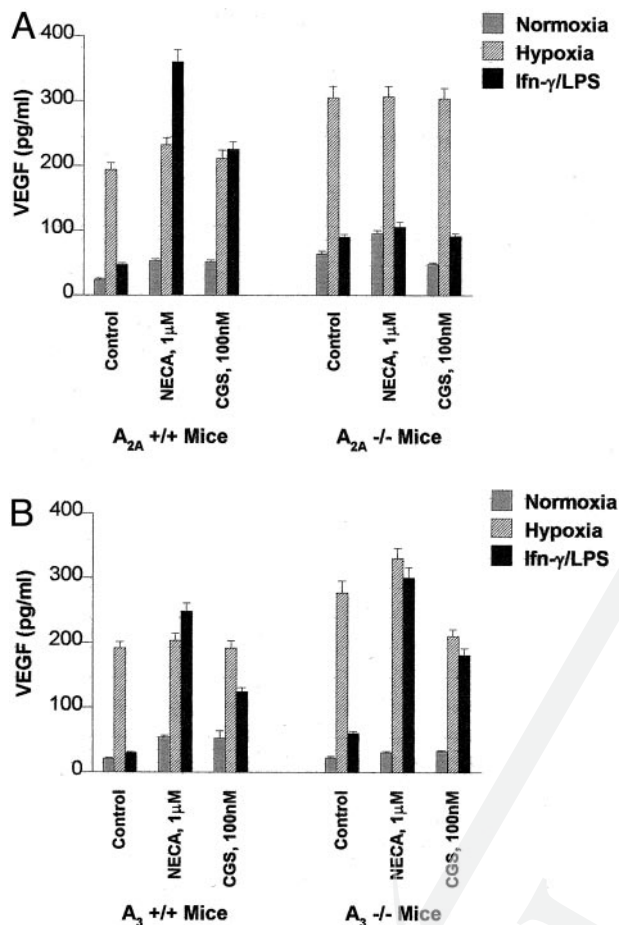


Figure 6. VEGF production by macrophages from normal mice *versus* mice with targeted disruptions in the genes for the adenosine A_{2A} or the adenosine A₃ receptors. Media were harvested after 24 hours. At least two replicates were studied for each condition, and each sample was analyzed in duplicate for VEGF by ELISA. Results of a typical experiment are shown, and are presented as means ± SD. **A:** Macrophages from control (A_{2A}^{+/+}) and A_{2A} receptor knockout (A_{2A}^{-/-}) mice were incubated under conditions of normoxia, hypoxia, or normoxia in the presence of IFN-γ and LPS. The cells were then treated with NECA (1 μmol/L) or CGS21680 (1 μmol/L). **B:** Macrophages from control (A₃^{+/+}) and A₃ receptor knockout (A₃^{-/-}) mice were treated as in **A**.

hypothesis that up-regulation of VEGF expression was mediated by signaling through the adenosine A_{2A} receptor. To test this hypothesis further, we examined production of VEGF by macrophages from adenosine receptor A_{2A} and A₃ knockout mice (A_{2A}^{-/-} and A₃^{-/-} mice). VEGF expression by normoxic and hypoxic macrophages from A_{2A}^{-/-} and A₃^{-/-} mice was similar to that observed in their wild-type littermate controls (Figure 6, A and B). These results clearly demonstrate that the adenosine A_{2A} and A₃ receptors are not involved in the hypoxic up-regulation of VEGF expression in these cells. The adenosine receptor agonist NECA in combination with IFN-γ/LPS did not up-regulate VEGF expression in macrophages from A_{2A}^{-/-} mice beyond that induced by IFN-γ/LPS-treatment alone (Figure 6A). In contrast, macrophages from A₃^{-/-} mice responded to IFN-γ/LPS and NECA in the same way as wild-type mice (Figure 6B). These results provide strong evidence that adenosine A_{2A} receptor occupancy mediates the increase in VEGF

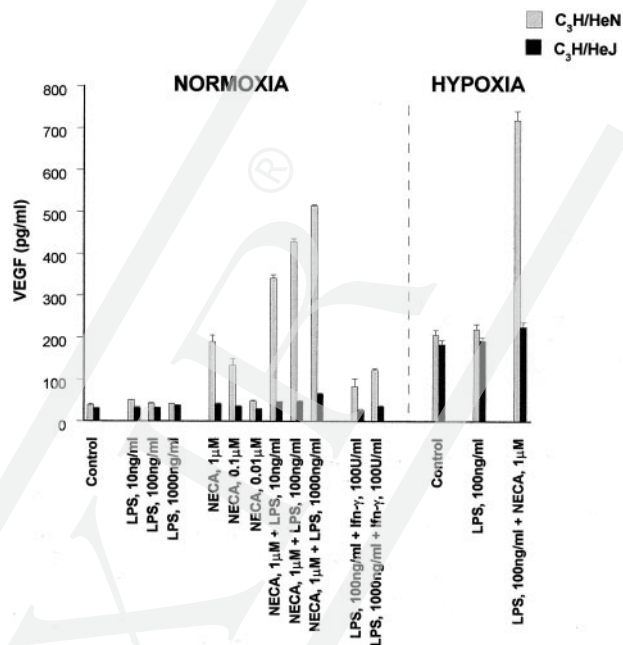


Figure 7. VEGF production by macrophages from C₃H/HeN mice (intact, functional Tlr4 receptor) *versus* macrophages from C₃H/HeJ mice (mutated, nonfunctional Tlr4 receptor). Cells were incubated under either normoxic or hypoxic conditions. Cells were treated with LPS, NECA, NECA plus LPS, or LPS plus IFN-γ, at the indicated concentrations. Media were harvested after 24 hours, and assayed for VEGF by ELISA. At least two replicates were studied for each condition, and each sample was analyzed for VEGF in duplicate. Results of a typical experiment are shown, and are presented as means ± SD.

production by IFN-γ/LPS- or LPS-stimulated macrophages.

VEGF Production by Macrophages from C3H/HeJ Mice that Lack Functional Tlr4 Receptors

LPS signaling in macrophages in terms of the induction of inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1 is mediated primarily through the CD14-Tlr4 receptor signaling pathway.⁴² However, other pathways mediated by β2 integrins,⁴⁶ scavenger receptors,⁴⁷ and L-selectin⁴⁸ have also been implicated in mediating some of the effects of LPS on macrophages. To determine the role of the Tlr4 receptor-mediated pathway in the synergistic interaction between LPS and adenosine A_{2A}-receptor agonists, we studied macrophages from C₃H/HeJ mice. C₃H/HeJ mice have a mutation in the cytoplasmic domain of Tlr4⁴² that functionally inactivates signaling by this receptor, resulting in a dominant-negative phenotype. C₃H/HeN mice contain the normal, nonmutated, functional Tlr4 receptor, and were used as controls. VEGF production by hypoxia was strongly induced in macrophages from both C₃H/HeN and C₃H/HeJ mice. Expression of VEGF by macrophages from C₃H/HeN mice was strongly up-regulated by NECA in combination with LPS (Figure 7). In contrast, macrophages from C₃H/HeJ mice did not respond to LPS and NECA beyond the response to NECA alone. This result indicates clearly that the role of LPS in the synergistic induction of VEGF with adenosine A_{2A} agonists is

mediated through the Tlr4 signaling pathway. It is of interest to note that the response of macrophages from C₃H/HeN mice to NECA was considerably stronger than that of macrophages from C57BL mice (Figure 7 *versus* Figure 1). Similarly, the C3H/HeN response was much stronger than that observed in the iNOS^{+/+} and iNOS^{-/-} mice (Figure 4), which are also bred on a C57BL background. The A_{2A}^{+/+} and A_{2A}^{-/-} mice, and the A₃^{+/+} and A₃^{-/-} mice are also bred on a C57BL background, and responded to NECA to the same extent as did C57BL mice (Figure 6).

Effects of Adenosine A_{2A} Receptor Agonists and LPS on VEGF mRNA Isoforms

Three isoforms of VEGF were produced by both nonactivated, IFN- γ /LPS-activated macrophages, and NECA/LPS and CGS/LPS-activated macrophages. These isoforms corresponded to VEGF-1 (652 bp), VEGF-2 (580 bp), and VEGF-3 (448 bp). Little change in the relative proportions of these isoforms was seen after activation (data not shown).

Effects of Adenosine A_{2A} Receptor Agonists and LPS on VEGF mRNA Steady-State Levels

VEGF steady-state mRNA levels were determined using an RPA with a 360-bp probe that recognizes all isoforms of murine VEGF. A typical example of this RPA is shown in Figure 8. LPS alone did not increase VEGF mRNA levels. NECA/LPS, on the other hand, induced a strong increase in VEGF steady-state mRNA levels. This increase was apparent by 4 hours, and peaked at 6 to 10 hours. By 24 hours, VEGF steady-state mRNA decreased to close to baseline levels.

Analysis of Adenosine A_{2A} Receptor Expression by Western Blotting

To determine whether treatment of macrophages with LPS up-regulates the expression of adenosine A_{2A} receptors in macrophages, the level of expression of A_{2A} receptors was determined by Western blot analysis. Crude membrane fractions were prepared from untreated macrophages, and from macrophages after overnight treatment with either LPS alone (100 ng/ml), NECA (1 μ mol/L), or LPS (100 ng/ml) plus NECA (1 μ mol/L). Equal amounts of protein from the crude membrane preparations were electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, transferred to nitrocellulose membranes, and the adenosine A_{2A} receptors were localized and quantified using a specific polyclonal antibody to adenosine A_{2A} receptors. A typical Western blot showing the results of this analysis is shown in Figure 9. The adenosine A_{2A} receptor localized as a single band with an apparent molecular size of 44 kd. Expression of A_{2A} receptors was virtually identical in untreated and LPS-treated macrophages. These results indicate that the synergistic up-regulation of VEGF expression by LPS

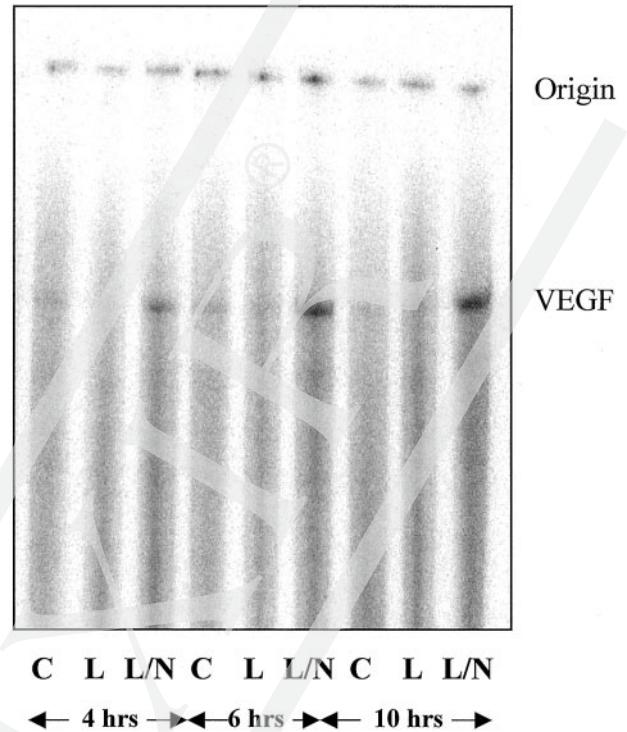


Figure 8. Ribonuclease protection assay (RPA) of VEGF mRNA levels in normoxic control (C), LPS-treated (100 ng/ml) (L), and LPS (100 ng/ml)- plus NECA (1 μ g/ml)-treated (L/N) murine peritoneal macrophages. Macrophages were harvested 4, 6, and 10 hours after stimulation. RNA was isolated and RPA was performed as described in Materials and Methods. The probe used to detect VEGF mRNA is a 360-bp anti-sense probe spanning exons 1 to 4, and detects all known isoforms of murine VEGF. Similar results were found in three separate experiments.

and adenosine A_{2A} receptor agonists is not simply because of the up-regulation of expression of adenosine A_{2A} receptors by LPS.

Angiogenic Activity of Macrophage-Conditioned Media

To determine whether the VEGF produced by macrophages treated with NECA (1 μ mol/L), LPS (100 ng/ml), or LPS (100 ng/ml) plus NECA (1 μ mol/L) is biologically

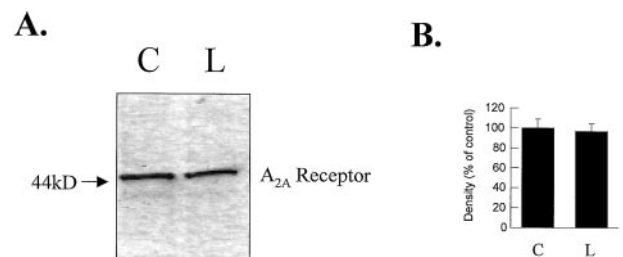


Figure 9. Western blot analysis of adenosine A_{2A} receptor expression. Crude membrane proteins were isolated from untreated and treated macrophages, as described in Materials and Methods, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred to nitrocellulose membranes and A_{2A} receptor expression was assessed using a polyclonal antibody against A_{2A} receptors. **A:** Representative Western blot for adenosine A_{2A} receptor protein. C, Untreated cells; L, LPS (100 ng/ml)-treated cells. **B:** Quantitation of protein expression by densitometric analysis of Western blots. Data are expressed as mean percentage of control \pm SD ($n = 3$). *, $P < 0.05$.

Table 1. Angiogenic Responses Induced in Rat Corneas by Conditioned Media from Macrophages Cultured under Various Conditions *in Vitro*

Group	Macrophage culture conditions*	Angiogenic score [†]
1	Normoxia	1
2	Hypoxia	9
3	NECA (1 μ mol/L) alone	5
4	LPS (100 ng/ml) alone	1
5	NECA (1 μ mol/L) + LPS (100 ng/ml)	12
6	rVEGF165 (20 ng)	11
7	Group 5 + anti-VEGF Ab (10 μ g/ml)	2
8	Group 6 + anti-VEGF Ab (10 μ g/ml)	2

*Macrophages were incubated for 24 hours under the indicated conditions, concentrated (20 \times) and diafiltered using Centricon 3 (3000 Mr cutoff) filters (Amicon). Samples were then combined with equal volumes of Hydron (Interferon Sciences Inc.). Ten μ l droplets were then allowed to dry on the cut ends of 2-mm diameter Teflon rods. These pellets were then implanted aseptically in the corneas of rats.

[†]Angiogenic responses were evaluated 7 days after implantation, and assessed on a graded scale. 0, No response, or slight budding of the limbal vasculature that regresses rapidly; 1, formation of a few capillary buds and sprouts that progress less than 0.2 mm from the limbus and start to regress; 2, persistent growth of a network of capillary buds and sprouts that grow at least 1 mm toward the implant, but do not reach and invade the implant; 3, strong growth of a network of capillary buds and sprouts that reaches and surrounds the implant. The angiogenic score represents the sum of the graded angiogenic responses from four individual corneas for each test sample. A maximal response would score 12, a minimal response 0.

active, conditioned media from the macrophage cultures were concentrated (20 times), diafiltered, and assayed for angiogenic activity using the rat corneal bioassay. Results are presented in Table 1. Medium from untreated macrophages did not induce angiogenesis. Medium from LPS-treated macrophages also failed to induce an angiogenic response. Medium from NECA-treated macrophages induced a mild angiogenic response, whereas medium from LPS/NECA-treated macrophages induced a strong angiogenic response. In both NECA-treated and LPS-NECA-treated macrophage-conditioned media, a neutralizing antibody to murine VEGF abrogated the angiogenic activity.

Discussion

Vascular endothelial growth factor (VEGF) is an endothelial cell mitogen and permeability factor that plays an important role in the regulation of angiogenesis in embryonic development, wound healing, inflammatory fibrosis, and the growth of solid tumors.^{49–55} VEGF is produced by several different cell types, and its expression is regulated by hypoxia via the expression of an oxygen-regulated transcription factor, HIF-1 α .^{56–58} We have shown previously that macrophages produce VEGF and that expression of VEGF by macrophages is strongly regulated by hypoxia.¹ VEGF expression is also regulated in macrophages by an iNOS-dependent pathway after activation by IFN- γ and LPS. NO-dependent regulation of VEGF expression has also been demonstrated in other cell types.^{59–61} In macrophages, iNOS-dependent up-

regulation of VEGF production is weaker than that observed with hypoxia. It has been suggested that this iNOS-dependent pathway is mediated at least in part through the hypoxia response element in the VEGF upstream promoter region that responds to the HIF-1 transcriptional regulator.⁶⁰

The purine nucleoside adenosine is produced and released into the extracellular milieu by cells under normoxic conditions, and its production is markedly increased under stress conditions such as hypoxia and ischemia, as a result of increased ATP turnover.^{6,7} Adenosine, acting at its receptors, is a potent immunomodulator, exerting effects both intracellularly (after uptake via specific transporters) and extracellularly via interaction with four different types of G-protein-coupled cell surface receptors, termed the A₁, A_{2A}, A_{2B}, and A₃ adenosine receptors. Adenosine up-regulates IL-10 and down-regulates IL-12 production in murine and human macrophages through the A_{2A} receptor.¹⁵ Adenosine also regulates TNF- α , IL-8, NO, free radical and arachidonic acid metabolite production in macrophages and human monocytes,^{9–15} and IL-6 expression in epithelial cells.⁶² In addition to its anti-inflammatory effects adenosine A_{2A} receptor ligation promotes more rapid wound closure in murine models.⁶³ Promotion of VEGF release by adenosine may play an important role in the promotion of wound healing as well as the anti-inflammatory effects of A_{2A} receptor agonists.

Previous studies have also implicated adenosine receptors in the regulation of VEGF expression in various cell types.^{35,36,39–41,64,65} In PC12 pheochromocytoma cells, the adenosine A_{2A} receptor agonist CGS21680 markedly depressed VEGF expression.⁶⁴ In bovine microvascular retinal endothelial cells, on the other hand, adenosine induced VEGF expression through the adenosine A_{2A} receptor, and activation of protein kinase-A was implicated in this induction.³⁹ In porcine brain-derived microvascular endothelial cells, the adenosine receptor antagonist 8-phenyl theophylline (8-PT) reduced the hypoxia-induced expression of VEGF by PK-C-dependent and -independent pathways.^{35,40} In bovine retinal pericytes and endothelial cells, adenosine induced VEGF expression via A_{2A} receptors through a PK-A-dependent pathway. In rat aortic smooth muscle cells, adenosine up-regulated VEGF expression 2.2-fold, and this up-regulation was independent of hypoxia-induced up-regulation of VEGF.³⁷ In canine myocardial vascular smooth muscle cells, adenosine A₂ agonists also stimulated VEGF protein levels by 51 to 132% and VEGF mRNA levels by 44 to 90%.³⁶ In a human macrophage cell line (U937 cells), adenosine A₂ receptor antagonists inhibited hypoxic induction of VEGF mRNA, suggesting that in these cells hypoxic induction of VEGF mRNA is mediated, at least in part, by endogenously released adenosine.⁴¹ Consistent with the capacity of A_{2A} receptor occupancy to promote VEGF expression, NECA also up-regulated VEGF mRNA in U937 cells. Interestingly, in U937 cells both hypoxia and adenosine increased VEGF mRNA expression posttranscriptionally by enhancing the stability of VEGF mRNA. Thus in most cell types studied, adenosine, acting usually at A_{2A} receptors, promotes

VEGF production and may play a role in hypoxia-induced VEGF release.

Normoxic macrophages treated with the adenosine A_{2A} -specific agonist CGS21680, showed a marked increase (50 to 150%) in their expression of VEGF. NECA, a nonselective adenosine A_2 receptor agonist, also stimulated VEGF production of normoxic macrophages. The adenosine A_1 receptor antagonist DPCPX had no effects on this VEGF production. The nonspecific adenosine A_2 receptor antagonist DMPX also had little effect, but the adenosine A_{2A} -specific antagonist ZM241385 strongly inhibited VEGF production. Alloxazine, an A_{2B} -specific antagonist also had no effect on VEGF production (data not shown). The concentrations of adenosine receptor agonists and antagonists required to stimulate or block VEGF production, as well as their relative potencies, were most consistent with occupancy of an A_{2A} receptor. This was clearly confirmed by the observation that the adenosine receptor agonists did not stimulate any increase in VEGF expression in A_{2A} receptor knockout mice, but stimulated a marked increase in VEGF expression in the wild-type littermate controls and the A_3 receptor-defective mice.

Whereas CGS21680 and NECA stimulated VEGF production in normoxic macrophages, this effect was greatly enhanced in the presence of concentrations of LPS that alone, did not stimulate any VEGF production. In the presence of LPS ($EC_{50} < 10$ ng/ml) the adenosine A_{2A} receptor agonists increased VEGF production by as much as 12-fold more than that produced by nontreated macrophages. Interestingly, IFN- γ played no role in stimulating this increased VEGF production, as demonstrated by the observation that treatment of macrophages with IFN- γ alone (100 to 300 U/ml), either with or without adenosine A_{2A} agonists had no effect on VEGF production beyond that of the agonists alone. We have recently observed that TNF- α and IL-1, agents that like LPS, provoke translocation of nuclear factor- κ B to the nucleus, increase expression and function of A_{2A} receptors, but IFN- γ diminishes both expression and function of the A_{2A} receptors. In other studies IFN- γ has been shown to up-regulate the expression of the adenosine A_{2B} receptor on macrophages, and this has been proposed as a mechanism for macrophage deactivation.¹¹ In our experiments, the up-regulation of VEGF expression seems to be mediated through the A_{2A} receptor rather than the A_{2B} receptor, and this effect is independent of IFN- γ . When IFN- γ (100 U/ml) was used together with LPS (100 ng/ml), in the presence of NECA or CGS21680, the up-regulation of VEGF expression was equivalent to that observed in cells treated with LPS alone. Also, increasing concentrations of IFN- γ (up to 300 U/ml) did not block the stimulatory effects of LPS together with NECA or CGS (data not shown). Thus, the enhancement of A_{2A} receptor expression and function stimulated by LPS is greater than any diminution induced by IFN- γ .

VEGF steady-state mRNA levels increased several fold 4 to 10 hours after treatment of macrophages with LPS (100 ng/ml) together with NECA (1 μ mol/L) in comparison to cells treated with LPS alone (Figure 8). This increase is comparable to or more than that observed after hypoxic

incubation of macrophages, which is mediated through the HIF-1 transcription factor. However, we do not yet know if this increase is mediated transcriptionally or post-transcriptionally. Preliminary results using a VEGF promoter construct regulating the expression of luciferase in an expression plasmid transfected into RAW264.7 cells (a macrophage cell line), indicate that whereas hypoxia and IFN- γ /LPS strongly up-regulate luciferase expression under the control of the VEGF promoter, LPS/NECA does not (data not shown). This result suggests that VEGF expression is regulated at least in part by posttranscriptional up-regulation.

Our results also suggest that the adenosine A_{2A} -mediated pathway does not play a significant role in the induction of VEGF by hypoxia and that the hypoxia-mediated and the LPS/ A_{2A} receptor-mediated pathways of induction of VEGF are independently regulated. Hypoxia induced a >500% increase in VEGF expression, and the adenosine receptor antagonists DPCPX, DMPX, alloxazine, and ZM241385 did not significantly reduce this induction. Also, in both $A_{2A}^{-/-}$ and $A_3^{-/-}$ mice, hypoxic up-regulation of VEGF expression was the same as that in control, wild-type mice, indicating that neither of these receptors plays a role in the hypoxic induction of VEGF expression. It will be of interest to determine whether the adenosine A_{2A} receptor/LPS-mediated pathway of VEGF induction also involves the hypoxia response element and HIF1, or whether this is an independent regulatory mechanism controlling VEGF expression. The induction of VEGF by IFN- γ together with LPS that we have reported previously was found to require at least in part, an iNOS-dependent pathway.¹ This was confirmed in the current experiments using macrophages from mice with a targeted disruption of the iNOS gene ($iNOS^{-/-}$). In these macrophages, IFN- γ /LPS does not induce VEGF expression. In contrast, LPS together with adenosine A_{2A} receptor agonists strongly induced VEGF expression in macrophages from $iNOS^{-/-}$ mice, to the same extent as that observed in macrophages from wild-type ($iNOS^{+/+}$) mice. Similarly, inhibitors of iNOS (L-NAME and aminoguanidine) had no effect on the LPS/NECA-induced VEGF production in macrophages from $iNOS^{+/+}$ mice, confirming the lack of dependence of this induction on iNOS and NO.

Induction of cytokines such as TNF- α and IL-1 by *E. coli* LPS is mediated through the CD-14-Tlr4 receptor-signaling pathway.⁴² However, LPS signaling through β 2 integrins,⁴⁶ scavenger receptors,⁴⁷ and L-selectin⁴⁸ has also been demonstrated. In this study, we used C_3H/HeJ mice that lack a functional Tlr4 receptor to study the role of Tlr4 in the synergistic up-regulation of VEGF expression by LPS and adenosine A_{2A} receptor agonists. In C_3H/HeJ mice, NECA/LPS did not induce VEGF expression beyond that induced by NECA alone, whereas in C_3H/HeN mice (with intact Tlr4 receptors), strong up-regulation of VEGF was observed. This clearly indicates that *E. coli* LPS signals through the Tlr4 pathway in this synergistic up-regulation. It will clearly be of interest to determine whether agonists of other Tlr receptors can also synergize with adenosine A_{2A} receptor agonists to up-regulate specific gene expression in macrophages or

other cell types. Interestingly, macrophages from C₃H/HeN mice show a stronger response to adenosine A_{2A} receptor agonists than do macrophages from C57BL mice or those bred on a C57BL background. This suggests that there are differences in the sensitivity of macrophages from different mouse strains to adenosine A_{2A} agonists. The basis for this differential sensitivity is as yet unclear.

The signal transduction pathways involved in the synergistic up-regulation of VEGF expression by LPS together with A_{2A} receptor agonists are not yet elucidated. We have investigated whether treatment of macrophages with LPS might up-regulate expression of adenosine A_{2A} receptors, thus synergistically increasing the response of the cells to adenosine A_{2A} receptor agonists. Western blot analysis indicated that this is not the case. The level of expression of adenosine A_{2A} receptor protein was found to be similar in untreated and in LPS-treated macrophages (Figure 9), suggesting that mechanisms other than the regulation of the level of receptor expression are involved in this synergistic interaction. In terms of signal transduction pathways involved in the downstream signaling, initial experiments indicate that U0126, a specific inhibitor of MEK1 and MEK2 (MAP kinase kinase), and SB203580, a p38 MAP kinase inhibitor, had no effect on VEGF production in this system. In addition 4-cyano-3-methyl-isoquinoline and KT5720, both specific inhibitors of PK-A, also had no effect on VEGF production, suggesting that the pathway of VEGF induction is PK-A-independent. Further experiments are required to elucidate the signal transduction pathways involved in this induction of VEGF expression.

The regulation of macrophage VEGF expression through the LPS/adenosine A_{2A} receptor-mediated pathway may be an example of an alternative pathway of macrophage activation, different from the classic IFN- γ /LPS-mediated pathway,⁶⁶ and may play a key role in the local, microenvironmental regulation of macrophage phenotype. As reviewed by Gordon⁶⁷ recently, activation of macrophages with IFN- γ /LPS results in an inflammatory, cytotoxic phenotype, with the up-regulation of expression of reactive oxygen species, IL-1, TNF- α , and iNOS. In contrast, the adenosine A_{2A} receptor-mediated activation results in an angiogenic, healing phenotype, with down-regulation of IL-12, TNF- α , iNOS, and reactive oxygen species, and the up-regulation of VEGF expression. This pathway is strongly enhanced by synergy with LPS. It will be interesting to determine what other factors are regulated through this LPS/adenosine receptor-mediated pathway, and the relationship of these factors to the angiogenic, healing phenotype apparent in these experiments.

In summary, we have identified a novel pathway of transcriptional up-regulation of VEGF expression in macrophages. This pathway involves a synergistic interaction between signaling through the adenosine A_{2A} receptor and LPS. Macrophages from mice lacking the adenosine A_{2A} receptor do not respond to LPS with adenosine receptor agonists with increased VEGF production, clearly indicating the critical role of the A_{2A} receptor in this pathway. In addition, macrophages from C₃H/HeJ mice,

which lack functional Tlr4 receptors, do not respond, indicating a critical role for the Tlr4 receptor in this pathway. This pathway is independent of hypoxia and NO production. We have previously demonstrated that application of topical adenosine A_{2A} receptor agonists promotes wound healing,⁶³ and the observation that occupancy of adenosine A_{2A} receptors stimulates VEGF production synergistically with LPS through the Tlr4 signaling pathway may be critical for this response.

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