Regulation of Vascular Endothelial Growth Factor Gene Expression in Murine Macrophages by Nitric Oxide and Hypoxia

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Vascular endothelial growth factor (VEGF) expression in murine peritoneal macrophages is strongly upregulated by hypoxia via transcriptional and posttranscriptional mechanisms. Interferon-γ (IFN-γ) with *Escherichia coli* lipopolysaccharide (LPS) also upregulates expression of VEGF, as well as of the inducible nitric oxide synthase (iNOS). Hypoxia (1% O_2) upregulates VEGF expression in macrophages from both wild-type and iNOS knockout mice, indicating that hypoxic upregulation of VEGF is independent of iNOS. However, the iNOS inhibitor aminoguanidine (AG) decreases the VEGF expression induced by LPS/IFN-γ, indicating an important role for NO. NO-dependent induction of VEGF is strongly dependent on cell density. LPS/IFN-γ treatment induces minimal VEGF protein expression in macrophages cultured at low cell densities (<0.25 × 10^6 cells/cm^2); at higher cell densities (>0.25 × 10^6 cells/cm^2) that lead to conditions of pericellular hypoxia, however, induction of VEGF expression was strong. Transient transfection of RAW 264.7 cells with luciferase reporter constructs of the murine VEGF promoter indicates that both hypoxia and LPS/IFN-γ independently induce VEGF promoter activity, irrespective of cell density. Although LPS/IFN-γ treatment induces transcriptional activation of the VEGF promoter, significant levels of VEGF protein are only expressed by cells at high density under conditions of pericellular hypoxia. This suggests an important regulatory role for hypoxia at the posttranscriptional level. Deletion analysis of the VEGF promoter shows that the hypoxia response element region and its immediate flanking sequences are essential for both hypoxia and LPS/IFN-γ-induced VEGF promoter activation. Exp Biol Med 228:697–705, 2003

Key words: macrophages; nitric oxide; hypoxia; vascular endothelial growth factor; cellular activation

Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen and a potent angiogenic factor. VEGF plays an important role in normal vascular development and in angiogenesis during embryonic development, wound healing, solid tumor growth, and certain chronic inflammatory diseases (1). Expression of VEGF is regulated at the transcriptional as well as the posttranscriptional level (2, 3). Macrophages play an important role in regulating angiogenesis and they produce VEGF in a tightly regulated manner (4). In wounds and solid tumors, macrophages are exquisitely sensitive to the microenvironment. Several *cis*-acting elements, including the hypoxia response element (HRE) and binding sites for transcription factors, including AP-1, AP-2, NF-κB, and SP-1, are present in the murine VEGF promoter (5). These elements are involved in the transcriptional activation of VEGF gene expression by numerous effectors, including hypoxia and growth factors and cytokines such as TGF-α, TGF-β, IL-1β, and IL-6 (6). Hypoxia has been shown to be an important factor that induces VEGF expression. Hypoxic upregulation of VEGF expression occurs at the transcriptional level (7–9), as well as at the posttranscriptional level (5). In hepatoma cells, hypoxic induction of VEGF is mediated by hypoxia inducible factor-1 (HIF-1) via its binding to the HRE in the VEGF promoter (2). However, in human epithelial cells and rat glial tumor cells, hypoxia upregulates VEGF expression posttranscriptionally by stabilization of mRNA (3, 10, 11). It has been reported recently that AP-1 DNA-binding activity is induced under acidic extracellular pH leading to increased VEGF expression in human glioblastoma cells (12). In macrophages, hydrogen peroxide induced VEGF promoter activity (13). Nitric oxide (NO) has also been shown to play a role in VEGF gene expression (14, 15). VEGF expression was either upregulated or downregulated by NO, depending on the cell type or the experimental conditions. NO triggered enhanced induction of VEGF in cultured keratinocytes (HaCaT) and *in vivo* during cutaneous wound repair in mice (15). Exogenous addition of NO donors or increased levels of endogenous NO enhanced VEGF synthesis in rat vascular smooth muscle cells

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(16, 17). However, in rat aortic smooth muscle cells, the NO donor *S*-nitrosoglutathione inhibited hypoxia-induced VEGF expression (18). NO was also shown to induce HIF-1 α (19). On the other hand, NO donors inhibited the DNA-binding activity of HIF-1 α (20) and hypoxic induction of the EPO gene (21, 22). NO induced VEGF gene transcription in glioblastoma and hepatoma cells (23, 24). Activation of VEGF transcription by NO was found to involve the HIF-1α-binding site and HIF-1 ancillary sequence (HAS) site within the HRE. AP-1 binding potentiated the HIF-1α-mediated hypoxia-induced transcriptional activation of the VEGF promoter in C6 glioma cells (25). Mutation in the AP-1 site just downstream of the HRE of the VEGF promoter partially inhibited the VEGF promoter activity, indicating that AP-1 plays a role in hypoxia- and NO-induced VEGF expression (24).

Although there are several reports on the role of hypoxia and NO in regulating VEGF gene expression, data on regulation of VEGF in macrophages are sparse. Macrophages play a key role in induction of angiogenesis, which is crucial for wound healing and solid tumor development, and express VEGF in response to both hypoxia and a combination of LPS and IFN-γ (14). LPS/IFN-γ treatment strongly upregulates expression of inducible NO synthase (iNOS) in macrophages (26). Because conditions of high cell density have been shown to create pericellular hypoxia (27), we investigated whether in murine peritoneal macrophages VEGF induction by LPS/IFN-γ might involve an interaction between NO and cell density-dependent pericellular hypoxia.

Materials and Methods

Reagents. *Escherichia coli* LPS and iNOS inhibitor, aminoguanidine (AG), were purchased from Sigma Chemical (St. Louis, MO). Murine recombinant IFN-γ was obtained from Invitrogen Life Technologies (Carlsbad, CA).

Animals. C57BL/6J mice (female, 7-8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice with a targeted disruption of the iNOS gene ($iNOS^{-/-}$ mice) were derived from an original homozygous breeding pair, and were kindly provided by Dr. John MacMicking and Dr. Carl Nathan (Cornell University Medical College, Ithaca, NY) and Dr. John Mudgett (Merck Research Laboratories, Rahway, NJ). These mice were derived from C57BL/6J × 129Sv/Ev lines originally generated at Merck, and were housed in the UMDNJ animal facility. The protocols were approved by the NJMS Animal Care and Use Committee.

Cell Culture. Mouse peritoneal macrophages were harvested using a procedure described earlier (14) with some modifications. C57BL/6J mice (7-8 weeks old) were injected intraperitoneally with 2.5 ml of thioglycollate broth, and 4 days later, peritoneal macrophages were harvested and cultured as a monolayer in RPMI 1640 medium (Cellgro; Mediatech Inc., Herndon, VA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Calabasas, CA), 2 m*M* L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Irvine Scientific, Santa Ana, CA). The cultures were found to be >98% pure as assessed by nonspecific esterase staining and staining with the macrophage specific F4/80 MAb. RAW 264.7 cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained in RPMI 1640 medium supplemented as described above. The cells were grown at 37°C in a humidified incubator in 5% CO_2 and 95% air.

Experiments with peritoneal macrophages were performed either in 24-well polystyrene plates, 60-mm polystyrene dishes, or 60-mm Permanox dishes (Nunc) at cell densities of 0.5×10^6 cells/cm^2, 0.25×10^6 cells/cm^2, or 0.125×10^6 cells/cm^2. Permanox dishes are made of a special polymer that allows rapid gas exchange (28). All experiments were performed at a fixed concentration of 1×10^6 cells/ml medium. Cells were plated 20 to 24 hr before stimulation. The medium used for stimulation contained 1% fetal bovine serum and cells were stimulated by hypoxia, a combination of LPS (100 ng/ml) and IFN-γ (100U/ml), or by a combination of LPS, IFN-γ, and AG (1.5 m*M*). Hypoxic conditions were obtained by placing the cells in a hypoxia chamber (Billups-Rothenberg, Del Mar, CA). The chamber was filled with a gas mixture of 1% O_2, 5% CO_2, and 94% N_2. The sealed chamber was then placed in a 37°C incubator. Conditioned media were harvested 24 hr after stimulation and were stored at −20°C.

VEGF Assay. VEGF levels in macrophage-conditioned media were assayed using Quantikine M murine VEGF ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Each sample was assayed for VEGF in duplicate, and results are presented as means ± SD.

Nitrite Assay. Nitrite levels were used as a measure of NO released into the conditioned media. Nitrite assay was performed using the Greiss reaction as described previously (14). Each sample was assayed for nitrite in duplicate, and results are presented as means ± SD.

Methyl Thiazole Tetrazolium (MTT) Assay. At the end of each experiment, the viability of the cells was assessed by MTT assay as described previously (29).

Plasmids and Constructs. pGL3-basic (promoterless) luciferase vector (Promega, Madison, WI) was used to prepare reporter constructs with a series of 5′-end deletions of the mouse VEGF promoter (GenBank TM/EMBL accession number U41393) (5). A 1091-bp fragment of the mouse VEGF promoter region (−975 to +116) and a series of its 5′-end deletions to −923, −857, −641, −107, and −40 bp were generated by PCR, using oligonucleotide primers designed with sites for the restriction enzymes Nhe I (forward primer) and Bgl II (reverse primer), and the PCR products were cloned into the Nhe I and Bgl II sites of the luciferase vector. The vector containing the 1091-bp fragment is designated pLUC-VEGF-975. The deletions from this promoter were designed to sequentially eliminate specific transcription factor-binding elements, namely the HRE (pLUC-

VEGF-923), AP-1 (pLUC-VEGF-857), AP-2 (pLUC-VEGF-641), NF-κB (pLUC-VEGF-107), and Sp-1 (pLUC-VEGF-40) (Fig. 5). The forward PCR primers used were as follows: −975: GCGCTAGCCTCTGTCTGCCCAGCAGT TGT; −923: GCGCTAGCCACTCCCCGCCACTGAC TAAC; −857: GCGCTAGCCCGTTCTCAGTGCCA CAAATT; −641: GCGCTAGCGTGTGTATGTCAGAAA CACGC, −107: GCGCTAGCGAAAGGCGGTGCCTG GCTCCA; and −41: GCGCTAGCTAGATTTCCTC TTTTTCTTTTTCTTCC. The reverse primer was GCGCA GATCTGTCCGCTGATAGTCTGCCTTG. All PCR products were sequenced and confirmed to be identical to published sequences within the mouse VEGF promoter.

Transient Transfections and Reporter Assays. To obtain endotoxin-free DNA for transfections, all plasmids were prepared using Endofree plasmid maxiprep kit (Sigma). RAW 264.7 cells were plated at a density of 4 × 10^6 cells/dish in 100-mm dishes. After a 22-hr incubation, cells were washed (×3) with phosphate-buffered saline (PBS). Transient transfections were carried out using Superfect (Qiagen, Valencia, CA) following the manufacturer's guidelines. DNA (10 μg) was diluted in 1 ml of serum-free RPMI 1640 and was incubated at room temperature for 5 min. Superfect (15 μl) was then added and the mixture was vortexed briefly (<10 sec) and incubated at room temperature for 10 to 15 min. Six milliliters of serum-free RPMI was then added to the mixture and the transfecting medium was then gently overlaid on cells that had been washed with PBS. Dishes were incubated at 37°C for 2.5 to 3 hr and the cells were again washed (×2) with PBS before returning them to RPMI 1640 supplemented with 10% fetal bovine serum. After an 18-hr incubation, the transfectants were harvested using fresh medium and were plated at two different densities, 0.0625 × 10^6 cells/cm^2 and 0.25 × 10^6 cells /cm^2, at a concentration of 0.5 × 10^6 cells/ml medium. These plating densities resulted in final cell densities at harvest of 0.125 × 10^6 and 0.5 × 10^6 cells/cm^2, comparable with those used for primary murine peritoneal macrophages. Cells were allowed to adhere for 6 to 7 hr at 37°C. The medium was changed to RPMI 1640 with 1% fetal bovine serum, and the cells were stimulated by hypoxia, a combination of LPS and IFN-γ, or by a combination of LPS, IFN-γ, and AG. After a 24-hr incubation, cells were lysed with cell culture lysis reagent (Promega) and luciferase assays were performed using a luciferase assay kit (Promega) following the manufacturer's instructions. Luciferase light units were normalized to total cell protein, determined using the Bradford protein assay reagent (Bio-Rad, Hercules, CA).

Statistics. The data presented are the results of a representative experiment of three independent experiments performed in duplicate and samples assayed in duplicate. The values are expressed as means ± SD. Statistical analysis was performed using the unpaired students *t* test. Differences with P values < 0.05 were considered significant.

Results

Effect of Cell Density on LPS/ IFN-γ-Induced VEGF Expression by Murine Peritoneal Macrophages. Murine peritoneal macrophages were plated at increasing cell densities in three different culture vessels, 60-mm Permanox dishes, 60-mm polystyrene dishes, and 24-well polystyrene plates. Cells were incubated under normoxic conditions in the absence or presence of LPS/IFN-γ or LPS/IFN-γ + AG, or under hypoxic conditions (1% O_2). Viability of the cells, as assessed by MTT assay, was not significantly affected by any of the treatments. The level of VEGF produced by the unstimulated cells was not significantly affected by cell density (Fig. 1, A–C). When stimulated with LPS/IFN-γ, the levels of VEGF produced were markedly dependent on cell density. VEGF production was greatest at the highest cell density (0.5 × 10^6 cells/cm^2) in all three culture vessel types, and decreased markedly at lower cell densities. The iNOS inhibitor AG strongly inhibited VEGF production, clearly indicating that NO production is essential for induction of VEGF. Under hypoxic conditions, VEGF production was strongly induced to a comparable level at all cell densities.

Nitrite levels in all the LPS/IFN-γ-treated cultures were significantly higher than in untreated cultures, and the addition of AG along with LPS/IFN-γ strongly downregulated nitrite production (Fig. 1, D–F). Levels of induced nitrite were slightly higher at higher cell densities, suggesting a possible involvement of pericellular hypoxia in NO production.

Expression of VEGF by Macrophages from $iNOS^{-/-}$ versus $iNOS^{+/+}$ Mice. To further investigate the role of the interaction between NO and lowered oxygen tension in LPS/IFN-γ-induced VEGF expression, macrophages from $iNOS^{-/-}$ and $iNOS^{+/+}$ mice were plated at high density (0.5 × 10^6 cells/cm^2), and at low cell density (0.125 × 10^6 cells/cm^2). Cells were incubated under normoxic conditions in the absence or presence of LPS/IFN-γ or LPS/IFN-γ + AG, or under hypoxic conditions (1% O_2). Viability of the cells, as assessed by MTT assay, was not significantly affected by any of the treatments. VEGF expression by both $iNOS^{-/-}$ and $iNOS^{+/+}$ macrophages was induced to a similar extent by hypoxia (Fig. 2, A and B). At low cell density, neither $iNOS^{-/-}$ nor $iNOS^{+/+}$ macrophages showed VEGF upregulation in response to LPS/IFN-γ (Fig. 2A); however, at high cell density, VEGF expression by LPS/IFN-γ was induced 2.6-fold in $iNOS^{+/+}$ macrophages and 1.7-fold in $iNOS^{-/-}$ macrophages (Fig. 2B). There were no detectable nitrites in the conditioned media of LPS/IFN-γ-treated $iNOS^{-/-}$ macrophages (Fig. 2, C and D).

Deletion Analysis of Murine VEGF Promoter. To investigate whether the interaction between NO and pericellular hypoxia in LPS/IFN-γ-mediated upregulation of VEGF expression by murine macrophages occurs at the transcriptional level, a 1091-bp fragment (−975 to +116) of the murine VEGF promoter and a series of sequential 5′-end

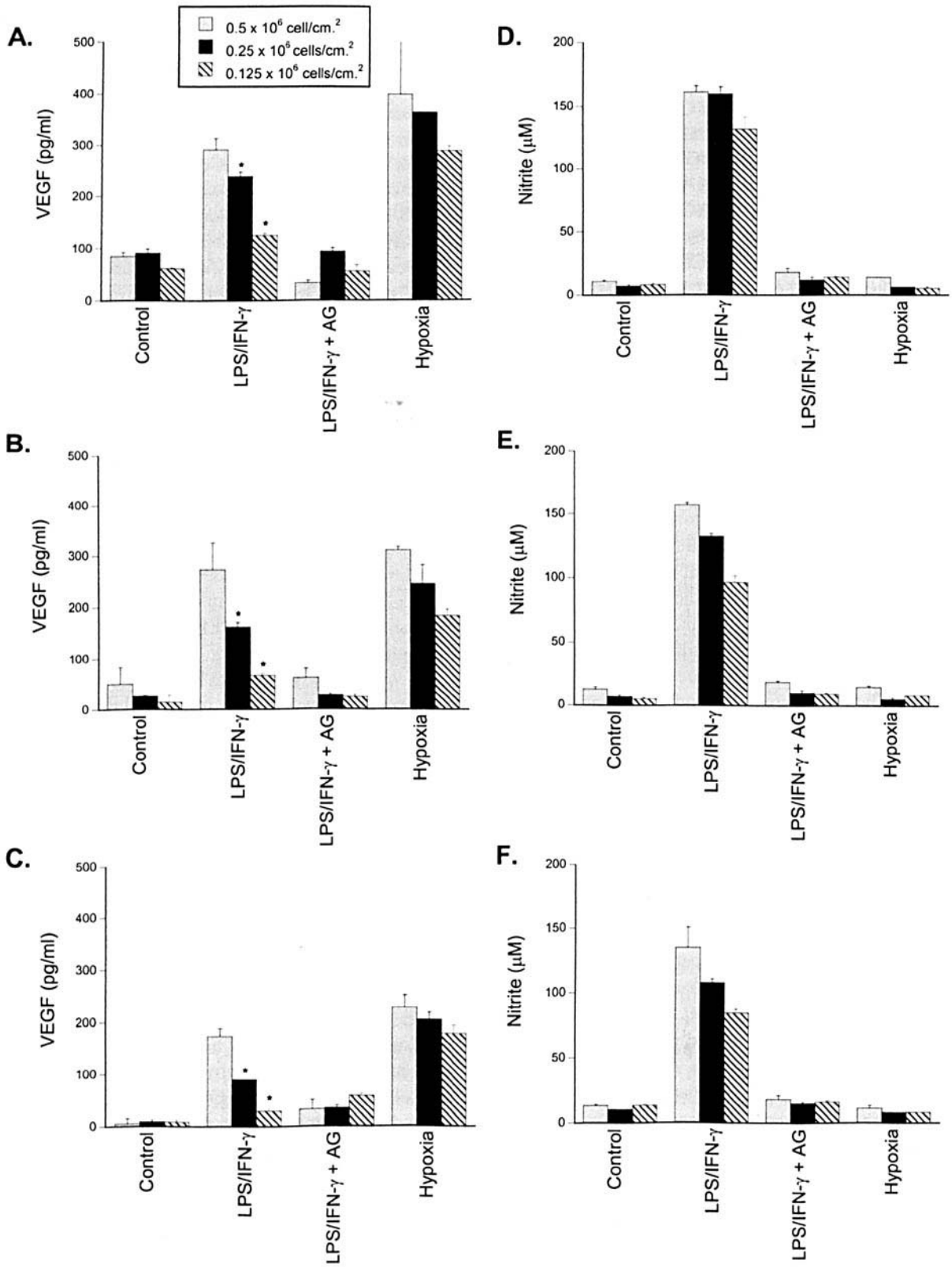

Figure 1. Effect of cell density on LPS/IFN-γ-induced VEGF protein expression by murine peritoneal macrophages. Murine peritoneal macrophages were plated in 60-mm Permanox dishes (A and D), 60-mm polystyrene dishes (B and E), or 24-well polystyrene plates (C and F) at cell densities of 0.5×10^6 cells/cm^2, 0.25×10^6 cells/cm^2, or 0.125×10^6 cells/cm^2. Cells were stimulated by hypoxia, a combination of LPS (100 ng/ml) and IFN-γ (100 U/ml), or by a combination of LPS, IFN-γ, and AG (1.5 m*M*). Conditioned media were harvested after 24 hr and were assayed in duplicate for VEGF (A-C) by ELISA and nitrites (D–F) by Greiss reaction. VEGF and nitrite levels were normalized to the number of viable cells as represented by MTT values. Results of a representative experiment are shown and are presented as means ± SD. * Indicates a *P* value of < 0.05 in comparison with cells plated at a density of 0.5×10^6 cells/cm^2.

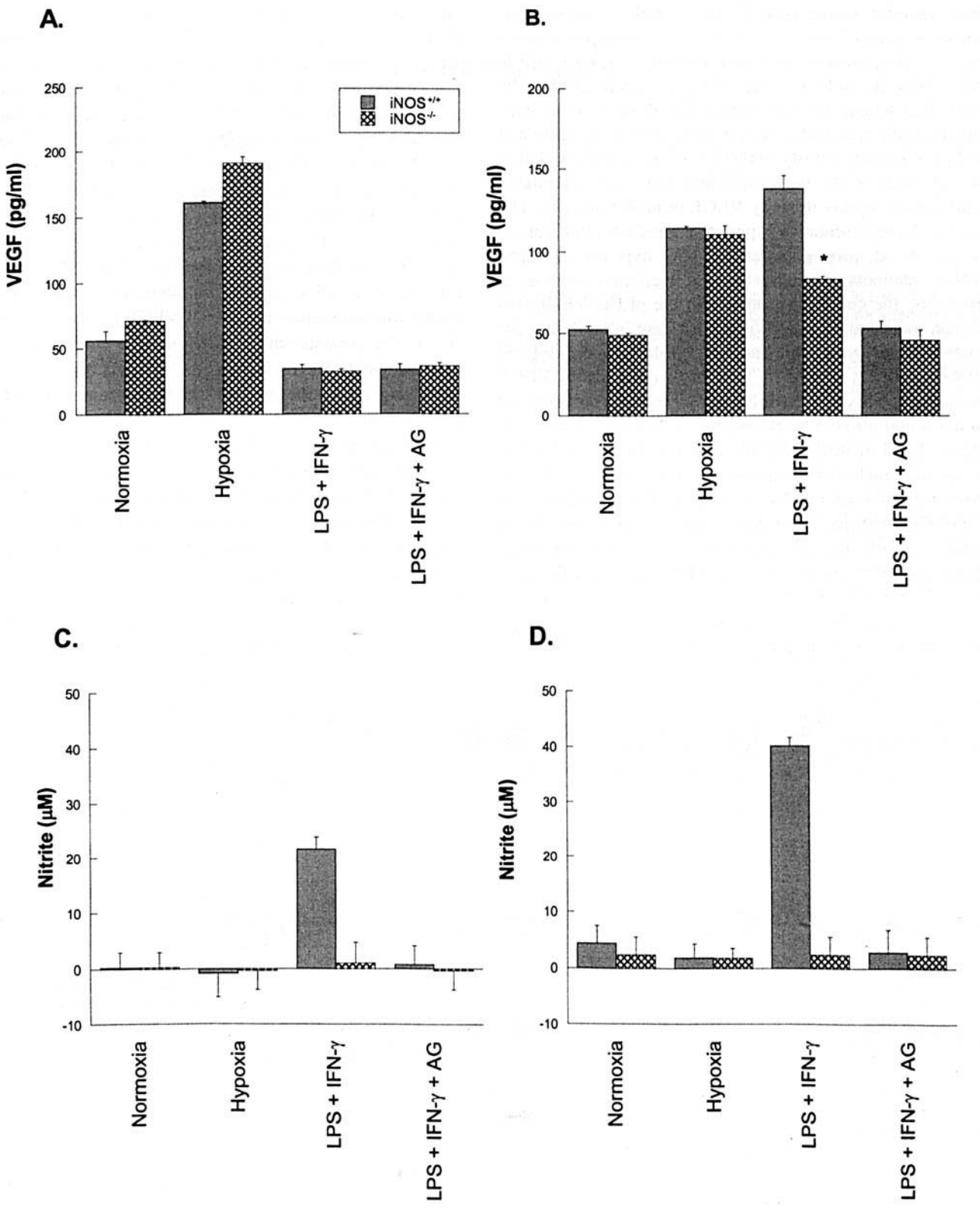

Figure 2. Expression of VEGF by macrophages from iNOS$^{-/-}$ versus iNOS$^{+/+}$ mice. Peritoneal macrophages from iNOS$^{-/-}$ and iNOS$^{+/+}$ mice were cultured at a density of 0.125 x 10^6 cells/cm^2 (A and C) and at a density of 0.5 x 10^6 cells/ cm^2 (B and D). Cells were stimulated by hypoxia, a combination of LPS (100 ng/ml) and IFN-γ (100 U/ml), or by a combination of LPS, IFN-γ, and AG (1.5 m*M*). Conditioned media were harvested after 24 hr and were assayed in duplicate for VEGF (A and B) by ELISA and for nitrites (C and D) by Greiss reaction. Results of a representative experiment are shown and are presented as means ± SD. * Indicates a *P* value of < 0.05 in comparison with cells from iNOS$^{+/+}$ mice.

deletion fragments were inserted into a promoterless luciferase reporter vector (pGL3-basic). These constructs involved sequential deletions of known transcription factor binding elements in the promoter, including the HRE, AP-1, AP-2, NFκ-B, and SP-1 sites (Fig. 3). RAW 264.7 cells were then transiently transfected with these reporter constructs. Cells transfected in the same dish were harvested and plated at low density (0.0625×10^6 cells/cm^2) and high density (0.25×10^6 cells/cm^2), and were then stimulated with various agents to study VEGF promoter activity. The results of a representative experiment are shown in Figure 4, A and B. At lower cell density, both hypoxia and LPS/IFN-γ treatments strongly induced luciferase expression driven by the complete promoter in the pLUC-VEGF-975 vector, indicated by the 28.5-fold increase in luciferase activity induced by hypoxia and the 29-fold increase by LPS/IFN-γ treatment (Fig. 4A). This major induction of VEGF promoter activity by LPS/IFN-γ, however, was not reflected in the VEGF protein levels expressed by RAW 264.7 cells cultured and treated under the same conditions used in the transient transfection experiments, suggesting a role for posttranscriptional regulation (Fig. 5). Downregulation of LPS/IFN-γ-induced luciferase activity by AG (from 29- to 4-fold) clearly indicates that NO mediates this transcriptional regulation. Deletion of the HRE decreased the hypoxic upregulation of VEGF promoter activity from 28.5- to 2.5-fold, suggesting that the HRE and/or regions flanking the HRE are involved in the hypoxia response. Deletion of the HRE also caused a decrease in LPS/IFN-γ-induced luciferase activity from 29.3- to 3.7-fold, indicating that the HRE is involved in the LPS/IFN-γ-induced activation of VEGF promoter. Further deletions of the *cis*-acting elements did not result in any further significant decrease in luciferase activity. When all the known *cis*-acting elements were deleted, luciferase activity was completely abolished.

When the cells transfected with pLUC-VEGF-975 (complete promoter) were plated at a density of 0.25×10^6 cells/cm^2, LPS/IFN-γ induced a 25.4-fold increase in luciferase activity, whereas hypoxic induction was 16.4-fold (Fig. 4B). AG decreased the LPS/IFN-γ-induced VEGF expression from 25.4- to 6.1-fold, suggesting that NO is a strong transcriptional activator of VEGF promoter activity. Again, the corresponding induction of VEGF protein expression by RAW 264.7 cells was significantly lower (9.3-fold), suggesting the posttranscriptional control of VEGF protein expression after LPS/IFN-γ-mediated transcriptional induction (Fig. 5). Levels of induction of VEGF protein and VEGF promoter activity by hypoxia were, however, comparable under both culture conditions. As observed in the cells plated at a lower density of 0.0625×10^6 cells/cm^2 (Fig. 4A), deletion of the HRE caused a significant decrease in LPS/IFN-γ induction (from 25.4- to 5.9-fold), as well as in hypoxic induction (from 16.4- to 1.9-fold) of VEGF promoter activity. Further deletions did not cause any significant change in luciferase levels. Deletion of

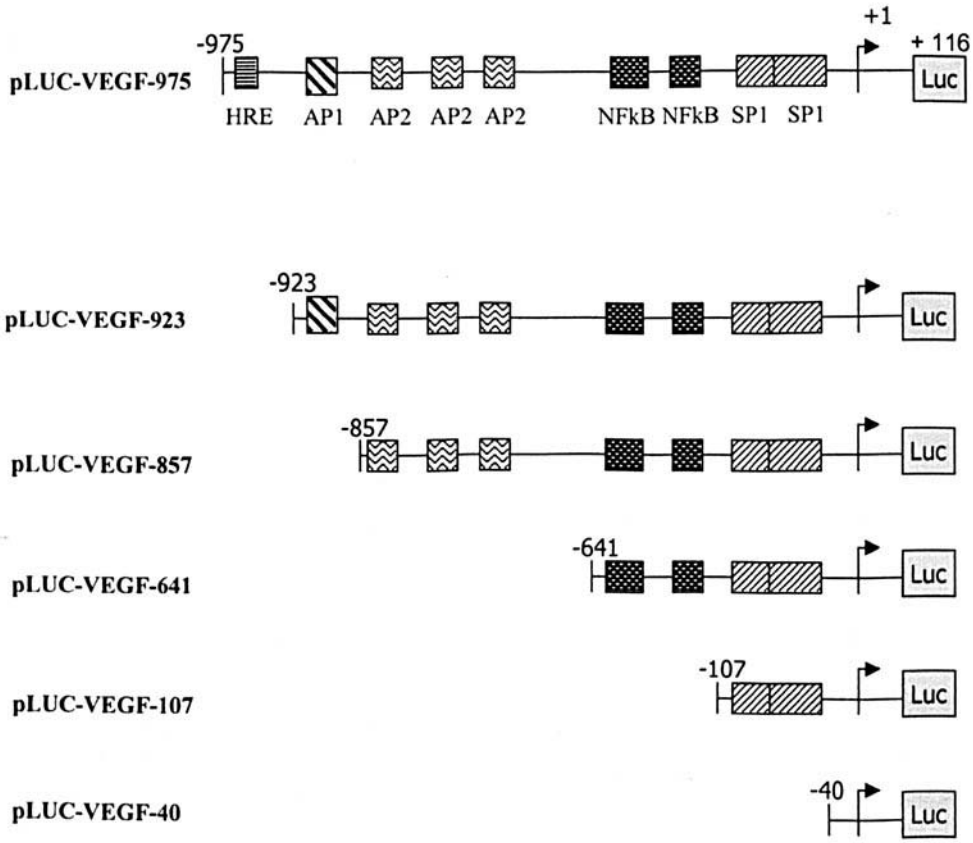

Figure 3. Luciferase reporter constructs of mouse VEGF promoter and its 5′-end deletions.

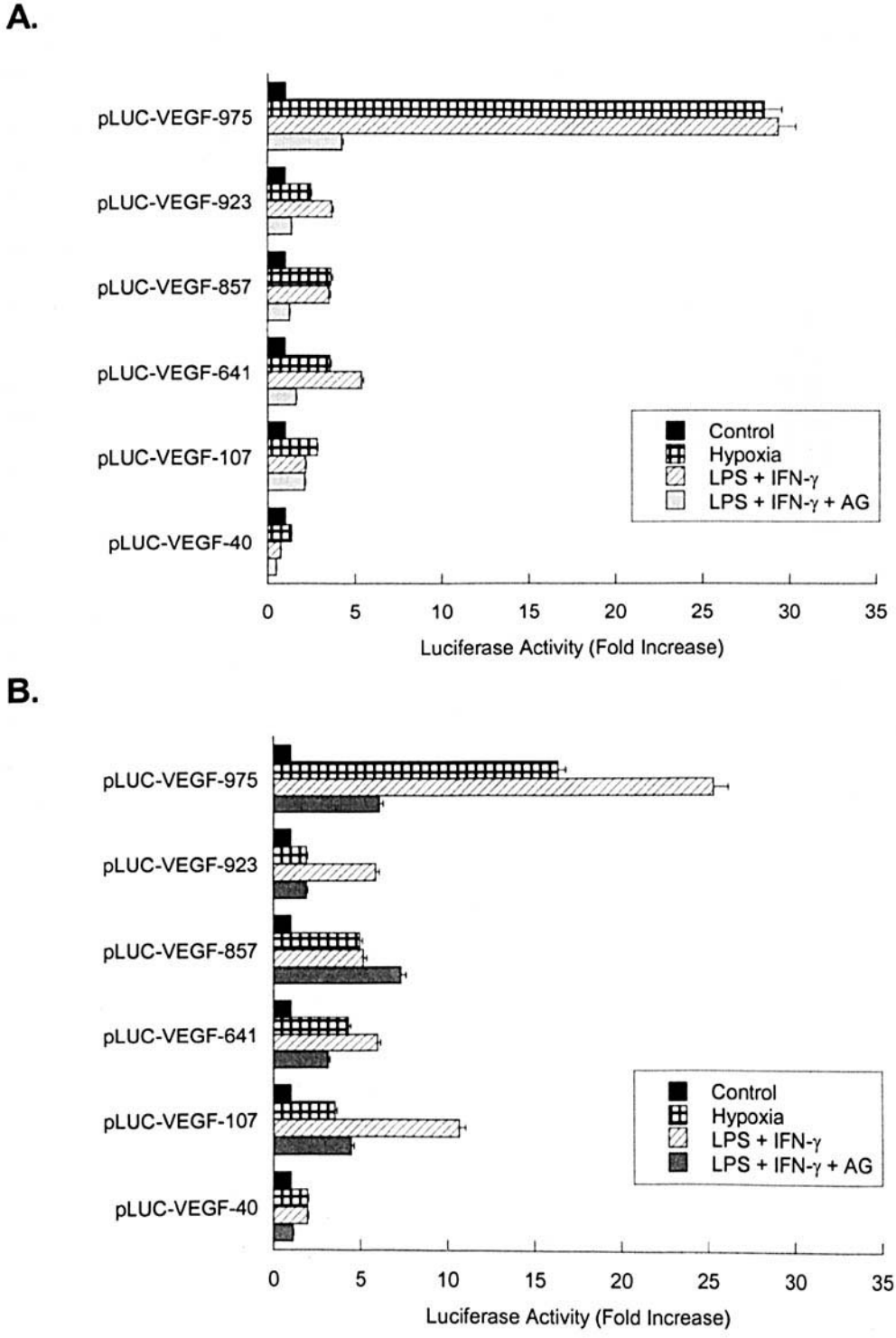

Figure 4. Deletion analysis of mouse VEGF promoter. A 1091-bp fragment (−975 to +116) of the murine VEGF promoter, and a series of sequential 5′-end deletion fragments were inserted into a promoterless luciferase reporter vector (pGL3 basic). RAW 264.7 cells were then transiently transfected with these reporter constructs. Cells transfected in the same dish were harvested and plated at a density of 0.0625×10^6 cells/cm^2 (A) and at a density of 0.25×10^6 cells/cm^2 (B). Transfectants were allowed to adhere for 6 hr and were then stimulated by hypoxia, a combination of LPS (100 ng/ml) and IFN-γ (100 U/ml), or by a combination of LPS, IFN-γ, and AG (1.5 mM). After a 24-hr incubation, cells were lysed with cell culture lysis reagent and luciferase assays were performed using luciferase assay kit. Luciferase light units were normalized to total cell protein determined using the Bradford protein assay. Results of a representative experiment are shown and are presented as means ± SD.

all *cis*-acting elements resulted in complete loss of induction of luciferase activity.

Discussion

In murine peritoneal macrophages, VEGF expression has been shown to be upregulated by both hypoxia and LPS/IFN-γ (14, 28, 29). In macrophages, LPS/IFN-γ treatment upregulates expression of iNOS and thus upregulates NO production. Although considerable research has been carried out to investigate the role of NO in VEGF induction, there has been little information on NO-mediated VEGF regulation in macrophages, which are an important source of VEGF in healing wounds. There are conflicting reports on the role of NO in regulation of VEGF expression (16–18).

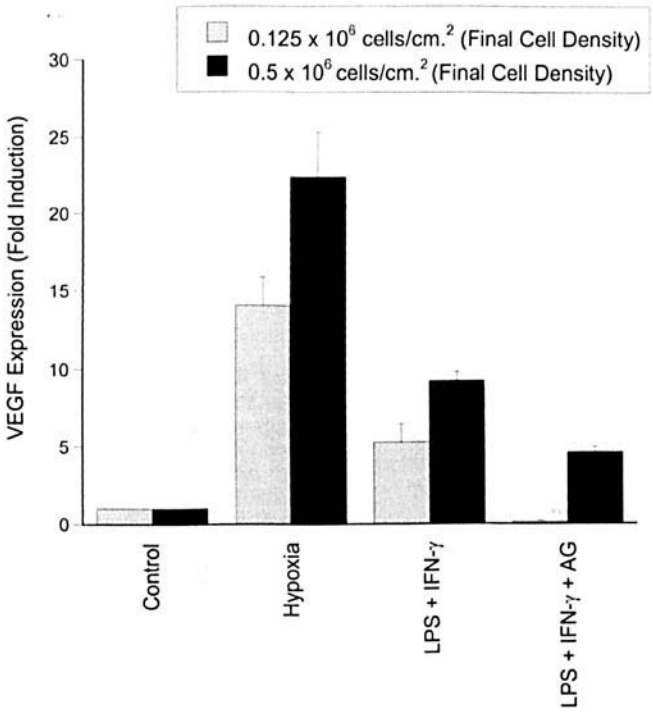

Figure 5. Expression of VEGF by RAW 264.7 cells. RAW 264.7 cells were plated at densities of 0.0625×10^6 cells/cm^2 and 0.25×10^6 cells/cm^2. Cells were allowed to adhere for 6 hr and were then stimulated by hypoxia, a combination of LPS (100 ng/ml) and IFN-γ (100 U/ml), or by a combination of LPS, IFN-γ, and AG (1.5 m*M*). After a 24-hr incubation, conditioned media were harvested and assayed in duplicate for VEGF by ELISA. At the end of the incubation period, wells were lysed using cell culture lysis reagent. VEGF levels were normalized to total cell protein determined using the Bradford protein assay. Results of a representative experiment are shown and are presented as means ± SD.

We have studied the role of NO in the regulation of VEGF expression in murine peritoneal macrophages and RAW 264.7 cells. Under hypoxic conditions, upregulation of VEGF expression was found to be independent of iNOS activity, as indicated by the hypoxic upregulation of VEGF in macrophages from iNOS$^{-/-}$ mice. Also, the iNOS inhibitor AG did not affect hypoxia-induced VEGF expression. In the present study, LPS/IFN-γ treatment produced high levels of VEGF expression when macrophages were cultured at increasing cell densities that lead to conditions of pericellular hypoxia (27). This induction of VEGF was strongly downregulated by AG, indicating that NO was critically involved in this upregulation of VEGF by LPS/IFN-γ at high cell densities. Levels of nitrite in cultures at high densities were slightly higher than those in cultures at lower densities. Hypoxia induces iNOS synergistically with IFN-γ (30, 31), and this may cause higher nitrite levels in LPS/IFN-γ-treated oxygen-deprived cultures. However, the levels of VEGF produced in the cultures at higher densities are much higher than the slight increase in NO production, suggesting that NO alone is insufficient to induce the strongly increased expression of VEGF protein, and that some degree of hypoxia is also required. On the other hand, unstimulated macrophages cultured at high cell density do not produce increased levels of VEGF, indicating that in the absence of stimulation by LPS/IFN-γ, conditions of pericellular hypoxia alone are not sufficient for VEGF gene induction. These observations suggest that stimulation of NO production in macrophages by LPS/IFN-γ, together with the conditions of pericellular hypoxia, leads to increased expression of VEGF protein by murine peritoneal macrophages.

The transient transfections using luciferase reporter constructs of the murine VEGF promoter showed that VEGF promoter activity was strongly upregulated both by hypoxia and by LPS/IFN-γ. The LPS/IFN-γ-induced luciferase expression was downregulated by AG, indicating a significant role for NO at the transcriptional level. Although VEGF promoter activity was strongly induced by LPS/IFN-γ, when RAW 264.7 cells were treated with LPS/IFN-γ under normoxic conditions at the same cell density, VEGF protein levels did not increase, as was also observed in murine peritoneal macrophages. This suggests that there are major posttranscriptional control mechanisms regulating VEGF expression in response to LPS/IFN-γ. These mechanisms result in the failure of the transcribed gene to produce increased levels of stable VEGF protein. This regulation could be at the level of mRNA or protein stability or at the translational level. There is extensive data implicating a role for VEGF mRNA stability in the regulation of VEGF expression (10, 32, 33). Hypoxia has been shown to upregulate VEGF by stabilizing VEGF mRNA (3, 10, 11). It is likely that LPS/IFN-γ-induced VEGF mRNA is also stabilized under reduced oxygen tension, thereby leading to increased expression of VEGF protein. However, strong hypoxic induction of the VEGF promoter was matched by comparable levels of VEGF protein, indicating that when macrophages are incubated in a hypoxic environment (1% O_2), induction of VEGF is at the transcriptional level. Deletion of the HRE caused a significant decrease in induction of VEGF promoter activity by LPS/IFN-γ as well as by hypoxia, indicating a crucial role for the HRE and its immediate flanking sequences in VEGF gene induction by these stimuli.

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