We investigated the possibility that vascular endothelial growth factor (VEGF) treatment could regulate KDR/Flk-1 receptor expression in endothelial cells. Blood adrenal cortex endothelial cells were incubated with 200 pm rhVEGF165 for 0–7 days. Western blot analysis showed a 3–5-fold increase in total KDR protein following 4-day VEGF treatment. Scatchard analysis revealed that VEGF induced a 2–3-fold increase in high affinity receptor number (5.0 × 10^5/cell versus 2.4 × 10^5/cell) without significantly affecting receptor binding affinity (K_d = 76 pm versus 72 pm). Quantitative polymerase chain reaction analysis demonstrated a 3-fold increase in KDR mRNA levels following VEGF exposure. VEGF-induced KDR expression primarily occurred at the transcriptional level as demonstrated by a luciferase reporter assay system. Receptor selective mutants with wild-type KDR binding and decreased Flt-1 binding also induced KDR up-regulation; in contrast, mutants with decreased KDR binding and wild-type Flt-1 binding did not, suggesting that KDR receptor signaling mediates the increase in KDR expression. Inhibition of tyrosine kinase, Src tyrosine kinase, protein kinase C, and mitogen-activated protein kinase activities all blocked VEGF-induced KDR up-regulation. Finally, co-incubation of nitric-oxide synthase inhibitors with VEGF had no significant effect on KDR expression, but 100 μM sodium nitroprusside, a NO donor, significantly inhibited VEGF-induced KDR up-regulation, indicating that NO negatively regulates KDR expression. In conclusion, our data demonstrate that VEGF binding to the KDR receptor tyrosine kinase results in an increase in KDR receptor gene transcription and protein expression. Thus, KDR up-regulation induced by VEGF may represent an important positive feedback mechanism for VEGF action in tumor and ischemia-induced angiogenesis.

Vascular endothelial growth factor (VEGF)^1 and its two tyrosine kinase receptors, the kinase insert-containing receptor (KDR/Flk-1) and Fms-like tyrosine kinase (Flt-1), play important roles in mediating physiological and pathophysiological angiogenesis (for a recent review see Ref. 1). Although VEGF is expressed in various cell types, KDR/Flk-1 and Flt-1 expression is primarily restricted to vascular endothelial cells (1–8). Up-regulation of VEGF and its receptors has been observed in tumors and in various conditions such as hypoxia and wound healing (1, 9–16), whereas relatively low levels are expressed in the blood vessels of normal adult tissues (17). More recently, Soker et al. (18) described a novel VEGF receptor called VEGF165R that only binds to VEGF165 but not VEGF121. This isoform-specific VEGF receptor has a sequence identical to that of human neuropilin-1 and seems to enhance the binding of VEGF165 to KDR and increases its bioactivity (18).

The signaling pathway following VEGF binding to its receptor tyrosine kinases is only partially understood but is known to initiate with intrinsic tyrosine kinase activation followed by autophosphorylation of tyrosine residues in the cytoplasmic domain of the receptors (4, 19, 20). The tyrosine-phosphorylated receptors are recognized by cytoplasmic signaling molecules that connect the activated receptor to transduction cascades and promote cellular responses including tyrosine phosphorylation of phospholipase C-γ (PLC-γ), increasing intracellular calcium, activation of protein kinase C (PKC), phosphatidylinositol 3'-kinase, and mitogen-activated protein kinase (MAPK) (7–8, 21–25). VEGF binding to endothelial receptors also induces production of NO, which is considered to be an important mediator of VEGF-induced vasodilatation and angiogenesis in vivo (26–31).

Biological mechanisms that result in the up-regulation of VEGF and Flt-1 receptor have been proposed (1, 11, 15, 32–34), but relatively little is known about the mechanism responsible for KDR up-regulation. A regulatory element involved in the activation of VEGF transcription in hypoxic cells has been localized upstream of the VEGF transcription initiation site (12). This element includes a binding site for the transcription factor hypoxia-inducible factor-1. Similarly, we have shown that the Flt-1 promoter also contains a hypoxia-inducible factor-1 binding sequence, thus providing a direct mechanism for the up-regulation of Flt-1 receptor in hypoxia (11). In contrast, no hypoxia-inducible factor-1-related elements were found in either the human or in the mouse KDR promoter sequences, suggesting that the hypoxia-induced up-regulation of KDR proceeds via an indirect mechanism.

In support of this, others have suggested that an unidentified paracrine factor released by hypoxic myoblasts or certain tumor cell lines was responsible for the indirect up-regulation of KDR mRNA in endothelial cells (34–36). Further studies implicated VEGF as being the paracrine factor responsible for the up-regulation of KDR in brain slices (36). Up-regulation of
VEGF and Flt-1 mRNA occurred within hours following the initiation of ischemia, whereas induction of KDR occurred on the order of days (15, 37, 38), suggesting a possible temporal regulation of VEGF and its receptors in response to ischemia in vivo.

These observations prompted us to further investigate the involvement of VEGF in regulating KDR receptor expression in endothelial cells. Here we show new evidence that VEGF is capable of directly up-regulating KDR receptor gene transcription and subsequently increasing KDR mRNA and protein expression via the activation of the KDR receptor tyrosine kinase in cultured endothelial cells. Because the KDR receptor has been previously shown to be necessary and sufficient for VEGF-induced endothelial cell proliferation (4, 19, 39), our finding may represent a physiologically important positive feedback mechanism for the amplification of the angiogenic response in vivo.

EXPERIMENTAL PROCEDURES

Cell Culture and Drug Treatment—Bovine adrenal cortex capillary endothelial (ACE) cells were prepared and maintained as described previously (40). Cells were plated onto 12-well tissue culture plates (Costar) and grown in low glucose Dulbecco's modified Eagle's medium, supplemented with 2 mM-glutamine, 10% bovine calf serum (HyClone Lab.), and 100 μg/ml penicillin/streptomycin (Life Technologies). Cultured cells were subsequently lysed in 1 ml of 0.1N NaOH and 0.1% SDS. The cell lysates were washed three times with ice-cold Dulbecco's modified Eagle's medium. The cell lysates were subsequently washed and lysed in 1 ml of column buffer. A specific activity of 60 Ci/m mole of VEGF was labeled with 1 mCi of Na[125]I (NEN Life Science Products) by the addition of 7.5 μl of a 100 μl/mole solution of bovine milk lactoperoxidase (Calbiochem) and 5 μl of a 0.02% solution of hydrogen peroxide. The reaction was incubated at room temperature for 5 min, and an additional 5 μl of 0.1% hydrogen peroxide was added. After 5 min, the reaction was quenched with 50 mM N-acetyl-L-tyrosine. Radioiodinated rhVEGF was separated from free iodine using a NAP-5 column (Amersham Pharmacia Biotech) that had been previously equilibrated with column buffer (5 mM sodium succinate, 130 mM sodium chloride, 0.01% Tween 20, pH 5). [125]VEGF was eluted with 1 ml of column buffer. A specific activity of 60 μCi/μg was obtained.

Radioiodination of VEGF—Iodination of VEGF was accomplished by a lactoperoxidase method in which 10 μg of VEGF was labeled with 1 μCi of Na[125]I (NEN Life Science Products) by the addition of 7.5 μl of a 100 μl/mole solution of bovine milk lactoperoxidase (Calbiochem) and 5 μl of a 0.02% solution of hydrogen peroxide. The reaction was incubated at room temperature for 5 min, and an additional 5 μl of 0.1% hydrogen peroxide was added. After 5 min, the reaction was quenched with 50 μM N-acetyl-L-tyrosine. Radioiodinated rhVEGF was separated from free iodine using a NAP-5 column (Amersham Pharmacia Biotech) that had been previously equilibrated with column buffer (5 mM sodium succinate, 130 mM sodium chloride, 0.01% Tween 20, pH 5). [125]VEGF was eluted with 1 ml of column buffer. A specific activity of 60 μCi/μg was obtained.

Competition Binding and Scatchard Analysis—ACE cell monolayers were incubated with 1 μl of Dulbecco's modified Eagle's medium binding buffer containing 200,000 cpm/ml of [125]VEGF, 0.1% bovine serum albumin along with increasing concentrations of unlabeled VEGF (5–100,000 pM) for 2 h at 4 °C. The binding was terminated by washing cells three times with ice-cold Dulbecco's modified Eagle's medium. The cells were subsequently lysed in 1 ml of 0.1N NaOH and 0.1% SDS. The radiolabeled bound to cells was quantitated using a gamma counter (Packard). In parallel experiments, cell monolayers were trypsinized, and cell numbers were determined using a hemocytometer. All experiments were carried out in duplicate or triplicate. Scatchard analysis was performed using NewLigand software, a modified version of that originally designed by Muson and Rodbard (42). Receptor number (R#) per cell was determined using the formula: 

\[
R_{cell} = \left( \frac{R_{total}}{L} \right) \times 6.023 \times 10^{23}
\]

where \( R_{cell} \) was the total number of receptors and \( L \) was liters.

Cell Lysis and Western Blot Detection of KDR—The methods for cell lysis and Western blot have been described in detail elsewhere (43). A rabbit anti-KDR polyclonal antibody (Santa Cruz Biotech, Santa Cruz, CA) was used to probe for KDR protein. A secondary antibody conjugated with horseradish peroxidase (Zymed Laboratories Inc.) and an enhanced chemiluminescent kit (Amersham Pharmacia Biotech) were used to visualize the KDR immunoreactive bands. Multiple exposures of films were obtained to determine the optimal exposure time. The protein bands were scanned by a densitometer, and the relative intensities were quantified using ImageQuant software (Molecular Dynamics).

RNA Extraction and Real Time RT-PCR Analysis—Total RNA was isolated using the TRIzol Reagent kit according to the supplier's protocol (Life Technologies, Inc.). KDR mRNA expression was quantified using real time RT-PCR analysis as described previously (11).

Assessment of KDR Promoter Activity Using a Luciferase Reporter Assay—To test whether VEGF affects KDR/Flk-1 gene transcription, ACE cells were transiently co-transfected with the luciferase reporter vector containing the mouse Flk-1, the mouse homologue of KDR, promoter, and pRL-CMV luciferase control vector (Promega) as described previously with some minor modifications (11). In brief, cells were plated onto 6-well plates (Costar) at the density of 18,000/cm² and cultured overnight at 37 °C. For preparation of transfection complexes, 1 μg of Luciferase reporter vector containing the mouse Flk-1 promoter, 0.1 μg/pL-CMV luciferase control vector (Promega), and 4 μl/pL of TargeTz Reagent (Targeting Systems, San Diego, CA) were mixed with 500 μl/well of OptiMEM (Life Technologies). The mix was let to stand at room temperature for 30 min to allow formation of transfection complexes.

Cells were washed twice with OptiMEM right before the addition of transfection mixes. 500 μl of transfection mix was added to each well and further incubated at 37 °C for 2 h. Then, 2 ml of medium was added to each well. Next day, transfected cells were either continuously incubated in regular medium or in medium containing 200 pM VEGF. Cells were lysed in a passive lysis buffer (Promega) and harvested at 1, 2, and 3 days post-VEGF exposure. Luciferase activity was measured in TurboTiter Design Model TD-20e Luminometer (Promega) using Dual Luciferase Assay system (Promega) according to the manufacturer's instructions. Signals from untransfected cells treated with or without VEGF were also measured and subtracted from transfected cells. The relative Flk-1 promoter expression was expressed as the ratio of Flk-1 promoter luciferase activity over the pRL-CMV luciferase activity.

RESULTS

VEGF treatment induced a time-dependent increase in KDR protein(s) (Fig. 1A). Under basal conditions, ACE cells primarily expressed the unglycosylated 150-kDa and mature 230-kDa forms with relatively low levels of the intermediate 200-kDa form. The KDR signals were further quantified by densitometry as shown in Fig. 1B. RhVEGF165 treatment caused an up-regulation of all KDR proteins with the maximal increase occurring 4 days post-VEGF treatment. This resulted in a 4.5-fold increase in the 230-kDa mature form, a 3-fold increase in the 200-kDa intermediate form, and a 2.5-fold increase in the 150-kDa unglycosylated form of KDR receptor (Fig. 1B). VEGF-induced KDR up-regulation was also dose-dependent over a range of VEGF concentration 10–500 pM (data not shown).

Blockade of VEGF with a neutralizing anti-VEGF monoclonal antibody abolished VEGF-induced KDR up-regulation (Fig. 2). Denaturing of VEGF by heating also blocked VEGF-induced KDR up-regulation (Fig. 2). These data establish that fully functional VEGF is required to induce KDR up-regulation.

To test whether VEGF treatment also resulted in an increase in receptor present on the endothelial cell surface, we conducted competition binding studies using [125]VEGF. The total [125]VEGF binding in VEGF-treated cells was 2-fold higher than that in control cells as shown in Fig. 3. The binding data were further analyzed using Scatchard plot, which revealed
that VEGF treatment (Fig. 3B, inset) induced a 2.2-fold increase in cell surface receptor number over control (Fig. 3A, inset) without significantly affecting receptor binding affinity.

**Fig. 1.** VEGF-induced up-regulation of KDR is time-dependent. Confluent ACE cells were incubated with 200 pM of VEGF<sub>165</sub> for 0–7 days at 37 °C. The medium was changed every 2 days. Cell lysates were prepared as described under “Experimental Procedures.” An equal amount of protein from each sample was denatured in SDS sample buffer, separated by SDS-polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride membrane. **A**, Western blot showing time-dependent increase in KDR proteins. Lane 1, control (no VEGF); lane 2, VEGF 1 day; lane 3, VEGF 3 days; lane 4, VEGF 4 days; lane 5, VEGF 5 days; lane 6, VEGF 6 days; lane 7, VEGF 7 days. Arrows indicate the fully glycosylated mature form of KDR (230 kDa), the partially glycosylated intermediate (200 kDa), and the unglycosylated immature form of KDR (150 kDa). **B**, histograms showing the optical densities of KDR bands at 230, 200, and 150 kDa by densitometry. Data shown reflect the means ± S.D. of four independent experiments.

**Fig. 2.** Neutralization of VEGF blocks KDR up-regulation. 200 pM VEGF was preincubated with 0–100 μg/ml of a neutralizing monoclonal anti-VEGF antibody for 60 min or was heat-denatured for 10 min before adding to cells. Cells were further cultured for 4 days. Lanes 1, no VEGF no antibody; lanes 2–6, 200 pM VEGF was preincubated with 0–100 μg/ml of anti-VEGF antibody, respectively; lane 7, cells were incubated with anti-VEGF antibody alone; lane 8, VEGF was heat-denatured. Data shown here are representative of two independent experiments.

**Fig. 3.** VEGF treatment increases high affinity binding sites for VEGF. Confluent ACE cells were incubated in the absence (A) and presence (B) of 200 pM VEGF for 4 days. Cells were washed prior to adding <sup>[125I]VEGF</sup><sub>165</sub> in the presence of increasing amounts of unlabeled rhVEGF<sub>165</sub> to develop a competition binding isotherm. New Li-gand software was used to generate a displacement plot and Scatchard plot (see inset). These data are representative of three independent experiments.
Homologous Up-regulation of KDR by VEGF

(200 μM VEGF for 4 days or control cells as described. 100 ng of reference pRL-CMV luciferase activity. was expressed as the ratio of the Flk-1 promoter luciferase activity over actin 1 and actin 2. The relative KDR promoter activity to actin 1 and actin 2.)

**Fig. 5. VEGF binding to the KDR receptor is required for KDR up-regulation.** VEGF variants were generated as described in the text. 200 μM of each variant was incubated with ACE cells for 4 days. Lane 1, control; lane 2, VEGF 165; lane 3, VFM24; lane 4, VFM23A, D63A/E64A/E67A; lane 5, VFM11, R82A/K84A/H86A; lane 6, VFM 17, R82E/K84E/H86E. The results shown here are representative of three independent experiments.

bind to KDR selectively or bind to Flt-1 selectively. Fig. 5 shows that the KDR selective binding mutants (VFM 23A and VFM 24) also increased KDR expression, whereas the Flt-1 selective binding mutants (VFM11 and VFM17) had no significant effect on KDR expression. These data and the effects of VEGF110, a heparin binding domain-deficient mutant with normal binding to KDR and Flt-1, and placental growth factor, a growth factor known to only bind to Flt-1 receptor, on KDR expression were further summarized in Table I. VEGF110 induced similar up-regulation (3–5-fold) of KDR as wild-type VEGF. VFM 24 and VFM 23A also induced a 3–4-fold increase in KDR protein; in contrast, VFM11 and VFM17 did not affect KDR expression. This was further confirmed by the fact that placental growth factor did not affect KDR expression either. These data indicate that KDR receptor is responsible in the signaling pathway for VEGF-induced receptor up-regulation.

Inhibitors of signaling molecules previously shown to lie downstream of KDR were used to further elucidate the mechanism responsible for receptor up-regulation. When genistein, a potent tyrosine kinase inhibitor, was co-incubated during VEGF exposure, a dose-dependent inhibition on VEGF-induced KDR expression resulted (Fig. 6A), suggesting that tyrosine kinase activity is required. The role of PLC-γ, PKC, and MAPK in VEGF-induced KDR up-regulation was also investigated. Fig. 6B shows that co-incubations of VEGF with PP1, a potent and selective inhibitor of Src family, completely blocked VEGF-induced KDR expression; similarly, inhibition of PKC with a pseudosubstrate sequence (PKCI) and staurosporine and inhibition of MAPK with PD-98059 also prevented KDR up-regulation. These data demonstrate that the activation of PLC-γ, PKC, and MAPK is necessary for VEGF-induced KDR up-regulation.

To study the role of NO in VEGF-induced KDR up-regulation, VEGF was either co-incubated with NOS inhibitors or with NO donor SNP. Inhibition of NOS with l-NAME or NNA did not affect VEGF-induced KDR up-regulation, whereas 100 μM SNP completely blocked VEGF-induced KDR up-regulation (Fig. 7), suggesting that NO negatively regulates KDR expression.

**DISCUSSION**

Up-regulation of VEGF and its two high affinity endothelium-specific receptors KDR/Flk-1 and Flt-1 has been documented under ischemic conditions and in tumors (1, 44, 45). In contrast to our knowledge about the various mechanisms by which VEGF and Flt-1 are up-regulated (1, 11, 15, 32, 33), little is known about the mechanism(s) governing KDR regulation.
In this paper, we have addressed the question of whether VEGF treatment of endothelial cells affects KDR expression and have partially elucidated the mechanism(s) by which VEGF increases KDR expression. Our results demonstrate that VEGF treatment directly up-regulates KDR protein expression as shown by Western blot and competition binding studies. Thus, two independent methods demonstrated that VEGF treatment results in a 2–5-fold increase in KDR expression in endothelial cells. The apparent discrepancy in the absolute increase between the two methods (4.5-fold increase in total KDR protein versus 2.2-fold increase in cell surface receptor) may be explained if not all of the mature KDR protein detected by Western blot in ACE cell lysates was accessible for VEGF binding in intact cells. The high affinity receptor detected by Scatchard analysis is presumed to represent KDR rather than Flt-1, because the ACE cells express predominantly KDR receptor and relatively low levels of Flt-1 receptor under control or VEGF stimulated conditions (data not shown). Inactivation of VEGF by a neutralizing monoclonal antibody or by heat denaturing completely blocked VEGF-induced KDR up-regulation, indicating that the up-regulation of KDR required biologically active VEGF in the cell culture medium. Previously, Brogi et al. (35) showed that unknown factor(s) in hypoxia-conditioned medium from myoblasts produced a 3-fold increase in [125I]VEGF binding and 13-fold increase in KDR receptor/cell in HuVECs without significantly affecting binding affinity. More recently, Kremer et al. (36) also showed that a hypoxia-inducible factor in the murine neuroectoderm was able to increase KDR receptor expression, which was mimicked by adding VEGF in a cerebral slice culture system, although the underlying mechanism was unknown. Barleon et al. (34) demonstrated that hypoxia-conditioned medium from various cancer cell lines was able to up-regulate Flt-1 mRNA and protein but not KDR mRNA in HuVECs. These effects were completely inhibited by VEGF-neutralizing extracellular domains. Our results clearly establish that VEGF directly up-regulates KDR expression and provide a detailed characterization of the time-dependent and receptor signaling pathway that leads to KDR up-regulation.

Regulation of receptor expression can occur at the transcriptional, post-transcriptional, or translational levels. To monitor the effect of VEGF on KDR gene expression, we used RT-PCR technology. This approach allows very accurate and reproducible quantification of gene expression, and unlike other quantification PCR methods, it does not require post-PCR sample handling, thus preventing product contamination (46, 47). Our data show that VEGF treatment resulted in a 3.3-fold increase in KDR mRNA (Fig. 4). Others have shown by Northern blot that VEGF treatment of mouse brain slices also resulted in an up-regulation of KDR mRNA (36). Conditioned medium from tumor cells increased KDR and Flt-1 mRNA (34, 35). Thus, our data are consistent with these observations. Increases in mRNA could be due to increased mRNA stability and/or increased gene transcription. Luciferase reporter assay further demonstrated that VEGF treatment induced a 5-fold increase in Flk-1/KDR promoter activity, indicating that the up-regulation of KDR was largely accounted for by an increase in KDR gene transcription. Whether VEGF also increases the stability

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**TABLE I**

| Identity | Mutation | Relative receptor binding | Fold increase in KDR expression |
|----------|----------|--------------------------|--------------------------------|
| Control  | NA       | NA                       | 1                               |
| VEGF<sub>160/165</sub> | NA       | 100                      | 3–5                            |
| VEGF<sub>110/110</sub> | heparin binding domain deficient | 100 | 3–4 |
| VFM24    | D63A/E64A/E67A | 10    | 100  | 3–4   |
| VFM11    | R82A/K84A/H86A | 33    | 1    | 1–2   |
| VFM27    | R232E/K84A/H86E | 10    | 0.1  | 1     |
| PIGF     | NA       | 100                      | 0                              |
of KDR/Flk-1 mRNA remains to be established. In this context, Waltenberger et al. (33) reported that hypoxia resulted in increased stability of the Flk-1/KDR mRNA.

Recent findings suggest that KDR and not Flt-1 is able to mediate the mitogenic and chemotactic effects of VEGF in endothelial cells (4, 19). In contrast, Flt-1 appears to be primarily involved in endothelial cell morphogenesis, at least during embryonic development (48). VEGF receptor selective mutants were used to identify which receptor signals for KDR up-regulation. VEGF mutants with wild-type KDR binding activity but with 10–100-fold decreased Flt-1 affinity (VFM24, D63A/E64A/E67A, and VFM23A, D63K/E64R/E67K) were still capable of inducing a 3–5-fold increase in KDR expression. Mutants (VFM11, R82A/K84A/H86A, and VFM 17, R82E/K84E/H86E) with a 100–1000-fold decreased KDR affinity and near normal (3–10-fold decreased) Flt-1 binding showed little or no effect on KDR expression. Consistent with this observation, placental growth factor, a member of the VEGF family known to bind Flt-1 only (49), had no effect on KDR expression either (Table I). These results suggested that VEGF binding to KDR, but not Flt-1, was required for elevated KDR expression. In a cerebral slice culture system, it was found that placental growth factor failed to induce KDR expression. Thus, the authors concluded that VEGF binding to KDR mediated VEGF-induced receptor up-regulation (33). This is in agreement with our observation that the KDR binding mutants but not Flt-1 binding mutants up-regulate the receptor expression. VEGF154 was also capable of this up-regulation, apparently ruling out the requirement for heparan sulfate proteoglycans or neuropilin binding.

The signaling cascade that follows VEGF interaction with receptor tyrosine kinases on endothelial cells is only partially understood but known to cause tyrosine phosphorylation of receptors, activation, and initiation of series cellular signaling events including PLC-γ, PKC, phosphatidylinositol 3-kinase, and MAPK (21). To understand whether VEGF-induced KDR up-regulation was also through this pathway, various inhibitors to tyrosine kinase, PKC, and MAPK were co-incubated with VEGF in the cells. Genistein, a potent tyrosine kinase inhibitor, completely blocked the VEGF-induced KDR up-regulation, suggesting that autophosphorylation of the KDR receptor or some downstream tyrosine phosphorylation event is an absolute requirement for VEGF-induced KDR up-regulation. Similarly, inhibition of Src kinase, PKC, and MAPK also blocked VEGF-induced KDR up-regulation, indicating that activation of PLC-γ, PKC, and MAPK are involved in the signaling cascade for KDR up-regulation. These factors have also been shown to be involved in VEGF-induced cell proliferation and migration (21). Therefore, VEGF-induced KDR up-regulation, at least in part, shares the same signal pathway as VEGF-induced cell proliferation. Recently, Abedi and Zachary (50) reported that p125 Fak and paxillin were components in a VEGF-stimulated signaling pathway. Mukhopadhay et al. (51) showed that in an in vivo model VEGF stimulated phosphorylation of PYK2 and focal adhesion kinase and activation of c-Jun-NH2 kinase with phosphorylation of c-Jun. The role of these molecules in VEGF-induced KDR up-regulation needs to be further investigated.

Several lines of evidence suggest that VEGF induces NO production, which mediates VEGF-induced vascular permeability, vasodilatation, and angiogenesis (26, 29, 52, 53). The role of NO was studied by co-incubation of VEGF with NOS inhibitors or NO donor SNP. Our data show that inhibition of NOS does not affect VEGF-induced KDR up-regulation, indirectly suggesting that the NO does not contribute to the VEGF-induced KDR up-regulation. Interestingly, SNP significantly reduced KDR expression (Fig. 7). Previously, Tuder et al. (15) showed that SNP decreased hypoxia-induced VEGF KDR and Flt-1 mRNA levels in isolated lungs, whereas l-NAME increased the VEGF mRNA level. Tsurumi et al. (54) proposed a reciprocal relation between VEGF and NO in the regulation of endothelial integrity and showed that the NO donor SNP inhibited PKC-induced VEGF up-regulation by interfering with binding of the transcription factor protein-1 to the VEGF promoter, suggesting that NO secreted by a restored endothelium functions as the negative feedback mechanism that down-regulates VEGF expression to basal levels. Thus, our data demonstrate that a similar reciprocal relation for NO/KDR as for NO/VEGF may exist and that NO negatively regulates KDR expression to maintain cellular homeostasis.

VEGF has also been shown to increase the cell surface expression of several other receptors. Mandriota et al. (55) showed that VEGF treatment induced a 2.8–3.5-fold increase in cell surface urokinase-type plasminogen activator receptor expression and urokinase-type plasminogen activator receptor mRNA in vascular endothelial cells. McCarthy et al. (56) reported that VEGF was able to increase endothelial receptor tyrosine kinase tie-1 expression at transcriptional level. Recently, Zucker et al. (57) proposed that VEGF induced tissue factor expression in HuVEC. Taken together, VEGF may modulate endothelial cell functions at multiple levels. It is reasonable to assume that the up-regulation of KDR plays a key role in these events.

Other angiogenic factors such as fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) have also been shown to increase KDR receptor expression (58–60). The mechanism responsible for the elevated expression of KDR following FGF and HGF treatment is currently unknown. However, the increased expression of KDR following FGF and HGF treatment may be due to an indirect effect of elevated endogenous VEGF, because HGF and FGF have also been shown to increase endogenous VEGF expression (58, 59, 61). The other factor such as transforming growth factor β1 has been reported to down-regulate Flk-1/KDR mRNA and protein expression (62), implying that transforming growth factor β1 may also regulate the VEGF/Flk-1 signal transduction pathway in endothelial cells.

In conclusion, our data strongly suggest that VEGF up-regulates its own KDR receptor expression through binding and activation of the KDR receptor tyrosine kinase. VEGF treatment of ACE cells increased KDR gene transcription, KDR mRNA, and the subsequent expression of KDR receptor on the cell surface. NO negatively regulates KDR expression. Future work will determine whether VEGF regulates KDR expression in vivo and if KDR up-regulation in tumors or in ischemic tissue is a result of the paracrine action of VEGF. If so, this finding may represent an important positive feedback mechanism involved in the control of tumor-associated angiogenesis, wound healing, and vessel growth.

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REFERENCES

1. Ferrara, N., and Davis-Smyth, T. (1997) Endocr. Rev. 18, 4–25
2. Klagesbrun, M., and D’Amore, P. A. (1996) Cytokine Growth Factor Rev. 7, 259–270
3. Jakeman, L. B., Winer, J., Bennett, G. L., Altar, A., and Ferrara, N. (1992) J. Clin. Invest. 89, 244–253
4. Millauer, B., Wizigmann-Voos, S., Schmurch, H., Martinez, R., Moller, N., Risau, W., and Ulrich, A. (1993) Cell 72, 835–846
5. Terman, B. I., Carrion, M. E., Kovacs, E., Rasmussen, B. A., Eddy, R. L., and Shows, T. B. (1991) Oncogene 6, 1677–1683
6. Breier, G., Clauss, M., and Risau, W. (1995) Dev. Dyn. 204, 228–239
