VEGF Receptor and Blood Pressure

Vascular Endothelial Growth Factor Receptor 2 Controls Blood Pressure by Regulating Nitric Oxide Synthase Expression

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Abstract—Drugs and antibodies that interrupt vascular endothelial growth factor (VEGF) signaling pathways improve outcomes in patients with a variety of cancers by inhibiting tumor angiogenesis. A major adverse effect of these treatments is hypertension, suggesting a critical role for VEGF in blood pressure (BP) regulation. However, the physiological mechanisms underlying the control of BP by VEGF are unclear. To address this question, we administered a specific antibody against the major VEGF receptor, VEGFR2, to normal mice and assessed the consequences on BP. Compared with vehicle-treated controls, administration of the anti-VEGFR2 antibody caused a rapid and sustained increase in BP of ∼10 mm Hg. This increase in BP was associated with a significant reduction in renin mRNA expression in the kidney (P<0.019) and in urinary excretion of aldosterone (P<0.05). Treatment with the anti-VEGFR2 antibody also caused a marked reduction in the expression of endothelial and neuronal NO synthases in the kidney. To examine the role of NO in the hypertension caused by blocking VEGFR2, mice were treated with L-NAME, an inhibitor of NO production. L-NAME administration abolished the difference in BP between the vehicle- and anti-VEGFR2–treated groups. Our data suggest that VEGF, acting via VEGFR2, plays a critical role in BP control by promoting NO synthase expression and NO activity. Interfering with this pathway is likely to be one mechanism underlying hypertension caused by antiangiogenic agents targeting VEGF. (Hypertension. 2009;54:652-658.)

Key Words: hypertension ■ angiogenesis ■ cancer ■ vascular endothelial growth factor ■ NO

Vascular endothelial growth factor (VEGF), a 45-kDa glycoprotein, is a powerful inducer of angiogenesis, also affecting vascular permeability, endothelial cell survival, and hematopoiesis.1 The 2 major receptors for VEGF signals are the structurally related tyrosine kinases, VEGFR1 (Flt-1) and VEGFR2 (Flk-1). VEGF also interacts with neuropilins 1 and 2, but their roles in VEGF signaling have yet to be clearly defined. Nonetheless, there is general agreement that the angiogenic, mitogenic, and permeability-enhancing effects of VEGF1 are primarily mediated by VEGFR2. Stimulation of angiogenesis by VEGF acting through VEGFR2 is a key factor in the propagation and spread of various cancers.2-4 Accordingly, antibodies and small molecules targeting VEGF and its associated signaling pathways are effective in treating a variety of human malignancies.

Along with its actions on blood vessel growth and permeability, VEGF also has acute hemodynamic effects impacting peripheral vascular resistance. For example, acute infusions of VEGF cause vasodilation and hypotension.5,6 These vasoactive responses are likely mediated by VEGFR2 and may involve stimulation of NO and vasodilator prostanoids, such as prostaglandin (PG) I2.1 However, the precise molecular mechanisms underlying the effects of VEGF on peripheral vascular resistance remain unclear. In addition to a capacity to influence acute vascular tone, a role for VEGF in chronic control of blood pressure (BP) has been suggested by clinical experiences with VEGF inhibitors. In this regard, hypertension has emerged as one of the most common adverse effects of these agents in patients treated for malignancies. For example, recent meta-analyses reveal substantial increases in the relative risk for hypertension by 6- to 22-fold in patients treated with anti-VEGF antibody or VEGFR kinase inhibitors.7-9 However, the pathophysiology of hypertension associated with anti-VEGF therapy has not been clearly delineated.

To investigate this issue, we used a specific monoclonal antibody to inhibit VEGFR2 in mice and examined the consequences on BP. We find that administration of the anti-VEGFR2 antibody caused robust hypertension, likely related to impaired capacity for the generation of NO.

Materials and Methods

Animals

Male 129S6/SvEv mice were purchased from Taconic (Hudson, NY). Animals were maintained in the animal facility of the Durham Veterans Medical Center.

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Veterans’ Affairs Medical Center and studied between 2 and 4 months of age. The experimental procedures described below were approved by the respective institutional animal care and use committees of the Durham Veterans’ Affairs and Duke University Medical Centers. Mice were fed a normal chow diet (0.4% NaCl, LabDiet) or a low-salt diet (<0.02% NaCl, Harlan Teklad), where indicated.

BP Measurements

Tail-Cuff Manometry

In the dose-finding experiments, systolic BPs were measured in conscious mice using a computerized tail-cuff system (Hatteras Instruments) after 2 weeks of daily training, as described previously. Data were recorded at baseline for 2 weeks and then 5 days per week throughout the study period. This method has been validated previously and correlates well with direct measurements of intra-arterial pressure.

Radiotelemetry Measurements of Intra-Arterial Pressure

BP was measured in conscious mice by radiotelemetry using TA11PA-C10 transmitters (Data Sciences International), as described previously. Briefly, mice were anesthetized with isoflurane, and a pressure-sensing catheter was implanted into the left carotid artery, as described. The transducer unit was then inserted into a subcutaneous pouch along the right flank generated by blunt dissection inferiorly from the original neck incision. Mice were allowed to recover for 7 days after surgery to regain their normal circadian rhythms before experiments were initiated. During BP measurements, mice were housed in a monitoring room in the animal facility where quiet is maintained and no other activities are permitted. Data were collected continuously with sampling every 5 minutes for 10-second intervals using Dataquest ART software (Data Sciences International).

Administration of the DC101 Antibody and Nω-Nitro-l-Arginine Methyl Ester

DC101-expressing hybridoma cells were obtained from American Type Culture Collection (ATCC No. HB-11534). These antibodies are specific for the murine tyrosine kinase receptor FLT-1 (VEGFR2) and inhibit tumor growth by suppressing tumor-induced neovascularization. DC101 production was performed at the Duke Cell Culture Facility. Cells were maintained in roller bottles and adapted to serum-free Hybridoma-SFM medium (Invitrogen). Cells were placed into a hollow-fiber cartridge system and maintained in serum-free medium at 37°C. DC101-containing medium was recovered when glucose levels decreased 50% below baseline and new serum-free medium was added to the cells. DC101-containing medium was concentrated using a 30-kDa Centricon concentrator (Amicon Corporation) and stored at −20°C until use. Protein was quantified by a modified Bradford assay (BioRad). cDNA and negative control (no reverse transcriptase or water) templates (1 μL) were added to 25-μL PCR mixtures consisting of 1× TaqMan Universal PCR master mix (Applied Biosystems) and 1× human eukaryotic 18S rRNA primer-probe mix (Applied Biosystems), 2 ng/μL each of renin forward and reverse primers and 800 nM renin probe, or 1× NOS1 or NOS3 primer-probe mix. Gene expression was quantified using the 2 standard curves method for relative quantitation.

Statistical Analysis

All of the data are presented as mean ± SEM. Differences between treatment groups were analyzed by unpaired \( t \) test or 1-way ANOVA followed by Newman-Keuls multiple comparison test, as indicated. Differences within groups, before and after l-NAME treatment, were analyzed by paired \( t \) test. A \( P \) value of <0.05 was considered significant.

Results

Dose-Dependent Effects of Anti-VEGFR2 Antibody on BP

To examine the capacity of VEGFR2 blockade to cause hypertension, we administered 2 different concentrations of anti-VEGFR2 antibody to normal 129/SvEv mice while monitoring their BPs by tail-cuff manometry. In preliminary studies, the higher dose (1000 μg) caused maximal inhibition of tumor angiogenesis in mice, whereas the lower dose caused moderate inhibition of tumor growth (data not shown). After 1 week, BPs were significantly increased in the mice treated with the higher dose (1000 μg) of antibody (152 ± 2 mm Hg) compared with controls receiving only vehicle (144 ± 2 mm Hg; \( P = 0.006 \)). By contrast, the lower dose of anti-VEGFR2 antibody had no effect on BP (143 ± 2 versus 144 ± 2 mm Hg; \( P \) value not significant). Thus, the dose of anti-VEGFR2 antibody that causes maximal inhibition of angiogenesis also caused a significant increase in BP.

Blockade of VEGFR2 Causes Hypertension in Mice

To more specifically evaluate the effects of inhibiting VEGFR2 on BP, radiotelemetry units were implanted into a separate group of 129/SvEv mice to directly measure intraarterial BP. After establishing baseline BPs, mice were given injections of the anti-VEGFR2 antibody (DC101, 1000 μg) or...
vehicle every 3 to 4 days. As shown in Figure 1, the anti-VEGFR2 antibody caused an immediate rise in BP, whereas BPs in vehicle-treated controls were unaffected. Within 2 days after beginning administration of the antibody, mean arterial pressure (MAP) was significantly higher in the mice receiving DC101 compared with controls (126 ± 2 versus 118 ± 3 mm Hg; *P = 0.03). Moreover, this difference in BP was sustained throughout the 2 weeks of antibody administration. Accordingly, average MAP during the 2-week period was significantly higher in the mice receiving the anti-VEGFR2 antibody than in controls (126 ± 1 versus 117 ± 2.4 mm Hg; *P = 0.016). The magnitude of BP increase (∼10 mm Hg) was very similar to that seen in the dose-finding experiments using tail-cuff BP measurements.

To determine whether the hypertension caused by the anti-VEGFR2 antibody could be modulated by changing dietary salt content, we fed vehicle- and DC101-treated mice a low-salt diet (<0.02% NaCl) while continuously monitoring BP by radiotelemetry. We observed a slight decrease in BP in both vehicle- and DC101-treated mice on a low-salt diet; however, this change was not statistically significant in either group (117 ± 4 versus 115 ± 4 mm Hg, vehicle, *P = 0.34; 126 ± 1 versus 124 ± 1 mm Hg, DC101, *P = 0.090; Figure 2). Furthermore, the difference in BP between the experimental groups was maintained during low-salt feeding (115 ± 4 versus 124 ± 1 mm Hg; *P = 0.019).

**Anti-VEGFR2 Antibody Treatment Suppresses the Renin-Angiotensin-Aldosterone System**

To begin to assess the pathogenesis of hypertension caused by blockade of VEGFR2, we measured kidney renin mRNA expression and urinary aldosterone excretion in mice treated with low (150 µg) or high doses of DC101 (1000 µg) or vehicle. Renin mRNA expression in kidneys from mice treated with either dose was reduced equivalently by ∼30% compared with mice treated with vehicle (0.072 ± 0.009 [low] or 0.075 ± 0.009 [high] versus 0.105 ± 0.009 arbitrary units; *P < 0.05; Figure 3A). Urinary aldosterone excretion was also reduced by ∼20% in the mice treated with anti-VEGFR2 antibody compared with controls (7165 ± 1920 [low] or 8530 ± 477 [high] versus 10 534 ± 744 pg/mg of creatinine; *P < 0.01 [low] or *P < 0.05 [high]; Figure 3B). Taken together, these findings suggest that activation of the renin-angiotensin-aldosterone system is not a primary mechanism of hypertension associated with VEGFR2 inhibition.

**Prostanoid Metabolism Is Unaffected by VEGFR2 Blockade**

Alteration of vasoactive prostanoid generation has been suggested to contribute to hypertension associated with VEGF inhibition.\(^{19}\) Therefore, we also measured the profile of urinary prostanoid excretion in mice treated with DC101 compared with controls. Specifically, we measured the stable metabolites of 3 prostanoids with well-characterized actions affecting BP: PGE\(_2\), PGI\(_2\), and thromboxane A\(_2\). We found no statistically significant differences in urinary excretion of PGE\(_2\) metabolites (432 ± 33 versus 447 ± 29 pg/mg of creatinine; *P value not significant), thromboxane B\(_2\) (856 ± 211 versus 1071 ± 149 pg/mg creatinine; *P = NS), or 6-keto-PGF\(_1\alpha\) (6002 ± 718 versus 5164 ± 438 pg/mg of creatinine; *P value not significant) between DC101- and vehicle-treated mice.

**Reduced Expression of NOS With Anti-VEGFR2 Antibody Treatment**

VEGF is known to modulate NO generation, and VEGFR2 activation affects the activity of eNOS.\(^{20}\) Thus, we measured the expression of 2 NO synthase isozymes, eNOS (NOS3) and nNOS (NOS1), in the kidneys of mice from the 2 experimental groups by real-time quantitative RT-PCR. Levels of mRNA for both eNOS and nNOS were reduced significantly by ∼20% in kidneys from DC101-treated mice compared with controls (0.808 ± 0.062- versus 1.000 ± 0.076-fold for eNOS, *P = 0.034, and 0.820 ± 0.061- versus 1.000 ± 0.072-fold for nNOS, *P = 0.038; Figure 4).

**1-NAME Abolishes BP Difference Associated With VEGFR2 Blockade**

To determine whether the reduced expression of NOS isoforms that we observed might contribute to changes in BP, we compared the effects of the NO synthesis inhibitor, 1-NAME,
on BP in the experimental groups. L-NAME (20 mg/kg per day) was administered in drinking water to DC101- and vehicle-treated mice beginning 5 weeks after antibody administration was initiated. In both groups, L-NAME caused a significant increase in BP (Figure 5). Compared with controls, the effect of L-NAME to increase BP was diminished in the mice receiving anti-VEGFR2 antibody, although the difference did not reach statistical significance (11±4% versus 6±1% increase; P=0.12). However, within 1 day after L-NAME was started, the difference in BP between the 2 groups of mice was abolished (132±3 versus 136±1 mm Hg; P=0.15; Figure 5).

**Discussion**

Angiogenesis inhibitors used as part of chemotherapy regimens improve outcomes for patients with several types of malignancies. However, hypertension has emerged as a common adverse effect of VEGF inhibition. These clinical observations have revealed a somewhat unexpected role for VEGF-associated signaling pathways to modulate BP. However, the mechanisms underlying the control of BP by VEGF have not been precisely defined. Elucidating these mechanisms will provide novel physiological insights but could also have clinical use for developing regimens to minimize the risk of significant hypertension, optimizing targeted antihypertensive drug therapies, and identifying those patients most susceptible to hypertension.

In clinical trials, hypertension has been observed after treatment with neutralizing antibodies against VEGF, as well as small molecules that inhibit multiple tyrosine kinase receptors, including the VEGF receptors and platelet-derived growth factor receptors. However, a precise linkage between specific angiogenic receptor pathways and BP control has not been established. In our studies, we found that blockade of VEGFR2 was sufficient to cause an immediate and sustained increase in BP. This finding suggests a tonic and nonredundant role for VEGFR2 and its associated signaling pathways to modulate BP in otherwise normal mice. The effect of VEGFR2 inhibition on BP is dose dependent, because a low

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*Figure 3. Renin mRNA expression (A) and urinary aldosterone excretion (B) in mice treated with DC101. Relative expression of renin mRNA (normalized to 18S rRNA) in whole kidneys was measured by real-time RT-PCR and aldosterone excretion by enzyme immunoassay in 24-hour urine samples from vehicle- and DC101-treated mice. Urinary aldosterone excretion was normalized to total creatinine (crt) excretion in the same sample. Mice treated with low-dose (150 µg) DC101 had BPs similar to vehicle-treated mice, whereas those treated with high-dose (1000 µg) DC101 were hypertensive. Data are mean±SEM for 9 to 10 mice per group. *P<0.05 vs vehicle by 1-way ANOVA followed by Newman-Keuls multiple comparison test.*

*Figure 4. Effect of VEGFR2 inhibition on NOS mRNA expression in kidney. Relative expression of NOS mRNA was measured by real-time RT-PCR in whole kidneys from mice treated with vehicle or DC101 (1000 µg). Levels of eNOS and nNOS were normalized to that of 18S rRNA in the same sample. Data are expressed as fold change vs vehicle for each gene. Data are mean±SEM for 9 to 10 mice per group. *P<0.05 vs vehicle by unpaired t test.*

*Figure 5. Effect of inhibiting NO synthesis on BP in vehicle- and DC101-treated mice. MAP (24 hours) was measured by radiotelemetry during administration of the NO synthesis inhibitor L-NAME (20 mg/kg per day). “DC101 mean” represents the average MAP of 14 days of treatment in vehicle- and DC101-treated mice. Data are mean±SEM for 7 to 8 mice per group. *P<0.05 vs vehicle by unpaired t test.*
dose of anti-VEGFR2 antibody, one with only modest effects on tumor growth, failed to elicit a hypertensive response. The general magnitude of the BP increase observed with a higher dose of anti-VEGFR2 antibody, shown previously to have potent antiangiogenic effects in mice (≈8 to 10 mm Hg), is consistent with observations from clinical trials of patients receiving antiangiogenic therapies. Moreover, this level of BP increase in humans, if sustained, would translate into a significant increase in the risk for cardiovascular morbidity and mortality. In addition, it is conceivable that these effects might be amplified in patients with preexisting hypertension, as suggested in other animal studies. Hypertension caused by blockade of VEGFR2 in mice was not affected by a reduction in dietary sodium, suggesting that it is relatively resistant to alterations in dietary salt intake, as shown in Figure 2.

The renin-angiotensin system (RAS) is a key regulator of BP, and previous studies have suggested that VEGF inhibition may exaggerate the severity of angiotensin II–dependent hypertension. Accordingly, we assessed a potential role for the RAS in hypertension caused by VEGFR2 inhibition. Because release of renin from juxtaglomerular cells in the kidney is a key rate-limiting step in the generation of angiotensin II, we compared renin mRNA expression in mice receiving the anti-VEGFR2 antibody with controls. Renin mRNA expression was reduced by ≈30% in mice treated with a dose of anti-VEGFR2 antibody (1000 μg) that was sufficient to cause hypertension. Furthermore, we saw a similar reduction of renin expression in mice treated with a lower dose of antibody (150 μg) that did not appreciably affect BP, suggesting that suppression of renin may be an early compensatory response to attenuate the increase in BP caused by VEGFR2 inhibition. Because aldosterone production by the adrenal gland is regulated by angiotensin II, we also measured urinary aldosterone excretion as an independent assessment of RAS activity. Consistent with the reduced renin expression, we found that urinary aldosterone excretion was also significantly diminished after administration of the anti-VEGFR2 antibody. Taken together, these data indicate that stimulation of the RAS is not a primary mechanism causing hypertension in this setting.

Suppression of vasodilator prostanoids, such as PGI2, relative to vasoconstrictor prostanoids, such as thromboxane A2, can cause sustained hypertension. Because endothelial cells are the major source of PGI2 in the circulation, it has been suggested that isolated inhibition of PGI2 production by endothelial cells might contribute to hypertension associated with inhibition of VEGF. We, therefore, examined the effects of VEGFR2 blockade on the general profile of prostanoid generation. Urinary excretion of 6-keto-PGF1α, the major metabolite of PGI2, was not affected by anti-VEGFR2 antibody. Likewise, excretion of thromboxane B2, the major metabolite of the vasoconstrictor eicosanoid thromboxane A2, was not different between DC101- and vehicle-treated mice. Urinary levels of the PGE2 metabolite were also similar between the groups. Thus, alteration in the balance of vasodilator and vasoconstrictor prostanoids does not explain the development of hypertension with VEGFR2 inhibition.

Infusions of VEGF cause acute vasodilation, and it has been suggested that this vasodilatory response is mediated by NO. Moreover, VEGF is known to stimulate calcium-independent NO synthesis in vascular endothelial cells by activating the phosphatidylinositol 3-kinase/Akt pathway downstream of VEGFR2, leading to serine phosphorylation and activation of eNOS. VEGF also upregulates eNOS mRNA and protein expression in human, bovine, and rodent endothelial cells. This upregulation is mediated by VEGFR2 and appears to be the result of a posttranscriptional effect on eNOS mRNA stability. Although acute regulation of the constitutive NOS enzymes (eNOS and nNOS) occurs through posttranslational mechanisms, chronic changes in NO synthesis are predominantly regulated by changes in eNOS or nNOS mRNA expression. Therefore, we examined the effects of VEGFR2 inhibition on NOS isozyme expression in kidneys. In mice receiving the specific anti-VEGFR2 antibody, eNOS mRNA levels were significantly reduced by ≈20%. Because eNOS-deficient mice are hypertensive, such a reduction in eNOS expression might be expected to affect BP homeostasis. Interestingly, we found a similar reduction in nNOS expression in mice after VEGFR2 blockade. The net contribution of this reduction of nNOS on BP is less clear, because BP is normal in nNOS-deficient mice. Nonetheless, recent data suggest that nNOS plays important roles in the regulation of both vascular tone and renal sodium handling under certain conditions.

Our findings of reduced expression of NOS isofoms in kidneys of mice treated with the anti-VEGFR2 antibody suggest that chronic inhibition of VEGFR2 signaling may reduce NO synthesis in the kidney and perhaps in other tissues, thereby promoting hypertension. To evaluate the role of impaired NO generation in the hypertension caused by VEGFR2 blockade, we examined responses to the NO synthesis inhibitor L-NAME. After administration of L-NAME, the 8- to 10-mm Hg difference in BP between the mice treated with the VEGFR2 blocker and controls was abolished. Taken together, these findings suggest that reduced levels of eNOS, and perhaps nNOS, with the coincident attenuation of NO generation are responsible for the increase in BP caused by VEGFR2 blockade.

In some patients with preeclampsia, levels of a soluble form of VEGFR1 are increased in the circulation. Soluble VEGFR1 specifically binds VEGF, decreasing its circulating levels, causing coincident endothelial dysfunction with hypertension, proteinuria, and glomerular endotheliosis. Moreover, a recent report suggests that NO formation is also impaired in this setting. In the context of our current findings, we suggest that impaired VEGFR2 signaling may contribute to hypertension and reduced NO generation associated with circulating VEGF inhibitors in preeclampsia.

The mechanism of reduced NOS expression caused by VEGFR2 blockade is not clear. The rapid increase in BP within days after the initial administration of the anti-VEGFR2 antibody suggests that acute interruption of signaling pathways with direct effects on NOS expression may play a role. Alternatively, the reduction in NOS expression might reflect diminution of microvessel density. In this regard, recent studies suggest that VEGF is required for maintenance
of normal blood vessels, in particular, for vessels containing fenestrated endothelia. In studies using mice, pharmacological inhibitors of VEGF signaling have been shown to cause vascular rarefaction, a reduction in microvessel density, in a number of tissues including the kidney, where fenestrated endothelial cells are found in glomerular and peritubular capillaries.

In summary, the purpose of this study was to examine the mechanism of hypertension caused by inhibition of VEGF signaling. We demonstrated that blockade of VEGFR2 in wild-type mice is sufficient to cause robust hypertension. The increased BP is not a result of RAS activation but rather appears to be a consequence of an impaired capacity for NO generation because of decreased NO synthase expression.

**Perspectives**

We demonstrate that blockade of VEGFR2 causes significant hypertension in normal mice that is likely mediated by reduced NO production. These findings indicate an important role for modulation of NO by VEGFR2 as a key signaling pathway in normal BP homeostasis. Our data suggest that blocking VEGF signaling via VEGFR2 is one mechanism by which anti-VEGF therapies cause hypertension. Moreover, these studies suggest that alterations of this pathway might contribute to hypertension in other circumstances. Additional work is needed to understand more completely the spatial and temporal contexts in which VEGFR2 signaling controls BP in health and disease.

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