Role of Endogenous Vascular Endothelial Growth Factor in Endothelium-Dependent Vasodilation in Humans


Abstract—Angiogenesis inhibitors have remarkably improved the outcome of patients with several types of cancer. Hypertension is the most reported side effect of angiogenesis inhibitors interfering with vascular endothelial growth factor signaling. In this study, we test the hypothesis that circulating vascular endothelial growth factor at physiological concentrations is essential to preserve normal endothelial control of vasomotor tone. In 7 healthy male volunteers, infusion of bevacizumab (monoclonal vascular endothelial growth factor antibody) into the brachial artery for 15 minutes (144 μg/dL forearm volume per minute) did not affect forearm vasodilator tone as measured with venous occlusion strain gauge plethysmography. In a separate group of 12 male volunteers, a similar bevacizumab infusion reduced the vasodilator response to 2 dosages of acetylcholine from (mean±SE) 440±157% and 926±252% to 169±40% and 612±154% (P<0.05). Finally, in a third group of 12 volunteers, bevacizumab did not alter the percentage increase in forearm blood flow during infusion of sodium nitroprusside at dosages equipotent to acetylcholine. Bevacizumab acutely and specifically reduced endothelium-mediated vasodilation at local concentrations that resemble plasma concentrations after systemic exposure to bevacizumab. This observation suggests a physiological role for vascular endothelial growth factor in maintaining normal endothelial control of vasomotor tone. The role of the endothelium in the mechanism of bevacizumab-induced hypertension deserves further exploration. (Hypertension. 2013;61:00-00.)

Key Words: angiogenesis inhibitors ■ endothelial function ■ human ■ vascular biology ■ VEGF

Angiogenesis, the formation of new capillaries from endothelial cells from existing vasculature, is essential for tumor growth. When tumor size exceeds a few millimeters, an angiogenic switch occurs as a consequence of hypoxia and nutrient deprivation. Subsequently, various proangiogenic factors are released by tumor cells to activate quiescent cells to promote vascular growth to the tumor. Vascular endothelial growth factor (VEGF) and its receptors play a key role in this process. However, also in normal physiology, VEGF may play a role in vascular homeostasis.

VEGF is an angiogenic growth factor which is mainly secreted from mesenchymal, stromal, and epithelial sources to act on endothelial cells. The angiogenic effects of VEGF are primarily mediated by VEGF receptor-2 (VEGFR-2).2

In 1971, Folkman proposed angiogenesis inhibition as an alternative strategy for the treatment of malignancies.3 Meanwhile, several drugs that target angiogenesis in tumors have been developed, almost all of them directed to VEGF or its receptors. Bevacizumab (BVZ), a humanized monoclonal antibody selectively binding VEGF, was the first VEGF inhibitor approved by the Food and Drug Administration.

BVZ in combination with chemotherapy has been approved for the treatment of metastatic colon carcinoma, unresectable or metastatic nonsmall cell lung cancer and glioblastoma, and in combination with interferon-α for metastatic renal cell carcinoma.

BVZ is generally considered to be safe and well tolerated but can be accompanied by a variety of side effects. An increased incidence of hypertension (defined as a blood pressure >150/100 mm Hg or a rise of 20 mm Hg in diastolic blood pressure) of up to 34% has been observed in BVZ-treated patients in clinical trials compared with up to 14% in those treated with standard therapy. The most serious reported adverse drug reactions are hemorrhage and arterial thrombo-embolism in patients treated with BVZ in combination with chemotherapy.4 The most frequently observed adverse drug reactions across clinical trials in patients receiving BVZ are hypertension, fatigue or asthenia, diarrhea, and abdominal pain.5

Pharmacological doses of VEGF stimulate endothelial nitric oxide (NO) formation in preclinical models6–8 and reduce blood pressure in animals and humans.9 In mice, inhibition of VEGF receptor-2 rapidly increased blood pressure and reduced the expression of endothelial and neuronal NO synthases in the kidney. Moreover, treatment with a NO antagonist abolished the effect of VEGF receptor-2 inhibition on blood pressure.1 It is tempting to speculate that anti-VEGF treatment will reduce endothelial NO release as the mechanism of BVZ-induced hypertension deserves further exploration.
hypertension. However, the role of exposure of endothelial cells to endogenous VEGF has never been studied in humans in vivo. Therefore, it is currently not known whether BVZ treatment results in endothelial dysfunction.

Therefore, the aim of this study was to explore the effect of specific inactivation of endogenous VEGF by binding to its monoclonal antibody BVZ on baseline vasomotor tone and on endothelium-dependent vasodilation in humans in vivo.

Materials and Methods

Subjects

After approval of the protocols (NCT00929058, NCT01125943) by the Institutional Review Board of the Radboud University Nijmegen Medical Center, a total of 31 healthy nonsmoking male volunteers were recruited after written informed consent. Female subjects were excluded to prevent the influence of menstrual cycle and related hormonal changes on vascular reactivity. Subjects were eligible if they had no clinical history of hypertension, hyperlipidemia, renal dysfunction, or diabetes mellitus. On specific request from our institutional ethical review board, subjects at increased risk for malignancy (first-degree relatives with cancer presented before age of 50 years) and subjects with first-degree relatives with premature atherosclerotic disease or recurrent (venous and arterial) thrombosis were excluded.

All studies were performed according to institutional and Good Clinical Practice guidelines.

Study Design

General Outline of the Procedures

The experiments were performed after at least 24 hours of caffeine abstinence, in the morning after an overnight fast. Volunteers were studied in supine position in a temperature-controlled room (23±1°C). At the start of each experiment, a 20-gauge cannula (Angiocath; Becton Dickinson, Sandy, UT) was inserted into the brachial artery of the nondominant arm for intra-arterial blood pressure measurement (Hewlett Packard, Böblingen, Germany) and infusion of drugs. During intra-arterial infusion of saline (NaCl, 0.9%) or drugs, forearm blood flow (FBF) was measured at both arms simultaneously, using ECG-triggered venous occlusion mercury in silastic gauge plethysmography (Hokanson EC4, Hokanson, Inc). During all FBF recordings, the hand circulation was completely occluded using wrist cuffs inflated to 200 mm Hg. At least 30 minutes after the cannulation experiments were started. Drugs or solvent (either saline 0.9% or glucose 5%) was infused at a constant rate of 50 L/dL forearm. The percentage changes in FBF to each dosage of a vasodilator were calculated as the difference from baseline FBF and averaged per FBF registration. Drug-induced effects were pitted into the wells and the plate was incubated overnight at 4°C. After washing, the plate was incubated with 100 µL mouse anti-Human IgG (Fc) peroxidase (dilution 1:25 000, SouthernBiotech, Birmingham, United Kingdom) during 2 hours at ambient temperature and again washed. Thereafter, incubation with 100 µL ready-to-use 3,3′,5,5′-tetramethyl-benzidine solution (Kem-En-Tec, Taasrup, Denmark) for 15 to 20 minutes took place for color development. The reaction was stopped by addition of 0.5 mol/L H₂SO₄, and optical density was measured at 450 nm in a Multiskan Ascent plate reader (Laboratory Systems, Oy, Helsinki, Finland). The analytic sensitivity, defined as the minimum BVZ concentration evoking a response significantly different from that of the zero calibrator, was 16 pg/mL. Plasma samples, diluted 4000- to 24 000-fold, exhibited excellent parallelism. To 6 plasma samples, known quantities of Avastin were added. The recoveries ranged in the plasma samples from 87% to 110% with a mean recovery of 98%. In each run the reference preparation, prepared from a pool of plasma from patients treated with BVZ, was used to monitor long-term performance of the assay. The concentration in reference preparation was 1670 ng/mL, the within-run coefficient of variation and the between-run coefficient of variation amounted to 7.0% and 10.0%, respectively.

Study 1: The Acute Vasomotor Response to BVZ

Seven volunteers were recruited for this study. FBF was measured using venous occlusion strain gauge plethysmography. First, baseline FBF was recorded for 5 minutes. Subsequently, the vasomotor effect of BVZ was studied during a 15-minute infusion of BVZ (144 µg/L forearm volume per minute) in the brachial artery of the nondominant arm. Thirty minutes after the BVZ infusion, FBF was assessed again for studying late effects of BVZ infusion. During the last minute of 15 minutes of BVZ infusion venous blood was collected from both arms to measure local and systemic concentrations of BVZ.

BVZ concentration was measured with a 1-site enzyme-linked immunosorbent assay (ELISA) developed by our laboratory. In short, 96-well plates (#655092, Greiner Bio-One, Alphen a/d Rijn, The Netherlands) were coated overnight at 4°C with 100 µL VEGF (0.50 µg/mL, Genentech Inc, San Francisco, CA), washed (96PW plate washer, Tican Group Ltd, Männedorf, Switzerland), and then blocked with 150 µL Superblock (#373515, Pierce, Rockford, IL) during 30 minutes at ambient temperature, washed and further blocked with 300 µL bovine serum albumin (#A-7906, Sigma Chemical, St. Louis, MO) for 4 hours at ambient temperature. Subsequently, the plate was washed, and standards (range, 0-10 ng/mL Avastin, Roche, Basel, Switzerland), study samples, and a reference sample were pipetted into the wells and the plate was incubated overnight at 4°C. After washing, the plate was incubated with 100 µL mouse anti-Human IgG (Fc) peroxidase (dilution 1:25 000, SouthernBiotech, Birmingham, United Kingdom) during 2 hours at ambient temperature and again washed. Thereafter, incubation with 100 µL ready-to-use 3,3′,5,5′-tetramethyl-benzidine solution (Kem-En-Tec, Taasrup, Denmark) for 15 to 20 minutes took place for color development. The reaction was stopped by addition of 0.5 mol/L H₂SO₄, and optical density was measured at 450 nm in a Multiskan Ascent plate reader (Laboratory Systems, Oy, Helsinki, Finland). The analytic sensitivity, defined as the minimum BVZ concentration evoking a response significantly different from that of the zero calibrator, was 16 pg/mL. Plasma samples, diluted 4000- to 24 000-fold, exhibited excellent parallelism. To 6 plasma samples, known quantities of Avastin were added. The recoveries ranged in the plasma samples from 87% to 110% with a mean recovery of 98%. In each run the reference preparation, prepared from a pool of plasma from patients treated with BVZ, was used to monitor long-term performance of the assay. The concentration in reference preparation was 1670 ng/mL, the within-run coefficient of variation and the between-run coefficient of variation amounted to 7.0% and 10.0%, respectively.

Study 2: The Effect of BVZ on Endothelium-Dependent Vasodilation

In 12 evaluable volunteers the vasodilator response to ACH was studied in the absence and presence of BVZ. The experiment started with measurement of baseline FBF during saline infusion during 5 minutes followed by 2 increasing dosages of ACH (0.5 and 2.0 µg/dL forearm volume per minute) 5 minutes each. After a washout period of 45 minutes, measurements of FBF response to saline and the 2 increasing dosages of ACH were repeated during continuous infusion of BVZ during 15 minutes. Subjects with <100% increase in FBF in the absence of BVZ were excluded, because of nonevaluable baseline response to ACH and replaced by newly recruited volunteers.

Study 3: The Effect of BVZ on Endothelium-Independent Vasodilation

In a separate group of 12 evaluable volunteers, the vasodilator response to SNP in the absence and presence of BVZ was studied. First baseline FBF was measured during glucose infusion followed by 2 increasing dosages of SNP (0.06 and 0.2 µg/dL forearm volume per minute). After a washout period of 45 minutes, measurements of FBF response to glucose and the 2 increasing dosages of SNP were repeated during continuous infusion of BVZ during 15 minutes.

Statistical Analysis

Data were analyzed using the SPSS (version 16.0) software packages. Values are reported as means±SE unless otherwise specified. Mean arterial pressure was measured continuously during each recording of FBF and averaged per FBF registration. Drug-induced effects were expressed as percentage of change from preceding saline or glucose infusion. The percentage changes in FBF to each dosage of a
vasodilator substance were averaged to 1 value for each vasodilator. These values were compared using a repeated measures ANOVA to assess the effect of BVZ. Before analysis, logarithmic transformation was performed to obtain a Gaussian distribution.

Results
In total, 46 subjects signed informed consent. Five subjects were excluded based on medical history or medication use. Another 5 subjects withdrew consent for personal reasons before start of the study.

Five subjects were excluded after completion of the study protocol because of an absent response to ACH as was pre-specified in our study protocol. In this study, we choose healthy volunteers to rule out other factors influencing endothelial function, such as medication, cardiovascular risk factors, or underlying disease, to assess the specific effect of BVZ on normal endothelial function. Subjects with an absent response to ACH measurement may have other unknown factors that already altered endothelial function and interfere with the response to BVZ. The percentage change in FBF in these 5 individuals compared with the mean FBF in the included group before and during BVZ is depicted in Figure 1. As shown in this figure, the lack of response to ACH was reproduced in the presence of BVZ, excluding regression to the mean as a potential confounder that could have been introduced by this exclusion.

Thirty-four male subjects (23.9±1.2 years) were included in the analysis.

Study 1: The Acute Vasomotor Response to BVZ

BVZ Concentrations
At the end of the 15 minutes infusion of BVZ, BVZ reached a concentration of 136±13.2 μg/mL (n=7) in the experimental arm, resembling plasma concentration after systemic exposure to BVZ in patients treated with 5 mg BVZ IV/kg. BVZ concentration in the control arm was 8.3±0.8 μg/mL (n=7).

Intra-arterial BVZ did not alter baseline FBF (Figure 2). Blood pressure did not change either (baseline mean arterial pressure, 77.1±2.7 mmHg; at the end of BVZ infusion, 79.5±2.2 mmHg).

Study 2: The Effect of BVZ on Endothelium-Dependent Vasodilation
In the experimental arm, FBF increased from 1.3±0.2 at baseline to 5.5±1.1 and 10.6±1.8 mL/dL per minute for the 2 increasing dosages of ACH (0.5 and 2.0 μg ACH/dL forearm per minute), respectively. In the presence of BVZ, ACH increased FBF from 1.4±0.2 to 3.8±0.8 and 8.9±1.8 mL/dL per minute, respectively (Figure 3). In the controlateral arm, FBF remained constant: 1.2±0.2, 1.1±0.2, and 1.2±0.2 mL/dL per minute in the absence of BVZ and 1.1±0.3, 1.0±0.2, and 1.2±0.2 mL/dL per minute in the presence of BVZ for baseline and 2 subsequent ACH doses, respectively (Figure 3). In the absence of BVZ, ACH increased FBF from baseline by 440±157% and 926±252%. In the presence of BVZ, ACH increased FBF from baseline by 169±40% and 612±154%, respectively (n=12; P<0.05 for the effect of BVZ, ANOVA for repeated measures on log-transformed data; Figure 4).

In the absence of BVZ, the percentage increase in FBF during SNP infusion was 270±45% (0.06 μg/dL per minute) and 671±162% (0.2 μg/dL per minute). In contrast to ACH, the vasodilator effect of SNP was not affected by simultaneous infusion of BVZ 248±64% and 679±156% (n=12; P>0.4; Figure 4). For the course in FBF, see Figure 3.

Blood pressure and heart rate did not change in response to the vasodilator agents (Table).

Side Effects
No side effects of BVZ were reported during infusion and 1 to 2 weeks after the experiment as evaluated by a questionnaire.

Discussion
In the present study, we show for the first time in humans that inactivation of circulating VEGF decreases
endothelium-dependent vasodilation within 15 minutes. This proves that circulating VEGF plays a role in maintaining normal endothelial control of vascular tone.

Previously, several clinical studies have shown decreases in endothelium-dependent but also endothelium-independent vasodilation during treatment with tyrosine kinase inhibitors targeting VEGF signaling. However, these studies have been conducted after at least 6 weeks of anti-VEGF treatment when rise in blood pressure already had occurred. Hypertension by itself decreases endothelial function. Prolonged treatment with tyrosine kinase inhibitor targeting the VEGF pathway has been reported to be associated with capillary rarefaction. Rarefaction increases vascular tone and interacts with both endothelium-dependent and endothelium-independent vasodilation. Rarefaction also occurs in idiopathic (essential) hypertensive patients and could, therefore, be a consequence rather than a cause of the rise in blood pressure that often occurs during treatment with these drugs. A recent study in swine showed an increase in blood pressure within a few hours after administration of sunitinib, a tyrosine kinase inhibitor targeting the VEGF pathway. The fast onset of changes in blood pressure makes rarefaction a less likely cause of VEGF targeting tyrosine kinase inhibitor-induced hypertension and suggests that rarefaction is a consequence rather than a cause of hypertension in this group of patients.

In this study, intra-arterial infusion of BVZ achieved a clinically relevant concentration of BVZ in the forearm, whereas systemic exposure to this drug was low. This allowed us to study the local effect of selective VEGF deprivation without interference of systemic effects, such as a rise in blood pressure. Bevacizumab selectively interrupts endogenous VEGF signaling. This contrasts with tyrosine kinase inhibitors, such as sunitinib, which inhibit not only VEGF signaling but also the response to other growth factors, such as platelet derived growth factor or c-Kit. Finally, we studied healthy volunteers, without any comedication that could interfere with vasomotor control such as chemotherapy. Therefore, a strength and unique feature of our study is the specificity of the used pharmacological intervention.

We did not observe an effect of BVZ on vascular tone. This suggests that the effect on the muscle vascular bed is not sufficient to cause hypertension. However, some remarks can be made about this conclusion. BVZ was infused for 15 minutes. The infusion time was based on previous investigations that showed a decrease in blood pressure in response to VEGF-infusion within a few minutes. More importantly, time was limited by the allowed exposure of healthy volunteers to this drug. The given dose was high enough to reach local concentrations mimicking concentrations after systemic exposure in patients, but the continuous arterial influx of free nonbound VEGF could have resulted in some residual VEGF receptor stimulation in our experimental set-up and could, therefore, have

**Figure 3.** Study protocol and actual forearm blood flow (FBF) in response to 2 increasing dosages of acetylcholine (ACH) and sodium nitroprusside (SNP). BVZ indicates bevacizumab.

**Figure 4.** Increases in forearm blood flow (FBF) in response to 2 increasing dosages of acetylcholine and sodium nitroprusside, depicted as percentage change from baseline. Error bars, SE of the mean. Statistical analysis was performed on the log-transformed data. BVZ indicates bevacizumab.
underestimated the effect as observed during systemic therapy with BVZ. In addition, regional differences in the role of VEGF on baseline vascular tone may exist.

Several previous studies (Kappers et al.1,16) have shown that activation of the endothelin axis may be involved in the onset of hypertension during inhibition of VEGF signaling. The reported decrease in endothelium-dependent vasodilation in our study could be because of diminished release of nitric oxide, as supported by the previously mentioned studies.7–10 It has been previously shown that the inhibition of baseline NO can enhance the release of endothelin-1.18,19 Thus, our observation provides a clue to a potential mechanism of increased endothelin-1 release in response to VEGF-targeted therapy.

Perspectives
Our observation does not exclude the possibility that BVZ increases vascular tone in other organs, such as the kidney, which is of particular interest with regard to the pathogenesis of hypertension. Our results indicate that local VEGF deprivation immediately reduces endothelium-dependent vasodilation; however, this study does not allow any conclusion on the mechanism of this altered endothelial function. Apart from a reduced release of NO, reduced formation of other endothelium-derived relaxing factors or an increased release of endothelium-derived contracting factors in response to acetylecholine may have contributed to this observation.

Conclusion
Our study is the first to investigate the acute effect of VEGF inhibition on vascular tone and endothelial function in healthy volunteers. It was performed in healthy volunteers to prevent a possible influence of previous and current medical treatment or disease on the vascular response to VEGF inhibition. Our study using a specific VEGF antibody suggests a role for circulating VEGF in normal endothelial control of vascular tone.

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Disclosures
None.

References

Table. Blood Pressure and Heart Rate

<table>
<thead>
<tr>
<th>Infusion Period</th>
<th>ACH MAP, mm Hg</th>
<th>Heart Rate, bpm</th>
<th>SNP MAP, mm Hg</th>
<th>Heart Rate, bpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>79.5±2.1</td>
<td>60±2</td>
<td>77.0±2.2</td>
<td>61±3</td>
</tr>
<tr>
<td>Vasodilator (low)</td>
<td>79.4±2.0</td>
<td>60±2</td>
<td>76.6±2.2</td>
<td>61±3</td>
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<tr>
<td>Vasodilator (high)</td>
<td>79.3±2.2</td>
<td>62±3</td>
<td>76.4±2.3</td>
<td>61±2</td>
</tr>
<tr>
<td>BVZ baseline</td>
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<td>60±2</td>
<td>81.8±2.2</td>
<td>60±3</td>
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<tr>
<td>BVZ vasodilator (low)</td>
<td>81.0±1.9</td>
<td>62±3</td>
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<td>60±3</td>
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<tr>
<td>BVZ vasodilator (high)</td>
<td>81.0±2.1</td>
<td>62±3</td>
<td>81.2±2.2</td>
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ACH indicates acetylcholine; BVZ, bevacizumab; MAP, mean arterial pressure; and SNP, sodium nitroprusside.


**What Is New?**
- Endogenous vascular endothelial growth factor (VEGF) plays a critical role in maintenance of normal endothelial function.
- Hypertension in patients who are treated with inhibitors of VEGF signaling could be a consequence of impaired endothelial function.

**What Is Relevant?**
- This observation provides mechanistic insight in the vascular effects of anticancer therapies that inhibit VEGF signaling, such as hypertension, heart failure, and proteinuria.

**Summary**
In healthy subjects, inactivation of circulating VEGF, by infusion of VEGF antibodies, acutely decreases endothelium-dependent vasodilation. This suggests a role for circulating VEGF in normal endothelial control of vascular tone.
Role of Endogenous Vascular Endothelial Growth Factor in Endothelium-Dependent Vasodilation in Humans