Suppression of the Nicotinic Oxide Pathway in Metastatic Renal Cell Carcinoma Patients Receiving Vascular Endothelial Growth Factor–Signaling Inhibitors

Emily S. Robinson, Eliyahu V. Khankin, Toni K. Choueiri, Mallika S. Dhawan, Miranda J. Rogers, S. Ananth Karumanchi, Benjamin D. Humphreys

Abstract—Therapies that target the vascular endothelial growth factor (VEGF) pathway cause hypertension, but the mechanism remains unknown. This cross-sectional study tested the hypothesis that VEGF inhibition causes hypertension by suppressing VEGF-mediated vasodilatory pathways. Urine was collected from 80 patients with metastatic renal cell carcinoma from 2002 to 2009, 40 at baseline and 40 while on VEGF inhibitors. Measured urinary biomarkers include albumin, metabolites of the nitric oxide (NO) pathway and its downstream effector cGMP, and prostaglandin pathway biomarkers prostaglandin E2, 6-keto prostaglandin F1α, and cAMP, all normalized to urinary creatinine. The mean age in both groups was 61.8 years, 76% were men, and urinary albumin was higher in patients receiving VEGF inhibitors (median: 18.4 versus 4.6 mg/g; P=0.009). cGMP/creatinine was suppressed in patients on VEGF inhibitors (0.28 versus 0.39 pmol/μg; P=0.01), with a trend toward suppression of nitrate/creatinine (0.46 versus 0.62 μmol/mg; P=0.09). Both comparisons were strengthened when patients on bevacizumab were excluded, and only those receiving small molecule tyrosine kinase inhibitors were analyzed (cGMP/creatinine: P=0.003; nitrate/creatinine: P=0.01). Prostaglandin E2, 6-keto prostaglandin F1α, and cAMP did not differ between groups. These results suggest that hypertension induced by VEGF inhibitors is mediated by suppression of NO production. Prospective studies are needed to explore whether these biomarkers may be useful predictors of efficacy in patients receiving VEGF-targeted therapies. (Hypertension. 2010;56:1131-1136.)

Key Words: hypertension ■ albuminuria ■ angiogenesis inhibitors ■ NO ■ VEGF

The use of vascular endothelial growth factor (VEGF)–targeted therapies, also called antiangiogenic therapies, to treat solid tumors is growing rapidly.1 Food and Drug Administration–approved antiangiogenic therapies include bevacizumab, a humanized monoclonal antibody directed against VEGF, and small molecule multitargeted kinase inhibitors, including sorafenib, sunitinib, and pazopanib. The small molecule antiangiogenic drugs target VEGF receptors, as well as the platelet-derived growth factor receptor RAS and c-KIT.2 Together, these new therapies have had a profound impact on the treatment of multiple tumor types.

VEGF-targeted therapies are strongly associated with the development of both hypertension and proteinuria. Some studies have noted hypertension in >80% of treated patients and an absolute elevation in blood pressure (BP) was observed in 93% of patients on sorafenib.3–5 Guidelines for the surveillance and management of hypertension in these patients were published recently by the National Cancer Institute, but they note that the mechanism of hypertension remains undetermined, and few data exist on which to base treatment recommendations.7 Proteinuria, measured as dipstick albuminuria, is also seen in 20% to 63% of patients, with a higher risk for renal cell carcinoma than for other tumor types.8–10 Importantly, the development of hypertension on VEGF-targeted therapy correlates with improved cancer outcomes and, therefore, could represent a biomarker useful for selecting the subset of patients with the best chance of responding to antiangiogenic therapy.11–14 Understanding the pathophysiology of hypertension on these agents may, therefore, lead to the development of other novel efficacy biomarkers.

Preclinical evidence implicates the inhibition of VEGF signaling in the development of hypertension on these agents. VEGF stimulates production of vasodilatory nitric oxide (NO) via activation of endothelial NO synthetase15,16 and also upregulates vasodilatory prostacyclin.17,18 Small molecule VEGF-targeted therapies inhibit the nitrate pathway in vitro,19 and in humans, hypertension often develops within the first few days of treatment, consistent with acute suppression of NO- and/or prostacyclin-mediated vasodilation.20–22 In mice, blockade of
VEGF signaling by administration of an antibody directed against VEGF receptor 2 also caused hypertension, but addition of an NO synthetase inhibitor abolished the BP difference between control and antibody groups, suggesting that VEGF inhibition causes hypertension by depressing NO levels.20 These observations led us to hypothesize that, in humans, VEGF-targeted antiangiogenic therapies suppress the nitrate and prostaglandin pathways, leading to hypertension. This cross-sectional study explored urinary biomarkers of the nitrate and prostaglandin pathways in patients with renal cell carcinoma, comparing patients receiving antiangiogenic therapy with patients not receiving antiangiogenic therapy.

Methods

Study Population

Urine samples were collected from 300 patients in the Dana Farber Cancer Institute Kidney Cancer Center between 2002 and 2009. The 80 patients for this particular study were all patients with metastatic renal cell carcinoma for whom we had a single urine collection. Forty patients were on antiangiogenic therapy at the time of the urine collection and were all included in this analysis, and 40 control patients were selected by a random selection method among the 60 patients who went on to initiate first-line VEGF inhibitors within the 6 months after collection of the urine sample, because we did not have enough candidates for controls to match on clinical factors. We assume that those who went on the medications shortly after the urine collection would likely have had similar tumor characteristics to the 40 patients on the medications at the time of the collections.

Inclusion criteria for the study were a biopsy-confirmed diagnosis of metastatic renal cell carcinoma and the VEGF inhibitor criteria listed above. Exclusion criteria included the use of nitrate medications for heart disease and uncontrolled baseline BP before starting VEGF-targeted therapy (systolic BP: >180 mm Hg; diastolic BP: >110 mm Hg).

Urine was collected by midstream clean catch at clinic visits, and within 4 hours of collection it was centrifuged at 2000 g for 10 minutes. The supernatant was aliquoted without addition of protease inhibitors and stored at −80°C until analysis. This study was approved by the institutional review board, and all of the patients signed consent forms for the urine sample collections for research use.

Measurement of Biomarkers

Commercially available kits were used to measure nitrates, the NO metabolite, and its downstream effector cGMP, as well as cAMP, prostaglandin E2 (PGE2), and 6-keto prostaglandin F (6-keto PGF 1α), a stable metabolite of prostacyclin (6-keto PGF 1α from Cayman Chemical; all others from R&D Systems). All of the biomarker values were normalized to urinary creatinine to account for differences in urinary concentration. Nitrates were measured by fluorescence spectroscopy and were measured as NO2. NO3 is the sum of NO2 + NO3, the stable metabolites of NO, after subtraction of endogenous nitrates. The coefficient of variation for the assay was 3.5%. cGMP, cAMP, and PGE2, and 6-keto PGF 1α were measured by ELISA, and the coefficients of variation for these assays were 9.0%, 8.2%, 12.4%, and 9.0%, respectively. Albumin and creatinine were measured using an Afinion microalbumin machine. Coefficients of variation for both of these assays were 3%. Albuminuria was calculated as the albumin:creatinine ratio (ACR), (albumin/creatinine)*100.

Measurement of BP

BP was measured at the time of the urine collections for the majority of the participants. For those in which there was no BP recorded at the time of the urine collection, the BP from the closest recorded clinic visit was used. For patients on VEGF inhibitors, baseline BPs were recorded from the medical chart as the BP immediately before the time at which they started the medication. Although BPs were measured at clinic visits, there was no standardized protocol for measurement of BPs in these patients. There was no difference in measurement technique between patients in the different groups. Because many patients receiving VEGF-targeted therapy were started on antihypertensive medications, we also recorded the number of BP medications that each patient was taking at the time of the urine collection.

Analysis of Covariates

Clinical information was collected by medical chart review by study coinvestigators. Covariates included age, body mass index, previous hypertension, family history of hypertension or cardiovascular disease, nephrectomy status, current smoking, and estimated glomerular filtration rate by the Chronic Kidney Disease Epidemiology Collaboration equation, chosen instead of the Modification of Diet in Renal Disease equation because of increased accuracy with values of estimated glomerular filtration rate >60 mL/min per 1.73 m2.23

Statistical Analysis

Wilcoxon rank-sum tests were used for linear comparisons between the 2 groups for albuminuria and for urinary biomarkers, which were not normally distributed, and t tests were used for all of the other linear comparisons. $\chi^2$ tests were used for binary comparisons. Spearman correlation coefficients for nonnormal data were used for correlations between ACR and other urinary biomarkers.

Nicotine is known to decrease urinary cGMP levels in nonsmokers but to increase urinary cGMP levels in smokers.24 Therefore, it would be important to look at smokers and nonsmokers separately. However, because there were not enough smokers for a separate analysis, we ran secondary analyses excluding smokers.

We also performed secondary analyses with log-transformed linear regression models to determine whether certain covariates confounded the relationship between VEGF inhibitor use and cGMP/creatinine (Cr) and 2-variable logistic regression models to detect any impact of covariates affecting the association between cGMP/Cr and macroalbuminuria. Additional analyses were performed comparing the urinary biomarkers in patients on bevacizumab separately from patients on small molecule inhibitors, as they have different mechanisms of action, and, therefore, their effect on vasodilatory pathways might be different.

Results

Patient Characteristics

The average age in both groups was 61.8 years, and 23% of the patients on VEGF inhibitors and 25% of those not on VEGF inhibitors were women (Table 1). This is reflective of the demographics of metastatic renal cell carcinoma. Baseline systolic BP was similar in both groups. Although the only statistically significant difference between the 2 groups was the frequency of previous hypertension, there were more nephrectomies and higher angiotensin-converting enzyme inhibitor use in the VEGF inhibitor group.

Comparison of Urinary Biomarkers

cGMP was suppressed in patients on VEGF inhibitors ($P = 0.01$), and nitrate levels also trended toward suppression in these patients, although this was not statistically significant ($P = 0.09$). cAMP, PGE2, and 6-keto PGF 1α were not different between the 2 groups (Table 2). After removing smokers from the analysis, cGMP was still suppressed in patients on VEGF inhibitors ($P = 0.02$). Because of the differences in nephrectomy status, angiotensin-converting enzyme inhibitor use, and previous hypertension between the 2 groups, secondary analyses were performed with log-transformed linear regres-
Comparison Between Classes of VEGF Inhibitor
Bevacizumab acts by binding and sequestering soluble VEGF, whereas the small molecule antiangiogenic therapies block intrinsic VEGF receptor tyrosine kinase activity. Because these separate mechanisms of action might influence vasodilatory pathways differently, we performed a stratified analysis and compared both groups with the non-VEGF inhibitor controls (Table 4). Comparing the patients receiving only small molecule inhibitors with patients not receiving any VEGF inhibitor strengthened the association between antiangiogenic therapy and suppression of cGMP/Cr and NOx/Cr ($P=0.003$ for cGMP/Cr; $P=0.01$ for NOx/Cr). However, there was no significant difference between these levels when comparing only patients on bevacizumab with controls and no differences in these associations for any of the other biomarkers. ACR values were higher in patients on both types of VEGF inhibitor compared with control patients.

We next compared urinary biomarkers in patients on the 2 classes of VEGF inhibitors (Table 5). NOx/Cr levels were higher in patients on bevacizumab (0.67 versus 0.36; $P=0.01$). There was no statistically significant difference between any of the other biomarkers, but given the low number of patients on bevacizumab we may lack power to detect a difference.

**BP Analysis**
BP values at baseline between the patients on VEGF inhibitors and those not on VEGF inhibitors were similar (Table 1). After starting therapy, the mean systolic BP in patients on VEGF inhibitors was 131.8 mm Hg compared with the mean systolic BP in patients not on VEGF inhibitors of 124.7 mm Hg ($P=0.09$). However, the median number of BP medications in patients receiving VEGF-targeted therapy was 1.5 (interquartile range: 0 to 2.5) compared with a median of 0 (interquartile range: 0 to 1) medications in patients not on VEGF-targeted therapy ($P=0.002$ for the comparison). There was no difference in the number of BP medications in patients on bevacizumab and those on other VEGF-targeted therapies (Table 4).

**Analysis of Albuminuria**
Spearman correlation coefficients were calculated between ACR and all of the urinary biomarkers (Table 6). Correlations were noted between ACR and both cGMP/Cr ($r=0.44$; $P=0.004$) and 6-keto PGF 1α/Cr ($r=0.31$; $P=0.05$). Although the small number of outcomes limited our ability to do multivariate regression, bivariate regression revealed that, even after adjustment for age, previous hypertension, nephrectomy status, diabetes mellitus, and angiotensin-converting enzyme inhibitor use in logistic regression models, cGMP/Cr was
Table 4. Urinary Biomarkers in Renal Cell Carcinoma Patients on Small Molecule VEGF Inhibitors Only (N=29) and Bevacizumab Only (N=11) Compared With Controls Not on VEGF Inhibitors With Median (Interquartile Range) and P Value for Comparison by Wilcoxon Test

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Non-VEGF Inhibitor Controls (N=40)</th>
<th>Small Molecule VEGF Inhibitors (N=29)</th>
<th>P vs Controls</th>
<th>Bevacizumab (N=11)</th>
<th>P vs Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOx/Cr, μmol/mg</td>
<td>0.62 (0.42 to 0.84)</td>
<td>0.36 (0.26 to 0.58)</td>
<td>0.01</td>
<td>0.67 (0.45 to 1.41)</td>
<td>0.52</td>
</tr>
<tr>
<td>cGMP/Cr, pmol/μg</td>
<td>0.39 (0.30 to 0.62)</td>
<td>0.25 (0.22 to 0.36)</td>
<td>0.003</td>
<td>0.47 (0.19 to 0.77)</td>
<td>0.66</td>
</tr>
<tr>
<td>PGE2/Cr, pg/μg</td>
<td>1.31 (0.99 to 1.69)</td>
<td>1.08 (0.78 to 1.82)</td>
<td>0.49</td>
<td>1.50 (0.80 to 2.54)</td>
<td>0.78</td>
</tr>
<tr>
<td>cAMP/Cr, pmol/μg</td>
<td>5.12 (3.88 to 6.17)</td>
<td>5.09 (4.68 to 7.43)</td>
<td>0.15</td>
<td>4.71 (4.27 to 6.49)</td>
<td>0.18</td>
</tr>
<tr>
<td>6-keto PGF 1α/Cr, pg/μg</td>
<td>1.13 (0.75 to 1.55)</td>
<td>1.03 (0.85 to 1.31)</td>
<td>0.86</td>
<td>1.26 (0.94 to 2.22)</td>
<td>0.98</td>
</tr>
<tr>
<td>ACR, mg/g</td>
<td>4.6 (0 to 35.6)</td>
<td>18.5 (0 to 232.3)</td>
<td>0.03</td>
<td>18.3 (7.9 to 607.9)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

NOx indicates nitric oxide.

Table 5. Comparison of Urinary Biomarkers and BPs in Patients on Bevacizumab and Patients on Small Molecule VEGF inhibitors, With Medians (Interquartile Range) and P Values by Wilcoxon Test for Comparisons

<table>
<thead>
<tr>
<th>Biomarker or Clinical Variable</th>
<th>Bevacizumab (N=11)</th>
<th>Small Molecule VEGF Inhibitors (N=29)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOx/Cr, μmol/mg</td>
<td>0.67 (0.45 to 1.41)</td>
<td>0.36 (0.26 to 0.58)</td>
<td>0.01</td>
</tr>
<tr>
<td>cGMP/Cr, pmol/μg</td>
<td>0.47 (0.19 to 0.77)</td>
<td>0.25 (0.22 to 0.36)</td>
<td>0.28</td>
</tr>
<tr>
<td>PGE2/Cr, pg/μg</td>
<td>1.50 (0.80 to 2.54)</td>
<td>1.08 (0.78 to 1.82)</td>
<td>0.48</td>
</tr>
<tr>
<td>cAMP/Cr, pmol/μg</td>
<td>4.71 (4.27 to 6.49)</td>
<td>5.09 (4.68 to 7.43)</td>
<td>0.30</td>
</tr>
<tr>
<td>6-keto PGF 1α/Cr, pg/μg</td>
<td>1.26 (0.94 to 2.22)</td>
<td>1.03 (0.85 to 1.31)</td>
<td>0.07</td>
</tr>
<tr>
<td>ACR, mg/g</td>
<td>18.3 (7.9 to 607.9)</td>
<td>18.5 (0 to 232.3)</td>
<td>0.55</td>
</tr>
<tr>
<td>No. of BP medications</td>
<td>2 (0 to 3)</td>
<td>1 (0 to 2)</td>
<td>0.82</td>
</tr>
<tr>
<td>Days of medication before collection</td>
<td>140 (80 to 245)</td>
<td>70 (21 to 168)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

NOx indicates nitric oxide.

Table 6. Spearman Correlation Coefficients Between Biomarkers and ACR in Patients on VEGF-Targeted Therapies (N=40)

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Spearman Correlation Coefficient</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOx/Cr</td>
<td>-0.08</td>
<td>0.61</td>
</tr>
<tr>
<td>cGMP/Cr</td>
<td>0.44</td>
<td>0.004</td>
</tr>
<tr>
<td>PGE2/Cr</td>
<td>0.12</td>
<td>0.46</td>
</tr>
<tr>
<td>cAMP/Cr</td>
<td>0.07</td>
<td>0.66</td>
</tr>
<tr>
<td>6-keto PGF 1α/Cr</td>
<td>0.31</td>
<td>0.05</td>
</tr>
</tbody>
</table>

NOx indicates nitric oxide.

significantly associated with macroalbuminuria (P<0.05 for all of the bivariate analyses).

Discussion

In this cross-sectional pilot study, urinary biomarkers of the NO pathway were suppressed in patients receiving VEGF-targeted chemotherapies. Although the suppression of nitrate levels was not statistically significant, its measurement can be affected by diet, and cGMP may be a more accurate reflection of NO pathway activity.26 These findings remained significant after adjusting for age, previous hypertension, angiotensin-converting enzyme inhibitor use, and nephrectomy status, although nephrectomy status did change the effect estimate. As expected, PGE2 and cAMP were not influenced by VEGF inhibition. Although VEGF can regulate vasodilatory prostacyclin production, 6-keto PGF 1α was not suppressed in this study. Together, these results support the theory that hypertension associated with VEGF-targeted therapies is caused by inhibition of NO-mediated vasodilation.4 VEGF inhibition may also contribute to hypertension by other mechanisms. For example, the proximal tubule natriuretic response to elevated BP is partially dependent on cGMP, and VEGF-targeted therapies might suppress this response, perpetuating the rise in BP.28–30 Our data do not rule out a contribution from capillary rarefaction to hypertension induced by VEGF blockade, as has been proposed,31,32 or from increased circulating endothelin 1, as reported recently.33

Although only 11 (28%) of 40 of patients were on bevacizumab and the rest were on small molecule VEGF receptor inhibitors, the difference in biomarkers between the 2 groups is striking. This is the first study reporting these comparisons, and inhibition of the NO pathway was much more profound in patients receiving small molecule VEGF inhibitors. Although not statistically significant, patients on bevacizumab had been in the study longer by the time of the urine collection (140 versus 70 days; P=0.09). However, they were similar with respect to previous hypertension (64% versus 75%; P=0.44), nephrectomy status (73% versus 86%; P=0.32), angiotensin-converting enzyme inhibitor use (36% versus 34%; P=0.82), diabetes mellitus (18% versus 17%; P=0.94), and median ACR values (18.3 versus 18.5 mg/g; P=0.55). The reason for these findings requires further investigation.

In both patients on bevacizumab and other types of VEGF inhibitors, ACR was elevated, and there was a higher incidence of macroalbuminuria than in patients not on VEGF inhibitors. These results are expected, because albuminuria is a well-described complication of antiangiogenic therapy reflecting inhibition of paracrine VEGF signaling between VEGF-producing glomerular podocytes and adjacent endothelial cells.34 Inhibition of podocyte-endothelial cell VEGF...
signaling, whether through genetic or pharmacological means, causes endotheliosis, thrombotic microangiopathy, and narrowing of the capillary lumen, the pathological lesion seen in human kidney biopsy specimens from patients with albuminuria receiving VEGF-targeted therapies.34,35

Because endothelial knockout of NO leads to renal thrombotic microangiopathy in mice36 and albuminuria from chronic VEGF inhibition likely reflects renal thrombotic microangiopathy in humans,37 we expected that patients receiving VEGF-targeted therapy with higher levels of albuminuria would also have suppressed NO pathway biomarkers. However, we observed that, although NO pathway activity was lower than in control patients not receiving these drugs, urinary cGMP positively correlated with a higher degree of albuminuria, indicating that individuals with albuminuria had the least degree of nitrate pathway inhibition. The reasons for this observation are unclear. Acute thrombotic microangiopathy syndromes can be associated with an upregulation of endothelial NO synthetase in rats, potentially as a vasodilatory compensation mechanism to maintain renal perfusion.37 Elevation of endothelial NO synthetase has been observed in renal biopsies of patients with diabetic nephropathy, a glomerular pathology characterized by endothelial damage.38 Finally, increased endothelial shear stress can activate endothelial NO synthetase activity directly, presumably through a VEGF-independent mechanism.39 Although clearly speculative, chronic VEGF inhibition may cause endothelial dysfunction with albuminuria with subsequent compensatory activation of endothelial NO signaling through VEGF-independent pathways.40,41 The increase in 6-keto PGF 1α with increasing ACR (Spearman correlation coefficient \( r = 0.31; P = 0.05 \)) also supports this theory, as prostacyclins can be upregulated in the setting of endothelial damage as well.42,43

There are inherent limitations to this analysis. BP measurements were not standardized. However, there was no difference in the way BP was measured between the 2 groups or from baseline to the time of the urine collection in the VEGF inhibitor group. This was a cross-sectional study, comparing patients on and off VEGF inhibitors. Although we tried to account for this by choosing patients for our control group only if they were started on VEGF inhibitors within 6 months of the collection, using these 2 groups instead of a prospective study is still not optimal. Urine specimens were collected from patients at different time points during their medication use. This may influence biomarkers if they change over time and would bias our results to the null when compared with measurements over standardized time intervals.

Perspectives
This pilot study demonstrates NO pathway suppression in patients on VEGF-targeted therapies. The results suggest a difference in pathophysiology between the mechanisms of hypertension and albuminuria induced by VEGF inhibition. Understanding the pathophysiology of these toxicities takes on increased importance as the clinical use of VEGF-targeted therapies grows. Because BP rise itself may represent a biomarker for clinical efficacy in individual patients, these results suggest that urinary cGMP might also be a useful efficacy biomarker less susceptible to the variations of office BP measurement.6 Future prospective studies will be required to correlate these novel biomarkers with cancer outcomes and the possible role of other pathways, such as prostacyclins or endothelin 1.44

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Disclosures
S.A.K. is listed as a coinventor on multiple patents held by the Beth Israel Deaconess Medical Center for the diagnosis and therapy of preeclampsia. These patents have been nonexclusively licensed to multiple companies. S.A.K. is a consultant to Beckman Coulter, Johnson & Johnson, Roche, and Abbott Diagnostics that are developing biomarkers for preeclampsia diagnosis/prediction.

References


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