HYPERTENSION AND RENAL TOXICITY DURING ANGIOGENESIS INHIBITION: SALT DEPENDENCY AND TREATMENT OPTIONS

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HYPERTENSION AND RENAL TOXICITY DURING ANGIOGENESIS INHIBITION: SALT DEPENDENCY AND TREATMENT OPTIONS

HYPERTENSIE EN NEFROTOXICITEIT TIJDENS ANGIOGENESEREMMING: ZOUTAFHANKELIJKHEID EN BEHANDELINGSOPTIES

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'It always seems impossible until it's done'

Nelson Mandela 1918 - 2013

CONTENTS

CHAPTER 1

General introduction part 1

MECHANISMS OF HYPERTENSION AND PROTEINURIA DURING ANGIOGENESIS INHIBITION: EVOLVING ROLE OF ENDOTHELIN-1

Stephanie Lankhorst, Mariëtte H.W. Kappers, Joep H.M. van Esch, A.H. Jan Danser, Anton H. van den Meiracker.

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ABSTRACT

Angiogenesis inhibition by blocking vascular endothelial growth factor (VEGF)-mediated signalling with monoclonal antibodies or tyrosine kinase inhibitors has become an established treatment of various forms of cancer. This treatment is frequently associated with the development of hypertension and proteinuria. Since VEGF increases the expression and the activity of nitric oxide synthase in endothelial cells, a decrease in the bioavailability of nitric oxide has been proposed as a key mechanism leading to hypertension during angiogenesis inhibition. However, results of clinical and experimental studies exploring this possibility are conflicting. Rarefaction, i.e. a structural decrease of microcirculatory vessels, has been reported during anti-angiogenic treatment, but evidence that it plays a role in development of hypertension is lacking. Elevated circulating and urinary levels of endothelin-1 have been observed in clinical and experimental studies with angiogenesis inhibitors. Furthermore, the observation that endothelin receptor blockers can prevent or revert the rise in blood pressure during angiogenesis inhibition and attenuate proteinuria, provides strong evidence that an activated endothelin-signalling pathway is a final common mediator of angiogenesis-inhibition-induced rise in blood pressure and renal toxicity.

INTRODUCTION

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is critical to solid tumor growth as well as metastasis. This process is regulated by various growth factors of which vascular endothelial growth factor (VEGF) with its corresponding receptors plays a prominent role.[1, 2] Inhibition of angiogenesis with humanized monoclonal antibodies to VEGF or with inhibitors of the VEGF receptors (RTKIs) has become an established treatment for various forms of cancer. Hypertension and proteinuria are common side effects of this treatment.[1, 3-7] These features are also characteristic for preeclampsia. Accumulating evidence indicates that an imbalance between pro- and anti-angiogenic factors is involved in the clinical features of preeclampsia. According to current insight, placental overproduction and release of the anti-angiogenic factors in the maternal circulation inhibit angiogenesis and like exogenous VEGF inhibition applied as anticancer treatment, results in hypertension and proteinuria.[8-10] How exogenous VEGF inhibition during anti-angiogenic treatment or endogenous VEGF inactivation during preeclampsia leads to hypertension and proteinuria is not precisely known. Recently, we and others have shown that activation of the endothelin pathway may be an important final mechanism.[11-13] In this article the effects of interference with VEGF signalling, either exogenously or endogenously, on the development of hypertension and proteinuria, will be reviewed.

VEGF, VEGFRs AND BIOLOGICAL EFFECTS OF VEGF SIGNALING

The mammalian VEGF belongs to a gene family that includes 5 different glycoproteins and the placental growth factors (PlGFs) 1 and 2 (Figure 1). Of the VEGF family VEGF-A, commonly referred to as VEGF, is best characterized.[14, 15] VEGF is produced and secreted by different cells including endothelial cells (ECs), podocytes, macrophages, and fibroblasts.[16, 17] VEGF produced and secreted by tumor cells targets ECs to promote tumor angiogenesis.[18] VEGF is up-regulated by hypoxia-inducible factor (HIF) 1α and several other factors.[2, 16] VEGF binds to three tyrosine kinase receptors: VEGF receptor 1 (VEGFR-1 or fms-like tyrosine kinase 1 (Flt-1) murine homologue), VEGF receptor 2 (VEGFR-2 or kinase domain region (KDR) human homologue or Flk-1 murine homologue) and VEGFR-3 (also known as FLT4). VEGFR-1 and VEGFR-2 are predominantly expressed on vascular ECs, whereas VEGFR-3, stimulated by VEGF-C, is largely restricted to lymphatic ECs.[19] VEGFRs contain an extracellular region, a transmembrane domain and a cytoplasmatic bipartite kinase domain with tyrosine residues (Figure 1). Neurophilins 1 and 2 serve as coreceptors for VEGF by increasing the affinity of ligands to the VEGFRs.[19] Under physiological conditions most of the biologically relevant VEGF-signaling in ECs is mediated by VEGFR-2.[20] The extracellular domain of Flt-1 or VEGFR-1 is also present at low concentrations as a soluble protein (sFlt-1). In preeclampsia the placental production of sFlt-1 is

considerably increased.[9, 21] In the maternal circulation sFlt-1 binds to VEGF acting as an endogenous VEGF antagonist.[9]

VEGF exerts many biological activities, including angiogenesis during embryogenesis, menstrual cycle and wound healing, but also has been recognized as a positive regulator of growth and metastasis of malignancies. Genetic ablation experiments in mice have shown that a single VEGF allele is insufficient to establish a proper vascular network and knockout mice die early in embryogenesis.[22] Besides its pro-angiogenic activity VEGF increases vascular permeability and is required for the maintenance of a differentiated phenotype of ECs and especially for the establishment and maintenance of endothelial fenestrae.[23-25] More recent studies have shown that autocrine VEGF signalling is a prerequisite for EC survival.[26] Genetic deletion of VEGF in ECs without interruption of the paracrine VEGF-signalling pathway, leads to progressive EC degeneration and sudden death in transgenic mice.[26] Finally, VEGF-signalling has shown to be essential for the maintenance of the glomerular filtration barrier.[27]

EXOGENOUS INHIBITORS OF THE VEGF SIGNALLING PATHWAY

Three approaches have been developed to inhibit the VEGF-signalling pathway (Figure 1).[2] Firstly, bevacizumab (Avastin, Genentech, South San Francisco, CA, USA) is a humanized monoclonal antibody that selectively binds VEGF. This compound has been approved for systemic use in various forms of cancer.[17, 28] In addition, bevazicumab is applied in ocular diseases.[29, 30] Secondly, RTKIs, of which sunitinib (SU011248, Sutent, Pfizer Inc., New York, NY, USA), sorafenib (Bay 43-9006, Nexavar, Bayer Pharma AG, Berlin, Germany) and pazopanib (Votrient, ClaxoSmithKline, United Kingdom) have been FDA-approved for treatment of various cancers, block the intracellular domain of the tyrosine kinase receptors.[31, 32] These agents have a low molecular size and can be taken orally. The RTKIs typically target a number of tyrosine kinases.[18] Thirdly, VEGF-trap (Aflibercept, Eylea, Regeneron Pharmaceuticals, Inc, Tarrytown, NY, USA) is a soluble receptor that binds to VEGF and therefore shares close similarity to sFlt-1. It has been approved for the intra-ocular treatment of age-related macular degeneration.[33]

Because under physiological conditions at least 99% of ECs is quiescent, adverse effects during anti-angiogenesis treatment were expected to be minimal. However, in line with knockout experiments in mice showing that VEGF is essential for EC survival.[26] and maintenance of glomerular filtration barrier function.[9, 27], inhibition of VEGF is associated with considerable cardiovascular and non-cardiovascular toxicity, including hypertension, left ventricular dysfunction, cardiac ischemia, myocardial infarction, proteinuria, renal function impairment, thrombosis, cerebral and intestinal haemorrhage, thyroid dysfunction and skin manifestations.[1, 34, 35]

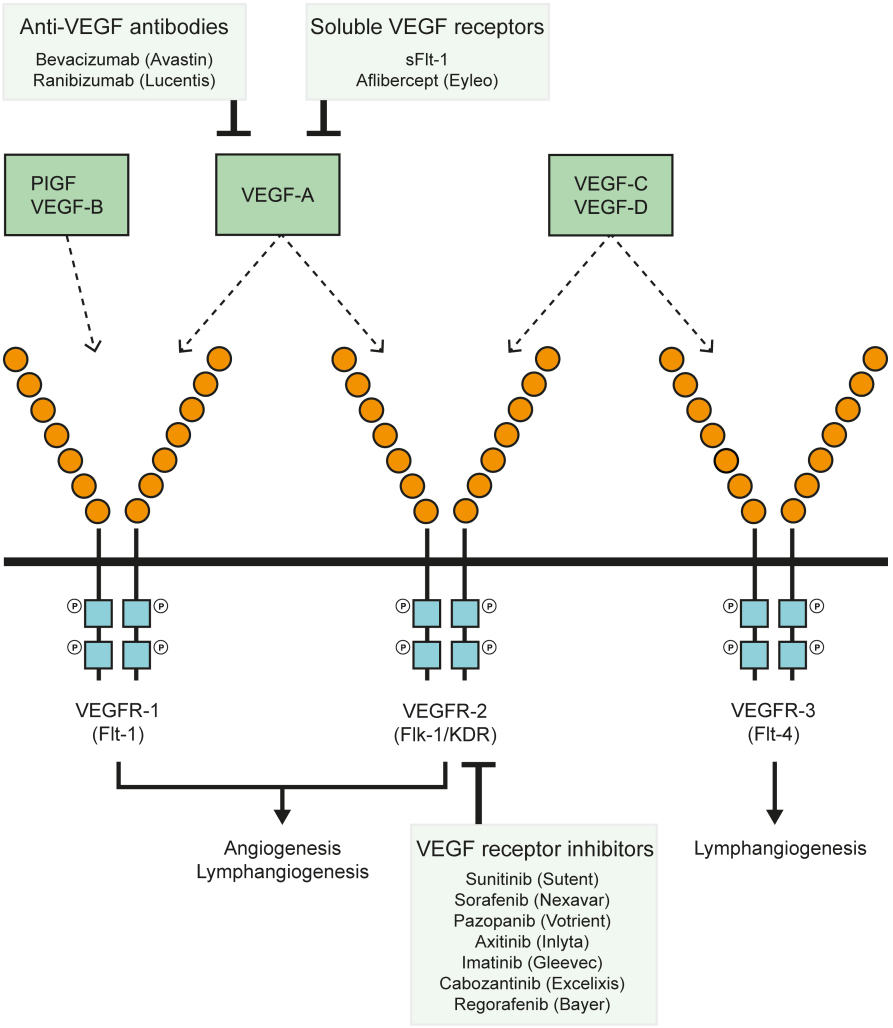

Figure 1. Overview of the VEGF family, VEGF receptors and possibilities of interference. PlGF, placental growth factor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

INCIDENCE, TIME COURSE AND SEVERITY OF HYPERTENSION

Blood pressure (BP) rises in virtually every patient treated with a VEGF inhibitor. The overall incidence of hypertension induced by bevacizumab and RTKIs ranges from 9% to 67%, whereas more severe hypertension (Grades 3, 4) has been reported in 3-18% of patients treated with these agents.[3] Compared to normotensive subjects, patients with a history of hypertension appear to be more at risk for the development of severe hypertension during angiogenesis inhibition.[36] According to the Common Toxicity Criteria for Adverse Events, hypertension in clinical trials is diagnosed when

BP is higher than 140/90 mmHg or diastolic BP (DBP) has increased by more than 20 mmHg (Table 1). These threshold values are higher than those used in guidelines of hypertension societies.[1, 37] According to the criteria of these guidelines the incidence of systolic hypertension in previously normotensive subjects was around 80% at the end of the second cycle of sunitinib.[38] The incidence of hypertension appears to be dose-related as has been shown for bevacizumab, and can increase to over 90% when bevacizumab and sunitinib are combined.[1, 3] In sporadic cases, hypertension is complicated by the posterior reversible encephalopathy syndrome.[39] Some clinical and experimental studies have investigated the time course of the rise in BP after starting anti-angiogenic therapy.[11, 38, 40-43] From these studies it appears that the rise in BP can develop within hours to days after treatment initiation.[11, 41-43] Although hypertension has initially been considered to be a toxic effect of anti-angiogenic therapy, evidence has accumulated that the development of hypertension is predictive for a favourable anti-tumor response and improved survival.[38, 44-49] A meta-analysis concerning patients with renal cancer treated with sunitinib has shown that the progression-free survival was 12.5 months in patients who developed hypertension compared to 2.5 months in patients who did not. For overall survival these values were respectively 30.9 and 7.2 months.[38, 48, 49] These findings support the view that identical mechanisms may underlie the anti-tumor response and the development of hypertension.[50] Although prospective studies are needed to confirm the association between the development of hypertension and the anti-tumor response during anti-angiogenic therapy, it has already been suggested that dose-titration with the objective to increase BP has the potential to result in a better anti-tumor effect and hence to improve clinical outcome.[38, 51]

INCIDENCE AND SEVERITY OF PROTEINURIA

The incidence of proteinuria associated with angiogenesis inhibition appears to be lower than that of hypertension and its severity may vary from <1 gram/day (Grade 1) (Table 1) to nephrotic range proteinuria.[5, 52] In a meta-analysis involving 1850 patients with different forms of cancer the relative risks to develop proteinuria and hypertension were respectively 1.4 and 3.0 for a low and 2.2 and 7.5 for a high dose of bevacizumab.[7] In a more recent meta-analysis involving 12.268 patients with various cancers the incidence of all grades of proteinuria was 13.3%, and of Grade 3 or 4 proteinuria (proteinuria >3.5 g/24h) 2.2%.[53] Compared to chemotherapy alone, the relative risk after addition of bevacizumab was 4.8 for high grade proteinuria and 7.8 for nephrotic syndrome range proteinuria. As observed for hypertension higher doses of bevacizumab were associated with a greater risk of proteinuria and regarding to the type of malignancy renal cell carcinoma was associated with the highest risk.[53] Information about the incidence and severity of proteinuria with the RTKIs sunitinib and sorafenib is limited to case reports.[54-56] In an open label phase II study of the RTKI cediranib, (AZD2171) involving 46 patients with ovarian cancer, 14 patients (30%) developed proteinuria (7 Grade 1 and 7 Grade 2).[57] In 7 of 14 women, proteinuria developed

within 2 weeks after initiation of treatment. As observed for bevacizumab, the risk to develop proteinuria was dose-related. In this study all patients who developed Grade 2 proteinuria also developed hypertension, but of the 20 women who developed Grade 3 hypertension only 7 developed proteinuria, indicating that these two toxicities are not superimposable. Similar observations have been made for bevacizumab.[58]

Table 1. Common terminology criteria for hypertension and proteinuria used in cancer trials

	Grade			
Adverse Event	**1**	**2**	**3**	**4**
Proteinuria	1+ proteinuria; urinary protein <1.0 g/24 hrs	2+ proteinuria; urinary protein 1.0 - 3.4 g/24 hrs	urinary protein ≥3.5 g/24 hrs	-
Hypertension	Prehypertension (systolic BP 120 - 139 mm Hg or diastolic BP 80 - 89 mm Hg)	Stage 1 hypertension (systolic BP 140 - 159 mm Hg or diastolic BP 90 - 99 mm Hg); medical intervention indicated; Recurrent or persistent (≥24hrs); symptomatic increase by >20 mm Hg (diastolic) or to >140/90 mm Hg if previously normotension; monotherapy indicated	Stage 2 hypertension (systolic BP ≥160 mm Hg or diastolic BP ≥100 mm Hg); medical intervention indicated; more than one drug or more intensive therapy than previously used indicated	Life-threatening consequences (e.g., malignant hypertension, transient or permanent neurologic deficit, hypertensive crisis); urgent intervention indicated

National Cancer Institute, Common Terminology Criteria for Adverse Events (v. 4.03)

http://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03_2010-06-14_QuickReference_5x7.pdf
(Last accessed on October 10th, 2012)

Renal biopsies performed in patients who developed severe proteinuria during angiogenesis inhibition have revealed different findings with thrombotic microangiopathy as the most commonly reported lesion.[59-62] In animals the glomerular lesion associated with anti-VEGF therapy is very similar to glomerular endotheliosis seen in preeclampsia.[9, 27, 41, 63-66] Glomerular endotheliosis is characterized by marked swelling of glomerular ECs and effacement of the podocyte food processes. It might be hypothesized that endotheliosis by obliterating the glomerular capillary tufts and changing the EC phenotype from an anti- to a prothrombotic state, precedes the development of thrombotic microangiopathy.

MECHANISM OF HYPERTENSION

Impairment of NO-signalling pathway

VEGF increases the expression of endothelial nitric oxide synthase (eNOS) and activates its activity through phosphorylation.[1, 20, 67-69] This phosphorylation occurs downstream of the VEGFR-2 and is mediated by the calcium-dependent phospholipase C/ phosphoinoside-3-kinase and the calcium-independent phosphatidylinositol 3-kinase/ Akt pathways.[1, 70] As eNOS is both upregulated and activated by VEGF, a decrease in NO bioavailability during angiogenesis inhibition, contributing to the vasoconstriction and rise in BP, is likely to occur. Meanwhile, clinical and experimental studies have been performed to explore whether VEGF inhibition is accompanied by a decrease in NO production and/or a decrease in the NO-mediated vasodilator tone.[44, 71] In a cross-sectional study in renal cell carcinoma patients, the excretion of urinary NO metabolites in patients with and without anti-angiogenic treatment was compared. Compared to controls the excretion of NO metabolites was identical in patients treated with bevacizumab but on average almost reduced by 50% in patients on RTKIs (0.36 vs 0.62 μmol per mg creatinine).[44] Urinary prostaglandin metabolites did not differ between the patients with and without anti-angiogenic therapy, likely excluding that prostacyclin production inhibition is involved in the BP rise with this treatment. In another study, plasma nitrate/nitrite levels were measured before and 6 weeks after treatment with the RTKI vandetanib in 17 breast cancer patients.[71] Compared to baseline levels plasma nitrate/nitrite levels modestly decreased, whereas BP had on average increased by 13 mmHg. In this study flow-mediated dilatation (FMD) of the brachial artery as a marker of NO-dependent vasodilation was also measured. FMD did not change (12.0% before and 13.8% after vandetanib). FMD before and during treatment with the RTKI telatinib has been investigated by Steeghs et al.[72] In 18 patients with advanced solid tumors, FMD after 5 weeks administration of the RTKI telatinib decreased from 6.0% to 3.9% (decrease of 35%), but nitroglycerine-induced brachial artery dilatation also decreased from 17.0% to 11.9% (decrease of 30%), indicating a diminished response of vascular smooth cells (VSMCs) to NO rather than a selective decrease in NO bioavailability.[72] In an *ex vivo* study, applying the Langendorff model to assess the coronary microcirculation, endothelium-dependent and endothelium-independent vasodilatory responses to respectively bradykinin and sodium nitroprusside were both impaired.[11] Remarkably, in this model we also observed a diminished response to the vasoconstrictor angiotensin II, suggesting generalized VSMC function impairment, at least in the coronary microvascular circulation, during sunitinib administration.[11] Finally, in a study performed in chronically instrumented awake swine exposed to sunitinib for 7 days, we found that acute BP rise to the NOS inhibitor N^g-nitro-L-arginine after 7 days of sunitinib administration was increased as compared to the rise without sunitinib (32 mmHg versus 24 mmHg, $P<0.05$), suggesting an increase rather than a decrease in NO-mediated vasodilator tone during sunitinib exposure.[73]

Taken together, the studies so far reported with regard to the effect of angiogenesis inhibition on the NO system are conflicting. The reason for these discrepant findings is not clear. Apparently the endothelial dysfunction and/or activation associated with exogenous VEGF inhibition may or may not be accompanied by a decrease in NO bioavailability or its responsiveness.

Rarefaction

VEGF acts as a survival signal to ECs, and in cancer xenograft models EC loss within tumors is observed within days after initiation of anti-angiogenic therapy. Consequently, rarefaction, i.e. the presence of a diminished number of microvessels, has frequently been proposed as a mechanism to explain the development of hypertension during anti-angiogenic therapy.[4, 26, 74] Clinical evidence that rarefaction occurs during anti-angiogenic therapy is limited to some small studies.[72, 75-77] Using intravital video-microscopy Mourad et al. found a small decrease in dermal capillary density at the dorsum of the fingers from 83 to 75 per mm^2 in 18 patients treated with bevacizumab for 6 months.[76] In another study, using side dark field video-microscopy, a decrease in oral mucosal capillary density from 20.8 capillaries per image at baseline to 16.7 capillaries per image after 5 weeks treatment with the RTKI telatinib has been reported in 7 patients.[72] In a study in 16 patients with metastatic renal cell carcinoma, nailfold capillary density decreased from 50 per mm^2 at baseline to 43 per mm^2 during sunitinib treatment.[77] After venous congestion, capillary density decreased from 69 to 65 per mm^2.[77] These studies indicate that rarefaction develops during anti-angiogenic therapy, but the magnitude rarefaction is limited. Moreover, it is unknown to what extent cutaneous rarefaction reflects rarefaction elsewhere in the body. For instance, in rats exposed to sunitinib for 7 days resulting in an increase in mean arterial pressure of 30 mmHg, we found no evidence for rarefaction in the myocardium (unpublished observation).

Although rarefaction occurs with angiogenesis inhibitors, its role in the development or maintenance of hypertension remains questionable.[78] First of all the rise in BP can occur within one day after initiation of anti-angiogenic therapy and it is unlikely that rarefaction at that time is already present.[41, 43, 73] Secondly, discontinuation of anti-angiogenic therapy is associated with a rapid normalisation of the increased BP. Thirdly, a mathematical model based on the hamster cheek pouch microcirculation indicates that 42% rarefaction of fourth order arterioles is necessary to increase resistance in that vascular bed by 5%.[79] This indicates that a considerable amount of rarefaction is required to induce an increase in vascular resistance and hence in BP, assuming that cardiac output remains unchanged. It is unlikely that such a rarefaction decrease ever occurs during anti-angiogenic therapy. Finally, the rarefaction observed in patients with untreated hypertension has been reported to disappear upon BP normalization with antihypertensive treatment.[80] Thus the observed rarefaction might be consequence rather than cause of the hypertension.[78]

Activation of the endothelin-signalling pathway

Endothelin-1 (ET-1) is the predominant member of the endothelin family and the most potent vasoconstrictor yet identified. ET-1 is secreted from endothelin secretory granules, also known as Weibel-Palade bodies.[81] Exocytosis of ET-1 from these bodies occurs in response to hypothermia, mechanical stress and agonists like histamine and thrombin.[82] A second pathway of ET-1 release is constitutive. ET-1 mediates vasoconstriction and proliferation by interacting with G-protein coupled membrane-bound ET_A and ET_B receptors on VSMCs. The ET_B receptor is also expressed on ECs. Activation of the ET_B receptor on ECs induces eNOS-mediated NO synthesis and stimulates the production of prostacyclin.[83] In addition, the ET_B receptor mediates ET-1 clearance. ET-1 synthesized by ECs is released toward the basolateral side of these cells and ET-1 is considered to act primarily as a paracrine/autocrine peptide.[84] Besides exerting vasoconstriction, ET-1 is also involved in cell growth and proliferation through activation of the mitogen-activated protein-kinase (MAPK) pathway.[85]

The first clinical evidence for the involvement of an activated endothelin-pathway in RTKI-induced hypertension stems from a study with sunitinib from our group.[11] In patients with renal cancer and gastrointestinal stromal cancer, administration of sunitinib for 4 and 10 weeks was associated with a doubling of plasma ET-1 concentration.[11] Furthermore, in a study in rats we found that administration of sunitinib for 7 days was also associated with a 3- to 4-fold increase in serum ET-1 concentration and in urinary ET-1 excretion and returning to pre-sunitinib values after its discontinuation.[41] Proof that ET-1 is involved in the rise in BP induced by angiogenesis inhibition has been provided by pharmacological studies in rats and swine.[41, 73, 86] In telemetry-instrumented rats the rise in BP induced by the experimental RTKI ABT-869 completely reversed with co-administration of the ET_A-receptor blocker atrasentan.[86], whereas the sunitinib-induced rise in BP was largely prevented by the mixed $ET_{A/B}$ receptor antagonist macitentan.[41] Furthermore, in chronically instrumented awake swine, the sunitinib-induced rise in BP and systemic vascular resistance could be completely reversed to pre-sunitinib values with the mixed $ET_{A/B}$ receptor antagonist tezosentan, while this compound did not lower BP under baseline conditions.[73]

Interestingly, in preeclampsia, increased circulating levels of ET-1 (1.5 to 2-fold) compared to normal pregnancies have repeatedly been reported.[87-91] Since treatment with endothelin receptor blockers is not allowed in preeclamptic women, insight into a pathophysiological role of the endothelin pathway in preeclampsia is restricted to experimental studies. Murphy et al. showed that infusion of sFlt-1 in pregnant rats for 6 days starting on the 13th day of gestation resulting in a preeclamptic phenotype was associated with a 3-fold increase in preproET-1 expression in the renal cortex.[92] In addition, these authors demonstrated that the sFlt-1-induced rise in BP could be completely abolished by the ET_A-receptor antagonist ABT 627. This study supports the hypothesis that ET-1, via activation of ET_A receptors, mediates hypertension in

response to excess sFlt-1 during pregnancy.

Mechanism of activation of the endothelin-signalling pathway

The observation that with both exogenous and endogenous VEGF inhibition the ET-1 pathway is activated is difficult to reconcile with findings showing that incubation of bovine aortic ECs with VEFG is associated with an enhanced preproET-1 mRNA expression and ET-1 production.[93] It is therefore possible that activation of the ET-1 pathway observed with anti-angiogenic therapy and in pregnancy is a consequence as well as a reflection of EC activation or dysfunction and not as a direct effect of VEGF inhibition.[94]

Already in 1990, Boulanger and Lüscher showed that inhibition of NO with L-NMMA potentiated the release of ET-1 from cultured ECs.[95] This potentiation was inhibited by the protein synthesis inhibitor cycloheximide, suggesting that de novo synthesis of ET-1 is involved. In eNOS deficient mice modest elevations of circulating ET-1 have been reported.[96] More recently, Weng et al. demonstrated that very low concentrations of NO suppressed the release of ET-1 from human umbilical vein ECs (HUVECs), in parallel with a decreased expression of preproET-1 mRNA.[97] Additional experiments showed that the NO-mediated suppression of ET-1 release from ECs is mediated by cGMP.[98] Apart from the observation that a decrease in vascular NO availability can result in increased ET-1 levels, there is also compelling evidence that the vasoconstriction induced by NOS blockade with L-arginine analogues is largely due to enhanced ET-1 signalling.[99-101] These studies indicate that a loss of EC-derived NO production results in increased ET-1 signalling, which because of the predominant abluminal secretion of ET-1 may or may not be reflected by elevated circulating ET-1 levels.

Salt sensitivity

Long-term BP control is governed by the kidneys via the regulation of extracellular fluid volume inherent to the regulation of sodium balance.[102] Obviously, renal injury occurring during anti-angiogenic therapy and in preeclampsia can contribute to the maintenance of hypertension.[36] Studies performed by Gu et al. have shown that subtle renal function impairment induced by a low dose of the RTKI sunitinib is associated with salt-dependent hypertension due to a rightward shift and a reduced slope of the pressure natriuresis curve.[103] In *in vitro* experiments, these authors showed a decrease in eNOS expression in cultured glomerular ECs and proximal tubular cells in response to sunitinib. A decreased expression of eNOS and neuronal NOS within the kidney and a rightward shift of the pressure natriuresis curve have also been reported during infusion of an antibody against the VEGF type 2 receptor.[20, 104] Based on these findings and the knowledge that impaired renal NO production contributes to salt-sensitive hypertension, both by inducing renal vasoconstriction and impairing renal sodium excretion, it is reasonable to assume that apart from structural changes, functional renal changes contribute to the development and maintenance of hypertension induced by

anti-angiogenic therapy.[105-107] The observed activation of the ET-1 pathway during anti-angiogenic therapy may contribute to this process by enhancing renal vasoconstriction (Figure 2).[108]

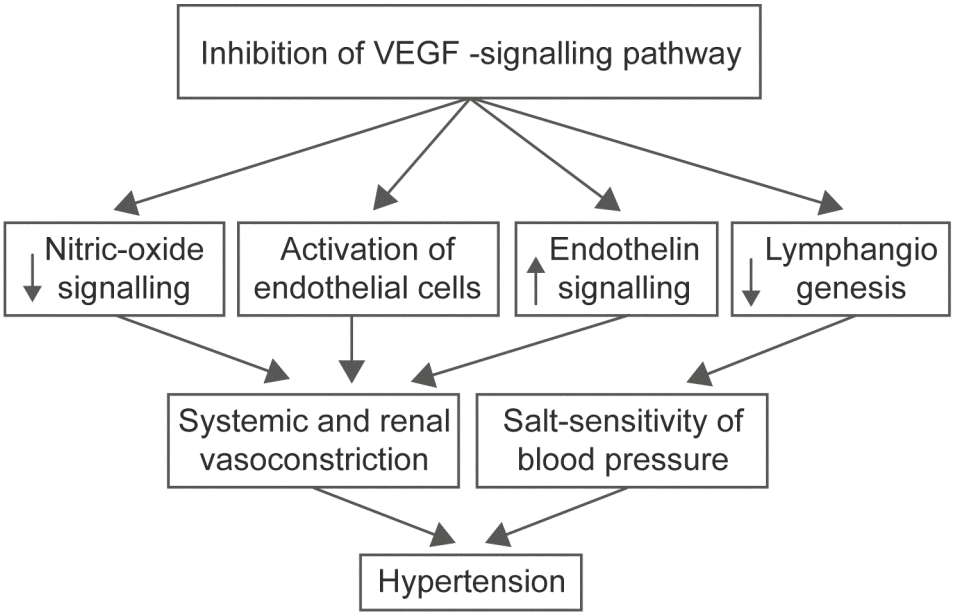

Figure 2. Proposed scheme of etiology of angiogenesis inhibition-induced hypertension. Of note, impairment of lymphangiogenesis only occurs with RTKIs that target the VEGF-3 receptor.

Recent studies have provided evidence that mononuclear phagocyte system (MPS) cells in the interstitial space regulate salt-dependent volume and BP by a VEGF-C-dependent buffering mechanism.[109] In response to a high-salt diet, hyperosmotic sodium storage through binding of sodium to proteoglycans occurs in the interstitial space. This storage stimulates MPS cells to produce VEGF-C via activation of osmotic stress receptors. VEGF-C in turn stimulates lymphangiogenesis and increases eNOS expression through activation of the respective VEGFR-2 and VEGFR-3. Inhibition of this system in combination with high salt intake induces excess interstitial salt retention and in rats it is associated with the development of hypertension.[109, 110] Thus, apart from impairment of renal function, blockade of the MPS-VEGF-C-lymphangiogenesis pathway may also contribute to the maintenance of hypertension during anti-angiogenic therapy with RTKIs, targeting the VEGFR-3 (Figure 2).

Oxidative stress

Oxidative stress may contribute to the development of hypertension during anti-angiogenic therapy in part through oxidation of NO to NO-peroxynitrite.[111] Our group

has explored the possibility whether the BP rise induced by sunitinib is accompanied by increased production of markers of oxidative stress and whether this BP rise can be prevented or reversed by antioxidants.[41, 73] Urinary excretion of thiobarbituric acid reactive substances (TBARS), as a measure of lipid peroxidation, did not increase in rats exposed to sunitinib and co-administration of the superoxide dismutase (SOD) mimetic Tempol only weakly attenuated the sunitinib-induced rise in BP. However, the sunitinib-induced proteinuria and increase in urinary ET-1 excretion were both markedly decreased. In a subsequent study performed in chronically-instrumented awake swine a mixture of antioxidants to achieve extensive reactive oxygen species (ROS) scavenging reduced BP by 13 mmHg under control conditions. After one week exposure to sunitinib MAP had increased by 14 mmHg. Administration of the antioxidant mixture at that time reduced MAP by only 5 mmHg.[73] Thus, conform the observations in rats, also in swine no evidence was found for an increase in ROS-mediated vasoconstriction during angiogenesis inhibition with sunitinib.

Other potential mechanisms

In an explorative clinical study, plasma levels of catecholamines, renin, aldosterone, urotensin II and endothelin have been measured in 20 patients treated with sorafenib.[40] After a 3-week treatment period, systolic BP (SBP) had increased by 20 mmHg in 60% of patients. Despite this BP rise, plasma concentrations of the above mentioned parameters remained unchanged. In contrast, in a clinical study with sunitinib, we found that BP rise was accompanied by a decrease in plasma renin concentration, whereas plasma aldosterone concentration remained unchanged.[11] A decrease in renal renin expression has also been reported in mice exposed to an anti VEGFR-2 antibody.[20] Like the study with sorafenib, no effect on plasma catecholamines was observed in our clinical study with sunitinib.[11] Based on the findings of these studies, activation of the renin-angiotensin system and/or the sympathetic nervous system as potential mechanisms for the development of hypertension during antiangiogenic therapy can be excluded.

MECHANISM OF PROTEINURIA DURING ANGIOGENESIS INHIBITION

Although hypertension may enhance the severity of proteinuria during anti-angiogenic therapy, experiments in mice with targeted disruption of VEGF production in podocytes indicate that the development of proteinuria is unrelated to the rise in BP.[60] VEGF produced by podocytes binds VEGFR on glomerular ECs and is critical for inducing and maintaining endothelial fenestrations in capillaries.[60, 112] Administration of VEGF neutralizing antibodies to mice as well as injection of adenovirus encoding for sFlt-1 in pregnant and non-pregnant rats have been reported to induce proteinuria.[9, 63] Selective depletion of VEGF in podocytes leads to proteinuria, accompanied by down-regulation of the slit diaphragm-associated protein nephrin, a major constituent of the glomerular

filtration barrier.[60, 63, 113] Since podocytes lack VEGF receptors one may wonder how anti-angiogenic therapy reduces podocyte nephrin production. Recently, Collino et al. have provided evidence for involvement of ET-1.[114] These investigators found that conditioned medium obtained from glomerular ECs incubated with preeclamptic sera induces nephrin and synaptodin loss from podocytes, which could be prevented by ET_A receptor antagonism.[114] Subsequent experiments showed that ET-1 triggered the nephrin shedding from cultured podocytes. On the baseis of these data not only loss of the protective effect of VEGF, but also the activation of the ET-1 pathway observed during angiogenesis inhibition and preeclampsia may contribute to the development of proteinuria by causing nephrin loss from podocytes.

TREATMENT OF VEGF-INHIBITION INDUCED HYPERTENSION

As no randomised controlled trials concerning the optimal treatment of angiogenesis-inhibition-induced hypertension are available, no clear recommendation for a particular antihypertensive agent or class of antihypertensive agents can be given at this stage.
Angiotensin II, via activation of the MAP-kinase pathway, exerts mitogenic activity, it has been suggested therefore that agents like angiotensin-converting enzyme inhibitors (ACEis) or angiotensin II type 1 receptor antagonists (ARBs) may have additional beneficial effects when used in the treatment of hypertension in patients with cancer.[115, 116] Since hypertension induced by angiogenesis inhibition is associated with renin suppression.[11], the antihypertensive efficacy of ACEis or ARBs may be less pronounced than that of diuretics or calcium channel blockers (CCBs).[11, 20] However, if the hypertension is accompanied by proteinuria, agents that interfere with the renin-angiotensin system may be preferred because of their renal-protective effect, which in part might be related to their ability to increase podocyte nephrin production as has been observed in diabetic nephropathy.[117]

The growing evidence that activation of the endothelin-signalling pathway is involved in the hypertension induced by angiogenesis inhibition may favour the use of endothelin receptor blockers in angiogenesis-inhibition-induced hypertension.[11, 41, 86] Endothelin-mediated ET_A receptor stimulation has shown to be mitogenic in cancer cells through activation of the MAPK pathway.[118] Thus, besides lowering BP, endothelin receptor antagonism may exert anti-tumor effects. Meanwhile, pilot studies exploring the anti-tumor effect of ET_A receptor blockade in prostate and renal cell carcinoma have been performed.[119, 120]

CONCLUSIONS

In this review evidence is provided that activation of the endothelin-signalling pathway is an important common final pathway for the development of hypertension and proteinuria associated with anti-angiogenic therapy. The mechanism by which anti-angiogenic therapy results in activation of the endothelin-signalling pathway is not yet known. Because in EC cultures VEGF has shown to stimulate preproET-1 mRNA expression and ET-1 production.[93], we suggest that activation of the endothelin-signalling pathway reflects the EC activation associated with anti-angiogenic treatment, but further studies are required to unravel the mechanism responsible for the increase in ET-1 production.

Rarefaction is frequently mentioned as a BP-rising mechanism, but in our view plays not or at best a marginal role in the maintenance of hypertension during anti-angiogenic therapy and considering the rapid onset of the BP rise after treatment initiation no role at all in the development of hypertension. Finally, the current knowledge that activation of the endothelin-signalling pathway plays a role in the development of hypertension during anti-angiogenic therapy may be incentive to design and to perform careful clinical studies to explore the efficacy and safety of endothelin receptor blockers in these conditions.

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CHAPTER 2

General introduction part 2

ENDOTHELIN-1 AND ANTIANGIOGENESIS

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SUMMARY

Antiangiogenesis, targeting vascular endothelial growth factor (VEGF), has become a well-established treatment for patients with cancer. This treatment is associated with NO-suppression and a dose-dependent activation of the endothelin-system, resulting in preeclampsia-like features, particularly hypertension and renal injury. Studies in eNOS-deficient mice and pharmacological treatment with endothelin receptor blockers and sildenafil indicate that an activated endothelin system, rather than NO suppression mediates the side effects of angiogenesis inhibitors. Activation of the endothelin-system is also observed in preeclamptic women, where it is related to the increased placental production of sFlt-1, the soluble form of the VEGF receptor-1. This receptors binds VEGF, thereby having the same consequences as antiangiogenic treatment with VEGF inhibitors. The side effects of antiangiogenic treatment in patients with cancer may be dose-limiting, thereby impairing its therapeuticpotential. In addition, because endothelin exerts proangiogenic effects, investigation of the effects of endothelin receptor blockade in patients with cancer treated with angiogenesis inhibitors is warranted.

INTRODUCTION

Angiogenesis, the formation of new vessels from pre-existing vasculature, is critical to tumor growth. It is a highly regulated process with vascular endothelial growth factor (VEGF) as one of the key stimulators.[1] This has resulted in the development of a large number of agents to inhibit the actions of VEGF as a new form of cancer treatment.[2] Soon after their introduction in the clinic it appeared that this treatment was associated with a number of adverse effects, particularly hypertension and renal injury, the latter reflected by proteinuria and renal function impairment. In the past several years evidence has accumulated that activation of the endothelin (ET)-system in response to anti-VEGF treatment is a major mediator of these side effects.[3]
In this review we first provide information about VEGF and the different treatment approaches by which the actions of VEGF can be inhibited. Subsequently, we discuss the frequency and time course of hypertension and renal injury associated with anti-VEGF treatment and the pivotal role of ET-1 in this process. In addition, when appropriate, we discuss findings in preeclampsia (PE) in view of the knowledge that PE also is an antiangiogenic condition with an activated ET-system.[4]

VEGF AND VEGF-RECEPTORS

The mammalian VEGF system belongs to a gene family that includes 5 different glycoproteins and the placental growth factors (PlGF) 1 to 4. Of the VEGF family, VEGF-A, commonly referred to as VEGF, is best characterized.[5] VEGF is produced and secreted by different cells, including endothelial cells (ECs), podocytes, macrophages, and fibroblasts. When produced and secreted by tumor cells, VEGF targets ECs to promote tumor angiogenesis. Hypoxia-inducible factors (HIF) 1α and 2α are a major up-regulators of VEGF. VEGF binds three tyrosine kinase receptors: VEGF receptor 1 (VEGFR-1 or fms-like tyrosine kinase 1 (sFlt-1) murine homologue), VEGF receptor 2 (VEGFR-2 or kinase domain region (KDR) human homologue or Flk-1 murine homologue) and VEGFR-3 (also known as Flt-4). VEGFR-1 and VEGFR-2 are predominantly expressed on vascular ECs, whereas VEGFR-3 is mainly restricted to lymphatic ECs.[6] VEGFRs contain an extracellular region, and a transmembrane and a cytoplasmatic bipartite kinase domain with tyrosine residues. Neurophilins 1 and 2 serve as coreceptors for VEGF, increasing the affinity of VEGF to the VEGFRs.[6] Although the affinity of VEGF-A is greater for VEGFR-1 than VEGFR-2, most of the effects of VEGF-A are mediated by the VEGFR-2.[7] VEGFR-1 may therefore act mainly as a decoy receptor. The extracellular domain of VEGFR-1, a splice variant of the gene encoding VEGFR-1, is also present at low concentrations in the circulation as a soluble protein (sFlt-1). Increased placental production of sFlt-1, acting as an endogenous inhibitor of VEGF and PlGF within the maternal circulation, has been recognized as an important mechanism in the pathogenesis of PE.[4]

The VEGF-system exerts many activities both in physiology and pathophysiology. For the formation of a well-functioning vascular network two VEGF alleles are required as has been demonstrated in partial knockout mice, whereas knockout of both VEGF alleles is incompatible with life.[8] In addition to its proangiogenic activity, VEGF increases vascular permeability and is required for the formation and maintenance of endothelial fenestrae.[9] Genetic deletion of VEGF in ECs without interruption of paracrine VEGF-signaling pathways leads to progressive EC degeneration and sudden death in transgenic mice, indicating that autocrine VEGF-signaling is required for the maintenance of the integrity of ECs.[10]

VEGF-SIGNALING

The mentioned activities of VEGF involve several signaling pathways, including activation of the phosphoinositide 3-kinase (PI3K)-Akt/protein kinase B (PKB) - mammalian target of rapamycin (mTOR) pathway.[11] This pathway stimulates nitric oxide (NO) production via phosphorylation of the three NO synthases (NOSs). The NOSs are ubiquitously expressed in malignant tumors and evidence is growing that NO has a function as a signaling molecule in the regulation of tumor genesis.[12] Other actions of VEGF include the activation of phospholipase C-g (PLC-g), protein kinase C (PKC), Raf-1, extracellular-signal-regulated protein kinase (ERK1/2), focal adhesion kinase (Fak) and mitogen-activated protein kinase (MAPK) pathways. Like the PI3K-Akt/PKB-pathway, the PLC-g pathway plays a crucial role in the production of NO by ECs. Additionally to the stimulation of NO production, prostacyclin production by ECs is stimulated by VEGF.[11]

PHARMACOLOGICAL APPROACHES TO INHIBIT THE VEGF PATHWAY

Already in 1971, Folkman proposed the inhibition of angiogenesis as a therapeutic strategy against malignancies.[13] Meanwhile a huge variety of drugs targeting VEGF or its receptors has been developed for the treatment of different forms of cancer.[2] These drugs can be divided in 1) monoclonal antibodies to VEGF, 2) monoclonal antibodies to the VEGFR-2 receptor, 3) agents that inhibit VEGF-receptors, usually denoted as receptor tyrosine kinase inhibitors (RTKIs) and 4) circulating VEGF receptors to trap VEGF. Bevacizumab (brand name Avastin) is an antibody that binds VEGF. It has been FDA-approved for various cancers, in addition, it is applied in age-related macular degeneration. RTKIs, of which a considerable number has been FDA-approved for the treatment of various cancers, block the phosphorylation of these receptors by interacting with their adenosine-triphosphate (ATP) pocket.[2] As a consequence RTKIs typically target a number of tyrosine kinases. For instance sunitinib (brand name Sutent), aside from inhibiting the three VEGF receptors, also inhibits platelet derived growth factor-α and β, c-kit, fms-like tyrosine kinase-3 (Flt-3), colony stimulating factor receptor type 1

and the glial cell-line derived neutrophic factor receptor RET. Aflibercept (brand name Eylea) is a recombinant fusion protein consisting of the VEGF-binding portions from the extracellular domains of the human VEGFR-1 and -2, fused to the Fc portion of human IgG1. It shares close similarity to sFlt-1, the extra-membranous part of the VEGF-1-receptor of which the placental production and levels in the maternal circulation are markedly increased in PE. Main indications of aflibercept are age and diabetes-related macula edema.

VEGF INHIBITION: HYPERTENSION AND RENAL INJURY

Since under physiological conditions ≥99% of ECs is quiescent, it was anticipated that adverse effects during anti-angiogenesis treatment were minimal. However, in line with knockout studies in mice showing that VEGF is essential for EC survival.[8] VEGF inhibition is associated with considerable cardiovascular and non-cardiovascular toxicity, including hypertension, left ventricular dysfunction, cardiac ischemia, myocardial infarction, renal injury, thyroid dysfunction, thrombosis, cerebral and intestinal hemorrhage, and skin manifestations. In virtually every patient exposed to anti-VEGF treatment blood pressure (BP) rises. The incidence of hypertension induced by anti-VEGF treatment ranges from 9% to 67%, whereas Grades 3 to 4 hypertension has been reported in 3-18% of patients.[11] The risk to develop severe hypertension is increased in patients with a history of hypertension. The incidence of hypertension is dose-dependent and increases to over 90% when bevacizumab and sunitinib are combined.[14] The rise in BP is caused by a rise in systemic vascular resistance and can develop within hours to days after treatment initiation.[3, 15] Of interest, the development of hypertension may be associated with improved survival. In patients with renal cancer treated with sunitinib the progression free survival was 12.5 versus 2.5 months and overall survival 30.9 versus 7.2 months in patients who did or did not develop hypertension.[16] These findings suggest that identical mechanisms underlie the anti-tumor response and the development of hypertension.

Proteinuria, sometimes causing nephrotic syndrome, is a key manifestation of renal damage induced by anti-VEGF treatment.[17] Kidney biopsies of patients who developed renal injury typically show glomerular thrombotic microangiopathy.[18] Likewise, kidneys of rats exposed to a relatively high dose of sunitinib for 1 week showed pronounced glomerular endotheliosis, a histopathological picture also present in PE, as well as intracapillary fibrin deposits.[19]

VEGF INHIBITION AND ACTIVATION OF THE ET-AXIS

ET-1 is the predominant member of the endothelin family and the most potent vasoconstrictor identified. ET-1 is a 21-amino acid cyclic peptide mainly produced by ECs. ET-1 is constitutively produced by ECs at low quantities. In addition, upon

activation of ECs, ET-1 is released from stores in the Weibel-Palade bodies. ET-1 mediates vasoconstriction and proliferation by interacting with G-protein coupled membrane-bound ET_A and ET_B receptors on vascular smooth muscle cells. The ET_B receptor is also expressed on ECs. Activation of the ET_B receptor on ECs induces eNOS-mediated NO production and prostacyclin synthesis.[11] In addition, the ET_B receptor mediates ET-1 clearance. ET-1 produced by ECs is predominantly released towards the basolateral side of these cells, acting primarily as a paracrine/autocrine peptide.[20] Besides exerting vasoconstriction, ET-1 is involved in cell growth and proliferation through activation of the MAPK pathway.[21] Because of this property, ET-1 may be involved in tumor angiogenesis.[22] In addition, ET-1 has been shown to induce oxidative stress within the placenta, which in turn may result in increased placental production of pro-inflammatory cytokines and sFlt-1.[23]

The first clinical evidence for the involvement of an activated ET-system during VEGF inhibition stems from a study with sunitinib by our group.[3] In patients with either metastatic renal cell cancer or imatinib-resistant gastrointestinal stromal tumors, administration of sunitinib was associated with a doubling of the circulating ET-1 concentration. In successive studies in rats we found that administration of sunitinib for 8 days was also associated with dose-dependent increase in serum ET-1 concentration, returning to pre-sunitinib values after its discontinuation.[24] In our initial study, using a relatively high dose of sunitinib, an increase in urinary ET-1 levels was also observed, but this could not be confirmed in a subsequent dose-finding study.[19] In contrast to several experimental models of PE, renal expression of the genes encoding for ET-1 or endothelin-converting enzyme was not increased.[19] In preeclamptic women, circulating ET-1 levels are increased compared to normotensive pregnant women. In a recent study we found that this rise in ET-1 closely relates to the elevated sFlt-1 levels.[25] Collectively, these data indicate that the rise in ET-1 is not unique for sunitinib, but a common response to exogenous or endogenous factors targeting VEGF (Table 1).

IS ET-1 INVOLVED IN THE BLOOD PRESSURE RISE DURING ANTIANGIOGENIC TREATMENT?

To address this point, preclinical studies with both ET_A selective and dual endothelin receptor blockers have been performed. In telemetry-instrumented rats exposed to the RTKI ABT-869, the rise in BP was abolished by an ET_A receptor blocker.[26] and in sunitinib-exposed rats the BP rise was prevented by macitentan, a dual endothelin receptor blocker.[27] Likewise, in instrumented awake swine, the sunitinib-induced rise in BP and systemic vascular resistance reversed to pre-sunitinib values by tezosentan.[28] In addition to its BP lowering effect, we also observed that the proteinuria induced by sunitinib could be largely prevented by the endothelin receptor blocker macitentan.[27] Importantly, this antiproteinuric effect of macitentan appears to be independent of its BP lowering effect, because prevention of the sunitinib-induced rise of BP with

amlodipine did not decrease the proteinuria, in fact proteinuria tended to increase.[27] This finding concurs with studies indicating that ET-1 can induce damage to glomerular podocytes. For instance, a sub-pressor dose of ET-1 administered to rats was found to increase glomerular permeability and inflammation as well as the excretion of the glomerular slit-diaphragm protein nephrin, effects that could be blocked by an ET_A receptor antagonist.[29] More recently, Buelli et al. have shown activation of the β-arrestin-1 signaling pathway by ET-1, causing transition of podocytes from an epithelial to mesenchymal cell type.[30] Thus besides glomerular VEGF depletion activation of the ET-system, as a consequence of this VEGF depletion, likely contributes to the proteinuria.

Table 1. Similarities of the endothelin system during anti-VEGF treatment and preeclampsia

	Anti-VEGF	**Preeclampsia**
Endothelial dysfunction	↑	↑
Circulating endothelin-1	↑	↑
Urinary endothelin-1	↑	↑/≈
Proteinuria	↑	↑
Glomerular endotheliosis	↑	↑
Expression of renal preproendothelin-1	↓/≈	↑
Expression of renal endothelin converting enzyme	↓	↑
Expression of ET_A and ET_B receptor	N.A.	↓ET_B
Effects of ET_A receptor blockade	↓BP	↓BP
Effects of ET_A/ET_B receptor blockade	↓BP, Proteinuria	N.A.

Table based on information from (2, 14, 20, 23). ≈ indicates no change, N.A. indicates not available

MECHANISM OF ACTIVATION OF ET-AXIS

As displayed in Figure 1, several mechanisms may account for the increased ET-1 levels during antiangiogenesis. Using a bovine arterial EC culture line, Matsuura et al. showed that VEGF stimulates the expression of preproET-1 mRNA as well as the secretion of ET-1.[31] The VEGF-induced increased expression of preproET-1 mRNA is mediated by the VEGFR-2. If VEGF stimulates the production of ET-1 by ECs, a decrease

in ET-1 levels during VEGF inhibition would be expected. Because of the opposite observation it is more likely that activation of ECs underlies the increase in ET-1 in response to VEGF deprivation. Indeed the glomerular endotheliosis associated with sunitinib administration and the increased glomerular EC expression of intercellular adhesion molecule I are clear manifestations of this EC activation.[19] Since the VEGF-signaling pathway stimulates NO production, anti-VEGF treatment is associated with a decrease in NO production.[27] Given that NO decreases (stimulated) ET-1 release in aortic ECs, this decrease in NO may also contribute to the increased ET-1 production.[32] Of note, a study in eNOS-deficient mice indicate that NO can mitigate the antiangiogenic-induced preeclamptic-like phenotype.[33] In these mice increased adenovirus-induced expression of sFlt-1 resulted in a more severe phenotype than in control mice. This more severe phenotype is mainly ET-1-mediated as it could be reversed by an ET_A receptor antagonist.[33] In our own studies, concurrent administration of sildenafil could not prevent the sunitinib rise BP in rats, supporting the view that NO deprivation is a much less important factor than activation of the ET-1 system for the rise in BP induced by sunitinib.[27] Moreover, in swine we observed that the rise in BP in response to N^{ω}-nitro-L-arginine was higher after than prior to sunitinib administration, even indicating an increase rather than a decrease in NO bioavailability during sunitinib in these animals.[28]

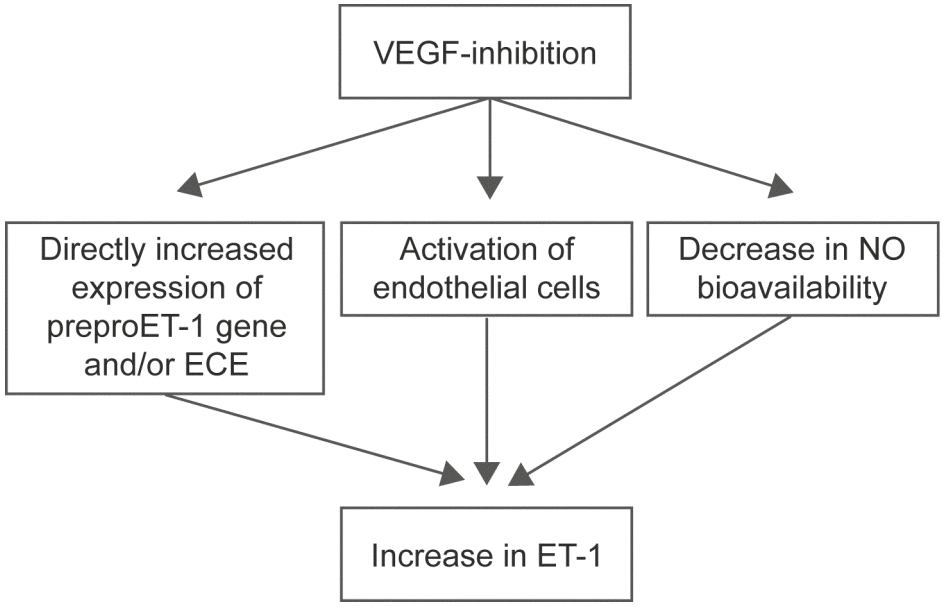

Figure 1. Putative mechanisms for the rise in ET-1 during antiangiogenesis. ECE denotes endothelin converting enzyme

PERSPECTIVES AND SIGNIFICANCE

Antiangiogenesis targeting VEGF, irrespective of its underlying mechanism, is associated with a rise in ET-1. This rise is most likely a consequence of activation of ECs and not a direct consequence of VEGF deprivation. Preclinical studies with endothelin receptor blockers have clearly shown that activation of the ET-axis plays a pathogenic role in the rise in BP as well the proteinuria associated with antiangiogenic treatment (Figure 2). Because adverse effects may be a reason to discontinue antiangiogenic treatment, thereby limiting their therapeutic potential, studies exploring the usefulness of an endothelin receptor blocker in patients with cancer treated with these agents are warranted, especially so because ET-1 may exert proangiogenic effects and stimulates VEGF production.[34]

Finally, it may be proposed that the magnitude of the ET-1 rise during antiangiogenic treatment may be useful biomarker of the efficacy of treatment, unless loss of the ET_B clearance receptor occurs during anti-angiogenic treatment as has been reported in experimental PE.[35] Although the rise in BP or the development of hypertension has also been suggested as an efficacy parameter, the level of BP is the result of a large number of stimulatory and counteracting factors, limiting its value as suitable biomarker.

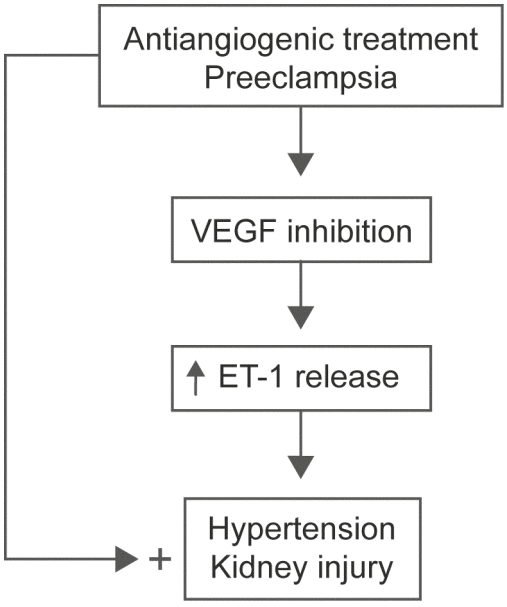

Figure 2. Role of ET-1 in the development of hypertension and proteinuria during antiangiogenesis. Note contributing role of VEGF inhibition in development of kidney injury.

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CHAPTER 3

AIMS OF THE THESIS

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Angiogenesis inhibition, by targeting VEGF or its receptors, has become an established treatment for various tumor types but is associated with adverse effects including hypertension, proteinuria and renal injury with activation of the endothelin-1 system. Sunitinib is an orally active angiogenesis inhibitor that blocks VEGF receptors -1, -2 and -3 and other tyrosine kinase receptors, including platelet derived growth factor (PDGF) and c-Kit receptors. VEGFR-1 and VEGFR-2 are predominantly expressed on vascular endothelial cells and peritubular capillary endothelial cells, whereas VEGFR-3, stimulated by VEGF-C, is restricted to lymphatic endothelial cells. Since the occurrence of side effects can be a reason to lower the sunitinib dose or even to discontinue anticancer therapy, thereby compromising its potential efficacy, exploring therapeutic approaches to counteract these side effects is important, not only from an academic point of view, but also from a clinical point of view.

To determine the most optimal way to prevent the sunitinib-induced adverse effects and to explore their interdependency, we investigate in **Chapter 4** the therapeutic potential of the dual endothelin receptor antagonist macitentan, the calcium channel blocker amlodipine, the angiotensin-converting enzyme inhibitor captopril and the phosphodiesterase type 5 inhibitor sildenafil. In **Chapter 5** we test the dose-dependency of these side effects, using a low, intermediate and high dose of sunitinib, aiming to find a dose that, with regard to hemodynamic and renal side effects, is comparable to the dose applied in patients. With this approach, we also want to explore whether the proteinuria observed during antiangiogenic treatment occurs irrespective of the presence of glomerular histological changes. In **Chapter 6** we aimed to explore the salt dependency of angiogenesis inhibition-induced hypertension by exposing animals to a low and high salt diet in combination with sunitinib, using a dose of sunitinib that does not impair renal function as based on the findings obtained in chapter 5. As a second aim, we explore whether sunitinib administration impairs the formation of lymph vessels within the skin, leading to skin sodium accumulation and salt-sensitivity of blood pressure.

In **Chapter 7**, we investigate the consequences of a high salt diet in combination with sunitinib administration on renal histopathology. Especially, we want to explore whether in addition to glomerular lesions, the combination of a high salt diet and sunitinib is accompanied by tubulointerstitial injury and/or peritubular rarefaction. The renal injury during angiogenesis inhibition closely resembles the renal abnormalities observed in preeclampsia and associates with podocyte injury. It has been suggested that the appearance of podocytes in urine, i.e., podocyturia, could be a sensitive marker for ongoing glomerular disease. In **Chapter 8** we aimed to examine whether podocyturia occurs in patients treated with the VEGF inhibitor bevacizumab, using a recently developed qPCR technique, and whether the podocyturia does or does not relate to the degree of proteinuria and cumulative dose of bevacizumab.

CHAPTER 4

TREATMENT OF HYPERTENSION AND RENAL INJURY INDUCED BY THE ANGIOGENESIS INHIBITOR SUNITINIB: PRECLINICAL STUDY

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ABSTRACT

Common adverse effects of angiogenesis inhibition are hypertension and renal injury. To determine the most optimal way to prevent these adverse effects and to explore their interdependency, the following drugs were investigated in unrestrained WKY rats exposed to the angiogenesis inhibitor sunitinib: the dual endothelin receptor (ET-R) antagonist macitentan, the calcium channel blocker (CCB) amlodipine, the angiotensin-converting enzyme inhibitor (ACEi) captopril and the phosphodiesterase type 5 (PDE5) inhibitor sildenafil. Mean arterial pressure (MAP) was monitored telemetrically. After 8 days, rats were sacrificed and blood samples and kidneys were collected. In addition, 24-hour urine samples were collected. After sunitinib start, MAP increased rapidly by about 30 mmHg. Co-administration of macitentan or amlodipine largely prevented this rise, whereas captopril or sildenafil did not. Macitentan, captopril and sildenafil diminished the sunitinib-induced proteinuria and endothelinuria and glomerular intra-epithelial protein deposition, whereas amlodipine did not. Changes in proteinuria and endothelinuria were unrelated. We conclude that in our experimental model ET-R antagonism and calcium channel blockade are suitable to prevent angiogenesis inhibition induced-hypertension, while ET-R antagonism, ACEi and PDE5 inhibition can prevent angiogenesis-inhibition induced proteinuria. Moreover, the variable response of hypertension and renal injury to different antihypertensive agents, suggests that these side effects are, at least in part, unrelated.

INTRODUCTION

Angiogenesis, the formation of new vessels from preexisting vasculature, is critical to solid tumor growth as well as to the development of metastasis.[1, 2] This process is regulated by numerous factors among which vascular endothelial growth factor (VEGF) plays a dominant role. Angiogenesis inhibition, by targeting VEGF or its receptors, has become an established treatment for various forms of cancer but is featured by adverse effects including hypertension and renal injury.[3] Hypertension has been reported in up to 36% of patients treated with bevacizumab, a monoclonal antibody against VEGF, and in up to 60% of patients treated with sunitinib, an orally active multitarget VEGF receptor tyrosine kinase inhibitor (RTKI).[3] Renal toxicity, mainly proteinuria, has been reported in 41-63% of patients treated with bevacizumab.[4] In a recent phase 3 randomized trial performed in patients with metastatic clear cell renal cell carcinoma, proteinuria occurred in 18% for patients randomized for pazopanib and in 14% for patients randomized for sunitinib.[5]

The development of hypertension and renal injury may compromise the use of VEGF-inhibition in patients with cancer who develop these side effects. Hence, exploration of therapeutic approaches to counteract these side effects is important, but clinical studies comparing different agents to manage these particular side effects are lacking and unlikely will be performed in this category of patients. In previous studies we have demonstrated that activation of the endothelin-axis is involved in sunitinib-induced hypertension and renal injury.[6] Moreover, we observed that concurrent administration of the dual ET_A/ET_B-receptor blocker macitentan in a sunitinib hypertensive rat model could to a large extent prevent the sunitinib-induced rise of blood pressure (BP) and proteinuria.[7] Since endothelin receptor blockers are not approved for the treatment of systemic hypertension and renal injury we explored here to what extent angiotensin converting enzyme (ACE) inhibition, calcium channel blockade (CCB) as well as phosphodiesterase 5 (PDE5) inhibition were able to prevent hypertension and renal injury in our animal model.

MATERIAL AND METHODS

In vivo study

Male Wistar Kyoto rats (WKY, 280-300 gram) obtained from Charles River, were housed in individual cages and maintained on a 12-h light/dark cycle, having access to standard laboratory rat chow and water ad libitum. Intra-aortic BP recordings were performed by radiotelemetry (Data Sciences International (DSI)) and the sunitinib and vehicle solution were prepared and administered by oral gavage as described previously.[6] Before and after implantation of the telemetry transmitters (DSI TA11PA-C40) using 2% isoflurane anesthesia, rats received analgesic treatment using Temgesic subcutaneously (0.05 mg/kg; RB Pharmaceuticals Limited) for 2 days. At the

end of each experiment, rats were euthanized with 60 mg/kg pentobarbital i.p. and blood was sampled for measurement of serum ET-1, serum creatinine, VEGF and sunitinib levels, and kidneys were rapidly excised. Five experiments were performed. In the first experiment rats were randomly administered sunitinib (26.7 mg/kg.day of sunitinib-L-malate; Sutent, Pfizer, n=10) or vehicle (n=10) by oral gavage (0.5 mL) for 8 days. The dose of sunitinib was based on initial experimental studies, investigating its effectiveness in a rat breast cancer model.[8] In the second experiment, rats (n=8) were orally administered the combination of sunitinib and macitentan (ACT-064992, kindly provided by Actelion) 30 mg/kg.day, for 8 days.[9] In the third experiment, rats were administered the combination of sunitinib and amlodipine 3 mg/kg.day by oral gavage for 8 days.[10, 11] In the fourth experiment, rats (n=9) were administered the combination of sunitinib by oral gavage and captopril at 3 mg/kg.day or 12 mg/kg.day (C4042, Sigma-Aldrich) using osmotic minipumps (Alzet 2ml2) for 8 days.[12, 13] In the final experiment, rats (n=6) were administered the combination of sunitinib and sildenafil 1.5 mg/kg.day (Revatio; Pfizer).[14] In all experiments, 6 days before (baseline) and 6 days after administration of the various agents, rats were housed in metabolic cages for 48 hours with free access to food and water; the first day to acclimatize and the second day to collect 24-hour urine samples for the determination of protein, ET-1, the nitric oxide (NO) metabolites (NO_2+NO_3 [NO_x]) and cyclic GMP (cGMP). BP was not monitored when rats were housed in metabolic cages due to the absence of telemetry receivers. Urine was collected on antibiotics (Antibiotic Antimycotic Solution, A5955, Sigma-Aldrich) to prevent formation of NO metabolites. Macitentan was dissolved in vehicle containing 0.5% methylcellulose aqueous solution and 0.05% Tween 80. Amlodipine besylate (Bioconnect, Huissen, The Netherlands) was suspended in 1% tragacanth gum solution. Sildenafil was suspended in 0.5% carboxymethylcellulose. All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC.

Renal histology

Details of the light and electron microscopy in this study are available in the Supplement. Briefly, transversely sliced kidney sections were stained for for haematoxilin-eosine (HE) and periodic acid Schiff (PAS). PAS-stained sections were blindly evaluated by a pathologist (F.M.M.S.) for the presence (1) or absence (0) of endothelial cell and epithelial cell swelling in 50 glomeruli, as well as scored for the presence of ischemia and intra-epithelial protein. For electron microscopy two glomeruli in each biopsy section were examined. The presence of glomerular endotheliosis and podocyte morphology were registered. Both reflection contrast and electron micrographs were obtained from reprocessed paraffin embedded tissue.

Biochemical measurements

ET-1 and VEGF were assessed using a chemiluminescent ELISA QuantiGlo®, R&D Systems) and Quantikine Immunoassay (R&D Systems) respectively. Urine NOx

concentration was determined by fluorimetric quantification of nitrite content (Cayman Chemicals, Ann Arbor, MI).[15] To investigate the systemic and local effects of sildenafil treatment, cGMP levels were determined in serum and urine respectively, using an ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA). Serum creatinine and urinary protein concentrations (Cobas c502 and c702, CREP2 and TP2/TPUC3, Roche Diagnostics) were measured at the clinical chemical laboratory of the Erasmus MC. Sunitinib levels were measured by a validated ultra-performance liquid chromatography/tandem mass spectrometry system.[16]

Statistical analysis

Data are presented as mean±SEM. Statistical analysis between groups was performed by unpaired *t*-testing or by repeated-measures ANOVA followed by Newman-Keuls or Dunnett's multiple comparison testing. For correlation analysis the Pearson *r* correlation coefficient was used. GraphPad Prism version 5.0 was used for all statistical analysis.

RESULTS

In vivo study

Baseline MAP and heart rate (HR) were 99.1±4.1mmHg and 357.9±5.8 bpm. Administration of sunitinib by oral gavage was associated with a rapid rise of intra-arterial BP (ΔMAP 31.6±0.9mmHg), whereas BP remained unchanged during administration of vehicle (Figure 1). The sunitinib-induced rise in BP was associated with a decrease in HR. Co-administration of macitentan (ΔMAP 12.3±1.5mmHg) or amlodipine (ΔMAP 11.4±1.7mmHg) attenuated the sunitinib-induced rise in BP by 73% ($P<0.001$) and 63% ($P<0.001$) respectively, whereas co-administration of both dosages of captopril and sildenafil had no BP-lowering effect. The sunitinib-induced decrease in HR was not prevented by each of the four compounds, and even aggravated by the low dose of captopril (Figure 1).

Sunitinib administration was associated with a 3-fold rise in serum creatinine concentration when compared with vehicle. This rise was not prevented by each of the four compounds (Figure 2A). Proteinuria was below the limit of detection during vehicle administration and increased to about 30 mg per day during sunitinib administration. The rise in proteinuria was attenuated by macitentan ($P<0.01$), both dosages of captopril ($P<0.001$) and sildenafil ($P<0.01$). Conversely, proteinuria tended to increase further with amlodipine (Figure 2B).

Circulating ET-1 concentration was 0.61±0.08 pg/ml during vehicle administration and increased during sunitinib administration ($P<0.01$). Amlodipine, captopril and sildenafil did not influence this rise. Because of a decrease in clearance caused by blockade of the ET_B receptor, ET-1 rose further during co-administration of macitentan (Figure 2C). Sunitinib administration was also associated with a rise in 24-hour urinary ET-1

excretion (Figure 2D). This rise was prevented by macitentan, captopril, and sildenafil but not by amlodipine (Figure 2D). Proteinuria and endothelinuria did not correlate (r= 0.17; P>0.05).

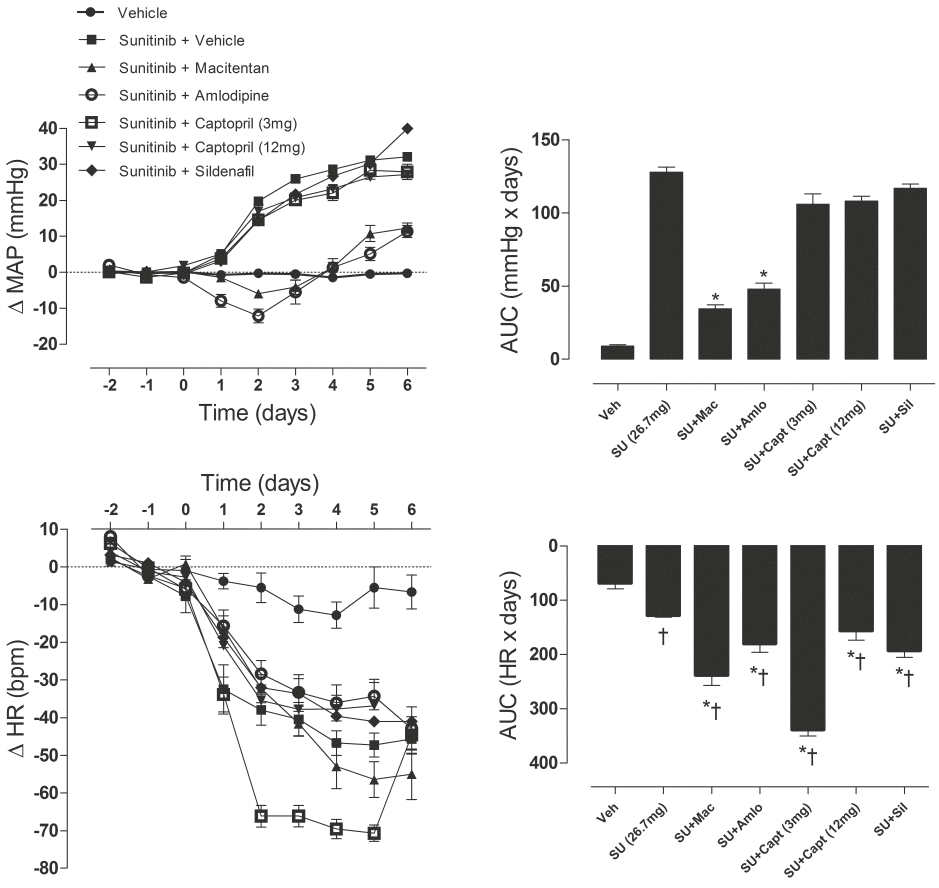

Figure 1. Changes in mean arterial pressure (MAP) and heart rate (HR) in response to administration of vehicle (n=6), sunitinib and vehicle (n=12), and co-administration of macitentan (n=8), amlodipine (n=8), captopril; 3 or 12 mg (n=7 each) or sildenafil (n=6). Left, Time course of changes in MAP and HR. Right, Areas under the curve (AUC). *P<0.05 vs sunitinib + vehicle; †P<0.05 vs vehicle. Data of macitentan from Kappers et al.[7]

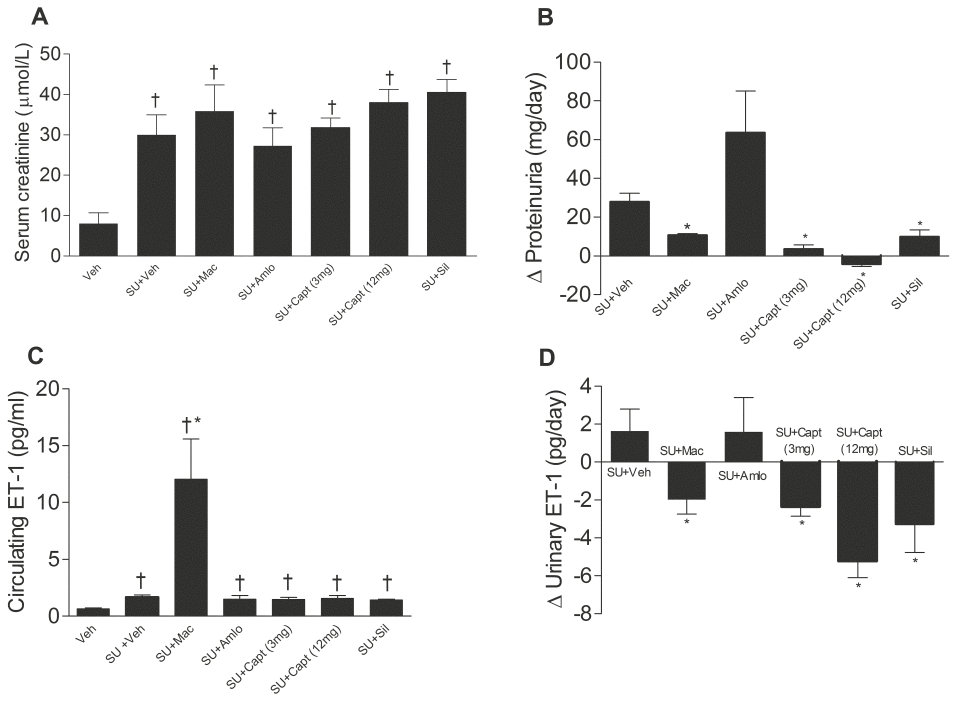

Figure 2. Serum creatinine (A), change in proteinuria (B), circulating ET-1 levels (C) and change in urinary ET-1 excretion (D) in rats after administration of vehicle (n=6), sunitinib and vehicle (n=16), and co-administration of macitentan (n=4-6), amlodipine (n=8), captopril; 3 or 12 mg (n=5-9), or sildenafil (n=6) for 8 days. *P<0.05 vs sunitinib + vehicle; †P<0.05 vs vehicle
Data of macitentan from Kappers et al.[7]

Urinary excretion of NOx per 100 g BW was 2.2±0.2 µmol per day during vehicle administration (Figure 3). Sunitinib administration was associated with a decrease in urinary NOx excretion (P<0.01). This decrease was not affected by macitentan or amlodipine and weakly attenuated by captopril (Figure 3). Changes in NOx excretion and endothelinuria were not correlated (r=0.14; P>0.05). Urinary cGMP decreased during sunitinib administration from 4563±190 pmol/mL during vehicle to 1387±105 pmol/mL per day (P<0.05). This decrease was prevented by concurrent administration of sildenafil. Circulating cGMP concentration was not affected by sunitinib administration nor by the combination of sunitinib and sildenafil (data not shown).

Mean serum concentration of sunitinib was 376±87 ng/mL. This concentration increased during treatment with sildenafil and by both dosages of captopril, to 800±151, 679±17 and 1082±238 ng/mL, respectively (P<0.05), confirming that their renoprotective effects were not due to a suppression of sunitinib bioavailability. Circulating VEGF increased from 4.9±2.3 pg/mL during vehicle to 947.8±45.1 pg/

mL during sunitinib administration. This rise was unaffected by sildenafil and the low dose of captopril (959±132 and 905±141 pg/mL), and attenuated by the high dose of captopril 689±67 pg/mL (P<0.05 vs sunitinib alone). Unfortunately, plasma to measure sunitinb and VEGF during treatment with amlodipine or macitentan was no longer available.

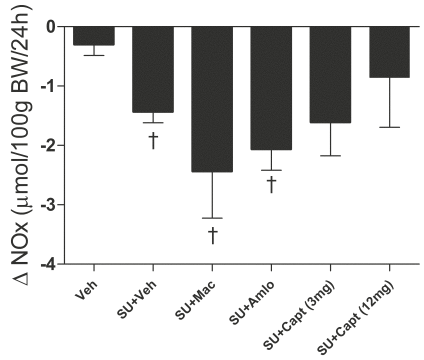

Figure 3. Change in nitric oxide metabolites (NOx) excretion in urine of WKY rats after administration of vehicle (n=4), sunitinib (n=9), and co-administration of macitentan (n=7), amlodipine (n=8) or captopril; 3 or 12 mg (n=7 each) for 8 days. *P<0.05 vs baseline, †P<0.05 vs sunitinib. Data of macitentan from Kappers et al.[7] BW indicates body weight.

Renal histology

Sunitinib administration was associated with a rise in kidney weight-to body weight ratio from 3.0±0.04 to 3.5±0.04 g/kg (P<0.001). This rise was not changed by any of the four agents.

Periodic acid Schiff-stained kidney sections showed marked glomerular changes, including intra-epithelial droplets and, epithelial and endothelial cell swelling with narrowing of the capillary lumina after administration of sunitinib (Table 1). Neither macitentan nor the low dose of captopril reversed the sunitinib-induced glomerular ischemia or endothelial and epithelial cell swelling. Only a high dose of captopril and sildenafil were more renoprotective. However, consistent with the decrease in proteinuria, glomerular intra-epithelial protein deposition diminished during co-administration of macitentan and captopril (Figure S1, Table 1). In rats co-administered sunitinib and amlodipine, glomerular ischemia and endothelial and epithelial cell swelling were more prominent compared to rats exposed to sunitinib alone, as was the glomerular intra-epithelial protein deposition (Table 1). Reflection contrast and electron microscopy showed intra-epithelial resorption droplets and severe glomerular endotheliosis during administration of sunitinib. This was partly prevented by macitentan, and reversed with a high dose of captopril and sildenafil (Figure S2).

Table 1. Light microscopic evaluation of kidney sections obtained from rats exposed to sunitinib and coadministration of macitentan, amlodipine, captopril, and sildenafil for 8 days with controls

	Glomerular ischemia (% of glomeruli)			Endothelial cell swelling (% of glomeruli)		Epithelial cell swelling (% of glomeruli)		Intra-epithelial protein (% of glomeruli)		
Treatment Group	None	Moderate	Severe	0	1	0	1	0	1	2
Control	60±13	32±10	8±4	100	0	100	0	99±1	1±1	0
Sunitinib	17±2*	54±4*	29±5*	78±3*	22±3*	63±5*	37±5*	29±4*	48±2*	23±4*
Sunitinib + Macitentan	15±6*	35±2†	51±4*†	85±3*	15±3*	77±6*	23±6*	33±8*	59±7*	8±3*†
Sunitinib + Amlodipine	12±3*	40±4†	47±6*†	53±10*†	47±10*†	16±4*†	84±4*†	7±2*†	18±4*†	75±5*†
Sunitinib + Captopril 3mg	4±2*	23±6†	73±7*†	n.a	n.a	54±6*	46±6*	77±5*†	16±4†	7±2†
Sunitinib + Captopril 12mg	73±3†	25±2†	2±1†	69±5*	31±5*	96±3†	4±3†	77±6*†	20±5*†	3±2†
Sunitinib + Sildenafil	64±10†	30±9†	6±2†	n.a	n.a	72±7*	28±7*	56±11*	29±8*	14±3*

Data of macitentan from Kappers et al.[7] n.a indicates not applicable.
*p<0.05 vs. control; †p<0.05 vs. sunitinib

DISCUSSION

Administration of the multi-target VEGF receptor blocker sunitinib is accompanied by a rise in BP, renal injury and proteinuria, activation of the ET-1 axis and renin suppression.[6, 7, 17] Previously, we found that the rise in BP and proteinuria could largely be prevented by the dual $ET_{A/B}$ receptor antagonist macitentan, indicating that activation of the ET-1 axis is critical for the development of these side effects. Here we explored whether alternative antihypertensive agents attenuate the occurrence of these side effects and to what extent they are interrelated.

Co-administration of amlodipine with sunitinib was associated with a marked attenuation of the rise in BP, comparable to the degree observed with macitentan. However, renal injury could not be prevented while proteinuria even tended to increase. In contrast, co-administration of captopril and sildenafil was associated with a decrease in proteinuria and renal injury, but without effect on BP. These findings strongly suggest that renal toxicity is a BP-independent adverse effect of short-term angiogenesis inhibition. Obviously, over more prolonged periods of time hypertension may further aggravate the renal injury by angiogenesis inhibition, whereas vice versa, renal injury may contribute

to a further rise in BP. Histological examination of the kidneys showed extensive glomerular ischemia in over 80% of the glomeruli during sunitinib administration. These histological abnormalities were largely prevented by the high dose of captopril and by sildenafil, but not by macitentan, the lower dose of captopril or amlodipine (Table 1). Unexpectedly, these beneficial effects of the high dose of captopril and of sildenafil on renal histology did not reflect in an attenuation of the sunitinib-induced rise in serum creatinine. Apparently serum creatinine concentration was not an accurate marker to reliably assess renal injury during the relatively short observation period in the present study. As an estimate of glomerular filtration rate we also measured endogenous creatinine clearances, but these provided identical results as the measurements of serum creatinine concentration (data not shown).

The beneficial effects of sildenafil and high dose of captopril on proteinuria occurred independently of a BP-lowering effect. A BP-independent beneficial effect of sildenafil on renal function has also been reported in DOCA-salt hypertensive rats.[18] This nephroprotective effect of sildenafil appears to be associated with anti-inflammatory, antifibrotic and antiapoptotic effects with down-regulation of transforming growth factor-β1 expression. Furthermore, sildenafil attenuates diabetic nephropathy in non-insulin-dependent Otsuka Long-Evans Tokushima fatty rats.[19] ACE-inhibitors are well-known for their antiproteinuric effect beyond BP reduction. In part this is related to a decrease in glomerular filtration pressure by preferential dilatation of efferent glomerular arterioles. In addition ACE inhibitors, like captopril, increase NO bioavailability by decreasing the breakdown of bradykinine. Finally, ACE inhibition can induce podocyte repopulation and thereby attenuating glomerular injury and proteinuria induced by anti-VEGF treatment.[20] The protective effect on renal injury was only seen with the high dose of captopril. This is in line with recommendations in hypertensive patients with proteinuria, in whom maximal ACE inhibition is advocated for optimal renal protection.[21]

In rats exposed to the RTKI sorafenib (20 mg/kg per day) for 4 weeks, a rise in systolic BP of almost 60 mmHg and marked albuminuria has been reported.[12] In this study concurrent administration captopril was associated with a marked reduction in both albuminuria and renal histological abnormalities and also with a 50% reduction of the rise in BP. Captopril in that study was given orally in a daily oral dose of 40 mg. Captopril in our study (3 and 12 mg/kg per day) was given subcutaneously by means of osmotic minipumps. The doses selected were based on previous studies performed in our lab in spontaneously hypertensive rats.[13] These studies showed that subcutaneously administered captopril at doses of 3 and 6 mg/kg per day for 1 week lowered mean arterial pressure (MAP) by respectively 14 and 28 mmHg. In addition, this BP reduction was associated with an about 10-fold rise in renin.[13] This rise is of comparable magnitude as that observed in young hypertensive patients exposed to ACE-inhibition or a change from a very high to a very low salt intake. Based on these findings we

are confident that the maximal dose of captopril of 12 mg/kg per day was sufficient to induce pronounced blockade of the RAS with beneficial renal, but not with BP-lowering effects. Interestingly, Curwen et al. observed in rats exposed to a relatively low dose of the RTKI cediranib, resulting in a BP rise of about 10 mmHg, that captopril (30 mg/kg orally) could completely reverse this rise in BP. In contrast, the same dose of captopril was without any effect in rats exposed to a relatively high dose of cediranib, resulting in a 40 mmHg BP rise.[22] These findings indicate that other factors than RAS activation are instrumental for the development of hypertension when higher doses of a RTKI are administered and that the RAS is likely down-regulated in an attempt to attenuate the development of severe hypertension. This probably also was the case in our rat model, where sunitinib administration caused a rise in MAP of 30 mmHg. This rise in BP related to the relatively high dose of sunitinib (resulting in plasma levels that were 5-10 times higher than those in humans) was considerably larger than observed in our clinical study.[6] In that study MAP rose by 12 mmHg, but interestingly, this moderate rise in BP was also already associated with >60% renin suppression, indeed suggesting that the BP elevation in response to RTKIs treatment in humans is not RAS-dependent and, consequently, less responsive to anti-RAS agents. Renin suppression during anti-angiogenic treatment might also be caused by an increase in aldosterone production. Only a limited number of studies has looked at the effect of RTKIs on aldosterone.[6, 23, 24] In these studies no increase in circulating aldosterone levels or urinary aldosterone excretion has been observed. Recently, VEGF-stimulated aldosterone release has been reported.[25] Therefore reduced rather than increased aldosterone production during anti-angiogenic treatment is to be expected.

As reflected by the decrease in urinary NO metabolites and cGMP, anti-angiogenic treatment is associated with a decrease in the activity of the NO system.[26, 27] Using the human forearm model it has recently been reported that intra-arterial infusion of bevacizumab inhibits the local vasodilator response to acetylcholine, but not to sodium nitroprusside, implying impairment of endothelium-dependent vasodilation.[28] This decreased activity of the NO system can contribute to the development of hypertension as well as renal injury.[7, 24] There are sporadic reports that an exogenous NO donor can lower BP in patients who develop hypertension during angiogenesis inhibition.[29] In the current study, the PDE5 inhibitor sildenafil was used to increase NO responsiveness. With this agent the sunitinib-induced reduction in urinary cGMP excretion was completely prevented, but this was not associated with any BP-lowering effect. Sildenafil was used in a daily dose of 1.5 mg/kg. This dose is equivalent to a daily dose of 100 mg in patients and has been shown to prevent the rise in systolic BP from 129±8 mmHg in control rats to 183±6 mmHg in rats exposed to the NOS inhibitor L-NAME.[14] The rise in BP induced by sunitinib could largely be prevented by the calcium channel blocker amlodipine, which is in line with an experimental study, demonstrating that nifedipine could completely reverse the rise in BP induced by the RTKI cediranib.[22]

As reported previously by our group, angiogenesis inhibition by sunitinib is associated with activation of the endothelin system.[7] During sunitinib administration, both the circulating ET-1 concentration and the 24h urinary excretion of ET-1 were increased. ET-1 within the kidney is produced by glomerular endothelial, mesangial cells as well as renal tubular cells, and urinary ET-1 excretion is considered to reflect the degree of renal ET-1 production.[30, 31] Recently, it has been shown that ET-1 produced by endothelial cells induces nephrin shedding from podocytes, which could be prevented by ET_A receptor antagonism.[32] Since mutations in the gene encoding for nephrin are associated with severe forms of the nephrotic syndrome, it has been speculated that increased glomerular endothelial cell production of ET-1 is one of the mediators of proteinuria.[33] Based on these data not only loss of the protective effect of VEGF on glomerular endothelial cells, but also the activation of the ET-1 system observed during angiogenesis inhibition may contribute to the development of proteinuria. To obtain further insight in this mechanism we explored whether endothelinuria and proteinuria were correlated. This seemed not to be the case.

Randomised controlled trials concerning the optimal treatment of angiogenesis-inhibition-induced hypertension are lacking, therefore no clear recommendation for a particular antihypertensive agent or class of antihypertensive agents can be given. Based on the present observations together with other experimental and clinical studies, showing that the hypertension induced by sunitinib is associated with renin suppression, dihydropyridine calcium channel blockers rather than anti-RAS agents are probably more effective for the treatment of hypertension.[34] Given their beneficial effects on the occurrence of proteinuria, an anti-RAS agent can be combined with a calcium channel blocker in case of the development of renal injury. In addition, it has been shown that an anti-RAS agent can enhance the effect of sunitinib in a murine xenograft tumor model.[35] Although NO-donors like nitrates or PDE5 inhibitors may also be beneficial, it has been suggested that these agents may potentially compromise the anti-angiogenic effect and therefore they can best be avoided.[22] The knowledge that activation of the endothelin-axis is involved in the hypertension induced by angiogenesis inhibition, may also favor the use of endothelin receptor blockers in angiogenesis-inhibition-induced hypertension.[6, 7, 36] ET_A-receptor stimulation has been shown to be mitogenic in cancer cells through activation of the MAPK pathway.[37] Thus, besides lowering BP, endothelin receptor antagonism may exert anti-tumor effects.[38, 39] However, due to adverse effects, single endothelin receptor blockers are currently not being marketed for the treatment of systemic hypertension, but only for pulmonary hypertension.[40] In addition, since macitentan is a strong inducer of the CYP3A4 enzyme, while sunitinib is metabolized by this enzyme, this combination should be avoided because of unwanted pharmacokinetic interaction.

PERSPECTIVES

Anti-angiogenic treatment targeting the VEGF-VEGFR pathway is complicated by the development of hypertension and renal injury. Occurrence of these particular side effects may compromise anti-cancer treatment, but the most optimal way to treat these remains to identified. Given the lack of large clinical studies, this study provides further insight into the mechanisms underlying sunitinib-induced hypertension and proteinuria as well as ways to counteract these adverse events. Translation of these findings to the clinic strongly suggests that dependent on the toxicity encountered, different classes of antihypertensive agents should preferably be used, i.e. a calcium channel blocker in the case of hypertension and a RAS blocker in the case of renal injury. Of interest, both captopril and sildenafil increased the steady-state sunitinib concentrations. The underlying pharmacokinetic interaction is currently unknown, but warrants further investigation in humans, not only because it may enhance the anti-cancer effectiveness of sunitinib, but also its side-effect profile.

NOVELTY AND SIGNIFICANCE

What is new?

This is the first study that provides information about treatment of hypertension and renal injury during anti-angiogenic treatment by comparing different antihypertensive agents.

What is relevant?

Our findings show that the effect of different antihypertensives to counteract hypertension and renal injury is variable, suggesting that different pathogenetic pathways underlie these side effects and that they require a dedicated treatment approach.

Summary

In a rat model of sunitinib-induced hypertension and renal injury we found evidence for beneficial antihypertensive and renoprotective effects with calcium channel blockade and ACE or phoshodiesterase inhibition respectively, thereby providing a rational basis for optimal treatment of the reno-cardiovascular side effects associated with angiogenesis inhibition.

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SUPPLEMENT

EXPANDED METHODS

Light microscopy

The left kidney was rapidly excised from euthanized rats, decapsulated, weighed and sliced transversely into 2-mm thick sections. Slices were fixed in a 3.5-4% formaldehyde solution for light microscopic evaluation. After fixation in the formaldehyde solution, tissue was dehydrated and paraffin-embedded. Deparaffinized 2-μm thick sections were stained for haematoxilin-eosine (HE) and periodic acid Schiff (PAS). PAS-stained sections were blindly evaluated by a pathologist (F.M.M.S.) for the presence (score 1) or absence (score 0) of endothelial cell and epithelial cell swelling. Glomerular ischemia was scored semiquantitatively and defined as the degree of open glomerular capillaries, wrinkling of the glomerular basement membrane and filling of Bowmans space. Wide open glomerular capillaries filling Bowman's space entirely corresponded with no ischemia. Partially open glomerular capillaries with mild wrinkling of the glomerular basement membrane and Bowman's glomerular space largely filled was classed as moderate ischemia. Totally collapsed glomeruli and extensive wrinkling of the glomerular basement membrane and only partial filling of Bowman's space corresponded with severe ischemia. Furthermore, the presence of glomerular intra-epithelial protein deposition was evaluated using a semiquantative scale: 0 (no protein), 1 (protein present in 1-50% of the epithelial cells) and 2 (protein present in >50% of the epithelial cells). Fifty glomeruli per kidney section (PAS staining) were evaluated. All images were obtained using a Reichert microscope and Leica DFC420 camera (40x objective) and Leica LAS software.

Electron microscopy

Formalin fixed paraffin embedded renal tissue was reprocessed for reflection contrast microscopy and electron microscopy. In brief, $1mm^3$ tissue blocks were deparaffinized, rehydrated, and post fixed with 1.5% glutaraldehyde, followed by incubation in 1% osmium tetroxide (OsO_4) in 0.1 M sodium cacodylate for 1 h. After each step of the fixation, the fish were rinsed twice with 0.1 M sodium cacodylate and finally dehydrated in a series of 70%, 80%, 90% and 3× 100% ethanol, prior to immersion in a 1:1 epon:propylene oxide solution for 1 h. The samples were washed afterwards with pure epon, embedded in pure epon LX112 and polymerized at 60 °C for 2 days. Sequential 100 um sections were placed on glass slides for reflection contrast microscopy or on grids for electron microscopy. The preparations were examined under a Leitz Orthoplan microscope (Leitz, Wetzlar, Germany) equipped for epi-illumination, which was adapted for reflection contrast microscopy as described previously. The slides were examined under a 100× objective lens. A JEOL JEM-1011 electron microscope equipped with a MegaView III digital camera was used for ultra-structural analysis.

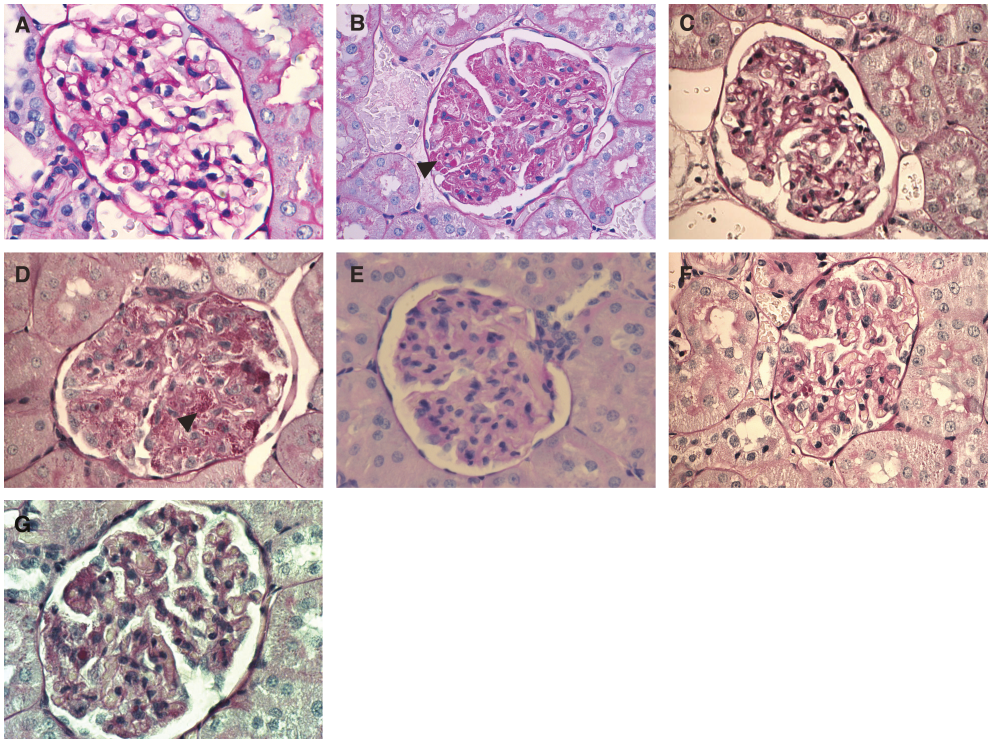
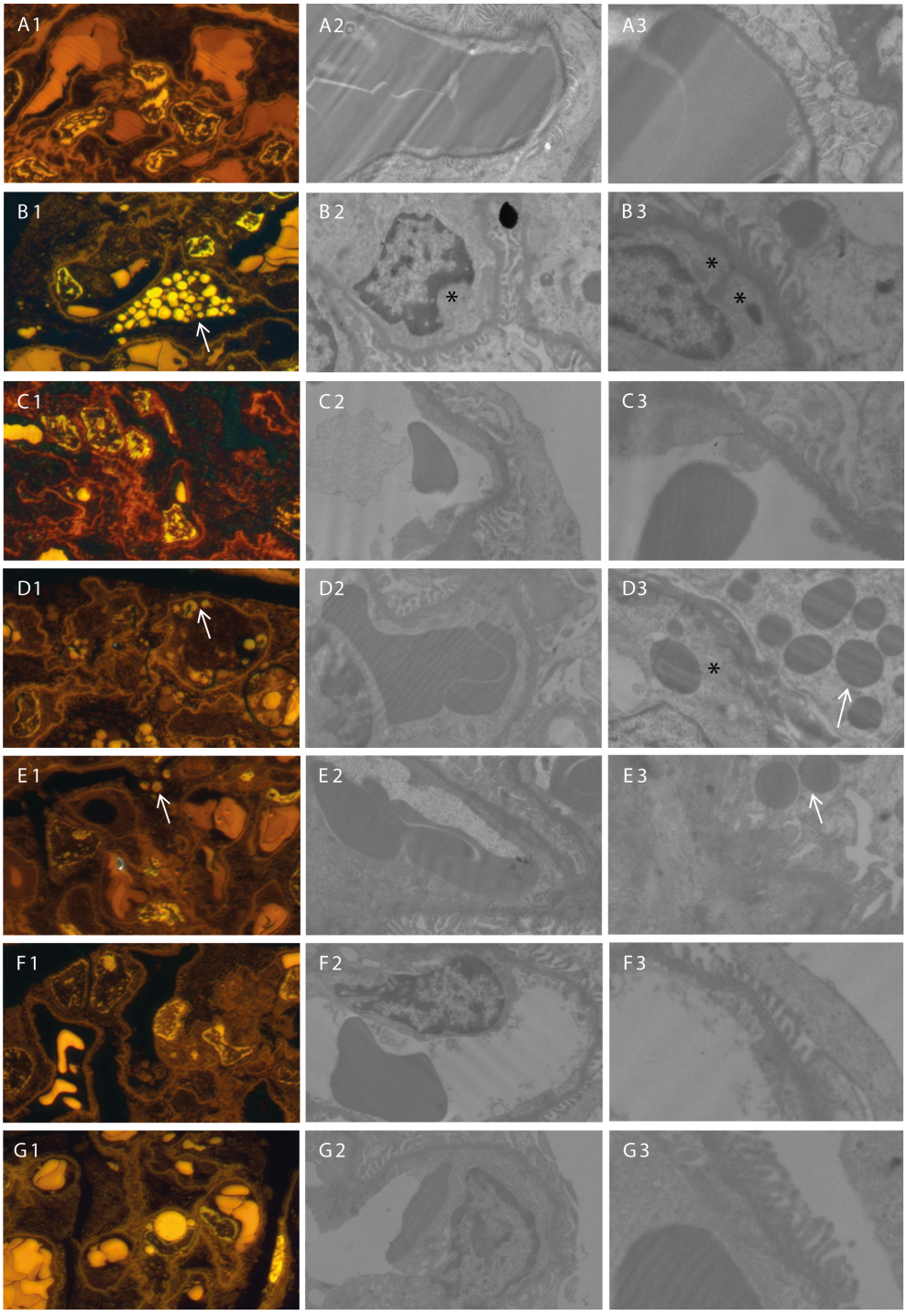


Figure S1. Kidney sections from WKY rats administered vehicle (A), sunitinib (B), macitentan (C), amlopidine (D), captopril (E/F) or sildenafil (G), stained with PAS stain (magnification x500). After administration of sunitinib for 8 days marked glomerular changes could be observed, including intra-epithelial droplets (arrowhead). Consistent with the decrease in proteinuria, glomerular intra-epithelial protein deposition diminished during co-administration of macitentan, captopril and sildenafil.

Figure S2. Reflection contrast and transmission electron micrographs of representative kidney sections from rats administered vehicle (A1; magnification x1500, A2; magnification x5000, A3; magnification 12000), sunitinib (B1; magnification x1500, B2; magnification x5000, B3; magnification x12000) or in combination with macitentan (C1; magnification x1500, C2; magnification x5000, C3; magnification x12000), amlodipine (D1; magnification x1500, D2; magnification x5000, D3; magnification x12000), low (E1; magnification x1500, E2; magnification x5000, E3; magnification x12000) and high dosage of captopril (F1; magnification x1500, F2; magnification x5000, F3; magnification x12000), or sildenafil (G1; magnification x1500, G2; magnification x5000, G3; magnification x12000). After sunitinib administration for 8 days intra-epithelial resorption droplets (white arrows) and glomerular endotheliosis (endothelial cell swelling; asterix) were observed. None of the abnormalities above could be observed in control kidney sections (A1-3) and they were partly prevented by macitentan, and reversed with a high dose of captopril and sildenafil.


A1
A2
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C2
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CHAPTER 5

GREATER SENSITIVITY OF BLOOD PRESSURE THAN RENAL TOXICITY TO TYROSINE-KINASE RECEPTOR INHIBITION WITH SUNITINIB

Stephanie Lankhorst, Hans J. Baelde, Mariëtte H.W. Kappers, Frank M.M. Smedts, Stefan Sleijfer, Ron H. Mathijssen, A.H. Jan Danser, Anton H. van den Meiracker.

Hypertension 2015; 66(3): 543-549

ABSTRACT

Hypertension and renal injury are off-target effects of sunitinib, a tyrosine-kinase receptor inhibitor used for the treatment of various tumor types. Importantly, these untoward effects are accompanied by activation of the endothelin system. Here, we set up a study to explore the dose-dependency of these side effects. Normotensive Wistar-Kyoto rats were exposed to 3 different doses of sunitinib or vehicle. After 8 days rats were sacrificed. Telemetrically measured blood pressure (BP) rose dose-dependently, from 13 to 30 mmHg. Proteinuria was present at all doses, but a rise in cystatin C occurred only at the intermediate and high dose. Compared to vehicle circulating endothelin-1 increased dose-dependently, whereas 24-hour urinary endothelin excretion decreased. Light and electron microscopy revealed glomerular endotheliosis and ischemia with the intermediate and high doses of sunitinib, but completely absent histological abnormalities with the low dose. Podocyte number per glomerular circumference did not change. Glomerular Nephrin, Neph1, podocin, and endothelin converting enzyme gene expression were downregulated in a dose-dependent manner. We conclude that the sunitinib-induced rise in BP requires lower doses than its induction of renal function impairment and that functional changes in glomerular filtration barrier contribute to the occurrence of proteinuria, given the lack of histopathological changes with the low dose of sunitinib.

INTRODUCTION

Neoangiogenesis, the formation of vessels from preexisting vasculature, is critical to solid tumor growth as well as to metastasis. Vascular endothelial growth factor (VEGF) plays a dominant role in this process. Therefore angiogenesis inhibition, by targeting VEGF or its receptors, has become an established treatment for several tumor types.[1] Sunitinib is an orally active angiogenesis inhibitor that blocks the VEGF receptors -1, -2 and -3 and other tyrosine kinase receptors, including platelet derived growth factor (PDGF) and c-Kit receptors. Off-target effects of sunitinib and other anti-angiogenic agents, sometimes necessitating discontinuation of treatment, are hypertension, proteinuria and renal failure.[2-4]

In the kidney, glomerular endotheliosis and thrombotic microangiopathy are the most frequently observed histological abnormalities seen during treatment with sunitinib.[5, 6] These abnormalities are comparable to those observed in preeclampsia likely due to the fact that both conditions share the same pathogenetic mechanism, i.e., disruption of the VEGF signalling pathway, which in preeclampsia is caused by increased placental production of the soluble Fms-like tyrosine kinase 1 (sFlt-1).[7]

In previous clinical and experimental studies we observed that activation of the endothelin system is involved in the rise in blood pressure (BP) during sunitinib treatment.[3, 8, 9] Recent studies have shown that ET-1 induces podocyte injury mediated by activation of the ET_A receptor on podocytes.[10, 11] It may be hypothesized therefore that the renal injury occurring during anti-angiogenic treatment is mediated both by a direct effect related to interruption of VEGF-signaling and by an indirect effect related to activation of the ET-1-system. In our previous studies, we used a sunitinib dose of 26.7 mg/kg.day by oral gavage. This high dose was associated with a rapid development of severe hypertension, proteinuria and irreversible glomerular renal injury, making it difficult to infer to what extent the renal injury is BP-independent.[12] Additionally, the extent of sunitinib-mediated side-effects in this particular model at this dose is much more severe than seen in patients, rendering it questionable whether this dose adequately reflected the off-target effects of sunitinib in clinical practice. Here, we tested the dose-dependency of these side effects, aiming to find a sunitinib dose that, with regard to hemodynamic and renal side effects, including ET-1 elevation, better resembles the dose applied in patients. With this approach, we also wanted to unravel whether the proteinuria observed during anti-angiogenic treatment occurs irrespective of the presence of glomerular histological changes.

MATERIALS AND METHODS

Animal study

Normotensive, male Wistar Kyoto rats (WKY, 280-300 gram) obtained from Charles River, were housed in individual cages and maintained on a 12-h light/dark cycle, having access to standard laboratory rat chow and water ad libitum. Intra-aortic BP recordings were performed by radiotelemetry and sunitinib (SU11248; Sutent, Pfizer) and vehicle solution were prepared and administered by oral gavage as described previously.[9] After implantation of the telemetry transmitters, rats received analgesic treatment using Temgesic subcutaneously (0.05 mg/kg; RB Pharmaceuticals Limited) for 2 days. At the end of each experiment, rats were euthanized with 60 mg/kg pentobarbital i.p. and blood was sampled for measurement of circulating ET-1, cystatin C levels, VEGF and sunitinib levels, and kidneys were rapidly excised. Rats were randomly administered a low (7 mg/kg.day) or intermediate (14 mg/kg.day) dose of sunitinib-L-malate (n=6 each), a high dose of sunitinib-L-malate (26.7 mg/kg.day; n=14), or vehicle (n=8) by oral gavage (0.5 mL) for 8 days. In all experiments, before (baseline) and after administration of sunitinib, rats were housed in metabolic cages for 48 hours with free access to food and water; the first day to acclimatize and the second day to collect 24-hour urine samples for the determination of protein, cyclic GMP and ET-1. BP was not monitored when rats were housed in metabolic cages due to the absence of telemetry receivers. All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC.

Biochemical measurements

ET-1 and VEGF were assessed using a chemiluminescent ELISA (QuantiGlo®, R&D Systems, range 0.34 - 250 pg/mL) and Solid Phase Sandwich ELISA (Quantikine, R&D Systems, range 31.2 - 2000 pg/mL) respectively. cGMP levels were determined in urine, using an ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA). Serum cystatin C and urinary protein concentrations (Cobas c502 and c702, CYSC and TP2/TPUC3, Roche Diagnostics) were measured at the clinical chemical laboratory of the Erasmus MC. Sunitinib levels were measured by a validated ultra-performance liquid chromatography/tandem mass spectrometry system, at the laboratory of Translational Pharmacology of the Erasmus MC Cancer Institute.[13]

Renal histology

Details of the light microscopy (LM) and electron microscopy (EM) in this study are available in the Supplement.

Podocyte and glomerular quantification

IHC staining for Wilms tumor protein (WT1), a podocyte-specific transcription factor, was performed to identify podocytes. In brief, three-micron paraffin sections were deparaffinized, rehydrated, and used for immunostaining after antigen retrieval

procedure. Endogenous peroxidase activity was blocked for 15 minutes in 0.1% H_2O_2 in water. After washing with PBS, sections were incubated with mouse anti WT1 mAb antibodies (WLM04, Abcam) diluted in 1% bovine serum albumen in PBS for 2 hours, 1 hour incubation with anti-mouse envision (DAKO), and the slides were developed with diaminobenzidine. Slides were counterstained with hematoxylin, dehydrated, and mounted. The number of positive nucleoli per glomerulus in 25 glomeruli was counted and the glomerular surface area was measured with the Philips Image Management System 2.3 software (Philips Medical Systems Nederland B.V., The Netherlands).
Intercellular adhesion molecule 1 (ICAM-1) staining was performed on frozen sections using an anti-rat CD54 monoclonal antibody (clone 1A29). An HRP labeled Anti-Mouse IgG (H+L) Antibody (KPL, Maryland, USA) was used as a secondary antibody.

mRNA expression

Quantitative polymerase chain reaction (qPCR) was performed to quantify mRNA expression. The RNA was isolated using the TRIzol method and reversed to cDNA using an AMV cDNA synthesis kit (Roche, Indianapolis, IN). For the qPCR reaction, iQ SYBR Green supermix (BioRad) was used. Relative transcription levels for Neph1, Nphs1, Nphs2 were determined and corrected to the podocyt specific gene Wt1 using the CFX manager software (BioRad) with primer sequences as described earlier.[14] The Edn1, Ece1, and Vegfa mRNA levels were measured and corrected to a general housekeeping gene hypoxanthine phosphoribosyltransferase 1 (Hprt1). The primer sequences used are shown in Table S1 (available in the Supplement).

Statistical analysis

Data are presented as mean±SEM. Statistical analysis between groups was performed by unpaired t-testing or by repeated-measures ANOVA followed by Newman-Keuls or Dunnett's multiple comparison testing. Correlations were performed by Pearson's testing. GraphPad Prism version 5.0 was used for all statistical analysis.

RESULTS

Plasma concentrations of sunitinib

Plasma concentrations of sunitinib increased dose-dependently with the three oral doses of sunitinib (Figure 1A). Sunitinib's plasma concentration at the lowest dose was comparable with the systemic concentrations reached in patients treated with a standard daily dose of sunitinib of 50 mg.[15] Sunitinib is metabolized by cytochrome P450 3A4 to the active compound N-desethyl sunitinib. Even at the lowest dose of sunitinib, the plasma concentration of this compound in rats was much higher than in patients during standard dosing (Figure 1B). The ratio metabolite/sunitinib was 5.1±0.7 compared to an average of 0.3 in humans (data not shown), implying a higher rate of N-de-ethylation in rodents.

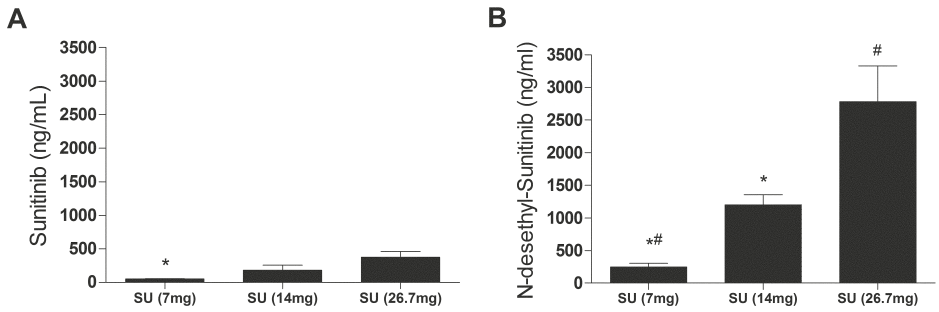

Figure 1. Trough plasma concentrations of sunitinib (A) and its active metabolite, N-desethyl sunitinib or SU12662, (B) in rats in response to administration of a low (7 mg/kg.day), an intermediate (14 mg/kg.day) and a high dose of sunitinib (26.7 mg/kg.day). *P<0.05 vs sunitinib (26.7 mg); #P<0.05 vs. sunitinib (14mg)

Dose-dependency of blood pressure rise, renal function impairment and proteinuria in sunitinib-exposed rats

Mean arterial BP (MAP) increased dose-dependently, ranging from 13.4±3.3 mmHg at the lowest to 31.1±0.9 mmHg at the highest dose versus vehicle (Figure 2A). With all three doses, the rise in MAP dose-dependently was accompanied by a decrease in heart rate (Figure 2B).

Compared to vehicle, cystatin C increased with the high and intermediate doses, but was identical to that in vehicle-treated rats with the low dose of sunitinib (Figure 2C). Proteinuria markedly increased with the high dose of sunitinib, whereas the intermediate and low dose of sunitinib, were associated with modest proteinuria (Figure 2D). Cystatin C and proteinuria were not related to MAP or ET-1.

Dose-dependent changes in cGMP, ET-1 and VEGF

Sunitinib was associated with dose-dependent decreases in urinary cGMP excretion (Figure 3A). In previous clinical and preclinical studies we have shown that sunitinib administration is associated with activation of the ET-1-axis.[8, 9, 12] At the different doses of sunitinib, circulating ET-1 increased dose-dependently (Figure 3B). Changes in urinary cGMP excretion and in ET-1 were unrelated (r=0.18, P>0.05). Circulating ET-1 and MAP were related (r=0.49; P<0.05). Urinary ET-1 excretion at baseline was 9.1±0.5 pg/day. This excretion slightly decreased by 2.3±0.7 pg/day with vehicle and by 5.4±1.9 pg/day at the low, 4.1±1.1 pg/day at the intermediate and 4.1±1.1 pg/day at the high sunitinib dose (P<0.05 suntinib versus vehicle).

Plasma VEGF dose-dependently and markedly increased in response to sunitinib administration (Figure 3C). Plasma VEGF was related to MAP, sunitinib and N-desethyl sunitinib (r=0.61, r=0.72 and r=0.83 respectively, P<0.01). The rise in circulating VEGF was not accompanied by a rise in the renal expression of the gene encoding VEGF-A

(Figure 4A). The expression of the gene encoding for ET-1 did not change with any of the doses, whereas the gene expression of endothelin-converting enzyme (ECE) decreased (Figure 4B and 4C).

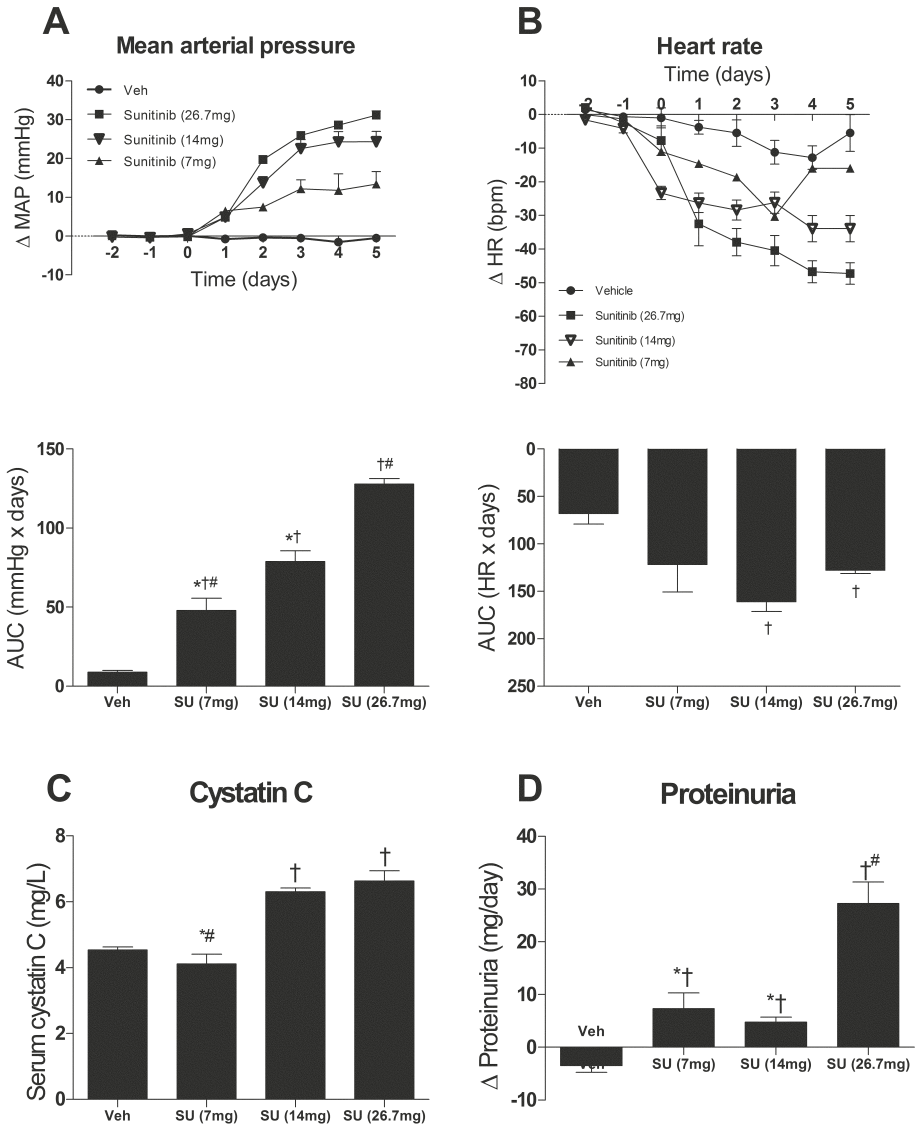

Figure 2. Time course of changes in mean arterial pressure (MAP; A) and heart rate (HR; B) in response to administration of vehicle, a low (7 mg/kg.day), an intermediate (14 mg/kg.day) and a high dose of sunitinib (26.7 mg/kg.day). Panels below, Areas under the curve (AUC). C and D, Serum cystatin C, and change in proteinuria (before and after treatment) respectively in rats after administration of vehicle, a low (7 mg/kg.day), an intermediate (14 mg/kg.day) and a high dose of sunitinib (26.7 mg/kg.day). *P<0.05 vs sunitinib (26.7 mg); †P<0.05 vs vehicle; #P<0.05 vs. sunitinib (14mg)

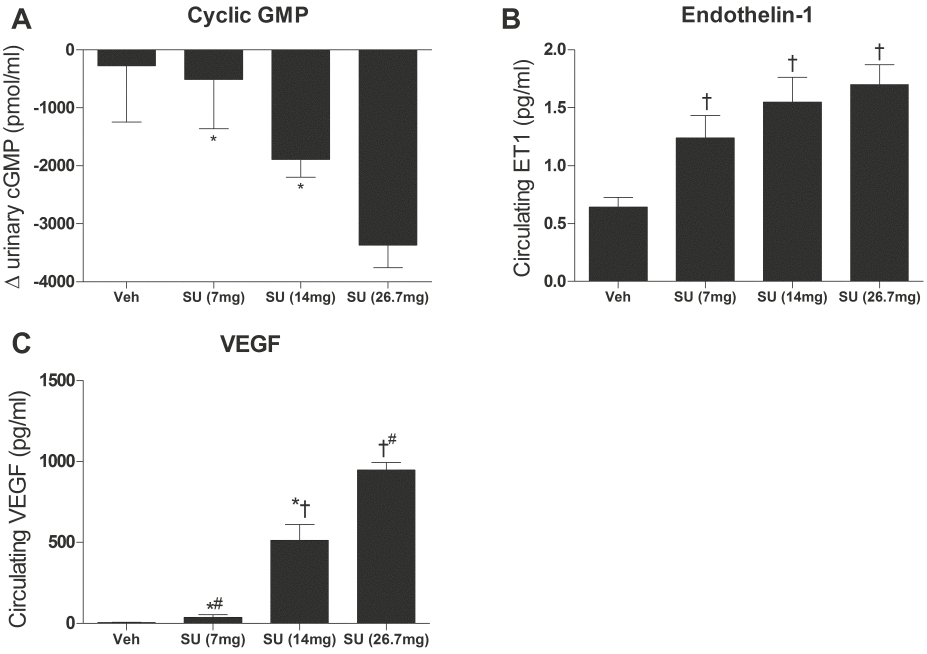

Figure 3. Change in urinary cyclic GMP (cGMP) excretion (A), circulating ET-1 levels (B) circulating VEGF levels (C) in rats after administration of vehicle, a low (7 mg/kg.day), an intermediate (14 mg/kg.day) and a high dose of sunitinib (26.7 mg/kg.day). *P<0.05 vs sunitinib (26.7 mg); †P<0.05 vs vehicle; #P<0.05 vs. sunitinib (14mg)

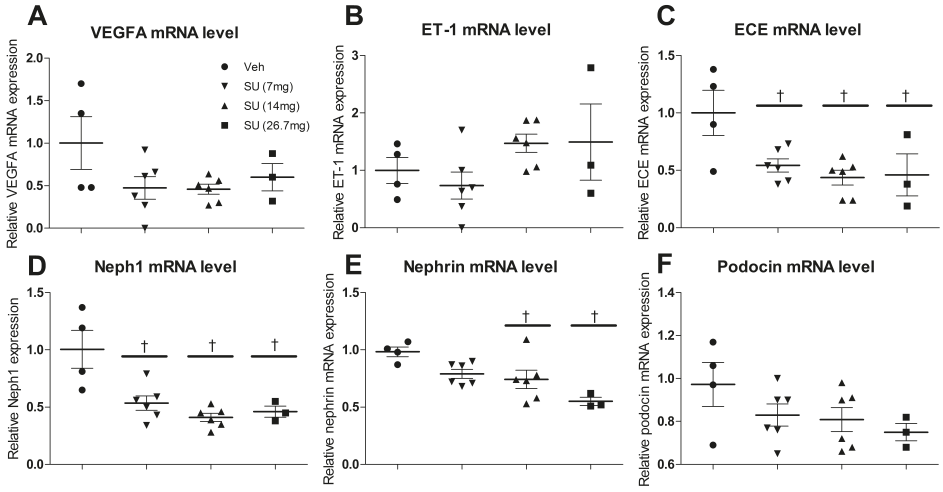

Figure 4. Relative renal mRNA expression level of VEGF-A (A), ET-1 (B) and ECE (C) after correction for the housekeeping gene Hprt. Relative renal mRNA expression of Neph1 (D), nephrin (Nphs1; E), and podocin (Nphs2; F). mRNA levels were corrected for the podocyte specific gene Wt1. †P<0.05 vs vehicle

Renal histopathology

Kidney weight-to-body weight ratio was 3.0±0.04 g/kg in vehicle-treated rats. This ratio increased to 3.3±0.2, 3.4±0.1 and 3.5±0.04 g/kg, respectively, at the low, intermediate and high dose of sunitinib ($P<0.05$ for all). LM of kidney sections revealed that the intermediate and high dose of sunitinib were associated with increases in endothelial cell swelling (Figure S1C and S1D). No endothelial cell swelling was present at the low dose of sunitinib (Figure S1B). PTAH staining showed fibrin deposits in glomerular capillaries (Figure S1E) and in small arteries at the high dose of sunitinib only (Figure S1F).

Quantification of renal histopathology based on LM images is displayed in Figure S2. The glomerular ischemia score in rats exposed to vehicle and low-dose sunitinib was identical, while this score increased dose-dependently with the intermediate and high dose of sunitinib. Comparable dose-dependent findings were obtained for the deposition of intraepithelial droplets. Peritubular capillary density or the presence of interstitial fibrosis and tubular atrophy between high dose of sunitinib and controls did not differ (data not shown).

Transmission EM of kidney sections showed normal foot processes and cytoplasmatic morphology at the low dose of sunitinib (Figure S4B). At the high dose of sunitinib, endothelial cell swelling with luminal obliteration and loss of intercellular fenestrations occurred (Figure S4C), whereas multiple intraepithelial droplets were present at the intermediate and high dose of sunitinib (Figure S4D).

Glomerular circumference, podocyte number and gene expression

Glomerular circumference tended to increase with the intermediate and high dose of sunitinib, but the number of podocytes per glomerular circumference remained unchanged (data not shown). The relative expression of the slit diaphragm mRNAs of genes encoding Neph1, nephrin and podocin decreased at the high and intermediate dose of sunitinib compared to vehicle (Figure 4D-F). Glomerular endothelium ICAM-1 expression was increased during sunitinib treatment (Figure S3).

DISCUSSION

Off-target effects of angiogenesis inhibitors that interfere with VEGF-signaling are the development of hypertension and renal injury.[3,16] By applying different doses of sunitinib, we explored the dose-dependency of these side effects and their interrelation. Our findings indicate that the severity of hypertension and the development of renal injury are dose-related. They also show a lower threshold dose for developing hypertension and proteinuria than for developing renal function impairment, as reflected by the rise in cystatin C concentration and the severity of glomerular ischemia. These findings concur well with clinical studies reporting a higher incidence of hypertension than renal

function impairment in patients treated with angiogenesis inhibitors.[3, 17] With the lowest dose of sunitinib applied, the BP rise of about 10 mmHg is of similar magnitude as the BP rise we have observed in patients after 2 and after 4 weeks administration of sunitinib, stressing the potential relevance of our low-dose model for the clinical situation.[9] Of note, the plasma sunitinib concentration with the low dose of sunitinib was comparable to the concentration measured in patients treated with a standard dose of sunitinib of 50 mg per day.[15] However, the concentration of its active metabolite n-desethyl sunitinib was markedly higher, indicating increased metabolism of the parent compound in rats, as has been reported previously.[18]

Glomerular endotheliosis, sometimes accompanied by thrombi, is a hallmark of angiogenesis inhibition-induced renal injury.[5] In the present study, glomerular endotheliosis with almost complete obliteration of glomerular capillaries was observed at the highest dose of sunitinib, whereas endotheliosis was less severe with the intermediate absent with the low dose of sunitinib. Using fibrin staining, fibrin clots in glomerular capillaries and small arteries, indicating thrombotic microangiopathy, were present at the high but not at the low and intermediate doses of sunitinib. Glomerular thrombotic microangiopathy has been reported in patients treated with anti-angiogenic treatment and can also occur in preeclampsia.[5, 6, 19] Exposure to sunitinib was not associated with a decrease in renal peritubular capillaries or interstitial fibrosis, indicating that the adverse renal effects are restricted to the glomeruli.

Genetic depletion studies in mice have shown that podocyte-specific heterozygosity for VEGF-A results in renal disease by 2.5 weeks of age characterized by proteinuria and endotheliosis.[20] Also rats or mice injected with a sFlt-1 or overexpressing sFlt-1 by virus injection develop endotheliosis and proteinuria.[7, 21, 22] Collectively, these findings indicate that interference with the VEGF-A signaling pathway, either genetically or by a soluble receptor trapping VEGF, or by a tyrosine kinase directly targeting VEGF-receptors, results in a similar glomerular phenotype.

In the kidney, VEGF is abundantly expressed in podocytes, whereas glomerular capillary endothelial cells preferentially express VEGF-receptors.[23] VEGF produced by podocytes is essential for the maintenance of the integrity of glomerular endothelial cells.[20, 24] In addition, VEGF is also required for the maintenance of podocyte function and slit-diaphragm proteins, implying that podocyte-derived VEGF exerts both paracrine and autocrine effects.[25] In preeclamptic patients and mice exposed to a VEGF-antibody or sFlt1, nephrin expression decreases.[22, 26] Moreover, in rats with progressive glomerulonephritis increased expression of sFlt1 resulted in massive proteinuria and down-regulated nephrin expression.[23] More recently, interaction of the cytoplasmatic domains of nephrin and VEGFR-2 has been demonstrated in both *in vivo* and *in vitro* studies.[27] Upon VEGF binding this nephrin-VEGFR-2 interaction diminishes, resulting in a change in shape and size of cultured podocytes.[27] Sunitinib administration in the

current study was also associated with a dose-dependent decreased expression of the gene encoding nephrin as well as a decrease in the genes expressing Neph1 and podocin, encoding for slit-diaphragm proteins that bind to nephrin (Figure 4).[28] Given that null mutations in the genes encoding nephrin, Neph1 and podocin are all associated with severe forms of congential nephrotic syndrome, it seems reasonable to conclude that the diminished expression of these genes is involved in the development of proteinuria during treatment with sunitinib as well as treatment with other agents interfering with VEGF signalling.[28, 29]

In line with our previous observations, sunitinib treatment was associated with activation of the circulating endothelin system, which was already present at the low dose of sunitinib.[3, 8, 9] The observed rise in ET-1 may be a direct consequence of VEGF inhibition, as shown in human lung microvascular endothelial cells.[30] The rise may also be related to the activation of the endothelium in response VEGF inhibition. This, however, was not supported by the present finding that despite activation of glomerular endothelial cells as reflected by the presence of glomerular endotheliosis and increased glomerular expression of ICAM-1, expression of gene encoding for ET-1 within the kidney was not increased. In addition to a direct adverse effect of VEGF inhibition on podocyte function and expression of slit-diaphragm proteins, ET-1 can exert negative effects on podocyte function, which additionally to the disruption of VEGF pathway, may contribute to glomerular injury and proteinuria.[10, 11, 31, 32] For instance, a sub-pressor dose of ET-1 administered to Sprague-Dawley rats was found to increase glomerular permeability and inflammation as well as nephrinuria, effects that could be blocked by an ET_A-receptor antagonist.[31] In cultured podocytes, ET-1 through activation of ET_A receptors induces nephrin shedding concomitant with a redistribution of the podocyte's cytoskeleton.[10] Very recently, Buelli et al. have shown activation of the β-arrestin-1 signalling pathway by ET-1, resulting in transition of podocytes from an epithelial to mesenchymal cell type.[11] Given the present observation that urinary ET-1 excretion was not increased during sunitinib administration or even decreased, the possibility that activation of ET-1 axis has contributed to the proteinuria in our rat model remains questionable. If ET-1 is involved it should, based on the present findings and an earlier study, reach the podocytes by ultrafiltration.[31]

Some limitations of our study should be mentioned. The duration of exposure to sunitinib was only 8 days. It would be interesting to see whether the rise in BP and renal pathology is progressive during more prolonged exposure. With the lowest dose of sunitinib the serum concentration of the active metabolite was considerably higher than observed in patients, yet the rise in blood pressure was comparable. Finally, although in the present study we have focussed on the renal effects of sunitinib, it is well known that VEGF inhibition can also negatively affect other organs, especially the myocardium and endocrine organs. In more long-term studies it would be interesting to see whether the lowest or even a still lower dose of sunitinib as used in the present

study still preferentially affects the kidney or also has adverse effects on the mentioned organs when administrated for prolonged periods.

Perspectives

This study shows activation of the circulating, but not of the renal ET-axis, already at a relatively low dose of sunitinib. This activation likely contributes to the off-target effects of sunitinib. In future experiments it should be explored whether blockade of the endothelin pathway can completely prevent the development of hypertension and renal injury in rats exposed to low doses of sunitinib for prolonged periods. This would provide evidence that activation of the endothelin pathway is an essential mediator for the rise in BP and renal toxicity associated with anti-angiogenic treatment.

ACKNOWLEDGMENTS

We want to thank Dr. J.A. Stoop for his excellent technical assistance.

NOVELTY AND SIGNIFICANCE

What is New?

This is the first study with the TKI sunitinib that explores the dose-dependency of hypertension and renal injury, aiming to find a sunitinib dose that, with regard to side effects, closely mimics the human situation

What is Relevant?

Our findings indicate that sunitinib-induced hypertension requires a lower dose than its induction of renal function impairment

Summary

This study shows activation of the systemic endothelin-axis, already at a low dose of sunitinib. This activation may not only contribute to the rise in blood pressure but also to the proteinuria and renal damage occurring during anti-angiogenic treatment

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SUPPLEMENT

EXPENDED METHODS

Light microscopy

The left kidney was rapidly excised from euthanized rats, decapsulated, weighed and sliced transversely. Slices were fixed in a 3.5-4% formaldehyde solution for light microscopic evaluation. After fixation in the formaldehyde solution, tissue was dehydrated and paraffin-embedded. Deparaffinized 2-µm thick sections were stained for haematoxilin-eosine (HE) and periodic acid Schiff (PAS). PAS-stained sections were blindly evaluated by a pathologist (F.M.M.S.) for the presence or absence of glomerular ischemia and intra-epithelial protein deposition in 50 glomeruli. Glomerular ischemia was scored semiquantitatively and defined as the degree of open glomerular capillaries, wrinkling of the glomerular basement membrane and filling of Bowmans space. Wide open glomerular capillaries filling Bowman's space entirely corresponded with no ischemia. Partially open glomerular capillaries with mild wrinkling of the glomerular basement membrane and Bowman's glomerular space largely filled was classed as moderate ischemia. Totally collapsed glomeruli and extensive wrinkling of the glomerular basement membrane and only partial filling of Bowman's space corresponded with severe ischemia. Furthermore, the presence of glomerular intra-epithelial protein deposition was evaluated using a semiquantative scale: 0 (no protein), 1 (protein present in 1-50% of the epithelial cells) and 2 (protein present in >50% of the epithelial cells). Fifty glomeruli per kidney section (PAS staining) were evaluated. Phosphotungstic acid-haematoxylin (PTAH) staining was used to identify fibrin deposits.

To assess the number of peritubular capillaries (PTC), 4µm formalin fixed paraffin-embedded tissue sections were cut and stained with CD31. Staining was performed following routine diagnostic procedures on the Benchmark Ultra stainer (Venatana Discovery), using buffers provided by Ventana that are validated for diagnostics. Antibody against CD31 (1:50 dilution, ab28364, Abcam, Cambridge, UK) were used to detect ptc's. The tissue sections were stained simultaneously to reduce inter-staining variation. Incubation with antibody was done for 60 minutes, after antigen retrieval of 64min at pH8.4. After staining, sections were blindly evaluated at 40x objective by a pathologist (M.C.v.G.) selecting 10 random, non-overlapping fields. Images were acquired using a Canon EOS 1100D camera and an Olympus BX40 microscope. The medulla and the subcapsular cortex with a width of 5 tubuli were excluded when images were acquired. Per image field, the number of PTC's and the number of tubuli were counted, and a ratio was made (ptc:tubuli). Evaluation of the staining has been adapted from Steegh *et al.*[1]

In addition, in order to assess the presence of interstitial fibrosis (ci) and tubulus atrophy (ct), 3µm sections were PAS stained following routine diagnostic procedures. Sections

were subsequently analyzed (M.C.v.G.) using a 20x objective and scored for ci and ct according to the Banff classification of renal allograft rejection.[2]

Electron microscopy

Kidney samples were processed for transmission EM as previously described.[3] Immediately after resection, cortical renal tissue was cut into blocks and immersed in Karnovsky´s fixative containing 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. After fixation, 1 mm^3 pieces were cut and postfixed in 1% OsO_4 in 0.1 M cacodylate buffer for 1 h. The specimens, were hereafter dehydrated in ethanol, immersed in acetone and embedded in Epon 812 R (Merck). Survey sections (2 μm) were stained with toluidine blue. Ultrathin (50-70 nm) sections with 2-3 randomly selected glomeruli were cut, mounted on copper grids and contrasted with uranyl acetate and lead citrate. Serial sections (8-10 sections per grid and 4-5 neighboring grids) were examined using a Philips Morgagni 261 EM microscope. Sections were blindly evaluated by a renal pathologist (A.H.) for the occurrence of glomerular endotheliosis (endothelial cell swelling, encroachment of the capillary spaces and loss of endothelial fenestration) and podocyte morphology as previously defined.[3]

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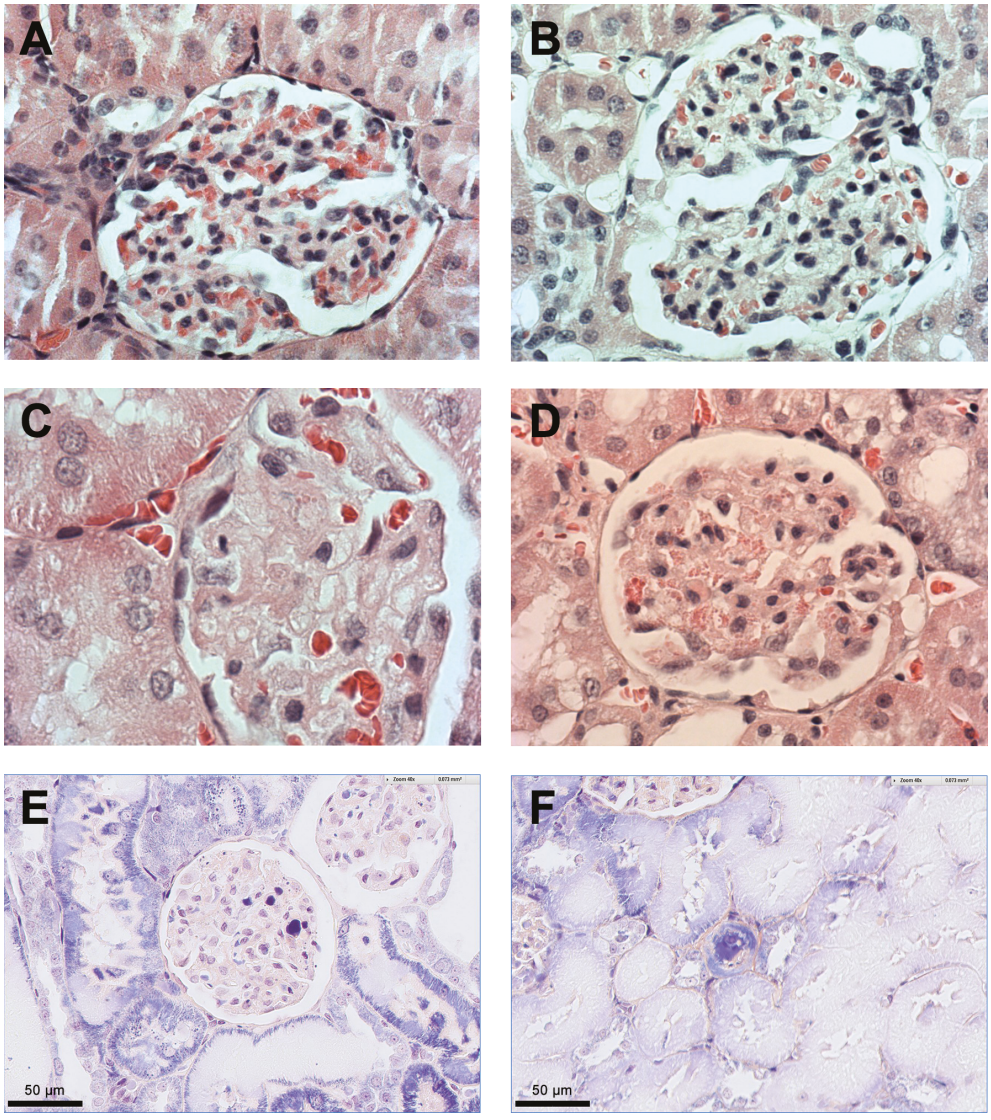


Figure S1. Figure S1. Kidney sections from WKY rats administered vehicle (A) or sunitinib; including a low dose (B), intermediate dose (C) or high dose (D), stained with HE stain (magnification x500). After administration of a high dose of sunitinib for 8 days marked glomerular changes could be observed, including intra-epithelial droplets and reduction in the number of capillaries. PTAH staining shows fibrin deposits in glomerular capillaries (E) and small arteries (F) with high dose of sunitinib (n=1).

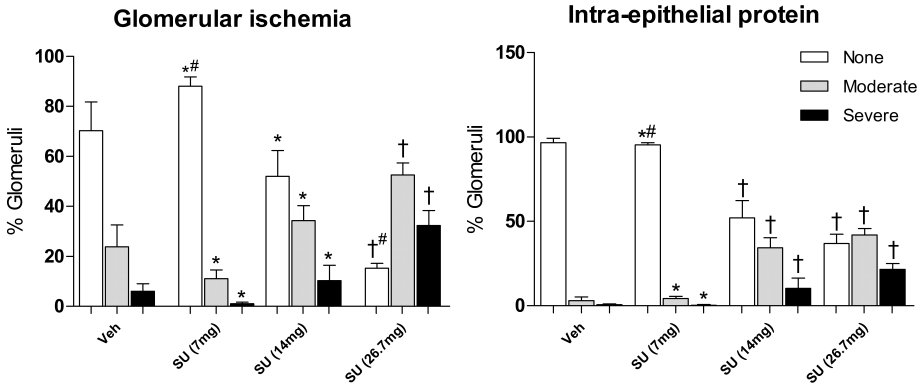


Figure S2. Light microscopic evaluation of kidney sections obtained from rats exposed to a low, an intermediate and a high dose of sunitinib for 8 days compared to controls. All evaluations were performed in 50 glomeruli of a PAS-stained section and the numbers of glomeruli with each score were counted. *P<0.05 vs sunitinib (26.7 mg); †P<0.05 vs vehicle; #P<0.05 vs. sunitinib (14mg)

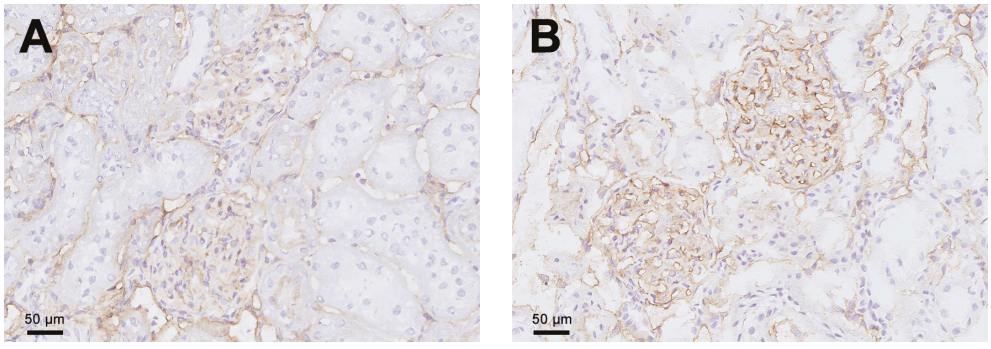


Figure S3. Kidney sections from WKY rats administered vehicle (A) or sunitinib (B). ICAM-1 staining shows increased glomerular endothelium staining, suggesting endothelium activation at all dosages of sunitinib (n=1).

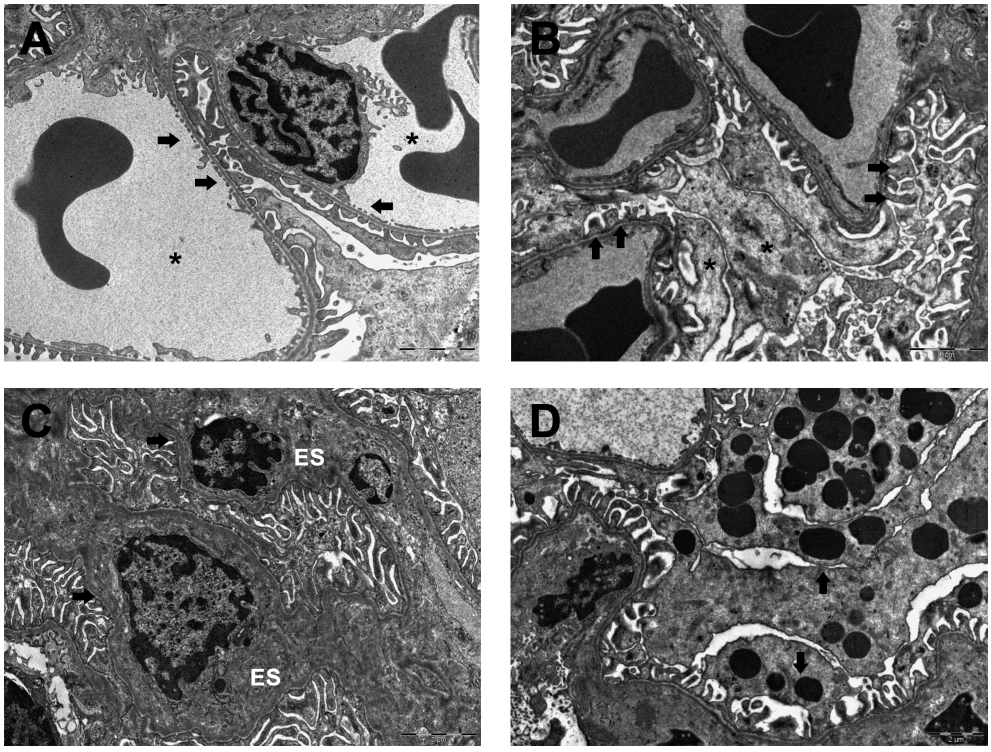


Figure S4. Transmission electron micrographs of representative kidney sections from Wistar Kyoto (WKY) rats treated with vehicle (A) or sunitinib (B-D). (A) Normal capillary lumina (asterix) and endothelial cells with preserved fenestration (arrows) in rat receiving vehicle. (B) Podocytes showing preserved foot processes (arrows) and normal cytoplasmic morphology (asterix) in low dose sunitinib-treated rat. (C) Glomerular endotheliosis with endothelial swelling (ES), loss of fenestration (arrows) and maximal luminal obliteration in high dose sunitinib-treated rat. (D) Podocyte containing multiple intracellular droplets (arrow) in intermediate dose sunitinib-treated rat. Scale bars = 2 μm.

Table S1. Real time PCR primers

Name	forward primer	reverse primer
VEGF-A	ACAGAAGGGGAGCAGAAAGCC	ACCGCATTAGGGGCACACAG
Endothelin-1	AGGGAAAACCCTGTCCCAAG	CACGGGGCTCTGTAGTCAAT
Endothelin converting enzyme	GAGCCTGAGCACCCTGAAAT	ACTTTGTCCAGCTCCTTGGG
Neph1	GGATGGCGGTAAGGTGGAGTG	CGTTATTGATGGTGAGAGTGGACAG
Nephrin	CGTCAGCATCAGCAGCAACC	AGCCACATCTTCCAGCCTCTC
Podocin	TGGACTCAGTGACCTGTGTTTGG	CAGCAATCACCCGCACTTTGG
WT-1	TGTGACTTCAAGGACTGCGAGAG	GGTGTGGGTCTTCAGGTGGTC
HPRT	GGCTATAAGTTCTTTGCTGACCTG	GGCTATAAGTTCTTTGCTGACCTG

CHAPTER 6

SALT-SENSITIVITY OF ANGIOGENESIS INHIBITION-INDUCED BLOOD PRESSURE RISE: ROLE OF INTERSTITIAL SODIUM ACCUMULATION?

Stephanie Lankhorst, David Severs, Lajos Marko, Natalia Rakova, Jens Titze, Dominik N. Müller, A.H. Jan Danser, Anton H. van den Meiracker.

In progress

ABSTRACT

In response to salt loading Na^+ and Cl^- accumulates in the skin in excess of water, stimulating skin lymphangiogenesis via activation of the mononuclear-phagocyte system (MPS) cell-derived VEGFC-VEGF type 3 receptor signalling pathway. Inhibition of this pathway results in salt-sensitive hypertension. Sunitinib is an antiangiogenic agent that blocks all three VEGF receptors and increases blood pressure. In the present study we explored whether sunitinib causes salt-sensitive hypertension and whether impairment of skin lymphangiogenesis is an underlying mechanism. Normotensive Wistar Kyoto rats were exposed to a normal or high salt (NS or HS) diet with or without sunitinib administration. Sunitinib induced a 15 mmHg rise in telemetrically measured blood pressure, which was aggravated by a HS diet, resulting in a decline of the slope of the pressure-natriuresis curve. Without affecting body weight, plasma sodium concentration or renal function, Na^+ and Cl^- skin content increased by 31 and 32% with the HS diet and by 49 and 50% with the HS diet plus sunitinib (P=NS), whereas skin water increased by 17 and 24% respectively. Skin MPS cells increased both during sunitinib and a HS diet. No further increment was seen when HS diet and sunitinib were combined. HS diet increased lymphangiogenesis whereas sunitinib treatment decreased lymphangiogenesis very modestly. We conclude that sunitinib administration is associated with salt-sensitive hypertension that can be only partly explained by impaired skin lymphangiogenesis, suggesting that other factors are involved.

INTRODUCTION

Angiogenesis, the formation of new vessels from pre-existing vasculature, is critical to solid tumor growth and metastasis. This process is regulated by numerous factors among which vascular endothelial growth factor (VEGF) plays a predominant role. Different strategies to inhibit the VEGF-signaling pathway by targeting VEGF or its receptors have been developed and are now established modalities for the treatment of a wide range of malignancies.[1-4] VEGF inhibition is associated with hypertension, proteinuria and renal function impairment in a substantial portion of patients, sometimes necessitating discontinuation of treatment.[5, 6] Sunitinib is an orally active angiogenesis inhibitor that blocks the VEGF receptors (VEGFR)-1, -2 and -3, and other tyrosine kinase receptors, including platelet derived growth factor (PDGF) and c-Kit receptors by interacting with their ATP-binding pockets.[7] VEGFR-1 and VEGFR-2 are predominantly expressed on vascular endothelial cells, whereas VEGFR-3, stimulated by VEGFC, is restricted to lymphatic endothelial cells.[7]

In a previous study, Gu et al. have shown that the multi-targeted VEGFR inhibitor SU5416 enhances dietary salt-induced hypertension and kidney injury in normotensive Sprague-Dawley rats.[8] The authors suggested that the SU5416-induced decrease in renal nitric oxide (NO) production in proximal tubular epithelial cells via inhibition of endothelial NO synthase (eNOS) underlies this salt-sensitive hypertension.[8] Recent evidence indicates that in response to a high salt diet, sodium and chloride accumulates in the skin in excess of water.[9] This results in a hypertonic interstitial fluid compartment leading to accumulation of mononuclear phagocyte system (MPS) cells.[10, 11] In response to the hypertonic environment, MPS cells produce increased amounts of the tonicity-responsive enhancer-binding protein (TonEBP), that initiate expression and secretion of VEGF-C. By activation of the lymph-endothelial VEGF-3 receptor, VEGF-C increases the density of the cutaneous lymph vessels as an adaptive mechanism to clear the excessive electrolytes.[10, 11] Interruption of this pathway in mice and rats is associated particularly with Cl^- accumulation and salt-sensitive hypertension.[9, 11] Since sunitinib, aside from blocking VEGFR-1 and VEGFR-2 signaling, also blocks the VEGFR-3 signaling that mediates lymphangiogenesis, we hypothesized that sunitinib administration impairs lymphangiogenesis in response to a high salt diet and that this impairment leads to Na^+ and especially Cl^- accumulation in the skin interstitium, contributing to salt-sensitive hypertension. To test this hypothesis, we measured blood pressure telemetrically, and determined skin electrolytes, MPS cell infiltration and skin lymphangiogenesis in normotensive Wistar-Kyoto rats exposed to a normal or a high sodium diet with and without addition of sunitinib.

METHODS

Animals

All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC. Male Wistar Kyoto rats (WKY, 180-200 gram, n=30), obtained from Harlan Laboratories, were individually housed and maintained on a 12-h light/dark cycle, having access to standard laboratory rat chow (normal salt diet (NS); 0.5-1.0% NaCl) and tap water ad libitum. Intra-aortic blood pressure (BP) recordings were performed by implanted radiotelemeters (PA-C40, Data Sciences International) and sunitinib (SU11248; Sutent, Pfizer) and vehicle solution were prepared and administered by oral gavage as described previously.[12] After implantation of the telemetry transmitters, rats received analgesic treatment using Temgesic subcutaneously (0.05 mg/kg; RB Pharmaceuticals Limited) for 2 days, followed by a recovery period of 10 days. After this, animals were randomly allocated to groups that received either a high salt (HS) diet (8% NaCl + 0.9% saline drinking water, n=15) or a normal salt diet for 2 weeks (n=15). Rats were then randomly administered a dose of sunitinib (7 mg/kg.day, n=8) or vehicle (n=7) by oral gavage (0.5 mL) for 8 days, on top of the NS or HS diet.

At the end of each experiment, rats were euthanized with 60 mg/kg pentobarbital i.p. Blood was sampled for measurement of creatinine, cystatin C levels and electrolytes, and kidneys were rapidly excised, and ears and skin were collected.
In all experiments, before the start of the specific diet (Baseline 1), and immediately before (Baseline 2) and after 8-day administration of vehicle or sunitinib, rats were housed in metabolic cages for 48 hours with free access to food and water; the first day to acclimatize and the second day to collect 24-hour urine samples. BP was not monitored when rats were housed in metabolic cages due to the absence of telemetry receivers.

Skin and ear samples were collected for histology. Chemical analysis of the skin included Na^+, K^+, Cl^-, and water measurements using a dry ashing method as described previously.[13]

Biochemical Measurements

Plasma creatinine and cystatin C (Cobas c502 and c702, CYSC, Roche Diagnostics), and plasma and urinary electrolytes were measured at the clinical chemical laboratory of the Erasmus MC.

Immunohistochemistry and immunofluorescence of lymph capillaries and MPS cells

Lymph vessel and MPS cell density were assessed in the right ear by thin-section histology using a podoplanin antibody as described previously.[11] Briefly, right ears of the rats were fixed in 5% formalin and 4% PFA respectively, and embedded in

paraffin. Podoplanin staining was performed using Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, USA) and the HRP super staining kit (ID Labs, Ontario, USA) according to the manufacturer's instructions. Deparaffinized slides were boiled two times for 5 min at 600 W in a microwave in 0.1 M citrate buffer (pH 6.0). After cooling down to room temperature, slides were incubated in 3% H_2O_2 for 10 min. Blocking was performed with the Avidin/Biotin Blocking according to the manufacturer's protocol and with SuperBlock for 7 min. After washing with phosphate-buffered saline (PBS) three times, slides were incubated with a podoplanin antibody (1:2000, RELIATech GmbH) for 1 h, followed by incubation with the polyvalent antibody and HRP for 10 min. Slides were washed three times with PBS between every step. Specific stainings were detected using AEC Chromogen/Substrate (3-Amino-9-ethylcarbazole, ID Labs). Lymph capillaries were counted at 100X amplification starting from the same edge of each ear in 5 consecutive fields.

MPS cells were visualized by indirect immunofluorescence. Nonspecific binding sites were blocked with 10% normal donkey serum (Jackson ImmunoResearch, West Grove, USA) for 30 min. Sections were then incubated with a CD68 antibody, a monocyte-macrophage marker (1:1000, AbD Serotec). All incubations were performed in a humid chamber. For fluorescence visualization of bound primary antibodies, sections were further incubated with Cy3-conjugated (Jackson ImmunoResearch) or Alexa Fluor 488-conjugated (Invitrogen, Paisley, UK) secondary antibodies for 1 h in a humid chamber at room temperature. Percentage of CD68 positive/total area was calculated in 5 consecutive fields and means of these values are presented.
Specimens were analyzed using a Zeiss Axioplan-2 imaging microscope with AxioVision 4.8 software (Zeiss, Jena, Germany), except for whole-mount stained samples, which were analyzed by multiphoton confocal microscope Zeiss LSM 710 with ZEN 2012 software (Zeiss). Investigators were blinded for treatment group assignments.

Statistical Analysis

Data are presented as mean±SEM. Statistical analysis between groups was performed by unpaired t-testing or by repeated-measures ANOVA followed by Newman-Keuls or Dunnett's multiple comparison testing. Correlations were performed using Pearson's testing. GraphPad Prism version 5.0 was used for all statistical analysis.

RESULTS

HS diet enhances the hypertensive effect of sunitinib and decreases the slope of the pressure-natriuresis curve

MAP during a NS diet was 101±0.9 mmHg (Figure 1A). A HS diet increased MAP by 27±3 mmHg ($P<0.05$ vs. NS diet). Sunitinib increased MAP by 15±1 mmHg during a NS diet ($P<0.05$ vs. NS diet alone) and by 23±4 mmHg during a HS diet ($P<0.05$ vs. HS diet alone). The sunitinib-induced increase in area under the curve under HS conditions

(85 mmHg x days) was enhanced by 124% (P<0.05) versus the increase under NS conditions (38 mm Hg x days) (Figure 1B), and this resulted in a decline in the slope of the pressure-natriuresis curve (Figure 1C).

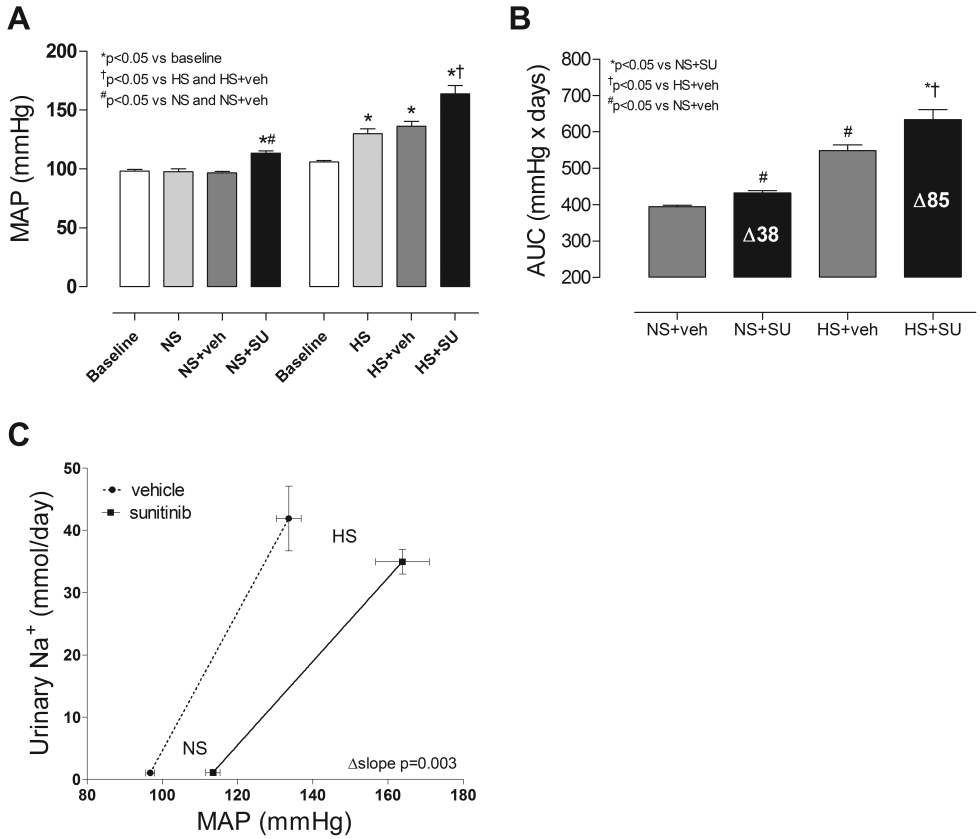


Figure 1. Mean arterial pressures (MAP; A) in rats after baseline (white bars), 2 weeks of specific diet (light grey bars) and in combination with or without VEGF inhibition (black and dark grey bars), area under the curve (consisting of MAP and number of treatment days) (B) and pressure-natriuresis curve during the specific diets with or without VEGF inhibition (C). NS, normal salt; HS, high salt, Veh, vehicle; SU, sunitinib. Δ=vs. respective vehicle group.

Body weight, plasma and skin electrolytes and kidney function

Urinary sodium excretions during a NS diet and HS diet were 1.0±0.2 mmol/day and 41.9±5.2 mmol/day respectively. Sunitinib did not affect these values significantly (1.1±0.1 mmol/day vs. 34.3±1.8 mmol/day). A HS diet or sunitinib administration had no effect on body weight or plasma Na^+ or K^+ concentration, whereas plasma Cl^- concentration modestly increased during the HS diet with and without sunitinib (Figure 2A and 2B and Table 1). A HS diet increased the content of Na^+ and Cl^- (mmol/g dry

weight) and of water content (ml/g dry weight) in the skin, and although sunitinib tended to increase both ions even further, these increases did not reach statistical significance versus HS alone (Table 1). The skin Na^+, Na^+ plus K^+ or Cl^- concentration per skin water (mmol/L) did not change either with the HS diet or with sunitinib (Table 1, Figure 3); however a linear trend was observed for Na^+ per skin water (Figure 2C). As displayed in Figure 4, both skin Na^+ (A) and Cl^- (B) concentrations correlated with blood pressure. Renal function as reflected by plasma creatinine and cystatin C concentration and creatinine clearance did not change in response to a HS diet with or without sunitinib (Table 1).

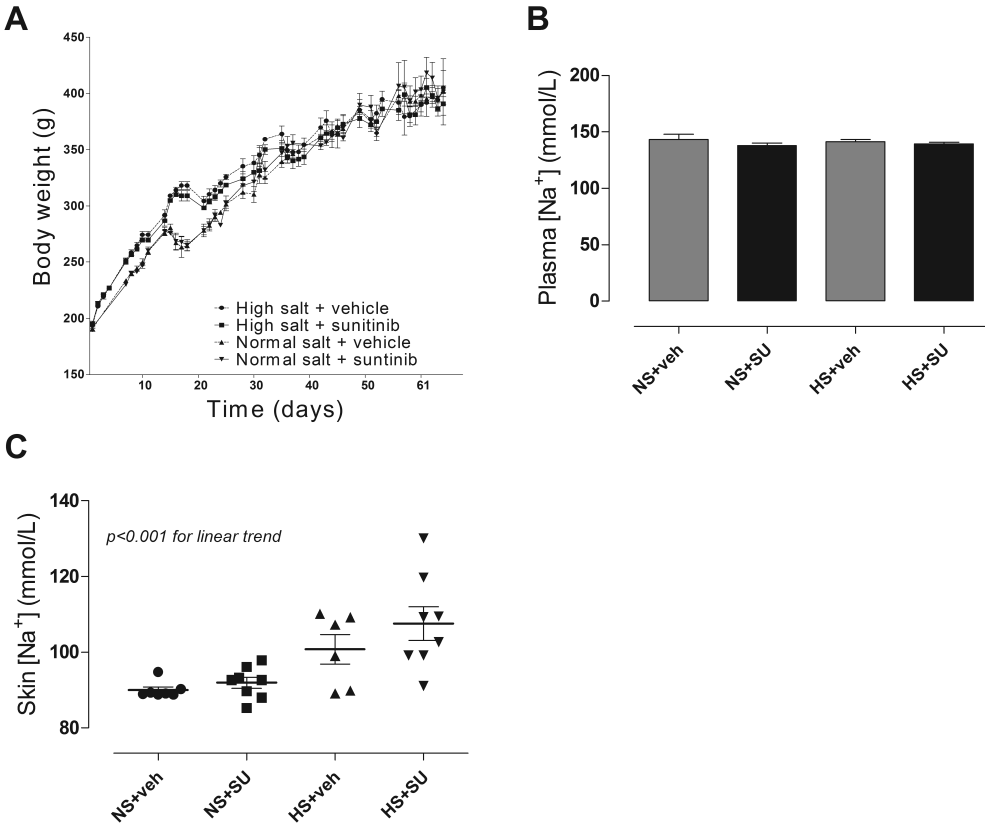


Figure 2. Body weight (A), plasma (B) and skin sodium concentrations (C) in rats after the specific diets. NS, normal salt; HS, high salt;Veh, vehicle; SU, sunitinib.

Table 1. Skin electrolyte and water distributions, plasma electrolytes and kidney function after specific diets with or without VEGF inhibition

Electrolyte and water content/concentration in skin	NS diet + Vehicle n=7	NS diet + sunitinib n=8	HS diet + Vehicle n=7	HS diet + sunitinib n=8
Skin Na^+ (mmol/g DW)	0.110±0.003	0.112±0.002	0.144±0.005#	0.164±0.009*#
Skin Cl^- (mmol/g DW)	0.051±0.003	0.052±0.003	0.068±0.006#	0.073±0.007*#
Skin water (mL/g DW)	1.223±0.032	1.208±0.019	1.432±0.032#	1.522±0.044*#
Skin (Na^+ + K^+)/skin water	0.170±0.001	0.167±0.003	0.176±0.008	0.171±0.004
Skin Na^+/skin water (mmol/L)	90±0.8	92±1.5	101±3.9	108±4.4
Skin Cl^-/skin water (mmol/L)	42±2.4	43±1.5	48±4.8	50±4.4
Skin K^+/skin water (mmol/L)	80±1.1	75±1.8#	71±3.0#	69±2.5
Plasma electrolytes				
Plasma [Na^+] (mM)	143.7±4.4	137.8±2.3	141.4±2.1	139.4±1.7
Plasma [K^+] (mM)	3.9±0.2	4.2±0.3	4.2±0.3	4.4±0.4
Plasma [Cl^-] (mM)	98.2±1.1	100.5±0.5	104.8±1.8#	109.1±1.0†*#
Kidney function				
Plasma creatinine (μM)	27.3±0.9	31.1±1.5	26.6±0.7	28.1±1.0
Plasma cystatin C (mg/L)	4.0±0.6	4.7±0.3	3.5±0.2	3.9±0.3
Creatinine clearance (ml/min)	3.1±0.5	3.1±0.5	3.5±0.3	3.5±0.2

NS, normal salt; HS, high salt; DW, dry weight. Data are presented as mean±SEM

*P<0.05 vs. NS+SU, †P<0.05 vs. HS+veh, #P<0.05 vs. NS+veh

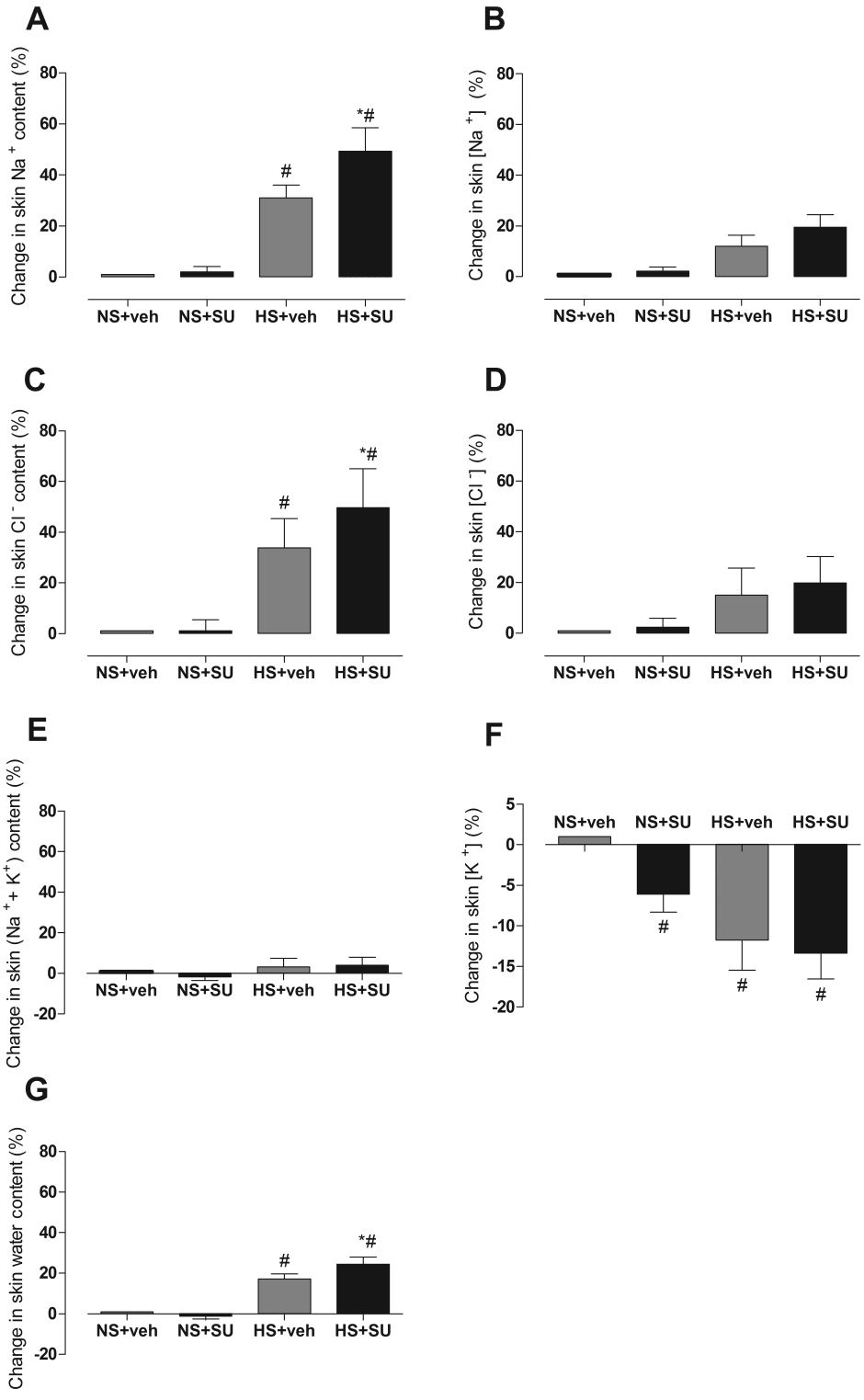
A HS diet and sunitinib increase skin MPS cell infiltration

As compared with the NS diet plus vehicle, both sunitinib administration and a HS diet were associated with an increase in CD68 positive cell area (Figure 5). These data confirm the notion that MPS cells respond to VEGFR blockade in an effort to restructure the lymphatic capillary network.

HS diet leads to increased lymph capillary density in the skin

In addition to the increased number of MPS cells, the number of podoplanin-positive lymph capillaries increased in response to a HS diet, and a HS diet plus sunitinib compared with a NS diet and NS diet plus sunitinib (Figure 6). Sunitinib treatment tended to decrease the number of podoplanin-positive lymph capillaries in the ears of rats fed with NS diet (2.6±0.2 vs. 1.9±0.3) and in the ears of rats fed with HS diet (4.4±0.5 and 3.6±0.4).

Figure 3. Change (%) in skin sodium content (A), chloride content (C), sodium-potassium ratio content (E), skin water content (G), and skin sodium concentration (B), chloride concentration (D) and potassium concentration (F) compared to a normal salt diet plus vehicle. NS, normal salt; HS, high salt, Veh, vehicle; SU, sunitinib.


A
Change in skin Na+ content (%)
80
60
40
20
0
#
*#
NS+veh
NS+SU
HS+veh
HS+SU
B
Change in skin [Na+] (%)
C
Change in skin Cl- content (%)
D
Change in skin [Cl-] (%)
E
Change in skin (Na+ + K+) content (%)
-20
F
Change in skin [K+] (%)
5
-5
-10
-15
G
Change in skin water content (%)

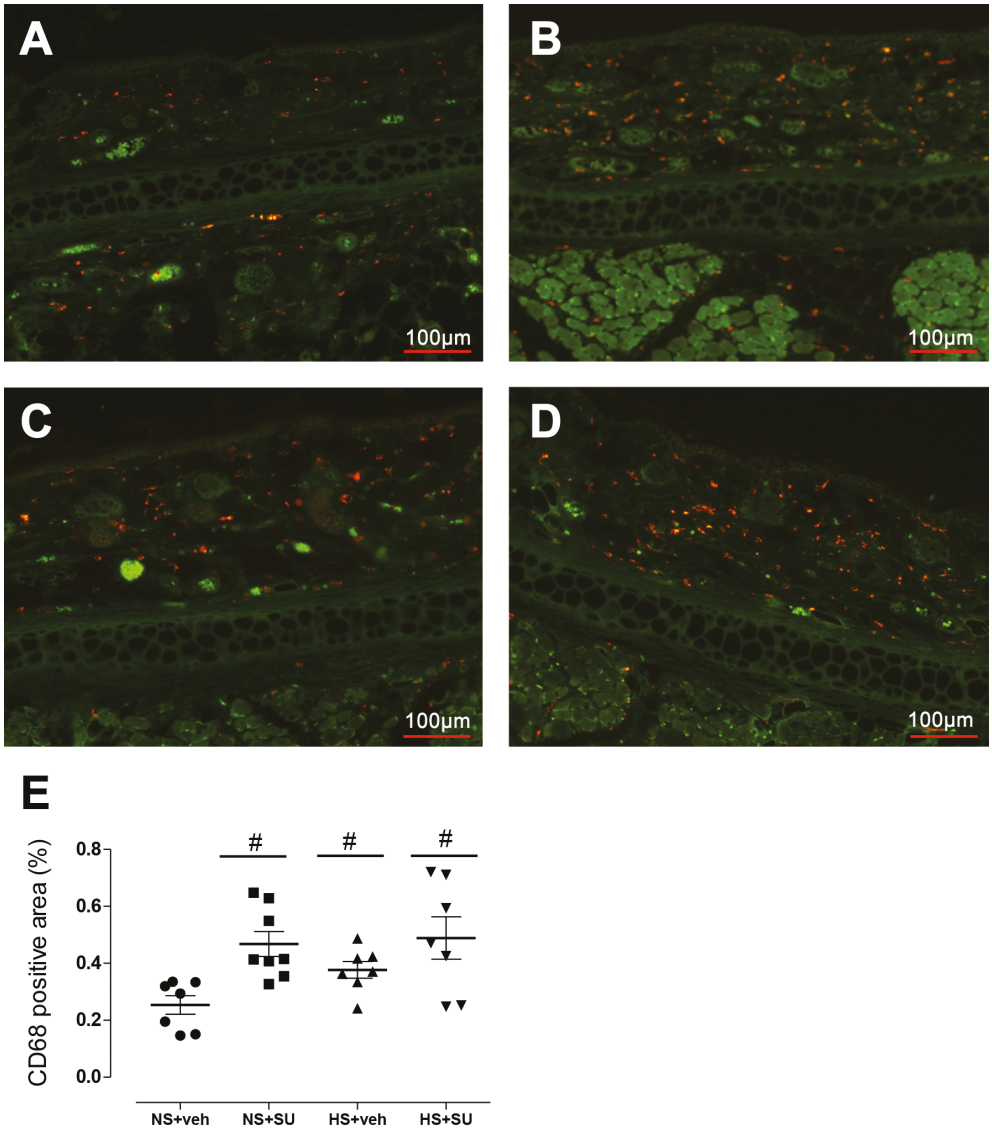


Figure 5. Representative pathological changes in rat ears with NS diet or HS diet in the absence (A or C) or presence (B or D) of sunitinib administration. MPS cells in rat ears visualized with a CD68-specific antibody (red), and quantification of the CD68 positive/total area (%) in rat ears after the specific diets (E). A, NS+veh; B, NS+SU; C, HS+veh; D, HS+SU. $^{\#}P<0.05$ vs. NS+veh

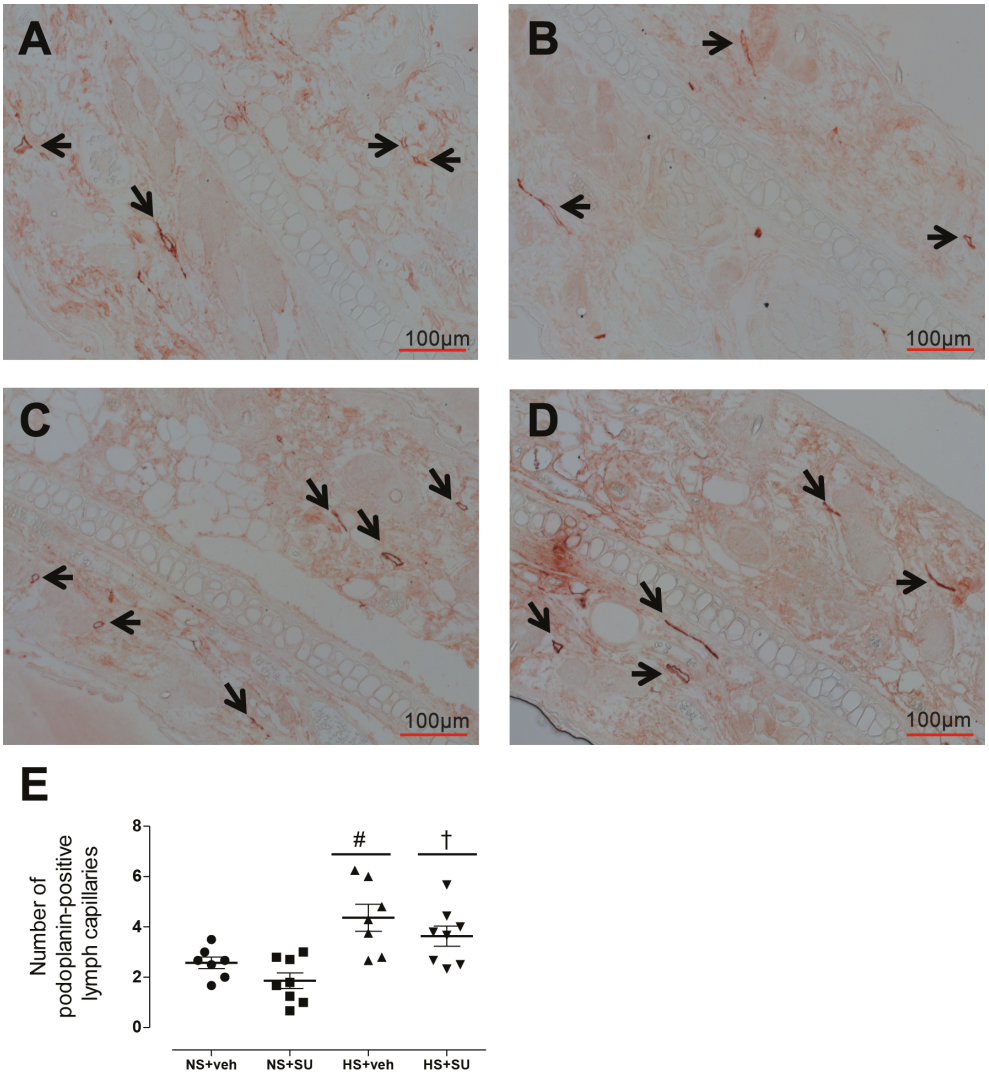


Figure 6. Representative pathological changes in rat ears with NS diet or HS diet in the absence (A or C) or presence (B or D) of sunitinib administration. Lymph capillary endothelial cells in rat ears visualized with a podoplanin-specific antibody (arrows), and quantification of the number of podoplanin-positive lymph capillaries in rat ears after the specific diets (E). A, NS+veh; B, NS+SU; C, HS+veh; D, HS+SU. †P<0.05 vs. HS+veh. #P<0.05 vs. NS+veh

DISCUSSION

In this study, we explored whether a HS diet augments the BP rise induced by the multi-targeted VEGF inhibitor sunitinib and whether this is associated with increased skin electrolyte accumulation concomitant with an attenuation of skin lymphangiogenesis. Our main findings are that 1) a HS diet in normotensive Wistar-Kyoto rats induces a marked rise in BP that is aggravated by sunitinib administration, 2) this rise in BP is not accompanied by an increase in body weight or changes in plasma sodium concentration or renal function impairment, 3) a HS leads to a proportionally comparable increase in skin Na^+ and Cl^- which is higher than the proportional increase in water content, 4) a HS diet associates with an accentuated MPS cells infiltration in the skin that is not affected by sunitinib administration, and 5) sunitinib treatment tended to decrease lymphangiogenesis independent of diet.

Our study shows that a HS diet in normotensive male WK rats for 2 weeks results in a 27 mmHg rise in BP, without increases in body weight or change in renal function. An identical rise in BP in response to a HS diet has been observed in normotensive Sprague-Dawley rats.[8] The dose of sunitinib applied in the present study induced a 15 mmHg rise in blood pressure while rats were on a NS diet. This dose of sunitinib was selected based on a previous study showing that when given for one week it did not impair renal function and induced a rise in blood pressure comparable to the rise observed in patients treated with sunitinib for four weeks at a daily oral dose of 50 mg.[14] In agreement with a previous study with the tyrosine kinase inhibitor SU5416, sunitinib administration aggravated the HS diet-induced rise in BP, as reflected by a decline in slope of the pressure-natriuresis curve.[8]

Evidence has mounted that in response to a HS diet, Na^+ and Cl^- in excess of water accumulates in tissues.[9-11] In a seminal study, Machnik et al. have shown that this skin electrolyte accumulation stimulates the formation of lymph capillaries, driven by MPS cells infiltrating the interstitium of the skin.[11] The authors also demonstrated that macrophage-derived VEGF-C, via activation of the lymph endothelium VEGFR-3, mediates this lymphangiogenesis and that interruption of this pathway in mice and in rats associates with salt-sensitive hypertension.[11] In the present study, performed in normotensive WKY rats, a HS diet was indeed accompanied by increased MPS cells infiltration and lymphangiogenesis. Since sunitinib has been shown to inhibit lymphangiogenesis by blocking VEGFR-3 signaling.[15], we anticipated an attenuation of this HS diet-induced lymphangiogenesis during sunitinib administration, and indeed a tendency for a decrease in skin lymphangiogenesis was observed.

In mice on anti-VEGFR-3 or VEGF-C trap treatment, or in mice genetically overexpressing soluble VEGFR-3 (K14-FLT4 mice) a HS diet caused a much more pronounced increase in skin Cl^- than in skin Na^+ content, leading to an increase in the

skin Cl^-/Na^+ ratio.[9, 16] This skin Cl^- accumulation, rather than skin Na^+ accumulation, was most strongly related to the rise in blood pressure in response to a HS diet.[9] A HS diet after 4 weeks in our rats was associated with an about 30% increase in skin interstitial Na^+ and Cl^- contents after vehicle administration, and the increase amounted to about 50% after sunitinib administration compared to a NS diet plus vehicle (Figure 3). This increase was higher than the increase in skin interstitial water, which was respectively 17% after 2 and 24% after 4 weeks, indeed confirming the occurrence of skin electrolyte accumulation in excess of water with salt loading.

One may wonder why sunitinib exerted only a small effect on lymphangiogenesis in the present study. Although an insufficient dose is the most obvious explanation, this is not supported by previous pharmacokinetic and in vitro studies.[14, 15] We previously reported that the same dose of sunitinib as used in the present study was associated with a plasma concentration of sunitinib and its active metabolite N-desethyl sunitinib of about 350 nanogram/mL or 875 nmol/L.[14] In an in vitro study, sunitinib concentrations as low as 30 nmol/L were shown to impair lymphangiogenesis.[15] Based on these data it is not very likely that the dose of sunitinib was the limiting factor to impair lymphangiogenesis.

Our findings do not support the idea that impaired skin lymphangiogenesis is a major contributor to the rise in blood pressure or salt-sensitivity observed with sunitinib. As reported earlier, administration of tyrosine kinase inhibitors is associated with activation of the endothelin system as well as suppression of the NO pathway and renin suppression.[12, 17, 18] As suggested by Gu et al., suppression of the NO pathway can contribute to the salt-sensitive blood pressure observed with anti-VEGF treatment.[8] Likewise, it is well established that renin suppression is associated with salt-sensitive hypertension.[19-21] To what extent activation of the endothelin system, occurring during sunitinib administration, contributes to salt-sensitive hypertension is not easy to answer because of the opposing effects on natriuresis mediated by the renal ET_A and ET_B receptors.[22] However, it has been reported that infusion of ET-1 in Sprague-Dawley rats induces hypertension when rats are on a high but not on a low salt diet.[23, 24] Similarly, transgenic mice overexpressing endothelial ET-1 had a significantly higher blood pressure when on a HS diet than their wild-type littermates.[25] These studies support the contention that an activated ET-1 system may contribute to salt-sensitive hypertension.

PERSPECTIVES

Our study shows that sunitinib administration to rats, in a relatively low dose not inducing renal function impairment, is associated with salt-sensitive hypertension. Impairment of the skin MPS cells-TonEBP-VEGF-VEGFR-3-lymphangiogenesis pathway has been recognized as a novel mechanism involved in salt-sensitive hypertension. In our study sunitinib treatment blocked lymphangiogenesis only modestly. Therefore, it is more likely that other mechanisms such as renin suppression, a decrease in NO

production and activation of the endothelin system largely contribute to the salt-sensitive hypertension during sunitinib administration. We acknowledge that sunitinib was administrated for a relatively short period. Whether prolonged administration of sunitinib impairs the formation of lymph vessels in response to a high salt diet remains to be established. Furthermore, given that interruption of the VEGF-signaling pathway is associated with activation of the endothelin system, it would be interesting to explore whether the sunitinib-induced salt-sensitive hypertension can be prevented by ET_A-selective or $ET_{A/B}$ endothelin receptor blockade.

NOVELTY AND SIGNIFICANCE

What is new?

This is the first study with the tyrosine kinase inhibitor sunitinib that explores whether the salt-sensitivity of VEGF inhibition-induced hypertension is related to impairment of skin lymphangiogenesis

What is relevant?

Our findings indicate that a high salt diet enhances the hypertensive effect of sunitinib, which may be not principally due to impaired skin lymphangiogenesis

Summary

Other mechanisms than impaired skin lymphangiogenesis, like renin suppression, a diminished NO production and/or activation of the endothelin system are likely to play a bigger role for the salt-sensitivity of blood pressure induced by sunitinib.

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CHAPTER 7

EFFECT OF HIGH SALT DIET ON BLOOD PRESSURE AND RENAL DAMAGE DURING VEGF INHIBITION WITH SUNITINIB

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ABSTRACT

Background

Antiangiogenic treatment with the multitargeted VEGF receptor inhibitor sunitinib associates with a blood pressure rise and glomerular renal injury. Recent evidence indicates that VEGF derived from tubular cells is required for the maintenance of the peritubular vasculature. In the present study we focused on tubular and glomerular pathology induced by sunitinib and explored whether a high salt (HS) diet augments the blood pressure rise and renal abnormalities.

Methods

Normotensive Wistar Kyoto (WKY) rats were exposed to a normal (NS) or HS diet for 2 weeks, and subsequently for 8 days to sunitinib or vehicle administration after which rats were euthanized and kidneys excised. Mean arterial pressure (MAP) was telemetrically measured. Urine was sampled for proteinuria and endothelinuria, and blood for measurement of endothelin-1, creatinine, and cystatin C.

Results

Compared to the NS diet, MAP rapidly rose by 27±3 mmHg with the HS diet. On sunitinib MAP rose further by 15±1 with the NS and by 23±4 mmHg with the HS diet ($P<0.05$). The HS diet itself had no effect on proteinuria, endothelinuria or the plasma levels of endothelin-1, creatinine, and cystatin C. Only with the HS diet, sunitinib administration massively increased proteinuria and endothelinuria and these 2 parameters were related ($r=0.50$, $P<0.01$). Likewise, renal glomerular pathology was enhanced during sunitinib with the HS diet, whereas tubulointerstitial injury or reduced peritubular capillary density did not occur.

Conclusions

A HS diet induces a marked blood pressure rise in WKY rats and exacerbates both the magnitude of the blood pressure rise and glomerular injury induced by sunitinib.

SUMMARY

VEGF inhibition is associated with activation of the endothelin system and renal injury that may aggravate during a high salt diet. In normotensive Wistar Kyoto rats the renal injury induced by sunitinib, a tyrosine-kinase inhibitor targeting all three VEGF receptors, was associated with a massive increase in proteinuria and endothelinuria and progression of histological glomerular damage.

INTRODUCTION

Vascular endothelial growth factor (VEGF) is a key mediator of angiogenesis that induces endothelial cell (EC) migration, growth, differentiation and regeneration through its VEGF receptors (VEGFRs).[1] In the kidney, VEGF is abundantly expressed in podocytes and tubular epithelial cells, whereas glomerular and peritubular ECs express VEGFRs.[2] The importance of renal VEGF in the kidney is evidenced by experiments in mice showing that depletion of one VEGF allele in podocytes induces severe glomerular pathology leading to proteinuria and impaired renal function.[3] In addition, selective embryonic excision of the gene encoding VEGF-A from renal tubular cells resulted in the formation of a smaller kidney with a striking reduction in peritubular capillaries, indicating that VEGF-A derived from tubular cells is essential for the maintenance of the peritubular vasculature.[4]

In patients with different forms of cancer, antiangiogenic treatment with agents inhibiting the VEGF pathway has become an approved treatment.[5, 6] In previous studies, we and others have shown that therapeutic VEGF inhibition in patients with cancer is associated with a rise in blood pressure (BP) and renal injury as well as activation of the endothelin system.[7-11] Histologic findings in kidney biopsies from patients who develop kidney injury during antiangiogenic agent exposure are mainly restricted to the glomerulus with thrombotic microangiopathy being most frequently reported.[12, 13] In normotensive rats, angiogenesis inhibition with the multitargeted VEGFR inhibitor sunitinib was associated with a dose-dependent rise in BP and renal injury, reflected by a dose-dependent rise in cystatin C, proteinuria and glomerular endotheliosis.[10] Whether sunitinib administration is also associated with tubular or peritubular renal injury and peritubular rarefaction remains to be established.

In previous studies, we have shown that the hypertension induced by sunitinib is accompanied by decreased nitric oxide (NO) production and renin suppression.[7-9] i.e. conditions related to an increased sensitivity of BP to salt.[14, 15] It might therefore be expected that a high salt (HS) diet results in augmentation of the BP rise induced by sunitinib, as has been demonstrated earlier for the tyrosine kinase inhibitor SU5416.[16] Whether this augmentation of BP rise translates into more pronounced renal damage remains to be established.

We set up the present study to investigate whether sunitinib administration to normotensive rats, in addition to glomerular lesions, induces tubulointerstitial injury and peritubular rarefaction and whether or not this is exacerbated by a HS diet.

METHODS

Animals

All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC. Male Wistar Kyoto rats (WKY, 180-200 gram, n=30), obtained from Harlan Laboratories, were housed in individual cages and maintained on a 12-h light/dark cycle, having access to standard laboratory rat chow (normal salt (NS) diet; 0.5% NaCl) and water ad libitum (tap water). Intra-aortic BP recordings were performed by implanted radiotelemeters (PA-C40, Data Sciences International) and sunitinib (SU11248; Sutent, Pfizer) and vehicle solution were prepared and administered by oral gavage as described previously.[8] After implantation of the telemetry transmitters, rats received analgesic treatment using Temgesic subcutaneously (0.05 mg/kg; RB Pharmaceuticals Limited) for 2 days, followed by a recovery period of 10 days. After 4 weeks, the group switched to either a HS diet (8% NaCl + 0.9% saline drinking water, n=15) or continuation of the NS diet for 2 weeks (n=15). Then, rats were randomly administered a dose of sunitinib (7 mg/kg.day, n=8) or vehicle (n=7) by oral gavage (0.5 mL) for 8 days, on top of the two specific diets.

At the end of each experiment, rats were euthanized with 60 mg/kg pentobarbital i.p. and blood was sampled for measurement of creatinine and cystatin C levels, and kidneys were rapidly excised. In all experiments, before start of the specific diet (Baseline 1), and immediately before (Baseline 2) and after 8-day administration of vehicle or sunitinib, rats were housed in metabolic cages for 48 hours with free access to food and water; the first day to acclimatize and the second day to collect 24-hour urine samples for the determination of sodium excretion, proteinuria and endothelinuria. BP could not be monitored when rats were housed in metabolic cages due to the absence of telemetry receivers under those conditions. A timeframe is given in Figure 1.

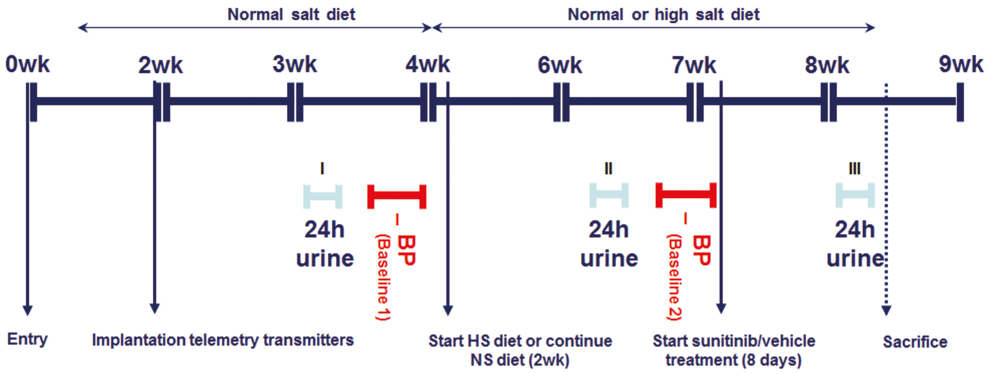

Figure 1. A timeframe of the study

Biochemical measurements

Endothelin (ET-1) was assessed using a chemiluminescent ELISA (QuantiGlo®, R&D Systems, range 0.34 - 250 pg/mL). Plasma creatinine, plasma cystatin C and urinary protein concentrations (Cobas c502 and c702, CYSC and TP2/TPUC3, Roche Diagnostics) were measured at the clinical chemical laboratory of the Erasmus MC.

Renal histology

Glomerular injury

The left kidney was rapidly excised from euthanized rats, decapsulated, weighed and sliced transversely. Slices were fixed in a 3.5-4% formaldehyde solution for light microscopic (LM) evaluation. After fixation, tissue was dehydrated and paraffin-embedded. Deparaffinized 2-μm thick sections were stained for haematoxilin-eosine (HE) and periodic acid Schiff (PAS). PAS-stained sections were blindly evaluated by a pathologist (F.M.M.S.) for the presence (1) or absence (0) of endothelial and epithelial cell swelling, glomerular ischemia and intra-epithelial protein in 50 glomeruli. Glomerular ischemia was scored semiquantitatively and defined as the degree of open glomerular capillaries, wrinkling of the glomerular basement membrane, and filling of Bowman's space. Wide open glomerular capillaries filling Bowman's space entirely corresponded with no ischemia. Partially open glomerular capillaries with mild wrinkling of the glomerular basement membrane and Bowman's glomerular space largely filled were classed as moderate ischemia. Totally collapsed glomeruli and extensive wrinkling of the glomerular basement membrane and only partial filling of Bowman's space corresponded with severe ischemia. Furthermore, the presence of glomerular intra-epithelial protein deposition was evaluated using a semiquantative scale: 0 (no protein), 1 (protein present in 1-50% of the epithelial cells) and 2 (protein present in >50% of the epithelial cells). Fifty glomeruli per kidney section (PAS staining) were evaluated.

Chronic and acute tubulointerstitial injury

Since peritubular capillary density correlates well with renal interstitial fibrosis and tubular atrophy (IF/TA), this parameter was used to assess capillary density.[17, 18] Transversely sliced 2-μm thick kidney sections were stained with periodic acid Schiff (PAS) to analyse kidney histomorphology. Subsequently, all sections were semi-quantitatively scored by a renal pathologist (M.C.v.G.) in a blinded manner using a 20x objective, and scored for IF (ci) and TA (ct) according to the Banff classification of renal allograft.[19] Each parameter was graded in 10 sequential fields with a score of 0-3 in which 0 meant no changes in pathology; Grade 1, <25% of change; Grade 2, 25-50% of change; Grade 3, >50% of tissue affected. Acute tubular ischaemic injury was also scored, and expressed by tubular dilatation, cast deposition, brush border loss and/or necrosis, using a 20x objective.[20] Each parameter was graded in 10 fields with a score of 0 to 5 in which Grade 0 meant no changes in pathology and Grade 5, extensive damage, involvement of >75% of the cortex.

mRNA expression

Quantitative polymerase chain reaction (qPCR) was performed to determine mRNA expression. RNA was isolated using the TRIzol method and reversed to cDNA using an AMV cDNA synthesis kit (Roche, Indianapolis, IN). For the qPCR reaction, iQ SYBR Green supermix (BioRad) was used. The Edn1, Ece1, Vegfa, and neutrophil gelatinase-associated lipocalin (NGAL) mRNA levels were measured and expressed relative to a general housekeeping gene hypoxanthine phosphoribosyltransferase 1 (Hprt1). The primer sequences used are shown in Table 1.

Table 1. Real time PCR primers

Name	forward primer	reverse primer
VEGF-A	ACAGAAGGGGAGCAGAAAGCC	ACCGCATTAGGGGCACACAG
Endothelin-1	AGGGAAAACCCTGTCCCAAG	CACGGGGCTCTGTAGTCAAT
Endothelin converting enzyme	GAGCCTGAGCACCCTGAAAT	ACTTTGTCCAGCTCCTTGGG
NGAL	GGAGCGATTCGTCAGCTTTG	CGCTCACCGTCTGTTCAGTT
HPRT	GGCTATAAGTTCTTTGCTGACCTG	GGCTATAAGTTCTTTGCTGACCTG

Statistical analysis

Data are presented as mean±SEM. Statistical analysis between groups was performed by unpaired t-testing or by repeated-measures ANOVA followed by Newman-Keuls or Dunnett's multiple comparison testing, or two-way ANOVA. GraphPad Prism version 5.0 was used for all statistical analysis.

RESULTS

Increased blood pressure rise during sunitinib and HS diet

No difference in (gain of) body weight between the four groups or rats was observed. As expected, urinary sodium excretion massively increased from 1.45±0.11 mmol/day during a NS diet to 43.57±3.09 mmol/day during a HS diet. Plasma sodium concentration did not differ between the four groups (data not shown). Mean arterial pressure (MAP) during the NS diet was 101±0.9 mmHg. The HS diet for 2 weeks increased MAP by 27±3 mmHg ($P<0.05$ vs. NS diet) (Figure 2A). The rise in MAP was already present within 1 week of the HS diet. Sunitinib increased MAP by 15±1 mmHg during the NS diet ($P<0.05$ vs. NS diet alone) and by 23±4 mmHg during the HS diet ($P<0.05$ vs. HS diet alone, $P<0.05$ vs NS diet+sunitinib) (Figure 2A). Compared to the respective vehicle groups, the delta AUC of MAP was 38 mmHg times days in NS diet+sunitinib group and 85 mmHg times days in HS diet+sunitinib group ($P<0.05$) (Figure 2B). Plasma creatinine and cystatin C levels in the four groups were similar. Creatinine clearance, irrespective of sunitinib administration, tended to be higher

during a HS diet. It changed from 3.11±0.26 mL/min during NS diet, to 3.48±0.46 mL/min during HS (P>0.05).

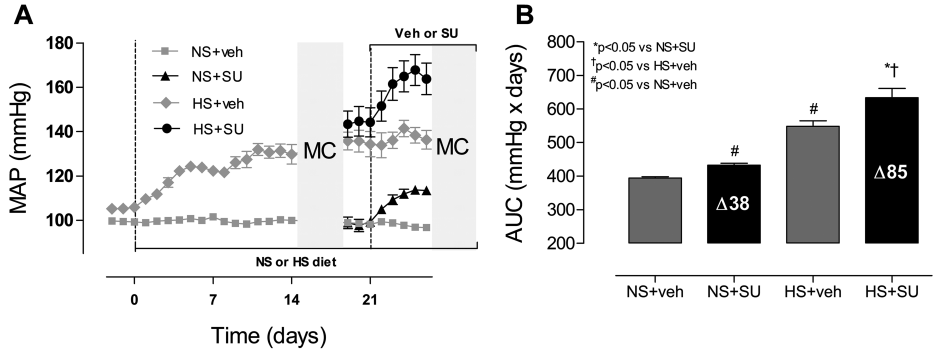

Figure 2. Mean arterial pressures (MAP) after 2 weeks of normal and high salt diet, adding vehicle or sunitinib treatment (A), and area under the curve (AUC; B) consisting of mean arterial pressure and number of treatment days. Abbreviations: NS, normal salt; HS, high salt; Veh, vehicle; SU, sunitinib; MC, metabolic cage. Δ=vs. respective vehicle group.

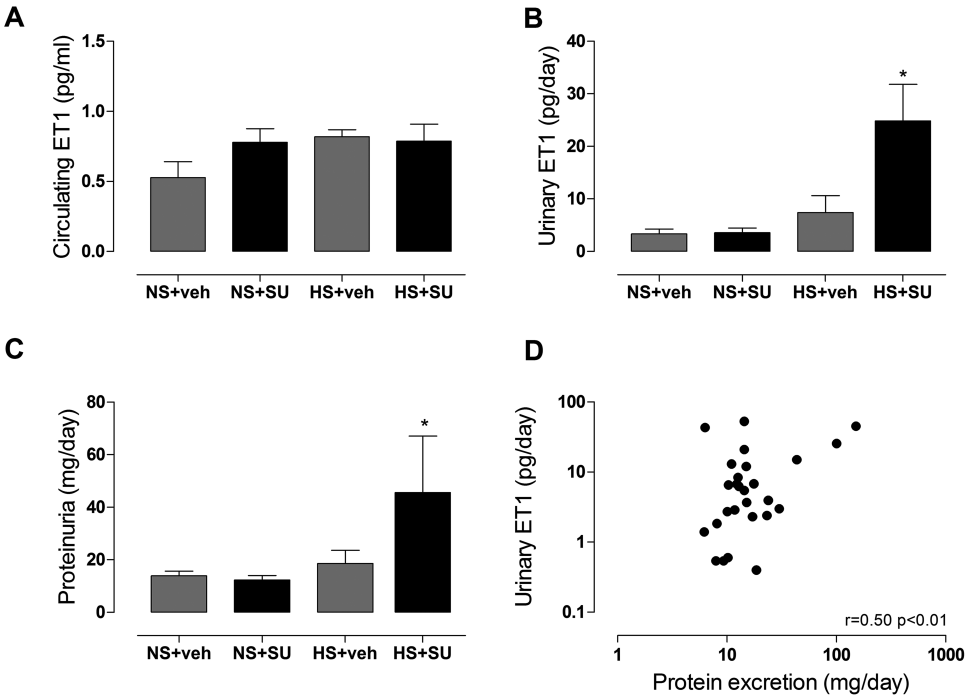

Figure 3. Circulating endothelin-1 (ET-1; A), endothelinuria (B), proteinuria (C) and the relation of urinary ET-1 and protein excretion (D) after sunitinib or vehicle treatment during the specific diets. Abbreviations: NS, normal salt; HS, high salt; Veh, vehicle; SU, sunitinib. *P<0.05 vs NS+SU

Dietary salt enhances sunitinib induced circulating and urinary endothelin and proteinuria

Compared to a NS diet, circulating ET-1 levels increased from 0.53±0.30 pg/mL to 0.78±0.28 pg/mL during NS diet+sunitinib, to 0.82±0.13 pg/mL during HS diet+vehicle and to 0.79±0.35 pg/mL, during HS diet+sunitinib (Figure 3A). Endothelinuria increased about 6-fold vs NS diet+sunitinib when sunitinib was administrated to rats during the HS diet (P<0.05, Figure 3B). Baseline protein excretion was 11.4±1.4 mg/day before NS diet and 9.9±1.3 mg/day before HS diet. The HS diet increased proteinuria to 18.6±1.1 mg/day (P=NS, Figure 3C). Proteinuria showed the same pattern as endothelinuria as it only increased massively to 45.6±21.5 mg/day (P<0.05) after sunitinib was added to the HS diet. The urinary proteinuria and endothelin excretions rates were correlated (Figure 3D).

Glomerular Injury

The glomerular ischemia scores in rats exposed to vehicle during the NS and the HS diet were identical, and the score increased during sunitinib administration irrespective of salt diet (P<0.05, Table 2). Similar findings were obtained for the deposition of intra-epithelial protein droplets. At most, the HS diet tended to enhance sunitinib-induced glomerular ischemia and intra-epithelial protein deposition. Epithelial cell swelling markedly increased during the HS diet with sunitinib, compared to the HS diet alone or the NS diet with sunitinib (P<0.05, Table 2). Representative pathological changes are shown in Figure 4 (figure legend on page 120).

Table 2. Light microscopic evaluation of kidney sections obtained from rats exposed to a normal and high salt diet and in combination with or without VEGF inhibition

	Glomerular ischemia (% of glomeruli)			Intra-epithelial protein (% of glomeruli)			Epithelial cell swelling (% of glomeruli)		Endothelial cell swelling (% of glomeruli)	
Treatment	None	Moderate	Severe	0	1	2	0	1	0	1
NS+veh	98±1	2±1	0	99±1	1±1	0	92±3	8±3	n.a	n.a
NS+SU	89±4	11±4#	0	95±1	5±1#	0	95±1	5±1	n.a	n.a
HS+veh	93±3	6±2	1±1	99±1	1±1	0	97±1	3±1	n.a	n.a
HS+SU	71±8	20±4†	8±4	82±6	12±4†	6±3	79±6†*	21±6†*	n.a	n.a

NS, normal salt; HS, high salt; Veh, vehicle; SU, sunitinib

*P<0.05 vs. NS+SU; #P<0.05 vs. NS+veh; †P<0.05 vs. HS+veh; n.a indicates not applicable.

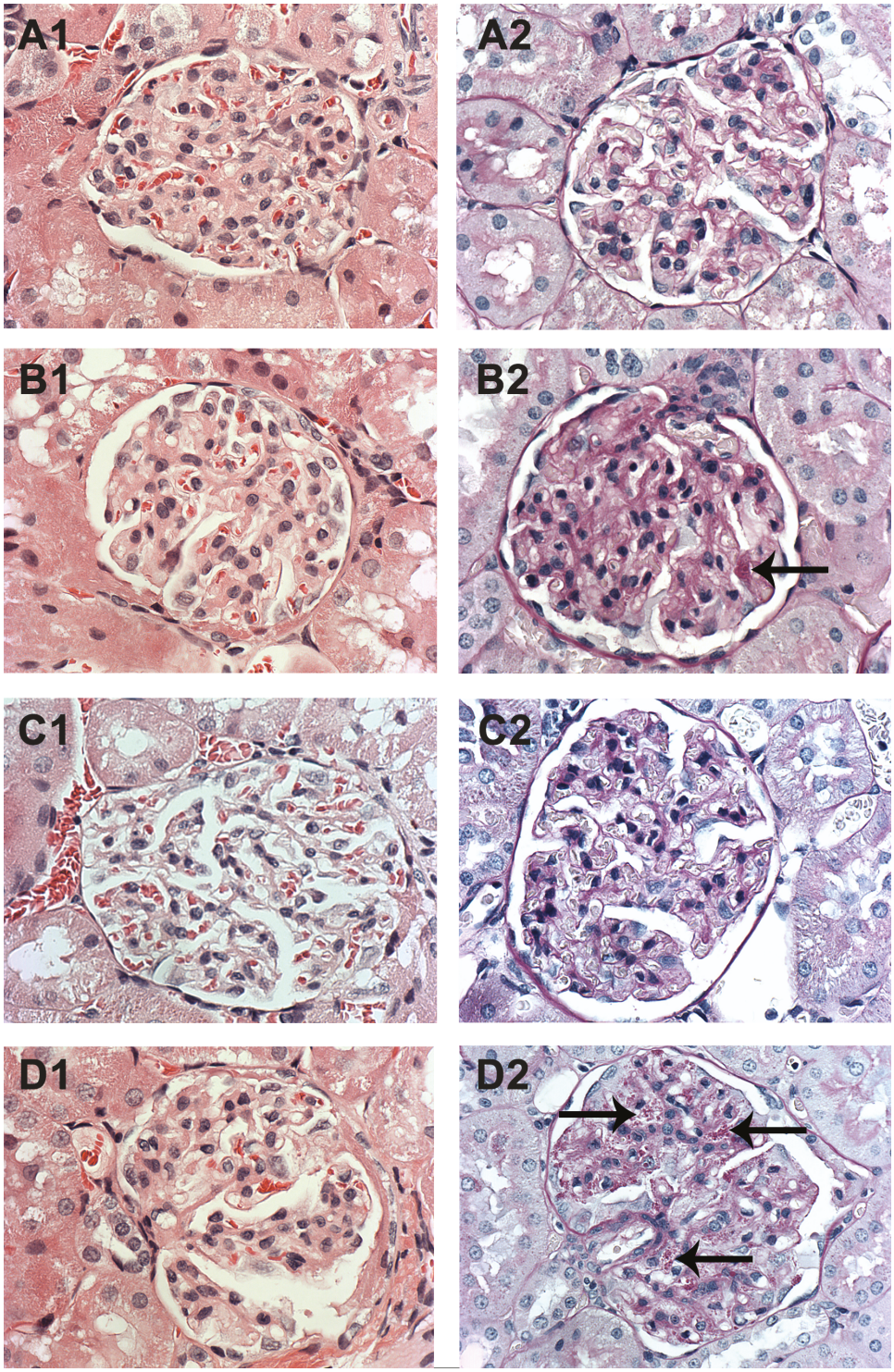
A1
A2
B1
B2
C1
C2
D1
D2

Figure 4. Kidney sections from WKY rats after receiving a normal salt with vehicle (A), a normal salt diet with sunitinib (B), a high salt diet with vehicle (C), and a high salt diet with sunitinib (D), stained with HE (left panel) and PAS stain (right panel, magnification x500). A1: showing no ischemia characterized by minor lobulation of the glomeruli which fill Bownman's space, and no presence of glycoproteins (A2). B1: showing distinct lobulation of the glomeruli indicating ischemia, and a very mild deposit of protein droplets (B2, arrow). C1: showing no ischemia, Bowman's space is entirely filled, but lobulation of the glomerulus is present. Protein droplets are hardly found (C2). D1: mild ischemia with distinct lobulation of the glomerulus which does not fill Bowman's space. PAS stain shows extensive deposits of protein droplets in epithelial cells (D2, arrows).

Chronic and acute tubulointerstitial injury

PAS-stained sections of the kidneys showed no marked renal abnormalities in any treatment group (Figure 5). In 50% of the NS diet+sunitinib group, mild tubular injury was observed. This percentage was slightly elevated during the HS diet (57%), but no further enhancement was observed after adding sunitinib on top of HS (38%). Sunitinib administration had no effect on the density of peritubular capillaries either during the NS or HS diet.

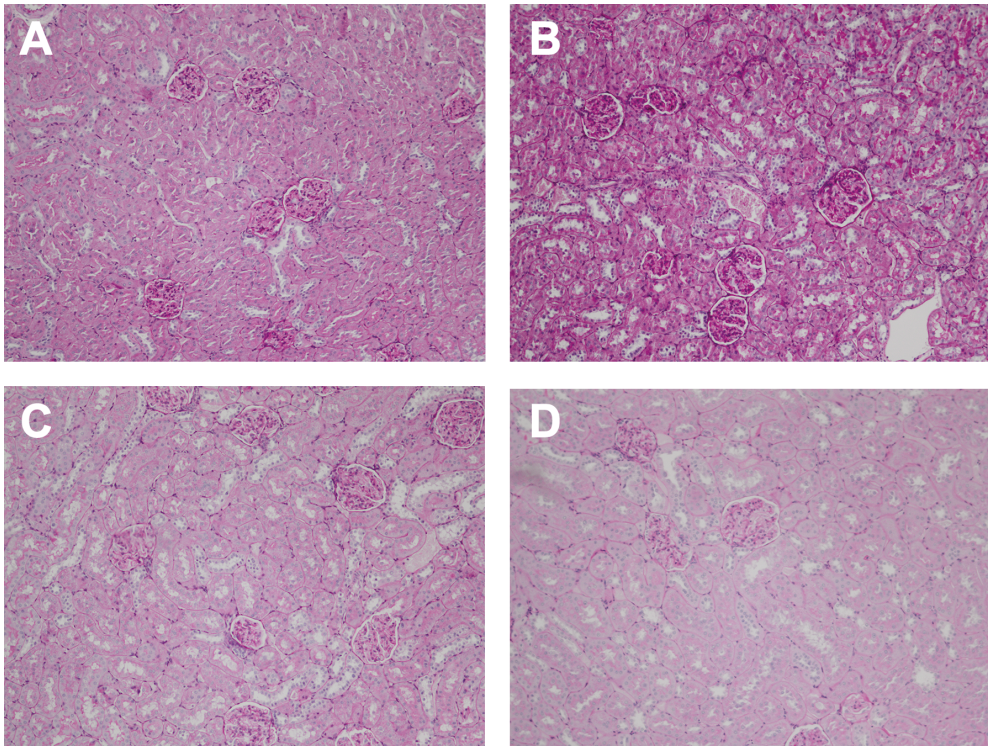

Figure 5. Cross section of PAS-stained kidney sections showing normal architecture, without any alterations. No interstitial fibrosis or tubular atrophy or globally sclerotic glomeruli are seen, therefore no indications of chronic renal scaring (magnification 10x). A, NS+veh; B, NS+SU; C, HS+veh; D, HS+SU

Decreased renal expression of Edn-1, Ece1 and Vegfa mRNA during HS diet and sunitinib

VEGF-A decreased during the HS and HS diet with sunitinib, compared to the NS diet ($P<0.05$). The rise in endothelinuria was not accompanied by a rise in the renal expression of the gene encoding for ET-1. In fact, compared to the NS diet ET-1 mRNA levels decreased during the NS diet with sunitinib, the HS diet and the HS diet with sunitinib ($P<0.05$), while endothelin converting enzyme (ECE) mRNA levels decreased during the NS diet with sunitinib only ($P<0.05$, Figure 6).

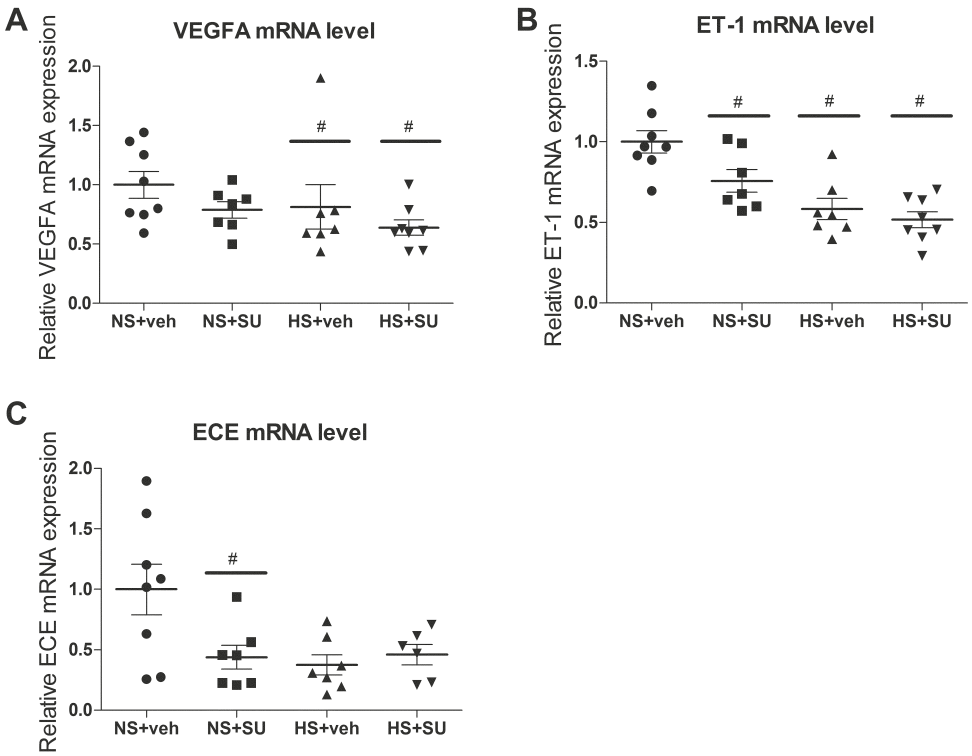

Figure 6. Relative renal mRNA expression level of vascular endothelial growth factor (VEGF-A; A), endothelin-1 (ET-1; B) and endothelin converting enzyme (ECE; C) after correction for the housekeeping gene Hprt. #$P<0.05$ vs NS+veh

Increased renal expression of NGAL mRNA during HS diet and sunitinib

The expression of the gene encoding for NGAL, a tubular injury marker, did not change after the HS diet with or without sunitinib. However, compared to the NS diet with sunitinib the expression of gene of NGAL increased during a HS diet with sunitinib (Figure 7).

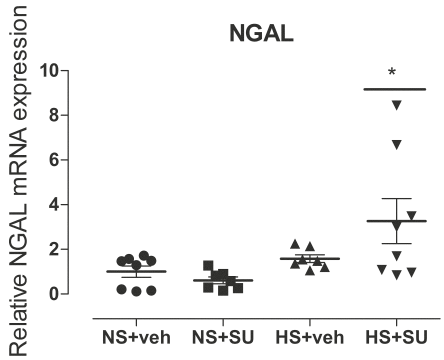

Figure 7. Relative renal mRNA expression level of NGAL after correction for the housekeeping gene Hprt. *P<0.05 vs. NS+SU

DISCUSSION

Hypertension and renal injury are well-recognized adverse side effects of agents targeting the VEGF-signalling pathway.[21, 22] In the present study, we focused on glomerular and tubulointerstitial pathology in response to the multitargeted VEGF inhibitor sunitinib and the potential aggravating effects of a HS diet. The new findings are that a HS diet exacerbates both the magnitude of the rise in BP and severity of glomerular injury induced by sunitinib, but that sunitinib administration for 8 days either with a normal or a HS diet is not associated with histological tubulointerstitial injury. In addition, we found that the HS-induced increase in urinary ET-1 excretion was markedly further augmented by sunitinib.

The dose of sunitinib of 7 mg/kg per day was associated with a BP rise of 15 mmHg, comparable to the BP rise observed in patients treated with sunitinib.[8, 10] Although a HS diet for 4 weeks amplified the sunitinib-induced rise in BP by 47 mmHg times days, the HS diet by itself already induced a considerable BP rise in WKY rats. This latter finding concurs well with a study in normotensive Sprague-Dawley (SD) rats, showing that a 8% HS diet for 8 weeks induced a 28 mmHg rise in MAP as measured by the tail cuff method.[23] Contrary to the present findings, the rise in BP in SD rats became only evident after 5 of the 8 weeks of the HS diet, whereas in the present study the marked rise in BP was already present within 1 week. These findings may imply a greater salt-sensitivity of BP in WKY than in SD rats. Moreover, BP in the present study was measured with the much more sensitive telemetry method.

The augmented salt-sensitivity of BP during sunitinib can be explained by earlier findings, showing that the rise in BP induced by angiogenesis inhibition is accompanied by renin suppression and a decrease in the NO synthase.[7-9] Administration of the NO synthase inhibitor N_{ω}-Nitro-L-arginine methyl ester (L-NAME) in rats results in a salt-

sensitive rise in BP, in part by preventing the adaptive rise in renal medullary blood flow and rise in renal interstitial pressure in response to a HS diet.[14] The fact that renin suppression is associated with salt-sensitive BP is known for many years. When renin is suppressed, it cannot further decline during salt loading and hence not counteract the volume-dependent rise in BP.[24] Another potential mechanism by which sunitinib administration leads to salt-sensitive hypertension is impairment of lymphangiogenesis by blockade of the VEGFR-3 signalling pathway.[25, 26] Rodent studies have shown that interstitial hypertonic volume retention during salt loading is associated with increased lymphangiogenesis in the skin. Blockade of this lymphangiogenesis by trapping VEGF-C, the agonist of the VEGF-3 receptor, results in a moderate salt-sensitive hypertension.[26] Whether sunitinib at the dose used in the present study inhibits salt-induced lymphangiogenesis remains to be established.

In SD rats, the HS diet-induced rise in BP was associated with a decreased renal expression of VEGF mRNA and VEGF protein levels and an increased urinary excretion of the endogenous VEGF inhibitor soluble FMS-like tyrosine kinase-1 (sFlt-1).[23] Likewise, the HS diet in our WKY rats was associated with a decreased renal Vegf expression. Given that a systemic infusion of angiotensin II increases renal Vegf expression, suppression of the renin-angiotensin system during a high salt intake may underlie the decrease in VEGF expression observed in the present study.[27]

Despite the marked rise in BP, a HS diet was not accompanied by proteinuria, impairment of renal function or histological glomerular or tubulointerstitial changes. However, with regard to renal injury, a clear interaction between the HS diet and sunitinib exposure was present. Administration of sunitinib to rats on a HS diet when compared to rats on a NS diet was associated with pronounced proteinuria and endothelinuria and glomerular epithelial cell swelling as well-increased expression of the gene encoding for NGAL, a marker of tubular injury. Administration of the tyrosine kinase inhibitor SU5416 at a dose of 10 mg/kg.day for 2 weeks in SD rats on a HS diet has been shown to induce a pronounced rise in BP (MAP 158 mmHg), severe proteinuria, glomerulosclerosis, extracellular matrix expansion and tubulointerstitial injury with infiltration of inflammatory cells.[16] Despite a comparable degree of hypertension, such pronounced renal histopathological changes were not observed in our WKY rats when exposed to sunitinib+HS. Possibly WKY rats are less prone to renal damage than SD rats. Also, the exposure of our rats to sunitinib lasted 8 days and the compound was given orally, whereas in SD rats, SU5416 was administered intra-peritoneally.

We and others have reported that administration of RTKIs or a rise in sFlt-1 in preeclamptic patients, is associated with activation of the endothelin system and that this activation plays a central role in the BP rise.[7, 8, 10, 28] In the present study, circulating ET-1 levels tended to be higher during sunitinib administration, but especially the combination of a HS diet and sunitinib was associated with a huge increase in urinary

ET-1 excretion. This increased urinary ET-1 excretion was not accompanied by renal overexpression of the genes encoding for preproET-1 or for endothelin converting enzyme. This finding suggests that this urinary ET-1 is mainly plasma-derived, reaching the urine by glomerular filtration, as also supported by the observed correlation between proteinuria and endothelinuria.

Selective, genetic, embryonic deletion of VEGF-A from renal tubules results in a decrease in peritubular capillaries and the formation of a small kidney.[4] Because of this crosstalk between tubular-derived VEGF-A and peritubular microvessels we explored whether administration of sunitinib was associated with a decrease in peritubular vascular density. This appeared not to be the case, suggesting that such a phenotype only occurs following embryonic interruption of the tubular VEGF pathway, which is supported by studies in patients who develop renal injury while treated with anti-angiogenic agents. In these patients by far the most frequently reported lesion is glomerular thrombotic microangiopathy and not tubulointerstitial injury.[13, 21, 29-31]

In conclusion, this study shows that a HS diet in normotensive WKY rats is associated with a marked rise in BP and exacerbation of renal glomerular renal injury in response to a dose of sunitinib that induces a rise in BP comparable to that observed in patients treated with this agent. We acknowledge that the duration of administration of sunitinib was relatively short. Whether more prolonged administration will eventually result in renal tubulointerstitial injury and peritubular rarefaction requires further investigation. The observation that a HS diet aggravates the rise in BP and renal injury may have implications for patients treated with angiogenesis inhibitors. These patients, especially when on long-term treatment with these agents as occurs in the neoadjuvant setting, should be advised to limit their dietary salt intake.

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CHAPTER 8

CUMULATIVE DOSE OF BEVACIZUMAB IS ASSOCIATED WITH ALBUMINURIA RATHER THAN PODOCYTURIA IN CANCER PATIENTS

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In progress

ABSTRACT

Angiogenesis inhibition with bevacizumab, a monoclonal antibody against VEGF, is an effective anti-cancer treatment associated with hypertension and renal glomerular toxicity referred to as a preeclampsia-like syndrome. In preeclampsia, podocyturia, i.e. shedding of viable podocytes in the urine, predates proteinuria and clinical features of preeclampsia, and as such is regarded as a biomarker of ongoing glomerular injury. Using a recently validated quantitative polymerase chain reaction of the podocyte-specific molecules nephrin, podocin and vascular endothelial growth factor A (VEGF-A) in the urine, we examined whether podocyturia is present in bevacizumab-treated cancer patients, and to what extent it relates to proteinuria, and the cumulative dose of bevacizumab. Urine samples were cross-sectionally collected from bevacizumab-treated patients (n=33), chemotherapy treated patients (n=19), and healthy controls (n=6). Urinary protein-to-creatinine ratio (geometric mean and range) was 20.09 mg/mmol [range 5.15-284.4] in the bevacizumab group, compared to 11.03 mg/mmol [1.14-28.57] in the chemotherapy group ($P<0.05$) and 6.80 mg/mmol [3.87-12.12] ($P<0.05$) in the healthy controls. For the urinary albumin-to-creatinine ratio values were respectively 2.72 mg/mmol [0.14-227.7], compared to 1.04 mg/mmol [0.20-3.51] and 0.21 mg/mmol [0.12-0.67] ($P<0.05$). The cumulative dose of bevacizumab ranged from 550 to 93628 mg. Urinary podocin mRNA expression was not detectable, urinary nephrin mRNA expression (expressed per mmol creatinine) ranged from 0.01 to 8.09 and urinary VEGF-A mRNA from 0.01 to 0.27. Urinary nephrin mRNA expression was not correlated to the albumin-to-creatinine ratio or the cumulative dose of bevacizumab in bevacizumab-treated patients, whereas the latter correlated with the albumin-to-creatinine ratio ($r=0.77$; $P<0.001$). Our results demonstrate that a cumulative dose of bevacizumab is closely correlated with albuminuria but not with podocyturia as measured with the qPCR technique, challenging the feasibility of this measurement to monitor ongoing glomerular injury in patients chronically treated with bevacizumab.

INTRODUCTION

Inhibition of angiogenesis with humanized monoclonal antibodies to vascular endothelial growth factor (VEGF) or with tyrosine kinase inhibitors targeting VEGF receptors (VEGFRs) has become an established treatment for various tumor types.[1-3] For example, in colorectal and breast cancer the monoclonal antibody bevacizumab is used in combination with chemotherapy.[1, 4]

Although successful, this therapy is associated with several side effects including hypertension and renal toxicity in a substantial proportion of patients. These side effects may especially become problematic with prolonged treatment and may even be a reason to withdraw treatment.[5, 6] The renal toxicity associated with angiogenesis inhibition includes proteinuria, renal function impairment and glomerular endotheliosis.[7, 8] Hypertension and proteinuria during angiogenesis inhibition are also referred to as a preeclampsia-like syndrome.[9, 10] Preeclampsia (PE) is a pregnancy-related disorder characterized by proteinuria and hypertension. One of the underlying pathogenetic mechanisms is an increased placental production of soluble fms-like tyrosine kinase 1 (sFlt-1).[11] Within the maternal circulation sFlt-1 binds VEGF thereby abrogating the VEGF-signaling pathway, and creating a condition similar to that induced by monoclonal antibodies to VEGF or VEGFRs currently used in cancer treatment.[12]

In the kidney, VEGF plays a dominant role in maintaining the integrity of the glomerular filtration barrier.[13, 14] VEGF is expressed in glomerular epithelial podocytes (epithelial cells) that form the final barrier to protein loss. Several podocyte proteins, including podocin, nephrin, synaptopodin and podocalyxin, maintain the structural and functional integrity of the glomerular slit diaphragm through complex interactions.[15] Podocyte loss, either due to apoptosis or through detachment from the glomerular basement membrane, followed by podocyturia, may cause disruption of the glomerular filtration barrier leading to proteinuria.[15] A prospective clinical study in pregnant women at the end of the second trimester has demonstrated that podocyturia predates proteinuria and clinical features of PE.[15] In this study podocyturia was assessed using the laborious cell culture and immunofluorescence technique. Recently, Kelder et al demonstrated that quantitative polymerase chain reaction (qPCR) of the podocyte-specific molecules nephrin, podocin and VEGF-A in urine distinguished preeclamptic patients from healthy pregnant and hypertensive controls and is a rapid and accurate tool for the detection of urinary podocyte excretion.[16]

Considering the comparable clinical and histopathological features of PE and VEGF-inhibition-associated toxicity, we examined whether podocyturia, based on the recently developed qPCR technique, is present in bevacizumab-treated cancer patients, and to what extent is relates to proteinuria, and the cumulative dose of bevacizumab.

MATERIAL AND METHODS

Clinical study

Using a cross-sectional study design, for the period of one year, all patients who were on bevacizumab treatment at the department of Oncology of the Amphia Hospital, were invited to participate in this study. Since treatment with bevacizumab is often combined with chemotherapeutic drugs such as paclitaxel, trastuzumab, oxaliplatin and capecitabine, a group, treated with one or more of these chemotherapeutics without bevacizumab, was included. A second control group consisted of healthy subjects who visited the outpatient clinic for other medical issues (e.g. fatigue) without malignancy. Healthy controls with abnormal kidney function and/or proteinuria, and patients with known renal insufficiency and/or proteinuria, previous therapy with bevacizumab and concomitant chemotherapy commonly (>10% of patients) associated with renal injury, were excluded. Abnormal kidney function was defined as an estimated glomerular filtration rate (eGFR)<60 ml/min, and proteinuria as a protein-to-creatinine ratio >45 mg/mmol or albumin-to-creatinine-ratio >30 mg/mmol. In all patients, morning spot urine samples were collected during routine visits at the outpatient clinic to measure albumin, protein, creatinine and podocyturia. Furthermore, a blood sample was collected for determination of serum levels of sodium, potassium, urea, creatinine, albumin and calcium which were measured at the clinical chemical laboratory of the Amphia hospital. eGFR was calculated using the MDRD formula. In addition, office blood pressure (BP) was measured. Patient's characteristics, including type of malignancy, use of an angiotensin-converting enzyme inhibitor or angiotensin receptor blocker, history of hypertension and diabetes mellitus, gender, age and cumulative dose of bevacizumab, were collected. Urine samples were coded and processed anonymously. The study was approved by the medical ethical review committee of the Amphia hospital. Written informed consent was obtained from each patient.

Urine Collection and Cell Isolation

Clean-catch urine samples were processed within 2 hours of collection. Urine samples were transferred to tubes and centrifuged at 500 g for 5 minutes. Centrifuged urine samples were stored at -20°C. Albumin, protein and creatinine levels were measured from these urine samples. Pellets of centrifuged urine samples were washed with phosphate buffered saline and centrifuged again at 500 g for 5 minutes. The pellets were suspended in RNAlater and stored at -20°C until RNA isolation. The RNA was isolated using the TRIzol method. Briefly, the cell suspension in RNAlater was centrifuged at 13 000 rpm for 2 minutes. Pellets were then dissolved with TRIzol, and RNA was isolated as described previously.[17]

Quantitative Polymerase Chain Reaction

cDNA was generated with avian myeloblastosis virus reverse transcriptase (20 U/μL) (Roche), according to manufacturer instructions. For the qPCR reaction, iQ SYBR Green super-mix (BioRad) was used. Expression of a podocyte-specific marker was

measured using gene-specific primers. The primer sequences used are shown in Table 1. Podocyturia was determined by measuring the expression of nephrin and podocin, coding for proteins that are localized to the slit diaphragm of podocyte foot processes. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a positive control. The levels of VEGF-A mRNA were also measured. In the kidney, VEGF is expressed in podocytes and in the proximal tubular epithelium. Relative mRNA levels detected by qPCR were corrected for creatinine concentration and calculated per mL of urine.[18]

Table 1. Human primer sequences used for qPCR analyses

Human gene	Forward primer	Reverse primer
Nephrin	AGGACCGAGTCAGGAACGAAT	CTGTGAAACCTCGGGAATAAGACA
Podocin	TGGACTCAGTGACCTGTGTTTGG	CAGCAATCACCCGCACTTTGG
VEGF	AAACCCTGAGGGAGGCTCC	TACTTGCAGATGTGACAAGCCG
GADPH	TTCCAGGAGCGAGATCCCT	CACCCATGACGAACATGGG

Statistical methods

Data are presented as mean±SEM or geometric mean and range for non-normally distributed values. Patient characteristics were compared using an unpaired t-test. mRNA levels of nephrin, podocin and VEGF-A were compared using repeated-measures ANOVA followed by Newman-Keuls or Dunnett's multiple testing. Data were logarithmically transformed before analysis in case of non-normal distribution. Correlations among the expression of the different markers and clinical characteristics were calculated using Spearman *r* coefficient. $P<0.05$ was considered significant. GraphPad Prism version 5.0 was used for all statistical analysis.

RESULTS

Patients characteristics

Patient characteristics are shown in Table 2. The cumulative dose of bevacizumab ranged from 550 to 93628 mg. As expected, patients treated with bevacizumab had higher systolic BP compared to patients on chemotherapy (P=NS). The systolic BP in the healthy control group was lower than in both other groups, likely related to their much younger age ($P<0.05$).

Albuminuria and Proteinuria

Mean protein-to-creatinine ratio was 20.09 mg/mmol [5.15-284.4] in the bevacizumab group, compared to 11.03 mg/mmol [1.14-28.57] in the chemotherapy group ($P<0.05$) and 6.80 mg/mmol [3.87-12.12] ($P<0.05$) in the healthy controls. Proteinuria was present in 18% of bevacizumab treated patients. Mean albumin-to-creatinine ratio

was 2.72 mg/mmol [0.14-227.7] in the bevacizumab group compared to 1.04 mg/mmol [0.20-3.51] in the chemotherapy group (P<0.05) and 0.21 mg/mmol [0.12-0.67] in healthy controls (P<0.05). Microalbuminuria was present in 18% of bevacizumab treated patients. The albumin-to-creatinine, but not the protein-to-creatinine ratio, was significantly correlated with cumulative dose of bevacizumab (Figure 1A).

Table 2. Patient characteristics

Characteristic	Bevacizumab + Chemotherapy	Chemotherapy	Healthy controls without malignancy
Patients involved in study, n(%)	33 (56.9)	19 (32.8)	6 (10.3)
Age, years			
Mean±SEM	61.4±1.8	58.3±1.9	30.8±3.5
Range	[40-77]	[46-73]	[19-41]
Gender, n(%)			
Female	21 (64)	14 (74)	5 (83)
Male	12 (36)	5 (26)	1 (17)
Malignancy, n(%)			
Breast carcinoma	12 (36.4)	12 (63.2)	
Colon carcinoma	9 (27.3)	7 (36.8)	
Sigmoid carcinoma	4 (12.1)		
Rectum carcinoma	6 (18.2)		
Ovarium carcinoma	1 (3.0)		
Unknown	1 (1.9)		
Cumulative dose of bevacizumab (mg)			
Mean±SEM	13741±3205		
Range	[550-93628]		
Blood pressure, mmHg			
Systolic, mean±SEM	142.6±3.0*	134.1±3.8*	119.4±1.9
Diastolic, mean±SEM	82.9±1.6	79.1±2.3	79.5±4.8
History of hypertension, n(%)	5 (15.2)	3 (15.8)	-
History of diabetes mellitus, n(%)	4 (12.1)	3 (15.8)	-
eGFR (ml/min/1.73m^2)			
Mean±SEM	77.0±3.3	83.8±8.7	87.0±2.9
Range	[36-90]	[65-90]	[75-90]
Medication use			
ACE inhibitor	7 (21.2)	6 (31.6)	
Angiotensin receptor blocker	2 (6.1)	-	

*P<0.05 vs. healthy controls

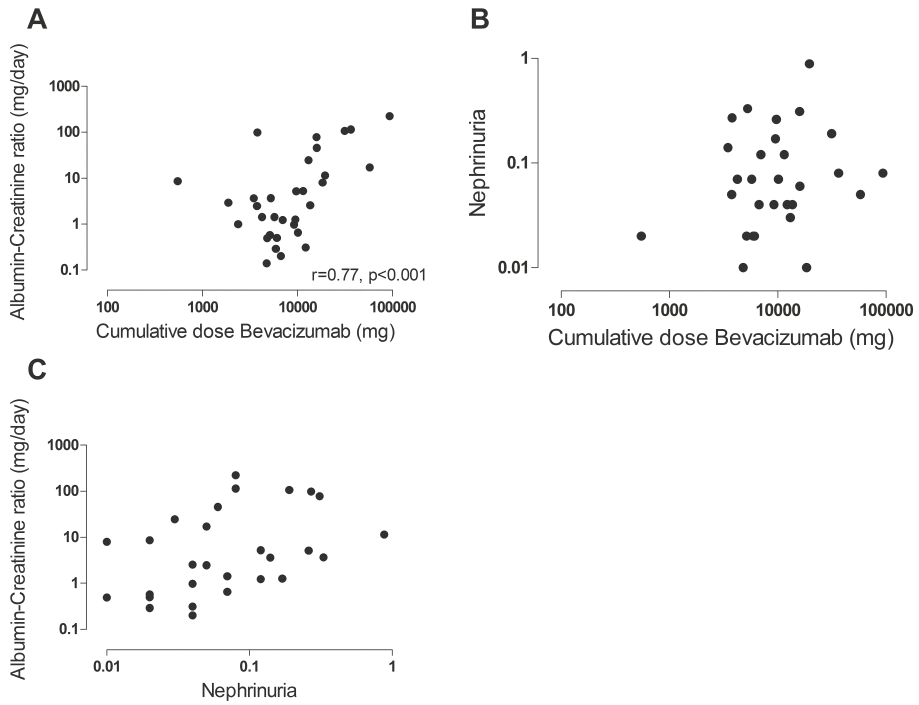

Figure 1. Relation of cumulative dose of bevacuzimab with albuminuria (A), with nephrinuria (B) and of albuminuria with nephrinuria in bevacuzimab treated patients (C). Nephrinuria is presented as mRNA expression/mmol creat.

Podocyturia

The house-keeping gene GADPH was detected in every sample, and its expression levels were similar among the three groups suggesting that the quality of RNA isolated from urinary cells was sufficient for amplification. Urinary nephrin mRNA expression (expressed per mmol creatinine) ranged from 0.01 to 8.09 and urinary VEGF-A mRNA from 0.01 to 0.27. Relative VEGF-A and nephrin mRNA expression between the groups did not differ (data not shown) and podocin mRNA was not detectable in any of the analyzed urine samples (data not shown). Relative VEGF-A and nephrin mRNA expression were correlated ($r=0.61$;$P<0.01$). Nephrin mRNA expression was not correlated with either the cumulative dose of bevacizumab or the albumin-to-creatinine ratio in bevacizumab-treated patients. In addition, no correlations were found between podocyturia and BP and eGFR (data not shown). VEGF-A mRNA expression was not correlated with either the cumulative dose of bevacizumab or the albumin-to-creatinine ratio in bevacizumab-treated patients.

DISCUSSION

To our knowledge, this is the first study to describe the use of qPCR to quantify podocyturia in the context of angiogenesis inhibition. In the present study, no elevated levels of nephrin mRNA or VEGF-A mRNA were detected in the urine of patients treated with the VEGF inhibitor bevacizumab when compared to patients treated with chemotherapy only or to healthy controls. Furthermore, there was no significant relationship between qPCR nephrinuria and albuminuria or between qPCR nephrinuria and the cumulative dose of bevacizumab. However, albuminuria was positively correlated to the cumulative doses of bevacizumab.

There is increasing evidence that podocyte injury plays a key role in the renal manifestations of PE, a pregnancy-related disease with similar renal pathological changes as angiogenesis inhibition-induced toxicity, including glomerular endotheliosis, and the presence of hypertension and proteinuria. Podocyte-specific conditional knockout of VEGF-A in mice is known to result in podocyte loss, proteinuria and glomerular thrombotic microangiopathy.[13] Likewise, anti-VEGF-treatment in patients is associated with comparable renal abnormalities as observed in preeclamptic women.[13] Yu et al. have shown that podocyturia may be a more sensitive marker of ongoing glomerular damage than proteinuria, due to the fact that the tubules are capable of reabsorbing filtered protein.[19] To date, several studies reported the presence of podocyturia in preeclamptic patients and observed that it can predict the development of PE.[15, 16, 20-22] Podocyturia has also been reported in proteinuric patients during anti-VEGF treatment.[23, 24] In a small cross-sectional study of 27 patients reported by Muller-Deile et al., albuminuria and podocyturia were found in 95% and 32% respectively, of bevacizumab treated patients, compared to 100% and 13% of sunitinib treated patients.[23] Remarkably, the incidence of albuminuria was much higher than that of podocyturia. A recent study of Garovic et al. showed that 60% of patients treated with a VEGF inhibitor had evidence of podocyturia.[24] In addition, they demonstrated a correlation between the cumulative dose of bevacizumab and both proteinuria and podocyturia, as well as a correlation between the number of urinary podocytes and the amount of proteinuria, suggesting that these variables are mechanistically related. It must be mentioned that patients treated with the combination of anti-VEGF therapy and cytotoxic agents known to induce renal injury in over 10% of patients, such as cisplatin, were also included in that study. The same holds true for patients on anti-VEGF agents in combination with other targeted therapies. Compared to our own study, in which patients on cytotoxic agents known to induce renal toxicity or on previous or dual-targeted therapy were excluded, the inclusion of these patients in the study of Garovic et al. might account for the higher incidence of proteinuria and podocyturia and the positive correlation between these two variables, despite the lower cumulative dose of bevacizumab.

In all of the mentioned studies, cell culture and immunofluorescence techniques by counting podocyte positive cells were used. A drawback of that method is that it is time-consuming with a test result obtained after 2 days at the earliest. Kelder et al., by using a more rapid qPCR method have demonstrated significantly elevated nephrin, podocin, and VEGF-A mRNA in the urine of preeclamptic women when compared to levels in healthy pregnant controls and healthy non-pregnant controls.[16] Cell culture and immunofluorescence techniques of the same urine samples revealed that the number of podocytes closely correlated with the levels of nephrin mRNA. However, similar to the present results, podocyturia and albuminuria were not correlated.[16] Since in our study only 18% of the bevacizumab-treated patients had albuminuria a correlation might be difficult to find, moreover podocyturia can be present without the appearance of proteinuria.[25] Considering these differences, and the fact that in the mentioned study of Muller-Deile et al. the incidence of albuminuria was much higher than that of podocyturia in their cancer patients on bevacizumab or sunitinib treatment, podocyturia and albuminuria might provide different information.

Why we did not observe any differences in podocyturia between the groups of subjects in contrast to previous studies on anti-VEGF therapies and studies in PE is not clear. The different anti-VEGF mechanisms, anti-VEGF monoclonal antibody (bevacizumab) versus receptor tyrosine kinase inhibitor (sunitinib) or circulating sFlt-1, might induce different degrees of VEGF inhibition. To further explore this, it would be interesting to determine podocyturia using the qPCR method in patients treated with one of the receptor tyrosine kinase inhibitors. Also, the qPCR-technique might be less specific than the cell culture and immunofluorescence technique, since it cannot differentiate between viable and non-viable podocytes. However, Kelder et al. demonstrated that mRNA levels of podocyte-specific markers correlated with the number of viable podocytes in patient's samples.[16]

In conclusion, our results demonstrate that a cumulative dose of bevacizumab is correlated with albuminuria but not with podocyturia as measured with the qPCR technique. This contrasts with observations in PE, notwithstanding the clinical and pathophysiological similarities of both conditions. Compared to angiogenesis inhibition with bevacizumab the development of sometimes severe proteinuria occurs in a much shorter time span in PE, indicating more intensive, acute renal damage in this condition. This likely implies more extensive podocyte damage as reflected by more pronounced podocyturia. Finally, podocyturia in our study was assessed while almost all patients were on bevacizumab treatment for extended periods. Possibly, the presence of podocyturia is more pronounced at the beginning of anti-VEGF treatment, predating the development of proteinuria, and attenuates during prolonged treatment. This could explain the absent association between the cumulative bevacizumab dose and podocyturia in our study. Prospective studies with repeated measurements of podocyturia are needed to further explore this.

SOURCES OF FUNDING

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CHAPTER 9

SUMMARY, DISCUSSION AND PERSPECTIVES

SUMMARY

Angiogenesis, the formation of new vessels from preexisting vasculature, is critical to solid tumor growth as well as metastasis. This process is regulated by numerous factors among which vascular endothelial growth factor (VEGF) plays a dominant role. Angiogenesis inhibition, by targeting VEGF or its receptors, has become an established treatment of various types of cancer. Bevacizumab is a humanized monoclonal antibody against VEGF, whereas sunitinib is an orally active angiogenesis inhibitor that blocks VEGF receptors -1, -2 and -3 and other tyrosine kinase receptors. VEGFR-1 and VEGFR-2 are predominantly expressed on vascular endothelial cells, whereas VEGFR-3, stimulated by VEGF-C, is restricted to lymphatic endothelial cells. Unfortunately, this therapy is associated with severe side effects, including hypertension and renal injury, the latter reflected by proteinuria and renal function impairment. Because the occurrence of these adverse effects can be a reason to lower the dose of the anti-VEGF agent or even to discontinue anticancer therapy, thereby compromising its potential efficacy, the present studies explored new therapeutic approaches, as well as the dose- and salt dependency of these side effects.

In virtually every patient exposed to anti-VEGF treatment blood pressure (BP) rises. The incidence of hypertension induced by anti-VEGF treatment ranges from 9% to 67%, whereas Grades 3 to 4 hypertension has been reported in 3-18% of patients.[1] The risk to develop severe hypertension is increased in patients with a history of hypertension. The incidence of hypertension is dose-dependent and increases to over 90% when bevacizumab and sunitinib are combined.[2] The rise in BP is caused by a rise in systemic vascular resistance and can develop within hours to days after treatment initiation.[3, 4]Although hypertension has initially been considered to be a toxic effect of anti-angiogenic therapy, evidence has accumulated that the development of hypertension is predictive for a favourable anti-tumor response and improved survival.[5-7] The incidence of proteinuria associated with angiogenesis inhibition appears to be lower than that of hypertension and its severity may vary from <1 gram/day (Grade 1) to nephrotic range proteinuria.[8, 9] **(Chapters 1 and 2).**

Since binding of VEGF to its receptors increases endothelial nitric oxide synthase (eNOS) and thus nitric oxide (NO) bioavailability, it has been suggested that inhibition of the VEGF-pathway reduced NO bioavailability resulting in vasoconstriction and rise in BP. Additionally, decreased NO bioavailability disturbs the balance between the vasodilator NO and the vasoconstrictor endothelin-1 (ET-1), favoring ET-1 production as well its action, thereby inducing further vasoconstriction and an additional rise in BP. The first clinical evidence for the involvement of an activated ET-1 system during VEGF inhibition stems from a study with sunitinib by our group.[3] In patients with either metastatic renal cell cancer or imatinib-resistant gastrointestinal stromal tumors, administration of sunitinib was associated with a doubling of circulating ET-1 levels. In

successive studies in rats, we found that administration of sunitinib was also associated with an increase in serum ET-1 concentration, returning to pre-sunitinib values after its discontinuation.[10] In addition, co-administration of the dual endothelin (ET_A/ET_B) receptor blocker macitentan could to a large extent prevent the sunitinib-induced BP rise and proteinuria **(Chapter 2).**

Since endothelin receptor (ET-R) blockers are not approved for the treatment of systemic hypertension and renal injury.[11], we determined the most optimal way to prevent the sunitinib-induced adverse effects and explored their interdependency, by investigating also other antihypertensive agents; the calcium channel blocker (CCB) amlodipine, the angiotensin-converting enzyme (ACE) inhibitor captopril and the phosphodiesterase type 5 (PDE5) inhibitor sildenafil in our sunitinib rat model. After sunitinib start, mean arterial pressure (MAP) increased rapidly by about 30 mmHg. Co-administration of macitentan or amlodipine attenuated the sunitinib-induced rise in BP by 73% and 63% respectively, whereas co-administration of both dosages of captopril and sildenafil had no BP-lowering effect. In rats, proteinuria increased to about 30 mg per day during sunitinib administration. The rise in proteinuria was attenuated by macitentan, captopril and sildenafil. Conversely, proteinuria tended to increase further with amlodipine. Of interest, both captopril and sildenafil increased the steady-state sunitinib concentrations. We conclude that in our experimental model ET-R antagonism and calcium channel blockade are suitable to prevent angiogenesis inhibition induced-hypertension, while ET-R antagonism, ACE and PDE5 inhibition can prevent angiogenesis-inhibition induced proteinuria **(Chapter 4).**

In our previous rat studies, we used a sunitinib dose of 26.7 mg/kg.day by oral gavage. This high dose was associated with a rapid development of severe hypertension, proteinuria and irreversible glomerular renal injury, making it difficult to infer to what extent the renal injury is BP-independent. Additionally, the extent of sunitinib-mediated side-effects in this particular model at this dose is much more severe than seen in patients, rendering it questionable whether this dose adequately reflected the off-target effects of sunitinib in clinical practice. By applying 3 different doses of sunitinib, we tested the dose-dependency of these side effects, aiming to find a sunitinib dose that, with regard to hemodynamic and renal side effects, including ET-1 elevation, better resembles the dose applied in patients. With this approach, we also wanted to unravel whether the proteinuria observed during antiangiogenic treatment occurs irrespective of the presence of glomerular histological changes. BP rose dose-dependently from 13 to 30 mmHg. Proteinuria was present at all doses, but a rise in cystatin C occurred only at the intermediate and high dose. Circulating ET-1 increased dose-dependently, whereas 24-hour urinary endothelin excretion did not. Glomerular endotheliosis and ischemia were present with the intermediate and high doses of sunitinib, but no histological abnormalities were observed with the low dose. From these findings we conclude that the sunitinib-induced rise in BP requires lower doses than its induction of

renal function impairment and that functional changes in glomerular filtration barrier contribute to the occurrence of proteinuria, given the lack of histopathological changes with the low dose of sunitinib **(Chapter 5).**

Recent evidence indicates that in response to a high salt (HS) diet, sodium and chloride accumulates in the skin in excess of water.[12] This results in a hypertonic interstitial fluid compartment, stimulating skin lymphangiogenesis via activation of the mononuclear-phagocyte system (MPS) cell-derived VEGF-C-VEGF type 3 receptor signalling pathway.[13, 14] Inhibition of this pathway results in salt-sensitive hypertension. Since sunitinib, aside from blocking VEGFR-1 and VEGFR-2 signaling, also blocks the VEGFR-3 signaling that mediates lymphangiogenesis, we hypothesized that sunitinib administration impairs lymphangiogenesis in response to a HS diet, and that this impairment leads to Na^+ and especially Cl^- accumulation in the skin interstitium, contributing to salt-sensitive hypertension. Rats were exposed to a normal (NS) or HS diet with or without sunitinib administration. Sunitinib induced a 15 mmHg rise in BP, which was aggravated by a HS diet, resulting in a decline of the slope of the pressure-natriuresis curve. This rise in BP was not accompanied by an increase in body weight or changes in plasma sodium concentration or renal function impairment. Furthermore, a HS led to a proportionally comparable increase in Na^+ and Cl^- which is higher than the proportional increase in water content in the skin, and associated with an accentuated MPS cells infiltration in the skin that was not affected by sunitinib administration. Independent of diet, sunitinib tended to decrease lymphangiogenesis. We therefore concluded that sunitinib administration is associated with salt-sensitive hypertension that can be explained by impaired skin lymphangiogenesis only partly, suggesting that other factors are involved **(Chapter 6).**

In previous studies we have shown that the hypertension induced by sunitinib is accompanied by a decreased NO production and renin suppression.[3, 10, 15] i.e. conditions related to an increased sensitivity of BP to salt.[16, 17] In the present study, we explored whether the augmented rise of BP during a HS diet, as obtained in chapter 6, also translates into more pronounced renal damage, and whether or not this is exacerbated by a HS diet. The HS diet itself had no effect on proteinuria, endothelinuria or plasma levels of ET-1, creatinine, and cystatin C. Only with the HS diet, sunitinib administration massively increased proteinuria and endothelinuria. Likewise, renal glomerular pathology was enhanced during sunitinib with the HS diet, whereas tubulointerstitial injury or reduced peritubular capillary density did not occur **(Chapter 7).**

The combination of hypertension and renal injury during angiogenesis inhibition is often referred to as a preeclampsia-like syndrome. Preeclampsia, a condition occurring after 20 weeks of pregnancy, is characterized by hypertension, proteinuria, elevated circulating ET-1 levels as well as glomerular endotheliosis. Indeed, we demonstrated that angiogenesis inhibition with sunitinib in rats induced similar clinical and renal

histopathological characteristics **(Chapters 4 and 5).** The renal injury during angiogenesis inhibition associates with podocyte injury. It has been suggested that the appearance of podocytes in urine, i.e., podocyturia, could be a sensitive marker for ongoing glomerular disease predating proteinuria. Using a recently validated quantitative polymerase chain reaction (qPCR) of the podocyte-specific molecules nephrin, podocin and VEGF-A in the urine.[18], we examined whether podocyturia is present in bevacizumab-treated cancer patients, and to what extent it relates to proteinuria, and the cumulative dose of bevacizumab. We observed no elevated levels of nephrin mRNA or VEGF-A mRNA in the urine of patients treated with the VEGF inhibitor bevacizumab when compared to patients treated with chemotherapy only or to healthy controls. Furthermore, there was no significant relationship between qPCR nephrinuria and albuminuria or between qPCR nephrinuria and the cumulative dose of bevacizumab. Only albuminuria was positively correlated to the cumulative doses of bevacizumab, challenging the feasibility of the qPCR measurement of podocyte-specific molecules to monitor ongoing glomerular injury in bevacizumab treated patients **(Chapter 8).**

DISCUSSION

Administration of the multi-target VEGF receptor blocker sunitinib is accompanied by a rise in BP, renal injury and proteinuria, activation of the ET-1 axis and renin suppression. Previously, we found that the rise in BP and proteinuria could be largely prevented by the dual (ET_A/ET_B) receptor antagonist macitentan, indicating that activation of the ET-1 axis is critical for the development of these side effects.[10]
Co-administration of amlodipine with sunitinib was associated with a marked attenuation of the rise in BP, comparable to the degree observed with macitentan. However, renal injury could not be prevented while proteinuria even tended to increase. In contrast, co-administration of captopril and sildenafil was associated with a decrease in proteinuria and renal injury, but without effect on BP. These findings strongly suggest that these side effects are, at least in part, unrelated. Obviously, over more prolonged periods of time hypertension may further aggravate the renal injury by angiogenesis inhibition, whereas vice versa, renal injury may contribute to a further rise in BP. Based on the present observations together with other experimental and clinical studies, showing that the hypertension induced by sunitinib is associated with renin suppression, dihydropyridine calcium channel blockers, rather than agents targeting the renin-angiotensin system (RAS), are probably more effective for the treatment of hypertension.[19] Given their beneficial effects on the occurrence of proteinuria, an anti-RAS agent can be combined with a calcium channel blocker in case of the development of renal injury. Although NO-donors like nitrates or PDE5 inhibitors may also be beneficial, it has been suggested that these agents may potentially compromise the anti-angiogenic effect and therefore they can best be avoided.[20] **(Chapter 4).**

With the lowest dose of sunitinib applied (7 mg/kg.day), the BP rise of about 10 mmHg is of similar magnitude as the BP rise we have observed in patients after 2 and after 4 weeks administration of sunitinib in rats, stressing the potential relevance of our low-dose model for the clinical situation.[3] Of note, the plasma sunitinib concentration with the low dose of sunitinib was comparable to the concentration measured in patients treated with a standard dose of sunitinib of 50 mg per day.[21] However, the concentration of its active metabolite n-desethyl sunitinib was markedly higher, indicating increased metabolism of the parent compound in rats, as has been reported previously.[22] In line with our previous observations, sunitinib treatment was associated with activation of the circulating endothelin system, which was already present at the low dose of sunitinib.[3, 10] The observed rise in ET-1 may be a direct consequence of VEGF inhibition, as shown in human lung microvascular endothelial cells.[23] The rise may also be related to the activation of the endothelium in response to VEGF inhibition. This, however, was not supported by the finding that, despite activation of glomerular endothelial cells as reflected by the presence of glomerular endotheliosis and increased glomerular expression of ICAM-1, expression of the gene encoding for ET-1 within the kidney was not increased. In addition to a direct adverse effect of VEGF inhibition on podocyte function and expression of slit-diaphragm proteins, ET-1 can exert negative effects on podocyte function, which, additionally to the disruption of VEGF pathway, may contribute to glomerular injury and proteinuria.[24-27] Given the present observation that urinary ET-1 excretion was not increased during sunitinib administration or even decreased, the possibility that activation of ET-1 axis has contributed to the proteinuria in our rat model remains questionable. If ET-1 is involved it should, based on the present findings and an earlier study, reach the podocytes by ultrafiltration.[26] **(Chapter5).**

Machnik and colleagues have shown that skin electrolyte accumulation, in response to a HS diet, stimulates the formation of lymph capillaries, driven by MPS cells infiltrating the interstitium of the skin.[14] The authors also demonstrated that macrophage-derived VEGF-C mediates this lymphangiogenesis and that interruption of this pathway leads to salt-sensitive hypertension. In our study, a HS diet was indeed accompanied by increased MPS cells infiltration and lymphangiogenesis. Yet, despite the fact that sunitinib has shown shown to inhibit lymphangiogenesis.[28], we observed only a very modest decrease in the HS diet-induced lymphangiogenesis during sunitinib administration. Although an insufficient dose is the most obvious explanation, this is not supported by previous pharmacokinetic and in vitro studies.[28] In Chapter 5 we reported that the same dose of sunitinib as used in the present study was associated with a plasma concentration of sunitinib and its active metabolite N-desethyl sunitinib of about 350 nanogram/mL or 875 nmol/L. In an in vitro study, sunitinib concentrations as low as 30 nmol/L were shown to impair lymphangiogenesis.[28] Based on these data it is not very likely that the dose of sunitinib was the limiting factor to impair lymphangiogenesis. As reported earlier, administration of tyrosine kinase inhibitors is associated with activation of the endothelin system as well as suppression of the NO pathway and renin suppression

(Chapter 4).[3, 10] As suggested by Gu et al., suppression of the NO pathway can contribute to the salt-sensitive BP observed with anti-VEGF treatment.[29] Likewise, it is well established that renin suppression is associated with salt-sensitive hypertension.[17, 30, 31] To what extent activation of the endothelin system, occurring during sunitinib administration, contributes to salt-sensitive hypertension is not easy to answer because of the opposing effects on natriuresis mediated by the renal ET_A and ET_B receptors.[32] However, it has been reported that infusion of ET-1 in Sprague-Dawley rats induces hypertension when rats are on a high - but not on a low salt diet.[33, 34] Similarly, transgenic mice overexpressing endothelial ET-1 had a significantly higher BP when on a HS diet than their wild-type littermates.[35] These studies support the contention that an activated ET-1 system may contribute to salt-sensitive hypertension **(Chapter 6).**

Despite the marked rise in BP, a HS diet was not accompanied by proteinuria, impairment of renal function or histological glomerular or tubulointerstitial changes. However, with regard to renal injury, a clear interaction between the HS diet and suntinib exposure was present. Administration of sunitinib to rats on a HS diet as compared to rats on a NS diet was associated with pronounced proteinuria and endothelinuria, as well as glomerular epithelial cell swelling. Although histologically tubulointerstitial injury was not apparent, sunitinib administration induced an increased expression of the gene encoding for NGAL, a marker of tubular injury, in rats exposed to the HS diet. In previous preclinical and clinical studies, we and others have reported that administration of tyrosine kinase inhibitors or a rise in sFlt-1 in preeclamptic patients, is associated with activation of the endothelin system and that this activated endothelin system plays a central role in the rise in BP.[3, 10, 36, 37] In the present study, circulating ET-1 levels tended to be higher during sunitinib administration, but especially the combination of a HS diet and sunitinib was associated with a huge increase in urinary ET-1 excretion. Remarkably, this increased urinary ET-1 excretion was not accompanied by an increased renal expression of the genes encoding for preproET-1 or endothelin converting enzyme. This suggests that this urinary ET-1 is plasma-derived. The observed glomerular injury may be due to enhanced filtration of ET-1, which is supported by the correlation between proteinuria and endothelinuria. Selective, genetic, embryonic deletion of VEGF from renal tubules results in a decrease in peritubular capillaries and the formation of a small kidney.[38] Because of this crosstalk between tubular-derived VEGF-A and peritubular microvessels we explored whether administration of sunitinib was associated with a decrease in peritubular vascular density. This appeared not to be case, suggesting that such a phenotype may only occur following embryonic interruption of the tubular VEGF pathway. This is indirectly supported by studies in patients treated with antiangiogenic agents who develop renal injury. In these patients the most frequently reported lesion is glomerular thrombotic microangiopathy, likely preceded by glomerular endotheliosis.[39-43] **(Chapter 7).**

Podocyte-specific conditional knockout of VEGF-A in mice is known to result in podocyte loss, proteinuria and glomerular thrombotic microangiopathy.[39] Likewise, anti-VEGF-treatment in patients is associated with comparable renal abnormalities as observed in preeclamptic women.[39] The first study which describes podocyturia during VEGF inhibition is reported by Muller-Deile et al.; here albuminuria and podocyturia were found in 95% and 32% respectively, of bevacizumab treated patients, compared to 100% and 13% of sunitinib treated patients.[44] Remarkably, the incidence of albuminuria was much higher than that of podocyturia. A recent study of Garovic et al. showed that 60% of patients treated with a VEGF inhibitor had evidence of podocyturia.[45] In addition, they demonstrated a correlation between the cumulative dose of bevacizumab and both proteinuria and podocyturia, as well as a correlation between the number of urinary podocytes and the amount of proteinuria, suggesting that these variables are mechanistically related. It must be mentioned that patients treated with the combination of anti-VEGF therapy and cytotoxic agents known to induce renal injury in over 10% of patients, such as cisplatin, were also included in that study. The same holds true for patients on anti-VEGF agents in combination with other targeted therapies. Compared to our own study, in which patients on cytotoxic agents known to induce renal toxicity or on previous or dual-targeted therapy were excluded, the inclusion of these patients in the study of Garovic et al. might account for the higher incidence of proteinuria and podocyturia and the positive correlation between these two variables, despite the lower cumulative dose of bevacizumab. Since in our study only 18% of the bevacizumab-treated patients had albuminuria a correlation might be difficult to find, moreover podocyturia can be present without the appearance of proteinuria.[46] Considering these differences, and the fact that in the above-mentioned study of Muller-Deile et al. the incidence of albuminuria was much higher than that of podocyturia in their cancer patients on bevacizumab or sunitinib treatment, podocyturia and albuminuria might provide different information.

Why we did not observe any differences in podocyturia between the groups of subjects in contrast to previous studies on anti-VEGF therapies and studies in PE is not clear. Our results demonstrate that a cumulative dose of bevacizumab is correlated with albuminuria but not with podocyturia as measured with the qPCR technique. This contrasts with observations in PE, notwithstanding the clinical and pathophysiological similarities of both conditions.[18] Compared to angiogenesis inhibition with bevacizumab, the development of sometimes severe proteinuria occurs in a much shorter time span in PE, indicating more intensive, acute renal damage in this condition. This likely implies more extensive podocyte damage as reflected by more pronounced podocyturia. Furthermore, podocyturia in our study was assessed while almost all patients were on bevacizumab treatment for extended periods. Possibly, the presence of podocyturia is more pronounced at the beginning of anti-VEGF treatment, predating the development of proteinuria, and attenuates during prolonged treatment. This could explain the absent association between the cumulative bevacizumab dose and podocyturia in our study **(Chapter 8).**

PERSPECTIVES

Given the lack of large clinical studies, we provided further insight into the mechanisms underlying sunitinib-induced hypertension and proteinuria as well as ways to counteract these adverse events using a recently developed rat model. Translation of the findings in this model to the clinic strongly suggests that dependent on the toxicity encountered, different classes of antihypertensive agents should preferably be used, i.e. a CCB in the case of hypertension and a RAS blocker in the case of renal injury. Of interest, both captopril and sildenafil increased the steady-state sunitinib concentrations. The underlying pharmacokinetic interaction is currently unknown, but warrants further investigation in humans, not only because it may enhance the anti-cancer effectiveness of sunitinib, but potentially also its side-effect profile. With respect to the relatively short observation period of 8 days during our previous studies, it would be interesting to see whether the rise in BP and renal pathology is progressive during more prolonged exposure to sunitinib **(Chapter 4).**

With the lowest dose of sunitinib the serum concentration of the active metabolite was considerably higher than observed in patients, yet the rise in BP was comparable. Finally, although in the present study we have focussed on the renal effects of sunitinib, it is well known that VEGF inhibition can also have negative effects on other organs, especially the myocardium and endocrine organs. In more long-term studies it would be interesting to see whether the lowest or even a still lower dose of sunitinib as used in the present study still preferentially affects the kidney or also has adverse effects on the myocardium and endocrine organs when administrated for prolonged periods. In future experiments it should be explored whether blockade of the endothelin pathway can completely prevent the development of hypertension and renal injury in rats exposed to low doses of sunitinib for prolonged periods. This would provide evidence that activation of the endothelin pathway is an essential mediator of the rise in BP and renal toxicity associated with antiangiogenic treatment. Moreover, it should be explored whether ET_A-selective receptor antagonism is more favourable than blockade of both the ET_A and ET_B receptor **(Chapter 5).**

Sunitinib administration to rats, in a relatively low dose not inducing renal function impairment, is associated with salt-sensitive hypertension. Impairment of the skin MPS cells-TonEBP-VEGF-VEGFR-3-lymphangiogenesis pathway has been recognized as a novel mechanism involved in salt-sensitive hypertension. Unexpectedly, because sunitinib is known to block the VEGFR-3-signaling pathway (thereby inhibiting lymphangiogenesis in *in vitro* studies), we only observed very modestly blocked lymphangiogenesis during sunitinib treatment. Therefore, it is more likely that other mechanisms such as renin suppression, a decrease in NO production and activation of the endothelin system contribute to a larger extent to the salt-sensitive hypertension during sunitinib administration. Whether more prolonged administration of sunitinib

impairs the formation of lymph vessels in response to a HS diet, and eventually results in renal tubulointerstitial injury and peritubular rarefaction requires further investigation. Furthermore, the observation that a HS diet aggravates the rise in BP and renal injury may have implications for patients treated with angiogenesis inhibitors. These patients, especially when on long-term treatments as occurs in the neoadjuvant setting, should be advised to limit their dietary salt intake. Finally, given that interruption of the VEGF-signaling pathway is associated with activation of the endothelin system, it would be interesting to explore whether the sunitinib-induced salt-sensitive hypertension can be prevented by ET_A-selective or ET_A/ET_B receptor blockade **(Chapters 6 and 7).**

Since only albuminuria was positively correlated to the cumulative doses of bevacizumab, it challenges the feasibility of the measurement of podocyturia to monitor ongoing glomerular injury in bevacizumab treated patients. The different anti-VEGF modalities, anti-VEGF monoclonal antibody (bevacizumab) versus receptor tyrosine kinase inhibitor (sunitinib) or circulating sFlt-1, might induce different degrees of VEGF inhibition. To further explore this, it would be interesting to determine podocyturia using the qPCR method in patients treated with one of the receptor tyrosine kinase inhibitors. Prospective studies with repeated measurements of podocyturia are needed to further explore this **(Chapter 8).**

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APPENDICES

NEDERLANDSE SAMENVATTING

Angiogenese, de vorming van nieuwe bloedvaten uit het reeds bestaande vaatstelsel, is essentieel voor de groei van tumoren en metastasering. Angiogenese wordt door meerdere factoren gereguleerd, met een dominante rol voor de vasculaire endotheliale groeifactor (VEGF). Angiogeneseremming, door remming van VEGF of VEGF-receptoren, heeft een vaste plaats verworven bij de behandeling van verschillende vormen van kanker. Bevacizumab is een gehumaniseerd monoclonaal antilichaam tegen VEGF, terwijl sunitinib een oraal werkzame angiogeneseremmer is die de VEGF-receptoren 1, 2 en 3 en andere tyrosinekinasereceptoren blokkeert. VEGF-receptoren 1 en 2 komen voornamelijk tot expressie in vasculaire endotheelcellen, terwijl de VEGF-receptor 3, die gestimuleerd wordt door VEGF-C, tot expressie komt in de endotheelcellen van de lymfevaten. VEGF-remming gaat gepaard met ernstige bijwerkingen, waaronder hypertensie en nierschade. Het optreden van deze bijwerkingen kan een reden zijn om de dosis van het anti-VEGF middel te verlagen of om het zelfs helemaal te stoppen, met belangrijke repercussies voor de kankerbehandeling. In onze studies hebben we gekeken naar de dosis- en zoutafhankelijkheid van de bijwerkingen van sunitinib en naar mogelijke therapieën om deze bijwerkingen te verminderen of te voorkomen.

Bij vrijwel iedere patiënt die behandeld wordt met een anti-VEGF middel stijgt de bloeddruk. De incidentie van hypertensie geïnduceerd door anti-VEGF behandeling varieert van 9% tot 67%. Ernstige (graad 3 en 4) hypertensie wordt gezien bij 3-18% van de patiënten. Het risico op het ontwikkelen van ernstige hypertensie neemt toe bij patiënten met hypertensie in de voorgeschiedenis. De incidentie van hypertensie is dosisafhankelijk en neemt toe tot meer dan 90% wanneer bevacizumab en sunitinib gecombineerd worden. De bloeddrukstijging wordt veroorzaakt door een toename van systemische vaatweerstand en kan binnen een paar uur na start van de behandeling ontstaan. Hoewel hypertensie een bijwerking is van anti-VEGF behandeling blijkt het ontstaan van hypertensie ook een voorspeller te zijn voor een gunstige antitumorrespons en verbeterde overlevingskans. De incidentie van proteïnurie geassocieerd met angiogeneseremming is lager dan die van hypertensie. De proteïnurie kan variëren van <1 gram/per dag (graad 1) tot massale proteïnurie **(Hoofdstukken 1 en 2).**

Binding van VEGF aan receptoren stimuleert het endotheliale stikstofmonoxide synthase (eNOS) en verhoogt daarmee de beschikbaarheid van stikstofmonoxide (NO). Remming van de VEGF-route zal de beschikbaarheid van NO verlagen, resulterend in vaatvernauwing en stijging van de bloeddruk. Daarnaast verstoort de verlaagde beschikbaarheid van NO de balans tussen het vaatverwijdende NO en het vaatvernauwende endotheline-1 (ET-1), waardoor de ET-1 productie en ET-1-gemedieerde vasoconstrictie de overhand krijgen, met verdere vaatvernauwing en bloeddrukstijging tot gevolg. Het eerste bewijs voor de betrokkenheid van een geactiveerd ET-1 systeem tijdens VEGF-remming kwam voort uit een klinische studie met sunitinib

uitgevoerd door onze groep. Bij patiënten met een gemetastaseerd niercelcarcinoom en imatinib-resistente gastrointestinale stromale tumoren, veroorzaakte sunitinib behandeling een verdubbeling van de ET-1 waarden in het bloed. In vervolgstudies in ratten vonden we dat de toediening van sunitinib leidde tot een toename van de serum ET-1-concentratie, dalend tot pre-sunitinib waarden na het stoppen van sunitinib. Bovendien kon het tegelijkertijd met sunitinib toedienen van de duale endotheline (ET_A/ET_B) receptorblokker macitentan de sunitinib-geïnduceerde bloeddrukstijging en proteïnurie grotendeels voorkomen **(Hoofdstuk 2).**

Omdat endotheline receptorblokkers niet geregistreerd zijn voor de behandeling van systemische hypertensie en nierschade, hebben we het effect van een aantal antihypertensiva op de sunitinib-geïnduceerde hypertensie en nierschade onderzocht in ratten. Toediening van sunitinib veroorzaakte een 30 mmHg stijging van de gemiddelde arteriële bloeddruk. Het tegelijkertijd toedienen van macitentan of de calciumantagonist amlodipine verminderde de sunitinib-geïnduceerde bloeddrukstijging met respectievelijk 73% en 63%, terwijl het tegelijkertijd toedienen van een lagere en hogere dosering van de ACE-remmer captopril en de fosfodiesterase-5-remmer sildenafil de bloeddruk niet verlaagde. Door sunitinib steeg de proteïnurie tot 30 mg per dag. Deze stijging werd grotendeels voorkomen door macitentan, captopril en sildenafil. Anderzijds leidde toediening van amlodipine juist tot een toename van de proteïnurie. Opvallend was dat captopril en sildenafil de steady-state sunitinibserumconcentratie verhoogden. Op grond van bovengenoemde bevindingen concluderen we dat in ons experimentele model endothelinereceptor- en calciumkanaalblokkade geschikt zijn om VEGF-remming-geïnduceerde hypertensie te voorkomen, terwijl endothelinereceptorblokkade, ACE-remming en fosfodiesterase-5-remming juist de VEGF-remming-geassocieerde proteïnurie kan voorkomen **(Hoofdstuk 4).**

In onze eerste rattenstudies gebruikten we sunitinib in een orale dosering van 26.7 mg/kg per dag. Deze relatief hoge dosis leidde binnen enkele dagen tot een ernstige hypertensie, proteïnurie en irreversibele glomerulaire nierschade, waardoor het moeilijk is om een uitspraak te doen in hoeverre de nierschade onafhankelijk is van de bloeddrukstijging. Daarnaast zijn de sunitinib-geïnduceerde bijwerkingen in dit specifieke model bij de gebruikte sunitinib dosering ernstiger dan de bijwerkingen die in patiënten worden waargenomen. We testten daarom de dosisafhankelijkheid van de bijwerkingen door het toedienen van verschillende doseringen van sunitinib, met als doel een sunitinib dosis te vinden die met betrekking tot de hemodynamische en renale bijwerkingen (waaronder ET-1 verhoging) beter overeenkomt met de bijwerkingen die we aantreffen bij patiënten. Met deze aanpak wilden we ook onderzoeken of de proteïnurie die tijdens VEGF-remming optreedt onafhankelijk is van het optreden van histologische afwijkingen in de glomerulus. Met de verschillende doseringen sunitinib steeg de bloeddruk dosis-afhankelijk van 13 tot 30 mmHg. Proteïnurie was aanwezig bij alle drie de doseringen, maar cystatine C als maat voor de glomerulaire filtratiesnelheid

steeg alleen met de middelste en hoogste sunitinibdosering. Circulerend ET-1, maar niet de 24-uurs urine endotheline-uitscheiding, steeg dosisafhankelijk. Glomerulaire endotheliose en ischemie werden gezien bij de middelste en hoogste dosis van sunitinib, maar niet bij de laagste dosering. Op grond van deze bevindingen concluderen we dat de drempeldosis van sunitinib voor het ontwikkelen van hypertensie lager is dan die voor het ontwikkelen van een verminderde nierfunctie en dat functionele veranderingen in de glomerulaire filtratiebarrière mede bijdragen aan het optreden van proteïnurie, gezien het ontbreken van histologische veranderingen bij de laagste dosering van sunitinib **(Hoofdstuk 5).**

Uit recent onderzoek is gebleken dat tijdens een hoog zout (HS) dieet er een relatief grotere ophoping is van natrium- en chloride- dan waterionen in de huid. Dit leidt tot een hypertonisch, interstitiëel vloeistofcompartiment met stimulatie van de lymfangiogenese in de huid via activering van het mononucleaire-fagocytaire celsysteem (MPS). Door het geactiveerde MPS wordt VEGF-C geproduceerd dat vervolgens via VEGF-receptor 3 de lymfangiogenese stimuleert. Remming van deze signaleringsroute in muizen en ratten leidt tot zout-gevoelige hypertensie. Omdat sunitinib, naast de blokkade van VEGF-receptoren 1 en 2, ook de VEGF-receptor 3 blokkeert, was onze hypothese dat sunitinib de lymfangiogenese in respons op een HS dieet remt, en dat deze remming leidt tot ophoging van natrium- en chloride-ionen in de huid, bijdragend aan zout-gevoelige hypertensie. Ratten werden blootgesteld aan een normaal (NS) of HS dieet met of zonder toediening van sunitinib. Sunitinib veroorzaakte een 15 mmHg bloeddrukstijging, die versterkt werd door een HS dieet en resulteerde in een afname van de hellingshoek van de bloeddruk-natriurese curve. Deze bloeddrukstijging ging niet gepaard met een toename van het lichaamsgewicht, veranderingen in plasma natriumconcentratie of verslechtering van de nierfunctie. Wel leidde een HS dieet tot een stijging van de hoeveelheid natrium- en chloride-ionen in de huid die hoger was dan de stijging van de hoeveelheid water. Deze stijging ging gepaard met een toename van MPS cellen in de huid die niet geremd werd door sunitinib. Onafhankelijk van het NS of HS dieet was er een tendens tot een verminderde lymfangiogenese tijdens sunitinib. Op grond van deze bevindingen concluderen we dat de toediening van sunitinib leidt tot een zout-gevoelige hypertensie, maar dat deze slechts zeer dele verklaard kan worden door een verminderde lymfangiogenese in de huid, erop wijzend dat andere factoren een rol spelen bij de zoutgevoelige hypertensie tijdens sunitinib toediening **(Hoofdstuk 6).**

In eerdere studies hebben we laten zien dat de sunitinib-geïnduceerde hypertensie gepaard gaat met een afname in NO-productie en renine onderdrukking; een situatie die gepaard gaat met een toegenomen zoutgevoeligheid. In de huidige studie onderzochten we of de sunitinib-geïnduceerde toegenomen bloeddrukstijging tijdens een HS dieet, zoals we zagen in hoofdstuk 6, ook leidt tot meer uitgesproken nierschade. Door het HS dieet alleen was er geen toename van de proteïnurie, endothelinurie, circulerend ET-1,

creatinine, en cystatine C. Alleen tijdens het HS dieet leidde toediening van sunitinib tot een aanzienlijke stijging van de proteïnurie en endothelinurie. Tevens namen tijdens het HS dieet in combinatie met sunitinib de histologische glomerulaire afwijkingen in de nier toe, maar tubulointerstitiële schade of een afname in peritubulaire capillaire dichtheid werd niet gezien **(Hoofdstuk 7).**

De combinatie van hypertensie en nierschade tijdens angiogeneseremming wordt ook wel een pre-eclampsie-achtig syndroom genoemd. Pre-eclampsie is een zwangerschapsgerelateerde aandoening die gekarakteriseerd wordt door hypertensie, proteïnurie, verhoogde circulerende ET-1 waarden en glomerulaire endotheliose optredend na de 20ste zwangerschapsweek. We lieten eerder zien dat angiogeneseremming met sunitinib afwijkingen in de nier veroorzaakt overeenkomend met de histologische afwijkingen die gezien worden bij pre-eclampsie **(Hoofdstukken 4 en 5).** Beschadiging van de podocyten speelt een belangrijke initiële rol bij de nierschade tijdens VEGF-remming. Gesuggereerd is dat het verschijnen van podocyten in de urine (podocyturie) een gevoelige marker is voor glomerulaire ziekteactiviteit, die voorafgaat aan de proteïnurie. Gebruikmakend van een recentelijk gevalideerde kwantitatieve polymerasekettingreactie (qPCR) van de podocyt-specifieke moleculen nefrine, podocine en VEGF-A in de urine, onderzochten we of podocyturie aanwezig is bij patiënten met kanker die behandeld worden met bevacizumab, en of deze gerelateerd is aan de proteïnurie en de cumulatieve doses van bevacizumab. We zagen geen verhoogde waarden van nefrine mRNA of VEGF-A mRNA in de urine van patiënten die behandeld werden met de VEGF-remmer bevacizumab in vergelijking met patiënten die behandeld werden met alleen chemotherapie of met gezonde controles. Bovendien was er geen relatie tussen de qPCR nefrinurie en albuminurie of tussen de qPCR nefrinurie en de cumulatieve doses van bevacizumab. Albuminurie en de cumulatieve doses van bevacizumab waren wel gerelateerd. Op grond van deze bevindingen concluderen we dat de qPCR meting van podocyt-specifieke moleculen ter monitoring van actieve glomerulaire schade in patiënten die met bevacizumab behandeld worden geen toegevoegde waarde heeft in vergelijking met het bepalen van proteïnurie **(Hoofdstuk 8).**

CURRICULUM VITAE

Stephanie Lankhorst werd geboren op 1 november 1986 te Amstelveen. Na het voltooien van het VWO atheneum aan het Hermann Wesselink College te Amstelveen, startte zij in 2006 met de opleiding Biomedische Wetenschappen aan de Vrije Universiteit van Amsterdam. Na een algemene bachelor van 3 jaar koos zij voor de klinisch georiënteerde specialisatie Cardiovascular Research aan het VU Medisch Centrum Amsterdam, waarin zij tijdens haar laatste stage tevens kennis leerde maken met de afdeling Inwendige Geneeskunde, sector Vasculaire Geneeskunde & Farmacologie in het Erasmus Medisch Centrum te Rotterdam. Na het behalen van haar Master diploma begon zij in 2011 onder leiding van Dr. Anton H. van den Meiracker en Prof. dr. A.H. Jan Danser aan haar promotieonderzoek, naar de behandelingsopties en zoutafhankelijkheid van angiogenese remming-geïnduceerde hypertensie en nierschade, dat beschreven is in het huidige proefschrift.

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PhD PORTFOLIO

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Research School:	Cardiovascular Research School (COEUR)
Promotor:	Prof. dr. A.H.J. Danser
Copromotoren:	Dr. A.H. van den Meiracker Dr. M.H.W. Kappers
PhD period:	2011-2015

COEUR Courses **7.5 ECTS**	**Year**
Intensive care research	2012
Arrhythmia research methodology	2012
Clinical cardiovascular epidemiology	2012
Cardiovascular pharmacology	2012
Vascular medicine	2013

Erasmus MC courses **5.1 ECTS**	**Year**
ERCATHAN masterclass cardio module	2012
Laboratory Animal Science	2012
Photoshop & Illustrator CS6 Workshop (MolMed)	2013
Basiscursus Regelgeving en Organisatie voor Klinisch Onderzoekers (BROK)	2014
CPO course: patient oriented research: design, conduct, analysis and clinical implications	2015

Courses from Dutch Heart/Kidney Foundation 4 ECTS	Year
Vascular Biology, Papendal	2012
Winterschool, Driebergen	2013
COEUR Research Seminars and lectures 3.3 ECTS	**Year**
Several	2012-2014
Didactic skills 5.9 ECTS	**Year**
Practical lessons Junior Med School, 5th year high school students (5VWO)	2012
VO Autonoom zenuwstelsel, 1st year medical students	2012-2014
Practical lessons cardiovascular pharmacology, 2nd year medical students	2013-2014
Education to clinical pharmacologists	2014
Practical lessons Junior Med School, 5th year high school students (5VWO)	2015
Symposia and conferences 17.4 ECTS	**Year**
Oral Presentations	
23rd annual scientific meeting of the European Society of Hypertension, Milan, Italy	2013
3rd ISH New Investigators' Symposium, New Orleans, United States	2013
Max-Delbruck Centre for Molecular Medicine, Charité Medical Faculty, Berlin, Germany	2013
Wetenschapsdagen Internal Medicine, Antwerp, Belgium	2014
24th/25thJoint meeting of the European and International Society of Hypertension, Athens, Greece	2014

Ledenvergadering Nederlandse Hypertensie Vereniging, Zeist	2015
Nederlandse Nefrologiedagen, Veldhoven	2015
25th annual scientific meeting of the European Society of Hypertension, Milan, Italy	2015

Work meetings 2012-2015

Poster Presentations

High Blood Pressure Research council of the American Heart Association, New Orleans, United States	2013
High Blood Pressure Research council of the American Heart Association, San Francisco, United States	2014
Wetenschapsdagen Internal Medicine, Antwerp, Belgium	2015
Hypertension council of the American Heart Association, Washington D.C, United States	2015

Attendance

Wetenschapsdagen Internal Medicine, Antwerp, Belgium	2012
PhD Day NAI, Rotterdam	2012
PhD Day NAI, Rotterdam	2013

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Jaap van Schouten Foundation travel grant to attend HBPR 2015 (Washington D.C, United States)

ESH accommodation grant to attend ESH 2015 (Milan, Italy)

ESH/ISH investigator grant to attend ESH/ISH 2014 (Athens, Greece)

ESH accommodation grant to attend ESH 2013 (Milan, Italy)

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