Salt Sensitivity of Angiogenesis Inhibition–Induced Blood Pressure Rise
Role of Interstitial Sodium Accumulation?

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

See Editorial Commentary, pp 785–786

Abstract—In response to salt loading, Na⁺ and Cl⁻ accumulate in the skin in excess of water, stimulating skin lymphangiogenesis via activation of the mononuclear phagocyte system cell-derived vascular endothelial growth factor–C–vascular endothelial growth factor type 3 receptor signaling pathway. Inhibition of this pathway results in salt-sensitive hypertension. Sunitinib is an antiangiogenic, anticancer agent that blocks all 3 vascular endothelial growth factor receptors and increases blood pressure. We explored the salt dependency of sunitinib-induced hypertension and whether impairment of skin lymphangiogenesis is an underlying mechanism. Normotensive Wistar–Kyoto rats were exposed to a normal or high salt with or without sunitinib administration. Sunitinib induced a 15 mmHg rise in telemetrically measured blood pressure, which was aggravated by a high-salt diet (HSD), resulting in a decline of the slope of the pressure–natriuresis curve. Without affecting body weight, plasma Na⁺ concentration or renal function, Na⁺ and Cl⁻ skin content increased by 31% and 32% with the high salt and by 49% and 50% with the HSD plus sunitinib, whereas skin water increased by 17% and 24%, respectively. Skin mononuclear phagocyte system cell density increased both during sunitinib and a HSD, but no further increment was seen when HSD and sunitinib were combined. HSD increased skin lymphangiogenesis, while sunitinib tended to decrease lymphangiogenesis, both during a normal-salt diet and HSD. We conclude that sunitinib induces hypertension that is aggravated by high salt intake and not accompanied by impaired skin lymphangiogenesis. (Hypertension. 2017;69:919-926. DOI: 10.1161/HYPERTENSIONAHA.116.08565.) ● Online Data Supplement

Key Words: angiogenesis inhibition ■ hypertension ■ lymphangiogenesis ■ Na⁺ storage ■ salt sensitivity ■ sunitinib ■ vascular endothelial growth factor

Angiogenesis, the formation of new vessels from preexisting vasculature, is critical to tumor growth and metastasis. This process is regulated by numerous factors, with vascular endothelial growth factor (VEGF) playing a predominant role. Different strategies to inhibit the VEGF signaling pathway by directly targeting VEGF or blocking its receptors have been developed and have become established modalities for the treatment of a wide range of malignancies. VEGF inhibition is associated with hypertension, proteinuria, and renal function impairment in a substantial proportion of patients, sometimes necessitating discontinuation of treatment. 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 receptors and c-Kit receptors, by interacting with their ATP-binding pockets. VEGFR-1 and VEGFR-2 are predominantly expressed on vascular endothelial cells, whereas VEGFR-3, stimulated by VEGF-C, is mainly restricted to lymphatic endothelial cells. A previous study has shown that the multitargeted VEGFR inhibitor SU5416 enhances dietary salt–induced hypertension and kidney injury in normotensive Sprague–Dawley rats. The authors speculated that the SU5416-induced decrease in renal nitric oxide (NO) production in proximal tubular epithelial cells via inhibition of endothelial NO synthase underlies this salt-sensitive hypertension. Recent reports indicate that in response to a high-salt diet (HSD), Na⁺ and Cl⁻ accumulate in the skin in excess of water. This results in a hypertonic interstitial fluid compartment and accumulation of mononuclear phagocyte system (MPS) cells. In response to the hypertonic environment, MPS cells secrete increased amounts of VEGF-C in a tonicity-responsive enhancer-binding protein (TonEBP), also known as nuclear factor of activated T-cells.

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5-dependent manner. VEGF-C, via activation of lymph-endothelial VEGF-3 receptors, in turn 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 with skin Cl− and Na+ accumulation and salt-sensitive hypertension. 10,11 Because 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 HSD and that this impairment leads to Na+ and Cl− accumulation in the skin interstitium, contributing to salt-sensitive hypertension. To test this hypothesis, we measured blood pressure (BP) telemetrically and determined skin electrolytes, MPS cell infiltration, and skin lymphangiogenesis in normotensive Wistar-Kyoto rats exposed to a normal-salt diet (NSD) or HSD with and without addition of sunitinib. In a previous study, we already reported that sunitinib exacerbates both the magnitude of the BP rise and glomerular injury in response to a HSD. 12

Methods

Animals

All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC.

Male Wistar-Kyoto rats (180–200 g; n=30), obtained from Harlan Laboratories, were individually housed and maintained on a 12-hour light/dark cycle having access to standard laboratory rat chow (NSD; 0.5%–1.0% NaCl) and tap water ad libitum. Intra-aortic BP recordings were performed by implanted ratemeters (PA-C40; Data Sciences International) and sunitinib (SU11248; Sutent, Pfizer), and vehicle solutions were prepared and administered by oral gavage as described previously. 11 After implantation of the telemetry transmitters, rats received analgesic treatment using buprenorphine 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 HSD (8% NaCl, Teklad, Envigo+0.9% saline drinking water, n=15) or an NSD for 2 weeks (n=15). Rats were then randomly administered a dose of sunitinib (7 mg/kg per day, n=8) or vehicle (n=7) by oral gavage (0.5 mL) 8 days, on top of the NSD or HSD.

At the end of each experiment, rats were euthanized with 60 mg/kg pentobarbital intraperitoneally. Blood was sampled for measurement of creatinine, cystatin C levels, and electrolytes; kidneys were rapidly excised; and ears and skin were collected.

In all experiments, before the start of the specific diet and immediately before 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 because of 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 podoplanin antibody as described previously. 11 Briefly, right ears of the rats were fixed in 5% formalin and 4% protein free albumin, respectively, and embedded in paraffin. Podoplanin staining was performed using Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame) and the horseradish peroxidase super staining kit (ID Labs, Ontario) according to the manufacturer’s instructions. Deparaffinized slides were boiled 2x for 5 minutes at 600 W in a microwave in 0.1 mol/L citrate buffer (pH 6.0). After cooling down to room temperature, slides were incubated in 3% H2O2 for 10 minutes. Blocking was performed using an Avidin/Biotin Blocking kit and the IDetect Super Staining kit (ID Labs) according to the manufacturer’s protocol. After washing with phosphate-buffered saline 3x, slides were incubated with a podoplanin antibody (1:2000; RELIATech GmbH) for 1 hour, followed by incubation with a horseradish peroxidase-conjugated secondary antibody for 10 minutes. Slides were washed 3x with phosphate-buffered saline 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) for 30 minutes. Sections were then incubated with a CD68 antibody, a monocoyte 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 conjugated to the manufacturer’s protocol. After staining with phosphate-buffered saline 3x, slides were incubated with a podoplanin antibody (SU11248; Sutent, Pfizer), and vehicle solutions were prepared and administered by oral gavage as described previously. 11 After implantation of the telemetry transmitters, rats received analgesic treatment using buprenorphine subcutaneously (0.05 mg/kg; RB Pharmaceuticals Limited) for 2 days, followed by a recovery period of 10 days. After this, animals were randomly selected to groups that received either a HSD (8% NaCl, Teklad, Envigo+0.9% saline drinking water, n=15) or an NSD for 2 weeks (n=15). Rats were then randomly administered a dose of sunitinib (7 mg/kg per day, n=8) or vehicle (n=7) by oral gavage (0.5 mL) for 8 days, on top of the NSD or HSD.

Expression of Skin Edn1, Ece1, Vegfc, TonEBP, Pdpn mRNA

Quantitative polymerase chain reaction was performed to determine mRNA expression. RNA was isolated using the TRIzol method and reverse transcribed to cDNA using an AMV cDNA synthesis kit (Roche Indianapolis, IN). For the quantitative polymerase chain reaction reaction, iQ SYBR Green supermix (BioRad) was used. Pro-endothelin-1 (Edn1), endothelin converting enzyme 1 (Ece1), vascular endothelial growth factor C (Vegfc), TonEBP, and podoplanin (Pdpn) mRNA levels were measured and expressed relative to 18s levels. The primers used are provided in Table S1 in the online-only Data Supplement.

Statistical Analysis

Data are presented as mean±SEM. Statistical analysis between groups was performed by 1-way analysis of variance followed by Tukey’s multiple comparison test or posttest for linear trend or by 2-way repeated measures analysis of variance. Correlations were performed using Pearson’s testing and differences in slope by linear regression analysis. GraphPad Prism version 5.0 was used for all statistical analysis. A P value <0.05 was considered to be statistically significant.

Results

HS Diet Enhances Sunitinib’s Hypertensive Effect and Decreases the Slope of the Pressure–Natriuresis Curve

Mean arterial pressure during an NSD was 101±0.9 mm Hg (Figure 1A). A HSD increased mean arterial pressure by 27±3 mm Hg (P<0.05 versus NS diet). Sunitinib increased mean
arterial pressure by 15±1 mm Hg during an NSD ($P<0.05$ versus NSD alone) and by 23±4 mm Hg during a HSD ($P<0.05$ versus HSD alone). As reported, HSD conditions increased the sunitinib-induced mean arterial pressure increase in area under the curve under NSD conditions by 124% (38 versus 85 mm Hg×days; $P<0.05$), resulting in a decline in the slope of the pressure–natriuresis curve (Figure 1B).

Body Weight, Plasma, and Skin Electrolytes and Kidney Function

Urinary sodium excretions during an NSD and HSD diet were 1.0±0.2 and 41.9±5.2 mmol/d, respectively. Sunitinib did not affect these values significantly (1.1±0.1 versus 34.3±1.8 mmol/d). A HSD or sunitinib administration had no effect on body weight or plasma Na$^+$ or K$^+$ concentration, whereas plasma Cl$^-$ concentration modestly increased during the HSD with and without sunitinib (Figure 2A and 2B and Table). A HSD increased the content of Na$^+$ and Cl$^-$ (mmol/g dry weight) and of water (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 HSD alone (Table). The skin Na$^+$, Na$^+$ plus K$^+$ or Cl$^-$ concentration per skin water (mmol/L) did not change either with the HSD or with sunitinib (Table; Figure S1); however, a linear trend was observed for Na$^+$ per skin water (Figure 2C). Both skin Na$^+$ (Figure 3A) and Cl$^-$ (Figure 3B) concentrations were associated with the BP rise in response to a HSD. Renal function as reflected by plasma creatinine and cystatin C concentrations and creatinine clearance did not change in response to a HSD with or without sunitinib (Table).
HS Diet and Sunitinib Increase Skin MPS Cell Infiltration

As displayed in Figure 4, a HSD and sunitinib administration led to a greater CD68-positive cell area than an NSD combined with vehicle, confirming 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 HSD and a HSD plus sunitinib compared with an NSD and NSD plus sunitinib (Figure 5). Sunitinib tended to decrease the number of podoplanin-positive lymph capillaries in the ears of rats fed with NSD (2.6±0.2 versus 1.9±0.3) or with a HSD (4.4±0.5 versus 3.6±0.4).

HS Diet and Sunitinib Lead to Increased Skin Edn1 and Ece1 mRNA Expression

Previous studies have shown that administration of sunitinib is accompanied by activation of the endothelin system. Administration of sunitinib and HSD was associated with increased skin expression of Edn1 and Ece1 mRNA (Figure S2). The combination of a HSD and sunitinib did not result in a further increase in expression of the 2 genes. TonEBP expression tended to increase and Vegfc expression increased during a HSD, whereas podoplanin expression tended to increase with a HSD (Figure S3).

Discussion

In this study, we explored whether a HS diet augments the BP rise induced by the multitargeted VEGF inhibitor sunitinib and whether this augmentation associates with increased skin electrolyte accumulation because of a concomitant attenuation...
of skin lymphangiogenesis. Our main findings are that the HS diet–induced rise in BP in male Wistar–Kyoto rats is aggravated by sunitinib and that this diet associates with an accentuated MPS cell infiltration and lymphangiogenesis in the skin that is not significantly impaired by sunitinib.

The applied HSD for 2 weeks resulted in a 27 mm Hg rise in BP, without increases in body weight or change in renal function. An identical rise in BP in response to a HSD has been observed in normotensive Sprague–Dawley rats. The dose of sunitinib applied in the present study induced a 15-mm Hg rise in BP while rats were on an NSD. This dose of sunitinib was based on our previous work, showing that when given for 1 week, it did not impair renal excretory function as reflected by unchanged cystatin C levels, albeit it was associated with mild proteinuria. Also in the present study, renal excretory function as estimated by plasma cystatin C levels and endogenous creatinine clearance remained unchanged during the normal salt condition with sunitinib and the high salt conditions with or without sunitinib. An early study performed in anesthetized rats using radiolabeled inulin as a glomerular filtration rate marker observed a 17% increase in glomerular filtration rate in response to a 1-week HSD. Because we did not use inulin, the possibility of missing a rise in glomerular filtration rate during HSD cannot be excluded. In agreement with a previous study with the tyrosine kinase inhibitor SU5416, sunitinib administration aggravated the HSD-induced rise in BP, as reflected by a decline in the slope of the pressure–natriuresis curve. An interesting point is whether this BP increase occurs only in response to a HSD or also in response to other BP-increasing stimuli like, for instance, angiotensin II or norepinephrine. To our knowledge, the possibility of such an interaction has not been explored yet.

Several studies have shown that in response to a HSD, Na+ and Cl− in excess of water accumulate in tissues. An earlier study showed that this skin electrolyte accumulation is accompanied by the formation of lymph capillaries, driven by MPS cells that infiltrate the interstitium of the skin. This study also demonstrated that macrophage-derived VEGF-C, via activation of lymph endothelial VEGFR-3, mediates this lymphangiogenesis and that interruption of this pathway in mice and in rats leads to salt-sensitive hypertension. In the present study, performed in normotensive Wistar–Kyoto rats, we confirmed that a HSD is accompanied by increased MPS cell infiltration and lymphangiogenesis. Because sunitinib has been shown to inhibit lymphangiogenesis by blocking VEGFR-3 signaling in a breast cancer model, we anticipated an attenuation of this HSD-induced lymphangiogenesis after sunitinib administration, but only a statistically insignificant trend toward a decrease in skin lymphangiogenesis was observed.

Because lymphangiogenesis is mediated by activation of the VEGFR-3 that is targeted by sunitinib, applying sunitinib in the present study was a straightforward choice. In addition,
we have obtained extensive experience with this agent in several previous studies.\textsuperscript{12,13,15,18,19} Of note, bevacizumab, a VEGF-A antibody, has been reported to block peritumoral lymphangiogenesis not inhibited by a VEGFR-3 neutralizing antibody.\textsuperscript{20} As a consequence, salt-sensitive hypertension because of impairment of skin lymphangiogenesis might theoretically also occur with bevacizumab.

In mice on anti-VEGFR-3 or VEGFC trap treatment or in mice genetically overexpressing soluble VEGFR-3 (K14-FLT4 mice) to trap VEGF-C, a HSD has shown to cause a more pronounced increase in skin Cl\textsuperscript{−} than in skin Na\textsuperscript{+} content, leading to an increase in the skin Cl\textsuperscript{−}/Na\textsuperscript{+} ratio.\textsuperscript{9,21} This skin Cl\textsuperscript{−} accumulation, rather than skin Na\textsuperscript{+} accumulation, was most strongly related to the BP rise in response to a HSD.\textsuperscript{9} In our rats, a HSD for 3 weeks was associated with a comparable increase in skin interstitial Na\textsuperscript{+} and Cl\textsuperscript{−} contents of ≈30\% after vehicle administration (Figure S1) and 50\% during concomitant sunitinib administration. This increase was higher than the increase in skin interstitial water, which was, respectively, 17\% and 24\%, confirming the occurrence of skin electrolyte accumulation in excess of water in response to salt loading.

One may wonder why the impairment in lymphangiogenesis induced by sunitinib was relatively small in the present study. Although an insufficient dose is an obvious explanation, this is not supported by previous pharmacokinetic and in vitro studies.\textsuperscript{15,17} We have reported that the same dose of sunitinib as applied in the present study was associated with a plasma concentration of sunitinib and its active metabolite N-desethyl sunitinib of ≈350 ng/mL (875 nmol/L).\textsuperscript{15} In an in vitro study, sunitinib concentrations as low as 30 nmol/L were shown to impair lymphangiogenesis.\textsuperscript{17} Based on these data, it is unlikely that the dose of sunitinib was the limiting factor to impair lymphangiogenesis. In our study, sunitinib was given for 8 days, and although this period is sufficient to induce a robust rise in BP, it would be interesting to explore whether sunitinib, when administrated for a more prolonged period, is able to significantly reduce HS diet–induced lymphangiogenesis. Sunitinib was applied after rats had been on the HSD for 2 weeks. In previously reported studies using an antibody that blocks VEGFR-3 or alternative methods to block skin lymphangiogenesis, this intervention was applied simultaneously with or prior to the initiation of a HSD. The possibility that sunitinib would have caused a more pronounced reduction in lymph vessel hyperplasia when administrated before or simultaneously with initiation of a HSD cannot be excluded.

Collectively, our findings do not support the notion that impaired skin lymphangiogenesis is a major contributor to the rise in BP observed with sunitinib under high salt conditions. As reported earlier, administration of tyrosine kinase inhibitors is associated with activation of the circulating and renal endothelin system, as well as suppression of the NO pathway and renin suppression.\textsuperscript{12,18,19} Renal NO, among others, is known to inhibit tubular Na\textsuperscript{+} transport in the medullary...
thick ascending limb. Suppression of the renal NO pathway during VEGF signaling inhibition may, therefore, contribute to the salt-sensitive BP observed with anti-VEGF treatment. Likewise, the association between renin suppression and salt-sensitive hypertension has been well established. Obviously, when renin is suppressed, the possibility for a further decrease in renin to enhance urinary sodium excretion during high salt conditions is limited. To what extent activation of the endothelin system, which interestingly was also demonstrable in the skin in the present study, contributes to salt-sensitive hypertension is difficult to answer because of the opposing natriuretic effects mediated by the renal ETA and ETB receptors. A low dose endothelin-1 (ET-1) infusion in Sprague-Dawley rats induces hypertension only when rats are on a HSD. Likewise, when on a HSD, BP is significantly higher in transgenic mice overexpressing endothelial ET-1 than in their wild-type littermates. These studies support the contention that an activated ET-1 system may contribute to salt-sensitive hypertension. Speed et al recently reported that a HSD in mice is associated with increased auricular ET-1 protein content. Vascular endothelial cells are most likely the source of this ET-1 because this increase is absent in vascular endothelial cell ET-1 knockout mice. Interestingly, in these knockout mice, skin Na+ concentration is also elevated in response to a HSD, supporting the view that extrarenal endothelial ET-1 production induced by HSD is required for removal of excess Na+ by the skin, like the renal ET-1 system is required for removal of excess circulating Na+ and water.

**Perspectives**

This study shows that salt intake significantly modulates the BP response to sunitinib. Impairment of the skin MPS cells–TonEBP–VEGF–VEGFR3–lymphangiogenesis pathway has been recognized as a novel mechanism involved in salt-sensitive hypertension. In our study, sunitinib treatment blocked lymphangiogenesis only modestly. Therefore, other mechanisms, such as suppression of renin release, a decrease in NO production, and possibly activation of the endothelin system, more likely contribute to the aggravated hypertension under high salt conditions during sunitinib administration. We acknowledge that sunitinib was administrated for a relatively short period and only after initiation of the HS diet. Whether prolonged administration of sunitinib impairs the formation of lymph vessels in response to a HS diet more extensively than presently observed 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 ETA-selective or ETA/B endothelin receptor blockade.

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**Disclosures**

None.

**References**


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**Novelty and Significance**

**What Is New?**

- This is the first study with the tyrosine kinase inhibitor sunitinib that explores whether the salt sensitivity of vascular endothelial growth factor 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 seems not principally because of impaired skin lymphangiogenesis.

**Summary**

Mechanisms other than impaired skin lymphangiogenesis, like suppression of renin release, a diminished nitric oxide production, and activation of the endothelin system, are likely to play a greater role for the salt sensitivity of blood pressure induced by sunitinib.
Salt Sensitivity of Angiogenesis Inhibition–Induced Blood Pressure Rise: Role of Interstitial Sodium Accumulation?
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Salt Sensitivity of Angiogenesis Inhibition-Induced Blood Pressure Rise: Role of Interstitial Sodium Accumulation?

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Abbreviations of primers are provided in tekst.
Figure S1: Change (%) in skin Na⁺ content (A), Cl⁻ content (C), Na⁺+K⁺ content (E), skin water content (G), and skin Na⁺ concentration (B), Cl⁻ concentration (D) and K⁺ concentration (F) compared to a normal salt diet plus vehicle. NS, normal salt; HS, high salt, Veh, vehicle; SU, sunitinib.

*p<0.05 vs NS+SU, # p<0.05 vs NS+Veh.
Figure S2: Expression of skin Edn 1 (left panel) and Ece 1 (right panel) mRNA after normal and high salt diet with or without sunitinib. *P<0.05 versus NS+veh.
Figure S3: Skin expression of *TonEBP*, *Vegfc*, and *Pdpn* mRNA.

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

*p*<0.05 vs NS+Veh