Vascular Endothelial Growth Factor-A and Aldosterone
Relevance to Normal Pregnancy and Preeclampsia


Abstract—Aldosterone levels are markedly elevated during normal pregnancy but fall even though volume contracts when preeclampsia occurs. The level of aldosterone in either condition cannot be explained solely by the activity of the renin–angiotensin II system. In normal gestation, vascular endothelial growth factor (VEGF) is thought to maintain vascular health, but its role in adrenal hormone production is unknown. We hypothesized that the role of VEGF in the adrenal gland is to maintain vascular health and regulate aldosterone production. Here, we demonstrate that supernatant of endothelial cells grown in the presence of VEGF enhanced aldosterone synthase activity in human adrenocortical cells. VEGF either alone or combined with angiotensin II increased aldosterone production in adrenal cells. These data suggest that endothelial cell–dependent and independent activation of aldosterone is regulated by VEGF. In contrast to angiotensin II, VEGF did not upregulate the steroidogenic acute regulatory protein. Consistent with this observation, angiotensin II stimulated both aldosterone and cortisol synthesis from progesterone, whereas VEGF stimulated selectively aldosterone production. In rats, overexpression of soluble fms-like tyrosine kinase-1, an endogenous VEGF inhibitor, led to adrenocortical capillary rarefaction and fall in aldosterone concentrations that correlated inversely with soluble fms-like tyrosine kinase-1 levels. These findings may explain why aldosterone increases so markedly during normal gestation and why preeclampsia, a condition characterized by high soluble fms-like tyrosine kinase-1, is associated with inappropriately low aldosterone levels in spite of relatively lower plasma volumes. (Hypertension. 2013;61:1111-1117.)

Key Words: adrenal cell ■ aldosterone ■ aldosterone synthase ■ preeclampsia ■ soluble fms-like tyrosine kinase-1 ■ steroidogenic acute regulatory protein ■ vascular endothelial growth factor

Circulating aldosterone is primarily produced in the adrenal cortical glomerulosa zone, its secretion dependent on angiotensin II (Ang II) with contributions from high potassium levels, endothelin, and poorly understood factors released from endothelial cells (ECs).1-5 Pregnancy is characterized by high circulating aldosterone, believed to play a role in the increase in maternal plasma volume, yet remaining high in spite of reaching a steady state.6 Additional properties of high aldosterone availability might contribute to an optimal fetal development. Recently, we demonstrated some important nonvolume-related effects of aldosterone in pregnancy, such as its contribution to placental trophoblast cell proliferation.7 Renin production, a major regulator of aldosterone production, also increases during pregnancy, but the increment seems as an insufficient explanation of high circulating aldosterone levels, as the ratio of aldosterone/renin is substantially increased throughout gestation.6,8 Similarly, aldosterone increases and the rise is greater than that of Ang II, as women move on from being nonpregnant, conceive, and progress to the third trimester.9-12 Infusion of increasing Ang II doses to both pregnant and nonpregnant women similarly lead to increments in plasma aldosterone concentrations arguing against significant receptor-dependent changes in Ang II-sensitivity.13 Finally the exogenous doses of Ang II required to increase blood pressure are greater in pregnant than in nonpregnant women,14 an observation supported by the fact that Ang II subtype 1 receptors in pregnancy are monomeric and inactivated by reactive oxygen species in contrast to their heterodimeric state of Ang II–sensitive conditions.15 Thus, pregnancy-related marked increases in aldosterone concentrations are insufficiently explained by the renin–Ang II system, and another mechanism awaits identification.

In contrast to normal pregnancy, the life-threatening hypertensive complication of pregnancy, preeclampsia (PE) is...
characterized by reduced plasma volume with paradoxically low aldosterone levels explained genetically in some patients but in most, the reason remains unclear. In this respect, the markedly increased production of factors that regulate angiogenesis and their further perturbation in PE, both differing markedly from what occurs in the nonpregnant state, combined with the fact that the adrenal cortex is highly vascularized led us to evaluate whether angiogenic factors may also regulate aldosterone production that is independent of renin–Ang II system.

Materials and Methods

Materials and Antibodies
Complete information on materials and antibodies used for the experiments is provided in the online-only Data Supplement.

Conditioning of EC Supernatant
Human umbilical vein endothelial cells (HUVECs) were cultured on collagen I–coated cell ware in their corresponding medium endothe-

Isolation of Primary Human Adrenocortical Cells
Primary human adrenal cells from the cortical glomerulosal zone were isolated from morphologically normal appearing adrenal tissue, which was obtained during tumor nephrectomy that also necessitated simultaneous adrenectomy following modified earlier protocols, which are detailed in the online-only Data Supplement.

Culture of Adrenal Cells With/Without ECCM
The human adrenal cell line H295R was cultured (in parallel to ECCM) with DMEM-F12, 0.1% ITS+, and 5% NU-I for 24 hours. After 24 hours, the medium was removed, and the cells were washed once with PBS. The ECCM was collected and transferred to the H295R cells and coincubated with either solvent or specific agonists for an additional 24 hours. Serum-free HUVEC medium EBM-2 with/without VEGF or H295R medium was transferred to the adrenal cell line without EC preconditioning.

Aldosterone Synthase (CYP11B2) and 11β-Hydroxylase (CYP11B1) Enzyme Activity in H295R
The functional analyses were performed with thin layer chromatogra-

Western Blot Analysis of CYP11B2
H295R cells were stimulated for 24 hours in serum-free DMEM-F12 with PBS, Ang II, VEGF, or the combination of Ang II and VEGF. Western blot analysis was performed as detailed in the online-only Data Supplement.

Real Time–Polymerase Chain Reaction for Fms-Like Tyrosine Kinase-1 (Flt-1), and Kdr, and TaqMan PCR of Steroidogenic Acute Regulatory Protein (StAR) and CYP11B2
H295R and HUVECs were cultured in their corresponding medium. After 24-hour incubation, medium was removed, and cells were washed once with PBS. Homology-based real-time–polymerase chain reaction and quantitative TaqMan PCR was performed for Flt-1, as well as Kdr, and StAR, respectively, as detailed in the online-only Data Supplement.

Immunofluorescence of VEGF Receptors in Cell Culture
Adrenal cells were cultured on glass cover slips for 24 hours in DMEM-F12 supplemented with 5% NU-I serum to identify the expression of the VEGFR1 and Kdr by immunofluorescence as detailed in the online-only Data Supplement.

Effect of VEGF Inhibition on Serum Aldosterone Levels in a Preeclamptic Rat Model
Soluble Flt-1 (sFlt-1) overexpression in pregnant rats was used as a model of PE. Pregnant rats were maintained on a standard chow (containing 0.42% NaCl) and had free access to tap water. Adenoviruses expressing sFlt-1 or control virus at a concentration of 9×1011 viral particles/kg were injected via tail vein on day 7 to 8 of gestation. Serum aldosterone concentrations and circulating sFlt-1 levels were determined from tail vein blood samples on day 18 of gestation by commercially available enzyme-linked immunosorbent assay kits.

Statistical Analysis
The statistical analysis is outlined in the online-only Data Supplement.

Results

EC-Dependent and -Independent Activation of Aldosterone Is Controlled by VEGF Availability
HUVECs were cultured either in DMEM-F12 + 5% NU-I serum (Figure 1A, n=5; suitable for cultured adrenal cells) or in EBM-2 + 5% fetal bovine serum (suitable for HUVECs, Figure 1B, n=5) in the presence or absence of VEGF (50 ng/mL). After 24 hours of preincubation of HUVECs, the cell culture supernatant was transferred to cultured adrenal cells. The presence of VEGF during the preincubation period of HUVECs stimulated with their appropriate medium (EBM-2) led to a pronounced increase in aldosterone production in the adrenal cells, which was still further increased by the addition of Ang II (10−6 mol/L; P<0.0003, n=5). The transfer of supernatant collected from HUVECs stimulated for 24 hours with the medium of adrenal cells (DMEM-F12)
Expression of CYP11B2 Transcripts in Human Adrenal Cells

CYP11B2 transcript concentration corrected for cyclophilin A serving as endogenous control was determined in cultured H295R cells after stimulation for 24 hours with either a constant concentration of Ang II (10^{-7} mol/L) coinubated with increasing concentrations of VEGF (10–200 ng/mL) or with a constant concentration of VEGF (100 ng/mL) in combination with increasing amounts of Ang II (10^{-5} to 10^{-4} mol/L), respectively. In the Ang II (Figure 3A, n=3–6 for each condition) as well as in the VEGF fixed dose experiments (Figure 3B; n=6–8 for each condition), the CYP11B2 transcript availability was dose-dependently enhanced during coincubation with either VEGF (up to 27-fold) or Ang II (up to 50-fold), correspondingly.

Western Blot Analysis of CYP11B2 in Human Adrenal Cells

H295R cells were cultured with serum-free medium and either vehicle (PBS), Ang II (10^{-6} mol/L), VEGF (100 ng/mL), or the combination thereof for 24 hours. CYP11B2 protein expression was assessed by Western blotting, whereas COS-7 cells overexpressing CYP11B2 were used as positive control. The baseline CYP11B2 enzyme levels were strongly enhanced, irrespective of whether Ang II or VEGF were used with a further increase if Ang II and VEGF were combined (Figure 4; n=3).

Specificity of VEGF on Steroid Hormone Synthesis in Cultured H295R Cells

To judge the specificity of VEGF-dependent activation of steroid hormone synthesis on aldosterone production, the stimulatory effect of VEGF (50 ng/mL) and Ang II (10^{-7} mol/L) on aldosterone and cortisol production was compared in H295R cells. Conversion of radioactive ^{3}H-progesterone to either ^{3}H-cortisol or to ^{3}H-aldosterone was measured by thin layer chromatography. Irrespective of the duration of stimulation between 2 and 8 hours, VEGF led to a higher aldosterone/cortisol ratio, indicating a more specific action on aldosterone production than Ang II (Figure 6, n=3–6).

Differential Regulation of StAR by Ang II and VEGF Parallels the Higher Selectivity of VEGF to Stimulate Aldosterone Production in Cultured H295R Cells

StAR expression is a global upregulator of steroid hormone synthesis. StAR was clearly upregulated on the transcriptional...
level by Ang II (10⁻⁷ mol/L), yet not by VEGF in various concentrations. Furthermore, no additive effect on StAR expression was observed by combining Ang II and VEGF (Figure 7; n=3–6). These data suggest that VEGF, although augmenting the Ang II response of the cells by increasing CYP11B2 expression, requires a given activation of this rate-limiting step to efficiently produce aldosterone.

**Impact of VEGF Inhibition on Adrenal Glands in a Nonpregnant and a Pregnant Rat Model of sFlt-1 Overexpression**

Nonpregnant rats were injected via tail vein with adenoviruses expressing sFlt-1, or control virus at a concentration of 9×10¹¹ viral particles/kg. After euthanizing animals on day 12, light microscopy and immunohistochemistry were performed to assess capillary density in the zona glomerulosa of the adrenal gland. Capillary density was reduced in rats overexpressing sFlt-1 as compared with control conditions (Figure 8A; n=6). These results were confirmed by immunohistochemical staining of the ECs with the endothelial markers CD31 and Kdr. (Figure 8B, n=6). Quantification of capillary density in the adrenal tissue of rats overexpressing sFlt-1 was done by immunohistochemical staining of endothelial (CD31) and epithelial adrenal cells (Kdr) of the zona glomerulosa. Reduced capillary density was confirmed in animals overexpressing sFlt-1 as compared with the controls (Figure 8C; n=6).

In pregnant rats injected with sFlt-1 adenovirus or control virus on day 7 to 8 of gestation, serum aldosterone levels were measured on gestational day 18 and 19. In line with the results obtained in cell culture, serum aldosterone concentrations...
The renin–Ang II system is considered as a major regulator of aldosterone secretion; however, the widely branched capillary bed of the adrenal cortex already suggested an interaction between ECs and adrenal epithelial cells. In line with our findings, ECCM has been shown to stimulate adrenal cell–derived aldosterone production.4,23–25 This phenomenon was neither inhibited by Ang II nor by endothelin blockers, yet was subject to proteolysis and heat inactivation26 and could well be locally, in the ECs, produced VEGF. PE, a disease characterized by a reduced VEGF availability with endothelial damage, also presents with lower aldosterone levels than in nonpreeclamptic pregnancies.27 VEGF enables ECs to enhance aldosterone production, and a lack of VEGF compromises this vascular-epithelial cross-talk, an assumption supported by our results.

VEGF has been described as the first example of a tissuespecific angiogenic factor coordinating adrenocorticotrophic hormone (ACTH)-stimulated growth and vascularization.28,29 ACTH stimulates secretion of VEGF by adrenal epithelial cells. VEGF receptors on adrenal cells in tissue and in culture clearly identified by us and others would thus allow for binding of circulating and also of locally produced VEGF.30,31 Local VEGF concentrations are high in pregnancy, thus effects of autocrine or systemic VEGF might account for the adrenal epithelial cell responses. The VEGF homolog placenta growth factor-1, also highly expressed in pregnancy, is capable of binding to Flt-1, yet not to Kdr. Because Flt-1 is not expressed in adrenocortical cells, VEGF, but not placenta growth factor-1, is able to stimulate adrenal epithelial cells rendering this VEGF effect specific and placenta growth factor-1 independent.

In line with earlier observations, in untreated pregnant rats, serum aldosterone levels are elevated compared with the endothelial marker CD31 was accompanied by a reduction in systemic aldosterone availability.

The angiogenic signaling molecule VEGF seems to enhance aldosterone availability either indirectly via the endothelium or directly via its actions on adrenal cells. VEGF was shown to act on adrenal epithelial cells in 2 ways; one independent of, whereas the second is synergistic to Ang II. As Ang II induces the activation of StAR, VEGF led primarily to an increased expression of the aldosterone synthesizing enzyme CYP11B2. In contrast to Ang II, we were able to demonstrate a highly specific stimulatory action of VEGF on aldosterone production when compared with cortisol synthesis. In a rat model of PE, overexpression of sFlt-1, which effectively traps circulating and local VEGF with high affinity,23 reduces capillarization of the adrenal zona glomerulosa and capillary integrity as assessed by a reduced expression of the endothelial marker CD31 was accompanied by a reduction in systemic aldosterone availability.
nonpregnant controls. Gravid rats overexpressing sFlt-1 not only had suppressed aldosterone levels but also reduced adrenal capillary density. These findings are consistent with previous work by Kamba et al who used various methods to inhibit VEGF signaling and also found decreased adrenal capillary density and loss of endothelial fenestrae. The increased CYP11B2 transcript availability in the human adenocarcinoma cell line H295R in response to VEGF translated into a corresponding rise in CYP11B2 protein expression, suggesting a primary impact on transcription. Although a transcriptional effect was also present in response to Ang II, in both, the cell line H295R and primary human adrenocortical epithelial cells, VEGF resulted in a higher aldosterone production than Ang II. Furthermore, both stimulatory factors in combination demonstrated an exaggerated response, suggesting independent but additive mechanisms. This might be explained by the ability of Ang II to upregulate steroidogenic enhancer mechanisms, such as StAR. Because this response is attributable to Ang II, but not to VEGF, it would provide the molecular basis for an additive effect of the combination of both Ang II and VEGF. This assumption is supported by our finding that VEGF does specifically enhance aldosterone production in contrast to Ang II, which also favors a slight increase in cortisol synthesis as well, probably via its effect on StAR.

In summary, we propose a novel regulatory pathway of aldosterone production by VEGF, which acts 2-fold: First, via maintaining endothelial integrity using endothelial humoral signals to promote adrenal aldosterone production and second, via a direct stimulatory effect on adrenocortical cells. Given this finding, the unexpected observation of low aldosterone availability in PE, despite a reduction in plasma volume, can be explained by the relative paucity of VEGF availability in this condition. sFlt-1 inhibits VEGF from binding to its cell surface receptors on adrenocortical cells and by this mechanism abrogates the VEGF-induced increase in aldosterone synthesis and plasma volume expansion observed in normal pregnancy. This effect might be even more pronounced in individuals with an already compromised aldosterone synthesis attributable to loss-of-function variants of the enzyme CYP11B2.

**Perspectives**

Our data suggest that VEGF causes renin-independent aldosterone stimulation, which explains the dissociation between aldosterone and renin with progressing pregnancy. Our observations further imply that VEGF contributes to the adaptation of plasma volume by an aldosterone-dependent mechanism, which is teleologically meaningful given the increased vascular space in pregnancy. These findings explain widely why aldosterone is present to such a huge extent in pregnancy and why aldosterone production is compromised in VEGF-deficient states, such as PE, thus contributing to a substantial plasma volume deficiency in this disease. Conversely, these findings support the concept of aldosterone providing the plasma volume in response to VEGF in highly angiogenic states, such as pregnancy to prepare for an appropriate filling of the vascular tree.

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**Disclosures**

Dr Karumanchi is a coinventor of multiple patents related to angiogenic proteins for the diagnosis and therapy of preeclampsia. These patents have been licensed to multiple companies. Dr Karumanchi reports having served as a consultant to Roche and Beckman Coulter and has financial interest in Agamin LLC.

**References**


Novelty and Significance

**What is New?**

- Vascular endothelial growth factor as a novel regulator of aldosterone synthesis.
- Antiangiogenic signals, such as soluble Flt-1, compromise adrenal aldosterone synthesis in vitro and in vivo in an animal model of preeclampsia.

**What is Relevant?**

- Vascular endothelial growth factor significantly stimulates aldosterone synthesis indirectly via endothelial cells and directly via adrenal cells.

**Summary**

A high vascular endothelial growth factor availability can enhance angiotensin II–induced, but also angiotensin II–independent, adrenal aldosterone production, a finding relevant to normal and hypertensive disease in pregnancy.
Vascular Endothelial Growth Factor-A and Aldosterone: Relevance to Normal Pregnancy and Preeclampsia


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VEGF-A and Aldosterone: Relevance to Normal Pregnancy and Preeclampsia

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**Materials**

Collagen I coated cell culture plates used for primary HUVEC and primary human adrenal cells were obtained from Becton Dickinson (Basel, Switzerland) and, those for the immortalized adrenocortical cell line H295R from Techno Plastic Products AG (Trasadingen, Switzerland). Cell culture media DMEM-F12 for H295R and DMEM high glucose for primary human adrenal cells were obtained from Gibco/Invitrogen (Basel, Switzerland) and the cell culture medium for HUVECs from Lonza (Basel, Switzerland), respectively. Cell culture media supplements and NU-I serum were obtained from Becton Dickinson (Basel, Switzerland) while FBS, VEGF, Ang_II, cAMP, cortisol, TNF-α, and progesterone were provided by Sigma (Buchs, Switzerland). ³H-DOC (specific activity 40-60Ci/mmol) and ³H-progesterone (specific activity 94Ci/mmol) were obtained from GE Healthcare (Glattbrugg, Switzerland) and TLC plates were purchased from Macherey-Nagel (Düren, Germany). The scintillation fluid, Irgasafe, was obtained from Zinsser Analytic (Frankfurt, Germany), the SV total RNA isolation kit was from Promega (Dübendorf, Switzerland) and Trizol from Invitrogen (Zug, Switzerland). The Pierce BCA Protein Assay Kit was from Socochim (Lausanne, Switzerland). All reagents for the PAGE and Western Blot were purchased from Bio Rad (Reinach, Switzerland) or Sigma (Buchs, Switzerland). Hybond-C nitrocellulose membrane and ECL detection solution was from Amersham Biosciences (GE Healthcare AG, Glattbrugg, Switzerland). Assay on demand primers and probes for CYP11B2 (Hs01597732_m1), cyclophilin A and GAPDH as endogenous controls were provided by Applied Biosystems (Rotkreuz, Switzerland). Specific primers for human Flt-1 (forward 5’-AGGGGAAGAAATCCTCCAGA-3’; reverse 3’-GCTGAACTTTCCACAGAGCC-5’), and human Kdr (forward 5’-CCCACGTTTCTCACAATGCT-3’; reverse 3’-CTACCGGTTTGGGACTCCAAT-5’) were obtained from Microsynth (Balgach, Switzerland). The human adrenocortical carcinoma cell line NCI-H295R (H295R) was from ATCC (Manassas, VA, USA). HUVECs were obtained from Lonza (Basel, Switzerland) and compared to fresh isolations provided by the Institute of Pathology of the University of Bern (Berne, Switzerland). Aldosterone in cell culture supernatants was measured with a commercial ELISA kit from Hözel Diagnostika (Cologne, Germany), whereas rat serum levels were obtained with the ELISA kit from Cayman Chemicals (Ann Arbor, USA). sFlt-1 levels were determined using the ELISA kit from R&D Systems (Abingdon, UK).

For the immunofluorescence studies, the following antibodies were used: Flt-1 from Abcam (Cambridge, UK), and Kdr from Santa Cruz (Heidelberg, Germany). For immunohistochemical studies, the following antibodies were used: CD31 (antibody against clone 1A10, R&D systems, Minneapolis, USA) and Kdr (sc-74002, Santa Cruz, USA). The human aldosterone synthase (CYP11B2) antibody used for Western Blot analysis was from Aviva Systems (San Diego, CA, USA). The anti-rabbit horse radish peroxidase-labeled secondary antibody was purchased from Santa Cruz (Heidelberg, Germany).

**Methods**

**Isolation of primary human adrenocortical cells**

Primary human adrenal cells from the cortical glomerulosal zone were isolated from morphologically normal appearing adrenal tissue obtained during tumor nephrectomy that also necessitated simultaneous adrenalectomy. This was performed by surgeons of the Division of Urology of the University of Bern (Berne,
Switzerland) following modified earlier protocols. The adrenal gland was washed with 1X PBS containing an antibiotic-antimycotic mixture and the capsule was removed. The subcapsular cell layer was employed for the isolation of the primary zona glomerulosa cells. Cells were seeded into collagen I coated cell ware containing DMEM high glucose + 10% FBS. Medium was renewed every other day. A positive staining for CYP11B2 proofed the purity of the culture. The cultures used for experiments showed a purity of 80-90%.

**Western Blot analysis of CYP11B2**

Protein lysates were stored in sucrose buffer (250mM Sucrose + 10mM Tris Base pH 7.5 + phenylmethanesulfonyl fluoride (100mM)). The protein amount was measured by the Pierce BCA Protein Assay Kit. A 10% sodium dodecyl sulfate-polyacrylamide gel was used to separate 50µg sample. A rabbit polyclonal anti-CYP11B2 antibody (1:1,000 in TBS containing 0.01% Tween (TBS-T) overnight at 4°C) was followed by a horseradish peroxidase conjugated anti-rabbit secondary antibody (1:10,000 in TBS-T, 1h at room temperature). Signals on Western blots were quantified by densitometry using Image J software.

**CYP11B2 and 11β-hydroxylase enzyme activity in H295R**

The functional analyses were performed with TLC following modified earlier own protocols. For CYP11B2 activity following 24h of incubation with the specified treatment in cell culture, the medium was changed, conditions replaced and 0.5µCi/mL ³H-DOC was added for another 24h. The supernatant was transferred into silanized glass vials and the hormones were extracted by vortexing with 5mL ethyl acetate for 1min and evaporated in streaming nitrogen at 37°C. Dried hormones were redissolved in 30µL unlabeled DOC/aldosterone mixture (10mg/mL) and 10µL of each vial were loaded on TLC plates with separation performed by dichloromethane:methanol:H₂O (150:10:1). The radioactive metabolites were located by detecting the cold location mixture under UV-light, and cut into scintillation vials containing scintillation fluid. Radioactive ³H-DOC and -aldosterone were analyzed in a β-counter (TRI-CARB Liquid scintillation analyzer 2000CA, Rüsselsheim, Germany). Apparent enzyme activity was calculated by the ratio of aldosterone/DOC. Assays were performed in triplicate.

A progesterone conversion assay was applied to account for the specificity of VEGF on CYP11B2 as compared with CYP11B1 activity following modified earlier protocols. In short, to H295R 0.5 µCi/mL ³H-progesterone was added for 2h, 4h, and 8h as described above for ³H-DOC in the presence and absence of Ang_II (10⁻⁷M) and VEGF (50ng/mL), respectively. ³H-aldosterone, -cortisol, and –progesterone were detected. The apparent enzyme activities of CYP11B2 and CYP11B1 are represented by the ratio of aldosterone to cortisol with high ratios indicating a predominating CYP11B2, low ratios an overly CYP11B1 activity.

**Western Blot analysis of CYP11B2**

H295R cells were stimulated for 24h in serum-free DMEM-F12 medium with either PBS, Ang II, VEGF or the combination of Ang II and VEGF Thereafter, cells were washed once with PBS and lysed. Protein lysates were stored in sucrose buffer (250mM Sucrose + 10mM Tris Base pH 7.5 + phenylmethanesulfonyl fluoride (100mM)). The protein amount was measured by the Pierce BCA Protein Assay Kit. A 10% sodium dodecyl sulfate-polyacrylamide gel was used to separate 50µg sample which was then transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk for 1h at room temperature and then probed
with rabbit polyclonal anti-CYP11B2 antibody (1:1,000 in TBS containing 0.01% Tween (TBS-T) overnight at 4°C). The membranes were washed with TBS-T and incubated with a horseradish peroxidase conjugated anti-rabbit antibody (1:10,000 in TBS-T, 1h at room temperature). Proteins were detected by enhanced chemiluminescence using an AGFA Curix 60 Image Station (Winterthur, Switzerland). Prestained standards were used as molecular weight markers. Signals on Western blots were quantified by densitometry using Image J software.

RT-PCR for Flt-1, and Kdr, and TaqMan PCR of steroidogenic acute regulatory protein (StAR) and CYP11B2 RNA was extracted using the SV total RNA isolation kit or by the Trizol method. Using 2µg of RNA, reverse transcription was done with the Super Script III RT-PCR system (Invitrogen, USA). PCR was performed with the specific primers. The PCR product was visualized on a 1% agarose gel.

Homology-based quantitative TaqMan PCR was performed in H295R with assay-on-demand primers and probes for StAR and CYP11B2 to identify and quantify the respective transcripts of StAR and CYP11B2. Negative controls for the RT and the PCR reagents were assessed. The cyclophilin A and the GAPDH signal served as endogenous controls. The assay was performed in triplicates.

Immunofluorescence of VEGF receptors in cell culture Following culture, adrenal cells were washed twice at room temperature with PBS and fixed in 4% formaldehyde for 15min. This was followed by repeated washes with 1X PBS (Inselspital Hospital Pharmacy, Berne, Switzerland) before and after addition of NH₄Cl (50mM) for 15min. Cell membranes were permeabilized with Triton X-100 (0.1%) for 5min and washed with 1X PBS before blocking in 10% FBS/PBS for 30min. Incubation with primary antibodies against Kdr (dilution 1:50 in 1% FBS/PBS) and VEGFR1 (dilution 1:250 in 1% FBS/PBS) was performed for 1h at room temperature. After washing with PBS, cells were incubated with the secondary antibodies (anti-mouse 1:200 in 1% FBS/PBS for Kdr and anti-rabbit 1:1000 in 1% FBS/PBS for VEGFR1) in the dark for 30min. Following repeated washes with 1X PBS, cover slips were analyzed with an immunofluorescence microscope (Nikon E600, Grenchen, Switzerland).

Light microscopy and immunohistochemistry of adrenal glands of non-pregnant rats overexpressing sFlt-1 After sacrificing the rats, adrenal glands were longitudinally bisected through the medulla. One-half adrenals were fixed in 10% buffered formalin for 72h at 4°C. Paraffin embedding, sectioning and hematoxylin & eosin staining were performed thereafter. The other half of one adrenal was prepared for frozen sectioning for immunohistochemistry and stored at -70°C. To identify the capillary space, the specific immunohistochemical marker CD31 was used with co-localization of CD31 and Kdr representing capillary endothelium.

Statistical analysis All data are presented as mean±SD. To determine statistically significant differences between more than two groups, one-way ANOVA was used, followed by Dunnett’s post-hoc tests for multiple comparisons. Unpaired t-test was used to analyze the difference observed between two groups. Linear regression analysis was used to correlate serum sFlt-1 levels to plasma volume and serum aldosterone
Significance was assigned at p<0.05. All statistical analyses were performed using SYSTAT version 12 (SPSS, Inc., Chicago, IL).

Correlation of aldosterone to sFlt-1 serum levels in a pregnant rat model measured by ELISA. Serum aldosterone levels, measured at day 18 of gestation in pregnant SD rats, negatively correlated to the respective serum sFlt-1 levels at day 18 of gestation upon linear regression analysis. The x-axis is a log scale. $r^2=0.1367$, $p=0.0406$, $n=29$. 