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Placental expression of the angiogenic placental growth factor is stimulated by both aldosterone and simulated starvation

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Aldosterone is an important factor supporting placental growth and fetal development. Recently, expression of placental growth factor (PlGF) has been observed in response to aldosterone exposure in different models of atherosclerosis. Thus, we hypothesized that aldosterone up-regulates growth-adaptive angiogenesis in pregnancy, via increased placental PlGF expression.

We followed normotensive pregnant women ($n = 24$) throughout pregnancy and confirmed these results in a second independent first trimester cohort ($n = 36$). Urinary tetrahydroaldosterone was measured by gas chromatography-mass spectrometry and corrected for creatinine. Circulating PlGF concentrations were determined by ELISA. Additionally, cultured cell lines, adrenocortical H295R and choriocarcinoma BeWo cells, as well as primary human third trimester trophoblasts were tested *in vitro*. PlGF serum concentrations positively correlated with urinary tetrahydroaldosterone corrected for creatinine in these two independent cohorts. This observation was not due to PlGF, which did not induce aldosterone production in cultured H295R cells. On the other hand, PlGF expression was specifically enhanced by aldosterone in the presence of forskolin ($p < 0.01$) in trophoblasts. A pronounced stimulation of PlGF expression was observed with reduced glucose concentrations simulating starvation ($p < 0.001$).

In conclusion, aldosterone stimulates placental PlGF production, enhancing its availability during human pregnancy, a response amplified by reduced glucose supply. Given the crucial role of PlGF in maintaining a healthy pregnancy, these data support a key role of aldosterone for a healthy pregnancy outcome.

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1. Introduction

Evidence accumulates for almost every physiological system to be closely embedded and regulated by environmental conditions and factors. In pregnancy, similar to the non-pregnant state, the renin-angiotensin II-aldosterone system is a mechanism closely related to salt and water availability [1]. As such, numerous effects beneficial to pregnancy have already been attributed to aldosterone. These include, but are not limited to maternal plasma volume expansion, improved fetal conditions and size, placental growth and lower maternal blood pressure [2–8]. We recently described that vascular endothelial growth factor (VEGF) alone and in combination with angiotensin II, directs augmented aldosterone production in pregnancy [9], suggesting a physiological survival benefit.

These advantageous characteristics of aldosterone are in marked contrast to its deleterious effects in the non-pregnant state. Organ

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fibrosis and atherosclerosis are promoted by excess aldosterone [10]. Upon exposure of vessels to aldosterone in models of atherosclerosis, enhanced placental growth factor (PlGF) expression has been observed, mediated by a mineralocorticoid responsive element in the promoter region of *plgf* [10,11]. In non-pregnant systemic vasculature, aldosterone-dependent PlGF expression leads to vascular injury, atherosclerosis, plaque formation and its inflammatory response [10,11]. PlGF is considered to be crucial in pregnancy to initiate and perpetuate placental angiogenesis (reviewed in Ref. [12]). As low levels compromise placental development, it also serves as early marker of pregnancies complicated by pre-eclampsia, a disease of placental origin [13,14]. The role of environmental conditions on PlGF expression in pregnancy is less clear. Upregulation of angiogenic factors, such as PlGF, in trophoblast seems not to be supported by hypoxia and hypoxia-induced factor-1α [15]. As such, other regulatory pathways must be considered such as glucose availability, which might play a role in this process. While maternal serum levels of PlGF are high in diabetic pregnancies, similar to certain vascular beds such as in the retina, experimental evidence in diabetic rats suggests low PlGF levels in the placenta; thereby a differential regulation between systemic and placental PlGF in response to altered glucose availability [16–18].

Given the high systemic availability of aldosterone during pregnancy [7,19], we hypothesized that aldosterone up-regulates growth-adaptive angiogenesis via placental PlGF expression. More specifically, we first aimed to identify trophoblast-derived aldosterone-sensitive PlGF expression; second to explore conditions most likely related to increased responsiveness such as starvation; and third, to investigate the relationship between aldosterone and PlGF in human pregnancy.

2. Methods

2.1. Patients

A set of healthy pregnant women selected from the Bernese pregnancy registry at the Department of Obstetrics and Gynecology, University Hospital of Bern, with a complete scheduled sampling of serum and urine were included in the study. Clinical data were prospectively collected including obstetric parameters, ultrasound data, standardized measurement of office blood pressure and pregnancy outcome. Only normotensive, healthy pregnant women were included in the analysis. Visits were at gestation week 11 ± 2, 20 ± 2, 28 ± 2 and at birth. The study was approved by the ethics committee of the Canton of Bern and adheres to the principles of the Declaration of Helsinki. Study subjects were only included in the study after obtaining written informed consent.

A confirmatory patient set ($n = 36$) was derived from a prospective pregnancy cohort (number initially screened $n = 3918$) at Maternity Units in Glasgow, UK. First trimester samples were taken at booking and those investigated who maintained normotensive throughout their pregnancy. The study was also approved by the West of Scotland Research ethics Committee and adheres to the principles of the Declaration of Helsinki. Study subjects were only included in the study after signing informed consent.

2.2. Material and cell lines

Collagen I-coated cell culture plates used for cell experiments were from Becton Dickinson (Basel, Switzerland). Cell culture media, L-glutamine, penicillin/streptomycin and HEPES were from Life Technologies, Inc./Invitrogen (Basel, Switzerland). Aldosterone was from Steraloids, Inc. (Brunschwig, Basel, Switzerland), forskolin, spironolactone, PlGF and fetal bovine serum (FBS) from Sigma (Buchs SG, Switzerland). Penicillin, streptomycin, and amphotericin B used for primary cell cultures were obtained from Invitrogen (Basel, Switzerland).

The human choriocarcinoma cell line BeWo and the human adrenocortical carcinoma cell line NCl-H295R (H295R) were obtained from American Type Culture Collection (Manassas, VA). Human primary term trophoblasts were isolated from term placentas (38–40 w) of healthy donors after obtaining informed consent from pregnant women at the Department of Obstetrics and Gynecology, University Hospital Bern, Switzerland. The isolation procedure was previously described [20]. Primary cytotrophoblasts were resuspended in DMEM:F12K (1:1) containing 10% FBS, penicillin, streptomycin, and amphotericin B and plated on collagen I-coated culture dishes. Cells were cultured at 37 °C and 5% CO_2.

H295R were cultured in DMEM-F12 containing 0.1% ITS+ and 5% NU-I. For the experiments, H295R cells were incubated for 24 h in serum-free DMEM-F12 containing angiotensin II, PlGF or the combination of both. RNA was then extracted.

Human primary term trophoblasts and BeWo cells were cultured for 24 h in DMEM:F12K (1:1) medium with 10% FBS, then cells were washed with PBS and fresh medium containing either 10% (for primary trophoblasts) or 0.1% (for BeWo) FBS supplemented with aldosterone, forskolin or spironolactone for 6 or 24 h. Following this incubation time, RNA was extracted from the cells and supernatant was taken for PlGF protein measurements.

2.3. Real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Basel, Switzerland). The RNA was then reverse transcribed (Prom II RT, Promega). For the real-time PCR, primers and probes for PlGF were obtained from Applied Biosystems (PlGF, Hs00182176_m1; CYP11B2, Hs01597732_m1; Applied Biosystems, Foster City, CA). GAPDH and cyclophilin A primers and probes (Applied Biosystems) were used as independent reference genes. Real-time PCR reactions were performed using the Universal ProbeLibrary Assay probes and Taqman Fast Universal PCR Master Mix (Invitrogen, Basel, Switzerland). All data were normalised to cyclopholin A or GAPDH and presented as fold-change compared to the mean of the controls.

2.4. PlGF ELISA

BeWo and human primary term trophoblasts conditioned media were taken for PlGF protein measurement using a commercially available quantitative sandwich enzyme-linked immunosorbent assays (ELISA, R&D Systems Europe Ltd., Abingdon, UK).

2.5. Collection of urine and serum/plasma

Morning urine samples were obtained from pregnant women in the longitudinal Bernese study and in the first trimester from the Glasgow cohort as has been described for population-based assessments [21]. In all urine collections, creatinine concentrations were determined using routine methods. Serum and urine aliquots were stored at −80 °C until further analysis.

2.6. Gas chromatography-mass spectrometry (GC-MS)

Urinary tetrahydro-aldosterone (TH-aldosterone) was analyzed by GC-MS according to the method originally described by Shackleton and applied by us as reported earlier [7,22]. Briefly, urine preparation consisted of pre-extraction, enzymatic hydrolysis, extraction from the hydrolysis mixture, derivatization, and gel filtration. Medroxyprogesterone (2.5 µg) was added as recovery

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