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Aldosterone deficiency adversely affects pregnancy outcome in mice

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ALDOSTERONE DEFICIENCY ADVERSELY AFFECTS PREGNANCY OUTCOME IN MICE

Abhijeet Todkar^{1,2,3}, Marianna Di Chiara^{1,3}, Dominique Loffing-Cueni^{1,3}, Carla Bettoni^{2,3}, Markus Mohaupt⁴, Johannes Loffing^{1,3,#}, Carsten A. Wagner^{2,3,#}

¹Institute of Anatomy, University of Zurich, Switzerland, ²Institute of Physiology, University of Zurich, Switzerland, ³Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Switzerland, ⁴Department of Nephrology/Hypertension, University Hospital Bern, Berne, Switzerland

J. Loffing and C.A. Wagner contributed equally to this work and share last authorship

Running title: Aldosterone affects feto-placental phenotype

Corresponding author
Carsten A. Wagner
Institute of Physiology
University of Zurich
Winterthurerstrasse 190
CH-8057 Zurich
Switzerland
Phone: +41-44-63 55023
Fax: +41-44-63 56814
Email: Wagnerca@access.uzh.ch

ABSTRACT

Circulating aldosterone levels are increased in human pregnancy. Inadequately low aldosterone levels as present in preeclampsia, a life-threatening disease for both mother and child, are discussed to be involved in its pathogenesis or severity. Moreover, inactivating polymorphisms in the aldosterone synthase gene have been detected in preeclamptic women. Here, we used aldosterone synthase deficient ($AS^{-/-}$) mice to test whether the absence of aldosterone is sufficient to impair pregnancy or even to cause preeclampsia. $AS^{-/-}$ and $AS^{+/+}$ females were mated with $AS^{+/+}$ and $AS^{-/-}$ males, respectively, always generating $AS^{+/-}$ offspring. With maternal aldosterone deficiency in $AS^{-/-}$ mice, systolic blood pressure was low before and further reduced during pregnancy with no increase in proteinuria. Yet, $AS^{-/-}$ had smaller litters due to loss of fetuses as indicated by a high number of necrotic placentas with massive lymphocyte infiltrations at gestational day 18. Surviving fetuses and their placentas from $AS^{-/-}$ females were smaller. High salt diet before and during pregnancy increased systolic blood pressure only before pregnancy in both genotypes and abolished the difference in blood pressure during late pregnancy. Litter size from $AS^{-/-}$ was slightly improved and the differences in placental and fetal weights between $AS^{+/+}$ and $AS^{-/-}$ mothers disappeared. Overall an increased placental efficiency was observed in both groups paralleled by a normalization of elevated HIF1 α levels in the $AS^{-/-}$ placentas. Our results demonstrate that aldosterone deficiency has profound adverse effects on placental function. High dietary salt intake improved placental function. In this animal model, aldosterone deficiency did not cause preeclampsia.

Key words: aldosterone, placenta, fetal growth, systolic blood pressure, high salt diet

INTRODUCTION

Pregnancy is characterized by a profound volume expansion due to high aldosterone levels meant to support fetal well-being [5]. Preeclampsia is a clinical syndrome in pregnant women defined as the new onset of hypertension and proteinuria after 20 weeks of gestation. It affects 3-5% of all pregnancies and is the leading cause of maternal and fetal morbidity and mortality [40]. The etiology of preeclampsia is multifactorial.

Insufficient placental implantation oxidative stress, genetic predisposition, natural killer cell dysfunction or preformed agonistic angiotensin II receptor subtype 1 autoantibodies may contribute to the development of preeclampsia [6, 29, 40]. Histologically, preeclampsia is characterized by a defect in the invasion of the endovascular trophoblast that may eventually cause reduced blood supply to the feto-placental unit [25, 28].

Placental ischemia may trigger the release of vasoactive, angiogenic and antiangiogenic factors such as vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) as well as an enhanced placental expression of the angiogenic receptors fms like tyrosine kinase 1 (Flt-1) and endoglin [22]. Likewise, their soluble forms sFlt-1 and sEng are released into systemic circulation leading to endothelial dysfunction and increased blood pressure.

Normal pregnancy is characterized by a marked expansion of plasma and extracellular volume associated with changes in renal hemodynamics as well as in the circulating level of adrenal steroid hormones [17, 27], which likely help adapting the maternal circulation to the increasing blood and substrate requirements of the growing placenta and fetus. Consistently, plasma aldosterone concentrations

increase during normal pregnancy coinciding with plasma volume expansion [33, 38]. In preeclampsia, a reduced plasma volume precedes the onset of and is consistently reported to be reduced during the disease associated with a shift of fluid from intravascular to interstitial space and edema [9, 12, 14, 38] while plasma aldosterone concentration and renin activity are suppressed [37]. This observation suggested preeclampsia to be caused or aggravated by an inadequate balance of extracellular volume and the activity of the renin-angiotensin-aldosterone system. As a substantial group of preeclamptic women also show reduced aldosterone synthase enzyme (*CYP11B2* gene) activity [35], aldosterone deficiency was implicated to play a role in the pathogenesis of preeclampsia. Aldosterone deficiency may lower extracellular volume and increase thereby the risk of placental hypoperfusion [35] thus indirectly causing the release of vasoactive factors leading to preeclampsia. The possible role of aldosterone was further evidenced by the finding that loss- and gain-of function due to distinct gene variants of *CYP11B2* may predispose for or protect from preeclampsia, respectively. In fact, the V386A variant of *CYP11B2* gene, which causes a deficiency in the rate-limiting step of aldosterone synthesis, the 18-hydroxycorticosterone methyl oxidase activity [30], was observed solely in a subgroup of preeclamptic women, but never in normal pregnant women [35]. In contrast, the gain of function variants of the *CYP11B2* gene in the SF-1 and Int2 (C) sites, which have been shown to be associated with hypertension in non-pregnant subjects [26], apparently reduce the risk of developing preeclampsia [8]. Recently, observations by Gennari-Moser et al. [16] suggested a role of aldosterone in fetal perfusion and also in placental growth. Taken together, these studies propose a role of aldosterone availability for placental size, fetal perfusion and also the development of preeclampsia. Whether these effects of aldosterone are mediated directly via the

mineralocorticoid receptor in placental tissue or more indirectly via the regulation of NaCl homeostasis and blood pressure regulation has not been fully clarified.

Aldosterone is closely linked to salt uptake. Of interest, an old clinical study forwards the observation of a reduced incidence of preeclampsia and fetal death on a high salt diet [32]. In addition, in a hypertensive woman homozygous for the V386A *CYP11B2* mutation and low aldosterone availability salt supplementation lowered SBP throughout pregnancy [10]. However, it is not clear whether aldosterone deficiency alone can lead to preeclampsia and whether aldosterone deficiency can be counterbalanced by a high salt diet.

Thus, we examined whether aldosterone deficiency would compromise pregnancy and even cause preeclampsia-like symptoms in mice. Using *CYP11B2*-deficient mice (*AS*^{-/-}) [21], we found that the absence of maternal aldosterone did not cause proteinuria or arterial hypertension, two major symptoms of preeclampsia, but reduced litter size due to loss of fetuses and severe placenta pathologies during the period of pregnancy. Feeding a high salt diet during pregnancy partially rescued this defect suggesting that reduced placental perfusion may play an important role.

METHODS

Animals

All experiments were performed with inbred 129 SvEv genetic background [24] aldosterone synthase wild type ($AS^{+/+}$) and aldosterone synthase-deficient ($AS^{-/-}$) female mice. This mouse model was generously provided by Prof. Oliver Smithies, University of North Carolina, Chapel Hill, NC, USA [21]. Unless indicated otherwise, animals were fed standard rodent chow (3430, KLIBA NAFAG, Kaiseraugst, Switzerland) with free access to normal tap water. All experiments were performed according to Swiss Animal Welfare laws.

Study design and treatment

Age matched female $AS^{+/+}$ and $AS^{-/-}$ mice, between 3.5 to 6.5 months old, were used for experiments. Animals were divided into two groups, a control group and an experimental group. In the control group, $AS^{-/-}$ male were mated with $AS^{+/+}$ female mice, whereas in the experimental group, $AS^{+/+}$ male mice were mated with $AS^{-/-}$ females to generate only heterozygous pups to have the same genotype for all pups. Both groups were fed with either the standard rodent chow powder, (3433 KLIBA NAFAG, Kaiseraugst, Switzerland) containing 0.20 % Na^+ and 0.36 % Cl^- (control diet) ($AS^{+/+}$ n = 9 and $AS^{-/-}$ mice n = 9) or with standard diet supplemented with NaCl (high salt diet, 5 % NaCl) ($AS^{+/+}$ n = 6-8 and $AS^{-/-}$ mice n = 8-10). To feed the mice with high salt diet, 100 g of standard rodent chow powder was mixed with 5 g NaCl and then mixed with an equal volume of deionised water to moisten the food for feeding. Male and female mice were kept together in individual cages for breeding and female mice were controlled daily in the early morning for the presence of copulation plugs and the following day was considered as gestational day 1.

Systolic blood pressure (SBP) was measured in female mice using the tail cuff method (see below). In another set of experiments mice were fed either the control diet ($AS^{+/+}$ n = 8, $AS^{-/-}$ n = 15) or high salt diet (5 % NaCl) ($AS^{+/+}$ n = 6, $AS^{-/-}$ n = 9) and sacrificed on day 18 of pregnancy by cervical dislocation. The number and weight of pups and placentas were measured, and tissues collected for further analysis. Placentas were collected and rapidly frozen in liquid nitrogen and stored at -80°C for RNA extraction. Some placentas were fixed in 4% paraformaldehyde for histology.

Tail cuff SBP measurements

Systolic BP (SBP) was measured by a non-invasive computerized tail cuff method (BP2000, Visitech Systems, Apex, NC, USA) as described previously [20]. Mice were adapted for 5 days before the start of the study. This period of adaption has been characterized previously for this specific tail cuff method and found to reduce stress induced side effects [20]. Blood pressure was measured at the same time everyday from 1 pm to 5 pm. Averages of SBP measurements having standard deviations less than 10 mmHg were considered for further analysis. Systolic blood pressure from female mice on control diet was measured on control conditions 2 to 3 days before pregnancy and from day 1 of pregnancy until the end of pregnancy up to 21 days. Mean SBP was calculated from each day's average SBP measurements. SBP for high salt diet experiments was measured on control diet, then mice were given high salt and SBP was measured again after 4 days of high salt diet. Thereafter, female mice were kept together with male mice for mating and SBP was measured at gestational days 5 to 7 and days 15 to 17. Mean SBP was calculated from 3 days of average SBP measurements.

Urinary protein concentration measurement:

Spot urine was collected from female mice during pregnancy everyday by gentle massage of the urinary bladder. Total urinary protein concentration was measured with a standard protein assay kit (Biorad D_c Protein Assay, Biorad, Hercules, CA, USA). Urinary creatinine concentration was measured by the Jaffe method [34].

RNA extraction and Semi-quantitative Real-time PCR

Semi-quantitative real time (qRT-PCR) was performed to assess the expression of vascular endothelial growth factor (VEGF), soluble fms-like tyrosine kinase-1 (sFlt-1), the hypoxia inducible factor 1 α (HIF1 α), Tumor Necrosis Factor 1 α (TNF1 α), and the monocyte chemo-attractant protein 1 (MCP-1). Snap frozen placentas (5 placentas per group) were homogenized in RLT buffer (Qiagen, Hilden, Germany) supplemented with 2-mercaptoethanol to a final concentration of 1 %. Total RNA was extracted from 200 μ l aliquots of each homogenized sample using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quality and concentration of the isolated RNA preparations were measured on a ND-1000 spectrophotometer (Thermo Fischer Scientific Inc., Wilmington, DE, USA). Total RNA samples were stored at -80°C until further use. Each RNA sample was diluted to 100 ng/ μ l and 3 μ l was used as a template for reverse transcription using the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Semi-Quantitative real time (qRT-PCR) was performed on the ABI PRISM 7500 Sequence Detection System (Applied Biosystem, Foster City, CA, USA). Primers for vascular endothelial growth factor (VEGF), soluble fms-like tyrosine kinase-1 (sFlt-1), and the

hypoxia inducible factor 1 α (HIF1 α) were designed using Primer 3 online software, whereas primers for hypoxanthine guanine phosphoribosyl transferase (HPRT) and GAPDH as housekeeping genes were designed using Primer Express software from Applied Biosystem. Sequences of primers are as follows: GAPDH forward 5'-GTCGTGGATCTGACGTGCC-3' and reverse 5'-GATGCCTG CTTCACCACCTT-3', VEGF forward 5'-CAGGCTGCTGTAACGATGAA-3' and reverse 5'-GCA TTCACATCTGCTGTGCT-3', sFlt forward 5'-GGGAAGACATCCTTCGGAAGA-3' and reverse 5'-TGTGGTACAATCATTCTCCTG-3', HIF1 α forward 5'-ATCTCGGCGAAGCAAAGAG and reverse 5'-CTGTCTAGACCACCGGCATC-3', HPRT forward 5'-TTATCAGACTGAAGAGCTACTGTAAGATC-3 and reverse 5'-TTACCAGTGTCAATTATATCTTCAACAATC-3'. The primers and probes used from Applied Biosystem are MCP-1 (Mm00441242) and TNF α (Mm99999068). Specificity of the primers was first tested in a standard PCR and always resulted in a single product of the expected size on 2% agarose gel. Real time PCR reactions were performed using the iQ SYBR Green Supermix (Biorad, Hercules, CA, USA). ROX Passive reference Dye (Biorad, Hercules, CA, USA) 3.3 μ l/ 1.25 ml of iQ SYBR Green Supermix was added. Briefly, 3 μ l cDNA, 0.8 μ l of each primer (10 μ M), 5.4 μ l RNase free water, 10 μ l iQ SYBR Green Supermix were mixed to 20 μ l final reaction volume. Reaction conditions were: denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 60°C for 60 s followed by dissociation stage (95°C for 15 s, 60°C for 15 s followed by a slow ramp to 95°C). All reactions were run in duplicate. The expression of gene of interest was calculated in relation to HPRT. Relative expression ratios were calculated as $R = 2^{[Ct(HPRT/-actin)-Ct(test\ gene)]}$.

Histology

Placentas were fixed by immersion in 4% paraformaldehyde (Applichem, Darmstadt, Germany). After tissue processing and embedding in paraffin, 5 µm sections were prepared. Paraffin sections were stained with hematoxylin-eosine (H&E) and scanned with a Mirax Midi Slide Scanner microscope (Zeiss, Jena, Germany).

Immunoblotting

Placentas, stored at -80°C, were homogenised in ice cold homogenisation buffer (0.27 M sucrose, 2 mM EDTA, 0.5 % NP40 prepared in 1x buffer A supplemented with protease and phosphatase inhibitor cocktails (Roche, Mannheim, Germany) (buffer A: 0.6 M KCl, 150 mM NaCl, 150 mM HEPES, pH 7.5,). Homogenates were overlaid on a sucrose cushion (30 % w/v sucrose, 2 mM EDTA, pH 8 prepared in 1x buffer A) and centrifuged at 3,000 rpm for 10 minutes at 4°C. After centrifugation, supernatant obtained were stored as a cytoplasmic fraction, and pellets of nuclei were resuspended in 1x nuclei extraction buffer (20 mM HEPES, pH 7.5, 400 mM NaCl, 1 mM EDTA, pH 8) and incubated for 15 minutes on ice with intermittent vortexing. Solubilised nucleated pellet fractions were centrifuged again at 15,000 rpm for 5 minutes at 4°C and supernatant collected as nuclear protein fraction. Protein concentration was measured by protein assay kit (Bio-rad protein assay, Munich, Germany) as per manufactures instructions. 60 to 80 µg of proteins were solubilised in 5X Laemmli sample buffer and run on 8% polyacrylamide gels.

On some blots, hypoxic (0.2 % oxygen for 16 hours) and normoxic (20 % oxygen for 16 hours) control samples collected from Mouse Embryonic Fibroblasts (MEFs) cell culture were loaded as positive controls (kindly provided by M. Kaufmann and R. Wenger, Univ. of Zurich). Briefly protein was separated by SDS-PAGE, transferred on nitrocellulose membrane by wet transfer method and incubated overnight with following primary antibodies: rabbit polyclonal anti-HIF1 α (1:1000, Novus Biologicals) and mouse monoclonal β -actin (1:10,000, Sigma). After washing nitrocellulose membranes were incubated with anti-rabbit secondary fluorescent antibody (goat anti-rabbit IRdye 800 1:10,000, or goat anti mouse IRDye 680, 1:20,000, Li-COR, Nebraska, USA). After washing the membranes three times, the protein signal was detected by scanning the membrane with the Odyssey Infrared Imaging System (Li-COR Biosciences). All images were analyzed using analysis software provided with the system to calculate the protein of interest/actin ratio.

Statistical analysis

Results are expressed as mean $\pm$ SEM. All data were tested for significance using paired or unpaired Student's test, one or two way ANOVA analysis followed by Tukey's multiple comparison post test where appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Absence of a maternal preeclamptic phenotype in $AS^{-/-}$ mice during pregnancy

To test the hypothesis that aldosterone deficiency during pregnancy may contribute to the pathogenesis of preeclampsia, we measured SBP and urinary protein excretion in $AS^{+/+}$ and $AS^{-/-}$ dams before and during pregnancy. In order to eliminate the genotype of the fetuses as confounding factor, $AS^{+/+}$ females were mated to $AS^{-/-}$ males and $AS^{-/-}$ females to $AS^{+/+}$ males producing only $AS^{+/-}$ offspring. Before and during pregnancy, SBP was significantly higher in $AS^{+/+}$ than in $AS^{-/-}$ dams (Figure 1A). While blood pressure of $AS^{+/+}$ remained constant at about 110 mmHg throughout pregnancy, SBP in $AS^{-/-}$ dams decreased from about 105 mmHg at day 1 to about 90 mmHg at day 21. Importantly, there was no difference in urinary protein excretion and no increase in $AS^{+/+}$ and $AS^{-/-}$ mice during pregnancy (Figure 1B). Thus, $AS^{-/-}$ dams did not become hypertensive and did not develop proteinuria during pregnancy.

Presence of a feto-placental phenotype in $AS^{-/-}$ mice on –control diet

Mean litter size at birth was lowered in $AS^{-/-}$ dams compared to $AS^{+/+}$ mice (3.0 ± 0.55 vs. 6.22 ± 0.66 , respectively; $p < 0.005$) representing a strong fetal phenotype (Figure 1C). To examine whether the reduced litter size at birth was due to intrauterine death of fetuses, dams were sacrificed on gestational day 18 (E18) and the number and weight of fetuses and placentas were measured. The mean litter size was again smaller in $AS^{-/-}$ compared to $AS^{+/+}$ dams (2.67 ± 0.54 vs. 7.13 ± 0.48 , respectively; $p < 0.0001$) (Figure 2A). The reduction of the number of pups

paralleled the presence of dark and necrotic placentas in $AS^{-/-}$ mice (Figures 2A and 2B). Necrotic placentas were mostly observed in series next to each other but sometimes also observed interspersed between healthy placentas. Necrotic placentas were not seen in any $AS^{+/+}$ dams on control diet.

When the mean litter and placenta weights were plotted against litter sizes (Figure 2C), it became evident that the data for the $AS^{-/-}$ mice were not only shifted to smaller litter sizes but also to reduced litter and placenta weights. This is most evident when litters of the same sizes in $AS^{-/-}$ and in $AS^{+/+}$ are compared (i.e. litter sizes of 5 and 7 pups in figure 2C). In contrast, in very small litters ($n=1-3$), placental weight appeared to be normal. Histological examination of the placentas further confirmed the reduced size of placentas in $AS^{-/-}$ dams, but did also reveal that most of the placentas were otherwise healthy with a similar structural organization of decidua basalis, junctional zone, and labyrinth as in $AS^{+/+}$ dams (Figure 3A and B, left and middle section). Only dark placentas from $AS^{-/-}$ dams showed severe coagulative necrosis with lymphocyte infiltrations in all three layers of the placenta (Figure 3A and B, right section).

High salt diet does not increase intrauterine survival, but improves fetal growth in $AS^{-/-}$ mice, and lowers SBP irrespective of the presence of aldosterone

Hypothesizing that aldosterone deficiency in $AS^{-/-}$ mice might compromise placental perfusion, we tested if replacing renal sodium losses by feeding a high salt diet (5 % NaCl) before and during pregnancy may improve SBP, the weight of placentas and pups, and litter sizes. $AS^{+/+}$ and $AS^{-/-}$ females were fed a high salt diet

starting 12 days before until the end of pregnancy. After 4 days on a high salt diet, SBP rose in both groups of mice, but this increase occurred in parallel and hence $AS^{-/-}$ female mice continued to have lower SBP than $AS^{+/+}$ female mice (Figure 4A). During the first days (days 5-7) of pregnancy, SBP dropped significantly in both $AS^{+/+}$ and $AS^{-/-}$ dams, but the decrease of SBP was much more pronounced in $AS^{+/+}$ mice than in $AS^{-/-}$ mice. Therefore, the SBP difference between genotypes became very small (days 5-7) and was no longer significant on days 15 to 17. This is in contrast to the measurements in dams on standard chow, where a significant difference in SBP was maintained throughout the entire pregnancy (Figure 1). In $AS^{-/-}$, blood pressure was lower towards the end of gestation irrespective of salt intake (Figure 4B). In $AS^{+/+}$ mice, SBP did not drop significantly in pregnancy on a normal salt diet. However, if a high salt diet was provided, blood pressure dropped reaching the lowest levels at the end of pregnancy (Figure 4C). Of interest, both a low and a high volume state led to a blood pressure reduction in pregnancy with the latter being even more consistent throughout pregnancy.

The high Na^+ diet did neither increase the mean litter size nor decrease the number of necrotic placentas in $AS^{-/-}$ dams (Figure 5A). However, the mean weights of litters and placentas were no longer different between the genotypes when litters of the same sizes were compared (Figure 5B). This contrasts with the standard salt intake, when both mean litter and placenta weights were smaller in $AS^{-/-}$ than in $AS^{+/+}$ dams (Figure 2C). Thus high salt intake does not increase the survival rate of fetuses, but appears to improve intrauterine growth of surviving pups.

The beneficial effect of a high salt intake was seen for both genotypes. Although the 5% NaCl intake did not increase mean placental weight, it significantly improved the weight of the fetuses in $AS^{+/+}$ and $AS^{-/-}$ dams (Figure 6A, B). Consistent with these

differential effects on fetal and placental growth, placental efficiency (ratio of fetal weight over placental weight) was significantly increased in both $AS^{+/+}$ and $AS^{-/-}$ dams on high salt diet compared to control diet (Figure 6C). Interestingly, placental efficiency was not significantly different between $AS^{+/+}$ and $AS^{-/-}$ dams, neither on control nor on high salt diet (Figure 6C).

Expression of the hypoxia inducible factor HIF1 α in placentas

Preeclampsia is associated with altered expression of key anti- and pro-angiogenic factors [40]. We did not detect any differences in the mRNA expression of HIF1 α (Figure 7A), VEGF (Figure 7B), and sFlt (Figure 7C) between healthy appearing placentas from $AS^{+/+}$ and $AS^{-/-}$ dams kept on either a control or a high salt diet. Since HIF1 α is mostly regulated at the post-translational level by stabilisation and degradation of the protein, we performed immunoblots. HIF1 α protein abundance was significantly increased in healthy placentas from $AS^{-/-}$ dams on control diet (Figure 9A). This difference disappeared on high salt diet (Figure 9B). HIF1 α protein was not detected in necrotic placentas (Figure 9A).

Expression of pro-inflammatory factors in necrotic placentas

The MCP-1 and TNF α are key pro-inflammatory cytokines involved in inflammatory process. Expression of MCP-1 and TNF α mRNA was significantly increased in necrotic placentas from $AS^{-/-}$ dams on control (Figure 8A) and high salt diet (Figure 8B) but not in healthy appearing placentas from both genotypes.

DISCUSSION

Plasma volume expansion in the presence of high aldosterone levels is considered crucial for healthy pregnancies, both of which are low in the most severe form of hypertensive pregnancies, preeclampsia. In order to examine the role of aldosterone in the development of preeclampsia, we used an aldosterone synthase knockout mouse model ($AS^{-/-}$). $AS^{-/-}$ mice have a compensatory increase of renin and angiotensin II to supraphysiological concentrations but no aldosterone [21] allowing to independently test the role of aldosterone in pregnancy. Additional effects of these high renin and angiotensin II may occur but could not be tested because survival of $AS^{-/-}$ mice depends on high angiotensin II levels and pharmacological blockade of AT_1 receptors is lethal in $AS^{-/-}$ mice (unpublished observations).

New-onset hypertension and proteinuria with feto-placental developmental disturbances are considered to be cardinal features of preeclampsia [40]. We found that $AS^{-/-}$ dams did not develop preeclampsia as suggested by the absence of hypertension and proteinuria during pregnancy. A previous study measured SBP by telemetry during pregnancy and showed no change in SBP during the first 5 days, followed by a drop in SBP from days 6 to 12, and again from day 15 to 17 of pregnancy [4]. In our hands, using the less sensitive tail cuff method, we found reduced SBP in wild type mice during pregnancy in response to salt loading. Though contra-intuitive, this observation is in line with some clinical observations during salt loading in pregnancy suggesting a beneficial systemic feedback on maternal hemodynamics upon optimal placental perfusion [10, 32]. Thus, preeclampsia is not caused by a lack of aldosterone alone, at least in mice.

However, the mouse model revealed a number of other interesting observations demonstrating an important role of aldosterone in normal pregnancy. $AS^{-/-}$ dams were hypotensive before pregnancy and became even more hypotensive during mid to term pregnancy. The resulting feto-placental phenotype of a decreased litter size and smaller or necrotic placentas without fetuses at term suggests a severe placental hypoperfusion. The sum of intact fetuses and necrotic placentas in $AS^{-/-}$ mice was similar to the average number of fetuses in $AS^{+/+}$ dams, indicating that $AS^{-/-}$ dams were able to conceive normally but had impaired placental development and function, predisposing to intrauterine death. The few normal weight placentas and fetuses observed in $AS^{-/-}$ dams carrying small litters would such provide a residual blood and nutrient supply to the remaining placentas.

Histological examination of healthy placentas from $AS^{-/-}$ dams demonstrated normal structural patterns but reduced size. One important reason for lower placental weight might be the absence of direct effects of aldosterone on the placenta in $AS^{-/-}$ dams. In-vitro experiments have revealed a direct role for aldosterone in the modulation of trophoblast growth and placental size [16] and in-vivo studies in ewes suggested a role of aldosterone in placental growth [19]. Placental size in rats and humans is positively correlated with plasma levels of aldosterone [16]. Thus, aldosterone may positively modulate placental growth, similar to its proliferative capabilities in organs such as the heart and the kidney [36, 39].

Reduced fetal weight in $AS^{-/-}$ dams might thus be due to placental insufficiency. In normal human pregnancy, plasma volume expansion is associated with increased plasma aldosterone level whereas both were decreased in pregnancies with intrauterine growth restriction and preeclampsia [33]. Preeclampsia is characterised by extracellular volume expansion but low intravascular volume

which is caused at least in part by a shift of fluid from the intravascular to the interstitial fluid space due to vasoconstriction and leaky endothelium [12-13, 31]. Lack of aldosterone in $AS^{-/-}$ dams might thus critically impair plasma volume expansion and thereby cause reduced uterine blood supply similar to functional aldosterone deficiency in mice chronically exposed to the mineralocorticoid receptor antagonist spironolactone during pregnancy which led to reduced fetal umbilical blood flow [16]. Moreover, modelling a reduced uterine blood supply by constriction of the uterine artery in rats from gestational day 14 caused placental insufficiency resulting in low-birth weight [2].

One common cause for the decreased litter size, reduced fetal and placental weight, and necrotic and inflammatory placentas in $AS^{-/-}$ dams could be hypoxia. In rats, hypoxia during the last 11 days of pregnancy results in smaller sized litters, decreased placental and fetal weights, and placental resorption [18]. Low oxygen tension affects cytotrophoblast proliferation and invasion and ultimately fetal growth [15]. Consistently, the hypoxia-induced factor HIF1 α , a master regulator in the adaption to hypoxia, is essential for mammalian placentation [1]. HIF1 α protein abundance was significantly higher in $AS^{-/-}$ mice on control diet, but was reduced to control levels on 5% NaCl diet in the normal appearing placentas suggesting hypoxia and rescue by high NaCl intake. Consequently, $AS^{-/-}$ necrotic placentas showed coagulative necrosis and severe lymphocyte infiltration and elevated levels of proinflammatory cytokines MCP-1 and TNF α .

Aldosterone plays a central role in the long-term control of extracellular volume by adapting renal and extrarenal reabsorption of sodium to blood pressure [3]. Thereby, extracellular NaCl and volume is enlarged, blood pressure and organ

perfusion increased. $AS^{-/-}$ mice are hypotensive despite highly elevated levels of renin and angiotensin II. High salt diet reverses renin and angiotensin II abnormalities without increasing blood pressure indicating that the extracellular volume status is at least partially normalized [23]. To rescue the possibly reduced plasma volume and lower uterine blood flow in $AS^{-/-}$ dams, we treated mice with high salt for 12 days before pregnancy until gestational day 18. Both $AS^{+/+}$ and $AS^{-/-}$ mice responded by increasing SBP suggesting that $AS^{-/-}$ dams also retained salt and fluid even though plasma volume was not directly measured.

High salt diet had several interesting implications. Next to the blood pressure lowering potency in pregnant wild-type mice, it partially rescued the fetal phenotype with a substantial effect on fetal weight, placental hypoxia, and placental efficiency in both $AS^{+/+}$ and $AS^{-/-}$ dams. Placental efficiency can be altered experimentally by manipulation of uterine blood flow, oxygen availability, and by the intake and composition of maternal diet. Increased efficiency is also seen in naturally small relative to large placentas in pigs, sheep, goats, rats and mice. Placental efficiency in these polytocous species is positively related to litter size [11]. We found that smaller placentas in $AS^{+/+}$ dams on high salt diet and $AS^{-/-}$ dams were associated with larger litter size indicating positive correlation between placental efficiency and litter size. Decreased placenta weight with larger litter size in $AS^{+/+}$ dams on high salt diet also suggests increased placental efficiency thus supporting an unrestricted fetal growth. While small, dark placentas were still observed in $AS^{-/-}$ dams on high salt diet similar to control diet it remains amenable a consequence of altered local aldosterone availability to support placental development.

Limitations of this model of aldosterone deficiency are the high renin and angiotensin II levels which may trigger local dysregulatory events in preeclampsia. In

the absence of aldosterone synthase, deoxycorticosterone, corticosterone and some 18-hydroxycorticosterone are still synthesized via 11 β -hydroxylase activity. Thus, the live-long inhibition of aldosterone synthesis in these animals potentially allowing for alternative mineralocorticoid active steroid hormones might have supplemented some extrarenal aldosterone effects or even enhanced the inhibition of proliferation of trophoblasts via the activation of glucocorticoid receptors.

Aldosterone availability appears an important prerequisite to support normal fetoplacental development. *AS*^{-/-} dams had a fetoplacental phenotype with decreased litter size, smaller placentas and fetuses, yet no overt preeclampsia. In the absence of aldosterone, the fetal phenotype might be rescued in part by high salt diet. In wild-type mice, the high salt diet led to profound blood pressure reduction in pregnancy which suggests an important role of plasma volume and placental perfusion for maternal well-being. Thus, the absence of aldosterone cannot trigger preeclampsia-like symptoms in mice, but might well contribute to the development of preeclampsia-related intrauterine growth restriction while preeclampsia needs to be triggered by additional factors. Thus, our study supports the importance of aldosterone in placental function in-vivo and a possible role for high salt supplementation in pregnancies specifically associated with hypoaldosteronism or reduced plasma volume expansion to maintain fetoplacental integrity. In contrast, in preeclampsia, the benefits of salt supplementation are still controversial and has no major impact on outcome as indicated by a recent meta-analysis [7].

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FIGURE LEGENDS

Figure 1. Systolic blood pressure (SBP), proteinuria and litter size on control diet.

A. SBP measured during the course of pregnancy from day 0 to day 21 in $AS^{+/+}$ (n=6-9 animals/ time point) and $AS^{-/-}$ (n=3-9 animals/time point) dams. **B.** Urine total protein concentration normalised to creatinine concentration measured from spot urine during pregnancy in $AS^{+/+}$ (n = 8) and $AS^{-/-}$ dams (n = 6). **C.** Number of pups counted on the day of delivery in $AS^{+/+}$ (n = 9) and $AS^{-/-}$ (n = 9) dams. Values are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ vs. $AS^{+/+}$.

Figure 2. Litter size, litter and placenta weights, and healthy and necrotic placentas in $AS^{+/+}$ and $AS^{-/-}$ dams on day 18 of pregnancy.

A. Number of fetuses and necrotic placentas were counted in the uterus from $AS^{+/+}$ (n = total number of fetuses and placentas from 8 females) and $AS^{-/-}$ dams (n = total number of fetuses and placentas from 15 females). **B.** Photos of fetuses and placentas in the uterus from $AS^{+/+}$ and $AS^{-/-}$ dams and comparison of healthy and necrotic placenta. **C.** Mean litter and mean placenta weight versus litter size in $AS^{+/+}$ (n = 8 litters) and $AS^{-/-}$ dams (n = 12 litters). Values are means $\pm$ SEM. ** $P < 0.005$, *** $P < 0.001$ vs. $AS^{+/+}$.

Figure 3. Histology of placentas

A. Cross section and HE stain of a placenta from an $AS^{+/+}$ dam, and healthy and necrotic placentas from $AS^{-/-}$ dams. Bar = 2000 μ M. **B.** Higher magnification of boxed area from figure 3A. Bar = 200 μ M. DB = decidua basalis, JZ = junctional zone and

LZ = labyrinth zone. * indicates lymphocyte infiltration and # indicates coagulative necrosis.

Figure 4. Effect of high salt diet on systolic blood pressure (SBP), litter size, litter and placenta weight and necrotic placentas at day 18 of pregnancy

A. SBP was measured at different time points (days) before and during pregnancy in $AS^{+/+}$ (n = 6-8 animals/time point) and $AS^{-/-}$ (n = 7-10 animals/ time point). **B.** In $AS^{-/-}$ mice, no differences in SBP was observed upon salt loading in pregnancy. **C.** In pregnant wild-type mice, salt loading led to a significant drop in SBP. For figure **B** and **C** SBP compared between control and high salt (5 % NaCl) diet at different time points just before pregnancy (-4 to -1 day for control and -10 to -8 for high salt), at gestational day 5 to 7 and 15 to 17. Values are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ vs. $AS^{+/+}$ (Figure 4A) or vs. control diet (Figure 4B, C) , ### $P < 0.005$ vs. high salt (5 % NaCl) at -10 to -1 days.

Figure 5. Effect of high salt diet on litter size, litter and placenta weight and necrotic placentas at day 18 of pregnancy

A. Number of fetuses and necrotic placentas detected in $AS^{+/+}$ (n = 6) and $AS^{-/-}$ dams (n = 9) at day 18 of pregnancy ($P = 0.088$). **B.** Mean litter and mean placenta weight versus litter size in $AS^{+/+}$ (n = 6) and $AS^{-/-}$ dams (n = 9) ($P = 0.015$). Values are means $\pm$ SEM. * $P < 0.05$ vs. $AS^{+/+}$.

Figure 6. Comparison of litter and placenta weights, and placental efficiency between control vs. high salt diet in $AS^{+/+}$ and $AS^{-/-}$ dams.

Mean litter weights (**A**) and mean placenta weights (**B**) on control diet vs. high salt diet in $AS^{+/+}$ (control diet n = 8 litters, high salt diet n = 6 litters) and $AS^{-/-}$ dams

(control diet n = 12 litters, high salt diet n = 9 litters). **C.** Placental efficiency in $AS^{+/+}$ (control diet n = 8 litters, high salt diet n = 6 litters) and $AS^{-/-}$ (control diet n = 13 litters, high salt diet n = 9 litters) dams on control and high salt diet. Values are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ vs. control diet, ### $P < 0.005$ vs. $AS^{+/+}$ on control diet, $\phi\phi\phi$ $P < 0.001$ vs. $AS^{-/-}$ on control diet.

Figure 7. Expression of VEGF, sflt and HIF1 α in placentas.

mRNA expression of HIF1 α (**A**) VEGF (**B**), and sFlt (**C**) in healthy placentas from $AS^{+/+}$ (n = 5) and $AS^{-/-}$ (n=4-5) dams on control and high salt (5 % NaCl) diet.

Figure 8. Expression of inflammatory markers MCP-1 and TNF α in placentas.

mRNA expression of MCP-1 and TNF α was tested by qPCR in healthy placentas from $AS^{+/+}$ (n = 5), and healthy and necrotic placentas from $AS^{-/-}$ (n = 5 each) dams on control (**A**) and high salt (5 % NaCl) (**B**) diet. Values are means $\pm$ SEM. * $P < 0.05$ and *** $P < 0.001$ vs. healthy placentas from $AS^{-/-}$, ** $P < 0.005$ and *** $P < 0.001$ vs. $AS^{+/+}$.

Figure 9. Protein expression of HIF1 α in placentas.

The protein abundance of HIF1 α was examined in placentas from $AS^{+/+}$ and $AS^{-/-}$ dams on control (**A**) and high salt (5 % NaCl) (**B**) diet. Hypoxic (0.2 % oxygen for 16 hours) and normoxic (20 % oxygen for 16 hours) control samples were collected from Mouse Embryonic Fibroblasts (MEFs) cell culture as positive controls. Bar graph summarize the results from two independent blots of n= 6 normalized to β -actin measured on the same membranes. Values are means $\pm$ SEM. ** $P < 0.005$ vs. $AS^{+/+}$.

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Figure 1

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Figure 2

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Figure 3

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Figure 4

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Figure 5

Figure 6

Figure 7

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Figure 8

Figure 9