Placenta 35 (2014) 953–961

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

The effects of gestational age and maternal hypoxia on the placental renin angiotensin system in the mouse

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ARTICLE INFO

Article history:
Accepted 3 September 2014

Keywords:
Placenta
Renin angiotensin system
Hypoxia

ABSTRACT

Introduction: The renin angiotensin system (RAS) is an important mediator of placental development. However, a comprehensive expression profile for 8 key components of the placental RAS throughout murine gestation has not been performed. Furthermore, maternal hypoxia induces dysregulation of RAS expression in fetal tissues but the effects on the murine placental RAS are less well known.
Methods: Placentas were collected from male and female CD1 mouse fetuses at seven gestational ages for qPCR analysis of *Agt*, *Ren1*, *Atp6ap2*, *Ace*, *Ace2*, *Agtr1a*, *Agtr2* and *Mas1*. mRNA localisation of *Agtr1* and *Mas1* and protein localisation of ACE and ACE2 was determined at E18.5. To determine the effects of maternal hypoxia on the placental RAS, mice were housed in 12% oxygen from E14.5–E18.5 and placentas examined at E18.5.
Results: All RAS genes were expressed in the placenta throughout pregnancy and expression varied with fetal sex and age. *Agtr1* was expressed within the labyrinth while Mas1 was expressed within the intraplacental yolk sac. ACE and ACE2 were localised to both labyrinth and junctional zones. In response to maternal hypoxia the expression of *Agt*, *Ace* and *Ace2* was decreased but expression of *Agtr1a* was increased. *Ace* and *Agtr1a* mRNA levels were affected to a greater extent in females compared to males.
Discussion: Collectively, the location within the placenta as well as the expression profiles identified, support a role for the placental RAS in labyrinth development. The placental RAS is disturbed by maternal hypoxia in a sexually dimorphic manner and may contribute to impairment of placental vascular development.

1. Introduction

The renin-angiotensin system (RAS) is an endocrine cascade well known for its role in regulating whole body sodium handling and fluid homeostasis. In addition, the RAS is an important system during development where it is involved in organogenesis of the kidney [1–3] and heart [4]. These effects are largely mediated by the actions of the classical RAS which involves angiotensin II binding to the angiotensin type 1 (AGTR1) or type 2 (AGTR2) receptors. Conversely, angiotensin II can be cleaved into angiotensin 1–7 by the actions of the angiotensin converting enzyme 2 (ACE2) and binds to AGTR2 as well as the more recently identified Mas-proto oncogene (MAS1). Signalling through AGTR1 contributes towards cell proliferation and hypertrophy while AGTR2/MAS1 activity regulates apoptosis, anti-proliferation and anti-hypertrophy [5]. While RAS receptor activation by angiotensin II primarily regulates RAS mediated developmental processes, a number of cleavage enzymes may also play a significant role. Angiotensin II is a peptide produced by cleavage of angiotensin I by ACE while angiotensin I is produced by the cleavage of angiotensinogen (AGT) by renin (Ren). Interestingly, renin itself must be activated from prorenin (either proteolytically or non-proteolytically) to be fully enzymatically active [6]. Non-proteolytical activation may occur in a low pH/low temperature environment or when bound to the prorenin receptor (encoded by the gene *Atp6ap2*) [7]. ATP6AP2 binding has also been shown to elicit independent downstream signalling [8] which may contribute to fetal development [9].

A functional RAS exists in various tissues as a complete local system, largely independent of the circulating RAS. A local RAS has been demonstrated in a number of fetal organs (reviewed by Ref. [10]) including the kidney [11] and heart [12]. Similarly, many components of the RAS are known to be expressed within the placenta [13]. The placental RAS is speculated to play an important role in the fine tuning of a number of placental physiological processes. Angiotensin II signalling through AGTR1 has been shown to decrease placental system A amino acid transport supporting a role

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http://dx.doi.org/10.1016/j.placenta.2014.09.004
0143-4004/

for this system in regulating placental nutrient transport [14]. Angiotensin II has been demonstrated to directly increase endothelial vascular permeability [15] and increase the synthesis and secretion of vascular endothelial growth factor (VEGF) in other tissues [16]. It has likewise been suggested that the RAS plays a key role in the angiogenesis and vascular permeability of the developing placenta [13]. Furthermore, the RAS is known to play a role in placental structural formation as knocking out AGTR1 disrupts the organisation of the definitive placenta [17]. Maternal perturbations such as hypoxia and dietary protein restriction have both been shown to alter placental renin levels [18,19], and placental insufficiency is associated with increased expression of many RAS genes [20]. Furthermore, an increase in the peptide levels of Angiotensin II and the mRNA levels of *Ren* and *Ace* in preeclamptic placentas suggests that the RAS may play a role in the pathophysiology of preeclampsia [21].

A recent study has examined the mRNA levels of many components of the RAS in the human placenta and shown expression levels differ between early and late gestation [22]. However, the expression of these genes and how they change over pregnancy has not been systematically investigated in the rodent. Identification of the expression profile of the RAS during pregnancy will provide a better understanding of the role of the RAS in placental development and function. Furthermore, the expression of various components of the RAS is sex dependent. The protective antiproliferative genes, *Agtr2* and *Ace2* are located on the X chromosome and renal expression of *Agtr2*, *Ace2* and *Mas1* are higher in adult females compared to males [23]. With recent studies demonstrating different growth strategies for placentas of male and female fetuses [24], sex differences in the placental RAS are of great interest. The aim of this study was to investigate the ontogeny of mRNA expression of components of the placental RAS, throughout pregnancy in the mouse and to localise RAS regional/cellular expression. Furthermore, we determined the impact of maternal hypoxia, a perturbation known to reduce fetal growth in association with sex specific alterations in placental development [25] on the expression of the placental RAS. We hypothesised that there would be changes across gestation in RAS mRNA expression in the mouse placenta and these would be altered by maternal oxygen status in a sex specific manner.

2. Methods

2.1. Animal treatment

Animal experiments were approved by the University of Queensland Animal Ethics Committee (AEC numbers 484/09 and 496/12) and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.1.1. Ontogeny studies

Pregnant untreated CD-1 mice were killed at embryonic day (E) 8.5, E9.5, E10.5, E12.5, E14.5, E16.5 and E18.5 for the collection of whole placentas. Placentas collected at E8.5, E9.5 and E10.5 were carefully separated from surrounding tissues to minimise risk of contamination and individual placentas in a litter were pooled for each age (n = three litters per age). At E12.5, E14.5, E16.5 and E18.5, fetal and placental weights were analysed, fetal sex determined [26] and individual placentas collected and frozen for QPCR analysis (n = 6 per sex, per age, from 3 to 6 litters).

2.1.2. Maternal hypoxia

Pregnant CD-1 mice were subjected to maternal normoxic (21%, n = 8) or hypoxic (12%, n = 8) conditions from E14.5 to E18.5 as described previously [25]. Briefly, dams were placed in a specialised hypoxia chamber which carefully monitors and automatically regulates oxygen content by oxygen displacement using nitrogen gas. Dams were killed and placentas collected at E18.5 and either frozen or fixed in 4% PFA.

2.2. mRNA gene expression studies

RNA was extracted from placentas collected at all-time points and from placentas of dams exposed to hypoxic conditions using the RNeasy minikit (QIAGEN, Chadstone Centre, VIC, Australia). RNA was extracted from pooled placentas at early embryonic time points (E8.5–E10.5, n = 3 litters per age) and from individual placental quarters (Ontogeny: E12.5–E18.5, n = 6 per sex per age, Hypoxia: E18.5, n = 10 per treatment per sex). All RNA was treated with deoxyribonuclease 1 and reverse transcribed into cDNA (iScript™, Bio-Rad, Gladesville, NSW, Australia). Relative mRNA levels were determined using multiplex reactions (50 ng of cDNA per 10 μl QPCR reaction) using Taqman reagents (Life Technologies, Mulgrave, VIC, Australia) and the 18s rRNA endogenous control (Life technologies, Cat. No. 4308329). The following probe and primers were used to detect *Agtr1a* gene expression; Forward P -GGGCTGTCTATACCGCTATGGAA, Probe-ACCGCTGGCCCTTCGGCAA, Reverse P- GCCGAAGCGATCTTACATAGGTG. Taqman Assay on Demand (AOD) assays were used to detect the following genes; *Agt* (Mm00599662_m1), *Ren1* (Mm02342887_mh), *Atp6ap2* (Mm00510396_m1), *Ace* (Mm00802048_m1), *Ace2* (Mm01159003_m1), *Agtr2* (Mm01341373_m1) and *Mas1* (Mm00627130_m1). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. For all ontogeny analyses, mRNA levels were normalized to the average of the E12.5 male group. For all hypoxia studies, mRNA levels were normalised to the average of the male normoxic group at E18.5.

2.3. In situ hybridisation (ISH)

Placental sections (7 μm paraffin sections) were collected from normoxic placentas at E18.5 for mRNA localisation using ISH. Primers were designed to generate ISH probes for total *Agtr1* (*FP*-TAATACGACTCACTATAGGGTGGCCCTTAACTCTTCTACTGAA, RP- AATTAACCCTCACTAAAGGGCTCGTAATGAAAAGCGCAAAC) and *Mas1* (*FP*- TAATACGACTCACTATAGGGCTGCTGACAGCCATCAGTGT, RP-AATTAACCCTCACTAAAGGGCCTGGGTTGCATTTCATCTT) receptors as described previously [27]. Anti-sense and sense probes (used as negative controls) were generated using the above primers and T3 and T7 promoter sequences. Hybridisation was carried out as described previously [27].

2.4. Western blotting

Total protein was extracted from E18.5 normoxic and hypoxic placentas and was subjected to SDS-PAGE and western blotting as described previously [28]. ACE (1:500, Cat. No ab75762, Abcam, Cambridge, UK) and ACE2 (1:333, Cat. No. Sc-20998, SantaCruz Biotechnologies, Dallas, TX, USA) were measured in placentas of female fetuses with an anti-β-actin antibody (ACTB, 1:10000, Cat. No. A1978, Sigma–Aldrich, St. Louis, MO, USA) used as a loading control.

2.5. Immunohistochemistry

Fixed placentas were sectioned as for ISH and subjected to antigen retrieval (Microwave heating to boiling point, for 5 × 5 min at 60% power) using a 10 mM sodium citrate solution made up in PBST. Slides were treated with 3% hydrogen peroxide in distilled water to block endogenous peroxidase activity, treated with 50% egg white in distilled H_2O, 5% skim milk in PBST and blocked in 2% BSA and 10% goat serum in PBST. Slides were incubated with ACE (1:500) or ACE2 (1:500) rabbit primary antibodies. Slides were subjected to a biotinylated goat anti-rabbit secondary antibody and stained using avidin–biotinylated enzyme complex (ABC Vectastain Elite kit, Vectorlabs). Slides were counterstained using haematoxylin.

2.6. Statistics

All data are expressed as mean ± SEM. qPCR data was analysed using two way analysis of variance (ANOVA) testing for both ontogeny (Sex and Gestational age) and hypoxia studies (Sex and Treatment) followed by Sidak or Fisher's LSD post hoc testing. When data was not normally distributed, data was appropriately transformed prior to testing.

3. Results

Male and female fetal body weight increased with advancing gestational age between E12.5 and E18.5 (P_{GA} < 0.001, Fig 1A). Placental weight increased from E12.5 to E14.5 and remained consistent thereafter (P_{GA} < 0.001, Fig 1B). The placental ratio (grams of fetus per gram of placenta) increased with advancing gestation (P_{GA} < 0.001, Fig 1C). Body weight, placental weight and the placental ratio were not affected by fetal sex.

3.1. Ontogeny of RAS receptor gene expression

Placental mRNA transcripts for *Atp6ap2*, *Mas1*, *Agtr1a* and *Agtr2*, were detected at all-time points examined. *Atp6ap2* mRNA levels were unchanged from E8.5 to E10.5 and were at levels similar to those detected in placentas from E12.5 males (Fig 2A). Gestational age affected *Atp6ap2* mRNA levels from E12.5–E18.5 (P_{GA} < 0.05). Although fetal sex did not affect the overall expression of *Atp6ap2*, expression peaked at E16.5 before decreasing at E18.5 in males