Placenta 31 (2010) 448–455

Contents lists available at ScienceDirect

Placenta

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

Expression of AT1R, AT2R and AT4R and Their Roles in Extravillous Trophoblast Invasion in the Human

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ARTICLE INFO

Article history:
Accepted 23 February 2010

Keywords:
Receptor
Angiotensin
Type 1
Type 2
Placenta

ABSTRACT

The placental renin-angiotensin system (RAS) is active from early pregnancy and may have a role in placentation. Angiotensin II (AngII) acts via binding to receptor types AT1R and AT2R. Recently smaller peptide members of the angiotensin family have been recognised as having biological relevance. Angiotensin (3-8) (AngIV) has a specific receptor (AT4R) and evokes hypertrophy, vasodilatation and vascular inflammatory response. The aim of this study was to characterise placental expression of AT1R, AT2R and AT4R, and to determine whether AngII and AngIV regulate extravillous trophoblast (EVT) invasion, apoptosis and proliferation. Placental samples were obtained from women undergoing elective surgical termination of pregnancy (TOP) at 8–10 weeks gestation (early TOP), 12–14 weeks gestation (mid TOP) or at delivery following normal pregnancy or with pre-eclampsia (PE). Immunohistochemistry and qRT-PCR were performed to determine placental mRNA and protein expression of AT1R, AT2R and AT4R at all gestational ages. EVT invasion following culture with AngII or AngIV was assessed in early placental tissue using Matrigel invasion assays. Invasion was assessed on day 6 of culture and placental explants were harvested for immunohistochemical analysis of apoptosis and proliferation. The results from qRT-PCR and immunohistochemistry showed placental AT1R expression which did not vary with gestation. The highest levels of expression of AT2R were found in early and mid TOP placentae compared to term pregnancy. Expression of AT4R was increased in term placentae, with a significant reduction in PE placentae. Moreover, culture with AngIV or AngII increased EVT invasion from placental explants, which showed increased trophoblast proliferation and reduced apoptosis. This study has characterised expression of AT4R and AT1R and AT2R in human placenta throughout normal pregnancy and in PE. Both AngIV and AngII may play an important role in normal pregnancy.

1. Introduction

The circulating renin-angiotensin system (RAS) is important for regulation of blood pressure and electrolyte balance. Until recently AngII was thought to be the major bioactive peptide, however recently it has emerged that the shorter peptides, produced by aminopeptidase mediated cleavage of AngII, including angiotensin (3-8) (AngIV), also have roles in regulating cardiovascular function. There are two major angiotensin receptors, angiotensin II receptor 1 (AT1R) and angiotensin II receptor 2 (AT2R), which have similar binding affinities for AngII [1]. AngII mediates most of its effects via binding with AT1R, ultimately triggering vasoconstriction, proliferation, angiogenesis or inflammation [1]. AT2R is predominantly expressed in fetal tissues [2], and AngII binding increases apoptosis, causes vasodilation and is involved in fetal tissue development [3].

AngIV appears to mediate various effects in different tissues via binding with high affinity to its specific receptor, the AT4R [4]. AngIV can also bind to AT1R and AT2R with low affinity. The active site of AT4R has been identified to be an insulin-regulated aminopeptidase (IRAP) [5], also known as cysteine aminopeptidase, oxytocinase or placental leucine aminopeptidase (P-LAP). AT4R expression has been localised in both endothelial [6] and smooth muscle cells [7] indicating physiological roles in regulating blood flow. AngIV can increase blood flow via a mechanism mediated by AT4R and nitric oxide [8]. Due to its localization in extravillous trophoblast (EVT) Ino et al. [9] suggested that this receptor may be involved in regulating the invasion of EVT during placentation.

In addition to the circulating RAS, tissue based systems are found in heart, brain and reproductive tissues. The fetal–maternal interface comprises both the fetal placental tissue RAS and the

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doi:10.1016/j.placenta.2010.02.014

maternal decidual tissue RAS. Factors involved in stimulating decidualisation, including oestrogen and progesterone, have been identified as playing a role in regulating the local RAS, which is thought to be important in spiral artery remodelling.

Plasma renin concentration and activity, as well as AngII levels are increased in pregnancy, but vascular responsiveness to AngII is decreased [10]. In contrast, pre-eclamptic patients exhibit exaggerated pressor responses to AngII, although circulating concentrations are lower compared to control pregnancies [10]. Although the underlying mechanism remains to be elucidated, there is growing evidence to indicate that dysregulation of both the tissue based and circulating RAS may be involved in the pathophysiology of PE [10].

The intervillous space is exposed to altering oxygen gradients during the first 10–12 weeks of gestation [11] during which time EVT invades through the decidua. Following this period of EVT invasion into uterine tissues, placental oxygen tension rapidly increases due to the maternal perfusion of the villous tips [12]. This occurs episodically, and is predicted to have effects similar to those seen in hypoxia/reperfusion, leading to the development of local placental oxidative stress. In the normal early placenta, antioxidants protect the placenta from undue damage due to oxidative stress [11].

The exact mechanism underlying the development of PE remains unknown, but it has been suggested that shallow trophoblast invasion resulting in inadequate spiral artery transformation may underlie its pathogenesis [13]. Increased mitochondrial generation of reactive oxygen species (ROS) and synthesis through xanthine oxidase (XO) [14] and NADPH oxidase result in increased levels of ROS in PE and this is combined with decreased expression of antioxidants [15]. Locally-generated AngII is a potent stimulus to NADPH oxidase secretion [16]. NADPH oxidase is composed of a number of subunits, the catalytic machinery of the enzyme being provided by $gp91^{phox}$. A family of genes homologous to $gp91^{phox}$ known as Nox 1-5 has been discovered; Nox 4 is expressed in fetal tissues, placenta and vascular cells [17].

The objectives of the current study were two-fold. The first was to examine the placental expression of AT1R, AT2R and AT4R, relate this to the expression of NADPH oxidase and XO throughout normal pregnancy and compare the expression with that in PE. The second was to investigate a potential role for these receptors in normal placental development.

2. Materials and methods

After local Ethical committee approval and with appropriate informed consent, placental tissue was obtained from women undergoing elective surgical termination of pregnancy (TOP) during the 1st trimester (early TOP; $n = 10$; gestational age 8.8 ± 0.9 weeks [Mean ± standard deviation]) and early 2nd trimester (mid TOP; $n = 10$; gestational age 12.9 ± 0.9 weeks) and at delivery in the third trimester from 10 women with normal term pregnancy (gestational age 39.4 ± 1.1 weeks) and 10 women with PE (gestational age 37.6 ± 2.6 weeks) who under went Caesarean section prior to labor, as previously described in Ref. [15]. Clinical data for normal term pregnancy and PE subjects is shown in Table 1. Biopsies for immunohistochemistry were fixed in formalin and wax embedded, and 5 μM serial sections were prepared. Biopsies for quantitative real-time PCR (qRT-PCR) were snap frozen and stored at −80 °C for generation of cDNA. Placental explants were prepared as outlined below.

2.1. Immunohistochemistry

Five rabbit polyclonal antibodies and one murine monoclonal antibody (HLA-G) were employed for the immunostaining of paraffin-embedded placental sections. The optimal dilution for each antibody was selected on the basis of maximal specific reactivity and minimal background staining. Single immunohistochemical labelling was performed using the Dako Envision visualisation system following the manufacturer's instructions (Dako UK Ltd, Cambridgeshire, UK). Following microwave heated pretreatment in trisodium citrate buffer (10 mM sodium citrate, pH 6.0) sections were incubated for 30 min in normal swine serum (Dako) to block non-specific binding. Slides were then incubated with either anti-angiotensin II type 1 receptor (Abcam PLC, Cambridge, UK; ab47162; 1:250 for 2 h), anti-angiotensin II type 2 receptor (Abcam; ab19134; 1:500 for 4 h), anti-IRAP (kind gift from Professor S Keller, University of Virginia; 1:500 for 1 h) anti-NOX4 (Abcam; ab60940; 1:1000 for 2 h), anti-xanthine oxidase (H-110) (Santa Cruz Biotechnology, Inc, Heidelberg, Germany; sc-20991; 1 in 200 for 2 h) or anti-HLA-G (Serotec; MCA2043; 1 in 200 for 1 h). Specificity of staining was confirmed using positive control tissue. Kidney tissue was used to confirm AT1R, AT2R and NOX4 staining, placental tissue was used for AT4R and HLA-G, liver for xanthine oxidase, breast carcinoma for caspase 3 and tonsil for Ki67. Negative controls were also performed in each staining run using no primary antibody.

Table 1
Clinical details of term pregnancy subject groups. Data are expressed as mean [standard deviation].

	Normal term pregnancy	Pre-eclampsia	*P* value
Number of subjects	10	10	n/a
Maternal age (years)	28 [8]	32 [6]	ns
Gestational age (weeks)	39.4 [1.1]	37.6 [2.6]	$P < 0.05$
Baby weight (kg)	3.4 [0.4]	2.9 [0.2]	ns
Placental weight (g)	708 [112]	618 [145]	ns
Systolic blood pressure (mmHg)	115 [4]	159 [8]	$P < 0.000$
Diastolic blood pressure (mmHg)	75 [3]	98 [5]	$P < 0.000$
Proteinuria (g/l)	n/a	1.0 [0.6]	n/a
Platelets ($\times 10^9$/l)	n/a	189 [43.9]	n/a
Creatinine (μmol/l)	n/a	64 [9.7]	n/a

2.2. Analysis

All slides were analysed by the same observer (PW) who was blinded to pregnancy stage or outcome. For analysis of placental sections, digital images of 5 randomly selected medium-power (×200) fields were captured on NIS-Elements F2.20 (Nikon UK Ltd, Surrey, UK). Using ImageScope (Aperio Technologies Ltd, Bristol, UK) the total percentage of positive labelled cells per ×200 magnification field was determined using the 'positive pixel count' function. Results are expressed as 'positivity' which takes into account both the number of positive pixels and the intensity of staining. Assessment was performed of both trophoblast and villous stroma. A visual check was performed to ensure accurate discrimination of immunolabelled regions.

2.3. Preparation of mRNA

Total RNA from placental biopsies (50–100 mg) was obtained using 1 ml of Tri-reagent (Sigma, Poole, UK) according to the manufacturer's instructions. Following RNA extraction, 1 μg of each sample was reverse transcribed in a 20 μl reaction using QuantiTect Reverse transcription kit (Qiagen, Crawley, UK) which included a genomic DNA elimination reaction, using a Primus 96 advanced gradient thermocycler (Peqlab Ltd, Fareham, UK). The conditions used to generate first strand cDNA were 42 °C (15 min) and 95 °C (3 min). RNA concentration and quality were verified by gel electrophoresis and spectrophotometrically using a standard conversion factor of one absorbance unit at 260 nm per 40 μg RNA/ml. RNA concentration was adjusted by diluting with nuclease-free water (Ambion Applied Biosystems, Warrington, UK) to a standard concentration of 1 μg/μl. All samples were stored at −80 °C until use.

2.4. Standard curve generation

Standards for AT1R, AT2R, AT4R and for the housekeeping gene TATA box binding protein (TBP) were made from cDNA obtained from a randomly selected control placenta using semiquantitative polymerase chain reaction (PCR). The method used oligonucleotide primers to AT1R (F- TCTCAGCATTGATCGATACC and R-TGACTTTGGC TACAAGCATT; Melting temperature 57 °C), AT2R (F-TATGGCCTGTTTGTCCTCAT and R-CATTGGGCATATTTCTCAGG; 55 °C), AT4R (F-TTCACCAATGATCGGCTTCAG and R-CTCCATCTCATGCTCACCAAG; 60 °C) and TBP (F-TATAATCCCAAGCGGTTTGC and R-GAATATGGTGGGGAGCTGTG; 58 °C) genes generating specific intron-spanning products.

The PCR program comprised an initial denaturation stage (95 °C, 15 min), amplification (stage I, 94 °C (30 s); stage II, melting temperature (30 s); stage III, 72 °C (1 min)), and final extension (72 °C, 7 min; 8 °C 'hold'). The PCR mixture (final volume 20 μl) contained 7 μl nuclease-free water (Ambion), 10 μl thermo-start PCR master mix (ABgene, Epsom, UK), 1 μl forward and reverse primers and 1 μl cDNA. The annealing temperature and cycle number of both primers were optimized and