Placenta 34 (2013) 1066–1071

Contents lists available at ScienceDirect

Placenta

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

No change in calreticulin with fetal growth restriction in human and rat pregnancies

K.E. Crawford [a,b,*], J.L. Stevenson [a], M.E. Wlodek [c], N.M. Gude [a,b]

[a] Department of Perinatal Medicine, Royal Women's Hospital, Parkville 3052, Victoria, Australia
[b] Department of Obstetrics and Gynaecology, University of Melbourne, Parkville 3052, Victoria, Australia
[c] Department of Physiology, The University of Melbourne, Parkville 3010, Victoria, Australia

ARTICLE INFO

Article history:
Accepted 29 July 2013

Keywords:
Calreticulin
Pregnancy
Fetal growth restriction
Human
Rat

ABSTRACT

Introduction: Calreticulin is a ubiquitously expressed protein that was detected in the circulation and is significantly increased in maternal blood during human pregnancy compared to the non-pregnant state. Calreticulin is further increased in the plasma of women with the pregnancy-related disorder pre-eclampsia compared to normotensive pregnancy. The aims of this study were to compare calreticulin in human pregnancy with calreticulin in rat pregnancy, and to compare calreticulin during fetal growth restriction with normal control pregnancies.
Methods: Women were recruited who either had normal pregnancies or had pregnancies complicated with fetal growth restriction; maternal blood samples and placentas were collected. Blood was also taken from women who were not-pregnant. Growth restriction was induced in pregnant rats by uterine vessel ligation; blood and placental samples were collected. Blood was also taken from non-pregnant rats. Western blot was used to quantify the placental expression of calreticulin and the concentrations of calreticulin in plasma.
Results: Although calreticulin was significantly increased in maternal plasma during human pregnancy compared to the non-pregnant state; it did not increase in plasma during rat pregnancy. These results suggest that there may be differences in the role of extracellular calreticulin in human compared to rat pregnancy. Calreticulin was not significantly altered in either placental extracts or maternal plasma in both the human and rat pregnancies complicated by fetal growth restriction compared to gestational matched control pregnancies.
Conclusion: This study found that there was no change in calreticulin during human pregnancy complicated with fetal growth restriction or when growth restriction is induced in rats.

1. Introduction

Calreticulin is a 46 kDa calcium-binding, chaperone protein originally identified in the endoplasmic reticulum. Calreticulin is present in the endoplasmic reticulum of most tissues and cells, including the placenta [1]. Calreticulin has three structurally and functionally distinct domains that contribute to the multi-functional nature of the protein [2]. Acting in association with calnexin, calreticulin is involved in the quality control systems of the endoplasmic reticulum for correct folding of newly synthesised glycoproteins [3]. The importance of calreticulin in cell function is evident by the observations that calreticulin coding sequence is highly conserved in most organisms and that calreticulin null-mice die of severe cardiac abnormalities by mid-gestation [4,5].

It is now well recognised that calreticulin is also located in other intracellular compartments, as well as the extracellular environment [6]. Although calreticulin lacks a transmembrane domain, recent studies identified calreticulin on the surface of many mammalian cells [7–9]. Outside the cell calreticulin has a diverse range of activities including regulation of cell adhesion, migration and facilitating the phagocytosis of apoptotic cells [6]. The addition of exogenous calreticulin can revive a number of calreticulin-dependent functions in calreticulin-null cells [6].

Knowledge about the extracellular function of calreticulin in human diseases is emerging. For example, calreticulin is associated with systemic lupus erythematosus and with celiac disease [10,11]. Circulating calreticulin significantly increases in maternal plasma during early pregnancy and then remains constant to term compared to the non-pregnant state [12]. Furthermore, calreticulin

* Corresponding author. Monash University, School of Nursing and Midwifery, PO Box 527, Frankston 3199, Australia. Tel.: +61 3 9904 4152; fax: +61 3 9904 4130.
E-mail address: kimberley.crawford@monash.edu (K.E. Crawford).

0143-4004/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.placenta.2013.07.068

was significantly increased in the plasma of women with pre-eclampsia between 26 and 39 weeks, in the absence of fetal growth restriction or any other confounding pregnancy complications, compared with age matched healthy pregnant women [12]. It is not known whether changes in either placental or circulating calreticulin are associated with other pregnancy disorders, such as fetal growth restriction.

Fetal growth restriction (FGR, also known as intrauterine growth restriction), like pre-eclampsia, can involve deficient trophoblast cell invasion and abnormal placentation. FGR is associated with reduced growth of the fetus such that the full fetal genetic growth potential is not achieved [13]. Pregnancies complicated by FGR are characterized by impaired placental blood flow, increased vascular resistance and placental dysfunction [14,15]. FGR can be induced in rats by bilateral ligation of the uterine artery and vein causing uteroplacental insufficiency [15,16]. This has been successfully used as a model for late-gestation placental insufficiency in humans, and is characterised by fetuses with asymmetric growth restriction and incomplete postnatal catch up growth [15].

The involvement of calreticulin during FGR has not been explored previously, however, studies on calreticulin-deficient mice reveal that the protein is vital for heart development and embryogenesis [17]. Calreticulin interacts directly with members of the nuclear hormone receptor superfamily of ligand-activated transcription factors, such as the glucocorticoid receptor [18,19]. Calreticulin binds to a conserved amino acid sequence found in the DNA binding domain of the glucocorticoid receptor and inhibits DNA binding and gene transcription [17]. Studies have demonstrated that a crosstalk exists between calreticulin and transcription factors; transcription factors can regulate calreticulin expression but calreticulin can also control the activity of the transcription factor [17]. Glucocorticoids have influential effects on tissue development and glucocorticoid receptors are expressed in most fetal tissues from early embryonic stages [19]. Glucocorticoid overexposure during late fetal life is associated with intra-uterine growth retardation and programmed hypertension in adulthood [20].

The aim of this study was to quantify the placental expression of calreticulin in FGR and the concentrations of calreticulin in plasma during human and rat pregnancy. Calreticulin was measured in placenta and in maternal plasma in human pregnancies that were either normal or complicated with FGR and in rat pregnancies that were either sham control or had induced fetal growth restriction by uterine vessel ligation. Calreticulin was also measured in human and rat plasma from subjects that were non-pregnant and compared to plasma taken during pregnancy.

2. Materials and methods

2.1. Patient recruitment and sample collection

Subjects for this study were women presenting to the Royal Women's Hospital for antenatal care or obstetric care. Clinical research midwives recruited all the women and written informed consent was obtained prior to inclusion in the study. The choice of each subject was based on a review of the patient's obstetric history obtained from routine antenatal data sheets and pregnancy outcome. Patients were assigned to three groups; those diagnosed with FGR ($n = 23$), normal control pregnancies ($n = 24$) or non-pregnant women ($n = 12$). Women diagnosed with FGR were not confounded by pre-eclampsia or other disorders. Ultrasound data were used to prospectively identify pregnancies complicated with FGR. FGR was classified as birth weight less than the 10th percentile for gestational age and at least one of the following: asymmetric fetal growth, abnormal umbilical artery end diastolic flow and reduced liquor volume. Women chosen for the control group did not have FGR, pre-eclampsia or any other confounding pathology. Women were invited to donate a specimen of blood or their placenta. Placental samples were taken from a mix of pre-term and term pregnancies.

For placental collection, deliveries were by Caesarean section, normal vaginal delivery or operative vaginal delivery. The indications for pre-term vaginal delivery or Caesarean section were either the presence of FGR or placenta praevia, spontaneous rupture of membranes with fetal distress and previous Caesarean section or poor past obstetric history. All control patients gave birth to normally formed babies with birth weights appropriate for gestational age and the placenta was normal.

Tissue was extracted from placentas within 20 min of delivery. Approximately 1 g of placental tissue was dissected randomly from multiple regions from the maternal side of each placenta and rinsed briefly in 0.9% saline, before being snap frozen in liquid nitrogen and stored at −80 °C prior to extraction.

Maternal blood was collected by the clinical research midwives during one of the women's visits to the hospital, not during delivery. The age of the women was not significantly different; samples were matched to an appropriate control and ages ranged from 17 to 40 years old. Blood was collected into EDTA tubes, centrifuged at 2400 rpm for 15 min at 4 °C and platelet-poor plasma was stored at −80 °C until required. Mean gestational ages are stated in the results section.

2.2. Uteroplacental insufficiency in the pregnant rat

Wistar Kyoto (WKY) rats (9–13 weeks of age) were exposed to 12 h light and 12 h darkness each day and housed in a temperature-controlled room at 19–22 °C. A vaginal impedance reader (Model MK-10B; MuKomachi Kikai, Osaka, Japan) was used to detect pro-oestrus in the rats. Rats in pro-oestrus were placed with a male overnight. A vaginal smear was performed the following morning to detect the presence of sperm and this was taken as Day 1 of pregnancy. On day 18 of gestation, pregnant rats were randomly assigned to the uteroplacental restriction (UPR) or control groups. Uterine vessel ligation was performed to induce FGR [15,16]. An intravenous tail vein injection containing a mixed solution containing ketamine (Parnell Laboratories, Alexandria, NSW, Australia; 50 mg kg^{-1} bodyweight) and Ilium Xylazil-20 (Troy Laboratories, Smithfield, NSW, Australia; 10 mg kg^{-1} bodyweight) was used to anaesthetise the rats. The UPR surgery involved a midline abdominal incision and only the cervical end of the uterus was exposed with clear access to the uterine artery and vein which were ligated. Surgery was also performed in the same manner on the sham control group but the uterine vessels were not ligated.

2.3. Rat tissue and plasma collection

At day 20 of pregnancy, pregnant rats were anaesthetised with intraperitoneal injection of ketamine (100 mg (kg body weight)$^{-1}$) and Ilium xylazil-20 (30 mg (kg body weight)$^{-1}$) [21]. Placentas and fetuses were removed and weighed separately. Placental tissue was separated into the zona basalis and the labyrinth, frozen in liquid nitrogen and stored at −80 °C until analysed. Maternal blood was removed at day 20 of pregnancy by cardiac puncture. Blood was centrifuged at 2400 rpm at 4 °C for 15 min and plasma was frozen in liquid nitrogen and stored at −20 °C until analysed.

2.4. Human and rat protein extraction

Human and rat snap frozen placental tissue was homogenised in 2 ml lysis buffer per 250 mg of tissue that contained 50 mM glycine buffer (BioRad, USA) with 0.5% Triton X-100 (BDH, Australia), 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (Sigma–Aldrich, USA) and 5 mM EDTA (Merck, Germany). The homogenised samples were centrifuged at 3500 g for 10 min at 4 °C to sediment any insoluble material. The protein concentration of the supernatant was determined by the Bradford Protein Assay (ThermoScientific) and bovine serum albumin was used as a standard.

2.5. Western immunoblotting

Plasma samples (0.75 μl) and placental extracts (0.75 μg) were diluted with tris-buffered saline (TBS) as required and mixed with XT sample loading buffer containing XT reducing agent (BioRad). Samples were heated at 95 °C and along with dual standard molecular weight markers (161-0374, BioRad) loaded onto a 4–12% Bis Tris Criterion XT Precast gel (BioRad). SDS PAGE was performed at 125 V for 2 h in XT MOPS Running Buffer (BioRad). Protein was transferred from the gel to PVDF membrane using a traditional wet transfer method. Following transfer, membranes with human samples were blocked in 5% Skim milk/TBS and with rat samples, the membrane was incubated with the TNB 0.5% (PerkinElmer Life Sciences, USA) blocking reagent overnight at 4 °C. All membranes were incubated overnight at 4 °C with the polyclonal rabbit anti-human calreticulin antibody (C4606 Sigma–Aldrich) at a concentration of 3.95 μg/ml in 2.5% skim milk/TBS. After washes in TBS, the membranes were incubated with the HRP-conjugated polyclonal swine-anti rabbit antibody (Dako, USA) (antibody concentration 1.3 μg/ml) in 2.5% skim milk/TBS for 1 h at room temperature. Lumi-Light Western Blotting Substrate (Roche Diagnostics Corporation, IN, USA) was added to the membranes and immunopositive bands captured on X-Ray film (Kodak, NY, USA). Semi-quantitative estimates of the protein bands were obtained by scanning films on a densitometer (GE Healthcare) and analysis of the relevant bands were conducted with ImageQuant 7.0 software (Molecular Dynamics Pty. Ltd). Western blots that compared placental samples from pregnancies with FGR with gestation matched controls were normalised to total protein load. Western blots that compared plasma samples were normalised according to plasma volume.