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Localisation and characterisation of uncoupling protein-2 (UCP2) in the human preterm placenta

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ABSTRACT

The increase in oxidative stress during pregnancy is associated with increased placental antioxidant enzyme activity and may additionally be limited by the uncoupling proteins (UCPs). There is little data on the expression and localisation of UCP2 in the human preterm placenta or on its role in the regulation of placental oxidative stress. Placentae were collected from women with singleton pregnancies who delivered between 24 and 36 weeks gestation ($n = 54$) and from a term reference group who delivered following uncomplicated pregnancy ($n = 11$). UCP2 expression and localisation was determined by quantitative real-time RTPCR using Taqman gene expression assays and immunohistochemistry. Placental lipid hydroperoxide and nitrotyrosine content was determined by ELISA. UCP2 mRNA expression increased from 24 to 41 weeks gestation ($p < 0.001$) and was positively correlated with placental weight ($p = 0.004$). While UCP2 expression was lower in small for gestational age infants ($p = 0.045$) it did not differ with respect to timing of antenatal betamethasone exposure nor with placental lipid hydroperoxide or nitrotyrosine content. UCP2 staining was identified in the cytotrophoblast in 34% of samples and in the syncytiotrophoblast in 63% of samples. Cytotrophoblast staining was more frequent in later gestations ($p = 0.03$) with syncytiotrophoblast UCP2 staining was not altered by gestation. In the preterm group, no association was observed with time since antenatal betamethasone exposure or placental lipid hydroperoxide or nitrotyrosine content. The current data supports gestation dependant alterations in UCP2 mRNA expression and immunohistochemical localisation in the human placenta but no evidence for an important role for UCP2 in protection against placental oxidative damage.

1. Introduction

Under normal conditions, a delicate balance exists between the production of reactive oxygen species (ROS) and the antioxidant defences that protect the human placenta and fetus [1,2]. The increase in oxidative stress that occurs during pregnancy is temporally associated with increases in the level and activity of placental antioxidant enzymes [3] with the physiological role of placental oxidative stress only beginning to be elucidated [4]. However, in conditions that complicate pregnancy and result in preterm birth, including inflammation and placental insufficiency, oxidative damage is exaggerated [5]. Further, excessive ROS production plays a significant role in the development of oxygen radical diseases of the newborn such as broncho-pulmonary dysplasia, peri-ventricular leukomalacia, and retinopathy of prematurity [6].

In addition to placental antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidise, ROS production may additionally be limited by the uncoupling proteins (UCPs). UCPs constitute a protein super-family of metabolite transporters of the mitochondrial inner membrane [7] which have been proposed to limit oxidative stress [8,9]. UCP1 is present in brown adipose tissue and is involved in non-shivering thermogenesis [10]. UCP2 is expressed in various tissues, including spleen, lung, intestine brain, pancreatic islets and placenta [11], while UCP3 is only found in skeletal muscle and brown adipose tissue. UCP4 and UCP5 are predominantly expressed in the nervous system.

UCP2 is up-regulated in a number of physiological situations-where there is oxidative stress yet the evidence for the involvement

Abbreviations: ROS, reactive oxygen species; UCP, uncoupling protein; PBS, phosphate buffered saline; IHC, immunohistochemistry; SGA, small for gestational age; HNE, 4-hydroxy-2-nonenal.

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of UCP2 as a defence mechanism against oxidative stress is largely circumstantial [12]. UCP2 mRNA is widely expressed yet UCP2 protein has been detected in only a few tissues [13] with the protein level of UCP2 not simply proportional to the mRNA level. In the placenta, UCP2 has been shown to increase across gestation in the rat and sheep [14,15] with placental expression altered by antenatal exogenous glucocorticoid exposure [15] and nutrient restriction [14,16]. Currently, there is little data on the expression of UCP2 and its localisation in the human placenta. Further, it is unknown if UCP2 is involved in the regulation of placental oxidative stress in humans or, indeed, if it is altered in human placental pathologies.

We have recently reported alterations in placental ROS production and antioxidant enzyme activities following antenatal glucocorticoid exposure in human pregnancies complicated by preterm birth [17]. In the current study we tested the hypothesis that human placental UCP2 expression would increase across gestation and would be altered by antenatal glucocorticoid exposure which is known to influence feto-placental ROS production and antioxidant defences [17,18].

2. Method

2.1. Participants

Women with singleton pregnancies who presented in preterm labour between 24 and 36 weeks gestation ($n = 54$) were recruited as part of a prospective cohort study at the John Hunter Hospital (NSW) as previously described [19]. In addition a term reference group ($n = 11$) were recruited following uncomplicated pregnancy. The study was approved by the human ethics committees of the University of Newcastle, the John Hunter Hospital, Newcastle, NSW, and the University of Adelaide, South Australia. Exclusion criteria included the presence of a major congenital malformation or receipt of no antenatal betamethasone therapy. Placentae were collected and processed within 45 min of delivery with multiple full thickness tissue blocks were collected prior to washing in ice-cold phosphate buffered saline. One portion was snap frozen in liquid nitrogen prior to storage at −80 °C for subsequent analysis, while the other portion was fixed in formalin and embedded in paraffin for immunohistochemical (IHC) analysis.

Betamethasone mediated effects on the fetus and placenta are known to be dependent upon the timing of exposure [19,20], with emerging evidence for increased oxidative stress [17,21] and impaired antioxidant defences [17,18] in those neonates and placenta delivered within 72 h of antenatal GC exposure. Therefore, preterm subjects were grouped a priori into those delivered within 72 h and those delivered greater than 72 h of the last maternally administered betamethasone dose. All women received at least one dose of betamethasone (11.4 mg Celestone). No participant received more than two doses of betamethasone, administered 24 h apart. Following delivery, infant sex, birth weight, and placental weight were recorded. Customised birth weight centiles were calculated using the perinatal network calculator (www.gestation.net, Australian version).

2.2. RNA extraction and relative mRNA quantification

Total placental RNA was extracted from pooled placental samples using previously described methods [22] and purified (RNeasy Minikit, Qiagen). Optical density was used to determine RNA concentration and RNA quality and integrity was confirmed using a bioanalyser (Agilent). RNA (10 ng) was reverse-transcribed using the Taqman RT kit, according to the manufacturer's directions (Applied Biosystems). The abundance of mRNA relative to the housekeeping gene (β-actin) was determined by quantitative real-time RTPCR using Taqman gene expression assays (UCP2 Hs01075227_m1; β-actin 4352935E). The PCR contained 10 ng reverse-transcribed sample, diluted in 4.5 μl RNase free water, 5 μl Taqman Master Mix (Applied Biosystems) and 0.5 μl of the appropriate Taqman gene assay (containing primer and Taqman probes; Applied Biosystems). The analysis was performed in duplicate samples using the 7500 Fast Real Time PCR Detection System. A duplicate no template control was used for each primer. The comparative CT (cycle threshold) method was used to determine relative mRNA abundance.

2.3. Protein extraction

Placental tissue (100 mg) was homogenised (Precellys, Bertin Technologies, France) in phosphate buffered saline with protease inhibitors (Roche). The protein concentration of the tissue homogenate was determined by Bradford assay (Biorad) and samples diluted to a final protein concentration of 10 μg/ml.

2.4. Lipid hydroperoxide assay

Lipid peroxidation in placental extracts was determined using a lipid hydroperoxide assay kit (Calbiochem) following the manufacturer's recommended protocol. All samples were assayed in duplicate with lipid hydroperoxide concentration expressed as μM/mg protein.

2.5. Nitrotyrosine assay

Placental 3-nitrotyrosine, produced following the reaction of superoxide with nitric oxide to form peroxynitrite with subsequent modification of protein tyrosine residues, was determined by commercial chemi-luminesence assay (Millipore). All samples were assayed in duplicate with the 3-nitrotyrosine concentration expressed as μg/mg protein.

2.6. Immunohistochemistry

Two micron paraffin-embedded sections were processed according to standard immunohistochemical protocol using the labelled streptavidin biotin method (LSAB2 System-HRP Kit, DAKO, Glostrup, Denmark). Briefly, following incubation on a heating block (at approximately 60 °C for 2 h), slides were deparaffinised in xylene, rehydrated in ethanol, and washed in phosphate buffered saline (PBS). Following PBS washes, endogenous peroxidase activity was quenched in 3% hydrogen peroxidase for 20 min. Additional PBS washes were followed by incubation with 5% fetal bovine serum for 30 min to prevent non-specific antibody binding. Primary antibody to UCP2 (C-20, Santa Cruz, diluted 1/200 in Dako antibody diluent) was applied to the sections and incubated overnight at 4 °C. Slides without primary antibody were included as negative controls. Additionally, mouse heart and hypothalamus were used as a negative and positive control respectively. After washing with PBS, the biotinylated secondary antibody, Rabbit Anti-Goat (Dako), was applied for 30 min. Following PBS washes, sections were incubated with Streptavidin-horseradish peroxidase (Dako LSAB2 kit) for 30 min. Immunoreactivity was visualized by incubation of sections for 6 min with 3,3′-diaminobenzidine in the presence of hydrogen peroxide (Dako LSAB2 kit). Sections were counterstained with 1/10 dilution of Lillie Mayer's haematoxylin. The sections were then dehydrated and a coverslip was applied using DPX mounting media. Intensity of staining in ten fields of vision was assessed by three blinded assessors into the categories 0 = no staining, 1 = low, 2 = moderate and 3 = high.

2.7. Statistics

All data was analysed using SPSS (v18). ANOVA was used to assess the effects of time since steroid exposure on normally distributed data. Chi-squared tests were used to assess frequency data, including the median immunohistochemistry scores. Spearman's correlations were used to assess the relationships between continuous, non-normally distributed data. Statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Clinical characteristics

All infants in the term reference group were delivered following uncomplicated pregnancy with a median (SD) gestation of 39 (2) weeks and birth weight centile of 48 (19). No infants in the term group were SGA or had been exposed to antenatal betamethasone earlier in gestation. The clinical characteristics of the 54 women and their infants who delivered preterm are described in Table 1, according to time of antenatal betamethasone exposure prior to delivery. There was no difference in gestational age, birth weight, birth weight centile or placental weight between those infants who delivered within 72 h compared to greater than 72 h after maternal betamethasone administration. The rate of maternal obstetric complications did not vary between the groups, nor did the incidence of significant neonatal morbidities or clinical indices of physiological stability (CRIB II score [23]). A significantly higher mortality rate was observed in those infants born within 72 h of last antenatal betamethasone exposure ($p = 0.03$).

3.2. Placental UCP2 mRNA expression

Placental UCP2 mRNA expression significantly increased from 24 to 41 weeks gestation (Spearman's rho = 0.525, $p < 0.001$) (Fig. 1A). A positive correlation was observed for placental UCP2 mRNA and