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Influence of speed of sample processing on placental energetics and signalling pathways: Implications for tissue collection☆

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ABSTRACT

Introduction: The placenta is metabolically highly active due to extensive endocrine and active transport functions. Hence, placental tissues soon become ischaemic after separation from the maternal blood supply. Ischaemia rapidly depletes intracellular ATP, and leads to activation of stress-response pathways aimed at reducing metabolic demands and conserving energy resources for vital functions. Therefore, this study aimed to elucidate the effects of ischaemia *ex vivo* as may occur during tissue collection on phosphorylation of placental proteins and kinases involved in growth and cell survival, and on mitochondrial complexes.

Methods: Eight term placentas obtained from normotensive non-laboured elective caesarean sections were kept at room-temperature and sampled at 10, 20, 30 and 45 min after delivery. Samples were analyzed by Western blotting.

Results: Between 10 and 45 min the survival signalling pathway intermediates, P-AKT, P-GSK3α and β, P-4E-BP1 and P-p70S6K were reduced by 30–65%. Stress signalling intermediates, P-eIF2α increased almost 3 fold after 45 min. However, other endoplasmic reticulum stress markers and the Heat Shock Proteins, HSP27, HSP70 and HSP90, did not change. Phosphorylation of AMPK, an energy sensor, was elevated 2 fold after 45 min. Contemporaneously, there was an ~25% reduction in mitochondrial complex IV subunit I.

Discussion and conclusions: These results suggest that for placental signalling studies, samples should be taken and processed within 10 min of caesarean delivery to minimize the impact of ischaemia on protein phosphorylation.

1. Introduction

Placental dysfunction lies at the heart of the 'Great Obstetrical Syndromes', including growth restriction, pre-eclampsia, pre-term delivery and stillbirth. These syndromes are related to a varying degree with a deficiency in deep trophoblast invasion, and subsequent remodelling of the uterine spiral arteries [1]. Over the last few years, considerable progress has been made in elucidating the pathophysiological changes within the placenta at the molecular level. Studies have revealed increased oxidative stress, and activation of stress-response signalling pathways consistent with malperfusion [2–4]. Unlike histological changes, which are relatively slow processes, alterations in transcript abundance and activation of signalling pathways occur rapidly in response to external stimuli. The placenta inevitably undergoes a period of ischaemia after delivery, following separation from the maternal arterial supply. Therefore, the speed of tissue sampling and processing is likely to be crucial in molecular studies in order to avoid the introduction of *ex vivo* artefacts.

The placenta has a high rate of oxygen and glucose consumption, reflecting its high metabolic activity [5–7]. This can be accounted for by the existence of numerous active transport systems for maternal–fetal transfer of nutrients, and its endocrine function. The majority of nutrients including amino acids, vitamins, Ca^{2+} and other biomolecules, such as antibodies, are reliant on

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primary or secondary active transport systems, which utilize energy either directly linked to the hydrolysis of adenosine triphosphate (ATP) or provided by ion gradients such as sodium, chloride, and protons [8]. In the sheep placenta, approximately 25% of total oxygen uptake is used to generate ATP to support cation co-transport systems, such as Na^+-K^+ ATPase, which creates a Na^+ gradient that is the basis for secondary active transport of amino acids and other substances [9].

Additionally, the placenta is one of the most active endocrine organs, synthesizing and secreting large quantities of polypeptide hormones, such as human chorionic gonadotrophin, human placental lactogen (hPL), and many growth factors, including insulin growth factor 2 and placental growth factor. As 4 ATP molecules are required for a single peptide bond formation, protein synthesis consumes a large fraction of cellular energy. Carter estimated that approximately 30% of the total placental oxygen consumption is used for protein synthesis [6].

This high metabolic activity suggests that the placenta is likely to undergo ischaemic changes rapidly following delivery, with depletion of its intracellular energy reserves. Indeed, the concentrations of high-energy phosphates and other cellular metabolites are reduced within 24 min of separation from the maternal blood supply [10]. This reduction could have a serious impact on energy-dependent cellular processes, including protein synthesis, active transport and ion transporters. As a result, stress-response signalling pathways aimed at restoring homoeostasis will be activated. A classic example is suppression of mRNA translation in an ATP-dependent manner that aids survival of cells under hypoxia [11,12].

In this study, our aim was to investigate the effects of *ex vivo* ischaemia, as would be experienced by delayed processing of placental samples, on activation of stress response pathways and suppression of growth and proliferation signalling, including the energy sensor AMP activated protein kinase (AMPK); cell growth, metabolism, and stress signalling pathways, such as AKT-mTOR and mitogen activated protein kinases (ERK1/2, p38 kinase and JNK); ER stress response pathways; and heat shock protein family members.

2. Materials and methods

All chemicals were from Sigma–Aldrich. Anti-IRE1α (phospho S724), anti-IRE1α, anti-ATF6, anti-KDEL and anti-HSP40 were from Abcam (Cambridge, UK). Anti-HSP90 and anti-HSP70 were from Enzo Life Science (Exeter, UK). Anti-GRP78 was from Transduction Laboratories (BD Biosciences, Oxford, UK), and anti-β-actin was from Sigma–Aldrich. Other antibodies were purchased from Cell Signalling Technology (New England BioLabs, Hitchin, UK).

2.1. Tissue collection

The study was approved by the Cambridge Local Research Ethics Committee and all participants gave written informed consent. Eight human term (38–40 weeks) placentas were obtained from normal uncomplicated singleton first pregnancies after elective non-laboured caesarean section.

Separation of the placenta from the uterus was designated as $t = 0$ min. As it is standard procedure in our hospital for the midwife to check placental integrity, the earliest time the placenta was accessible was between 5 and 10 min after separation. Therefore, for consistency, the placentas were kept at room temperature and repetitively sampled at 10, 20, 30 and 45 min after separation. To minimize possible contamination with maternal decidual tissue, the surface of the basal plate was removed with scissors to a depth of approximately 1–2 mm. To avoid regional variations, all samples were taken from the same placental lobule. At each time-point approximately a 1 cm^3 piece of villous tissue was taken from the selected lobule. This was rinsed twice in ice-cold PBS, and several small pieces (~10 mg and ~50 mg) were quickly cut off and further washed in cold PBS. These were blotted dry and snap frozen in liquid nitrogen. This post-sampling procedure took approximately 2 min. All tissues were stored at −80 °C freezer for further analysis.

2.2. Western blotting

Details of the procedures have been previously described [13]. Briefly, a tissue lysate, was prepared using Matix D and FastPrep Homogenizer (MP Biomedicals UK, Cambridge, UK) and the Bicinchoninic Acid (BCA) used to determine protein concentration. Equal amounts of protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes. After incubation with primary and secondary antibodies, enhanced chemiluminescence (ECL, GE Healthcare, Little Chalfont, UK) and X-ray film (Kodak, Hempstead, UK) were used to detect the bands. Multiple exposure times were employed when necessary. Unsaturated bands were scanned using HP Scanjet G4050 (HP, UK) and band intensities quantified by Image J (Freeware).

2.3. Statistical analysis

Given the number of placentas and time points, it was necessary to run 2 gels per analyte. In order to combine data across the gels, densitometric values were normalized to the mean of the 10 min sample values for all the samples run on the same gel. The distribution of the normalized values was assessed using the Shapiro–Wilk test. If the distribution was non-normal, seven arithmetic transformations were evaluated and the optimal method for generating a normal distribution selected. These tests were carried out using the statistical language R (version 3.0.1). For each analyte, differences between the means of the transformed data for each time point were tested using one-way ANOVA with repeated measures, with the Tukey correction for multiple comparisons. These analyses were carried out using Prism GraphPad version 6.0. We used the method of Benjamini and Hochberg to control the false discovery rate due to multiple testing when groups comprised 10 or more analytes [14]. Significance levels were set at $p < 0.05$ or adjusted $p < 0.05$.

3. Results

3.1. Activation of AMPK is associated with a reduction in mitochondrial complex IV subunit I protein level

Activation of the energy sensor AMPK [15] by phosphorylation was investigated in the time series of placental samples. Although the levels of phosphorylated and total AMPKα did not change significantly (Suppl. Fig. 1A), the relative ratio of phosphoryated to total AMPKα (P-AMPKα/AMPKα) showed a 2-fold significant increase ($p = 0.022$) at the 45 min time point (Fig. 1A and C).

Mitochondria are the major source of intracellular ATP production in eukaryotic cells. Therefore, accumulation of AMP, which activates AMPK, suggests that mitochondrial activity in the placentas may be compromised by *ex vivo* ischaemia. Indeed, the electron acceptor complex IV subunit I in the electron transport chain (ETC) was reduced significantly ($p = 0.016$) at 45 min after separation, although the protein level of other subunits in complexes I, II, III and V were not affected (Fig. 1B and D; Suppl. Fig. 1B). Loss of subunits of the ETC complexes could result in reduction of mitochondrial activity, which in turn compromises intracellular energy production.

3.2. Activation of ER stress signalling

In response to intracellular energy depletion, stress pathways are activated in an attempt to restore cellular homoeostasis. Therefore, we investigated changes in phosphorylation or protein level of key members of the most common stress signalling pathways, including the ER stress response, MAPK stress kinases, and heat shock proteins.

Protein synthesis is highly energy demanding. Therefore, we first looked at the ER stress response pathway. There was a significant ($p = 0.0075$) and incremental elevation of phosphorylation of eukaryotic initiation factor 2 subunit alpha (P-eIF2α); ~1.5 fold by 30 min, and almost 3 fold by 45 min compared to either 20 and 10 min respectively (Fig. 2A). However, other ER stress markers, including phosphorylation of IRE1α and the levels of ATF6, GRP78 and GRP94, were relatively constant (Fig. 2A; Suppl. Fig. 2A).

Next, we examined MAPK stress kinases and HSPs pathways. In the MAPK family, the relative ratios between phosphorylated and total proteins of two JNK isoforms, p54 and p46, exhibited a significant reduction (p54, $p = 0.033$ and p46, $p = 0.02$) following delayed processing (Fig. 2B). Another MAPK, p38 kinase, did not change (Suppl. Fig. 2B). Cytosolic heat shock proteins P-HSP27, HSP27, HSP70 and HSP90 also remained relatively constant throughout the 45 min period studied (Suppl. Fig. 2C).