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

H.W. Yung [a,*], F. Colleoni [a], D. Atkinson [a], E. Cook [b,c], A.J. Murray [a], G.J. Burton [a,c], D.S. Charnock-Jones [a,b,c]

[a] Centre for Trophoblast Research, Department of Physiology, Development and Neuroscience, University of Cambridge, Downing Street, Cambridge CB2 3EG, UK
[b] Department of Obstetrics and Gynaecology, University of Cambridge, Rosie Hospital, Robinson Way, Cambridge CB2 0SW, UK
[c] National Institute for Health Research Cambridge Comprehensive Biomedical Research Centre, UK

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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

* Corresponding author. Tel.: +44 1223 333862.
E-mail address: hwy20@hermes.cam.ac.uk (H.W. Yung).

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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).

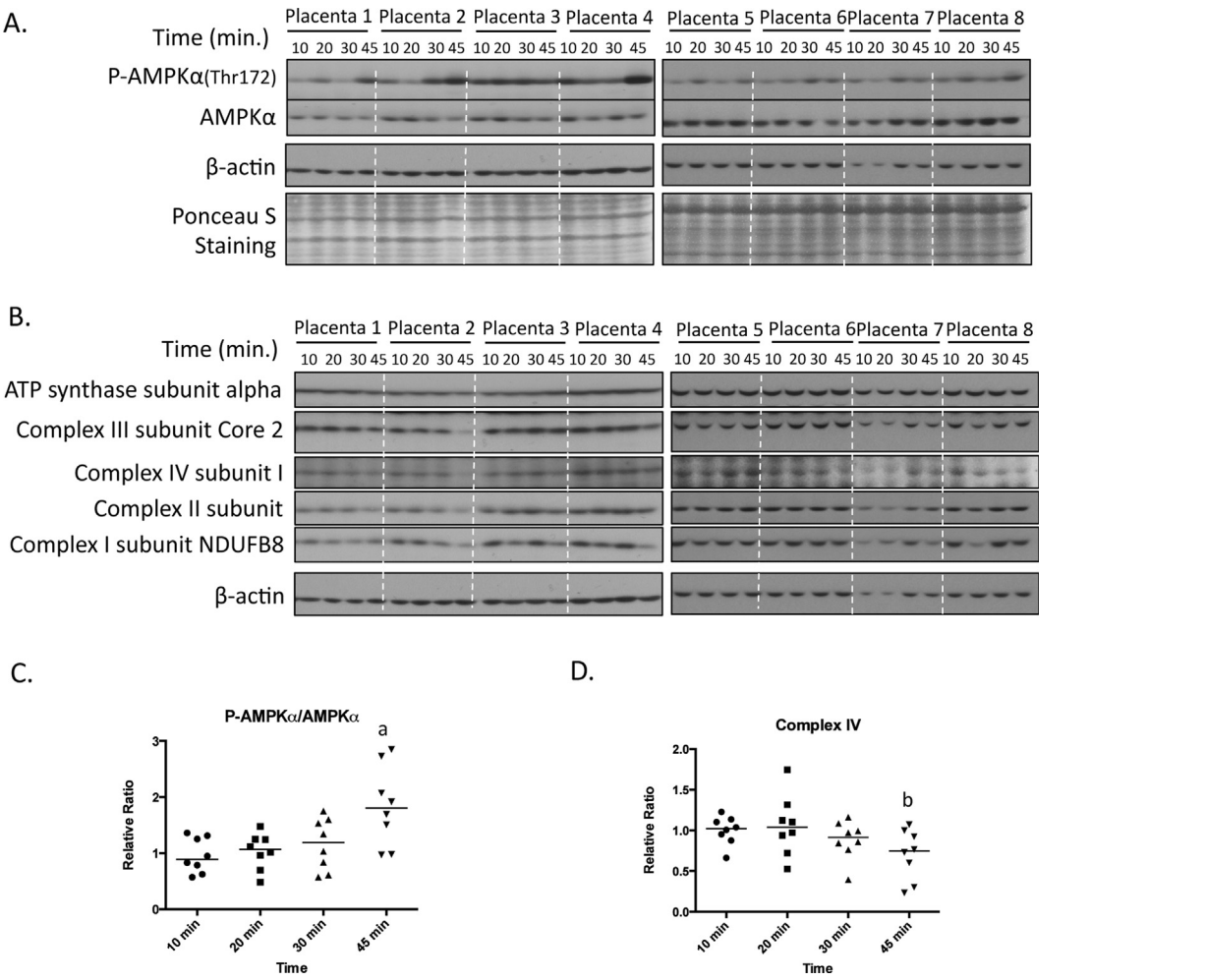

Fig. 1. AMPK phosphorylation and mitochondrial ETC complexes protein level following ischaemia *ex vivo*. Western blotting analysis was used to assess the phosphorylation of AMPKα and level of subunits in ETC complexes. Both β-actin and Ponceau S staining were used to show equal loading among samples. A) AMPKα; B) Mitochondrial complexes subunits. C & D) Densitometry was used to quantify band intensities. Each data point was calculated relative to the mean value of the 10 min values. In the dot plot graphs, the median of the group is shown, $n = 8$. "a" and "b" denote significance $p < 0.05$ compared to 10 and 20 min respectively. C) P-AMPKα/AMPKα. D) Mitochondrial complex IV subunit I.

These data confirm that specific stress-response pathways are activated and change progressively in placental tissues as time elapses after separation.

3.3. Rapid loss of AKT-mTOR signalling

Signalling pathways regulating cell growth and metabolism are sensitive to cellular energy levels. We therefore examined the phosphorylation status of kinases and proteins in the AKT-mTOR pathway, a central regulatory pathway for cell growth and metabolism. Full activation of AKT is reliant on phosphorylation of residues at both Ser473 and Thr308 [16]. Interestingly, phosphorylation of AKT at Ser473 was reduced significantly throughout the period ($p = 0.006$), and by 60% and 65% at 30 min and 45 min respectively. In contrast, phosphorylation of the Thr308 residue, which is phosphorylated by upstream PI-3K-PDK1 signalling [17], was relatively constant compared to the 10 min control (Fig. 3; Suppl. Fig. 3).

Glycogen synthase kinase 3 (GSK-3) is a downstream target of the AKT pathway whose activity is inhibited by AKT-mediated phosphorylation at Ser21 of GSK-3α and Ser9 of GSK-3β. The loss of AKT phosphorylation was associated with ~30% ($p = 0.014$) and ~40% ($p = 0.006$) decrease in phosphorylation of GSK-3α and β respectively (Fig. 3B).

Furthermore, phosphorylation of AKT at the Ser473 residue is regulated by mTOR complex 2 (mTORC2) [18,19]. Therefore, the reduction in Ser473 phosphorylation might indicate a decrease in mTOR signalling. Indeed, the phosphorylation level of two downstream substrates of mTOR complex 1 (mTORC1), 4E-binding protein 1 (4E-BP1) and p70 S6 kinase [20], were inhibited by over 60% ($p = 0.0056$) and ~40% ($p = 0.0064$) respectively after 45 min (Fig. 3A and B). The other survival kinase in the MAPK pathway, ERK, was not changed after 45 min from separation (Suppl. Fig. 3).

These results indicate that the activity of the AKT/mTOR pathway was rapidly suppressed by the ischaemic insult.

4. Discussion

The majority of cellular processes, such as phosphorylation, protein translation and ion transporter activity, are sensitive to intracellular energy levels. Our results show a reduction in phosphorylation of proteins and kinases involved in AKT-mTOR signalling, including 4E-BP-1, GSK-3, and AKT. There are also changes in the eIF2α and JNK stress signalling pathways with increasing duration of ischaemia (Figs. 2 and 3). The changes in phosphorylation of 4E-BP1 and eIF2α are two key molecular mechanisms involved in the inhibition of protein synthesis [21], and were observed at 30 min after separation. Coincidently, the level of

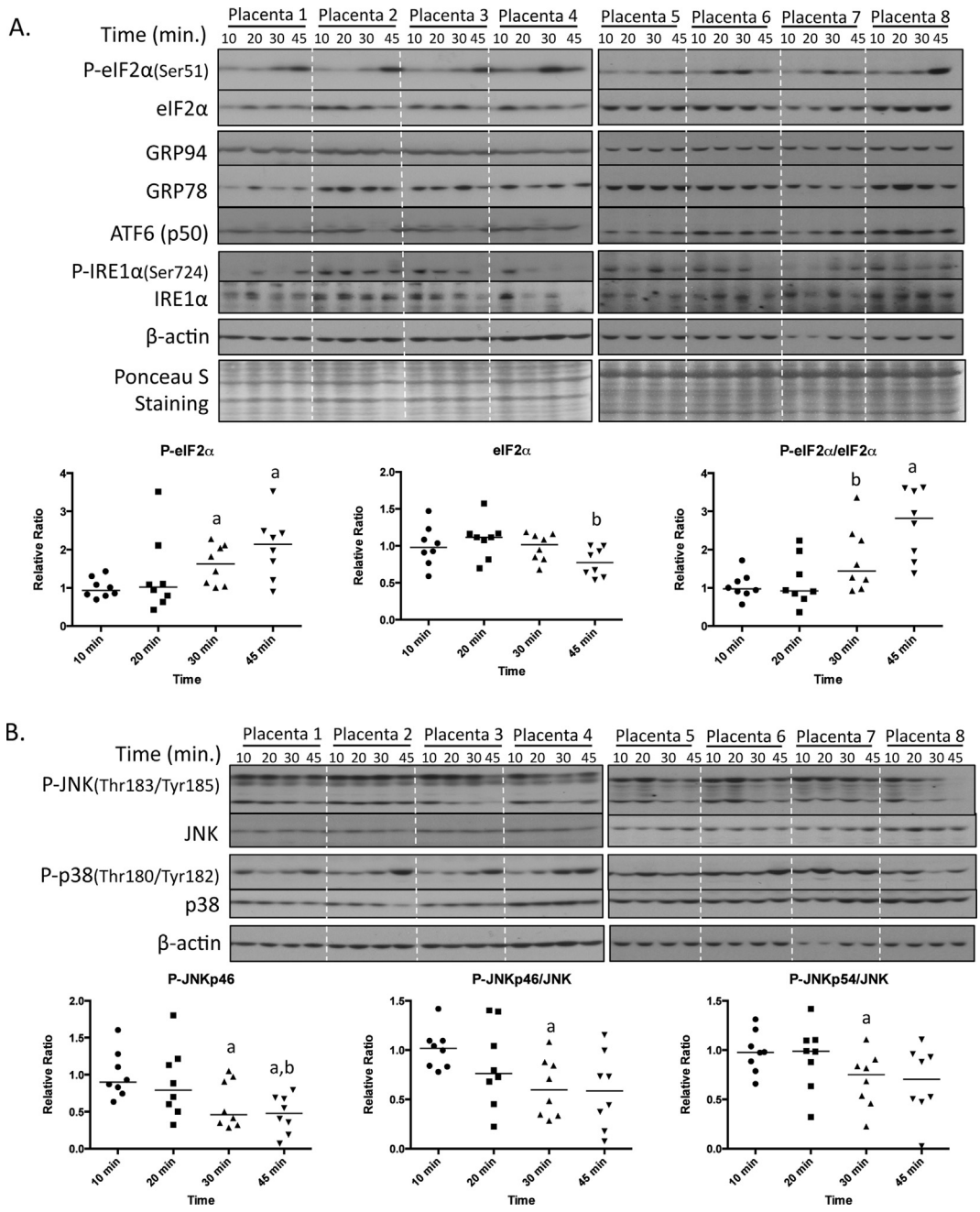

Fig. 2. Phosphorylation of eIF2α and JNK. Upper panel, protein lysates were resolved by SDS-PAGE and probed with antibodies specific for total and phosphorylated kinases or proteins: ER stress markers, and MAPK family members. Both β-actin and Ponceau S staining were used to show equal loading among samples. Lower panel, densitometry was used to quantify band intensity. Each data point was calculated relative to the mean value of the 10 min values. In the dot plot graph, the median of the group is shown, $n = 8$. "a" and "b" denote significance $p < 0.05$ compared to 10 min or 20 min respectively. A) ER stress response pathways; B) MAPK pathways.

mitochondrial ETC complex IV subunit I was reduced by over 25% at 45 min. Further experiments, including measurement of the transcript encoding complex IV subunit I and their turnover rate, will be required to confirm any role of protein synthesis inhibition in the reduction of that subunit. However, we recently demonstrated that increased phosphorylation of eIF2α following treatment of JEG3 cells with salubrinal, a specific eIF2α phosphatase inhibitor, was sufficient to down-regulate complex IV subunit I, indicating a potential direct regulation of translation of those proteins by the eIF2α pathway [22]. Furthermore, swelling of mitochondria and other organelles has been reported at 10 min after placental separation [23], indicating loss of ionic homoeostasis most likely due to compromised ion transporter activity. Taken together, these mitochondrial impairments could serve as a positive feedback loop to further reduce cellular energy production. Therefore, prolonged ischaemia (over 45 min) will eventually induce necrotic cell death due to severe energy depletion, resulting in loss of cell integrity and tissue damage.

To conclude, the phosphorylation status of specific placental kinases and proteins involved in cell growth, metabolism, and stress signalling is changed by 20 min after placental separation from the uterine wall. Our findings indicate that to avoid ischaemia-induced artefacts in studies focussing on these pathways, placental samples must be collected and processed rapidly following delivery, preferably within 10 min. Furthermore, our previous work has shown that stress-response pathways are

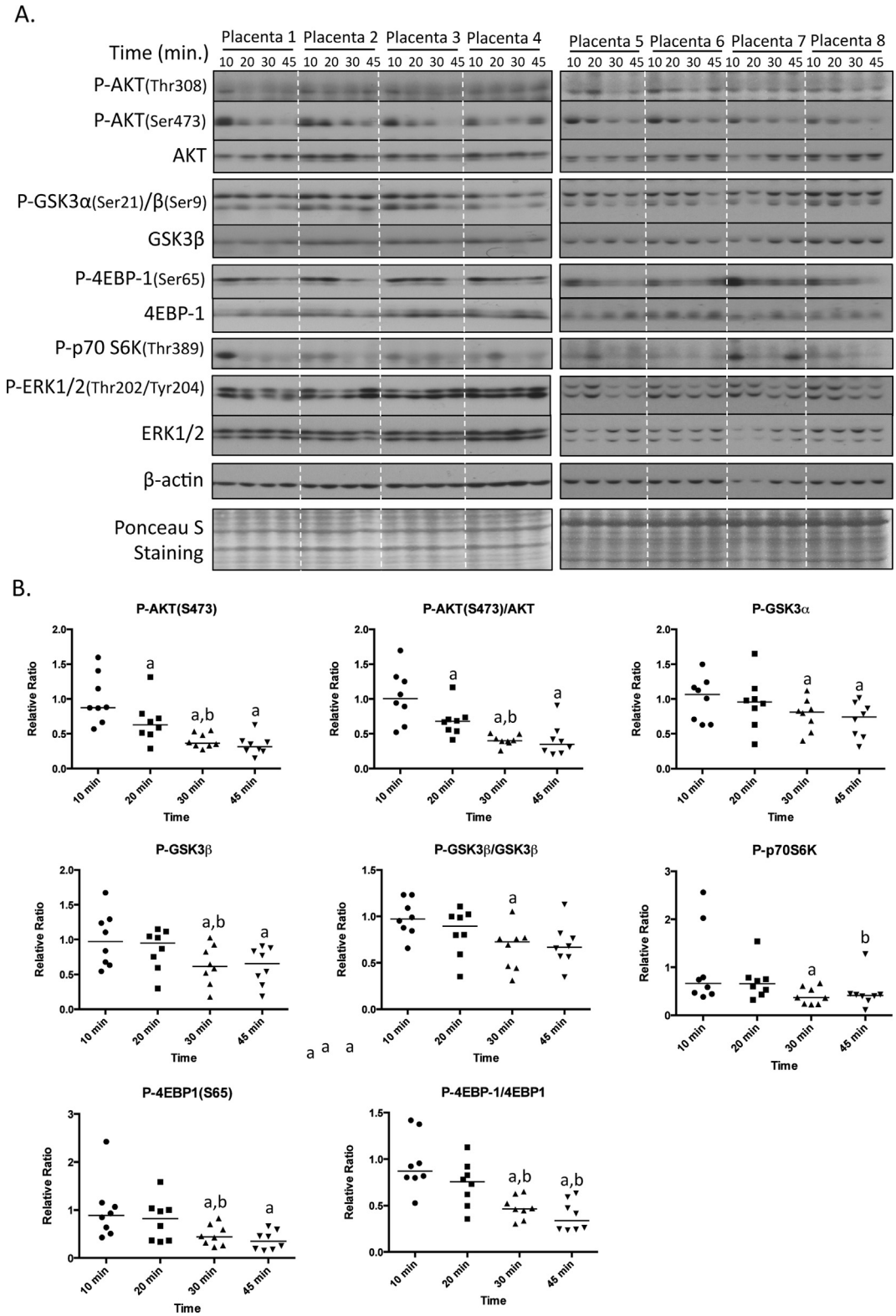

Fig. 3. Phosphorylation of AKT, GSK-3, 4EBP-1 and p70S6K. A) Phosphorylation levels of kinases and proteins in the AKT-mTOR pathway were measured by Western blot. Both β-actin and Ponceau S staining were used to show equal loading among samples. B) Densitometry was used to quantify band intensities. Each data point was calculated relative to the mean value of the 10 min values. In the dot plot graph, the median of the group is shown, $n = 8$. "a" and "b" denote significance adjusted $p < 0.05$ compared to 10 min or 20 min respectively.

activated during a vaginal delivery [24], and so only non-laboured caesarean delivered placentas are appropriate for such studies.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2013.11.016.

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