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Regulation of fatty acid binding proteins by hypoxia inducible factors 1α and 2α in the placenta: Relevance to pre-eclampsia

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A B S T R A C T

Pre-eclampsia is characterized by placental hypoxia and dyslipidemia. Arachidonic and docosahexanoic acids are essential maternal nutrients for fetal development. They are transported via placental trophoblast cells by membrane and cytosolic fatty acid binding proteins. Others report the expressions of these proteins which are increased in hypoxic trophoblasts. Using bioinformatics, BeWo cells, reporter assays, quantitative real-time PCR and immunoblotting we tested the hypothesis that hypoxia inducible factors 1α (HIF-1α) and/or 2α (HIF-2α) regulate the expressions of FABP1, FABP3, FABP4 and FATP2 proteins. Three hypoxia responsive elements (HRE) were identified in *FABP*1 which cumulatively responded strongly to HIF-1α and weakly to HIF-2α. FABP3 expression partially responded to HIF-1α. Two putative HRE were validated in *FABP*4 both of which responded weakly to HIF-1α and HIF-2α. FATP2 protein expression reacted positively to hypoxia. Thus, fetal essential fatty acid supply via the placenta is protected under hypoxia. It will be interesting to determine if our findings are replicated in human pre-eclamptic placenta.

1. Introduction

Pre-eclampsia affects 2–7% of pregnancies and is associated with fetal growth restriction, premature birth and fetal and maternal morbidity [1]. The condition is characterized by a sudden rise in blood pressure and proteinuria. It affects the liver, kidneys and cardiovascular and clotting systems. These symptoms are alleviated after delivery of the baby and placenta. The etiology of the disorder is not completely understood. However, abnormal placentation, hypoxia, inflammation and endothelial damage are implicated [1].

Maternal and fetal concentrations of long chain polyunsaturated fatty acids are lower in plasma [2] and red blood cells [3] from pregnancies complicated by pre-eclampsia compared with controls. Arachidonic acid (ARA) and docosahexanoic acid (DHA) are long chain polyunsaturated fatty acids required for fetal development. They are formed by metabolism of essential dietary fatty acids α-linolenic and linoleic by elongases and desaturases [4]. Since the fetus and human placenta have limited abilities to desaturate and elongate fatty acids [5], the fetal demand for ARA and DHA has to be satisfied by the mother. The placenta transports ARA and DHA from the mother to the fetus. The syncytiotrophoblast keeps maternal and fetal circulation separate while allowing nutrient exchange.

Proteins that bind and transport ARA and DHA have been identified in the trophoblast. These include three intracellular liver, heart and adipocyte fatty acid binding proteins (FABP1, FABP3, and FABP4 respectively) and membrane associated fatty acid transport protein 2/very long chain acyl CoA synthetase 1 (FATP2) [6]. The protein levels of FABP1, FABP3 and FABP4 increase in hypoxic human trophoblasts [7]. The mRNA levels of FATP2 increase in primary human trophoblasts cultured under hypoxia [8]. We therefore hypothesized that in the trophoblast the expressions of FABP1, FABP3, FABP4 and FATP2 are under the control of HIF-1α and/or HIF-2α.

The heterodimeric hypoxia-inducible factor (HIF) transcription factors regulate cellular responses to hypoxia [9]. Each heterodimer consists of a specific α subunit (HIF-1α, HIF-2α and HIF-3α) which complexes with the HIF-1β (aryl hydrocarbon nuclear translocator) subunit under hypoxic conditions. HIF-1α and HIF-2α are the best characterized of the three HIF α subunits and possess overlapping but differing roles in hypoxic gene response. Each protein forms a dimer with HIF-1β to activate transcription of target genes by binding to a consensus hypoxic response element (HRE) that is comprised of a consensus G/ACGTG sequence. Together they regulate the expression of approximately 200 genes implicated in angiogenesis, energy metabolism, cell proliferation, and apoptosis. While some targets are shared, others are preferentially regulated by HIF-1α or HIF-2α [9].

Here we employed the BeWo choriocarcinoma cell line, a frequently used and well described model of the syncytiotrophoblast,

Abbreviations: HIF, hypoxia-inducible factor; HRE, hypoxic response element; qPCR, quantitative real-time PCR; SDHA, succinate dehydrogenase complex, subunit A

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and combined bioinformatics, cell biology and biochemical approaches to address the above hypothesis. Our results identified three HRE sequences in the *FABP*1 gene that responded additively and strongly to HIF-1α and weakly to HIF-2α. HIF-1α partially induced FABP3 expression, possibly via an intronic HREsequence. Two putative HRE sequences were identified and validated in the *FABP*4 gene, and FATP2 reacted positively to hypoxic stimulation. These findings suggest that the supply of long chain fatty acids is protected under hypoxia.

2. Materials and methods

2.1. Bioinformatics

The genomic nucleotide sequences (2500 bp upstream of the TSS) were extracted from the ENSEMBL human database (assembly GRCh37) (http://www.ensembl.org) using Biomart software at the ENSEMBL web site. To locate putative HRE genes, sequences were analyzed using bioinformatics tools that included Mulan (http://mulan.dcode.org/), Matinspector (http://www.genomatix.de/), TFsearch (http://www.cbrc.jp/research/db/TFSEARCH.html), P-Match (http://www.gene-regulation.com) and JASPAR (http://jaspar.genereg.net/). Motif sampling programs were employed to evaluate further any promoter regions identified by Promoterbase. These programs included Weeder (http://159.149.109.16:8080/weederWeb/), MotifSampler (http://ccmbweb.ccv.brown.edu/gibbs/gibbs.html) and MEME (http://meme.nbcr.net/meme/). Any HRE genes predicted in the human sequences in the database TRANSFAC (v10.2) (http://www.gene-regulation.com) were subsequently analyzed using MULAN for further validation.

2.2. Plasmid constructs

Putative promoter regions upstream of each of our four target genes were amplified from human genomic DNA by PCR and cloned into the luciferase reporter vector pGL3-Basic (Promega) between NheI/HindIII or BglII/MluI and upstream of the luciferase reporter gene. Successful clones were sequenced to confirm 100% base pair accuracy.

2.3. Cell culture and hypoxia

BeWo cells were cultured in F-12 Nutrient mixture (Sigma) supplemented with 10% FBS and 100 units/ml penicillin, 10 mg/ml streptomycin until 80% confluent, as assessed by light microscopy. Different experimental approaches were used to mimic hypoxia including the hypoxia-inducing agent cobalt chloride, transfection with HIF-1α and HIF-2α overexpression vectors and 1% oxygen tension. BeWo cells (at passage number < 20) were maintained at 37 °C in 20% O_2, 5% CO_2 and 75% N_2 in either the presence or absence of effectors (0.4 mM $CoCl_2$, 0.8 μg HIF-1α or HIF-2α over expression vectors) for 24 h or in a hypoxic chamber gassed with 1% oxygen over a time course from 4 to 24 h. $CoCl_2$ induced hypoxia did not decrease cell viability compared with control cells nor did the addition of up to 1.5 μg DNA.

2.4. Transient transfection and luciferase activity

To assay transcriptional activity of HIF, the pGL3-Basic vector containing six copies of the HRE sequence RCGTG upstream of the firefly luciferase gene was used as a positive control and the "empty" pGL3-Basic vector was used as a negative control. 0.5 μg test construct DNA was transfected using 4 μl per well of lipofectamine 2000 (Invitrogen) in a 12 well plate format. A pRL-TK vector expressing the renilla luciferase protein was co transfected in all experiments as an internal control for normalization. Briefly, 3.5×10^4 BeWo cells were seeded in 12-well culture plates. The DNA mixture containing test construct and internal control (20:1) was delivered to cells. At 24 h post-transfection, cells were incubated under normoxia or hypoxia for further 24 h. Cells were lysed and reporter gene expression assessed using the Dual Luciferase Reporter Assay System (Promega). Following normalization of firefly to renilla luciferase fluorescence, changes in reporter gene expression were calculated by normalizing the ratios of each test construct measured under the hypoxic state to the normoxic state. The student *t*-test was used to assess significant differences.

2.5. RNA isolation and quantitative real-time PCR (qPCR)

BeWo cells that underwent exposure to gaseous hypoxia were dissolved in TRIzol reagent (Invitrogen) at the appropriate time point and total RNA was extracted following the manufacturer's guidelines. 1.2 μg total RNA was converted to cDNA using the high capacity RNA-to-cDNA Kit (Applied Biosystems) in the presence of oligo$(dT)_{18}$. A 1:3 dilution of the cDNA was used in qPCR reactions using the Universal Probe Library System (Roche) with gene specific primers and fluorescent probes designed using the online assay design tool (http://www.roche-applied-science.com). The Fast Start Universal Probe Master (Rox) mix (Roche) was used and assays for individual gene transcripts run in triplicate in 96 well plates. Water and minus reverse transcriptase samples were used as negative controls. Data was collected using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) and analyzed using 7000 System Software (Applied Biosystems). All tested transcripts were amplified efficiently making their use in comparative analyses valid. The relative abundance of each gene transcript was quantified using the ΔΔCt method [10] with succinate dehydrogenase complex, subunit A (SDHA) as a reference gene [11].

2.6. Protein extraction and immunoblotting

Proteins from BeWo cells that had been exposed to gaseous hypoxia were extracted using RIPA buffer in the presence of protease inhibitor cocktail (Roche) and phosphatase inhibitor (Roche); 1×10^6 BeWo cells were resuspended in 300 μl of RIPA buffer. Total isolated protein was quantified using the BCA assay and 27 μg total extracted protein from each time point were separated on 4–20% SDS-PAGE (Biorad). Proteins were transferred onto a PVDF membrane via wet transfer overnight at 18 V and 4 °C. Ponceau S staining of membranes ensured equal protein loading. Primary antibodies against the following proteins were used: HIF-1α (BD Biosciences), SDHA (New England Biolabs), FABP3 (a kind gift from Prof Jan Glatz), and FATP2 (a kind gift from Prof Andreas Stahl). HRP-conjugated secondary antibodies were used. Immunoblots were developed using the Pierce ECL Plus Western Blotting Substrate (Thermo Scientific).

3. Results

3.1. Functional hypoxia-response element(s) in the human FABP1 gene

We examined the sequence of the *FABP*1 gene for matches to the consensus HRE sequence (Section 2). Three matches were found within the *FABP*1 gene (Fig. 1). To determine if these were functional HRE, the putative promoter region was amplified and inserted into the pGL3-Basic vector upstream of the luciferase gene. The region from −1315 to–648 bp (containing all three putative HRE) of the *FABP*1 gene increased luciferase activity under all hypoxic conditions tested (Fig. 2). A small increase in luciferase activity was observed under $CoCl_2$ induced hypoxia although this was negligible compared with