Placenta 36 (2015) 427–432

Contents lists available at ScienceDirect

Placenta

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

Strong hypoxia reduces leptin synthesis in purified primary human trophoblasts

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ARTICLE INFO

Article history:
Accepted 24 January 2015

Keywords:
Placenta
Trophoblast
Hypoxia
Leptin/leptin receptor

ABSTRACT

Introduction: Oxygen availability severely affects placental function. During placental hypoxia, stabilization of hypoxia inducible factors (HIFs) affects transcription, and leptin gene expression concomitantly increases *in vivo* and *in vitro*. However, a causal relationship is uncertain.
Methods: We investigated the effect of oxygen availability on HIF-1 alpha (HIF1A) and leptin regulation in primary human trophoblasts isolated from six normal term placentae cultured at 0.1%, 1%, 3%, and 8% oxygen for 6 h, 24 h and 48 h. Gene expressions of leptin (*LEP*), leptin receptors (*LEPR*), *HIF1A*, insulin receptor (*INSR*) and further genes relevant in hypoxia (*VEGFA, EPO, NOS2*) or apoptosis (*BCL2, BAX, Tp53*) were examined. Leptin, HIF1A, INSR, phospho-AKT/AKT (insulin receptor signaling), caspase 3 and cleaved caspase 3 (apoptosis) proteins were measured.
Results: A hypoxic reaction with stabilization of HIF1A protein as well as up-regulation of *HIF1A* and *VEGFA* gene expressions, but without any hint for apoptosis, was present at 0.1% and 1% oxygen. However, leptin protein concentration (cell supernatants) peaked at 8% oxygen (normoxia) and was significantly reduced at 0.1% oxygen. There was no significant correlation between leptin and HIF1A, neither on the gene nor on the protein level.
Discussion: Elevated leptin gene expression in hypoxic placentas may not originate from trophoblasts, but from other placental cells, or from interaction of trophoblasts with other cells. Not only fetal hyperleptinemia, but also fetal hypoleptinemia under hypoxic conditions is conceivable. Strategies to prevent leptin dysregulation during pregnancy should be elucidated to protect the offspring from fetal programming of leptin resistance and adiposity in later life.

1. Introduction

Leptin is an essential component in the regulation of energy metabolism and indispensible for fertility and reproduction [1]. During gestation, the human placenta produces leptin in high amounts and releases it both to the maternal and to the fetal circulation [2,3]. In complicated pregnancies, including intrauterine growth restriction and preeclampsia, leptin production in the human placenta is dysregulated [4–6]. Unlike in normal pregnancies, fetal plasma leptin concentrations are positively correlated to maternal leptin concentrations [7,8]. Consequently, placental leptin production and trans-placental leptin transport, the latter possibly mediated by the short isoform of the placental leptin receptor [9], both have critical impact on fetal leptin exposure in certain gestational pathologies.

Oxygen availability severely affects placental function in all stages of pregnancy, and hypoxia inducible factors (HIFs) are major mediators of oxygen dependent placental adaptations [10]. During hypoxia, HIFs are stabilized and act as transcription factors [11,12]. In pregnancies complicated by intrauterine growth restriction, preeclampsia or birth asphyxia, either acute or chronic hypoxia has been described, and increased placental leptin gene expression at birth is a common feature [13,14]. In JAr and in BeWo cells, two

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http://dx.doi.org/10.1016/j.placenta.2015.01.191
0143-4004/

choriocarcinoma cell lines used to study placenta biology, hypoxia upregulates leptin gene expression *in vitro* [4,15,16]. The leptin gene promoter indeed is transactivated by HIF1 [17,18]. It was therefore suggested that oxygen is a major regulator of placental leptin expression via HIF-dependent activation of the leptin gene during hypoxia [18]. This mechanism is of high clinical interest, as it may strongly affect fetal leptin availability, a key factor contributing to perinatal programming of metabolic disease in later life [5,6,19,20]. Furthermore, trophic effects of leptin on placenta and fetus have been demonstrated [21–24].

However, a causal relationship between hypoxia-induced HIF1 activation and leptin elevation in the placenta has not been proven. In above mentioned clinical study, hypoxia inducible factor-1 alpha (HIF1A) protein could not be quantified [13]. In a rat model of reduced uteroplacental blood flow, placental leptin gene expression was even reduced at term [20]. Regarding *in vitro* studies, both JAr and BeWo cells are cancer cell lines and markedly differ from primary human trophoblasts. Most importantly, they are adapted to room oxygen whereas 8% oxygen concentration represents physiologic ("normoxic") conditions for primary human trophoblasts [25].

Thus, the focus of our study was to examine the effect of different oxygen concentrations on HIF1A protein stabilization, leptin gene expression and leptin protein synthesis in primary human trophoblasts. Additionally, leptin receptor gene expressions were examined because of their assumed implication in transplacental leptin transport. Secondarily, we studied the effect of oxygen availability on insulin receptor signaling as an important modulator of leptin gene and protein expression [26,27]. To ensure data reliability, we examined common pro- and anti-apoptotic markers to address the question whether primary human trophoblasts survive *in vitro* even at very low oxygen tensions (i.e. 0.1% hypoxia for 48 h).

2. Material and methods

The study was reviewed and approved by the local institutional review board (ethics committee) and performed in accordance with the Declaration of Helsinki. Informed consent was given by all participants.

2.1. Trophoblast isolation and cell culture

Six human placentae from uncomplicated singleton term pregnancies (37–40 weeks of pregnancy) were collected immediately after elective caesarean section. Presence of hypertension, proteinuria, diabetes mellitus, heart insufficiency, HIV-infection and amniotic infection was excluded. Trophoblast cells were isolated using a modified trypsin-DNase-dispase/percoll method [28]. Further purification was performed using a negative immunomagnetic bead-separation leading to a final reduction of CD45-and HLA-ABC positive cells of less than 1% [29]. Within a maximum of 5 min after purification, cells were transferred to freezing-medium [70% Dulbecco's Modified Eagle's Medium (D-MEM/F-12; Invitrogen), 20% Dimethyl sulfoxide (DMSO; D8418, Sigma) and 10% fetal calf serum (PAA Laboratories)] and placed at −80 °C overnight. Then, cells were transferred to liquid nitrogen for storage. Storage time was similar in all samples and did not exceed 4 weeks. Time for defrosting and transfer to fresh medium did not exceed 5 min. For RNA-isolation, 2.5×10^6 cells were seeded in 6 cm dishes and for protein isolation, 1×10^7 cells were seeded in 10 cm dishes. Cells were cultured in D-MEM/F-12 supplemented with 10% fetal calf serum and 1% Antibiotic-Antimycotic (100×) liquid (Invitrogen) at 37 °C in an incubator supplied with 5% CO_2 and the respective oxygen concentration (0.1%, 1%, 3%, 8%). Oxygen concentration was monitored by an oxygen sensor. Additionally, mean oxygen partial pressures (mmHg) in cell supernatants were measured by a routine blood gas analyzer (ABL800 Basic, Radiometer) within 3 min after sample collection as follows: 0.1%, 6 h, 37.3; 0.1%, 48 h, 35.0; 1%, 6 h, 44.3; 1%, 48 h, 41.6; 3%, 6 h, 51.3; 3%, 48 h, 64.4; 8%, 6 h, 72.8; 8%, 48 h, 81.0. Cells were lysed after 6 h, 24 h and 48 h for RNA and protein quantification. Cell supernatants were used to measure leptin protein concentrations after 6 h and 48 h. The time points 6 h and 48 h were chosen to examine acute or chronic hypoxic effects, respectively. At 24 h, we analyzed gene expression only.

2.2. RNA isolation and PCR techniques

We measured 1) genes relevant for regulation of/by leptin [leptin (*LEP*), full length leptin receptor (full *LEPR*), short leptin receptor (short *LEPR*), insulin receptor (*INSR*)], 2) genes indicating cellular hypoxia [hypoxia inducible factor-1 alpha (*HIF1A*), vascular endothelial growth factor (*VEGFA*), erythropoietin (*EPO*), inducible NO-synthase (*NOS2*), as well as 3) pro-apoptotic [Bcl2-associated X protein (*BAX*), tumor protein p53 (*Tp53*)] and 4) anti-apoptotic [B-cell CLL/lymphoma 2 (*BCL2*)] genes (Table 1). Expression levels of target genes were normalized to the expression of five different housekeeping genes [*YWAHZ, SDHA, TBP*, beta2-microglobulin (*B2M*), beta-Actin (Table 1)], which were all tested for reliability. All normalizations showed similar results. Thus, gene expression results (Fig. 2A, Table 2) are shown normalized to YWAHZ (most reliable housekeeping gene).

RNA was isolated using guanidine–thiocyanate acid phenol (TRIzol®, Invitrogen). RNA concentrations were determined spectro-photometrically. One microgram of DNase-treated RNA was reversely transcribed in a volume of 26 µl (37 °C, 60 min, Finnzymes oligonucleotides). Quantitative RT-PCR was performed on a 7500 Real-Time PCR-System (Applied Biosystems). Primers and probes (Table 1) were designed using Primer Express Software™ (Applied Biosystems). All of the primers and probes were purchased from Eurofins MWG. Commercial reagents (Eurogentec) and conditions were applied according to the manufacturer's protocol. 2.5 µl of complementary DNA (reverse transcription mixture), 2.5 µl of 200 µM TaqMan hybridization probe and 2.5 µl of each primer respectively were analyzed in a 25 µl-volume reaction mix. For quantification, the CT values of the samples were interpolated to an external standard curve (serial dilution) of a known template.

2.3. Protein isolation and western blot techniques

Cells were lysed in extraction buffer (10 mM Tris pH 6.8; 6.65 M Urea, 10% Glycerol, 1% SDS, 10 µl/ml 0.5 M DTT, 10 µl/ml 50 mM PMSF), incubated on ice (1 h) and centrifuged (14.000 rpm, 15 min, 4 °C). Subsequently, protein concentration was determined using a commercial kit (BCATM Protein Assay Kit, Thermo Scientific). For protein detection, 80 µg of protein were separated on 10% acrylamide SDS-PAGE under reducing conditions and transferred onto a nitrocellulose membrane for 120 min at 1.2 mA/cm^2 using towbin buffer. Membranes were subsequently blocked (5% milk powder, 2% BSA, TRIS-buffered saline containing 0.05% Tween-20) and probed overnight with the primary antibody in blocking buffer at 4 °C. Primary antibodies used were β-actin mAb #3700, Caspase-3 Rabbit Antibody #9662, cleaved caspase-3 Rabbit Antibody #9661, IRS-1 Rabbit mAb #3407, Akt Antibody #9272, Phospho-Akt Rabbit mAb #4058 (all CellSignaling), and HIF1A Rabbit Antibody NB100-449 (Novus Biologicals). After washing and incubation in HRP-conjugated secondary antibody, membranes were developed using Amersham ECL Plus-Solution, GE-Healthcare. Secondary antibodies used were Anti-mouse IgG, HRP-linked Antibody #7076 and Anti-rabbit IgG, HRP-linked Antibody #7074 (all CellSignaling).

2.4. Statistical analysis

All data was checked for outliers by Grubb's test (significance level $p < 0.05$). A maximum of one outlier was excluded from some data sets. The effect of different oxygen concentrations on either protein concentration or gene expression was analyzed by a global Friedman test (each time point separately) in the majority of analyses. In case of missing values due to excluded outliers, a Kruskal-Wallis-test (each time point separately) with Dunn's posttests was performed, because a Friedman test does not tolerate missing values. A p-value of <0.05 was considered to be statistically significant. The effect of incubation time was tested by comparing values with the same oxygen concentrations at 6 h with values at 48 h by a Mann-Whitney-test with a Bonferroni-adjusted p-value of <0.01 considered to be significant. Additionally, we performed Spearman correlation analyses of HIF1A as well as leptin gene expressions with the expression of the other measured genes at all oxygen concentrations analyzed in one analysis each, and of HIF1A/actin densitometric protein data with either leptin ELISA data or InsR/actin densitometric protein data.

3. Results

3.1. Primary human trophoblast cells react to 0.1% oxygen with immediate stabilization of HIF1A protein and subsequent up-regulation of VEGFA, HIF1A and full LEPR gene expressions

Strong hypoxia acutely stabilized HIF1A protein. Subsequently, gene expressions of *HIF1A* and *VEGFA* were induced. In the chronic hypoxic phase, HIF1A protein concentrations decreased, but gene expressions of *VEGFA*, *HIF1A* and full *LEPR* increased. In detail, HIF1A protein was stabilized at 0.1% and − to a lesser degree − 1% oxygen after 6 h of incubation and showed a double band at ~120 kDa. After 48 h, HIF1A protein stabilization was detectable at 0.1% oxygen only (Fig. 1). Densitometric HIF1A/beta-actin protein ratios, which were available from P3, P4 and P6 ($n = 3$), showed a negative correlation with oxygen concentrations (due to low sample number only