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Upregulation of leptin-receptor in placental cells by Hypoxia

Daniela Klaffenbach [a,*,1], Udo Meißner [a,1], Michael Raake [a], Fabian Fahlbusch [a], Miguel A. Alejandre Alcazar [a], Ida Allabauer [a], Jürgen Kratzsch [b], Wolfgang Rascher [a], Jörg Dötsch [a]

[a] Department of Pediatrics and Adolescent Medicine, University Erlangen- Nuernberg, Erlangen, Germany
[b] Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics, University of Leipzig, Leipzig, Germany

1. Introduction

The *ob*-gene product leptin is a pleiotropic molecule that regulates food intake and body weight as well as metabolic and endocrine functions [1,2]. There is increasing evidence that leptin also exerts proliferative and antiapoptotic effects in a variety of cell types and plays a pivotal role in tissue repair. Furthermore, leptin has been suggested to be involved in fetal and placental development during pregnancy [3–6]. It is a known fact that maternal plasma leptin concentrations undergo large changes during pregnancy – out of proportion with change in maternal adipose tissue mass – returning to normal levels rapidly after delivery [7,8]. These observations suggest that leptin is released into the maternal circulation from the placenta [9]. Estrogens and insulin have been identified as positive regulators of placental leptin production [10]. In addition to type I diabetic mothers with markedly increased leptin mRNA and protein levels in placenta, preeclampsia is another pathological situation with enhanced placental leptin synthesis.

During placental development, oxygen environment of placental cells undergoes large changes and cells may face rather hypoxic conditions. Such changes in oxygen availability may modulate leptin synthesis during pregnancy in the placenta [11,12]. Grosfeld et al. and our group showed a significant induction of leptin transcription via a hypoxia-inducible-factor 1 (HIF1) dependent mechanism. At least one hypoxia-responsive element, located −120 bp to −116 bp in the leptin promoter, is involved in this HIF1 mediated effect on the transcriptional regulation. These observations are in line with further studies on cells of placental origin demonstrating that the human leptin gene is transcriptionally up-regulated by hypoxia [13–15]. In addition, the fact that leptin and its receptor (Ob-R) are co-expressed in human placenta suggests auto- and paracrine mechanisms of action beside leptins endocrine functions [16].

The human leptin receptor has several isoforms resulting from alternative mRNA splicing during transcription. The long form of the human leptin receptor (Ob-Rb) shares a large homology to the intracellular signalling domain of the type I cytokine receptor family, using the JAK/STAT signal transduction pathway in most cells [17,18]. In human placental tissue, however, it seems that leptin signal is transduced via the MAPK pathway instead of the JAK/STAT cascade [19].

Recent studies were able to show an overexpression of leptin and its receptor in cells of human endometrial and colorectal cancer [20,21]. In this tissue, the expression of leptin and its receptor seem to correlate with hypoxia and the abundance of hypoxia-inducible-factor-1α (HIF-1α). This raised the hypothesis, that the leptin receptor might be regulated by hypoxia. So far it is unknown, however, if low oxygen tension may influences the expression of the leptin receptor in the placenta.

* Corresponding author. Department of Pediatrics and Adolescent Medicine, University Erlangen-Nuernberg, Loschgestrasse 15, Erlangen, 91054 Germany. Tel.: +49 9131 8533118; fax: +49 9131 8533113.
E-mail address: daniela.klaffenbach@uk-erlangen.de (D. Klaffenbach).
[1] Both authors contributed equally to this work.

Therefore, the main purpose of this study was to characterize the effect of hypoxia on leptin receptor transcription and translation in placental cells. Moreover the influence of low oxygen tension on the putative leptin signal transduction pathways and the effect on placental – cell proliferation was investigated. Along with primary villous trophoblast cells, the BeWo and JAR chorioncarcinoma cell lines were employed as a model for placental biology due to their expression of all leptin receptor isoforms as well as leptin [22].

2. Materials and methods

2.1. Cell culture

JAR and BeWo cell lines were purchased from DSMZ (Braunschweig, Germany) and cultured in D-MEM:F-12 (Gibco-BRL, Eggenstein, Germany) supplemented with 10% FCS, 100 µg/ml penicillin and 1000 U/ml streptomycin (Clontech, BD Biosciences, Heidelberg, Germany) in the presence or absence of stimuli as indicated. Cells were cultured under normoxic conditions at 37 °C under a humidified atmosphere containing 5% unless stated otherwise. Before plating, cells were trypsinized and washed twice in cold PBS, followed by resuspension in their culture medium. Human recombinant leptin was purchased from R&D Systems (Minneapolis, MN, USA).

2.2. Placental tissue and cell preparation

Human placentas were obtained in collaboration with the department of Gynecology and Obstetrics at the University Hospital of Erlangen from

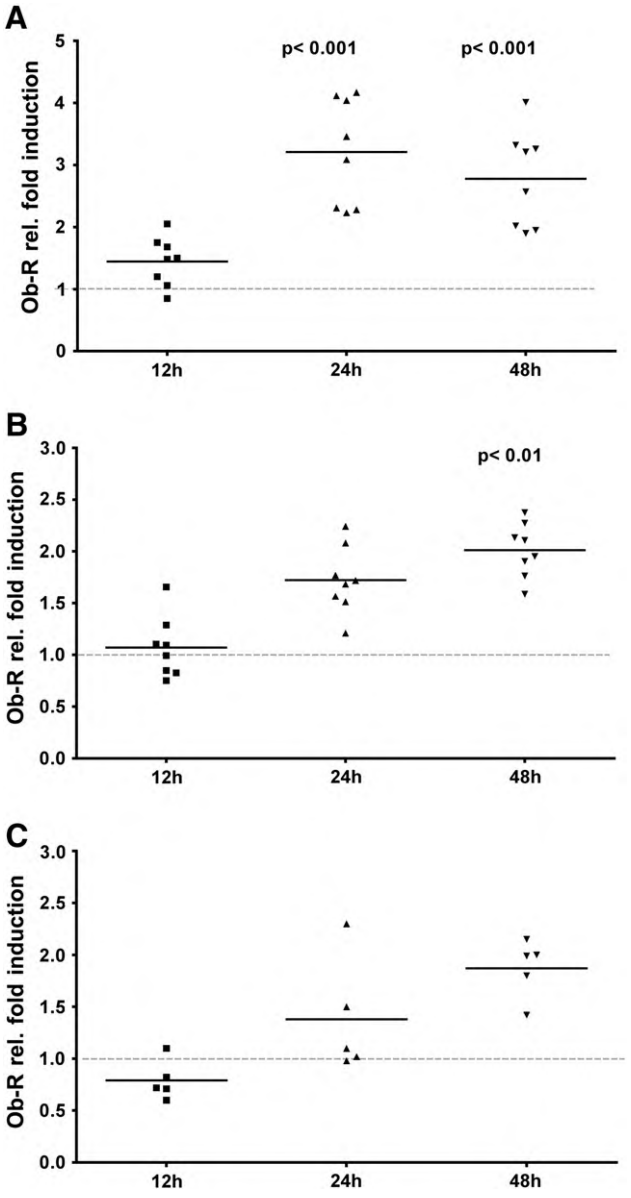

Fig. 1. Expression of leptin receptor mRNA under hypoxic conditions. BeWo (A), JAR cells (B) and villous trophoblasts (C) were incubated for 12 h, 24 h and 48 h hypoxic (H) conditions (1% O_2). Induction of Ob-R mRNA expression was compared and normalized to normoxic controls (N). The grey, dashed lines represent the normalized control. Number of experiments n = 8 (BeWo/JAR), n = 5 (VT). Statistical analysis was performed using one-way-ANOVA with Bonferroni post test for selected comparisons (12 h N vs. 12 h H, 24 h N vs. 24 h H, 48 h N vs. 48 h H). *p* values are indicated if $p<0.05$.

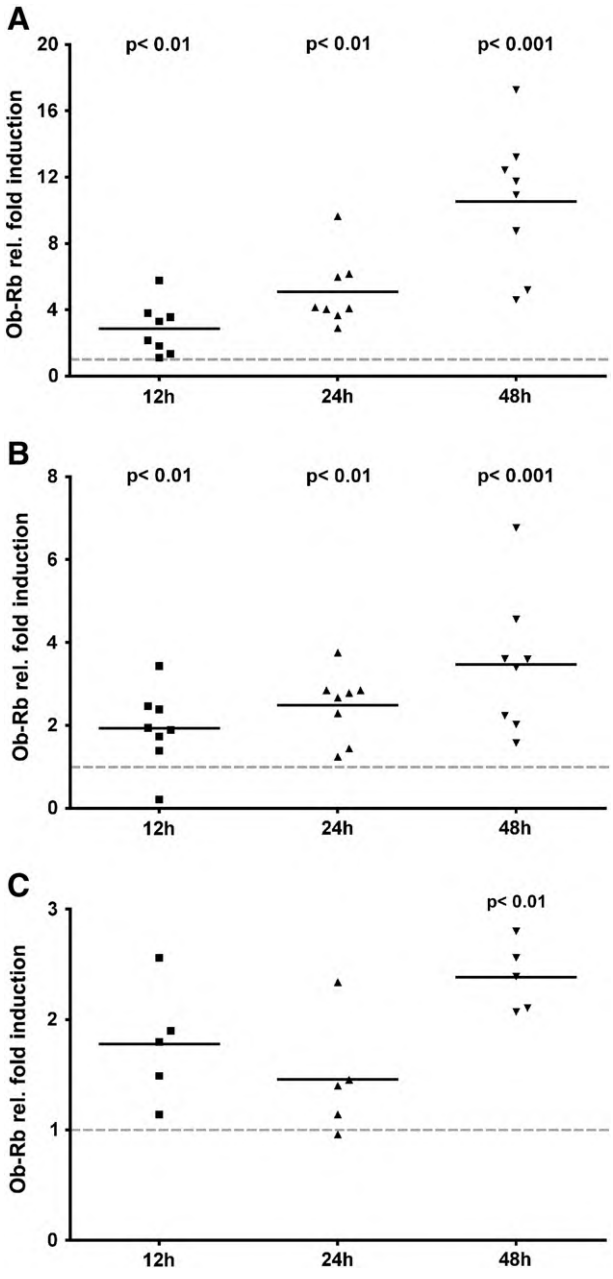

Fig. 2. Expression of leptin receptor long isoform mRNA under hypoxic conditions. BeWo (A), JAR cells (B) and villous trophoblasts (C) were incubated for 12 h, 24 h and 48 h under hypoxic (H) conditions (1% O_2). Induction of Ob-Rb mRNA expression was compared and normalized to normoxic controls (N). The grey, dashed lines represents the normalized control. Number of experiments n = 8 (BeWo/JAR), n = 5 (VT) respectively. Statistical analysis was performed using one-way-ANOVA with Bonferroni post test for selected comparisons (12 h N vs. 12 h H, 24 h N vs. 24 h H, 48 h N vs. 48 h H). *p* values are indicated if $p<0.05$.

uncomplicated term pregnancies after elective caesarean section in accordance with the local ethic committee. Specimens from placental villi were taken immediately after delivery and washed in physiologic saline solution to remove blood. Villous trophoblasts (VT) were isolated by the modified trypsin-DNase, Percoll (Sigma, Deisenhofen, Germany) gradient centrifugation method initially described by Kliman et al. [23]. Further purification of trophoblast cells was carried out using negative immunomagnetic bead-separation with a final reduction of CD45- and HLA-ABC positive cells to less than 1%.

2.3. Hypoxic conditions

Hypoxic treatment was carried out in an incubator (Invivo 400, Ruskinn Technology Ltd/Ruskinn Life Sciences Ltd, Pencoed, UK) supplied with 1% O_2 and 5% CO_2 . Oxygen concentration was monitored by measurement of oxygen partial pressure in cell medium and an oxygen sensor in the chamber.

2.4. RNA isolation and PCR techniques

Total RNA was isolated from cells using TRIzol (Invitrogen, Karlsruhe, Germany) after 12, 24 and 48 h under hypoxic (1% O_2) and normoxic (21% O_2) conditions. After DNase treatment 1 μg RNA was transcribed into cDNA using MMLV-RT (Promega, Mannheim, Germany), Oligo (dT) and random hexamer primer. DNase treatment and cDNA synthesis were carried out according to the manufacturers recommendations. The cDNA expression levels were quantified by real time polymerase chain reaction (qRT-PCR) using the SYBR-Green incorporation method (iCycler IQ5, Bio-Rad, Hercules, California, USA) according to the suppliers protocol. For amplification of all leptin receptor isoforms (Ob-R) and the long isoform (Ob-Rb) we used the following primers: Ob-R: forward primer: 5′-GTAAGAGGCTAGATGGACTGGGATAT-3′, reverse primer 5′-ATTCTCCAAAATTCAGGTCCTCTCA-3′, Ob-Rb: forward primer 5′-AGGCTGAGGGTACTGAGGTAACC-3′, reverse primer: 5′-GATCAGCGTGGCGTATTTAACA-3′. Expression levels were normalized to the expression of the human housekeeping genes HPRT and beta-Actin. Primer pairs for HPRT were as follows: forward primer: 5′-CCGGCTCCGTTATGGC-3′, reverse pimer: 5′-GGTCATAACCTGGTTCATCATCA-3′ and for beta-Actin: forward primer 5′-GATGAGATTGGCATGGCTTT-3′ and reverse primer 5′-CACCTTCACCGTTCCAGTTT-3′. All calculations were based on the $^{\Delta\Delta}$Ct-method as previously described in detail [24].

2.5. Immunoblot experiments

Cells were incubated in the presence of the indicated stimuli, washed twice with ice-cold PBS and lysed in modified tris buffer (50 mM Tris HCl, 150 mM NaCl, 1% Triton X, 0.25% Na-desoxycholate, 1 mM EDTA, 1 mM Phenylmethylsulfonylfluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/ml pepstatin-A, 1 mM NaF, 1 mM Na_3VO_4). Lysates were immunoblotted by separating the protein by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. After transfer to a nitrocellulose membrane (Whatman GmbH, Dassel, Germany) the activation of p42/p44 (ERK 1/2) and STAT3 was analysed with a phospho-specific anti ERK 1/2 (Promega, Mannheim, Germany, Catalogue number V80319, dilution 1:750) and phospho-specific STAT3 antibody (CellSignaling, Minneapolis, MA, USA, Catalogue number 9131, dilution 1:1000), visualized by chemoluminescence using ECL plus (Amersham Pharmacia biotech, Freiburg, Germany). Equal protein loading was verified after stripping the nitrocellulose membrane and adding an antibody directed against the total protein of either ERK 1/2 (Promega, Catalogue number V114, dilution 1:1000) or STAT3 (Cell Signaling, Catalogue number 9139, dilution 1:1000). Leptin receptor antibody was purchased from R&D Systems (Catalogue Number MAB 389, dilution 1:125).

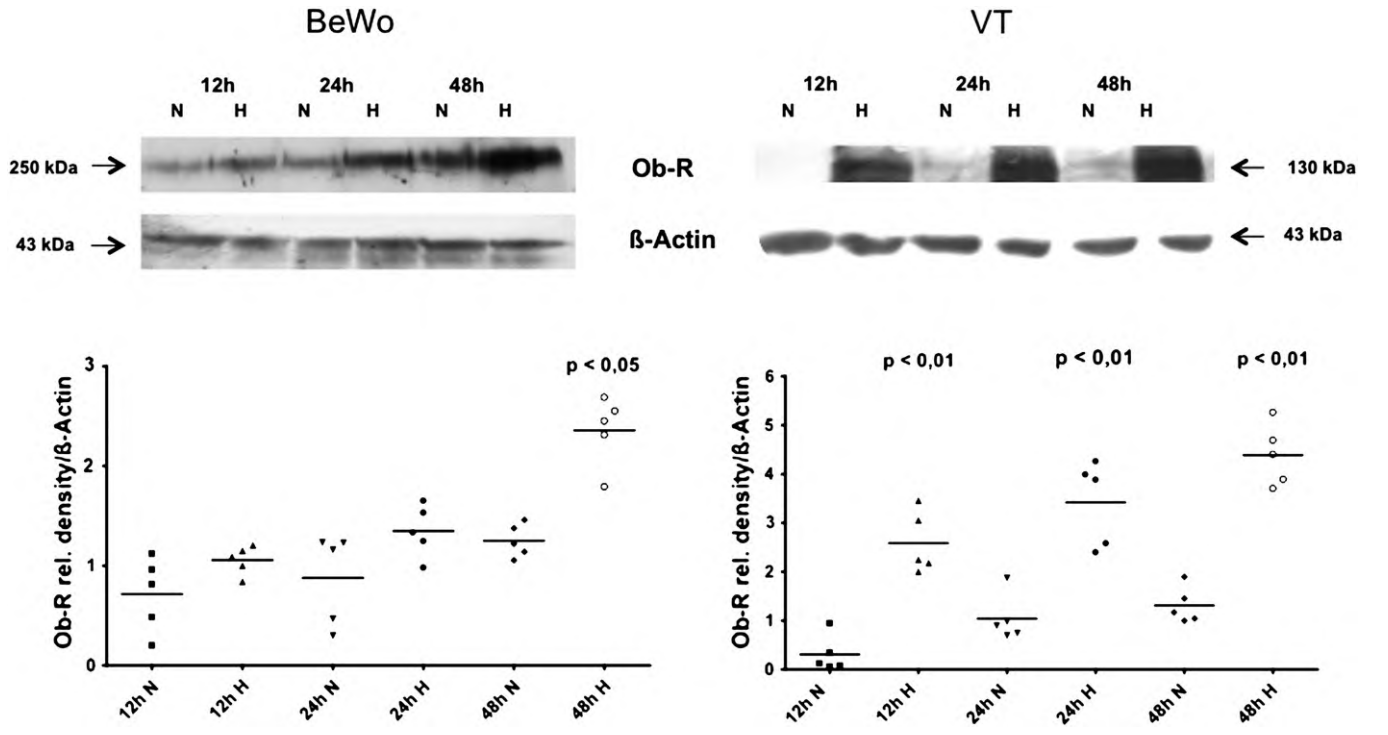

Fig. 3. Western Blot analysis of Leptin receptor expression under hypoxic conditions. Cells were incubated as indicated under hypoxic conditions (H, 1% O_2). Afterwards they were lysed and subjected to Western Blot analysis of Leptin receptor (Ob-R). Lysates of untreated cells were used as normoxic controls (N). Top: Immunoblot analysis of Ob-R (dependent on its grade of glycosylation 250 kDa or 130 kDa) and β-Actin as loading control. Bottom: Densitometric analysis of Western Blots. Similar results were obtained using JARs (data not shown). Number of independent experiments n = 5. The one-way-ANOVA was used for statistical analysis. Bonferroni post testing revealed a significant impact of hypoxia in late BeWo incubation (48 h) and all hypoxic incubation tested in VT. *p* values are indicated if $p<0.05$.

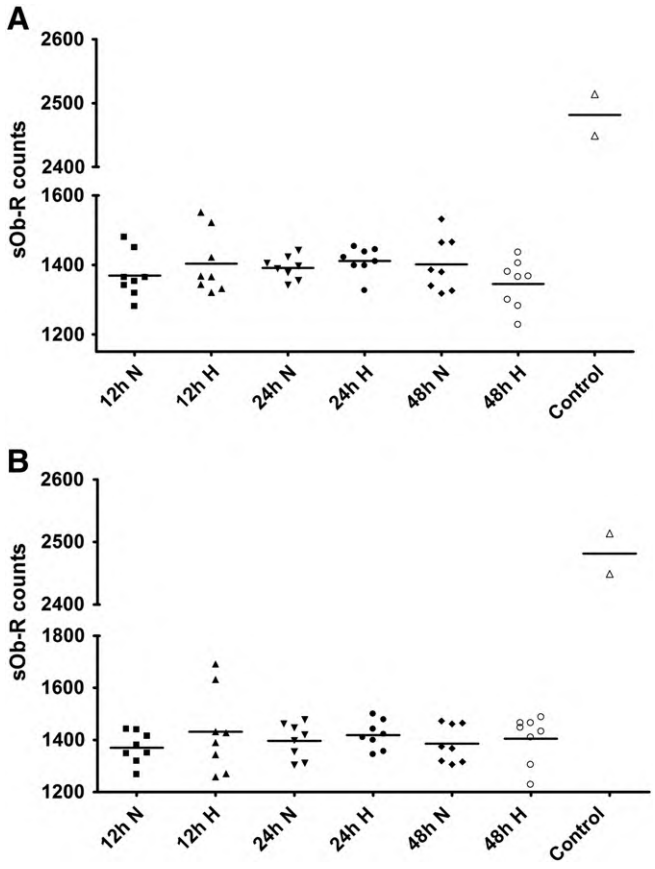

Fig. 4. Release of soluble leptin receptor isoform under hypoxic conditions. After incubation of BeWo (A) and JAR cells (B) under normoxic (N) and hypoxic conditions (H) as indicated, concentrations of sOb-R in the supernatants were investigated. Recombinant sOb-R with a concentration of 1.56 ng/ml was used as positive control ("Control"). Number of independent experiments n = 8. One-way-ANOVA was used for testing and showed no significant difference between these groups.

2.6. Densitometry

Densitometric analysis of the bands was performed using Advanced Image Data Analyzer-Software (Fuji Photo Film Co., Omiyama, Japan) and normalized to total amount of ß-actin per lane. Pixel densities were corrected for background staining of the same film.

2.7. sOb-R analysis

After incubating the cells under hypoxia or normoxia, levels of the soluble leptin receptor (sOb-R) were measured by a sensitive ligand-immunofunctional assay [25]. For the determination, samples were dried by vacuum centrifugation and then resolved using cell medium to only one tenth of the former volume.

2.8. Cell proliferation assay

BeWo and JAR cells were transferred to microtiter plates at a density of 3×10^3 cells/well. After adhesion medium was changed to D-MEM:F-12 supplemented with 0.5% FCS to starve the cells. Experiments were also performed by blocking internal leptin with anti-leptin antibody (R&D Systems, Catalogue Number AF398) and goat anti-mouse as control (Promega, catalogue number W402B). After 24 h cells were stimulated with 0.1 µg/ml, 0.5 µg/ml or 1 µg/ml leptin in the culture medium or left untreated. Afterwards 5-bromo-2′-deoxyuridine (BrdU) was added to the cells and they were incubated for 24 h or 48 h under normoxic or hypoxic conditions. The cell proliferation assay was purchased from *Roche* (Mannheim, Germany) and carried out according to the manufacturers recommendations.

2.9. Statistical analysis

Statistical analyses as indicated were performed using GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego California USA. $p<0.05$ was considered statistically significant.

3. Results

3.1. Expression of leptin receptor under hypoxic conditions

To analyze if placental cells regulate Ob-R under hypoxic conditions, the expression of leptin receptor mRNA was investigated using chorioncarcinoma cell lines as a model of placenta biology and primary trophoblasts for *in vitro* assays. Comparing the hypoxic cells (1% oxygen) to normoxic controls, a time-dependent induction of Ob-R mRNA was observed in hypoxic primary trophoblasts as well as the cell lines (Fig. 1). For more detailed analysis, the regulation of the long isoform of the receptor (Ob-Rb) being responsible for its signal transduction was investigated. Therefore cells were incubated again in a time dependent manner under hypoxia following mRNA analysis. We were able to detect an up to ten-fold induction of Ob-Rb mRNA expression in the hypoxic placental cells compared to the normoxic controls (Fig. 2).

In a second approach, we analyzed whether the results obtained from the mRNA expression analysis were followed by an enhanced protein synthesis. For this purpose we performed leptin receptor immunoblots. As shown in Fig. 3, hypoxic induction of Ob-R mRNA resulted in an increased expression of the protein in primary trophoblasts as well as the chorioncarcinoma cells lines.

Finally, we investigated if an increased expression of Ob-R results in an augmented release of the soluble leptin receptor isoform. Therefore, we collected supernatants of hypoxic as well as normoxic cell cultures. Interestingly, sOb-R was unaltered comparing hypoxic cell culture supernatants to those of normoxic controls (Fig. 4).

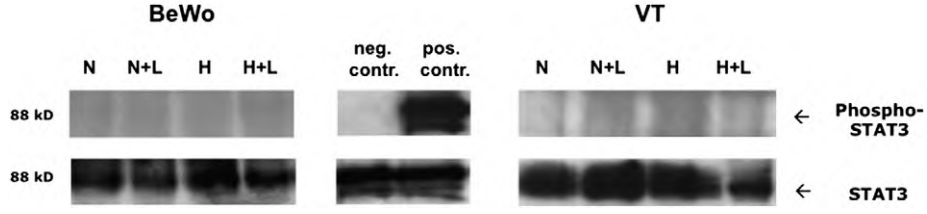

Fig. 5. STAT 3 signal transduction in placental cells after leptin incubation. BeWo (left) cells and primary villous trophoblasts (right, VT) were preincubated under normoxic (N) and hypoxic 1% oxygen (H) conditions for 24 h and lysed after stimulation for 15 min using 1 µg/ml Leptin (L). Lysates were subjected to a Phospho-STAT3/STAT 3 Western Blot. HeLa cells treated with Interferon-alpha were used as positive/negative control (middle). Comparable results were obtained using different leptin concentrations, different lengths of stimulation or Jar cells. Number of independent experiments n = 5.

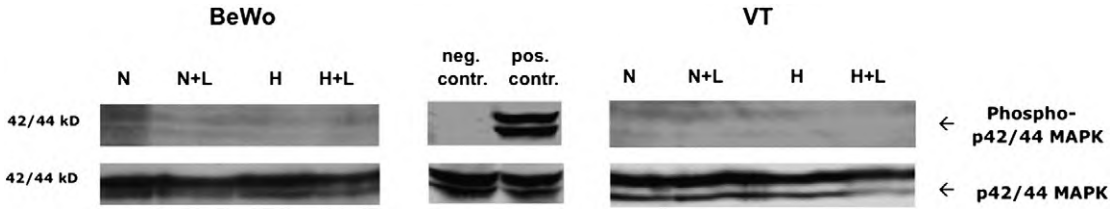

Fig. 6. p42/44 MAPK signal transduction in placental cells after leptin incubation. BeWo (left) cells and primary villous trophoblasts (right, VT) were preincubated under normoxic (N) and hypoxic (H, 1% oxygen) conditions for 24 h and lysed after stimulation for 15 min using 1 μg/ml Leptin (L). Lysates were subjected to a Phospho-p42/44 MAPK and p42/44 MAPK Western Blot. Cells were treated with TPA as positive control (middle). Comparable results were obtained using different leptin concentrations, different length of stimulation or JAR cells. Number of independent experiments n = 5.

3.2. Signal transduction of leptin receptor under hypoxia

We next analyzed if p42/p44 ERK or STAT3-pathways are recruited in placental cells after incubation under hypoxic conditions and stimulation with recombinant leptin. First STAT3 phosphorylation in primary trophoblasts and chorioncarcinoma cell lines was investigated. Here, we were not able to activate the STAT3 pathway after leptin stimulation, independently of oxygen tension (Fig. 5). Interestingly, p42/44 MAPK phosphorylation did not change after addition of 0.1 μg/ml respectively 1 μg/ml or 10 μg/ml leptin, neither in BeWo/JAR cells nor in primary trophoblasts (Fig. 6) also.

3.3. Effect of hypoxia and leptin on cell proliferation in placental cells

In this study we investigated the effect of leptin on placental cell proliferation. Cells were incubated under normoxic or hypoxic conditions in the presence or absence of different leptin concentrations. When focusing on the effects after 24 and 48 h, no effect of either leptin or leptin in combination with hypoxia on the proliferative capacities of cells tested could be detected (Fig. 7). Incubating the placental cells with anti-leptin antibody (10 μg/ml) 24 h prior to stimulation for blocking internal leptin activity had no effect on the proliferation rate (data not shown).

4. Discussion

Besides the adipose tissue, the placenta is another side of leptin synthesis. Increased placental leptin production has been reported in a number of gestational pathologies resulting in an increased risk for preterm- and stillbirths [26,27]. Although little is known about the exact physiological role of leptin during human pregnancy, recent observations suggest that this hormone could be a key player in the regulation of embryonic implantation as well as the maintenance of pregnancy. The presence of leptin receptors in trophoblasts may

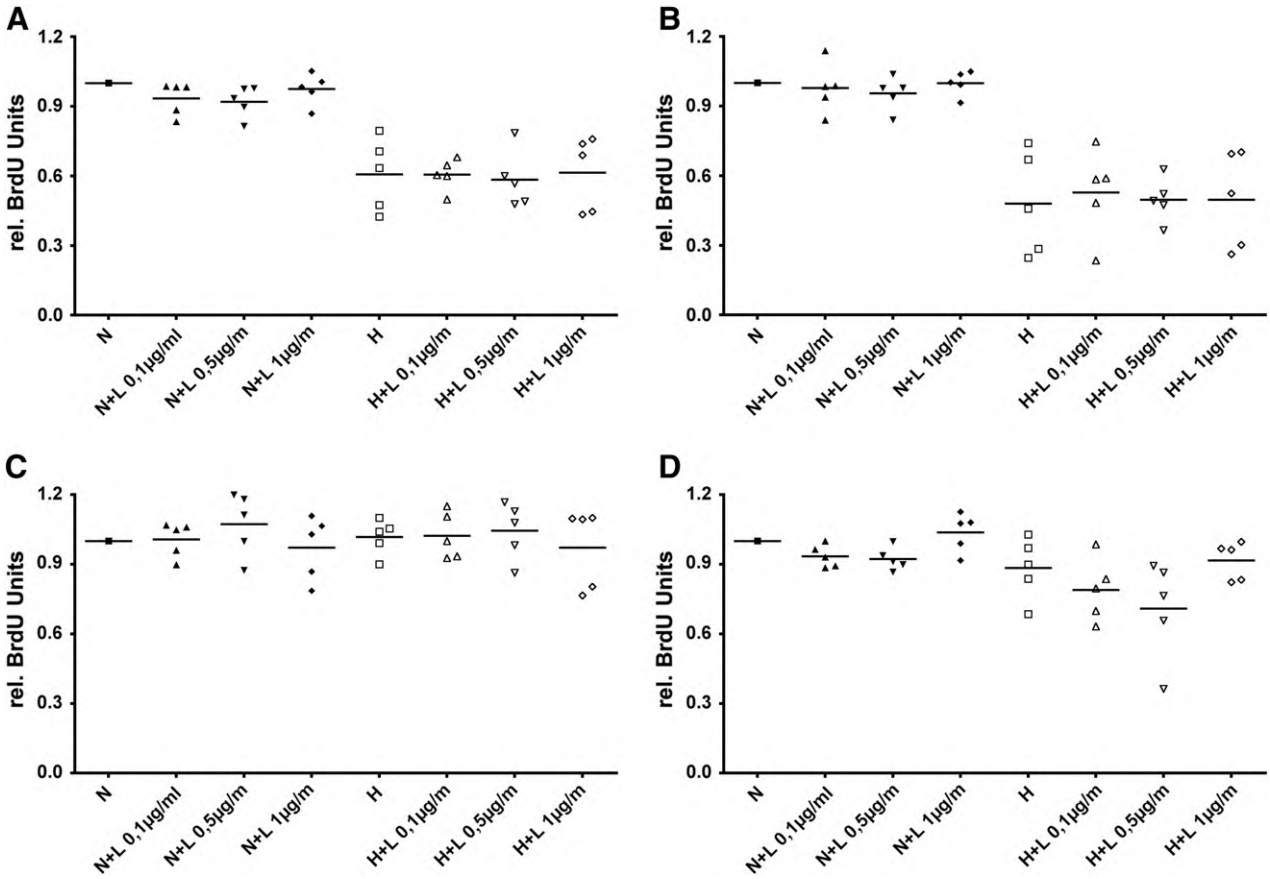

Fig. 7. BrdU incorporation of leptin stimulated BeWo (A/B) and JAR cells (C/D). Cells were incubated for 24 h (A/C) and 48 h (B/D) under normoxic (N) or hypoxic (H) conditions in the presence or absence of 0.1 μg/ml, 0.5 μg/ml or 1 μg/ml leptin (L). Number of experiments n = 5. Two-way-ANOVA was used for testing the independent variables of hypoxia/normoxia or variable leptin concentrations and showed no significant difference between these groups.

indicate that placental leptin exerts its biological effects by recruiting local intraplacentar effectors via auto- or paracrine pathways. Since leptin gene expression is up-regulated under hypoxic conditions in placental cells, this raised the hypothesis that low oxygen tension not only induces leptin production — it may in fact modulate the effects of leptin on placental cells by induction of its receptor.

Several isoforms of the leptin receptor have been identified [28,29]. These various isoforms can be divided into three groups: a single long receptor which has an intracellular domain of 306 amino acids, several short receptors with intracellular domains of 32 to 40 amino acids, or, lacking a transmembrane domain, being a soluble leptin receptor. Our data suggest that hypoxia leads to an induction of the long isoform of the leptin receptor in placental cells but does not induce a release of the soluble receptor. Therefore, based on our data the induction of the leptin receptor by hypoxia does not appear to be involved in modulation of free active leptin within the placental *milieu* as discussed here [30].

In line with previous findings, we were showed that the JAK/STAT pathway is not recruited after incubating primary trophoblasts as well as placental cell lines. Yet, in contrast to reports by Caüzac et al. [19], no activation of the MAPK-pathway after leptin treatment could be detected, neither in the chorioncarcinoma cell lines used nor in primary trophoblasts. As leptin synthesis itself is enhanced in placental cells due to hypoxia [15], further investigations will be needed to dissect the question if receptor synthesis is enhanced by the hormone or by hypoxia-related pathways.

As we found no direct effect of leptin added to our cell culture – being independent of the augmented leptin receptor expression driven by hypoxia in our cell culture – several explanations for the use of the upregulation of leptin receptor under hypoxic conditions may be discussed:

A) Chorioncarcinoma cells are morphologically similar to their cells of origin, the trophoblast of the first trimester placenta and may serve as a valid and convenient model system for studying the cellular activities and regulation of leptin *in vitro*. Studies on BeWo cells from other groups suggest that the *in vivo* system is well mimicked by this cell line regarding its syncytialization and hormone secretion [31,32]. Perhaps it may be possible that the cell culture model might not reflect *in vivo* situation with regard to signal transduction. On the other hand we could show that the MAPK-pathways itself may be stimulated in our cell lines using TPA. However, in primary trophoblasts, we similarly were not able to activate the MAPK- or JAK-pathway after leptin treatment.
B) Maternal decidual cells, but not trophoblast cells might be the target of placental leptin. Both leptin-receptor mRNA and protein have been detected in the decidua [33]. These findings suggest that the decidua may be target tissue for leptin action indicating a function in the blastocyst-endometrial-dialogue [34]. Based on these data one might speculate that leptin may be a factor being essential for embryonic and fetal development and less for the development of maternal tissues [35,36].
C) Placental leptin synthesis might preferentially be intended for executing endocrine functions within the placental *milieu* and not as a paracrine factor influencing proliferation. This notion is supported by previous studies from our group indicating that in contrast to non placental cells chorioncarcinoma cells do not show an altered rate of apoptosis after stimulation with leptin [37].

In conclusion, the presence of leptin and the leptin receptor isoforms in human placenta from very early stage of development until term suggests that leptin has definite biological effects within the feto-placental milieu. We were able to show that hypoxia up-regulates leptin receptor in placental cells without enhancing the release of the soluble receptor isoform. It is striking that leptin-induced signal transduction as well as cell proliferation remained unchanged in our experimental setting. Further investigations are needed to redefine the role of placental leptin synthesis.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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