EXPERIMENTAL STUDY

Hypoxia-induced leptin production in human trophoblasts does not protect from apoptosis

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Abstract

Objective: The *ob*-gene product, leptin, is an important regulator of placental and fetal development during pregnancy. Leptin, being induced by hypoxia in the placenta, is a known pro-apoptotic molecule in adipose tissue but is also known to inhibit apoptosis in other tissues like neuroblastoma cells. Based on these findings, we investigated if leptin has a pro- or anti-apoptotic effect on a trophoblastic cell line (JAR cells) in the presence or absence of oxygen.

Methods and results: Measurement of leptin in the supernatant by using ELISA showed hypoxia-induced leptin production in JAR cells *in vitro*. This could be confirmed by a leptin-specific RT-PCR. By analyzing leptin and/or hypoxia exposed cells with FACS cytometry we found that JAR cells can cope with hypoxia down to oxygen tensions of 1%. At this level, only a small number of cells underwent apoptosis. Interestingly, leptin added to the culture medium in high concentrations was not able to interfere with the rate of proliferation or apoptosis in these cells independent of the oxygen tension. Finally, an anti-caspase-3 and anti-caspase-9 Western blot was performed. Again, no difference in the expression of caspase-3 and -9 under the conditions tested was seen.

Conclusions: These results show that leptin, produced by placental cells after hypoxia *in vitro*, has no influence on the rate of proliferation of these cells. Furthermore, it does not influence apoptotic pathways in the trophoblastic cell line tested under hypoxic and non-hypoxic conditions.

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Introduction

The discovery of the adipose-derived hormone, leptin, has generated increased interest in its involvement in the regulation of feeding by interacting with central nervous structures. There is increasing evidence that leptin has systemic effects apart from those related to energy homeostasis (1). These include the regulation of neuroendocrine and immune function, modulation of the cell cycle, and a role in fetal development or tissue repair (2–5). Mammalian cells have multiple responses to decreased oxygen tension. By far the most common reaction to low or zero oxygen concentrations is cell death through apoptosis. Apoptotic signaling during oxygen deprivation occurs through the release of cytochrome c and the activation of caspase-9 mediated by Bcl-2 family members such as Bax or Bak (reviewed in detail in (6)). Trophoblasts are responsible for the maternal–fetal exchange of gas, nutrients and waste products. Therefore, placental apoptosis is a key cellular interface in the control of placental homeostasis (7). Several authors have provided evidence for a connection

between pathological states during pregnancy (e.g. pre-eclampsia) and increased placental apoptosis (8–10). Leptin plays a dual role regarding apoptosis as it can act either as a pro- or an antiapoptotic messenger. First, leptin was reported to be responsible for the induction of lipolysis. Thus, this is an effect mediated through apoptosis of the adipose tissue (11). In contrast, Fujita *et al.* demonstrated that leptin in fasted mice is able to prevent the apoptotic reduction of lymphocyte numbers in steroid-injected mice via a *bcl-xL*-dependent mechanism (12). This anti-apoptotic effect of leptin is in line with evidence that the hormone protects SK-N-SH-SY5Y neuroblastoma cells from apoptosis in serum-free medium by a STAT/JAK-dependent down regulation of caspase-10 and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (13). It is well known that leptin contributes to a physiological pregnancy by regulation of implantation, energy balance of placenta and conceptus, as well as an angiogenic factor (14–16). During pregnancy, leptin expression undergoes dramatic changes in maternal plasma in parallel with the rising of cytokine levels during the first trimester (17) returning to normal

levels after delivery (18). The production of placental leptin is reported to be increased by interleukin-1 α , 17 β -estradiol and hypoxia (19–21). Preeclampsia is a disorder associated with maternal hypertension, reduced placental blood flow and placental hypoxia (22). Elevated concentrations of leptin are found when comparing sera and placental tissues from women with preeclampsia with sera of mothers with uncomplicated pregnancies (23–25). Apart from the placenta, many peripheral tissues including heart, liver, kidneys and muscle react to hypoxia with an increased transcription of leptin which is mediated by a hypoxia-inducible factor-1 α (HIF-1 α)-dependent mechanism (26–32).

Based on these findings and the dual role of leptin as a pro- or anti-proliferative factor, we were interested in how leptin could influence hypoxia-induced cell death in JAr cells, a trophoblastic cell line. Therefore, we set out to ask the following questions. (i) Are JAr cells expressing the functional variants of the leptin receptor? (ii) Is hypoxia able to induce leptin production in JAr cells? (iii) How does hypoxia influence the viability and death of the cells? (iv) Is the exposure to hypoxia *in vitro* followed by an increased rate of apoptosis? (v) How does leptin interact with β -human chorionic gonadotropin (β -hCG) secretion, and the proliferation and apoptosis of these cell lines under normoxic and hypoxic conditions?

Materials and methods

Cell culture and reagents

JAr cells were purchased from DSMZ (Braunschweig, Germany), and cultured in D-MEM:F-12 (purchased from Gibco-BRL, Eggenstein, Germany) supplemented with 10% FCS, 100 μ g/ml penicillin, and 1000 U/ml streptomycin (purchased from Clonotech, BD Biosciences, Heidelberg, Germany), in the presence or absence of different stimuli and chemicals as indicated. Cells were cultured under normoxic conditions at 37 °C under a humidified atmosphere containing 5% CO₂ unless stated otherwise. Leptin was purchased from R&D Systems (Minneapolis, MN, USA).

Treatment of cells

Before plating, cells were trypsinized and washed twice in cold PBS followed by resuspension in the respective culture medium. They were plated into 6-well plates and stimulated with various concentrations of leptin (1.0 μ g/ml to 0.25 μ g/ml) or incubated under hypoxic conditions (1% or 5% O₂) for the time period indicated.

RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from 5×10^5 JAr cells using TRIzol (Gibco-BRL, Life Technologies). After DNase

treatment, 1.5 μ g RNA were transcribed into cDNA using M-MLV-RT (Promega, Mannheim, Germany) and Oligo(dT). DNase treatment and cDNA synthesis were carried out according to the manufacturer's recommendations. The cDNA expression level was quantified by conventional PCR analysis. A 25 μ l reaction mixture contained the following ingredients: 2 μ l cDNA, 1 $\times$ Taq reaction buffer, 2.5 mmol/l MgCl₂, 1 mmol/l dNTP, 0.25 μ mol/l forward primer, 0.25 μ mol/l reverse primer, and 1 U Taq Polymerase (Promega). For amplification of the two different isoforms of the leptin receptor we used the following primers: short leptin receptor (ObRs): sense 5'-GTAAGAGGCTAGATGGACTGGG-ATAT-3', ObRs antisense 5'-ATTCTCCAAAATTCAGGTCCTCTCA-3'; long leptin receptor sense (ObRl): 5'-AGGCTGAGGGTACTGAGGTAACC-3', ObRl antisense 5'-GATCAGCGTGGCGTATTTAAC A-3'.

Real-time PCR

For analyzing the gene expression (mRNA) of leptin and of the hypoxanthine-guanine phosphoribosyltransferase (HPRT), as well as of porphobilinogen deaminase (PBGD) as housekeeping genes, we used the primers as previously described (21). All assays were performed using a quantitative real-time polymerase chain reaction (TaqMan PCR, PerkinElmer Life Sciences Inc. Boston, MA, USA). All calculations were based on the $\Delta\Delta C_t$ – method as described in detail elsewhere (26).

Leptin ELISA

Concentrations of leptin in the culture supernatants were measured by using the Human Leptin DuoSet ELISA Development System (R&D Systems) following the recommendations of the manufacturer.

β -hCG ELISA

Concentrations of β -hCG in the culture supernatants were measured by using the DRG- β -hCG-ELISA kit (DRG Instruments, Marburg, Germany) following the recommendations of the manufacturer.

Flow cytometry and annexin-V/propidium iodide apoptosis assay

Apoptotic cell detection was performed using fluorescein-isothiocyanate (FITC)-conjugated annexin-V-Fluos (Responsif, Erlangen, Germany) and propidium iodide (Sigma, Deisenhofen, Germany), following the manufacturer's instruction. Briefly, 10^6 cells were collected for each condition, trypsinized, washed with cold PBS and resuspended in 100 μ l incubation buffer (10 mmol/l HEPES/NaOH, pH 7.4, 140 mmol/l NaCl, 3.3 mmol/l CaCl₂) containing 2 μ l annexin V-Fluos and 2 μ l propidium iodide (PI). After 15 min of incubation in the dark at room temperature, 400 μ l cold

incubation buffer were added and cells were analyzed by flow cytometry (FACSCalibur, Becton-Dickinson, Heidelberg, Germany), using 488 nm excitation and band pass filters of 530/30 nm (for FITC detection) and 585/42 nm (for PI detection). For each cell type, appropriate electronic compensation of the instrument was performed to avoid overlapping of the two emission spectra. Data analyses were performed with Cell Quest software (Becton-Dickinson). Cells were counted using CALTAG Counting Beads (Caltag Laboratories, Burlingame, CA, USA) as recommended by the manufacturer.

SDS-PAGE and Western blot

Cells were lysed in buffer containing Igepal-630 and sodium desoxycholate as detergents (all chemicals purchased from Sigma). Equal amounts of protein were separated by 12% SDS-PAGE, blotted to a Protran nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), and detected with a rabbit anti-human-caspase-3 or -9 polyclonal antiserum (Cell Signaling Technology/Upstate Biotech, Lake Placid, NY, USA) followed by a visualization using a horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology) and ECL Plus on Kodak BioMax (both purchased from Amersham Pharmacia Biotech, Freiburg, Germany).

Statistical analysis

If not otherwise stated, all values are given as means $\pm$ standard errors. A Kruskal-Wallis one-way ANOVA was used to calculate the differences between the different groups (control vs hypoxia vs leptin vs hypoxia + leptin). Given a significant *P* value ($P < 0.05$), a Dunn's multiple comparison followed the ANOVA test. All calculations were performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA).

Results

In this study we tested the hypothesis that leptin influences hypoxia-induced apoptosis in trophoblasts and human kidney cells. We used the human choriocarcinoma cell line JAr, which has previously been proved to be a useful model for the study of placenta biology for *in vitro* assays (33). When testing these cells for the expression of the different leptin receptor isoforms by RT-PCR, two types, the short and the long functional variants of the leptin receptor, could be detected (Fig. 1). One aim was to study to what extent endogenous leptin is produced by these cells after exposure to hypoxia. Therefore, JAr cells have been tested on their ability to produce leptin after incubation under various hypoxic conditions. When incubating JAr cells under hypoxic conditions (1% O₂ for 6 to 48 h) they reacted with a significant increase in leptin-specific mRNA. The amount of leptin-specific mRNA rose up to

Figure 1 Expression profiles of leptin receptor isoforms. JAr cells express both isoforms (long, ObRI variant and short, ObRs variant) of the leptin receptor. H₂O as template served as a negative control (-).

10-fold when comparing hypoxic cells to untreated controls (Fig. 2A). The augmented leptin mRNA was followed by a significant increase of the hormone in the culture supernatants of the stimulated cells throughout the whole period studied. All specimens taken from hypoxic cell cultures had more leptin than those of untreated, normoxic controls (Fig. 2B). To analyze the changes in the viability of the cells and the rate of apoptosis, cells were subjected to flow cytometry using the annexin V/PI method to divide them into apoptotic, viable and dead cells. By using the annexin V staining, the cells can be analyzed regarding their rate of apoptosis prior to morphological changes associated with apoptosis. Cells negative for the annexin V and the PI dye were regarded as viable cells (Fig. 3, R2). In contrast, cells staining positive for annexin V and the non-vital dye PI were interpreted as being dead (Fig. 3, R3). In between, two groups of intact cells could be discriminated by this method: (a) the early apoptotic (EA) cells are annexin V positive but negative for PI (Fig. 3, R4), and (b) the late apoptotic (LA) cells (Fig. 3, R5) that are positive for both dyes, but could be distinguished clearly from the highly PI-positive (dead) cells. We established this method, which had previously been described for leukocytes, and used it for the analysis of trophoblasts. With the following assays we were able to get an insight into the effects that leptin might exert on apoptosis and proliferation in the presence or absence of oxygen. JAr cells were incubated for 24 h or 48 h under normoxic or hypoxic (1% or 5% oxygen) conditions, in the presence of leptin (250 ng/ml to 1.0 μ g/ml) or in the presence of a combination of leptin and hypoxia. When focusing on the effects after 24 h, no significant effect of either leptin or hypoxia on vitality or apoptosis could be detected by flow cytometry. The results obtained from assays using 1.0 μ g/ml leptin and 1% O₂ are depicted in Table 1. These effects were independent of the dose of leptin and the hypoxic conditions, as reducing the leptin concentration to 500 or 250 ng/ml (data not shown) had no effect. After 48 h, a significant decrease in the number of annexin V and PI double negative (vital) cells could be measured in JAr cells. The percentage of dead JAr cells was about three

Figure 2 Elevated concentrations of leptin-specific mRNA and protein in lysates and supernatants of hypoxia-incubated trophoblastic cells. JAR cells were incubated under hypoxic conditions at an oxygen tension of 1% O₂ for 6 to 48 h. Cells incubated without a stimulus under normoxic conditions were used as controls. After the period indicated, cells were subjected to cDNA synthesis, and supernatants were collected for ELISA analysis. The amount of leptin mRNA, relative to the levels of two housekeeping genes, was measured to calculate the relative change in induction of leptin mRNA; both housekeeping genes gave similar results. Data normalized to PBGD as the housekeeping gene are displayed as fold induction compared with unstimulated controls (A). Leptin protein in supernatants was measured using a specific two-site ELISA with a standard of known concentration (B). Open bars, normoxic cells; solid bars, hypoxic cells. The data represent the mean of 3 identical experiments. **P* < 0.05, ***P* < 0.01 compared with normoxic controls.

times higher than those of the normoxic controls (Table 1). Exposure of JAR cells to hypoxia for 48 h initiated a significant increase in early and late apoptotic cells compared with normoxic controls. Thus, the addition of leptin to these cells had no effect either on the percentage of apoptotic cells or on the differentiation between EA and LA (Table 1). In the following steps, we tested if the addition of leptin (at high concentrations of 1 µg/ml to 250 ng/ml) or incubation under hypoxic conditions had a significant effect on the proliferation of the two different cell lines as determined by counting the cells after the incubation

period using flow cytometry. A small (but non-significant) increase in absolute cell numbers could be detected when leptin was added to the cells for 24 h; hypoxic conditions influenced proliferation in a negative manner. This effect could not be overruled by the addition of leptin to the JAR cells (Fig. 4). To determine if the expression of caspases is influenced by exposure to hypoxia in the presence or absence of leptin, lysates from JAR cells were subjected to Western blot analysis. No difference in the expression of caspase-3 and caspase-9 could be detected when the cells were incubated for 24 h under 1% oxygen with or without 1 µg/ml leptin (Fig. 5).

Discussion

Human embryogenesis takes place in an hypoxic environment because the trophoblast shell prevents the entry of oxygen via maternal blood. Throughout gestation the oxygen tension rises (34). As anerobic conditions are a common feature in the placenta, at least within the first trimester, it is clear that differentiation of placental cells and the maintenance of the feto-placental unit is dependent on hypoxia-induced signals (35, 36). Thus, a variety of pathophysiological conditions during pregnancy are accompanied by hypoxia (10, 34, 37). It was shown by Esterman *et al.* that *in vitro* cultures of trophoblasts can survive extreme hypoxia and that they react rapidly to restitution of normal oxygen tension by readopting their reduced metabolism and cell cycle (38). This is in line with the data from the present study. Here, the trophoblastic choriocarcinoma cell line, JAR, showed a better survival following hypoxia than 293HEK cells, an embryonic kidney cell line (data not shown). Programmed cell death plays a pivotal role in maintaining placental development and function throughout gestation (7, 35). But even here, the border between 'good' and 'bad' apoptosis has to be drawn tightly as it is known that preeclampsia is linked to an increased rate of apoptotic death within trophoblasts (8–10). We tested if apoptosis could be induced in JAR cells by incubating the cells under hypoxic conditions (1% O₂) for up to 48 h. Cells were analyzed using FACS and Western blot. No significant changes, either in the number of apoptotic (annexin V-positive) cells or in the expression of proapoptotic proteins such as caspase-3 or -9 could be seen. These data are supported by publications demonstrating an increased resistance of placental cells to hypoxia-induced apoptosis (39). Other reports focus on the increasing numbers of apoptotic placental cells in the course of hypoxia (40, 41). Preeclampsia is a severe disorder of human pregnancy characterized by placental hypoxia and followed by activation of maternal endothelial cells as a final step. Hung *et al.* showed that reoxygenation after a state of hypoxia is the final key for the induction of apoptosis in syncytiotrophoblast cells whilst hypoxia alone is more likely to cause necrosis

Figure 3 Flow cytometric analysis of apoptosis in JAr cells. Cells were stimulated for 48 h in the presence of 1 $\mu\text{g/ml}$ leptin (+Leptin) or under hypoxic conditions (1% O_2 , +Hypoxia). In addition, hypoxic cells were stimulated using 1 $\mu\text{g/ml}$ leptin (+Leptin + Hypoxia). Afterwards, annexin V/propidium iodide (PI) staining was used to determine early (annexin V^+/PI^- , R4) and late (annexin V^+/PI^+ , R5) apoptotic cells. In addition, the viable cells (lower left quadrant of the cytogram, R2) and necrotic cells (upper right quadrant of the cytogram, R3) were counted.

(42). It still remains unclear what protected the JAr cells in our assays from undergoing apoptosis; also it is not known what caused the increased rate of apoptotic cell death in the reoxygenated cells. Leptin is thought to play a pivotal role in placental development and maintenance during pregnancy (5, 43). Comparing sera from women with preeclampsia with sera of mothers with uncomplicated pregnancies (44), elevated concentrations of this cytokine can be found in the

preeclamptic mothers. This effect is mediated by the fact that leptin production could be enhanced by hypoxia (20, 21, 26, 29). Conflicting results have been found regarding the ability of the *ob*-gene product, leptin, to interfere with apoptosis (11–13). We have chosen to administer quite high leptin concentrations, from 250 ng/ml up to 1.0 $\mu\text{g/ml}$, because it has been shown in previous studies by Wolf and colleagues that a single dose of at least 500 $\mu\text{g/ml}$ leptin (~ 30 nmol/l) is necessary to induce a significant proliferation of mesangial cells in *in vitro* cultures (45). To our surprise, leptin added to

Table 1 Flow cytometric analysis of apoptosis in JAr cells. Cells were stimulated for 24 or 48 h in the presence of 1 $\mu\text{g/ml}$ leptin (Leptin) or under hypoxic conditions (1% O_2 , Hypoxia). In addition, hypoxic cells were stimulated using 1 $\mu\text{g/ml}$ leptin (L + H). Annexin V/propidium iodide staining was used to determine early (EA) and late (LA) apoptotic cells. In addition, the viable (Living) and necrotic (Dead) cells were counted. The cells counted in the respective region (as depicted in Fig. 3) were divided by the absolute number of accepted cells. The numbers represent the mean $\pm$ S.E.M. of 5 independent assays.

	Living	EA	LA	Dead
24 h				
Control	87.1 $\pm$ 2.6	4.9 $\pm$ 1.3	2.8 $\pm$ 0.9	4.5 $\pm$ 0.4
Leptin	86.5 $\pm$ 2.7	4.9 $\pm$ 1.4	3.0 $\pm$ 0.9	5.0 $\pm$ 0.6
Hypoxia	79.0 $\pm$ 2.6	7.2 $\pm$ 1.1	4.1 $\pm$ 0.6	8.8 $\pm$ 2.4
L + H	80.3 $\pm$ 3.1	7.3 $\pm$ 1.2	3.7 $\pm$ 0.8	7.9 $\pm$ 1.8
48 h				
Control	85.1 $\pm$ 1.4	4.5 $\pm$ 0.9	3.0 $\pm$ 0.6	6.7 $\pm$ 0.8
Leptin	84.9 $\pm$ 1.4	4.6 $\pm$ 0.6	3.2 $\pm$ 0.6	6.5 $\pm$ 1.1
Hypoxia	63.2 $\pm$ 3.8§	9.0 $\pm$ 3.8§	5.6 $\pm$ 0.8§	18.3 $\pm$ 3.5§
L + H	65.8 $\pm$ 3.4§	10.4 $\pm$ 1.8§	5.5 $\pm$ 0.6§	16.6 $\pm$ 2.6§

§ $P < 0.05$, § $P < 0.01$ compared with control (one-way ANOVA).

Figure 4 Cell counts of stimulated JAr cells. Cells were stimulated for 24 h in the presence of 1 $\mu\text{g/ml}$ leptin (Leptin) or under hypoxic conditions (1% O_2 , Hypoxia). In addition, hypoxic cells were stimulated using 1 $\mu\text{g/ml}$ leptin (L + H). Afterwards, cells were counted by flow cytometry using beads.

Figure 5 Equal expression of caspase-3 and caspase-9 in JAr cells after exposure to hypoxia in the presence or absence of leptin. Cells were stimulated for 48 h in the presence of 1 µg/ml leptin (L) or under hypoxic conditions (1% O₂, H) or with a combination of both stimuli (H/L). Afterwards, cells were lysed and subjected to Western blot analysis for the expression of caspase-3 (Casp 3) and -9 (Casp 9). Lysates of untreated cells were used as controls (C).

the culture medium was unable to interfere either with the rate of proliferation of the trophoblasts or with their rate of apoptosis, although we could demonstrate the existence of both leptin receptor isoforms in both cell lines. Previous work from several authors has shown that an oxygen tension of at least 2% or lower is necessary to induce a sufficient (HIF-1 α -dependent) response (46, 47). We tested oxygen tensions of 5% to 1%. Here, a lower production of leptin in the culture supernatant could be found when incubating the cells with more oxygen. The exposure to 5% oxygen had no effect on the survival of the cells or on leptin added to the cell culture (in comparison to normoxic controls, or the hypoxic cells kept at 1% oxygen tension). Another attribute of placenta cells is the secretion of β -hCG. When JAr cells were tested, no significant effect of leptin or short term (24 h) hypoxia on β -hCG concentration could be measured when analyzing the culture supernatants in an ELISA. After 48 h, a decreased β -hCG concentration in the culture supernatants of the hypoxic cells was found. This has been observed before (48). Thus, leptin added to the hypoxic cells had no significant effect on the hypoxia-dependent reduction of hCG secretion (data not shown).

Our data suggest that placental leptin, induced by hypoxia, may exert functions in the placenta other than preventing the cells (e.g. trophoblasts) from undergoing (hypoxia driven) apoptosis. In addition, the hormone does not seem to play a role as an additional proliferative factor for placental cells during hypoxia. Thus, additional assays (e.g. in animal studies) are needed to discover the physiological role of leptin in the placenta during hypoxia.

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