Published in final edited form as:

Placenta. 2012 January ; 33(1): 31–38. doi:10.1016/j.placenta.2011.09.021.

Global protein synthesis in human trophoblast is resistant to inhibition by hypoxia

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Abstract

Placental growth and function depend on syncytial cell processes which require the continuing synthesis of cellular proteins. The substantial energy demands of protein synthesis are met primarily from oxidative metabolism. Although the responses of individual proteins produced by the syncytiotrophoblast to oxygen deprivation have been investigated previously, there is no information available on global protein synthesis in syncytiotrophoblast under conditions of hypoxia. These studies were designed to test the hypothesis that syncytial protein synthesis is decreased in a dose-dependent manner by hypoxia. Experiments were performed to measure amino acid incorporation into proteins in primary syncytiotrophoblast cells exposed to oxygen concentrations ranging from 0 to 10%. Compared to cells exposed to normoxia (10% O₂), no changes were observed following exposure to 5% or 3% O₂, but after exposure to 1% O₂, protein synthesis after 24 and 48 h decreased by 24% and 23% and with exposure to 0% O₂, by 65% and 50%. As a consequence of these results, we hypothesized that global protein synthesis in conditions of severe hypoxia was being supported by glucose metabolism. Additional experiments were performed therefore to examine the role of glucose in supporting protein synthesis. These demonstrated that at each oxygen concentration there was a significant, decreasing linear trend in protein synthesis as glucose concentration was reduced. Under conditions of near-anoxia and in the absence of glucose, protein synthesis was reduced by >85%. Even under normoxic conditions (defined as 10% O₂) and in the presence of oxidative substrates, reductions in glucose were accompanied by decreases in protein synthesis. These experiments demonstrate that syncytiotrophoblast cells are resistant to reductions in protein synthesis at O₂ concentrations greater than 1%. This could be explained by our finding that a significant fraction of protein synthesis in the syncytiotrophoblast is sustained by glycolytic metabolism. This suggests that with increasing degrees of chronic hypoxia there is a shift from oxidative to glycolytic pathways, allowing a substantial degree of protein synthesis to be maintained.

Keywords

Protein synthesis; Hypoxia; Human; Trophoblast; Glucose

1. Introduction

Placental growth and function depend on syncytial cell growth and differentiation, processes which require the continuing synthesis of cellular proteins. Protein synthesis is necessary for the process of differentiation and fusion of mononuclear cytotrophoblast cells into the syncytiotrophoblast, allowing for the continuous development of the non-proliferative syncytial barrier. Syncytial protein synthesis is also necessary for the continued regeneration of the microvillous and basal membranes, which carry out the vital transport functions required for fetal nutrition and for the secretion of the array of hormones and growth factors produced by the placenta. Since sustained protein synthesis by the syncytium is a key component of fetoplacental growth and well-being, it is important to determine the circumstances and the mechanisms by which synthetic function is inhibited in fetal growth pathologies.

Protein synthesis consumes a large fraction of cellular energy [1]. The energy that drives protein synthesis is derived primarily from the metabolism of O₂ and glucose. One of the initial results of the decreased intervillous blood flow common to both intrauterine growth restriction (IUGR) and preeclampsia (PE) is a reduction in the supply of O₂ to the placenta [2,3], leading to chronic (feto) placental hypoxia [4,5]. In the placenta, since protein synthesis accounts for ~30% of placental O₂ consumption [1], it seems likely that a diminished O₂ supply will result in a reduced supply of the energy needed for protein synthesis. The general cellular response to hypoxia consists of several elements which function to preserve the energy supply and increase O₂ supply to the affected cells.

Energy supply is sustained via an increase in anaerobic glycolysis while the increase in cellular O₂ availability is accomplished by a process known as O₂ sparing. This process is mediated by HIF-1 (hypoxia-inducible factor-1) and stimulates active, reversible inhibition of oxidative metabolism [6–8]. These processes have been observed to operate in the placenta under conditions of mild to moderate hypoxia leading to reductions in fetal, but not placental growth [9,10]. However in clinically significant IUGR there is generally severe hypoxia with marked reductions in both fetal and placental growth [11]. This suggests that severe O₂ deficiency may stimulate another hypoxia response, the cessation of non-essential, energy-requiring mechanisms such as protein synthesis, a process known as “demand reduction”.

The effect of hypoxia on protein synthesis is cell-specific and dependent on both the degree and duration of hypoxia. For example in HEK293 cells, 1.5% O₂ for 24 h did not decrease protein synthesis, but 25% and 50% reductions were seen at 32 and 48 h [12]. Incubation of PC-3 cells in 1% O₂ for 20 h reduced protein synthesis by 90% [13]. In contrast, in rat hepatocytes, 60 min of mild hypoxia (6.5% O₂) reduced protein synthesis by 70% [14]. Hypoxia has been shown to alter the synthesis of individual proteins in trophoblast cells. GLUT1, VEGF, sFlt-1 and eNOS, proteins which form part of the hypoxia response [6], show increased levels of syncytial translation under hypoxic conditions [15–18] whereas a number of other proteins such as 11- β hydroxysteroid dehydrogenase-2, the SNAT1 and SNAT2 amino acid transporters, PIGF and hCG show decreased synthesis as a result of limited O₂ supply [16,19,20]. There is, however, no information on global protein synthesis in this cell type either in the normal physiologic state or under conditions of chronic hypoxia. Global protein synthesis is crucial for placental growth and function and thus provides a measure of the capacity of the placenta to function when hypoxia occurs. These studies were designed to investigate the effects of chronic hypoxia on protein synthesis in syncytiotrophoblast cells, testing the hypothesis that syncytial protein synthesis is decreased with increasing degrees of hypoxia. Secondly we investigated the hypothesis that glucose metabolism supports protein synthesis under conditions of increasing hypoxia.

2. Methods

2.1. Cell isolation and culture

Human placental tissue was obtained from normal, term singleton pregnancies at elective cesarean section using a protocol approved by the Institutional Review Board of UMDNJ-New Jersey Medical School, with written, informed consent. Subjects were excluded if there was evidence of diabetes, hypertension, anemia, tobacco or drug use or other medical or obstetric complications which might affect cell function (see Table 1). Cytotrophoblast cells were prepared as described previously [21,22]. Briefly, after removal of the membranes, cord and maternal surface, villous tissue was washed, dissected free of connective tissue and vessels then incubated three times for 30 min each with 0.25% trypsin-EDTA. The supernatant from the first incubation was discarded; the supernatants from the second and third incubations were combined, filtered through a 100 μ m nylon mesh, pelleted and resuspended in Ham's-Weymouth's medium containing 10% FBS. Cytotrophoblasts were purified by isolation on a 40% Percoll gradient, plated at 0.4×10^6 cells/cm² in 6-well plates and incubated in keratinocyte growth medium (KGM; Lonza, Walkersville, MD) containing 10% FBS under a humidified air/5% CO₂ atmosphere. Medium was changed every 24 h.

Purity of the syncytiotrophoblast cultures was assessed following extraction of cell proteins after 72 h of culture by Western blotting for vimentin. Equal amounts of protein from the cell extracts and from a placental homogenate sample were subjected to SDS-PAGE then transferred to nitrocellulose and membranes were blotted using a vimentin monoclonal antibody (1:5000; MP Biomedicals, Solon, OH). Bands were detected using an HRP-conjugated secondary antibody (1:20,000; Sigma Chemical Co., St Louis, MO) and chemiluminescence (SuperSignal Western Pico; Pierce, Rockford IL). Syncytialization of the primary cytotrophoblast cells was monitored by measuring hCG production in cell culture supernatants using an hCG ELISA (BioQuant, San Diego, CA).

2.2. Hypoxia exposure

Apart from the time course experiment in which cells were incubated for either 48 or 72 h prior to hypoxic exposure, the cells used in all other experiments were incubated for 72 h before hypoxic exposure, as described above. Cells in 6-well plates were placed in a sealed hypoxia chamber and the chamber was equilibrated with the desired gas mixture (0, 1, 3, 5 or 10% O₂/5% CO₂/balance N₂) for 15 min at which time the culture medium was exchanged with fresh culture medium previously gassed with 95% N₂/5% CO₂. The culture medium comprised

DMEM/10% FBS, 5.5 mM glucose. Specific reagents such as the protein synthesis inhibitor cycloheximide (CHX) were also added at this point. In experiments examining the effects of glucose concentration, the medium was DMEM/10% FBS with glucose adjusted to the indicated concentrations. The culture plates were then placed into separate sealed hypoxia incubation units (Billups-Rothenburg, Del Mar, CA), flushed with the appropriate gas mixture for 2 min and placed in a humidified 37 °C incubator. O₂ levels in the pre-gassed medium, hypoxia chamber or hypoxic incubation units were measured using a galvanic O₂ electrode (Cole Palmer, Vernon Hills, IL) coupled to an analog-to-digital converter (ADInstruments, Colorado Springs, CO).

2.3. Measurement of protein synthesis rates

Two methods were employed for the measurement of protein synthesis rates, radiolabel incorporation and a more recent, non-radioactive technique. In the radiolabeling experiments [³H]L-Leu was added to cultures (1 mCi/ml) at the time of exposure to 10, 5, 3, or 1% O₂. After an 18 h incubation, medium was removed, wells were washed twice with cold PBS

and the cell proteins were extracted with RIPA buffer (200 µl/well). Two volumes of PBS containing 0.1 mg/ml BSA, 0.01% NaN₃ were added to the extract and mixed followed by another two volumes of 20% trichloroacetic acid. The mixture was filtered through glass microfiber disks (Whatman GF/C) which were washed twice with 4 ml of cold 10% trichloroacetic acid followed by two washes with 100% EtOH. Disks were placed in a scintillation vial, 5 ml of scintillant was added and the radioactivity was counted. The counts per min were normalized to total protein, measured by the Bradford protein assay (QuickStart, Bio-Rad, Hercules, CA) in extracts from parallel wells.

To assess protein synthesis after cellular equilibration to chronic hypoxia we employed a second type of measurement which reflects protein synthesis rates at the end of a hypoxic incubation. This second measure utilized the Click-iT AHA Protein Labeling Kit (Invitrogen, Carlsbad, CA). In this labeling reaction L-azidohomoalanine (AHA), a methionine analog containing a reactive azido group, is incorporated into proteins and later derivatized with a fluorophore to label the protein. In our experiments after 20 or 44 h of hypoxic incubation, cells were incubated in methionine-free media for 1 h and then AHA (final concentration 50 µM) was added to the cell cultures for a further 3 h incubation. Cells were cultured under the same experimental conditions for the last 4 h of the incubation as for the preceding period except for the absence of methionine and the presence of AHA. Cell proteins were extracted after the AHA incubation and extracts were derivatized using the Click-iT Tetramethylrhodamine (TAMRA) Protein Analysis Detection Kit (Invitrogen) according to the manufacturer's instructions. After labeling, proteins were chloroform/methanol precipitated, resuspended in Tris-buffered saline (TBS; 100 mM Tris HCl, 150 mM NaCl, pH 7.5) and slot blotted (Bio-Dot SF-1, Bio-Rad) on to nitrocellulose (Hybond ECL, GE Healthcare, Piscataway, NJ). AHA incorporation was determined by measurement of TAMRA fluorescence on a Typhoon 9410 imager (GE Healthcare) using an excitation of 532 nm. Following this the slot blot was washed, fixed and stained with Sypro Ruby protein blot stain (Invitrogen) according to manufacturer's instructions and the fluorescence was visualized on the Typhoon imager using an excitation of 488 nm. Image density was measured using ImageJ (NIH). AHA incorporation results obtained from the TAMRA measurements were normalized to total protein as determined by Sypro Ruby staining. Values for AHA incorporation in the presence of the protein synthesis inhibitor cycloheximide (CHX, 0.1 mg/ml) were subtracted from values for AHA incorporation in other experiments to correct for residual fluorescence which did not reflect protein synthesis.

For incorporation experiments, the medium over the cells was switched from KGM/10% FBS to DMEM/10% FBS for the incubation period. This allowed for use of methionine-free medium in the AHA incorporation experiments and for the use of glucose-free medium in the experiments investigating responses to hypoglycemia. Initial experiments were performed to investigate the effects of hypoxia (10, 5, 3, or 1% O₂) after incubation for 18 h. The effects of a 24 or 48 h hypoxic incubation were determined in cells placed in a hypoxic environment 48 or 72 h after plating (10, 1 or 0% O₂) and incubated for a total of 48 or 24 h respectively. The effects of hypoxia (10, 1 or 0% O₂) combined with altered glycemic status (5.5, 2 or 0 mM glucose) were measured 72 h after plating in a 24 h incubation. In other experiments cells were exposed to cycloheximide (CHX; 0.1 mg/ml), dimethyloxallylglycine (DMOG; 2.0 mM), tunicamycin (Tm; 1 µM) or 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR; 1 mM) for 24 h.

2.4. Statistical analysis

Data are presented as mean ± SEM. The notation "n" is used to indicate the number of experimental trophoblast cell preparations obtained. In each experiment a minimum of three replicates was obtained for each experimental point from each placental preparation and the average of these replicates was used in the analyses. Statistical analysis was performed using

the Graphpad InStat software package (v3.0b). Repeated measures ANOVA with TukeyKramer multiple comparisons post hoc test was used for analysis of data in which syncytiotrophoblast obtained from different placentae were subjected to the same set of multiple conditions, such as variation in the levels of oxygenation or extracellular glucose concentrations. Differences in the mean values for hCG secretion and protein synthesis were analyzed by repeated measures ANOVA and subjected to post tests; Dunn's multiple comparison post test for nonparametric data, the TukeyKramer post test for parametric data or the post test for a linear trend. Comparisons of protein synthesis in control syncytiotrophoblast and those exposed to cycloheximide (CHX), dimethyloxallylglycine (DMOG), tunicamycin (Tm) or 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) were performed using a paired *t* test. Comparison of AHA incorporation between syncytiotrophoblast exposed to 10% and 21% O₂ was carried out using a Wilcoxon matched-pairs signed-ranks test. Statistical analysis was performed on raw experimental data however in Figs. 2–5, for purposes of visualization, the experimental data were transformed and are presented as a percent of control, where the 10% O₂ control value was set to 100%.

2.5. Materials

Cell culture reagents were obtained from Invitrogen (Carlsbad, CA) with the exception of DNase which was obtained from Roche Diagnostics (Indianapolis, IN) and fetal bovine serum (FBS) which was obtained from Thermo HyClone (Waltham, MA). [³H]L-Leu was purchased from American Radiolabel Chemicals (St. Louis, MO), dimethyloxallylglycine (DMOG) from Cayman Chemical (Ann Arbor, MI), AHA labeling kits from Invitrogen and Hybond ECL nitrocellulose from GE Healthcare (Piscataway, NJ). The anti-vimentin antibody was obtained from MP Biomedicals (Solon, OH). All other chemicals were obtained from Sigma Chemical Company (St Louis, MO).

3. Results

3.1. Cell culture

Cell culture purity was confirmed by Western blotting of syncytiotrophoblast cell protein extracts for vimentin. Blotting of equal amounts of cell protein extract from cells cultured for 72 h and a placental homogenate sample (Fig. 1A) showed low staining for vimentin, demonstrating minimal contamination by non-trophoblast cell types. Syncytialization of the primary cytotrophoblast cells over the time course of the 72 h incubation was tested by measuring the extent of hCG secretion. As shown in Fig. 1B, although there was minimal hCG secreted in cultures incubated for 24 h, at 48 and 72 h the cultures secreted significant quantities of hCG, demonstrating syncytialization.

3.2. Oxygenation conditions

To ensure that cells were exposed to the intended O₂ concentrations and did not experience either the slow alterations in oxygenation or hypoxia-reoxygenation described previously [23], testing was performed to measure the O₂ concentrations in the atmospheres of the hypoxia chamber, the hypoxia incubation units and in the culture media (data not shown). Flushing the hypoxia chamber in which experimental manipulations were performed with reduced O₂ mixtures resulted in alteration of the O₂ concentration in the chamber to the required level within 5 min, as measured using an O₂ electrode. Flushing the incubation units reduced the O₂ level similarly within 1 min. To verify that the medium achieved the required level of oxygenation, culture medium was bubbled with a series of gas mixtures containing 5% CO₂ and different levels of O₂ (0–10%, balance nitrogen). This reduced the O₂ level of previously air-equilibrated medium to that of the gas mixture within 15 min. These O₂ levels were maintained throughout the experimental period. An exception was observed following gassing with 0% O₂, when medium oxygenation was not reduced to 0%

O₂ but rather to a level < 0.4% O₂, suggesting the possibility of a residual O₂ in those incubations.

3.3. Protein synthesis and hypoxia

We compared the extent of protein synthesis taking place in cells exposed to 10% O₂ and those exposed to room air (21% O₂). We chose to test an atmosphere of 10% O₂ since this is closer to the probable level of O₂ in the intervillous space than the room air level frequently used in cell culture experiments [24]. Although syncytiotrophoblast cultures exposed to room air showed no significant differences in protein synthesis ($125 \pm 8\%$ in 21% O₂ vs. $100 \pm 13\%$ in 10% O₂; NS, $n = 5$) with exposed those to 10% O₂ (set to 100%), we chose to use a 10% O₂ concentration as the control for the hypoxia experiments as it is the more physiological concentration of O₂. We performed initial experiments to measure the effects of hypoxia on protein synthesis by exposing cells to O₂ concentrations of 1, 3, 5 or 10%. Cultures were incubated for 18 h under conditions of differing oxygenation in the presence of tracer [³H]L-Leu. Protein synthesis measured following incubation in 5% or 3% O₂, did not differ from control (10% O₂) as shown in Fig. 2A (5% O₂, $88 \pm 3\%$ of control; 3% O₂, $95 \pm 4\%$; $n = 4$, N.S.). in 1% O₂ reduced by Incubation protein synthesis 20% ($80 \pm 6\%$ of control; $n = 4$, $p < 0.05$). As positive controls, we simultaneously incubated cells for the same time period in the presence of the proteinsynthesis inhibitor cycloheximide (CHX, 0.1 mg/ml) or in the presence of the hypoxia mimetic, dimethyloxallylglycine (DMOG, 2.0 mM) [25]. CHX treatment resulted in a decrease in protein synthesis of 97% ($3.1 \pm 0.5\%$ of control; $n = 4$, $p < 0.0001$) as in Fig. 2B. This confirms that protein synthesis in this cell type is abolished by the synthesis inhibitor, CHX. DMOG treatment resulted in a decrease in protein synthesis of nearly 40% ($62 \pm 6\%$ of control; $p < 0.05$, $n = 5$).

In light of the modest response of protein synthesis to hypoxia described above, we modified our protocols. In addition to increasing the incubation period in hypoxia from 18 to 24 h, we employed a new measure of protein synthesis. The new assay of protein synthesis used incorporation of a synthetic amino acid, azidohomoalanine (AHA) to provide a means of labeling newly synthesized proteins with a fluorescent label. The primary advantage of this method over that of [³H]L-Leu incorporation is that the latter took place over the entire 18 h incubation period, including an initial period during which cellular equilibration to hypoxia is occurring, whereas AHA incorporation takes place in the last 3 h of the incubation when rates of synthesis are stable at levels dictated by the level of hypoxia.

3.4. Verification of the hypoxia response

As there was only a modest decrease in leucine incorporation at the lowest O₂ concentration used in the experiments described above (1%), we decided to investigate specific hypoxia-responsive pathways to verify that protein synthesis was inhibitable via these pathways in trophoblast cells. We tested the effect of agents known to inhibit protein synthesis via hypoxia-responsive pathways. Hypoxia has been shown to inhibit protein synthesis in other cells types through activation of protein kinase-like endoplasmic reticulum kinase (PERK) and phosphorylation of eIF2a (eukaryotic initiation factor 2a), preventing its participation in the ribosomal initiation complex [26]. Hypoxia has also been shown to inhibit protein synthesis by activation of AMP kinase (AMPK), preventing ribosomal S6 protein phosphorylation and promoting phosphorylation of eukaryotic elongation factor 2 (eEF2) [27,28], actions which inhibit the initiation and elongation respectively of nascent proteins. Tunicamycin (1 μ M) was used to generate an endoplasmic reticulum stress response, activating PERK, while 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR, 1 mM) was used to stimulate AMPK. Treatment of cells exposed to these agents resulted in decreased protein synthesis compared to control, as shown in Fig. 3. Tunicamycin reduced

protein synthesis by nearly 80% ($22 \pm 6\%$ of control; $p < 0.005$, $n = 3$) while AICAR decreased protein synthesis by 70% ($28 \pm 6\%$ of control; $p < 0.001$, $n = 5$).

3.5. Effects of severe hypoxia

Following the verification of the hypoxia-responsive pathways, we repeated the hypoxia experiments using the AHA incorporation assay. We extended the duration of hypoxia exposure to include both a 24 and a 48 h period. In addition, we altered the profile of hypoxic conditions to include a 0% O₂ exposure while removing the incubations at 3% and 5% O₂, since they gave results similar to the 10% O₂ exposure. We incubated cells in 0, 1 or 10% O₂ for either 24 or 48 h. As shown in Fig. 4, after exposure to 1% O₂ for 24 and 48 h, protein synthesis decreased by 24% and 23% ($76 \pm 12\%$, $77 \pm 7\%$ of control; $p < 0.05$, $n = 4$) and with exposure to 0% O₂ after 24 $\pm$ h and 48 h, by 66% and 50% ($34 \pm 12\%$, $50 \pm 9\%$ of control; $p < 0.05$, $n = 4$). At both 0% and 1% O₂, there were no differences between the 24 h and the 48 h incubations. These data show that there was a reduction in protein synthesis in conditions of severe hypoxia (0% O₂), however a longer duration did not increase the effect observed at 24 h.

3.6. Role of glucose

Despite severe hypoxia, verging on anoxia, protein synthesis in the previous experiments was still only reduced to 34% of control and thus we asked whether protein synthesis was being sustained by glucose metabolism, as observed in other cell types [29]. We therefore investigated the effect of differing extracellular glucose concentrations on protein synthesis under varying conditions of hypoxia. The experiments described in the previous section were repeated using conditions of severe hypoxia (0% and 1%) and compared to control (10% O₂) using glucose concentrations of 0 mM, 2 mM and 5.5 mM for each oxygenation condition and using a 24 h incubation. We chose these concentrations because mean fetal circulating glucose concentrations can decrease to as low as 1.2 mM in severe IUGR [30,31]. Thus the glucose concentrations used here encompass the fetal (patho)physiological range. When compared with 10% O₂/5.5 mM glucose (control), protein synthesis was decreased by both hypoxia and hypoglycemia (Fig. 5). At each O₂ concentration there was a significant decreasing linear trend in protein synthesis as glucose concentration was reduced from 5.5 to 2.0 to 0 mM ($p < 0.05$, $n = 5$). Under conditions of near-anoxia and in the absence of glucose, protein synthesis was reduced by > 85% ($14 \pm 6\%$ of control; $p < 0.01$, $n = 5$).

To ensure that cell death was not a factor in these experiments, we compared the protein content of the cell culture extracts, obtained after washing of the cultures to remove extracellular protein and cellular debris (x 3 with cold PBS), by Sypro Ruby staining of the blotted protein. There was no significant difference in protein content of the extracts between any of the groups, ranging from the control (10% O₂, 5.5 mM glucose) to the extreme hypoxic, hypoglycemic incubation (0% O₂, 0 mM glucose) over the 24 h incubation (data not shown). This confirmed the absence of changes observed on visual inspection of the cultures. It is also possible that under hypoxic conditions, an increased rate of glycolysis might lead to reductions in the extracellular glucose concentration such that hypoglycemia might contribute to cellular energy deprivation. We determined whether glucose concentrations were decreased over a 24 h incubation. Samples of medium were drawn from each well before and after hypoxic incubation of cells incubated in initial glucose concentrations of 5.5 and 2.0 mM. Samples were assayed for glucose. The results show that for three separate placental preparations the glucose concentrations were not significantly different in medium from cultures incubated at 0, 1, 3, 5, 10 or 21% O₂ for 24 h (data not shown).

4. Discussion

These experiments demonstrate that syncytiotrophoblast cells are strikingly resistant to reductions in protein synthesis at O₂ concentrations greater than 1%. Use of agents such as tunicamycin and AICAR to stimulate specific hypoxia-responsive pathways showed that protein synthesis can be reduced by at least 75%, thus clearly these pathways are intact and are capable of mediating the inhibition of protein synthesis. Extending the duration of severe hypoxic exposure to 48 h did not increase protein synthesis inhibition compared to a 24 h incubation. Reduction of O₂ levels to 0% decreased protein synthesis after 24 h to 34% of the value obtained from cultures incubated in 10% O₂. The resistance to hypoxia is partially explained by our finding that protein synthesis in the syncytiotrophoblast is also supported by glycolytic metabolism, whether O₂ levels are normal or diminished. Increasing degrees of hypoglycemia at any level of O₂ were accompanied by progressive reductions in protein synthesis that exceeded those seen with O₂ deprivation alone. These results show that protein synthesis in human syncytiotrophoblast is supported by energy derived from both oxidative metabolism and anaerobic glycolysis. This supports our hypothesis that global protein synthesis is decreased by hypoxia and secondarily, that the energy generated by glycolytic metabolism ensures that protein synthesis continues to be fully supported in conditions of mild-to-moderate hypoxia.

One of the important aspects to note concerning the experiments conducted here is the O₂ concentration. We used 10% O₂ as the control condition since this appears to be at the upper level of the O₂ concentration observed in the intervillous space [32,33]. It is difficult to determine the intervillous O₂ concentration during the third trimester, however it is clear that the 10% level used here is more physiologic than the 21% usually used in cell culture experiments. Despite this, protein synthesis in cells exposed to 21% O₂ for 24 h was no different from that observed in cells exposed to 10% O₂.

With respect to the O₂ concentrations used to produce hypoxia, we started with 1%, 3% and 5% O₂ (equivalent to 7.6, 22.8 and 38.0 μ m Hg). The 1% concentration is approximately half of that observed at < 10 weeks gestation [34], whilst 5% O₂ approximates the lower end of normal third trimester intervillous O₂ tension [35]. However, as we did not see any change in protein synthesis with either 3% or 5% compared to 10% O₂ over an 18 h period, we conducted additional studies focused on more severe levels of hypoxia (0% and 1% O₂). Many studies have exposed trophoblast cells to a low O₂ concentrations, but as demonstrated by Newby et al. [23], this is done frequently in a manner which leads to a slow decline of the O₂ level in the culture medium such that there is a gradually changing level of exposure. "Pre-gassing" the media, a technique we used here, produces the desired initial oxygenation level without fluctuations that might compromise the response. Measurement of O₂ levels in both the atmosphere over the cells and in the cell culture medium verified that, with the exception of cells exposed to 0% O₂, the procedures used in our experiments achieved the intended levels of oxygenation. In the case of cells exposed to 0% O₂, we found that we could not confirm levels of O₂ in the culture medium below 0.4% and it is possible therefore that residual traces of O₂ might have supported oxidative metabolism in those cells nominally exposed to 0% O₂.

A number of controls were used to confirm the operation of hypoxia-responsive translational pathways in the syncytial cells. Treatment of the cells with the protein synthesis inhibitor cycloheximide essentially eliminated label incorporation into proteins, demonstrating virtually complete inhibition of protein synthesis. Treatment with DMOG, a well-described hypoxia mimetic which increases HIF-1 protein levels by inhibiting HIF-1 α degradation, reduced protein synthesis by w40%. This suggests that while the HIF-1 mediated response to hypoxia functions in these cells [15,36], effects due to HIF-1 are not sufficient to

completely suppress protein synthesis. Administration of either tunicamycin or AICAR inhibited protein synthesis by $\pm 75\%$, demonstrating that hypoxia-responsive pathways described in other cells [12,26,37,38] are functional in syncytial cells. It should be noted of course that these experiments examine global protein synthesis however they provide no evidence concerning individual proteins or groups of proteins which may show a different response.

We found it necessary to reduce O_2 concentration to 1% or less to obtain a significant degree of inhibition of protein synthesis in syncytiotrophoblast cells, an effect which was not enhanced by doubling the incubation time. Syncytiotrophoblast cells thus appear to display a marked resistance to hypoxia, prompting us to ask whether continued translation was being sustained by anaerobic glycolysis. Although O_2 is the dominant source for energy for cellular processes, when O_2 supply is limited, increased anaerobic glycolysis substitutes as a source of energy. Augmenting the hypoxic insult by reducing glucose concentrations caused a decrease in protein synthesis over that observed in the presence of hypoxia alone, with almost complete cessation of protein synthesis at the most extreme levels of hypoxia and hypoglycemia. Interestingly even under “normoxic” conditions (i.e. 10% O_2), dropping the glucose concentration caused reductions in protein synthesis, suggesting that a substantial fraction of syncytial protein synthesis is normally supported by glucose, despite the presence of oxidative substrates such as pyruvate and glutamine/glutamate. The resistance of syncytiotrophoblast to hypoxia-associated inhibition of protein synthesis is supported by data from perfusion experiments. Uncoupling oxidative phosphorylation produced only a minor effect on ATP concentrations and a modest decrease in hCG synthesis. In contrast iodoacetate, a glycolytic inhibitor, combined with a mitochondrial uncoupler caused a loss of ATP and a substantial decrease in protein synthesis [39].

What do these results mean for protein synthesis in vivo, under conditions where O_2 delivery is likely reduced, conditions such as fetal growth restriction or preeclampsia which are frequently associated with chronic hypoxia? Assuming that the maternal glucose supply is not decreased, syncytial protein synthesis is unlikely to be affected unless the placenta experiences severe hypoxia ($\pm 1\% O_2$). Low PO_2 and high lactate are seen in umbilical vein samples from severe IUGR pregnancies, those with abnormal fetal heart patterns and Doppler indices of reduced fetal blood flow, suggesting a shift from oxidative to glycolytic metabolism [40]. The increase in the maternal-fetal glucose gradient seen in IUGR pregnancies also supports that the fetus and the placenta have shifted towards anaerobic glycolysis as an energy source [41]. Using in vivo umbilical levels of O_2 and glucose as the lower limit to which the placenta is exposed, it is apparent that, although the most extreme conditions used in this study (0% O_2 , 0 mM glucose) are not encountered in vivo, in severe IUGR the placenta can be exposed to levels of O_2 well below 1% O_2 , as measured in the umbilical artery [42,43], while umbilical venous glucose concentrations down to almost 1 mM have been reported [30,31]. It is thus probable therefore that there will be inhibition of syncytial protein synthesis at the lower ranges of O_2 and glucose concentrations occurring in vivo. We hypothesize that there is a transition zone at these lower values where the fetus moves from moderate to severe growth restriction and in which, because of decreasing O_2 and glucose supply, global syncytial protein synthesis and hence placental function begin to decline, contributing further to fetal growth restriction.

We speculate that the resistance of protein synthesis to hypoxia may ensure preservation of placental growth and function and therefore ensure fetal survival, perhaps at the cost of reducing fetal growth, as demonstrated by the reduction in fetal weight but maintenance of placental weight in conditions of chronic hypoxia [9]. The minimal change in protein synthesis with a decreasing O_2 supply is likely a component of this mechanism, a component of “hypoxia tolerance” [44]. In the hierarchy of hypoxia responses, sustained

reduction in energy demand is an extreme response, invoked only following the initial responses which result in increasing O₂ supply to the placenta, augmentation of energy production via glycolysis and mechanisms that contribute to cellular O₂ sparing in the placenta [10]. Demand reduction involves a decrease or cessation of protein synthesis and other energy-intensive processes coupled to transcription and translation. The progression from earlier stages of the hypoxia response to demand reduction marks a cellular shift from adaptation to survival mode, with the shutdown of synthetic functions and the consequent decrease in demand for metabolic energy. The fact that this occurs only under relatively extreme conditions of deprivation suggests that demand reduction and the inhibition of protein synthesis is not the mechanism for fetoplacental growth reduction which takes place under more moderate conditions of substrate limitation.

In summary, these investigations demonstrate that protein synthesis in cultured human syncytiotrophoblast is supported by both oxidative and glycolytic metabolism. Increasing degrees of hypoxia cause a progressive shift from the former to the latter, allowing protein synthesis to be fully maintained under conditions of moderate hypoxia. Severe hypoxia coupled with an increasing degree of hypoglycemia is required, at least in vitro, before significant reductions in placental protein synthesis occur, highlighting the adaptive capacity of the placenta in the face of adverse conditions. The transitional stage in which placental function is still relatively normal but there is an increased reliance on anaerobic glycolysis may be the window for initiation of fetal growth restriction. The timing in gestation at which placental protein synthesis declines may determine the severity of compromise in fetal growth. Finding a means of identifying this transition point clinically would be beneficial in prenatal monitoring of fetoplacental well-being.

Acknowledgments

The authors would like to acknowledge the support of NIH in funding this research (HD046982, N.P.I.; HD042737, S.Z.).

Abbreviation

AHA L-azidohomoalanine.

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Fig. 1.

Measures of trophoblast purity and syncytialization. (A) Western blot of cell proteins obtained 72 h after cytotrophoblast plating. Protein extracts of syncytial cultures (ST) and placental homogenates (H) were blotted for vimentin. (B) hCG release into syncytial culture medium was measured by ELISA for the 0–24 h, 24–48 h and 48–72 h periods following plating. Culture supernatant hCG concentrations at 48 and 72 h were greater than 24 h ($p < 0.05$, $p < 0.001$; $n = 6$, repeated measures ANOVA).

Fig. 2.

Effects of hypoxia on syncytial protein synthesis. Data in this figure represents the incorporation of [³H]L-Leu into protein, as a percent of control (10% O₂). After 72 h of culture, cells were exposed for 18 h to (A) O₂ levels of 1, 3, and 5% O₂ (*n* = 4; 1% < 10%, *p* < 0.05, repeated measures ANOVA) or to (B) the protein synthesis inhibitor, cycloheximide (CHX, 10 mg/ml; < 10%, *p* < 0.0001, *n* = 3, paired *t* test) or the hypoxia mimetic, dimethyloxallylglycine (DMOG, 2.0 mM; < 10%, *p* < 0.005, *n* = 5, paired *t* test).

Fig. 3. Effects of hypoxia-responsive pathway inhibitors on syncytial protein synthesis. Protein synthesis measured by the incorporation of the methionine analog, azidohomoalanine (AHA) as a percent of control (21% O₂). After 72 h in culture, cells were exposed for 24 h to tunicamycin (1 mM; $p < 0.001$, $n = 3$, paired t test) or 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR, 1 mM; $p < 0.001$, $n = 5$, paired t test).

Fig. 4.

Effects of severe hypoxia. Cells were exposed to hypoxia (0% or 1% O₂) for 24 or 48 h and protein synthesis was measured by AHA incorporation, given as percent of control (10% O₂). Primary cytotrophoblast cells cultured for 48 h were exposed to 0%, 1% or 10% O₂ for a further 48 h (light blue bars). Cells cultured for 72 h were exposed to 0%, 1% or 10% O₂ for a further 24 h (dark blue bars). Cells exposed to 0% O₂ showed decreases compared to control ($p < 0.05$, $n = 4$, repeated measures ANOVA) but there were no differences between values at 24 and 48 h.

Fig. 5.

Hypoxic and hypoglycemic effects on syncytial protein synthesis. The figure shows the effects of hypoxia and hypoglycemia on AHA protein incorporation (measured as percent of control; 10% O₂). After 72 h in culture, syncytial cells were exposed to 0%, 1% or 10% O₂ for 24 h in the presence of 0, 2 or 5.5 mM D-glucose. Incorporation at 0 mM glucose was lower than at 5.5 mM glucose at all three O₂ concentrations ($p < 0.05$, $n = 5$, repeated measures ANOVA). At each O₂ concentration protein synthesis decreased progressively as glucose levels declined ($p < 0.05$, repeated measures ANOVA).

Table 1

Clinical characteristics of pregnancies for placentas studied: Table shows the clinical characteristics of the pregnancies from which placentas were obtained for the purpose of preparing primary cytotrophoblast cells ($n = 11$).

Parameter	Clinical characteristics of pregnancies for placentas studied			
Gravidity	Median = 3	Range 2–5		
Parity	Median = 1	Range 1–4		
Gestational age (weeks)	Average = 38.7	SD = 0.5		
Maternal age (years)	Average = 25.2	SD = 4.5		
Race	Black $n = 4$	White $n = 7$		
Prenatal medications	Iron, $n = 1$	Other (list): $n = 1$ (Prednisone)	None, $n = 9$	
Drugs	Cigarettes, $n = 0$	Alcohol, $n = 0$		
Previous prenatal admission(s)	Yes, $n = 0$	No, $n = 11$		
Blood pressures <140/90 mm Hg	Yes, $n = 11$	No, $n = 0$		
Screened for diabetes	Yes, $n = 11$	No, $n = 0$		
Beta strep status	Positive, $n = 3$	Negative, $n = 8$		
Antenatal steroids:	Yes, $n = 0$	No, $n = 11$		
Magnesium sulfate	Yes, $n = 0$	No, $n = 11$		
Anesthesia	Epidural, $n = 9$	Narcotics, $n = 0$	General, $n = 2$	
Labor	No, $n = 11$			
Delivery mode (N = # patients)	C-section, Repeat, no labor: $n = 9$	C-section, Repeat, with labor: $n = 0$	C-section, primary, no labor: $n = 2$	C-section, primary, with labor: $n = 0$
Birth weight (g)	Average = 3469	SD = 364		
Placental weight (g)	Average = 627	SD = 55		
Baby's sex	Female = 4	Male = 7		
Delivery to processing (mins)	Average < 15 min			