Placenta 34 (2013) 690–698

Contents lists available at SciVerse ScienceDirect

Placenta

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

Oxygen tension modulates AQP9 expression in human placenta

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ARTICLE INFO

Article history:
Accepted 24 April 2013

Keywords:
Hypoxia
AQP9
Syncytiotrophoblast
Placenta
Preeclampsia

ABSTRACT

Placental hypoxia has been implicated in pregnancy pathologies such as preeclampsia. We have previously reported that AQP9 is highly expressed in syncytiotrophoblast from normal placentas and shows an overexpression in preeclamptic placentas, with a lack of functionality for water transport. Up to now, the response of AQP9 to changes in the oxygen tension in trophoblast cells is still unknown.

Objective: Our aim was to establish whether alterations in oxygen levels may modulate AQP9 expression in human placenta.

Methods: A theoretical analysis of the human AQP9 gene to find conserved DNA regions that could serve as putative HIF-1 binding sites. Then, explants from normal placentas were cultured at different concentrations of oxygen or with 250 μM $CoCl_2$. AQP9 molecular expression and water uptake was determined.

Results: Fourteen consensus HIF-1 binding sites were found in the human AQP9 gene, but none of them in the promoter region. However, placental AQP9 decreased abruptly when HIF-1α is expressed by deprivation of oxygen or $CoCl_2$ stabilization. In contrast, after reoxygenation, HIF-1α was undetectable while AQP9 increased significantly and changed its cellular distribution, showing the same pattern as that previously described in preeclamptic placentas.

Accordingly with the decrease in AQP9 expression, water uptake decreased in explants exposed to hypoxia or treated with $CoCl_2$. Conversely as we expected, after reoxygenation, water uptake decreased dramatically compared to the control and was not sensitive to $HgCl_2$.

Conclusion: Our findings suggest that oxygen tension may modulate AQP9 expression in human placenta. However, the role of AQP9 still remains uncertain.

1. Introduction

The human placenta is a unique organ in terms of oxygenation as it undergoes a transition from a low to a more oxygenated environment. Placentation occurs in a relatively hypoxic environment which is essential for appropriate embryonic development. Intervillous blood flow increases at around 10–12 weeks of gestation and results in exposure of the trophoblast to increased oxygen tension. Prior to this time, low oxygen appears to prevent trophoblast differentiation towards an invasive phenotype. This physiological switch in oxygen tension is a prerequisite for proper placental development [1,2]. Therefore, failure of the oxygen-associated developmental events contributes to placental diseases such as preeclampsia.

One of the most common characteristic features in placentas from pregnancies complicated by preeclampsia is an insufficient trophoblast invasion of maternal endometrial spiral arteries [1–4]. As a result, perfusion of the placenta is impaired, and oxygen concentration within the intervillous space is more variable in comparison to a healthy pregnancy, resulting in an ischemia-reperfusion (hypoxia-reoxygenation [H/R])-type injury [3,4]. The human syncytiotrophoblast (hST), the specialized epithelium that comprises the barrier between the mother and the fetus, is known to be extremely sensitive to changes in oxygen tension early in placental development [5–7]. However, trophoblast responses to hypoxia later in pregnancy are much less clear.

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http://dx.doi.org/10.1016/j.placenta.2013.04.017

We have previously reported that aquaglyceroporins (AQPs) permselective to urea and glycerol (such as AQP3) as well as to a broad range of small solutes (such as AQP9) are expressed in hST [8]. We also observed an increased expression and a different cellular distribution of placental AQP9 in preeclampsia [9].

It is well known that hypoxia regulates the expression of a number of genes that enable cells to adapt to this stress condition [10–12]. In brain, it has been demonstrated that the hypoxia-inducible transcription factor-1α (HIF-1α) participates in the transcriptional regulation of AQPs [13–15]. For instance, in the cerebellum of rats subjected to hypoxia, increases in mRNA and protein levels of vascular endothelial growth factor (VEGF) and aquaporin-4 (AQP4) have been found to be closely associated with an increase in HIF-1α expression [13]. In an ischemic/hypoxic model, traumatic brain injury induces HIF-1α, which, in turn, up-regulates the expression of AQP4 and AQP9. Additionally, inhibition of HIF-1α by 2-methoxyestradiol reduces the up-regulated levels of both of these AQPs [14].

Although AQP9 upregulation is a widely recognized response to hypoxia in multiple cell and tissue types [13–20], its response in trophoblast cells is still unknown.

Our aim was to elucidate the effects of changes in oxygen tension on AQP9 expression in placental villous tissue fragments and to determine whether its regulation is mediated by HIF-1α.

Our hypothesis is that hypoxia may be responsible for the alteration in AQP9 expression, distribution and function as we previously reported preeclamptic placentas [9].

2. Materials and methods

2.1. In silico analysis of the human AQP9 gene

We performed a theoretical analysis of the promoter region of the AQP9 gene (GenBank accession number NG_011975), the 5′ flanking region and the complete sequence of the gene, to identify putative recognition sites by transcription factors, using the MatInspector® tool from Genomatix® [21]. Sequence alignment was performed to locate the promoter with the Dialin® tool from Genomatix® and typical sequences of promoters were investigated in *Homo sapiens* and vertebrate matrices using the PromoterInspector® tool from Genomatix.

2.2. Tissue collection

This study was approved by the local ethics committee of the Hospital Nacional Dr. Prof. Alejandro Posadas, Buenos Aires, Argentina, and written consent was obtained from the patients before the collection of samples.

Full-term normal ($n = 15$) placental tissues were obtained after cesarean section. Clinical data are shown in Table 1.

2.3. Tissue culture

Placental tissue was gently separated by sterile dissection from different cotyledons, excluding chorionic and basal plates, minced with scalpel blades, and washed repeatedly with 0.9% sodium chloride to remove blood from the intervillous space. Whole villous tissue (~50 mg/well) was incubated in 24-well polystyrene tissue culture dishes in 2 mL of serum-free Dulbecco modified Eagle medium (DMEM; Life Technologies, Inc.) containing 100 IU/mL penicillin, 100 mg/mL streptomycin, 32 mg/mL gentamicin at 37 °C for 2 h under standard tissue culture conditions of 5%CO_2-balance room air to equilibrate the cultures and allow for recovery from isolation procedures.

First, we investigated the viability of the explants up to 7 days of culture. Cultures with apparent bacterial contamination were interrupted and excluded.

Table 1
Clinical characteristics of normal pregnant women. Values are mean ± SD.

	Normal pregnant women
Number of pregnant women	15
Parity	
Primiparous	8
Multiparous	7
Maternal age, yr	22.3 ± 1.5
Gestational age, wk	38.7 ± 1.0
Mean blood pressure, mm Hg	
Systolic	110 ± 3.9
Diastolic	63 ± 2.3
Proteinuria	Negative
Body Mass index (BM), kg/m^2	24 ± 3
Birth weight, g	3090 ± 240
Fetal sex	
Male	9
Female	6

2.4. Treatments

After changing the medium, some plates were incubated at 37 °C for 18 h under standard conditions (or "normoxia") in the cell culture incubators.

Hypoxic exposures (2%O_2–5%CO_2-balance nitrogen) were carried out in a hypoxic chamber/glove box (Billups-Rothenberg Inc.) for 3 h or 18 h (after stabilization) or for 3 h after 7 days of culture.

Hypoxia-reoxygenation [H/R]: At the end of the 3 h of hypoxic period, some explants were again exposed to standard conditions during 15 h.

Explants were also treated with $CoCl_2$, which is known to activate hypoxia-dependent pathways under normal oxygen levels by inhibiting prolyl-hydroxylase domain-containing enzymes, a family of enzymes that play a key role in the oxygen-dependent degradation of HIF-1α and consequently stabilizing HIF-1 [22].

Treatments with 250 μmol/L $CoCl_2$ (Sigma–Aldrich Corp.) were performed to induce chemical hypoxia for 18 h incubation period under standard conditions.

Supernatants were collected for hCG and LDH analysis and the explants were examined for functional and morphological evaluation.

2.5. Biochemical assays

Tissue viability was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay as described previously [23]. Tissue samples were collected in triplicate at 0 (after the 2 h of stabilization), 1, 2, 3, 4, 5, 6 and 7 days, exposed to MTT and the formation of the formazan product of MTT was measured by monitoring relative absorbance at 595 nm.

In addition, the production of β-human chorionic gonadotrophin (β-hCG) was also tested after 6 and 20 h of culture [9]. The concentration of β-hCG by hour in the culture medium was assessed by quantitative immunoradiometric determination (IRMA) using a commercially available kit (hCG solid phase component system, Coat-ACoat hCG IRMA, EURO/DPC Ltd., UK). The β-hCG assay uses the "sandwich technique" where the solid phase binds the alpha subunit of hCG and a radiolabeled antibody in the liquid phase binds to the beta one.

Integrity of explants was verified by the release of the intracellular enzyme lactate dehydrogenase (LDH) into the incubation medium after 4, 6 and 20 h of incubation. LDH concentrations were determined with a Lactate Dehydrogenase Assay kit (Sigma–Aldrich Corp.), according to the manufacturer's protocol.

2.6. Semiquantitative RT-PCR

Total RNA was isolated using an SV Total RNA isolation system (Promega Co., USA) and reverse-transcribed as previously described [8]. PCR was carried out using 5 μM of a specific oligonucleotide primer designed on the basis of a highly conserved region flanked by Asn-Pro-Ala (NPA) in the aquaporin family (sense 5′-CATCAACCCAGCTGTGTCT-3′, antisense 5′-CAGCCACTGTTCAGTCCCA-3′), amplifying a 393-bp fragment of human AQP9 [8]. β-actin primers were used as internal standards. The densitometry of the bands was quantified by the ImageJ 1.45s software package.

2.7. Immunoblotting

Treated and untreated explants from normal term placentas were processed according to the method previously described [8]. Briefly, explants were kept in a buffer containing 10 mM HEPES-KOH, 0.1 mM EGTA, 250 mM sucrose, pH 7.4, with protease inhibitors (0.2 mM PMSF, 25 mg/mL p-aminobenzamidine, 20 mg/mL aprotinin, 10 mg/ml leupeptin, 10 mg/mL pepstatin), homogenized (Ultra-Turrax homogenizer) and centrifuged at 3100 g for 10 min. The supernatants were collected and protein concentration of each sample was measured by the BCA assay (Pierce).

For immunoblotting studies, 100 μg of protein were loaded and resolved on a 15% polyacrylamide gel, and electrotransferred onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech Ltd.). After blocking, membranes were incubated overnight with the primary antibody anti-AQP9 (Alpha Diagnostic International Inc.; 1:500) and then with a goat anti-rabbit immunoglobulin G ([IgG] Jackson ImmunoResearch Laboratories, Inc.; 1:10,000) conjugated to peroxidase.

Immunoreactivity was detected using the Enhanced Chemiluminescence (ECL) Western Blotting Analysis System (ECL plus, Amersham Pharmacia Biotech Ltd.) according to the manufacturer's instructions.

The densitometry of the bands was quantified by the ImageJ 1.45s software package.