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Oxygen tension modulates AQP9 expression in human placenta

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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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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.

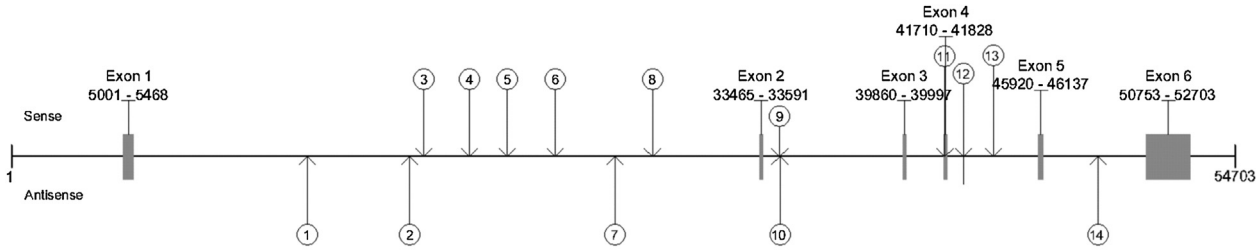
2.7. Immunoblotting

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.



ASSIGNED NUMBER	STARTING POSITION	END POSITION	STRAND	MSS	CSS	SEQUENCE
1	13226	13242	-	0.975	1.0	GGCTTCCA**CGTG**GGCTT
2	17823	17839	-	0.896	1.0	TGTTAATA**CGTG**GTTAT
3	18444	18460	+	0.97	1.0	CATATATA**CGTG**TGTAC
4	20490	20506	+	0.922	1.0	CAGGGTTA**CGTG**CAATA
5	22167	22183	+	0.946	1.0	TGTGCAGA**CGTG**ATAGA
6	24297	24313	+	0.953	1.0	AGGGATGA**CGTG**CCACC
7	26994	27010	-	0.92	1.0	TGCTAAAA**CGTG**CTGCC
8	28667	28683	+	0.985	1.0	TTCATGGA**CGTG**CAGAT
9	34355	34371	+	0.949	1.0	CTCTTTCA**CGTG**CATAC
10	34356	34372	-	0.972	1.0	TGTATGCA**CGTG**AAAGA
11	41730	41746	+	0.986	1.0	ACAAAGGA**CGTG**AGTGT
12	42597	42613	+	0.969	1.0	AGAACTCA**CGTG**AAGGA
13	43894	43910	+	0.972	1.0	GAAGAGGA**CGTG**TACCC
14	48592	48608	-	0.972	1.0	CAGTAAGA**CGTG**TGTGT

Fig. 1. Schematic drawing of potential HRE motifs in the human AQP9 gene. Genomic structure was taken from GenBank accession no. NG_011975. Identification of putative HIF-1 binding sites is derived from the HRE consensus sequence model of Wenger and Gassmann [25], which represents the deca base region (T,G,C)(A,G)CGTG(C,G,A)(G,T,C)(G,T,C)(C,T,G), and allowing for only one base mismatch outside the CGTG core structure (in bold). HRE sites in the schematic drawing are indicated by lowercase numbers within circles. Analysis was performed in both sense and antisense orientation. Matrix similarity score (MSS) and core similarity score (CSS) are indicated for comparison.

2.8. Immunoperoxidase and immunofluorescence assays

Treated and untreated explants were fixed overnight in 10% formaldehyde-0.1 mol/L sodium phosphate buffer (PBS), pH 7.4, dehydrated, and embedded in paraffin as previously described [8,9]. Then, thin sections (4–5 mm) were cut, dewaxed, rehydrated and incubated in 3% hydrogen peroxide/methanol for 5 min to block endogenous peroxidase. After blocking nonspecific binding sites with DAKO reagent (DAKO LSAB kit, Dako Corp.), tissue slices were incubated overnight (4 °C) with anti-AQP9 (1:50). Later, the samples were placed in prediluted link antibody, and incubated in a solution of streptavidin-conjugated horse-radish peroxidase. Staining was conducted with Vectastain kit (Vector Laboratories) and labeling was visualized by reaction with diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin.

For immunofluorescence experiments [24], after incubation with the primary antibody, tissue slices were treated with anti-rabbit IgG antibody conjugated with fluorescein (dilution 1:400) (Santa Cruz Biotechnology, USA) and the labeling was observed in an epifluorescent microscope (Nikon, Eclipse E200) provided with a green filter.

Negative controls were performed by omitting the primary antibody.

2.9. Water uptake

After each treatment placental explants were incubated at room temperature, in 0.5 mL hypo-osmolar saline solution containing ^{3}HOH (New England Nuclear Corp.). The uptake was stopped after different times, by adding ice-cold Ringer solution containing 1 mM unlabeled solute. At the end of the incubations, explants were quickly washed in cold Ringer's solution and solubilized with 1.0 ml of 1 M sodium

hydroxide overnight at 37 °C. Aliquots of the solubilized explants were vortex-mixed with 2.0 ml of scintillant (Optiphase "HiSafe", Wallc Oy) and counted on a scintillation counter [9,24–26]. Other aliquots were kept for determination of protein concentration by the bicinchoninic acid (BCA) assay (Pierce), according to the manufacturer's protocol.

Inhibition studies were carried out in placental explants previously incubated for 10 min in Ringer solution containing 0.3 mM $HgCl_2$. Solute uptake experiments were then performed as described above.

The uptake data (pmol mg^{-1} min^{-1}) obtained from each group were compared by one-way analysis of variance (ANOVA) followed by Fisher LSD test.

2.10. Statistical analysis

Statistical analysis was carried out with *Statistica software version 6.0* (Statistica, StatSoft, Inc. Tulsa). The criterion for statistical significance was $p < 0.05$.

3. Results

3.1. Identification of putative hypoxia-response element (HRE) sites in the human AQP9 gene

Since it is known that HIF-1α-mediated gene transactivation involves HIF-1α binding to distinct nucleic acid motifs, namely HREs, we used the MatInspector® tool of Genomatix® [21] to analyze the human AQP9 gene (GenBank accession number NG_011975) for appropriate HRE sequences. In this analysis, we followed the HRE consensus motif proposed by Wenger & Gassmann [27]: (T,G,C)(A,G)CGTG(C,G,A)(G,T,C)(G,T,C)(C,T,G), which has been constructed from the nucleotide sequence of HIF-1α binding sites of 13 oxygen-dependent genes, and allowed for no more than a single base mismatch outside the CGTG core sequence. We analyzed not only the 5′-promoter region but also the 3′-flanking region, introns/exons, and looked for consensus motifs in both sense and antisense strands, because these genomic areas have been previously shown to have functional HRE sites in other HIF-1α-inducible genes [27–29]. These premises allowed us to find fourteen putative HRE sites (Fig. 1), but none of them in the promoter region of the gene.

This finding allowed us to conjecture that AQP9 may be modulated by HIF-1α as a consequence of oxygen tension variations.

3.2. Biochemical assays

The biochemical status of the tissue was assessed by studying MTT incorporation, LDH release and hCG secretion.

MTT incorporation, a measure for mitochondrial dehydrogenase enzymatic activity was high over the first 24 h in culture but began to decrease at further time points up to 5 days (Fig. 2). After 7 days of culture, MTT incorporation increased to the initial values, suggesting that the syncytium was regenerated as it was previously described by Siman and coworkers [30].

The syncytial secretory capacity was tested by the estimation of β-hCG in the culture medium after 6 and 20 h of culture. Table 2A shows that the production of β-hCG by hour was preserved during culture.

Finally, cell integrity was measured by the release of LDH in the culture medium at 4, 6 and 20 h of culture. We observed that the levels LDH by hour declined at 20 h of culture as it was previously described [23,31] (Table 2B).

3.3. Effect of oxygen tension on AQP9 expression

After having established the cellular viability of the placental explants, we examined the expression of AQP9 by exposing explants to different oxygen conditions.

Placental AQP9 is expressed as a faint unglycosylated form of 28 kDa and a major glycosylated form at ~37 kDa, but the functional consequence of their glycosylation is not clear [32,33]. First, we observed that after 3 h, 18 h or 7 days of culture under standard conditions basal levels of AQP9 expression were similar and only the glycosylated form dramatically decreased by low-oxygen tension (Fig. 3A).

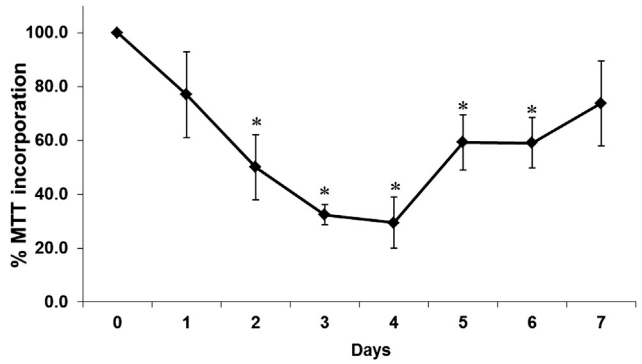


Fig. 2. Effect of time in culture on the viability of the villous tissue studied by MTT incorporation. Explants remained viable up to 24 h and after 7 days of culture when syncytium is regenerated. The data are shown as mean ± SEM obtained from 6 normal placental explants ($p < 0.05$ compared to 0 day of culture).

Immunoblotting analysis showed that the culture of the explants under hypoxic conditions resulted in a 1.4-fold decrease in the glycosylated form of AQP9. Explants treated with 250 μM $CoCl_2$ also showed a significant decrease in AQP9 expression. However, when we examined the effect of hypoxia/reoxygenation, AQP9 protein expression showed a 1.8-fold increase. In all cases, the 28-kDa band corresponding to the non-glycosylated form of AQP9 was unchanged (Fig. 3B).

We also determined whether low-oxygen conditions affected HIF-1α protein expression in our experimental system. Explants cultured under both hypoxic and $CoCl_2$ treatments showed an increase in HIF-1α protein expression compared with those cultured under standard conditions (Fig. 3B).

To investigate whether the changes in AQP9 protein levels were due to enhanced expression at the transcriptional level, total RNA was extracted from explants and RT-PCR analysis was performed using specific pairs of primers to amplify the NPA–NPA region of AQP9.

Table 2
Effect of time in culture on the biochemical status of the villous tissue. A) Release of β-hCG by term villi in culture medium per 6 and 20 h of culture. The data in each group are shown as mean ± SEM obtained from 8 normal placental explants ($p < 0.05$). B) Release of lactic dehydrogenase (LDH) by placental villi in culture medium per 4, 6 and 20 h of culture. Treatments did not decrease villous explant viability, as measured by the release of LDH ($p > 0.05$ compared to untreated controls). The data in each group are shown as mean ± SEM obtained from 10 normal placental explants.

A)	β-hCG (mUI × h^{-1} × g^{-1} explant)	
	After 6 h of culture	After 20 h of culture
Normoxia	381 ± 161	366 ± 29
Hypoxia	308 ± 50	320 ± 53
Hypoxia/reoxygenation	428 ± 150	314 ± 62
+$CoCl_2$	429 ± 138	314 ± 48

B)	LDH (U × h^{-1} × g^{-1} explant)		
	After 4 h of culture	After 6 h of culture	After 20 h of culture
Normoxia	0.550 ± 0.040	0.452 ± 0.030	0.261 ± 0.017*
Hypoxia	0.571 ± 0.033	0.581 ± 0.035	0.302 ± 0.020*
Hypoxia/reoxygenation	0.703 ± 0.045	0.610 ± 0.028	0.270 ± 0.015*
+$CoCl_2$	0.690 ± 0.039	0.500 ± 0.034	0.283 ± 0.013*

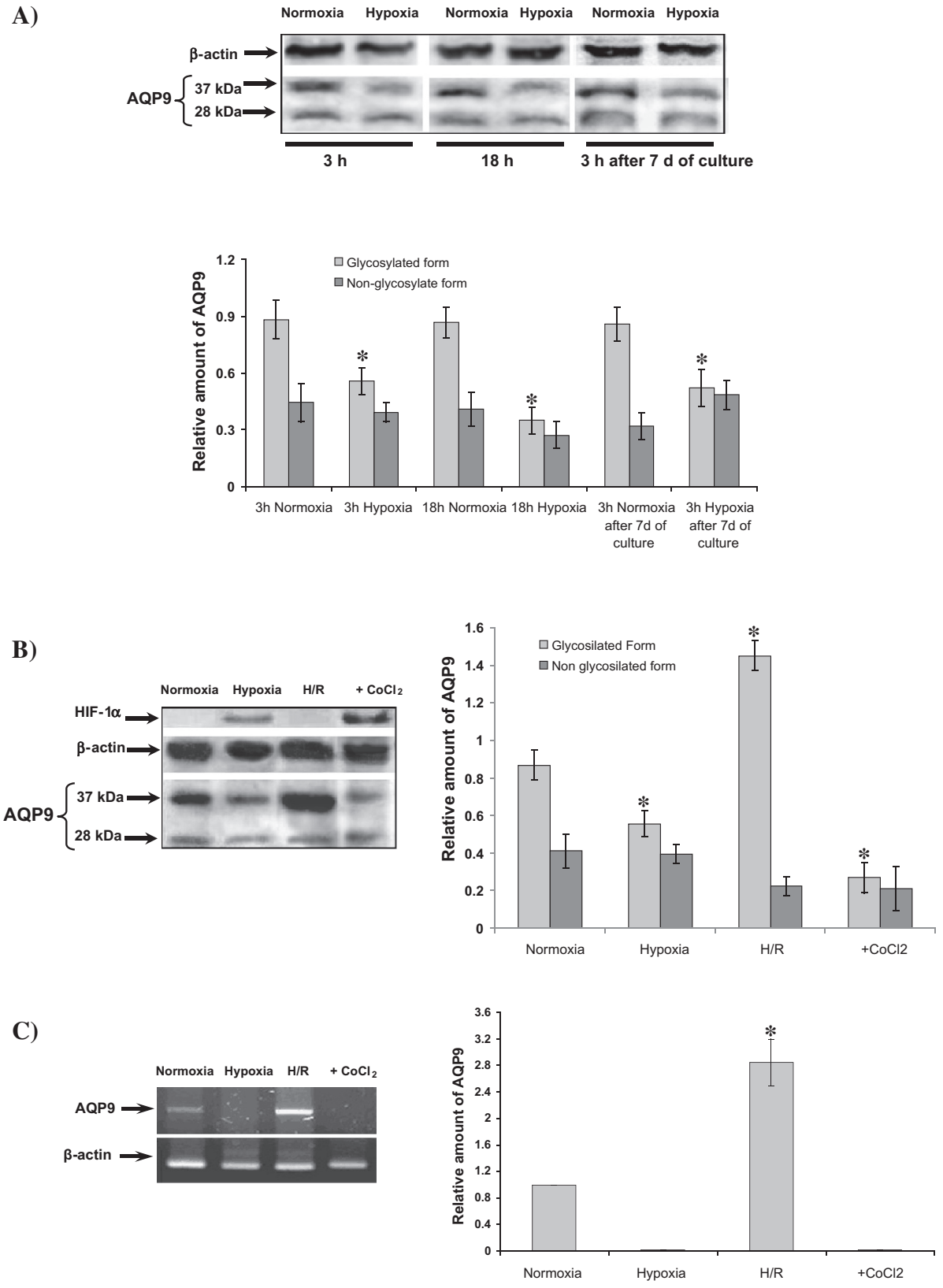


Fig. 3. AQP9 expression in placental explants exposed to $CoCl_2$ (chemical hypoxia) or cultured under different O_2 tensions. A) A representative immunoblot for AQP9 in explants exposed to normoxia and hypoxia during 3 h, 18 h or 7 days showed the two characteristic bands of AQP9 corresponding to the glycosylated (37-kDa) and non-glycosylated (28-kDa) forms of the protein. Densitometry of immunoblots containing AQP9 protein level expression was performed, and after normalization for β-actin, the values were plotted as AQP9/β-actin relative ratio. AQP9 protein of explants cultured in normoxia showed the same basal level after 3 h, 18 h or 7 days of culture. All hypoxia treatments (3 h, 18 h or 3 h after 7 days of culture) significantly decreased the glycosylated form of AQP9 ($n = 6$, $p < 0.05$). B) Semiquantitative immunoblot analysis of AQP9 expression in explants cultured under normoxia, hypoxia (3 h), hypoxia (3 h) followed by reoxygenation (15 h), and 250 μM $CoCl_2$. In all cases, we observed the bands of 37-kDa and 28-kDa of AQP9. In explants exposed to hypoxia or $CoCl_2$ treatments the band of 37-kDa decreased 1.4- and 1.3-fold respectively while in those explants cultured under hypoxia/reoxygenation this band increased

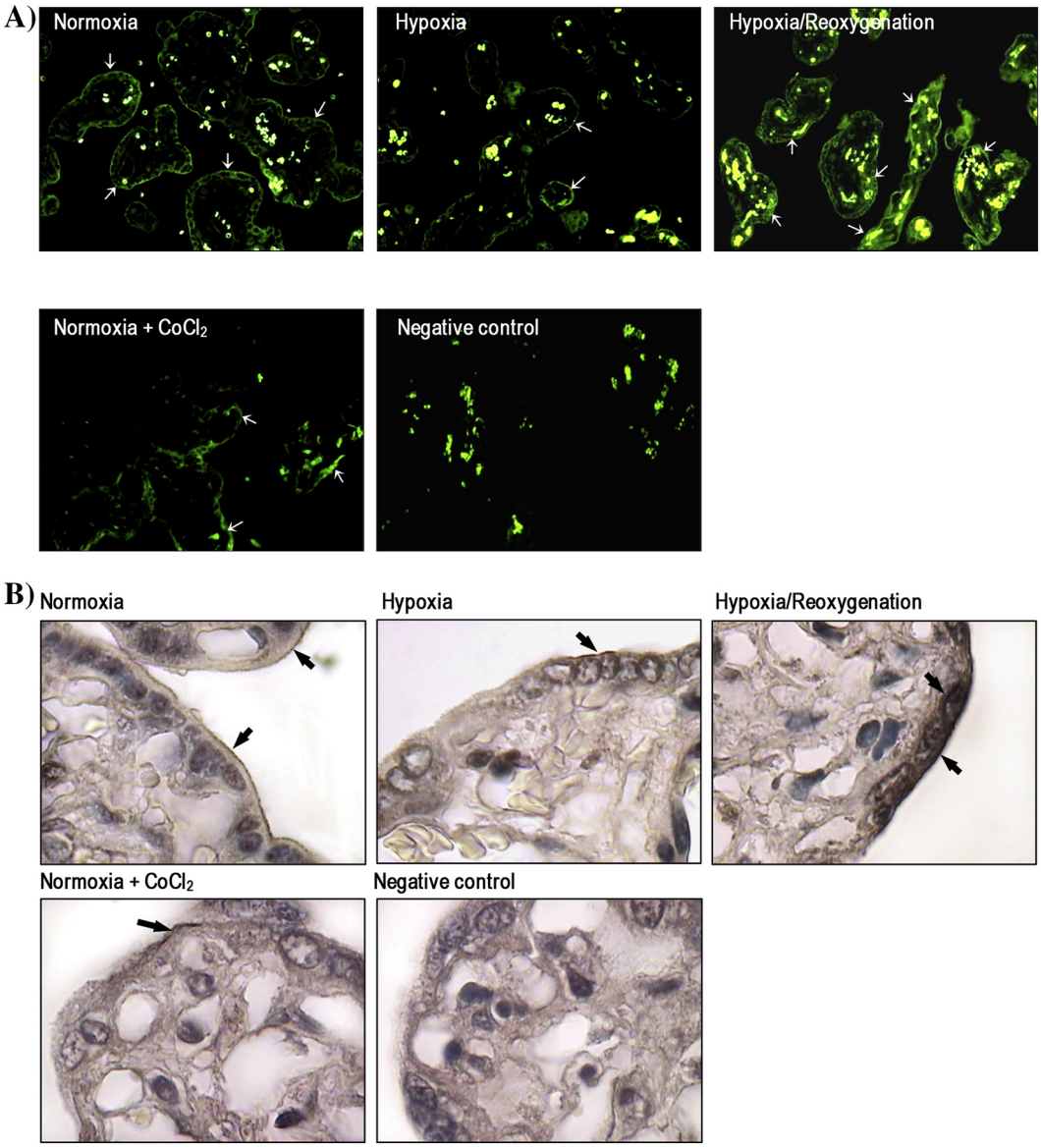


Fig. 4. Localization of AQP9 in placental explants exposed to $CoCl_2$ or cultured under different O_2 tensions by immunoflurescence (A) and immunoperoxidase assays (B). A) Immunofluorescence assay showed that AQP9 signal was weakly detectable after hypoxia or chemical hypoxia treatments and it was notably increase after reoxygenation. Negative controls were performed by omitting the primary antibody. Magnification: ×400. B) Immunostaining with an anti-AQP9 antibody revealed specific labeling in the apical membrane of hST from normal placentas. This labeling was almost undetectable at low oxygen tension and when the explants were treated with $CoCl_2$. However, in explants exposed to hypoxia/reoxygenation AQP9 localized not only in the apical and basal membranes of hST but also in the cytoplasmic region. Negative controls were performed by omitting the primary antibody and replaced by a non-immune rabbit serum. Magnification: ×1000.

An expected band of 393-bp was obtained in explants cultured in normoxia and hypoxia/reoxygenation. The semiquantitative PCR analysis, which used β-actin as internal control, showed a 2.9-fold increase in explants exposed to hypoxia/reoxygenation in comparison with the explants cultured in normoxic conditions (Fig. 3C). No bands were detected when the explants were cultured under hypoxic conditions or after the treatment of $CoCl_2$.

Consistent with these findings, immunofluorescence studies showed that the AQP9 signal was weakly detectable after deprivation of oxygen or $CoCl_2$ treatments and markedly increased after reoxygenation (Fig. 4A).

Immunolocalization of AQP9 showed labeling in the apical membrane of hST of chorionic villi exposed to normoxia. No staining was observed in cytotrophoblast, stroma or endothelial cells. In agreement with the immunoblotting and PCR assays, the label of AQP9 was weakly detected in the explants cultured under hypoxia and in those treated with 250 μM $CoCl_2$. On the other hand, in explants exposed to hypoxia followed by reoxygenation, AQP9 was strongly found in the apical membrane and in the cytoplasm of hST cells. No immunoreactivity was observed in control sections when the primary antibody was omitted (Fig. 4B).

significantly. In all the conditions tested, the non-glycosylated form of AQP9 showed no change ($n = 12$, $p < 0.05$). We also tested the expression of HIF-1α. We observed that only in those explants cultured under hypoxia or chemical hypoxia HIF-1α was expressed. Each plotted value corresponds to the mean ± SEM obtained from 12 normal placental explants ($p < 0.05$). C) Densitometry analysis of semiquantitative RT-PCR showed that AQP9 was almost undetectable in explants cultured in hypoxia and chemical hypoxia. However, the expression of AQP9 increased nearly 3–fold in those explants cultured under hypoxia/reoxygenation ($n = 12$, $p < 0.05$).

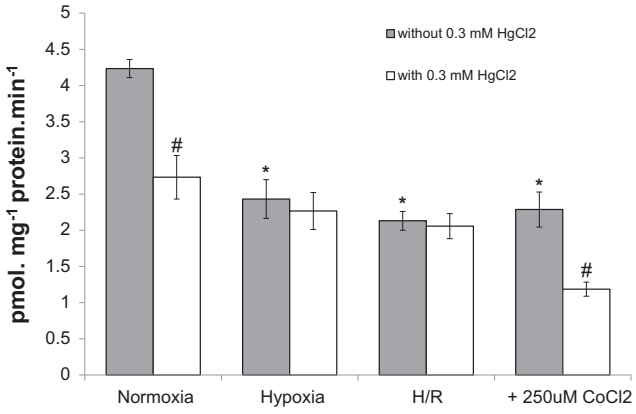


Fig. 5. Water uptake in explants under the different treatments. Explants from normal term placentas ($n = 12$) were cultured under normoxia, hypoxia, hypoxia followed by reoxygenation and chemical hypoxia (250 μM $CoCl_2$). The uptake data was expressed as pmol mg^{-1} min^{-1}. Water uptake decreased significantly and was not sensitive to $HgCl_2$ when the explants were cultured in hypoxia and in hypoxia/reoxygenation ($^*p < 0.01$). Explants treated with $CoCl_2$, showed a decrease in water uptake similar to those cultured under hypoxia ($^*p < 0.01$), however it was sensitive to $HgCl_2$ suggesting that water is passing through AQPs ($\#p < 0.05$).

3.4. Water uptake

Explants exposed to hypoxia and those exposed to hypoxia/reoxygenation showed not only a significant decrease in water uptake compared with those cultured under standard conditions but also lack of sensitivity to $HgCl_2$. Although explants treated with $CoCl_2$ showed a decreased water uptake similar to that observed in hypoxia and hypoxia/reoxygenation, they were sensitive to $HgCl_2$ (Fig. 5).

4. Discussion

Placental hypoxia has been implicated in pregnancy pathologies such as preeclampsia. However, the mechanism by which the trophoblast responds to hypoxia has not been adequately explored. Insufficient uteroplacental oxygenation in preeclampsia is believed to be responsible for the molecular events leading to the clinical manifestations of this disease [1–4]. Explant culture studies on the morphological effects of hypoxia on the full-term trophoblast have shown that hypoxia causes morphological changes similar to those reported in trophoblast cells of preeclamptic placentas [3]. However, the assumption that the placental changes are induced only by hypoxia may be overly simplistic. Recently, it has been proposed that intermittent placental perfusion, secondary to deficient trophoblast invasion of the endometrial arteries, could provide the basis for an ischemia-reperfusion (hypoxia-reoxygenation)-type injury [3,4].

In mammals, the adaptive response to hypoxia is accompanied by an increase in the expression of a variety of genes such as erythropoietin, VEGF, glycolytic enzymes, and the gene for the inducible isoform of the nitric oxide synthase (iNOS). Most of these genes are regulated by a common pathway which involves the formation of a protein complex: HIF-1 [28,29,34]. It has been reported that HIF-1α is significantly overexpressed in preeclamptic placentas but that villous explants from preeclamptic placentas fail to adequately downregulate HIF-1α upon oxygenation *in vitro* [35]. Thus, it is likely that this overexpression of HIF-1α contributes to a dysregulation of numerous genes, which perturbs placental function.

The response of AQP9 to changes in the oxygen tension seems to be related to its tissue distribution. Some authors have described that AQP9 increases after traumatic brain injury, playing a crucial role in its aggravation [14]. In addition, it has been recently reported that AQP9 is also upregulated in ischemic retina, probably via HIF-1α, but in this case, the increase in AQP9 expression might prevent lactic acidosis and subretinal edema [19]. In contrast, Arena and coworkers have recently found that, in varicocele testis, AQP9 is downregulated as a consequence of hypoxia, which leads to lactate deprivation and subsequent hypospermatogenesis [20].

Up to now, the effects of hypoxia on placental AQP9 expression and its consequences remain to be examined. Therefore, we decided to use villous explants to interrogate the influence of hypoxia exposure on AQP9 expression.

First, we analyzed the human AQP9 gene to find conserved DNA regions that could serve as putative HIF-1 binding sites. We searched the available gene sequence for a hexanucleotide element (5′-ACGTGC-3′) representing the consensus HIF-1 binding site and found fourteen putative HRE sites. At the light of this result, we hypothesized that the increased expression of HIF-1α as a consequence of the low levels of oxygen should regulate placental AQP9.

Our findings revealed that AQP9 protein decreased abruptly when HIF-1α is expressed by deprivation of oxygen or $CoCl_2$ stabilization. In contrast, in explants exposed to hypoxia followed by 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 [9].

We know that our studies are limited by the number of experimental conditions tested, which do not fully capture variability in oxygen levels, duration of exposure, and alternating patterns of oxygen. However, even if all oxygenation conditions are modeled *in vitro*, it is clear that results from such experiments should not be simply extrapolated to villous injury *in vivo*, where trophoblasts are exposed to blood, not medium, and are attached to the villous basal membrane in the vicinity of stromal and endothelial cells. Nonetheless, trophoblast cell culture is an invaluable tool for mechanistic analysis of cellular and molecular processes.

Here, we reported the presence of 14 putative HRE sites in the AQP9 gene, but none of them was found in the promoter region. Although, in other HIF-1α-inducible genes functional HRE sites were localized outside the promoter region [27–29], it is possible that HREs in the promoter region should be required to induce an upregulation of AQP9 transcription. In addition, even though AQP9 protein can be observed, the absence of AQP9 mRNA after hypoxia suggests that during the deprivation of oxygen the novo synthesis of AQP9 may be repressed.

Recently, it has been reported that leptin, a hypoxia-inducible gene, downregulates AQP9 in adipocytes and hepatocytes [36]. Therefore, we speculated that HIF-1α may enhance the expression of some intermediate which impacts directly downregulating AQP9 expression. Thus, the subsequent reoxygenation not only stimulates the synthesis of new AQP9 protein but may also modify the lipid membrane composition of the hST as we observed in preeclamptic placentas [37]. This may probably contribute to create an unfavorable environment for AQP9 insertion in the plasma membrane changing its cell distribution.

Regarding the role of AQP9 in the water exchange between the mother and the fetus, we have earlier reported that water uptake in preeclamptic placental explants is significantly reduced and that $HgCl_2$ is unable to block it [9]. Here, in explants exposed to hypoxia or treated with $CoCl_2$, we found a decrease in water flux which correlates to a decrease in AQP9 expression. Nevertheless, water

flux after $CoCl_2$ treatment and not after hypoxia seems to be sensitive to $HgCl_2$. Even more, in explants exposed to H/R, despite the increase in AQP9 expression, water uptake decreased dramatically compared to the control and was not sensitive to $HgCl_2$. A possible explanation for this discrepancy is that $CoCl_2$ only affects HIF-1α expression while the low-oxygen tension may also modify the intracellular pH (pH_i) of hST cells [38]. Emerging evidence shows that changes in pH may alter the permeability of AQPs [39–42]. Therefore; we can suppose that after the deprivation of oxygen the pH_i could not be restored properly disturbing the transcellular water transport mediated by AQPs. However, further experiments are needed to elucidate this point.

On the other hand, we have recently found that in normal placental explants treated with insulin, the decrease in the molecular expression of AQP9 shows no relationship with the functional experiments, suggesting that water is not passing through AQP9 [24]. In addition, in this work we found that water is not passing through AQPs despite the increase in AQP9 after H/R treatment. Taken into account that in addition to being permeable to water, AQP9 is permeable to neutral solutes [31], we can also assume that this protein might not have a key role in water transport in human placenta. Using AQP9-null mice, it was recently reported that the primary function of liver AQP9 is the glycerol uptake for glycerol gluconeogenesis [43]. In same model, Carbrey and coworkers have also found that AQP9 play a role in the excretion of arsenic by the liver [44]. However, these hypotheses concerning the function of placental AQP9 are still speculative. In conclusion, our findings suggest that oxygen tension may modulate placental AQP9 expression and distribution. Nevertheless, the role of AQP9 in human placenta and the consequences of its dysregulation by changes in oxygen tension still remain uncertain.

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