HIGHLIGHTS

- AQP4 protein decreases in preeclamptic placentas.
- Hypoxia enhances AQP4 mRNA but reduces AQP4 protein expression in human placenta.
- Oxygen regulates placental AQP4 expression through a HIF-1α dependent pathway.
- Placental AQP4 degradation occurs via the lysosomal pathway.

RBMO

ARTICLE

Oxygen regulation of aquaporin-4 in human placenta

BIOGRAPHY

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KEY MESSAGE

Aquaporin (AQP)4 expression was decreased in pre-eclamptic placentae. Fluctuations in oxygen tension may explain this alteration through a hypoxia inducible factor 1α-dependent mechanism. As AQP4 expression is higher at the beginning of pregnancy, when the basis of pre-eclampsia is established, its decrease might have a key role in the cause of this syndrome.

ABSTRACT

Research question: We recently reported that blocking of placental aquaporins (AQP) abrogates the apoptotic response of the trophoblast. As trophoblast apoptosis is exacerbated in pre-eclampsia, we hypothesized that changes in AQP in these placentae may trigger programmed cell death. We analysed AQP4 expression in pre-eclamptic placentae and its regulation by oxygen tension.

Design: AQP4 expression was studied in placentae from non-pathological and pre-eclamptic pregnancies by reverse transcription polymerase chain reaction (RT-PCR), Western blot, immunofluorescence and immunohistochemistry. Explants from non-pathological placentae were cultured in normoxia, hypoxia, hypoxia–reoxygenation and $CoCl_2$. AQP4 expression was investigated by RT-PCR and Western blot. Hypoxia responsive elements sites on AQP4 promotor were investigated by *in-silico* analysis. AQP4 degradation was studied in the presence of proteosomal and lysosomal inhibitors.

Results: AQP4 protein expression was weakly detectable in pre-eclamptic placentae, but its mRNA was elevated compared with non-pathological placentae. In non-pathological explants cultured in hypoxia, AQP4 mRNA and protein were increased compared with placentae cultured in ambient oxygen but decreased after reoxygenation. Incubation with $CoCl_2$, that stabilizes hypoxia inducible factor (HIF)-1α, also increased AQP4 levels. *In-silico* analysis showed three putative binding sites for HIF-1α in AQP4 promotor.

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Conclusions: Oxygen may regulate AQP4 expression in human placenta, possibly through HIF-1α. Therefore, the decrease in AQP4 throughout pregnancy, previously reported, is consistent with changes in HIF-1α, and suggests that AQP4 might have a crucial role during placentation. Therefore, the abnormal expression of AQP4 may be involved in the cause of pre-eclampsia, but it does not seem to take part in the apoptotic events.

INTRODUCTION

The human placenta develops initially in a low-oxygen environment, which is essential for appropriate embryonic development and placental angiogenesis. Oxygen tension rises at about 10–12 weeks' gestation, after the intervillous circulation is established (*Jauniaux et al., 2003; James et al., 2006*).

The placenta adapts to these profound changes in oxygenation by modulation of the hypoxia inducible factor (HIF)−1α and by increasing cellular antioxidant defenses (*Patel et al., 2001; Burton and Jauniaux, 2004*). Aberrations in the remodelling of the spiral arteries lead to fluctuations in the oxygen tension within the placenta, resulting in an ischaemia reperfusion (hypoxia-reoxygenation) type injury (*Burton and Jauniaux, 2004; Hung and Burton, 2006*).

Pre-eclampsia is a multisystem syndrome exclusively described in human pregnancy, in some cases associated with shallow trophoblast invasion and poor remodelling of the maternal spiral arteries leading to persistent placental hypoxia and enhancing trophoblast apoptosis (*Roberts, 2014*). The exacerbated trophoblast apoptosis contributes to the release of various mediators into the maternal circulation, which are thought to cause the endothelial dysfunction that finally results in the maternal manifestations of this syndrome (*Myatt, 2002*).

Several studies have shown that HIF-1α is significantly overexpressed in placentae from pregnancies complicated by pre-eclampsia (*Rajakumar et al., 2003; Rajakumar et al., 2004*). In addition, it has been found that villous explants from pre-eclamptic placentae fail to adequately downregulate HIF-1α upon oxygenation *in vitro* (*Rajakumar et al., 2008*). Therefore, the overexpression of HIF-1α may contribute to dysregulation of numerous genes, which perturbs placental functions. Accumulated evidence suggests that the expression of a variety of syncytiotrophoblast transporters, such as aquaporins (AQP), is abnormal in pre-eclamptic placentae (*Damiano et al., 2006; Del Monaco et al., 2006; Graves et al., 2007; Castro-Parodi et al., 2009; Marino and Kotsias, 2013; Martínez et al., 2016; Szpilbarg and Damiano, 2017*).

Aquaporins are a family of small integral membrane proteins that play critical roles in controlling the water content of cells. Mammals have at least 13 AQP, which are widely distributed in different organs (*Agre et al., 1998; Gonen and Watlz 2006; Carbrey and Agre 2009*). It is well known that AQP are involved in several physiological processes and in multiple and diverse clinical dysfunctions (*Verkman, 2013*). Although the classic participation of AQP in the facilitation of trans-epithelial fluid transport is widely known, recent studies have revealed unexpected cellular roles of AQP, including in the physiology of organelles, proliferation, apoptosis and cell migration (*Verkman, 2005; Verkman, 2013*). All these processes require transient changes in cell volume and activity of certain ion transporters.

Recently, we reported that blocking of placental AQP abrogates the apoptotic response of the trophoblast. As trophoblast apoptosis is exacerbated in pre-eclampsia, we hypothesized that changes in AQP in these placentae may trigger the programmed cell death (*Szpilbarg et al., 2016*). We previously reported that, AQP9 was increased and AQP3 was decreased in placentae complicated by pre-eclampsia (*Damiano et al., 2006; Szpilbarg and Damiano, 2017*). In subsequent experiments, we found that oxygen fluctuations increased placental AQP9 protein expression and changed its cellular distribution. Meanwhile, oxygen deprivation reduced AQP3 protein expression significantly, and the subsequent reoxygenation failed to restore AQP3 to basal levels (*Szpilbarg et al., 2016*).

De Falco et al. (2007) described the expression of AQP4 in the trophoblast at the initial stages of gestation and its decrease at term, but little is known about its regulation. Furthermore, the expression of AQP4 in placentae from pre-eclamptic pregnancies and its response to hypoxia in trophoblast cells are still unknown. In the present study, we investigate the expression of AQP4 in placentae from pre-eclamptic pregnancies and the effects of changes in oxygen tension on AQP4 expression in placental villous tissue.

MATERIALS AND METHODS

Tissue collection and study design
This study was conducted at the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina, and samples were obtained from the Hospital Nacional Prof. Alejandro Posadas between April 2015 and November 2017. It was approved by the Internal Board Review of the Hospital Nacional Prof. Alejandro Posadas (initial approval 08-27-2010, and renewed annually) and Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, (CIEC FFyB 12,072,017–55); written consent was obtained from patients before the collection of samples.

Inclusion criteria for the selection of 'normal' pregnant women were as follows: maternal blood pressure 110/70 mmHg or lower, no proteinuria and no other complications. Severe pre-eclampsia was defined as high maternal blood pressure (≥160/110 mmHg) and proteinuria (≥300 mg/24 h) after 20 weeks' gestation in previously normotensive women. Exclusion criteria were as follows: comorbidity, multiple gestation, use of assisted reproduction techniques, women with eclampsia, HELLP syndrome (hemolysis, elevated liver enzymes and low platelet count syndrome) and previous hypertension. Third-trimester placentae from normal pregnant women (n = 13) and from women with severe pre-eclampsia (n = 13) were obtained after caesarean section. Clinical data are presented in TABLE 1. All women belonged to the white Hispanic ethnic group.

TABLE 1 CLINICAL CHARACTERISTICS OF NORMAL WOMEN AND PREGNANT WOMEN WITH SEVERE PRE-ECLAMPSIA[a]

	Normotensive pregnant women	Pregnant women with severe pre-eclampsia	P-values
Number of pregnant women	13	13	–
Parity			
Primiparous	7	9	
Multiparous	6	4	NS
Maternal age, years	24.3 ± 2.1	26.7 ± 6.3	NS
Gestational age, weeks	38.9 ± 1.5	35.1 ± 3.1	0.0010
Body mass index, kg/m^2	25 ± 3	24 ± 4	NS
Birth weight, g	3110 ± 240	2580 ± 113	0.0001
Fetal sex			
Male	7	6	
Female	6	7	NS

[a] Values are number or mean ± SD.

Tissue culture

Fragments of cotyledons were gently separated by dissection from different areas of each placenta midway between the chorionic and basal plate. Villous tissue was further dissected into explants of about 50 mg. Placental tissue from non-pathological pregnancies was cultured under the different oxygen conditions as previously described (*Castro-Parodi et al., 2013; Szpilbarg et al., 2016*). Briefly, villous tissue (about 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., Carlsbad, CA, USA) containing 100 IU/ml penicillin (Life Technologies, Inc., Carlsbad, CA, USA), 100 mg/ml; streptomycin (Life Technologies, Inc., Carlsbad, CA, USA), and 32 mg/ml gentamicin (Life Technologies, Inc., Carlsbad, CA, USA) at 37 °C for 2 h under standard tissue culture conditions of 5% CO_2-balanced room air to equilibrate the cultures and allow for recovery from isolation procedures. After providing fresh DMEM medium, 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 or glove box (Billups-Rothenberg Inc., San Diego, CA, USA) for 18 h. For the hypoxia–reoxygenation condition, at the end of a 15-h hypoxic incubation period, explants were transferred to culture plates with DMEM medium that had been saturated

at 37 °C with room air/5% CO_2 and cultured up to 18 h.

In addition, explants exposed to normoxia, hypoxia and hypoxia–reoxygenation were cultured in the presence and in the absence of the proteasome inhibitor MG-132 (10 μM) (Sigma-Aldrich Corp., Saint Louis, MO, USA) or the lysosomal inhibitor NH_4Cl (10 mM) (Sigma-Aldrich Corp.) to evaluate whether the proteasome-dependent proteolytic pathway or the lysosome degradation pathway are involved in the degradation of AQP4.

In another set of experiments, villous explants were treated for 18 h with 250 μM $CoCl_2$ (Sigma-Aldrich Corp.), a hypoxia-mimicking agent that inhibits HIF-1α degradation. These explants were previously incubated for 30 min with 300 μM $ZnCl_2$ (Sigma-Aldrich Corp.), an inducer of HIF-1α proteasome degradation or 1 μM 6-aminoflavone (Sigma-Aldrich Corp.) to block HIF-1α messenger RNA (mRNA) expression (*Chun et al., 2001; Terzuoli et al., 2010; Aban et al., 2016*).

Biochemical assays

Tissue viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich Corp.) assay as described previously (*Castro-Parodi et al., 2013*). Tissue samples (about 50 mg, fresh) were exposed to MTT and the formation of the formazan product of MTT was measured by monitoring relative absorbance at 595 nm. The experiments were independently

conducted in triplicate at least three times.

The syncytial secretory capacity of explants in terms of hormone release, was determined by the production of beta-hCG after 6 and 24 h of culture (*Castro-Parodi et al., 2013*). The concentration of beta-hCG in the culture medium was assessed by quantitative immunoradiometric determination using a commercially available kit (beta-hCG solid phase component system, Coat-A-Count hCG immunoradiometric determination [IRMA], EURO/DPC Ltd., Caernarfon, UK) (*Damiano et al., 2006*).

Integrity of explants was verified by the release of the intracellular enzyme lactate dehydrogenase (LDH) into the incubation medium after 4, 6 and 24 h of incubation. A Lactate Dehydrogenase Assay kit (Sigma-Aldrich Corp.) was used to determine LDH concentrations, according to the manufacturer's protocol (*Castro-Parodi et al., 2013*).

Semi-quantitative reverse transcription polymerase chain reaction

Total RNA from non-pathological, pre-eclamptic and cultures of unaffected placental explants was isolated using an SV Total RNA isolation system (Promega Corp., San Luis Obispo, CA, USA) and reverse transcribed as previously described (*Damiano et al., 2001*). Polymerase chain reaction (30 cycles) was carried out using 5 μM of a specific oligonucleotide primer. It's design was based on the region flanked by the two

highly conserved Asn-Pro-Ala (NPA) motifs in the aquaporin family (sense 5′-GGAATTTCTGGCCATGCTTA-3′, antisense 5′-TTGCAATGCTGAGTCCAAAG-3′), amplifying a 128-bp fragment of human AQP4. Human ribosomal protein L30 (L30, sense 5′-CCGCAAAGAAGACGAAAAAG-3′, antisense 5′-AAAGCTGGGCAGTTGTTAGC- 3′) was used as internal standard (*Szpilbarg and Damiano, 2017*). The ImageJ 1.45 s software package (Wayne Rasband, National Institutes of Health, USA) was used to define the densitometry of the bands.

Optical density curves as a function of the number of cycles were carried out to select the number of cycles that allowed us to detect in the exponential phase of amplification both the amplified fragment of *AQP4* gene and *L30* gene. In addition, we used a lower concentration of L30 primers in attempt to favour the amplification of AQP4, which is expressed at lower levels, and the two sets of primers did not compete between each other.

Western blotting

Human placental villi from non-pathological term and pre-eclamptic placentae and cultured explants were processed according to the method previously described (*Damiano et al., 2006; Castro-Parodi et al., 2011*). Briefly, samples 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, IKA® Works Inc., Wilmington, NC, USA) and centrifuged at 3100 g for 10 min. The supernatants were collected, and protein concentration of each sample was measured by the Pierce® BCA assay (Thermo Fisher Scientific Inc., Waltham, MA, USA).

For immunoblotting studies, 100 µg of protein was loaded and resolved on a 15% polyacrylamide gel, and electrotransferred onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech Ltd., Pittsburgh, PA USA). After blocking with 5% non-fat powered milk, membranes were incubated overnight with the primary antibody anti-AQP4 (Alpha Diagnostic International Inc., San Antonio, TX, USA; 1:1000) and then with goat anti-rabbit immunoglobulin G (IgG) Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA; 1:10,000) conjugated to peroxidase.

Immunoreactivity was detected using the Enhanced Chemiluminescence (ECL) Western Blotting Analysis System (ECL plus, Amersham Pharmacia Biotech Ltd., Pittsburgh, PA USA) according to the manufacturer's instructions. The densitometry of the bands was quantified by the ImageJ 1.45 s software package. To confirm equal loading, each membrane was analysed for beta-actin protein expression with monoclonal anti-beta-actin (Alpha Diagnostic International Inc.).

To study whether the experimental conditions affected HIF-1α protein expression in our system, we also analysed HIF-1α protein expression using a monoclonal anti-HIF-1α antibody (Alpha Diagnostic International Inc.) (*Castro-Parodi et al., 2013*). Equal loading was confirmed by staining the membrane with a Ponceau S solution (Sigma-Aldrich Corp., Saint Louis, MO, USA; 0.1% [weight per volume] in 5% acetic acid) as a general protein marker (*Lanoix et al., 2012*). The membrane was incubated for up to an hour in the staining solution with gentle agitation and then rinsing the membrane in distilled water until the background was clean.

Immunohistochemistry

Placental tissues were fixed overnight in 10% formaldehyde-0.1 M sodium phosphate buffer, pH 7.4, dehydrated, and embedded in paraffin as previously described (*Castro-Parodi et al., 2011*). Then, thin sections (4–5 µm) were cut, dewaxed, rehydrated, and incubated in 3% hydrogen peroxide and methanol for 5 min to block endogenous peroxidase. After blocking non-specific binding sites with DAKO reagent (DAKO LSAB kit, Dako Corp., Carpinteria, CA, USA), tissue slices were incubated overnight (4 °C) with anti-AQP4 (Alpha Diagnostic International Inc., San Antonio, TX, USA; 1:100). Fulfilled this step, the slides were washed with phosphate buffered saline (PBS) for three times, 5 min each. Samples were then incubated with prediluted secondary universal biotinylated anti-mouse/rabbit IgG (Vector Laboratories), for 1 h and incubated in a solution of streptavidin-conjugated horseradish peroxidase (VECTASTAIN Elite ABC HRP Kit, Vector Laboratories, USA). The slides were rinsed with PBS three times, 5 min each and then, staining was conducted with a Vectastain kit (Vector Laboratories Inc., Burlingame, CA, USA) and labelling was visualized by reaction with diaminobenzidine tetrahydrochloride (Dako Corp.) and counterstained with haematoxylin (Sigma-Aldrich Corp., Saint Louis, MO, USA).

For immunofluorescence experiments, after overnight incubation at 4 °C with the AQP4 primary antibody (Alpha Diagnostic International Inc., San Antonio, TX, USA; 1:100) tissue slices were rinsed with PBS during 5 min three times and treated with secondary anti-rabbit IgG antibody conjugated with Texas Red® (Vector Laboratories Inc. 1:200) for 2 h at room temperature. After washing three times with PBS during 5 min each, slices were mounted with Fluoroshield mounting medium with DAPI (Abcam, Cambridge, MA, USA) to label nuclei and analysed with an epifluorescent microscope (Nikon, Eclipse E:200).

For negative controls, the primary antibody was blocked with the specific AQP4 control–blocking peptide (Alpha Diagnostic International Inc., San Antonio, TX, USA; 10 µg control peptide per 1 µg of AQP4 antibody).

In-silico analysis of the human *AQP4* gene

A theoretical analysis of the promoter region of the *AQP4* gene (GenBank accession number NG_029560.1) was conducted to identify putative recognition sites by transcription factors, using the MatInspector® tool from Genomatix® (*Cartharius et al., 2005*). Sequence alignment was carried out 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® (*Cartharius et al., 2005; Castro-Parodi et al., 2013*).

The core similarity of a matrix is defined as the consecutive highest conserved positions of the matrix. The maximum core similarity of 1.0 is only reached when the highest conserved bases of a matrix match exactly in the sequence. The matrix similarity considers all bases over the whole matrix length. Compared

with the theoretical matrix, matrix similarity is represented by a score value over 0.85.

Statistical analysis

The GraphPad Prism 7.02 (GraphPad Software, Inc., La Jolla, CA, USA) was used for all statistical analysis. One-way analysis of variance followed by Fisher LSD test or t-test were applied. Data are shown as mean ± SD or SE. AQP4 expression data were assessed for normality using the D' Agostino test. Fisher's exact test was used to analyse fetal sex and parity. The criterion for statistical significance was $P < 0.05$.

RESULTS

Abundance of AQP4 mRNA and protein in pre-eclamptic placenta

In the present study, the expression of AQP4 in placentae from pregnancies complicated by pre-eclampsia and unaffected pregnancies was investigated. AQP4 mRNA was found to be significantly increased in these placentae compared with non-pathological ones ($n = 13$; $P < 0.001$) (FIGURE 1A). When the expression of the protein was analysed, AQP4 significantly decreased (66%; $n = 13$; $P < 0.01$) (FIGURE 1B). No bands were observed when the AQP4 antibody was pre-absorbed with the specific peptide (data not shown).

Immunolocalization and immunofluorescence assays showed that AQP4 was present in the apical membrane of syncytiotrophoblast and in endothelial cells. A weak AQP4 staining, however, was observed in placentae from pregnant women with pre-eclampsia, in agreement with the immunoblotting assay (FIGURE 1C and D).

Effect of oxygen tension on AQP4 expression

We checked the viability of the placental villous explants from non-pathological placentae cultured under the different conditions (normoxia, hypoxia and hypoxia–reoxygenation) by MTT incorporation. We observed that MTT incorporation, a measure for mitochondrial dehydrogenase enzymatic activity, did not change over the first 24 h in culture ($n = 10$ per condition; data not shown).

In addition, the syncytial secretory capacity, tested by the measurement of beta-hCG in the culture medium, was preserved during the first 24 h

of culture and was similar among the groups (FIGURE 2A). Finally, cell integrity was measured by the release of LDH in the culture medium. As expected, we found higher levels of LDH per hour during the first hours, possibly owing to tissue cellular damage during isolation procedures, which declined at 24 h of culture and were similar among the groups (FIGURE 2B).

After establishing the cellular viability of the placental explants, we examined the expression of AQP4 by exposing explants to different oxygen conditions. To investigate the expression of AQP4, RT-PCR and Western blot analyses were carried out. The semi-quantitative PCR analysis showed a 1.6-fold increase in explants exposed to hypoxia compared with the explants cultured in normoxic conditions ($n = 8$; $P < 0.001$) (FIGURE 3A). In agreement with this finding, the immunoblotting analysis showed a 1.7-fold increase in AQP4 protein levels in explants exposed to hypoxia compared with controls ($n = 8$; $P < 0.001$) (FIGURE 3B). In contrast, the subsequent reoxygenation restored AQP4 mRNA to control levels and induced a significantly decreased in AQP4 protein expression ($n = 8$; $P < 0.05$ versus normoxia) (FIGURE 3A and 3B).

Identification of putative hypoxia-response element sites in the human *AQP4* gene promotor

As it is known that HIF-1α -mediated gene transactivation involves HIF-1α binding to distinct nucleic acid motifs, namely hypoxia-response elements (HRE), we used the MatInspector® tool of Genomatix® (*Cartharius et al., 2005*) to analyse the human *AQP4* gene promotor (GenBank accession number NG_029560.1 for appropriate HRE sequences. In this analysis, we followed the HRE consensus motif proposed by Wenger and Gassmann (*Wenger and Gassmann, 1997*), (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.

Following these premises, we identified three putative HRE sites (TABLE 2) in the promoter region of the gene. All sites showed a high core similarity and matrix similarity compared with the theoretical matrix, represented by a score value of over 0.85. This finding allowed us to surmise that placental AQP4 may be

modulated by HIF-1α because of oxygen deprivation.

### Effect of CoCl$_2$ treatment on AQP4 expression

To activate hypoxia-dependent pathways, explants from non-pathological placentae were treated with 250 µM CoCl$_2$, which stabilizes HIF-1α under ambient 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 (*Vengellur and LaPres, 1997*). First, we determined whether low-oxygen conditions affected HIF-1α protein expression in our experimental system. As we expected, HIF-1α protein was detected in explants cultured under both hypoxic and CoCl$_2$ treatments, whereas HIF-1α was almost undetectable in explants exposed to normoxia and hypoxia–reoxygenation (FIGURE 4A).

With the expression of AQP4, we observed that after CoCl$_2$ treatment, AQP4 mRNA ($n = 8$; $P < 0.01$) and protein ($n = 8$; $P < 0.001$) increased 1.8-fold compared with the control (FIGURE 4B and C). To confirm that this effect was mediated by HIF-1α, before CoCl$_2$ treatment, explants were incubated with ZnCl$_2$ or 6-aminoflavone, both inhibitors of HIF-1α. As shown in FIGURE 4B and FIGURE 4C, treatments with ZnCl$_2$ or 6-aminoflavone reversed the increase of AQP4 mRNA and protein produced by CoCl$_2$, suggesting that upregulation of AQP4 may be mediated by HIF-1 α. HIF-1α inhibitors per se did not modify AQP4 expression (data not shown).

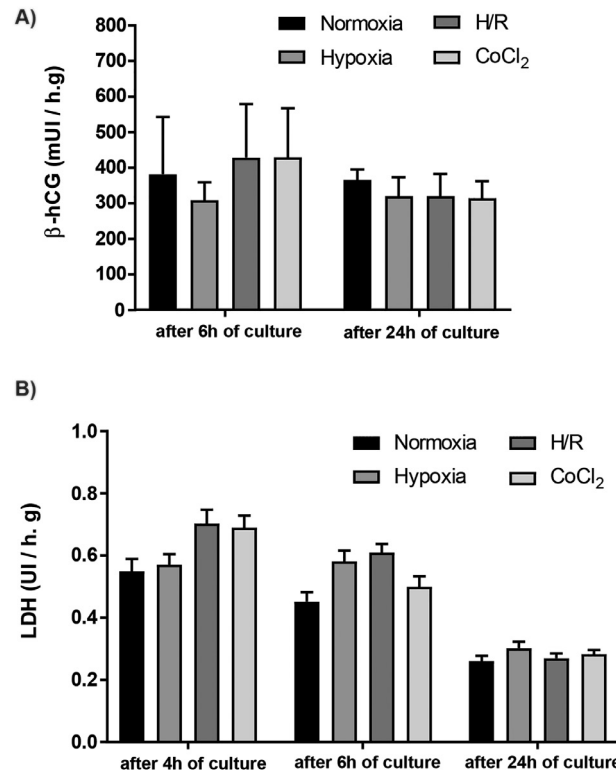### Effect of proteasome or lysosome inhibitors on AQP4 expression

To investigate whether the proteasome-dependent proteolytic pathway or the lysosome degradation pathway plays a role in the effect of hypoxic stress on AQP4 expression, we cultured explants under normoxia, hypoxia and hypoxia–reoxygenation in the presence and in the absence of MG-132, an inhibitor for 26S proteasome, and the lysosomal inhibitor NH$_4$Cl respectively. We found that the addition of the proteasome inhibitor did not modify the hypoxia–reoxygenation-induced decrease in AQP4 protein expression (FIGURE 5A). The addition of the lysosome inhibitor maintained AQP4 expression at hypoxic levels, preventing its degradation, in the explants exposed to the hypoxia–reoxygenation treatment ($n = 6$; $P < 0.001$) (FIGURE 5B).
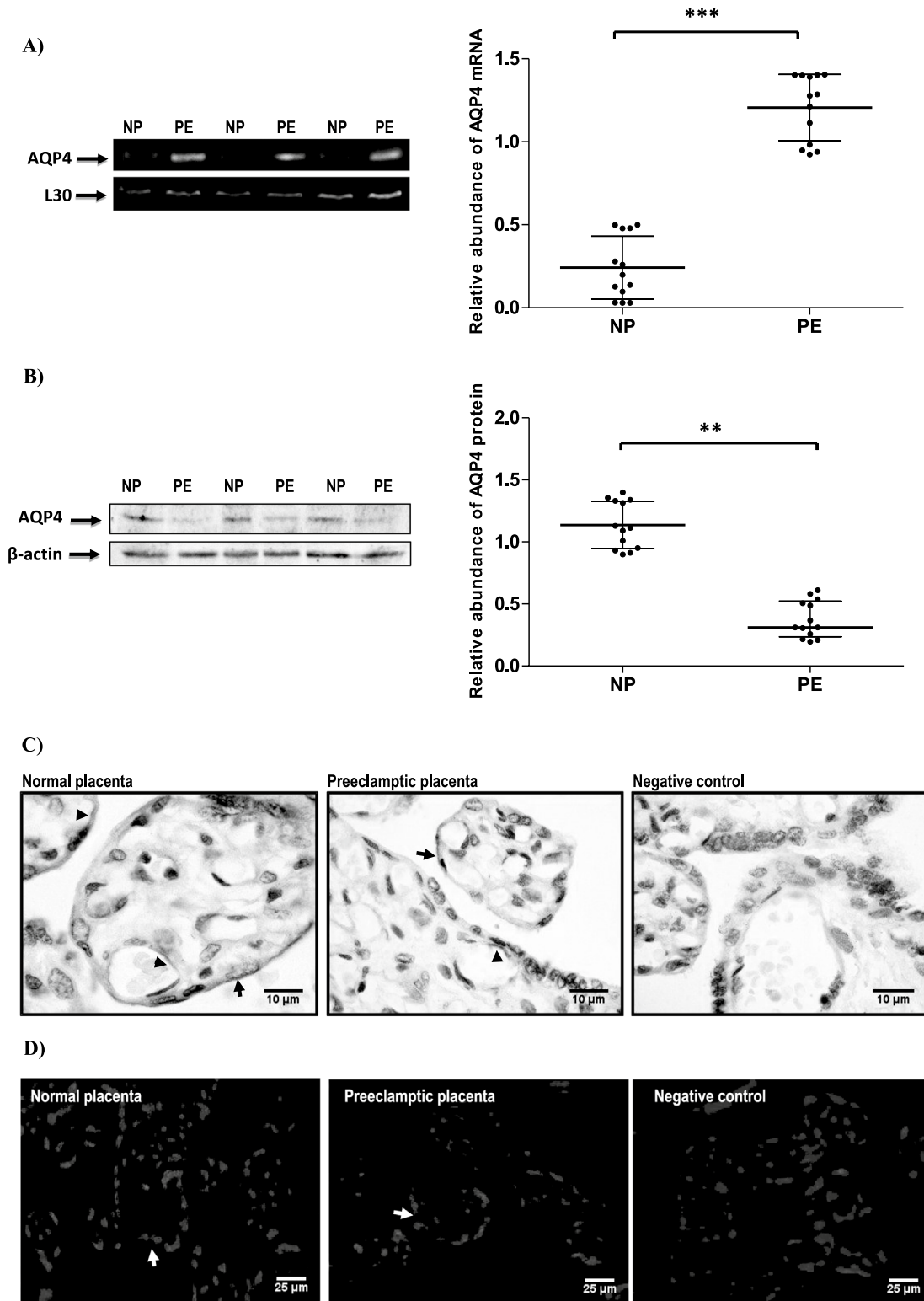
FIGURE 1 Aquaporin (AQP)4 expression and localization in human placentae from pre-eclamptic and non-pathological pregnancies. (A) AQP4 mRNA was measured by semi-quantitative reverse transcription polymerase chain reaction in placentae from non-pathological (NP) pregnancies (*n* = 13) and pre-eclamptic (PE) pregnancies (*n* = 13). L30 expression was determined to control for equal loading. Densitometry was carried out, and after normalization for L30, the values of mRNA relative abundance were plotted as AQP4–L30 relative ratio. Values are plotted for each sample and mean ± SD are indicated. AQP4 protein expression follows normal distribution (D′Agostino normality test). ****P* < 0.001, ***P* < 0.01; (B) AQP4 protein expression was determined by immunoblotting in NP (*n* = 13) and PE (*n* = 13). Beta-actin expression was determined to control for equal loading. Densitometry was performed and after normalization for beta-actin, the values of protein relative abundance were plotted as AQP4/beta-actin relative ratio. Values are plotted for each sample and mean ± SD are indicated AQP4 protein expression follows normal distribution (D′Agostino normality test). AQP4 protein expression was 66% lower in PE versus NP (*P* < 0.01); (C) Immunostaining with an anti-AQP4 antibody

(continued)

FIGURE 2 Effect of time in culture on the biochemical status of villous explants of placentae from non-pathological pregnancies. Explants of non-pathological placentae were exposed to normoxia, hypoxia, hypoxia–reoxygenation (H/R) and $CoCl_2$ for 4, 6 or 24 h (A) Release of beta-hCG by placental villi in culture medium per hour at 6 and 24 h of culture. The data in each group are shown as mean ± SEM obtained from 10 non-pathological placental explants. No statistically significant differences were found between the normoxia group and the other conditions after 6 h or 24 h. (B) Release of lactate dehydrogenase (LDH) by placental villi in culture medium per hour at 4, 6, and 24 h of culture. The data in each group are shown as mean ± SEM obtained from 10 non-pathological placental explants. Treatments did not significantly decrease villous explant viability, as measured by the release of LDH.

DISCUSSION

One of the most common features in placentae from pregnancies complicated by pre-eclampsia is shallow trophoblast invasion and abnormal placentation. Therefore, the perfusion of these placentae is impaired, and oxygen concentration within the intervillous space is intermittent. These fluctuations in oxygen tensions can affect the functions of the syncytiotrophoblast and increase oxidative stress and apoptosis of the tissue (*Myatt, 2002; Burton and Jauniaux, 2004; Hung and Burton, 2006*).

Recently, we explored the role of AQP3, AQP4, and AQP9 in placental programmed cell death. We observed that inhibition of these proteins abrogates the apoptotic response in non-pathological human placenta (*Szpilbarg et al., 2016*).

Consequently, we have explored here the expression of AQP4 in pre-eclamptic placentae and its behaviour in non-pathological placental explants exposed to oxygen changes. We hypothesized that changes in AQP4 expression may contribute to the development of pre-eclampsia by increasing apoptosis of the villous trophoblast. Interestingly, we found that AQP4 mRNA was significantly increased in pre-eclamptic placentae, but AQP4 protein expression significantly decreased. This dysregulation of AQP4 protein in pre-eclampsia would be expected to reduce the apoptosis of the trophoblast; however, apoptosis is exacerbated in pre-eclampsia (*Leung et al., 2001*). Consequently, our findings suggest that AQP4 would not take part in the increased apoptotic events in pre-eclampsia.

In addition, we studied the effect of variation in the oxygen tension on the expression of AQP4. The oxygen concentration that is physiological for villi *in vivo*, and the concentration that should be mimicked in vitro, is a topic of debate that is unresolved. Evidence shows that the oxygen concentration that may be considered 'normal' for *in-vitro* experiments ranges between 5 and 20% (*Miller et al., 2005; Bainbridge et al., 2006; Cindrova-Davis et al., 2007; Imperatore et al., 2010; Barsoum*
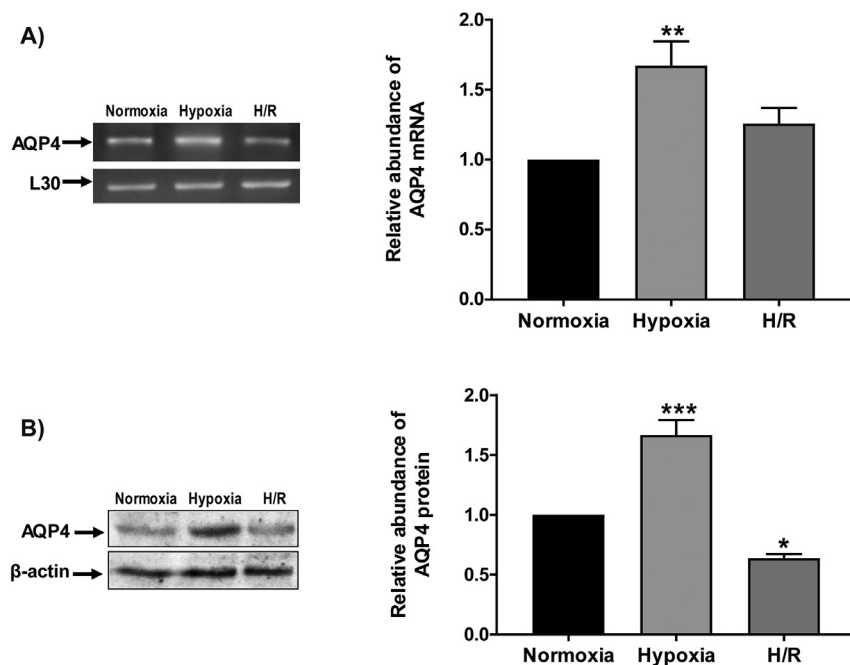
FIGURE 3 Aquaporin (AQP)4 expression in human non-pathological placental explants exposed to different O_2 tensions. (A) Semi-quantitative revere transcription polymerase chain reaction (left panel) and densitometry analysis (right panel) showed a 1.6-fold increase of AQP4 mRNA levels in explants exposed to hypoxia in comparison with the explants cultured in normoxic and hypoxia–reoxygenation (H/R) conditions. L30 expression was determined to control for equal loading. Densitometry was conducted, and after normalization for L30, the values of mRNA relative abundance were plotted as AQP4/L30 relative ratio. Each plotted value corresponds to the mean ± SEM ($n = 8$ placentae per group; **$P < 0.01$ versus normoxia); (B) representative Western blot (left panel) and densitometry analysis (right panel) showed that AQP4 protein expression showed a 1.7-fold increase in explants exposed to hypoxia compared with explants cultured in normoxia. The subsequent reoxygenation H/R decreased significantly AQP4 protein expression. β-actin expression was determined to control for equal loading. Densitometry was conducted, and after normalization for beta-actin, the values of protein relative abundance were plotted as AQP4/beta-actin relative ratio. Each plotted value corresponds to the mean ± SEM ($n = 8$ placentae per group, ***$P < 0.001$; *$P < 0.05$. versus normoxia).

TABLE 2 POTENTIAL HYPOXIA-RESPONSE ELEMENTS MOTIFS IN THE HUMAN *AQP4* GENE PROMOTOR

Sequence name	Start position	End position	Strand	Matrix similarity[c]	Core similarity[b]	Sequence
GXP_85336	151	167	+	0.974	1	cctatgga**CGTG**ttcct[a]
GXP_85336	289	305	–	0.934	1	cagccccg**CGTG**ccagg[a]
GXP_5124502	317	333	–	0.964	1	attaaaca**CGTG**ttttc[a]

[a] Genomic structure was taken from GenBank accession NG_029560.1. Identification of putative HIF-1 binding sites is derived from the HRE consensus sequence model of Wenger and Gassmann (*Wenger and Gassmann, 1997*), which represents the CGTG core structure (in bold).

[b] The core similarity of a matrix is defined as the consecutive highest conserved positions of the matrix. The maximum core similarity of 1.0 is only reached when the highest conserved bases of a matrix match exactly in the sequence.

[c] The matrix similarity takes into account all bases over the whole matrix length.

et al., 2011; Tuuli et al., 2011). In the present study, we used 20% oxygen in the normoxia condition. Our results show that variations in oxygen tension affect the expression of AQP4, without significant changes in cell viability. We observed that AQP4 protein and mRNA expression increased in explants exposed to hypoxia, and significantly decreased after the subsequent reoxygenation.

In light of these results, we investigated the human AQP4 gene to find conserved DNA regions that could serve as putative HIF-1 binding sites. The *in-silico* analysis showed three putative binding sites for HIF-1α in AQP4 promotor region. Moreover, treatment with $CoCl_2$, a well-known stabilizer of HIF-1α protein, enhanced AQP4 expression, leading to the same result shown by hypoxia treatment. Both $CoCl_2$ and hypoxia treatment enhanced HIF-1α expression in placental explants, as we had previously described (*Castro-Parodi et al., 2013*).

The subsequent reoxygenation, however, induced a decrease in HIF-α corresponding with a decrease in AQP4 expression. Our results suggest that HIF-1α has a role in AQP4 upregulation in human placenta. Although AQP4 is one of the least abundant AQPs expressed in human placenta, it was found in syncytiotrophoblast throughout gestation (*De Falco et al., 2007*; *Saadoun et al., 2013*). *De Falco et al. (2007)* found a 10% reduction from the first to the third trimester whereas *Saadoun et al. (2013)* found changes from moderate and intense levels in the second trimester to weak or undetectable in the third trimester. In fact, abundance of AQP4 seems to be low in third-trimester human placenta, and this is reflected by the

A)

B)

C)
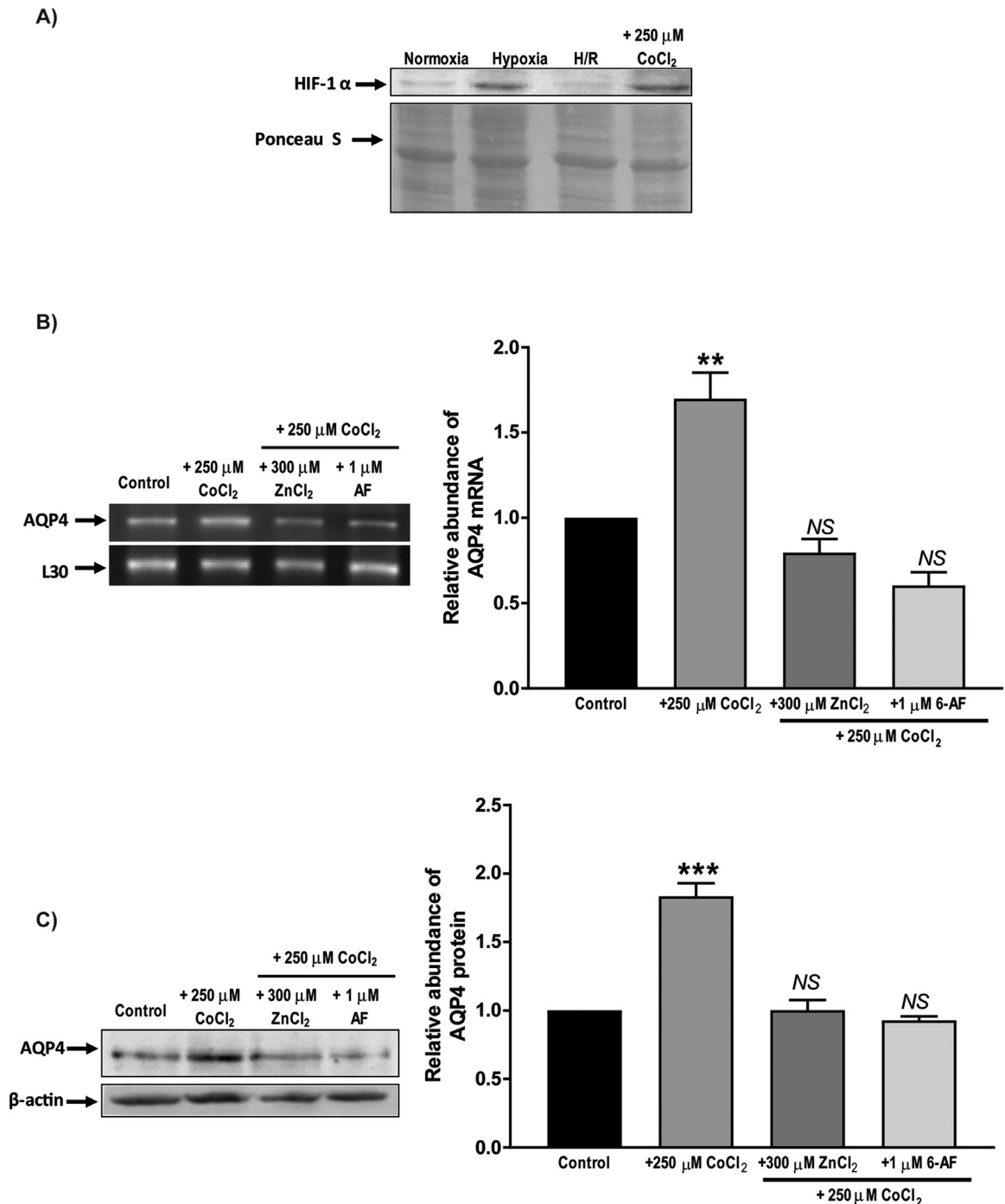
FIGURE 4 Aquaporin (AQP)4 expression in human non-pathological placental explants after hypoxia inducible factor (HIF)−1α stabilization with $CoCl_2$. (A) Explants from non-pathological placentae cultured under both hypoxic and $CoCl_2$ treatments. Representative Western blot revealed a band corresponding to HIF-1α. In normoxia and hypoxia–reoxygenation (H/R), HIF-α was almost undetectable. To confirm equal loading, the membrane was stained with a Ponceau S solution. The same results were obtained in eight independent experiments; (B) semiquantitative reverse transcription polymerase chain reaction analysis showed a 1.8-fold increase of AQP4 levels in explants treated with $CoCl_2$ compared with normoxic explants. The addition of 300 μM $ZnCl_2$ or 1 μM 6-aminoflavone (6-AF) restored AQP4 mRNA to control levels. L30 expression was determined to control for equal loading. Densitometry was conducted and, after normalization for L30, the values of mRNA relative abundance were plotted as AQP4–L30 relative ratio. Each plotted value corresponds to the mean ± SEM (n = 8 placentae per group; **$P < 0.01$, NS, non-significant. P-values reflect comparison with control); (C) representative Western blot (left panel) and densitometry analysis (right panel) showed that $CoCl_2$ treatment showed a significant increase of AQP4 protein expression compared with the control. Explants treated with 300 μM $ZnCl_2$ or 1 μM 6-AF for 30 min before $CoCl_2$ addition, showed no changes in AQP4 levels compared with controls. Beta-actin expression was determined to control for equal loading. Densitometry was conducted and, after normalization for beta-actin, the values of protein relative abundance were plotted as AQP4/beta-actin relative ratio. Each plotted value corresponds to the mean ± SEM (n = 8 placentae per group; ***$P < 0.001$, NS, non-significant. P-values reflect comparison with control). $CoCl_2$, addition of 250 μM Cobalt chloride, a hypoxia mimic agent; $ZnCl_2$, addition of 300 μM Zinc Chloride an inducer of HIF-1α proteasome degradation; AF, addition of 1 μM 6-aminoflavone to block HIF-1α expression.
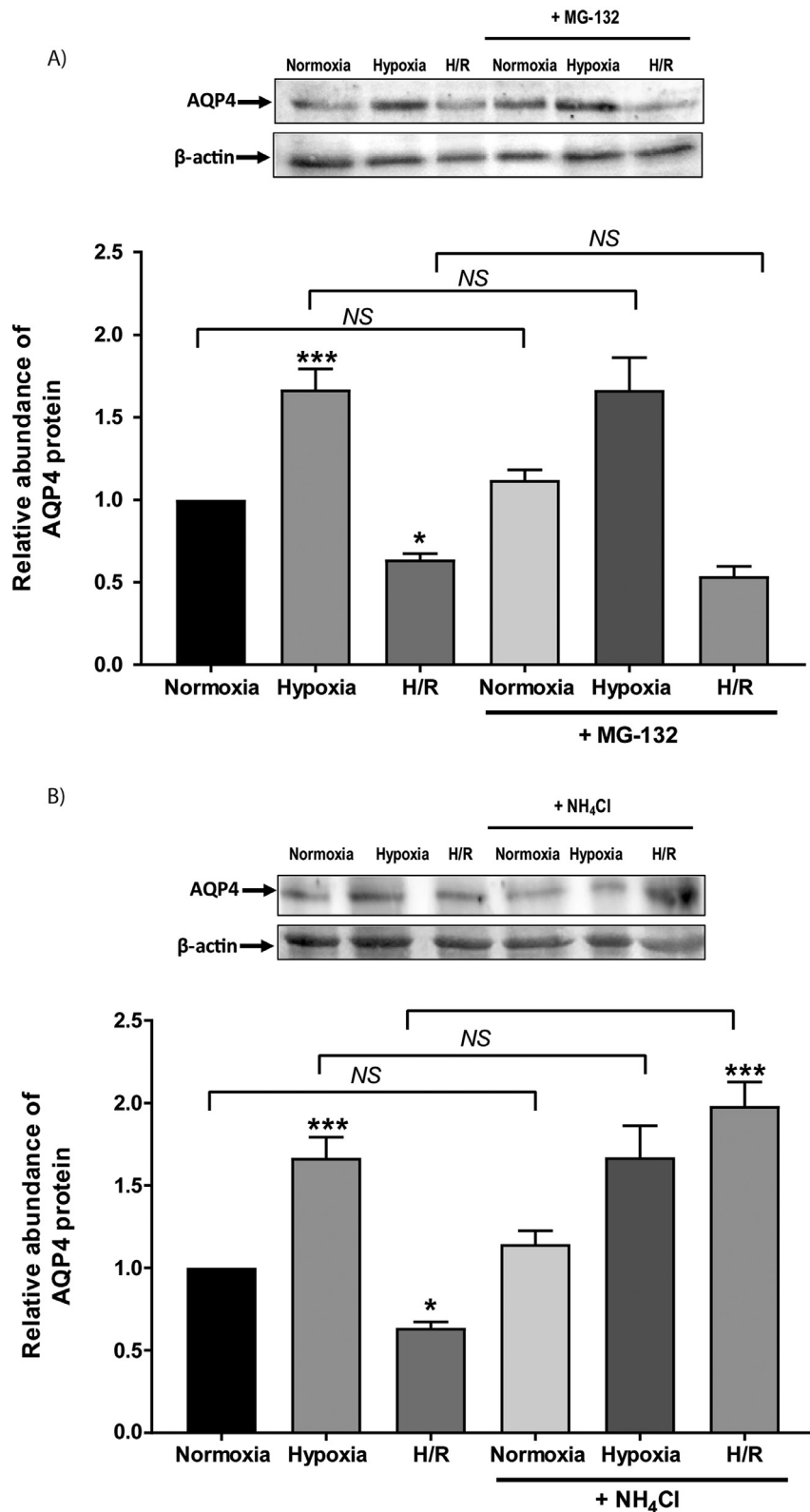
FIGURE 5 Proteasomal and lysosomal degradation of aquaporin (AQP)4 in explants from non-pathological placentae cultured under different O_2 tensions. (A) Explants exposed to different O_2 tensions were cultured in the presence and in the absence of a proteasome inhibitor, MG-132. Representative Western blot (upper panel) and densitometry (lower panel) showed that the addition of MG-132 was not able to prevent the decrease in AQP4 protein levels in explants cultured in hypoxia–reoxygenation (H/R). No significant difference was observed in AQP4 levels in each condition before and after the addition of MG-132. Each plotted value corresponds to the mean ± SEM (n = 6 placentae per group; NS, non-significant; hypoxia versus normoxia ***P < 0.001; H/R versus normoxia *P < 0.05); (B) explants exposed to different O_2 tensions were cultured in the presence and in the absence of a lysosome inhibitor, NH_4Cl. Representative Western blot (upper panel) and densitometry analysis _(continued)_

FIGURE 5   *(continued)*   (lower panel) showed that the addition of the lysosome inhibitor maintained AQP4 protein expression at hypoxic levels in the explants exposed to the H/R treatment. Each plotted value corresponds to the mean ± SEM (n = six placentae per group; NS, non-significant; H/R versus normoxia *P < 0.05 in absence of NH_4Cl; H/R versus normoxia ***P < 0.001 in presence of NH_4Cl; H/R in absence of NH_4Cl versus hypoxia–reoxygenation in presence of ***P < 0.001). In both cases, beta-actin expression was determined to control for equal loading. Densitometry was conducted and, after normalization for beta-actin, the values of protein relative abundance were plotted as AQP4/beta-actin relative ratio.

discrepancy of some reports in which AQP4 was not found in human placenta (*Fagerberg et al., 2014*; *Sober et al., 2015*).

Along with these reports, we propose that De Falco and Saadoun's observations may be consistent with the changes in HIF-1α expression throughout gestation and suggest that AQP4 might have a relevant role at the beginning of gestation. In placentae from pre-eclamptic pregnant women, despite an increase in HIF-1α protein (*Rajakumar et al., 2003; 2004*) and AQP4 mRNA, AQP4 protein expression decreased. It is well accepted that HIF-1α is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Degradation of HIF-1α, however, is impaired in placentae from pre-eclamptic women owing to proteasome dysfunction (*Rajakumar et al., 2008*).

In the present study, we showed that the lysosomal but not the proteasomal proteolytic pathway participates in the placental AQP4 protein degradation. The lysosomal degradation of AQP4 was observed in other tissues (*Dibas et al., 2008; Gan et al., 2012; Huang et al., 2013; Willermain et al., 2014*). These results may explain why, in these poorly perfused placentae from pre-eclamptic pregnancies, despite the proteasomal dysfunction, AQP4 decreases.

We recently reported that the pre-eclamptic apical membranes of syncytiotrophoblast are more rigid than non-pathological ones, possibly owing to an increase of sphingomyelin that may be altering the membrane lipid order of the trophoblastic cells (*Levi et al., 2016*). As cell membranes display a tremendous complexity of lipids and proteins designed to perform the functions cells require, an altered lipid composition may disrupt the ability of sphingomyelin and cholesterol to assemble into lipid rafts in the luminal leaflet of the bilayer, affecting protein expression and cell signalling. Therefore, even if in placentae from pre-eclamptic women, HIF-1α enhances the expression of AQP4, the potential

damage to the syncytiotrophoblast membranes produced by the intermittent hypoxia may contribute to create an unfavourable environment for AQP4 insertion into the plasma membrane, increasing its lysosomal degradation.

In conclusion, we propose that oxygen may regulate the expression of placental AQP4 possibly through a HIF-1α dependent pathway. Therefore, the decrease of this protein in syncytiotrophoblast toward the end of gestation, consistent with changes in HIF-1α expression, suggests that AQP4 might have a crucial role at the first stages of placental development. In addition, the abnormal expression of AQP4 in placentae from pregnancies complicated by pre-eclampsia, attributable to fluctuations in oxygen tension within the placenta, might have a key role in the cause of this gestational disorder.

The consequences of AQP4 dysregulation in the physiopathology of pre-eclampsia, however, remain uncertain.

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