Increased Expression of Aquaporin 9 in Trophoblast From Gestational Diabetic Patients

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- gestational diabetes mellitus
- aquaporins
- AQP9
- placenta

Abstract

Gestational diabetes mellitus is the most frequent pathophysiological alteration in pregnancy, increasing the incidence of complications in both mother and fetus. The macrosomia that occurs in these fetuses may be related with some changes in nutrient transport mechanism in placenta. The presence of aquaporin 9, an aquaglyceroporin, has previously been demonstrated in placenta. We raised the question whether aquaporin 9 expression may be upregulated in placenta from gestational diabetes, thus providing a faster transport of glycerol and water through placenta. We studied 21 placentas (13 controls and 8 gestational diabetes) from cesarean delivery at term. The expression of aquaporin 9 was analyzed by quantitative PCR, immunoblot, and immunohistochemistry. The median values from quantitative PCR were compared by nonparametric tests for independent samples (Mann-Whitney U-test). We have found that trophoblast from gestational diabetes express higher amount of aquaporin 9, which was found statistically significant ($p<0.05$). The increase in aquaporin 9 expression was confirmed by immunoblot, and localization in the syncytiotrophoblast was checked by immunohistochemistry. The increase in aquaporin 9 expression in placenta from gestational diabetes may contribute to the higher transport rate in this pathology of pregnancy.

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Introduction

Aquaporins (AQPs) are a family of integral membrane proteins. There are 13 known AQPs in mammals. Their structure is tetrameric with monomers (<30 kDa) containing 6 membrane-spanning helical domains surrounding an aqueous pore [1,2]. Three subgroups of AQPs have been described according to their structure and functional properties: The "classical aquaporins", which only permeate water; the "aquaglyceroporins" that comprise AQP3, 7, 9, and 10, which are also permeable to urea and glycerol, and the "super-aquaporins", AQP11 and 12, which are localized in the cytoplasm and whose permeability has not yet been fully determined [3]. In the particular case of AQP9, it can also facilitate the flux of neutral solutes such as monocarboxylates, purines, and pyrimidines.

AQPs are also expressed in placenta and fetal membranes where they seem to play an important role in amniotic fluid volume regulation. Previously, Damiano and colleagues demonstrated that the localization of AQP9 was not only in apical and basal membranes but also in the cytoplasm of human preeclamptic placenta, which was found increased 2.5-fold compared with normal term placentas. Moreover, they found that there was a lack of functionality of AQP9 for water and mannitol transport, but there is evidence that this aquaporin is involved in the excretion of urea across syncytiotrophoblast of human placenta from mother to fetus [4,5]. It is known that a proper development of pregnancy depends on high-quality ovulation, successful fertilization, normal embryonic and fetal development, and homeostasis of amniotic fluid. Throughout pregnancy, AQPs are expressed in placenta, uterus, adnexa, brain, urinary system, and the lacrimal gland. The regional and temporal regulation of AQPs plays important roles in normal pregnancy, fetal growth, and homeostasis of amniotic fluid volume. The pregnant phenotypes of aquaporin-knockout mice provide direct evidence that AQPs deficiency results in adverse outcome of pregnancy. Changes in the expression regulation of aquaporins are seen in preeclampsia, abnormal amniotic fluid volume,

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chorioamnionitis, and maternal undernourished pregnancy [6]. Gestational diabetes mellitus (GDM) is the most frequent pregnancy alteration, affecting 4–8% of all pregnancies [7,8], and increasing the risk of both the mother and the fetus for adverse events. In vitro studies showed an upregulation in transport system for some amino acids in placenta. The alteration of placenta function may be the reason for abnormal fetal growth [9] observed in this pathology of pregnancy [10]. Women with GDM have increased plasma leptin levels [11]. Besides, insulin levels are also increased in GDM, and hyperinsulinemia may mediate increase of leptin synthesis in placenta [12]. In this sense, our group has recently described increased leptin and leptin receptor expression in placentas obtained from GDM [13], and insulin induces leptin expression in trophoblastic cells, enhancing the activity of leptin promoter region [14]. Increased insulin and leptin levels may modulate the expression of AQP9, as it was previously described in placenta [15], as well as adipocytes and hepatocytes [16]. Even though the functional role of AQPs in placenta remains to be elucidated, its expression and regulation in abnormal pregnancy may point to new potential therapeutic targets for the pathology of pregnancy. In this context, increased AQP9 expression has been found in preeclamptic placenta [5]. We raised the hypothesis that AQP9 expression may also be increased in GDM. In the present study, we have examined the expression of AQP9 in human syncytiotrophoblast of placentas from normal and GDM pregnancies.

Subjects and Methods

Subjects

This study was approved by the local ethical committee and informed written consent was obtained from all subjects before the collection of samples. The reported investigations have been carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000.

Term placentas (n = 21, 13 from healthy pregnancies and 8 from GDM) were obtained after programed cesarean section by the Obstetric-Gynecology service of the Hospital Virgen Macarena. The patients were diagnosed with GDM based on ADA criteria: Glycemia fasting > 92 mg/dl (5.1 mmol/l); glycemia 1-h post glucose intake > 180 mg/dl (10 mmol/l); glycemia 2-h post glucose intake > 153 mg/dl (8.5 mmol/l). Clinical data are shown in **Table 1**.

Human placentas were immediately suspended in ice-cold phosphate-buffered saline (PBS) and transported to the laboratory (10–20 min) for being processed within 1–2 h after delivery as previously described [17]. Briefly, placentas were washed 2–3 times in sterile PBS to remove excess blood. Villous tissues, free of visible infarct, calcification, or hematoma were sampled from at least 5 cotyledons at a distance midway between the chorionic and basal plates. These core parts of cotyledons were cut into multiple cubic segments (10–15 mg wet weight) and thoroughly rinsed with cold DMEN-F12 medium pH 7.4 (137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, and 4 mM $NaHCO_3$).

Table 1 Subject characteristics of healthier cases and GDM.

Characteristics	Healthy (n = 13)	GDM (n = 8)	p-Value
Maternal age at delivery (years)	28.1 ± 6.0	34.8 ± 5.0	< 0.05
Gestational age at delivery (weeks)	39.5 ± 0.7	37.6 ± 1.4	ns
Infant weight (g)	3 120 ± 67.2	3 545 ± 285	< 0.05
Placenta weight (g)	430 ± 57	575 ± 88	< 0.05
IMC (kg/cm^2)	26	28.4	ns

Values are mean ± SD. ns: Not significant

Quantitative real time PCR assay

Total RNA was extracted from placental samples using Trisure reagent, according to the manufacturer's instructions (Bioline Co). Concentration and purity of the isolated RNA were estimated by spectrophotometry at 260 and 280 nm with purity in A_{260}/A_{280} ratio around 2.0.

For cDNA synthesis, 5 µg of total RNA was reverse-transcribed at 55 °C during 1 h using the Transcriptor first Strand cDNA synthesis Kit (Roche). qPCR reaction was performed using the following primers sequences: AQP-9 sense: 5′GAAAAGACTGAGCCAGAG GAA3′, AQP-9 Antisense: 5′AGACCCTCATTGTCTGGGTCTA3′ and cyclophylin, forward primer: 5′CTTCCCCGATACTTCA3′ and reverse primer: 5′TCTTGGTGCTACCTC3′.

RT-qPCR Master Mix Reagent kit was "SensiMixTM Plus SYBR Kit" (Quantace), and PCR reactions were performed on MJ Mini BioRAD (Bio Rad).

The reaction was initiated by preheating at 95 °C for 10 min. Subsequently, 45 amplification cycles were carried out as follows: denaturation 15 s at 95 °C, 30 s annealing at 58 °C, and 30 s extension at 72 °C. The threshold cycle (CT) from each well was determined by the Bio RAD CFX Manager Program. Relative quantification was calculated using the $2^{-\Delta CT}$ method, using cyclophylin as control expression. For *q*PCR, samples were run in triplicates with 5% intra-assay variability, and 11% interassay variability.

Western blot analysis

Total protein level was determined with "BCA Protein Assay Kit" (Pierce, Rockford, IL, USA) by the bicinchoninic acid method (Thermo Scientific), using bovine serum albumin as standard (BSA, Sigma Chemical Co, St. Louis, USA).

Supernatants were mixed with Laemmli's sample buffer containing 2% SDS and 30 mM β-mercaptoethanol, boiled for 5 min, resolved by SDS-PAGE on a 10% gel, using Prestained Molecular Markers Broad Range (Bio Rad) as PM marker. After that, the samples were electrophoretically transferred to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences).

Membranes were equilibrated in 1 × PBS and nonspecific binding sites were blocked by albumin solution (5%) in PBS at room temperature for 30 min. Membranes were then immunoblotted with polyclonal antibodies that detect human AQP9 (1:1 000, AlphaDiagnostic). Loading controls were performed by immunoblotting the same membranes with monoclonal anti-Tubulin (1:1 000, Santa Cruz Biotechnology Inc.). Membranes were then incubated with secondary antibodies using horseradish peroxidase-linked anti-mouse/anti-rabbit immunoglobulins. Bound horseradish peroxidase was visualized by a highly sensitive chemiluminescence system (Super Signal from Pierce). The bands obtained in the blots were scanned and analyzed by the PCBAS 2.0 program (Raytest, Straubenhardt, Germany). The relative optical density of the different bands was normalized by the corresponding intensity of the GAPDH immunoblot in each individual experiment.

Immunohistochemistry

Human villous tissues were cut into small pieces, fixed overnight in 10 per cent formaldehyde in 0.1 M sodium phosphate

buffer, pH 7.4, dehydrated, and embedded in paraffin. Paraffin sections (4 µM) were cut and mounted on 2% silanized slides, dried, dewaxed and rehydrated. Tissue slices were incubated 30 min in 3% H_2O_2/methanol to block endogenous peroxidase, and washed with PBS. All subsequent steps were carried out in a humidified chamber. Nonspecific binding sites were blocked by incubation at room temperature in serum solution 1:20 for 20 min. Before removal of the serum solution, sections were incubated 18–24 h at 2–8 °C with anti-AQP9 antibodies at a dilution of 1:500. The sections were then washed in PBS (3×, 5 min each) and incubated with their respective secondary antibodies. The manufacturer's instructions were followed for the sequential incubation and durations for the exposure to the secondary antibodies. After washing with PBS, the sections were incubated with diaminobenzidine substrate kit (Dako, Carpinteria, CA, USA) for 5 min that resulted in a brown-colored precipitate at the antigen-antibody binding sites and the reaction was stopped in distilled water. After removing the slides from water, all the sections were dehydrated and one drop of aqueous mounting medium (Dako Faramount) was applied, and the sections were cover-slipped.

Finally, the immunohistochemical specimens were examined using a Leica Laborlux S Microscope (Leica Microsystem GmbH, Wetzlar, Germany) with a Nikon DSL2 photo digital system (Nikon Corp, Tokyo, Japan). Each sample was analyzed with a double-blind system by 2 different operators under magnifications up to 100×. A cell was considered as positive when it demonstrated distinct brown surface staining. Representative sites in each sample were photographed at 100× magnification and captured with a software system (CS3, version 10.0.1; Adobe Photoshop, San Jose, CA, USA)

Statistical analysis

In placental explants, immunoblot are a representative experiment from the 21 placentas studied (13 from healthy pregnancies and 8 from GDM). Results are expressed as mean ± SD. The statistical significance was assessed by ANOVA followed by Bonferroni's multiple comparison post hoc test and was calculated using the GraphPad Instat computer program (GraphPad, San Diego, CA, USA). A p-value less than 0.05 was considered statistically significant.

Results

Since we have hypothesized that AQP9 may be upregulated in placenta from gestational diabetes, we have investigated the AQP9 expression in placenta from gestational diabetic women compared to that from control women, using quantitative PCR. PCR data were analyzed using the CFX Manager™ Software Version 1.5 of BIO RAD program and then were subjected to statistical analysis by GraphPad Instat computer program. As shown in **Fig. 1**, samples of placentas of women with GDM have a significant higher expression of AQP9 gene compared with control placentas.

In order to assess whether the increase in AQP9 gene expression produced higher amount of AQP9 protein, we carried out immunoblot analysis. **Fig. 2** shows a representative immunoblot of 2 gestational diabetic samples compared with 2 control samples. We have found that trophoblast from gestational diabetes patients express higher amount of aquaporin 9 than trophoblast

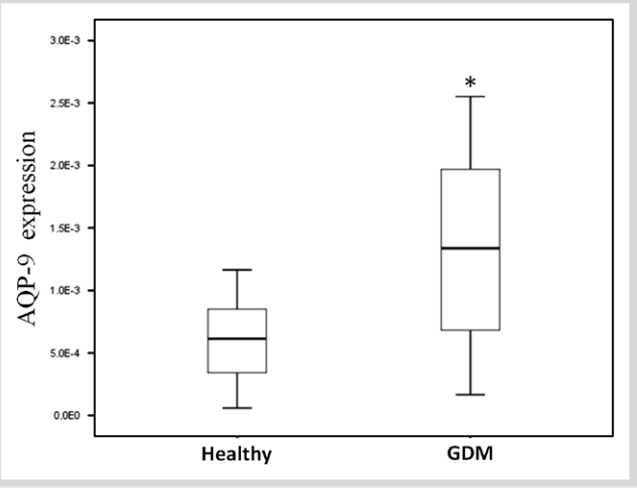

Fig. 1 Increased expression of Aquaporin 9 (AQP9) in placentas from pregnancies complicated with gestational diabetes mellitus (GDM) compared with placentas from healthy pregnancies: Placental samples were obtained from 13 control placentas and 8 placentas from GDM. AQP-9 mRNA was quantified with qRT-PCR. RNA was extracted as described in Materials and Methods. Cyclophylin was used as internal standard. Statistical analyzes were performed by ANOVA. Asterisks indicate significant differences from the control according to Bonferroni's multiple comparison post hoc test. * $p < 0.05$ indicates significant differences from the control.

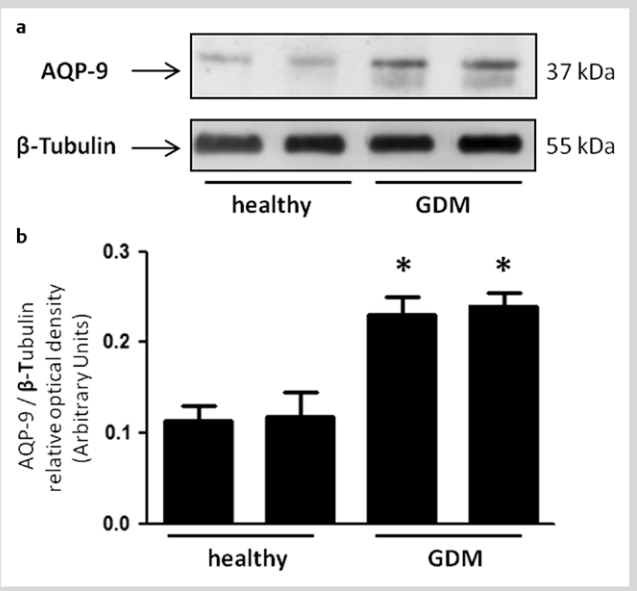

Fig. 2 AQP9 expression in healthy and gestational diabetic placental (GDM) explants**: a** Placental extracts were prepared and proteins were separated on SDS-PAGE gels. AQP-9 was determined by Western blot analysis. Molecular weights were estimated using standard protein markers. Molecular mass (kDa) is indicated at the right of the blot. Loading controls were performed by immunoblotting in the same membranes with anti-tubulin. A representative immunoblot shows a 37 kDa band corresponding to the glycosylated form of AQP9 in normal and GDM placental explants. **b** Densitometry of immunoblots containing AQP9 protein level expression was performed, and after normalization for β-tubulin, the values were plotted as AQP9/β-tubulin relative ratio. Each plotted value corresponds to the mean ± SD obtained from for 3 independent experiments ($p < 0.05$).

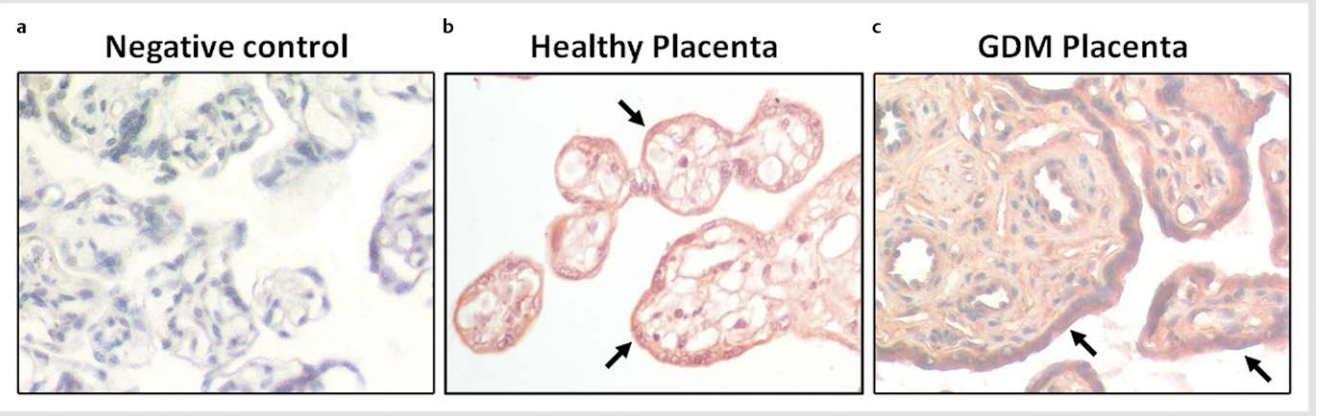

Fig. 3 Localization of AQP-9 in healthy and GDM placenta: Representative sections from Healthy and GDM placental explants evidencing the AQP-9 expression. **a** Negative controls were performed by omitting the primary antibody and replaced by a nonimmune rabbit serum. **b** Immunohistochemistry revealed specific labeling in the apical membrane of syncytiotrophoblast cells from healthy placental explants. **c** In placentas from patients with GDM, AQP-9 labeling was stronger in the apical membrane. Magnification ×400. (Color figure available online only).

from control patients. These results are in line with those obtained by qPCR.

The localization of leptin expression in control and GDM women trophoblasts was verified by immunohistochemistry. AQP9 was expressed in the syncytiotrophoblast as previously described. **Fig. 3** shows immunohistochemistry analysis for one placenta cut from a healthy sample and one section of placenta from women with GDM. Data reveal that the expression level of AQP9 is present in the syncytiotrophoblast, and placenta samples from women with GDM express higher amount of AQP9 than those obtained from healthy placentas.

Discussion

Gestational diabetes is a health problem that affects both the mother and the child, increasing the risk of death during the perinatal period. The main pathophysiological complications of GDM are due to fetal macrosomia, which is accompanied by a larger size and weight of the placenta to support the increased needs of the macrosomic fetus. Fetal macrosomia requires a greater availability of nutrients, which are provided by various transport systems, such as amino acids, fatty acids, and glucose. Another important nutrient for energy provision is glycerol, transported by some AQPs, such as AQP3, AQP7 and AQP9.

Our research group has been working on the role of leptin in the growth and metabolism of the placenta [18] where the trophic action of leptin may mediate an increase in the size of the placenta from DMG, since both the expression of leptin and its receptor are increased [13]. These effects appear to be mediated by activation of protein synthesis by acting on the PI3K and MAPK pathways, which converge at the point of activation of protein translation signaling [18]. Moreover, leptin seems to increase AQP9 expression in trophoblast explants in vitro (data not shown).

In this context, it seemed reasonable to consider that the transport system of glycerol, namely AQP9 trophoblast was overexpressed in placentas from women with GDM, and the leptin/leptin receptor system could play an important role, since the system is activated in the placenta from GDM. We have found that, indeed, the amount of AQP9 mRNA, as well as protein amount in trophoblasts from placentas of women with GDM is higher than that observed in placental trophoblasts from control women. Besides, the AQP9 localization was confirmed in syncytiotrophoblast as previously described [5].

The increased expression of AQP9 in the syncytiotrophoblast from placentas with GDM could mediate the increased transport of glycerol to the fetus, thus contributing to attend the increased energy intake requirements in the macrosomic fetus. On the other hand, we cannot exclude the possible role of other AQPs, such as AQP3, which is known to be present in the syncytiotrophoblast. In any case, the increased expression of AQP9 may contribute to the increase in water and/or glycerol transport. Regarding the underlying mechanisms producing this increase in AQP9, we may speculate that a possible candidate may be leptin, whose plasma levels are increased in GDM. In this context, the possible effect of leptin on AQP9 expression in vitro is being currently investigated. Regardless of the mechanism, with the limitation of the number of samples, the present data strongly suggest the increased expression of AQP9 in trophoblast from GDM and thus, the increased glycerol transport to the fetus may help to cover the increase in energy needs that may occur in GDM.

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Conflict of Interest

The authors declare that they have no conflicts of interest in the authorship or publication of this contribution.

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