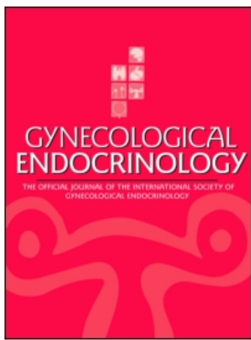

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ORIGINAL ARTICLE

Leptin upregulates aquaporin 9 expression in human placenta *in vitro*

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ABSTRACT
Aquaporins are integral membrane proteins that have permeability functions in many tissues. Aquaporin 9 may transport not only water but also small molecules, such as glycerol, monocarboxylates, purines and pyrimidines. Aquaporin 9 is expressed in syncytiotrophoblast of human term placenta, and it may contribute to the embryonic/fetal growth and survival. We have previously found that Aquaporin 9 expression levels seem to be increased in placenta from gestational diabetes. Since leptin plasma levels and leptin expression are increased in placenta from gestational diabetes, we aimed to study the possible role of leptin on Aquaporin 9 expression in human placenta *in vitro*. The present work shows that leptin produces a dose-dependent increase of Aquaporin 9 expression, resulting in an increase in Aquaporin-9 protein in human trophoblast explants.

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Introduction

Placenta is a critical organ responsible for nutrient uptake, waste elimination, and gas exchange between mother and fetus [1]. Consequently, placental dysfunction can lead to a number of adverse fetal outcomes, including abnormal fetal growth [2,3]. Gestational diabetes mellitus (GDM) is the most frequent pregnancy alteration, affecting 4–8% of all the pregnancies [4,5]. Leptin is a peptide hormone that centrally regulates energy metabolism [6], but also regulates other systems including reproduction [7]. Leptin is produced by adipocytes, but also by trophoblast cells, acting as a trophic factor [8,9]. That is why plasma leptin levels are increased during pregnancy [10]. Besides, plasma leptin levels have been found increased in women with GDM [1,11]. Hyperinsulinemia observed in GDM may contribute to the increase of leptin synthesis in placenta [12,13], since insulin increases the expression of leptin in trophoblastic cells. We have previously studied the role of leptin in the growth and metabolism of the placenta [8], and both leptin and its receptor are overexpressed in placenta from GDM [14]. Previously, our group had found that the AQP9 expression is also increased in syncytiotrophoblast from placentas of GDM patients [15]. Aquaporins (AQPs) are a family of integral membrane proteins with water permeability functions. AQP9 can also facilitate the flux of neutral solutes such as glycerol, monocarboxylates, purines and pyrimidines [16,17]. AQPs are expressed in placenta and fetal membranes, where they seem to play an important role in amniotic fluid regulation. There is evidence that AQP9 is also involved in the excretion of urea across syncytiotrophoblast of human placenta from mother to fetus [18–20]. AQP9 expression has been found to be positively regulated by insulin [21]. Changes in the expression regulation of AQPs are seen in preeclampsia, abnormal amniotic fluid volume, chorioamnionitis and maternal undernourished pregnancy [22], thus pointing to a new therapeutic target in pathological pregnancies. In this context, our group has found that the amount of AQP9 mRNA as well as AQP9 protein level in trophoblast from placentas of women with GDM is higher than that observed in control placenta trophoblast [15]. Since leptin plasma levels and leptin expression are increased in placenta from GDM [8,14], now we aimed to study the possible role of leptin on AQP9 expression in human placenta *in vitro*.

Material and methods

The present research has been performed in accordance with the Declaration of Helsinki and has been approved by the local Institutional Review Board (Virgen Macarena University Hospital IRB) and samples were obtained with prior informed written consent from all subjects. All mandatory laboratory health and safety procedures have been complied with in the course of conducting the experimental work reported in this paper.

Placental explants collection and processing

Term placentas from non-GDM patients and non-hypertense patients, were obtained after programed cesarean section. Placentas were immediately suspended in ice-cold phosphate-buffered saline (PBS) and transported to the laboratory for being processed within 1–2 h after delivery as previously described [23].

In vitro *studies with leptin stimulation*

The experiments were performed in duplicate from five independent placentas. The explants were incubated during 6 h at

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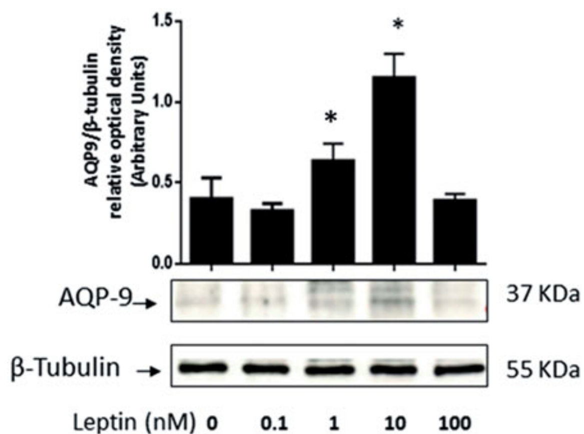

Figure 1. Leptin treatment increases the amount of AQP9 in human placental trophoblast explants. Placental explants were incubated with increasing concentrations of leptin (0, 0.1, 1, 10, 100 nM) for 6 h. Placental extracts were prepared and proteins were separated on SDS-PAGE gels. AQP9 was determined by Western blot analysis. Loading controls were performed by immunoblotting in the same membranes with anti-β-tubulin. A representative immunoblot shows a 37 kDa band corresponding to the glycosylated form of AQP9 placental explants. 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. Results shown are from a representative experiment and are expressed as means ± SD for three independent experiments $^*p < .05$ vs. control.

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37 °C in isotonic medium DMEN F12 (0% SBF) with increasing leptin concentrations (0, 0.1, 1, 10 y 100 nM). For the immunoblotting, samples were lysed, denatured and resolved by SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane (Hybond ECL, AmershamBiosciences), and then incubated with polyclonal antibodies that detect human AQP9 (AlphaDiagnostic). Loading controls were performed by immunoblotting the same membranes with monoclonal anti-β-Tubulin (Santa Cruz Biotechnology Inc, Dallas, TX). The antibodies were detected using ECL, SuperSignal® (Thermo Scientific, Waltham, MA).

For the expression analysis by RT-PCR, total RNA was extracted using Trisure® (Bioline Co, London, UK). Five micrograms of total RNA was reverse-transcribed at 55 °C during 1 h using the Transcriptor first Strand cDNA synthesis Kit (Roche, Basel, Switzerland). qPCR reaction was performed using the primers sequences and protocol described in previous work [15]. Data were analyzed using CFX Manager™ Software version 1.5 program BIO RAD and subsequently underwent statistical analysis using the GraphPad Prism software.

Statistical analysis

In placental explants, immunoblot are a representative experiment from the 5 placentas studied. Results are expressed as mean ± SD. For the expression analysis data by RT-PCR, the statistical significance was assessed by ANOVA followed by Bonferroni's multiple comparisons *post hoc* test and was calculated using the GraphPad Instat computer program (GraphPad, San Diego, CA). It was considered statistically significant a p values less than .05.

Results

To study the effect of leptin on AQP9 expression in human trophoblasts, explants were incubated with increasing

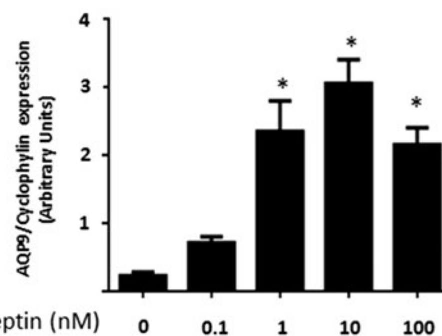

Figure 2. Leptin treatment increases the AQP9 expression in human placental trophoblast explants. Placental explants were incubated with increasing concentrations of leptin (0, 0.1, 1, 10, 100 nM) for 6 h. Total RNA was extracted and AQP-9 mRNA was quantified with qRT-PCR in independent experiments. Cyclophilin was used as internal standard. Results shown are from a representative experiment and are expressed as means ± SD for three independent experiments $^*p < .05$ vs. control.

concentrations of leptin (from 0 to 100 nM) for 6 h. The analysis of protein expression was referenced as β-Tubulin control protein. As shown in Figure 1, leptin stimulated AQP9 expression in trophoblast explants, in a dose-dependent manner, with maximal effect achieved with a concentration of 10 nM and observing an effect of saturation at 100 nM. Similar dose-response results have previously been found in leptin and insulin effects on human trophoblast explants such as protein synthesis [11,24].

To test whether leptin induces AQP9 gene expression, trophoblast explants were incubated in the same manner as described above, but the expression level was quantified by qRT-PCR. As shown in Figure 2, the expression of AQP9 is dose-dependently increased by leptin, reaching a maximum effect at 10 nM, and showing a saturation effect at 100 nM. Therefore, leptin seems to transcriptionally activate AQP9 expression in placenta.

Discussion

AQP9 has previously been found in the apical membranes of the syncytiotrophoblasts of human term placenta [19]. AQP9 is likely fundamental to the regulation of fetal water and solutes flow both in intramembranous absorption and in placental water transfer from mother to fetus. Nevertheless, it should be taken into account that AQP9 is not only permeable to water, but also to neutral solutes [25], suggesting that this channel may also be involved in metabolite diffusion and may, therefore, have a role in placenta/fetal energy metabolism.

Our previous results about the role of leptin in the growth and metabolism of the placenta trophoblastic cells [8], and the overexpression of both leptin and its receptor in placenta from GDM [14,26], suggest that leptin may mediate the increase in size of the placenta by activating both PI3K and MAPK signaling pathways [11]. We have previously found increased expression of AQP9 in the syncytiotrophoblast from placentas with GDM, and thus, AQP9 could mediate the increased transport of glycerol to the fetus to attend the increased energy requirements in the macrosomic fetus [15]. The typical macrosomia observed in newborn from GDM mother requires a greater availability of nutrients provided by different transport systems, including an important nutrient for energy provision such as glycerol, which may be transported by AQP3, AQP7 and AQP9.

In this context, our results demonstrate that leptin produces an increase in AQP9 expression by trophoblast explants *in vitro*, providing a molecular mechanism for a better nutrient transfer

and therefore, AQP9 may be a mediator of the metabolic action of leptin in placenta, and finally, leptin could mediate the increased AQP9 expression observed in GDM.

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Disclosure statement

The authors report no conflicts of interest.

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