Activated Translation Signaling in Placenta from Pregnant Women with Gestational Diabetes Mellitus: Possible Role of Leptin

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Key words
- leptin
- LEPR
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- translation
- gestational diabetes

Abstract

Placentas from gestational diabetes (GDM) suffer from structural and functional changes including overgrowth. That is why we aimed to study [3H]-leucine incorporation into protein in addition to translation signaling in placenta from GDM. Thus, we investigated the expression of leptin and leptin receptor (LEPR), as well as the activation state of signaling proteins regulating protein synthesis, such as mTOR, S6 Kinase, EIF4E-BP1, EIF4E, and eEF2 by measuring protein phosphorylation by immunoblot. [3H]-Leucine incorporation into protein also was determined in trophoblastic placenta explants from GDM and control pregnancy. We found that leptin and LEPR expression are increased in placentas from GDM and the translation machinery activity as well as [3H]-leucine incorporation into protein were higher in placentas from GDM compared with placentas from control pregnancy. In conclusion, protein synthesis rate is increased in placenta from GDM patients, and this may be due, at least in part, by the activation of translation signaling. The increased expression of leptin and LEPR may contribute to these effects. These results may provide a possible mechanism for the previously observed increase in placenta growth in GDM.

Introduction

Gestational diabetes mellitus (GDM) is defined as carbohydrate intolerance of variable severity with onset or first recognition during pregnancy [1]. It affects 4–8% of all pregnancies depending on the population studied and the diagnostic tests employed [2]. Increased placenta weight has been previously found in women with GDM compared with normal pregnancies [3]. GDM is associated with high perinatal mortality and morbidity including macrosomia, neonatal metabolic disorders, respiratory distress syndrome, and newborn death [2]. Additionally, GDM possess an increased risk for development of type 2 diabetes mellitus later in life for both child and mother [2], which is even greater if obesity is present [4–6].

The placenta is a vitally important endocrine organ during pregnancy. In addition to the production of a wide variety of steroids, peptide hormones, and other regulatory factors, the placenta is also an endocrine target tissue, expressing a broad spectrum of hormone receptors and growth factor receptors [7]. In this sense, leptin and leptin receptors are expressed in placenta [8,9] in a regulated way [10–12], with an autocrine role controlling growth, proliferation and preventing apoptosis in trophoblast cells [8,13]. Abnormalities in the placenta of women with GDM have been well described; these include placental overgrowth, villous immaturity, and vascular hyperplasia of the chorionic villi [3,14,15]. Increased leptin expression in placenta from GDM patients have been previously described [16]. Moreover, we have found that leptin stimulates protein synthesis in both trophoblastic cell lines and placenta explants by activating different translation factors, which are involved in the initiation and elongation stages of translation [9,17,18]. Briefly, the initiation factor eIF4E binds to the cap structure at the 5′-end of the mRNA and mediates the assembly of the initiation-factor complex eIF4F. The assembly of this complex is inhibited by EIF4E-binding proteins (EIF4E-BPs), such as EIF4E-BP1 (PHAS-I) [19]. Phosphorylation of these EIF4E-BPs releases EIF4E from inactive EIF4E-BP1-EIF4E complex, allowing EIF4E to bind to EIF4G, and EIF4A to form the active EIF4F complex [20]. The activity

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of this complex is also regulated by phosphorylation of EIF4E [19,21]. Interestingly, the intracellular signaling cascade leading to EIF4E-BP1 phosphorylation involves the activation of p70S6k. Moreover p70 S6 kinase is mainly activated by mammalian target of rapamycin (mTOR) [22,23]. Besides, there are other signaling pathways involved in the regulation of protein synthesis such as eukaryotic elongation factor 2 kinase (eEF2). eEF2 catalyzes the translocation of peptidyl-tRNA from the A site to the P site on the ribosome. The phosphorylation of eEF2 at threonine 56 inhibits its activity [24,25], and thus to inhibit peptide-chain elongation. In this context, we raised the hypothesis that [^{3}H]-leucine incorporation into protein rate might be increased and translation signaling might be activated in placenta from GDM, providing a molecular mechanism for the placenta overgrowth previously observed in GDM. We aimed to study the expression of leptin and leptin receptor (LEPR), [^{3}H]-leucine incorporation into protein, as well as to investigate the possible activation of translation signaling by studying the phosphorylation levels of mTOR, S6 Kinase, EIF4E-BP1, EIF4E, and eEF2 in placenta trophoblastic explants from GDM patients compared with control pregnancy.

Materials and Methods

Placentas

Term placentas from pregnancies with GDM (n = 10; 5 patients who were diet-controlled and 5 patients who required insulin therapy) and from uncomplicated pregnancies (n = 10) were obtained after Cesarean section delivery following normal term pregnancies in the Virgen Macarena University Hospital. None of the patients had previous history of diabetes mellitus or any known endocrinopathy. Subject characteristics of controls and GDM cases were mean maternal age at delivery (27.1 years ± 6.0 vs. 34.8 years ± 5.0), mean infant birth weight (3 170.4 g ± 87.2 vs. 3 245.8 g ± 467.2), mean placenta weight (564 g ± 105 vs. 670 g ± 122). Subject characteristics of controls and GDM cases were similar with regards to gestational age (39.5 weeks ± 0.7 vs. 38.6 weeks ± 1.4) and nulliparity (5 vs. 4). The patients were diagnosed with GDM based on either 1) a value > 200 mg/dl on a 50 g 1-h post-glucose blood sugar or 2) 2 or more elevated values on a 100 g 3-h glucose tolerance test. The placental tissues were collected at the Cesarean delivery and immediately transferred on ice to the laboratory for preparations of protein lysate as well as [^{3}H]-leucine incorporation experiments.

This study was approved by the local ethical committee (Comité Local de Etica e Investigación del Hospital Universitario Virgen Macarena); and informed written consent was obtained from all subjects. The reported investigations have been carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000.

Placental samples

Human placentas were immediately suspended in ice-cold phosphate-buffered saline (PBS) and transported to the laboratory (10–20 min), where they were washed 2–3 times in sterile PBS to remove excess blood. Villous tissue free of visible infarct, calcification, or hematoma was 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 (50 mg weight) and washed with phosphate buffer saline. Next, explants were resuspended in 500 µl of lysis buffer [1 × PBS, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 10 % glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 50 mM NaF, 10 mM pyrophosphate, and protease inhibitor cocktail] during 30 min at 4 °C on an orbital shaker and later centrifuged at 10 000 *g* for 20 min. Supernatants were analyzed by Western blot. For leucine incorporation experiments, after thoroughly washing with phosphate buffer saline, the explants were placed in tubes containing 1 ml of a HAM F-12 medium and the experiment were performed immediately.

Western blot analysis

Total protein levels were determined by the bicinchoninic acid method (Thermo Scientific), using bovine serum albumin as standard. Fifty µg protein was loaded in each lane. 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 12 % gel, and electrophoretically transferred to a nitrocellulose membrane (Hybond, Amersham Pharmacia), thereafter. Membranes were equilibrated in 1 × PBS and nonspecific binding sites were blocked by 5 % nonfat milk in PBS at room temperature for 1 h. Membranes were then immunoblotted with polyclonal antibodies that detect human leptin Y20 (1:1000, Santa Cruz), and monoclonal mouse anti-leptin receptor (B-3) recognizing the membrane-bound LEPR (1:1000, Santa Cruz) to study the amount of leptin and leptin receptor. We also used polyclonal rabbit that detect the mammalian target of rapamycin (mTOR) only when phosphorylated at Ser2448 (1:1000, Cell signaling), polyclonal rabbit anti-phospho-p70S6 kinase (Thr421/Ser424) (1:3000, Sigma), polyclonal rabbit anti-phospho-EIF4EBP1 (Thr37/46) (1:2000, Cell signaling), anti-phospho-EIF4E (Ser209) (1:2000, Cell signaling) and anti-phospho-eEF2 (Thr56) (1:2000, Cell signaling) antibodies. Loading controls for the immunoblots of leptin and leptin receptor were performed by immunoblotting the same membranes with monoclonal anti-β-tubulin. For the translation machinery experiments we normalized samples with polyclonal rabbit anti-total-mTOR (1:2000, Cell signaling) polyclonal rabbit anti-total-S6K (1:3000, Santa Cruz), with polyclonal rabbit anti-total-eEF2 (1:2000, Cell signaling), with polyclonal rabbit anti-total-EIF4EBP1 (1:3000, Santa Cruz) and with polyclonal rabbit anti-total-EIF4E (1:3000, Santa Cruz). The antibodies were detected using horseradish peroxidase-linked anti-rabbit/anti-mouse immunoglobulin (1:10 000, Santa Cruz) and visualized using a highly sensitive chemiluminescence system (Supersignal; Pierce).

Quantitative real-time PCR assay

Abundance of LEPR mRNA was determined by quantitative real time PCR reaction (*q*RT-PCR). Total RNA was extracted from placental samples using Trisure reagent, according to the manufacture's instructions (Bioline Co). Concentration and purity of the isolated RNA were estimated spectrophotometrically at 260 and 280 nm with a purity in A_{260}/A_{280} ratio around 1.8. For cDNA synthesis, 5 µg of total RNA was reverse-transcribed at 50 °C during 1 h using the Transcriptor first Strand cDNA synthesis Kit (Roche). Quantitative real time PCR reaction was performed using the following primers based on the sequences of the NCBI GenBank database: LEPR, forward primer, 5′ATAGTTCAGTCACCAAGTGC3′; reverse primer, 5′GTCCTGGAGAACTCTGATGTCC3′ and cyclophilin, forward primer, 5′CTTCCCCGATACTTCA3′; reverse primer, 5′TCTTGGTGCTACCTC3′. *q*RT-PCR Master Mix Reagent kit was obtained from Roche (Fast Start universal SYBR

Green) and PCR reactions were performed on a Chromo 4 DNA Engine (Bio-Rad). A typical reaction contained 10 µM of forward and reverse primer, 3 µl of cDNA and the final reaction volume was 25 µl. The reaction was initiated by preheating at 50 °C for 2 min, followed by heating at 95 °C for 10 min. Subsequently, 41 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 Opticon Monitor 3 Program. Relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method [26]. For the samples, evaluation of $2^{-\Delta\Delta CT}$ indicates the fold change in gene expression, normalized to a housekeeping gene (cyclophylin), and relative to the control sample. For quantitative PCR, samples were run in triplicates with 5% intra-assay variability, and 11% interassay variability.

[^{3}H]-Leucine incorporation experiments

Placental samples (50 mg) were placed in tubes containing 1 ml of a Ham's F-12 medium (triplicate per each placenta), within 1 h after delivery. Incubation of placental samples started in a shaking water bath at 37 °C for an equilibration period of 5 min. Then, [^{3}H]-leucine (1 µCi/ml), from Amersham Pharmacia, was added and incubation continued for 4 h. Next, explants were then washed and solubilized in 1 ml of 1 M NaOH. After, they were neutralized with 1 ml of 1 M HCl and the lysate was precipitated with 20% trichloroacetic acid. The pellet was resuspended in 200 µl 1 M NaOH and 0.1% SDS. The incorporated radioactivity was quantified by scintillation counting. Total protein levels were determined as described above. Assessment of explant viability was routinely monitored by measuring the release of LDH into medium relative to a 1% Triton X-100 (Sigma)-lysed positive control.

Data Analysis

Experiments were repeated separately at least 3 times to guarantee representative results. Data are expressed as the mean ± standard deviation (S.D.). The statistical significance was assessed by 1-way ANOVA followed by Bonferroni's multiple comparison post hoc test and was calculated using the Graph Pad Instat computer program (San Diego, CA, USA). A p-value of <0.05 was considered statistically significant. Quantification of protein bands was determined by densitometry using Scion Image software (Scion Corporation, Washington, DC, USA).

Results

Placentas from GDM overexpress leptin and its receptor compared with placentas from healthy donors

We have previously demonstrated leptin and leptin receptor expression not only in BeWo and JEG-3 cells but also in human placental samples from healthy donors, suggesting that leptin is probably exerting autocrine/paracrine effects in these systems. Moreover, deregulation of autocrine/paracrine function of leptin at the feto-placento-maternal interface may be implicated in the pathogenesis of several disorders of pregnant women with GDM. In the present study, leptin and leptin receptor expression were characterized in placental samples from GDM by Western blot and qRT-PCR analysis compared with healthy control pregnancies.

As assessed by Western blot, results shown in **Fig. 1a** demonstrate that leptin protein is increased in placentas from patients with GDM, compared with placentas from normal pregnancies.

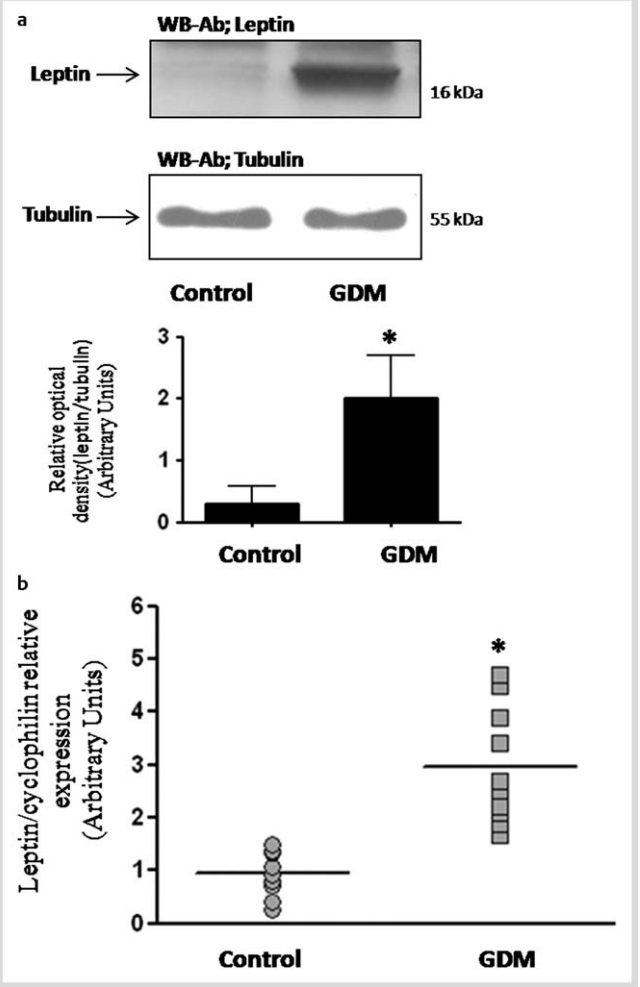

Fig. 1 Increased expression of leptin (LEP) in placentas from pregnancies complicated with gestational diabetes mellitus (GDM) compared with placentas from healthy pregnancies. **a** Placental samples (10 from control pregnancies, 10 from gestational diabetic pregnancies) were lysed and analyzed by Western blot to determine leptin expression. Loading controls were performed by immunoblotting the same membranes with anti-β-tubulin. Results shown in the immunoblot are from a representative experiment. Densitograms show mean ± SD from control and gestational diabetic samples, and expressed as arbitrary units. Statistical analyses were performed by ANOVA test. *$p<0.05$ indicates significant differences from the control. **b** Placental samples were obtained from 10 control placentas and 10 placentas from GDM. Leptin mRNA was quantified with qRT-PCR. Cyclophilin was used as internal standard. Data from GDM represent fold change from the mean values of control placentas. Results shown are from a representative experiment and are expressed as means ± SD. *$p<0.05$ indicates significant differences from the control.

To examine whether this effect was due to the upregulation of leptin expression, qRT-PCR analysis was carried out. Thus, as shown in **Fig. 1b**, placental expression level of leptin gene was found to be increased about 2-fold in placentas from GDM. These data are in agreement with those obtained by other authors [27,28].

We also studied the leptin receptor (LEPR) protein and mRNA content in placentas from GDM. As assessed by Western blot (**Fig. 2a**) and qRT-PCR (**Fig. 2b**), LEPR is also upregulated in placentas from GDM patients, which expresses twice as much LEPR than placentas from control pregnancies. The increased expression of leptin receptor points to an activation of the signaling pathways recruited by leptin.

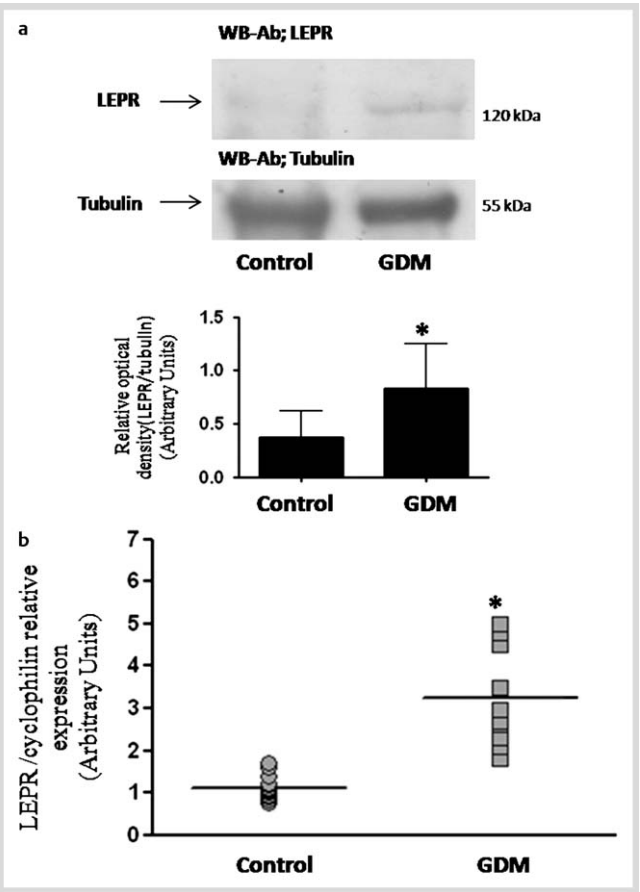

Fig. 2 Increased expression of leptin receptor (LEPR) in placentas from pregnancies with gestational diabetes mellitus (GDM) compared with placentas from healthy pregnancies. **a** Placental samples (10 from control pregnancies, 10 from gestational diabetic pregnancies) were lysed and analyzed by Western blot to determine leptin receptor expression. Loading controls were performed by immunoblotting the same membranes with anti-β-tubulin. Results shown in the immunoblot are from a representative experiment. Densitograms show mean ± SD from control and gestational diabetic samples, and expressed as arbitrary units. Statistical analyses were performed by 1-way ANOVA followed by Bonferroni's multiple comparison post hoc test.* $p < 0.05$ indicates significant differences from the control. **b** Placental samples were obtained from 10 control placentas and 10 placentas from GDM. Leptin receptor mRNA was quantified with qRT-PCR. Cyclophilin was used as internal standard. Data from GDM represent fold change from the mean values of control placentas. Results shown are from a representative experiment and are expressed as means ± SD. * $p < 0.05$ indicates significant differences from the control.

Activation of protein synthesis signaling pathway in placentas from GDM compared with control placentas

On the basis of the previously described effect of leptin on protein synthesis pathway in different systems [29,30], including placental samples from healthy donors [9,18,31], we checked whether the kinases implicated in the translation machinery, are overactivated in placentas from GDM. As shown in **Fig. 3a**, the phosphorylation of mTOR is significantly increased in placentas from GDM compared with control placentas. The amount of mTOR in each sample was controlled using anti-tubulin antibody. p70 S6 kinase is the target of mTOR, which participate in the cell growth. Since p70 S6 kinase activity is correlated with its phosphorylation state, specifically Thr421 and Ser424 [32], we employed polyclonal antibodies that detects p70 S6 kinase only when phosphorylated at Thr421/Ser424. As shown in **Fig. 3b**, the phosphorylation of p70 S6k is significantly increased in placentas from GDM compared with control placentas. The immunoblot was controlled using anti-S6K antibody. Similarly, we observed an increase in the phosphorylation of EIF4EBP1 (**Fig. 3c**), which is known to release EIF4E to be activated. The phosphorylation of EIF4E, which is proportional to the activation of this factor, was also increased in placentas from GDM (**Fig. 3d**). We controlled the amount of loaded protein by immunoblot using anti-EIF4EBP1 and anti-EIF4E respectively antibodies. Eukaryotic elongation factor 2 (eEF2) is an important check point in the regulation of protein translation initiation, which catalyzes the translocation of peptidyl-tRNA from the A site to the P site on the ribosome. It has been shown that phosphorylation of eEF2 at threonine 56 by eEF2 kinase inhibits its activity and thus inhibiting peptide-chain elongation. As shown in **Fig. 3e**, the phosphorylation of eEF2 is significantly decreased in placentas from GDM compared with control placentas. We controlled the amount of loaded protein by immunoblot using total anti-eEF2 antibody.

Increased [^{3}H]-leucine incorporation into protein in placentas from GDM

We next wanted to assess whether the activation of the translation signaling in placenta from GDM correlated with an increase in [^{3}H]-leucine incorporation into protein, as previously found in vitro in trophoblast explants stimulated with leptin [9,18]. As shown in **Fig. 4**, villous explants from GDM had almost a 2-fold increase in the rate of [^{3}H]-leucine incorporation into protein, as compared with control pregnancies.

Discussion

Placentas from women with GDM have usually functional and structural abnormalities such as overgrowth [3,15]. In fact, the placental dysfunction in women with GDM is associated with increased amino acid transport [33]. That is why, in the present work, we hypothesized an increased phosphorylation state of different proteins implicated on the initiation stage of translation, and as a result, the [^{3}H]-leucine incorporation into protein might be increased in placentas from GDM. Numerous factors could be implicated in this growth, including leptin, which is an important local mediator in placenta during pregnancy [13]. Possible physiological effects of placenta-derived leptin include angiogenesis, growth, and immunomodulation. These effects may be of physiological relevance since trophoblastic cells are an important source of leptin production [34], and circulating leptin levels are increased in pregnancy [35]. It was demonstrated that the human leptin gene is actively engaged by hypoxia through mechanisms that are common to other hypoxia-inducible genes [36,37]. This is relevant under stressful conditions for placenta cells, such as some pathophysiological pregnancy disorders – mainly gestational diabetes [35]. The leptin overproduced by placenta under stressful circumstances may be helpful to prevent the stress-mediated apoptosis of the trophoblastic cells. In this context, we have demonstrated that leptin promotes proliferation and cell survival as well as protein synthesis of human peripheral blood mononuclear cells and JEG-3 trophoblastic cells [8,38,39]. However, conflicting results have been found regarding the ability of the ob-gene product, leptin, to interfere with hypoxia-induced apoptosis [40] and therefore, both the physiological and pathophysiological role of leptin in

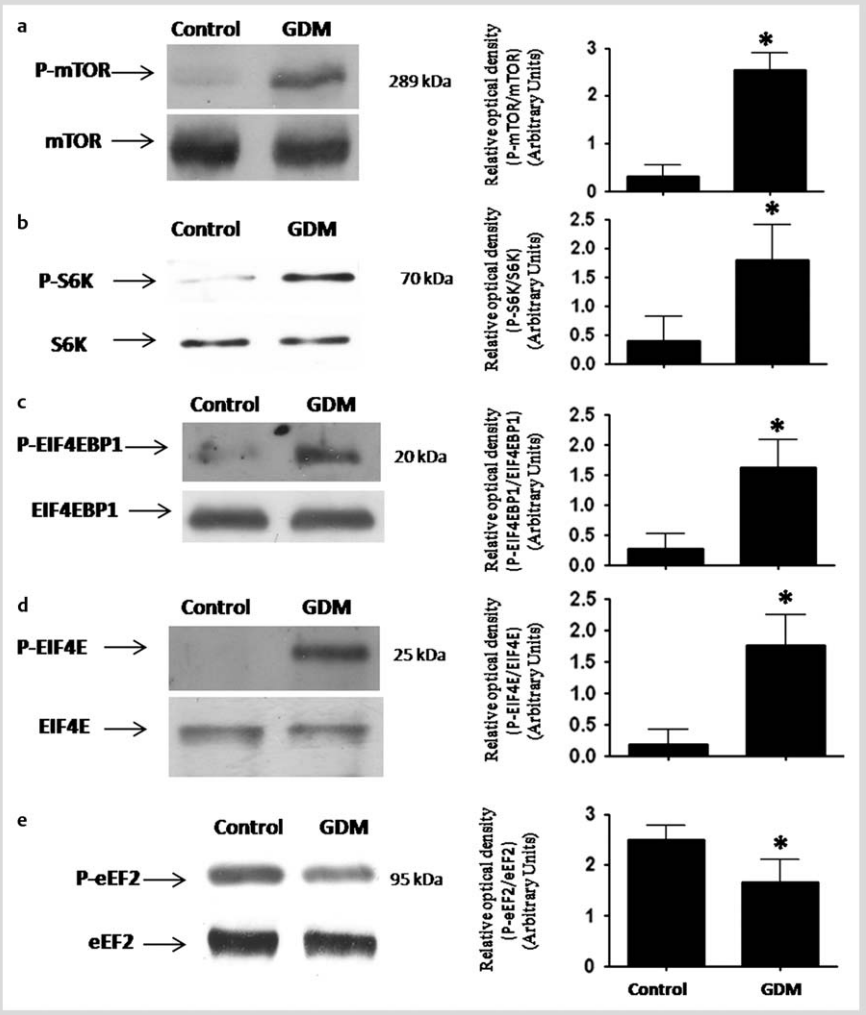

Fig. 3 Increased phosphorylation state of kinases implicated on protein synthesis signaling in placentas from gestational diabetes patients (GDM) compared with placentas from healthy control pregnancies. Placental samples were obtained from control placentas (n = 10) and placentas from GDM (n = 10) and were lysed and analyzed by immunoblot with antibodies that specifically recognize phosphorylated mTOR (Ser2448) **a** p70S6 kinase (Thr421/Ser424) **b** EIF4EBP1 (Thr37/Thr46) **c** EIF4E (Ser209) **d** and phosphorylated eEF2 (Thr56) **e** The amount of protein was controlled by immunoblotting the same membranes with monoclonal anti-β-tubulin, with polyclonal rabbit anti-total-S6K, with polyclonal rabbit anti-total-EIF4BP1 and anti-total-EIF4E. Results shown are from a representative experiment. Densitograms show mean ± SD from control and gestational diabetic samples, and expressed as arbitrary units. Statistical analyses were performed by 1-way ANOVA followed by Bonferroni's multiple comparison post hoc test. *$p < 0.05$ indicates significant differences from the control.

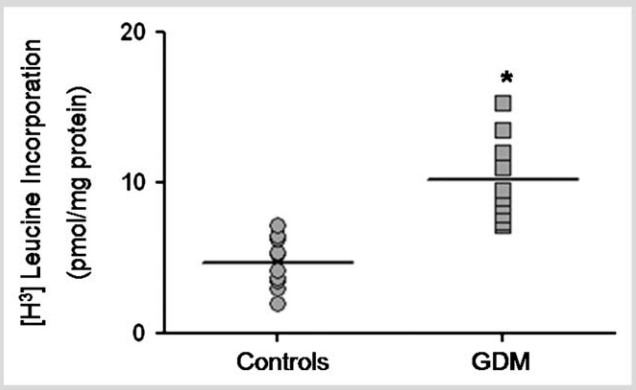

Fig. 4 Increased [^{3}H]-leucine incorporation in placentas from gestational diabetes mellitus (GDM) compared with placentas from healthy control pregnancies. Villous explants from control (n = 10) and gestational diabetic (n = 10) placentas were placed in tubes containing 1 ml HAMF-12 medium and were incubated with [^{3}H]-leucine (1 μCi/ml), for 4 h and then explants were processed as described in Materials and Methods. The protein synthesis was increased significantly in gestational diabetic placentas vs. control. Statistical analyses were performed by 1-way ANOVA followed by Bonferroni's multiple comparison post hoc test. *$p < 0.05$ indicates significant differences from the control. Results are means ± SD. Protein synthesis is estimated as absolute units (pmol/mg protein).

this regard remains to be clarified. We have also previously studied the signaling pathways activated by leptin receptor in different systems including placenta [9, 39, 41].

Numerous studies have reported that circulating leptin levels are significantly higher in pregnant women with GDM compared to healthy control women with uncomplicated pregnancy [3, 16]. We have demonstrated an increased phosphorylation state of different proteins implicated on the initiation stage of translation, and as a result, an increased protein synthesis rate in placentas from GDM. Thus, phosphorylation of mTOR, p70S6 kinase as well as phosphorylation of the EIF4EBP1, EIF4E are significantly increased in placentas from GDM compared to control placentas, while phosphorylation of the eEF2 is decreased in placentas from GDM compared to control placentas. These data suggest that this mechanism might contribute to the increased [^{3}H]-leucine incorporation into protein and placenta overgrowth observed in samples from GDM women.

This is the first time that the phosphorylation of kinases is reported to implicate in the activation of the translation machinery in placenta from GDM. Moreover, we have demonstrated that there is an increase on basal [^{3}H]-leucine incorporation into protein. This may be relevant for the increased placental growth and transport, trophoblast invasion and placental angiogenesis in GDM. In fact, it has been reported that under diabetic conditions, the placenta undergoes structural and functional changes, including overgrowth [3, 42]. Besides, a decrease in EIF4EBP1 phosphorylation has been recently found in intrauterine growth restriction of the fetus resulting from impaired placental devel-

opment [43]. Nevertheless, even though the activation of translation signaling was consistently found in GDM samples, it should be pointed out that this is not always correlated with an increase in infant birth weight. Therefore, other factors may account for the macrosomia in GDM in addition to the increased weight in placenta.

In conclusion, we have found that the placenta from pregnant women with GDM has upregulated the translation signaling machinery and this may be due, at least in part, by the increased leptin expression found in this placenta. Nevertheless, other factors may also contribute to the increase in translation signaling in GDM placenta. Finally, we have found an increase in basal [^{3}H]-leucine incorporation into protein that may mediate the increased growth previously observed in placenta from GDM. These data provide new molecular mechanisms that might explain, at least in part, the increased growth of placenta in gestational diabetes.

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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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