Molecular and Cellular Endocrinology 332 (2011) 221–227

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Leptin receptor activation increases Sam68 tyrosine phosphorylation and expression in human trophoblastic cells

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ARTICLE INFO

Article history:
Received 20 July 2010
Received in revised form
27 September 2010
Accepted 19 October 2010

Keywords:
Leptin receptor
Sam68
Signal transduction
Placenta

ABSTRACT

Leptin is produced in placenta where it has been found to be an important autocrine signal for trophoblastic growth during pregnancy, promoting antiapoptotic and trophic effects. Leptin receptor is present in trophoblastic cells and leptin may fully activate signaling. We have previously implicated the RNA-binding protein Sam68 in leptin signal transduction in immune cells. In the present work, we have studied the possible role of Sam68 in leptin receptor signaling in trophoblastic cells (JEG-3 cells). Leptin dose-dependently stimulated Sam68 phosphorylation in JEG-3 cells, as assessed by immunoprecipitation and immunoblot with anti-phosphotyrosine antibodies. As previously observed in other systems, tyrosine phosphorylation of Sam68 in response to leptin inhibits its RNA binding capacity. Besides, leptin stimulation dose-dependently increases *Sam68* expression in JEG-3 cells, as assessed by quantitative PCR. Consistently, the amount of Sam68 protein is increased after 24 h of leptin stimulation of trophoblastic cells. In order to study the possible role of Sam68 on leptin receptor synthesis, we employed antisense strategy to knockdown the expression of *Sam68*. We have found that a decrease in *Sam68* expression leads to a decrease in leptin receptor amount in JEG-3 cells, as assessed both by quantitative PCR and immunoblot.

These results strongly suggest the participation of Sam68 in leptin receptor signaling in human trophoblastic cells, and therefore, Sam68 may mediate some of the leptin effects in placenta.

1. Introduction

Leptin hormone, the protein product of the *LEP* gene (Zhang et al., 1994), is a multifunction hormone with a tertiary structure resembling that of members of the long-chain helical cytokine family (Flier, 1995). The pleiotropic nature of leptin is supported by the universal distribution of leptin receptor (LEPR), which shows structural similarity to the class I cytokine receptor family (Lee et al., 1996; Lollmann et al., 1997; Myers, 2004; Tartaglia et al., 1995; Tartaglia, 1997). Several alternatively spliced isoforms of leptin receptor have been identified, differing in the lengths of their cytoplasmic regions (Lee et al., 1996; Myers, 2004). Short isoforms ($LEPR_S$) are distributed in almost all peripheral tissues and show distinct signaling capabilities that include the activation of mitogen-activated protein kinase (MAPK) pathway (Yamashita et al., 1998; Bjorbaek et al., 1997). A long form ($LEPR_L$), which predominates in the hypothalamus, exhibits consensus aminoacid sequences to bind Janus tyrosine kinases (JAK/STAT) promoting the complete activation of this isoform (Ghilardi and Skoda, 1997).

Similar to other receptors of the family, the JAK (Janus kinases)/STAT (signal transducers and activators of transcription) pathway is one of the main signaling cascades activated by leptin in $LEPR_L$ (Ahima and Osei, 2004; Sahu, 2003; Sweeney, 2002). In this context, we have found constitutive association of JAK2 and JAK3 with $LEPR_L$ in human peripheral blood mononuclear cells, with the subsequent activation by tyrosine phosphorylation of STAT3 in both human peripheral blood mononuclear cells and trophoblastic cells (Sanchez-Margalet and Martin-Romero, 2001; Perez-Perez et al., 2008). Different pathways in addition to STATs are known to be involved in LEPR signaling. Thus, the mitogen-activated protein kinase (MAPK) family and the phosphatidylinositol 3-kinase (PI3K) signaling cascade become also activated by leptin, as we have previously found in peripheral blood mononuclear cells (Santos-Alvarez et al., 1999; Najib and Sanchez-Margalet, 2002a) and trophoblastic cells (Perez-Perez et al., 2008, 2009, 2010).

Leptin has been found to play a role in reproduction and pregnancy (Sagawa et al., 2002), particularly in placenta, where it may act as an autocrine hormone (Hauguel-de Mouzon et al., 2006).

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doi:10.1016/j.mce.2010.10.014

Expression of leptin and the different isoforms of leptin receptor are found in placenta (Li et al., 2004; Smith and Waddell, 2002). We have previously demonstrated that both human trophoblastic explants and human trophoblastic JEG-3 cells express the long isoform of leptin receptor, mediating the activation of signaling (Perez-Perez et al., 2008). Possible physiological effects of placenta-derived leptin include angiogenesis, growth, and immune modulation. In addition, leptin is a trophic and mitogenic factor for trophoblastic cells by virtue of inhibiting apoptosis and promoting growth and proliferation (Santos-Alvarez et al., 1999) via MAPK activation (Perez-Perez et al., 2008, 2009, 2010; Najib and Sanchez-Margalet, 2002a). In fact, we have reported that human chorionic gonadotropin (Maymo et al., 2009) and estradiol (Gambino et al., 2010) up-regulates leptin expression most likely by using the MAPK signal transduction pathway. Insulin has also growth promoting effects in JAr placental cells (Bifulco et al., 2002; Taylor and Shalloway, 1994) suggesting potential crosstalks between insulin and leptin signalling pathways.

Sam68 (Src-associated in mitosis) was originally identified as the only known substrate for Src family tyrosine kinases during mitosis (Taylor and Shalloway, 1994; Fumagalli et al., 1994) and is a member of the STAR (signal transduction and activation of RNA metabolism) family (Taylor and Shalloway, 1994). Based on the domain organization of the protein, Sam68 could connect signal transduction pathways and RNA metabolism. In fact, there are many data demonstrating that signaling mediated phosphorylation of Sam68 may alter its ability to bind RNA, thus providing a direct way to modify the RNA metabolism by the activation of signaling pathways (Coyle et al., 2003; Derry et al., 2000; Wang et al., 1995).

It has been proposed that Sam68 may provide a link between the PI3K and Ras pathways via the interaction of phosphorylated tyrosine and proline-rich motifs with proteins containing SH2 and SH3 domains respectively (Najib et al., 2005). Thus, Sam68 may be part of a multifunctional module required for the activation of overlapping pathways. In fact, Sam68 can be used by insulin and leptin receptors (Martin-Romero and Sanchez-Margalet, 2001; Sanchez-Margalet and Najib, 1999, 2001), in a similar way to other tyrosine kinase substrates that may function as adaptor proteins in different signaling pathways. In this sense, we have reported in human peripheral blood mononuclear cells, that human leptin time and dose dependently stimulates tyrosine phosphorylation of Sam68, promoting the association with STAT3, probably by interacting with the SH2 domains (Sanchez-Margalet and Martin-Romero, 2001).

However, the precise biological role of Sam68 is unknown, specially in trophoblastic cells, where it has not been studied. Even though Sam68 is a ubiquitous protein, its expression levels vary from tissue to tissue, with testis and ovary expressing higher levels of the protein (Sette et al., 2010). However there is limited information regarding expression of Sam68 in placenta and whether it may be recruited by the activated leptin receptor. The aim of this study was to investigate whether acute leptin administration affects phosphorylation (function) and expression of *Sam68* in JEG-3 trophoblastic cells and, in addition, whether downregulation of Sam68 may affect the expression of *LEPR*, as it has been previously reported in C2C12 muscle cells, in which, the positive regulation of *LEPR* mRNA by leptin is one of the early events triggered by the hormone, which depends on both Sam68 and ERK activity (Maroni et al., 2009).

2. Materials and methods

2.1. Antibodies and reagents

The recombinant human leptin was provided by Sigma (Sigma Chemical); Antibodies against leptin receptor (LEPR), the anti-Sam68 (C-20 and N-terminal), anti-tubulin from Santa Cruz Biotechnology; the anti-actin and anti-leptin antibodies from Sigma, the anti-phosphotyrosine (RC20) antibodies cross-linked to horseradish peroxidase from Transduction Laboratories. Agarose immobilized poly(U) and protein A-sepharose were purchased from Pharmacia. Oligonucleotide antisense of Sam68 sequence 5′-cagTGGCaCCTCtGGTgatg-3′ and standard control sequence 5′-catcACCaGAGGtGCCActg-3′ were purchased from Biomedal S.L.

2.2. Cell culture and treatments

The human choriocarcinoma cell line JEG-3 was generously provided by Susana Genti-Raimondi, (Universidad Nacional de Córdoba, Argentina). Cells were grown in DMEM-F12 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 g/mL streptomycin at 37 °C in 5% CO_2. JEG-3 cells were treated at different times in the absence or presence of different concentrations of leptin (0–10 nM), corresponding to the physiological range of leptin response previously tested in both normal and JEG-3 trophoblastic cells (Perez-Perez et al., 2008, 2009). The lisates were washed with cold PBS and solubilized for 30 min at 4 °C in lysis buffer containing 20 mM Tris, pH 8, 1% Nonidet P-40, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 0.4 mM sodium orthovanadate. Total protein levels were determined by the bicinchoninic acid method (Smith et al., 1985) using bovine serum albumin as standard.

2.3. Immunoprecipitation and poly(U)-binding assay

Soluble cellular lysates (0.5 mg of protein) were precleared with 50 μL of protein A–sepharose for 2 h at 4 °C by end-over-end rotation. The precleared cellular lysates were incubated with appropriate antibodies for 3 h at 4 °C (Sanchez-Margalet and Martin-Romero, 2001). Next, 50 μL of protein A–sepharose was added to immune complexes and incubation was continued for 2 h at 4 °C. For the poly(U)-binding assay, 60 μL of a 50% solution of agarose–poly(U) beads (Pharmacia) were added to 0.5 mg of proteins of cellular lysates. The mixture was rotated for 2 h at 4 °C (Martin-Romero and Sanchez-Margalet, 2001).

2.4. Western blotting analysis

The immunoprecipitates and poly(U)-affinity precipitates were washed three times with lysis buffer. We added 40 μL of SDS-stop buffer containing 100 mM of DTT to the immunoprecipitates followed by boiling for 5 min. The soluble supernatants were then resolved by 8–16% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes (Martin-Romero and Sanchez-Margalet, 2001; Sanchez-Margalet and Najib, 1999). The membranes were blocked with buffered saline–0.05% Tween 20 (PBST) containing 5% nonfat dry milk for 1 h at 23 °C. The blots were then incubated with primary antibody for 1 h, washed in PBST, and further incubated with secondary antibodies using horse radish peroxidase-linked anti-rabbit/anti-mouse immunoglobulin (Santa Cruz). Bound horseradish peroxidase was visualized by a highly sensitive chemiluminescence system (SuperSignal from Pierce), (Sanchez-Margalet et al., 2003a,b). The bands obtained in the blots were scanned and analyzed by the PCBAS2.0 program. Values are expressed as means ± SEM. Student's *t*-test was used for comparisons, with differences being considered statistically significant at $p < 0.05$.

2.5. Transfection experiments

For transfection experiments, JEG-3 cells were plated at a density of 2.5×10^5 cells/ml onto six-well dishes containing 2 mL of DMEM-F12 plus 10% FCS. Cells were incubated for 24 h. Medium was replaced, and transfection of cells was performed according to the standard liposome-mediated method optimized for JEG-3 cells (Zhang et al., 2007). Typically, 1.3 μg of the Sam68 antisense or sense (control) was transfected using 5 μL of LipofectAMINE (Life Technologies). The medium was replaced after 8 h with DMEM-F12 without FCS for 24 h. Transfection analysis was performed by duplicate in each of at least three independent experiments.

2.6. RNA extraction and quantitative real-time -PCR (qRT-PCR) assay

Abundance of Sam68, and *LEPR* mRNA was determined by qRT-PCR. Total RNA was extracted from JEG-3 using TRISURE reagent (Chomczynski, 1993). Concentration and purity of the isolated RNA were estimated spectrophotometrically at 260 and 280 nm. 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). qRT-PCR was performed using the following primers based on the sequences of the National Center for Biotechnology Information GenBank database: *LEPR*: forward, 5′-ATAGTTCAGTCACCAAGTGC-3′; reverse, 5′-GTCCTGGAGAACTCTGATGTCC-3′; cyclophilin: forward, 5′-CTTCCCCGATACTTCA-3′; reverse, 5′-TCTTGGTGCTACCTC-3′ and Sam68: forward, 5′-TTTGTGGGGAAGATTCTTGG-3′; reverse, 5′-GGGGGTCCAAAGACTTCAAT-3′. Quantitative RT-PCR Master Mix Reagent kit was obtained from Roche (Fast Start universal SYBR Green), and PCRs 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 and 1 min annealing and extension at 58 °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. For the treated samples, evaluation of $2^{-\Delta\Delta CT}$ indicates the fold change in gene expression, normalized to a housekeeping gene (cyclophilin), and relative to the untreated control.

2.7. Data analysis

Experiments were repeated separately at least three times to assure reproducible results. Results are expressed as means ± standard deviation (SD) in arbitrary units (AU). Arbitrary units were calculated as normalized band intensity in Western blot analysis. Statistical analysis was performed using the Graph Pad Prism computer program (GraphPad Software). Statistical significance was assessed by ANOVA followed by different post hoc tests, as indicated in each figure. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Effect of leptin treatment on Sam68 tyrosine-phosphorylation in JEG-3 trophoblastic cells

We have previously implicated Sam68 as a signaling molecule in the LEPR system in human monocytes and lymphocytes (Sanchez-Margalet et al., 2003a,b). We have now tested this possible implication of Sam68 in leptin signaling in trophoblastic cells. Using JEG-3 trophoblastic cells, we studied the tyrosine phosphorylation of Sam68 in response to leptin stimulation. Cells were stimulated with increasing concentrations of leptin (0–10 nM) for 5 min, and lysed. Total extracts from control and leptin-treated trophoblastic cells were immunoprecipitated with N-terminal anti-Sam68 antibodies, and analysed by means of immunoblotting with anti-phosphotyrosine antibodies. As shown in Fig. 1a, immunoprecipitated Sam68 was tyrosine phosphorylated when the cells were incubated with leptin for 5 min. Maximal phosphorylation of Sam68 was observed at 1 nM leptin, but 0.1 nM was enough to partially increase tyrosine-phosphorylation of Sam68, thus indicating that the leptin signaling involves this protein in trophoblastic cells. The phosphorylated band corresponded with an apparent molecular mass of about 68 kDa, consistent with previously reported data in different systems. The amount of protein immunoprecipitated in every lane was controlled by immunoblot with the same immunoprecipitating antibody.

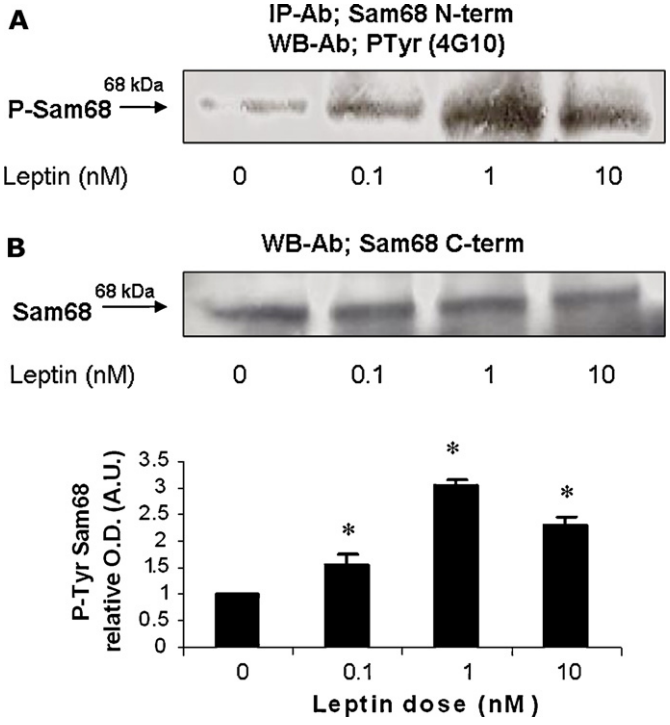

Fig. 1. Leptin stimulates Sam68 tyrosine-phosphorylation in JEG-3 trophoblastic cells. JEG-3 cells were incubated in the presence of increasing concentrations of leptin (0–10 nM) for 5 min. Cells were lysed and soluble clarified cell lysates were subjected to immunoprecipitation with anti-Sam68 antibodies. Immunoprecipitates were resolved by SDS-PAGE and Western blot with anti-phosphotyrosine antibodies (A). The same lysates were analyzed by immunoblot using the Sam68 antibodies to control the amount of protein in every lane (B). An experiment representative of three independent experiments is shown and densitograms with standard error are shown. *p < 0.05 versus control.

3.2. Tyrosine phosphorylation of Sam68 by leptin inhibits the binding to poly(U)

Since leptin stimulation promotes the tyrosine phosphorylation of Sam68 we sought to check the possible regulation of Sam68 association with RNA by leptin. Thus, we used immobilized poly(U) polymer, since it has been previously shown that Sam68 binds this polymeric RNA specifically (Taylor and Shalloway, 1994; Wang et al., 1995; Martin-Romero and Sanchez-Margalet, 2001).

As shown in Fig. 2, leptin stimulation increased the tyrosine phosphorylation level (A) and resulted in an inhibition of the binding efficiency of Sam68 to poly(U) in a dose-dependent manner (B). Thus, at 10 nM leptin almost no Sam68 is able to bind RNA.

3.3. Effect of leptin treatment on Sam68 mRNA expression in JEG-3 trophoblastic cells

Many different receptors positively modulate the expression of the signaling proteins. Thus, we have previously shown an increase of Sam68 expression in response to insulin in CHO-IR cells and adipocytes (Sanchez-Margalet et al., 2003a,b). In order to check the effect of leptin on Sam68 expression in trophoblastic cells, JEG-3 cells were incubated in the absence of serum with and without leptin (0–10 nM) for 16 h. We evaluated mRNA expression by means

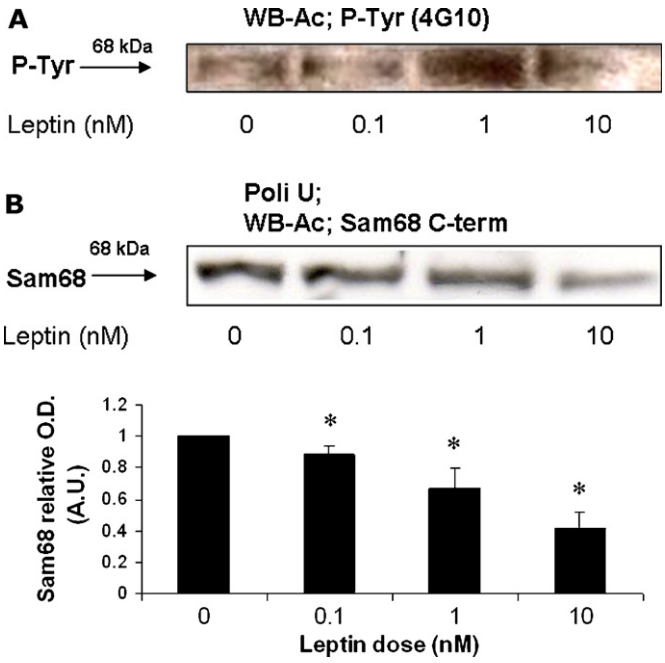

Fig. 2. Tyrosine phosphorylation of Sam68 by leptin stimulation inhibits the Sam68 binding to poly(U). JEG-3 cells were incubated in the presence of different increasing concentrations of leptin for 5 min and lysed. Precleared lysates were then incubated with Sepharose-coupled poly(U) beads for Sam68 affinity precipitation. Samples were then washed and analyzed by immunoblot with anti-phosphotyrosine (A) or anti-Sam68 (B) antibodies. A representative experiment of three is shown and densitograms are expressed as means with standard error *p < 0.05 versus control in Sam68 binded to poly(U).

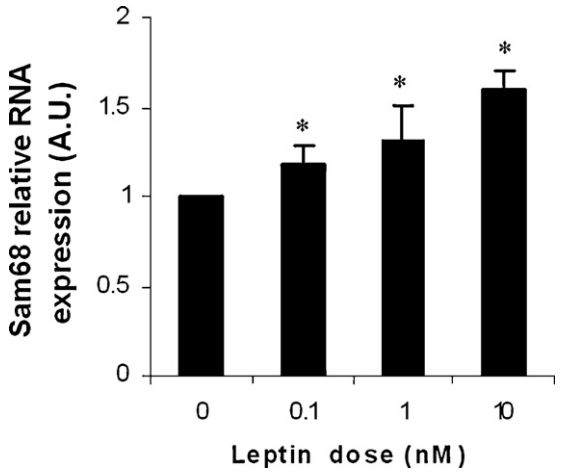

Fig. 3. Leptin up-regulates *Sam68* RNA expression level in JEG-3 trophoblastic cells. Cells were obtained and treated with increasing leptin dose (0–10 nM) during 16 h. Total RNA was extracted as previously described in Section 2. Leptin mRNA was quantified with qRT-PCR. Cyclophilin was used as internal standard. Results shown are from a representative experiment and are expressed as means ± SD for three independent experiments $^{*}p < 0.05$ versus control.

of qRT-PCR, using cyclophilin as an internal control for reaction efficiency. As shown in Fig. 3, leptin stimulation for 16 h increased *Sam68* expression about two times the basal levels in trophoblastic cells. Similar experiments were performed using Western blot to further analyze the leptin effect on *Sam68* expression by studying the amount of protein. As shown in Fig. 4, leptin dose-dependently stimulated *Sam68* expression by using antibodies that specifically recognize Sam68. The amount of total protein in every sample was controlled using anti-actin antibodies.

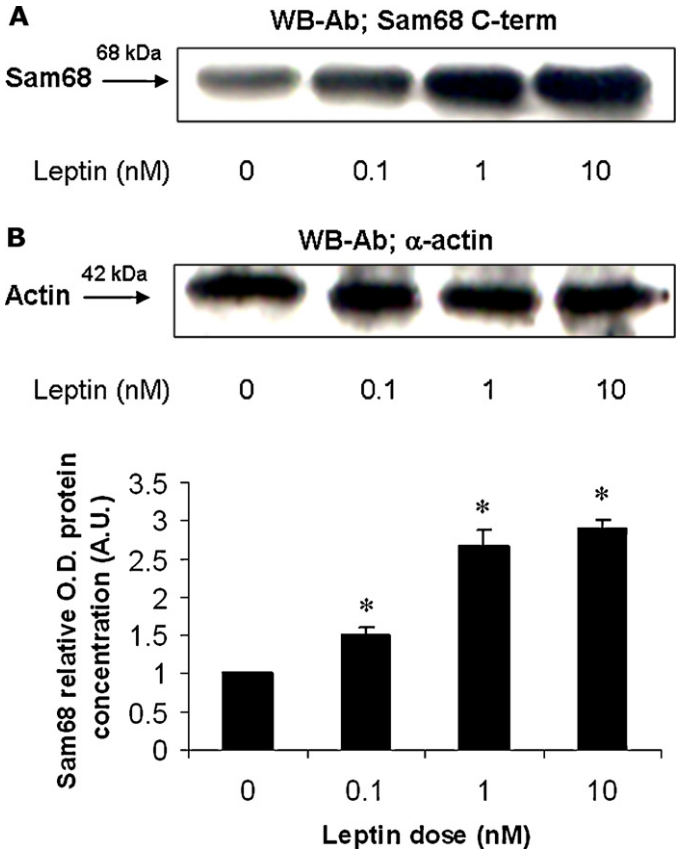

Fig. 4. Leptin increases Sam68 protein abundance in JEG-3 trophoblastic cells. JEG-3 cells were treated with increasing leptin doses (0–10 nM) during 16 h. Cells were then lysed and proteins were separated on SDS-PAGE gels. Sam68 expression was determined by Western blot analysis using specific anti-Sam68 antibodies. Loading controls were performed by immunoblotting the same membranes with anti-β-actin. Bands densitometry is shown in a representative experiment of three independent experiments with standard error, $^{*}p < 0.05$ versus control.

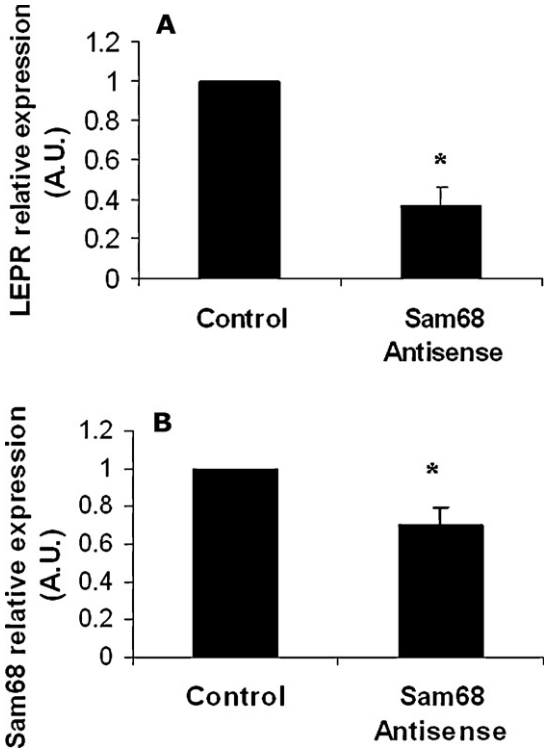

Fig. 5. Downregulation of Sam68 diminishes *LEPR* expression, determined by qRT-PCR in trophoblastic cells. JEG-3 cells were transfected with antisense of *Sam68* for 24 h. After transfection, cells were treated with leptin 10 nM during 12 h. Total RNA was extracted as indicated in Section 2 and cyclophilin was used as internal standard and quantified with qRT-PCR. (A) *LEPR* expression in the absence (control) or presence of *Sam68* antisense. (B) The downregulation of *Sam68* expression with the antisense was also checked by qRT-PCR. We show a representative experiment with the means ± SD of three independent experiments. $^{*}p < 0.05$ versus control.

3.4. Study of the effect of the downregulation of Sam68 on LEPR expression in trophoblastic cells

We next studied the relationship between Sam68 and LEPR in the opposite direction. Thus, we checked the effect of downregulation of Sam68 on *LEPR* expression. Thus, we reduced Sam68 levels by means of an antisense strategy, transfecting JEG-3 cells with an antisense oligonucleotide of *Sam68* for 36 h, using the sense oligonucleotide as a control. Then, we evaluated *LEPR* expression in both conditions by using qRT-PCR. As shown in Fig. 5, *LEPR* mRNA was significantly reduced when Sam68 was downregulated. The amount of *Sam68* mRNA was measured to check the downregulation method. Next, the amount of LEPR was measured by Western blot, using antibodies that specifically recognize LEPR. As shown in Fig. 6, Sam68 knock-down significantly diminished LEPR protein amount, thus providing consistent results to those obtained by qRT-PCR. The total amount of protein in every sample was controlled using anti-tubulin antibodies and the transfection control was checked by using anti-Sam68 antibodies.

4. Discussion

Previously published results suggested that leptin has a role in reproduction, particularly in the fetoplacental physiology. Tro-

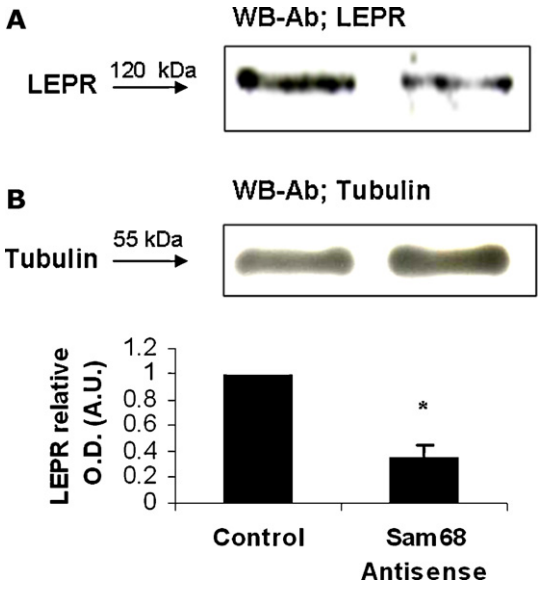

Fig. 6. Downregulation of Sam68 diminishes LEPR protein abundance, determined by Western blot, in trophoblastic cells. After JEG-3 transfection with Sam68 antisense or Sam68 sense as control (0) for 24 h, cells were treated with leptin 10 nM during 12 h. They were lysed and proteins were separated on SDS-PAGE gels. LEPR protein amount was determined by Western blot analysis. Total amount of protein in each sample was controlled by immunoblotting the same membranes with anti-tubulin antibodies. Bands densitometry for LEPR in Sam68 antisense-treated cells and control cells is shown in a representative experiment of three independent ones. Densitograms with standard error are shown. $^{*}p < 0.05$ versus control.

phoblastic cells produce leptin locally, and the autocrine action of leptin may be important for trophoblastic cell survival, growth and proliferation. Insulin has also growth promoting effects in JAr placental cells (Bifulco et al., 2002; Boileau et al., 2001) suggesting potential crosstalks between insulin and leptin signaling pathways. However, the molecular mechanisms underlying the effects of leptin on trophoblastic cells are not completely understood (Sagawa et al., 2002). To gain insight into the mechanisms of leptin action, we sought to identify cellular proteins that may be pertinent to its function. In this context, one possible candidate for integrating signal transduction pathways is Sam68, a member of the STAR protein family that not only contains KH domains, but also proline- and tyrosine-rich sequences that respectively interact with RNA and proteins (Lukong and Richard, 2003). Previous data from other authors indicated that Sam68 could play a role in proliferation and apoptosis in an RNA-binding dependent manner (Taylor et al., 2004). Sam68 may therefore play a role in RNA metabolism including alternative splicing (Gorla et al., 2006; Rajan et al., 2008). In addition, Sam68 has been found to function as an adaptor protein in signal transduction systems, including insulin receptor in hepatic and adipose cells (Najib et al., 2005; Sanchez-Margalet and Najib, 1999, 2001; Sanchez-Margalet et al., 2003a,b; Najib and Sanchez-Margalet, 2002b) as well as leptin receptor itself in mononuclear cells (Martin-Romero and Sanchez-Margalet, 2001; Martin-Romero et al., 2000). Moreover, we have found that tyrosine phosphorylation of Sam68 in response to leptin in mononuclear cells inhibits Sam68 ability to bind RNA (Martin-Romero and Sanchez-Margalet, 2001). In this context, a role for Sam68 linking signal transduction with RNA metabolism has been proposed (Najib et al., 2005; Lukong and Richard, 2003). However, there are no data regarding the role of Sam68 in leptin signaling in placenta, or the regulation of *Sam68* expression in placenta in response to leptin or any other signal. That is why, in the present study, we have investigated the possible participation of Sam68 in leptin receptor signaling and the regulation of *Sam68* expression by leptin in JEG-3 human choriocarcinoma cells. JEG-3 cells express both leptin and its receptor (Magarinos et al., 2007) and maintain many characteristics of human trophoblast cells. They have been widely used to study placental cellular signaling (Perez-Perez et al., 2008; Standley and Standley, 2002; Zygmunt et al., 1998) showing comparable results.

In the present work, we have first reported that leptin indeed upregulates phosphorylation of Sam68 in placental cells. Moreover, this effect is dependent on the dose, in a similar way to that previously observed in other systems in response to insulin (Sanchez-Margalet and Najib, 1999) or leptin (Martin-Romero and Sanchez-Margalet, 2001). As a result of this tyrosine phophorylation of Sam68 in response to leptin, we have found that the RNA binding capacity of Sam68 is diminished in trophoblastic JEG-3 cells. This result is consistent with previous reported data demonstrating that tyrosine phosphorylation of Sam68 by kinases of the Src (Wang et al., 1995) and Brk (Derry et al., 2000; Haegebarth et al., 2004) family negatively regulates its RNA binding function. More precisely, we have found that leptin stimulation of mononuclear cells promotes tyrosine phosphorylation of Sam68 mediating an inhibition on the Sam68 RNA binding activity, determined as binding to poly(U) (Martin-Romero and Sanchez-Margalet, 2001). Thus, Sam68 may play a role in the transduction of the leptin signal from the plasma membrane to RNA metabolism via a rapid mechanism mediated by phosphorylation. Therefore, the leptin-stimulated tyrosine phosphorylation of Sam68 and its subsequent inhibition on RNA binding capacity might contribute to the leptin effects observed in trophoblastic cells. In fact, we have previously found that Sam68 participates in leptin and insulin signaling, showing dose-dependent phosphorylation and interaction with different signaling proteins. Thus, in different systems, Sam68 has been shown participate in different pathways including JAK-STAT (Sanchez-Margalet and Martin-Romero, 2001), MAPK and PI3K (Martin-Romero and Sanchez-Margalet, 2001; Sanchez-Margalet and Najib, 1999, 2001; Najib and Sanchez-Margalet, 2002b). Nevertheless, the possible participation of Sam68 in these signaling pathways in trophoblastic cells remains to be investigated.

Other receptor systems have been found to positively modulate the expression of kinases substrates and other molecules recruited to the receptor signaling upon stimulation with the specific ligand (Montezano et al., 2010). In this context, we have also found that leptin stimulation of JEG-3 cells increases the expression of *Sam68* in a similar way to that observed in response to insulin (Sanchez-Margalet et al., 2003a,b).

To further investigate the relationship between Sam68 and LEPR in trophoblastic cells, by using Sam68 antisense strategy, we studied the effect of Sam68 on *LEPR* mRNA expression levels, as well as the amount of LEPR protein. We have found that Sam68 does play a role in the *LEPR* expression in trophoblastic cells, as we clearly observed a significant inhibition of *LEPR* expression after 36 h of transfection with *Sam68* antisense oligonucleotide, as assessed both by qRT-PCR and Western blot analysis. Even though the role of Sam68 mediating the increase in *LEPR* expression has been recently reported in muscle cell line (Maroni et al., 2009), this is the first time that Sam68 has been implicated in the expression of *LEPR* in JEG-3 cells, where it also functions as a leptin signaling protein. Therefore, Sam68 may positively regulate *LEPR* expression in a similar way to that observed in other signaling proteins and receptor systems (Edvinsson, 2009; Rice and Garner, 1999). Thus, we have provided some evidence for the possible role of Sam68 in leptin signaling produced by JEG-3 cells and that may be extensible for *in vivo* trophoblastic cells, describing its effect on the positive regulation of *LEPR* expression.

We have previously demonstrated the role of leptin as a trophic factor for placenta promoting growth and preventing apoptosis of human trophoblastic cells (Perez-Perez et al., 2008; Magarinos

et al., 2007). Since Sam68 has been found to be involved in cell proliferation and survival (Barlat et al., 1997; Li et al., 2002; Busa et al., 2007), the recruitment of Sam68 to LEPR signaling in JEG-3 cells may be useful to mediate these effects of leptin on human placenta. However, this hypothesis should be confirmed in future studies by using a more physiological system such as trophoblastic explants from healthy donors.

In conclusion, leptin dose-dependently stimulates expression and phosphorylation of Sam68 in JEG-3 trophoblastic cells, and we have found that down-regulation of Sam68, by antisense strategy, reduces *LEPR* expression levels, suggesting a link between leptin signaling and Sam68, probably contributing to signal transduction complexes in JEG-3 trophoblastic cells.

Acknowledgements

This work was supported by a Grant from the Instituto de Salud Carlos III (ISCIII PS09/00119). Flora Sánchez-Jiménez and Antonio Pérez-Pérez are research fellow supported by the Instituto de Salud Carlos III (Contrato Post-formación especializada, Río Hortega), ISCIII CM07/00025, Spain. We are grateful to Beatriz Puche for providing language help.

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