

The SHP-1 protein tyrosine phosphatase negatively modulates Akt signaling in the ghrelin/GHSR1a system

Maria Lodeiro^{a,b}, Begoña O. Alén^{a,b}, Carlos S. Mosteiro^{a,b}, Daniel Beiroa^{b,c}, Rubén Nogueiras^{b,c}, Marily Theodoropoulou^d, María Pardo^{a,b}, Rosalía Gallego^e, Yolanda Pazos^{a,b}, Felipe F. Casanueva^{a,b,f}, and Jesus P. Camiña^{a,b}

^aÁrea de Endocrinología Molecular y Celular, Instituto de Investigación Sanitaria de Santiago, Hospital Clínico Universitario de Santiago, Servicio Gallego de Salud, 15706 Santiago de Compostela, Spain; ^bCIBER Fisiopatología de la Obesidad y Nutrición, Spain; ^cDepartamento de Fisiología, Universidad de Santiago de Compostela, 15705 Santiago de Compostela, Spain; ^dDepartment of Endocrinology, Max Planck Institute of Psychiatry, Munich, Germany; ^eDepartamento de Ciencias Morfológicas and ^fDepartamento de Medicina, Universidad de Santiago de Compostela, 15705 Santiago de Compostela, Spain

ABSTRACT The aim of the present study was to identify the signaling mechanism(s) responsible for the modulation of growth hormone secretagogue receptor type 1a (GHSR1a)-associated Akt activity. Ghrelin leads to the activation of Akt through the interplay of distinct signaling mechanisms: an early $G_{i/o}$ protein-dependent pathway and a late pathway mediated by β -arrestins. We found that the Src homology 2-containing protein tyrosine phosphatase (SHP-1) was an essential molecule in both $G_{i/o}$ protein-dependent and β -arrestin-mediated pathways. More specifically, the role of SHP-1 in the $G_{i/o}$ protein-dependent pathway was demonstrated by the fact that the overexpression of a catalytically defective SHP-1 augments tyrosine phosphorylation of the PI3K regulatory subunit p85, leading to an increase in the phosphorylation of cSrc and phosphoinositide-dependent protein kinase 1, and finally activating Akt. The presence of SHP-1 in the β -arrestin-scaffolded complex and its attenuating effect on the cSrc and Akt activities verified that SHP-1 regulates not only the $G_{i/o}$ protein-dependent pathway but also the β -arrestin-mediated pathway. Assays performed in preadipocyte and adipocyte 3T3-L1 cells showed SHP-1 expression. According to our results in HEK-GHSR1a cells, ghrelin stimulated SHP-1 phosphorylation in 3T3-L1 cells. The increase in ghrelin-induced Akt activity was enhanced by small interfering RNA of SHP-1 in preadipocyte 3T3-L1 cells. These results were reproduced in white adipose tissue obtained from mice, in which SHP-1 exhibited higher expression in omental than in subcutaneous tissue. Furthermore, this pattern of expression was inverted in mice fed a high-fat diet, suggesting a role for SHP-1 in controlling ghrelin sensitivity in adipose tissue. Indeed, SHP-1 deficiency was associated with augmented ghrelin-evoked Akt phosphorylation in omental tissue, as well as decreased phosphorylation under overexpression of SHP-1 in subcutaneous tissue. These findings showed a novel role for SHP-1 in the regulation of Akt activity through the modulation of the ghrelin/GHSR1a system signaling.

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Address correspondence to: Jesus P. Camiña (jesus.perez@usc.es; jesus.perez.camina@sergas.es).

Abbreviations used: GHSR1a, growth hormone secretagogue receptor 1a; GPCR, G protein-coupled receptor family; HFD, high-fat diet; HM, hydrophobic motif; IHC, immunohistochemistry; mTORC2, mammalian target of rapamycin complex 2; PDK1, phosphoinositide-dependent protein kinase 1; PI3K, phosphatidylinositol

3-kinase; pPDK1(S241), PDK1 A-loop(S241) phosphorylation; PTP, protein tyrosine phosphatase; SHP-1, Src homology-2 domain-containing phosphatase-1; siRNA, small interfering RNA; WAT, white adipose tissue.

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INTRODUCTION

The main physiological functions of ghrelin as a growth hormone secretagogue, an orexigenic peptide, and a long-term regulator of energy homeostasis are critically regulated through the growth hormone secretagogue receptor type 1a (GHSR1a), a receptor belonging to the G protein-coupled receptor family (GPCR). This receptor has traditionally been linked to heterotrimeric G proteins and downstream second-messenger molecules (Castañeda *et al.*, 2010). However, for a number of years, biochemical and cellular studies have suggested that some aspects of GHSR1a-mediated signaling occur in parallel to G-protein activation via multifunctional adaptor proteins known as β -arrestins (Camiña *et al.*, 2007; Lodeiro *et al.*, 2009). The β -arrestins constitute a small family of gene products originally identified as molecules that desensitize GPCR signaling (Kovacs *et al.*, 2009). On ghrelin binding, GHSR1a undergoes conformational changes that result in the activation of heterotrimeric G proteins. This step is followed by receptor phosphorylation that leads to β -arrestin recruitment, which then sterically interdicts further coupling to G proteins, leading to receptor desensitization. β -Arrestins also mediate endocytosis of GHSR1a, leading to receptor recycling (Camiña *et al.*, 2004; Holliday *et al.*, 2007). In addition to their role in terminating G-protein signaling, recent studies demonstrated that β -arrestins also function as scaffold molecules for ghrelin-activated signaling networks, such as ERK1/2 (Camiña *et al.*, 2007) and Akt/protein kinase B (Lodeiro *et al.*, 2009), which are defined as "signalosomes" (Kovacs *et al.*, 2004). The signaling mechanisms that underlie the activation of the mitogenic ERK growth response by the GHSR1a result from both classical G protein-regulated effectors and β -arrestin-dependent ERK1/2 recruitment (Camiña *et al.*, 2007). One pathway is $G_{i/o}$ -dependent, and it is mediated by phosphatidylinositol 3-kinase (PI3K), protein kinase C ϵ , and the nonreceptor tyrosine kinase, cSrc. The second pathway is $G_{q/11}$ -dependent and involves the activation of protein kinase C α/β and cSrc. A third pathway involves the recruitment of GHSR1a, cSrc, Raf-1, and ERK1/2 into a β -arrestin-scaffolded complex. This complex persists for extended periods, and it is probably determined by its stability.

Over the past decade, the serine/threonine kinase Akt emerged as a critical signaling node in the regulation of metabolism, apoptosis, transcription, and the cell cycle, placing it at the center of human physiology and disease (Manning and Cantley, 2007). The GHSR1a is implicated in Akt signaling through a complex interplay of distinct signaling mechanisms: an early $G_{i/o}$ protein-dependent pathway and a late pathway mediated by β -arrestins. The starting point is the $G_{i/o}$ protein-dependent PI3K activation that leads to the membrane recruitment of Akt, which is phosphorylated at Y by cSrc, with subsequent phosphorylation at the A loop (T308) and the hydrophobic motif (HM; S473) by phosphoinositide-dependent protein kinase 1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2), respectively. Once the receptor is activated, a second signaling pathway mediated by β -arrestins 1 and 2 is activated, involving the recruitment of cSrc and Akt. This β -arrestin-scaffolded complex leads to full activation of Akt through PDK1 and mTORC2, which are not physically associated to the complex. Both G protein- and β -arrestin-mediated pathways are temporally different and act in a sequential way where β -arrestin-scaffolded signaling complex persists for prolonged periods (Lodeiro *et al.*, 2009).

The present study focuses on the mechanisms regulating GHSR1a-associated Akt activity, and emphasizes the implication of reversible tyrosine phosphorylation of upstream targets of Akt through the cytoplasmic protein Src homology-2 domain-containing phosphatase-1 (SHP-1), a protein tyrosine phosphatase (PTP). SHP-1 is a well-known inhibitor of activation-promoting signaling

cascades in hematopoietic cells (Tonks, 2006; Lorenz, 2009) and insulin tissue targets (Dubois *et al.*, 2006), but its potential role in ghrelin/GHSR1a system is unknown. These molecular mechanisms were investigated in HEK 293 cells stably expressing the GHSR1a (HEK-GHSR1a) as a model system to follow the interaction of specific proteins and the roles, if any, played by G proteins and β -arrestins. Results were confirmed in 3T3-L1 cells, as both preadipocyte and adipocytes cells, and potential physiological repercussions based on ghrelin signaling to Akt were studied in white adipose tissue (WAT) obtained from mice fed a high-fat diet (HFD).

RESULTS

Figure 1A shows that SHP-1 activity, measured as SHP-1 phosphorylation in the C-terminal Y536 residue (pSHP-1(Y536); Zhang *et al.*, 2003), reached maximal levels within 10 min of ghrelin stimulation (100 nM), staying at this level for at least 60 min in HEK-GHSR1a cells. The role of SHP-1 on ghrelin-induced phosphorylation of the Akt HM (S473; pAkt(S473)) and the A loop (T308; pAkt(T308)) was evaluated by transient transfection of dominant-negative catalytically inactive SHP-1 (SHP-1dn). Ghrelin induced Akt phosphorylation at both pAkt(S473) and pAkt(T308) after 5 min of treatment (Figure 1B). In cells overexpressing SHP-1dn, the ghrelin-induced pAkt(S473) and pAkt(T308) levels were significantly higher after 5 and 10 min of treatment (Figure 1B). This effect was more prominent in the case of pAkt(T308) and remained up to 20 min after ghrelin treatment, while no difference was observed in ghrelin-induced pAkt(S473) levels in SHP-1dn-transfected cells and control cells at this time point.

The role of SHP-1 on PI3K, cSrc, and PDK1, key nodes for Akt A loop (T308) phosphorylation, was evaluated in SHP-1dn-overexpressing cells. SHP1 associates with PI3K and dephosphorylates the kinase at Y688, a residue that maps within the p85 subunit (Cuevas *et al.*, 2001). Control and SHP-1dn-transfected were evaluated for p85 Y phosphorylation under ghrelin stimulation (100 nM, 5 min). Results from p85 immunoprecipitation analysis revealed the level of p85 Y phosphorylation induced by ghrelin (100 nM, 5 min) to be markedly higher in SHP-1dn-transfected cells compared with control cells (Figure 1C). cSrc is regulated by GHSR1a through a $G_{i/o}$ -dependent pathway involving PI3K and PKC ϵ by phosphorylating the Y416 residue within the kinase domain (pcSrc(Y416)) which is important for its tyrosine kinase activity (Lodeiro *et al.*, 2009). Results from immunoblot analysis revealed markedly higher pcSrc(Y416) levels in cells overexpressing SHP-1dn than in control cells under ghrelin stimulation (100 nM; Figure 1D), providing evidence that cSrc activity is regulated by Y416 dephosphorylation by SHP-1. The effect of SHP-1 on PDK1 A-loop(S241) phosphorylation (pPDK1(S241)) was next evaluated. Consequently, overexpression of SHP-1dn increased ghrelin-induced pPDK1(S241) levels (Figure 1D). These data indicated that ghrelin-induced p85, cSrc, PDK1, and, subsequently, Akt A-loop (T308) phosphorylation depended on tyrosine phosphorylation events restrained by SHP-1.

The finding that Akt phosphorylation on HM(S473) was also affected by SHP-1dn led us to analyze the effect of this tyrosine phosphatase on mTORC2 (mTORC2: Rictor, mLST8, mSin1 variants, Protor/PRR5 and mTOR kinase). While the catalytic activity of mTORC2 is stimulated by ghrelin (Lodeiro *et al.*, 2009), the molecular mechanisms regulating its cellular activities remain unknown. Therefore mTORC2 was immunoprecipitated with antibody to Rictor from lysates of ghrelin-treated cells (100 nM, 5 min), transfected with SHP-1dn or controls, and subjected to immunoblot with anti-phosphotyrosine. However, the lack of differences between ghrelin-treated and control cells discarded

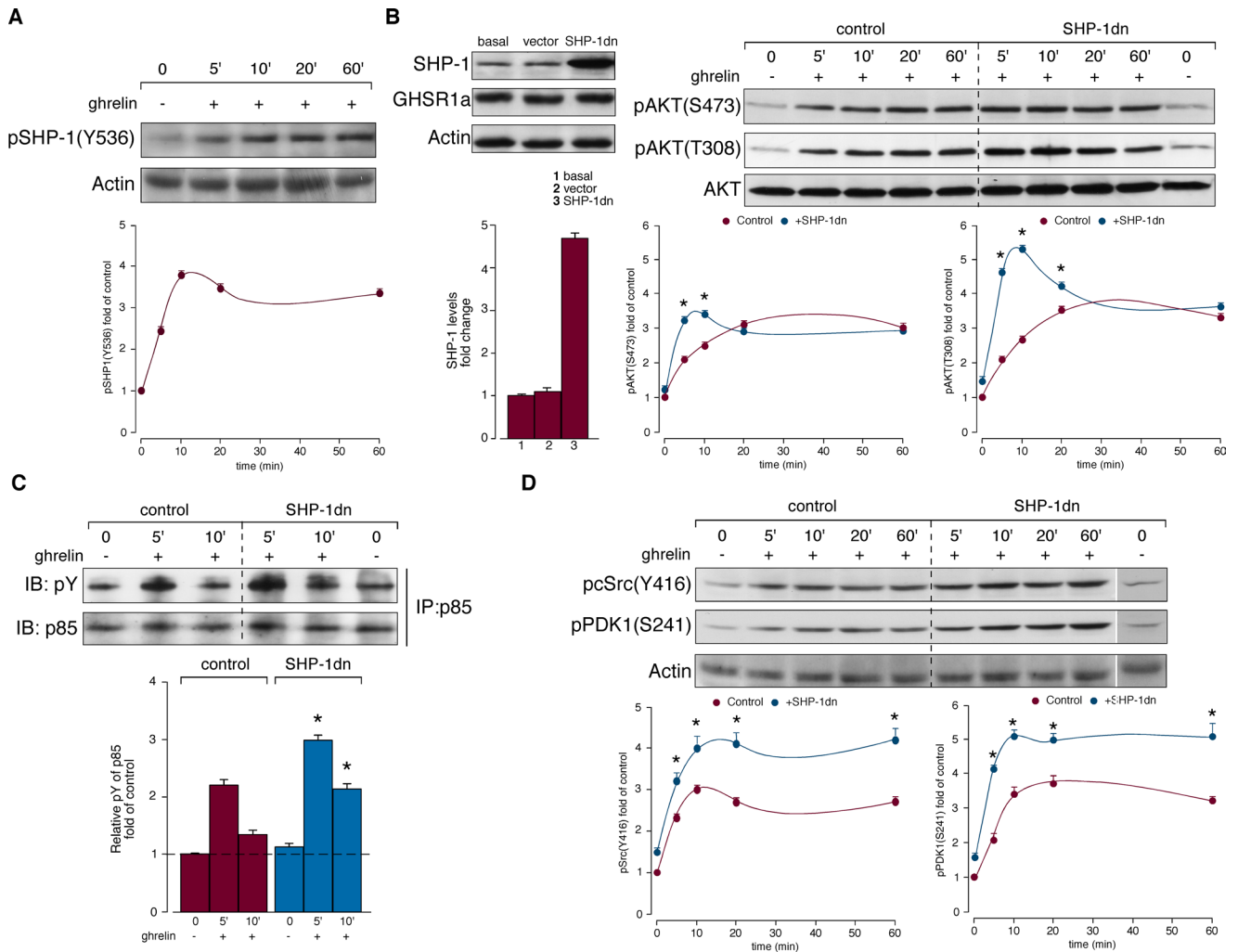


FIGURE 1: Effects of overexpression of dominant-negative SHP-1 (SHP-1dn) on ghrelin-activated Akt signaling cascade. (A) Time course of the effect of ghrelin (100 nM) on pSHP-1(Y536). (B) Ghrelin-induced Akt HM (S473) and A-loop (T308) phosphorylation in the absence (vector control: control) or presence of SHP-1dn. Effect of vector control (vector) and SHP-1dn on SHP-1 and GHSR1a expression in HEK-GHSR1a cells is shown on the left. (C) Basal and ghrelin-induced Y phosphorylation and expression of p85 regulatory subunit of PI3K in vector control and SHP-1dn-transfected HEK-GHSR1a cells. IP, immunoprecipitation; IB: immunoblot. (D) Ghrelin-induced pcSrc(Y416) and pPDK1(S241) measured in control (vector control) and SHP-1dn-transfected HEK-GHSR1a cells. Immunoblots in (A), (B), (C), and (D) are representative of three independent experiments. Data are expressed as mean \pm SE obtained from intensity scans of three independent experiments. * $p < 0.05$ vs. control values.

the modulation of SHP-1 on mTORC2 activity (Supplemental Figure S1).

Once the GHSR1a is activated, a second signaling pathway is mediated by β -arrestins 1 and 2, involving the recruitment of at least the β -arrestins, cSrc, and Akt. Therefore the effects of small interfering RNA (siRNA)-mediated suppression of β -arrestins 1 and 2 expression were examined on the kinetics of pSHP-1(Y536) following ghrelin stimulation. siRNA experiments targeting β -arrestin 1 or β -arrestin 2 reduced their expression by $50 \pm 2\%$ and $65 \pm 4\%$, respectively (Figure 2A). β -arrestin 1 and β -arrestin 2 siRNAs decreased ghrelin-activated pSHP-1(Y536) with respect to siRNA control, with maximal inhibitory effect at the later time points ($62 \pm 3\%$ and $51 \pm 5\%$ at 10 min for β -arrestin 2 and β -arrestin 1 siRNA, respectively [Figure 2A]). To further validate the connection between β -arrestin-scaffolded complex and SHP-1, immunoprecipitations of β -arrestins were assayed at 10 min corresponding to the maximum of β -arrestin/GHS-1a complex formation (Camiña *et al.*, 2007; Lodeiro *et al.*,

2009) in SHP-1dn-transfected cells. Immunoprecipitation of β -arrestin 1 or β -arrestin 2 revealed a physical association between β -arrestins and pSHP-1(Y536), which increased substantially under ghrelin stimulation to control cells (Figure 2B). A 10-min ghrelin stimulation of the cells overexpressing SHP-1dn effectively enhanced the ghrelin response on β -arrestin-associated pAkt(S473) and pcSrc(Y416) phosphorylation, verifying that SHP-1 regulates not only the $G_{i/o}$ protein-dependent pathway but also the pathway mediated by β -arrestin-associated complex (Figure 2B).

Src is able to phosphorylate SHP-1 on Y536 *in vitro*, leading to an increase in the activity of the phosphatase (Somani *et al.*, 1997; Frank *et al.*, 2004). To investigate this possibility, ghrelin-stimulated cells with siRNA targeting cSrc were evaluated for SHP-1 activity. As indicated in Figure 2C, siRNA experiments targeting cSrc reduced its expression by $54 \pm 2\%$. cSrc siRNA decreased ghrelin-activated SHP-1(Y536) phosphorylation ($73 \pm 2\%$ at 10-min ghrelin stimulation with respect to siRNA control) for all times

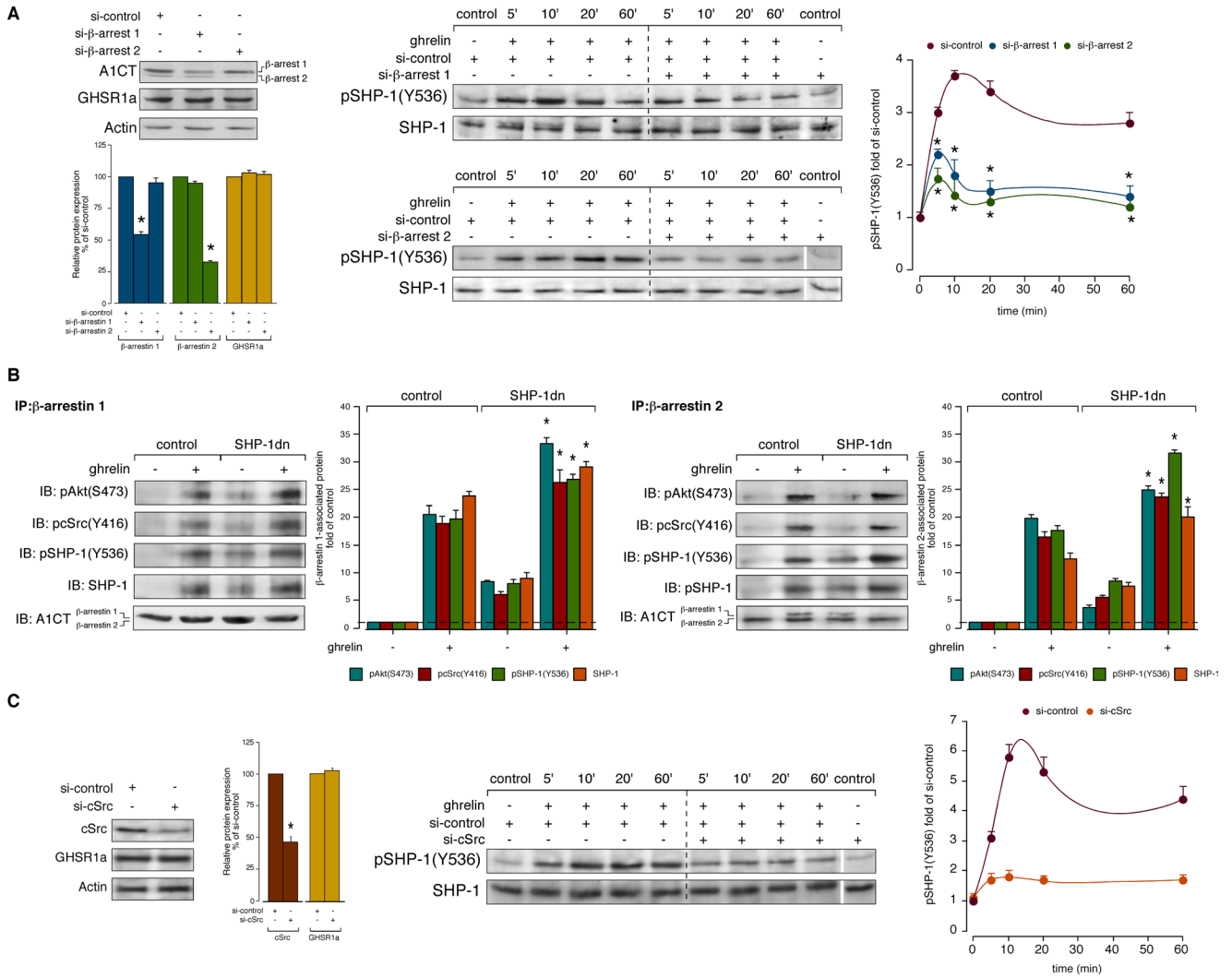


FIGURE 2: Functional relation between GHSR1a-associated, β-arrestin-scaffolded complex and SHP-1 activity.

(A) Ghrelin-induced pSHP-1(Y536) in the absence (–) or presence (+) of β-arrestin 1 or 2 siRNA. Left, the immunoblot analysis of β-arrestin and GHSR1a expressions in control siRNA and β-arrestin 1 or 2 siRNA-transfected HEK-GHSR1a cells. (B) Effect of ghrelin (100 nM, 10 min) on the assembly of complexes containing β-arrestins 1 (left) and 2 (right), pAkt(S473), pcSrc(Y416), and pSHP-1(Y536) measured in control (vector control) and SHP-1dn-transfected HEK-GHSR1a cells. IP, immunoprecipitation; IB: immunoblot. (C) Ghrelin-induced pSHP-1(Y536) in the absence (–) or presence (+) of cSrc siRNA. Left, shows the immunoblot analysis of cSrc and GHSR1a expressions in control siRNA and cSrc siRNA-transfected HEK-GHSR1a cells. Immunoblots in (A), (B), and (C) are representative of three independent experiments. Data are expressed as mean ± SE obtained from intensity scans of three independent experiments. *p < 0.05 vs. control values.

tested, suggesting that SHP-1 activation is almost entirely mediated by cSrc.

SHP-1 is largely expressed in hematopoietic cells, but has also been detected in liver and skeletal muscle (Plutzky *et al.*, 1992; Norris *et al.*, 1997; Dubois *et al.*, 2006). Results of immunoblot analysis demonstrated moderate SHP-1 expression in 3T3-L1 cells and WAT compared with liver or spleen (Figure 3A). In preadipocyte and adipocyte 3T3-L1 cells, SHP-1 was also detected by immunohistochemistry (IHC) in the cytoplasm, with enrichment in the perinuclear region (Figure 3B). Determination of pSHP-1(Y536) in ghrelin-treated (100 nM) 3T3-L1 cells confirmed the stimulatory ghrelin effect on SHP-1 activity in preadipocytes and adipocytes (Figure 3C). Ghrelin-stimulated 3T3-L1 preadipocyte cells with siRNA targeting SHP-1 were evaluated for Akt activity. As indicated in Figure 3D, siRNA experiments targeting SHP-1 reduced its ex-

pression by 58 ± 1%. SHP-1 siRNA increased ghrelin-activated pAkt(T308) (91 ± 3% at 10 min of ghrelin stimulation) and pAkt(S473) (36 ± 1% at 10 min of ghrelin stimulation) with respect to siRNA control. SHP-1 was detected immunohistochemically in omental and subcutaneous WAT obtained from mice under normal chow (control) and HFD (Figure 4A). pSHP-1(Y536) was significantly increased in ghrelin-stimulated omental and subcutaneous WAT explants from control mice in vitro (100 nM, 10 min). This activation was higher in omental than in subcutaneous tissue, correlating with a higher expression for the SHP-1 in the omental tissue (Figure 4B). Similarly, pSHP-1(Y536) was significantly increased in ghrelin-stimulated omental and subcutaneous WAT explants from HFD mice in vitro (100 nM, 10 min). This activation, however, was higher in subcutaneous than in omental tissue, correlating with a higher expression for the SHP-1 in the subcutaneous tissue from HFD mice

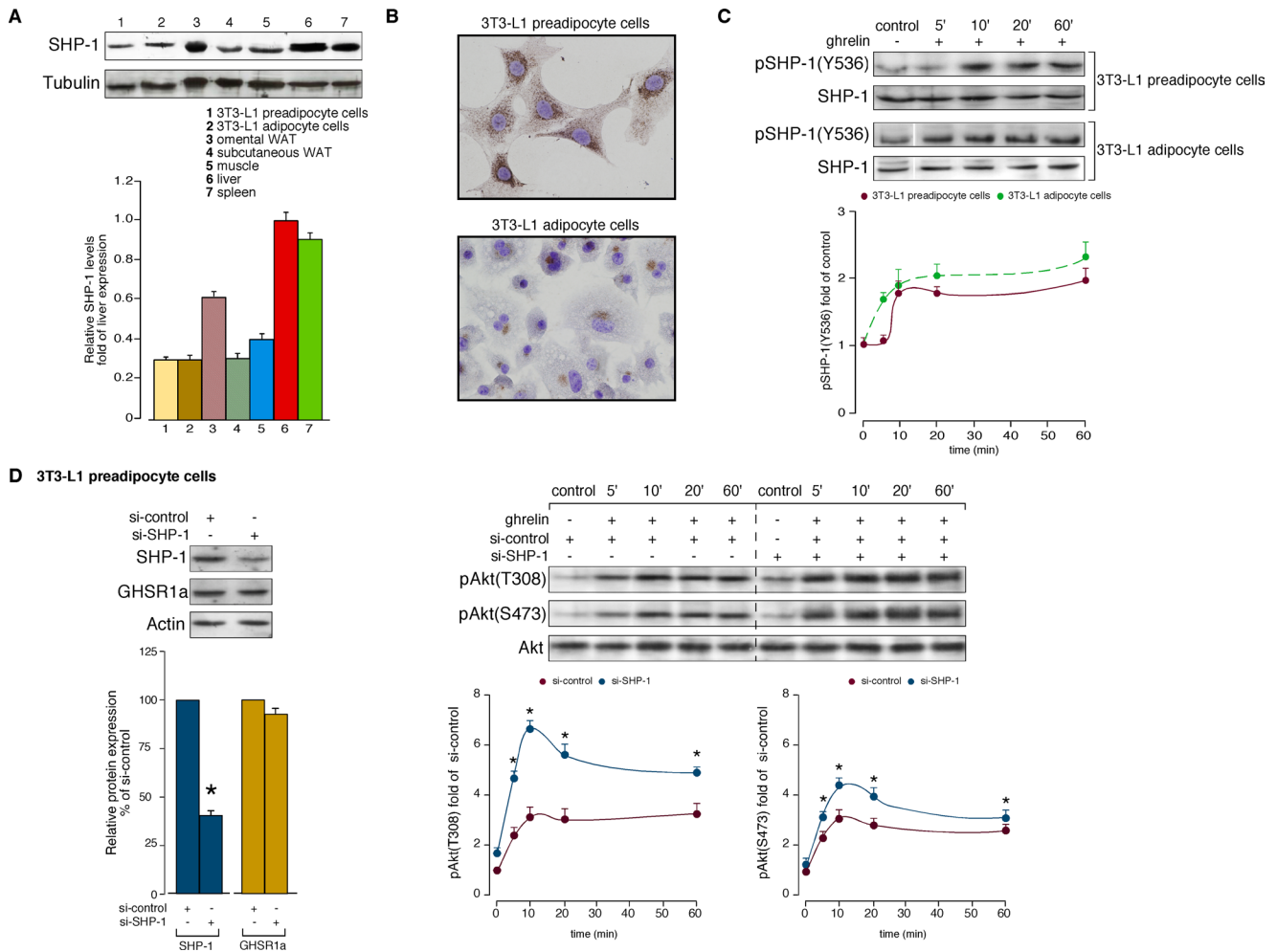


FIGURE 3: Role of SHP-1 in 3T3-L1. (A) Immunoblot analysis of SHP-1 in extracts from 3T3-L1 cells, WAT, skeletal muscle, liver, and spleen of control mice ($n = 10$). Same amount of protein ($40 \mu\text{g}$) was used for this analysis. Results were expressed as fold increase of SHP-1 level in liver. (B) Immunocytochemical detection of SHP-1 in 3T3-L1 preadipocyte and adipocyte cells (objective magnification: $40\times$). SHP-1 immunostaining was mainly concentrated in perinuclear compartments in both 3T3-L1 cells. (C) Time course of the effect of ghrelin (100 nM) on pSHP-1(Y536) in 3T3-L1 preadipocyte and adipocyte cells. pSHP-1(Y536) was expressed as a fold increase of control (unstimulated) cells. Immunoblots are representative of three independent experiments. (D) Ghrelin-induced Akt HM (S473) and A-loop (T308) phosphorylation in the absence (-) or presence (+) of SHP-1 siRNA in 3T3-L1 preadipocyte cells. Left, the immunoblot analysis of SHP-1 and GHSR1a expressions in control siRNA and SHP-1 siRNA-transfected HEK-GHSR1a cells. Data are expressed as mean \pm SE obtained from intensity scans of three independent experiments. * $p < 0.05$ vs. control values.

(Figure 4B). This was followed by increased ghrelin-stimulated pAkt(T308) and pAkt(S473) levels in omental WAT of HFD mice compared with omental WAT of control mice (~ 2.0 - and 0.8 -fold higher, respectively). In contrast, ghrelin-induced pAkt(T308) and pAkt(S473) levels were $\sim 38\%$ and $\sim 33\%$ less in subcutaneous WAT of HFD mice relative to subcutaneous WAT of control mice, respectively (Figure 4C). No changes were detected in total Akt, GHSR1a, and p85 levels in all instances (Figure 4, C–E). cSrc expression was markedly enhanced in omental and subcutaneous WAT of HFD mice compared with WAT of control mice (~ 1.9 - and ~ 1.6 -fold, respectively; Figure 4F). PDK1 levels were enhanced in subcutaneous WAT of HFD mice relative to control (~ 0.9 -fold), although they were not altered in omental WAT of HFD mice (Figure 4G). On the contrary, mTOR levels were augmented in omental WAT of HFD mice and were diminished in subcutaneous WAT of HFD mice (Figure 4H).

DISCUSSION

Over the past several years, much progress has been made in elucidating intracellular signaling events mediating metabolic actions of the ghrelin/GHSR1a system. However, the molecular processes that mediate the inactivation of these events remain less defined. We previously have demonstrated that the GHSR1a-associated signaling pathways trigger Akt activation, with cSrc functioning as a switch that initiates the Akt pathway associated to both the $G_{i/o}$ protein-dependent pathway and the β -arrestin-scaffolded complex (Lodeiro *et al.*, 2009). The current study indicates that SHP-1 is a critical metabolic piece in the ghrelin/GHSR1a signal transduction pathway. More specifically, our work offers four major findings related to the regulation of Akt activity in response to ghrelin. First, activation of SHP-1 was cSrc-dependent and involved the interplay of G protein- and β -arrestin-dependent signaling pathways. Second, SHP-1 attenuated the action of ghrelin by dephosphorylating PI3K and cSrc,

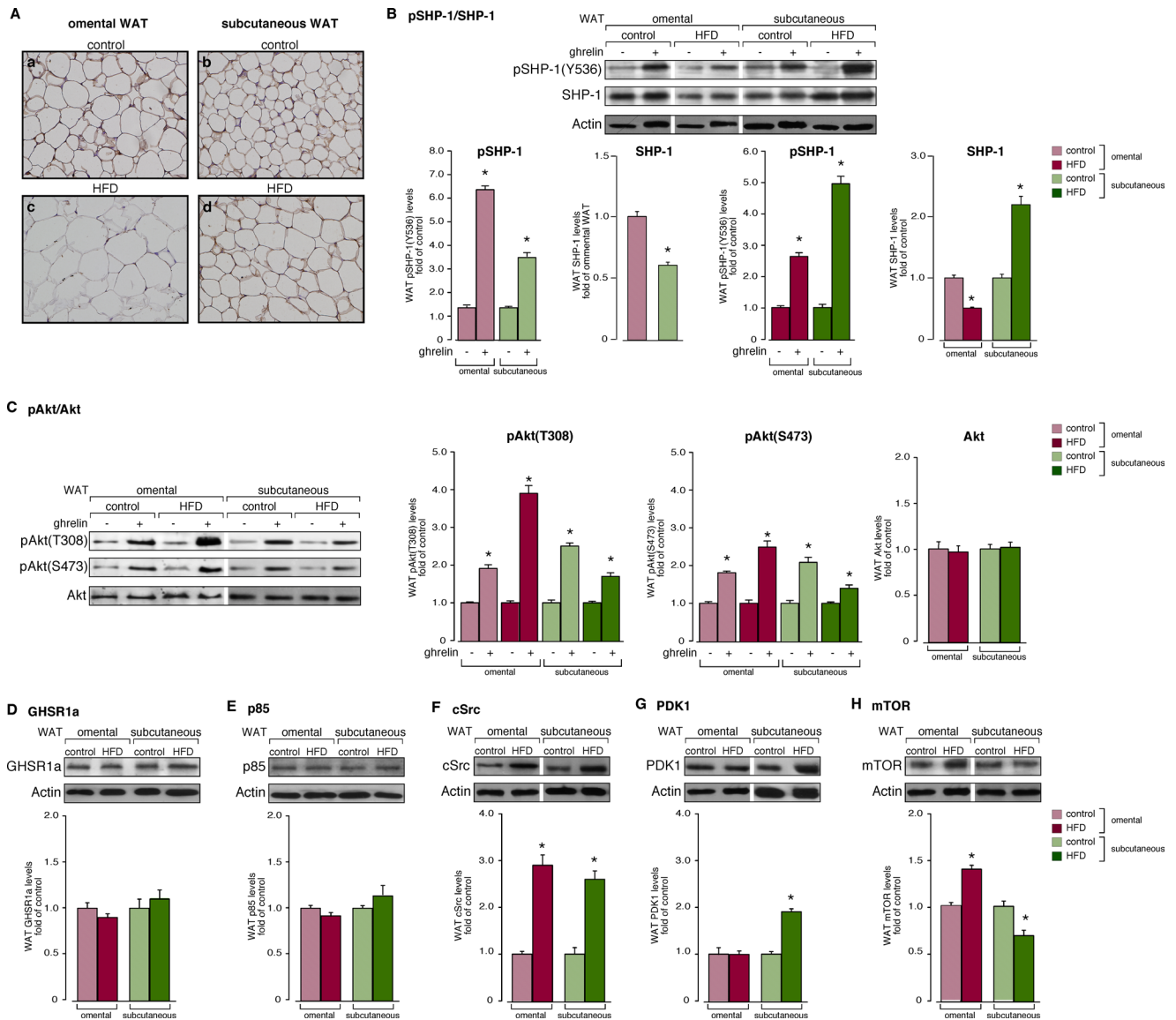


FIGURE 4: Role of SHP-1 in WAT. (A) Immunohistochemical detection of SHP-1 in subcutaneous and omental WAT obtained from control and HFD-treated mice (objective magnification: 20 \times). Decreased SHP-1 immunostaining was evident in omental WAT compared with control mice. (B) Effect of ghrelin (100 nM, 10 min) on pSHP-1(Y536) in omental and subcutaneous WAT explants in vitro from control and HFD mice. pSHP-1(Y536) levels were expressed as a fold increase of control (unstimulated) WAT ($n = 10$ per group). Expression levels of SHP-1 in omental and subcutaneous WAT obtained from control and HFD mice. (C) Effect of ghrelin (100 nM, 10 min) on pAkt(T308) and pAkt(S473) in omental and subcutaneous WAT explants in vitro from control and HFD mice. pAkt levels were expressed as a fold increase of control (unstimulated) WAT ($n = 10$ per group). Expression levels of Akt in omental and subcutaneous WAT obtained from control and HFD mice. (D–E) Expression levels of GHSR1a (D), p85(E), cSrc (F), PDK1 (G), and mTOR (H) in omental and subcutaneous WAT obtained from control and HFD mice ($n = 10$ per group). Data are expressed as mean \pm SE obtained from intensity scans of animal groups (control and stimulated WAT from SD and HFD mice). * $p < 0.05$ vs. control values.

thereby inhibiting Akt signaling. Third, SHP-1 was expressed and stimulated by ghrelin in WAT. Fourth, SHP-1 expression in WAT was dynamically regulated in response to high-fat feeding. Thus SHP-1 would define the impact of the ghrelin/GHSR1a-associated biological response, at least on Akt signaling.

SHP-1 plays a variety of roles in multiple signal transduction events via Y dephosphorylation (Neel and Tonks, 1997; Neel *et al.*, 2003; Zhang *et al.*, 2000), acting as a negative (Klingmüller *et al.*, 1995; Somani *et al.*, 1997; Kozlowski *et al.*, 1998; Feng *et al.*, 2002)

or positive (Krautwald *et al.*, 1996; Wishcamper *et al.*, 2001) modulator of a great variety of receptors. The results presented here are in favor of the role of SHP-1 as a negative regulator of the ghrelin/GHSR1a signaling. The expression of catalytically inactive SHP-1 increased ghrelin-induced Akt phosphorylation, with a greater repercussion on the Akt A loop (T308) phosphorylation. While much of the characterization of SHP-1 is centered on its function as a PTP, the regulation of its activation is still a matter of controversy (Lorenz *et al.*, 1994; Uchida *et al.*, 1994; Zhang *et al.*, 2003). Although this

uncertainty remains, it was demonstrated that the Y phosphorylation of the C-terminal (Y536) on SHP-1 increases its PTP activity (Uchida *et al.*, 1994; Frank *et al.*, 2004). Our results showed that ghrelin is able to phosphorylate SHP-1 at the C-terminus Y536 in intact cells and WAT. Additionally, our study showed that the ablation of cSrc by siRNA impaired ghrelin-stimulated C-terminus Y536 phosphorylation of SHP-1. With this result, cSrc was located as an upstream signal that regulates the phosphorylation and activation of SHP-1, which is in agreement with previous reports showing that Src activates SHP-1 (Frank *et al.*, 2004). This is also consistent with recent works in which angiotensin II AT₂ receptor regulates SHP-1 activity through Src activation (Feng *et al.*, 2002; Alvarez *et al.*, 2008). This new data, together with our previous findings (Lodeiro *et al.*, 2009), support the idea that cSrc operates as a “switch,” in concert with PI3K, for the modulation of Akt activity in response to ghrelin.

Analysis of the upstream targets in ghrelin signaling for Akt activation under the expression of catalytically inactive SHP-1 revealed increased ghrelin-induced Y phosphorylation of p85 regulatory subunit of PI3K. Our data confirm that Y-phosphorylated p85 is a target of SHP-1, and cells lacking SHP-1 increased PI3K activity, as previously described for other factors (Imani *et al.*, 1997; Yu *et al.*, 1998; Cuevas *et al.*, 1999; Theodoropoulou *et al.*, 2006). The inhibitory effect of SHP-1 on PI3K activity is a consequence of its Y dephosphorylation (Y688), a residue that, when phosphorylated, interacts with the p85 N-terminal SH2 domain releasing the inhibitory activity of p85 on the p110 catalytic subunit (Imani *et al.*, 1997; Yu *et al.*, 1998; Cuevas *et al.*, 1999). In fact, activation of downstream PI3K targets, PDK1 [pPDK1(S241)] and cSrc [pcSrc(Y416)], is increased in cells lacking SHP-1 under ghrelin stimulation. This is also consistent with the increase of ghrelin-stimulated A-loop (T308) phosphorylation of Akt in the context of SHP-1 deficiency.

The presence of a pathway involving receptor endocytosis is supported by the fact that depletion of β -arrestins 1 and 2 using specific siRNAs reduced the magnitude of the ghrelin-stimulated C-terminus Y536 phosphorylation of SHP-1. Coimmunoprecipitation assays revealed that the β -arrestins function as adaptors, recruiting SHP-1 to the ghrelin-occupied receptor through the formation of β -arrestin complexes. This is also consistent with the increase of ghrelin-stimulated Akt phosphorylation in the context of functional SHP-1 deficiency. The mechanism of activation of SHP-1 associated to β -arrestins is not completely delineated. Available data suggest that cSrc is essential for Akt activation, even when cSrc is associated to β -arrestins (Lodeiro *et al.*, 2009). Given that cSrc is recruited to GHSR1a- β -arrestin complex to initiate Akt phosphorylation upon ghrelin stimulation (Lodeiro *et al.*, 2009), it was plausible to hypothesize that SHP-1 interacts with the cSrc associated to this complex, exerting a modulation on cSrc activity, as p85 is not associated to the GHSR1a- β -arrestin complex (Camiña *et al.*, 2007). Interestingly, both β -arrestin 1 and β -arrestin 2 are required to promote the activation of SHP-1. The requirement of both proteins supports the need to form heterodimers to activate the β -arrestin-dependent signaling pathway for GHSR1a.

Ghrelin plays a significant role in the regulation of adipogenesis and lipid storage in WAT (Wells, 2009). At the cellular level, ghrelin-activated Akt is both necessary and sufficient to drive these processes, as Akt plays an essential role in adipogenesis (Xu and Liao, 2004; Zhang *et al.*, 2009). The implication that SHP-1 is a negative regulator of ghrelin activity raises the possibility that this PTP is one of the critical signaling components for ghrelin action and essential for proper regulation of Akt-dependent processes. In fact, the dissimilar SHP-1 expression in subcutaneous and omental fat suggests that the ghrelin sensitivity of these specific depots may be modu-

lated by this PTP. According to our results in cell culture models, ghrelin activated SHP-1 in WAT. Furthermore, the level of SHP-1 phosphorylation correlated to the SHP-1 levels associated to specific WAT depots. Taken together, these results point to a higher ghrelin effect in subcutaneous than omental WAT in normal conditions. Our study took advantage of signaling defects triggered by modification of the SHP-1 expression in HFD mice to determine the contribution of this PTP. While the low SHP-1 levels in omental WAT from HFD mice led to the augmentation of ghrelin-evoked Akt response, the high SHP-1 levels in subcutaneous depots led to a partial attenuation of Akt response, showing a differential fine-tuning of the signal transduction between individual WAT depots. This effect was more evident on the A loop (T308) than in the HM (S473) phosphorylation. This differential effect was also observed in HEK-GH-SR1a and 3T3-L1 cells, providing a suitable model for the analysis of the signaling events of this process. SHP-1 attenuated PDK1 activity through PI3K with no apparent effect on mTORC2. This is further supported by the fact that both the A-loop (T308) and the HM (S473) phosphorylations are independent (Lodeiro *et al.*, 2009). Analysis of initial steps in ghrelin signaling to Akt revealed modified levels for cSrc, PDK1, and mTOR in WAT from mice fed with HFD relative to control mice, while no changes in Akt, GHSR1a, and p85 expression were detected. However, this pattern does not appear to explain the differences in Akt activity detected between both groups. In fact, enhanced cSrc and PDK1 expression in subcutaneous WAT from mice fed with HFD does not counteract the attenuation of ghrelin-evoked Akt phosphorylation.

One of the main characteristics of adipose tissue is its extreme plasticity, which allows it to respond promptly to changes in nutrient intake through adipocyte hypertrophy and/or hyperplasia, depending on the fat depot and fat-cell size (Wajchenberg, 2000). In this regard, omental adipose tissue accumulation is correlated to a cluster of diabetogenic, atherogenic, prothrombotic, and proinflammatory metabolic abnormalities known as the metabolic syndrome (Després, 2006). Due to the close ties between the SHP-1 expression pattern in WAT and its regulation in a positive energy balance situation, it is possible to speculate about its role in the enlargement of WAT in obesity. Accumulation of fat, and particularly omental fat, is associated with decreased SHP-1 levels, with the consequent magnification of ghrelin response, resulting in increased size and number of fat cells. Therefore SHP-1 may contribute to the dissimilar responses to overfeeding in the different fat depots.

In conclusion, and as summarized in Figure 5, we have identified the SHP-1 tyrosine phosphatase as a negative regulator of ghrelin signaling. Our data are consistent with a model in which ghrelin-activated Akt translocates to the plasma membrane through the binding of its pleckstrin homology domain to the second messenger PtdIns(3,4,5)P₃ generated by PI3K, which is activated through the G_{i/o} protein-dependent signaling pathway. Akt is phosphorylated at Y by the membrane-bound cSrc. This Y phosphorylation is followed by phosphorylation of the Akt A loop (T308) and the HM (S473) by PDK1 and mTORC2, respectively. Once the receptor is activated, a second signaling pathway is mediated by β -arrestins 1 and 2, involving the recruitment of GHSR1a, cSrc, and Akt into a β -arrestin-scaffolded complex. In both signaling pathways, cSrc phosphorylates the SHP-1 C-terminus (Y536), which exerts an inhibitory effect on activity of PI3K and Akt. Consequently, cSrc functions as a switch that controls the Akt pathways associated to both the G_{i/o} protein-dependent pathway and β -arrestin-scaffolded complex. Although further studies are required, the current data show that SHP-1 is expressed in WAT, one of the target tissues of ghrelin, modulating

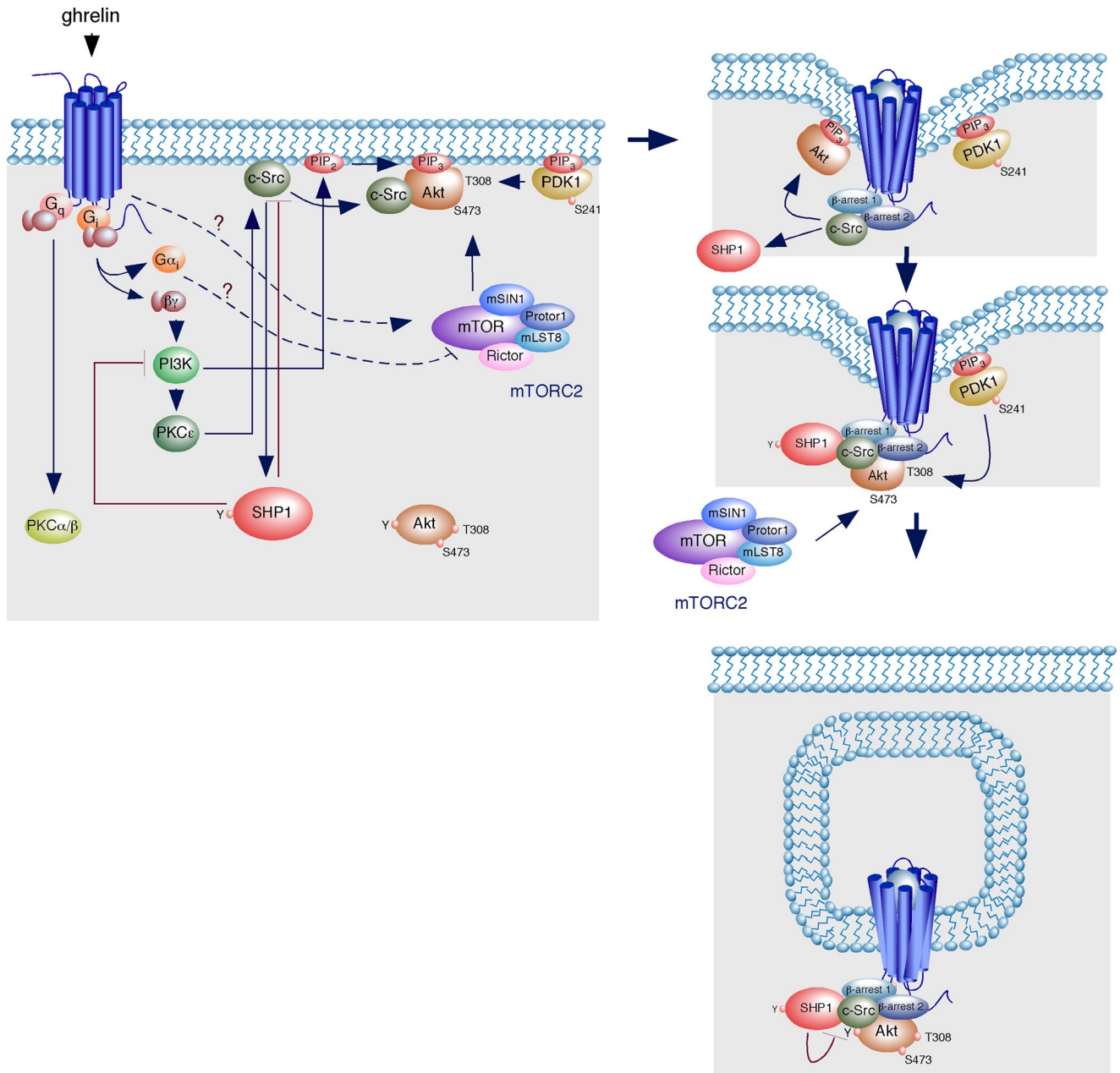


FIGURE 5: Model of SHP-1 activation and modulation on ghrelin signaling to Akt. Ghrelin binding to GHS-R1a activates the generation of the second messenger PtdIns(3,4,5)P₃ (PIP₃) by PI3K activation through G_{i/o} protein-dependent signaling pathway. Akt translocates to the plasma membrane by binding to PIP₃, where it is phosphorylated at Y by the membrane-bound cSrc. Activation of cSrc induced phosphorylation of SHP-1, attenuation of p85 subunit from PI3K and PDK1, and phosphorylation of Akt. SHP-1 activation regulates cSrc activity by attenuation of Y416 phosphorylation. Once the receptor is activated, a second signaling pathway is mediated by β-arrestins 1 and 2, involving the recruitment of GHS-R1a, cSrc, SHP-1, and Akt into a β-arrestin-scaffolded complex.

ghrelin signaling to Akt and determining ghrelin sensitivity in WAT based on adipose depot differences.

MATERIALS AND METHODS

Materials

Human ghrelin was obtained from Global Peptide (Fort Collins, CO). Anti-p44/42 MAPK, anti-pcSrc (Y416), anti-pAkt HM (S473), anti-pAkt A-loop (T308), and anti-pPDK1 (S241) rabbit polyclonal antibodies were from Cell Signaling Technology (Beverly, MA). Anti-Rictor rabbit polyclonal was from Bethyl Laboratories (Montgomery, TX). Anti-pY rabbit polyclonal antibody was from

Upstate Technology (Lake Placid, NY). Anti-β-arrestin 1 goat polyclonal, anti-β-arrestin 2 mouse monoclonal, anti-SHP-1, anti-pSHP-1 (Y536), and anti-cSrc rabbit polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). β-arrestin 1 siRNA, β-arrestin 2 siRNA, cSrc siRNA, SHP-1 siRNA, and siRNA control were from Santa Cruz Biotechnology. Anti-rabbit horseradish peroxidase was from GE-Amersham (Buckinghamshire, UK), while anti-goat horseradish peroxidase was from Santa Cruz Biotechnology. Rabbit anti-rat β-arrestin 1C-terminal (A1CT) antiserum was provided by R. J. Lefkowitz (Duke University Medical Center, Durham, NC).

Cell culture

HEK 293 cells, which stably express the human ghrelin receptor 1a (HEK-GHSR1a), were cultured as previously described (Lodeiro et al., 2009). 3T3-L1 preadipocyte cells were propagated and differentiated according to the protocol previously described (Gurriarán-Rodríguez et al., 2011). In brief, confluent 3T3-L1 cells were treated with 0.5 mM isobutylmethylxanthine, 25 mM dexamethasone, and 861 nM (5 µg/ml) insulin for 3 d and maintained in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin, and supplemented with 172 nM (1 µg/ml) insulin for 10 d after the beginning of differentiation.

Animals

This study was carried out in strict accordance with the recommendations in the "Guide for the Care and Use of Laboratory Animals" of the Faculty Animal Committee at the University of Santiago de Compostela, and the experiments were performed in agreement with the rules of laboratory animal care and international law on animal experimentation; all efforts were made to minimize suffering. Adult Swiss male mice (8 wk old) were housed in air-conditioned rooms (22–24°C) under a 12:12 h light/dark cycle and fed with standard chow (SD) or HFD for 12 wk (D12451, 45 kcal% fat, Research Diets, New Brunswick, NJ). Food intake and body weight were measured weekly during the experimental phase. Animals were killed by decapitation between 10:00 and 12:00 h when they were 20 wk old. Excised WAT, omental and subcutaneous, was immediately transported to the laboratory in ice-cold Krebs-Ringer-HEPES buffer (HEPES, pH 7.4, 25 mM; NaCl, 125 mM; KCl, 5 mM; MgSO₄, 1.2 mM; CaCl₂, 2 mM; KH₂PO₄, 2 mM; glucose, 6 mM). After blood vessels and conjunctive tissue were removed, adipose tissue was washed with sterile Krebs-Ringer-HEPES buffer. Tissue fragments were placed in six-well dishes containing DMEM supplemented with penicillin (100 U/ml) and streptomycin sulfate (100 µg/ml). After a preincubation period of 1 h at 37°C under a humidified atmosphere of 95% air–5% CO₂, the media were aspirated, and fresh medium, with or without ghrelin (100 nM, 10-min), was dispensed into each well. The media were then aspirated, and tissue samples were directly lysed in ice-cold RIPA buffer (Tris-HCl, pH 7.2, 50 mM; NaCl, 150 mM; ethylenediaminetetraacetic acid [EDTA], 1 mM; NP-40, 1% [vol/vol]; Na-deoxycholate, 0.25% [wt/vol]; protease inhibitor cocktail [Sigma-Aldrich; St. Louis, MO]; phosphatase inhibitor cocktail [Sigma]) for immunoblot analysis.

Plasmids and cell transient transfection

The catalytically inactive SHP-1/C453S (SHP-1dn; kind gift of T. Florio, University of Genova, Genova, Italy) mutant was used (Pagès et al., 1999). Cells (3 × 10⁵) were transfected with 1 µg SHP-1dn plasmid or vector control (pcDNA3) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as previously described (Theodoropoulou et al., 2006). Immunoblot analysis was used to confirm the SHP-1 incorporation.

Immunoblot analysis

Serum-starved cells were stimulated with ghrelin for the indicated time period at 37°C. The medium was then aspirated and the cells were lysed in ice-cold RIPA buffer. Tissue samples were directly lysed in ice-cold RIPA buffer. The solubilized lysates were transferred into centrifuge tubes and left at 4°C for 15 min, then precleared by centrifuging at 13,000 × g for 15 min. Protein concentration was evaluated with the QuantiPro BCA assay kit (Sigma). Subsamples (same amount of protein) of each sample were separated on

SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The blots were incubated with 5% nonfat dry milk in TBST (Tris-HCl, pH 8.0, 20 mM; NaCl, 150 mM; Tween-20, 0.1% [vol/vol]; used for all incubation and washing steps) for 1 h. Next blots were incubated with the corresponding antibodies according to the manufacturer's instructions. The blots were subsequently incubated with the corresponding peroxidase-conjugated IgG antibody. After washing, signals were visualized using an enhanced chemiluminescence detection system (GE-Amersham).

Immunoprecipitation

Serum-starved cells were stimulated with ghrelin for the indicated time period at 37°C and lysed in ice-cold nondenaturing NP-40 solubilization buffer (immunoprecipitation lysis buffer [ILB]: Tris-HCl, pH 7.5, 20 mM; NaCl, 150 mM; EDTA, 1 mM; NP-40, 1% [vol/vol]; protease inhibitor cocktail [Sigma]; phosphatase inhibitor cocktail [Sigma]). Total protein (500 µg) was prewashed with 20 µl of 50% protein A/G-agarose (Santa Cruz Biotechnology) for 30 min at 4°C, and then incubated with 1 µg of the corresponding antibody overnight at 4°C, which was followed by addition of 40 µl of 50% protein A/G for 2 h at 4°C. After being washing twice with ILB, the pelleted beads were resuspended in Laemmli sample buffer. Proteins were then analyzed by 10% SDS-polyacrylamide gels, which was followed by immunoblotting.

siRNA silencing of gene expression

Chemically synthesized double-stranded siRNA duplexes (with 3' dTdT overhangs) were purchased from Santa Cruz Biotechnology for the following targets: β-arrestin 1 (5'-AAAGCCUUCUGCGCGGAGAAU-3'), β-arrestin 2 (5'-AAGGACCGCAAAGUGUUUGUG-3', 5'-AAAGCCUUCUGCGCGGAGAAU-3', 5'-AAGGACCGCAAAGUGUUUGUG-3'), cSrc (5'-CUCGGCUCAUUGAAGACA-3', 5'-UGACUGAGCUCACCACAAA-3', 5'-CCUCAUCAUAGCAAUAACA-3', 5'-GUAGAUUUCAGAUACUUAU-3') and SHP-1. A nonsilencing RNA duplex was used as a control for all siRNA experiments. Cells were transfected with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Silencing was quantified by immunoblotting. Only experiments with verified silencing were used.

IHC

3T3-L1 cells were cultured on coverslips and differentiated into adipocytes. Cells were fixed on coverslips in 96% ethanol. WAT samples were fixed by immersion in 10% buffered formalin for 24 h, dehydrated, and embedded in paraffin using a standard procedure. Sections (5 µm thick) were mounted on Histobond Adhesion Microslides (Paul Marienfeld, Lauda-Königshofen, Germany), dewaxed, and rehydrated. Slides were consecutively incubated with: 1) anti-SHP-1 rabbit polyclonal antibody at a dilution of 1:100 in Dako ChemMate antibody diluent (Dako, Glostrup, Denmark); 2) EnVision peroxidase rabbit (Dako, Glostrup, Denmark), which was used as the detection system; 3) 3,3'-diaminobenzidine-tetrahydrochloride (Dako Liquid DAB + Substrate-chromogen system). Cell and WAT sections were faintly counterstained with Harris's hematoxylin. Substitution of the primary antibody with PBS was used as a negative control.

Data analysis

The data from the immunoblot analysis were normalized to control bands, and the results were expressed as the mean ± SE. Differences between means were evaluated by one-way analysis of variance (ANOVA); *p < 0.05 versus control values.

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