UNIVERSIDAD DE SANTIAGO DE COMPOSTELA

FACULTAD DE MEDICINA

DEPARTAMENTO DE FISIOLOGÍA

LABORATORIO DE ENDOCRINOLOGÍA MOLECULAR

Characterization of the intracellular signaling mechanisms activated by ghrelin through GHSR-1a: role of β-arrestins

MARÍA LODEIRO POSE

Santiago de Compostela, mayo de 2011

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Memoria que para optar al Grado de Doctor en Biología
por la Universidad de Santiago de Compostela presenta:

María Lodeiro Pose

Santiago de Compostela, mayo de 2011

La memoria adjunta titulada "**Characterization of the intracellular signaling mechanisms activated by ghrelin through GHSR-1a: role of β-arrestins"** que para optar al Grado de Doctor en Biología presenta Dña. María Lodeiro Pose, ha sido realizada bajo nuestra dirección en el Área de Endocrinología Molecular y Celular del Instituto de Investigación Sanitaria de Santiago (Complejo Hospitalario Universitario de Santiago de Compostela).

Considerando que constituye trabajo de Tesis Doctoral, autorizamos su presentación en la Universidad de Santiago de Compostela.

Y para que así conste, firmamos la presente en Santiago de Compostela en mayo de 2011.

Dr. Jesús Pérez Camiña
Investigador SERGAS

Prof. Dr. Felipe Casanueva Freijo
Catedrático de Medicina

Santiago de Compostela, mayo de 2011

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“Si no estás dispuesto a equivocarte, nunca llegarás a nada”.

Ken Robinson

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ABBREVIATIONS

7TM: seven transmembane

7TMR: seven transmembrane receptor

AC: adenylato cyclase

ACTH: adrenocorticotrope hormone

AG: acylated ghrelin

Akt: serine /threonine kinase (Protein kinase B)

AMP: adenosine monophosphate

AMPK: AMP-activated protein kinase

ANOVA: analysis of variance

AP-2: adaptor protein 2

AT1AR: angiotensin II type 1A receptor

AT2R: angiotensin II type 2 receptor

ATM: ataxia telangengiectasia mutated gene product

β1AR: β-1 adrenergic receptor

β2AR: β-2 adrenergic receptor

BHK: baby hamster kidney cells

BSA: bovine serum albumin

cAMP: cyclic adenosine monophosphate

cGMP: cyclic guanosine monophosphate

CON A: concanavalin A

CPM: counts per minute

CPT-1: carnitine palmitoyltransferase 1

CST: cortistatin

DAG: diacylglycerol

DEX: dexamethasone

DMEM: Dulbecco´s Modified Eagle´s Medium

DNAPK: double-stranded DNA-dependent protein kinase

ER: endoplasmic reticulum

ERK 1/2: extracellular signal-regulated kinases 1/2

FAS: fatty acid synthase

FBS: fetal bovine serum

FURA-2 AM: fura-2 acetoxymethylester

GC: guanylyl ciclase

GH: growth hormone

GHRH: growth hormone releasing hormone

GHRH-R: growth hormone releasing hormone receptor

GHS: growth hormone secretagogues

GHS-R: growth hormone secretagogue receptor

GHSR-1a: growth hormone secretagogue receptor type 1a

GHSR-1b: growth hormone secretagogue receptor type 1b

GOAT: ghrelin O-acyl-transferase

GPCR: G-protein-coupled receptor

GPR39: G-protein 39-coupled receptor

GRB2: growth factor receptor-bound protein 2

GRK: G-protein-coupled receptor kinase

HEK: human embrionary kidney cells

HFD: high fat diet

IBMX: 3-isobutyl-1-methylxanthine

ILB: immunoprecipitation lysis buffer

ILK: integrin-linked kinase

IGF-1: insulin-like growth factor 1

IHC: immunohistochemistry

IP_3: inositol (1, 4, 5)-triphosphate

IRS-1: insulin receptor substrate 1

KDa: KiloDaltons

KRH: Krebs-Ringers Henseleit

MAPK: mitogen-activated protein kinase

mRNA: messenger RNA

MBOAT: membrane-bound O-acyl transferase

MrgX2: mass related gene X2

mTOR: mammalian target of rapamycin

mTORC2: mammalian target of rapamycin complex 2

NO: nitric oxide

NOS: nitric oxide synthase

PAM: peptidyl glycine α-amidating monooxygenase

PAMP12: proadrenomedullin N-terminal peptide 12

PC 1/3: proprotein convertase 1/3

PCR: polymerase chain reaction

PDK-1: 3-phosphoinositide-dependent kinase-1

PH: pleckstrin homology domain

PHLPP1: PH domain leucine-rich repeat protein phosphatase 1

PHLPP2: PH domain leucine-rich repeat protein phosphatase 2

PI3K: phosphatidylinositol 3´-kinase

PI-PLC: phosphatidylinositol-specific phospholipase C

PKA: protein kinase A

PKC: protein kinase C

PMA: phorbol 12-myristate 13-acetate

PP2A: protein phosphatase 2A

PRL: prolactin

PTH1R: parathyroid hormone type 1 receptor

PTP: protein tyrosine phosphatase

PTX: pertussis toxin

qRT-PCR: quantitative real time PCR

RNA: ribonucleic acid

RTK: receptor tyrosine kinase

SDS: sodium dodecyl sulfate

SHP-1: Src homology-2 domain containing phosphatase-1

SHP-1dn: SHP-1 dominant negative

siRNA: small interfering RNA

SST: somatostatin

SSTR: somatostatin receptor

TCA: trichloroacetic acid

UAG: unacylated ghrelin

V2R: vasopressin type 2 receptor

VMH: ventromedial nucleus of the hypothalamus

VIP: vasoactive intestinal peptide

WAT: white adipose tissue

WORT: wortmannin

WT: wild type

1. OBJECTIVES

The ghrelin/ghrelin receptor (GHSR-1a) system directs diverse array of physiological responses and hence has broad relevance to numerous diseases, among them obesity and anorexia. Recent studies suggest that signaling through GPCR is far more diverse than original thought, as GPCR can couple to multiple G-proteins as well as other adaptor proteins, among them the β-arrestin family. Based on it, the work hypothesis for this Thesis was that, for ghrelin/GHSR-1a system, β-arrestins constitute a relevant and separable signaling arm from the classical heterotrimeric G-proteins in addition to their role in the ending of G-protein/GHSR-1a signaling.

The global objective is to delineate the β-arrestin signaling for the ghrelin receptor GHSR-1a. This general objective is divided into the following specific objectives:

1. To determine the protein partners of both β-arrestin 1 and 2 under basal and ghrelin-stimulated conditions in the regulation of metabolism, apoptosis, transcription and cell-cycle. This point is divided in:

    1.1. To determine the role of β-arrestins on ERK 1/2-dependent signaling pathway that mediate the effect of ghrelin on cell survival, growth and proliferation.

    1.2. To determine the role of β-arrestins on Akt-dependent signaling pathway that mediate the effect of ghrelin on metabolism, apoptosis, transcription and cell-cycle.

2. To define the mechanism for regulating GHSR-1a-associated Akt activity, with emphasis in the implication of the cytoplasmic protein Src homology-2 domain containing phosphatase-1 (SHP-1).

3. To determine the role of β-arrestins on Akt-and ERK 1/2-dependent signaling pathways that mediate the effects of CST-17 through GHSR-1a.

These molecular mechanisms were investigated in HEK 293 cells, stably expressing the GHSR-1a (HEK-GHSR-1a) as model system to follow the interaction of specific proteins and the roles, if any, played by G-proteins and β-arrestins. In addition, some results were confirmed in 3T3-L1 cells and white adipose tissue.

2. INTRODUCTION

The transmission of extracellular signals inside the cells represents one of the basic cellular processes. Most of them are mediated by membrane receptors, where the seven transmembrane receptors (7TMR), also called G-protein-coupled receptors (GPCR), form the largest, most versatile and most ubiquitous group of integral membrane receptor proteins. This is a process that needs to be tightly regulated and controlled to achieve an appropriate functioning. These 7TMR comprise more than 800 members in the human genome and respond to a great variety of extracellular stimuli including neurotransmitters, hormones, phospholipids, photons, odorants, certain taste ligands and growth factors; controlling all vital physiologic functions and maintaining homeostasis. [1, 2] In addition, they also constitute the most common target of therapeutic drugs. [3, 4]

Ligand binding to the extracellular domain of the receptor triggers a conformational change in the receptor allowing it to couple, via heterotrimeric G-proteins, to the regulation of a variety of effector systems (Figure 1). In addition, ligand binding to GPCR initiates a series of regulatory processes that contributes to GPCR desensitization, endocytosis and down-regulation. GPCR desensitization is an adaptative process that prevents receptor overstimulation in response to prolonged agonist stimulation. Similarly, to prevent prolonged desensitization, many GPCR exhibit the ability to become resensitized following agonist removal, a process that appears to require receptor endocytosis. Consequently, GPCR-mediated cell signaling represents a delicate balance between mechanisms regulating receptor activation, desensitization and resensitization. [5]

[1] Lefkowitz RJ. Seven transmembrane receptors: something old, something new. Acta Physiol. 2007; 190: 9-19.
[2] Pierce KL, Premont RT, Lefkowitz RJ. Seven-transmembrane receptors. Nat Rev Mol Cell Biol. 2002; 3: 639-50.
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[5] Lefkowitz RJ, Shenoy SK. Transduction of receptor signals by β-arrestins. Science. 2005; 308: 512-7.

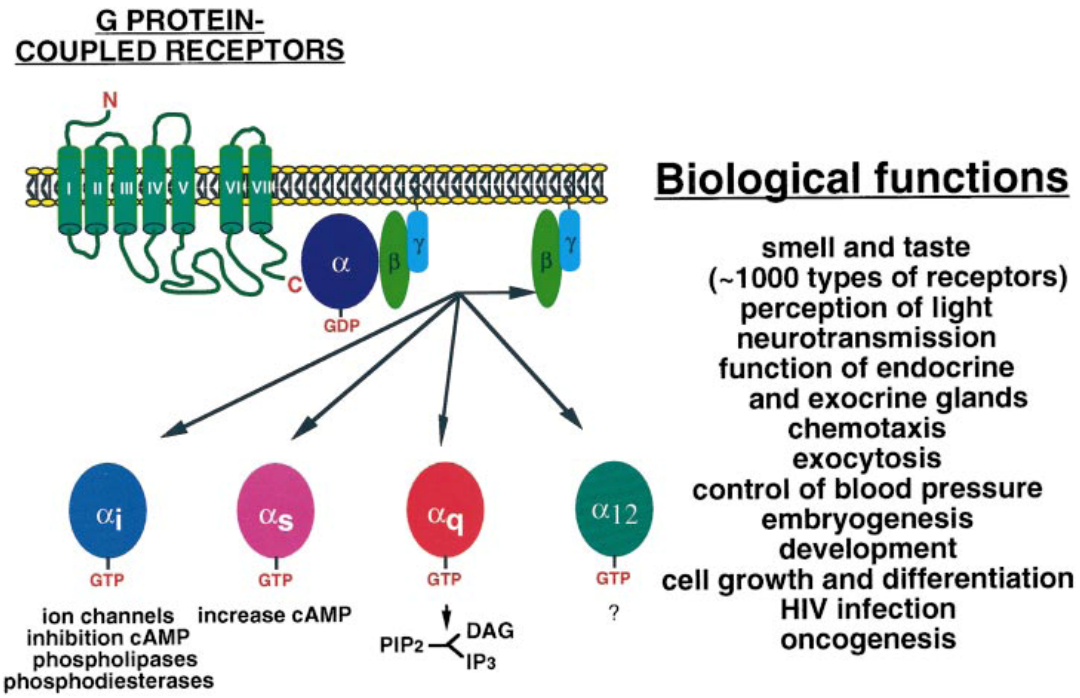

Figure 1. Picture showing the diversity of the G-protein-coupled receptor signal transduction system. Each GPCR can interact with different G-proteins which results in the activation or inhibition of second messengers molecules, resulting in the control of multiple biological functions. (Figure extracted from J Biol Chem. 1998; 273: 1839-42).

2.1. The ghrelin system

2.1.1. Ghrelin

The ghrelin hormone was discovered in gastric extracts as the natural ligand of the orphan growth hormone secretagogue receptor type 1a (GHSR-1a) using "reverse pharmacology". [6] Ghrelin is a 28 amino acidic peptidic hormone with a unique post-translational modification of *O*-*n*-octanoylation at serine 3. [7] It is mainly produced in the stomach by the enteroendocrine X/A-like cells in the oxyntic mucosa and in other organs and tissues, to a lesser extent, as other parts of gastrointestinal tract, pancreas, kidney, pituitary and hypothalamus.[8, 9] The acylation is essential for the binding to the GHSR-1a, for the release of growth hormone (GH) both *in vivo* and *in vitro* and for the most of the other endocrine actions. [6] In addition to central effect on food intake and GH release, ghrelin possess other no endocrine activities such as the control of body energy, gastric motility, acid-gastric secretion, influence on pancreatic activity and glucose metabolism, cardiovascular effects and proliferative and antiproliferative effects on different cell lines. [10]

In humans, the ghrelin gene is located on the chromosome position 3q 25-26 and it has 4 exons and 3 introns. [10, 11] This structure gives 2 types of ghrelin molecules by alternative splicing: ghrelin and des-Gln14-ghrelin, which posses a deletion on glutamine on amino acid number 14. It also has been identified and described different and multiple ghrelin-derived molecules produced by post-translational processing, on the basis of amino acid length and by type of acylation at Ser. [12]

The precursor molecule of ghrelin, pre-proghrelin, is composed of 117 amino acids, with 23 amino acids conforming the signal sequence and 94 the proghrelin (28 amino acids from ghrelin plus 66 amino acids from the tail). The signal sequence is eliminated subsequently in

[6] Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature. 1999; 402: 656-60.
[7] Kojima M, Hosoda H, Matsuo H, Kangawa K. Ghrelin: discovery of the natural endogenous ligand for the growth hormone secretagogue receptor. Trends Endocrinol Metab. 2001; 12: 118-22.
[8] Horvath TL, Diano S, Sotonyi P, Heiman M, Tschop M. Minireview: ghrelin and the regulation of energy balance: a hypothalamic perspective. Endocrinology. 2001; 142: 4163-9.
[9] Inui A. Ghrelin: an orexigenic and somatotrophic signal from the stomach. Nat Rev Neurosci. 2001; 2: 551-60.
[10] Camina JP. Cell biology of the ghrelin receptor. J Neuroendocrinol. 2006; 18: 65-76.
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the endoplasmic reticulum (ER) and then a proteolitic processing occurs giving the mature ghrelin and a 66 amino acids C-terminal fragment named the C-ghrelin (Figure 2). [13]

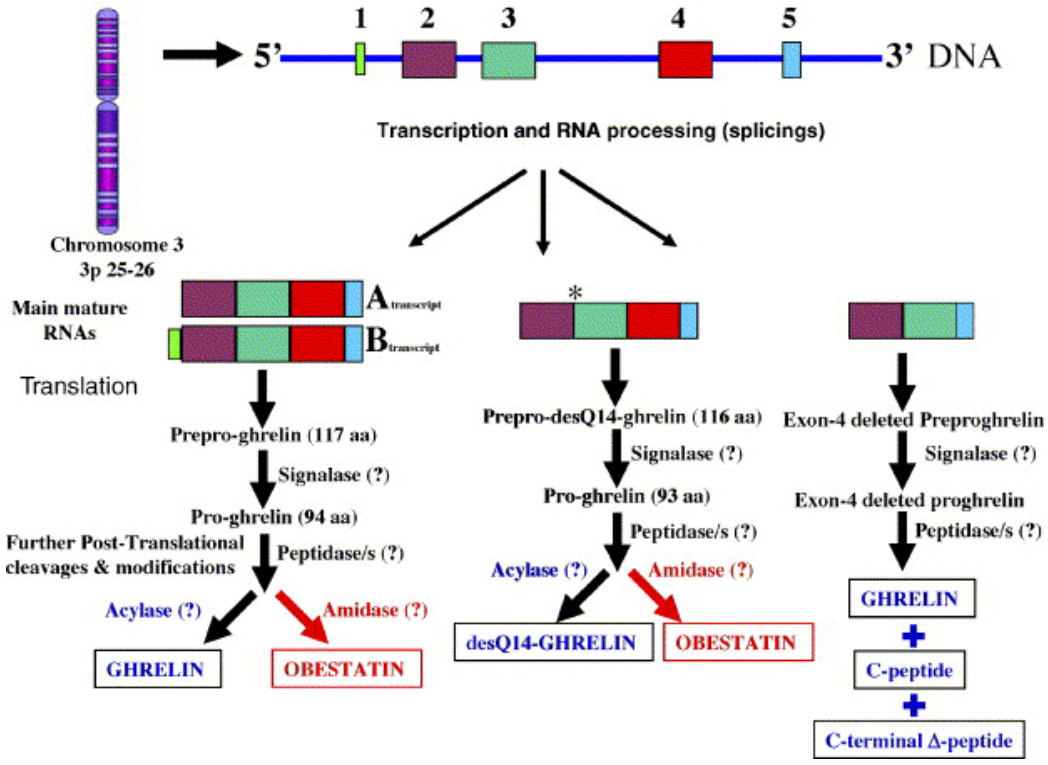

Figure 2. Structure of the ghrelin gene. Schematic representation of transcription, splicing, translation and post-translational modifications by which ghrelin, obestatin and other different peptides are generated. (Figure extracted from Mol Cell Endocrinol, 2006; 256: 1-8).

In addition to this acylated ghrelin, exists another form of ghrelin, commonly named des-acyl ghrelin (or unacylated ghrelin, UAG), which circulates in a greater amount respect to acylated ghrelin, it does not present the acylation on serine 3 but nevertheless it has biological functions. Des-acyl ghrelin has different no endocrine functions like cardiovascular and antiproliferative effects [14, 15] and numerous reports point to the existence of another receptor, different to the GHSR-1a, for the des-acyl ghrelin. [16]

[13] Gualillo O, Lago F, Casanueva FF, Dieguez C. One ancestor, several peptides post-translational modifications of preproghrelin generate several peptides with antithetical effects. Mol Cell Endocrinol. 2006; 256: 1-8.
[14] Korbonits M, Goldstone AP, Gueorguiev M, Grossman AB. Ghrelin: a hormone with multiple functions. Front Neuroendocrinol. 2004; 25: 27-68.

Recently, the enzyme that catalyzes ghrelin acylation has been discovered using a bioinformatic approach. [17, 18] The ghrelin O-acyl transferase (GOAT) belongs to the membrane bound O-acyl transferases (MBOAT), specifically MBOAT4. In mouse, GOAT is located to the ER and its distribution is well defined across the gastrointestinal tract and testis. In humans, it is expressed in stomach and pancreas. Another difference between rodents and humans is that GOAT catalyzes the binding of n-octanoic acid to the serine 3 in a specific way in the former one meanwhile in humans this binding can occur with other different fatty acids. [18] The acylation occurs before pro-ghrelin is transported to the Golgi and it starts after the signal sequence is cleaved by a signal peptide peptidase. Then, GOAT, which is located in the membrane of the ER compartment, mediates the translocation of the octanoyl-CoA from the cytosolic side to the ER lumen. Once the pro-ghrelin precursor reaches the trans-Golgi compartment, it is cleaved by PC1/3 proprotein convertase, packaged in vesicles, and released to the blood (Figure 3). [19]

It is known that the acylation of ghrelin by GOAT is regulated by nutrient availability and depends on specific dietary lipids as acylation substrates. [20] In addition, different *in vitro* experiments showed that the first five amino acids of the ghrelin sequence with the C-terminus region amidated (GSSFLNH2) can be used as a substrate for the enzyme GOAT nearly as well as for pro-ghrelin. This is consistent with the idea that proghrelin remains bound to the enzyme along the acylation process. [19]

According to the published data, it is reasonable to hypothesize that the synthesis of the ghrelin transcript and the secretion of acylated ghrelin (AG) constitute two independent processes because the GOAT–ghrelin system is only activated when certain fatty food is consumed to inform the brain about food availability, whereas regulation of ghrelin transcript synthesis may be mediated by different inputs.[19] There are different publications where the

[15] Bowers CY. Unnatural growth hormone-releasing peptide begets natural ghrelin. J Clin Endocrinol Metab. 2001; 86: 1464-9.
[16] Baldanzi G, Filigheddu N, Cutrupi S, Catapano F, Bonissoni S, Fubini A, Malan D, Baj G, Granata R, Broglio F, Papotti M, Surico N, Bussolino F, Isgaard J, Deghenghi R, Sinigaglia F, Prat M, Muccioli G, Ghigo E, Graziani A. Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. J Cell Biol. 2002; 159: 1029-37.
[17] Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. Cell. 2008; 132: 387-96.
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[19] Romero A, Kirchner H, Heppner K, Pfluger PT, Tschöp MH, Nogueiras R. GOAT: the master switch for the ghrelin system? Eur J Endocrinol. 2010; 163: 1-8.
[20] Kirchner H, Gutierrez JA, Solenberg PJ, Pfluger PT, Czyzyk TA, Willency JA, Schürmann A, Joost HG, Jandacek RJ, Hale JE, Heiman ML, Tschöp MH. GOAT links dietary lipids with the endocrine control of energy balance. Nat Med. 2009; 15: 741-5.

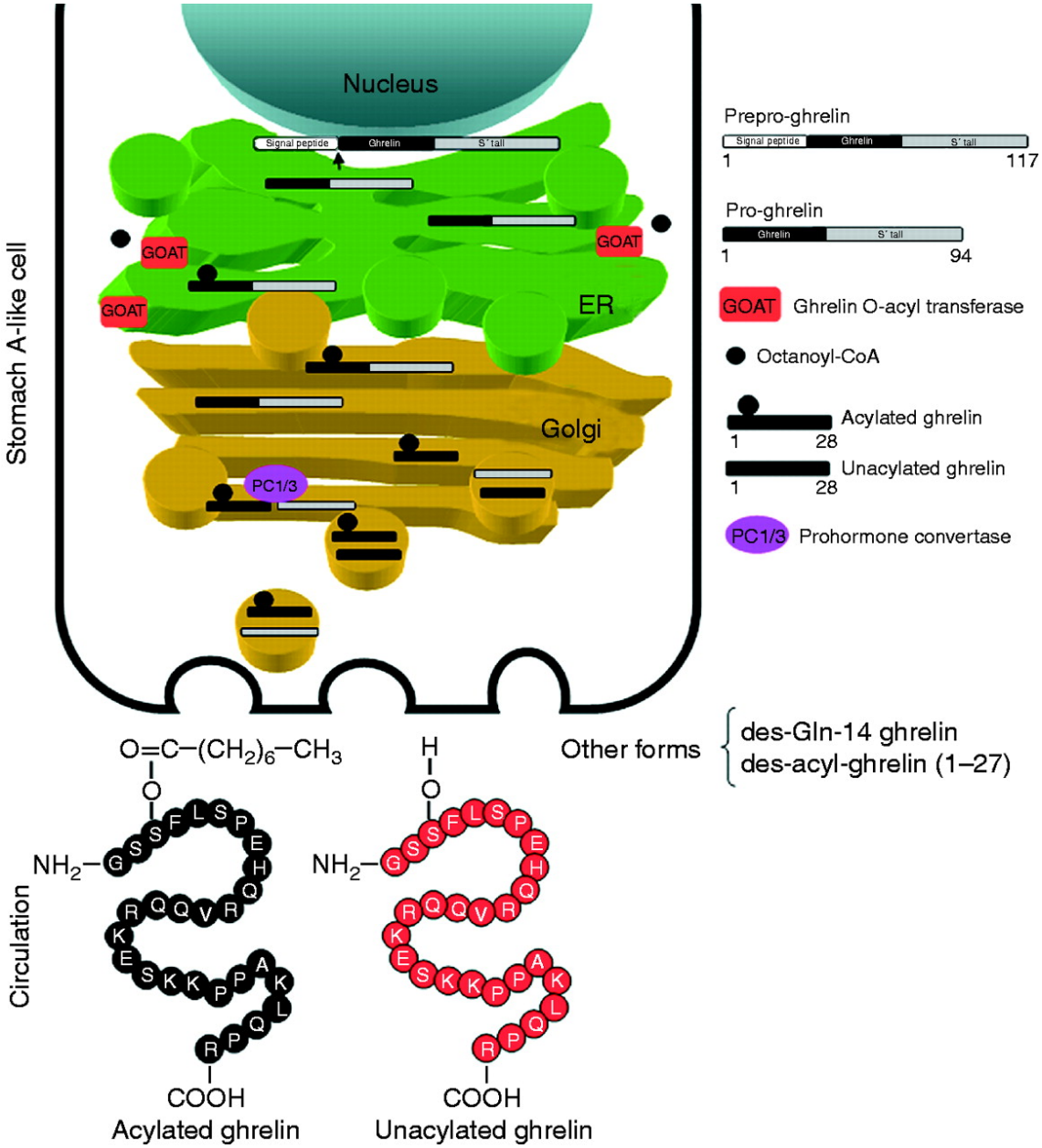

Figure 3. Post-translational processing and acylation of the pro-ghrelin peptide. After the signal sequence is cleaved by a signal peptide peptidase the acylation of pro-ghrelin occurs. For this purpose, GOAT, which is located at the ER compartment, mediates the translocation of the octanoyl-CoA from the cytosolic side. Later, in the trans-Golgi compartment, the pro-ghrelin precursor is cleaved by PC1/3 proprotein convertase, packaged in vesicles, and released to the blood. Different forms of ghrelin can be found into the circulation: acylated (AG), unacylated (UAG), and other shorter forms whose role is still unknown. (Figure extracted from Eur J Endocrinol. 2010; 163: 1-8).

role of GOAT has been studied under different conditions such as nutritional status, age, sex and lactating status. [21, 22] This suggests that the ghrelin-GOAT system act as a lipidic sensor that informs the brain about the high fat meal availability to achieve an optimal energetic storage. [20] This also suggests that the physiological function of ghrelin may not necessarily, or at least not exclusively, be a hunger signal reflecting an empty stomach. Because GOAT is the unique enzyme that acylates ghrelin in a highly conserved manner, it only binds and activates its receptor GHSR-1a when acylated, and its inhibition or stimulation would not affect physiological processes other than ghrelin acylation. It is also important to mention that GOAT has important implications in terms of developing drugs to target the acylation process and, consequently, the physiological effects of AG. [19]

In 2005, using genomic comparative analysis and bioinformatic predictions, a peptide encoded by the ghrelin gene was discovered and it was called obestatin, a contraction of obese, from the Latin "obedere," meaning to devour, and "statin," denoting suppression. [23] This new peptide derives from proghrelin and it has 23 amino acids with a glycine residue on the carboxyterminal domain that can be amidated (Figure 4). It was originally isolated from rat stomach, showing to be a circulating peptide whose secretion is pulsatile and displays an ultradian rhythmicity similar to ghrelin and growth hormone secretion, and then it was shown that it was also found in other tissues like duodenum, pancreas, spleen, mammary glands and plasma. [24, 25] It was originally reported to be the ligand for the orphan receptor GPR39, which belongs to the family of the ghrelin receptor GHSR-1a and the motilin receptor. [24] Despite the initial enthusiasm about the potential of this molecule as a physiological opponent of ghrelin, several observations related to this point have set its effectiveness into question. [26, 27]

[21] González CR, Vázquez MJ, López M, Diéguez C. Influence of chronic undernutrition and leptin on GOAT mRNA levels in rat stomach mucosa. J Mol Endocrinol. 2008; 41: 415-21.
[22] Al-Massadi O, Crujeiras AB, González RC, Pardo M, Diéguez C, Casanueva FF, Seoane LM. Age, sex, and lactating status regulate ghrelin secretion and GOAT mRNA levels from isolated rat stomach. Am J Physiol Endocrinol Metab. 2010; 299: E341-50.
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[24] Zhang JV, Ren PG, Avsian-Kretchmer O, Luo CW, Rauch R, Klein C, Hsueh AJ. Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. Science. 2005; 310: 996-9.
[25] Grönberg M, Tsolakis AV, Magnusson L, Janson ET, Saras J. Distribution of obestatin and ghrelin in human tissues: immunoreactive cells in the gastrointestinal tract, pancreas, and mammary glands. J Histochem Cytochem. 2008; 56: 793-801.
[26] Nogueiras R, Pfluger P, Tovar S, Arnold M, Mitchell S, Morris A, Perez-Tilve D, Vázquez MJ, Wiedmer P, Castañeda TR, DiMarchi R, Tschöp M, Schurmann A, Joost HG, Williams LM, Langhans W, Diéguez C. Effects of obestatin on energy balance and growth hormone secretion in rodents. Endocrinology. 2007; 148: 21-6.
[27] Zhang JV, Jahr H, Luo CW, Klein C, Van Kolen K, Ver Donck L, De A, Baart E, Li J, Moechars D, Hsueh AJ. Obestatin induction of early-response gene expression in gastrointestinal and adipose tissues and the mediatory role of G protein-coupled receptor, GPR39. Mol Endocrinol. 2008; 22: 1464-75.

Consequently, the state-of-knowledge on obestatin suffers from serious gaps, especially for the lack of reproducibility of its central activities. [28, 29]

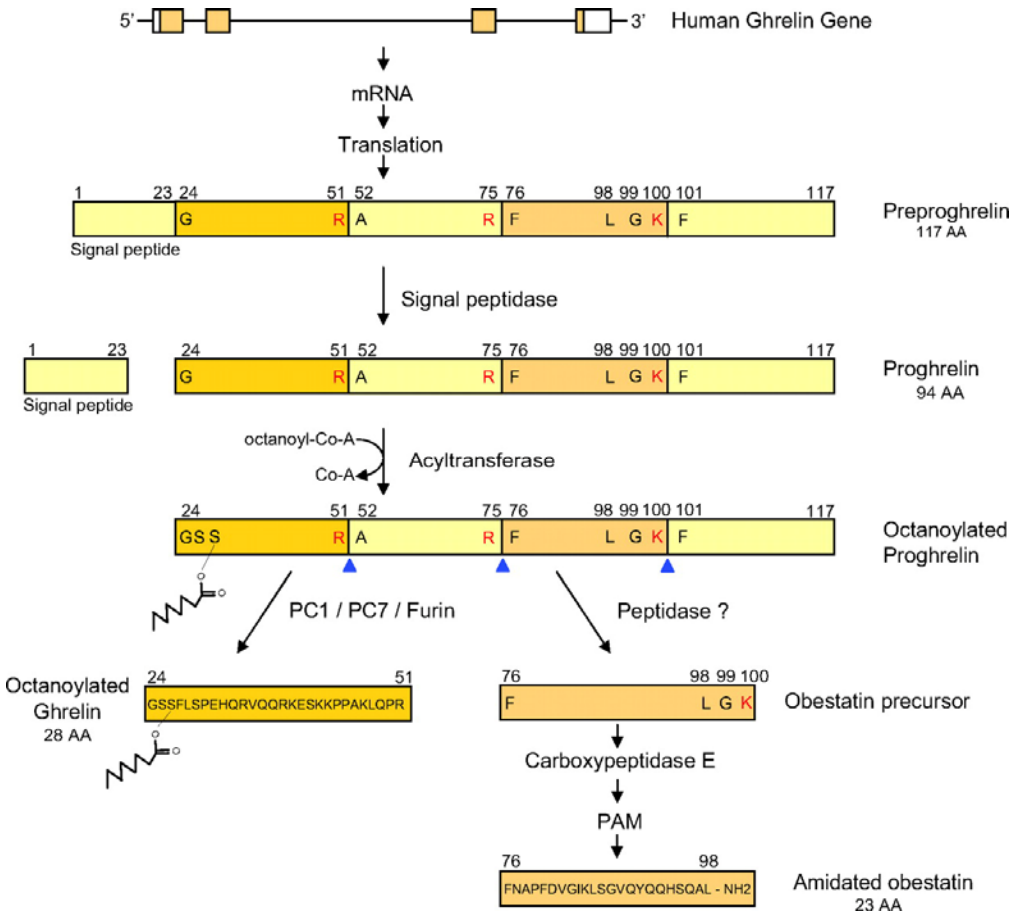

Figure 4. Proposed post-translational processing of preproghrelin to mature ghrelin or mature obestatin. The human *GHRL* gene consists of four exons and three introns. Formation of mRNA and further translation yields a polypeptide of 117 amino acids called preproghrelin. This polypeptide contains a signal peptide of 23 amino acids at the amino terminus, which is first cleaved by a putative signal peptidase resulting in proghrelin with 94 amino acids. Further processing of proghrelin to ghrelin involves proteolytic cleavage by proprotein convertase .Then ghrelin is acylated at the serine 3 by GOAT enzyme. Obestatin results from proteolysis of proghrelin at two cleavage sites. After the cleavage, the carboxy-terminal leucine residue is amidated by a bifunctional enzyme, peptidyl glycine α-amidating monooxygenase (PAM). (Figure extracted from J Clin Endocrinol Metab. 2007; 92: 3396-8.)

[28] Seoane LM, Al-Massadi O, Pazos Y, Pagotto U, Casanueva FF. Central obestatin administration does not modify either spontaneous or ghrelin-induced food intake in rats. J Endocrinol Invest. 2006; 29: RC13-RC15.

[29] Chartrel N, Alvear-Perez R, Leprince J, Iturrioz X, Reaux-Le Goazigo A, Audinot V, Chomarat P, Coge F, Nosjean O, Rodriguez M, Galizzi JP, Boutin JA, Vaudry H, Llorens-Cortes C. Comment on "Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake". Science. 2007; 315: 766.

Keeping aside its controversial anorexigenic activity, there are data suggesting a relevant biological role, like the mitogenic effect described in 3T3-L1 preadipocyte, [27] human gastric carcinoma [30, 31] and pancreatic β-cells.[32] Furthermore obestatin induced *c-fos* expression in gastrointestinal and white adipose tissues through binding to GPR39. [27] Of interest, GPR39 expression in white adipose tissue of rats was up-regulated during fasting whereas GPR39 levels were decreased in cultured mouse embryonic fibroblast cell lines during adipogenesis.[33] In human adipose tissue, decreased GPR39 expression was found in patients with obesity-associated type 2 diabetes mellitus. [34]

Recently a role for obestatin as a regulator of adipocyte metabolism and adipogenesis was proposed, pointing to a putative role of obestatin in the pathogenesis of metabolic syndrome through an autocrine/paracrine manner. [35] It is also shown that obestatin stimulates preadipocyte proliferation, adipocyte differentiation, and fatty acid uptake meanwhile it inhibits lipolysis in 3T3-L1 adipocytes, suggesting that obestatin could stimulate adipose tissue hyperplasia and hypertrophy by various mechanisms. [36] It is also known that obestatin protects cardiac cells against myocardial injury and apoptosis induced by ischemia-reperfusion. These effects are likely to be initiated by specific obestatin binding to receptors present on cardiomyocytes and involve the activation of PI3K and ERK 1/2 pathways. This fact suggests that circulating obestatin also might play a crucial role in protecting the myocardium from

[30] Pazos Y, Alvarez CJ, Camiña JP, Casanueva FF. Stimulation of extracellular signal-regulated kinases and proliferation in the human gastric cancer cells KATO-III by obestatin. Growth Factors. 2007; 25: 373-81.
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prolonged and excessive stress, having potentially important implications in clinical conditions of cardiodegenerative disease and/or ischemic injury. [37]

2.1.2. Ghrelin receptor

The growth hormone secretagogue receptor (GHS-R) was originally discovered in 1997 and it was an orphan receptor until the discovery of ghrelin, its natural ligand. [38] It is mainly expressed in the pituitary and hypothalamus, where ghrelin acts releasing GH and modulating food intake respectively. [6] It is also detected in other organs [39] and tissues [40, 41] presenting a broader expression as for example in thyroid, pancreas, spleen, myocardium, adrenal glands, testis, ovary, placenta and stomach. The human GHSR-1a gene is located on the cromosome position 3q 26.2. [42] There are two types of cDNA after an alternative splicing mechanism, namely receptor type 1a and 1b (Figure 5). [43] The type 1a cDNA codes a 366 amino acids receptor with seven transmembrane domains; it has a molecular weight of 41 KDa and presents high affinity and specifity for ghrelin and the growth hormone secretagogues (GHS). On the other hand, the type 1b cDNA only presents five transmembrane domains and 289 amino acids and it does not allow ligand binding. [44]

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Ghrelin binding to GHSR-1a promotes important changes in the transmmebrane alpha-helix, affecting to its conformation and facilitating the G-protein binding site and its interaction. [10] With regard to GHSR-1a expression level, both GH and leptin hormones inhibit its expression meanwhile ghrelin and growth hormone releasing hormone (GHRH) increase it. [45, 46] In this way, ghrelin and leptin have complementary roles with regard to food intake control and body energetic homeostasis. [47]

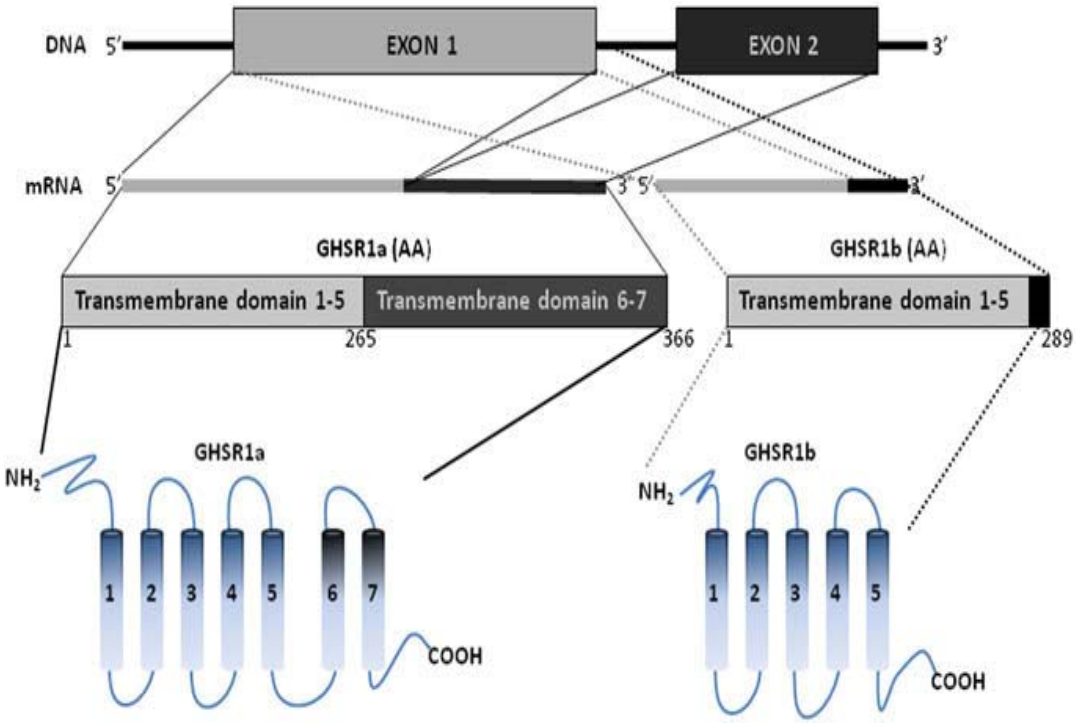

Figure 5. Ghrelin receptors structure. After an alternative splicing mechanism, two types of cDNA encoding ghrelin receptor are generated, named type 1a and 1b. (Figure extracted from Neuropharmacology. 2010; 58: 2-16).

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GHSR-1a levels are also down-regulated by GHS in the pituitary, but in the arcuatus nucleus, ghrelin is able to up-regulate the receptor expression levels. [48, 49]

It is known that GHSR-1a is internalizated after ligand binding promoting its desensitization. These experiments were shown in HEK 293 cell line stably transfected with the ghrelin receptor through radioligand binding assays and confocal microscopy.[50] The results indicate that GHSR-1a is mainly localized at the plasma membrane under unstimulated conditions and rapidly desensitizes after stimulation. The ghrelin/GHSR-1a complex progressively disappears from the plasma membrane after 20 minutes of exposure to ghrelin and accumulates in the perinuclear region after 60 minutes via clathrin-coated pits. In addition GHSR-1a show slow recycling and in this way, its internalization may explain the characteristic physiological responses mediated by this receptor.[50]

The ghrelin receptor belongs to a family that includes the GPR39 as well as receptors for the peptides motilin and neurotensin. For these receptors a high agonist-independent activity was displayed resulting from spontaneous adoption of an activated seven transmembrane receptor conformation (7TM). For example ghrelin receptor signals with 50%, depending on the signal transduction pathway, of its maximal signaling capacity without the presence of any hormone. [51] This phenomenon was shown not only in heterologous expressions systems but also for endogenous GHSR-1a, detected by specific antibodies in both central and gastrointestinal neurons, endothelial cells and astrocytoma cells. Constitutive activity has been demonstrated for many 7TMR *in vitro*, but whether or not constitutive signaling has physiological relevance *in vivo* remains unclear. [52, 53] However, this fact was recently clarified with the identification of naturally occurring human mutations which selectively eliminate its constitutive activity without affecting the affinity, potency, or efficacy of the ghrelin hormone. Importantly, this

[48] Bresciani E, Nass R, Torsello A, Gaylinn B, Avallone R, Locatelli V, Thorner MO, Müller EE.Hexarelin modulates the expression of growth hormone secretagogue receptor type 1a mRNA at Hypothalamic and Pituitary Sites. Neuroendocrinology. 2004; 80: 52-9.

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[52] Holst B, Mokrosinski J, Lang M, Brandt E, Nygaard R, Frimurer TM, Beck-Sickinger AG, Schwartz TW. Identification of an efficacy switch region in the ghrelin receptor responsible for interchange between agonism and inverse agonism. J Biol Chem. 2007; 282: 15799-811.

[53] Holst B, Cygankiewicz A, Jensen TH, Ankersen M, Schwartz TW. High constitutive signaling of the ghrelin receptor--identification of a potent inverse agonist. Mol Endocrinol. 2003; 17: 2201-10.

mutation, which selectively eliminated the constitutive signaling, segregated with the development of short stature and the development of obesity. [54] The high constitutive signaling activity of the GHSR-1a indicates that a compound acting as an efficient inverse agonist could be an interesting antiobesity agent and many efforts are followed in this way. [55]

Because many 7TMR undergo internalization and intracellular trafficking after agonist exposure, often initiated by receptor phosphorylation and subsequent recruitment of β-arrestin proteins, is important to understand how constitutive activity affects GHSR-1a signaling, and how the effects of agonists and inverse agonists are influenced by processes that regulate receptor activity. In this sense, it is known that GHSR-1a undergoes both constitutive and agonist-induced receptor internalization and the molecular determinants of the trafficking profiles reside within the receptor C-terminal domains and that constitutive activity drives GHSR-1a endocytosis, in part by β-arrestin independent mechanisms. [51]

Another interesting point with regard to GHSR-1a is the existence of alternative ligands for this receptor. The GHSR-1a transduces information provided not only by ghrelin but also by the group of GHS not related structurally to ghrelin. [44, 56] This fact is explained by the existence of a common binding domain demonstrated by molecular modeling and site-directed mutagenesis studies developed with GHS peptide and nonpeptide agonists. [57] This binding site might determine that a conserved structure of agonists recognizes a complementary conserved binding pocket which directs the variable part of the ligand and interacts with specific agonist-associated regions determining and overlapping in the agonist-binding site. [58]

A few years ago a role for adenosine as endogenous ligand for GHSR-1a was proposed, showing an intracellular calcium response in HEK 293 and baby hamster kidney (BHK) cells which expressed the GHSR-1a but without any effect with regard to GH release from pituitary

[54] Pantel J, Legendre M, Cabrol S, Hilal L, Hajaji Y, Morisset S, Nivot S, Vie-Luton MP, Grouselle D, de Kerdanet M, Kadiri A, Epelbaum J, Le Bouc Y, Amselem S. Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature. J Clin Invest. 2006; 116: 760-8.

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cells *in vitro*. [59, 60] However this idea was finally rejected although a role for adenosine signaling in controlling the release of ghrelin from the mouse stomach has been recently proposed. [61]

Another proposed ligand for the GHSR-1a is cortistatin. [62, 63] Cortistatin (CST) is a hormone originally described in the rat, mouse, and human cerebral cortex that displays structural and functional similarities to somatostatin (SST). It binds to all five somatostatin receptors (SSTR) and, differently from SST, also binds to MrgX2, which has recently been identified as its specific receptor. [64] Among its functions, CST inhibits GH release from human fetal and adenoma pituitary cells and prolactin (PRL) secretion from cultured prolactinomas. [65] CST also posses other endocrine actions like inhibition of GH and insulin secretion in physiological conditions and in acromegaly and a similar inhibitory effect on PRL and adrenocorticotropic hormone (ACTH) secretion was shown in acromegaly, prolactinoma or in Cushing's disease. [66] Recently, a role for CST as therapeutic agent to autoimmune diseases has been proposed. [67]

Another candidate as GHSR-1a ligand is growth hormone releasing hormone (GHRH). GHRH, a 44-amino acid hypothalamic peptide, is a key regulator of GH secretion (from the pituitary gland together with somatostatin) in which ghrelin is also involved. [68, 69] Ghrelin stimulates GH release both directly, acting at the level of the anterior pituitary gland, and by enhancing GHRH release. [70] The GH-releasing activity of ghrelin is lower than that of GHRH however ghrelin and

[59] Smith RG, Griffin PR, Xu Y, Smith AG, Liu K, Calacay J, Feighner SD, Pong C, Leong D, Pomés A, Cheng K, Van der Ploeg LH, Howard AD, Schaeffer J, Leonard RJ. Adenosine: A partial agonist of the growth hormone secretagogue receptor. Biochem Biophys Res Commun. 2000; 276: 1306-13.

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GHRH show an additive or synergistic effect to stimulate GH secretion when administered *in vivo* [71]. Moreover, it has been reported that for the full GH-releasing effect of ghrelin, the vagus nerve is essential and that also requires a functional hypothalamus-pituitary connection. [72] The growth hormone releasing hormone receptor (GHRH-R) is a seven transmembrane receptor of 52 KDa that belongs to the secretin/glucagon/vasoactive intestinal peptide (VIP) subfamily of GPCR. [73] It stimulates adenylyl cyclase through a G_s protein to increase the production of the cellular second messenger cAMP which will stimulate PKA activation. [74] In addition, in pituitary cells from pigs, ghrelin requires activation of the NOS/NO route, and its subsequent GC/cGMP signal transduction pathway, as necessary steps to induce GH secretion. [75] Recently, it has been shown that GHRH may directly activate the ghrelin receptor modulating the ghrelin-associated intracellular signaling pathways, which open new possibilities on the study of the molecular circuits involved in the regulation of GH release and other ghrelin-mediated actions. [76]

2.1.3. Functions of the ghrelin/GHSR-1a system.

With regard to the signaling systems evoked by ghrelin/GHSR-1a system, the GH release is the most studied. In this particular case, it is well known that after ligand binding the phosphatidylinositol-specific phospholipase C (PI-PLC) is activated, [43, 44] producing inositol (1, 4, 5)-triphosphate (IP_3) and diacylglycerol (DAG). The IP_3 activates calcium release from IP_3-sensitive stores at the ER whereas DAG induces the activation of protein kinase C (PKC) in the plasma membrane. This PKC inhibits the potassium channels causing a depolarization on the plasma membrane promoting an opening of the voltage-dependent L-and- T-type calcium channels. [77] Lately the increase of the intracellular calcium promotes the release of GH to the

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extracellular medium. [70, 78] However, the calcium mobilization can be achieved by an alternative mechanism as in the case of the adenylato cyclase (AC)/cAMP/PKA pathway in porcine somatotropes [79, 80] or chondrocytes. [81] In addition, it has been reported a role for ghrelin in proliferative processes, activating the mitogen-activated protein kinase (MAPK) pathway in different cellular systems. It has been described that ghrelin stimulates the growth of cultured human adrenal zone glomerulosa cells through PKA and PKC-independent mechanisms. [82, 83] In the 3T3-L1 cell line, the mitogenic effect of ghrelin is mediated by the PI3K/Akt and MAPK pathways. [84] In accordance with these pathways, it has been published that ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK and Akt activation. [16] In addition, a role for ghrelin in hepatoma cells has also described, acting through the insulin receptor substrate-1 (IRS-1) phosphorylation. [85] Recently, it was demonstrated that ghrelin also has a positive effect on nitric oxide (NO) synthesis and that several of its activities, such as the GH-releasing effect, [86] food intake, [87] endothelial relaxation, [88] the gastric positive effect [89] and the control of insulin and glucagon secretion [90]

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seem to use the NO pathway. With regard to ghrelin functions in food intake, in 2008 it was demonstrated that the physiological orexigenic response to ghrelin involves specific inhibition of fatty acid biosynthesis induced by AMP-activated protein kinase (AMPK) resulting in decreased hypothalamic levels of malonyl-CoA and increased carnitine palmitoyltransferase-1 (CPT-1) activity (Figure 6). [91] In addition to the complexity around the ghrelin-activated intracellular pathways, the GHSR-1a displays a high degree of ligand-independent signaling activity and receptor trafficking. This constitutive activity leads to the production of inositol phosphate and the activation of cAMP-response element–driven transcription element. Moreover, it is also suggested that GHSR-1a is constitutively internalized, pointing that the control of the receptor expression level is directly correlated to signal activity. [53, 92]

There also are evidences for other receptors than the GHSR-1a, since alternative binding sites have been characterized in the pituitary, thyroid and other tissues. [93, 94] This reveals that peripheral binding sites do not reflect the peripheral distribution of the classic GHSR-1a and that new receptor subtypes may exist which seem specific for peptide GHS but not for ghrelin or non peptide GHS. [95] In addition, binding sites for des-acylghrelin, different receptor subtypes from GHSR-1a for ghrelin and common binding sites for ghrelin and des-acylghrelin have been identified. [16, 56, 81, 96]

[90] Qader SS, Lundquist I, Ekelund M, Håkanson R, Salehi A. Ghrelin activates neuronal constitutive nitric oxide synthase in pancreatic islet cells while inhibiting insulin release and stimulating glucagon release. Regul Pept. 2005; 128: 51-6.

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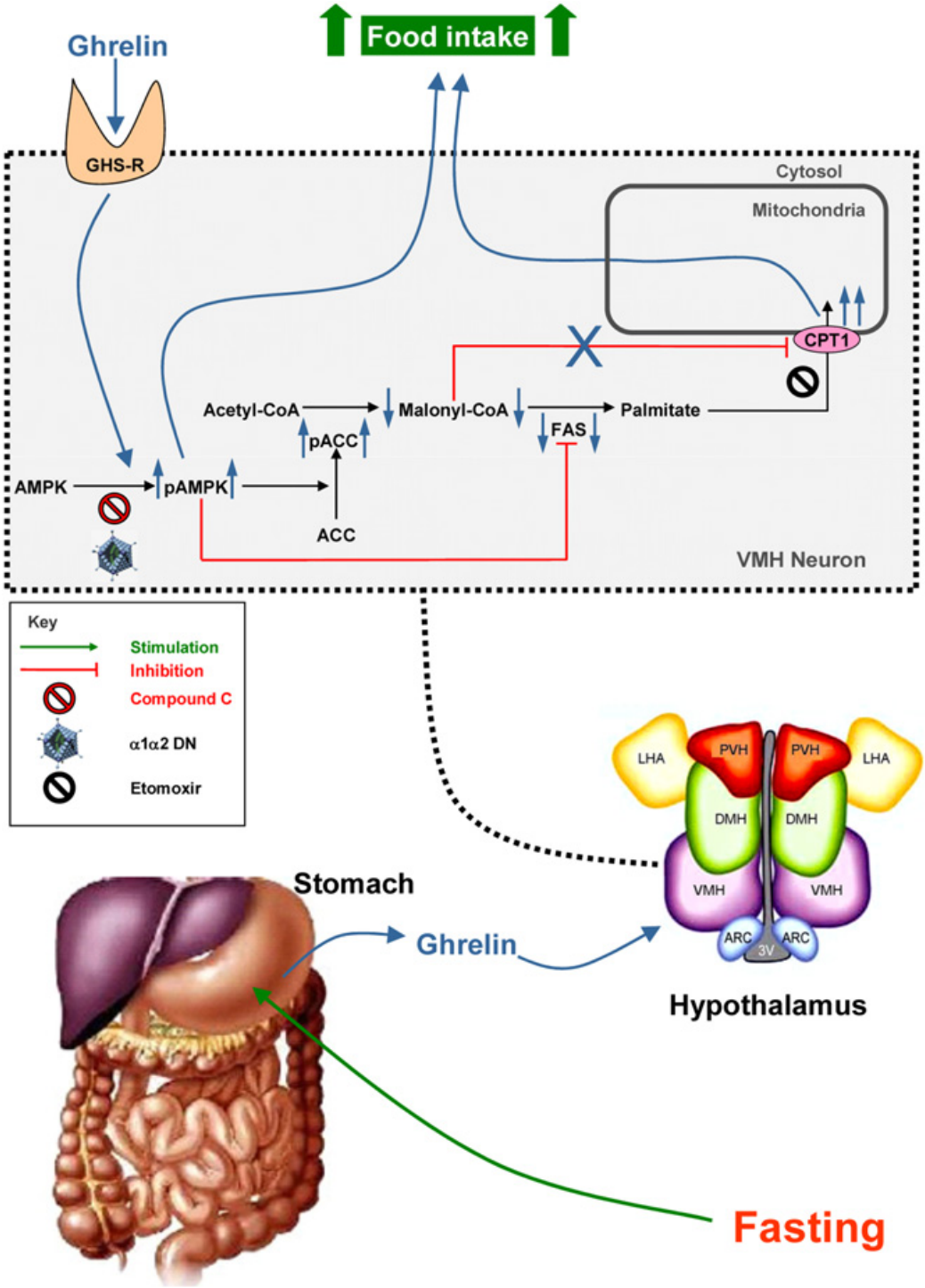

Figure 6. Proposed model of ghrelin actions on hypothalamic fatty acid metabolism. The increase in ghrelin secretion produced by fasting involves the activation of hypothalamic AMPK and inactivation of enzymatic steps of the de novo fatty acid biosynthetic pathway, resulting in decreased malonyl-CoA, leading to activation of CPT-1. Fasting also down regulates FAS in the VMH through an AMPK and ghrelin-dependent mechanisms. (Figure extracted from Cell Metabolism 2008; 7: 389-399).

2.2. The β-arrestins

2.2.1. The β-arrestin family

Arrestins constitute a small family with only four members that interact with G-protein-coupled receptors (GPCR) when these have been activated and phosphorylated by G-protein-coupled receptor kinases (GRK), promoting its desensitization. There are four types of arrestins: visual arrestins (arrestin 1 and 4) and β-arrestins 1 and 2 (arrestin 2 and 3, respectively). Visual arrestin is 60% and 65% identical in amino acid composition to β-arrestin 1 and β-arrestin 2 respectively and predominantly localized in rod photoreceptor cells of the retina but they can also be found in other tissues. [97]

The β-arrestins are 78% identical in amino acid composition and widely expressed in tissues, but their expression level varies in a cell type-specific fashion. The variations in arrestin homology, localization, and expression level suggest that arrestin family members may differ in their abilities to regulate GPCR signaling. [97]

They were originally discovered in the desensitization processes of rhodopsin and β-2 adrenergic receptors (β2AR), acting through the blockade between the receptor and the G-protein. [5] Nowadays, in addition to their role in desensitization, this is, the waning of 7TMR-mediated signals after prolonged exposure to agonist uncoupling the receptors from G-proteins and preventing further signaling, they also play important roles in receptor endocytosis and in signaling, acting as multifunctional adaptor proteins (Figure 7).[4, 97]

The structure of β-arrestins posses two main domains: an amino terminal domain (residues 8-180) and a carboxyl terminal domain (residues 188-362), which is composed of seven β-lamins each one. Both domains are linked by a phosphate sensor which maintains arrestin in an inactive conformation, process that is reversed when the arrestin binds to the phosphorylated receptor. When this happens, there are conformational changes and transductional modifications like dephosphorylation and ubiquitination. [97] This is the reason why there are multiple conformational states of β-arrestins, induced by the binding to phosphorylated receptors in different places or in different combination of places, so β-arrestins would act stabilizing these changes in the receptors. [98] The interaction between the receptor and the β-arrestins is necessary to the function of these one. At the beginning it was believed that there were two states of the receptor, active and inactive, but nowadays it is known that this is not

[97] DeWire SM, Ahn S, Lefkowitz RJ, Shenoy SK. β-arrestins and cell signaling. Annu Rev Physiol. 2007; 69: 483-510.
[98] Shenoy SK, Lefkowitz RJ. Multifaceted roles of β-arrestins in the regulation of seven-membrane spanning receptor trafficking and signalling. Biochem J. 2003; 375: 503-15.

so simple. Two classes of GPCR, designated A and B, are identified and differ in their affinities for the arrestin isoforms. [99] Class A receptors, such as the β2AR, do not interact with visual arrestins and bind β-arrestin 1 with less affinity than β-arrestin 2. Class B receptors, such as the angiotensin II type 1A receptor (AT1AR), interact with visual arrestins and bind both β-arrestin 1 and β-arrestin 2 with similar high affinities. The molecular determinants underlying this classification appear to reside in specific serine residues located in the receptor carboxyl-terminal tail. [100]

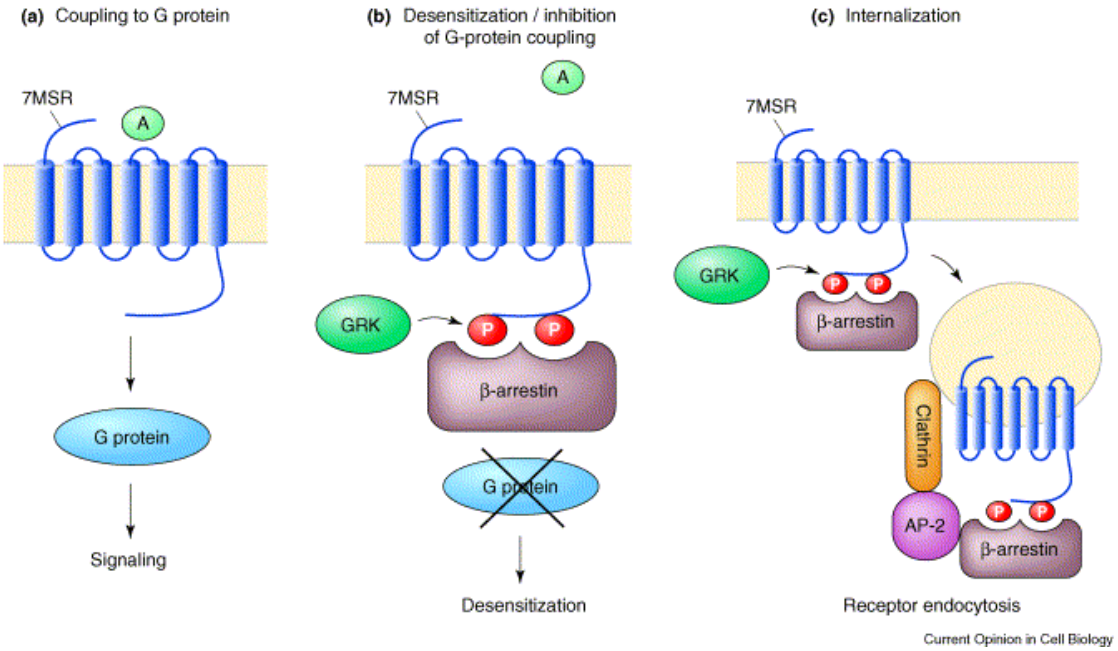

Figure 7. Activation and signaling of GPCR. Ligand binding to the GPCR promotes G-protein activation which lately will produce the activation of a signaling pathway (a). Other option is the binding of β-arrestins to the receptor, after the receptor becomes phosphorylated by GRK, uncoupling the G-protein signaling pathways and promoting its desensitization (b). A third mechanism involves the formation of an endocytosis vesicle through the role of β-arrestins acting as scaffolds, which promotes GPCR internalization (c). (Figure extracted from Curr Opin Cell BIol. 2004; 16: 162-8).

[99] Oakley RH, Laporte SA, Holt JA, Barak LS, Caron MG. Association of β-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. J Biol Chem. 1999; 274: 32248-57.

[100] Oakley RH, Laporte SA, Holt JA, Caron MG, Barak LS. Differential affinities of visual arrestin, β arrestin 1, and β-arrestin2 for G protein-coupled receptors delineate two major classes of receptors. J Biol Chem. 2000; 275: 17201-10.

These findings reveal a potential role for visual arrestins in the regulation of GPCR outside the visual system. Moreover, they suggest that the particular cellular complement of arrestins isoforms and their distinct interactions with intracellular proteins will play a critical role regulating the pattern of GPCR desensitization, sequestration, and resensitization.

2.2.2. Functions

2.2.2.1. Desensitization

Receptor desensitization is an adaptive process that constitutes a basic regulation mechanism that prevents receptor overstimulation in response to prolonged agonist stimulation. This mechanism starts a few seconds after ligand exposure and involves GPCR phosphorylation by second messenger-dependent kinases (PKA, PKC), leading the process known as heterologous desensitization, or by G-protein-coupled receptor-specific serin/threonine kinases (GRK), and leading to homologous desensitization. [2, 101] Second messenger-dependent protein kinase-mediated phosphorylation of serine and threonine residues within the intracellular domain avoids further coupling to G-protein and in the other hand, GRK-mediated phosphorylation increases β-arrestin binding to the receptors, sterically blocking their interaction with G-protein.

2.2.2.2. Endocytosis

An important part of the regulation of GPCR signaling is the agonist-induced internalization of activated GPCR from the plasma membrane into internal compartments. GPCR internalization, also called endocytosis, is initiated within seconds to minutes of agonist exposure and contributes to both desensitization and resensitization of GPCR responsiveness. [98] Endocytosis constitutes a process by which receptors are eliminated from the cellular surface and it also seems not to be necessary for desensitization, but it is for phosphorylation and resensitization of activated receptors. The endocytosis process may be mediated by clathrin-coated pits, caveloae or other uncoated vesicles. [98]

The predominant endocytic route utilized by GPCR is the clathrin-mediated endocytic pathway. This route involves both GRK-mediated phosphorylation and β-arrestin binding, where β-

[101] Shenoy SK, Lefkowitz RJ. Seven-transmembrane receptor signaling through β-arrestin. Sci STKE. 2005; 2005: cm10.

arrestins, in addition to desensitize GPCR, act as a intermediary endocytic adaptor proteins that target GPCR to coated pits via their association with both adaptor protein 2 (AP-2) and clathrin. [97] The interaction between β-arrestin, clathrin and AP-2 and also with phosphoinositides, defines the places where clathrin coated pits will be make up. In the last step, the action of dynamin is required. [102]

The endocytic function of β-arrestin 1 is regulated by phosphorylation, being dephosphorylated when is translocated to the plasma membrane. On the other hand, β-arrestin 2 is regulated by post-transductionals modifications after its binding to the receptor. [103] Once internalizated, GPCR can be degradated in lysosomes or dephosphorylated and recycled back to the plasma membrane as fully functional receptors. [104] It has to be mentioned that there exist a tight relationship between this fact and β-arrestins ubiquitination. [100] β-arrestins modulate post-endocytic GPCR trafficking patterns and appear to be specific in GPCR recycling; therefore, β-arrestins are involved, not only in terminating receptor G-protein coupling but also in initiating processes that regulate re-establishment of receptor responsiveness.

2.2.2.3. Down-regulation

Down-regulation is a process characterized by a decrease of the total number of binding sites evoked by prolonged exposure to agonist. The lately purpose of down regulation is the GPCR resensitization, for which dephosphorylation and ligand dissociation are required. There are evidences that internalization may also be required. [105] In a physiological approach, it is not probable that cells are continuously exposed to hormones due the existence of mechanisms that avoid it. However, this situation can occur in pathophysiological states, as continuous hormone release in tumoral process. [106] The down-regulation mechanism involves two

[102] Barki-Harrington L, Rockman HA. β-arrestins: multifunctional cellular mediators. Physiology. 2008; 23: 17-22.
[103] Shenoy SK, Lefkowitz RJ. Trafficking patterns of β-arrestin and G protein-coupled receptors determined by the kinetics of β-arrestin deubiquitination. J Biol Chem. 2003; 278: 14498-506.
[104] Anborgh PH, Seachrist JL, Dale LB, Ferguson SS. Receptor/β-arrestin complex formation and the differential trafficking and resensitization of β2-adrenergic and angiotensin II type 1A receptors. Mol Endocrinol. 2000; 14: 2040-53.
[105] Luttrell LM. Transmembrane signaling by G protein-coupled receptors. Methods Mol Biol. 2006; 332: 3-49.
[106] Collins S, Bouvier M, Lohse MJ, Benovic JL, Caron MG, Lefkowitz RJ. Mechanisms involved in adrenergic receptor desensitization. Biochem Soc Trans. 1990; 18: 541-4.

different mechanisms: pre-existing receptors degradation and decreasing and blocking of receptors from *de novo* synthesis. [107]

2.2.2.4. Signaling

In recent years, a previously unappreciated mechanism has been elucidated for β-arrestins. In a β-arrestin-dependent mechanism, receptor internalization initiates new sequences of events from receptor desensitization and internalization to scaffolding of kinases to controlling intracellular trafficking of GPCR following endocytosis and signaling. That is, β-arrestins would act as transducers signals (Figure 8).[102, 108]

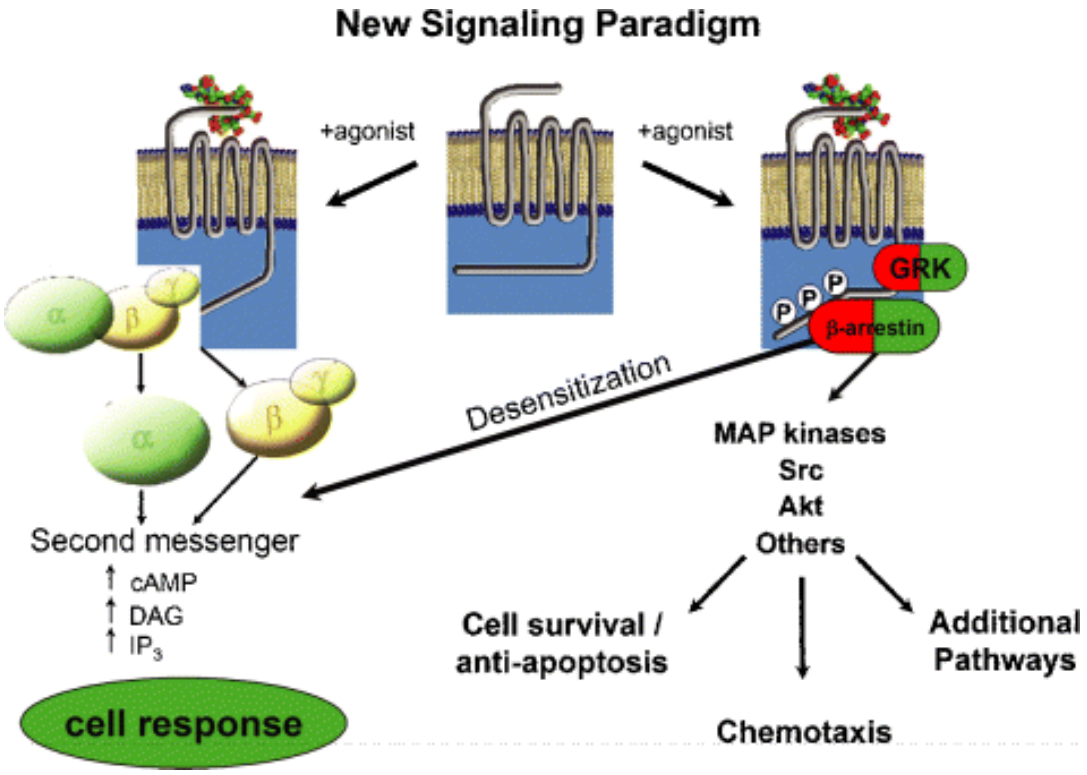

Figure 8. **New paradigm signaling in GPCR.** The binding of agonist to its cognate receptor initiates not only classical G-protein-mediated signalling via second messengers, but also GRK and β-arrestin dependent signals. New pathways with β-arrestin activating proteins such as ERK and Src affect cell responses including anti-apoptotic signalling, cardiac contractility, and dopaminergic behaviours among others. These roles are in addition to the historically defined roles for the GRK and β-arrestins in 7TM receptor desensitization and internalization. (Figure extracted from Acta Physiol. 2007; 190: 9-19).

[107] Böhm SK, Grady EF, Bunnett NW. Regulatory mechanisms that modulate signalling by G-protein-coupled receptors. Biochem J. 1997; 322: 1-18.
[108] Gether U. Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocr Rev. 2000; 21: 90-113.

It is well known that β-arrestins can form complexes whit different signaling proteins including cSrc family tyrosin kinases and components of MAPK pathway.[97, 109] In this way, β-arrestins act as adaptor proteins that provide GPCR-mediated MAPK activation, increase efficiency signaling between the different kinases of the pathway and they also direct them to specific locations inside the cells. This fact supposes important functional roles.

A well documented example is the receptor tyrosin kinase–mediated ERK 1/2 activation. [110] In this example, after ligand binding, the receptor tyrosin kinase (RTK) reclutes Sos protein to plasma membrane, where it activates Ras and this one activates Raf-1, being translocated to plasma membrane. Lately, MAPK are activated and ERK 1/2 is translocated to the nucleus, where mitogenesis will be stimulated, or will be retain into the cytosol, where it will phosphorylate different cytosolic targets. [111] The receptor tyrosin kinase activation also initiates the clathrin coated pits formation, allowing the recycling of the receptor or its degradation. There are multiple examples where many GPCR activate MAPK signaling pathways and where endocytosis is a mechanism required. [112, 113] Nevertheless, there are also other reports where MAPK activation can occur in an independent way to the endocytosis mechanism. [112] The explanation to this question is exposed by two hypotheses closely related. The first one suggests that endocytosis would add an additional specific information layer to signaling pathways through its compartmentalization. The second one proposes that endocytosis would allow transporting the signaling complexes to specific locations inside the cells.

The G-protein-mediated signaling is characterized by a rapid and transient mechanism which involves nuclear translocation of ERK 1/2. On the other hand, β-arrestin-mediated signaling is a slow, persistent one which involves the cytosolic retention of ERK 1/2. This supposes different physiological consequences. [102] In addition, it is well established that activation of a GPCR can promote G-protein-independent/β-arrestin-mediated signaling, suggesting that receptors can assume distinct conformational states that initiate multiple signaling pathways. This represents

[109] Ahn S, Shenoy SK, Wei H, Lefkowitz RJ. Differential kinetic and spatial patterns of β-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. J Biol Chem. 2004; 279:35518-25.
[110] Ferguson SS. Receptor tyrosine kinase transactivation: fine-tuning synaptic transmission. Trends Neurosci. 2003; 26: 119-22.
[111] Peyssonnaux C, Eychène A. The Raf/MEK/ERK pathway: new concepts of activation. Biol Cell. 2001; 93: 53-62.
[112] Kim SJ, Kim MY, Lee EJ, Ahn YS, Baik JH. Distinct regulation of internalization and mitogen-activated protein kinase activation by two isoforms of the dopamine D2 receptor. Mol Endocrinol. 2004; 18: 640-52.
[113] Qian H, Pipolo L, Thomas WG. Association of β-Arrestin 1 with the type 1A angiotensin II receptor involves phosphorylation of the receptor carboxyl terminus and correlates with receptor internalization. Mol Endocrinol. 2001; 15: 1706-19.

an opportunity for development of novel therapeutics targeting selective biological effects of a given receptor. Moreover, it now appears that, for some receptors, multiple ligands can differentially direct signaling down β-arrestin instead of G-protein signaling pathways. [4] This concept is called the biased agonism. Biased agonism is a property of the ligand-receptor complex and so, a ligand or a receptor may be biased. A biased ligand favors one response over another (either G-protein or β-arrestin) compared with the endogenous ligand, which is considered to be neutral. A biased receptor is only capable of signaling through a restricted subset of pathways that are typically available to that class of receptor.[102, 114] For example, some receptors such as AT1R, several chemokine receptors and μ-opioid receptors have ligands that are biased, that is, compounds that stimulate only β-arrestin-dependent pathways. [115, 116] Such ligands are of considerable interest in physiological and pathological settings because of their potential benefit to drive receptor signaling in desired directions.

Although biased agonism was recently proposed, there are still gaps in the understanding of bias and with regard to the different receptor conformations that are responsible for signaling to G-proteins and β-arrestins. Some hypothesis point to a role for GRK or cofactors controlling G-protein versus β-arrestin signaling but more studies are required. [117] These ones will serve to a better understanding of the different contributions of specific pathways to the regulation of physiological functions and also as targeted agents for clinical therapies. [4] In a similar way it is unclear whether such selective G-protein uncoupling is caused by a lack of ability to interact with G-proteins or rather by an increased ability of the receptor to recruit β-arrestins (Figure 9). Since uncoupling of G-proteins by increased ability to recruit β-arrestins could lead to different cellular or *in vivo* outcomes than lack of ability to interact with G-proteins, it is essential to distinguish between these two mechanisms. These findings have important implications for drug discovery and 7TMR biology and illustrate the necessity of uncovering the exact molecular determinants for G-protein coupling and β-arrestin recruitment, respectively. [118]

[114] Violin JD, Lefkowitz RJ. β-arrestin-biased ligands at seven-transmembrane receptors. Trends Pharmacol Sci. 2007; 28: 416-22.
[115] Whistler JL, von Zastrow M. Morphine-activated opioid receptors elude desensitization by β-arrestin. Proc Natl Acad Sci U S A. 1998; 95: 9914-9.
[116] Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM, Lefkowitz RJ. Independent β-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. Proc Natl Acad Sci U S A. 2003; 100: 10782-7.
[117] Whalen EJ, Foster MW, Matsumoto A, Ozawa K, Violin JD, Que LG, Nelson CD, Benhar M, Keys JR, Rockman HA, Koch WJ, Daaka Y, Lefkowitz RJ, Stamler JS. Regulation of β-adrenergic receptor signaling by S-nitrosylation of G-protein-coupled receptor kinase 2. Cell. 2007; 129: 511-22.
[118] Bonde MM, Hansen JT, Sanni SJ, Haunsø S, Gammeltoft S, Lyngsø C, Hansen JL. Biased signaling of the angiotensin II type 1 receptor can be mediated through distinct mechanisms. Plos One. 2010; 5: e14135.

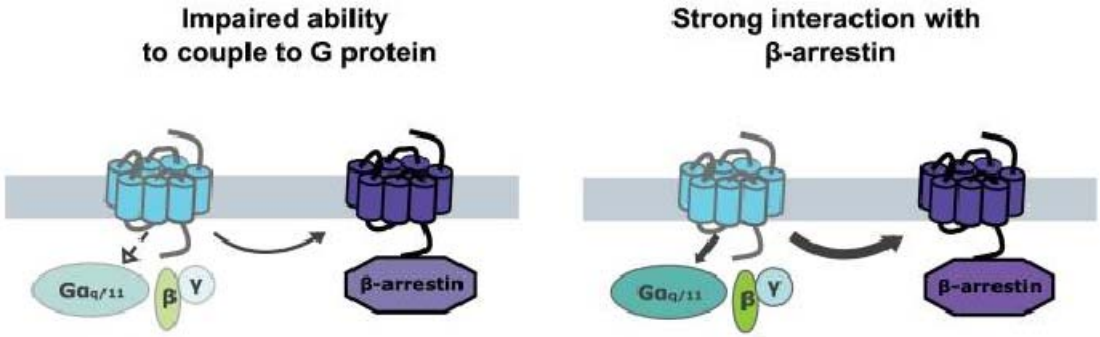

Figure 9. Schematic presentation of possible mechanism underlying differential activation. Hypothetically, a receptor mutant selectively activating β-arrestin induced pathways could either be impaired in G-protein coupling (right) or show very strong interaction with β-arrestins (left). (Figure extracted from Plos One, 2010; 5: e14135).

3. METHODS

3.1. Materials

Human ghrelin was obtained from Global Peptide (Fort Collins, CO, USA). Pertussis toxin (PTX), concanavalin A (Con A) and Phorbol 12-myristate 13-acetate (PMA) were from Sigma (St. Louis, MO, USA). Wortmannin, BAPTA-2 AM, calphostin C, Gö6976, PP2, PP3, genistein and cortistatin-17 were purchased from Calbiochem (San Diego, CA, USA). Fura-2 acetoxymethylester (FURA-2 AM) was obtained from Molecular Probes (Eugen, OR, USA).

Anti-phospho-p44/42 MAPK, anti-p44/42 MAPK rabbit polyclonal, anti-phospho-PKC α/β rabbit polyclonal, anti-phospho-PKC μ rabbit polyclonal, anti-phospho-cSrc (Tyr 416) rabbit polyclonal, anti-phospho cSrc (Tyr 527) rabbit polyclonal, anti-pAkt HM (S473), anti-pAkt A-loop (T308), anti-Akt rabbit polyclonal, anti-Rictor rabbit polyclonal, anti-mTOR rabbit polyclonal and anti-pPDK-1 (S241) rabbit polyclonal antibodies were from Cell Signaling Technology (Beverly, MA, USA). Anti-β-arrestin 1 goat polyclonal, anti-β-arrestin 2 rabbit monoclonal, anti-GHSR (H-80) rabbit polyclonal, anti-Raf-1 rabbit polyclonal, anti-cSrc rabbit polyclonal, anti-PI3K (p85) rabbit polyclonal, anti-phospho-PKC ε rabbit polyclonal, anti-phospho-PKC δ rabbit polyclonal, anti-phospho-PKC ζ rabbit polyclonal, anti-SHP-1 (H-65) mouse polyclonal, anti-pSHP-1 (Y536) rabbit polyclonal, anti-actin (H-300) rabbit polyclonal antibodies and protein A/G agarose were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-tyrosine rabbit polyclonal antibody was from Upstate Technology (Lake Placed, NY, USA). β-arrestin 1 siRNA, β-arrestin 2 siRNA, cSrc siRNA and siRNA control were likewise from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SSTR2 siRNA was provided by Dharmacon (CO, USA). Rictor siRNA was synthesized by Cell Signaling (Beverly, MA, USA). Anti-rabbit horseradish peroxidase was from GE-Amersham (Buckinghamshire, UK), while anti-goat horseradish peroxidase was from Santa Cruz (Santa Cruz, CA, USA). Rabbit anti-rat β-arrestin 1C-terminal (A1CT) antiserum was provided by Prof. R.J. Lefkowitz (Duke University Medical Center, Durham, NC, USA).

Protease and phosphatase inhibitor cocktails were obtained from Sigma (St. Louis, Mo, USA). Pro-Q Diamond phosphoprotein gel stain, SYPRO® Ruby and Lipofecamine 2000 were from Invitrogen (Carlsbad, CA, USA).

[^{125}I]-ghrelin and QuickPrepTM Micro mRNA Purification Kit were from GE Healthcare Amersham Pharmacia (Ailington Heghts, IL, USA). [^{125}I]-CST-17 was from Phoenix Pharmaceutical (Burlingame, CA, USA).

3.2. Cell culture

HEK 293 cells, which stably express the human ghrelin receptor 1a (HEK-GHSR-1a), were cultured in 100-mm diameter dishes in high glucose DMEM containing 10% (v/v) fetal calf serum plus 500 μg/ml geneticin G-418 to 70–80% confluence for 3 days as described previously. [50] Nontransfected HEK 293 were seeded in 100-mm dishes and cultured to 80% confluence for 2 days in DMEM supplemented with 10% (v/v) fetal calf serum. Media were supplemented with penicillin G (100 U/ml) and streptomycin sulfate (100 μg/ml). Cells were grown under a humidified atmosphere of 95% air, and 5% CO_2 at 37°C.

3T3-L1 preadipocyte cells were obtained from American Type Culture Collection and maintained in DMEM containing 10% calf serum, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were grown under a humidified atmosphere of 95% air, and 5% CO_2 at 37°C. Confluent 3T3-L1 cells were treated with 0.5 mM isobutylmethylxantine (IBMX), 25 μM dexamethasone (DEX), and 861 nM (5 μg/mL) insulin for 3 days and maintained in DMEM containing 10% FBS, 100 U/mL penicillin, 100 U/mL streptomycin and supplemented with 172 nM (1 μg/mL) insulin for 10 days after the beginning of differentiation as described previously.[35]

3.3. Immunoblotting analysis

Serum-starved cells were stimulated for the indicated time period at 37°C. The media was then aspirated and the cells were lysed in ice-cold RIPA buffer [Tris-HCl (pH 7.2), 50 mM; NaCl, 150 mM; EDTA, 1 mM; NP-40, 1% (v/v); Na-deoxycholate, 0.25% (w/v); protease inhibitor cocktail; phosphatase inhibitor cocktail]. The solubilized lysates were transferred into centrifuge tubes and left at 4°C for 15 minutes, then pre-cleared by centrifuging at 13,000g for 15 minutes. Protein concentration was evaluated with the QuantiProTM BCA assay kit. Subsamples (same amount of protein) of each sample were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The blots were incubated with 5% non-fat dry milk in TBST [Tris-HCl (pH 8.0), 20 mM; NaCl, 150 mM; Tween-20, 0.1% (v/v); used for all incubation and washing steps] for 1 hour. Next, blots were incubated for 1 hour with the corresponding antibodies according to the manufacturer's instructions. Blots were subsequently incubated with the corresponding peroxidase-conjugated IgG antibody. After washing, signals were visualized using an enhanced chemiluminescence detection system.

3.4. [^{125}I]-ghrelin internalization assays

Confluent HEK-GHSR-1a monolayer cells (70-80%) were resuspended in binding buffer [containing, DMEM (pH 7.4) plus 1% (w/v) BSA], centrifuged at 500g for 3 minutes at room temperature, washed twice and then resuspended (1 x 100-mm plate/1.5 mL; 5-6 x 10^5 cells per aliquot) in binding buffer supplemented with 100.000 cpm/aliquot of [^{125}I]-ghrelin for 2 hours at 4°C. The media containing labeled ghrelin were removed and cells were washed twice with ice-cold binding buffer. Fresh binding buffer (0.5 mL) was added and cells were incubated at 37°C for periods from 0 to 60 minutes. At each time point, cells were pelleted and the incubation medium was treated with 10% (w/v) trichloroacetic acid (TCA) at 4°C for 1 hour. The TCA-insoluble materials were pelleted by centrifugation at 10,000g for 10 minutes at 4°C. Supernatant and insoluble fractions were counted to determine postactivation releasable label and TCA-insoluble material, respectively. Cell-surface-bound ghrelin was removed by resuspending the cells in ice-cold acid buffer for 15-20 minutes at 4°C. The cells were pelleted by centrifugation and the supernatants, corresponding to cell-surface-bound ghrelin, were counted. Finally, the pellet obtained was solubilized in lysis buffer (Nonidet P-40, 1%; Triton X-100, 0.5% and NaOH, 1M) and radioactivity, corresponding to internalized ghrelin, was measured as previously described. [50]

3.5. Assays in K^+-depleted medium

HEK-GHSR-1a cells were resuspended in HEPES-buffered saline [HEPES (pH 7.4), 100 mM; NaCl, 120 mM; $MgSO_4$, 1.2 mM; EDTA, 1 mM; CH_3COONa, 15 mM; $CaCl_2$, 1 mM; glucose, 10 mM] complemented with 1% (w/v) BSA. Intracellular potassium depletion was carried out by incubating the cells for 5 minutes at 37°C in hypotonic medium [HEPES-buffered saline: water, 1:1 (v/v)], followed by 60 minutes of incubation in HEPES-buffered saline supplemented with (control) or without 10 mM K^+ at 37°C. For [^{125}I]-ghrelin internalization experiments, cells were then washed and resuspended in HEPES-buffered saline supplemented with 100,000 cpm/aliquot of [^{125}I]-ghrelin for 2 hours at 4°C. After incubation, cells were washed and then incubated at 37°C for various periods of time. At each time point, cells were pelleted and the internalized ghrelin was measured as previously described. [50] For immunoblotting analysis, cells were preincubated in medium with or without K^+ and then the cells were stimulated with ghrelin at 37°C for the time periods indicated, lysed and analyzed by immunoblotting analysis.

3.6. Immunoprecipitation

Serum-starved cells were stimulated for the indicated time period at 37°C and lysed in ice-cold non-denaturing NP-40 solubilization buffer [immunoprecipitation lysis buffer (ILB), Tris-HCl (pH 7.5), 20 mM; NaCl, 150 mM; EDTA, 1 mM; NP-40, 1% (v/v); protease inhibitor cocktail, phosphatase inhibitor cocktail]. 500 µg of total protein was pre-washed with 20 µL of 50% protein A/G-agarose for 30 minutes at 4°C and then incubated with 1 µg of the corresponding antibody (overnight at 4°C) followed by addition of 40 µL of 50% protein A/G (2 hours at 4°C). After washing two times with ILB, the pelleted beads were resuspended in Laemmli sample buffer. Proteins were analyzed by 10% SDS-polyacrylamide gels, followed by immunoblotting analysis or visualized in acrylamide gels using Pro-Q Diamond (phosphoproteins) and SYPRO® Ruby staining solutions.

3.7. Small interfering RNA (siRNA) silencing of gene expression

Chemically synthesized double-stranded siRNA duplexes (with 3´ dTdT overhangs) were purchased from Santa Cruz Biothecnology 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´-GUAGAUUUCAGAUGACUAU-3´) or from Cell Signaling for the following target: Rictor (5´-CACUUCGAUUAGUCAGAAA-3´, 5´-CGCUUACUUUGCCUAACAA-3´, 5´-CCAACUGAGUGCAAUAUGU-3´). A non-silencing RNA duplex was used as a control for all siRNA experiments. Cells were transfected with Lipofectamine 2000, according to manufacturers' instructions. Silencing was quantified by immunoblotting. Only experiments with verified silencing were used.

The sequence of SSTR2 siRNA was not provided.

3.8. Plasmid and cell transient transfection

The cDNA encoding Gβγ sequester β-ARK-CT (gift of P. Voigt, Institute of Pharmacology, Charite´-Medical Universiy, Campus Benjamin Franklin, Berlin, Germany) was transfected into subconfluent HEK-GHSR-1a cells using Lipofectamine 2000 following the manufacture's

protocol. The β-ARK-CT incorporation was confirmed by means of intracellular calcium measurements in transfected cells before and after treatment, as previously described. [119]

SHP-1/C453S (SHP-1dn) dominant negative mutant was used. [120] Cells ($3x10^5$) were transfected with 1 μg of SHP-1dn plasmid using Lipofectamine 2000 as previously described. [119] To confirm the SHP-1 incorporation immunoblot analysis was used.

3.9. Calcium measurements

Intracellular calcium measurements were performed in HEK-GHSR-1a cell suspensions using the fluorescent calcium probe FURA-2 AM as described previously. [50] Briefly, cells were resuspended in Krebs-Ringer-HEPES [KRH; containing HEPES (pH 7.4), 25 mM; NaCl, 125mM; KCl, 5 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 2 mM; glucose, 6 mM] and loaded with 3 μM FURA-2 AM for 45 minutes at room temperature under gentle continuous mixing. Cell suspensions were diluted 1:4 with KRH and maintained at room temperature until use. For each measurement, around 5-6 x 10^5 cells were resuspended in 2 mL KRH and then placed in a cuvette positioned in a holder and thermostatically controlled at 37°C. The fluorescence signal was measured under continuous stirring in an LS-50B fluorometer (PerkinElmer, Boston, Ma) in ratio mode, using 345 nm as λ_{1ex}, 380 nm as λ_{2ex} and 490 nm as λ_{em}. Each measurement was calibrated by the cell lysis method. [50]

3.10. Immunohistochemistry

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 hours, dehydrated and embedded in paraffin by a standard procedure. Sections, 5 μm thick, were mounted on Histobond Adhesion Microslides (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;

[119] Theodoropoulou M, Zhang J, Laupheimer S, Paez-Pereda M, Erneux C, Florio T, Pagotto U, Stalla GK. Octreotide, a somatostatin analogue, mediates its antiproliferative action in pituitary tumor cells by altering phosphatidylinositol 3-kinase signaling and inducing Zac1 expression. Cancer Res. 2006; 66: 1576-82.

[120] Pagès P, Benali N, Saint-Laurent N, Estève JP, Schally AV, Tkaczuk J, Vaysse N, Susini C, Buscail L. sst2 somatostatin receptor mediates cell cycle arrest and induction of p27(Kip1). Evidence for the role of SHP-1. J Biol Chem. 1999; 274: 15186-93.

Glostrup, Denmark); 2) EnVision peroxidase rabbit (Dako; CA, USA) used as the detection system; 3) 3, 3´-diaminobenzidine-tetrahydrochloride (Dako Liquid DAB + Substrate-chromogen system). Cells were faintly counterstained with Harris' haematoxylin.

3.11. RNA isolation and qRT-PCR

Total RNA from HEK 293 or HEK-GHSR-1a cells was extracted using the Trizol reagent (Life Technologies, Gaithersburg, MD, USA) following the manufacturer's instructions. The amount of RNA recovered was determined by the Ribogreen RNA quantification kit (Molecular Probes). Total RNA (1 μg) was reverse transcribed (RT) in a 20 μl volume using the reagents supplied in the cDNA First-Strand Synthesis kit (Fermentas) and cDNA was treated with RNAase H. One microliter aliquots of the resulting cDNA were amplified by real-time PCR using the SYBR PCR Master Mix (Bio-Rad) and the following primers: SSTR1: sense (5´-CACATTTCTCATGGGCTTCCT-3´), antisense (5´-ACAAACACCATCACCACCATC-3´); SSTR2: sense (5´-GGCATGTTTGACTTTGTGGTG-3´), antisense (5´-GTCTCATTCAGCCGGGATTT-3´); SSTR3: sense (5´-TGCCTTCTTTGGGCTCTACTT-3´), antisense (5´-ATCCTCCTCCTCAGTCTTCTCC-3´); SSTR4: sense (5´-CGTGGTCGTCTTTGTGCTCT-3´), antisense (5´-AAGGATCGGCGGAAGTTGT-3´); SSTR5: sense (5´-CTGGTGTTTGCGGGATGTT-3´), antisense (5´-GAAGCTCTGGCGGAAGTTGT-3´); sst5TMD5: sense (5´-GCGCCGTCTTCATCATCTAC-3´), antisense (5´-CAGGAAAAGCTGGTGTTTGG-3´); sst5TMD4: sense (5´-TACCTGCAACCGTCTGCC-3´), antisense (5´-AGCCTGGGCCTTTCTCCT-3´); β-actina: sense (5´- ACTCTTCCAGCCTTCCTTCCT-3´), antisense (5´-CAGTGATCTCCTTCTGCATCCT-3´). Thermal cycling profile consisted of a preincubation step at 95°C for 10 min, followed by 40 cycles of denaturation (95°C, 30 seconds), annealing (61–64°C, 1 minute), and extension (72°C, 30 seconds). The amplified products generated a single 165-bp product for SSTR1, a single 185-bp product for SSTR2, a single 190-bp product for SSTR3, a single 174-bp product for SSTR4, a single 183-bp product for SSTR5, and a single 176-bp product for β-actin. The thermocycling and fluorescence detection were performed using a Stratagene Mx3000p Real-Time PCR machine (Stratagene, La Jolla, CA, USA).

3.12. Whole cell binding assay

HEK-WT or HEK-GHSR-1a cells were resuspended in binding buffer [containing, DMEM (pH 7.4) plus 1% (w/v) BSA], centrifuged at 500g for 5 minutes at room temperature, washed twice and

resuspended in proportion 1x100 mm plate/1.5 mL (500,000 cells/aliquot) in binding buffer supplemented with the radioligand [[^{125}I]-CST-17, 100.000 cpm/aliquot; [^{125}I]-ghrelin; 100.000 cpm/aliquot] in the presence or absence of unlabeled competitor for 2 hours at 4° C. After incubation, media were removed and the pellet was washed twice with binding buffer at 4°C. Cell surface radioligand was determined by incubating the cells in 0.5 ml ice cold acid buffer [containing, acetic acid (pH 2.0), 0.2 M and NaCl, 0.5 M] for 10 minutes at 4°C. Finally, the cells were centrifuged and the supernatants were counted in a γ counter.

3.13. Animals

Adult swiss male mice (8 weeks-old) were housed in air-conditioned rooms (22-24°C) under a 12:12 hours light/dark cycle and fed with standard chow or high fat diet during twelve weeks. (D12451, 45 kcal % fat, Research Diets, Inc, NJ, USA). Food intake and body weight were measured weekly during the experimental phase. Animals were sacrificed by decapitation between 10:00 and 12:00 hours when they were 20 weeks-old. Animal experiments were conducted in accordance to the standards approved by 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. Excised adipose tissue was immediately transported to the laboratory in ice-cold Krebs-Ringer-HEPES buffer [KRH; containing HEPES (pH 7.4), 25 mM; NaCl, 125 mM; KCl, 5 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 2 mmM; KH_2PO_4, 2 mM; glucose, 6 mM]. After removing blood vessels and conjunctive tissue, adipose tissue was washed with sterile Krebs-Ringer-HEPES. 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 hour at 37°C under a humidified atmosphere of 95% air and 5% CO_2, the media were aspirated, and fresh medium, with or without ghrelin (100 nM, 1 hour), were 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; EDTA, 1 mM; NP-40, 1% (v/v); Na-deoxycholate, 0.25% (w/v); protease inhibitor cocktail, phosphatase inhibitor cocktail] for immunoblotting analysis.

3.14. Data analysis

The results were expressed as the mean±SE. Differences between means were evaluated by one-way analysis of variance (ANOVA). (*, #, $P<0.05$).

4. RESULTS

CHAPTER 1

Stimulation by ghrelin of p42/p44 mitogen-activated protein kinase through the GHSR-1a receptor: role of G-proteins and β-arrestins.

Since the temporal pattern of ERK 1/2 activation is one of the key determinants that establish its biological responses, first of all we wanted to investigate the pattern of ERK 1/2 activation induced by ghrelin through the binding to its receptor, the GHSR-1a. For this purpose, we examinated the time-course of ghrelin-induced ERK 1/2 activation (100 nM) at different time points in HEK-GHSR-1a cells. The result obtained is shown in Figure 1 where we can observe a maximal ERK 1/2 phosphorylation within 5-10 minutes of ghrelin stimulation, decreasing to approximately 50% of the maximum by 60 minutes after stimulation. This result suggest the existence of different pathways by which ghrelin, through GHSR-1a, activates ERK 1/2, pointing to a role for the G-proteins and to the receptor internalization process.

To assess the role of receptor internalization in the ghrelin-induced ERK 1/2 activation we first tested the effect of potassium-depleted medium, a condition that significantly affects the formation of clathrin-coated pits and internalization through this pathway. Figure 2 A shows this condition inhibited the internalization process of [^{125}I]-ghrelin. Under the same conditions the impact of GHSR-1a internalization on ERK 1/2 activation was also evaluated, showing an inhibition of ghrelin-induced ERK 1/2 phosporylation (Figure 2 B), demonstrating that the internalization process contributes primarily to the late time component of ERK 1/2 activation. By subtracting the control curve (with potassium; blue line) from the internalization-independent curve (without potassium; red line) an estimation of the internalization-dependent pathway can be obtained (dotted curve, green line).

Additionally, the impact of internalization of GHSR-1a on ERK 1/2 activation was checked using two independent experimental treatments previously demonstrated to block internalization by clathrin coated pit pathways, pretreatment with hypertonic sucrose or Concanavalin A (Con A). Hypertonic sucrose pretreatment was a non-viable strategy in this cell line, since it caused ERK 1/2 activation without ghrelin treatment (data not shown). The second approach, pretreatment with Con A (250 mg/ml, 30 min), which blocks receptor clustering, inhibited the ghrelin-induced ERK 1/2 phosphorylation (45% inhibition; data not shown). These results suggest the existence of at least two pathways by which GHSR-1a might activate ERK 1/2, one mediated by G-proteins and the other one related to GHSR-1a endocytosis.

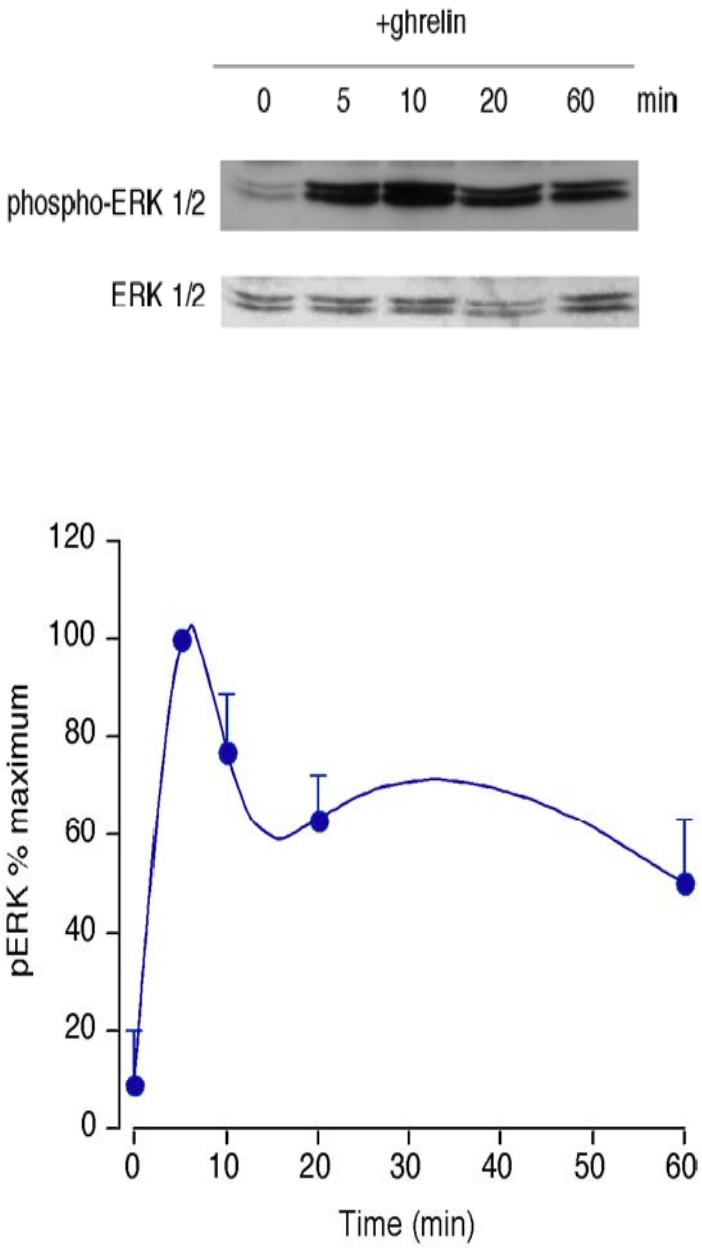

Figure 1. Time-course of the effect of ghrelin on phosphorylation of ERK 1/2. Serum-starved HEK-GHSR-1a cells were stimulated with ghrelin (100 nM) for the time periods indicated. Cells were lysed and analyzed by immunoblotting using specific antibodies against phospho-ERK 1/2 and ERK 1/2. ERK 1/2 phosphorylation was quantified by densitometry and expressed as a percentage of the maximal phosphorylation (mean±SE). Immunoblots are representative of three independent experiments.

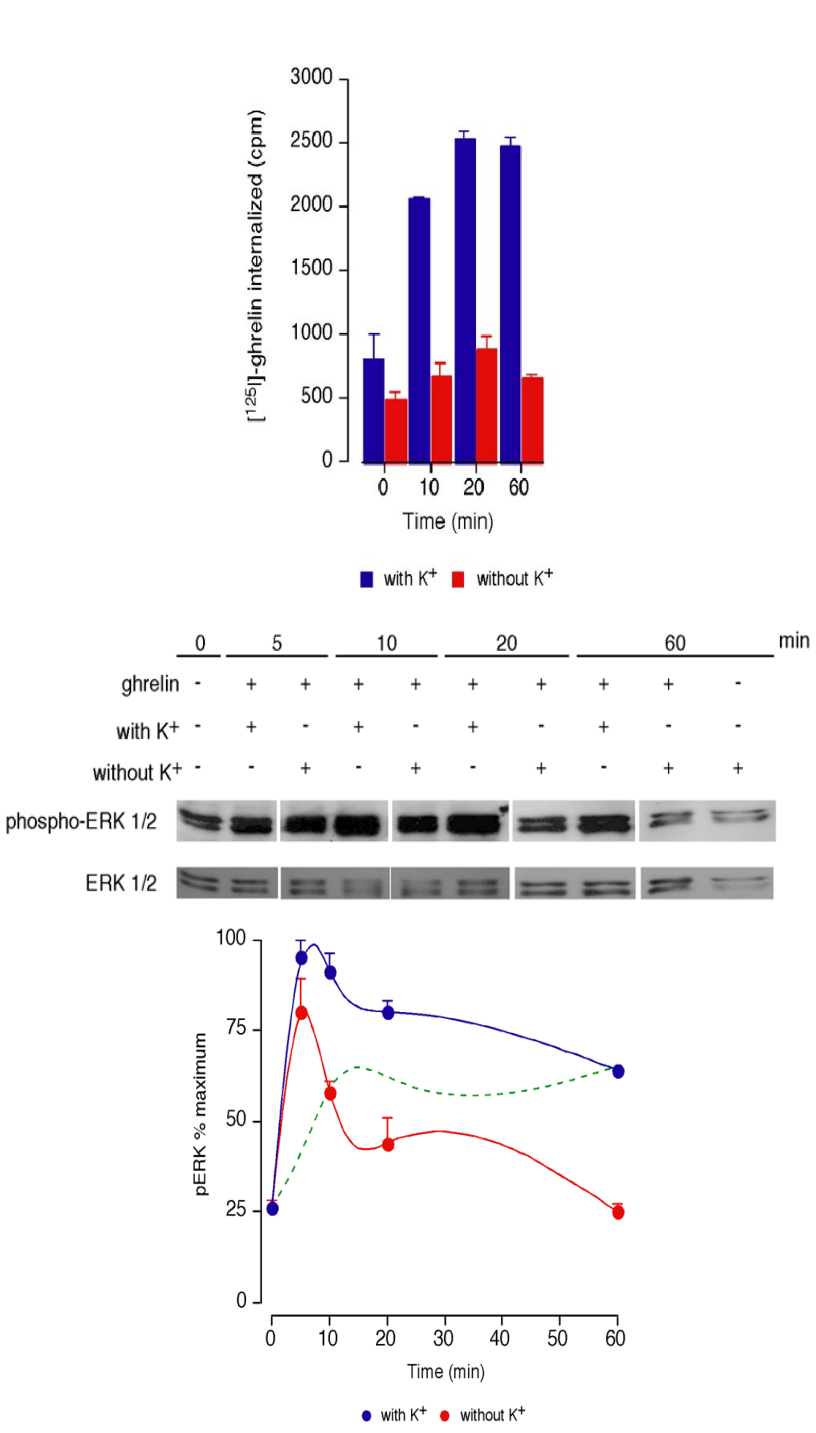

Figure 2. Effect of potassium depletion on the internalization of [^{125}I]-ghrelin and on the phosphorylation of ERK 1/2. A. Cells were preincubated with [^{125}I]-ghrelin for 2 h at 4°C and then incubated at 37°C in medium with or without K^+ for the time periods indicated. At each time point, cells were washed and surface [^{125}I]-ghrelin was removed by acid stripping, and the remaining cells were then solubilized in NaOH ([^{125}I]-ghrelin internalized). B. Cells were preincubated in medium with or without K^+, then stimulated with ghrelin (100 nM) for the time periods indicated and analyzed by immunoblotting using specific antibodies against phospho-ERK 1/2 and ERK 1/2. ERK 1/2 phosphorylation was quantified by densitometry and expressed as a percentage of the maximal phosphorylation of ERK 1/2 (mean±SE). Immunoblots are representative of three independent experiments.

A candidate for a molecule connecting the GHSR-1a internalization to the activation of ERK 1/2 might be β-arrestins. To this end, we used RNA interference to reduce the expression of endogenous β-arrestins in HEK-GHSR-1a cells. In these experiments siRNA targeting β-arrestin 1 or β-arrestin 2 reduced their expression by 48% and 54%, respectively (Figure 3 A). In the presence of a non-targeting control siRNA, ERK 1/2 phosphorylation measured at 10 minutes after ghrelin treatment was identical to that observed without any transfection (data not shown). β-arrestin 1 and β-arrestin 2 siRNA reduced ERK 1/2 phosphorylation at 10 minutes by 65% and 55%, respectively (Figure 3 B), suggesting that part of the ERK 1/2 activation requires the simultaneous presence of both isoforms. Taking together, the results confirm that the ghrelin-induced activation of ERK 1/2 involves GHSR-1a internalization, and that the observed reduction in the ghrelin-induced activation following β-arrestin depletion reflects the requirement of β-arrestins for this internalization.

Next, we evaluated the role of G-protein-dependent pathways. For this purpose, we first investigated the possible role of $G_{i/o}$-proteins by means of pretreatment with pertussis toxin (PTX, 100ng/mL, 12 h). As Figure 4 A shows, PTX reduced the ghrelin-induced activation of ERK 1/2 by 50% approximately at 5-10 minutes of stimulation with ghrelin, corresponding to the early time component. We also observed that at later time points, the effect of PTX on ERK 1/2 activation was more sensitive. Subtraction of the time-course obtained from the stimulation with ghrelin in the presence of PTX (green line) from the control curve (blue line) predicts the time-course for the $G_{i/o}$-dependent activation of ERK 1/2 (dotted curve; red line).

We also tested the effects on ghrelin-induced ERK 1/2 activation of the Ca^{+2} chelator BAPTA-AM. Figure 4 B shows that the pretreatment with BAPTA-AM (30 μM, 30 min) reduced the ERK 1/2 activation induced by ghrelin (100 nM, 5 min) by 65%. The effect of the PI3K inhibitor wortmannin was also assayed. Figure 4 B shows that pretreatment with wortmannin (1 μM, 30 min) decreased the ghrelin-induced ERK 1/2 activation by 78%.

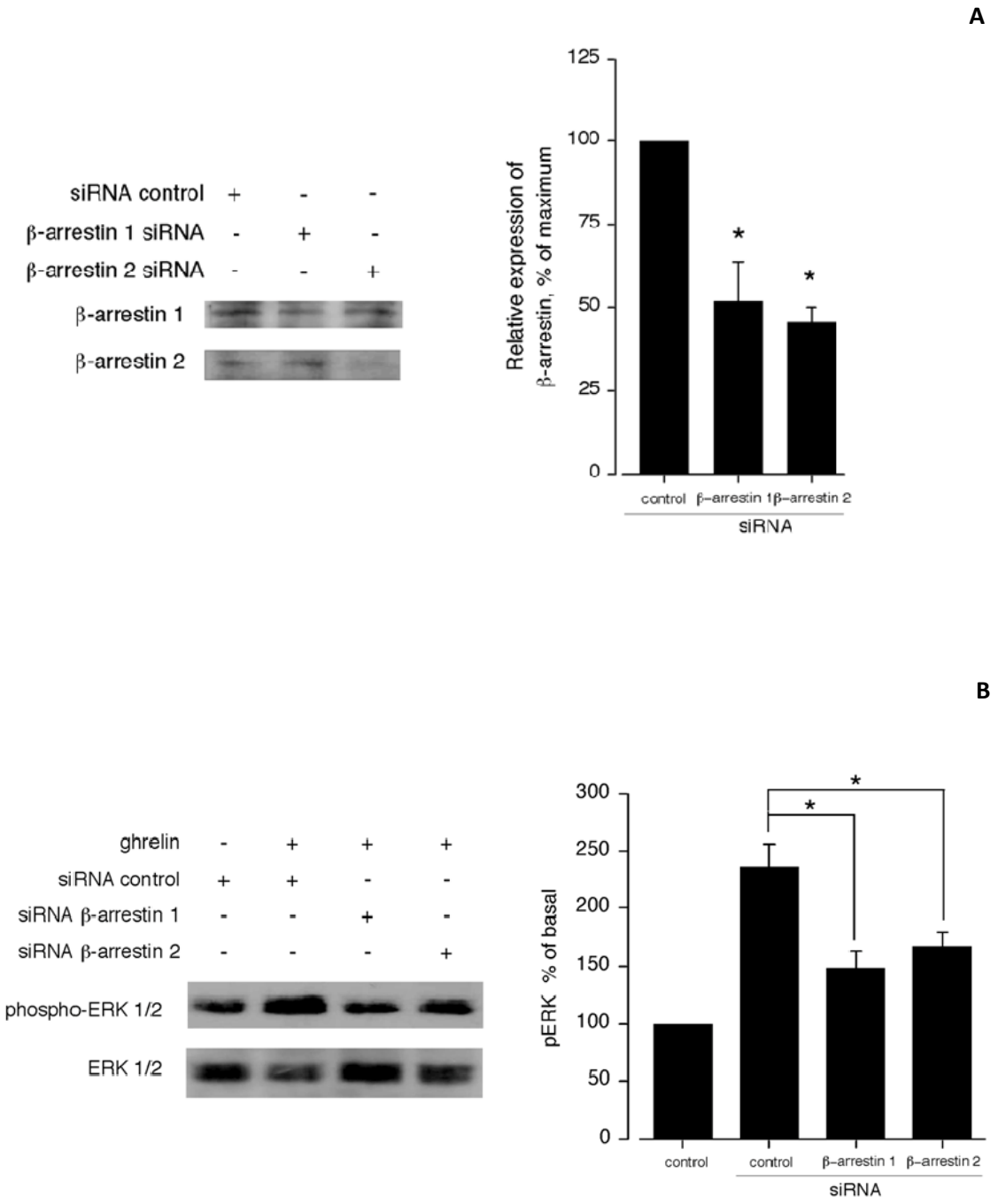

Figure 3. Effect of siRNA depletion of β-arrestin1 or β-arrestin 2 on ghrelin-induced ERK 1/2 phosphorylation. HEK-GHSR-1a cells transfected with β-arrestin 1 or β-arrestin 2 siRNA were serum-starved and then stimulated with ghrelin (100 nM, 5 min). After stimulation, cell extracts were prepared as described in methods. Equal amounts of protein in each sample were used to assess the expression of β-arrestin1 or β-arrestin 2 (A) or ERK 1/2 phosphorylation (B) by immunoblotting analysis. Expression of β-arrestin1 or β-arrestin 2 was quantified by densitometry. Values shown are percentages of the level of β-arrestins in control siRNA-transfected cells. ERK 1/2 phosphorylation was quantified by densitometry and expressed as a percentage of the basal phosphorylation of ERK 1/2 obtained 5 min after ghrelin addition to control siRNA transfected cells (mean±SE). Immunoblots are representative of three independent experiments. *P<0.05.

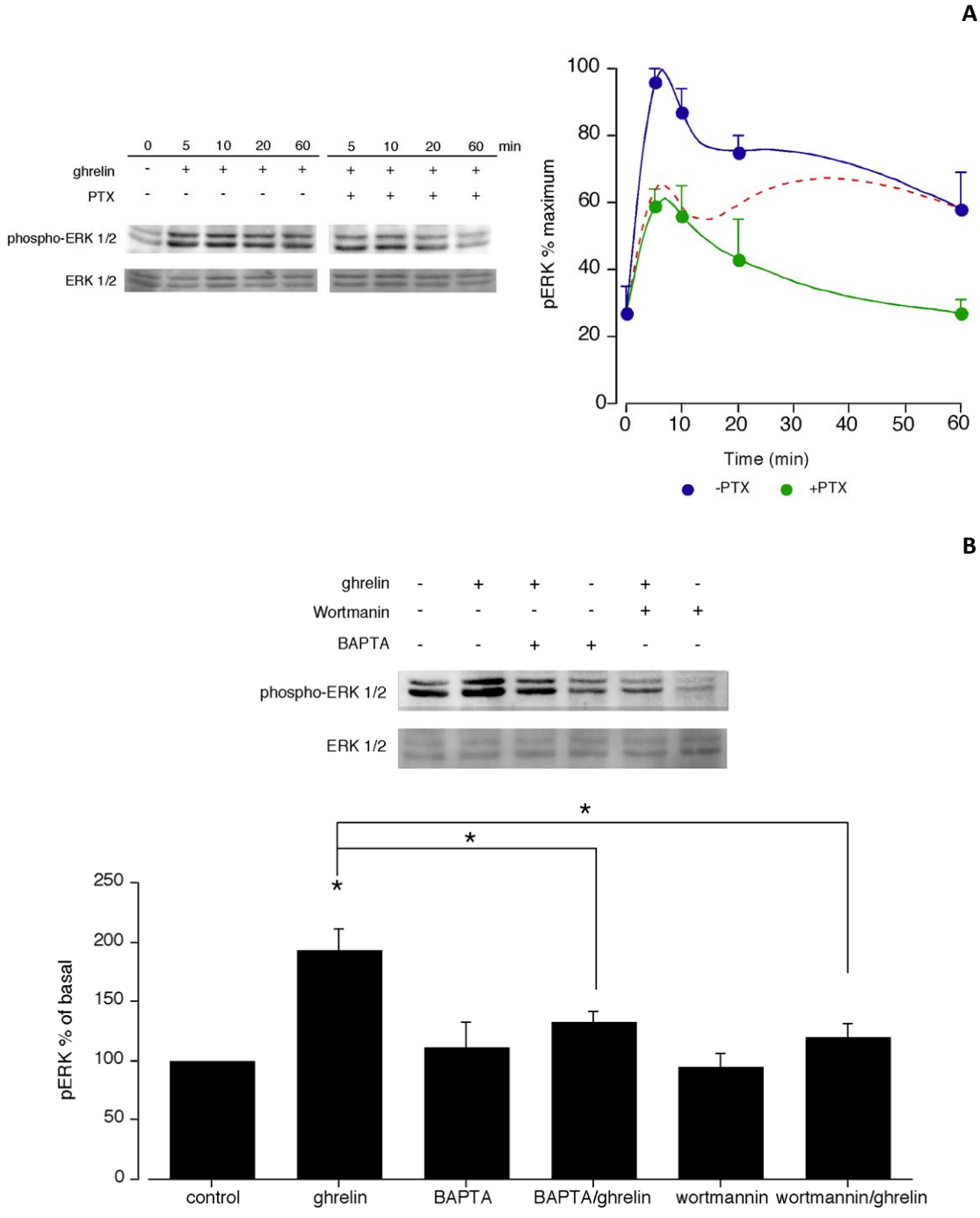

Figure 4. A. Ghrelin-induced ERK 1/2 phosphorylation in the absence or presence of PTX (100 ng/ml, 12 h). Serum-starved cells were stimulated with ghrelin (100 nM) for the time periods indicated. ERK 1/2 phosphorylation was quantified by densitometry and expressed as a percentage of the maximal phosphorylation of ERK 1/2 (mean±SE). Dotted curve predicts the time-course for the $G_{i/o}$-dependent activation of ERK 1/2. B. Effects of BAPTA and wortmannin on ERK 1/2 phosphorylation. Serum-starved cells were pretreated with BAPTA (30 µM, 30 min) or wortmannin (1 µM, 30 min) before ghrelin stimulation (100 nM, 5 min). ERK 1/2 phosphorylation was quantified by densitometry and expressed as a percentage of the basal phosphorylation of ERK 1/2 obtained in control cells (mean±SE). Immunoblots are representative of three independent experiments. *P<0.05.

Next we explored the role of PKC, using the PKC inhibitors Gö6976 and calphostin C. Ghrelin-induced ERK 1/2 activation was reduced 41% by pretreatment with Gö6976 (100 nM, 30 min), a selective inhibitor of Ca^{+2} dependent PKC α and PKC βI isoenzymes, and 67% by pretreatment with calphostin C (10 μM, 30 min), a non- selective PKC inhibitor (Figure 5 A). To determine the temporal contribution of PKC α/β on ERK 1/2 activation, cells were treated with Gö6976 prior to stimulation with ghrelin. As shown in Figure 5 B, Gö6976 partially reduced the early time component of ghrelin-induced ERK 1/2 activation (30%, 5–10 min). Unexpectedly, the greatest effect on the time-course was observed on the sustained ERK 1/2 activation at 20–60 minutes after ghrelin stimulation. Subtraction of the time-course obtained from the Gö6976 treatment curve, to the control curve, predicts the time-course for the $G_{q/11}$/PKC α/β-dependent activation of ERK 1/2 (Figure 5 B, dotted curve). Taken together with the potassium depletion medium and PTX data above, these results suggest that β-arrestin-mediated component of ERK 1/2 activation is dependent of ''interrelated'' components activated by $G_{i/0}$-and $G_{q/11}$-proteins.

Important roles of non-receptor tyrosine kinases (RTK), for example members of the cSrc family, have been reported during GPCR-mediated ERK 1/2 activation in various cell types. Ghrelin-induced ERK 1/2 activation was strongly inhibited by genistein (2 μM, 30 min) and PP2 (5 μM, 30 min), a selective cSrc inhibitor (Figure 6 A). This inhibition was specific, since pretreatment with PP3 (5 μM, 30 min), a negative control for PP2, had no effect on the ghrelin-induced ERK 1/2 phosphorylation (Figure 6 A), supporting a role for cSrc protein in ghrelin signaling. To investigate the activation of cSrc, we compared the phosphorylation of both cSrc regulatory tyrosines, namely Tyr 527 and Tyr 416. Phosphorylation of Tyr 416 displayed an early increase at 5 minutes of ghrelin stimulation and remained greater than over twofold basal for at least 60 minutes after stimulation. Conversely, the phosphorylation of inhibitory Tyr 527 showed a decrease concurrent with the dynamic of phosphorylation of Tyr 416 (Figure 6 B). Furthermore, PP2 attenuated the stimulatory effect of ghrelin on phosphorylation of cSrc at Tyr 416 (data not shown).

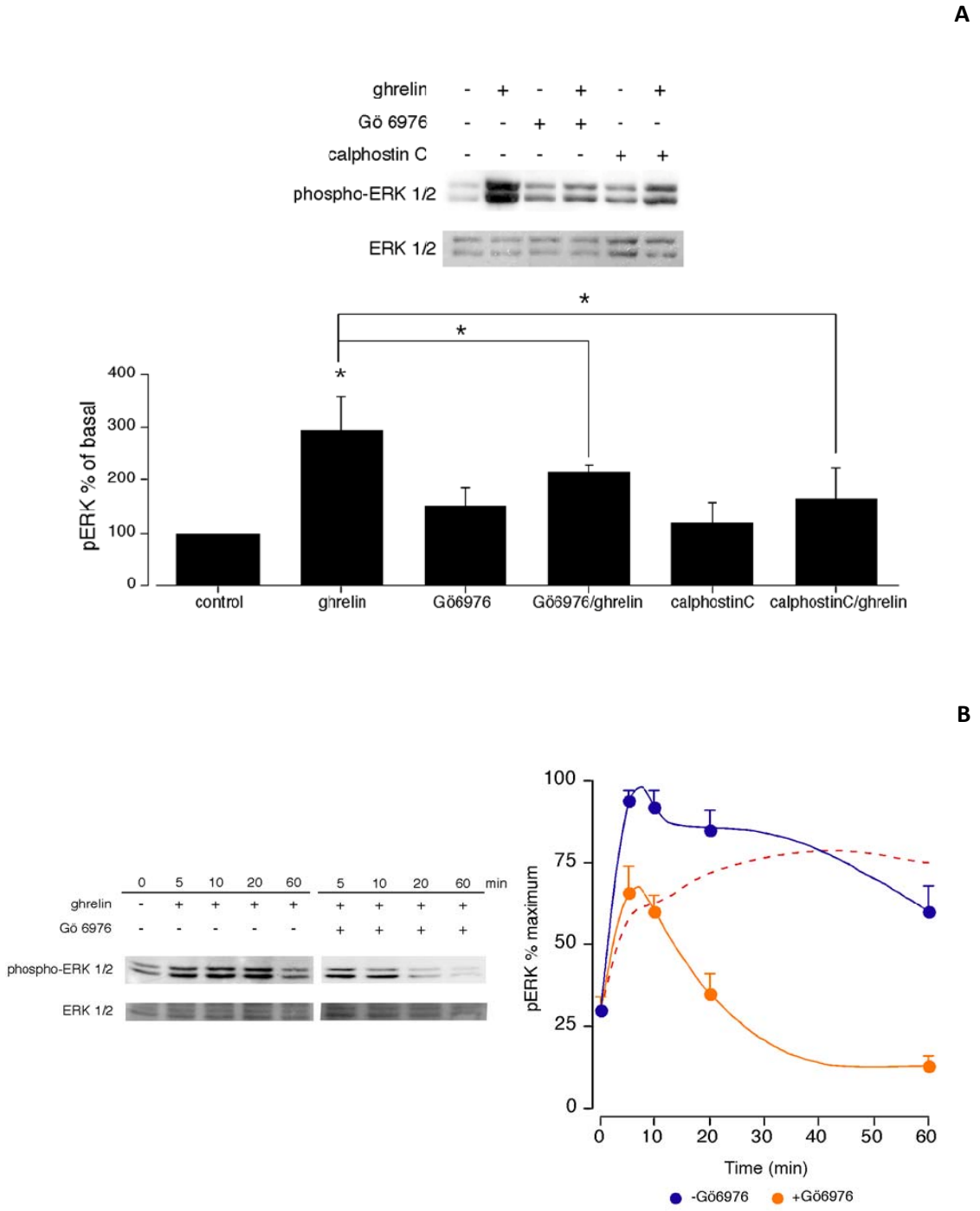

Figure 5. A. Effect of the PKC inhibitors Gö6976, specific for PKC α and PKC β, and calphostin C, a non-specific PKC inhibitor, on ghrelin-induced ERK 1/2 response. Serum-starved cells were pretreated with Gö6976 (100 nM, 30 min) or calphostin C (10 µM, 30 min) before ghrelin stimulation (100 nM, 5 min). Cellular extracts were prepared to visualize phosphorylation of ERK 1/2 by immunoblotting. ERK 1/2 phosphorylation was quantified by densitometry and expressed as a percentage of the basal phosphorylation of ERK 1/2 (mean±SE). B. Ghrelin-induced ERK 1/2phosphorylation in the absence or presence of PKC inhibitor Gö6976. Serum-starved cells were stimulated with ghrelin (100 nM) for the time periods indicated. ERK 1/2 phosphorylation was quantified by densitometry and expressed as a percentage of the maximal phosphorylation of ERK 1/2 (mean±SE). Dotted curve predicts the time course for the $G_q/_{11}$-dependent activation of ERK 1/2. Immunoblots are representative of three independent experiments. *P<0.05.

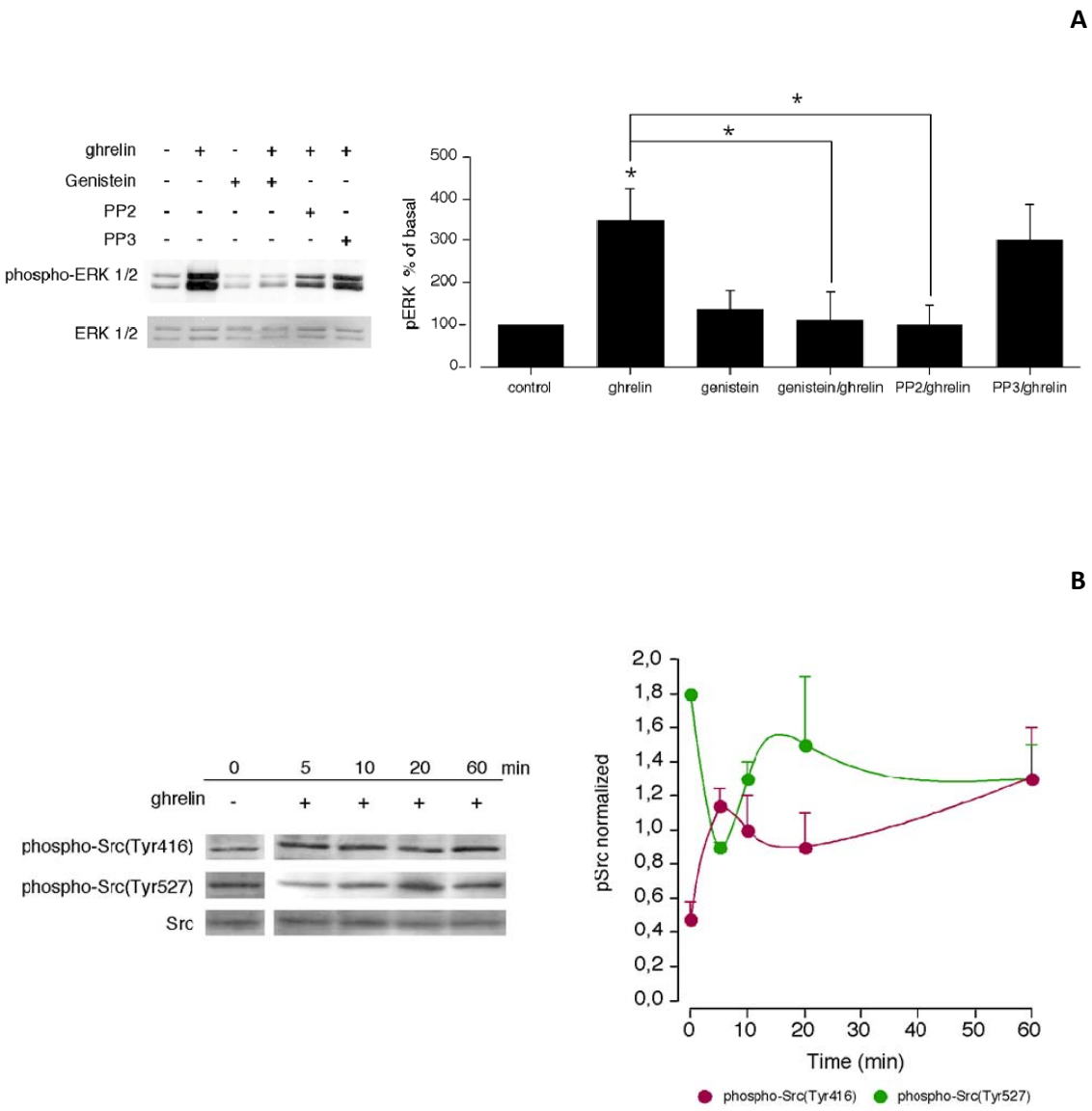

Figure 6. A. Effect of the tyrosine kinase inhibitors genistein, PP2 and PP3 on the ghrelin-induced ERK 1/2 response. Serum-starved cells were pretreated with genistein (2 µM, 30 min), PP2 (5 µM, 30 min) or PP3 (5 µM, 30 min) before ghrelin stimulation (100 nM, 5min). Cell extracts were prepared to visualize phosphorylation of ERK 1/2. Values were obtained by densitometry and are expressed as a percentage of the basal phosphorylation obtained in control cells (mean±SE). B. Time-course for the ghrelin-induced phosphorylation of different cSrc tyrosine residues. Cells were stimulated with ghrelin (100 nM) for the time periods indicated. Phospho-cSrc (Tyr 416 and Tyr 527) and cSrc were detected by immunoblotting and quantified by densitometry for normalization (mean±SE). Immunoblots are representative of three independent experiments. *P<0.05.

In addition, the effect of PTX on cSrc Tyr 416 phosphorylation was evaluated since $G_{i/o}$–coupled receptors can activate cSrc. Pretreatment of the cells with PTX (100 ng/mL, 12 h) inhibited significatively the ghrelin-induced phosphorylation of cSrc at Tyr 416 (Figure 7 A). The contribution of PI3K signaling on ghrelin-induced cSrc Tyr 416 phosphorylation was also evaluated by means of wortmannin pretreatment (1 μM, 30 min). The data in figure 7 B showed a reduction on ghrelin-induced cSrc Tyr 416 phosphorylation, indicating that this process involves PI3K. In the same line, but to check the contribution of G_q-dependent pathways, the effect of BAPTA-AM was evaluated. The result obtained shows that BAPTA-AM pretreatment (30 μM, 30 min) also reduced the ghrelin-induced cSrc Tyr 416 phosphorylation (Figure 7 B).

Due to the involvement of calcium on ghrelin-induced cSrc Tyr 416 phosphorylation, we tried to determine a role, if any, of PKC. To this purpose, an experiment with Gö6976, the selective PKC α/β inhibitor, and calphostin C, a non-selective PKC inhibitor, was performed. The results obtained showed that calphostin C (10 μM, 30 min) inhibited ghrelin-induced cSrc Tyr 416 phosphorylation and Gö6976 (100 nM, 30 min) partially did it (Figure 8).

To try to identify which PKC was involved in ghrelin-induced cSrc Tyr 416 phosphorylation, we perform a time-course on ghrelin-induced PKC activation using specific antibodies against the activated forms of classical PKC (PKC α/β), novel PKC (PKC ε, PKC δ, PKC θ) and atypical PKC (PKC ζ). The results in Figure 9 showed that classic PKC were activated by ghrelin, with activation peaking 5-10 minutes after ghrelin addition. Novel PKC were likewise activated by ghrelin but with different time-courses: PKC ε reached a maximum 5 minutes after ghrelin stimulation and then gradually declined, while PKC δ and PKC θ peaked about 10 minutes after ghrelin addition. On the other hand, atypical PKC ζ was inhibited by ghrelin.

The previous result pointed to a role of PKC α/β and PKC ε on cSrc activation acting as upstream mediators. To explore the implication of PKC ε, we evaluated the possible role of the $G_{i/o}$ /PI3K pathway in its activation. As shown in Figure 10 A, pretreatment of cells with PTX (100 ng/mL, 12 h) had a significant inhibitory effect on ghrelin-induced phosphorylation of PKC ε. Furthermore, phosphorylation of PKC ε was abolished by wortmannin pretreatment (1 μM, 30 min) to the same extent (Figure 10 B).

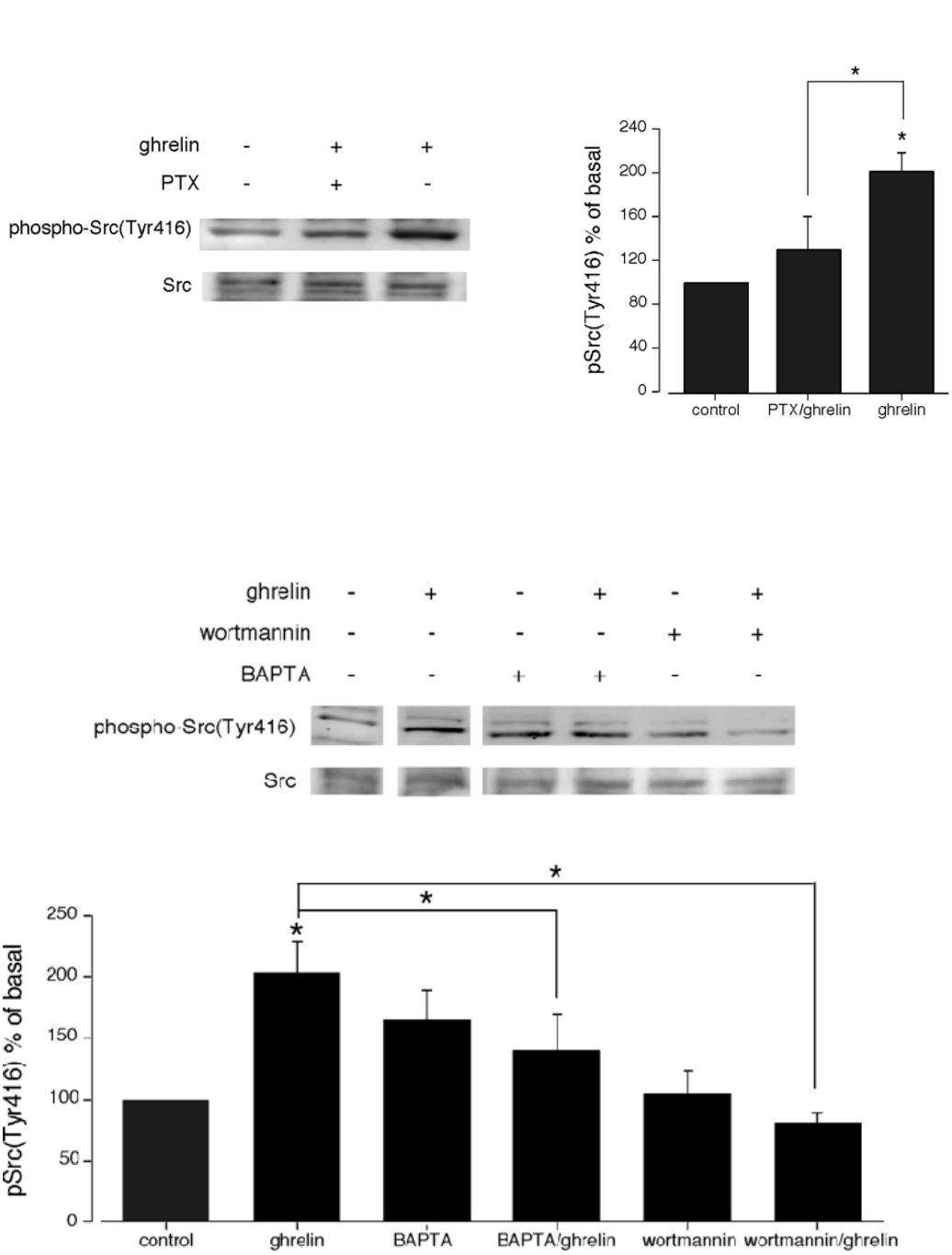

Figure 7. A. Ghrelin-induced cSrc phosphorylation (Tyr416) (100 nM, 5 min) in the absence or presence of PTX (100 ng/ml, 12 h). Phospho-cSrc (Tyr 416) was quantified by densitometry and expressed as a percentage of the basal phosphorylation obtained in control cells (mean±SE). B. Mechanism of cSrc activation by ghrelin. Serum-starved cells were pretreated with the PI3K inhibitor wortmannin (1 µM, 30 min) or the Ca^{2+} chelator BAPTA-AM (30 µM, 30 min) before ghrelin stimulation (100 nM, 5 min). Cellular extracts were prepared to visualize phospho-cSrc (Tyr 416) protein band, which was quantified by densitometry and expressed as the percentage of the basal phosphorylation obtained in control cells (mean±SE). Immunoblots are representative of three independent experiments. **P*<0.05.

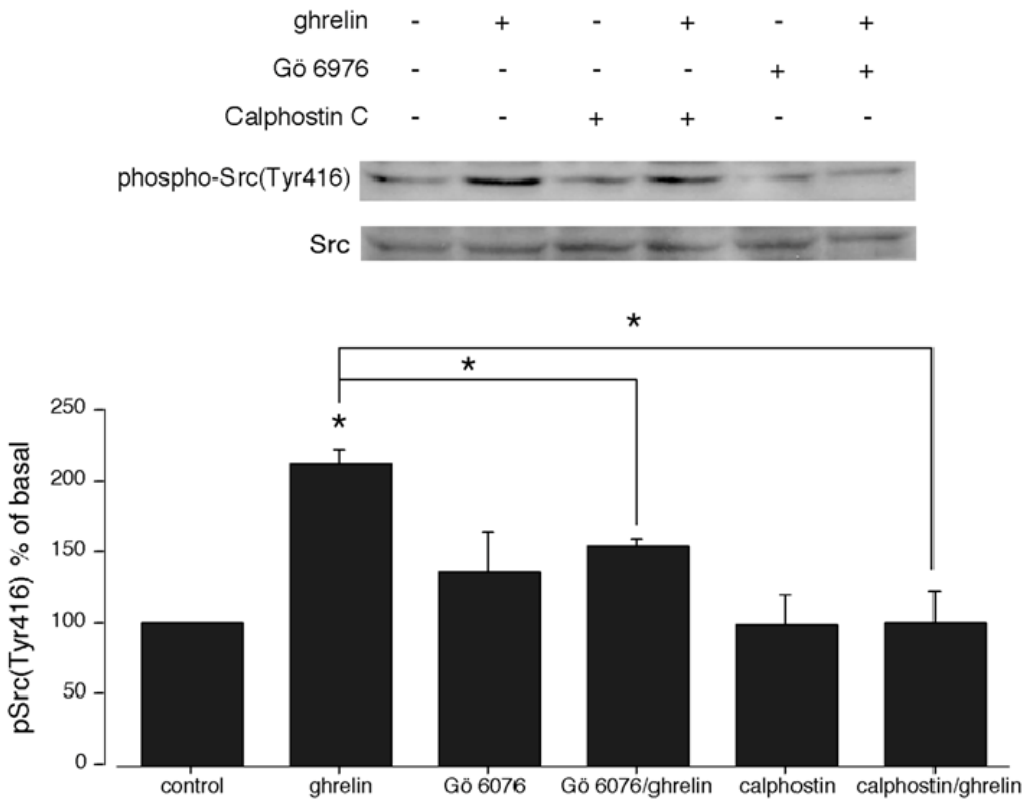

Figure 8. Mechanism of cSrc activation by ghrelin. Serum-starved cells were pretreated with the PKC α/β specific inhibitor Gö6976 (100 nM, 30 min) and the non-specific PKC inhibitor calphostin C (10 µM, 30 min) before ghrelin stimulation (100 nM, 5 min). Cellular extracts were prepared to visualize phospho-cSrc (Tyr 416) protein band, which was quantified by densitometry and expressed as the percentage of the basal phosphorylation obtained in control cells (mean±SE). Immunoblots are representative of three independent experiments. **P*<0.05.

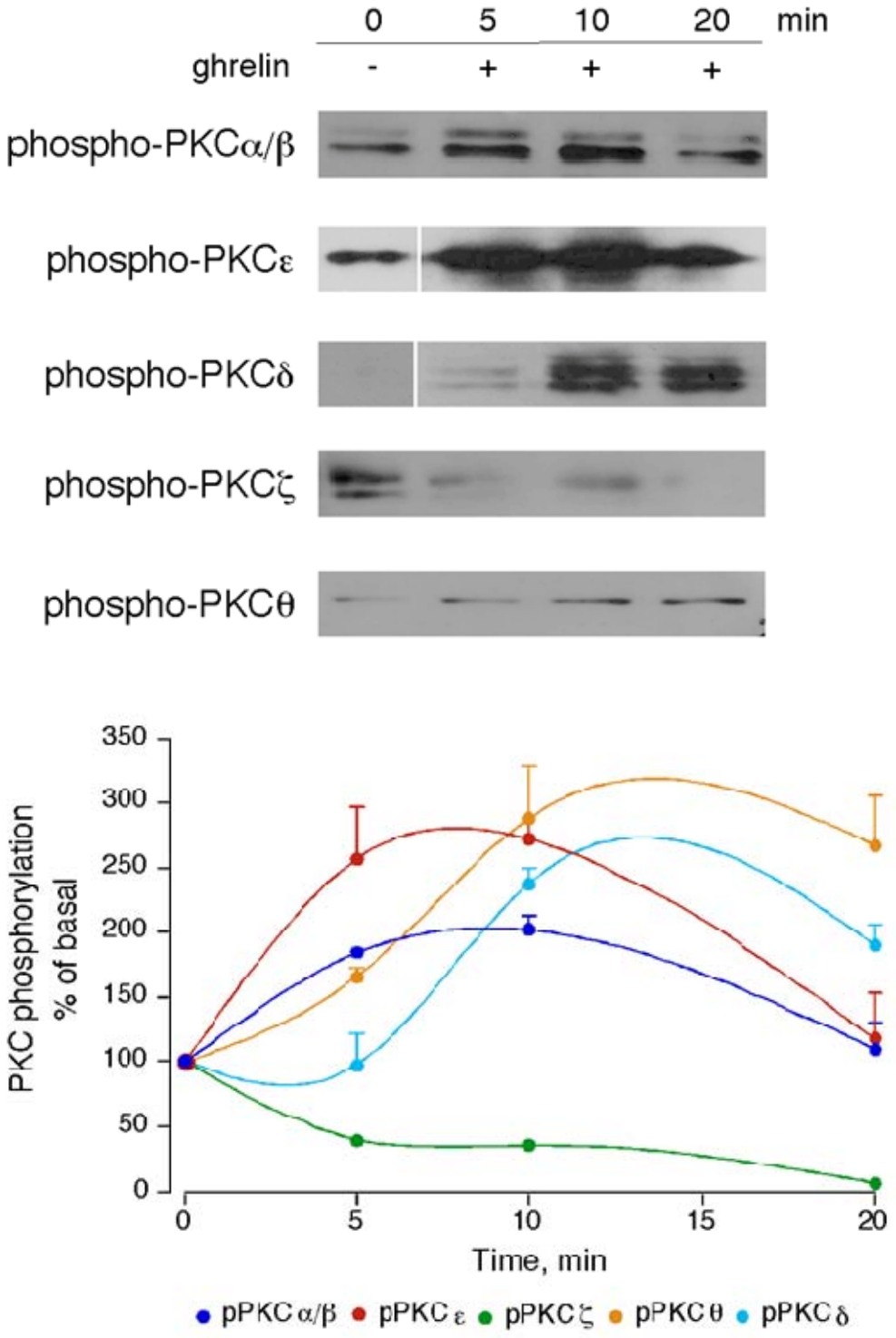

Figure 9. Time-course of PKC activation by ghrelin stimulation of HEK-GHSR-1a cells. Serum-starved cells were stimulated with ghrelin (100 nM) for the time periods indicated. Phosphorylation of PKC α/β, ε, δ, ζ and θ was visualized by immunoblotting using specific antibodies against the different phospho-PKC. Values shown are percentages of the basal phosphorylation obtained in unstimulated cells (mean±SE). Immunoblots are representative of three independent experiments.

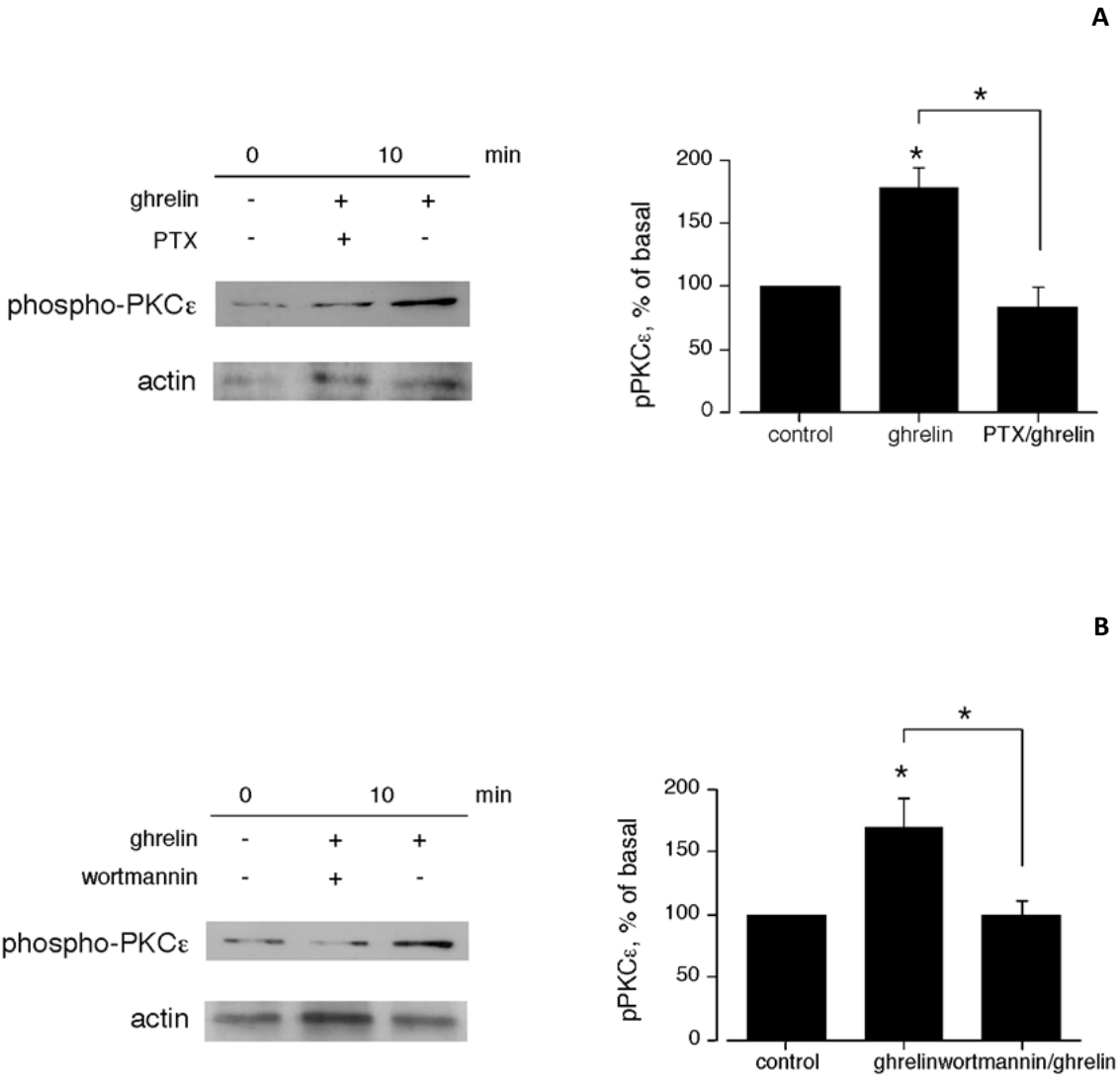

Figure 10. Mechanism of PKC ε activation by ghrelin. Serum-starved cells were pretreated with (A) PTX (100 ng/ml, 12 h) or (B) PI3K inhibitor wortmannin (1 μM, 30 min) and then stimulated with ghrelin (100 nM, 10 min). PKC ε phosphorylation was quantified by densitometry and expressed as percentages of the basal phosphorylation obtained in control cells (mean±SE). Inmunblots are representative of three independent experiments. *P<0.05.

After determine that β-arrestins and cSrc play a role in the GHSR-1a-mediated ERK 1/2 activation, we next investigated whether ghrelin induces formation of a complex including β-arrestins, the internalizated receptor and other kinases. To address this question immunoprecipitation experiments were made. The results obtained showed that immunoprecipitation of ghrelin-stimulated cells with antibodies to phospho-ERK 1/2 co-precipitates cSrc, Raf-1 and GHSR-1a. Similarly, immunoprecipitation with antibodies to β-arrestin 1 or β-arrestin 2 co-precipitate phospho-ERK 1/2 and GHSR-1a (Figure 11).

With the previous data obtained, we wanted to test the hypothesis of cSrc binding to β-arrestins, which then binds to receptor, to form the complex previously described. To this purpose, the ghrelin-induced cSrc activation was evaluated after reducing cellular levels of β-arrestin 1 or β-arrestin 2 by transfecting with siRNA. The results showed that in the presence of β-arrestin 1 siRNA (47% reduction in β-arrestin 1 expression); activation of cSrc by ghrelin (100 nM) was reduced by 45%. In the presence of β-arrestin 2 siRNA (57% reduction in β-arrestin 2 expression) activation of cSrc by ghrelin (100 nM) was reduced by 60% (Figure 12).

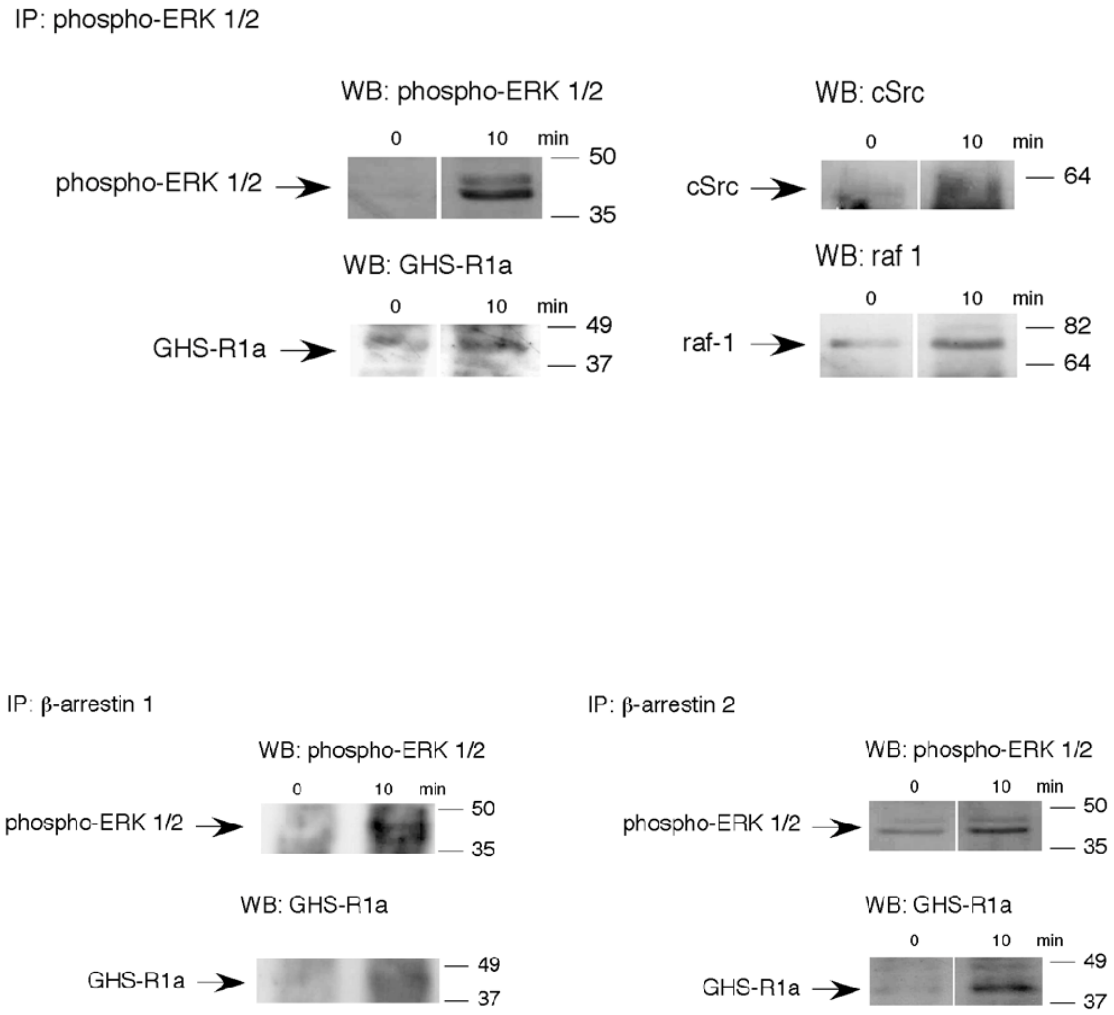

Figure 11. Effect of ghrelin on the assembly of complexes containing GHSR-1a, β-arrestins 1 and 2, cSrc, Raf 1, and phospho-ERK 1/2. Serum-starved cells were stimulated with ghrelin (100 nM) for the time periods indicated, then lysed and immunoprecipitated (IP) with antibodies to phospho-ERK 1/2 (upper panel) or β-arrestin 1 or β-arrestin 2 (lower panel), then analyzed by immunoblotting with antibodies to cSrc, Raf-1, GHSR-1a and phospho-ERK 1/2. Immunoblots are representative of three independent experiments.

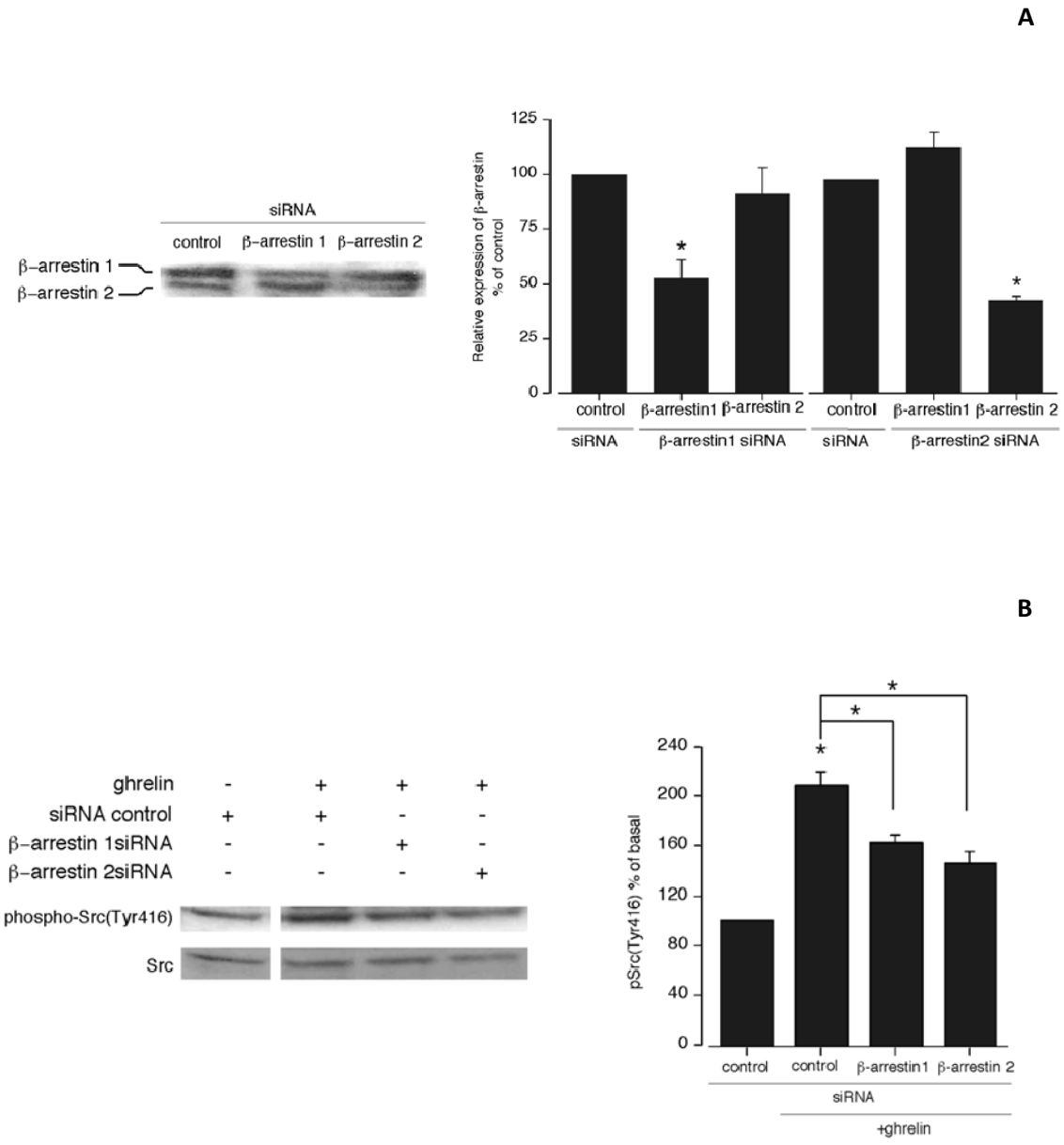

Figure 12. Activation of cSrc by β-arrestins. Cells transfected with β-arrestin 1 or β-arrestin 2 siRNA were serum-starved and then stimulated with ghrelin (100 nM, 5 min). After stimulation, cell extracts were prepared and equal amounts of protein in each sample were used to visualize expression of β-arrestins 1 and 2 (A1CT antibody) (A) and phospho-cSrc (Tyr 416) protein band (B) by immunoblotting analysis. Expression of β-arrestins was determined by densitometry. Values shown are percentages of the level of the corresponding β-arrestins in control siRNA transfected cells. Phospho-cSrc (Tyr 416) was quantified by densitometry and expressed as the percentage of the basal phosphorylation obtained in control siRNA-transfected cells (mean±SE). Immunoblots are representative of three independent experiments. *P<0.05.

CHAPTER 2

cSrc regulates Akt signaling in response to ghrelin via β-arrestin signaling-independent and-dependent mechanisms.

First of all, the time-course of Akt activation was defined after the stimulation of HEK-GHSR-1a cells with ghrelin (100 nM). As Figure 1 shows, ghrelin induced maximal levels of Akt phosphorylation in both the activation loop within the kinase domain [A-loop (T308)] and the hydrophobic motif in the C-terminal region [HM (S473)] within 20 minutes keeping the maximum by 60 minutes.

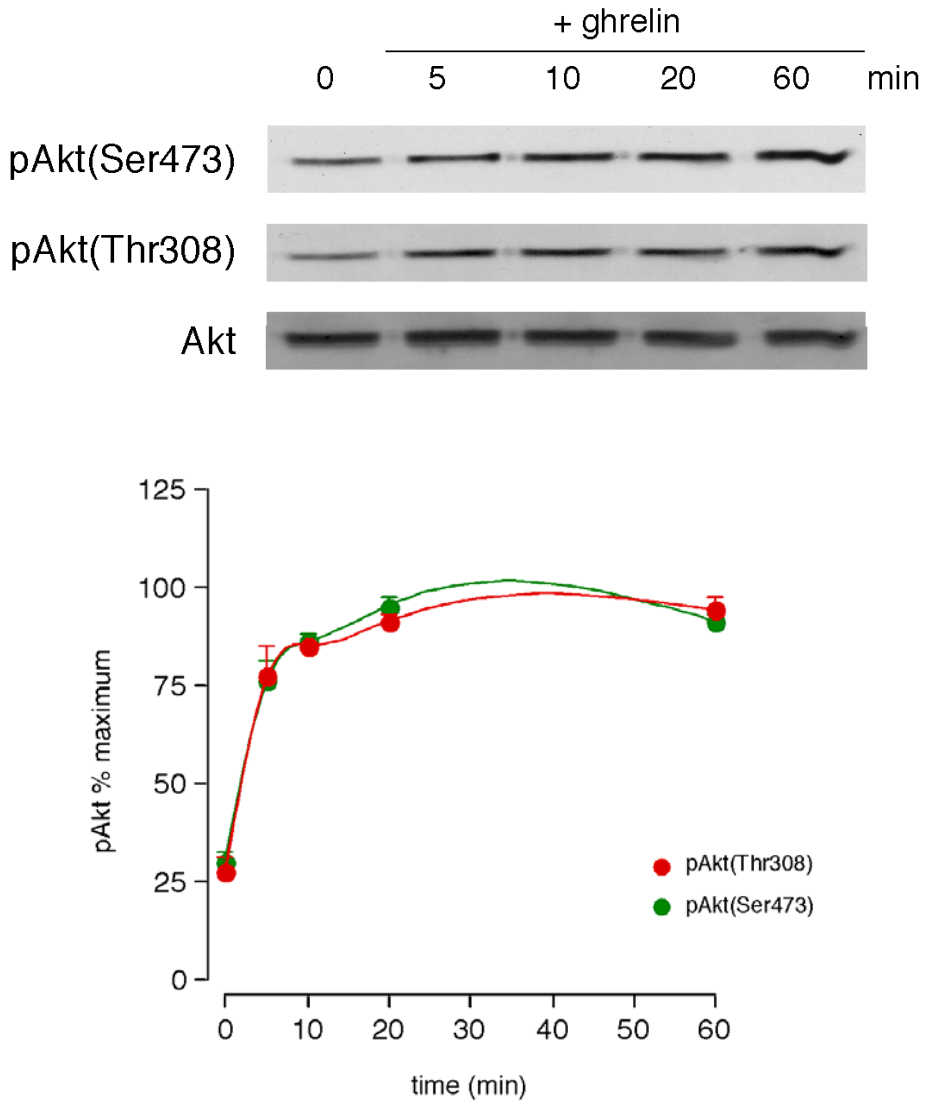

Figure 1. Time-course of the effect of ghrelin on Akt HM (S473) and A-loop (T308) phosphorylation. Serum-starved HEK-GHSR-1a cells were stimulated with ghrelin (100 nM) for the time periods indicated. Cells were lysed and analyzed by immunoblotting using specific antibodies against pAkt (S473) and (T308). Akt phosphorylation was quantified by densitometry and expressed as a percentage of the maximal phosphorylation obtained for each residue (mean±SE). Immunoblots are representative of three independent experiments.

The involvement of $G_{i/o}$-proteins was evaluated by means of pretreatment with PTX (100 ng/mL, 12 h). The result shows that PTX reduced the ghrelin-induced phosphorylation of the Akt A-loop (T308). Surprisingly, the effect of PTX on the other residue, (S473), was increased. Meanwhile, the effect of PI3K inhibitor wortmannin (1 μM, 30 min) showed a decrease of ghrelin-induced Akt phosphorylation at both residues. It was also checked the involvement of the βγ-subunit of G-proteins through the use of the construct β-ARK-CT, which also decreased the effect of ghrelin on Akt phosphorylation at both residues (Figure 2).

Akt phosphorylation was strongly inhibited at both residues by the selective cSrc inhibitor PP2 (5 μM, 30 min) for all time tested. This inhibition was specific, since pretreatment with PP3 (5 μM, 30 min), a negative control for PP2, had no effect on ghrelin-induced Akt phosphorylation (data not shown). The siRNA experiments targeting cSrc reduced its expression by 57±2%. cSrc siRNA decreased ghrelin-activated Akt phosphorylation with respect to siRNA control for all time tested and for both residues [54±3% at HM (S473); and 45±6% at A-loop(T308)] (Figure 3).

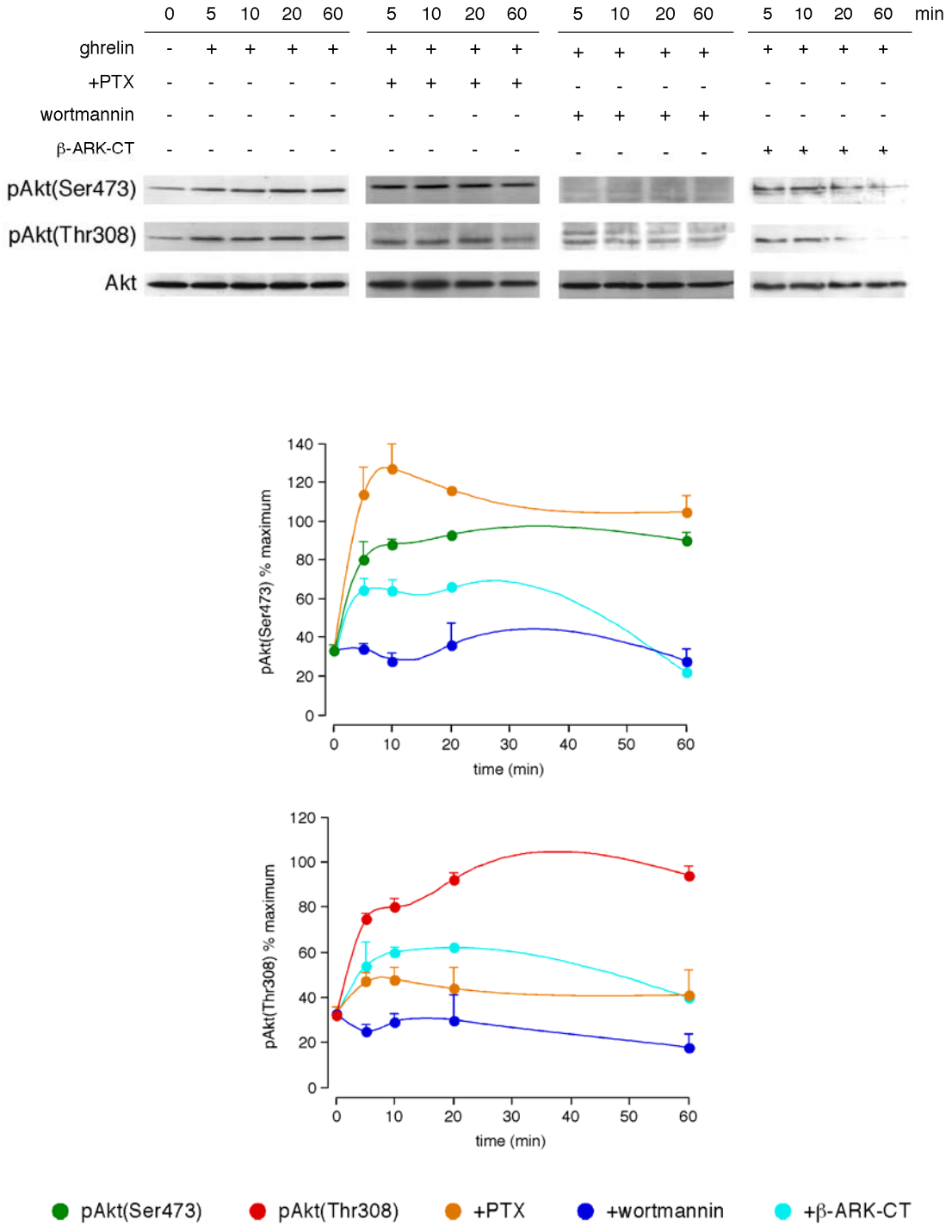

Figure 2. Role of $G_{i/o}$-proteins, Gβγ dimmers and PI3K in Akt phosphorylation in response to ghrelin. Ghrelin-induced Akt phosphorylation in the absence or presence of PTX (100 ng/mL, 12 h), PI3K inhibitor wortmannin (1 µM, 30 min) and βγ sequester β-ARK-CT. Serum-starved HEK-GHSR-1a cells were stimulated with ghrelin (100 nM) for the time periods indicated. Cells were lysed and analyzed by immunoblotting using specific antibodies. Akt phosphorylation was quantified by densitometry and expressed as a percentage of the maximal phosphorylation obtained for each residue (mean±SE). Immunoblots are representative of three independent experiments.

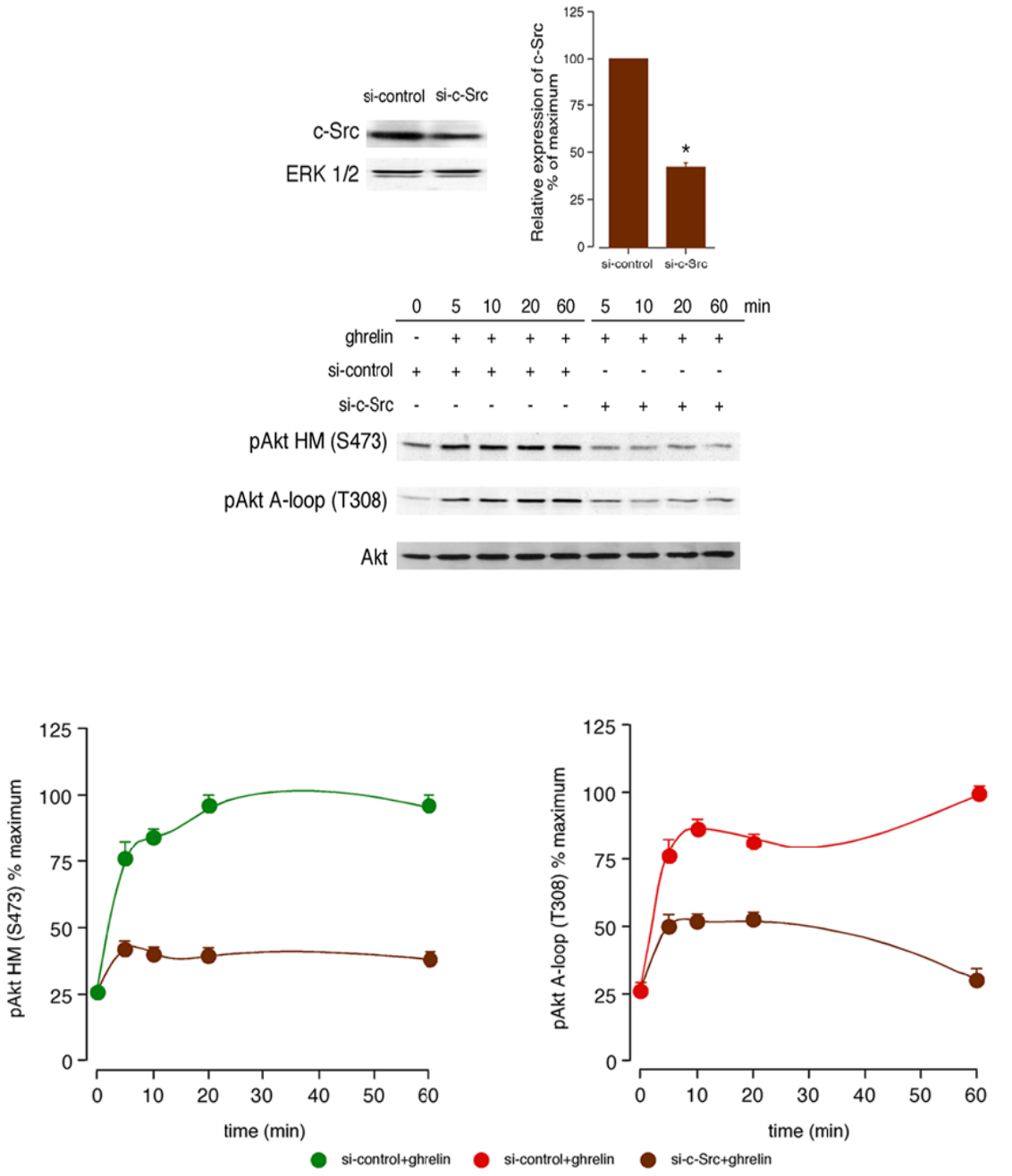

Figure 3. Effect of siRNA depletion of cSrc on ghrelin-induced Akt phosphorylation. HEK-GHSR-1a cells transfected with cSrc siRNA were serum-starved and then stimulated with ghrelin (100 nM). After stimulation, equal amounts of protein in each sample were used to assess the expression of cSrc (upper panel) and Akt phosphorylation (lower panel) by immunoblotting. Expression of cSrc was quantified by densitometry and expressed as percentages of the level of cSrc in control siRNA transfected cells (mean±SE). Akt phosphorylation was quantified by densitometry and expressed as a percentage of the maximal phosphorylation obtained for each residue after ghrelin addition to control siRNA-transfected cells (mean±SE). Immunoblots are representative of three independent experiments. *P<0.05.

Immunoprecipitation assays were made to evaluate the effect of ghrelin on tyrosine phosphorylation of Akt. As Figure 4 shows, immunoprecipitation of Akt showed an increase of tyrosine phosphorylation of this kinase in ghrelin-stimulated cells (100 nM, 5 min) in comparison with unstimulated cells. Furthermore, the activated form of cSrc, pcSrc (Tyr 416), co-immunoprecipitated with Akt demonstrating a direct interaction between both kinases.

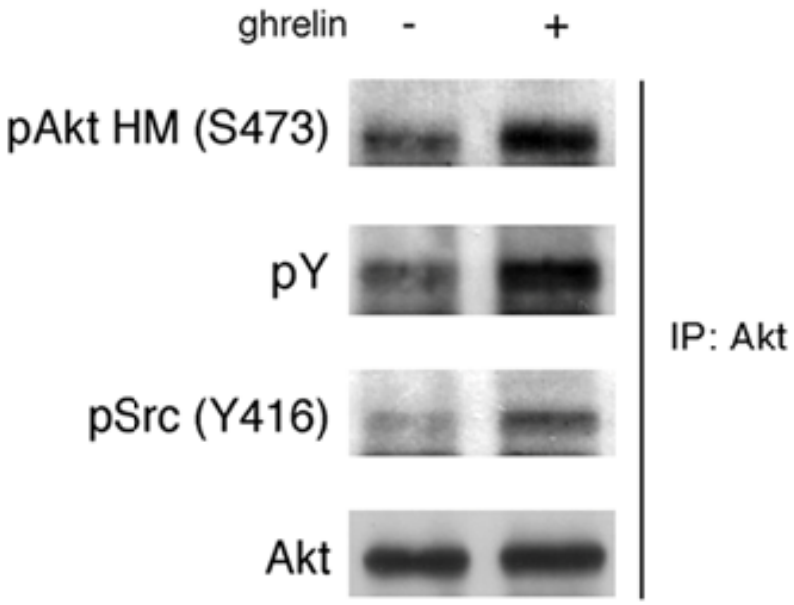

Figure 4. Effect of ghrelin on tyrosine phosphorylation of Akt and interaction between Akt and cSrc. Serum-starved cells were stimulated with ghrelin (100 nM, 5 min), lysed and immunoprecipitated (IP) with antibodies to Akt, and then analyzed by immunoblotting with pAkt HM (S473), pY, pcSrc (Y416) antibodies. Immunoblots are representative of three independent experiments.

The immunoprecipitation assays of Akt made in the presence of cSrc siRNA (66±2% reduction in cSrc expression) clearly decreased tyrosine phosphorylation of this kinase in ghrelin-stimulated cells (100 nM, 5 min) in comparison with non-targeting control siRNA cells (Figure 5). cSrc siRNA decreased ghrelin-activated Akt phosphorylation and the co-immunoprecipitation of pcSrc (Tyr 416) with respect to siRNA control (Figure 5).

Figure 6 A shows that pPDK-1 reached maximal levels within 5-10 minutes of ghrelin stimulation (100 nM), decreasing to approximately 50% of the maximum by 60 minutes after stimulation. This time-course was parallel to the phosphorylation of Akt A-loop (T308). Pretreatment of the cells with PTX (100 ng/mL, 12 h) had a significant inhibitory effect on ghrelin-induced pPDK-1 (S241) phosphorylation (100 nM, 5 min) as shown in Figure 6 B. The inhibition of PI3K caused by wortmannin pretreatment (1 μM, 30 min) showed a decreased on ghrelin-induced PDK-1 phosphorylation (Figure 6 B).

The involvement of mammalian target of rapamycin-insensitive complex 2 (mTORC2) was evaluated through the targeting of one of its components named Rictor. Working in presence of Rictor siRNA (50±2% reduction in Rictor expression), Akt HM (S473) phosphorylation was reduced by 50±4%, whereas A-loop (T308) phosphorylation was not affected (Figure 7).

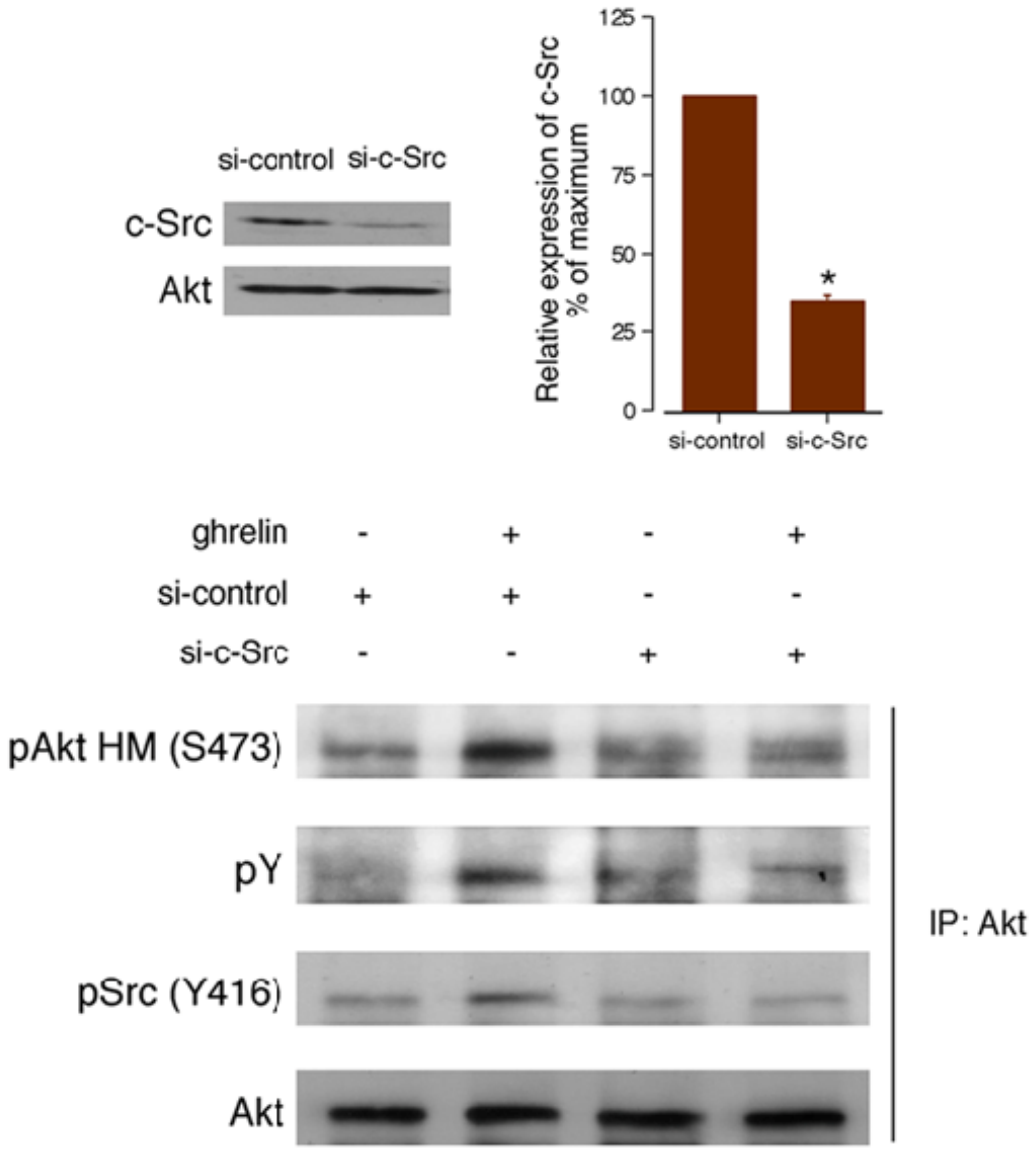

Figure 5. Effect of ghrelin on tyrosine phosphorylation of Akt in the presence of cSrc siRNA. HEK-GHSR-1a cells transfected with cSrc siRNA were serum-starved and then stimulated with ghrelin (100 nM, 5 min). Equal amounts of protein in each sample were used to assess the expression of cSrc [upper panel; values shown (mean±SE) are percentages of the level of cSrc in control siRNA-transfected cells]. Cells were lysed and immunoprecipitated (IP) with antibodies to Akt, and then analyzed by immunoblotting with pAkt HM (S473), pY and pcSrc (Y416) antibodies (lower panel). Immunoblots are representative of three independent experiments. *P<0.05.

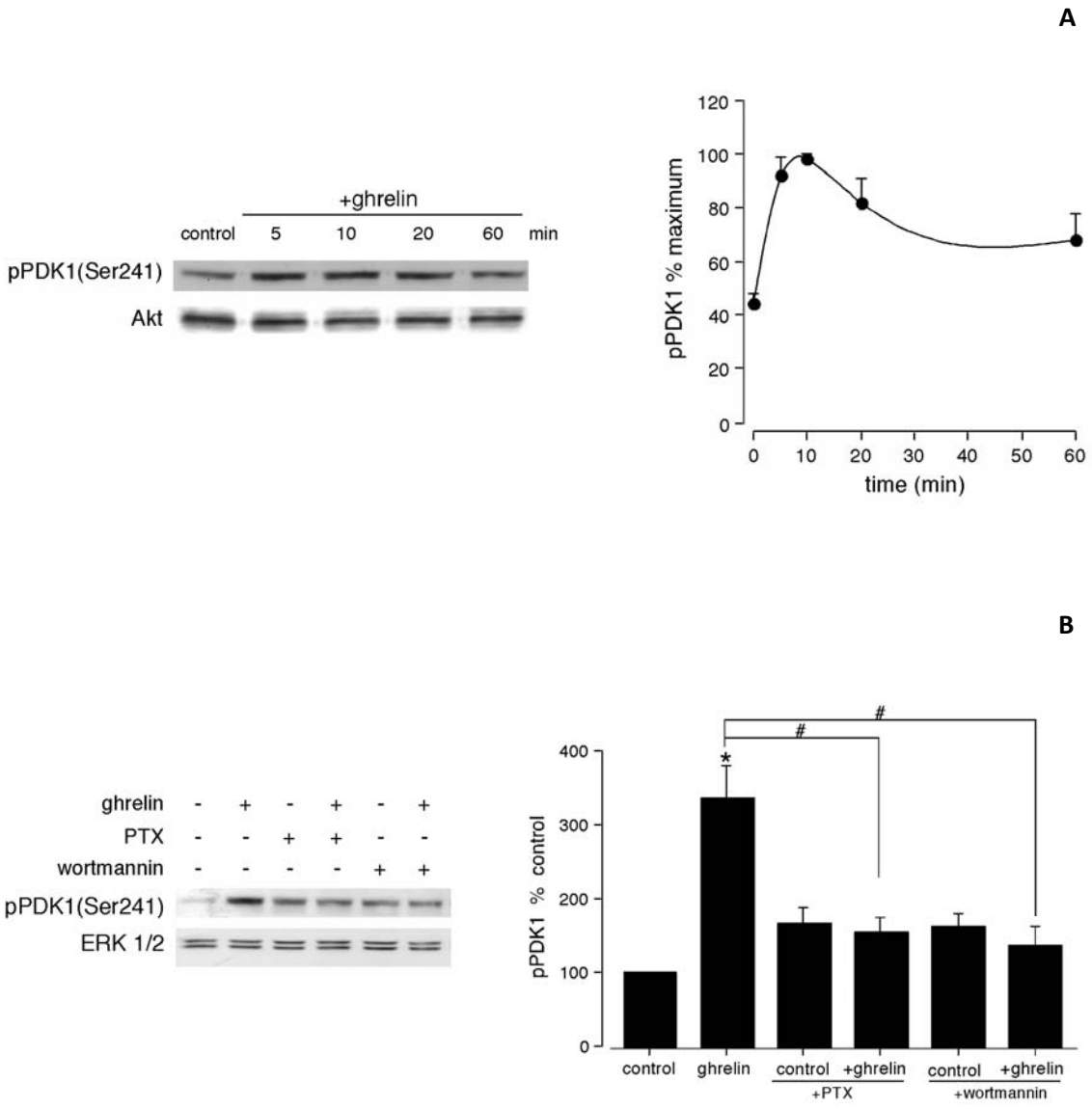

Figure 6. Mechanism of PDK-1 activation by ghrelin. A. Time-course of the effect of ghrelin on phosphorylation of PDK-1**.** Serum-starved cells were stimulated with ghrelin (100 nM), lysed and then analyzed by immunoblotting with antibodies against pPDK-1 (S241) and Akt. PDK-1 phosphorylation was quantified by densitometry and expressed as a percentage of the maximal phosphorylation (mean±SE).B. PDK-1 phosphorylation induced by ghrelin (100 nM, 5 min) in the absence or presence of PTX (100 ng/mL, 12 h) and the PI3K inhibitor wortmannin (1 µM, 30 min). PDK-1 phosphorylation was quantified by densitometry and expressed as the percentage of the basal phosphorylation obtained in control cells (means±SE). Immunoblots are representative of three independent experiments. (*, #, $P<0.05$).

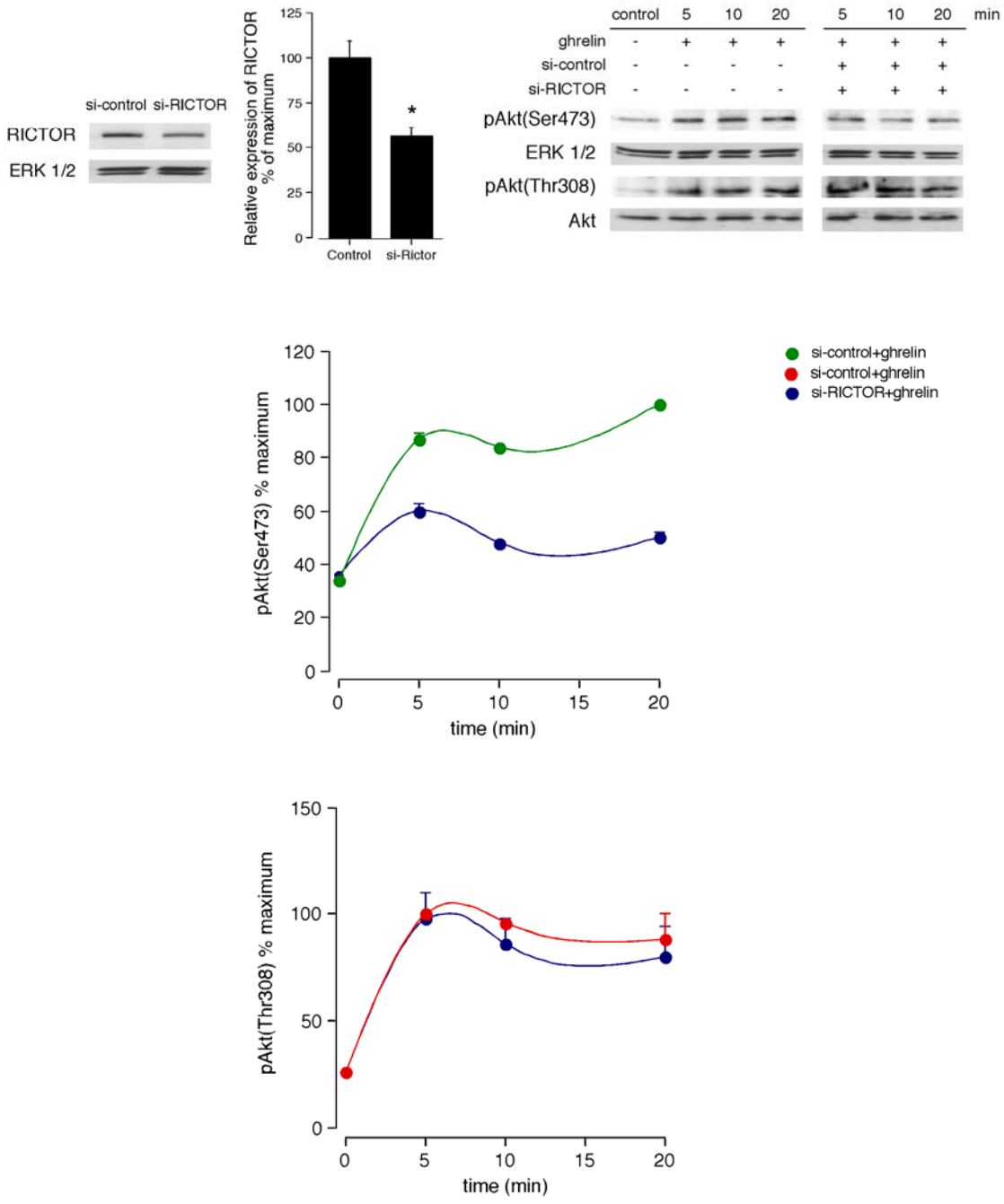

Figure 7. Effect of siRNA depletion of Rictor on ghrelin-induced Akt phosphorylation. HEK-GHSR-1a cells transfected with Rictor siRNA were serum-starved and then stimulated with ghrelin (100 nM). Equal amounts of protein in each sample were used to assess the expression of Rictor and Akt phosphorylation by immunoblotting. Expression of Rictor was quantified by densitometry. Values shown are percentages of the level of Rictor in control siRNA-transfected cells (mean±SE). Akt phosphorylation was quantified by densitometry and expressed as a percentage of the maximal phosphorylation of Akt HM (S473) and A-loop (T308) after ghrelin stimulation to control siRNA-transfected cells (mean±SE). Immunoblots are representative of three independent experiments. (*, P<0.05).

The next step was dilucidate the role of β-arrestins in Akt phosphorylation. The siRNA experiments targeting β-arrestin 1 or β-arrestin 2 (45±2% and 55±4% reduction respectively) showed that β-arrestin 1 and β-arrestin 2 siRNA led to rapid Akt phosphorylation, which decreased after 10 minutes of ghrelin stimulation (100 nM) with respect to siRNA control [41±2% and 39±3% at HM (S473) for β-arrestin 1 and β-arrestin 2 siRNA respectively; and 47±5% and 40±4% at A-loop (T308) for β-arrestin 1 and β-arrestin 2 siRNA respectively]. This inhibitory effect stayed on both residues for at least 60 minutes (Figure 8).

Immunoprecipitation of ghrelin-stimulated cells (100 nM, 10 min) with antibodies to β-arrestin 1 or β-arrestin 2 co-precipitated pAkt (T308, S473) while failed to co-precipitated pPDK-1 (S241), Rictor and mTOR (Figure 9). The β-arrestin-dependent Akt phosphorylation was dependent on cSrc as no immunoprecipitation of pAkt was obtained with antibodies to β-arrestin 1 or β-arrestin 2 in ghrelin-stimulated cells (100 nM, 10 min) after pretreatment with PP2 (5 μM, 30 min). This inhibition was specific, since pretreatment with PP3 (5 μM, 30 min) had no effect on the β-arrestin-dependent Akt phosphorylation induced by ghrelin (data not shown).

Figure 10 shows that in 3T3-L1 cells, ghrelin induced Akt phosphorylation at both residues and at different time points, reaching maximal levels within 20–60 minutes of ghrelin stimulation (100 nM). This time-course was similar to the time-course observed in HEK-GHSR-1a cells. In a similar way, the effect of PP2 (5 μM, 30 min) reduced Akt phosphorylation at both residues, meanwhile PP3 pretreatment (5 μM, 30 min), a negative control for PP2, showed no effect (Figure 10).

Immunoprecipitation experiments performed on ghrelin-stimulated cells (100 nM, 10 minutes) with antibodies to β-arrestin 1 and β-arrestin 2 co-precipitated full-activated Akt showing an increase on tyrosine phosphorylation compared to unstimulated cells. In addition, the activated form of cSrc, pcSrc (Tyr 416) co-immunoprecipitated with β-arrestin 1 and β-arrestin 2 (Figure 11).

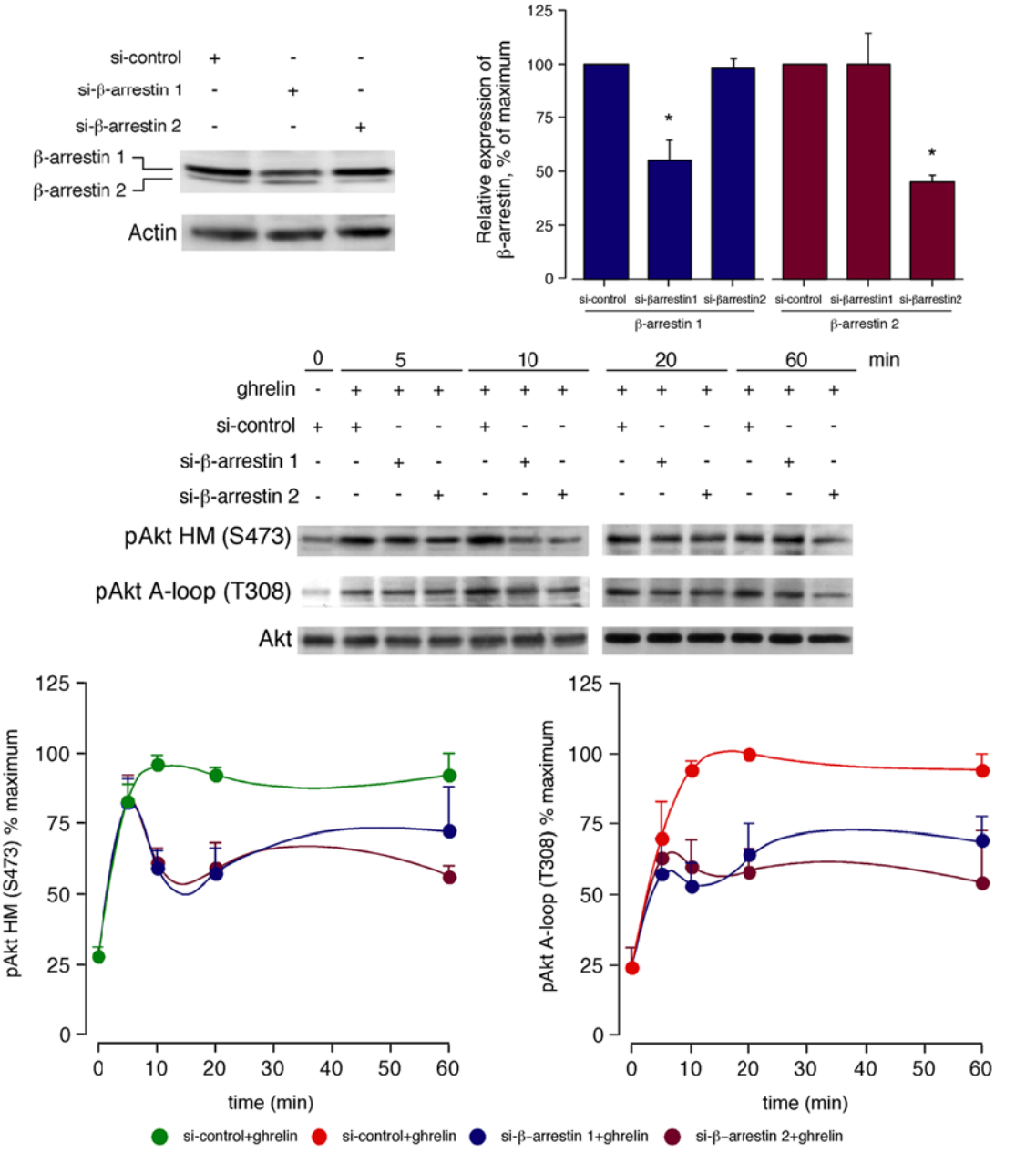

Figure 8. Effect of siRNA depletion of β-arrestin 1 or β-arrestin 2 on ghrelin-induced Akt phosphorylation. HEK-GHSR-1a cells transfected with β-arrestin 1 or β-arrestin 2 siRNA were serum starved and then stimulated with ghrelin (100 nM). Equal amounts of protein in each sample were used to assess the expression of β -arrestin 1 or β-arrestin 2 (upper panel) and Akt phosphorylation (lower panel) by immunoblotting. Expression of β-arrestin 1 or β-arrestin 2 was quantified by densitometry. Values shown are percentages of the level of β-arrestins in control siRNA-transfected cells (mean±SE). Akt phosphorylation was quantified by densitometry and expressed as a percentage of the maximal phosphorylation at HM (S473) and A-loop (T308) after ghrelin addition to control siRNA-transfected cells (mean±SE). Immunoblots are representative of three independent experiments. (*, P<0.05).

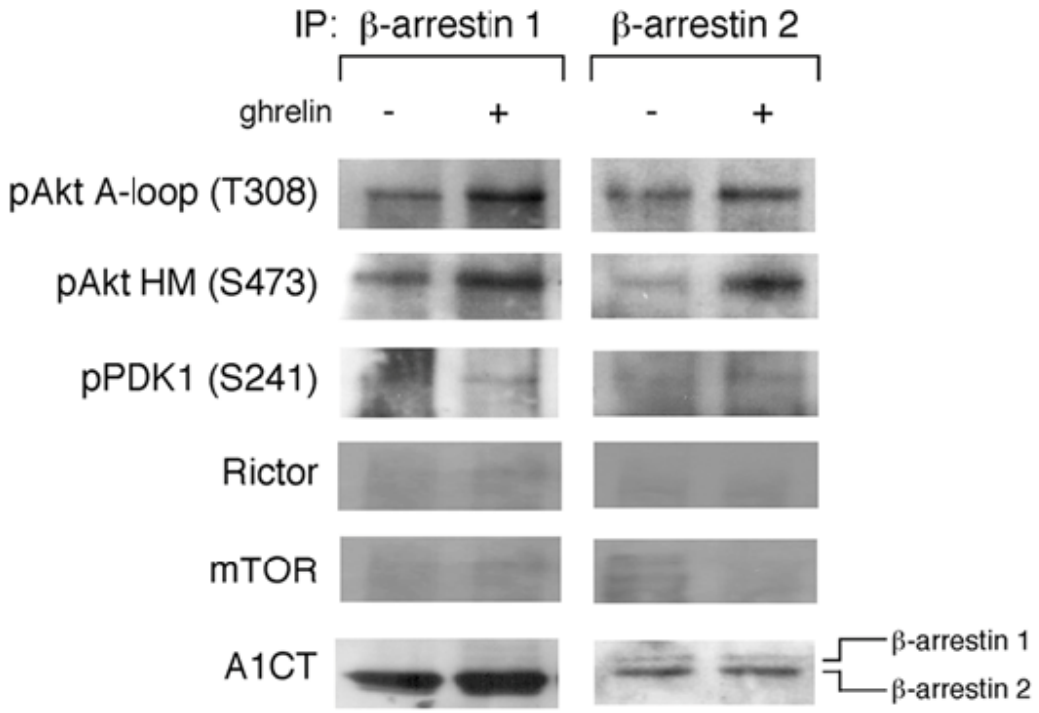

Figure 9. Effect of ghrelin on the assembly of complexes containing β-arrestins 1 and 2 and pAkt. Serum-starved HEK-GHSR-1a cells were stimulated with ghrelin (100 nM, 10 min), lysed and immunoprecipitated (IP) with antibodies to β-arrestin 1 (left panel) or β-arrestin 2 (right panel), and then analyzed by immunoblotting with pAkt [HM (S473), A-loop (T308), pPDK-1 (S241)], Rictor, mTOR, and β-arrestin (A1CT) antibodies. Immunoblots are representative of three independent experiments.

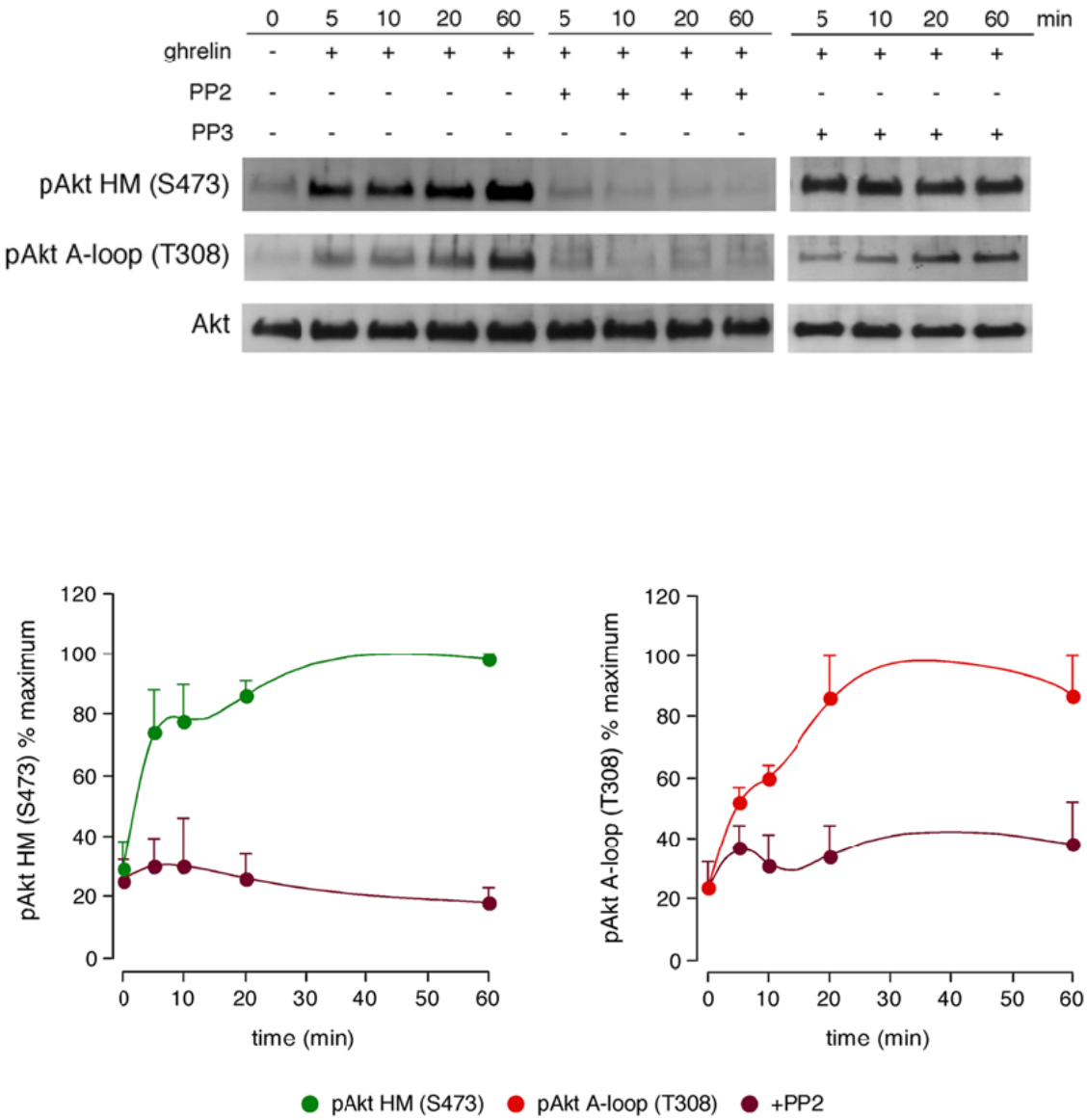

Figure 10. Effect of the cSrc inhibitor PP2 and PP3, on the ghrelin-induced Akt activation in 3T3-L1 preadipocyte cells. Serum-starved 3T3-L1 cells were pretreated with PP2 (5 µM, 30 min) or PP3 (5 µM, 30 min) before ghrelin stimulation (100 nM) for the indicated time periods at 37°C. Then, cells were lysed and analyzed by immunoblotting with pAkt HM (S473) and A-loop (T308). Akt phosphorylation was quantified by densitometry and expressed as a percentage of the maximal phosphorylation at HM (S473) and A-loop (T308) (mean±SE). Immunoblots are representative of three independent experiments.

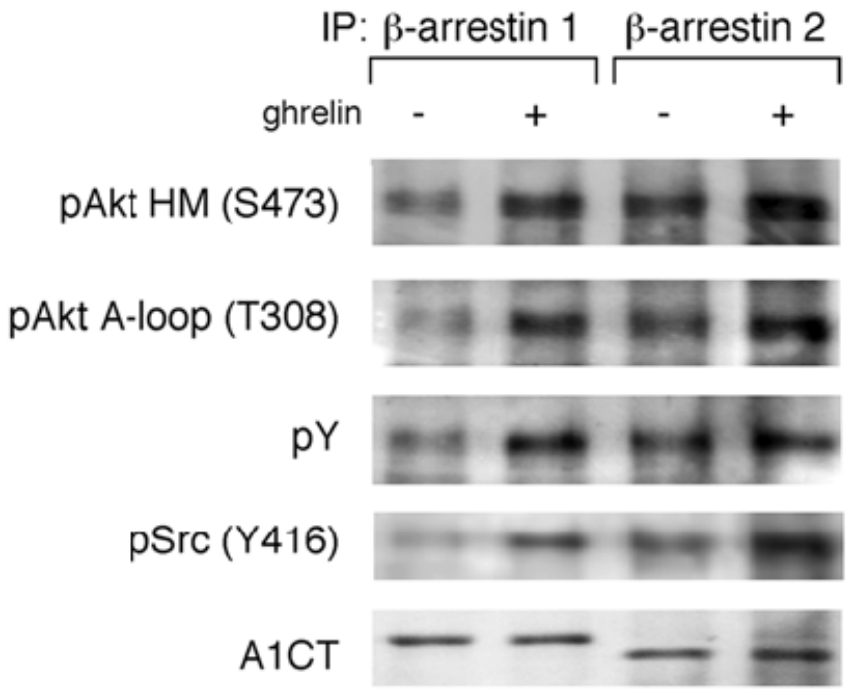

Figure 11. Effect of ghrelin on the assembly of complexes containing β-arrestins 1 and 2 and pAkt in 3T3-L1 preadipocyte cells. Serum-starved 3T3-L1 cells were stimulated with ghrelin (100 nM, 10 min), lysed and immunoprecipitated (IP) with antibodies to β-arrestin 1 and 2 and then analyzed by immunoblotting with pAkt HM (S473), pAkt A-loop (T308), pY and pcSrc (Y416) antibodies. Immunoblots are representative of three independent experiments.

CHAPTER 3

SHP-1 protein tyrosine phosphatase negatively modulates Akt signaling in ghrelin/GHSR-1a system. Implications in white adipose tissue.

Phosphorylation of the C-terminal Y536 residue of SHP-1 [pSHP-1 (Y536)] after ghrelin stimulation in HEK-GHSR-1a cells was evaluated. As Figure 1 shows, ghrelin (100 nM) increased pSHP-1 (Y536), reaching maximal levels within 10 minutes, keeping this degree by at least 60 minutes.

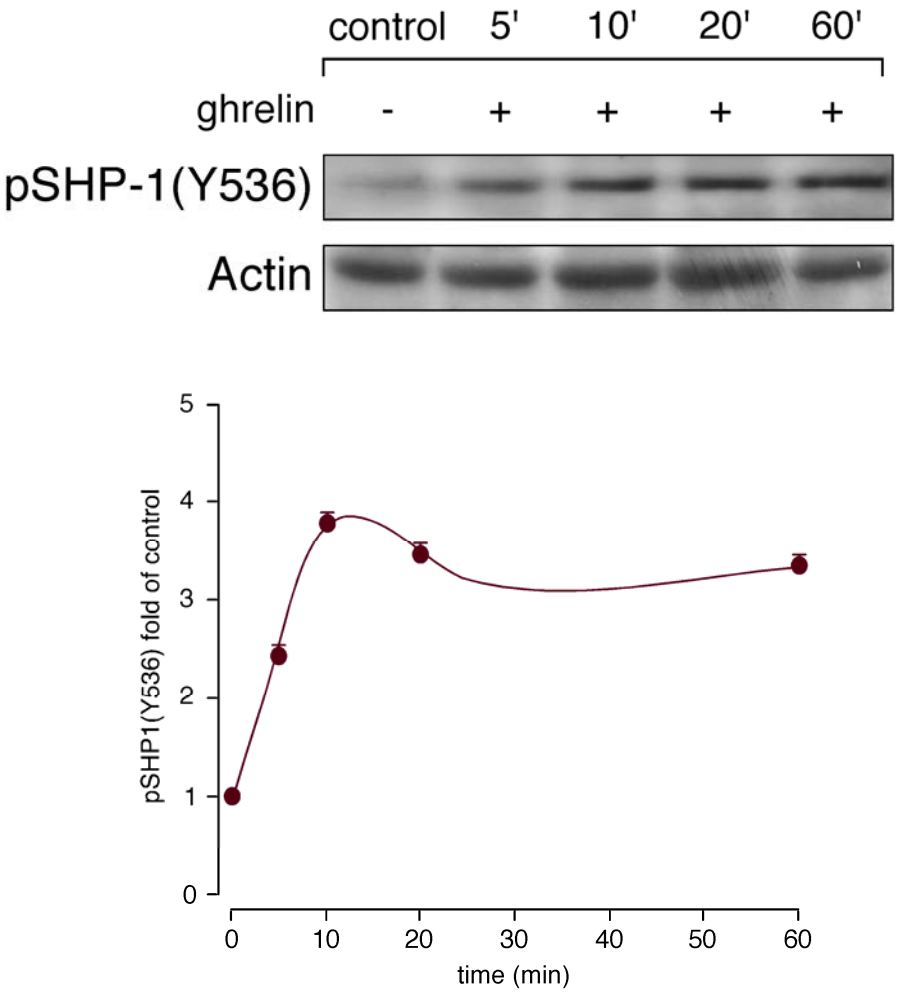

Figure 1. Time-course of the effect of ghrelin on SHP-1 C-terminal Y536 residue phosphorylation [pSHP-1(Y536)]. Serum-starved HEK-GHSR-1a cells were stimulated with ghrelin (100 nM) for the time periods indicated. Cells were lysed and analyzed by immunoblotting using specific antibodies against pSHP-1 and actin. SHP-1 phosphorylation was quantified by densitometry and expressed as a fold of control cells (mean±SE). Immunoblots are representative of three independent experiments.

The role of SHP-1 on ghrelin-induced phosphorylation of the Akt A-loop (T308) and HM (S473) was evaluated by means of overexpression of a dominant-negative SHP-1 mutant (SHP-1dn). Ghrelin-induced Akt activity was increased in SHP-1dn-transfected cells, with higher effect on Akt A-loop (T308) phosphorylation than that observed for Akt HM (S473) phosphorylation within 20 minutes of ghrelin stimulation (100 nM) (Figure 2). Akt phosphorylation at both residues was not altered after 20 minutes of ghrelin treatment in the SHP-1dn-transfected cells (Figure 2).

PI3K activation was evaluated by means of overexpression of SHP-1dn. For this purpose, control and SHP-1dn-transfected cells were immunoprecipitated with p85 and the tyrosine phosphorylation (Y), under ghrelin stimulation was evaluated. Results in Figure 3 showed that the level of p85-tyrosine phosphorylation induced by ghrelin (100 nM, 5 min) was markedly higher in SHP-1dn-transfected cells compared with control cells.

cSrc activation was also evaluated through overexpression of SHP-1dn. Ghrelin-stimulated cSrc phosphorylation [pSrc (Y416)] was measured in control and SHP-1dn-transfected cells. As shown in Figure 4, the level of pcSrc (Y416) is markedly higher in cells overexpressing SHP-1dn compared with control cells. In addition, the effect of SHP-1dn on PDK-1 phosphorylation at its conserved serine residue, S241, was also evaluated. Overexpression of SHP-1dn increased ghrelin-induced PDK-1 phosphorylation [pPDK-1 (S241)] (Figure 4).

The effect of siRNA-mediated suppression of β-arrestins 1 and 2 expression on the kinetics of pSHP-1 following ghrelin stimulation was examined. siRNA experiments targeting β-arrestin 1 or β-arrestin 2 reduced their expression by 50±2% and 65±4%, respectively (Figure 5). In the presence of a non-targeting control siRNA, ghrelin-activated pSHP-1(Y536) was identical to that observed without any transfection (data not shown). β-arrestin 1 and β-arrestin 2 siRNA decreased ghrelin-activated pSHP-1(Y536) with respect to siRNA control, with maximal inhibitory effect at the later time points [34±3% and 60±9% at 10 min for β-arrestin 1 and β-arrestin 2 siRNA, respectively (Figure 5)].

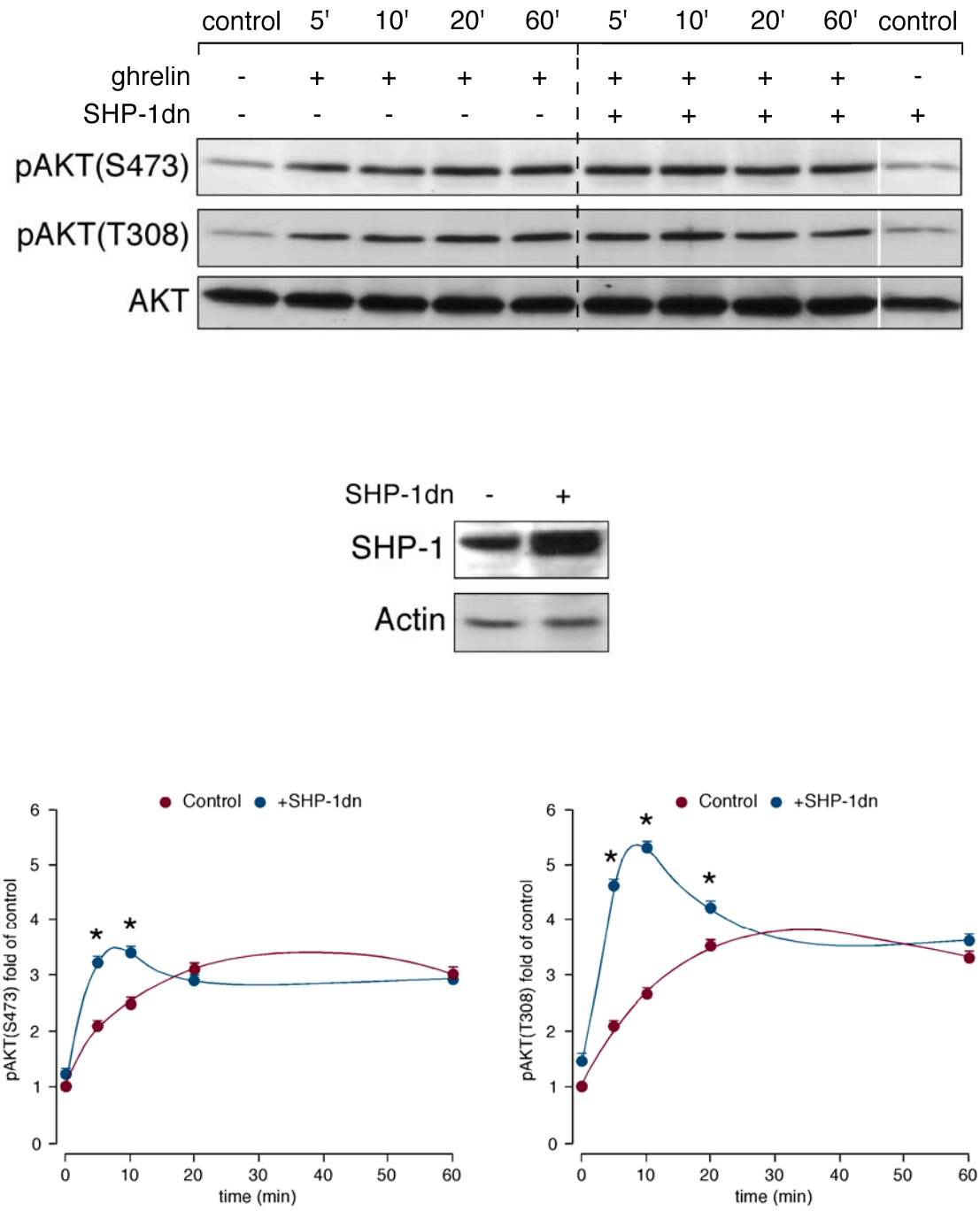

Figure 2. Effect of overexpression of dominant-negative SHP-1 mutant (SHP-1dn) on ghrelin-mediated Akt activation. Serum-starved HEK-GHSR-1a cells were stimulated with ghrelin (100 nM) for the time periods indicated. Cells were lysed and analyzed by immunoblotting using specific antibodies against pAkt (S473) and (T308). Akt phosphorylation was quantified by densitometry and expressed as a fold of control cells (mean±SE). Immunoblots are representative of three independent experiments. Immunoblot analysis of SHP-1 expression in control and SHP-1dn-transfected HEK-GHSR-1a cells is shown in the center of the panel. (*, P<0.05).

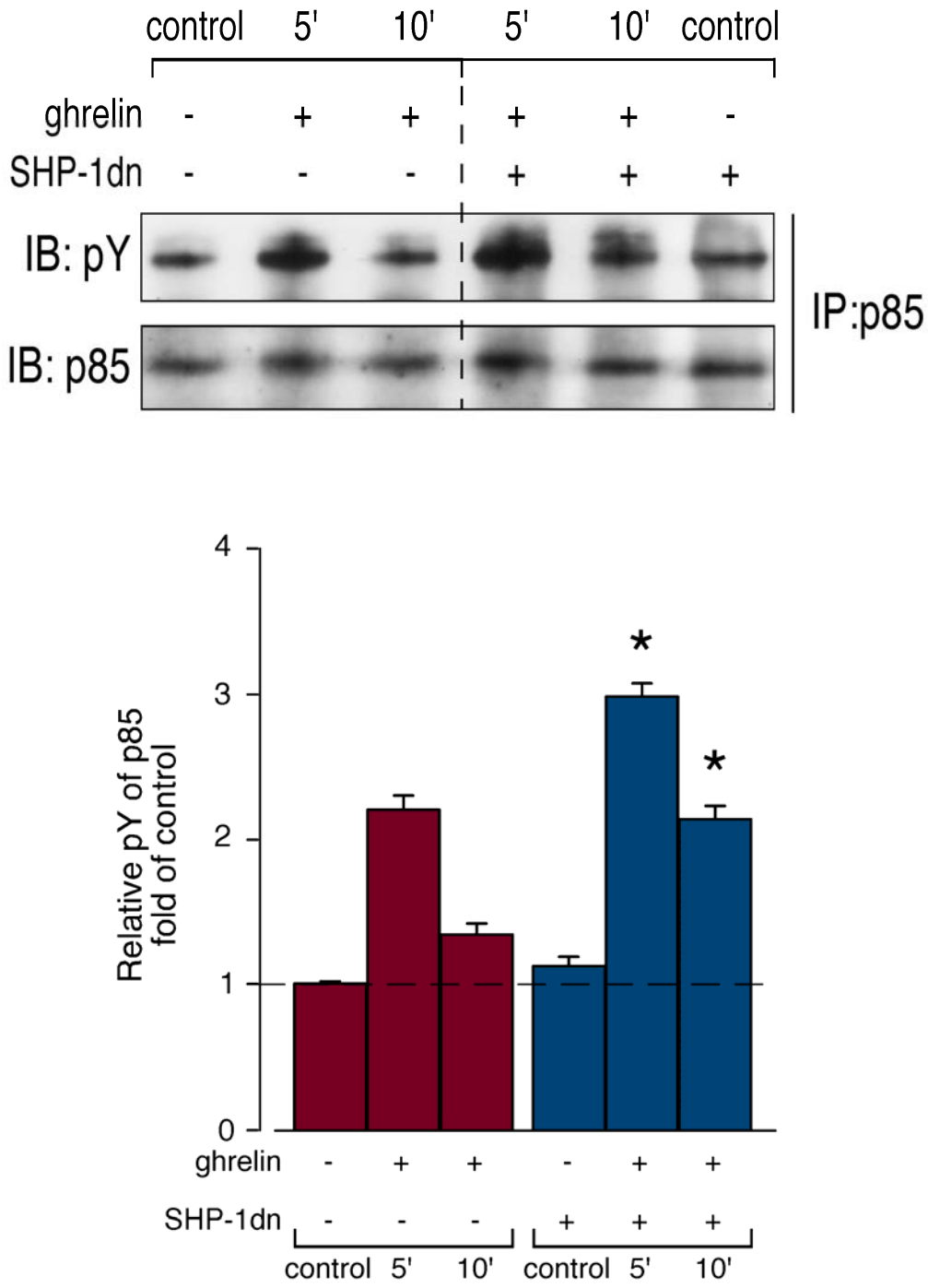

Figure 3. Basal and ghrelin-induced Y phosphorylation and expression of p85 regulatory subunit of PI3K in SHP-1dn-transfected and control cells. Serum-starved HEK-GHSR-1a cells were stimulated with ghrelin (100 nM) for the time periods indicated. Cells were lysed and immunoprecipitated (IP) with antibodies to p85 and then analyzed by immunoblotting with antibodies to phospho-tyrosine (pY) and p85. Tyrosine phosphorylation of PI3K was quantified by densitometry and expressed as a fold of control cells (mean±SE). Immunoblot is representative of three independent experiments. (*, P<0.05).

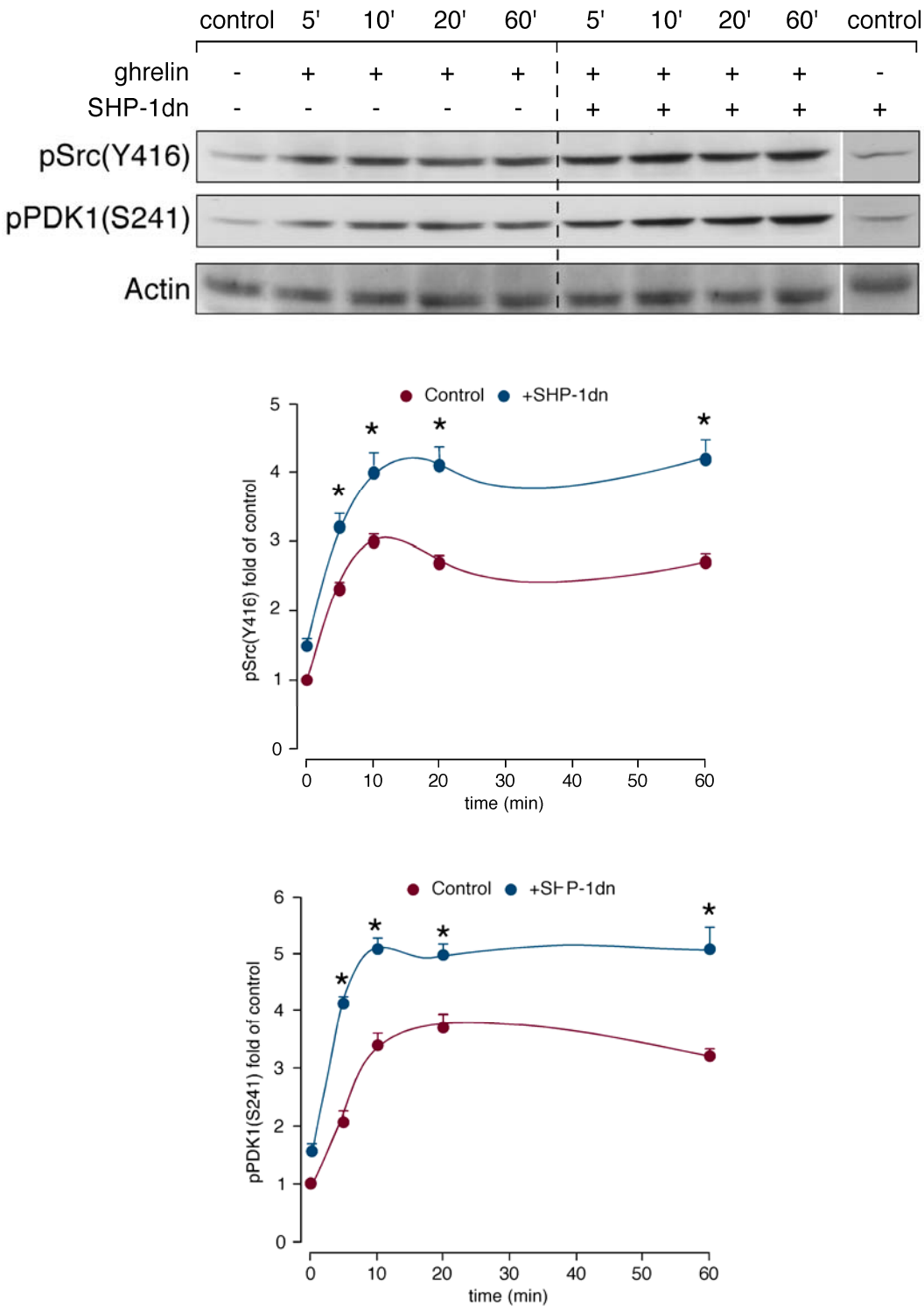

Figure 4. Ghrelin-induced cSrc (Y416) and PDK-1 (S241) phosphorylation in control and SHP-1dn-transfected cells. Serum-starved HEK-GHSR-1a cells were stimulated with ghrelin (100 nM) for the time periods indicated. Cells were lysed and analyzed by immunoblotting using specific antibodies against cSrc (Y416) and PDK-1 (S241). Phosphorylation was quantified by densitometry and expressed as a fold of control cells (mean±SE). Immunoblots are representative of three independent experiments. (*, $P<0.05$).

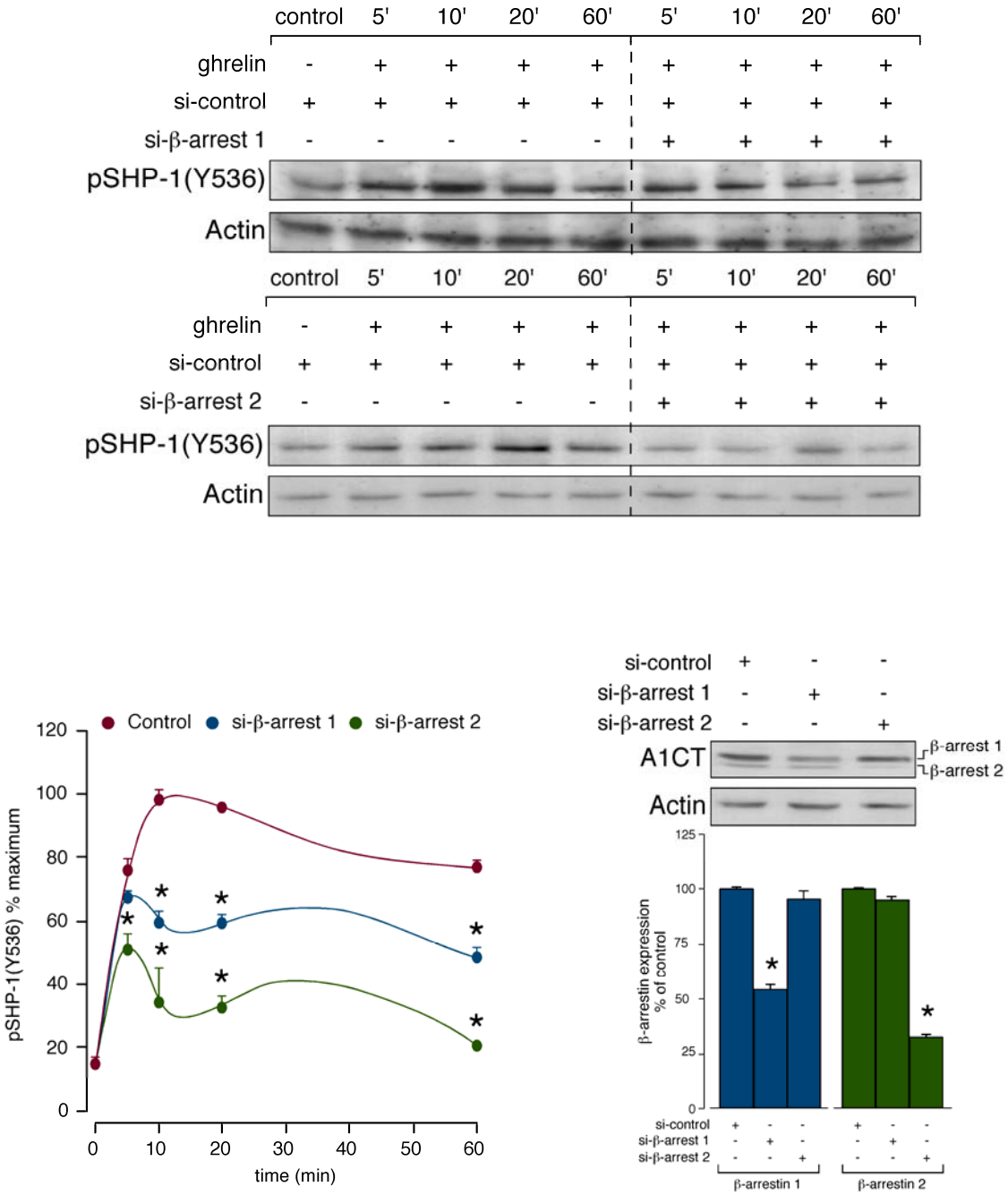

Figure 5. Effect of siRNA depletion of β-arrestin 1 and 2 on ghrelin-induced pSHP-1(Y536) HEK-GHSR-1a cells transfected with β-arrestin 1 or β-arrestin 2 siRNA were serum starved for 12 h and then stimulated with ghrelin (100 nM) at 37°C. After stimulation, equal amounts of protein in each sample were used to assess the expression of β -arrestin 1 or β-arrestin 2 (lower panel) and SHP-1 phosphorylation (upper panel) by immunoblotting. Expression of β-arrestin 1 or β-arrestin 2 was quantified by densitometry. Values shown are percentages of the level of β-arrestins in control siRNA-transfected cells (mean±SE). SHP-1 phosphorylation was quantified by densitometry and expressed as a percentage of the maximal phosphorylation after ghrelin addition to control siRNA-transfected cells (mean±SE). Immunoblots are representative of three independent experiments. (*, P<0.05).

Immunoprecipitation assays of β-arrestin 1 and 2 in SHP-1dn-transfected cells, co-immunoprecipitated pSHP-1 (Y536) under ghrelin stimulation (100 nM, 10 min) by an average of 4.4±0.3 or 4.4.±0.2-fold relative to control cells, respectively (Figure 6). In addition, ghrelin stimulation (100 nM, 10 min) of cells overexpressing SHP-1dn, enhanced the ghrelin response on β-arrestin associated pAkt (S473) and pcSrc (Y416) phosphorylation, verifying that SHP-1 regulates not only $G_{i/o}$-dependent pathway but also the pathway mediated by β-arrestins-associated complex (Figure 6).

Because cSrc is able to phosphorylate SHP-1 on Y536 *in vitro*, experiments of ghrelin-stimulated siRNA targeting cSrc were made. As Figure 7 shows, siRNA targeting cSrc reduced its expression by 69±3%. cSrc siRNA decreased ghrelin-activated SHP-1 (Y536) phosphorylation by over 80% with respect to siRNA control for all time tested, suggesting that SHP-1 activation is entirely mediated by cSrc (Figure 7).

Immunoblot analysis demonstrated moderate SHP-1 expression in 3T3-L1 cells and WAT but at lower levels than in liver (Figure 8 A). In preadipocyte and adipocyte 3T3-L1 cells, SHP-1 was detected by immunohistochemistry (IHC) in the cytoplasm with enrichment in the perinuclear region (Figure 8 B).

Determination of pSHP-1 (Y536) by ghrelin stimulation (100 nM) in 3T3-L1 cells confirmed the stimulatory ghrelin effect on SHP-1 activity in preadipocytes and adipocytes (Figure 9).

SHP-1 was also detected immunohistochemically in omental and subcutaneous WAT obtained from mice under normal chow (control) and high fat diet (HFD) (Figure 10). pSHP-1 (Y536) was significantly increased in ghrelin-stimulated omental and subcutaneous WAT explants from control mice *in vitro* (100 nM, 1h). This activation is higher in omental than in subcutaneous tissue correlating with a higher expression for the SHP-1 in the omental tissue (Figure 11 A). Similarly, pSHP-1(Y536) was significantly increased in ghrelin-stimulated omental and subcutaneous WAT explants from HFD mice *in vitro* (100 nM, 1h). This activation, however, is higher in subcutaneous than in omental tissue correlating with a higher expression for the SHP-1 in the subcutaneous tissue from HFD mice (Figure 11 B).

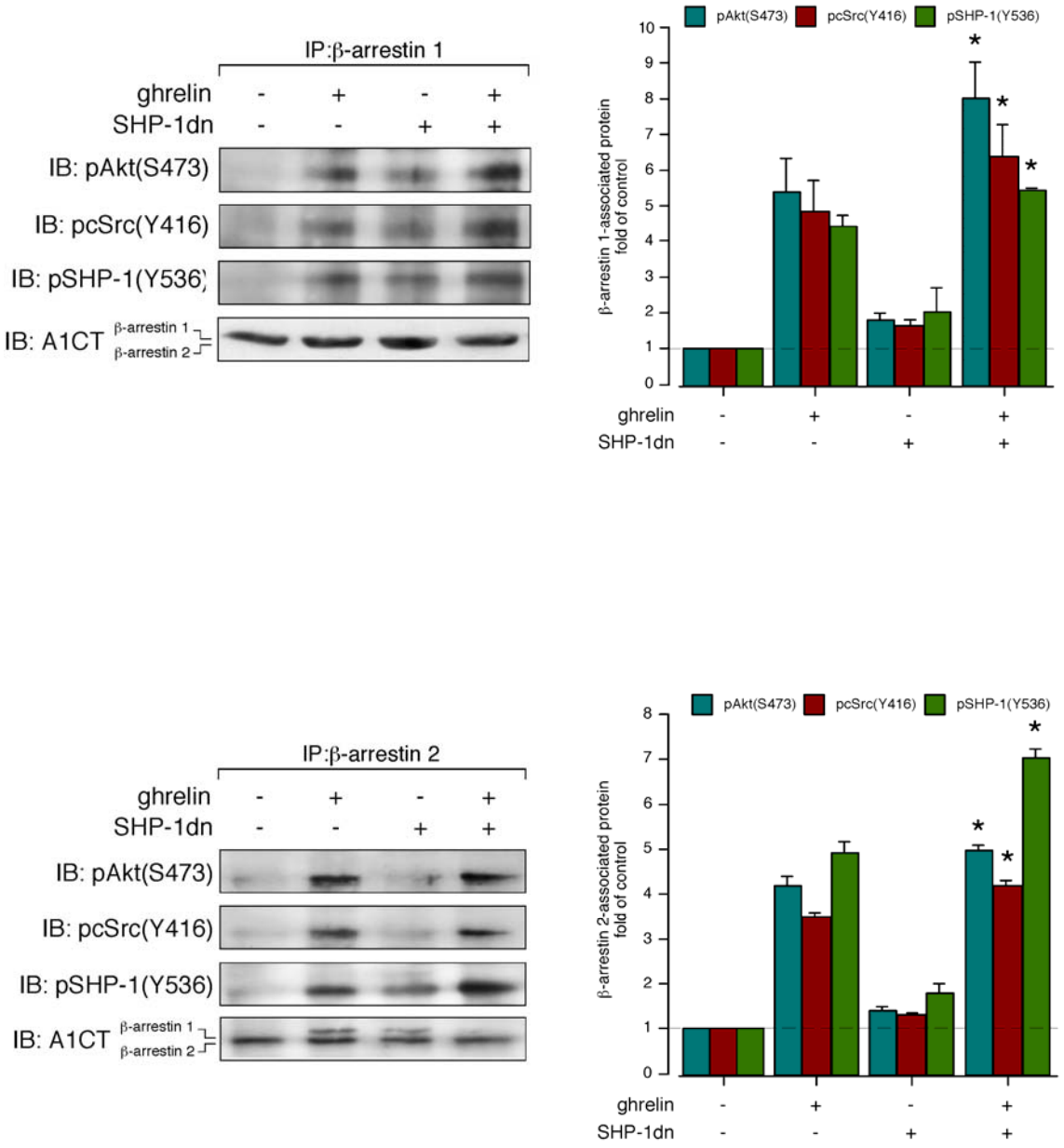

Figure 6. Effect of ghrelin on the assembly of complexes containing β-arrestins 1 and 2 pAkt (S473), pcSrc (Y416) and pSHP-1 (Y536) in control and SHP-1dn-transfected cells. Serum-starved HEK-GHSR-1a cells were stimulated with ghrelin (100 nM, 10 min), lysed and immunoprecipitated (IP) with antibodies to β-arrestin 1 (upper panel) and β-arrestin 2 (lower panel), and then analyzed by immunoblotting with pAkt HM (S473), pcSrc (Y416) and pSHP-1 (Y536) antibodies. Phosphorylation was quantified by densitometry and expressed as a fold of control cells (mean±SE). Immunoblots are representative of three independent experiments. (*, P<0.05).

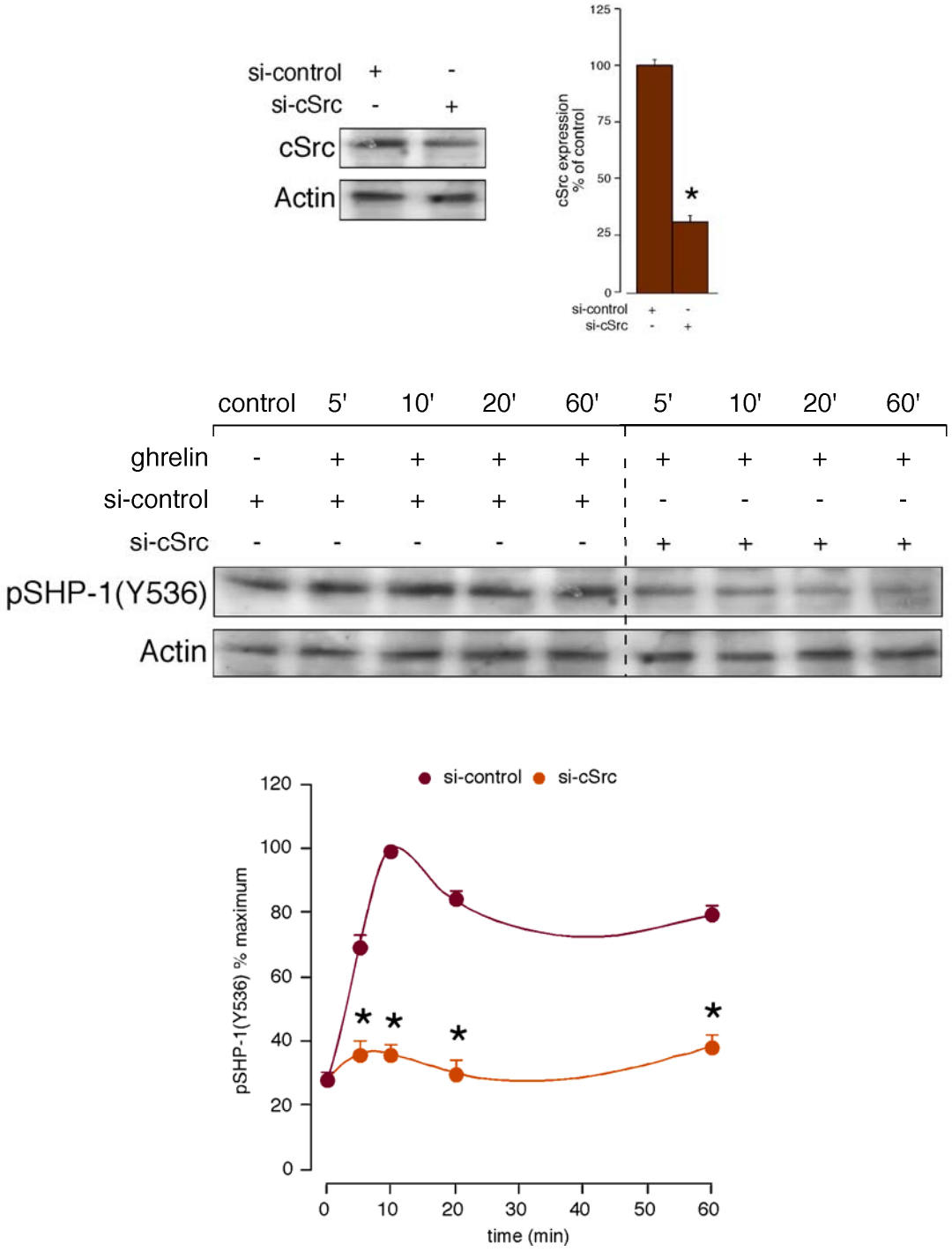

Figure 7. Effect of siRNA depletion of cSrc on ghrelin-induced pSHP-1(Y536). HEK-GHSR-1a cells transfected with cSrc siRNA were serum starved and then stimulated with ghrelin (100 nM). Equal amounts of protein in each sample were used to assess the expression of cSrc (upper panel) and SHP-1 phosphorylation (lower panel) by immunoblotting. Expression of cSrc was quantified by densitometry. Values shown are percentages of the level of cSrc in control siRNA-transfected cells (mean±SE). SHP-1 phosphorylation was quantified by densitometry and expressed as a percentage of the maximal phosphorylation after ghrelin addition to control siRNA-transfected cells (mean±SE). Immunoblots are representative of three independent experiments. (*, P<0.05).

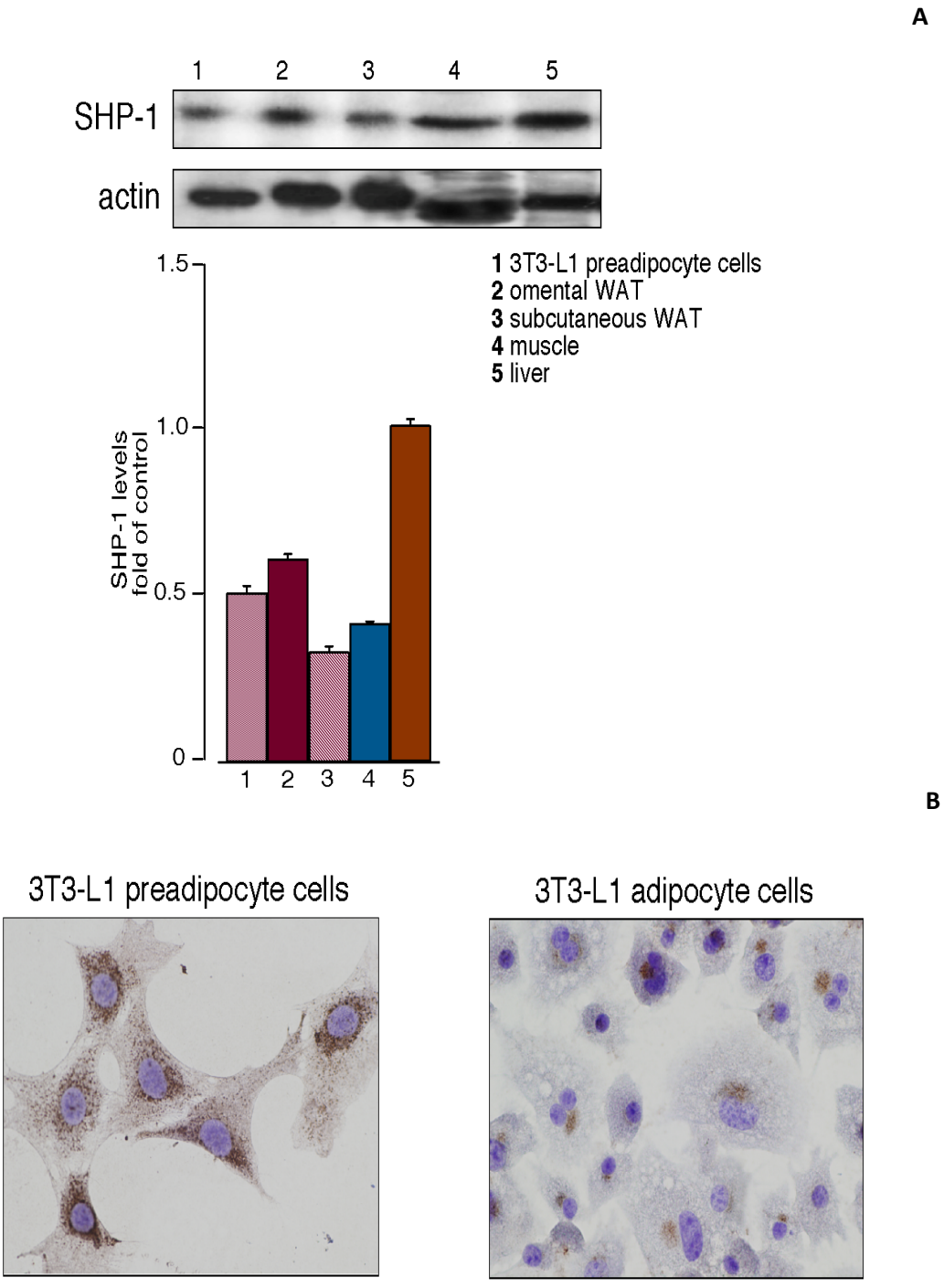

Figure 8. A. Immunoblot analysis of SHP-1 in extracts from 3T3-L1 cells, WAT, skeletal muscle and liver of control mice. Same amount of protein (40 µg) was used for this analysis. SHP-1 was detected by immunoblotting and normalized for actin.Results were expressed as a fold of respective control (mean±SE; n=10). Immunoblots are representative of three independent experiments. B. Immunocytochemical detection of SHP-1 in 3T3-L1 preadipocyte and adipocyte cells (objective magnification 40x). SHP-1 immunostaining was higher in preadipocyte than adipocyte cells being mainly concentrated in perinuclear compartments in both 3T3-L1 cells.

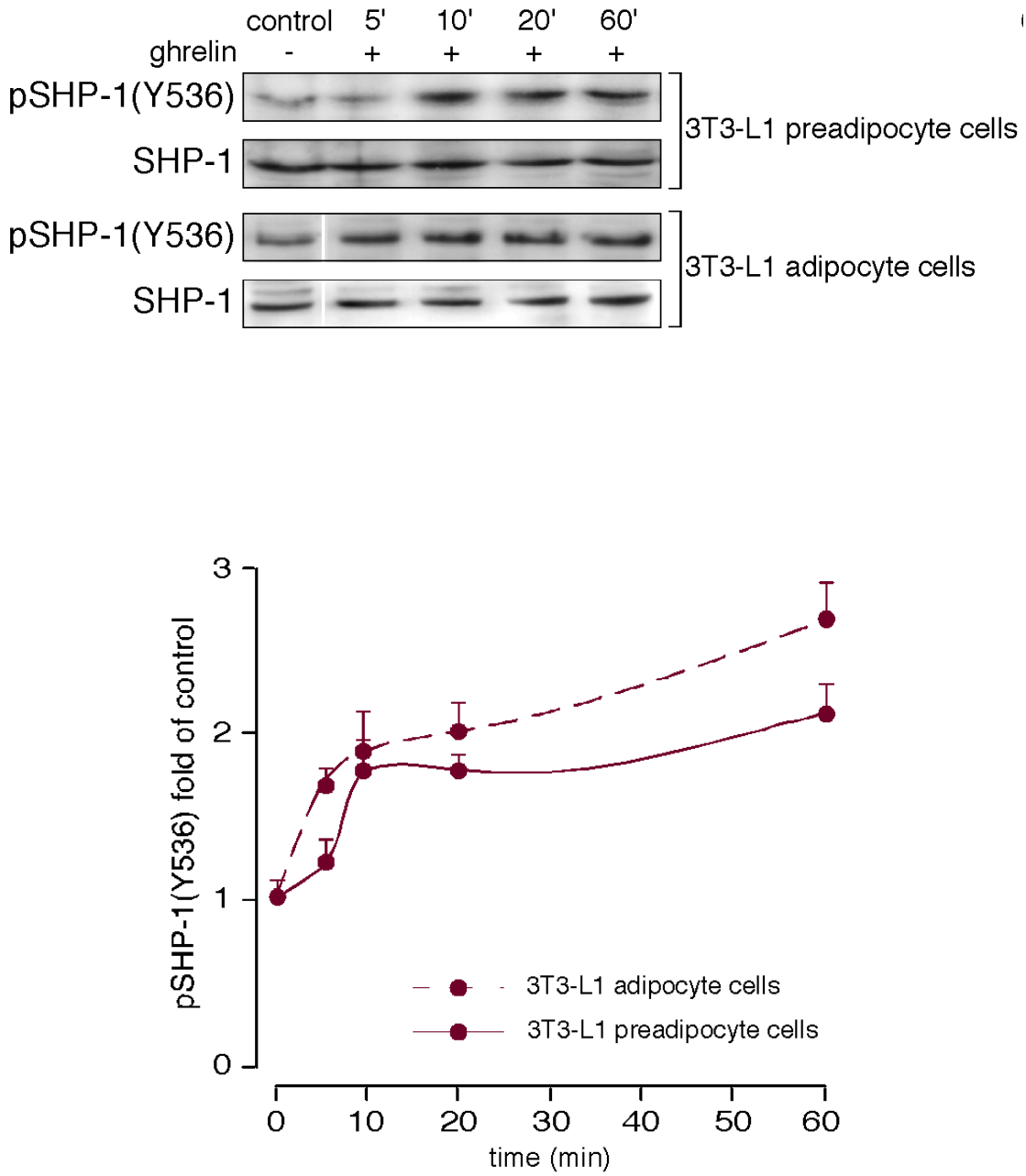

Figure 9. Time-course of the effect of ghrelin on pSHP-1 (Y536) in 3T3-L1 preadipocyte and adipocyte cells. Serum-starved 3T3-L1 cells were stimulated with ghrelin (100 nM) for the time periods indicated. Cells were lysed and analyzed by immunoblotting using specific antibodies against pSHP-1 and SHP-1. SHP-1 (Y536) phosphorylation was quantified by densitometry and expressed as a fold of control cells (mean±SE). Immunoblots are representative of three independent experiments.

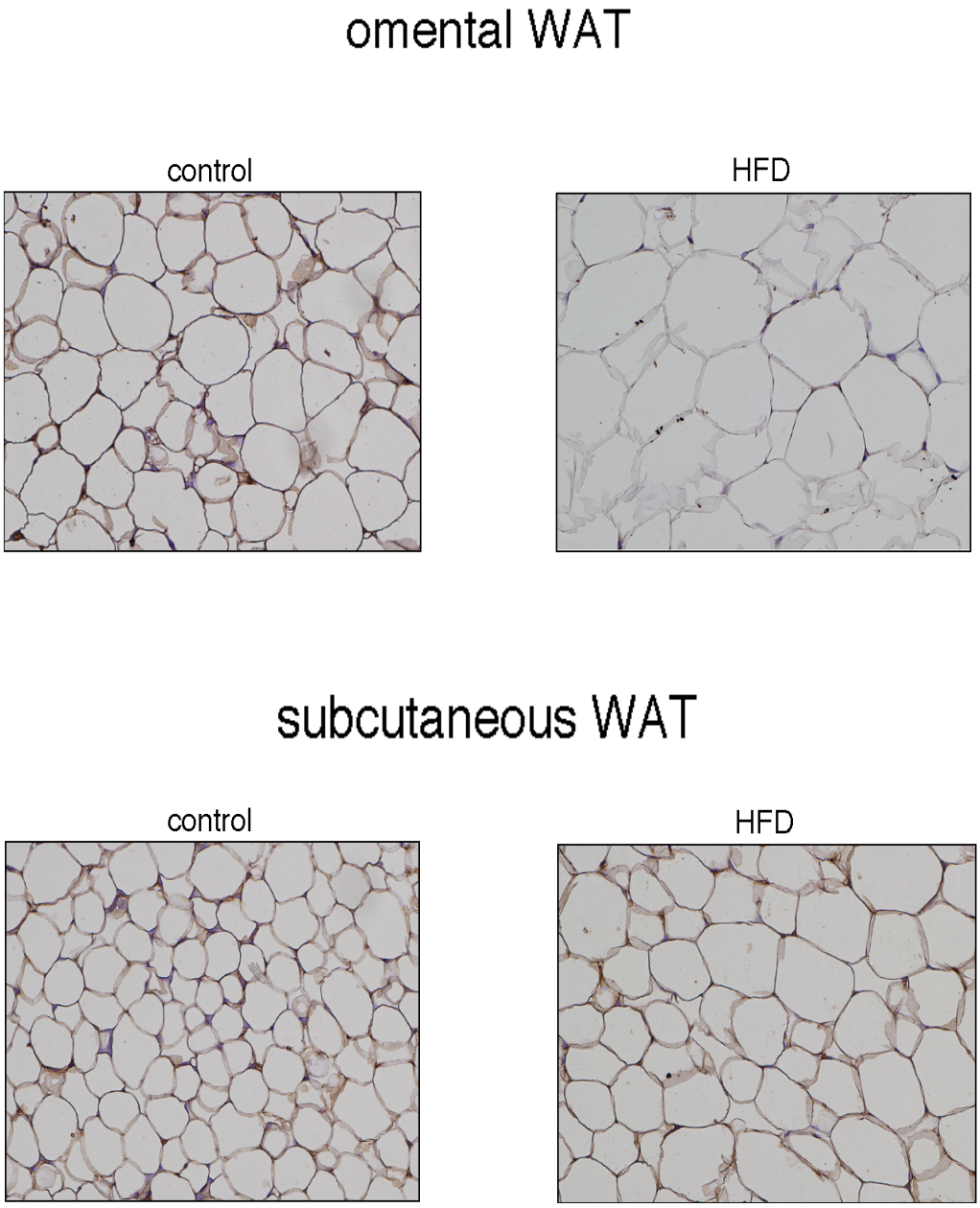

Figure 10. Immunohistochemical detection of SHP-1 in omental (upper panel) and subcutaneous (lower panel) WAT obtained from control and HFD-treated mice (objective magnification 20x). Decreased SHP-1 immunostaining was evident in omental WAT compared to control mice.

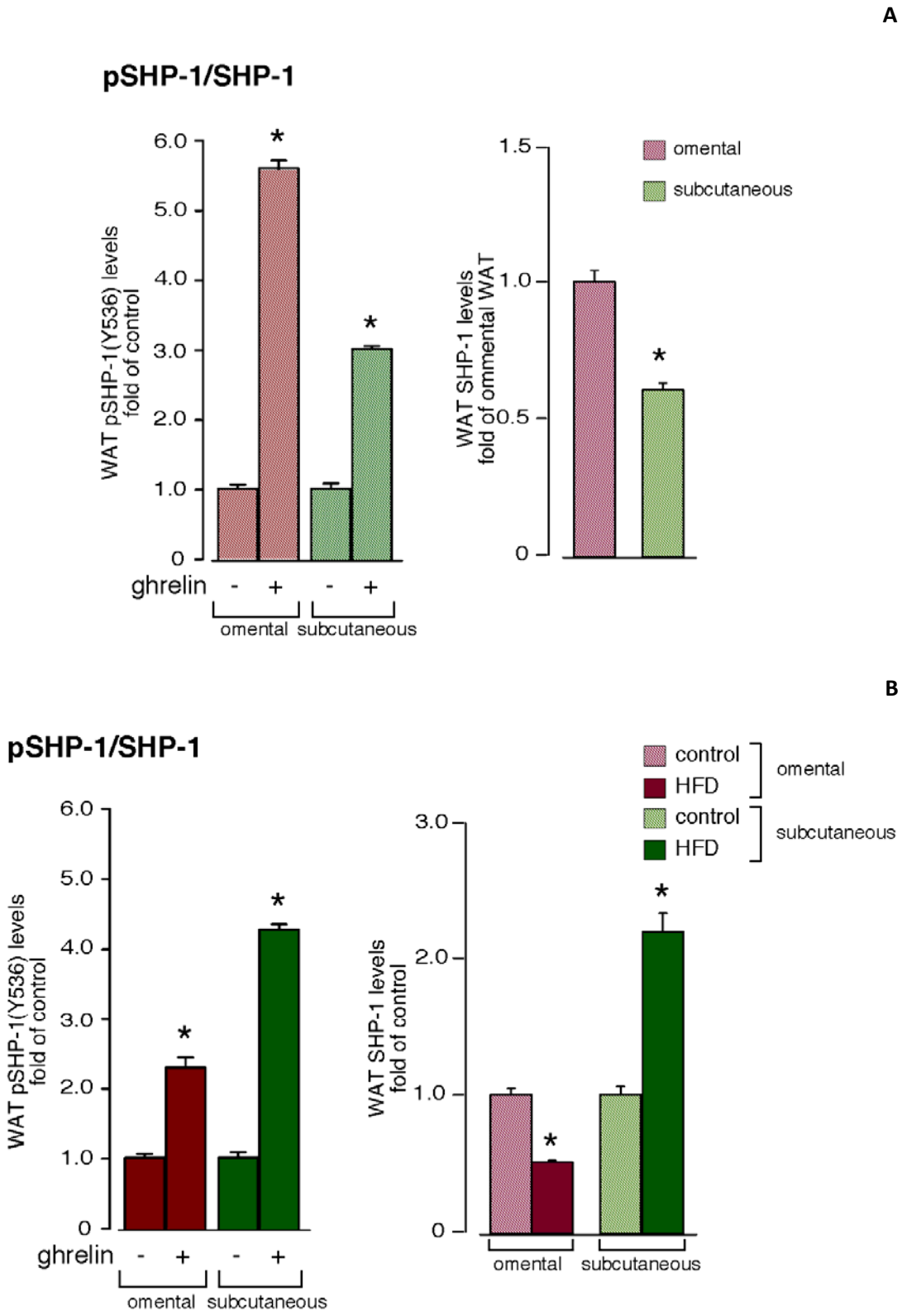

Figure 11. A. Effect of ghrelin (100 nM, 1h) on pSHP-1(Y536) in omental and subcutaneous WAT explants *in vitro* from control (A) and HFD (B) mice. pSHP-1 (Y536) levels were expressed as a fold of control (unstimulated) WAT (n=10 per group). In A, subcutaneous SHP-1 level was expressed as fold of omental WAT. In B, SHP-1 level was expressed as fold of WAT from control mice. (*, P<0.05).

To further define the role for SHP-1 in modulating ghrelin action, GHSR-1a signaling to Akt was further examined in WAT tissue explants *in vitro*. Ghrelin-stimulated Akt activity (100 nM, 1h) was increased on both A-loop (T308) and HM (S473) regulatory sites in omental WAT of HFD mice compared to omental WAT of control mice (~1.4- and 1.5-fold higher, respectively). In contrast, ghrelin-induced pAkt (T308) levels were 37% less in subcutaneous WAT of HFD relative to subcutaneous WAT of control mice (Figure 12 A). However the extent of pAkt (S473) stimulation after ghrelin treatment in subcutaneous WAT of HFD was quite similar to its respective control (Figure 12 A). Total Akt levels were comparable in all instances (Figure 12 B).

No changes were detected in GHSR-1a and p85 levels (Figure 13 A and B, respectively) in all instances. cSrc expression was markedly enhanced in omental and subcutaneous WAT of HFD compared to WAT of control mice (~1.9- and ~1.6- fold, respectively; Figure 14 A). PDK-1 levels were enhanced in subcutaneous WAT of HFD mice relative to control (~0.9 fold), although were not altered in omental WAT of HFD mice (Figure 14 B). On the contrary, mTOR levels were increased in omental WAT of HFD mice and were diminished in subcutaneous WAT of HFD mice (Figure 14 C).

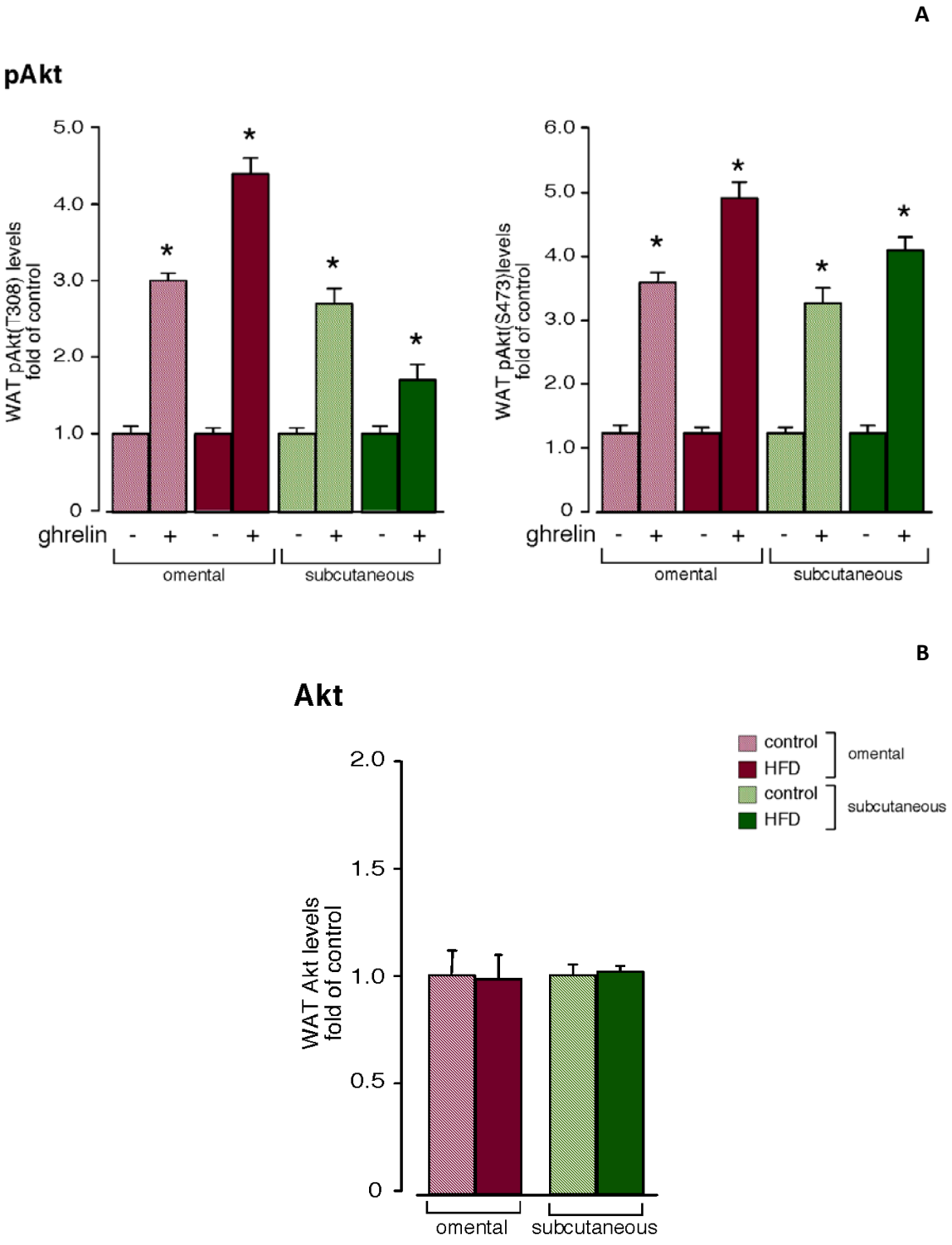

Figure 12. Effect of ghrelin (100 nM, 1h) on pAkt (T308) and pAkt (S473) (A) and Akt (B) in WAT explants *in vitro* in omental and subcutaneous WAT obtained from control and HFD-treated mice (n=10 per group). Protein expression was normalized for actin and results were expressed as a fold of respective control as mean±SE. (*, P<0.05).

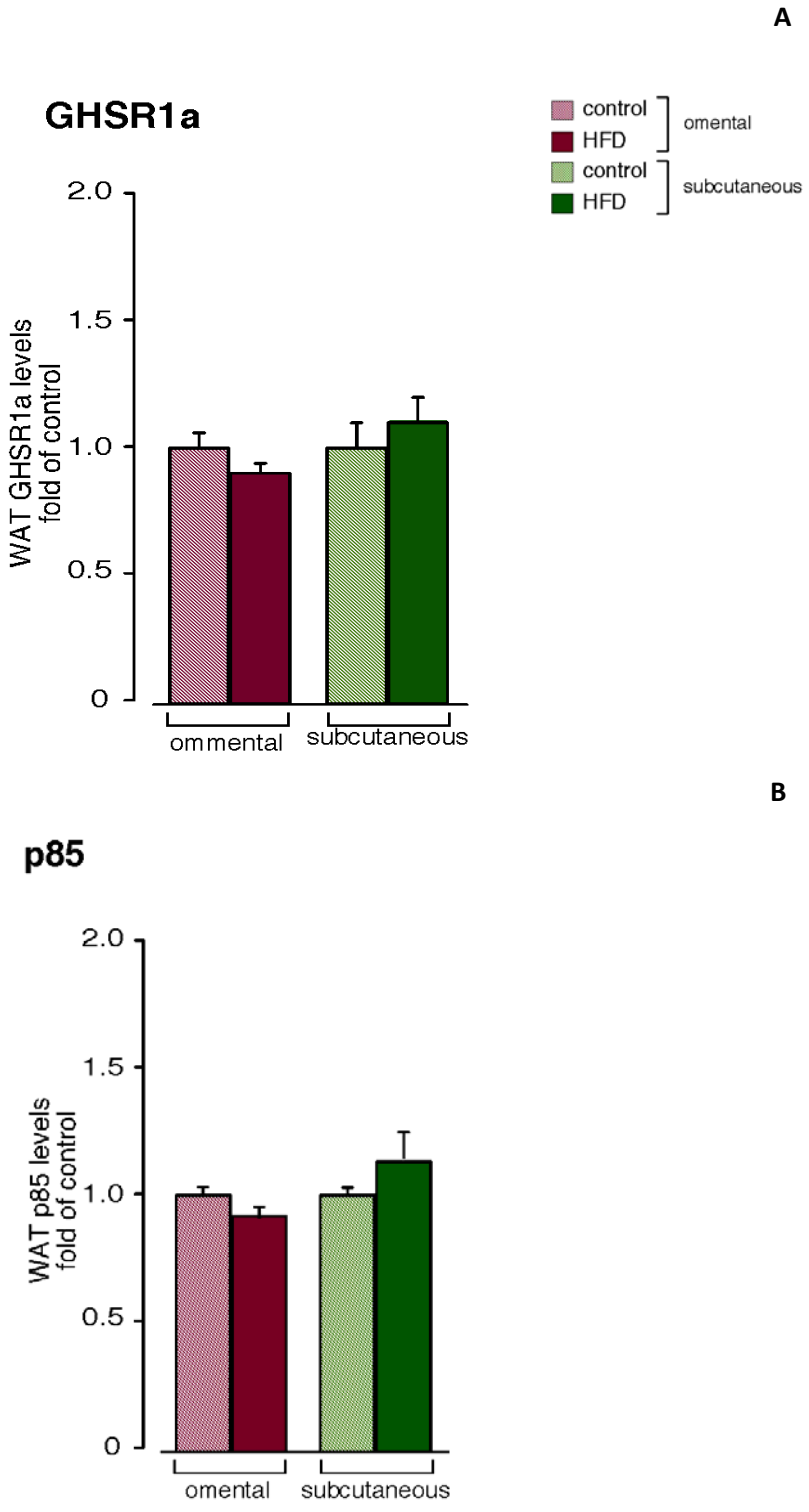

Figure 13. Effect of ghrelin (100 nM, 1h) on GHSR-1a (A) and p85 (B) in WAT explants *in vitro* in omental and subcutaneous WAT obtained from control and HFD-treated mice (n=10 per group). Protein expression was normalized for actin and results were expressed as a fold of respective control (mean±SE).

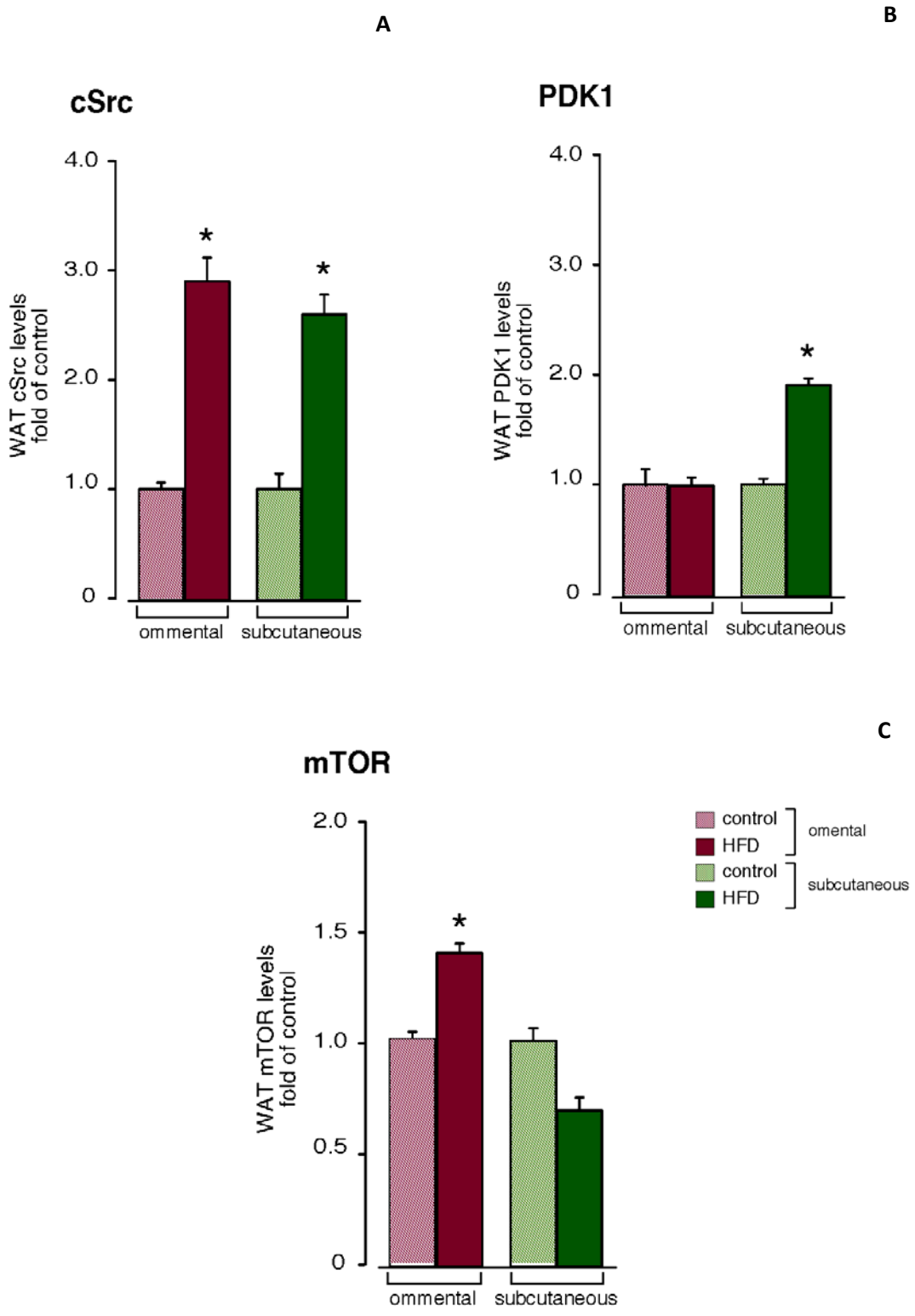

Figure 14. Effect of ghrelin (100 nM, 1h) on cSrc (A), PDK-1 (B) and mTOR (C) in WAT explants *in vitro* in omental and subcutaneous WAT obtained from control and HFD-treated mice (n=10 per group). Protein expression was normalized for actin and results were expressed as a fold of respective control (mean±SE).(*,P<0.05).

CHAPTER 4

Cortistatin-17 modulates ghrelin receptor GHSR-1a.

[^{125}I]-CST-17 binding to HEK-WT and HEK-GHSR-1a cells were assayed. As shown in Figure 1 A, HEK-WT cells exhibited a high non-specific binding for [^{125}I]-CST-17 that was no displaced by saturating dose of CST-17 (1 μM). Higher binding capacity of [^{125}I]-CST-17 was showed in HEK-GHSR-1a cells which was displaced by both CST-17 (1 μM) and ghrelin (1μM). Figure 1 B shows that [^{125}I]-ghrelin binding to HEK-GHSR-1a cells was completely displaced in a dose-dependent manner, by ghrelin and to a lesser extent by CST-17.

The effect of CST-17 on intracellular calcium mobilization was evaluated in HEK-GHSR-1a cells. As Figure 2 A shows, the ghrelin-induced $[Ca^{2+}]_i$ rise was dose-dependent, with a half maximal response at 0.5 nM and with saturation at 100 nM. By contrast, CST-17 failed to induce $[Ca^{2+}]_I$ rise for all dose tested. The effect of CST-17 on ERK 1/2 and Akt phosphorylation was evaluated in HEK-WT and HEK-GHSR-1a cells. As shown in Figure 2 B, CST-17 stimulation (200 nM) had no effect neither ERK 1/2 nor Akt phosphorylation for all time tested in HEK-WT. By contrast, ERK 1/2 and Akt phosphorylation reached maximal levels within 20 minutes of CST-17 stimulation (200 nM) in HEK-GHSR-1a cells (Figure 3). siRNA experiments targeting β-arrestin 1 and 2 (50+4% and 70+3% reduction in β-arrestin 1 and 2, respectively) clearly decreased both patterns of activation in CST-17-stimulated-HEK-GHSR-1a cells (Figure 3). Immunoprecipitation assays of GHSR-1a showed an increase of GHSR-1a phosphorylation in both ghrelin-and CST-17-stimulated cells (200 nM, 10 min) in comparison with unstimulated cells (Figure 4). Furthermore β-arrestin 1 and 2 co-immunoprecipitated with GHSR-1a, which demonstrates a direct interaction of these proteins after GHSR-1a desensitization (Figure 4).

Somatostatin receptors expression, SSTR1, SSTR2, SSTR3, SSTR4, SSTR5, SST5MD5 and SST5MD4, was examined at mRNA by qRT-PCR in HEK-WT and HEK-GHSR-1a cells (Figure 5). The amount of SSTR2 mRNA showed a significative presence of this type in both HEK-WT and HEK-GHSR-1a cells. Working in the presence of SSTR2 siRNA (47+2% reduction in SSTR2 expression) CST-17-induced ERK 1/2 and Akt phosphorylation were affected. CST-17 led to rapid Akt phosphorylation in the presence of SSTR2 siRNA, which decreased 5 minutes post-stimulation (Figure 6). Curiously, SSTR2 siRNA led to a complete recover of ERK 1/2 phosphorylation showing the same dynamic that was previously described for ghrelin (Figure 6).

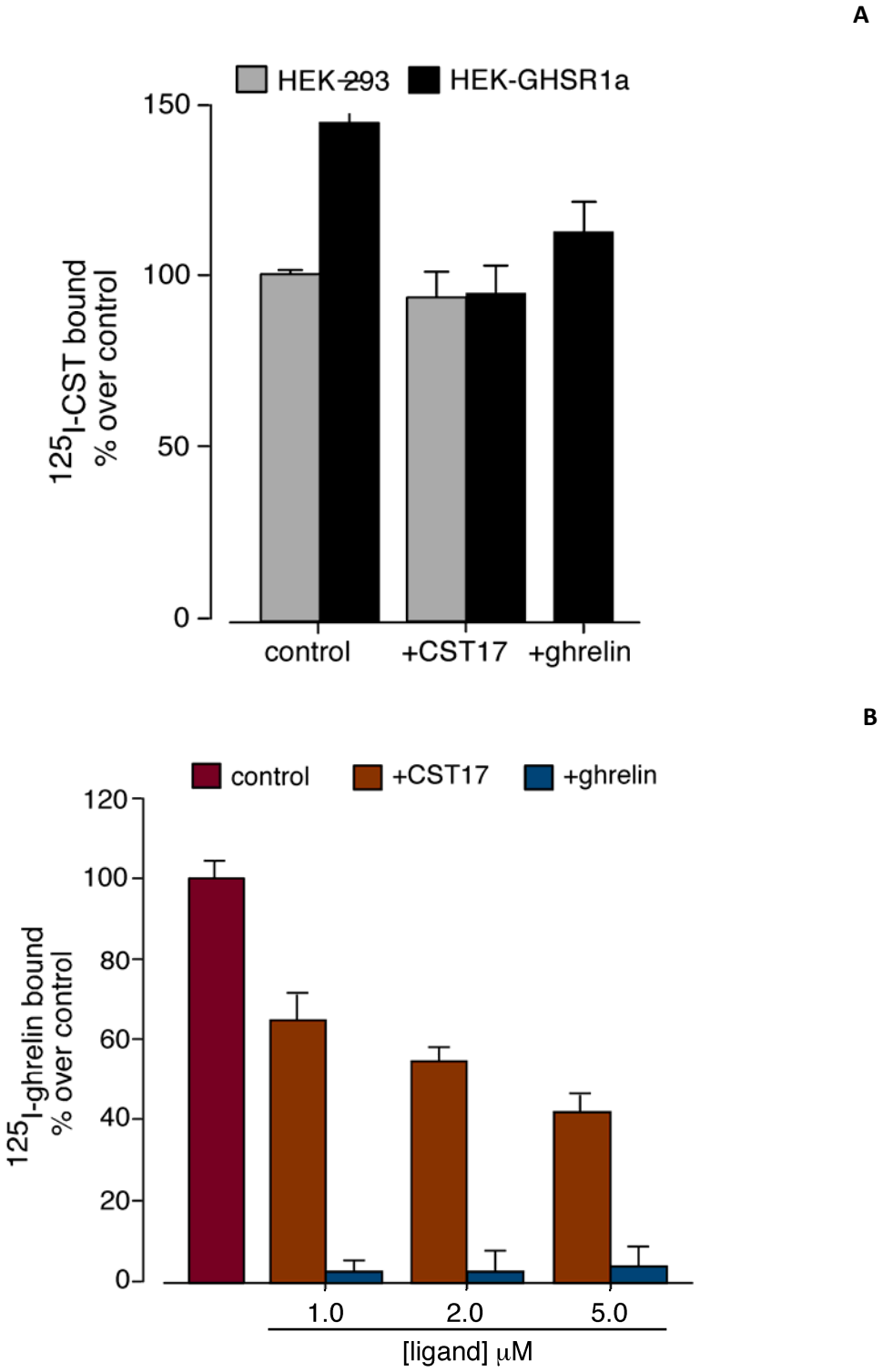

Figure 1. A. Binding property of [^{125}I]-CST-17 in the presence of CST-17 (1μM) or ghrelin (1μM) in HEK-WT and HEK-GHSR-1a cells. B. Binding property of [^{125}I]-ghrelin in the presence of CST-17 (1.0-5.0 μM) or ghrelin (1.0-5.0 μM) in HEK-GHSR-1a cells. Data are represented as percentage over control, binding in the absence of unlabeled competitor (mean±SE of three independent experiments performed in duplicate).

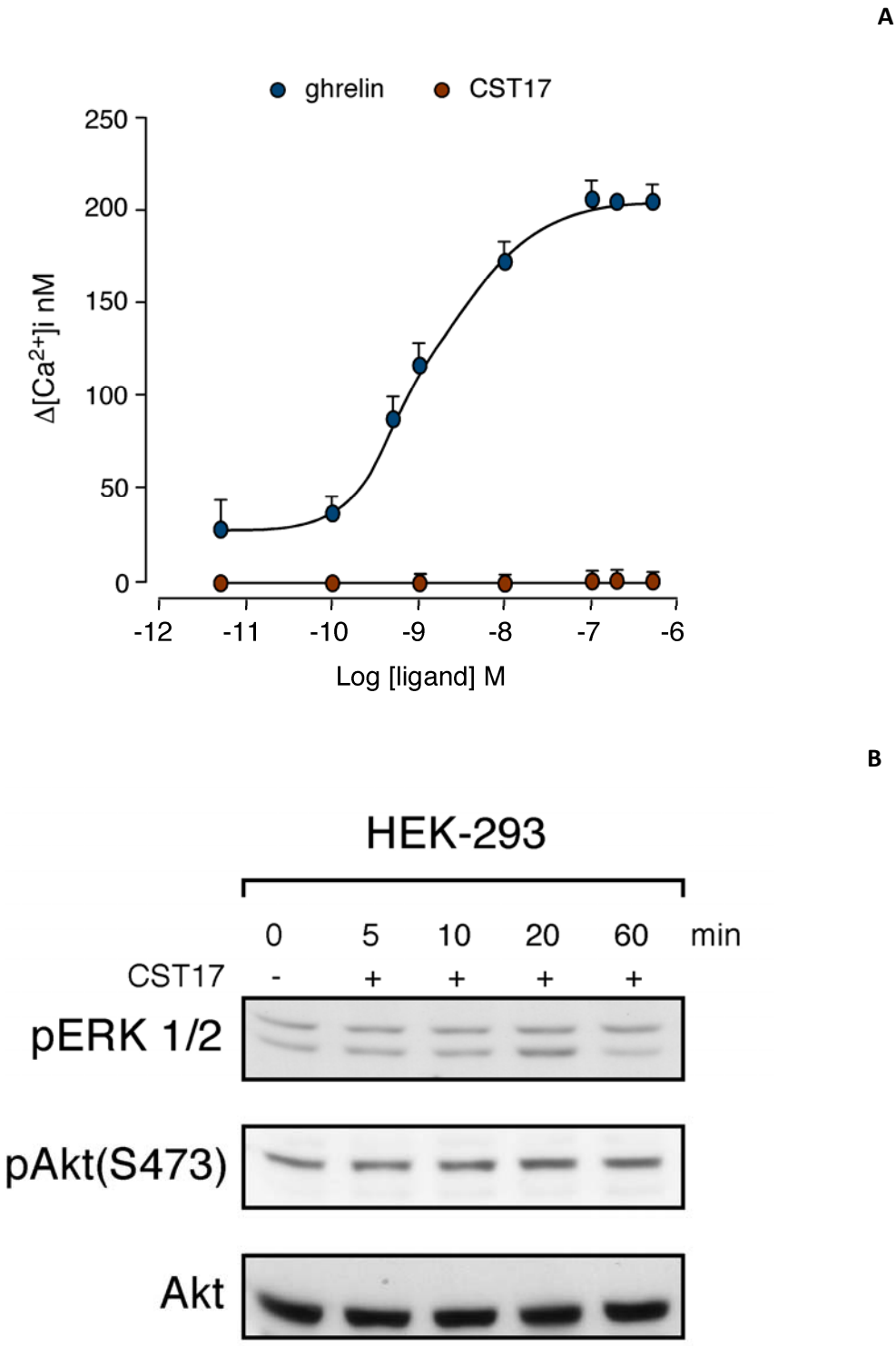

Figure 2. A. Dose-response of CST-17-and ghrelin-induced $[Ca^{+2}]_i$ mobilization. Calcium mobilization is expressed as mean±SE of three independent experiments. B. Effects of CST-17 on ERK 1/2 and Akt HM (S473) phosphorylation in HEK-WT cells. HEK-WT cells were serum-starved, stimulated with CST-17 (200 nM) for the time periods indicated and analized by immunoblotting using antibodies against pERK 1/2 and pAkt (S473). Immunoblots are representative of three independent experiments.

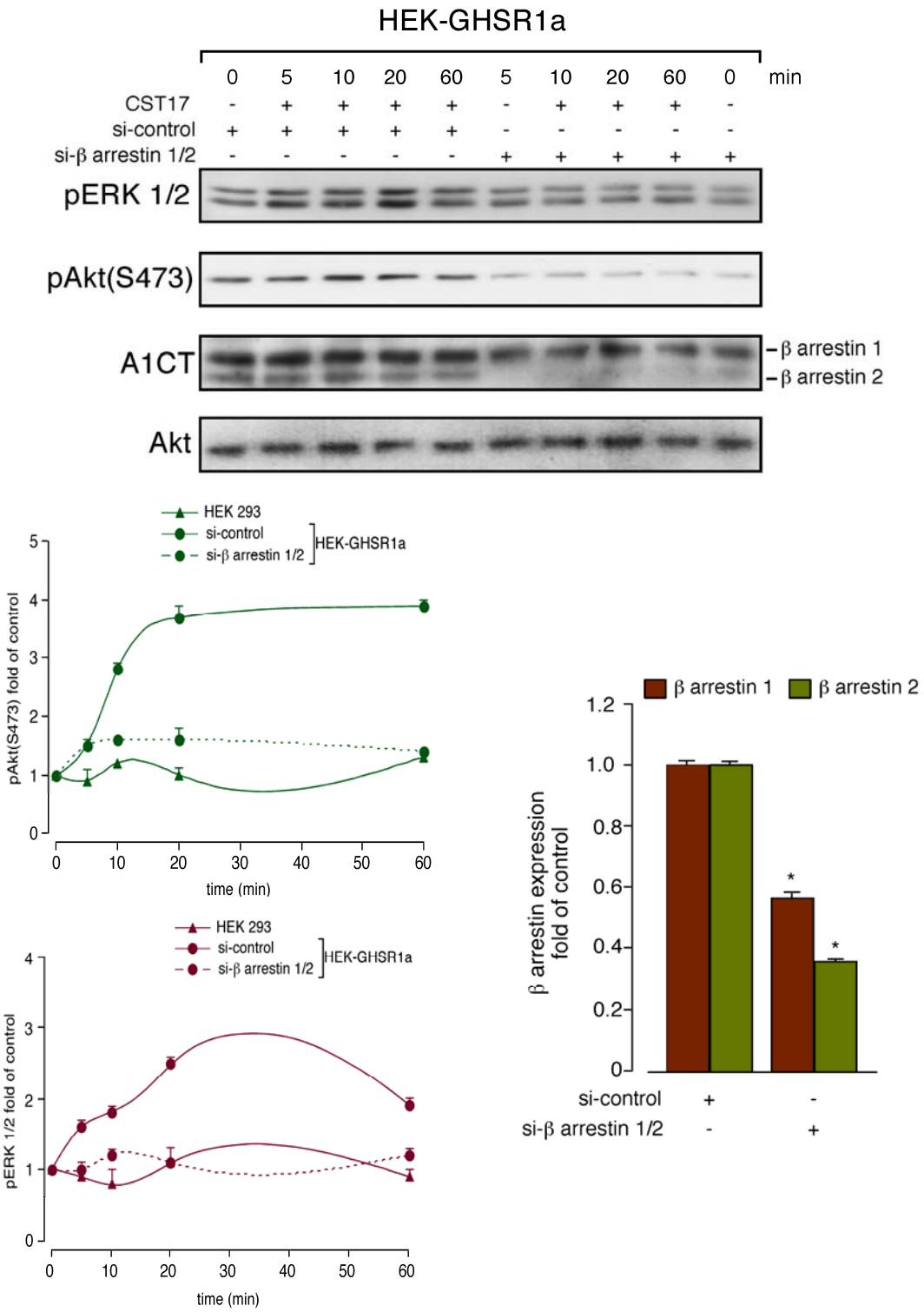

Figure 3. Effect of siRNA depletion of β-arrestin 1 and 2 on CST-17-induced ERK 1/2 and Akt HM (S473) phosphorylation. HEK-GHSR-1a cells transfected with β-arrestin 1 or β-arrestin 2 siRNA were serum-starved and then stimulated with CST-17 (200 nM) for the time periods indicated. Cells were lysed and analyzed by immunoblotting. ERK 1/2 and Akt phosphorylation were quantified by densitometry and expressed as percentage of the basal phosphorylation (mean±SE). Expression of β-arrestin1 or β-arrestin 2 was quantified by densitometry. Values shown are percentages of the level of β-arrestins in control siRNA-transfected cells (mean±SE). Immunoblots are representative of three independent experiments. (*, P<0.05).

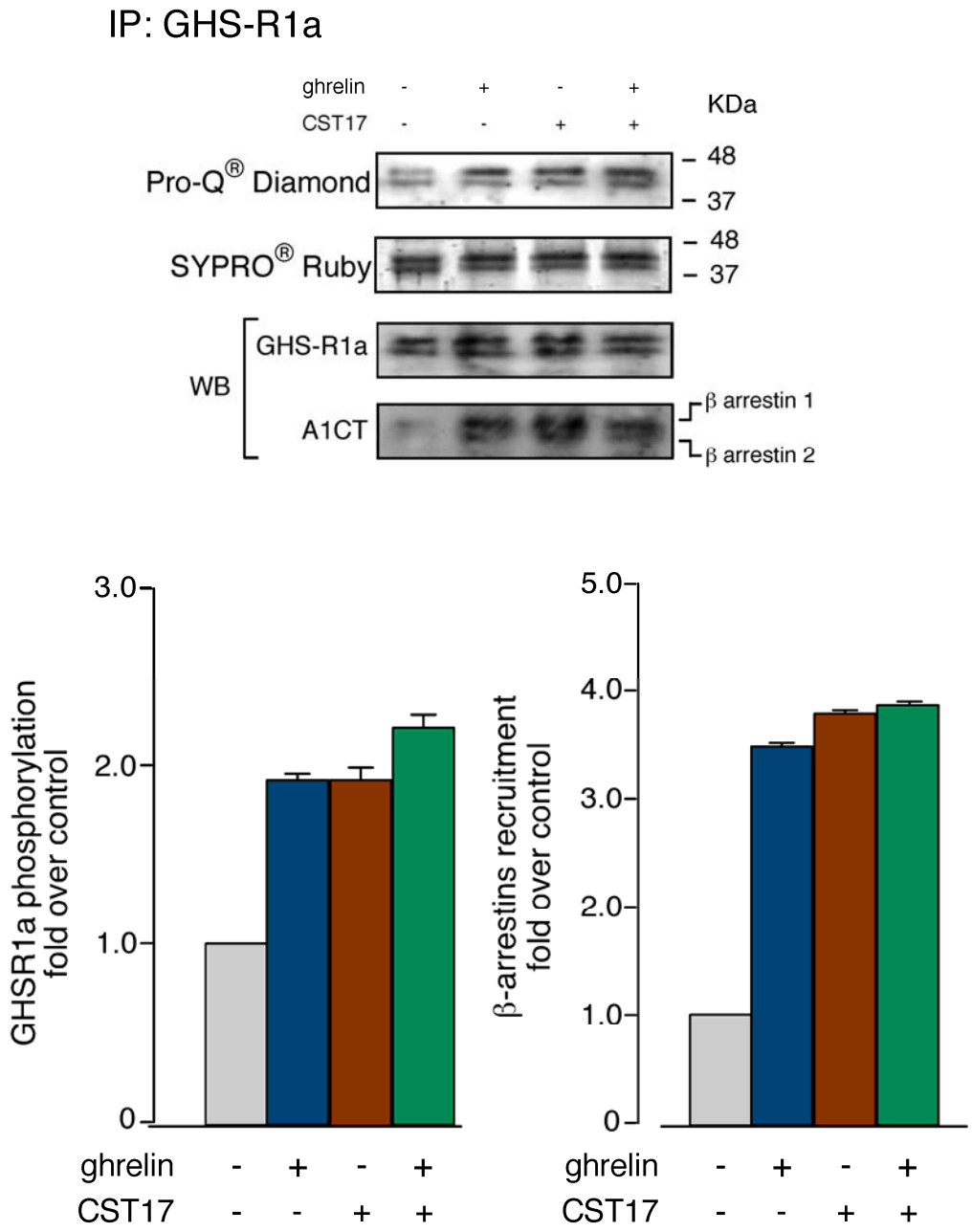

Figure 4. Effect of ghrelin and CST-17 on GHSR-1a phosphorylation. HEK-GHSR-1a cells were serum-starved, stimulated with ghrelin (200 nM), CST-17 (200 nM) or combination of both for 10 minutes. Then cells were lysed and immunoprecipitated (IP) with antibody to GHSR-1a. Phosphoproteins were detected by Pro-Q Diamond phosphoprotein gel staining in acrylamide gels followed by immunoblotting using the anti-GHSR-1a and anti-β-arrestin (AC1T) antibodies. Results were expressed as fold over control after densitometry analysis (mean±SE). Immunoblots are representative of three independent experiments (mean±SE).

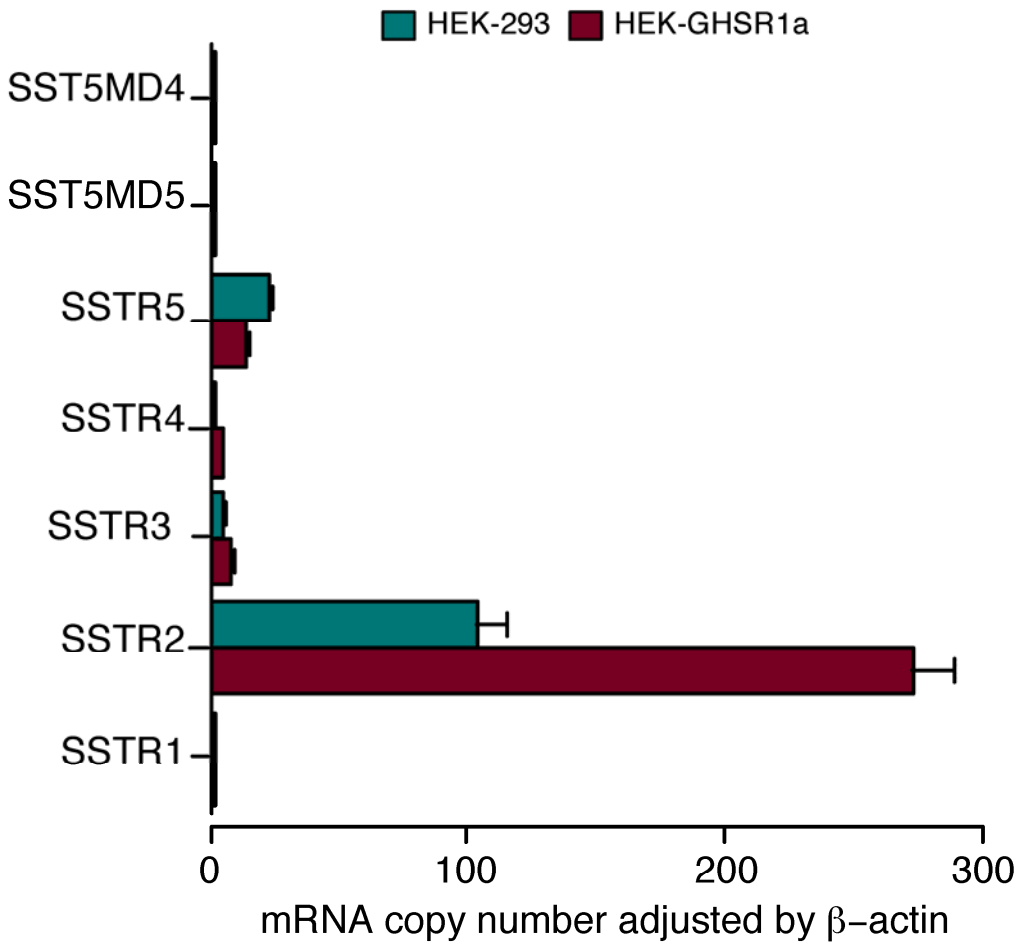

Figure 5. Expression levels of SSTR in HEK-WT and HEK-GHSR-1a cells. Absolute mRNA copy number of SSTR1, SSTR2, SSTR3, SSTR4, SSTR5, SST5MD5 and SST5MD4 measured by qRT-PCR. Values indicate mean mRNA copy number±SE of each transcript adjusted by β-actin mRNA copy number.

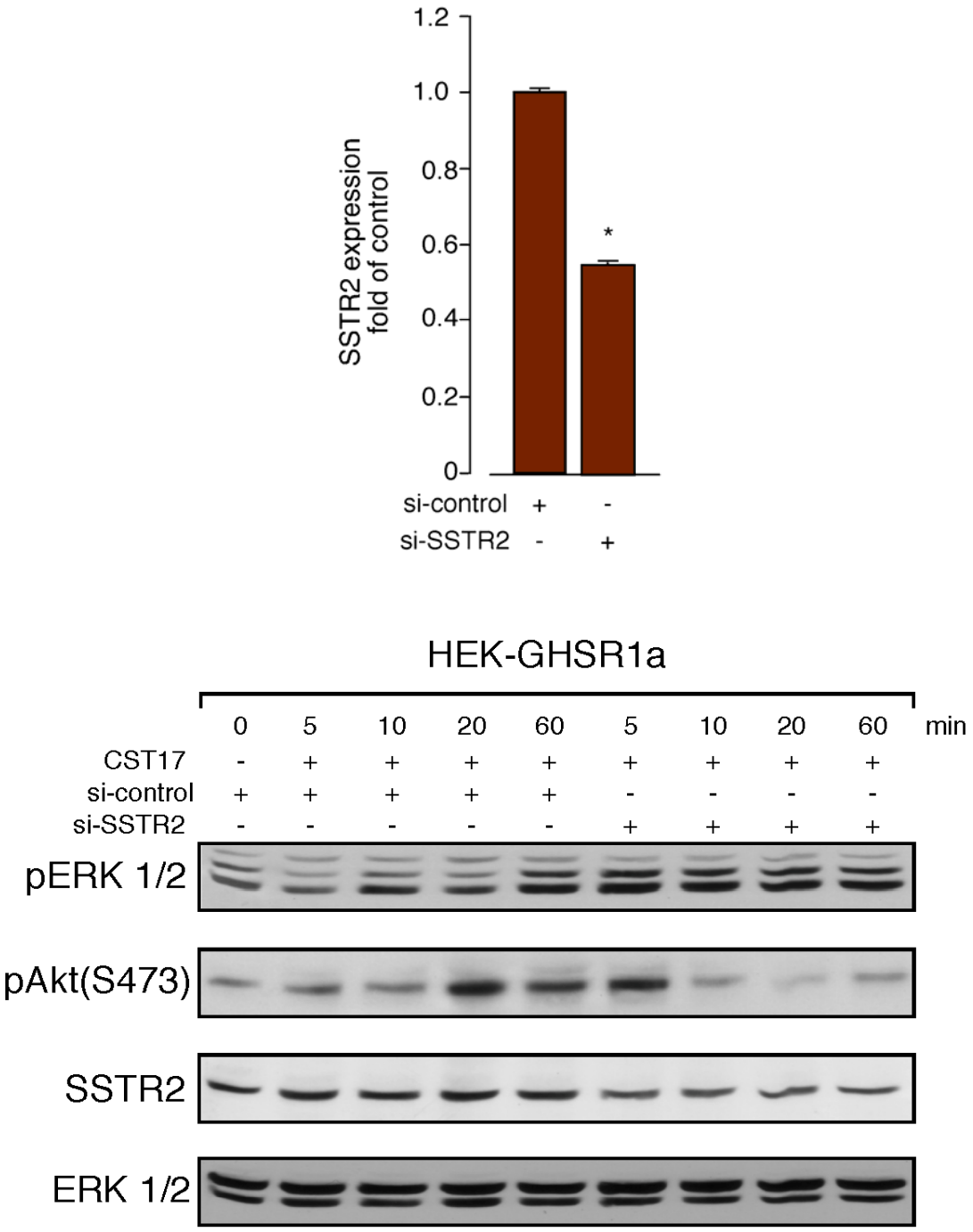

Figure 6. Effect of siRNA depletion of SSTR2 on CST-17-induced ERK 1/2 and Akt HM (S473) phosphorylation. HEK-GHSR-1a cells transfected with SSTR2 siRNA were serum-starved and then stimulated with CST-17 (200 nM) for the time periods indicated. Equal amounts of protein in each sample were used to assess the expression of SSTR2 (upper panel) or ERK 1/2 and Akt (S473) phosphorylation (lower panel) by immunoblotting analysis. Expression of SSTR2 was quantified by densitometry and expressed as percentages of the level of SSTR2 in control siRNA-transfected cells (mean±SE). Immunoblots are representative of three independent experiments. (*, $P<0.05$).

Ghrelin-induced $[Ca^{2+}]_i$ rise was inhibited after pre-stimulation with CST-17 or CST-14 (200 nM, 30 sec) (Figure 7 A). This inhibitory effect was dose-dependent, exceeding 50% at a dose of 500 nM for both CST-17 and CST-14. However the effect did not share with SST pretreatment (1.0 µM, 30 sec before ghrelin stimulus) (Figure 7 A). Having established previously that CST-17 exerts an inhibitory role in ghrelin signaling, the role of SSTR2 in this inhibitory effect was evaluated. To this end, we first investigated the possible role of $G_{i/o}$-proteins by means of pretreatment with pertussis toxin (PTX, 100 ng/mL, 12h). As shown in Figure 7 B, PTX, which uncouples $G_{i/o}$ from receptors, reversed the inhibitory effect of CST-17 on ghrelin-induced calcium rise. The inhibitory effect of CST-17 on ghrelin-induced calcium mobilization was not reversed after a pretreatment with PMA (1 µmol/l, 5 min before ghrelin stimulus), a characteristic shared with ghrelin-activated calcium mobilization through GHSR-1a (Figure 7 C).

The effect of CST-17 on ERK 1/2 phosphorylation compared to the effect of ghrelin, was also evaluated and is shown in Figure 8. CST-17 pretreatment (200 nM, 30 sec before ghrelin stimulus) inhibited ghrelin-induced ERK 1/2 phosphorylation (200 nM, 5 min). However ghrelin-induced ERK 1/2 phosphorylation (200 nM, 5 min) was not modified by SST (200 nM, 30 sec before ghrelin stimulus). It was also shown that in HEK-GHSR-1a cells, SST activates phosphorylation of ERK 1/2 (data not shown).

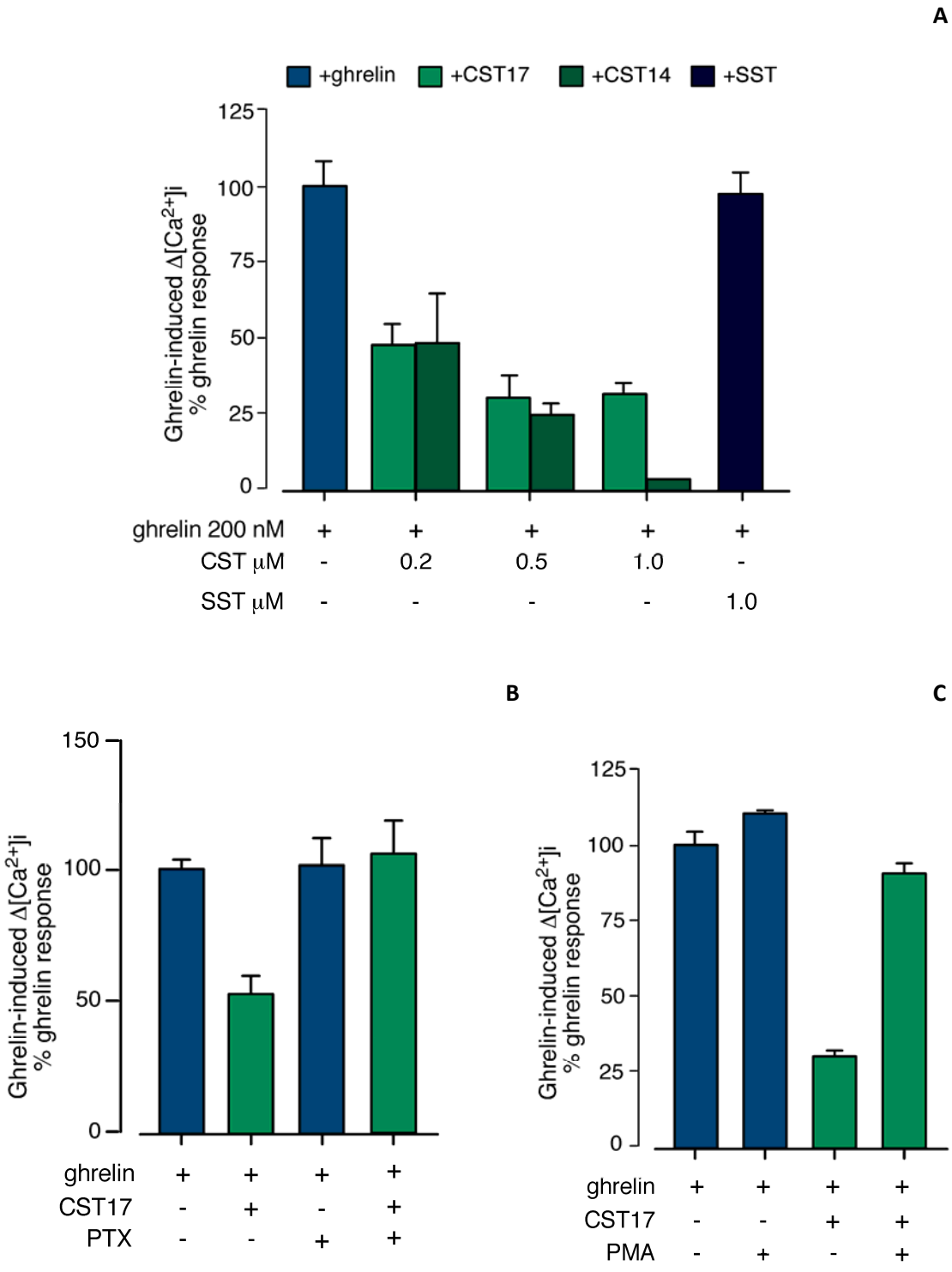

Figure 7. A. Effect of CST-17, CST-14 or SST on ghrelin-induced $[Ca^{+2}]_I$ in HEK-GHSR-1a cells. Results are expressed as % of maximal ghrelin response (200 nM; mean±SE; n=3). B. Effect of CST-17 (200 nM) on ghrelin-induced $[Ca^{2+}]_i$ (200 nM) in the absence or presence of PTX (100 ng/mL, 12h). C. Effects of PMA (1 μM, 5 minutes before stimulus) on the inhibitory effect of CST-17 (200 nM) on ghrelin-induced $[Ca^{2+}]_I$ (200 nM). For A, B and C results are expressed as % of maximal ghrelin response (mean+SE; n=6).

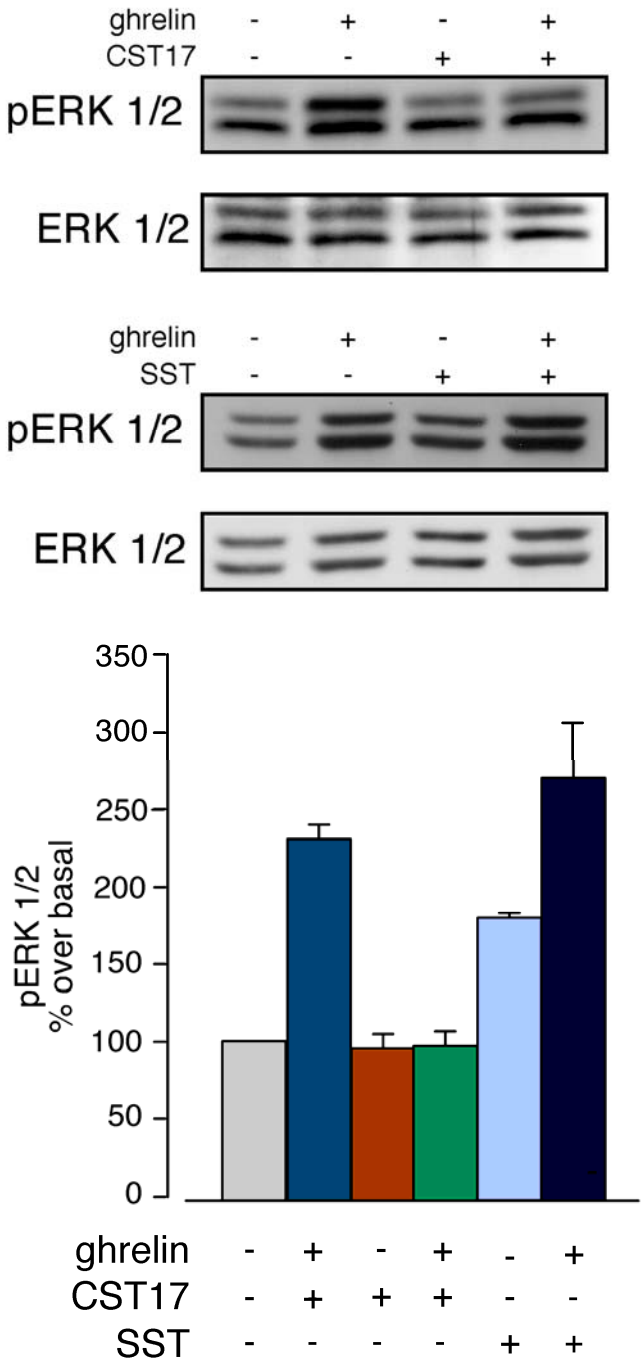

Figure 8. Effect of ghrelin, CST-17 and SST on basal-and ghrelin-induced ERK 1/2 phosphorylation. HEK-GHSR-1a cells were serum starved and then stimulated with ghrelin alone (200 nM), CST-17 alone (200 nM), SST alone (200 nM), ghrelin plus CST-17 (200 nM, 30 seconds prior to ghrelin stimulation) or ghrelin plus SST (200 nM, 30 seconds prior to ghrelin stimulation) for 5 minutes. Cells were lysed and analyzed by immunoblotting using specific antibodies against pERK 1/2. ERK 1/2 phosphorylation was quantified by densitometry and expressed as the percentage of the basal phosphorylation (mean±SE). Immunoblots are representative of three independent experiments.

5. DISCUSSION

Since ghrelin discovery, more than a decade ago, numerous publications have been reported with the different roles of this acylated peptidic hormone, ranging from regulation of appetite and food intake to the release of distinct hormones, effects on glucose and lipid metabolism, cardiovascular effects, modulation of cell proliferation or effects on reproductive system.[10]

Only understanding in detail the basics mechanisms that control and regulate the response of this hormone through its receptor, will allow us a better knowledge to develop new drugs acting on GHSR-1a or in its targets.

Ghrelin/MAPK pathway

With regard to the role of ghrelin in cell proliferation, the MAPK pathway represents the main pathway involved in mitogenic processes. [10] The classical mechanism by which G-protein-coupled receptors (GPCR) activate MAPK is through heterotrimeric G-proteins. These receptors stimulate G-protein subunits, which activate the Ras-dependent cascade, leading to the activation of Raf-1 and MAP kinase kinase 1, a specific activator of extracellular signal-regulated kinases 1 and 2 (ERK 1/2).

For example, in 3T3-L1 preadipocytes, the mitogenic effect of ghrelin is mediated via activation of the MAPK and phosphoinositide 3-kinase (PI3K) pathways through a pertussis toxin (PTX)-sensitive G-protein ($G_{i/o}$). [84] The PI3K/Akt pathway has also been implicated in the mitogenic effects of ghrelin on pancreatic adenocarcinoma cells. [121] Meanwhile, in human and rat adrenal zone glomerulosa cells, the mitogenic effect of ghrelin involves activation of a tyrosine kinase-dependent MAPK p42/43 mechanism and seems to be independent of cAMP-dependent protein kinase (PKA) and protein kinase C (PKC). [82, 83] It was also seen that in hepatoma cells, ghrelin modulates the downstream stages of insulin signaling by stimulating cell proliferation through the tyrosine phosphorylation of insulin receptor substrate-I (IRS-I), leading association of IRS-I with the adaptor molecule growth factor receptor-bound protein 2 (GRB2) and thus activating MAPK activity. [85]

Thus, there are different papers where the ghrelin-induced MAPK activation has been described and the intracellular signaling mechanisms have been elucidated.

Recent evidence has suggested that ERK 1/2 activation may also be achieved via pathways involving β-arrestins. The β-arrestins, originally thought only to be involved in receptor

[121] Duxbury MS, Waseem T, Ito H, Robinson MK, Zinner ML, Ashley SW, Whang EE. Ghrelin promotes pancreatic adenocarcinoma cellular proliferation and invasiveness. Biochem Biophys Res Commun. 2003; 309: 464–68.

uncoupling and internalization, serve as multifunctional adaptor, scaffolds and signal transducers that connect activated receptors with diverse signaling molecules within the cell. [5] β-arrestin-mediated activation of ERK 1/2 appears to be linked to the function of β-arrestins in mediating endocytosis of a number of GPCR, in which they act as scaffolds for the component kinases that activate ERK 1/2. [5, 122] In pathways mediated by the class-B GPCR, the β-arrestin scaffolded signaling complex persists for prolonged periods while in pathways mediated by class-A GPCR, the scaffold is much less persistent. [5, 122] These differences are determined by the stability of the β-arrestin/receptor complexes. [100, 123, 124] These scaffolding complexes can determine the subcellular location and specificity of activated ERK 1/2, promoting phosphorylation of diverse cytosolic substrates and thereby having different physiological consequences, like the angiotensin II type 1a receptor (AT1AR) for example. [116] This is owing to the fact that G_q-protein-mediated activation is rapid and transient leading to nuclear translocation of the activated ERK 1/2 with consequent mitogenic activity. In contrast, β-arrestin-mediated activation is characterized by retention of the activated ERK 1/2 in endocytic vesicles, so there is no induction of mitogenesis, as it was reported for the type 2 vasopressin receptor (V2R). [125] Thus, it may be concluded that the physiological consequences were different since the activated ERK 1/2 pools generated by the two pathways would have different spatial and temporal distributions and subcellular locations. Another important thing to mention is related to the action of these pathways. It is not clear the interplay between the β-arrestin-dependent and G-protein-dependent pathways. There are examples where the G-protein and β-arrestin-dependent pathways act sequentially [125] and cases where both pathways can act in parallel, as described for AT1AR [116] and β-2 adrenergic receptor (β2AR). [126]

It is well-known that ERK 1/2 activation via the GHSR-1a receptor has been reported to involve G_i-mediated signaling and PI3K, PKC, tyrosine kinase phosphorylations. However, it is unclear how this receptor couple to MAPK signaling pathway and, furthermore, whether there are subtype-related regulations in this signaling pathway. The results presented here allow us to

[122] Reiter E, Lefkowitz RJ. GRKs and β-arrestins: Roles in receptor silencing, trafficking and signaling. Trends Endocrinol Metab. 2006; 17: 159–65.
[123] Ferguson SS. Evolving concepts in G-protein-coupled receptor endocytosis: The role in receptor desensitization and signaling. Pharmacol Rev. 2001; 53: 1–24.
[124] Luttrell LM, Lefkowitz RJ. The role of β-arrestins in the termination and transduction of G-protein-coupled receptor signals. J Cell Sci. 2002; 115: 455–65.
[125] Ren XR, Reiter E, Ahn S, Kim J, Chen W, Lefkowitz RJ. Different G-protein-coupled receptor kinases govern G-protein and β-arrestin-mediated signaling of V2 vasopressin receptor. Proc Natl Acad Sci USA. 2005; 102: 1448–53.
[126] Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S, Reiter E, Premont RT, Lichtarge O, Lefkowitz RJ. β-arrestin-dependent, G-protein-independent ERK1/2 activation by the β2 adrenergic receptor. J Biol Chem. 2006; 281: 1261–73.

delineate the pathway of MAPK activation induced by ghrelin in response to binding to GHSR-1a in HEK-GHSR-1a cells. The signaling mechanisms that underlie the phosphorylation and activation of ERK 1/2 are complex and result from the interplay of three different signaling pathways which depend on both classical G-protein-regulated effectors and β-arrestins-dependent mechanisms. One pathway is $G_{q/11}$-dependent and involves a Ca^{+2}-dependent PKC (PKC α/β) and cSrc. A second pathway is $G_{i/o}$-dependent and involves PI3K, PKC ε, and cSrc. The third pathway is mediated by β-arrestins 1 and 2 and requires the entry of the receptor into a multiprotein complex with the β-arrestins, cSrc, Raf-1, ERK 1/2, and perhaps other components of the MAPK cascade. In addition, it has been shown that the $G_{q/11}$-and the $G_{i/o}$-proteins are crucially involved and converge in the β-arrestin 1 and 2-mediated ERK 1/2 activation. Another point to highlight is the crucial role of cSrc playing as a node linking both pathways. Thus, these findings support the view that ghrelin activates different ERK 1/2 pools that differ in their temporal and spatial distributions, and thus probably have different physiological consequences.

Starting from the previous data reported where ERK 1/2 activation via the GHSR-1a involved a G_i-protein-mediated mechanism we continued to explore the involvement of other G-proteins which might be mediating ERK 1/2 activation. The discovery of the $G_{q/11}$-dependent pathway was achieved after testing the effect of BAPTA, which partially decreased the ERK 1/2 activation after ghrelin stimulation, pointing to a Ca^{+2}-dependent signaling pathway. It is well-known that $G_{q/11}$-proteins activate phosphatidylinositol-specific phospholipase C (PI-PLC) which generates IP_3 and DAG from phosphatidylinositol 4,5-biphosphate with the subsequent liberation of Ca^{+2} from IP_3-sensitive stores and activation of Ca^{+2}-dependent PKC α/β. In addition, the use of a selective inhibitor of the Ca^{+2}-and DAG-dependent PKC α and PKC β isoforms, Gö6976, caused a similar decrease in the ghrelin-induced ERK 1/2 activation. Besides, the data obtained from PKC α and PKC β activation in response to ghrelin parallel the time-course of ghrelin-induced ERK 1/2 activation. It was also determined the role of cSrc, confirming its participation in this route downstream the PKC α/β, since Gö6976 pretreatment had a significant effect on cSrc activation.

The other pathway described belongs to the $G_{i/o}$-dependent and was indicated by the inhibitory effect on ERK 1/2 activation achieved after PTX pretreatment, which disables $G_{i/o}$-protein. It also has been shown that cSrc-tyrosine phosphorylation was reduced by this pretreatment, involving this non-receptor tyrosine kinase in the signaling pathway. Similarly, the PI3K blocker wortmannin, inhibited cSrc phosphorylation. In addition, the time-course of PKC ε was closely similar with the dynamics of ghrelin-induced ERK 1/2 activation and a control

of PKC ε by PI3K via a PTX-sensitive G-protein was also observed. Thus these results suggest that in this $G_{i/o}$-dependent pathway the cSrc might be activated by PKC ε, as it has been suggested for other systems. [127, 128]

The kinetic of cSrc activation in response to ghrelin shows two phases that correlate with the kinetic of ERK 1/2 activation. This result supports that ghrelin employs different mechanisms to activate cSrc in which PKC proteins function as upstream mediators of such activation at least for early activation. From the data obtained, PKC α/β and PKC ε are candidates to direct cSrc activation, at least for early cSrc activation. The ability of PKC to activate cSrc is not due to direct interactions between the two kinases. Although PKC can phosphorylate cSrc (serines 12 and 48) [129, 130] *in vitro*, studies using purified cSrc and PKC demonstrated that PKC were unable to directly activate cSrc. [131] Therefore, the ability of PKC to activate cSrc is likely due to the activity of other proteins that relayed signals from PKC, which in turn direct the activation of cSrc. The most important regulatory activation of cSrc is through dephosphorylation of Tyr 527 [132, 133] and phosphorylation of Tyr 416. [134, 135, 136] In principle, reduced phosphorylation could result from either decreased Tyr 416-directed kinase activity [137, 138] or increased Tyr 527-

[127] Song C, Vondriska TM, Wang GW, Klein JB, Cao X, Zhang J, Kang YJ, D'Souza S, Ping P. Molecular conformation dictates signaling module formation: Example of PKCε and Src tyrosine kinase. Am J Physiol Heart Circ Physiol. 2002; 282: H1166–71.

[128] Piiper A, Elez R, You SJ, Kronenberger B, Loitsch S, Roche S, Zeuzem S. Cholecystokinin stimulates extracellular signal-regulated kinase through activation of the epidermal growth factor receptor, Yes, and protein kinase C. Signal amplification at the level of Raf by activation of protein kinase C ε. J Biol Chem. 2003; 278: 7065–72.

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[138] Frame MC. Src in cancer: Deregulation and consequences for cell behaviour. Biochim Biophys Acta. 2002; 1602: 114–30.

directed phosphatase activity. [139, 140] At least in the proliferative activity, it is most likely that activation results from increased phosphatase activity. [141, 142] A candidate for a physiological Tyr 527 phosphatase are protein tyrosine phosphatases (PTP) [143] which can be phosphorylated by PKC at Ser 180 and 204 resulting in stimulation of its activity. [144, 145]

The third pathway described involved in ghrelin-induced ERK 1/2 activation is related to receptor endocytosis. This fact is supported by the fact that β-arrestin depletion using siRNA reduced the magnitude of ERK 1/2 activation by ghrelin. In addition, the experiments made in K^+-depleted medium, [50] which affects the formation of clathrin-coated pits and internalization through this pathway, point to the same direction since they show a different kinetic and spatial pattern of ERK 1/2 activation. Besides, the immunoprecipitation assays shows that β-arrestins act as scaffolds that recruit diverse proteins to the ghrelin-occupied receptor, among then cSrc, which leads to the activation of a Raf-1-dependent pathway, [146] the formation of a GHSR-1a/ β-arrestins/ cSrc/ Raf-1 complex and ultimately ERK 1/2 activation. This β-arrestin-scaffolded complex allows to the different components of MAPK cascade be in close proximity to each other, ensuring substrate specify and avoiding the translocation of activated ERK 1/2 to the nucleus, phosphorylating then only cytosolic substrates. [5]

As it also been reported for other receptors such the parathyroid hormone type 1 receptor (PTH1R) [147], protease-activated receptor 2, [148] neurokinin-1 receptor [149] and β2AR, [126] both β-

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arrestin 1 and β-arrestin 2 are required to promote activation of MAPK cascade. This might indicate a need to form heterodimers to activate the β-arrestin-dependent signaling pathway, hypothesis supported by structural studies of visual arrestins, proteins analogous to the β-arrestins, which show dimerization under physiological conditions [150, 151] giving rise to both homo- and hetero-oligomers in living cells. It is also possible that these dimerizations may control, at least to some extent, the biological functions of these proteins [152] but this cannot be generalizated since in other systems β-arrestins show opposing effects: for example, in the case of the AT1AR receptor, β-arrestin 1 down-regulates ERK 1/2 activation, while β-arrestin 2 has a positive effect.[109] It seems reasonable to speculate that β-arrestins dimerization is determined by the receptor controlling β-arrestin-associated functions.

In addition, the results showed that β-arrestins mediate cSrc activation, as shown by the partial inhibition of cSrc phosphorylation at Tyr 416 after depletion of β-arrestins with siRNA. This activation might result from a conformational change induced by β-arrestins binding, as reported for the β2AR [153] and the neurokinin 1 receptor. [149] Thus, these data suggest that β-arrestins determine the recruitment and activation of a second pool of cSrc that delineate the initiation of a Raf-dependent pathway with the consequent β-arrestin-scaffolded ERK 1/2 activation. Binding of β-arrestins to the activated GHSR-1a ends a first wave of cSrc activation via G-protein-dependent signaling, but at the same time initiates a second wave of cSrc activation via β-arrestins-mediated signaling, pointing to these pathways are not only mechanistically distinct, but also perform different biological functions. [97]

The presence of multiple intracellular mechanisms is determined by the specific array of intracellular second messengers available in each tissue. Depending on cell type, one mechanism may predominate or multiple mechanisms may be activated simultaneously. However, it seems reasonable to think that ERK 1/2 activation has different physiological consequences. ERK 1/2 activated by G-proteins generally accumulates in the nucleus where it phosphorylates and activates various transcription factors. [154] In contrast, ERK 1/2 activated by

[150] Granzin J, Wilden U, Choe HW,Labahn J,Krafft B, Buldt G. X-ray crystal structure of arrestin from bovine rod outer segments. Nature. 1998; 391: 918–21.
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β-arrestins is presumably not transported to the nucleus but is instead confined to a cytoplasmic compartment in which it phosphorylates a distinct set of effectors. [5, 122]

In summary, the data indicate the existence of different pathways by which ERK 1/2 can be activated by the GHSR-1a. One pathway is mediated by β-arrestins 1 and 2, and involves the recruitment of GHSR-1a, cSrc, Raf-1, and ERK 1/2 (and possibly other proteins) into a β-arrestin-scaffolded complex. The second pathway is $G_{q/11}$-dependent and involves the activation of PKC α/β and cSrc. The third pathway is $G_{i/o}$-dependent and is mediated by PI3K, PKC ε, and cSrc. Moreover, $G_{i/o}$-and $G_{q/11}$-protein-dependent signaling pathways are involved in the β-arrestin-mediated ERK 1/2 activation.

Ghrelin/Akt pathway

The Akt pathway is another one of the routes involved in cell proliferation, although it also exerts a central role in the regulation of metabolism, apoptosis, transcription or cell cycle regulation. These roles are cell-type-specific and are mediated by a variety of down-stream targets. [155]

The GHSR-1a has been implicated in Akt signaling as it was described the activation of Akt in endothelial, [156, 157] skeletal muscle, [158] thyroid, [159] preadipocytes [84] and pancreatic cells. [121]

Akt pathway is activated by different targets but the classical mechanism involves the translocation to the plasma membrane through the binding of its pleckstrin homology (PH) domain to the second messenger phosphatidylinositol-3, 4, 5-triphosphate (IP_3) generated by PI3K. At the membrane, Akt becomes phosphorylated on A-loop (T308) and HM (S473) by PDK-1 and mTORC2 respectively. Once fully activated, Akt will activate different targets to exert its biological functions.

From the results obtained, we can mention three major findings related to the activation of Akt in response to ghrelin in HEK-GHSR-1a and 3T3-L1 preadipocyte cells. First of all is that Akt

[155] Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell. 2007; 129: 1261–1274.
[156] Iantorno M, Chen H, Kim JA, Tesauro M, Lauro D, et al. Ghrelin has novel vascular actions that mimic PI 3-kinase-dependent actions of insulin to stimulate production of NO from endothelial cells. Am J Physiol Endocrinol Metab. 2007; 292: E756–64.
[157] Zhao H, Liu G, Wang Q, Ding L, Cai H, et al. Effect of ghrelin on human endothelial cells apoptosis induced by high glucose. Biochem Biophys Res Commun. 2007; 362: 677–81.
[158] Barazzoni R, Zanetti M, Cattin MR, Visintin L, Vinci P, et al. Ghrelin enhances *in vivo* skeletal muscle but not liver AKT signaling in rats. Obesity (Silver Spring). 2007; 15: 2614–23.
[159] Park YJ, Lee YJ, Kim SH, Joung DS, Kim BJ, et al. Ghrelin enhances the proliferating effect of thyroid stimulating hormone in FRTL-5 thyroid cells. Mol Cell Endocrinol. 2008; 285: 19–25.

is phosphorylated by the interplay of two signaling pathways; the $G_{i/o}$-protein-dependent pathway and a β-arrestin-scaffold complex. One pathway is $G_{\beta\gamma}$-dependent and involves the activation of PI3K. The second pathway is mediated by the β-arrestins 1 and 2, and requires the entry of the receptor into a multiprotein complex. Second, PDK-1 and mTORC2 are essential for A-loop and HM phosphorylation of Akt, respectively. Third, cSrc plays an essential role in the activation of Akt mediated by ghrelin. Thus, as it was seen for MAPK pathway, [160] it is reasonable to think that ghrelin activates Akt pools that differ in their temporal and spatial distributions, suggesting different physiological targets and consequences.

From the data obtained on Akt time-course we can see that Akt phosphorylation at both residues reaches maximum levels within 20 minutes after ghrelin stimulation keeping the maximum by 60 minutes. The PI3K is involved in this activation through the βγ subunits of a $G_{i/o}$–protein, since expression of Gβγ sequestrant inhibited both phosphorylation residues. [161] The second messenger generated by PI3K, IP_3, allows membrane translocation of proteins containing PH domains such as PDK-1 and Akt. Then, PDK-1 is autophosphorylated at Ser241 leading to its own activation and consequently phosphorylates Akt A-loop (T308). [162, 163] Finally, the full activation of Akt involves the phosphorylation of HM (S473). Several kinases, designated as PDK-2 kinases, have been proposed to phosphorylate the Akt HM (S473) domain, including integrin-linked kinase (ILK), PKC α, double-stranded DNA-dependent protein kinase (DNAPK), ataxia telangengiectasia mutated (ATM) gene product and the mTORC2. [164] From all the proposed candidates, evidence supports mTORC2 as the most compelling. mTORC2 complex contains Rictor, mLST8, mSin1 variants and the mTOR kinase [165] and data show that ablating mTORC2 function, by siRNA targeting Rictor, the ghrelin-stimulated HM (S473) phosphorylation of Akt was impaired, but not at the A-loop (T308). The fact that the Akt A-loop (T308) phosphorylation is unaffected by loss of HM (S473) phosphorylation in mTORC2-deficient cells supports that the phosphorylation of HM (S473) is not critical for that of A-loop

[160] Camiña JP, Lodeiro M, Ischenko O, Martini AC, Casanueva FF. Stimulation by ghrelin of p42/p44 mitogen-activated protein kinase through the GHS-R1a receptor: role of G-proteins and β-arrestins. J Cell Physiol. 2007; 213: 187–200.
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[165] Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. Cancer Cell. 2007; 12: 9–22.

(T308). [166, 167] Thus, it is reasonable to deduce that the PDK-1-mediated Akt A-loop (T308) phosphorylation is not dependent on prior HM (S473) phosphorylation by mTORC2. [168, 169] Another fact to highlight is that the way mTORC2 is activated remains unknown. Based on the PH-like domain of mSIN1, mTORC2 and Akt might interact as consequence of colocalization at plasma membrane when PI3K is activated. [170] This does not exclude the possibility that other upstream signals regulate activation of the mTORC2 in response to different growth factors. Indeed mSIN1 contains a Ras-binding domain raising the possibility that Ras regulates mTORC2. [170, 171] A regulatory role of $G_{i/o}$-protein on Akt phosphorylation can be draw, maybe through the regulation of PH domain leucine-rich repeat phosphatase (PHLPP1 and PHLPP2) activation, since PTX treatment selectively enhanced earliest time points of Akt HM (S473) phosphorylation. PHLPP1 and PHLPP2, as their name indicate, are phosphatases that selectively dephosphorylate the HM of Akt, resulting in decreased kinase activity. [172, 173]

In addition to these findings, and as it was described for MAPK activation by ghrelin, [160] cSrc protein acts upstream of Akt, since cSrc depletion through the use of siRNA reduced the ghrelin-induced Akt phosphorylation at both residues. It is known that Akt requires tyrosine phosphorylation for its full activation. [174, 175] From the data obtained it is clear that ghrelin activates tyrosine phosphorylation of Akt. In addition, the co-immunoprecipitation assays showed that cSrc interacts with Akt in response to ghrelin. Because cSrc contains a SH3

[166] Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, Brown M, Fotzgerald KJ, Sabatini DM. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCα, but not S6K1. Dev Cell. 2006; 11: 859–71.

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[171] Lee S, Comer FI, Sasaki A, McLeod IX, Duong Y, Okumura K, Jates JR 3rd, Parent CA, Firtel Ra. TOR complex 2 integrates cell movement during chemotaxis and signal relay in Dictyostelium. Mol Biol Cell. 2005; 16: 4572–83.

[172] Gao T, Furnari F, Newton AC. PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. Mol Cell. 2005; 18: 13–24.

[173] Brognard J, Sierecki E, Gao T, Newton AC. PHLPP and a second isoform, PHLPP2, differentially attenuate the amplitude of Akt signaling by regulating distinct Akt isoforms. Mol Cell. 2007; 25: 917–31.

[174] Chen R, Kim O, Yang J, Sato K, Eisenmann KM, McCarthy J, Chen H, Qiu Y. Regulation of Akt/PKB activation by tyrosine phosphorylation. J Biol Chem. 2001; 276: 31858–62.

[175] Conus NM, Hannan KM, Cristiano BE, Hemmings BA, Pearson RB. Direct identification of tyrosine 474 as a regulatory phosphorylation site for the Akt protein kinase. J Biol Chem. 2002; 277: 38021–28.

domain, it was proposed that cSrc interacts with the PXXP motif of Akt [176] which is consistent with this, that is, Akt translocates to the plasma membrane through the binding of its PH domain to IP_3 generated by PI3K. This allows the interaction of membrane bound cSrc and Akt through its PXXP motif in the C-terminal regulatory region and the SH3 domain of cSrc. Then cSrc phosphorylates Akt at tyrosine residue(s), which triggers Akt A-loop (T308) and HM (S473) phosphorylation by PDK-1 and mTORC2 respectively. Altogether these data support the idea that cSrc operates as a ''switch'' in concert with PI3K for activation of Akt in response to ghrelin.

On the other side, the data obtained show a role of β-arrestins in ghrelin-induced Akt activation, role that is related to receptor endocytosis. This is clear since β-arrestin depletion using siRNA reduced the magnitude of Akt activation in response to ghrelin. Besides, the co-immunoprecipitation assays showed that β-arrestins, acting like adaptor proteins, recruit Akt to the ghrelin-occupied receptor. Although the mechanism of activation of Akt-associated to β-arrestins is not clear, from the data obtained it can be concluded that cSrc is essential for Akt activation even when is associated to β-arrestins. As it already was described previously, [160] it would be possible that Akt interacts with cSrc associated to GHSR-1a-β-arrestin complex as consequence of colocalization at plasma membrane when PI3K is activated in response to ghrelin. This Akt-cSrc association is presumed to initiate Akt phosphorylation by allowing tyrosine phosphorylation. Furthermore, in line with its function, β-arrestin-scaffolded complex places the different components of the Akt cascade in close proximity to each other, ensuring substrate specificity. This fact is supported by the data showing that neither PDK-1 nor mTORC2 co-immunoprecipitated with β-arrestins. It is also noteworthy that both β-arrestins seem to be required to promote Akt activation. This could be indicative of a need to form heterodimers, as it was described for MAPK activation. [160] However, this mechanism cannot be generalizated since in other systems β-arrestins show opposite effects. This is the case of D_2-class receptors where β-arrestin 2 facilitates the dephosphorylation of Akt by phosphatase 2A (PP2A) in response to dopamine. [177, 178] Other examples are the prostaglandin E2, [179] IGF-1, [180]

[176] Jiang T, Qiu Y. Interaction between Src and a C-terminal proline-rich motif of Akt is required for Akt activation. J Biol Chem. 2003; 278: 15789–93.
[177] Beaulieu JM, Marion S, Rodriguiz RM, Medvedev IO, Sotnikova TD, Ghisi V, Wetsel WC, Lefkowitz RJ, Gainetdinov RR, Caron MG. A β-arrestin 2 signaling complex mediates lithium action on behavior. Cell. 2008; 132: 125–36.
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[179] Buchanan FG, Gorden DL, Matta P, Shi Q, Matrisian LM, DuBois RN. Role of β-arrestin 1 in the metastatic progression of colorectal cancer. Proc Natl Acad Sci USA. 2006; 103: 1492–97.

α-thrombin [181] and β-1 adrenergic receptors [182, 183] which activate Akt pathway through β-arrestin 1 dependent mechanisms. Thus, it seems that each receptor determine the β-arrestin-associated functions on basis of the receptor-associated isoforms.

As it is reported in the bibliography, ghrelin regulates Akt activity through the GHSR-1a in different cellular systems. [156-159] Particularly, the mitogenic effect of ghrelin in 3T3-L1 preadipocytes is mediated by the PI3K/Akt and MAPK pathways via a G_i-protein. [84] In line with the previous data obtained in HEK-GHSR-1a cells, co-immunoprecipitation assays performed in 3T3-L1 cells indicated that β-arrestins recruit Akt, leading to its activation. In addition, cSrc acts upstream of Akt, as PP2 experiments abolished the ghrelin-induced Akt activation. Furthermore, the co-immunoprecipitation assays also show that cSrc interacts with Akt in response to ghrelin, allowing the phosphorylation of Akt at tyrosine residue(s).

In summary, the results presented here show that Akt translocates to the plasma membrane through the binding of its PH domain to the second messenger IP_3 generated by PI3K which is activated through $G_{i/o}$-protein-dependent signaling pathway. Akt is phosphorylated at tyrosine by the membrane bound cSrc via the interaction between its C-terminal proline-rich motif and the SH3 domain of cSrc. This tyrosine phosphorylation is followed by phosphorylation of Akt A-loop (T308) and HM (S473) by PDK-1 and mTORC2 respectively. Once the receptor is activated, a second signaling pathway is mediated by β-arrestins 1 and 2 involving the recruitment of GHSR-1a, cSrc and Akt into a β-arrestin-scaffolded complex. Thus cSrc functions as a switch that initiates the Akt pathway associated to both the $G_{i/o}$-protein-dependent pathway and β-arrestin-scaffolded complex.

[180] Povsic TJ, Kohout TA, Lefkowitz RJ. β-arrestin1 mediates insulin-like growth factor 1 (IGF-1) activation of phosphatidylinositol 3-kinase (PI3K) and anti-apoptosis. J Biol Chem. 2003; 278: 51334–39.

[181] Goel R, Phillips-Mason PJ, Raben DM, Baldassare JJ. α-Thrombin induces rapid and sustained Akt phosphorylation by β-arrestin1-dependent and -independent mechanisms, and only the sustained Akt phosphorylation is essential for G1 phase progression. J Biol Chem. 2002; 277: 18640–48.

[182] Noma T, Lemaire A, Naga Prasad SV, Barki-Harrington L, Tilley DG, Chen J, Le Corvoisier P, Violin JD, Wei H, Lefkowitz RJ, Rockman HA. β-arrestin- mediated β1-adrenergic receptor transactivation of the EGFR confers cardioprotection. J Clin Invest. 2007; 117: 2445–58.

[183] Morisco C, Marrone C, Galeotti J, Shao D, Vatner DE, Vatner SF, Sadoshima J. Endocytosis machinery is required for β1-adrenergic receptor-induced hypertrophy in neonatal rat cardiac myocytes. Cardiovasc Res. 2008; 78: 36–44.

Akt pathway/SHP-1

Over the past several years, much progress has been made in elucidating intracellular signaling events mediating metabolic actions by the ghrelin/GHSR-1a system. However, the molecular processes that mediate the inactivation of these events remain less defined. The results obtained here offer three major findings related to the regulation Akt activity in response to ghrelin. First, SHP-1 is one of the negative regulators of GHSR-1a-mediated Akt activation. Second, activation of SHP-1 is cSrc-dependent and involves the interplay of G-protein-and β-arrestins-dependent signaling pathways. Third, white adipose tissue (WAT) expresses relatively high levels of SHP-1 and this expression is dynamically regulated in response to high fat feeding. Thus, SHP-1 attenuates the action of ghrelin by dephosphorylating PI3K and cSrc and thereby inhibiting ghrelin signaling to the Akt pathway.

The protein tyrosine phosphatase SHP-1 plays a variety of roles in multiple signal transduction events [184] by tyrosine dephosphorylation. [185, 186] This events comprise signaling of cytokine receptors, [187, 188] GPCR [119, 189,] and RTK. In these and many other cases, SHP-1 regulates signaling in a negative manner; in other pathways SHP-1 exerts a positive function. [190, 191]

The results presented here are in favor of the role of SHP-1 as a negative regulator of ghrelin/GHSR-1a signaling. The expression of catalytically inactive SHP-1 increased ghrelin-induced Akt phosphorylation, with greater repercussion on Akt A-loop (T308) phosphorylation.

[184] Neel BG, Tonks NK. Protein tyrosine phosphatases in signal transduction. Curr Opin Cell Biol. 1997; 9: 193-204.
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[191] Krautwald S, Büscher D, Kummer V, Buder S, Baccarini M. Involvement of the protein tyrosine phosphatase SHP-1 in Ras-mediated activation of the mitogen-activated protein kinase pathway. Mol Cell Biol. 1996; 16: 5955-63.

While much of the characterization of SHP-1 is centered on its function as a PTP, [192] the regulation of its activation is confused. [193, 194] Despite such puzzlement, it was demonstrated that tyrosine phosphorylation (Y) of the C-terminal Y536 in SHP-1 increases its PTP activity.[193, 195] This is consistent with these results, which showed that ghrelin is able to phosphorylate SHP-1 at C-terminus Y536 in intact cells and WAT. Additionally, the results show that ablating cSrc by siRNA, impaired ghrelin-stimulated C-terminus Y536 phosphorylation of SHP-1. This result locates to cSrc as an upstream signal that regulate activation of SHP-1. Indeed a previous report showed that cSrc phosphorylates SHP-1 on Y *in vitro*, leading to an increase in the activity of the phosphatase. [195] This is also consistent with recent works in which angiotensin II type 2 receptor (AT2R) regulates SHP-1 activity through cSrc activation.[189, 196] This data support the idea that cSrc operates as a "switch" in concert with PI3K for 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 an increase of ghrelin-induced tyrosine phosphorylation of p85 regulatory subunit of PI3K. Under resting conditions, p85 acts to both stabilize and inactivate the p110 catalytic subunit PI3K of the N-terminal domain. [197] Our data confirm that tyrosine-phosphorylated p85 is a target of SHP-1 and cells lacking SHP-1 increase PI3K activity as it was previously described for other factors. [198, 199] The inhibitory effect of SHP-1 on PI3K activity is consequence of its tyrosine dephosphorylation (Y688), [200] a residue that, when phosphorylated, interacts with the p85 N-terminal SH2 domain releasing the inhibitory activity

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of p85 on the p110 catalytic subunit. [198-200] In fact, activation of downstream PI3K targets, PDK-1 [pPDK-1 (S241)] and cSrc [pSrc (Y416)] are increased in cells lacking SHP-1 under ghrelin stimulation. This is also consistent with the increase on 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 β-arrestin 1 and 2 depletion using specific siRNA reduced the magnitude of ghrelin-stimulated C-terminus Y536 phosphorylation of SHP-1. Co-immunoprecipitation assays reveal that 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 on 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 is associated to β-arrestins. [201] Given that cSrc is recruited to GHSR-1a-β-arrestin complex to initiate Akt phosphorylation upon ghrelin stimulation, [160] it would be possible that SHP-1 interacts with cSrc associated to this complex, exerting a modulatory role on cSrc activity as p85 is not associated to GHSR-1a-β-arrestin complex. [160] Interestingly, both β-arrestin 1 and β-arrestin 2 are required to promote the activation of SHP-1. The requirement of both proteins might be indicative of a need to form heterodimers to activate the β-arrestin-dependent signaling pathway for GHSR-1a.

PTP have emerged as main regulators of key metabolic processes such as insulin sensitivity [202, 203] and glucose homeostasis. [204, 205, 206] From this group, the metabolic role of SHP-1 has remained unstudied, probably as result of the observation that it is mainly expressed in

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hematopoietic lineages. [207] However, SHP-1 expression is also found in peripheral tissues including insulin target tissues such as liver and muscle, [208, 209] suggesting a more widespread involvement of SHP-1. In fact, SHP-1 regulates glucose homeostasis through modulation of insulin signaling in liver and muscle. [208] Furthermore, SHP-1 expression was also found in other cell types and tissues such as RPE and perivascular cells (glial cells) in the retina and in renal podocytes, suggesting the involvement of SHP-1 in diabetic pathology. [210] As reported here, WAT expresses SHP-1 albeit to a lesser extent than liver. The subcellular distribution of SHP-1 is primarily cytosolic and concentrated in perinuclear compartments in preadipocyte and adipocyte 3T3-L1 cells. According to the results in HEK-GHSR-1a cells, ghrelin stimulated C-terminus Y536 phosphorylation of SHP-1 in both 3T3-L1 cells and WAT. Analysis of SHP-1 levels in control mice exhibited higher expression in omental than subcutaneous WAT. Furthermore, this pattern of expression is inverted in HFD-treated mice, suggesting a role of SHP-1 in controlling ghrelin sensitivity in this organ. Indeed, deficiency of SHP-1 was associated here with an increase in ghrelin-evoked pAkt (T308) in omental WAT as well as a decrease in activation of T308 phosphorylation under abundance of SHP-1 in subcutaneous WAT. Intriguingly, the inhibitory effect of SHP-1 on pAkt (S473) showed lower repercussion as it was described in HEK-GHSR-1a cell model. The SHP-1-regulated targets associated to the phosphorylation of A-loop (T308) and HM (S473) might explain this difference. Indeed, SHP-1 attenuates PDK-1 activity through PI3K with no apparent effect on mTORC2. This is further supported by the fact that both A-loop (T308) and HM (S473) phosphorylations are independent. [201] Analysis of initial steps in ghrelin signaling to Akt revealed modified levels for cSrc, PDK-1 and mTOR in WAT from HFD-treated mice relative to control mice, while no change in Akt and GHSR-1a expressions were detected. However this differential pattern does not appear to explain the differences of Akt activity detected between both groups. In fact, enhanced cSrc and PDK-1 expression in subcutaneous WAT from HFD mice does not counteract the attenuation of ghrelin-evoked pAkt (T308). Based on this information, it is possible to propose that ghrelin action in WAT is, at least in part, under regulation of SHP-1. As

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is consistent with data from cell model studies of SHP-1 functions, deficiency of SHP-1 is associated with enhanced activation of PI3K, PDK-1, cSrc and Akt thereby increasing ghrelin control on growth and metabolism in WAT and *vice versa*. [211, 212] These data are in line with the attenuating role of SHP-1 on Akt signaling in liver and muscle, which determines insulin sensitivity and glucose metabolism.

In conclusion, we have identified the SHP-1 tyrosine phosphatase as a negative regulator of ghrelin signaling. Data are consistent with a model in which ghrelin-activated Akt translocates to the plasma membrane through the binding of its PH domain to the second messenger IP_3 generated by PI3K which is activated through the $G_{i/o}$-protein-dependent signaling pathway. Akt is phosphorylated at tyrosine by the membrane bound cSrc. This tyrosine phosphorylation is followed by phosphorylation of Akt A-loop (T308) and HM (S473) by PDK-1 and mTORC2 respectively. Once the receptor is activated, a second signaling pathway is mediated by β-arrestins 1 and 2, involving the recruitment of GHSR-1a, cSrc and Akt into a β-arrestin-scaffolded complex. In both signaling pathways cSrc phosphorylates the SHP-1 C-terminus (Y536) which exerts an inhitory effect on the activity of PI3K and Akt. Consequently cSrc functions as a switch that controls the Akt pathways associated to both the Gi/o-protein dependent pathway and β-arrestin-scaffolded complex. Although further studies are required, the results show that SHP-1 is expressed in white adipose tissue, one of the ghrelin target tissue, and that it modulates ghrelin signaling to Akt, determining ghrelin sensitivity in WAT, based on adipose depot differences.

Ghrelin/CST-17

Elucidation of the intracellular signaling events mediating GHSR-1a actions constitutes an important point to a better knowledge about ghrelin/GHSR-1a system. GHSR-1a shows a broad binding capacity that allows the interaction of peptidic/nonpeptidic GHS, not related structurally to ghrelin, and other natural ligands such as adenosine [59, 60] , CST [62] GHRH. [76]

CST-17 is a hormone originally described in the rat, mouse, and human cerebral cortex that displays structural and functional similarities to SST, binding to all five somatostatin receptors

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(SSTR). [213, 214] Among its functions, CST inhibits GH and insulin secretion in physiological conditions and in acromegaly, [66] it also inhibits prolactin (PRL) secretion from cultured prolactinomas [65] and, recently, a role for CST as therapeutic agent to autoimmune diseases has been proposed. [67] Because of these different biological activities, it was proposed the existence of a CST specific receptor. In fact, an orphan receptor called MrgX2 has been shown to bind CST with selective affinity over SST. [64, 215] However, MrgX2 was found to bind additional peptides as proadrenomedullin N-terminal peptide 12 (PAMP12) with similar affinity to that described for CST. [216]

The idea that CST might be a ligand of GHSR-1a emerges from binding studies that demonstrates a cross competition with ghrelin binding in membranes from human hypothalamus and pituitary gland [62, 217] suggesting a potential functional interaction between CST and ghrelin system. To consider a true ligand-receptor interaction, two requirements must be fulfilled: specific binding and biological effect. In the present study, we have provided that CST-17 shared specific binding sites in HEK-GHSR-1a cells, binding that were displaceable with unlabeled CST-17 and ghrelin. Contrarily, WT cells exhibited a non-specific binding for [^{125}I]-CST-17 that was not displaced by saturating dose of CST-17. On the other side, [^{125}I]-ghrelin binding to HEK-GHSR-1a cells was completely displaced in a dose-dependent manner by ghrelin and to a lesser extent by CST-17. Despite its potential binding capacity to GHSR-1a, CST-17 did not show ghrelin-associated signaling pathway. CST-17 failed to induce intracellular calcium release for all dose tested in HEK-GHSR-1a cells. However the ghrelin-induced $[Ca^{2+}]_i$ rise was inhibited when cells were pre-stimulated with CST-17, effect that was dose-dependent and not shared with SST pretreatment. In addition, CST-17 did activate neither ERK 1/2 nor Akt phosphorylation for any of the time or doses tested in HEK-WT cells. However, the effect in HEK-GHSR-1a cells was different; since CST-17 activated Akt and ERK 1/2 phosphorylation reaching maximal levels within 20 minutes of CST-17 stimulation. This activation was dependent on β-arrestins, since siRNA targeting both β-arrestin 1 and β-arrestin 2 abolished completely the pattern of ERK 1/2 and Akt activation. Besides, surprisingly, CST-17

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[216] Kamohara M, Matsuo A, Takasaki J, Kohda M, Matsumoto M, Matsumoto S, Soga T, Hiyama H, Kobori M, Katou M. Identification of MrgX2 as a human G-protein-coupled receptor for proadrenomedullin N-terminal peptides. Biochem Biophys Res Commun. 2005; 330: 1146-52.
[217] Muccioli G, Papotti M, Locatelli V, Ghigo E, Deghenghi R. Binding of ^{125}I-labeled ghrelin to membranes from human hypothalamus and pituitary gland. J Endocrinol Invest. 2001; 24: RC7-9.

pre-stimulation before ghrelin stimulus inhibited ghrelin-induced ERK 1/2 phosphorylation, action that was not modified by SST, establishing that CST-17 exerts an inhibitory role in ghrelin signaling. In addition, the immunoprecipitation assays demonstrated that CST-17 promotes GHSR-1a phosphorylation at the same level that ghrelin, and to a great extent when is combinated to ghrelin. It was also proved that CST-17 promotes β-arrestin recruitment, demonstrating a direct interaction of these proteins after GHSR-1a desensitization.

Therefore, present data seem to indicate that CST-17 inhibits the mechanisms of signaling stimulated by ghrelin, both in its G-protein-dependent pathway and in the β-arrestin-dependent one. This might be considered the biological effect of the CST-17.

To explain the above discrepancy, we decided to explore the SSTR expression on our cell model. SSTR expression in HEK-WT and HEK-GHSR-1a showed that SSTR2 mRNA is significatively present in both cell types. Experiments of siRNA targeting SSTR2 in HEK-GHSR-1a cells affected to the ERK 1/2 and Akt dynamics stimulated by CST-17 and are alike the observed ones for the case of the ghrelin. This fact points to a role for SSTR2 subtype in CST-17 signaling, possibly through a dimerization process between both receptors. These results seem to establish reasons to propose CST-17 as a modulator of ghrelin signaling, inhibiting the ghrelin-mediated signaling. On the basis of the obtained results it seems reasonable to propose two hypotheses to explicate these results. First, CST-17 might be considered as a true ligand for the GHSR-1a acting through the modulation of the ghrelin activity. Second, CST-17 acts as a SSTR2 ligand promoting the formation of dimmers with the GHSR-1a and, therefore, modifying the action of this one. According to the first hypothesis, binding and displacement assays would support the CST-17 union to the GHSR-1a, fulfilling therefore one of two necessary requirements to consider a true ligand-receptor interaction. In addition, the effect on the activation of ghrelin targets, as ERK 1/2 and Akt, in HEK-GHSR-1a respect to WT would assure the second point necessary of the ligand-receptor interaction. Furthermore, the fact that CST-17 modifies the ghrelin-induced Akt and ERK 1/2 activation reveals that, somehow, it concerns the signaling pathways of this one. These facts, together with the results of targeting of β-arrestins and GHSR-1a phosphorylation, point to CST-17 acting through the modification or modulation of the signaling pathways mediated by ghrelin through GHSR-1a.

The results proving the expression of SSTR2 in HEK-GHSR-1a cells would support the second hypothesis. It is known that SSTR2 signals through G_i-dependent proteins, so it would be possible that CST-17 would exercise its functions on ghrelin targets through this mechanism, which would also justify the data obtained. In addition, the intracellular calcium mobilization assays in presence of PTX reversed the inhibitory effect of CST-17 on ghrelin-induced calcium

rise (effect that it was not reversed after a pretreatment with PMA). Data about GHSR-1a phosphorylation and β-arrestin would also explain this hypothesis, through the effect that SSTR2 presence would cause on the GHSR-1a desensitization.

Therefore, CST-17 might bind to the ghrelin receptor, acting as a potential ligand for the GHSR-1a as it has been described in other cases as for example for GHRH, [76] the GABA β2 receptor [218] or the non–peptidyl growth hormone secretagogue L-692,429. [219] On the other hand, CST-17 also could bind to SSTR2, forming a dimmer with the GHSR-1a and affecting in this way to the ghrelin-mediated signaling as it was described for other cases. [220]

In summary, CST-17 shows to modulate ghrelin signaling through SSTR2. Furthermore, CST-17 showed the capacity to activate GHSR-1a in absence of SSTR2 displaying a pattern of intracellular signaling different to that described for ghrelin, which suggest a biased ligand for this receptor.

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6. CONCLUSIONS

The conclusions from the present work are the following:

1. In the characterization of the intracellular signal transduction mechanisms activated by ghrelin through GHSR-1a to induce ERK 1/2 phosphorylation it has to be mentioned that:

   1.1. The ERK 1/2 phosphorylation is achieved by the interplay of three different signaling pathways, both G-protein and β-arrestin–dependent.

      1.1.1. The first pathway is mediated by β-arrestins 1 and 2 an requires the entry of the receptor into a multiprotein complex with the β-arrestins, cSrc, Raf-1, ERK 1/2 and perhaps other components of the MAPK cascade.

      1.1.2. A second pathway is $G_{q/11}$–dependent and involves a Ca^{+2}-dependent PKC (PKC α/β) activation and cSrc.

      1.1.3. The third one is $G_{i/o}$-dependent and involves PI3K, PKC ε and cSrc activation.

   1.2. $G_{i/o}$ and $G_{q/11}$–protein-dependent signaling pathways are crucially involved in the β-arrestin-mediated ERK 1/2 activation.

   1.3. G-protein and β-arrestin-mediated ERK 1/2 pathways are both temporally different and act in a sequential way.

2. With regard to the mechanism of Akt activation and regulation, it is noteworthy to mentioned three major findings.

   2.1. Akt protein is phosphorylated and activated by ghrelin stimulation in HEK-GHSR-1a cells through the interplay of two signaling pathways.

      2.1.1. One pathway is Gβγ-dependent and involves the activation of PI3K.

      2.1.2. The second pathway is mediated by β-arrestins 1 and 2 and requires the entry of the receptor into a multiprotein complex.

   2.2. PDK-1 and mTORC2 are essential for T308 and S473 phosphorylation of Akt respectively.

   2.3. cSrc plays an essential role in the full activation of Akt

   2.4. In 3T3-L1 preadipocyte cells, the role of cSrc and β-arrestins in Akt activation has also been corroborated.

3. In the characterization of the mechanism for regulating GHSR-1a-associated Akt activity, it is necessary to notice:

   3.1. Identification of the SHP-1 tyrosine phosphatase as a negative regulator of ghrelin-mediated Akt activation through the GHSR-1a. This tyrosine phosphatase regulates both the G-protein and the β-arrestin-dependent pathways.

   3.2. SHP-1 activation is dependent on cSrc activation, which supposes the interplay of both the $G_{i/o}$-protein dependent pathway and the β-arrestin one, through the dephosphorylation of PI3K and cSrc.

   3.3. SHP-1 is expressed in white adipose tissue and it modulates, at least in part, ghrelin signaling to Akt, determining ghrelin sensitivity in this organ based on adipose depot differences.

4. Regarding the effects of CST-17 on ghrelin/GHSR-1a system, we have demonstrated the folllowing data:

   4.1. CST-17 attenuates ghrelin signaling through SSTR2. This effect involves GHSR-1a desensibilization through GHSR-1a phosphorylation and β-arrestin recruitment. This desensibilization process appears to be related to GHSR-1a-associated β-arrestin pathway, at least, for ERK 1/2 and Akt.

   4.2. CST-17 activates GHSR-1a in absence of SSTR2 displaying a pattern of intracellular signaling different to that described for ghrelin, which suggest a biased ligand for this receptor.

7. ANEXO

La ghrelina es una hormona peptídica de 28 aminoácidos que presenta un residuo de ácido octanoico en la serina 3. Fue descubierta como el ligando natural del receptor de secretagogos de hormona de crecimiento tipo 1a (GHSR-1a). Es producida principalmente en el estómago aunque también otros órganos, como son el tracto gastrointestinal, páncreas, hipófisis e hipotálamo sintetizan dicha hormona pero en menor cantidad.

La modificación post-traduccional que presenta la ghrelina es esencial para su unión al GHSR-1a y para sus actividades biológicas. Entre ellas podemos destacar la secreción de hormona de crecimiento (GH) tanto *in vivo* como *in vitro* y la regulación de la ingesta así como otras actividades no endocrinas como la motilidad y secreción gástrica, la influencia en la actividad pancreática y el metabolismo de la glucosa, efectos cardiovasculares y efectos proliferativos y antiproliferativos en distintas líneas celulares. En humanos el gen de la ghrelina se localiza en la posición cromosómica 3q 25-26 y presenta 4 exones y 3 intrones. Esta estructura da lugar a dos tipos de moléculas distintas por un mecanismo de splicing alternativo: ghrelina y des-gln14-ghrelina. La molécula precusora de la ghrelina, llamada preproghrelina, contiene 117 aminoácidos, 23 de ellos formando la secuencia señal y 94 la proghrelina (28 aminoácidos de la ghrelina y 66 de la cola). Posteriormente la secuencia señal es eliminada en el retículo endoplasmático y luego un procesamiento proteolítico dará lugar a la ghrelina madura y a un fragmento C-terminal de 66 aminoácidos, llamado C-ghrelina. La ghrelina que se produce es desacilada por lo que posteriormente tiene que ser acilada, mecanismo recientemente descrito y que es llevado a cabo por la enzima GOAT (ghrelin-O-acyl transferase). Además de la ghrelina existe otro péptido codificado por el mismo gen llamado obestatina. Este péptido deriva de la proghrelina y presenta 23 aminoácidos con un residuo C-terminal que puede ser amidado. Originalmente se aisló también del estómago aunque su expresión se encuentra distribuida en diferentes tejidos como el duodeno, páncreas, bazo, glándulas mamarias y plasma. Inicialmente se propuso como ligando del receptor huérfano GPR39, que pertenece a la misma familia que el GHSR-1a pero más tarde se demostró que no era así. Hasta el momento son numerosas las publicaciones en las que se han descrito diferentes funciones de la obestatina desde la implicación en procesos mitogénicos, regulador del metabolismo del adipocito y la adipogénesis hasta su implicación en la protección de las células cardíacas, entre otras.

Respecto al GHSR-1a cabe mencionar que se localiza en la posición cromosómica 3q 26.2. Existen dos tipos de cDNA, que por un mecanismo de splicing alternativo, dan lugar al GHSR tipo 1a y tipo 1b. El tipo 1a codifica un receptor de 7 dominios transmembrana y presenta gran afinidad y especifidad por la ghrelina y el resto de secretagogos de GH (GHS). Por su parte, el

tipo 1b sólo posee 5 dominios transmembrana y no permite la unión del ligando. EL GHSR-1a se expresa principalmente en hipófisis e hipotálamo, aunque también se ha detectado en otros órganos y tejidos como tiroides, páncreas, bazo, miocardio, glándulas adrenales, testículos, ovaries, placenta y estómago. Tras la unión de la ghrelina al GHSR-1a, éste es internalizado por un mecanismo dependiente de vesículas recubiertas de clatrina promoviendo su desensibilización. Hay que mencionar también que el GHSR-1a presenta actividad constitutiva, esto es, produce una activación del mismo en ausencia de ligando. Si la actividad constitutiva posee alguna función *in vivo* está por descubrir, aunque recientemente se ha visto que mutaciones que eliminan selectivamente esta actividad constitutiva segregan con el desarrollo de una baja estatura y el desarrollo de obesidad. Otro hecho importante en relación al GHSR-1a es la existencia de diferentes ligandos. Además de los ya descritos GHS, también se ha demostrado la unión de la cortistatina (CST-17), una hormona presente en el cortex cerebral que muestra similitud con la somatostatina (SST). Otro candidato es la hormona liberadora de la hormona de crecimiento (GHRH), hormona clave en la secreción de GH por parte de la hipófisis junto con la SST y la ghrelina.

En relación a los sistemas de señalización activados por la ghrelina y su receptor, cabe mencionar que la secreción de GH y la regulación de la ingesta son los más estudiados. En el primer caso, el mecanismo implica la activación de una fosfolipasa C dependiente de fosfatidilinositol (PI-PLC), cuyos segundos mensajeros provocan la liberación de calcio intracelular y la activación de la proteína quinasa C (PKC) que en última instancia provocará la liberación de GH al medio extracelular. En el segundo caso, la respuesta orexigénica de la ghrelina se consigue a través de la inhibición de la síntesis de ácidos grasos inducida por la proteína quinasa activada por AMP (AMPK). También son numerosos los trabajos donde se ha visto un papel de la ghrelina en los procesos de proliferación celular a través de la activación de la ruta de las proteínas quinasas activadas por mitógenos (MAPK). Este es el caso de células 3T3-L1, cardiomiocitos, células endoteliales y células de hepatoma.

Las arrestinas constituyen una pequeña familia de proteínas que interacciona con los receptores acoplados a proteínas G (GPCR), una vez que estos han sido activados y fosforilados por las quinasas de los receptores acoplados a proteínas G (GRK), promoviendo su desensibilización. Esta familia comprende 4 miembros: las arrestinas 1 y 4 (arrestinas visuales) y las arrestinas 2 y 3 (llamadas β-arrestinas 1 y 2 respectivamente). La composición entre ambas es hasta un 65% idéntica pero las arrestinas visuales se localizan en los conos y bastones de las células de la retina mientras que las β-arrestinas tienen una expresión más ubicua. Además de su papel en los procesos de desensibilización también participan en

mecanismos de endocitosis y de señalización intracelular actuando como proteínas de "andamiaje". Poseen una estructura característica y también sufren de procesos de fosforilación/defosforilación y ubiquitinación, lo que provoca la existencia de múltiples estados conformacionales, inducidos por la unión a los receptores fosforilados en diferentes lugares o combinaciones de lugares.

Existen dos tipos de GPCR, designados A y B, que difieren en su afinidad por las β-arrestinas. Los receptores clase A, como el receptor adrenérgico tipo β2 (β2AR), se unen con más afinidad a la β-arrestina 1 que a la 2. Los clase B, como el receptor tipo 1A de angiotensina II (AT1AR), se unen con igual afinidad a ambas β-arrestinas. Entre las funciones de las β-arrestinas se encuadran la desensibilización de los GPCR, la endocitosis de los mismos, down-regulación y la señalización. La desensibilización se define como un proceso que previene la sobreactivación del receptor en respuesta a una estimulación prolongada. Comienza tras la exposición al ligando y supone la fosforilación del mismo por GRK o PKC y la posterior unión de las β-arrestinas, las cuales bloquean su interacción con las proteínas G. La endocitosis constituye un proceso mediante el cual los receptores son eliminados de la superficie celular a través de un mecanismo de internalización del receptor hacia compartimentos intracelulares, contribuyendo al mismo tiempo a la desensibilización y resensibilización de los mismos. El proceso de endocitosis suele estar mediado a través de vesículas recubiertas de clatrina y una vez que el receptor ha sido internalizado, éste puede ser degradado en los lisosomas o defosforilado y reciclado de vuelta a la membrana plasmática. La down-regulación es un proceso que se caracteriza por una disminución del número total de receptores tras una exposición prolongada al ligando. El propósito último es la resensibilización del receptor para la cual debe existir defosforilación del receptor y disociación del ligando. La down-regulación implica dos mecanismos diferentes: la degradación de los receptores preexistentes y la disminución de la síntesis *de novo*.

Con respecto a la señalización, se ha descrito que tras la internalización del receptor se produce una secuencia de eventos, a través de la función de las β-arrestinas como proteínas de andamiaje, para controlar la señalización intracelular. Las β-arrestinas incrementarían la eficiencia de señalización entre las distintas proteínas de una ruta determinada y también dirigiéndolas a lugares específicos dentro de la célula. El ejemplo más estudiado lo constituye la activación de la ruta de las MAPK en el cual esta activación se consigue por mecanismos dependientes de proteínas G y por mecanismos dependientes de las β-arrestinas. El primero de ellos suele ser rápido y transitorio e implica la traslocación nuclear de ERK. EL segundo es lento, persistente en el tiempo y supone la retención citosólica de ERK. Esto implica diferentes

consecuencias fisiológicas. Recientemente se ha descubierto que para algunos GPCR múltiples ligandos pueden, de un modo diferenciado, activar rutas de señalización a través de las β-arrestinas en vez de las rutas mediadas por proteínas G. Esto es lo que se denomina "biased ligands" y que se caracteriza porque se favorece una respuesta frente a otra comparado con el ligando endógeno. Este es el caso por ejemplo del AT1AR. Su interés es importante en el desarrollo de nuevos fármacos que conduzcan a una activación celular en una dirección deseada y no en otra.

El sistema ghrelina/GHSR-1a está implicado en diversas respuestas fisiológicas, por lo que tiene relevancia en diferentes enfermedades, entre ellas la obesidad y anorexia. Las recientes publicaciones sugieren que la señalización mediada por los GPCR es más diversa de lo que originalmente se pensaba, ya que aquéllos se pueden acoplar a distintas proteínas G así como a otras proteínas adaptadoras como son la familia de las β-arrestinas. En base a esto, la hipótesis de trabajo para la presente Tesis se estableció en que para el sistema ghrelina/GHSR-1a, las β-arrestinas constituyen una vía de señalización importante y diferente de la ruta clásica mediada por las proteínas G, además de la función, ya descrita, de terminación de la señalización.

El objetivo global se centra en delinear el papel de las β-arrestinas en la señalización mediada por el GHSR-1a. Este objetivo general se puede dividir en los siguientes objetivos específicos:

1. Determinación de la señalización mediada por las β-arrestinas 1 y 2, bajo condiciones basales y estimuladas por la ghrelina, implicadas en la regulación del metabolismo, apoptosis, transcripción y ciclo celular. Este apartado se divide a su vez en:

    1.1. Determinación del papel de las β-arrestinas en la ruta de señalización dependiente de ERK 1/2 que media el efecto de la ghrelina en la supervivencia celular, crecimiento y proliferación.

    1.2. Determinación del papel de las β-arrestinas en la ruta de señalización dependiente de Akt que media el efecto de la ghrelina en el metabolismo, apoptosis, transcripción y ciclo celular.

2. Caracterización del mecanismo de regulación de la activación de Akt asociada al GHSR-1a, destacando la implicación de la proteína tirosina fosfatasa SHP-1.

3. Determinación del papel de las β-arrestinas en las rutas de señalización dependientes de ERK 1/2 y Akt que median los efectos de la CST-17 a través del GHSR-1a.

Los mecanismos propuestos en esta hipótesis se realizaron en la línea celular HEK 293 transfectada de forma estable con el GHSR-1a (HEK-GHSR-1a) como modelo para estudiar la interacción de proteínas específicas y el papel, en su caso, tanto de las rutas dependientes de las proteínas G como de las β-arrestinas. Algunos de los resultados fueron corroborados en células 3T3-L1 y tejido adiposo.

Ghrelina/MAPK

En este trabajo los resultados muestran que la ghrelina activa la ruta de las MAPK, ruta principal de los procesos mitogénicos, en la línea celular HEK 293 transfectada de modo estable con el GHSR-1a. Este hecho también se ha descrito en otras líneas celulares pero el mecanismo por el cual se producía esta activación no se conocía. Los datos demuestran que la activación de MAPK inducida por la ghrelina se consigue por la interrelación de 3 vías de señalización diferentes, que dependen tanto de las proteínas G como de las β-arrestinas. Una de estas rutas depende de una proteína $G_{q/11}$ e implica la activación de una PKC dependiente de Ca^{+2} (PKC α/β) y de cSrc. La segunda ruta es dependiente de una proteína $G_{i/o}$ y supone la activación de PI3K, PKC ε y cSrc. Por último, la tercera vía depende de las β-arrestinas 1 y 2 y requiere de la formación de un complejo multiproteico formado por el GHSR-1a, las β-arrestinas, cSrc, Raf-1, ERK 1/2 y quizás otros componentes de la cascada de señalización de las MAPK. También cabe destacar que las vías dependientes de $G_{q/11}$ y $G_{i/o}$ están implicadas y convergen en la vía mediada por las β-arrestinas. Además, la proteína cSrc juega un papel clave en este proceso, uniendo ambas rutas. Hay que matizar el papel de las β-arrestinas, pues originalmente se pensaba que sólo participaban en los procesos de desensibilización e internalización de los receptores aunque se ha demostrado que también actúan como moléculas adaptadoras y transductoras de señales. Un hecho que no queda claro es si ambas rutas actúan secuencialmente o en paralelo como ha sido descrito por ejemplo para el AT1AR o el β2AR. Aún así, parece razonable suponer que las consecuencias fisiológicas de esta activación sean distintas por lo que la ghrelina activaría diferentes "pools" de ERK 1/2 que diferirían tanto en su localización como en su perfil de activación. La activación de ERK 1/2 mediada por las proteínas G se acumularía en el núcleo donde fosforilaría y activaría diferentes factores de transcripción mientras que la mediada por las β-arrestinas estaría confinada al citoplasma donde fosforilaría diferentes proteínas efectoras.

Ghrelina/Akt

La ruta de Akt, además de su papel en procesos proliferativos, también ejerce otras varias funciones que abarcan desde la regulación del metabolismo, la transcripción, la regulación del ciclo celular o la apoptosis. Estas funciones suelen ser específicas del tipo celular y están mediadas por diferentes dianas. El mecanismo clásico de activación de Akt implica su traslocación a la membrana plasmática donde será fosforilada en los residuos T308 y S473 por la proteína PDK-1 y el complejo mTORC2, respectivamente. Una vez activada, Akt activa diferentes dianas dentro de la célula para ejercer sus funciones biológicas.

De los datos obtenidos podemos destacar tres hechos principales relacionados con la activación de Akt en respuesta a la ghrelina en las células HEK-GHSR-1a y 3T3-L1. El primer hecho es que Akt se fosforila por la acción de dos rutas de señalización: la primera de ellas es dependiente de una proteína $G_{i/o}$ e implica la activación de la PI3K y la segunda está mediada por las β-arrestinas 1 y 2. El segundo muestra que PDK-1 y mTORC2 son esenciales para la fosforilación y activación de Akt en los residuos T308 y S473, respectivamente. El tercero supone el papel clave que presenta la proteína cSrc en la activación de Akt puesto que inicia la fosforilación de Akt asociada tanto a la ruta dependiente de proteínas G como a la de las β-arrestinas.

Akt/SHP-1

Con la caracterización de la ruta de activación de Akt mediada por la ghrelina, se quiso estudiar más en detalle la regulación de esta activación. Para ello los experimentos realizados mostraron los siguientes resultados.

En primer lugar, se identificó a la proteína tirosina fosfatasa SHP-1 como uno de los reguladores de la activación de Akt mediada a través del GHSR-1a. Previamente se había descrito que la SHP-1 interviene en distintas rutas de señalización, mediadas tanto por GPCR como por receptores tirosina quinasa (RTK), a través de la defosforilación de proteínas, ejerciendo tanto un efecto negativo (en la mayoría de los casos) como positivo. Este papel regulador de SHP-1 sobre Akt se observó al tratar las células HEK-GHSR-1a con un mutante dominante negativo de SHP-1 (SHP-1dn) lo que provocó un aumento en la fosforilación de Akt inducida por la ghrelina. En segundo lugar se procedió al estudio en detalle de este mecanismo, encontrándose que la activación de SHP-1 es dependiente de la proteína cSrc, que

a su vez implica la convergencia de las rutas dependientes tanto de las proteínas G como de las β-arrestinas. Por lo tanto, la SHP-1 atenúa la acción de la ghrelina sobre Akt a través de la defosforilación de la PI3K y de la cSrc. Por último, dado que las SHP regulan procesos metabólicos claves como la sensibilidad a la insulina y la homeostasis de la glucosa, a través de la modulación de la señalización mediada por la insulina en hígado y músculo, se comprobó la expresión de SHP-1 en células 3T3-L1, tanto en preadipocitos como adipocitos maduros, y en tejido adiposo de ratones. Los resultados obtenidos mostraron que SHP-1 se expresa tanto en preadipocitos como en adipocitos, mostrando una distribución principalmente citosólica y concentrada en la región perinuclear. Por su parte, el tejido adiposo, omental y subcutáneo, de ratones expresa niveles relativamente altos de SHP-1 y su expresión es regulada en respuesta a dietas ricas en grasas. Es decir, el tejido adiposo omental muestra una disminución de la expresión de SHP-1 comparada con la misma en una dieta regular, mientras que el tejido subcutáneo, muestra un incremento en la expresión SHP-1 comparado con la misma en una dieta regular. En base a estos resultados se puede especular que el aumento de la expresión de SHP-1 en el tejido subcutáneo resulta en una atenuación de la señalización dependiente de Akt con la consecuente reducción de la sensibilidad de la ghrelina. Por contra, la down-regulación de SHP-1 en el tejido omental reforzaría la sensibilidad a la ghrelina, promoviendo el almacenamiento de grasa.

Ghrelina/CST-17

Con respecto a este último apartado, el trabajo se centró en el estudio de la CST-17, una hormona que muestra similitud funcional y estructural con la SST, como posible ligando del GHSR-1a, modificando la respuesta de la ghrelina a través de éste. Las dos hipótesis que se propusieron fueron por una parte, la posible unión de la CST-17 al GHSR-1a y por otro la posibilidad de dimerización entre el GHSR-1a y el SSTR2. Previamente, ya se había demostrado, a través de ensayos de unión ligando/receptor, que la CST-17 mostraba capacidad de unión al receptor de ghrelina, sugiriendo una potencial interacción funcional entre ambos. Sin embargo, hasta la fecha no se ha podido demostrar una interacción directa entre ambas.

Para que se considere una interacción verdadera ligando-receptor, se requieren dos hechos. El primero de ellos es una unión específica y el segundo es un efecto biológico. En primer lugar, se demostró que la CST-17 muestra sitios de unión específicos en células transfectadas con el GHSR-1a. Esta unión es específica y desplazable por la ghrelina. Con respecto a la activación de proteínas como Akt o ERK 1/2 se observó que la CST-17 produce una activación de Akt y ERK

1/2 en células HEK-GHSR-1a, la cual no se observa en células HEK-WT. La activación de estas proteínas se vio que dependían de las β-arrestinas, pues cuando éstas se silenciaban, el perfil de activación de ERK 1/2 y Akt se perdía. Además, la CST-17 modifica, inhibiendo, la señalización mediada por la ghrelina implicada en la ruta de activación de Akt y ERK 1/2. Asimismo también se observó una inhibición de la respuesta de movilización de calcio inducida por la ghrelina cuando se pre-estimulaba con la CST-17; inhibición que no se observó para el caso de la SST. Por otra parte, se vio también que la CST-17, al igual que la ghrelina, provoca un aumento en la fosforilación del receptor de ghrelina (aumento que es mayor cuando ambas hormonas se combinan) y en el reclutamiento de las β-arrestinas, proteínas claves, como hemos visto, en la desensibilización del GHSR-1a. Por lo tanto, los datos parecen indicar que la CST-17 afecta a los mecanismos de señalización mediados por la ghrelina, tanto en su parte dependiente de las proteínas G como en la dependiente de las β-arrestinas. Esto podría considerarse como el efecto biológico de la CST-17.

Por otra parte, se determinó también la expresión del subtipo 2 del receptor de somatostatina (SSTR2) en las células HEK-GHSR-1a, lo que pondría de manifiesto la posible dimerización entre ambos receptores y explicaría también los datos obtenidos. Sorprendentemente, cuando se silencia el SSTR2, los perfiles de activación de Akt y ERK 1/2 mediados por la CST-17 se ven afectados y se asemejan a los observados para el caso de la ghrelina. Todo ello hace pensar que la CST-17 actúa fundamentalmente a través del receptor SSTR2, atenuando la acción del GHSR-1a a través de un proceso de desensibilización que implica la fosforilación del mismo y la consiguiente unión de las β-arrestinas. Este proceso de desensibilización lleva consigo la activación de la vía dependiente de las β-arrestinas. Por otra parte, en ausencia del SSTR2, la CST-17 muestra capacidad de unión por el GHSR-1a. Dicha unión activa la maquinaria intracelular asociada pero con una dinámica diferente a la mostrada por la ghrelina. Ello nos hace pensar en la posibilidad que CST-17 se comporte como un ligando selectivo o “biased ligand” para el GHSR-1a.

Las conclusiones del presente trabajo se resumen a continuación.

1. En la caracterización del mecanismo de señalización intracelular activado por la ghrelina a través de su receptor, el GHSR-1a, para inducir la fosforilación y activación de ERK 1/2 hay que mencionar:

   1.1. La fosforilación de ERK 1/2 se consigue por la interrelación de 3 vías de señalización diferentes, que dependen tanto de las proteínas G como de las β-arrestinas.

      1.1.1. La primera vía está mediada por las β-arrestinas 1 y 2 y requiere la presencia del GHSR-1a en un complejo multiproteico junto con las β-arrestinas, Raf-1, cSrc, ERK 1/2 y quizás otros componentes de la ruta de las MAPK.

      1.1.2. La segunda ruta depende de una proteína $G_{q/11}$ e implica la activación de una PKC dependiente de calcio (PKC α/β) y de la proteína cSrc.

      1.1.3. La tercera ruta depende de una proteína $G_{i/o}$ e implica la activación de PKC ε y cSrc.

   1.2. Las rutas de señalización dependientes de las proteínas $G_{i/o}$ and $G_{q/11}$ están implicadas y convergen en la activación de ERK 1/2 mediada por las β-arrestinas.

   1.3. Las distintas vías de activación de ERK 1/2 son ambas temporalmente diferentes y actúan de un modo secuencial.

2. Respecto al mecanismo de activación de Akt y su regulación, hay que destacar tres hechos principales:

   2.1. Akt se fosforila y activa tras la estimulación por la ghrelina a través de dos rutas.

      2.1.1. Una ruta es dependiente de las subunidades βγ de las proteínas G e implica la activación de la PI3K.

      2.1.2. La segunda está mediada por las β-arrestinas 1 y 2 y requiere la entrada del receptor en un complejo multiproteico.

   2.2. PDK-1 y mTORC2 son proteínas esenciales en la fosforilación de los residuos T308 y S473 de Akt, respectivamente.

   2.3. cSrc juega un papel muy importante en la completa activación de Akt.

2.4. EL papel de cSrc y las β-arrestinas en la activación de Akt se corroboró en células 3T3-L1 preadipocitarias.

3. En la caracterización del mecanismo de regulación de Akt asociado al GHSR-1a hay que destacar tres hechos:

   3.1. SHP-1 es una de las proteínas reguladoras negativas de la activación de Akt mediada por el GHSR-1a. SHP-1 regula tanto las rutas dependientes de proteínas G como de las β-arrestinas.

   3.2. La activación de SHP-1 es dependiente de cSrc, lo que supone la interrelación de las rutas dependientes de proteínas G y de las β-arrestinas, a través de la defosforilación de la PI3K y cSrc.

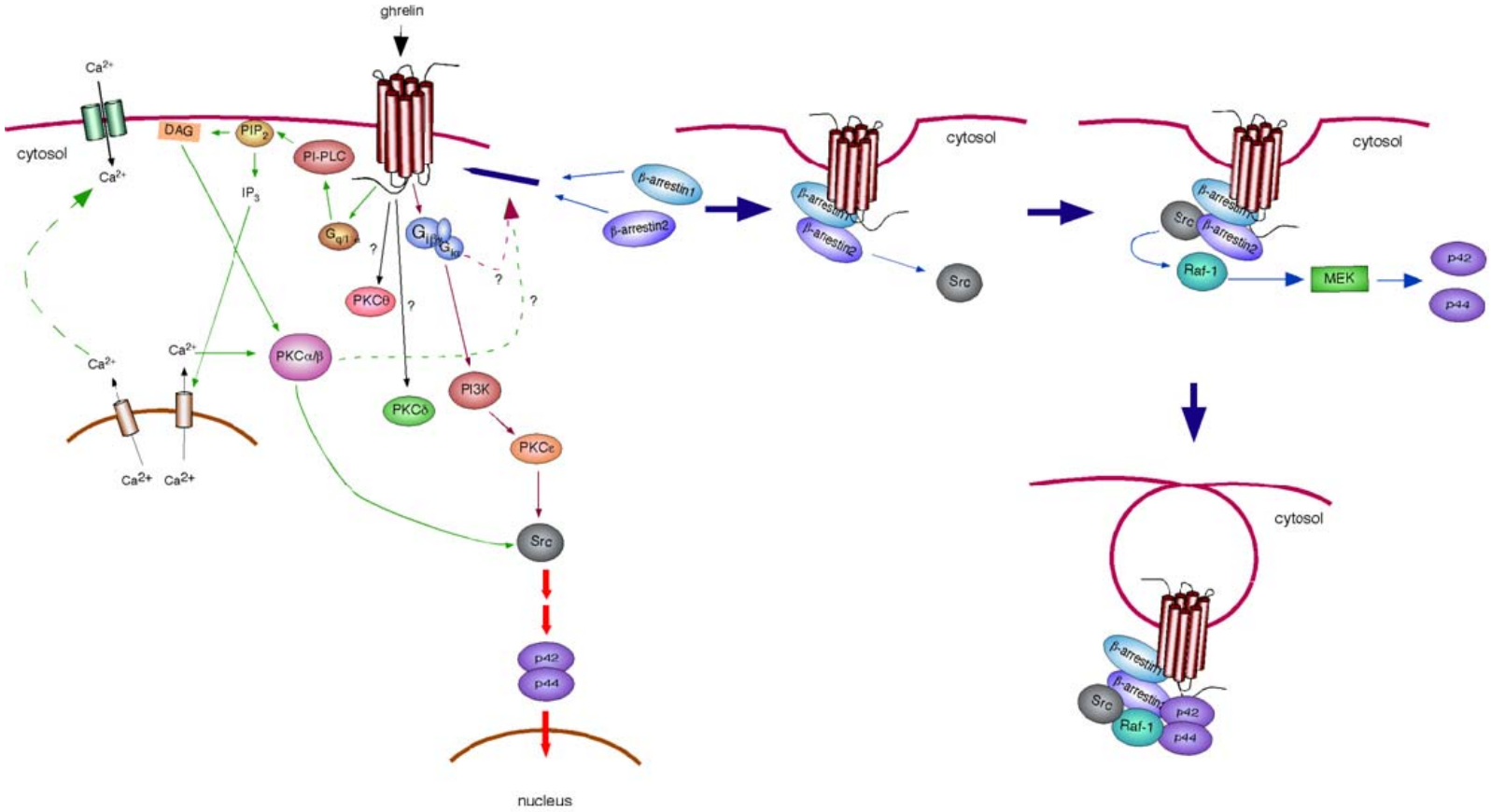
   3.3. SHP-1 se expresa en el tejido adiposo y modula, al menos en parte, la activación de Akt mediada por la ghrelina, determinando la sensibilidad de la misma en este órgano, en base a las diferencias encontradas entre los depósitos de tejido adiposo.

4. Por último, respecto a los efectos de la CST-17 en el sistema ghrelina/GHSR-1a se han demostrado los siguientes datos:

   4.1. La CST-17 atenúa la señalización mediada por la ghrelina a través del SSTR2. Este efecto implica la desensibilización del GHSR-1a a través de su fosforilación y del reclutamiento de las β-arrestinas. El proceso de desensibilización está relacionado con la ruta dependiente de las β-arrestinas asociada al GHSR-1a, al menos, para la activación de ERK 1/2 y Akt.

   4.2. La CST-17 activa el GHSR-1a en ausencia del SSTR2, mostrando un perfil de señalización intracelular diferente al descrito para la ghrelina, lo que sugiere que la CST-17 podría actuar como un “biased ligand” para el GHSR-1a.

Proposed model of the ghrelin-stimulated signaling pathway for ERK 1/2 activation



Proposed model for Akt activation by ghrelin and its regulation through SHP-1