UNIVERSIDAD DE SANTIAGO DE COMPOSTELA

FACULTAD DE MEDICINA

DEPARTAMENTO DE FISIOLOGÍA

Fundamentos estructurales y moleculares de la acción de Ghrelina y Obestatina

JUAN CARLOS ÁLVAREZ PÉREZ

Santiago de Compostela, diciembre de 2009

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

Juan Carlos Álvarez Pérez

Santiago de Compostela, diciembre de 2009

La memoria adjunta titulada "**Fundamentos estructurales y moleculares de la acción de Ghrelina y Obestatina"** que para optar al Grado de Doctor en Biología presenta D. Juan Carlos Álvarez Pérez, ha sido realizada bajo nuestra dirección en los laboratorios del Instituto de Investigación Sanitaria (Complejo Hospitalario Universitario de Santiago de Compostela).

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

Y para que así conste, expedimos el presente certificado en Santiago de Compostela en diciembre de 2009.

Prof. Dr. Felipe Casanueva Freijo
Catedrático de Medicina

Dra. Yolanda Pazos Randulfe
Investigadora SERGAS

Santiago de Compostela, diciembre de 2009

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"Algunas personas nunca aprenden nada, porque todo lo comprenden demasiado pronto."

Alexander Pope

ÍNDICE

RESUMEN DE CALIDAD DE PUBLICACIONES

La presente Tesis Doctoral consiste en un compendio de trabajos publicados y no publicados por el doctorando Juan Carlos Álvarez Pérez sobre el proyecto de Tesis Doctoral titulado **"**Fundamentos estructurales y moleculares de la acción de Ghrelina y Obestatina**".**

A continuación se indican las referencias de los trabajos publicados que forman parte de esta Tesis Doctoral, el Factor de Impacto y los cuartiles en la relación de revistas internacionales en que han sido publicados según el *Journal Citation Report*.

Categoría y referencia de los artículos	FI	Cuartil
Bioquímica y Biología Molecular		2º
FEBS Journal 247: 5714-5726 (2007)	3.396	
Endocrinología y Metabolismo		2º
Biología Celular		2º
Growth Factors 25: 373-381 (2007)	3.742	
Endocrinología y Metabolismo		1º
Oncología		1º
Endocrine-Related Cancer 16: 599-611 (2009)	5.236	
Bioquímica y Biología Molecular		2º
Biofísica		2º
Biochemical and Biophysical Research Communication 390: 1377-1381	2.648	
Bioquímica y Biología Molecular		2º
Química Medicinal		2º
Química Orgánica		1º
Bioorganic & Medicinal Chemistry (enviado)	3.075	

ABREVIATURAS

2D: dos dimensiones

AC: adenilato ciclasa

ACTH: hormona adenocorticotropina

AgRP: peptido relacionado con la proteína agoutí

AGS: línea celular de adenocarcinoma gástrico humano

AMPc: adenosín monofosfato cíclico

AMPK: proteína quinasa estimulada por AMP

AP-2: proteína adaptadora 2

ARC: núcleo arcuato

CART: péptido regulado por cocaína y anfetamina

CCK: colecistoquinina

CD: dicroísmo circular

CHO: línea celular de ovario de hámster chino

CPT-1: carnitina palmitoiltransferasa 1

CRF: factor liberador de corticotropina

CSI: índice de desplazamientos químicos

CSP: cambios de desplazamiento químico

DAG: diacilgliderol

DMN: núcleo dorsomedial

EGF: factor de crecimiento epidérmico

EGFR: receptor de EGF

ERK: quinasa regulada por señales extracelulares

GABA: ácido γ-aminobutírico

GC: línea celular de tumor somatotropo de rata

GH: hormona de crecimiento

GHRH: hormona liberadora de GH

GHRP-6: hexarelina o hexapéptido secretagogo de GH

GHS: secretagogos de GH

GHS-R1a: receptor de secretagogos de GH tipo 1a

GHS-R1b: receptor de secretagogos de GH tipo 1b

Gln: glutamina

GLP-1: péptido similar a glucagón 1

GLPR: receptor del péptido similar a glucagón

Glu: glutámico

GOAT: O-aciltransferasa de ghrelina

GPCR: receptor acoplado a proteínas G

GPR39: receptor acoplado a proteína G 39

GRB2: proteína enlazada al receptor de factores de crecimiento tipo 2

GRK: quinasas de receptores acoplados a proteínas G

HDL: lipoproteínas de alta densidad

HEK: línea celular de riñón de embrión humano

***icv*:** intracerebroventricular

***ip*:** intraperitoneal

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

IRS-1: sustrato del receptor de insulina 1

***iv*:** intravenosa

Leu: leucina

LHA: área hipotalámica lateral

LPA: ácido lisofosfatídico

Lys: lisina

MAPK: proteína quinasa activada por mitógenos

MMP: metaloproteinasa

MSH: hormona estimulante de los melanocitos

mTOR: diana de rapamicina de mamíferos

NOE: efecto overhauser nuclear

NPY: neuropéptido Y

OXM: oxintumodulina

PBS: tampón fosfato salino

PDE: fosfodiesterasa

PDK-1: quinasa dependiente de fosfoinosítido 1

PFA: área perifornical

Phe: fenilalanina

PI3k: quinasa de fosfatidil inositol 3

PIP_2: fosfoinositol 4,5-difosfato

PKA: proteína quinasa A

PKC: proteína quinasa C

PLC: fosfolipasa C

POMC: pro-opiomelanocortina

PP: polipéptido pancreático

PTX: toxina pertúsica

PVN: núcleo paraventricular

PYY: polipéptido YY

RMN: resonancia magnética nuclear

RTK: receptores tirosina quinasa

SCE: cambio conformacional lento

SDS: dodecil sulfato sódico

Ser: serina

SNC: sistema nervioso central

SRIF: factor inhibidor de la liberación de somatotropinas, somatostatina

SS: somatostatina

TOCSY: espectroscopía de correlación total

TSC: complejo de esclerosis tuberosa

UAG: des-acil ghrelina

Val: valina

VMN: núcleo ventromedial

WT: tipo salvaje

1. Introducción

1. INTRODUCCIÓN

1.1. Balance energético

En las últimas décadas, nuestro conocimiento acerca de los mecanismos que regulan la homeostasis corporal ha aumentado considerablemente. La caracterización de redes neuronales hipotalámicas y de neurotransmisores, junto con el descubrimiento de péptidos circulantes que envían señales al sistema nervioso central (SNC) con respecto al estado nutricional del cuerpo, supone un gran avance científico.

El hipotálamo juega un papel crucial en la regulación del apetito y del balance energético. En concreto, existen diversos núcleos hipotalámicos implicados en el control de la homeostasis energética, siendo el núcleo arcuato (ARC) uno de los más importantes. El ARC está constituido fundamentalmente por dos subpoblaciones neuronales que integran señales implicadas en el control de la ingesta de alimentos y la homeostasis energética; las neuronas neuropéptido Y (NPY)/péptido relacionado con la proteína agouti (AgRP) y las neuronas pro-opiomelanocortina (POMC)/tránscrito relacionado con cocaína y anfetamina (CART)[1] (Figura 1).

Neuronas NPY/AgRP: constituyen un circuito neuronal que estimula la ingesta a través de la expresión y posterior liberación de NPY y de AgRP.[2] Estas neuronas conectan con otros núcleos hipotalámicos (paraventricular, PVN).[3] Dentro del propio ARC, una subpoblación de neuronas NPY libera ácido γ-aminobutírico (GABA),

[1] Cone RD, Cowley MA, Butler AA, Fan W, Marks DL, Low MJ. The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. Int J Obes Relat Metab Disord. 2001;25 Suppl 5:S63-7.

[2] Broberger C, Johansen J, Johansson C, Schalling M, Hökfelt T. The neuropeptide Y/agouti gene-related protein (AGRP) brain circuitry in normal, anorectic, and monosodium glutamate-treated mice. Proc Natl Acad Sci U S A. 1998;95:15043-8.

[3] Bai FL, Yamano M, Shiotani Y, Emson PC, Smith AD, Powell JF, Tohyama M. An arcuato-paraventricular and -dorsomedial hypothalamic neuropeptide Y-containing system which lacks noradrenaline in the rat. Brain Res. 1985;331:172-5.

un neurotransmisor que inhibe a neuronas adyacentes que liberan POMC.

Neuronas POMC: inhiben la ingesta a través de la expresión de CART y de POMC, siendo este último procesado hasta originar la hormona estimuladora de melanocitos α (α-MSH).[4,5] Estas poblaciones neuronales proyectan sus axones hacia otras zonas del SNC que también están implicadas en la regulación del balance energético, como son PVN, el núcleo dorsomedial (DMN), el núcleo ventromedial (VMN) y las áreas hipotalámicas lateral y perifornical (LHA/PFA).[6]

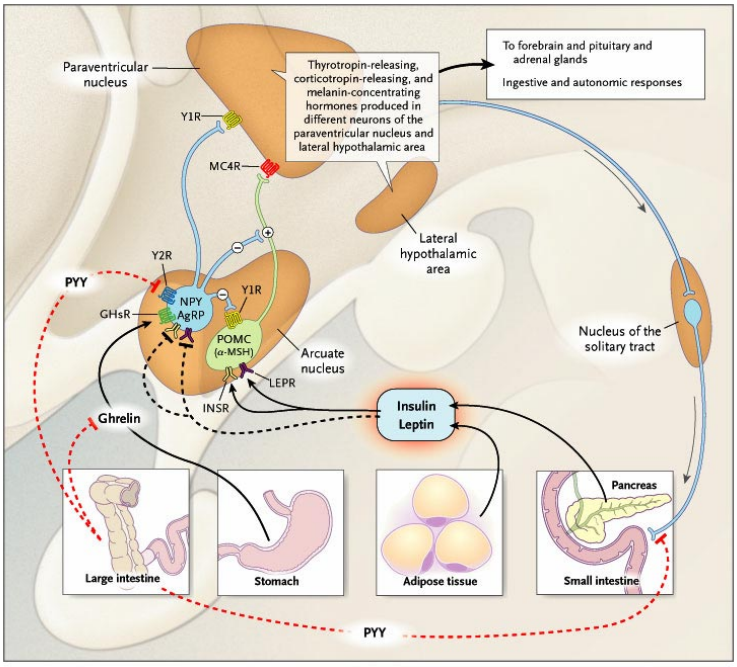

Figura 1. Interacciones entre vías hormonales e hipotalámicas que regulan la ingesta y la masa grasa. El ARC y el PVN contienen neuronas que son capaces tanto de estimular como de inhibir la ingesta. Y1R y Y2R representan los subtipos de receptores de NPY. Figura extraída de: N Engl J Med 2003;349:941.

[4] Elias CF, Lee C, Kelly J, Aschkenasi C, Ahima RS, Couceyro PR, Kuhar MJ, Saper CB, Elmquist JK. Leptin activates hypothalamic CART neurons projecting to the spinal cord. Neuron. 1998;21:1375-85.

[5] Ellacott KL, Cone RD. The central melanocortin system and the integration of short- and long-term regulators of energy homeostasis. Recent Prog Horm Res. 2004;59:395-408.

[6] Elmquist JK, Maratos-Flier E, Saper CB, Flier JS. Unraveling the central nervous system pathways underlying responses to leptin. Nat Neurosci. 1998;1:445-50.

Además de los reguladores hipotalámicos del balance energético entre los que se encuentran el NPY (el más potente factor orexigénico conocido), el sistema de la melanocortina (α–MSH, principal ligando endógeno del sistema de la melanocortina, que inhibe la ingesta, mientras que los agonistas selectivos del receptor 4 de melanocortinas hipotalámico, MC4R, producen hiperfagia[7]), CART y las orexinas (A y B que se expresan en neuronas del LHA/PFA y que estimulan la ingesta)[8], existen una serie de reguladores periféricos:

- Hormonas del tejido adiposo - Leptina, adiponectina y resistina.
- Hormonas pancreáticas – Insulina y polipéptido pancreático (PP).
- Hormonas gastrointestinales – Polipéptido YY (PYY), péptido similar al glucagón tipo 1 (GLP-1), oxintomodulina (OXM), colecistoquinina (CCK), ghrelina y obestatina.

1.2. Ghrelina

En 1999, se descubrió la ghrelina en extractos gástricos como el ligando natural del receptor huérfano de secretagogos de hormona de crecimiento (GH) tipo 1a (GHS-R1a). Este receptor se expresa, principalmente, en hipotálamo e hipófisis donde la ghrelina actúa como un péptido liberador de la hormona de crecimiento y modulador de ingesta.[9] La ghrelina se produce en las células enteroendrocrinas

[7] Benoit SC, Schwartz MW, Lachey JL, Hagan MM, Rushing PA, Blake KA, Yagaloff KA, Kurylko G, Franco L, Danhoo W, Seeley RJ. A novel selective melanocortin-4 receptor agonist reduces food intake in rats and mice without producing aversive consequences. J Neurosci. 2000;20:3442-8.

[8] Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell. 1998;92:573-85.

[9] 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.

X/A del estómago que representan la mayor población endocrina de la mucosa oxíntica.

La ghrelina es un péptido de 28 aminoácidos con un ácido graso de ocho carbonos en el tercer aminoácido (Ser) del extremo *N*-terminal. Es la primera hormona natural en la cual el grupo hidroxilo de una de sus serinas está acilado con un grupo *n*-octanoico (Figura 2). Esta acilación es esencial para la unión al receptor GHS-R1a, para la capacidad liberadora de GH, y para la mayoría de sus otras acciones endocrinas.[10,11] Alrededor del 10-20% de la ghrelina circulante se encuentra acilada, pero el 80-90% restante está desacilada. Sin embargo, los tejidos estomacales humanos contienen una relación ~2:1 de desacilada frente acilada. Esta relación indica que en el momento de entrar en la circulación sanguínea, la ghrelina acilada se convierte en desacilada.[12] El mecanismo de desacilación de la ghrelina no se conoce, pero se sabe que la ghrelina acilada en plasma, se une a lipoproteínas de alta densidad (HDL) que tienen actividad esterasa y paraxonasa.[13] Además, se identificó a la lisofosfolipasa I como la enzima responsable de la desacilación de la ghrelina en estómago de rata, pero no se encontró actividad de esta enzima en plasma.[14] En el caso de los humanos, se sabe que la enzima encargada de desacilar la ghrelina tiene actividad serina proteasa/esterasa y butirilcolinesterasa.[15]

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[10] Muccioli G, Papotti M, Locatelli V, Ghigo E, Deghenghi R. Binding of 125I-labeled ghrelin to membranes from human hypothalamus and pituitary gland. J Endocrinol Invest. 2001;24:RC7-9.

[11] Matsumoto M, Hosoda H, Kitajima Y, Morozumi N, Minamitake Y, Tanaka S, Matsuo H, Kojima M, Hayashi Y, Kangawa K. Structure-activity relationship of ghrelin: pharmacological study of ghrelin peptides. Biochem Biophys Res Commun. 2001;287:142-6.

[12] Hosoda H, Kojima M, Mizushima T, Shimizu S, Kangawa K. Structural divergence of human ghrelin. Identification of multiple ghrelin-derived molecules produced by post-translational processing. J Biol Chem. 2003;278:64-70.

[13] Beaumont NJ, Skinner VO, Tan TM, Ramesh BS, Byrne DJ, MacColl GS, Keen JN, Bouloux PM, Mikhailidis DP, Bruckdorfer KR, Vanderpump MP, Srai KS. Ghrelin can bind to a species of high density lipoprotein associated with paraoxonase. J Biol Chem. 2003;278:8877-80.

[14] Shanado Y, Kometani M, Uchiyama H, Koizumi S, Teno N. Lysophospholipase I identified as a ghrelin deacylation enzyme in rat stomach. Biochem Biophys Res Commun. 2004;325:1487-94.

[15] De Vriese C, Gregoire F, Lema-Kisoka R, Waelbroeck M, Robberecht P, Delporte C. Ghrelin degradation by serum and tissue homogenates: identification of the cleavage sites. Endocrinology. 2004;145:4997-5005.

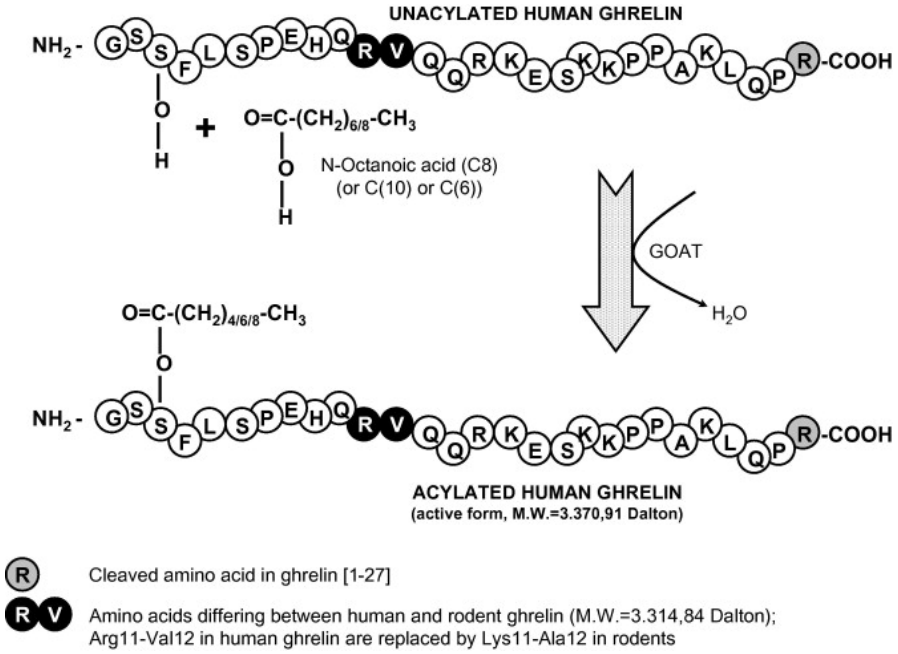

Figura 2. Estructura de la ghrelina. La ghrelina se puede encontrar en el organismo como una molécula de 28 aminoácidos (C-terminal Arg) o de 27 (C-terminal Pro). La ghrelina de rata y ratón son idénticas y difieren de la humana en dos aminoácidos (Arg11-Val12 por Lys11-Ala12). La ghrelina está acilada en la serina 3 por acción la enzima *ghrelin O-Acyltransferase* (GOAT). La ghrelina acilada de 28 aminoácidos es la forma activa predominante. Figura extraída de: Front Neuroendocrinol 2009.doi:10.1016/j.yfrne.2009.10.008

Inicialmente se pensaba que la forma desacilada de la ghrelina era inactiva porque no se une al receptor GHS-R1a, pero hay evidencias que le confieren actividad. Tanto la ghrelina como la desacilghrelina inhiben la apoptosis inducida por doxorrubicina en cardiomiocitos adultos H9c2 y en células endoteliales mediante la activación de las vías de ERK 1/2 y Akt,[16] tienen acciones adipogénicas periféricas

[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.

directas en médula ósea de tibia[17] y, además de otras acciones biológicas, la desacilghrelina puede estimular la proliferación de osteoblastos humanos a través de un mecanismo que implica las vías de señalización de MAPK y PI3k.[18] La mayoría de las evidencias experimentales indican que las acciones de la desacilghrelina son independientes de la activación del GHS-R1a.

El gen de la proghrelina humana esta localizado en el cromosoma 3 en la posición p25-26,[19] mientras que el gen para el GHS-R está en la posición q26-27 del mismo cromosoma.[20]

La estructura del gen de la ghrelina de ratón comprende cinco exones y la ghrelina madura está codificada por los exones 2 y 3, mientras que el resto de la secuencia de la proghrelina se codifica en los exones 4 y 5.[21] El mRNA de la ghrelina humana codifica para 117 aminoácidos (prepro-ghrelina), de los cuales 23 aminoácidos son la secuencia señal y 94 la pro-ghrelina (28 aminoácidos de la ghrelina madura y 66 de cola). El retículo endoplasmático elimina el péptido señal y un posterior tratamiento proteolítico del precursor de la pro-ghrelina resulta en la producción de la ghrelina madura (28 aminoácidos) y de un fragmento C-terminal de 66 aminoácidos, C-ghrelina (Figura 3).

[17] Thompson NM, Gill DA, Davies R, Loveridge N, Houston PA, Robinson IC, Wells T. Ghrelin and des-octanoyl ghrelin promote adipogenesis directly in vivo by a mechanism independent of the type 1a growth hormone secretagogue receptor.Endocrinology. 2004;145:234-42.

[18] Delhanty PJ, van der Eerden BC, van der Velde M, Gauna C, Pols HA, Jahr H, Chiba H, van der Lely AJ, van Leeuwen JP. Ghrelin and unacylated ghrelin stimulate human osteoblast growth via mitogen-activated protein kinase (MAPK)/phosphoinositide 3-kinase (PI3K) pathways in the absence of GHS-R1a. J Endocrinol. 2006;188:37-47.

[19] Kishimoto M, Okimura Y, Nakata H, Kudo T, Iguchi G, Takahashi Y, Kaji H, Chihara K. Cloning and characterization of the 5(')-flanking region of the human ghrelin gene. Biochem Biophys Res Commun. 2003;305:186-92.

[20] Smith RG, Leonard R, Bailey AR, Palyha O, Feighner S, Tan C, Mckee KK, Pong SS, Griffin P, Howard A. Growth hormone secretagogue receptor family members and ligands. Endocrine. 2001;14:9-14.

[21] Tanaka M, Hayashida Y, Iguchi T, Nakao N, Nakai N, Nakashima K. Organization of the mouse ghrelin gene and promoter: occurrence of a short noncoding first exon. Endocrinology. 2001;142:3697-700.

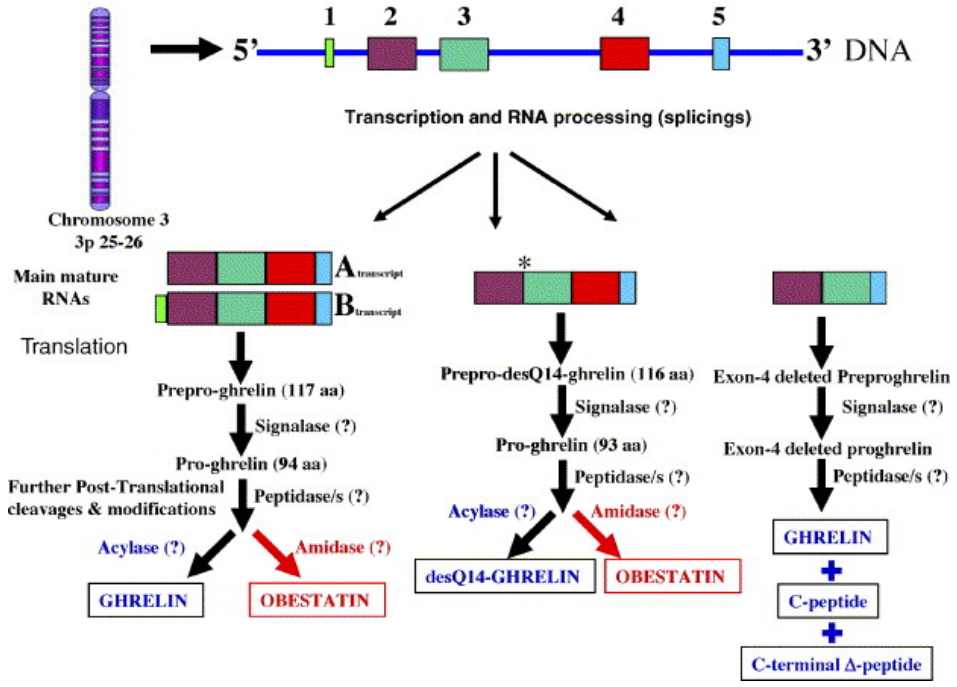

Figura 3. Estructura del gen de la ghrelina. Representación esquemática de los procesos de transcripción, traducción y modificación postraduccional en los que se generan la ghrelina, la obestatina, diferentes isoformas de ghrelina y los péptidos terminales. Figura extraída de: Mol and Cell Endocrinol 2006;256:1-8.

Recientemente, utilizando un modelo bioinformático,[22] se identificó la enzima que cataliza la unión del ácido *n*-octanoico a la serina 3 de la ghrelina (O-aciltransferasa de ghrelina, GOAT).[23,24] Se trata de una enzima que pertenece a la familia de las O-aciltransferasas unidas a membrana (MBOATs), concretamente la MBOAT4. En ratones, GOAT se localiza en el retículo endoplasmático, y su distribución está bien delimitada dentro del tracto gastrointestinal y los

[22] Hofmann K. A superfamily of membrane-bound O-acyltransferases with implications for wnt signaling.Trends Biochem Sci. 2000;25:111-2.
[23] 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.
[24] Gutierrez JA, Solenberg PJ, Perkins DR, Willency JA, Knierman MD, Jin Z, Witcher DR, Luo S, Onyia JE, Hale JE. Ghrelin octanoylation mediated by an orphan lipid transferase. Proc Natl Acad Sci USA. 2008;105:6320-5.

testículos. Sin embargo, en humanos se expresa en estómago y páncreas (Figura 4). En ratones, GOAT cataliza de manera específica la unión covalente de ácido *n*-octanoico a la serina 3 de la ghrelina, mientras que en humanos, GOAT puede también acilar a la ghrelina con otros ácidos grasos.[22,21]

El descubrimiento de la importancia de la acilación de la ghrelina para su actividad, confiere un papel más determinante a las modificaciones postraduccionales y, con el descubrimiento de GOAT, se abre la posibilidad de valorar el papel fisiológico de ghelina y des-acil ghrelina (UAG).

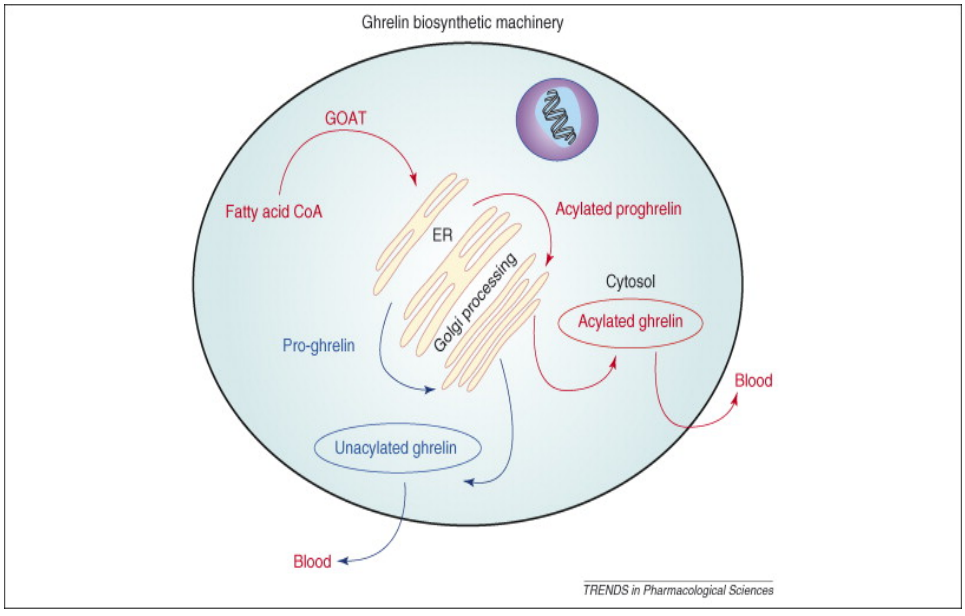

Figura 4. **Maquinaria de síntesis para la ghrelina**. GOAT añade terminaciones carboxílicas, coenzimas A, desde el citosol hacia el lumen del retículo endoplasmático, donde se acila la pro-ghrelina. Figura extraída de: Trends Pharmacol Sci 2008;29:398-401.

Con objeto de entender el sistema ghrelina-GOAT, recientemente se ha publicado un estudio en el que se realizan experimentos

valorando le expresión de GOAT en respuesta a diferentes situaciones nutricionales. Además, en este estudio también analizan la ingesta y niveles de ghrelina y desacilghrelina en modelos de ratones MBOAT4-/- y otros modelos de sobreexpresión génica, mostrándonos al sistema ghrelina-GOAT como un sensor lipídico que informa al cerebro de la disponibilidad de comida rica en grasa, conduciendo así a una optimización metabólica y al almacenamiento energético (Figura 5).[25] Esta observación sugiere un papel distinto al tradicional para la ghrelina, ya que en este modelo, la ghrelina informaría al sistema nervioso central sobre la disponibilidad de calorías y no de su ausencia, es decir, sería una señal preparatoria ante la comida más que un factor iniciador de la ingesta.

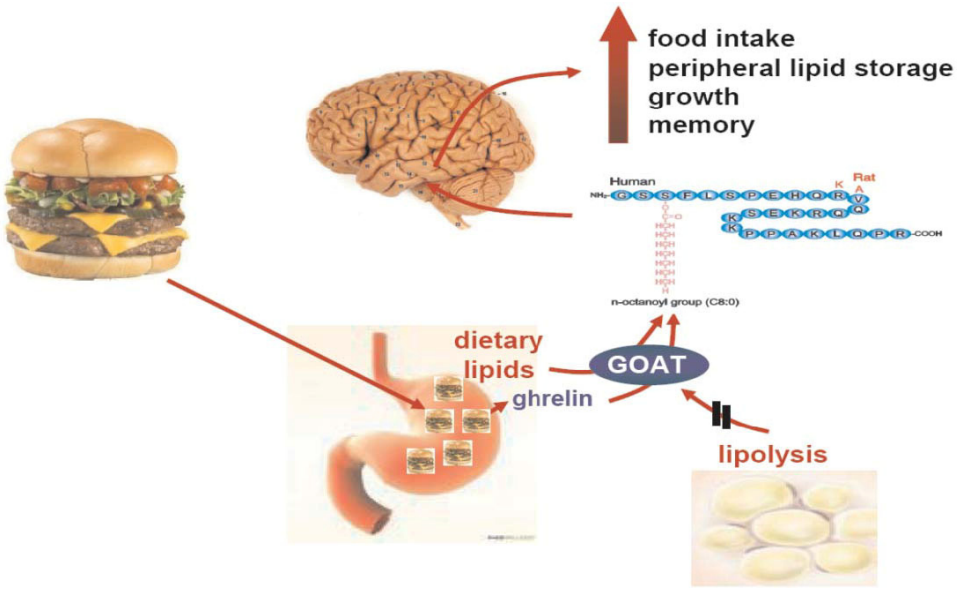

Figura 5. **Modelo del sistema ghrelina/GOAT**. Este sistema actúa como un sensor lipídico que informa al SNC sobre la disponibilidad de calorías para optimizar el almacenamiento de lípidos y permitir el crecimiento. Figura extraída de: Nat Med 2009;15:741-5.

[25]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.

En cuanto a la estructura de la ghrelina, existen algunos datos sobre las características estructurales que necesita la ghrelina para unirse a su receptor. Estas aproximaciones se realizaron mediante estudios de *binding* y activación de la movilización de calcio intracelular. De este modo, los péptidos modificados con cadenas alifáticas más largas que el ácido *n*-octanoico en la Ser3, mostraban una activación del receptor a la ghrelina. Por el contrario, cuando se reemplazaba el grupo octanoilo por un grupo acetilo (mucho más pequeño), se obtenía un agonista más pobre. Este hecho revela la importancia de la interacción hidrofóbica en el reconocimiento y activación del receptor.

Utilizando esta misma aproximación, se pudo comprobar que la secuencia mínima necesaria para la activación del receptor humano GHS-R1a lo componían los cinco primeros aminoácidos de la secuencia de la ghrelina [N-terminal Gly-Ser-Ser(n-octanoyl)-Phe-Leu-COOH]. Estos 5 residuos son suficientes para inducir movilización de calcio *in vitro*,[26] aunque la afinidad por el receptor GHS-R1a es 200 veces menor y no muestra actividad liberadora de GH *in vivo*.[27,28] Esta discrepancia podría atribuirse a que la movilización de calcio inducida por los análogos truncados de ghrelina no refleje una activación completa de todos los sistemas de señalización y, con ello, no active la secreción de GH.

Por otro lado, existen pocos datos sobre la estructura tridimensional de la ghrelina en disolución. Mediante experimentos de resonancia magnética nuclear (RMN) realizados a pH ácido, se comprobó que la ghrelina se comporta como un péptido sin estructura definida (*random*

[26] Bednarek MA, Feighner SD, Pong SS, McKee KK, Hreniuk DL, Silva MV, Warren VA, Howard AD, Van Der Ploeg LH, Heck JV. Structure-function studies on the new growth hormone-releasing peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a. J Med Chem. 2000;43:4370-6.

[27] Tolle V, Zizzari P, Tomasetto C, Rio MC, Epelbaum J, Bluet-Pajot MT. In vivo and in vitro effects of ghrelin/motilin-related peptide on growth hormone secretion in the rat. Neuroendocrinology. 2001;73:54-61.

[28] Torsello A, Ghe' C, Bresciani E, Catapano F, Ghigo E, Deghenghi R, Locatelli V, Muccioli G. Short ghrelin peptides neither displace ghrelin binding in vitro nor stimulate GH release in vivo. Endocrinology. 2002;143:1968-71.

coil).[29] Empleando otro método de aproximación, como son los estudios de dinámica molecular realizados en agua y a pH neutro, se sugiere la formación de hélice-α desde el residuo Pro7 a Glu13.[30] Basándose en modelos informáticos, se puede concluir que la ghrelina tiene una estructura definida en disolución acuosa y a pH neutro, y que además, la cadena de ácido *n*-octanoico no es un simple anclaje lipídico, sino que se une directamente al GHS-R1a, con lo que el sitio de unión consistiría en un bolsillo hidrofóbico capaz de acomodar la cadena de ácido *n*-octanoico de la ghrelina. Sin embargo, se necesitan nuevas aproximaciones experimentales para entender la estructura de la ghrelina en la unión a su receptor.

1.2.1. Funciones

1.2.1.1. Actividad liberadora de GH.

La ghrelina y otros secretagogos sintéticos tienen una potente y dosis-dependiente actividad liberadora de GH, siendo ésta más pronunciada en humanos que en animales.[31,32,33] Puesto que la actividad liberadora de GH de la ghrelina se inhibe fuertemente con antagonistas o anticuerpos de GHRH, así como con la desconexión hipotálamo-hipófisis, parece que tanto la ghrelina como otros secretagogos de GH (GHS) actúan principalmente a nivel hipotalámico

[29] Silva Elipe MV, Bednarek MA, Gao YD. 1H NMR structural analysis of human ghrelin and its six truncated analogs. Biopolymers. 2001;59:489-501.
[30] Beevers AJ, Kukol A. Conformational flexibility of the peptide hormone ghrelin in solution and lipid membrane bound: a molecular dynamics study. J Biomol Struct Dyn. 2006;23:357-64.
[31] Seoane LM, Tovar S, Baldelli R, Arvat E, Ghigo E, Casanueva FF, Dieguez C. Ghrelin elicits a marked stimulatory effect on GH secretion in freely-moving rats. Eur J Endocrinol. 2000;143:R7-9.
[32] Arvat E, Di Vito L, Broglio F, Papotti M, Muccioli G, Dieguez C, Casanueva FF, Deghenghi R, Camanni F, Ghigo E. Preliminary evidence that Ghrelin, the natural GH secretagogue (GHS)-receptor ligand, strongly stimulates GH secretion in humans. J Endocrinol Invest. 2000;23:493-5.
[33] Ghigo E, Arvat E, Giordano R, Broglio F, Gianotti L, Maccario M, Bisi G, Graziani A, Papotti M, Muccioli G, Deghenghi R, Camanni F. Biologic activities of growth hormone secretagogues in humans. Endocrine. 2001;14:87-93.

y tal vez, sobre neuronas secretoras de GHRH (Figura 6). [34,35] También existen evidencias de que tanto la ghrelina como otros GHS pueden actuar como antagonistas funcionales de la somatostatina a nivel hipotalámico e hipofisario.[36,37]

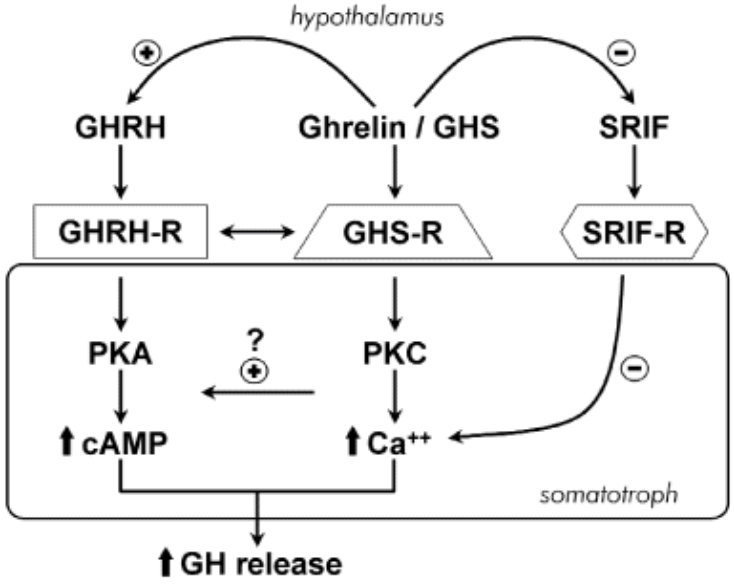

Figura 6. **Secreción de GH mediada por ghrelina**. Modelo esquemático de las posibles interacciones entre GHRH, ghrelina/GHS y somatostatina (SRIF) a nivel hipotalámico e hipofisario. Figura extraída de: Arq Bras Endocrinol Metab 2006;50:1.

[34] Pandya N, DeMott-Friberg R, Bowers CY, Barkan AL, Jaffe CA. Growth hormone (GH)-releasing peptide-6 requires endogenous hypothalamic GH-releasing hormone for maximal GH stimulation. J Clin Endocrinol Metab. 1998;83:1186-9.

[35] Popovic V, Miljic D, Micic D, Damjanovic S, Arvat E, Ghigo E, Dieguez C, Casanueva FF. Ghrelin main action on the regulation of growth hormone release is exerted at hypothalamic level. J Clin Endocrinol Metab. 2003;88:3450-3.

[36] Tannenbaum GS, Bowers CY. Interactions of growth hormone secretagogues and growth hormone-releasing hormone/somatostatin. Endocrine. 2001;14:21-7.

[37] Di Vito L, Broglio F, Benso A, Gottero C, Prodam F, Papotti M, Muccioli G, Dieguez C, Casanueva FF, Deghenghi R, Ghigo E, Arvat E. The GH-releasing effect of ghrelin, a natural GH secretagogue, is only blunted by the infusion of exogenous somatostatin in humans. Clin Endocrinol. 2002;56:643-8.

Además, la ghrelina y GHS presentan otras acciones endocrinas como la liberación de prolactina y ACTH.[38,39,40]

1.2.1.2. Orexigénica.

La administración de ghrelina produce adiposidad (actividad adipogénica) debido al incremento de la ingesta (actividad orexigénica), así como una reducción en el gasto energético.[41] Estas actividades adipogénica y orexigénica son independientes de la capacidad de liberar GH, y están mediadas por mecanismos centrales específicos.

Existen dos grupos de células que responden a la ghrelina en el ARC. Unas son orexigénicas y coexpresan NPY y AgRP. El otro grupo de neuronas, conocido a menudo como anorexigénicas, se caracterizan por la coexpresión de POMC y de CART. El receptor de ghrelina se expresa principalmente en las neuronas NPY/AgRP y, en menor número, en las neuronas POMC/CART.[42,43]

La ghrelina se une directamente a su receptor para activar las neuronas NPY/AgRP, y las dos poblaciones de neuronas del núcleo ARC interactúan. Así, la activación de las neuronas NPY/AgRP conlleva, al menos, tres efectos relevantes: 1) secreción de NPY provocando un efecto orexigénico con incremento de la ingesta y reducción del gasto energético; 2) liberación de AgRP, el cual actúa

[38] Arvat E, Maccario M, Di Vito L, Broglio F, Benso A, Gottero C, Papotti M, Muccioli G, Dieguez C, Casanueva FF, Deghenghi R, Camanni F, Ghigo E. Endocrine activities of ghrelin, a natural growth hormone secretagogue (GHS), in humans: comparison and interactions with hexarelin, a nonnatural peptidyl GHS, and GH-releasing hormone. J Clin Endocrinol Metab. 2001;86:1169-74.

[39] Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, Mori K, Komatsu Y, Usui T, Shimatsu A, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, Nakao K. Ghrelin strongly stimulates growth hormone release in humans. J Clin Endocrinol Metab. 2000;85:4908-11.

[40] Korbonits M, Goldstone AP, Gueorguiev M, Grossman AB. Ghrelin, a hormone with multiple functions. Front Neuroendocrinol. 2004;25:27-68.

[41] Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. Nature. 2000;407:908-13.

[42] Zigman JM, Elmquist JK. Minireview: From anorexia to obesity--the yin and yang of body weight control. Endocrinology. 2003;144:3749-56.

[43] Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW. Central nervous system control of food intake and body weight. Nature. 2006;443:289-95.

como antagonista competitivo y agonista inverso del MC4R inhibiendo así las vías de la melanocortina y, reforzando por tanto, el efecto orexigénico; y 3) incremento en la liberación del neurotransmisor inhibidor GABA en las inmediaciones de las neuronas POMC/CART, provocando una inhibición en la liberación del ligando endógeno de MC4R, α-MSH, y contribuyendo así a la reducción del tono melanocortinérgico (Figura 7).

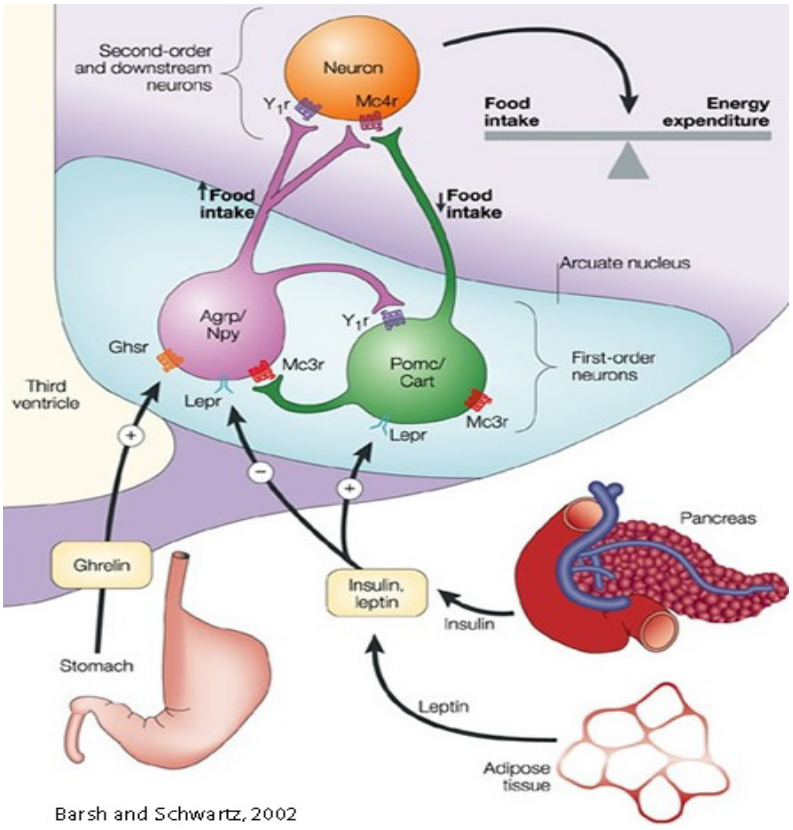

Figura 7 Regulación de la ingesta a nivel hipotalámico. Existen dos grupos de neuronas en el ARC (AgRP/NPY y POMC/CART) que están reguladas por hormonas circulantes. AgRP y NPY son neuropéptidos que estimulan la ingesta y disminuyen el gasto energético, mientras que α-MSH y CART son neuropéptidos que inhiben la ingesta e incrementan el gasto energético. La ghrelina es secreta por el estómago y activa neuronas AgRP/NPY estimulando la ingesta. Figura extraída de: Nat Rev Genet 2002;3:589-600.

1.2.2. Receptor de secretagogos de GH tipo 1a (GHS-R1a)

El receptor GHS-R humano, que puede ser considerado como el receptor de ghrelina, está codificado por un gen localizado en la posición cromosomal 3q26.2. Hay dos tipos de cDNAs del receptor GHS-R que surgen, presumiblemente, como resultado de un procesamiento alternativo del pre-mRNA y que se indentificaron y designaron como receptores 1a y 1b.[44,45] El cDNA del 1a codifica un receptor de 366 aminoácidos con siete segmentos transmembrana y una masa molecular de ~ 41 kDa, llamado GHS-R1a. Y el cDNA del 1b codifica un receptor más corto, con 289 aminoácidos y cinco segmentos transmembrana, llamado GHS-R1b[46] (Figura 8).

El GHS-R1a pertenece a la familia de receptores de neuropéptidos y hormonas peptídicas, la cual incluye al receptor de motilina, los receptores 1 y 2 de neurotensina, el GPR-39 y los receptores 1 y 2 de neuromedina.

La expresión del GHS-R1a es muy intensa en hipófisis anterior donde la ghrelina ejerce su función sobre la regulación de la liberación de GH y en el núcleo arcuato, zona hipotalámica crucial para las acciones neuroendocrinas y orexigénicas de la ghrelina.[47,48] Sin

[44] Smith RG, Van der Ploeg LH, Howard AD, Feighner SD, Cheng K, Hickey GJ, Wyvratt MJ Jr, Fisher MH, Nargund RP, Patchett AA. Peptidomimetic regulation of growth hormone secretion. Endocr Rev. 1997;18:621-45.

[45] McKee KK, Palyha OC, Feighner SD, Hreniuk DL, Tan CP, Phillips MS, Smith RG, Van der Ploeg LH, Howard AD. Molecular analysis of rat pituitary and hypothalamic growth hormone secretagogue receptors. Mol Endocrinol. 1997;11:415-23.

[46] Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou M, Liu KK, McKee KK, Pong SS, Chaung LY, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsinghji DJ, Dean DC, Melillo DG, Patchett AA, Nargund R, Griffin PR, DeMartino JA, Gupta SK, Schaeffer JM, Smith RG, Van der Ploeg LH. A receptor in pituitary and hypothalamus that functions in growth hormone release. Science. 1996;273:974-7.

[47] Shintani M, Ogawa Y, Ebihara K, Aizawa-Abe M, Miyanaga F, Takaya K, Hayashi T, Inoue G, Hosoda K, Kojima M, Kangawa K, Nakao K. Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. Diabetes. 2001;50:227-32.

[48] Guan XM, Yu H, Palyha OC, McKee KK, Feighner SD, Sirinathsinghji DJ, Smith RG, Van der Ploeg LH, Howard AD. Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. Brain Res Mol Brain Res. 1997;48:23-9.

embargo, también se observa un amplio patrón de expresión en tejidos periféricos como la tiroides, el páncreas, el bazo, el miocardio, las glándulas adrenales, los testículos, los ovarios y el estómago.[49,50,51] Esta distribución periférica sugiere que la ghrelina debe estar implicada en otras funciones, además de la regulación de la secreción de GH y la homeostasis energética.

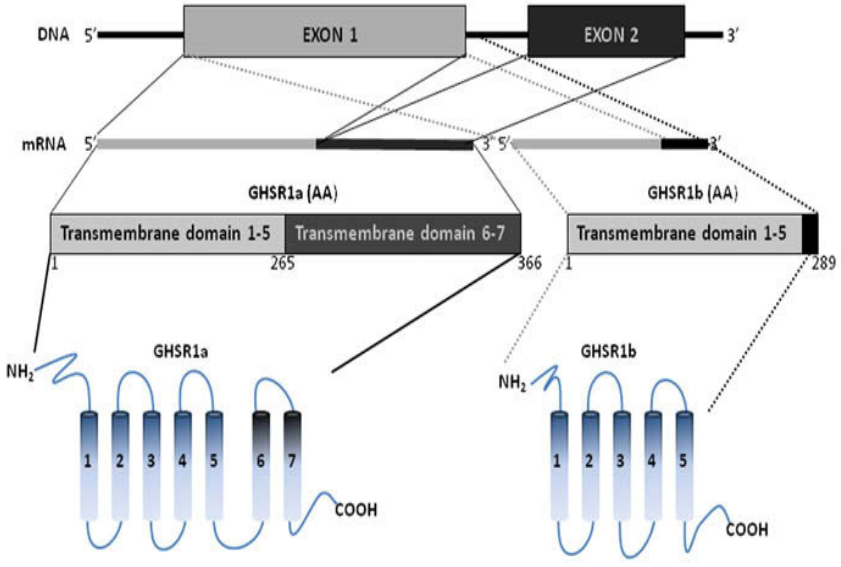

Figura 8. **Estructura de los receptores de ghrelina**. Surgen dos tipos de cDNA como resultado de un *splicing* alternativo del pre-mRNA dando lugar a dos receptores distintos que se denominaron 1a y 1b. Figura extraída de: Neuropharmacology 2010;58:2-16.

[49]Gaytan F, Barreiro ML, Chopin LK, Herington AC, Morales C, Pinilla L, Casanueva FF, Aguilar E, Diéguez C, Tena-Sempere M. Immunolocalization of ghrelin and its functional receptor, the type 1a growth hormone secretagogue receptor, in the cyclic human ovary. J Clin Endocrinol Metab. 2003;88:879-87.

[50] Gaytan F, Barreiro ML, Caminos JE, Chopin LK, Herington AC, Morales C, Pinilla L, Paniagua R, Nistal M, Casanueva FF, Aguilar E, Diéguez C, Tena-Sempere M. Expression of ghrelin and its functional receptor, the type 1a growth hormone secretagogue receptor, in normal human testis and testicular tumors. J Clin Endocrinol Metab. 2004;89:400-9.

[51] Kageyama H, Funahashi H, Hirayama M, Takenoya F, Kita T, Kato S, Sakurai J, Lee EY, Inoue S, Date Y, Nakazato M, Kangawa K, Shioda S. Morphological analysis of ghrelin and its receptor distribution in the rat pancreas. Regul Pept. 2005;126:67-71.

Un hecho destacable referente a los niveles de expresión de GHS-R1a lo supone la observación de que tanto la GH como la hormona anorexigénica leptina, inhiben estos niveles en el ARC. Por el contrario, la ghrelina aumenta los niveles de expresión del GHS-R1a. Consecuentemente, parece que la ghrelina y la leptina desempeñan papeles complementarios en un sistema regulador que informa al sistema nervioso central sobre el balance energético.[52,53,54]

En cuanto a los sistemas de señalización activados por el GHS-R1a, el mecanismo de transducción de señales más estudiado es el involucrado en la secreción de GH. Este comienza con la unión ligando-receptor, la disociación de la subunidad $G_{q/\alpha 11}$ y la consecuente estimulación de la fosfolipasa C (PLC).[55] Seguidamente, la PLC escinde el lípido de membrana fosfoinositol 4,5-difosfato [PtdIns(4,5)P_2; PIP_2] en diacilglicerol (DAG) e inositol (1,4,5)-trifosfato (IP_3). El IP_3 generado activa la liberación de calcio de los depósitos del retículo endoplasmático, mientras que el DAG activa la proteína quinasa C (PKC) en la membrana plasmática. La PKC inhibe los canales de potasio causando una despolarización que induce la apertura de los canales de calcio voltaje-dependientes (Figura 9). La elevada

[52] Bennett PA, Thomas GB, Howard AD, Feighner SD, van der Ploeg LH, Smith RG, Robinson IC. Hypothalamic growth hormone secretagogue-receptor (GHS-R) expression is regulated by growth hormone in the rat. Endocrinology. 1997;138:4552-7.

[53] Nass R, Gilrain J, Anderson S, Gaylinn B, Dalkin A, Day R, Peruggia M, Thorner MO. High plasma growth hormone (GH) levels inhibit expression of GH secretagogue receptor messenger ribonucleic acid levels in the rat pituitary. Endocrinology. 2000;141:2084-9.

[54] Nogueiras R, Tovar S, Mitchell SE, Rayner DV, Archer ZA, Dieguez C, Williams LM. Regulation of growth hormone secretagogue receptor gene expression in the arcuate nuclei of the rat by leptin and ghrelin. Diabetes. 2004;53:2552-8.

[55] Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou M, Liu KK, McKee KK, Pong SS, Chaung LY, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsinghji DJ, Dean DC, Melillo DG, Patchett AA, Nargund R, Griffin PR, DeMartino JA, Gupta SK, Schaeffer JM, Smith RG, Van der Ploeg LH. A receptor in pituitary and hypothalamus that functions in growth hormone release. Science. 1996;273:974-7.

concentración de calcio intracelular provoca la liberación exocítica de GH al medio extracelular.[56,57,58]

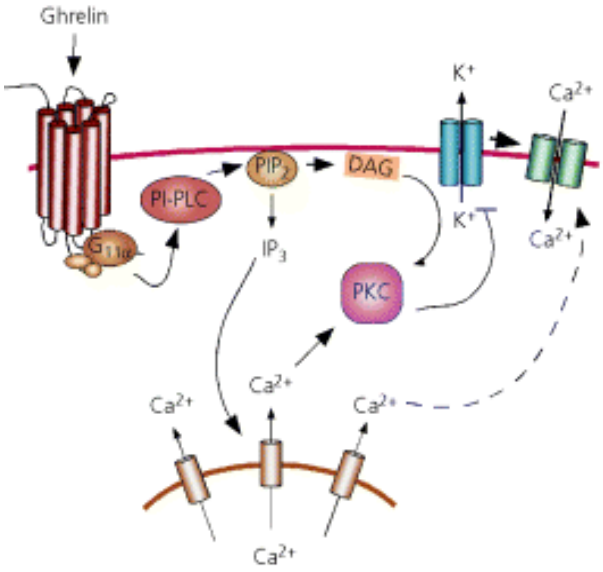

Figura 9. **Modelo de transducción de señales propuesto para la activación de la movilización de calcio inducida por ghrelina**. La ghrelina activa la PLC generando DAG e IP_3 con la consecuente liberación de Ca^{+2} del retículo y la activación de PKC. La PKC inhibe canales de K^+ lo que causa una despolarización y una apertura posterior de canales de Ca^{+2}. Figura extraída de: J Neuroendocrinol 2006;18:65-76.

No obstante, la movilización de calcio inducida por ghrelina puede deberse a mecanismos alternativos a la vía $G_{q/\alpha 11}$/PI-PLC. De este modo, los experimentos llevados a cabo en células somatotropas porcinas demostraron que la administración de la ghrelina estimulaba

[56] Chen C, Zhang J, Vincent JD, Israel JM. Sodium and calcium currents in action potentials of rat somatotrophs: their possible functions in growth hormone secretion. Life Sci. 1990;46:983-9.
[57] Balla T. Phosphoinositide-derived messengers in endocrine signaling. J Endocrinol. 2006;188:135-53.
[58] Anderson LL, Jeftinija S, Scanes CG. Growth hormone secretion: molecular and cellular mechanisms and in vivo approaches. Exp Biol Med (Maywood). 2004;229:291-302.

la vía de la adenilato ciclasa (AC)/cAMP/PKA con la consecuente entrada de calcio a través de canales tipo N.[59,60] Es importante reseñar que la vía AC/PKA podría ser específica del receptor porcino, puesto que los experimentos realizados en células embrionarias humanas HEK-293 que sobreexpresan el receptor GHS-R1a no muestran dicha activación.[61]

Además, la ghrelina ejerce una actividad proliferativa activando la cascada de las MAP quinasas (MAPK) en diversos sistemas celulares. En células adrenales de la glomerulosa de ratas y humanos, la ghrelina activa la proliferación a través de las MAPK de manera independiente de la PKA y la PKC.[62,63] En preadipocitos, el efecto mitogénico de la ghrelina está mediado por la activación de PI3k/Akt y MAPK usando una proteína G_i.[64] De especial interés es el hecho de que la ghrelina estimule la proliferación en células de hepatoma a través de la fosforilación del sustrato del receptor de insulina 1 (IRS-1)[65] (Figura 10).

[59] Glavaski-Joksimovic A, Jeftinija K, Scanes CG, Anderson LL, Jeftinija S. Stimulatory effect of ghrelin on isolated porcine somatotropes. Neuroendocrinology. 2003;77:367-79.

[60] Malagón MM, Luque RM, Ruiz-Guerrero E, Rodríguez-Pacheco F, García-Navarro S, Casanueva FF, Gracia-Navarro F, Castaño JP. Intracellular signaling mechanisms mediating ghrelin-stimulated growth hormone release in somatotropes. Endocrinology. 2003;144:5372-80.

[61] Carreira MC, Camiña JP, Smith RG, Casanueva FF. Agonist-specific coupling of growth hormone secretagogue receptor type 1a to different intracellular signaling systems. Role of adenosine. Neuroendocrinology. 2004;79:13-25.

[62] Mazzocchi G, Neri G, Rucinski M, Rebuffat P, Spinazzi R, Malendowicz LK, Nussdorfer GG. Ghrelin enhances the growth of cultured human adrenal zona glomerulosa cells by exerting MAPK-mediated proliferogenic and antiapoptotic effects. Peptides. 2004;25:1269-77.

[63] Andreis PG, Malendowicz LK, Trejter M, Neri G, Spinazzi R, Rossi GP, Nussdorfer GG. Ghrelin and growth hormone secretagogue receptor are expressed in the rat adrenal cortex: Evidence that ghrelin stimulates the growth, but not the secretory activity of adrenal cells. FEBS Lett. 2003;536:173-9.

[64] Kim MS, Yoon CY, Jang PG, Park YJ, Shin CS, Park HS, Ryu JW, Pak YK, Park JY, Lee KU, Kim SY, Lee HK, Kim YB, Park KS. The mitogenic and antiapoptotic actions of ghrelin in 3T3-L1 adipocytes. Mol Endocrinol. 2004;18:2291-301.

[65] Murata M, Okimura Y, Iida K, Matsumoto M, Sowa H, Kaji H, Kojima M, Kangawa K, Chihara K. Ghrelin modulates the downstream molecules of insulin signaling in hepatoma cells. J Biol Chem. 2002;277:5667-74.

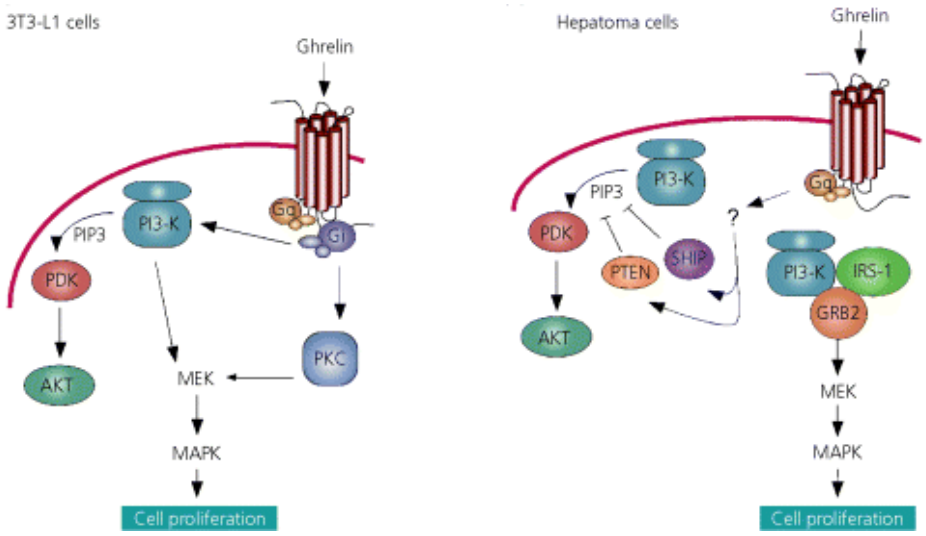

Figura 10. **Rutas usadas por ghrelina para activar MAPK**. Modelo de activación de las MAPK en células 3T3-L1 vía PI3k y PKC (izquierda). Vía de transducción de señales en células de hepatoma para activar las MAPK a través de la asociación de IRS-1, GRB2 y PI3k (derecha). Figura extraída de: J Neuroendocrinol 2006;18:65-76.

Como recientemente se ha demostrado, la ghrelina es el único factor aferente endocrino conocido que depende del metabolismo intraneuronal de ácidos grasos. En dicho estudio se pudo comprobar que el efecto orexigénico de la ghrelina implica la activación hipotalámica de AMPK y la inactivación de diversos pasos enzimáticos de la síntesis de novo de ácidos grasos en el VMN, resultando en un descenso de los niveles de malonyl-CoA que llevan a la activación de CPT1 (Figura 11). [66]

[66] López M, Lage R, Saha AK, Pérez-Tilve D, Vázquez MJ, Varela L, Sangiao-Alvarellos S, Tovar S, Raghay K, Rodríguez-Cuenca S, Deoliveira RM, Castañeda T, Datta R, Dong JZ, Culler M, Sleeman MW, Alvarez CV, Gallego R, Lelliott CJ, Carling D, Tschöp MH, Diéguez C, Vidal-Puig A. Hypothalamic fatty acid metabolism mediates the orexigenic action of ghrelin. Cell Metab. 2008;7:389-99.

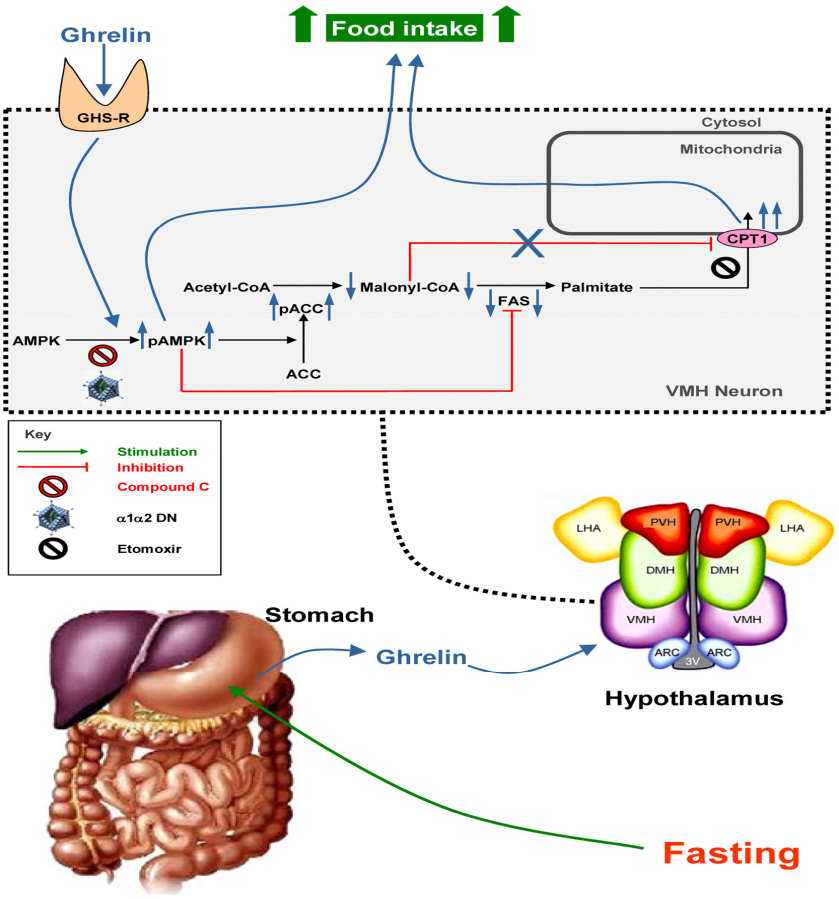

Figura 11. **Modelo propuesto para las acciones de la ghrelina sobre el metabolismo de ácidos grasos hipotalámico**. Los efectos orexigénicos de la ghrelina comprometen la activación a nivel hipotalámico de AMPK y la inactivación de los pasos enzimáticos de la vía de síntesis *de novo* de ácidos grasos. Esto resulta en una disminución de los niveles de malonyl-CoA lo que conduce a la activación de CPT1. Figura extraída de: Cell Metab 2008;7:389-399.

Una parte importante de la regulación de los GPCR es la internalización inducida por el agonista desde la membrana plasmática a los compartimentos intracelulares.[67] Los GPCRs pueden ser desensibilizados, tras su activación por agonistas, a causa de la

[67] Freedman NJ, Lefkowitz RJ. Desensitization of G protein-coupled receptors. Recent Prog Horm Res. 1996;51:319-51.

fosforilación por miembros de la familia de quinasas de receptores acoplados a proteínas G (GRKs). A estos receptores fosforilados se unen arrestinas que evitan estimulaciones sucesivas de las proteínas G y, por tanto, el desencadenamiento de nuevas vías de señalización. El complejo GPCR/arrestina recluta componentes de la maquinaria de endocitosis como son la proteína adaptadora 2 (AP-2) y la clatrina llevando a la internalización del receptor. Dependiendo de si el receptor internaliza con o sin las arrestinas, determina la cinética de reciclaje y desensibilización (Figura 12).

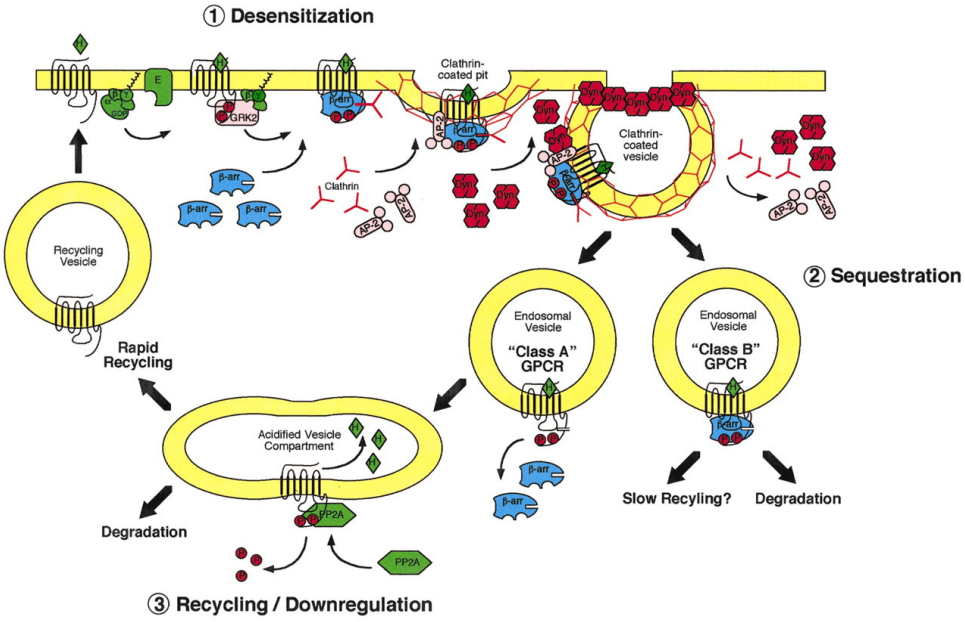

Figura 12. **Desensibilización, secuestro y tráfico intracelular de GPCRs**. Los receptores de clase A se disocian rápidamente de las β-arrestinas, el ligando se separa en las vesículas endosomales y el receptor es desfosforilado para reciclarse a la membrana plasmática. Los receptores clase B establecen complejos estables con las β-arrestinas, son degradados en las vesículas endocíticas y se reciclan lentamente a la membrana. Figura extraída de: J Cell Sci 2002;115:455-465.

En el caso concreto de la ghrelina, ésta estimula la internalización del receptor GHS-R1a lo que implica una desensibilización homóloga. Los estudios de unión de radioligando y de microscopía confocal llevados a cabo en células HEK293 que expresan el receptor de ghrelina de manera estable, demostraron que el GHS-R1a se internaliza de

manera tiempo-dependiente con un máximo de internalización a los 20 minutos tras la estimulación con ghrelina. El complejo ligando-receptor se internaliza en vesículas de clatrina vía endosomas y posteriormente el receptor GHS-R1a se clasifica en endosomas para su reciclaje a la membrana. Así, el nivel de receptores en la superficie celular alcanza de nuevo el 100% a los 360 minutos tras la estimulación con ghrelina.[68]

1.3. Obestatina

Aunque el principal producto activo del gen de la ghrelina es la propia ghrelina, descubrimientos recientes muestran que dicho gen puede generar diversas moléculas bioactivas además de ésta. Así, des-acil ghrelina y obestatina surgen de un proceso de *splicing* alternativo o como consecuencia de modificaciones postraduccionales.

En 2005, mediante análisis genómicos comparativos, se propuso la existencia de un nuevo péptido derivado del extremo C terminal de la proghrelina. Este nuevo péptido se llamó obestatina debido a su principal característica como supresor del apetito. Se trata de un péptido de 23 aminoácidos [pro-ghrelina (53-75)] con un residuo de glicina conservado en el extremo C-terminal y susceptible de ser amidado[69] (Figura 13). Originalmente se extrajo de estómagos de rata y luego se comprobó que era un péptido circulante que fluctuaba en sangre como la ghrelina.[70,71] Por tanto, el principal productor de esta

[68] Camiña JP, Carreira MC, El Messari S, Llorens-Cortes C, Smith RG, Casanueva FF. Desensitization and endocytosis mechanisms of ghrelin-activated growth hormone secretagogue receptor 1a. Endocrinology. 2004;145:930-40.
[69] 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.
[70] Harada T, Nakahara T, Yasuhara D, Kojima S, Sagiyama K, Amitani H, Laviano A, Naruo T, Inui A. Obestatin, acyl ghrelin, and des-acyl ghrelin responses to an oral glucose tolerance test in the restricting type of anorexia nervosa. Biol Psychiatry. 2008;63:245-7.
[71] Zizzari P, Longchamps R, Epelbaum J, Bluet-Pajot MT. Obestatin partially affects ghrelin stimulation of food intake and growth hormone secretion in rodents. Endocrinology. 2007;148:1648-53.

nueva hormona es el estómago y la mayoría de las células productoras de obestatina se distribuyen por la parte basal de la mucosa oxíntica. Además, se comprobó que la eliminación del estómago mediante gastrectomía, reduce los niveles circulantes de obestatina en torno a un 50-80%.[72]

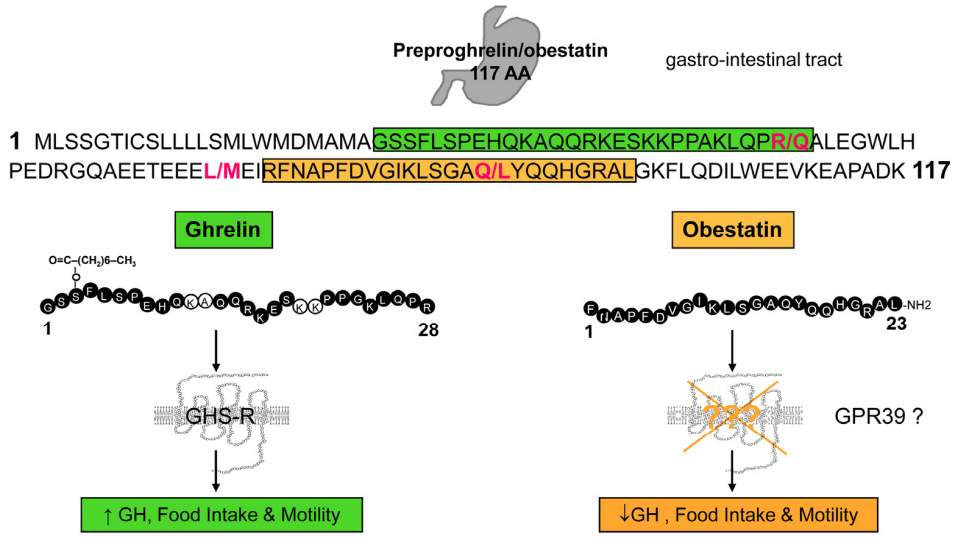

Figura 13. **Ghrelina y Obestatina**. Dos péptidos con acciones antagónicas y derivados de la misma prohormona. Figura extraída de: Mol Cell Endocrinol 2009. doi:10.1016/j.mce.2009.

La obestatina también se encuentra en el duodeno, yeyuno, colon, páncreas, intestinos, bazo, glándulas mamarias, leche materna y plasma.[73] En rata, la expresión de obestatina se encontró en estómago, plexo mientérico, páncreas y células de Leydig.[74] Además, la inmunoreactividad en el páncreas de rata se correlaciona

[72] Furnes MW, Stenstrom B, Tømmerås K, Skoglund T, Dickson SL, Kulseng B, Zhao CM, Chen D. Feeding behavior in rats subjected to gastrectomy or gastric bypass surgery. Eur Surg Res. 2008;40:279-88.
[73] 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.
[74] Dun SL, Brailoiu GC, Brailoiu E, Yang J, Chang JK, Dun NJ. Distribution and biological activity of obestatin in the rat. J Endocrinol. 2006;191:481-9.

positivamente con la secreción de insulina, sugiriendo que la obestatina pancreática contribuye a la función de las células β.[75]

1.3.1. Receptor de la obestatina

Inicialmente, la obestatina se describió como el ligando endógeno del receptor huérfano acoplado a proteínas G 39 (GPR-39), un receptor que comparte homología con el GHS-R1a (Figura 14). Sin embargo, existen una serie de estudios recientes que indican que la obestatina no es el ligando endógeno del GPR39 puesto que no se encuentra *binding* específico en células que lo expresan ni activación de vías de transducción de señales.[76,77,78] Otros autores muestran al Zn^{+2} como un eficaz activador de rutas de señalización de este receptor, lo que podría indicar la validez de este ion metal como agonista o modulador fisiológico del GPR39.[79]

Los propios autores del descubrimiento de la obestatina, al no poder reproducir sus ensayos originales de *binding* y activación del GPR39, ofrecieron una posible explicación. En este sentido, la ausencia de *binding* observada podría deberse a la pérdida de la bioactividad de la obestatina tras su yodación, ya que se incorporan cuatro o más moléculas de yodo. Así, usando obestatina mono-yodada, se observó que ésta es capaz de unirse a células HEK293T transfectadas con

[75] Chanoine JP, Wong AC, Barrios V. Obestatin, acylated and total ghrelin concentrations in the perinatal rat pancreas. Horm Res. 2006;66:81-8.
[76] 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.
[77] Lauwers E, Landuyt B, Arckens L, Schoofs L, Luyten W. Obestatin does not activate orphan G protein-coupled receptor GPR39. Biochem Biophys Res Commun. 2006;351:21-5
[78] Tremblay F, Perreault M, Klaman LD, Tobin JF, Smith E, Gimeno RE. Normal food intake and body weight in mice lacking the G protein-coupled receptor GPR39. Endocrinology. 2007;148:501-6.
[79] Holst B, Egerod KL, Schild E, Vickers SP, Cheetham S, Gerlach LO, Storjohann L, Stidsen CE, Jones R, Beck-Sickinger AG, Schwartz TW. GPR39 signaling is stimulated by zinc ions but not by obestatin. Endocrinology. 2007;148:13-20.

plásmidos que codifican el GPR39 humano o de ratón.[80] Estos autores también comprobaron que el tratamiento de células de estómago con obestatina induce la expresión de c-fos en ratones WT, pero no en ratones *knockout* para el GPR39. Además, el análisis inmunohistoquímico, usando anticuerpos contra el tercer *loop* extracelular del GPR39, indica la colocalización del GPR39 con c-fos en diferentes células diana de obestatina. Todos estos datos apuntan, claramente, en la dirección de que el GPR39 es el receptor al que se une la obestatina para ejercer sus funciones.

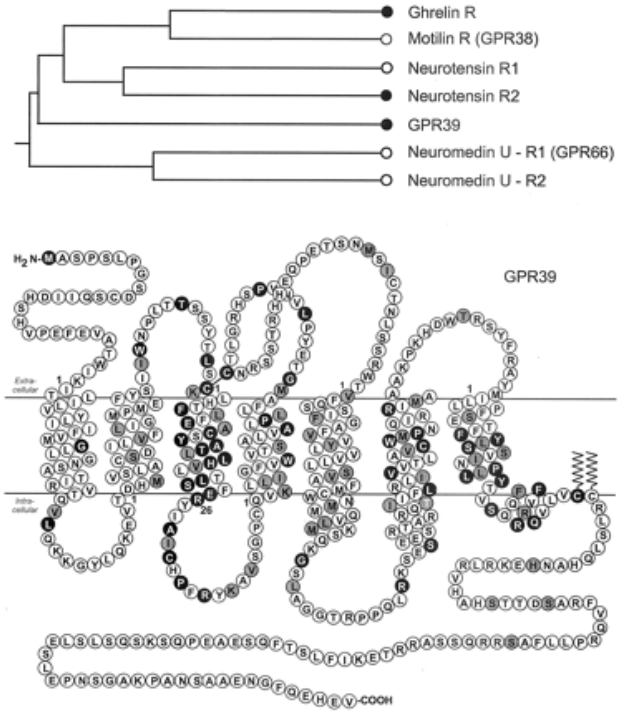

Figura 14. **GPR39**. Representación esquemática del árbol filogenético de la familia de receptoresde ghrelina. Modelo del receptor GPR39. Figura extraída de: J Biol Chem 2004;279:53806-17.

[80] 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.

Se ha detectado la expresión de RNAm de GPR39 en varios tejidos de rata y humanos como son el estómago, el duodeno, el yeyuno, el íleon y el tejido adiposo o de riñón entre otros,[81,82] pero existen discrepancias en lo que a expresión hipotalámica e hipofisaria se refiere. Así, mientras algunos estudios no detectan la expresión del GPR39 en estas áreas,[83,84] otros sí muestran su expresión.[69,81] Cabe destacar que los niveles de expresión de RNAm de GPR39 en tejido adiposo están reducidos en pacientes obesos con diabetes tipo 2.[85]

1.3.2. Funciones

Inicialmente, la obestatina se describió como un péptido con la capacidad de inhibir la ingesta en ratones cuando se administraba intracerebroventricular (*icv*) o periféricamente (Figura 15).

A día de hoy, existe una gran controversia sobre los efectos de la obestatina sobre la ingesta y así, algunos estudios sugieren que puede inhibir la ingesta y disminuir el peso corporal tanto en condiciones basales como de estímulo de ghrelina,[69,71,86,87] y otros sugieren que no existe efecto alguno.[84,,88,89]

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[81] McKee KK, Tan CP, Palyha OC, Liu J, Feighner SD, Hreniuk DL, Smith RG, Howard AD, Van der Ploeg LH. Cloning and characterization of two human G protein-coupled receptor genes (GPR38 and GPR39) related to the growth hormone secretagogue and neurotensin receptors. Genomics. 1997;46:426-34.

[82] Egerod KL, Holst B, Petersen PS, Hansen JB, Mulder J, Hökfelt T, Schwartz TW. GPR39 splice variants versus antisense gene LYPD1: expression and regulation in gastrointestinal tract, endocrine pancreas, liver, and white adipose tissue. Mol Endocrinol. 2007;21:1685-98.

[83] Jackson VR, Nothacker HP, Civelli OGPR39 receptor expression in the mouse brain. Neuroreport. 2006;17:813-6.

[84] 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.

[85] Catalán V, Gómez-Ambrosi J, Rotellar F, Silva C, Gil MJ, Rodríguez A, Cienfuegos JA, Salvador J, Frühbeck G. The obestatin receptor (GPR39) is expressed in human adipose tissue and is down-regulated in obesity-associated type 2 diabetes mellitus. Clin Endocrinol (Oxf). 2007;66:598-601.

[86] Green BD, Irwin N, Flatt PR. Direct and indirect effects of obestatin peptides on food intake and the regulation of glucose homeostasis and insulin secretion in mice. Peptides. 2007;28:981-7.

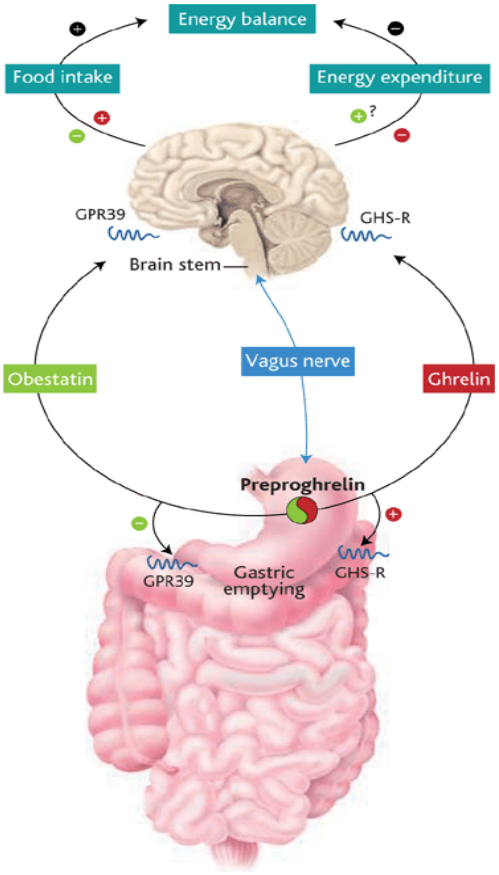

Figura 15. **Efectos antagónicos de ghrelina y obestatina**. Ambas hormonas derivan del mismo precursor y son secretadas mayoritariamente por el estómago. Actúan sobre receptores distintos y tienen efectos opuestos sobre ingesta, peso corporal y motilidad gástrica. Figura extraída de: Science 2005;310:985-986.

[87] Lagaud GJ, Young A, Acena A, Morton MF, Barrett TD, Shankley NP. Obestatin reduces food intake and suppresses body weight gain in rodents. Biochem Biophys Res Commun. 2007;357:264-9.

[88] 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:RC13-5.

[89] Kobelt P, Wisser AS, Stengel A, Goebel M, Bannert N, Gourcerol G, Inhoff T, Noetzel S, Wiedenmann B, Klapp BF, Taché Y, Mönnikes H. Peripheral obestatin has no effect on feeding behavior and brain Fos expression in rodents. Peptides. 2008;29:1018-27.

Parte de esta controversia podría ser explicada por un estudio en el que se comprobó que la obestatina intraperitoneal (*ip*) inhibe la ingesta y la ganancia de peso corporal en ratones de manera dosis-dependiente con una gráfica en forma de U.[87] Por lo tanto, la explicación a esta diversidad de resultados podría residir en la forma de dosificación de la obestatina a la hora de realizar los experimentos. También se sabe que la administración *icv* de obestatina inhibe la ingesta de agua, tanto en ratas con libre acceso a la comida y al agua como en ratas con restricciones.[90] Podría ser que la obestatina tuviese un efecto biológico, pero que su acción sobre la ingesta fuese secundaria al fenómeno de supresión de la sed o deshidratación. Por otro lado, la obestatina tiene un tiempo de vida media muy corto en sangre y no atraviesa significativamente la barrera hematoencefálica, por lo que parece que las acciones de la obestatina estarían restringidas a un ámbito local.[91,92]

Atendiendo a la capacidad de la obestatina como modulador de la motilidad gástrica, nos encontramos con las mismas discrepancias. Está descrito que la ghrelina acelera el tránsito intestinal a través de mecanismos dependientes de los receptores de los neuropéptidos hipotalámicos Y_1 y el factor liberador de corticotropina 1 (CRF_1)[93] Curiosamente, en ratas con libre acceso a la comida, la obestatina inhibe la motilidad gastroduodenal, pero no en ratas en condiciones de ayuno. En el cerebro, se activan neuronas que contienen CRF y urocortina por la inyección de obestatina, y a este nivel, la obestatina puede ejercer su función inhibidora de la motilidad gastroduodenal involucrando a los receptores 1 y 2 de CRF. La aferencia vagal puede

[90] Samson WK, White MM, Price C, Ferguson AV. Obestatin acts in brain to inhibit thirst. Am J Physiol Regul Integr Comp Physiol. 2007;292:R637-43.
[91] Pan W, Tu H, Kastin AJ. Differential BBB interactions of three ingestive peptides: obestatin, ghrelin, and adiponectin. Peptides. 2006;27:911-6.
[92] Vergote V, Van Dorpe S, Peremans K, et al. In vitro metabolic stability of obestatin: kinetics and identification of cleavage products. Peptides. 2008;29:1740-1748.
[93] Tebbe JJ, Mronga S, Tebbe CG, Ortmann E, Arnold R, Schäfer MK. Ghrelin-induced stimulation of colonic propulsion is dependent on hypothalamic neuropeptide Y1- and corticotrophin-releasing factor 1 receptor activation. J Neuroendocrinol. 2005;17:570-6.

estar participando, sólo en parte, en este proceso.[94] Por el contrario, otros estudios encontraron que la obestatina ni afecta al vaciado gástrico ni inhibe las propiedades movilizadoras de la ghrelina en ratones y ratas.[95]

Los datos existentes sobre el efecto de la obestatina en la secreción de insulina también son controvertidos. Hay estudios que describen tanto la estimulación,[96] como la inhibición[97] o la ausencia de efecto.[86] En este sentido, recientemente se comprobó que la ausencia del GPR39 daña la secreción de insulina *in vivo*. El mecanismo por el cual se ejerce dicha acción está sin determinar, pero surge la posibilidad de que los activadores del GPR39 podrían ser utilizados en el tratamiento de la diabetes tipo 2.[98]

En relación a la capacidad proliferativa de la obestatina, se demostró que es capaz de influir en procesos proliferativos y apoptóticos. En este sentido, la obestatina induce la proliferación de células de cultivos primarios del epitelio pigmentario retiniano humano mediante la fosforilación de MEK/ERK1/2[99] y, también promueve la supervivencia de células de islotes pancreáticos humanos a través del incremento de cAMP y activación de la vía AC/cAMP/PKA.[96] Sin embargo, la obestatina no es capaz de modificar el ciclo celular o la viabilidad de cardiomiocitos de ratón HL-1 ni de

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[94] Fujimiya M, Asakawa A, Ataka K, Kato I, Inui A. Different effects of ghrelin, des-acyl ghrelin and obestatin on gastroduodenal motility in conscious rats. World J Gastroenterol. 2008;14:6318-26.

[95] De Smet B, Thijs T, Peeters TL, Depoortere I. Effect of peripheral obestatin on gastric emptying and intestinal contractility in rodents. Neurogastroenterol Motil. 2007;19:211-7.

[96] Granata R, Settanni F, Gallo D, Trovato L, Biancone L, Cantaluppi V, Nano R, Annunziata M, Campiglia P, Arnoletti E, Ghè C, Volante M, Papotti M, Muccioli G, Ghigo E. Obestatin promotes survival of pancreatic beta-cells and human islets and induces expression of genes involved in the regulation of beta-cell mass and function. Diabetes. 2008;57:967-79.

[97] Qader SS, Håkanson R, Rehfeld JF, Lundquist I, Salehi A. Proghrelin-derived peptides influence the secretion of insulin, glucagon, pancreatic polypeptide and somatostatin: a study on isolated islets from mouse and rat pancreas. Regul Pept. 2008;146:230-7.

[98] Tremblay F, Richard AM, Will S, Syed J, Stedman N, Perreault M, Gimeno RE. Disruption of G protein-coupled receptor 39 impairs insulin secretion in vivo. Endocrinology. 2009;150:2586-95.

[99] Camiña JP, Campos JF, Caminos JE, Dieguez C, Casanueva FF. Obestatin-mediated proliferation of human retinal pigment epithelial cells: regulatory mechanisms. J Cell Physiol. 2007;211:1-9.

prevenir la apoptosis inducida por citarabina.[100] También cabe destacar que la obestatina puede inhibir la proliferación y diferenciación de preadipocitos 3T3-L1, efecto en este caso, contrario al observado para la ghrelina.[101]

En lo que se refiere a la secreción de GH, la mayoría de los estudios muestran que ni la administración intracerebroventricular ni intravenosa afectan la secreción de GH en ratas.[102,103] Sin embargo, bajo ciertas condiciones, la obestatina puede inhibir la acción de la ghrelina exógena sobre la liberación de GH.[71]

En un estudio reciente, realizado en corazón aislado de rana, se observa como la obestatina, al contrario que la ghrelina, aumenta la fuerza de contracción del corazón. Dicho efecto inotrópico positivo resulta de la liberación de epinefrina por medio de un mecanismo dependiente de G_i y de la activación de PKA.[104]

Se ha demostrado que todas las células de los tejidos por los que se distribuyen la ghrelina y la obestatina muestran inmunoreactividad para ambos péptidos, además, la localización subcelular de ambos es esencialmente idéntica, indicando que la obestatina y la ghrelina se almacenan en las mismas vesículas secretoras. Existe una gran correlación entre niveles de RNAm de ghrelina y obestatina en tejidos normales, apoyando la hipótesis de que ambas hormonas surgen como consecuencia de modificaciones postraduccionales más que por procesos de *splicing* alternativo. Cuando se analizan tumores

[100] Iglesias MJ, Salgado A, Piñeiro R, Rodiño BK, Otero MF, Grigorian L, Gallego R, Diéguez C, Gualillo O, González-Juanatey JR, Lago F. Lack of effect of the ghrelin gene-derived peptide obestatin on cardiomyocyte viability and metabolism. J Endocrinol Invest. 2007;30:470-6.

[101] Zhang Z, Zou DJ, Chen Y, Wang M, Wu J, Guo ZF. Obestatin inhibits proliferation and differentiation of 3T3-L1 preadipocytes. Acad J Second Mil Med Univ. 2007;28:929-32.

[102] Bresciani E, Rapetti D, Donà F, Bulgarelli I, Tamiazzo L, Locatelli V, Torsello A. Obestatin inhibits feeding but does not modulate GH and corticosterone secretion in the rat. J Endocrinol Invest. 2006;29:RC16-8.

[103] Yamamoto D, Ikeshita N, Daito R, Herningtyas EH, Toda K, Takahashi K, Iida K, Takahashi Y, Kaji H, Chihara K, Okimura Y. Neither intravenous nor intracerebroventricular administration of obestatin affects the secretion of GH, PRL, TSH and ACTH in rats. Regul Pept. 2007;138:141-4.

[104] Lliyana V Sazdova, Bilyana M Llieva, Ignat B Minkov, Rudolf Schubert, Hristo S Gagov. Obestatin as contractile mediator of excised frog heart. Cent Eur J Biol. 2009;4:327-334.

endocrinos, y aunque los niveles de expresión de RNAm de ambas hormonas se correlacionan, en la mayoría de los casos la expresión de obestatina se restringe a células o grupos de células aisladas que representan una pequeña fracción del tumor productor de ghrelina. Así, en condiciones neoplásicas, los eventos postraduccionales parecen ser los responsables de la diferente producción de ghrelina y obestatina.[105,106]

[105]Volante M, Rosas R, Ceppi P, Rapa I, Cassoni P, Wiedenmann B, Settanni F, Granata R, Papotti M. Obestatin in human neuroendocrine tissues and tumours: expression and effect on tumour growth. J Pathol. 2009;218:458-66.

[106] Tsolakis AV, Grimelius L, Stridsberg M, Falkmer SE, Waldum HL, Saras J, Janson ET. Obestatin/ghrelin cells in normal mucosa and endocrine tumours of the stomach. Eur J Endocrinol. 2009;160:941-9.

2. Objetivos

El presente trabajo de Tesis Doctoral se marcó los siguientes objetivos:

1. Estudio de los mecanismos intracelulares que modulan la secreción celular de ghrelina. Papel de factores atípicos en la regulación de esta secreción.

2. Estudio de los mecanismos intracelulares que regulan las acciones biológicas de la obestatina.

3. Evaluación del papel de la obestatina sobre la secreción de GH.

4. Estudio de la obestatina como regulador de la proliferación celular.

5. Determinación de la conformación bioactiva de la ghrelina cuando interactúa con su receptor (GHS-R1a) mediante resonancia magnética nuclear (RMN) con células vivas.

3. Trabajos publicados

the FEBS Journal

Lysophosphatidic acid inhibits ghrelin secretion in the human gastric adenocarcinoma AGS cell line – role of mitogenic activated protein kinase signaling pathway

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3 Department of Medicine, Santiago de Compostela University, Spain

Keywords
c-Src; epidermal growth factor receptor; ERK1/2; ghrelin; lysophosphatidic acid

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Ghrelin, the endogenous ligand for the growth hormone secretagogue receptor type 1a (GHS-R1a), is a 28 amino acid residue with a post-translational octanoyl modification on Ser3. Despite the biomedical interest in this hormone, the fine details of its regulation and the mechanisms controlling its secretion are largely unknown. The present study analyzes the molecular steps involved in the full lysophosphatidic acid (LPA) receptor-mediated activation of the mitogenic extracellular signal-regulated kinase (ERK) pathway and its consequent role as an inhibitor of ghrelin secretion in the gastric adenocarcinoma cell line AGS. ERK1/2 phosphorylation mediated by LPA proceeds via activation of the type 2 LPA receptor, activation of the nonreceptor tyrosine kinase c-Src, and subsequent transactivation of the epidermal growth factor receptor. Furthermore, LPA-induced ERK activation was found to be independent of matrix metalloproteinases; thus, c-Src acted as the scaffold-transactivating epidermal growth factor receptor. Finally, a correlation was observed between the mitogenic effects of LPA and ghrelin secretion in the human gastric adenocarcinoma cell line AGS. These data suggest a possible physiological role of LPA in ghrelin secretion. The relationship found between LPA and ghrelin secretion might explain the low circulating levels of ghrelin observed in obese patients, as a bona fide reflex of the energetic stores.

Ghrelin, the endogenous ligand for the growth hormone secretagogue receptor type 1a [1], is a 28 amino acid residue with a post-translational octanoyl modification on Ser3 that was first discovered in rat and human stomach tissues [2]. This hormone is mainly produced in the stomach by the enteroendocrine X/A-like cells, but substantially lower amounts of ghrelin were detected in bowel, pancreas, kidney, the immune system, placenta, testes, pituitary, lung and hypothalamus [3–7]. Functionally, ghrelin stimulates growth hormone secretion from pituitary somatotropes [2,8] and increases food intake and body weight [9]. It has been proposed that ghrelin acts directly on the hypothalamic regulatory nuclei that control energy homeostasis, acting as an orexigenic peptide [10]. The ghrelin-induced increase in body weight and adipose

Abbreviations
ATX, type II ectonucleotide pyrophosphatase phosphodiesterase (autotaxin); BAPTA-AM, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetra(acetoxymethyl) ester; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated kinases 1/2; GPCR, G-protein-coupled receptor; h-HPRT, human hypoxanthine-phosphoribosyl-transferase; LPA, lysophosphatidic acid; LPA1, type 1 lysophosphatidic acid receptor; LPA2, type 2 lysophosphatidic acid receptor; LPA3, type 3 lysophosphatidic acid receptor; MAPK, mitogen-activated protein kinase; MDC, monodansylcadaverine; MEK, mitogenic extracellular kinase; MMP, matrix metalloproteinase; PI3k, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PMA, 12-*o*-tetradecanoylphorbol 13-acetate-4β,9α,12β,13α,20-pentahydroxytiglia-1,6-dien-3-one 12-tetradecanoate 13-acetate; PTX, pertussis toxin.

tissue may be induced indirectly through stimulation of the appetite and directly through a change in the respiratory quotient that enhances the preferential consumption of carbohydrates over fat [11]. It has also been reported that basal concentrations of ghrelin are reduced in obese subjects and that diet-induced weight loss raises them [12,13]. Published data indicate that ghrelin antagonizes leptin action in rats through the activation of the hypothalamic neuropeptide Y-Y1 receptor pathway [14]. The fact that leptin and ghrelin display mirroring changes in relation to body mass index suggests that long-term weight regulation may involve their interplay. Although this appears to be an appropriate physiological adaptation, ghrelin levels are not suppressed in obese subjects postprandially and, hence, ghrelin itself may be involved in the perpetuation of obesity [15]. On the basis of the currently available data, ghrelin seems to be part of a molecular regulatory interface between energy homeostasis, glucose metabolism and physiological processes regulated by the classic endocrine axes, such as growth and reproduction [6,16].

It has become evident that adipose tissue is a secretory organ of considerable complexity that is highly integrated into the overall physiological and metabolic control systems of mammals. There is a wide range of protein signals and factors secreted by white adipocytes, now generally known as adipokines. These include peptidergic factors (tumor necrosis factor-α, transforming growth factor-β, adipsin, angiotensinogen, leptin), lipidic factors (fatty acids, prostaglandins) [17], and glycerophospholipids such as lysophosphatidic acid (LPA) [18]. LPA, in particular, regulates the development and function of numerous organ systems, including the cardiovascular [19], nervous [20], immune [21] and reproductive systems [22]. Altered lysophospholipid signaling has been implicated in the etiology of disorders such as inflammation, autoimmune diseases, neuropathic pain, atherosclerosis, cancer, and obesity [18].

Despite the biomedical interest raised by this hormone, the fine details of its regulation and the mechanisms controlling its secretion are largely unknown. It is necessary to gain further insight into the cell biology steps leading to enhanced or reduced ghrelin release to plasma. To date, the possible interaction between adipose tissue and the stomach in the regulation of ghrelin has not been addressed.

The aim of this study was to analyze the regulation of ghrelin secretion and the role played by LPA. The specific targets were to evaluate: (a) the role of LPA in the reduction of ghrelin secretion; (b) the intracellular signaling that mediates such regulation, in particular activation; and (c) the effect of LPA and epidermal growth factor (EGF) on such actions.

Results

The expression of prepro-ghrelin and *in vitro* ghrelin secretion by the AGS cell line were studied in order to characterize the cell model. Prepro-ghrelin (13 kDa) was detected using anti-prepro-ghrelin (86–117) (human)-purified rabbit IgG (Fig. 1A). RT-PCR analysis using specific primer pairs demonstrated the expression of the genes encoding ghrelin (327 bp) in the AGS cell line (Fig. 1B, lanes 3 and 4). No products were obtained after omitting reverse transcription in the reaction (Fig. 1B, lane 2). The RIA-measured time-course concentration of ghrelin ($pg{\cdot}mL^{-1}$, 1×10^6 cells) following 24 h of starvation in cell culture medium showed a time-dependent pattern that flattened at 6–8 h (Fig. 1C). Culture medium after 24 h of starvation was subjected to peptide extraction and subsequent RP-HPLC analysis, followed by RIA. Two immunoreative peaks, detected by RIA, eluted at identical retention times to octanoylated and desoctanoylated ghrelin (data not shown), in 55% and 45% of total ghrelin, respectively.

As can be seen in Fig. 2A, LPA ($1\ \mu g{\cdot}mL^{-1}$) induced a $[Ca^{2+}]_i$ increase, which was completely abolished after treatment with 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA-AM) (30 min, 30 μM). The expression of mRNA for the different LPA receptor subtypes as determined by RT-PCR with specific primers for type 1 LPA receptor (LPA1) (432 bp), type 2 LPA receptor (LPA2) (352 bp) and type 3 LPA receptor (LPA3) (481 bp) (Fig. 2B) showed that the AGS cell line expresses mRNA for LPA2 but not for LPA1 or LPA3. No products were obtained after omitting reverse transcription in the reaction (data not shown). The above results demonstrate that AGS cells are endowed with functional LPA receptors.

The AGS cells were incubated without fetal bovine serum with increasing concentrations of LPA for 3 min. After medium renewal, the secretion was collected at specified times to measure ghrelin secretion. The time-course of the treatment presented a parabolic shape with the maximal inhibitory effect at 5 h after LPA treatment (Fig. 3A). The effect was transitory, as cells recovered basal levels at 9 h post-treatment. The maximal inhibitory effect of LPA on ghrelin secretion was observed at $1\ \mu g{\cdot}mL^{-1}$ LPA and reached 60% reduction ($P < 0.05$). No further reduction was observed with higher doses (Fig. 3B).

Treatment of the AGS cells with the potent protein kinase A (PKA) inhibitor H89 (30 min, 10 μM) did not

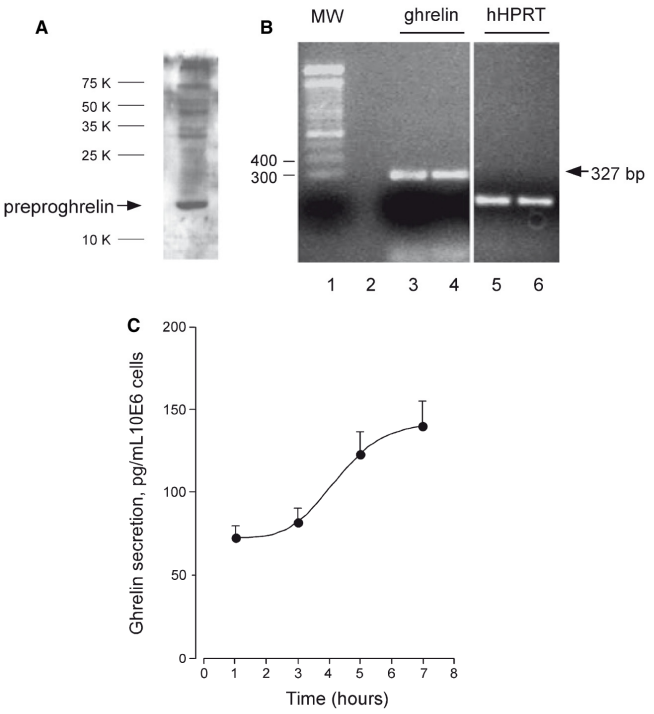

Fig. 1. Identification of ghrelin in the AGS cell line. (A) Detection of prepro-ghrelin by western immunoblot in AGS cells with antibody to human prepro-ghrelin. The band observed between the 10 and 15 kDa size markers corresponds to the prepro-ghrelin (13 kDa). (B) Detection of mRNA for ghrelin by RT-PCR in AGS cells. The amplified product for ghrelin was 327 bp (lanes 3 and 4). The omission of cDNA in the PCR mixture was used as negative control (lane 2). h-HPRT was used as an internal control (amplified product 285 bp, lanes 5 and 6; MW, molecular weight markers, lane 1). (C) Mean ± SE of ghrelin secretion to the incubation medium from nonstimulated AGS cells. Ghrelin secretion was detected in the culture medium using a human ghrelin RIA. The values are presented as the cumulative ghrelin release by 1×10^6 cells for the indicated times.

affect the LPA-mediated inhibition of ghrelin secretion (Fig. 4A). Similarly, no effect on secretion was observed when AGS cells were pretreated with the selective protein kinase C (PKC) inhibitor staurosporine (Fig. 4B). Furthermore, PKC expression was downregulated by pretreating cells with 1 μM 12-*o*-tetradecanoylphorbol 13-acetate-4β,9α,12β,13α,20-pentahydroxytiglia-1,6-dien-3-one 12-tetradecanoate 13-acetate (PMA) for 24 h, and under these conditions, the same inhibition caused by LPA treatment (1 $\mu g \cdot mL^{-1}$) was observed (Fig. 4B). The inhibitory action of LPA (1 $\mu g \cdot mL^{-1}$) was fully reversed by pretreatment with wortmannin (2 μM), indicating the possible participation of phosphatidylinositol 3-kinase (PI3k) (Fig. 4C). Finally, when cells were pretreated with the specific mitogenic extracellular kinase (MEK) inhibitor PD098059, ghrelin secretion was not significantly inhibited by LPA (Fig. 4C). These results suggest a significant involvement of mitogen-activated protein kinases (MAPKs) in the inhibitory action of LPA.

In order to understand the intracellular signaling mechanisms involved in LPA inhibition, serodeprived AGS cells were treated with 1 $\mu g \cdot mL^{-1}$ LPA. This revealed time-dependent phosphorylation of the extracellular regulated kinases 1/2 (ERK1/2) (Fig. 5A). The LPA-induced ERK1/2 activation was detectable as early as 1 min after LPA administration, being most prominent at 10 min (Fig. 5A). There was no change in protein amounts of MAPK as assessed by immunoblots using p44/p42 MAPK antibodies. LPA effects were completely blocked by pretreatment with the specific MEK inhibitor PD098059 (50 μM, 30 min). This agent also reduced basal ERK1/2 levels in control cells (Fig. 5B). To assess the results obtained in ghrelin secretion analysis for PI3k and PKC, the cells were pretreated with wortmannin (2 μM, 30 min) and staurosporine (1 μM, 5 min), inhibitors of PI3k and PKC, respectively. Despite the fact that both inhibitors raised basal levels of phospho-ERK1/2, neither diminished LPA-induced activation of ERK1/2 (Fig. 5B).

The activation of ERK1/2 phosphorylation occurred in a pertussis toxin (PTX)-insensitive manner (100 $ng \cdot mL^{-1}$, 4 h), which ruled out G_i-protein involvement in the LPA-induced activation of MAPK isoforms (Fig. 5C).

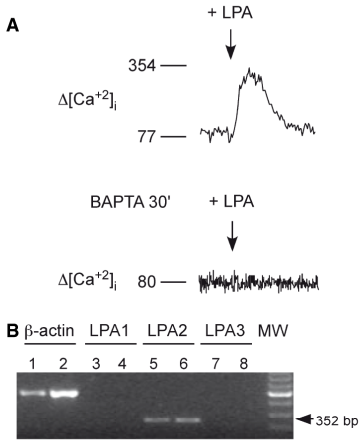

Fig. 2. (A) Calcium mobilization ($[Ca^{2+}]_i$) in the AGS cell line. Effect of LPA (1 μg·mL^{-1}) on intracellular calcium mobilization in AGS cells. Results (mean ± SE, $n = 3$) are expressed as variation of intracellular calcium concentration. This effect is completely abolished after treatment with BAPTA-AM (30 min, 30 μM). (B) Expression of LPA receptor mRNA in AGS cells determined by RT-PCR. β-Actin was used as an internal control (amplified product bp, lanes 1 and 2). Lanes 3 and 4: LPA1 (432 bp). Lanes 5 and 6: LPA2 (352 bp). Lanes 7 and 8: LPA3 (481 bp). This figure is representative of four independent experiments.

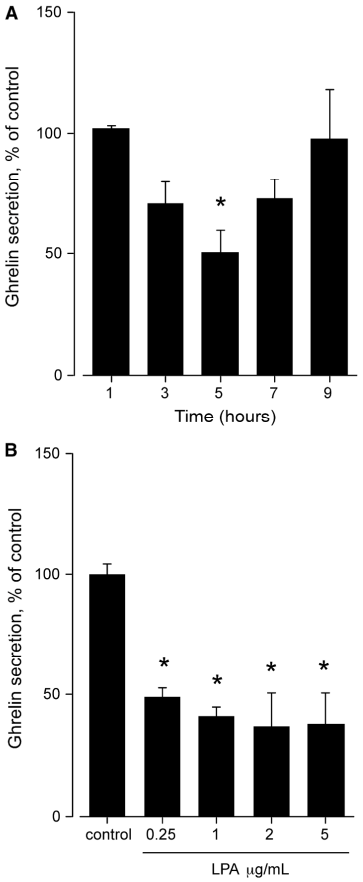

Fig. 3. Regulation of ghrelin secretion by LPA. Ghrelin in culture medium is expressed relative to untreated control cells (100%) and represented as mean ± SE of three independent experiments. *$P < 0.05$ when comparing LPA-treated with untreated control cells. (A) Time-dependent inhibition of ghrelin secretion by LPA (1 μg·mL^{-1}). Serum-deprived AGS cells were stimulated with LPA for the indicated periods of time (1–9 h). (B) Dose-dependent inhibition of ghrelin secretion by LPA. AGS cells were serum-deprived for 48 h before various concentrations of LPA were added (0.25–5 μg·mL^{-1}) for 5 h.

To determine whether ligand-induced LPA2 internalization was necessary for ERK1/2 activation, AGS cells were pretreated with monodansylcadaverine (MDC) (300 μM, 30 min) and stimulated with LPA (1 μg·mL^{-1}). Although the basal levels of phospho-ERK1/2 were increased by MDC pretreatment, LPA-induced activation of ERK1/2 was not significantly modified (Fig. 5D), thus ruling out the possibility of receptor endocytosis.

When LPA-stimulated cells were pretreated with specific protein tyrosine kinase inhibitors, both genistein (2 μM, 1 h) and AG18 (25 μM, 1 h) counteracted the LPA-mediated phospho-ERK1/2 increase (Fig. 6A). Furthermore, pretreatment with the specific inhibitor of c-Src, PP2 (5 μM, 30 min), produced a reduction of LPA-mediated ERK1/2 phosphorylation, and corroborated previous results of c-Src involvement in this process (Fig. 6B). To investigate the activation of Src, the phosphorylation of both Src regulatory tyrosines, namely Tyr527 and Tyr416, was compared. Phosphorylation of Tyr416 displayed an early increase at 1 min after LPA stimulation and a decline at 3 min poststimulation. Conversely, the phosphorylation of inhibitory Tyr527 showed a decrease concurrent with the dynamics of phosphorylation of Tyr416 (Fig. 6C).

To test whether EGF receptor (EGFR) transactivation had a role in the effects of LPA on ghrelin secretion, AGS cells were treated with LPA (1 μg·mL^{-1}), EGF (100 nM) or both. As can be seen in Fig. 7A, the two compounds had a similar effect on ERK1/2

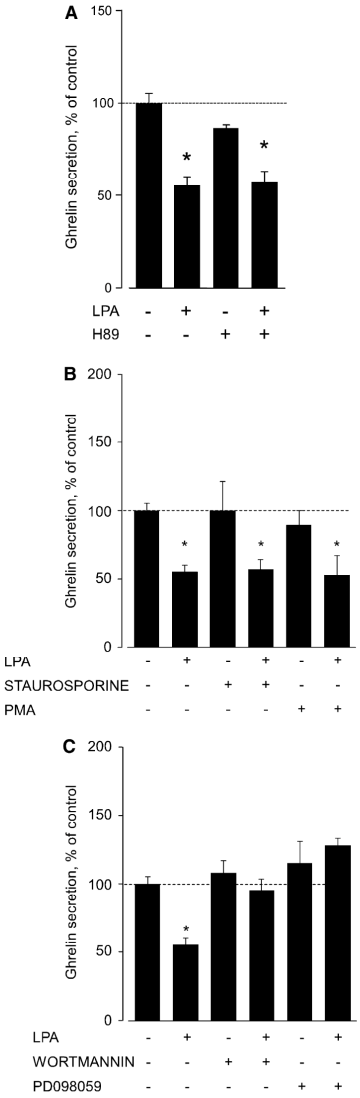

Fig. 4. Effect of LPA signaling pathway inhibitors on LPA-mediated ghrelin secretion inhibition. The experiment shown is representative of two independent assays. Data for ghrelin secretion in culture media are expressed relative to untreated control cells (100%) and represented as mean ± SE of three independent experiments. $*P < 0.05$ when comparing LPA-treated with untreated control cells. (A) Effect of the protein kinase inhibitor H89 (10 μM, 30 min). (B) Effect of the PKC inhibitor staurosporine (1 μM, 5 min) and downregulation of PKC with PMA (1 μM, 24 h) on LPA (1 $\mu g \cdot mL^{-1}$, 2 min) regulation of ghrelin secretion. (C) Effect of the PI3k inhibitor wortmannin (2 μM, 30 min) and the MEK inhibitor PD098059 (50 μM, 30 min) on LPA (1 $\mu g \cdot mL^{-1}$, 2 min) regulation of ghrelin secretion.

activation, which was augmented when they were administered together. Pretreatment of AGS cells with a highly potent and specific inhibitor of EGFR, tyrphostin AG1478 (50 μM, 30 min), completely suppressed the previously described effect on ERK1/2 phosphorylation of treatment with LPA, EGF or both. Blockade of G-protein-coupled receptor (GPCR)-activated matrix metalloproteinase (MMP) action by the broad-spectrum inhibitor GM6001 had no significant inhibitory effect on agonist-induced ERK1/2 responses, even at increased doses (data not shown).

Figure 7B shows the effect of the c-Src inhibitor PP2 on MAPK phosphorylation after EGF (100 nM) treatment. c-Src is shown to be the scaffold that transactivates EGFR, as PP2 treatment did not diminish EGF-mediated ERK1/2 phosphorylation (Fig. 7B), but did diminish LPA-mediated phosphorylation (Fig. 6C).

Finally, to test whether the effect on EGF-mediated ERK1/2 phosphorylation could extend to ghrelin secretion, AGS cells were treated with LPA (1 $\mu g \cdot mL^{-1}$), EGF (100 nM) or both. In fact, analogous results were obtained for ghrelin secretion inhibition (Fig. 7C).

Discussion

The highest level of ghrelin expression has been demonstrated in the fundus of the stomach [23,24]. It has been reported that circulating ghrelin levels in patients with gastroenteropancreatic tumors are similar to those in healthy individuals [25] and that gastrointestinal carcinoids express ghrelin [26]. The capacity of the AGS cell line to produce ghrelin [27] made this gastric adenocarcinoma a suitable model for a thorough assessment of the mechanisms regulating ghrelin secretion. For its part, LPA is recognized as an extracellular lipid mediator that evokes growth factor-like responses in almost every cell type [28,29]. Moreover, the study of LPA as a regulator of ghrelin secretion was also prompted by the widespread action of LPA on secretion [30–33] and the high-level expression of LPA2 [34] in the AGS cell line. The results obtained did indeed show that LPA, at a dose of 1 $\mu g \cdot mL^{-1}$, had a powerful inhibitory effect on ghrelin secretion, exhibiting a parabolic shape and a maximum at 5 h.

Screening for different inhibitors was carried out to analyze the LPA signal transduction pathway acting

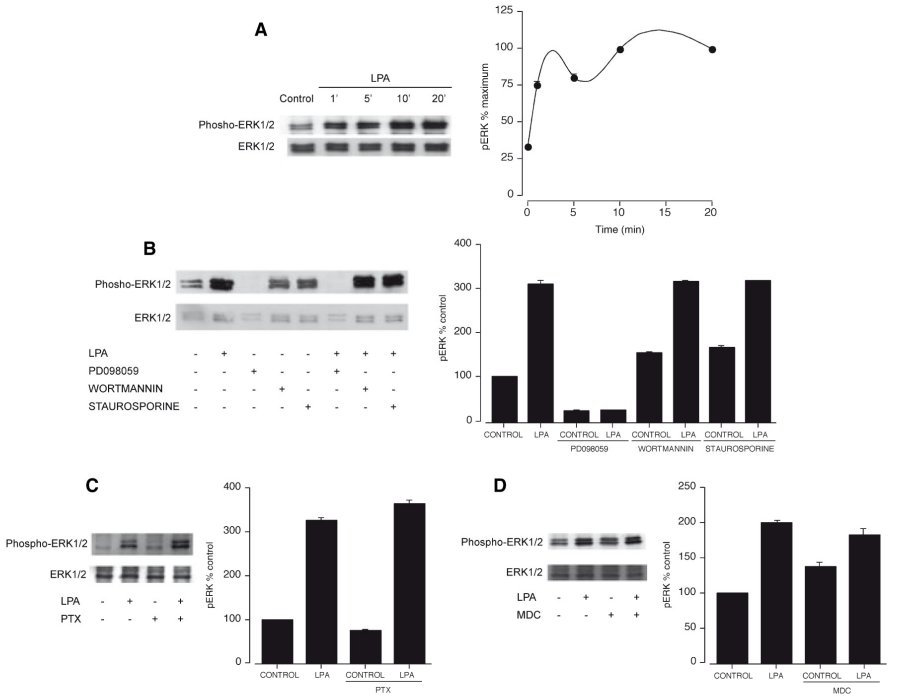

Fig. 5. LPA effect on ERK1/2 phosphorylation. Effect of LPA signaling pathway inhibitors on LPA-mediated ERK1/2 phosphorylation. (A) Time-course of LPA effect on ERK1/2 phosphorylation. Serum-deprived AGS cells were stimulated with LPA (1 μg·mL^{-1}). ERK1/2 phosphorylation was quantified by densitometry and expressed as a percentage of the maximal phosphorylation of ERK1/2 (mean ± SE). (B) Effect of the MEK inhibitors PD098059 (50 μM, 30 min), wortmannin (2 μM, 30 min) and staurosporine (1 μM, 5 min) on LPA-mediated (1 μg·mL^{-1}, 2 min) ERK1/2 phosphorylation. Serum-deprived AGS cells were stimulated with LPA after pretreatment with PD098059, wortmannin or staurosporine. (C) Effect of the G_i inhibitor PTX on LPA-mediated ERK1/2 phosphorylation. Serum-deprived AGS cells were stimulated with LPA (1 μg·mL^{-1}, 2 min) after pretreatment with PTX (100 ng·mL^{-1}, 4 h). (D) Internalization. Serum-deprived AGS cells were stimulated with LPA (1 μg·mL^{-1}, 2 min) after pretreatment with MDC (300 μM, 30 min). For (B), (C) and (D), ERK1/2 phosphorylation was quantified by densitometry and expressed as percentage of the basal phosphorylation of ERK1/2 obtained in control cells (mean ± SE of three independent experiments).

on ghrelin secretion. The PKA inhibitor H89 had no effect on ghrelin secretion. Neither downregulation of PKC expression by PMA treatment, nor use of the selective PKC inhibitor staurosporine, had an effect on the inhibition. The addition of the PI3k inhibitor wortmannin diminished the LPA effect no more than the inhibitor alone, so the role of PI3k in LPA-mediated inhibition remains unclear. However, when cells were pretreated with the p44/p42 MAPK inhibitor PD098059, LPA exposure did not significantly inhibit ghrelin secretion, suggesting that LPA activates the MAPK cascade through the LPA2 receptor to regulate ghrelin secretion, with no participation by PKA or PKC.

GPCRs employ multiple mechanisms to activate the MAPK cascade. The signaling mechanisms are complex, and may result from activation of classic G-protein-regulated effectors such as PKC and PI3k, from cross-talk between GPCR and receptor tyrosine kinases or focal adhesion complexes, or from β-arrestin scaffolding directly on the GPCR [35]. Depending on receptor and cell type, one mechanism may predominate or multiple mechanisms may be activated simultaneously. It has been shown that LPA receptors couple to a variety of G-proteins, namely G_q, G_i and $G_{12/13}$ [36]. Our data pointed to an activation of ERK1/2 in a PTX-insensitive fashion, suggesting a null role for G_i. Although our results pointed to a role for Ca^{2+} ($G\alpha_q$) in MAPK

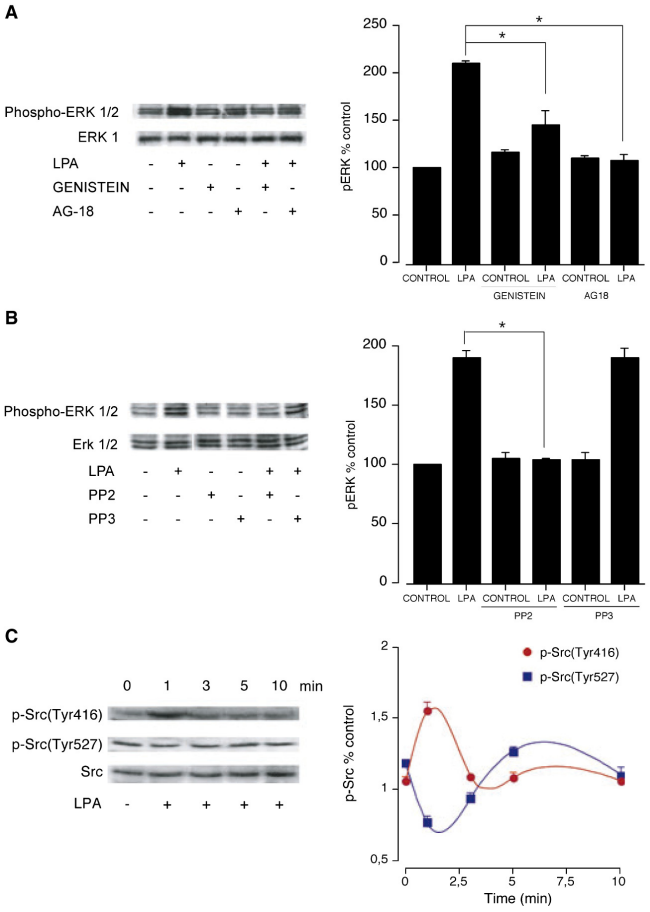

Fig. 6. Nonreceptor tyrosine kinase involvement in LPA-mediated ERK1/2 phosphorylation. (A) Serodeprived AGS cells were pretreated with specific protein tyrosin kinase inhibitors, genistein (2 μM, 1 h) and AG-18 (25 μM, 1 h). (B) Effect of the tyrosine kinase c-Src family specific inhibitor PP2 (5 μM, 30 min) on LPA-mediated ERK1/2 phosphorylation. (C) Time-course for LPA-induced phosphorylation at different Src tyrosine residues. Cells were stimulated with LPA (1 $\mu g \cdot mL^{-1}$) at 37 °C for the indicated periods. Phospho-Src (Tyr416 and Tyr527) and Src were detected by inmunoblotting and quantified by densitometry for normalization. Normalized values for phosphorylation of each tyrosine are represented in the plot (mean ± SE of three independent experiments). (A, B) ERK1/2 phosphorylation was quantified by densitometry and expressed as percentage of the basal phosphorylation of ERK1/2 obtained in control cells (mean ± SE of three independent experiments).

phosphorylation, it was not possible to pinpoint the role of $G\alpha_q$. BAPTA-AM pretreatment stimulated MAPK phosphorylation *per se*. Additionally ghrelin secretion was restored, but treatment led to cell death, which was unreliable. PKC and PI3k were not found to participate in MAPK cascade activation. We also showed that inhibition of receptor internalization by treatment with MDC did not modify LPA-induced ERK1/2 phosphorylation. This result suggested no contribution of β-arrestin-mediated ERK1/2 activation to LPA2 [37,38].

It has been proposed that LPA and other GPCR agonists exploit EGFR as an intermediary to trigger mitogenic signaling [35,39]. One possibility involves the activation of c-Src and the phosphorylation of EGFR by this nonreceptor tyrosine kinase [40]. The secretion data for AGS cells established that both LPA and EGF inhibit ghrelin secretion in a MAPK-dependent fashion. Thus, when AGS cells were pretreated with the specific inhibitors for tyrosine kinases, genistein and AG18, ERK1/2 phosphorylation showed a clear reduction in intensity. Moreover, the kinetics of c-Src activation in response to LPA, represented by dephosphorylation of Tyr527 and phosphorylation of Tyr416 [41–44], showed a maximum of Tyr416 phosphorylation at 1 min. This feature was corroborated by the reduction of ERK1/2 phosphorylation observed when LPA-stimulated cells were pretreated with the specific c-Src inhibitor PP2. Given that this reduction was not detected when cells were stimulated with EGF and pretreated with PP2, c-Src was considered to be the

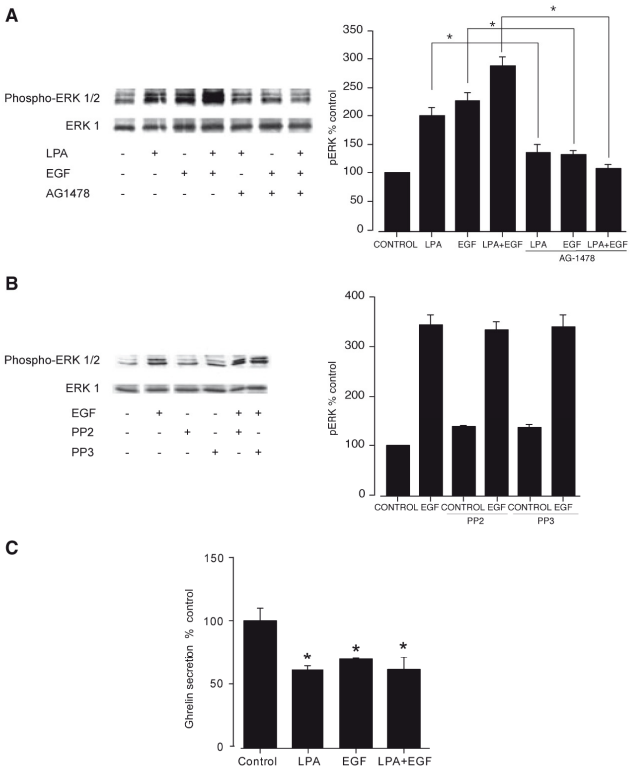

Fig. 7. EGF receptor involvement in the LPA-induced MAPK pathway and in ghrelin secretion. (A) Serodeprived AGS cells were treated or not treated with LPA (1 $\mu g \cdot mL^{-1}$), EGF (100 nM) or both, and pretreated or not treated with tyrphostin AG1478 (50 μM, 30 min). (B) Serodeprived AGS cells were treated or not treated with EGF (100 nM) and pretreated or not treated with PP2 (5 μM, 30 min). ERK1/2 phosphorylation was quantified by densitometry and expressed as percentage of the basal phosphorylation of ERK1/2 obtained in control cells (mean ± SE of three independent experiments). (C) Cells starved for 24 h were stimulated with LPA (1 $\mu g \cdot mL^{-1}$), EGF (100 nM) or both; the medium was collected after 5 h, and ghrelin was measured by RIA. The experiment shown is representative of two independent assays. Data for ghrelin secretion in culture media are expressed relative to untreated control cells (100%) and represented as mean ± SE of three independent experiments.

connecting nexus between LPA2 stimulation and EGFR activation. The other possibility is that activation of the LPA receptor leads to the proteolytic cleavage of a latent agonist for EGFR. Accordingly, a GPCR-activated MMP cleaves the heparin-binding EGF [45], which binds to and activates the EGFR [46]. However, when we blocked the MMP action with GM6001 [47], there was no significant inhibitory effect on agonist-induced ERK1/2 responses. The role of EGFR participation was assessed by pretreating AGS cells with AG 1478 [48]. Treatment of these cells with LPA, EGF or both clearly inhibited ERK1/2 phosphorylation. This finding is in line with previous research [49], although in some systems, positive crosstalk between LPA and EGF signaling cascades that does not necessarily involve receptor transactivation can occur at various levels [50]. In addition, Src or Src-like kinases have been found to mediate the phosphorylation of Shc by GPCRs and $\beta\gamma$-subunits [40], and inhibitors of Src-like kinases diminish the activation of MAPK by G_q- and G_i-coupled receptors [51]. In light of this, we propose that LPA-mediated ghrelin secretion inhibition in AGS cells may be prompted by LPA2 activation mediated by $G\beta\gamma_q$, and subsequent phosphorylation of c-Src. This nonreceptor protein kinase transactivates EGFR and thus activates the downstream signaling pathway to ERK1/2 phosphorylation, possibly via Ras activation after recruitment of Sos–Grb2 complexes (Fig. 8).

The results of the present study suggest a possible physiological role for LPA in ghrelin secretion. This hypothesis seems to be consistent with the secretion of LPA by adipocytes [52], which controls the mobility and proliferation of preadipocytes through LPA1 receptor. This idea is supported by the fact that autotaxin (type II ectonucleotide pyrophosphatase phosphodiesterase, ATX), a lysophospholipase D involved in the synthesis of LPA, has been found to be expressed and released by adipocytes [53]. ATX exerts paracrine control on preadipocyte growth via an LPA-dependent mechanism. Upregulation of ATX expression and, in consequence, increase of LPA biosynthesis with adipocyte differentiation and genetic obesity

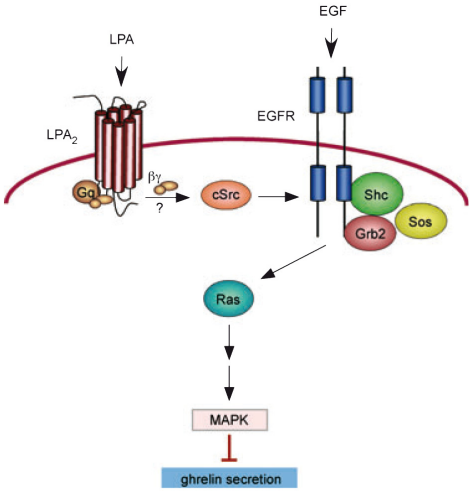

Fig. 8. Proposed model for the LPA-stimulated signaling pathway to ERK1/2 activation. LPA-mediated ghrelin secretion inhibition in AGS cells is promoted by LPA2 activation by its ligand LPA with activation of $G\beta\gamma_q$, and subsequent phosphorylation of c-Src. This nonreceptor protein kinase transactivates EGFR, which activates the downstream signaling pathway to ERK1/2 phosphorylation, possibly via Ras activation after recruitment of Sos–Grb2 complexes.

suggest that the established relationship between LPA and ghrelin secretion may shed new light on the low circulating levels of ghrelin observed in obese patients, as a bona fide reflex of the energetic stores [54].

In conclusion, we present data describing the molecular steps involved in the LPA receptor-mediated activation of the ERK pathway and its ultimate role as an inhibitor of ghrelin secretion in the gastric adenocarcinoma cell line AGS. Induction of ERK by LPA proceeds via LPA2-mediated activation of the nonreceptor tyrosine kinase c-Src and therefore transactivation of EGFR. Furthermore, LPA-induced ERK activation is independent of MMPs, suggesting that c-Src is the scaffold that transactivates EGFR. Finally, we present data demonstrating a correlation between the mitogenic effects of LPA and ghrelin secretion in the human gastric adenocarcinoma cell line AGS.

Experimental procedures

Reagents

LPA, EGF, PMA, PD098059, staurosporine, PTX and MDC were obtained from Sigma Chemical Co. (St Louis, MO). Wortmannin, BAPTA-AM, PP2, PP3, genistein, AG18, tyrphostin AG1478, GM6001, H89 and MDL were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). LPA receptor primers were purchased from Invitrogen (Carlsbad, CA). Rabbit anti-prepro-ghrelin (86–117) (human) IgG and ghrelin (human) RIA kits were obtained from Phoenix Pharmaceuticals Inc. (Belmont, CA). Rabbit polyclonal IgG antibodies to phospho-p44/42-MAPK, p44/42 MAPK, phospho-Src(Tyr416) and phospho-Src(Tyr527) were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal IgG antibody to c-Src was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit IgG, horseradish peroxidase linked whole antibody (from donkey) was purchased from Amersham Pharmacia (Arlington Heights, IL).

Cell culture

The human gastric adenocarcinoma cell line AGS was cultured as described by the supplier (ECACC, European Collection of Cell Cultures, Porton Down, UK). Briefly, cells were seeded in 100 mm dishes and cultured in F-12 Ham medium supplemented with 10% (v/v) fetal bovine serum, 100 $U \cdot mL^{-1}$ penicillin G, 100 $mg \cdot mL^{-1}$ streptomycin sulfate and 2.5 mM L-glutamine with 5% CO_2 at 37 °C. The subculture routine was as follows: split subconfluent cultures (70–80%) 1 : 4 seeding at 2–5 × 10 000 $cells^{-2}$ using 0.25% trypsin and 0.05% EDTA.

Ghrelin RIA

Ghrelin levels in AGS culture media were determined using specific RIA kits for total ghrelin from Phoenix Pharmaceuticals Inc., according to the protocol provided by the supplier. Ghrelin determination was performed in serum-free conditions after a starvation period of 24 h.

RNA isolation and RT-PCR in AGS cells

Expression of ghrelin or LPA receptors in AGS cells was determined by RT-PCR. mRNA from AGS cell line was extracted using a QuickPrep Micro mRNA Purification Kit (Amersham Biosciences, GE Healthcare, Fairfield, CT) following the manufacturer's instructions. Approximately 1 μg of mRNA was reverse-transcribed into cDNA using the conditions previously described [55] in a total volume of 30 μL. The PCR amplification was carried out using 3 μL of cDNA template for each reaction, and 150 ng of human ghrelin antisense primer 5′-TGA GCC CTG AAC ACC AGA GAG-3′ and 150 ng of human ghrelin sense primer 5′-AAA GCC AGA TGA GCG CTT CTA-3′ [55], or 50 pmol of human LPA1 antisense primer 5′-AAT CGA GAG GCA CAT TAC GG-3′ and 50 pmol of human LPA1 sense primer 5′-TGT GGA CAG CAC ACG TCT

AG-3′, or 50 pmol of human LPA2 antisense primer 5′-CAT CAT GCT TCC CGA GAA CG-3′ and 50 pmol of human LPA2 sense primer 5′-GGG CTT ACC AAG GAT ACG CAG-3′, or 50 pmol of human LPA3 antisense primer 5′-AGG ATG CGG GTC CAT AGC AA-3′ and 50 pmol of human LPA3 sense primer 5′-GAT GAT GGG GTT CAC GAC GG-3′ [56], in a total volume of 50 μL. The amplification was performed in an automatic thermal cycler (Mastercycler gradient; Eppendorf AG, Westbury, NY). For ghrelin, amplification was carried out with 35 cycles of the following conditions: denaturation at 98 °C for 20 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, with an additional step at 72 °C for 10 min. Human hypoxanthine-phosphoribosyl-transferase (h-HPRT) amplification was used as an internal control: sense primer 5′-AGC AAG ACG TTC AGT CCT GTC-3′, and antisense primer 5′-CAG CCC TGC CGT CGT GAT TA-3′. For LPA receptors, the mixture was subjected to 40 amplification cycles: denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 74 °C for 1 min, with an additional step at 74 °C for 10 min. β-Actin amplification was used as an internal control: sense primer 5′-GGC ATC GTG ATG GAC TCC G-3′, and antisense primer 5′-GCT GGA AGG TGG ACA GCG A-3′. The amplified products were resolved in 2% agarose gels and visualized with ethidium bromide. The PCR reaction generates a single 327 bp product for human ghrelin and a single 139 bp product for h-HPRT, a single 432 bp product for LPA1, a single 352 bp product for LPA2, a single 481 bp product for LPA3, and a single 615 bp product for β-actin [57].

Calcium measurements

Intracellular calcium measurement was performed in cell suspensions using the fluorescent calcium indicator fura-2/AM as previously described [58]. Briefly, cells were resuspended (2×100 mm plates/mL) in Krebs/Ringer/Hepes solution and loaded with 3 fura-2/AM. For each measurement, around 2×10^6 cells were resuspended in 2 mL of Krebs/Ringer/Hepes solution and then placed in a cuvette positioned in a holder at 37 °C. The fluorescence signal was measured under continuous stirring in an LS-50B fluorimeter (Perkin-Elmer, Boston, MA) in ratio mode ($\lambda_{ex1} = 345$, $\lambda_{ex2} = 380$, and $\lambda_{em} = 490$ nm) and calibrated by the cell lysis method [59].

Peptide extraction and RP-HPLC separation

Ghrelin determination was performed in serum-free conditions after a starvation period of 24 h. The acidified medium (pH 4–5 with HCl) was loaded onto a Sep-Pak C18 column (Waters, Milford, MA) pre-equilibrated with H_2O/CH_3CN/trifluoroacetic acid 96.9 : 3 : 0.1. After washing with H_2O/CH_3CN/trifluoroacetic acid 89.9 : 10 : 0.1, the peptides were eluted with H_2O/CH_3CN/trifluoroacetic acid 39.9 : 60 : 0.1. The eluate was concentrated in a Speed-Vac and subjected to RP-HPLC analysis on a Symmetry300 C18 column (3.9×150 mm; Waters) using a linear gradient from A to B for 40 min (H_2O/CH_3CN/10% trifluoroacetic acid, 90 : 10 :1 for A; (H_2O/CH_3CN/10% trifluoroacetic acid, 40 : 60 :1 for B), at a flow rate of 1 mL·min^{-1}, with detection at 210 nm. RP-HPLC fractions were collected, concentrated and submitted to RIA.

Immunoblotting analysis

Serum-starved AGS cells were stimulated with LPA for the indicated time period at 37 °C. The medium was then aspirated, and the cells were lysed in ice-cold lysis buffer (RIPA buffer). Samples were transferred into centrifuge tubes and left at 4 °C for 15 min. The protein concentration was evaluated with a Quanty Pro BCA Assay Kit (Sigma). The same amount of protein of each sample was separated on 10% SDS/polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The blots were incubated with 5% albumin (Fraction V, Sigma) in NaCl/Tris with Tween 20 (TBST) for 1 h (this solution was also used for all incubation and washing steps). Then, blots were incubated for 1 h with either rabbit anti-prepro-ghrelin (86–117) (human)-purified IgG (1 : 1000), anti-phospho-p44/42 MAPK (Thr202/Tyr204) rabbit polyclonal IgG (1 : 1000), anti-p44/42 MAPK rabbit polyclonal IgG (1 : 2000), anti-c-Src rabbit polyclonal IgG (1 : 500), anti-phospho-Src(Tyr416) rabbit polyclonal (1 : 1000) and anti-phospho-Src(Tyr527) rabbit polyclonal IgG (1 : 1000); this was followed by extensive washing. The blots were subsequently incubated with the corresponding peroxidase-conjugated IgG antibody. After washing, signal was visualized using ECL plus a western blotting detection system (Amersham Biosciences). Densitometry was performed using the IMAGEJ 1.36 program.

Statistical analysis

Ghrelin secretion in each experimental group was compared with that of non-LPA-treated controls in the same experiment. In total, three different experiments were included in each group for statistical analysis. Each experiment was performed in triplicate. Results are expressed as the percentage of ghrelin production compared with that of controls in the same experiment. The mean percentage of control ghrelin secretion in each experimental condition is shown with SE. Student's *t*-test was performed when comparing groups ($P < 0.05$).

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Stimulation of extracellular signal-regulated kinases and proliferation in the human gastric cancer cells KATO-III by obestatin*

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Abstract
Obestatin, the ghrelin-associated peptide, activates cell proliferation in the gastric cancer cell line KATO-III. The results showed that this peptide induced cell proliferation by mitogen-activated kinase kinase/extracellular signal-regulated kinases1/2 (ERK1/2) phosphorylation. A sequential analysis of the obestatin transmembrane signalling pathway indicated that the ERK1/2 activity is partially blocked after preincubation of the cells with pertussis toxin, as well as by wortmannin (an inhibitor of phosphoinositide 3-kinase (PI3K)), staurosporine (an inhibitor of protein kinase C (PKC)) and 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2, which inhibits the non receptor tyrosine kinase Src). Upon administration of obestatin, the intracellular levels of phospho-PKCε- and θ-isoenzymes rise with similar time-courses, from which PKCε appears to be the responsible for ERK1/2 response. Based on the experimental data, a signalling pathway involving the consecutive activation of G_i, PI3K, novel PKCε and Src for ERK1/2 activation is proposed. These results point to a functionally active peptide that regulates proliferation of the gastric cancer cells KATO-III.

Keywords: *Obestatin, MAPK, KATO-III*

Abbreviations: *AC, adenylyl cyclase; ACTH, adrenocorticotrophic hormone; ANOVA, analysis or variance; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; EDTA, ethylenediaminetetraacetic acid; ERK, extracellular signal-regulated kinases; FBS, fetal bovine serum; GH, growth hormone; GHS-R1a, growth hormone secretagogues receptor type 1a; GPR39, G protein coupled receptor 39; icv, intracerebroventricular; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinases; MEK, mitogen-activated kinase kinase; PD098059, 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; PI3K, phosphoinositide 3-kinase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PMA, phorbol myristate acetate; PP2, 4-Amino-5-(4-chlorophenyl)-7-(*t-butyl)pyrazolo[3,4-d]pyrimidine; PP3, 4-Amino-7-phenylpyrazol[3,4-d]pyrimidine; PS, phosphatidylserine; PTX, pertussis toxin; RIPA, radioimmuno precipitation assay; hRPE, human retinal pigment epithelium cells; SDS, sodium dodecyl sulphate; SEM, standard error of the mean; TBST, Tris buffered solution/Tween-20.*

Introduction

Gut peptides have received growing attention for their ability to regulate food intake and energy homeostasis (Cummings and Overduin 2007). Among them, ghrelin has been characterized as one of the most prominent peptides to regulate energy homeostasis (van der Lely et al. 2004). Ghrelin is a 28-amino acid acylated peptide, mainly synthesized in the entero-endocrine A-like cells of the oxintic mucosa of the

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stomach, which interacts with the growth hormone secretagogue receptor type 1a (GHS-R1a; Kojima et al. 1999). In this context, a novel 23-amino acid peptide derived from the ghrelin peptide precursor (preproghrelin) has been recently identified as a physiological opponent of ghrelin (Zhang et al. 2005). This peptide was initially termed as obestatin for its ability to inhibit food intake in mice when peripherally or icv injected. Obestatin was originally isolated from stomach and has subsequently been shown to be a circulating peptide whose secretion is pulsatile and displays an ultradian rhythmicity similar to ghrelin and growth hormone (GH) secretion (Zhang et al. 2005). Moreover, obestatin was characterized to bind selectively to the orphan receptor G protein coupled receptor 39 (GPR39), which belongs to the family of the ghrelin receptor GHS-R1a and the motilin receptor (Zhang et al. 2005). Curiously, the subsequent studies (Gourcerol et al. 2006; Seoane et al. 2006; Nogueiras et al. 2007; Yamamoto et al. 2007; Zizzari et al. 2007) with the exception of four reports (Bresciani et al. 2006; Carlini et al. 2007; Lagaud et al. 2007; Tremblay et al. 2007) were unsuccessful to reproduce the anorexigenic property of obestatin initially reported (Zhang et al. 2005). Furthermore, several groups were unable to confirm that obestatin binds to GPR39 or activates signalling in transfected cells, suggesting that obestatin is unlikely to be the cognate ligand for this receptor (Chartrel et al. 2007; Holst et al. 2007). Consequently, the present state-of-knowledge on obestatin is leaving significant unanswered issues, specially the basis for the lack of reproducible biological actions for this ghrelin-associated peptide on feeding. Throughout this period, additional actions for this peptide have been reported providing evidence of biological functionality. Thus, obestatin showed to suppress drinking responses (Samson et al. 2007), improve memory (Carlini et al. 2007), decrease GH secretion *in vivo* (Zizzari et al. 2007), regulate sleep (Szentirmai and Krueger 2006), activate cortical neurons (Dun et al. 2006) and stimulate proliferation of retinal pigment epithelial cells (Camiña et al. 2007). Thus, obestatin seems to be a functional peptide, though its precise function remains to be properly established.

In the present work, the ability of obestatin to modulate cell proliferation on gastric cells, one of the main sources of this peptide, was evaluated. As a model, the human gastric carcinoma cell line KATO-III was used to assess the obestatin mitogenic action and to characterize the intracellular signalling pathway.

Materials and methods

Materials

Obestatin was obtained from Phoenix Pharmaceuticals Inc. (Belmont, CA, USA). Pertussis toxin (PTX), staurosporine, PD98059 were obtained from Sigma Chemical Co. (St Louis, MO, USA). Wortmannin, genistein, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2) and 4-amino-7-phenylpyrazol[3,4-d]pyrimidine (PP3) were purchased from Calbiochem (Merk KGaA, Darmstadt, Germany). Rabbit polyclonal IgG antibodies to phospho-p44/42 mitogen-activated protein kinases (MAPK), p44/42 MAPK, phospho-Src(Tyr416) and phospho-Src(Tyr527) were obtained from Cell Signalling Technology (Beverly, MA, USA). Rabbit polyclonal IgG antibodies to phospho-PKCε-, δ-, θ-, ζ-, μ- and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit IgG, horseradish peroxidase linked whole antibody (from donkey) was obtained from Amersham Pharmacia (Arlington Heights, IL, USA).

Cell culture

Human gastric cancer cell line, KATO-III, was cultured as described by the supplier (ECACC, Wiltshire, UK). Briefly, cells were seeded in 100-mm dishes and culture in RPMI-1640 medium supplemented with 20% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin G, 100 mg/ml streptomycin sulphate and 2.5 mM L-glutamine with 5% CO_2 and 37°C. Subculture routine was as follows: split sub-confluent cultures (70–80%) 1:4 seeding at 2–5 × 10,000 cells/cm^2 using 0.25% trypsin, 0.05% ethylenediaminetetraacetic acid (EDTA).

Proliferation assays

KATO-III cells were seeded in 24-well plates at a density of 20 × 10^3 cells per well in 500 μl of RPMI-1640 medium. After 2 days, the medium was renewed and cells were cultured in a serum-free medium for 24 h. Cells were then treated with different doses of obestatin, ghrelin (500 nM), lysophosphatidic acid (LPA; 2.3 μM) or FBS (10% v/v) in fresh RPMI-1640. After 48 h, cells were trypsinized and counted using a Coulter Counter. In all cases, triplicate dishes were used for each experiment point.

Immunoblotting analysis

Serum-starved KATO-III cells were stimulated with obestatin for the indicated time period and doses at 37°C. The medium was then aspirated and cells were lysed in ice-cold lysis buffer radioimmuno precipitation assay (RIPA) buffer: 50 mM Tris–HCl pH 7.2, 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 0.25% (w/v) Na-deoxycholate, protease inhibitor cocktail (Sigma), phosphatase inhibitor cocktail (Sigma)). Samples were transferred into centrifuge tubes and left at 4°C for 15 min. The soluble cell lysates were pre-cleared by centrifuging at 13,000*g* for 15 min.

The protein concentration was evaluated with the QuantiPro™ BCA Assay Kit (Sigma). The same amount of protein of each sample was separated on 10% sodium dodecyl sulphate (SDS)/polyacrilamide gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The blots were incubated with 5% non-fatty milk in Tris buffered solution/Tween-20 (TBST) (20 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% (v/v) Tween-20, used for all incubation and washing steps) for 1 h. Then, blots were incubated for 1 h with either anti-phospho-p44/42 MAPK (Thr202/Tyr204) rabbit polyclonal IgG (1:1000), anti-p44/42 MAPK rabbit polyclonal IgG (1:1000), anti-phospho-PKCε rabbit polyclonal IgG (1:1000), anti-phospho-PKCθ rabbit polyclonal IgG (1:1000), anti-phospho-PKCμ rabbit polyclonal IgG (1:1000), anti-phospho-PKCδ rabbit polyclonal IgG (1:1000), anti-phospho-PKCζ rabbit polyclonal IgG (1:1000), anti-β-actin rabbit polyclonal IgG (1:1000) or anti-phospho-tyrosine Src (Tyr416 or Tyr527) rabbit polyclonal IgG (1:1000) antibobodies followed by extensive washings. Blots were subsequently incubated with the corresponding peroxidase-conjugated IgG antibody. After washing, signal was visualized using ECL plus Western Blotting Detection System (Amersham Biosciences). The blots shown are representative of three experiments. Densitometry was performed using IMAGEJ 1.36 program.

Data analysis

SPSS 14.00 software for Windows (SPSS Inc. Chicago, IL, USA) was used for all statistical analyses. Parameters were expressed as mean ± standard error of the mean (SEM). Statistical differences among means of percentages were identified with one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. P values of 0.05 or smaller were considered significant. $\star$ denotes $P < 0.05$ when comparing obestatin-treated with untreated control cells. # denotes $P < 0.05$ when comparing inhibitor + obestatin-treated with obestatin-treated cells.

Results

Initially, the action of obestatin on KATO-III cells was evaluated by means of cell proliferation. As shown in Figure 1(A), obestatin showed a significative proliferative effect [$F = 166.44$, df (8;29), $P < 0.001$] on KATO-III cells for the range of doses tested (0.050–1.00 μM). This effect appears to be mediated by MEK, since control cells treated with the specific MEK inhibitor PD98059 (50 μM, 30 min) had a significantly blockade over the obestatin effect at 1.00 μM comparing to untreated cells. Treatment of the cells with LPA (2.3 μM) showed a comparable mitogenic response (Figure 1(A)). Additionally, maximum proliferation capacity was determined by stimulation with FBS (10%, v/v;). By contrast, treatment of the cells with ghrelin (500 nM) failed to modify the mitogenic response of KATO-III cells (Figure 1(A)).

The kinetic of ERK1/2 activation following stimulation of KATO-III cells with obestatin showed maximal levels of ERK1/2 phosphorylation within 5 min of obestatin treatment (100 nM), which decreased to basal levels after 20 min (Figure 1(B)). By contrast, non-amidated obestatin did not induce ERK1/2 phosphorylation (data not shown). Inhibition of MEK by treatment with PD98059 (50 μM, 30 min) blocked obestatin-dependent activation of ERK1/2 (data not shown). These data strongly suggest that MEK/ERK1/2 are involved in mediating signalling for obestatin-activated KATO-III cell proliferation.

Previous results demonstrated that obestatin stimulates the production of the second messenger cyclic adenosine monophosphate (cAMP; Zhang et al. 2005). Among the effectors of cAMP, it is included the cAMP-dependent protein kinase (PKA) and cAMP-dependent calcium channels. However, no intracellular calcium rise was observed after obestatin stimulation of KATO-III cells (data not shown), ruling out the implication of a Ca^{2+}-dependent signalling pathway. Pretreatment of the KATO-III cells with pertussis toxin (PTX; 100 ng/ml, 12 h), which uncouples receptors from $G_{i/o}$, reduced the obestatin-dependent significant activation of ERK1/2 [$F = 18.14$, df (3;8), $P < 0.05$] by ~80% (Figure 1(C)). $G_{i/o}$ protein is known to mediate downstream signals through activation of PI3k in different cell types (Hawes et al. 1996; Yart et al. 2002). As shown in Figure 1(D), pretreatment with wortmannin (1 μM, 30 min) caused a clear-cut blockade (~100%) of the ERK1/2 activation induced by obestatin (100 nM). In addition, the significant activation of ERK1/2 by obestatin (100 nM) [$F = 138.70$, df (5;12), $P < 0.001$] was also inhibited ~100% by pretreatment with staurosporine (1 μM, 30 min), a non-selective PKC inhibitor (Figure 1(D)). To verify the role of PKC, the time course of obestatin-induced PKC activation was analyzed using specific antibodies against the activated forms of: (i) novel PKCs (PKCε, PKCδ, PKCμ and PKCθ), which are Ca^{2+} independent but still regulated by phosphatidylserine (PS) and diacylglycerol (DAG); and (ii) atypical PKC (PKCζ), which is Ca^{2+} independent and do not require PS or DAG for activation (Parker and Murray-Rust 2004). As shown Figure 2(A), obestatin (100 nM) considerably activated PKCε, PKCθ and PKCζ. PKCε reached a maximum 3 min after obestatin stimulation (~2.5-fold baseline) reaching prestimulatory level within 10 min; PKCθ reached a maximum 5 min after obestatin treatment (~3.0-fold baseline); while PKCζ peaked about 10 min after obestatin addition (~2.4-fold baseline). In contrast, PKCμ and PKCδ were not significantly modified after obestatin treatment (Figure 2(A)).

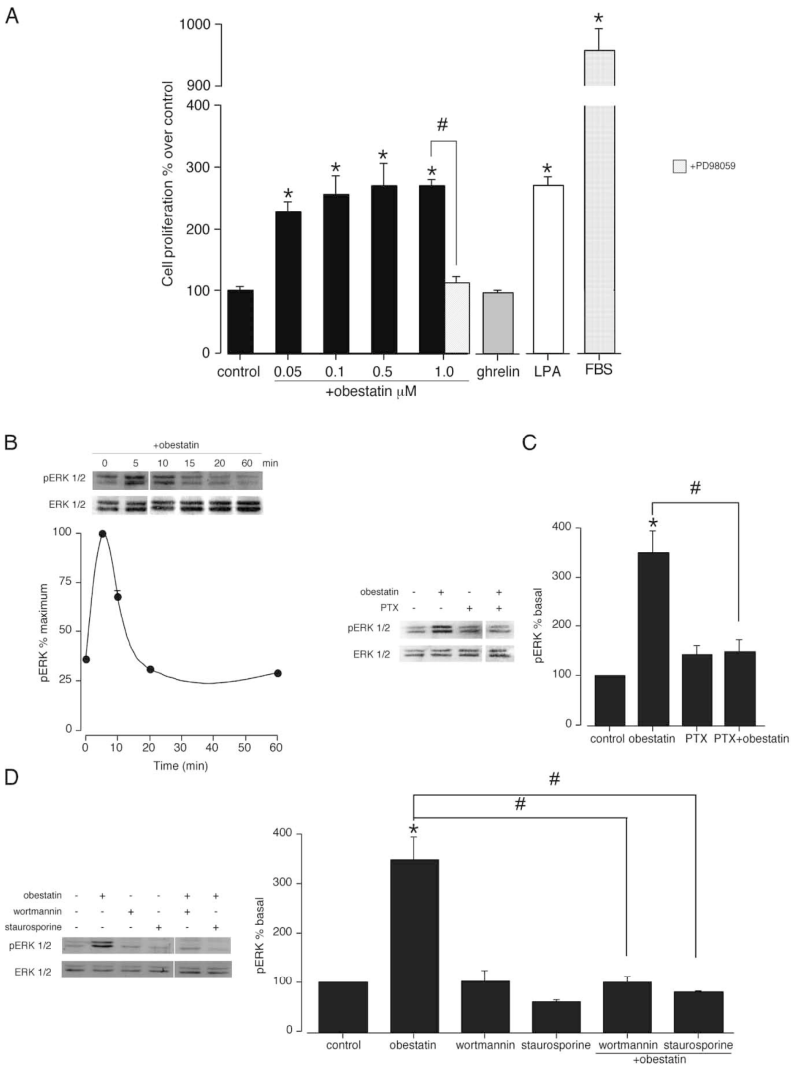

Figure 1. (A) Mitogenic effect of obestatin on KATO-III cells. The cells were treated with different doses of obestatin, obestatin (1.0 μM) pretreated with PD98059 (50 μM, 30 min), LPA (2.3 μM), ghrelin (500 nM) or FBS (10%, v/v), and cell proliferation was evaluated after 48 h. Data are expressed as a percentage of the basal proliferation of untreated cells (Mean ± SEM). (B) Temporal pattern of ERK1/2 activation. Serum-starved KATO-III cells were stimulated with obestatin (100 nM) for the indicated periods. (C) Dependence of obestatin-induced ERK1/2 phosphorylation on PTX-sensitive G protein. KATO-III cells were pretreated with PTX (100 ng/ml, 12 h) and stimulated with obestatin (100 nM). (D) Dependence of obestatin-induced ERK1/2 phosphorylation on PI3k and PKC. Cells were pretreated with wortmannin (1 μM, 30 min) or staurosporine (1 μM, 30 min) and stimulated with obestatin (100 nM). ERK1/2 phosphorylation was quantified by densitometry and expressed as a percentage of the maximal phosphorylation of ERK1/2 obtained after obestatin stimulation (B) or as a percentage of the basal phosphorylation of ERK1/2 obtained in control cells (100%) (C–D) (Mean ± SEM). ⋆ denotes $P < 0.05$ when comparing obestatin-treated with untreated control cells. # denotes $P < 0.05$ when comparing inhibitor + obestatin-treated with obestatin-treated cells. Western blots are representative of three independent experiments (A–D).

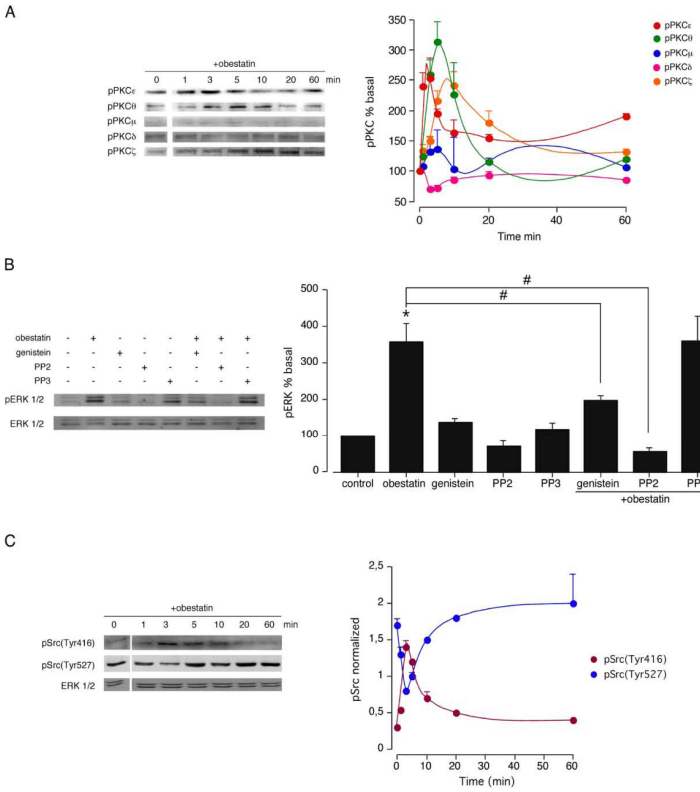

Figure 2. (A) Activation of novel and atypical PKCs by obestatin in KATO-III cells. Time course of obestatin-induced phosphorylation of PKCε-,δ-,ζ-,θ- and μ-isoenzymes. Serum-starved cells were treated with obestatin (100 nM) for the indicated periods, and then cellular extracts were prepared to visualized phosphorylation of different PKCs by means of specific antibodies. Values were obtained by densitometry and are expressed as a percentage of the basal phosphorylation (Mean ± SEM). (B) Effects of the tyrosine kinase inhibitors genistein, PP2 and PP3 on the obestatin-induced ERK1/2 response. Serum-starved cells were pretreated with genistein (2 μM, 30 min), PP2 (5 μM, 30 min) or PP3 (5 μM, 30 min) before obestatin stimulation (100 nM). ERK1/2 phosphorylation was quantified by densitometry and expressed as a percentage of the basal phosphorylation (Mean ± SEM). ⋆ denotes $P < 0.05$ when comparing obestatin-treated with untreated control cells. # denotes $P < 0.05$ when comparing inhibitor + obestatin-treated with obestatin-treated cells. (C) Time-course for the obestatin-induced phosphorylation of different Src tyrosine residues. Cells were stimulated with obestatin (100 nM) for the indicated periods. Phospho-Src (Tyr416 and Tyr527) and ERK1/2 were detected by inmunoblotting and quantified by densitometry for normalization. Normalized values for phosphorylation of each tyrosine are represented in the plot (Mean ± SEM). Western blots are representative of three independent experiments (A–C).

When obestatin-stimulated cells were pretreated with the specific protein tyrosine kinase inhibitor genistein (2 μM, 30 min), this inhibitor counteracted the significant obestatin-mediated phospho-ERK1/2 increase [$F = 94{,}92$, df (7;16), $P < 0.001$]. Also, obestatin-induced ERK activation was strongly inhibited by PP2 (5 μM, 30 min), a selective Src inhibitor (Figure 2(B)). This inhibition was specific, since pretreatment with PP3 (5 μM, 30 min), a negative control for PP2, had no effect on the obestatin-induced ERK1/2 phosphorylation (Figure 2(B)), supporting a role for Src protein in obestatin signalling. To investigate the activation of Src, we compared the phosphorylation of both Src regulatory tyrosines, namely Tyr527 and Tyr416. Phosphorylation of Tyr416 displayed an early increase at 3 min of obestatin stimulation (100 nM). Conversely, the phosphorylation of inhibitory Tyr527 showed a decrease concurrent with the dynamic of phosphorylation of Tyr416 (Figure 2(C)). Furthermore, PP2

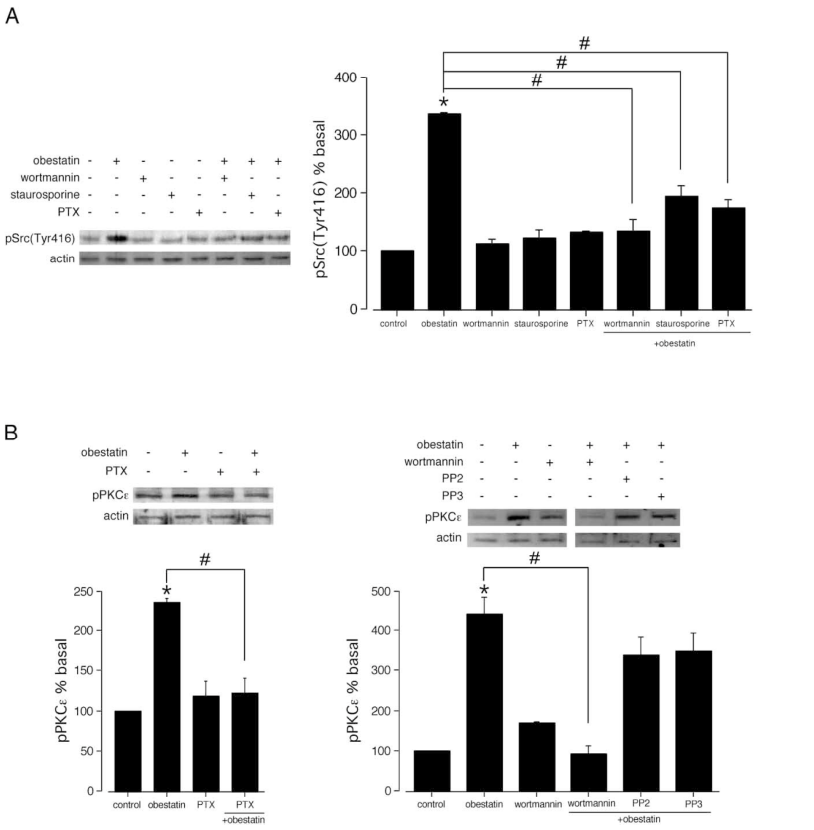

Figure 3. (A) Mechanism of Src activation by obestatin. Serum-starved cells were pretreated with PTX (100 ng/ml, 12 h), staurosporine (1 μM, 30 min) and wortmannin (1 μM, 30 min) followed by stimulation with obestatin (100 nM). Cellular extracts were prepared to visualize phospho-Src(Tyr416) protein band. (B) Dependence of obestatin-induced PKCε phosphorylation on PTX-sensitive G protein and PI3k. Serum-starved cells were pretreated with PTX (100 ng/ml, 12 h), PP2 (5 μM, 30 min), PP3 (5 μM, 30 min) or wortmannin (1 μM, 30 min) and stimulated with obestatin (100 nM) and then cellular extracts were prepared to visualized phospho-PKCε. Phospho-proteins were quantified by densitometry and expressed as a percentage of the basal phosphorylation obtained in control cells (Mean ± SEM). * denotes $P < 0.05$ when comparing obestatin-treated with untreated control cells. # denotes $P < 0.05$ when comparing inhibitor + obestatin-treated with obestatin-treated cells. Western blots are representative of three independent experiments.

attenuated the stimulatory effect of obestatin on phosphorylation of Src at Tyr416 (data not shown). Since $G_{i/o}$-coupled receptors can activate Src (Piiper et al. 2003), the effect of PTX on obestatin receptor-induced phosphorylation of Src was evaluated. As shown in Figure 3(A), pretreatment of cells with PTX (100 ng/ml, 12 h) have a clear-cut blockade on the significant obestatin-induced tyrosine 416 phosphorylation of Src [$F = 126.90$, df (7;16), $P < 0.001$]. Inhibition of PI3K by wortmannin pretreatment (1 μM, 30 min) revealed a PI3K-dependence for obestatin-activated Src. A possible link between the PKC and Src pathway was evaluated in KATO-III cells. The Src inhibitor PP2 inhibited phorbol myristate acetate (PMA)-induced ERK1/2 activation, showing that Src is activated downstream of PKC in this cell line (data not shown). Furthermore, obestatin-induced Src phosphorylation (100 nM) was completely inhibited by pretreatment with the non-selective PKC inhibitor staurosporine (1.0 μM, 30 min; Figure 3(A)). Taken together with the dynamic of Src activation, maximum of activation

3 min after obestatin treatment, PKCε might be the upstream mediators for this non-receptor tyrosine kinase. To test this hypothesis, the possible role of the $G_{i/0}$/PI3K pathway in the activation of PKCε was evaluated. As shown in Figure 3(B), phosphorylation of PKCε was inhibited by PTX (100 ng/ml, 12 h; ~84% inhibition; $F = 20.92$, df (3;8), $P < 0.001$) and wortmannin (1 μM, 30 min; ~100% inhibition; $F = 20.55$, df (5;12), $P < 0.001$) pretreatments. By contrast, no significant inhibition was observed after PP2 (5 μM, 30 min) pretreatment. These data show that the $G_{i/0}$/PI3K pathway arbitrates the obestatin-induced PKCε, consistent with a role for PKCε in Src activation in these cells.

Discussion

The data reported here allow delineating the signal transduction mechanisms activated by obestatin to induce ERK1/2 phosphorylation in KATO-III cells, and, in consequence, cell proliferation. The coupling between the obestatin receptor and the intracellular effectors is mediated, at least in part, by a non-receptor tyrosine kinase, Src, as shown by the blockade of the ERK1/2 response after PP2 treatment of KATO-III cells. Indeed, the kinetic of Src activation in response to obestatin showed a maximum of activation through dephosphorylation of Tyr527 and phosphorylation of Tyr416, which correlates with the kinetic of ERK1/2 activation. In principle, the coupling between obestatin and Src activation is mediated, by a PTX-sensitive G-protein, as shown by the inhibitory effect of PTX pretreatment. In a similar way, the PI3k blocker wortmannin was able to suppress the activation of Src. This pathway appears to be under regulation by PKC, because unspecific PKC inhibitor staurosporine caused an inhibition of the Src phosphorylation of Tyr416. Furthermore, obestatin failed to activate Ca^{2+} mobilization, which ruled out the action Ca^{2+}-dependent PKC activation. In our study, obestatin activated PKCε-, PKCθ- and ζ-isoenzymes. The measurement of the time course of PKCs activation suggests that PKCε (maximum 1–3 min post-stimulation) is upstream for Src (maximum 3 min post-stimulation) in the activation of ERK1/2 (maximum 5 min post-stimulation). In fact, the activation of PKCε appears to be controlled by PI3k via a PTX-sensitive G-protein. Regarding this point, the ability of PKC to activate Src is not due to direct interactions between the two kinases. Although, PKC can phosphorylate Src (serines 12 and 48) (Gould et al. 1985; Moyers et al. 1993), *in vitro* studies using purified Src and PKCs demonstrated that PKCs were unable to directly activate Src (Brandt et al. 2003). Therefore, the ability of PKC to activate Src is likely due to the activity of other proteins that relayed signals from PKC, which in turn direct the activation of Src. In principle, reduced phosphorylation could result from either decreased Tyr527-directed kinase activity or increased Tyr527-directed phosphatase activity (Thomas and Brugge 1997; Frame 2002; Yeatman 2004; Roskoski 2005). At least in the proliferative activity, it is most likely that activation results from increased phosphatase activity (Bagrodia et al. 1991; Kaech et al. 1991; Zheng et al. 1992, 2002). Taken together, the data obtained demonstrate that obestatin receptor activates PI3k through a PTX-sensitive G-protein. The activation of PI3k results in the activation of novel PKCε that would be the responsible for the consecutive activation of MAPK through activation of Src dependent pathway (Figure 4). These findings resemble with the $G_{i/o}$-

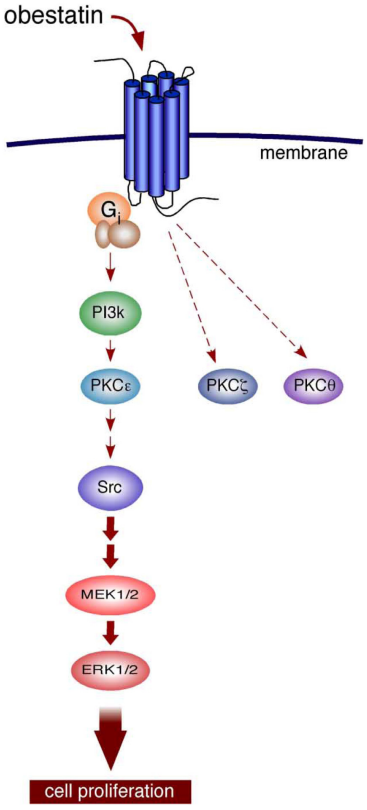

Figure 4. Proposed model for the obestatin-stimulated signalling pathway to ERK1/2 activation. Obestatin-mediated cell-proliferation in KATO-III cells is promoted by an obestatin-related GPCR activation with subsequent activation of Gi, PI3k, novel PKCε and subsequent phosphorylation of Src for ERK1/2 phosphorylation.

dependent ERK1/2 activation seen in human retinal pigment epithelium cells (hRPE; Camiña et al. 2007).

Since the discovery of obestatin, several observations have decreased the initial enthusiasm about the potential of this molecule putting the effectiveness of obestatin as an anorexigenic peptide into question. This situation is no so different to that with PYY3-36 where some investigators found significant effects on inhibition of food intake, while others not (Batterham et al. 2002, 2003; Boggiano et al. 2005). At this moment, further clarity has to come from additional experimental data collected under rigorous conditions with sample sizes sufficient to produce definitive conclusions. In the middle of this 'storm', several groups have been showing different biological effects for obestatin. Our present study demonstrates that stimulation of KATO-III cells with obestatin results in a significant increase of cell proliferation through activation of ERK cascade, a fact not observed for ghrelin. Because obestatin and ghrelin are derived from the same peptide precursor, this lack of functional correlation supports the concept that obestatin is a biologically relevant peptide and not only a non-functional connective peptide. This may also be the case of a polypeptide precursor which products would be differentially generated through successive cell-specific processing steps and post-translational modifications as happen for proopiomelanocortin, the precursor of adrenocorticotrophic hormone (ACTH), endorphins, melanotropins, and lipotropins (Raffin-Sanson et al. 2003; Coll et al. 2004). Regarding the receptor for obestatin, the data presented strongly suggest a G protein-coupled receptor as the responsible to mediate the mitogenic action. It is noteworthy that despite KATO-III cells expressed GPR39 (data not shown), the initial proposed receptor for obestatin (Zhang et al. 2005), recent studies do not support this notion (Chartrel et al. 2007; Holst et al. 2007). In this sense, it is more prudent to talk about an obestatin receptor, as a "generic" concept. Aside from obestatin receptor, this peptide might be involved in processes such as repair gastric mucosal damage or as fuel for gastric cancer cell proliferation. Elucidation of the pathological role of obestatin signalling and the presence of this peptide in tumor environments are intriguing areas for further research.

Acknowledgements

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Obestatin stimulates Akt signalling in gastric cancer cells through β-arrestin-mediated epidermal growth factor receptor transactivation

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Abstract

Obestatin was identified as a gut peptide encoded by the ghrelin gene that interacts with the G protein-coupled receptor, GPR39. In this work, a sequential analysis of its transmembrane signalling pathway has been undertaken to characterize the intracellular mechanisms responsible for Akt activation. The results show that Akt activation requires the phosphorylation of T308 in the A-loop by the phosphoinositide-dependent kinase 1 (PDK1) and S473 within the HM by the mammalian target of rapamycin (mTOR) kinase complex 2 (mTORC2: Rictor, mLST8, mSin1, mTOR kinase) with participation neither of $G_{i/o}$-protein nor $G\beta\gamma$ dimers. Obestatin induces the association of GPR39/β-arrestin 1/Src signalling complex resulting in the transactivation of the epidermal growth factor receptor (EGFR) and downstream Akt signalling. Upon administration of obestatin, phosphorylation of mTOR (S2448) and p70S6K1 (T389) rise with a time course that parallels that of Akt activation. Based on the experimental data obtained, a signalling pathway involving a β-arrestin 1 scaffolding complex and EGFR to activate Akt signalling is proposed.

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Introduction

Obestatin, a 23-amino acid peptide encoded by the ghrelin gene, was originally isolated from stomach showing to be a circulating peptide whose secretion is pulsatile and displays an ultradian rhythmicity similar to ghrelin and GH secretion (Zhang *et al*. 2005). Obestatin was initially characterized as an anorexigenic peptide being a physiological opponent of ghrelin. This action appeared to be mediated by the orphan receptor GPR39 that belongs to the family of the ghrelin receptor (GH secretagogues receptor type 1a, GHS-R1a) and the motilin receptor (Zhang *et al*. 2005). Since the discovery of obestatin, several observations have decreased the initial enthusiasm about the potential of this molecule putting the effectiveness of obestatin as an anorexigenic peptide into question. Several studies (Gourcerol *et al*. 2006, Seoane *et al*. 2006, Nogueiras *et al*. 2007, Yamamoto *et al*. 2007, Zizzari *et al*. 2007) with the exception of four reports (Bresciani *et al*. 2006, Carlini *et al*. 2007, Lagaud *et al*. 2007, Tremblay *et al*. 2007) were unsuccessful to reproduce the anorexigenic property of obestatin initially reported. Furthermore, several groups were unable to confirm that obestatin binds to GPR39 or activates signalling in transfected cells, suggesting that obestatin is unlikely to be the cognate ligand for this receptor (Chartrel *et al*. 2007, Holst *et al*. 2007). In spite of it, a recent work demonstrated that obestatin is a metabolic hormone capable of binding to GPR39 to regulate the functions of diverse gastrointestinal and adipose tissues (Zhang *et al*. 2008). Thus, the state-of-knowledge on obestatin leaves

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significant unanswered issues, specially the basis for the lack of reproducible biological actions for this ghrelin-associated peptide. Throughout this period, additional actions for this peptide have been reported providing evidence of biological functionality (Dun *et al.* 2006, Szentirmai & Krueger 2006, Carlini *et al.* 2007, Samson *et al.* 2007, Zizzari *et al.* 2007). Among them, it is remarkable that the mitogenic effect described for obestatin in human retinal pigment epithelial cells (hRPE; Camiña *et al.* 2007*a*), human gastric carcinoma cell line KATO-III (Pazos *et al.* 2007), pre-adipocytes (Zhang *et al.* 2008) and pancreatic β-cells (Granata *et al.* 2008). A signalling pathway involving the consecutive activation of G_i, phosphatidylinositol 3-kinase, novel protein kinase Cε (PKCε), and Src for extracellular signal-regulated kinases 1/2 (ERK1/2) activation mediates this effect in hRPE and KATO-III cells (Camiña *et al.* 2007*a*, Pazos *et al.* 2007). The fact that obestatin modulates cell proliferation of gastric cells, one of the main sources of this peptide, points to the involvement of this peptide in diverse processes such as repair gastric mucosal damage or as fuel for gastric cancer cell proliferation. It is intriguing that this mitogenic effect is not observed for ghrelin. Because obestatin and ghrelin are derived from the same peptide precursor, this lack of functional correlation supports the concept that obestatin is a biologically relevant peptide and not only a non-functional connective peptide.

The present work addresses the role of obestatin to stimulate Akt signalling, a serine/threonine kinase that acts as a central player in the regulation of metabolism, apoptosis, transcription and cell cycle (Manning & Cantley 2007). As a model, the human gastric carcinoma cell line KATO-III was used to characterize the intracellular signalling pathway. Results were reproduced on the gastric adenocarcinoma cell line AGS, which also endogenously expresses the GPR39 receptor.

Materials and methods

Materials

Human obestatin was obtained from Phoenix Pharmaceuticals Inc. (Belmont, CA, USA). Pertussis toxin (PTX) was obtained from Sigma Chemical Co. Wortmannin, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo [3,4-d]pyrimidine (PP2), 4-amino-7-phenylpyrazol [3,4-d]pyrimidine (PP3), 4-(3-chloroanilino)-6, 7-dimethoxyquinazoline (AG1478) and *N*-[(2*R*)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide (GM6001) were purchased from Calbiochem (Merck KGaA). Rabbit polyclonal IgG antibodies to phospho-p44/42 mitogen-activated protein kinases (MAPK), p44/42 MAPK, phospho-Src(Y416), phospho-Akt HM(S473), phospho-Akt A-loop(T308), Akt, Rictor, phospho-mTOR (S2448), phospho-p70S6K1(T389), phospho-PDK1 (S241), Rictor siRNA and control siRNA were purchased from Cell Signalling Technology (Beverly, MA, USA). Anti-phosphotyrosine rabbit polyclonal IgG antibody was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Goat polyclonal IgG antibodies to β-arrestin 1 and HRP, rabbit polyclonal IgG antibodies to β-actin and EGFR, β-arrestin 1 siRNA and control siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-GPR39 rabbit polyclonal IgG antibody was from Abcam (Cambridge, MA, USA). Anti-rabbit IgG HRP was purchased from Amersham Pharmacia. Prof. R J Lefkowitz (Duke University Medical Center, Durham, NC, USA) kindly provided rabbit anti-rat β-arrestin 1C-terminal (A1CT) antiserum. The cDNA encoding the Gβγ sequester β-ARK-CT was a gift of Dr P Voigt (Institute of Pharmacology, Charité-Medical University, Campus Benjamin Franklin, Berlin, Germany).

Cell culture

Human gastric cancer cell lines, KATO-III and AGS, were cultured as described by the supplier (ECACC, Wiltshire, UK). Briefly, KATO-III cells were seeded in 100 mm dishes and culture in RPMI-1640 medium supplemented with 20% (v/v) foetal bovine serum (FBS), 100 U/ml penicillin G, 100 mg/ml streptomycin sulphate and 2.5 mM L-glutamine with 5% CO_2 and 37 °C. AGS cells were seeded in 100 mm dishes and cultured in F-12 Ham medium supplemented with 10% (v/v) FBS, 100 U/ml penicillin G, 100 mg/ml streptomycin sulphate and 2.5 mM L-glutamine with 5% CO_2 at 37 °C.

Cell transient transfection

The cDNA encoding Gβγ sequester β-ARK-CT was transfected into subconfluent KATO-III cells using Lipofectamine (Invitrogen) following the manufacturer's protocol. The β-ARK-CT incorporation was confirmed by means of intracellular calcium mobilization in transfected cells before and after treatment as previously described (Theodoropoulou *et al.* 2006).

Immunoblotting analysis

Serum-starved cells were stimulated with obestatin for the indicated time period and doses at 37 °C. The medium was then aspirated and cells were lysed in

ice-cold lysis buffer (RIPA buffer: 50 mM Tris–HCl pH 7.2, 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 0.25% (w/v) Na-deoxycholate, protease inhibitor cocktail (1:100, Sigma), phosphatase inhibitor cocktail (1:100, Sigma)). The soluble cell lysates were pre-cleared by centrifuging at 13 000 ***g*** for 15 min. The protein concentration was evaluated with the Quanti-Pro BCA Assay Kit (Sigma). The same amount of protein of each sample was separated on 10% SDS/polyacrilamide gels and transferred to nitrocellulose membranes (Bio-Rad). The blots were incubated with 5% non-fatty milk in Tris buffered solution/Tween 20 (TBST) (20 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% (v/v) Tween-20, used for all incubation and washing steps) for 1 h. Then, blots were incubated for 1 h with corresponding antibodies, according to the manufacturer's instructions and were subsequently incubated with the corresponding peroxidase-conjugated IgG antibody. After washing, signals were visualized using ECL plus Western Blotting Detection System (Amersham Pharmacia Biotech). The blots shown are representative of three experiments. Densitometry was performed using IMAGEJ 1.40 g program.

Immunoprecipitation procedure

Serum-starved KATO-III cells were stimulated with obestatin 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 (1:100, Sigma); phosphatase inhibitor cocktail (1:100, Sigma)). Five hundred micrograms of total protein were pre-washed with 20 µl of 50% protein A/G-agarose (Santa Cruz) for 30 min at 4 °C, and then, incubated with 1 µg corresponding antibody (overnight at 4 °C) followed by addition of 40 µl of 50% protein A/G (2 h at 4 °C). After washing two times with ILB, the pelleted beads were resuspended in Laemmli sample buffer. Proteins were analyzed by SDS-PAGE, followed by western blotting.

Small interfering RNA (siRNA) silencing of gene expression

Chemically synthesized double-stranded siRNA duplexes (with 3′ dTdT overhangs) were purchased from Santa Cruz Biotechnology for the following targets: β-arrestin 1 (5′-AAAGCCUUCUGCGCGGA-GAAU-3′) and Rictor (5′-CACUUCGAUUAGUCA-GAAA-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 (Invitrogen) according to manufacturer's instructions. Silencing was quantified by immunoblotting. Only experiments with verified silencing were used.

Data analysis

Parameters were expressed as mean ± S.E.M. Statistical differences among means of percentages were identified with one-way ANOVA followed by Bonferroni *post hoc* test. P values of 0.05 or smaller were considered significant. *Denotes $P<0.05$ when comparing obestatin-treated with untreated control cells. #Denotes $P<0.05$ when comparing treatment + obestatin-treated with obestatin-treated cells.

Results

Obestatin-induced Akt activity is PTX-insensitive and not mediated by Gβγ dimmers

The dose response curves of Akt phosphorylation, at both the A-loop(T308) and the HM(S473), following stimulation of KATO-III cells with obestatin showed a dose-dependent pattern, being maximal at 200 nM (data not shown), so this concentration was used in subsequent experiments. Figure 1A shows that 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)) reached maximal levels within 10 min of obestatin stimulation (200 nM), keeping the sustained activity by at least 60 min. The role of $G_{i/o}$ protein was evaluated by pre-treatment with PTX, which uncouples $G_{i/o}$ protein from receptors (PTX; 100 ng/ml, 4 h). As shown in Fig. 1B, PTX did not modify phosphorylation at both the A-loop(T308) and the HM(S473) within 10 min of obestatin addition (200 nM). Overexpression of a peptide that contains the Gβγ binding domain (Gβγ sequesters, β-ARK-CT) had no effect on Akt phosphorylation at both residues, ruling out the involvement of the βγ-subunit of G proteins (Fig. 1C). Pre-treatment with the PI3K inhibitor wortmannin (1 µM, 30 min) completely inhibited the obestatin-induced Akt phosphorylation at both residues (data not shown).

PDK1 and mTORC2 mediate Akt phosphorylation activated by obestatin

Figure 2A shows that PDK1 phosphorylation at S241 (pPDK1(S241)) reached maximal levels within 5–10 min of obestatin stimulation (200 nM), keeping

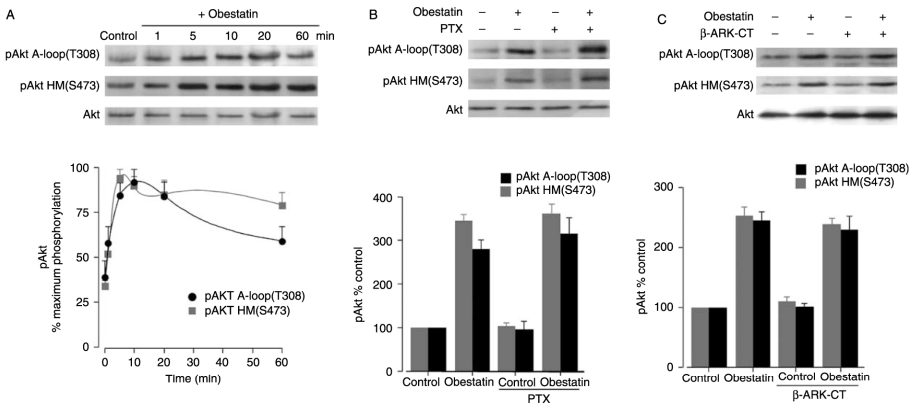

Figure 1 (A) Time-course of the effect of obestatin (200 nM) on Akt phosphorylation at A-loop(T308) and HM(S473). (B) Effect of PTX (100 ng/ml, 4 h) on obestatin-induced Akt phosphorylation (200 nM, 10 min). (C) Obestatin-induced Akt phosphorylation (200 nM, 10 min) in the absence or presence of βγ sequester β-ARK-CT. Akt phosphorylation was quantified by densitometry and expressed as a percentage of the maximal phosphorylation obtained for each residue (A) or as the percentage of the basal phosphorylation obtained in control cells for each residue (B and C) (mean±S.E.M. of three independent experiments). Blots were representative of three independent experiments developed in KATO III cells.

a sustained phosphorylation for 60 min after stimulation. This increase was parallel to the phosphorylation of the Akt A-loop(T308). Pre-treatment of the cells with PTX (100 ng/ml, 4 h) had no effect on PDK1 phosphorylation in response to obestatin (200 nM, 10 min; Fig. 2B). Transient transfection with β-ARK-CT did not inhibit the obestatin-induced PDK1(S241) phosphorylation (Fig. 2C). On the other hand, ablating mTORC2 function by siRNA targeting Rictor (75% reduction in Rictor expression), Akt HM(S473) phosphorylation was reduced by 63% (Fig. 2D).

Src, MMP and EGFR regulate Akt activity in response to obestatin

Akt phosphorylation at both residues was strongly inhibited by the selective non-receptor tyrosine kinase Src inhibitor PP2 (5 μM, 30 min; Fig. 3A) in response to obestatin (200 nM, 10 min). This inhibition was specific, since pre-treatment with PP3 (5 μM, 30 min), a negative control for PP2, had no effect on the obestatin-induced Akt phosphorylation at both residues. Pre-treatment with GM6001 (1 μM, 1 h), an inhibitor of Zn^{2+}-activated metalloproteinases that catalyzed the shedding of the EGF-like factors, inhibited Akt phosphorylation at both residues (Fig. 3A). Pre-treatment with AG1478 (1 μM, 30 min), the potent and specific inhibitor of the EGFR, also inhibited the Akt phosphorylation by obestatin (200 nM, 10 min; Fig. 3B). By contrast, GM6001 (1 μM, 1 h) and AG1478 (1 μM, 30 min) had no effect on ERK1/2 phosphorylation in response to obestatin (Fig. 3A and B respectively). Obestatin-stimulated EGFR phosphorylation was assessed by immunoprecipitation of the EGFR followed by immunoblotting with anti-phosphotyrosine antibody (pY). As shown in Fig. 3C, obestatin induced a rapid EGFR phosphorylation that resembled with the dynamic of Akt activation. Furthermore, pre-treatment with PP2 (5 μM, 30 min) or GM6001 (1 μM, 1 h), inhibited the increase in tyrosine phosphorylation of EGFR in response to obestatin (200 nM, 5 min; Fig. 3D).

Regulation of Akt phosphorylation by β-arrestin 1

Immunoprecipitation assays of β-arrestin 1 showed the association with the activated form of Src (pSrc(Y416)) and GPR39 in response to obestatin (200 nM, 10 min; Fig. 4A), indicating that obestatin does induce the association of GPR39/β-arrestin 1/Src complex. Next, the role of β-arrestin 1 on Akt phosphorylation was evaluated by siRNA technique to down-regulate the expression of endogenous β-arrestin 1 in KATO III cells. siRNA experiments targeting β-arrestin 1 reduced its expression by

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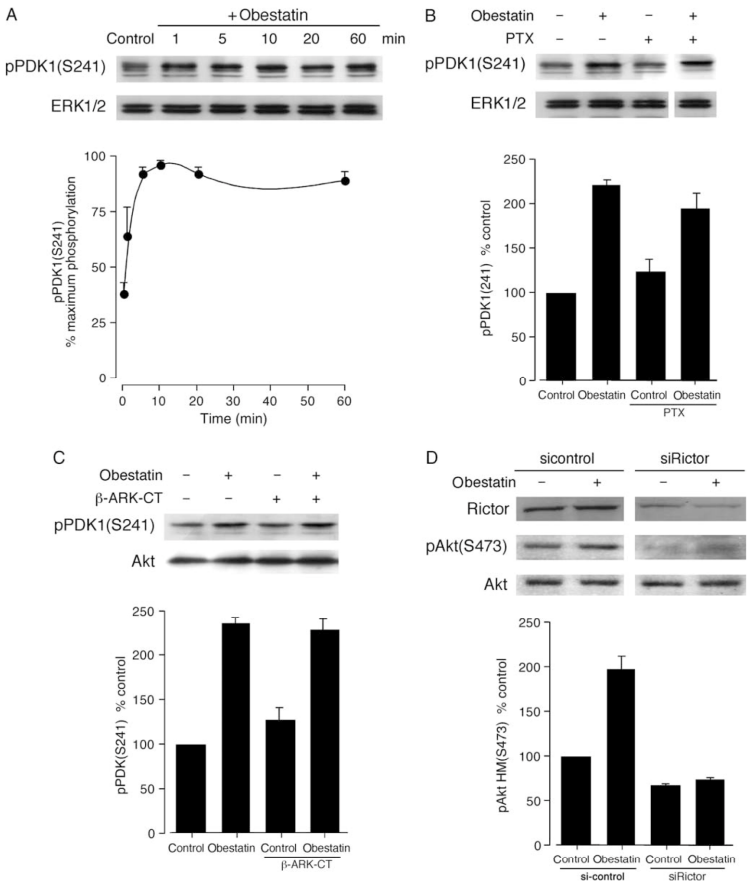

Figure 2 (A) Time-course of the effect of obestatin (200 nM) on phosphorylation of PDK1. Cell extracts were analyzed by SDS-PAGE using specific antibody against pPDK1(S241). PDK1 phosphorylation was quantified by densitometry and expressed as a percentage of the maximal phosphorylation (mean ± S.E.M. of three independent experiments). (B) PDK1 phosphorylation induced by obestatin (200 nM, 10 min) in the absence or presence of PTX (100 ng/ml, 4 h). PDK1 phosphorylation was quantified by densitometry and expressed as the percentage of the basal phosphorylation obtained in control cells (means ± S.E.M). (C) Obestatin-induced PDK1 phosphorylation (200 nM, 10 min) in the absence or presence of βγ sequester β-ARK-CT. (D) Effect of siRNA depletion of Rictor on obestatin-induced Akt HM(S473) phosphorylation. KATO III cells transfected with Rictor siRNA were stimulated with obestatin (200 nM, 10 min) at 37 °C. After stimulation, cell extracts were prepared as described in Experimental Procedures. Equal amounts of protein in each sample were used to assess the expression of Rictor and Akt HM(S473) phosphorylation by western blotting. Akt phosphorylation was quantified by densitometry and expressed as the percentage of the basal phosphorylation obtained in control cells (means ± S.E.M). In A, B, C and D, blots were representative of three independent experiments developed in KATO III cells.

67 ± 2%. In the presence of a non-targeting control siRNA, obestatin-activated Akt phosphorylation was identical to that observed without any transfection (data not shown). Under these conditions, β-arrestin 1 siRNA decreased both the A-loop(T308) and the HM(S473) phosphorylation after 10 min of obestatin stimulation (200 nM) with respect to siRNA control (90 ± 1% at S473; 90 ± 3% at T308). Furthermore, β-arrestin 1 siRNA significantly reduced the phosphorylation of Src(Y416) by 55 ± 5% (Fig. 4B).

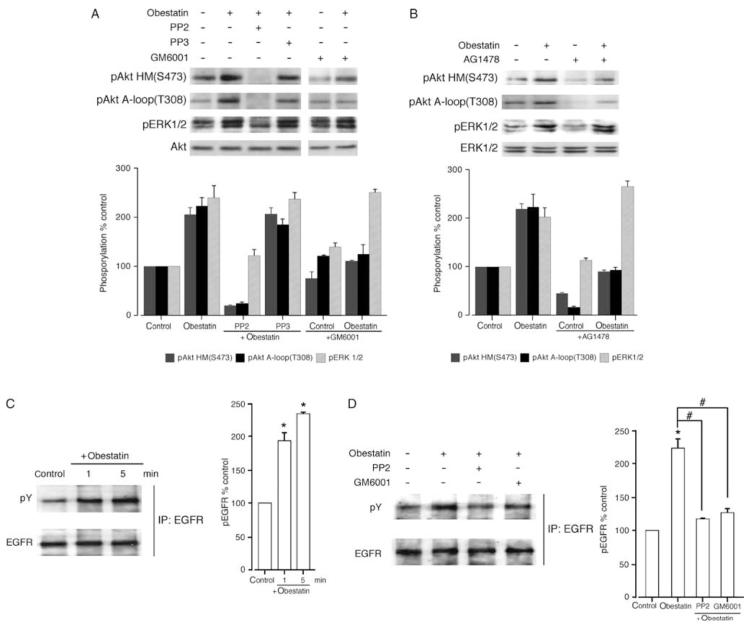

Figure 3 (A) Effects of the Src inhibitor PP2 and the Zn^{2+}-activated metalloproteinases inhibitor GM6001 on the obestatin-induced Akt and ERK1/2 phosphorylations. (B) Effect of the EGFR inhibitor AG1478 on the obestatin-induced Akt and ERK1/2 phosphorylations. Serum-starved cells were pre-treated with PP2 (5 μM, 30 min), PP3 (5 μM, 30 min), GM6001 (1 μM, 1 h) or AG1478 (1 μM, 30 min) previous to obestatin stimulation (200 nM, 10 min). Akt and ERK1/2 phosphorylations were quantified by densitometry and expressed as the percentage of the basal phosphorylation obtained in control cells (mean ± S.E.M. of three independent experiments). (C) Effect of obestatin on EGFR phosphorylation. Cells were incubated with obestatin (200 nM) at 37 °C, lysed and immunoprecipitated (IP) with antibodies to EGFR, and then analyzed by western blotting with pY and EGFR antibodies. (D) Effects of the Src inhibitor PP2 and the Zn^{2+}-activated metalloproteinases inhibitor GM6001 on the obestatin-induced EGFR phosphorylation. Cells were incubated with obestatin (200 nM, 5 min) at 37 °C, immunoprecipitated (IP) with antibodies to EGFR and then analyzed by western blotting with pY and EGFR antibodies. In (C) and (D) EGFR phosphorylation was quantified by densitometry and expressed as the percentage of untreated cells (control; mean ± S.E.M. of three independent experiments; *denotes $P < 0.05$ when comparing obestatin-treated with untreated control cells; # denotes $P < 0.05$ when comparing obestatin-treated with PP2/GM6001 + obestatin-treated cells). In A, B, C and D, blots were representative of three independent experiments developed in KATO III cells.

Obestatin regulates mTOR and p70S6K1 phosphorylation

Obestatin (200 nM) promoted rapid increases (maximal in 5–10 min) in mTOR phosphorylation at S2448 (Fig. 5A). Furthermore, obestatin (200 nM) evoked an increase in p70S6K1 phosphorylation at T389 (Fig. 5B). The dynamic of this phosphorylation showed a maximum at 10 min that was followed by a 50% reduction over 20 min period post-stimulus. Pre-treatment of cells with rapamycin (50 nM, 30 min) inhibited the p70S6K1(T389) phosphorylation in response to obestatin (200 nM, 10 min), implicating mTOR as an upstream mediator in this pathway (Fig. 5C).

Regulation of Akt phosphorylation by β-arrestin 1, Src and EGFR in AGS cells

Obestatin activated Akt phosphorylation at both the A-loop(T308) and the HM(S473) in a dose-dependent manner being maximal at 200 nM and 10 min post-stimulation, keeping the sustained activity by at least 60 min (data not shown). Akt phosphorylation was inhibited by GM6001 (1 μM, 1 h), AG1478 (1 μM, 30 min) and PP2 (5 μM, 30 min) pre-treatments in response to obestatin (200 nm, 10 min; Fig. 6A and B). Furthermore, siRNA experiments targeting β-arrestin 1 decreased both the Akt (84 ± 9% at S473; 77 ± 3% at T308; reduction of β-arrestin 1 expression 77 ± 1%)

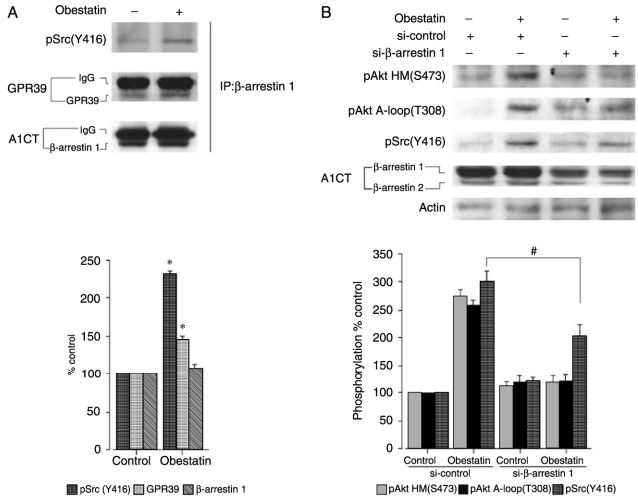

Figure 4 (A) Effect of obestatin on the assembly of complexes containing pSrc(Y416), GPR39 and β-arrestin 1. Cells were incubated with obestatin (200 nM, 10 min) at 37 °C, lysed and immunoprecipitated (IP) with antibodies to β-arrestin 1, and then analyzed by western blotting with pSrc(Y416), GPR39 and β-arrestin (A1CT) antibodies. pSrc(Y416), GPR39 and β-arrestin 1 were quantified by densitometry in IP and expressed as the percentage of the proteins obtained in control cells (mean ± S.E.M. of three independent experiments; *denotes $P<0.05$ when comparing obestatin-treated with untreated control cells). (B) Effect of siRNA depletion of β-arrestin 1 on obestatin-induced Akt and Src phosphorylations. Cells transfected with β-arrestin 1 siRNA were serum-starved for 12 h and then stimulated with obestatin (200 nM, 10 min) at 37 °C. After stimulation, equal amounts of protein in each sample were used to assess Akt A-loop(T308) and HM(S473) phosphorylation, pSrc(Y416) and β-arrestins by western blotting. Akt and Src phosphorylations were quantified by densitometry and expressed as the percentage of the basal phosphorylation obtained in control siRNA cells (mean ± S.E.M.; # denotes $P<0.05$ when comparing β-arrestin 1 siRNA obestatin-treated with si-control + obestatin-treated cells). In A and B, blots are representative of three independent experiments developed in KATO III cells.

and Src (63 ± 13% at Y416) phosphorylations at 10 min after obestatin stimulation (200 nM) with respect to siRNA control (Fig. 6C).

Discussion

The present study offers three major findings related to the activation of Akt in response to obestatin. First, obestatin-induced Akt phosphorylation requires EGFR transactivation and MMP activity through a mechanism that does involve neither $G_{i/o}$-proteins nor Gβγ dimers. Second, obestatin induces the association of GPR39/β-arrestin 1/Src signalling complex resulting in the transactivation to the EGFR and downstream Akt signalling. Third, PDK1 and mTORC2 are essential for A-loop(T308) and HM(S473) phosphorylation of Akt respectively. Thus, results shown provide support for the notion that obestatin activates in parallel the EGFR/Akt and the $G_{i/o}$/MEK/ERK pathway.

The protein kinase Akt exerts a key signalling node that regulates the control of cell proliferation, survival, metabolism and nutrient uptake in a cell-type-specific manner through a variety of down-stream targets (Manning *et al.* 2007). From the results presented so far, obestatin activates Akt by two distinct phosphorylation events, both of which depend on PI3K. Obestatin receptor activates PI3K to regulate several downstream signalling pathways through the generation of the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 allows membrane translocation of proteins containing PH domain such as PDK1 and Akt. Then, PDK1 autophosphorylates at S241 leading to its own activation and consequently phosphorylates Akt A-loop(T308) (Casamayor *et al.* 1999, Storz & Toker 2002). Finally, the full activation of Akt involves the phosphorylation of HM(S473). Our study shows that ablating mTOR complex 2 function by siRNA targeting Rictor, impaired obestatin-stimulated HM(S473) phosphorylation of Akt. This fact supports the model by which both Akt and PDK1

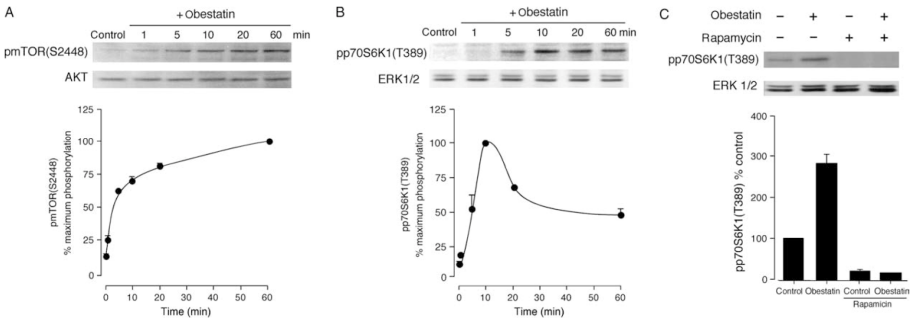

Figure 5 Time-course of the effect of obestatin (200 nM) on mTOR (A) and p70S6K1 (B) phosphorylations. pmTOR(S2448) and pp70S6K1(T389) were quantified by densitometry and expressed as a percentage of the maximal phosphorylation (mean±S.E.M. of three independent experiments). (C) Effect of rapamycin (50 nM, 30 min) on obestatin-induced p70S6K1 phosphorylation (200 nM, 10 min). pp70S6K1(T389) was quantified by densitometry and expressed as the percentage of the basal phosphorylation obtained in control cells (mean±S.E.M. of three independent experiments). In A, B and C, blots are representative of three independent experiments developed in KATO III cells.

interact by colocalization at the plasma membrane, where Akt is further phosphorylated by mTORC2 (Guertin & Sabatini 2007). Despite being a key element in the Akt activation, the way mTORC2 is activated remains unknown. Based on the PH-like domain of mSIN1, mTORC2 and Akt may interact as consequence of colocalization at plasma membrane when PI3K is activated (Schroder *et al*. 2007). Other possibility would be that other upstream signals regulate the activation of the mTORC2 complex, for instance such Ras as mSIN1 contains a Ras-binding domain (Lee *et al*. 2005, Schroder *et al*. 2007). In addition to mTORC2 activation, obestatin mediates the phosphorylation of mTOR at S2448, an important site for regulation of mTOR function (Kenerson *et al*. 2002, Reynolds *et al*. 2002), and the phosphorylation of p70S6K1 at T389, an event blocked by rapamycin, thereby implicating mTOR complex 1 (mTORC1: Raptor, mLST8, PRAS40, mTOR kinase). Therefore, obestatin regulates the activity of mTORC1 and mTORC2, and this fact involves this peptide in cell growth and G1 cell cycle progression through activation of p70S6K1 (Pullen & Thomas 1997).

We first described that obestatin stimulates cell proliferation by MEK/ERK1/2 phosphorylation in the KATO-III cells (Pazos *et al*. 2007). Although obestatin activates ERK1/2 through $G_{i/o}$-protein dependent signalling pathway, the lack of effect of PTX and the Gβγ sequester on Akt phosphorylation rules out $G_{i/o}$-protein and Gβγ dimers as upstream signals for Akt activation. The blocking effect of AG1478 and GM6001 indicate that obestatin-mediated Akt activation requires EGFR transactivation and MMP activities. Indeed, obestatin induced a striking increase in EGFR tyrosine phosphorylation, an effect inhibited by GM6001 pre-treatment. This is congruent with EGFR transactivation through the proteolytic release of EGF-like ligands at the cell surface that activate EGFR by autocrine or paracrine stimulation (Carpenter 2000, Olayioye *et al*. 2000, Prenzel *et al*. 2001, Ohtsu *et al*. 2006, Higashiyama *et al*. 2008). Little is known regarding the detailed upstream mechanisms that link G protein-coupled receptors and their effectors for MMPs activation. Besides phosphorylation, MMPs are regulated through protein–protein interactions. In fact, several kinases such as PKC, PYK2 and Src were identified as direct MMP interacting proteins (Ohtsu *et al*. 2006). Our results showed that Src acts upstream of Akt, as PP2 inhibited the obestatin-induced Akt phosphorylation at both A-loop(T308) and HM(S473). Because Src contains a SH3 domain, it is proposed that Src interacts with the PXXP motif of MMPs to transactivate EGFR (Seals & Courtneidge 2003). This model is supported by the fact that coimmunoprecipitation assays show that β-arrestin 1 recruits Src through the formation of a β-arrestin complex. Furthermore, β-arrestin 1 mediates Src and Akt activation, as shown by the inhibition of Src phosphorylation at Y416 and Akt phosphorylation at both residues after depletion of β-arrestin 1 by siRNA. Src activation by β-arrestin might result from a conformational change induced by β-arrestin binding, as reported for the β_2 adrenergic receptor (Luttrell *et al*. 1999), neurokinin 1 receptor (DeFea *et al*. 2000) and

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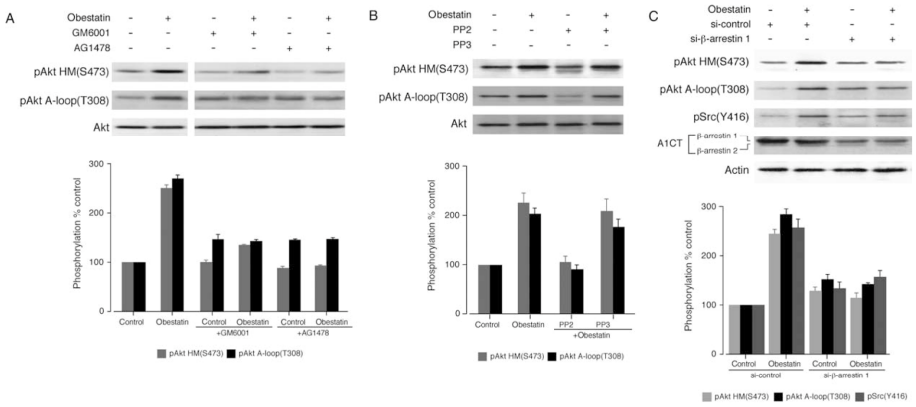

Figure 6 Effects of the Zn^{2+}-activated metalloproteinases inhibitor GM6001, EGFR inhibitor AG1478 (A) and Src inhibitor PP2 (B) on the obestatin-induced Akt phosphorylation in AGS cells. Serum-starved AGS cells were pre-treated with GM6001 (1 μM, 1 h), AG1478 (1 μM, 30 min), PP2 (5 μM, 30 min) or PP3 (5 μM, 30 min) previous to obestatin stimulation (200 nM, 10 min). (C) Effect of siRNA depletion of β-arrestin 1 on obestatin-induced Akt and Src phosphorylations in AGS cells. After siRNA transfections, cells were stimulated with obestatin (200 nM, 10 min) at 37 °C and then equal amounts of protein in each sample were used to assess Akt A-loop(T308) and HM(S473) phosphorylation, pSrc(Y416) and β-arrestins (A1CT) by western blotting. In A, B and C, phosphorylations were quantified by densitometry and expressed as the percentage of the basal phosphorylation obtained in control cells (mean ± S.E.M). Blots are representative of three independent experiments.

ghrelin receptor (Camiña *et al.* 2007*b*). To date, this β-arrestin mechanism was not suggested to be EGFR dependent. Thus, β-arrestin 1 functions as an adaptor that recruits Src to GPR39, leading to the activation of MMP through a GPR39/β-arrestin 1/Src complex, and ultimately, EGFR transactivation. This model is further supported by the fact that PP2 pre-treatment inhibited EGFR tyrosine phosphorylation in response to obestatin. The interplay between G-protein and β-arrestin to transactivate EGFR was described for prostaglandin E2 receptor and β1-adrenergic receptor transactivation of EGFR (Buchanan *et al.* 2006, Noma *et al.* 2007). It seems reasonable to speculate that MMP activation by obestatin involves receptor endocytosis and compartmentalization determining spatial regulation for specific activation of Akt signalling.

Adding to the complexity and obvious cell type specificity of EGFR transactivation pathways, we found that the activation of both ERK1/2 and Akt signalling pathways act in parallel in KATO III cells. This is supported by the fact the suppression of EGFR tyrosine kinase by AG1478 or MMP activity by GM6001 treatment did not inhibit the obestatin-stimulated ERK1/2 phosphorylation. Furthermore, PTX treatment had no effect on obestatin-induced Akt and PDK1 activation. In this way, EGFR transactivation is the link between obestatin receptor and Akt signalling pathway, whereas $G_{i/o}$ proteins regulates ERK1/2 pathway. A similar signalling network was described for ANG II-stimulated mitogenesis in intestinal epithelial cells (Chiu *et al.* 2005) where ErbB/PI3K/Akt/mTOR/p70S6K1 and G_q/PLC/PKC/MEK/ERK pathways act in parallel. It is becoming clear that the signalling specificity depends not only on the presence of a specific ErbB receptor from EGFR family, but also on the biochemical characteristics of the individual EGF-like ligand (Olayioye *et al.* 2000, Ohtsu *et al.* 2006). These ligands are bivalent, a property that determines which homo- or heterodimer combinations are formed and the downstream signalling to be activated (Olayioye *et al.* 2000). Furthermore, β-arrestin-scaffolded complex presumably places the different components in close proximity ensuring substrate specificity or MMP activity. Such elaborate mechanisms reflect the importance of this transactivation process in obestatin signalling.

Recent studies have shown that Zn^{2+} induces EGFR phosphorylation through the extracellular release of EGF-like ligands that are mediated by MMPs (Wu *et al.* 2004, Hwang *et al.* 2005). Zn^{2+} exposure has been shown to activate MAPKs and PI3K/Akt pathways through activation of EGFR in various cell types (Wu *et al.* 2002, 2005, Samet *et al.* 2003).

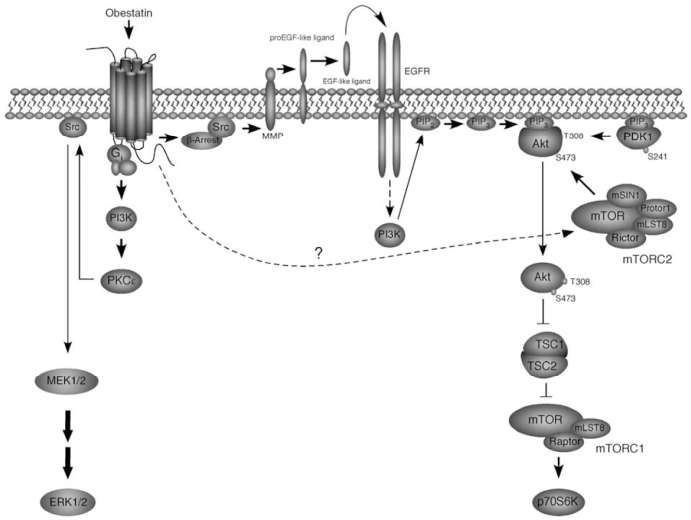

Figure 7 Proposed model of signalling pathway for Akt and ERK1/2 activation in response to obestatin. Translocation of β-arrestins 1 to obestatin receptor (GPR39) allows its association with Src. β-arrestin 1 activates Src (phosphorylation at Y416) that initiates the transactivation of EGFR and subsequent downstream Akt signalling.

These findings also suggested that there are marked cell-type-specific differences in the mechanism of EGFR activation induced by Zn^{2+} exposure. In view of this fact, the stimulatory effects of Zn^{2+} on GPR39 signalling might be due to the activation of MMP-EGFR since obestatin requires EGFR transactivation and MMP activities. It remains to determine the function of Zn^{2+} on GPR39 signalling lacking MMP and/or EGFR to clearly define its function as ligand or ago-allosteric modulator on this receptor (Storjohann *et al*. 2008).

Taken together, our data in gastric cancer cells (KATO-III and AGS) are consistent with a model in which Akt signalling pathway is activated by EGFR transactivation. Once obestatin receptor is activated, a signalling pathway is mediated by β-arrestin 1 involving the recruitment and activation of Src into a β-arrestin-scaffolded complex. Thus, Src functions as a switch that activates MMPs to initiate the proteolytic release of EGF-like ligands at the cell surface and then bind to EGFR. Ligand binding drives receptor dimerization, leading to activation of the intrinsic kinase and autophosphorylation of specific docking sites, among them for PI3K. Activation of PI3K generates the second messenger PIP3 that allows the translocation of Akt to the plasma membrane through the binding of its PH domain. Akt is phosphorylated at Akt A-loop(T308) and HM(S473) by PDK1 and mTORC2 respectively. Then, activated Akt inactivates the heterodimer TSC1/TSC2 leading the activation of mTORC1 and the phosphorylation of downstream targets, p70S6K1 among them (Fig. 7). mTORC1 mediates phosphorylation of p70S6K1(T389) within the hydrophobic motif, whereas PDK1 is responsible for phosphorylation of the p70S6K1 at the T loop. This signalling network adds a new component to the intracellular signalling targets regulated by obestatin. Furthermore, obestatin is added to the group of MMPs regulator factors, which have been implicated in diverse human diseases, such as inflammatory diseases and cancer (Seals & Courtneidge 2003, Huovila *et al*. 2005). It is quite likely that obestatin-induced EGFR transactivation could be a key mechanism by which MMPs regulate these diseases. In support of this hypothesis, numerous studies demonstrate that altered expression and/or mutations in EGFR/ErbB receptor family members are observed in tumours and the cell lines derived from these tumours, and these alterations may contribute to cancer progression (Normanno *et al*. 2006, Ohtsu *et al*. 2006, Bhola & Grandis 2008). Elucidation of the detailed activation/regulation mechanism of EGFR transactivation by obestatin and the pathophysiological significance of the signalling events are intriguing areas for further research.

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Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Role of obestatin on growth hormone secretion: An *in vitro* approach

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ABSTRACT

Obestatin, the ghrelin-associated peptide, showed to activate MAPK signaling with no effect on Akt nor cell proliferating activity in rat tumor somatotroph cells (growth cells, GC). A sequential analysis of the obestatin transmembrane signaling pathway indicated a route involving the consecutive activation of G_i, PI3k, novel PKCε, and Src for ERK1/2 activation. Furthermore, obestatin treatment triggers growth hormone (GH) release in the first 30 min, being more acute at 15 min. At 1 h, obestatin treated cells showed the same levels in GH secretion than controls. Added to this functionality, obestatin was secreted by GC cells. Based on the capacity to stimulate GH release from somatotroph cells, obestatin may act directly in the pituitary through an autocrine/paracrine mechanism.

Introduction

Obestatin, a 23-amino acid peptide encoded by the ghrelin gene was originally isolated from stomach showing to be a circulating peptide whose secretion is pulsatile and displays an ultradian rhythmicity similar to ghrelin and growth hormone secretion [1]. Obestatin was initially characterized as an anorexigenic peptide being a physiological opponent to ghrelin. This action appeared to be mediated by the orphan receptor GPR39, which belongs to the family of the ghrelin receptor growth hormone secretagogue receptor type 1a (GHS-R1a) and the motilin receptor [1]. Since the discovery of obestatin, several observations have decreased the initial enthusiasm about the potential of this molecule putting the effectiveness of obestatin as an anorexigenic peptide into question. Throughout this period, additional actions for this peptide have been reported providing evidence of biological functionality [2–6]. Among them, it is remarkable the mitogenic effect described for obestatin in human retinal pigment epithelial cells (hRPE) [7], human gastric carcinoma cell line KATO-III [8], pre-adipocytes [9] and pancreatic β-cells [10].

In addition to its orexigenic effect, it is well established that ghrelin is a potent GH secretagogue. Due to the opposition to the effects of obestatin on food intake, some works have been carried out aiming to show a potential antagonism of obestatin of the ghrelin-stimulated GH secretion. To our knowledge, only one of these publications showed an effect of this peptide on growth hormone secretion, pointing to an inhibition of the stimulating effect of ghrelin on GH *in vivo* [4].

In the present work, the ability of obestatin to modulate growth hormone secretion in cultured somatotroph cells was evaluated. As a model, the GC cells derived from a rat somatotroph tumor were used to evaluate the action of obestatin and to characterize the intracellular signaling pathway.

Materials and methods

Materials. Mouse obestatin was obtained from California Peptide Research (Napa, CA, USA). Pertussis toxin (PTX) and cholera toxin (ChTx) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Wortmannin and 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2) and calphostin C were purchased from Calbiochem (Merck KGaA, Darmstadt, DE). Rabbit

Abbreviations: AC, adenylyl cyclase; ADP, adenosine diphosphate; cAMP, cyclic adenosine monophosphate; ChTX, cholera toxin; DAG, diacylglycerol; ERK, extracellular signal-regulated kinases; FBS, fetal bovine serum; GC, growth cells; GH, growth hormone; GHS-R1a, growth hormone secretagogue receptor type 1a; GPR-39, G protein coupled receptor 39; *icv*, intracerebroventricular; *ip*, intraperitoneal; *iv*, intravenous; MAPK, mitogen-activated protein kinases; PKC, protein kinase C; PI3k, phosphoinositide 3-kinase; PKA, cAMP-dependent protein kinase; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine; PP3, 4-amino-7-phenylpyrazol[3,4-d]pyrimidine; PS, phosphatidyl serine; PTX, pertussis toxin; hRPE, human retinal pigment epithelium cells

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polyclonal IgG antibodies to phospho-p44/42 mitogen-activated protein kinases (MAPK), p44/42 MAPK, phospho-Akt(S473), phospho-PKCε and epidermal growth factor receptor (EGFR) were purchased from Cell Signalling Technology (Beverly, MA, USA). Anti-goat IgG horseradish peroxidase and anti-β-actin rabbit polyclonal IgG antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit IgG horseradish peroxidase was purchased from Amersham Pharmacia (Arlington Heights. IL. USA). Anti-rabbit polyclonal IgG GPR39 antibody was obtained from Abcam plc (Cambridge, UK). Prof. Dr. I.C.A.F. Robinson (Division of Neurophysiology, National Institute for Medical Research, The Ridgeway, Mill Hill, London, UK) kindly provided GC cell line.

Cell culture. The GC cell line [11] was maintained routinely as a monolayer in complete DMEM medium supplemented with 15% (v/v) horse serum, 2.5% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin G, 100 mg/mL streptomycin sulfate and 2.5 mM L-glutamine. Cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Calcium measurements. Intracellular calcium measurements were performed in cell suspensions using fluorescent calcium indicator fura-2/AM as previously described [12]. Briefly, cells were resuspended (2×100 mm plates/mL) in Krebs–Ringer–Hepes solution (KRH) and loaded with 3 M fura-2/AM. For each measurement, around 2×10^6 cells were resuspended in 2 mL of KRH and then placed in a cuvette positioned in a holder at 37 °C. The fluorescence signal was measured under continues stirring in a LS-50B fluorimeter (Perkin-Elmer, Boston, MA) in ratio mode (λ_{ex1} = 345, λ_{ex2} = 380, and λ_{em} = 490 nm) and calibrated by the cell lysis method [13].

Radioimmunoassays. Obestatin levels in GC culture media were determined using specific RIA kits for total obestatin from Phoenix Pharmaceuticals Inc. (Belmont, CA, USA), according materials and protocol provided by the supplier. Growth hormone levels in GC culture media were determined by double antibody RIA using materials supplied by the National Hormone and Peptide Program (NHPP) as previously described [14]. Obestatin and GH determination was performed in serum-free conditions after a starvation of 24 h.

Immunoblotting analysis. Serum-starved GC cells were stimulated with obestatin for the indicated time period and doses at 37 °C. The medium was then aspirated and cells were lysed in ice-cold lysis buffer [RIPA buffer: 50 mM Tris–HCl pH 7.2, 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 0.25% (w/v) Na–deoxycholate, protease inhibitor cocktail (1:100; Sigma Chemical Co., St. Louis, MO, USA), phosphatase inhibitor cocktail (1:100, Sigma)]. The soluble cell lysates were pre-cleared by centrifuging at 13.000*g* for 15 min. The protein concentration was evaluated with the QuantiPro™ BCA Assay kit (Sigma Chemical Co., St. Louis, MO, USA). The same amount of protein of each sample was separated on 10% sodium dodecyl sulfate (SDS)/polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The blots were incubated with 5% non-fatty milk in Tris buffered solution/Tween-20 (TBST) [20 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% (v/v) Tween-20, used for all incubation and washing steps] for 1 h. Then, blots were incubated for 1 h with corresponding antibodies, according to the manufacturer's instructions and were subsequently incubated with the corresponding peroxidase-conjugated IgG antibody. After washing, signals were visualized using ECL plus Western Blotting kit (GE-Amersham, Buckinghamshire, UK). The blots shown are representative of three experiments. Image processing was performed using the NIH Image Software ImageJ 1.38×.

Proliferation assays. GC cells were seeded in 24-well plates at a density of 20×10^3 cells per well in 500 μL of DMEM medium. After 2 days, the medium was renewed and cells were cultured in a serum-free medium for 24 h. Cells were then treated with different doses of obestatin (100, 200 and 500 nM) or FBS (10% v/v) in fresh DMEM. After 48 h, cells were trypsinized and counted using a Coulter Counter. In all cases, triplicate dishes were used for each experiment point.

Data analysis. A total of three different experiments were included on each group for statistical analysis. Each experiment was performed in sextuplicate. The Student's *t* test was performed when comparing groups. *P* values of 0.05 or smaller were considered significant. $*$ denotes $P < 0.05$ and $**$ denotes $P < 0.001$, when comparing obestatin treated with untreated control cells.

Results

Initially, the action of obestatin on GC cells was evaluated by means of extracellular signal-regulated kinases (ERK1/2) phosphorylation. The kinetic of ERK1/2 activation following stimulation of GC cells with obestatin (200 nM) showed maximal levels of ERK1/2 phosphorylation after 15 min of obestatin treatment, which decreased to basal levels after 60 min (Fig. 1A). Previous results demonstrated that obestatin stimulates the production of the second messenger cyclic adenosine monophosphate (cAMP) [1]. Among the effectors of cAMP, it is included the cAMP-dependent protein kinase (PKA) and cAMP-dependent calcium channels. However no intracellular calcium rise was observed after obestatin stimulation of GC cells (data not shown), ruling out the implication of a Ca^{2+}-dependent signaling pathway. Pretreatment of the GC cells with pertussis toxin (PTX; 100 ng/mL, 12 h), which uncouples receptors from $G_{i/o}$, reduced the obestatin-dependent activation of ERK1/2 by ~85% (Fig. 1B). Nevertheless, pretreatment with cholera toxin (ChTX; 2 μg/mL, 4 h) that stimulates adenylyl cyclase (AC) by catalyzing adenosine diphosphate ribosylation (ADP-ribosylation) of the alpha chain (alpha s) of Gs, showed no significant modification on the ERK1/2 phosphorylation mediated by obestatin. $G_{i/o}$ protein is known to mediate downstream signals through activation of phosphoinositide 3-kinase (PI3k) in different cell types [15,16]. As shown in Fig. 1C, pretreatment with wortmannin (1 μM, 30 min) lead to an important reduction (~72%) of the ERK1/2 activation induced by obestatin (200 nM). Also, obestatin-induced ERK activation was strongly inhibited by PP2 (5 μM, 30 min), a selective Src inhibitor (Fig. 1C). This inhibition was specific, since pretreatment with PP3 (5 μM, 30 min), a negative control for PP2, had no effect on the obestatin-induced ERK1/2 phosphorylation (data not shown), supporting a role for Src protein in obestatin signaling.

In addition, the significant activation of ERK1/2 by obestatin (200 nM) was also inhibited ~64% by pretreatment with calphostin C (10 μM, 1 h), a cell permeable highly specific inhibitor of protein kinase C (PKC, Fig. 1D). As previously published [8], obestatin activates ERK1/2 phosphorylation by activating the novel PKCε, which is Ca^{2+} independent but still regulated by phosphatidyl serine (PS) and diacylglycerol (DAG) for activation [17]. As shown in Fig. 1E, obestatin (200 nM) considerably activated PKCε and reached a maximum 1 min after obestatin stimulation (~2-fold baseline) reaching prestimulatory level within 10 min. Since it was previously described that obestatin activates Akt phosphorylation in gastric cancer cell lines and this action was mediated by the GPR39 receptor [18], we tested whether Akt was involved in obestatin action in this cell line and the time-course curve of Akt phosphorylation at the hydrophobic motif (S473) [pAkt(S473)] was performed. Remarkably, obestatin (200 nM) failed to activate Akt phosphorylation although it decreased basal pAkt(S473) at the time tested (~57% inhibition, 5 min post-stimulation; Fig. 1F). Obestatin-induced Akt activation requires a signaling pathway involving EGFR transactivation in AGS cells [18]. Due to the lack of obestatin-evoked Akt activity, the expression of EGFR was tested

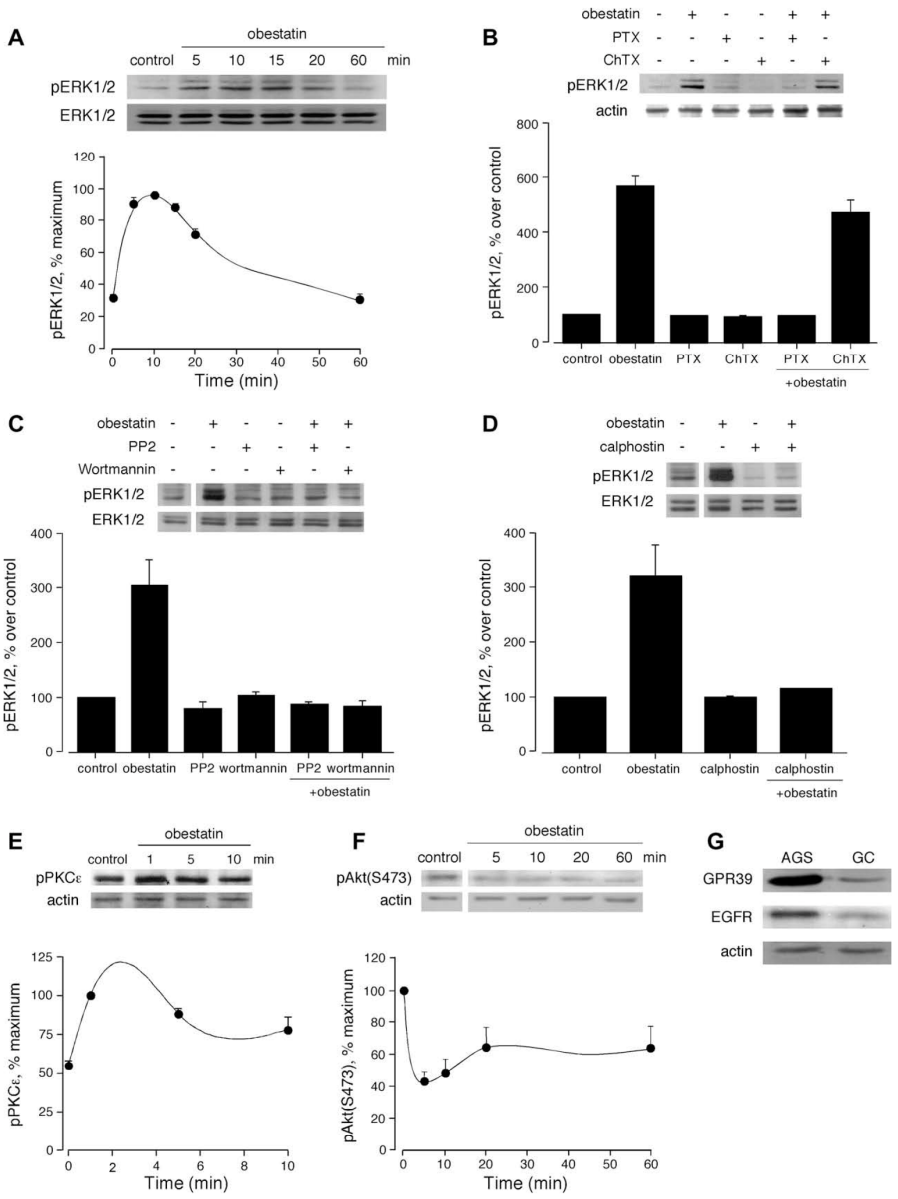

Fig. 1. (A) Time-course of the effect of obestatin on ERK1/2 phosphorylation. (B) Obestatin-induced ERK1/2 phosphorylation in the absence or presence of PTX (100 ng/mL, 12 h) and ChTX (2 μg/mL, 4 h). (C) Effects of the Src inhibitor PP2 (5 μM, 30 min) and PI3k inhibitor wortmannin (1 μM, 30 min) on the obestatin-induced ERK1/2 activation. (D) Effect of PKC inhibitor calphostin (10 μM, 1 h) on ERK1/2 phosphorylation. (E) Time-course of the effect of obestatin on PKCε phosphorylation. (F) Time-course of the effect of obestatin on Akt HM (S473) phosphorylation. (G) GPR39 and EGFR expression in AGS and GC cells. (A–F) Serum-starved GC cells were treated with obestatin (200 nM) at 37 °C for the indicated times and under the indicated pretreatments. Cells were lysed and analyzed by SDS–PAGE using specific antibodies. ERK, PKCε and Akt phosphorylation were quantified by densitometry and expressed as a percentage of the maximal phosphorylation (A, E and F) or as a percentage of the basal phosphorylation of ERK1/2 obtained in control cells (B–D). Results are expressed a Mean ± SE of three independent experiments. Blots are representative of three independent experiments.

at protein level in GC cells using the gastric adenocarcinoma AGS cell line as positive control. As shown in Fig. 1G, AGS cells showed high EGFR expression whereas a slight expression was detected in GC cells. Besides, in this cell line obestatin had no effect on cell proliferation for the range of doses tested (100–500 nM). Additionally, maximum proliferation capacity was determined by stimulation with FBS (10%, v/v; ~6.4-fold control) (data not shown). Although the GPR39 was reported to be present in pituitary [19], the expression of this receptor in GC cell line was tested. Fig. 1G shows a low expression of the GPR39 in GC cells compared to that in AGS cells.

To evaluate the GC model, the obestatin and GH spontaneous secretion were measured. Fig. 2A shows the time-course of obestatin secretion with a maximum at 1 h, with an acute drop at 3 h and a gradual fall up to 7 h (Fig. 2A). On the contrary, GH secretion was increasing almost linearly with time (Fig. 2B). As shown in Fig. 2C, obestatin treatment (200 nM) caused an increase in growth hormone secretion in the first 30 min, being more acute at 15 min. At 1 h, obestatin treated cells showed the same levels in growth hormone secretion than controls.

The implication of MAPK signaling on obestatin-evoked GH secretion was evaluated by means of the inhibitor of MAPK activity, PD-98,058. However, this treatment was a non-viable strategy in GC cells, since it caused cellular lysis (data not shown).

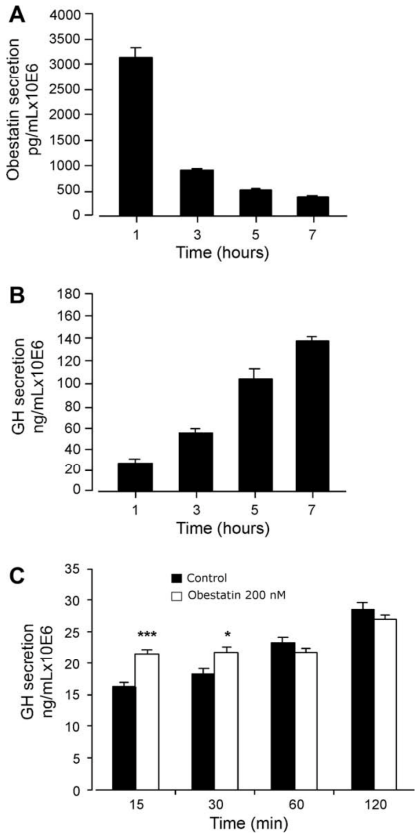

Fig. 2. (A) Mean ± SE of obestatin secretion to the incubation medium from non-stimulated GC cells. Obestatin secretion was detected in the culture medium using a rat obestatin RIA. The values are presented as the cumulative obestatin release by 1×10^6 cells for the indicated times. (B) Mean ± SE of GH secretion to the incubation medium from non-stimulated GC cells. GH secretion was detected in the culture medium by double antibody RIA. The values are presented as the cumulative GH release by 1×10^6 cells for the indicated times. (C) Regulation of GH secretion by obestatin. GH secretion to the incubation medium from non-stimulated GC cells (black bars) and from obestatin treated cells (white bars) and represented as Mean ± SE of three independent experiments.* denotes $P < 0.05$ and *** denotes $P < 0.001$ when comparing obestatin treated (white bars) with untreated control cells (black bars).

Discussion

The present study offers three major findings related to the action of obestatin on somatotroph GC cells. First, obestatin activated ERK 1/2 signaling with no concomitant effect on Akt activation and cell proliferating action, demonstrating a functionality in these cells. Second, exogenous obestatin increased GH release. Third, these cells secreted obestatin, being detectable at protein level in culture medium. Thus, obestatin has a functional role on somatotroph cells, exerting a stimulatory action on GH secretion. Furthermore the secretion of this peptide by somatotroph cells prompted us to speculate for an autocrine/paracrine mechanism that engages obestatin receptor-GH secretion at pituitary level.

The coupling between the obestatin receptor and the intracellular effectors is mediated, at least in part, by PI3K through a PTX-sensitive G protein. PI3k action results in the phosphorylation of novel PKCε that would be the protein responsible for the consecutive activation of MAPK through a Src-dependent pathway. This observation is consistent with the ERK1/2 activation observed in human retinal pigment epithelial cells [7], gastric cell lines [8], pre-adipocyte 3T3-L1 cells [9] and pancreatic β-cell lines [10]. A remarkable observation is that obestatin failed to promote cell proliferation in GC cells, probably associated to the lack of Akt activity. Obestatin-evoked Akt activation requires a signaling pathway involving a β-arrestin 1 scaffolding complex, Zn^{2+}-activated metalloproteinase (MMP) regulator factors and EGFR, acting in parallel to ERK1/2 signaling [18]. Numerous studies demonstrate that altered expression and/or mutations in EGF receptor family members are observed in tumors and the cell lines derived from these tumors, and these alterations contribute to cell growth progression. Furthermore the signaling specificity depends not only on the presence of a specific EGFR family, but also on the biochemical characteristics of the individual EGF-like ligand [20,21]. It is quite likely that lack of Akt activity may be associated to the level of MMPs and/or EGFR expression in GC cells determining the specificity, length and intensity of obestatin signaling. In this regard it is important to note that despite PI3k activity is involved in ERK1/2 signaling, this ERK-related enzyme did not regulate Akt activity [18]. This specificity is linked to their structural and functional homologies determining three major 'Classes' from which Class IB are associated to GPCRs, while Class IA are associated to receptor tyrosine kinases [22]. Based on it, obestatin receptor might regulate PI3k (Class IB) by interaction and its consequent inhibition through the Giα-associated obestatin receptor, as happen for somatostatin receptor in pituitary cells [23]. Thus, inhibition of PI3k activity would decrease basal Akt activity observed in GC cells.

Little is known about the effect of obestatin on growth hormone secretion. On this topic, a few works have been published in the last years. For instance, no significant effect of obestatin on spontaneous GH secretion was found in freely moving rats. Furthermore, *iv* administration of a substantial dose of obestatin failed to influence ghrelin-induced GH secretion [24,25]. In the same direction,

ip administration of obestatin did not modify GH secretion in 10-day-old rats and did not antagonize the GH-releasing effects of hexarelin [26]. These findings suggest that obestatin has no effect on pituitary hormone secretions despite the presence of GPR39, a receptor for obestatin, in the pituitary [19]. However, Zizzari and co-workers found that obestatin was only effective *in vivo* to inhibit ghrelin stimulation of GH levels [4]. These results could be explained by the short half-life that obestatin presents in plasma [27] and by the incapability of this peptide to cross the blood brain barrier [28]. In *in vitro* experiments, unlike ghrelin, obestatin did not increase GH secretion by cultured rat pituitary cells of anterior pituitaries [1]. Also the effects of obestatin were monitored in superfused pituitary explants without any effect on spontaneous or ghrelin-induced GH release [4]. In pituitary cell cultures log molar concentrations of obestatin ranging from 1.0 pM to 100 nM failed to alter basal growth hormone (GH) secretion, concluding that obestatin does not act in pituitary gland to regulate GH secretion [2]. In all of these *in vitro* experiments GH secretion was measured 1-h post-stimuli, clearly showing that obestatin does not perform any change on GH secretion. In our experiences with the GC cell line, when secretion was collected at times shorter than 1 h a clear effect was observed. At 15 and 30 min post-stimuli, obestatin showed an important increase in growth hormone secretion. This fact could be explained as a possible action of this peptide emptying the GH stores in the first 30 min. Later than this time the effect is not observed to any further extent, and obestatin stimulated GH secretion is practically identical than in controls. It is remarkable but not surprising that obestatin spontaneous secretion in GC cell line was diminishing with time. The reason for this acute diminution could be that obestatin was degraded in the cell medium due to its low stability (~22 min), as it has been previously reported [27].

Obestatin stimulates GH release from somatotroph cells, which indicates that this peptide can act directly on the pituitary. Based on the incapability of obestatin to cross the blood brain barrier [28], it is not possible to talk about a peripheral control of GH secretion by obestatin. However the expression of preproghrelin at hypothalamic level [29] prompted us to speculate about the involvement of the hypothalamus in obestatin-mediated stimulation of GH release. A direct involvement would suppose that hypothalamic obestatin was secreted into the portal system to exert its action on somatotroph cells that modulate GH secretion. An indirect involvement would imply that other factor(s) might be involved in the regulation of the pituitary expression of preproghrelin and obestatin secretion that would modulate GH synthesis and/or secretion. Thus, obestatin might be included in the series of stimulatory releasing factors that control the GH release from somatotrophs.

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4. Trabajos enviados

Interaction between ghrelin and the ghrelin receptor (GHS-R1a), a NMR study using living cells[#]

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Abstract

The study of the interaction of ghrelin (**1**), the endogenous ligand for the GH secretagogues receptor (GHS-R1a), and desacyl-ghrelin (**2**) with the GHS-R1a by NMR using living cells is presented, using GHS-R1a stably transfected cell lines (CHO and HEK 293) and wild type cells. Therefore, the interaction of **1** and **2** with the GHS-R1a receptor has been performed using quasi-physiological conditions. Ghrelin (**1**), showed a higher number of residues affected by chemical shift perturbation (CSP) or chemical shift exchange (CSE) effects: Ser3, Phe4, Leu5, Val12, Gln13/Gln14, Lys16/Lys19, Glu17 and Lys24 were much more affected in **1** than in des-acyl ghrelin (**2**). The chemical shift index CSI values indicated the presence of a possible α-helical region between Glu8 and Lys20 for ghrelin (**1**). After analyzing the NMR data, two possible structures have arisen, which present different proline rotamers: the *EEZE* and the *EZEZ* conformers, at positions Pro7, Pro21, Pro22 and Pro27, respectively, keeping a left-handed α-helix from Glu8 to Lys20. These experimental evidences might imply that the GHS-R1a receptor is acting as a prolyl-*cis/trans* isomerase.

Keywords: ghrelin, des-acyl ghrelin, GHS-R1a, NMR spectroscopy.

1. Introduction

Ghrelin (**1**), the endogenous ligand for the growth hormone secretagogue receptor,[1] is a 28-amino residue peptide with a post-translational octanoyl modification on Ser3 (Figure 1), which was first discovered in rat and human stomach tissues.[2] This hormone is mainly synthesized in the stomach, but substantially lower amounts have been detected in other tissues.[3] Functionally, ghrelin (**1**) stimulates growth hormone (GH) secretion from pituitary somatotropes[2,4] and increases food intake and body weight.[5] Indeed, it has been proposed that **1** acts directly on the hypothalamic regulatory nuclei that control energy homeostasis acting as an orexigenic peptide.[6] On the other hand, desacyl-ghrelin (**2**), which presents the same structure with the exception of the n-octanoyl modification on Ser-3, does not show the same functionalities.[7] The receptor GHS-R1a transduces the information provided by ghrelin (**1**) and the group of growth hormone secretagogues (GHS), not structurally related to it. These striking properties have been explained on the basis of the existence of a common binding domain, as demonstrated, using GHS peptide and non-peptide agonists, by site-directed mutagenesis studies assisted by molecular modelling procedures.[8]

Since the discovery of ghrelin (**1**), there have been several studies aimed at determining which are the minimal structural requirements that permit to detect ghrelin receptor biological activity. Bednarek et al. reported the first ghrelin-based minimally active structure-activity studies, demonstrating that the minimum sequence necessary for GHS-R1a activation encompassed the first five residues, with the octanoyl modification on Ser-3. The employed protocol involved binding assays and activation of GHS-R1a by measuring intracellular calcium mobilization, using HEK 293 cells transfected with GHS-R1a.[9] However, it was later demonstrated that this truncated analogue was not capable of stimulating GH secretion from somatotrope cells.[10] In principle, this discrepancy might be attributed to the fact that the rise in calcium obtained for the truncated analogues does not reflect the complete activation of the signal transduction systems as required, for example, to activate GH secretion. At the present moment, no data are available regarding the bioactive conformation of ghrelin (**1**) and its mode of interaction of GHS-R1a at the key binding site. In principle, given the presence of four Pro residues within the 28-amino acid primary sequence, as well as the n-octanoylation at Ser3, one could expect the presence of conformational heterogeneity and a fair amount of flexibility. To the best of our knowledge, three publications have reported approaches to determine the 3D structure of ghrelin (**1**) in solution. The ^{1}H-NMR studies performed by Silva et al.[11] showed that ghrelin behaves as an unstructured and/or fast interconverting peptide at acidic pH. Later, Kukol et al.[12] reported a molecular dynamics (MD) simulation study at neutral pH in water and in the presence of a lipid bilayer, proposing the existence of stable secondary structural features for **1** in the latter case. In particular, the presence of a short α-helix from Pro7 to Glu13 and a hairpin structure with Glu17 to Lys20 in the bending region. Very recently, Dehlin et al. have reported on the CD study of ghrelin (**1**) and des-acyl ghrelin (**2**) in the presence of Tris pH 7.4 and of the α-helix stabilizing solvent, TFE.[13] Although rather qualitative, it was described that the helical content in **1** and **2** was enhanced from 12 to 24 and 50%, respectively.

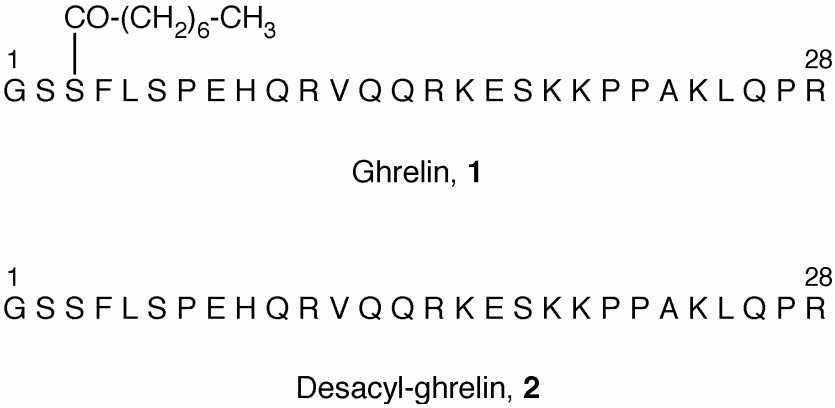

Figure 1. Primary structure of human ghrelin (**1**) and of its des-acylated related peptide, **2**.

In any case, the proper structure-activity relationship study should be based on the knowledge of the bioactive conformation of **1** and **2** when bound to the receptor. NMR techniques are suitable to this end when working with isolated protein receptors,[14-16] and even recently, new NMR experiments using receptor-rich living cells have been reported.[17] Provided that the system holds the right kinetic features, these experiments, with living cells, could avoid the requirement for isolating the protein receptor. On this basis, the approach presented herein aims at the determination of the bioactive conformation of ghrelin (**1**) when interacting with its receptor, at neutral pH. Indeed, since the NMR study of ghrelin (**1**) is performed with living cells highly decorated with the GHS-R1a receptor, physiological conditions similar to those taking place in Nature are kept. Moreover, as the n-octanoyl modification of **1** has been shown to be essential for its physiological role, the des-acyl ghrelin analogue (**2**) has also been analyzed using the same approach. Thus, the NMR analysis of the interaction of **1** and **2** with the ghrelin receptor (GHS-R1a) was performed in CHO and HEK 293 cell lines.

2. Results and Discussion

2.1. Preliminary ^{1}H NMR experiments

To assure time stability of the samples, and therefore reliability and reproducibility of the NMR experiments, a series of 1D ^{1}H NMR spectra of the samples with living cells were collected at regular intervals during the course of one day. These spectra were recorded for ghrelin (**1**) and des-acyl ghrelin (**2**) in the presence of wild type and GHS-R1a-containing cells. The ^{1}H NMR spectra of the **1**:CHO-GHSR1a sample and of the **1**:CHO sample showed drastic changes for most of the exchangeable amide protons (data not shown). In fact, many signals gradually broadened and eventually disappeared within ca. 7 hours after sample preparation. This was not the case for cell-free samples and, since buffer conditions (pH 7.0) were employed, the observed changes could, in principle, be attributed to the interaction of the peptides with the cells (probably through the receptor), thus affecting the water-exchange process for the amide protons. Nevertheless, the ^{1}H NMR spectra also showed significant changes for some of the non-exchangeable aliphatic resonances, especially for ghrelin (**1**) in the presence of transfected cells, even with the appearance of new sets of signals (Figure 2).

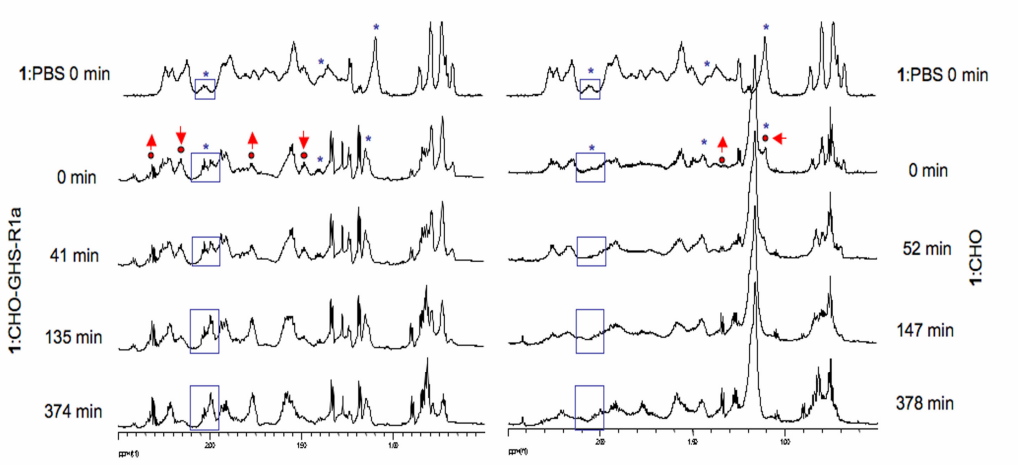

Figure 2. Time-course of the aliphatic section of the ^{1}H NMR spectra of **1**:CHO-GHS-R1a (left) and **1**:CHO (right) compared to the **1**:PBS data at time 0 min. The ^{1}H NMR spectra show important changes (red arrows) for some of the non-exchangeable aliphatic resonances and the appearance of new sets of signals for ghrelin (**1**) in the presence of the receptor (blue squares). The signals for the octyl group are marked with blue asterisks.

After analyzing the ^{1}H-NMR experiments taken at regular intervals of time for **1** and **2** in the presence of wild type cells and GHS-R1a transfected cells, it could be deduced that changes of the NMR signals only took place for ghrelin (**1**), and only when the transfected version of the cells was employed. No effect was observed for **2**. Additionally,

no effects were observed for the wild-type cells. Even more important, the changes in the NMR spectra were shown to be reversible. When the transfected cells were eliminated from the sample also containing **1**, the original chemical shifts for **1** in PBS buffer were recovered.

2.2. NMR cell titration experiment

Additional experiments were performed by increasing the number of cells in the NMR tube containing either **1** or **2**. The results of the cell titration study are shown in Figure 3. The signals of the amide protons disappeared gradually from the spectra and some of the non-exchangeable aromatic protons between 6.5 and 7.5 ppm (Figure 3A, B and C) had a gradual change in their chemical shifts with the increase in the number of cells. Besides, new sets of signals were progressively appearing in the aliphatic region of the spectrum (Figure 3D, E, and F), with the increment in the number of cells in the NMR tube.

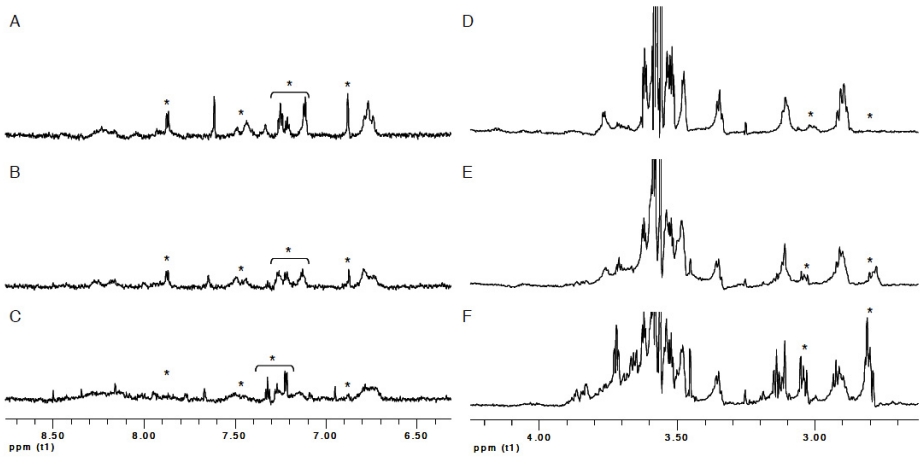

Figure 3. ^{1}H-NMR titration study of **1**:CHO-GHSR1a. Spectra A, B and C correspond to the amide/aromatic region with 0, 2x10^{6} and 4x10^{6} cells, respectively. Spectra D, E and F correspond to the aliphatic region with 0, 2x10^{6} and 4x10^{6} cells, respectively. The evident and significant changes in some signals have been marked with an asterisk.

2.3. Comparison of the data for GHSR1a-containing cells with those for wild type cells with 2D-TOCSY experiments

For each of the two peptides studied, **1** and **2**, 2D-TOCSY spectra were acquired for different NMR samples and prepared under different conditions. Namely, spectra were recorded for **1** and **2** in PBS (without cells), and also in the presence of CHO cells or HEK cells. In each case, two types of cells were employed, either wild type or GHSR1a-transfected. Exclusive chemical shift perturbations (CSP) and slow conformational exchange (SCE) effects occurring in the two transfected cell samples were identified by comparison among TOCSY spectra: the two obtained for transfected and wild type cells

of the same cell line, and the spectrum of the peptide in PBS, for which no CSP or SCE effects were obviously detected.

2.4. CSP and SCE effects in ghrelin (1)

Figure 4 shows part of the amide region 2D TOCSY spectra of **1** with CHO-GHSR1a cells (A) and CHO cells (B). Many of the correlations involving the amide protons have been drastically affected in the presence of the receptor-containing cells.

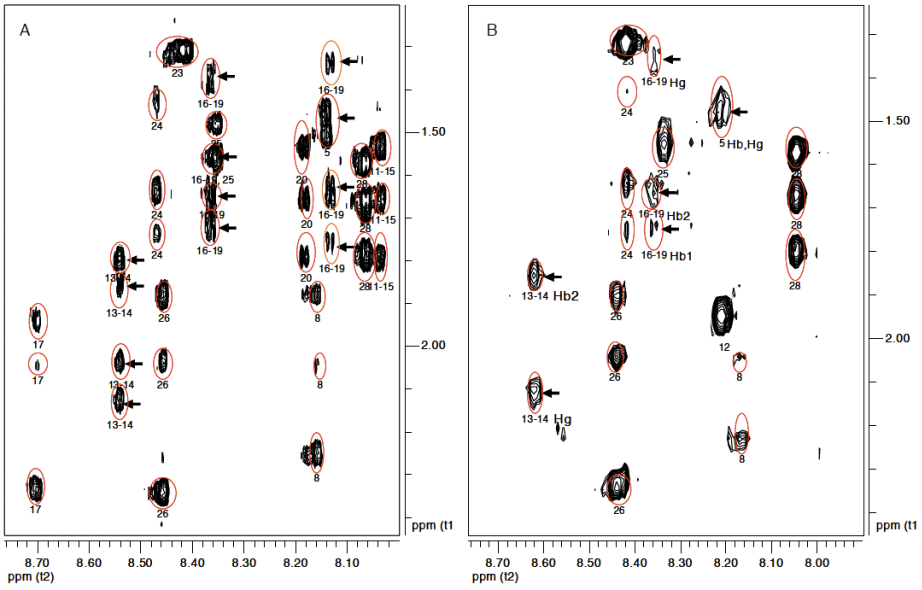

Figure 4. Amide/aromatic proton region in the 2D-TOCSY spectra of **1**:CHO-GHSR1a (A) and **1**:CHO (B). The numbering is indicated under the cross peaks. Differences between peaks are marked with an arrow.

The evaluation of the spectra (using the spectrum acquired in absence of cells, **1**:PBS, as reference, Figure S1) revealed that the following changes were exclusive for sample **1**:CHO-GHSR1a: i) there are two sets of signals having SCE for protons Hβ and/or Hγ of Gln13 (and/or Gln14), in addition to a CSP of -0.08 ppm for the amide proton of Gln13 (and/or Gln14); ii) there is a negative CSP effect for the amide proton of Leu5, which resonates ca. 0.07 ppm lower than in the **1**:CHO sample (Figure 4B) or in the **1**:PBS sample (Figure S1); and, iii) the signals of the amide proton/s of Lys16 (and/or Lys19) are separated in two different sets. In the new set of signals for Lys16 (and/or Lys19), there was a CSP of -0.11, +0.01 and -0.04 ppm for Hα, Hβ1 and Hγ, respectively. The amide proton of Glu-17 had a positive CSP effect of +0.1 ppm in **1**:CHO-GHSR1a respect to **1**:PBS. A negative CSP effect of -0.16 ppm was measured for the aromatic signals of Phe4. The amide proton of Lys24 displayed a CSP of +0.03 ppm in **1**:CHO-GHSR1a.

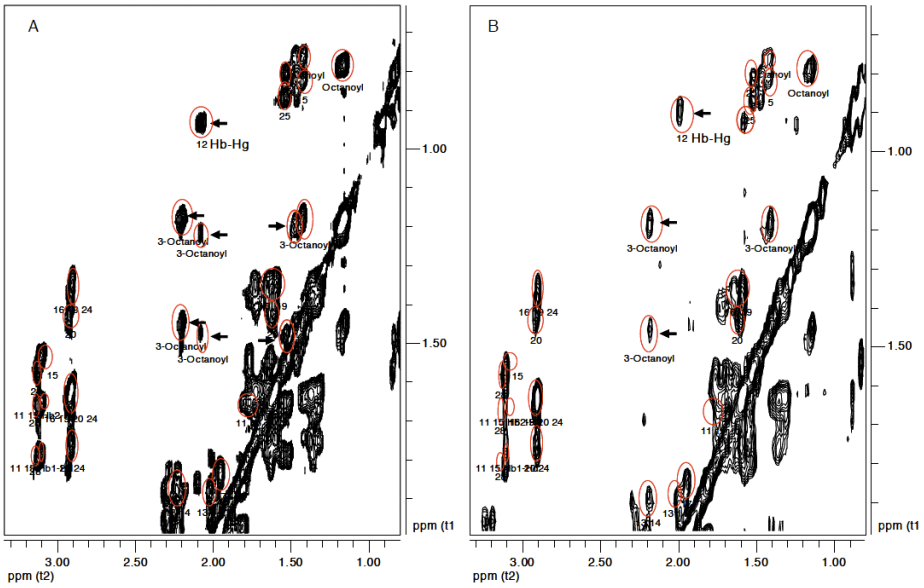

Figure 5. H-alpha/aliphatic proton region in the 2D-TOCSY spectra of **1**:CHO-GHSR1a (A) and **1**:CHO (B). The numbering is indicated under the cross peaks. Differences between peaks are marked with an arrow.

The 2D-TOCSY spectra shown in Figure 5 compare the aliphatic region for **1**:CHO-GHSR1a (A) and **1**:CHO (B) samples. Significant differences were observed for the **1**:CHO-GHSR1a spectrum, which did not occur for the **1**:CHO or for the **1**:PBS analogues: i) two sets of signals generated by a SCE effect are observed for the n-octanoyl group attached to Ser3; ii) the cross peak corresponding to protons Hβ and Hγ of Val12 presented a CSP effect of -0.10 and +0.05 ppm, respectively, with respect to the same peak in the **1**:CHO sample (Figure 5B), or to the **1**:PBS sample (Figure S2). These observed SCE or CSP effects matched the evolutions observed in the preliminary ^{1}H NMR experiments described above.

The authenticity of the conformational exchange previously found for ghrelin (**1**) was assessed by variable temperature 2D TOCSY experiments. The spectra were focussed in the double set of signals for the first methylene of the octyl group attached to Ser3. Four different temperatures of 8, 15, 25 and 35 ^{o}C were tested for **1** in the presence of HEK GHS-R1a enriched cells. These TOCSY spectra exhibited a modulation of the intensities for these methylene signals with the temperature. While at 8 ^{o}C the intensity of the new methylene signal was rather small, at 35 ^{o}C the two signals had practically the same intensity. As the temperature was raised, the intensity of the new methylene signal, which did not appear in cell-free ghrelin (**1**) sample, consistently increased at expenses of the former, indicating the presence of two conformers in the equilibrium (Figure 6).

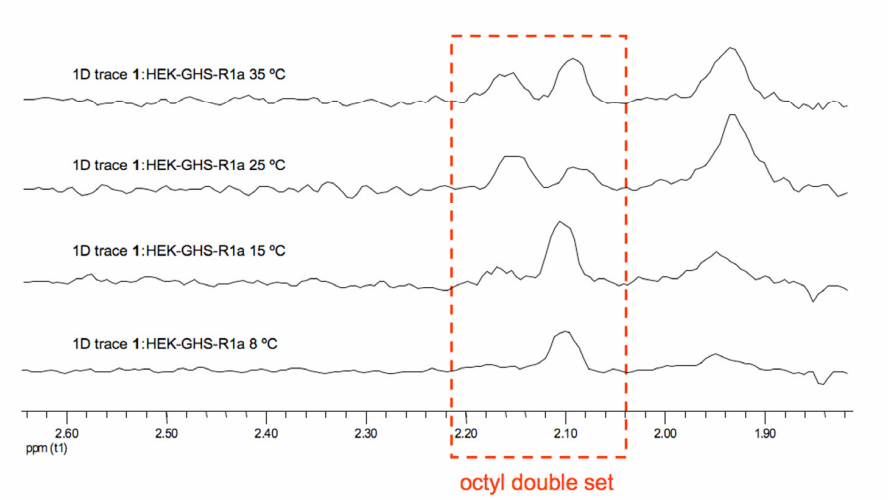

Figure 6. Traces from the variable temperature 1H 2D TOCSY experiments performed in the sample **1**:HEK-GHSR1a. The experiments were not performed consecutively with the raising in temperature.

In order to check the consistency of the obtained results with the CHO cell line, the same study was performed with another cell line, HEK 293. Most of the aforementioned effects were reproduced again with this new HEK cell line. The unique difference observed between the two cell lines was the presence of two sets of signals for the Hβ and Hγ protons of Val12 due to a SCE effect in the **1**:HEK-GHSR1a version, instead of the CSP effect previously described for the **1**:CHO-GHSR1a sample (Figure S3).

2.5. CSP and SCE effects in des-acyl ghrelin (2)

The amide protons region of **2**:HEK-GHSR1a (A) and **2**:HEK (B) samples is represented in Figure S4. Many amide protons have vanished or completely disappeared. The only clear difference exclusive to the spectrum of the **2**:HEK-GHSR1a sample (Figure S4A) refers to the amide proton of Leu5, which has a large CSP effect of +0.19 ppm respect to that of **2**:HEK (Figure S4B), or **2**:PBS (Figure S6). There are other changes in the amide region (Figure S4) that occur in both spectra with HEK-GHSR1a and HEK cells, which differ from those for the **2**:PBS sample (Figure S6). These changes were identified as a double (or triple) set of signals for the amide proton of Ala23 and Glu17/Gln26 (Figure S4A). For **2**:HEK (Figure S4B) there was a double set of signals occurring in the amide proton of residue Val12 (as seen in the correlation 12NH/12Hγ). Nevertheless, this correlation could not be seen in **2**:HEK-GHSR1a due to a fast exchange of the Val12 amide proton (Figure S4A).

Figure S5 gathers the comparison between samples **2**:HEK-GHSR1a (A) and **2**:HEK (B) in the Hα region. No exclusive modification for **2**:HEK-GHSR1a sample was found. There was one common variation to both spectra, the appearance of a double set of signals in SCE for the Hα of Ala23, which was not seen in the **2**:PBS spectrum (Figure S7).

There was also an exclusive SCE effect in the **2**:HEK spectrum (Figure S5B), a double set of signals for the Hα of Val12, which did not occur in the **2**:HEK-GHSR1a (Figure S5A) or in the **2**:PBS sample (Figure S7).

Therefore, different effects are observed for both peptides, some being unique for ghrelin (**1**) in the transfected cell samples and others being observed for des-acyl ghrelin (**2**), but common to the wild type or transfected cells. The exclusive CSP and SCE effects detected for ghrelin (**1**) occurring only in the transfected cell samples are presumably due to the interaction of **1** with the GHS-R1a receptor.

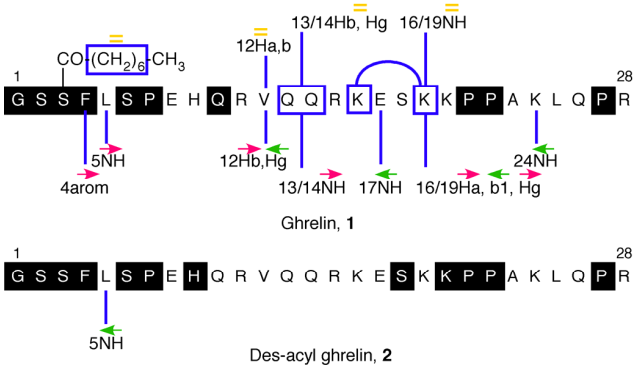

Figure 7. Description of the exclusive interactions of ghrelin (**1**) and des-acyl ghrelin (**2**) with the GHS-R1a receptor. Positive and negative CSP are indicated with arrows pointing to the left and right respectively. SCE effects are indicated with equal signs. No information is available for the NH amide protons of the residues shown in black background.

In contrast, those effects occurring for **2** but for both wild type and transfected cells (especially in the HEK 293 cell line) could probably reflect the interaction of this peptide with other receptors at the cell membrane. Indeed, it has been previously described that this peptide exerts its biological actions through an unknown receptor, which is distinct from the GHS-R1a receptor,[18] and could be present in the HEK 293 cell line. The scheme of Figure 7 summarizes all the SCE and CSP effects observed in the TOCSY spectra of **1**, which are exclusive for GHS-R1a transfected cells. The SCE effects are possibly reflecting a change in the relative population of some of the conformers available to this flexible peptide. The large, both positive and negative, CSP effects detected enhance the signal dispersion of the amide protons of **1**. Both results could be related to the selection of a preferred conformation upon binding with the GHS-R1a receptor. The average lifetime of this preferred conformation should be stable within the chemical shift time scale to be observed in slow exchange in the NMR spectrum. The scheme of Figure 7 represents a reduced and conservative map of the changes observed for ghrelin (**1**) with the GHS-R1a receptor, because there were a number of residues for which no information could be obtained. The reasons for the absence of information are twofold: on one hand, the ambiguities introduced in the assignment of the lateral chains due to the extensive changes occurred in the presence of the cells; and, on the other hand, the fast NH amide

exchange phenomenon that caused that many residues did not give correlations in the key amide region of the TOCSY spectrum. These residues have been represented in black background in the scheme of Figure 7. Nevertheless, the relative large number of SCE and CSP effects detected in the interaction of ghrelin (**1**) with the GHS-R1a receptor suggests that the presence of its receptor causes a significant conformational change in the peptide.

The only change observed for des-acyl ghrelin (**2**) in the GHS-R1a transfected cells corresponded to the CSP effect of the amide proton of Leu5, which is shown in the scheme of Figure 7. As for ghrelin (**1**), the residues for which it was not possible to obtain information are shown in black background. However, the large number of differences found between both schemes is evident and, very probably, indicates that the interactions of des-acyl ghrelin (**2**) with GHS-R1a are not as extended or specific as those taking place for ghrelin (**1**).

The analysis of the Chemical Shift Index (CSI), a semi empirical protocol to determine the secondary structure of a peptide or protein was then employed.[19] This approach compares the Hα chemical shifts of every aminoacid within the peptide of interest with those described for the corresponding residue in an average of random coil structures. Although the method has its drawbacks, it accurately matches the data determined by X-ray crystallography, even for small proteins. Thus, in the absence of extensive NOE data, we decided to apply this protocol to the data obtained for the different ghrelin (**1**) samples to verify whether the different CSI suggested, or not, the presence of α-helix between Pro7 and Pro21. Indeed, residues showing α-helix preference, with the exception of Val12 (CSI = 0) that is bulky and awkward due to branched beta carbon, basically compose that region of the molecule. The CSI values for the **1**:H_2O sample showed the characteristics of an unstructured peptide (data not shown), while those for the **1**:PBS analogue showed a high-field shift for all of the Hα of the previously described region, with the related CSI's suggesting the presence of certain population of α-helix. This discrepancy between the two samples could be explained by the presence of the phosphate ion, a known α-helix stabilizing osmolyte.[20] The **1**:CHO sample showed approximately the same chemical shifts as the PBS sample, while those measured for the **1**:CHO-GHSR1a sample were even more shifted to high field, which could indicate further stabilization of the putative α-helix. These data are in agreement with the idea that the loop from Ser18 to Lys20 described by Kukol could be incorporated in a longer α-helix.[21] The NMR studies performed might indicate that ghrelin (**1**) in PBS is only partially structured, with a low percentage of α-helix from Glu8 to Lys20. This secondary structure is further stabilized in the presence of the GHS-R1a. In a parallel manner, the experiments performed in the presence of the receptor showed the development of new sets of ^{1}H NMR signals in several residues. Since this was shown to be a reversible process (see above), this fact could be explained by the isomerization of any of the prolines present in the ghrelin (**1**) skeleton: Pro7, Pro21, Pro22, or/and Pro27. Although the work of Schubert et al has shown that Δ^{13}C is an accurate indicator of the proline N-terminal peptide bond isomerization state,[22] it was impractical herein due to the nature of our samples. As an alternative, simulated annealing calculations were

performed by restricting the torsion angles for adopting a α-helix structure from Glu8 to Lys20. When a α-helix was forced in this peptide, there was only one possibility to adopt this conformation between these residues, namely a left-handed α-helix. Nevertheless, although the prolines could adopt two possible conformations E (ω = 180º) and Z (ω = 0º), the results showed two structures with left-handed α-helix, with the prolines adopting the following conformations: *EEZE* and *EZEZ*, for Pro7, Pro21, Pro22, and Pro27, respectively, as the best among the 32 possible structures (Figure 8).

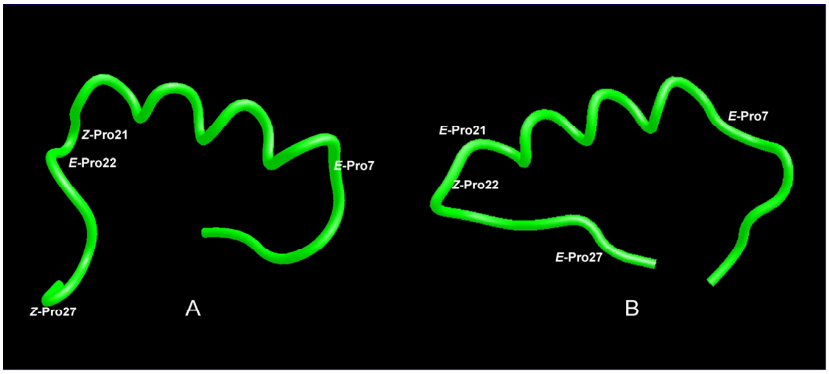

Figure 8. Possible conformations of ghrelin (**1**) in the presence of its receptor GHS-R1a. The most stable conformations were EEZE (A) and EZEZ (B) with a pre-defined left-handed α-helix from Glu8 to Lys20.

Peptides usually adopt several conformations in solution. Therefore, in most of the cases, the assessment of one single 3D structure is unrealistic and, indeed, NMR may easily generate virtual conformations, when ensemble averages are not properly taken into account. A good strategy to decrease conformational exchange is the use of low temperatures before the freezing point (0-8 ºC). It has been speculated that these temperatures may induce the formation of structure that is similar to the conformation of the peptide in the receptor bound state.[23-25] Although in all the experiments described herein, the temperature was set at 5 ºC, we could only infer a low population of α-helix in the PBS sample, and not in the H_2O sample. Additionally, the (reversible) appearance of new sets of signals, indicative of the possible proline cis/trans isomerizations, exclusively arose in the presence of the receptor. Indeed, additional NMR experiments carried out under experimental conditions mimicking membrane-like environments (SDS-micelles) did not show the presence of additional NMR signals, but just suggested, on the basis of chemical shifts and NOEs (data not shown), the existence of an additional percentage, although still small, of helical structure. These pieces of information might suggest that the GHS-R1a receptor is acting as a prolyl-cis/trans isomerase and that, prior to bind to its receptor, ghrelin (**1**) needs to adopt a specific conformation.

2.6. Conclusion

The NMR study described above has shown that ghrelin (**1**) displays a large number of residues affected by CSP or SCE effects by interaction with the GHS-R1a receptor. In contrast, des-acyl ghrelin (**2**) only presents one residue with a marked CSP effect, a result that is consistent with the known higher affinity of **1**. [2,11,26] Moreover, the n-octanoic group at Ser3 of **1** was seen to be clearly involved and necessary for the interaction with the GHS-R1a receptor, a fact coherent with previous studies of ghrelin analogues that claimed that this pendant chain plays a defining role in its bioactivity.[2,11] The lack of CSP or SCE effects observed for des-acyl ghrelin (**2**) with the GHS-R1a receptor in the NMR experiments corroborated this piece of information. This result also supports the conclusion that NMR data using living cells accurately report on the functional interaction of these peptides.

Besides, the CSI obtained could indicate the presence of certain population of α-helix for the region between Glu8 and Lys20 in ghrelin (**1**) in PBS solution, which is further stabilized in the presence of the GHS-R1a receptor. After analyzing the possible conformations with modelling calculations, two possible structures have arisen: the *EEZE* and the *EZEZ* conformers with a left-handed α-helix from Glu8 to Lys20.

3. Experimental Section

3.1. Reagents. Ghrelin (**1**) was purchased from Global Peptides (Fort Collins, Co, USA). Des-acyl ghrelin (**2**) was obtained from Bachem AG (Bubendorf, CH). F-12 Ham was purchased from Sigma Chemical Co (St. Louis, MO, USA). DMEM was purchased from Cambrex Bio Science (Walkersville, MD, USA). D_2O was purchased from Spectra Stable Isotopes (Columbia, MD, USA).

3.2. Cell cultures. HEK 293 and CHO cell lines were cultured as described by the supplier (ECACC, Wiltshire, UK). Briefly, cells were seeded in 100-mm dishes and cultured in DMEM or F-12 Ham medium, respectively, supplemented with 10% foetal bovine serum (FBS), 100U/mL penicillin G, 100 µg/mL streptomycin sulphate and 2.5 µM L-glutamine with 5% CO_2 and 37 ^oC. Subculture routine was as follows: split sub-confluent cultures (70-80%) 1:4 seeding at 2-5x10.000 cells/cm^2 using 0.25% trypsin, 0.05% EDTA. Stably transfected cell lines HEK 293-GHS-R1a and CHO-GHS-R1a were cultured as described for the parent wild type cell line and selected on the basis of resistance to geneticin sulphate G-418 (500 µg/mL).[26,27]

3.3. NMR experiments. NMR spectra were acquired on a Varian INOVA spectrometer operating at 750 MHz and processed with MestRe-C v3.x software[28] using a standard inverse detection triple-resonance and triple-axis gradient probe. Temperature was set at 5 ^oC in all experiments to avoid internalization of the ligand-receptor complex in these cells. It has been described that the complete desensitization-resensitization process takes for about 6 hours.[26] A soft-watergate solvent suppression scheme was used to suppress the H_2O solvent signal in all the experiments.[29] All the spectra were acquired with an external reference of 3-(trimethylsilyl)propionic acid-d_4 sodium salt (TSP, 0.0 ppm).

3.4. NMR sample preparation. NMR samples of the pure peptides **1** or **2** were prepared by dissolving 400 µg of each peptide in 0.5 mL of a mixture PBS buffer (pH 7.2):D_2O (95:5, v/v). These samples are referred in the text as **1**:PBS and **2**:PBS, respectively. To perform the NMR experiments with living cells, $4x10^6$ cells were counted, washed, and dissolved in phosphate-buffered saline (PBS, pH 7.2). Samples were prepared by placing 400 µg of each peptide in 0.5 mL of a suspension of the cells in a mixture PBS (pH 7.2):D_2O (95:5, v/v). Different samples of peptides **1** and **2** were prepared as a suspension with the following cell lines: HEK 293, HEK 293-GHS-R1a, CHO, and CHO-GHS-R1a; and they are referred in the text as **1**:HEK, **1**:HEK-GHSR1a, **1**:CHO, **1**:CHO-GHSR1a, **2**:HEK and **2**:HEK-GHSR1a. Parallel experiments (Tripan Blue Dye) probed that, under the NMR experimental conditions, cells viability was about 80% at 24 hours. The exact amount of receptors on the cell surface is unknowable. However, we estimate the binding sites concentration present in the samples and, therefore, the

GHS-R1a:**1** ratio. Ghrelin (**1**) concentration in the samples is $237x10^{-6}$ M, which means a GHS-R1a:**1** ratio of 1:119.

3.5. 1D ^{1}H-NMR experiments. Fifteen 1D ^{1}H-NMR spectra were acquired for each NMR sample for 24 hours, at regular time intervals, after sample preparation. Each 1D ^{1}H-NMR spectrum was acquired with 256 scans (6 min).

3.5.1. NMR titration. A NMR titration study with **1**:CHO-GHSR1a was performed. Seventeen 1D ^{1}H-NMR spectra were acquired for samples prepared with a crescent number of cells (0 to $4x10^6$ cells) in $0.25x10^6$ cells steps. The complete titration study was performed in a relative short time (c.a. 4 h) to avoid cell sedimentation. Each 1D ^{1}H-NMR spectrum was acquired with 256 scans (6 min).

3.6. 2D ^{1}H-TOCSY experiments. 2D ^{1}H-TOCSY was acquired for each NMR sample. NMR samples were prepared as previously described for each peptide using $4x10^6$ cells. For each sample, three 2D ^{1}H-TOCSY experiments were acquired at different times after preparation to check sample stability. The experiments were acquired at time 0, 7 and 24 hours after sample preparation. Each spectrum was acquired in c.a. 2.5 hours with 24 scans and 128 complex points in the t1 dimension using the states phase sensitive mode. The spectra were processed with a 90^o shifted sinebell apodization function and Fourier transformed to 2048x512 real points in dimensions F2 and F1, respectively.

3.6.1. Variable temperature 2D-^{1}H TOCSY experiments. Variable temperature 2D-^{1}H TOCSY experiments were adquired with the same conditions described above with a sample of 400 μg ghrelin and $4x10^6$ cells transfected cells in 0.5 mL PBS (pH 7.2):D_2O (95:5, v/v). The experiments were acquired at the following temperatures: 8, 15, 25 and 35 ^oC.

3.7. 2D gradient ^{1}H-COSY magnitude experiments. 2D gradient ^{1}H-COSY magnitude experiments were acquired for the PBS samples. NMR samples were prepared as previously described for each peptide. Each spectrum was acquired in 1.25 h with 24 scans and 128 points in the t1 dimension. The spectra were processed with a cosinebell apodization function and Fourier transformed to 2048x512 real points in dimensions F2 and F1, respectively. 2D-TOCSY and 2D-COSY experiments were used for the signal assignment of the peptides **1** and **2**. This assignment was based in the assignment of the pure peptides in the PBS buffer at pH 7.2. The assignment of these peptides in H_2O at pH 3.0 had been previously described.[11]

3.8. CYANA calculations. Simulated annealing calculations were performed with CYANA v2.1.[30] Ghrelin (**1**) was built with the appropriated configuration for the prolines and the acylated serine at position 3. Simulated annealing calculations were performed starting from 100 different random structures that were submitted to 10.000 simulated

annealing iterations. During the annealing, the torsion angles ϕ/ψ of residues 8 to 20 were restrained around the typical angles either of the right-handed α-helix ($-60^{o}/-50^{o}$) or of the left-handed α-helix ($60^{o}/50^{o}$). The ω torsion angles of prolines 7, 21, 22, and 27 were restrained to either E ($\omega = 180^{o}$) or Z ($\omega = 0^{o}$). Each of the sixteen possible combinations of the prolines was tested in different CYANA calculations (Table S1 and Table S2).

4. Acknowledgements

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21. The further stabilization of the helical region could be indeed due to the presence of the receptor. Indeed, recent NMR data (including extensive NOE analysis) gathered in our labs demonstrates that both **1** and **2** do not show a significant α-helix population when included in membrane-like environments, such as SDS micelles (manuscript in preparation).

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6. Legends

Figure 1. Primary structure of human ghrelin (**1**) and of its des-acylated related peptide, **2**.

Figure 2. Time-course of the aliphatic section of the ^{1}H NMR spectra of **1**:CHO-GHS-R1a (left) and **1**:CHO (right) compared to the **1**:PBS data at time 0 min. The ^{1}H NMR spectra show important changes (red arrows) for some of the non-exchangeable aliphatic resonances and the appearance of new sets of signals for ghrelin (**1**) in the presence of the receptor (blue squares). The signals for the octyl group are marked with blue asterisks.

Figure 3. ^{1}H-NMR titration study of **1**:CHO-GHSR1a. Spectra A, B and C correspond to the amide/aromatic region with 0, $2x10^{6}$ and $4x10^{6}$ cells, respectively. Spectra D, E and F correspond to the aliphatic region with 0, $2x10^{6}$ and $4x10^{6}$ cells, respectively. The evident and significant changes in some signals have been marked with an asterisk.

Figure 4. Amide/aromatic proton region in the 2D-TOCSY spectra of **1**:CHO-GHSR1a (A) and **1**:CHO (B). The numbering is indicated under the cross peaks. Differences between peaks are marked with an arrow.

Figure 5. H-alpha/aliphatic proton region in the 2D-TOCSY spectra of **1**:CHO-GHSR1a (A) and **1**:CHO (B). The numbering is indicated under the cross peaks. Differences between peaks are marked with an arrow.

Figure 6. Traces from the variable temperature 1H 2D TOCSY experiments performed in the sample **1**:HEK-GHSR1a. The experiments were not performed consecutively with the raising in temperature.

Figure 7. Description of the exclusive interactions of ghrelin (**1**) and des-acyl ghrelin (**2**) with the GHS-R1a receptor. Positive and negative CSP are indicated with arrows pointing to the left and right respectively. SCE effects are indicated with equal signs. No information is available for the NH amide protons of the residues shown in black background.

Figure 8. Possible conformations of ghrelin (**1**) in the presence of its receptor GHS-R1a. The most stable conformations were *EEZE* (A) and *EZEZ* (B) with a pre-defined left-handed α-helix from Glu8 to Lys20.

Supporting Information

Interaction between ghrelin and the ghrelin receptor (GHS-R1a), a NMR study using living cells[#]

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Supporting Information

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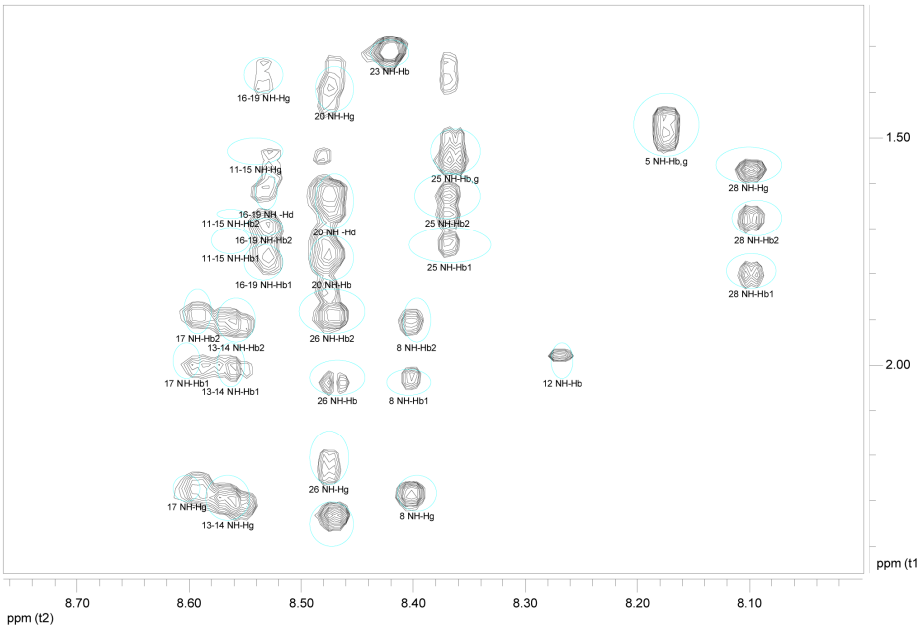

Figure S1. Amide proton region in the 2D-TOCSY spectrum of sample **1**:PBS showing the assignment.

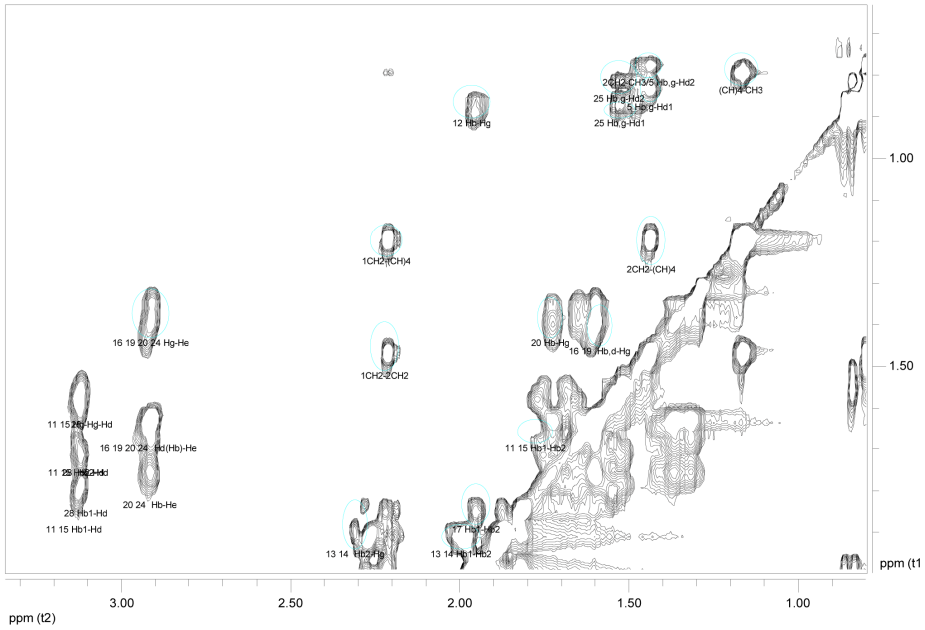

Figure S2. H-alpha/aliphatic proton region in the 2D-TOCSY spectrum of **1**:PBS showing the assignment.

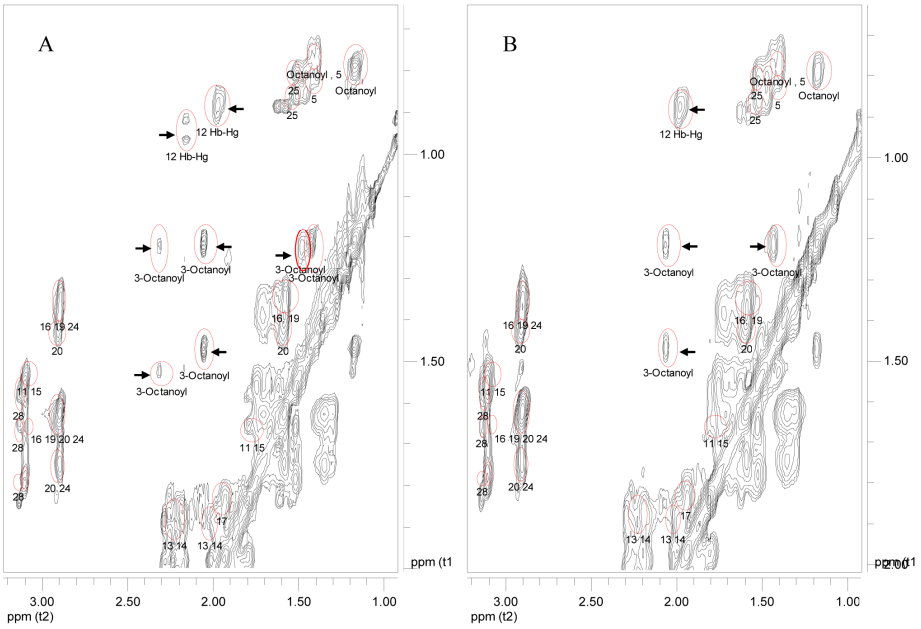

Figure S3. H-alpha/aliphatic proton region in the 2D-TOCSY spectra of **1**:HEK-GHSR (A) and **1**:HEK (B). The numbering is indicated under the cross peaks. Differences between peaks are marked with an arrow.

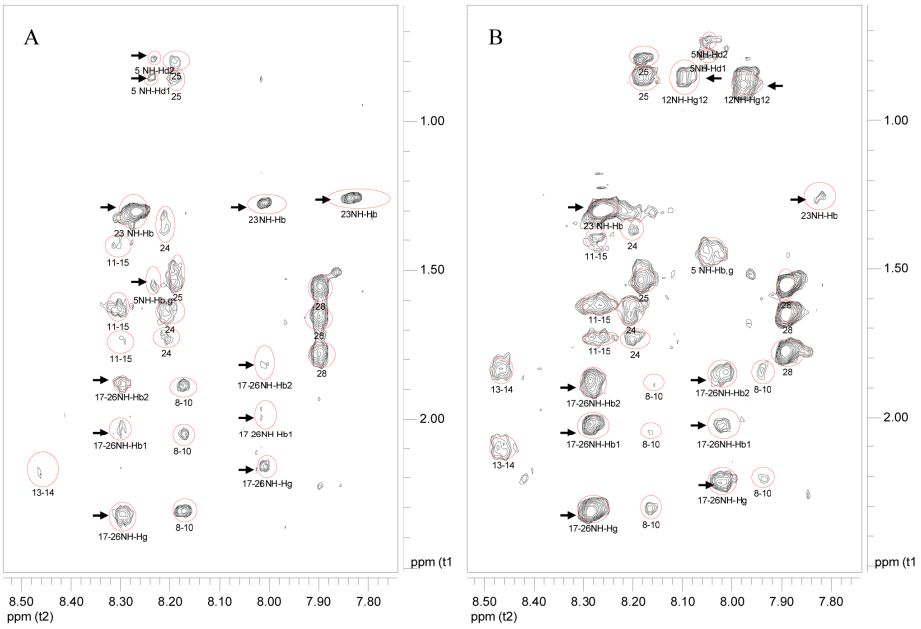

Figure S4. Amide proton region of the 2D-TOCSY spectra of **2**:HEK-GHSR (A) and **2**:HEK (B). The numbering is indicated under the cross peaks. Differences between peaks are marked with an arrow.

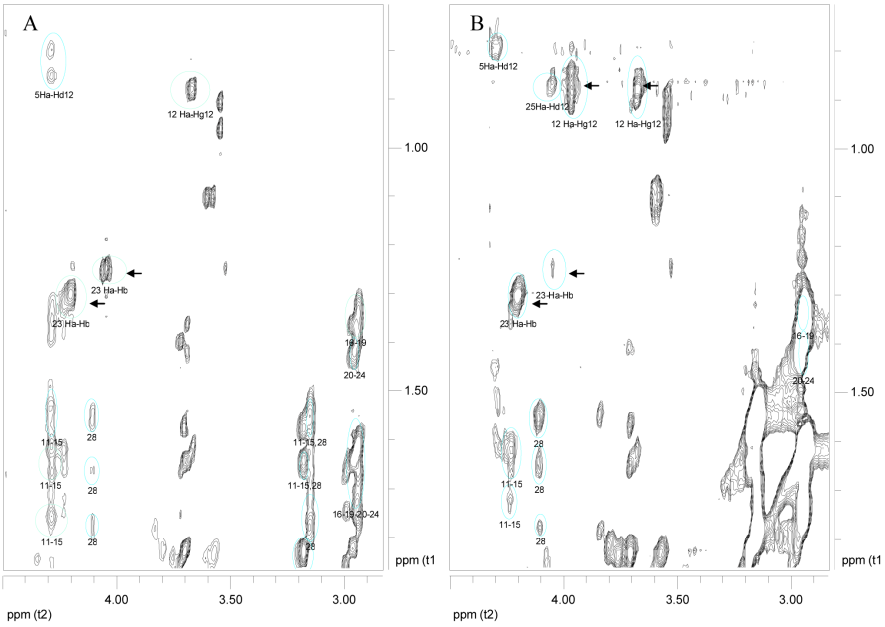

Figure S5. H-alpha/aliphatic proton region in the 2D-TOCSY spectra of **2**:HEK-GHSR (A) and **2**:HEK (B). The numbering is indicated under the cross peaks. Differences between peaks are marked with an arrow.

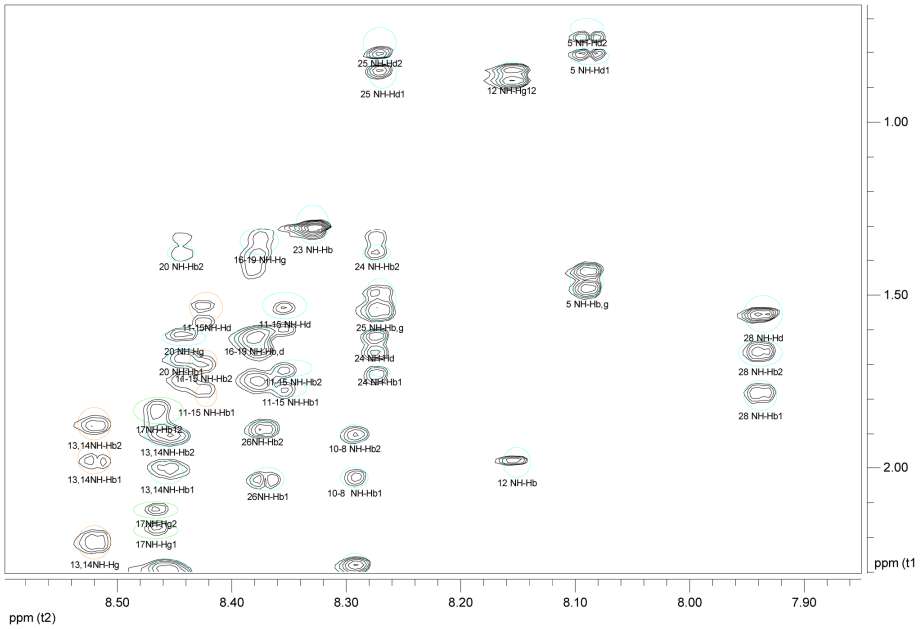

Figure S6. Amide proton region in the 2D-TOCSY spectrum of **2**:PBS showing the assignment.

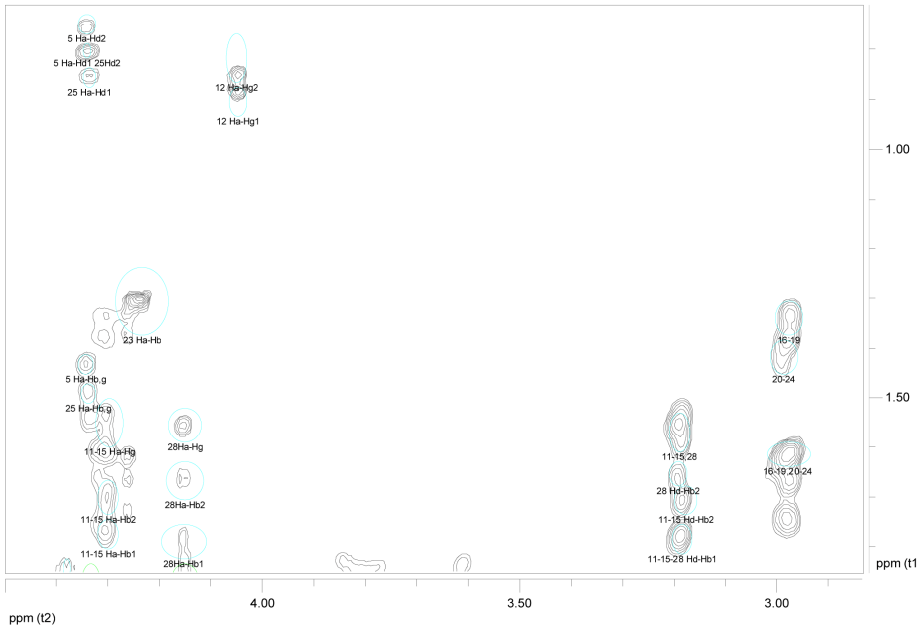

Figure S7. H-alpha/aliphatic region in the 2D-TOCSY spectrum of **2**:PBS showing the assignment.

CYANA calculations.

Proline configuration	Minimun target function	Average Backbone RMSD to mean
EEEE	3.97E-4	6.32
EEEZ	0.11	6.53
EEZE	0.10	6.37
EEZZ	0.22	5.90
EZEE	0.12	5.71
EZEZ	0.23	5.79
EZZE	0.25	5.76
EZZZ	0.36	6.25
ZEEE	0.11	6.48
ZEEZ	0.22	6.15
ZEZE	0.22	5.93
ZEZZ	0.33	6.45
ZZEE	0.24	6.03
ZZEZ	0.37	5.49
ZZZE	0.37	6.26
ZZZZ	0.47	6.22

Table S1. Test for right-handed alpha-helix from Glu8 to Lys20. Torsional restrictions of backbone phi/psi angles: -60º/-50º: restraint ϕ (-55º, -65º), restraint ψ (-45º, -55º). Sixteen combinations of ω torsion angles *Z*/*E* of prolines: Pro7, Pro21, Pro22, Pro27.

Proline configuration	Minimun target function	Average Backbone RMSD to mean
EEEE	4.27E-4	3.93
EEEZ	0.11	3.50
EEZE	**0.10**	**2.90**
EEZZ	0.22	3.24
EZEE	0.13	4.81
EZEZ	**0.24**	**2.50**
EZZE	0.25	3.97
EZZZ	0.37	4.65
ZEEE	0.13	3.42
ZEEZ	0.24	3.57
ZEZE	0.24	3.42
ZEZZ	0.34	3.83
ZZEE	0.26	4.57
ZZEZ	0.39	4.21
ZZZE	0.38	4.93
ZZZZ	0.51	4.26

Table S 2. Test for left-handed alpha-helix from Glu8 to Lys20. Torsional restrictions of backbone phi/psi angles: +60º/+50º: restraint ϕ (55º, 65º), restraint ψ (45º, 55º). Sixteen combinations of ω torsion angles *Z*/*E* of prolines: Pro7, Pro21, Pro22, Pro27.

5. Discusión

5.1. LPA y secreción de ghrelina

Aunque la ghrelina se distribuye por una serie de tejidos (páncreas, cerebro, riñon, testículos, placenta,…), los niveles en plasma están controlados por su secreción desde el estómago en respuesta al hambre o al ayuno y sirviendo como señal periférica al sistema nervioso central para estimular la ingesta. Existen una gran variedad de sustancias, además del estado nutricional, que pueden modular los niveles de ghrelina en plasma (Tabla 1). Uno de los objetivos de esta tesis fue encontrar algún factor capaz de modificar la secreción de ghrelina y dilucidar su mecanismo de acción.

Estimuladores	Inhibidores
	Ingesta, BMI alto
Ayuno, BMI bajo	Glucosa
Leptina	Insulina
GHRH	Somatostatina
Hormonas tiroideas	GH
Testosterona	GHS, ghrelin
Actividad parasimpática	PYY
	Urocortina-1

Tabla 1. Reguladores de la secreción de ghrelina. Extraída de: Front. Neuroendocrinol. 2004;25:27-68.

Los niveles de ghrelina en plasma se incrementan durante el ayuno y decrecen a niveles mínimos en la primera hora tras las comidas. La administración oral o intravenosa de glucosa o lípidos disminuye los niveles de ghrelina en plasma, pero no ocurre así con la carga oral de

proteína.[107,108] Un factor importante, identificado como el primer compuesto que disminuye fuertemente los niveles de ghrelina circulante, es la exendina-4. Este péptido se considera un agonista del receptor de GLP[109,110] y reduce los nieveles de ghrelina circulante durante períodos largos y de una manera dosis-dependiente en ratas en condiciones de ayuno. Además, esta inhibición parece ser un mecanismo específico e independiente de la activación del GLPR.[111]

La expresión y secreción de ghrelina están reguladas principalmente por cambios en el balance energético y en la homeostasis de la glucosa. Además, las alteraciones en los ejes endocrinos, como puede ser un incremento en los niveles de GH, pueden influenciar dichos procesos. Por lo tanto, la ghrelina parece actuar como un agente regulador integrador de la homeostasis energética, el metabolismo de la glucosa y los procesos fisiológicos clásicos endocrinos como son el crecimiento y la reproducción.

En personas con obesidad los niveles de ghrelina circulante son bajos, pero estos niveles aumentan en mayor medida de lo que lo hacen en personas sin sobrepeso en condiciones de ayuno. De igual modo, cuando los sujetos obesos pierden peso sus niveles de ghrelina aumentan.[112] Sin embargo, en estos individuos los niveles de ghrelina disminuyen en menor medida tras las comidas, con lo que su ganancia de peso se mantiene. Los estudios llevados a cabo con material hipotalámico anterior de autopsias de individuos obesos muestran una

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elevada expresión de ghrelina y NPY (proteína y RNAm) en comparación a individuos normopesos. Por lo tanto, los sujetos obesos, presentan elevada la ghrelina hipotalámica, mientras que sus niveles circulantes son bajos.[113,114]

Se ha descrito que los niveles de ghrelina circulante en pacientes con tumores gastroenteropancreáticos son similares a los de individuos sanos y que algunos carcinomas gastrointestinales expresan ghrelina.[115,116] La línea celular de adenocarcinoma gástrico AGS produce ghrelina por lo que puede ser empleada como modelo para valorar sistemas que regulen su secreción.[117] Además, el ácido lisofosfatídico (LPA) es un lípido mediador extracelular que provoca respuestas similares a las de un factor de crecimiento en casi todos los tipos celulares.[118] Todo esto, junto con el amplio espectro de acciones que tiene el LPA sobre secreción y el elevado nivel de expresión del receptor tipo 2 del LPA (LPA2) en las células AGS,[119,120] nos llevó al planteamiento del estudio del LPA como regulador de la secreción de ghrelina. De hecho, los resultados obtenidos con LPA (1 µg/mL)

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mostraron una potente inhibición de la secreción de ghrelina con un perfil parabólico y un máximo a las 5 h.

Cuando las células se pretrataron con el inhibidor de la MAPK p44/p42, PD098059, la secreción de ghrelina no se inhibió de manera significativa tras el estímulo con LPA, sugiriendo que el LPA activa la cascada de las MAPK a través del receptor LPA2 para regular la secreción de ghrelina sin necesidad de la participación de PKA, PKC o PDE y con un papel poco claro para la PI3k y el Ca^{+2}.

Los receptores acoplados a proteínas G (GPCR) emplean multitud de mecanismos para activar las cascada de las MAPK. De esta forma, los mecanismos de señalización pueden resultar de la activación directa de efectores regulados por las proteínas G clásicas como PKC o PI3k, del señalamiento cruzado entre GPCR y receptores con actividad tirosina quinasa o por mediación directa de las β-arrestinas en los GPCR.[121] Los receptores de LPA pueden estar acoplados a varios tipos de proteínas G distintas; G_q, G_i y $G_{12/13}$, las cuales pueden activar múltiples sistemas de efectores.[122] El LPA es el modelo de ligando de GPCR que activa la vía de las MAPK a través de Ras de manera dependiente de G_i y de tirosina quinasas. Sin embargo, nuestros datos mostraron una activación de ERK1/2 insensible a PTX, evidenciando la independencia de G_i en la mediación de este efecto.

Tanto el LPA como el EGF inhibieron la secreción de ghrelina de manera dependiente de las MAPK. Se ha propuesto que el LPA y otros agonistas de GPCRs activan señales mitogénicas usando el receptor de EGF (EGFR) como intermediario. El receptor de EGF puede ser fosforilado por tirosina quinasas no asociadas a receptor.[123] Nuestros datos indicaron la participación de c-Src en la activación de ERK1/2, así como la participación de esta tirosina quinasa no asociada a receptor en el paso de transactivación de EGFR. La otra posibilidad

[121] Gutkind JS. Regulation of mitogen-activated protein kinase signaling networks by G protein-coupled receptors. Sci STKE. 2000;11:RE1.
[122] van Leeuwen FN, Giepmans BN, van Meeteren LA, Moolenaar WH. van Leeuwen FN, Giepmans BN, van Meeteren LA, Moolenaar WH. Biochem Soc Trans. 2003;31:1209-12.
[123] Luttrell LM, Della Rocca GJ, van Biesen T, Luttrell DK, Lefkowitz RJ. Gβγ subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. A scaffold for G-protein-coupled receptor-medaited Ras activation. J Biol Chem. 1997 272:4637-44.

sería que la activación del receptor de LPA llevase a una liberación proteolítica de un agonista del EGFR. Sin embargo, cuando bloqueamos la acción de las metaloproteinasas (MMP) con GM6001,[124] no observamos ninguna inhibición significativa sobre la fosforilación de ERK1/2. Además, se sabe que Src es capaz de mediar la fosforilación de Shc iniciada por subunidades βγ de GPCRs.[125] Teniendo esto en cuenta, proponemos que la inhibición de la secreción de ghrelina mediada por el LPA puede comenzar con la activación de $G_{\beta\gamma q}$ y la consecuente fosforilación de Src. Esta quinasa transactiva al EGFR y así desencadena la vía de la fosforilacion de ERK, posiblemente a través de la activación de Ras tras la formación del complejo SOS-GRB2.

Estos datos nos permiten especular sobre un posible papel fisiológico del LPA sobre la secreción de ghrelina. Dicho papel sería consistente con la secreción de LPA por parte de los adipocitos que controlan la movilidad y proliferación de los preadipocitos a través de los receptores de LPA.[126] De esta forma, la relación establecida entre el LPA y la ghrelina, podría proporcionar una explicación a los bajos niveles circulantes de ghrelina observados en pacientes obesos.

5.2. Obestatina y proliferación de las células KATO-III

Desde que se identificó la obestatina y se postuló como el oponente fisiológico de la ghrelina, las funciones que se le atribuyen para tal consideración han sido puestas en entredicho en multitud de estudios. Diversos grupos de investigación no han podido reproducir las

[124] Santiskulvong C, Rozengurt E. Galardin (GM 6001), a broad-spectrum matrix metalloproteinase inhibitor, blocks bombesin- and LPA-induced EGF receptor transactivation and DNA synthesis in rat-1 cells. Exp Cell Res. 2003;290:437-46.

[125] Luttrell LM, Della Rocca GJ, van Biesen T, Luttrell DK, Lefkowitz RJ. Gbetagamma subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. A scaffold for G protein-coupled receptor-mediated Ras activation. J Biol Chem. 1997;272:4637-44.

[126] Pagès C, Daviaud D, An S, Krief S, Lafontan M, Valet P, Saulnier-Blache JB. Endothelial differentiation gene-2 receptor is involved in lysophosphatidic acid-dependent control of 3T3F442A preadipocyte proliferation and spreading. J Biol Chem. 2001 276:11599-605.

acciones anorexigénicas de la obestatina ni la capacidad de unión al GPR39. Además, un estudio reciente sobre la pureza de los péptidos de obestatina muestra que, la calidad de dos tercios de estos péptidos vendidos por diferentes empresas a los grupos de investigación es insuficiente para experimentos *in vitro* e *in vivo*.[127] Esto podría ser otra explicación a la enorme variabilidad de efectos atribuídos a la obestatina.

A lo largo de este período, se han descrito acciones adicionales de la obestatina (supresión de la sed,[90] mejora de la memoria,[128] regulación del sueño,[129] disminución de la secreción de GH *in vivo,*[71] activación de neuronas corticales[74] y estimulación de la proliferación[99]) que nos muestran a este péptido como una molécula funcional. En esta tesis, tratamos de evaluar la capacidad de la obestatina para modular la proliferación de una línea celular gástrica, ya que el sistema gástrico es uno de los productores principales de este péptido. Como modelo, hemos utilizado la línea de carcinoma gástrico humano KATO-III para valorar la acción mitogénica de la obestatina y para caracterizar la vía de señalización intracelular.

Los datos mostrados permiten dibujar el mecanismo de transducción de señales activado por la obestatina para inducir la fosforilación de ERK1/2 en KATO-III y, como consecuencia, su proliferación. De esta forma, tras la unión del ligando, el receptor de obestatina activa PI3k a través de una proteína G_i sensible a PTX. La activación de PI3k provocaría la activación de la PKCε novel que llevaría a la activación de las MAPK a través de un mecanismo dependiente de Src. La activación de Src a cargo de la PKC no es un proceso de interacción directa entre las dos quinasas. A pesar de que la PKC puede fosforilar a Src,[130] estudios *in vitro* demuestran que las

[127] De Spiegeleer B, Vergote V, Pezeshki A, Peremans K, Burvenich C. Impurity profiling quality control testing of synthetic peptides using liquid chromatography-photodiode array-fluorescence and liquid chromatography-electrospray ionization-mass spectrometry: the obestatin case. Anal Biochem. 2008;376:229-34.
[128] Carlini VP, Schiöth HB, Debarioglio SR. Obestatin improves memory performance and causes anxiolytic effects in rats. Biochem Biophys Res Commun. 2007;352:907-12.
[129] Szentirmai E, Krueger JM. Obestatin alters sleep in rats. Neurosci Lett. 2006;404:222-6.
[130] Gould KL, Woodgett JR, Cooper JA, Buss JE, Shalloway D, Hunter T. Protein kinase C phosphorylates pp60src at a novel site. Cell. 1985;42:849-57.

PKCs no son capaces de activar de forma directa a Src.[131] Por consiguiente, la PKC activa Src por medio de otras proteínas que reducen la fosforilación de Src en la Tyr527, ya sea disminuyendo la actividad quinasa o incrementando la actividad fosfatasa de este residuo.[132,133] En procesos proliferativos, es más usual que la activación resulte de un incremento en la actividad fosfatasa.[134,135]

Dentro de la confusión creada por diversos grupos mostrando diferentes acciones para la obestatina, nuestros datos muestran que la estimulación de células KATO-III con obestatina conduce a un aumento en la proliferación celular mediada por la activación de la vía de ERK, un hecho no observado para la ghrelina. Ya que estas dos moléculas derivan del mismo precursor peptídico, esta falta de correlación funcional apoya el concepto de que la obestatina es un péptido relevante desde el punto de vista biológico y no un mero péptido de unión sin funcionalidad. Aunque nuestros datos apuntan claramente a un receptor acoplado a proteínas G como mediador de la acción mitogénica, y a pesar de que la línea KATO-III expresa el receptor GPR39, no tenemos ninguna prueba concluyente de que sea el GPR39 el que media esta acción proliferativa.

Los datos aquí presentados hacen pensar en la posibilidad de que la obestatina pueda estar involucrada en procesos como la reparación de daños en la mucosa gástrica o como un factor potenciador de la proliferación en cáncer gástrico. Sería de especial interés el estudio y determinación del papel patológico de la señalización de la obestatina, así como su presencia en procesos neoplásicos.

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[131] Brandt DT, Goerke A, Heuer M, Gimona M, Leitges M, Kremmer E, Lammers R, Haller H, Mischak H. Protein kinase C delta induces Src kinase activity via activation of the protein tyrosine phosphatase PTP alpha. J Biol Chem. 2003;278:34073-8.
[132] Yeatman TJ. A renaissance for SRC. Nat Rev Cancer. 2004;4:470-80.
[133] Roskoski R Jr. Src kinase regulation by phosphorylation and dephosphorylation. Biochem Biophys Res Commun. 2005;331:1-14.
[134] Bagrodia S, Chackalaparampil I, Kmiecik TE, Shalloway D. Altered tyrosine 527 phosphorylation and mitotic activation of p60c-src. Nature. 1991;349:172-5.
[135] Kaech S, Covic L, Wyss A, Ballmer-Hofer K. Association of p60c-src with polyoma virus middle-T antigen abrogating mitosis-specific activation. Nature. 1991;350:431-3.

5.3 Activación de Akt en células de cáncer gástrico por parte de la obestatina

Una vez observada la capacidad proliferativa de la obestatina, decidimos profundizar en el estudio de las proteínas clave para la regulación del control de la proliferación y supervivencia celular. Por tanto, nos propusimos estudiar el papel de la obestatina sobre Akt, una proteína serina/treonina quinasa que juega un papel central en la regulación del metabolismo, apoptosis, transcripción y ciclo celular.[136] Empleamos la línea KATO-III como modelo para caracterizar los mecanismos intracelulares y reproducimos los resultados en la línea de adenocarcinoma gástrico humano AGS.

La obestatina activa Akt por medio de dos procesos de fosforilación que dependen de la activación de PI3k. La activación de PI3k por el receptor de la obestatina genera PIP_3 el cual permite el anclaje a la membrana de proteínas con dominios PH como son Akt y PDK1. Esta colocalización, junto con la activación de PDK-1 por autofosforilación en el residuo S241, [137] permite la fosforilación de Akt en T308 (A-loop) a cargo de PDK-1.[138] La activación de Akt se completa con la fosforilación en S473 (HM). Demostramos que la inactivación del complejo mTOR2, por medio del silenciamiento del RNAm de la proteína Rictor, reduce la fosforilación de Akt en HM(S473). Esto, apoya el modelo por el cual Akt y PDK1 interactúan a nivel de membrana, donde posteriormente Akt es fosforilada por mTORC2.[139] A pesar de ser un elemento central en la activación de Akt, el modo de activación de mTORC2 aún es desconocido. Además, la obestatina media la fosforilación de mTOR en S2448, un sitio importante para su

[136] Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell. 2007;129:1261-74.
[137] Casamayor A, Morrice NA, Alessi DR. Phosphorylation of Ser-241 is essential for the activity of 3-phosphoinositide-dependent protein kinase-1: identification of five sites of phosphorylation in vivo. Biochem J. 1999;342:287-92.
[138] Storz P, Toker A. 3'-phosphoinositide-dependent kinase-1 (PDK-1) in PI 3-kinase signaling. Front Biosci. 2002;7:886-902.
[139] Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. Cancer Cell. 2007;12:9-22.

regulación,[140] y la fosforilación de p70S6K1 en T398, proceso bloqueado por rapamicina lo que implica al complejo mTOR1 (mTORC1: Raptor, mLST8, PRAS40, mTOR quinasa). Consecuentemente, la obestatina regula la actividad de los complejos mTOR 1 y 2 lo que la implica en procesos de crecimiento y división celular (progresión en G1) a través de la activación de p70S6K1.[141]

Aunque anteriormente habíamos demostrado la participación de Gi en la activación de ERK1/2 tras el estímulo de obestatina en la línea celular KATO-III, esta proteína G no juega ningún papel en la fosforilación de Akt. Sin embargo, la fosforilación de Akt provocada por la obestatina sí requiere la participación de metaloproteinasas (MMP) y la transactivación del EGFR. Esta transactivación respondería a la liberación proteolítica (por parte de las MMP) de EGF de la superficie celular y posterior activación del EGFR por estimulación autocrina o paracrina.[142,143] Nuestros datos mostraron la mediación de Src en la activación de Akt. Posiblemente, Src interactúa con el motivo PXXP de las MMPs para transactivar el EGFR.[144] Los ensayos de coinmunoprecipitación que muestran que β-arrestina 1 recluta a Src, apoyaron este modelo. Además, el silenciado del RNAm de β-arrestina 1 implicaba una disminución en la fosforilación de Akt y Src (Y416). La formación de complejos entre β-arrestina 1 y Src puede desencadenar la activación de esta última a causa de los cambios conformacionales producidos por la unión de β-arrestina 1.[145] Por tanto, β-arrestina 1 actuaría como adaptador que recluta Src al GPR39 llevando a la

[140] Kenerson HL, Aicher LD, True LD, Yeung RS. Activated mammalian target of rapamycin pathway in the pathogenesis of tuberous sclerosis complex renal tumors. Cancer Res. 2002;62:5645-50.
[141] Pullen N, Thomas G. The modular phosphorylation and activation of p70s6k. FEBS Lett. 1997;410:78-82.
[142] Carpenter G. EGF receptor transactivation mediated by the proteolytic production of EGF-like agonists. Sci STKE. 2000;15:PE1.
[143] Higashiyama S, Iwabuki H, Morimoto C, Hieda M, Inoue H, Matsushita N. Membrane-anchored growth factors, the epidermal growth factor family: beyond receptor ligands. Cancer Sci. 2008;99:214-20
[144] Seals DF, Courtneidge SA. The ADAMs family of metalloproteases: multidomain proteins with multiple functions. Genes Dev. 2003;17:7-30.
[145] 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 beta-arrestins. J Cell Physiol. 2007;213:187-200.

activación de las MMP a través del complejo GPR39/ β-arrestina 1/Src, y transactivando al EGFR en última estancia.

La activación de las vías de señalización de ERK1/2 y Akt actúan en paralelo en las células KATO-III. En este sentido, la transactivación del EGFR es el nexo entre el receptor de obestatina y Akt, mientras que la proteína G_i regula la vía de ERK1/2.

Estudios recientes muestran que el Zn^{+2} induce la fosforilación del EGFR por medio de la liberación extracelular de ligandos parecidos al EGF provocada por MMPs.[146] Además, la exposición al Zn^{+2} se vio que es capaz de activar las vías de PI3k/Akt y MAPK a través de la activación del EGFR en diversos tipos celulares.[147] A la vista de estos datos, los efectos estimulatorios del Zn^{+2} sobre el señalamiento del GPR39 pueden ser debidos a la activación de las MMP y EGFR, ya que la obestatina requiere la transactivación del EGFR y la actividad de las MMP. Sería interesante determinar la función del Zn^{+2} sobre la señalización del GPR39 en ausencia de MMPs y EGFR para definir su función como ligando o modulador alostérico de este receptor.[148]

En conclusión, nuestros datos en células tumorales gástricas (KATO-III y AGS) son consistentes con un modelo en el que la ruta de señalización de Akt estimulada por obestatina está activada a través de la transactivación del EGFR. Cuando se activa el receptor de obestatina, β-arrestina 1 se encarga de reclutar y activar Src. De este modo, Src funciona como un interruptor que se encarga de activar las MMPs que liberan ligandos parecidos a EGF en la superficie celular. La unión de estos ligandos al EGFR desencadena su dimerización y autofosforilación de sitios específicos de unión entre ellos para la unión de PI3k. La activación de PI3k genera segundos mensajeros como el PIP3 que permite la translocación a la membrana de Akt por medio de sus dominios PH. Esta localización de Akt, hace que sea fosforilada en

[146] Hwang JJ, Park MH, Choi SY, Koh JY. Activation of the Trk signaling pathway by extracellular zinc. Role of metalloproteinases. J Biol Chem. 2005;280:11995-2001.
[147] Samet JM, Dewar BJ, Wu W, Graves LM. Mechanisms of Zn(2+)-induced signal initiation through the epidermal growth factor receptor. Toxicol Appl Pharmacol. 2003;191:86-93.
[148] Storjohann L, Holst B, Schwartz TW. Molecular mechanism of Zn2+ agonism in the extracellular domain of GPR39. FEBS Lett. 2008;582:2583-8.

A-loop(T308) y HM(S473) por PDK1 y por mTORC2 respectivamente. Una vez activada Akt, ésta inactiva el heterodímero TSC1/TSC2 con la consecuente activación de mTORC1 y fosforilación de otras dianas como p70S6K1(T389).

Esta vía de señalización añade un nuevo componente al conjunto de dianas intracelulares reguladas por la obestatina. Además, la obestatina se puede añadir al grupo de factores reguladores de las MMPs, las cuales han sido implicadas en diversas patologías humanas como son los procesos inflamatorios y el cáncer.[144,149] La transactivación de EGFR inducida por la obestatina podría ser un mecanismo por el cual las MMPs regularan estas enfermedades. Apoyando esta hipótesis, numerosos estudios muestran que en tumores y en líneas celulares derivadas de esos tumores, se observan alteraciones en la expresión o mutaciones en la familia de receptores EGFR/ErbB.[150,151,152]

La determinación detallada de los mecanismos de activación/regulación de la transactivación del EGFR por la obestatina y su implicación patofisiológica representan interesantes áreas de estudio.

La existencia de dos hormonas (ghrelina y obestatina) derivadas del mismo precursor proteico, actuando a través de receptores distintos y mediando efectos opuestos, es un hecho de gran interés y sugiere la posibilidad de que estudios previos de expresión de RNAm de ghrelina deberían ser reconsiderados con una nueva perspectiva.

[149] Huovila AP, Turner AJ, Pelto-Huikko M, Kärkkäinen I, Ortiz RM. Shedding light on ADAM metalloproteinases. Trends Biochem Sci. 2005;30:413-22.
[150] Normanno N, De Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR, Carotenuto A, De Feo G, Caponigro F, Salomon DS. Epidermal growth factor receptor (EGFR) signaling in cancer. Gene. 2006;366:2-16.
[151] Bhola NE, Grandis JR. Crosstalk between G-protein-coupled receptors and epidermal growth factor receptor in cancer. Front Biosci. 2008;13:1857-65.
[152] Ohtsu H, Dempsey PJ, Eguchi S. ADAMs as mediators of EGF receptor transactivation by G protein-coupled receptors. Am J Physiol Cell Physiol. 2006;291:C1-10.

5.4. Obestatina y secreción de GH.

El presente estudio ofrece tres hechos relevantes relacionados con la acción de la obestatina sobre las células somatotropas GC: Primero, la obestatina activa la vía de ERK1/2 sin mostrar efecto sobre la activación de Akt ni sobre la proliferación, demostrando una funcionalidad de la obestatina en estas células. Segundo, la administración exógena de obestatina incremeanta la liberación de GH. Tercero, esta línea celular secreta obestatina, siendo detectable a nivel proteico en el medio de cultivo. Por lo tanto, la obestatina tiene una funcionalidad en las células somatotropas, ejerciendo una acción estimuladora sobre la secreción de GH. Además, dado que estas células somatotropas secretan obestatina, nos permite especular sobre la existencia de un mecanismo autocrino/paracrino que relacione al sistema obestatina/receptor con la secreción de GH a nivel hipofisario.

El nexo de unión entre el receptor de obestatina y los efectores intracelulares es, al menos en parte, la PI3k activada a través de una proteína G sensible a PTX. La activación de la PI3k conlleva la fosforilación de la PKCε, que podría ser responsable de la activación consecutiva de las MAPK por medio de una vía dependiente de Src. Esta observación es consistente con la activación de ERK1/2 en células del epitelio retiniano pigmentario humano,[99] en líneas celulares gástricas,[153] en pre-adipocitos 3T3-L1[80] y en líneas de células β pancreáticas.[96] Un hecho interesante es que la obestatina no estimula la proliferación en las células GC, probablemente asociado a la ausencia de actividad de Akt. La activación de Akt inducida por la obestatina requiere un mecanismo de señalización en el que participan β-arrestina 1, factores reguladores de metaloproteinasas activadas por Zn^{+2} (MMP) y EGFR, actuando en paralelo a la activación de ERK1/2.[154] Ha sido demostrado en numerosos estudios la existencia de alteraciones en la

[153] Pazos Y, Alvarez CJ, Camiña JP, et al. Stimulation of extracellular signal-regulated kinases and proliferation in the human gastric cancer cells KATO-III by obestatin. Growth Factors. 2007;25:373-381.

[154] Alvarez CJ, Lodeiro M, Theodoropoulou M, Camiña JP, Casanueva FF, Pazos Y. Obestatin stimulates Akt signalling in gastric cancer cells through beta-arrestin-mediated epidermal growth factor receptor transactivation. Endocr Relat Cancer. 2009;16:599-611

expresión o mutaciones en la familia del receptor de EGF en tumores y en líneas celulares derivadas de esos tumores, y que estas alteraciones contribuyen al crecimiento celular. Además, la especificidad de la cascada de señalización no sólo depende del tipo de receptor ErbB de la familia de EGFR, sino también de las características bioquímicas individuales del ligando similar a EGF.[151,152] Es probable que la falta de actividad de Akt en las células GC pueda estar relacionada con el nivel de expresión de MMPs y/o EGFR, determinando la especificidad, duración e intensidad de la acción de la obestatina. En relación a esto, es importante destacar que a pesar de que la actividad de la PI3k está presente en la activación de ERK1/2, esta proteína no regula la actividad de Akt.[154] Esta especificidad de la PI3k está asociada a sus características estructurales y funcionales determinando tres grandes "Clases" principales, de las cuales la Clase IB está asociada a GPCRs, mientras que la Clase IA está asociada a RTK.[155] Basándonos en esto, el receptor de la obestatina puede regular la PI3k (Clase IB) inhibiéndola a través de una proteína Giα, como ocurre para el receptor de somatostatina (SS) en células de hipófisis.[156] De este modo, la inhibición de la actividad de PI3k podría conllevar la disminución da la actividad de Akt observada en las células GC.

Poco se conoce sobre el efecto de la obestatina en la secreción de GH. Sobre este tema, se han publicado unos pocos trabajos en los últimos años. Por ejemplo, no se ha encontrado efecto alguno de la obestatina sobre la secreción espontánea de GH en ratas libres. Además, la administración *iv* de dosis elevadas de obestatina no afecta a la secreción de GH inducida por ghrelina.[84,103] En la misma dirección, la administración *ip* de obestatina no modifica la secreción de GH ni antagoniza el efecto liberador de GH de la hexarelina en ratas de 10 días de edad.[102] Estos datos sugieren que la obestatina no tiene efecto alguno sobre la secreción hormonal hipofisaria a pesar de la presencia del GPR39 en la hipófisis.[81] Sin embargo, Zizzari encontró que la

[155] Williams R, Berndt A, Miller S, et al. Form and flexibility in phosphoinositide 3-kinases. Biochem Soc Trans. 2009;37:615-626.

[156] Theodoropoulou M, Zhang J, Laupheimer S, et al. 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;66:1576-1582.

obestatina, sólo *in vivo,* inhibe los niveles de GH estimulados por ghrelina.[71] Estos resultados podrían explicarse por la escasa vida media de la obestatina en plasma[92] y por la incapacidad de ésta para cruzar la barrera hematoencefálica.[91] En los experimentos *in vitro*, y a diferencia de lo que ocurre con la ghrelina, la obestatina no es capaz de incrementar la secreción de GH en cultivos de células de hipófisis anterior de rata.[69] La obestatina tampoco tiene efecto alguno sobre la secreción espontánea de GH, ni sobre la inducida por ghrelina en explantes de hipófisis.[71] En cultivos celulares de hipófisis, la obestatina en concentraciones logarítmicas desde 1.0 pM hasta 100 nM es incapaz de alterar la secreción basal de GH, concluyendo que no es capaz de actuar sobre la glándula hipofisaria para regular la secreción de GH.[90] En todos estos experimentos *in vitro,* la secreción de GH se valora una hora después del estímulo, mostrando claramente que la obestatina no es capaz de producir ningún cambio sobre dicha secreción. En nuestros experimentos con las células GC, cuando recogíamos la secreción a tiempos más cortos que una hora, observábamos un efecto claro. La secreción de GH se incrementaba significativamente a los 15 y 30 min tras el estímulo de obestatina. Una explicación plausible a este hecho podría ser que la obestatina actuase como un inductor del vaciado de los depósitos de GH en los primeros 30 min. A tiempos más largos no se observa este efecto y la secreción de GH estimulada por obestatina es prácticamente idéntica a la de los controles. Un hecho destacable aunque no sorprendente es que la secreción espontánea de obestatina en las células GC disminuya con el tiempo. La razón podría residir en la rápida degradación de la obestatina en el medio de cultivo debido a su baja estabilidad (~22min), como ha sido establecido previamente.[92]

La obestatina estimula la liberación de GH de las células somatotropas, lo cual indica que este péptido puede actuar directamente sobre la hipófisis. Teniendo en cuenta la incapacidad de la obestatina para cruzar la barrera hematoencefálica,[91] no es posible hablar de un control periférico de la obestatina sobre la secreción de GH. Sin embargo, la expresión de preproghrelina a nivel

hipotalámico[157] permite especular sobre un posible papel del hipotálamo en la liberación de GH estimulada por obestatina. Un papel directo supondría que la obestatina hipotalámica se secretase en el sistema portal para ejercer su acción sobre las células somatotropas que modularían las secreción de GH. Un papel indirecto implicaría que otros factores pudiesen regular la expresión hipofisaria de preproghrelina y la secreción de obestatina la cual modularía la síntesis y/o secreción de GH. De acuerdo con esto, la obestatina podría formar parte del conjunto de hormonas liberadoras que controla la secreción de GH de las células somatotropas.

5.5. Interacción entre ghrelina y GHSR-1a.

Desde el descubrimiento de la ghrelina, se han desarrollado diversos estudios en los que se perseguía determinar cuáles eran los requerimientos estructurales mínimos que permitirían detectar la actividad biológica del receptor de ghrelina, GHS-R1a. Bednarek y col. llevaron a cabo los primeros estudios de relación estructura-actividad, describiendo la secuencia mínima activa para la activación del GHS-R1a. Esta secuencia se componía de los cinco primeros aminoácidos de la molécula, así como la modificación *n*-octanoilo de la Ser3. El protocolo llevado a cabo comprendía estudios de *binding* al GHS-R1a así como la medida de la activación de este receptor mediante ensayos de movilización de Ca^{+2} intracelular utilizando células HEK-293 transfectadas con el receptor GHS-R1a.[26] Sin embargo, posteriormente se demostró que este análogo truncado no era capaz de estimular la secreción de GH de células somatotropas.[28] En principio, esta discrepancia puede atribuirse al hecho de que la movilización de calcio obtenida para los análogos truncados no refleja una activación completa del sistema de transducción de señales como se necesita, por ejemplo, para activar la secreción de GH. En este momento, no existen

[157] Cowley MA, Smith RG, Diano S, et al. The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron. 2003;37:649-661.

datos acerca de la conformación bioactiva de ghrelina y de su modo de interacción con el GHS-R1a en el sitio de unión. En principio, dada la existencia de cuatro residuos Pro en la estructura primaria de los 28 aminoácidos, así como el *n*-octanoilo en la Ser3, uno podría esperar la presencia de heterogeneidad conformacional y una justa cantidad de flexibilidad. A nuestro entender, tres grupos de investigación han llevado a cabo aproximaciones para determinar la estructura 3D de ghrelina en disolución. Los estudios de RMN realizados por Silva y col.[29] mostraron que la ghrelina se comportaba como un péptido desestructurado y/o en rápido equilibrio de interconversión a pH ácido. Más tarde, Kukol y col.[30] describían un estudio de simulación de dinámica molecular (MD) a pH neutro en agua y en presencia de una bicapa lipídica, proponiendo la existencia de características de estructura secundaria estables para la ghrelina en el segundo caso. En particular, la presencia de una hélice-α corta desde la Pro7 hasta la Glu13 y una estructura de horquilla de Glu17 a Lys20 en la región de plegamiento. Recientemente, Dehlin y col. han descrito un estudio de dicroismo circular de ghrelina y des-acil ghrelina en presencia de Tris pH 7.4 y de un disolvente estabilizador de hélice-α como es el trifluoroetanol (TFE).[158] Aunque es una aproximación bastante cualitativa, describieron que el contenido de hélice de ghrelina y des-acil ghrelina aumentaba de un 12% a un 24 y un 50 % respectivamente.

En cualquier caso, el estudio de la relación estructura-actividad debería basarse en el conocimiento de la conformación bioactiva de ghrelina y des-acil ghrelina cuando se unen al receptor. De esta forma, las técnicas de resonancia magnética nuclear (RMN) son adecuadas para este fin trabajando con receptores aislados,[159,160,161] e incluso recientemente, se han llevado a cabo estudios utilizando células vivas

[158] Dehlin E, Liu J, Yun SH, Fox E, Snyder S, Gineste C, Willingham L, Geysen M, Gaylinn BD, Sando JJ. Regulation of ghrelin structure and membrane binding by phosphorylation. Peptides. 2008;29:904-11.
[159] Meyer B, Peters T. NMR spectroscopy techniques for screening and identifying ligand binding to protein receptors. Angew Chem Int Ed Engl. 2003;42:864-90.
[160] Lepre CA, Moore JM, Peng JW. Theory and applications of NMR-based screening in pharmaceutical research. Chem Rev. 2004;104:3641-76.
[161] Zartler ER, Yan J, Mo H, Kline AD, Shapiro MJ. ID NMR Methods in ligand-receptor interactions. Curr Top Med Chem. 2003;3:25-37.

enriquecidas con los receptores.[162] Dado que el sistema posee los hechos cinéticos adecuados, estos experimentos con células vivas evitarían la necesidad de aislar el receptor. Con estas bases, hemos llevado a cabo una aproximación a la determinación de la conformación bioactiva de ghrelina cuando interacciona con su receptor a pH neutro. Asimismo, ya que el estudio de RMN de ghrelina se lleva a cabo con células vivas (CHO y HEK 293) transfectadas de forma estable con el receptor GHS-R1a, se mantienen la condiciones fisiológicas que tienen lugar en la Naturaleza. Además, como la modificación *n*-octanoilo se ha mostrado esencial para su papel fisiológico, se ha analizado el análogo des-acilado utilizando la misma aproximación.

De esta forma, comparando los espectros 2D-TOCSY de resonancia magnética nuclear (RMN) realizados en las distintas muestras de ambos péptidos (estado libre en disolución de tampón fosfato (PBS), en presencia de células wild type y en presencia de células transfectadas), se observaron tanto cambios en desplazamientos químicos (CSP), como intercambios conformacionales lentos (SCE). Precisamente, se observan diferentes efectos para ambos péptidos, algunos siendo únicos para la ghrelina en las muestras transfectadas y otros para la des-acil ghrelina, pero comunes a las células wild type o transfectadas con el GHS-R1a. Los efectos CSP y SCE exclusivos observados para la ghrelina y que ocurren únicamente en las muestras de células transfectadas con el GHS-R1a se deben presumiblemente a la interacción de la ghrelina con el receptor.

Por el contrario, los efectos observados para la des-acil ghrelina tanto en células wild type como en células transfectadas (especialmente en las células HEK 293) podrían reflejar la interacción de este péptido con otros receptores presentes en la membrana celular. De hecho, se ha descrito que este péptido induce sus acciones biológicas a través de un

[162] Mari S, Serrano-Gómez D, Cañada FJ, Corbí AL, Jiménez-Barbero J. 1D saturation transfer difference NMR experiments on living cells: the DC-SIGN/oligomannose interaction. Angew Chem Int Ed Engl. 2004;44:296-8.

receptor desconocido, distinto del GHS-R1a,[163] y que podría estar presente en la línea celular HEK 293.

Los efectos SCE posiblemente reflejen un cambio en la población relativa de alguno de los confórmeros posibles para este péptido tan flexible. La gran cantidad de efectos CSP detectados (tanto positivos como negativos) aumenta la dispersión de la señal de los protones amida de la ghrelina. Ambos resultados podrían estar relacionados con la selección de una conformación preferida en la unión al receptor GHS-R1a. El tiempo de vida medio de esta conformación debería ser estable en la escala de tiempos de los desplazamientos químicos y así poder ser observada en intercambio lento en el espectro de RMN. Para algunos residuos no ha sido posible obtener información, debido posiblemente a dos factores principales: por un lado, la ambigüedad que se introduce en la asignación de las cadenas laterales dado el gran número de cambios que se observan en presencia de las células; y, por otro lado, el rápido fenómeno de intercambio de los NH amida que provoca la pérdida de las correlaciones en la importante región amida del espectro TOCSY. Sin embargo, la gran cantidad de efectos SCE y CSP detectados en la interacción de ghrelina con el receptor GHS-R1a sugiere que la presencia de su receptor provoca un cambio conformacional significativo en el péptido. En el caso de la des-acil ghrelina, el único cambio observado en presencia del receptor corresponde a un efecto CSP del protón amida de la Leu5. Debido a la gran diferencia existente en los efectos observados para ambos péptidos, es evidente, y muy probablemente indican que las interacciones de des-acil ghrelina con el GHS-R1a no son tan amplias ni tan específicas como las que tienen lugar para la ghrelina.

El análisis del índice de los desplazamientos químicos (*Chemical Shift Index*, CSI) es un método semiempírico para determinar la estructura secundaria de un péptido o una proteína.[164] Esta aproximación compara los desplazamientos químicos de los Hα de

[163] Zhang W, Chai B, Li JY, Wang H, Mulholland MW. Effect of des-acyl ghrelin on adiposity and glucose metabolism. Endocrinology. 2008;149:4710-6.
[164] Wishart DS, Sykes BD. Chemical shifts as a tool for structure determination. Methods Enzymol. 1994;239:363-92.

cada aminoácido en el péptido de interés con aquellos descritos para el residuo correspondiente en un promedio de estructuras *random coil*. Aunque el método tiene sus inconvenientes, describe exactamente los datos determinados mediante cristalografía de rayos X, incluso para proteínas pequeñas. Así, dada la ausencia de suficientes datos de NOE (*Nuclear Overhauser Effect*), decidimos aplicar este protocolo a los datos obtenidos para las distintas muestras de ghrelina y, de esta forma, verificar si los diferentes CSI sugerían o no la presencia de una hélice α entre la Pro7 y la Pro21. De hecho, esta zona de la molécula está compuesta de residuos que muestran una preferencia de hélice α, con la excepción de Val12 (CSI = 0) que es voluminoso y poco flexible debido a la ramificación en el carbono beta. Los valores de CSI para la muestra de ghrelina en agua mostraron las características de un péptido desestructurado, mientras que en PBS mostró un desplazamiento a campo alto para todos los Hα de dicha zona, con unos valores de CSI's sugiriendo la presencia de una cierta población de hélice α. Esta discrepancia entre las dos muestras se podría explicar por la presencia del ión fosfato, que se ha descrito como un osmolito estabilizador de hélice α.[165] La muestra de ghrelina en presencia de células *wild type* mostró aproximadamente los mismos desplazamientos químicos que la muestra en PBS, mientras que los medidos para la ghrelina en presencia de células transfectadas, estaban incluso más desplazados hacia campo alto, lo que podría indicar una estabilización posterior de la supuesta hélice α. Estos datos están de acuerdo con la idea de que el loop de la Ser18 a la Lys20 descrito por Kukol podría incorporarse en una hélice α más larga. Los estudios de RMN llevados a cabo podrían indicar que la ghrelina está sólo parcialmente estructurada, con un porcentaje bajo de hélice α del Glu8 a la Lys20. Esta estructura secundaria estaría más estabilizada en presencia del receptor GHS-R1a. De forma paralela, los experimentos llevados a cabo en presencia del receptor mostraron la aparición de nuevos juegos de señales de RMN de ^{1}H en diversos residuos. Ya que se comprobó que era un proceso reversible, este hecho podría explicarse basándonos en

[165] Celinski SA, Scholtz JM. Osmolyte effects on helix formation in peptides and the stability of coiled-coils. Protein Sci. 2002;11:2048-51.

la isomerización de alguna de las prolinas presentes en el esqueleto de la ghrelina: Pro7, Pro21, Pro22, y/o Pro27. Aunque los trabajos de Schubert y col. demostraron que $\Delta^{13}C$ es un indicador muy exacto del estado de isomerización del enlace peptídico *N*-terminal de la prolina,[166] fue imposible medirlo dada la naturaleza de nuestras muestras. Como alternativa, se llevaron a cabo cálculos de simulación restringiendo los ángulos de torsión para adoptar una estructura en hélice α desde Glu8 a Lys20. Cuando se fuerza este tipo de estructura, sólo existe una posibilidad de adoptar esta conformación entre estos residuos, y esto es, una hélice α a izquierdas. Sin embargo, aunque las prolinas podrían adoptar dos posibles conformaciones *E* ($\omega = 180^o$) y *Z* ($\omega = 0^o$), los resultados mostraron dos estructuras con una hélice α a izquierdas, con las prolinas adoptandos las siguientes conformaciones: *EEZE* y *EZEZ*, para Pro7, Pro21, Pro22, y Pro27, respectivamente, como las mejores entre las 32 posibles estructuras.

Los péptidos en disolución normalmente adoptan diversas conformaciones. De esta forma, y en la mayoría de los casos, la descripción de una única estructura 3D para ellos es irreal y, de hecho, la resonancia magnética nuclear puede fácilmente generar conformaciones virtuales cuando no se tienen en cuenta de forma adecuada las diversas contribuciones de las distintas conformaciones. Una buena estrategia para disminuir la velocidad del intercambio conformacional es la utilización de bajas temperaturas antes del punto de congelación (0-8 ^oC). Se ha especulado que estas temperaturas podrían inducir la formación de una estructura similar a la conformación del péptido cuando se encuentra unido al receptor.[167,168,169] Aunque en todos los experimentos llevados a cabo, la

[166] Schubert M, Labudde D, Oschkinat H, Schmieder P. A software tool for the prediction of Xaa-Pro peptide bond conformations in proteins based on 13C chemical shift statistics. J Biomol NMR. 2002;24:149-54.
[167] Slupsky CM, Sykes DB, Gay GL, Sykes BD. The HoxB1 hexapeptide is a prefolded domain: implications for the Pbx1/Hox interaction. Protein Sci. 2001;10:1244-53.
[168] Booth V, Slupsky CM, Clark-Lewis I, Sykes BD. Unmasking ligand binding motifs: identification of a chemokine receptor motif by NMR studies of antagonist peptides. J Mol Biol. 2003;327:329-34.
[169] Langelaan DN, Bebbington EM, Reddy T, Rainey JK. Structural insight into G-protein coupled receptor binding by apelin. Biochemistry. 2009;48:537-48.

temperatura se fijó en 5 ^{o}C, sólo podemos inferir una pequeña población de hélice α en las muestras de PBS, y no en la muestra de agua. Además, la aparición (reversible) de nuevos juegos de señales, indicativos de las posibles isomerizaciones *cis*/*trans* de las prolinas, sólo surgen en presencia del receptor. De hecho, los experimentos de RMN adicionales llevados a cabo bajo condiciones que imitan las membranas (micelas de dodecil sulfato de sodio, SDS) no mostraron la presencia de señales adicionales en los espectros de RMN, sugiriendo así, basándonos en los datos de desplazamientos químicos y NOEs, la existencia de un porcentaje adicional, aunque pequeño, de estructura helicoidal. Toda esta información podría sugerir que el receptor GHS-R1a actúa como una prolil-*cis*/*trans* isomerasa y que, antes de unirse a su receptor, la ghrelina necesita adoptar una conformación específica.

6. Conclusiones

1. El LPA inhibe la secreción de ghrelina en células AGS, a través de la activación de ERK. La fosforilación de ERK procedente de la activación del receptor 2 del LPA, requiere la participación de c-Src y la transactivación del EGFR. Además, dicha activación es independiente de MMP. La correlación observada entre los efectos mitogénicos del LPA y la secreción de ghrelina podría explicar los bajos niveles circulantes de ghrelina observados en pacientes obesos.

2. La obestatina estimula la proliferación de las células de cáncer gástrico KATO-III a través de la activación de la vía de ERK. La estimulación del receptor de obestatina conlleva la activación de PI3k mediante una proteína G sensible a PTX. PI3k activaría una PKC novel (PKCε) que acabaría por desencadenar la ruta de las MAPK a través de la activación de Src. Estos datos hacen pensar en la posibilidad de que la obestatina pueda estar involucrada en procesos como la reparación de daños en la mucosa gástrica o como un factor potenciador de la proliferación en cáncer gástrico.

3. La obestatina activa Akt en células de cáncer gástrico. Esta fosforilación de Akt requiere la transactivación del EGFR y la actividad de las MMPs a través de un mecanismo independiente de proteínas G. La obestatina induce la formación de un complejo de señalización GPR39/β-arrestina 1/Src que transactiva el EGFR. Además, PDK1 y mTORC2 se mostraron como indispensables para la fosforilación de Akt en A-loop(T308) y HM(S473) respectivamente. Por lo tanto, la obestatina se puede añadir al grupo de factores reguladores de las MMPs, las cuales han sido implicadas en diversas patologías humanas como son los procesos inflamatorios y el cáncer.

4. La obestatina activa la secreción de GH en células GC de tumor somatotropo de rata. Las evidencias experimentales muestran una activación consecutiva de G_i, PI3k, PKCε y Src antes de la fosforilación de ERK. Dado que esta línea celular produce obestatina, cabe la

posibilidad de que ésta pueda estar actuando como un factor autocrino/paracrino para inducir la secreción de GH.

5. La grelina muestra un número elevado de residuos afectados por CSP o SCE en presencia de células transfectadas con su receptor: Ser3, Phe4, Leu5, Val12, Gln13, Lys16, Lys19, Glu17 y Lys24. Por el contrario, la des-acil ghrelina sólo presenta un residuo con un CSP evidente. Además, se comprobó que el grupo *n*-octanoico de la Ser3 era claramente necesario para la interacción de la ghrelina con el receptor GHS-R1a, un hecho coherente con los estudios previos de análogos de ghrelina que conferían un papel decisivo de esta cadena para la bioactividad de la ghrelina. La ausencia de CSP o SCE para el caso de la des-acil ghrelina corroboraba esta idea. Estos resultados también apoyaban la conclusión de que los datos de RMN usando células vivas constituyen una buena aproximación para el estudio funcional de la interacción de estos péptidos.

Los valores de CSI obtenidos podrían indicar la presencia de cierta hélice α entre Glu8 y Lys20 para la ghrelina en PBS, la cual sería estabilizada en presencia del receptor GHS-R1a. Dos estructuras posibles surgen por cálculos de modelización tras analizar las posibles conformaciones: los confórmeros *EEZE y EZEZ* con una hélice α a izquierdas desde Glu8 a Lys20. Toda esta información podría sugerir que el receptor GHS-R1a actúa como una prolil-*cis*/*trans* isomerasa y que, antes de unirse a su receptor, la ghrelina necesita adoptar una conformación específica.