Universidad de Santiago de Compostela

Regulación de la secreción gástrica de ghrelina, acción hormonal, control neuroendocrino y desarrollo postnatal

Laboratorio de Endocrinología Molecular
Departamento de Fisiología
Facultad de Medicina

Omar Al-Massadi Iglesias **Diciembre 2009**

El profesor Felipe Casanueva Freijo Catedrático del Departamento de Medicina de la Universidad de Santiago de Compostela y la Doctora Luisa María Seoane Camino, Investigadora Carlos III-SERGAS

Certifican:

Que la presente Tesis Doctoral titulada: "**Regulación de la secreción gástrica de ghrelina, acción hormonal, control neuroendocrino y desarrollo postnatal**" que presenta Omar Al-Massadi Iglesias ha sido realizada bajo su dirección en el Laboratorio de Endocrinología Molecular en la Facultad de Medicina de la Universidad de Santiago de Compostela, estimando que se encuentra concluida y en condiciones de ser presentada para optar al grado de Doctor en Biología.

Y para que conste, firmamos la presente autorización en Santiago de Compostela, a 1 de Diciembre de 2009.

Fdo: Felipe Casanueva Freijo Fdo: Luisa María Seoane Camino

Fdo: Omar Al-Massadi Iglesias

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Índice:

Abreviaturas:

ACTH:	hormona adrenocorticotropa.
AgRP:	péptido relacionado con Agouty.
CART:	tránscrito relacionado con cocaína y anfetamina.
CCK:	colecistokinina.
CHO:	línea celular de ovario de hamster chino.
CRH:	hormona liberadora de corticotropina.
ER(α) y ER(β):	receptores de estrógenos tipo α y β.
GC:	células somatotropas de rata.
GH:	hormona de crecimiento.
GHRH:	hormona liberadora de hormona de crecimiento.
GHRH-R:	receptor de la hormona liberadora de la hormona de crecimiento.
GHRP:	péptidos liberadores de la hormona de crecimiento.
GHS:	secretagogos de la hormona de crecimiento.
GHS-R:	receptor de secretagogos de la hormona de crecimiento.
GLP-1:	péptido similar al glucógeno tipo 1.
GOAT:	ghrelin O-acil transferasa.
HEK-293:	línea celular de embrión de riñón humano.
i.c.v.:	intracerebroventricular.
IgG:	inmunoglobulina G.
IGF-1:	factor de crecimiento insulínico tipo 1.
i.v.:	intravenoso.
KDa:	Kilo Daltons.
KRH:	Krebs Ringer Hepes.
MBOAT:	O-acil transferasa asociada a membrana
MCFA:	ácidos grasos de cadena media.
MCH:	hormona concentradora de melanina.
NPY:	neuropéptido Y hipotalámico.
POCS:	síndrome de ovario poliquístico.
POMC:	proopiomelanocortina.
PRL:	prolactina.
PYY:	péptido YY.

RIA:	radioinmunoensayo.
SNC:	sistema nervioso central.
SNE:	sistema nervioso entérico.
SS:	somatostatina.
TG:	transgénico.
TRH:	hormona estimuladora de la tiroides o tirotropina.
WT:	*wild-type*, no modificado genéticamente.

INTRODUCCIÓN

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La ghrelina es un péptido de 28 aa que se aisló a partir de extractos de tejido gástrico procedentes de roedores (Kojima M et al 1999). Es el ligando endógeno para el receptor de secretagogos de la hormona de crecimiento (GHS-R). Su nombre proviene del dialecto Protoindoeuropeo, donde la raíz *ghre* significa crecimiento y el sufijo *relin* significa liberación, haciendo referencia a su capacidad de liberación de hormona de crecimiento. Aunque la principal fuente de producción de esta hormona es el estómago de donde procede el 65% de la ghrelina circulante (Ariyasu et al H 2001, Dornonville DlC et al 2001, Pekic S et al 2006, Popovic V et al 2005), su síntesis tiene lugar también en otros tejidos. Entre las diferentes funciones atribuidas a la ghrelina destacan su capacidad de estimular la secreción de GH y su potente efecto orexigénico.

La ghrelina fue descubierta como resultado de la llamada farmacología reversa. Inicialmente se sintetizaron una serie de compuestos artificiales que fueron denominados secretagogos de la hormona de crecimiento (GHS), por el grupo de Bowers, y colaboradores (Momany FA et al 1981, Momany FA et al 1984, Bowers CY 1993, Bowers CY 1998), con el fin de investigar posibles alternativas a la administración de hormona de crecimiento en pacientes con deficiencia en GH.

El desarrollo de los GHS empezó con la síntesis de análogos peptídicos modificados de la encefalina, incluyendo GHRP-1, GHRP-2 y GHRP-6. Seguidamente un largo número de secretagogos peptídicos y no peptídicos fueron desarrollados para mejorar la baja bioactividad y especificidad de los GHS (Camanni F et al 1998).

El análisis comparativo inicial de los efectos de la hormona liberadora de hormona de crecimiento (GHRH) y del GHRP-6 sobre la liberación de GH, sugirió que actuaban a través de un mecanismo de acción diferente y complementario.

Este hallazgo se confirmó tras el clonaje por el grupo de Howard de un receptor específico para secretagogos de GH (GHS-R) distinto del receptor de GHRH (Howard AD et al 1996).

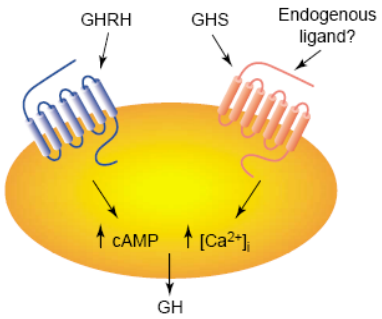

Figura 1: Receptor de secretagogos de GH (Modificada de Kojima M et al 2005).

1. Ghrelina gástrica:

La ghrelina fue originalmente aislada en el estómago, esta hormona se encuentra en el *fundus* gástrico, en la llamada glándula oxíntica (la zona del estómago que secreta ácido). Existen un gran número de células endocrinas en este órgano, el 20% de estas células endocrinas expresan ARNm de ghrelina. Las células que contienen ghrelina son equivalentes a aquellas previamente conocidas como células X/A (Date Y et al 2000a). Existen dos tipos de células productoras de ghrelina, las células cerradas y las células abiertas que están en contacto con el lumen (Sakata I et al 2002a).

En las células productoras de ghrelina, la desacilghrelina o ghrelina no acilada está principalmente localizada en el perinúcleo, mientras que la ghrelina acilada está localizada principalmente en la periferia del citoplasma. Está aceptado que las células endocrinas abiertas productoras de ghrelina en el tracto gastrointestinal están principalmente reguladas por señales relacionadas con el contenido luminal, mientras que las células cerradas productoras de ghrelina reciben modulación por hormonas, estimulación neuronal y distensión mecánica (Solcia E et al 2000).

En humanos se ha descrito así mismo un descenso de un 65% en los niveles circulantes de ghrelina en pacientes sometidos a una gastrectomía (Ariyasu H et al 2001, Pekic S et al 2006, Popovic V et al 2005), hecho que ocurre también en roedores (Dornonville dlC et al 2001). Esto sugiere que la mucosa oxíntica es la mayor fuente de producción de ghrelina del organismo aunque se ha encontrado también producción de ghrelina a menor escala en el intestino delgado (Hosoda H et al 2000a).

2. Receptor de secretagogos de GH:

El receptor de ghrelina se expresa en un único gen localizado en la región cromosómica 3q26.2. Se producen dos tipos de ADN complementario a partir del ARNm del GHS-R como resultado del procesamiento alternativo de su gen, lo que da lugar a dos subtipos del receptor, tipo 1a y tipo 1b. El GHS-R1a está compuesto por 366 aa, presenta siete dominios transmembrana y tiene un peso molecular de 41 KDa, mientras que el GHS-R1b está compuesto por 289 aa con solo cinco dominios transmembrana (Howard AD et al 1996, Mc Kee KK et al 1997a).

El receptor de ghrelina tipo 1a es activado tanto por ghrelina como por secretagogos de GH, sin embargo el tipo b no es activado por ninguno de estos compuestos, y además no está claro que sea un receptor funcional (Smith RG et al 1997, Howard AD et al 1996, Smith RG et al 2001, Mc Kee KK et al 1997a, Gnanapaban S et al 2002).

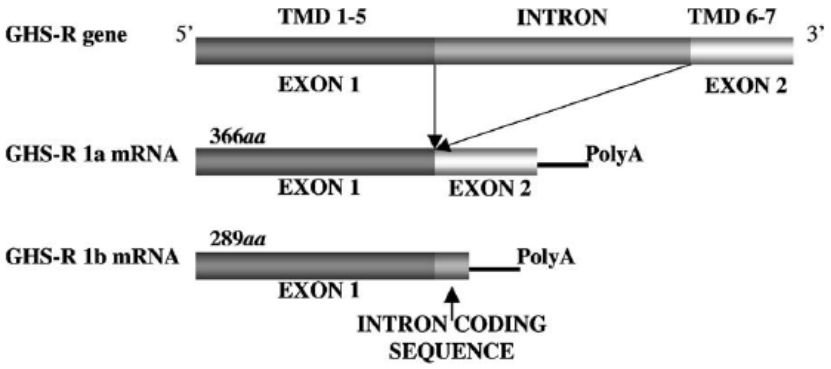

Figura 2: Gen del receptor de secretagogos de GH (Modificada de Smith RG et al 2005).

Existen evidencias de la expresión de GHS-R1a a nivel central, tanto en hipófisis como en hipotálamo (Smith RG et al 1997, Gnanapavan S et al 2002) esta localización es consistente con distintas funciones centrales atribuidas a la ghrelina como el control del apetito, el balance energético y la liberación de GH. Se ha encontrado expresión de este receptor en otras áreas del SNC que afectan a los ritmos biológicos como conciencia, memoria y aprendizaje (Van Der Lely AJ et al 2004), así como en múltiples órganos periféricos como estómago, intestino, páncreas, tiroides, gónadas, glándula adrenal, corazón y en varias líneas celulares tumorales (Gnanapavan S et al 2002, Gaytan F et al 2004).

El receptor de ghrelina está activado de forma constitutiva y esta situación podría tener importancia fisiológica en su papel como regulador de la ingesta y modulador de la secreción de GH (Holst B et al 2003). Se ha demostrado como este receptor activa la vía de la fosfolipasa C a través de la subunidad Gq, dando lugar a la activación de la proteína kinasa C y produciendo un incremento de la concentración de Ca^{+2} intracelular (Kojima M et al 2001).

Recientemente se ha postulado la idea de la existencia de un receptor adicional aún desconocido para la ghrelina, basándose en estudios realizados con una sustancia sintética (BIM-28163) que presenta la capacidad de inhibir la secreción de GH y a la vez produce por otro lado un aumento del peso corporal (Halem HA et al 2005).

3. Estructura de la ghrelina:

El gen de la ghrelina humana está localizado en el cromosoma 3 (3p25-26) y consta de 4 exones y 3 intrones**.** La proteína madura es codificada en los exones 1 y 2 (Wajnrajch MP et al 2000).

El precursor de la ghrelina es un péptido de 117 aa llamado preproghrelina, que tras distintos procesos enzimáticos da lugar a la secuencia final de 28 aa que compone la ghrelina (Kojima M et al 1999, Jeffery PL et al 2005).

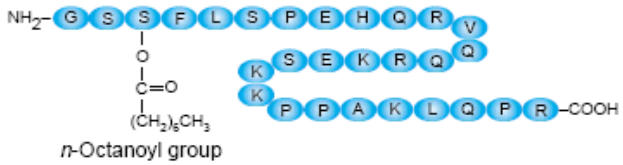

Figura 3: Estructura de la ghrelina (Modificada de Kojima M et al 2005).

Antes de secretarse, la ghrelina sufre una esterificación en el citoplasma, concretamente una n-octanoilación en el residuo 3 de Serina. La enzima que cataliza este proceso es la recientemente descubierta ghrelin O-acil transferasa (GOAT) (Yang J et al 2008, Gutierrez JA et al 2008). La ghrelina constituye el primer ejemplo de acilación de una proteína secretada y parece que esta octanoilación es esencial para la unión al receptor y por tanto para su actividad biológica (Kojima M et al 1999) al menos en lo que se refiere a la secreción de GH**.**

Algunos análogos truncados en el extremo carboxilo terminal de la ghrelina son también capaces de unirse y activar el GHS-R1a. Esos hallazgos muestran que no solamente el grupo acilo, sino también los primeros 7 aa (los cuales muestran similitudes estructurales con algunos GHS peptídicos como GHRP-6 y hexarelina) son esenciales para la activación del GHS-R1a (Bednarek MA et al 2000).

4. Ghrelin O-acil tranferasa (GOAT):

El gen de la ghrelina da lugar a diferentes productos de los cuales los más abundantes son las formas acilada y no acilada de este péptido. La forma acilada de la ghrelina se caracteriza por la presencia de un acido n-octanoico en el residuo de la Ser^3.

Esta acilación es esencial para su unión al GHSR-1a y por lo tanto para llevar a cabo sus funciones endocrinas, aunque se han descrito diferentes efectos ejercidos por la forma no acilada de la ghrelina. Recientemente se ha descrito por dos grupos distintos que esta modificación de la ghrelina se produce por acción de la ghrelin O-acil transferasa (GOAT), una enzima anteriormente conocida como MBOAT4 (Gutiérrez JA et al 2008, Yang J et al 2008). Se ha postulado que esta acción se produce en el retículo endoplasmático, previo paso al aparato de Golgi donde la prohormona convertasa 1/3 va a dar lugar a la forma madura de ghrelina. Mediante estudios de inmunohistoquímica se ha demostrado que existe colocalización entre GOAT y ghrelina sugiriendo que esta acilación se produce en las mismas células donde se sintetiza la ghrelina. En estas células se ha encontrado que los niveles de ARNm de GOAT son dos veces menores que los de ghrelina (Sakata I et al 2009).

GOAT se expresa en cantidades variables en: estómago, páncreas, colon, corazón, hígado, músculo, hipófisis, glándula salivar, testículo, timo, hipotálamo, tejido adiposo, glándula adrenal, placenta y ovario dependiendo de la especie estudiada (Sakata I et al 2009, Gonzalez CR et al 2008, Gutierrez JA et al 2008, Yang J et al 2008).

El sustrato que utiliza GOAT para acilar la ghrelina son ácidos grasos de cadena media procedentes de la dieta (MCFAs). Los MCFAs se encuentran de forma natural en el coco, el aceite, la mantequilla, en aceites de palma y están presentes también en la leche de roedores y humanos (Nishi Y et al 2005, Kirchner H et al 2009).

Existe una gran controversia en cuanto a la regulación de esta enzima por el estado nutricional. Unos autores demuestran que en ratón sigue un patrón de expresión similar al del gen de la ghrelina (Xu G et al 2009), con una elevada expresión en estados energéticos negativos y baja en estados energéticos positivos. Por otra parte otros autores encontraron en rata que la expresión de esta enzima permanece inalterada en

condiciones de ayuno agudo sin embargo se incrementa en casos de malnutrición prolongada (González CR et al 2008). A raíz de estos datos se ha sugerido un posible papel de esta enzima mediando la respuesta fisiológica a la malnutrición crónica, lo cual representaría una respuesta adaptativa para prevenir posibles alteraciones a largo plazo de la homeostasis energética y del peso corporal en estados de balance energético negativo (González CR et al 2008). Por otra parte, un tercer grupo encontró que se producía un aumento en la expresión de esta enzima en estados energéticos positivos en ratones. Existen resultados obtenidos con ratones *knock-out* para GOAT donde se demuestra que presentan un fenotipo normal, sin embargo cuando ingieren una dieta alta en MCFAs alcanzan un peso corporal significativamente menor que los *wild-type* (WT) alimentados con este tipo de dieta. Los autores demostraron también que el ratón TG (sobreexpresa ghrelina y GOAT) cuando se alimenta con una dieta alta en MCFAs presenta una mayor masa grasa y peso corporal que el ratón WT, debido a que el gasto energético es significativamente menor, lo que indica que los ratones TG presentan una tasa catabólica de lípidos menor que los WT. Se comprobó que este modelo es inducible y reversible cuando se modifica la dieta alta en MCFAs por una dieta normal (Kirchner H et al 2009). Así algunos autores proponen que el sistema de ghrelina es el responsable de preparar al organismo para metabolizar óptimamente el alimento y almacenar energía, más que un factor de iniciación de la ingesta o señal de apetito como se había propuesto hasta el momento. Se ha propuesto también que la expresión o síntesis de GOAT podría actuar como un represor translacional del gen de ghrelina lo cual vendría justificado por los elevados niveles plasmáticos de esta hormona encontrados en ratones *knock-out* para GOAT.

En cuanto a un posible dimorfismo sexual o variaciones dependientes de la edad de GOAT existen muy pocos datos disponibles, solo hay constancia en la bibliografía de

un trabajo donde estudian la expresión de esta enzima en roedores macho de 10, 25, 60 días de edad y no ven ninguna variación entre los grupos estudiados (González CR et al 2008).

5. Funciones principales de ghrelina:

5.1. Liberación de GH:

La administración de ghrelina estimula la secreción de GH tanto *in vitro* como *in vivo* (Kojima M et al 1999, Seoane LM et al 2000) en prácticamente todas las especies estudiadas. La potencia de liberación de GH es comparable a la de GHRH *in vitro* y es superior a esta en animales en libre movimiento y humanos (Seoane LM et al 2000, Peinó R et al 2000, Álvarez-Castro P et al 2004, Arvat E et al 2001).

La inyección intravenosa (i.v.) e intracerebroventricular (i.c.v.) de ghrelina estimula la secreción de GH a dosis mínimas de 1.5 nmol y 10 pmol respectivamente (Date Y et al 2000a), demostrando una mayor potencia en el caso de la administración i.c.v. La inyección i.v de ghrelina en humanos, induce la secreción de GH de una manera dosis dependiente, con una dosis mínima de 0.2 µg/Kg (Date Y et al 2002a, Takaya K et al 2000). Aunque tanto la ghrelina como algunos secretagogos de GH son capaces de inducir la secreción de GH en cultivos primarios de células somatotropas (Kojima M et al 1999), también se ha demostrado que esta acción es mediada por estructuras suprapituitarias hipotalámicas (Popovic V et al 2003, Popovic V et al 1995, Pombo M et al 1995), por lo que no está muy claro a qué nivel actúa la ghrelina para producir la secreción de GH.

La coadministración de ghrelina con GHRH, produce un efecto sinérgico en la secreción de GH (Hataya Y et al 2001, Arvat E et al 2001, Broglio F et al 2002) y la infusión de GHRH en ratas en libre movimiento resulta en un incremento significativo

de la expresión de los genes que codifican la ghrelina y sus receptores en la glándula hipofisiaria (Kamegai J et al 2004). Finalmente tanto los anticuerpos como los antagonistas de R-GHRH producen una atenuación de la respuesta secretora de GH a secretagogos de ghrelina (Pandya N et al 1998), así como en pacientes con una mutación del R-GHRH (Gondo RG et al 2001, Maheshwari HG et al 2002) lo cual demuestra que para que se produzca el efecto estimulador de la secreción de GH por ghrelina es necesario que el sistema de GHRH permanezca intacto (Maghnie M et al 2007, Popovic V et al 1995, Pombo M et al 1995, Popovic V et al 2003).

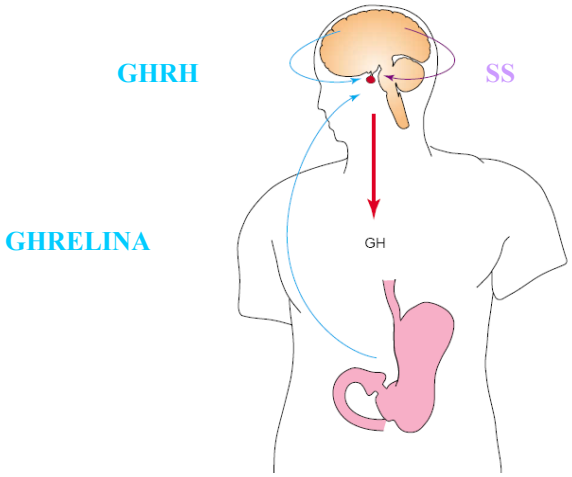

Figura 4: Regulación de la secreción de GH (Modificada de Korbonits M et al 2004).

5.2. Acciones orexigénicas:

La primera evidencia del efecto orexigénico de ghrelina fue obtenida por Arvat E et al, en el año 2000 el cual en un estudio de liberación de GH, encontró que en un 75% de los voluntarios sanos se producía un aumento del apetito como efecto colateral a la inyección de ghrelina. Estos hallazgos fueron confirmados en estudios posteriores (Nakazato M et al 2001, Wren AM et al 2001). Al mismo tiempo otros estudios reforzaron esta implicación de la ghrelina sobre la regulación del balance energético (Tschop M et al 2000, Shintani M et al 2001). La composición corporal de ratones tratados con ghrelina crónicamente muestra un aumento de la masa grasa (Tschop M et

al 2000). Análisis inmunohistoquímicos indican que las células neuronales que contienen ghrelina se encuentran en el núcleo arcuato del hipotálamo, una región implicada en la regulación del apetito (Kojima M et al 1999, Hosoda H et al 2002).

Además publicaciones recientes indican que la ghrelina también ha sido detectada en neuronas que no habían sido previamente caracterizadas, adyacentes al tercer ventrículo entre el núcleo dorsal, el núcleo ventral, el núcleo paraventricular y núcleo arcuato del hipotálamo (Cowley MA et al 2003).

Las neuronas que contienen ghrelina interactúan con neuronas que contienen neuropéptido Y (NPY), proteína asociada a Agouti (AgRP) y Orexina (Nakazato M et al 2001, Kamegai J et al 2001, Toshinai K et al 2003) y pueden estimular la liberación de péptidos orexigénicos. Por otra parte pueden inhibir la expresión de neuronas que expresan POMC (proopiomelanocortina) y CART (transcrito relacionado con cocaina y anfetamina) las cuales presentan una acción anorexigénica (Chen HY et al 2004, Shioda S et al 2008, Nogueiras R et al 2008).

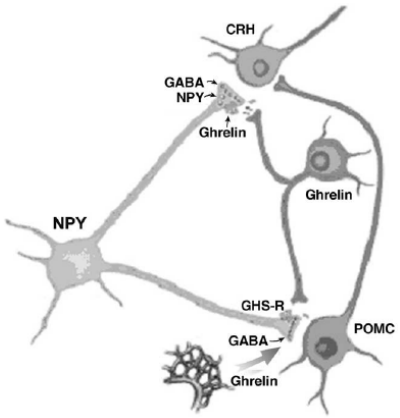

Figura 5: Células neuronales del núcleo arcuato hipotalámico (Modificada de Smith RG et al 2005).

El núcleo arcuato hipotalámico es el principal sitio activo de la ghrelina y es también una diana para la leptina, principal hormona supresora del apetito, producida por el tejido adiposo. NPY y AgRP son producidos en las mismas neuronas de este

núcleo hipotalámico, y los efectos estimuladores del apetito de estos péptidos son directamente inhibidos por la leptina (Mizuno TM et al 2000). Como sugiere la distribución de las neuronas que contienen ghrelina en el hipotálamo, la inyección i.c.v. e i.v. de ghrelina induce la expresión de proteína c-fos en células neuronales NPY e incrementan la cantidad de ARNm de NPY y AgRP en el núcleo arcuato (Seoane LM et al 2003, Nakazato M et al 2001). La inyección i.c.v. de antagonistas del receptor de NPY, IgG anti-NPY, inhibidores de AgRP e IgG anti-AgRP inhiben los efectos orexigénicos de la ghrelina. Estos resultados indican que la ghrelina ejerce una actividad estimuladora del apetito en el hipotálamo promoviendo la producción y secreción de los péptidos NPY, AgRP y Orexina.

Teniendo en cuenta que la administración periférica de ghrelina estimula la ingesta (Nakazato M et al 2001, Tschop M et al 2000) y que las hormonas peptídicas en la circulación sanguínea generalmente no cruzan la barrera hematoencefálica, debería haber otra vía indirecta a través de la cual la ghrelina periférica pueda activar las neuronas hipotalámicas reguladoras del apetito (Date Y et al 2002a, Williams DL et al 2003a). Se sugiere por tanto que la ghrelina secretada por el estómago, podría actuar en las terminacuiones del nervio vago a nivel gástrico y transmitir la señal al núcleo arcuato hipotalámico estimulando el apetito (Kojima M et al 2004).

Recientemente se ha descrito un mecanismo de acción alternativo de la ghrelina en el hipotálamo según el cual el efecto orexigénico de la ghrelina estaría mediado por el metabolismo de los ácidos grasos (López M et al 2008). En otro estudio se propone a la ghrelina como una vía de señalización que informa al sistema nervioso central de la presencia de calorías en la dieta más que de la ausencia como es comúnmente aceptado (Kirchner H et al 2009).

6. Otras funciones de la ghrelina:

La ghrelina ha sido identificada en un gran número de especies diferentes, incluyendo humano, cerdo, rata, ratón y pollo (Parhar IS et al 2003). El efecto sobre la ingesta de la ghrelina es opuesto en el pollo con respecto al observado en rata y humano (Saito ES et al 2002). Se ha encontrado expresión de ghrelina en múltiples tejidos como la hipófisis (Caminos JE et al 2003a), hipotálamo (Kojima M et al 1999), células inmunes (Hatori N et al 2001), placenta (Gualillo O et al 2001a), ovario (Caminos JE et al 2003b, Gaytan F et al 2003), testículos (Tena-Sempere M et al 2002), pulmón (Volante M et al 2002a), riñón (Mori K et al 2000), condrocitos (Caminos JE et al 2005), cardiomiocitos (Iglesias MJ et al 2004) y páncreas (Volante M et al 2002b, Date Y et al 2002b) lo cual pone de manifiesto la diversidad y complejidad de funciones de la ghrelina.

Así esta hormona presenta diferentes acciones: liberación de ACTH y prolactina, regulación de la secreción ácida del estómago (Date Y et al 2001), de la motilidad gástrica (Masuda Y et al 2000), del sueño, de la vasodilatación y de la proliferación celular. Sus funciones fisiológicas están por dilucidar.

En cuanto a la regulación del sueño la ghrelina modifica los patrones de sueño-vigilia disminuyendo la duración de los períodos de sueño REM (Bona G et al 2003). La respuesta de la ghrelina al estrés está mediada a través de CRH, así la administración de esta hormona incrementa la expresión de ARNm y secreción de Prolactina, ACTH y Cortisol (Arvat E et al 2001, Wren AM et al 2000, Asakawa A et al 2001**).**

Parece que la ghrelina tiene un efecto importante en la proliferación celular. La ghrelina en cultivos celulares puede causar la inhibición del crecimiento de cáncer de mama (Cassoni P et al 2001), cáncer de tiroides (Cassoni P et al 2000) y cáncer de

pulmón (Ghe C et al 2002), sin embargo induce efectos proliferativos en líneas celulares de hepatoma (Murata M et al 2002) y de cáncer de próstata (Jeffery PL et al 2002).

7. Regulación de la secreción de ghrelina:

Se ha descrito que el principal foco productor de ghrelina es el estómago, donde se sintetiza el 65% del total de la ghrelina circulante del organismo (Ariyasu A et al 2001, Pekic S et al 2006, Popovic V et al 2005). La desacilghrelina es la forma mayoritaria constituyendo el 90% de la ghrelina circulante total (Hosoda H et al 2000a).

Dado que además del estómago se ha encontrado expresión de ghrelina en diferentes tejidos del organismo, la regulación de la secreción de ghrelina y sus efectos pueden ocurrir a diferentes niveles.

La regulación de los niveles de ghrelina circulante se encuentra influenciada de manera muy acusada por la ingesta. Así se ha descrito un aumento en los niveles circulantes de ghrelina inmediatamente antes de la ingesta de alimentos y caídas postpandriales (Tolle V et al 2002, Seoane LM et al 2007b). Estos niveles presentan un ritmo circadiano, con una variación diurna caracterizada por niveles generalmente elevados durante la noche, seguida por una disminución a las 2-4 h (Cummings DE et al 2001, Chan JL et al 2004). No se ha observado una sincronización con el pulso de GH en estudios en roedores (Okimura Y et al 2003).

El estado nutricional es un importante regulador de los niveles de ghrelina endógena, observándose niveles elevados de ghrelina circulante en condiciones de ayuno.

La elevación encontrada durante el ayuno es el resultado del incremento en la frecuencia de su pulso secretor, así como de la amplitud del mismo, esto se produce de manera sincronizada con el bajo pulso de leptina que se observa en condiciones de

ayuno y los dos factores dan lugar a una fuerte acción orexigénica (Bagnasco M et al 2003). El ayuno produce un aumento de la expresión de ghrelina en estómago, sin embargo este aumento no se observa en el hipotálamo ni en la hipófisis (Torsello A et al 2003). Cuando se restablece la alimentación tras el ayuno, los niveles de ghrelina disminuyen de forma drástica a través de un mecanismo desconocido hasta el momento. Dado que la mayoría de la ghrelina circulante tiene un origen gástrico, es razonable pensar que la disminución en los niveles de ghrelina observada tras la ingesta así como la sensación de saciedad tiene su origen en el estómago.

Tanto la actividad del sistema nervioso autónomo como la secreción hormonal participan en el control de la respuesta gastrointestinal a la ingesta de nutrientes.

Esta respuesta se subdivide en fase cefálica, fase gástrica y fase intestinal. En humanos la fase cefálica preabsortiba se produce como consecuencia de oler y ver el alimento, así como del estímulo orofaríngeo producido al masticar la comida; la fase gástrica resulta de la acción del alimento en el estómago. La primera es producida mayoritariamente por la activación eferente vagal y la concomitante liberación de hormonas gastroenteropancreaticas, y puede influir en la subsiguiente respuesta gastrointestinal (Strube JH et al 1992, Ramirez I et al 1985).

Recientemente se ha sugerido la existencia de la estimulación cefálica vagal sobre la regulación de los niveles de ghrelina por la ingesta en animales (Sujino T et al 2002), sin embargo en humanos existen datos contradictorios (Erdmann J et al 2003, Arosio M et al 2004) debido a que este tipo de estudios resultan difíciles de realizar dada la capacidad de condicionamiento de nuestra especie.

Los niveles de ghrelina son bajos en sujetos obesos comparados con sujetos con peso normal, mientras que pacientes con bajo índice de masa corporal, como la anorexia nerviosa, tienen niveles más altos (Ariyasu H et al 2001, Shiiya T et al 2002, Miljic D et

al 2006). Los sujetos obesos incrementan sus niveles de ghrelina circulante cuando pierden peso (Hansen TK et al 2002) y estos niveles disminuyen en pacientes con anorexia nerviosa cuando recuperan el peso normal (Otto B et al 2001). Una excepción a este comportamiento lo constituye el síndrome de Prader-Willi, que es el síndrome más común de obesidad humana, caracterizado por una hiperfagia severa, deficiencia de GH e hipogonadismo. Pacientes con este síndrome presentan niveles de ghrelina circulante muy elevados a pesar de la obesidad severa que padecen (Haqq AM et al 2003a, Goldstone AP et al 2004).

7.1 Efecto de la insulina y de la glucosa sobre la secreción de ghrelina:

Como ya se ha dicho anteriormente, la ghrelina es una hormona que varía de forma importante dependiendo del estado nutricional del individuo. Así la ingesta de nutrientes provoca una caída en los niveles circulantes de ghrelina. Actualmente se acepta que tanto la insulina como la glucosa tienen un efecto inhibitorio sobre los niveles de ghrelina (Williams LM et al 2003, Briatore R et al 2003, Barber TM et al 2008, Flanagan ED et al 2001) este efecto se ha sugerido que puede ser mediado al menos en parte por el nervio vago (Pekic S et al 2006).

Existe un estudio en el cual se evaluó el efecto de la infusión de glucosa sobre los niveles de ghrelina en animales en los cuales se había colocado una válvula pilórica que permitía o impedía el vaciamiento gástrico. Se demostró como la infusión de glucosa disminuye los niveles de ghrelina circulante tras 30 minutos en el caso de los animales que presentaban la válvula abierta, permitiendo el vaciamiento gástrico, mientras que la infusión de agua no producía ningún efecto (Williams DL et al 2003b). Por el contrario cuando la válvula permanecía cerrada, impidiendo el vaciamiento gástrico, los niveles de ghrelina plasmática no se veían afectados por ninguno de los dos tratamientos.

Estos datos ponen de manifiesto la existencia de un mecanismo postgástrico mediado por la ingestión de glucosa que regula los niveles circulantes de ghrelina (Williams DL et al 2003b).

Además existen numerosos estudios donde se demuestra que la administración, tanto oral como i.v. de glucosa disminuye los niveles de ghrelina. Un factor adicional a tener en cuenta es el incremento en los niveles de insulina que se produce como consecuencia de la administración de glucosa.

Se ha postulado que es la glucosa únicamente la que ejerce esta inhibición en los niveles de ghrelina (Briatore R et al 2003). Sin embargo existen estudios que apoyan la teoría de que es la insulina la que produce este efecto independientemente de la glucosa (Flanagan ED et al 2003).

Diversos trabajos de investigación apuntan el hecho de que la ghrelina puede estar induciendo un balance energético positivo. Se ha descrito como la administración de ghrelina disminuye la utilización de los depósitos grasos e incrementa el metabolismo de los carbohidratos (Tschop M et al 2000). Estos hallazgos apoyan la hipótesis de que ya que la ghrelina ejerce un balance energético positivo, debe encontrarse disminuida en condiciones de hiperglicemia para compensar este exceso de energía, formando parte de un mecanismo de *feed-back* para mantener la homeostasis energética.

7.2 Efecto de la SS sobre la secreción de ghrelina:

La somatostatina (SS) es un péptido producido en el cerebro, en el tracto gastrointestinal y páncreas, que ejerce un amplio espectro de actividades biológicas a través de mecanismos endocrinos, paracrinos y neuroendocrinos (Reichlin S et al 1983, Reisine T et al 1995). Este péptido en las células gástricas D funciona de una forma paracrina, suprimiendo la secreción de histamina y de gastrina de las células

enterocromafines y células G respectivamente (Schubert ML et al 1987, Chiba T et al 1988). La SS, así como sus análogos, inhiben la secreción de ghrelina en sujetos normales, en sujetos acromegálicos y en pacientes con el síndrome de Prader-Willi (Haqq AM et al 2003b). Sin embargo el mecanismo a través del cual ejercen estos efectos no ha sido determinado.

Recientemente ha sido publicado que la infusión de somatostatina en sujetos normales reduce los niveles plasmáticos de ghrelina en un 70-80% de los valores control (Broglio F et al 2002, Norrelund H et al 2002). La administración subcutánea de octreotide, un análogo de somatostatina, también disminuye los niveles de ghrelina en pacientes con acromegalia (Freda PU et al 2003). La infusión sistémica de SS afecta a un amplio rango de hormonas además de la ghrelina, como GH, insulina, glucagón y otras hormonas gastroentéricas. Estos hallazgos pueden reflejar un efecto indirecto de esta hormona sobre la secreción de ghrelina.

Se sabe poco sobre la regulación directa de la secreción de ghrelina por parte del estómago. Las células inmunoreactivas para ghrelina aparecen desde el cuello hasta la base de la glándula oxíntica de la rata, las células inmunoreactivas para SS están también presentes en la glándula oxíntica. Una porción de las células inmunoreactivas para ghrelina contactan con las células inmunoreactivas para somatostatina, lo cual sugiere la existencia de algún tipo de interacción entre ghrelina y SS a nivel gástrico (Norrelund H et al 2002).

Los efectos de la SS sobre las funciones gastrointestinales son exclusivamente inhibitorios, actuando de forma opuesta a otros péptidos gastrointestinales y afectando a la secreción gástrica, motilidad, crecimiento y secreción hormonal (Shimada M et al 2003).

7.3 Efecto de GH sobre la secreción de ghrelina:

La ghrelina es un potente estimulador de GH tanto en humanos como en animales de laboratorio (Peino R et al 2000, Seoane LM et al 2000).

Este hecho ha puesto de manifiesto la posibilidad de la existencia de un eje hipófisis-estómago. En ese caso, cualquier reducción o elevación en los niveles de GH sistémica afectaría a la ghrelina gástrica y a su secreción.

En un principio se vio como la administración de GH a ratas, disminuía los niveles de ARNm y niveles plasmáticos de ghrelina hasta aproximadamente un tercio de los niveles control, posiblemente como consecuencia de un *feedback* negativo ejercido por la propia GH sobre la secreción de ghrelina (Qi X et al 2003, Tschop M et al 2002).

Se ha demostrado que los ratones transgénicos que sobreexpresan GHRH presentan unos niveles plasmáticos de GH aproximadamente 10 veces más altos que los observados en animales no transgénicos (Debeljuk L et al 1999, Hammer RE et al 1985), mientras que los niveles de expresión de ghrelina gástrica en estos animales se encuentran disminuidos de manera importante (Qi X et al 2003). En la rata adulta de edad avanzada, los niveles de expresión, los depósitos de péptido y los niveles plasmáticos de ghrelina se incrementan significativamente cuando se comparan con ratas jóvenes, mientras que la secreción de GH disminuye con la edad en roedores (Sonntag WE et al 1980, Floríni JR 1981). Teniendo en cuenta que la administración exógena de GH produce un *feed-back* negativo sobre la ghrelina en estómago, el aumento en la producción y secreción de ghrelina con la edad en ratas viejas puede ser debido a la reducción en el *feed-back* negativo producido por la caída de GH endógena.

Estos hallazgos apoyan la hipótesis de la existencia de un eje GH-hipófisis-ghrelina-estómago (Qi X et al 2003, Tschop M et al 2002), sin embargo el mecanismo

por el cual actuaría esa realimentación negativa en la homeostasis y en la secreción de ghrelina es aún desconocido. Por otro lado existen estudios que proponen que esta regulación de GH sobre la secreción de ghrelina por parte del estómago no se produce con una administración crónica de GH (Janssen JA et al 2001).

Así usando diferentes modelos de ratones transgénicos se detectó que una exposición a altos niveles de GH desde la edad temprana no afectaba a los niveles de ARNm de ghrelina en el estómago, ni a sus niveles plasmáticos, este grupo de investigación propone que la regulación de GH sobre la secreción de ghrelina en el estómago no se ve afectada por cambios en la acción periférica de GH (Nass R et al 2004).

Por lo tanto existe una controversia sobre el efecto que ejerce la GH sobre la regulación de la ghrelina circulante.

7.4 Efecto de IGF-1 sobre la secreción de ghrelina:

El IGF-1 (factor de crecimiento insulínico tipo 1) es sintetizado principalmente en el hígado por acción de la GH. Se sabe poco sobre la interacción entre esta hormona y la ghrelina. Existen estudios que muestran una correlación negativa entre estas dos hormonas en adolescentes y niños (Whatmore AJ et al 2003, Bellone S et al 2003, Bellone S et al 2002, Kitamura S et al 2003). También se ha propuesto la existencia de una regulación negativa del IGF-1 sobre la expresión del receptor de ghrelina en la hipófisis de rata (Kamegai J et al 2005). Por el contrario estudios en humanos adultos no muestran la existencia de correlación alguna (Dall R et al 2002, Malik IA et al 2004, Rigamonti AE et al 2002, Tschop M et al 2002).

Es por tanto muy pobre el conocimiento a cerca del efecto que el IGF-1 ejerce sobre la regulación de ghrelina y los datos que existen hasta el momento son controvertidos.

7.5. Efecto de los estrógenos sobre la secreción de ghrelina:

El receptor α de estrógenos (ERα) se expresa en el estómago, concretamente en la mucosa gástrica en las células productoras de ghrelina (Campbell-Thompson ME et al 2001). Este dato sugiere la posibilidad de que exista una interrelación a nivel gástrico entre los estrógenos y la ghrelina. Distintos estudios tanto en animales de experimentación como en humanos, demuestran que variaciones en los niveles de estrógenos están involucradas en la regulación de la secreción de ghrelina.

Existe un estudio que propone que son los estrógenos sintetizados en el propio estómago los que regulan directamente la ghrelina gástrica (Sakata I et al 2006).

Estudios *in vitro* demuestran que los estrógenos incrementaban la secreción gástrica de este péptido de una forma dosis-dependiente en células aisladas de estómago de roedores mientras que el pretratamiento con antagonistas del receptor de estrógenos bloquean ese incremento en la secreción de ghrelina (Sakata I et al 2006).

Existen datos contradictorios en la bibliografía sobre el efecto de la ovariectomía sobre los niveles de ghrelina, mientras que Gualillo et al determinaron que este procedimiento no afectaba a los niveles circulantes de ghrelina (Gualillo O et al 2001b), Matsubara M et al mostraron como tanto la ghrelina plasmática, el ARNm y el número de células productoras de ghrelina se incrementan en animales sometidos a una ovariectomía y este aumento era revertido tras el restablecimiento de los niveles fisiológicos de estrógenos mediante la administración de β-estradiol (Matsubara M et al 2004).

Estudios realizados en humanos demuestran que el tratamiento con β-estradiol a mujeres menopáusicas produce un incremento en los niveles plasmáticos de ghrelina total (Di CC et al 2007) y acilada (Kellokoski E et al 2005). Además pacientes con

anorexia nerviosa presentan una elevación en los niveles de ghrelina plasmática tras la administración de estrógenos (Grispoon S et al 2004). En otro trabajo no se encuentra ninguna relación entre estas dos hormonas en un estado hipoestrogénico (Purnell JQ et al 2003).

Aunque los datos existentes sobre el tema pueden parecer contradictorios en un primer momento, los diferentes resultados pueden explicarse por las diferencias metodológicas entre esos trabajos experimentales.

7.6. Efecto de la testosterona sobre la secreción de ghrelina:

Hasta la fecha existen muy pocas evidencias experimentales del efecto que produce la testosterona sobre la secreción de ghrelina en animales de experimentación.

Se ha demostrado que existen receptores de andrógenos en el estomago y tejido gastrointestinal humano (Wilson CM et al 1996) y de primate no humano (Winborn WB et al 1987) lo que indica que es posible una relación directa entre estas hormonas.

Estudios en humanos han buscado esta posible relación en pacientes con hipogonadismo, a los que se les administró un suplemento de testosterona o mujeres diagnosticadas de ovario poliquístico, que suelen presentar unos niveles superiores a lo normal de andrógenos debido a su patología. Datos experimentales muestran una controversia en cuanto al efecto que produce la suplementación con testosterona en personas del sexo masculino. Algunos autores defienden que esta suplementación en pacientes con hipogonadismo aumenta los niveles séricos de ghrelina (Pagotto U et al 2002). Sin embargo otro estudio en pacientes adolescentes muestra una disminución de la secreción plasmática de ghrelina tras la administración de hormonas sexuales (Lebenthal Y et al 2006).

Existen datos conflictivos sobre los niveles de ghrelina en mujeres con síndrome de ovario poliquístico (PCOS), mientras unos trabajos defienden que los niveles en estas

mujeres están significativamente disminuidos (Pagotto U et al 2002, Schofl C et al 2002, Moran LJ et al 2004), otros defienden que están significativamente aumentados (Wasko R et al 2004).

Por último otros estudios muestran que no existe correlación alguna entre los niveles séricos de ghrelina y los niveles de testosterona en mujeres con esta patología.

Esos datos tan contradictorios tienen difícil explicación.

7.7 Regulación de los niveles de ghrelina con la edad:

La distribución de los péptidos neuroendocrinos varía con la edad en diferentes localizaciones del tracto gastrointestinal. Es posible que la ghrelina gástrica cambie con la edad para adaptar el organismo a los requerimientos metabólicos demandados en cada etapa de la vida.

Varios trabajos han estudiado las variaciones de los niveles de ghrelina durante el desarrollo postnatal, sin embargo estos trabajos muestran resultados muy controvertidos. En un estudio llevado a cabo en ratones, no se encontraron cambios significativos en la expresión de ARNm de ghrelina con la edad (Tanaka M et al 2001). Por el contario, la mayoría de estudios en roedores muestran que la expresión de ghrelina gástrica es detectable justo después del nacimiento y aumenta gradualmente hasta la edad adulta (Sakata I et al 2002b, Gualillo O et al 2001b). Existen estudios histológicos en los cuales se detecta un patrón similar al de los niveles de expresión de ARNm de ghrelina en células gástricas inmunopositivas para ghrelina (Sakata I et al 2002b). Sorprendentemente este patrón de expresión de la ghrelina es completamente opuesto al encontrado en la hipófisis, donde los niveles de expresión de este péptido son elevados en el periodo fetal y disminuyen después del nacimiento (Kamegai J et al 2001). Sin embargo la expresión del receptor GHS-R1 en hipófisis sigue el mismo patrón que la expresión gástrica de ghrelina.

Desde un punto de vista metabólico, uno de los procesos más relevantes en el desarrollo postnatal de la rata es el destete. El destete está asociado con cambios drásticos tanto en la dieta como en el entorno, y está caracterizado por alteraciones en la morfología del tracto gastrointestinal, por lo cual todas las modificaciones relacionadas con este periodo pueden estar afectando a la producción y expresión de ghrelina. Un estudio en crías de especie porcina mostró que la administración de ghrelina exógena durante el destete, produce una rápida recuperación de la disminución de peso corporal producida por este fenómeno (Salfen BE et al 2004).

Los niveles de ghrelina en animales lactantes están regulados de diferente manera que en animales adultos, así en animales de una semana de edad, ocho horas de restricción de leche disminuyen la concentración de ghrelina en el estómago sin embargo los niveles de ghrelina plasmática aumentan como es característico en el estado de restricción calórica (Hayashida T et al 2002).

La pubertad es otra etapa en la cual los requerimientos energéticos y consecuentemente los niveles de hormonas circulantes están alterados de manera notable. Varios trabajos demuestran que los cambios más relevantes en los niveles de ghrelina son producidos en la etapa puberal, en paralelo con variaciones en los niveles de hormonas sexuales. Estas alteraciones asociadas a la edad afectan a la expresión de ARNm de ghrelina, células inmunopositivas para ghrelina y ghrelina plasmática (Matsubara M et al 2004).

Aunque se han publicado datos sobre los niveles de ARNm de ghrelina y contenido proteico, no existen datos sobre secreción de ghrelina desde tejido gástrico a lo largo del desarrollo postnatal.

8. Subproductos del gen de ghrelina:

Existen diferentes subproductos del gen de ghrelina entre los que destacan la obestatina (Zhang JV et al 2005), la desacilghrelina y la desglut-14ghrelina (Hosoda H et al 2000b). La obestatina procede de una modificación postraduccional a partir de la preproghrelina, la desglut-14-ghrelina se produce por un diferente procesamiento del ARNm del gen de ghrelina y la desacilghrelina no presenta la acilación postraduccional de la forma octanoilada.

8.1. Obestatina:

La obestatina es un péptido de 23 aa que se descubrió en el año 2005 (Zhang JV et al 2005). Su descubrimiento se produjo a partir de predicciones bioinformáticas partiendo de la hipótesis de la existencia de posibles señales metabólicas derivadas de propéptidos de hormonas conocidas, así la obestatina proviene del mismo gen que la ghrelina y mediante diferentes modificaciones da lugar a dicho péptido (Zhang JV et al 2005). Su nombre procede de una contracción de la palabra obese del latín *obedere* que significa devorar y *statin* que denota supresión. Se postuló que esta hormona presenta la capacidad de inhibir la ingesta y disminuir el peso corporal, el vaciamiento gástrico y la motilidad intestinal (Zhang JV et al 2005). Hasta el momento se ha encontrado expresión de obestatina a nivel del sistema nervioso central (Zhang JV et al 2005), en el tracto gastrointestinal, así como en otros órganos periféricos de roedores como el bazo, el páncreas (Chanoine JP et al 2006) o en testículo (Dun SL et al 2006). El gen de la ghrelina codifica un pre-propéptido de 117aa (pre-proghrelina), que mediante una serie de modificaciones produce un propéptido, la proghrelina (1-94aa) donde se incluye la ghrelina madura, que corresponde a los primeros 28aa y una cola de 66aa (29-94) (Kojima M et al 1999, Jeffery PL et al 2005), la cual puede circular como péptido con

su longitud total (C-ghrelin) o como péptidos pequeños principalmente como obestatina. Mientras la ghrelina presenta una modificación postraduccional (ácido octanoico), la obestatina presenta una amidación en su extremo C-terminal con Gly-Lys siendo esta amidación necesaria para su actividad biológica (Zhang JV et al 2005).

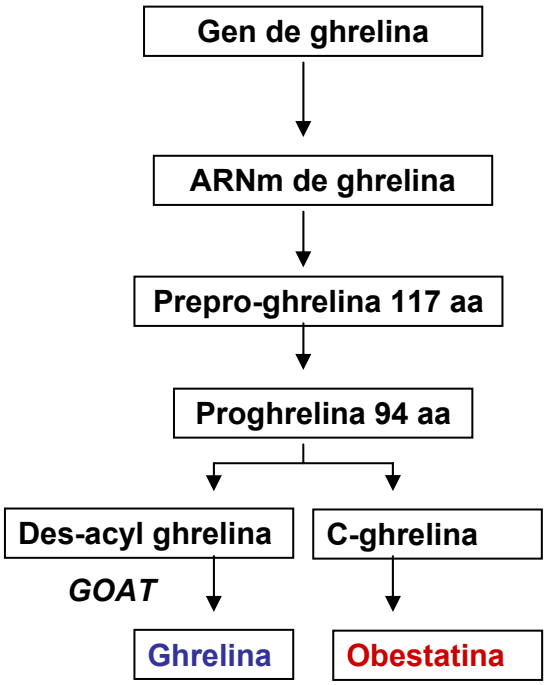

Figura 6: Representación esquemática de la síntesis de obestatina y de ghrelina.

8.1.1. ¿GPR-39, receptor de obestatina?:

En un principio el grupo de Zhang et al en 2005 describe el receptor huérfano GPR-39 como el receptor de la obestatina. Tras ese hallazgo no tardaron en aparecer nuevos trabajos que corroboraban esos datos (Moechars D et al 2006, Zhang JV et al 2008) aunque paradójicamente aparecieron también trabajos que los contradecían (Chartrel N et al 2007, Holst B et al 2007, Lauwers E et al 2006, Zhang JV et al 2007) lo cual estableció una fuerte controversia sobre este tema que en el momento actual no se ha resuelto.

El receptor GPR-39 presenta un peso molecular de 52 kDa y está localizado en el cromosoma 2q21-q22 que codifica una proteína de 453 aa, presenta 7 dominios

transmembrana característicos de los receptores asociados a proteínas G (GPC-R). El receptor GPR-39 pertenece a una familia de receptores en los que se incluyen los receptores para la motilina y la ghrelina (McKee KK et al 1997b).

El GPR-39 presenta una amplia distribución, se encuentra en ciertas zonas del sistema nervioso central, así como en otros órganos periféricos como el hígado, páncreas, pulmones, ovarios y testículos y en grandes cantidades en el tracto gastrointestinal (Zhang JV et al 2005).

8.1.2. Funciones principales de obestatina:

8.1.2.1 Acciones anorexigénicas:

Al contrario que la ghrelina, la obestatina se propone como señal supresora del apetito y del peso corporal. Así el artículo original de Zhang y colaboradores justifican estos efectos argumentando que existe expresión del supuesto receptor de obestatina en el hipotálamo, un área cerebral implicada en la regulación del apetito, al que se uniría la obestatina para ejercer sus acciones reguladoras del metabolismo. Estos resultados fueron corroborados por una serie de grupos (Zhang JV et al 2005, Zhang JV et al 2007, Lagaud GJ et al 2007, Nagaraj SR et al 2008).

A pesar de los estudios realizados en los últimos años indicando un papel de la obestatina sobre la regulación de la ingesta tanto *per se* como de forma indirecta, la gran mayoría de trabajos hechos en distintos modelos experimentales (animales en ayuno, sin restricción de comida, *knock-out* para el receptor GPR-39, ratas obesas y normopesas) en los que se utiliza un amplio rango de dosis y rutas de administración, no fueron capaces de reproducir esos resultados (Gourcerol G et al 2007, Holst B et al 2007, Nogueiras R et al 2007, Samson WK et al 2007, Tremblay F et al 2007, Yamamoto D et al 2007, Zizarri P et al 2007, Seoane LM et al 2006, Goucerol G et al 2006).

Una posible explicación a esta controversia sería un reciente estudio que propone que la relación dosis respuesta de la obestatina con la ingesta y el peso corporal sigue una curva en forma de U en la que altas y bajas dosis no tienen efecto, sin embargo sí lo tienen las dosis intermedias (Lagaud GJ et al 2007).

8.1.2.2 Regulación del balance energético y motilidad gástrica:

En un trabajo publicado en el año 2007 por Nogueiras R y colaboradores que estudia el impacto de la administración de obestatina sobre diversos factores relacionados con el metabolismo energético (ingesta, peso corporal, composición corporal, gasto energético, actividad motora, cociente respiratorio y neuropéptidos hipotalámicos) se concluye que este péptido no afecta a ninguno de estos parámetros (Nogueiras R et al 2007).

Al igual que ocurre con la ingesta los resultados del efecto de la obestatina sobre la motilidad gástrica son controvertidos y dudosos (Zhang et al JV 2005, Zhang JV et al 2007, Ataka K et al 2008, De Smet B et al 2007, Bassil AK et al 2007).

8.1.2.3 Secreción hormonal:

Zhang et al describen en su artículo de 2005 que la obestatina no altera la secreción de GH en un cultivo de células hipofisarias. Por otra parte Zizarri P et al en 2007 encontraron que la obestatina aunque es incapaz de regular la secreción basal de GH en experimentos *in vitro* e *in vivo*, sí es capaz de inhibir la secreción de GH inducida por la ghrelina en ratas en libre movimiento. El resto de estudios experimentales no son capaces de demostrar una relación entre estas dos hormonas (Samson WK et al en 2007, Nogueiras R et al 2007, Yamamoto D et al 2007).

Recientemente se ha descrito la capacidad de liberación de GH por parte de la obestatina en un estudio realizado en células somatotropas tumorales (Pazos Y et al 2009).

Diferentes grupos de investigación tampoco hallaron relación entre la obestatina y otras hormonas de diferentes ejes endocrinos como son: PRL, TSH, ACTH (Yamamoto D et al 2007), leptina (Zhang Z et al 2005) y corticosterona (Bresciani E et al 2006).

Sin embargo sí se demostró que la obestatina i.c.v. era capaz de disminuir los niveles de AVP (Samson WK et al 2007). Con respecto a la interacción entre obestatina e insulina hay diferentes acciones descritas (Green BD et al 2007, Granata R et al 2007) y en cuanto a la relación entre obestatina y hormonas de la función reproductora se ha detectado una estimulación de la secreción de progesterona en un cultivo primario de células granulosas de ovario porcino (Mészárosová M et al 2007).

8.1.2.4 Proliferación celular y apoptosis:

En cuanto a la regulación de la proliferación celular se han publicado datos que demuestran un papel estimulador de la obestatina sobre la proliferación celular (Camiña JP et al 2007, Mészárosová M et al 2007, Pazos Y et al 2007), un efecto inhibitorio (Zhang Z et al 2007) o la ausencia de efecto sobre este parámetro (Iglesias MJ et al 2007). Estas acciones se podrían explicar debido a las diferencias en cuanto al origen de la línea celular utilizada en cada trabajo, lo cual implicaría una acción diferente dependiendo del tejido en el que actúa la obestatina.

8.2. Des-acil-ghrelina:

La desacilghrelina presenta idéntica estructura que la ghrelina pero no posee el grupo acilo en la Ser^3. Debido a que este grupo acilo es necesario para la unión al GHS-

R1a, en un principio se pensó que era un péptido inactivo aunque más tarde se demostró que es capaz de regular el metabolismo y presenta diferentes funciones que en algún caso también son controvertidas. El hecho de que no se una al GSH-R1a puso de manifiesto la posible existencia de un receptor específico mediante el cual realice sus funciones fisiológicas aunque hasta el momento no se ha identificado. La ghrelina y la desacilghrelina presentan una distribución muy similar, siendo el tracto gastrointestinal y más concretamente el estómago los tejidos donde estas proteínas son más abundantes (Hosoda H et al 2000b).

A nivel gástrico la relación entre estas dos hormonas es de 2:1, mientras que en la circulación la desacilghrelina representa el 90% de la ghrelina total (Bang AS et al 2007, Hosoda H et al 2000b).

Dependiendo de la condiciones experimentales la desacilghrelina presenta acciones orexigénicas (Toshinai K et al 2006) o anorexigénicas (Asakawa A et al 2005, Chen CY et al 2005, Matsuda K et al 2006). Sin embargo existe una tendencia general a considerar que esta hormona inhibe la ingesta, siendo este efecto específico de una administración central y mediado por un incremento en la expresión de CART y urocortina en el núcleo paraventricular y en el núcleo arcuato hipotalámico (Asakawa A et al 2005). Además el ratón transgénico para desacilghrelina presenta una disminución en el peso corporal acompañado por una ligera disminución del crecimiento lineal, sugiriendo que desacilghrelina al contrario que la ghrelina, induce un estado energético negativo (Asakawa A et al 2005). La ghrelina no acilada parece que no está implicada en la secreción ácida del estómago (Dornonville de la Cour C et al 2004, Sibilia V et al 2006) sin embargo es capaz de inhibir el vaciamiento gástrico (Asakawa A et al 2005, Chen CY et al 2005).

En cuanto a la secreción de GH la desacilghrelina no es capaz de unirse al GHS-R1a y por lo tanto no presenta una acción directa sobre GH. Sin embargo es capaz de disminuir la respuesta de GH a ghrelina en ratones transgénicos que sobreexpresan desacilghrelina y además estos ratones presentan un fenotipo carcterizado por un menor tamaño corporal con respecto a sus correspondientes *wild-types* (Ariyasu H et al 2005). También se ha demostrado que la desacilghrelina es capaz de inhibir el efecto sobre la insulina de la acil-ghrelina *in vitro* (Qader SS et al 2007). Sin embargo presentaría el mismo efecto que la acil-ghrelina sobre la LH en ratas en libre movimiento (Martini AC et al 2006).

Recientes estudios *in vitro* muestran que la desacilghrelina presenta actividades biológicas relevantes sobre la proliferación celular (Bang AS et al 2007, Granata R et al 2006, Sato M et al 2006, Nakahara K et al 2006, Filigheddu N et al 2007, Cassoni P et al 2004, Cassoni P et al 2003).

9. El estómago:

El estómago es un segmento dilatado del tracto digestivo. Entre sus funciones más relevantes destaca la de proseguir con la digestión iniciada en la boca, mediante el almacenamiento transitorio del alimento que será transportado posteriormente en cantidades más pequeñas al intestino para su posterior absorción. Este órgano ejerce funciones exocrinas y endocrinas mediante la secreción de hormonas en respuesta a la ingestión de alimentos (Lippert H et al 1996).

Tradicionalmente el estómago se divide en cuatro segmentos sin límites netos: la porción cardíaca, llamada tradicionalmente cardías que es la entrada al estómago, el cuerpo del estómago, el fondo o *fornix* del estómago que es el lugar donde se produce la ghrelina y finalmente la porción pilórica, que constituye la parte final del estómago comunicada con el intestino (Lippert H et al 1996).

9.1. Estructura:

La pared gástrica tiene cinco capas (Lippert H et al 1996, Young B et al 2004):

- Mucosa: epitelio de revestimiento monoestratificado.
- Submucosa: tejido conjuntivo laxo con vasos sanguíneos de calibre relativamente grande que facilita la formación de pliegues de la mucosa al contraerse la musculatura gástrica.
- Muscular: presenta tres estratos, el externo con fibras musculares longitudinales, el estrato intermedio con fibras musculares circulares y el estrato interno de fibras oblicuas.
- Subserosa: fina capa de tejido conjuntivo.
- Serosa: peritoneo.

La superficie de la mucosa está estructurada en áreas constituidas por prominencias verruginosas, en las cuales desembocan las fosas gástricas o conjunto de conductos excretores procedentes de las glándulas gástricas (Lippert H et al 1996, Young B et al 2004).

9.1.1. Glándulas:

Las glándulas gástricas están formadas por una población mixta de células:

En el cuerpo y fundus se encuentran las glándulas principales, elongadas, rectas, poco ramificadas y de luz estrecha que ocupan la mayor parte de la mucosa gástrica.

Tres tipos de células componen estas glándulas (Young B et al 2004);

- Células mucosas superficiales: cubren la superficie luminal del estómago y revisten las faveolas gástricas. Secretan principalmente bicarbonato.
- Células mucosas del cuello: que se encuentran entre las células parietales en las regiones del cuello y la base de las glándulas gástricas.

- Células parietales u oxínticas: distribuidas a lo largo de toda la longitud de las glándulas. Secretan principalmente ácido gástrico.
- Células principales, pépticas o zimogénicas Cp localizadas hacia la base de las glándulas gástricas. Secretan pepsina.
- Células neuroendocrinas, secretan serotonina y otras hormonas.
- Células precursoras que se encuentran sobre todo en el cuello de las glándulas gástricas.

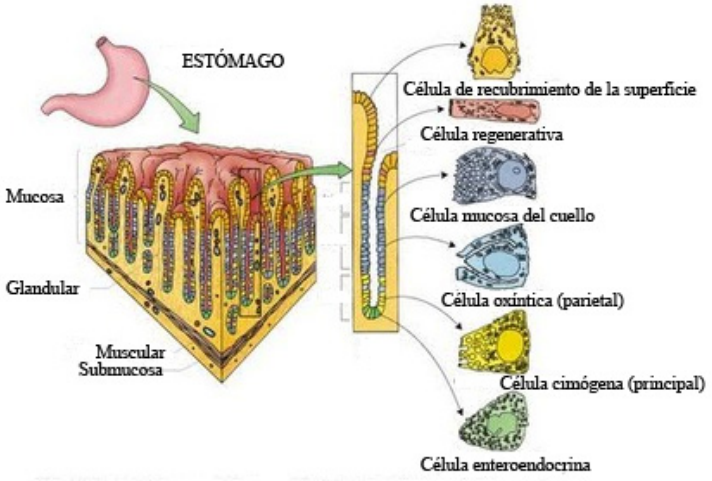

Figura 7: Esquema del estómago y su composición celular (Modificada de Young B et al 2004).

9.2. Control gástrico:

Una vez que los nutrientes son ingeridos interaccionan con diferentes áreas del tracto gastrointestinal informando al cerebro sobre el estado nutricional. El control de la función digestiva es ejercido a dos niveles diferentes. El control neural y el control hormonal. Esos mecanismos son de crucial importancia para el proceso de ingestión de nutrientes y tienen como resultado final la regulación del balance energético (Konturek SJ et al 2004).

9.2.1. Control neural del estómago, sistema nervioso entérico (SNE):

El control neural del tracto gastrointestinal se produce a través del sistema nervioso autónomo que recibe una inervación dual, una inhibitoria desde la división parasimpática y otra excitatoria desde la división simpática.

La división parasimpática está compuesta por los nervios vagales y pélvicos mientras que la división simpática incluye los nervios esplácnicos (Konturek SJ et al 2004). El nervio vago es un nervio craneal con fibras aferentes y eferentes.

Las fibras aferentes del nervio vago son las principales uniones neuroanatómicas entre el tracto alimentario y el núcleo del tracto solitario en el cerebro (Sawchenko PE 1983, van der Kooy D et al 1984, Ritter S et al 1994, Schwartz MW et al 2000). Algunas de esas fibras se encuentran en la mucosa y submucosa gástrica y están en contacto con sustancias liberadas por células enteroendocrinas. Los metabolitos relacionados con la comida, péptidos y estímulos químicos y mecánicos transmiten las señales de saciedad al núcleo del tracto solitario a través de los nervios aferentes vagales o al hipotálamo por la circulación sanguínea.

Una tercera división del sistema nervioso autónomo está constituida por una red de neuronas intrínsecas localizadas en el tracto gastrointestinal constituyendo su propio sistema nervioso denominado sistema nervioso entérico. El sistema nervioso entérico es el principal controlador de la función gastrointestinal y es necesario para la motilidad gástrica, secreción y un flujo sanguíneo efectivo. Las células nerviosas que componen el sistema nervioso entérico están localizadas en el plexo mientérico y submucoso, el primero de ellos está localizado entre la capa muscular circular media y la longitudinal, mientras que la segunda está entre la capa circular media y la mucosa (Konturek SJ et al 2004). El plexo submucoso está involucrado mayoritariamente en la emisión de señales cómo respuesta a la ingestión de nutrientes mientras que el plexo mientérico está

principalmente involucrado en la coordinación de los patrones de motilidad (Konturek SJ et al 2008). Algunas evidencias sugieren una posible interacción entre las terminaciones vagales aferentes y las neuronas entéricas vecinas (Burdyga G et al 2004).

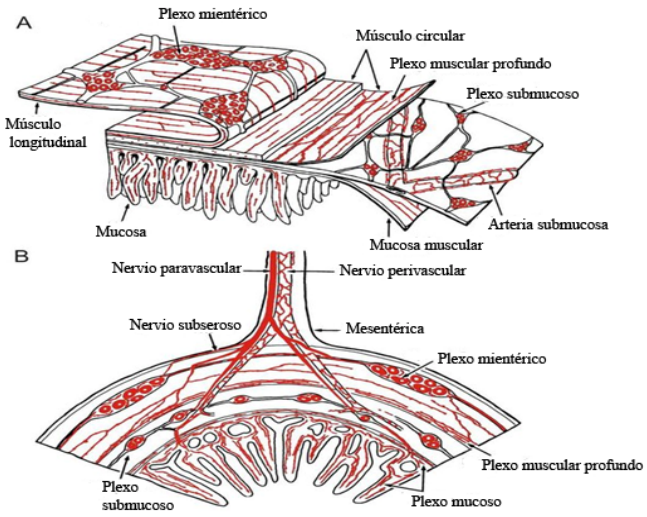

Figura 8: Composición gástrica e inervación nerviosa (Modificada de Young B et al 2004).

9.2.2 Control hormonal del estomago:

El tracto gastrointestinal es uno de los mayores órganos endocrinos del organismo y produce alrededor de 30 hormonas peptídicas diferentes, las cuales actúan en diferentes tejidos como glándulas exócrinas y SNC. Los péptidos gastrointestinales son secretados desde las células enteroendocrinas en respuesta a la ingesta, actuando normalmente en la regulación a corto plazo de la ingesta de alimentos y la homeostasis energética, aunque pueden interactuar a varios niveles con las hormonas involucradas en la regulación a largo plazo del peso corporal como por ejemplo leptina o insulina (Konturek SJ et al 2008). Después de ingerir alimentos, varias hormonas son liberadas desde diferentes áreas del tracto gastrointestinal: colecistokinina (CCK) (Rehfeld JF et al 1998) desde la parte proximal del intestino delgado, péptido similar al glucógeno tipo

1 (GLP-1) (Herrmann C et al 1995) y péptido YY (PYY) (Adrian TE et al 1985), desde la parte distal del intestino delgado. Esas hormonas gastrointestinales juegan un papel fundamental en varias funciones digestivas como el vaciamiento gástrico, así como en la regulación del apetito (Inui A et al 2004).

Diversas patologías entre las cuales se encuentran la diabetes, la insuficiencia renal y desordenes neurológicos como distrofias miotónicas, enfermedad de Parkinson o enfermedades desmielinizantes como la esclerosis múltiple, están asociadas con la reducción del tránsito gastrointestinal (Hellström PM et al 2009). Por esa razón los péptidos derivados de esa zona involucrados en el control del balance energético han adquirido un gran protagonismo en los últimos tiempos, entre esos péptidos la ghrelina está considerada como la más relevante.

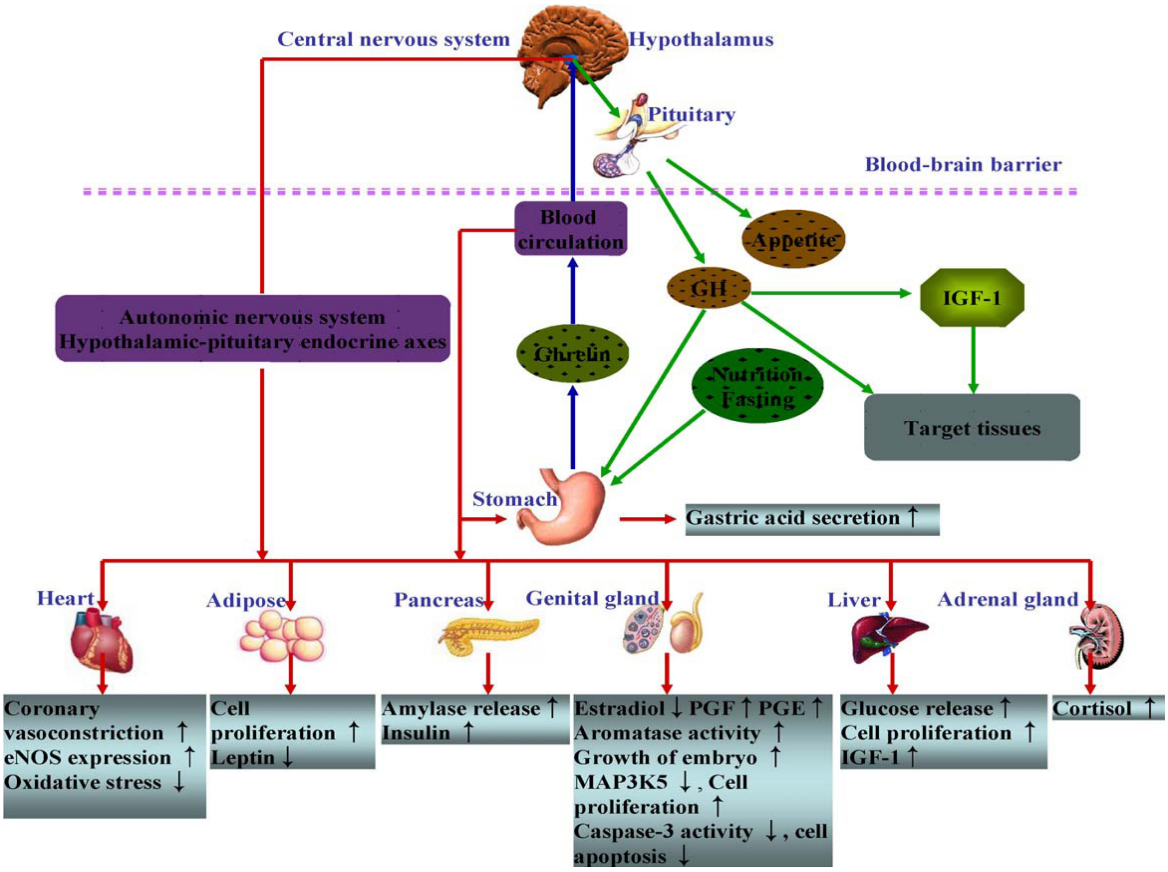

Figura 9: Acciones de la ghrelina (Modificado de Xiao-Ying Dong et al 2009).

OBJETIVOS

La regulación de la secreción de ghrelina y sus niveles plasmáticos han sido exhaustivamente estudiados tanto en animales de experimentación como en seres humanos. Dado que el 70% de la ghrelina circulante procede del estómago, se ha asumido de una forma automática que todo cambio plasmático refleja un cambio en la secreción gástrica. Se ha ignorado de esta forma, las condiciones secretoras del estómago y el papel jugado por otros tejidos intestinales, así como el aclaramiento de la ghrelina circulante por otros órganos.

Dado que la regulación de la secreción de ghrelina por el tejido gástrico no ha sido estudiada y se carecía de un modelo *in vitro* adecuado, en el presente trabajo nos hemos planteado los siguientes objetivos:

1. Desarrollar y validar un modelo *in vitro* que permita estudiar directamente la secreción gástrica de ghrelina en cualquier situación experimental. Modelo simple, reproducible y de coste razonable.

2. Establecer la acción de los componentes del eje somatotropo: GH, GHRH, IGF-1 y SS, sobre la secreción de ghrelina directamente a nivel gástrico.

3. Observar de que forma el desarrollo asociado a la edad y el sexo modulan la secreción de ghrelina y niveles de expresión de GOAT por el tejido gástrico.

4. Determinar de que forma el estado de lactancia influye en el desarrollo de la capacidad secretora de ghrelina y niveles de expresión de GOAT por el estómago.

5. Observar como el estado de ayuno o alimentación regulan la secreción de ghrelina directamente a nivel gástrico.

6. Determinar si es el estómago directamente o alternativamente el SNC quien detecta los cambios de ingesta que regulan la secreción de ghrelina.

7. Evaluar el papel que puede jugar el nervio vago y el plexo mientérico en la regulación de la secreción de ghrelina por células X/A.

RESULTADOS

J. Endocrinol. Invest. 30: RC22-RC25, 2007

RAPID COMMUNICATION

Growth hormone and somatostatin directly inhibit gastric ghrelin secretion. An *in vitro* organ culture system

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ABSTRACT. Ghrelin is a 28-amino-acid hormone produced mainly by the stomach which strongly promotes food intake. It is the only known peripheral orexigenic hormone that induces the release of GH. Ghrelin has been proposed as a link between the enteric system and central regulation of energy balance and growth. Although it has recently been the focus of extensive study, the secretion mechanism is not yet well characterized. The aim of this study was to test the direct effect of hormones from the somatotropic axis on ghrelin release directly from the stomach. To this end, an organ culture model of gastric tissue explants from rat donors was used. These stomach explants were incubated in 6 well plates for 1, 2, and 3 h after treatment with either GH, GHRH, SS or IGF-I, all them at 10^{-6} M. After incubation, the medium was collected and the amount of ghrelin secreted by the gastric tissue was measured by radioimmunoassay. It was observed that GH and SS significantly decreased gastric ghrelin secretion, while GHRH and IGF-1 had no effect on the present model. These results would confirm the capacity of GH and SS to act directly upon gastric level, inhibiting ghrelin secretion *in vitro*.
(J. Endocrinol. Invest. 30: RC22-RC25, 2007)

INTRODUCTION

The main function of the somatotropic axis is the regulation of GH secretion and general metabolism (1). The somatotropic axis is made up of GH, GHRH, somatostatin (SS), and IGF-I, and the recently incorporated ghrelin. This 28-amino-acid peptide (2) is expressed in a large number of tissues (3-5) but mainly in endocrine cells within the oxyntic gland of the stomach (6).

There is evidence suggesting a relation between ghrelin and the other components of the somatotropic axis (7, 8). GH is controlled by metabolic status and is profoundly altered in states such as obesity, malnutrition, fasting, and diabetes mellitus (1). For its part, plasma ghrelin is also affected by nutritional status, being enhanced in the fasting state and reduced in obesity and situations of positive energy balance (9-11). In light of this, ghrelin may well be the link between the somatotropic axis and metabolism.

There is currently controversy over the effect of GH on the regulation of ghrelin secretion. It is difficult to explain as the interplay between GHRH, GH, and ghrelin levels has not yet been established. Exogenous treatment with another component of the somatotropic axis SS and its analogues has been reported to produce inhibition of plasma ghrelin levels in normal subjects (12). Systemic administration of SS suppresses the secretion of a wide range of splanchnic hormones, such as insulin, glucagon, and gastroentero-pancreatic hormones and ghrelin may be part of that list.

IGF-I is a primary mediator of GH functions as a negative feed-back regulator of GH secretion *in vivo*. However, little is known about how IGF-I affects the regulation of ghrelin secretion. A negative correlation between these two hormones has been reported in humans (13), and there have been reports of a down regulation of IGF-I on ghrelin receptor (GHS-R) in rat pituitary (14).

Although it is commonly assumed that any variation of plasma ghrelin reflects changes in the rate of secretion by the stomach, this has not been looked into. The intimate mechanisms governing the biosynthesis and secretion of

Key-words: Ghrelin, somatotropic axis, GH, GHRH, SS, IGF-I.

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stomach-derived ghrelin are unknown. The purpose of the present study was to evaluate the interaction between the components of the somatotropic axes such as GH, GHRH, SS, and IGF-I and gastric ghrelin released directly by the stomach without interferences from other organs. To do so, a model of gastric tissue explant cultures validated by our group was used.

MATERIALS AND METHODS

Animals and drugs

Adult Sprague Dawley rats were used for all experiments. Experimental animals were housed in 12 h light/12 h dark cycles with free access to food and water (no.=10). Research was conducted according to protocols approved by the Animal Care Committee of Santiago de Compostela University.

SS and IGF-I were purchased from Sigma Chemical Co. (St Louis, MO, USA), GHRH was obtained from Serono (Madrid, Spain), GH was supplied by Pfizer (Sant Cugat del Vallés, Barcelona).

Tissue-explant cultures

Gastric explant cultures were performed as previously described (15). In brief, immediately after euthanasia, the stomachs were rapidly excised and transported to the incubator in sterile Krebs-Ringer-Hepes buffer. After blood vessels and connective tissue were removed, stomach tissue was washed. Approximately 1 g of tissue, mostly gastric fundus, was then placed in each of 6 well dishes containing 2.5 ml of Dulbecco's modified Eagle's medium supplemented with penicillin (100 U/ml) and streptomycin sulphate (100 µg/ml) and incubated at 37 C under a humidified atmosphere of 95% air-5% CO_2. After a pre-incubation period of 1 h, the media was discarded and 2.5 ml of fresh medium was dispensed into each well. Culture medium was finally collected at 1, 2 or 3 h and tissue was weighed with a precision scale. Media was stored at –20 C until ghrelin assay.

Biochemical analysis

Total ghrelin levels were determined by means of a double antibody radioimmunoassay (RIA) using reagents kits and methods provided by Phoenix Pharmaceuticals Inc. (Belmont, CA), as previously described (16). The limit of assay sensitivity was 1 pg/ml. Results were expressed as pg/ml of ghrelin per g of tissue in culture media. Data is expressed as mean±SE and assessed by the Mann-Whitney test. $p<0.05$ was considered significant.

RESULTS

In order to assess the action of the hormones making up the somatotropic axis on gastric ghrelin secretion *in vitro*, several kinds of treatment were carried out. Solutions of 10^{-6} M of GH, GHRH, SS or IGF-I or vehicle were added to the culture plate incubation medium.

The treatment of gastric tissue explants with GHRH 10^{-6} M did not affect gastric ghrelin secretion at any time tested (GHRH 10^{-6} M: 1671±84 pg/ml per g of tissue *vs* control: 1698±34 pg/ml/g of tissue at 2 h of incubation) (Fig. 1).

Similarly, IGF-I treatment also had no effect on gastric ghrelin secretion (IGF-I 10^{-6} M: 1686 pg/ml/ per g of tissue *vs* control 1511 pg/ml/per g of tissue) (Fig. 1).

In contrast, SS treatment significantly decreased basal ghrelin secretion from tissue explants at 2 and 3 h of incubation (SS 10^{-6} M: 1963±89 pg/ml/per g of tissue *vs* 1814±100 pg/ml/per g of tissue control at 1 h; 1381±61 pg/ml/per g of tissue *vs* 1698±34 pg/ml/g of tissue control, $p<0.01$; 1415±221 pg/ml/g of tissue *vs* 1959±63 pg/ml/g of tissue control at 3 h of incubation, $p<0.05$) (Fig. 1). The same occurred regarding treatment with GH (GH 10^{-6} M: 1512±54 pg/ml/per g of tissue *vs* 1814±100 pg/ml/per g of tissue control at 1 h; 1184±122 pg/ml/per g of tissue *vs*

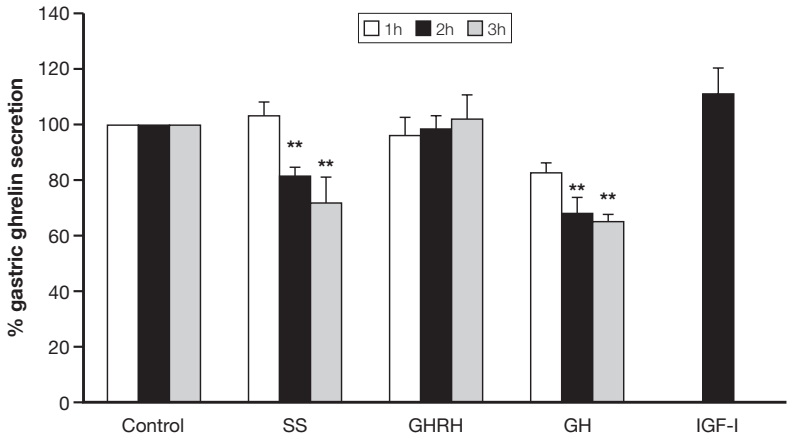

Fig. 1 - *Ghrelin secretion directly from gastric tissue explants presented as % over control (mean±SE) after incubation for 1, 2 and 3 h of SS, GHRH, GH, and IGF-I at doses of 1 µM. Samples were measured by duplicate, no.=10. (**p<0.01 vs control).*

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control: 1698±34 pg/ml/per g of tissue at 2 h of incubation, $p<0.01$; 1280±51 pg/ml/per g of tissue *vs* 1960±9 pg/ml/per g of tissue control at 3 h, $p<0.01$) (Fig. 1).

DISCUSSION

The present study was conducted using an organ culture model of gastric tissue capable of assessing the direct regulation of ghrelin secretion by the stomach (15). With this model, a 2-3 h incubation with GH and SS was found to significantly decrease ghrelin secretion directly from the stomach *in vitro*, thus suggesting for the first time that the observed plasma ghrelin changes induced by these hormones would be directly due to changes in gastric ghrelin release. On the other hand, both GHRH and IGF-I failed to induce modifications on basal gastric ghrelin secretion.

The stimulating effect of ghrelin administration on GH secretion (2, 6, 7) has been widely reported. This, together with the fact that the receptor for ghrelin (GHS-R 1a) is expressed in the pituitary and that the main source of ghrelin is the stomach, suggests that a stomach-pituitary-GH axis may exist. The aim of the present study was to determine whether the classical components of the somatotropic axes could, in turn, be acting at the gastric level to modulate ghrelin secretion.

GHRH, the main hypothalamic stimulatory peptide from the somatotropic axis, has been directly related to ghrelin since the co-administration of GHRH and ghrelin produces a synergistic effect on GH secretion (17). In the present study, it was found that the treatment of gastric tissue explants with a dose of 10^{-6} M of GHRH had no effect on gastric ghrelin secretion from the stomach. This absence of the effect is supported by the fact that GHRH-R expression has never been reported in the stomach. Recently, the expression of a splice variant of GHRH-R, called SV1, has been described at the gastric level (18). However the results here presented seem to indicate that SV1-mediated GHRH actions may include other effects, but not ghrelin secretion.

SS, the inhibitory hypothalamic peptide, is produced locally at the gastric level and suppresses secretion of several gastrointestinal peptides in a paracrine fashion. It has been demonstrated that some SS-producing cells make direct cellular contact with ghrelin-producing cells in the gastric fundus (19, 20), suggesting a regulatory interaction. It was found that treatment with 10^{-6} M of SS induced a clear decrease of gastric ghrelin secretion. This would be the first demonstration that the previously reported *in vivo* reduction of plasma ghrelin by SS might reflect a direct action of ghrelin on the stomach. Expression of SS receptors in the gastric mucosa has been reported (21), which suggests that gastric ghrelin release may be modulated by a direct activation of gastric SS receptors.

Several data in the literature suggest a negative GH feedback on stomach ghrelin (22). This is supported by the fact that GH receptors are present in the stomach and intestine (23). However a review of the bibliography reveals an apparent contradiction regarding the effect of GH levels on ghrelin (24, 25). The findings in the present study showing that treatment with a dose of 10^{-6} M of GH directly on gastric tissue explants induced an inhibitory effect on ghrelin release from the stomach should shed some light on this dilemma. The indication that variations in circulating ghrelin induced by changes in circulating GH levels are due to a direct action of GH on the stomach would be the first evidence of a direct GH effect on gastric ghrelin secretion.

The mechanism of action behind the GH-mediated reduction of ghrelin levels may involve the release of gastric SS produced locally in gastric tissue. The increase in GH levels could be activating gastric GH receptors and up-regulating SS tone in the stomach in order to reduce ghrelin release as well as consequent ghrelin plasma levels.

IGF-I is a primary mediator of GH functions as a negative feedback regulator of GH secretion and has an inhibitory action directly on the pituitary expression of GHS-R 1a (14). At gastrointestinal level, a positive expression of IGF-I as well as IGF-I receptor has been described (26), which may play a role in the regeneration of intestinal mucose (27). The relation between IGF-I and gastric ghrelin regulation has not yet been definitively elucidated. In fact, some controversy exists as some authors report a negative correlation while others report no correlation at all (13). In the present study, a positive effect of IGF-I directly on stomach regulation has been discarded.

In conclusion, to the best of our knowledge this result might constitute the first demonstration that both GH and SS reduce plasmatic ghrelin by a direct inhibitory action on the stomach. GHRH and IGF-I were devoid of action in this model.

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TITLE: DIRECT GHRELIN SECRETION FROM THE STOMACH AND GOAT mRNA LEVELS ARE INFLUENCED BY AGE, SEX AND LACTATION STATUS.

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Running title: "Gastric ghrelin, GOAT and age"

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Abstract

Ghrelin is a stomach derivate peptide involved in energy homeostasis regulation. Ghrelin O-acyltransferase (GOAT) is the enzyme responsible for ghrelin acylation. Puberty is a period characterized by changes in the metabolic requirements and notable variations of sexual hormone levels. On the other hand, the weaning process is a fundamental modification of the diet, which implicates several adaptations of the gastrointestinal tract morphology. Until now GOAT variations and the direct secretion of ghrelin from the stomach under these conditions, without interferences from other organs, has never been studied.

The main objective of this paper was to investigate how the stomach modulates ghrelin production and GOAT expression, through each period of life.

Using a model of gastric explants developed by our group, ghrelin secretion directly from the stomach was studied through different stages of life and especially in puberty and weaning. The effects of estrogen, testosterone and also breastfeeding in young animals on gastric ghrelin were tested *ex vivo* and *in vitro*. The mRNA variations and the plasmatic levels of ghrelin were determined by real-time-qPCR and radioimmunoassay respectively. Gastric ghrelin secretion is regulated by estrogens and present an inverse pattern to those found in gastric GOAT mRNA. The weaning affects to gastric ghrelin secretion, body weight and sexual hormone levels.

In conclusion, the stomach itself regulates its own ghrelin and GOAT production in order to adapt the organism to the metabolic requirements demanded through each stage of life.

Keywords: ghrelin, estrogens, testosterone, weaning, GOAT.

INTRODUCTION:

Ghrelin is a 28-aminoacid peptide that has been isolated from the stomach as an endogenous ligand for the growth hormone secretagogues orphan receptor (GHSR)(24). It was found that circulating ghrelin levels were decreased by 65% after gastrectomy in humans and rodents (1;9;27) suggesting that the main source of ghrelin in the organism is the stomach, especially X/A-like cells. In addition, ghrelin expression has also been found along the gastrointestinal tract (19) and also detected in other tissues such as the hypothalamus (8), testis (2), pituitary (5), ovary (6;12), heart (20) and placenta (14), although their contribution to the ghrelin circulating levels is ancillary, if any.

The 30% of ghrelin circulating levels presents Ser^3 residue acylated by an n-octanoic acid; the remainder circulates as unacylated ghrelin (19). The acyl modification of ghrelin is necessary for ghrelin to bind the ghrelin receptor GHS-R-1a (24). The acyltransferase that catalyzes ghrelin octanoylation has recently been identified as ghrelin O-acyltransferase (GOAT)(16;36). This enzyme is expressed in stomach and was concretely colocalized with ghrelin in mouse oxyntic mucosa (30). GOAT is essential for ghrelin octanoylation as probed by the fact that GOAT knockout mice do not have the octanoylated ghrelin form and show an increase in des-acyl ghrelin levels compare to wild type mice. It was found that GOAT is regulated by nutritional status (35), and their levels are highly influenced by the nature of the lipids in the diet (23).

It is widely shown that neuroendocrine peptide regulation changes with age in different locations such as the gastrointestinal tract (10). Ghrelin is the only stomach-derived peptide with orexigenic action that communicates the gastrointestinal tract and the brain, contributing to energy homeostasis regulation (4). It might also be possible that gastric ghrelin changes with age in order to adapt the organism to metabolic requirements demanded through each stage of life.

Several works have studied the variations of the stomach ghrelin expression with age, and controversial data about this topic is present in the bibliography. In one study performed in mice, it was reported that non-significant changes in stomach mRNA ghrelin occur with age (34). On the contrary, the majority of the studies done in rats, showed detectable gastric ghrelin mRNA expression just after birth which gradually increases to the adulthood stage (15;28). According to the mRNA expression findings, histological studies proved that inmunopositive cells for ghrelin in the stomach present a parallel pattern with mRNA (28). Surprisingly, the gastric pattern of ghrelin expression is completely opposite to that described in the pituitary, where the mRNA

levels are elevated through the fetal period and decrease after birth (21). However, the expression of ghrelin receptor (GHS-R) at pituitary level, it was found to be in line with gastric ghrelin expression (22).

From a metabolic point of view, one of the most relevant processes in rat development is the weaning period, which is associated in the offspring with drastic dietary and environmental changes. In addition, this period is characterized by alterations in the gastrointestinal tract morphology and these weaning-related effects might possibly be affecting ghrelin production (3). A study in pigs shows that exogenously administered ghrelin to animals during the weaning period, rapidly regain their temporary body weight decrease associated to this nutritional change (31). Ghrelin levels in suckling animals are regulated in a different manner than in adult animals. It was shown in 1 week old animals that eight hours of milk restriction significantly decrease ghrelin concentration in the stomach while at the same time plasmatic ghrelin levels increased as expected during the fasting period (18).

In addition, puberty is a life stage characterized by noticeably high energy requirements and alterations in circulating hormone levels. Available studies have shown that most relevant changes in ghrelin levels are produced in the pubertal stage under different interventions associated to estrogen levels variations (e.g., ovariectomy and estradiol treatment). These approaches affect gastric ghrelin mRNA, the number of positive ghrelin cells and plasma ghrelin (26)

Although several articles have been published about gastric mRNA levels and protein content, the mechanism of gastric ghrelin regulation is still unknown and the factor/s regulating the secretion has not been identified yet. The recently identified enzyme for ghrelin acylation (GOAT) has been studied in relation to nutritional status but not with the developments. Under this context, there is no data available about the regulation of ghrelin secretory function of the stomach and GOAT expression by age, puberty and breastfeeding. The deep study of these topics might be of great relevance for the possible role of gastric ghrelin in neonatal and pubertal development.

The three objectives of this paper are: first to gain insight into the control of ghrelin secretion directly from the stomach in response to different stages of life and the role of lactation, second the role of gonadal hormones on ghrelin secretion and third the regulation of GOAT levels by age, sex and lactation.

MATERIALS AND METHODS:

ANIMALS AND EXPERIMENTAL DESIGN

For all experiments, Sprague Dawley rats were used. Animal research was conducted according to protocols approved by the Animal Care Committee of Santiago de Compostela University. Rats were housed in air conditioned rooms (22-24 C) under controlled light/dark cycle (12 hours light, 12 hours darkness) with free access to food and water (n=10). Surgical procedures were performed under anesthesia by intraperitoneal (ip) injection of a mixture of ketamine/xylazine (Ketamine 100 mg/Kg body weight + Xylazine 15mg/kg body weight). Animals were euthanatized by decapitation. Trunk blood was collected and immediately centrifuged and plasma stored at -20ºC for the biochemical measurements.

Experiment 1: Age related variations in gastric ghrelin secretion.

To investigate if differential secretion of gastric ghrelin takes place during development, male and female rats (1-9 weeks of age), were used to measure gastric ghrelin secretion, and levels of ghrelin and testosterone in plasma (n=10). Upon decapitation, trunk blood was collected and the uterus was dissected out of the surrounding fat and its weight recorded after a brief drying with cellulose paper. Due to the difficulty of measuring basal estrogen levels directly by RIA, the measurements of the uterus weight were used as an index of pubertal maturation and activation of the reproductive axis. It was shown that this value is strongly associated with estrogen circulating levels (25). The variations in ghrelin mRNA levels were tested by real time PCR in male and female rats (2, 4, 6 and 8 weeks of age).

Experiment 2: Effect of estrogen and testosterone *in vitro* on gastric ghrelin secretion.

Explants of gastric tissue obtained from adult female rats with the method described below were incubated for 3 hours with 17β-estradiol at dose of 1 μM (β-estradiol 3 benzoato, Sigma Aldrich, Steinheim, Germany). On the other hand, the gastric explants obtained from male animals were incubated with testosterone propionate (Fluka Biochemika, Buchs, Switzerland) at a dose of 1 μM for 3 hours. The medium was collected and ghrelin levels in the culture medium were measured by radioimmunoassay (RIA).

Experiment 3 and 4: Effects of the estrogen and testosterone *ex vivo* on gastric ghrelin secretion.

To study the role of sexual hormones of gastric ghrelin secretion, young animals (4 weeks-old), females and males, were bilaterally ovariectomized, orchidectomized or sham operated respectively, under ketamine-xylazine anesthesia. After 3 days, rats were killed by euthanasia. One group ovariectomized animals was implanted subcutaneously with a Silastic tubing (i.d. 1.0mm, o.d. 1.5 mm, 20 mm in length) cannula, containing 17 β estradiol (sigma Aldrich; Steinheim, Germany). One group of male, orquidectomized was implanted with a similar cannula to those described above but containing testosterone propionate.

Experiment 5: Breastfeeding effect on gastric ghrelin secretion

With the aim of testing the weaning effect on gastric ghrelin secretion, the model of delay weaning (DW) was developed by preventing pups from eating solid food from day 21 (3 weeks of age) to day 28 (4 weeks of age), leaving the pups with the mum for this period. The corresponding controls (4 weeks) that were previously weaned at 3 weeks of age were used.

The pup body weight was monitored. Testosterone levels were measured by RIA and uterus weight recorded.

Experiment 6: Regulation of gastric GOAT mRNA expression by age and breast feeding.

In order to test the possible changes in the ghrelin O-acyl tranferase (GOAT) with age, the mRNA levels of this enzyme were studied by real time qPCR in the same experimental groups were mRNA ghrelin levels were tested (2, 4, 6, 8 weeks of age). To test the effect of the weaning in gastric GOAT expression, real time qPCR for GOAT was performed in stomach mucosa from animal groups of the experiment 5.

RNA ISOLATION AND REAL-TIME QUANTITATIVE RT- PCR

Total RNA was isolated from rat stomach mucosa using Trizol (Invitrogen, CA, USA), according to the manufacturer's recommendations. Extracted total RNA was purified with DNase treatment by means of DNA-free kit as a template (Ambion, USA) to generate first-strand cDNA synthesis using High-capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). Quantitative real-time PCR was performed using an ABI PRISM 7300 HT Sequence Detection System (Applied Biosystems) with specific Taqman qRT-PCR primers and probes (Table 1). For the analysis, gene expression levels of ghrelin and GOAT were normalized using as a housekeeping gene 18S rRNA

(TapMan: Applied Biosystems) and Hypoxanthine phosporibosyltransferase 1 (HPRT1), respectively, and were expressed in respect to the average value for the control group (13).

TISSUE EXPLANTS CULTURE

A model of tissue explants culture was developed as described. In brief, to obtain *ex-vivo* tissue, the stomachs were immediately excised and transported to the incubator in sterile Krebs-Ringer-Hepes buffer (NaCl, 125 mmol/l; KCl, 5 mmol/l; MgSO4, 1.2 mmol/l; KH2PO4, 1.3 mmol/l; CaCl2, 2 mmol/l; glucose, 6 mmol/l; Hepes 25 mmol/l; pH=7.4). After blood vessel and connective tissue elimination, stomach tissue was washed with sterile Krebs-Ringer-Hepes. Tissue explants, mostly gastric fundus, were placed in six well dishes containing 2.5 ml Dulbecco´s modified Eagle´s medium supplemented with penicillin (100 U/ml) and streptomycin sulphate (100 μg/ml) and incubated at 37 ºC under a humidified atmosphere of 95% air-5% CO2. After a pre-incubation period of 1 hour, the media was discarded and 2.5 ml of fresh medium was dispensed into each well. Culture medium was then collected at 2 hours and tissue was weighed with a precision scale. Media was stored at -20ºC until ghrelin assay.

BIOCHEMICAL ANALYSIS:

Total ghrelin levels were determined by means of a double antibody RIA using reagents kits and methods provided by Linco Reseach (St Charles, Misssouri). Samples for measuring the secretions from the tissue explants were obtained directly by collecting the culture medium. Samples were analyzed by RIA, as previously described. The limit of assay sensitivity was 93 pg/ml. Results were expressed as ng/ml of ghrelin per gram of tissue in the culture media.

Testosterone concentrations in serum samples were measured using commercial kit purchased from ICN Biomedical, Inc. (Costa Mesa, CA) following the manufacturer's instructions. The sensitivity of the assay was 100 pg/ml.

STATISTICAL ANALYSIS

Data were expressed as mean ± SEM. Statistical significance was determined by in GraphPad InStat software using a Mann-Whitney test where * $p \leq 0,05$, ** $p \leq 0,01$ were considered significant and very significant respectively.

RESULTS

1. AGE RELATED VARIATIONS IN GASTRIC GHRELIN SECRETION COMPARED WITH PLASMA GHRELIN LEVELS AND mRNA

Plasmatic ghrelin levels were unchanged by age in both male and female (Figures 1 A and E). The total gastric ghrelin secretion increased with age as long as the body weight was increasing. However when gastric ghrelin secretion was corrected per gram of gastric tissue, the data showed interesting variations, specifically at two points of the study: At 4-weeks of age the levels of gastric ghrelin secretion dramatically decreased in both male (30.8±2.1 ng/ml at 3 weeks old; 11.2±0.5 ng/ml 4weeks old; 19.1±1.2 ng/ml at 5 weeks old; *$p \leq 0.05$, **$p \leq 0.01$ *vs* 3 weeks old (Figure 1B) and female (20.4±1.5 ng/ml at 3 weeks old; 7.9 ±0.5 ng/ml 4weeks old; 20.3±12.9 ng/ml in 5 weeks old; (*$p \leq 0.05$, **$p \leq 0.01$*vs* 3 weeks old) animals (Figure 1F).

The other outstanding finding was a peak in gastric ghrelin secretion found in 6 weeks old males (19.1±12.3 ng/ml at 5 weeks old, 27.6±2.5 ng/ml at 6 weeks old, 19.6±2.4 at 7 weeks old; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ *vs* 6 weeks old) (Figure 1B). However, in the 6 week old female group, gastric ghrelin secretion was increased to values characteristic of the adult stage, remaining constant trough the adulthood (Figure 1F). The age of 4 weeks coincide with the initial pubertal stage in females, as it was shown by the uterus weight data. Between the first and the third week of life the uterus remained small in size (0.05-0.02 g), however in the fourth week it experienced a considerable increase in their volume (0.2 g at 4 and 5 weeks), and after the sixth week the uterus remained constant throughout adult stage (Figure 1H). This parameter can be considered as an index of pubertal maturation and it is directly related with circulating levels of estrogen (25). The latter indicates that at 4 weeks of age the animals began the pubertal stage with an increase in estrogen levels.

On the other hand the measurements of testosterone levels in males showed a peak at just 6 weeks of age coinciding with the pubertal stage in males. The levels of testosterone measured by RIA were of 0.02±0.002 ng/ml between the first and the fifth week of age and exhibited a noticeable increase at 6 weeks of age (0.12±0.05 ng/ml) through the adult age (Figure 1D).

The mRNA levels for gastric ghrelin determined by real-time PCR, showed that gastric mRNA ghrelin raised in 4 weeks old females (2 weeks old females. 100 ±17 arbitrary

units *vs* 4 weeks old: 217±45 arbitrary units; * p≤0.05) (Figure 1G). On the contrary, in the male it was not found mRNA ghrelin variations with age (Figure 1 C).

2. EFFECT OF *IN VITRO* TREATMENT WITH ESTROGENS AND TESTOSTERONE ON GASTRIC GHRELIN SECRETION

Treatment with testosterone propionate (1 μM) directly on gastric tissue explants did not affect gastric ghrelin secretion (Figure 2A). On the contrary, the treatment of gastric tissue explants *in vitro* with 1μM of estradiol induced a significant decrease in gastric ghrelin secretion (14.5±0.5 ng/ml in control animals *vs* 11.0±0.7 ng/ml in estradiol treated ones; **p≤0.01) (Figure 2B).

3. EFFECT OF *EX VIVO* TESTOSTERONE ON GASTRIC GHRELIN SECRETION

Testosterone influence on stomach ghrelin secretion was tested by ghrelin measurements in the medium secreted from gastric tissue explants extracted from young male rats of several experimental groups: three days orquidectomized rats, testosterone propionate treated rats after three days orquidectomized and the corresponding sham-operated controls.

Young animals presented low levels of gastric ghrelin secretion as well as low levels of testosterone. Orquidectomy did not affect gastric ghrelin secretion in the same way that testosterone treatment had no effect in orquidectomized males (Figure 3B). These effects at gastric level were reflected in the circulating levels of the hormone (Figure 3A).

4. EFFECT OF ESTROGENS *EX VIVO* ON GASTRIC GHRELIN SECRETION

Taking into account that the key age for gastric ghrelin regulation was young age (4 weeks old), this parameter was studied in young female rats under several experimental approaches: animals 3 days after being subjected to surgical ovariectomy, animals with estrogen replacement after 3 days of ovariectomy and the sham-operated control animals (26). It was found that estrogen treatment in young ovariectomized females reversed the stimulatory effect on gastric ghrelin secretion exerted by the ovariectomy (control animals: 23.3±1.1 ng/ml, ovariectomized animals: 38.1±2.6 ng/ml, ovariectomy plus estradiol treatment: 26.8±2.1 ng/ml; **p≤0.01 *vs* control, $^{\#\#}$p≤0.01 vs ovariectomy) (Figure 3D).

5. BREASTFEEDING EFFECT ON GASTRIC GHRELIN SECRETION

Two experimental models were performed in order to determine what the implication of maternal food intake has on gastric ghrelin secretion. The first of those included young

animals of 4 weeks old which has been weaned at the end of the third week of life (21 days old). The second group was comprised of young animals that were still not weaned, named delay weaning model (DW). In the female group delay weaning did not alter the gastric secretion neither the plasmatic ghrelin levels (Figure 4 A and B). On the other hand, in the male group the delay of the weaning produced an increase in gastric ghrelin secretion (control animals: 51.6±2.9 *vs* delay weaning 61.3±3.3 ng/ml; *p≤0.05) (Figure 4 B). In circulating ghrelin levels the variation was not statistically significant (Figure 4A).

Gastric mRNA levels of ghrelin were measured by quantitative real-time RT-PCR. It was found a decrease in gastric mRNA levels in females subjected to delay weaning (control: 100±12.8 vs delay weaning: 44.9±8.9 arbitrary units, **p≤0.01). Contrarily, gastric mRNA in males was not affected by delay weaning. (Figure 4C).

Body weight was measured in the different groups and surprisingly it was found that delay weaning was associated with a decrease in body weight in both, males and females (control male: 74.5±2.6 g, delay weaning male: 62.5±2.8 grams, *p≤0.05; control female: 67.9±2.5 g, delay weaning female: 54.6±1.5 g, *p≤0.05) (Figure,5C).

Estrogen and testosterone levels were calculated in each one of these experimental groups and it was found that delay weaning caused a diminution of the uterus weight in females (control: 0.058±0.007 g vs DW: 0.035±0.003 g, *p≤0.05) (Figure 5B). In addition, the delay weaning decreased the testosterone circulating levels in male animals (control: 0.02±0.002 ng/ml vs DW: 0.007±0.001 ng/ml; *p≤0.05) (Figure 5A).

6. **AGE AND BREASTFEEDING EFFECT ON GOAT mRNA IN STOMACH MUCOSA.**

In the male groups an increase in GOAT mRNA levels was found with age: 2 weeks old females: 100 ±12 arbitrary units; 4 weeks old: 366±66 arbitrary units; 6 weeks old: 740±91.5 arbitrary units, **p<0.01 vs 2 weeks old; 8 weeks old: 814±155 arbitrary units, **p<0.01 vs 2 weeks old; * p<0.05)(Figure 6A). On the contrary the female group showed a peak in GOAT mRNA levels at 6 week of age: 320±43 arbitrary units vs 2 weeks old: 100±27 arbitrary units, **p<0.001 (Figure 6 B).

By the other way, in the male group the breastfeeding induces a decrease in gastric GOAT mRNA levels as probed by the delay weaning group values (control: 100±14 arbitrary units vs delay weaning: 66±11 arbitrary units, *p<0.05). In contrast, in four weeks old male the daly of weaning did not affect GOAT mRNA levels (Figure 6C).

DISCUSSION

An organ culture model of gastric tissue developed and validated by our group (32;33), has been used in the present study to assess the direct regulation of ghrelin secretion by the stomach during postnatal life. With this model, it has been shown that age, sexual steroids and dietary modifications regulate the stomach ghrelin secretory function, independently of the mRNA and circulating levels of this hormone. The most relevant findings in this paper were four: First, puberty is a key stage in the regulation of gastric secretory activity. The hormonal modifications associated to the pubertal period, i.e. variations of estrogen circulating levels, directly regulate gastric ghrelin secretion. Second, weaning is strongly implicated in gastric ghrelin secretion and body weight regulation. Third, the weaning delay affects sexual hormone levels, weight and ghrelin secretion. Fourth, all the mentioned factors are closely implicated in gastric GOAT mRNA regulation.

Ghrelin expression shows age-related changes and sexual dimorphism. Several studies have reported that ghrelin producing cells are differentiated in a sexual dimorphic manner (28). In the present study, it was shown that plasmatic ghrelin levels were not affected in a significant way by age (Figure 1 A and E). According to the published results, our data indicate an increase in mRNA ghrelin expression in young (4 weeks old) female rats (Figure 1G). However, the most novel results showed that ghrelin secretion from the stomach was strongly altered in both male and female animals at 4 weeks of age and in 6 week old male animals (Figure 1 B and F). In the females, the beginning of the pubertal stage started at 4 weeks of life, and coincided with an increase in the uterus weight (Figure 1H); while in males it started at 6 weeks of life as shown by the increase in testosterone levels (Figure 1D). Under this context, the relevant variations in ghrelin secretion by the stomach were found in puberty, a period characterized by noticeable variations in estrogen and testosterone circulating levels, suggesting that both hormones may act on the stomach to regulate ghrelin production. To confirm this affirmation, a culture system of gastric tissue explants previously established by our group (33) was used in both *ex vivo* and *in vitro* studies. With this approach, the direct *in vitro* effect of estrogen treatment on this tissue induced a significant decrease in ghrelin secretion (figure 2B). On the contrary, direct treatment with testosterone on the stomach did not affect gastric ghrelin secretion (figure 2A). Our data is supported by the fact that estrogen receptor α (ERα) expression has been

reported in the stomach mucosa of rats; concretely in ghrelin producing cells (7). A previous study, using isolated stomach cells, found that estrogen treatment significantly stimulates ghrelin mRNA expression and the number of inmunopositive cells for ghrelin (29). These results are in agreement with our findings, as proved by an increase in gastric ghrelin mRNA in 4 week old females (Figure 1 G) which coincided with an increase in uterus weight (Figure 1 H), indicating high levels of estrogen during this stage of life. Together all these results suggested a new mechanism for gastric ghrelin production regulation, proposing that the decrease in ghrelin secretion from the stomach may be reflected in a greater amount of protein storage in ghrelin positive cells, as consequence of the increase in the mRNA expression found after estrogen treatment.

In this paper, the effect of estrogen and testosterone modulation on gastric ghrelin secretion was tested *ex vivo*. It was found that estrogens act by regulating gastric ghrelin secretion. In a first phase after birth, basal estrogen levels are low, and around the fourth week of age, these levels increase noticeably reaching the threefold value at which they act as a triggered signal of the puberty onset and dropped gastric ghrelin secretion. This physiological regulation was reproduced in the lab through different approaches of estrogen manipulation. The increase in estrogen levels produced in early stage pubertal animals (4 weeks old) was blocked by surgical ovariectomy, and because of it manipulation, the drop in gastric ghrelin secretion was prevented as well. This kept the secretion within the range of basal values found between the 1st and the 3rd week of life (Figure 3C). Moreover, when the estrogen values were replaced by exogenous treatment, the ovariectomy effect was reverted. Gastric mRNA and plasma ghrelin modifications have been described in response to ovariectomy in several works (26). Previous data from other groups showed no specific changes in mRNA ghrelin levels in adult rat stomachs after ovariectomy (15), however another showed that gastric ghrelin expression increased in 4 weeks old animals with ovariectomy. This change is reverted by administration of 17-β estradiol. Our findings help to understand the contradictory data reported in the bibliography since it is confirmed that estrogen plays an important role in the regulation of ghrelin through a mechanism dependent upon age.

The decreased gastric ghrelin secretion found in 4 week old males (Figure 1B) can not be explained as a consequence of variations in testosterone levels since the puberty in males was not reached until the sixth week of life. This fact was confirmed in the lab since these animals were subjected to orquidectomy and did not show variations in gastric ghrelin secretion (Figure 3B)

A modification of diet, the weaning, takes place immediately prior to the fourth week of life. This process is characterized by the step from breastfeeding to solid food ingestion and takes place at the end of the 3rd week of life. Possibly gastric ghrelin secretion modifications found at 4 weeks might be produced as a consequence of the weaning. This is supported by previous data showing that weaning appears to be important for gastric ghrelin expression regulation; this process has several effects on the maturation of ghrelin producing cells and morphology in the gastrointestinal tract (3). The direct ghrelin secretion by the stomach was measured in animals subjected to a delay in weaning in order to determine a possible association between weaning and gastric ghrelin production. This approach consists of preventing pups from eating solid food from day 21 (3 weeks of age) to day 28 (4 weeks of age), the pups remained with the mum for this period. In the female pups, the delay of weaning did not affect gastric ghrelin secretion (Figure 4A), probably due to the powerful effect of estrogen, which may be masking the effect of the diet modification. However the mRNA ghrelin was strongly inhibited as a consequence of the delay in weaning (Figure 4C), which is in agreement with the increase in ghrelin mRNA levels found in weaned females (figure 1C).

Opposite to females, the delay weaning in male pups prevented the drop in gastric ghrelin secretion evoked in 4 week old weaned animals (Figure 4B). Although the tendency of the gastric ghrelin secretion was reflected in the circulating ghrelin (Figure 4A), the variation was not statistically significant. However, ghrelin mRNA was not affected by the weaning in males (figure 4C), and this is supported by the lack of effect of age in ghrelin mRNA (figure 1C). Our results are in contrast with previous data from another group which found a decrease in plasma ghrelin levels, ghrelin mRNA and ghrelin cell density in delayed weaning rats (11)but this group did not test the direct ghrelin secretion from the stomach. In addition the discrepancy with the present data could be explained because there are several methodological differences (e.g., the age of weaning). The possibility that maternal ghrelin could be crossing to the pup through ingestion of milk, should be considered since it is suggested by the data of plasmatic ghrelin levels (figure 4A), where it found that both, males and females under maternal feeding present increased ghrelin levels. However, with our explants model, direct secretion from the pup stomach was measured (figure 4B) and together with the data of mRNA (figure 4C), there is evidence that the stomach has its own mechanism of ghrelin regulation under diet modifications.

Body weight was also affected by delayed weaning which induced a decrease in this parameter in both males and females (Figure 5C). This last finding was previously described by another group and it is in agreement with the fact that a prolonged period of breastfeeding has been shown to reduce the risk of developing obesity, although the mechanism behind this is still unknown (17).

In the present work for the first time it was shown a diminution in testosterone levels in males as well as in uterus weight in female pups with the delay of weaning (Figure 5 A and B). This data might indicate a delay in the onset of puberty in rats subjected to delay weaning but this topic deserves future investigation.

The existence of acyl and desacyl forms of ghrelin was known from 1999, when ghrelin was discovered, nevertheless the mechanism for ghrelin acylation was only recently known. In 2008 two different groups characterized the enzyme in charge of ghrelin acylation (GOAT) (16;36). Since this moment several studies were carried out in order to determine GOAT distribution as well to identify its possible modifications under nutritional status variations although controversial data are recorder in the bibliography about this topic. By one way, one group showed that in *ad libitum* fed mice GOAT mRNA levels increased compare to mice with different periods of fasting, in which only desacyl ghrelin was increased keeping the acyl ghrelin levels constant for 12, 24 and 48 h fasting (23). On the other hand, another group found that 48 hours of fasting in mice decrease GOAT mRNA levels in gastric mucosa (35). However in rats under food restriction, it was found by another group that GOAT mRNA levels remained relatively stable during periods of decreased food intake but when the animals reached a determined body weight loss, GOAT started to increase (13). These findings suggested a role of GOAT producing an adaptive response to prevent alterations in energy balance and body weight homeostasis. Under this context, it is possible that GOAT mRNA levels change through the different periods of life in order to adjust the organism to energetic requirements variations with age. In the best of our knowledge, the data presented in this work showed for the first time age-related changes in the GOAT mRNA pattern at gastric level in both male and female. In males (Figure 6A) GOAT mRNA increases linearly with age in parallel to body weight gain (data not shown). However, in the females, the maximum value for goat mRNA was found in 6 weeks old females (Figure 6B), which is the initial adult age and the moment when gastric ghrelin secretion is stabilized remaining at values characteristic of the adult stage thereafter (Figure 1F). The fact that the growing period in female finish in a younger age than in

males (data not shown), might be linked to the differences in goat mRNA pattern between males and females. During the growing period and especially during the puberty the energetic requirements are higher than in other periods of life and it is just at this time when the GOAT production is more elevated in both male and female. The main function of GOAT consists in the acylation of ghrelin in order to produce the active form of this peptide with orexigenic and adiposity-inductor actions by its binding to the GHSR-1a. Under this context it is possible that in a periods of life characterized by a negative energy balance as a consequence of the elevated energetic requirements, GOAT increases in order to save energy to counteract this negative energy balance. In this model GOAT it is proposed as a mechanism of body weight defense allowing the organism the adaptation to the different necessities of each period of life by the switch of the rate of acyl/desacyl ghrelin.

By the other way, GOAT mRNA levels were tested as well in 4 weeks old male and female rats subjected to a delay weaning and compare with their controls. As occur with gastric ghrelin secretion (Figure 4 B) in the female group the delay of weaning did not affect GOAT mRNA levels, probably due to if any effects take place is masked with the strong action of the puberty at this age. In the same way that gastric ghrelin secretion (Figure 4B), in the male group GOAT mRNA levels are highly affected by delay weaning (Figure 6C). Surprisingly GOAT mRNA levels presented an inverse pattern respect to those of gastric ghrelin secretion; while the delay of the weaning increases gastric ghrelin secretion the gastric GOAT levels are strongly decreased. This model was corroborated by the data of GOAT changes with age since the decrease in gastric ghrelin secretion found in the period between 4 and 6 weeks of age in both males (Figure 1B) and females (Figure 1F) occurs in parallel with a increase in GOAT mRNA levels at this period (Figure 6 A, B).

In summary, the present study demonstrates for the first time that gastric ghrelin secretion is regulated through postnatal life in an independent way of gastric expression and circulating levels of this hormone. All together, the present findings indicate a strong regulation of gastric ghrelin secretion by estrogens. The weaning strongly regulates gastric ghrelin secretion. Furthermore, animals subjected to delayed weaning present a lower body weight than the corresponding controls. For the first time it is shown that a noticeable decrease in testosterone and estrogens circulating levels is

associated with delay of weaning. GOAT mRNA levels in the stomach are strongly regulated by age and breastfeeding in an inverse manner that gastric ghrelin secretion.
In conclusion, the data presented in this paper might be indicating that the stomach itself could be regulating its own ghrelin production throughout life independently of other organs in order to adapt the organism to the metabolic requirements demanded through each stage of life.

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FIGURE LEGENDS:

FIGURE 1: Plasmatic ghrelin concentration in male (A) and female (E) rats of different ages (1-9 weeks old). n=10

Gastric ghrelin secretion from tissue explants from male (B) and female (F) rats of different ages (1-9 weeks old), to the incubation medium *p≤0.05, **p≤0.01 vs 3 weeks old; # p≤0.05, ## p≤0.01 vs 6 weeks old, n=10.

Ghrelin mRNA expression in gastric mucosa by quantitative real-time RT-PCR, standardized by 18S mRNA levels in males (C) and females (G) with different ages (2, 4, 6, 8 weeks old), *p≤0.05, n=10.

Testosterone levels from the male animals (1-9 weeks old) (D) and uterus weight from the female animals (H) of different age (1,3,4,5,7 and 9 weeks old).Values are mean ± SEM. *P≤0.05. **p≤0.01. n=10.

FIGURE 2: Changes in gastric ghrelin secretion in A) Gastric explants from adult male rats treated *in vitro* with testosterone propionate. B) Gastric explants from adult female rats treated *in vitro* with 17β-estradiol. Values are mean ± SEM of ten individual dishes. **p ≤0.01.

FIGURE 3: Plasmatic ghrelin levels from male (A) and female rats (C) and gastric ghrelin secretion from tissue explants from male (B) and female rats (D) under different approaches (sham: control 4 week old rats; OVX/ORQX: 4 week old rats subjected to surgical orquidectomy; ORQX+T: 4 week old rats treated with testosterone propionate and previously subjected to surgical orquidectomy and OVX+E: 4 weeks old rats treated with 17 β estradiol and previously subjected to surgical ovariectomy). Values are mean ± SEM . *p≤0.05, **p≤0.01, n=10.

FIGURE 4: (A) Plasmatic ghrelin concentration in 4 week old male and female rats (B) Gastric ghrelin secretion from tissue explants from 4 week old male and female rats to the incubation medium (C) Ghrelin mRNA expression in gastric mucosa by quantitative real-time RT-PCR, standardized by 18S mRNA levels in 4 week old males and females C: control; DW: delay weaning. Values are mean ± SEM. *p≤0.05, **p≤0.01. n=10.

FIGURE 5:(A) Testosterone levels (ng/ml) in control and delay weaning young male rats (B) Uterus weight in grams in control and delay weaning young female rats (C) Body weight in grams measured in males and females 4 weeks old rats, control and subjected to delay weaning. Values are mean ± SEM .*p≤0.05, n=10.

FIGURE 6: GOAT m RNA levels in gastric mucosa by quantitative real-time RT-PCR, standardized by Hypoxanthine phosporibosyltransferase 1 (HPRT1) RNA levels in males (A) and females (B) with different ages (2, 4, 6, 8 weeks old) and (C) 4 weeks old male and female rats C: control; DW: delay weaning. Values are mean ± SEM. *p< 0.05, **p< 0.001 vs 2 weeks, # p< 0.05 vs 4 weeks. n=10.

Table 1: Primers and probes for real time-qPCR analysis.

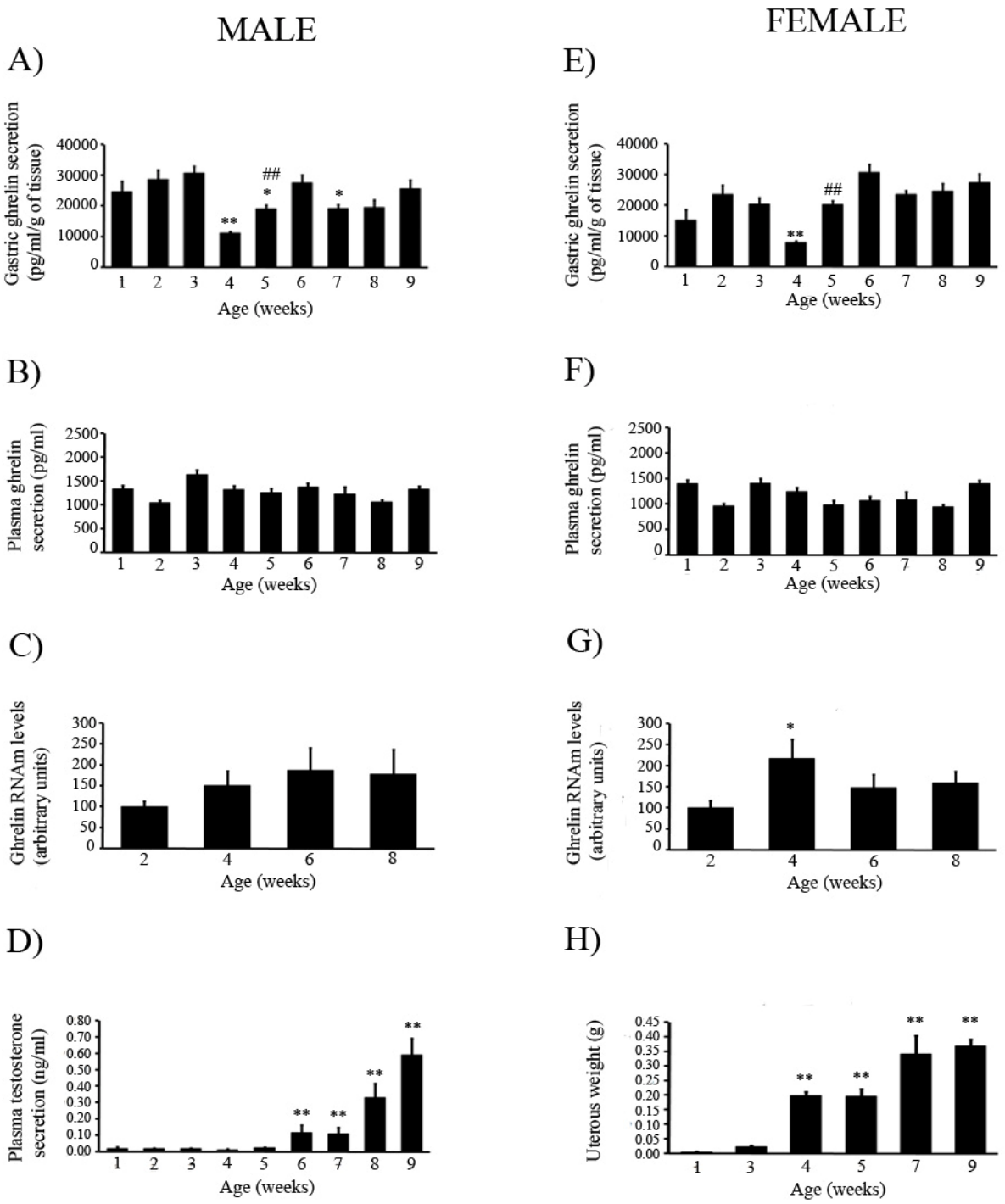
MALE
FEMALE
A)
E)
Gastric ghrelin secretion (pg/ml/g of tissue)
Age (weeks)
B)
F)
Plasma ghrelin secretion (pg/ml)
Age (weeks)
C)
G)
Ghrelin RNAm levels (arbitrary units)
Age (weeks)
D)
H)
Plasma testosterone secretion (ng/ml)
Uterous weight (g)
Age (weeks)

Figure 1

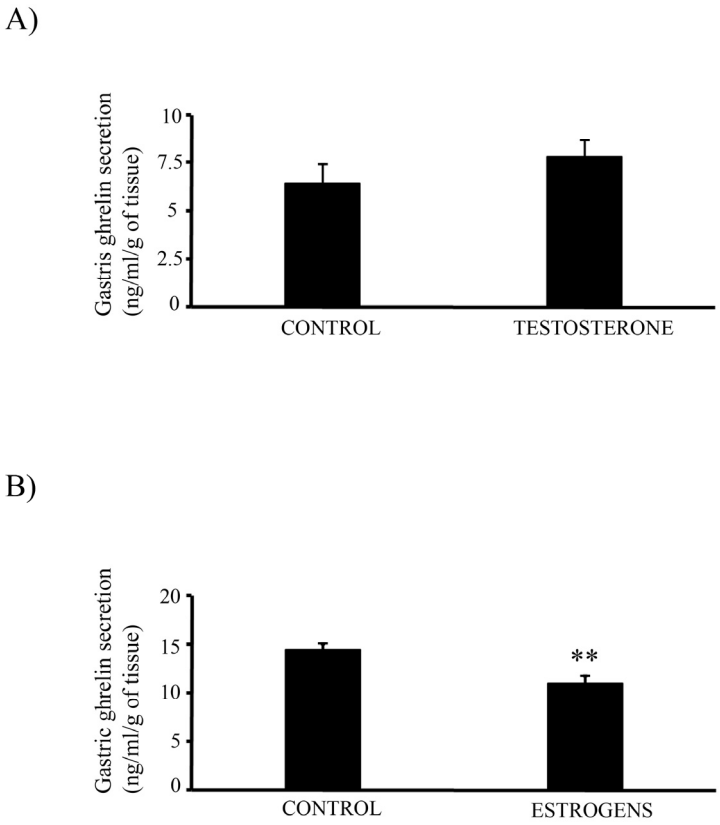

Figure 2

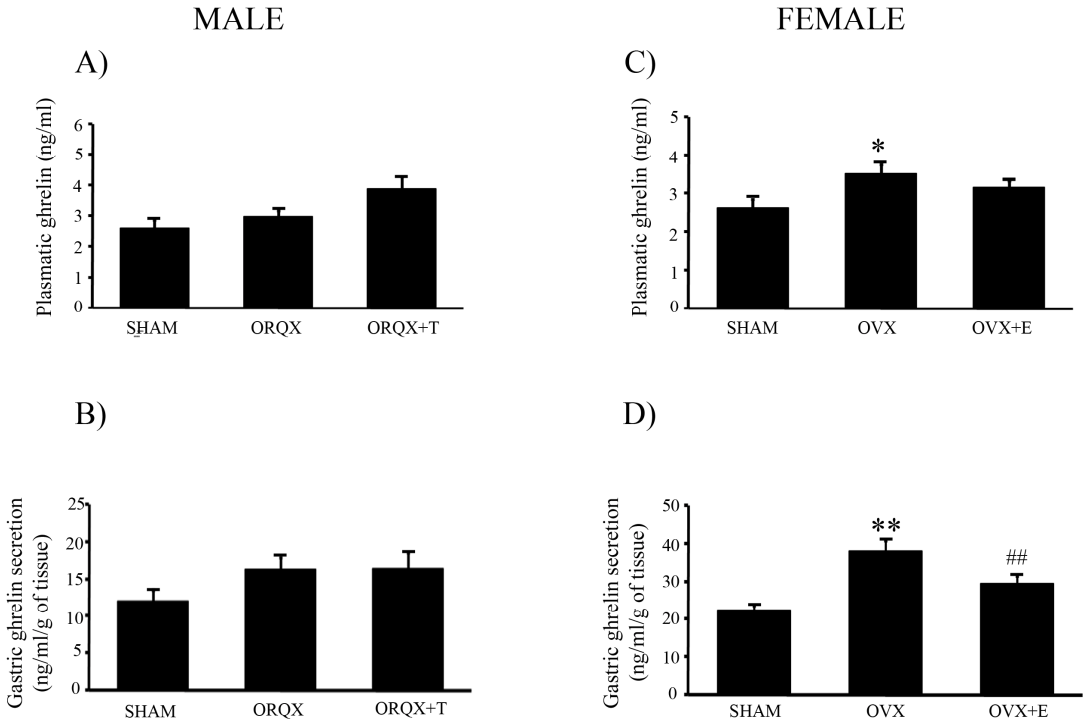

Figure 3

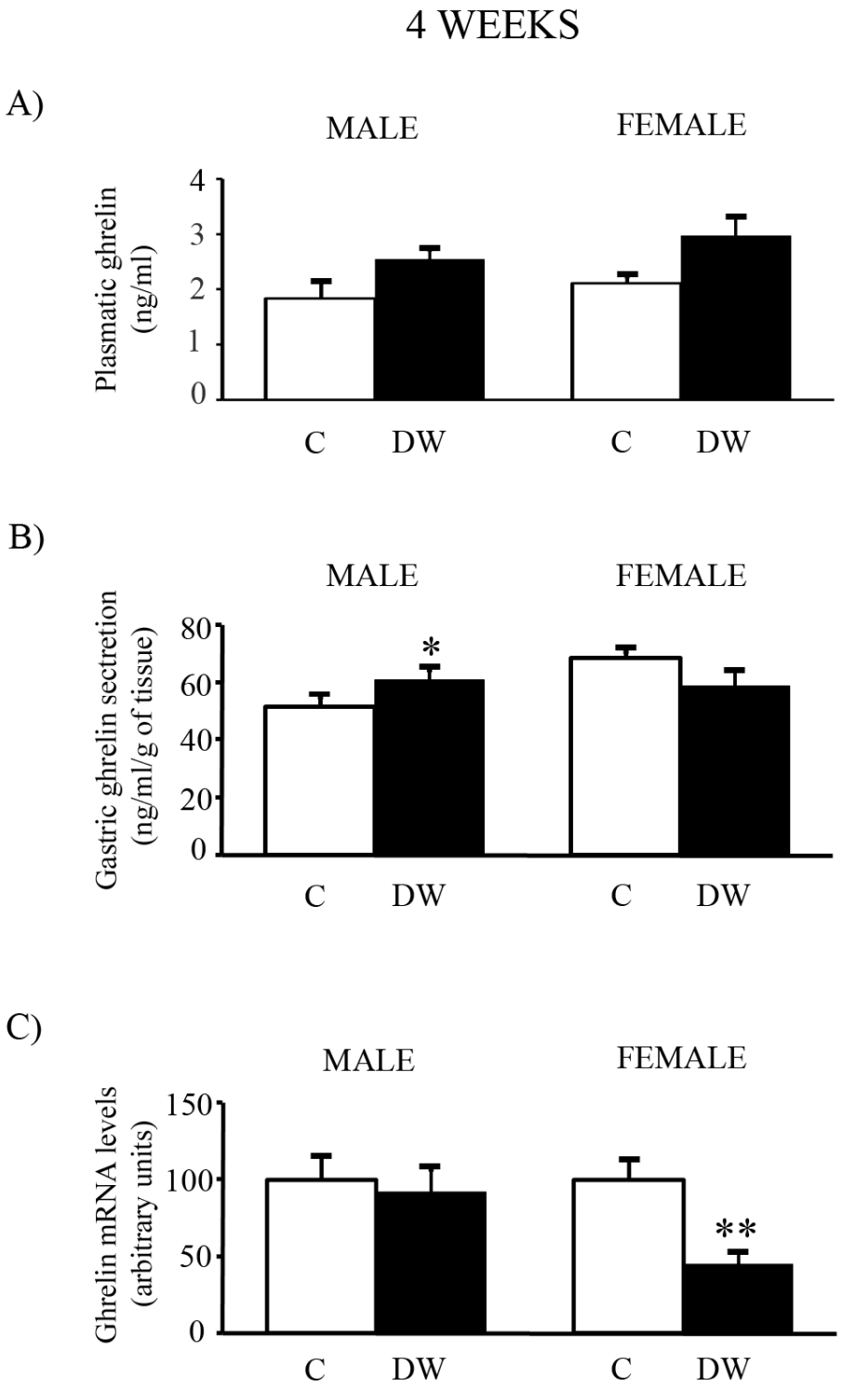
4 WEEKS
A)
MALE
FEMALE
Plasmatic ghrelin (ng/ml)
4
3
2
1
0
C
DW
C
DW
B)
MALE
FEMALE
Gastric ghrelin sectretion (ng/ml/g of tissue)
80
60
40
20
0
*
C
DW
C
DW
C)
MALE
FEMALE
Ghrelin mRNA levels (arbitrary units)
150
100
50
0
**
C
DW
C
DW

Figure 4

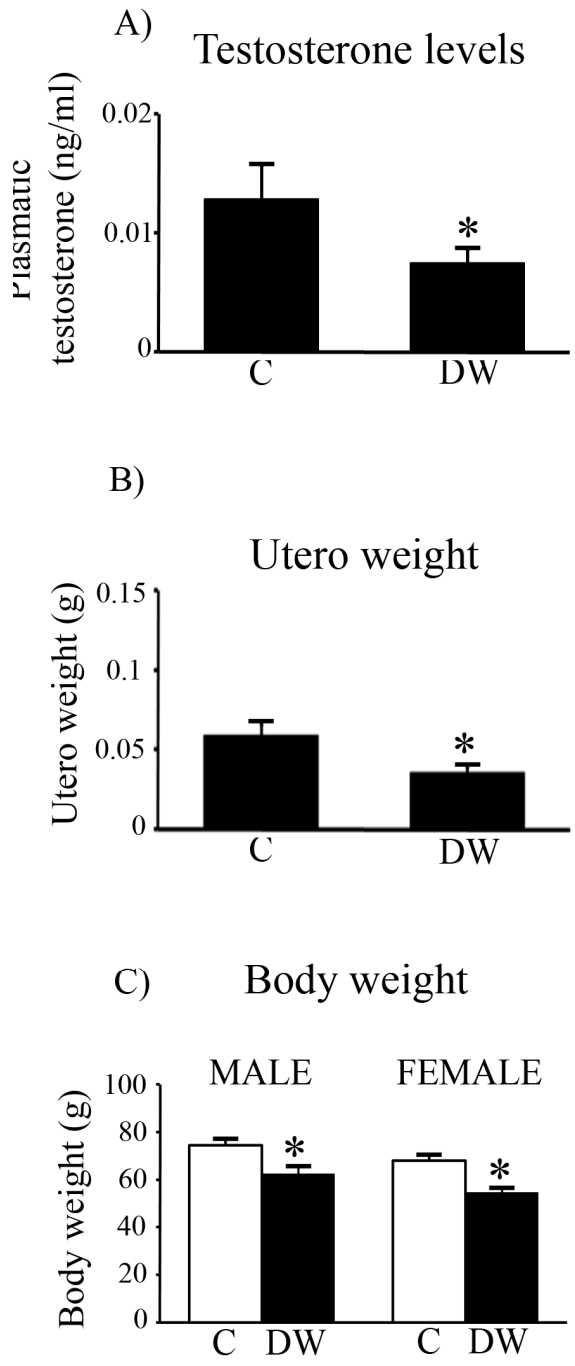
A)
Testosterone levels
Plasmatic testosterone (ng/ml)
0.02
0.01
0
*
C
DW
B)
Utero weight
Utero weight (g)
0.15
0.1
0.05
0
*
C
DW
C)
Body weight
MALE
FEMALE
Body weight (g)
100
80
60
40
20
0
*
*
C
DW
C
DW

Figure 5

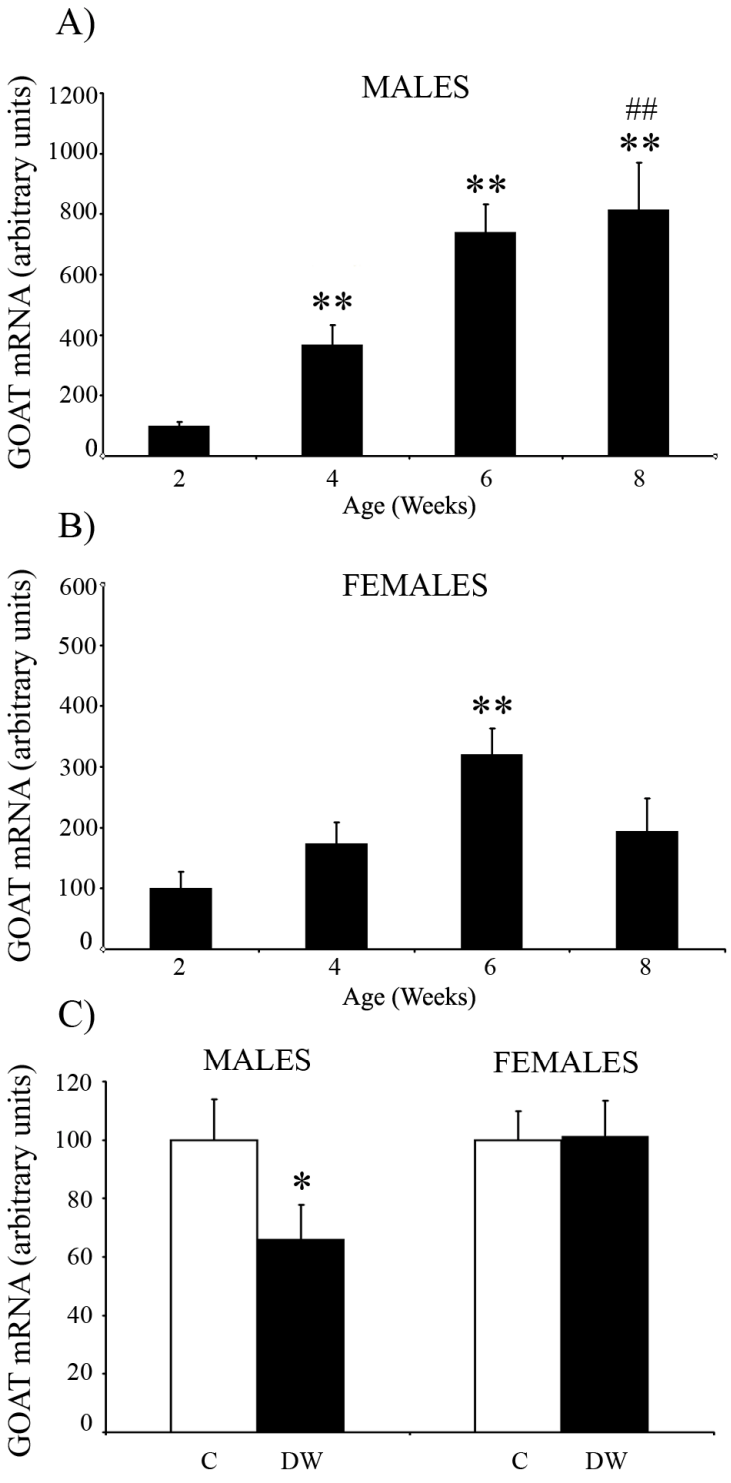
A)
MALES
GOAT mRNA (arbitrary units)
1200
1000
800
600
400
200
0
**
**
##
**
2
4
6
8
Age (Weeks)
B)
FEMALES
GOAT mRNA (arbitrary units)
600
500
400
300
200
100
0
**
2
4
6
8
Age (Weeks)
C)
MALES
FEMALES
GOAT mRNA (arbitrary units)
120
100
80
60
40
20
0
*
C
DW
C
DW

Figure 6

Primers and probes for real time-qPCR analysis

mRNA	GenBank accession number		Sequence
Ghrelin	AB029433.1	**Forward Primer**	5´GAGCCCAGAGCACCAGAAAG-3´
		Reverse Primer	5´GCTCGTGGCTGCAGTTTAGC3´
		Probe	FAM5´CCAGCAGAGAAAGGAATCCAAGAAGCCA-3´TAMRA
GOAT	NM_001107317	**Forward Primer**	5´GGCCGGAGCTTTTCCTCTCT-3´
		Reverse Primer	5´AAAGGCAGTACGTTACAGGGGAAG-3´
		Probe	FAM 5´-TGCCGGCTGTGCTGTTCTTACAACA-3´TAMRA
HPRT1	NM_012583	**Forward Primer**	5´AGCCGACCGGTTCTGTCAT-3´
		Reverse Primer	5´GGTCATAACCTGGTTCATCATCAC-3´
		Probe	FAM 5´CGACCCTCAGTCCCAGCGTCGTGAT-3´TAMRA

Table 1

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Sensory Stimuli Directly Acting at the Central Nervous System Regulate Gastric Ghrelin Secretion. An *ex Vivo* Organ Culture Study

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Ghrelin, a novel gastrointestinal hormone involved in GH regulation, has been postulated as a relevant orexigenic peptide released by splanchnic tissues. Descriptive studies have shown that plasma ghrelin levels increase in states of negative energy balance or fasting, while decreasing in obesity and after feeding. In the present study, a novel organ-culture model of gastric tissue explants obtained from rat donors has been validated for *ex vivo* experiments. Fasting induced gastric ghrelin release as well as ghrelin mRNA expression that were reflected in plasma. Interestingly, those changes were fully reverted by 15 min of refeeding before stomach extraction. Unexpectedly, when animals were allowed 15 min before explant extraction to see or smell, but not eat, the food (tease feeding), ghrelin secretion was suppressed just like in gastric explants from refed animals. This effect was blocked when the animals were subjected to surgical vagotomy or treated with atropine sulphate. In conclusion, gastric explants were a suitable model for testing ghrelin mechanism of secretion *in vitro*, and they were found to maintain memory of the previously received signals. Similar to feeding, tease feeding resulted in suppression of ghrelin discharge by explants. (*Endocrinology* 148: 3998–4006, 2007)

GHRELIN, THE ENDOGENOUS ligand for the GH secretagogue receptor, is a 28-amino-acid peptide with a serine three residue n-octanoylated strongly involved in the regulation of GH secretion and energy homeostasis (1). Although ghrelin is expressed in a large number of tissues such as pituitary, hypothalamus, thyroid, and placenta (2–4). It is found in greatest quantity in the gastric fundus (5). Ghrelin mRNA expression, as well as the peptide itself, have been localized in the X/A-like cells within the acid-producing oxyntic glands of rat stomachs. However, ghrelin immunoreactive cells are not strictly confined to oxyntic mucosa because ghrelin is also synthesized and secreted from the duodenum, ileum, cecum, and colon (6). Ghrelin is considered an incretin, *i.e.* a gastrointestinal hormone regulated by and regulating nutritional status.

Several studies have shown that intracerebroventricular and/or ip ghrelin administration stimulates food intake as well as adiposity (7, 8). Ghrelin activates the expression of orexigenic neuropeptides such as neuropeptide Y and agouti-related peptide, in the hypothalamic arcuate nucleus. This leads to an increase in food intake and body weight (9).

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Abbreviations: CNS, Central nervous system; HPRT, hypoxanthine-guanine phosphoribosyltransferase; NS, nonsignificant.

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Gastric ghrelin expression is conditioned by nutritional status. Thus, hypoglycemia, leptin, and fasting up-regulate ghrelin (7, 10). On the other hand, in obesity, ghrelin plasma levels are low and increase after hypocaloric diet treatment (11). Exceptions to this are patients with Prader-Willi syndrome, who present elevated ghrelin levels that may contribute to their voracious appetite, hyperphagia, and obesity (12, 13). In patients with anorexia nervosa, plasma ghrelin levels are elevated and return to normal levels after partial weight recovery (14). Human studies have reported a preprandial increase and a postprandial decline in plasma ghrelin levels, suggesting that it may play a physiological role in hunger and meal initiation (7, 11). However, there is still much controversy regarding the mechanisms regulating these changes (15).

The cephalic phase of gastrointestinal responses to food intake interacts with the gastric and intestinal phases to promote the absorption and use of incoming substrates. The cephalic response is activated by the thought, sight, smell, and taste of food, which produce an appetizing effect (16). When subjects are exposed to food-related sensory stimuli, vagal efferent fibers from the solitary tract nucleus are activated, and some gastrointestinal hormones are released; these hormones are considered cephalic phase reflexes (17). As one of these, ghrelin has been cited for its potential role in the anticipatory meal response (18). This is reminiscent of insulin, which presents a preprandial surge of secretion to minimize the prandial increases in blood glucose (19). The

mechanisms controlling ghrelin secretion after food exposure have not yet been described (18).

The objectives of the present paper were three. First, the aim was to validate an *ex vivo* model suitable for assessing direct ghrelin secretion from the stomach and the ghrelin regulation mechanism by nutrients. Second, it was to gain insight into the control of ghrelin secretion directly by the stomach and, third, to observe whether ghrelin is secreted in anticipation of actual food intake.

Materials and Methods

Animals and experimental design

For all experiments, adult Sprague-Dawley rats were used. Animal research was conducted according to protocols approved by the Animal Care Committee of Santiago de Compostela University. Rats were housed in 12-h light/12-h dark cycles with free access to food and water. The animals were assigned to one of four weight-matched experimental groups (n = 10): 1) *ad libitum*-fed group with 24-h access to food and water; 2) a fasting group in which rats were deprived of food for 36 h before euthanasia; 3) the refeeding group in which the animals were deprived of food for 36 h but were allowed to have free access to food 15 min before euthanasia, and finally, 4) a tease feeding group, in which after 36 h of deprivation, the animals were allowed to smell and see food, but not to eat it, for 15 min before euthanasia.

Additional experimental groups were used to assess the effects of surgical vagotomy and cholinergic blockade on ghrelin levels. Two different groups of vagotomized animals were studied: one of them under fasting conditions (b) and the other under tease feeding conditions (d). Sham operated rats were used as controls. To test the effect of cholinergic blockade, two experimental groups were treated with atropine sulfate (0.5 mg/kg ip, dissolved in sterile saline; Sigma-Aldrich, St. Louis, MO) 30 min before the beginning of fasting conditions or tease feeding conditions. Experiments were performed during the first 2 h of the light cycle. After euthanasia, a blood sample for ghrelin analysis was obtained, and stomachs were excised.

Tissue explants culture

To obtain *ex vivo* tissue, the stomachs were immediately excised and transported to the incubator in sterile Krebs-Ringer-HEPES buffer [NaCl, 125 nmol/liter; KCl, 5 nmol/liter; $MgSO_4$, 1.2 nmol/liter; KH_2PO_4, 1.3 nmol/liter; $CaCl_2$, 2 nmol/liter; glucose, 6 nmol/liter; and HEPES 25 nmol/liter (pH = 7.4)]. After blood vessels and connective tissue were removed, stomach tissue was washed with sterile Krebs-Ringer-HEPES. Tissue explants, mostly gastric fundus, with an approximate weight of 2 g, were placed in six-well dishes containing 2.5 ml DMEM supplemented with penicillin (100 U/ml) and streptomycin sulfate (100 μg/ml), and incubated at 37 C under a humidified atmosphere of 95% air-5% CO_2. After a preincubation period of 1 h, the medium was discarded, and 2.5-ml fresh medium was dispensed into each well. Culture medium was then collected at 1, 2, or 3 h, and tissue was weighted with a precision scale. Media and plasma were stored at −20 C until ghrelin assay.

Surgical vagotomy

The surgical procedure was performed aseptically, and all surgical instruments were sterilized before use. Animals were operated under ketamine-xylazine anesthesia. Rats were placed on their backs, and a midline abdominal incision was made. The liver was carefully moved to the right to expose the esophagus. Dorsal and ventral branches of the vagus nerve were then exposed and dissected from the esophagus. Each branch of the nerve was ligated with surgical suture at two points, as distally as possible to prevent bleeding, and cauterized between the sutures. The abdominal muscles and the skin were then sutured with surgical silk. Sham surgeries were also performed, in which each trunk of the nerve was exposed, but not tied or cauterized. One week after vagotomy, the animals were euthanized, and the stomachs were excised as described previously. The effectiveness of the vagotomy was assessed by postmortem stomach observation. Only the rats that showed an increase in stomach size after vagotomy were included.

RT-PCR

RT-PCR protocol conditions and quantitative real-time PCR amplification and detection for gastric mucosa ghrelin were performed on a Roche Light Cycler system (Roche Molecular Biochemicals, Mannheim, Germany), as previously described (20). RNA extraction was performed on the samples according to the manufacturer's protocol (Invitrogen, Barcelona, Spain). RNA was resuspended in diethylpyrocarbonate water, and the integrity of total RNA was checked by agarose gel electrophoresis and 28S and 18S rRNAs visualized after ethidium bromide staining (data not shown). Two micrograms of total RNA were reverse transcribed with SuperScript III Reverse Transcriptase (Invitrogen) into cDNA, as previously described (21).

PCR was performed using 3-μl cDNA template with specific primers for ghrelin. Rat hypoxanthine-guanine phosphoribosyltransferase (HPRT), designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3__www.cgi), was used as an internal control gene. The fluorescence spectra were recorded during the elongation phase of each PCR cycle. Contamination with genomic DNA was excluded by using total RNA samples as template in which no amplification product was detected. Furthermore, intron-spanning primers for ghrelin and HPRT housekeeping were used to support further the absence of genomic DNA (3). After the final cycle, the melting curve was determined to check that only one product had been produced, and the PCR product was electrophoresed on a 1.5% agarose/0.5× Tris-borate EDTA gel containing ethidium bromide to confirm that the product was the expected size. Relative quantification of PCR products was then based on value differences using the comparative cycle threshold method (20). Ghrelin mRNA levels were normalized with respect to the HPRT level in each sample. This experiment was performed on eight animals per group.

Time-course studies

To perform plasmatic ghrelin and insulin time-course studies, intracardiac cannulas were implanted under ketamine-xylazine anesthesia, as previously described (9). After surgery, the animals were placed directly in isolation test chambers for 5 d and given free access to food and water. The day of the experiment, the animals were assigned to one of the four experimental groups described previously, and blood samples (0.3 ml) were withdrawn at the appropriate times: 15, 30, 45, and 60 min. Serum was kept at −20 C until RIA analysis.

Biochemical analysis

Total ghrelin levels were determined by a double antibody RIA using reagents kits and methods provided by Phoenix Pharmaceuticals Inc. (Belmont, CA). Samples for tissue explants were obtained directly from culture medium. For testing plasma ghrelin levels, the samples were obtained from trunk blood by decapitation, and were collected in tubes containing EDTA (1 mg/ml blood) and aprotinin (500 U/ml blood; Sigma-Aldrich). Samples were immediately centrifuged and then subjected to RIA, as previously described (22). The limit of assay sensitivity was 1 pg/ml. Results were expressed as picograms per milliliter of ghrelin per gram of tissue in culture media or as picograms per milliliter in plasma.

Plasma insulin levels were determined by a double antibody RIA using insulin RIA kits provided by Phoenix Pharmaceuticals Inc.

Plasma glucose concentrations were determined by a glucose autoanalyzer (Accu-Chek sensor, Roche Diagnostics GmbH, Mannheim, Germany). The rats were weighed on two occasions: at the start of fasting and just before euthanasia. Data were expressed as mean ± SE and assessed by the Mann-Whitney *U* test. $P < 0.05$ was considered significant.

Results

To determine the adequate parameters for the organ culture model used here, two variables were analyzed:

fasting period and incubation time. Stomach explants from animals with *ad libitum* food intake (control group) were compared with tissue from rats deprived of food for 12, 36, and 60 h. All groups were compared after 1 h of incubation, 2 h, and 3 h. No meaningful changes were detected in any of the groups at 1 h. However, differences were found at 2 h of incubation, but only in donors that had fasted for 36 h or more (Fig. 1B). Thus, the model was set at 2-h incubation of explants from donors deprived of food for 36 h. The secretion from *ad libitum* animals explants was 2366 ± 217-pg/ml/g tissue, and after 36 h of fasting was of 3501 ± 338-pg/ml/g tissue. This represents 148% of the control group ($P < 0.01$).

Having established a proper model, the focus became the determination of whether ghrelin changes in plasma under different metabolic situations were due to direct variations in gastric release. Groups of experimental animals with either *ad libitum* food intake or 36-h fasting were studied (Fig. 2A). As expected, fasting significantly increased plasmatic ghrelin values to 300% over controls (620 ± 66 pg/ml fasting animals *vs.* 225 ± 45 pg/ml in controls; $P < 0.01$). In the groups that had fasted for 36 h, 15 min of refeeding previous to explants extraction induced a nonsignificant (NS) reduction in ghrelin levels (529 ± 96 pg/ml) *vs.* fasting group.

It is noteworthy that the plasmatic ghrelin changes were a direct reflection of the stomach secretion of ghrelin into the incubation media (Fig. 2B). Fasting for 36 h enhanced stomach release of ghrelin (3549 ± 343 pg/ml/g of tissue), which is 150% *vs.* control ($P < 0.01$), and that increase was significantly and strikingly reduced by just 15 min of refeeding (2058.44 ± 205.6 pg/ml/g), which is 87% over control ($P < 0.01$). The model was considered validated because this refeeding was able to counteract the fasting induced increase in ghrelin secretion. The changes in ghrelin release were

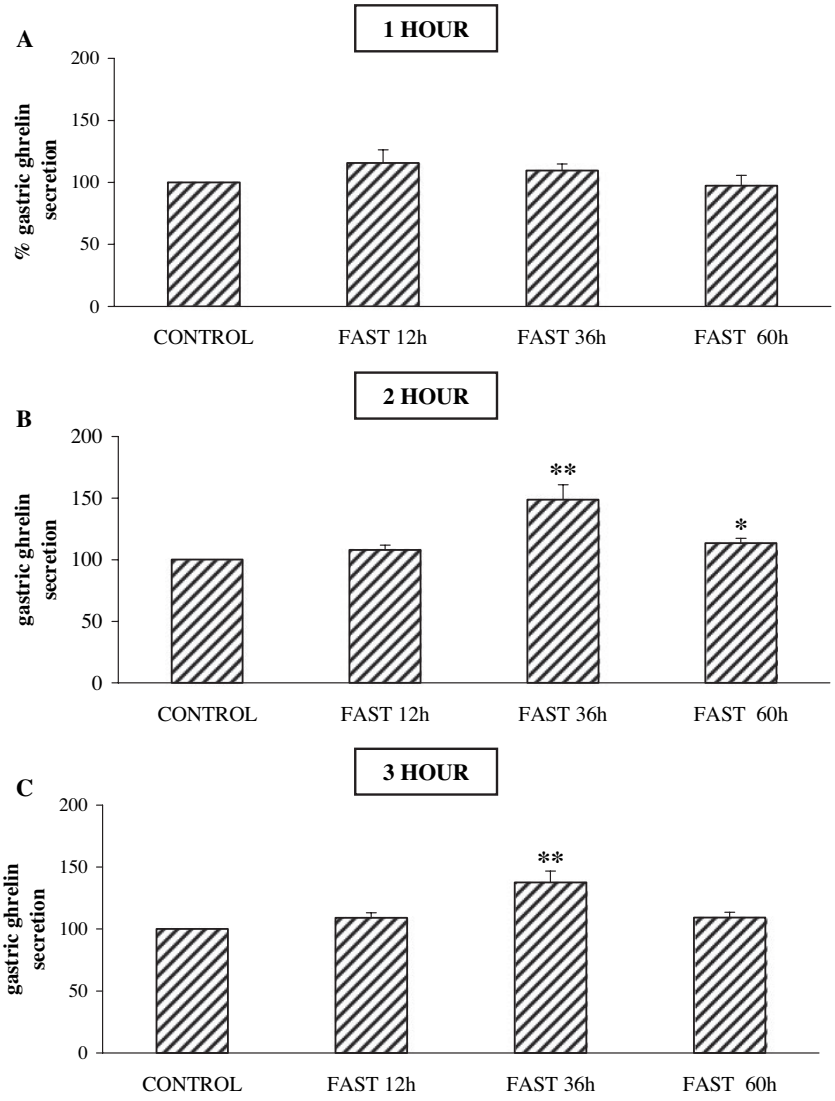

FIG. 1. Gastric ghrelin secretion directly from tissue explants at 1 (A), 2 (B), and 3 h (C) of incubation presented as percent over control (mean ± SE). Samples were measured in duplicate (n = 10–15). *, $P < 0.05$; **, $P < 0.01$ *vs.* control.

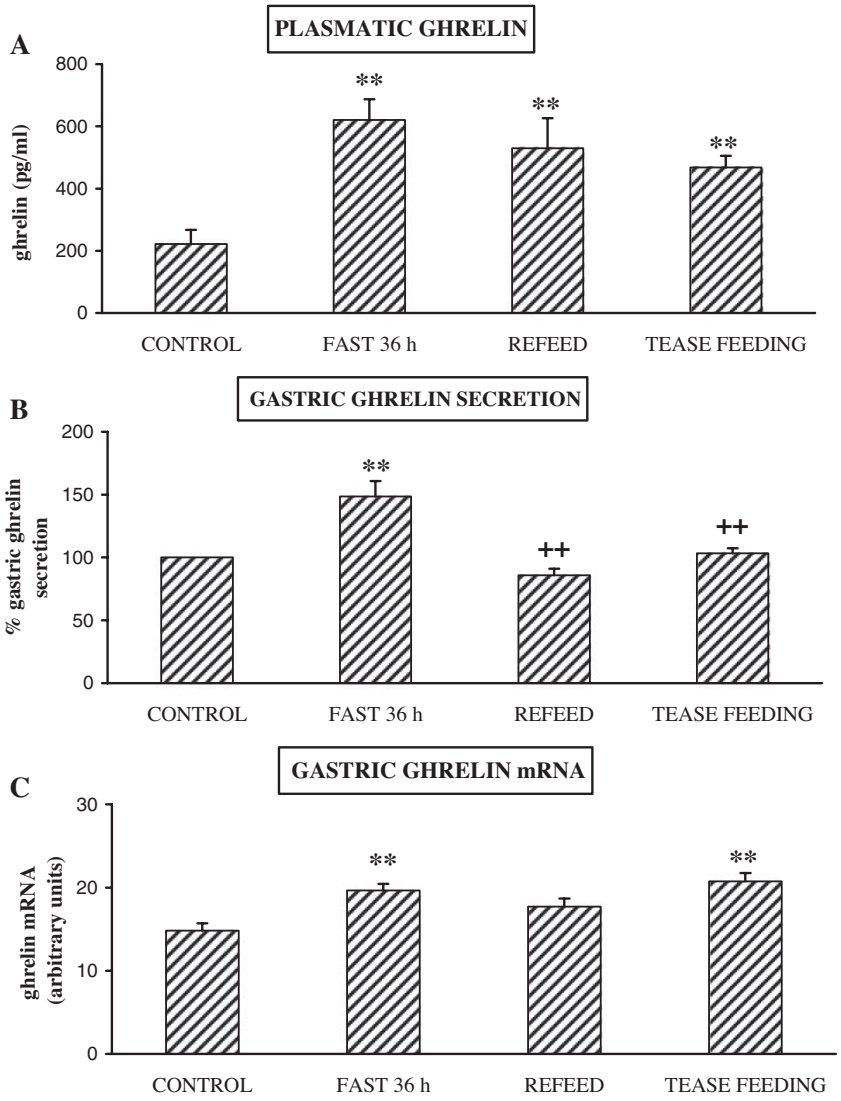

FIG. 2. A, Plasmatic ghrelin concentration (n = 10–15) in the different experimental conditions. B, Gastric ghrelin secretion from tissue explants to the incubation medium (**, $P < 0.01$ *vs.* control group; ++, $P < 0.01$ *vs.* fasting group). C, Ghrelin mRNA expression in gastric mucosa by real-time RT-PCR, standardized by HPRT mRNA levels (**, $P < 0.01$ *vs.* control group).

supported by changes in gastric ghrelin mRNA (Fig. 2C) (fasting 36 h 19 ± 0.7 arbitrary units *vs.* control 14 ± 0.8 arbitrary units; $P < 0.01$), but refeeding did not induce significant alteration in mRNA (refeeding 17 ± 0.9 arbitrary units, NS *vs.* fasting). The NS change was most probably due to the fact that 15 min of refeeding was too short a time to modify the previously enhanced mRNA expression induced by fasting.

The most unexpected finding was the effect of tease feeding on gastric ghrelin secretion (Fig. 2A). In the tease feeding group, which was allowed to watch and smell, but not to eat food, for 15 min before euthanasia, a reduction was found in ghrelin release after incubation similar to that obtained for the *ad libitum* feeding group (tease feeding: 2484 ± 505 pg/ml/g of tissue; $P < 0.01$ *vs.* fasting). This change, unrelated to food intake, was most probably due to an inhibition of the gastric release, seeing as ghrelin mRNA levels were not modified with respect to the fasting group (Fig. 2C). On the other hand, animals subjected to surgical vagotomy did not show any significant difference between tease feeding and fasting, in either plasmatic ghrelin levels or gastric ghrelin secretion. In the tease feeding group, gastric ghrelin levels were 83.1% of values obtained for fasting animals, and plasmatic ghrelin levels were 100% (Fig. 3). Identical results were obtained in animals treated with atropine sulfate 30 min before exposure to the tease feeding stimulus: the gastric ghrelin values of secretion were 100.94%, and the plasmatic ghrelin levels were 115.7% of the fasting group.

As proof that animals complied with the experimental model, plasma glucose was found to lower significantly in both the fasting and tease feeding rats but recovered normal values in the refeeding group (Fig. 4A). Insulin levels in plasma were slightly increased in the tease feeding rats with

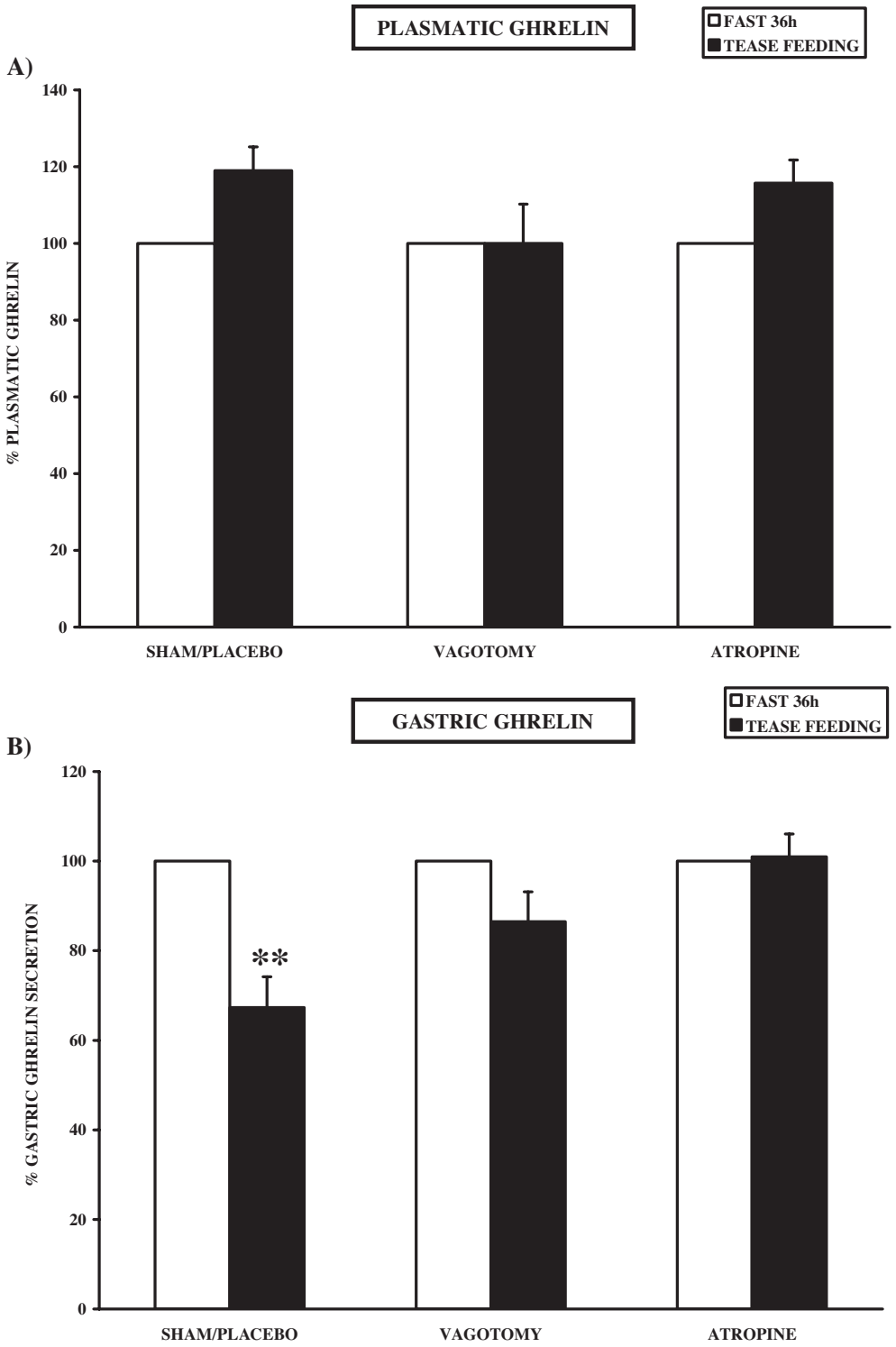

FIG. 3. A, Plasmatic ghrelin concentration (n = 10) and gastric ghrelin secretion (B) from tissue explants to the incubation medium in rats fasted 36 h without (*white bars*) or with tease feeding (*black bars*) in animals subjected to either vagotomy or atropine administration. **, $P < 0.01$ *vs.* fast group.

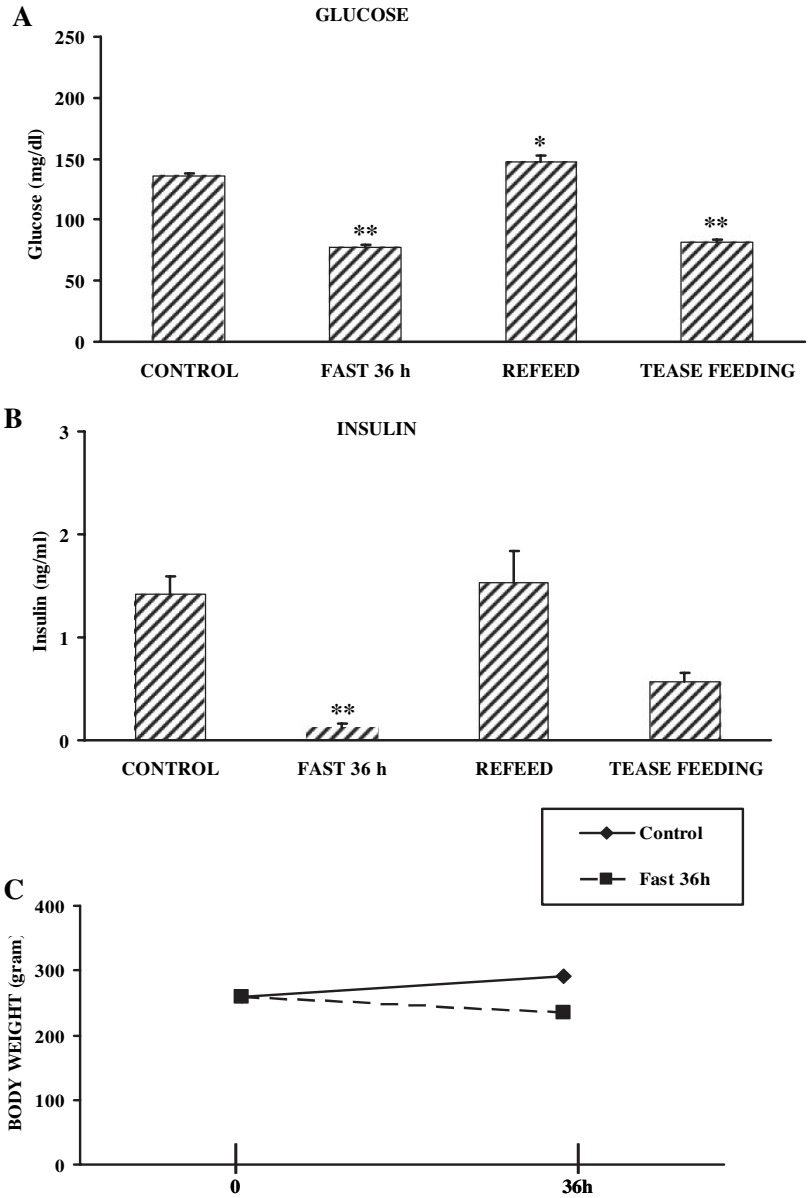

FIG. 4. A, Plasma glucose levels in: *ad libitum* animals (control), fast 36 h, 15 min refeed after fasting, and tease feeding for 15 min after fasting expressed as mean ± SE (n = 10) (**, $P < 0.01$; *, $P < 0.05$). B, Plasma insulin levels in: *ad libitum* animals (control), fast 36 h, 15 min refeed after fasting, and tease feeding for 15 min after fasting expressed as mean ± SE (n = 10) (**, $P < 0.01$; *, $P < 0.05$). C, Body weight of experimental animals before and after fasting for 36 h.

respect to fasting, but in the same way than glucose, increased with refeed to normal values (Fig. 4B). Body weight also showed compliance with the fasting protocol (Fig. 4C). To control the short-term time dynamic of the plasmatic changes observed, plasma ghrelin was measured at shorter intervals in freely moving cannulated rats (Fig. 5A). In the fasting group, plasma ghrelin remained high throughout, while refeeding induced a reduction in ghrelin levels that was evident at 15 and 30 min [620 ± 66 pg/ml fast *vs.* 222 ± 45 pg/ml control and 529 ± 96 pg/ml refeed at 15 min (**, $P < 0.01$ *vs.* control); 573 ± 76 pg/ml at 36 h fasting *vs.* 250 ± 34 pg/ml control and 441 ± 82 pg/ml in refeed at 30 min (**, $P < 0.01$ *vs.* control)]. On the other hand, tease feeding induced a dramatic reduction in ghrelin levels evident at 15 min (468 ± 37 pg/ml tease feeding, NS *vs.* refeed) and 30 min (450 ± 27 pg/ml tease feeding, NS *vs.* refeed), but the reduction disappeared at 60 min (45 min: 296 ± 62 pg/ml control, 487 ± 87 pg/ml fast, 317.5 ± 73 pg/ml refeed, and

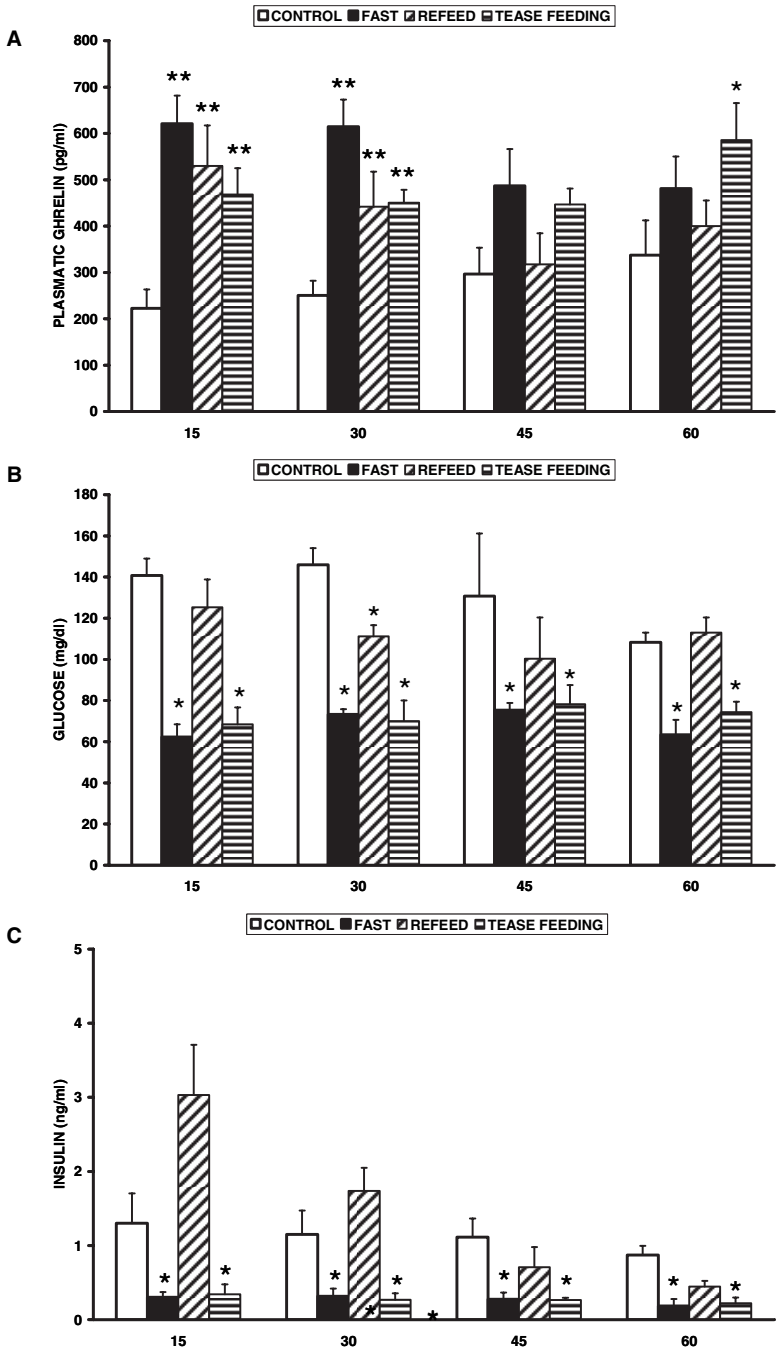

FIG. 5. A, Plasmatic ghrelin levels (pg/ml) at 15, 30, 45, and 60 min [**, $P < 0.01$; *, $P < 0.05$ (n = 10)]. B, Plasma glucose levels (pg/ml) at 15, 30, 45, and 60 min [*, $P < 0.05$ (n = 10)]. C, Plasma insulin levels (pg/ml) at 15, 30, 45, and 60 min [*, $P < 0.05$ *vs.* control groups (n = 10)].

426 ± 36 pg/ml tease feeding; and 60 min: 312 ± 62 pg/ml control, 481 ± 69 pg/ml fast, 400 ± 60 pg/ml refeed, and 585 ± 103 tease feeding).

It was measured insulin and glucose plasma levels in parallel to ghrelin to assess that the changes observed in plasma ghrelin were not mediated by changes in insulin. It was found that insulin (Fig. 5B) and glucose concentration were not affected by tease feeding because the values obtained were similar to that obtained in the fasting group.

Discussion

To assess the direct regulation of ghrelin secretion by the stomach, an organ culture model of gastric tissue has been standardized in the present study. With this model, it has been proved that the food-mediated changes in plasma ghrelin levels are due to variations in ghrelin release by the stomach. However, there were two findings that were more surprising. First, excised tissues incubated for a total of 3 h, despite no longer being under central nervous system (CNS) control, still maintained the previous CNS conditioning. In fact, 15 min of feeding or even tease feeding, led to a reduction in gastric ghrelin secretion that was maintained 1–3 h after excision of the explant. Second, tease feeding (smell and sight of food, but not intake) was able to reduce gastric secretion of ghrelin in a way similar to true feeding. These findings strongly suggest that the influence of food on ghrelin secretion is partly mediated by nondigestive sensory signals, perhaps involving the vagus nerve. The findings also indicate that the neuronal network of the myenteric plexus is endowed with medium-term memory.

As the first known orexigenic peptide coming from splanchnic tissues, ghrelin has raised considerable interest in the scientific community. A large number of communications have described the variations in ghrelin plasma levels in diverse clinical and experimental situations (1, 7, 12). It is well known that plasma ghrelin increases in states of negative energy balance, like fasting, and that it normalizes after refeeding (10). However, the mechanism of regulation has not been described, leading to the unproved assumption that any changes in plasma ghrelin are a reflection of changes in gastric release (23). A model that allows direct assessment of gastric ghrelin secretion would improve knowledge of ghrelin regulation. In the present study, a model for assessing gastric ghrelin secretion directly from gastric tissue explants was validated, and the optimal incubation period for the gastric explants was found to be 2 h. In states of fasting and refeeding, the observed ghrelin changes reported here in plasma, incubation media, and mRNA unambiguously validate this model for use in further studies.

Fasting induced an increase in both gastric ghrelin secretion and mRNA content, generating an increase in ghrelin plasma levels. The fact that refeeding rapidly reversed those changes demonstrates, for first time, that the often-cited ghrelin changes in plasma under such conditions (24) are a direct reflection of changes in the gastric release of ghrelin. However, the effect of food intake on circulating ghrelin levels was found to involve a more complex mechanism of action than was previously thought. The time-course study of plasmatic ghrelin levels in freely moving rats showed that refeeding produced a strong and time-dependent reversion of the fasting-induced increase in plasma ghrelin levels; at 45 min of refeeding, ghrelin values were identical to those obtained in fed animals. Refeeding for just 15 min was enough to induce a quick blockade of ghrelin secretion from the stomach, but the time of refeeding was too short to affect enhanced ghrelin synthesis. These data suggest that ghrelin secretion directly from the stomach is the first target of ingested food and that inhibitory signals stop the release of previously synthesized ghrelin.

The cephalic phase reflexes are anticipatory changes in the digestion process that allow a more efficient use of food. These complex changes are mostly mediated by enteric hormones whose release is dependent on CNS-generated neural stimuli, more than on nutrient-induced stimulation (25). It has been reported in many species that the cephalic response to sensory stimulations of food produces an increase in insulin, glucagon, and other incretins mediated by vagus nerve action on the pancreas (26). The present study aimed to analyze whether something similar could be controlling gastric ghrelin secretion. Human studies on this kind of anticipatory responses are limited, and some were not able to show clear results because psychological, cognitive, and social attitudes toward food influence individual responses (27). Similarly, studies have been performed in animals to assess the cephalic phase reflexes, but their mechanisms were not elucidated (25). The tease feeding used in this study is a model to study whether ghrelin is secreted as an anticipatory response to an imminent feeding or is activated by food stimuli; the model has been validated in the present work by the measure of insulin levels in which it was found a anticipatory response of insulin to sensory stimulus of food in the tease feeding group (Fig. 4B), although this change is not relevant to mediate the effect of this stimulus on plasma ghrelin as it was observed in insulin time-course study (Fig. 5B). Surprisingly, it was found that tease feeding and true refeeding produced similar effects on gastric ghrelin secretion and ghrelin plasma levels. The great difference between the two models was that in refeeding, the restored circulating ghrelin levels were maintained through the time, whereas in tease feeding, there was just a transient effect. These changes affected gastric ghrelin secretion but did not alter expression because enhanced ghrelin mRNA was unaltered.

Interestingly, previous studies in sheep with pseudofeeding, in which the animals were provided with food wrapped in a nylon bag that could be swallowed but not digested, reported that the preprandial pulse of circulating ghrelin was reverted (25). This suggests that the regulation of circulating ghrelin is not merely due to absorption of nutrients, although in that model, the mechanical stimuli to esophagus and gastric wall were fully operative. In the tease feeding model presented here, there was no direct mechanical contact between food and esophagus or stomach; the only operative factor was sensorial stimuli acting at the CNS level because the animals could smell and see the food but not swallow, taste, or chew it. Nevertheless, a clear effect of blockade in ghrelin secretion was found. The present study suggests that ghrelin secretion directly from the stomach is not only due to direct mechanical contact with the gastric wall, digestion, or absorption of nutrients. Another relevant factor involved in this process is the CNS sensorial stimuli, which induce the cephalic responses. The implication of vagus nerve mediating the cephalic response of ghrelin to food-related stimuli is supported in the present study by the fact that both vagotomy and cholinergic blockade with atropine sulfate prevented the tease feeding-mediated ghrelin reduction because the values of gastric ghrelin secretion and plasmatic ghrelin levels in this group did not differ from those observed in fasting animals. We propose that ghrelin could be a neurally mediated integrative factor, constituting a link among the sensory qualities of food, neural activation, and nutrient metabolism. Moreover, ghrelin could be considered an incretin hormone.

Gastric tissue excised from the organism maintained the previous secretory status for a period of 3 h after excision, sug-

gesting that the neurons from the enteric system of the gastric tissue have medium-term memory. Previous studies showed that there exists a molecular memory of synaptic activity at the enteric nervous system (27). Electrophysiological studies provide evidence that the prolonged activation of myenteric plexus neurons of the guinea pig contribute to a slowly developing, sustained increase in excitability of the neurons associated with depolarization and that this increased excitability lasted for up to 3.5 h after stimulation (28).

In conclusion, the present study presents a suitable model for studying ghrelin secretion directly from the stomach, which eliminates interference produced by other organs. This new model demonstrated that food intake regulation of plasmatic ghrelin is mediated by gastric tissue because changes in nutritional status first affect ghrelin secretion before expression or circulating levels. For the first time, it has been shown that sensorial stimuli related to food, but without food ingestion, are able to modify gastric ghrelin secretion and circulating ghrelin levels in the same way that true food ingestion does, demonstrating that factors other than those caused by nutrient digestion or absorption are involved in ghrelin regulation. This regulation of ghrelin secretion from the stomach by food-related stimuli is mediated by a medium-term memory mechanism from the system of sensory neurons integrated in the enteric nervous system.

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DISCUSIÓN

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En los últimos años se han descubierto una gran cantidad de hormonas producidas en tejidos periféricos del organismo diferentes de los órganos endocrinos "clásicos". Entre estos tejidos destacan el adiposo y el tracto gastrointestinal por lo cual algunos autores los han definido como auténticos órganos endocrinos.

Aunque la ghrelina ha despertado desde el primer momento una gran expectación y se ha profundizado mucho en su conocimiento desde diversos campos científicos, aun no se ha aclarado por completo cual es su mecanismo de acción y de regulación.

La mayoría de los trabajos relacionados con el tema versan sobre la variación de los niveles de ARNm, contenido gástrico de proteína a partir de extractos celulares y niveles plasmáticos o séricos de ghrelina (Tschop MH et al 2000, Van der Lely AJ et al 2004, Cummings DE et al 2001), dando por supuesta la teoría no probada de que cualquier cambio en la secreción plasmática de esta hormona se debe a cambios en la secreción gástrica (Sánchez J et al 2004). De esta manera un modelo que permitiese el estudio directo de la secreción gástrica de ghrelina podría mejorar el conocimiento de la regulación de esta hormona.

En el presente trabajo se ha puesto a punto un sistema de cultivo de explantes de tejido gástrico, con el fin de determinar cómo es regulada la secreción de ghrelina directamente desde el estómago, sin interferencias de otros órganos. Se ha utilizado este sistema para determinar el efecto de los componentes del eje somatotropo, del desarrollo asociado a la edad, del estado de lactancia y de diferentes estímulos externos relacionados con la ingesta sobre la secreción gástrica de ghrelina.

Mediante experimentos *in vitro*, en un primer estudio, se ha estudiado el efecto de los componentes clásicos del eje somatotropo (GHRH, SS, GH e IGF-1) sobre la secreción gástrica de ghrelina.

Demostramos como la adición de GH y SS a la placa de cultivo donde se encuentran los explantes gástricos es capaz de producir una disminución en la secreción gástrica de ghrelina a las dos y tres horas de incubación. Por otra parte la adición de IGF-1 y GHRH no alteraban los niveles basales de ghrelina gástrica (Seoane LM et al 2007a; Figura 1).

En este trabajo se observó que la administración de GHRH a una concentración de 10^{-6} M no modificaba la secreción basal de ghrelina, esto puede justificarse por el hecho de que no se han encontrado hasta la fecha receptores de GHRH en el estómago por lo que esta hormona no tendría efectos relevantes a este nivel. Recientemente se ha encontrado un nuevo subtipo de receptor para esta hormona denominado SVI (Busto R et al 2002) pero los datos de este trabajo parecen indicar que los efectos de este receptor no incluyen la regulación de la secreción de ghrelina.

En cuanto a la SS se sabe desde hace tiempo que se produce localmente en el estómago y que suprime la acción de varias hormonas entéricas de forma paracrina. Existen receptores para esta hormona en la mucosa gástrica lo cual sugiere que estas células productoras de SS en contacto con las células productoras de ghrelina podrían estar interaccionando a nivel del *fundus* gástrico (Di Vito L et al 2002, Shimada M et al 2003, Patel YC et al 1999).

Varios trabajos sugieren una regulación negativa por parte de la GH sobre la ghrelina gástrica (Lee HM et al 2002, Qi X et al 2003, Tschop M et al 2002), apoyado este argumento en el hecho de la existencia de receptores de GH en el estómago e intestino (Nagano R et al 1995).

El mecanismo de acción bajo el cual se produce la disminución de los niveles de ghrelina mediados por GH puede ser aquel en el que el aumento de GH puede activar sus receptores en el estómago y aumentar los niveles de SS en el mismo, para reducir la secreción de ghrelina gástrica y posteriormente los niveles circulantes.

La relación entre IGF-1 y ghrelina aun no está clara, de hecho hay una controversia sobre este tema en el que algunos autores postulan un aumento (Dall R et al 2002, Malik IA et al 2004, Rigamonti AE et al 2002) y otros un descenso (Whatmore AJ et al 2003, Bellone S et al 2003, Bellone S et al 2002, Kitamura S et al 2003) en los niveles de ghrelina tras una infusión de IGF-1. En este estudio se ha descartado un efecto positivo de IGF-1 directamente sobre la regulación del estómago.

La ghrelina es un potente péptido orexigénico derivado del estómago que está implicado en la regulación del balance energético. Es razonable pensar que los niveles de este péptido varían con la edad para atender a las demandas energéticas de cada período de la vida. En un segundo estudio se demostró que aunque los niveles plasmáticos de ghrelina no están afectados de manera significativa por la edad, si lo está la secreción de ghrelina por el estómago (Al-Massadi O et al submmited, Figura 1). Se observó que las mayores variaciones en la secreción de esta hormona tanto en machos como en hembras se produjeron a la 4 y 6 semanas de edad. De acuerdo con los datos publicados hasta el momento también se observó un aumento de los niveles de expresión de ARNm de ghrelina en ratas hembra de 4 semanas, etapa que coincide con el inicio del periodo puberal (Sakata I et al 2002b).

Los resultados sugieren que la pubertad es una etapa clave en la regulación de la secreción gástrica de ghrelina y que está regulada de manera independiente a la expresión de ARNm de ghrelina (Al-Massadi O et al submmited, Figura 1).

La medida del peso del útero seco es un índice de desarrollo y madurez sexual, se ha demostrado una correlación directa entre el tamaño del útero y los niveles circulantes de estrógenos (Marriot LK et al 2007). Este método ha sido usado en el presente trabajo debido a la dificultad de medir los niveles basales de estrógenos directamente mediante radioinmunoensayo. Por otra parte, se han medido también los niveles circulantes de testosterona en ratas macho de los diferentes grupos experimentales. En las hembras, el inicio de la etapa puberal empieza a las cuatro semanas de edad como se deduce en la diferencia en el peso del útero detectado a esa edad, mientras que en ratas macho este proceso comienza alrededor de las seis semanas de edad, donde es detectado un incremento muy significativo en los niveles de testosterona. Con estos datos, se puede sugerir que las variaciones en la secreción gástrica de ghrelina están fuertemente asociadas con los períodos de la vida que se caracterizan por un marcado cambio en los niveles circulantes de hormonas sexuales (Al-Massadi O et al submmited, Figura 1).

Se ha demostrado una acción directa de los estrógenos sobre el tejido gástrico, provocando una significativa reducción de la secreción gástrica de ghrelina, este efecto podría ser mediado por los receptores α de estrógenos (α ER) de la mucosa gástrica (Campbell-Thompson M et al 2001) (Al-Massadi O et al submmited, Figura 2). Aunque los trabajos publicados sobre este tema sugieren que los estrógenos producidos localmente en el estómago pueden desempeñar un papel fundamental en la regulación de la ghrelina gástrica (Sakata I et al 2006), hasta el momento no existían datos sobre este efecto.

Por el contrario un estudio previo, usando células aisladas de estómago, mostró como el tratamiento con estrógenos estimula tanto la expresión de ARNm como el número de células inmunopositivas para ghrelina (Sakata I et al 2002).

Estos datos junto con los resultados aquí presentados, propondrían un mecanismo de acción para la regulación de la ghrelina gástrica, así la disminución en su secreción podría dar lugar a una acumulación de ghrelina a nivel celular lo cual se vería potenciado por el incremento en la expresión de ARNm de ghrelina en el estómago. Por el contrario, el tratamiento con testosterona *in vitro* directamente en el estómago no afecta a la secreción gástrica de ghrelina (Al-Massadi O et al submmited, Figura 2A) a pesar de las variaciones encontradas en animales de 6 semanas de edad *ex vivo* (Al-Massadi O et al submmited, Figura 1A). Estos datos sugieren un mecanismo indirecto que podría estar regulando ese proceso. En el presente trabajo el efecto de los estrógenos y la testosterona sobre la secreción de ghrelina fue estudiada en hembras y machos respectivamente a través del tratamiento subcutáneo con estradiol y propionato de testosterona, en animales de 4 semanas previamente sometidos a ovariectomia/orquidectomia (Al-Massadi O et al submmited, Figura 3). Se ha observado un efecto del estradiol sobre la secreción gástrica de ghrelina. Cuando los niveles de estrógenos se incrementan en la etapa puberal, la secreción de ghrelina gástrica sufre una caída. Si este incremento en los niveles de estrógenos característico de esta etapa es bloqueado mediante la realización de una ovariectomia quirúrgica, la caída en la secreción gástrica de ghrelina no se produce, por el contrario la secreción desde el estómago se incrementa, además el tratamiento exógeno con estrógenos revierte el efecto de la ovariectomia, posiblemente debido al restablecimiento de los niveles basales de estrógenos (Al-Massadi O et al submmited, Figura 3).

Nuestros hallazgos justifican los datos contradictorios publicados hasta el momento (Matsubara M et al 2004, Gualillo O et al 2001). Entre los trabajos publicados sobre ese tema uno de ellos encuentra que la expresión de ARNm de ghrelina gástrica solo se incrementa en ratas sometidas a ovariectomía y ese incremento es revertido tras la

administración de 17β-estradiol. Sin embargo no se encontraron cambios en animales adultos. La diversidad de efectos recogidos en la bibliografía sugiere que el mecanismo por el cual la expresión de ARNm de ghrelina es regulada, difiere dependiendo de la edad y todo ello junto con los resultados mostrados en el presente trabajo, confirman que los estrógenos juegan un papel importante en la regulación de la expresión de ghrelina en la etapa peripuberal.

En ratas macho de 4 semanas de edad los niveles de testosterona no sufrieron variaciones significativas, permaneciendo bajos hasta las 6 semanas de edad (Al-Massadi O et al submmited, Figura 1D). Este hecho sugiere que la disminución de la secreción de ghrelina gástrica encontrada a las cuatro semanas de edad en machos no es una consecuencia de la variación en los niveles de testosterona. Esto fue confirmado cuando esos animales sujetos a orquidectomía no mostraron variaciones en la secreción gástrica de ghrelina.

Las cuatro semanas de edad en la rata coinciden con una importante modificación de la dieta, como es el destete. Esto implica el cambio de la dieta líquida materna a la dieta sólida. Este proceso afecta a la maduración y morfología de las células productoras de ghrelina (Bjorkqvist M et al 2002) así como a la expresión de ghrelina gástrica. Para determinar si existe una asociación entre el cambio de alimentación y las alteraciones encontradas en la producción de ghrelina, la secreción directa de ghrelina desde el estómago fue medida en animales sometidos a un retraso del destete. Este experimento consiste en retrasar el paso a la alimentación sólida desde el día 21 al día 28, manteniendo a las crías con la madre en este período. En las hembras de cuatro semanas el retraso del destete no afecta a la secreción gástrica de ghrelina (Al-Massadi O et al submmited, Figura 4), debido probablemente a que son tan drásticas las variaciones en este parámetro asociado al inicio de la pubertad que estarían enmascarando el efecto del

destete. Sin embargo, en crías macho el retraso del destete bloquea la caída en la secreción gástrica de ghrelina que se observa con este cambio de alimentación (Al-Massadi O et al submmited, Figura 4). Aunque la tendencia en la secreción gástrica de este péptido se ve reflejado en los niveles de ghrelina circulante no es estadísticamente significativo. Los resultados aquí obtenidos son opuestos a datos previos de otro grupo que encontró una disminución en los niveles plasmáticos, expresión de ARNm y densidad de células de ghrelina cuando prolongaban el periodo de lactancia (Fak F et al 2007) pero este grupo no estudió la secreción directa de ghrelina del estómago, y además hay diferencias metodológicas como la edad en la cual se produce el destete.

El peso corporal así como las hormonas sexuales son otros parámetros que están también afectados por este proceso, observándose una disminución en ambos sexos (Al-Massadi O et al submmited, Figura 5C).

Este último hallazgo está en concordancia con el hecho de que un período prolongado de lactancia materna produce una reducción en el riesgo de desarrollar obesidad, aunque el mecanismo por el que este hecho tiene lugar es hasta el momento desconocido (Harder T et al 2005).

El gen de ghrelina presenta diferentes productos entre los que destacan las formas acilada y no acilada, estos dos péptidos se conocen desde el año 1999 cuando se descubrió esta hormona (Kojima M et al 1999), sin embargo el mecanismo mediante el cual se producía esta n-octanoilación se ha descubierto recientemente. En el año 2008 dos grupos independientes caracterizaron la enzima responsable de este proceso, denominándola GOAT (ghrelin O-acil transferasa) conocida anteriormente como MBOAT4 (Gutiérrez JA et al 2008, Yang J et al 2008).

Desde ese momento varios grupos de investigación han intentado determinar la distribución así como las variaciones de este enzima con respecto a los cambios en el estado nutricional.

Uno de estos grupos demuestra que la expresión de GOAT es similar a la de ghrelina, es decir está aumentada en estados energéticos negativos y disminuye en estados energéticos positivos como son el ayuno y la obesidad respectivamente (Xu G et al 2009). Por otra parte otro grupo de investigación determina que la expresión de GOAT en ratones alimentados *ad libitum* aumenta con respecto a ratones sometidos a diferentes periodos de ayuno, en los cuales solamente la ghrelina no acilada presentaba un aumento mientras que los valores de la ghrelina acilada se mantenían constantes (Kirchner H et al 2009). Sin embargo un tercer grupo de investigación determinó que en ratas sometidas a restricción calórica crónica los niveles de expresión de ARNm de GOAT se mantenían estables hasta que se producía un grado determinado de pérdida de peso corporal, donde los niveles de GOAT aumentaban. Estos hallazgos sugieren un papel de GOAT como una respuesta adaptativa que previene alteraciones en el balance energético y la homeostasis energética (González CR et al 2008). Bajo este contexto, es posible que los niveles de expresión de GOAT cambien a lo largo de diferentes periodos de la vida para ajustar el organismo a los distintos requerimientos energéticos de cada etapa. Los resultados presentados en este trabajo muestran por primera vez cambios relacionados con la edad en la expresión de ARNm de GOAT a nivel gástrico en ambos sexos. En machos (Al-Massadi O et al submmited, Figura 6A) los niveles de ARNm de GOAT se incrementan de forma lineal con la edad de forma paralela al aumento del peso corporal (datos no mostrados). Sin embargo, en hembras, el valor máximo de los niveles de ARNm de GOAT se encontró en 6 semanas de edad (Al-Massadi O et al submmited, Figura 6B) cuando empieza la edad adulta en la rata y que coincide también

con la estabilización de la secreción gástrica de ghrelina (Al-Massadi O et al submmited, Figura1F). El hecho de que la maduración en hembras se produzca antes que en machos (datos no mostrados), puede estar relacionado con las diferencias en el patrón de expresión de GOAT entre ambos sexos. Durante el periodo de crecimiento y especialmente en la pubertad los requerimientos energéticos son mayores que en otros periodos de la vida y es justo en ese momento cuando la producción de GOAT está más elevada tanto en machos como en hembras. La principal función de GOAT consiste en la acilación de ghrelina mediante la cual se produce la forma acilada de esta hormona, que realiza sus acciones orexigenicas e inductoras de adiposidad a través de su unión al GHSR-1a. Bajo este contexto es posible que en periodos de la vida caracterizados por un balance energético negativo como consecuencia de unos elevados requerimientos energéticos, GOAT se incremente para almacenar energía y así contrarrestar ese balance energético negativo. En este modelo GOAT es propuesto como un mecanismo de defensa del peso corporal permitiendo al organismo la adaptación a las diferentes necesidades de cada periodo de la vida modificando la relación acil/desacil ghrelina.

Por otra parte, los niveles de ARNm de ghrelina fueron estudiados también en el modelo de retraso del destete en ratas de 4 semanas de edad. Como ocurre con la secreción gástrica de ghrelina (Al-Massadi O et al submmited, Figura 4B) en el grupo de las hembras el retraso del destete no afecta a los niveles de expresión de ARNm de ghrelina, probablemente debido a un enmascaramiento por el efecto de la pubertad.

De la misma manera que en la secreción gástrica de ghrelina en machos la secreción de GOAT está fuertemente afectada por el retraso del destete (Al-Massadi O et al submmited, Figura 6C). Inesperadamente los niveles de expresión de ARNm de GOAT presentan un patrón inverso con respecto a los de la secreción gástrica de ghrelina; mientras que el retraso del destete incrementa los niveles de la secreción gástrica de esta

hormona los niveles de GOAT disminuyen. Así mismo los niveles de GOAT se incrementan en el periodo entre 4-6 semanas de edad (Al-Massadi O et al submmited, Figura 6A, B) en paralelo con la disminución en la secreción gástrica de ghrelina (Al-Massadi O et al submmited, Figura 1B, F).

Los hallazgos más relevantes de este apartado son: primero, la etapa puberal es clave en la regulación de la actividad secretora de ghrelina a través de las modificaciones hormonales asociadas a este periodo, como por ejemplo variaciones en los niveles circulantes de estrógenos; segundo, las modificaciones producidas en la dieta como consecuencia del destete están fuertemente implicadas en la regulación de la secreción gástrica de ghrelina; tercero, la prolongación de la lactancia en roedores afecta a las hormonas sexuales y el peso corporal; cuarto, los niveles de ARNm de GOAT están regulados fuertemente por la edad y el destete de manera inversa a la secreción gástrica de ghrelina.

En conclusión, todos estos datos podrían indicar que el estómago por él mismo puede regular su propia producción de ghrelina y de GOAT durante el desarrollo postnatal independientemente de otros órganos, para adaptar el organismo a los requerimientos metabólicos demandados en cada etapa de la vida.

Por otra parte utilizando el mismo modelo de explantes de tejido gástrico se estudió mediante experimentos *ex vivo*, el comportamiento de esta hormona bajo cuatro estados nutricionales diferentes como son: *ad libitum* (libre acceso al alimento), ayuno de 36 h, realimentación tras un periodo de ayuno, y finalmente un grupo menos común en el que establecimos un simulacro de alimentación (tease feeding) donde el animal únicamente percibía los estímulos relacionados con el alimento (visión y olor) pero sin ingestión del mismo (Seoane LM et al 2007b; Figura 2B).

En este último estudio se hicieron medidas de expresión de ARNm, secreción desde tejido y niveles circulantes de ghrelina (Seoane LM et al 2007b; Figura 2). Dado que los niveles de ghrelina aumentan en estados energéticos negativos como el ayuno y que estos niveles se normalizan tras la realimentación, en el presente trabajo el ayuno produjo un aumento de los niveles de expresión de ARNm de ghrelina, así como de la secreción de ghrelina por el estómago, generando por tanto un aumento en los niveles de ghrelina plasmática (Seoane LM et al 2007b; Figura 2). El hecho de que la realimentación revierta de forma rápida esos niveles constituye la prueba de que los cambios en los niveles plasmáticos de ghrelina que se producen en esas condiciones son una consecuencia directa de los cambios en la secreción gástrica. Sin embargo, el efecto del alimento sobre los niveles de ghrelina circulante supone unos mecanismos de acción más complejos de los que previamente se pensaba.

Un experimento que permitió un seguimiento del comportamiento de la ghrelina plasmática en el tiempo en ratas en libre movimiento, mostró que la realimentación produce una inhibición sostenida en el tiempo del incremento de los niveles plasmáticos de ghrelina inducido por el ayuno, así 45 minutos después de la realimentación, los niveles de ghrelina fueron idénticos a los valores obtenidos para las ratas alimentadas *ad libitum*. Además la realimentación durante 15 minutos tras un ayuno de 36 horas produce un bloqueo de la secreción gástrica de ghrelina pero no afecta a la síntesis de ghrelina que se encontraba incrementada por el ayuno (Seoane LM et al 2007b; Figura 5A).

Este dato sugiere que la secreción de ghrelina directamente por el estómago es la primera diana del alimento ingerido.

El simulacro de alimentación (tease feeding) que se ha utilizado es un modelo para estudiar como la ghrelina es secretada como respuesta anticipatoria a una alimentación

inminente; el modelo ha sido validado en este trabajo por la medida de los niveles de insulina en el cual encontramos una respuesta anticipatoria de insulina a los estímulos sensoriales en el simulacro de alimentación (Seoane LM et al 2007 b; Figura 4B) aunque este cambio no es relevante en la mediación del efecto de estos estímulos en la ghrelina plasmática como se observa en la cinética de insulina plasmática (Seoane LM et al 2007 b; Figura 5C).

En este trabajo, por primera vez, se demuestra como los estímulos sensoriales relacionados con el alimento, pero sin que exista ingesta real del mismo, son capaces de modificar la secreción gástrica y los niveles circulantes de ghrelina de la misma manera que lo hace la verdadera ingesta de nutrientes. Este hecho muestra que otros factores diferentes de aquellos causados por la digestión o absorción de nutrientes están involucrados en la regulación de esta hormona. Esta regulación de la secreción gástrica de ghrelina por estímulos relacionados con el alimento está mediada por un mecanismo de *memoria* a medio plazo. Prueba de ello es el hecho de que tanto 15 minutos de realimentación como incluso 15 minutos de simulacro de alimentación tras el ayuno, dan lugar a una reducción en la secreción gástrica de ghrelina que se mantiene al menos durante 3 horas tras la extirpación del órgano, probablemente como consecuencia de la existencia de una red neuronal que compone el plexo mientérico del estómago (Seoane LM et al 2007 b; Figura 2B)

Debido a la más que probable implicación del nervio vago en la mediación de los efectos de la ghrelina, se eliminó la conexión nerviosa que conecta el SNC y el estómago mediante una vagotomía quirúrgica bilateral o mediante un bloqueo farmacológico del impulso colinérgico en el grupo de ayuno y en el simulacro de alimentación para ver si esta inhibición en los niveles gástricos de ghrelina producida por los estímulos sensoriales era transmitida por esta vía. Al ver que la secreción

gástrica de ghrelina era idéntica en ambos grupos tras la operación quirúrgica y el bloqueo químico colinérgico se llegó a la conclusión de que los estímulos sensoriales eran capaces de regular la secreción gástrica de ghrelina y que esta regulación era mediada a través del nervio vago (Seoane LM et al 2007 b; Figura 3).

En resumen**,** el presente trabajo presenta un modelo adecuado para el estudio de la secreción de ghrelina directamente a partir del estómago, el cual elimina posibles interferencias producidas por otros órganos. Constituye la primera demostración de que tanto GH como SS reducen la ghrelina plasmática por una acción inhibitoria directamente sobre el estómago, mientras IGF-1 y GHRH no producen ningún efecto en este modelo. También se ha probado que la secreción gástrica de ghrelina está regulada por la edad, esteroides sexuales y modificaciones en la dieta de forma independiente a los niveles circulantes y expresión de ARNm de este péptido. Por primera vez se muestra en el presente estudio que los niveles de testosterona en ratas macho así como el tamaño del útero en crías hembra disminuyen con el retraso del destete. Por otra parte los niveles de ARNm de GOAT están regulados fuertemente por la edad y el destete. En este nuevo modelo la regulación de los niveles de ghrelina plasmática por los estímulos sensoriales relacionados con la ingesta de alimentos es mediada por el tejido gástrico, teniendo en cuenta que cambios en el estado nutricional afectan a la secreción de ghrelina antes que a la expresión o a la ghrelina circulante y estos cambios son mediados por el nervio vago

CONCLUSIONES

CONCLUSIONES:

a) Se ha validado un nuevo modelo de explantes de tejido gástrico simple y reproducible que permite evaluar directamente la secreción tisular de ghrelina sin interferencias de otros tejidos o del aclaramiento metabólico.

b) La GH y la SS inhiben la secreción gástrica de ghrelina actuando directamente a nivel gástrico. IGF-1 y GHRH no tienen actividad en este modelo.

c) Existe una regulación género y edad dependiente de la secreción gástrica de ghrelina y de los niveles de ARNm de GOAT de alta relevancia.

d) La lactancia modula la secreción gástrica de ghrelina y los niveles de ARNm de GOAT.

e) Los estrógenos actúan directamente sobre el tejido gástrico disminuyendo la secreción gástrica de ghrelina mientras que la testosterona no presenta acciones evidentes en este modelo.

f) Tanto el ayuno como la realimentación se traducen en un ajuste de larga duración en la secreción gástrica de ghrelina, que aumenta y disminuye respectivamente.

g) El SNE que regula la secreción de ghrelina opera funcionalmente a través de un mecanismo de *memoria* de duración horaria.

h) La secreción gástrica de ghrelina está completamente subordinada a las órdenes del SNC. Una falsa alimentación contrarresta el efecto que ejerce el ayuno sobre la misma operando mediante señales que se transmiten por el nervio vago.

ANEXO

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RAPID COMMUNICATION

Central obestatin administration does not modify either spontaneous or ghrelin-induced food intake in rats

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ABSTRACT. The isolation of ghrelin unveiled a new system implicated in food intake regulation. The recently isolated hormone obestatin derives from the same precursor of ghrelin and seems to perform opposite actions. It could be part of a dual system connecting gut and brain to regulate energy homeostasis. The ability of intracerebroventricular administration of obestatin to modify food intake was evaluated. Obestatin had no effect on spontaneous food intake in both *ad libitum* and food restricted rats. The obestatin injection was not able to antagonize the ghrelin-stimulated increase in food intake either. In conclusion, the present work does not support a role for obestatin on the regulation of food intake in any model studied.
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INTRODUCTION

Ghrelin is mainly produced from the stomach and intestinal area (1). In addition to its powerful actions on GH regulation, the main role of ghrelin could be the regulation of food intake and energy homeostasis. In rodents and humans, ghrelin powerfully stimulates food intake when administered, either centrally or peripherally (2-4). *In vivo* ghrelin administration to *ad libitum* fed rats leads to a clear-cut increase in Agouti-related peptide (AgRP) and neuropeptide Y (NPY) mRNA contents in the medial arcuate nucleus, which indicates that AgRP/NPY neurons are the primary targets of ghrelin orexigenic actions at the hypothalamus (5). Human studies have found a preprandial rise and a post-prandial decline in plasma ghrelin levels, suggesting that ghrelin plays a physiological role in hunger and meal initiation, and studies in rodents have shown elevations in ghrelin levels with acute and chronic food deprivation (2, 6).

A 23 amino acid new hormone, named obestatin, has been recently isolated from the stomach. Curiously, obestatin comes by alternative splicing from preproghrelin, the ghrelin precursor (7). This peptide is highly expressed in the central nervous system and activates the GPR39 receptor. Zhang et al. (7) found a very low expression of GPR39 receptor at pituitary and hypothalamus, but recent published data did not show any hypothalamic expression by *in situ* hybridization (8). It has been reported that obestatin effects are the opposite of those exerted by ghrelin and that central (icv) and peripheral (iv) obestatin administration decreases food intake in mice and rats, decreasing body weight gain (2). On the other hand, leptin serum levels are not affected after obestatin treatment, suggesting that the decrease in body weight is not mediated by changes in adipocyte signalling (7). Obestatin, at gastrointestinal level, decelerates gastric emptying and decreases contractility in mice, and circulating obestatin levels are not affected by fasting or food intake conditions (7). Obestatin, a ghrelin-related peptide, could be part of a new system, implicated in the regulation of food intake and energy homeostasis.

In the present work, the ability of obestatin to modify either spontaneous or ghrelin-mediated food intake was evaluated in rats, in order to gain insights into its actions.

Key-words: Obestatin, ghrelin, food-intake, energy homeostasis, appetite.

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MATERIALS AND METHODS

Adult male rats (10-12 weeks old) were housed at 23 C under a 12 h light-dark cycle. Animal experiments were conducted in accordance with the standards approved by the Animal Committee at the University of

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Santiago de Compostela. Rat obestatin was purchased from Peptides International, Inc (Louisville, KY, US) and human ghrelin was obtained from Global Peptides (Fort Collins, CO, US).

Rats were anesthetized by ip injection of ketamine-xylazine (50 mg/kg) and a chronic icv cannula was implanted as previously described (9, 10). The amount of food daily ingested by each rat was measured the days previous to the experiment. One group of rats had available food *ad libitum* (fed rats). The second group was food-deprived for 12 h before the experiment (nocturnal fasted rats), and the third group was 50%-deprived for 12 h before the experiment (restricted rats). Each of the 3 groups was divided in four different icv treatments (no. 8-10): human ghrelin (6 µg/rat), vehicle (saline, control rats), rat obestatin (5 µg/rat) and rat obestatin (5 µg/rat) plus human ghrelin (6 µg/rat). Total food intake, measured from time 0, was assessed at 1, 2, 4 and 6 h post-injection.

Statistical analysis was performed comparing groups by the non-parametric Mann-Whitney test.

RESULTS

As expected, during the first hour of observation the ingested food was superior in the nocturnal fasted rats to *ad libitum* rats, since the latter were satiated. The food intake in the 50%-deprived was intermediate. No changes in spontaneous food intake between obestatin treated rats and control rats were observed, in any of the three groups studied (Fig. 1). In *ad libitum* animals the same stimulatory effect of ghrelin was found at all times tested. Ghrelin treatment showed the expected orexigenic effect, at 1 h post-injection, in *ad libitum* (ghrelin: 2.44±0.7 g *vs* control: 0.34±0.22* g, *$p<0.05$) and in nocturnal fasted animals (ghrelin: 7.15±0.39 g *vs* control: 5.87±0.35* g, *$p<0.05$). In the 50%-restricted rats the ghrelin effect was significant at 6 h post-injection. In the three experimental models, obestatin administration did not modify ghrelin-mediated food intake (Fig. 1).

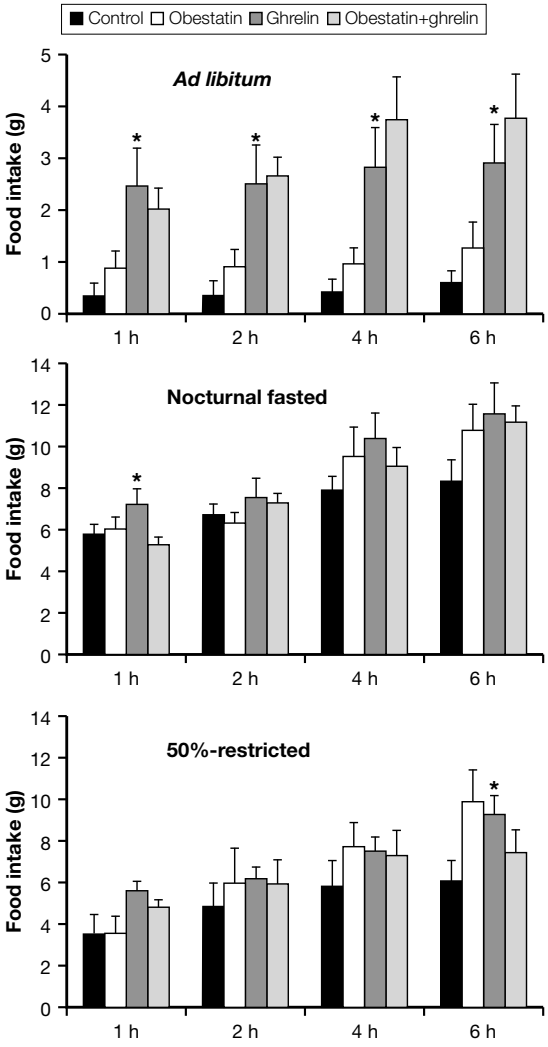

Fig. 1 - *Food intake (g) measured hourly after intracerebroventicular (icv) treatment in male rats. Results are expressed as mean±SE; no. 10-20.* *p*<0.05* vs *control. A)* Ad libitum *fed rats. B) Nocturnal fasted rats. C) 50%-restricted rats.*

DISCUSSION

In the present work, obestatin was unable to modify either fast-induced or ghrelin-mediated food intake, seriously challenging the role of ghrelin antagonist proposed for this new peptide. Central injection of ghrelin increased food intake in all the models used, the effect being less important in nocturnal fasted and 50%-restricted rats than in *ad libitum* animals. This could be explained because in fed animals, circulating ghrelin levels are low (6), whereas in fasted rats, circulating ghrelin levels are elevated. It is then possible that in food deprivation or restricted food conditions, the circulating ghrelin levels were so elevated that the exogenous ghrelin effect was at least partially masked. It has been previously reported that obestatin circulating levels were not influenced by food intake (7), and that these levels remain unaltered in animals subjected to food restriction or fasting, and that could explain why obestatin did not modify food intake at any time in the three models. The results here reported are opposite to those published by Zhang et al. (7), as they found that the obestatin icv treatment in mice produces a severe decrease in food intake. It should be taken into account that the animal species is different, although the dose of obestatin here used in rats (9 nmol/kg of body weight) is equivalent to that used in mice (8 nmol/kg of

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body weight). Another point of difference is the source of the injected obestatin as in this work rat obestatin was used while human obestatin was used in mice.

Also, contrarily to Zhang's report (7), the acute administration of rat obestatin in rats was not able to antagonize the orexigenic effect of ghrelin. They had shown that chronic injection of obestatin produced a decrease in body weight and was able to block the increase in body weight induced by chronic ghrelin administration in rats. The present results may indicate that the decrease in body weight, induced by chronic obestatin, is not a consequence of a decrease in food intake. A plausible explanation could be that this ghrelin-related peptide has *in vivo* powerful effects at gastrointestinal level, suppressing gastric emptying activity, and *in vitro* decreasing the contractile activity of jejunum muscle, thus antagonizing the ghrelin effect. These facts may help to understand why rats under obestatin injection three times/day, for several days lose weight (7), although appetites were not affected, accordingly with the present work.

The existence of the obestatin receptor, GPR39, was reported in the central nervous system but not in the hypothalamus (8). These data are not completely concordant to those published describing GPR39 receptor expression in the hypothalamus, although at very low levels (7). The hypothalamus is one of the most important centers implicated in appetite regulation, and our group has shown that the orexigenic effect of ghrelin administration was centrally mediated by hypothalamic neuropeptides implicated in appetite, such as NPY and AgRP (5). The absence of the obestatin receptor at hypothalamic level could explain the lack of effect of central obestatin in food intake here reported.

In conclusion, it was found that acute central obestatin administration to rats did not affect spontaneous food intake and, also, it was not able to revert the orexigenic effect of central ghrelin administration. Future experiments are needed in order to elucidate the precise role of obestatin on food intake and energy homeostasis.

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