RAPID COMMUNICATION

Preproghrelin expression is a key target for insulin action on adipogenesis

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Abstract

This study aimed to investigate the role of preproghrelin-derived peptides in adipogenesis. Immunocytochemical analysis of 3T3-L1 adipocyte cells showed stronger preproghrelin expression compared with that observed in 3T3-L1 preadipocyte cells. Insulin promoted this expression throughout adipogenesis identifying mTORC1 as a critical downstream substrate for this profile. The role of preproghrelin-derived peptides on the differentiation process was supported by preproghrelin knockdown experiments, which revealed its contribution to adipogenesis. Neutralization of endogenous *O*-acyl ghrelin (acylated ghrelin), unacylated ghrelin, and obestatin by specific antibodies supported their adipogenic potential. Furthermore, a parallel increase in the expression of ghrelin-associated enzymatic machinery, prohormone convertase 1/3 (PC1/3) and membrane-bound *O*-acyltransferase 4 (MBOAT4), was dependent on the expression of preproghrelin in the course of insulin-induced adipogenesis. The coexpression of preproghrelin system and their receptors, GHSR1a and GPR39, during adipogenesis supports an autocrine/paracrine role for these peptides. Preproghrelin, PC1/3, and MBOAT4 exhibited dissimilar expression depending on the white fat depot, revealing their regulation in a positive energy balance situation in mice. The results underscore a key role for preproghrelin-derived peptides on adipogenesis through an autocrine/paracrine mechanism.

Journal of Endocrinology (2011) **210,** R1–R7

Introduction

The concept of adipose tissue as an endocrine organ redefined the understanding of the mechanisms by which adipocytes affect whole-body energy homeostasis (Scherer *et al.* 2006, Trujillo *et al.* 2006). Adipose cells not only serve as inert storage compartments for triglycerides but also synthesize and secrete a variety of peptide and nonpeptide factors. These factors allow communication among the adipose tissue with other organs as well as inside the adipose tissue. This cross talk coordinates a variety of biological processes including energy metabolism, neuroendocrine function, and immune function (Scherer *et al.* 2006, Virtue & Vidal-Puig 2010). Added to this capacity, adipose tissue expresses a broad spectrum of receptors that allow it to respond to signals from hormone systems as well as from the central nervous system. Although regulation of adipocyte homeostasis by endocrine factors has been extensively studied, the local regulation of lipid accumulation and adipogenesis by auto/paracrine factors remains relatively unstudied. Observations that preproghrelin expression increased during adipogenesis (Gurriaran-Rodriguez *et al.* 2010) prompted us to examine the possible role for this peptide (Castañeda *et al.* 2010) as source of potential auto/paracrine factors, acylated ghrelin (AG), unacylated ghrelin (UAG), and obestatin, in the regulation of adipogenesis.

DOI: 10.1530/JOE-11-0233
0022–0795/11/0210–R1
Online version via http://www.endocrinology-journals.org

Materials and Methods

Peptides and reagents

AG and UAG were obtained from Global Peptide (Fort Collins, CO, USA). Obestatin was obtained from California Peptide Research (Napa, CA, USA). All other chemical reagents were from Sigma.

Cell culture and differentiation induction of 3T3-L1 preadipocytes

3T3-L1 preadipocytes were maintained in DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin. For differentiation assays, group A was treated with 0·5 mM isobutylmethylxanthine (IBMX) and 25 mM dexamethasone (DEX) in DMEM containing 10% FBS for 3 days and then maintained in DMEM supplemented with 10% FBS for 7 days. Group B was treated with 0·5 mM IBMX, 25 mM DEX, and 861 nM insulin in DMEM containing 10% FBS for 3 days and then for 7 days with insulin (172 nM). Differentiation was assessed by staining formalin-fixed adipocytes with Oil Red O (Gurriaran-Rodriguez *et al.* 2010).

Immunoblot analysis

The cells were lysed on ice-cold RIPA buffer (Tris–HCl (pH 7·2), 50 mM; NaCl, 150 mM; EDTA, 1 mM; NP-40, 1% (v/v); Na-deoxycholate, 0·25% (w/v); protease inhibitor cocktail (Sigma); phosphatase inhibitor cocktail (Sigma)), clarified by centrifugation (14 000 ***g*** for 15 min at 4 °C), and the protein concentration was quantified using the QuantiPro BCA Assay Kit (Sigma). Cellular proteins were fractionated by SDS–PAGE before immunoblotting against preproghrelin, membrane-bound *O*-acyltransferase 4 (MBOAT4), prohormone convertase 1/3 (PC1/3), GHSR1a, tubulin, ERK1/2, (Santa Cruz Biotechnology, Santa Cruz, CA, USA), actin or GPR39 (Abcam, Cambridge, UK). Immunoreactive bands were detected by ECL Plus Western Blotting Kit (GE, Pittsburgh, PA, USA). Protein expression was analyzed using ImageJ Software (National Institutes of Health, Bethesda, MD, USA) and normalized for corresponding loading controls (ERK1/2, actin, or tubulin).

Quantitative real-time PCR

Total RNA was extracted using Trizol (Invitrogen) and purified with DNA-free Kit (Applied Biosystems, Foster City, CA, USA). Preproghrelin, *MBOAT4* and *PC1/3* gene expression was quantified after reverse transcription using High-capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed using an ABI PRISM 7300 HT Sequence Detection System with specific Taqman probes (Applied Biosystems). All results were normalized to levels of *ACTB* as a housekeeping gene.

Immunocytochemistry/immunohistochemistry

Cell and white adipose tissue (WAT) samples were processed using standard procedures (Gurriaran-Rodriguez *et al.* 2010). Slides were consecutively incubated with 1) anti-preproghrelin, anti-MBOAT4, or anti-PC1/3 rabbit polyclonal antibody (Santa Cruz Biotechnology) at a dilution of 1:100 in Dako ChemMate antibody diluent (Dako, Glostrup, Denmark); 2) EnVision peroxidase rabbit (Dako, Carpinteria, CA, USA) used as the detection system; 3) 3,3′-diaminobenzidine tetrahydrochloride (Dako Liquid DAB + Substrate-chromogen system). Cell and tissue sections were faintly counterstained with Harris' hematoxylin.

Small-interfering RNA silencing of gene expression

Double-stranded small-interfering RNA (siRNA) duplexes for preproghrelin were as follows (Santa Cruz Biotechnology): 3′-CAGAGAAAGGAAUCCAAGA-5′, 3′-CCUUCGAUGUUGGCAUCAA-5′, 3′-CUCUCCUACCACUUUAAGA-5′. A non-silencing RNA duplex was used as a control for all siRNA experiments. GPR39-targeted siRNAs (Ambion siRNA sequence numbers s89441, s89442, s89443) were selected from Silencer Pre-designed sequences from Applied Biosystems. 3T3-L1 cells were transfected with Lipofectamine 2000 (Invitrogen), prior to induction of adipocyte differentiation.

Animals

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Faculty Animal Committee at the University of Santiago de Compostela and the experiments were performed in agreement with the Rules of Laboratory Animal Care and International Law on Animal Experimentation and all efforts were made to minimize suffering. Adult Swiss male mice (8 weeks old) were housed in air-conditioned rooms (22–24 °C) under a 12 h light:12 h darkness cycle and fed with standard diet (SD, control) or high-fat diet (HFD) for 12 weeks (D12451, Research Diets, Inc., New Brunswick, NJ, USA). Food intake and body weight were measured weekly during the experimental phase. Animals were killed by decapitation between 1000 and 1200 h when they were 20 weeks old. Excised WAT, omental and subcutaneous, were washed with ice-cold Krebs–Ringer–HEPES buffer (HEPES (pH 7·4), 25 mM; NaCl, 125 mM; KCl, 5 mM; $MgSO_4$, 1·2 mM; $CaCl_2$, 2 mM; KH_2PO_4, 2 mM; glucose, 6 mM). Tissue samples were directly lysed in ice-cold RIPA buffer for immunoblot analysis.

Data analysis

All data represent mean ± S.E.M. Statistical analysis was carried out by ANOVA, using ANOVA with Bonferroni *post hoc* test. ⋆,#$P<0·05$.

Results

Expression of preproghrelin, PC1/3, and MBOAT4 throughout adipogenesis in 3T3-L1 cells

Preproghrelin, PC1/3, and MBOAT4 proteins were found to predominate in 3T3-L1 adipocyte cells compared to preadipocyte cells as indicated in Fig. 1A. To ascertain whether insulin determines this pattern of expression, mRNA and protein expression of preproghrelin, PC1/3, and MBOAT4 were analyzed in insulin-induced adipogenesis and compared to that with no insulin treatment (group B: insulin, DEX and IBMX; and group A: DEX, IBMX). Whereas the preproghrelin mRNA showed a maximum at 144 h in group A, insulin-stimulated group showed two peaks of expression at 6 and 144 h being ∼1·7- and 1·5-fold higher compared with the former (Fig. 1B). The *PC1/3* mRNA levels significantly augmented for all times tested in the group A, although this pattern of expression was clearly regulated under insulin treatment in group B with a maximal display at 144 h (∼3·5-fold). The *MBOAT4* mRNA levels increased 24 h after treatment to return to near basal levels over 144 h in group A (Fig. 1B). By contrast, insulin treatment inhibited *MBOAT4* mRNA throughout adipogenesis to return to basal levels in terminal differentiation (Fig. 1B). Results of immunoblot analysis (Fig. 1C) showed that the preproghrelin protein level markedly increased at 6 h (∼4·5-fold) which was sustained along terminal differentiation in group B. A similar temporal profile was observed for PC1/3 protein showing two maximal expression levels concomitant with the maximum of preproghrelin expression (∼2·5- and 2·0-fold at 6 and 144 h respectively), whereas MBOAT4 protein was apparent within 24 h and increased in abundance throughout adipogenesis reaching maximal values in terminal differentiation (∼3·0-fold at 240 h). In marked

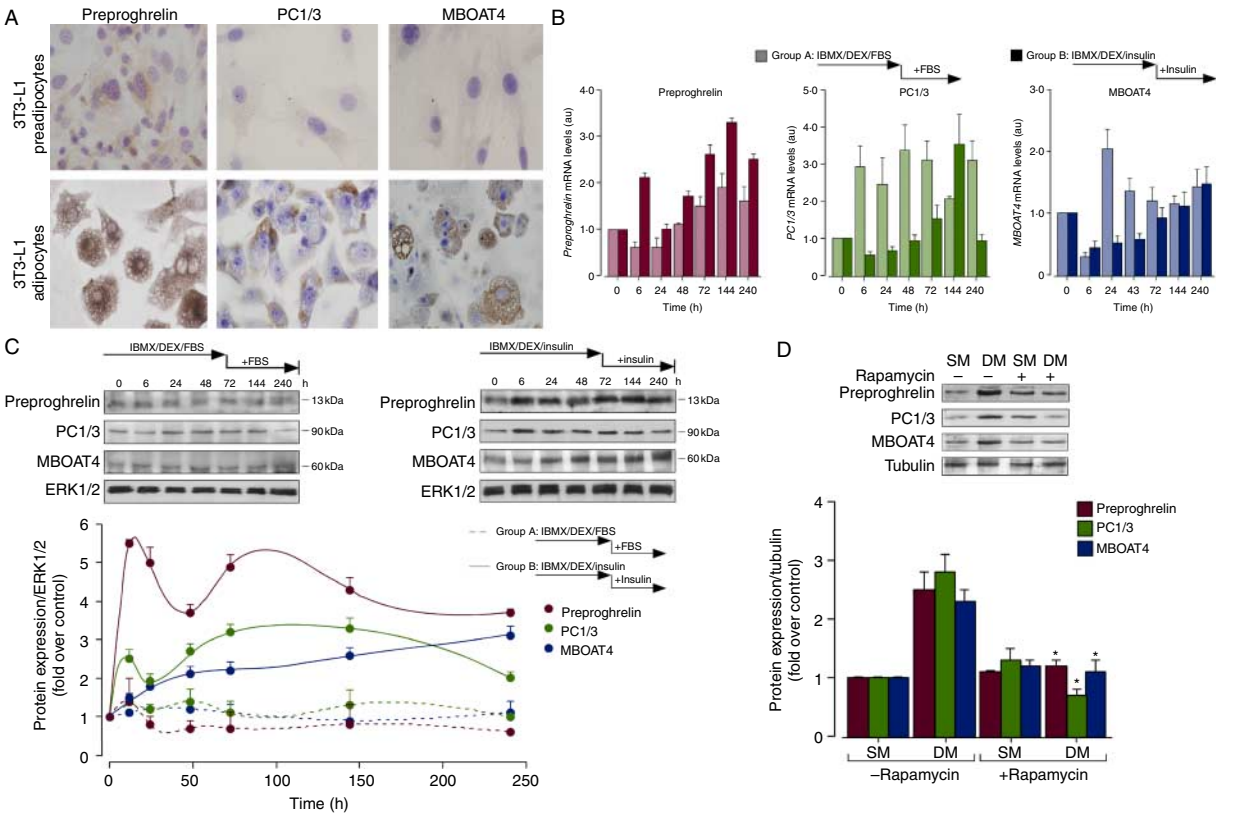

Figure 1 (A) Immunocytochemical detection of preproghrelin, PC1/3, and MBOAT4 in 3T3-L1 preadipocyte and adipocyte cells (objective magnification 40×). (B) Preproghrelin, *PC1/3*, and *MBOAT4* mRNA levels in the course of adipogenesis. Data were normalized to levels of *ACTB* and expressed as mean±S.E.M. ($n=6$). (C) Western blot analysis of preproghrelin, PC1/3, and MBOAT4 in adipogenesis. mRNA or protein levels were examined in group A (3T3-L1 preadipocyte cells maintained in DMEM containing 10% FBS for 7 days after treatment for 3 days with 0·5 mM IBMX, 25 mM DEX) and group B (3T3-L1 preadipocyte cells maintained in DMEM containing 10% FBS+insulin (172 nM) for 7 days after induction for 3 days (0·5 mM IBMX, 25 mM DEX, and 861 nM insulin)). (D) Preproghrelin, PC1/3, and MBOAT4 protein expression in absence or presence of rapamycin. After pretreatment with rapamycin (25 nM, 2 h), 3T3-L1 preadipocyte cells were maintained for 72 h in DMEM containing 10% FBS, 0·5 mM IBMX, 25 mM DEX, 861 nM insulin (differentiation medium, DM), or DMEM containing 10% FBS (standard medium, SM). For B–D, data are expressed as mean±S.E.M. ($n=3$). *$P<0·05$ versus control values. Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-10-0233.

contrast, the amount of preproghrelin, PC1/3, and MBOAT4 protein was not significantly affected in the absence of insulin treatment (group A). To further explore the mechanism involved in the control of protein translation, the effect of rapamycin treatment (25 nM) was examined 2 h prior to insulin-induced adipocyte differentiation. Inhibition of mTORC1/S6K1 pathway significantly reduced the preproghrelin, MBOAT4, and PC1/3 protein expression at 72 h after insulin-activated adipogenesis (Fig. 1D). Rapamycin treatment did not significantly alter the expression of analyzed proteins in 3T3-L1 preadipocyte-treated controls.

Auto/paracrine role of preproghrelin-derived peptides in adipogenesis in 3T3-L1 cells

To define the auto/paracrine role of preproghrelin-derived peptides in adipogenesis, the effect of preproghrelin siRNA was first examined. Transfection of 3T3-L1 preadipocytes prior to induction of adipogenesis with siRNA designed against preproghrelin reduced target protein expression by 51±2% (Fig. 2A). The effect of preproghrelin deficiency decreased MBOAT4 and PC1/3 with respect to siRNA control (49±3 and 61±3% respectively; Fig. 2A). Oil Red O staining revealed that preproghrelin knockdown significantly reduced insulin-mediated adipogenesis by at least 30±1 and 50±5% for 3T3-L1 preadipocyte cells under treatment with IBMX, DEX, and insulin for 3 days and then maintained in DMEM containing 10% FBS or insulin for 7 days respectively (Fig. 2A). Similar results were obtained by neutralization of endogenous obestatin or UAG/AG using anti-obestatin (5 μg/ml) or anti-ghrelin (5 μg/ml; antibody raised against a peptide mapping within an internal region of ghrelin) antibodies (Ab; Fig. 2B). The differentiation-associated lipid accumulation was significantly reduced by anti-obestatin Ab (25±4%). A larger effect was observed with anti-ghrelin Ab (39±2%). These effects were reverted neutralizing the primary antibody with its respective antigen peptide

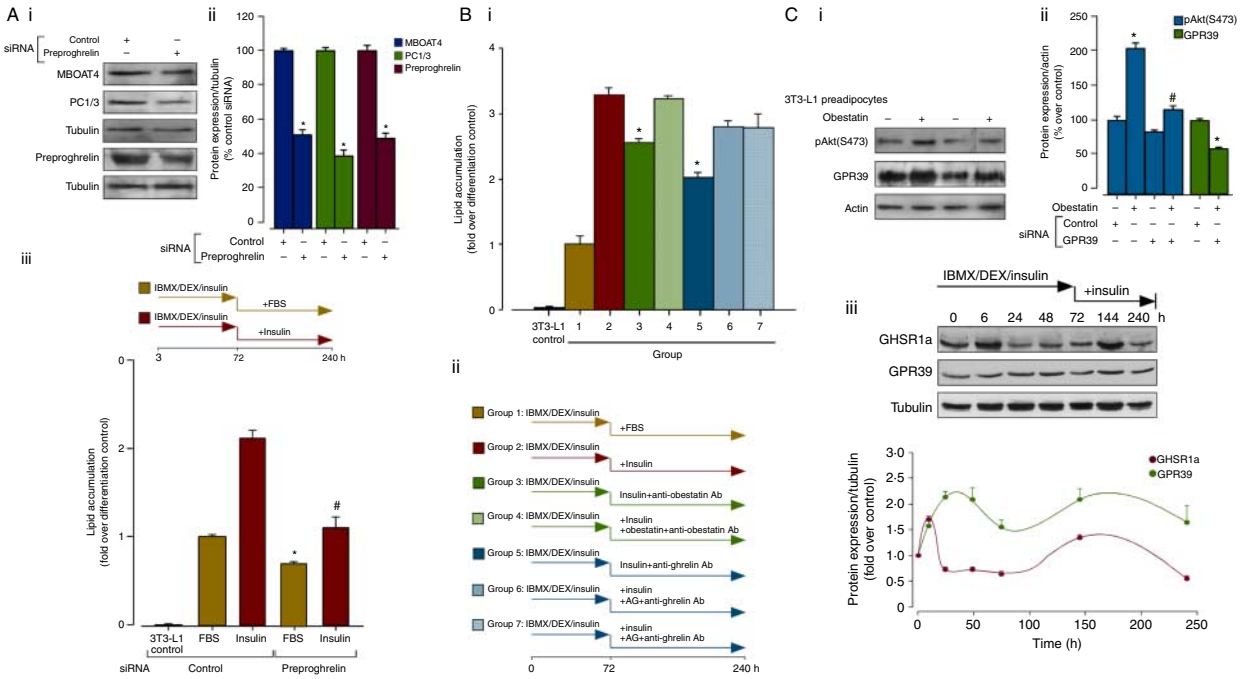

Figure 2 (A) Effect of siRNA depletion of preproghrelin on adipogenesis. 3T3-L1 cells transfected with preproghrelin siRNA prior to induction of adipocyte differentiation (DMEM/10% FBS or DMEM/10% FBS with insulin (172 nM) for 7 days after induction for 3 days under 0·5 mM IBMX, 25 μM DEX, and 861 nM insulin). Equal amounts of protein in each sample were used to assess the expression of MBOAT 4, PC1/3, and preproghrelin (Ai and ii) by western blotting. Lipid droplet accumulation was analyzed by spectrophotometry at 520 nm by Oil red O staining (Aiii panel). (B) Autocrine/paracrine role of obestatin and ghrelin/*O*-acyl ghrelin on adipogenesis. 3T3-L1 preadipocyte cells were maintained in DMEM containing 10% FBS (group 1), 10% FBS+insulin (172 nM; group 2), 10% FBS+insulin (172 nM)+anti-obestatin Ab (5 μg/ml; group 3), 10% FBS+insulin (172 nM)+anti-obestatin Ab (5 μg/ml)+obestatin (5 μg/ml; group 4), 10% FBS+insulin (172 nM)+anti-ghrelin Ab (5 μg/ml; group 5), 10% FBS+insulin (172 nM)+anti-ghrelin Ab (5 μg/ml)+AG (5 μg/ml; group 6) or 10% FBS+insulin (172 nM)+anti-ghrelin Ab (5 μg/ml)+UAG (5 μg/ml; group 7) for 7 days after induction of differentiation by treatment with 0·5 mM IBMX, 25 μM DEX, and 861 nM insulin for 3 days. For A and B, data are mean of three independent experiments. (C) Effect of siRNA depletion of GPR39 on obestatin-activated pAKT (S473; 100 nM, 5 min) in 3T3-L1 preadipocyte cells. Expression of pAKT (S473) and GPR39 were expressed as fold of their levels in control siRNA-transfected cells (Ci and ii panel). Western blot analysis of GHSR1a and GPR39 in adipogenesis (Ciii panel). For A–C, data are mean±S.E.M. of three independent experiments ($^{*,\#}P<0{\cdot}05$ versus control values). Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-10-0233.

(obestatin, AG or UAG, 5 μg/ml). The expression pattern of GHSR1a and GPR39 was further analyzed in group B. The GHSR1a protein levels were concomitant with the maximum of preproghrelin expression (∼1·7- and 1·4-fold at 6 and 144 h respectively; Fig. 2Cii). The GPR39 levels showed a more sustained expression throughout adipogenesis with two maximal levels (∼2·3- and 2·2-fold at 48 and 144 h respectively; Fig. 2Cii). The effect of acute GPR39 deficiency was determined by means of siRNA in 3T3-L1 preadipocyte cells. The constructs decreased GPR39 expression by 44±2% (Fig. 2Cii). Silencing of GPR39 in preadipocyte cells decreased obestatin-activated AKT(S473) with respect to siRNA control (55±4% under treatment with obestatin (100 nM) for 5 min) in accordance with previous results (Gurriaran-Rodriguez *et al.* 2010).

Preproghrelin, PC1/3, and MBOAT4 expression in WAT

Preproghrelin, PC1/3, and MBOAT4 were detected immunohistochemically in omental and subcutaneous WAT obtained from mice under normal chow (control; Fig. 3A). Immunoblot analysis comparing WAT obtained from mice under normal chow and HFD showed that preproghrelin expression was enhanced in subcutaneous WAT of HFD mice relative to WAT of control mice (∼0·5-fold), although its expression was not altered in omental WAT of HFD mice (Fig. 3B). PC1/3 levels were enhanced in both omental and subcutaneous WAT of HFD mice (∼1·5- and 0·6-fold respectively). MBOAT4 levels were also augmented in both omental and subcutaneous WAT of HFD mice (∼0·8- and 0·4-fold respectively; Fig. 3B).

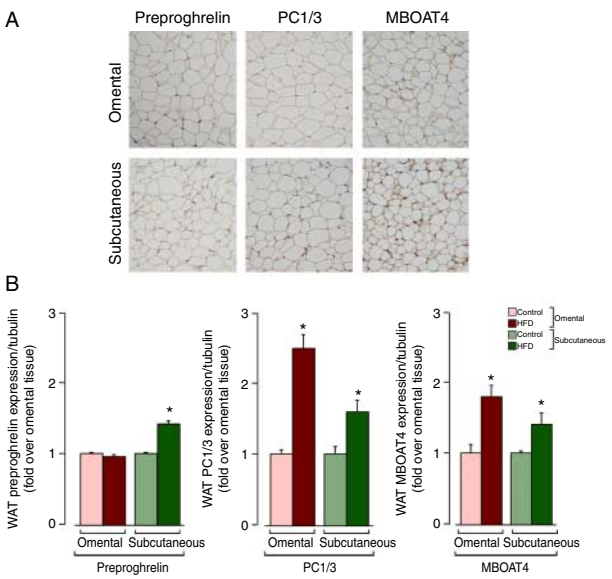

Figure 3 (A) Immunohistochemical analysis of preproghrelin, PC1/3, and MBOAT4 in omental and subcutaneous WAT obtained from control mice (objective magnification 20×). (B) Immunoblot analysis of preproghrelin, PC1/3, and MBOAT4 in omental and subcutaneous WAT obtained from control and HFD mice ($n=10$ per group). Data are expressed as mean±S.E.M. $^*P<0·05$ versus control values. Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-10-0233.

Discussion

In this study, we have demonstrated that preproghrelin plays a critical role in the process of adipogenesis. We have shown that insulin-induced adipocyte conversion is impaired in the absence of preproghrelin. Furthermore, neutralization of preproghrelin-derived peptides displays defective accumulation of lipid droplets. Thus, preproghrelin is a key source of autocrine peptides involved in adipocyte differentiation. Furthermore, we have identified that preproghrelin and the enzymatic machinery associated to the processing of this peptide into UAG and AG are upregulated during adipose conversion. This enzymatic system includes PC1/3, which cleaves after arginine-28 of proghrelin leading to the production of the mature 28 amino acid peptide (Zhu *et al.* 2006) and MBOAT4, a polytopic membrane-bound enzyme that attaches the octanoyl residue to serine-3 in AG (Gutierrez *et al.* 2008, Yang *et al.* 2008). The changes in mRNA and protein expression were further associated with insulin action, although transcription and translation of these targets showed different regulatory systems. In addition, acute inhibition of mTORC1 activity in the presence of rapamycin led to decreased levels of these proteins. These data suggest the involvement of the mTORC1/S6K1 pathway in the regulation of preproghrelin, PC1/3, and MBOAT4 translation during the early steps of adipogenesis. This point disagree with the findings that inhibition of mTORC1 stimulates gastric and pancreatic preproghrelin expression (Xu *et al.* 2009, An *et al.* 2010). Indeed, it is likely that the mTORC1/S6K1 pathway and preproghrelin interact in a tissue-specific manner. In addition, the type of pharmacological treatment, chronic versus acute, might also explain this paradigm. In this sense, it is important to note that chronic rapamycin treatment can inhibit both mTORC1 and mTORC2 activities (Sarbassov *et al.* 2006, Laplante & Sabatini 2009).

The potential role of preproghrelin as source of auto/paracrine peptides has remained unstudied in adipose tissue, possibly as a result of the perception that preproghrelin is not substantially expressed in this tissue. As reported here and elsewhere (Gnanapavan *et al.* 2002, Knerr *et al.* 2006), however, adipose tissue expresses relatively high levels of preproghrelin. This peptide undergoes stepwise processing to produce UAG, AG, and obestatin (Zhu *et al.* 2006). AG is widely known for its role in adipogenesis and lipid storage in WAT in both rodents (Wells 2009) and humans (Rodriguez *et al.* 2009). It was recently reported that obestatin also promotes adipogenesis, being a regulator of adipocyte metabolism (Gurriaran-Rodriguez *et al.* 2010). By contrast, the role of UAG in adipogenesis is not clear, because there are

reports showing that UAG suppressed adipogenesis and lipid accumulation (Zhang *et al.* 2008, Delhanty *et al.* 2010), whereas others showed that UAG increased adipogenesis and lipid accumulation in both rodents (Thompson *et al.* 2004) and human adipocytes (Rodriguez *et al.* 2009). In this study, we found that the net effect of these secreted preproghrelin-derived peptides favors adipocyte conversion. This fact appears to be a consequence of the AKT activation by both AG and obestatin in adipose tissue (Lodeiro *et al.* 2009, Gurriaran-Rodriguez *et al.* 2010). There is strong evidence, including results obtained from genetic mouse models, supporting the concept that AKT is a key node for regulation of the transcriptional program required for adipogenesis (Cho *et al.* 2001, Garofalo *et al.* 2003, Xu *et al.* 2004, Manning & Cantley 2007, Berggreen *et al.* 2009, Zhang *et al.* 2009). Interestingly, knockdown experiments by preproghrelin siRNA displayed defective adipocyte differentiation. We propose that insulin activates preproghrelin expression and consequent secretion of preproghrelin-derived peptides, which intensify insulin signaling to AKT in an autocrine manner in preadipocyte cells. The coexpression of GHSR1a and GPR39 supports this autocrine function. These peptides further determine the progressive expression of PC1/3 and MBOAT4 related to adipocyte differentiation defining the secretion rate between AG and UAG. Through a paracrine action, preproghrelin-derived peptides impact on adipocytes leading to AKT signaling favoring regulation processes such as hypertrophy. Thus, it is likely that the insulin effect is mediated by expression and secretion of these peptides on the late stage of adipogenesis.

In agreement with our data obtained *in vitro*, where the expression of preproghrelin, PC1/3, and MBOAT4 was increased during adipogenesis, we found that, in both omental and subcutaneous fat of mice fed on HFD, the expression of these factors is higher than in the WAT of mice fed on SD. The only exception was the expression of preproghrelin in omental fat that remained unchanged between SD and HFD mice, indicating that preproghrelin is regulated in a fat depot-specific manner. Overall, our findings showing higher levels of preproghrelin-related factors in the WAT of animals where adipogenesis is stimulated suggest that preproghrelin, PC1/3, and MBOAT4 might play an important role in the accumulation of lipids during diet-induced obesity.

In conclusion, we have shown that insulin triggers the expression of preproghrelin, and have demonstrated that preproghrelin is a critical target for insulin-induced adipogenesis. The mechanisms of regulation of these targets by insulin await further investigation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by grants from Instituto de Salud Carlos III (Ministerio de Ciencia e Innovación, Spain; PS09/02202, PS09/02075), Ministerio de Ciencia e Innovación (SAF2010-20451), and Xunta de Galicia (INCITE09PXIB918374PR). The work of J P C, Y P, and L M S are funded by the Instituto de Salud Carlos III and SERGAS through a research staff stabilization contract. A B C is funded by the Instituto de Salud Carlos III and SERGAS through a research staff contract Sara Borrell. U G-R is funded by Xunta de Galicia through a research staff contract Isabel Barreto.

Author contribution statement

Y P and J P C designed research. U G R, O A M, D B, C S M, and M A D performed cellular and animal research. C S M and R G performed immunohistochemical assays. U G R, O A M, A B C, L M S, R N, R G, Y P, F F C, and J P C analyzed data. J P C wrote the paper.

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Received in final form 8 June 2011
Accepted 20 June 2011
Made available online as an Accepted Preprint
20 June 2011