RAPID COMMUNICATION

Preproghrelin expression is a key target for insulin action on adipogenesis

Uxía Gurriarán-Rodríguez1,2, Omar Al-Massadi1,2, Ana Belén Crujeiras1,2, Carlos S Mosteiro1,2, María Amil-Diz1,2, Daniel Beiroa2,3, Rubén Nogueiras2,3, Luisa María Seoane1,2, Rosalía Gallego4, Yolanda Pazos1,2, Felipe F Casanueva1,2,5 and Jesús P Caminha1,2

1Área de Endocrinología Molecular y Celular, Instituto de Investigación Sanitaria de Santiago (IDIS), Hospital Clínico Universitario de Santiago, Servicio Gallego de Salud (SERGAS), Santiago de Compostela, Spain
2CIBER Fisiopatología de la Obesidad y Nutrición, Santiago de Compostela, Spain
3Departamento de Fisiología, 4Departamento de Ciencias Morfológicas and 5Departamento de Medicina, Universidad de Santiago de Compostela, Santiago de Compostela, Spain

(Correspondence should be addressed to J P Caminha at Laboratorio de Endocrinología Celular (Lab.4), Área de Endocrinología Molecular y Celular, Instituto de Investigación Sanitaria de Santiago (IDIS), Hospital Clínico Universitario de Santiago, Choupana s/n 15706 Santiago de Compostela, Spain; Email: jesus.perez@usc.es; jesus.perez.camina@sergas.es)

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 (Gurriarán-Rodríguez 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.
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 isobutylmethyl-xanthine (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 C), and the protein concentration was quantified using the QuantiPro BCA Assay Kit (Sigma). Cellular proteins were fractionated by SDS–PAGE before immunoblot analysis against preproghrelin, membrane-bound O-acyltransferase 4 (MBOAT4), prohormone convertase 1/3 (PC1/3), GHSR1a, tubulin, ERK1/2, -acyltransferase 4 (MBOAT4), prohor-
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).
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.
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).

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 preproghrelin 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 autocrine and 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

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.
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 preadipocytes. 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.

References


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


Downloaded from Bioscientifica.com at 09/09/2019 06:44:16AM
via free access


Scherer PE 2006 Adipose tissue: from lipid storage compartment to endocrine organ. Diabetes 55 1537–1545. (doi:10.2337/db06-0263)


Xu J & Liao K 2004 Protein kinase B/AKT1 plays a pivotal role in insulin-like growth factor-1 receptor signaling induced 3T3-L1 adipocyte differentiation. Journal of Biological Chemistry 279 35914–35922. (doi:10.1074/jbc.M402297200)


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