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RESEARCH PAPER

Effects of glucagon-like peptide-1 on the differentiation and metabolism of human adipocytes

Correspondence Dr Francisco J. Tinahones, Dr Maria M. Malagón and Dr Rajaa El Bekay, Laboratory of Biomedical Research, Virgen de la Victoria Clinical University Hospital, Campus de Teatinos s/n, Málaga, 29010, Spain. E-mail: fjtinahones@hotmail.com; bc1mapom@uco.es; elbekay@gmail.com

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Rajaa El Bekay[1,2]*, Leticia Coín-Aragüez[1,2]*, Diego Fernández-García[1,3], Wilfredo Oliva-Olivera[1,2], Rosa Bernal-López[1,2], Mercedes Clemente-Postigo[1,2], Javier Delgado-Lista[4], Alberto Diaz-Ruiz[5], Rocío Guzman-Ruiz[5], Rafael Vázquez-Martínez[5], Said Lhamyani[1,2], María Mar Roca-Rodríguez[1,2], Sonia Fernandez Veledo[6], Joan Vendrell[6], María M. Malagón[5] and Francisco José Tinahones[1,3]

[1]CIBER Pathophysiology of Obesity and Nutrition CB06/03, Carlos III Health Institute, Malaga, Spain, [2]Laboratory of Biomedical Research, Virgen de la Victoria Clinical University Hospital, Málaga, Spain, [3]Endocrinology Service, Virgen de la Victoria Clinical University Hospital, Malaga, Spain, [4]Lipids and Atherosclerosis Unit, Department of Medicine, IMIBIC/Reina Sofia University Hospital/University of Córdoba, Córdoba, Spain, [5]Department of Cell Biology, Physiology, and Immunology, IMIBIC/Reina Sofia University Hospital/Universidad de Cordoba, CIBERobn, Córdoba, Spain, and [6]CIBERDEM, Joan XXIII University Hospital, Pere Virgili Institute, Tarragona, Spain

*L.C.A. and R.E.B. contributed equally to this manuscript.

BACKGROUND AND PURPOSE

Glucagon-like peptide-1 (GLP-1) analogues improve glycaemic control in type 2 diabetic (T2D) patients and cause weight loss in obese subjects by as yet unknown mechanisms. We recently demonstrated that the GLP-1 receptor, which is present in adipocytes and the stromal vascular fraction of human adipose tissue (AT), is up-regulated in AT of insulin-resistant morbidly obese subjects compared with healthy lean subjects. The aim of this study was to explore the effects of *in vitro* and *in vivo* administration of GLP-1 and its analogues on AT and adipocyte functions from T2D morbidly obese subjects.

EXPERIMENTAL APPROACH

We analysed the effects of GLP-1 on human AT and isolated adipocytes *in vitro* and the effects of GLP-1 mimetics on AT of morbidly obese T2D subjects *in vivo*.

KEY RESULTS

GLP-1 down-regulated the expression of lipogenic genes when administered during *in vitro* differentiation of human adipocytes from morbidly obese patients. GLP-1 also decreased the expression of adipogenic/lipogenic genes in AT explants and mature adipocytes, while increasing that of lipolytic markers and adiponectin. In 3T3-L1 adipocytes, GLP-1 decreased free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). GLP-1-induced responses were only partially blocked by GLP-1 receptor antagonist exendin (9–39). Moreover, administration of exenatide or liraglutide reduced adipogenic and inflammatory marker mRNA in AT of T2D obese subjects.

CONCLUSIONS AND IMPLICATIONS

Our data suggest that the beneficial effects of GLP-1 are associated with changes in the adipogenic potential and ability of AT to expand, via activation of the canonical GLP-1 receptor and an additional, as yet unknown, receptor.

Abbreviations

ADRP, adipocyte differentiation-related protein; AT, adipose tissue; ATGL, adipose triglyceride lipase; BMI, body mass index; FABP4, fatty acid binding protein 4; FASN, fatty acid synthase; GLP-1, glucagon-like peptide-1; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; MO, morbidly obese; SAT, subcutaneous AT; SREBP1, sterol regulatory element-binding transcription factor 1; T2D, type 2 diabetic; VAT, visceral AT

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Tables of Links

TARGETS	
Other protein targets[a]	**Enzymes**[d]
FABP4	Acetyl CoA carboxylase
TNF-α	Adenylate cyclase
GPCRs[b]	Akt (PKB)
GLP-1 receptor	ERK1
Nuclear hormone receptors[c]	ERK2
PPARγ	FASN
Transporters[e]	Hormone sensitive lipase (HSL)
GLUT4	PKA

LIGANDS	
Adiponectin	IBMX
cAMP	IL-6
Dexamethasone	Indomethacin
Exenatide (exendin-4)	Insulin
Exendin (9-39)	Liraglutide
GLP-1	Metformin

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c,d,e]Alexander *et al.*, 2015a,b,c,d,e).

Introduction

Glucagon-like peptide-1 (GLP-1) is a potent physiological regulator of blood glucose that stimulates insulin secretion, which underlies its use for the treatment of type 2 diabetes mellitus (T2D) (Nathan *et al.*, 1992; Nauck *et al.*, 1997). Circulating levels of GLP-1 rise in association with the postprandial increase in blood glucose concentration. This peptide hormone binds to a specific GPCR, the GLP-1 receptor, which is expressed in several cell types, including pancreatic beta cells (Baggio and Drucker, 2007; McIntosh *et al.*, 2009). GLP-1 enhances glucose-induced insulin synthesis and secretion upon binding to GLP-1 receptors in beta cells, thus increasing beta cell sensitivity to glucose (Drucker, 2006; Yusta *et al.*, 2006). This effect, referred to as the incretin effect, plays a critical role in the maintenance of systemic glucose homeostasis (Kreyman *et al.*, 1987). Furthermore, GLP-1 also improves alpha-cell glucose sensing in patients with T2D and controls food intake by increasing satiety in these patients (Flint *et al.*, 1998; Gutzwiller *et al.*, 1999; Dunning *et al.*, 2005).

The effect of GLP-1 on adipose tissue (AT) has been poorly studied, and few results have been published. Studies performed in isolated rat and human adipocytes indicate that GLP-1 may activate both lipogenic and lipolytic mechanisms (Ruiz-Grande *et al.*, 1992; Perea *et al.*, 1997; Villanueva-Peñacarrillo *et al.*, 2001a; Azuma *et al.*, 2008; Majumdar and Weber, 2010). Recent studies in murine 3T3-L1 cells indicate that GLP-1 promotes pre-adipocyte differentiation (Challa *et al.*, 2012; Yang *et al.*, 2013). The effects of GLP-1 in fat, as in the liver or muscle, seem to be exerted through a GLP-1-specific receptor that is structurally and/or functionally distinct from that expressed in the pancreas (Montrose-Rafizadeh *et al.*, 1997; Villanueva-Peñacarrillo *et al.*, 2001b). Recently, we obtained evidence demonstrating the presence of the GLP-1 receptor in AT and showed that its mRNA and protein levels are increased in visceral AT (VAT) from morbidly obese (MO) patients with a high degree of insulin resistance (IR) (Montrose-Rafizadeh *et al.*, 1997; Vendrell *et al.*, 2011) and that, in 3T3-L1, GLP-1 enhances lipolysis in a receptor-dependent manner (Vendrell *et al.*, 2011). Taken together these data support the view that the GLP-1/GLP-1 receptor system in AT may play a role in improving insulin sensitivity in obese patients (Vendrell *et al.*, 2011).

Adipocyte differentiation is a tightly regulated process orchestrated by the temporal expression of key transcription factors, such as PPARγ, adipocyte differentiation-related protein (ADRP) and fatty acid binding protein 4 (FABP4), which result in cytoskeletal changes as well as in the induction of key genes involved in lipogenesis [lipoprotein lipase (LPL), fatty acid synthase (FASN), sterol regulatory element-binding transcription factor 1 (SREBP1) and forkhead box protein O1(FOXO1)] and lipolysis, such as α-2-glycoprotein 1, zinc binding, a potent inducer of lipolysis (Russell *et al.*, 2004); adipose triglyceride lipase (ATGL); hormone-sensitive lipase (HSL); and perilipin (Hunt *et al.*, 1986; Gregoire *et al.*, 1998; Rosen *et al.*, 1999; Gao *et al.*, 2000; Large *et al.*, 2004; Russell *et al.*, 2004; Farmer, 2006; Kolditz and Langin, 2010). These last two genes are known to be primarily regulated via activation of cAMP/PKA (Kolditz and Langin, 2010). The relative balance of these processes in mature adipocytes is crucial in determining adipocyte size and hence fat mass.

To gain further insights into the effects and mechanisms of GLP-1 action on AT, herein, we explored the effects of *in vitro* and *in vivo* administration of GLP-1 and its analogues on AT and adipocyte functions from T2D MO subjects.

Methods

Patients and AT collection

All participants gave their informed consent, and the study was reviewed and approved by the Hospital Ethics and Research Committee.

In vitro studies

VAT and subcutaneous AT (SAT) samples were obtained from healthy obese subjects [body mass index (BMI) = 49.09 ± 2.69 kg·m^{-2}] with a low degree of IR [homeostatic model assessment IR (HOMA-IR) < 4] (n = 27) undergoing elective surgery (cholecystectomy, surgery for abdominal hernia) at the Virgen de la Victoria Hospital (Malaga, Spain). Exclusion criteria were dyslipidaemia, arterial hypertension, cardiovascular diseases and drug treatment.

Transversal pilot study: treatment of T2D MO subjects with the GLP-1 analogue exenatide

MO subjects (BMI = 48.09 ± 2.69) (n = 18) with T2D who were under treatment with metformin were separated into two groups, receiving (n = 9) or not (n = 9) a daily administration of exenatide (10 μg twice a day) for a duration of 6 months. At the end of the treatment, patients underwent bariatric surgery, and SAT samples were collected at the site of surgical incision from the abdominal wall, while VAT samples were obtained from the omentum. Exclusion criteria were glycated haemoglobin Hba1c > 8%, the use of other treatments apart from metformin and exenatide, a history of cardiovascular disease, and kidney, hepatic or cardiac failure.

The anthropometrical and biochemical characteristics of the patients included in this study are shown in Table 2.

Prospective pilot study: treatment of T2D MO subjects with the GLP-1 agonist liraglutide

Liraglutide (1.2 mg) was administered daily for 1 month to three T2D patients (BMI > 30 and HA1c > 7.5%) who were under metformin treatment. SAT biopsies were obtained using Tru-Cut® Soft Tissue Biopsy Needles (CareFusion, Waukegan, IL), with local anaesthesia, before and 1 month after treatment.

The anthropometric and biochemical characteristics are shown in Table 3.

Effect of GLP-1 on AT explants

VAT and SAT explants (5 mg) from healthy obese subjects (BMI = 49.09 ± 2.69 kg·m^{-2}) with a low degree of IR (HOMA-IR < 4) were incubated for 30 min in PBS supplemented with 5% BSA (3 mL·g^{-1}) and then in M199-medium, 10% FBS, 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin for 1 h at 37°C. GLP-1 100 nM and/or exendin (9–39) 100 nM were added, and tissue explants were incubated at 37°C for 1, 3 or 6 h for mRNA analysis.

Isolation and expansion of cells derived from the stromal vascular fraction of human VAT and SAT

Isolation and expansion of human cells derived from Stromal Vascular Fraction cells (SVFCs) of AT was carried out using a procedure modified from Moreno-Navarrete *et al.* (2012). Briefly, AT samples were incubated in 0.150% collagenase type I (MMP1) and 1.0% BSA for 70 min at 37°C. SVFC was resuspended in DMEM/F12, 10% FBS, 100 μg·mL^{-1} streptomycin, 100 U·mL^{-1} penicillin, 2 mM L-glutamine and 1 μg·mL^{-1} of amphotericin B. Cells were incubated at 37°C, in 5% CO_2 for 7 days until 90% confluence was reached.

Analysis of the effect of GLP-1 on adipocyte differentiation

SVFCs (20 000 cells cm^{-2}) were differentiated in adipogenic medium (expansion medium supplemented with 0.5 mM IBMX, 1.0 μM dexamethasone, 10 μM insulin and 200 μM indomethacin) in the absence or presence of 10 nM GLP-1 for 1, 3, 6, 9, 12 and 15 days. Treatment was renewed every 2 days.

Effects of GLP-1 and exendin (9–39) on in vitro differentiated human adipocytes

Fourteen-day differentiated adipocytes were exposed to different doses of GLP-1 (10, 100 and 1000 nM) in the presence or absence of the GLP-1 receptor antagonist, exendin (9–39) 100 nM, for 12 h at 37°C. Cells were immediately processed for RNA extraction as described below.

Effect of GLP-1 on human mature adipocytes

Mature adipocytes were obtained by enzymatic digestion of SAT as indicated above. Adipocytes were resuspended in 400 μL DMEM/F12 containing 10 mg·mL^{-1} BSA and then placed in an Eppendorf tube containing 600 μL DMEM/F12 supplemented with BSA. Cells were cultured for 4 h in the absence or presence of 10 or 100 nM GLP-1 at 37°C. Cells were processed for RNA extraction as described below.

Effects of GLP-1 on differentiated 3T3-L1 adipocytes

3T3-L1 cells were differentiated into adipocytes as previously described (Pulido *et al.*, 1999) and processed for $[Ca^{2+}]_i$, measured by microfluorimetry as previously described (Moreno-Navarrete *et al.*, 2012). Briefly, 3T3-L1 cells were cultured in DMEM, 10% FBS, 4 mM glutamine and 1% antibiotic–antimycotic solution. At 100% confluence (day 0), cells were incubated in DMEM containing 10% FBS, 0.5 mM IBMX, 0.25 μM dexamethasone and 10 μg·mL^{-1} insulin for 72 h (day 3). The culture medium was replaced by DMEM with 10% FBS and 10 μg·mL^{-1} insulin for an additional 72 h period (day 6) and was then exchanged for DMEM without insulin until days 9–10.

In vitro experiments with 3T3-L1 were repeated at least three times on different cell preparations, and a minimum of three replicate wells per treatment were tested in each experiment. In another set of experiments, differentiated 3T3-L1 cells were treated with 100 nM GLP-1 to assess the effects of the peptide on the activation of signalling intermediates (after 5 and 30 min of GLP-1 treatment) and on the expression of several lipogenesis and lipolysis markers (after a 24 h treatment with the peptide).

RNA extraction and real-time qPCR

RNA extraction and real-time qPCR were carried out as previously described (Vendrell *et al.*, 2011). Briefly, total RNA was isolated from whole AT samples using the TRIzol® RNA isolation method (Invitrogen, Carlsbad, CA, USA) and subsequently purified with the RNeasy® Lipid kit (Qiagen, Valencia, CA, USA). Total RNA from cell cultures was obtained using the RNA-Stat 60 Reagent (Ams Biotechnology, Abingdon, UK). RT-qPCR reactions were carried out using specific TaqMan® Gene Expression Assays (Applied Biosystems by Thermofisher

Scietific, Spain). During PCR, the Ct values for each amplified product were determined using a threshold value of 0.1. The specific signals were normalized by constitutively expressed cyclophilin signals using the formula $2^{-\Delta\Delta Ct}$. References for TaqMan® probes are presented in Table S1.

Protein extraction and western blot analysis

Protein extraction from 3T3-L1 cells was obtained as described previously (Guzmán-Ruiz *et al.*, 2014). Protein preparations were stored at −80°C until used.

For immunoblotting analysis, 15–30 μg of protein were loaded onto 4–20% precasted SDS-PAGE gels and transferred onto nitrocellulose membranes. Proteins were detected using antibodies against specific proteins. Antibody references are presented in the figure legends. Anti-ß-actin was employed as a loading control. Optical densities of the immunoreactive bands were measured using ImageJ analysis software.

$[Ca^{2+}]_i$ measurements

3T3-L1 cells were processed as previously described (Moreno-Navarrete *et al.*, 2012). Briefly, at day 10 of differentiation, cells were loaded with 2.5 μM Fura-2AM (Molecular Probes, Eugene, OR, USA) and 0.02% Pluronic F127 (Molecular Probes) in phenol red-free DMEM containing 20 mM $NaHCO_3$ (pH 7.4) for 30 min at 37°C. Cells were then sequentially epi-illuminated at 340 and 380 nm for 100 ms every 5 s for 8–10 min, and the fluorescent emission was captured at 505/510 nm before (basal line) and after the addition of 100 nM GLP-1. In another series of coverslips, cells were pre-incubated for 30 min in the presence of the GLP-1 receptor antagonist exendin (9–39) (100 nM) and subsequently exposed to 100 nM GLP-1. Image acquisition was controlled using MetaFluor PC software (Universal Imaging Corp., West Chester, PA, USA), and the fluorescence emission was captured using a back-thinned CCD cooled digital camera (ORCA II BT; Hamamatsu Photonics, Hamamatsu, Japan) running in 1 bit mode. Changes in $[Ca^{2+}]_i$ were recorded as the ratio of the corresponding excitation wavelengths (F340/F380).

Statistical analyses

The statistical analysis was carried out with the SPSS software programme (version 15.0 for Windows; SPSS, Chicago, IL, USA). Statistical comparisons of the densitometric data and the differences between the different treatments used were carried out using Student's *t*-test. Comparisons between normalized mRNA expression levels of different tissues were performed using the ANOVA test and Duncan's *post hoc* test. Levene's test was used to assess the equality and homogeneity of variances. No statistical analysis was performed on data from the prospective pilot study due to the small sample size ($n = 3$). Results are expressed as means ± SEM. For $[Ca^{2+}]_i$ measurements, unpaired *t*-tests were used. Statistical significance level was set at $P < 0.05$. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

Results

Effects of GLP-1 in differentiated 3T3-L1 cells

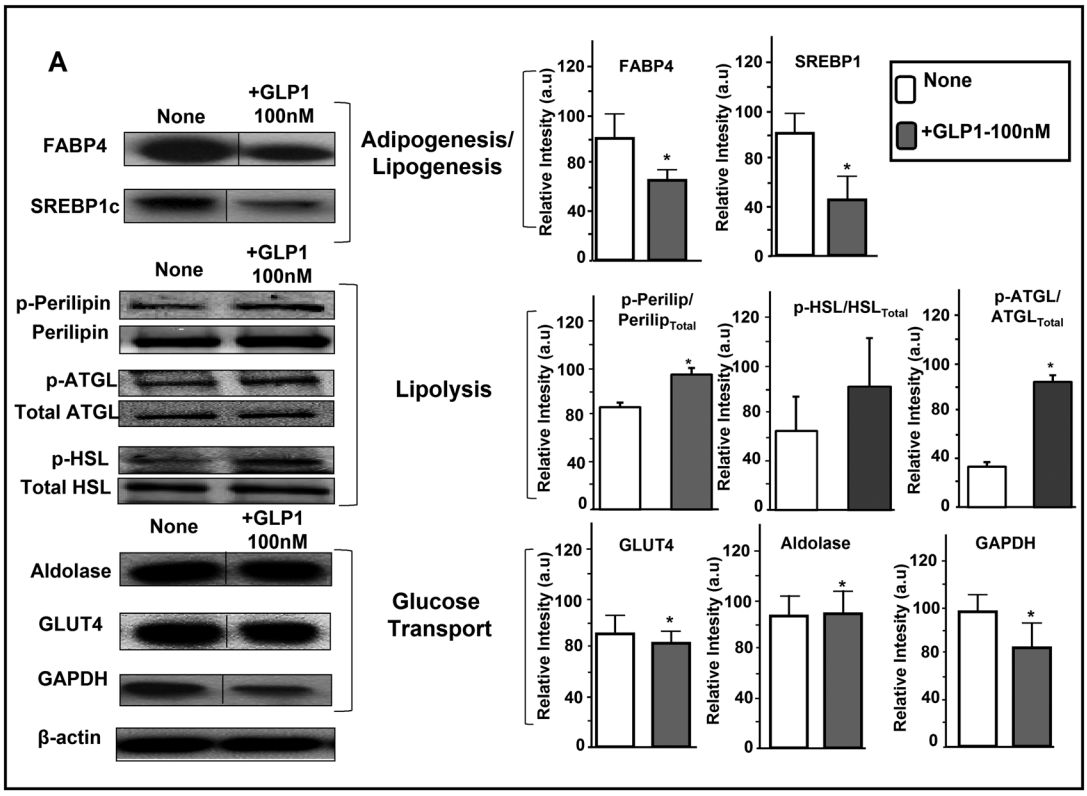
The effects of GLP-1 were studied in the adipocyte murine cell model, 3T3-L1 cells. Specifically, we measured the protein content of selected adipogenic and lipolytic markers in differentiated 3T3-L1 adipocytes after short-term (4 h) or long-term (24 h) exposure to 100 nM GLP-1. No effects were observed at 4 h of treatment (data not shown), while exposure of cells to GLP-1 for 24 h decreased the protein levels of FABP4, SREBP1 and GAPDH, while increasing those of phosphorylated forms of perilipin and of ATGL. GLUT4 and aldolase protein levels remain unchanged after GLP-1 treatment (Figure 1A).

It has been shown that changes in free cytosolic Ca^{2+} concentration in adipocytes affect both lipogenesis and basal and stimulated lipolysis (Xue *et al.*, 1998; Gericke *et al.*, 2009). Therefore, we subsequently investigated the effects of GLP-1 and exendin (9–39) on Ca^{2+}_i dynamics in single, differentiated 3T3-L1 cells following a protocol optimized by ourselves for this cell type (Moreno-Navarrete *et al.*, 2012). Exposure of 3T3-L1 cells to 100 nM GLP-1 induced a significant decrease in $[Ca^{2+}]_i$ in 86.4% of the cells recorded (45 out of 52 cells; $n = 3$ independent experiments) (Figure 1B). Specifically, GLP-1 decreased $[Ca^{2+}]_i$ by 13.3 ± 0.6% as compared with that observed before the administration of the peptide. Maximal $[Ca^{2+}]_i$ inhibition was observed after 17.4 ± 1.5 s of exposure to GLP-1. Interestingly, when cells were pre-incubated with exendin (9–39) (100 nM) prior to GLP-1 exposure, the peptide decreased $[Ca^{2+}]_i$ only in 43.4% of the cells (33 out of 76 cells; $n = 3$ independent experiments) (Figure 1B). In these cells, GLP-1 evoked a 17.5 ± 1.1% decrease in $[Ca^{2+}]_i$, and maximal $[Ca^{2+}]_i$ inhibition was reached later than in cells treated with GLP-1 alone (Figure S1A). In all the experiments, cells in the coverslips displayed significant $[Ca^{2+}]_i$ increases in response to the Ca^{2+} ionophore ionomycin.

Finally, we also investigated the effect of GLP-1 on Akt and ERK, intracellular signalling pathways that have been reported to be activated by GLP-1 in adipocytes and other cell types (Challa *et al.*, 2012; Yang *et al.*, 2013). Neither Akt nor ERK1/2 phosphorylation rates were modified after 5 or 30 min of exposure of differentiated 3T3-L1 cells to GLP-1 (Figure S1B).

Effects of GLP-1 on expression of adipogenic, lipogenic and lipolytic genes in differentiated human adipocytes in vitro

Differentiated human adipocytes from VAT and SAT were incubated in the presence of 10–1000 nM GLP-1 for 12 h. Figure 2A shows that exposure of differentiated human adipocytes obtained from SAT samples to GLP-1 *in vitro* decreased mRNA levels of a variety of genes involved in adipogenesis (*PPARγ* and *FABP4*), lipogenesis (*LPL* and *FASN*) and lipolysis (*ATGL*), as well as *adiponectin* gene expression. In the *in vitro* differentiated VAT adipocytes, GLP-1 caused a significant down-regulation of *adiponectin* mRNA when administered at 100 or 1000 nM, while it increased *AZGP1* mRNA at the highest dose tested (Figure 2A). Other lipogenic genes (*SREBP1*, *acetyl CoA carboxylase* and *VLDL-R*), lipolytic genes such as *HSL* and *perilipin* as well as genes involved in



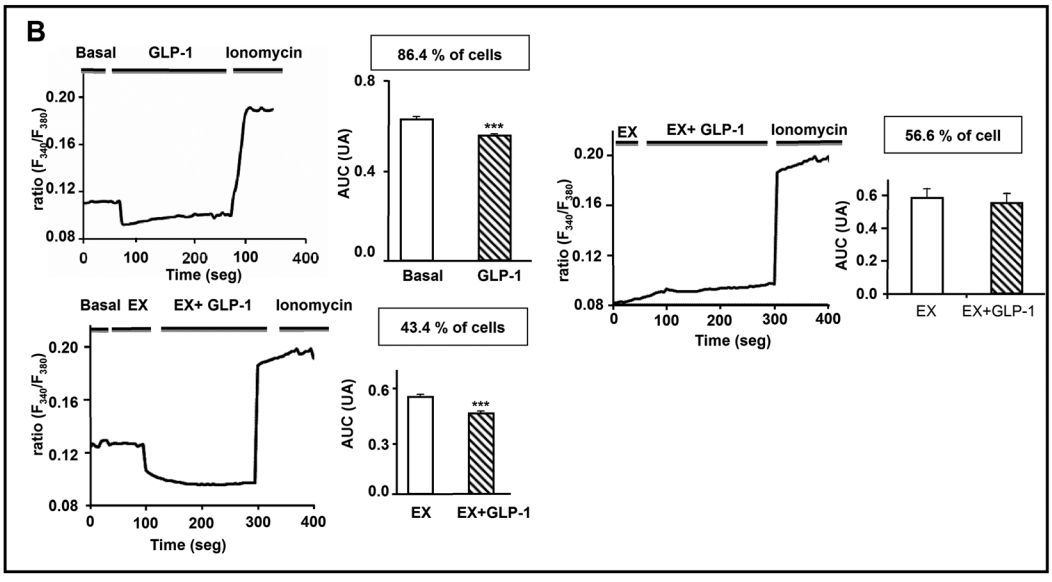


Figure 1

Effects of GLP-1 on the protein expression levels of adipogenic, lipogenic and lipolytic markers and $[Ca^{2+}]_i$ profile in 3T3-L1 differentiated cells. (A) 3T3-L1 ($n = 6$) were incubated for 24 h in the presence or absence of GLP-1 100 nM at 37°C. Proteins were extracted and loaded (30 μg) into 4–20% precast SDS-PAGE gels and transferred to nitrocellulose membranes using the Trans-Blot Turbo transfer system (Bio-Rad, Hercules, CA, USA) for western blot analysis. The blots presented are representatives of samples comparing various treatments, which were run on the same blot with their loading controls. Also, these blots are representative of five blots that showed similar results. β-Actin protein was used as a loading control to ensure that similar quantities of proteins were loaded in each line. Comparisons of the densitometric data were carried out using Student's *t*-test. Values for relative intensity obtained after densitometry of the bands are means ± SEM. Student's *t*-test was used for comparisons between GLP-1 treatment and untreated controls. Dividing lines are combined from a single electrophoresis gel, because duplicated results were removed from the figure (lines). (B) Representative profiles of the effects of GLP-1 (100 nM) on $[Ca^{2+}]_i$ in 3T3-L1 differentiated cells in basal conditions and in those pre-incubated with exendin (9–39) 100 nM. A Fura-2 dual-wavelength fluorescence imaging system was used to measure $[Ca^{2+}]_i$ as described in Methods. The AUC of the $[Ca^{2+}]_i$ responses elicited by GLP-1 in responsive 3T3-L1 cells is presented in arbitrary units. Data are the mean ± SEM of three different experiments for both conditions, basal and pre-incubated with exendin (9–39) at 100 nM (Student's *t*-test).

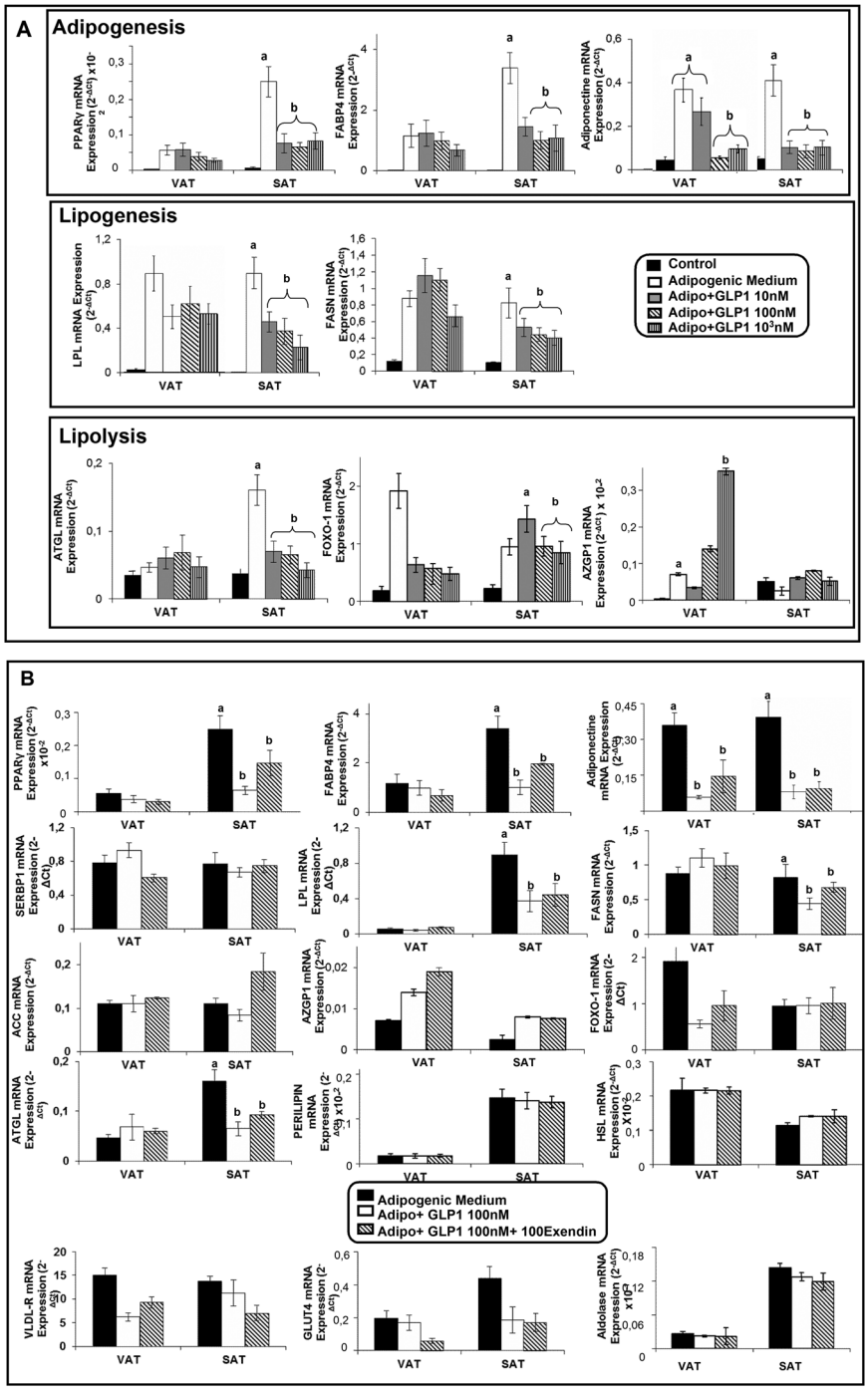


Figure 2

Effects of GLP-1 and GLP-1 receptor antagonist exendin (9–39) on GLP-1-induced changes in the mRNA expression levels of adipogenic, lipogenic and lipolytic markers in *in vitro* differentiated human adipocytes from SAT and VAT. (A) SVFCs were isolated from human VAT and SAT samples obtained from obese subjects ($n = 6$); 14-day differentiated adipocytes were exposed to GLP-1 (10, 100 and 1000 nM) for 12 h. PPARγ, FABP4, adiponectin, LPL, FASN, α-2-glycoprotein 1, zinc binding (AZGP1), ATGL and forkhead box protein O1 (FOXO-1) mRNA levels were normalized to *cyclophilin* mRNA levels. Results were obtained in triplicate for each patient and expressed as the mean ± SEM. Bars with different letters are significantly different according to ANOVA and Duncan's *post hoc* test. (B) *In vitro* differentiated adipocytes obtained from VAT and SAT samples of obese patients ($n = 6$) were incubated in the presence of 100 nM GLP-1 in combination with 100 nM exendin (9–39) for 12 h at 37°C. The gene expression levels of *PPARγ*, *FABP4*, *SREBP1*, *adiponectin*, *LPL*, *FASN*, *acetyl CoA carboxylase* (*ACC*), *AZGP1*, *ATGL*, *VLDL-R*, *FOXO-1*, *perilipin*, *HSL*, *GLUT4* and *aldolase* were measured by qRT-PCR. mRNA levels of the genes investigated were normalized to *cyclophilin* mRNA levels. Results were obtained in triplicate for each patient and expressed as the mean ± SEM. Levene's test showed the homogeneity of variance. There were differences between treatments according to ANOVA and Duncan's *post hoc* test, and bars with different letters represent a significant difference according to Student's *t*-test for independent samples.

glucose transport (*GLUT4*) and metabolism (*aldolase*) did not show any significant change upon exposure of cells from SAT or VAT to GLP-1 (Figure S2).

Exendin (9–39) did not reverse the effects induced by GLP-1, *in vitro*, on the expression levels of adipogenic, lipogenic or lipolytic genes in differentiated adipocytes obtained from human VAT and SAT (Figure 2B). Exposure of differentiated adipocytes to exendin (9–39) alone caused no effects on the genes examined (data not shown).

Effect of GLP-1 on expression levels of adipogenic, lipogenic and lipolytic gene markers in human mature adipocytes

Mature adipocytes obtained by enzymatic digestion of SAT were cultured in suspension and treated with GLP-1 at 10 or 100 nM for 4 h. The mRNA expression levels of *ADRP*, *FABP4*, *LPL*, *VLDL-R* and *SCD1* were significantly lower in adipocytes exposed to either 10 or 100 nM GLP-1 than in adipocytes cultured in medium alone (Figure 3A). No significant changes were observed in *PPARγ*, *FASN* or *SREBP1* mRNA expression in the presence of GLP-1 (data not shown). In contrast, GLP-1 treatment increased *adiponectin* mRNA expression levels in adipocytes. Likewise, this peptide enhanced the mRNA levels of *HSL* and perilipin at both 10 and 100 nM (Figure 3A). In contrast, exposure to GLP-1 had no effect on the expression levels of *GLUT4* or the glycolytic enzymes *aldolase* and *GAPDH* in mature adipocytes (data not shown).

Effect of GLP-1 on human adipocyte differentiation

The expression levels of *ADRP*, *FABP4*, *perilipin* and *HSL* were evaluated at 1, 3, 6, 9, 12 and 15 days of differentiation of SVFCs cultured in the presence or absence of 10 nM GLP-1. Gene expression levels of the adipogenic markers *ADRP* and *FABP4* were significantly down-regulated in the presence of GLP-1 as compared with cells exposed to differentiation medium alone. These effects were already observed at day 6 of differentiation. In contrast, the presence of GLP-1 (10 nM) in the differentiation medium increased *HSL* and *perilipin* mRNA levels in the cell cultures, especially between days 6 and 9 of differentiation, as compared with cells that were differentiated in the absence of this peptide (Figure 3B).

Effect of GLP-1 on expression of adipogenic, lipogenic and lipolytic genes in VAT and SAT explants from obese subjects

GLP-1 significantly decreased the expression levels of two key markers of adipocyte differentiation, *PPARγ* and *FABP4*, as well as that of *adiponectin* in VAT explants compared with untreated controls, especially after 3 h of exposure to this peptide. Incubation of AT explants with GLP-1 receptor antagonist, exendin (9–39), reversed the effect of this peptide on *PPARγ* and *FABP4* mRNA expression levels (Table 1). GLP-1 decreased mRNA of genes promoting lipogenesis and triglyceride accumulation in adipocytes, including *SCD1*, *LPL* and *VLDL-R*, although no changes were observed in *FASN* expression levels (data not shown). In contrast, GLP-1 increased the expression of several markers of lipolysis in VAT explants, including *ATGL*, *HSL and perilipin*, which are the markers responsible for TAG hydrolysis in adipocytes (Smirnova *et al.*, 2006; Kolditz and Langin, 2010) (Table 1). In SAT explants, GLP-1 had no significant effect on *adiponectin* and *FABP4* expression and only reduced *PPARγ* transcript content after 6 h of treatment. *SCD1* expression decreased at both 3 and 6 h of GLP-1 treatment, and *VLDL-R* was significantly reduced at all the times tested as compared with the corresponding controls. Similar to that observed for VAT explants, GLP-1 also increased *perilipin* and *HSL*. No significant changes were observed in the expression levels of genes involved in glucose metabolism in either the VAT or SAT explants treated with GLP-1 (data not shown).

Effect of GLP-1 supplementation with the GLP-1 analogues exenatide and liraglutide in metformin-treated MO T2D patients

T2D subjects with BMI >45 (n = 18) who were being treated with metformin received a supplemental treatment with the GLP-1 analogue exenatide (10 μg twice a day) for 6 months and were compared with the diabetic/MO metformin-treated subjects who did not receive any supplementary treatment. As shown in Table 2, the HOMA index and glucose levels tended to decrease albeit without reaching statistical significance in the exenatide-treated group as compared with patients not treated with this GLP-1 analogue. No significant differences were found in any other clinical or biochemical variables between the two groups.

qPCR analysis showed that *PPARγ* gene expression was numerically lower in both SAT and VAT from exenatide-treated subjects than in non-treated patients. Similarly, *FABP4*, *FASN* and *SREBP-1* expression levels were significantly lower in exenatide-treated subjects compared with the control group. In contrast, adiponectin gene expression levels were higher in exenatide-treated subjects than in controls, yet this increase was statistically significant in VAT but not in SAT. Gene expression of the inflammatory cytokine *IL-6* was also lower in the exenatide-treated group. No changes were observed in the other genes examined, except for *perilipin*, which was increased in VAT of exenatide-treated patients (Table 2).

In a prospective pilot study in which three patients were treated for 30 days with liraglutide, no clear effects of this GLP-1 analogue were apparent on most clinical parameters, although in all three patients their weight decreased by about 2 kg. Expression levels of adipogenic and lipogenic markers in SAT, in particular, *ADRP*, *FABP4* and *LPL*, showed a clear decrease, while that of *perilipin* tended to increase in response to liraglutide. In addition, *TNF-α* was decreased in AT from liraglutide-treated patients compared with control values (Table 3).

Discussion

In the present work, we showed that GLP-1 reduces the expression of both adipogenic and lipogenic genes and enhances those of lipolytic markers in human AT explants. These effects were also observed when the peptide was administered to differentiated human adipocytes or to freshly isolated human mature adipocytes *in vitro*, thus supporting the view that GLP-1 impairs adipogenesis and lipogenesis while increasing lipolysis in human AT. Remarkably, these

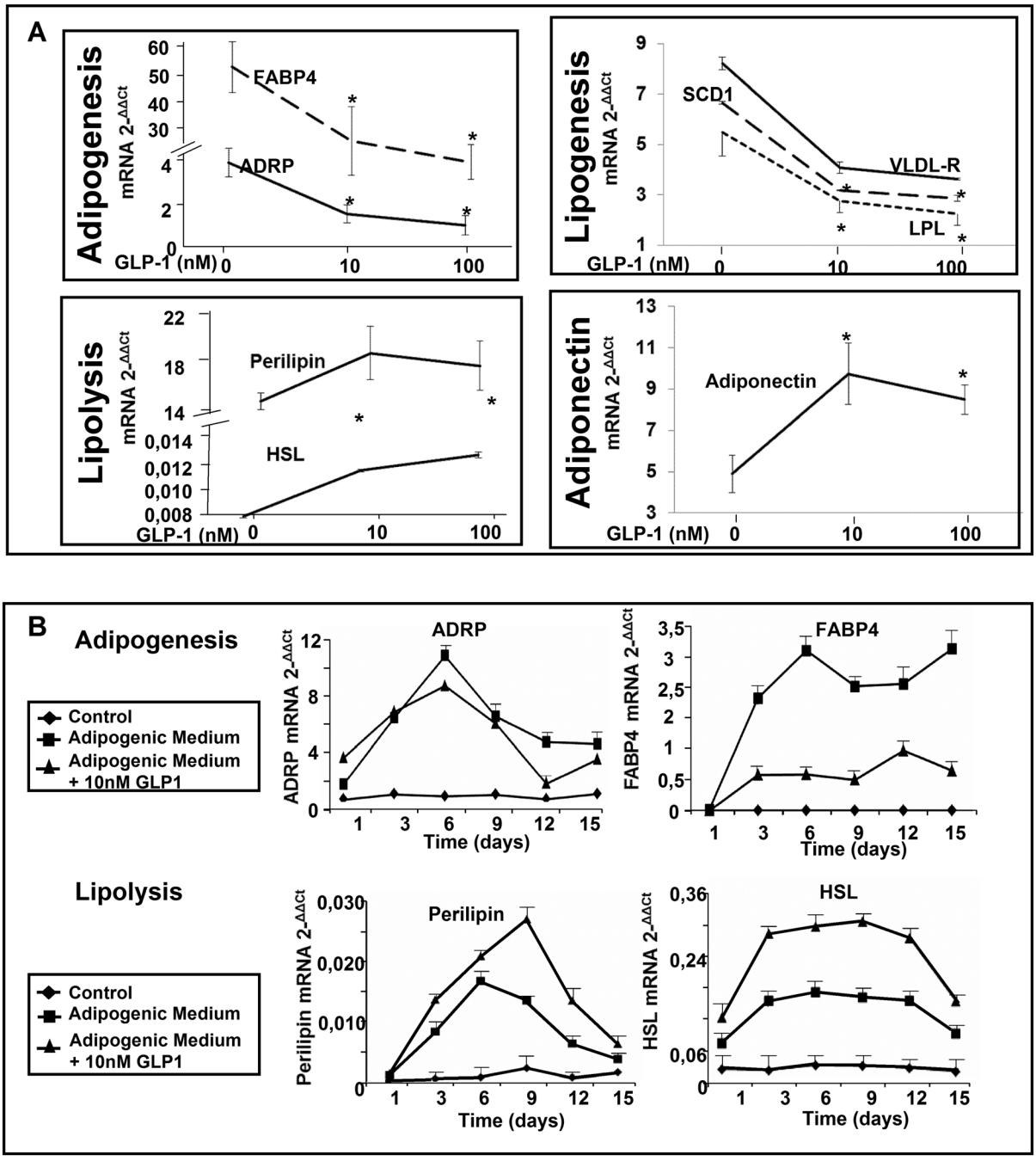


Figure 3

Effects of GLP-1 on the mRNA expression levels of adipogenic, lipogenic and lipolytic markers in human mature adipocytes and in human adipocytes during differentiation. (A) Human mature adipocytes, obtained from dispersion of subcutaneous fat (n = 4), were cultured in suspension and exposed to 10 or 100 nM GLP-1 for 4 h. Gene expression levels of *ADRP*, *adiponectin*, *PPARγ*, *FABP4*, *FASN*, *LPL*, *SREBP1*, *SCD1*, *VLDL-R*, *acetyl CoA carboxylase*, *perilipin*, *HSL*, *ATGL*, *forkhead box protein O1*, *GLUT4*, *aldolase* and *GADPH* mRNA expression levels were measured by RT-PCR, and signals were normalized to cyclophilin gene expression using the formula $2^{-\Delta Ct}$. ANOVA and Duncan's *post hoc* test were used to analyse the association between mRNA expressions because Levene's test showed homogeneity of variance. In the figure, only genes that showed significant changes are presented. Data are the mean ± SEM. (B) Effects of GLP-1 on *ADRP*, *FABP4*, *perilipin* and *HSL* mRNA expression during human adipocyte differentiation. mRNA expression levels were evaluated in human adipocytes differentiated *in vitro* from SVFC (n = 3 patients) in the presence or absence of 10 nM GLP-1 for 1, 3, 6, 9, 12 or 15 days. mRNA levels of test genes were normalized to cyclophilin mRNA levels. Results were obtained in triplicate for each patient and expressed as the mean ± SEM. No statistical analysis was performed due to small sample size.

Table 1

Effect of GLP-1 on adipogenic/lipogenic and lipolytic markers, mRNA expression in human VAT and SAT explants

			1 h		3 h			6 h	
AT	**Gene**	**Control**	**+GLP-1**	**+Ex(9–39) +GLP-1**	**+GLP-1**	**+Ex(9–39) +GLP-1**	**+Ex (9–39)**	**+GLP-1**	**+Ex(9–39) +GLP-1**
SAT	*AdipQ*	1	—	—	1.18 ± 0.21	1.10 ± 0.12	2.06 ± 1.06$^{\#}$	1.02 ± 0.22	1.12 ± 0.13
	PPARγ	1	—	—	0.99 ± 0.33	0.96 ± 0.18	1.75 ± 0.36$^{\#}$	0.85 ± 0.009$^{\#}$	1.04 ± 0.36
	FABP4	1	—	—	1.10 ± 0.18	1.02 ± 0.047	1.41 ± 0.63$^{\#}$	0.94 ± 0.34	0.99 ± 0.46
	SCD1	1	—	—	0.86 ± 0.06*	1.08 ± 0.13	1.01 ± 0.02	0.79 ± 0.005$^{\#}$	0.90 ± 0.001
	VLDL-R	1	—	—	0.79 ± 0.07*	0.87 ± 0.19	1.00 ± 0.05	0.79 ± 0.05$^{\#}$	0.74 ± 0.008
	Perilipin	1	—	—	1.76 ± 0.19*	1.53 ± 0.23	1.09 ± 0.02$^{\#}$	1.71 ± 0.11	1.41 ± 0.25
	HSL	1	—	—	1.69 ± 0.15*	1.42 ± 0.28	1.01 ± 0.09$^{\#}$	1.78 ± 0.19$^{\#}$	1.45 ± 0.15
VAT	*AdipQ*	1	0.90 ± 0.23	0.88 ± 0.28	0.77 ± 0.05*	0.96 ± 0.10	1.7 ± 0.48	1.04 ± 0.03	1.10 ± 0.09
	PPARγ	1	0.98 ± 0.29	0.83 ± 0.34	0.48 ± 0.07*	0.90 ± 0.19$^{\#}$	0.95 ± 0.20$^{\#}$	0.83 ± 0.22	1.43 ± 0.23
	FABP4	1	1.16 ± 0.37	0.82 ± 0.39	0.65 ± 0.007*	0.80 ± 0.006$^{\#}$	1.09 ± 0.35$^{\#}$	0.92 ± 0.10	1.07 ± 0.07$^{\#}$
	LPL	1	0.67 ± 0.06*	0.63 ± 0.24	0.53 ± 0.12*	0.56 ± 0.19	0.97 ± 0.52$^{\#}$	0.71 ± 0.08$^{\#}$	0.78 ± 0.11
	SCD1	1	0.64 ± 0.09*	0.81 ± 0.19	0.89 ± 0.27	1.01 ± 0.11	0.93 ± 0.06	0.71 ± 0.07$^{\#}$	0.82 ± 0.13
	VLDL-R	1	0.84 ± 0.12	0.88 ± 0.23	0.73 ± 0.08*	0.82 ± 0.16	0.92 ± 0.09	0.77 ± 0.25	0.81 ± 0.29
	Perilipin	1	1.32 ± 0.03*	1.14 ± 0.011	1.56 ± 0.04*	1.43 ± 0.22	1.11 ± 0.12$^{\#}$	1.15 ± 0.21	1.01 ± 0.35
	HSL	1	1.48 ± 0.012*	1.51 ± 0.09	1.64 ± 0.12*	1.55 ± 0.08	1.06 ± 0.09$^{\#}$	1.48 ± 0.09$^{\#}$	1.39 ± 0.12
	ATGL	1	1.21 ± 0.23	1.33 ± 0.15	1.32 ± 0.11*	1.24 ± 0.12	1.11 ± 0.18	1.19 ± 0.18	1.23 ± 0.23

VAT and SAT explants ($n = 3$) were incubated with GLP-1 (100 nM) and/or exendin fragment (9–39) (100 nM) for 1, 3 or 6 h, and then *adiponectin* (*AdipQ*), *PPARγ*, *FABP4*, *SREBP1*, *LPL*, *SCD1*, *FASN*, *VLDL-R*, *perilipin*, *HSL*, *ATGL*, *GLUT4* and *aldolase* mRNA expression was measured by RT-PCR. Only those genes that showed significant changes are presented in the Table. All mRNA expressions were compared with their respective controls, which represents a positive control of gene expression. GLP-1 effects at different times were compared with adipogenic controls. Exendin (9–39) effects were compared with GLP-1. Signals were normalized by constitutively expressed cyclophilin signals using the formula $2^{-\Delta\Delta Ct}$. Data are the mean ± SEM. Student's *t*-test was used to analyse the association between mRNA expressions.

*$P < 0.05$ with respect to control.

$^{\#}P < 0.05$ with respect to GLP-1 effect.

Table 2

Effects of exenatide in T2D MO subjects treated with metformin

	Metformin-treated patients (n = 9)	Metformin- and exenatide-treated patients (n = 9)
Age (in surgery, years)	52.33 ± 3.78	45.88 ± 3.65
BMI (kg·m^{-2})	48.09 ± 2.69	49.19 ± 2.31
Waist/hip	0.95 ± 0.04	0.99 ± 0.03
HOMA-IR	11.86 ± 2.94	7.07 ± 2.051
Glucose (mmol·L^{-1})	10.84 ± 1.50	7.73 ± 0.77
Triglycerides (mmol·L^{-1})	1.61 ± 0.26	1.90 ± 0.24
Cholesterol (mmol·L^{-1})	4.95 ± 0.28	4.89 ± 0.41
SBP	141.00 ± 5.25	133.00 ± 9.82
DBP	88.88 ± 2.90	81.50 ± 9.95
Adiponectin VAT($2^{-\Delta Ct}$)	3.827 ± 0.780	7.021 ± 1.145*
Adiponectin SAT ($2^{-\Delta Ct}$)	6.778 ± 1.436	7.992 ± 1.302
FABP4 VAT ($2^{-\Delta Ct}$)	13.921 ± 1.094	4.532 ± 1.384*
FABP4 SAT ($2^{-\Delta Ct}$)	20.379 ± 3.047	11.843 ± 2.948*
FASN VAT ($2^{-\Delta Ct}$)	0.765 ± 0.174	0.404 ± 0.096
FASN SAT ($2^{-\Delta Ct}$)	0.664 ± 0.094	0.301 ± 0.064*
SREBP1 VAT ($2^{-\Delta Ct}$)	0.221 ± 0.035	0.120 ± 0.015*
SREBP1 SAT ($2^{-\Delta Ct}$)	0.179 ± 0.179	0.122 ± 0.015*
Perilipin VAT ($2^{-\Delta Ct}$)	1.885 ± 0.532	2.871 ± 0.495*
Perilipin SAT ($2^{-\Delta Ct}$)	2.995 ± 0.392	2.577 ± 0.157
HSL VAT ($2^{-\Delta Ct}$)	0.121 ± 0.088	0.218 ± 0.013
HSL SAT ($2^{-\Delta Ct}$)	0.009 ± 0.002	0.008 ± 0.004
ATGL VAT ($2^{-\Delta Ct}$)	0.812 ± 0.177	0.575 ± 0.142
ATGL SAT ($2^{-\Delta Ct}$)	0.786 ± 0.106	0.519 ± 0.164
TNF-α VAT ($2^{-\Delta Ct}$)	0.014 ± 0.007	0.017 ± 0.006
TNF-α SAT ($2^{-\Delta Ct}$)	0.010 ± 0.005	0.008 ± 0.001
IL-6 VAT ($2^{-\Delta Ct}$)	0.334 ± 0.271	0.056 ± 0.027*
IL-6 SAT ($2^{-\Delta Ct}$)	0.343 ± 0.199	0.112 ± 0.697*
PPARγ VAT ($2^{-\Delta Ct}$)	0.157 ± 0.036	0.111 ± 0.033
PPARγ SAT ($2^{-\Delta Ct}$)	0.132 ± 0.028	0.104 ± 0.023

MO subjects (n = 9) with T2D being treated with metformin received a supplementary treatment of exenatide (10 μg twice a day) for 6 months, and other MO patients (n = 9) were only treated with metformin. Anthropometric parameters and gene expression of adipogenic, lipogenic, lipolytic and inflammatory markers in VAT and SAT were evaluated in both groups. RNA from patients was isolated from VAT and SAT, and then *PPARγ*, *FABP4*, *adiponectin*, *FASN*, *SREBP1*, *acetyl CoA carboxylase*, *ATGL*, *perilipin*, *HSL*, *GLUT4*, *aldolase*, *GADPH*, *TNF-α* and *IL-6* gene expression was measured by RT-PCR. In the table, only genes that showed significant changes are presented. Signals were normalized by constitutively expressed *cyclophilin* using the formula $2^{-\Delta Ct}$. Data are the mean ± SEM. Student's t-test was used to analyse the association between mRNA expressions. SBP, systolic blood pressure; DBP, diastolic blood pressure.

*$P < 0.05$.

effects were more pronounced in SAT than in VAT. In line with our present results, we previously found that downstream adenylate cyclase/cAMP signalling is involved GLP-1-stimulated lipolysis in differentiated 3T3-L1 adipocytes (Vendrell *et al*., 2011). These results, together with those obtained in the present study in both human and 3T3-L1 adipocytes, strongly support the notion that lipolysis is a major target of for the effects of GLP-1 in this cell type. In our previous study, we demonstrated that GLP-1 receptor expression showed a different behaviour, depending on AT depot, obesity and extent of IR. In SAT, no differences in GLP-1 receptor expression were noted in obese subjects with a low degree of IR. In the case of MO, a substantial increase in GLP-1 receptor expression was observed when compared with the non-MO cohort. Indeed, functional classification of obese subjects according to IR status revealed that GLP-1 receptors in VAT depots were markedly up-regulated when the degree of IR was very high (Vendrell *et al*., 2011). Moreover, we found that GLP-1 receptors are expressed in 3T3-L1, and their mRNA levels were increased after GLP-1 treatment (Figure S1A).

Notably, a negative role of GLP-1 in human adipogenesis is also supported by our findings; on continuous exposure

Table 3

Prospective pilot study for effect of liraglutide treatment on adipogenic, lipogenic and inflammatory marker gene expression in SAT from MO subjects treated with metformin

	Liraglutide treatment					
	Before	**After**	**Before**	**After**	**Before**	**After**
Age (in surgery, years)	44	44	48	48	51	51
Weight (kg)	137.5	136	146	143	158	156
BMI ($kg \cdot m^{-2}$)	54.5	54	50.1	49.8	56	55
Waist	139	138	132	130	149	147
HOMA-IR	9.5	9.2	9.6	9.4	9.1	9
HbA1c	8.6	8.3	7.8	7.1	8.4	7.8
Glucose ($mmol \cdot L^{-1}$)	8.60	7.66	7.99	6.66	8.44	7.77
Triglycerides ($mmol \cdot L^{-1}$)	4.59	3.39	2.58	2.26	2.14	1.99
Cholesterol ($mmol \cdot L^{-1}$)	5.44	5.23	4.82	4.66	4.45	4.66
SBP	142	125	138	130	155	140
DBP	88	84	80	78	98	92
Adiponectin ($2^{-\Delta Ct}$)	6.037	3.715	7.853	3.995	9.229	9.050
PPARγ ($2^{-\Delta Ct}$)	0.081	0.071	0.089	0.079	0.097	0.086
FABP4 ($2^{-\Delta Ct}$)	20.739	9.756	20.557	14.367	20.355	16.299
ADRP ($2^{-\Delta Ct}$)	1.374	0.441	1.159	0.432	0.482	0.446
LPL ($2^{-\Delta Ct}$)	1.419	0.987	1.606	1.298	1.792	1.225
Perilipin ($2^{-\Delta Ct}$)	7.697	10.570	8.652	10.362	25.036	26.021
TNF-α ($2^{-\Delta Ct}$)	0.012	0.006	0.009	0.005	0.005	0.007

MO subjects ($n = 3$) with T2D being treated with metformin received a supplementary treatment of liraglutide for 1 month. Anthropometric parameters and gene expression of adipogenic, lipogenic and inflammatory markers in SAT were evaluated before and after 1 month of liraglutide treatment. RNA from patients was isolated from SAT, and then *PPARγ*, *FABP4*, *ADRP*, *adiponectin*, *LPL*, *perilipin* and *TNF-α* gene expression was measured by RT-PCR. Signals were normalized to constitutively expressed *cyclophilin* using the formula $2^{-\Delta Ct}$. SBP, systolic blood pressure; DBP, diastolic blood pressure.

of human pre-adipocytes from obese patients to the peptide during differentiation, the expression levels of several adipogenic/lipogenic factors were reduced in these cells. Other studies have shown that GLP-1 stimulates adipogenesis in 3T3-L1 adipocytes (Challa *et al.*, 2012; Yang *et al.*, 2013). GLP-1 did not exert any significant effect either on adipogenesis or on lipolysis in adipocytes from AT of lean subjects (data not shown), which is in agreement with our previous studies showing that the GLP-1 receptor is mainly expressed in adipocytes from obese subjects and nearly non-existent in adipocytes from lean subjects (i.e. those employed in the present study) (Vendrell *et al.*, 2011). Taken together, these findings suggest that the effects of GLP-1 on adipogenesis may vary depending on the origin of the adipocytes.

It is well known that AT expansion during the development of obesity is initially characterized by fat cell hypertrophy followed by rises in fat cell number (Hausman *et al.*, 2001), and this is involved in the increased adipogenesis and lipogenesis within AT (Kim *et al.*, 2007; Gealekman *et al.*, 2014). In severely obese subjects, body weight loss involves a decrease in adipocyte size and fat mass together with a parallel improvement in circulating adipokine and metabolic profiles (Varady *et al.*, 2009). Likewise, it is well established that GLP-1 decreases body weight and restores metabolic parameters that are impaired in obesity and T2D (Gutzwiller *et al.*, 2004; Sancho *et al.*, 2006). Our *in vitro* data suggest that GLP-1 may reduce fat storage capacity and adiposity by inhibiting both adipocyte differentiation and lipogenesis and stimulating lipolysis in adipocytes, which, together, would contribute to body weight loss and metabolic improvement.

In line with this hypothesis, exenatide and liraglutide, two GLP-1 analogues that have been found to ameliorate glycaemic concentration, glycosylated haemoglobin and arterial pressure and to reduce body weight in diabetic subjects (Hajer *et al.*, 2008), showed similar effects on adipogenesis, lipogenesis and lipolysis in AT from obese subjects to those observed *in vitro* with GLP-1. Specifically, the treatment of T2D MO subjects with exenatide significantly decreased the expression of adipogenic markers as well as that of enzymes involved in fatty acid biosynthesis, in parallel with a concomitant increase in adiponectin mRNA levels. We have to include the caveat that the T2D MO subjects included in our study were receiving metformin therapy. Given that metformin has been shown to regulate lipolysis in AT (Castro Cabezas *et al.*, 2012) and to increase GLP-1 production in response to food (Mannucci *et al.*, 2001), we cannot exclude the possibility that this drug might have influenced the

response observed in exenatide-treated patients. Notwithstanding, our *in vitro* data on GLP-1 support a targeted effect of exenatide on the different AT markers evaluated in this study. Indeed, similar to that observed in human mature adipocytes exposed to GLP-1, administration of exenatide increased the expression of the insulin-sensitizing adipokine, adiponectin, in AT of T2D MO subjects. When viewed together, these results suggest that exenatide-evoked enhancement of adiponectin expression may be responsible, at least in part, to the beneficial effects of this GLP-1 analogue on the HOMA index observed in these patients. Interestingly, two out of three patients included in the prospective study (i.e. treated with liraglutide for 30 days) showed a marked decrease in adiponectin after treatment. In general, liraglutide treatment showed similar results, although this pilot study must be viewed with caution given the low sample size. Remarkably, exenatide decreased the expression levels of *TNF-α*, which is known to interact with adipogenic markers and to promote AT dysfunction (Hajer *et al.*, 2008). These data provide a novel mechanism underlying the beneficial effects of GLP-1 on AT and further support the crucial role of this peptide in improving lipid metabolism and endocrine function in AT in T2D patients.

We previously showed that GLP-1 stimulates lipolysis in differentiated 3T3-L1 in a receptor-dependent manner that involves downstream adenylate cyclase/cAMP signalling (Vendrell *et al.*, 2011). Recent studies have shown that both Akt and ERK1/2 are activated by GLP-1 in 3T3-L1 when the peptide is administered at early stages of adipogenesis, which could account for the increased adipogenic rate observed in these cells (Challa *et al.*, 2012; Yang *et al.*, 2013). In contrast, we observed that this peptide did not evoke the activation of these signalling intermediates in differentiated 3T3-L1, wherein the peptide did decrease the amount of FABP4 and SREBP1. Taken together, these results suggest that the effects of GLP-1 may differ according to the differentiation stage of the murine adipocytes. Herein, we have shown that GLP-1 decreases $[Ca^{2+}]_i$ in differentiated 3T3-L1 adipocytes. An inhibitory effect of GLP-1 or its analogues on Ca^{2+} signalling has also been observed previously in other cell types (Montrose-Rafizadeh *et al.*, 1997). In contrast, it has been shown that increasing $[Ca^{2+}]_i$ stimulates lipogenesis and inhibits basal and agonist-stimulated lipolysis in both human and murine adipocytes (Xue *et al.*, 1998; Gericke *et al.*, 2009). Thus, it is tempting to speculate that the down-regulation of lipogenic markers and the up-regulation of lipolytic factors caused by GLP-1 treatment may be related, at least in part, to the inhibitory effect exerted by this peptide on $[Ca^{2+}]_i$ dynamics in adipocytes, although further measurements of $[Ca^{2+}]_i$ in SAT samples and human mature adipocytes are needed to confirm this hypothesis.

Notably, exendin (9–39) only partially prevented GLP-1-induced $[Ca^{2+}]_i$ decrease. However, the stimulating effects of GLP-1 on cAMP production in 3T3-L1 were fully abolished in the presence of exendin (9–39) (Vendrell *et al.*, 2011). Together, these results are consistent with the presence of an additional, as yet unknown, receptor, distinct from the GLP-1 receptor, in adipocytes, as has been previously suggested for this and other cell types (Merida *et al.*, 1993; Márquez *et al.*, 1998; 2001; Xie *et al.*, 2006; Wicki *et al.*, 2007; Connolly *et al.*, 2012; Wang *et al.*, 2012). Given the 'glucagon-like' effect of GLP-1, the glucagon receptor could be a potential receptor for the effects of GLP-1 in adipocytes, although further experiments are needed to confirm this hypothesis. Identification of other putative GLP-1 receptors deserves more work and may help in the understanding of the mechanisms by which this peptide facilitates adipocyte function.

Conclusion

In summary, our study provides new clues on the effects and mechanisms activated by GLP-1 in AT (summarized in Figure 4), which may help elucidate how this peptide improves the metabolic profile of obese patients, thus paving the way for developing novel therapies for the treatment of obesity and T2D.

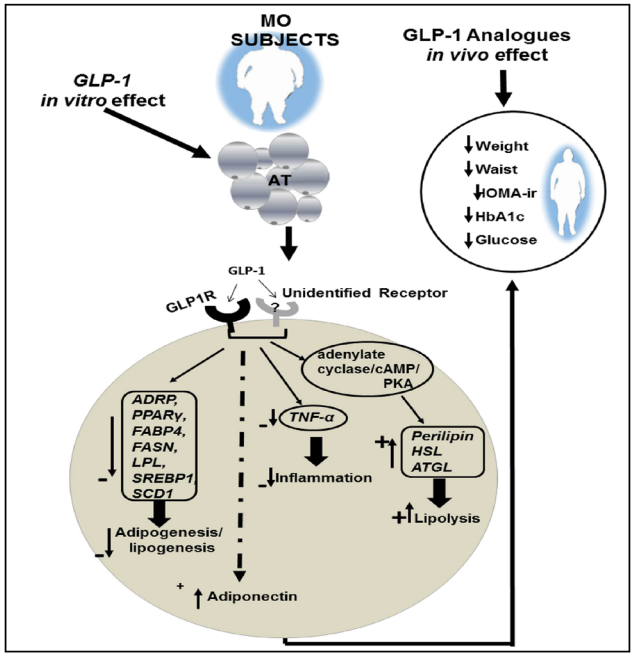


Figure 4

Schematic drawing outlining the main *in vivo* and *in vitro* effects evoked by GLP-1 in AT of obese subjects. The cartoon summarizes data obtained from the analysis (mRNA and protein) of a variety of genes involved in adipogenic/lipogenic, lipolytic and inflammation processes (*PPARγ*, *FABP4*, *FASN*, *LPL*, *SREBP1*, *SCD1*, *ATGL*, *perilipin*, *HSL*, *ATGL*, *TNF-α* and *adiponectin*) in human AT and adipocytes in response to GLP-1. The GLP-1 receptor antagonist, exendin (9–39), did not fully block GLP-1-induced effects, which suggests that GLP-1 might bind to another receptor different from the specific GPCR, GLP-1 receptor. Given that HSL and perilipin are known to be primarily regulated via activation of cAMP/PKA (Kolditz and Langin, 2010) and that GLP-1-stimulated lipolysis occurs in a receptor-dependent manner involving downstream adenylate cyclase/cAMP signalling (Vendrell *et al.*, 2011), we propose that GLP-1 would promote lipolysis by acting on HSL and perilipin via adenylate cyclase/cAMP/PKA signalling. As discussed in the text, a role for Ca^{2+} signalling in GLP-1 actions on lipid metabolism in adipocytes is also proposed. All these molecular changes in AT could underlie the clinical improvement observed in morbid subjects treated with GLP-1 analogues.

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Author contributions

R.E.B., F.J.T., D.F.G. and M.M.M. designed the research; L.C. A., W.O.O., R.B.L., M.C.P., J.D.L., A.D.R., R.G.R., J.V., S.H., M.M.R. and R.V.M performed the research; R.E.B., F.J.T. and M.M.M. analysed the data and wrote the paper.

Conflict of interest

There are no conflicts of interest to be declared. F.J.T., M.M.M. and R.E. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of pre-clinical research recommended by funding agencies, publishers and other organizations engaged with supporting research.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13481

Table S1 TaqMan probe's references from Applied Biosystems.

Figure S1 Effect of GLP-1 on GLP-1 receptor expression, on $[Ca^{2+}]_i$ and on AkT/ERK phosphorylation in 3T3-L1 differentiated preadipocytes. (A) 3T3-L1 (n = 6) were incubated for 24 h in the presence or absence of GLP-1 100 nM at 37°C; mRNA levels were normalized to β-actin mRNA levels. Results were obtained in triplicate and expressed as the mean ± SEM. $^{*}P < 0.05$. (B) Data are the mean ± SEM of the AUC, time for maximal response and percentage of $[Ca^{2+}]_i$ inhibition evoked by GLP-1 (100 nM) alone or in combination with exendin (100 nM) (n = 3 independent experiments). $^{***}P <$ 0.001 versus basal $[Ca^{2+}]_i$ levels and $^{\#}P < 0.001$ versus GLP-1 100 nM alone. Student's t-test. (C) 3T3-L1 (n = 6) were incubated for 5 and 30 min in the presence or absence of GLP-1 100 nM at 37°C. Proteins were extracted and loaded **(**30 μg) into SDS-PAGE gels and transferred to nitrocellulose membranes using the Trans-Blot Turbo transfer system (Bio-Rad) for western blot analysis. Blots are representative of five blots that showed similar results. Total Akt and total ERK proteins were used as loading control to insure that similar quantities of proteins are loaded in each line. Values for relative intensity obtained after densitometry of the bands are means ± SEM.

Figure S2 Effect of GLP-1 receptor antagonist exendin (9–39) on GLP-1-induced changes in the expression of adipogenic, lipogenic and lipolytic markers in human differentiated adipocytes. *In vitro* differentiated adipocytes obtained from VAT and SAT samples of obese patients (n = 6) were incubated in the presence of 100 nM GLP-1 alone or in combination with 100 nM exendin (9–39) for 12 h at 37°C. The gene expression levels of *PPARγ*, *FABP4*, *SERBP1*, *adiponectin*, *LPL*, *FASN*, *ACC*, *AZGP1*, *ATGL*, *VLDL-R*, *FOXO-1*, *perilipin*, *HSL*, *GLUT4* and *aldolase* were measured by qRT-PCR. mRNA levels of test genes were normalized to *cyclophilin* mRNA levels. Results were obtained in triplicate for each patient and expressed as the mean ± SEM. Bars with different letters have a significant difference ($P < 0.05$).