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Effect of Desacyl Ghrelin, Obestatin and Related Peptides on Triglyceride Storage, Metabolism and GHSR Signaling in 3T3-L1 Adipocytes

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ABSTRACT

Acyl-ghrelin (AG), desacyl-ghrelin (DAG) and obestatin are all derived from the same gene transcript; however their plasma levels do not necessarily change in parallel. The influence of these peptides towards the development of obesity and their direct effects on adipocyte physiology has not been thoroughly investigated. This study was designed to evaluate the direct effects of peptides of the ghrelin family on preadipocyte proliferation, differentiation and adipocyte lipid and glucose metabolism in 3T3-L1 cells. 3T3 cells were treated with physiological peptide concentrations for 1 h to 9 days, and the relevant assays measured. In preadipocytes, AG, GHRP-6 and DAG stimulated proliferation, measured as ^{3}H-thymidine incorporation (up to 200%, $P < 0.05$), while all peptides stimulated differentiation (up to 300%, $P < 0.01$) as compared to standard differentiation conditions. In adipocytes, FA uptake was increased in a concentration-dependent manner especially with obestatin (three- to fourfold, $P < 0.001$) and DAG (three- to fivefold, $P < 0.001$). By contrast, glucose transport was unchanged. DAG and obestatin significantly decreased lipolysis measured as non-esterified fatty acid and glycerol release by 50%, $P < 0.05$–0.01 and 51%, $P < 0.01$, respectively. Interestingly, DAG stimulation of FA uptake was blocked with GHSR1 antagonist (D-lys^{3})-GHRP-6 ($P < 0.05$), phospholipase C inhibitor U73122 and phosphatidylinositol-3-kinase inhibitor wortmannin ($P < 0.001$). Finally, in omental but not subcutaneous human adipose tissue, GHSR1 correlated with BMI ($r = 0.549$, $P < 0.05$) and insulin ($r = 0.681$, $P < 0.01$). Taken together, these results suggest that ghrelin-related peptides may directly affect adipose tissue metabolism. J. Cell. Biochem. 112: 704–714, 2011.

KEY WORDS: DESACYL GHRELIN; GHRELIN; OBESTATIN; GHSR RECEPTOR; TRIGLYCERIDE STORAGE

The prevalence of obesity and its related disorders (insulin resistance, type 2 diabetes, and cardiovascular disease) is a global social health concern. Biologically, both hypertrophy and hyperplasia of adipocytes are primary characteristics of obesity [Allende-Vigo, 2010], and a growing body of evidence suggests that several hormonal factors could have an influence on these processes. Insulin is one well-characterized effector of preadipocyte proliferation and differentiation, and fat cell metabolism. Adipokines (adipose tissue hormones) such as Acylation Stimulating Protein (ASP), leptin and others are also implicated. Recently, gastrointestinal hormones, known for their intestinal effects on motility and exocrine/endocrine secretion have also been reported to have broader effects both peripherally (cardiovascular, reproduction) and centrally (food intake, memory) [Muccioli et al., 2007; Soares and Leite-Moreira, 2008; Taub, 2008].

Previous studies mostly focused on the role of gastrointestinal hormones in the regulation of mechanisms that govern food intake and weight gain but have not thoroughly explored the potential direct role for gastrointestinal peptides on adipocyte functions. Three gastrointestinal peptides, obestatin, desacyl ghrelin (DAG) and acyl ghrelin (AG), all derived by posttranslational processing from a common prohormone, preproghrelin, originate from endocrine cells in the oxyntic gland of the stomach [Soares and Leite-Moreira, 2008]. AG administrations were reported to induce adipogenic effects in rats, and the endogenous circulating hormone levels are altered in insulin resistance and obesity [Gutierrez-Grobe et al.,

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2010]. Taken together, these elements provide relevance for evaluating their role in the regulation of adipocyte physiology.

Desacyl ghrelin and acyl ghrelin are the two major molecular forms of ghrelin gene products in circulation. In addition to gastrointestinal functions, AG has been shown to increase or have no effect on food intake and body weight [Druce et al., 2006; Neary et al., 2006; Pfluger et al., 2008; Kirchner et al., 2009]. Further, AG has been shown to mediate a number of functions including cardiomyocyte survival, enhancement of memory and neurogenesis as well as have effects on adipogenesis [Taub, 2008]. Derived mainly from the gastric mucosa, AG can be desacylated to the DAG form in the plasma. The concentration of the DAG form is approximately 5 times more important than the one of AG in the circulation. Interestingly, DAG, was initially thought to be an inactive form of AG but recent studies have indicated that it promotes proliferation and inhibits apoptosis of pancreatic beta-cells and human islets, inhibits cell proliferation in human breast carcinoma cell lines [Cassoni et al., 2001], cell death in cardiomyocytes and endothelial cells [Baldanzi et al., 2002]. The direct effect of DAG on food intake is more controversial, with both decreased food intake [Asakawa et al., 2005; Chen et al., 2009], or no effects [Neary et al., 2006] reported. Further, the role of DAG in preadipocyte proliferation and adipogenesis remains unclear [Zhang et al., 2004; Giovambattista et al., 2008].

In the 1980s, different synthetic peptides, such as the growth hormone releasing peptide 6 (GHRP-6), have been shown to stimulate GH secretion, but the growth hormone secretagogue receptor 1a (GHSR1a) mediating these effects was not isolated and characterized before 1997. The GHSR1a remained an orphan receptor until AG was identified as its endogenous ligand [Gauna et al., 2007; Giovambattista et al., 2008; Davies et al., 2009]. Prior to this, growth hormone releasing peptide 6 (GHRP-6) had been identified as an agonist for GHSR1a [Dass et al., 2003], although GHRP6 also appears to bind an alternate receptor CD36. However the ability of DAG to bind and signal through GHSR1a is still questioned with data presented both for [Gauna et al., 2007] and against [Zhang et al., 2004; Patel et al., 2006; Giovambattista et al., 2008]. An additional receptor, GHSR1b, produced through translational processing has now been identified [Colinet et al., 2009]. The presence of these receptors within adipose tissue has been both demonstrated [Choi et al., 2003; Kim et al., 2004; Patel et al., 2006; Rodríguez et al., 2009], and refuted [Gnanapavan et al., 2002; Fontenot et al., 2007]. Further, it has been suggested that additional, as yet unidentified, receptors for AG and DAG may also exist [Muccioli et al., 2007].

Obestatin is a 23-amino acid peptide co-secreted with ghrelin from the stomach [Zhang et al., 2004]. Although different groups have reported that obestatin is able to reduce appetite, gastric emptying, jejunal motility and exert proliferative, survival, and antiapoptotic effects in β-cells, the biological effects of obestatin remain highly controversial and need to be thoroughly investigated [Gourcerol et al., 2007; Tremblay et al., 2007]. This peptide is suggested to be a natural ligand of GRP39, a receptor with 52% identity with GHSR-1a [Holst et al., 2004; Zhang et al., 2008], although again controversial [Pemberton and Richards, 2008]. It has been reported that the biologically active form of the receptor, GRP39-1a is present in the adipose tissue and that its expression is either increased in the fasting condition or decreased during adipocyte differentiation and obesity [Catalan et al., 2007; Fontenot et al., 2007; Zhang et al., 2008], providing evidence that it can be considered as a potential target tissue, although the effects it mediates remain unclear.

Much of the current research on gastrointestinal peptides is focused on their effects on food intake and body weight regulation. With respect to fat cell metabolism, AG clearly binds GHSR1a, stimulates preadipocyte proliferation and differentiation, and influences adipocyte metabolism [Muccioli et al., 2007; Soares and Leite-Moreira, 2008; Taub, 2008]. However there is little information on the effects of DAG or obestatin on these parameters, with substantial controversy in the literature. The aim of the present study was to comprehensively evaluate the biologic effects of DAG and obestatin versus AG on preadipocyte proliferation and differentiation, as well as adipocyte metabolism using 3T3-L1 cells. Further, the expression and the potential role of GHSR1 in mediating these effects in human adipose tissue was evaluated.

MATERIALS AND METHODS

REAGENTS

Insulin-like growth factor I (IGF1), insulin, U73122, and growth hormone secretagogue receptor antagonist (D-lys^3)-GHRP-6 were purchased from Sigma–Aldrich (Oakville, ON, CA). Quaternary beta-cylodextrin was from Supelco (Bellefonte, PA). 2-(4-,5-dimethyl-2-thiazolyl)-3-(4-sulfophenyl) (MTS) was purchased from Promega Corporation (Madison, WI). DMEM/F12, phosphate buffered saline (PBS), bovine calf serum (BCS) and fetal bovine serum (FBS) were from GIBCO (Burlington, ON, CA). Isobutyl-methylxanthine (IBMX), dexamethasone, penicillin–streptomycin and trypsin were from Life Technologies (Carlsbad, CA), 2-deoxy-D[1-^{3}H]glucose and ^{3}Hthymidine were purchased from GE Healthcare (Montreal, QC, CA). Wortmannin was obtained from Invitrogen (Camorillo, CA).

GHRELIN-RELATED PEPTIDES PREPARATION BY SOLID PHASE SYNTHESIS

Acylated and desacylated ghrelin, obestatin and GHRP-6 were generously provided by Peptidec Technologies Ltd. and assembled by the solid-phase method as previously described [Merrifield, 1963] using either Rink amide resin (C-terminal amidated peptides) or Wang resin (C-terminal free-carboxyl peptides) as solid supports with the Fmoc protection protocol. Peptides were cleaved from the resin with TFA in the presence of scavengers (*m*-cresol, triisopropylsilane, anisole) and purified by reversed-phase HPLC using 0.1% TFA:acetonitrile gradients. Peptide purity (<95%) and identity were confirmed by analytical reverse-phase HPLC on C18 3 micron columns and by LC–Mass Spectrometry. Peptide content was greater than 85%. Peptides were reconstituted in phosphate buffered saline (PBS) and stored at −80°C until use. As previously reported in 3T3-L1 cells, peptides were used between concentrations of 10^{-9} and 10^{-12} M [Kim et al., 2004].

CELL CULTURE OF 3T3-L1 PREADIPOCYTES AND STANDARD DIFFERENTIATION OF 3T3 ADIPOCYTES

Murine preadipocytes (3T3-L1) cells were purchased from the American Type Culture Collection (Manassas, VA). Preadipocytes were cultured using DMEM/F12 supplemented with 10% bovine calf serum (BCS) medium and maintained at 37°C in 5% CO_2 incubator. Cells were maintained at low passage number and subdivided at <70% confluence.

For standard 3T3-L1 adipocyte differentiation, preadipocytes were seeded in 48-well culture plates (15×10^3 cells/cm^2) and grown to confluence. Two days post-confluence, differentiation was initiated with medium containing 1.721 μM insulin, 1 μM dexamethasone and 0.5 mM isobutylmethylxanthine (IBMX) in DMEM/F12 with 10% BCS. Two days later, medium was replaced with DMEM/F12 containing 10% BCS and 1.721 μM insulin. Two days later, media was changed to DMEM/F12, 10% FBS. Differentiated cells (at least 90% with lipid droplet accumulation) were used on day 8–12 after initiation of differentiation.

ASSESSMENT OF METABOLIC ACTIVITY AND CELL PROLIFERATION IN 3T3 PREADIPOCYTES

Metabolic activity was evaluated using Cell titer 96 aqueous one-solution (Promega Corp.) as described by the manufacturer. 3T3-L1 preadipocytes (5×10^3/well) were plated in 96-well culture plates. Cells were then changed to serum-free media for 2 h (75 μl), then supplemented with ghrelin-related peptides, for 24 h at 37°C. MTS solution (20 μl of 2-(4-,5-dimethyl-2-thiazolyl)-3-(4-sulfophenyl)) was added and incubated for 3 h, 37°C. Absorbance at 490 nm indicates formazan production, an indicator of mitochondrial activity.

Preadipocyte proliferation was evaluated based on [Boney et al., 2000]. Cells were seeded (10×10^3 cells/well, 48-well plate). Cells were incubated in serum-free media for 2 h, then treated with ghrelin-related peptides for 24 h at 37°C, followed by incubation with ^{3}H-thymidine for 15 h at 37°C incubator. After removal of the labeling solution, cells were rinsed three times with cold PBS, solubilized in 250 μl 0.3 N NaOH for 3 h, and a 50 μl aliquot taken for protein assay. TCA solution (50 μl, 20%) was added, incubated for 30 min at room temperature, centrifuged and supernatant counted by liquid scintillation counting.

EFFECT OF GHRELIN-RELATED PEPTIDES ON 3T3-L1 DIFFERENTIATION ASSESSED BY OIL RED O QUANTITATION

3T3-L1 preadipocytes were differentiated in the presence of ghrelin-related peptides using a modification of the methodology as described above. Two-day post-confluent 3T3-L1 preadipocytes were treated with the standard differentiation medium (1.721 μM insulin, 1 μM dexamethasone and 0.5 mM isobutylmethylxanthine (IBMX) in DMEM/F12 with 10% FBS) with supplementation of the indicated ghrelin-related peptides for 3 days. Media was changed to DMEM/F12, 10% FBS, 1.721 μM insulin, with supplementation of the indicated ghrelin-related peptides for 3 days. Media was then changed to DMEM/F12, 10% FBS, with/without peptides for 3 days. On day 9, 3T3-L1 adipocytes were washed with phosphate-buffered saline (PBS), fixed with 10% formalin for 30 min, stained for 1 h at room temperature in freshly diluted Oil Red O solution. Cells were rinsed three times with PBS, stained lipids were visually assessed by microscopy. For quantitative analysis of OilRed O staining, stained lipids were extracted with 200 μl heptane–isopropanol (3:2) overnight at 4°C, and absorbance measured at 540 nm.

EFFECT OF GHRELIN-RELATED PEPTIDES ON LIPOLYSIS IN 3T3 ADIPOCYTES

3T3 adipocytes (standard differentiation) were preincubated in serum-free media for 2 h, then treated with ghrelin-related peptides in serum-free DMEM/F12. Media was collected after 3 and 24 h and frozen at −80°C. Non-esterified fatty acid (NEFA) and glycerol concentrations were measured using colorimetric enzyme assays (Wako, Osaka, Japan, and Roche Diagnostics, Indianapolis, IN, respectively).

EFFECT OF GHRELIN-RELATED PEPTIDES ON FATTY ACID TRANSPORT AND INCORPORATION IN 3T3-L1 ADIPOCYTES

3T3 adipocytes (standard differentiation) were preincubated for 2 h in serum free DMEM/F12, then treated with the indicated concentrations of ghrelin-related peptides for 1 h (200 μl, in serum-free DMEM/F12), followed by fatty acid uptake measurement. For experiments with the inhibitors growth hormone secretagogue receptor antagonist (D-lys^3)-GHRP-6, caveolae disruptor Beta-cyclodextrin, phospholipase C inhibitor U73122 and PI3 kinase inhibitor wortmannin, inhibitors at the indicated concentrations were added 30 min prior to the addition of ghrelin-related peptides.

Fatty acid uptake and incorporation using QBT^{TM} fluorescent fatty acid uptake assay kit (Molecular Devices, Sunnyvale, CA) was performed according to the manufacturer's instructions. As previously described [Cui et al., 2009], BODIPY reagent (200 μl, diluted in 0.2% BSA in Hanks Balanced salt solution) was added directly to cells containing incubation media and BODIPY-fatty acid (FA) uptake was measured in real-time every 20 s over 120 min in a bottom-reading fluorescent microplate reader (Synergy HT, Biotek). Following measurement of BODIPY-FA uptake, solutions were removed, cells washed twice gently with ice-cold PBS, and neutral lipids extracted with 200 μl of heptane–isopropanol (3:2) overnight at 4°C, and rinsed with an additional 500 μl of the same solvent mix. Lipid extracts were evaporated to dryness in a centrifuge-evaporator (Canberra-Packard Canada) and redissolved in 50 μl heptane–isopropanol (3:2). TG mass was measured using enzymatic colorimetric assay (Roche Diagnostics). Incremental-area-under-the curve of FA incorporation into adipocytes was calculated by trapezoidal method (Prism, Graphpad Software, San Diego, CA) and normalized to cell TG mass.

EFFECT OF GHRELIN-RELATED PEPTIDES ON GLUCOSE TRANSPORT IN 3T3-L1 ADIPOCYTES

3T3 adipocytes (standard differentiation) were preincubated for 2 h in serum free DMEM/F12, then treated with the indicated concentrations of ghrelin-related peptides for 1 h (200 μl, in serum-free DMEM/F12), followed by glucose transport measurement. Glucose transport assays were performed as previously described [Wen et al., 2008]. Following stimulation, cells were rinsed with warm (37°C) PBS, and glucose free DMEM media was added.

Cells were incubated with ^{3}H-2-deoxy-glucose (50 μmol/L, final specific activity 60–190 dpm/pmol) in serum-free glucose-free media at 37°C for exactly 10 min. In all experiments, zero-time controls were performed to subtract background ^{3}H-2-deoxyglucose. Following incubation, uptake was stopped with rapid washing of the cells with cold PBS, and cells were dissolved in 0.1 N NaOH. Aliquots were taken for scintillation counting. Cell protein was measured by Bradford assay (BioRad, Mississauga, ON). Glucose transport was measured as pmol ^{3}H-2-deoxy-glucose uptake per mg soluble cell protein, and expressed relative to the basal untreated cells set as 100%.

MICROARRAY ANALYSIS

Subcutaneous and omental adipose tissues were collected at time of operation from 16 men and women, non-obese and obese, as described in detail elsewhere [MacLaren et al., 2008]. Average age was 45.6 ± 11.5 (5 men, 11 women), average body mass index as 45.5 ± 17.2 (range 20–80). Omental tissue was not available from two women. Plasma lipid, glucose, and insulin values as well as detailed methodology for microarray analysis are reported elsewhere [MacLaren et al., 2008]. Ethics approval for this project was obtained from the McGill University Health Centre (Royal Victoria Hospital) ethics review committee (Montreal, QC) and subjects signed an informed consent form prior to participation.

STATISTICAL ANALYSIS

For all assays, each experiment was assayed in triplicate and repeated at least three times. Results are presented as an average of all data. Groups were compared by *t*-tests (for comparisons between two groups) or one-way ANOVA followed by Dunnett's post-hoc test for several groups using Prism software (Graphpad Software) for graphs and statistical analysis. Statistical significance was set as $P < 0.05$.

RESULTS

The effects of ghrelin-related peptides on proliferation, metabolic activity and differentiation were evaluated in 3T3-L1 preadipocytes. Four different peptides were evaluated: acyl-ghrelin (AG), desacyl-ghrelin (DAG) as well as growth-hormone-releasing peptide-6 (GHRP6) and obestatin (also derived from the same gene). Based on the plasma concentrations of obestatin, acyl ghrelin and desacyl ghrelin [Kojima and Kangawa 2005; Nakahara et al., 2008; Yano et al., 2009], physiologically relevant concentrations were used experimentally.

EFFECT OF GHRELIN-RELATED PEPTIDES ON CELL METABOLIC ACTIVITY AND CELL PROLIFERATION

Evaluation of metabolic activity, based on mitochondrial response, demonstrated that none of the ghrelin-related products affected mitochondrial activity (Fig. 1A), although the positive control insulin, at 50 nM, significantly increased mitochondrial activity. Additionally, co-treatment with both insulin and each peptide did not affect metabolic activity (data not shown).

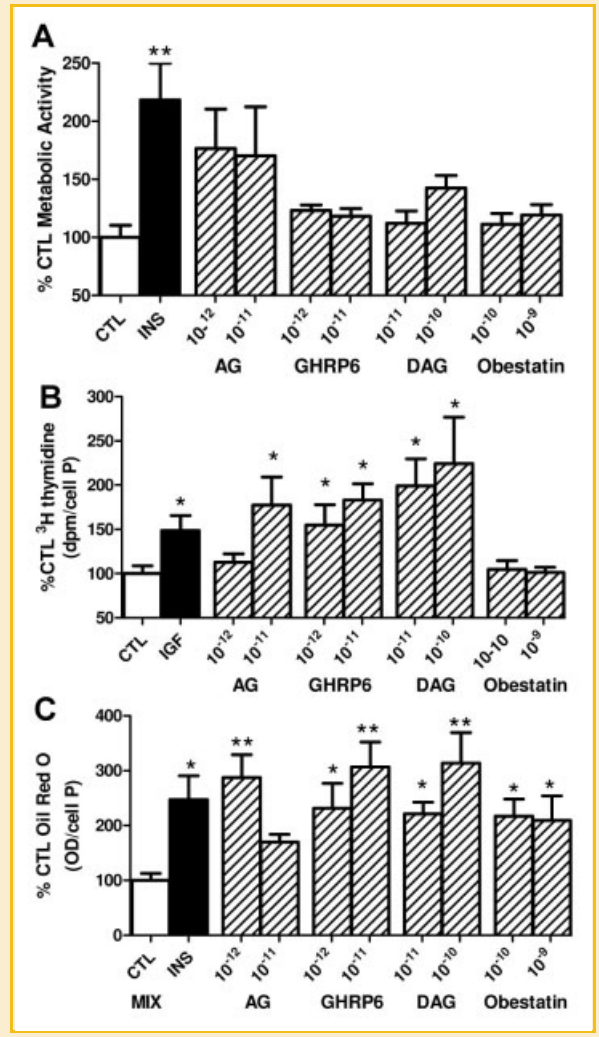

Fig. 1. Effect of ghrelin-related products on 3T3-L1 preadipocyte metabolic activity, proliferation and differentiation. A,B: Preadipocytes were preincubated with serum free media for 2 h, then treated with acylated-ghrelin (AG), GHRP-6, desacyl-ghrelin (DAG) or obestatin at the indicated concentrations for 24 h, followed by (A) incubation with MTS reagent for 3 h followed by measurement of absorbance (490 nm) or (B) incubation with ^{3}H-thymidine for 15 h followed by cell solubilization and scintillation counting. C: Preadipocytes were differentiated with standard differentiation mixture (CTL MIX), or supplemented with additional insulin (50 nM), or AG, GHRP-6, DAG or obestatin at the indicated concentrations for 9 days. Differentiation was measured based on OilRedO staining of lipids, and quantitation by absorbance (540 nm). Values were normalized to cell protein and expressed as mean ± SEM versus control (CTL, set as 100%), where n = 3–4 independent experiments with individual triplicate cells in each experiment) where $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus CTL.

As presented in Figure 1B, the effect of a treatment with DAG on cell proliferation measured by the incorporation of ^{3}H-thymidine over a 15 h period (199% and 254%, $P < 0.05$–0.01) was shown to be greater than that of the positive control, IGF-1 at 50 nM (149%, $P < 0.05$). The higher dose of AG (10 pM) and GHRP6 were also able to stimulate cell proliferation (up to 186%; $P < 0.05$), however this effect could not be detected for obestatin.

EFFECT OF GHRELIN-RELATED PEPTIDES ON ADIPOCYTE DIFFERENTIATION

As indicated in Figure 1C, the differentiation of 3T3-L1 was enhanced either by adding peptides of the ghrelin family or increasing insulin in the standard differentiating cocktail over a 9-day period. There was a significant increase in lipid accumulation (247%; $P < 0.01$) at insulin concentrations considered above standard (50 nM) for differentiation stimulation. Only the lower concentration of AG significantly increased lipid accumulation (287%, $P < 0.01$). Both 1 and 10 pM GHRP-6 led to increased lipid accumulation ($232 \pm 45\%$, $P < 0.05$ and $307 \pm 45\%$, $P < 0.01$, respectively, Fig. 1C). Similarly, DAG induced a significant increase of lipid accumulation compared to MIX control ($221 \pm 21\%$, $P < 0.05$ and 314 ± 55, $P < 0.01$, respectively). Obestatin, at both concentrations, significantly increased lipid accumulation by $217 \pm 31\%$ and $209 \pm 45\%$, respectively, $P < 0.05$. Overall, the effects on preadipocyte proliferation and differentiation-induced lipid accumulation are similar across the ghrelin-related peptides (within physiological concentrations).

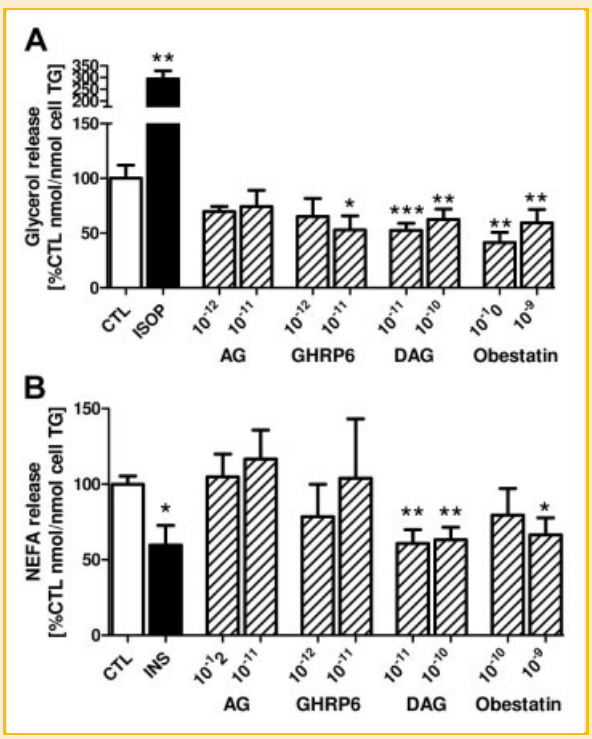

Fig. 2. Effect of ghrelin-related products on 3T3-L1 adipocyte lipolysis. 3T3-L1 adipocytes were differentiated with standard differentiation media. Adipocytes were preincubated in serum free media for 2 h, then treated with acylated-ghrelin (AG), GHRP-6, desacyl-ghrelin (DAG) or obestatin at the indicated concentrations for 3 h (A,B) or 24 h (C). Cell culture media were collected, glycerol and non-esterified fatty acid (NEFA) levels were determined and expressed relative to cell TG mass. Values are mean ± SEM normalized to control (CTL = 100%) where n = 3–4 independent experiments with individual triplicate cells in each experiment) where $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus CTL.

EFFECT OF GHRELIN-RELATED PEPTIDES ON INTRACELLULAR TG LIPOLYSIS IN DIFFERENTIATED 3T3-L1 ADIPOCYTES

Lipolysis of intracellular TG stimulates the extracellular release of glycerol and NEFA. The effects of peptides of the ghrelin family were tested over a period of 3 and 24 h treatment. As depicted in Figure 2A, following 3 h incubation, both DAG and obestatin significantly decreased glycerol release from −40% to −60% ($P < 0.05$–0.001). While AG had no effect on glycerol release, GHRP-6 at the higher concentration tested significantly decreased glycerol release by −50% ($P < 0.05$). As expected, the treatment with the positive control isoproterenol (a β-adrenergic agonist) significantly increased glycerol release ($P < 0.05$). Results on NEFA release (Fig. 2B) were similar to those of glycerol, DAG and obestatin both significantly decreased NEFA release (−40%), comparable to the decrease in glycerol. While insulin significantly inhibited NEFA release (positive control), AG and GHRP-6 had no significant effect. Similar results were obtained after 24 h incubation (data not shown).

EFFECT OF GHRELIN PEPTIDE FAMILY ON LIPID AND GLUCOSE UPTAKE IN DIFFERENTIATED 3T3-L1 ADIPOCYTES

The effects of ghrelin-related peptides on lipid storage activities (fatty acid uptake and glucose transport) were also evaluated. Following acute short term (1 h) stimulation, as shown in Figure 3A, both DAG and obestatin increased fatty acid uptake (Fig. 3A) up to 506% and 383%, respectively ($P < 0.001$). AG at the lower concentration significantly increased fatty acid uptake (265%, $P < 0.001$), while GHRP6 had an effect only at the higher concentration tested (198%, $P < 0.05$). Insulin, a known positive stimulator, significantly increased real-time fatty acid uptake by twofold ($P < 0.01$) relative to treatment with PBS (vehicle) only (CTL). Of note, the DAG and obestatin effects were significantly greater than the insulin effects when compared directly (DAG vs. insulin 2.5-fold $P < 0.001$, obestatin vs. insulin 2.3-fold $P < 0.05$).

More detailed evaluation of DAG demonstrated a concentration dependent saturating effect (Fig. 3B), while evaluation of AG, indicated a concentration dependent decrease at higher concentrations (data not shown). Evaluation of fatty acid transport after 24 h stimulation by insulin or ghrelin-related peptides demonstrated similar stimulatory effects (data not shown).

By contrast with fatty acid uptake, preincubation of 3T3 adipocytes with ghrelin-related peptides did not alter glucose transport (Table I), although, as expected, insulin treatment did significantly increase glucose transport ($P < 0.01$).

INVOLVEMENT OF GROWTH HORMONE SECRETAGOGUE RECEPTOR (GHSR1) AND DOWNSTREAM SIGNALING COMPONENTS ON DAG STIMULATION OF ADIPOCYTES

The potential involvement of the growth hormone secretagogue receptor (GHSR1) in the DAG response was evaluated using specific inhibitors of GHSR1 and of the downstream signaling components. For this purpose, [D-lys^3]-GHRP-6, shown to be a GHSR1 antagonist [Sun et al., 2010] was used. As shown in Figure 4A, 30 μM [D-lys^3]-GHRP-6 alone had no effect on basal FA uptake. However, presence of [D-lys^3]-GHRP-6 completely blocked the maximal stimulation by both concentrations of DAG ($P < 0.001$ +inhibitor vs. no inhibitor).

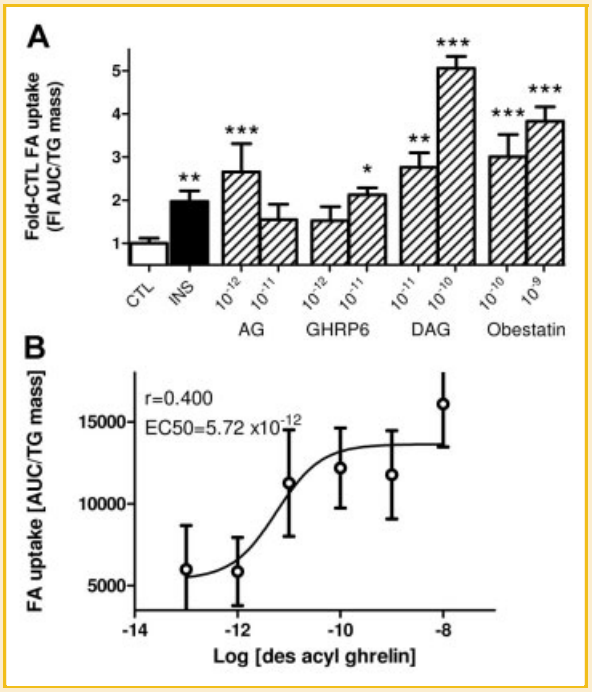

Fig. 3. Effect of ghrelin-related products on 3T3-L1 adipocyte fatty acid uptake and incorporation into lipids. 3T3-L1 adipocytes were differentiated with standard differentiation media. Adipocytes were preincubated in serum free media for 2 h, then treated with (A) acylated-ghrelin (AG), GHRP-6, desacyl-ghrelin (DAG) or obestatin at the indicated concentrations for 1 h, or (B) increasing concentrations of DAG from 10^{-13} to 10^{-7} M, followed by assessment of fatty acid (FA) uptake in 3T3-L1 adipocytes in real-time over 2 h using QBT fluorescent fatty acid (FI-FA) assay. Results are calculated as incremental area-under-the-curve (AUC) of FA uptake per nmol cell triglyceride content as mean ± SEM (B) or expressed versus control (A: CTL = 1), where n = 3 independent experiments with individual triplicate cells in each experiment) where $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus CTL. For DAG concentration curve (B), $EC_{50} = 5.72 \times 10^{-12}$ M.

Phospholipase C and PI3 kinase have all been implicated in the signaling pathway for GHSR1, while fatty acid uptake is partially dependent on maintenance of lipid rafts. 3T3-L1 adipocyte pretreatment with quaternary beta-cylodextrin (50 μM) a lipid raft disruptor, did not affect basal FA uptake when compared to control. However, 10^{-11} and 10^{-10} M DAG stimulated-FA uptake was significantly inhibited ($P < 0.001$, Fig. 4B). Incubation of unstimulated 3T3-L1 adipocytes with 40 μM U73122, a phospholipase C inhibitor, had no effect on basal FA uptake, however U73122 completely blocked DAG stimulation at both 10^{-11} and 10^{-10} M concentrations ($P < 0.001$, Fig. 4C). Similarly, wortmannin, an inhibitor of the PI3kinase catalytic site, had no effect on basal FA uptake (100 nM wortmannin, Fig. 4D), although it significantly reduced the DAG mediated stimulation of FA uptake at both DAG concentrations ($P < 0.001$).

TABLE I. Effect of Insulin, Acyl-Ghrelin, GHRP6, Desacyl-Ghrelin and Obestatin on Glucose Transport in 3T3-L1

Addition	Concentration	Mean ± SEM	ANOVA, $P < 0.0001$
CTL		21.4 ± 3.1	P value vs. CTL
Insulin	5×10^{-8}	34.1 ± 2	$P < 0.01$
Acyl-ghrelin	1×10^{-12}	24.7 ± 1.9	NS
	1×10^{-11}	22.2 ± 1.9	NS
GHRP-6	1×10^{-12}	16.1 ± 1.4	NS
	1×10^{-11}	24.6 ± 1.8	NS
Desacyl-ghrelin	1×10^{-11}	19.1 ± 3.4	NS
	1×10^{-10}	26.1 ± 2.2	NS
Obestatin	1×10^{-10}	13.2 ± 1.4	NS
	1×10^{-9}	18 ± 3.3	NS

Adipocytes. 3T3-L1 adipocytes were differentiated with standard differentiation media. Adipocytes were preincubated in serum free media for 2 h, then treated with acyl-ghrelin, GHRP-6, desacyl-ghrelin or obestatin at the indicated concentrations for 1 h followed by assessment of glucose transport. Results are calculated as pmol ^{3}H-2-deoxy-glucose uptake/μg cell protein as mean ± SEM of multiple replicates in four independent experiments (n = 12).

MICROARRAY EXPRESSION OF GHSR IN HUMAN OMENTAL AND SUBCUTANEOUS ADIPOSE TISSUE

Expression of GHSR1 in human omental and subcutaneous adipose tissue was evaluated across a weight range extending from normal body weight to severely obese, based on body mass index (BMI). Subjects included both men (n = 5) and women (n = 11), with an average age of 45.6 ± 11.5 years, however omental tissue was not available in two women. As shown in Figure 5, GHSR1 expression in omental adipose tissue correlated with BMI (Fig. 5A, r = 0.549, $P = 0.04$) as well as plasma insulin (Fig. 5B, r = 0.681, $P = 0.007$). However, there was no significant correlation of GHSR1 expression in subcutaneous adipose tissue with any physiological or plasma parameter. Further, GHSR1 expression (subcutaneous and omental tissue combined) also correlated with a number of genes involved in fatty acid metabolism although there was little correlation with glucose related genes (Table II).

DISCUSSION

There are strong indications suggesting that specific GI hormones influence important metabolic parameters. Although it may be of major relevance, their effects on the functions of specific tissues have not been thoroughly investigated. AG, DAG and obestatin, all derived from the preproghrelin transcript, have been reported to be involved in the neuroendocrine regulation of food intake and energy homeostasis, but there is little evidence of their effect on adipocyte functions. Therefore, the present study was designed to investigate effect of peptides of the ghrelin family on adipocyte functions such as proliferation, differentiation, fatty acid uptake and triglyceride synthesis. The salient results presented here are (i) in preadipocytes, DAG, AG and GHRP6 significantly increased proliferation and differentiation; (ii) in adipocytes, both DAG and obestatin had pronounced effects on stimulation of FA uptake and inhibition of NEFA and glycerol release, (iii) the effect of DAG on fatty acid uptake was blocked using GHSR1 antagonist D-(lys^{3})-GHRP6, lipid raft disruptor beta-cyclodextrin, U73122 (PLC inhibitor) and wortmannin (PI3kinase inhibitor), suggesting a GHSR1-mediated pathway, and (iv) expression of GHSR1 in omental adipose tissue was observed to be increased in obesity. The results presented here suggest that they may also play a very direct role in obesity development and the consequences thereof.

Previous studies have demonstrated that AG stimulates proliferation of a number of cell types, such as pituitary and fetal spinal

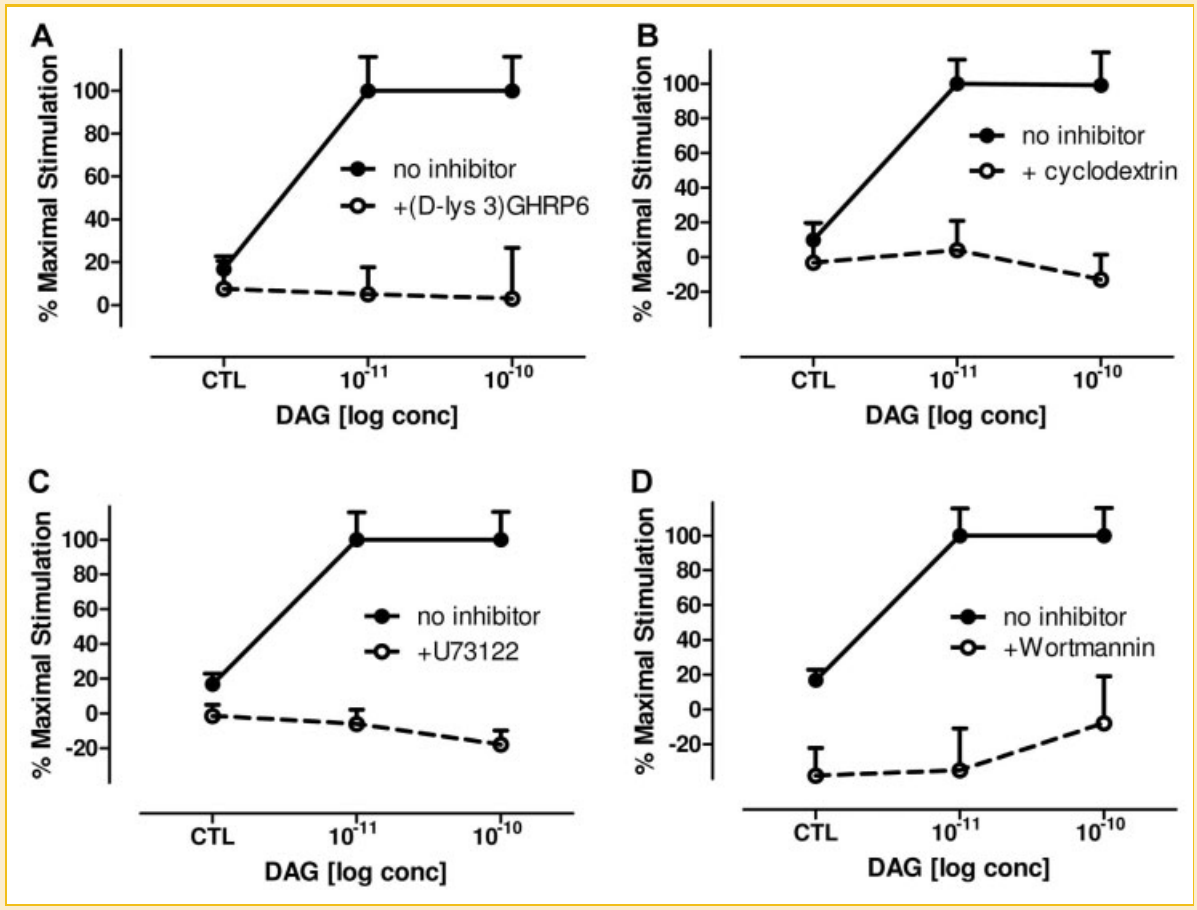

Fig. 4. Effect of GHSR1 antagonists and inhibitors on desacyl-ghrelin-induced fatty acid uptake stimulation in 3T3-L1 adipocytes. 3T3-L1 adipocytes were differentiated with standard differentiation media. Adipocytes were preincubated in serum free media for 2 h, with addition of GHSR1 antagonist, 30 μM (D-lys^3)-GHRP6 (A), caveolae inhibitor Beta-cyclodextrin (B), phospholipase C inhibitor, 40 μM U73122 (C) or phosphatidylinositol 3-kinase inhibitor, 100 nM Wortmannin (D) for the last 30 min before adding desacyl-ghrelin (DAG) at the indicated concentrations for 1 h stimulation. Fatty acid (FA) uptake in 3T3-L1 adipocytes was assessed in real-time over 2 h using QBT fluorescent fatty acid (FI-FA) assay. All inhibitors were present throughout the experiment. Results are calculated as incremental area-under-the-curve (AUC) of FA uptake per nmol cell triglyceride content as mean ± SEM, and normalized to % maximal DAG stimulation where n = 3 independent experiments with individual triplicate cells in each experiment) where $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus CTL.

cord cells [Sato et al., 2006], including 3T3-L1 preadipocytes [Kim et al., 2004; Zwirska-Korczala et al., 2007; Liu et al., 2009]. Further AG has also been shown to stimulate differentiation of adipocytes [Choi et al., 2003; Rodríguez et al., 2009] including 3T3-L1 adipocytes [Kim et al., 2004; Zhang et al., 2004; Liu et al., 2009]. The present results confirm this, at least at physiological AG concentrations. With respect to DAG, in a number of situations, the effects paralleled those of AG: stimulation of fetal spinal cord proliferation, inhibition of DU-145 cell proliferation, biphasic effect in PC-3 cells, differentiation of C2C12 skeletal myoblasts [Cassoni et al., 2004; Sato et al., 2006].

By contrast, published results with DAG in preadipocytes are far from consistent. No studies have tested the effects of DAG on proliferation of preadipocytes. With adipocyte differentiation studies, there was either no DAG tested [Kim et al., 2004; Liu et al., 2009], or DAG results were negative [Zhang et al., 2004], although two studies have suggested a positive effect on differentiation [Giovambattista et al., 2008; Rodríguez et al., 2009]. In the present study, in parallel comparisons with AG and GHRP6, we demonstrate clearly that DAG, at several concentrations, does stimulate proliferation of preadipocytes as well as differentiation of 3T3-L1 adipocytes in a manner comparable to both AG and GHRP6, and this effect is therefore consistent with activation mediated via GHSR1a.

In adipocytes, published AG and DAG functional data vary. There are evidences suggesting that AG and DAG have some similar biological effects and others that are opposite. For example, while a study by Muccioli et al. [2004] demonstrated that AG and DAG decrease lipolysis, by contrast, a study by Kos et al. [2009] demonstrated that while DAG decreased lipolysis, AG did not. Similarly, while AG alone had no effect on glucose transport, addition of AG with insulin enhanced the insulin effect, while DAG had no effect in any situation [Patel et al., 2006]. In one study, AG and DAG both stimulated leptin secretion, however, only the AG effect was blocked with GHSR1 antagonist [Giovambattista et al., 2008]. By contrast, DAG has also been shown to inhibit leptin

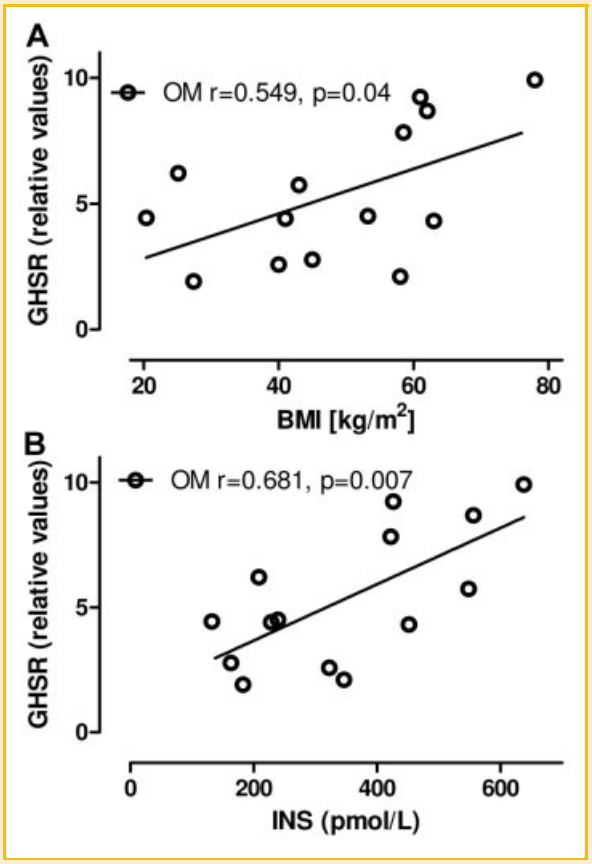

Fig. 5. Expression of GHSR1 in subcutaneous and omental human adipose tissue. Expression of GHSR1 in human omental and subcutaneous adipose tissue was evaluated by microarray analysis in 16 subjects from normal body weight to severely obese, based on body mass index (BMI:45.5 ± 17.2 kg/m^2). Subjects included 5 men and 11 women, average age 45.6 ± 11.5 years (omental samples were unavailable in 2 women). GHSR1 expression in omental adipose tissue correlated with BMI (A) and plasma insulin (B). There was no significant correlation in subcutaneous adipose tissue with any physiological or plasma parameter.

secretion [Kos et al., 2009]. Finally, while in vivo infusion of AG increased retroperitoneal adipose tissue, DAG had no effect [Davies et al., 2009]. It was suggested that this effect was mediated through GHSR1, as floxed null mice were unresponsive. In several other in vitro and in vivo studies, these peptides displayed opposite functional effects [Gauna et al., 2005]. While it is difficult to explain these discrepancies, the small number of studies, each one evaluating a different functional parameter, and the differences in hormone concentrations tested, may all have an impact.

In the present study, evaluation of the effect of peptides related to the ghrelin family demonstrated that in 3T3-L1 adipocytes, DAG, increased fatty acid uptake and triglyceride storage, while decreasing lipolysis as evidenced by the reduction in extracellular NEFA and glycerol release. To our knowledge, this is the first study to directly evaluate the effects on triglyceride metabolism.

Overall, the effects of DAG on preadipocyte proliferation and differentiation, and on adipocyte lipid storage are all consistent with a pro-lipogenic adipogenic effect. By contrast, in adipocytes,

TABLE II. Correlation of GHSR With Microarray Expression of Housekeeping, Lipid Metabolism, and Glucose Metabolism Genes in Adipose (Omental and Subcutaneous) Tissue

Gene	ACCN#	r	*P*
Housekeeping genes			
Beta-actin	M10277	0.358	NS
TFRC	NM_003234	0.167	NS
Lipid metabolism genes			
AGPAT1	NM_006411	−0.257	NS
CES1	NM_001266	−0.377	0.048
DGAT1	NM_012079	−0.575	0.001
DGAT2	NM_032564	−0.439	0.019
ELOVL1	NM_022821	−0.651	0
ELOVL2	NM_017770	0.663	0
ELOVL4	NM_022726	0.433	0.021
FABP1	NM_001443	0.053	NS
FABP2	NM_000134	0.563	0.002
FABP3	NM_004102	−0.635	0
FABP3	U17081	0.014	NS
FABP4	NM_001442	0.509	0.006
FABP5	NM_001444	0.025	NS
FABP6	NM_001445	0.504	0.006
FABP7	NM_001446	0.535	0.003
FACL2	NM_021122	−0.681	0
FACL3	NM_004457	−0.788	0
FACL4	NM_022977	−0.839	0
FACL5	NM_016234	−0.682	0
FADS2	NM_004265	0.865	0
FADS3	NM_021727	0.492	0.008
FASN	NM_004104	−0.41	0.03
LIPC	NM_000236	0.358	0.05
LIPE	NM_005357	−0.541	0.003
LPAP	NM_016361	−0.086	NS
LPL	NM_000237	0.215	NS
PDHB	NM_000925	−0.158	NS
PLIN	NM_002666	−0.273	NS
PNLIP	1255094	0.593	0.001
PPAP2A	NM_003711	−0.779	0
SCD	AB032261	−0.638	0
SLC27A4	NM_005094	0.481	0.01
TGL	NM_022034	0.556	0.002
ADFP	NM_001122	−0.639	0
ADIP-N	NM_025225	0.966	0
Glucose metabolism genes			
ME1	NM_002395	−0.183	NS
ME2	NM_002396	−0.218	NS
ME3	NM_006680	−0.287	NS
GLUT1	NM_006516	0.277	NS
GLUT2	NM_000340	−0.01	NS
GLUT3	NM_006931	0.223	NS
GLUT4	NM_001042	0.79	0
HK1	NM_000188	−0.779	0
HK2	NM_000189	0.638	0
Insulin signaling genes			
IP4	AF368319	−0.488	0.008
IRS1	NM_005544	0.538	0.003
P101-PI3K	NM_014308	−0.344	0.05
PI3KP85*	M61906	−0.387	0.042
PIK3C2G	NM_004570	0.562	0.002
PIK3CA	NM_006218	−0.707	0
PIK3CB	NM_006219	−0.481	0.01
PIK3CG	NM_002649	0.638	0
PIK3R2	NM_005027	−0.651	0
PIK3R3	NM_003629	0.628	0
PTPN1	NM_002827	0.555	0.002
Adipocyte differentiation related genes			
CEBPA	NM_004364	−0.642	0
CEBPB*	NM_005194	−0.585	0.001
CEBPD	NM_005195	−0.007	NS
CEBPE	NM_001805	0.66	0
CEBPG	NM_001806	0.587	0.001
PPARA	NM_005036	−0.013	NS
PPARD	NM_006238	0.7	0
PPARG	NM_005037	−0.628	0
PPARGC1	NM_013261	0.594	0.001

the effects of AG are clearly different (little effect on fatty acid uptake).

The question of whether DAG binds GHSR1, and whether GHSR1 is expressed in adipose tissue are two issues that have received substantial scrutiny. In the present study we therefore investigated the expression of the GHSR1 in adipose tissue. As expected, GHSR1 expression was detected both in subcutaneous and omental human adipose tissue. Furthermore the expression of the GHSR1 in omental tissue positively correlates with BMI as well as insulin levels, and a number of expressed genes. Meanwhile a recent review by Muccioli et al. [2007] has proposed that there may be up to four different receptors for ghrelin-related peptides, including GHSR1a, GHSR1b, and two additional putative GHS receptors (currently uncloned) based on published ligand-binding groupings, cell signaling, and functional in vitro and in vivo responses. It is still debated as to whether DAG binds GHSR1. However, a recent detailed study of AG and DAG ligand-binding, using HEK cells transfected with GHSR1a Gauna et al. [2007], clearly demonstrated that (i) DAG binds GHSR1a and displaces AG binding; (ii) DAG binding was blocked with specific antagonists BIM28163 and [D-lys^3]GHRP6; and (iii) DAG was active. Several groups have failed to detect GHSR1a in adipose tissue, while others have reported its presence, binding properties and increased expression during adipocyte differentiation. The presence of GHSR1b in adipose tissue has also been demonstrated [Gnanapavan et al., 2002]. Further, it has been suggested that GHSR1a may signal through Ca^{2+}, while GHSR1b signals through phospholipase C [Giovambattista et al., 2008]. The present results are consistent with DAG stimulation mediated through GHSR1, as the specific antagonist [D-lys^3]GHRP6 blocked DAG induced stimulation. Further, inhibition of phospholipase C and PI3Kinase pathways and disruption of lipid rafts also blocked DAG-mediated effect, again consistent with a GHSR1 pathway.

The role of obestatin and its interaction with its putative receptor GPR39, remain controversial [Gourcerol et al., 2007]. Following the claim that GPR39 was a receptor for obestatin, numerous groups were unable to replicate this data [reviewed in Epelbaum et al., 2010]. However it has been recently suggested that inconsistencies in the binding of ^{125}I-obestatin to GPR39 may be due to variations in the bioactivity of iodinated obestatin [Zhang et al., 2008]. Regardless, obestatin binds in a specific and saturable manner to cells [Epelbaum et al., 2010]. However, as with demonstration of the nature of its receptors, the physiological effects of obestatin appear difficult to reproduce. The present results demonstrate that obestatin does not display a proliferative effect in preadipocytes, in contrast to the published proliferative effects in ovarian, retinal pigment epithelial cells, beta-cells and human islets [Tang et al., 2008]. However, we demonstrate clearly that the effects of obestatin generally paralleled those of DAG, stimulating preadipocyte differentiation, adipocyte fatty acid uptake and inhibiting lipolysis.

CONCLUSIONS

In this study, we show for the first time that DAG and obestatin stimulated preadipocyte proliferation, adipocyte differentiation, fatty acid uptake and inhibited lipoysis in 3T3-L1 adipocytes. These results highlight the fact that these gastrointestinal peptides variously affect cell metabolism. This study also supports that DAG may be acting through the GHRS1 pathway in adipocytes. Together, these results suggest that DAG and obestatin could stimulate adipose tissue hyperplasia and hypertrophy by various mechanisms. This study provides evidence that ghrelin-related peptides might simultaneously interact to maintain and exacerbate an obese phenotype.

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REFERENCES

Allende-Vigo MZ. 2010. Adipocytes and Cardiometabolic Risk. Endocr Pract 3:1–21.

Asakawa A, Inui A, Fujimiya M, Sakamaki R, Shinfuku N, Ueta Y, Meguid MM, Kasuga M. 2005. Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. Gut 54(1):18–24.

Baldanzi G, Filigheddu N, Cutrupi S, Catapano F, Bonissoni S, Fubini A, Malan D, Baj G, Granata R, Broglio F, Papotti M, Surico N, Bussolino F, Isgaard J, Deghenghi R, Sinigaglia F, Prat M, Muccioli G, Ghigo E, Graziani A. 2002. Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. J Cell Biol 159(6): 1029–1037.

Boney CM, Gruppuso PA, Faris RA, Frackelton AR, Jr. 2000. The critical role of Shc in insulin-like growth factor-I-mediated mitogenesis and differentiation in 3T3-L1 preadipocytes. Mol Endocrinol 14(6):805–813.

Cassoni P, Papotti M, Ghè C, Catapano F, Sapino A, Graziani A, Deghenghi R, Reissmann T, Ghigo E, Muccioli G. 2001. Identification, characterization, and biological activity of specific receptors for natural (ghrelin) and synthetic growth hormone secretagogues and analogs in human breast carcinomas and cell lines. J Clin Endocrinol Metab 86(4):1738–1745.

Cassoni P, Ghé C, Marrocco T, Tarabra E, Allia E, Catapano F, Deghenghi R, Ghigo E, Papotti M, Muccioli G. 2004. Expression of ghrelin and biological activity of specific receptors for ghrelin and des-acyl ghrelin in human prostate neoplasms and related cell lines. Eur J Endocrinol 150(2):173–184.

Catalan V, Gomez-Ambrosi J, Rotellar F, Silva C, Gil MJ, Rodriguez A, Cienfuegos JA, Salvador J, Fruhbeck G. 2007. The obestatin receptor (GPR39) is expressed in human adipose tissue and is down-regulated in obesity-associated type 2 diabetes mellitus. Clin Endocrinol (Oxf) 66:598–601.

Chen CY, Asakawa A, Fujimiya M, Lee SD, Inui A. 2009. Ghrelin gene products and the regulation of food intake and gut motility. Pharmacol Rev 61(4):430–481.

Choi K, Roh SG, Hong YH, Shrestha YB, Hishikawa D, Chen C, Kojima M, Kangawa K, Sasaki S. 2003. The role of ghrelin and growth hormone secretagogues receptor on rat adipogenesis. Endocrinology 144(3):754–759.

Colinet FG, Vanderick S, Charloteaux B, Eggen A, Gengler N, Renaville B, Brasseur R, Portetelle D, Renaville R. 2009. Genomic location of the bovine growth hormone secretagogue receptor (GHSR) gene and investigation of genetic polymorphism. Anim Biotechnol 20(1):28–33.

Cui W, Lapointe M, Gauvreau D, Kalant D, Cianflone K. 2009. Recombinant C3adesArg/acylation stimulating protein (ASP) is highly bioactive: A critical evaluation of C5L2 binding and 3T3-L1 adipocyte activation. Mol Immunol 46(16):3207–3217.

Dass NB, Hill J, Muir A, Testa T, Wise A, Sanger GJ. 2003. The rabbit motilin receptor: Molecular characterisation and pharmacology. Br J Pharmacol 145(5):948–954.

Davies JS, Kotokorpi P, Eccles SR, Barnes SK, Tokarczuk PF, Allen SK, Whitworth HS, Guschina IA, Evans BA, Mode A, Zigman JM, Wells T. 2009. Ghrelin induces abdominal obesity via GHS-R-dependent lipid retention. Mol Endocrinol 23(6):914–924.

Druce MR, Neary NM, Small CJ, Milton J, Monteiro M, Patterson M, Ghatei MA, Bloom SR. 2006. Subcutaneous administration of ghrelin stimulates energy intake in healthy lean human volunteers. Int J Obes (Lond) 30(2):293–296.

Epelbaum J, Bedjaoui N, Dardennes R, Feng DD, Gardette R, Grouselle D, Loudes C, Simon A, Tolle V, Yang SK, Zizzari P. 2010. Role of the ghrelin/obestatin balance in the regulation of neuroendocrine circuits controlling body composition and energy homeostasis. Mol Cell Endocrinol 314(2):244–247.

Fontenot E, DeVente JE, Seidel ER. 2007. Obestatin and ghrelin in obese and in pregnant women. Peptides 28(10):1937–1944.

Gauna C, Delhanty PJ, Hofland LJ, Janssen JA, Broglio F, Ross RJ, Ghigo E, Van Der Lely AJ. 2005. Ghrelin stimulates, whereas des-octanoyl ghrelin inhibits, glucose output by primary hepatocytes. J Clin Endocrinol Metab 90(2):1055–1060.

Gauna C, van de Zande B, van Kerkwijk A, Themmen AP, Van Der Lely AJ, Delhanty PJ. 2007. Unacylated ghrelin is not a functional antagonist but a full agonist of the type 1a growth hormone secretagogue receptor (GHS-R). Mol Cell Endocrinol 274(1–2):30–34.

Giovambattista A, Gaillard RC, Spinedi E. 2008. Ghrelin gene-related peptides modulate rat white adiposity. Vitam Horm 77:171–205.

Gnanapavan S, Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, Bhattacharya S, Carpenter R, Grossman AB, Korbonits M. 2002. The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. J Clin Endocrinol Metab 87(6):2988.

Gourcerol G, Coskun T, Craft LS, Mayer JP, Heiman ML, Wang L, Million M, St-Pierre DH, Taché Y. 2007. Preproghrelin-derived peptide, obestatin, fails to influence food intake in lean or obese rodents. Obesity (Silver Spring) 15(11):2643–2652.

Gutierrez-Grobe Y, Villalobos-Blasquez I, Sánchez-Lara K, Villa AR, Ponciano-Rodríguez G, Ramos MH, Chavez-Tapia NC, Uribe M, Méndez-Sánchez N. 2010. High ghrelin and obestatin levels and low risk of developing fatty liver. Ann Hepatol 9(1):52–57.

Holst B, Holliday ND, Bach A, Elling CE, Cox HM, Schwartz TW. 2004. Common structural basis for constitutive activity of the ghrelin receptor family. J Biol Chem 279(51):53806–53817.

Kim MS, Yoon CY, Jang PG, Park YJ, Shin CS, Park HS, Ryu JW, Pak YK, Park JY, Lee KU, Kim SY, Lee HK, Kim YB, Park KS. 2004. The mitogenic and antiapoptotic actions of ghrelin in 3T3-L1 adipocytes. Mol Endocrinol 18(9):2291–2301.

Kirchner H, Gutierrez JA, Solenberg PJ, Pfluger PT, Czyzyk TA, Willency JA, Schürmann A, Joost HG, Jandacek RJ, Hale JE, Heiman ML, Tschöp MH. 2009. GOAT links dietary lipids with the endocrine control of energy balance. Nat Med 15(7):741–745.

Kojima M, Kangawa K. 2005. Ghrelin: Structure and function. Physiol Rev 85:495–522.

Kos K, Harte AL, O'Hare PJ, Kumar S, McTernan PG. 2009. Ghrelin and the differential regulation of des-acyl (DSG) and oct-anoyl ghrelin (OTG) in human adipose tissue (AT). Clin Endocrinol (Oxf) 70(3):383–389.

Liu J, Lin H, Cheng P, Hu X, Lu H. 2009. Effects of ghrelin on the proliferation and differentiation of 3T3-L1 preadipocytes. J Huazhong Univ Sci Technol Med Sci 29(2):227–230.

MacLaren R, Cui W, Simard S, Cianflone K. 2008. Influence of obesity and insulin sensitivity on insulin signaling genes in human omental and subcutaneous adipose tissue. J Lipid Res 49(2):308–323.

Merrifield RB, 1963. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J Am Chem Soc 85:2149.

Muccioli G, Pons N, Ghè C, Catapano F, Granata R, Ghigo E. 2004. Ghrelin and des-acyl ghrelin both inhibit isoproterenol-induced lipolysis in rat adipocytes via a non-type 1a growth hormone secretagogue receptor. Eur J Pharmacol 498(1–3):27–35.

Muccioli G, Baragli A, Granata R, Papotti M, Ghigo E. 2007. Heterogeneity of ghrelin/growth hormone secretagogue receptors. Toward the understanding of the molecular identity of novel ghrelin/GHS receptors. Neuroendocrinology 86(3):147–1164.

Nakahara T, Harada T, Yasuhara D, Shimada N, Amitani H, Sakoguchi T, Kamiji MM, Asakawa A, Inui A. 2008. Plasma obestatin concentrations are negatively correlated with body mass index, insulin resistance index, and plasma leptin concentrations in obesity and anorexia nervosa. Biol Psychiatry 64(3):252–255.

Neary NM, Druce MR, Small CJ, Bloom SR. 2006. Acylated ghrelin stimulates food intake in the fed and fasted states but desacylated ghrelin has no effect. Gut 55(1):135.

Patel AD, Stanley SA, Murphy KG, Frost GS, Gardiner JV, Kent AS, White NE, Ghatei MA, Bloom SR. 2006. Ghrelin stimulates insulin-induced glucose uptake in adipocytes. Regul Pept 134(1):17–122.

Pemberton CJ, Richards AM. 2008. Biochemistry of ghrelin precursor peptides. Vitam Horm 77:13–30.

Pfluger PT, Kirchner H, Günnel S, Schrott B, Perez-Tilve D, Fu S, Benoit SC, Horvath T, Joost HG, Wortley KE, Sleeman MW, Tschöp MH. 2008. Simultaneous deletion of ghrelin and its receptor increases motor activity and energy expenditure. Am J Physiol Gastrointest Liver Physiol 294(3):G610–G618.

Rodríguez A, Gómez-Ambrosi J, Catalán V, Gil MJ, Becerril S, Sáinz N, Silva C, Salvador J, Colina I, Frühbeck G. 2009. Acylated and desacyl ghrelin stimulate lipid accumulation in human visceral adipocytes. Int J Obes (Lond) 33(5):541–552.

Sato M, Nakahara K, Goto S, Kaiya H, Miyazato M, Date Y, Nakazato M, Kangawa K, Murakami N. 2006. Effects of ghrelin and des-acyl ghrelin on neurogenesis of the rat fetal spinal cord. Biochem Biophys Res Commun 350(3):598–603.

Soares JB, Leite-Moreira AF. 2008. Ghrelin, des-acyl ghrelin and obestatin: Three pieces of the same puzzle. Peptides 29(7):1255–1270.

Sun Q, Zang WJ, Chen C. 2010. Growth hormone secretagogues reduce transient outward K+ current via phospholipase C/protein kinase C signaling pathway in rat ventricular myocytes. Endocrinology 151(3):1228–1235.

Tang SQ, Jiang QY, Zhang YL, Zhu XT, Shu G, Gao P, Feng DY, Wang XQ, Dong XY. 2008. Obestatin: Its physicochemical characteristics and physiological functions. Peptides 29(4):639–645.

Taub DD. 2008. Novel connections between the neuroendocrine and immune systems: The ghrelin immunoregulatory network. Vitam Horm 77:325–346.

Tremblay F, Perreault M, Klaman LD, Tobin JF, Smith E, Gimeno RE. 2007. Normal food intake and body weight in mice lacking the G protein-coupled receptor GPR39. Endocrinology 148(2):501–506.

Wen Y, Wang H, MacLaren R, Wu J, Lu H, Cianflone K. 2008. Palmitate and oleate induction of acylation stimulating protein resistance in 3T3-L1 adipocytes and preadipocytes. J Cell Biochem 104(2):391–401.

Yano Y, Toshinai K, Inokuchi T, Kangawa K, Shimada K, Kario K, Nakazato M. 2009. Plasma des-acyl ghrelin, but not plasma HMW adiponectin, is a useful cardiometabolic marker for predicting atherosclerosis in elderly hypertensive patients. Atherosclerosis 204(2):590–594.

Zhang W, Zhao L, Lin TR, Chai B, Fan Y, Gantz I, Mulholland MW. 2004. Inhibition of adipogenesis by ghrelin. Mol Biol Cell 15(5):2484–2491.

Zhang W, Chai B, Li JY, Wang H, Mulholland MW. 2008. Effect of des-acyl ghrelin on adiposity and glucose metabolism. Endocrinology 149(9):4710–4716.

Zwirska-Korczala K, Adamczyk-Sowa M, Sowa P, Pilc K, Suchanek R, Pierzchala K, Namyslowski G, Misiolek M, Sodowski K, Kato I, Kuwahara A, Zabielski R. 2007. Role of leptin, ghrelin, angiotensin II and orexins in 3T3 L1 preadipocyte cells proliferation and oxidative metabolism. J Physiol Pharmacol 58(Suppl 1):53–64.