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Effects of the ghrelin gene-derived peptides on adipose browning and thermogenesis

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ABSTRACT

Introduction: White adipose tissue (WAT) stores excess energy as triglycerides, while brown adipose tissue (BAT) dissipates energy through heat, acting as a defence against cold and obesity and as a positive regulator of metabolic functions. BAT thermogenic functions are mainly induced by mitochondrial uncoupling protein-1 (UCP-1), which induces uptake of lipids and glucose to sustain oxidation and thermogenesis in both brown and beige adipocytes. The ghrelin gene-derived peptides, acylated ghrelin (AG), unacylated ghrelin (UAG) and obestatin (Obe), are key regulators of energy homeostasis, as well as glucose and lipid metabolism. *In vivo* studies suggest that AG, via the growth hormone (GH) secretagogue receptor type 1a (GHSR-1a), exerts inhibitory effects on UCP-1 mRNA expression and noradrenaline release in BAT; moreover, ablation of GHSR-1a in mice increased BAT thermogenic functions. However, the role of the ghrelin peptides in adipose browning and BAT function is yet unknown. Thus, we aimed to assess the role of AG, UAG and Obe in promoting adipocyte browning. **Methods:** 3T3-L1 murine preadipocytes were differentiated into white adipocytes for 7 days and, to induce browning, the cells were further incubated for 72 h with 0.5 μM rosiglitazone and 1 μM insulin (transdifferentiation medium). Mature adipocytes isolated from subcutaneous (SC) and omental (OM) adipose tissue were placed into culture with DMEM/F12 containing FBS 1% and insulin 17 nM and incubated at 37 °C for 24 h. To assess the effects of ghrelin gene peptides on browning of adipocytes, the cells were treated with AG, UAG and Obe (500 nM) during transdifferentiation and then we evaluated the mRNA expression of BAT-specific genes and lipolytic/lipogenic genes (by real-time PCR), as well as proteins by Western blot analysis, and lipid droplets accumulation by Oil-Red O staining. Lipolysis was also studied by ELISA and regulation of mitochondrial fatty acid uptake and oxidation by real time PCR. **Results:** UAG and Obe, but not AG, increased the mRNA levels of BAT genes (*Ucp-1*, *Pgc-1α*, *Prdm16*, *Cd137* and *Dio2*) and

proteins (UCP1, PGC-1α and PRDM16). Furthermore, UAG and Obe increased the expression of lipolytic genes (*Cpt-1a* and *Sirt1*), while reducing the lipogenic gene *C/ebp* in 3T3-L1 and human adipocytes. Moreover, in 3T3-L1 UAG and Obe increased the number of small lipid droplets, characteristics of brown adipocytes, induced the expression of lipolytic enzymes and increased the isoproterenol-induced glycerol release, suggesting a role of this peptides in mitochondrial activity and fatty acids oxidation. Finally, both UAG and Obe, but not AG, promoted the mRNA expression of the main browning genes in human SC and OM adipose tissue. **Conclusions:** Our findings indicate that UAG and Obe, but not AG, promote browning of adipocytes and suggest that these peptides could represent novel potential therapeutic candidates for the treatment of obesity and metabolic diseases.

INTRODUCTION

1. ADIPOSE TISSUE

In a world struggling with a pandemic of obesity, there is a constant need to seek for new therapeutic strategies for its treatment. The pathogenesis of simple obesity assumes an imbalance between the energy intake and expenditure that leads to the accumulation of energy surplus in the form of adipose tissue (AT). Therefore, the available methods for obesity treatment are based on either reducing calorie intake (diets, pharmacological approaches, bariatric surgery) or increasing energy expenditure (physical activity). AT is comprised of different cells located in the intercellular matrix. These cells include adipocytes, preadipocytes, adipose stem cells (ASCs), fibroblasts as well as endothelial, nerve and immune cells whose interactions are fundamental for AT homeostasis [1], [2], [3]. AT is composed mainly by adipocytes, which are predominantly white adipocytes in the white adipose tissue (WAT), and brown adipocytes in the brown adipose tissue (BAT). Consequently, WAT and BAT have different structures and biological roles. White adipocytes have a single large lipid droplet occupying most of the cell volume with few mitochondria, dislocating the nucleus peripherally. Brown adipocytes are polygonal cells containing several small lipid droplets (therefore, called multilocular adipose tissue), with a central nucleus surrounded by a clear cytoplasm and large amounts of mitochondria [4]. Also, WAT and BAT have different origins and progenitor cells, and many adipogenesis mediators [5]. WAT is found throughout the body, being divided into visceral (around organs – mesenteric, perigonadal, omental) and SC (under the skin – inguinal) depots. BAT is found in specific regions that comprises interscapular, subscapular, axillary, perirenal and periaortic regions in rodents, and cervical, supraclavicular, paravertebral, mediastinal and perirenal regions in humans [6]. WAT represents the main energy reservoir of the body,

while BAT is characterized by energy dissipation through thermogenesis. Both WAT and BAT function as endocrine tissues, signaling to other organs through *adipokines* (WAT) and *batokines* (BAT) [7], [8]. *Brite* adipocytes were reported as a new type of adipocytes set in WAT but resembling to brown adipocytes phenotype. In the basal state, *brite* adipocytes act as white adipocytes, but under the adequate stimulus they might transform into brown-like adipocytes [9]. Recent studies have indicated that human BAT is a *brite* adipocyte that was originally white, but, under stimulation acquired a brown-like phenotype [10]. Thus, human white adipocytes can be converted into *brite* adipocytes with beneficial metabolic consequences [11]. In newborns, nonshivering thermogenesis in brown adipose tissue (BAT) plays a significant role in maintaining body temperature, while in older individuals this mechanism seems to be less pronounced since BAT was thought to fade with age. However, the development of modern imaging techniques such as positron emission tomography (PET) has enabled the location of BAT deposits in various areas of the adult body. It has also been found that brown adipocyte-like cells can be dispersed in WAT. Moreover, the amount of BAT positively correlates with the energy expenditure, and in obese subjects is significantly lower than in slim individuals. Therefore, strategies aimed at induction and/or activation of BAT could be potentially useful in the treatment of obesity [12]

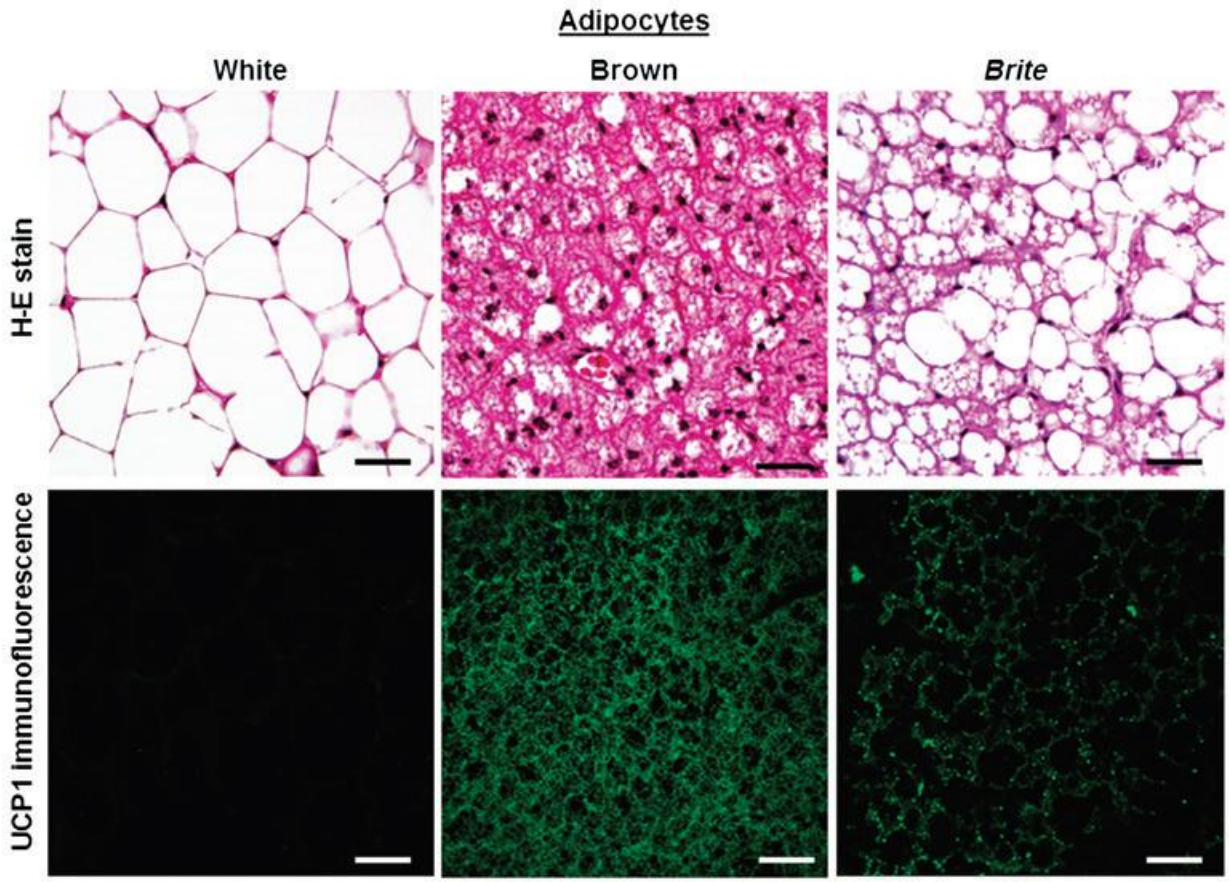

Figure 1: Adipocytes (Bargut et al.: Browning in experimental models, 2017).

1.1 Browning of adipocytes and underlying mechanisms

BAT has become central in obesity research because of adaptive thermogenesis, the process of regulated heat production that is mediated by the catabolism of energy substrates without the release of chemical energy from the breakdown of adenosine triphosphate. This process is piloted by UCP1, a transmembrane protein located in the mitochondrial membrane in brown adipocytes; it participates in adaptive thermogenesis by uncoupling the production of adenosine triphosphate from the catabolic pathways of lipids and carbohydrates [13]. The energy produced is released by brown adipocytes in form of heat that diffuses in the body, thanks to the rich vascularization of BAT. UCP1

is exclusively expressed in BAT and an increase in BAT mass in obese patients may improve energy dissipation. A possible way to increase the expression of UCP1 in AT is the conversion of white (pre)adipocytes into brown-like fat cells, known as browning of WAT[14]. The browning of white adipose tissue is mainly driven by sympathetic stimulation and by the interaction of norepinephrine (NE) with β3-adrenergic receptors (β3-ARs) present on the plasma membrane of white adipocytes. This interaction triggers a signal transduction cascade leading to the overexpression of uncoupling protein 1 (UCP1) and other thermogenic proteins [13].

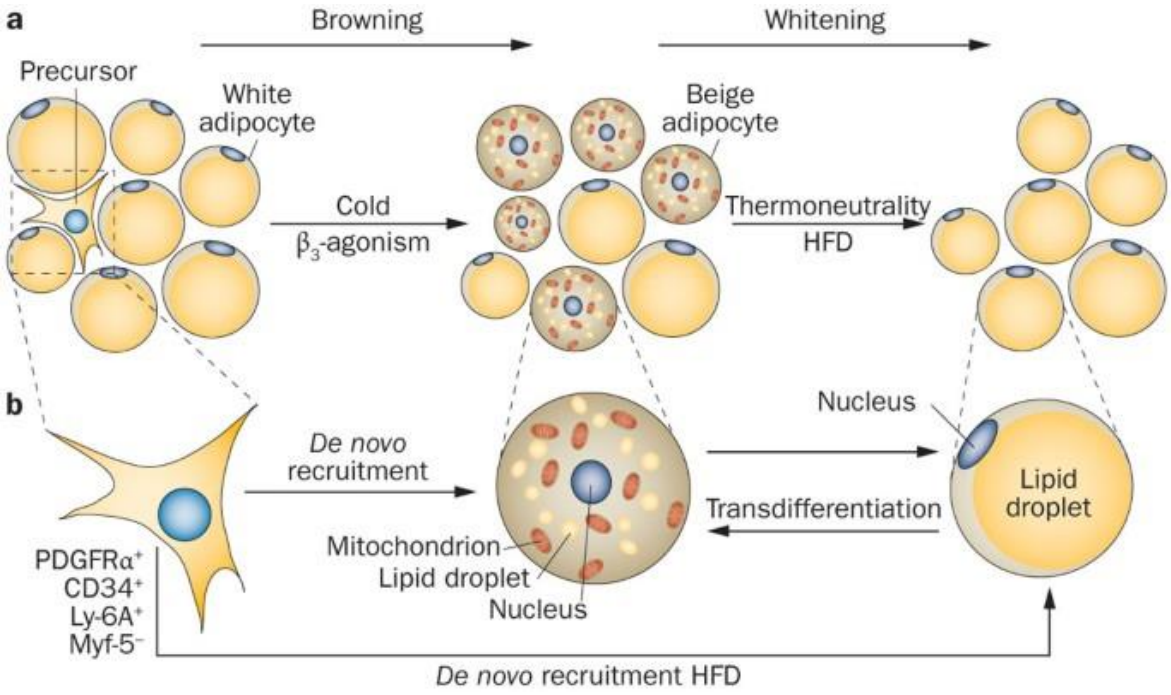

Figure 2: Browning of adipose tissue is an adaptive and reversible response to environmental challenges

1.2 Endogenous browning factors

As previously mentioned, UCP1 expression is under adrenergic control: the activation of β3-ARs by NE triggers a signal transduction cascade that involves a number of enzymes and transcription factors that, either directly or indirectly, affect the expression level of UCP1 [15]. The interaction of NE with β3-ARs causes the activation of a linked stimulatory G protein (Gs) that, in turn, activates the membrane enzyme adenylyl cyclase (AC). AC stimulates the production of cyclic adenosine monophosphate (cAMP), which is the necessary activator of protein kinase A (PKA). The PKA-dependent transduction pathways that lead to the overexpression of UCP1 are p38 mitogen-activated protein kinase (MAPK), which stimulates the expression of the PPARγ coactivator-1α (PGC-1α) and the activating transcription factor 2, that are directly involved in the overexpression of UCP1; cAMP response element binding protein, which directly binds on UCP1 promoter in a p38 MAPK independent manner [15], [16]. The MAPK signalling pathway is linked to phosphorylation of a set of important transcription factors, including cAMP-response element binding protein (CREB), which controls expression of type 2 iodothyronine deiodinase (DIO2) [15], [17]. DIO2, in turn, converts inactive tetraiodothyronine (T4) to triiodothyronine (T3) in brown adipose tissue and thereby increases the activation of thyroid hormone receptors as well as UCP1 expression. The MAPK pathway also phosphorylates cAMP-dependent transcription factor ATF-2, which in turn initiates transcription of *Ucp1* and *Ppargc1a* (encoding the PPARγ cofactor PGC-1α) [18]. Surprisingly, primary white adipocytes isolated from inguinal and epididymal fat depots, but not primary brown adipocytes, increase *Ucp1* and *Ppargc1a* mRNA levels *in vitro* at temperatures below 37 °C, independently of the CREB pathway [19]. This phenomenon was also observed in cold-exposed mice that lack all three β-adrenergic receptor types, which suggests that a certain proportion of the

thermogenesis that occurs in WAT could result from a novel, as yet undefined mechanism [19].

1.3 Regulation mediated by the PGC-1α–PPAR complex

PGC-1α is differentially expressed in BAT and skeletal muscle upon cold exposure [20], and is probably considered the most important regulatory protein in thermogenesis. PGC-1α activated the expression of UCP1 and other key mitochondrial enzymes of the respiratory chain, such as Cox4, increasing mitochondrial biogenesis [20]. PGC-1α binds to complexes of PPARα or PPARγ and retinoid X receptor (RXR), which both activate UCP1 expression by binding to a PPAR response element in the UCP1 promoter. Thus, treating adipocytes with PPARα, PPARγ or RXR agonists results in increased transcription of UCP1 [21], [22]. Importantly, activation of UCP1 expression via PPAR response elements in its promoter involves a positive feedback loop, resulting from the induction of Ppargc1a [23]. This mechanism might also be fine-tuned by direct negative regulators of PGC-1α, such as twist basic helix-loop-helix transcription factor 1, which is co-induced by PPARδ [23].

1.4 PR domain zinc finger protein 16 (PRDM16) - mediated regulation

The activity of the PGC-1α–PPAR complex is modulated by another BAT-specific cofactor, PR domain zinc finger protein 16 (PRDM16). This cofactor is highly enriched in brown adipocytes compared with white adipocytes and is essential for the development of brown adipocytes. In mice, PRDM16 suppresses classic white adipocyte genes by interacting with C-terminal-binding proteins, and stimulates the transcription

of several proteins involved in thermogenesis in WAT (including PGC-1α, UCP1 and DIO2) [24], [25]. When expressed in adipose tissue, PRDM16 contributes to the high levels of UCP1 in inguinal WAT [26]. Notably, PRDM16 is also required for the WAT remodelling induced by β3-adrenergic signalling [26]. Moreover, chronic rosiglitazone-mediated activation of PPARγ induces PGC-1α [27] and stabilizes PRDM16 protein, which increases browning [28].

2 THE GHRELIN GENE PEPTIDES

Ghrelin is a 28-amino acid acylated peptide identified by Kojima et. al in 1999 in rat stomach as endogenous ligand for growth-hormone secretagogues-receptor1 a (GHSR-1a). It is mainly synthesized by the endocrine cells of the oxyntic mucosa of the stomach and largely found in other tissues like intestine, pituitary and hypothalamus [29]. Ghrelin was originally identified as a growth hormone secretagogue, but further studies underlined its important role in the regulation of body weight and appetite. Ghrelin shows many effects, in particular it regulates gastric acid secretion, blood glucose control, increase drug and food addiction, promotes renal protection and has various effects on cardiovascular system [30]. The clear preprandial rise and postprandial fall in plasma ghrelin levels support the hypothesis that ghrelin plays a physiological role in meal initiation [31]. The effects of this hormone result from the activation of central, vagal and enteric neural receptors as well as receptors on immune cells, underlining ghrelin involvement in neuroprotection and in immune function improvement [32]. Recently, a new literature shows some involvement of ghrelin genetic variants in several neoplastic conditions [33]. GHSR-1a is a G protein-coupled receptor (GPCRs) involved in many regulatory effects on physiology and behavior. Stimulation of GHSR-1a by acylated ghrelin triggers the phospholipase C (PLC) signal, leading to increase inositol

phosphate (IP) turnover and protein kinase C (PKC) activation, resulting in the release of calcium from intracellular stores. GHSR-1a activation also inhibits K+ channels, allowing the entry of calcium through voltage-gated L- and T-type channels [34]. Human ghrelin derives from a 117-residue prepropeptide by post-translational cleavage. It exists in two different forms in our body: unacylated ghrelin (UAG) and acylated (AG). Acylation on the third serine residue is necessary to activate GHSR-1a, thanks to ghrelin O-acyltransferase (GOAT) enzyme [35]. Surprisingly, AG comprises <10% of the total circulating ghrelin but performs most of the central ghrelin functions [36], [37]. Further studies on the precursor peptide, searching for putative hormone derived from the same prepropeptide, discovered another secreted and bioactive peptide named obestatin. Obestatin, a 23-amino acid peptide hormone, was isolated in 2005 by Zhang and colleagues from the rat stomach. It is a peptide with a molecular mass of 2516.3 Da and its amino-acid sequence is FNAPFDVGIKLSGAQYQQHGRAL [37]. Zhang and colleagues compared preproghrelin gene in 11 species and, on the basis of bioinformatic prediction, identified that the predicted sequence of obestatin, is 100% identical in rat and mice. This is an evidence that this hormone peptide plays key role in metabolic process common to all these species and it is preserved through the evolution [38]. Noteworthy, and similar to ghrelin, the biological activity of the ghrelin-associated peptide obestatin also depends on modification: obestatin requires amidation at its C-terminal glycine residue to exert its effects [37]. To characterize endogenous obestatin effects, Zhang and colleagues synthesized the peptide and performed radioimmunoassays on rat-tissue extract with obestatin-specific antibody. They showed its function on food intake in adult mice, by intraperitoneal and intracerebroventricular injection of obestatin and in association with ghrelin. Treatment with obestatin revealed a reduction of body-weight and suppression of emptying activity and jejunal motility, antagonized the stimulatory effect of ghrelin in rodent [37]. This initial investigation on

food intake gave the hormone its name: the word obestatin is a contraction of obese and derives from Latin “obedere”, meaning “to devour” and “statin”, denoting suppression [39]. Although obestatin and ghrelin derived from the same gene growth hormone secretagogue receptor ligand (GHRL), they showed opposite effects on food intake, body weight and metabolic control. Obestatin appears to function as part of a complex gut-brain network whereby hormones and substances from the stomach and intestines signal the brain about satiety or hunger [40]. In contrast to ghrelin, obestatin seems to exert orexigenic activity, decreasing food intake and reducing ghrelin stimulatory effect on growth hormone (GH) secretion. However, various experimental conditions *in vivo* showed that obestatin itself or in co-administration with ghrelin has no inhibitory effect on food intake and body weight gain; this suggested controversial behavior of this hormone [41].

2.1 Preproghrelin gene

The human ghrelin gene is located on the chromosome 3p25–26 (Fig.3) and it is constituted by five exons and three introns in 141, 258, 367 of a 511bp DNA [30]. The 5’flanking is 2000 bp region upstream the start codon and contains putative binding sites for several transcription factors, such as activator protein 2 (AP2), basic helix–loop–helix (bHLH), polyomavirus enhancer activator 3 (PEA-3), hepatocyte nuclear factor-5, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and half sites for estrogen and glucocorticoid response elements [42]. Two different transcripts (forms A and B) were originated from two distinct transcriptional initiation sites: one at -80 and another at -555 relative to the ATG initiation codon. Studies revealed that transcript A is the main form of human ghrelin mRNA in vivo, an alternative splicing product from exon 2 to exon 4.

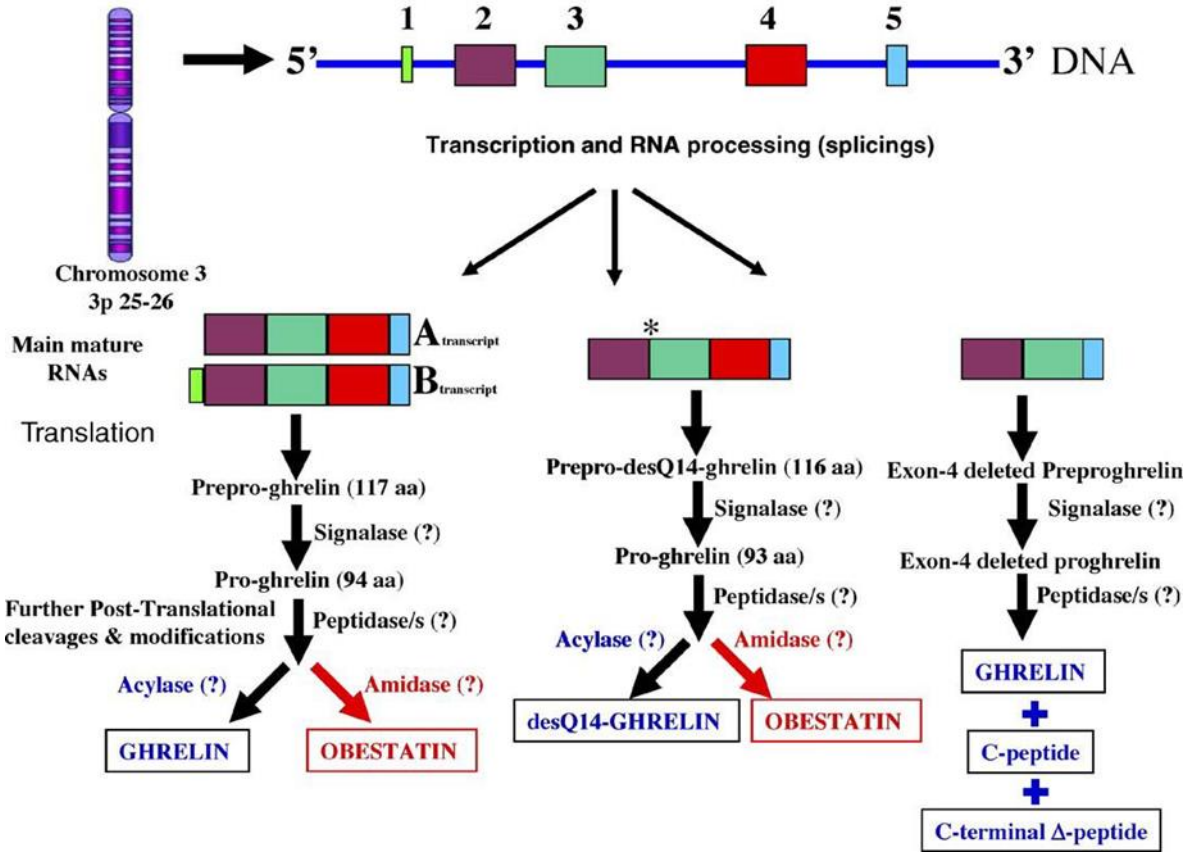

Figure 3. One ancestor, several peptides post-translational modifications of preproghrelin generate several peptides with antithetical effects. (Adapted from Gualillo et al., 2006)

In rodent, pig and human ghrelin genes, complementary DNA analyses revealed a truncated splicing variant lacking the codon Gln14(CAG) encodes a des-Gln14-ghrelin precursor. Des-Gln14-ghrelin is identical to ghrelin, except for the deletion of Gln14. However, human des-Gln14-ghrelin plasma level is low and negligible [38]. Human ghrelin mRNA, form A, codes for a 117 amino acid peptide, called preproghrelin. Ontogenetic studies on ghrelin gene products underlined the key role of prohormone convertase 1/3 (PC 1/3) processing from preproghrelin to ghrelin and possibly obestatin in rat pancreas and stomach [43].

2.4 Ghrelin receptor gene

The GHSR is encoded by a conserved single-copy gene (*GHSR*) located on chromosome 3 in humans [44], and is composed of two exons, whose alternative splicing can generate two mRNA species, named GHSR1a and GHSR1b. GHSR1a mRNA encodes a 366-amino acid G protein-coupled receptor (GPCR) with seven transmembrane domains (TMDs). Conversely, GHSR1b mRNA is formed by retention of the intron between exons 1 and 2, which generates a 289-amino acid GPCR isoform with only five TMDs and having a dissimilar 24-amino acid sequence at the C-terminal region compared with the GHSR1a sequence. To date, the functional activity of truncated GHSR1b remains to be fully elucidated [44], while it is well-established that GHSR1a is the receptor responsible for transducing the signal of AG and the family of synthetic GH secretagogues (GHSs; [45]). In fact, the interaction of GHSR1a and AG is determined by the conformational flexibility introduced by ghrelin when it is modified with Ser3 acylation.

2.3 Additional receptors for ghrelin gene-derived peptides

The family of receptors that mediate the actions of ghrelin gene-derived peptides is yet to be fully identified. The presence of specific uncharacterized GHSR(s), different from GHSR1a, has been previously suggested in cardiomyocytes, human erythroleikemic HEL cells and chondrocytes [44]. Furthermore, the existence of a common receptor that mediates the biological effects shared by AG and UAG has also been postulated in breast cancer cell lines, cardiomyocytes, human prostatic tumors, and related cell lines [46]. Also, the receptor(s) for obestatin are still not determined. This peptide was initially reported to bind and activate the orphan G-protein-coupled receptor 39 (GPR39; [37]),

this notion is still a matter of debate [47],[48]; more recently, the glucagon-like peptide-1 receptor (GLP-1R) has been postulated as an alternative receptor for obestatin ([49][50]). Similarly, the receptor(s) that mediate(s) the biological actions of the recently identified splicing variants (In1-ghrelin, Ex3-deleted ghrelin, etc.) is/are still unknown.

2.4 Ghrelin-O-acyl-transferase

Ghrelin Ser3 can be post-translationally modified by GOAT [51][35], an enzyme that belongs to the super-family of membrane bound O-acyltransferases (MBOATs; [52]), which is also named MBOAT4, located on chromosome 8p12 . GOAT is a polytopic integral membrane protein that octanoylates Ser3 of proghrelin in the endoplasmic reticulum (ER) lumen after signal peptide cleavage [53]. GOAT enzyme can process a range of fatty acids, being the most likely acyl donors for the acyl-coAs, infact GOAT has been postulated to be a putative transporter of acyl-coA from the cytosol to the ER lumen [35]. GOAT mRNA is expressed in human tissues such as pancreas, intestine, skeletal muscle, heart, bone and stomach [54]. Indeed, GOAT protein and mRNA transcripts exist in individual ghrelin-containing cells as is the case of gastric mucosal oxyntic cells [55]. Moreover, some studies have shown that GOAT mRNA expression levels do not exactly correlate with ghrelin expression levels, but they can be compared to the expression levels of the In1-ghrelin variant [56].

2.5 Ghrelin Gene-Derived Peptides and Adipocytes

Expression of the ghrelin gene products, both at the mRNA and protein level, has been demonstrated in human and rodent adipocytes [57][58][59]. Similarly, GHSR-1a is expressed in adipocytes and in rodent and human adipose tissue [60][61][59], although

opposite findings have been also shown [62][47]. AG and UAG also bind to common receptor(s) in adipocytes, suggesting the existence of a yet unknown receptor, likely different from GHSR-1a [62]. In vivo, both intracerebroventricular and intraperitoneal administration of AG in rodents induces adiposity [63][64] and promotes the increase of fat storage-promoting enzymes in WAT [65]. Also UAG, like AG, has been shown to promote adiposity in vivo and the effects of both peptides were found to be either independent of GHSR-1a [66] or of the AG orexigenic effect [22][67]. Interestingly, mice overexpressing UAG in adipose tissue showed reduced fat mass and resistance to obesity induced by high fat diet; moreover, these mice demonstrated improved insulin sensitivity and glucose tolerance, suggesting positive effects of UAG in glucose metabolism [68]. Furthermore, in GHSR-1A deleted mice UAG suppressed the expression of genes involved in lipid metabolism, particularly those involved in lipogenesis, in WAT and muscle, suggesting direct, GHSR-independent action of UAG to improve insulin sensitivity and metabolic profile [69]. AG adipogenic action was also observed in brown adipose tissue (BAT), where AG either decreased or increased the expression of uncoupling protein (UCP) mRNA [65]. Another study failed to demonstrate any effect of AG on BAT accumulation, although increased mitogen-activated protein kinase activity and decreased adiponectin gene expression was observed [70]. In rats, both intracerebroventricular and intraperitoneal administration of AG reduced noradrenaline release in BAT, suggesting an important role of AG in the regulation of energy metabolism by suppression of the sympathetic nervous system innervating brown adipocytes [71][72]. AG has been also found to promote leptin secretion from rat white adipocytes [73], whereas UAG exhibited opposite effects in human adipocytes [58]. GHSR-1a ablation in aging mice was shown to reduce glucose/lipid uptake and lipogenesis in WAT and to increase thermogenic capacity in BAT, suggesting that GHSR-1a is an important regulator of lipid metabolism during

normal aging [74]. In agreement with most in vivo data, different studies have reported ghrelin-induced adipocyte differentiation in vitro [60][61][75]. In 3T3-L1 preadipocytes, AG promotes cell proliferation and glucose uptake, inhibits apoptosis, activates PI3K/Akt and ERK1/2 signaling and induces adipocyte differentiation [75]. Conversely, ghrelin overexpression in 3T3-L1 adipocytes and AG administration to these cells was also reported to reduce adipocyte differentiation, through increased cell proliferation and mitogen-activated protein kinase activity, and inhibition of peroxisome proliferator activator receptor-γ (PPAR-γ) [76]. In human adipocytes isolated from subcutaneous abdominal fat UAG, but not AG, showed inhibition of lipolysis [58]. Accordingly, recent data indicated that UAG improves glucose metabolism and reduces lipolysis in vivo in healthy volunteers [77]. Furthermore, AG infusion in humans acutely induced lipolysis and insulin resistance independently of GH and cortisol secretion [78]. Recently, obestatin effects have been demonstrated in adipocytes, both in vitro and in vivo. These studies suggested that obestatin plays a positive role on adipocyte function and glucose metabolism [50], [59], [61]. Obestatin stimulates proliferation and inhibits apoptosis in 3T3-L1 adipocytes, and its antiapoptotic effect involves activation of PI3K/Akt and ERK1/2 signaling [49], [61]. Obestatin promotes differentiation of both 3T3-L1and human subcutaneous (SC)- and omental (OM)-derived adipocytes and increases expression of adipogenic genes [49], [57], [59]. Furthermore, it inhibits lipolysis in 3T3-L1 cells [49], [59]and human SC and OM adipocytes isolated from both lean and obese individuals [49]. Similar inhibitory results were obtained for nonesterified fatty acid (NEFA) release in 3T3-L1 cells [61]. Furthermore, in human SC adipocytes, obestatin, used in the nanomolar range, increased adiponectin and reduced leptin secretion [49] . Moreover, it promoted glucose uptake in both 3T3-L1 [49], [57] and human SC adipocytes, as well as translocation of the glucose transporter GLUT4 [50]. Sirtuin 1 (SIRT1), which has been reported to increase glucose transport and insulin signaling in

adipocytes [79], was increased by obestatin and involved in obestatin stimulatory effect on glucose uptake, as demonstrated by studies using small interfering RNA (siRNA) [50]. Obestatin also increased PI3K/Akt phosphorylation in 3T3-L1 and human SC adipocytes, as well as the Akt downstream pathways GSK-3β, mTOR and S6K1. Moreover, Akt activation was demonstrated in vivo in WAT of rats infused with obestatin [50] [57]. Obestatin also potently increased free fatty acid uptake in 3T3-L1 adipocytes [61]. Interestingly, obestatin showed binding specificity for both 3T3-L1 and human SC preadipocytes and differentiated adipocytes, and its binding was displaced by both the GLP-1R agonist exendin-4 (Ex-4) and the antagonist exendin-9 (Ex-9). Notably, obestatin binding in 3T3-L1 preadipocytes and adipocytes was prevented by siRNA against GLP-1R [50]. In agreement with the previous findings in pancreatic β-cells [49], these results suggest specific obestatin interaction with GLP-1R in adipocytes. Obestatin secretion in both 3T3-L1 and human SC and OM adipocytes was increased during differentiation [50], suggesting that obestatin may be a novel adipokine exerting autocrine/paracrine effects, in line with other studies [59].

AIM OF THE STUDY

WAT stores excess energy as triglycerides, while BAT dissipates energy through heat, acting as defence against cold and obesity and as a positive regulator of metabolic functions. BAT activity and browning of WAT are increased by cold exposure, exercise and catecholamines, and promote fatty acid oxidation and UCP-1 activity through β-adrenergic receptors. The ghrelin gene peptides, AG, UAG and Obe, play an important role in regulating energy metabolism, lipid metabolism, adipogenesis and energy expenditure. *In vivo* studies have demonstrated that AG, through its binding with the growth hormone (GH) secretagogue receptor type 1a (GHSR-1a), exert inhibitory effects on UCP-1 mRNA expression and noradrenaline release in BAT. Moreover, ablation of GHSR-1a in mice increased thermogenic capacity of BAT by enhancing mitochondrial biogenesis. Despite the evidences about the effects of AG on BAT, little is known on the actions of UAG and Obe, that have been often shown to antagonize or exert opposite metabolic effects compared to AG. However, there are still no data about the role of these peptides in browning of adipocytes. Thus, aim of this study was to investigate the effects of AG, UAG and Obe in promoting the browning of WAT and the regulation of BAT activity. In particular we studied the effects of the ghrelin gene peptides on browning, lipolysis and fatty acids oxidation in 3T3-L1 preadipocytes transdifferentiated in brite adipocytes. Moreover, we analyzed the browning effects of AG, UAG and Obe in human mature adipocytes isolated from subcutaneous (SC) and omental (OM) adipose tissue of obese individuals.

MATERIALS AND METHODS

3T3-L1 cell culture

3T3-L1 murine preadipocytes (ATCC) were maintained in Dulbecco Modified Eagle's Medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS) (Life Technologies). Differentiation was induced in confluent cells by replacing DMEM with differentiation medium (DM) consisting of 5 µg/ml insulin, 1 µM dexamethasone (Dexa), and 0.5 mM IBMX in DMEM with 10% fetal bovine serum (FBS) (Sigma-Aldrich). After 2 days, cells are switched to maintenance medium consisting of DMEM with 10% FBS and 1 µg/ml insulin (Sigma-Aldrich), for up to day 7. For differentiation of white adipocytes 3T3-L1 in brown adipocytes, at day 7 of differentiation, cells were treated with transdifferentiation medium (0.5 µM rosiglitazone (Sigma-Aldrich) and 1 µM insulin) for 72 h with or without Dexamethasone (Sigma-Aldrich). The cells were cultured al 37 °C in a 5% CO2 humidified atmosphere.

Human individuals

Subcutaneous (SAT) and omental (OAT) AT explant from obese individuals were obtained during bariatric surgery at the Department of Surgical Sciences, University of Turin. SAT explants were also obtained from elective plastic surgery of lean adult individuals at the Department of Reconstructive and Aesthetic Plastic Surgery, University of Turin.

Isolation of human mature adipocytes

Human subcutaneous and omental adipose tissue was washed and minced carefully, followed by digestion in DMEM/F12 containing 750 µg/ml Collagenase II, 2% BSA and 15 mM Hepes at 37°C for 2 h. Undigested tissue is removed by filtration using 100 µm nylon

filter. After centrifugation, floating cells are considered mature adipocytes while the pellet contains preadipocytes. The floating mature adipocytes were washed three times with PBS 1X and after centrifugation were put into culture with DMEM/F12 containing FBS 1% and Insulin 17 nM and incubate at 37 °C for 24 h with or without AG, UAG or Obe (500 nM).

Oil Red O Staining

Transdifferentiated 3T3-L1 adipocytes were fixed with 3% paraformaldehyde (Thermofisher) for 20 min and washed twice with phosphate buffered saline (PBS) 1X. The fixed cells were then stained using oil red O solution (Sigma-Aldrich) for 30 min and washed with distilled water. After drying, the fixed cells were imaged under light microscopy at 20X.

Real-Time PCR

Total RNA isolation and reverse transcription to cDNA (1 μg RNA) from 3T3-L1 and human adipocytes treated with TRIzol reagent (Life Technologies) were performed as described previously [80]. cDNAs were treated with DNA-free DNase (Life Technologies) and the reaction performed with 50 ng cDNA, 100 nM of each primer and SYBR-green Mastermix (Euroclone) using the ABI-Prism 7300 (Applied Biosystems). 18S rRNA was used as endogenous control. Relative quantification was performed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. Primers were designed with the Primer 3 Software (http://www.primer3.org/). Sequences of primer sets used in this study are listed in Table 1.

Table 1:

Gene	Forward	Reverse
3T3-L1		
Ucp1	5'-GGTTTTGCACCACACTCCTG-3'	5'-GTACATGGACATCGCACAGC-3'
Prdm16	5'ATCCACAGCACGGTGAAGCCAT-3'	5'-ACATCTGCCCACAGTCCTTGCA-3'
Pgc-1α	5'-ATGTGCAGCCAAGACTCTGTA-3'	5'-CGCTACACCACTCTAACTCAC-3'
Tmem26	5'-GATGGCTTTGGACTTGGGTA-3'	5'- GTCAACGGTCCCGTAAATTG-3'
Cd137	5' -GGTCTGTGCTTAAGACCGGG-3'	5'-TCTTAATAGCTGGTCCTCCCTC-3'
Dio2	5'-GTGTCAACACGCATTTCAT-3'	5'-GCTCATCTCCTATGACCTACGG-3'
Cpt-1α	5'GGCATAAACGCAGAGCATTCCTG-3'	5'-CAGTGTCCATCCTCTGAGTAGC-3'
C/ebp	5'-GCAAAGCCAAGAAGTCGGTGGA-3'	5'-CCTTCTGTTGCGTCTCCACGTT-3'
Atgl	5'-GGAACCAAAGGACCTGATGACC-3'	5'-ACATCAGGCAGCCACTCCAACA-3'
Hsl	5'-GCTCATCTCCTATGACCTACGG-3'	5'-TCCGTGGATGTGAACAACCAGG-3'
Human		
UCP1	5'-AGTTCCTCACCGCAGGGAAAGA-3'	5'-GTAGCGAGGTTTGATTCCGTGG-3'
PRDM16	5'-CAGCCAATCTCACCAGACACCT-3'	5'-GTGGCACTTGAAAGGCTTCTCC-3'
PGC-1a	5'-CCAAAGGATGCGCTCTCGTTCA -3'	5'-CGGTGTCTGTAGTGGCTTGACT-3'
18s	5'-GTGGAGCGATTTGTCTGGTT-3'	5'-CGCTGAGCCAGTTCAGTGTA-3'

Western blotting

3T3-L1 adipocytes were lysed in RIPA buffer (Sigma-Aldrich) and protein concentrations were calculated as previously described (2). Proteins (70 μg) were resolved in 11% SDS-PAGE and transferred to a nitrocellulose membrane. After blocking with 5% Bovine Serum Albumin (BSA) in Tris-buffered saline with 0.1% Tween (Sigma-Aldrich) for 1 h at room temperature, membranes were incubated overnight at 4 °C with the specific antibody UCP-1, PGC-1α, PRDM16 and ATGL (diluition 1:1000). Blots were reprobed with actin (diluition 1:500) for protein normalization. Immunoreactive proteins were visualized using horseradish peroxidase-conjugated anti-rabbit or anti-mouse (1:4000) secondary antibodies by enhanced chemiluminescence using ChemiDoc XRS (Bio-Rad, Milan, Italy). Each experiment was performed in triplicate. Densitometric analysis was performed with Quantity One Software (Bio-Rad, Milan, Italy).

Lipolysis assay

Lipolysis was evaluated by measuring the amount of glycerol released into the medium. Transdifferentiated 3T3-L1 adipocytes were treated with AG, UAG or Obe (500 nM) in the presence or absence of isoproterenol (ISO, 100 nM) for three hours. Following the manufacturer's instructions, 50 microliters of medium was then transferred into a new 96-well plate for glycerol measurement on a microplate reader at 570 nm (nm (Eti System Fast Reader ELX; BioTek Instruments, Winooski, VT, USA). The amount of glycerol release was calculated as micromoles per milligram of protein and expressed as percentage of ISO-induced lipolysis.

Statistical Analysis

Results are presented as mean ± SEM. Significance was calculated by unpaired two-tailed Student's *t*-test or one-way ANOVA followed by followed by Dunnett's or Tukey's *post-hoc* test, as appropriate, using GraphPad Prism v.5 (GraphPad Software, SanDiego, CA, USA).

Study approval

The study protocol for the use of human adipose tissue explants was approved by the local ethics committee [Ethics Committee of A.O.U. Città della Salute e della Scienza of Turin, Turin, Italy (Protocol number 0021413, February 26, 2021)] and all the individuals provided informed written consent before surgery.

RESULTS

The ghrelin gene derived peptides induce browning of adipocytes

To investigate the possible browning effect of the ghrelin gene peptides, 3T3-L1 adipocytes were treated with different doses of AG, UAG and Obe (0-500 nM), and expression levels of *Ucp1* were examined by Real-time PCR. Our results show that all AG, UAG and Obe induced mRNA expression of *Ucp1*, with the most relevant effect observed at concentration 500 nM, a concentration that was used for the next experiments. In particular, as expected, AG had less effect on *Ucp1* mRNA expression than UAG and Obe (**Fig. 1A**). Next, 3T3-L1 adipocytes were treated with 500 nM of AG, UAG and Obe, and the expression levels of beige-specific genes were determined. We used 1 μM β-3 adrenergic agonist CL316246 as positive control for all the experiments. The results revealed that UAG and Obe, but not AG, increased the mRNA levels of distinct beige markers such as *Pgc-1α, Prdm16, Cd137* and *Dio2* (**Fig. 1B-E**). The increased expression of UCP1, PGC-1α and PRDM16 was also confirmed at the protein level by Western blotting analysis (**Fig. 1F**).

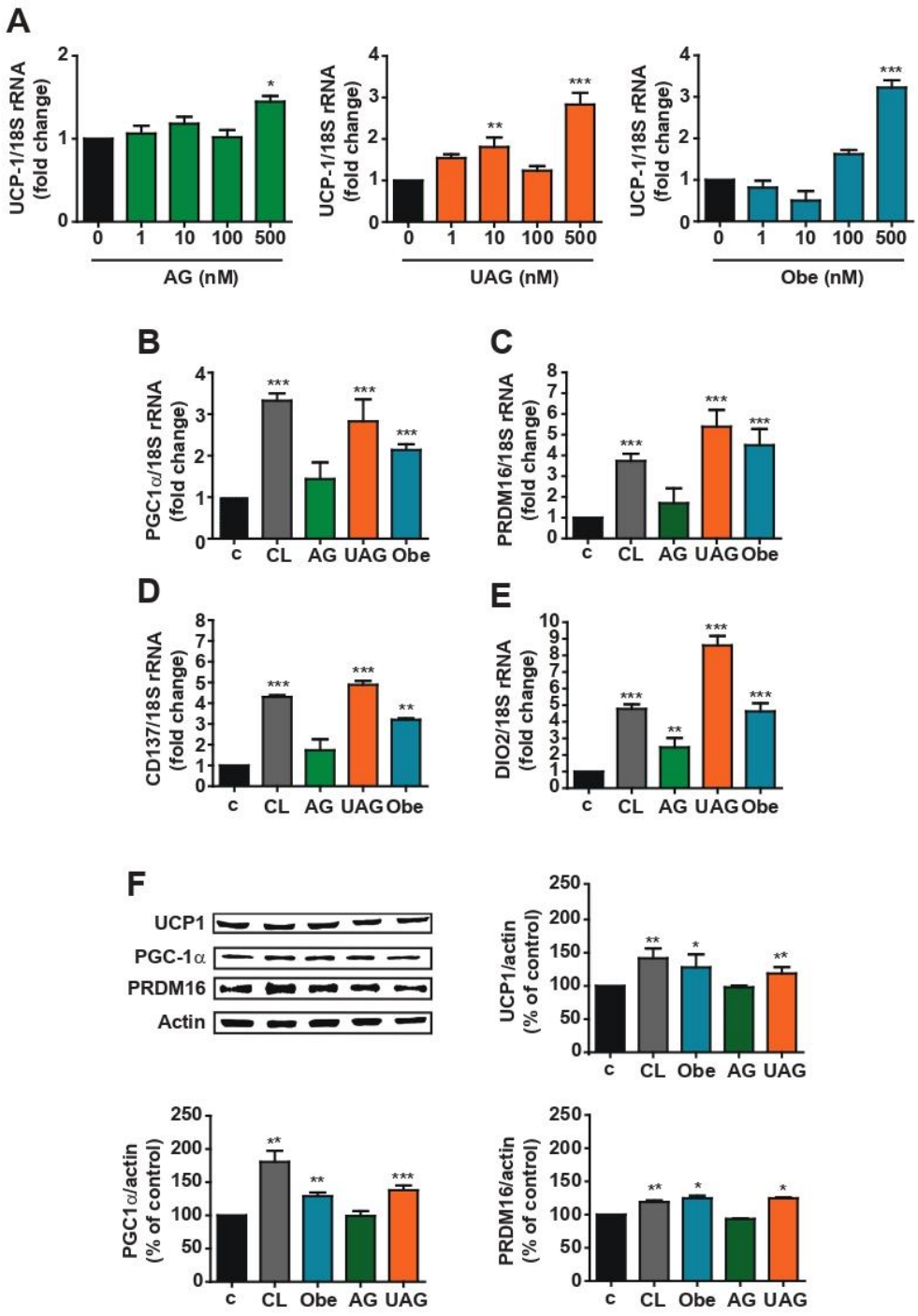

Figure 1: Effect of the ghrelin gene peptides on expression of brown adipocyte-specific genes and proteins. 3T3-L1 adipocytes were treated with the β3-AR agonist CL316246 (CL,1µM), AG, UAG or Obe (500 nM) during transdifferentiation. The mRNA expression levels of browning genes were evaluated by real-time PCR (n=5), 1-way ANOVA and Tukey's post hoc test. (**A-E**) and protein expression by Western blot (n=3), 1-way ANOVA and Tukey's post hoc test. (**F**). Results are mean ± SEM. (*P<0.05; **P<0.01; ***P<0.001 vs c).

The ghrelin gene peptides regulate lipid metabolism in 3T3-L1 adipocytes

To determine the effects of the ghrelin gene peptides on lipid accumulation, the formation of lipid droplets was detected using Oil Red O (ORO) staining. ORO staining showed a reduction in the formation of lipid droplets in 3T3-L1 adipocytes treated with UAG and Obe, but not with AG (**Fig. 2A**). Furthermore, our data demonstrate that UAG and Obe led to significant elevation of marker of β-oxidation *Cpt1α* and *Sirt1* (**Fig. 2B-C**), while decrease the mRNA expression of lipogenetic gene *C/ebp* (**Fig. 2D**), suggesting enhanced fat oxidation upon UAG and Obe treatment. Lipolysis was further measured by the content of glycerol released in the culture medium. As expected, UAG and Obe, but not AG, increased the Isoproterenol-induced glycerol release, suggesting a role of these peptides in mitochondrial activity (**Fig. 2E**).

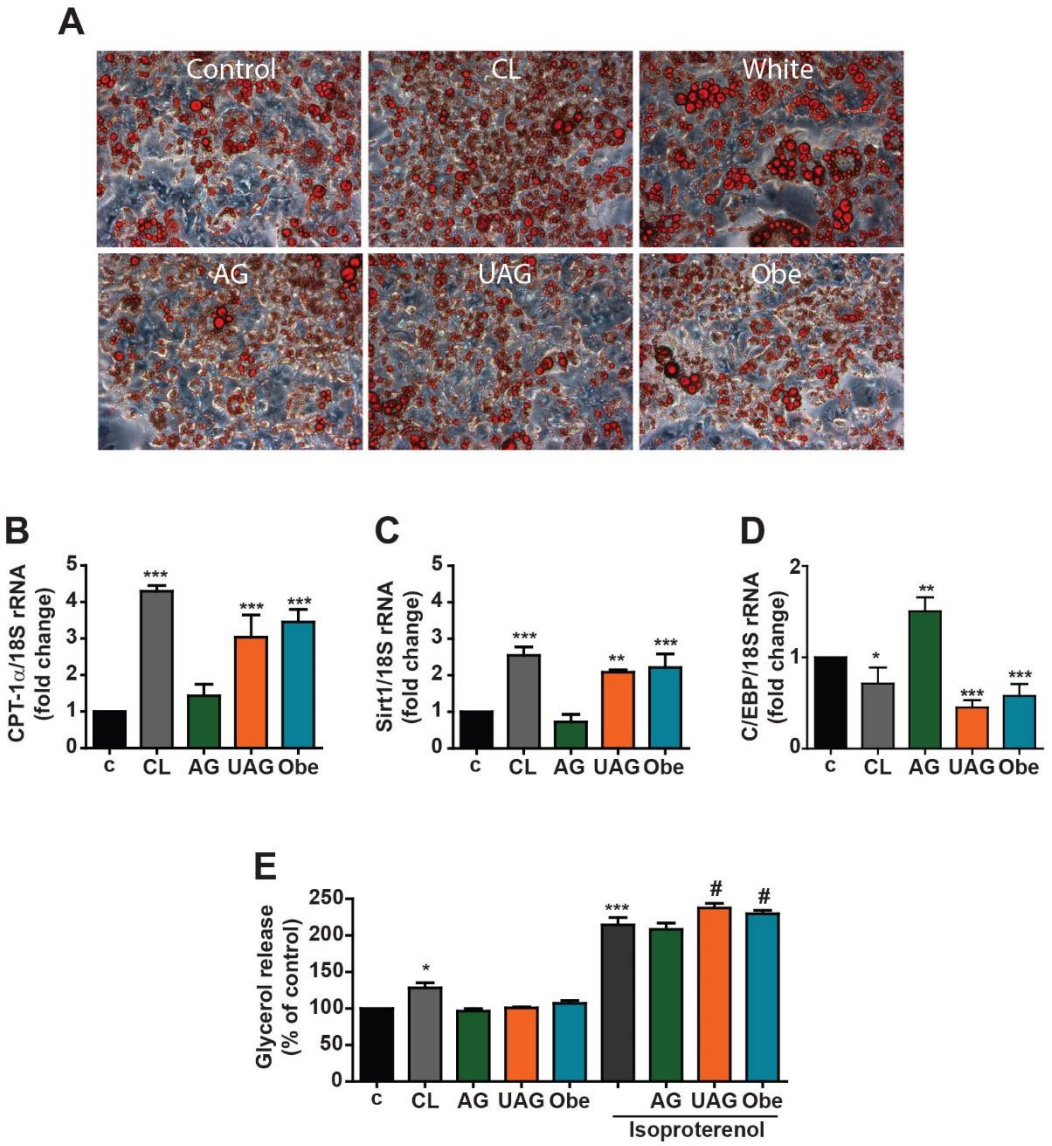

Figure 2: Effect of ghrelin gene peptides on lipid metabolism. (A) Representative images of Oil Red O staining were taken at 20 X and *scale bars* = 50 µm. (**B-D**) The mRNA expression of *Cpt-1α, Sirt1* and *C/ebp* was evaluated by real-time PCR (n=4), 1-way ANOVA and Tukey's post hoc test. Results are mean ± SEM. (*P<0.05; **P<0.01; ***P<0.001 vs c). (**E)** Lipolysis was measured in the presence or absence of 100 nM Isoproterenol (ISO) which was added for 3 hours with or without β3-AR agonist CL 316243 (1 µM) or AG, UAG or Obe 500 nM) The results are expressed as percentage of glycerol release compared to the control (n = 4), 1-way ANOVA and Tukey's post hoc test. Results are mean ± SEM. (*P<0.05; **P<0.01; ***P<0.001 vs c; #P<0.05 vs Isoproterenolo).

The ghrelin gene peptides induce the expression of lipolytic enzymes HSL and ATGL

In order to better understand the influence of the ghrelin gene peptides on lipid metabolism, we evaluated the expression levels of hormone-sensitive lipase (*Hsl*) and adipocyte triglyceride lipase (*Atgl*). As shown in Fig. 3, UAG and Obe increased the mRNA expression of *Hsl* and *Atgl*, while AG had no effect on *Hsl* and it had a significant reduction on *Atgl* (**Fig. 3A-B**). The protein expression of ATGL was also confirmed by Western blot analysis (**Fig. 3C**).

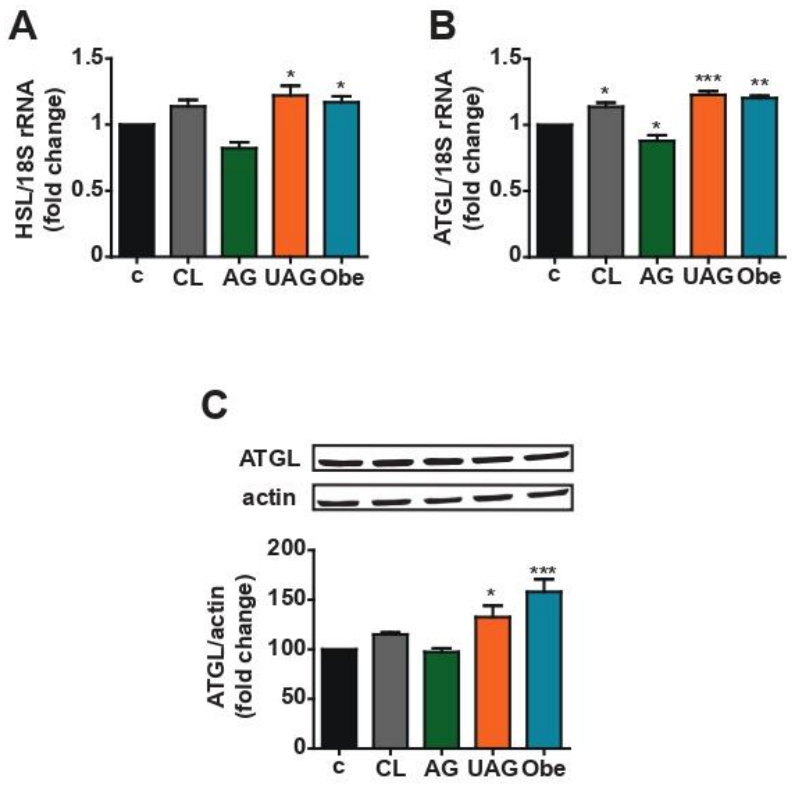

Figure 3: Effects of the ghrelin gene peptides on lipolytic enzymes. The mRNA expression of *Hsl* and *Atgl* was evaluated by real-time PCR (**A-B**) and protein expression by Western blot (**C**). Results are mean ± SEM (n=3), 1-way ANOVA and Tukey's post hoc test. (*P<0.05; **P<0.01; ***P<0.001 vs c).

Involvement of growth hormone secretagogue receptor (GHSR-1a) and glucagon-like peptide 1 receptor (GLP-1R) on UCP1 mRNA expression

To evaluate the direct effect of GHSR-1a and GLP-1R on thermogenic regulation, we stimulated 3T3-L1 adipocytes with [D-lys3]-GHRP-6 to antagonize the GHSR-1a and Exendin-9 to antagonize GLP-1R. As shown in Figure 4, 10 µM [D-lys3]-GHRP-6 abolished the stimulatory effect of AG on UCP1 mRNA expression (**Fig. 4A**), while had no effect on the stimulatory effect of UAG, suggesting that the AG receptor is not involved in the effects of UAG (**Fig. 4B**). Furthermore, 10 nM Exendin-9 abolished the stimulatory effect of Obe on UCP1 mRNA expression in 3T3-L1 adipocytes (**Fig. 4C**). These results suggest that AG, and Obe, increase the browning of adipocytes through binding to GHSR-1a and GLP-1R, respectively, while UAG effects likely involve a different receptor that remains to be identified.

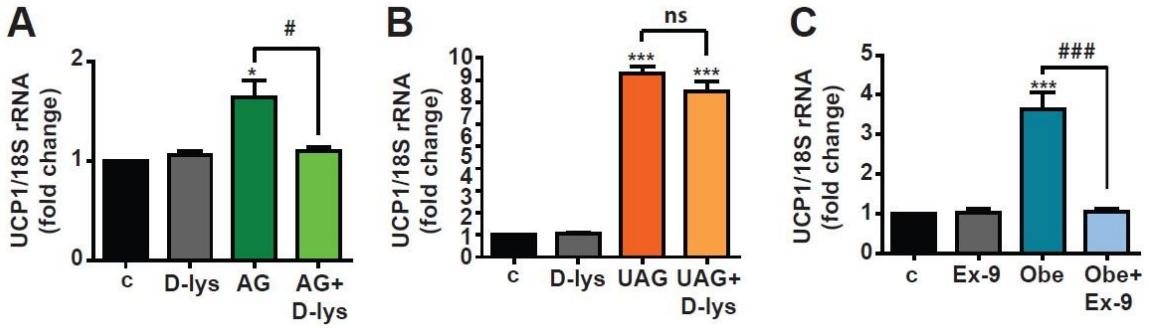

Figure 4: Effects of GHSR-1a and GLP-1 inhibitors on UCP-1 mRNA expression. 3T3-L1 adipocytes were treated with [D-lys3]-GHRP-6 (D-lys, 10 µM), Exendin-9 (Ex-9, 10 nM) AG, UAG or Obe (500 nM) during transdifferentiation. UCP-1 mRNA expression was evaluated by real-time PCR (**A-C**) (n=3), 1-way ANOVA and Tukey's post hoc test. Results are mean ± SEM. (*P<0.05; ***P<0.001 vs c; #P<0.05 vs AG; ###P<0.01 vs Obe, ns=no significant).

The ghrelin gene peptides increase beige/brown fat specific markers in human adipocytes

We next aimed to verify the effects of the ghrelin gene peptides on human primary adipocytes derived by subcutaneous (SAT) and omental (OAT) adipose tissue derived by obese individuals. We treated mature adipocytes extracted by SAT and OAT with AG, UAG and Obe for 24 hours and next we assessed the expression of the main browning genes by real-time PCR. As shown in the figure 5, UAG and Obe, but not AG, were able to increase the mRNA expression of UCP1, PGC-1a and PRDM16 both in SAT (**Fig. 5A**) and OAT (**Fig. 5B**).

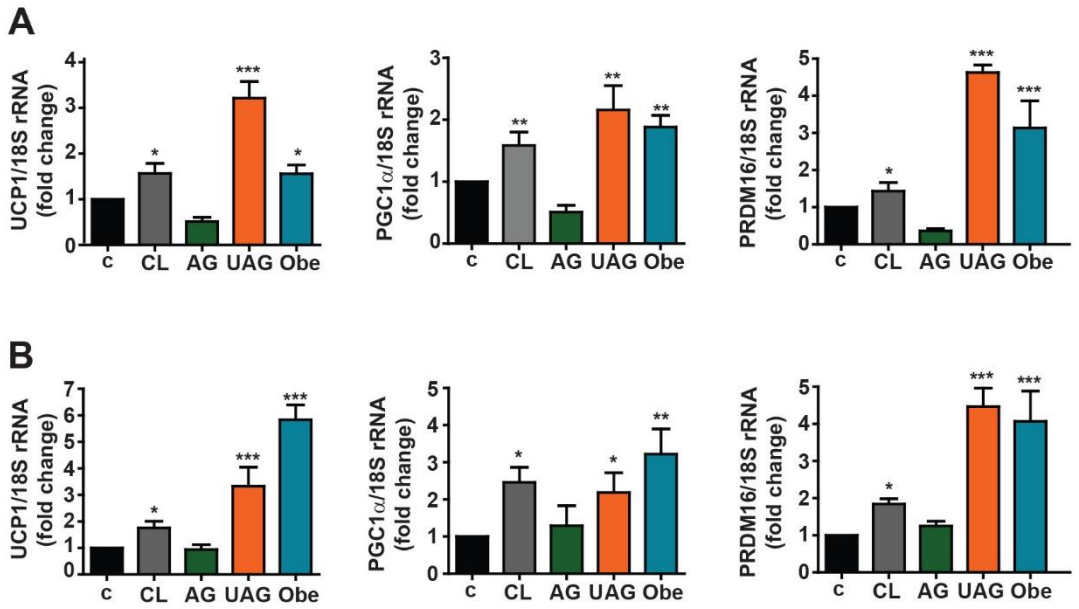

Figure 5. Effects of ghrelin gene peptides on browning genes in SAT and OAT. Mature adipocytes explanted by SAT and OAT were treated with β3-AR agonist CL316246 (CL,1μM), AG, UAG or Obe (500 nM) for 24 h. The mRNA expression of UCP1, PGC-1A and PRDM16 for SAT (**A**) and OAT (**B**) was evaluated by real-time PCR (n=3), 1-way ANOVA and Tukey's post hoc test. Results are mean ± SEM. (*P<0.05; ***P<0.001 vs c).

DISCUSSION

Adipose tissue is an essential organ that is related with the modulation of energy metabolism and insulin sensitivity [81]. It includes various cell types, such as white and brown adipocytes. Each of these types of adipocytes has unique cell-autonomous functions, and they differ at the molecular and morphological levels [85]. WAT principally stores lipids in the form of TGs, thereby acting as a repository of surplus energy in the body. Conversely, BAT utilizes stored lipids for nonshivering thermogenesis, involving β-oxidation and uncoupling of oxidative phosphorylation in mitochondria. Consistent with this function, the cytoplasm of BATs contains numerous mitochondria and small lipid bodies [82]. Thus, enhancing BAT activity or transition to brown-like cells of WAT can improve energy expenditure and in turn has the potential to ameliorate or prevent the development of metabolic diseases that are linked with obesity. The results of the present study demonstrate for the first time that UAG and Obe can induce the brown fat phenotype in 3T3-L1 and human primary adipocytes.

The modulatory roles of the ghrelin gene peptides in inducing the brown adipocyte-like phenotype in 3T3-L1 adipocytes and human primary adipocytes were established based on enhanced expression of brite-specific and thermogenic markers such as *Cd137* and *Dio2* as well as PRDM16, PGC-1α, and UCP1, respectively. *Cd137* and *Dio2* are considered to be distinct molecular signatures of inducible brown adipocyte-like cells and are highly enriched in beige adipocytes [83]. It is well recognized that PRDM16 and PGC-1α play predominant roles in determining the fates of precursor cell lineages of adipocytes, as well as brown adipocyte development and function [84]. PGC-1α is considered as a master regulator of mitochondrial biogenesis, which is one of the characterized features of brown adipocytes [85]. Overall, our results demonstrate that UAG and Obe, but not AG, stimulate the brown adipocyte-like phenotype in 3T3-L1 adipocytes.

Fatty acids released by lipolysis can be oxidized in mitochondria. CPT1 is a rate-limiting enzyme in β-oxidation because it is required for the transfer of free fatty acids (FFa) into the mitochondria. CPT1 overexpression leads to greater fatty acids oxidation, lipolysis, UCP1 expression and mitochondrial activity, and its expression is regulated transcriptionally by PPARγ and PGC1α [86]. Sirt1 plays a key role in thermogenesis and is a master regulator of mitochondrial biogenesis in adipose tissue by deacetylation of PPARγ, increasing PGC1α promoter activity and then driving UCP1 transcription [87]. Therefore, enhanced expression of these two genes is likely to reflect the stimulatory action of ghrelin gene peptides regarding fat oxidation. We have demonstrated that both UAG and Obe increase the expression of these molecules in 3T3-L1 adipocytes, implying that treated cells adopt a more pronounced brown/beige phenotype. C/EBP is recognized as a major player in obesity and adipogenesis. Inhibiting the expression of this important transcription factor has been shown to reduce adipocyte differentiation in 3T3-L1 preadipocytes [88]. We found that both UAG and Obe reduced the mRNA expression of C/EBP in 3T3-L1 adipocytes, while AG, in line with the literature, significantly increased its mRNA expression, indicating a reduction in fat deposition for UAG and Obe conversely to AG.

The lipolytic process occurs through consecutive steps that require the action of at least three different lipases: adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol lipase (MGL). ATGL is responsible for the initial step of lipolysis to catalyse the conversion of triacylglycerols (TGs) into diacylglycerols (DGs), whereas HSL is predominantly responsible for the hydrolysis of DGs to monoacylglycerols (MGs), and MGL hydrolyses MGs. However, ATGL and HSL are responsible for more than 95% of TG hydrolysis in adipocytes [89]. Enhancing lipolysis not only reduces adipocyte size and lipid accumulation but also impairs insulin sensitivity by increasing the level of free fatty acids. However, free fatty acids from lipolysis can act as substrates for β-oxidation and upregulate

UCP1 activity [87]. The present study shows that the treatment with UAG and Obe, contrarily to AG, increased the mRNA expression of HSL and ATGL in 3T3-L1 adipocytes, which would result in the hydrolysis of TG to form diglycerol (DG), monoglycerol (MG), and FFA.

As previously described, AG, which contains an *n*-octanoic acid at the third Ser residue, activates GHSR-1a. *In vivo*, administration of AG in rodents induces adiposity [62] and promotes the increase of fat storage-promoting enzymes in WAT [65]. In contrast UAG, which does not activate GHSR-1a, improves glucose metabolism and reduces lipolysis [77]. Furthermore, GHSR-1a ablation in aging mice was shown to reduce glucose/lipid uptake and lipogenesis in WAT and to increase thermogenic capacity in BAT, suggesting that GHSR-1a is an important regulator of lipid metabolism during normal aging [74]. Regarding obestatin, its receptor is still not determined. This peptide was initially reported to bind and activate the orphan G-protein-coupled receptor 39 (GPR39) [37], but more recently our group demonstrated that obestatin at least partly interacts with glucagon-like peptide-1 receptor (GLP-1R) in pancreatic cells and in adipocytes [49][50]. In this study, to determine whether GHSR-1a and GLP-1 has direct effects on adipocytes browning and lipid metabolism, we treated 3T3-L1 adipocytes [D-lys3]- GHRP-6, an antagonist of GHSR-1a, and with Exendin-9 (Ex-9), an antagonist of GLP1-R. D-Lys3 inhibited *Ucp1* mRNA expression induced by AG but had no effects on the increase of *Ucp1* by UAG, suggesting that effects of UAG are not due to GHSR-1a activation. Furthermore, Ex-9 inhibited *Ucp1* mRNA expression induced by Obe, suggesting the involvement of GLP-R in the browning effects of Obe.

Finally, we wanted to analyse the effect of the ghrelin gene peptides on human primary adipocytes obtained by SC and omental OM adipose tissue explants. These cells mimic more likely what happens in patients with obesity. We treated human mature adipocytes with of

AG, UAG or Obe and evaluated the mRNA expression of the main browning genes. UAG and Obe significantly enhanced brown adipogenesis in the bot SAT and OAT, as demonstrated by increased UCP1, PGC-1a and PRDM16 and mRNA expression. AG on the contrary decreased the mRNA expression of the main browning genes in both SAT and OAT. Future studied are needed to examine the ghrelin gene peptides effects on protein expression and mitochondrial respiration in the human mature adipocytes.

In conclusion, the results of this study demonstrate that UAG and Obe, but not AG, promote the expression of browning specific genes and proteins, regulate lipid metabolism and promote mitochondrial biogenesis in 3T3-L1 adipocytes and human SC and OM adipose tissue, suggesting novel potential therapeutic roles for these peptides in obesity and metabolic diseases.

BIBLIOGRAPHY

[1] K. Almind, M. Manieri, W. I. Sivitz, S. Cinti, and C. R. Kahn, "Ectopic brown adipose tissue in muscle provides a mechanism for differences in risk of metabolic syndrome in mice," Proc. Natl. Acad. Sci. U. S. A., vol. 104, no. 7, pp. 2366–2371, 2007, doi: 10.1073/pnas.0610416104.

[2] Rosen ED, Spiegelman BM. Adipocytes as regulators of energy balance and glucose homeostasis. Nature 2006; 444:847-853

[3] M. E. Frigolet and R. Gutiérrez-Aguilar, "The colors of adipose tissue," Gac. Med. Mex., vol. 156, no. 2, pp. 142–149, 2020, doi: 10.24875/GMM.M20000356.

[4] M. J. Betz and S. Enerbäck, "Human brown adipose tissue: What we have learned so far," Diabetes, vol. 64, no. 7, pp. 2352–2360, 2015, doi: 10.2337/db15-0146.

[5] J. A. Timmons et al., "Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages," Proc. Natl. Acad. Sci. U. S. A., vol. 104, no. 11, pp. 4401–4406, 2007, doi: 10.1073/pnas.0610615104.

[6] A. Park, "Distinction of white, beige and brown adipocytes derived from mesenchymal stem cells," World J. Stem Cells, vol. 6, no. 1, p. 33, 2014, doi: 10.4252/wjsc.v6.i1.33.

[7] R. K. Baboota, S. M. Sarma, R. K. Boparai, K. K. Kondepudi, S. Mantri, and M. Bishnoi, "Microarray Based gene expression analysis of murine brown and subcutaneous adipose tissue: Significance with human," PLoS One, vol. 10, no. 5, pp. 1–20, 2015, doi: 10.1371/journal.pone.0127701.

[8] A. Booth, A. Magnuson, J. Fouts, and M. T. Foster, “Adipose tissue: An endocrine organ playing a role in metabolic regulation,” Horm. Mol. Biol. Clin. Investig., vol. 26, no. 1, pp. 25–42, 2016, doi: 10.1515/hmbci-2015-0073.

[9] J. Wu et al., “Beige Adipocytes Are a Distinct Type of Thermogenic Fat Cell in Mouse and Human,” Cell, vol. 150, no. 2, pp. 366–376, Jul. 2012, doi: 10.1016/J.CELL.2012.05.016.

[10] P. Lee, C. D. Werner, E. Kebebew, and F. S. Celi, “Functional thermogenic beige adipogenesis is inducible in human neck fat,” Int. J. Obes., vol. 38, no. 2, pp. 170–176, 2014, doi: 10.1038/ijo.2013.82.

[11] V. Barquissau et al., “White-to-brite conversion in human adipocytes promotes metabolic reprogramming towards fatty acid anabolic and catabolic pathways,” Mol. Metab., vol. 5, no. 5, pp. 352–365, May 2016, doi: 10.1016/J.MOLMET.2016.03.002.

[12] T. C. L. Bargut, M. B. Aguila, and C. A. Mandarim-de-Lacerda, “Brown adipose tissue: Updates in cellular and molecular biology,” Tissue Cell, vol. 48, no. 5, pp. 452–460, Oct. 2016, doi: 10.1016/J.TICE.2016.08.001.

[13] J. Nedergaard and B. Cannon, “The browning of white adipose tissue: Some burning issues,” Cell Metab., vol. 20, no. 3, pp. 396–407, 2014, doi: 10.1016/j.cmet.2014.07.005.

[14] Jeremic N, Chaturvedi P, Tyagi SC. Browning of White Fat: Novel Insight Into Factors, Mechanisms, and Therapeutics. J Cell Physiol. 2017 Jan;232(1):61-8. doi: 10.1002/jcp.25450. Epub 2016 Jun 21. PMID: 27279601; PMCID: PMC6567990.

[15] M. L. Bonet, P. Oliver, and A. Palou, “Pharmacological and nutritional agents promoting browning of white adipose tissue,” Biochim. Biophys. Acta - Mol. Cell Biol. Lipids, vol. 1831, no. 5, pp. 969–985, May 2013, doi: 10.1016/J.BBALIP.2012.12.002.

[16] B. Cannon and J. Nedergaard, “Brown Adipose Tissue: Function and Physiological Significance,” Physiol. Rev., vol. 84, no. 1, pp. 277–359, 2004, doi: 10.1152/physrev.00015.2003.

[17] J. Himms-Hagen, A. Melnyk, M. C. Zingaretti, E. Ceresi, G. Barbatelli, and S. Cinti, “Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes,” Am. J. Physiol. - Cell Physiol., vol. 279, no. 3 48-3, pp. 670–681, 2000, doi: 10.1152/ajpcell.2000.279.3.c670.

[18] M. Rosenwald and C. Wolfrum, “The origin and definition of brite versus white and classical brown adipocytes,” Adipocyte, vol. 3, no. 1, pp. 4–9, 2014, doi: 10.4161/adip.26232.

[19] J. G. Granneman, P. Li, Z. Zhu, and Y. Lu, “Metabolic and cellular plasticity in white adipose tissue I: Effects of β3-adrenergic receptor activation,” Am. J. Physiol. - Endocrinol. Metab., vol. 289, no. 4 52-4, pp. 608–616, 2005, doi: 10.1152/ajpendo.00009.2005.

[20] P. Puigserver, Z. Wu, C. W. Park, R. Graves, M. Wright, and B. M. Spiegelman, “A Cold-Inducible Coactivator of Nuclear Receptors Linked to Adaptive Thermogenesis,” Cell, vol. 92, no. 6, pp. 829–839, Mar. 1998, doi: 10.1016/S0092-8674(00)81410-5.

[21] E. Hondares et al., “Peroxisome Proliferator-activated Receptor α (PPARα) Induces PPARγ Coactivator 1α (PGC-1α) Gene Expression and Contributes to Thermogenic Activation of Brown Fat: INVOLVEMENT OF PRDM16,” J. Biol. Chem., vol. 286, no. 50, pp. 43112–43122, Dec. 2011, doi: 10.1074/JBC.M111.252775.

[22] E. Hondares et al., “Thiazolidinediones and rexinoids induce peroxisome proliferator-activated receptor-coactivator (PGC)-1α gene transcription: An autoregulatory loop controls PGC-1α expression in adipocytes via peroxisome proliferator-activated

receptor-γ coactivation," Endocrinology, vol. 147, no. 6, pp. 2829–2838, 2006, doi: 10.1210/en.2006-0070.

[23] D. Pan, M. Fujimoto, A. Lopes, and Y. X. Wang, "Twist-1 Is a PPARδ-Inducible, Negative-Feedback Regulator of PGC-1α in Brown Fat Metabolism," Cell, vol. 137, no. 1, pp. 73–86, Apr. 2009, doi: 10.1016/J.CELL.2009.01.051.

[24] S. Kajimura, P. Seale, and B. M. Spiegelman, "Transcriptional Control of Brown Fat Development," Cell Metab., vol. 11, no. 4, pp. 257–262, Apr. 2010, doi: 10.1016/J.CMET.2010.03.005.

[25] P. Seale et al., "Transcriptional Control of Brown Fat Determination by PRDM16," Cell Metab., vol. 6, no. 1, pp. 38–54, Jul. 2007, doi: 10.1016/J.CMET.2007.06.001.

[26] P. Seale et al., "Prdm16 determines the thermogenic program of subcutaneous white adipose tissue in mice," J. Clin. Invest., vol. 121, no. 1, pp. 96–105, 2011, doi: 10.1172/JCI44271.

[27] L. Wilson-Fritch et al., "Mitochondrial remodeling in adipose tissue associated with obesity and treatment with rosiglitazone," J. Clin. Invest., vol. 114, no. 9, pp. 1281–1289, 2004, doi: 10.1172/JCI21752.

[28] H. Ohno, K. Shinoda, B. M. Spiegelman, and S. Kajimura, "PPARγ agonists Induce a White-to-Brown Fat Conversion through Stabilization of PRDM16 Protein," Cell Metab., vol. 15, no. 3, pp. 395–404, Mar. 2012, doi: 10.1016/J.CMET.2012.01.019.

[29] Granata R, Settanni F, Biancone L, Trovato L, Nano R, Bertuzzi F, Destefanis S, Annunziata M, Martinetti M, Catapano F, Ghè C, Isgaard J, Papotti M, Ghigo E, Muccioli G. Acylated and unacylated ghrelin promote proliferation and inhibit apoptosis of pancreatic beta-cells and human islets: involvement of 3',5'-cyclic adenosine monophosphate/protein kinase A, extracellular signal-regulated kinase 1/2, and phosphatidyl inositol 3-Kinase/Akt

signaling. Endocrinology. 2007 Feb;148(2):512-29. doi: 10.1210/en.2006-0266. Epub 2006 Oct 26. PMID: 17068144.

[30] K. K. Masayasu Kojima, “Structure and function of ghrelin,” Seikagaku, no. 79(9):853–67, 2007, [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/17969325/.

[31] K. C. Campbell, “A Preprandial Rise in Plasma Ghrelin Levels Suggests a Role in Meal Initiation in Humans,” J. Lab. Clin. Med., vol. 20, no. 5, pp. 520–522, 1935, doi: 10.1016/s0002-9378(37)90859-7.

[32] A. J. Van Der Lely, M. Tschöp, M. L. Heiman, and E. Ghigo, “Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin,” Endocr. Rev., vol. 25, no. 3, pp. 426–457, 2004, doi: 10.1210/er.2002-0029.

[33] M. V. Mauro Papotti, Eleonora Duregon, “Ghrelin and tumor,” Endocr dev, no. 25:122-34.

[34] M. Kojima, H. Hosoda, H. Matsuo, and K. Kangawa, “Ghrelin: Discovery of the natural endogenous ligand for the growth hormone secretagogue receptor,” Trends in Endocrinology and Metabolism, vol. 12, no. 3. Elsevier Current Trends, pp. 118–122, Apr. 01, 2001, doi: 10.1016/S1043-2760(00)00362-3.

[35] J. Yang, M. S. Brown, G. Liang, N. V. Grishin, and J. L. Goldstein, “Identification of the Acyltransferase that Octanoylates Ghrelin, an Appetite-Stimulating Peptide Hormone,” Cell, vol. 132, no. 3, pp. 387–396, Feb. 2008, doi: 10.1016/J.CELL.2008.01.017.

[36] R. Granata, A. Baragli, F. Settanni, F. Scarlatti, and E. Ghigo, “Unraveling the role of the ghrelin gene peptides in the endocrine pancreas,” J. Mol. Endocrinol., vol. 45, no. 3, pp. 107–118, 2010, doi: 10.1677/JME-10-0019.

[37] J. V. Zhang et al., “Medicine: Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin’s effects on food intake,” Science (80-.)., vol. 310, no. 5750, pp. 996–999, 2005, doi: 10.1126/science.1117255.

[38] O. Gualillo, F. Lago, F. F. Casanueva, and C. Dieguez, “One ancestor, several peptides: Post-translational modifications of preproghrelin generate several peptides with antithetical effects,” Mol. Cell. Endocrinol., vol. 256, no. 1–2, pp. 1–8, Aug. 2006, doi: 10.1016/J.MCE.2006.05.007.

[39] C. M. Zhao, M. W. Furnes, B. Stenström, B. Kulseng, and D. Chen, “Characterization of obestatin- and ghrelin-producing cells in the gastrointestinal tract and pancreas of rats: An immunohistochemical and electron-microscopic study,” Cell Tissue Res., vol. 331, no. 3, pp. 575–587, 2008, doi: 10.1007/s00441-007-0514-3.

[40] E. Gargantini, C. Grande, L. Trovato, E. Ghigo, and R. Granata, “The role of obestatin in glucose and lipid metabolism,” Horm. Metab. Res., vol. 45, no. 13, pp. 1002–1008, 2013, doi: 10.1055/s-0033-1351325.

[41] I. Depoortere, T. Thijs, D. Moechars, B. De Smet, L. Ver Donck, and T. L. Peeters, “Effect of peripheral obestatin on food intake and gastric emptying in ghrelin-knockout mice,” Br. J. Pharmacol., vol. 153, no. 7, pp. 1550–1557, 2008, doi: 10.1038/sj.bjp.0707683.

[42] Kojima, M., Hosoda, H., Matsuo, H., & Kangawa, K. (2001). Ghrelin: Discovery of the natural endogenous ligand for the growth hormone secretagogue receptor. Trends in Endocrinology and Metabolism. Elsevier Inc. https://doi.org/10.1016/S1043-2760(00)00362-3

[43] P. Walia, A. Asadi, T. J. Kieffer, J. D. Johnson, and J. P. Chanoine, “Ontogeny of ghrelin, obestatin, preproghrelin, and prohormone convertases in rat pancreas and stomach,” Pediatr. Res., vol. 65, no. 1, pp. 39–44, 2009, doi: 10.1203/PDR.0b013e31818bc134.

[44] J. P. Camiña, “Cell biology of the ghrelin receptor,” J. Neuroendocrinol., vol. 18, no. 1, pp. 65–76, 2006, doi: 10.1111/j.1365-2826.2005.01379.x.

[45] Cocchi D, Maccarinelli G, Sibilia V, Tulipano G, Torsello A, Pazzaglia UE, Giustina A, Netti C. GH-releasing peptides and bone. J Endocrinol Invest. 2005;28(8 Suppl):11-4. PMID: 16323823.

[46] P. Cassoni et al., “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., vol. 150, no. 2, pp. 173–184, 2004, doi: 10.1530/eje.0.1500173.

[47] E. Lauwers, B. Landuyt, L. Arckens, L. Schoofs, and W. Luyten, “Obestatin does not activate orphan G protein-coupled receptor GPR39,” Biochem. Biophys. Res. Commun., vol. 351, no. 1, pp. 21–25, Dec. 2006, doi: 10.1016/J.BBRC.2006.09.141.

[48] A. K. Bassil et al., “Little or no ability of obestatin to interact with ghrelin or modify motility in the rat gastrointestinal tract,” Br. J. Pharmacol., vol. 150, no. 1, pp. 58–64, 2007, doi: 10.1038/sj.bjp.0706969.

[49] R. Granata et al., “Obestatin promotes survival of pancreatic β-cells and human islets and induces expression of genes involved in the regulation of β-cell mass and function,” Diabetes, vol. 57, no. 4, pp. 967–979, 2008, doi: 10.2337/db07-1104.

[50] R. Granata et al., “Obestatin regulates adipocyte function and protects against diet-induced insulin resistance and inflammation,” FASEB J., vol. 26, no. 8, pp. 3393–3411, 2012, doi: 10.1096/fj.11-201343.

[51] J. A. Gutierrez et al., “Ghrelin octanoylation mediated by an orphan lipid transferase,” Proc. Natl. Acad. Sci. U. S. A., vol. 105, no. 17, pp. 6320–6325, 2008, doi: 10.1073/pnas.0800708105.

[52] S. C. Chang and A. I. Magee, “Acyltransferases for secreted signalling proteins (Review),” Mol. Membr. Biol., vol. 26, no. 1–2, pp. 104–113, 2009, doi: 10.1080/09687680802706432.

[53] M. S. Taylor et al., “Architectural Organization of the Metabolic Regulatory Enzyme Ghrelin O-Acyltransferase,” J. Biol. Chem., vol. 288, no. 45, pp. 32211–32228, Nov. 2013, doi: 10.1074/JBC.M113.510313.

[54] C. T. Lim, B. Kola, and M. Korbonits, “The ghrelin/GOAT/GHS-R system and energy metabolism,” Rev. Endocr. Metab. Disord., vol. 12, no. 3, pp. 173–186, 2011, doi: 10.1007/s11154-011-9169-1.

[55] I. Sakata et al., “Colocalization of ghrelin O-acyltransferase and ghrelin in gastric mucosal cells,” Am. J. Physiol. - Endocrinol. Metab., vol. 297, no. 1, pp. 134–141, 2009, doi: 10.1152/ajpendo.90859.2008.

[56] M. D. Gahete et al., “A novel human ghrelin variant (in1-ghrelin) and ghrelin-O-acyltransferase are overexpressed in breast cancer: Potential pathophysiological relevance,” PLoS One, vol. 6, no. 8, 2011, doi: 10.1371/journal.pone.0023302.

[57] M. Kojima, H. Hosoda, Y. Date, M. Nakazato, H. Matsuo, and K. Kangawa, “Ghrelin is a growth-hormone-releasing acylated peptide from stomach,” Nature, vol. 402, no. 6762, pp. 656–660, 1999, doi: 10.1038/45230.

[58] K. Kos, A. L. Harte, P. J. O’Hare, S. Kumar, and P. G. McTernan, “Ghrelin and the differential regulation of des-acyl (DSG) and oct-anoyl ghrelin (OTG) in human adipose tissue (AT),” Clin. Endocrinol. (Oxf)., vol. 70, no. 3, pp. 383–389, 2009, doi: 10.1111/j.1365-2265.2008.03321.x.

[59] U. Gurriarán-Rodríguez et al., “Obestatin as a regulator of adipocyte metabolism and adipogenesis,” J. Cell. Mol. Med., vol. 15, no. 9, pp. 1927–1940, 2011, doi: 10.1111/j.1582-4934.2010.01192.x.

[60] U. Gurriarán-Rodríguez et al., “Preproghrelin expression is a key target for insulin action on adipogenesis,” J. Endocrinol., vol. 210, no. 2, pp. 1–7, 2011, doi: 10.1530/JOE-11-0233.

[61] P. Miegueu, D. St Pierre, F. Broglio, and K. Cianflone, “Effect of desacyl ghrelin, obestatin and related peptides on triglyceride storage, metabolism and GHSR signaling in 3T3-L1 adipocytes,” J. Cell. Biochem., vol. 112, no. 2, pp. 704–714, 2011, doi: 10.1002/jcb.22983.

[62] G. Muccioli, N. Pons, C. Ghè, F. Catapano, R. Granata, and E. Ghigo, “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., vol. 498, no. 1–3, pp. 27–35, Sep. 2004, doi: 10.1016/J.EJPHAR.2004.07.066.

[63] M. Tschop, D. L. Smiley, and M. L. Heiman, “Ghrelin induces adiposity in rodents,” Nature, vol. 407, no. 6806, pp. 908–913, 2000, doi: 10.1038/35038090.

[64] A. M. Wren et al., “Ghrelin Causes Hyperphagia and Obesity in Rats . Corresponding author : Address : Tel :,” Am Diabetes Assoc, vol. 50, no. November, pp. 1–22, 2001.

[65] C. Theander-Carrillo et al., “Ghrelin action in the brain controls adipocyte metabolism,” J. Clin. Invest., vol. 116, no. 7, pp. 1983–1993, 2006, doi: 10.1172/JCI25811.

[66] N. M. Thompson et al., “Ghrelin and des-octanoyl ghrelin promote adipogenesis directlyin vivo by a mechanism independent of GHS-R1a,” Endocrinology, vol. 145, no. 1, pp. 234–242, 2004, doi: 10.1210/en.2003-0899.

[67] A. Rodríguez et al., “Acylated and desacyl ghrelin stimulate lipid accumulation in human visceral adipocytes,” Int. J. Obes., vol. 33, no. 5, pp. 541–552, 2009, doi: 10.1038/ijo.2009.40.

[68] W. Zhang, B. Chai, J. Y. Li, H. Wang, and M. W. Mulholland, “Effect of des-acyl ghrelin on adiposity and glucose metabolism,” Endocrinology, vol. 149, no. 9, pp. 4710–4716, 2008, doi: 10.1210/en.2008-0263.

[69] P. J. D. Delhanty et al., “Unacylated ghrelin rapidly modulates lipogenic and insulin signaling pathway gene expression in metabolically active tissues of GHSR deleted mice,” PLoS One, vol. 5, no. 7, 2010, doi: 10.1371/journal.pone.0011749.

[70] V. Ott et al., “Direct peripheral effects of ghrelin include suppression of adiponectin expression,” Horm. Metab. Res., vol. 34, no. 11–12, pp. 640–645, 2002, doi: 10.1055/s-2002-38261.

[71] A. Mano-Otagiri, H. Ohata, A. Iwasaki-Sekino, T. Nemoto, and T. Shibasaki, “Ghrelin suppresses noradrenaline release in the brown adipose tissue of rats,” J. Endocrinol., vol. 201, no. 3, pp. 341–349, 2009, doi: 10.1677/JOE-08-0374.

[72] T. Tsubone, T. Masaki, I. Katsuragi, K. Tanaka, T. Kakuma, and H. Yoshimatsu, “Ghrelin regulates adiposity in white adipose tissue and UCP1 mRNA expression in brown adipose tissue in mice,” Regul. Pept., vol. 130, no. 1–2, pp. 97–103, Aug. 2005, doi: 10.1016/J.REGPEP.2005.04.004.

[73] A. Giovambattista, J. Piermaria, M. O. Suescun, R. S. Calandra, R. C. Gaillard, and E. Spinedi, “Direct effect of ghrelin on leptin production by cultured rat white adipocytes,” Obesity, vol. 14, no. 1, pp. 19–27, 2006, doi: 10.1038/oby.2006.4.

[74] L. Lin et al., “Ablation of ghrelin receptor reduces adiposity and improves insulin sensitivity during aging by regulating fat metabolism in white and brown adipose tissues,” Aging Cell, vol. 10, no. 6, pp. 996–1010, 2011, doi: 10.1111/j.1474-9726.2011.00740.x.

[75] S. K. Min et al., “The mitogenic and antiapoptotic actions of ghrelin in 3T3-L1 adipocytes,” Mol. Endocrinol., vol. 18, no. 9, pp. 2291–2301, 2004, doi: 10.1210/me.2003-0459.

[76] Zhang W, Zhao L, Lin TR, Chai B, Fan Y, Gantz I, Mulholland MW. Inhibition of adipogenesis by ghrelin. Mol Biol Cell. 2004 May;15(5):2484-91. doi: 10.1091/mbc.e03-09-0657. Epub 2004 Mar 19. PMID: 15034137; PMCID: PMC404039.

[77] A. Benso et al., “Metabolic effects of overnight continuous infusion of unacylated ghrelin in humans,” Eur. J. Endocrinol., vol. 166, no. 5, pp. 911–916, 2012, doi: 10.1530/EJE-11-0982.

[78] E. T. Vestergaard et al., “Ghrelin infusion in humans induces acute insulin resistance and lipolysis independent of growth hormone signaling,” Diabetes, vol. 57, no. 12, pp. 3205–3210, 2008, doi: 10.2337/db08-0025.

[79] T. Yoshizaki et al., “SIRT1 Exerts Anti-Inflammatory Effects and Improves Insulin Sensitivity in Adipocytes,” Mol. Cell. Biol., vol. 29, no. 5, pp. 1363–1374, 2009, doi: 10.1128/mcb.00705-08.

[80] D. Gallo et al., “GH-releasing hormone promotes survival and prevents TNF-α-induced apoptosis and atrophy in C2C12 myotubes,” Endocrinology, vol. 156, no. 9, pp. 3239–3252, 2015, doi: 10.1210/EN.2015-1098.

[81] T. Montanari, N. Pošćić, and M. Colitti, “Factors involved in white-to-brown adipose tissue conversion and in thermogenesis: a review,” Obes. Rev., vol. 18, no. 5, pp. 495–513, 2017, doi: 10.1111/obr.12520.

[82] J. Yu et al., “Lipid droplet remodeling and interaction with mitochondria in mouse brown adipose tissue during cold treatment,” Biochim. Biophys. Acta - Mol. Cell Res., vol. 1853, no. 5, pp. 918–928, May 2015, doi: 10.1016/J.BBAMCR.2015.01.020.

[83] L. Z. Sharp et al., “Human BAT Possesses Molecular Signatures That Resemble Beige/Brite Cells,” PLoS One, vol. 7, no. 11, 2012, doi: 10.1371/journal.pone.0049452.

[84] P. Seale et al., “Transcriptional Control of Brown Fat Determination by PRDM16,” Cell Metab., vol. 6, no. 1, pp. 38–54, 2007, doi: 10.1016/j.cmet.2007.06.001.

[85] C. Tiraby et al., “Acquirement of brown fat cell features by human white adipocytes,” J. Biol. Chem., vol. 278, no. 35, pp. 33370–33376, 2003, doi: 10.1074/jbc.M305235200.

[86] Y. J. Seo et al., “Cardamonin suppresses lipogenesis by activating protein kinase A-mediated browning of 3T3-L1 cells,” Phytomedicine, vol. 65, p. 153064, Dec. 2019, doi: 10.1016/J.PHYMED.2019.153064.

[87] C. C. Chen, C. H. Kuo, Y. L. Leu, and S. H. Wang, “Corylin reduces obesity and insulin resistance and promotes adipose tissue browning through SIRT-1 and β3-AR activation,” Pharmacol. Res., vol. 164, p. 105291, 2021, doi: 10.1016/j.phrs.2020.105291.

[88] G. Wang, B. Wu, W. Xu, X. Jin, K. Wang, and H. Wang, “The Inhibitory Effects of Juglanin on Adipogenesis in 3T3-L1 Adipocytes,” 2020, doi: 10.2147/DDDT.S256504.

[89] S.-O. Kim et al., “Illudins C 2 and C 3 Stimulate Lipolysis in 3T3-L1 Adipocytes and Suppress Adipogenesis in 3T3-L1 Preadipocytes,” 2014, doi: 10.1021/np400520a.