Original Article

MOLECULAR METABOLISM

Temporal patterns of lipolytic regulators in adipose tissue after acute growth hormone exposure in human subjects: A randomized controlled crossover trial

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ABSTRACT

Objective: Growth hormone (GH) stimulates lipolysis, but the underlying mechanisms remain incompletely understood. We examined the effect of GH on the expression of lipolytic regulators in adipose tissue (AT).

Methods: In a randomized, placebo-controlled, cross-over study, nine men were examined after injection of 1) a GH bolus and 2) a GH-receptor antagonist (pegvisomant) followed by four AT biopsies. In a second study, eight men were examined in a 2 × 2 factorial design including GH infusion and 36-h fasting with AT biopsies obtained during a basal period and a hyperinsulinemic-euglycemic clamp. Expression of GH-signaling intermediates and lipolytic regulators were studied by PCR and western blotting. In addition, mechanistic experiments in mouse models and 3T3-L1 adipocytes were performed.

Results: The GH bolus increased circulating free fatty acids ($p < 0.0001$) together with phosphorylation of signal transducer and activator of transcription 5 (STAT5) ($p < 0.0001$) and mRNA expression of the STAT5-dependent genes *cytokine-inducible SH2-containing protein* (*CISH*) and *IGF-1* in AT. This was accompanied by suppressed mRNA expression of *G0/G1 switch gene 2* (*G0S2*) ($p = 0.007$) and fat specific protein 27 (FSP27) ($p = 0.002$) and upregulation of *phosphatase and tensin homolog* (*PTEN*) mRNA expression ($p = 0.03$). Suppression of G0S2 was also observed in humans after GH infusion and fasting, as well as in GH transgene mice, and *in vitro* studies suggested MEK-PPARγ signaling to be involved.

Conclusions: GH-induced lipolysis in human subjects *in vivo* is linked to downregulation of *G0S2* and FSP27 and upregulation of *PTEN* in AT. Mechanistically, *in vitro* data suggest that GH acts via MEK to suppress PPARγ-dependent transcription of *G0S2*. ClinicalTrials.gov NCT02782221 and NCT01209429.

Keywords Growth hormone; Lipolysis; G0S2; PTEN; FSP27

1. INTRODUCTION

Lipolysis is the process whereby triglycerides are hydrolyzed to free fatty acids (FFA) and glycerol. In adipocytes, this involves activation of three enzymes: adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoglyceride lipase. The primary stimulator is β-adrenergic receptor-mediated cAMP-dependent activation of PKA, whereas insulin is the major inhibitor of lipolysis via Akt-dependent suppression of cAMP and PKA activity [1].

Growth hormone (GH) also stimulates lipolysis especially during fasting, thereby promoting utilization of lipids from adipose tissue (AT) as an energy substrate at the expense of glucose and protein [2,3]. This effect is evident *in vivo* as an increase in circulating levels of FFA approximately one hour after GH exposure, peaking after 2–3 h followed by a gradual return to baseline within 5–6 h [4]. This temporal pattern suggests that the effect involves regulation of gene expression in AT. We have consistently demonstrated that systemic GH acutely activates the STAT5 signaling pathway, which is considered the canonical pathway whereby GH regulates transcription of target genes [5], in human AT *in vivo* [6–11]. In support of this, studies in rodents indicate that STAT5 is involved in GH-induced lipolysis [12–15], but

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Abbreviations

AT	adipose tissue
ATGL	adipose triglyceride lipase
bGH	bovine GH
cAMP	cyclic AMP
CGI-58	comparative gene identification-58
CISH	cytokine-inducible SH2-containing protein
CIDEC	cell death inducing DFFA like effector C
EE	energy expenditure
FFA	free fatty acid
FSP27	fat-specific protein 27
G0S2	G0/G1 switch gene 2
GH	growth hormone
HEC	hyperinsulinemic-euglycemic clamp
HSL	hormone sensitive lipase
PI3K	phosphoinositide 3-kinases
PKA	protein kinase A
PPARγ	peroxisome proliferator-activated receptors γ
PTEN	phosphatase and tensin homolog
PDE3B	phosphodiesterase 3B
RER	respiratory exchange rates
SC	subcutaneous
SOCS	suppressor of cytokine signaling
STAT5	signal transducer and activator of transcription 5

there are also *in vitro* studies to suggest that GH stimulates lipolysis via activation of the MAPK pathway [16,17].

GH-induced lipolysis in human subjects *in vivo* is abrogated by co-administration of acipimox, a niacin derivative that inhibits cAMP-dependent lipolysis [18,19], but the exact mechanisms remain unclear. Recently, we reported suppression of fat specific protein 27 (FSP27), also known as cell death-inducing DFFA-like effector C (CIDEC), a LD-associated protein that suppresses ATGL expression and activity [20], after a GH infusion in healthy men [21]. We have also reported that fasting suppresses the ATGL inhibitor G0/G1 switch gene 2 (G0S2) [1,22] in healthy men [23,24]. Since fasting is associated with an increase in endogenous GH levels [24], we hypothesize that G0S2 suppression may be involved in GH-induced lipolysis, and the objective of the present study was to examine the expression of lipolytic regulators in AT in response to GH.

A general shortcoming of previous human *in vivo* studies is that AT biopsies were obtained shortly after GH exposure, which may not appropriately capture changes in gene and protein expression [8,11,23,25,26], and several studies have included only a single AT biopsy after GH exposure [8,10,23,25,26]. Therefore, in the present study, we obtained multiple AT biopsies in nine obese but otherwise healthy, human subjects before and 60, 180, and 300 min following a single GH bolus to encompass the temporal effect of GH on the expression of lipolytic regulators. The study included a separate control day preceded by pharmacological blockade of the GH receptor (pegvisomant). In addition, we analyzed gene and protein expression of G0S2 in AT biopsies in healthy, lean subjects during fasting and GH infusion alone and in combination. The human studies were complemented by mechanistic studies in mice and mouse cell lines.

2. MATERIAL AND METHODS

2.1. Study design and participants

Two *in vivo* studies in human subjects were conducted in accordance with the Declaration of Helsinki II, approved by the regional Ethics Committee System, and reported at http://www.clinicaltrials.gov (NCT02782221 and NCT01209429). Written and oral consent was obtained from all participants, who were healthy according to a medical interview and a physical examination, including routine blood chemistry tests. Two days prior to the study day, the participants were instructed to refrain from vigorous exercise and alcohol intake. Both studies were carried out in the Medical Research Laboratory, Aarhus University.

2.1.1. Study 1 (GH bolus study)

Nine obese men with a mean ± SEM BMI of 31.8 ± 2.2 kg/m2 in the age range of 21–48 years were studied in a single-blinded, randomized, placebo-controlled, crossover study. Inclusion criteria: age >18 years old, male, healthy, BMI between 30 kg/m2 and 40 kg/m2.

2.1.2. Study 2 (GH infusion study)

Eight lean males with a mean ± SEM BMI 22.5 ± 1.5 kg/m^2 in the age range of 19–23 years were studied in a single-blinded, randomized 2 × 2 factorial design. Inclusion criteria: age >18 years old, male, healthy, BMI between 19 kg/m2 and 25 kg/m2.

2.2. Study protocol

2.2.1. GH bolus study

All participants were examined on two occasions separated by a minimum of three weeks: (1) after an IV bolus of GH (Genotropin® Miniquick, 0.6 mg) [GH], and (2) after an IV bolus of saline preceded by a subcutaneous (SC) injection of a GH receptor antagonist, pegvisomant (Somavert®, 30 mg), 38 h prior to the study day in order to block the peripheral effects of endogenous GH. A SC injection of saline was injected 38 h prior to the GH day. The participants were studied in a quiet, thermo-neutral indoor environment in the supine position for 5.5 h (t = -30-300 min) after an overnight fast. At the onset of each study day, an IV cannula was inserted into a dorsal hand vein for blood sampling every 30 min. The hand was placed in a heat pad in order to obtain arterialized blood samples. The GH/saline bolus was given at t = 0 preceded by the first AT biopsy, blood sampling and indirect calorimetry. Four SC AT biopsies were performed at t = 0, t = 60, t = 180, and t = 300, respectively. Indirect calorimetry was performed for 30 min with a canopy system (Oxycon Pro; Intramedic, Gentofte, Denmark) at baseline (t = -30 – 0 min), t = 150–180 min and t = 270–300 min to estimate energy expenditure (EE), respiratory exchange rates (RER) and substrate oxidation rates as previously described [27]. Urine was collected throughout the day to estimate urea excretion rates and protein oxidation.

The primary outcome was serum FFA concentrations as a measure of lipolysis. Secondary outcomes were biomarkers of intracellular lipolytic activity and GH signaling. Sample size was determined based on a power calculation.

2.2.2. GH infusion study

All participants were examined on four occasions separated by a minimum of one week: (1) during a saline infusion following an overnight fast (Control), (2) during a GH infusion (30 ng/kg/min,

Genotropin, Pfizer) following an overnight fast [GH], (3) during a saline infusion following a 36-hour fast (Fasting), and (4) during a GH infusion following a 36-hour fast (GH + Fasting). An IV cannula was placed in the antecubital vein for infusion. For blood sampling, a second cannula was placed in a heated dorsal hand vein. The participants were studied in a quiet, thermo-neutral indoor environment in the supine position for 4.5 h (t = 0–270 min) including a 150-minutes basal period followed by a two-hour hyperinsulinemic-euglycemic clamp (HEC) with an insulin infusion rate of 0.6 mU/kg/min (Actrapid; Novo Nordisk A/S, Copenhagen, Denmark). SC AT biopsies were taken at t = 120 (basal state) and at t = 270 (HEC). Data from this study have been published previously [21,28].

2.3. Blinding and randomization

The participants and the lab technicians, but not the investigator, were blinded regarding the interventions. Following simple randomization procedures (computerized random numbers), the participants were assigned to start with either GH or pegvisomant. The randomization and the enrollment of participants were done by the investigator. All participants received the intended intervention and completed the study.

2.4. Blood samples

Plasma glucose and lactate levels were measured at bedside (YSI 2300 STAT Plus glucose analyzer; YSI, Burlington, VT). Insulin and glucagon (Merkodia, Uppsala, Sweden) and cortisol (DRG Diagnostics, Marburg, Germany) were analyzed using commercial ELISA kits. Serum FFA was determined by a colorimetric method employing a commercial kit (Wako Chemicals, Neuss, Germany). Serum GH concentrations were determined using chemiluminescence technology (IDS-iSYS human GH; Immunodiagnostic Systems, Boldon, UK). Plasma epinephrine and norepinephrine concentrations were measured by electrochemical detection following HPLC.

2.5. Adipose tissue biopsies

The SC AT biopsies were obtained from the periumbilical region by liposuction under sterile conditions using lidocaine as local anesthetic. The samples were immediately washed free of blood, frozen in liquid nitrogen, and stored at −80 °C until use.

2.6. Quantitative PCR (human AT biopsies)

qPCR analyses were performed as previously described [25]. TRIzol (Gibco BRL/Life Technologies, Roskilde, Denmark) were used to extract RNA. The PCR reactions were performed in duplicate using a LightCycler SYBR Green master mix (Roche Applied Science, Penzberg, Germany). β2 microglobulin was used as the housekeeping gene. Messenger RNA levels of β2 microglobulin were similar in both interventions. The fold change was calculated using the $2^{-\Delta\Delta Ct}$ method; ΔΔCt is the difference between the average Ct for the target gene and the house keeping gene at time x minus the difference between Ct for the target gene and the house keeping gene at baseline (GH bolus study) or at the control day (GH infusion study). Detailed information regarding primer sequences is shown in the supplementary data.

2.7. Western blotting

Western blot analyses were performed using standard protocols and commercially available antibodies. All samples were homogenized in a buffer with a 7.4 pH level and containing 50 mM HEPES, 20 mM NaF, 2 mM Na3VO4, 5 mM EDTA, HALT, 5 mM NAM, 10 μM TSA, SDS 5%, and demineralized water. Samples were centrifuged at 14,000 g for 20 min. SDS-PAGE was performed on 4–15% Criterion TGX Stain-Free Precast Gels (Bio-Rad). Detailed information regarding the antibodies is shown in the supplementary data. PKA phosphorylation of PLIN1 as well as overall PKA substrate phosphorylation were analyzed. Protein levels are expressed as a ratio to β-actin content in the sample. Protein phosphorylation is expressed as a ratio to total protein level.

2.8. Capillary electrophoresis immunoassay (Wes system)

The levels of ERK 1/2 phosphorylation and FSP27 were measured by capillary electrophoresis immunoassay (Wes; ProteinSimple, Santa Clara, CA) as previously described [29,30]. Information regarding the antibodies is shown in the supplementary data. All antibodies were diluted 1:10 in Antibody Dilution (ProteinSimple). AT biopsies were homogenized in a buffer containing 50 mM HEPES, 137 mM NaCl, 10 mM Na4P2O7, 20 mM NaF, 5 mM EDTA, 1 mM $MgCl_2$, 1 mM $CaCl_2$, NP-40, 2 mM NaOV, 5 mM NAM, 10 μM TSA, NP-40, HALT, glycerol and demineralized water. After 1 h of agitation at 37 °C, the samples were spun at 1300 RPM for 20 min and the infranatant was used. Protein concentration was measured with a BCA assay. Samples were vortex mixed and heated at 95 °C for 5 min. Protein expression was quantified as peak area for the protein of interest. FSP27 is expressed as a ratio to β-actin content in the sample. ERK phosphorylation is expressed as a ratio to total protein expression of ERK.

2.9. Mice

Bovine GH (bGH) transgene mice, Stat5ΔN/ΔN mice [31] and wild type mice were housed at 22 °C under a 14-h light, 10-h darkness cycle, 3–4 mice per cage and *ad libitum* access to water and standard laboratory chow (ProLab RMH 3000). All mice were males, 6 weeks of age, with a C57BL/6 background, and raised at the Ohio University animal facility. The control mice were age-matched C57BL/6 mice. All mice experiments were approved by the Ohio University Institutional Animal Care and Use Committee.

2.10. Cell culture

3T3-L1 adipocytes were grown and differentiated as previously described [32]. 3T3-L1 (from ATCC: CL-173; passages 4–12) were grown in DMEM, high glucose (4.5 g/L), Glutamax, Pen-Strep and 10% FBS and then seeded at 200,000 cells/well in six-well plates for differentiation into adipocytes. The medium was replaced by differentiation medium, and, after 2 days, the medium was replaced with growth medium. Glycerol release was measured as an estimation of lipolysis.

2.11. Quantitative PCR (mice and cell cultures)

Analyses of gene expression were conducted as previously described [32]. RNA was extracted by TRIzol Reagent (Life Technologies) and quantified by measuring absorbance at 260 and 280 nm. TBP primers: 5′-ACCCTTCACCAATGACTCCTATG-3′ 5′-TGACTGCAGCAAATCGCTTGG-3′.

2.12. Reporter assays

293T cells were transfected with polyethylenimine (PEI) transfection reagent while still in suspension in 96-well plates. Each well was transfected with 50 ng of a previously described G0S2 luciferase construct [33], together with a total of 50 ng of expression plasmids containing STAT5A, STAT5B or empty vector controls and 10 ng of Renilla-TK plasmid. The cells were harvested 24 h later and luciferase activity was measured using a Dual Renilla Luciferase II Assay Kit and normalized to Renilla luciferase measurements (Promega). Site-directed mutagenesis of the *G0S2* constructs by PCR was

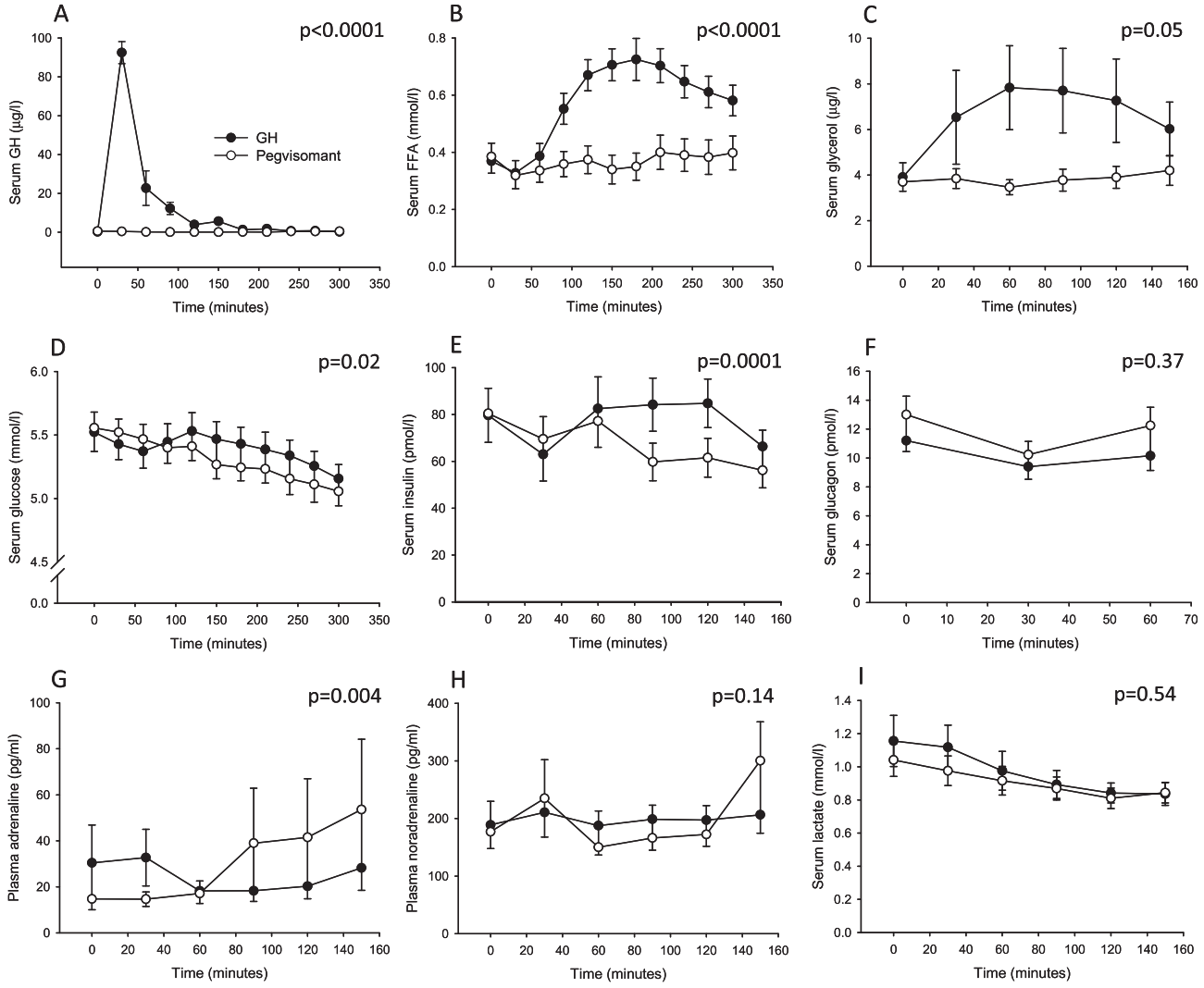

Figure 1: Circulating hormones and metabolites. Data are analyzed by a repeated measurement mixed effects model analysis. Mean ± SEM of raw data. The p-values indicate interaction between time and intervention (GH vs. Pegvisomant). Black dots: GH, white dots: Pegvisomant. GH, Growth hormone.

performed. The reporter vector was co-transfected with either a vector control or 25 ng of PPARγ expression vector and 25 ng of the obligate heterodimer RXRα.

2.13. Statistics

Effects of GH over time in the human studies were analyzed by a repeated measurement mixed effects model analysis using the Restricted Maximum Likelihood method in STATA (version 14.2, StataCorp). In the GH bolus study, the model included intervention (GH versus pegvisomant), sample time (t = 0–300), visit (first or second study day), order of intervention, and the interaction between intervention and sample time as fixed effects. In the GH infusion study, the model included infusion (GH versus control), fasting (overnight versus 36 h fasting), sample time (basal or clamp), visit, and all possible interactions between infusion, fasting and sample time as fixed effects. In both studies, visit nested within subject was used as random effect, and time nested within visit was treated as repeated measurements using a covariance matrix with independent and identically distributed Gaussian residuals with one common variance within visit nested within subject. Data from mouse and *in vitro* studies were analyzed by t tests (compared to WT, baseline or control). Normal distribution was assessed by inspecting QQ-plots of the residuals. To obtain normal distribution of the residuals, variables were transformed by logarithmic transformation if necessary.

To account for unbalanced data sets because of missing observations, Kenward Roger's approximation was used for calculation of degrees of freedom in all models. Linear pairwise comparisons based on t-tests were performed to compare differences within and between treatment groups. Adrenaline data from one visit of one subject were excluded from the data analysis due to a vasovagal reaction from which the participant rapidly recovered.

Data from the blood samples are presented as mean ± SEM from raw data, data from q-PCR and WB are presented as estimated means ± the confidence intervals (CI) and data from mouse and *in vitro* experiments are presented as mean ± SEM from raw data. The graphical presentations were performed with SigmaPlot (version 11.0, Systat Software Inc.). All analyses were performed as two-tailed tests and p-values <0.05 were considered statistically significant.

3. RESULTS

3.1. A single GH bolus stimulates FFA release and induces insulin resistance in obese human subjects *in vivo*

A peak in serum GH was measured 30 min after the GH bolus (Figure 1A). Serum FFA levels increased 90 min after GH exposure and peaked after 180 min (Figure 1B) followed by a gradual decline towards baseline levels. On the pegvisomant day, no increase in serum FFA levels was recorded. Serum glycerol levels changed in a pattern similar to FFA (Figure 1C). An overall decline in plasma glucose levels with time occurred on both study days; following GH exposure, however, a transitory significant increase was recorded (Figure 1D). In parallel with this, an increase in serum insulin levels was observed at $t = 60$ on the GH day as opposed to a gradual decline on the pegvisomant day (Figure 1E). Circulating glucagon levels were comparable on the two study days (Figure 1F) with a decrease at $t = 180$ (main effect of time: $p = 0.0001$). Plasma adrenaline levels (pg/ml) at baseline were higher on the GH day (30.5 ± 16.4 vs. 14.8 ± 4.7, $p = 0.006$) and did not change significantly with time as opposed to a gradual increase recorded on the pegvisomant day (Figure 1G). No differences in circulating noradrenaline levels were noted after GH (Figure 1H). Serum lactate levels decreased with time (main effect of time: $p < 0.0001$) with no difference between the GH and the pegvisomant day (Figure 1I).

Lipid oxidation rates increased with time during both study days (main effect of time: $p < 0.001$), and, at the time point of peak serum FFA levels ($t = 180$), lipid oxidation tended to be higher on the GH day ($p = 0.08$) (Figure 2A). Glucose oxidation rates decreased significantly with time on both study days (main effect of time: $p < 0.001$) with no significant effect of GH (Figure 2B). GH did not significantly affect protein oxidation rates (Figure 2C) or resting EE (Figure 2D).

3.2. GH signaling in AT is recorded after a single GH bolus

A pronounced increase in STAT5 phosphorylation occurred 60 min after GH exposure (Figure 3A). No significant STAT5 activation was detectable at other time points. The expression of *cytokine-inducible SH2-containing protein* (*CISH*) mRNA increased 60 min after GH and peaked at $t = 180$ followed by a return to baseline at $t = 300$ (Figure 3B). The mRNA levels of *suppressor of cytokine signaling* (*SOCS*)1, *SOCS2* and *SOCS3* were low on both days, without a detectable GH effect (data not shown). *IGF*-1 mRNA expression increased 1.5 fold at $t = 180$ after GH followed by a return to baseline at $t = 300$ (Figure 3C). Moreover, we measured sporadic thr^{202} and tyr^{204} phosphorylation of ERK that did not appear to be GH-dependent (data not shown).

3.3. GH downregulates *G0S2* mRNA expression and FSP27 protein levels in human subjects *in vivo*

Expression of *G0S2* mRNA was significantly reduced after GH exposure (Figure 4A), characterized by a two-fold reduction at $t = 300$ without detectable changes in protein levels (Figure 4B). To corroborate this observation, we analyzed gene and protein expression of G0S2 in AT biopsies from a study in lean human subjects during GH infusion as well as fasting [28]. As previously reported from that study, serum FFA

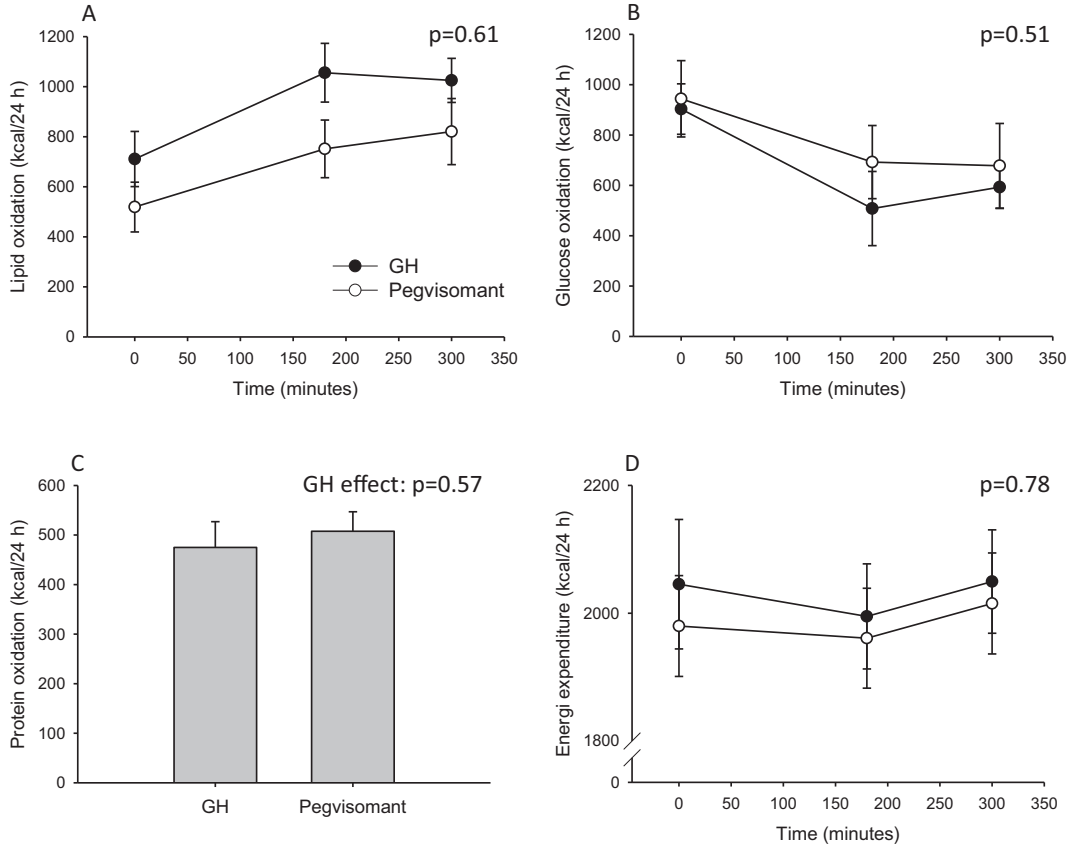

Figure 2: Substrate metabolism and energy expenditure. (A) Lipid oxidation, (B) glucose oxidation, (C) protein oxidation and (D) resting energy expenditure. Data are analyzed by a repeated measurement mixed effects model analysis. Mean ± SEM of raw data. The p values indicate interaction between time and intervention. Black dots: GH, white dots: Pegvisomant. GH, growth hormone.

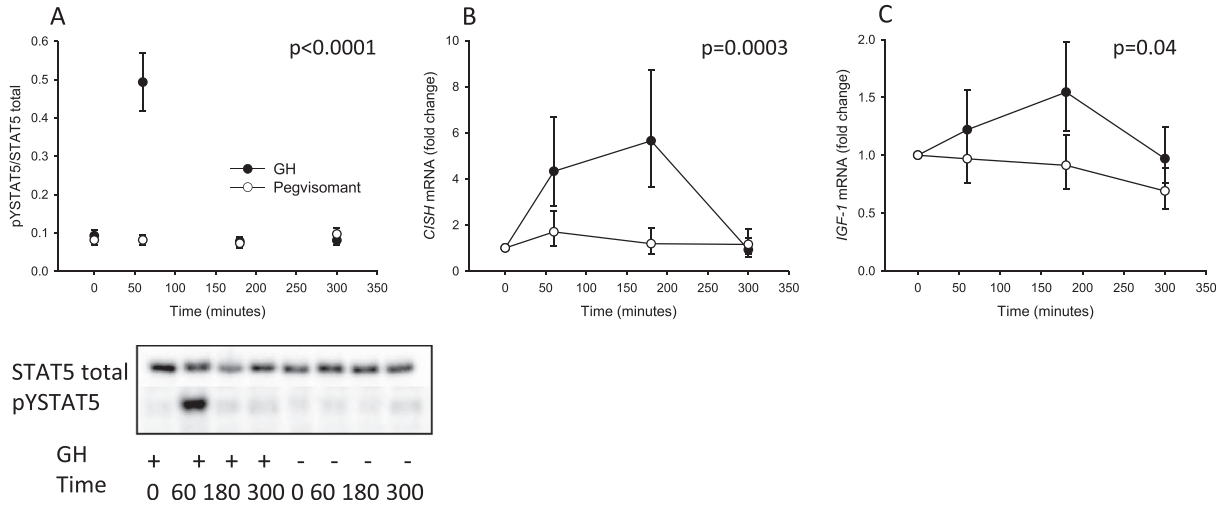

Figure 3: GH signaling i adipose tissue. (A) pYSTAT5/STAT5 total including a representative western blot, (B) *CISH* mRNA levels (C) and *IGF-1* mRNA levels. Data are analyzed by a repeated measurement mixed effects model analysis. q-PCR data are relative to baseline. Estimated means ± CI. The p values indicate interaction between time and intervention (GH vs. Pegvisomant). Black dots: GH, white dots: Pegvisomant. GH, Growth hormone; CISH, cytokine-inducible SH2-containing protein.

levels and *CISH* mRNA-expression in AT increased significantly in response to both GH and fasting [21]. GH infusion also reduced *G0S2* mRNA-expression in the basal state ($p = 0.07$) and during the HEC ($p = 0.02$) compared to the control day (Figure 4C). Suppression of *G0S2* mRNA expression also occurred during fasting alone (basal: $p < 0.0001$, HEC: $p = 0.04$) and during GH infusion together with fasting (basal: $p < 0.0001$, HEC: $p < 0.0001$). *G0S2* mRNA expression increased during the HEC on the fasting day ($p < 0.0001$) and during GH together with fasting ($p = 0.007$). No significant difference in protein levels of G0S2 could be demonstrated in response to either GH or fasting (Figure 4D). Since *G0S2* is a PPAR-γ target gene [33], we measured phosphorylation of PPAR-γ Ser^{273}, which is associated with PPARγ degradation, in the biopsies from the GH bolus study without detecting a distinct GH effect (data not shown).

Protein levels of FSP27 declined 1 h after the GH bolus and were reduced by 50% after 3 h, followed by an increase at $t = 300$ (Figure 4E). No effect of GH on *FSP27* mRNA expression could be detected (Figure 4F). The mRNA gene expression of *phosphatase and tensin homolog* (*PTEN*), which acts to suppress PI3K stimulation of Akt [34], was significantly upregulated 180 min after GH (Figure 4G), whereas PTEN protein expression was unchanged (Figure 4H). The protein levels of comparative gene identification-58 (CGI-58), which is a potent activator of ATGL [1] did not change in response to GH (data not shown).

Neither PKA-phosphorylation of PLIN1 nor PKA-phosphorylation on all detectable proteins >15 kDa changed significantly in response to GH. Likewise, phosphorylation of HSL Ser^{563}, Ser^{565}, and Ser^{660} did not increase after GH. Phosphorylation of Akt Ser^{473} and *phosphodiesterase 3B* (*PDE3B*) mRNA expression were also unchanged after GH.

3.4. *G0S2* mRNA expression are suppressed in bovine GH (bGH) transgenic mice and in 3T3-L1 adipocytes in response to GH

Consistent with the human data, *G0S2* mRNA was significantly reduced in SC AT of bGH transgenic mice compared with WT mice (Figure 5A). In support of a direct suppressive effect, differentiated 3T3-L1 adipocytes exhibited a reduction of *G0S2* mRNA 1−2 h after GH exposure (Figure 5B). The GH-induced decrease in *G0S2* mRNA levels was dose-dependent (Figure 5C), and this was accompanied by glycerol release, as published previously [32].

3.5. Inhibition of MEK and activation of PPARγ abrogate GH-induced suppression of *G0S2* mRNA expression

To investigate the roles of MEK/PPARγ for GH-induced *G0S2* mRNA suppression, GH-treated 3T3-L1 adipocytes were pre-treated with U0126, a MEK inhibitor, and rosiglitazone, a PPARγ agonist. Glycerol release was completely suppressed by U0126 and rosiglitazone, as previously reported [32]. U0126 pretreatment abrogated the GH-mediated suppression of *G0S2*, while rosiglitazone led to a two-fold increase in *G0S2* mRNA expression that was not suppressed by GH (Figure 5D). Further supporting a role for PPARγ in the regulation of *G0S2*, luciferase activity of 293T cells transfected with the 2.2-kb WT G0S2 luciferase reporter or with a mutation in the PPARγ response element (ΔPPRE) indicated that the expression of the G0S2 promoter is increased by PPARγ through its consensus PPRE. This was not further increased by co-transfection with STAT5 (Figure 5E). Furthermore, qPCR analysis of RNA isolated from SC and perigonadal (PG) adipose tissue of mice, which express hypomorphic forms of both STAT5a and STAT5b (STAT5ΔN/ΔN mutant mice) [31], indicated that *G0S2* mRNA remains unchanged by reduced STAT5 activity.

4. DISCUSSION

The overarching finding from the present study is that GH-induced lipolysis involves downregulation of *G0S2* and FSP27, both of which are important suppressors of ATGL. These observations were made in our pivotal human experiment involving consecutive AT biopsies from healthy, obese, male subjects after a GH bolus. The suppression of *G0S2* mRNA became evident 3 h after GH and prevailed for 5 h. We corroborated this observation by analyzing AT biopsies in a separate experiment employing GH infusion in lean subjects, and by studying bGH transgenic mice, and in a subsequent *in vitro* experiment, we demonstrated that downregulation of *G0S2* mRNA is a direct effect of GH, which may involve MEK-induced suppression of PPARγ-dependent *G0S2* gene transcription.

In accordance with previous data, lipolysis was stimulated concomitantly with induction of insulin resistance [2]. Notably, the increase in serum FFA levels appeared 90 min after GH, whereas the suppression of *G0S2* mRNA became evident only after 180 min, which indicates

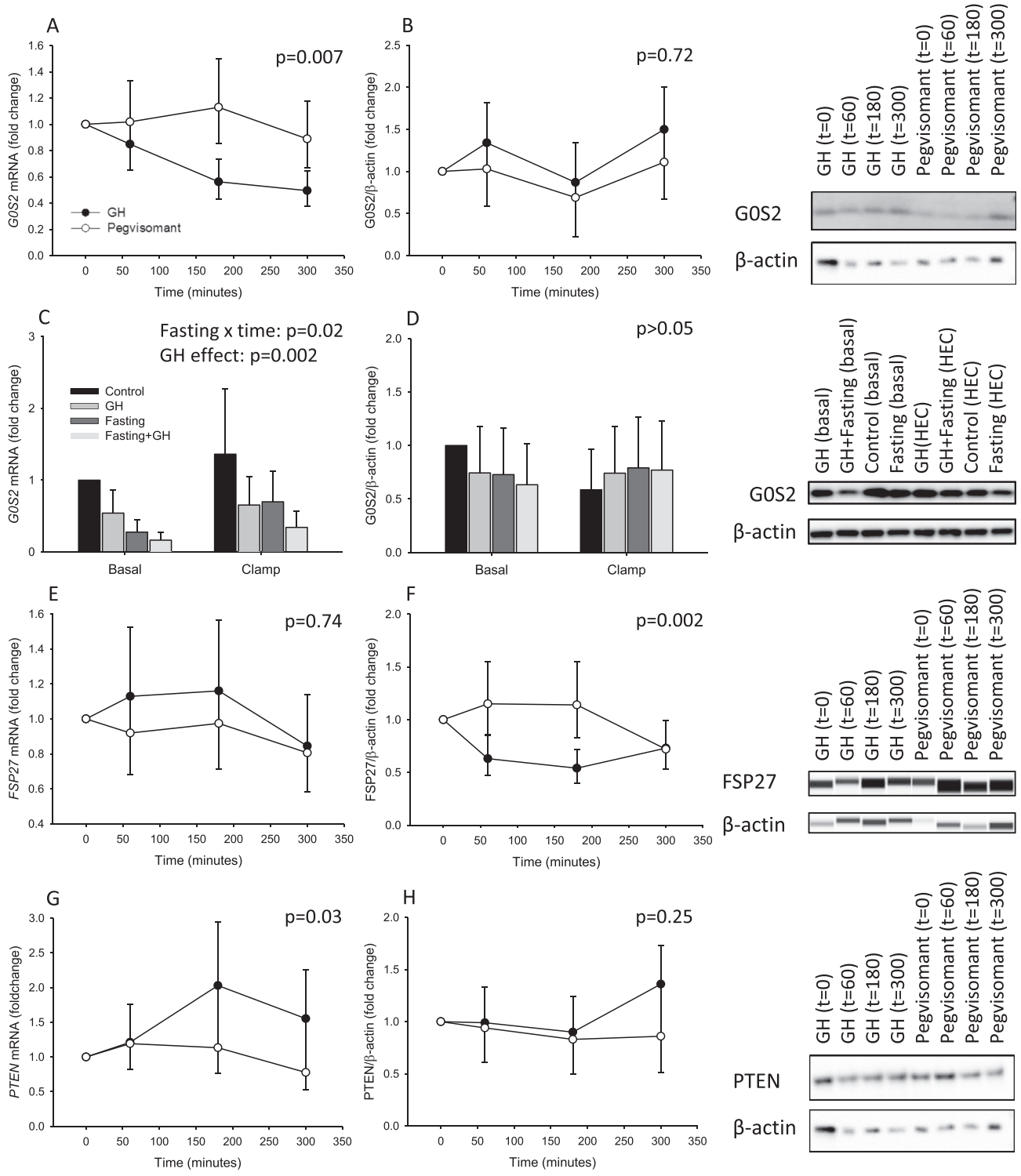

Figure 4: Expression of lipolytic regulators in adipose tissue. (A) *G0S2* mRNA and (B) protein expression from the GH bolus study. (C) *G0S2* mRNA and (D) protein expression from the GH infusion study. (E) *FSP27* mRNA and (F) protein expression from the bolus study. (G) *PTEN* mRNA and (H) protein expression from the bolus study. Data are analyzed by a repeated measurement mixed effects model analysis. Geometric mean ± CI. Representative western blots are presented. Data are relative to baseline (bolus study) or basal control (infusion study). The p values indicate interaction between time and intervention (bolus study) or interaction between GH, fasting and time and main effect of GH, fasting or time (if no interaction) (infusion study). Black dots: GH, white dots: Pegvisomant. Black bar: control, light grey: GH, dark grey: Fasting, lightest grey: GH + fasting. GH, growth hormone; G0S2, G0/G1 switch gene 2; FSP27, fat specific protein 27; PTEN, phosphatase and tensin homolog.

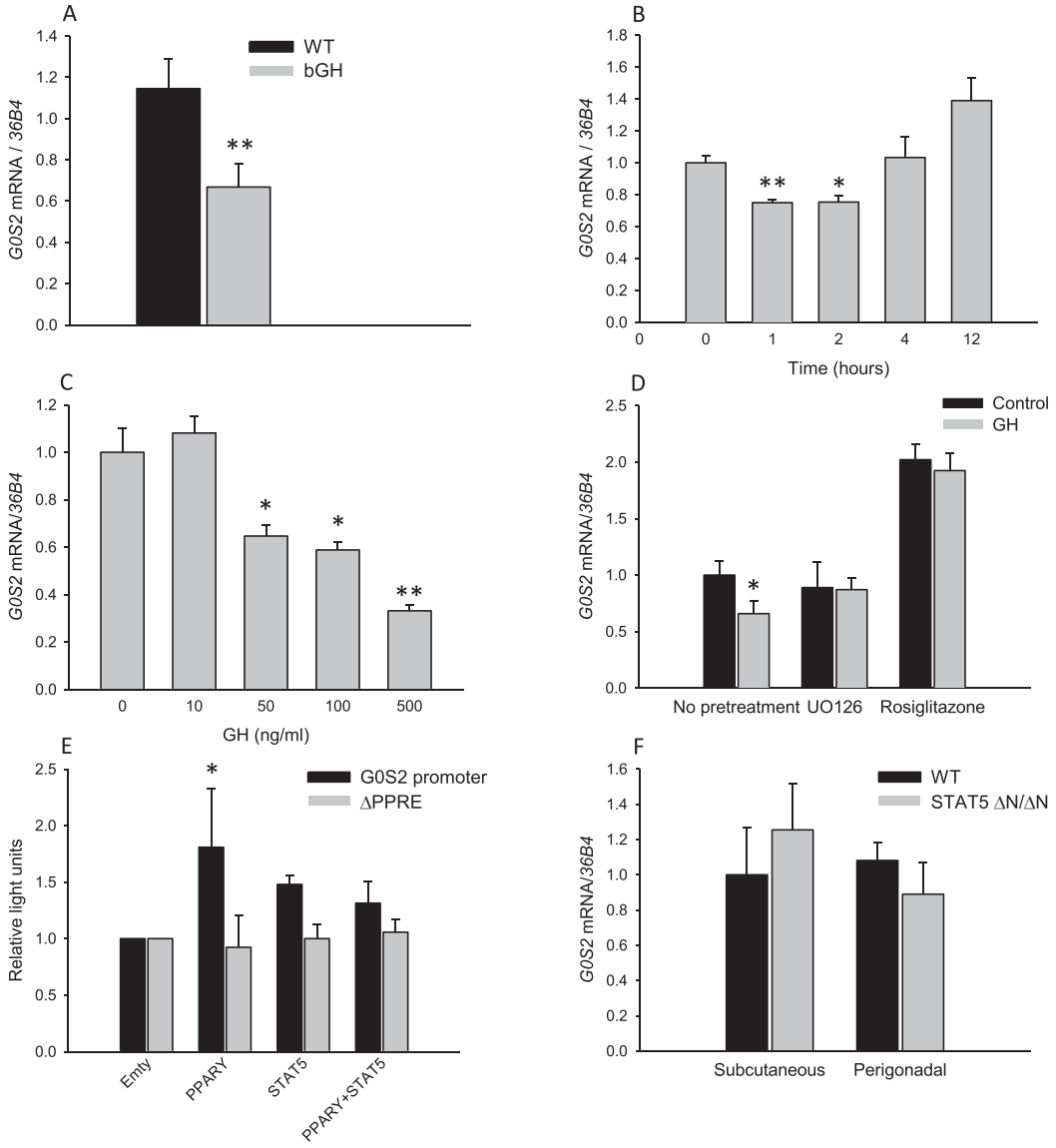

Figure 5: Mice and *in vitro* experiments. (A) G0S2 mRNA expression in subcutaneous adipose tissue of 4 months old male WT and bovine growth hormone-transgenic (bGH) mice. N = 10. (B) G0S2 mRNA expression in RNA isolated from 3T3-L1 adipocytes treated with 500 ng/mL recombinant bovine GH (bGH). N = 3. (C) G0S2 mRNA expression in RNA isolated from 3T3-L1 adipocytes treated with bGH for 2 h. N = 3. (D) G0S2 mRNA expression in RNA isolated from 3T3-L1 adipocytes treated with vehicle (control) or 500 ng/mL bGH (GH) for 2 h after 2 h pre-treatment with 10 μM U0126 (MAPK/ERK inhibitor), 1 μM rosiglitazone (PPARγ agonist) or no pretreatment. N = 3. (E) Luciferase activity of 293T cells transfected with the 2.2-kb WT G0S2 luciferase reporter (G0S2 promoter) or with the PPARγ response element mutated (ΔPPRE). The reporter vector was co-transfected with either a vector control, 25 ng of PPARγ expression vector and 25 ng of the obligate heterodimer retinoid X receptor α (RXRα) or 25 ng of a STAT5a expression vector. N = 6. (F) G0S2 mRNA expression in RNA isolated from subcutaneous and perigonadal fat of 4 months old male WT mice and mice expressing hypomorphic forms of both STAT5a and STAT5b (STAT5ΔN/ΔN mutant mice). N = 6–10. Mean ± SEM. Data are analyzed by t-tests (WT vs. bGH; 1, 2, 4, and 12 h vs. baseline; 10, 50, 100 and 500 ng/ml vs. baseline; GH vs. control; PPARγ, STAT5, PPARγ+STAT5 vs. control; WT vs. STAT5ΔN/ΔN). *p < 0.05, **p < 0.01. GH, growth hormone; G0S2, G0/G1 switch gene 2.

that GH-induced lipolysis is not initiated by *G0S2* suppression. This is compatible with other data reporting that G0S2 mainly plays a role as a long-term regulator of lipolysis [1]. In support of this, suppression of G0S2 mRNA [23–25] and protein levels [23,24] occurs after prolonged fasting but not after short-term exercise [24]. In further support of a suppressive effect of GH on G0S2, lipopolysaccharide (LPS) infusion in healthy subjects generates lipolysis in concomitance with increased endogenous GH secretion and suppressed *G0S2* mRNA levels in AT, whereas no such effects are detected in GH-deficient hypopituitary patients [26].

We were not able to detect a decrease in the protein level of G0S2 after GH, which could reflect that the timing of our biopsies were outside the window of opportunity, since protein levels at a given time point depend on transcription as well as degradation. Another explanation, however, is methodological challenges. We have previously detected *in vivo* G0S2 protein expression with WB in AT from human subjects during fasting [24], when we used an antibody (Santa Cruz, cat. no. sc-133424) that is no longer available.

The suppression of FSP27 after the GH bolus is compatible with a recent publication from our group that included data on the human GH

infusion experiment from which we presently report G0S2 data, as well as data in bGH transgene mice and cultured adipocytes [21]. The FSP27 decrease after the GH bolus was followed by a rebound increase at t = 300 (Figure 3F), which may represent a feedback mechanism to restrain excessive lipolysis, as suggested previously [35]. GH did not downregulate *FSP27* mRNA expression in this study, which was unexpected but may be explained by the timing of the biopsies and the use of a GH bolus as opposed to a GH infusion [21]. It is also possible that FSP27undergoes post-translational modifications in response to GH, but this remains to be tested.

In addition to the suppression of *G0S2* and FSP27, we observed GH-induced upregulation of the gene expression of *PTEN*, which is a negative regulator of insulin signaling [34]. In accordance with this, a previous study from our group reported increased *PTEN* mRNA in humans after fasting [25], and upregulation of *PTEN* mRNA expression has also been demonstrated in GH-treated mice [36]. However, we were unable to detect increased protein levels of PTEN and suppressed insulin signaling at the level of Akt phosphorylation after GH exposure. PTEN was initially recognized as a tumor suppressor regulating cell growth and survival [37], and even though PTEN is shown to suppress Akt signaling in 3T3-L1 adipocytes [38], the physiological role of PTEN as a regulator of lipolysis remains unclear. Downregulation of *PDE3B* mRNA in AT has also been recorded after fasting in humans [25], and this protein acts via Akt to suppress PKA activity by decreasing cAMP [1]. In the present study, no effect of GH on *PDE3B* mRNA expression was demonstrated.

We found no difference in protein levels of CGI-58 after GH exposure, which is in consistence with previous studies [21,32]. On the other hand, CGI-58 mRNA expression is increased by prolonged fasting in human subjects [23], and adipocyte-specific STAT5 deficiency in mice induces reduced CGI-58 levels in concomitance with reduced lipolysis and increased adiposity [12]. It remains to be tested if more prolonged GH stimulation may increase CGI-58 expression in human AT.

In vitro studies show that GH increases phosphorylation of HSL [39], and AT HSL phosphorylation has also been recorded in human subjects *in vivo* after exercise [40], hypoglycemia [41], LPS infusion [42], and fasting [23]. In the present GH bolus study, we did not detect a stimulatory effect on PKA activity and HSL phosphorylation, and in the GH infusion study, fasting (but not GH) increased HSL phosphorylation [21]. Prolonged GH treatment in obese women also does not increase HSL activity [43], and PKA and HSL phosphorylation are unaltered in adipocyte-specific STAT5 knockout compared to WT mice [12].

Consistent with previous human studies [6–11], we recorded STAT5 phosphorylation in AT 60 min after the GH bolus together with upregulated gene expression of *IGF-1* and *CISH*. JAK2-STAT5 signaling is considered the dominant GH signaling pathway [44], and studies in adipocyte-specific STAT5 and JAK2 knockout mice imply that the JAK2-STAT5 pathway also mediates GH-induced lipolysis [12,45]. Our *in vitro* studies indicate that MEK inhibition as well as PPARγ agonism abolish GH-induced downregulation of G0S2 mRNA, which is in line with previous *in vitro* studies [16,17]. Both G0S2 and FSP27 are known PPARγ target genes [33,46], and our luciferase activity assay data support the involvement of PPARγ. Our data in Stat5ΔN/ΔN mice suggest that suppressed STAT5 signaling does not reduce G0S2 expression, but it would be relevant to measure G0S2 expression in adipocyte-specific STAT5 knock out mice. By contrast, we have so far not been able to demonstrate MAPK signaling in response to GH in our human *in vivo* models [8] including the present study, and taken together, the individual roles of the two pathways for GH-induced lipolysis remain to be further studied.

We used pegvisomant rather than saline as a control experiment in order to suppress endogenous GH activity and lipolysis, but were surprised to see that adrenaline levels were lower after pegvisomant and increased significantly over time as compared to the GH day (Figure 1G). The reason for this is unexplained, but it is noteworthy that the absolute difference in adrenaline levels between the two study days was moderate and thus of dubious physiological significance compared to e.g. adrenaline changes in response to hypoglycemia [41].

Certain limitations of this study merit attention. First, assessment of gene and protein expression in crude AT biopsies obtained at certain intervals may not be sufficiently sensitive to capture temporal and dynamic changes in signaling pathways and enzymatic activity. On the other hand, we consider it a strength that we combined human *in vivo* data with *in vitro* experiments. Second, our human, clinical study involved healthy, obese, male subjects. It is not necessarily generalizable to women and lean subjects; however, we are analyzing AT biopsies from lean subjects, and a similar pattern is demonstrated, supporting the assumption that this pattern of *G0S2* expression is also evident in lean subjects. At last, our sample size was relatively small and the risk of a type 2 error is therefore a possibility.

In conclusion, GH-induced lipolysis is associated with suppression of *G0S2* and FSP27 in human, obese, male subjects. Furthermore, upregulation of *PTEN* mRNA expression was recorded. Subsequent *in vitro* data suggest that GH-induced downregulation of *G0S2* may involve the MEK/ERK pathway leading to suppressed PPARγ-mediated *G0S2* gene transcription. Based on the temporal changes in serum FFA levels, we suggest that additional signals may act to trigger lipolysis, but this remains to be investigated.

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CONFLICT OF INTEREST

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2019.08.013.

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