Insulin resistance induced by growth hormone is linked to lipolysis and associated with suppressed pyruvate dehydrogenase activity in skeletal muscle: a 2 × 2 factorial, randomised, crossover study in human individuals

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Abstract

Aims/hypothesis Growth hormone (GH) causes insulin resistance that is linked to lipolysis, but the underlying mechanisms are unclear. We investigated if GH-induced insulin resistance in skeletal muscle involves accumulation of diacylglycerol (DAG) and ceramide as well as impaired insulin signalling, or substrate competition between fatty acids and glucose.

Methods Nine GH-deficient male participants were randomised and examined in a 2 × 2 factorial design with and without administration of GH and acipimox (an anti-lipolytic compound). As-treated analyses were performed, wherefore data from three visits from two patients were excluded due to incorrect GH administration. The primary outcome was insulin sensitivity, expressed as the AUC of the glucose infusion rate (GIR_{AUC}), and furthermore, the levels of DAGs and ceramides, insulin signalling and the activity of the active form of pyruvate dehydrogenase (PDHa) were assessed in skeletal muscle biopsies obtained in the basal state and during a hyperinsulinaemic–euglycaemic clamp (HEC).

Results Co-administration of acipimox completely suppressed the GH-induced elevation in serum levels of NEFA (GH versus GH+acipimox, $p < 0.0001$) and abrogated GH-induced insulin resistance (mean GIR_{AUC} [95% CI] [mg min⁻¹ kg⁻¹] during the HEC: control, 595 [493, 718]; GH, 468 [382, 573]; GH+acipimox, 654 [539, 794]; acipimox, 754 [618, 921]; GH vs GH+acipimox: $p = 0.004$). GH did not significantly change either the accumulation of DAGs and ceramides or insulin signalling in skeletal muscle, but GH antagonised the insulin-stimulated increase in PDHa activity (mean ± SEM [% from the basal state to the HEC]: control, 47 ± 19; GH, -15 ± 21; GH+acipimox, 3 ± 21; acipimox, 57 ± 22; main effect: $p = 0.02$).

Conclusions/interpretation GH-induced insulin resistance in skeletal muscle is: (1) causally linked to lipolysis; (2) not associated with either accumulation of DAGs and ceramides or impaired insulin signalling; (3) likely to involve substrate competition between glucose and lipid intermediates.

Trial registration [ClinicalTrials.gov](https://clinicaltrials.gov) NCT02782208

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Keywords Acipimox · Ceramides · Diacylglycerols · Fatty acids · Growth hormone · Insulin resistance · Insulin signalling · Pyruvate dehydrogenase activity

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Abbreviations

ACN	Acetonitrile
AS160	Akt substrate of 160 kDa
DAG	Diacylglycerol
EGP	Endogenous glucose production
GH	Growth hormone
GHD	GH deficiency
GIR	Glucose infusion rate

Research in context

What is already known about this subject?

- Growth hormone (GH)-induced insulin resistance is linked to the concomitant increase in circulating NEFA

What is the key question?

- Is GH-induced insulin resistance associated with (1) accumulation of diacylglycerols and ceramides in skeletal muscle and impaired insulin signalling or (2) substrate competition between fatty acids and glucose?

What are the new findings?

- GH-induced insulin resistance is not associated with either accumulation of diacylglycerols and ceramides or impaired insulin signalling
- The GH-induced suppression of insulin-stimulated active pyruvate dehydrogenase activity suggests that substrate competition between fatty acids and glucose is involved in GH-induced insulin resistance in skeletal muscle

How might this impact on clinical practice in the foreseeable future?

- These data provide fundamental insight into fatty acid-driven insulin resistance, which is of importance for understanding the metabolic syndrome, insulin resistance and type 2 diabetes

GIR _{AUC}	AUC of the GIR
GS	Glycogen synthase
GSK	Glycogen synthase kinase
HEC	Hyperinsulinaemic–euglycaemic clamp
IPA	Isopropanol
NOGD	Non-oxidative glucose disposal
PDH	Pyruvate dehydrogenase
PDHa	The active form of PDH
PDK	Pyruvate dehydrogenase kinase
R _a	Glucose rate of appearance
R _d	Glucose rate of disappearance
UHPLC	Ultra-high-performance liquid chromatography system

Introduction

Growth hormone (GH) directly stimulates lipolysis [1], which is detectable as an increase in circulating levels of NEFA 1–2 h after a GH bolus [2, 3]. Concomitantly, GH induces insulin resistance in skeletal muscle as evidenced by a decrease in muscle glucose uptake [2, 4].

It is well known that fatty acids induce insulin resistance in skeletal muscle [5–9] and the mechanisms have been suggested to involve impaired insulin signalling induced by intramyocellular lipid accumulation [10–14] and/or substrate competition between fatty acids and glucose [8].

Substrate competition, or the glucose–fatty acid cycle, as proposed by Randle et al, implies that enhanced fatty acid oxidation increases mitochondrial acetyl-CoA and NADH, which inhibits pyruvate dehydrogenase (PDH) activity, and thereby the conversion of pyruvate into acetyl-CoA. This ultimately leads to accumulation of glucose 6-phosphate and reduced glucose

uptake [8]. Other studies, however, reported that intravenous infusion of lipid induced insulin resistance by reducing insulin signalling and action in human skeletal muscle [11–13]. This led to the hypothesis that fatty acids inhibit insulin-stimulated glucose uptake secondary to intramyocellular accumulation of certain lipid intermediates that directly impair insulin signalling [10, 14].

Several experimental studies have shown that pharmacological inhibition of lipolysis resulting in low serum NEFA levels abolishes GH-induced insulin resistance in human individuals [15–18]. Furthermore, we have observed GH-induced suppression of active PDH (PDHa) activity in healthy human volunteers concomitantly with normal insulin signalling [19].

Taken together, this suggests that GH-induced insulin resistance is causally linked to lipolysis, but the underlying mechanisms remain to be experimentally tested. We therefore designed a study to elucidate the metabolic effects of GH in the presence and absence of activated lipolysis. To this end, nine hypopituitary adults with GH deficiency (GHD) were studied in a 2 × 2 factorial design including GH replacement therapy and treatment with acipimox, a nicotinic acid derivative that blocks protein kinase A-dependent lipolysis via suppression of adenylyl cyclase [20]. The outcomes included insulin sensitivity, basal and insulin-stimulated insulin signalling, intramuscular content of diacylglycerols (DAGs) and ceramides, and PDHa activity in skeletal muscle.

Methods

Participants and study design

In a 2 × 2 factorial, randomised, crossover design, nine hypopituitary men with documented GHD receiving stable GH

replacement therapy were studied. Both participants and investigators were blinded to the intervention, excluding GH administration. Eligibility criteria included age over 18, male sex and documented GHD. None of the patients had diabetes or any overt chronic disease. The participants were in the age range of 29–71 years and had a mean $\pm$ SEM BMI of 28.2 ± 0.9 kg/m². The study was carried out in the Medical Research Laboratory, Aarhus University, and was conducted in accordance with the Declaration of Helsinki II, approved by the regional Ethics Committee System and reported at [ClinicalTrials.gov](https://www.clinicaltrials.gov) (registration no. NCT02782208). Oral and written consents were obtained from all participants prior to inclusion. Data on serum NEFA levels, lipoprotein lipase activity and expression of *ANGPTL4* mRNA (which encodes angiopoietin-like 4) from this study have been published previously [21].

Protocol

Each participant was studied on four separate occasions after: (1) discontinuation of GH injections for 36 h plus oral placebo ('control'); (2) continuation of GH injections plus oral placebo ('GH'); (3) continuation of GH injections plus oral acipimox ('GH+acipimox'); and (4) discontinuation of GH injections for 36 h plus oral acipimox ('acipimox'), respectively. Oral acipimox (Olbetam, Orifarm Generics, Denmark) in a dose of 250 mg or placebo was administered at 20:00 and 23:00 hours on the evening before and at 06:00 and 10:00 hours on each of the four study days (Fig. 1). The pharmacy at Aarhus University Hospital undertook randomisation, blinding and

provision of acipimox/placebo. Block randomisation was used and the random allocation sequence was stored in sealed envelopes until the study was unblinded after all participants had completed the study. GH was administered as subcutaneous self-injections at 22:00 hours. During the acipimox and control experiments, GH injections were discontinued 2 days prior to the study day. Replacement therapy with other hormones was continued.

The metabolic studies were performed between 8:00 and 14:00 hours ($t = 0$ –360) after an overnight fast. The participants were instructed to refrain from alcohol intake and vigorous exercise for 2 days prior to the study day. On each study day, an intravenous catheter for infusions was inserted in the antecubital vein of one arm, and another catheter was placed in a contralateral hand vein, which was heated to obtain arterialised blood. Blood samples were drawn at $t = 0, 60, 120, 160, 170, 180, 240, 300, 340, 350$ and 360 min. A 3 h basal period (0–180 min) was followed by a 3 h (180–360 min) hyperinsulinaemic–euglycaemic clamp (HEC) with continuous insulin infusion (0.6 mU min⁻¹ kg⁻¹, Humulin Regular, Eli Lilly, Denmark). During the HEC, plasma glucose concentrations were measured at bedside every 10 min and the glucose infusion rate (GIR) was adjusted with a 20% glucose infusion to maintain euglycaemia (5 mmol/l). The M value was calculated as mean GIR ($t = 340$ –360 min) per kg body mass.

Tracer kinetics

A bolus (0.74 MBq) of [3 - 3 H]-glucose (GE Healthcare, Denmark) was given at $t = 0$ followed by a continuous

Fig. 1 Study design. The participants were studied after: (1) discontinuation of GH injections plus oral placebo tablets (control); (2) continuation of GH plus oral placebo tablets (GH); (3) continuation of GH plus oral acipimox tablets (GH+acipimox); and (4) discontinuation of GH plus oral acipimox tablets (acipimox). Oral acipimox was administered at 20:00 and 23:00 hours the evening before and at 06:00 and 10:00 hours on the study day and GH was administered as subcutaneous self-injections at

22:00 hours. The study day commenced with a 3 h basal period (0–180 min) followed by a 3 h HEC (180–360 min). Biopsies were obtained in the basal state and during the HEC. Blood samples were drawn at $t = 0, 60, 120, 160, 170, 180, 240, 300, 340, 350$ and 360 min (large red arrows), and furthermore, plasma glucose concentrations were measured at the bedside every 10 min to adjust the GIR during the HEC (small red arrows)

infusion (0.44 MBq/h) at $t = 0$ –360 min. In addition, [3 - 3 H]-glucose was added to the glucose infused during the HEC to avoid rapid dilution (3.7 MBq [3 - 3 H]-glucose in 500 ml of 20% glucose). Specific activities of [3 - 3 H]-glucose were measured in triplicates at the end of the basal period ($t = 160$ –180 min) and the HEC ($t = 340$ –360 min). Glucose rate of appearance (R_a) and glucose rate of disappearance (R_d) were calculated using the non-steady-state equation of Steele [22]. Endogenous glucose production (EGP) equals R_a in the basal period, whereas EGP during the HEC was calculated by subtracting GIR from R_a . Non-oxidative glucose disposal (NOGD) was calculated by subtracting oxidative glucose disposal (derived from indirect calorimetry, see below) from total R_d .

To obtain a measure of protein degradation, [13 C]-urea was administered in the basal state ($t = 0$ –180 min) as a 390.6 mg bolus followed by a continuous infusion of 42 mg/h and was measured in triplicates at the end of the basal period ($t = 160$ –180 min).

Indirect calorimetry

Indirect calorimetry was performed with a canopy system (Oxycon Pro; Intramedic, Gentofte, Denmark) in the basal state ($t = 120$) and during the HEC ($t = 300$) to estimate energy expenditure, respiratory exchange ratio and substrate oxidation rate as previously described [23]. Urine was collected in the basal period and during the HEC to estimate protein oxidation based on urea excretion.

Biopsies

Skeletal muscle biopsies were obtained in the basal state ($t = 30$) and during the HEC ($t = 360$). The biopsies were obtained from the vastus lateralis of the quadriceps femoris muscle under sterile conditions using a Bergström needle. Lidocaine was used as local anaesthetic and the biopsies were immediately washed free of blood, frozen in liquid nitrogen and stored at -80°C until analysis.

Blood samples

Plasma glucose and lactate levels were measured at bedside (YSI 2300 STAT Plus glucose analyser; YSI, Burlington, VT, USA). Commercial ELISA kits were used to analyse insulin (Merkodia, Uppsala, Sweden), glucagon (Merkodia, Uppsala, Sweden) and cortisol (DRG Diagnostics, Marburg, Germany). Serum NEFA levels were determined by a colorimetric method using a commercial kit (Wako Chemicals, Neuss, Germany). Serum GH concentrations were determined by a modified chemiluminescence technology (IDS-iSYS human GH; Immunodiagnostic Systems, Boldon, UK). Plasma adrenaline (epinephrine) and noradrenaline (norepinephrine)

concentrations were measured by electrochemical detection following HPLC separation.

Quantitative PCR

Quantitative PCR analyses were performed as previously described [24]. *B2M* (encoding $\beta 2$ microglobulin) was used as housekeeping gene, as mRNA levels were similar between interventions. Primer sequences of *B2M* were GAGGCTATCCAGCGTACTCC and AATGTCGGATGGATGAAACC C (length 111), primer sequences of *PDK2* were AGCCGATTACATGGTCTACGTC and AGTCGCCC TCATGGCATTCTTG (length 80), and primer sequences of *PDK4* were ATTTAAGAATGCAATGCGGGC and GCGGTCAATAATTCTCAGGG (length 151). The fold change was calculated using the $2^{-\Delta\Delta C_t}$ method, where $\Delta\Delta C_t$ is the difference between the mean C_t for the target gene and the housekeeping gene at time x , minus the difference between the mean C_t for the target gene and the housekeeping gene in the basal state on the control day. The efficiency of the PCR reaction was similar for *B2M* and each of the target genes, verifying that the $\Delta\Delta C_t$ method can be used.

Capillary electrophoresis immunoassay (Wes system)

Phosphorylation of signalling proteins was measured by capillary electrophoresis immunoassay (Wes; ProteinSimple, Santa Clara, CA, USA) as previously described [25, 26]. Detailed information regarding the antibodies is provided in the electronic supplementary material (ESM) Table 1. All antibodies were diluted 1:25 in Antibody Dilution (ProteinSimple), and the tissue samples were homogenised in a buffer containing 50 mmol/l HEPES, 137 mmol/l NaCl, 10 mmol/l $\text{Na}_4\text{P}_2\text{O}_7$, 20 mmol/l NaF, 5 mmol/l EDTA, 1 mmol/l MgCl_2 , 1 mmol/l CaCl_2 , NP-40, 2 mmol/l NaOV, 5 mmol/l NAM, 10 $\mu\text{mol/l}$ TSA, HALT, glycerol and demineralised water. After 1 h of agitation at 4°C , the samples were spun at 16,200 g for 20 min and the supernatant was used. Protein concentration was measured with a bicinchoninic acid (BCA) assay. Samples were vortex mixed with mastermix (ProteinSimple) and heated at 95°C for 5 min. Protein content and phosphorylation were quantified as peak area for the protein of interest, and phosphorylation levels were expressed as the ratio between phosphorylation and total content of the protein.

PDHa activity

PDHa activity was measured as previously described [19]. In short, 8–15 mg of the muscle tissue was homogenised on ice for 50 s using a micro glass homogeniser (Kontes, Vineland, NJ, USA) and snap-frozen in liquid nitrogen. The PDHa activity in the homogenate was determined from the rate of acetyl-

CoA production using a radioactivity assay [27, 28] and normalised to the content of creatine in the sample.

Measurement of metabolite levels by mass spectrometry

Metabolite extractions Metabolites were extracted from skeletal muscle tissues using a modified method of Folch et al [29]. Briefly, 30 mg wet weight of tissue was homogenised in chloroform/methanol (2:1, vol./vol., 750 µl) using a TissueLyser (Qiagen, Manchester, UK). Samples were sonicated for 15 min and demineralised water and chloroform were added (200 µl of each). Separation of the aqueous and organic fractions was performed following centrifugation (13,000 *g* for 20 min). The organic phase (lower phase) extracts containing lipids were dried under a stream of nitrogen gas. A mixture of ^2H -labelled internal standards was added to the fractions and dried again under nitrogen (ESM Table 2).

Analysis of intact lipids using orbitrap mass spectrometry The organic fraction was reconstituted in 100 µl of chloroform/methanol (1:1, vol./vol.), and 10 µl of the resulting solution was added to 190 µl of isopropanol (IPA)/acetonitrile (ACN)/water (2:1:1, vol./vol.). Samples were analysed using an LTQ Orbitrap Elite Mass Spectrometer (Thermo Scientific, Hemel Hempstead, UK). Sample (5 µl) was injected onto a C18 CSH column (Waters, Manchester, UK; 1.7 µmol/l pore size, 2.1 mm × 50 mm), which was held at 55°C in a Dionex Ultimate 3000 ultra-high-performance liquid chromatography system (UHPLC; Thermo Scientific). A gradient (flow rate 0.5 ml/min) of mobile phase A (ACN/water, 60:40) and B (LC-MS-grade ACN/IPA, 10:90), with either 10 mmol/l ammonium formate (positive mode) or 10 mmol/l ammonium acetate (negative mode), was used. In both positive and negative ion modes, the gradient run began at 40% B; increased to 43% B at 0.8 min, 50% B at 0.9 min, 54% B at 4.8 min, 70% B at 4.9 min, 81% B at 5.8 min; peaked at 99% B at 8 min for 0.5 min; and subsequently returned to the starting conditions for another 1.5 min to re-equilibrate the column. The UHPLC was coupled to an electrospray ionisation (ESI) source, which ionised the analytes before entering the mass spectrometer. Spectra were collected in both positive and negative ion modes in the range of 110–2000 *m/z*. Default instrument-generated optimisation variables were used (ESM Table 3).

The spectra files were converted to mzML format and features picked using XCMS (<https://xcmsonline.scripps.edu/> version 3.5) [30], after retention time alignment. Lipid identification was performed using an in-house R script. Peak areas of each metabolite were normalised to the appropriate internal standard and tissue weight.

Statistics

Data were analysed to investigate GH and acipimox effects in the basal state and during the HEC; the relative change from basal to HEC as an additional measure of insulin responsiveness was also tested. Circulating hormones and metabolites were analysed as AUC during the basal state and the HEC.

The effects of GH and acipimox were assessed by repeated measurement mixed effects model analysis using the Restricted Maximum Likelihood method in STATA (<https://www.stata.com/> version 14.2, StataCorp). The model included GH (GH vs no GH treatment), acipimox (acipimox vs placebo), visit order and the interaction between GH and acipimox as fixed effects, and visit nested within-subject as random effect. To correct for multiple testing in the analyses of the content of ceramides and DAGs in skeletal muscle, the Holm–Bonferroni method was used.

To obtain homogeneous variance, unequal variance variables were logarithmically transformed. To account for missing values, Kenward Roger’s approximation was used for calculation of degrees of freedom in all models. The results of the statistical analyses are given by the *p* values. All analyses were performed as two-tailed tests. When an interaction or a main effect was present, pertinent pairwise comparisons were made. A *p* value of <0.05 was considered statistically significant. As-treated analyses were performed, and therefore data from three visits from two different patients were excluded due to incorrect GH administration, resulting in *N* = 9 on the control day, *N* = 7 on the GH day, *N* = 9 on the GH+acipimox day and *N* = 8 on the acipimox day. Furthermore, three skeletal muscle biopsies were excluded because of fat contamination, two from the GH+acipimox day and one from the acipimox day, resulting in *N* = 9 on the control day, *N* = 7 on the GH day, *N* = 7 on the GH+acipimox day and *N* = 7 on the acipimox day in the protein and quantitative PCR analyses.

All graphical presentations were performed with SigmaPlot (<http://www.sigmaplot.co.uk/>, version 14.0, Systat Software). Data are presented as mean ± SEM (blood analyses and substrate oxidation), or as geometric mean ± 95% CI.

The primary outcome was insulin sensitivity, expressed as the AUC of the GIR (GIR_{AUC}) during the HEC and the *M* value. Secondary outcomes were insulin signalling, content of DAGs and ceramides, and PDHa activity in the skeletal muscle. The sample sizes were defined based on a previous study [15].

Results

Circulating hormones

Serum GH levels increased during GH replacement therapy both in the basal state (main effect: *p* < 0.0001) and during the HEC (main effect: *p* < 0.0001), and acipimox evoked a

minute, but detectable, increase in endogenous GH secretion in the basal state (main effect: $p = 0.02$) (Fig. 2a). Serum glucagon levels were elevated by acipimox in the basal state (main effect: $p = 0.0002$) and suppressed in response to insulin (main effect: $p < 0.0001$) (Fig. 2b). Basal plasma lactate levels were similar in the four study arms and increased in response to insulin irrespective of GH and acipimox (main effect: $p < 0.0001$) (Fig. 2c). Circulating cortisol levels decreased with time (main effect: $p < 0.0001$) with no significant effect of either GH or acipimox (Fig. 2d). Basal and insulin-stimulated adrenaline (Fig. 2e) and noradrenaline (Fig. 2f) levels were similar between the four interventions.

Lipid metabolism

Data on serum NEFA levels are previously described [21]. In short, basal serum NEFA levels were increased by GH (control vs GH: $p = 0.03$), whereas NEFA levels were potently

suppressed by acipimox (GH vs GH+acipimox: $p < 0.0001$, control vs acipimox: $p < 0.0001$) (Fig. 3a). In the presence of acipimox, GH exerted no residual effect on NEFA (GH+acipimox vs acipimox: $p = 0.43$). Insulin suppressed serum NEFA levels during all four interventions (main effect: $p < 0.0001$). Lipid oxidation in the basal state was decreased by acipimox (main effect: $p = 0.0008$), whereas during the HEC, lipid oxidation decreased (main effect: $p < 0.0001$) to comparable levels irrespective of treatment (Fig. 3b).

Glucose metabolism and insulin sensitivity

Basal plasma glucose levels were decreased by acipimox (main effect: $p = 0.005$) (Fig. 4a), whereas comparable and stable glucose levels were obtained in all four interventions at the end of the HEC ($t = 340\text{--}360$). GH increased basal serum insulin levels relative to control ($p = 0.02$), and this increase was abrogated by acipimox (GH vs GH+acipimox:

Fig. 2 Circulating hormones. Circulating levels of GH (a), glucagon (b), lactate (c), cortisol (d), adrenaline (e) and noradrenaline (f) in the basal state and during the HEC, presented as mean $\pm$ SEM, $N = 7\text{--}9$. Data were analysed by repeated measurement mixed effects model analysis. Main effect of GH: $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$; main effect of acipimox: $^{\dagger}p < 0.05$, $^{\dagger\dagger\dagger}p < 0.001$

Fig. 3 NEFA metabolism and lipid oxidation. Serum NEFA levels (a) and lipid oxidation (b) in the basal state and during the HEC, presented as mean $\pm$ SEM, $N = 7$ –9. Data were analysed by repeated measurement mixed effects model analysis. Interaction between GH and acipimox:

* $p < 0.05$; main effect of acipimox: ‡ $p < 0.05$, ‡‡ $p < 0.001$

$p = 0.002$). During the HEC, there was no significant difference in serum insulin levels between the four interventions (Fig. 4b).

Assessment of insulin sensitivity was based on GIR_{AUC} and the M value. GIR_{AUC} (control, 595 [493, 718]; GH, 468 [382, 573]; GH+acipimox, 654 [539, 794]; acipimox, 754 [618, 921]) was decreased by GH (control vs GH: $p = 0.04$) and increased by acipimox (GH vs GH+acipimox: $p = 0.004$; control vs acipimox: $p = 0.03$), with no effect of GH during acipimox treatment (GH+acipimox vs acipimox: $p = 0.2$) (Fig. 4c). The M value was reduced by GH as compared with control ($p = 0.005$), which was abolished by acipimox (GH vs GH+acipimox: $p = 0.02$) (Fig. 4d). There was no significant difference in the M value between control, GH+acipimox and acipimox alone.

Glucose disposal expressed by the R_d was similar on all study days in the basal state. During the HEC, GH decreased R_d (main effect: $p = 0.01$), whereas acipimox evoked a non-significant increase (main effect: $p = 0.06$) (Fig. 4e). Glucose oxidation in the basal state was increased by acipimox irrespective of GH (main effect: $p = 0.004$); during the HEC, glucose oxidation increased to similar levels in all four interventions (Fig. 4f). NOGD in the basal state was suppressed by acipimox (main effect: $p = 0.0009$). During the HEC, a non-significant increase in NOGD was observed after acipimox (main effect: $p = 0.06$) (Fig. 4g). EGP in the basal state was similar on all study days, whereas acipimox induced a decrease in EGP during the HEC ($p = 0.05$) (Fig. 4f).

Energy expenditure and protein metabolism

Basal and insulin-stimulated energy expenditure did not change significantly in response to either GH or acipimox (data not shown). GH decreased protein oxidation during the HEC (main effect: $p = 0.03$), whereas an increase was recorded after acipimox (main effect: $p = 0.02$) (Fig. 5a). Similarly, urea turnover increased after acipimox (main effect: $p = 0.01$) (Fig. 5b).

DAG and ceramide content in skeletal muscle

A total of 19 different species of DAGs and ceramides were identified in skeletal muscle (Table 1). Basal

DAG44:1 and ceramide CER(41:1) content decreased after GH irrespective of acipimox ($p = 0.002$). By contrast, the level of CER(42:1) was unaffected by GH and acipimox in the basal state but increased after GH irrespective of acipimox during the HEC ($p = 0.007$). However, when adjusted for multiple testing, there was no difference between the four interventions.

Insulin signalling in skeletal muscle

Phosphorylation of Akt Ser⁴⁷³ was unaffected by GH and acipimox in the basal state as well as during the HEC (Fig. 6a). Basal phosphorylation of Akt substrate of 160 kDa (AS160) Thr⁶⁴² was also comparable across all study days. During the HEC, however, AS160 phosphorylation was more pronounced after GH alone compared with control ($p = 0.02$) and GH+acipimox ($p = 0.03$) (Fig. 7b). Basal phosphorylation of glycogen synthase kinase (GSK)-3 α Ser²¹ was similar between interventions, but during the HEC, GH increased GSK-3 α phosphorylation (main effect: $p = 0.02$) (Fig. 6c). Phosphorylation of GSK-3 β Ser⁹ was similar between the interventions both in the basal period and during the HEC (Fig. 6d). Basal and insulin-stimulated phosphorylation of glycogen synthase (GS) 3a was unaffected by GH and acipimox (Fig. 6e). As expected, insulin increased the phosphorylation of Akt (main effect: $p < 0.0001$), AS160 (main effect: $p < 0.0001$), GSK-3 α (main effect: $p < 0.0001$) and GSK-3 β (main effect: $p < 0.0001$) and reduced GS phosphorylation (main effect: $p < 0.0001$).

PDHa activity, PDK2 and PDK4 mRNA expression, and PDH Ser²⁹³ phosphorylation in skeletal muscle

PDHa activity was similar between the four interventions both in the basal state and during the HEC (Fig. 7a). In response to insulin, PDHa activity increased $47 \pm 19\%$ on the control day ($p = 0.02$) and $57 \pm 22\%$ with acipimox alone ($p = 0.02$), but this increase was abrogated by GH (GH, -15 ± 21 ; GH+acipimox, 3 ± 21 ; GH vs control: $p = 0.05$; GH vs acipimox: $p = 0.03$) (Fig. 7b).

Fig. 4 Glucose metabolism and insulin sensitivity. Circulating levels of glucose (**a**) and insulin (**b**) in the basal state and during the HEC presented as mean $\pm$ SEM. GIR (**c**) and M value (**d**) presented as geometric mean $\pm$ 95% CI. R_d (**e**), glucose oxidation (**f**), NOGD (**g**) and EGP (**h**) presented as mean $\pm$ SEM. $N = 7-9$. The data were analysed by repeated measurement mixed effects model analysis. Interaction between GH and acipimox: * $p < 0.05$; main effect of GH: † $p < 0.05$; main effect of acipimox: ‡ $p < 0.05$, §§ $p < 0.01$, §§§ $p < 0.001$

Fig. 5 Protein metabolism. Protein oxidation in the basal state and during the HEC (**a**) and urea turnover in the basal state (**b**). Data are analysed by repeated measurement mixed effects model analysis and presented as mean $\pm$ SEM, $N = 7-9$. Main effect of GH: † $p < 0.05$; main effect of acipimox: ‡ $p < 0.05$

Table 1 Content of DAGs and ceramides in the skeletal muscle

Lipid	Basal, mean (95% CI)				HEC, mean (95% CI)			
	Control	GH	GH+acipimox	Acipimox	Control	GH	GH+acipimox	Acipimox
DAG32:0	1.0 (0.6, 1.5)	1.5 (1.0, 2.2)	1.9 (1.3, 2.8)	1.4 (0.9, 2.1)	1.0 (0.7, 1.5)	1.3 (0.8, 1.9)	1.2 (0.8, 1.7)	1.5 (1.0, 2.3)
DAG34:0	1.0 (0.7, 1.4)	1.3 (0.9, 1.9)	1.2 (0.9, 1.7)	1.2 (0.8, 1.8)	0.9 (0.9, 1.4)	1.1 (0.7, 2.0)	1.1 (0.7, 1.9)	1.4 (0.8, 2.4)
DAG34:1	1.0 (0.6, 1.8)	1.1 (0.6, 2.1)	1.7 (1.0, 3.0)	1.3 (0.7, 2.6)	0.9 (0.7, 1.1)	0.8 (0.6, 1.1)	0.9 (0.7, 1.2)	1.1 (0.8, 1.5)
DAG36:0	1.0 (0.6, 1.6)	1.2 (0.7, 2.0)	1.3 (0.8, 2.1)	1.0 (0.6, 1.6)	0.8 (0.4, 1.4)	1.3 (0.6, 2.7)	0.9 (0.5, 1.7)	1.2 (0.7, 2.3)
DAG36:1	1.0 (0.7, 1.4)	1.1 (0.8, 1.5)	1.3 (0.9, 1.8)	1.4 (1.0, 2.0)	0.9 (0.7, 1.3)	1.0 (0.7, 1.3)	1.1 (0.8, 1.5)	1.1 (0.8, 1.5)
DAG36:2	1.0 (0.6, 1.7)	1.4 (0.8, 2.5)	1.7 (1.0, 2.9)	1.5 (0.8, 2.8)	1.0 (0.7, 1.4)	1.3 (0.9, 1.8)	1.2 (0.9, 1.7)	1.1 (0.8, 1.5)
DAG38:0	1.0 (0.8, 1.2)	1.1 (0.9, 1.3)	1.1 (0.9, 1.3)	1.2 (1.0, 1.5)	1.0 (0.9, 1.2)	0.9 (0.8, 1.0)	1.0 (0.9, 1.2)	1.0 (0.9, 1.2)
DAG38:1	1.0 (0.7, 1.3)	1.2 (0.9, 1.6)	1.4 (1.0, 1.9)	1.1 (0.8, 1.6)	1.0 (0.8, 1.3)	1.1 (0.8, 1.4)	1.1 (0.9, 1.4)	1.1 (0.9, 1.4)
DAG42:0	1.0 (0.7, 1.5)	0.7 (0.5, 1.1)	0.7 (0.5, 1.1)	1.3 (0.8, 2.0)	0.8 (0.6, 1.1)	0.7 (0.5, 1.1)	0.9 (0.6, 1.3)	1.0 (0.7, 1.4)
DAG42:1	1.0 (0.7, 1.5)	1.0 (0.6, 1.6)	0.8 (0.5, 1.3)	1.6 (1.0, 2.6)	1.0 (0.6, 1.4)	0.9 (0.6, 1.4)	1.1 (0.7, 1.7)	1.1 (0.7, 1.7)
DAG42:2	1.0 (0.5, 2.0)	0.8 (0.4, 1.9)	0.9 (0.4, 1.8)	1.3 (0.6, 2.8)	0.8 (0.5, 1.5)	0.7 (0.4, 1.3)	1.1 (0.6, 1.9)	0.9 (0.5, 1.6)
DAG44:0	1.0 (0.7, 1.4)	0.7 (0.5, 1.0)	0.8 (0.6, 1.1)	1.0 (0.7, 1.5)	0.7 (0.5, 1.0)	0.7 (0.5, 1.0)	0.8 (0.6, 1.1)	0.9 (0.6, 1.3)
DAG44:1	1.0 (0.7, 1.4)	0.7 (0.5, 1.1)	0.7 (0.5, 1.0)	1.3 (0.8, 1.9)	0.8 (0.5, 1.1)	0.7 (0.5, 1.1)	0.9 (0.6, 1.3)	0.9 (0.6, 1.3)
DAG44:2	1.0 (0.7, 1.5)	0.9 (0.6, 1.4)	0.8 (0.6, 1.2)	1.6 (1.0, 2.4)	0.9 (0.6, 1.3)	1.0 (0.6, 1.5)	1.2 (0.8, 1.8)	1.0 (0.6, 1.4)
CER(40:1)	1.0 (0.9, 1.2)	1.0 (0.9, 1.2)	0.9 (0.7, 1.0)	1.1 (0.9, 1.3)	0.9 (0.8, 1.1)	1.0 (0.9, 1.2)	1.0 (0.9, 1.2)	1.0 (0.8, 1.1)
CER(41:1)	1.0 (0.8, 1.2)	0.9 (0.7, 1.0)	0.7 (0.6, 0.9)	1.0 (0.8, 1.1)	1.1 (0.9, 1.2)	1.0 (0.9, 1.1)	1.1 (0.9, 1.2)	1.0 (0.9, 1.1)
CER(41:2)	1.0 (0.8–1.2)	1.0 (0.8, 1.3)	1.1 (0.9, 1.3)	1.0 (0.8, 1.3)	1.0 (0.8, 1.3)	0.8 (0.6, 1.1)	1.0 (0.8, 1.3)	1.0 (0.8, 1.3)
CER(42:1)	1.0 (0.9, 1.1)	1.1 (0.9, 1.2)	0.9 (0.8, 1.1)	1.1 (0.9, 1.3)	0.9 (0.8, 1.0)	1.0 (0.9, 1.1)	1.1 (1.0, 1.2)	0.9 (0.8, 1.0)
CER(42:2)	1.0 (0.8, 1.2)	1.0 (0.9, 1.2)	1.0 (0.8, 1.2)	1.1 (0.9, 1.3)	1.0 (0.8, 1.2)	1.0 (0.8, 1.2)	1.1 (0.9, 1.3)	1.0 (0.9, 1.2)

Pyruvate dehydrogenase kinase (PDK) 2 and 4 phosphorylate and thereby inactivate PDH in skeletal muscle [31]. Expression of *PDK2* mRNA in the basal state was decreased by GH+acipimox compared with control ($p = 0.006$), GH ($p = 0.01$) and acipimox ($p = 0.004$). During the HEC, *PDK2* mRNA content was similar in all four interventions with no effect of insulin (Fig. 7c). Basal *PDK4* mRNA content was reduced by GH+acipimox as compared with control ($p = 0.03$), GH ($p = 0.008$) and acipimox ($p = 0.02$) (Fig. 7d). The content of *PDK4* mRNA decreased on all study days in response to insulin (main effect of time: $p < 0.0001$). During the HEC, *PDK4* mRNA expression was still decreased by GH+acipimox as compared with GH alone ($p = 0.009$) and acipimox alone ($p = 0.03$) (Fig. 7d). Phosphorylation of PDH Ser²⁹³ was comparable between all study days in the basal state and during the HEC (Fig. 7e).

Discussion

It is well documented that GH-induced insulin resistance depends on concomitant lipolysis [15, 16, 18], and the present study was undertaken to elucidate the underlying mechanisms. Our results suggest that suppression of PDHa activity, rather than impairment of insulin signalling, promotes GH-induced insulin resistance in skeletal muscle in humans.

The PDH complex controls the entry of carbohydrate-derived pyruvate into the tricarboxylic acid (TCA) cycle [32]. In line with this, we found an increase in PDHa activity in response to insulin, which is supported by most [33–36], but not all [19], previous studies. Our current observation that this increase is abrogated by GH suggests that GH-induced insulin resistance involves PDH-mediated inhibition of glucose uptake. In support of this, it has previously been reported that PDHa activity is suppressed after GH exposure, fasting and high NEFA levels [19, 37, 38]. The suppressive effect of GH on PDHa activity prevailed to a minor extent during acipimox co-administration, which suggests the existence of NEFA-independent effects of GH that remain elusive.

The activity of the PDH complex is suppressed by reversible PDK-induced phosphorylation of the E1 subunits, whereas activation is mediated by PDH phosphatases [31]. Accordingly, the *PDK4* mRNA content increases with fasting and exercise [37, 39, 40] in a NEFA-dependent manner [40, 41] and the insulin-induced decrease in *PDK4* mRNA expression in the present study is in line with previous reports [19, 34, 36]. The lack of GH-associated regulation of *PDK4* mRNA and PDH Ser²⁹³ phosphorylation in response to GH or insulin in the present study may suggest that one of the other known phosphorylation sites is involved or that other post-translational modifications such as acetylation caused the change in PDHa activity, as previously suggested [42].

Fig. 6 Insulin signalling in skeletal muscle. p-Akt Ser⁴⁷³/total Akt (a), p-AS160 Thr⁶⁴²/total AS160 (b), p-GSK3 α Ser²¹/total GSK3 α (c), p-GSK3 β Ser⁹/total GSK3 β (d) and p-GS 3a Ser⁶⁴¹/total GS 3a (e) in the basal state and during the HEC, presented as geometric mean $\pm$ 95% CI, with representative western blots (f), $N=7-9$. The data were analysed by repeated measurement mixed effects model analysis. Interaction between GH and acipimox: * $p < 0.05$; main effect of GH: † $p < 0.05$

Intramyocellular DAGs and ceramides are suggested to be the cause of fatty acid-driven insulin resistance by the activation of protein kinase C ϵ and θ [10] which, in turn, phosphorylate and inactivate insulin receptor substrate 1 and thereby decrease Akt activity [43]. In the present study, we therefore evaluated the content of DAGs and ceramides in human skeletal muscle during GH exposure with and without activation of lipolysis, and these results imply that GH-induced insulin resistance in skeletal muscle is unlikely to be caused by the accumulation of DAGs or ceramides. Taken together with the observation of unaltered or even, in some cases, increased phosphorylation of Akt, AS160 and GS, we conclude that GH does not suppress insulin signalling in human skeletal muscle [19, 44–46].

The observation that acipimox increases protein turnover and oxidation, which is reversed by GH, supports

previous studies [47, 48]. Gluconeogenesis is decreased by nicotinic acid-induced suppression of plasma NEFA [49], and is therefore not likely to contribute to acipimox-induced ureagenesis. The protein-sparing effect of GH during fasting is partly attributed to lipolysis [47], but the present data support that GH also possesses direct protein anabolic effects.

The inclusion of GH-replaced hypopituitary patients provides a unique model for studying the interplay between GH and anti-lipolysis. Not only does it provide a model of fixed GH exposure, it also makes it possible to obtain sustained anti-lipolysis with acipimox, which otherwise is hampered by a rebound increase in endogenous GH secretion (triggered by low NEFA levels) that, in turn, stimulates lipolysis [50]. At the same time, we believe that our data contribute

Fig. 7 PDH activity. Activity of PDH in skeletal muscle in the basal state and during the HEC presented as geometric mean $\pm$ 95% CI (a) and the relative change in PDH activity from the basal state to the HEC presented as mean $\pm$ SEM (b). mRNA expression of *PDK2* (c) and *PDK4* (d) in the basal state and during the HEC presented as geometric mean $\pm$ 95% CI. p-PDH-E α 1 (Ser²⁹³)/total PDH-E α 1 presented as geometric mean $\pm$ 95% CI (e) and representative western blot (f). The data were analysed by repeated measurement mixed effects model analysis, $N = 7$ –9. Interaction between GH and acipimox: * $p < 0.05$; main effect of GH: † $p < 0.05$

to the understanding of fatty acid-associated insulin resistance in general and we dare to challenge the hypothesis that direct impairment of insulin signalling is a crucial mechanism in human individuals in vivo.

Certain limitations of this study merit attention. First, we recorded a minor increase in serum GH levels on the acipimox day due to residual endogenous GH secretion. However, the GH levels on the acipimox day were significantly lower than on both the GH and the GH+acipimox day. Second, although mass spectrometry allowed identification of all DAGs and ceramides in the specimen, it did not discriminate between intra- and extra-myocellular lipids. Finally, our sample size was relatively small, which is associated with an increased risk of a type 2 error.

In conclusion, the present study confirms that GH-induced insulin resistance is causally linked to lipolysis and provides novel evidence that this is not mediated by accumulation of DAGs and ceramides or impaired insulin signalling in skeletal muscle. Instead, the observed suppression of insulin-stimulated PDH activity suggests that substrate competition between fatty acids and glucose is involved in GH-induced insulin resistance.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on request.

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Contribution statement AJH had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. AJH, NJ and JOLJ designed the study. AJH, EC, SBP, AG and HP acquired the data and carried out the data analysis. AJH and JOLJ drafted the manuscript. All authors made substantial contributions to the interpretation of the results and critical revision of the manuscript. All authors approved the final version of the manuscript.

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