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Growth hormone-induced insulin resistance in human subjects involves reduced pyruvate dehydrogenase activity

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Abstract

Aim: Insulin resistance induced by growth hormone (GH) is linked to promotion of lipolysis by unknown mechanisms. We hypothesized that suppression of the activity of pyruvate dehydrogenase in the active form (PDHa) underlies GH-induced insulin resistance similar to what is observed during fasting.

Methods: Eight healthy male subjects were studied four times in a randomized, single-blinded parallel design: Control, GH, Fasting (36 h) and GH + Fasting. GH (30 ng $\times$ kg^{-1} $\times$ min^{-1}) or saline was infused throughout the metabolic study day. Substrate metabolism and insulin sensitivity were assessed by indirect calorimetry and isotopically determined rates of glucose turnover before and after a hyperinsulinemic euglycemic clamp. PDHa activity, PDH-E1α phosphorylation, PDK4 expression and activation of insulin signalling proteins were assessed in skeletal muscle.

Results: Both fasting and GH promoted lipolysis, which was associated with $\approx$50% reduction in insulin sensitivity compared with the control day. PDHa activity was significantly reduced by GH as well as fasting. This was associated with increased inhibitory PDH-E1α phosphorylation on site 1 (Ser^{293}) and 2 (Ser^{300}) and up-regulation of PDK4 mRNA, while canonical insulin signalling to glucose transport was unaffected.

Conclusion: Competition between intermediates of glucose and fatty acids seems to play a causal role in insulin resistance induced by GH in human subjects.

Keywords fasting, growth hormone, pyruvate dehydrogenase, substrate oxidation.

Insulin resistance in skeletal muscle is a pivotal feature of the metabolic syndrome and related disorders. The aetiology is complex including genetic disposition combined with environmental cues such as physical inactivity and chronic overnutrition (Muoio & Newgard 2008, Samuel *et al.* 2010). Cytosolic accumulation of lipid-derived metabolites in skeletal muscle is considered an important pathogenic factor but the mechanistic links are uncertain. Randle *et al.* (1963) suggested a mechanism by which oxidation of fatty acids (FA) suppresses pyruvate dehydrogenase (PDH) activity. Activity of PDH in the active form (PDHa) is regulated by inhibitory phosphorylations on the PDH-E1α subunit controlled by PDH kinases (PDK) and PDH phosphatases (Pilegaard & Neufer 2004). Suppressed PDH activity leads to an increase in citrate that inhibits phosphofructokinase and elevates cytosolic concentrations of glucose-6-phosphate, which

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prevents further glucose uptake and disposal (Randle *et al.* 1963). Such a mechanism has been proposed to contribute to fat-induced insulin resistance. Alternatively, it is suggested that accumulation of diacylglycerol and/or ceramides inhibits insulin signalling through the phosphatidylinositol 3-kinase (PI3K)–AKT pathway and thereby impairs downstream signalling proteins critical for GLUT4 translocation (Schmitz-Peiffer *et al.* 1997, Chavez *et al.* 2003, Powell *et al.* 2004).

Growth hormone (GH) also causes pronounced skeletal muscle insulin resistance, which is reversible (Krusenstjerna-Hafstrom *et al.* 2011a) and causally linked to the concomitant GH-induced stimulation of lipolysis (Nielsen *et al.* 2001). Moreover, short-term high-dose GH treatment in healthy subjects increases intramyocellular triglyceride content (Krag *et al.* 2007). The secretory pattern of GH is reciprocal to that of insulin with elevation in response to fasting and exercise, where GH is considered to promote lipid utilization at the expense of glucose (Moller & Jorgensen 2009). The molecular mechanisms underlying this effect of GH are poorly understood. In particular, there is no distinct evidence of impaired insulin signalling in either the basal (Jorgensen *et al.* 2006) or insulin-stimulated state (Krusenstjerna-Hafstrom *et al.* 2011b). The impact of GH on pyruvate dehydrogenase complex (PDC) is unknown, which is intriguing because PDHa activity is suppressed by FA (Spriet *et al.* 2004) and PDK4 is up-regulated in skeletal muscle by short-term fasting (Pilegaard *et al.* 2003) and prolonged exercise (Pilegaard & Neufer 2004).

The present study was undertaken to elucidate the mechanism underlying insulin resistance induced by GH in human subjects. Eight healthy male subjects were studied on four occasions in a 2 × 2 factorial design involving GH and 36 h fasting. On each occasion, the subjects were studied both in the basal state and during a hyperinsulinemic euglycemic clamp. Skeletal muscle biopsies were obtained for assessment of PDHa activity and PDK4 expression in addition to expression and phosphorylation of signalling proteins of insulin and GH.

Materials and methods

Subjects

We studied eight healthy young men (21.2 ± 1.1 year) with a body mass index of 22.5 ± 1.5 kg × m^{-2} (mean ± SE). The Regional Scientific Ethics Committee of Denmark approved the study protocol (Central Denmark Region Ethics Committee, M-20080053), and written informed consent was obtained before participation in the study. The study was conducted in accordance with the Helsinki Declaration and registered in a public database (www.clinicaltrials.gov; NCT01209429).

Experimental design

In a single-blinded, randomized crossover design, subjects were studied on four occasions separated by 3–4 weeks: saline infusion after an overnight fast ('Control'), GH infusion after an overnight fast ('GH'), saline infusion after 36 h fasting ('Fasting') and GH infusion after 36 h fasting ('GH + Fasting'). The fasting period started at 2000 h, and the infusion of either GH (30 ng × kg^{-1} × min^{-1}, Genotropin, Pfizer) or saline started at 0800 h (Fig. 1). Participants abstained from exercise and alcohol intake for 48 h before each study day. One intravenous cannula was placed in the antecubital vein for infusion. For blood sampling, a second cannula was placed in a dorsal hand vein, which was placed in a heat pad of 65 °C for arterialization of the blood. Each study day included a 150-min basal period followed by a 120-min hyperinsulinemic euglycemic clamp (0.6 mU × kg^{-1} × min^{-1}, Actrapid; Novo Nordisk A/S, Copenhagen, Denmark).

Circulating levels of hormones and metabolites

Plasma glucose was measured instantly on YSI 2300 STAT Plus glucose analyzer (YSI, Hampshire, England). Serum samples were frozen and stored at −20 °C until analysed. The specific activity (SA) of [3-^{3}H]glucose was measured as previously described (Moller *et al.* 1989). Insulin, GH and cortisol were analysed using time-resolved fluoroimmunoassay (TR-IFMA; AutoDELFIA, PerkinElmer, Turku, Finland), and glucagon was analysed with an in-house RIA. Free FA (FFA) was analysed with a commercial kit (Wako Chemicals, Neuss, Germany).

Glucose metabolism and insulin sensitivity

At t = 0 min, a priming dose of [3-^{3}H]glucose (20 μCi, Lægemiddelstyrelsen; The Isotope Agency, Copenhagen, Denmark) was given, followed by a continuous infusion of [3-^{3}H]glucose (12 μCi × h^{-1}) for 270 min. At t = 150 min, the hyperinsulinemic euglycemic clamp began (insulin infusion rate: 0.5 mU × kg^{-1} × min^{-1}). Plasma glucose was measured every 10 min and kept at 5 mM by adjusting the glucose infusion rate (GIR) (DeFronzo *et al.* 1979). Glucose rates of appearance (Ra) were estimated by dividing the [3-^{3}H]glucose infusion rate by the measured (SA) and corrected for non-steady state (Debodo *et al.* 1963). A pool fraction of 0.65 was used.

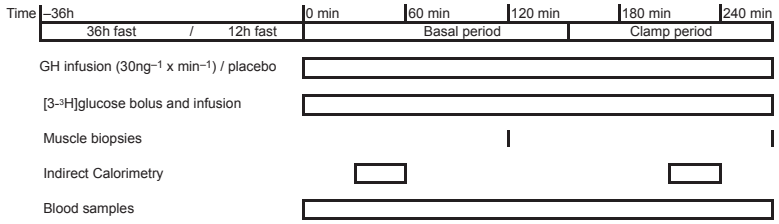

Figure 1 Outline of the study protocol.

Non-oxidative glucose disposal (NOGD) was calculated by subtracting oxidative glucose disposal as assessed by indirect calorimetry from whole body glucose disposal. Basal endogenous glucose production (EGP) was estimated as the mean of Ra calculated during the last 30 min of the basal period. The amount of infused glucose required to maintain blood glucose at 5.0 mM during the clamp reflects peripheral insulin sensitivity. The M-value was calculated as the GIR during the last 30 min of the clamp per kg body weight. EGP during the clamp was estimated by subtracting the GIR from the Ra the last 30 min of the clamp.

Indirect Calorimetry

The respiratory exchange ratio (RER) and resting energy expenditure (REE) were measured by indirect calorimetry (Deltatrac monitor; Dantes Instrumentarium, Helsinki, Finland) performed at time: 30–60 min ('basal') and at time: 210–240 min ('clamp'). Net lipid and glucose oxidation rates were calculated from the above measurements after correction for protein oxidation (Ferrannini 1988).

Biopsies

At time = 120 min ('basal') and time = 270 min ('clamp'), singleton muscle biopsies with a size of ≈200 mg were obtained from vastus lateralis using a Bergström biopsy needle under local anaesthesia. The tissue was quickly cleaned of blood, frozen in liquid nitrogen and stored at −80 °C until further preparations.

Measurement of insulin signalling and PDH regulation

Western blot (WB) analyses were used to assess total and phosphorylated levels of pertinent proteins. Phospho-specific antibodies against pAkt Thr^{308}, pAkt Ser^{473}, PAS, pAS160 Ser^{588}, pAS160 Thr^{642}, pGS Ser^{641}, pSTAT5 Tyr^{699} and antibodies against STAT5, Akt, AS160 and GS were all from Cell Signaling Technology, Beverly, CA, USA. An antirabbit IgG horseradish peroxidase (Amersham, GE-Healthcare, Pittsburgh, PA, USA) was used as secondary antibody. Muscle biopsies were pulverized in a frozen steel mortar. Proteins were purified from ~50 mg muscle by homogenization in an ice-cold buffer [20 mM Tris-HCl, 50 mM NaCl, 50 mM NaF, 5 mM natrium pyrophosphate, 250 mM sucrose, 1% (vol/vol) Triton X-100, 2 mM DTT, 50 μg/mL soybean trypsin inhibitor, 4 μg/mL leupeptin, 100 μM benzamidine and 500 μM PMSF]. Insoluble materials were removed by centrifugation at 28 000 ***g*** for 20 min at 4 °C. The supernatant was collected, frozen in liquid nitrogen and stored at −80 °C until analyses were performed. WB was performed using 15-μg protein per sample. Proteins were visualized by chemiluminescence (LumiGLO reagent and peroxide; Cell Signaling Technology) and quantified using UVP BioImaging System (UVP, Upland, CA, USA). To assess PDK4 protein and PDH-E1α content and phosphorylation, skeletal muscle was homogenized in a Tissue LyserII (Qiagen, Hilden, Germany). Muscle lysates were prepared as previously reported (Pilegaard *et al.* 2006), and protein concentration was determined using the bicinchoninic acid method (Pierce, Rockford, IL, USA). Total protein content of PDH-E1α and PDK4 as well as protein phosphorylation of PDH-E1α Ser^{293} and Ser^{300} was determined. The antibodies have previously been described (Pilegaard *et al.* 2006, Kiilerich *et al.* 2010) and were kindly provided by Prof. Hardie, University of Dundee, Scotland.

Protein phosphorylation data were normalized to the total amount of the specific protein and given relative to the control biopsy.

PDHa activity

Muscle tissue (9–10 mg) was homogenized on ice for 50 s using a glass homogenizer (Kontes, Vineland, NJ, USA) and quick frozen in liquid nitrogen as previously described (Pilegaard *et al.* 2006). The PDHa activity in the homogenate was then determined from the rate of acetyl-CoA production and determination of the produced acetyl-CoA by the use of a radioactivity assay as previously described (Cederblad *et al.* 1990,

Constantin-Teodosiu *et al.* 1991, Putman *et al.* 1993). The PDHa activity was normalized to the total creatine content in each sample (St Amand *et al.* 2000); in addition, the creatine-normalized PDHa activity measure was also expressed relative to PDH-E1α protein content as assessed by WB.

Glycogen content

Muscle samples were hydrolysed in 2M HCl at 100 °C for 2 h, followed by neutralization with 2 M NaOH, and glucose content was measured by the hexokinase enzymatic method using a glucose hexokinase reagent (Eagle Diagnostics, Desoto, TX, USA) (Bondar & Mead 1974).

Real-time RT-PCR for mRNA analysis

For real-time reverse transcriptase PCR, complementary DNA was constructed using random hexamer primers as described by the manufacturer (Verso cDNA kit from VWR, Herlev, Denmark). PCR-master mix was added, and real-time quantification of genes was performed by SYBR-green real-time reverse transcription PCR assay (KAPA SYBR Fast Universal kit from Ken-En-Tec, Taastrup, Denmark) in 384-well formats in a LightCycler from Roche (RocheApplied Science, 68298 Mannheim, Germany).

The following primer (DNA Technology, Risskov, Denmark) pairs were used as follows:

SOCS1: 5′-ACACGCACTTCCGCACATTC-3′ and 5′-CGAGGCCATCTTCACGCTAAG-3′

SOCS2: 5′-GGTCGAGGCGATCAGTG and 5′-TCC TTGAAGTCAGTGCGAATC-3′

SOCS3: 5′-GCCCTTTGCGCCCTTT-3′ and 5′-CG GCCACCTGGACTCCTATGA-3′

CIS: 5′-GCCCTGAGCCCTGGTAGTCC-3′ and 5′-GACACATTCACAGACGGGTGG-3′

B2 microglobulin: 5′-AATGTCGGATGGATGAAA CC-3′ and 5′-TCTCTCTTTCTGGCCTGGAG-3′ (housekeeping)

cDNA with specific primers was amplified in separate tubes, and the increase in fluorescence measured in real time. The relative gene expression was calculated using the advanced relative quantification module in the LightCycler software. All samples were amplified in duplicate.

Statistical analysis

Statistical comparisons between the study days were examined by two-way repeated-measurements ANOVA. Data were analysed to investigate potential fasting effects and GH effects in the basal state and in the insulin-stimulated state respectively. Moreover, potential insulin-induced changes were analysed by separate two-way repeated-measurements ANOVA with all four groups together. Where the ANOVA revealed significant over-all effect, the Student–Newman–Keuls method was used as post hoc test. Unless stated otherwise, data are presented as mean ± SE, and P-value <0.05 was considered significant. Normality was tested by qq-plots of the raw data and the residuals.

Results

Circulating hormones

GH was significantly elevated during GH and GH + Fasting as compared with Control, and as expected, Fasting was associated with elevated endogenous GH levels (Table 1). Basal serum insulin levels declined significantly with Fasting and GH + Fasting. Unexpectedly, steady-state insulin levels during the clamp in the Fasting and GH + Fasting studies were significantly decreased despite identical infusion rates of insulin on all four occasions (Table 1). Fasting with and without GH was associated with elevated glucagon levels in the basal state, whereas a suppression of glucagon levels in response to the clamp was observed in all four settings. Basal cortisol levels did not differ between the four experiments, but a significant suppression in response to insulin stimulation during the clamp was recorded (Table 1).

Insulin sensitivity and substrate metabolism

GH, Fasting and GH + Fasting induced significant insulin resistance as evidenced by reduced GIR and M-values on these study days compared with Control (Fig. 2a). This was also evident when the M-values were adjusted for plasma insulin levels (Fig. 2a, inserted histogram).

No differences were found in REE, neither between basal vs. clamp data nor when comparing the four experiments (Table 1). Basal serum FFA levels increased significantly with GH and Fasting as compared to the control day and were suppressed during the clamp in all experiments; however, this suppression was more pronounced in the control experiment as compared to the three other experiments (Fig. 2b). The corresponding rates of lipid oxidation were significantly elevated during Fasting, whereas the insulin-stimulated suppression of lipid oxidation was attenuated after GH, Fasting and GH + Fasting (Fig. 2c). EGP in the basal state was significantly lower after Fasting and GH + Fasting than in Control and GH, whereas the degree of suppression of EGP during the clamp was similar in all four experiments (Fig. 2d).

Table 1 Mean ± SE circulating levels of insulin, GH, cortisol and glucagon, and resting energy expenditure in the basal state and during the clamp after an overnight fast (C), GH infusion (GH), 36 h fasting (F) and GH infusion in combination with 36 h fasting (GH + F)

	C	GH	F	GH + F
Insulin (pM)				
Basal	31.4 ± 3.9	39.6 ± 6.3	17.8 ± 2.3*,†	14.2 ± 2.3*,†
Clamp	205.3 ± 7.2	196.5 ± 9.4	167.2 ± 12.2*,†	171.7 ± 9.5*,†
GH (μg/L)				
Basal	0.43 ± 0.28	8.07 ± 0.79*	4.79 ± 0.93*,†	14.62 ± 1.22*
Clamp	0.90 ± 0.30§	9.68 ± 0.58*	3.67 ± 1.55*,†	11.80 ± 0.62*,‡
Glucagon (pmol/mL)				
Basal	70.6 ± 9.0	73.5 ± 7.6	176.6 ± 13.6*,†	167.5 ± 23.8*,†
Clamp	46.4 ± 9.7§	40.9 ± 7.4§	56.0 ± 9.0†,§	50.4 ± 9.6§
Cortisol (nM)				
Basal	173.1 ± 9.8	156.5 ± 18.9	172.4 ± 16.5	174.6 ± 16.4
Clamp	73.2 ± 8.4§	68.2 ± 8.6§	69.2 ± 5.9§	69.3 ± 8.7§
REE (kcal/24 h)				
Basal	1841 ± 49	1856 ± 71	1768 ± 37	1796 ± 48
Clamp	1839 ± 34	1820 ± 46	1893 ± 48	1890 ± 42

$P < 0.05$: *vs. C, †vs. GH, ‡vs. F, §basal vs. clamp.

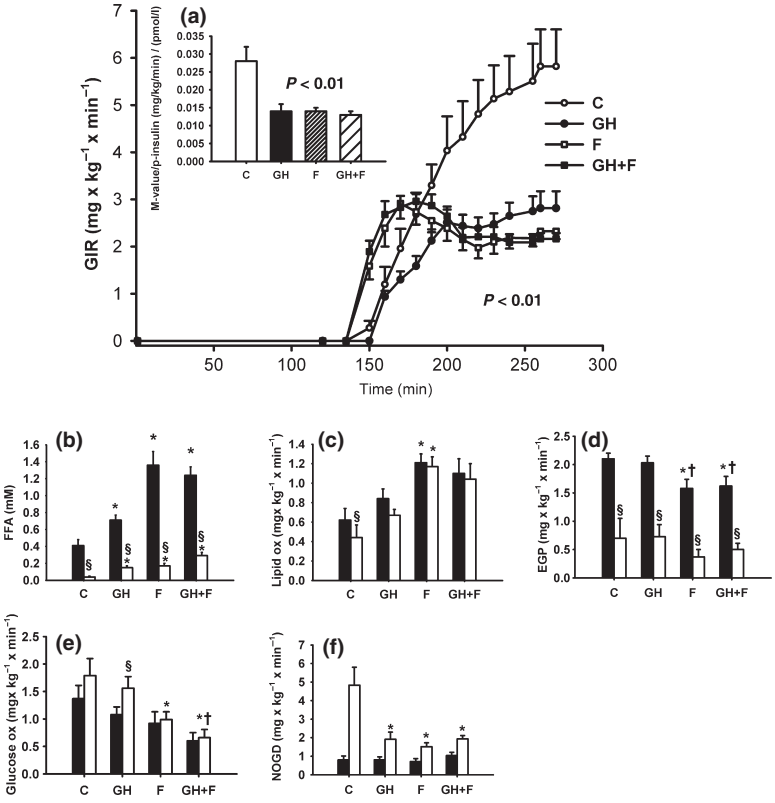

Figure 2 Insulin Sensitivity. Panel a: Mean ± SE glucose infusion rate levels during the clamp; the insert depicts the corresponding M-value corrected for plasma insulin levels. Panels b–f: Mean ± SE levels of serum FFA (b), lipid oxidation rates (c), endogenous glucose production rates (d), glucose oxidation rates (e) and non-oxidative glucose disposal rates (f) in the basal state (black bar) and during the clamp (white bar). All participants were studied on four occasions: after an overnight fast (C), after an overnight fast with GH infusion (GH), after 36 h fasting (F), after 36 h fasting with GH infusion (GH + F). The levels of GIR and M-value during C were significantly different compared with GH, F and GH + F (panel a). The following symbols indicate post hoc statistics: $P < 0.05$: * vs. C, † $P < 0.05$ compared with GH (panels b–f), § insulin effect.

Glucose oxidation was reduced by Fasting and GH + Fasting, and an attenuation of the clamp-induced increase in glucose oxidation was recorded during Fasting with no additional effect of GH (Fig. 2e). NOGD during the clamp was significantly elevated in the Control study as compared to the three other study days (Fig. 2f).

Insulin signalling to glucose uptake and glycogen synthase

Activation of the serine/threonine kinase Akt is crucial for most of the metabolic actions of insulin (Taniguchi *et al.* 2006). Phosphorylation of both Thr^{308} (Fig. 3a) and Ser^{473} residues (Fig. 3b) on Akt was stimulated by insulin in all four experiments ($P < 0.01$). Moreover, Akt Ser^{473} phosphorylation in the basal state tended to be increased during Fasting (overall effect $P = 0.05$, post hoc analysis non-significant). Akt substrate of 160 kDa (AS160), also known as tre-2/USP6, BUB2 and cdc16 domain family member (TBC1D4), has been identified as a downstream regulator of insulin-stimulated glucose uptake (Sakamoto & Holman 2008). Similar to Akt, AS160 phosphorylations were stimulated by insulin (for all three antibodies $P < 0.002$). For Ser^{588}, a small but significant effect of Fasting was observed with slightly reduced basal levels during GH+Fasting ($P < 0.01$) (Fig. 3c). By contrast, basal Thr^{642} phosphorylation levels were increased with GH and GH + Fasting ($P = 0.03$) (Fig. 3d). No distinct effects of either GH or Fasting were, however, observed using the PAS antibody, which detects phosphorylation on multiple Akt-motifs on AS160 (Fig. 3e). Glycogen synthesis is regulated by the enzyme glycogen synthase (GS), and this enzyme is inactivated by phosphorylation. GS Ser^{641} phosphorylation was suppressed by insulin stimulation ($P < 0.01$), whereas basal GS phosphorylation tended to be up-regulated by Fasting ($P = 0.09$) (Fig. 3f). Further, glycogen content in the skeletal muscle was down regulated by Fasting in the insulin-stimulated state ($P = 0.01$) (Fig. 3g).

STAT5 phosphorylation showed an apparent increase in response to GH, Fasting and GH + Fasting, but was subject to a wide variation and failed to reach statistical significance [pSTAT5 (%): $Control_{basal}$ 100 ± 0, $Control_{clamp}$ 122 ± 41, GH_{basal} 216 ± 66, GH_{clamp} 147 ± 46, $Fasting_{basal}$ 161 ± 52, $Fasting_{clamp}$ 138 ± 29, GH + $Fasting_{basal}$ 150 ± 15, GH + $Fasting_{clamp}$ 137 ± 37 (NS)]. A pronounced increase in mRNA expression of cytokine-inducible SH2-containing protein (CIS; a member of the SOCS family) was recorded after Fasting and GH+Fasting ($P < 0.001$) in addition to a borderline insulin effect ($P = 0.08$) (Fig. 3h). No significant changes in mRNA expression of SOCS1, SOCS2 or SOCS3 were detected (data not shown).

PDH regulation

PDHa activity normalized to creatine (Fig. 4a) exhibited a non-significant decrease in response to both GH and fasting (Fig. 4a), but PDHa activity during the clamp decreased significantly in response to GH ($P < 0.05$), Fasting ($P = 0.01$) and GH+Fasting ($P = 0.01$) compared with Control (Fig. 4a). Suppression of insulin-regulated PDHa activity after GH and Fasting prevailed when normalized to total PDH-E1α content, whereas it failed to reach statistical significance during GH+Fasting (Fig. 4b). PDH-E1α protein content was unaffected by fasting, GH treatment and insulin stimulation [Total PDH-E1α protein (A.U.): $Control_{basal}$ 0.22 ± 0.4, $Control_{clamp}$ 0.24 ± 0.05, GH_{basal} 0.23 ± 0.06, GH_{clamp} 0.14 ± 0.03, $Fasting_{basal}$ 0.19 ± 0.04, $Fasting_{clamp}$ 0.16 ± 0.04, GH + $Fasting_{basal}$ 0.18 ± 0.04, GH + $Fasting_{clamp}$ 0.14 ± 0.04 (NS)]. Fasting and GH + Fasting increased the phosphorylation on PDH-E1α site 1 (Ser^{293}) (Fig. 4b) and 2 (Ser^{300}) (Fig. 4c) in the insulin-stimulated state ($P < 0.01$). Ser^{300} phosphorylation also tended to be increased in the basal period after Fasting and GH + Fasting (over-all effect $P = 0.05$, post hoc analysis non-significant), and a similar non-significant trend was observed for Ser^{293} phosphorylation ($P = 0.07$).

The expression of PDK4 mRNA was significantly up-regulated in the basal state during Fasting and GH + Fasting; this up-regulation was abrogated during the clamp period, and the levels during the clamp were significantly reduced during GH and Fasting when compared to Control (Fig. 4d). PDK4 protein expression did not differ significantly between the experiments (Fig. 4e).

Discussion

The present study was designed to elucidate mechanisms underlying GH-induced insulin resistance in skeletal muscle in human subjects. The main findings of the study are that GH as well as fasting induces marked insulin resistance concomitant with stimulated lipolysis and suppression of PDHa activity but no distinct suppression of insulin signalling pathways. These results suggest that substrate competition is involved in insulin resistance induced by GH.

We included experiments with fasting for two reasons. First, fasting is associated with elevated endogenous GH levels in concomitance with stimulated lipolysis and insulin resistance, and this condition can be considered the natural domain for the direct metabolic effect of GH (Moller & Jorgensen 2009).

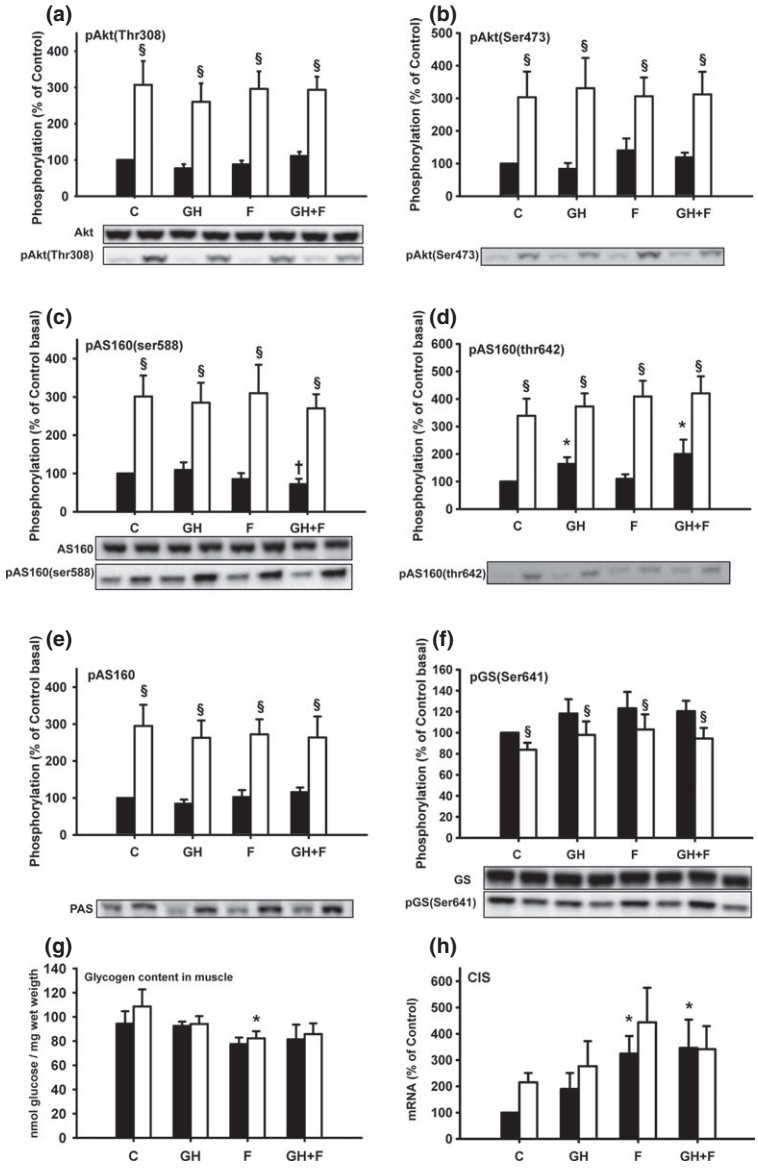

Figure 3 Western Blot. Phosphorylation of Akt Thr^{308} (a) and Akt Ser^{473} (b) and the downstream protein AS160 pAS160 Ser^{588} (c), Thr^{642} (d) and PAS (e) as well as GS Ser^{641} (f) assessed by WB; representative blots are shown below with the total protein only represented once and the corresponding phosphorylation sites by each histogram. Glycogen content in muscle (g). Cytokine-inducible SH2-containing protein (CIS) mRNA expression (h). C = overnight fast, GH = overnight fast with GH infusion, F = after 36 h fasting, GH + F = 36 h fasting + GH infusion. Black bar: basal period, white bar: clamp period. $P < 0.05$: *vs. C, † $P < 0.05$ compared with GH, § insulin effect.

Second, PDHa activity is known to be suppressed during fasting in human subjects (Sugden & Holness 2003, Spriet *et al.* 2004, Cahill 2006). In the present study, insulin sensitivity was suppressed to almost identical levels by GH, Fasting and GH+Fasting, and this was accompanied by reduction in insulin-stimulated glucose disposal.

The metabolic effects of GH were not identical to those observed after Fasting, but the combination of GH and Fasting did not seem to add to the effects recorded during Fasting alone. The diminution of insulin-stimulated glucose oxidation was more pronounced during Fasting than during GH (Fig. 2e), and basal EGP was reduced during Fasting and GH + Fasting but not during GH. Moreover, lipolysis and lipid oxidation rates both in the basal state and during the clamp were more elevated during Fasting and GH + Fasting than with GH (Fig. 2b,c). This was accompanied by a significant elevation of basal glucagon levels and reciprocal changes in insulin levels after Fasting and GH + Fasting relative to both Control and GH alone (Table 1). It is plausible that

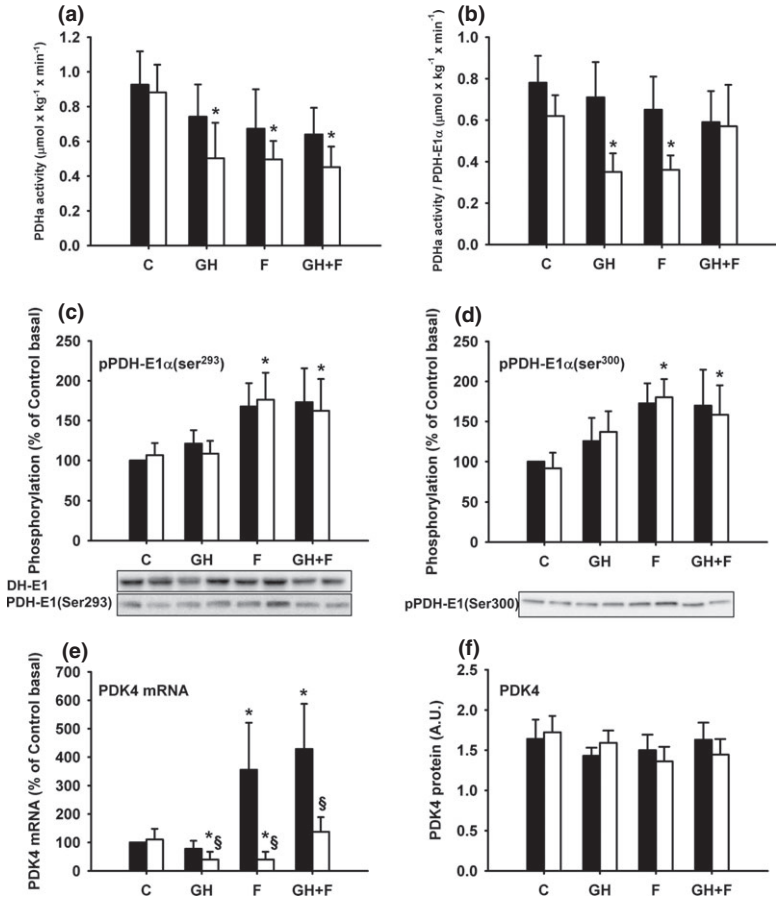

Figure 4 PDHa, PDHa-E1α phosphorylations, PDK4. Changes in PDHa activity (a) and PDHa activity corrected for total PDH-E1α protein level (b), as well as corresponding changes in phosphorylations on PDHa site 1 Ser^{293} (c) and site 2 Ser^{300} (d), as well as expression of PDK4 mRNA (e) and PDK4 protein (f). Representative blots are provided with the total protein represented once, and the corresponding phosphorylation sites below each histogram. C = overnight fast, GH = overnight fast with GH infusion, F = after 36 h fasting, GH + F = 36 h fasting + GH infusion. Black bar: basal period, white bar: clamp period. $P < 0.05$: * vs. C, § insulin effect.

the differences in basal glucagon and insulin levels between GH and fasting observed in our study were causally linked to the corresponding differences in glucose and lipid metabolism (Cahill 2006).

The PDC controls the entry of carbohydrate into the citric acid cycle for complete oxidation and energy production, and the subsequent increase in malonyl CoA inhibits CPT-I and thereby mitochondrial FA oxidation (Muoio & Newgard 2008). PDH activity is inhibited via phosphorylation of the E1 component of the enzyme, a reaction catalysed by PDK. Our observation that fasting suppresses PDHa activity (Fig. 4a) and increases PDK4 mRNA (Fig. 4d) content in skeletal muscle in human subjects is in accordance with previous studies (Pilegaard *et al.* 2003, Spriet *et al.* 2004). In the present study, neither GH nor 36 h fasting (Fasting) was associated with a detectable increase in PDK4 protein expression (Fig. 4e), which contrasts findings of increased mitochondrial PDK4 protein content in skeletal muscle from starved rats (Wu *et al.* 1999, Sugden *et al.* 2000). At present, we have no explanation for this discrepancy, but we speculate that PDK4 protein expression as assessed by WB from crude muscle specimen rather than isolated mitochondrial protein is less sensitive or alternatively that species-specific differences exist. Phosphorylation of PDH-E1α is determined by the balance between the activity of PDK and PDP. Furthermore, PDK and PDP activities are not only regulated by changes in protein expression, but also through acute regulation. While PDP1 activity has been reported to be regulated by Ca^{2+} and insulin (Sugden & Holness 2003), PDK activity is regulated by the ratio of acetyl-CoA/CoA, NADH/NAD+, ATP/ADP and the levels of pyruvate (Sugden & Holness 2003). Hence, a discrepancy between PDK4 protein content and PDH phosphorylation may be due to acute regulation of PDK activity, but also to regulation of PDP expression or PDP activity.

We also recorded a suppressive effect of insulin on PDK4 mRNA content with GH and Fasting separately (Fig. 4d). The ability of insulin to suppress PDK4 gene expression in skeletal muscle is in agreement with both *in vitro* experiments and human studies (Tsintzas *et al.* 2007). Tsintzas *et al.* (2007) observed that intravenous co-infusion of lipid blunted the insulin-stimulated suppression of PDK4 mRNA, which was associated with intramuscular accumulation of long-chain acyl-CoA, a fatty acid metabolite speculated to down-regulate PDK4 gene transcription. Interestingly, no changes in skeletal muscle PDK4 protein content in

the latter experiments were recorded (Tsintzas *et al.* 2007). In accordance with the increase in PDK4 mRNA content and the suppression of PDHa activity, we recorded increased phosphorylation of PDH-E1α at sites 1 and 2 both of which are recognized targets of PDK4 (Sugden & Holness 2003, Pilegaard & Neufer 2004).

Our observation that PDHa activity did not increase during insulin stimulation was not expected and disagrees with some (Mandarino *et al.* 1987, Tsintzas *et al.* 2007) but not all (Kelley & Mandarino 1990) existing human data. A rather pronounced increase in PDHa activity was observed by Tsintzas *et al.* (2007) after 6 h of insulin infusion at a rate of ≈85 mU/min. In the study by Mandarino *et al.* (1987), the increase in PDHa activity during a clamp was only moderate. The explanation for these controversies remains to be scrutinized in studies involving prolonged infusion rates of insulin in combination with serial muscle biopsies. It is also noteworthy that PDH-E1α phosphorylation as well as PDK4 mRNA content only changed to a significant degree during Fasting and GH + Fasting, but not with GH alone, despite the fact that the degree of suppression of PDHa activity was comparable between the three settings. We currently do not have an explanation for this, but an alternative means for targeting isolated metabolic effects of GH in human subjects could be administration of pegvisomant, which is a specific GH antagonist, licensed for the treatment for acromegaly (Moller *et al.* 2009).

In accordance with previous data, GH-induced insulin resistance was not associated with detectable suppression of insulin signalling in skeletal muscle (9, 16, 17). The present experiments included measurements of phosphorylation of Ser^{473} and Thr^{308} on Akt in addition to phosphorylation of the downstream target AS160 (Fig. 3). Inhibition of the GTPase activity via phosphorylation of AS160 is considered to facilitate translocation of GLUT4 to the cell surface (Sakamoto & Holman 2008). AS160 is phosphorylated at multiple sites, but the exact role of the individual phosphorylation on the regulation of GLUT4 translocation is unknown (Sakamoto & Holman 2008). Reduced insulin-stimulated phosphorylation of Ser^{588} has previously been observed in muscle from type 2 diabetic patients (Vind *et al.* 2012) and in insulin resistant muscle after 72 h fasting (Vendelbo *et al.* 2012). However, insulin resistance after 36 h of fasting in our study was not associated with impaired insulin-stimulated Ser^{588} phosphorylation. Interestingly, GH infusion was associated with slightly increased basal Thr^{642} phosphorylation (Fig. 3d), but this did not translate into increased PAS phosphorylation (Fig. 3e). It is suggested that Thr^{642} is the only phosphorylation site recognized by PAS antibody (Kane *et al.* 2002, Geraghty *et al.* 2007), but using these two antibodies does not necessarily produce matching results in human skeletal muscle (Vind *et al.* 2012), agreeing with the present study.

Some additional ambiguous findings pertain to this study. First, up-regulation of CIS mRNA content was more pronounced during 36 h of fasting than GH; CIS belongs to the SOCS family of proteins, whose expression is induced by GH and subsequently act as negative regulators of GH signalling in a classic feedback way (Flores-Morales *et al.* 2006). It could be speculated that amplification of endogenous GH secretion as observed during fasting constitutes a more powerful stimulation of CIS expression as compared to an exogenous GH infusion, but this remains to be experimentally tested. Second, the steady-state serum levels of insulin during the clamp were lower during Fasting than in Control and GH despite identical insulin infusion rates. Whether this reflects differences in the clearance rate or distribution of insulin and to which degree, it impacts the overall effect of insulin in the target tissues is uncertain, but it should be taken into consideration when comparing the four experiments.

Taken together, however, our data suggest that GH-induced insulin resistance in skeletal muscle is causally linked to metabolic feedback. The exact molecular mechanisms remain to be further characterized, but the suppressive impact of lipid infusion on PDHa activity (Hoy *et al.* 2009) and the observation that GH-induced insulin resistance is abrogated when the concomitant stimulation of lipolysis is blocked in human subjects (Nielsen *et al.* 2001) support that GH-induced lipolysis plays a causal role. It is also plausible that GH may directly stimulate PDK4 gene expression as previously reported in adipose tissue *in vitro* (White *et al.* 2007). The accumulating amount of data indicating that metabolic overload in terms of by-products of lipid oxidation plays a key role in the development of insulin resistance in skeletal muscle (Muoio & Newgard 2008, Watt & Hevener 2008) also applies to the effects observed with GH and merits further studies. In a physiological context, our data add to the importance of GH as a regulator of substrate metabolism by promoting the utilization of lipid at the expense of glucose (Moller & Jorgensen 2009). This effect is of importance for the metabolic adaptation to fasting, where insulin resistance may be favourable.

Conflict of interest

The authors declare no conflict of interest.

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