Title: Acute hypoglycemia in healthy humans impairs insulin stimulated glucose uptake and glycogen synthase in skeletal muscle; a randomized clinical study.

Short title: Hypoglycemia and insulin action in skeletal muscle.

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Abstract

Hypoglycemia is the leading limiting factor in glycemic management of insulin-treated diabetes. Skeletal muscle is the predominant site of insulin-mediated glucose disposal and our study was designed to test to what extent insulin induced hypoglycemia affects glucose uptake in skeletal muscle and whether hypoglycemia counter-regulation modulates insulin and catecholamine signaling and glycogen synthase activity in skeletal muscle.

Nine healthy volunteers were examined on three randomized study days in a crossover design: i) hyperinsulinemic hypoglycemia (bolus insulin), ii) hyperinsulinemic euglycemia (bolus insulin and glucose infusion) and iii) saline control with skeletal muscle biopsies taken just before, 30 min and 75 min after insulin/saline injection.

During hypoglycemia glucose levels reached a nadir of ~2.0mmol/l and epinephrine rose to ~900pg/ml.

Insulin stimulated glucose disposal and glucose clearance in skeletal muscle were impaired whereas insulin signaling to glucose transport was unaffected by hypoglycemia. Insulin-stimulated glycogen synthase activity was completely ablated during hyperinsulinemic hypoglycemia and catecholamine signaling via PKA as well as phosphorylation of inhibiting sites on glycogen synthase all increased.
Hypoglycemia is the most common side-effect to insulin treated diabetes, and the leading factor precluding optimal glucose management (1). Hypoglycemia is defined by Whipple’s triad: symptoms related to hypoglycemia, low plasma glucose measured at the time of symptoms and relief of symptoms when glucose levels are raised to normal. Furthermore hypoglycemia is differentiated by severity of symptoms (or lack hereof) and glucose levels into: asymptomatic hypoglycemia – no symptoms but plasma glucose <3.9mmol/l (70mg/dl), symptomatic hypoglycemia – symptoms and plasma glucose <3.9mmol/l (70mg/dl), and severe hypoglycemia – an event requiring aid from another person and low plasma glucose (2). Insulin induced hypoglycemia occurs rapidly (nadir plasma glucose within 30 min) (3). In healthy subjects, hypoglycemia leads to a decrease in insulin and increases in circulating levels of the counter-regulatory hormones: glucagon, nor-epinephrine, epinephrine, growth hormone (GH) and cortisol (4). Catecholamines and glucagon inhibit glycolysis and increase glycogenolysis as well as gluconeogenesis via increased adenylyl cyclase (AC) and cAMP dependent protein kinase (PKA)-activity (5). The combined effects of these hormones effectively and rapidly increase endogenous glucose production during hypoglycemia (6).

Insulin-stimulated glucose disposal is primarily governed by skeletal muscle (7). Intracellular signaling to this process is initiated by insulin binding to its receptor and subsequent signaling through the IRS1/PI3K signaling pathway. This activates Akt and stimulates phosphorylation of AS160 and TBC1D1 (8). This stimulates translocation of the glucose transporter, GLUT4, to the cell surface and allows for facilitated transport of glucose into the muscle. Once glucose is taken up, glucose is metabolized either by oxidative or non-oxidative pathways (6).

The effects of insulin are inhibited during hypoglycemia (9) plausibly due to action of counter regulatory hormones and a reduced glucose concentration gradient (10).
Catecholamines stimulate G-protein coupled β-receptors, present on skeletal muscle, and thereby activate protein kinase A (PKA) (11). Increased PKA activity can inhibit insulin action and may thereby suppress glucose uptake during hypoglycemia (12). Epinephrine administration to isolated rat skeletal muscle decreases insulin-stimulated glucose-uptake by decreasing GS-activity leading to accumulation of G-6-P and subsequent inhibition of hexokinase activity (13). In humans, epinephrine administration in adrenalectomized subjects during exercise decreases glucose disposal (14). However, catecholamine-mediated signaling in skeletal muscle during acute hypoglycemia has not been determined. Similarly to β-receptors, GH receptors are expressed on skeletal muscle and GH stimulation potently inhibits insulin-stimulated glucose transport after latency through unknown mechanisms (15). The aim of this study was to study insulin action in skeletal muscle during acute hypoglycemia under the hypothesis, that intracellular insulin action is impaired during this condition.
**Research Design & Methods:**

The study protocol was approved by the local scientific ethics-committee (1-10-72-113-13) and registered at clinicaltrials.gov (ID NCT01919788). The study was conducted in accordance to the Helsinki Declaration II and all subjects gave their oral and written informed consent.

The design is a randomized crossover study of three study-days previously described in detail (16). Shortly, each subject was allocated to i) CTR (bolus 2ml NaCl i.v.) ii) HH (bolus insulin 0.1 U actrapid/kg in 2ml NaCl i.v.) and iii) HE (bolus insulin 0.1 U actrapid/kg in 2 ml NaCl i.v. and glucose infusion 20% (varying amounts) i.v.).

The study days were separated by at least 21 days, commenced at 9.00am (t=0 min), and ended at 10.45 am (t=105 min).

**Our primary outcome** for this study was forearm glucose-disposal. **Secondary outcomes** were glucose oxidation rates, insulin signaling to glucose transport, glycogen synthesis in skeletal muscle and energy expenditure (EE).

**Muscle biopsies** from vastus lateralis of the quadriceps muscle were obtained under sterile conditions 15 min after local anesthesia (lidocaine 1%, 10 ml) was applied. Biopsies were obtained from the right leg just before t=0 min and at t=75 min (separated by > 10cm) and from the left leg at t=30 min. Biopsies were immediately snap-frozen in liquid nitrogen and subsequently stored at -80°C until analyzed. All samples were homogenized for 2x30 sec at 5000rpm, in a buffer containing (in mmol/l) 50 HEPES, 20 NaF, 2 NaOV, 5 EDTA, 5 NAM, 137 NaCl, 10 Na₄P₂O₇, 1 MgCl₂, 1 CaCl₂, 0.01 TSA, HALT™ protease inhibitor cocktail (100x), 1%NP-40, and 10% Glycerol in a Precellys Homogenizer (Bertin Instruments, Montigny-le-Bretonneux,France). Then samples rotated at 4°C for 30 min before being centrifuged at 14,000g for 20 min and the supernatant collected.
**Western Blot (WB)** analyses were performed using 4–15% CriterionXTBis-Tris gels (Bio-Rad, Hercules, CA, USA). Primary antibodies used: from Cell Signaling: AKT, pAKTser473, pAKTthr308, AS160, pAS160ser642, pAS160ser588, GS, pGSser641 (site 3a), GSK-3, pGSK-3α+β, mTOR, pmTORser2448, AMPK, pAMPKthr172, 4EBP1, non-phos thr46 4EBP1, PKA phospho-substrate (Cat No: #4691, #9271, #9275, #5676, #4288, #8730, #3886, #3891, #5676, #9331, #2972, #5536, #2532, #2531, #9644, #4923, #9624), from EMD Millipore: ACC, pACCser79, GLUT4 (Streptavidin, Upstate 077303, 0771404), from BD Biosciences: GSK-3β (#610202). Antibodies detecting pGS ser7+10 (site2+2a) were raised against peptide PLSRSL(Sx)MS(Sx)LPGLED (residues 1-16 of rat GS), pGS ser697 (site1a) and pGS ser710 (site1b) were raised against the peptides CEWPRRASpSCTSSTG (692-703 residues of human GS) and CSGSKRNSpVDTATS (704-716 residues of human GS), the specificity of these antibodies have been reported previously (17, 18). Phosphorylation levels are expressed as a ratio to the targeted protein, measured on the same membrane. Stain-free protein technology was used to control for equal loading (19). GLUT4 and PKA phospho-substrate levels are expressed as a ratio to the protein content quantified by stain-free protein technology. PKA phospho-substrate antibody was used as a measure of PKA activation by detection of PKA-phosphorylation on all detectable proteins >15kDa. This antibody detects proteins containing a phospho-serine/threonine residue with arginine at the -3 position which is a bone fide PKA-motif (20). Glycogen phosphorylase a and b antibodies were used as previously described (21). Proteins were visualized and quantified by enhanced chemiluminescence after incubation with horseradish peroxidase-conjugated anti-rabbit/anti-mouse secondary antibodies, using Image Lab 5.0 (Bio-Rad). Protein measurements are expressed as ratio to mean CTR value at t=0 min (RU).

**Muscle glycogen concentration** was determined as glycosyl units after acid hydrolysis of muscle lysate by fluorometric method (22) **Glycogen synthase** activity was measured using a
modified version (18) of the method described by Thomas et al. (23). The activity was measured at 37°C and the mixture contained uridine diphospho-glucose (UDPG) at a concentration of 1.7mmol/l and G-6-P at concentrations of 0.02mmol/l (=Zero), 0.167mmol/l (=Low), and 8.0mmol/l (=High). GS-activity at a concentration of G-6-P of (Zero/High)*100 is termed I-Form, whereas (Low/High)*100 is termed Fractional Velocity (FV).


**Indirect calorimetry** (Deltatrac monitor; Dantes Instrumentarium, Helsinki, Finland) was used from t=90-105min, to determine total EE, respiratory quotient and glucose oxidation-rates (24). Protein oxidation was set at 12% of EE (25).

**Forearm blood flow (FBF)** was measured using strain-gauge plethysmography (26) and forearm glucose-disposal was assessed by using the product of FBF and arterio-venous (a-v) differences in glucose concentrations. Arterialization of venous blood was obtained as described previously (16). When looking at differences in glucose disposal using a-v differences* FBF, possible differences in glucose disposal - due to differences in glucose gradient across the cell membrane (glucose conc.) - are not accounted for. To account for these differences in glucose levels, plasma glucose clearance in ml plasma*100ml forearm⁻¹*min⁻¹ was calculated as: glucose disposal (mmol*100ml forearm⁻¹*min⁻¹) divided by arterial glucose concentration (mmol/l) * 1000.
Statistical analyses were performed using a repeated-measurements mixed model with visit order, visit number, time, intervention, and the interaction between intervention and time as factors (Stata 13.0, College Station, TX, USA). When only one daily measure was applied, a repeated-measurements mixed model was used, with visit order, visit number, and intervention as factors. Unequal correlations and standard errors, when detected, were taken into account in the analysis. Model validation was performed, by inspection of qq-plots of the residuals, and of scatterplots of the predicted versus the fitted values. If residuals were not normally distributed, logarithmic transformation was performed. P<0.05 was considered statistically significant. Graphs are geometric mean values with standard error of the mean. Numerical data are geometric mean values with 95% CI.
Results

Subjects characteristics
These have in part been published previously in a study describing lipid metabolism and adipose tissue signaling during acute insulin induced hypoglycemia (16) and are summarized in table 1.

Glucose disposal in skeletal muscle
Plasma glucose and insulin levels have been published previously in detail (16). Comparable peak insulin-levels of ~900pmol/l were reached during both HH and HE (p=0.87) at t=15 min. Insulin-levels remained slightly elevated during the rest of the study during HE (overall p<0.001) (fig 1a). This was likely due to a mild overdosing of glucose during HE leading to increased endogenous insulin-production – confirmed by overall ~ 2.7 times elevated c-peptide levels during HE compared with HH (p<0.001) and ~1.3 times elevated levels compared with CTR (p<0.001).

Forearm blood flow showed no difference among interventions at any time point.

Forearm glucose disposal was markedly larger (~2.5times overall) during HE compared with HH (overall p<0.001, fig. 1c). These differences were present already at t=20min

Plasma glucose clearance (fig. 1e) was reduced ~35% during HH compared with HE (overall p< 0.01). Differences were evident at t=40min. (p=0.01), t=60min. (p=0.04), and t=80min. (p<0.01), thus confirming reduced insulin-stimulated glucose uptake in skeletal muscle during hypoglycemia.
**Insulin signaling to GLUT 4 translocation and protein synthesis**

Insulin increased phosphorylation of Akt ser473 (fig.2a) and Akt thr308 (fig.2b) 30 min after injection by ~ten fold compared to the saline control. There were no differences between the hypo- and euglycemic insulin-stimulated conditions. These findings were associated with increased signaling at the downstream target AS160 at thr642 (fig.2c) and ser588 (fig.2d) at the same time point, during both HE and HH. 75 min after insulin injection Akt phosphorylations at ser473 and thr308 were still elevated during HH and HE conditions compared to saline control, but only about four fold. Phosphorylation on AS160 ser588 remained elevated during both HH and HE, but thr642 phosphorylation was increased during HE only.

We did not detect any differences in Akt, AS160 or GLUT4 protein levels among all interventions.

Insulin stimulates protein synthesis through activation of the mTOR pathway (27). Insulin increased phosphorylation of mTOR at ser2448 ~2.5 fold 30 min after injection during both HE and HH compared with CTR (both p<0.001; fig. 2e) while remaining stable throughout CTR. At t=75 min the increased mTOR phosphorylation during HH was ~75% higher than CTR (p<0.001) and ~35% higher than HE (p<0.001). Similar regulation by insulin was evident at the downstream target protein 4EBP1 (fig. 2f).

**Counter-regulatory hormones and signaling:**

Hypoglycemia profoundly increased, circulating levels of epinephrine, nor-epinephrine, GH and glucagon. For clarity epinephrine and glucagon, which have been published previously (16), are shown again.
Epinephrine (fig. 3a) levels were low and comparable between HE and CTR at all time-points (p=0.14). During HH, epinephrine levels rose rapidly to peak level of 891pg/ml (560; 1221pg/ml) at t=30min. Overall comparison revealed differences between both HH and CTR (p<0.001) as well as between HH and HE (p<0.001).

Nor-epinephrine (fig. 3b) quickly rose during HH to peak level of 387pg/ml (349; 425pg/ml) at t=30min, and was clearly increased compared with both HE (p<0.001) and CTR (p<0.001). No overall difference was detected between CTR and HE (p=0.07).

Glucagon (fig. 3c) levels increased during HH, to peak level of 133pg/ml (119; 147pg/ml) at t=45min. and were restored to CTR levels at t=90min and t=105min (p>0.05). During HE, glucagon levels were suppressed compared with CTR (p<0.001). Differences between these two study days were evident from t=30min. Glucagon levels were stable during CTR with an overall mean of 52pg/ml (47; 56pg/ml).

GH (fig. 3d) was comparable between HE and CTR at all times. During HH, GH-levels increased from t=45min and were elevated throughout the study day, with peak level of 15.6µg/l (6.3; 38.7µg/l) at t=60min. Overall comparison between interventions revealed increased GH-levels during HH. HH and CTR (p=0.001), HH and HE (p<0.001).

PKA-activity (fig. 3e): phospho PKA-substrate as a surrogate marker of catecholamine stimulation increased by ~75% during HH at t=30min compared with both CTR (p<0.001) and HE (p<0.001). At t=75min PKA substrate phosphorylations decreased to just ~10% more than during CTR (NS), and to ~30% above HE levels (p<0.01).
AMPK-signaling (fig. 3f): No differences in 5’AMP-activated protein kinase (AMPK) thr172 phosphorylation were detected among all interventions. Neither did we detect any effect of interventions on phosphorylation of the downstream target acetyl-coA carboxylase (ACC) data not shown.

Regulation of glycogen synthesis and breakdown, total glycogen, glucose oxidation and energy expenditure

GS-activity:

FV (fig 4a): Insulin potently increased GS FV by ~35% during HE at both t=30min and t=75 min to compared to CTR (p<0.001). Hypoglycemia totally abolished the effect of insulin on GS-activity and thus no differences were detected comparing HH with CTR at t=30min or t=75min.

I-Form (fig 4b): The same pattern was seen using I-Form, which was increased by ~75% at t=30min (p<0.001) and by ~65% at t=75min (p<0.001) compared with CTR. Again hypoglycemia completely impaired GS-activity employing I-Form and no differences were detected comparing HH with CTR.

These findings confirm that storage of glucose as glycogen in muscle tissue was inhibited by hypoglycemia. To unfold possible explanatory mechanisms for this decrease in GS activity, we also investigated known regulatory phosphorylation sites on GS and the upstream GSK-3.

GS phosphorylations:

Site 3a (ser641; fig 4c): Insulin induced activating de-phosphorylation on site 3a, leading to ~30% reduced phosphorylation during HE (p<0.001) whereas the combined effects of insulin and hypoglycemia during HH decreased phosphorylation on site 3a by ~13% (NS) compared
with CTR at t=30min. At t=75min the insulin effects during HE were a little smaller, and phosphorylation on site 3a was now decreased ~25% (p<0.01) compared with CTR. The effects of insulin were totally abrogated by hypoglycemia at t=75min when comparing HH with CTR.

Sites 2+2a (ser7+ser10; fig 4d): Insulin decreased phosphorylation on site 2+2a with ~20% (NS) at t=30min and with ~45% at t=75min (p=0.001) during HE compared with CTR. The effects of hypoglycemia totally abolished the effects on insulin on GS site 2+2a, and thus increased phosphorylation with~ 90% (p<0.001) at t=30min and with ~60% (p<0.01) at t=75min comparing HH with CTR. Comparing HH with HE revealed a ~ 2.5fold increase in phosphorylations on site2+2a during HH at both t=30min and t=75min (both p<0.001).

Sites 1a and 1b (fig 4e and 4f): phosphorylation on site1a and site1b revealed no statistical significant interaction (time x intervention, p=0.09 and 0.06 respectively) and thus, results were analyzed accordingly. A main effect of intervention was found regarding both sites 1a and 1b, between HH and CTR, with ~25% (p=0.01) increased site 1a phosphorylation and ~15% (p<0.05) increased site 1b phosphorylation during HH.

GSK-3 phosphorylations (α and β) (figures 5a and 5b): Insulin increased phosphorylation of GSK-3α ~ 40% (p<0.001) and GSK-3β ~ 25% (p<0.01) during both HE and HH compared with CTR at t=30min. No statistically significant differences in both GSK-3α and GSK-3β phosphorylations were detected comparing HE with HH at t=30min or at t=75min. At t=75min, GSK-3α phosphorylations had normalized during HH, whereas GSK-3β phosphorylation was increased ~ 20% compared with CTR (p=0.04). No statistically
significant differences were detected in GSK-3β phosphorylation comparing HE with both HH and CTR.

**Glycogen phosphorylase a (GPa) and b (GPb) (figures 5c and 5d):**

Glycogen phosphorylase is present in two forms, GPa and GPb. They differ in phosphorylation and GPb is converted into GPa by phosphorylation at two sites (28).

*GPa*: No statistical significant interaction (time x intervention p=0.3) or main effect of any intervention (p>0.05) was observed.

*GPb*: at t=30min, GPb decreased during CTR and increased during HH, revealing a ~75% increase in GPb levels during HH compared with CTR (p=0.003). Levels were stable during HE and not statistically significantly different from either CTR or HH at t=30min and t=75min (p>0.05).

**Total glycogen (figure 5e)** in skeletal muscle biopsies was not statistical significant different between any timepoints among interventions.

**Glucose oxidation** was determined using indirect calorimetry. Oxidation rates were at their lowest during CTR=669kcal/day and highest during HE=1278kcal/day (p<0.001). During HH=924kcal/day, glucose oxidation rates were in between CTR (NS) and HE (p=0.07).

**Energy expenditure** increased during hypoglycemia to 1826kcal/day compared with 1717kcal/day during HE (p<0.01). Energy expenditure during CTR was 1762kcal/day and this was not statistically different from HE or HH.
Discussion

The key finding of this study is that insulin action in skeletal muscle is profoundly inhibited during acute symptomatic hypoglycemia in humans. This is determined using a design that reflects a clinically relevant situation. Our study therefore allows for direct comparisons between acute insulin action during eu- and hypoglycemia. In contrast, previous investigations of hypoglycemia have employed insulin-clamp conditions (9, 29-32) where glucose levels are reduced for longer periods. The clamp conditions allow for steady state estimates of glucose disposal, but the condition is far from the acute hypoglycemia experienced by insulin-treated patients. A previous investigation comparing hypoglycemia induced by either a 10 min or a 12 hour insulin infusion found substantial reduction of glucose disposal during prolonged but not acute hypoglycemia (33). This is in contrast to the findings in the present study, but several differences in the models could explain the discrepancies. Most importantly, the level of hypoglycemia was more pronounced in the present study where glucose levels reached a nadir of ~2.0mmol/l compared to ~2.8mmol/l in the previous study. The lower glucose levels in the present study were associated with a substantially higher epinephrine response at ~900pg/ml compared to a peak <400pg/ml in the previous investigation. A similar, but less pronounced pattern was observed comparing nor-epinephrine levels between the two studies. Furthermore, it is interesting that the increase in adrenaline concentration was much larger than the limited increase in noradrenaline, which indicates adrenal effect rather than regulation by the sympathetic nervous system. This may implicate a role of beta2 adrenergic receptors in muscles for counter regulation.

Due to the above mentioned differences, our results do not necessarily conflict with the existing data, but demonstrate the mechanisms involved during a more pronounced counter-regulatory response.
The threshold for autonomic symptoms to hypoglycemia is ~ 3.2 mmol/l and for neuroglycopenic symptoms ~ 2.8 mmol/l (4). Population based data demonstrate that 30-40% of individuals with type 1 DM experience between one and three episodes of severe (acquiring assistance from another person) hypoglycemia per year (34), indicating that in a clinical setting, glucose levels lower than 2.8 mmol/l (and probably even much lower) are often experienced in type 1 DM. Our data therefore demonstrate that decreased glucose disposal in skeletal muscle in addition to increased endogenous glucose production, can be an important contributing factor to glucose counter-regulation during symptomatic hypoglycemia.

When circulating glucose levels are within the physiological range and metabolism is normally regulated, transport across the cell membrane is the limiting factor for glucose uptake in skeletal muscle (35). During insulin-stimulation, glucose is primarily disposed into skeletal muscle by facilitated transport through GLUT4 (36). Under these conditions, the amount of GLUT4 in the cell membrane and the glucose gradient into the cell determines uptake. Therefore the decreased glucose gradient during HH contributes significantly to the decreased forearm glucose disposal as observed in this and other studies (10). Km for GLUT4 is ~4.3 mmol/l (37), and glucose clearance defined as the ratio between glucose disposal and circulating glucose levels can be assumed constant (33). In the present study, glucose clearance was significantly reduced during hypoglycemia, and this could indicate reduced GLUT4 content in the cell membrane. We were not able to measure GLUT4 translocation in skeletal muscle biopsies in the present study, but as expected, the interventions did not affect the total amount of GLUT4 protein. Neither did we observe impaired activation of signaling intermediates regulating GLUT4 trafficking 30 min after insulin stimulation where glucose disposal peaked. In fact, phosphorylation of Akt and AS160 tended to be higher during HH compared with HE although these differences did not reach statistical significance. At t=75 min where glucose levels were nearly normalized,
phosphorylation of AS160 at thr642 was lower during HH compared to HE. This phosphorylation site on AS160 has consistently been shown to regulate glucose uptake (38), but the lower phosphorylation level at this time point is unlikely to explain differences in glucose disposal during acute hypoglycemia. It may instead be caused by the slightly elevated insulin levels during HE. Therefore, a lower glucose gradient through the plasma membrane during HH decreases glucose transport during these conditions. In addition, reduced GLUT4 content in the cell membrane cannot be excluded as a mechanism for decreased glucose clearance during symptomatic hypoglycemia, but it is not a consequence of reduced insulin signaling to GLUT4 translocation.

Under non-physiological conditions in mouse models, glucose metabolism and not transport across the cell membrane can become the limiting factor for glucose uptake in skeletal muscle (39). Similar mechanisms may lower glucose clearance during hypoglycemia in situations where insulin-stimulated GLUT4 translocation to the cell membrane is normal. Unlike the subtle effects of hypoglycemia on insulin signaling to glucose transport, we found profound effects of acute hypoglycemia on important regulators of glycogen synthesis. GS is the key enzyme responsible for glycogen synthesis in muscle tissue and under physiological conditions, GS activity is stimulated by insulin (40). Reduced glycogen synthesis can impair glucose uptake by mechanisms originally described by Phillip Randle (41) and may contribute to reduced glucose clearance during hypoglycemia. The impaired glycogen synthesis could be caused by the observed effects of hypoglycemia on several regulating phosphorylation sites on GS. Insulin signals to GS via activation of Akt and subsequent phosphorylation of GSK-3. This reduces the activity of GSK-3 and inhibits phosphorylation of especially the inactivating site 3a on GS (42, 43). Hypoglycemia did not affect insulin-stimulated Akt and GSK-3 phosphorylation, but instead we found evidence of direct inhibition of GS activity by catecholamine signaling, these findings are supported by the data
from a previously conducted study, investigating the effects of insulin and adrenaline on Akt, GSK-3, GS-activity and phosphorylations (44). PKA directly inhibits GS-activity via phosphorylation of sites 1a, 1b and 2 (45). In addition, PKA possibly increases phosphorylation on site 3a indirectly via inhibition of the GM protein phosphatase 1-complex (GM-PP1) (12). The increased GS phosphorylations on sites 3a and 2+2a during hypoglycemia were therefore likely caused by catecholamine-stimulated PKA-activity. This was further supported by the trends to inhibition of phosphorylation at the sites 1a and 1b although these differences failed to reach statistically significance (p = 0.09 and 0.06, respectively).

Hypoglycemia did not only decrease GS activity, but also decreased glucose oxidation despite an increase in total energy expenditure. In agreement with the reduced glucose oxidation, we did not find evidence for measurable glycogen degradation, and GPa, the active form of glycogen phosphorylase was not increased during hypoglycemia (46). We were not able to measure all important regulators of glycogen metabolism including Protein Phosphatase I (PP1) complexes and glucose-6-phosphate in our biopsy material (46). However, our data suggest that glycogenolysis alone was not able to compensate for the reduced glucose uptake at the time point where the biopsy was obtained. This indicates that lipids become the primary metabolic substrate during hypoglycemia, which is supported by our previously published observations from the same subjects (16). Under physiological conditions, insulin lowers free fatty acid (FFA) levels. During hypoglycemia, counter-regulatory hormones abrogate insulin inhibition and lipolysis in adipose tissue is potently stimulated leading to increases lipid availability (16). An increase in circulating FFA induces skeletal muscle insulin resistance in a dose-dependent manner, but only hours after FFA levels increase (47). Activation of lipolysis in adipose tissue by catecholamines has been suggested to have protein sparing/anabolic effects in skeletal muscle (11). In rat skeletal
muscle, this has been shown to be associated with catecholamine-induced Akt and mTOR activation (11). Our data extend these findings to human skeletal muscle and indicate that catecholamines may affect protein synthesis via mTOR signaling. It is therefore likely that the increase in lipid oxidation affects protein synthesis, but lipid-induced insulin resistance is unlikely to be involved in the acute response to hypoglycemia.

This study examined the effects of hypoglycemia in healthy volunteers, and this ensured homogeneous counter-regulatory hormone responses. Unlike healthy individuals, diabetic subjects treated with insulin often have impaired glucagon responses to hypoglycemia. In such cases, the catecholamine response is crucial in attempts to restore circulating glucose levels (48). The pronounced increase in catecholamine signaling to GS and the associated inhibition of glycogen synthesis observed in this study therefore demonstrate mechanisms that are likely intact among patients with impaired glucagon responses. This could indicate that treatment with beta-receptor antagonist may dampen counter-regulation in skeletal muscle in addition to the well-known shift in glycemic thresholds for symptoms to lower plasma glucose concentrations (49). The preserved glucagon response in the healthy subjects is expected to increase endogenous glucose production, but it is not expected to directly target skeletal muscle due to the absence of glucagon receptors in this tissue (50). It is therefore likely that our data from insulin action in skeletal muscle from healthy volunteers can be extrapolated to diabetic patients even with long disease duration and impaired glucagon response.

In conclusion, we find that acute symptomatic hypoglycemia decreases insulin stimulated skeletal muscle glucose disposal. These findings are not associated with decreased insulin signaling to glucose transport. Instead, insulin stimulated glycogen synthesis is potently
inhibited. This is, at least partly, explained by increased counter-regulatory catecholamine levels leading to increased PKA signaling and subsequently to GS inactivation.
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Author contributions: NM, UK, MHV, NJ and TSV designed the study; TSV recruited the participants; TSV conducted the trial; NJ, NM, MVS, JRH, JW, and TSV collected the data; TSV conducted the statistical analyses; NJ and TSV wrote the manuscript; and NM, MHV, MVS, UK, JRH, JW reviewed and edited the manuscript. NJ is the guarantor of this work. All authors approved the final version of the manuscript.

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Duality of interest

The authors declare no duality of interest.
References


Table 1: Baseline characteristics of the 9 subjects. Data are mean (95% CI) or median (range) Most of these data have been published previously (16)
Legend fig 1: Glucose disposal in skeletal muscle during; CTR (bolus 2ml NaCl i.v.) - hollow squares with solid line; HH (bolus insulin 0.1 U actrapid/kg) - filled circles with dash-dot dot; and HE (bolus insulin 0.1 U actrapid/kg and glucose 20%) - filled triangles with tight dot. a) insulin concentrations in pmol/l (published previously in (16)); b) Glucose infusion rate (ml/h) during HE; c) Arterio-venous glucose differences (mmol/l); d) Forearm glucose disposal (µmol/100ml forearm/min); e) Glucose clearance (ml/100ml forearm/min). Symbols indicate p<0.05: † HH vs CTR; ‡ HE vs CTR; § All.

Legend fig 2: Insulin signaling to glucose transport and protein synthesis during; CTR (bolus 2ml NaCl i.v.) - hollow squares with solid line; HH (bolus insulin 0.1 U actrapid/kg) - filled circles with dash-dot dot; and HE (bolus insulin 0.1 U actrapid/kg and glucose 20%) - filled triangles with tight dot. Phosphorylation of proteins at t=0, 30 and 75 min. Values are shown relative to CTR level at t=0 min. a) and b) ratio of ser473 and thr308 phospho Akt to Akt protein level. c) and d) ratio of thr642 and ser588 phospho AS160 to AS160 protein level e) ratio of ser2448 phospho mTOR to mTOR protein level f) ratio of non-phosphorylated thr 46 4EBP1 and 4EBP1 protein level. Symbols indicate p<0.05: *HH vs HE; † HH vs CTR; ‡ HE vs CTR; § All.

Legend fig 3: Plasma levels of counter-regulatory hormones and PKA signaling in skeletal muscle; during; CTR (bolus 2ml NaCl i.v.) - hollow squares with solid line; HH (bolus insulin 0.1 U actrapid/kg) - filled circles with dash-dot dot; and HE (bolus insulin 0.1 U actrapid/kg and glucose 20%) - filled triangles with tight dot.

a-d) Counter-regulatory hormone concentrations in plasma; e) Phosphorylation of a range of proteins >15kDa at t=0, 30 and 75 min using an antibody directed against phosphorylated
PKA-motif peptide sequences. Values are shown relative to CTR level at t=0 min. f) Ratio of thr172 phospho-AMPK to AMPK protein level (WB shown below); g) representative picture of WB for p-PKA substrate >15 kDa. Symbols indicate p<0.05: *HH vs HE; †HH vs CTR; ‡HE vs CTR; §All.

**Legend fig 4**: Glycogen synthase activity and phosphorylation during; CTR (bolus 2ml NaCl i.v.) - hollow squares with solid line; HH (bolus insulin 0.1 U actrapid/kg) - filled circles with dash-dot dot; and HE (bolus insulin 0.1 U actrapid/kg and glucose 20%) - filled triangles with tight dot. Glycogen synthase activity expressed as a) fractional velocity (%) and b) I-Form (%). c-f) Phosphorylation of GS site 3a, 2+2a, 1a and 1b relative to total GS-protein at t=0, 30 and 75 min. Values are shown relative to CTR level at t=0 min (representative WB pictures shown below graphs). Symbols indicate p<0.05: *HH vs HE; †HH vs CTR; ‡HE vs CTR; §All.

**Legend fig 5**: Regulators of glycogen synthase phosphorylation and glycogen breakdown, and total glycogen during CTR (bolus 2ml NaCl i.v.) - hollow squares with solid line; HH (bolus insulin 0.1 U actrapid/kg) - filled circles with dash-dot dot; and HE (bolus insulin 0.1 U actrapid/kg and glucose 20%) - filled triangles with tight dot. a) pGSK-3alpha relative to GSK-3alpha b) pGSK-3beta relative to GSK-3beta c+d) glycogen phosphorylase a and b, and e) glycogen. Values are shown relative to CTR level at t=0 min (representative WB pictures shown below graphs). Symbol indicates p<0.05: †HH vs CTR.