Insulin resistance after a 72-h fast is associated with impaired AS160 phosphorylation and accumulation of lipid and glycogen in human skeletal muscle


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Prolonged fasting is associated with reduced sensitivity to insulin (30). This phenomenon was first described in 1859 by Claude Bernard (3) and was termed “starvation diabetes.” Insulin resistance in skeletal muscle plays a key role in this adaptive response (4). During fasting, skeletal muscle switches its energy substrate metabolism predominantly to oxidation of lipids (30), and insulin-stimulated glucose uptake in skeletal muscle is reduced (4). The intracellular mechanisms that cause insulin resistance in skeletal muscle have been investigated intensely. In 1963, Randle et al. (36) proposed a theory of direct substrate competition where accumulation of citrate and glucose 6-phosphate (G6P) causes enzyme inhibition and reduced glucose uptake. This paradigm was challenged when NMR spectroscopy demonstrated reduced intramuscular G6P content in insulin-resistant muscle (38, 39). Instead, inhibition of proximal insulin signaling through insulin receptor substrate-1 (IRS-1) and reduced Akt phosphorylation was suggested as an underlying mechanism behind insulin resistance (23, 38, 39). However, this finding has not been consistent, and several reports have demonstrated that insulin sensitivity in skeletal muscle can be modulated despite normal phosphorylation of Akt (13, 18, 20, 21, 46, 47, 51). This indicates that, in addition to defects in proximal insulin signaling, alternative mechanisms downstream of Akt may cause insulin resistance.

The Rab-GTPase-activating protein Akt substrate of 160 kDa (AS160), also known as tre-2/USP6, BUB2, and cdc16 domain family member (TBC1D4), and the AS160 paralog TBC1D1 have been identified recently as regulators of insulin-stimulated glucose uptake (40). Of the two proteins, AS160 is the most extensively studied, and AS160 phosphorylation is reduced in patients with type 2 diabetes and their first-degree offspring (26, 27, 53). AS160 is thought to control the translocation of the glucose transporter 4 (GLUT4) from intracellular vesicles to the cell surface (40) by promoting hydrolysis of GTP to GDP on Rab proteins. The signal from the insulin receptor to AS160 is mediated through the serine/threonine kinase Akt (40), but AS160 can also be phosphorylated independently of Akt by the energy-sensing enzyme AMP-activated protein kinase (AMPK) (28, 48). Previously, most investigations of AS160 have used the phospho-Akt substrate (PAS) antibody. Although AS160 contains several Akt residues, the PAS antibody has been reported to recognize only phosphorylation on Thr242 (17, 25), and important information on AS160 regulation may not be detected with this antibody. The Ser431 site on AS160 has been shown to affect 14-3-3 binding (17), and with the use of phosphospecific antibodies it has been shown that improved insulin sensitivity is associated with increased insulin-stimulated AS160 phosphorylation at Ser218, Ser431, and Ser751 (49). Furthermore, insulin-stimulated phos-
phorylation of AS160 at site Ser\textsuperscript{318}, Ser\textsuperscript{588}, and Ser\textsuperscript{751} is reduced in patients with type 2 diabetes (53). This indicates that site-specific AS160 phosphorylation may be an important regulator of insulin-stimulated glucose uptake. TBC1D1 phosphorylation on AMPK consensus motifs in human skeletal muscle has been shown recently to be increased by physical exercise (15, 24). TBC1D1 also contains a full Akt consensus motif (Thr\textsuperscript{596}) that is phosphorylated in response to insulin in mouse skeletal muscle (35, 52). However, it is not known whether insulin regulates TBC1D1 in human skeletal muscle.

The effects of a prolonged fast on insulin signaling to glucose transport in human skeletal muscle are incompletely understood. In a recent publication, insulin-stimulated Akt Ser\textsuperscript{473} and AS160 PAS phosphorylation were reduced after a 62-h fast (44). However, the subjects were clamped at significantly higher insulin levels in the control situation compared with the 62-h fasting condition (44), making it difficult to determine whether the decreased signaling was due to fasting or the lower insulin concentrations. Another report has examined insulin sensitivity after a 48-h fast (2). Muscle biopsies were obtained after a hyperinsulinemic euglycemic clamp was stopped, but at this time point insulin did not increase signaling under control or fasting conditions (2). Thus, studies of insulin signaling in human skeletal muscle after a prolonged fast are warranted, and AMPK regulation under these conditions remains to be investigated.

In the current study, we determined the effects of prolonged fasting on insulin signaling. For this purpose, we studied eight healthy subjects on two occasions that consisted of either an overnight fast as the control condition or a 72-h fast. We determined insulin-stimulated glucose uptake, substrate oxidation, and lipid accumulation. Glycogen content, glycogen synthase (GS) regulation, and phosphorylation levels of AS160, TBC1D1, Akt, and AMPK were determined in skeletal muscle biopsies. Our findings indicate that skeletal muscle insulin resistance after a 72-h fast is associated with site-specific impairment of AS160 phosphorylation and accumulation of intramyocellular lipids and glycogen. This was not associated with changes in the phosphorylation of the upstream kinases Akt and AMPK, suggesting that, under physiological conditions, insulin sensitivity can be modulated by specific regulation of AS160 phosphorylation independently of Akt and AMPK.

METHODS

Ethical approval. All participants gave their written, informed consent after being given oral and written information regarding the study, in accordance with the Declaration of Helsinki II. The Aarhus County local ethics scientific committee approved the study.

Subjects. Eight healthy men with no family history of diabetes participated. The average age was 26 ± 4 yr, body weight was 82.9 ± 8.8 kg, and body mass index was 23.8 ± 1.6 kg/m\(^2\). The subjects did not take prescription medicine.

Protocol. In a randomized crossover design, subjects were examined on 2 days separated by >1 mo (1) after an overnight fast of 10 h (control condition) and 2) after 72-h fasting, during which subjects were allowed to drink tap water and perform normal ambulatory activities, excluding physical exercise. At 0800 (t = 0) the subjects were placed in a quiet, thermoneutral room on both examination days. For blood sampling, one intravenous catheter (Venflon; Viggo, Helsingborg, Sweden) was placed in a dorsal hand vein, and the hand was placed in a 65°C heated box for arterialization of the blood. Subjects were studied for 6 h from 0800 to 1400 (t = 0 to 360). After a 4-h basal period (t = 0–240), the blood glucose levels were clamped at ~5 mM with an insulin (Actrapid; Novo Nordisk) infusion of 0.8 mU·kg\(^{-1}\)·min\(^{-1}\) for the last 2 h of the study (t = 240–360). At t = 30 and 270 min, a 5- to 7-mm incision was made, using local anesthesia and sterile conditions, 12–15 cm proximal to the superior border of the patella, and a muscle specimen was obtained from the superficial border of the vastus lateralis of the quadriceps femoris muscle using the Bergström needle. The muscle tissue was immediately dissected free from connective tissue and placed in liquid nitrogen.

Blood analysis. Plasma glucose was immediately measured in duplicate on a Beckman Glucometeranalyzer (Beckman Instruments, Palo Alto, CA) except during fasting, where whole blood glucose measurements were performed in duplicate on an Accu-Chek inform (F. Hoffmann-La Roche, Basel, Switzerland). Serum samples were frozen and stored at ~20°C, and insulin was analyzed using time-resolved fluoroimmunoassay (TR-IFMA, AutoDELFIA; PerkinElmer, Turku, Finland). C-peptide was measured by ELISA (DakoCytomation, Cambridgeshire, UK), and free fatty acids (FFA) were analyzed by a commercial kit (Wako Chemicals, Neuss, Germany).

MRS spectroscopy. \(^1\)H-magnetic resonance spectroscopy was performed using a Sigma Excite 1.5 tesla twin speed scanner (GE Medical Systems). The subjects were examined at 0, 12, and 60 h of fasting. For logistic reasons, the last scan was performed in the evening before the examination day. To obtain muscle spectroscopy, subjects were positioned in supine position with feet first in the magnet bore, and the left calf was placed in a standard radio frequency transmit-receive extremity coil for radio frequency transmission and signal reception. A transversal plane T1-weighted gradient echo pulse sequence with a repetition time (TR) of 140 ms and an echo time (TE) of 2.2/4.4 ms was performed to enable identification of the area of interest. The volume of interest was carefully positioned in a homogeneous part of the tibialis anterior muscle, avoiding visible vessels and connective tissue. Autoshimming was performed to optimize the magnetic field homogeneity. A water-suppressed, point-resolved spectroscopy sequence (TE: 30 ms; TR: 2,000 ms; no. of acquisitions: 128) was applied in triplicate, using water as the autocenter frequency. Full width at half-maximum of the unsuppressed water peak was 11.95 ± 0.097 Hz.

Indirect calorimetry. The respiratory exchange ratio (RER) and resting energy expenditure were estimated by indirect calorimetry (Deltatrac monitor; Dantec Instrumentarium, Helsinki, Finland) performed at t = 180–210 and t = 300–330 of the basal and clamp period, respectively. The mean values of the last 25 min were used for calculations. Urine was collected during the basal period, and urea content was measured using the urease-Berthelot method (11). Oxidative glucose disposal was estimated after correction for protein oxidation, which was calculated on the basis of urea nitrogen excretion (12).

Glucose metabolism. During the entire examination day a primed, continuous infusion of [\(^3\)H]glucose (bolus 20 μCi, 0.12 μCi/min·kg\(^{-1}\); NEN Life Science Products, Boston, MA) was performed. To minimize rapid dilution of the [\(^3\)H]glucose during the hyperinsulinemic euglycemic clamp, [\(^3\)H]glucose was added to the infused glucose (100 μCi [\(^3\)H]glucose/500 ml 20% glucose).

Glucose rate of appearance (\(R_{\text{A}}\)) and disappearance (\(R_{\text{D}}\)) was estimated by Steele’s equation (45). Endogenous glucose production (EGP) equals \(R_{\text{A}}\) of glucose under basal conditions, and during the clamp EGP is calculated by subtracting mean rate of exogenous glucose from \(R_{\text{A}}\).

Nonoxidative glucose disposal was estimated by subtracting oxidative glucose disposal from total glucose disposal (glucose \(R_{\text{D}}\)).

Glycogen. Muscle samples were hydrolyzed in 2 M HCl at 100°C for 2 h, followed by neutralization with 2 M NaOH (54), and glucose
content was measured by the hexokinase enzymatic method using a glucose hexokinase reagent (Eagle Diagnostics, Desoto, TX) (5).

Muscle GS activity was measured in the presence of 0.002, 0.17, and 8 mM G6P (unfilter 350 plates; Whatman, Cambridge, UK). GS activity is presented as G6P-independent GS activity (%I-form = 100 × activity in the presence of 0.02 mM G6P/activity in the presence of 8 mM G6P) and as percent of fractional velocity (%FV = 100 × activity in the presence of 0.17 mM G6P/activity in the presence of 8 mM G6P).

Western blotting. Muscle biopsies were homogenized in an ice-cold buffer containing (in mM) 50 HEPES, 137 NaCl, 10 Na2PO4, 10 NaF, 2 EDTA, 1 MgCl2, 1 CaCl2, and 2 Na2VO4 and 1% (vol/vol) Nonidet P-40, 10% (vol/vol) glycerol, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 10 μg/ml antipain, 1.5 mg/ml benzamidine, and 100 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride, and hydrochloride (pH 7.4), and samples were rotated for 60 min at 4°C. Insoluble materials were removed by centrifugation at 16,000 g for 20 min at 4°C. Western blot analyses were used to assess protein and phosphorylation levels of various proteins. Antibodies to AMPKα1, AMPKα2, and phosphospecific (Ser279) Acetyl-CoA carboxylase (ACC) antibody were from Millipore (Billerica, MA). Polyclonal anti-COOH-terminal peptide GLUT4 antibody was produced as described previously (29). Phosphospecific TBC1D1 (Ser237 and Thr642) was generated as described previously (52). Phosphospecific AMPK (Thr172), Akt, phosphospecific Akt (Ser473 and Thr308), GS, phosphospecific GS (Ser411), GSK-3 (α and β), cytochrome c, and AS160 antibodies were from Cell Signaling Technology (Beverly, CA). ACC expression was assessed using horseradish peroxidase-conjugated streptavidin (Pierce Chemical, Rockford, IL). Phosphospecific AS160 (Ser341, Ser588, Thr642, Ser704, and Ser751) antibodies were generated as described previously (17, 50). Proteins were visualized by BioWest enhanced Chemiluminescence (Pierce) and quantified using UVP BioImaging System (UVP, Upland, CA). Quantifications of protein phosphorylation are expressed as a ratio of total protein expression measured on the same membranes.

Quantitative PCR. Total RNA was isolated from muscle biopsies using Trizol (Gibco BRL, Life Technologies, Roskilde, Denmark); RNA was quantified by measuring absorbance at 260 and 280 nm with a ratio ≥ 1.8. Integrity of the RNA was checked by visual inspection of the two ribosomal RNA, 18S and 28S, on an agarose gel. cDNA was synthesized with the TaqMan Gold RT-PCR kit (PerkinElmer, Boston, MA). Real-time PCR for GS was done with mRNA levels of β-actin as internal control. The following primers were used: GS (ACC TGG CTT ATT CCC AAC TGC TC and AGT GAC CTC AGG TTC TGG ATC ATG, 128 bp) and β-actin (ACG GGG TCA CCC ACA CTG TGC and CTA GAA GCA TTT GCG GTG GAC GAT G, 658 bp). The analyses were performed in duplicate using the KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Woburn, MA) in an iCycler from Bio-Rad (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis. Results are expressed as means ± SE. Normal distribution was assessed by inspection of QQ plots, and the Levene Median test was used to test for equal variance. Comparisons between the main effects of 72 h of fasting and insulin stimulation were assessed by a two-way repeated-measures ANOVA. When the two-way repeated-measures ANOVA revealed significant differences, post hoc test multiple comparison procedures using Student-Newman-Keul’s method were performed. Fasting effects before examination day were assessed by a one-way repeated-measurement ANOVA. Isolated comparisons between control and 72 h of fasting were assessed by a paired t-test. P < 0.05 was considered significant. The coefficient of variation of the intraindividual MR spectroscopy data was calculated as the standard deviation divided by the mean of triplicate measurements.

RESULTS

Substrate oxidation and insulin sensitivity. The subjects lost 3.4 ± 1.2 kg during fasting, and this was associated with an ~100% increase in plasma FFA levels, an ~25% decrease in blood glucose concentrations, and an ~50% reduction in insulin levels compared with the control day. Energy expenditure was not changed after a 72-h fast (Tables 1 and 2). Urine urea excretion rates were 13.9 ± 1.46 and 14.11 ± 0.78 mmol·kg−1·min−1 in the control and fasting condition, respectively. This was used to correct the relative contribution of lipid and glucose in substrate oxidation assessed from the RER. As shown in Fig. 1A, a 72-h fast decreased the RER to 0.76 compared with 0.83 in the control condition. This decrease in RER indicates a further shift in substrate metabolism toward lipid oxidation after 72 h of fasting compared with a 10-h overnight fast. Insulin stimulation increased the RER under the control condition but had no effect on the RER after 72 h of fasting. We measured protein expression of cytochrome c, a component of the electron transport chain in mitochondria and a marker of mitochondrial mass, but did not observe any changes in expression after 72 h of fasting (96.1 ± 7.1% of control, P = 0.60).

Insulin sensitivity was assessed by a hyperinsulinenic euglycemic clamp. Insulin was infused at a rate of 0.8 mU·kg−1·min−1. This increased plasma insulin concentrations to ~270 pM, with no significant differences between control

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<th>Table 1. Hormone levels and energy expenditure under control and fasting conditions</th>
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Values are means ± SE. FFA, free fatty acids. Glucose, insulin, C-peptide, and FFA levels in plasma were determined during examination days at t = 0 and t = 360 after an overnight fast of 10 h (control) and after a 72-h fast (fasting). Energy expenditure was estimated by indirect calorimetry at t = 180–210 and t = 300–330 (*P < 0.01 fasting vs. control).

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<th>Table 2. Plasma measurements during fasting</th>
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Glucose, insulin, C-peptide, and FFA levels in plasma were monitored during the 72-h fasting period. *P < 0.01, †P < 0.05, 0- vs. 72-h fast.
and fasting conditions. The glucose infusion rate in the last 30 min of the clamp was \( \sim 60\% \) lower after a 72-h fast (Fig. 1B). This was associated with a decreased \( R_d \) of glucose after a 72-h fast (Fig. 1C), a clear indication of skeletal muscle insulin resistance. However, insulin stimulation still increased \( R_d \) also after a 72-h fast, albeit at a significantly lower level (Fig. 1C). Glucose oxidation was decreased significantly during a 72-h fast, and insulin increased glucose oxidation only under control

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**Fig. 1.** A: the respiratory exchange ratio (RER) was assessed before and after insulin stimulation after an overnight fast of 10 h (control condition) and after a 72-h fast. Throughout the figure, open bars indicate no insulin stimulation and filled bars indicate insulin stimulation. There was a significant interaction between insulin stimulation and fasting conditions \( (P < 0.05) \), and post hoc test showed that insulin increased RER only in the control condition \( (\ast P < 0.05 \text{ vs. basal within experimental day}) \), and RER was reduced during fasting under both basal and insulin-stimulated situations \( (\dagger P < 0.05 \text{ vs. control within insulin or basal}) \). B: insulin sensitivity was assessed by a hyperinsulinemic euglycemic clamp. Insulin was infused at a rate of 0.8 mU·kg\(^{-1}\)·min\(^{-1}\). The glucose infusion rate (GIR) under the last 30 min of the clamp was \( \sim 60\% \) lower after a 72-h fast \( (\ddagger P < 0.05) \). C: the reduced GIR after 72 h of fasting was associated with a reduced rate of disappearance (\( R_d \)) for glucose. There was a significant interaction between insulin stimulation and fasting conditions \( (P < 0.01) \), and post hoc test showed that \( R_d \) during fasting was reduced by \( \sim 60\% \) \( (\S P < 0.01 \text{ vs. control within insulin/basal}) \). Although the effect of insulin was reduced after 72 h of fasting, there was still a significant increase in \( R_d \) during control and fasting conditions. Basal \( R_d \) was not reduced by fasting. D: 72-h fasting reduced glucose oxidation. There was a significant interaction between insulin stimulation and fasting conditions \( (P < 0.05) \), and insulin increased glucose oxidation only in the control condition. Glucose oxidation was decreased under both basal and insulin-stimulated conditions during fasting. E: similarly to \( R_d \), endogenous glucose production (EGP) was reduced during fasting, and there was a significant interaction between insulin stimulation and fasting conditions \( (P < 0.01) \). Post hoc test showed that this was associated with reduced basal EGP during fasting, whereas insulin suppressed EGP in both situations. The EGP during insulin stimulation was similar after 72-h fasting compared with the control situation.
conditions (Fig. 1D). The reduced peripheral glucose uptake was not due to reduced expression of GLUT4 in skeletal muscle (93.5 ± 8.6% of control, \(P = 0.58\)). EGP was assessed on the basis of measurements of the specific activity of the [3-\(^3\)H]glucose tracer (Fig. 1E). The EGP was reduced after 72 h of fasting. However, when stimulated with insulin, EGP was suppressed to \(~\)0.2 mg·kg\(^{-1}\)·min\(^{-1}\) under both control and fasting conditions. This indicates that the observed insulin resistance after a 72-h fast can be attributed primarily to reduced peripheral glucose uptake and not liver insulin resistance.

**Akt and AMPK phosphorylation.** Both Akt and AMPK are known upstream kinases of AS160 in skeletal muscle (40). Therefore, we first assessed phosphorylation of the two activating sites Ser\(^{473}\) and Thr\(^{308}\) on Akt. Insulin stimulation increased phosphorylation on both sites, and this was not affected by fasting (Fig. 2, A and B). We next assessed whether 72 h of fasting would affect the phosphorylation of AMPK, a positive regulator of insulin-independent glucose uptake. AMPK is activated by phosphorylation of the catalytic \(\alpha\)-subunit, but the 72-h fast did not increase AMPK phosphorylation (Fig. 2C). Consistent with the AMPK results, phosphorylation of ACC, a well-known target of AMPK activity, was also not affected by 72 h of fasting (Fig. 2D). Insulin stimulation did not affect AMPK and ACC phosphorylation (Fig. 2, C and D) and protein expression of Akt, AMPK, and ACC was similar among all conditions.

**AS160 and TBC1D1 phosphorylation.** We determined AS160 phosphorylation on five sites: Ser\(^{341}\), Ser\(^{588}\), Thr\(^{642}\), Ser\(^{704}\), and Ser\(^{751}\). In addition to the site-specific antibodies, we used the PAS antibody to assess AS160 phosphorylation. Insulin increased site-specific phosphorylation of all sites investigated as well as PAS phosphorylation (Fig. 3, A–F) under both control and fasting conditions. There were main effects of 72 h of fasting on AS160 phosphorylation on Ser\(^{341}\), Ser\(^{588}\), Ser\(^{704}\), and Ser\(^{751}\) but not on Thr\(^{642}\) or AS160 PAS phosphorylation.

![Fig. 2](http://ajpendo.physiology.org/) Phosphorylation (p) of the intracellular kinases Akt, AMP-activated protein kinase (AMPK), and acetyl-CoA carboxylase (ACC) in muscle biopsies taken before and during a hyperinsulinemic euglycemic clamp was assessed by Western blot analysis. Throughout the figure, open bars indicate no insulin stimulation and filled bars indicate insulin stimulation. A and B: there was a main effect of insulin stimulation, but not fasting, on Akt Ser\(^{473}\) and Thr\(^{308}\) under both control and fasting conditions (*\(P < 0.001\)). Post hoc tests showed no difference between experiment days within basal or insulin-stimulated situations. C and D: 72 h of fasting and insulin stimulation did not affect phosphorylation of the AMPK or the downstream target ACC.
Fig. 3. Site-specific phosphorylation of Akt substrate of 160 kDa (AS160) and phospho-Akt substrate (PAS) phosphorylation in muscle biopsies taken before and during a hyperinsulinemic euglycemic clamp was assessed by Western blot analysis. Throughout the figure, open bars indicate no insulin stimulation and filled bars indicate insulin stimulation. Representative blots show from left to right control situation before and after insulin stimulation and fasting conditions before and after insulin stimulation. A: there was main effect of a 72-h fast (\(* P < 0.05\)) and insulin stimulation (\(† P < 0.01\)) on Ser341 phosphorylation. Post hoc test showed a decrease in basal phosphorylation during fasting (\(‡ P < 0.05\) vs. control within insulin or basal). B: there was a main effect of a 72-h fast and insulin stimulation on Ser588 phosphorylation. Post hoc test showed that both basal and insulin-stimulated phosphorylation were reduced during fasting. C: there was a main effect of a 72-h fast and insulin stimulation on Ser704 phosphorylation. D: there was a main effect of a 72-h fast and insulin stimulation on Ser751 phosphorylation. E and F: there was a significant interaction of insulin and a 72-h fast (\(§ P < 0.001\)) on Thr642 or AS160 PAS phosphorylation. Post hoc test showed a significant insulin effect (\(§ P < 0.001\) vs. basal within experimental day). Basal phosphorylation was reduced during fasting, whereas insulin-stimulated phosphorylation was similar under fasting and control conditions. G: phosphorylation of the Akt consensus site Thr596 on TBC1D1 was increased during insulin stimulation (main effect of insulin \(P < 0.05\)), but there was no main effect of fasting. H: TBC1D1 phosphorylation at Ser237, an AMPK consensus site, was not affected by fasting or insulin.
Post hoc tests showed decreased insulin-stimulated phosphorylation on Ser\textsuperscript{588}, Ser\textsuperscript{704}, and Ser\textsuperscript{751} during fasting, and consistent with the lower plasma insulin levels after 72 h of fasting, basal phosphorylation of AS160 on Thr\textsuperscript{642}, Ser\textsuperscript{341}, Ser\textsuperscript{588}, Ser\textsuperscript{704}, and Ser\textsuperscript{751}, and AS160 PAS phosphorylation was reduced (Fig. 3, E and F).

Insulin stimulated TBC1D1 phosphorylation of the Akt consensus motif Thr\textsuperscript{596}, and fasting did not modify this effect (Fig. 3G). Phosphorylation of the AMPK consensus motif Ser\textsuperscript{237} was not affected by insulin stimulation or 72-h fasting (Fig. 3H).

**Skeletal muscle lipid content.** Accumulation of lipids and glycogen in skeletal muscle is associated with the development of insulin resistance (42, 43). Therefore, we determined the intramuscular lipid levels by MRI spectroscopy before and after 12 and 60 h of fasting. As shown in Fig. 4, the 60-h fasting was associated with an approximately twofold increase in skeletal muscle lipid content, whereas no change was observed between prefasting and the 12-h fasting. The median within-subject variation coefficient was 9.5\% with a range of 1.7–41.6\%.

**Skeletal muscle glycogen content and regulation of glycogen synthesis.** Seventy-two hours of fasting increased glycogen content by ~10\% \((P < 0.05)\), and, as expected, we did not see a significant change in glycogen content after only 30 min into the hyperinsulinemic euglycemic clamp (Fig. 5A). Glycogen synthesis is inhibited by accumulation of glycogen in the muscle in a classical feedback loop, and in agreement with the increased glycogen level, nonoxidative glucose disposal decreased during fasting compared with control under insulin stimulation (Fig. 5B). Glycogen synthesis is regulated by the enzyme GS, and this enzyme is inactivated by phosphorylation. During fasting conditions GS activity was decreased, but insulin increased GS activity under both fasting and control conditions (Fig. 5, C and D). After 72 h of fasting, GS protein expression did not change, but GS gene expression was reduced by 33\% \((67.0 \pm 6.1 \% \text{ of control}, P < 0.05)\). Glycogen synthesis is also regulated by insulin through Akt and GSK3, which is inactivated by phosphorylation. In agreement with the insulin-stimulated Akt phosphorylation, phosphorylation of GSK-3\(\alpha\) and -\(\beta\) increased during insulin stimulation, and this was not affected by fasting (Fig. 5, E and F). Consistent with the GS activity measurements, GS phosphorylation was increased significantly during fasting (Fig. 5G). Insulin tended to reduce GS phosphorylation, but this decrease did not reach statistical significance (main effect of insulin: \(P = 0.051\); power of performed test: 0.44).

**DISCUSSION**

Insulin resistance in skeletal muscle plays an important role in the adaptive response to fasting (4). The current study demonstrates that a 72-h fast is associated with reduced phosphorylation of the Akt substrate AS160 but not TBC1D1. Using site-specific antibodies, we have determined that phosphorylation of AS160 at Ser\textsuperscript{341}, Ser\textsuperscript{588}, and Ser\textsuperscript{751} is reduced in skeletal muscle, whereas TBC1D1 Thr\textsuperscript{596} was normal. This was associated with a modest increase in glycogen content and accumulation of intramuscular lipids.

Several lines of evidence suggest an important role of AS160 in glucose uptake in skeletal muscle (40), but little is known about the importance of the individual phosphorylation sites on AS160. The association with the reduced glucose uptake observed in the present study could indicate that Ser\textsuperscript{588}, Ser\textsuperscript{704}, and Ser\textsuperscript{751} are involved in regulating GAP activity and subsequent GLUT4 translocation. This is supported by a recent finding in patients with type 2 diabetes. In these insulin-resistant patients, insulin-stimulated phosphorylation of sites
Fig. 5. Glycogen content and regulation of glycogen metabolism were determined before and after a hyperinsulinemic euglycemic clamp. Throughout the figure, open bars indicate a non-insulin-stimulated condition and filled bars indicate insulin stimulation. A: there was a main effect of fasting on glycogen levels (*P < 0.05) with increased glycogen content in skeletal muscle after a 72-h fast. B: this was associated with a decreased, insulin-stimulated, nonoxidative glucose disposal after 72 h of fasting (fasting-insulin interaction, P < 0.05). Post hoc test showed that insulin increased nonoxidative glucose disposal (†P < 0.001 vs. basal within experimental day), but the insulin-stimulated level was significantly lower during fasting (§P < 0.05 vs. control within insulin or basal). There were no differences between experimental days in nonoxidative glucose disposal within the non-insulin-stimulated condition. C and D: glycogen synthase (GS) activity was reduced during fasting when measured as both %I-form (glucose 6-phosphate-independent GS activity) and as %fractional velocity (main effect of fasting, P < 0.05). Insulin stimulated GS activity under both fasting and control conditions (main effect of insulin, P < 0.05). Post hoc tests showed that both basal and insulin-stimulated GS activity as %I-form were reduced during fasting, whereas %fractional velocity was reduced only in the insulin-stimulated form. E and F: representative blots show from left to right control situation before and after insulin stimulation and fasting conditions before and after insulin stimulation. Insulin increased phosphorylation of the upstream kinase glycogen synthase kinase-3 (GSK-3; main effect of insulin: ¶P < 0.001), but there was no effect of 72 h of fasting. G: phosphorylation of GS increased after fasting. There was a trend toward reduced GS phosphorylation after insulin stimulation, but this did not reach statistical significance (main effect of insulin: P = 0.051), and post hoc test showed a significant reduction only with insulin stimulation within the control situation (‖P < 0.05 vs. basal within experimental day).
similar observations have been done in C2C12 cells incubated with muscles, AMPK is activated under fasting conditions, and in human skeletal muscle during a prolonged fast. In rodent liver, muscle glycogen content was increased after a 72-h fast. This is likely a result of decreased glucose oxidation during fasting in the non-insulin-stimulated condition. The capacity of skeletal muscle to perform high-intensity exercise is highly dependent on glycogen content (41), and from an evolutionary perspective it appears advantageous that glycogen levels are preserved during a prolonged fast. Glycogen synthesis in skeletal muscle is the principal pathway of glucose disposal during insulin stimulation (43). After 72 h of fasting, nonoxidative glucose disposal was decreased, and glycogen synthase was in its inactive phosphorylated form. The inhibition of glycogen synthesis was likely in part due to the increased glycogen content after 72 h of fasting, since glycogen is a negative regulator of glycogen synthesis (55). This accumulation of glycogen may also contribute to the reduced insulin-stimulated glucose uptake. Elevated levels of glycogen can reduce insulin-stimulated GLUT4 translocation (10), and increased glycogen content in combination with reduced phosphorylation of AS160 at Ser341, Ser588, Ser704, and Ser751 in healthy individuals 4 h after an exercise bout (49). This could indicate that the reduced phosphorylation observed after fasting could be a detraining phenomenon. However, the subjects performed normal daily activities during the fast, and the expression of GLUT4 and cytochrome c was not changed. Both proteins are known to be downregulated during prolonged physical inactivity (6). In addition, we do not expect that differences in physical activity in the preclamp period affected AS160 phosphorylation since the clamp was performed after the subjects had rested >4 h under both control and fasting conditions at the research facilities. Therefore, it does not seem likely that the reduced Ser341, Ser588, Ser704, and Ser751 phosphorylation was due to acute or long-term differences in physical activity.

Endogenous glucose production was reduced in the basal state during a 72-h fast, most likely reflecting depletion of liver glycogen (33). In agreement with previous observations, insulin-stimulated glucose production in the non-insulin-stimulated condition (2), and there was thus no indication of hepatic insulin resistance. Unlike the liver, muscle glycogen content was increased after a 72-h fast. This is likely a result of decreased glucose oxidation during fasting in the non-insulin-stimulated condition. The capacity of skeletal muscle to perform high-intensity exercise is highly dependent on glycogen content (41), and from an evolutionary perspective it appears advantageous that glycogen levels are preserved during a prolonged fast. Glycogen synthesis in skeletal muscle is the principal pathway of glucose disposal during insulin stimulation (43). After 72 h of fasting, nonoxidative glucose disposal was decreased, and glycogen synthase was in its inactive phosphorylated form. The inhibition of glycogen synthesis was likely in part due to the increased glycogen content after 72 h of fasting, since glycogen is a negative regulator of glycogen synthesis (55). This accumulation of glycogen may also contribute to the reduced insulin-stimulated glucose uptake. Elevated levels of glycogen can reduce insulin-stimulated GLUT4 translocation (10), and increased glycogen content in combination with reduced phosphorylation of AS160 at Ser341, Ser588, Ser704, and Ser751 in healthy individuals 4 h after an exercise bout (49). This could indicate that the reduced phosphorylation observed after fasting could be a detraining phenomenon. However, the subjects performed normal daily activities during the fast, and the expression of GLUT4 and cytochrome c was not changed. Both proteins are known to be downregulated during prolonged physical inactivity (6). In addition, we do not expect that differences in physical activity in the preclamp period affected AS160 phosphorylation since the clamp was performed after the subjects had rested >4 h under both control and fasting conditions at the research facilities. Therefore, it does not seem likely that the reduced Ser341, Ser588, Ser704, and Ser751 phosphorylation was due to acute or long-term differences in physical activity.

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In conclusion, insulin resistance induced by a 72-h fast is associated with reduced phosphorylation of AS160 at Ser841, Ser858, Ser751, and Thr642 whereas neither AMPK phosphorylation nor insulin-stimulated phosphorylation of Akt was affected. Instead, prolonged fasting is associated with the accumulation of lipid and glycogen in muscle, and a combination of these factors and a site-specific impaired phosphorylation of AS160 are distinct features of insulin resistance under these conditions.

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DISCLOSURES

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS


REFERENCES


