Original Article

Increased Lipid Availability Impairs Insulin-Stimulated ATP Synthesis in Human Skeletal Muscle

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Insulin resistance correlates with intramyocellular lipid content (IMCL) and plasma free fatty acids (FFAs) and was recently linked to mitochondrial dysfunction. We examined the underlying relationships by measuring skeletal muscle ATP synthase flux, glucose transport/phosphorylation, and IMCL in response to different plasma insulin and plasma FFA concentrations. Healthy men were studied twice during hyperinsulinemic-euglycemic clamps with (LIP) or without (CON) lipid infusion (plasma FFA: CON ~36 vs. LIP ~1,034 μmol/l, $P < 0.001$). ATP synthase flux, glucose-6-phosphate (G6P), and IMCL were determined before and during the clamp in calf muscle using ^{31}P and ^{1}H magnetic resonance spectroscopy. Plasma lipid elevation resulted in ~46% reduced whole-body glucose metabolism (180–360 min; $P < 0.0001$ vs. CON) and a 70% lower rise of G6P ($P < 0.05$ vs. CON) without significant changes in IMCL (LIP 117 ± 12% vs. CON 93 ± 3% of basal, $P = 0.073$). During the clamp, ATP synthase flux increased by ~60% under control conditions ($P = 0.02$ vs. baseline) and was 24% lower during lipid infusion (LIP 11.0 ± 0.9 vs. CON 14.6 ± 1.2 μmol · g muscle^{-1} · min^{-1}, $P < 0.05$). Physiologically increased plasma FFA concentrations reduce insulin-stimulated muscle ATP synthase flux in parallel with induction of insulin resistance. *Diabetes* 55:136–140, 2006

Insulin resistance is an essential feature of type 2 diabetes and frequently precedes the onset of the disease by decades. Skeletal muscle accounts for the majority of insulin-stimulated whole-body glucose uptake, thereby rendering this tissue essential for the development of insulin resistance. This goes along with impaired insulin-stimulated glucose transport/phosphorylation, decreased glycogen synthesis, and reduced glucose oxidation (1,2). In parallel, insulin-resistant humans display increased intramyocellular lipid content (IMCL), correlating with the degree of insulin resistance (3,4).

Several environmental factors leading to increased accumulation and availability of fat contribute to impaired skeletal muscle insulin sensitivity. This applies in particular to elevated plasma concentrations of free fatty acids (FFAs), which increase IMCL (5,6) and impair glucose transport/phosphorylation, glycogen synthesis, and glucose oxidation similar to the defects observed in type 2 diabetes (7–11). These effects are currently attributed to increased intramyocellular concentrations of fatty acid metabolites and interference with insulin signaling (10,12,13).

Skeletal muscle strongly relies on mitochondrial oxidative phosphorylation for ATP production. Accordingly, decreased oxidative capacity and mitochondrial aberrations can be major contributors to the development of insulin resistance and type 2 diabetes and are thus seen in skeletal muscle biopsies of insulin-resistant humans (14). In parallel, mitochondria obtained from skeletal muscle of obese and type 2 diabetic patients exhibit structural and functional perturbations correlating with the degree of insulin resistance (15). These alterations are associated with the failure of insulin to increase transcript levels, protein, and ATP synthesis in mitochondria of skeletal muscle of insulin-resistant humans (16,17).

Impaired ATP synthase flux and increased IMCL are also present in lean glucose-tolerant offspring of type 2 diabetes and have been attributed to dysregulation of intramyocellular fatty acid metabolism and to inborn defects of oxidative phosphorylation (18). However, no data have been reported on skeletal muscle ATP synthase flux in nutrient-induced insulin resistance.

The present study therefore aimed to test the interference of increased lipid availability with skeletal muscle ATP synthase flux in humans. To this end, we assessed intramyocellular glucose transport/phosphorylation by measuring glucose-6-phosphate (G6P), skeletal muscle ATP synthase flux by magnetization saturation transfer experiments, and IMCL by combined ^{31}P and ^{1}H magnetic resonance spectroscopy (MRS) during euglycemic clamp tests.

RESEARCH DESIGN AND METHODS

Eight healthy male volunteers (aged 26 ± 1 years, 73 ± 3 kg body wt, and BMI 22.5 ± 0.7 kg/m^2) without family history of diabetes were included. None were glucose intolerant or taking any medication on a regular basis. They were physically active but not trained. Their physical activity was assessed with a questionnaire of habitual physical activity, in which values can theoretically range from 1.0 to 5.0, with higher values indicating greater activity (mean activity index: 2.6 ± 0.1) (19). All participants were instructed to ingest an isocaloric diet (carbohydrate/protein/fat: 60/20/20%) and refrain from any physical exercise during the 3 days preceding the studies and then fasted overnight for 12 h before the experiments. The protocol was approved by the local institutional ethics board, and informed consent was obtained from all subjects after the nature and possible consequences of the studies were explained to them.

Euglycemic clamp tests. Using public transportation, participants arrived between 0545 (−230 min) and 0600 (−215 min) at the research facility and

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FFA, free fatty acid; G6P, glucose-6-phosphate; IMCL, intramyocellular lipid content; MRS, magnetic resonance spectroscopy.

remained in bed from then until 0700 (−155 min), when they were transported by wheelchair to the MRS unit. At 0630 (−185 min), catheters (Vasofix; Braun, Melsungen, Germany) were placed in antecubital veins of each arm for blood sampling and infusions, respectively. Starting at 0930 (−5 min), somatostatin was infused (0.1 $\mu g \cdot kg^{-1} \cdot min^{-1}$; UCB Pharma, Vienna, Austria) throughout the clamp tests. Conditions of fasting insulinemia and insulinemia in the high physiological range were mimicked during euglycemic clamp tests for 360 min, when insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was replaced from 0935 to 1235 (0–180 min) at a constant rate of 6 mU · m body surface $area^{-2} \cdot min^{-1}$ (low insulin) and from 1235 to 1535 (180–360 min) at a rate of 40 mU · m body surface $area^{-2} \cdot min^{-1}$ (high insulin). Plasma glucose concentration was maintained at ~5.6 mmol/l using a variable intravenous glucose infusion (20 g/dl) (20).

To examine the role of circulating lipids for skeletal muscle ATP synthesis, all participants were studied by a random cross-over design under two experimental conditions: *1*) insulin-suppressed FFA concentrations during normal saline infusion (0935–0945, 0–10 min: 10 ml/h; 0945–1535, 10–360 min: 90 ml/h) and *2*) elevation of plasma FFA concentrations to ~1 mmol/l corresponding to physiological concentrations observed during high-fat diet, moderate exercise, or to fasting plasma concentrations in obese nondiabetic and type 2 diabetic patients (12) induced by intravenous infusion of a triglyceride emulsion (0935–0945, 0–10 min: 10 ml/h; 0945–1535, 10–360 min: 90 ml/h; 20% Intralipid; Pharmacia and Upjohn, Vienna, Austria).

Experimental protocols were spaced by 4- to 8-week intervals during which the participants' body weights and lifestyle remained unchanged. Blood samples were drawn at timed intervals for measurement of hormone and metabolite concentrations.

MRS. MRS measurements were performed on a 3-T (80 cm bore) magnetic resonance spectrometer (Medspec S300-DBX; Bruker, Ettlingen, Germany). Subjects remained supine inside the nuclear magnetic resonance spectrometer with the right lower leg positioned so that the isocenter of the magnetic field was placed ~2 cm into the medial head of the gastrocnemius muscle. All measurements were performed using a 10-cm circular double resonant surface coil for $^{1}H/^{31}P$ (125.6/50.8 MHz).

^{31}P MRS. Skeletal muscle ATP synthase flux ($\mu mol \cdot g\ muscle^{-1} \cdot min^{-1}$) was determined in the gastrocnemius-soleus muscle complex by ^{31}P MRS using the saturation transfer experiment applied to the exchange between inorganic phosphate (P_i) and ATP (18,21–25). Unidirectional rates of ATP synthesis from P_i are the product of the rate constant of ATP synthesis and the concentration of intracellular P_i. The steady-state P_i magnetization, M_z, was measured with selective continuous wave irradiation of the γATP resonance and compared with the equilibrium P_i magnetization M_o with the selective irradiation placed symmetrically down field from the P_i frequency (T_R = 15 s, pulse length 150 μs, NS = 32, signal width = 10 kHz, 2 k data). The fractional reduction of P_i magnetization upon γATP saturation $(M_o - M_z)/M_o$ was measured from the spectra and was used in the calculation of the pseudo–first-order rate constant according to the equation of Forsen and Hoffman: $k_1 = [(M_o - M_z)/M_o] \times (1/T_1^*)$, where T_1^* is the spin lattice relaxation time for P_i when ATP is saturated. T_1^* was measured using an inversion recovery experiment ($\tau_1 - 180° - \tau_2 - 90° -$ acquire, T_R = 15 s, NS = 8, SW = 10 kHz, 2 k data) in the presence of a steady-state continuous wave saturation of γATP during the τ_1 and τ_2 interpulse delays. A B_1-insensitive pulse (WURST shape, duration 5 ms, bandwidth 3,700 Hz) was used for the inversion. The repetition time was set to 15 s in all ^{31}P experiments according to previously measured T_1 relaxation times of ^{31}P metabolites at 3-T (26). The eight variable τ_2 delay lengths used in the inversion recovery experiment ranged from 500 ms to 7 s, whereas the repetition time of the experiment was kept constant. T_1^* was evaluated using a nonlinear least-squares fitting method. Unidirectional flux of ATP synthesis was then calculated by multiplying the constant k_1 by the P_i concentration.

Baseline intramyocellular concentrations of G6P (μmol/l muscle) and P_i were assessed in the gastrocnemius-soleus muscle complex from the ratio of the integrated respective peak intensities and βATP resonance intensity in two sets (NS = 16) of spectra without inversion and saturation (pulse length 150 μs/90°, 2 k data, eight averages, repetition time of 15 s), assuming a constant ATP concentration of 5.5 mmol/l muscle (20,27). Changes of G6P from baseline values were determined from difference spectra (11,20). Average root mean-square noise measured from a 1-kHz bandwidth without any resonance gives a variation (1 SD) of G6P concentration of 26 μmol/l muscle.

^{31}P spectra were acquired at baseline from 0730 to 0930 (−125 to −5 min) and during the final 2 h of the high-insulin period of the clamp tests (from 1335 to 1525, 240–350 min), respectively.

^{1}H MRS measurements. IMCL in soleus muscle was determined by ^{1}H MRS as described (20). Scout images were acquired using the proton channel of the surface coil to position the cubic volume of interest within soleus muscle (1.73 cm^3), and the magnetic field was shimmed on the localized water signal (line width = 12–15 Hz). The stimulated echo acquisition mode sequence with chemical shift selective water suppression (echo time, 20 ms; repetition time, 4 s; 32 averages; 2,048 data points) was used. Spectra were line broadened and phase and baseline corrected, and the resonances of interest were quantified using a line-fitting procedure using the MacNuts PPC software package (AcornNMR, Livermore, CA). After T_2 relaxation correction (28), the IMCL was assessed from the intensity of the (CH_2) n = resonance (1.25 ppm), which was compared with the water resonance intensity obtained from spectra without water suppression (20).

^{1}H spectra were acquired at baseline from 0720 to 0730 (−135 to −125 min,) and at the end of the high-insulin period (from 1525 to 1535, 350–360 min) of the clamp tests, respectively.

Analytical procedures. Plasma glucose was measured by the glucose oxidase method (Glucose analyzer II; Beckman Instruments, Fullerton, CA). Plasma FFAs were assayed with a microfluorimetric method (Wako Chem USA, Richmond, VA). In vitro lipolysis was prevented by collecting blood into vials containing orlistat (20,29). Plasma triglycerides were hydrolyzed by lipase, and the released glycerol was measured by a peroxidase-coupled colorimetric assay (Roche, Vienna, Austria). Plasma insulin and glucagon were determined by double-antibody radio immunoassay (20). Plasma lactate and β-hydroxy-butyrate were determined using enzymatic methods (Roche) (20).

Data analysis and statistics. Statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA) or SPSS 11.0 software (SPSS, Chicago, IL). Data are presented as means ± SE. Statistical comparisons between measurements on CON and LIP study days were performed using two-tailed paired Student's *t* tests. Changes of sequential data within experiments were evaluated by repeated-measures one-way ANOVA with post hoc Tukey correction. Linear correlations are Pearson product-moment correlations. Differences were considered significant at the 5% level.

RESULTS

Hormones and metabolites. Mean plasma glucose concentrations were comparable between both study days during basal (CON vs. LIP: 4.9 ± 0.1 vs. 5.0 ± 0.1 mmol/l) and the high-insulin period of the clamp test (5.4 ± 0.1 vs. 5.5 ± 0.1 mmol/l). Plasma insulin concentrations (CON vs. LIP in pmol/l) were not different between the study days during fasting (CON: 44 ± 3 vs. 45 ± 5 pmol/l) and the high-insulin period (CON: 440 ± 25 vs. 429 ± 12 pmol/l). Fasting plasma FFA concentrations were similar on both study days (CON: 425 ± 87 vs. 501 ± 73 μmol/l). Lipid infusion resulted in higher plasma FFA concentrations during the high-insulin period of the clamp (CON: 12 ± 2 vs. 1,037 ± 29 μmol/l, $P < 0.001$). During fasting plasma, triglyceride concentrations were comparable on both study days (CON: 0.86 ± 0.09 vs. 0.96 ± 0.13). Plasma triglycerides decreased on CON days to 0.38 ± 0.03 mmol/l during the high-insulin period but increased on LIP days to 8.12 ± 1.05 mmol/l during the high-insulin period of the clamp tests (CON vs. LIP, $P < 0.0001$). Fasting plasma concentrations of C-peptide (CON: 0.48 ± 0.03 vs. 0.48 ± 0.04 nmol/l), glucagon (CON: 71 ± 6 vs. 73 ± 6 ng/l), lactate (CON: 0.8 ± 0.1 vs. 0.7 ± 0.1 mmol/l), and β-hydroxy-butyrate (CON: 122 ± 15 vs. 155 ± 26 μmol/l) did not differ between both study days. Somatostatin infusion effectively suppressed endogenous insulin and glucagon secretion as indicated by similar decreases in plasma concentrations of C-peptide (CON: 0.07 ± 0.02 vs. 0.09 ± 0.02 nmol/l) and glucagon (CON: 45 ± 5 vs. 47 ± 4 ng/l) during the high-insulin period of the clamps on both study days. Plasma lactate concentrations tended to be higher on CON days during the high-insulin period (CON: 1.5 ± 0.1 vs. 1.3 ± 0.2 mmol/l, $P = 0.09$). Plasma β-hydroxy-butyrate concentrations increased ~2.5-fold during the clamps on LIP days only (CON: 96 ± 0 vs. 349 ± 98 μmol/l, $P < 0.05$).

Glucose metabolism. Throughout the high-insulin period of the clamp tests, glucose infusion rates were ~46% lower on LIP days (mean 180–360 min: CON 51.9 ± 2.0 vs. LIP 27.9 ± 3.3 $\mu mol \cdot kg^{-1} \cdot min^{-1}$, $P < 0.0001$).

Intramyocellular concentrations of G6P did not differ at baseline between both study days (CON 137 ± 26 vs. LIP

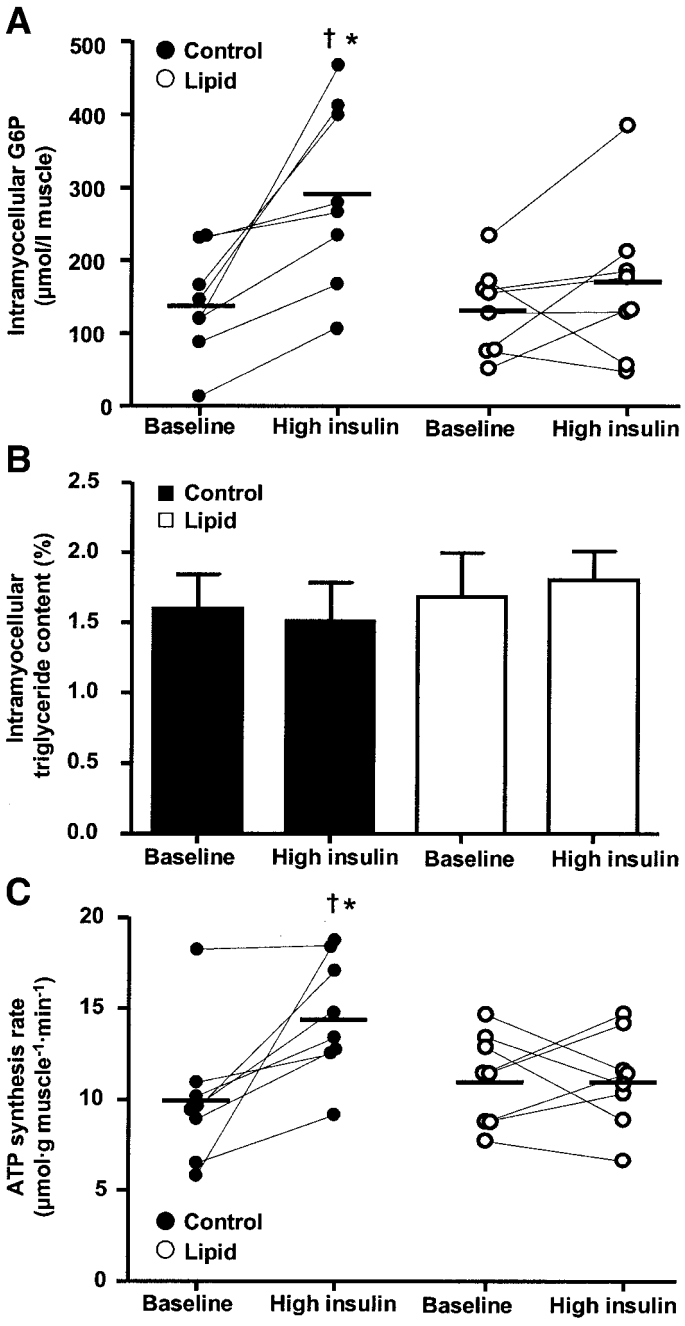

FIG. 1. Intramyocellular G6P concentrations (*A*), intramyocellular triglyceride content of soleus muscle (*B*), and flux through ATP synthase in skeletal muscle (*C*) under overnight-fasted resting conditions (baseline) and during the high-insulin period of the euglycemic clamp tests on control (Control, ■) and triglyceride emulsion infusion days (Lipid, □). *A* and *C*: Data are individual data points, and solid lines connect data points from each individual from baseline and during the high-insulin period on LIP and CON days, respectively. The thick horizontal lines represent the means. *B*: Data are means ± SE. **P* < 0.05 for LIP vs. CON; †*P* < 0.05 for LIP or CON vs. baseline.

131 ± 22 μmol/l muscle) (Fig. 1*A*). During the high-insulin period on CON days, G6P rose by ~112% ($P < 0.01$ vs. baseline) but remained unchanged (P = NS) from baseline concentrations on LIP days (Fig. 1*A*). Thus, G6P was ~41% lower during the high-insulin period on LIP days than on CON days (CON 291 ± 37 vs. LIP 173 ± 28 μmol/l muscle, $P < 0.05$).

IMCL. Soleus muscle IMCL was not different at baseline between both study days and remained unchanged from

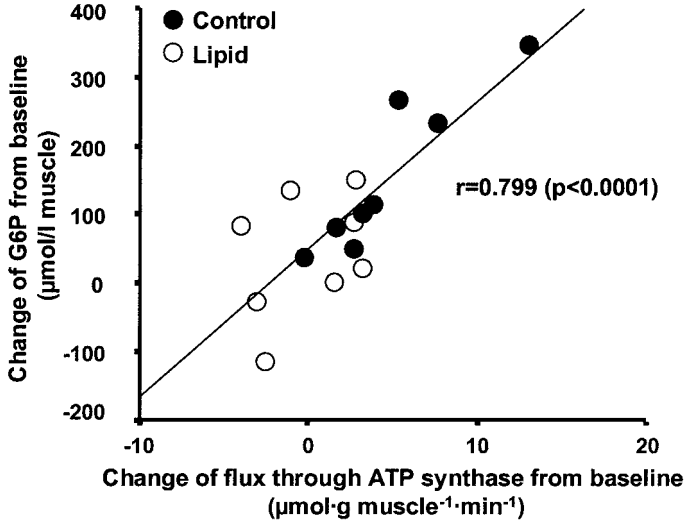

FIG. 2. Relationship between the changes of intramyocellular G6P concentrations and the changes of skeletal muscle ATP synthesis rates during the high-insulin period (180–360 min) of the euglycemic clamp tests with (Lipid, ○) and without (Control, ●) infusion of a triglyceride emulsion.

baseline during the high-dose insulin period of the clamp tests (CON 93 ± 3% vs. LIP 117 ± 12% of baseline, P = 0.073, Fig. 1*B*).

Skeletal muscle ATP synthase flux. At baseline, skeletal muscle ATP synthase flux was similar on both study days (Fig. 1*C*). During the high-insulin period, flux through ATP synthase increased by ~60% to 14.6 ± 1.2 μmol · g muscle^{-1} · min^{-1} on CON days (P = 0.02 vs. baseline) but remained unchanged from baseline at 11.0 ± 0.9 μmol · g muscle^{-1} · min^{-1} on LIP days. Thus, flux through ATP synthase was ~24% lower on LIP days ($P < 0.05$ vs. CON).

Relationship between ATP synthase flux and metabolic parameters. Flux through ATP synthase in skeletal muscle did not correlate with IMCL in soleus muscle, plasma insulin, or plasma FFA concentrations at any time on LIP or CON days. Skeletal muscle ATP synthase flux positively correlated with glucose infusion rates during the clamp tests (r = 0.496, $P < 0.005$). During the high-insulin period, changes from baseline in flux through ATP synthase and in G6P concentrations were positively correlated (r = 0.799, $P < 0.0001$) (Fig. 2).

DISCUSSION

These data show that short-term changes in plasma insulin and lipid concentrations within the physiological range affect ATP synthase flux in resting skeletal muscle. Under insulin-stimulated conditions, increased lipid concentrations reduce the increase in insulin-stimulated ATP synthase flux and impair glucose transport/phosphorylation.

Effect of insulin per se. During the insulin-somatostatin clamp, suppression of endogenous insulin secretion was comparable on both study days. Plasma glucagon concentration similarly decreased by ~36% on both study days, which is in line with previous somatostatin-insulin clamp studies (30,31). During the high-insulin period, increased glucose infusion rates almost exclusively result from an increase in skeletal muscle glucose uptake (31). This rise in glucose uptake/phosphorylation is mirrored by the observed ~2.1-fold increase of the intramyocellular G6P concentration (9,20). Under these conditions, ATP synthase flux increased by ~60%, most likely resulting from

insulin-stimulated energy-consuming and energy-generating processes. Such insulin-stimulated energy-consuming processes include glucose phosphorylation by hexokinase II, conversion of glucose-1-phosphate to uridine-diphosphate glucose for glycogen synthesis, and stimulation of protein synthesis (17,32), whereas energy-providing processes in skeletal muscle include glucose oxidation and glycolysis (33).

Of note, evidence has been provided that insulin upregulates mitochondrial-encoded gene expression, which also correlates with insulin-stimulated skeletal muscle glucose uptake (34). Stump et al. (17) assessed protein synthesis, enzyme activities, and the in vitro capacity of ATP production in mitochondria obtained from vastus lateralis muscle biopsies after low- and high-dose insulin infusion. Similar to the present results on in vivo ATP synthase flux, high-degree insulinemia increased mitochondrial capacity of the ATP production, protein synthesis, and activities of tricarboxylic acid cycle and electron transport chain enzymes (17). These data suggest that the increase in flux through ATP synthase as measured with ^{31}P MRS most likely results from augmented oxidative phosphorylation under hyperinsulinemic conditions.

Effects of lipid elevation. Elevation of plasma FFA concentrations is known to induce skeletal muscle insulin resistance by reduction of glucose transport/phosphorylation, glycogen synthesis, and glucose oxidation with a time delay of up to 180 min (7–11). These effects are currently attributed to a rise in intramyocellular concentrations of fatty acid metabolites and subsequent interference with various steps of insulin signaling (10,12,13). The present study found that lipid infusion impaired glucose transport/phosophorylation, as indicated by lower intramyocellular G6P concentration in parallel with reduced increase in flux through ATP synthase.

The metabolic processes contributing to these effects of increased lipid availability during hyperinsulinemia include *1*) impairment of insulin signaling, *2*) activation of the mTOR/S6 kinase nutrient sensing pathway, *3*) reduced stimulation of energy-consuming or energy-generating processes, and *4*) direct alteration of mitochondrial functions such as uncoupling reactions.

First, FFA-induced insulin resistance is associated with a rise in intramyocellular long-chain fatty acyl-CoA, diacylglycerol, and ceramide (13) and impaired insulin signaling (10). Increased lipid availability can result in activation of signal transduction pathways including atypical protein kinase C isoforms (35), IκB kinase/nuclear factor κB system (13), and c-Jun N-terminal kinase (36), which lead to serine/threonine phosphorylation of insulin signaling proteins and finally inhibition of glucose transport activity. Thus, it seems feasible that such lipid-induced impairment of insulin signaling (10,13) could jeopardize insulin-dependent mitochondrial processes and thereby reduce flux through ATP synthase.

Second, experimental plasma FFA elevation for ~48 h in nondiabetic humans not only induces skeletal muscle insulin resistance but also leads to decreased mRNA expression of nuclear encoded mitochondrial genes and peroxisome proliferator–activated receptor-γ cofactor 1 (37). Peroxisome proliferator–activated receptor-γ cofactor 1 can be activated by nutrients via the mTOR/S6 kinase system and serves as a transcriptional coactivator driving the expression of many genes involved in energy metabolism (38). Activation of this pathway could either directly impair mitochondrial function and energy expenditure or interfere with insulin signaling. Of note, these alterations of skeletal muscle gene expression are similar to those observed in skeletal muscle of insulin-resistant humans (39,40).

Third, increased lipid availability ultimately leads to a reduction in insulin-stimulated skeletal muscle glucose transport/phosphorylation and glycogen synthesis (9–11). As these processes consume energy exceeding the energy demand of resting muscle during fasting, any inhibition of these processes likely contributes to lower energy demand during combined hyperlipidemia and hyperinsulinemia. Supporting this contention, this study also found a strong correlation between the changes of intramyocellular G6P and flux through ATP synthase, suggesting a causal relationship between insulin-stimulated glucose disposal and ATP synthase flux. However, in the absence of simultaneous measurement of metabolic fluxes, insulin signaling, and gene expression, the present results do not allow to draw the conclusion that lipid-mediated impairment of insulin signaling is responsible for the blunted flux through ATP synthase.

Finally, FFAs act as mild uncouplers of respiration from ATP synthesis in vitro (41), which may also apply to in vivo conditions. Even smaller increases in plasma FFAs than achieved in the present study resulted in augmented expression of uncoupling protein-3, but not uncoupling protein-2 in skeletal muscle of healthy nondiabetic humans (42). This effect was observed after prolonged lipid infusion and abolished by concomitant hyperinsulinemia, suggesting that alterations in ucoupling protein expression unlikely explain the effects of the present study.

One limitation must be considered in the interpretation of the results of the present study. Flux through ATP synthase and G6P concentrations were measured in the gastrocnemius-soleus muscle complex, whereas IMCL were quantified in the soleus muscle only. Soleus muscle contains two- to threefold amounts of IMCL compared with gastrocnemius muscle, which contains relatively more of fast-twitch fibers (type II muscle fibers) (43). The differences in fiber composition and IMCL might result in variable responses to metabolic conditions in these muscles (5,6,44,45). Thus, the absence of an effect of lipid infusion on IMCL in soleus muscle in the present study does not exclude the possibility of IMCL changes in gastrocnemius muscle, which could have escaped detection in our experimental design.

Taken together, in insulin-sensitive humans short-term elevation of lipid availability reduces insulin-stimulated increase of ATP synthase flux in skeletal muscle. This is line with the impairment of flux through ATP synthase reported for insulin-resistant humans under basal (18,25) and insulin-stimulated conditions (16,17).

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