Effects of Acute Changes of Plasma Free Fatty Acids on Intramyocellular Fat Content and Insulin Resistance in Healthy Subjects

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The reason for the 3- to 4-h delay between a rise in plasma free fatty acid (FFA) levels and the development of insulin resistance remains unknown. In the current study, we have tested the hypothesis that the delay may be caused by the need for plasma FFAs to first enter muscle cells and to be re-esterified there before causing insulin resistance. To this end, we have determined intramyocellular triglyceride (IMCL-TG) content with proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy in healthy volunteers before and 4 h after lowering of plasma FFAs (with euglycemic-hyperinsulinemic clamping) or after increasing plasma FFAs (with lipid plus heparin infusions). Increasing plasma FFAs (from 516 to 1,207 μmol/l or from 464 to 1,857 μmol/l, respectively) was associated with acute increases in IMCL-TG from 100 to 109 ± 5% ($P < 0.05$) or to 133 ± 11% ($P < 0.01$), respectively, and with a significant increase in insulin resistance ($P < 0.05$ after 3.5 h). Lowering of plasma FFAs from 560 to 41 μmol/l was associated with a tendency for IMCL-TG to decrease (from 100 to 95 ± 3%). Changes in plasma FFAs correlated linearly with IMCL-TG ($r = 0.74$, $P < 0.003$). The demonstration that acute changes in plasma FFAs were accompanied by corresponding changes in IMCL-TG and with the development of insulin resistance, taken together with previous reports of a close correlation between IMCL-TG and insulin resistance, supported the notion that accumulation of IMCL-TG is a step in the development of FFA-induced insulin resistance. *Diabetes* 50:1612–1617, 2001

Obesity, which has reached epidemic proportions in the U.S. (1), is associated with insulin resistance (2), a well-established risk factor not only for type 2 diabetes but also for atherosclerotic vascular disease (3). Plasma free fatty acids (FFAs), which are generally elevated in obese subjects (4,5), have been demonstrated to be a major link between obesity and insulin resistance (rev. in 6). Thus, lowering plasma FFAs decreases insulin resistance (7), whereas raising plasma FFAs increases insulin resistance (after a delay of 3–4 h) in nondiabetic and diabetic subjects (8–10). The mechanism by which FFAs cause insulin resistance, however, is not completely understood. The long (3–4 h) delay between the rise in plasma FFAs and the onset of inhibition of insulin-stimulated glucose uptake makes a direct effect of FFAs on insulin action unlikely. On the other hand, an explanation for the delayed onset of insulin resistance may be that FFAs need to accumulate first as triglycerides (TGs) inside muscle fibers. In support of this notion, several studies in animals and humans have demonstrated a close relationship between muscle fat content and insulin resistance (11–16). Most of these studies, however, were unable to differentiate between extramyocellular (EMCL) fat, i.e., fat located in adipocytes between muscle fibers, and intramyocellular (IMCL) fat, i.e., fat located within muscle fibers. It has recently been shown that IMCL fat content can be quantitated with proton-nuclear magnetic resonance (^{1}H-NMR) spectroscopy (17,18). In this study, we have used ^{1}H-NMR spectroscopy to test the hypothesis that FFA-induced inhibition of insulin-stimulated glucose uptake is accompanied by IMCL accumulation of fat by assessing effects of acutely raising of plasma FFA levels on insulin resistance and on IMCL-TG content in soleus muscles of young healthy volunteers.

RESEARCH DESIGN AND METHODS

Subjects. A total of 15 healthy young volunteers (8 males and 7 females) participated in three different studies. One subject participated in all three studies; 4 participated in two studies (3 in studies 1 and 2 and 1 in studies 2 and 3); 10 participated in one study (3 in study 1, 2 in study 2, and 5 in study 3). The subjects' ages, weights, heights, and body compositions are shown in Table 1. None of the subjects had a family history of diabetes or any other endocrine disorder, and none was taking any medication. Their weights were stable for at least 2 months, and their diets contained a minimum of 250 g/day of carbohydrate for at least 2 days before the studies. Informed written consent was obtained from each participant after explanation of the nature, purpose, and potential risks of these studies. The study protocol was approved by the Institutional Review Board of Temple University Hospital. Subjects were admitted to Temple University Hospital's General Clinical Research Center on the evening before the studies. The studies began at ~8:00 A.M after an overnight fast with the subjects reclining in bed. A short polyethylene catheter was inserted into an antecubital vein for infusion of test substances. Another catheter was placed into a contralateral forearm vein for blood sampling. This arm was wrapped with a heating blanket (~70°C) to arterialize venous blood.

Study design. Study 1 was a 4-h euglycemic-hyperinsulinemic clamp. Plasma FFAs decreased to very low levels because of insulin-induced inhibition of

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ANOVA, analysis of variance; EMCL, extramyocellular; FFA, free fatty acid; fov, field of vision; GIR, glucose infusion rate; ^{1}H-NMR, proton nuclear magnetic resonance; IMCL, intramyocellular; IRS-1, insulin receptor substrate-1; LC-CoA, long-chain acyl-CoA; MRI, magnetic resonance imaging; npRQ, nonprotein respiratory quotient; PKC, protein kinase C; TE, echo time; TG, triglyceride; TR, repetition time.

TABLE 1
Study subjects

	Insulin	Insulin + lipid	Lipid
Sex (M/F)	4/3	3/4	4/3
Age (years)	25.7 ± 1.1	24.4 ± 0.3	25.9 ± 1.9
Height (cm)	171.7 ± 4.8	169.3 ± 3.9	174.3 ± 3.0
Weight (kg)	70.1 ± 5.0	67.3 ± 4.2	71.3 ± 4.3
Body fat (%)	23.8 ± 1.3	25.0 ± 3.0	ND
BMI (kg/m^2)	23.6 ± 0.9	23.7 ± 1.0	23.3 ± 0.7

Data are means ± SD. ND, not determined.

lipolysis. ^{1}H-NMR spectroscopy was performed within 15 min before and after the clamps.

Study 2 was a 4-h euglycemic-hyperinsulinemic clamp with simultaneous IV infusion of lipid plus heparin. Plasma FFA levels rose because heparin-mediated lipolysis from the infused fat exceeded insulin-mediated antilipolysis in adipose tissue. ^{1}H-NMR spectroscopy was performed as in study 1.

Study 3 was a 4-h infusion of lipid plus heparin (as in study 2) without infusion of insulin. Plasma FFA levels rose to higher levels than those in study 2, presumably because FFAs released from adipose tissue, which continued uninhibited, were added to FFAs released from the infused fat. ^{1}H-NMR spectroscopy was performed as in studies 1 and 2.

Studies were performed in random order separated by 1–2 months.

Euglycemic-hyperinsulinemic clamping with and without lipid/heparin. Regular human insulin (Humulin R; Eli Lilly, Indianapolis, IN) was infused intravenously at a rate of 7 pmol/kg per min for 4 h, and plasma glucose concentrations were clamped at ~5 mmol/l by a feedback-controlled glucose infusion (study 1).

In study 2, euglycemic-hyperinsulinemic clamping was performed as described above. In addition, Liposyn II (Abbott Laboratories, North Chicago, IL), a 20% triglyceride emulsion (10% safflower and 10% soybean oil), plus heparin (0.4 U/kg per min) were infused at a rate of 1.5 ml/min for 4 h.

^{1}H-NMR spectroscopy

Anatomical imaging. Subjects were brought to the Temple University Hospital Outpatient Imaging Center and placed in the magnetic resonance imaging (MRI) scanner (Signa 1.5T; GE Medical Systems, Waukashsaw, WI) in the supine position. Each subject's calf was placed on padding in a transmit/receive extremity coil. The calf muscle was centered in the anterior-posterior and left-right directions. The superior-inferior center was chosen at the largest diameter of the calf. Care was taken to ensure that the calf was not angled in the coil to avoid the magic angle variation in the IMCL-TG peak (17). The calf was marked with ink at the position of the aligning crosshairs from the scanner to ensure that the repeat scan was performed in the same position. Anatomical axial T1-weighted spin-echo images (repetition time [TR] = 400 ms, echo time [TE] = 10 ms, field of view [FOV] = 16 cm, 256×256) were acquired with high contrast between muscle tissue and the hypointense facial planes. The soleus muscle was easily distinguished on these images.

NMR spectrum acquisition. The voxel for NMR spectrum acquisition was placed in the slice that showed the largest diameter of the soleus muscle; typically this slice was at isocenter. The voxel was either ~8 or 1 cm^3, depending on the homogeneity of the tissue in the soleus muscle. Voxels were placed in homogeneous muscle tissue, avoiding vessels and facial planes. The 3D reformatting capabilities at the scanner console were used to check for tissue homogeneity in all three planes. The spectra were acquired using a standard clinical PROBE pulse sequence with water suppression (TR = 1,174 ms, TE = 35 ms, number of excitations = 8, scantime = 1:47, FOV = 16 cm, 1,024 spectral points).

Data analysis. The SAGE spectroscopy software package (GE Medical Systems, Fremont, CA) was used to analyze the spectroscopy data. Raw data files were reconstructed using the PROBE quantification algorithm. The following steps were performed: pure water subtraction, convolution filtering, apodization, zero-filling, and fast Fourier transformation. After reconstruction, the resulting spectra were phase-corrected with a zero-order phase correction and baseline was subtracted. For baseline subtraction, points were chosen at 0 and 8 ppm and a spline was calculated and subtracted.

Peak areas were measured using 0.1 ppm widths centered on the following peaks in the water-suppressed spectrum: creatine, EMCL methylene, and IMCL methylene peaks. A width of 0.1 ppm was chosen to measure the ratio of the peak areas pre- and postinfusion because the spectral resolution using wider areas was inadequate to resolve the methyl and methylene EMCL and IMCL peaks in some subjects. Normalization of spectra taken at pre- and postinfusion studies was carried out by using the ratio of the creatine peak at 3.2 ppm as an internal standard because that peak has been shown to be stable over time (17). At least 94% of the water signal was suppressed in all subjects and full-width half-maximum spectra of the water peaks were equal to or less than 11 Hz. This permitted resolution of the methylene EMCL peaks at 1.6 ppm and the IMCL peaks at 1.3 ppm in all subjects.

Body composition. Body composition was determined by bioimpedance analysis (19).

Glucose infusion rates. The rate of glucose infusion needed to maintain euglycemia during hyperinsulinemia was used as an estimate of insulin-stimulated total-body glucose uptake. As endogenous glucose production is suppressed by only 80–85% with the insulin infusion rates used (7 pmol/kg per min), the glucose infusion rate (GIR) may have underestimated true glucose uptake rates by ≤20%.

Indirect calorimetry. Respiratory gas exchange rates were determined with a metabolic measurement cart (Deltatrac II; Sensor Medics, Yorba Linda, CA), as previously described (20), before and at 30-min intervals during the clamps. Rates of protein oxidation were estimated from urinary nitrogen excretion after correction for changes in urea nitrogen pool size (21). Rates of protein oxidation were used to determine the nonprotein respiratory quotient (npRQ). Rates of carbohydrate oxidation were determined with the tables of Lusk, which are based on an npRQ of 0.707 for 100% fat oxidation and 1.00 for 100% carbohydrate oxidation.

Analytical procedures. Plasma glucose was measured with a glucose analyzer (YSI, Yellow Springs, OH). Serum insulin was determined by radioimmunoassay with a specific antibody that crossreacts only minimally (<0.2%) with proinsulin (Linco, St. Charles, MO). Total plasma FFAs were determined enzymatically in plasma containing EDTA and the lipoprotein lipase inhibitor Paroxam (0.275 mg/ml blood; Sigma, St. Louis, MO) with a kit from Wako (Richmond, VA).

Statistical analysis. All data are expressed as the means ± SE. Statistical significance was assessed using analysis of variance (ANOVA) with repeated measures and paired Student's two-tailed t test when applicable.

RESULTS

Plasma glucose, insulin, and FFAs. Basal glucose concentrations were 5.2 ± 0.2, 5.1 ± 0.1, and 4.5 ± 0.1 mmol/l in the insulin only, the insulin plus lipid, and the lipid only groups, respectively. Mean clamp glucose concentrations were 5.2 ± 0.2, 5.4 ± 0.2, and 4.5 ± 0.2 mmol/l, respectively, in the three groups. The differences were not statistically significant (Fig. 1).

Basal insulin levels were 42 ± 4, 44 ± 6, and 58 ± 5 pmol/l in the insulin only, the insulin plus lipid, and the lipid only groups, respectively. Mean insulin levels during the clamps were 421 ± 4, 472 ± 21, and 60 ± 5 pmol/l, respectively.

In the insulin only group, plasma FFA concentrations decreased from 548 ± 65 (at 0 min) to 42 ± 10 μmol/l (at 240 min) ($P < 0.001$). In the insulin plus lipid group, plasma FFA rose from 516 ± 75 (at 0 min) to 1,206 ± 142 μmol/l (at 240 min). In the lipid only group, plasma FFAs increased from 464 ± 108 to 1,893 ± 242 μmol/l (at 240 min).

GIR and fat oxidation. GIR rose from 0 (at 0 min) to 48.7 ± 5 μmol/kg per min (at 240 min) in the insulin only group and from 0 to 27.8 ± 2.1 μmol/kg per min in the insulin plus lipid group. The difference between the two groups became significant at 210 min ($P < 0.05$) and was 43% ($P < 0.01$) at 240 min. No glucose needed to be infused to maintain euglycemia in the lipid only group (Fig. 2).

In the insulin only group, fat oxidation decreased from 0.87 ± 0.14 μmol/kg per min at 0 min to 0.26 ± 0.10 μmol/kg per min after 4 h of hyperinsulinemia (−70%, $P < 0.001$).

In the insulin plus lipid group, rates of fat oxidation remained essentially unchanged throughout the 4-h experimental period (0.88 ± 0.10 μmol/kg per min at 0 min vs. 0.97 ± 0.19 μmol/kg per min at 240 min, NS). Fat oxidation was significantly higher in the insulin plus lipid infused

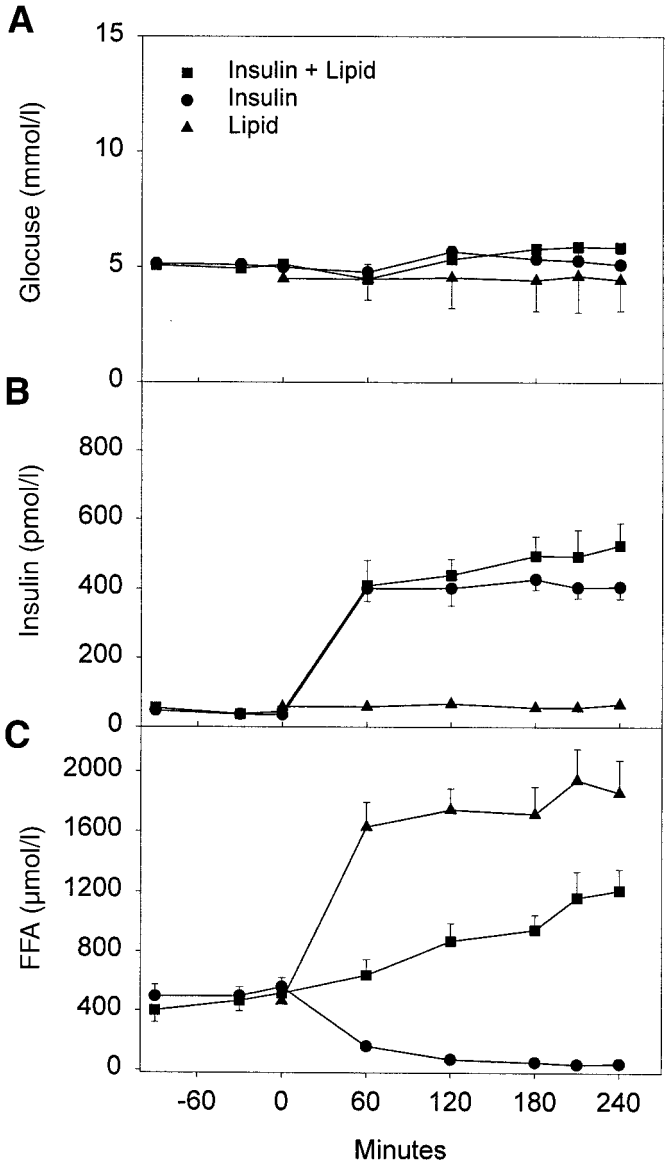

FIG. 1. Plasma concentrations of glucose (*A*), insulin (*B*), and FFAs (*C*) during euglycemic-hyperinsulinemic clamping (insulin), euglycemic-hyperinsulinemic clamping plus lipid/heparin infusion (insulin plus lipid), and during lipid/heparin infusion without insulin (lipid). Data are means ± SE.

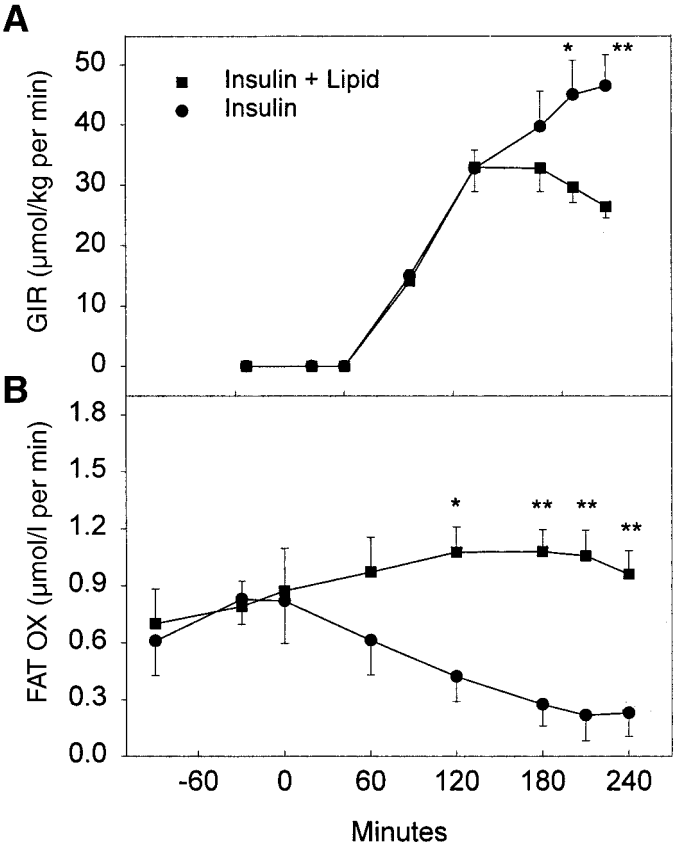

FIG. 2. GIRs needed to maintain euglycemia (*A*) and fat oxidation rates (FAT OX) (*B*) during euglycemic-hyperinsulinemic clamping with infusion of lipid/heparin (insulin plus lipid) and without lipid infusion (insulin). Data are means ± SE. **P* < 0.05; ***P* < 0.01, insulin vs. insulin plus lipid.

versus the insulin alone group starting at 180 min ($P < 0.01$) (Fig. 2). Fat oxidation was not determined in the lipid only group.

Changes in IMCL-TG and plasma FFAs. In the insulin only group, hyperinsulinemic-euglycemic clamping for 4 h tended to decrease IMCL-TG (100 ± 0 vs. 95 ± 3%, NS). In the insulin plus lipid group, IMCL-TG rose from 100 ± 0 to 109 ± 5% ($P < 0.04$). Individually, IMCL-TG rose in six of seven subjects (range 5–38%). In the lipid only group, IMCL-TG rose from 100 ± 0 to 133 ± 11% ($P < 0.02$). Individually, IMCL-TG rose in six of seven subjects (range 5–50%) (Fig. 3).

These increases in IMCL-TG content were associated with similar increases in plasma FFA levels (during the preceding 3 h), whereas decreasing IMCL-TG content was associated with decreasing plasma FFA levels (Fig. 3*B*). This resulted in a highly significant and close linear correlation between plasma FFA levels and IMCL-TG content ($r = 0.74$, $P < 0.0003$) (Fig. 4).

EMCL-TG. There were no significant changes in EMCL-TG in response to insulin alone (100 ± 0 vs. 1.22 ± 34%), to insulin plus lipid (109 ± 43%), or to lipid alone (146 ± 23%).

DISCUSSION

It was the objective of this study to test whether changes in insulin resistance produced by acute changes in plasma FFA levels involved corresponding changes in IMCL-TG. We found that IMCL-TG changed acutely, in what appeared to be a dose-dependent way, with changes in plasma FFA concentration ($r = 0.74$). Increases in plasma FFA levels of ~700 and ~1,400 μmol/l, were accompanied by increases in IMCL-TG of 9% ($P < 0.04$) and 33% ($P < 0.02$), respectively, over the 4-h study period. A decrease in plasma FFAs by ~500 μmol/l was associated with a 5% decrease in IMCL-TG, which, however, was not statistically significant. There may be several reasons why it was easier to demonstrate an increase, rather than a decrease, in IMCL-TG. First, the increases in plasma FFA levels were larger than the decrease (+700 and +1,400 vs. −500 μmol/l). Second, it is possible, in fact it is likely, that a decrease in IMCL-TG takes more time than an increase because the decrease depends on the rate of TG consumption in muscle, which, in resting muscle, is probably quite low.

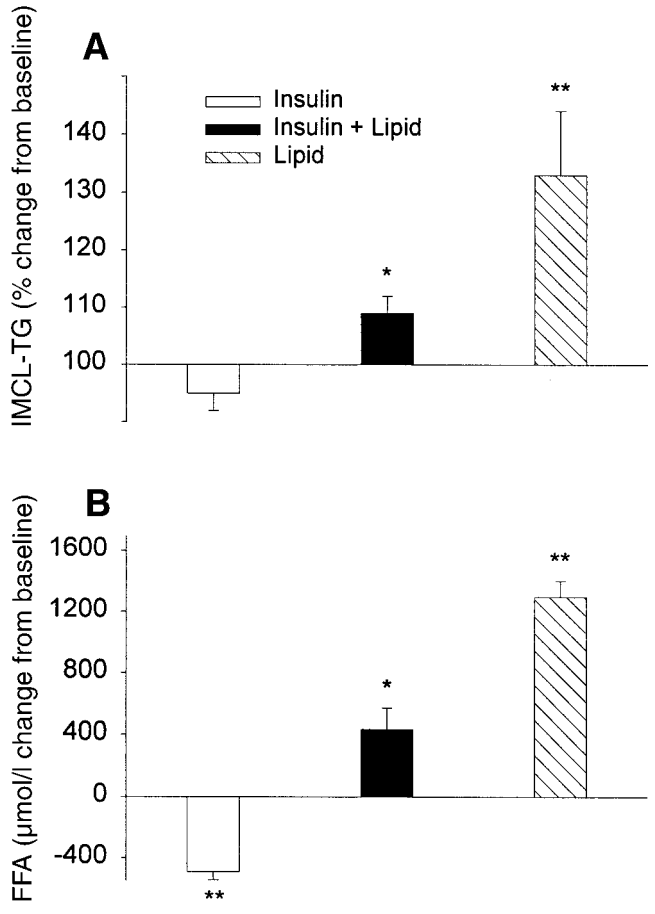

FIG. 3. Changes from baseline of IMCL-TG (*A*) and plasma FFA (*B*) concentrations after 4 h of euglycemic-hyperinsulinemic clamping without lipid/heparin infusion (insulin) and with lipid/heparin infusion (insulin plus lipid) and with lipid/heparin infusion alone (lipid). IMCL-TG was determined immediately before and after the 4-h studies. FFA values represent the mean FFA levels between 60 and 240 min. IMCL-TG: **P* < 0.05; ***P* < 0.02. FFA: **P* < 0.02; ***P* < 0.0001 vs. baseline.

It is worth noting that the increase in IMCL-TG in response to a rise in plasma FFAs (during lipid/heparin plus insulin infusions) was associated with a ~40% increase in whole-body insulin resistance, confirming previous reports (8–10). On the other hand, it remains to be shown whether a decrease in IMCL-TG is associated with a decrease in insulin resistance.

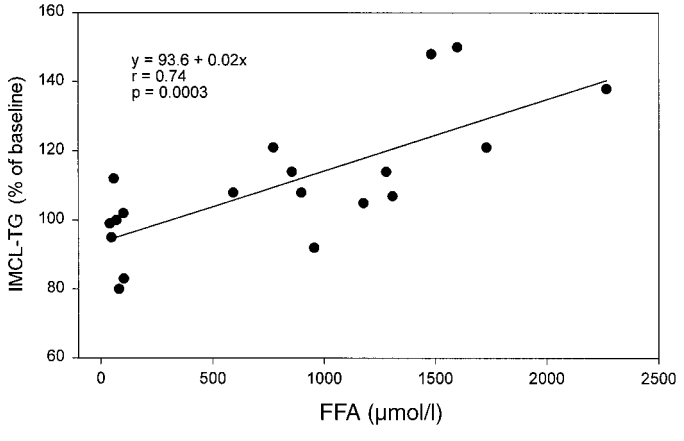

FIG. 4. Correlation between IMCL-TG (expressed as percentage of baseline) and plasma FFA levels (mean of FFA levels between 60 and 240 min) of the insulin alone, lipid alone, and insulin plus lipid/heparin infusion studies.

The validity of the IMCL-TG measurements is supported by evidence demonstrating that the proton resonances at ~1.3 ppm (used here to represent IMCL-TG) are produced by methylene groups of acyl chains of IMCL-TG (17,18). This evidence includes the demonstration that tissues that did not have adipocytes, including liver and heart muscle, had single resonances at 1.3 ppm (22–24), whereas adipose tissue had single resonances at ~1.6 ppm (17,18,24). Further support that the proton resonances at 1.3 ppm represented IMCL-TG was provided by findings that *1*) the resonances at 1.3 ppm correlated with markers of muscle mass (for instance, creatine), whereas the resonances at 1.6 ppm did not (17); *2*) the resonances at 1.3 ppm decreased after exercise and recovered after resting and after eating (17); and *3*) patients with generalized lipodystrophy, a disorder characterized by almost complete absence of adipose tissue, had abundant signals at 1.3 but none at 1.6 ppm (18). It also needs to be pointed out that the methylene groups of circulating TGs or FFAs do not contribute to the generation of the IMCL-TG resonances, as only stationery tissues or fluid is detected by ^{1}H-NMR spectroscopy (25).

The acute changes in IMCL-TG observed here were compatible with results of two previous studies in which FFA turnover was determined (26,27). In one study, the rate of FFA release after 4 h of hyperinsulinemia (comparable with the insulin only group in the current study), was 1.6 μmol/kg per min, and the rate of FFA oxidation was 1.7 μmol/kg per min; hence, the rate of FFA re-esterification was slightly negative (26). This was entirely compatible with the trend in the current study toward a decrease in IMCL-TG. In a second study, when lipid/heparin was infused (comparable with the insulin plus lipid group in the current study), the rate of FFAs released by lipolysis from the infused lipid was ~17 μmol/kg per min, while FFA oxidation was ~3 μmol/kg per min; hence, the rate of whole-body FFA re-esterification was ~14 μmol/kg per min (27), which is compatible with the 9% increase in IMCL-TG observed in the current study.

The demonstration that the IMCL-TG content and the level of insulin resistance were both regulated by the level of plasma FFAs does not alone prove that there was a cause-and-effect relationship between changes in IMCL-TG and changes in insulin resistance. Nevertheless, the close association between IMCL-TG and insulin resistance, reported in several recent studies (15,28,29), supported the hypothesis that plasma FFA levels first needed to enter muscle cells and to be re-esterified to IMCL-TG before they caused insulin resistance. This may also explain the 3- to 4-h delay in the onset of FFA-mediated insulin resistance (4,8,9). Presumably, IMCL-TG started to increase at the beginning of the insulin plus fat infusions. The rather sudden development of insulin resistance after ~2 h, therefore, suggested that IMCL-TG (or unidentified factors associated with IMCL-TG) may need to reach certain threshold concentrations before they can interfere with insulin action.

IMCL-TG accumulation may not, however, be the only mechanism by which FFAs cause insulin resistance. For

instance, it seems quite possible that lipid/heparin infusion could also induce changes in fatty acid composition in muscle membrane phospholipids, which could then lead to changes in membrane fluidity and insulin resistance (30).

How could IMCL-TG cause insulin resistance? IMCL-TG has been shown to be a metabolically active pool of fat (31–33), consisting of small oil droplets that are located in close proximity to mitochondria, thereby providing fuel for oxidation (34,35). There is, however, good evidence indicating that changes in fat oxidation are not the immediate reason for the development of insulin resistance. As shown before (8,9) and again in this study, lipid/heparin infusion inhibited fat oxidation long before it inhibited insulin-stimulated glucose uptake. This makes it unlikely that the decrease in fat oxidation was directly responsible for the insulin resistance, as originally postulated by Randle et al. (36). In addition, Han et al. (37) have shown that insulin resistance produced by high-fat feeding in rats was unrelated to changes in fat oxidation. A more likely cause for insulin resistance may have been an increase in cytosolic long-chain acyl-CoA (LC-CoA) concentration associated with the synthesis or the lipolysis of IMCL-TG. LC-CoA is known to increase diacylglycerol and protein kinase C (PKC). The latter can inhibit insulin action via serine-threonine phosphorylation of insulin receptor substrate-1 (IRS-1) (38). In support of this notion, muscle concentration of LC-CoA have been reported to correlate well with insulin resistance and to be increased in the high-fat feeding rat model (39). Moreover, Griffin et al. (40) have recently shown blunting of insulin-stimulated IRS-1 tyrosine phosphorylation and a fourfold increase in inactive (membrane-bound) PKC in lipid/heparin–infused rats.

In summary, we have previously demonstrated that acute increases of plasma FFAs cause insulin resistance with a 3- to 4-h delay (8,9,41). Here, we show that IMCL-TG content and insulin resistance change together within 4 h in response to changes in plasma FFA concentrations. Taken together with studies demonstrating that IMCL-TG content correlated well with insulin resistance, our findings support the claim that an increase in IMCL-TG content comprises a step in the FFA-induced development of insulin resistance.

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