Discordant effects of a chronic physiological increase in plasma FFA on insulin signaling in healthy subjects with or without a family history of type 2 diabetes

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insulin resistance; free fatty acids; insulin signal transduction

Type 2 diabetes mellitus (T2DM) is an insulin-resistant state characterized by impaired insulin action in muscle, liver, and fat (19). Adipose tissue insulin resistance is manifested by an excessive rate of lipolysis, and the resultant chronic increase in the plasma free fatty acid (FFA) level is believed to play an important role in the development of T2DM (7, 19, 43). “Lipotoxicity” refers to tissue disease (i.e., skeletal muscle insulin resistance) that may occur when fatty acid spillover in excess of the oxidative needs of those tissues enhances the flux of lipid into harmful pathways of nonoxidative metabolism (62). Intramyocellular lipid (IMCL) accumulation has been associated with decreased insulin sensitivity (33, 37, 47, 51, 60) and impaired insulin signaling (64) in nondiabetic subjects. In these studies adipose tissue insulin resistance was evident by an increased (33, 37) or “normal” (i.e., inadequate suppression) (60, 64) plasma FFA concentration despite marked hyperinsulinemia.

Our group (13, 24) and others (4, 10, 11, 21, 40, 42, 54–56, 63, 67) have shown that an acute (2- to 6-h) pharmacological elevation in plasma FFA concentration induces insulin resistance in healthy individuals. In these early studies, plasma FFA were increased 5- to 10-fold above fasting to levels ranging from ~1,800 to ~4,000 μmol/l. Furthermore, in already insulin-resistant T2DM subjects, we (3) and others (6, 8) have reported that insulin action may be further impaired by a supraphysiologic increase in plasma FFA concentration (~1,500–2,500 μmol/l). Taken together, these studies (3, 4, 6, 8, 11, 13, 21, 24, 40, 42, 54–56, 63, 67) have served as “proof-of-principle” for the cross talk between glucose and lipid metabolism in skeletal muscle. However, their clinical relevance to insulin resistance in humans requires further study, because the plasma FFA concentration under normal living conditions is rarely so high, usually being in lean healthy subjects in the range of ~300–400 μmol/l (4, 11, 13, 21, 24, 40, 42, 54–56, 63, 67) and ~500–800 μmol/l in insulin-resistant states such as obesity or T2DM (3, 6–8, 19, 43).

A more complete picture may emerge for the role of lipotoxicity in human disease by increasing the plasma FFA level within the physiological range (~500–800 μmol/l) and by infusing lipid for a longer period of time (i.e., ~90 h rather than 4–6 h). The few studies that have raised plasma FFA within the physiological range (9, 35, 38) have reported a decrease in insulin action and glucose oxidation, with glycogen synthase (GS) activity (a rather distal step of...
insulin signaling) being either unaffected (35) or slightly impaired (9, 38) by an acute physiological lipid infusion. It remains unclear whether the FFA-induced defect in GS activity (9, 38) was due to a direct effect of FFA on the enzyme or associated with abnormalities at earlier steps of the insulin-signaling pathway. A number of recent novel mechanisms have been proposed to explain FFA-induced insulin resistance beyond the glucose-lipid substrate competition hypothesis originally proposed by Randle et al. (53). A plasma FFA of ~3,000 μmol/l for 5–6 h may cause insulin resistance by altering the glucose transport/phosphorylation system (21, 54, 55). Insulin receptor substrate (IRS)-1-associated phosphatidylinositol (PI) 3-kinase activity is impaired when the plasma FFA concentration is increased to supraphysiological levels by three- to sixfold (21, 41), and intramyocellular lipid (IMCL) accumulates rapidly after a 5- to 6-h lipid infusion (plasma FFA ~3,000 μmol/l) (2, 14). Marked storage of IMCL may be associated with accumulation of intracellular signaling molecules [i.e., ceramide, diacylglycerol, protein kinase C with activation of the Ikβ-kinase (1KK-β)/Ikβ/nuclear factor (NF)-κβ pathway (16, 27, 32, 48)] that may feed back to inhibit PI 3-kinase activity associated with IRS-1. However, no study has examined early insulin-signaling steps in skeletal muscle in humans during a physiological increase in plasma FFA concentration.

We (28, 36, 52) and others (22, 49, 66) have demonstrated that normal individuals with a strong family history of T2DM (FH+) are insulin resistant and have impaired insulin-stimulated IRS-1 tyrosine phosphorylation and PI 3-kinase activity associated with IRS-1 (52). FH+ subjects frequently have an increased plasma FFA concentration and/or a blunted suppression of lipolysis in response to chronic endogenous hyperinsulinemia or to an exogenous insulin infusion (28, 36, 49, 52). Therefore, one could argue that, in such genetically predisposed individuals, lipotoxicity (62) already is maximally or near maximally established and that further elevation in the plasma FFA concentration would have a blunted impact on insulin signaling in muscle. However, there is no information on the effect of elevated plasma FFA levels on early insulin signal transduction steps in normal glucose-tolerant offspring of diabetic parents (FH+).

The aims of the present study were to examine the effect of lipotoxicity within the physiological range of plasma FFA elevation. In control subjects, we wanted to establish whether a subtle increase in plasma FFA (as seen in obesity and T2DM) would be sufficient to induce insulin resistance, as in earlier studies at pharmacological levels (4, 11, 13, 21, 24, 40, 42, 54–56, 63, 67), and potentially induce insulin-signaling defects in skeletal muscle similar to those of nondiabetic subjects genetically predisposed to T2DM (52). In FH+ subjects, we wanted to examine whether inducing “lipotoxicity” with a low-dose lipid infusion would further impair insulin action at signaling steps known to be already altered in these subjects (i.e., insulin receptor tyrosine phosphorylation, IRS-1-associated PI 3-kinase activity, and GS activity) (52). Testing the role of lipotoxicity at plasma FFA levels typically seen in obesity and in T2DM (~500–800 μmol/l) would provide the ideal setting to assess the clinical relevance of FFA-induced insulin resistance in humans, and in particular, in populations at high risk of developing T2DM.

### METHODS

**Subjects.** Seven Mexican-American subjects with a strong (≥2 first-degree relatives) family history of T2DM (FH+) and 10 age-, gender-, and weight-matched control subjects (8 Mexican-Americans, 2 Caucasians) without any family history of T2DM (CON) participated in the study. Their clinical and laboratory characteristics are shown in Table 1. Four FH+ subjects had two parents with T2DM, and three FH+ subjects had one diabetic parent and two or more siblings with T2DM. All subjects had a normal 75-g oral glucose tolerance test (OGTT) before enrollment. Body mass index was similar in FH+ and CON groups. Total body fat content, determined by bioimpedance, was similar in the two groups. No subject was excessively sedentary or participated in any strenuous physical activity in the days before testing or between study admissions. Body weight was stable in all subjects for ≥3 mo before enrollment. No subjects had any clinical or laboratory evidence of cardiac, hepatic, renal, or any other organ system disease, as determined by a complete medical history, physical examination, electrocardiogram, routine blood work, and urinalysis. Plasma lipids and blood pressure were within normal limits in all subjects. No participants were receiving any medications known to affect carbohydrate metabolism. Five control and four FH+ subjects had insulin secretion measurements that have been reported elsewhere (36). Each subject gave written informed consent before participation. The study protocol was approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio, TX.

**Experimental design.** After the OGTT, all subjects were admitted to the General Clinical Research Center (GCRC) at 1700 of day 0 on two separate occasions for the infusion, in random order, of normal saline or lipid (Liposyn III, a 20% triglyceride emulsion largely composed of soybean oil). Lipid or saline was infused at a rate of 0.5 ml/min (30 ml/h) for a total of 4 nights and 5 days (90 h). To avoid artificially stimulating lipoprotein lipase and altering the physiological removal of lipid by the enzyme, heparin was not confused. Before the start of the Liposyn or saline infusion, body composition was determined in all subjects by bioimpedance (RJL Bio-106 Spectrum Body Composition Analyzer; RJL Systems, Detroit, MI). On days 0–4, all subjects were given a eucaloric, weight-maintaining diet with a caloric distribution of 30% (0800), 30% (1200), 30% (1730), and 10% (2100). Meals consisted of 50% carbohydrate, 30% fat, and 20% protein. Subjects consumed identical meals during each hospital stay.

| Table 1. Clinical and laboratory characteristics of subjects |
|-----------------|-----------------|-----------------|
| Controls | FH+ | P |
| n (M/F) | 10 (5/5) | 7 (3/4) |
| Ethnicity (MA/C) | 8/2 | 70 |
| Age, yr | 37±3 | 44±3 | NS |
| BMI, kg/m² | 26.6±1.1 | 27.5±1.1 | NS |
| LBM, % | 71±2 | 71±3 | NS |
| FPG, mg/dl | 91±2 | 95±3 | NS |
| 2-h Glucose, mg/dl | 107±7 | 123±5 | NS |
| Hb A₁c, % | 4.9±0.1 | 5.0±0.1 | NS |
| Fasting plasma insulin, μU/ml | 6±1 | 14±3 | <0.01 |
| 2-h Insulin, μU/ml | 25±6 | 59±5 | <0.001 |
| Fasting FFA, μmol/l | 580±76 | 631±77 | NS |
| 2-h FFA, μmol/l | 151±22 | 151±39 | NS |
| Triglyceride, mg/dl | 129±17 | 112±14 | NS |
| HDL-cholesterol, mg/dl | 43±3 | 49±5 | NS |
| Systolic BP, mmHg | 124±5 | 120±4 | NS |
| Diastolic BP, mmHg | 71±3 | 72±4 | NS |

Data are means ± SE. FH+, subjects with a family history of type 2 diabetes; M/F, males/females; MA/C, Mexican-American/Caucasian; BMI, body mass index; LBM, lean body mass; FPG, fasting plasma glucose; Hb A₁c, hemoglobin type A₁c; FFA, free fatty acids; HDL-cholesterol, high-density lipoprotein cholesterol; BP, blood pressure; NS, not significant.
admission. Complete food intake was confirmed by a research nurse after each meal. On day 4, a 40 mU·m⁻²·min⁻¹ euglycemic insulin clamp (20) was performed in combination with titrated glucose, muscle biopsies (17), and indirect calorimetry (58), as described in Euglycemic insulin clamp. After the insulin clamp, subjects were discharged. Within 2–3 wk, volunteers were readmitted to the GCRC and given either Liposyn or normal saline to complete their participation in the study. All repeat procedures were performed in an identical fashion to those described above.

Euglycemic insulin clamp. The euglycemic insulin clamp was performed at 0800 on day 4 of admission after a 12-h overnight fast, as previously described (20). An antecubital vein was cannulated for infusion of [3-³H]glucose, 20% glucose, and insulin. A hand vein was cannulated retrogradely, and the hand was placed in a heated box (65°C) for sampling of arterialized blood. A primed [25 μCi·min⁻¹·100 fasting plasma glucose (FGP⁻¹)] continuous (0.25 μCi/min) infusion of [3-³H]glucose was begun 2 h before the start of the insulin infusion to allow for isotopic equilibration. Blood was drawn every 10 min during the last 30 min of the isotopic equilibration period for measurement of plasma glucose, insulin, and FFA concentrations and titrated glucose radioactivity. After 60 min of bed rest (0900), a percutaneous muscle biopsy was obtained with a Bergstrom cannula from the vastus lateralis muscle under local anesthetic (17). Muscle biopsy specimens immediately bled free of blood, frozen in liquid nitrogen, and stored under liquid nitrogen until processing. After the 2-h isotopic equilibration period, insulin was started (at 1000) at a rate of 40 mU·min⁻¹·100 kg body weight. A primed and continuous (0.25 μCi/min) infusion of [3-³H]glucose was begun 2 h before the start of the insulin infusion to allow for isotopic equilibration. Blood was drawn every 10 min during the last 30 min of the isotopic equilibration period for measurement of plasma glucose, insulin, and FFA concentrations and titrated glucose radioactivity. After the 2-h isotopic equilibration period, insulin was started (at 1000) at a rate of 40 mU·min⁻¹·100 kg body weight. A primed and continuous (0.25 μCi/min) infusion of [3-³H]glucose was begun 2 h before the start of the insulin infusion to allow for isotopic equilibration. Blood was drawn every 10 min during the last 30 min of the isotopic equilibration period for measurement of plasma glucose, insulin, and FFA concentrations and titrated glucose radioactivity. Thirty minutes after the start of insulin infusion (90 min after the initial biopsy), a second percutaneous muscle biopsy was obtained from the opposite vastus lateralis muscle. The insulin infusion was continued for a total of 120 min to obtain a measure of the rate of insulin-stimulated whole body glucose disposal (Ra) during the last 40 min of the study (80–120 min time period). Continuous indirect calorimetry (Deltatrac; Sensormedics, Anaheim, CA) was performed for the determination of oxygen consumption and carbon dioxide production during the last 40 min of the baseline (40–0 min) and euglycemic insulin clamp (80–120 min) periods (58). Patients were fed at the conclusion of the study and discharged from the hospital.

Insulin receptor signaling and enzyme activity assays. Insulin receptor tyrosine phosphorylation was assayed using immunoprecipitation and immunoblot analyses, as previously described (17). Insulin receptor tyrosine phosphorylation was determined by antiphosphotyrosine immunoblot analysis of anti-insulin receptor immunoprecipitates of muscle lysates. Insulin stimulation of the association of PI 3-kinase activity with IRS-1 was assayed by determining the ability of anti-IRS-1 immunoprecipitates to incorporate [³²P]ATP into phosphatidylinositol (17). Polyclonal anti-IRS-1 antibody and protein A-Sepharose beads were used for immunoprecipitation of IRS-1-associated PI 3-kinase (250 μg protein). The PI-3 product was identified by its comigration with a PI-4 standard and sensitivity to wortmannin. PI 3-kinase activity was calculated as a value relative to PI 3-kinase activity in a positive control (rat liver standard).

GS activity was assayed as described earlier (17) in subjects in whom there was sufficient muscle sample left for analysis after the previous assays. Activity was determined in the presence of 0.1 (GS10) and 10 (GS100) mM glucose 6-phosphate (G-6-P). GS fractional velocity (GSfV) is the ratio of GS10 to GS100 and is an indicator of dephosphorylation and, thus, activation of the enzyme.

Analytical determinations and calculations. The plasma glucose concentration was determined in duplicate by the glucose oxidase method with a Beckman Glucose Analyzer II (Beckman Instruments).

Plasma insulin concentration was determined by radioimmunoassay (Diagnostics Products, Los Angeles, CA). Plasma FFA concentration was assayed by an enzymatic method (NEFA-C kit, Wako Pure Chemicals, Osaka, Japan). Intra- and interassay coefficients of variation (CV) were for insulin 4.0 and 4.9% and for FFA, 1.1 and 3.3%. For the determination of plasma glucose radioactivity, plasma was deproteinized using barium hydroxide-zinc sulfate, and samples were centrifuged for 30 min at 3,500 g, and the clear supernatant was evaporated to dryness at 55°C in a Speed-Vac Evaporator (Savant, Farmindale, NY). The pellet was resuspended in 1 ml of distilled water mixed with 5 ml of Scintiverse II (Fisher Scientific, Pittsburgh, PA) and counted in a Beckman LS 600IC scintillation counter.

During the postabsorptive period, the rate of glucose appearance equals the rate of disappearance and was calculated as the titrated insulin glucose infusion rate (dpmin/min) divided by the plasma titrated glucose specific activity (dpmin/mg). During the euglycemic insulin clamp, non-steady-state conditions prevail, and rates of glucose appearance and disappearance were calculated with Steele’s non-steady-state equation by use of a glucose distribution volume of 0.65 (17), considered to be appropriate for both groups because participants were not overweight.

Statistical analysis. All values are presented as means ± SE. Within-group differences were determined by the paired two-tailed Student’s t-test. Differences between basal and insulin clamp periods and between FH+ and CON were tested by two-way ANOVA for repeated measures. Insulin receptor tyrosine phosphorylation, PI 3-kinase activity associated with IRS-1, and GSfV data are expressed relative to 100% stimulation in control subjects during saline infusion. Normal distribution was checked before all analyses, and nonparametric estimates were used when appropriate. Comparisons were considered statistically significant if the P value was <0.05. Where appropriate, regressions were calculated by least squares linear correlation coefficients analysis.

RESULTS

Fasting substrate and hormone concentrations. Table 2 summarizes the plasma glucose, insulin, C-peptide, and FFA concentrations during the 3 days of saline or lipid infusion preceding the metabolic studies.

<table>
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<th>Day 2</th>
<th>Day 3</th>
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<td>95±4</td>
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<td>98±4</td>
<td>96±4</td>
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<td>103±4</td>
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<td>Controls</td>
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</table>

Data represent means ± SE. *P < 0.01, lipid vs. saline infusion; †P < 0.01, FH+ vs. control subjects.
On the morning of the euglycemic insulin clamp with muscle biopsies (day 4), the fasting plasma glucose, insulin, and FFA concentrations in the CON group were 91 ± 2 mg/dl, 6 ± 1 µU/ml, and 580 ± 76 µmol/l, respectively, before lipid infusion and rose to 95 ± 3 mg/dl (P < 0.02), 10 ± 1 µU/ml (P < 0.05), and 731 ± 70 µmol/l (P < 0.01) after 4 days of lipid infusion. Saline infusion for 4 days did not alter the fasting glucose, insulin, or FFA concentrations in CON (93 ± 2 mg/dl, 7 ± 1 µU/ml, and 573 ± 61 µmol/l, respectively). In the FH+ group on day 4, the FPG, insulin, and FFA concentrations were 95 ± 3 mg/dl [not significant (NS) vs. CON], 14 ± 3 µU/ml (P < 0.01 vs. CON), and 631 ± 51 µmol/l (NS vs. CON) and rose to 98 ± 2 mg/dl (P < 0.02), 18 ± 3 µU/ml (P < 0.01), and 800 ± 100 µmol/l (P < 0.01 vs. baseline; P = NS vs. CON). Saline infusion had no effect on the fasting glucose, insulin, or FFA levels in the FH+ group (97 ± 2 mg/dl, 13 ± 1 µU/ml, and 602 ± 89 µmol/l, respectively).

Euglycemic insulin clamp: plasma glucose, insulin, and FFA concentrations. During the euglycemic insulin clamp studies, the steady-state plasma glucose concentrations during the saline (CON = 96 ± 2 vs. FH+ = 94 ± 3 mg/dl, P = NS) and lipid infusion (CON = 95 ± 2 vs. FH+ = 98 ± 2 mg/dl, P = NS) were similar in the two groups. The CV in plasma glucose was <5% in every study with saline and lipid infusion. The increments in plasma insulin concentration during the euglycemic insulin clamp were similar during saline and lipid infusion in CON (50 ± 2 vs. 49 ± 4 µU/ml, respectively, P = NS) and FH+ (53 ± 2 vs. 54 ± 4 µU/ml, respectively, P = NS). Suppression of plasma FFA concentration during the insulin clamp was greater in CON than in FH+ during both the saline (to 86 ± 12 vs. 138 ± 15 µmol/l, P < 0.02) and lipid (to 301 ± 26 vs. 398 ± 30 µmol/l, P < 0.03) infusion studies.

Glucose metabolism and substrate oxidation. In the CON group, there was a 25% decline in whole body insulin-mediated glucose disposal (Rd; Fig. 1) during lipid infusion [from 8.0 ± 0.6 to 6.0 ± 0.3 mg·kg·lean body mass (LBM)−1·min−1, P < 0.01]. This decrease was accounted for primarily by a reduction in nonoxidative glucose disposal (4.2 ± 0.7 to 2.7 ± 0.2 mg·kg·LBM−1·min−1, P < 0.01) and a small but significant decrease in glucose oxidation (3.8 ± 0.2 to 3.3 ± 0.2 mg·kg·LBM−1·min−1, P < 0.04). In the FH+ group, Rd (4.2 ± 0.5 mg·kg·LBM−1·min−1) was 48% lower than in the CON group (P < 0.001) during saline infusion. Lipid infusion in FH+ subjects caused no further worsening of Rd (4.2 ± 0.6 mg·kg·LBM−1·min−1, P = NS). Compared with CON, both glucose oxidation (3.2 ± 0.2 mg·kg·LBM−1·min−1, P < 0.02) and nonoxidative glucose disposal (1.1 ± 0.4 mg·kg·LBM−1·min−1, P < 0.01) were lower in FH+ subjects during saline infusion. Lipid infusion did not further worsen either glucose oxidation or nonoxidative glucose disposal in the FH+ group (Fig. 1).

Under fasting conditions, lipid oxidation (Fig. 2) was similar in CON and FH+ groups (1.01 ± 0.17 vs. 0.95 ± 0.07 mg·kg·LBM−1·min−1, respectively, P = NS) during saline infusion and was suppressed similarly in the two groups (0.32 ± 0.11 vs. 0.40 ± 0.07 mg·kg·LBM−1·min−1, respectively, P = NS) during the euglycemic insulin clamp (Fig. 2). Four days of lipid infusion in FH+ significantly increased the basal rate of lipid oxidation from 0.95 ± 0.07 to 1.34 ± 0.18 mg·kg·LBM−1·min−1 (P < 0.04) and impaired the suppression of lipid oxidation during the euglycemic insulin clamp (from 0.40 ± 0.07 to 1.02 ± 0.20 mg·kg·LBM−1·min−1, P < 0.01). In CON, 4 days of lipid infusion did not significantly alter the basal rate of lipid oxidation (1.01 ± 0.17 vs. 1.00 ± 0.13 mg·kg·LBM−1·min−1, P = NS) but impaired the suppression of lipid oxidation during the euglycemic insulin clamp (0.32 ± 0.11 vs. 0.66 ± 0.15 mg·kg·LBM−1·min−1, P < 0.01).

During the saline studies, basal endogenous glucose production (EGP) was not significantly different between the two groups (CON = 2.6 ± 0.1 vs. FH+ = 2.4 ± 0.1 mg·kg·LBM−1·min−1, P = NS). Lipid infusion caused small but significant increases in basal EGP in FH+ (from 2.4 ± 0.1 to 2.7 ± 0.2 mg·kg·LBM−1·min−1, P < 0.03). There was a trend for an increase in basal EGP in CON during LIP infusion (from 2.6 ± 0.1 to 2.9 ± 0.1 mg·kg·LBM−1·min−1, P = 0.06). EGP was nearly completely suppressed by insulin during the euglycemic insulin clamp during saline infusion in both groups (CON = 0.2 ± 0.1 vs. FH+ = 0.2 ± 0.1 mg·kg·LBM−1·min−1, P = NS). Lipid infusion significantly impaired the suppression of EGP in FH+ (0.7 ± 0.2 mg·kg·LBM−1·min−1, P < 0.04 vs. SAL) but not in CON (0.5 ± 0.02 mg·kg·LBM−1·min−1, P = 0.2 vs. SAL).
Insulin signaling. In CON subjects, insulin significantly \((P < 0.03)\) increased insulin receptor tyrosine phosphorylation by \(45 \pm 21\%\) (Fig. 3). Lipid infusion in CON substantially prevented insulin stimulation of insulin receptor tyrosine phosphorylation \((P < 0.05;\ Fig. 3)\). In FH+, insulin failed to significantly increase insulin receptor tyrosine phosphorylation \((P < 0.05\; vs.\; CON)\) during the saline infusion study \((Fig. 3)\), and lipid infusion did not further worsen the preexisting defect in insulin-stimulated insulin receptor tyrosine phosphorylation.

In CON subjects, insulin infusion significantly increased PI 3-kinase activity associated with IRS-1 \((P < 0.01;\ Fig. 4)\). After 4 days of lipid infusion in CON, insulin stimulation of PI 3-kinase activity associated with IRS-1 was decreased significantly compared with the saline infusion study \((P < 0.05)\). Although in absolute terms PI 3-kinase activity associated with IRS-1 was only slightly lower than in control subjects, FH+ subjects were insulin resistant at the level of PI 3-kinase, because the increase above baseline in insulin-stimulated PI 3-kinase associated with IRS-1 was significantly decreased compared with control subjects \((17 \pm 5 \; vs. \; 37 \pm 3\% \; increment \; over \; basal, \; P < 0.05;\ Fig. 4)\). After 4 days of lipid infusion in FH+, insulin stimulation of IRS-1-associated PI 3-kinase activity was slightly reduced and the increase over baseline no longer statistically significant.

In CON subjects during saline infusion, insulin significantly increased \(\Delta G_{SV} \) \((0.112 \pm 0.016 \; vs. \; 0.154 \pm 0.014, \; P < 0.0004)\). The 44% increase in \(\Delta G_{SV} \) after insulin stimulation during the euglycemic insulin clamp during saline infusion contrasted with the modest 15% stimulation of \(\Delta G_{SV} \) with insulin after 4 days of lipid infusion \((P = 0.2 \; vs. \; baseline; \; P < 0.05 \; saline \; vs. \; lipid \; infusion \; in \; insulin-stimulated \; \Delta G_{SV})\). In FH+ subjects during saline infusion, baseline \(\Delta G_{SV} \) was reduced by 35% compared with CON subjects \((P = 0.06)\), and insulin was unable to increase \(\Delta G_{SV} \) significantly \((0.072 \pm 0.016 \; vs. \; 0.090 \pm 0.025, \; P = NS, \; P < 0.03 \; vs. \; CON)\). Lipid infusion in FH+ subjects did not further worsen the preexisting defect in \(\Delta G_{SV} \) \((0.083 \pm 0.022 \; vs. \; 0.102 \pm 0.009, \; P = NS)\).

Correlations. The fasting plasma FFA concentration during saline infusion correlated inversely with insulin stimulated whole body \(R_d \) \((r = -0.69, \; P = 0.05)\), glucose oxidation \((r = -0.79, \; P = 0.03)\), and nonoxidative glucose disposal \((r = -0.81, \; P = 0.02)\). During lipid infusion, the basal FFA concentration in CON also correlated inversely with \(R_d \) \((r = -0.70, \; P = 0.05)\), insulin-stimulated glucose oxidation \((r = -0.83, \; P = 0.01)\), and nonoxidative glucose disposal \((r = -0.71, \; P < 0.05)\). In CON, the increase in fasting plasma FFA concentration above baseline during lipid infusion correlated directly with the decrements in glucose oxidation \((r = 0.81, \; P = 0.02)\), the decrease in nonoxidative glucose disposal \((r = 0.66, \; P < 0.05)\), and the reduction in insulin-stimulated insulin receptor tyrosine phosphorylation \((r = 0.56, \; P = 0.05)\) during the euglycemic insulin clamp. There was a strong positive correlation between the decrease of \(R_d \) after lipid infusion and the reduction in insulin-stimulated nonoxidative glucose disposal \((r = 0.94, \; P < 0.01)\). This was not entirely unexpected, as \(R_d \) during insulin stimulation was significantly reduced by lipid infusion, and both variables are interdependent (nonoxidative glucose disposal is estimated as the difference between \(R_d \) and glucose oxidation as measured by indirect calorimetry). However, the correlation between the decrease in GS activity and nonoxidative glucose disposal in CON \((0.62, \; P < 0.05)\) supports the view that the glycogen synthetic pathway was impaired by a chronic lipid oversupply, as reported in earlier acute lipid infusion studies \((9, \; 11, \; 21, \; 38)\).

There was also a positive correlation between the impairment in insulin-stimulated PI 3-kinase activity associated with IRS-1 and the reduction in \(R_d \) \((r = 0.70, \; P = 0.05)\) and decrease in nonoxidative glucose disposal \((r = 0.69, \; P < 0.05)\).

In FH+ subjects, the plasma basal FFA concentration during saline infusion correlated inversely with \(R_d \) \((r = -0.70, \; P = 0.05)\) and oxidative glucose disposal \((r = -0.94, \; P < 0.01)\).
There was no correlation between basal FFA concentration (absolute or incremental change) during lipid infusion and $R_d$, oxidative glucose disposal, nonoxidative glucose disposal, insulin receptor tyrosine phosphorylation, PI 3-kinase activity associated with IRS-1, or $G_{SV}$. 

**DISCUSSION**

This study examines for the first time 1) the role of a chronic (90-h) lipid infusion in humans 2) at a physiological increase in plasma FFA concentration to levels seen in obesity and in T2DM of $\sim$600–800 μmol/l [most previous studies have used acute 2- to 6-h lipid infusions at pharmacological doses ranging from $\sim$1,800 to 4,000 μmol/l (3, 4, 6, 8, 11, 13, 21, 24, 40, 42, 54–56, 63, 67); 3) normal glucose-tolerant subjects with a strong family history of T2DM (FH+), a group never examined before under the current experimental conditions; and 4) insulin action/insulin signaling at physiological increases in plasma FFA in FH+ vs. control subjects without any family history of T2DM, another novel aspect of this study. Thus this study design allows us for the first time to learn of the clinical relevance of an increase in plasma FFA concentration in human disease by means of inducing a sustained “physiological lipotoxicity” in a population at high risk of developing T2DM.

Recent studies have provided evidence that an acute supraphysiological elevation in the plasma FFA concentration (from $\sim$1,200 to 3,000 μmol/l) may inhibit glucose transport (40, 54, 55) and insulin signal transduction (21, 41) in association with an impairment in insulin action in humans. However, plasma FFA rarely approaches levels above $\sim$1,000 μmol/l under normal living conditions in humans (3, 4, 6–9, 11, 13, 19, 21, 24, 35, 38, 40, 42, 43, 54–56, 63, 67), and no previous study has examined the effect of a physiological increase or a more chronic (>6 h) elevation in the plasma FFA concentration on the insulin-signaling pathway in healthy subjects without any family history of diabetes and compared it with insulin-resistant FH+ individuals. In control subjects, we wanted to establish whether plasma FFA at levels observed in insulin-resistant states (i.e., obesity and T2DM) would be sufficient to cause insulin resistance, as in earlier studies at pharmacological levels (3, 4, 6, 8, 11, 13, 21, 24, 40, 42, 43, 54–56, 63, 67). If lipotoxicity is an important mechanism in human disease, the insulin-signaling defects in skeletal muscle would be similar to those of nondiabetic subjects genetically predisposed to T2DM (52). In FH+ subjects, who are known to be insulin resistant (22, 28, 33, 36, 49, 52, 66), we wanted to examine whether inducing lipotoxicity with a low-dose lipid infusion would further impair insulin action at the level of insulin receptor tyrosine phosphorylation, IRS-1-associated PI 3-kinase activity, and GS activity (52). We believed that if there were a preexisting lipotoxicity (62) or severe insulin resistance due to other genetic defects in insulin action, a physiological increase in plasma FFA would have few, if any, additional effects to impair insulin signaling.

In insulin-sensitive healthy subjects, lipid infusion increased fasting and day-long plasma FFA concentration by $\sim$150–200 μmol/l but failed to change the basal rate of lipid oxidation. However, suppression by insulin of both the plasma FFA concentration and whole body lipid oxidation rate during the euglycemic insulin clamp was clearly impaired after 4 days of lipid infusion. The elevated fasting plasma FFA concentrations after 4 days of lipid infusion correlated strongly and inversely with $R_d$ ($r = -0.70, P = 0.05$) and with insulin-stimulated oxidative ($r = -0.83, P = 0.01$) and nonoxidative ($r = -0.71, P = 0.05$) glucose disposal. The increase in plasma FFA concentration of only $\sim$150–200 μmol/l in this study caused a 25% reduction in whole body (primarily muscle) insulin-mediated $R_d$. This was somewhat greater than that observed during acute experiments at similar plasma FFA levels by us (5, 38) and by others (9, 35) and may represent the full effect of lipotoxicity from a more prolonged lipid infusion. FFA-induced insulin resistance primarily involved the nonoxidative pathway of glucose disposal and was associated with a blunting of insulin’s ability to stimulate GS activity. After lipid infusion, there was a strong correlation between the reduction of nonoxidative glucose disposal and $R_d$ ($r = 0.94, P < 0.01$) and an inverse correlation with the increase in plasma FFA ($r = -0.66, P < 0.05$). These findings are consistent with low- (5, 9, 38) and high-dose (3–6, 8, 11, 13, 21, 24, 40, 42, 54–56, 63, 67) lipid infusions in humans.

A novel finding of the present study is that a low-dose lipid infusion inhibits insulin stimulation of insulin receptor tyrosine phosphorylation. The decrease in insulin-stimulated insulin receptor tyrosine phosphorylation correlated inversely with the increase in fasting plasma FFA concentration brought about by lipid infusion ($r = 0.56, P < 0.05$). This suggests that the abnormality in PI 3-kinase activation seen here and in earlier studies (21) could be due, at least in part, to a more proximal lipid-induced inhibition of insulin receptor tyrosine phosphorylation. However, because there was no correlation between the impairment in IRS-1-associated PI 3-kinase activity and the defect in insulin receptor tyrosine phosphorylation, this raises the possibility that FFA exert separate defects at the level of the insulin receptor and association of PI 3-kinase with IRS-1. Gumbiner et al. (29) and Kruszynska et al. (41) reported no impairment on insulin receptor tyrosine phosphorylation after acute fourfold elevations in plasma FFA concentration in healthy volunteers, suggesting that downregulation of insulin receptor function may be more a function of chronic exposure to elevated FFA than to the plasma level achieved.

In healthy control subjects, 4 days of modest elevation in the plasma FFA concentration markedly impaired the ability of insulin to increase the association of PI 3-kinase activity with IRS-1 (Fig. 4). The defect in insulin-stimulated PI 3-kinase activity associated with IRS-1 was related to the impairments in insulin-stimulated whole body $R_d$ ($r = 0.70, P < 0.05$) and nonoxidative glucose disposal ($r = 0.94, P < 0.01$), providing support for a physiological link between the molecular defect (impaired PI 3-kinase activity) induced by elevated plasma FFA and induction of in vivo insulin resistance. Previous studies in humans (21, 41) have demonstrated that an acute supraphysiological elevation in the plasma FFA concentration impairs the ability of insulin to increase PI 3-kinase activity associated with IRS-1. Our results support the notion that even a very small, but chronic, physiological increment in the plasma FFA concentration in vivo can induce a pattern of insulin resistance at whole body, organ (muscle), and cellular/molecular levels that closely reproduces defects reported in subjects genetically predisposed to develop T2DM later in life, i.e., FH+ subjects (52) and individuals with T2DM (17).
Consistent with previous studies from our laboratory (28, 36, 52) and others (22, 46, 49, 66), nondiabetic FH+ subjects were severely insulin resistant (\(R_d = 4.2 \pm 0.5 \text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}\)) compared with age/gender/obesity-matched control subjects without a family history of diabetes (8.0 ± 0.6 mg · kg \(-1 \cdot \text{min}^{-1}\)), and the decrease in insulin-mediated glucose disposal was accounted for primarily by a reduction in nonoxidative glucose disposal (glycogen synthesis), although glucose oxidation was also mildly impaired (Fig. 1). A particularly striking observation of the present study was that 4 days of lipid infusion in FH+ subjects did not cause any further worsening of insulin-mediated whole body glucose disposal, nonoxidative glucose disposal, and glucose oxidation, or of preexisting defects in insulin-stimulated insulin receptor tyrosine phosphorylation (Fig. 3), PI 3-kinase activity associated with IRS-1 (Fig. 4), or GS activity. Consistent with these findings, there was no correlation between fasting plasma FFA with glucose disposal or insulin-signaling defects in FH+ subjects.

These results can be interpreted in one of two ways. J) Lipotoxicity develops early in life and is fully established in adult insulin-resistant FH+ subjects; consequently, lipid infusion cannot cause any further reduction in insulin action [except when lipid is given at pharmacological doses (3, 6, 8)]. This would be in agreement with the elevated plasma FFA levels and/or rates of lipid oxidation (28, 33, 37, 49) reported in previous studies in FH+ individuals. In this study, as in earlier reports from our laboratory (52) and others (23, 34, 46, 60, 64), preexisting adipose tissue insulin resistance was more subtle: our FH+ subjects needed a two- to threefold higher plasma insulin concentration to suppress the fasting and 2-h plasma FFA concentration during the OGTT (Table 1) and the 72-h in-hospital profile (Table 2) to levels of CON subjects. In the studies by Sinha et al. (60) and Virkamaki et al. (64), adipose tissue insulin resistance (i.e., “normal” plasma FFA concentration despite severe hyperinsulinemia) was associated with increased IMCL, indicating lipotoxicity (i.e., fat deposition in muscle) from an imbalance between lipid supply and lipid oxidation. Of note, a small but sustained elevation in plasma FFA during the 6-h studies. The additional triglycerides and calories from the lipid infusion could have led to compensatory hyperinsulinemia and contributed to impair insulin sensitivity in control subjects. This confounding variable deserves more careful examination, because in our experience mild 72-h hyperinsulinemia per se can cause insulin resistance in healthy subjects (18, 31). However, lipid-induced insulin resistance in nondiabetic subjects happens beyond variable changes in plasma insulin concentration (4, 10, 11, 13, 21, 24, 40, 42, 54–56, 63, 67), and ICML accumulation has been reported in the absence of any change in plasma insulin levels (12) in short-term (≤6-h) studies. Unfortunately, we did not measure skeletal muscle fatty tissue infiltration in this study but are actively examining it in ongoing work. Impaired insulin sensitivity (33, 37, 47, 51, 60) and insulin signaling (64) are associated with increased IMCL.

During acute lipid infusion studies, IMCLs keep a close temporal relationship with the increase in plasma FFA concentration (2, 12, 14). During the infusion of lipid emulsions in healthy humans, triacylglycerol-rich particles may be hydrolyzed by muscle lipoprotein lipase and transported into muscle without their fatty acids necessarily spilling over to the systemic circulation (i.e., contributing to the plasma FFA pool) (44). To the extent that this happens, it may contribute to impair insulin action (i.e., by IMCL formation) beyond the plasma FFA levels achieved. Although in the present study, as in prior reports (2, 4, 5, 10–14), the decrease in insulin sensitivity and insulin-signaling defects were most likely due to the increase in plasma FFA concentration, further studies are needed to learn more about the role of muscle LPL and disposition of triglyceride particles during acute and chronic lipid infusions in humans. However, factors other than triglyceride oversupply are involved in the deterioration of muscle insulin sensitivity and ICML formation, such as the rate of muscle lipid oxidation (25, 50), the intramyocellular localization of the fat droplets in relationship to mitochondria (65), the level of expression/function of uncoupling protein-1 (30), differences in genetic determinants of peroxisome proliferator-activated receptor-γ and retinoid X receptor expression in muscle (15), plasma FFA and insulin levels, and the duration of lipid oversupply such that a modest but chronic lipid overload may be sufficient to impair insulin sensitivity in humans, as shown in this study.

Recent studies suggest that elevated plasma FFA are transported into the cell by a specific fatty acid transport protein (FATP) and accumulate in excessive amounts as long-chain fatty acyl-CoA derivatives (LCFA-CoAs) (48). The importance of this step has been highlighted by the finding that FATP1 knockout mice lacking the fatty acid transporter are protected from lipotoxicity (39). LCFA-CoAs increase the synthesis of diacylglycerol (45) and, either directly (48) or acting through diacylglycerol, increase the activity of protein kinase C (PKC), especially the δ-isofrom (1, 27). PKCδ causes serine phosphorylation of both the insulin receptor and IRS-1, impairing insulin-stimulated tyrosine phosphorylation (27, 48). PKCδ activation also enhances IKK-β activity, which impairs insulin-stimulated IRS-1 tyrosine phosphorylation (32). In addition, LCFA-CoAs increase the formation of ceramide (16), which, acting through PKB or IKK-β, can impair the activation of GS by insulin (1, 27, 32, 57). Thus there are a number of established mechanisms by which elevated plasma FFA concentrations can interfere with the insulin signal transduction.
system to induce insulin resistance. However, our results demonstrate for the first time that a physiological increment in the plasma FFA concentration, by impairing insulin receptor tyrosine phosphorylation (Fig. 3) and PI 3-kinase activity associated with IRS-1 (Fig. 4), leads to defects in insulin-stimulated nonoxidative glucose disposal (glycogen synthesis) (Fig. 1) and GS activity. The lack of correlation between changes in lipid oxidation and either insulin-signaling step indicates that the original FFA-glucose substrate competition cycle originally proposed by Randle et al. (53) must be expanded to take into account the important effects of LCFA-CoAs on the insulin-signaling pathway (Figs. 3 and 4).

Lipid infusion had strikingly opposite effects on insulin secretion in the two groups. After mixed meals, day-long plasma C-peptide levels increased with LIP in control subjects but decreased significantly in FH+ subjects (Table 2, P < 0.01). As reported by Kashyap et al. (36), a chronic lipid infusion increases glucose-stimulated insulin secretion in subjects without a family history of T2DM but impairs it in FH+ subjects. This was more evident when the prevailing insulin resistance was taken into account [ISRra = ISR / (1/IRa)], so that first- and second-phase ISRra values were 25 and 42% of the value in control subjects (both P < 0.001 vs. controls). Therefore, whether insulin resistance in FH+ subjects or T2DM is due to lipotoxicity or other unrelated mechanisms, acquired lipotoxicity of obesity in genetically predisposed individuals may tip the balance toward β-cell failure and hyperglycemia in a system with already limited capacity for further adaptation.

In conclusion, a sustained physiological increase in the plasma FFA concentration to levels seen in T2DM and obesity decreases insulin-stimulated glucose uptake and insulin receptor signaling in normal glucose-tolerant, insulin-sensitive subjects without a family history of diabetes, but it does not worsen insulin resistance or insulin signaling in subjects genetically predisposed to develop T2DM later in life. Whether the lack of effects of elevated plasma FFA levels in the offspring of T2DM parents is due to already established lipotoxicity or other mechanisms remains to be determined.

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