

Dose-Response Effect of Elevated Plasma Free Fatty Acid on Insulin Signaling

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The dose-response relationship between elevated plasma free fatty acid (FFA) levels and impaired insulin-mediated glucose disposal and insulin signaling was examined in 21 lean, healthy, normal glucose-tolerant subjects. Following a 4-h saline or Liposyn infusion at 30 ($n = 9$), 60 ($n = 6$), and 90 ($n = 6$) ml/h, subjects received a 2-h euglycemic insulin ($40 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$) clamp. Basal plasma FFA concentration ($\sim 440 \text{ } \mu\text{mol/l}$) was increased to 695, 1,251, and 1,688 $\mu\text{mol/l}$ after 4 h of Liposyn infusion and resulted in a dose-dependent reduction in insulin-stimulated glucose disposal (R_d) by 22, 30, and 34%, respectively (all $P < 0.05$ vs. saline control). At the lowest lipid infusion rate (30 ml/h), insulin receptor and insulin receptor substrate (IRS)-1 tyrosine phosphorylation, phosphatidylinositol (PI) 3-kinase activity associated with IRS-1, and Akt serine phosphorylation were all significantly impaired ($P < 0.05$ – 0.01). The highest lipid infusion rate (90 ml/h) caused a further significant reduction in all insulin signaling events compared with the low-dose lipid infusion ($P < 0.05$ – 0.01) whereas the 60-ml/h lipid infusion caused an intermediate reduction in insulin signaling. However, about two-thirds of the maximal inhibition of insulin-stimulated glucose disposal already occurred at the rather modest increase in plasma FFA induced by the low-dose (30-ml/h) lipid infusion. Insulin-stimulated glucose disposal was inversely correlated with both the plasma FFA concentration after 4 h of lipid infusion ($r = -0.50$, $P = 0.001$) and the plasma FFA level during the last 30 min of the insulin clamp ($r = -0.54$, $P < 0.001$). PI 3-kinase activity associated with IRS-1 correlated with insulin-stimulated glucose disposal ($r = 0.45$, $P < 0.01$) and inversely with both the plasma FFA concentration after 4 h of lipid infusion ($r = -0.39$, $P = 0.01$) and during the last 30 min of the insulin clamp ($r = -0.43$, $P < 0.01$). In summary, in skeletal muscle of lean, healthy subjects, a progressive increase in plasma

FFA causes a dose-dependent inhibition of insulin-stimulated glucose disposal and insulin signaling. The inhibitory effect of plasma FFA was already significant following a rather modest increase in plasma FFA and develops at concentrations that are well within the physiological range (i.e., at plasma FFA levels observed in obesity and type 2 diabetes). *Diabetes* 54:1640–1648, 2005

Type 2 diabetes is characterized by insulin resistance in muscle, adipocytes, and liver and by impaired β -cell function (1–4). Both genetic (5–8) and acquired (7,8) disturbances contribute to the defects in insulin action and insulin secretion. Among the acquired defects, lipotoxicity (7–10), glucotoxicity (11,12), and obesity (3,4) all have been shown to exacerbate insulin resistance and contribute to the demise in β -cell function.

Randle et al. (13) were the first to demonstrate that incubation of muscle with free fatty acids (FFAs) decreased insulin-stimulated glucose uptake. According to the originally proposed Randle cycle, oxidation of fatty acids led to inhibition of the Krebs cycle/glucose oxidation, impaired glycolytic flux, and eventually product inhibition of hexokinase and glucose transport. Early studies in healthy humans supported this notion because lipid infusion rapidly reduced glucose oxidation (14–17), increased muscle acetyl-CoA content (18), and inhibited muscle pyruvate dehydrogenase activity (19), as would be expected from elevated muscle acetyl-CoA levels.

However, additional mechanisms, such as a direct effect of FFAs on early steps of glucose metabolism (i.e., at the level of glucose transport and/or phosphorylation), were suggested from studies showing the discordance between the rapid reduction in glucose oxidation and the delay in the inhibition of insulin-stimulated glucose uptake (18), as well as by inconsistencies in the temporal inhibition by FFAs of glucose uptake, glycogen synthesis, and glycolysis (20,21). Subsequent studies consistently failed to observe the expected rise (according to the Randle hypothesis) in skeletal muscle glucose-6-phosphate (G6P) levels at a time when lipid infusion had already decreased insulin-stimulated glucose uptake (22–24). Taken together, these observations indicated that mechanisms other than impaired glycolytic flux with intramyocellular accumulation of G6P must account for the defect in insulin-mediated glucose disposal following lipid infusion to elevate the plasma FFA concentration.

Recent studies suggest that FFAs interfere with the

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CR, Clinical Research Center; EGP, endogenous glucose production; FFA, free fatty acid; G6P, glucose-6-phosphate; IRS, insulin receptor substrate; PI, phosphatidylinositol.

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initial steps of the insulin signaling cascade. Inhibition of insulin-stimulated glucose transport has been reported in vitro when myotubes are exposed to high concentrations of palmitate (25–27), together with a decrease in glycogen synthesis (26), insulin-induced phosphorylation of the insulin receptor, and insulin receptor substrate (IRS)-1 (25) and/or of protein kinase B/Akt (25–27). In rats, a 5-h lipid infusion reduced both glucose oxidation and glycogen synthesis in association with impaired glucose transport (28). This was accompanied by decreased insulin-stimulated tyrosine phosphorylation of IRS-1, reduced IRS-1-associated phosphatidylinositol (PI) 3-kinase activity, and increased protein kinase C θ activity. In humans, the same group demonstrated that elevated plasma FFAs impaired IRS-1-associated PI 3-kinase activity in skeletal muscle and inhibited insulin-stimulated glucose transport and phosphorylation (23). Similar results on early steps of insulin signaling have been reported by Kruszynska et al. (29) but not when lipid is infused to already insulin-resistant obese subjects (30). In vitro (26,27,31,32) and in vivo studies in animals (28,33,34) and humans (34–38) have demonstrated that elevated FFA levels lead to an increase in intramyocellular lipids and of a variety of fat-derived toxic metabolites, including fatty acyl CoAs, ceramides, and diacylglycerol, which are believed to activate serine kinases in which serine phosphorylate and inhibit key insulin signaling molecules. Both the protein kinase C and I κ B/NF κ B pathways have been implicated in the FFA-induced impairment of IRS-1 tyrosine phosphorylation (28,37,39–41).

However, most but not all (14,18–20,54) of the previous human studies have used high lipid infusion rates, which elevated the plasma FFA >1,500 $\mu\text{mol/l}$, levels that are considerably higher than the usual FFA levels observed in obese and type 2 diabetic subjects (1–4). Fasting plasma FFA levels in healthy subjects usually range between ~300 and 400 $\mu\text{mol/l}$ but may increase to ~800–1,100 $\mu\text{mol/l}$ under certain extreme conditions, such as fasting for 2–3 days (42–44). In obese nondiabetic individuals (45,46) and in patients with type 2 diabetes (46–49), fasting and day-long plasma FFA levels are usually elevated (~600–800 $\mu\text{mol/l}$) because of resistance to the antilipolytic effect of insulin, but plasma FFAs usually remain \leq 1,000 $\mu\text{mol/l}$ even in the presence of severe hypertriglyceridemia (either with [50] or without [51] concomitant diabetes) or in poorly controlled diabetes (52,53). When the plasma FFA levels of lean, healthy subjects are increased within the physiological range to levels seen in obesity and type 2 diabetes (14,18–20,54), insulin resistance and impaired glucose oxidation develop, while glycogen synthase activity (a rather distal step in insulin signaling) has been reported to be either normal (54) or impaired (19,20). However, the molecular pathways in muscle responsible for the development of insulin resistance by FFAs remain incompletely understood, and their dose response to FFA inhibition has yet to be systematically studied.

The aim of the present study was to evaluate the effect of an increase in plasma FFA levels on insulin-stimulated whole-body glucose metabolism and early steps of muscle insulin signaling, spanning from FFA levels typically seen in obesity and type 2 diabetes (~600–800 $\mu\text{mol/l}$), through the upper range of the physiological spectrum (~1,000–

TABLE 1
Clinical and laboratory characteristics of subjects

	Lipid infusion rate (ml/h)		
	30	60	90
<i>n</i>	9	6	6
Sex (M/F)	6/3	3/3	4/2
Age (years)	31 \pm 4	25 \pm 1	26 \pm 2
BMI (kg/m ²)	24 \pm 1	24 \pm 1	24 \pm 1
Lean body mass (%)	75 \pm 3	74 \pm 3	73 \pm 3
Glucose (mg/dl)*	87 \pm 2	87 \pm 2	86 \pm 2
Insulin ($\mu\text{U/ml}$)*	5 \pm 1	7 \pm 1	7 \pm 2
FFA ($\mu\text{mol/l}$)*	434 \pm 54	377 \pm 62	383 \pm 27

Data are means \pm SE. *Fasting plasma levels on the day of the oral glucose tolerance test (mean of three basal values).

1,200 $\mu\text{mol/l}$), to plasma FFA concentrations within the pharmacological range (~1,700 $\mu\text{mol/l}$).

RESEARCH DESIGN AND METHODS

Twenty-one lean, healthy subjects without family history of type 2 diabetes participated in the study. Their clinical characteristics are shown in Table 1. All participants had a normal 75-g oral glucose tolerance test according to World Health Organization criteria. None of the subjects were taking any medication. Body weight was stable in all subjects for at least 3 months before enrollment. None of the subjects participated in strenuous physical activities. All subjects gave their written informed consent before participation, and the study protocol was approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio.

To examine the dose-response effect of elevated plasma FFAs on insulin-mediated glucose disposal (R_d) and insulin signaling, volunteers were divided into three groups, which were matched for age, sex, ethnicity, BMI, and lean body mass (Table 1), which was determined by bioelectrical impedance (Cyprus 1.2, Quantum II; RJL Systems). Following documentation of normal glucose tolerance, subjects were admitted to the Clinical Research Center (CRC) at 1700 on two occasions for the infusion in random order of Liposyn III (20% triglyceride emulsion, primarily composed of soybean oil) or normal saline (control study). Subjects were fed a eucaloric standardized diet at 1800 and remained fasting, except for water after 2100. At 0500 on the following morning, a 20-gauge catheter was placed into an antecubital vein and lipid or normal saline infusion was started. Participants were randomly divided into three groups that received lipid infusions at the rate of 30, 60, or 90 ml/h. This rate was maintained constantly during the following 8 h (until 1300). At 0700, a second catheter was inserted retrogradely into a vein on the dorsum of the hand for collection of blood samples, and the hand was placed in a temperature-regulated box at 65°C to achieve arterialization of venous blood. At 0700, a primed (25 $\mu\text{Ci/min}$)-continuous (0.25 $\mu\text{Ci/min}$) infusion of [$3\text{-}^3\text{H}$]glucose (DuPont-New England Nuclear, Boston, MA) was started and continued until the end of the study. At 0900, subjects underwent a 4-h, 40-mU \cdot min⁻² \cdot min⁻¹ euglycemic insulin clamp as described below. Within 2–4 weeks, individuals were readmitted to the CRC and given either lipid or normal saline. During the repeat study, all procedures were performed in an identical fashion to the initial study.

At 60 min before the start of the insulin clamp (at 0800), a percutaneous muscle biopsy was obtained with a Bergstrom cannula from the vastus lateralis muscle under local anesthesia as previously described (55). Muscle specimens were blotted free of blood, frozen in liquid nitrogen within 30 s, and stored in liquid nitrogen until assays were performed. At 0900, after allowing 120 min for isotopic equilibration, a 4-h euglycemic insulin (40 mU \cdot min⁻² \cdot min⁻¹) clamp was started using a primed-continuous insulin infusion (56). The plasma glucose concentration was measured every 5 min, and a variable feedback principle (56) to maintain each subject's glucose concentration constant at the basal level. Blood was obtained every 10–15 min during the insulin clamp for measurement of plasma insulin and FFA concentrations and tritiated 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 contralateral vastus lateralis muscle. Carbohydrate and lipid oxidation were measured by continuous indirect calorimetry (Deltatrac; Sensormedics, Anaheim, CA) during the last 40 min of the baseline (-40- to 0-min) and insulin clamp (200- to 240-min) periods (57). Patients were fed at the conclusion of the study and discharged from the CRC.

TABLE 2
Metabolic parameters

	Saline			Lipid		
	Fasting	Basal	Clamp	Fasting	Basal	Clamp
Glucose (mg/dl)						
30 ml/h	93 ± 3	96 ± 2	96 ± 2	93 ± 1	95 ± 3	96 ± 2
60 ml/h	92 ± 2	91 ± 2	96 ± 1	90 ± 3	92 ± 2	94 ± 3
90 ml/h	88 ± 3	88 ± 3	95 ± 2	91 ± 3	92 ± 3	91 ± 3
Insulin (μU/ml)						
30 ml/h	6 ± 1	5 ± 1	57 ± 3	8 ± 1	7 ± 1	57 ± 3
60 ml/h	8 ± 2	7 ± 1	60 ± 4	9 ± 2	9 ± 2	53 ± 5
90 ml/h	7 ± 2	6 ± 2	68 ± 15	7 ± 2	8 ± 2	55 ± 11
FFA (μmol/l)						
30 ml/h	474 ± 56	434 ± 54	74 ± 15	401 ± 45	695 ± 50*	269 ± 32*
60 ml/h	401 ± 66	402 ± 154	72 ± 25	386 ± 62	1,251 ± 140*†	945 ± 217*†
90 ml/h	457 ± 64	383 ± 27	54 ± 15	416 ± 77	1,688 ± 308*†	1,260 ± 252*†

* $P < 0.05$, lipid vs. saline study; † $P < 0.05$, 60- and 90-ml/h lipid infusion compared with 30-ml/h lipid infusion.

Insulin receptor signaling and enzyme activity assays. Insulin receptor tyrosine phosphorylation was assayed using immunoprecipitation and immunoblot analyses as previously described (55). 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 32 P-ATP into PI. 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).
Immunoblot analysis of Akt Ser⁴⁷³ phosphorylation. Frozen muscle specimens were homogenized as described (55), and protein concentration was determined by the Lowry method (58). Akt activation was assessed using immunoblot analysis of 100 μg total muscle protein by determining the phosphorylation of serine⁴⁷³ using a specific anti-phospho-Akt antibody. All blots were stripped and reprobed for protein expression, and protein phosphorylation was expressed relative to protein levels. Anti-phospho Akt antibody was purchased from Upstate Biotechnology (Waltham, MA). Polyclonal anti-Akt was purchased from Cell Signaling Technology (Beverly, MA).
Analytical determinations. The plasma glucose concentration was determined by the glucose oxidase method with a Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA). Plasma insulin was determined by radioimmunoassay (Diagnostic Products, Los Angeles, CA). Plasma FFA concentration was measured by standard colorimetric methods (Wako Chem-

icals, Neuss, Germany). Plasma glucose radioactivity was determined on barium hydroxide/zinc sulfate-precipitated plasma extracts.

Endogenous glucose production (EGP) and insulin-stimulated whole-body R_d . During the basal period, the rate of endogenous glucose appearance (which primarily represents hepatic glucose production) equals the rate of glucose disappearance and was calculated by dividing the [3 H]glucose infusion rate (dpm/min) by the steady-state plasma-tritiated glucose specific activity (dpm/mg) during the last 30 min of tracer equilibration. Since the infusion of insulin results in non-steady-state conditions, the rate of plasma glucose appearance was calculated using Steele's non-steady-state equation (59). During the euglycemic insulin infusion period, EGP was computed as the difference between the exogenous glucose infusion rate and the isotopically measured rate of plasma glucose appearance. The rate of total-body insulin-mediated glucose disposal was calculated by adding the residual rate of EGP to the rate of exogenous glucose infusion. Net glucose and lipid oxidation rates were calculated from oxygen consumption (V_{O_2}) and carbon dioxide production (V_{CO_2}) using standard calorimetric equations (57). Nonoxidative glucose disposal, which primarily represents glycogen synthesis (60), was calculated by subtracting the rate of glucose oxidation (measured by indirect calorimetry) from the rate of total-body glucose disposal.

Statistical analysis. All values represent the mean ± SE. Differences between basal and insulin clamp periods were tested by paired two-tailed Student's t test using StatView for Windows (version 5.0; SAS Institute, Cary, NC). Differences between saline and lipids within a group were determined by the paired Student's t test. Between-group differences (i.e., 30- vs. 60- vs. 90-ml/min lipid infusion rates) were tested by two-way ANOVA for repeated measures. Insulin receptor tyrosine phosphorylation, IRS-1 tyrosine phosphorylation, PI 3-kinase activity associated with IRS-1, and Akt phosphorylation

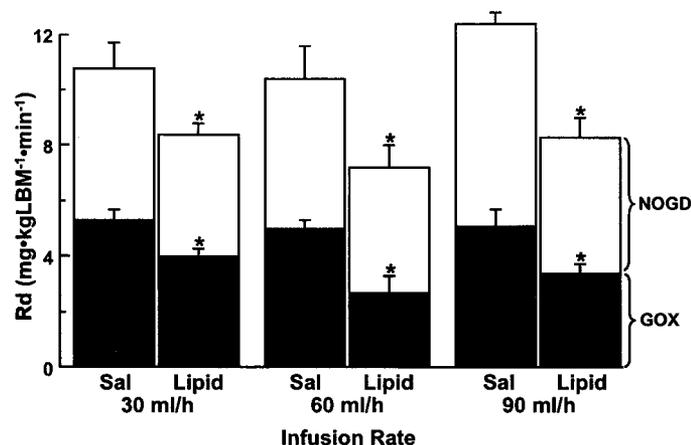


FIG. 1. Effect of saline (Sal) versus lipid (Liposyn III, a 20% triglyceride emulsion) infusion at rates of 30, 60, and 90 ml/h on total-body insulin-mediated glucose disposal (total height of bars), glucose oxidation (GOX; solid portion of bars), and nonoxidative glucose disposal (NOGD), which primarily represents muscle glycogen synthesis (open portion of bars). * $P < 0.05$ – 0.01 , lipid vs. saline infusion. LBM, lean body mass.

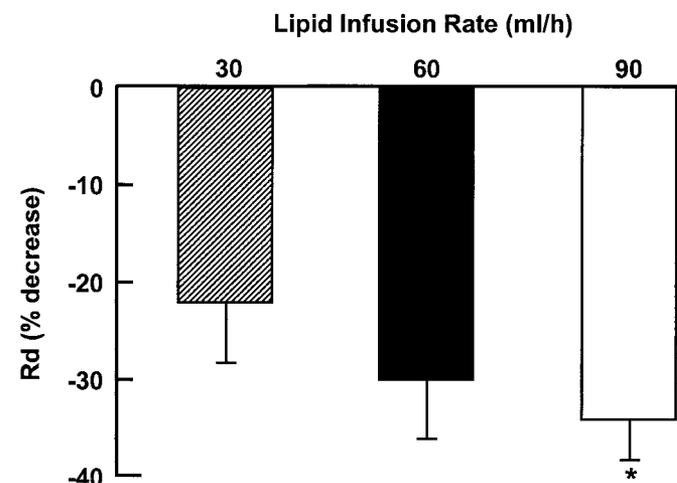


FIG. 2. Effect of lipid infusion (Liposyn III, a 20% triglyceride emulsion) at rates of 30, 60, and 90 ml/h on the percent decrease (compared with their respective saline control study) in insulin-stimulated total-body R_d . * $P < 0.05$, 90- vs. 30-ml/h lipid infusion rates.

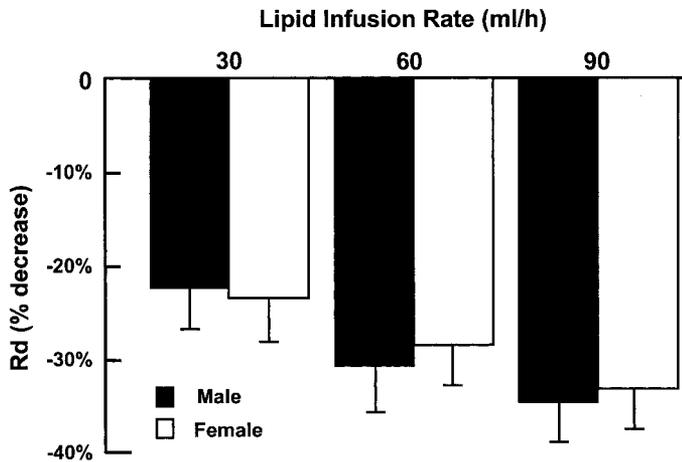


FIG. 3. Percent decrease (compared with their respective saline control study) in insulin-stimulated total-body R_d analyzed by sex following lipid infusion at 30, 60, or 90 ml/h. There was also no effect of sex when all subjects were analyzed together (data not shown).

results are expressed relative to 100% stimulation in control subjects during saline infusion. Comparisons were considered statistically significant if the P value was <0.05 . Normal distribution was checked before all analyses, and nonparametric estimates were used where deemed appropriate. Regression analysis was performed using least squares linear correlation coefficients.

RESULTS

Fasting substrate and hormone concentrations. Before the start of lipid or saline infusion (0500), the fasting plasma glucose, insulin, and FFA concentrations were similar in all groups. The plasma glucose concentration did not change significantly following the 4 h (–30- to 0-min time period) of saline or lipid infusion and was maintained close to the basal level during the 2-h insulin clamp in all groups. The plasma insulin concentration remained unchanged following 4 h of saline or lipid infusion and was increased to similar levels (53–57 $\mu\text{U/ml}$)

during the insulin clamp studies in all three groups. The fasting plasma FFA concentration (0500) was similar in all groups and did not change significantly in the basal state with saline in any group (Table 2). Four hours (–30- to 0-min time period) of lipid infusion at 30, 60, or 90 ml/h significantly increased the plasma FFA concentration by 1.7-fold ($P < 0.01$), ~3.0-fold ($P < 0.001$), and 4.2-fold ($P < 0.0001$), respectively. During the saline infusion studies, insulin similarly suppressed plasma FFA levels by ~85% in all three groups. During 30-, 60-, and 90-ml/h lipid infusion rates, the plasma FFA concentrations during the last 30 min of the insulin clamp were 269 ± 32 , 945 ± 217 , and $1,260 \pm 252 \mu\text{mol/l}$, respectively (all $P < 0.001$ vs. saline control).

Basal and insulin-mediated glucose and lipid metabolism. Basal EGP was not different between groups during saline infusion (low, intermediate, and high lipid infusion = 2.7 ± 0.1 , 2.5 ± 0.1 , and $3.0 \pm 0.3 \text{ mg} \cdot \text{kg lean body mass}^{-1} \cdot \text{min}^{-1}$, respectively, NS; Fig. 1). Lipid infusion did not increase basal EGP in any group (2.7 ± 0.1 , 2.7 ± 0.1 , and $2.9 \pm 0.1 \text{ mg} \cdot \text{kg lean body mass}^{-1} \cdot \text{min}^{-1}$, respectively, NS). During the last 30 min of the insulin clamp, EGP was similar and nearly completely suppressed ($>90\%$) in all groups (data not shown).

Insulin-stimulated R_d was similar in all three groups during the saline infusion studies. Lipid infusion reduced R_d in a dose-dependent manner by 22, 30, and 34% at the low, intermediate, and high lipid infusion rates, respectively (all $P < 0.05$ vs. respective saline studies; Fig. 1). Basal glucose oxidation (5.3 ± 0.4 , 5.0 ± 0.3 , and $5.1 \pm 0.6 \text{ mg} \cdot \text{kg lean body mass}^{-1} \cdot \text{min}^{-1}$) was similar between groups during saline studies and was significantly reduced by lipid infusion (4.0 ± 0.3 , 2.7 ± 0.6 , and $3.4 \pm 0.3 \text{ mg} \cdot \text{kg lean body mass}^{-1} \cdot \text{min}^{-1}$; all $P < 0.01$). During the insulin clamp, both glucose oxidation and nonoxidative glucose disposal were impaired by all three lipid infusion rates

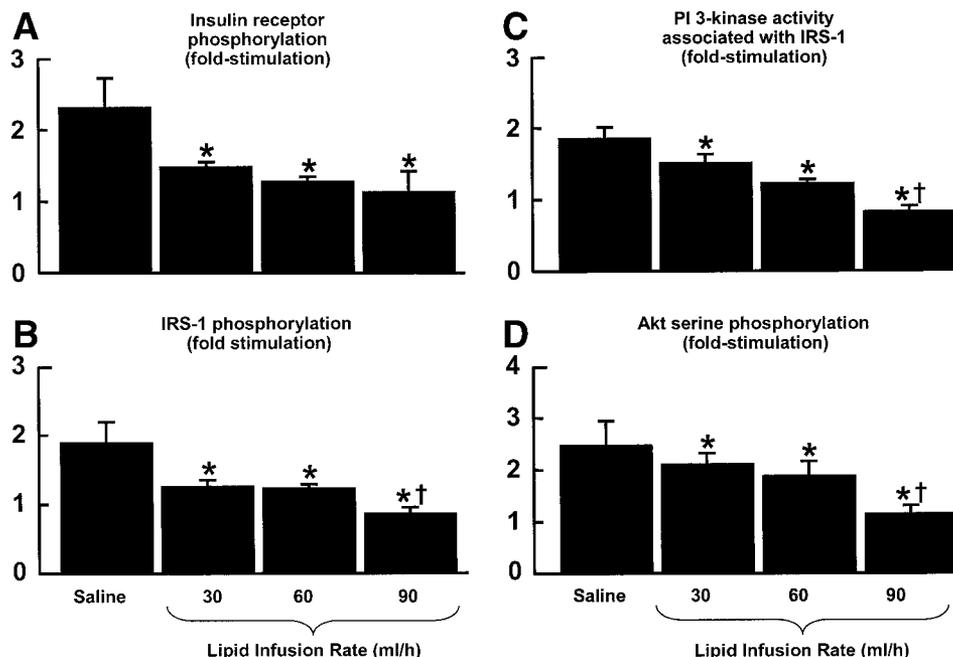


FIG. 4. Effect of saline versus lipid (Liposyn III, a 20% triglyceride emulsion) infusion at 30, 60, and 90 ml/h on the percent reduction in insulin receptor (A) and IRS-1 tyrosine phosphorylation (B), PI 3-kinase activity associated with IRS-1 (C), and serine phosphorylation of Akt (D). * $P < 0.01$ – 0.05 , lipid vs. saline; † $P < 0.05$, 90- vs. 30-ml/h lipid infusion rates.

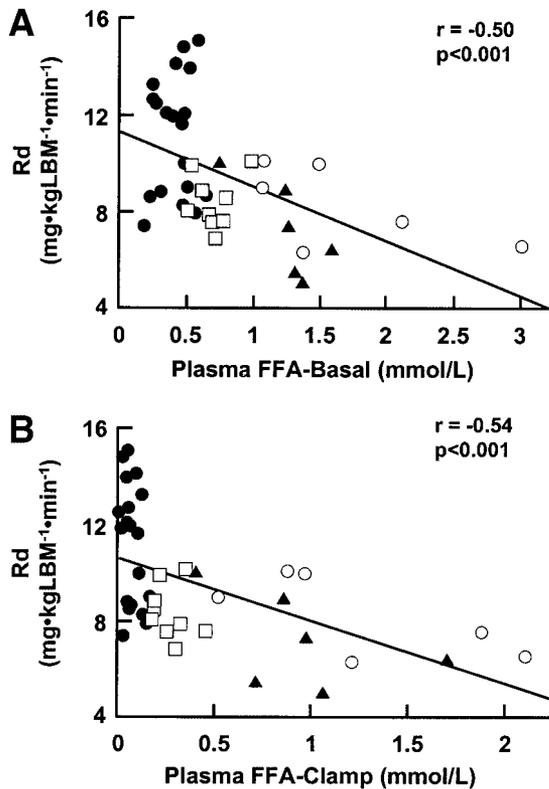


FIG. 5. Inverse correlation between total-body insulin-mediated glucose disposal and plasma FFA concentration during basal (fasting) conditions (A) and during the euglycemic insulin clamp (B). ●, saline infusion; ○, lipid infusion at 90 ml/h; ▲, lipid infusion at 60 ml/h; □, lipid infusion at 30 ml/h. LBM, lean body mass.

(Fig. 1, all $P < 0.01$ vs. saline studies). The percent reduction in total-body glucose disposal paralleled the reduction in absolute rate of total body glucose disposal (Fig. 2). There were no sex differences in the magnitude of the reduction of R_d by lipid infusion (men: from 11.2 ± 0.7 to 8.0 ± 0.5 vs. women: from 11.0 ± 1.0 to 8.1 ± 0.5 $\text{mg}\cdot\text{kg lean body mass}^{-1}\cdot\text{min}^{-1}$) or in the reduction of insulin-stimulated glucose oxidation, nonoxidative glucose disposal, or insulin signaling. The same results held true when possible sex differences in FFA-induced insulin resistance were examined and divided by low, intermediate, or high lipid infusion rates (Fig. 3).

Insulin signaling. During saline studies, insulin increased insulin receptor tyrosine phosphorylation, IRS-1 tyrosine phosphorylation, PI 3-kinase activity associated with IRS-1, and Akt serine phosphorylation by 2.3-fold, 1.9-fold, 1.8-fold, and 2.5-fold above baseline, respectively (all $P < 0.01$) (data from all three groups combined for simplicity of presentation) (Fig. 4). The low-dose lipid infusion significantly inhibited insulin receptor and IRS-1 tyrosine phosphorylation, PI 3-kinase activity associated with IRS-1, and Akt serine phosphorylation compared with the respective saline control ($P < 0.05$ – 0.01). The high-dose lipid infusion caused a significantly greater inhibition of IRS-1 tyrosine phosphorylation, PI 3-kinase activity associated with IRS-1, and Akt serine phosphorylation compared with the low-dose lipid infusion (all $P < 0.05$). The inhibition of the insulin signaling cascade with the intermediate dose infusion was slightly greater ($P < 0.05$ for PI 3-kinase associated with IRS-1) compared with the low-

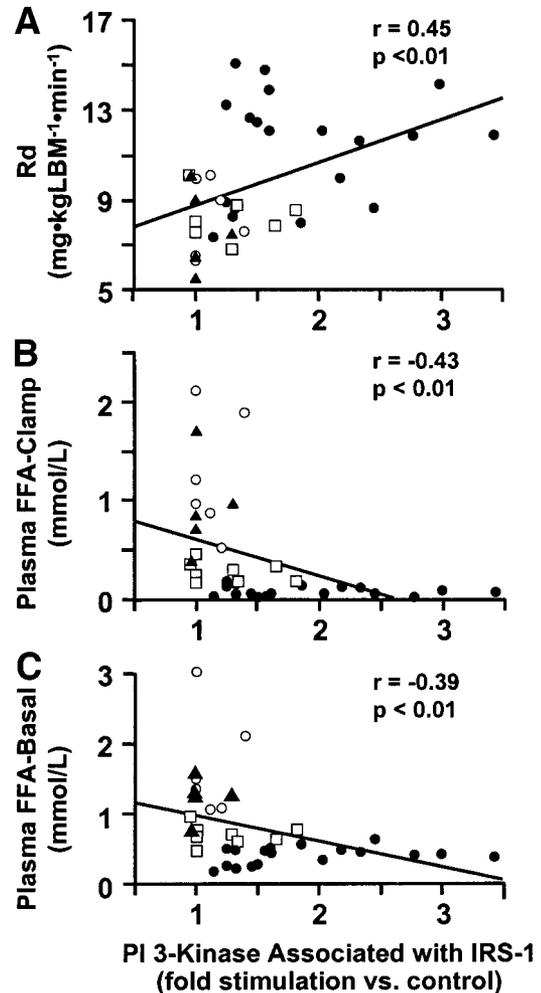


FIG. 6. Correlations between PI-3 kinase activity associated with IRS-1 and insulin-stimulated total-body R_d (A), plasma FFA concentration during the insulin clamp (B), and basal (fasting) plasma FFA concentration (C) performed with saline infusion (●) and lipid infusion at 90 (○), 60 (▲), and 30 (□) ml/h.

dose lipid infusion and significantly less than the high-dose lipid infusion.

Insulin-stimulated glucose uptake (R_d) correlated inversely with both the fasting plasma FFA concentration ($r = -0.50$, $P < 0.001$) and the plasma FFA concentration during the euglycemic insulin clamp ($r = -0.54$, $P < 0.001$) (Fig. 5). Of the insulin signaling elements, PI 3-kinase activity associated with IRS-1 displayed the strongest correlation with R_d ($r = 0.45$, $P < 0.01$) (Fig. 6). Insulin receptor tyrosine phosphorylation ($r = 0.34$, $P < 0.05$), IRS-1 tyrosine phosphorylation ($r = 0.31$, $P < 0.05$), and Akt serine phosphorylation ($r = 0.32$, $P < 0.05$) all showed a weaker but significant correlation with R_d . Both the fasting plasma FFA concentration ($r = -0.39$, $P = 0.01$) and the FFA concentration during the insulin clamp ($r = -0.43$, $P < 0.01$) were inversely correlated with PI 3-kinase associated with IRS-1 during the insulin clamp (Fig. 6).

DISCUSSION

In the present study, we have examined the dose-response relationship between an increase in plasma FFA concentrations and the inhibition of insulin-stimulated glucose disposal and activation of the insulin signaling cascade.

Our results demonstrate that throughout the physiological and pharmacological range, an elevation in plasma FFA concentration inhibits insulin-stimulated whole-body glucose disposal, which primarily reflects muscle glucose metabolism (61). Most importantly, we have demonstrated for the first time that a physiological increase in the plasma FFA concentration from 401 to 695 $\mu\text{mol/l}$ inhibited the ability of insulin to activate the insulin signal transduction cascade. Studies increasing the plasma FFA levels within the physiological range (14,18–20,54) reported the development of insulin resistance and impaired glucose oxidation, but only glycogen synthase activity (a rather distal step in insulin signaling) was examined (19,20,54), and none of the studies examined the FFA dose-response inhibition of molecular pathways of insulin action in skeletal muscle. Prior studies in lean, healthy subjects have shown that elevated FFAs inhibit the insulin signaling system but employed FFA levels that were within the pharmacological range ($>1,500 \mu\text{mol/l}$) (22–24). In this study, at the lowest lipid infusion rate, which increased the plasma FFA concentration only by $\sim 300 \mu\text{mol/l}$, insulin-stimulated whole-body glucose disposal was reduced by 22%, whereas insulin receptor and IRS-1 tyrosine phosphorylation, PI 3-kinase activity associated with IRS-1, and Akt phosphorylation were decreased by 36, 33, 19, and 15%, respectively. At the highest lipid infusion rate (mean plasma FFA 1,688 $\mu\text{mol/l}$), insulin receptor and IRS-1 tyrosine phosphorylation, PI 3-kinase activity associated with IRS-1, and Akt serine phosphorylation were reduced by 52, 54, 55, and 54%, respectively ($P < 0.05$ – 0.01 vs. low-dose lipid infusion), yet insulin-stimulated whole-body glucose disposal was decreased only by an additional 12% (from 22 to 34%). These results indicate that most of the inhibitory effect of elevated plasma FFA on insulin-mediated glucose disposal was observed with a physiological increase in plasma FFA concentration.

FFAs impacted proportionally more insulin signaling (maximally inhibited by 52–55%) than insulin action (reduced by as much as 34%). Such discrepancy has been reported in vitro in myotubes after exposure to palmitate (25–27) and in rats (28) as well as in humans (21) following lipid infusion. Because the inhibitory effect of FFAs on insulin-stimulated glucose disposal is not only dose but also time dependent (17,18,20,22), it is possible that the discrepancy between the effect of FFAs on insulin signaling versus insulin action may be related to a time lag between stimulus and effect. Therefore, one may speculate that if the insulin clamp would have been extended beyond 4 h, the reduction of insulin-stimulated glucose disposal over time might have been of comparable magnitude to that observed on the insulin signaling pathway. Alternatively, given the well-known redundancy of the insulin signaling pathways (62), it may be possible that even an ~ 50 – 55% inhibition of early steps of insulin signaling may be insufficient to impair, to a similar degree, insulin-stimulated glucose disposal. More clinically relevant, while a pharmacologic elevation in plasma FFA produced the greatest inhibition of insulin signaling (Fig. 4), most of the inhibitory effect on insulin-mediated glucose disposal (~ 60 – 70%) was already observed at plasma FFA levels that were well within the physiological range (Fig. 1).

As predicted by the Randle cycle (13), the increase in

lipid oxidation brought about by the increase in plasma FFA concentration was inversely correlated with the decrease in glucose oxidation before ($r = -0.80$, $P < 0.001$) and after ($r = -0.57$, $P < 0.01$) insulin infusion. However, we failed to observe any correlation between lipid oxidation and insulin-stimulated glucose disposal. In contrast, the increase in plasma FFA concentration before ($r = -0.50$, $P = 0.001$) and during ($r = -0.54$, $P < 0.001$) insulin infusion inversely correlated with insulin-stimulated glucose disposal (Fig. 5). Moreover, the increase in plasma FFA concentration was inversely correlated with the decrease in PI 3-kinase activity associated with IRS-1 ($r = -0.43$, $P < 0.01$). No association between increased lipid oxidation and PI 3-kinase associated with IRS-1 was observed. These results serve to further emphasize that it is not the increase in lipid oxidation but the increase in plasma FFA and associated increase in intramyocellular metabolites of FFA metabolism that are responsible for the induction of muscle insulin resistance. The current findings that elevated plasma FFA inhibited IRS-1 tyrosine phosphorylation and PI 3-kinase associated with IRS-1 are in agreement with previous studies from our group (8) and others (22–24), in which lipid infusion inhibited the rise in insulin-stimulated G6P (22–24) and stimulation of early steps of insulin signaling (23,28,29). However, in obese (BMI 32 kg/m^2) insulin-resistant subjects with either normal glucose tolerance or impaired glucose tolerance and a family history of type 2 diabetes, an acute (2-h) or sustained (24-h) lipid infusion failed to cause a worsening in insulin signaling (30). This is in agreement with our recent results in nonobese, insulin-resistant, normal glucose-tolerant subjects with both parents with type 2 diabetes after a physiological increase in plasma FFA for 4 days (8). Discrepancies with the current dose-response study are likely related to differences in 1) patient population (obese insulin-resistant subjects with an already blunted insulin signaling response [30] vs. lean control subjects in the current study), 2) plasma FFA levels achieved (~ 500 – $600 \mu\text{mol/l}$ [1.1- to 1.6-fold increase above basal FFA] [30] vs. ~ 700 to $\sim 1,700 \mu\text{mol/l}$ in our low- to high-dose lipid infusion [1.7- to 4.0-fold increase above basal FFA]), and 3) study design (duration of hyperinsulinemia [2 vs. 4 h], biopsy times [120 vs. 30 min], etc.).

The present results are also consistent with previous findings from our lab of an inhibition of insulin receptor tyrosine phosphorylation following a physiological increase in the plasma FFA concentration (8). Palmitate has been reported to have such an effect in vitro (25) and in vivo in Wistar rats (63), but two previous humans studies (29,64) failed to find any inhibitory effect of elevated plasma FFA on insulin receptor tyrosine phosphorylation. However, in these two studies, the muscle biopsy was performed 4 h (64) and 5 h (29) after the start of insulin. We have previously shown that stimulation of the early steps of the insulin signaling cascade by insulin is maximal at 30 min and begins to wane thereafter (55). Therefore, it is likely that any effect of FFA on insulin receptor tyrosine phosphorylation would have dissipated by performing the muscle biopsy 4–5 h after the start of insulin.

The mechanism by which elevated plasma FFA causes an inhibition of the insulin signaling cascade is most likely related to an increase in intramyocellular metabolites of

FFA and triglyceride metabolism. Using magnetic resonance spectroscopy, Brechtel et al. (35) have demonstrated that lipid infusion for as little as 5 h causes a significant increase muscle lipid content. Increased intramyocellular lipid has been reported to be associated with impaired insulin-stimulated glucose disposal, insulin receptor tyrosine phosphorylation, and PI 3-kinase associated with IRS-1 tyrosine phosphorylation in healthy, normal glucose-tolerant subjects (65). Studies in vitro (25–27,31,66) and in vivo (28,29,32,33,37,38) in animals and humans have provided evidence that increased levels of long-chain fatty acyl CoAs, ceramides, and diacylglycerol can all inhibit the insulin signaling cascade. Both long-chain fatty acyl CoAs and diacylglycerol have been shown to activate protein kinase C, which is phosphorylated by serine and inactivates IRS-1 (28,33,37,67–69). Activation of another serine kinase cascade, the I κ B/NF κ B pathway, also has been implicated in the FFA-induced inhibition of the insulin signaling system (39–41).

The present study is the first to demonstrate that elevated plasma FFA levels inhibit serine phosphorylation of Akt. This observation is consistent with in vitro studies in myotubes in which elevated intracellular ceramide levels, following increased fat supply, have been shown to inhibit serine phosphorylation and activation of Akt (25–27,31,32,70,71). Akt is a downstream effector of PI 3-kinase and when activated enhances glycogen synthase activity and GLUT4 translocation (72,73). Ceramide content is increased in skeletal muscle from obese insulin-resistant humans (38). Taken together, our results indicate that increased plasma FFA may cause insulin resistance in humans by altering skeletal muscle insulin signaling at multiple sites, ranging from early (i.e., insulin receptor phosphorylation, PI 3-kinase activity associated with IRS-1) to rather distal (i.e., Akt activation) steps of insulin action. Finally, in contrast to one report (74), we observed no sex differences in FFA-induced insulin resistance or inhibition of insulin signaling at any lipid infusion dose (30, 60, or 90 ml/h) (Fig. 3), which is consistent with results from our database during either acute (1,3) or chronic (7,8) lipid infusion studies, as well as results from Homko et al. (75).

In summary, an increase in plasma FFA concentrations by lipid infusion causes a dose-dependent decrease in insulin-mediated glucose disposal and inhibition of the insulin signaling cascade. As much as ~60–70% of the maximal inhibition of insulin signaling was observed at plasma FFA levels that are well within the physiological range and involved decreased insulin receptor and IRS-1 tyrosine phosphorylation, reduced association of PI 3-kinase with IRS-1, and impaired serine phosphorylation of Akt. These results confirm the important role of lipid metabolism and of plasma FFA concentrations on muscle glucose homeostasis in humans.

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