# Mechanism of Free Fatty Acid–induced Insulin Resistance in Humans

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## Abstract

**To examine the mechanism by which lipids cause insulin resistance in humans, skeletal muscle glycogen and glucose-6-phosphate concentrations were measured every 15 min by simultaneous $^{13}C$ and $^{31}P$ nuclear magnetic resonance spectroscopy in nine healthy subjects in the presence of low ($0.18 \pm 0.02$ mM [mean$\pm$SEM]; control) or high ($1.93 \pm 0.04$ mM; lipid infusion) plasma free fatty acid levels under euglycemic ($\sim$ 5.2 mM) hyperinsulinemic ($\sim$ 400 pM) clamp conditions for 6 h. During the initial 3.5 h of the clamp the rate of whole-body glucose uptake was not affected by lipid infusion, but it then decreased continuously to be $\sim$ 46% of control values after 6 h ($P < 0.00001$). Augmented lipid oxidation was accompanied by a $\sim$ 40% reduction of oxidative glucose metabolism starting during the third hour of lipid infusion ($P < 0.05$). Rates of muscle glycogen synthesis were similar during the first 3 h of lipid and control infusion, but thereafter decreased to $\sim$ 50% of control values ($4.0 \pm 1.0$ vs. $9.3 \pm 1.6$ $\mu$mol/[kg $\cdot$ min], $P < 0.05$). Reduction of muscle glycogen synthesis by elevated plasma free fatty acids was preceded by a fall of muscle glucose-6-phosphate concentrations starting at $\sim$ 1.5 h ($195 \pm 25$ vs. control: $237 \pm 26$ $\mu$M; $P < 0.01$). Therefore in contrast to the originally postulated mechanism in which free fatty acids were thought to inhibit insulin-stimulated glucose uptake in muscle through initial inhibition of pyruvate dehydrogenase these results demonstrate that free fatty acids induce insulin resistance in humans by initial inhibition of glucose transport/phosphorylation which is then followed by an $\sim$ 50% reduction in both the rate of muscle glycogen synthesis and glucose oxidation. (*J. Clin. Invest.* 1996. 97:2859–2865.) Key words: free fatty acids • muscle glycogen • glucose transport • nuclear magnetic resonance spectroscopy • glucose-6-phosphate**

## Introduction

Non–insulin-dependent diabetes mellitus (NIDDM)[1] is frequently associated with obesity and/or elevation of plasma free fatty acids (1, 2). Over thirty years ago Randle et al. (3, 4) demonstrated that free fatty acids (FFA) effectively compete with glucose for substrate oxidation in isolated rat heart muscle and diaphragms and therefore speculated that increased fat oxidation might cause the insulin resistance associated with diabetes and obesity. The postulated mechanism has been that increased free fatty acid oxidation causes elevation of the intramitochondrial acetyl-CoA/CoA and NADH/$NAD^+$ ratios with subsequent inactivation of pyruvate dehydrogenase (Fig. 1). This in turn causes citrate concentrations to increase which leads to inhibition of phosphofructokinase and subsequent accumulation of glucose-6-phosphate. Finally, increased concentrations of glucose-6-phosphate would inhibit hexokinase II resulting in decreased glucose uptake (2–4).

Subsequent studies in healthy humans have revealed that lipid/heparin infusions, which raise plasma FFA levels, inhibit whole-body glucose disposal during hyper- and euglycemic-hyperinsulinemia (5–7) and insulin-dependent glucose uptake by human forearm tissues in vivo (8). These data support the operation of Randle's glucose-fatty acid cycle in humans since the decrease in insulin-dependent glucose uptake can be accounted for by a fat-induced defect in carbohydrate oxidation (6–8). However, several other studies could not detect an inhibitory effect of fatty acids on insulin-mediated glucose uptake in healthy humans (9), obese humans (10), and patients with NIDDM (11, 12). The failure to demonstrate this effect has been attributed to the insufficient duration of the triglyceride/heparin infusion, since fat-induced inhibition of glucose uptake might develop after more than 3 h of fat infusion (13, 14). However, other studies (8, 11, 15) have found no inhibitory action of fatty acids on nonoxidative glucose metabolism even when triglyceride/heparin was infused for 4 h.

Recently, Boden et al. (13, 14) have provided evidence that a reduction in carbohydrate oxidation is responsible for only one-third, while impairment of nonoxidative glucose metabolism, which mostly reflects glycogen synthesis (16, 17), accounted for two-thirds of the fatty acid–dependent decrease in glucose uptake. These workers have suggested that two different defects contribute to the impairment of glycogen synthesis depending on the FFA concentration. At FFA concentrations of $\sim$ 0.75 mM they found increased intramuscular glucose-6-

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1. *Abbreviations used in this paper:* NMR, nuclear magnetic resonance spectroscopy; NIDDM, non–insulin-dependent diabetes mellitus; RF, radio frequency.

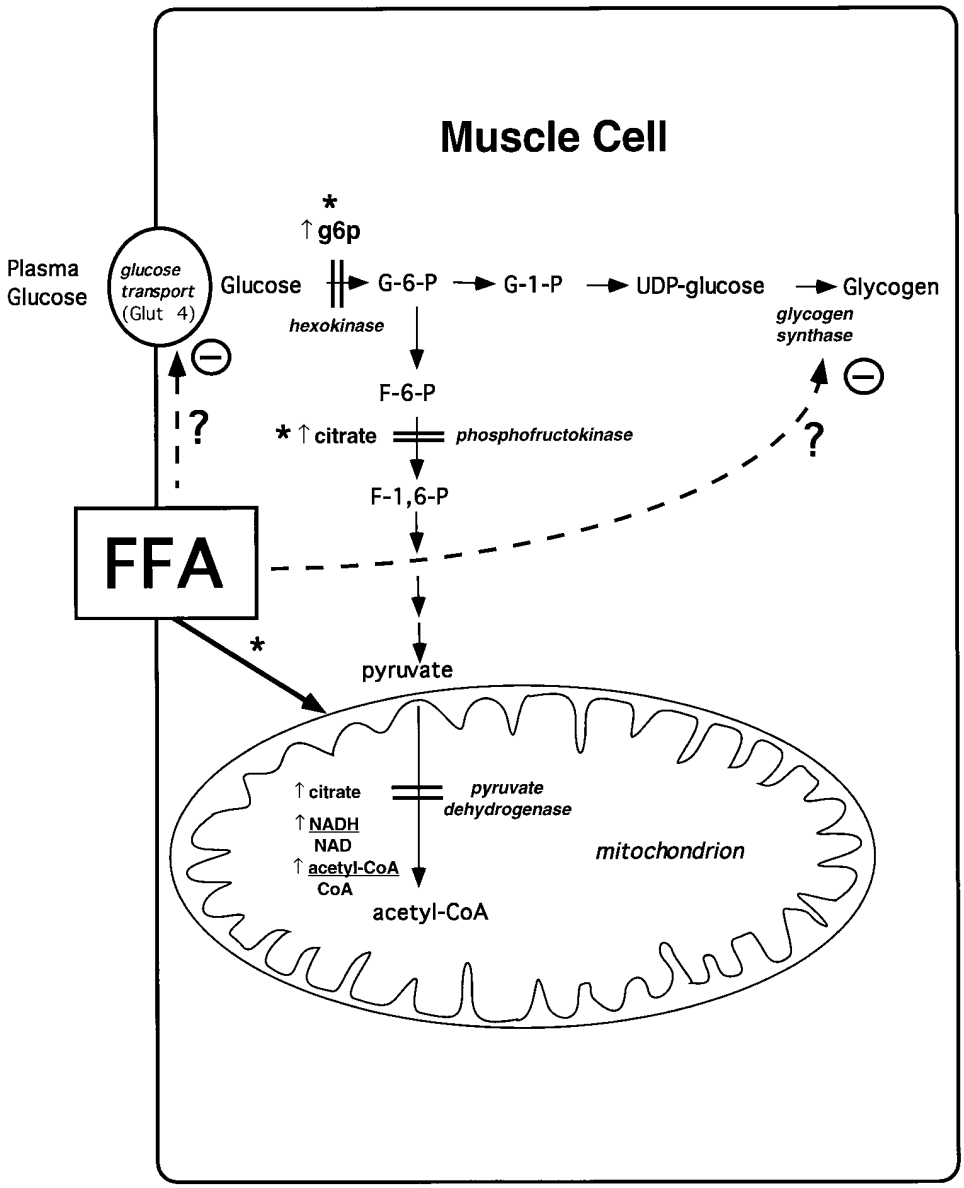


*Figure 1.* Schema of potential sites of free fatty acid action on insulin mediated glucose metabolism in skeletal muscle and those sites (*) hypothesized to be affected by Randle et al. (3). *g6p*, glucose-6-phosphate; *G-1-P*, glucose-1-phosphate; *F-6-P*, fructose-6-phosphate, *F-1,6,-P*; fructose 1,6 biphosphate.

phosphate concentrations, as measured in muscle biopsies, suggesting a FFA-induced inhibition of glycogen synthase, whereas at FFA concentrations of $\sim$ 0.5 mM they were not able to find any difference in intramuscular glucose-6-phosphate. Nevertheless these workers went on to speculate that at this lower FFA concentration, decreased muscle glucose transport/phosphorylation accounted for the reduced rates of muscle glucose uptake. In contrast, no inhibitory action of fatty acids on nonoxidative glucose metabolism was found by other authors (8, 11, 15), nor did fat infusion affect the activities of glycogen synthase (15, 18) or of pyruvate dehydrogenase (15) in biopsies from human m. vastus lateralis muscle. The interpretation of the results of enzyme activities measured in vitro is obscured by several limitations: (*a*) using muscle biopsies, measurements are limited to a few time points; (*b*) enzyme activities do not necessarily reflect in vivo substrate flux; and (*c*) measurements of glucose-6-phosphate concentrations in human muscle biopsies are artifactually high due to glycogen breakdown between sample excision and freezing (19). The experiments performed in this report were designed to overcome these limitations by performing in vivo nuclear magnetic resonance (NMR) spectroscopy on the gastrocnemius muscle of young healthy subjects during long-term infusion of triglycerides/heparin in the presence of euglycemic-hyperinsulinemia. $^{13}$C-NMR spectroscopy was used to provide continuous quantitative information on the rate of muscle glycogen synthesis (17) while simultaneous $^{31}$P-NMR spectroscopy was used to monitor changes in glucose-6-phosphate concentrations in gastrocnemius muscle in vivo (20).

## Methods

*Subjects.* Nine healthy volunteers (eight males, one female; age range: 22–46 yr; body wt: 70.3±2.5 kg; body surface area: 1.85±0.5 m$^2$; body mass index: 23.0±0.5 kg/m$^2$) without family history of diabetes mellitus, dyslipidemia, or bleeding disorders were given an isocaloric diet [35 kcal/(kg · d); carbohydrate/protein/fat: 60/20/20%] for 2 d and then fasted overnight for 12 h before the studies. Informed

consent was obtained from all subjects after the nature and possible consequences of the studies were explained to them. The protocol was reviewed and approved by the Human Investigation Committee of the Yale University School of Medicine.

*Euglycemic-hyperinsulinemic clamps.* All studies were begun at 7:30 a.m. with insertion of Teflon® catheters in antecubital veins of the right and left arm for blood sampling and glucose/lipid/hormone infusions, respectively. To study insulin-dependent effects on glucose/glycogen metabolism, euglycemic-hyperinsulinemic glucose clamps (21) were performed to create conditions of standardized hyperinsulinemia of $\sim$ 400 pM during constant basal plasma concentrations of glucose (euglycemia; $\sim$ 5.2 mM). Insulin (Humulin Regular; Eli Lilly and Co., Indianapolis, IN) was administered as a primed-continuous infusion [1 mU/(kg · min)] and the infusion of [1-$^{13}$C] glucose (10–20% enriched) was periodically adjusted to maintain euglycemia based on the plasma glucose concentrations, which were obtained in 5–8-min intervals. To test the effects of free fatty acids, the plasma concentration of FFAs was increased by intravenous infusion of a triglyceride emulsion (1.5 ml/min; Liposyn II, Abbott Laboratories, North Chicago, IL) combined with heparin [bolus: 200 IU; continuous infusion: 0.2 IU/(kg · min)], which was used to stimulate lipoprotein lipase and thereby to catalyze hydrolysis of triglycerides. During the control experiments glycerol [0.7 mg/(kg · min)], which is also contained in the triglyceride emulsion, was infused to control for effects of glycerol per se. All subjects participated in both experimental protocols, which were spaced by an interval of 3–8 wk.

*In vivo $^{13}$C-NMR spectroscopy.* To allow simultaneous, repetitive monitoring of muscle glycogen and glucose-6-phosphate, the subjects remained in supine position with the right leg placed inside a 4.7 T Biospec NMR spectrometer system (Bruker Instruments, Inc., Billerica, MA) with a 30-cm diameter magnet bore to obtain interleaved $^{13}$C- (17) and $^{31}$P-spectra (20) from the gastrocnemius muscle before and every 15-min from time 0 to 360 min during the clamp, respectively. The spectrometer was equipped with a modified radio frequency (RF) relay switch that allowed the hardware to switch the transmit RF power between $^{13}$C (50.4 MHz) and $^{31}$P (81.1 MHz) channels with a 10-μs switching time. A modified pulse sequence allowed switching of the acquisition parameters and preamplifiers between the two channels during the 10-μs switching time. A 5.1-cm diameter circular $^{13}$C-$^{31}$P double-tuned surface coil RF probe was used for interleaved acquisitions (22, 23). The double-tuned circuit was optimized for the $^{31}$P channel so that the NMR sensitivity would be enhanced to detect glucose-6-phosphate. Shimming, imaging, and $^{1}$H decoupling at 200.4 MHz were performed with a 9 × 9 cm series butterfly coil. Proton water linewidths were shimmed to $<$ 50 Hz. A microsphere containing $^{13}$C and $^{31}$P reference standards was fixed at the center of the double-tuned RF coil for calibration of RF pulse widths. Subjects were positioned by an image-guided localization routine that employed a $T_1$-weighted gradient-echo image (TR = 82 ms, TE = 21 ms). The subjects' lower legs were typically positioned so that the isocenter of the magnetic field was $\sim$ 1 cm into the medial head of the gastrocnemius muscle. By determining the 180° flip angles at the center of the observation coil from the microsphere standard, RF pulse widths were set so that the 90° pulse was at the center of the muscle. This maximized suppression of the lipid signal that arises from the subcutaneous fat layer and optimized the signal derived from the muscle. The interleaved $^{1}$H decoupled $^{13}$C-$^{31}$P RF pulse sequence was designed so that 72 $^{31}$P transients were acquired during the same period that 2,736 $^{13}$C transients were obtained (38 $^{13}$C transients per $^{31}$P relaxation period). The repetition time for $^{31}$P acquisition was 4.6 s to allow for relaxation of the long $T_1$ $^{31}$P resonances. Power deposition, assessed by magnetic vector potential specific absorption rate calculation was $<$ 4 W/kg. The total scan time for each interleaved spectrum was 5.5 min. Intramuscular glycogen concentrations were determined by comparison with an external standard solution (150 mM glycogen + 50 mM KCl) in a cast of the subject's leg that electrically loaded the RF coil the same as subject legs (24). $^{13}$C spectra were processed by methods that have been described in detail previously (24). Briefly, Gaussian broadened spectra (30 Hz) were baseline corrected ±1,000 Hz on either side of the 1-$^{13}$C glycogen resonance of both subject spectra and standard spectra. Integrated peak areas were then assessed ±200 Hz about the resonance. The $^{13}$C-NMR technique for assessing intramuscular glycogen concentrations has been validated by comparison with biopsied human gastrocnemius muscle tissue samples (25). Intramuscular glucose-6-phosphate was quantified by comparison with the β-ATP resonance as an internal reference standard assuming a constant concentration of 5.5 mM for resting muscle ATP (20). This method has previously been validated in an animal model (26).

*Respiratory exchange measurements.* Continuous indirect calorimetry was performed for 20 min before and at 90–110, 150–170, 210–230, 270–290, and 330–350 min during the clamp to determine rates of whole-body glucose oxidation. From these data and from the amount of nitrogen excreted in the urine, nonoxidative glucose metabolism and rates of lipid and protein oxidation were calculated (17).

*Analytical procedures.* Plasma glucose concentrations were measured by the glucose oxidase method (Glucose analyzer II; Beckman Instruments Inc., Fullerton, CA). Plasma concentrations of lactate and of triglycerides (Sigma Chemical Co., St.Louis, MO) were measured by using enzymatic methods. Plasma concentrations of FFAs and of glycerol were determined using microfluorimetric methods. Plasma immunoreactive insulin was determined by a double antibody RIA (Diagnostic Systems Laboratories, Inc., Webster, TX). Plasma [1-$^{13}$C] glucose atom percent excess was measured using gas chromatography-mass spectrometry (17, 24). Plasma alanine concentrations were determined by an automated amino acid analyzer (Dionex, Sunnyvale, CA).

*Calculations and data analysis.* Increments in muscle glycogen concentration were determined from the change in [1-$^{13}$C] glycogen concentration and the plasma [1-$^{13}$C] glucose atom percent excess as described previously (17, 24). Rates of glycogen synthesis were then calculated from the slope of the least-square linear fit to the glycogen concentration curve during the given time periods. All data are presented as means±SEM. Statistical comparisons between control and lipid infusion experiments were performed by using the paired Student's *t* test.

## Results

Basal plasma concentrations of glucose, insulin, and free fatty acids were not different between control and lipid infusion studies (Fig. 2). During the euglycemic-hyperinsulinemic clamps plasma glucose concentrations remained constant compared to the basal period and were not different between control and lipid infusion studies (Fig. 2 *A*). Plasma insulin concentrations increased similarly to $\sim$ 400 pM in both studies (Fig. 2 *B*). In the control study the plasma concentration of FFAs dropped by $\sim$ 70% once the euglycemic clamp was started, but it increased approximately fourfold within 90 min of the lipid infusion (Fig. 2 *C*). Basal plasma concentrations of triglycerides (lipid infusion: 1.15±0.14, control: 1.13±0.20 g/liter) and glycerol (0.27±0.01 vs. 0.28±0.02 mM) were not different between the studies however during the lipid infusion plasma concentrations of triglycerides (7.02±0.85 vs. 0.93±0.16 g/liter; $P < 0.0001$) and of glycerol (1.29±0.08 vs. 0.39±0.02 mM; $P < 0.0001$) increased. Basal plasma concentrations of lactate were 0.70±0.07 vs. 0.57±0.04 mM (lipid infusion vs. control) and alanine were 0.36±0.03 vs. 0.30±0.03 mM (lipid infusion vs. control). During the last hour of lipid infusion (300–360 min) plasma concentrations of lactate (lipid infusion; 0.71±0.23 vs. control; 1.28±0.13 mM, $P < 0.02$) and alanine (lipid infusion; 0.22±0.01 vs. control; 0.31±0.02 mM,

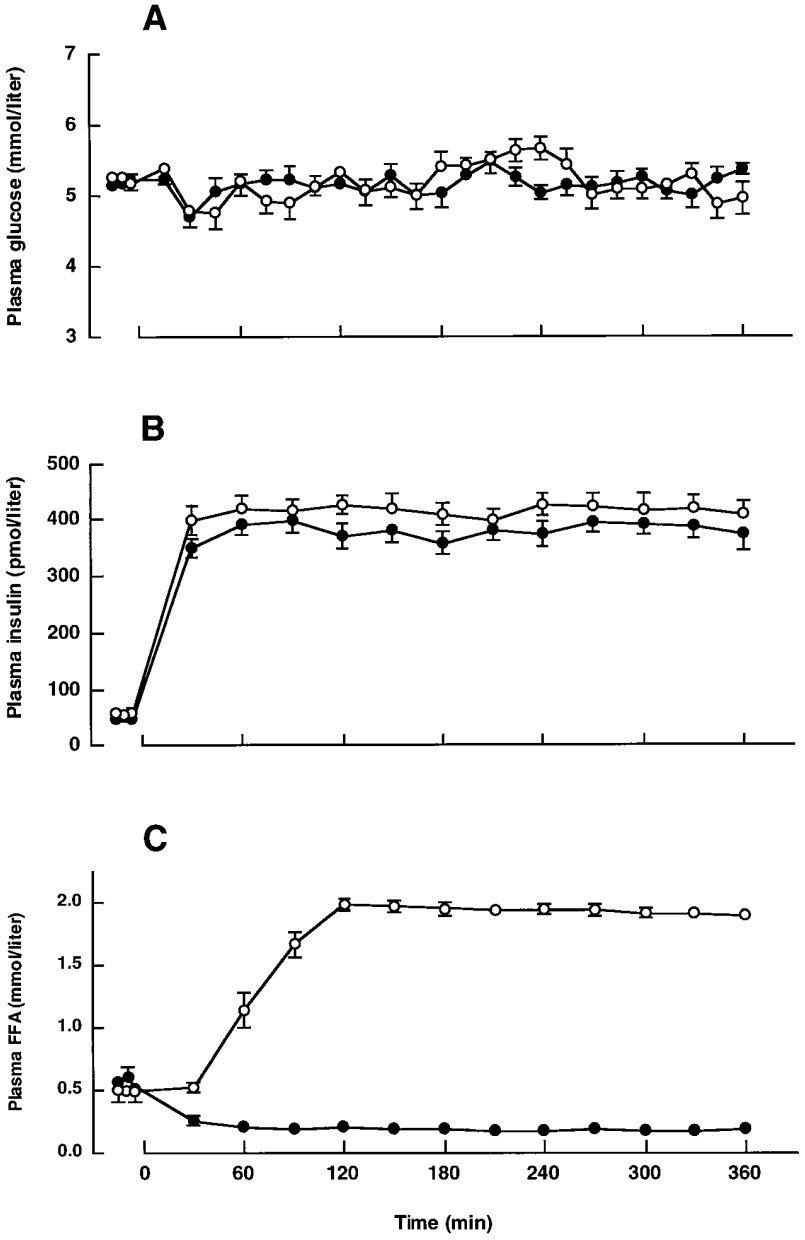


*Figure 2.* Plasma concentrations of glucose (*A*), insulin (*B*), and free fatty acids (*C*) at low (*closed symbols*) and at elevated plasma free fatty acid concentrations (*open symbols*). All units expressed as means±SEM of nine paired studies.

$P < 0.001$) were both significantly lower than during the control study.

During the first 3.5 h of the clamp test, whole-body glucose metabolism as reflected by the glucose infusion rates was stimulated in a similar fashion by insulin at low and at high plasma FFA concentrations (Fig. 3 *A*). After that time glucose infusion rates decreased progressively during lipid infusion (210–230 min: lipid infusion; 40.3±3.9 vs. control; 52.1±3.1 μmol/[kg · min], $P < 0.01$) declining to ~46% of the corresponding control values during the last hour of the clamp (330–350 min: lipid infusion; 25.7±2.6 vs. control; 57.2±3.5 μmol/[kg · min], $P < 0.00001$).

During lipid infusion the mean respiratory quotient (RQ) was lower starting with the first recording between 90 and 110 min (lipid infusion; 0.84±0.02 vs. control; 0.90±0.01, $P < 0.05$), and it continued to decrease until the last hour (lipid infusion; 0.79±0.03 vs. control; 0.96±0.01, $P < 0.00001$) reflecting increased lipid oxidation (90–110 min: lipid infusion; 0.53±0.13 vs. control; 0.11±0.03 mg/[kg · min], $P < 0.05$, 330–350 min: lipid infusion; 0.82±0.08 vs. control; 0±0.06 mg/[kg · min], $P < 0.05$). In contrast to the progressive increase in glucose oxidation rate observed in the control study augmented lipid oxidation in the lipid infusion study blunted the increase in glucose oxidation rate resulting in a significantly decreased rate of glucose oxidation compared to the control study [t = 150 ($P < 0.02$), t = 210 ($P = 0.005$), t = 270 ($P < 0.0002$), t = 330 ($P < 0.00005$)] (Fig. 3 *B*). It is of note that decreased glucose oxidation failed to affect whole-body glucose metabolism before 210 min (glucose oxidation, 150–170 min: lipid infusion: 7.1±1.7 vs. control: 12.1±1.3 μmol/[kg · min], $P < 0.05$). The rate of protein oxidation also gradually decreased during the lipid infusion (330–350 min, lipid infusion; 0.89±0.07 vs. control; 1.08±0.07 mg/[kg · min], $P < 0.01$).

Basal concentrations of skeletal muscle glycogen were

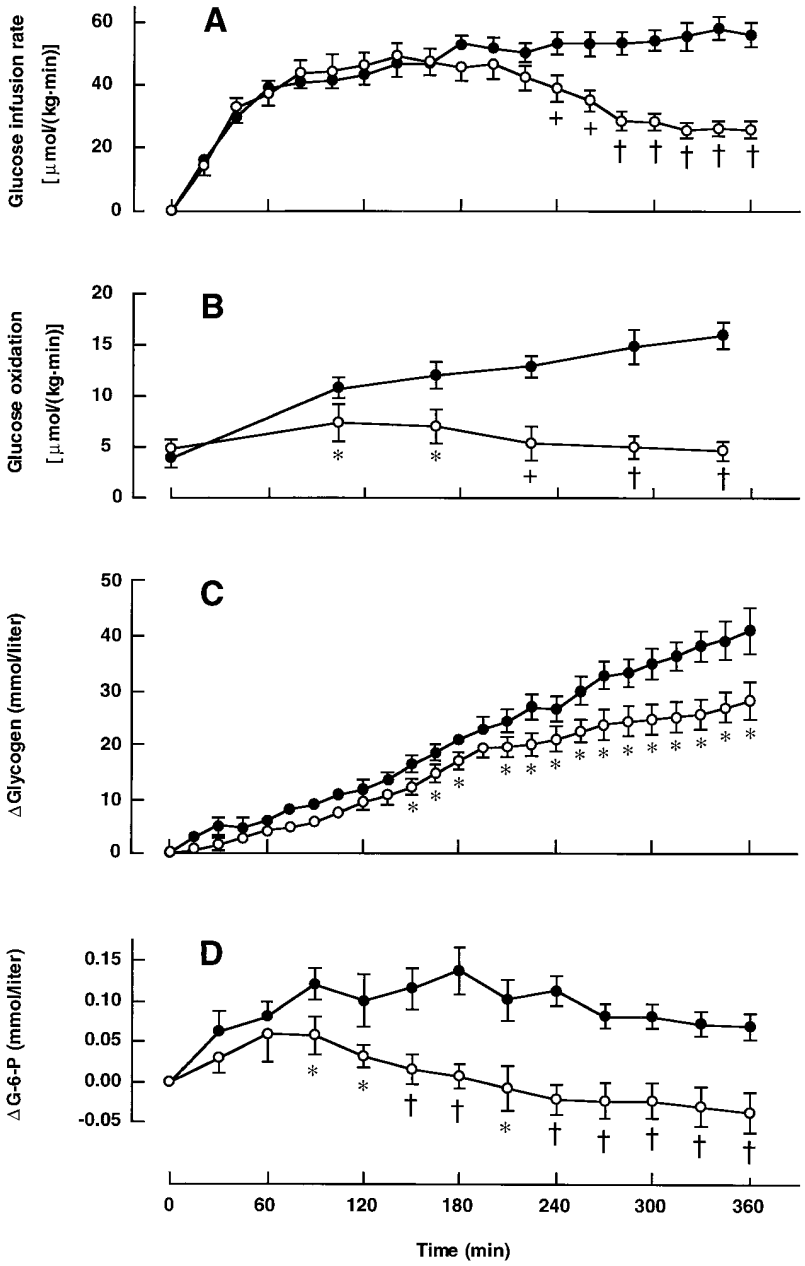


*Figure 3.* Glucose infusion rate (*A*), glucose oxidation rate (*B*), increase in calf muscle glycogen (*C*), and increase in calf muscle glucose-6-phosphate (*D*) at low (*closed symbols*) and at elevated plasma free fatty acid concentrations (*open symbols*). All units expressed as means±SEM of nine paired studies. $^{*}P < 0.05$, $^{+}P < 0.01$, $^{\dagger}P < 0.001$.

85±5 and 72±6 mM in the control and lipid infusion studies, respectively. The insulin-dependent increase in skeletal muscle glycogen concentration was not different during the first 210 min, but thereafter declined during the lipid infusion to ~ 44% ($P < 0.05$) of the corresponding control values at 360 min (Fig. 3 *C*). Net rates of muscle glycogen synthesis (Vsyn) were: 94±11 (0–90 min), 134±10 (90–180 min), 122±13 (180–270 min), and 93±16 μmol/[liter.min] (270–360 min) under conditions of low plasma concentrations of free fatty acids. Elevated FFA concentrations did not affect net glycogen synthetic rates during the first 3 h (0–90 min: 85±10, 90–180: 120±15 μmol/[liter · min]), whereas thereafter Vsyn decreased by ~ 50% compared with the control studies (180–270 min: 65±20, $P < 0.05$, 270–360 min: 40±10 μmol/[liter · min], $P < 0.05$). Similarly, nonoxidative glucose metabolism was not different between lipid infusion and control experiments during the first 4 h: lipid infusion; 30.9±2.8 vs. control; 38.1±5.2, NS (90–110 min), lipid infusion; 39.7±3.5 vs. control; 38.4±3.3, NS (150–170 min), lipid infusion; 34.8±3.6 vs. control; 39.1±3.3 μmol/[kg · min], NS (210–230 min). Thereafter, nonoxidative glucose metabolism decreased to ~ 50% during the lipid infusion: lipid infusion; 22.7±2.8 vs. control; 39.0±4.1, $P < 0.005$ (270–290 min) and lipid infusion; 21.0±2.2 vs. control; 41.2±3.9 μmol/[kg · min], $P < 0.0005$ (330–350 min).

During lipid infusion absolute concentrations of muscle glucose-6-phosphate were always lower than those of the control experiments (Fig. 3 *D*): lipid infusion: 195±25 vs. control: 237±26 μM, $P < 0.01$ (90–110 min), lipid infusion: 152±16 vs. control: 231±31 μM, $P < 0.01$ (150–170 min), lipid infusion: 129±22 vs. control: 217±20 μM, $P < 0.01$ (210–230 min), lipid

infusion: 113±14 vs. control: 197±15 μM, $P < 0.00005$ (270–290 min), and lipid infusion: 107±16 vs. control: 188±12 μM, $P < 0.0005$ (330–350 min).

## Discussion

The time course of nonoxidative glucose metabolism paralleled that of muscle glycogen synthesis, consistent with muscle glycogen synthesis being the predominant route of insulin-stimulated nonoxidative glucose metabolism (16, 17). The observed reduction in nonoxidative glucose metabolism/muscle glycogen synthesis could be due to an FFA-induced reduction in muscle glycogen synthase activity, which would be expected to result in an increase in intracellular glucose-6-phosphate concentration. In support of this possibility some studies have found decreased insulin-stimulated fractional velocities of glycogen synthase in skeletal muscle biopsies obtained after 4–6 h of lipid infusion (7, 13, 14), while others have reported no change in glycogen synthase activity (15, 18). Alternatively, inhibition of muscle glycogen synthesis could be accounted for by impaired glucose transport/phosphorylation, which would be mirrored by a decrease of skeletal muscle glucose-6-phosphate concentration. In support of this latter possibility we found that the insulin-dependent increase in intramuscular glucose-6-phosphate concentration was lower ($P < 0.01$) at 90 min and was followed by a continuous decrease to and even below baseline values in the presence of elevated plasma free fatty acid concentration (Fig. 3 *D*). The finding that intramuscular glucose-6-phosphate concentrations were lower during the lipid infusion study than during the control study does not support the original mechanism as proposed by Randle and co-workers in which glucose uptake is decreased by increased FFA levels due to inhibition of hexokinase II resulting from an increased intracellular concentration of glucose-6-phosphate (2–4, 27, 28). Instead, these results suggest an inhibitory effect of fatty acids on glucose transport/phosphorylation. As a result of its location between glucose transport/phosphorylation and synthase enzymes in the pathways of glycogen synthesis the concentration of muscle glucose-6-phosphate is sensitive to the relative activities of these enzymes and the rate of glycolysis. While it is theoretically possible that an increased rate of glycolysis in the lipid infusion studies could explain the lower glucose-6-phosphate concentrations observed in these studies, this is very unlikely since any increase in glycolytic lactate production would be included in the measured rate of nonoxidative glucose metabolism which was ~ 50% lower during the last 90 min of the lipid infusion studies. Furthermore, the lower plasma concentrations of alanine and lactate observed in the lipid infusion studies suggest that rates of glycolysis were lower in these studies compared to the control studies. Finally a higher rate of oxidative glycolysis would be reflected by an increase in rates of glucose oxidation which were also significantly lower in the lipid infusion studies compared to the control studies.

Whether FFA induces its inhibitory effect on glucose uptake through inhibition of glucose transport and/or hexokinase cannot be discerned from the present data. GLUT4 expression has been shown to be suppressed in skeletal muscle from high fat-fed rats whose FFA levels are not suppressed during insulin infusion but to be unchanged in genetically obese Zucker rats which present with increased plasma FFA levels (29). Decreased glucose transport could also be accounted for by reduced intrinsic activity of GLUT4 despite normal insulin-stimulated GLUT4 translocation as reported for high fat–fed rats (30). The FFA-induced decrease in skeletal muscle glucose-6-phosphate concentration could also be due to impaired glucose phosphorylation by hexokinase II, which is insulin-dependent and could become a rate controlling step (31). However, no changes in insulin-stimulated hexokinase activity in biopsies from human vastus lateralis muscle were found after 6 h of lipid infusion combined with a 4-h hyperinsulinemic-euglycemic clamp (18).

In conclusion, contrary to the classical mechanism of free fatty acid–induced insulin resistance as proposed by Randle et al. (2, 27, 28) in which free fatty acids exert their effect through initial inhibition of pyruvate dehydrogenase, we found that elevation in plasma free fatty acid concentration causes insulin resistance by inhibition of glucose transport and/or phosphorylation with a subsequent reduction in rates of glucose oxidation and muscle glycogen synthesis. This reduction in insulin-inducible glucose transport/phosphorylation is similar to what is observed in patients with NIDDM (20) and their normoglycemic-insulin–resistant offspring (24) and suggests that alterations in intramuscular FFA metabolism may play an important role in the pathogenesis of the insulin resistance observed in patients with NIDDM.

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