The effects of increased plasma free fatty acids (FFA) on insulin-dependent whole body glucose disposal, skeletal muscle glycolysis, glycogen synthesis, pyruvate versus FFA/ketone oxidation, and glucose 6-phosphate (Glu-6-P) were investigated in the awake rat. A control group (glycerol-infused) and high plasma FFA group (Liposyn-infused) were clamped at euglycemia (−6 mEq)-hyperinsulinemia (10 milliunits/kg/min) throughout the experiment (180–240 min). In the initial experiment, 13C NMR was used to observe [1-13C]glucose incorporation into [1-13C]glycogen in the rat hindlimb for glycogen synthesis calculations and into [3-13C]lactate and [3-13C]alanine for glycolytic flux calculations. These experiments were followed by 31P NMR measurements of Glu-6-P changes under identical conditions of the initial experiment. Plasma FFA concentrations were 2.25 ± 0.36 and 0.20 ± 0.03 mEq in the high plasma FFA and control groups respectively (p < 0.0005). Glucose infusion rates (Ginf) decreased significantly in the Liposyn-control groups respectively (0.36 and 0.20 mEq/min) versus 0.03 mEq/min in the high plasma FFA and control groups, respectively at 15 min to 30.7 ± 2.3 and 17.7 ± 1.3 mg/kg/min, respectively, at the end of the experiment, p < 0.002. Glycogen synthesis rates were 163 ± 32 and 104 ± 17 nmol/g/min, and glycolytic rates were 57.9 ± 8.0 and 19.5 ± 3.6 nmol/g/min (p < 0.002) in the control and high plasma FFA groups, respectively. The relative flux of pyruvate versus free fatty acids and ketones entering the tricarboxylic acid cycle was greater in the control (57 ± 9%) versus high plasma FFA group (25 ± 4%) (p < 0.005) as assessed by [4-13C]glutamate/3-13C]lactate steady state isotopic enrichment measurements. Finally, Glu-6-P concentrations increased by 29.8 ± 7.0 and 52.8 ± 12.3% (p < 0.05) in the control and high plasma FFA groups, respectively, above their basal concentrations by 180 min.

In conclusion, we have demonstrated the ability to use in vivo NMR to elucidate the metabolic fate of glucose within skeletal muscle of an awake rat during a euglycemic-hyperinsulinemic clamp and increased levels of plasma FFA. These data suggest that increased concentrations of plasma FFA inhibit insulin-stimulated muscle glucose metabolism in the rat through inhibition of glycolysis.

13C and 31P NMR Studies on the Effects of Increased Plasma Free Fatty Acids on Intramuscular Glucose Metabolism in the Awake Rat

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Increased levels of plasma free fatty acids (FFA) are prevalent in people with non-insulin-dependent diabetes mellitus and may play an important role in mediating the insulin resistance associated with this disease. A decrease in insulin-dependent glucose uptake by increased FFA was originally observed in vitro in heart and diaphragm muscle by Randle et al. (1). This phenomenon was postulated to be a result of pyruvate dehydrogenase (PDH) enzyme allosteric control by increased intramitochondrial acetyl-CoA/CoA and NADH/NAD+ ratios that activates pyruvate dehydrogenase kinase enzyme which subsequently deactivates the PDH enzyme complex (2). Intramitochondrial citrate also increases under conditions of increased FFA oxidation and decreased flux through isocitrate dehydrogenase. Citrate is a potent allosteric effector of phosphofructokinase (3) and therefore inhibits this step which will increase glucose 6-phosphate (Glu-6-P). Increased Glu-6-P will allosterically inhibit hexokinase (4). Therefore, increased intracellular glucose will diminish the glucose gradient, and glucose transport into the muscle cell will also be inhibited. This metabolic phenomenon, also known as the glucose-free fatty acid cycle, has more recently come into question in skeletal muscle as conflicting results have been presented.

Previous studies in rat have shown that an increase in plasma FFA concentration caused an increase (5), decrease (6–8), or no change (9, 10) in skeletal muscle or whole body glucose uptake. Discrepancies within these previous findings may in large be due to the vastly different techniques used to elucidate skeletal muscle and/or whole body glucose uptake.

Most in vivo studies (6, 11–15) that have examined the effects of increased plasma FFA on whole body glucose uptake have relied on indirect calorimetry or measurements of plasma tritium derived from [3-3H]glucose to provide indirect measurements of glycolysis and fat oxidation (indirect calorimetry) and reflect average whole body glycolytic and FFA oxidation measurements. Due to the quantitatively important contribution of other organs to whole body glycolysis (brain) and whole body FFA oxidation (liver and heart), rates of glycolysis and FFA oxidation must be measured directly in skeletal muscle to accurately assess the effects of FFA on these processes in this tissue.

To address these issues, we have developed a method using in vivo 13C NMR to directly measure rates of glycolysis and glycogen synthesis simultaneously in skeletal muscle of awake rats, and additional measurements in skeletal muscle tissue extracts were used to calculate relative rates of pyruvate versus FFA/ketone oxidation. These measurements were combined with 31P NMR temporal measurements of Δ[Glu-6-P] to assess the mechanism for decreased insulin-dependent glucose uptake.

1 The abbreviations used are: FFA, free fatty acid; PDH, pyruvate dehydrogenase; HGP, hepatic glucose production rate; APE, atom percent excess.

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in rat muscle under conditions of euglycemia-hyperinsulinemia and increased plasma FFA.

**EXPERIMENTAL PROCEDURES**

**Animals**—Harlan Sprague Dawley rats (Charles River, Raleigh, NC) were maintained on standard rat chow (Ralston Purina Co., St. Louis, MO) and housed in an environmentally controlled room with a 12-h light/dark cycle. At weights between 250 and 300 g, rats were chronically catheterized in the right jugular vein and carotid artery. The catheters were externalized through an incision in the skin flap behind its head (16). The rats were allowed to recuperate after surgery for the indwelling catheters until they were at least of preoperative weight (approximately 5–10 days). All rats were fasted 24 h before the infusion experiment. Rats (250–350 g) were placed in a customized restraining tube that allowed their left hindlimb to be secured to the outside of the tube in a manner to limit free movement of the leg for NMR measurements. The rats were transiently anesthetized (<30 s) with a low dose (2.5 mg) of thiopental (Sigma) to place them in the restraining tube. Two groups were studied. The control group (n = 15) was infused with glycerol to eliminate any contribution of glycerol from triglycerides to metabolic changes in the muscle, and the high plasma FFA group (n = 14) was infused with Liposyn II (1.5 v/v with saline, Abbott), a 20% triglyceride emulsion combined with heparin (continuous infusion, 0.0975 IU/min). Heparin was used to activate lipoprotein lipase and thereby catalyze the hydrolysis of triglycerides. At the end of the in vivo NMR experiment, rats were anesthetized with thiopental (50 mg/kg).

Superficial skin was rapidly removed from the left hindquarter followed by in situ freeze clamping of the gastrocnemius and quadriceps muscles. Rats were euthanized with a lethal dose of thiopental.

**Euglycemic-Hyperinsulinemic Clamps**—Euglycemic-hyperinsulineemic (10 milliunits/kg/min, Humulin Regular, Lilly) clamps were performed using [1-13C]glucose (99% enriched, 20% w/v, Cambridge Isotope Laboratories, Cambridge, MA) infused simultaneously with glycerol (continuous infusion, 2.5 mg) of thiopental (Sigma) to place them in the restraining tube. Animals were allowed to recuperate after surgery for the indwelling catheters until they were at least of preoperative weight (approximately 5–10 days). All rats were fasted 24 h before the infusion experiment. Rats (250–350 g) were placed in a customized restraining tube that allowed their left hindlimb to be secured to the outside of the tube in a manner to limit free movement of the leg for NMR measurements. The rats were transiently anesthetized (<30 s) with a low dose (2.5 mg) of thiopental (Sigma) to place them in the restraining tube. Two groups were studied. The control group (n = 15) was infused with glycerol to eliminate any contribution of glycerol from triglycerides to metabolic changes in the muscle, and the high plasma FFA group (n = 14) was infused with Liposyn II (1.5 v/v with saline, Abbott), a 20% triglyceride emulsion combined with heparin (continuous infusion, 0.0975 IU/min). Heparin was used to activate lipoprotein lipase and thereby catalyze the hydrolysis of triglycerides. At the end of the in vivo NMR experiment, rats were anesthetized with thiopental (50 mg/kg).

Superficial skin was rapidly removed from the left hindquarter followed by in situ freeze clamping of the gastrocnemius and quadriceps muscles. Rats were euthanized with a lethal dose of thiopental.

**In Vivo NMR Spectroscopy**—All in vivo NMR experiments were performed on a Bruker Biospec 7.0 tesla system (horizontal 22-cm diameter bore magnet). In the initial experiment (control: n = 8, high plasma FFA: n = 9), 13C observe1H decoupled NMR was performed in both rats. The 1H coil (30 mm) was tuned to 300.68 MHz, and the inner 13C coil (18 mm) was tuned to 75.65 MHz. The radio frequency isolation between the two coils was 43 db. The rat hindlimb was placed in the 13C coil and placed in magnet isocenter. The sample was positioned over the13C coil and placed in magnet isocenter. Broadband carbon decoupling. The 4-H2 glutamate triplet that appears at 2.33 ppm has overlapping signal contribution from malate and lactate. The signal at 2.33 ppm was set to 93.0 ppm. The C-3 lactate (21.0 ppm) was clamped using a Gaussian filter followed by Fourier transformation. All spectra were baseline subtracted prior to peak analysis, and the (α) (1-13C)glucose peak was set to 93.0 ppm. The C-1 glycogen peak (100.5 ppm) was followed by a nonselective hard pulse (approximately 5–10 days). All rats were fasted 24 h before the infusion experiment. Rats (250–350 g) were placed in a customized restraining tube that allowed their left hindlimb to be secured to the outside of the tube in a manner to limit free movement of the leg for NMR measurements. The rats were transiently anesthetized (<30 s) with a low dose (2.5 mg) of thiopental (Sigma) to place them in the restraining tube. Two groups were studied. The control group (n = 15) was infused with glycerol to eliminate any contribution of glycerol from triglycerides to metabolic changes in the muscle, and the high plasma FFA group (n = 14) was infused with Liposyn II (1.5 v/v with saline, Abbott), a 20% triglyceride emulsion combined with heparin (continuous infusion, 0.0975 IU/min). Heparin was used to activate lipoprotein lipase and thereby catalyze the hydrolysis of triglycerides. At the end of the in vivo NMR experiment, rats were anesthetized with thiopental (50 mg/kg).

Superficial skin was rapidly removed from the left hindquarter followed by in situ freeze clamping of the gastrocnemius and quadriceps muscles. Rats were euthanized with a lethal dose of thiopental.

**Global 1H Shimming was followed by localized shimming with a STEAM sequence (17) over a 1 × 2 × 2-cm volume of the leg to optimize magnetic field homogeneity over the volume observed by the 13C surface coil. Water line widths of 35–45 Hz were obtained.**

**Decoupled 13C NMR spectroscopy was performed in the following manner. An initial frequency-selective sinc pulse (20 ms) set on the low frequency was placed between C-4 glutamate and C-3 alanine (121.72 MHz). A hard pulse (45° flip angle) was optimized and was used as the internal concentration standard (7.2 ppm) (18). All spectra were integrated over a frequency window of 7.13–7.36 ppm which corresponds to the downfield side of Glu-6-P and then multiplied by 2. This was done to minimize error in the measurement resulting from a-glycerol phosphate peak overlap at 6.92 ppm (19). The S/N for the basal Glu-6-P peak was determined to be 4.3 ± 0.2.**

**Tissue Extract Analysis—**Muscle tissue extracts were prepared for high field NMR analysis by homogenizing approximately 1 g of combined quadriceps and gastrocnemius muscle with a variable high speed electric homogenizer after sample was placed in a vortex tube filled with 0.9% perchloric acid (3 v/v) and 100 μl of 1 N sodium formate. The homogenization was performed over ice to keep the sample cold. After homogenization, the sample was centrifuged (10,000 rpm) for 10 min (3400 rpm).

The supernatant was extracted and the pellet saved for glycogen enrichment measurements. Minimal additional [1-13C]glycogen was detected in the supernatant as determined by high field 13C NMR but was not different between groups. KOH (4 N, 0.675 v/v) was added to the supernatant to precipitate excess chloride ions. The sample was centrifuged once at 4 °C for 15 min (3400 rpm). The supernatant was extracted, and 500 μl phosphate buffer, pH = 7, was added to neutralize the sample. The sample was dried in a speed-vac (Savant, Farmingdale, NY) overnight. and 1 ml of D2O was added to the dried powder before placing it in a 5-mm NMR tube for NMR analysis at 8.4 tesla (Bruker WB-360 NMR spectrometer). Proton observed-carbon-enhanced spectroscopy was performed on tissue extract samples for fractional enrichment calculations (20). The broadband13C inversion pulse frequency was placed between C-4 glutamate and C-3 alanine (−26.2 ppm) and turned on during alternate transients with raw data separated into two data sets providing spectra with heteronuclear coupled spins inverted (spectrum B) and non-inverted (spectrum A). The fractional enrichments (APE) of glutamate, lactate, and alanine were calculated from their respective resonances in spectrum A and B as follows in Equation 1:

\[
\text{APE} = 0.5 \frac{A - B}{A} \times 100 - 1.1 \quad \text{(Eq. 1)}
\]

Spectra were acquired with TR = 6 s, NS = 512, 16k data, and broadband carbon decoupling. The 4-H2 glutamate triplet that appears at 2.33 ppm has overlapping signal contribution from malate and β-hydroxybutyrate. Malate should be similarly enriched at steady state and is low in concentration, and negligible β-hydroxybutyrate was detected at 1.19 ppm (4H4). Therefore, these co-resonating signals are expected to have little effect on the glutamate enrichment calculation. For quantitation, a correction factor was calculated when a TR = 19 s was used. Glutamate, glutamine, and alanine were quantitated by comparing signal intensity with a known internal concentration standard (sodium formate) that was added during the extraction procedure. High pressure liquid chromatography was used to validate amino acid concentrations measured by NMR (mean 3.8% error). Intramuscular lactate concentration was calculated by extrapolation of in vivo NMR data (C-3 lactate and C-3 alanine) after correcting for T1 differences.

**Analytical Procedures—**Glucose plasma concentrations were measured by the glucose oxidase method (Glucose Analyzer II, Beckman Instruments, Fullerton, CA). Plasma immunoreactive free insulin was measured with a double antibody radioimmunossay technique (Linco Research Inc., St. Charles, MO) (21). 13C enrichment of plasma glucose and alanine was determined by gas chromatography-mass spectroscopy using a HP gas chromatograph (HP-1 capillary column, 12 m × 0.2 mm × 0.5 μm film, coupled to a Hewlett-Packard 5971 A mass selective detector operating in the positive chemical ionization mode with methane as a reagent gas. 100-ml aliquots of plasma from each time point were deproteinized with equal amounts of Ba(OH)2 and ZnSO4. The samples were centrifuged, and the supernatant was run over a cation exchange chromatography column (AG50W-X8 100–200 mesh Bio-Rad). Both amino acid
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FIG. 1. Schematic of skeletal muscle glucose metabolism following [1-13C]glucose precursor infusion. [1-13C] label from glucose becomes incorporated into [1-13C]glycogen and [3-13C]pyruvate which can be reduced to [3-13C]lactate or converted to [3-13C]alanine via aminotransferase reaction. The rate at which substrate enters the lactate + pyruvate + alanine) pool is 2 \times 2\text{G_2}, >\text{glycolytic rate (2V_Gly)}. Under steady state metabolic conditions 2\text{G_2} is equal to pyruvate dehydrogenase (V_{PDH}) + pyruvate carboxylase (V_p). Subsequent labeling of [4-13C]glutamate/glutamine occurs from label entering the tricarboxylic acid (TCA) cycle via pyruvate dehydrogenase. Label entering the tricarboxylic acid cycle via pyruvate carboxylase will label C-3 oxaloacetate (OAA). V_{PPP} = pentose phosphate pathway flux (stippled due to loss of label).
turnover as the intramuscular lactate and alanine label turnover, so label dilution from blood was excluded from Equation 2 for the calculation of $V_{\text{Gly}}$. Due to the large contribution of skeletal muscle to total body weight and high proportion of insulin-stimulated GLUT4 transporters, skeletal muscle has been measured to account for the majority (~88%) of insulin-stimulated whole body glucose uptake in man (28). Therefore, although the cell membrane transport mechanisms for lactate (29) and alanine (30) have been characterized and influx and efflux activities are high for both substrates, the plasma-labeled lactate and alanine turnover under euglycemic-hyperinsulinemic conditions should be dictated by the skeletal muscle-labeled lactate and alanine turnover rather than by label turnover of these pools in smaller organs (e.g. heart) that have higher glycolytic activity than skeletal muscle.

Statistical Analysis—All data are reported as the mean ± S.E. Student’s two-tailed $t$ test was performed on data to determine significance at a minimum $p \leq 0.05$ threshold.

RESULTS

Euglycemic-hyperinsulinemic Clamp—In both control and high plasma FFA groups, the plasma glucose concentrations were clamped at approximately 6 mM, and there was no significant difference between the groups (Fig. 2A). Plasma insulin concentrations in both groups started at 102 ± 20 and 130 ± 38 pm and increased to a level of 1230 ± 174 and 1356 ± 84 pm in the control and high plasma FFA groups, respectively, at 60 min and were clamped throughout the experiment (Fig. 2B).

Basal plasma free fatty acid concentrations were 0.91 ± 0.11 and 0.96 ± 0.19 mM in the control and high plasma FFA group, respectively (Fig. 2C). At the first measurement (60 min), the plasma FFA concentrations were 0.28 ± 0.03 and 2.25 ± 0.29 mM in the control and high plasma FFA group ($p < 0.005$) and remained there for the duration of the study.

Plasma lactate concentrations increased rapidly in the control group from 0.86 ± 0.15 mM at base line to 1.42 ± 0.22 mM at 15 min (Fig. 2D) but less rapidly in the high plasma FFA group (0.92 ± 0.07 mM at base line to 1.45 ± 0.24 at 90 min).

Glucose infusion rates ($G_{\text{inf}}$) were 29.5 ± 0.7 and 27.2 ± 1.2 mg/kg/min at 15 min for control and high plasma FFA groups, respectively. While the $G_{\text{inf}}$ in the high plasma FFA group continued to decrease throughout the experiment to 17.7 ± 1.3 mg/kg/min at 240 min (Fig. 2E), it remained relatively stable in the control group throughout the experiment (30.7 ± 2.3 mg/kg/min at 180 min, $p < 0.002$). The whole body glucose disposal is defined as the glucose infusion rate plus HGP. The HGP rate has been shown to increase when glycerol was infused simultaneously with a euglycemic-hyperinsulinemic clamp over controls (31). The glycerol infusion rate chosen in our control group was set to match the plasma glycerol concentration produced by lipolysis during the triglyceride infusion, and no significant differences in estimated hepatic glucose production rates between the control and high plasma FFA groups were calculated ($7.3 ± 0.7$ and $7.6 ± 0.6$ mg/kg/min, respectively) at 180 min when the plasma glucose enrichment was at steady state. At 240 min, the whole body glucose disposal rate in the high plasma FFA group was 63% of the control group at 180 min ($23.9 ± 1.8$ versus $37.9 ± 2.8$ mg/kg/min, $p < 0.002$).

Glycogen Synthesis Rates—Twenty four-hour fasted basal muscle glycogen concentrations were 23.7 ± 2.5 and 23.8 ± 2.0 μmol/g for control and high plasma FFA groups, respectively. 

Fig. 3 illustrates a typical time course for the relative in vivo net glycogen production in a series of 15-min $^{13}$C NMR collected spectra. At base line, no basal glycogen was detected by NMR due to the low sensitivity with only 1.1% $^{13}$C natural abundance. Once the $[1^{-13}\text{C}]$glucose infusion was begun, the β- and α-anomer of glucose was observed at 96.8 and 93.0 ppm, respectively. $[1^{-13}\text{C}]$Glycogen appeared by 15 min at 100.5 ppm and continued to increase at a constant rate throughout the experiment in both groups yielding a net synthesis rate of 163 ± 32 and 104 ± 17 nmol/g/min for control and high plasma FFA groups, respectively (Fig. 4).

Glycolytic Flux ($V_{\text{Gly}}$) Calculations—A series of base line-subtracted $^{13}$C NMR spectra of $[3^{-13}\text{C}]$lactate and $[3^{-13}\text{C}]$ala-
nine turnover are illustrated in Fig. 5. Peaks appearing at 16.9 and 21.0 ppm are indicative of C-3 alanine and C-3 lactate, respectively. The peaks were integrated and normalized for plasma glucose fractional enrichments before fitting the label turnover data (Fig. 6A). Line fitting of the data was done with $R^2 = 0.90$. There was a distinct difference in rate of turnover with the high plasma FFA group being much slower than the control group ($1.44 \pm 0.25 \times 10^{-2}$ versus $3.35 \pm 0.30 \times 10^{-2}$ min⁻¹, $p < 0.0005$). Therefore, an additional 60 min experimental time was required for NMR detection of lactate and alanine label turnover in the high plasma FFA group. The lactate and alanine concentrations were measured from muscle tissue extracts, and the concentrations were determined to be $1.51 \pm 0.09$ and $1.88 \pm 0.12$ μmol/g, respectively, for the control group and $1.27 \pm 0.16$ and $1.67 \pm 0.21$ μmol/g for the high plasma FFA group. The value of $\alpha$ used in Equation 2 that corresponds to the label dilution factor resulting from unlabeled substrate contribution via glycogenolysis and pentose phosphate pathway was $0.68 \pm 0.03$ and $0.60 \pm 0.02$ in the control and high plasma FFA groups, respectively ($p < 0.02$). $V_{Gil}$ (Fig. 6B) was significantly lower in the high plasma FFA group ($19.5 \pm 3.6$ and $57.9 \pm 8.0$ nmol/g/min in the high plasma FFA and control groups respectively, $p < 0.002$).

Steady State Isotopic Enrichment—Label enrichments were calculated for lactate, alanine, and glutamate in tissue extracts. Table I depicts the differences in these metabolite enrichments between groups. C-3 alanine and C-3 lactate fractional enrichments were $27.4 \pm 0.9$ and $26.5 \pm 1.2\%$ for control
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and 23.2 ± 0.9 and 21.8 ± 1.3% for high plasma FFA groups, respectively (p < 0.005). Assuming one pool of pyruvate labels both alanine and lactate, then the pyruvate enrichment must equal that of C-3 lactate and C-3 alanine at isotopic steady state. The C-4 glutamate fractional enrichment was 15.0 ± 2.4 and 5.6 ± 1.0% for the control and high plasma FFA groups, respectively (p, 0.005). The ratio of C-4 glutamate, which becomes labeled when label from C-3 pyruvate enters the tricarboxylic acid cycle via PDH (Fig. 1), to C-3 lactate reflects the percent of flux entry via PDH versus FFA and ketone oxidation under steady state metabolic conditions. We assume a single compartment model and cell type for this analysis. Therefore, the pyruvate pool in exchange with lactate and alanine will label glutamate in the mitochondria. C-4 Glu/C-3 Lac was 56.9 ± 8.7 and 25.4 ± 4.0% for control and high plasma FFA groups, respectively, reflecting an approximate 2-fold increase in the relative rate of intracellular FFA/ketone oxidation in the high plasma FFA versus control group.

Glucose 6-Phosphate—31P NMR was used with the same experimental conditions as the 13C NMR experiments. Fig. 7 contains a 15-min acquired base-line subtracted 31P spectra of the rat hindlimb during a euglycemic-hyperinsulinemic clamp in a control rat are staggered to illustrate the 13C label turnover in [3-13C]lactate (21.0 ppm) and [3-13C]alanine (16.9 ppm). Spectra were base-line subtracted to minimize lipid distortion in measurements of the peaks.

control and high plasma FFA groups, respectively. A rapid increase in Glu-6-P in the control group of 0.04 ± 0.01 μmol/g occurred during the initial 30 min of the experiment. This was followed by a moderate additional increase of 0.02 μmol/g before slightly decreasing at 150–180 min. In the high plasma FFA group, the ΔGlu-6-P concentration continued to increase throughout the experiment to 0.10 ± 0.01 μmol/g at 210–240 min. Glu-6-P concentrations increased by 29.8 ± 7.0 and 52.8 ± 12.3% (p, 0.05) in the control and high plasma FFA groups, respectively, by 180 min.

DISCUSSION

Previous studies in rats have shown that an increase in plasma FFA concentration resulted in an increase (5), decrease (6–8), or no change (9, 10) in muscle glucose uptake. In our studies, whole body glucose disposal rates decreased from approximately 60 min on throughout the experiment in the high plasma FFA group (Fig. 2E). It is difficult to assess changes in skeletal muscle glucose uptake per se from changes in whole body glucose metabolism since the brain (32) accounts for a significant proportion of whole body glucose uptake under rest-
ing conditions. Measurements of whole body glycolysis also suffer from the inability to reflect tissue-specific glycolytic measurements. Currently, most of these measurements are determined by indirect calorimetry (11, 12, 33, 34) or measurements of tritiated water production from [3-3H]glucose (13, 25, 35). The significant advantage of the NMR technique for glycolytic flux measurements is that it provides a direct assessment of intramuscular oxidative glycolytic metabolism.

Using this technique, we found the glycolytic flux decreased (67%) in the high plasma FFA versus control group (19.5 ± 3.6 and 57.9 ± 8.0 nmol/g/min, respectively). Kim et al. (6) estimated glycolysis in rat skeletal muscles as a difference between glucose uptake measured by 2-deoxyglucose uptake and glycogen synthesis. Their results also showed a significant reduction in glycolysis (41–50%) in Intralipid-infused versus control rats, although their absolute glycolytic fluxes were higher ranging from 67 to 316 nmol/g/min in various muscle types of the rat hindlimb at euglycemia and insulinemia similar to that in our experiments. Effects of oleate on rates of glycolysis in isolated soleus muscle following incubation with [14C]glucose showed a similar trend (~50% decrease) but in general were much lower (decreasing from ~12 to 7 nmol/g/min) (7) which may be attributed to differences between the in vitro and in vivo preparations.

Pyruvate dehydrogenase flux (V_{PDH}) was estimated to be ~2V_{Glyc}V_{Pc} under steady state metabolic conditions assuming that net lactate efflux from muscle due to anaerobic glycolysis was negligible. Anaplerotic substrate contribution to the tricarboxylic acid cycle was determined to be minor with respect to citrate synthase flux under similar conditions in the heart (36). Therefore assuming negligible anaplerosis in muscle, our mean PDH rates were 58 versus 28 nmol/g/min in FFA versus control, respectively, as calculated from their 24-h fasted PDH_{Total} and % PDH active data.

Furthermore when using PDH flux data in combination with steady state isotopic enrichments of lactate and glutamate in muscle extracts, one may calculate the combined FFA and ketone oxidation rates. We estimate that the rate of intramuscular FFA/ketone oxidation almost doubled in the high plasma

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**TABLE I**

| Group              | C-3 lactate APE | C-3 alanine APE | C-4 glutamate APE | C-4 GluAPE/C-3 LacAPE |
|--------------------|----------------|----------------|------------------|-----------------------|
| Control            | 26.5 ± 1.2     | 27.4 ± 0.9     | 15.0 ± 2.4       | 56.9 ± 8.7            |
| High plasma FFA    | 21.8 ± 1.3     | 23.2 ± 0.9     | 5.6 ± 1.0        | 25.4 ± 4.0            |

APE, atom percent excess; Glu, glutamate; Lac, lactate.

p < 0.005 control versus high plasma FFA group.

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**FIG. 7. In vivo 31P NMR spectra.** 15 min acquired spectrum processed with Gaussian filtering. Peaks present are phosphocreatine set to 0 ppm, adenosine triphosphate (γ-ATP, α-ATP, β-ATP), inorganic phosphate (Pi), and phosphomonoester region. The expanded phosphomonoester region include peaks corresponding to glucose 6-phosphate (G-6-P) at 7.13 ppm, and α-glycerol phosphate at 6.92 ppm.
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0.12
0.1
0.08
0.06
0.04
0.02
0

Glu-6-P (nmol/g)
time (min)
0 30 60 90 120 150 180 210 240

FIG. 8. Net increase in intramuscular Glu-6-P concentration. 30 min time averaged measurements of ΔGlu-6-P were made in control (○) and high plasma FFA (●) groups during a euglycemic-hyperinsulinemic clamp. *, p < 0.05, control versus high plasma FFA group at respective times.

FFA versus control group, and substrate entry via glycolysis into the tricarboxylic acid cycle accounted for 57% in controls and decreased to 25% (p < 0.005) in the high plasma FFA group.

Our in vivo NMR measurements were obtained on the rat hindlimb which is comprised of both type I and type II muscle fibers. Therefore, the calculated glycolytic flux, glycogen synthesis rate, and Glu-6-P concentration represent a weighted average of these respective measurements in the individual muscles. Although localized NMR spectroscopy of specific muscles is possible, the signal sensitivity is such that these measurements would be extremely difficult in the rat. The $V_{\text{Gly}}$ calculations were based upon steady state metabolic kinetic modeling as was used by Mason et al. (27) to measure tricarboxylic acid and glycolytic flux in brain. To perform these calculations, we assumed that the intramuscular lactate, pyruvate, and alanine concentrations did not change throughout the duration of the experiment and that glucose entering the [lactate + pyruvate + alanine] pool through glycolysis was at a steady state rate. In support of this assumption, we measured intramuscular [lactate + alanine] before and after the euglycemic-hyperinsulinemic clamp and found them to be similar (3.17 ± 0.49 versus 3.39 ± 0.25 μmol/g). Since steady state metabolic conditions were assumed to simplify the glycolytic flux calculation, $V_{\text{Gly}}$ was possibly overestimated in the high plasma FFA group, because the intramuscular glucose uptake as reflected by the glucose infusion rate was decreasing throughout the experiment. This would result in a slight overestimation in $V_{\text{Gly}}$ calculated from fitting the label turnover of lactate and alanine with emphasis on the earlier time points when glycolysis may be greater before the whole body glucose disposal initially begins to decrease. Nevertheless, Fig. 6A depicts a significant difference in lactate and alanine label turnover illustrating the ability of increased plasma FFA to inhibit $V_{\text{Gly}}$ in skeletal muscle.

Muscle glycogen synthesis rates tended to be lower, although not significantly, in the high plasma FFA versus control groups (104 ± 17 and 163 ± 32 nmol/g/min, respectively). There was no significant change in these rates during the time course of the experiment (Fig. 4). Therefore, the predominant mechanism by which increased concentrations of plasma FFA decrease muscle glucose uptake in rat is through inhibition of glycolysis. Li et al. (38) also showed no difference in $^{13}$C label incorporation into glycogen in rat soleus or extensor digitorum longus muscle with the addition of palmitate. This technique was used to obtain similar results in rhesus sartorius muscle (39). In contrast Jenkins et al. (5) found an increase in total glucose label incorporation into glycogen in soleus and red and white gastrocnemius muscles in the rat hindquarter at 2 or 4 mst plasma FFA concentrations. Kim et al. (6) also observed significantly increased glucose label incorporation into glycogen in soleus, extensor digitorum longus, and tibialis anterior at physiological insulin concentrations and only in soleus at maximal insulin concentrations of rats receiving Intralipid infusion. The advantage of the $^{13}$C NMR method over that of these previous studies is that it allows one to make temporal measurements of muscle glycogen synthesis as opposed to a single time point measurement.

To assess the effects of FFA on potential rate-controlling steps in muscle glucose metabolism, glucose 6-phosphate was measured by in vivo $^{31}$P NMR (13). Glu-6-P concentrations increased 30% ($p < 0.01$) and 53% ($p < 0.005$) above basal values in the control and high plasma FFA groups, respectively, by the end of the euglycemic-hyperinsulinemic clamp (Fig. 8). It is interesting to note that while Δ[Glu-6-P] increased rapidly by 30 min and slowed thereafter in the control group, it continued to increase throughout the experiment in the high plasma FFA group. These data lend support to the mechanism that Randle et al. (1) proposed to explain FFA inhibition of insulin-stimulated glucose uptake which predicts that Glu-6-P should increase as a result of inhibition of phosphofructokinase. If glucose transport/hexokinase were rate controlling for insulin-stimulated glucose uptake, then a decrease in Glu-6-P would be expected (11). Previous studies found an increase in
Glu-6-P measured in rat soleus (6, 7), extensor digitorum longus (6), and vastus lateralis (6) but not epitrochlearis muscle (7) under high plasma FFA conditions. Therefore, Randle’s cycle appears to operate in predominantly slow twitch skeletal muscles in rat.

We may analyze metabolic flux control (40) with knowledge of changes in whole body glucose disposal, glycogen synthesis, glycolysis, and Glu-6-P after an increase in plasma FFA. At the end of the experiment, the rate of whole body glucose disposal and glycolysis decreased by 37 and 67%, respectively, in the high plasma FFA versus control group. This was accompanied by a 60% increase in absolute $\Delta$(Glu-6-P) (0.05 versus 0.08 mmol/g in control versus high plasma FFA at 150–180 min) and no significant change in glycogen synthesis rate in the high plasma FFA versus control group. The ratio of glycolytic to total glucose disposal rates in muscle ($V_G/(V_G + V_{glycogen})$) decreased 40% in the high plasma FFA versus control group. Thus, there is good correlation between the decreased flux through glycolysis as a fraction of total muscle glucose disposal (−40%) and decreased whole body glucose disposal (−37%). In addition, the Glu-6-P data provide substrate data required for flux control analysis. From the traditional notion of metabolic flux control, the enzyme whose activity is less sensitive to substrate change exerts greater control in a metabolic pathway, so phosphofructokinase activity controls Glu-6-P levels that subsequently exert allosteric control on hexokinase activity and glucose transport.

There are a number of studies performed in humans that suggest, as in animals, that increased plasma FFA does reduce whole body glucose disposal (11–14, 31, 34, 41). This phenomenon was also observed at the tissue-specific level by the arterial–venous balance technique in leg muscle (41) and by positron enon was also observed at the tissue-specific level by the arterio-venous balance technique in leg muscle (41) and by positron emission tomography. These studies confirmed that increased plasma FFA reduces muscle glucose uptake due to decreased glucose transport/phosphorylation at 550 μM FFA (medium), although no significant change in intramuscular glycogen (medium), although no significant change in intramuscular glycogen synthesis simultaneously in skeletal muscle of awake rats, and measurements in skeletal muscle tissue extracts provided relative rates of FFA/ketone versus pyruvate oxidation. Additionally, in vivo $^{31}$P NMR was used to make temporal $\Delta$(Glu-6-P) measurements to assess the mechanism for decreased insulin-dependent glucose uptake in rat muscle under conditions of euglycemia-hyperinsulinemia and increased plasma FFA. The results are consistent with the mechanism proposed by Randle and colleagues (1) to explain FFA inhibition of insulin-stimulated muscle glucose uptake. This in vivo NMR approach is directly applicable to humans and should prove useful for examining the regulation of intramuscular glucose metabolism in normal and diabetic humans.

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REFERENCES

1. Randle, P. J., Garland, P. B., Hales, C. N., and Newsholme, E. A. (1963) Lancet i, 785–789
2. Randle, P. J., Priestman, D. A., Mistry, S., and Halsall, A. (1994) Diabetologia 37, Suppl 2, S155–S161
3. Newsholme, E. A., Sugden, P. H., and Williams, T. (1977) Biochem. J. 166, 123–129
4. Lueck, J. D., and Fromm, H. J. (1974) J. Biol. Chem. 249, 1341–1347
5. Jenkins, A. B., Storlien, L. H., Chisholm, D. J., and Kruegen, E. W. (1988) J. Clin. Invest. 82, 293–299
6. Kim, J. K., Wi, J. K., and Youn, J. H. (1996) Diabetes 45, 446–453
7. Nolte, L. A., Galasla, D., Martin, I. K., Geraath, J. R., and Naliberg-Henriksson, H. (1994) Acta Physiol. Scand. 151, 51–59
8. Kruszynska, Y. T., McCormack, J. G., and McIntyre, N. (1991) Diabetes 33, 396–402
9. Goodman, M. N., Berger, M., and Ruderman, N. B. (1974) Diabetes 23, 881–888
10. Berger, M., Hagg, S. A., Goodman, M. N., and Ruderman, N. B. (1976) Biochem. J. 156, 191–202
11. Boden, M., Price, T. B., Perseghin, G., Petersen, K. F., Rothman, D. L., Cline, G. W., and Shulman, G. L. (1990) J. Clin. Invest. 97, 2859–2865
12. Boden, G., and Jahadi, F. (1991) Diabetes 40, 686–692
13. Boden, G., Xinohar, C., Rast, J., White, J. V., and Rossetti, L. (1994) J. Clin. Invest. 93, 2438–2446
14. Boden, G., Jahadi, F., Liang, Y., Mozoli, M., Chen, X., Coleman, E., and Smith, C. (1991) J. Clin. Invest. 88, 960–966
15. Sidossis, L. S., and Wolfe, R. R. (1996) Am. J. Physiol. 270, E733–E738
16. Smith, D., Rossetti, L., Ferrerannini, E., Johnon, C. M., Cobelli, L., Toffolo, G., Katz, L. D., and DeFronzo, R. A. (1987) Metabolism 36, 1167–1174
17. Früh, J., Merhold, K., and Hänicke, W. (1990) Acta Physiol. Scand. 140, 522–528
18. Bloch, G., Chase, J. R., Avison, M. J., and Shulman, R. G. (1993) Magn. Reson. Med. 30, 347–350
19. Rothman, D. L., Shulman, G. L., and Shulman, G. L. (1992) J. Clin. Invest. 90, 1069–1075
20. Fitzpatrick, S. M., Hetherington, H. P., Behar, K. L., and Shulman, R. G. (1980) J. Cereb. Blood Flow Metab. 10, 170–179
21. Rosselin, G., Assan, R., Yalow, R. S., and Berson, S. A. (1966) Nature 212, 355–358
22. Cline, G. W., and Shulman, G. L. (1991) J. Biol. Chem. 266, 4984–4988
23. Leimer, K. R., Rice, R. H., and Gehrke, C. W. (1977) J. Chromatogr. 141, 121–144
24. Keppler, D., and Decken, K. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed) pp. 1127–1131, Verlag Chemie Weinheim, Academic Press, New York
25. Rossetti, L., and Gasparini, A. (1999) J. Clin. Invest. 85, 1750–1752
26. Bloch, G., Chase, J. R., Meyer, D. B., Avison, M. J., Shulman, G. L., and Shulman, R. G. (1994) Am. J. Physiol. 266, E55–E61
27. Mason, G. F., Rothman, D. L., Behar, K. L., and Shulman, R. G. (1992) J. Cereb. Blood Flow Metab. 12, 434–447
28. DeFronzo, R. A., Ferrerannini, E., Sato, Y., Felig, P., and Wahren, J. (1981) J. Clin. Invest. 68, 1468–1474
29. Roth, D. A., and Brooks, G. A. (1993) J. Appl. Physiol. 75, 1559–1565
30. Haudal, H. S., Rennie, M. J., Watt, P. W. (1989) J. Physiol. (London) 408, 83–114
31. Ferrerannini, E., Barrett, E. J., Bevilacqua, S., and DeFronzo, R. A. (1988) J. Clin. Invest. 72, 1737–1747
32. Mason, G. F., Rutter, R., Rothman, D. L., Behar, K. L., Shulman, R. G., and Novotny, E. J. (1996) J. Cereb. Blood Flow Metab. 15, 12–25
33. Bevilacqua, S., Buzigoli, G., Bonadonna, R., Brandi, L. S., Oleggini, M., Boni, C., Geloni, M., and Ferrerannini, E. (1990) Diabetologia 39, 383–389
34. Wolfe, B. M., Klein, S., Peters, E. J., Schmidt, B. F., and Wolfe, R. R. (1988) Metabolism 37, 323–329
35. Rossetti, L., Lee, Y.-T., Ruiz, J., Aldridge, S. L., Shulman, J., and Boden, G. (1993) Am. J. Physiol. 265, E761–E769
36. Malloy, C. R., Sherry, A. D., and Jeffrey, P. M. (1988) J. Biol. Chem. 263, 5964–5971
37. Kruszynska, Y. T., McCormack, J. G., and McIntyre, N. (1991) Diabetologia 34, 205–211
38. Li, J., Stillman, J. S., Clore, J. N., and Blackard, W. G. (1993) *Metabolism* **42**, 451–456
39. Beatty, C. H., and Bocek, R. M. (1971) *Am. J. Physiol.* **220**, 1928–1934
40. Shulman, R. G., Bloch, G., and Rothman, D. L. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 8535–8542
41. Kelley, D. E., Mokan, M., Simoneau, J. A., and Mandarino, L. J. (1993) *J. Clin. Invest.* **92**, 91–98
42. Nuutila, P., Koivisto, V. A., Kuusniemi, J., Ruotsalainen, U., Teräs, M., Haaparanta, M., Bergman, J., Solin, O., Voipio-Pulkki, L.-M., Wagenarius, U., and Yki-Järvinen, H. (1992) *J. Clin. Invest.* **89**, 1767–1744
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