# Differential Effects of Safflower Oil Versus Fish Oil Feeding on Insulin-Stimulated Glycogen Synthesis, Glycolysis, and Pyruvate Dehydrogenase Flux in Skeletal Muscle

## A $^{13}$C Nuclear Magnetic Resonance Study

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**To examine the effects of safflower oil versus fish oil feeding on in vivo intramuscular glucose metabolism and relative pyruvate dehydrogenase (PDH) versus tricarboxylic acid (TCA) cycle flux, rats were pair-fed on diets consisting of *1*) 59% safflower oil, *2*) 59% menhaden fish oil, or *3*) 59% carbohydrate (control) in calories. Rates of glycolysis and glycogen synthesis were assessed by monitoring [1-$^{13}$C]glucose label incorporation into [1-$^{13}$C]glycogen, [3-$^{13}$C]lactate, and [3-$^{13}$C]alanine in the hindlimb of awake rats via $^{13}$C nuclear magnetic resonance (NMR) spectroscopy during a euglycemic (~6 mmol/l) hyperinsulinemic (~180 µU/ml) clamp. A steady-state isotopic analysis of lactate, alanine, and glutamate was used to determine the relative PDH versus TCA cycle flux present in muscle under these conditions. The safflower oil–fed rats were insulin resistant compared with control and fish oil–fed rats, as reflected by a markedly reduced glucose infusion rate ($G_{inf}$) during the clamp (21.4 ± 2.3 vs. 31.6 ± 2.8 and 31.7 ± 1.9 mg · kg$^{-1}$ · min$^{-1}$ in safflower oil versus control and fish oil groups, respectively, $P$ < 0.006). This decrease in insulin-stimulated glucose disposal in the safflower oil group was associated with a lower rate of glycolysis (21.7 ± 2.2 nmol · g$^{-1}$ · min$^{-1}$) versus control (62.1 ± 10.3 nmol · g$^{-1}$ · min$^{-1}$, $P$ < 0.001) and versus fish oil (45.7 ± 6.7 nmol · g$^{-1}$ · min$^{-1}$, $P$ < 0.04), as no change in glycogen synthesis (103 ± 15, 133 ± 19, and 125 ± 14 nmol · g$^{-1}$ · min$^{-1}$ in safflower oil, fish oil, and control, respectively) was detected. The intramuscular triglyceride (TG) content was increased in the safflower oil group (7.3 ± 0.8 µmol/g) compared with the control group (5.2 ± 0.8 µmol/g, $P$ < 0.05) and the fish oil group (3.6 ± 1.1 µmol/g, $P$ < 0.01). Conversely, the percent PDH versus TCA cycle flux was decreased in the safflower oil (43 ± 8%) versus the control (73 ± 8%, $P$ < 0.01) and fish oil (64 ± 6%, $P$ < 0.05) groups. These data suggest that the reduced insulin-stimulated glucose disposal attributed to safflower oil feeding was a consequence of reduced glycolytic flux associated with an increase in relative free fatty acid/ketone oxidation versus TCA cycle flux, whereas fish oil feeding did not alter glucose metabolism and may in part be protective of insulin-stimulated glucose disposal by limiting intramuscular TG deposition.** ***Diabetes*** **48:134–140, 1999**

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ANOVA, analysis of variance; APE, atom percent excess; FFA, free fatty acid; $G_{inf}$, glucose infusion rate; NMR, nuclear magnetic resonance; PDH, pyruvate dehydrogenase; TCA, tricarboxylic acid; TG, triglyceride; $V_{gly}$, rate of glycolysis; $V_{glyc}$, rate of glycogen synthesis.

It is well established that chronic high-fat feeding can induce insulin resistance in rats (1–9). Furthermore, it has been demonstrated that the dietary fat composition is a critical factor in this process because a diet high in long-chain ω-3 fatty acids (fish oil) has been found to prevent the development of insulin resistance, although the mechanism behind this protective effect remains unclear (10,11). Although many studies have demonstrated that the insulin resistance associated with high-fat feeding is secondary to decreased muscle glycolysis (1,3,6,9,11,12), no studies to date have assessed its impact on pyruvate dehydrogenase flux because of the difficulty in measuring this parameter. This is a critical factor because the classic mechanism (glucose–free fatty acid [FFA] cycle) proposed by Randle and colleagues (13,14) demonstrates that an increase in acetyl-CoA resulting from increased β-oxidation of fat inhibits pyruvate dehydrogenase (PDH) flux by inactivating the PDH complex. Furthermore, the effect of ω-3 fatty acids on this flux is unknown.

To address these questions, we used in vivo $^{13}$C nuclear magnetic resonance (NMR) spectroscopy in awake rats to examine the impact of contrasting high-fat feeding protocols that either produce insulin resistance (safflower oil) or protect against insulin resistance (fish oil) on the following factors in insulin-stimulated muscle: *1*) glycolysis, *2*) glycogen synthesis, and *3*) relative PDH versus tricarboxylic acid (TCA) cycle flux.

## RESEARCH DESIGN AND METHODS

**Animals.** Sprague-Dawley rats (Charles River, Raleigh, NC) were housed in an environmentally controlled room with a 12-h light/dark cycle. Rats (35–42 days old) were begun on a chronic high-fat feeding protocol that consisted of one of three different powdered diets containing, by calorie, 59% safflower oil, 23% carbohydrate, and 18% protein (safflower oil diet, $n$ = 10); 59% menhaden fish oil,

23% carbohydrate, and 18% protein (fish oil diet, $n$ = 10); or 59% carbohydrate, 17% soybean oil, and 24% protein (control diet, $n$ = 10) (diets #112245, #112246, and #110700, respectively, Dyets, Bethlehem, PA). Mineral mix #210025 and vitamin mix #310025 as required for AIN-93G standard diet were used. All diets contained a trace amount of t-butylhydroquinone as a stabilizer. Major fatty acid composition of the dietary fats are as follows: soybean oil (16:0 10.2%, 18:0 4.5%, 18:1 22.7%, 18:2 54.8%, 18:3 7.8%); safflower oil (16:0 6.9%, 18:0 2.9%, 18:1 12.2%, 18:2 78%); menhaden fish oil (14:0 9.0%, 16:0 17.1%, 16:1 12.5%, 18:1 11.4%, 18:2–4 6.6%, 20:5 15.5%, 22:6 9.1%, other 18.8%). A pair-feeding protocol that matched protein and vitamin/mineral content between the three groups was used. Rats were fed ~100 kcal/day from individually vacuum packaged and refrigerated portions of diet for 4–5 weeks so as to match weight gain within the three groups. After this feeding period, rats were chronically catheterized as described elsewhere (15), and the dietary regimen was continued for another 5–10 days.

**In vivo experiments.** All rats were fasted 24 h before the infusion experiment. Rats (weighing between 300 and 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 limiting 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, St. Louis, MO) to place them in the restraining tube. A euglycemic-hyperinsulinemic (10 mU · $kg^{-1}$ · $min^{-1}$ Humulin Regular; Eli Lilly, Indianapolis, IN) clamp was begun using [1-$^{13}$C]glucose (99% enriched, 20% wt/vol; Cambridge Isotope Laboratories, Cambridge, MA) at 2.5 min after the commencement of the primed-continuous insulin infusion. Plasma glucose concentrations were clamped at ~6 mmol/l. All clamps lasted for 180 min. Blood samples were drawn during the baseline NMR measurement, at 7.5 min, at 15 min, and at every 15 min thereafter for immediate assessment of plasma glucose and lactate concentrations. 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 killed with a lethal dose of thiopental. The protocol was reviewed and approved by the Yale Animal Care Use Committee.

**In vivo NMR spectroscopy.** All in vivo $^{13}$C NMR experiments were performed on a Bruker Biospec system (horizontal/22 cm diameter bore 7.0 tesla field strength magnet), as previously described (16).

**Tissue extract analysis.** Muscle tissue extracts were prepared for high-field NMR analysis by homogenizing approximately 1 g of combined quadriceps and gastrocnemius muscle, as previously described (16). NMR analysis was performed at 8.4 tesla (Bruker WB-360 NMR spectrometer). Proton observed–carbon enhanced spectroscopy was performed on tissue extract samples for fractional enrichment calculations (16). For quantitation, a correction factor was calculated when a TR (pulse repetition time) = 19 s was used. Alanine was quantitated by comparing its signal intensity with a known internal concentration standard (lactate), which was measured in tissue extracts using a 2300 Stat Plus lactate analyzer (Yellow Springs Instrument, Yellow Springs, OH). Intramuscular lactate concentration before freeze-clamping was calculated by extrapolation of in vivo NMR data ([3-$^{13}$C]lactate and [3-$^{13}$C]alanine) after correcting for $T_1$ (longitudinal relaxation time) differences.

**Analytical procedures.** Plasma glucose concentrations were measured by the glucose oxidase method (Glucose Analyzer II; Beckman, Fullerton, CA). Plasma immunoreactive free insulin was measured with a double antibody radioimmunoassay technique (Linco Research, St. Charles, MO). $^{13}$C enrichment of plasma glucose was determined using a Hewlett-Packard 5890 gas chromatography (HP-1 capillary column, 12 m × 0.2 mm × 0.33 mm film thickness) interfaced to a Hewlett-Packard 5971A mass selective detector operating in the positive chemical ionization mode with methane as a reagent gas (17).

Glycogen $^{13}$C fractional enrichments were determined using the precipitated glycogen from the initial muscle perchloric acid extraction (16), and absolute glycogen concentrations were measured on a separate portion of muscle, as previously described (18).

Plasma free fatty acids were determined using an acyl-CoA oxidase based colorimetric kit (Wako NEFA-C; Wako Pure Chemical Industries, Osaka, Japan). Plasma triglyceride (TG) concentrations were determined enzymatically (Sigma Diagnostics, St. Louis, MO). Plasma lactate concentrations were measured using a 2300 Stat Plus lactate analyzer.

Skeletal muscle TG content was determined using a method adapted from Storlien et al. (5) and Frayn and Maycock (19). Approximately 0.5 g of frozen tissue was ground under liquid $N_2$ using a mortar and pestle. All measurements were made in triplicate by placing a weighed portion of the powdered muscle into three separate tubes. Chloroform/methanol (3 ml, 2:1 vol/vol) was added to each tube before homogenizing (~10,000 rpm) over ice for ~1 min. Tubes were shaken for 4 h at room temperature before 2 ml of 1 mmol/l $H_2SO_4$ was added. Samples were shaken and subsequently centrifuged (1,000 rpm for 10 min) to separate phases. The organic bottom layer containing TG was separated and was assumed to be 2 ml in volume. This portion was used in the plasma TG assay discussed above. Recovery of the TG standard used was 101 ± 3%. The remaining organic volume was dried down with $N_2$ gas and resuspended in 0.5 ml chloroform for $^{13}$C NMR analysis.

**Endogenous glucose production calculation.** Endogenous glucose production was estimated by taking the difference of the glucose infusion rate ($G_{inf}$) and the tracer-determined rate of whole-body glucose appearance using the steady-state plasma [1-$^{13}$C]glucose enrichment at 90–120 min after correction for label scrambling in the triose pool, which produces [6-$^{13}$C]glucose (20).

**Glycogen synthesis rate ($V_{glyc}$) calculation.** The incremental change in C-1 glycogen peak intensity from [1-$^{13}$C]glucose incorporation was measured at 100.5 ppm. Incremental plasma glucose $^{13}$C fractional enrichment and final glycogen $^{13}$C enrichment and concentrations were used to back-extrapolate the glycogen concentration (micromoles per gram, which represents micromoles of glucosyl units per gram of muscle wet weight) at each measured time point to baseline as described by Bloch et al. (21). Glycogen synthesis rates were determined using a linear regression analysis over the individual time-point glycogen concentrations.

**Glycolytic flux ($V_{gly}$) calculations.** Metabolic steady-state conditions were assumed for calculating carbon flux through the glycolytic pathway into the intermediate triose pool of lactate, pyruvate, and alanine. Differential equations were developed from steady-state rate equations and solved for $V_{gly}$ (22). $^{13}$C label incorporation from glucose into lactate and alanine in the hindlimb muscles was observed by $^{13}$C NMR as an indirect marker of pyruvate labeling. Label incorporation into lactate and alanine is a qualitative indicator of glycolytic flux ($V_{gly}$) (Fig. 1).

**Statistical analysis.** All data are reported as means ± SE. Analysis of variance (ANOVA) was performed on data to determine significance at a minimum $P$ < 0.05 threshold between the three groups. A multiple comparison Fisher's projected least significant difference post hoc test was used when necessary to determine significance between groups. The correlation data was subjected to a Fisher's $r$-to-$z$ transformation to determine significance.

## RESULTS

**Basal measurements.** The initial weights of the rats in the three groups were similar at the onset of the dietary feeding and were not significantly different at the end of the experiment,

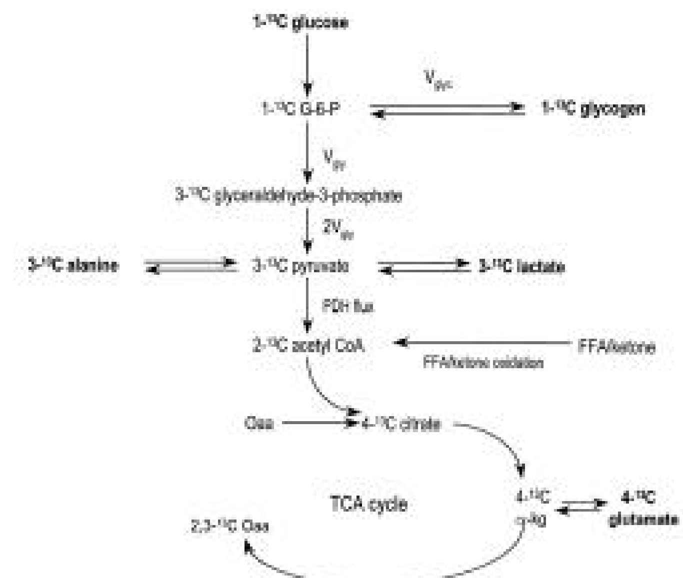


**FIG. 1. Schematic of skeletal muscle metabolite labeling after [1-$^{13}$C]glucose precursor infusion. $^{13}$C label from glucose becomes incorporated into [1-$^{13}$C]glycogen and [3-$^{13}$C]pyruvate, which can be reduced to [3-$^{13}$C]lactate or converted to [3-$^{13}$C]alanine via aminotransferase reaction. The rate at which substrate enters the [lactate + pyruvate + alanine] pool is 2× the glycolytic rate ($2V_{gly}$). Subsequent labeling of [4-$^{13}$C]glutamate occurs from the label entering the TCA cycle via PDH. The degree to which the [4-$^{13}$C]glutamate enrichment is diluted with respect to [3-$^{13}$C]pyruvate is determined by the ratio of unlabeled flux through FFA/ketone oxidation versus labeled flux through PDH. G-6-P, glucose-6-phosphate; Oaa, oxaloacetate; α-kg, α-ketoglutarate.**

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TABLE 1
Weights and basal measurements

| Group | Weight gain (g) | Feeding duration (days) | Experimental weight (g) | Insulin (μU/ml) | Glucose (mmol/l) | Plasma FFA (mmol/l) | Plasma TG (mg/dl) |
|---|---|---|---|---|---|---|---|
| Control | 181 ± 11 | 32 ± 1 | 326 ± 8 | 10.6 ± 1.7 | 6.3 ± 0.2 | 1.2 ± 0.06 | 34.8 ± 2.8 |
| Safflower oil | 189 ± 7 | 31 ± 1 | 339 ± 4 | 9.0 ± 1.9* | 6.9 ± 0.4 | 1.1 ± 0.07 | 41.7 ± 5.8 |
| Fish oil | 180 ± 12 | 36 ± 2 | 327 ± 11 | 14.5 ± 1.4 | 6.6 ± 0.3 | 1.1 ± 0.06 | 36.2 ± 4.0 |

Data are means ± SE. *$P$ < 0.001 vs. control group. Significance was determined by ANOVA post hoc test.

but the fish oil group required a slightly longer (4–5 days) feeding duration to achieve the same weight (Table 1). Basal insulin concentrations were significantly higher in the safflower oil group (19.0 ± 1.9 μU/ml) than in the fish oil and control groups (14.5 ± 1.4 and 10.6 ± 1.7 μU/ml, respectively, $P$ < 0.001). Basal plasma glucose, FFA, and TG concentrations were not significantly different between the three groups (Table 1).

**Euglycemic-hyperinsulinemic clamp.** During the euglycemic-hyperinsulinemic clamp experiment, plasma glucose concentrations were maintained at 6.5 ± 0.3 in the safflower oil group, 6.3 ± 0.3 in the fish oil group, and 6.3 ± 0.3 mmol/l in the control group (Fig. 2*A*). Plasma insulin concentrations increased to 201 ± 35 in the safflower oil group, 175 ± 37 in the fish oil group, and 170 ± 25 μU/ml in the control group. Plasma FFA decreased in all groups (0.4 ± 0.1, 0.3 ± 0.1, and 0.4 ± 0.1 mmol/l in the safflower oil, fish oil, and control groups, respectively). Plasma TG decreased only in the control and fish oil groups during the infusion protocol to 18.0 ± 2.4 ($P$ < 0.01) and 20.9 ± 2.7 ($P$ < 0.01), respectively. The glucose infusion rate ($G_{inf}$) was stable throughout the duration of the clamp period, but it was significantly lower in the safflower oil group as shown in Fig. 2*B* (21.4 ± 2.3 mg · kg$^{-1}$ · min$^{-1}$) than in the fish oil or control groups (31.7 ± 1.9, $P$ < 0.006, and 31.6 ± 2.8 mg · kg$^{-1}$ · min$^{-1}$, $P$ < 0.005, respectively). As reflected in Table 2,

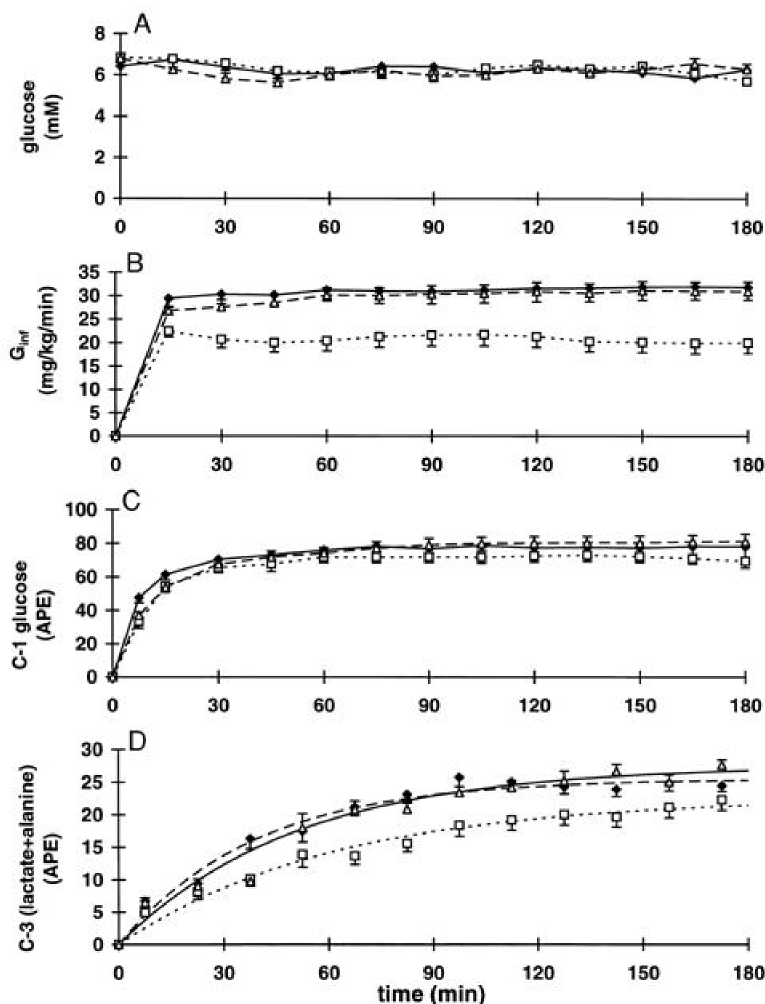


**FIG. 2. Glucose concentration and kinetic data in safflower oil (□), fish oil (△), and control (◆) groups during a euglycemic-hyperinsulinemic clamp. *A*: Plasma glucose concentration (mmol/l). *B*: Glucose infusion rate ($G_{inf}$) (mg · kg$^{-1}$ · min$^{-1}$). *C*: Plasma [1-$^{13}$C]glucose enrichment (APE). *D*: [3-$^{13}$C](lactate + alanine) enrichment (APE).**

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TABLE 2
Euglycemic-hyperinsulinemic clamp

| Group | $G_{inf}$ (mg · kg$^{-1}$ · min$^{-1}$) | EGP (mg · kg$^{-1}$ · min$^{-1}$) | $V_{glyc}$ (nmol · g$^{-1}$ · min$^{-1}$) | $V_{gly}$ (nmol · g$^{-1}$ · min$^{-1}$) |
|---|---|---|---|---|
| Control | 31.6 ± 2.8 | 4.3 ± 0.6 | 125 ± 14 | 62.1 ± 10.3 |
| Safflower oil | 21.4 ± 2.3* | 3.5 ± 0.4 | 103 ± 15 | 21.7 ± 2.2† |
| Fish oil | 31.7 ± 1.9 | 3.2 ± 1.0 | 133 ± 19 | 45.7 ± 6.7 |

Data are means ± SE. EGP, endogenous glucose production. *$P < 0.005$ vs. control group; $P < 0.006$ vs. fish oil group. †$P < 0.001$ vs. control group; $P < 0.04$ vs. fish oil group. Significance was determined by ANOVA post hoc test.

endogenous glucose production in the three groups did not differ during the euglycemic-hyperinsulinemic clamp. The plasma [1-$^{13}$C]glucose atom percent excess (APE) increased rapidly during the clamp and reached steady state in approximately 30–45 min (Fig. 2*C*).

**In vivo $^{13}$C NMR.** Rates of glycogen synthesis ($V_{glyc}$) and glycolysis ($V_{gly}$) were assessed by in vivo $^{13}$C NMR. Figure 3 illustrates a typical $^{13}$C NMR spectrum in a control rat taken at 120 min, when significant $^{13}$C label incorporation into metabolite intermediates was achieved. The β- and α-anomer peaks of [1-$^{13}$C]glucose appear at 96.8 and 93.0 ppm, respectively; and the large peak slightly downfield at 100.5 ppm corresponds to the C-1 glucosyl unit of the glycogen polymer. [3-$^{13}$C]lactate and [3-$^{13}$C]alanine may also be observed at 21.0 and 16.9 ppm, respectively. The steady-state $V_{glyc}$ was determined to be 103 ± 15, 133 ± 19, and 125 ± 14 nmol · g$^{-1}$ · min$^{-1}$ in the safflower oil, fish oil, and control groups, respectively (Table 2). Although $V_{glyc}$ was lower in the safflower oil group than in the control and fish oil groups, these rates were not significantly different. $V_{gly}$ was calculated using the metabolic steady-state turnover kinetics of $^{13}$C label in [3-$^{13}$C]lactate and [3-$^{13}$C]alanine pools (Fig. 2*D*) as a marker of pyruvate glycolytic intermediate turnover (22). $V_{gly}$ was similar in the fish oil and control groups (45.7 ± 6.7 and 62.1 ± 10.3 nmol · g$^{-1}$ · min$^{-1}$, respectively) but was significantly lower in the safflower oil group (21.7 ± 2.2 nmol · g$^{-1}$ · min$^{-1}$, $P < 0.001$ vs. control and $P < 0.04$ vs. fish oil) (Table 2). This may also be qualitatively inferred from Fig. 2*D*, which depicts the [3-$^{13}$C]lactate and [3-$^{13}$C]alanine pool turnover as much slower than that of the fish oil or control group. Therefore, the decreased glucose disposal observed in the safflower oil–fed rats was associated with a ~65% reduction in glycolytic flux, whereas the fish oil and control groups had similar $V_{glyc}$ and $V_{gly}$. Additionally, a positive correlation between $G_{inf}$ and $V_{gly}$ was found ($r = 0.54$, $P < 0.005$).

**Isotopomer analysis.** In vitro NMR analysis was used to determine the skeletal muscle [3-$^{13}$C]lactate, [3-$^{13}$C]alanine, and [4-$^{13}$C]glutamate enrichments for determining the relative contribution of flux entering the TCA cycle originating from PDH flux versus FFA/ketone oxidation (Fig. 1). $^{13}$C label entering the TCA cycle via PDH incorporates label into [4-$^{13}$C]glutamate as a result of the first turn of the TCA cycle. This labeled pool is diluted to the degree by which FFA/ketone oxidation versus PDH flux occurs during the clamp. The results in Table 3 show that the ratio of [4-$^{13}$C]glutamate APE versus the average [3-$^{13}$C]lactate and [3-$^{13}$C]alanine APE was significantly lower in the safflower oil group than in the fish oil and control groups (43 ± 8 vs. 64 ± 6%, $P < 0.05$, and 73 ± 8%, $P < 0.01$, respectively). Positive correlations were found between the PDH/TCA cycle flux ratio and $G_{inf}$ ($r = 0.66$, $P < 0.0005$) and $V_{gly}$ ($r = 0.51$, $P < 0.005$), suggesting that increased FFA/ketone oxidation was associated with decreased glycolysis and glucose disposal in all groups.

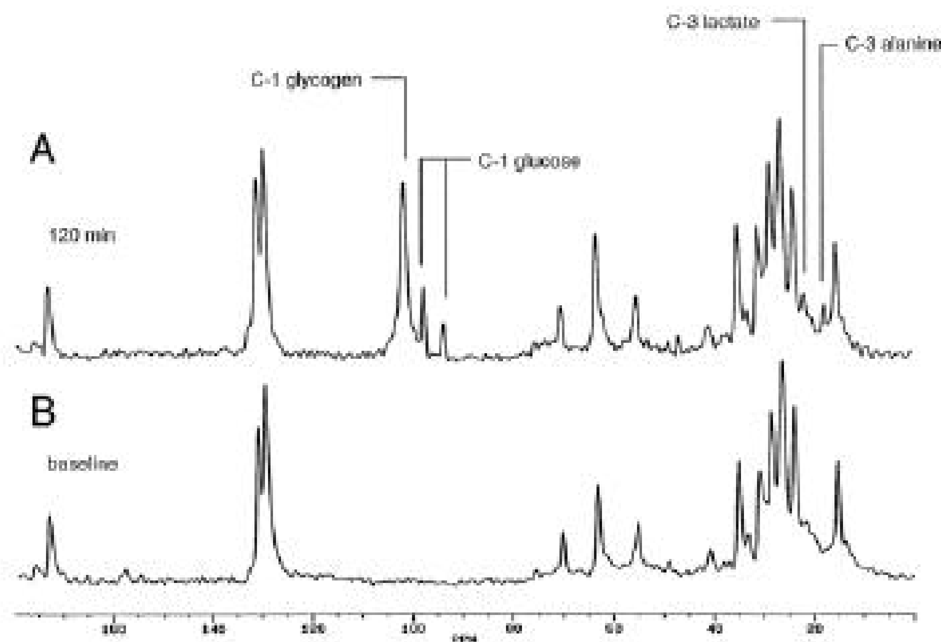


**FIG. 3. In vivo $^{13}$C NMR spectra of [1-$^{13}$C]glucose label incorporation into [1-$^{13}$C]glycogen, [3-$^{13}$C]lactate and [3-$^{13}$C]alanine. *A*: A 15-min acquired spectrum taken at 120 min into the euglycemic-hyperinsulinemic clamp. [1-$^{13}$C]glucose (β-anomer: 96.8 ppm; α-anomer: 93.0 ppm), [1-$^{13}$C]glycogen (100.5 ppm), [3-$^{13}$C]lactate (21.0 ppm), and [3-$^{13}$C]alanine (16.9 ppm) are visible where indicated. *B*: A 15-min acquired baseline $^{13}$C NMR spectrum from the rat hindlimb.**

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TABLE 3
Steady-state $^{13}$C isotopic enrichments in [3-$^{13}$C]lactate, [3-$^{13}$C]alanine, [4-$^{13}$C]glutamate, and PDH/TCA cycle flux ratio in skeletal muscle

| Group | [3-$^{13}$C]lactate APE (%) | [3-$^{13}$C]alanine APE (%) | [4-$^{13}$C]glutamate APE (%) | PDH/TCA cycle flux (%) |
|---|---|---|---|---|
| Control | 23 ± 2 | 24 ± 1 | 18 ± 3 | 73 ± 8 |
| Safflower oil | 21 ± 3 | 20 ± 3 | 9 ± 2* | 43 ± 8† |
| Fish oil | 27 ± 2 | 25 ± 1 | 17 ± 1 | 64 ± 6 |

Data are means ± SE. For PDH/TCA cycle flux, [4-$^{13}$C]glutamate APE/[([3-$^{13}$C]lactate APE + [3-$^{13}$C]alanine APE)/2]. *$P < 0.02$ vs. control group; $P < 0.04$ vs. fish oil group. †$P < 0.01$ vs. control group; $P < 0.05$ vs. fish oil group. Significance was determined by ANOVA post hoc test.

**Intramuscular TG.** Because of the significant increase in relative FFA/ketone oxidation in the rats fed safflower oil but not fish oil, we examined the relationship between fatty acid composition in the diet and insulin sensitivity. Therefore, the baseline $^{13}$C NMR spectrum was used to obtain data regarding the fatty acid profiles of the intramuscular TG and surrounding subcutaneous adipose tissue. To ensure that the lipid signal in the in vivo spectrum reflected that of the intramuscular TG, the chloroform/methanol extracted intramuscular lipid was also analyzed by $^{13}$C NMR. Additionally, the lipid from the diet alone was extracted and analyzed by $^{13}$C NMR. The results demonstrate that both in vivo and muscle extract spectra exhibited lipid profiles that are very similar to that of their respective diets and that these lipid profiles are unique with respect to the different diets. However, when a correlation analysis was performed on the NMR-based lipid profiles with respect to ω-3, polyunsaturated, monounsaturated, and [poly+monounsaturated]/polyunsaturated content versus insulin sensitivity ($G_{inf}$), no significant correlations were determined. Therefore, the TG profile (ω-3, or degree of unsaturation) detected in the muscle by NMR could not be used as a corollary measure of insulin sensitivity. When total TG content in the muscle was measured enzymatically, significant differences in TG content between diets were found (control = 5.2 ± 0.8, fish oil = 3.6 ± 1.1, safflower oil = 7.3 ± 0.8 μmol/g; $P < 0.01$ safflower oil vs. fish oil, $P < 0.05$ safflower oil vs. control). Additionally, there was a significant negative correlation found between muscle TG content and $G_{inf}$ when all three feeding groups were pooled together ($r = -0.566$, $P < 0.005$, Fig. 4).

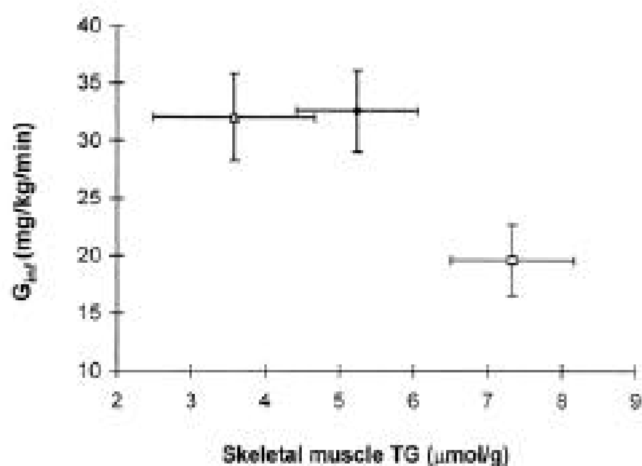


**FIG. 4. Correlation of insulin sensitivity ($G_{inf}$) and intramuscular TG. There was a significant negative correlation determined between intramuscular $G_{inf}$ and TG when all three groups (safflower oil [□], fish oil [△] and control [◆]) were pooled together ($r = -0.566$, $P < 0.005$).**

## DISCUSSION

In this study, rats chronically fed one of two high-fat diets (safflower oil or fish oil) to produce contrasting degrees of whole-body insulin responsiveness were studied using in vivo $^{13}$C NMR to elucidate the intracellular partitioning of glucose flux (glycogen synthesis, glycolysis, and relative flux through the PDH versus the TCA cycle) in skeletal muscle. We found that the insulin resistance in skeletal muscle associated with chronic safflower oil feeding could primarily be attributed to a reduction in glycolysis, whereas fish oil feeding had no effect on any of the major intramuscular pathways of glucose metabolism. Additionally, FFA/ketone oxidation versus PDH flux as well as intramuscular TG were greater in the safflower oil group than in the control or fish oil groups. These data suggest that the elevated intramuscular TG observed in the rats fed safflower oil correlates with increased fat oxidation and decreased PDH flux and that the protective effect of fish oil feeding may occur through its ability to limit TG deposition in skeletal muscle.

The underlying metabolic consequence of insulin resistance associated with chronic high-fat feeding has previously been linked to a reduction in glycolysis (1,3,6,9,11,12). The effect of chronic high-fat feeding on rates of insulin-stimulated muscle glycogen synthesis is considerably more variable, with some studies reporting no differences (1,3,6,9) and others reporting a decrease in the high-fat–fed rats (12,23). Skeletal muscle tissue type, duration of high-fat feeding, and degree of hyperinsulinemia may all factor into the differences reported. Although we did not detect significant differences in $V_{glyc}$ per se, the reduction in glycolysis in the safflower oil versus the control group (40.4 nmol · g$^{-1}$ · min$^{-1}$) could maximally account for a 22% reduction in the muscle glucose disposal rate of the control group (146.7 vs. 187.1 nmol · g$^{-1}$ · min$^{-1}$), whereas the $G_{inf}$ was reduced 32% in the safflower oil versus the control group (21.4 vs. 31.6 mg · kg$^{-1}$ · min$^{-1}$). Therefore, $V_{glyc}$ may be reduced in the safflower oil group but not detected in this study possibly because of a type II error.

Storlien et al. (5) and others (12,23) have postulated the involvement of the glucose-FFA cycle after measuring elevated intramuscular TG and decreased glucose uptake in chronic high-fat–fed rats. However, one cannot directly attribute the decrease in glycolysis observed in high-fat–fed

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rats (1,3,6,9,11,12) to the classic glucose-FFA cycle as the mechanism for glycolytic inhibition unless PDH flux is actually shown to be decreased. This is because high-fat feeding might cause a selective decrease in anaerobic glycolytic flux independent of any effect on PDH flux. To date, no studies have examined the impact of high-fat feeding directly on PDH flux. Oakes et al. (12), using a β-oxidation inhibitor (etomoxir), demonstrated an increase in insulin-stimulated glucose uptake in skeletal muscle of high-fat–fed rats and therefore indirectly established the role of the glucose-FFA cycle in insulin resistance induced by chronic fat feeding. In contrast, Han et al. (24) showed that insulin-stimulated glucose transport was reduced ~40% in skeletal muscle of high-fat–fed rats under anoxic conditions where β-oxidation should not occur. We found significant correlations between the PDH/TCA cycle flux ratio and $V_{gly}$ and $G_{inf}$, which suggests that increased fat oxidation (i.e., low PDH/TCA cycle flux ratio) is coupled with decreased $V_{gly}$ and $G_{inf}$. This is consistent with the classic inhibitory mechanism of fat-induced insulin resistance proposed by Randle et al. (13), although direct effects of intramuscular fat on insulin signaling/glucose transport (25–29) cannot be ruled out.

Previous studies found that long-chain polyunsaturated ω-3 FFAs, abundant in marine oils, have both a hypotriglyceridemic effect (5,7) and a protective effect on insulin sensitivity (7,11). Storlien et al. (5) studied the relationship of specific dietary fats to insulin sensitivity in skeletal muscle and concluded that the particular fatty acid profiles of diets can determine insulin sensitivity. We also found a protective effect of the fish oil diet on insulin sensitivity, as there was no significant difference in $G_{inf}$ between groups (31.7 ± 1.9 and 31.6 ± 2.8 mg · kg$^{-1}$ · min$^{-1}$ for fish oil and control groups, respectively). Additionally, there were no differences in glycogen synthesis and glycolysis between the control and fish oil groups in our study. FFA/ketone oxidation relative to TCA cycle flux was also the same in the fish oil and the control groups, therefore suggesting the possibility of preferential discrimination against long-chain/highly unsaturated FFA for cellular oxidation. When a correlation analysis was performed on lipid profiles determined from NMR (ω-3, polyunsaturated, monounsaturated, and [polymonounsatrated]/poly unsaturated) compared with $G_{inf}$, no significant correlations were found. Therefore, the FFA profile of the intramuscular TG could not be used as a marker for insulin sensitivity. Only the absolute amount of intramuscular TG inversely correlated with $G_{inf}$ (Fig. 4). Indeed, the most insulin-resistant fish oil–fed rat ($G_{inf}$ = 23.1 mg · kg$^{-1}$ · min$^{-1}$) in our experiment had an elevated intramuscular TG content of 6.6 µmol/g. It has been postulated that fish oil may prevent hyperlipidemia, possibly by decreasing fatty acid synthase activity (10) or VLDL (30) secretion in the liver. Additionally, rats fed fish oil tend to have less total body fat and less intra-abdominal fat than other groups of high-fat–fed rats (7). Another possible explanation for the hypolipidemia observed in rats fed fish oil may be that the resulting increase in the fluidity of the plasma membrane due to the incorporation of long-chain, highly unsaturated fatty acids into phospholipids could cause cells to become "leaky" and therefore less efficient, as more energy is required for maintenance of proper cell ion gradients (5). This energy-wasting mechanism would result in lower weight gain (observed in our study) and increased fat oxidation. However, we did not observe a relative increase in fat oxidation in the fish oil group versus controls during hyperinsulinemic conditions. Thus, it appears that the protective effect of fish oil on insulin-stimulated muscle glucose disposal occurs via a relative reduction of FFA oxidation compared with the rats fed safflower oil, possibly as a result of lowered intramuscular TG and FFA availability.

In conclusion, the results from our studies demonstrate that *1*) the diminished insulin-stimulated glucose disposal in muscle observed with chronic safflower oil feeding can be primarily attributed to a reduction in glycolysis associated with decreased intramuscular pyruvate oxidation and increased fat oxidation and *2*) chronic fish oil feeding spares the muscle from becoming insulin resistant in part by lowering intramuscular TG availability for oxidation and increasing PDH versus TCA cycle flux relative to safflower oil feeding.

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Author Queries (please see Q in margin and underlined text)

Q1: Edits in sentence beginning “To address these questions” OK?
Q2: For the sentence beginning “Major fatty acid composition” please clarify what the numbers in parenthesis indicate. Which percentages go with which of the three diets studied? What do the ratios signify?
Q3: Please spell out PCA.
Q4: Please spell out PLSD.
Q5: In Table 1, please spell out TAG.
Q6: Please give a reference(s) for Randle and coworkers in the sentence beginning “This is consistent.”

For Ref. 18, it looks like there are 2 publishers. Which is the correct one? Also, are Keppler and Decker the authors of the whole book? If so, why is there also an editor listed? If Keppler and Decker are the authors of a chapter of the book, please list the chapter title.
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