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Muscle Oxidative Capacity Is a Better Predictor of Insulin Sensitivity than Lipid Status

CLINTON R. BRUCE, MITCHELL J. ANDERSON, ANDREW L. CAREY, DAVID G. NEWMAN, AREND BONEN, ADAMANDIA D. KRIKETOS, GREGORY J. COONEY, AND JOHN A. HAWLEY

Exercise Metabolism Group (C.R.B., D.G.N., J.A.H.) and Skeletal Muscle Research Laboratory (A.L.C.), School of Medical Sciences, RMIT University, Bundoora, Victoria 3083, Australia; Exercise Physiology and Metabolism Laboratory, Department of Physiology (M.J.A.), The University of Melbourne, Parkville, Victoria 3052, Australia; Department of Kinesiology (A.B.), University of Waterloo, Waterloo, Ontario, Canada N2L 3G1; and Garvan Institute of Medical Research (A.D.K., G.J.C.), St. Vincent's Hospital, Sydney, New South Wales 2010, Australia

We determined whole-body insulin sensitivity, long-chain fatty acyl coenzyme A (LCACoA) content, skeletal muscle triglyceride (TG_m) concentration, fatty acid transporter protein content, and oxidative enzyme activity in eight patients with type 2 diabetes (TYPE 2); six healthy control subjects matched for age (OLD), body mass index, percentage of body fat, and maximum pulmonary O_2 uptake; nine well-trained athletes (TRAINED); and four age-matched controls (YOUNG). Muscle biopsies from the vastus lateralis were taken before and after a 2-h euglycemic-hyperinsulinemic clamp. Oxidative enzyme activities, fatty acid transporters (FAT/CD36 and FABPpm), and TG_m were measured from basal muscle samples, and total LCACoA content was determined before and after insulin stimulation. Whole-body insulin-stimulated glucose uptake was lower in TYPE 2 ($P < 0.05$) than in OLD, YOUNG, and TRAINED. TG_m was elevated in TYPE 2 compared with all other groups ($P < 0.05$). However, both basal and insulin-stimulated skeletal muscle LCACoA content were similar. Basal citrate synthase activity was higher in TRAINED ($P < 0.01$), whereas β-hydroxyacyl CoA dehydrogenase activity was higher in TRAINED compared with TYPE 2 and OLD. There was a significant relationship between the oxidative capacity of skeletal muscle and insulin sensitivity (citrate synthase, $r = 0.71$, $P < 0.001$; β-hydroxyacyl CoA dehydrogenase, $r = 0.61$, $P = 0.001$). No differences were found in FAT/CD36 protein content between groups. In contrast, FABPpm protein was lower in OLD compared with TYPE 2 and YOUNG ($P < 0.05$). In conclusion, despite markedly elevated skeletal muscle TG_m in type 2 diabetic patients and strikingly different levels of whole-body glucose disposal, both basal and insulin-stimulated LCACoA content were similar across groups. Furthermore, skeletal muscle oxidative capacity was a better predictor of insulin sensitivity than either TG_m concentration or long-chain fatty acyl CoA content. (*J Clin Endocrinol Metab* 88: 5444–5451, 2003)

INSULIN RESISTANCE IN skeletal muscle is a major factor in the pathogenesis of type 2 diabetes. Studies in both rodents (1) and humans (2–6) have demonstrated a strong negative relationship between im triglyceride (TG_m) content and insulin action. The mechanisms that result in excess accumulation of TG_m in insulin-resistant states remain unclear but may result from increased availability and uptake of circulating free fatty acids (FFA), diminished fatty acid (FA) oxidation, or a combination of both processes (7, 8). However, because triglyceride (TG) is a relatively inert intracellular metabolite, it seems doubtful that it interferes directly with insulin action. Instead, it is more likely that TG acts as a surrogate marker for some other FA-derived entity such as long-chain fatty acyl coenzyme A (LCACoA) esters (9–11). This metabolically active form of lipid may influence metabolism acutely by changing substrate availability or by altering key enzyme activities involved in glucose flux (12), or chronically by interfering with signal transduction, membrane trafficking, and gene regulation (for review, see Ref. 13).

Abbreviations: BMI, Body mass index; CoA, coenzyme A; DAG, diacylglycerol; FA, fatty acid; FFA, free FA; GIR, glucose infusion rate; β-HAD, β-hydroxyacyl CoA dehydrogenase; HbA_{1C}, glycosylated hemoglobin; LCACoA, long-chain fatty acyl CoA; LCFA, long-chain FA; TG, triglyceride; TG_m, im TG; v. lateralis, vastus lateralis; VO_2max, maximum pulmonary O_2 uptake.

In humans, a negative relationship between insulin action and skeletal muscle LCACoA content has been reported, with LCACoA content a better predictor of whole-body insulin sensitivity than TG_m (9, 14). Additionally, recent findings suggest a reduction in LCACoA content may, in part, be responsible for enhanced insulin action after weight loss in obese individuals (10). In rodents, insulin administration reduces LCACoA content (9, 15), although the ability of acute insulin stimulation to suppress LCACoAs is diminished in the muscle of insulin-resistant, fat-fed rats (15). To date, no study has investigated the effect of acute insulin stimulation on muscle LCACoA content in humans. Accordingly, one aim of the current investigation was to determine the effect of insulin stimulation on skeletal muscle LCACoA content in type 2 diabetic and healthy control subjects. We hypothesized that individuals with enhanced insulin sensitivity would have less accumulation of LCACoA, and that insulin stimulation would suppress muscle LCACoA content to a greater degree in these subjects than in those with impaired insulin action. In addition, we reexamined the premise that muscle lipid content (*i.e.* TG_m and LCACoA) is strongly associated with insulin action in human skeletal muscle. This was achieved by determining lipid status in muscle from individuals with a wide range of insulin sensitivity. Finally, other markers of muscle lipid status (FA transport proteins) were measured to ascertain their putative role in the distur-

bances in lipid metabolism observed in insulin-resistant muscle.

Subjects and Methods

Experimental subjects

A total of 27 volunteers (eight patients with type 2 diabetes, 10 healthy but sedentary control subjects, and nine well-trained endurance athletes) participated in this cross-sectional investigation. The experimental protocol was approved by the Human Ethics Committee of RMIT University. The purpose, nature, and potential risks of the study were explained to all subjects, and written informed consent was obtained before participation. The 10 control subjects were divided into two groups on the basis of their age. One group of control subjects (OLD) was matched to the eight patients with type 2 diabetes (TYPE 2) for age, body mass index (BMI), percentage of body fat, and maximum pulmonary O_2 uptake (VO_2max). An unrelated group of healthy subjects served as age-matched control subjects (YOUNG) for the well-trained individuals (TRAINED). The well-trained individuals were either cyclists or triathletes currently completing approximately 15 h/wk of endurance training. The physical characteristics of all study participants are presented in Table 1. Normal glucose tolerance was confirmed in control subjects and well-trained endurance athletes by an oral glucose tolerance test (75 g glucose). The diabetic subjects had a mean time of 4 ± 1 yr since diagnosis of disease (range, 6 months to 10 yr). The diabetic patients were being treated with diet (n = 2) or oral hypoglycemic agents (sulfonylureas, n = 1; metformin, n = 1; a combination of sulfonylureas and metformin, n = 4). Other than diabetes, none of these subjects had any significant medical problems; all were nonsmokers; and, apart from type 2 diabetic patients, none were taking medications known to alter carbohydrate metabolism. All subjects had maintained a constant body mass during the 6 months preceding the experiment. All subjects were instructed to abstain from any form of vigorous physical activity for 36 h before an experiment (described subsequently) and to maintain their normal diet. The subjects reported to the laboratory for the experimental trial after a 12- to 14-h overnight fast, and in the case of the type 2 diabetic patients, before the administration of any antidiabetic medication. All subjects were studied under free living conditions.

Assessment of body composition

On their first visit to the laboratory, whole-body dual-energy x-ray absorptiometry (Lunar DPX, Lunar Radiation Corp., Madison, WI) was used to measure whole-body fat mass and fat-free mass. Before each scan, the dual-energy x-ray absorptiometry was calibrated with known phantoms.

Determination of VO_2max

After assessment of body composition, subjects performed a progressive, incremental cycling test to volitional fatigue on an electronically braked ergometer (Lode, Groningen, The Netherlands) for the determination of VO_2max. Throughout the test, subjects breathed through a mouthpiece attached to a Medgraphics metabolic cart (Medical Graphics Corp., St. Paul, MN). Expired gas was passed through a flow meter, an O_2 analyzer, and a CO_2 analyzer. The flow meter was calibrated with a 3L Hans Rudolph syringe. The gas analyzers were calibrated with gases of known concentrations (4.00% CO_2 and 16.00% O_2). The flow meter and gas analyzers were connected to a computer, which calculated minute ventilation, O_2 consumption, carbon dioxide production, and respiratory exchange ratio.

Euglycemic-hyperinsulinemic clamp

On a subsequent visit to the laboratory, whole-body insulin-stimulated glucose uptake was determined during a 120-min euglycemic-hyperinsulinemic clamp. An anticubital vein was cannulated for infusion of glucose and insulin, and a hand vein was cannulated retrogradely and heated for sampling of arterialized blood. After collection of baseline blood samples, a percutaneous biopsy of the vastus lateralis (v. lateralis) muscle was obtained. Briefly, local anesthesia [1% lignocaine (lidocaine)] was administered to the skin, sc tissue, and fascia of the v. lateralis, and an incision was made. At this time, a second site on the same leg (5-cm distal) was prepared for the postclamp biopsy. Then, a resting muscle biopsy was obtained using a 6-mm Bergström needle modified to include suction. Muscle biopsy specimens (100–150 mg) were immediately frozen in liquid nitrogen and stored at −80 C until subsequent analysis. After resting for 5 min, a primed (9 $mU \cdot kg^{-1}$) continuous infusion of insulin (Actrapid, Novo Nordisk, Sydney, New South Wales, Australia) was commenced at a rate of 40 $mU \cdot m^{-2} \cdot min^{-1}$, and blood glucose concentration was measured at 5-min intervals throughout the clamp. A variable rate infusion of 20% glucose was used to maintain euglycemia (5 mM) for the duration of the clamp (120 min). The blood glucose concentration in the diabetic subjects was allowed to decrease during the insulin infusion to 5 mM and then maintained at this concentration for the remainder of the clamp. Samples (2 ml) for subsequent determination of plasma insulin concentration were obtained every 10 min, centrifuged, and stored at −80 C. Whole-body glucose uptake was calculated from the glucose infusion rate (GIR; milligrams per kilogram fat-free mass per minute) required to maintain a blood glucose concentration of 5 mmol/liter as determined during the final 30 min of the clamp. A second muscle biopsy was obtained 120 min after the initiation of the insulin infusion. To prevent a decrease in plasma potassium concentration during the clamp, 30 mmol KCl (Slow-K, Novartis, North Ryde, New South Wales, Australia) was administered orally.

Blood biochemistry

Blood glucose concentration was measured using an automated analyzer (2300 Stat Plus Glucose and L-Lactate Analyzer; Yellow Springs Instruments, Yellow Springs, OH). Plasma insulin concentration was determined by RIA using a commercially available kit (Phadeseph, Insulin RIA, Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden). Glycosylated hemoglobin (HbA_{1C}) was determined by specific ion-exchange chromatography (Sigma Diagnostics, Castle Hill, New South Wales, Australia). Plasma FFA concentration was measured using an enzymatic colorimetric method (NEFA C test kit, Wako, Richmond, VA). Plasma cholesterol and TGs were analyzed using an automated analyzer (Cholestech LDX, Cholestech, Hayward, CA).

TABLE 1. Subjects' physical characteristics

	TYPE 2	OLD	YOUNG	TRAINED
Men/women	7/1	5/1	3/1	9/—
Age (yr)	46 ± 3	50 ± 2	25 ± 2[a,b]	25 ± 1[a,b]
Body mass (kg)	90.4 ± 7.8	85.7 ± 3.1	86.2 ± 7.3	73.3 ± 2.1
BMI (kg/m^2)	29.1 ± 1.8	27.6 ± 1.0	26.2 ± 1.8	23.2 ± 0.3[a]
% Body fat	27.1 ± 3.4	26.0 ± 3.9	26.8 ± 1.9	8.7 ± 0.6[a,b,c]
VO_2max (liters/min)	2.6 ± 0.2	3.3 ± 0.2	3.5 ± 0.3[d]	5.0 ± 0.2[a,b,c]

Data are means ± SEM.
[a] Different from TYPE 2, $P < 0.01$.
[b] Different from OLD, $P < 0.01$.
[c] Different from YOUNG, $P < 0.01$.
[d] Different from TYPE 2, $P < 0.05$.

TG_m

Approximately 30 mg muscle was freeze-dried under vacuum for 24 h. The sample (~10 mg) was viewed under a microscope (×6.3) at room temperature for dissection and removal of all traces of adipose tissue, connective tissue, and blood contaminants. This procedure yielded approximately 7–8 mg dry-weight, dissected muscle from which a direct measure of TG_m content was determined, as previously described (3). Total lipids were extracted according to the method of Folch *et al.* (16) from the dried and dissected skeletal muscle in 4 ml chloroform: methanol (2:1) and left to rotate at room temperature overnight. Sodium chloride (0.6%) was added, and centrifugation (2000 rpm for 10 min) resulted in a separation of the aqueous and organic phases. The organic phase containing the TG was transferred to a glass vial and air dried. The isolated lipids were resuspended in 250 μl ethanol, and the TG concentration was determined spectrophotometrically at 490 nm using an enzymatic colorimetric test kit (Triglycerides GPO-PAP, Roche Molecular Biochemicals, Sydney, New South Wales, Australia). The within-assay coefficient of variation for this assay in our laboratory is approximately 8%.

Tissue LCACoA measurement

The total amount of LCACoA in muscle biopsy samples (~50 mg) was measured using an assay adapted from Antinozzi *et al.* (17). Tissue was homogenized in 10% trichloroacetic acid. The acid-insoluble pellet (which includes proteins, some lipids, and LCACoAs) was washed with ether and water to remove unwanted lipids and water-soluble contaminants. The pellet was resuspended in 10 mM dithiothreitol, and the pH was raised to 11.5 with KOH. The sample was incubated at 55 C for 10 min to hydrolyze the ester bond between FA and coenzyme A (CoA). After hydrolysis, the sample was neutralized with 1 M HCl and centrifuged at 13,000 rpm, and 250 μl of the supernatant was used for the determination of CoA. The supernatant was added to a reaction buffer containing 50 mM KH_2PO_4, 100 μM α-ketoglutarate, and 50 μM nicotinamide adenine dinucleotide$^+$. After a basal reading, 20 mU α-ketoglutarate dehydrogenase was added, and the reaction rate was followed to completion using a fluorometer (excitation, 340 nm; emission, 460 nm). The concentration of CoA in the sample was calculated with reference to a standard curve of known concentrations of CoA. The within-assay coefficient of variation for this assay in our laboratory is 10%.

Immunoblot analysis

Approximately 25 mg muscle tissue was used to determine the expression of FAT/CD36 and FABPpm. For detection of FAT/CD36 and FABPpm, we used a monoclonal antibody against human CD36 (provided by Dr. N. N. Tandon, Otsuka Research Institute, Maryland, Rockville, MD) and a rabbit polyclonal anti-FABPpm antiserum (provided by Dr. J. Calles-Escandon, SmithKline Beecham, Miami, FL), as previously described (18). Briefly, muscle samples were solubilized, and proteins were separated on 10% SDS-polyacrylamide gels. This was followed by transfer onto Immobilon polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were incubated for 2 h with either the monoclonal CD-36 antibody or the polyclonal FABPpm antibody. Secondary complexes were generated using an antimouse IgG horseradish peroxidase secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for FAT/CD36 and donkey antirabbit IgG horseradish peroxidase-conjugated secondary antibody (1:3000; Amersham, Arlington Heights, IL) for FABPpm.

Citrate synthase and β-hydroxyacyl CoA dehydrogenase (β-HAD) activity

Muscle (5–10 mg) was homogenized in 1:50 dilution (wt/vol) of a 175 mM potassium buffer solution, and citrate synthase activity was assayed spectrophotometrically at 25 C. β-HAD activity was assayed spectrophotometrically at 25 C, measuring the disappearance of reduced nicotinamide adenine dinucleotide using the same homogenate as for citrate synthase (19).

Statistics

Data are presented as mean ± SEM. Differences between experimental groups were determined using a one-way ANOVA. Significant differences were located using Tukey's *post hoc* test. A two-factor repeated-measures ANOVA was performed to determine differences in LCACoA content. Associations between variables were investigated using simple or multiple regression analyses, as appropriate. Statistical significance was accepted at $P < 0.05$.

Results

Subject characteristics

BMI and percentage of body fat were lower in TRAINED than in TYPE 2, OLD, and YOUNG ($P < 0.01$; Table 1). VO_2max was 2-fold greater in TRAINED than in TYPE 2 ($P < 0.01$) and also significantly higher than that of OLD and YOUNG ($P < 0.01$). VO_2max of YOUNG was significantly higher than that of TYPE 2 ($P < 0.05$).

The clinical characteristics of all subjects are shown in Table 2. Fasting blood glucose, plasma insulin, and FFA concentrations were higher in TYPE 2 than in OLD, YOUNG,

TABLE 2. Subjects' clinical characteristics

	TYPE 2	OLD	YOUNG	TRAINED
Basal				
Blood glucose (mM)	8.2 ± 1.3	4.4 ± 0.1[a]	4.3 ± 0.1[a]	4.1 ± 0.1[a]
Plasma insulin (pM)	106.3 ± 18.1	61.1 ± 4.6[a]	47.8 ± 7.3[a]	34.9 ± 2.7[b]
Plasma FFA (mM)	1.11 ± 0.11	0.79 ± 0.07[a]	0.74 ± 0.06[a]	0.74 ± 0.06[b]
TGs (mM)	1.89 ± 0.31	1.31 ± 0.20	0.77 ± 0.07[a]	0.81 ± 0.08[a]
Cholesterol (mM)	3.90 ± 0.15	4.38 ± 0.24	3.64 ± 0.08	3.33 ± 0.19[c]
HDL cholesterol (mM)	1.11 ± 0.09	1.09 ± 0.10	1.38 ± 0.18	1.38 ± 0.06
LDL cholesterol (mM)	1.98 ± 0.18	2.69 ± 0.21	1.90 ± 0.15	1.58 ± 0.17[c]
VLDL cholesterol (mM)	0.86 ± 0.14	0.60 ± 0.09	0.33 ± 0.03[a]	0.37 ± 0.04[a]
HbA_{1C} (%)	8.0 ± 0.6	5.1 ± 0.4[b]	4.4 ± 0.1[b]	4.7 ± 0.1[b]
Clamp				
Insulin-stimulated glucose uptake (mg·kg FFM^{-1}·min^{-1})	4.3 ± 1.3	10.7 ± 1.3[a]	12.5 ± 1.5[a]	18.2 ± 1.3[b,c,d]
Glucose (mM)	5.0 ± 0.1	5.1 ± 0.1	4.9 ± 0.2	4.9 ± 0.1
Insulin (pM)	487.1 ± 34.5	576.1 ± 42.5	489.7 ± 82.5	412.6 ± 25.2

Data are means ± SEM. HDL, High-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein.
[a] Different from TYPE 2, $P < 0.05$.
[b] Different from TYPE 2, $P < 0.01$.
[c] Different from OLD, $P < 0.01$.
[d] Different from YOUNG, $P < 0.05$.

and TRAINED. Similarly, HbA_{1C} levels were significantly elevated in TYPE 2 ($P < 0.01$).

Whole-body insulin-stimulated glucose uptake

Whole-body insulin-stimulated glucose uptake determined during the euglycemic-hyperinsulinemic clamp was significantly impaired in TYPE 2 (60% reduction compared with OLD, $P < 0.05$; 66% reduction compared with YOUNG, $P < 0.05$; 76% reduction compared with TRAINED, $P < 0.01$; Table 2). GIR was higher in TRAINED compared with OLD (41%; $P < 0.01$) and YOUNG (31%; $P < 0.05$).

Skeletal muscle lipids

TG_m content in TYPE 2 was approximately 150% higher compared with OLD, approximately 200% higher compared with YOUNG, and approximately 180% compared with TRAINED ($P < 0.05$; Fig. 1). Total skeletal muscle LCACoA content at rest was not significantly different between groups (Fig. 2). Furthermore, insulin stimulation had no detectable effect on total LCACoA content (Fig. 2).

FA transporters

FABPpm was lower in OLD compared with TYPE 2 and YOUNG ($P < 0.05$; Fig. 3A). FAT/CD36 protein was not different between groups (Fig. 3B).

Skeletal muscle oxidative capacity

Citrate synthase activity was significantly higher in the muscle of TRAINED subjects (232% higher compared with TYPE 2, $P < 0.01$; 162% higher than OLD, $P < 0.01$; 81% higher than YOUNG, $P < 0.01$; Table 3). There was no difference in citrate synthase activity between TYPE 2, OLD, and YOUNG. β-HAD activity was 94% higher in TRAINED compared with TYPE 2, and 76% higher than OLD ($P < 0.01$ for both; Table 3). β-HAD activity also tended to be higher in TRAINED compared with YOUNG (28%), but this difference did not reach statistical significance. The activity of β-HAD from muscle of TYPE 2, OLD, and YOUNG was not significantly different.

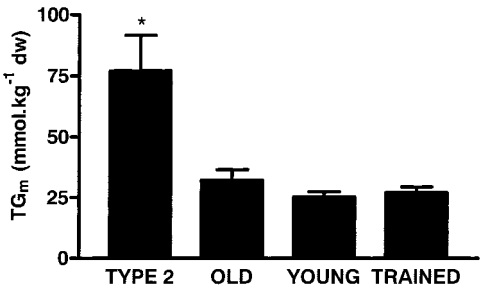

FIG. 1. TG_m content in biopsy samples obtained from the v. lateralis. Values are means ± SEM. *, Significantly different to OLD, YOUNG, and TRAINED ($P < 0.05$).

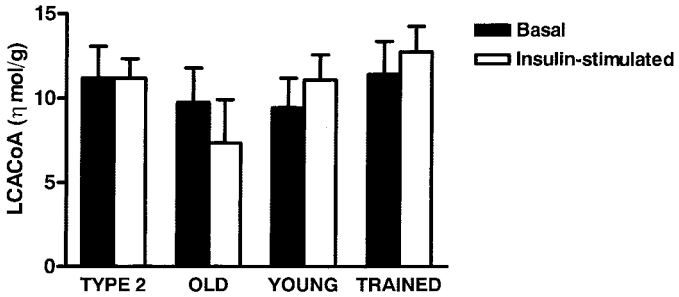

FIG. 2. Basal and insulin-stimulated LCACoA content in biopsy samples obtained from the v. lateralis. Values are means ± SEM.

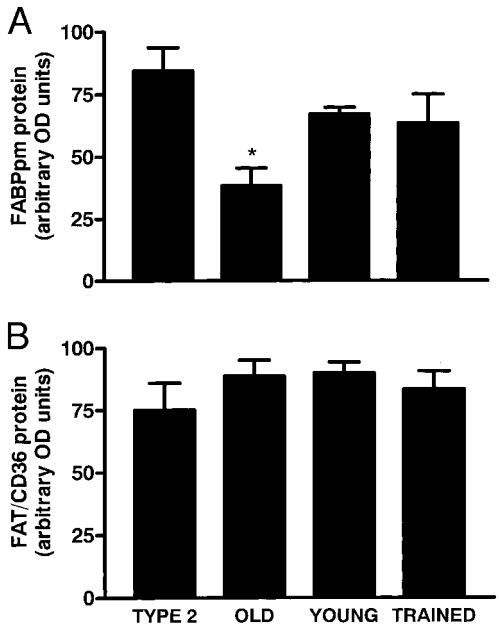

FIG. 3. Skeletal muscle FABPpm (A) and FAT/CD36 (B) protein content in biopsy samples obtained from the v. lateralis. Values are means ± SEM. *, Significantly different to TYPE 2 and YOUNG ($P < 0.05$).

TABLE 3. Citrate synthase and β-HAD activity

	TYPE 2	OLD	YOUNG	TRAINED
Citrate synthase	4.1 ± 0.5	5.2 ± 0.6	7.5 ± 0.9	13.6 ± 1.1[a,b,c]
β-HAD	4.9 ± 0.5	5.4 ± 1.0	7.4 ± 1.0	9.5 ± 0.8[a,b]

Values are micromoles per gram per minute. Data are means ± SEM.

[a] Different from TYPE 2, $P < 0.01$.
[b] Different from OLD, $P < 0.01$.
[c] Different from YOUNG, $P < 0.01$.

Relationship between anthropometric and muscle markers with insulin sensitivity

There was a significant negative correlation between percentage of body fat and GIR ($r = -0.61$; $P = 0.001$; Fig. 4A) and between BMI and GIR ($r = -0.69$; $P = 0.001$; Fig. 4B). The relationship between BMI and GIR remained significant even when the TRAINED subjects were omitted from the analysis ($r = -0.63$; $P = 0.01$). Fasting FFA levels were also negatively correlated with insulin sensitivity ($r = -0.57$; $P = 0.003$; Fig. 4C). Such a relationship was still evident even when the TRAINED individuals were excluded from analyses ($r = -0.52$; $P = 0.03$). There was a strong positive relationship between VO_2max and GIR for all subjects under investigation ($r = 0.74$; $P < 0.0001$; Fig. 4D). However, when the TRAINED subjects were excluded from the analysis, no such

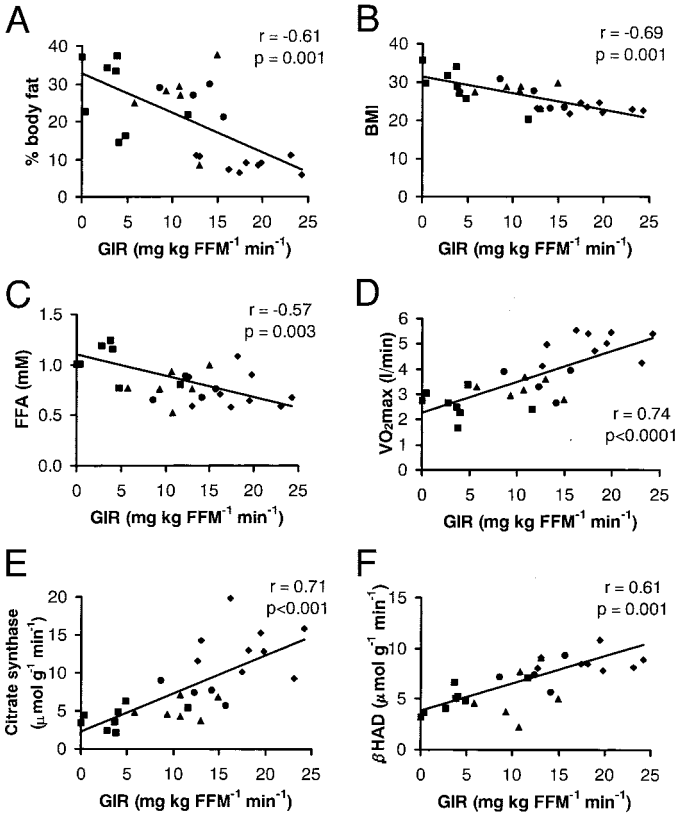

FIG. 4. Relationship between whole-body insulin-stimulated glucose uptake and percentage of body fat (A); BMI (B); plasma FFA concentration (C); VO_2max (D); citrate synthase activity (E); and β-HAD activity (F). ■, TYPE 2; ▲, OLD; ●, YOUNG; ◆, TRAINED.

association existed between VO_2max and GIR. Markers of skeletal muscle oxidative capacity were strongly associated with insulin sensitivity. Both citrate synthase activity ($r = 0.71$; $P < 0.0001$; Fig. 4E) and β-HAD activity ($r = 0.61$; $P < 0.001$; Fig. 4F) were significantly correlated to GIR. Even when the TRAINED individuals were excluded from the analysis, the association between citrate synthase activity and GIR ($r = 0.55$; $P = 0.02$) and between β-HAD activity and GIR ($r = 0.57$; $P = 0.01$) was robust. In contrast, there was no relationship between GIR and TG_m or LCACoA content, with or without inclusion of the TRAINED group from the analysis. Furthermore, no correlation was observed between insulin sensitivity and FABPpm or FAT/CD36 protein content. Stepwise regression analysis revealed that VO_2max accounted for 53% of the variance in GIR ($P < 0.001$). The addition of BMI to the regression model was able to explain a further 13% of the variance, such that the two factors combined accounted for 66% of the variance of GIR ($P < 0.001$).

Discussion

A growing body of evidence has emerged in support of the hypothesis that abnormal accumulation of lipid in skeletal muscle (and other tissues) plays an important role in the etiology of insulin resistance. Indeed, a number of independent research groups using a variety of different measurement techniques have found negative correlations between muscle lipid stores (*i.e.* TG_m) and whole-body insulin sensitivity (3–6). Here, for the first time in a single study, we determined a multitude of interdependent markers of lipid status in muscle from individuals with a wide range of insulin sensitivity. Accordingly, a major finding was that, despite TG_m being significantly elevated in type 2 diabetic patients (Fig. 1), there was no relationship between TG_m and insulin sensitivity, even when the well-trained (insulin-sensitive) individuals were excluded from the analysis. This observation is in agreement with the paradoxical findings of Goodpaster *et al.* (20), who have previously reported no association between TG_m and insulin sensitivity in a group of individuals with a range of insulin sensitivity.

A second finding from the present investigation was the failure to observe an elevated TG_m content in our highly trained subjects. Previously, Goodpaster *et al.* (20) noted that individuals involved in regular endurance training had higher TG_m despite being markedly insulin sensitive. The GIR of the endurance-trained individuals in the present investigation was significantly higher (~30%) than that of our age-matched (young) subjects, despite similar TG_m and LCACoA concentrations. On the other hand, Goodpaster *et al.* (20) reported no difference in insulin sensitivity between untrained, healthy, lean individuals and their trained subjects. Differences in results between the present study and that of Goodpaster *et al.* (20) may be due to several reasons. First, it is tempting to speculate that the methodological techniques used to quantify TG_m in the two investigations may explain some of the difference in results. It has been argued that the biochemical extraction technique could result in the inadvertent contamination of fat and connective tissue with the sample (20), whereas the histochemical method allows for direct quantitative analysis of biopsy specimens. However, it should be noted that the biochemical technique used in the present study includes a microscopic visualization of samples to carefully remove adipose tissue and other contaminants from the muscle specimens before extraction of lipid for TG quantification (3). Second, there may be subtle but important differences in the training status of the subjects under study: the well-trained male subjects in the present investigation had VO_2max values approximately 12% higher than those reported by Goodpaster *et al.* (20), had a prolonged history of endurance training (3–7 yr), and were competitive athletes. Indeed, the GIR for these individuals was approximately 80% higher than that observed by Goodpaster *et al.* (20) for their trained men and women. Thus, it is possible that adaptations induced by chronic, intense exercise training, such as increased GLUT4 protein content, may mediate the enhanced insulin sensitivity observed in our trained subjects. Finally, there is the possibility that the 2-h clamp used in the present study may be inadequate time for subjects with insulin resistance to reach steady-state insulin-stimulated glucose uptake at an infusion rate of 40 $mU/m^2 \cdot min$. In the study of Goodpaster *et al.* (20), a 4-h clamp was performed. During a 2-h clamp, hepatic glucose production may not be completely suppressed in individuals with marked insulin resistance. However, this potential limitation would not confound the conclusions drawn.

Although a cause-effect relationship cannot be conclusively determined from investigations that find TG_m and

insulin sensitivity to be statistically associated, it seems unlikely that TG_m *per se* interferes with insulin action. Instead, it has recently been suggested that TG_m alone does not confer insulin resistance, but acts as a surrogate for increased availability of FA-derived metabolites that have a more mechanistic and direct link with the pathophysiology of insulin resistance (9, 10, 12). One such metabolite is the LCACoA esters (11, 21). Accumulation of cytosolic LCACoAs may enhance synthesis of diacylglycerol (DAG), a potent activator of protein kinase C (22), which can lead to serine phosphorylation of the insulin receptor and insulin receptor substrate-1 (23) interfering with the ability of insulin to activate phosphatidylinositol 3-kinase, thereby inducing insulin resistance (24). LCACoAs also regulate nuclear transcription factors involved in carbohydrate and lipid metabolism (25) and alter the activity of key enzymes involved in glucose metabolism, such as hexokinase (12) and glycogen synthase (26). Elevated LCACoA content has previously been reported in skeletal muscle from insulin-resistant, high-fat-fed rodents (9, 15). Investigations of the measurement of LCACoA in humans are scarce, but a recent study reported that total LCACoA content measured in muscle biopsy samples taken from a group of older men with varying degrees of glucose tolerance was a better indicator of insulin action than measurements of TG_m (9). Hence, the second novel finding of the present investigation was that, despite the strikingly different levels of insulin sensitivity (Table 2) and elevated TG_m content in patients with type 2 diabetes (Fig. 1), total LCACoA at rest was similar between all groups under investigation (Fig. 2).

There are several possible explanations for the discrepancy in results between the current study and those from previous investigations. First, due to limited biopsy sample size, we were only able to determine total muscle LCACoA content: such a measure may not reflect changes in LCACoAs of different chain lengths and/or degrees of saturation (10). In this regard, Houmard *et al.* (10) recently reported that, in morbidly obese individuals who underwent weight loss (~60 kg), there were reductions in palmityl, stearate, and linoleate CoA species despite no significant differences in total muscle LCACoA content. Of particular interest was the tendency for a greater improvement in insulin sensitivity to be associated with a larger decline in palmityl CoA after weight loss, indicating that saturated FA and their derivatives may be involved in the pathogenesis of insulin resistance (10, 27). In the present cross-sectional investigation, it is possible that skeletal muscle from type 2 diabetic patients contained a higher proportion of saturated fatty acyl CoAs, and, conversely, that the enhanced insulin action in the endurance-trained subjects was due to a lower proportion of this species. In support of this contention, the proportion of palmitic acid found in the skeletal muscle membranes of endurance-trained subjects is lower than that of untrained individuals (28, 29), suggesting that regular exercise modifies the FA profile of skeletal muscle in favor of enhanced insulin sensitivity.

It is also possible that other FA-derived moieties, such as DAG and ceramide, play a role in the development of insulin resistance. DAG levels are elevated in a number of models of insulin resistance and, in many cases, are associated with increased protein kinase C activity (22, 30). Ceramide content has also been shown to be elevated in muscle of insulin-resistant animals (31). Ceramide can inhibit insulin-stimulated glucose transport, glycogen synthesis, and Akt activation (32, 33). Due to insufficient muscle sample, we were unable to determine DAG and ceramide content in the present study. However, it is tempting to speculate that these lipid metabolites may have been elevated in skeletal muscle from patients with type 2 diabetes. Further work is required to investigate the potential role of DAG and ceramides in the pathogenesis of insulin resistance.

Hyperinsulinemia has previously been reported to decrease the content of skeletal muscle LCACoAs in rodents (15). However, the ability of acute insulin stimulation to suppress LCACoA content is severely diminished in the muscle of insulin-resistant rats (15). Short-term interventions that ameliorate insulin resistance in fat-fed rats, such as a low-fat meal, overnight fasting, or prior exercise, are associated with a reduction in LCACoA levels during a 2-h glucose clamp, suggesting a close association between insulin suppressibility of LCACoA and enhanced glucose metabolism (15). Based on these findings, we hypothesized that acute insulin stimulation would suppress LCACoA content to a greater extent in healthy muscle compared with that of insulin-resistant muscle. Here, for the first time, we report that acute insulin stimulation does not lead to a reduction in muscle LCACoA content (Fig. 2). To the best of our knowledge, this is the first study to determine the effect of insulin stimulation on LCACoA content. Hence, we do not know whether 2 h is the best time point to detect any potential suppression of muscle LCACoA. Future investigations should employ longer (3–5 h) periods of insulin stimulation to assess the impact of such a perturbation on muscle lipid content. However, it should be noted that, although 2 h of hyperinsulinemia failed to suppress LCACoA content in skeletal muscle from type 2 diabetic patients, it had little effect on LCACoAs in muscle from highly trained individuals who presented with markedly superior insulin sensitivity.

The precise mechanisms that cause muscle lipids to accumulate in type 2 diabetic individuals are unclear, but may involve increased uptake of blood-borne FA, decreased FA oxidation, or a combination of both processes (7, 8). It is well known that long-chain FAs (LCFAs) are an important substrate for skeletal muscle. LCFAs taken up by the muscle cell are esterified to triacylglycerols both at rest and during exercise (34). LCFA transport across the sarcolemma is believed to occur via a carrier-mediated mechanism involving a number of FA transport proteins (35). Accordingly, it is possible that the derangements in fat metabolism observed in insulin-resistant skeletal muscle are associated with changes in the content of FA transport proteins. In this regard, FABPpm protein content is increased in skeletal muscle of obese humans (8), but not in the genetically obese Zucker rat (18). Additionally, FABPpm is negatively related to insulin sensitivity (8). In the present study, FABPpm content was higher in the muscle of type 2 diabetic patients compared with the age-matched control group (Fig. 3). However, no difference was observed between the TYPE 2, YOUNG, and TRAINED groups. Although speculative, this suggests that skeletal

muscle from type 2 diabetic individuals may possess the capacity for enhanced FA uptake.

In the present study, the oxidative capacity of skeletal muscle, as reflected by the activities of the enzymes citrate synthase and β-HAD, was elevated in muscle from the highly trained individuals compared with all other groups under investigation. A higher training-induced skeletal muscle oxidative capacity reflects an increased mitochondrial density (36) and an enhanced capacity for oxidative phosphorylation toward total energy production (37). In contrast, skeletal muscle from obese and insulin-resistant subjects is characterized by reduced oxidative capacity (8, 38, 40) and lower fasting rates of FA oxidation (40, 41). In accordance with a previous report (38), we observed a strong association between oxidative enzyme capacity and insulin sensitivity for all subjects under investigation. This association was robust: even when the highly trained (insulin-sensitive) subjects were excluded from the analysis, relationships between markers of skeletal muscle oxidative capacity (citrate synthase and β-HAD activity) and insulin action remained strong. Accordingly, in the present study, markers of skeletal muscle oxidative capacity were better correlates of insulin sensitivity than muscle lipid status (Fig. 4). Gerbitz *et al.* (42) have previously suggested that disturbed oxidative phosphorylation capacity could be a direct cause of insulin resistance, whereas Kelley *et al.* (43) have recently reported impaired bioenergetic capacity and mitochondrial function in skeletal muscle from type 2 diabetics. Whether these perturbations to normal mitochondrial function are a result of a sedentary lifestyle in subjects with impaired insulin action can only be addressed by interventional exercise-training studies.

In conclusion, for the first time in a single investigation we determined a number of interdependent markers of lipid status in skeletal muscle from individuals with a wide range of insulin sensitivity. Despite dramatically elevated TG in the muscle of type 2 diabetic patients, total LCACoA content was not altered. Furthermore, insulin stimulation had no detectable effect on muscle LCACoA levels in any group under investigation, despite strikingly different levels of insulin sensitivity. Muscle oxidative capacity and whole-body maximal aerobic power were better predictors of insulin sensitivity than a range of markers of lipid status determined in this study.

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Address all correspondence and requests for reprints to: John A. Hawley, Ph.D., School of Medical Sciences, RMIT University, P.O. Box 71, Victoria 3083, Australia. E-mail: john.hawley@rmit.edu.au.

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Current address for A.B.: Department of Human Biology and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada N1G 2W1.

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