

Prolonged Inhibition of Muscle Carnitine Palmitoyltransferase-1 Promotes Intramyocellular Lipid Accumulation and Insulin Resistance in Rats

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Cross-sectional studies in human subjects have used ^1H magnetic resonance spectroscopy (HMRS) to demonstrate that insulin resistance correlates more tightly with the intramyocellular lipid (IMCL) concentration than with any other identified risk factor. To further explore the interaction between these two elements in the rat, we used two strategies to promote the storage of lipids in skeletal muscle and then evaluated subsequent changes in insulin-mediated glucose disposal. Normal rats received either a low-fat or a high-fat diet (20% lard oil) for 4 weeks. Two additional groups (low-fat + etoxomir and lard + etoxomir) consumed diets containing 0.01% of the carnitine palmitoyltransferase-1 inhibitor, R-etomoxir, which produced chronic blockade of enzyme activity in liver and skeletal muscle. Both the high-fat diet and drug treatment significantly impaired insulin sensitivity, as measured with the hyperinsulinemic-euglycemic clamp. Insulin-mediated glucose disposal (IMGD) fell from 12.57 ± 0.72 in the low-fat group to 9.79 ± 0.59 , 8.96 ± 0.38 , and $7.32 \pm 0.28 \mu\text{mol} \cdot \text{min}^{-1} \cdot 100 \text{g}^{-1}$ in the low-fat + etoxomir, lard, and lard + etoxomir groups, respectively. We used HMRS, which distinguishes between fat within the myocytes and fat associated with contaminating adipocytes located in the muscle bed, to assess the IMCL content of isolated soleus muscle. A tight inverse relationship was found between IMGD and IMCL, the correlation ($R = 0.96$) being much stronger than that seen between IMGD and either fat mass or weight. In conclusion, either a diet rich in saturated fat or prolonged inhibition of fatty acid oxidation impairs IMGD in rats via a mechanism related to the accumulation of IMCL. *Diabetes* 50:123–130, 2001

Evidence indicates that abnormalities of lipid metabolism can negatively impact insulin-mediated glucose disposal (IMGD) in skeletal muscle. The elevated skeletal muscle lipid stores found in many models of insulin resistance are consistent with this idea (1–10). For example, muscle triglyceride levels (as measured

in biopsy samples) are increased in patients with type 2 diabetes (11) and, in nondiabetic individuals, appear to correlate with insulin resistance better than measures of adiposity, such as BMI or percent body fat (9). Because the measurement of total triglycerides in tissue extracts cannot discriminate between fat within the myocyte and fat associated with surrounding adipose tissue, we have used ^1H magnetic resonance spectroscopy (HMRS) to provide a noninvasive measure of the lipid within the muscle cells (12,13). Distinguishing intramyocellular lipid (IMCL) from extramyocellular lipid (EMCL) concentrations provides a quantitative measure of true cell-associated lipids that can only be qualitatively assessed in muscle biopsy samples by oil-red O staining or electron microscopy. It also greatly reduces the variability inherent in the measurement of cell-associated lipids by use of conventional chemical analyses of biopsy samples (13,14).

In cross-sectional studies with humans, we found that insulin resistance correlates more tightly with IMCL than with any other factor, including BMI, percent body fat, waist-to-hip ratio, or age (12). Other groups have recently reported similar findings (15–17). Furthermore, an acute intervention, such as infusion of a lipid emulsion to augment tissue uptake of fatty acids in normal humans, antagonizes insulin-stimulated uptake and phosphorylation of glucose in muscle (18,19). The same is true in rat muscle previously exposed to elevated free fatty acid (FFA) levels (20). Importantly, more prolonged exposure of rats to a diet containing large quantities of fat elicits insulin resistance that is associated with the accumulation of fatty acyl-CoA (8) and triglycerides (1,10) in skeletal muscle. Because rodents receiving high-fat diets have an increased adipose tissue mass, it is evident that variability in the amount of adipose tissue within the muscle bed could bias measurements of muscle triglycerides made from extracts of whole muscle (1,10). We deemed it important, therefore, to verify that high-fat diets elicit insulin resistance in association with the accumulation of IMCL when it is distinguished from adipose tissue by HMRS.

Although excessive dietary fat provides a strong impetus for the accumulation of lipids in muscle and other tissues, the same effect is theoretically achievable by pharmacological blockade of fatty acid oxidation with the R-isomer of ethyl-2-[6-(4-chlorophenoxy)hexyl]-oxirane-2-carboxylate (etomoxir). After conversion to its CoA ester, R-etomoxir covalently inhibits carnitine palmitoyltransferase (CPT)-1 and blocks the entry of fatty acids into mitochondria (21,22). The racemic mixture of R- and S-etomoxir has hypoglycemic effects in humans and animal models with diabetes and has been proposed as a potentially useful therapeutic agent

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CPT, carnitine palmitoyltransferase; EMCL, extramyocellular lipid; FFA, free fatty acid; HMRS, ^1H magnetic resonance spectroscopy; IMCL, intramyocellular lipid; IMGD, insulin-mediated glucose disposal.

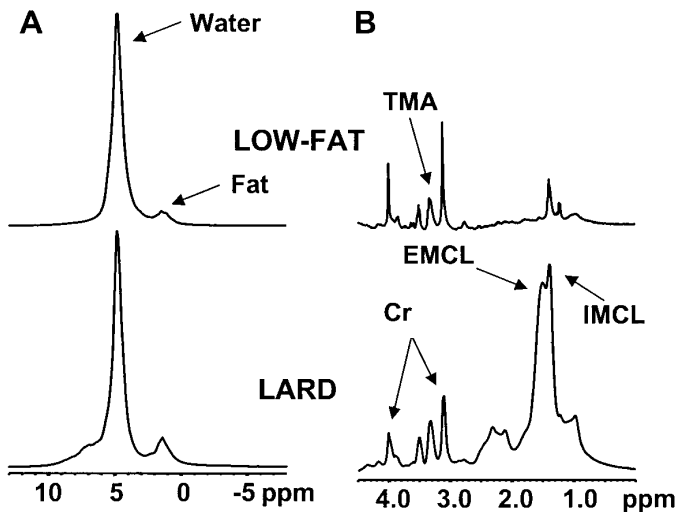


FIG. 1. A: Whole-body proton magnetic resonance spectra (HMRS) showing fat and water resonances from rats receiving low-fat and lard diets. **B:** HMRS of individual muscles taken from rats receiving low-fat and lard diets. The labeled peaks represent the resonances of protons from trimethylammonium (TMA) compounds, creatine (Cr), and the EMCL or IMCL components of the methylene peak.

(23–25). Because prolonged treatment with high doses of etomoxir promotes triglyceride accumulation within tissues (as determined by chemical analysis of biopsy samples [26]), a second objective of the present study was to determine what effect such treatment might have on both whole-body insulin sensitivity and IMCL in rats.

RESEARCH DESIGN AND METHODS

Animals. Male Sprague-Dawley rats weighing 125–150 g were placed in metabolic cages at 22°C and maintained on a 12-h light-dark cycle (lights on from 10:00 A.M. to 10:00 P.M.) with ad libitum access to water and specially prepared diets (Harlan Teklad, Madison, WI). To investigate whether a high-fat diet could alter insulin sensitivity in association with changes in IMCL content, all animals received either a high-fat diet enriched with highly saturated lard oil or a low-fat diet containing only essential fatty acids for 4 weeks. The low-fat diet (TD94128) contained primarily cornstarch and had a caloric density of 3.5 kcal/g with a distribution of 4% fat, 75% carbohydrate, and 21% protein. The lard diet (TD96001) had a caloric density of 4.4 kcal/g and contained 41% fat, 40% carbohydrate, and 19% protein. To investigate the impact of etomoxir on insulin sensitivity and IMCL, two separate groups of animals (low-fat + etomoxir and lard + etomoxir) received diets supplemented with 0.01% R-etomoxir. Food intake was monitored daily, and body weights were recorded twice a week.

Isolation of mitochondria and assay of CPT-1 activity. To verify suppression of CPT-1 activity by etomoxir, rats in both the fed and 18 h-fasted states were killed, and mitochondria were isolated from the liver and gastrocnemius muscle by method A of McGarry et al. (27). They were suspended in 150 mmol/l KCl, 5 mmol/l Tris-HCl, pH 7.2, and CPT-1/CPT-2 activity was assayed as described previously in the direction palmitoyl-CoA + L-[¹⁴C]carnitine → palmitoyl-[¹⁴C]carnitine + CoASH (28). Enzyme activity was normalized for mitochondrial protein content (29).

Hyperinsulinemic-euglycemic clamps. Rats were anesthetized with ketamine (55 mg/kg) and xylazine (10 mg/kg) and fitted with carotid artery and jugular venous catheters after 3 weeks of diet treatment (30). They were allowed 6–8 days to recover before experiments were performed. Animals were fasted starting at 4:00 P.M. and were studied in the conscious unrestrained state at ~10:00 A.M. the following day. Catheters were flushed with 25 U/ml heparinized saline (Elkins-Sinn, Cherry Hill, NJ), and the venous line was connected for infusion of 3-[³H]glucose, insulin, and replacement blood (1.5 ml/h). Replacement blood was obtained via cardiac puncture from a littermate of the study animal (31). The arterial line was used for blood sampling and was kept patent by the infusion of heparinized saline. Studies lasted 210 min and were conducted according to a modification of the procedure outlined by Rossetti and colleagues (31–33). A 3- μ Ci bolus of 3-[³H]glucose was

given to initiate the experiment, followed by infusion of 0.25 μ Ci/min for 90 min. From 60–90 min, blood was sampled at 5-min intervals for determination of plasma [³H]glucose-specific activity and content of ³H₂O after precipitation with BaOH and ZnSO₄. The tracer infusion was discontinued after 90 min and insulin was infused at 60 mU/kg body wt for 2 min before being decreased to 3 mU · kg⁻¹ · min⁻¹. Plasma glucose concentrations were measured frequently, and 25% glucose was infused to maintain a constant level of ~6 mmol/l. Thirty minutes after the insulin was started, the 3-[³H]glucose tracer infusion was bolused again and continued at 0.25 μ Ci/min. From 180–210 min, blood was drawn at 5-min intervals for determination of plasma tritiated glucose-specific activity and content of tritiated water. Additional samples were obtained at 75, 90, 150, 180, and 210 min for assay of plasma FFA and triglyceride levels. Remaining plasma from the 60- to 90-min and 180- to 210-min samples was pooled and frozen for subsequent assays of basal and steady-state plasma insulin levels.

Body composition. After 4 weeks of diet treatment, ¹H spectra of the entire animal (Fig. 1A) were collected using a 4.7T Omega system with a single pulse sequence and the following parameters: repetition time of 5 s, spectral width of 3 kHz, and 2,048 data points (34–37). Water and fat resonances were quantified after line-fitting with NUTS software (Acorn NMR, Fremont, CA). Fat mass and fat-free mass were computed from the ratio of fat and water signal areas and known ratios of proton densities of fatty acid chains and water, assuming that water comprised 72% of fat-free mass (38). This technique was validated by previous investigators; the correlation coefficient between total body lipid and water content measured by HMRS and classical carcass analysis was 0.97 (34). **IMCL.** Overnight-fasted rats were killed for excision of the liver and bilateral soleus muscles. All tissues were immediately frozen in liquid N₂ except for the right soleus muscle. It was placed in saline so that a small slice (15–20 mg) could be excised, stripped of all visible fat under 10 \times magnification and placed in a 5-mm NMR tube filled with deuterated saline. High-resolution proton spectra were obtained at 37°C using a 300-MHz Varian-INOVA system and were processed to estimate total lipid, IMCL, and EMCL content (13) by comparing the methylene signal intensity to that of a 2- μ mol formic acid standard corrected for T₁ relaxation (39). Triglycerides from the liver and contralateral soleus muscle were extracted by the method of Folch et al. (40). Aliquots of the liver extract were saved for the measurement of total tissue triglycerides by colorimetric assay (41), whereas the soleus muscle was fractionated using silica Sep-Pak columns (Waters, Milford, MA) (42) for analysis of fatty acid composition by gas-liquid chromatography (43). The average molecular weight and number of methylene protons in the muscle triglyceride pool was used for converting proton signal intensities into milligrams of lipids.

Figure 1B provides an example of the high-resolution spectra and highlights the two components of the methylene peak designated EMCL and IMCL. Despite the great care taken in preparing muscle slices, spectroscopy detected EMCL in some samples. Such interfascial adipose tissue is inevitably included in analyses of muscle triglyceride obtained from tissue extracts (1,10), but it can be distinguished from true IMCL when proton spectra are obtained in intact muscle tissue (13,44–46). A detailed discussion of theoretical principles and the practical application of HMRS for quantitative assessment of total tissue lipids and IMCLs is beyond the scope of the current article and is provided elsewhere (44–46).

The validity of the method we used in the current studies was assessed in soleus muscle biopsies obtained from six animals consuming a standard 4% fat diet. The HMRS measurement was completed 25–30 min after the muscle had been excised, and the tissue was subsequently frozen at –70°C until the neutral lipids were extracted with chloroform:methanol for biochemical measurement of the total muscle triglyceride content. For comparison, an adjacent section was immediately frozen and stored for biochemical determinations. The correlation between the total muscle lipid value obtained by spectroscopy and the triglyceride content assayed biochemically is shown in Fig. 2. A strong linear relationship existed between the two independent techniques ($R = 0.99$) and the absolute difference for any value ranged between 0.23 and 1.05 mg/g tissue. The concordance between spectroscopy and biochemical assay of a single sample was much stronger than that between biochemical determinations of triglyceride for two separate samples taken from a single muscle (data not shown). Figure 2 also highlights the advantage of collecting spectra from an intact muscle preparation to differentiate between triglyceride contained in adipocytes and myocytes. In most cases, adipose tissue contributed only a small portion of the total lipids in the specimen, but the contribution varied quite markedly.

Analytical procedures. Perfusate glucose concentrations were measured by the glucose oxidase method on a Beckman Glucose Analyzer II machine. Plasma FFA levels were determined by colorimetric assay (catalog no. 1381375; Boehringer Mannheim, Indianapolis, IN). Plasma triglyceride concentrations and tissue triglyceride content were measured using a colorimetric assay that was

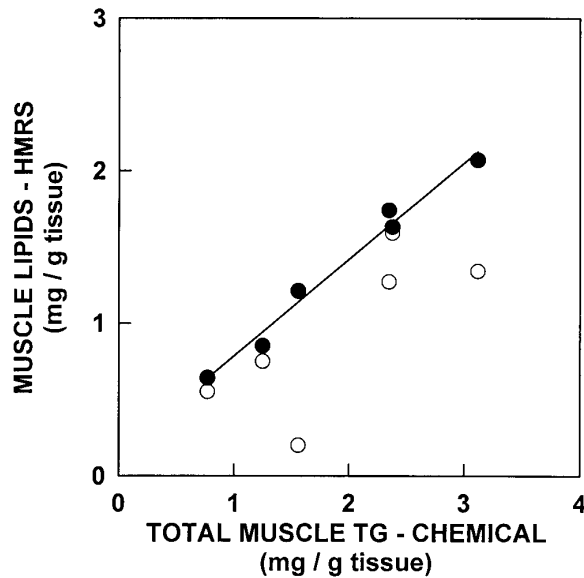


FIG. 2. Comparison of total muscle lipids (●) and IMCLs (○), as determined by HMRS of intact muscle tissue, with total muscle triglyceride (TG) content assayed chemically after chloroform:methanol extraction. Soleus muscle samples from six overnight-fasted rats that had received a 4% fat diet were analyzed.

corrected for free glycerol contamination (procedure no. 337; Sigma, St. Louis, MO). Plasma and perfusate insulin concentrations were assayed with a double-antibody radioimmunoassay kit obtained from Linco (St. Charles, MO).

Materials. The sodium salts of R- and S-etomoxir were kindly provided by Novo Nordisk Pharmaceuticals (Copenhagen). Radiolabeled 3-³H]glucose was obtained from NEN Life Science Products (Boston, MA) and L-[¹⁴C]carnitine was purchased from American Radiolabeled Chemical (St. Louis, MO).

Statistical analysis. Experimental results were analyzed for effects of the diet and the drug using two-way multiple analysis of variance. The association between IMGD and other measured parameters was assessed by linear regression analysis. All calculations were made with SigmaStat software for Windows (SPSS, Chicago).

RESULTS

Food intake and body weight. Food intake was similar in all diet groups, although there was a trend toward a decline in the rats receiving R-etomoxir that did not reach statistical signifi-

cance. The average daily caloric intake was 69.6 ± 1.9 , 65.2 ± 1.9 , 69.7 ± 1.9 , and 66.5 ± 1.0 kcal/day for animals on the low-fat, low-fat + etomoxir, lard, and lard + etomoxir diets, respectively. Weight gain was similar in all cases, so that after 4 weeks, the average weights of the animals in the 4 groups were 276 ± 5 , 292 ± 7 , 288 ± 9 , and 270 ± 5 g, respectively.

CPT-1 activity. In animals receiving the low-fat and high-fat diets for 4 weeks, mitochondrial CPT-1 activity in the fed state was similar in both skeletal muscle (12.1 ± 0.1 vs. 11.8 ± 1.8 nmol \cdot min⁻¹ \cdot mg protein⁻¹, respectively) and liver (8.7 ± 0.7 vs. 8.3 ± 0.2 nmol \cdot min⁻¹ \cdot mg protein⁻¹). When 0.01% R-etomoxir was added to the diets, CPT-1 activity in both fed and fasted animals was suppressed by >95% in liver and 80–90% in skeletal muscle. CPT-2 was not inhibited in either tissue. Comparable results were obtained after 10 and 20 days of etomoxir treatment, indicating that the inhibitor effectively suppressed mitochondrial fatty acid oxidation in both liver and skeletal muscle over the time frame of these experiments.

Hyperinsulinemic-euglycemic clamps. Studies were undertaken to determine the insulin sensitivity of the liver and skeletal muscle of rats that had consumed low-fat and high-fat diets with or without etomoxir. Table 1 lists the concentrations of plasma glucose, insulin, FFA, and triglycerides after an overnight fast and at the end of the insulin infusion period. The fasting plasma glucose concentration was reduced in animals receiving etomoxir, which was consistent with diminished glucose production (Fig. 3C) via gluconeogenesis (23–25,47–50) because glycogenolysis in rats is completely suppressed after an 18-h fast. Exogenous insulin infusion elicited a similar increase in plasma insulin levels in all of the groups, and plasma glucose concentrations were maintained at euglycemic levels. Acute administration of oxirane CPT-1 inhibitors has been known to augment plasma triglyceride levels (50). However, we saw no changes in fasting plasma triglyceride concentrations after prolonged CPT-1 inhibition, although fasting plasma FFA levels fell significantly.

Figure 3 illustrates the marked alterations in glucose kinetics resulting from the consumption of a high-fat diet and treatment with etomoxir. The glucose infusion rate during the hyperinsulinemic period provided an index of whole-body insulin sensitivity. The value of 13.33 ± 1.34 μ mol \cdot min⁻¹ \cdot 100 g⁻¹ in the low-fat animals was much greater than that seen in any

TABLE 1

Influence of dietary fat content and R-etomoxir on plasma parameters before and during hyperinsulinemic-euglycemic clamps

	Low-fat	Low-fat + etomoxir	Lard	Lard + etomoxir
<i>n</i>	7	6	6	6
Glucose (mmol/l)				
Basal	5.98 ± 0.09	$5.28 \pm 0.15^*$	6.18 ± 0.26	$5.87 \pm 0.10^*$
Clamp	6.18 ± 0.13	5.85 ± 0.10	6.26 ± 0.06	6.15 ± 0.14
Insulin (pmol/l)				
Basal	25 ± 5	20 ± 5	40 ± 10	15 ± 5
Clamp	320 ± 30	330 ± 10	335 ± 15	300 ± 10
FFA (mmol/l)				
Basal	0.68 ± 0.04	$0.55 \pm 0.09^*$	0.65 ± 0.04	$0.50 \pm 0.02^*$
Clamp	0.22 ± 0.04	0.31 ± 0.08	0.35 ± 0.04	0.30 ± 0.04
Triglycerides (mg/dl)				
Basal	9.3 ± 2.2	6.0 ± 2.8	8.5 ± 1.4	12.2 ± 2.2
Clamp	9.1 ± 2.4	5.3 ± 3.4	8.4 ± 1.1	12.4 ± 3.1

Data are means \pm SE. **P* < 0.05 vs. rats not receiving etomoxir.

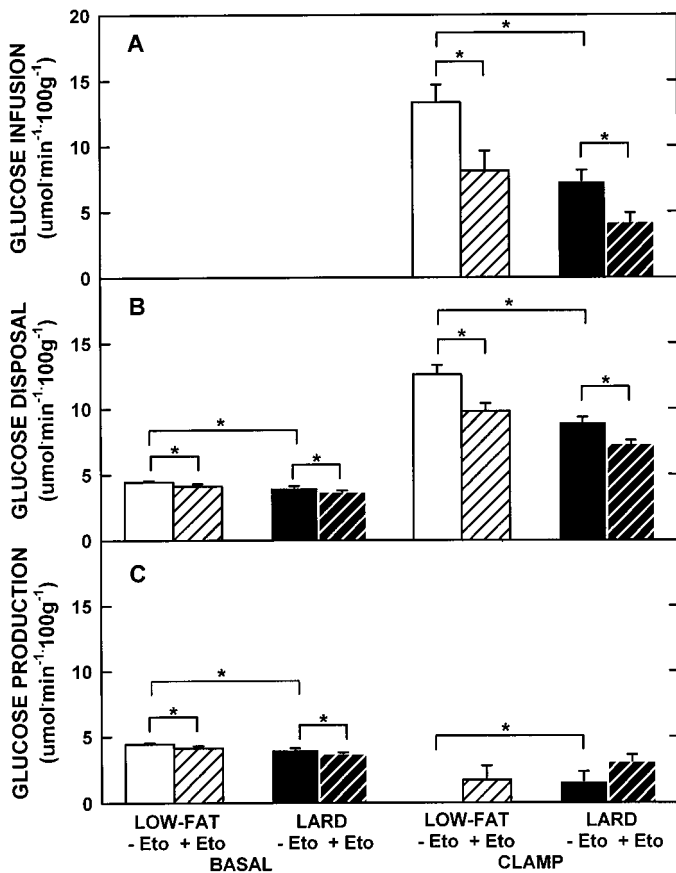


FIG. 3. Influence of dietary fat content and etomoxir (Eto) on the rates of glucose infusion (A), glucose disposal (B), and endogenous glucose production (C) in overnight-fasted rats before and during insulin infusion at 3 mU · kg⁻¹ · min⁻¹. Each value represents the mean ± SE for six to seven determinations. *P < 0.05 between linked groups.

other group (Fig. 3A). Both IMGD (Fig. 3B) and insulin-induced suppression of endogenous glucose production (Fig. 3C) were impaired in the lard group. Glucose utilization was 8.96 ± 0.38 μmol · min⁻¹ · 100 g⁻¹, and glucose production declined to only 1.61 ± 0.73 μmol · min⁻¹ · 100 g⁻¹. For comparison, glucose utilization in the low-fat rats was 12.57 ± 0.72 μmol · min⁻¹ · 100 g⁻¹, whereas production was completely suppressed. Adding R-etomoxir to the diets impaired insulin sensitivity regardless of dietary fat intake. Glucose was infused at a rate of 8.19 ± 1.53 μmol · min⁻¹ · 100 g⁻¹ in the low-fat + etomoxir group and 4.23 ± 0.67 μmol · min⁻¹ · 100 g⁻¹ in

the lard + etomoxir group. The overall reduced insulin sensitivity imparted by etomoxir clearly reflected a reduction of glucose utilization coupled with a trend toward impaired suppression of endogenous glucose production. The ability of R-etomoxir to alter glucose kinetics was specific for inhibition of CPT-1 activity, because no changes were evident when we supplemented the lard diet with S-etomoxir, the form lacking the ability to block CPT-1 (data not shown).

Measures of whole-body fat and tissue lipid content. The foregoing data clearly indicate that altering lipid dynamics, either by increasing the dietary intake of saturated fat or by inhibiting the clearance of lipids via the mitochondrial β-oxidation pathway, resulted in insulin resistance. Therefore, additional experiments were undertaken to characterize exactly what effects the various diet treatments had upon whole-body fat mass, muscle lipid content, and hepatic triglycerides. Values for these parameters are listed in Table 2. Fat mass estimated utilizing proton magnetic resonance spectroscopy was reduced in the low-fat rats, whereas the other three groups displayed similar degrees of adiposity. It was not surprising that rats consuming the lard diet gained significantly more fat than their counterparts in the low-fat group, but the impact of CPT-1 inhibition on adiposity was more difficult to interpret. The addition of etomoxir to the low-fat diet significantly augmented body-fat content; this effect was not apparent in rats consuming the high-fat diet. It has been suggested that a defect in muscle lipid oxidation would favor storage of lipids in adipose tissue and initiate a cascade of events culminating in obesity and insulin resistance (2,3,51). The development of obesity in the low-fat + etomoxir group fits this scenario, but it would seem that other regulatory mechanisms such as marked insulin resistance or subtle changes in food intake prevented additional adiposity in the lard + etomoxir group.

In keeping with previous studies with human subjects (13,15–17), we used HMRS to quantify the lipid contained in the soleus muscle. This muscle is primarily composed of slow-twitch oxidative fibers (type I) that have increased insulin sensitivity and lipid content compared with other muscle fiber types (16,17). Table 2 reveals that the amount of lipid measured within the muscle increased when either lard oil or etomoxir was added to the diet. The total lipid content of soleus muscle excised from rats in the fasted state was significantly augmented by both dietary fat intake and CPT-1 inhibition. The unique information revealed by HMRS was that the elevated muscle lipid stores resulted from increases in both the extramyocellular and intramyocellular compartments. Whereas no EMCLs were detected in the animals receiving the low-fat diet, the more obese animals in the

TABLE 2
Influence of dietary fat content and R-etomoxir on fat mass, muscle lipid content, and hepatic triglyceride content

	Low-fat	Low-fat + etomoxir	Lard	Lard + etomoxir
Fat-mass (g)	12.1 ± 0.7	23.3 ± 4.3	29.9 ± 4.5	21.2 ± 3.0
Total muscle lipid (mg/g wet wt)	3.09 ± 0.37	4.98 ± 0.36†	5.91 ± 1.42*	6.19 ± 1.48*†
Extramyocellular lipid (mg/g wet wt)	0.00 ± 0.00	1.36 ± 0.14	1.83 ± 0.97	1.23 ± 0.63
Intramyocellular lipid (mg/g wet wt)	3.09 ± 0.37	3.68 ± 0.37†	4.09 ± 0.86*	4.91 ± 1.06*†
Hepatic triglyceride (mg/g wet wt)	10.6 ± 2.2	22.6 ± 3.4†	13.6 ± 1.0*	56.9 ± 18.0*†

Data are means ± SE for four to eight determinations. *P < 0.05 vs. rats receiving the low-fat diets. †P < 0.05 vs. rats not receiving etomoxir.

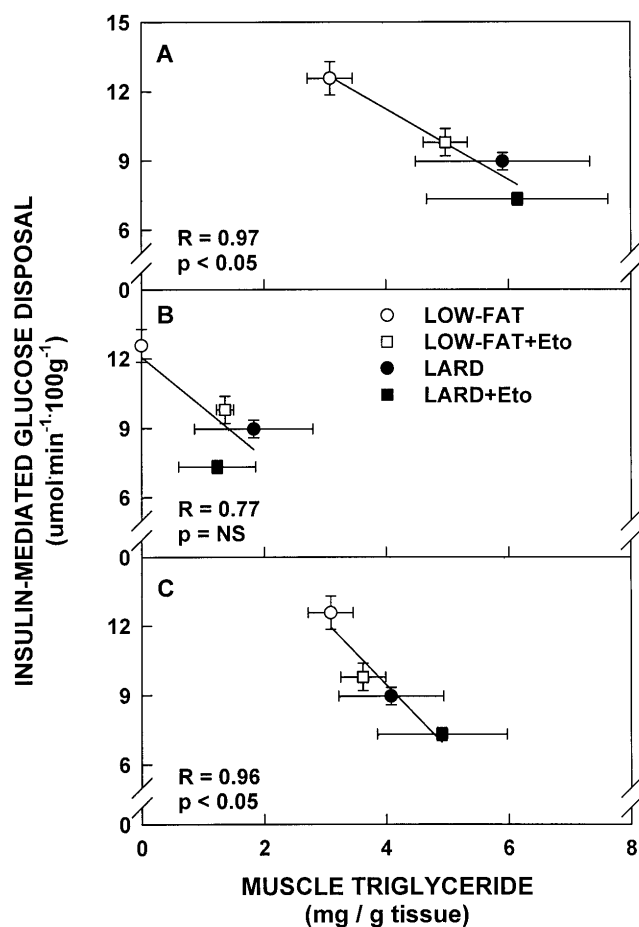


FIG. 4. Correlation between IMGD and soleus muscle lipid content in rats receiving low-fat and lard diets either alone or supplemented with the CPT-1 inhibitor, R-etomoxir (Eto). *A*, *B*, and *C* represent total lipid, EMCL, and IMCL contents, respectively. Each value represents the mean \pm SE for four to seven determinations.

other diet groups predictably exhibited greater muscle adiposity. After adjusting for artifact from adipose tissue, the IMCLs in muscle samples excised from rats were again profoundly influenced by dietary fat intake and CPT-1 inhibition. Figure 4A illustrates the steep inverse relationship that exists between IMGD, a commonly utilized index of muscle insulin sensitivity, and total muscle lipid content. EMCL content was not independently correlated with deteriorating insulin resistance, and it appeared to play a marginal role in the link between IMGD and total muscle lipid content. On the other hand, it is evident that the lipid actually contained within muscle cells played a more pivotal role in determining the association with IMGD (Fig. 4C). The apparent association between IMGD and whole-body fat mass shown in Fig. 5 did not quite reach statistical significance because of the limited number of data points. These data are entirely consistent with results of clinical studies showing that insulin resistance correlates more tightly with muscle lipid content than with percent body fat or BMI (9,12,15,52).

Hepatic triglyceride content was only measured from chloroform:methanol extracts because all of the fat in the liver is contained within hepatocytes (13). Inhibition of CPT-1 resulted in a marked elevation of hepatic triglycerides exceeding the level with increased dietary fat (Table 2). Thus, prolonged CPT-1

blockade caused fat to accumulate in the liver without any increase in plasma FFA or triglyceride concentrations.

DISCUSSION

There is growing evidence that excessive accumulation of fat in skeletal muscle and other tissues is an important factor in the genesis of whole-body insulin resistance, a hallmark feature of obesity/type 2 diabetes syndromes. Although insulin sensitivity, defined in terms of glucose production and disposal rates, can be accurately quantified using the hyperglycemic-euglycemic clamp procedure, skeletal muscle triglyceride content has traditionally been chemically analyzed on the basis of chloroform:methanol extraction of total lipids from biopsy samples. This approach does not distinguish between IMCLs and EMCLs, both of which may vary independently, particularly in a skeletal muscle sample in which infiltration by adipocytes may be extensive. Recently, we and others (13) have overcome this limitation with the use of *in vivo* proton magnetic resonance spectroscopy, a procedure that readily discriminates between IMCL and EMCL within the muscle bed. After applying this technique noninvasively in a cross-sectional study of healthy volunteers, the degree of insulin resistance was found to bear a tight relationship to the amount of IMCLs (12,15–17). Because cross-sectional studies cannot truly establish a cause-effect relationship between IMCL accumulation and insulin resistance, we designed separate interventions to answer two questions in the current work. First, is the insulin resistance that is known to follow high-fat feeding in rats accompanied by elevation of IMCL stores, as measured by HMRS? Second, if the IMCL pool can be expanded by pharmacological blockade of mitochondrial fatty acid oxidation, can insulin sensitivity also be lowered? Both of these questions, neither of which has been addressed as of yet, can now be answered in the affirmative, as discussed below.

Sequential measurement of the total triglyceride content of a single muscle sample, first using HMRS and then by conventional means following chemical extraction showed strong agreement. However, as demonstrated with humans *in vivo* (13,44,45), the primary advantage of HMRS proved to be its ability to quantify both IMCLs and EMCLs. Thus, it was possible to show that the EMCL component represented a substantial fraction of total lipid content, even when great efforts were made to strip all visible fat from samples. As a result, the triglyceride level measured from the tissue extract didn't correlate as well with IMCL as it did with the sum of IMCL and EMCL. We believe that the variability of the EMCL fraction likely contributes significantly to the scatter in biochemically determined triglyceride levels found in different samples taken from a single muscle (14).

Provision of a high-fat diet caused a twofold increase in whole-body fat mass and a notable elevation of adipose tissue in the muscle bed. Inhibition of CPT-1 with R-etomoxir also doubled adiposity in the low-fat animals but did not further increase fat mass in the high-fat group. Similarly, R-etomoxir significantly increased the EMCL component of soleus muscle in the low-fat but not the lard-fed rats. As predicted, an increase in dietary fat intake elicited a striking reduction (45%) in whole-body insulin sensitivity that was reflected in impairment of both IMGD and suppression of endogenous (mainly hepatic) glucose production during the hyperinsulinemic-euglycemic clamp. Importantly, CPT-1 inhibition negatively impacted both parameters, regardless of diet.

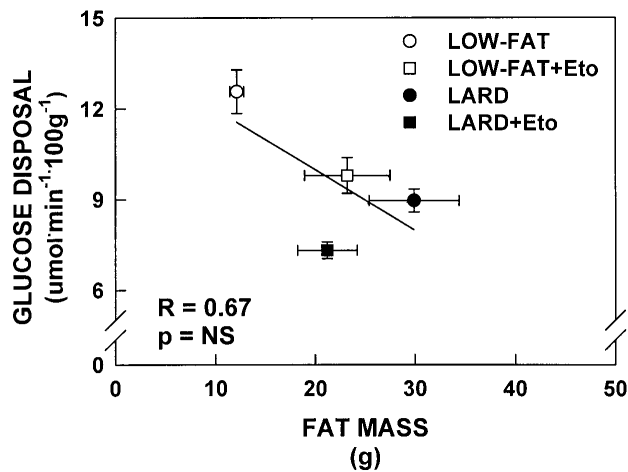


FIG. 5. Poor correlation between glucose disposal and whole-body fat mass in rats receiving low-fat and lard diets either alone or supplemented with the CPT-1 inhibitor, etomoxir (Eto). Each value represents the mean \pm SE for five to eight determinations.

Fasting gluconeogenesis was modestly reduced after inhibition of CPT-1. Limiting the oxidation of fatty acids during a fast limits gluconeogenesis by depleting NADHs and ATPs that are required for key steps of the gluconeogenic pathway and by reducing the level of acetyl-CoA, which serves as an allosteric activator of pyruvate carboxylase. During the hyperinsulinemic period, glucose production was paradoxically increased after prolonged inhibition of CPT-1. High concentrations of insulin normally suppress gluconeogenesis via direct effects on the liver and an indirect mechanism linked to the inhibition of lipolysis and a fall in hepatic fatty acid oxidation (53). Because mitochondrial fatty acid oxidation was already blocked in animals treated with R-etomoxir, we assume that some other factor must have offset the direct effects of insulin on hepatic gluconeogenesis in this setting. It is known that etomoxir treatment causes triglyceride and long-chain fatty acids to accumulate in hepatocytes, where they activate peroxisome proliferator-activated receptor α and enhance the expression of target genes involved in peroxisomal fatty acid oxidation (54). Accumulated lipids might also regulate the expression of factors participating in insulin signaling or key enzymes of gluconeogenesis, such as glucose-6-phosphatase, fructose 1,6-bisphosphatase, or phosphoenolpyruvate carboxykinase. Clearly, additional work is needed before the complex interaction between CPT-1 inhibition and gluconeogenesis can be fully understood.

What emerges from the present study is that the impairment in IMGD after high-fat feeding or CPT-1 blockade correlated strongly and inversely with the IMCL content of the soleus muscle. We suspect that the same was true for other muscles, although this hypothesis was not examined. It has been difficult to decipher whether the buildup of IMCL either leads to or results from insulin resistance using cross-sectional studies, because subjects with the highest lipid content likely had a genetic predisposition favoring the development of insulin resistance. In the current work, IMCLs were varied in a group of genetically homogeneous rats. The results favor a model in which an initial defect of lipid oxidation followed by the accumulation of muscle lipids, particularly the intramyocellular component, can lead to insulin resistance.

It seems doubtful that muscle triglycerides interfere with insulin action in the muscle cells. Rather, we favor the view that the triglyceride level serves as a surrogate marker for some other fatty acid-derived entity that acts to impair insulin signaling. Long-chain fatty acyl-CoA is an attractive candidate because its intracellular concentration will invariably increase with fat loading or after inhibition of CPT-1 (8). Long-chain fatty acyl-CoA, perhaps through its conversion into diacylglycerol, might alter the activity of one or more forms of protein kinase C with resultant impairment in insulin receptor substrate-1-mediated activation of phosphatidylinositol 3-kinase and, thus, GLUT4 translocation to the cell membrane (19,55–57). It is also possible that a high concentration of one or more fatty acyl-CoA species alters transcription/translation or acylation of a protein(s) involved in the insulin signaling cascade. The paradox that trained athletes are highly insulin-sensitive despite having elevated muscle triglyceride content (58) provides further evidence that muscle triglyceride cannot be the sole determinant of insulin sensitivity. However, lipid deposition resulting from extensive physical training could have different metabolic consequences than those associated with inhibition of mitochondrial lipid oxidation. Physical training increases the proportion of type I muscle fibers in the stressed muscle, and these fibers generally have greater lipid content, higher oxidative capacity, and enhanced insulin-stimulated glucose transport relative to type 2b fibers (59,60). Prolonged aerobic exercise depletes endogenous triglyceride stores (45,61), and cyclical depletion and repletion of lipids in skeletal muscle with a high capacity for lipid oxidation may protect against the adverse effects of muscle lipid accumulation (58). Lipid deposition in the current model is associated with a diminished activity of CPT-1 in skeletal muscle similar to that recognized in human obesity (62). In a setting in which lipid metabolism in skeletal muscle is skewed toward storage rather than oxidation, any maneuver capable of lowering the IMCL content should promote an increase in insulin sensitivity. Accordingly, it is tempting to speculate that the salutary effect of an intervention, such as the reduction of dietary fat intake on whole-body insulin sensitivity, operates (at least in part) by limiting lipid accumulation in muscle.

Finally, it should be emphasized that in these studies we purposely chose a high dose of etomoxir to assure a major degree of CPT-1 suppression in skeletal muscle as well as liver. Because the muscle isoform of CPT-1 is far less sensitive to inhibition by etomoxir-CoA than the liver isoform (21), it is possible, using much lower doses of etomoxir, to arrange conditions so that liver but not skeletal muscle CPT-1 is significantly inhibited (unpublished observations). Accordingly, the current findings do not necessarily contraindicate the strategic use of CPT-1 inhibitors in certain clinical situations. For example, such agents might still prove useful in the acute reversal of diabetic ketoacidosis (63), in the prevention of myocardial injury or arrhythmia during reperfusion of the heart after ischemia (64), or in the treatment of congestive heart failure (65).

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