Evidence for Increased and Insulin-Resistant Lipolysis in Skeletal Muscle of High-Fat–Fed Rats

ZengKui Guo and Lianzhen Zhou

The metabolic and isotopic profiles of glycerol in skeletal muscle were examined using awake, fasted lean and high-fat–induced obese rats, and hyperinsulinemic-euglycemic clamp was performed to assess the effect of insulin. During the clamp, Intralipid (no heparin; Fresnius Kabi Clayton, Clayton, NC), free fatty acids, glycerol, and glucose were coinfused to maintain their respective basal plasma levels in both groups. At steady-state, [U-^{14}C]glycerol was infused intravenously for 120 minutes followed by muscle biopsy. The classical phenotypic characteristics of obesity, namely, reduced insulin-stimulated glucose uptake, a failure to suppress systemic lipolysis by insulin, and elevated plasma fatty acid concentration, were observed in the obese rats. Novel observations showed that in the basal state, the isotopic specific activity (S.A.) of glycerol (dpm/nmol) in gastrocnemius (0.03 *v* 0.12), soleus (0.05 *v* 0.12), and tibialis anterior (0.03 *v* 0.12) was significantly lower (all $P < .003$) in obese than in lean rats despite similar concentrations, indicating an active basal intramyocellular lipolysis. In addition, the lipolysis appeared resistant to insulin because the suppression of muscle glycerol during the clamp was 8%, 12%, and 8% in obese compared to 67%, 71%, and 63% in the lean control for gastrocnemius ($P = .001$), soleus ($P = .007$), and tibialis anterior ($P = .004$), respectively. The active intracellular lipolysis likely disturbs metabolic functions that may contribute to insulin resistance.

SKELETAL MUSCLE insulin resistance is a characteristic of obese[1] and type 2 diabetic[2] humans. Unfortunately, the mechanism remains poorly understood. Because skeletal muscle is the primary site for insulin-stimulated glucose uptake,[3] skeletal muscle insulin resistance, combined with pancreatic β-cell failure, is largely responsible for the hallmark hyperglycemia seen in obesity that has diverse negative health consequences. It is well established that obesity is a risk factor for developing type 2 diabetes.[4] Therefore, understanding and treatment of skeletal muscle insulin resistance in obesity is of high importance for preventing and reducing the prevalence of type 2 diabetes and other diseases.

Intramyocellular triglycerides (imcTG) that reside inside the myocytes are increasingly recognized as a chemical entity that may bear importance in the development and/or maintenance of skeletal muscle insulin resistance.[5] The content of imcTG in obese and diabetic humans and animals is abnormally high and it is inversely correlated with insulin sensitivity.[6,7] On the other hand, beyond this correlative relationship with insulin resistance, the metabolism and regulation of imcTG is not well understood. For example, the hydrolysis and turnover of imcTG and its hormonal regulation has not been studied in detail.

This lack of understanding of imcTG metabolism is largely attributable to the previous inability to obtain pure muscle specimens for metabolic studies because skeletal muscle naturally contains a large number of adipocytes which are difficult to eliminate by common techniques. Recently, we have investigated in detail the histology and anatomic location of intramuscular adipocytes and developed a procedure for removing the adipocytes in order to obtain pure muscle specimens.[8,9] Applying this procedure, we are now able to sample pure skeletal muscle specimens for imcTG metabolic studies. Our effective elimination of intramuscular adipocytes greatly improved the reliability of metabolic data and increased our ability to delineate imcTG kinetics.

In the present investigation, we studied the metabolic and isotopic profiles of glycerol, the backbone of imcTG molecules and the immediate product of hydrolysis, in an obese rat model that is induced by high-fat feeding. Our findings suggest that the hydrolysis of imcTG in the diet-induced obesity rat model is accelerated and its regulation by insulin is impaired.

MATERIALS AND METHODS

Animals

Sprague Dawley male rats, all littermates with the same birth date, weighing 100 g were purchased from Harlan Sprague Dawley, Inc (Harlan, IN). On delivery, all rats were fed on regular rat chow (see below) for 3 days before half of the rats were switched to a high-fat pellet diet (55% of calories from lard) ad libitum. The other half of the animals were continued on rat chow. Water was available at all times. The rats were housed in a facility with temperature and humidity control and a 12/12 light/dark cycle. When the high-fat–fed rats were at least 20% heavier, they were studied after an overnight fast, awake. At the time of study, all of the animals were 8 months old and the obese rats had been on the high-fat diet for 7 months. All study protocols were approved by the Mayo Institutional Animal Care and Use Committee.

Diets

The diet fed to the lean rats was Rat Laboratory Chow 5001 from Purina Mills, Inc (Richmond, IN), which contains 4% fat (wt/wt). The high-fat diet was Diet 101078 from Dyets, Inc (Bethlehem, PA), which is grain-based (Purina RLC 5001) and contains 32% lard (wt/wt) with 55% of the energy from lard. The micronutrients of the high-fat diet were the same or similar to the rat chow diet.

From the Endocrine Research Unit, Mayo Foundation, Rochester, MN.

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Address reprint requests to ZengKui Guo, PhD, 5-194 Joseph, Mayo Foundation, Rochester, MN 55905.

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Table 1. Body Weight, Plasma Insulin, and Substrate Concentration and Flux in Lean and Obese Rats

	N	Insulin (nmol/L)	Glycerol (μmol/L)	Glycerol Flux (μmol/kg/min)	FFA (μmol/L)	Glucose (mmol/L)	Glucose Infusion Rate (μmol/kg/min)
Saline							
Lean	9	0.032 ± 0.01	331 ± 45	5.1 ± 0.5	598 ± 61	5.8 ± 0.1	None
Obese	9	0.031 ± 0.01	334 ± 30	4.5 ± 0.5	704 ± 27	5.9 ± 0.1	None
P		.9	.35	.5	.05	.9	
Hyperinsulinemia							
Lean	6	2.8 ± 0.5	533 ± 79	2.7 ± 1.1	572 ± 95	5.7 ± 0.1	172 ± 7
Obese	8	3.3 ± 0.8	399 ± 51	5.0 ± 1.1	639 ± 61	5.6 ± 0.1	132 ± 5
P		.8	.11	.09	.10	.9	.0005

NOTE. Values are mean ± SEM, determined during or at the end of the 120-minute infusion protocols. All of the lean and obese rats were littermates and raised in identical conditions, except for different diets, for 7 months. Therefore, at the time of the study, rats used in the saline and clamp studies were metabolically the same.

Abbreviation: FFA, free fatty acids.

Experimental Design

Lean and obese rats were used in 2 series of experiments: saline (control) and hyperinsulinemic-euglycemic clamp studies.

Saline studies. In the morning of the study, the rats (n = 9 for both groups) were transferred to a metabolic cage. After an adaptation period of at least 30 minutes, a continuous, unprimed intravenous tracer infusion was initiated through a sterile intravenous infusion line into a lateral tail vein. A Harvard infusion pump (Harvard Apparatus, Holliston, MA) was used to infuse [U-^{14}C]glycerol for 120 minutes continuously at a constant rate of 0.1 μCi/kg/min. Normal saline was coinfused at 20 μL/min. The rats were awake and relaxed throughout the study without the use of anesthetics. At the end of the infusions, pentobarbital was injected intravenously at a dose of 50 mg/kg to anesthetize the animal and a blood sample was taken from the left ventricle by percutaneous cardiopuncture. Then the animals were bled to death to reduce blood in muscle. Gastrocnemius, soleus, and tibialis anterior were swiftly exposed and biopsied, immediately immersed in liquid N_2, and later transferred to a −80°C freezer until analyzed. The blood sample was centrifuged on site to separate the plasma and saved in the freezer.

Hyperinsulinemic-euglycemic clamp studies. In this protocol, 6 lean and 8 obese rats were used. Prior to the study, a catheter was installed in the ventral tail artery under local anesthesia with lidocaine.[10] Then the animals were put in a spacious metabolic cage for recovery for 30 minutes before the experiments began. All experimental procedures were identical to the saline studies, except that insulin was infused at 100 pmol/kg/min at least for 20 minutes before and throughout the tracer infusion. Blood glucose level was monitored using a Accu-Check Advantage glucose monitor (Boehringer Mannheim, Indianapolis, IN) every 15 minutes on a drop of blood drawn from the tail arterial catheter and was kept at the basal level by infusion of glucose at varying rates. Meanwhile, Intralipid (20%, no heparin; Fresnius Kabi Clayton, Clayton, NC), free fatty acids, and free glycerol were coinfused at predetermined rates to maintain the basal plasma levels of free fatty acids, glycerol, and triglycerides in both lean and obese rats during the course of the study.

Sample Processing and Analysis

Muscle biopsy. The frozen-stored muscle biopsies were thawed and thoroughly dissected under a stereo microscope for extramyocellular adipocytes, blood vessels, and fascia. The muscle specimen processing and microdissection techniques have been described in detail.[8,9] The dissected muscle samples were then pulverized into fine powder using a steel mortar and pestle at −100°C. The muscle powder was immediately extracted for intracellular free glycerol and total lipids by Folch's method.[11] imcTG was then separated from the extracted total lipids by high-performance liquid chromatography (HPLC).[12] The purified imcTG were directly transmethylated by incubating at 100°C for 2 hours in 2.5% H_2SO_4 in methanol to separate the glycerol (imcTG-glycerol) and fatty acid moieties. The imcTG-glycerol and intracellular free glycerol, present in the upper phase of the Folch extract, were quantified by an enzymatic method.[13] The ^{14}C labels in the 2 glycerol fractions were determined by a liquid scintillation counter and the ^{14}C specific activity (S.A.) of glycerol was calculated.

Plasma. Plasma concentrations of triglycerides, free fatty acids, and free glycerol were determined using the above enzymatic methods. For plasma free glycerol S.A., an aliquot of plasma was treated with equal volume of 3N $ZnSO_4$ and 3N BaOH to precipitate proteins. Plasma free glycerol was then separated by subjecting the diluted serum to an ion-exchange column containing 1 g of AG1x8 resin (hydroxide form, 200 to 400 mesh; Bio-Rad Laboratories, Hercules, CA). Glycerol was eluted with 3 mL of water. The separated glycerol was used to determine the S.A. of ^{14}C. Plasma insulin was measured using a HPLC method.[14]

Calculations. Glucose infusion rate during the clamp studies was the average of the weighted infusion rate taking into account the length of time for each glucose infusion. Endogenous glycerol flux was determined by [U-^{14}C]glycerol infusion rate divided by the ^{14}C S.A. of plasma glycerol, expressed as μmol/kg body weight/min. The infused exogenous glycerol was subtracted from the calculated glycerol flux.

Statistics. All values are expressed as the mean ± SEM. Type 2 Student's *t* test was used for comparisons between lean and obese rats. When no actual *P* value is given, a significance level at .05 is implied.

RESULTS

Metabolic Characteristics

At the time of study, the body weight of obese rats was significantly greater ($P = .0001$) than their lean littermate control for both saline (584 ± 16 g *v* 487 ± 11 g) and hyperinsulinemic-euglycemic clamp (646 ± 16 g *v* 515 ± 15 g) studies. The metabolic profiles of the lean and obese rats during the studies are given in Table 1. The 2 groups' plasma insulin concentrations were not different in either protocol, nor were the concentrations of plasma glycerol or glucose. The concentration of plasma free fatty acid for the obese rats in the saline studies was higher than that in the lean rats by 106 μmol/L (18%) ($P = .05$).

During the clamp studies, unlike that seen in the lean rats,

Table 2. Effects of Hyperinsulinemia on Intramyocellular Free Glycerol Concentration in Skeletal Muscles of Lean and Obese Rats (μmol/g wet weight)

	Gastrocnemius	Soleus	Tibialis Anterior
Saline			
Lean	0.27 ± 0.03	0.39 ± 0.06	0.32 ± 0.04
Obese	0.20 ± 0.02	0.29 ± 0.05	0.23 ± 0.02
P	.06	.21	.03
Hyperinsulinemia			
Lean	0.09 ± 0.02	0.11 ± 0.02	0.12 ± 0.02
Obese	0.18 ± 0.01	0.26 ± 0.03	0.21 ± 0.02
P	.001	.007	.004

NOTE. Values are mean ± SEM. The numbers of rats in each group and procedure (saline or insulin infusion) are the same as in Table 1. Muscles were biopsied immediately before the termination of the experiments when the infusions were still ongoing.

glycerol flux in the obese rats was not suppressed by insulin, and therefore tended to be higher than that in the lean control group. The glucose infusion rate required to maintain euglycemia was significantly lower (by 40 μmol/kg/min, or 23%) in obese than in lean rats ($P = .0005$).

Basal Muscle Glycerol

In the saline studies, the concentration of muscle free glycerol was somewhat lower in obese than in lean rats, although the difference was not statistically significant except for tibialis anterior ($P = .03$) (Table 2). Despite its lower concentration, the ^{14}C S.A. values of muscle free glycerol in obese rats were 2 to 3 times lower than that in lean rats for all 3 muscles (Table 3). After correction for plasma glycerol S.A. (the ratio of muscle glycerol S.A. to plasma glycerol S.A.), the differences remained ($P = .0005$, .003, and .0006 for gastrocnemius, soleus, and tibialis anterior, respectively).

Insulin Effect on Muscle Glycerol

The data in Table 3 show the changes in concentration of muscle free glycerol in the clamp studies. Muscle free glycerol was suppressed by 67%, 71%, and 63% by insulin in gastrocnemius, soleus, and tibialis anterior, respectively, of the lean rats. In contrast, the corresponding suppressions were only 8% ($P = .00004$), 12% ($P = .002$), and 8% ($P = .0002$) in obese rats. As a result, muscle glycerol concentrations in obese rats were approximately 2 times that in lean rats ($P = .001$, .007, and .004, respectively, for the same muscles).

Table 3. Basal Specific Activity of Intramyocellular Free Glycerol in Skeletal Muscles of Lean and Obese Rats (dpm/nmol)

	Gastrocnemius	Soleus	Tibialis Anterior
Raw data			
Lean	3.24 ± 0.3	3.25 ± 0.22	3.32 ± 0.26
Obese	1.24 ± 0.07	1.83 ± 0.13	1.23 ± 0.08
P	.000005	.00006	.000001
Corrected for plasma glycerol S.A. (muscle-glycerol S.A./plasma glycerol S.A.)			
Lean	0.12 ± 0.02	0.12 ± 0.03	0.12 ± 0.02
Obese	0.03 ± 0.004	0.05 ± 0.01	0.03 ± 0.003
P	.0005	.003	.0006

NOTE. Values are mean ± SEM. Data are from the same experiments as in Table 3.

Intramyocellular Triglycerides

In the saline studies, imcTG content of gastrocnemius ($P = .05$) and tibialis anterior ($P = .01$) was significantly higher in obese than in lean rats, although there was no difference for soleus. In the clamp studies, imcTG content increased by 0.86 (2.8-fold) and 0.7 (2.4-fold) μmol/g wet weight for gastrocnemius and tibialis anterior, respectively, in the lean rats, whereas the corresponding increases in obese rats were only 0.31 (1.4-fold) and 0.4 (1.4-fold) μmol/g wet weight. The increase in imcTG content for soleus was 1.2-fold in the lean and 1.4-fold in the obese rats.

DISCUSSION

Because glycerol is the backbone of triglycerides, and its metabolic profile directly represents those of triglycerides. Therefore, its use as a tracer for triglyceride metabolism is justified. This is substantiated by our recent finding that blood glycerol is the predominant precursor for the synthesis of imcTG in fasting rats.[15] Glycerol is also an active precursor for imcTG synthesis in human myocytes.[16]

The high-fat–induced obese rats employed in the present studies manifested insulin resistance during their early life (8 months old, equivalent to the late 20s in humans). This included the failure for insulin to suppress glycerol flux (lipolysis), reduced insulin-stimulated glucose uptake, and elevated basal plasma free fatty acids (Table 1). These are all well-established phenotypic characteristics of obesity.[17] The failure to suppress glycerol flux and the virtually unchanged plasma and muscle glycerol concentration in obese rats during insulin infusion suggest that glycerol uptake by skeletal muscle was perhaps not altered by insulin either.

The insulin effects on muscle glycerol concentration in obese and lean rats were distinct. In the hyperinsulinemic-euglycemic clamp studies, muscle glycerol in obese rats did not change appreciably despite large increase in insulin concentration coupled with unchanged glycerol flux and plasma glycerol. In contrast, the relative insulin suppression of muscle glycerol in the lean rats was 6- to 8-fold greater. This suggested an insuppressible and thus continued imcTG lipolysis during insulin infusion in the obese rats. Consistent with this, muscle glycerol S.A. in obese rats was 2- to 4-fold lower (Table 2) despite similar concentrations (Table 3), suggesting an already accelerated imcTG hydrolytic activity in this group in the basal state.

The unsuppressed imcTG lipolysis was also supported by the imcTG content data (Table 4). While imcTG content in lean rats in the clamp studies was 2 to 3 times that in the saline studies for gastrocnemius and tibialis anterior, the differences for these muscles in the obese rats were only moderate. This appears to have been caused by a continued, active imcTG hydrolysis in obese rats. Insulin inhibits lipolysis, as seen in the lean rats, and this appears to be blunted in the muscles of dietary obese rats. Interestingly, this lack of insulin suppression

Table 4. Intramyocellular Triglycerides Concentrations in Lean and Obese Rats During Saline and Hyperinsulinemic Clamp (μmol/g wet weight)

	Gastrocnemius	Soleus	Tibialis Anterior
Saline			
Lean	0.49 ± 0.09	2.46 ± 0.31	0.51 ± 0.07
Obese	0.76 ± 0.13*	2.39 ± 0.29	1.05 ± 0.17†
Hyperinsulinemia			
Lean	1.35 ± 0.32	2.93 ± 0.49	1.20 ± 0.20
Obese	1.07 ± 0.13	3.26 ± 0.34	1.45 ± 0.09

NOTE. Values are mean ± SEM. Data are obtained from the same muscle biopsies as in Table 3.

*$P = .05$, †$P = .01$, significantly higher than that in the same muscle of lean rats in saline studies.

of muscle lipolysis was also observed in non-obese women.[18] In contrast, low insulin reportedly suppressed glycerol release in tibialis anterior of healthy, lean humans.[19] However, the method (microdialysis) used in that study could not differentiate glycerol releases from subcutaneous adipose tissue and from muscle, which may have confounded the results.

In comparison to that seen for white muscle (gastrocnemius, tibialis anterior), the clear differences in imcTG content and in the pattern of changes during insulin infusion for soleus muscle from the present studies appeared to indicate different glycerol/imcTG kinetics in this oxidative muscle. This conforms to the fact that soleus is different in almost every aspect from other less oxidative muscle types such as gastrocnemius and tibialis anterior. Unfortunately, there is an overall lack of information on insulin regulation of imcTG metabolism, and thus a comparison or discussion is impossible, especially as related to different muscle types.

It has been reported that skeletal muscle possesses the same lipolytic enzyme hormone-sensitive lipase (HSL) that is homologous to adipocyte HSL[20] to which insulin is inhibitory. Therefore, insulin normally inhibits imcTG lipolysis.[21] Thus a lack of insulin inhibition of imcTG lipolysis appears to be another abnormality of obesity. Hopp et al reported that the hydrolysis of imcTG in muscle strips from diabetic rats was inhibited by insulin plus glucose in vitro.[22] However, the effect was probably also contributed by glucose, which has an independent spare effect on the utilization of lipids.[23] Alternatively, the difference in the models employed may have played a role. The rats used in their study[22] were acutely made diabetic by streptozotocin injection and thus the intrinsic biology of skeletal muscle remained largely unaltered, whereas the rats used in the present studies were made obese chronically by high-fat feeding for 7 months during which time phenotypic changes most likely occurred.[24]

The quantitative relationship between imcTG and glycerol in muscle appears to be a complementary one: while the basal imcTG content in obese rat is greater than that in lean rats (Table 4), the glycerol concentration showed an opposite relationship (Table 3). Similarly, in the clamp studies glycerol concentration in lean rats decreased significantly (Table 3), whereas the content of imcTG increased by almost 3-fold. Muscle glycerol in obese rats changed little during the clamp and this was echoed by the only modest increase in imcTG content during the same period. This seems to suggest that the size of total muscle glycerol pool (free glycerol plus imcTG-glycerol) is balanced between the 2 glycerol sub-pools. These observations experimentally confirmed the traditional view that glycerol is the primary source of glycerol-3-phosphate and a direct product of imcTG hydrolysis. The complementary relationship between imcTG and muscle free glycerol also suggested that these 2 glycerol sub-pools comprise the bulk of the dynamic glycerol pool in the myocyte.

In summary, for the first time the present studies evaluated the dynamics and metabolic profiles of glycerol in the skeletal muscle of lean and high-fat–fed obese rats. In this obesity model, an accelerated lipolytic activity was suggested to operate in the myocyte and it is resistant to insulin suppression. The resulting high intracellular glycerol traffic (eg, imcTG-glycerol cycling) may interfere with glucose metabolism and thus contribute to insulin resistance.

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