Lipolysis in Skeletal Muscle Is Decreased in High-Fat–Fed Rats

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The intracellular triglyceride content in skeletal muscle is increased in insulin-resistant states such as obesity or high-fat feeding. It has been hypothesized that increased fatty acid oxidation resulting from increased lipolysis of intramyocellular triglycerides may be responsible for this insulin resistance. This study was undertaken to examine whether insulin resistance is associated with increased lipolysis in skeletal muscle in rats fed a high-fat diet. Sprague-Dawley rats were fed a high-fat diet for 5 weeks. Lipolysis in skeletal muscle and adipose tissue was determined by measuring the interstitial glycerol concentrations using a microdialysis method in basal and hyperinsulinemic-euglycemic clamp conditions. In the basal state, plasma free fatty acid (FFA) levels were higher in high-fat–fed rats than in low fat-fed rats ($P < .05$). In contrast, plasma glycerol levels ($P < .001$) and interstitial glycerol concentrations of skeletal muscle ($P < .05$) and adipose tissue ($P < .01$) were lower in high fat-fed rats than in low fat-fed rats. Plasma ($P < .05$) and interstitial glycerol concentrations ($P < .05$ for skeletal muscle, $P < .01$ for adipose tissue) during the hyperinsulinemic euglycemic clamps were also lower in the high-fat diet group. These results do not support the idea that increased fatty acid oxidation resulting from increased lipolysis of intramyocellular triglycerides is responsible for the insulin resistance in high fat-fed rats.

INSULIN RESISTANCE is a major characteristic of type 2 diabetes and obesity. Skeletal muscle is one of the major sites responsible for peripheral insulin resistance.[1,2] Since the original proposal of the glucose–fatty acids cycle by Randle et al,[3] many in vivo and in vitro studies have repeatedly demonstrated that an increased provision of free fatty acids (FFAs) inhibits insulin-stimulated glucose uptake and glucose oxidation in skeletal muscle.[4-6] However, the relationship between insulin sensitivity and plasma FFA concentration remains controversial,[7,8] which casts doubt on the in vivo role of FFAs in the pathogenesis of insulin resistance. The correlation coefficients between plasma FFA concentrations and insulin sensitivity indices have been reported to be less than 0.6.[5,8,9] Therefore, the pathogenesis of insulin resistance cannot be explained solely by elevated plasma FFA concentrations. One possible explanation for such a discrepancy is that plasma FFA concentrations may not precisely reflect the fatty acid availability in the body.

The rate of fatty acid oxidation in skeletal muscle is determined not only by plasma FFA concentrations released from adipose tissue, but also by fatty acids supplied by local lipolysis of triglycerides stored in skeletal muscle itself.[10-12] Microdialysis measurement of interstitial glycerol concentrations permits the separate and simultaneous examination of local lipolysis in various tissues.[13] By employing this technique, it has been demonstrated that a substantial amount of lipolysis occurs in human skeletal muscles.[12-14] However, it has not been established whether the rate of lipolysis in skeletal muscle is increased in insulin-resistant states such as obesity or high-fat feeding. In the present study, we quantitatively examined lipolysis in the skeletal muscle and adipose tissue of insulin-resistant, high fat-fed rats by microdialysis. Hormone-sensitive lipase (HSL) catalyses the rate-limiting step of lipolysis in adipocytes. The presence of HSL mRNA and protein has been demonstrated in skeletal muscle cells.[15,16] Therefore, we also measured HSL activity in the skeletal muscle from high fat-fed rats.

MATERIALS AND METHODS

Animals and Diets

Male Sprague-Dawley rats weighing 300 to 330 g were used in this study. Sixteen rats from 3 litters were divided randomly into 2 groups (n = 8 per group), and were given either a low-fat diet (LFD) or a high-fat diet (HFD) (Harlan Teklad, Madison, WI) for 5 weeks. The LFD (15 kJ/g) consisted of 12.5% fat (by calories), 66.5% carbohydrate, and 21% protein (60% corn starch, 21.5% casein, 5.0% sucrose, 3.7% cellulose, 2.5% corn oil, and 2.2% shortening by weight), and the HFD (21 kJ/g) consisted of 66.5% fat (by calories), 12.5% carbohydrate, and 21% protein (33% shortening, 30.1% casein, 10.5% corn starch, 10.2% cellulose, 5.0% sucrose, and 3.6% corn oil by weight). Animals were housed under controlled temperature (22 ± 2°C) and lighting (12-hour light, 6 AM to 6 PM; 12-hour dark, 6 PM to 6 AM) with free access to water and rat chow. All experimental procedures were approved by the Animal Care and Use Committee at the Asan Institute for Life Sciences and Technology.

Microdialysis

Following a 5-hours fast, rats were anesthetized with an intraperitoneal injection of 25 mg sodium pentobarbital. The animals were kept anesthetized with continuous intravenous infusion of sodium pentobarbital (120 μg/min), and rectal temperature was maintained at 36 to 37°C on a small electric heating pad throughout the course of the experiment. Two tail vein infusion catheters (PE-10, Intramedic, Clay Adams, Parsippany, NJ) and 1 tail artery blood-sampling catheter were inserted, and patency of the arterial catheter was maintained by a slow (15 μL/min) infusion of normal saline.

Microdialysis probes (CMA Microdialysis AB, Stockholm, Sweden) were inserted into the gastrocnemius muscle and perinephric fat

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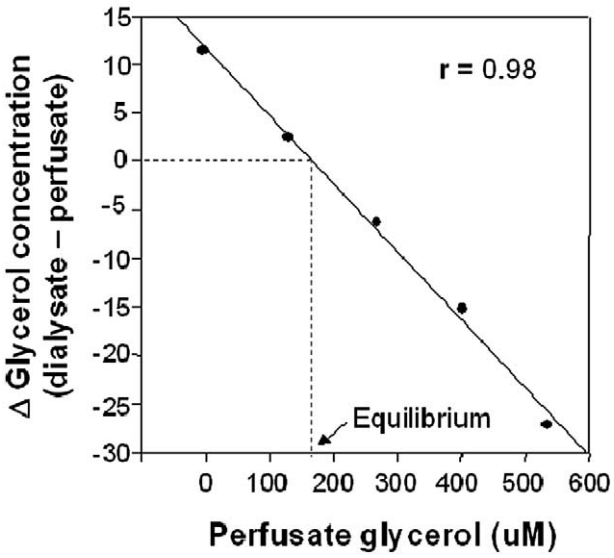

Fig 1. An example of estimation of the interstitial glycerol concentration by a no net flux protocol. There was a linear, inverse relationship between the glycerol concentration in the incoming perfusate and the net glycerol concentration change in the outgoing dialysate. Equilibrium (no change in the dialysate concentration) is achieved when the perfusate glycerol concentration equals that of the interstitial fluid. This point, where net flux across the dialysis membrane is zero, can be determined by simple linear regression analysis.

through a small incision in the lateral abdominal wall. A 30-minute equilibration period was allowed after probe insertion with perfusion of 1.0 μL/min normal saline. We measured interstitial glycerol levels by a no net flux protocol.[12] The probes were perfused at 2 μL/min with normal saline containing 5 different concentrations of glycerol (0, 200, 400, 600, 800 μmol/L in the basal state; 0, 100, 200, 300, 400 μmol/L during the hyperinsulinemic euglycemic clamp) using a microdialysis pump (CMA Microdialysis AB). At each stage, dialysate was collected at 30-minute intervals with 10-minute wash-out periods between each change in the perfusate content. Samples were immediately frozen and stored at −80°C. Blood samples were also taken for measurements of serum glycerol at 30-minute intervals. To estimate the interstitial glycerol concentrations, the net changes of glycerol concentration (outgoing dialysate − incoming perfusate) were plotted against the glycerol concentration of the incoming perfusate. The equilibrium point at which net flux across the dialysis membrane is zero and the perfusate glycerol concentration is the same as that of the interstitial fluid was determined by simple linear regression analysis (Fig 1).

The basal period was followed by a 3-hour hyperinsulinemic euglycemic clamp (clamp period) in which human insulin (Novo-Nordisk, Gentofte, Denmark) was continuously infused at a rate of 24 pmol/kg/min. Blood samples (40 μL) were collected at 10-minute intervals for the immediate measurement of plasma glucose, and 25% dextrose was infused at variable rates to maintain plasma glucose at basal concentrations (~7.8 mmol/L). Microdialysis measurement of interstitial glycerol was performed again during the steady state (final 2 hours) of the hyperinsulinemic clamp. Additional blood samples (100 μL) were obtained at 0, 180, and 360 minutes for the determination of plasma insulin and FFA concentrations.

Blood flow changes around the microdialysis probes were measured according to the method of Hickner et al[17] using ethanol at 20 mmol/L. Ethanol concentrations were measured using an enzymatic method (Sigma, St Louis, MO). Changes in blood flow are presented as a percentage of the baseline, ie, (outflow/inflow) × 100.

HSL Activity Assay

An additional 12 rats were used to measure HSL activity and triglyceride content in gastrocnemius muscle. The rats (n = 6 per group) were given either a LFD or a HFD for 5 weeks before being killed. Following a 5-hour fast, the rats were anesthetized and the gastrocnemius muscle was quickly removed. HSL activity was measured by Osterlund's method.[18] The excised muscles were homogenized on ice in 10 vol of 0.25 mol/L sucrose, 1 mmol/L dithioerythritol, 40 mmol/L β-glycerophosphate, 10 mmol/L sodium pyrophosphate, 31 nmol/L okadaic acid, 20 μg/mL leupeptin, 10 μg/mL antipain, and 1 μg/mLl pepstatin, pH 7.0. The homogenate was centrifuged at 15,800 × *g* in an Eppendorf tube at 4°C for 45 seconds. The supernatant was recovered and the pellets were resuspended in a volume of homogenized buffer equal to the recovered volume. Upon centrifugation, the infranatant and pellets were recovered. Both the supernatants and pellets were stored at −80°C until analysis within 7 days. HSL activity was assayed by measuring the release of [^{3}H]oleic acid from tri[^{3}H]olein (referred to as TO activity), which represents the activity of the activated form of HSL. TO substrates were emulsified with phospholipids by sonication as described previously,[15] and bovine serum albumin (BSA) was used as a fatty acid acceptor. Fourteen-microliter samples of muscle supernatant or pellet were incubated for 30 minutes at 37°C with 100 μL 5 mmol/L TO substrate (1.25 × 10^6 cpm) and enzyme dilution buffer to a total volume of 200 μL. Hydrolysis was stopped by the addition of 3.25 mL methanol:chloroform:heptane (10:9:7 by volume) followed by 1.1 mL 0.1 mmol/L potassium carbonate/0.1 mol/L boric acid (pH 10.5). The mixture was vortexed vigorously for 10 seconds and centrifuged at 1,100 × *g* for 20 minutes. One milliliter of supernatant containing the released fatty acids was mixed with 10 mL scintillation liquid. Radioactivity was determined in a scintillation counter (Packard Instrument Co, Meriden, CT).

Measurement of Muscle Triglyceride Content

Fat tissue attached to the gastrocnemius muscle sample was carefully removed by microdissection under a stereomicroscope.[19] Then the samples (50 mg) were extracted with saturated $CHCl_3$–MeOH (1:2 vol/vol) and homogenized. Homogenates were centrifuged at 3,000 rpm for 30 minutes at 4°C, washed with 500 μL ice-cold phosphate-buffered saline (PBS) and centrifuged as above. After the addition of 2 mL double-distilled H_2SO_4, the tubes were vortexed and centrifuged at 1,000 rpm for 10 minutes at 4°C. The upper phase was discarded, and 100 mg $Na_2S_2O_3$ was added to the lower phase. The samples were vortexed and centrifuged at 1,000 rpm for 5 minutes at 4°C. The upper phase was removed, and the lower phase was evaporated under N_2. The samples were dissolved with 70% isopropanol for 10 seconds. Measurements were made in duplicate using the Sigma Triglyceride (GPO-Trinder) kit.

Analytical Methods

Plasma glucose was measured by the glucose oxidase method using a glucose analyzer (Beckman Instruments, Palo Alto, CA). Glycerol concentrations of the dialysate and plasma were measured by an enzymatic assay kit (Sigma). Plasma FFA was measured by an enzymatic assay using a kit from Eiken Chemical Co (Tokyo, Japan). Plasma insulin was measured by radioimmunoassay using commercial kits for rat (basal insulin; Linco, St Charles, MO) and human insulin (clamp insulin; Dainabot, Tokyo, Japan).

Statistical Analysis

Data are presented as means ± SE. The significance of the difference between the 2 groups was assessed by unpaired Student's *t* test.

Table 1. Body Weight and Basal Metabolic Parameters in LFD- and HFD-Fed Rats

	Body Weight (g)	Glucose (mmol/L)	Insulin (nmol/L)	FFA (μmol/L)
LFD	416 ± 19	7.6 ± 0.8	1.7 ± 0.36	456 ± 38
HFD	457 ± 28*	7.5 ± 0.6	2.1 ± 0.54	527 ± 44*

NOTE. Values are mean ± SE.
*$P < .05$ *v* LFD group.

RESULTS

Table 1 shows the metabolic characteristics of the LFD and HFD groups after the 5-week period of dietary treatment. The average body weight was significantly higher in the HFD group than in the LFD group ($P < .05$). Basal plasma glucose and insulin concentrations did not significantly differ between the 2 groups. Basal plasma FFA concentrations were elevated in the HFD group compared to the LFD group ($P < .05$).

During the clamps, plasma insulin concentrations were raised to similar levels (~1,200 pmol/L) in both groups, and plasma glucose levels were maintained at a basal (~7.8 mmol/L) level (Fig 2A). However, the glucose infusion rate required to maintain euglycemia was significantly lower in the HFD group than in the LFD group (55 ± 14 *v* 190 ± 17 μmol/kg/min, $P < .001$) (Fig 2B).

In the basal state, plasma glycerol levels were lower in HFD rats than in LFD rats (137 ± 11 *v* 173 ± 18 μmol/L, $P < .001$) (Fig 2C). The interstitial glycerol concentrations of skeletal muscle (258 ± 98 *v* 382 ± 92 μmol/L, $P < .05$) and adipose tissue (293 ± 75 *v* 510 ± 166 μmol/L, $P < .01$) were also lower in HFD rats than in LFD rats (Fig 3A and 3B). During the hyperinsulinemic euglycemic clamp, the steady-state mean plasma glycerol concentrations were also lower in the HFD group (111 ± 14 *v* 134 ± 21 μmol/L, $P < .05$) (Fig 2C). The interstitial glycerol concentrations in skeletal muscle (152 ± 61 *v* 215 ± 72 μmol/L, $P < .05$) and adipose tissue (187 ± 85 *v* 312 ± 32 μmol/L, $P < .01$) were also lower in the HFD group during the clamp (Fig 3A and 3B). Although glycerol release in each tissue under hyperinsulinemia was suppressed in both groups, the degree of suppression was lower in the HFD group than in the LFD group (muscle: −106 ± 38 *v* −167 ± 42 μmol/L, $P < .05$; adipose tissue: −104 ± 29 *v* −198 ± 57 μmol/L, $P < .05$, Fig 3C). Local blood flow around the probes was not significantly changed throughout the experiment in both skeletal muscle and adipose tissue, and was not different between HFD and LFD groups (Fig 3D).

Muscle HSL activity in the basal state was lower in HFD rats than in LFD rats (387 ± 29 *v* 504 ± 25 dpm, $P < .05$, Fig 4A). Muscle triglyceride content was higher in HFD rats than in LFD rats (0.45 ± 0.03 *v* 0.31 ± 0.04 mmol/mg wet weight, $P < .05$, Fig 4B).

DISCUSSION

Microdialysis is a useful technique for determining interstitial substrate concentrations. However, determination of absolute concentration of the substrate is not easy, since the recovery characteristics of the dialysis membranes vary considerably from membrane to membrane in vivo. In the present study, we used "no net flux" method, which has been validated by several previous studies.[12,20] Five different concentrations of the glycerol were added to the perfusion liquids and the concentration resulting in no net change of glycerol content in vivo was estimated by regression analysis. As shown in Fig 1, there was

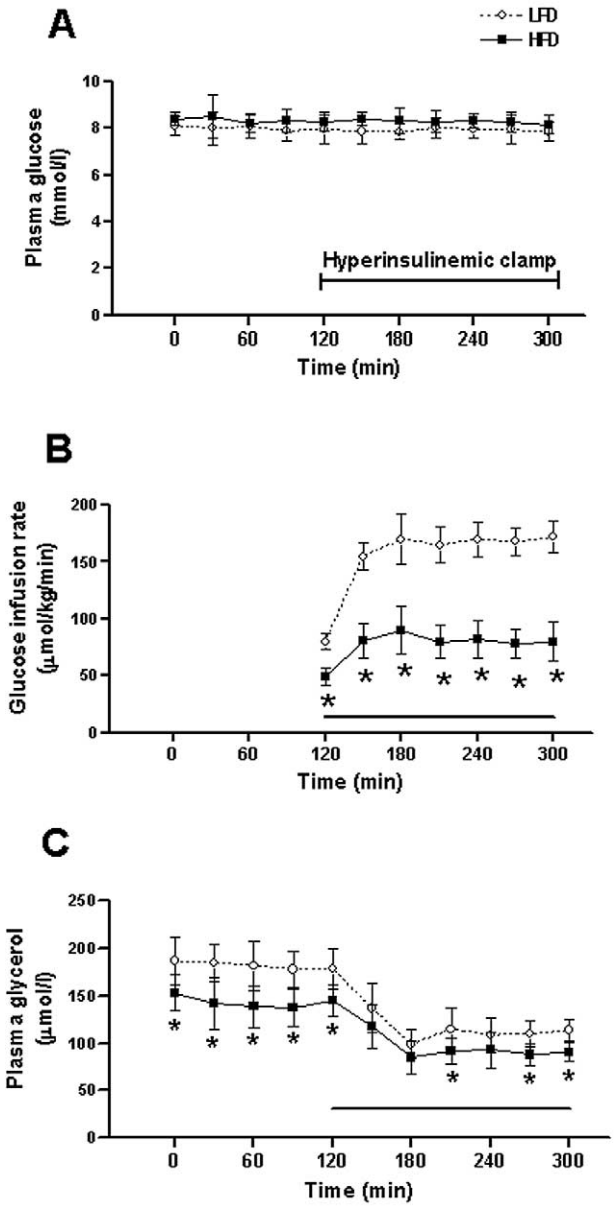

Fig 2. (A) Plasma glucose levels, (B) glucose infusion rates, and (C) plasma glycerol concentrations during basal (0-120 min) and hyperinsulinemic euglycemic clamp (120-300 min) conditions in LFD- and HFD-fed rats (n = 8 per group).

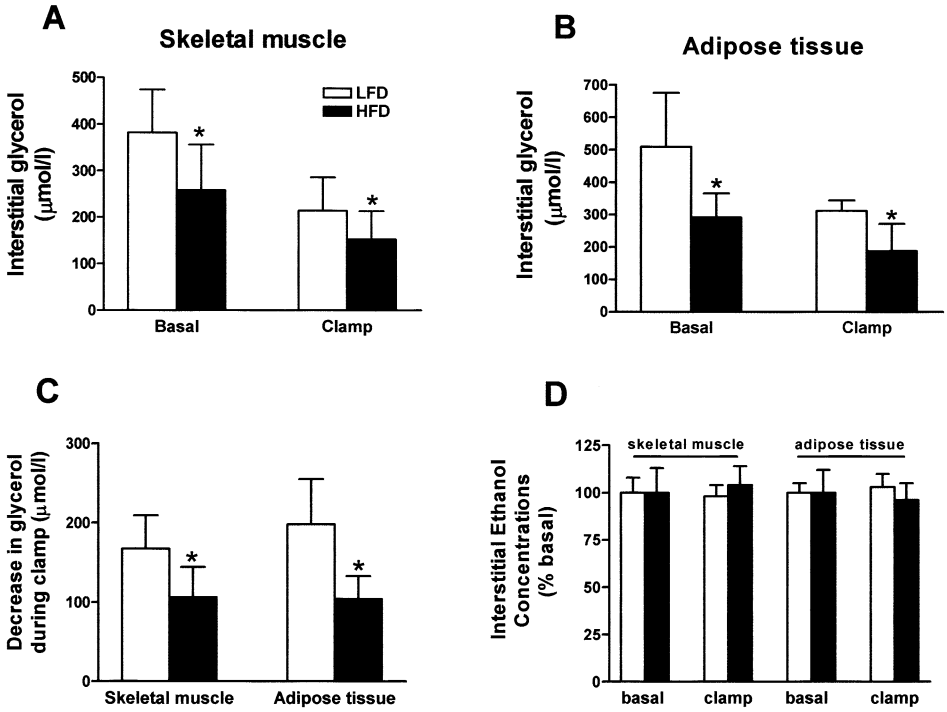

Fig 3. Interstitial glycerol concentrations in (A) skeletal muscle and (B) adipose tissue under basal and hyperinsulinemic euglycemic clamp conditions, and (C) net change in the interstitial glycerol concentrations during clamp (n = 8 per group). (D) Interstitial ethanol concentrations in skeletal muscle and adipose tissue during microdialysis.

a linear, inverse relationship between the glycerol concentration in the incoming perfusate and the net glycerol concentration change in the outgoing dialysate. The correlation coefficients were sufficiently high, indicating the validity of this method. By employing this method, the present study confirms the occurrence of a substantial amount of lipolysis in skeletal muscle. The interstitial glycerol concentration of skeletal muscle was slightly lower than that of adipose tissue, but higher than that of plasma. This finding indicates the importance of a local supply of FFA as a metabolic fuel.[12,14] Contrary to expectations, however, we found that lipolysis was decreased in the skeletal muscle of obese, insulin-resistant animals. Together with decreased lipolysis, we found a decrease in HSL activity in skeletal muscle from high fat-fed animals. Although the enzymatic regulation of triglyceride breakdown in muscle is poorly understood, a good accordance between HSL activities and lipolysis in our study may imply a physiologic role for HSL in the regulation of lipolysis within muscle.

Increased lipolysis from adipose tissue is thought to be a primary source of elevated plasma FFAs, which is known to induce insulin resistance in skeletal muscle. However, in the present study, lipolysis in the adipose tissue of high fat-fed rats was reduced compared to that of low-fat–fed rats. This is consistent with previous studies showing decreased lipolytic response to adrenergic agents in the adipose tissue from rats fed a HFD.[21-23] In contrast, other investigators reported that adi-

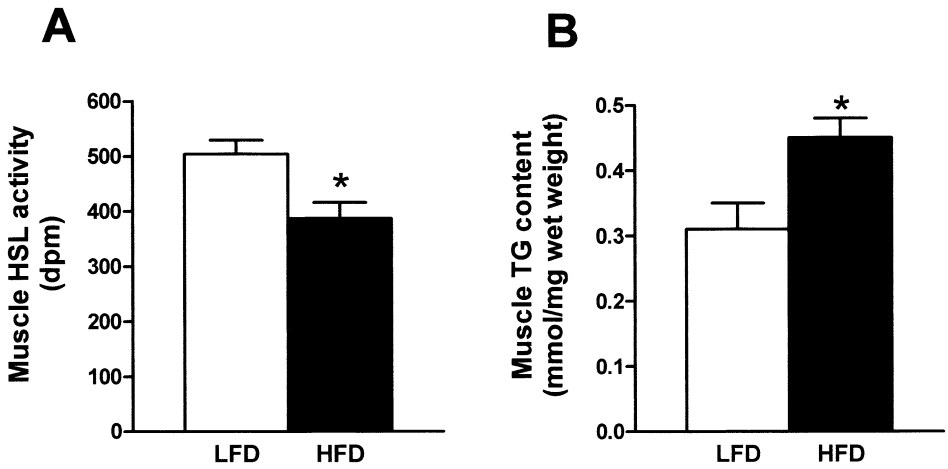

Fig 4. (A) HSL activities and (B) triglyceride contents in skeletal muscle in the fasting state (n = 6 per group). *$P < .05$ *v* LFD group.

pose tissue lipolysis and HSL activity were increased in high-fat–fed rats and obese subjects.[14,24,25] This discrepancy may be due to the differences in the duration of high-fat feeding, anatomical location of fat tissue studied, and pharmacological stimulation used in measuring lipolysis.

It has been reported that increased intracellular triglyceride content in skeletal muscle is associated with insulin resistance.[9-12] Insulin sensitivity was inversely correlated with intramuscular triglyceride content in high-fat–fed rats[10,26] and nondiabetic Pima Indians.[11] In accordance with this, the present study shows higher muscle triglyceride content and lower insulin sensitivity in high-fat–fed rats. According to the glucose–fatty acids cycle hypothesis, increased fatty acid oxidation resulting from either increased lipolysis or FFA uptake in skeletal muscle should decrease glucose oxidation and cause insulin resistance.[10] However, our results showing decreased lipolysis in skeletal muscle do not support this hypothesis. Recent studies[27-29] have found that skeletal muscle utilization of FFA was decreased, rather than increased, in insulin-resistant subjects. Furthermore, administration of β_3-adrenoreceptor agonist, which increases fat oxidation, increased insulin activity in both rodents[30] and humans.[31] Taken together, these observations suggest that intracellular triglyceride accumulation in the insulin-resistant state is the consequence of diminished fatty acid oxidation capacity rather than the cause of insulin resistance. Indeed, diminished fatty acid oxidation capacity increases cytosolic long-chain fatty acyl-CoA (LCAC),[26] which has been shown to impair insulin signaling.[32,33] Increased cytosolic LCAC may also decrease intramyocellular lipolysis by inhibition of HSL.[34]

The mechanism of decreased lipolysis and/or fatty acid oxidation in the skeletal muscles of insulin-resistant animals is not known. It has been suggested that excess glucose could inhibit lipid oxidation.[35,36] Increased muscle glucose metabolism could potentially increase intracellular malonyl conenzyme A (CoA) concentration, which inhibits carnitine palmitoyl transferase (CPT)-1 and blocks fatty acid oxidation.[37] However, plasma glucose levels were similar in the 2 groups in our study. Therefore, the above argument cannot explain the decreased lipolysis in high-fat–fed rats.

Another possible mechanism to explain this decreased lipolysis is a decrease in β-adrenergic responsiveness in obese subjects or those with energy excess. Adipose tissue from rats fed a HFD showed decreased lipolytic response to adrenergic agents,[21-23] and fat cell plasma membranes from obese patients were less responsive to isoproterenol than those from normal-weight subjects.[38] Furthermore, it has been suggested that depressed expression of adipocyte β_3-adrenergic receptors is a common feature of congenital and diet-induced obesity in rodents.[39] The β_3-adrenergic system is a primary regulator of lipolysis in adipose tissue,[40] and it has been implicated in the regulation of fatty acid oxidation in brown adipose tissue and skeletal muscle through the transcriptional regulation of peroxisome proliferator activated receptor (PPAR)-gamma coactivator-1.[41] Therefore, reduced β-adrenergic activity in obese human or animals may cause a decrease in both lipolysis and fat oxidation. While the role of a β-adrenergic system in skeletal muscles that do not express β_3-adrenergic receptors may seem unlikely,[42] the β_3-adrenergic agonist BRL37344 was able to improve insulin sensitivity in this tissue,[43] suggesting the existence of another as yet unidentified β-adrenergic receptor.

During the hyperinsulinemic euglycemic clamp, plasma and interstitial glycerol concentrations of muscle and adipose tissue were reduced in both the HFD and LFD groups. As in the basal state, interstitial glycerol concentrations during the hyperinsulinemic euglycemic clamp were lower in the HFD group. However, the magnitude of glycerol concentration suppression was lower in the HFD group than in the LFD group, suggesting a resistance to antilipolytic action of insulin in high-fat–fed rats. This finding is consistent with a recent proposal by Kelley and Mandarino[36] that a reduced capacity to switch between fuels, the so-called metabolic inflexibility, is a key aspect of insulin resistance in skeletal muscle. It was found that obese subjects manifested less lipid oxidation during the fasting state and greater lipid oxidation during insulin-stimulated conditions relative to the lean volunteers, and the absolute rate of lipid oxidation remained fixed in obese subjects.

There are several potential limitations in our study. First, measurement of interstitial glycerol concentrations may not wholly reflect the lipolysis state in the skeletal muscle. In contrast to fat tissue, muscle can reutilize a certain amount of glycerol for on-site lipogenesis.[44] Thus, the interstitial glycerol concentration in the skeletal muscle represents a net effect of glycerol release and uptake. However, Hagstrom-Toft et al[45] estimated that the skeletal muscle tissue glycerol uptake is at most 20% to 30% of infused glycerol under the conditions of markedly elevated (~8 times of basal level) circulating glycerol. Therefore, enhancement of glycerol uptake, if any, may not be a major determinant of reduced basal interstitial glycerol concentration in the high-fat–fed rats. Second, these experiments were conducted during pentobarbital anesthesia. General anesthesia may stimulate catecholamine production, thus affecting lipolysis and glucose metabolism. However, a recent study investigating the effects of anesthesia on norepinephrine kinetics indicated that pentobarbital anesthesia did not change plasma norepinephrine concentration.[46] Third, we measured muscle triglyceride content in total muscle tissue. We tried to remove extramyocellular fat by microdissection under a stereomicroscope, but some contaminating extramyocellular triglyceride as well as the intramyocellular triglyceride could have been measured. Thus we cannot exclude the possibility that increased muscle triglyceride in high-fat–fed rats is due to an increase in lipid accumulation outside the myocytes. In this regard, Commerford et al[47] reported that intracellular FFA cycling is increased in high-fat–fed rats, suggesting a tendency to accumulate lipids inside the fat cell.

In summary, lipolysis in skeletal muscle is decreased in high-fat–fed rats. These results do not support the idea that increased fatty acid oxidation resulting from increased lipolysis of intramyocellular triglyceride is responsible for insulin resistance in high-fat–fed rats. Instead, it can be suggested that diminished lipolysis and lipid oxidation during a fast may facilitate lipid accumulation within skeletal muscle and increased lipid metabolites may in turn contribute to insulin resistance in obese humans and animals.

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