Paradoxical coupling of triglyceride synthesis and fatty acid oxidation in skeletal muscle overexpressing DGAT1

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**Objective:** Transgenic expression of diacylglycerol acyltransferase-1 (DGAT1) in skeletal muscle leads to protection against fat-induced insulin resistance despite accumulation of intramuscular triglyceride (TG), a phenomenon similar to what is known as the “athlete paradox”. The primary objective of this study is to determine how DGAT1 affects muscle fatty acid (FA) oxidation in relation to whole-body energy metabolism and insulin sensitivity.

**Research Design and Methods:** We first quantified insulin-sensitivity and the relative tissue contributions to the improved whole-body insulin-sensitivity in muscle creatine kinase (MCK)-Dgat1 transgenic mice by hyperinsulinemic-euglycemic clamps. Metabolic consequences of Dgat1-overexpression in skeletal muscles were determined by quantifying TG synthesis/storage (anabolic) and FA oxidation (catabolic), in conjunction with gene expression levels of representative marker genes in FA metabolism. Whole-body energy metabolism including food consumption, body weights, oxygen consumption, locomotor activity, and respiration exchange ratios were determined at steady states.

**Results:** MCK-Dgat1 mice were protected against muscle lipoptoxicity, although they remain susceptible to hepatic lipotoxicity. While augmenting TG synthesis, Dgat1-overexpression also led to increased muscle mitochondrial FA-oxidation efficiency, as compared with wild-type muscles. On a high-fat diet (HFD), MCK-Dgat1 mice displayed higher basal metabolic rates and 5-10% lower body weights compared with wild-type littermates, while food consumption was not different.

**Conclusions:** DGAT1-overexpression in skeletal muscle led to parallel increases in TG synthesis and FA oxidation. Seemingly paradoxical, this phenomenon is characteristic of insulin sensitive myofibers and suggests that DGAT1 plays an active role in metabolic “remodeling” of skeletal muscle coupled with insulin sensitization.
Despite the observation that higher fat content in skeletal muscle is associated with insulin resistance as commonly seen in obesity and type 2 diabetes, increased muscle fat content is also associated with exercise training, in which it is “paradoxically” coupled with enhanced insulin sensitivity (the “athlete paradox”) (1-3). In fact, higher fat content is also seen in “oxidative” (with greater FA oxidation capacity), slow-twitch type I myofibers compared with “glycolytic”, fast-twitch type II myofibers. Interestingly, type I myofibers appear to be inherently more insulin sensitive than type II myofibers (9). In human skeletal muscle, insulin-stimulated glucose transport directly correlates with the percentage of oxidative type I myofibers (4-6). Furthermore, exercise enhances muscle insulin sensitivity and is associated with increased muscle fat content and/or mitochondrial FA oxidation capacity (1,3,7,8). In contrast, a decrease in oxidative, and conversely an increase in glycolytic, capacity is characteristic of skeletal muscle seen in sedentary obese individuals (10,11).

Exercise increases myocytic DGAT activity, and transgenic overexpression of DGAT1 in skeletal muscle in mice is sufficient to replicate the exercise paradox characterized by increased muscle fat content coupled with increased muscle insulin sensitivity (3). Exercise also increases skeletal muscle DGAT1 expression and improves insulin sensitivity in humans (12). DGAT1 catalyzes the last step of TG synthesis from diacylglycerol (DAG) and fatty acyl-CoA. By channeling FA substrates into the TG synthesis pathway, DGAT1 effectively decreases levels of muscle DAG and ceramide (3), two FA derivatives that are believed to be causative in the development of obesity-related insulin resistance (3,13-17). In sedentary humans or rodents with fat-induced insulin resistance, higher muscle TG content is also associated with elevated myocytic DAG and/or ceramide levels as a result of FA overload (12,13,15); lipid metabolites and storage products are both increased in this substrate-driven process. Increased FA load and consequent increases in intramyocellular FA metabolites are lipotoxic and cause muscle insulin resistance (18). Increased myocytic DAG, for example, causes insulin resistance via activation of protein kinase Cθ (13,14) leading to serine-phosphorylation of IRS-1 (13). Thus, while increased DAG is causal, high myocytic TG-content is not always a marker for muscle insulin resistance, as long as lipotoxic FA metabolites are kept at low levels (3).

Given the parallel increases in both muscle TG stores and FA oxidation activity as exemplified by oxidative type I myofibers or the exercise-trained muscle model, we hypothesized that TG synthesis and FA oxidation capacities are intrinsically interconnected and both are upregulated in insulin sensitive muscles. The present study demonstrates upregulation of both TG synthesis and FA oxidation capacities in the insulin sensitive, DGAT1-overexpressing muscle model. This study also suggests that Dgat1-overexpression is a cause of such a “paradoxical” change in fat metabolism. We postulate that this coordinated change enables the myocytes to keep the intracellular levels of FA metabolites below a potentially lipotoxic level. By channeling lipotoxic FA metabolites into storage (as TG) and into a pathway for terminal oxidation, such a change may lessen FA-induced insulin resistance that is often associated with acquired obesity (3,12,13,19).

**RESEARCH DESIGN AND METHODS**

Mice, diets, and exercise: MCK-Dgat1 transgenic mice and WT littermates were fed regular chow or HFD as previously described (3). The swimming exercise
regimen was the same as described (3). Male mice were used in this study. Animal protocols were approved by the Columbia University IACUC.

Hyperinsulinemic-euglycemic clamps: Three month-old mice were fed HFD or normal chow diet for 8 weeks before the clamp study. The clamp was conducted in awake mice as previously described (19). All procedures were approved by the Yale University IACUC. Briefly, after an overnight fast, [3-3H]-glucose (HPLC purified; Perkinelmer) was infused at 0.05 μCi/min for 2 hours to assess the basal glucose turnover. Hyperinsulinemic-euglycemic clamp was conducted for 120 min with primed/continuous infusion of human insulin (Novo Nordisk; 105 pmol/kg prime, 15 pmol/kg/min infusion), keeping plasma glucose at ~6.7 mM (basal level). [3-3H]-glucose was infused at 0.1 μCi/min throughout the clamp to estimate insulin-stimulated whole-body glucose fluxes. Calculations of hepatic glucose output (HGO), whole-body glycolysis, and whole-body glycogen synthesis were also as described (20). To assess muscle glucose uptake, separate groups of mice were clamped, and 2-deoxy-D-[1-14C]glucose (2-[14C]DG; Perkinelmer) was injected as a bolus at the 75th minute of the clamp as described (20).

Mitochondrial FA oxidation: Four month-old mice pretreated with HFD for 8 weeks were used for these experiments. Mitochondria were isolated from muscle tissues using Mitochondria Isolation Kits from Sigma-Aldrich (St Louis, MO) according to the manufacturer’s instructions. Mitochondrial FA oxidation was performed by measuring the rate of 3H2O production from the substrate [3H]palmitate present in excess (final concentrations: 0.2 mM palmitate with 10 μCi/ml of 9,10(n)-[3H]palmitate and 1.25% BSA) in a mitochondria incubation medium (containing 20 μg mitochondria protein) as described (21).

Mitochondrial DNA copy number, ATP production rate (MAPR), citrate synthase (CS), β-hydroxyacyl-CoA dehydrogenase (β-HAD), and carnitine palmitoyl transferase I (CPT-I) activities: Four-month-old mice pretreated with HFD for 8 weeks were used for these experiments. Mitochondrial DNA copy number was measured using quantitative real-time PCR as previously described (22). Mitochondrial DNA for NADH dehydrogenases 1 and 4 (ND1 & ND4) and nuclear DNA for β-actin were co-amplified within the same reaction wells (for primer sequences, see Supplemental Table 1 in the online appendix available at http://diabetes.diabetesjournals.org).

Mitochondrial ATP production rates were measured in isolated mitochondria through time-course monitoring of bioluminescence in response to a luciferin-luciferase ATP-monitoring reagent (Biotherma, Haninge, Sweden) as described (23). Maximal activity of citrate synthase (CS) was measured in muscle homogenates by photospectrometry as described (24). β-HAD activity was also measured in muscle homogenates by following spectrophotometrically the conversion of acetoacetyl-CoA to β-hydroxybutyryl-CoA by β-HAD as described (25). Total CPT activity was assayed radiochemically in isolated mitochondria by following the conversion of palmitoyl-L-carnitine from palmitoyl-CoA and radiolabeled L-carnitine as described (25, 26). CPT-I activity was assayed from the total CPT activity after subtraction of CPT-II activity, which was measured in the presence of 0.2 mM malonyl-CoA that inhibits CPT-I.

Transmission Electron Microscopy: Four month-old mice pretreated with HFD for 8 weeks were used. Muscles were isolated, fixed with 2.5% glutaraldehyde in 0.1M Sorenson’s buffer (PH 7.2), treated with 1% OsO4 also in Sorenson’s buffer for one hour. After dehydration and embedment in Lx-112
(Ladd Research Industries, Inc.), thin sections (60 nm) were cut using a MT-7000 ultramicrotome, stained with uranyl acetate and lead citrate, and examined under a JEOL JEM-1200 EXII electron microscope. Pictures were captured by an ORCA-HR digital camera (Hamamatsu) and recorded with an AMT Image Capture Engine.

**Indirect calorimetry:** Twenty-week-old weight-matched male MCK-Dgat1 mice and WT littermates that had been on a HFD for 8 weeks were singly housed in metabolic chambers. After 2-4 days of adaptation, oxygen consumption, locomotor activity, and respiration exchange ratio were measured continuously over at least 5 days during 12:12 light-dark cycles, using a CLAMS (Columbus Instruments, Columbus, OH) open circuit indirect calorimetry system as previously described (27).

**Real-time PCR, Western blot, DGAT activity and TG-contents:** Four-month-old mice with 8-week HFD-pretreatment were used for these experiments. RT-PCR (3,28) and Western blot (3,30) were as described. Gene-specific primer sets are listed in Supplemental Table 1. Primary antibodies to PDK4, UCP3, CD36 and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) were obtained from Abcam (Cambridge, MA). DGAT activity was measured in vitro using [14C]-labeled palmitoyl-CoA as previously described (29,30). The method to quantify muscle TG-content was as described (3,30).

**Statistical analysis:** Statistical differences were first analyzed by ANOVA to determine overall treatment effects (genotype or exercise), followed by post hoc comparisons between concerned study groups (Statistica V6.0, StatSoft, OK). A two-tailed p value of <0.05 was used to indicate statistical significance. For indirect calorimetry measurements, ANOVA repeated measures (Prism, GraphPad Software, San Diego, CA) were used. Post-hoc analyses were conducted using Tukey post-hoc or Bonferroni comparisons. Data are expressed as means with standard errors.

**RESULTS**

**MCK-Dgat1 transgenic mice demonstrate improved resistance to diet-induced lipotoxicity, but they remain susceptible to hepatic insulin resistance on HFD.** Using glucose tolerance and insulin tolerance tests, we showed previously that MCK-Dgat1 mice were protected against HFD-induced insulin resistance (3). Ex vivo 2-deoxyglucose uptake in skeletal muscles isolated from HFD-fed animals demonstrated that insulin sensitivity was greater in DGAT1-overexpressing muscles than in the wild-type muscles (3). In this study, whole-body and tissue insulin sensitivities were assessed in vivo by hyperinsulinemic-euglycemic clamps in combination with radioisotope-labeled glucose infusions. Three month-old male MCK-Dgat1 and WT mice were fed HFD for 8 weeks (WT-HF and Dgat1-HF, respectively) before the clamp study. Age- and gender-matched WT mice on normal chow (WT-NC) were used as baseline control. The major findings are depicted in Fig 1, and other energetic and clamp parameters are summarized in Supplemental Table 2. As expected, WT-HF mice showed marked insulin resistance with the mean whole-body glucose infusion rate at only ~16 mg/kg/min (Fig 1A). Dgat1-HF mice were more insulin-sensitive than WT-HF mice, achieving a mean whole-body glucose infusion rate of ~29 mg/kg/min (Fig 1A). Dgat1-HF mice were more insulin-sensitive than WT-HF mice, achieving a mean whole-body glucose infusion rate of ~29 mg/kg/min (Fig 1A). However, the baseline whole-body glucose infusion rate in the control WT-NC mice was ~45 mg/kg/min (Fig 1A), indicating that Dgat1-overexpression provided ~45% protection against HFD-induced insulin resistance. Transgenic mice were not protected from HFD-induced hepatic insulin resistance. Compared with a >50% suppression of hepatic glucose output (HGO) in WT-NC
mice during the clamping, insulin-mediated suppression of GHO was completely absent in both WT-HF and Dgat1-HF mice (Fig 1B). To directly assess muscle insulin sensitivity under the same clamping conditions, a bolus infusion of 2-deoxy-D-[1-14C]glucose was given. Uptake of 2-deoxy-D-[1-14C]glucose in skeletal muscle was ~30% greater in Dgat1-HF mice than in WT-HF mice (Fig 1C), confirming that increased lipotoxicity-tolerance in skeletal muscle was the physiological basis for the protection of MCK-Dgat1 mice, albeit incomplete, against HFD-induced whole-body insulin resistance.

**Dgat1-overexpression in skeletal muscle leads to improved mitochondrial FA oxidation efficiency as well as increased TG synthesis and storage.** As expected, muscle DGAT activity was 3-fold increased in MCK-Dgat1 (Fig 2A), which was associated with a ~70% increase in muscle TG-content (Fig 2B). To characterize skeletal muscle FA oxidation capacity, we first determined mitochondrial DNA (mtDNA) copy number by assessing the ratio of mtDNA NADH dehydrogenases 1 and 4 (ND1 & ND4) to the nuclear DNA β-actin gene in soleus and anterior tibial muscles. There were 30-45% fewer mitochondria per copy genome DNA in Dgat1-overexpressing tibial and soleus muscles than those of the WT controls (Fig 2C). However, analyses of mitochondrial ATP production rates (MAPR) per unit muscle protein revealed no reduction in transgenic mice as compared with WT mice (Fig 2D). Maximal tissue CS activity was significantly greater in the Dgat1-overexpressing soleus muscle than the WT counterpart, although no significant difference was detected in tibialis anterior (Fig 2D). Maximal tissue β-HAD activity per unit muscle protein was also significantly greater in the transgenic muscle than the WT muscle (Fig 2E). Similarly, mitochondrial CPT-I activities per unit mitochondrial protein was significantly greater in the Dgat1-overexpressing muscle than the WT muscle (Fig 2E). The unchanged or potentially enhanced mitochondrial oxidative capacity in the presence of fewer mitochondria suggested a higher mitochondrial efficiency in Dgat1-overexpressing muscles. Direct examination of soleus muscles by electron microscopy confirmed that fewer, but bigger, mitochondria were present in the Dgat1-overexpressing muscle (Fig 2F) than in the WT muscle (Fig 2G). Additionally, in vitro measurement of maximal FA oxidation rates demonstrated a 20% increase in FA oxidation capacity in the MCK-Dgat1 muscle compared with the WT muscle (1217 versus 1019 nmol/min/gram mitochondrial protein, p<0.01; Fig 2H).

**Upregulation of PDK4 and UCP3 in skeletal muscle of MCK-Dgat1 transgenic mice.** Messenger RNA levels of marker genes in fat metabolism were examined by real-time PCR in soleus muscle from 4 month-old male WT and MCK-Dgat1 transgenic mice after 8 week-HFD feeding (Fig 3A). DGAT1 mRNA levels were ~3-fold as high in MCK-Dgat1 mice as in WT mice (p<0.05) as expected, and mRNA levels for PDK4 and UCP3 were doubled in MCK-Dgat1 compared with WT mice (p<0.05). There was also a trend of ~doubling of mRNA levels for CD36 (p=0.051), CPT1 (p=0.120), ACC2 (p=0.072), and GLUT4 (p=0.117) in MCK-Dgat1 mice. No significant changes were detected in mRNA levels for DGAT2, FAS, ACS1, ADRP, SREBP1c, PPARα, PPARδ, PPARγ, PGCβ, CRAT, ECHS1, UCP1, UCP2, and PEPCK.

PDK4 is believed to play a critical role in regulating the fuel partitioning between fat and glucose oxidation (31,32). To further understand the changes in PDK4 expression in MCK-Dgat1 muscles, we compared a group of DGAT1-overexpressing mice with a group of WT mice after one week swimming exercise (3). Using sedentary WT mice as a baseline control, both DGAT1-overexpression
and exercise led to ~1.5-fold increases (p<0.05) in PDK4 mRNA levels in skeletal muscle (Fig 3B, 3C), providing two independent cases, in which upregulation of PDK4 mRNA expression was dissociated from glucose intolerance. Western blot of CD36, UCP3, and PDK4 showed 1.4-1.8 fold increases of these proteins in Dgat1-overexpressing muscle relative to WT muscle (Fig 3D).

**MCK-Dgat1 mice display increased energy expenditure without changes in food intake.** To determine the physiological significance of the above in vitro findings and to help interpret the physiological consequences of the increased TG synthesis and increased mitochondrial FA oxidation capacity (and higher expression levels of the uncoupling protein UCP3) in Dgat1-overexpressing skeletal muscle, we measured whole-body energy metabolism in vivo. Four month-old weight- and adiposity-matched (Fig 4A), male MCK-Dgat1 and WT mice were analyzed in metabolic chambers after 8-week HFD feeding. MCK-Dgat1 mice appeared less physically active than WT mice (Fig 4B), although the changes seemed to be unrelated to food-seeking behavior, as food intake was the same between MCK-Dgat1 and WT mice (Fig 4G). In contrast to the changes in physical activity, oxygen consumption rates were significantly higher in the transgenic mice than in WT mice (Fig 4C, WT vs MCK-Dgat1, p<0.05, repeated measures ANOVA). No significant difference was detected in respiration exchange rates (RER) under these conditions (Fig 4D). Further experimental maneuver with two consecutive 24-hour measurements of VO2 during fasting followed by refeeding revealed that the difference in energy expenditure was accentuated during refeeding. As expected, energy expenditure reduced and increased in response to prolonged fast and refeeding, respectively, in both WT and MCK-Dgat1 mice (Fig 4E). However, the increase during refeeding was markedly greater in the transgenic mice than in WT mice (p<0.05, repeated measures ANOVA) (Fig 4F). As was during regular ad lib feeding, no difference in food intake was observed during the 24-hour refeeding period between MCK-Dgat1 and WT mice (Fig 4G).

Long-term food intake (FI) was followed in mice fed a chow diet (total 11 weeks) or a HFD (total 8 weeks), starting at 2 months of age. Periodic fluctuations in FI were observed in both transgenic and WT mice, more so in chow-fed mice (Fig 5A). However, no difference was found between the two genotypes either on chow or on HFD (Fig 5A and 5B). A small but significant body weight difference (5-10%) was found between WT and MCK-Dgat1 mice on HFD, which became evident after 5 weeks of HFD feeding and persisted throughout the remaining study period (Fig 5C). In the absence of reduced FI or increased physical activities, this finding indicates that metabolic energy expenditure in Dgat1-HF mice was higher than that of the WT-HF mice. No differences in weight were observed between WT and MCK-Dgat1 mice on chow diet (data not shown).

**DISCUSSION**

In this study, we first demonstrated in vivo that while skeletal muscle of MCK-Dgat1 mice was more resistant to developing HFD-induced lipotoxicity, their liver was as susceptible for lipotoxic insulin resistance as that of WT mice. Under hyperinsulinemic euglycemic clamp conditions, MCK-Dgat1 mice were ~45% protected against HFD-induced insulin resistance at the whole-body level compared with WT mice. The greater whole-body insulin sensitivity in HFD-fed MCK-Dgat1 mice (compared with HFD-fed WT mice) was associated with ~30% higher uptake of bolus-injected 2-deoxyglucose in skeletal muscle under the same feeding and clamp conditions, whereas insulin suppression of HGO was similarly impaired in these mice,
indicating that liver did not contribute to the improved whole-body insulin sensitivity.

We then showed that while TG synthesis and storage capacities were increased in DGAT1-overexpressing muscle, mitochondrial FA oxidation efficiency was also improved. The latter was initially recognized by decreased mitochondrial copy number in combination with the uncompromised muscle CS activity and increased maximal β-HAD and CPT-I activities in Dgat1-overexpressing muscles. This was then confirmed by increased maximal mitochondrial FA oxidation capacity per unit mitochondrial protein in Dgat1-overexpressing muscles. Interestingly, MAPR per unit mitochondrial proteins was similar in the two genotypes, suggesting that the increased FA oxidation efficiency does not lead to increased energy efficiency. Gene expression levels of muscle PDK4 and UCP3, normalized to β-actin expression levels, were ~100% greater in HFD-fed MCK-Dgat1 mice than in age-, gender-, and diet-matched WT mice. While increased PDK4 has been associated with greater fuel partition into FA oxidation (31,32), higher levels of UCP3 may also signify increased use of fat as fuel substrate and/or conversion of fat to thermal energy (33,34). It is plausible that the lack of increase in MAPR in Dgat1-overexpressing muscle, in the presence of increased FA oxidation rates, is due to increased uncoupling of oxidative phosphorylation leading to increased heat production. Although heat production was not directly measured, whole-body 24-hour VO2 production rates were greater in Dgat1-HF than in WT-HF mice in the absence of increased physical activity in the former. The increased energy expenditure in MCK-Dgat1 mice was further substantiated by the modest but significant lower body weight in the transgenic mice relative to WT mice without any difference in energy intake; long-term follow-up of the growth curve showed that MCK-Dgat1 mice weighed 5-10% less than WT mice on an at lib HFD-feeding regimen, while food consumption was the same in both groups. The reduced body weight may contribute to the improved insulin sensitivity shown in this study. However, the small weight difference appears to result from changed fuel metabolism and is unlikely a primary cause of the observed protection against lipotoxicity. Marked differences in insulin sensitivity were seen between “weight-matched” WT and MCK-Dgat1 mice, and between isolated Dgat1-muscle and WT muscle in ex vivo experiments, where weight was not a factor (3).

Insulin resistance is associated with mitochondrial dysfunction in type 2 diabetes (23,35,36) and in the offspring of parents with type 2 diabetes (37). Reduced mitochondrial function and insulin resistance occur in aging in association with reduced mitochondrial DNA copy number (23). However, the issue regarding mitochondrial deficiency and insulin resistance is unresolved (38). The present study does not support the hypothesis that reduction in mitochondrial DNA copy number per se results in insulin resistance. In our model, despite a reduction in mitochondrial number, mitochondrial ATP production is maintained (and heat production probably increased) because of increased FA oxidation efficiency. These findings suggest that mitochondrial quality is more critical. In this regard, a recent study by Koves et al demonstrated that stressed mitochondria with greater but incomplete FA oxidation are only detrimental to insulin sensitivity (39). The present study further suggests that greater FA oxidation capacity associated with increased uncoupling activity may also be important in maintaining insulin sensitivity. Of course, maintaining adequate oxidative phosphorylation is necessary for muscle contractility, particularly during exercise.

We have previously shown that skeletal muscle overexpressing Dgat1 behaved similarly to exercise-trained muscle
in that it had higher TG-content but was more insulin-sensitive, a phenomenon referred to as the “athlete paradox”, or “exercise paradox”. The decreased myocytic DAG and ceramide levels with increased myocytic TG-content are the expected effects of DGAT1 actions (3). By converting fatty acyl-CoA and DAG to TG, DGAT1 is believed to be directly responsible for lowering the levels of the lipotoxic FA derivatives. The key finding of the present study is that Dgat1-overexpressing muscle, like exercised muscle, had increased FA oxidation capacity, which was associated with increased steady-state metabolic rates at the whole body level and a smaller HFD-induced weight gain compared with WT mice. Importantly, since these findings are made in the muscle-specific Dgat1 transgenic mice, DGAT1 overexpression must be primarily responsible for these paradoxical changes. Since increased DGAT1 expression is also observed with exercise, we further postulate that DGAT1 may play a key mediatory role in exercise-induced augmentation of both TG synthesis and, indirectly, mitochondrial FA oxidation. Together, the increased anabolic (TG synthesis/storage) and catabolic (FA oxidation/degradation) capacities may both help remove FA intermediates that can be over-produced during FA overload. Reducing intramyocellular DAG levels to below the “threshold of lipotoxicity” is believed necessary to prevent the induction of a cascade of signaling events including activation of PKC isoforms and JNK1, and inhibition of IRS-1, Akt, and GLUT4 in FHD-fed mice (3).

Parallel upregulated TG synthesis and FA oxidation seemed perplexing at the first glance because anabolic and catabolic pathways usually undergo changes in the opposite directions, determined by cellular substrate levels and energy status. However, examination of typical myofibers does show interdependency between the two aspects of FA metabolism: myofibers that have a high TG-content are typically the “oxidative” type characterized by a high mitochondrial capacity, whereas those with low TG-content (and high glycogen-content) are typically “glycolytic”. Importantly, compared with glycolytic myofibers, oxidative myofibers with greater capacities for TG synthesis and FA oxidation appear to be intrinsically more insulin sensitive (9). The present study provided an example of the “paradoxical” coupling of lipid synthesis and oxidation in the Dgat1-overexpressing muscles.

Clearly, these are not the usual reciprocal regulations of the two aspects of the FA metabolism driven by the FA substrate availability. Dgat1-overexpression in skeletal muscle increased the capacities of both TG synthesis and FA oxidation and enabled the myocytes to endure fat-overload without developing significant lipotoxicity or insulin resistance. However, instead of increasing mitochondrial densities, Dgat1-overexpressing muscle appeared to have greater mitochondrial FA oxidation efficiency. Whether the improved FA oxidation is coupled with increased FA transport (as suggested by upregulation of CD36 and CPT-1) and/or potentially increased de novo FA synthesis will require further investigation. A “futile cycle” of FA metabolism which consists of increased FA oxidation coupled with increased de novo FA synthesis has been proposed as a thermogenic mechanism in muscle (40).

PDK is a key regulator of fuel partitioning between FA and glucose oxidation, and PDK4 is the major isoform in muscle (41, 42). PDK is acutely regulated, in general, by small molecular effectors generated from FA β-oxidation. Gene expression of PDK4 also responds to fuel substrate availability and insulin levels (43-46). Additionally, PDK4 gene expression appears to be modulated by several more complex biological processes, such as insulin resistance or exercise. Insulin resistance is
generally associated with upregulation of PDK4 (43,45), which may be due in part to elevated FA overload (47,48) or deficient/impaired insulin action (45,46,48). While promoting muscle insulin sensitivity, exercise also “paradoxically” upregulates PDK4 gene expression in skeletal muscle (49,50). Therefore, it appears that a different mechanism for upregulating PDK4 is involved in exercise-trained skeletal muscle and that this mechanism is not substrate-driven (49). Exercise upregulates PDK4 and of FA oxidation, but does not inhibit insulin-stimulated glucose oxidation. In the present study, we showed that Dgat1-overexpression produced a result similar to exercise with regard to PDK4 upregulation, increased FA-oxidation and insulin sensitivity, suggesting that the change in PDK4 expression in this model is also not a secondary response to reduced insulin actions. It is currently unclear, in either the exercise model or the Dgat1-overexpression model, what mediates upregulation of PDK4 gene expression and whether increased mitochondrial FA oxidation is directly caused by upregulated PDK4.

In summary, transgenic expression of DGAT1 in skeletal muscle results in a series of “paradoxical” metabolic changes often seen in the exercise-trained muscle, including increased TG synthesis, increased FA oxidation, and preserved insulin-sensitivity during the challenging of fat overload. While a high TG-content and PDK4 expression level may serve as markers of insulin resistance in sedentary and obese individuals, the same parameters may reflect a greater capacity for fat metabolism. In the latter situation, insulin-sensitivity is preserved due to reduced levels of intracellular FA derivatives. Since DGAT1 is induced in exercise, and since the MCK-Dgat1 model presents a set of metabolic features similar to the exercise model, we hypothesize that DGAT1 mediates a significant part of exercise-induced metabolic remodeling in skeletal muscle.

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muscle during recovery from exercise
Figure legends

Figure 1. Whole-body, hepatic, and muscle insulin sensitivity assessed by hyperinsulinemic euglycemic clamp. **A.** Steady-state whole-body glucose infusion rates in age-, gender-, and genetic background, and breeding environment matched wild-type mice on normal Chow diet (WT-NC), wild-type mice pretreated with ad lib HFD feeding for 8 weeks (WT-HF), and MCK-Dgat1 mice pretreated with ad lib HFD feeding for 8 weeks (Dgat1-HF). **B.** Steady-state hepatic glucose output before (basal HGO) and after (clamp HGO) insulin infusion at the rate of 3 mU/kg/min in WT-NC, WT-HF, and Dgat1-HF mice under the same clamp conditions as in A. **C.** 2-deoxyglucose (2-DG) uptake in skeletal muscle after bolus injection of the [14C]-labeled 2-DG under the same clamp conditions as in A and B. Data are presented as means +/- SE; p and n are as indicated; * denotes p<0.05.

Figure 2. Assessment of anabolic and catabolic FA metabolism in skeletal muscle of MCK-Dgat1 and control WT mice. Male mice pretreated with 8-week HFD as described in Fig 1. **A.** DGAT activity in soleus muscles isolated from the 4-month-old WT and MCK-Dgat1 (Dgat1) mice (n=4, each group). **B.** TG-content in soleus muscle from the WT (n=5) and MCK-Dgat1 (n=6) mice. **C.** Mitochondrial copy numbers as determined by PCR quantification of ND1 or ND4 as mitochondrial DNA (using β-actin as reference for nuclear DNA) in anterior tibial and soleus muscles of the WT (n=7) and MCK-Dgat1 (n=6) mice. **D.** Mitochondrial ATP production rate (MAPR) (determined in isolated mitochondria) and citrate synthase (CS) activity (measured in muscle homogenates) of the anterior tibial and soleus muscles from the WT (n=7) and MCK-Dgat1 (n=6) mice. **E.** β-HAD activity (measured in muscle homogenates) and CPT-I activity (measured in isolated mitochondria) of the soleus muscles from the WT and MCK-Dgat1 mice (n=6, each group). **F.** and **G.** Representative low- and high-power (inserts) views of the electron microscopic fields of the soleus muscle from the WT (**F**) and MCK-Dgat1 (**G**) mice. Quantification of 6000x EM micrographs using an image analysis system (Imagine-Pro Plus 5.0, Media Cybernetics, Inc.) showed that WT and Dgat1 mice have 149 +/- 3.6 and 98 +/- 7.7 mitochondria per 10x10 micron muscle area, respectively (p<0.001). The average mitochondrial size is 34432 +/- 4246 pixels for WT, and 62418 +/- 938 pixels for Dgat1 mice (p=0.014). **H.** Maximal mitochondrial FA oxidation rates measured in isolated mitochondria of the soleus muscles from the WT and MCK-Dgat1 mice (n=6, each group). Values are expressed as means +/- SE; NS, no statistical significance (p>0.05); * denotes p<0.05 and ** denotes p<0.01 (n as indicated); ND1, NADH dehydrogenases-1; ND4, NADH dehydrogenases-4; 28S, 28S ribosomal RNA.

Figure 3. Effects of Dgat1-overexpression and exercise on relative levels of gene expression in soleus muscle. **A.** Gene expression levels were measured by real-time PCR in soleus muscles isolated from WT and MCK-Dgat1 mice (Dgat1) pretreated with 8-week HFD as described in Fig 1, using primer sets listed in Table 1. Gene abbreviations are the same as listed in Table 1. *, p<0.05 (n=5-7 in each group). **B.** Relative gene expression levels of PDK4 in
soleus muscles from HFD pretreated WT versus MCK-Dgat1 mice (Dgat1). C. Relative gene expression levels of PDK4 in soleus muscles from sedentary (Sed) versus exercised (Exe) WT mice. The swimming exercise regimen is as previously described (3); p values and n are as indicated. D. Western blot analysis of CD36, UCP3, and PDK4 in soleus muscle from the 8-week HFD pretreated WT and MCK-Dgat1 mice.

**Figure 4. Physical activity and whole-body oxygen consumption.** Two-month-old male WT and MCK-Dgat1 mice were treated with 8-week HFD as in Fig 1 prior to indirect calorimetry study. Weight-matched mice were used in this study to avoid the need to correct for differences in weight and body composition across the groups (WT vs MCK-Dgat1 mice). A. Body composition by MRI. B. Locomotor activity. C. 24-hr oxygen consumption (VO₂). D. Respiration exchange ratio (RER). The above were measured during the study period while the mice had ad lib access to HFD. In a separate experiment, VO₂ was measured in the same WT and MCK-Dgat1 mice during a 24-hr fasting period (E), followed by a 24-hr period of ad lib refeeding (F). Food intake during the 24-hr period of ad lib feeding and during the 24-hr refeeding period were also measured (G). Data are expressed as means +/- SE; p values are as indicated, or denoted by “NS” for no statistical significance or “***” for p<0.01.

**Figure 5. Food consumption and growth curves.** Food intake per day was averaged weekly and plotted over time in separate groups of age-matched male WT and MCK-Dgat1 mice either on normal Chow diet (A) or on HFD (B) starting at age of 2 months. The growth curve is plotted using weekly measured body weights in WT and MCK-Dgat1 mice on HFD for 10 weeks starting at age of 2 months (C). p values are denoted by “NS” for no statistical significance, “*” for p<0.05, and “***” for p<0.01 (n as indicated).
Figure 1

(A) Whole Body

(B) Liver

(C) Muscle

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**Whole Body**

Chart showing glucose infusion rates for WT-NC (n=7), WT-HF (n=8), and Dgat1-HF (n=10).

**Liver**

Chart showing hepatic glucose output for WT-NC (n=7), WT-HF (n=8), and Dgat1-HF (n=10), comparing Basal HGO and Clamp HGO.

**Muscle**

Chart showing 2-DG uptake for WT-HF (n=9) and Dgat1-HF (n=9).
Figure 2

A. DGAT activity (soleus)

B. TG content (soleus)

C. mtDNA copy number

D. MAPR and CS

E. β-HAD and CPT-1

F. EM-mitochondria

G. EM-mitochondria

H. MLFA oxidation
Figure 3

A. Relative mRNA expression levels in soleus muscle

B. Dgat1 overexpression on Muscle PDK4 expression

C. Exercise on Muscle PDK4 expression

D. Western blot – soleus muscle

- CD36: WT 1:1.4 (WT Dgat1)
- UCP3: WT 1:1.8 (WT Dgat1)
- PDK4: WT 1:1.6 (WT Dgat1)
- GAPDH (loading control)
Figure 4

A. Body composition by MRI (G):
- WT
- DGAT
- NS

B. Beam breaks (X1000):
- WT
- DGAT
- NS

C. VO2 (mL/kg/hr) over time (hr):
- WT
- DGAT
- ad lib feeding

D. RER:
- WT
- DGAT
- NS

E. Fasting VO2 (mL/kg/hr) over time (hr):
- WT
- DGAT
- NS

F. Re-feeding VO2 (mL/kg/hr) over time (hr):
- WT
- DGAT

G. HFD Food intake (g/d/mouse) over time:
- WT
- DGAT
- Ad lib
- Re-feeding

Figure 5

A. Food intake (g/day) over time (week):
- Chow
- WT (n=5)
- Dgat1 (n=6)

B. Food intake (g/day) over time (week):
- HFD
- WT (n=4)
- Dgat1 (n=5)

C. Growth curve:
- Body weight (g) over age (week):
- WT (n=10)
- Dgat1 (n=20)