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Rosiglitazone Enhances Glucose Tolerance by Mechanisms Other than Reduction of Fatty Acid Accumulation within Skeletal Muscle

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We hypothesized that improved glucose tolerance with rosiglitazone treatment would coincide with decreased levels of im triacylglycerol (IMTG), diacylglycerol, and ceramide. Obese Zucker rats were randomly divided into two experimental groups: control (n = 9) and rosiglitazone (n = 9), with lean Zucker rats (n = 9) acting as a control group for obese controls. Rats received either vehicle or 3 mg/kg rosiglitazone for 6 wk. Glucose tolerance was impaired ($P < 0.01$) in obese compared with lean rats, but was normalized after rosiglitazone treatment. IMTG content was higher in obese compared with lean rats (70.5 ± 5.1 *vs.* 27.5 ± 2.0 μmol/g dry mass; $P < 0.05$) and increased an additional 30% ($P < 0.05$) with rosiglitazone treatment. Intramuscular fatty acid composition shifted toward a higher proportion of monounsaturates ($P < 0.05$) in obese rosiglitazone-treated rats due to an increase in palmitoleate (16:1; $P < 0.05$). Rosiglitazone treatment increased ($P < 0.05$) skeletal muscle diacylglycerol and ceramide levels by 65% and 100%, respectively, compared with obese rats, but elevated muscle diacylglycerol was not associated with changes in the total or membrane contents of the diacylglycerol-sensitive protein kinase C isoforms θ, δ, α, and β. In summary, we observed a disassociation among skeletal muscle IMTG, diacylglycerol and ceramide content, and glucose tolerance with rosiglitazone treatment in obese Zucker rats. Our data suggest, therefore, that rosiglitazone enhances glucose tolerance by mechanisms other than reduction of fatty acid accumulation within skeletal muscle. (*Endocrinology* 145: 5665–5670, 2004)

INSULIN RESISTANCE IN skeletal muscle is a characteristic feature of obesity and precedes the development of type 2 diabetes and its secondary complications (1). The direct cause of skeletal muscle insulin resistance in obese individuals remains unclear; however, accumulation of im triacylglycerol (IMTG) is strongly associated with both whole body and skeletal muscle insulin resistance (2; reviewed in Refs. 3 and 4). The molecular mechanisms linking IMTG accumulation and impaired insulin sensitivity have not been fully elucidated, but it has been proposed that IMTG acts as a marker for the presence of other, more metabolically active lipid intermediates, which are directly linked to defects in insulin signaling and may play a causative role in obesity-induced insulin resistance (5).

One such lipid intermediate is diacylglycerol, which is elevated in both genetic and diet-induced insulin resistance (6–8). Diacylglycerol is proposed to induce insulin resistance by activating diacylglycerol-sensitive protein kinase C (PKC) isoforms (9–11), which results in serine phosphorylation of insulin receptor substrate-1 (12, 13). Ceramide, which is a second messenger in the sphingomyelin signaling pathway, is also elevated in the muscle of obese, insulin-resistant humans (14, 15) and rodents (16), and its accumulation affects downstream insulin signaling by preventing insulin-induced Akt phosphorylation and activation (17, 18).

Rosiglitazone (RSG) belongs to a class of insulin-sensitizing drugs that are ligands for peroxisome proliferator-activated receptor γ (PPAR-γ). PPAR-γ is a critical transcription factor that influences numerous genes related to lipid homeostasis, suggesting that changes in lipid metabolism may mediate the therapeutic effects of RSG. In addition to improving insulin sensitivity, chronic RSG treatment results in decreased blood triglyceride (19, 20) and free fatty acid (FA) levels (21, 22) and increased FA uptake and oxidation in cultured skeletal muscle (23). Because there is strong evidence for the role of several lipid species in the development of skeletal muscle insulin resistance, and PPAR-γ antagonism results in altered lipid homeostasis, the primary aim of the current investigation was to determine the effects of RSG on the skeletal muscle lipid profile.

In this regard, previous studies examining IMTG content after chronic RSG treatment have reported increased (19), decreased (28–30), and unchanged (28, 31) levels. The changes observed in IMTG content with RSG treatment may result from alterations in regulatory enzymes of lipid turnover such as hormone-sensitive lipase (HSL) and glycerol-3-phosphate acyltransferase (GPAT). However, the effect of

Abbreviations: CON, Control; FA, free fatty acid; GPAT, glycerol-3-phosphate acyltransferase; HSL, hormone-sensitive lipase; IMTG, im triacylglycerol; LN, lean; MUFA, monounsaturated fatty acid; OB, obese; PKC, protein kinase C; PPAR-γ, peroxisome proliferator-activated receptor γ; RSG, rosiglitazone.

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RSG treatment on the protein content and activities of these regulatory enzymes are unresolved.

The present investigation examined the effect of chronic RSG treatment on glucose tolerance, the skeletal muscle content of lipid metabolites, and the activities of lipid regulatory enzymes in a rodent model of insulin resistance. We hypothesized that muscle lipids would be elevated in obesity, and that improved glucose tolerance after RSG treatment would coincide with decreased levels of IMTG, diacylglycerol, and ceramide.

Materials and Methods

Animals

Female obese Zucker (*fa/fa*) and age-matched lean (*fa/?*) rats were obtained from Monash Animal Services (Monash, Australia) at 19 wk of age. Rats were housed under controlled light (12-h light, 12-h dark) at an ambient temperature of 21 C. Animals had free access to standard rat chow and water except when overnight fasting was required for blood measurements. All procedures were approved by the RMIT University animal ethics committee. The obese rats were randomly divided into two experimental groups: control (OB CON; n = 9) and RSG (OB RSG; n = 9). The lean rats were treated with vehicle (LN CON; n = 9) and acted as a CON group for OB CON. Rats were treated daily for 6 wk by oral gavage with either vehicle, which consisted of 0.5% carboxymethylcellulose (100 μl/100 g body mass) or 3 mg/kg RSG (GlaxoSmithKline, Worthing, West Sussex, UK) suspended in an equal volume of carboxymethylcellulose.

Oral glucose tolerance test and surgery

After the 6-wk experimental period, rats were overnight fasted, weighed, and underwent an oral glucose tolerance test. To assess oral glucose tolerance, glucose (70% wt/vol, solution) was administered by oral gavage at a dose of 2.5 g/kg body weight, and tail blood was obtained at −1, 10, 15, 30, 60, and 90 min after administration of the glucose load. Three days later, rats were euthanized by CO_2 asphyxia, followed by exsanguination. The soleus and red gastrocnemius muscles were rapidly dissected from the rat hindlimbs, snap-frozen in liquid nitrogen, and stored at −80 C for later analysis.

Analytical procedures

Blood metabolite measurements. Blood was analyzed for fasting concentrations of glucose, insulin, triglyceride, and cholesterol. Plasma was obtained by centrifugation of whole blood collected at the time of euthanasia and stored at −80 C until analysis. Blood glucose levels were monitored with the MediSense2 Blood Glucose Testing System (MediSense Australia Pty. Ltd., Melbourne, Victoria, Australia). Plasma insulin levels were determined using an enzyme immunoassay kit (Ultrasensitive Rat Insulin ELISA, Mercodia AB, Uppsala, Sweden), and plasma triglycerides and total cholesterol were measured using the enzymatic assay kits GPO-PAP and CHOD-PAP (Roche, Basel, Switzerland), respectively.

Analysis of muscle lipids. IMTG content was analyzed as previously described (32). Freeze-dried muscle was powdered and cleaned of all visible connective tissue and blood under magnification. Lipid was extracted by a Folch extraction (33), the triacylglycerol was saponified in an ethanol/potassium hydroxide solution at 60 C, and glycerol content was determined fluorometrically.

The FA composition of muscle triacylglycerols was determined on a separate portion of powdered skeletal muscle. Skeletal muscle lipids were extracted (33), and triacylglycerols were separated from phospholipids by solid phase extraction on Sep-Pak silica cartridges (Waters Division, Millipore Corp., Bedford, MA) as described by Pan and Storlien (34). Triacylglycerol fractions were transmethylated with 14% (wt/vol) boron trifluoride in methanol, and FA methyl esters were separated by gas-liquid chromatography on a HewlettPackard 5890 series II gas chromatograph (HewlettPackard, Palo Alto, CA) with a fused silica capillary column. Individual FA were identified by comparing each peak's retention time to those of external standards and are expressed as the molar percentage of total FA. The five major FA in the triglyceride pool (16:0, 16:1, 18:0, 18:1, and 18:2), which represent approximately 95% of total muscle FA, are presented.

Diacylglycerol and ceramide were extracted and quantified according to the methods described by Preiss *et al.* (35). Briefly, lipids were extracted from freeze-dried, powdered soleus muscle using chloroform/methanol/PBS and 0.2% sodium dodecyl sulfate (1:2:0.8). Diacylglycerol kinase and [γ-^{32}P]ATP (15 μCi/μmol cold ATP) were added to extracts, and the reaction was stopped using chloroform/methanol (2:1). Samples were spotted onto thin layer chromatography plates and developed to two thirds of the total plate length. Bands corresponding to diacylglycerol and ceramide were identified against standards after phosphorimaging, dried, scraped from the thin layer chromatography plate, and counted in a liquid scintillation analyzer (Tri-Carb 2500TR, Packard, Canberra, Australia).

Analysis of HSL activity. An aliquot of freeze-dried muscle was used to determine HSL activity as described previously (36). Briefly, the powdered muscle was homogenized, and after centrifugation the supernatant was removed and analyzed for HSL activity against a triolein substrate. All measurements were made in triplicate, and the mean of these values is reported.

Analysis of GPAT activity. Skeletal muscle GPAT activity was determined as described by Muoio *et al.* (37). After homogenization, total GPAT activity was measured with 900 mM [^{3}H]glycerol-3-phosphate (ARC, St. Louis, MO) and 90 mM palmitoyl coenzyme A. The reaction was run for 20 min at 37 C and stopped with 1% $HClO_4$ and chloroform/methanol (2:1). After a series of washes with $HClO_4$, 1 ml of the organic phase containing the labeled glycerol-3-phosphate incorporated into lysophosphatidic acid was dried, and 4 ml scintillation fluid were added. Radioactivity was determined with a liquid scintillation analyzer.

Subcellular fractionation and Western blotting protocol. Soleus muscle was homogenized in 8× (wt/vol) ice-cold buffer containing 20 mM HEPES (pH 7.4), 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM Na_3VO_4, 10% glycerol, 3 mM benzamidine, 10 μM leupeptin, 5 μM pepstatin A, and 1 mM phenylmethylsulfonylfluoride. The homogenate was centrifuged at 100,000 × *g* for 30 min at 4 C, and the supernatant was collected as the cytosolic fraction. The pellet was resuspended by agitation in 4× (wt/vol) ice-cold homogenization buffer to which 1% Triton X was added. The resuspended pellet was then centrifuged at 15,000 × *g* for 10 min at 4 C. The supernatant, representing the total membrane fraction, was collected. Both the membrane and cytosolic fractions were stored at −80 C. A separate portion of soleus muscle was homogenized, centrifuged at 16,000 × *g* for 60 min, and later analyzed for total cellular HSL protein.

The protein concentration of the muscle lysates was determined (Pierce Chemical Co., Rockford, IL). Muscle lysates (100 μg) from both fractions were solubilized in Laemmli buffer and separated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane by electrophoresis, and the membranes were blocked, then incubated overnight at 4 C with antibodies specific for HSL (provided by Fredric Kraemer, Stanford University, CA) or for PKCθ, -α/β, or -δ isoforms (1:1000; Cell Signaling, Beverley, MA). The immunoreactive proteins were detected with enhanced chemiluminescence (PerkinElmer, Rowville, Australia) and quantified by densitometry.

Statistical analysis

Results are presented as the mean ± SE. All statistical analyses were performed using the unpaired *t* test. $P < 0.05$ was considered significant.

Results

OB CON rats at 25 wk of age had a greater body mass and elevated fasting plasma insulin, triglyceride, and cholesterol levels compared with LN CON rats (Table 1; $P < 0.05$). RSG treatment in OB rats resulted in additional increases in body mass and plasma cholesterol, but lower plasma insulin and triglyceride levels compared with OB CON rats (Table 1; $P <$ 0.05). Compared with LN rats, OB rats had elevated ($P <$

0.05) plasma glucose levels throughout the oral glucose challenge (Fig. 1). The area under the curve during the oral glucose challenge averaged 123 ± 3 and 152 ± 8 mM for LN and OB CON rats ($P < 0.05$), respectively. RSG treatment improved glucose tolerance in OB rats, as indicated by a reduced area under the curve (126 ± 4 mM; $P < 0.05$; Fig. 1).

IMTG content in soleus muscle was higher in OB compared with LN rats (70.5 ± 5.1 *vs.* 27.5 ± 2.0 μmol/g dm; $P < 0.001$), and increased an additional 30% ($P = 0.04$) after RSG treatment (Fig. 2). Soleus muscle diacylglycerol and ceramide levels were not elevated when comparing LN with OB CON rats, but were increased by 65% ($P < 0.001$) and 100% ($P = 0.02$), respectively, after RSG treatment (Fig. 2). IMTG content in a glycolytic muscle, the extensor digitorum longus, was also increased with obesity (LN CON, 9.3 ± 2.4; OB CON, 35.2 ± 8.4; $P < 0.05$), but was not increased after RSG treatment.

To determine the potential mechanism(s) mediating the changes in IMTG and diacylglycerol levels with RSG treatment, the basal activities of HSL and GPAT were assessed. There was no difference in HSL activity between LN and OB CON rats, whereas HSL activity was decreased by about 30% after RSG treatment in OB rats (Fig. 3A; $P = 0.02$). The decreased HSL activity in OB RSG was not associated with changes in total HSL protein as determined by Western blot analysis (Fig. 3, B and C). Skeletal muscle GPAT activity was not altered by obesity or RSG treatment (Fig. 3D).

Diacylglycerol is known to translocate PKC from the cytosolic to the membrane fraction resulting in its activation, and PKC has been implicated in the insulin resistance of skeletal muscle (10, 11). Accordingly, we measured the cytosolic and membrane fraction protein contents of several diacylglycerol-sensitive PKC isoforms. There was no change in total PKCθ, -α/β, or -δ protein or in the membrane fractions of these PKC isoforms with obesity or RSG treatment (Fig. 4 and Table 2).

Increased skeletal muscle lipid content with obesity and RSG were associated with changes in FA composition (Fig. 5). The proportion of monounsaturated FA (MUFA) was elevated in OB (38.0 ± 0.8%) compared with LN (29.1 ± 0.8%; $P < 0.0001$) rats and was also elevated by RSG (43.7 ± 2.5%; $P = 0.05$). The elevation in percent MUFA observed in OB rats was due to specific increases in palmitoleate [16:1(n-7); $P < 0.0001$] and oleate [18:1(n-9)] ($P < 0.0001$) FA, in conjunction with decreases in the proportions of stearate (18:0; $P < 0.0001$) and linoleate [18:2(n-6); $P < 0.0001$]. The additional increase in percent MUFA observed after RSG treatment was solely due to an increase in 16:1(n-7) (Fig. 5; $P = 0.02$).

TABLE 1. Body mass and plasma characteristics of LN CON, OB CON, and OB RSG rats

	LN CON	OB CON	OB RSG
Mass (g)	211 ± 7.6	388 ± 9.4[a]	444 ± 13.3[b]
Plasma			
Glucose (mM)	6.3 ± 0.5	7.1 ± 0.5	6.4 ± 0.3
Insulin (pM)	62 ± 18	986 ± 291[a]	103 ± 49[b]
Triglyceride (mM)	0.42 ± 0.07	2.93 ± 0.64[a]	1.15 ± 0.19[b]
Cholesterol (mM)	1.13 ± 0.06	1.57 ± 0.05[a]	1.84 ± 0.11[b]

[a] $P < 0.05$, LN CON *vs.* OB CON.
[b] $P < 0.05$, OB CON *vs.* OB RSG.

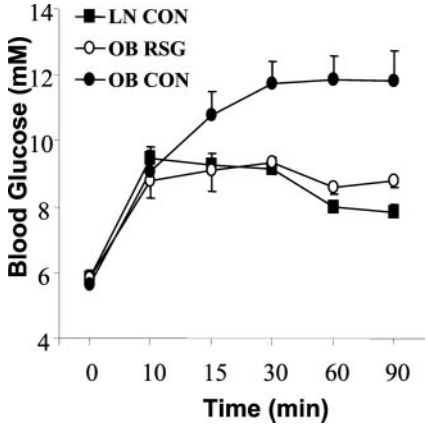

FIG. 1. Oral glucose tolerance test. Glucose was administered by oral gavage at a dose of 2.5 g/kg body weight, and blood glucose was measured for 90 min. *, $P < 0.01$, OB CON *vs.* OB RSG.

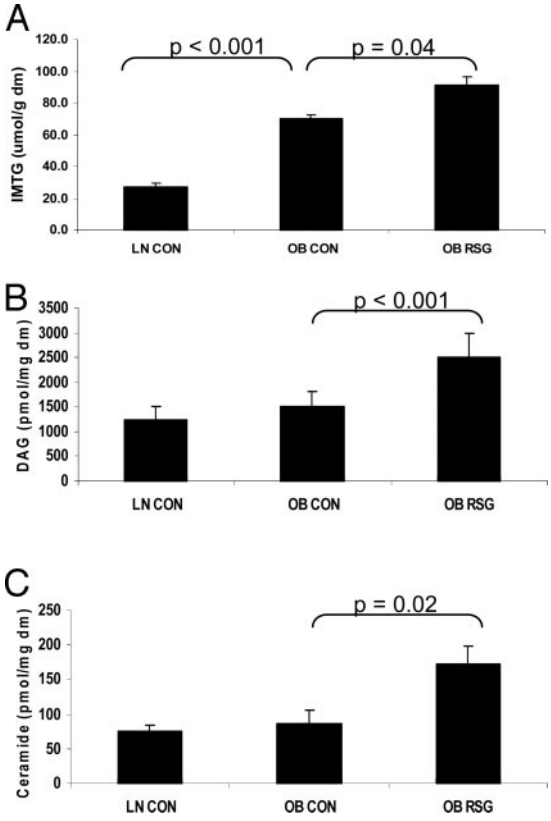

FIG. 2. Intramyocellular lipids. Changes in skeletal muscle IMTG (A), diacylglycerol (B), and ceramide (C) with obesity (LN CON *vs.* OB CON), and RSG treatment (OB CON *vs.* OB RSG). Significant differences ($P < 0.05$) between groups are indicated by the P values listed on the figure.

Discussion

The novel finding of the current study was that despite a normalization of glucose tolerance, 6 wk of RSG treatment increased IMTG (1.3-fold), diacylglycerol (1.7-fold), and ceramide (2-fold) in the skeletal muscle of OB, insulin-resistant rats. Our results suggest, therefore, that the improvements in

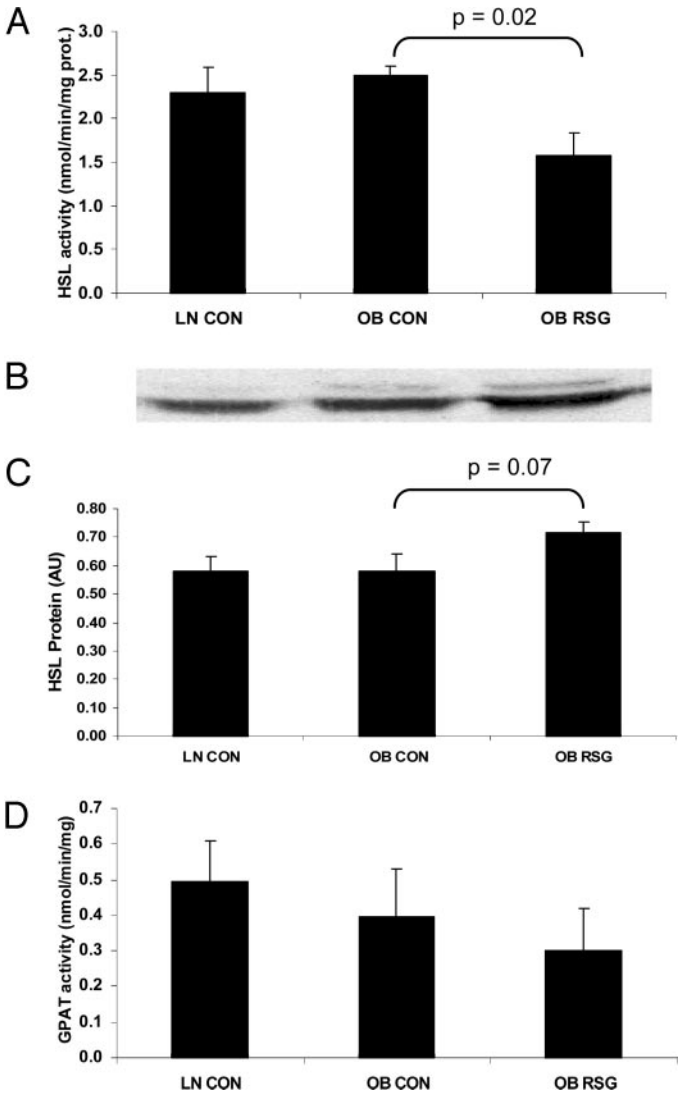

FIG. 3. Activity and protein levels of lipid regulatory enzymes. A, HSL activity with obesity (LN CON *vs.* OB CON, not significant) and RSG treatment (OB CON *vs.* OB RSG, $P = 0.02$). B, A representative Western blot of total HSL protein. C, Mean values [arbitrary units (AU)] ± SEM of HSL protein with obesity (LN CON *vs.* OB CON, not significant) and RSG treatment (not significant). D, GPAT activity with obesity (LN CON *vs.* OB CON, not significant) and RSG treatment (OB CON *vs.* OB RSG, not significant).

glucose tolerance seen with RSG treatment are not mediated by reductions in the total content of these muscle lipids. Furthermore, increased diacylglycerol was not associated with increased membrane-associated PKCθ, -α/β, or -δ content (Fig. 4 and Table 2). Because diacylglycerol-induced insulin resistance in skeletal muscle is thought to be mediated by PKC translocation to the sarcolemma (9–11), this provides additional evidence that the insulin-sensitizing effects of RSG in skeletal muscle are not lipid mediated.

It is important to note that the improved glucose tolerance with RSG observed in the current study was not necessarily due to increased skeletal muscle glucose uptake and may involve increased hepatic or adipose glucose uptake, decreased gluconeogenesis, and/or increased insulin secretion during the glucose challenge. However, direct measurement

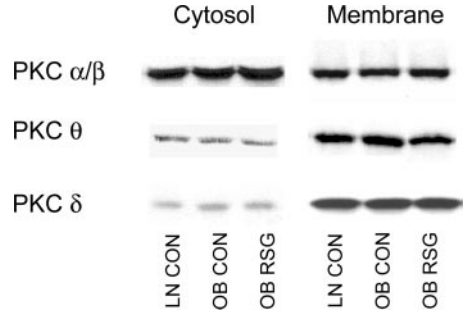

FIG. 4. PKC distribution. Representative immunoblots of PKCθ, -δ, and -α/β Soleus muscles from LN CON, OB CON, and OB RSG rats were homogenized, and the cytosolic and membrane fractions were separated using subcellular fractionation (see *Materials and Methods*). Proteins were quantified by Western blot analysis and densitometry.

TABLE 2. Membrane/total protein ratio of diacylglycerol-sensitive PKC isoforms from LN CON, OB CON, and OB RSG rats

	LN CON	OB CON	OB RSG
PKCα/β	0.31 ± 0.05	0.35 ± 0.04	0.38 ± 0.04
PKCθ	0.90 ± 0.01	0.95 ± 0.02	0.93 ± 0.02
PKCδ	0.76 ± 0.02	0.80 ± 0.01	0.82 ± 0.03

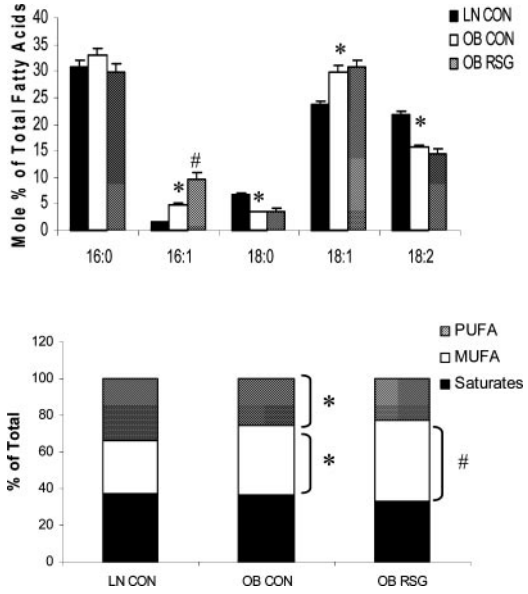

FIG. 5. FA composition of muscle triacylglycerols. A, Molar percentage of total FA for specific fatty acyl species (palmitate 16:0, palmitoleate 16:1, stearate 18:0, oleate 18:1, linoleate 18:2). B, Proportion of polyunsaturated species (PUFA), monounsaturated species (MUFA), and saturates within the muscle lipid pool. *, $P < 0.05$, LN CON *vs.* OB CON; #, $P < 0.05$, OB CON *vs.* OB RSG.

of insulin-stimulated glucose uptake after chronic RSG treatment has previously shown an increase in skeletal muscle glucose transport despite increased IMTG content (19).

The effect of RSG treatment on skeletal muscle lipid storage is unclear, because previous investigations have reported a decrease (20, 24, 28–30), an increase (19), or no effect (28, 31) on IMTG content. Most studies demonstrating decreased IMTG content with RSG have analyzed muscle with a large proportion of type II fibers (20, 29, 30). In contrast, we chose to analyze muscle with predominantly (>90%) type I com-

position because type I muscle fibers contain more ectopic lipids (38), have a greater role in lipid utilization (39), and are more insulin sensitive than type II fibers (40). The conflicting reports among laboratories analyzing different muscle groups present the possibility that RSG action may be fiber type dependent. To test this, we also analyzed the IMTG content of a more highly glycolytic muscle (extensor digitorum longus; >50% type IIB fibers) and found that there was no increase in IMTG with RSG treatment. This suggests that oxidative fibers may be more responsive to RSG treatment than glycolytic fibers. This also raises the possibility of contamination by extramyocellular adipocytes when using chemical extraction to measure IMTG, and a previous study using ^{1}H-nuclear magnetic resonance spectroscopy demonstrated increased extramyocellular lipid content in human skeletal muscle after RSG treatment (31). However, contamination of a muscle sample by extramyocellular adipocytes can be avoided if a thorough removal of adipose tissue under magnification is performed (27). We are confident that our samples were not contaminated with extramyocellular adipocytes, because thorough microsdissection was performed. Furthermore, HSL activity, which was measured using the same aliquot of freeze-dried tissue as IMTG, was decreased in OB RSG-treated rats. If there was significant adipocyte contamination, one would expect HSL activity to be elevated because of its high expression in adipocytes compared with myocytes. Similarly, GPAT is also highly expressed in adipose tissue and was not different between treatment groups.

The decrease in HSL activity (Fig. 3) is a potential mechanism for increased IMTG and diacylglycerol after RSG treatment. HSL is a regulatory enzyme for skeletal muscle IMTG degradation (41), and RSG treatment caused a significant decrease in HSL activity that was not associated with changes in total protein content. We also examined GPAT activity, because it is the first committed step in glycerolipid synthesis, and found that GPAT activity was similar in all groups and was unaffected by RSG. Taken together, these data are consistent with the increased IMTG observed after RSG treatment. HSL is also an important diacylglycerol lipase, as demonstrated by diacylglycerol accumulation in $HSL^{-/-}$ mice (42). The down-regulation of HSL in the present study is also likely to be responsible for diacylglycerol accretion after RSG treatment.

Our consistent observation of increased lipid levels with RSG may also be due to altered FA transport and oxidation. RSG increases sarcolemmal FA transport in primary skeletal muscle cell culture, which is associated with increased FA translocase expression (23). Such increases in sarcolemmal FA transport, if present during chronic RSG treatment, would probably result in skeletal muscle lipid deposition if they were not matched by similar increases in FA oxidation. RSG does not appear to alter whole body FA oxidation (31); however, other PPAR-γ agonists (*e.g.* troglitazone) have been reported to increase (25, 43) or cause no change (44) in skeletal muscle FA oxidation in isolated preparations. The effect of RSG on skeletal muscle FA oxidation has yet to be examined and may provide additional insight into the observed changes in lipid levels after RSG treatment.

Elevated intramyocellular lipid levels in conjunction with increased insulin sensitivity appear counterintuitive given the abundance of evidence linking lipid accumulation and insulin resistance. However, the composition of im lipid stores may provide a protective effect against insulin resistance in the face of increased lipid levels. Because skeletal muscle insulin resistance has been directly correlated with the proportion of saturated FA present in the triacylglycerol pool (45), we investigated whether changes in specific FA species occur in the muscle lipid pool with RSG treatment. Although there was no decrease in total saturated species with RSG, we observed a shift toward a higher proportion of MUFA, which was specifically due to an increase in palmitoleate [16:1 (n-7)]. Incubation of C2C12 myocytes with 16:1(n-7) had no deleterious effect on insulin-stimulated Akt phosphorylation (18); however, incubation with the saturated species palmitate (16:0) and oleate (18:0) resulted in the incorporation of these FA into ceramide (18) and diacylglycerol (9, 18) and a decrease in insulin sensitivity. Therefore, a shift toward the storage of monounsaturated species may provide protection from ceramide- and diacylglycerol-induced insulin resistance. Additional investigation of the effect of RSG treatment on the incorporation of FA into specific diacylglycerol and ceramide molecular species are warranted.

In summary, we found that improved glucose tolerance after 6 wk of RSG treatment was associated with increased skeletal muscle triacylglycerol, diacylglycerol, and ceramide content. RSG-induced increases in lipid levels were associated with decreased HSL activity and changes in the FA composition of muscle lipids. Our results suggest that reducing im lipid accumulation is not the mechanism by which RSG improves insulin sensitivity.

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