Effects of Retinoids on Glucose Transport and Insulin-mediated Signaling in Skeletal Muscles of Diabetic (db/db) Mice*

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Rexinoids and thiazolidinediones (TZDs) are two classes of nuclear receptor ligands that induce insulin sensitization in diabetic rodents. TZDs are peroxisome proliferator-activated receptor γ (PPARγ) activators, whereas rexinoids are selective ligands for the retinoid X receptors (RXRs). Activation of both the insulin receptor substrates (IRSs)/Akt and the c-Cbl-associated protein (CAP)/c-Cbl pathways are important in regulating insulin-stimulated glucose transport. We have compared the effects of a rexinoid (LG268) and a TZD (rosiglitazone) on these two signal pathways in skeletal muscle of diabetic (db/db) mice. The results we have obtained show that treatment of db/db mice with either LG268 or rosiglitazone for 2 weeks results in a significant increase in insulin-stimulated glucose transport activity in skeletal muscle. Treatment with LG268 increases insulin-stimulated IRS-1 tyrosine phosphorylation and Akt phosphorylation in skeletal muscle without affecting the activity of the CAP/c-Cbl pathway. In contrast, rosiglitazone increases the levels of CAP expression and insulin-stimulated c-Cbl phosphorylation without affecting the activity of IRS-1/Akt pathway. The effects of LG268 on the IRS-1/Akt pathway were associated with a decrease in the level of IRS-1 Ser307 phosphorylation. Taken together, these data suggest that rexinoids improve insulin sensitivity via changes in skeletal muscle metabolism that are distinct from those induced by TZDs. Rexinoids represent a novel class of insulin sensitizers with potential applications in the treatment of insulin resistance.

Activation of either peroxisome proliferator-activated receptor γ (PPARγ)1 or retinoid X receptors (RXRs) has been shown to lower serum glucose in rodent models of type 2 diabetes (1). The thiazolidinediones (TZDs) such as rosiglitazone and pioglitazone are PPARγ ligands. The mechanisms that contribute to the insulin-sensitizing activity of the TZDs have been extensively characterized. PPARγ is expressed abundantly in adipose tissue (2, 3), and TZDs activate gene expression in adipose tissue, altering fatty acid metabolism and lowering circulating levels of free fatty acids in ways that are thought to lead to increased skeletal muscle insulin sensitivity (4, 5). In addition, TZDs have been shown to increase the production of insulin-sensitizing adipokines such as adiponectin and to suppress the circulating levels and/or the activity of insulin resistance-triggering adipokines such as tumor necrosis factor-α (TNF-α) and resistin. These alterations in adipokine expression may also contribute to skeletal muscle insulin sensitization (6–8). There is controversy as to the extent to which TZDs may also have direct effects on skeletal muscle metabolism. Studies in several experimental models, including “fat free” mice, have suggested that part of the insulin-sensitizing activity of TZDs is likely to be due to their direct effects on muscle gene expression (9–12).

In contrast to the TZDs, much less is known about the cellular and molecular basis for the insulin-sensitizing activity of RXR agonists (rexinoids). Rexinoids are a class of nuclear receptor ligands that are structurally and functionally distinct from the TZDs. Because the RXRs serve as the obligate partners in a large number of nuclear receptor heterodimers, including the PPARs (13), it has been suggested that the insulin-sensitizing activity of rexinoids could be due to the activation of RXR/PPARγ heterodimers. In this model, rexinoids would function as “TZD-mimetics,” activating the same RXR/PPARγ heterodimers as the TZDs, inducing similar alterations in gene expression and producing comparable pharmacological effects. However, a direct comparison of the effects of rexinoids and TZDs on gene expression in diabetic animals has revealed marked differences in the patterns of gene expression produced by these two classes of nuclear receptor ligands. Although TZDs have their most marked effects on gene expression in adipose tissue, the primary effects of rexinoids involve alterations in gene expression in liver and skeletal muscle (14). These results suggest that rexinoids and TZDs might produce insulin sensitization via distinct alterations in insulin-mediated metabolic signaling.

Skeletal muscle accounts for the majority of insulin-regulated whole body glucose disposal. Glucose transport is the rate-limiting step in this process (15). Insulin promotes glucose transport by activating at least two distinct intracellular signal transduction pathways, the insulin receptor (IR)/insulin receptor substrate (IRS)/phosphatidylinositol 3-kinase (PI3K)/Akt pathway, and the IR/c-Cbl-associated protein (CAP)/c-Cbl pathway (16). The present study was undertaken to compare the effects of a prototypic rexinoid, LG268, and a prototypic TZD, rosiglitazone, on these two signaling pathways in the muscles...
of diabetic (db/db) mice. The results we have obtained show that the treatment of db/db mice with rexinoids or TZDs for 2 weeks resulted in a significant increase in insulin-stimulated glucose transport activity in skeletal muscle. Rexinoids increased the activity of components of the IRS-1/Akt pathway without alterations in the CAP/c-Cbl pathway. TZDs, on the other hand, increased the levels of expression and activity of components of the CAP/c-Cbl pathway without significant effects on the IRS-1/Akt pathway. The effects of rexinoids on the IRS-1/Akt pathway were associated with a decrease in the level of IRS-1 Ser^307 phosphorylation as well as qualitative and quantitative alterations in the levels of intramyocellular fatty acyl-CoAs. Taken together, these findings suggest that rexinoids produce insulin sensitization via changes in muscle glucose c/o and/or lipid metabolism that are distinct from those induced by the TZDs. Rexinoids appear to represent a novel class of insulin-sensitizing agents with potential applications in the treatment of insulin-resistant states.

EXPERIMENTAL PROCEDURES

MATERIALS—Insulin and bovine serum albumin (BSA, RIA grade) were purchased from Sigma (St. Louis, MO). Complete protease inhibitor mixture tablets were purchased from Roche Diagnostics (Mannheim, Germany). The BCA protein assay kit was from Pierce (Rockford, IL). Protein A-Sepharose (4 fast flow) was from Amersham Biosciences (Uppsala, Sweden). Antibodies against CAP, c-Cbl, IR β subunit, and IRS-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal anti-phospho-rsine (clone 4G10), rabbit anti-phospho-IRS-1 (Ser^307) antisemur and rabbit anti-PTEN antisemur were from Upstate Biotechnology Inc. (Lake Placid, NY); antibodies against Akt, phospho-Akt (Ser^473), phospho-44/42 MAPK, and phospho-p44/42 MAPK (Thr^202/Tyr^204) were obtained from New England Biolabs (Beverly, MA); mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase was from Integrated DNA Technologies, Inc. (Coralville, IA). Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-goat secondary antibodies were from Bio-Rad Laboratories (Hercules, CA); HRP-labeled anti-rabbit secondary antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA), and the enhanced chemiluminescence (ECL) detection system was from Amersham Biosciences (Little Chalfont Buckinghamshire, England).

Animals and Drug Administration—5-week-old male db/db mice and lean littermates (Jackson Laboratories, Bar Harbor, ME) were housed in plastic cages on a 12:12 light/dark cycle with free access to water and food (Purina 5008). After 2 weeks of acclimation, animals were weighed, bled via the tail vein, and assigned to treatment groups based on starting glucose values (first criterion) and initial body weights (second criterion). Mice were gavaged once daily with vehicle, LG268 (30 mg/kg), or rexinoid solution containing 4 mg/mL LG268 before each meal. On day 15, animals were weighed, blood was collected in the fed state 3 h after dosing, and serum glucose and triglyceride levels were measured using the glucose oxidase Trinder protocol and Sigma triglyceride (GPO-Trinder) reagents. At the end of the experiments, animals were sacrificed by cervical dislocation under isoflurane anesthesia, and skeletal muscles (soleus, white gastrocnemius, and quadriceps) were rapidly excised, flash frozen in liquid nitrogen, and stored at −80 °C for RNA and protein analysis and determination of intramyocellular long chain fatty acyl-CoAs. For insulin signaling studies, paired extensor digitorum longus (EDL) and soleus muscles were used for ex vivo incubation.

Long Chain Fatty Acyl-CoA Measurement—Skeletal muscles (soleus and gastrocnemius) were assayed to determine the intramyocellular content of LCFAs using mass spectrometry as previously described (17, 18). Briefly, after removing visible adipose and connective tissues, the muscle samples (~50 mg) were frozen in liquid nitrogen and stored at −80 °C until analyzed. LCFAs were extracted from samples by solid-phase extraction and C^17 CoA was added as an internal standard. A tandem mass spectrometer (API 3000, PerkinElmer Life Sciences) interfaced with a Turbolonspray ionization source was used for mass spectrometry/mass spectrometry analysis. LCFA-CoAs were ionized in a negative electrospray mode, and the transition pairs [M−H]^-/(M-H-80)^- were monitored in multiple reaction-monitoring mode. Doubly charged ions and corresponding product ions (precursor minus phosphate group) were selected as a transition pair for multiple reaction monitoring for quantitation.

RNA Isolation and Real-time Quantitative Reverse Transcription and PCR—Total RNA extraction and real-time qRT-PCR were performed as described previously (14). Briefly, muscles were homogenized in Tri-reagent ( Molecular Research Center, Inc., Cincinnati, OH) with a Polytron, precipitated with isopropanol, and the homogenates were applied to RNAeasy spin columns (Qiagen Inc., Valencia, CA). RNA was eluted and then treated with DNase (Roche Diagnostics GmbH, Mannheim, Germany) for 30 min at 37 °C, followed by heat inactivation at 75 °C for 10 min, and storage at −80 °C. 10-ng aliquots of total RNA were reverse-transcribed in triplicate using 10 units of Superscript II (Invitrogen, Gaithersburg, MD) in the presence of 1 × RT buffer (Invitrogen), 500 nm reverse primer, 10 nm dithiothreitol, 500 μM dNTPs, and 4 μM MgCl_2 at 50 °C for 30 min, followed by 72 °C for 5 min. An additional “no-RT” sample without reverse transcriptase was run in parallel as a control. The reverse transcription reaction (10 μl) was added to 40-μl PCR mix containing 1 × PCR buffer (Invitrogen), 300 nM forward and reverse primers, 100 μM fluorogenic probe, 1.25 unit of Taq polymerase (Roche Applied Science, Indianapolis, IN), 500 μM dNTPs and 4 μM MgCl_2. Real-time PCR was carried out by amplification of samples in 96-well plates in an ABI Prism 7700 (Applied Biosystems, Norwalk, CT) at 95 °C for 1 min, followed by 40 cycles of 95 °C for 12 s and 60 °C for 1 min. Data were analyzed by the use of the Sequence Detection Application software, and the absolute values of transcripts were generated by interpolating the C_{T} value (number of cycles required to reach the threshold) of an unknown sample on a standard curve of a series of dilutions of a known amount of a synthetic amplicon (single-stranded DNA) run in parallel on the same 96-well plate. All values were corrected for RNA input by normalization to the level of cyclophilin mRNA. For each transcript, specific PCR primer pairs and a dual fluorochrome-labeled hybridization probe (TaqMan probe) were designed using ABI Primer Express software (Table I) and synthesized by Integrated DNA Technologies, Inc. (Corvalle, IA).

Ex Vivo Muscle Incubation—Intact soleus and EDL muscles were rapidly dissected from both hindlimbs, and at the end of each muscle were tied with suture before mounting on stainless steel clips to maintain consistent resting muscle length and tension throughout the incubation. The incubation solution was performed in stopped flasks gassed with 5% CO_2 and 95% O_2 in a shaking water bath at 30 °C. Muscles were first preincubated in 3 ml of oxygenated Krebs-Henseleit bicarbonate buffer (KHB, 104 mM NaCl, 22 mM NaHCO_3, 4 mM KCl, 1.1 mM CaCl_2, 1.2 mM KH_2PO_4, 1 mM MgSO_4, and 6.7 mM Hepes, pH 7.4) supplemented with 5.5 mM glucose and 1% BSA (RIA grade). After 40 min, the preincubation solution was exchanged with the incubation solution, and the muscles were incubated for 20 min in 3 ml of incubation buffer in the presence or absence of 10^4 micromolar/mL insulin. The incubation was terminated by immediately freezing the muscles in liquid nitrogen. Frozen muscles were then placed into ice-cold lysis buffer consisting of 20 mM Tris, pH 8.0, 135 mM

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**Table I**

| Assay                             | Primers (5'-3') | TaqMan probe (5'-3') | Accession no. |
|-----------------------------------|-----------------|----------------------|---------------|
| Cyclophilin                        |                 |                      |               |
| CAP                               |                 |                      |               |
| Insulin receptor                   |                 |                      |               |
| PTEN                              |                 |                      |               |
| SHIP2                             |                 |                      |               |
| Liver and muscle mass measurements |                 |                      |               |

*5' 'F, reporter dye FAM (6-carboxyfluorescein); T, quencher dye TAMRA (6-carboxytetramethylrhodamine).
Rexinoids, Insulin Signaling, and Skeletal Muscle

TABLE II

| Body weight | Serum glucose | Serum triglyceride |
|-------------|---------------|-------------------|
| g           | mg/dl         |                   |
| Db           |              |                   |
| Vehicle     | 31.8 ± 1.9°  | 370 ± 119°        |
| LG268       | 39.9 ± 2.7   | 531 ± 108°        |
| ROS         | 43.2 ± 4.0°  | 250 ± 64°         |
| Lean         |              |                   |
| Vehicle     | 22.3 ± 2.0   | 129 ± 14          |
| LG268       | 24.2 ± 2.2   | 122 ± 14          |
| ROS         | 25.0 ± 1.6   | 166 ± 22          |

*p < 0.05 versus untreated lean mice.  
* p < 0.05 versus vehicle-treated db/db mice using Student’s t test.  
* ND, not determined.

NaCl, 2.7 mm KCl, 1 mm MgCl₂, 1% Triton X-100, 10% glycerol, 1× complete protease inhibitor mixture, 10 mM NaF, and 5 mM Na₂VO₄. The muscles were homogenized using four 10-s bursts of a Polytron at maximum speed. The homogenates were allowed to sit on ice for 30 min, followed by centrifugation at 14,000 × g for 20 min. The supernatants were collected, proteins were determined using the BCA protein assay kit, and aliquots were stored at −80 °C until analyzed.

Immunoprecipitation and Western Blotting Procedures—For detection of tyrosine phosphorylation of IR, IRS-1, and c-Blk, muscle lists containing equal amounts of protein were subjected to immunoprecipitation for 1 h (IR) or overnight (IRS-1 and c-Blk) at 4 °C with rabbit polyclonal anti-IR-β, anti-IRS-1, or anti-c-Blk antibodies in a final volume of 160 μl. 100 μl of 20% protein A-Sepharose slurry was added to the immunoprecipitation reaction, and incubation was continued for another 1 h at 4 °C with rotation. Immune complexes bound to Sepharose were collected by brief centrifugation and washed three times with washing buffer (20 mM Tris, pH 8.0, 135 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1% Triton X-100, 10% glycerol, 10 mM NaF, and 5 mM Na₂VO₄). The samples were resuspended in 2× Laemmlı sample buffer, boiled for 5 min, and separated by 6.0 or 7.5% SDS-PAGE under reducing conditions. Proteins on the gels were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA). Following blocking with 5% nonfat milk or BSA at room temperature for 2 h, the membranes were immunoblotted with anti-phosphotyrosine 4G10 (1:1000) overnight at 4 °C. After three washes with Tris-buffered saline/0.1% Tween 20, the membranes were incubated at room temperature for 40 min with HRP-conjugated secondary antibodies. Bands of interest were visualized by ECL, scanned, and quantified by densitometry.

For assessment of Ser307 phosphorylation of IRS-1, muscle homogenates (400 μg) were immunoprecipitated with 2 μg of mouse monoclonal IRS-1 antibody overnight at 4 °C, followed by immunoblotting with rabbit anti-phospho-IRS-1 (Ser307) antisera. The bands of interest were detected as described above. To determine protein levels of CAP, IR-β, Akt, phospho-Akt, p44/p42 MAPK, phospho-p44/p42 MAPK, and PTEN, equal amounts of protein were prepared from muscle homogenates and subjected to SDS-PAGE and immunoblotting procedures as described above.

2-Deoxyglucose Transport Activity in Muscle—Briefly, intact soleus and EDL muscles were preincubated for 40 min at 30 ºC in 3 ml of oxygenated KHB buffer containing 5.5 mM glucose, 5 milliunits/ml insulin, and 1% BSA (RIA grade). Afterward, the muscles were incubated for 30 min in 3 ml of incubation medium containing 0.5 μCi/ml 2-[14C]deoxy-o-glucose (2-[14C]deoxyglucose/glycose = 19.5 nm/5.5 mm) and 0.1 μCi/ml [U-13C]sucrose, in the presence or absence of 10 mM diazoxide/ml insulin. At the end of incubation, the muscles were blotted on Kimwipes, snap frozen in liquid nitrogen, weighed, and homogenized in 0.5 ml of ice-cold 6% perchloric acid and centrifuged at 3000 × g for 15 min at 4 °C to remove precipitated protein. A portion of the supernatant was counted on a liquid scintillation counter pre-configured for 3H and 14C dual channel counting. The rates of transport of 2-[14C]deoxyglucose into the muscle cells were calculated after correcting for any 2-[14C]deoxyglucose trapped in the extracellular space, which was determined using the [U-14C]sucrose. Results were expressed as micromoles of 2-deoxyglucose transported per hour per gram wet muscle weight (micromoles/h/g of tissue).

Statistical Analysis—All data are expressed as means ± S.E. The significance of the effects of treatment within groups was determined by using Student’s t test, with p < 0.05 considered to be statistically significant.

RESULTS

Effects of LG268 and Rosiglitazone on Body Weight, Serum Glucose, and Triglyceride Levels in db/db Mice—The goal of our studies has been to treat diabetic db/db mice with a rexinoid, LG268, or a well characterized T2D, rosiglitazone, and then to compare the effects of the two treatments on glucose transport activity and several components involved in insulin-mediated signaling in skeletal muscle. To ensure that the effects observed were conducted under comparable conditions, we treated the db/db mice with either LG268 or rosiglitazone both at a dose of 30 mg/kg/day, because previous investigations had shown that both compounds at this dose produced a maximal and an equivalent suppression of hyperglycemia in diabetic rats (14). Table II compares the body weight, serum glucose, and triglyceride levels of a group of db/db mice and their lean littermates at baseline and after 2 weeks of treatment with vehicle, LG268, or rosiglitazone. The db/db mice were significantly heavier than their lean littermates at baseline (31.8 ± 1.9 versus 20.0 ± 1.3 mg/dl in diabetic and lean mice, respectively, p < 0.05), and they gained significantly more weight during the 2-week treatment period. The weight gain in the LG268-treated db/db animals was not different from the vehicle-treated controls, whereas rosiglitazone treatment resulted in a significant increase in weight gain for the db/db mice (11.4 versus 5.4 mg/d per week for rosiglitazone and vehicle treatments, respectively, p < 0.05). Neither LG268 nor rosiglitazone affected the weight gain of the lean animals.

At 7 weeks of age, db/db mice are both insulin-resistant and hyperglycemic (1). Under the conditions of our studies, the baseline serum glucose level in the db/db mice was 370 ± 119 mg/dl, compared with a baseline level of 129 ± 14 mg/dl in the lean mice. In addition, the degree of hyperglycemia in the db/db mice increased significantly to 531 ± 108 mg/dl during the 2-week study period. Both LG268 and rosiglitazone markedly decreased the hyperglycemia of the db/db animals (by 68 and 83%, respectively). Neither drug affected the serum glucose level of the lean animals. In addition to being hyperglycemic, db/db mice were also hypertriglyceridemic (119 ± 20 versus 79 ± 6 mg/dl in diabetic and lean mice, respectively, p < 0.05). Although RXR ligands produce sustained hypertriglyceridemia in diabetic rats and in humans (19, 20), in db/db mice 15 days...
of treatment with either LG268 or rosiglitazone resulted in a normalization of serum triglyceride levels.

**Effects of LG268 and Rosiglitazone on Insulin-stimulated Glucose Transport Activity in Skeletal Muscle of db/db Mice**—We first compared the ability of isolated skeletal muscle to transport 2-deoxyglucose in db/db mice and their lean littermates. For these studies, paired soleus and EDL muscles were rapidly excised from both hindlimbs of diabetic and lean mice, incubated in physiological buffer containing 2-[3H]deoxyglucose in the presence or absence of 10^4 microunits/ml insulin at 30 °C for 30 min. 2-Deoxyglucose transport was then determined as described under “Experimental Methods.” There was no significant difference between the diabetic and lean mice in the basal level of glucose transport in either soleus (Fig. 1A) or EDL muscle (Fig. 1B). There was, however, a moderate reduction in the values of insulin-stimulated glucose transport in both soleus and EDL muscles from db/db mice compared with lean controls. The insulin-stimulated 2-deoxyglucose transport was decreased by 30% in diabetic soleus (2.7 ± 0.4 versus 4.0 ± 0.4 μmol/g/h for diabetic and lean mice, respectively, p < 0.05) and 22% in diabetic EDL (2.9 ± 0.2 versus 3.7 ± 0.4 micromoles/g/h for diabetic and lean mice, respectively, p = 0.09).

To determine whether treatment with either the rexinoid or the TZD affected the glucose transport activity of skeletal muscle, db/db mice were treated for 2 weeks with either vehicle, LG268, or rosiglitazone, paired EDL muscles were excised and basal and insulin-stimulated 2-deoxyglucose transport was measured as described above (Fig. 1C). LG268 had no effect on basal glucose transport (2.8 ± 0.3 versus 2.3 ± 0.2 μmol/g/h for LG268- versus vehicle-treated animals, p = 0.22), but it did induce a significant increase in insulin-stimulated glucose transport (4.5 ± 0.3 versus 3.0 ± 0.2 μmol/g/h for LG268-versus vehicle-treated animals, 50% increase, p < 0.05). Treatment with rosiglitazone, on the other hand, resulted in a significant increase in both basal and insulin-stimulated glucose transport. The basal glucose transport in muscle from rosiglitazone-treated diabetic mice was 33% higher than the vehicle controls (3.1 ± 0.3 versus 2.3 ± 0.2 μmol/g/h for rosiglitazone-versus vehicle-treated animals, p < 0.05). The effect of rosiglitazone on insulin-stimulated glucose transport was even more pronounced. Insulin-stimulated glucose transport in muscle from rosiglitazone-treated db/db mice (4.4 ± 0.3 μmol/g/h) was 47% higher than the vehicle controls (3.0 ± 0.2 μmol/g/h). Thus, the impaired rate of insulin-stimulated glucose transport in skeletal muscles of db/db mice was normalized by treatment with either LG268 or rosiglitazone.

**Effects of LG268 and Rosiglitazone on Intramyocellular LCFA-CoAs**—Interventions that alter intramyocellular LCFA-CoAs levels have been associated with changes in insulin-mediated signaling and insulin sensitivity (17, 18). To determine whether the insulin-sensitizing activity of LG268 or rosiglitazone was linked to alterations in the amounts or types of fatty acid metabolites in muscle, we measured the contents of saturated and unsaturated LCFA-CoAs in extracts of skeletal muscle (soleus and gastrocnemii) from vehicle-, LG268-, and rosiglitazone-treated db/db mice. Muscle from vehicle-treated lean littermates served as controls (Fig. 2 and Table III). Diabetes was associated with a large increase in the levels of total saturated (14.0-fold increase, p < 0.05) and unsaturated (9.0-fold increase, p < 0.05) LCFA-CoAs in the soleus (2.57 ± 0.21 versus 0.19 ± 0.03 nmol/g of tissue for saturated LCFA-CoAs in diabetic and non-diabetic muscles, respectively; 19.50 ± 1.55 versus 2.14 ± 0.20 nmol/g of tissue for total unsaturated LCFA-CoAs, Fig. 2A). Treatment of db/db mice with rosiglitazone resulted in a substantial decrease in both saturated and unsaturated LCFA-CoAs in the soleus muscle (by 67 and 65%, respectively, both p < 0.05 versus vehicle-treated diabetic mice, Fig. 2A). In contrast, treatment with LG268 had no effect on the levels of either saturated or unsaturated LCFA-CoAs in the soleus muscle (Fig. 2A). As was noted for the soleus muscle, gastrocnemii muscle from db/db mice also contained significantly higher levels of both saturated and unsaturated LCFA-CoAs relative to lean controls (2.8- and 2.4-fold increase for saturated and unsaturated LCFA-CoAs, respectively, both p < 0.05, Fig. 2B). However, the response of gastrocnemii to the insulin-sensitizing drugs was very different from that seen in soleus. In gastrocnemii, rosiglitazone had
very little effect on the levels of both saturated and unsaturated LCFA-CoAs. LG268, on the other hand, had very little effect on the saturated LCFA-CoAs, but it induced a substantial increase in the level of total unsaturated LCFA-CoAs (28.1 ± 2.2 versus 18.9 ± 3.1 nmol/g of tissue for muscles from LG268- versus vehicle-treated diabetic mice, p < 0.05, Fig. 2B).

Table III shows the effects of LG268 and rosiglitazone on the levels of individual LCFA-CoAs in both soleus and gastrocnemius muscles from diabetic mice. The level of each of the LCFA-CoAs species examined in soleus was significantly decreased by rosiglitazone (27, 42, 36, 29, and 40% of diabetic control for palmitoyl-CoA (16:0), stearoyl-CoA (18:0), palmitoleoyl-CoA (16:1), oleoyl-CoA (18:1), linoleoyl-CoA (18:2), and linolenoyl-CoA (18:3), respectively). LG268 produced the greatest increases in the levels of palmitoyl-CoA (16:1) and oleoyl-CoA (18:1) (231 and 173% of diabetic control, respectively) with smaller increases in linoleoyl-CoA (18:2) and linolenoyl-CoA (18:3) in the gastrocnemius muscle.

**Insulin Receptor Expression and Tyrosine Phosphorylation**—To investigate the effects of rexinoids and TZDs on insulin receptor expression, total RNA and protein were prepared from the quadriceps muscle of db/db mice that had been treated with vehicle, LG268, or rosiglitazone for 2 weeks. The level of insulin receptor mRNA was measured by real-time Q-RT-PCR with PCR primers and a probe specific for the mouse receptor. The level of insulin receptor protein was measured by immunoblotting with a polyclonal insulin receptor-β antibody. Receptor transcripts were normalized to the transcripts of the housekeeping gene cyclophilin, and insulin receptor protein levels were normalized to glyceraldehyde-3-phosphate dehydrogenase. There was no difference in either the level of insulin receptor mRNA (Fig. 3A) or insulin receptor protein (Fig. 3B) in muscles obtained from the vehicle, LG268-treated, and rosiglitazone-treated animals.

To determine whether treatment with the insulin-sensitizing drugs altered insulin receptor tyrosine kinase activity, we measured both the basal level of receptor tyrosine phosphorylation and the response of the receptor to exogenous insulin stimulation. For these studies, paired EDL muscles removed from the hindlimbs of either vehicle-, LG268-, or rosiglitazone-treated db/db mice were incubated for 20 min in media with or without 10⁴ microunits/ml insulin. Homogenates of these muscles were immunoprecipitated with anti-insulin receptor-β antibody and phosphotyrosyl receptor was measured by immunoblotting with 4G10 anti-phosphotyrosine antibody followed by densitometric quantitation. Neither LG268 nor rosiglitazone showed any effect on either the basal level of insulin receptor tyrosine phosphorylation or the level of phosphorylation following insulin stimulation (Fig. 3C).

**Comparison of the Effects of LG268 and Rosiglitazone on the Insulin Receptor Adaptor Proteins IRS-1 and CAP**—The IRSs and CAP are two families of adaptor proteins that couple the

![Fig. 2. Intramyocellular LCFA-CoAs contents in skeletal muscle of db/db mice. Db/db mice were treated with vehicle, LG268, or rosiglitazone for 2 weeks. Soleus (A) and gastrocnemius (B) muscles were dissected and assayed to determine the concentrations of individual intramyocellular LCFA-CoAs species using mass spectrometry. Total saturated LCFA-CoA was calculated as the sum of C16:0 and C18:0. Total unsaturated LCFA-CoAs was calculated as the sum of C16:1, C18:1, C18:2, and C18:3. n = 2 for soleus for each treatment group; n = 8–10 for gastrocnemius for each treatment group.

**Table III**

Profiles of long chain fatty acyl-CoAs in skeletal muscles of db/db mice

| LCF-CoAs content | Treatment groups | Lean/VH | db/VH | db/LG268 | db/ROS |
|------------------|------------------|---------|-------|----------|--------|
| Saturated LCFA-CoAs |                  |         |       |          |        |
| C16:0            | S:               | 0 ± 0   | 1.1 ± 0.2 | 1.0 ± 0.2 | 0.3 ± 0.0* |
|                  | G:               | 0.39 ± 0.19 | 1.7 ± 0.5 | 2.0 ± 0.2 | 1.0 ± 0.1 |
| C18:0            | S:               | 0.19 ± 0.03 | 1.4 ± 0.1 | 1.5 ± 0.2 | 0.6 ± 0.1* |
|                  | G:               | 1.28 ± 0.10 | 2.8 ± 0.3 | 3.6 ± 0.3* | 2.4 ± 0.1 |
| Unsaturated LCFA-CoAs |               |         |       |          |        |
| C16:1            | S:               | 0.31 ± 0.01 | 1.7 ± 0.2 | 1.8 ± 0.5 | 0.9 ± 0.1* |
|                  | G:               | 0.52 ± 0.17 | 1.6 ± 0.3 | 3.7 ± 0.3* | 2.1 ± 0.2 |
| C18:1            | S:               | 0.80 ± 0.09 | 9.0 ± 0.4 | 9.6 ± 0.8 | 3.3 ± 0.2* |
|                  | G:               | 3.85 ± 0.44 | 8.0 ± 1.3 | 13.9 ± 0.9* | 8.5 ± 0.5 |
| C18:2            | S:               | 0.85 ± 0.08 | 7.9 ± 1.0 | 5.2 ± 0.6 | 2.3 ± 0.2* |
|                  | G:               | 2.62 ± 0.18 | 5.5 ± 0.9 | 8.1 ± 0.6* | 5.4 ± 0.4 |
| C18:3            | S:               | 0.23 ± 0.03 | 1.0 ± 0.1 | 0.7 ± 0.1 | 0.4 ± 0.1* |
|                  | G:               | 0.85 ± 0.04 | 1.9 ± 0.2 | 2.4 ± 0.2* | 1.6 ± 0.1 |

* p < 0.05 versus vehicle-treated db/db mice using Student’s t test.
lin-sensitizing drugs on IRS-1 expression and phosphorylation, paired EDL muscles were collected and incubated as described above. IRS-1 was immunoprecipitated from homogenates of the muscles and the levels of IRS-1 and phospho-IRS-1 were determined by immunoblotting with antibodies to IRS-1 and phosphotyrosine, respectively (Fig. 4). The level of IRS-1 protein in skeletal muscle was unchanged by treatment of the mice with either LG268 or rosiglitazone. However, insulin-stimulated tyrosine phosphorylation of IRS-1 was greater in the LG268-treated diabetic mice than in the vehicle-treated controls (5.0-fold increase versus 3.1-fold increase for LG268 and vehicle treatment, respectively, p < 0.05). This effect was not observed in the muscle from the rosiglitazone-treated animals, the insulin-induced increase in IRS-1 tyrosine phosphorylation in these animals (3.3-fold) was not different from the vehicle controls (3.1-fold).

There is evidence that the level of expression of CAP is a critical factor in the regulation of the CAP/Cbl pathway (21). To examine the effects of the insulin-sensitizing drugs on CAP mRNA expression, total RNA was prepared from the quadriceps muscle of db/db mice that had been treated with vehicle, LG268, or rosiglitazone for 2 weeks, and CAP transcripts were quantitated by real-time Q-RT-PCR. In agreement with previous reports (22), rosiglitazone was an effective inducer of CAP gene expression. Chronic treatment with rosiglitazone resulted in a marked increase in the level of CAP transcripts (3.5-fold) compared with vehicle controls, p < 0.05, Fig. 5A). Interestingly, under the same conditions, LG268 had no effect on the level of CAP gene expression (Fig. 5A). To determine whether the alterations in CAP gene expression resulted in alterations in the level of the CAP protein, proteins from the quadriceps of vehicle-, LG268-, and rosiglitazone-treated db/db mice were immunoblotted with an antibody against the CAP protein. The level of the CAP protein was 2- to 3-fold higher in the muscle from the rosiglitazone-treated mice than in the muscles from either the vehicle- or LG268-treated animals (Fig. 5B). In summary, in skeletal muscle of db/db mice LG268-treatment selectively increased insulin-dependent IRS-1 tyrosine phosphorylation, whereas rosiglitazone increased the expression of the CAP adaptor protein.
Comparison of the Effects of LG268 and Rosiglitazone on the Phosphorylation of the Akt and c-Cbl—Insulin signaling, particularly in the context of glucose transport, is mediated at least in part by the phosphorylation and activation of key downstream effector molecules such as Akt and c-Cbl (21, 23–25). To determine whether the insulin-sensitizing activity of rexinoids or TZDs involved alterations in the activation of these enzymes, diabetic and non-diabetic (lean littermates) mice were treated with vehicle, LG268, or rosiglitazone for 2 weeks, paired EDL and soleus muscles were incubated as described previously, and the levels of expression and phosphorylation of both Akt and c-Cbl were determined by immunoprecipitation and immunoblot analysis. In the case of Akt, treatment of db/db mice with either LG268 or rosiglitazone had no effect on the level of Akt protein (Fig. 6A). There was however a marked increase in the level of insulin-stimulated Akt Ser473 phosphorylation in the muscles from the LG268-treated animals (Fig. 6A). In the EDL muscle from LG268-treated animals, insulin induced a 10.1-fold increase in Akt phosphorylation compared with a 4.7-fold increase in the muscle from the vehicle controls (p < 0.05). A similar trend was also apparent in the soleus muscle from the LG268- and vehicle-treated animals, the insulin-induced increase in Akt phosphorylation was substantially higher in the LG268-treated animals (7.9-fold) than in the vehicle controls (4.5-fold), but in this case the difference failed to achieve statistical significance (p = 0.09), probably due to the small sample size (n = 3). In contrast, treatment with rosiglitazone had no effect on insulin-stimulated Akt phosphorylation in either the EDL or the soleus muscles in db/db mice (Fig. 6A). To determine whether the effect of LG268 on insulin-stimulated Akt phosphorylation was restricted to diabetic animals, we carried out a similar analysis of the effects of LG268 on insulin-stimulated Akt phosphorylation in non-diabetic mice (Fig. 6B). In both the EDL and the soleus muscles, LG268 treatment resulted in a significantly greater degree of insulin-stimulated Akt phosphorylation than
in muscles from animals treated with vehicle alone. Also, as in the diabetic mice, rosiglitazone had no effect on insulin-stimulated Akt phosphorylation in muscles of lean animals.

With respect to the effects of LG268 and rosiglitazone on expression and activation of c-Cbl, neither had any effect on the level of c-Cbl protein (Fig. 7). LG268 also had no effect on either the basal or insulin-stimulated c-Cbl tyrosine phosphorylation. Treatment with rosiglitazone, on the other hand, caused a small but significant increase in the levels of c-Cbl phosphorylation in both basal and insulin-stimulated muscles (Fig. 7). In summary, treatment of db/db mice with LG268 resulted in an increased level of insulin-stimulated Akt phosphorylation with no effect on c-Cbl phosphorylation, whereas rosiglitazone did not affect Akt phosphorylation but induced a modest increase in insulin-stimulated c-Cbl phosphorylation.

**Effects of LG268 and Rosiglitazone on IRS-1 Serine Phosphorylation**—Recent studies have demonstrated that increased phosphorylation of IRS-1 at Ser307 results in a decrease in insulin-stimulated IRS-1 tyrosine phosphorylation and insulin resistance (17, 26). To evaluate the effects of LG268 and rosiglitazone on IRS-1 Ser307 phosphorylation, we used an IRS-1 Ser307 phospho-specific antibody to quantitate the level of the serine phosphoprotein in IRS-1 immunoprecipitated from the quadriceps muscles of vehicle-, LG268-, and rosiglitazone-treated db/db mice (Fig. 8). Treatment with LG268 resulted in a small (21%) but significant decrease in the level of Ser307-phosphorylated IRS-1. The decrease in IRS-1 Ser307 phosphorylation in muscle of LG268-treated db/db mice lowered the phosphoprotein to the same level as was present in muscle from non-diabetic control mice. Treatment of db/db mice with rosiglitazone had no effect on the level of Ser307-phosphorylated IRS-1.

**MAPK Activation**—In addition to its effects on metabolic processes, mediated in part by the IRS-1/Akt and CAP/c-Cbl pathways, insulin also affects cell proliferation. The mitogenic activities of insulin are, in part, dependent on the activation of the MAPK signal pathways (27). To determine whether insulin sensitizers such as rexinoids and TZDs affected these mitogenic pathways, we measured both the basal and insulin-stimulated levels of p44/42 MAPK phosphorylation in the EDL muscles obtained from diabetic animals treated with vehicle, LG268, or rosiglitazone (Fig. 9). There was no difference in either the basal value or insulin-stimulated level of p44/42 MAPK phosphorylation among any of the treatment groups.
DISCUSSION

Rexinoids are synthetic agonists for the RXRs. They represent a second class of nuclear receptor ligands that have insulin-sensitizing activity. Previous studies have shown that, in diabetic rats under conditions of a euglycemic-hyperinsulinemic clamp, rexinoids decrease hepatic glucose production and increase the rate of glucose disposal, suggesting that they increase both hepatic and peripheral insulin sensitivity. In line with this, rexinoids have been shown, in vivo, to have marked effects on metabolic gene expression in both liver and skeletal muscle (14). To gain further insight into the mechanism of the insulin-sensitizing activity of rexinoids, we have focused on their effects on glucose metabolism and insulin-mediated signaling in diabetic skeletal muscle. We have treated diabetic animals for a sufficient time and with sufficient doses of both rexinoids and TZDs to be sure that we have obtained a maximal degree of insulin sensitization. The results we obtained show that the deficit in insulin-stimulated glucose transport in diabetic muscle can be corrected by treatment with either a rexinoid or a TZD.

Akt and c-Cbl play an essential role in transducing the effects of insulin on glucose metabolism in skeletal muscle (16). The next goal of our study was to determine the extent to which alterations in the activity of either of these enzymes contributed to the ability of rexinoids to increase muscle glucose transport in diabetic mice. The results we obtained were clear-cut. In the case of c-Cbl, rexinoid treatment had no effect on either the level of expression or the degree of phosphorylation of c-Cbl. Rexinoids also had no effect on the expression of the adaptors for various downstream signal molecules such as the regulatory subunit of PI3K, p85. Serine phosphorylation of IRS-1 may hamper its interaction with downstream p85, or substrate for the receptor (40). In addition, serine phosphorylation of IRS-1 by the tyrosine kinase activity of the insulin receptor, allows it to serve as an adaptor protein for various downstream signal molecules such as the regulatory subunit of PI3K, p85. Serine phosphorylation of IRS-1, on the other hand, impairs its association with the activated insulin receptor and thereby renders IRS-1 a less efficient substrate for the receptor (40).

There are several potential mechanisms that could account for the increased level of insulin-stimulated Akt phosphorylation in the muscle of rexinoid-treated diabetic mice. An increase in Akt phosphorylation could simply be due to increased Akt expression. However, neither the level of Akt transcripts nor Akt protein was affected by rexinoid treatment. The activation of Akt is dependent on the presence of phosphorylated inositol 1,4,5-trisphosphate. Alterations in the expression or activation of lipid phosphatases, such as the 5'- phosphatase SH2 domain-containing inositol 5-phosphatase 2 and the 3'- phosphatase PTEN that degrade phosphorylated inositol 1,4,5-trisphosphate can therefore affect Akt activation (37–39). We do not think that these enzymes contribute to the effects of rexinoids on Akt phosphorylation, because there was no change in the expression of either at the transcript or protein level in vehicle versus rexinoid-treated animals (data not shown). We believe that the most likely explanation for the increased Akt phosphorylation in rexinoid-treated animals is a reflection of increased IRS-1 tyrosine phosphorylation. Phospho-IRS-1 is a key regulator of Akt activation in skeletal muscle (via PI3K activation), and the 60% increase in phosphotyrosyl IRS-1 in the rexinoid-treated animals would be expected to have a substantial effect of Akt activation.

IRS-1 is a complicated protein that undergoes phosphorylation at numerous tyrosine and serine/threonine residues. Tyrosine phosphorylation of IRS-1, by the tyrosine kinase activity of the insulin receptor, allows it to serve as an adaptor protein for various downstream signal molecules such as the regulatory subunit of PI3K, p85. Serine phosphorylation of IRS-1, on the other hand, impairs its association with the activated insulin receptor and thereby renders IRS-1 a less efficient substrate for the receptor (40).

Increased IRS-1 Ser[Ser] phosphorylation has been found in insulin resistance induced by a variety of agents, including the pro-inflammatory cytokine TNF-α, insulin, and fatty acids (17, 26, 44). There is only one report, prior to the studies reported here, in which increased IRS-1 Ser[Ser] phosphorylation has been associated with insulin sensitization. Salicylates and pharmacological inhibitors of the c-Jun N-terminal kinase (JNK) can decrease IRS-1 Ser[Ser] phosphorylation and prevent insulin resistance in HEK293 cells exposed to TNF-α and phorbol 12-myristate-13-acetate (45). The studies we have reported here show that rexinoids also appear to be...
Rexinoids, Insulin Signaling, and Skeletal Muscle

able to decrease IRS-1 Ser307 phosphorylation in vivo in skeletal muscle of diabetic animals. Our results show that IRS-1 Ser307 phosphorylation is modestly increased in muscle of db/db mice and treatment with the rexinoid reverses this effect, generating a muscle in which the fraction of IRS-1 available for interaction with insulin receptor is similar to that in non-diabetic animals. We believe that it is this decrease in IRS-1 Ser307 phosphorylation that leads to an increase in insulin-stimulated IRS-1 tyrosine phosphorylation, which in turn increases Akt activation and eventually causes normalization of muscle glucose transport activity.

A key question that remains to be answered is how rexinoids are able to suppress IRS-1 Ser307 phosphorylation in diabetic muscle. As pointed out, inflammation is a powerful stimulus for insulin resistance, and at least some of its effects are mediated by TNF-α and other adipo- kines that can impair the insulin sensitivity of adjacent muscle cells (46). Inhibition of the excessive secretion or aberrant activity of these paracrine effectors in diabetic muscle thus could result in decreased level of IRS-1 Ser307 phosphorylation. However, we have previously shown that, unlike TZDs, rexinoids do not suppress TNF-α expression in muscle from obese and diabetic rats (14). We also examined an alternative explanation for the effect of rexinoids on IRS-1 Ser307 phosphorylation, namely that changes in intramyocellular lipid metabolites might be linked to this effect. Elevated circulating levels of free fatty acids, increased delivery of fatty acids into muscle, and decreased intramyocellular fatty acid metabolism have long been implicated in the insulin resistance observed in obesity and type 2 diabetes (47, 48). We did observe that rexinoid treatment significantly increased the levels of unsaturated LCFA-CoAs in gastrocnemius, a muscle composed of a mixture of slow-twitch and fast-twitch myofibers. However, rexinoids had no effect on the levels of either saturated or unsaturated LCFA-CoAs in the slow-twitch soleus muscle under conditions of insulin resistance in obese animals (data not shown). We speculate that the beneficial effects of LG268 on insulin sensitivity might reflect the activation of the PPAR-α or PPAR-δ regulated pathway or possibly pathways specifically regulated by RXR homodimers. Future studies on the detailed effects of RXR heterodimeric ligands on insulin-mediated signal pathways will be required to distinguish between these possibilities.

In conclusion, our results demonstrate that in db/db mice rexinoids improve insulin sensitivity via mechanisms different from those of TZDs. Rexinoids increase the activity of components of the insulin-mediated IRS-1/Akt signal pathway in skeletal muscle. This effect may be associated with a decrease in the level of IRS-1 Ser307 phosphorylation. Rexinoids appear to represent a novel class of insulin sensitizers, with potential applications for the treatment of type 2 diabetes.

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Rexinoids, Insulin Signaling, and Skeletal Muscle
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