Rosiglitazone Improves Muscle Insulin Sensitivity, Irrespective of Increased Triglyceride Content, in *ob/ob* Mice

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The present study was performed to examine the effects of rosiglitazone treatment on tissue-specific insulin sensitivity. Therefore, we used obese, insulin-resistant *ob/ob* mice and measured the effects of rosiglitazone treatment on insulin sensitivity and simultaneously tissue-specific uptake of glucose and free fatty acids (FFA) under hyperinsulinemic euglycemic clamp conditions. Rosiglitazone treatment resulted in significantly higher body weight and decreased plasma levels of glucose, insulin, and triglyceride (TG). Glucose tolerance, as well as insulin sensitivity, was improved upon rosiglitazone treatment, as assessed by glucose tolerance and insulin sensitivity tests. Hyperinsulinemic euglycemic clamps showed increased glucose infusion rates with increased whole body insulin sensitivity. Rosiglitazone treatment resulted in increased glucose uptake by cardiac and skeletal muscle under hyperinsulinemic euglycemic clamp conditions, while no differences were observed in FA uptake. Measurement of TG content showed that rosiglitazone treatment resulted in decreased TG content of cardiac muscle, but increased TG content of skeletal muscle. We conclude that rosiglitazone treatment leads to strong improvement of insulin sensitivity, irrespective of increased muscle TG content, in *ob/ob* mice.

THIAZOLIDINEDIONES ARE synthetic ligands for the peroxisome proliferator-activated receptor-γ (PPAR-γ), which is expressed predominantly in adipose tissue.[1,2] PPAR-γ activation leads to activation of genes that are involved in transport and sequestration of fatty acids (FA). Activation of PPAR-γ also results in differentiation of adipocytes, leading to an increased number of small adipocytes and a decreased number of large adipocytes.[3-5] Thiazolidinediones are drugs that are used in the treatment of type 2 diabetes mellitus, and treatment with thiazolidinediones results in (1) lowering of plasma levels of glucose and insulin; (2) reduced plasma levels of triglycerides (TG) and free fatty acids (FFA); and (3) improvement of sensitivity to the action of insulin.[6,7] Although thiazolidinediones have been studied extensively during the last years, the exact mechanism of action has not been fully elucidated. A body of evidence suggests that improvement of insulin sensitivity and hypertriglyceridemia are, at least in part, a direct result of decreased FFA supply to insulin-sensitive target tissues, particularly liver and skeletal muscle,[8,9] leading to lower TG contents in these tissues.

Studies in obese diabetic Zucker fa/fa rats by Oakes et al[10] showed that treatment with thiazolidinediones, such as rosiglitazone and darglitazone, results in (1) an increased ability to take up and store plasma FFA in white adipose tissue concomitant with a reduced uptake in the liver; (2) an augmented capacity to mobilize FFA under fasting conditions; and (3) a greatly enhanced ability of postprandial levels of insulin to suppress FFA mobilization. Thus, treatment with rosiglitazone and darglitazone results in improved FA utilization. Furthermore, thiazolidinediones improve insulin sensitivity of glucose metabolism. The focus of their study was the effect on FFA and TG metabolism under basal and hyperinsulinemic euglycemic clamp conditions, but it remains obscure in what way rosiglitazone influences the insulin sensitivity of distinct tissues.

The aim of our study was to examine the effects of rosiglitazone on tissue-specific insulin sensitivity. To that extent, we used obese, insulin-resistant *ob/ob* mice and measured the effect of rosiglitazone treatment on whole body insulin sensitivity and simultaneously tissue-specific uptake of glucose and FA under hyperinsulinemic euglycemic clamp conditions.

We found that rosiglitazone treatment resulted in a major improvement of whole body insulin sensitivity, confined to cardiac and skeletal muscles. Remarkably, in skeletal muscle, TG content was increased upon rosiglitazone treatment, arguing against a positive relationship between muscle TG content and insulin resistance, at least in *ob/ob* mice.

MATERIALS AND METHODS

Animals

At the age of 4 to 5 months *ob/ob* mice on a C57BL/6J background were housed individually and received either a standard chow diet (CHOW) or chow containing rosiglitazone maleate (rosiglitazone) (Avandia: SmithKline Beecham, Brentford, UK). Littermates were equally divided over both groups, which were matched for body weight. Body weight was measured weekly during the 10 weeks of treatment. All animal experiments were approved by the institutional committee on animal welfare of TNO.

Treatment and Diet

Powdered standard chow diet (Hope Farms, Woerden, The Netherlands) was used to prepare the 2 diets. Rosiglitazone was added to obtain a daily dose of 3 mg/kg per mouse. After mixing rosiglitazone with the powdered chow, 2% (wt/wt) agar in 1 L warm water was added to the mixture. After kneading, little chocks were made, which were freeze-dried and stored at −20°C until being fed to the mice. Control diet (CHOW) without rosiglitazone was prepared the same way.

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Blood Chemistries

After 5 weeks of treatment, blood samples were taken from the mice by tail bleeding, after a 4-hour fast. The blood was collected in capillary tubes (Hawksly & Sons, CAT. no. 01603, Lancing, Sussex, UK) that were coated with paraoxon (diethyl p-nitrophenyl phosphate; Sigma Chemical, St Louis, MO) to prevent hydrolysis of TG[11] and kept on ice. The samples were spun (13,000 rpm) at 4°C for 3 minutes, and the separated plasma was immediately used to determine glucose, FFA, TG, and total cholesterol (TC) concentrations. The remaining plasma was frozen in liquid nitrogen and stored at −20°C for later measurement of insulin.

Plasma glucose was determined by a commercially available kit (#315-500, Sigma). FFA was measured enzymaticaly with a NEFA-C kit (Wako Chemicals, Neuss, Germany). Levels of TG, corrected for free glycerol, and total cholesterol were determined by using commercially available enzymatic kits (#2336691, Boehringer Mannheim, Mannheim, Germany and Sigma Glucose Oxidase [GO]-trinder kit, 337-B). Insulin levels were measured by using a radioimmunoassay kit (Sensitive Rat Insulin Assay; Linco Research, St Charles, MO).

Glucose Tolerance and Insulin Sensitivity Test

Mice were subjected to glucose tolerance and insulin sensitivity tests after 3 and 5 weeks of treatment, respectively. For the glucose tolerance test, mice were fasted for 4 hours. After collection of baseline (t = 0) blood samples, the mice received an intraperitoneal (IP)-injected bolus (2 g/kg) of a 25% (wt/vol) D-glucose solution, and additional blood samples were taken at 15, 30, 60, and 120 minutes after injection. Blood samples were treated as mentioned above, and the plasma glucose concentration was measured immediately. The area under the glucose tolerance curve (AUC) was used as a measurement of glucose tolerance.

The insulin sensitivity test was performed similarly as the glucose tolerance test, but in this test, 1.0 U insulin per kilogram body weight was administered IP as a 0.2 U/mL solution. The insulin sensitivity was determined by the lowering of plasma glucose concentration after insulin administration.

Hyperinsulinemic Euglycemic Clamp Analysis

After 5 weeks of treatment with rosiglitazone or CHOW (as mentioned above), tissue-specific uptake of glucose and FA was determined under hyperinsulinemic euglycemic conditions using ^{3}H-2-deoxy-glucose (2-DG) (Amersham, Little Chalfont, UK) and ^{14}C-palmitate (Amersham). The clamp experiments were performed as described earlier.[12] After an overnight fast, animals were anesthetized (0.5 mL/kg Hypnorm; Janssen Pharmaceutica, Berchem, Belgium and 12.5 mg/g midazolam; Genthon BV, Nijmegen, The Netherlands) and an infusion needle was placed in 1 of the tail veins. Subsequently, a bolus of insulin (200 mU/kg; Actrapid, Novo Nordisk, Chartres, France) was given, and a hyperinsulinemic euglycemic clamp was started with a continuous infusion of insulin (7.0 mU/min · kg) and a variable infusion of 12.5% D-glucose (in phosphate-buffered saline [PBS]) to maintain blood glucose level at ~ 7.5 mmol/L. Blood samples were taken every 5 to 10 minutes to monitor plasma glucose levels using a hand-glucose meter (FreeStyle; Disetronics, Vianen, The Netherlands). Glucose infusion rate was adjusted if necessary as described by Voshol et al.[12] When the blood glucose concentration was in steady state for 20 minutes, ~1.5 hours after starting the clamp, a bolus (100 μL) of ^{3}H-2-DG (5 μCi) was administered, followed by a bolus (100 μL) ^{14}C-palmitate (3 μCi) 29 minutes later. One minute after administering the ^{14}C-palmitate bolus, blood was collected, and the animal was killed. Liver, cardiac muscle, skeletal muscle (quadriceps), and white adipose tissue (WAT, reproductive fat pads) were rapidly collected, snap frozen in liquid nitrogen, and kept at −20°C for analysis. During preparation of the quadriceps muscle, excess extramyocellular fat tissue was removed in principle as described by Guo et al[13] to obtain liable measurements in muscle tissue. The collected blood was used to measure plasma glucose and insulin.

Tissue Homogenates

To determine the protein content and the uptake of palmitate and 2-DG, tissue samples (± 250 mg) were homogenized in 1 mL demineralized water (demi-water). Tissue protein was measured according to the method of Lowry et al[14] using bovine serum albumin (BSA; Sigma, Deisenhofen, Germany]) as standard. To determine the uptake of ^{14}C-palmitate in liver and WAT, lipids were extracted by a modification of the method of Bligh and Dyer.[15] In short, 200 μL tissue homogenate was added to 1 mL demi-water, then 4.5 mL chloroform: methanol (2:1) was added and mixed thoroughly, after which 1.5 mL demi-chloroform was added and mixed. Finally, 1.5 mL demi-water was added and mixed. After centrifugation, the chloroform layer was collected, evaporated, and resuspended again in 50 μL chloroform: methanol (5:1), from which 10 μL was used for determination of ^{14}C radioactivity. Thin-layer chromatography (TLC) analyses showed that 90% of the label was in the FA fraction.

For determination of tissue 2-DG uptake, homogenates of WAT, heart, and skeletal muscle were boiled, centrifuged, and the supernatant subjected to an ion-exchange column to separate 2-DG-6-phosphate (2-DG-P) from 2-DG, as previously described.[16,17]

Uptake of palmitate was expressed as percent uptake by the tissue of total administered ^{14}C activity per gram tissue protein and 2-DG as percent uptake by the tissue of total administered ^{3}H activity per gram tissue.

Tissue TG Content

Total TG content was determined in homogenates of heart, liver, WAT, and skeletal muscle retrieved from mice after 10 weeks of treatment with or without rosiglitazone. Lipids were extracted and separated by high-performance thin layer chromatography (HPTLC) as described previously.[18] Quantification was performed by scanning the plates and by integrating the density areas using TINA version 2.09 software (Raytest, Straubenhardt, Germany).

Statistical Analyses

For statistical analysis, SPSS version 11.0 (SPSS, Chicago, IL) was used. The Mann-Whitney nonparametric test for 2 independent samples was used to define plasma parameters differences between the groups of mice. Because we matched for body weight by the formation of the experimental groups, we used the Wilcoxon nonparametric test for 2 related samples to define body weight differences between the groups. The criterion for significance was set at $P < .05$.

RESULTS

Body Weight and Blood Parameters

During treatment, animals that were treated with rosiglitazone gained significantly more body weight than the controls (14.7 ± 3.5 *v* 9.8 ± 2.4 g, $P < .05$), as shown in Fig 1. Despite this, plasma glucose (8.9 ± 2.0 *v* 6.8 ± 0.4 mmol/L, $P < .05$) and insulin levels (19.9 ± 5.7 *v* 4.5 ± 1.1 ng/mL, $P < .05$) declined significantly in response to rosiglitazone treatment (Table 1). Also, plasma TG concentration (0.25 ± 0.1 *v* 0.12 ± 0.02 mmol/L, $P < .05$) was reduced compared with controls, although the levels were low for both groups, whereas circulating FFA and TC were not affected.

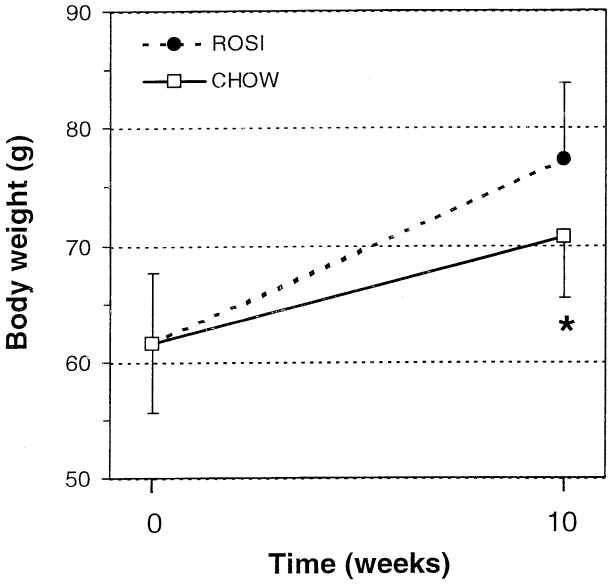

Fig 1. Body weight during treatment. Mice, 4 to 5 months of age, were treated with (ROSI) or without rosiglitazone (CHOW) for 10 weeks. Values represent mean body weight ± SD of 6 mice per group after treatment. *$P < .05$ CHOW *v* ROSI using Mann-Whitney nonparametric test for 2 independent samples.

Glucose Tolerance and Insulin Sensitivity

Glucose tolerance, as estimated by the AUC in response to IP glucose administration, was significantly improved by rosiglitazone treatment (Fig 2). Also, insulin sensitivity appeared to be increased by rosiglitazone treatment, as evidenced by a considerable lowering of plasma glucose levels in response to IP insulin administration (Fig 3)

Hyperinsulinemic Euglycemic Clamp and Tissue Glucose and FA Uptake

Plasma glucose concentrations in steady state (~7.5 mmol/L) were similar in rosiglitazone treated mice and controls (Table 2). Glucose infusion rate, to maintain this concentration, was significantly higher in the rosiglitazone-treated mice than in the controls (60.3 ± 8.9 *v* 43.3 ± 3.4 μmol/kg/min, $P < .05$, respectively), indicating that whole body insulin sensitivity was enhanced by rosiglitazone treatment.

Table 1. Plasma Glucose, Insulin, FFA, TG, and TC After Treatment With or Without Rosiglitazone

	CHOW ($n = 6$)	ROSI ($n = 6$)
Glucose (mmol/L)	8.9 ± 2.0	6.8 ± 0.4*
Insulin (ng/mL)	19.9 ± 5.7	4.5 ± 1.1*
FFA (mmol/L)	0.88 ± 0.22	0.73 ± 0.25
TG (mmol/L)	0.25 ± 0.11	0.12 ± 0.02*
TC (mmol/L)	5.4 ± 1.5	3.7 ± 1.4

Abbreviations: FFA, free fatty acids; TG, triglyceride; TC, total cholesterol; ROSI, with rosiglitazone; CHOW, without rosiglitazone.

*$P < .05$ CHOW *v* ROSI using Mann-Whitney nonparametric test for 2 independent samples.

In steady state, uptake of glucose was significantly increased in heart and skeletal muscle after rosiglitazone treatment, whereas no difference was observed in WAT (Fig 4A). There were no changes in the uptake of FA by liver and WAT in the rosiglitazone-treated *ob/ob* mice compared with the chow-fed *ob/ob* mice (Fig 4B).

Tissue TG Content

After 10 weeks of treatment, TG content was measured in heart, liver, WAT, and skeletal muscle. (Fig 5). TG content of liver and WAT were not different between control and rosiglitazone-treated *ob/ob* mice. In skeletal muscle, TG content was increased, while it was decreased in cardiac muscle during rosiglitazone treatment.

DISCUSSION

Type 2 diabetes is a complex disease, which is associated with strong impairment of insulin action with respect to glucose uptake, ie, insulin resistance. Alterations in FA metabolism in tissues, such as muscle and liver, seem to be involved in the development of tissue-specific insulin resistance. Increased intramuscular TG content was found to be inversely associated with insulin action in type 2 diabetic patients. In type 2 diabetes mellitus, thiazolidinediones (eg, rosiglitazone) are used as a treatment, and its main action is thought to involve lowering of FA supply to insulin-sensitive tissues.[19] The present study was performed to address the question as to whether rosiglitazone

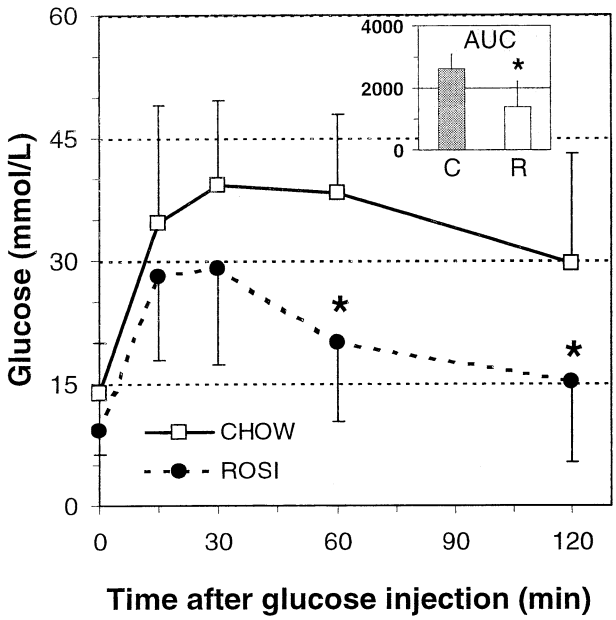

Fig 2. Glucose tolerance test. Mice were fasted for 4 hours and glucose (2 g/kg as a 25% D-glucose solution) was administered IP. Plasma glucose levels were monitored in time as described in Materials and Methods. Values represent mean glucose levels ± SD of 6 mice per group. *$P < .05$ CHOW *v* ROSI using Mann-Whitney nonparametric test for 2 independent samples. Bars in the upper right corner represent the AUC during the glucose tolerance test for chow-fed (C) and rosiglitazone-treated mice (R). *$P < .05$ CHOW *v* ROSI using Mann-Whitney nonparametric test for 2 independent samples.

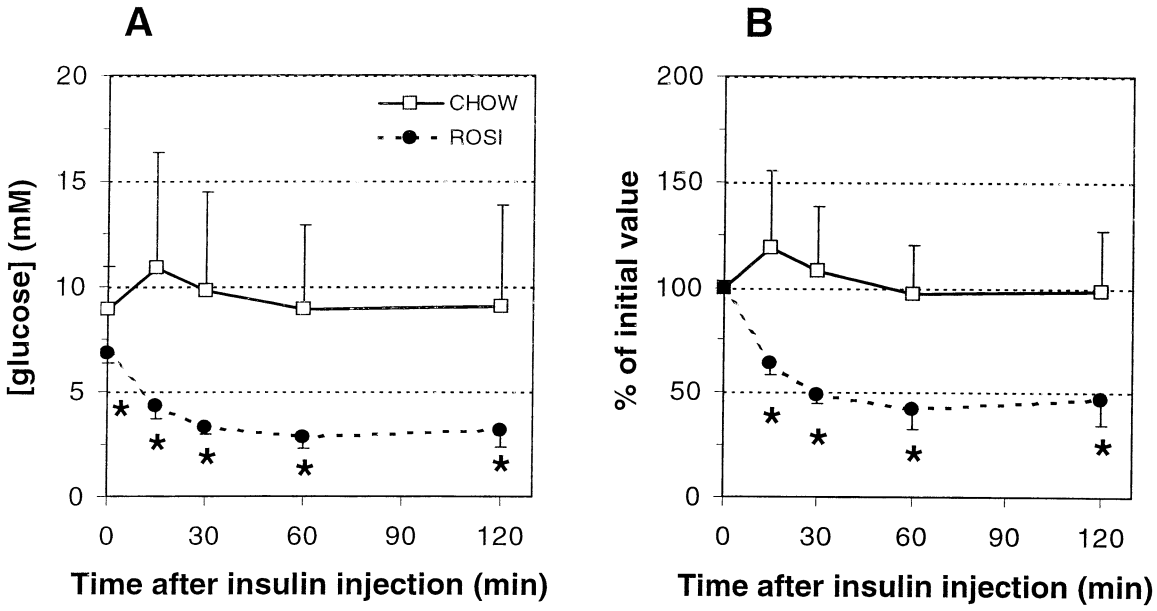

Fig 3. Insulin sensitivity test. Mice were fasted for 4 hours and insulin (1.0 U/kg as a 0.2 U/mL solution) was administered IP. Changes in plasma glucose were monitored in time as described in Materials and Methods. Values represent (A) mean plasma glucose ± SD and (B) relative changes in plasma glucose (t[0] = 100%) ± SD. Number of mice: CHOW n = 5 and ROSI n = 4. *$P < .05$ CHOW *v* ROSI using Mann-Whitney nonparametric test for 2 independent samples.

treatment leads to improvement of insulin sensitivity in liver and muscle tissue concomitant with alterations in FA handling in obese, insulin-resistant *ob/ob* mice.

Compared with mice maintained on chow, rosiglitazone treatment in *ob/ob* mice led to marked increase in body weight due to increased adipose tissue mass, an observation also seen in humans treated with rosiglitazone.[20] Rosiglitazone activates PPAR-γ resulting in differentiation of adipocytes leading to an increased number of small adipocytes.

The significant decrease in plasma glucose and insulin levels, as well as in plasma TG levels in rosiglitazone-treated *ob/ob* mice, are in line with the effects of rosiglitazone treatment in humans and rodents.[4,10,21] In the current study, we directly assessed increased whole body glucose uptake using hyperinsulinemic clamps and clearly showed increased insulin sensitivity in rosiglitazone-treated *ob/ob* mice compared with untreated mice, which is in line with findings of others.[22,23] Our primary goal was, however, to measure tissue-specific glucose and FA uptake. Insulin-mediated glucose uptake in both skeletal and heart muscle was significantly increased upon rosiglitazone treatment, whereas no effect was seen on glucose uptake in WAT. In addition to insulin-mediated glucose uptake, we analyzed insulin-mediated FA acid uptake in the 2 major organs involved in FA handling, ie, liver and WAT. We did not observe any significant effect on insulin-mediated FA uptake in liver or WAT upon rosiglitazone treatment. These results seem to contradict the findings of Oakes et al,[10] who reported an increased FA uptake in WAT and decreased uptake in liver tissue. A possible explanation for the differences between our results using *ob/ob* mice and those of Oakes et al (using Zucker fa/fa rats), could be the duration of the treatment period. Oakes et al used a 3-week period, while we treated our mice for 5 weeks before measurement of tissue-specific uptake. Of course, species differences as explanation for the observed differences cannot be excluded. The fact that we did not observe changes in fasted plasma FA levels in rosiglitazone-treated *ob/ob* mice compared with untreated *ob/ob* mice sustains our results for unaltered FA uptake upon rosiglitazone treatment in *ob/ob* mice.

It is commonly assumed that intracellular TG accumulation is associated with decreased insulin sensitivity/signaling.[24,25] We questioned whether the improved insulin sensitivity observed in rosiglitazone-treated *ob/ob* mice was associated with decreased TG content in liver, muscle, and adipose tissue. We found an increased TG content in skeletal muscle of rosiglitazone-treated compared with nontreated *ob/ob* mice. However, this increase in TG content did not decrease insulin sensitivity in the muscle. Hence, our results argue against a direct inverse correlation between TG content and insulin sensitivity in skeletal muscle. The absence of an inverse correlation between muscle TG content in muscle and insulin sensitivity was found previously.[12] We recently found no effects on insulin-mediated glucose uptake in skeletal muscle with strongly increased TG content as a consequence of muscle-specific overexpression of

Table 2. Glucose Infusion Rate During and Plasma Glucose and Insulin Levels at the End of the Clamp Experiments

	CHOW (n = 6)	ROSI (n = 5)
Glucose infusion rate (μmol/kg/min)	43.3 ± 3.4	60.3 ± 8.9*
Glucose (mmol/L)	8.1 ± 1.6	6.9 ± 1.1
Insulin (ng/mL)	86.2 ± 29.7	67.8 ± 17.4

*$P < .05$ CHOW *v* ROSI using Mann-Whitney nonparametric test for 2 independent samples.

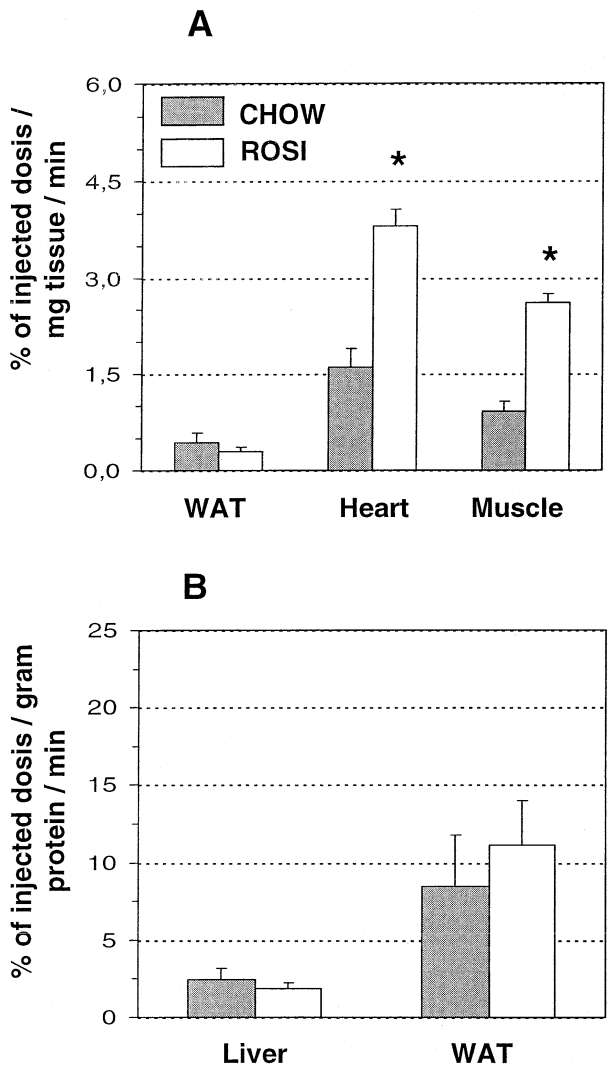

Fig 4. Tissue-specific uptake of glucose and FA. Mice were fasted overnight and tissue-specific uptake of (A) ^{3}H-labeled 2-deoxyglucose in WAT, cardiac, and skeletal muscle and (B) ^{14}C-labeled palmitate in liver and WAT was measured under hyperinsulinemic euglycemic conditions in mice treated with (ROSI) or without rosiglitazone (CHOW) after 5 weeks of treatment. Data are means ± SD for n = 4 mice per group. *P < .05 CHOW *v* ROSI using Mann-Whitney nonparametric test for 2 independent samples.

lipoprotein lipase (LPL).[12] Kim et al[26] and Ferreira et al[27] showed decreased muscle insulin sensitivity in muscle-specific LPL-overexpressing mice. Various obvious differences can be found between their and our studies, eg, genetic background, dietary fat content, body weights, muscle and liver TG content, and insulin levels during the hyperinsulinemic euglycemic clamp. Differences in genetic backgrounds are responsible for several metabolic and physiologic differences between different mouse strains. However, it remains to be elucidated what explains the observed differences among the 3 studies.

Although we carefully removed excess extramyocellular fat pads, essentially as described by Guo et al,[13] we cannot exclude that extramyocellular adipocytes, at least in part, contribute to the observed increase in TG content in muscle. Rosiglitazone treatment leads to proliferation and differentiation of adipocytes also in extramyocellular fat pads. In this respect, Mayerson et al[19] also observed an increased extramyocellular TG content in type 2 diabetic subjects after rosiglitazone treatment. Rosiglitazone is a PPAR-γ agonist and leads to activation of genes involved in FA transport, oxidation, and sequestration, eg, TG accumulation.[1,2,28] Muscle of obese mice contains more TG as compared with lean mice,[29] and the current data show that in the muscle of rosiglitazone-treated *ob/ob* mice also leads to further increased TG content (Fig 5). Muscle-specific LPL-overexpressing mice showed increased muscle TG content.[12,26,27] It has been reported that PPAR-γ agonists induce LPL activity.[30] Thus, increased LPL activity in muscle could, at least partly, explain the increased muscle TG content observed in rosiglitazone-treated *ob/ob* mice.

We measured increased TG content with increased insulin sensitivity in muscle tissue after rosiglitazone treatment, although an inverse relation between TG content and insulin sensitivity is commonly assumed. This implicates that TG content per se does not affect tissue-specific insulin sensitivity. It seems likely that FA metabolites, eg, diacylglycerols, fatty acyl CoAs, ceramide, play a crucial role in the link between muscle TG accumulation and insulin signaling. These metabolites are implicated in mechanisms involved in the development of insulin resistance[31] and are probably a better determinant of tissue (muscle) insulin resistance/sensitivity. It seems most likely that in our study, rosiglitazone not only affects intracellular TG levels, but also FA metabolites, eg, diacylglycerols, fatty acyl CoAs, ceramide. Therefore, future experiments are necessary to elucidate the tissue-specific effects of rosiglitazone on these FA metabolites, with respect to insulin sensitivity.

In conclusion, rosiglitazone treatment leads to strong improvement of insulin sensitivity in obese *ob/ob* mice, with respect to muscle insulin-mediated glucose uptake, irrespective of increased muscle TG content. On the other hand, rosiglitazone treatment had no effect on insulin-mediated FA uptake in WAT and liver of *ob/ob* mice.

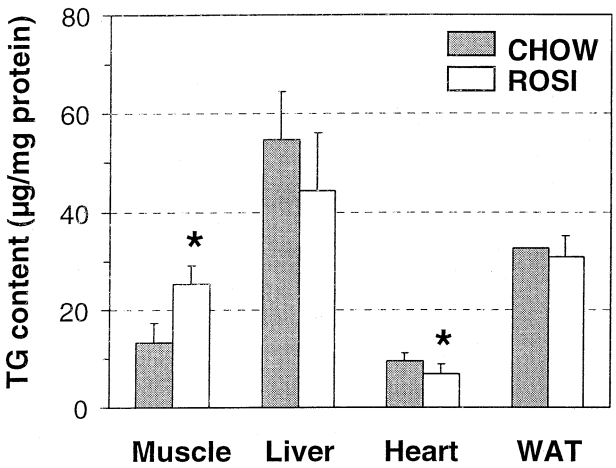

Fig 5. Tissue TG content of skeletal muscle, liver, cardiac muscle, and WAT. TG content was determined after 10 weeks of treatment. Data are means ± SD for n = 5 per group. *P < .05 CHOW *v* ROSI using Mann-Whitney nonparametric test for 2 independent samples.

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