# The Insulin Sensitizer, BRL 49653, Reduces Systemic Fatty Acid Supply and Utilization and Tissue Lipid Availability in the Rat

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**Thiazolidinediones are oral insulin-sensitizing agents that may be useful for the treatment of non–insulin-dependent diabetes mellitus (NIDDM). BRL 49653 ameliorates insulin resistance and improves glucoregulation in high-fat–fed (HF) rats. It is known that thiazolidinediones bind to the peroxisome proliferator–activated receptor (PPARγ) in fat cells, but the extent to which the improved glucoregulation and hypolipidemic effects relate to adipose tissue requires clarification. We therefore examined BRL 49653 effects on lipid metabolism in HF and control (high-starch–fed [HS]) rats. The diet period was 3 weeks, with BRL 49653 (10 μmol/kg/d) or vehicle gavage administered over the last 4 days. Studies were performed on animals in the conscious fasted state. In HF rats, rate constants governing $^3$H-palmitate clearance were unaffected by BRL 49653. This finding, taken with a concurrent decrease of fasting plasma nonesterified fatty acids (NEFA) ($P < .01$, ANOVA), demonstrated that systemic NEFA supply and hence absolute utilization are reduced by BRL 49653. Hepatic triglyceride (TG) production (HTGP) assessed using Triton WR1339 was unaffected by diet or BRL 49653. In liver, BRL 49653 increased insulin-stimulated conversion of glucose into fatty acid in both HF (by 270%) and HS (by 30%) groups ($P < .05$). Relative to HS rats, HF animals had substantially elevated levels of muscle diglyceride (diacylglycerol [DG] by 240%, $P < .001$). BRL 49653 significantly reduced muscle DG in HF (by 30%, $P < .05$) but not in HS rats. The agent did not reduce the intake of dietary lipid. In conclusion, these results are consistent with a primary action of BRL 49653 in adipose tissue to conserve lipid by reducing systemic lipid supply and subsequent utilization. The parallel effects of diet and BRL 49653 treatment on insulin resistance and muscle acylglyceride levels support the involvement of local lipid oversupply in the generation of muscle insulin resistance.**


INSULIN RESISTANCE, the characteristic metabolic disorder of non–insulin-dependent diabetes mellitus (NIDDM) and obesity, is strongly associated with hyperlipidemia. Early in vitro experiments demonstrating that fatty acid oxidation has an inhibitory effect on glucose oxidation in muscle[1] and a stimulatory effect on gluconeogenesis and hepatic glucose production (HGP)[2] led to the suggestion that insulin resistance associated with hyperlipidemia could be mediated by an oversupply of lipid to the tissues. Thiazolidinediones are a class of structurally related oral agents that may be useful for the treatment of NIDDM and related disorders, by enhancing insulin-stimulated glucose metabolism and decreasing circulating lipid levels without stimulating insulin secretion.[3,4] Mechanisms of the insulin-sensitizing actions of these compounds remain unclear. Their hypolipidemic effects raise the possibility that the improvements in glucoregulation are mediated by reduced tissue lipid availability. In vitro study[5] has established a member of the nuclear receptor superfamily of ligand-activated transcription factors, peroxisome proliferator–activated receptor γ (PPARγ), as a molecular target of thiazolidinediones. Since ligand activation of PPARγ plays an important role in triggering adipocyte differentiation of preadipocytes in vitro,[6] the question arises as to whether the hypolipidemic effects of thiazolidinediones are a result of modulation of systemic fatty acid supply from adipose tissue, or also involve altered fatty acid clearance.

In rats, long-term consumption of diets high in fat can induce profound insulin resistance in the liver and skeletal muscle.[7,8] Tissue lipid overload is manifested in these animals by substantially elevated triglyceride (TG) stores in liver and muscle, the major site of insulin-stimulated glucose disposal. Muscle lipid overload could reduce muscle glucose utilization via several mechanisms, including the classic glucose–fatty acid cycle. Insulin resistance could also result from attenuation of the insulin signal at any of the steps between the insulin receptor and the downstream metabolic enzymes. Evidence is accumulating that activation of specific members of the protein kinase C (PKC) family of isozymes can cause insulin resistance by disruption of the insulin signal.[9-11] The so-called conventional and novel PKC subfamilies are activated by diglycerides (diacylglycerols [DGs]),[12] which are also intermediates of TG synthesis. In severely hyperlipidemic Zucker obese rats, skeletal muscle insulin resistance is associated with elevated DG,[13] although its status in muscle of high-fat–fed rats appears to be unknown.

We recently reported that BRL 49653, a thiazolidinedione derivative, substantially ameliorated the hepatic and skeletal muscle insulin resistance in high-fat–fed rats.[14] In the present studies, we assessed the effect of BRL 49653 on plasma TG and nonesterified fatty acid (NEFA) turnover to investigate whether the compound decreases systemic lipid supply. In addition, we examined the influence of high-fat feeding and BRL 49653 on the levels of DG in skeletal muscle. Studies were performed in both insulin-sensitive (high-starch–fed) and insulin-resistant (high-fat–fed) rats. Our studies confirm that BRL 49653 decreases the systemic supply and utilization of lipid in the high-fat–fed rat. In addition, our data support a possible role for the DG-PKC signaling system in the generation of muscle insulin resistance induced by high-fat feeding and its amelioration with BRL 49653.

## MATERIALS AND METHODS

### Overview

Three separate series of experiments were performed. In the first, radiolabeled palmitate was administered to investigate the effects of

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BRL 49653 on whole-body NEFA metabolism. In the second series, Triton WR1339 was used to examine the effects of BRL 49653 on hepatic very–low-density lipoprotein (VLDL) TG production. In the third study, hepatic lipogenesis was assessed by measuring radiolabeled glucose incorporation into liver lipid under conditions of insulin stimulation. The first study was conducted in high-fat–fed rats only, and the second and third in both high-fat–and high-starch–fed animals.

## Experimental and Measurement Procedures

### *Animal Preparation and Dietary and BRL 49653 Treatment*

All surgical and experimental procedures performed for this study were approved by the Animal Experimentation Ethics Committee (Garvan Institute) and were in accordance with National Health and Medical Research Council of Australia guidelines on animal experimentation.

Adult male Wistar rats maintained in a temperature-controlled (22° ± 1°C) room with a 12-hour light:dark cycle (lights on at 6:00 AM) had free access to rodent chow and water until commencement of the dietary treatment. Rats (≈250 g) were then housed individually and fed isocaloric rations of either a high-starch or high-fat diet for 3 weeks up to the study day. The macronutrient composition of the fat diet expressed as a percent of total dietary calories was as follows: 59% fat, 21% protein, and 20% carbohydrate.[8] The starch diet consisted of 10% fat, 21% protein, and 69% carbohydrate.[15] Rats on both diets received equal quantities of vitamins, minerals, and trace minerals as recommended by the American Institute of Nutrition.[16] The fixed energy intake (350 kJ/d) approximated the normal intake in ad libitum chow-fed rats. Diets were freshly prepared every 3 to 4 days and were stored at 4°C. One week before the study, rats were fitted with jugular and carotid cannulae under ketamine hydrochloride (90 mg/kg)/xylazine (10 mg/kg) anesthesia. Cannulae were exteriorized via a small skin incision between the ears, and patency was maintained as previously described.[15] Each diet group was divided into BRL 49653–treated and control subgroups. Treated rats were administered four doses of BRL 49653 (10 μmol/kg/d) at 7:30 AM by gastric gavage commencing 3 days before and finishing on the study day. BRL 49653 was prepared daily for administration by dissolving 10 μmol/kg in 0.7 mL normal saline. A total of 1.4 mL was administered, including a 0.7-mL normal saline flush, via a pediatric feeding tube. Control rats received an equal volume of vehicle (saline).

### *Study 1: NEFA Disposal*

Studies were conducted in 5-hour fasted animals. Blood samples (200 μL) taken via the carotid cannula were collected into tubes containing potassium-EDTA 1, 2, 4, 8, 12, 16, 25, 35, 45, and 60 minutes after injection of a $^3$H-palmitate-albumin complex bolus via the carotid cannula (description follows). Plasma from these samples was stored immediately at −20°C for later measurement of tracer concentrations. Red blood cells (RBCs) from alternate samples were resuspended carefully in normal saline and returned to the rat to minimize blood loss. Lack of appreciable color in the plasma supernatant throughout the experiment indicated the absence of significant hemolysis resulting from this procedure. Cannula patency was maintained without the use of heparin by continuously infusing normal saline (1 mL/h) between blood sampling. Following collection of the final sample, rats were killed with an overdose of pentobarbitone (60 mg/kg intravenously [IV]), and the hindlimb muscle, red gastrocnemius, was rapidly removed and frozen by metal tongs precooled in liquid $N_2$. Tissues were stored at −70°C until assayed for TG and DG measurement.

*Preparation of the $^3$H-palmitate-albumin complex for bolus injection.* [9,10(*n*)-$^3$H]Palmitic acid ($^3$H-palmitate; Amersham, Amersham, England) was first dissolved with unlabeled palmitate and then complexed to bovine serum albumin ([BSA] 99% fatty acid–free; Sigma Chemicals, St Louis, MO). The injected bolus of 100 μL consisted of 4% (wt/vol) BSA and 0.5 mmol/L palmitate in normal saline.

*Plasma processing.* One aliquot of diluted plasma was counted directly to determine total $^3$H activity ($^3$H-Tot). Another aliquot was dried under a hot air stream before counting ($^3$H-Dry). Lipid extraction[17] was performed on a third aliquot. This procedure separated charged ($^3$H-NEFA) and uncharged ($^3$H-EFA) lipid species subfractions. The difference between $^3$H-Tot and $^3$H-Dry was assumed to represent $^3H_2O$.

### *Study 2: Hepatic TG Production Rate*

Studies were conducted following a 15-hour fast. Three basal blood samples (vol, 0.6, 0.18, and 0.6 mL) were taken 10 minutes apart for determination of plasma insulin and TG levels. RBCs from the two large blood samples (0.6 mL) were resuspended in normal saline and returned to the rat. Rats then received a 1-mL IV bolus of 20% (wt/wt) Triton WR1339 (polymeric *p*-isoctylpolyoxyethylenephenol, Tyloxapol; Sigma Chemical) in normal saline.[18] The cannula used to deliver the bolus was immediately flushed with 0.8 mL normal saline. Four blood samples (vol, 0.18, 0.6, 0.18, and 0.6 mL) were then taken at 30, 60, 90, and 120 minutes following Triton administration for TG and insulin determinations. RBCs from the 60-minute sample were returned to the rat in saline. Plasma samples were stored at −20°C until assayed.

### *Study 3: Hepatic Lipogenesis From Glucose*

Studies were conducted in 5-hour fasted animals at midphysiological plasma insulin levels (500 pmol/L) under conditions of the euglycemic-hyperinsulinemic glucose clamp. Details of the clamp procedure have been reported previously.[19] Briefly, insulin was infused at a constant rate (1.8 nmol/kg/h) and arterial glucose was maintained at basal levels by a variable infusion of 30% glucose. D-[U-$^{14}$C]glucose (Amersham) was administered as an IV bolus about 75 minutes after commencement of insulin infusion. At the end of the clamp (≈120 minutes insulin infusion), rats were killed with an overdose of pentobarbitone (60 mg/kg IV) and the liver was rapidly removed, frozen by metal tongs precooled in liquid $N_2$, and stored at −70°C. $^{14}$C incorporation into total nonpolar lipid and a saponified fatty acid extract were measured separately. The difference between these measures was assumed to represent $^{14}$C incorporated into glycerol.

### *General Tissue and Plasma Analytic Methods*

Plasma glucose and insulin levels were determined as previously described.[20] Plasma free fatty acids were determined using an acyl-coenzyme A oxidase–based colorimetric kit (NEFA-C; WAKO Pure Chemical Industries, Osaka, Japan). Plasma TG levels were measured using enzymatic colorimetric techniques (Triglyceride INT, procedure 336; Sigma). Plasma and tissue levels of $^3$H- and $^{14}$C-labeled tracers were measured as previously described.[20]

Total muscle neutral lipids were extracted.[21] Individual lipid fractions were separated using thin-layer chromatography (TLC) Silica Gel 60 F254 TLC plates (Merck, Darmstadt, Germany) in petroleum ether: diethyl ether:acetic acid (82:18:2 for TG and 82:18:4 for DG). DG and TG spots were stained with Coomassie Blue G solution (0.03% in 20% methanol for 30 minutes).[22] The plates were scanned with a Macintosh (Apple, Cupertino, CA) OneScanner, and the images were analyzed using the National Institutes of Health (NIH) Image software (NIH, Bethesda, MD).

## Mathematical Methods

### *Study 1: NEFA Disposal*

The NEFA-lowering effect of BRL 49653 (reported previously[14] and confirmed in the current study) could result from reduced entry of fatty acid into the plasma (from reductions in either lipolysis or intracellular

reesterification), or alternatively, increased clearance of plasma NEFA (from accelerated oxidation or extracellular fatty acid reesterification) might be involved.

Here, we have isolated the effect of BRL 49653 on the clearance processes alone by comparing the kinetics of labeled substrate and products (EFA and water) following bolus administration of $^3$H-palmitate in drug-treated and control animals. Because fatty acid oxidation is the only significant pathway for irreversible loss of plasma NEFA, plasma appearance of $^3H_2O$ should more accurately reflect irreversible disposal than $^3$H-palmitate kinetics alone. Plasma concentrations of corresponding labeled species in treated and untreated rats were compared directly by repeated-measures ANOVA.[23] A significant difference here would have indicated an effect of BRL 49653 on fatty acid disposal.

A corroborative (model-specific) analysis, in which measured plasma $^3$H-palmitate and $^3H_2O$ activities were jointly used to estimate irreversible $^3$H-palmitate clearance in individual animals, was also used. These calculations are outlined in the Appendix.

*Study 2: Estimation of Hepatic TG Production Rate*

IV administration of Triton WR1339 effectively blocks the clearance of plasma TG. The hepatic TG production (HTGP) rate can then be calculated from the linear rate of accumulation of TG in plasma.[18] (In the present study, linear regression of plasma TG on time in individual animals over the 120-minute exposure period revealed a mean $R^2$ of .987 with no tendency to plateau). The plasma TG clearance rate, an index of the tissues combined ability to remove TG from the circulation, was calculated as HTGP/$C_{TG}$, where $C_{TG}$ refers to the basal plasma TG concentration sampled immediately before Triton administration. The moderate duration of fasting imposed (15 hours) was chosen to minimize biases resulting from gut absorption while avoiding effects on HTGP occurring with extended fasting.[24]

*Study 3: Estimation of Hepatic Lipogenesis From Glucose*

Indices of the rates of plasma glucose incorporation into the glycerol and fatty acid lipid moieties (GI) were calculated as follows:

$$GI = Cp \cdot Cm(T) \Big/ \int_0^T Cp^*dt.$$

Cp is the plasma glucose concentration, Cm(T) is the lipid moiety $^{14}$C content per unit tissue mass at the time of tissue collection ($t = T$), and Cp* is the plasma $^{14}$C-glucose activity.[25]

## Statistical Analysis

Differences in plasma disappearance/appearance curves in study 1 were tested for significance between drug-treated and control groups by repeated-measures ANOVA.[23] Other data were analyzed as follows. Data were arranged into the following four groups: untreated fat-fed (fat control), BRL 49653–treated fat-fed (fat-treated), untreated starch-fed (starch control), and BRL 49653–treated starch-fed (starch-treated) groups. Analysis of group data was based on three a priori contrasts designed to specifically examine the principal comparisons of the study: fat control versus starch control (diet effect), fat control versus fat drug, and starch control versus starch drug (treatment effects). Statistical significance of the three contrasts was evaluated on the basis of F tests using the error mean square resulting from two-factor (diet and drug treatment) ANOVA.[23] Statistical calculations were performed using a commercial software package, Statview (Abacus Concepts/Brainpower, Berkeley, CA). Results are reported as the mean ± SEM. $P$ less than .05 was considered statistically significant.

## RESULTS

The high-fat diet for 3 weeks resulted in greater final body weight, due to greater weight gain, than the isocaloric starch diet (body weight, 369 ± 7 *v* 347 ± 6 g for fat *v* starch controls, $P < .05$). BRL 49653 treatment for 4 days did not reduce either food intake or final body weight (data not shown). Some of the basal plasma parameter responses to diet and BRL 49653 in 7-hour fasted rats are presented in Table 1. Fat feeding significantly elevated plasma glucose and reduced plasma NEFA and TG levels compared with starch feeding ($P < .01$; Table 1). BRL 49653 decreased plasma NEFA in both fat- and starch-fed rats ($P < .01$), but had no effect on plasma levels of glucose or TG.

*Study 1: NEFA Disposal*

Plasma $^3$H-species kinetics following bolus administration of $^3$H-palmitate are shown in Fig 1. An initial rapid disappearance of $^3$H-palmitate activity was reversed at later time points (12 to 35 minutes) by a leveling-off or a distinct increase in activity peaking at about 20 minutes. (Variability in the timing of this peak masks its presence in the group data shown in Fig 1.) This delayed recycling feature has been noted in previous studies in rodents,[26,27] and kinetic analysis (not shown) suggests that it is generated by labeled fatty acid that is taken up by the liver, incorporated into VLDL TG, and exported from the liver, and subsequently reappears in the plasma via VLDL hydrolysis. $^3$H-EFA kinetics (Fig 1) are qualitatively consistent with the appearance of esterified $^3$H-palmitate in plasma VLDL TG following initial uptake of $^3$H-palmitate by the liver. Oxidation of $^3$H-palmitate produces $^3H_2O$, which accumulates in the plasma (Fig 1).

There was no suggestion that BRL 49653 altered the plasma kinetics of any of the three species shown in Fig 1. Repeated-measures ANOVA yielded $P$ values for a main drug effect of .58, .97, and .48 for $^3$H-palmitate, $^3$H-EFA, and $^3H_2O$, respectively. The equivalence of these metabolite plasma concentrations in the two groups indicates that the efficiency of clearance and transfer processes between species is unchanged by BRL 49653 treatment. These results (particularly those relating to efficiency of individual transfer processes) were confirmed by the more detailed modeling analysis described in the Appendix. For example, irreversible NEFA clearance ($F_{21}$) estimated from the modeling study was equivalent at 20.2 ± 0.7 and 20.9 ± 1.6 $mL \cdot min^{-1} \cdot kg^{-1}$ for control and BRL 49653–treated rats, respectively.

A reduction in the release of fatty acid into the circulation, rather than enhanced clearance, is therefore the likely mechanism for the observed BRL 49653–induced decrease of cold

**Table 1. Basal Plasma Parameter Responses to Diet and BRL 49653 Treatment**

| | Fat | | Starch | |
|---|---|---|---|---|
| Parameter | Control | Treated | Control | Treated |
| Glucose (mmol/L) | 8.0 ± 0.2* | 8.1 ± 0.1 | 7.4 ± 0.1 | 7.6 ± 0.1 |
| NEFA (mmol/L) | 0.56 ± 0.03* | 0.44 ± 0.04‡ | 0.68 ± 0.03 | 0.51 ± 0.02§ |
| TG (mmol/L) | 0.49 ± 0.05† | 0.50 ± 0.04 | 0.88 ± 0.08 | 0.71 ± 0.08 |

NOTE. Data were obtained in basal 7-hour fasted, BRL 49653–treated or untreated, high-fat– or high-starch–fed rats. Results are expressed as the mean ± SEM (n = 8 to 15).

Fat control *v* starch control: *$P < .01$, †$P < .001$.

Fat control *v* fat treated: ‡$P < .01$.

Starch control *v* starch treated: §$P < .001$.

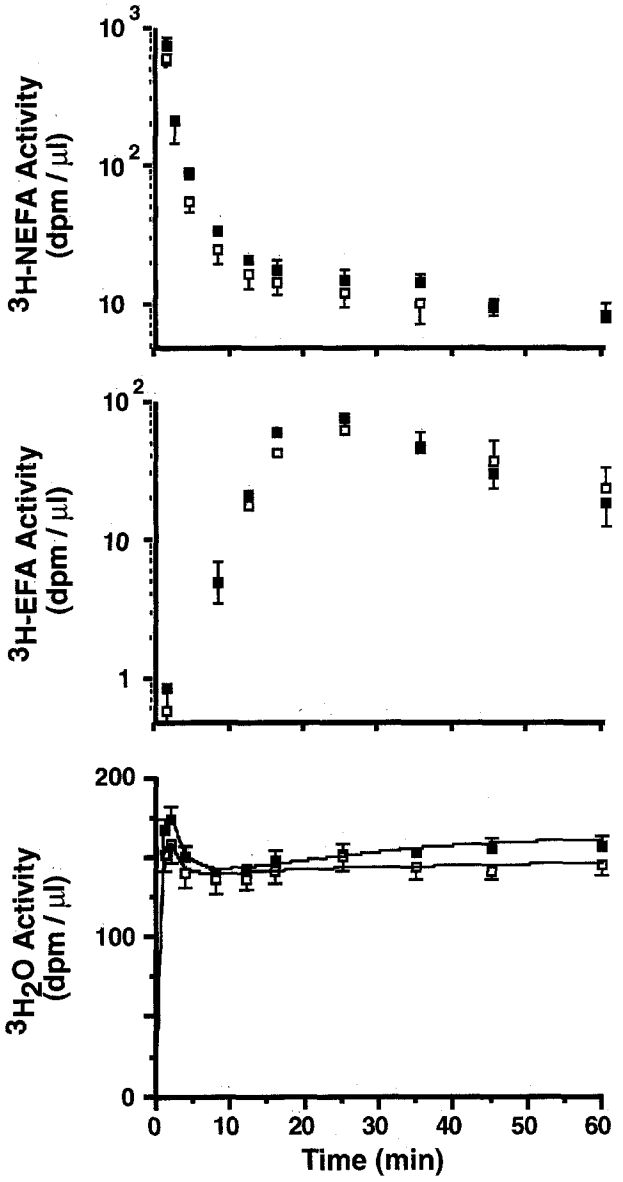


**Fig 1. Plasma $^3$H-species kinetics following IV administration of a $^3$H-palmitate bolus in high-fat–fed rats. Time course data are for BRL 49653–treated (□) and untreated (■) rats. $^3$H-NEFA and $^3$H-EFA represent, respectively, charged and uncharged lipid-soluble species. Least-square fits to $^3H_2O$ data were generated using $^3$H-NEFA data in combination with a model of systemic NEFA kinetics (see Appendix).**

NEFA plasma concentrations. Under these circumstances, lower circulating levels imply lower absolute irreversible disposal (oxidation) rates of NEFA (control BRL 49653–treated, 11.2 ± 0.6 *v* 8.7 ± 1.0 μmol/kg/min, $P = .06$).

*Study 2: HTGP Rate*

Prolonged fasting (15 to 18 hours) in the rats used for estimation of TG production significantly decreased plasma TG compared with the levels in 7-hour fasted rats (TG data in Table 1 *v* Table 2, $P < .01$, length of fasting effect, three-factor ANOVA). Consistent with results from 7-hour fasted rats, basal plasma TG (levels immediately before Triton WR1339 administration) were lower in high-fat–fed animals compared with

**Table 2. Effects of Diet and BRL 49653 on Plasma TG and HTGP**

| | Fat | | Starch | |
|---|---|---|---|---|
| | Control | Treated | Control | Treated |
| Plasma TG (mmol/L) | 0.37 ± 0.04 | 0.39 ± 0.05 | 0.44 ± 0.03 | 0.48 ± 0.03 |
| HTGP (μmol/kg/min) | 2.29 ± 0.17 | 2.06 ± 0.21 | 2.06 ± 0.19 | 2.22 ± 0.18 |

NOTE. Results are expressed as the mean ± SEM (n = 6). Experiments were conducted in 15-hour fasted rats. HTGP was determined from the accumulation of TG in plasma after an injection of Triton WR1339.

starch-fed animals ($P < .05$, diet main effect, two-factor ANOVA). Following Triton WR1339 administration, plasma TG increased linearly over time (mean $R^2 = .987$), with no tendency to plateau over the 120-minute study period. Estimated rates of HTGP were not significantly affected by diet or BRL 49653 treatment (Table 2). Plasma TG clearance rate was higher in fat-fed compared with starch-fed rats (6.1 ± 0.4 *v* 4.7 ± 0.3 mL/kg/min, $P < .01$, two-factor ANOVA), but was unaffected by BRL 49653 treatment (results not shown).

*Study 3: Hepatic Lipogenesis From Glucose*

Dietary and BRL 49653 treatment effects on glucose incorporation into lipid moieties in liver are displayed in Fig 2. Compared with starch feeding, fat feeding resulted in much less glucose incorporation into the glycerol fraction ($P < .01$) and almost total suppression of incorporation into the fatty acid fraction ($P < .001$). Averaged over both diet groups, BRL 49653 increased glucose incorporation into fatty acid ($P < .05$, BRL 49653 main effect, ANOVA) but had no effect on incorporation into glycerol.

*Muscle DG and TG*

High-fat–fed animals had substantially elevated red gastrocnemius DG (by 240%, $P < .001$) and TG (350% of starch-fed controls, $P < .001$) levels. BRL 49653 treatment of high-fat–fed rats significantly reduced DG content (by 31%, $P < .05$) and tended to decrease accumulation of TG (by 24%), although

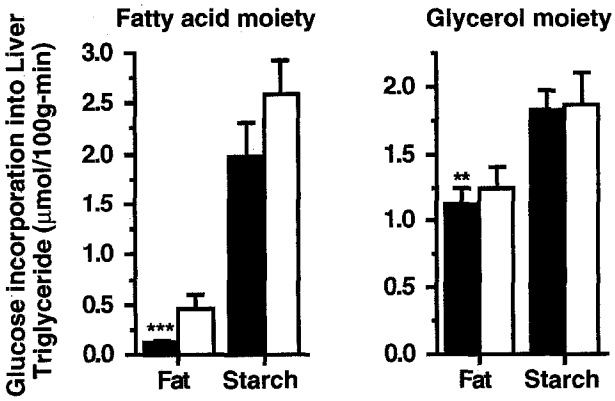


**Fig 2. Rates of $^{14}$C-glucose incorporation into liver lipid assessed under conditions of the hyperinsulinemic-euglycemic clamp in untreated (■) and BRL 49653–treated (□) groups. Fat control *v* starch control, $^{**}P < .01$ and $^{***}P < .001$.**

this did not achieve statistical significance. BRL 49653 did not significantly affect either DG or TG levels in starch-fed rats.

Previous studies have linked changes in muscle TG stores to the presence or amelioration of muscle insulin resistance[28,29]; muscle insulin-stimulated glucose utilization varies inversely with muscle TG content. Figure 3 shows the relationship of skeletal muscle insulin-stimulated glucose metabolism (previously reported[14]) with red gastrocnemius DG and TG content. The glucose metabolic index shown on the vertical axes estimates the rate of glucose uptake by muscle and is based on entrapment of 2-deoxyglucose tracer by muscle. The measurements were made under conditions of the euglycemic-hyperinsulinemic clamp in rats that had dietary and BRL 49653 treatment identical to that used in the present study.

## DISCUSSION

This study examined the effects of a thiazolidinedione derivative, BRL 49653, on lipid metabolism in rat dietary models of normal and impaired insulin sensitivity. We present strong evidence that in insulin-resistant (high-fat–fed) animals, the compound reduced the level and oxidation rate of circulating NEFA. Since NEFA oxidation is the only pathway for irreversible loss of the body's lipid stores and because BRL 49653 did not affect intake of dietary lipid, we suggest that the compound may act to contain fatty acid in adipose tissue TG stores. We postulate that the previously reported effects of BRL 49653 on glucoregulation of high-fat–fed rats, including reduced insulin levels, reduced HGP, and increased insulin-stimulated muscle glucose metabolism, are a consequence of a reduced systemic lipid supply leading to reduced tissue lipid availability.

Our analysis of fatty acid tracer kinetic data shows that BRL 49653 treatment reduces plasma NEFA levels in high-fat–fed rats without altering irreversible NEFA clearance. It was previously known that a general action of the thiazolidinediones, including BRL 49653, is to decrease plasma NEFA[14,30-33]; however, to our knowledge, the nature of this action has not been investigated. Plasma NEFA levels represent a balance between release of fatty acids from TG stores and their

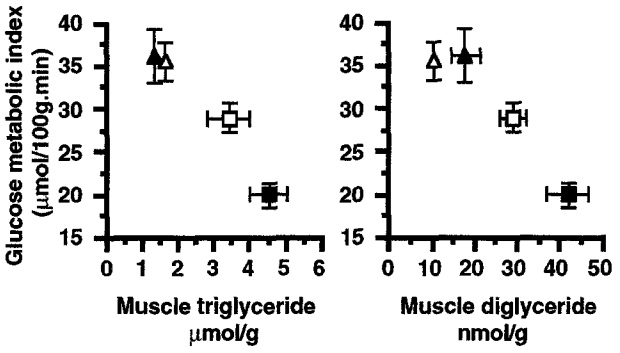


**Fig 3. Relationship between insulin-stimulated muscle glucose metabolism and red gastrocnemius TG and DG content. Points represent the mean ± SEM; muscle glucose metabolic index (reported previously[13]) and glyceride measurements were performed in separate groups (n ≥ 6) of rats. Squares denote fat-fed and triangles starch-fed results; closed symbols are control and open symbols BRL 49653–treated.**

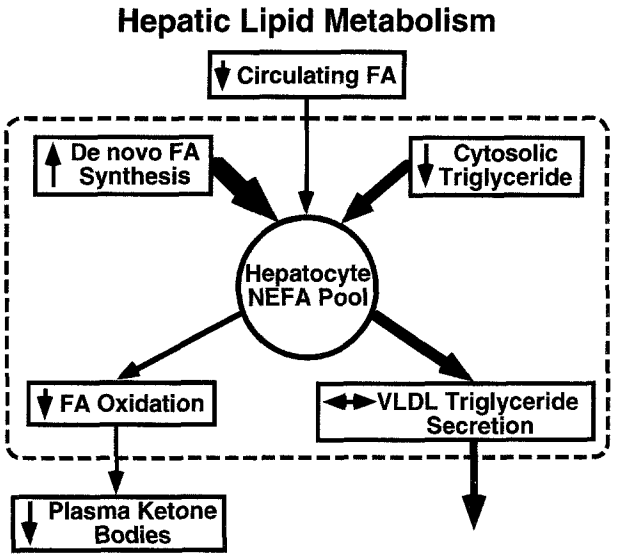


**Fig 4. Postulated effects of BRL 49653 on major sources and sinks of hepatic fatty acid (FA) metabolism in high-fat–fed rats. Net lipid fluxes are represented by the linking arrows. Postulated BRL 49653–induced changes in these fluxes are represented by arrow width: narrow signifies reduced, intermediate signifies unchanged, and broad indicates a BRL 49653–induced increase in flux. BRL 49653–induced reductions in hepatic TG stores and circulating ketone body levels were reported previously.[13]**

clearance into tissues. Reduced plasma NEFA could be a consequence of reduced mobilization, increased tissue clearance, or a combination of these processes. Effects on NEFA utilization therefore cannot be inferred from plasma levels alone. The absence of a BRL 49653 effect on $^{3}$H-palmitate disappearance in the present study (Fig 1), combined with our previously reported finding that plasma glycerol is reduced in parallel to the decrease in plasma NEFA,[14] suggests the involvement of reduced mobilization of fatty acid in the BRL 49653–induced decrease of NEFA utilization.

Reduced plasma NEFA levels might be expected to decrease HTGP because circulating fatty acids represent a major precursor source for hepatic TG synthesis.[34] It was therefore surprising that BRL 49653 treatment had no significant effect on HTGP. We hypothesize (Fig 4) that the reduced systemic NEFA supply was counteracted by increased hepatic de novo synthesis of fatty acid (as evidenced by the enhanced $^{14}$C-glucose incorporation into hepatic TG fatty acid), net utilization of hepatic TG stores over the drug treatment period (in high-fat–fed rats only[14]), and reduced hepatic fatty acid oxidation (suggested by the BRL 49653–induced reduction of circulating ketone bodies[14]). Confirming previous studies, high-fat feeding profoundly suppressed hepatic lipogenesis,[8] and since existing hepatic TG stores could not be reduced indefinitely, extension of the treatment period may have eventually decreased HTGP in high-fat–fed rats. The relatively short BRL 49653 treatment period used in this study (4 days) might explain why our data contrast with the reported effects of other thiazolidinediones to reduce hypertriglyceridemia in several states of insulin resistance.[30-32,35,36]

Thiazolidinediones are agents that enhance insulin action. Despite considerable differences in potency, the reported lipid

and glucose metabolic actions for individual agents (including ciglitazone, pioglitazone, englitazone, and BRL 49653) appear to be generally conserved for this class of compounds.[37] We have assessed BRL 49653 action in the high-fat–fed rat model of insulin resistance. The effects of BRL 49653 reported here and previously[14] in this model are consistent with general thiazolidinedione effects both in genetic rodent models of NIDDM,[30-32] and in NIDDM patients.[36] These effects include reduced basal plasma insulin, reduced basal HGP, reduced plasma NEFA, increased insulin suppressibility of HGP, and amelioration of skeletal muscle insulin resistance.

We have demonstrated here and previously[14] that BRL 49653 reduces lipid availability, which could play a major role in these glucoregulatory improvements. Reduced lipid oxidation could theoretically improve whole-body glucose regulation by enhancing skeletal muscle glucose oxidation (via the glucose–fatty acid cycle[1]) and by decreasing HGP (through reduced stimulation of liver gluconeogenesis[2]). Lipids available for oxidation are derived from circulating NEFA and TG or intracellular stored TG. Reduced NEFA oxidation may be the basis for the tendency of BRL 49653 to decrease basal HGP. However, it is unlikely to explain the compound's improvement of skeletal muscle insulin action in fat-fed animals assessed under conditions of physiological hyperinsulinemia, where differences in circulating NEFA induced by BRL 49653 were abolished. Therefore, other possibilities need to be investigated.

A link between muscle insulin resistance and lipid availability is supported by previous studies from this Institute revealing a strong inverse association between insulin-stimulated glucose uptake and local accumulation of intramuscular TG.[28,29] When combined, results of this and a companion study[14] also conform to this relationship (Fig 3). Muscle TG could theoretically be linked to local glucose oxidation through operation of the glucose–fatty acid cycle. However, this classic mechanism fails to predict effects on nonoxidative glucose disposal, which accounts for the majority of insulin-stimulated glucose uptake into skeletal muscle.

Results of the present study extend the relationship between muscle insulin resistance and local lipid availability beyond TG to include muscle DG content (Fig 3). DGs are activators of specific PKC isoforms, which in turn can modulate insulin action.[10] Increased DG and altered PKC expression in skeletal muscle of insulin-resistant animal models have been reported.[38] The DG-PKC signaling system could therefore provide a mechanistic link between states of increased intramuscular lipid availability and insulin resistance. The parallel effects of BRL 49653 and diet on DG content and insulin resistance are consistent with involvement of this mechanism in the changes in skeletal muscle insulin resistance. However, the DG-lowering effect of BRL 49653 is in contrast to results from a recent study showing that skeletal muscle DG levels of $KKA^Y$ mice were increased by pioglitazone treatment.[39] This discrepancy is not readily resolved, but may be related to differential effects of the thiazolidinedione treatments on synthetic versus degradative pathways of DG metabolism in the two models of insulin resistance.

From a mechanistic viewpoint, the variation of muscle DG and circulating TG with diet is enlightening. Starch feeding is associated with high circulating TG and low muscle DG levels, whereas low circulating TG and high muscle DG result from fat feeding. (Analysis of data common to both Tables 1 and 3 revealed a significant negative relationship between DG and TG, n = 15, $R^2 = .817$, $P < .0001$.) We therefore suggest that muscle uptake of circulating TG fatty acid is a major determinant of muscle DG content. This interpretation is based on the following considerations. HTGP was unaffected by dietary or BRL 49653 treatment. Therefore, the lower plasma TG associated with high-fat feeding versus starch feeding probably resulted from enhanced clearance into the tissues. Increased muscle uptake was a likely component of this enhancement, because fat feeding elevates muscle lipoprotein lipase (LPL).[40,41] This would have the effect of diverting a greater fraction of plasma TG fatty acid into muscle, as tissue uptake of fatty acids from circulating TG is directly related to the amount of LPL in the capillary beds of the tissue.[42] Finally, the relationship between plasma TG and muscle DG combined with the possible mechanistic link between elevated DG and insulin-stimulated glucose metabolism suggest that an elevated flux of fatty acid derived from circulating TG may be important in the etiology and maintenance of muscle insulin resistance in the high-fat–fed rat.

We believe our present findings regarding BRL 49653 effects on lipid metabolism and our previous findings regarding effects on glucose metabolism are consistent with a primary site of action of BRL 49653 in adipose tissue. Details of the cellular mechanisms of thiazolidinedione action are emerging, with recent in vitro study showing BRL 49653 to be a potent inducer of adipogenesis through activation of a specific PPARγ, a member of the nuclear receptor superfamily.[5] Adipogenesis, which can occur throughout the life of a rat or a human,[43] commits the differentiating cells to a process of TG filling, which could potentially sequester lipid, thereby reducing systemic lipid supply and utilization. The current findings that BRL 49653 administration reduced lipid oxidation in rats with a fixed daily intake of diet implies reduced energy expenditure and increased adiposity with prolonged treatment. A recent study has demonstrated increased adiposity in normal rats treated with BRL 49653, but this was associated with increased food intake, probably effected via PPARγ-induced downregulation of the satiety factor leptin.[44] A study has also been performed in animal models of NIDDM, the obese Zucker rat and the ob/ob mouse, which are able to produce leptin but cannot respond to it.[45] Consistent with our findings, thiazolidinedione treatment induced substantial adiposity in these animals without increased food intake.[45] However, species differences may exist, because clinical trials with another thiazolidinedione, troglitazone, have generally failed to reveal significant treatment-induced weight gain.[36,46,47] Finally, as discussed here and previously, the glucoregulatory actions of BRL 49653 (including reduced basal insulin secretion, reduced basal HGP, increased insulin suppressibility of HGP, and increased insulin-stimulated skeletal muscle glucose metabolism) could be indirect effects of reduced tissue lipid availability resulting from reduced systemic lipid supply.

In conclusion, our results support the hypothesis that the antidiabetic agent BRL 49653 acts to conserve adipose tissue TG stores through reduced systemic lipid supply and utilization.

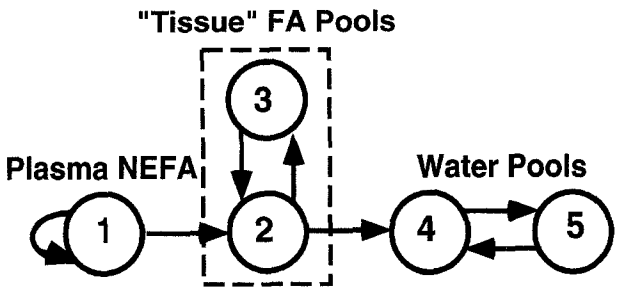


**Fig 5. Model of systemic NEFA kinetics comprising a plasma measurement pool serving as an input into a 2-pool "tissue" subsystem from which $^3$H-palmitate is irreversibly converted to $^3H_2O$. Pool 4 represents the $^3H_2O$ plasma measurement pool that undergoes equilibrative exchange with pool 5.**

Future study is needed to define the in vivo role of adipogenesis. However, the compound may be triggering a response in fat tissue that is normally activated by an overload of dietary lipid. Finally, the associations between glucose metabolism and systemic, as well as muscle, lipid availability provide further support for the role of elevated lipid availability in the development and maintenance of insulin resistance.

## APPENDIX: CALCULATION OF NEFA OXIDATION RATE

The methods used here for analysis of $^3$H-palmitate disappearance and $^3H_2O$ appearance in plasma are based on compartmental analysis[27] for analysis of plasma $^{14}$C-palmitate disappearance and $^{14}CO_2$ appearance in the breath. The present model is shown in Fig 5. Compartments representing $CO_2$ pools in the previously described model were replaced by a standard compartmental representation of whole-body water.[48]

The general approach was to obtain the model-predicted plasma $^3H_2O$ activity time course by using the measured plasma $^3$H-palmitate activity as a forcing function, providing an input to a tissue subsystem from which $^3$H-palmitate was irreversibly lost via oxidation to $^3H_2O$. Mathematically, the modeling involved convolving the $^3$H-palmitate curve with the plasma $^3H_2O$ response function. The plasma $^3H_2O$ response function is defined as the response in the plasma $^3H_2O$ pool (compartment 4, Fig 5) to a $\Delta$ function input in the plasma $^3$H-palmitate pool (compartment 1). The plasma $^3H_2O$ response function was calculated from the analytical solution to the system of linear differential equations governing mass balance in the individual compartments.[48] $^3$H-palmitate activity between sampling time points was calculated using quadratic interpolation. Numerical convolution was performed using fourth-order Runge-Kutta integration.

Tissue $^3$H-palmitate kinetics were represented by a "NEFA" pool and an "EFA" pool (Fig 5, compartments 2 and 3, respectively). Water kinetics were modeled using a two-compartment representation with input of newly formed $^3H_2O$ directly into the plasma pool (compartment 4). A whole-body water distribution volume (70% of body weight) was assumed. Nonlinear regression was used to estimate the fractional clearance coefficient governing transfer from compartment 1 to compartment 2 ($F_{21}$). Tissue pool uptake of NEFA was represented as an irreversible process (Fig 5), and therefore the NEFA oxidation rate can be expressed simply as $= F_{21}\ C_P$, where $C_P$ refers to plasma NEFA concentration. Goodness-of-fit was assessed quantitatively using a chi-square test.[49]

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## REFERENCES

1. Randle PJ, Garland PB, Hales CN, et al: The glucose fatty acid cycle: Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. Lancet 1:785-794, 1963

2. Ruderman NB, Toews CJ, Shafrir E: Role of free fatty acids in glucose homeostasis. Arch Intern Med 123:299-313, 1969

3. Hofmann CA, Colca JR: New oral thiazolidinedione antidiabetic agents act as insulin sensitizers. Diabetes Care 15:1075-1078, 1992

4. Bressler R, Johnson D: New pharmacological approaches to therapy of NIDDM. Diabetes Care 15:792-803, 1992

5. Lehmann JM, Moore LB, Smith-Oliver TA, et al: An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator–activated receptor $\gamma$ (PPAR$\gamma$). J Biol Chem 270:12953-12956, 1995

6. Schoonjans K, Staels B, Auwerx J: The peroxisome proliferator activated receptors (PPARs) and their effects on lipid metabolism and adipocyte differentiation. Biochim Biophys Acta 1302:93-109, 1996

7. Grundleger ML, Thenen WS: Decreased insulin binding, glucose transport, and glucose metabolism in soleus muscle of rats fed a high fat diet. Diabetes 31:232-237, 1982

8. Storlien L, James DE, Burleigh KM, et al: Fat feeding causes widespread in vivo insulin resistance, decreased energy expenditure, and obesity in rats. Am J Physiol 251:E567-E583, 1986

9. Ahmad Z, Lee FT, De Paoli-Roach A, et al: Phosphorylation of glycogen synthase by the $Ca^{2+}$ and phospholipid-activated protein kinase (protein kinase C). J Biol Chem 267:12673-12681, 1984

10. Considine RV, Caro JF: Protein kinase C: Mediator or inhibitor of insulin action? J Cell Biochem 52:8-13, 1993

11. Danielsen AG, Liu F, Hosomi Y, et al: Activation of protein kinase C inhibits signaling by members of the insulin receptor family. J Biol Chem 270:21600-21605, 1995

12. Nishizuka Y: Protein kinase C and lipid signaling for sustained cellular responses. FASEB J 9:484-496, 1995

13. Cooper DR, Watson JE, Dao ML: Decreased expression of protein kinase-C, $\alpha$, $\beta$, $\epsilon$ in soleus muscle of Zucker obese (fa/fa) rats. Endocrinology 133:2241-2247, 1993

14. Oakes ND, Kennedy CK, Jenkins AB, et al: A new antidiabetic agent, BRL 49653, reduces lipid availability and improves insulin action in the rat. Diabetes 43:1203-1210, 1994

15. Kraegen EW, Clark PW, Jenkins AB, et al: Development of muscle insulin resistance after liver insulin resistance in high-fat–fed rats. Diabetes 40:1397-1403, 1991

16. Reeves PG: AIN-76 diet: Should we change the formulation? J Nutr 119:1081-1082, 1986

17. Hagenfeldt L: A gas chromatographic method for the determination of individual free fatty acids in plasma. Clin Chim Acta 13:266-268, 1966

18. Otway S, Robinson DS: The use of non-ionic detergent (Triton WR-1339) to determine rates of triglyceride entry into the circulation of the rats under different physiological conditions. J Physiol (Lond) 190:321-332, 1967

19. Kraegen EW, James DE, Bennet SP, et al: In vivo insulin sensitivity in the rat determined by the euglycaemic clamp. Am J Physiol 245:E1-E7, 1983

20. Kraegen EW, James DE, Jenkins AB, et al: Dose-response curves for in vivo insulin sensitivity in individual tissues in rats. Am J Physiol 248:E353-E362, 1985

21. Folch J, Lees M, Sloane Stanley GH: A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497-509, 1957

22. Nakamura K, Handa S: Coomassie brilliant blue staining of lipids on thin-layer plates. Anal Biochem 142:406-410, 1984

23. Sokal RR, Rohlf FJ: Biometry. San Francisco, CA, Freeman, 1969

24. Duerden JM, Gibbons GF: Secretion and storage of newly synthesized hepatic triacylglycerol fatty acids in vivo in different nutritional states and diabetes. Biochem J 255:929-935, 1988

25. Jenkins AB, Storlien LH, Chisholm DJ, et al: Effects of nonesterified fatty acid availability on tissue-specific glucose utilization in rats in vivo. J Clin Invest 82:293-299, 1988

26. Laurell S: Recycling of intravenously injected palmitic acid-1-$^{14}$C as esterified fatty acid in the plasma of rats and turnover rate of plasma triglycerides. Acta Physiol Scand 47:218-232, 1959

27. Baker N, Gan-Elepano M, Guthrie BA, et al: Delayed recycling of plasma FFA in mice: Revised model of turnover and oxidation. Am J Physiol 253:R746-R755, 1987

28. Storlien LH, Jenkins AB, Chisholm DJ, et al: Influence of dietary fat composition on development of insulin resistance in rats. Diabetes 40:280-289, 1991

29. Storlien LH, Oakes ND, Pan DA, et al: Syndromes of insulin resistance in the rat. Inducement by diet and amelioration with benfluorex. Diabetes 42:457-462, 1993

30. Fujita T, Sugiyama Y, Taketomi S, et al: Reduction of insulin resistance in obese and/or diabetic animals by 5-[4-(1-methylcyclohexylmethoxy)benzyl]-thiazolidine-2,4-dione (ADD-3878, U-63,287, ciglitazone), a new antidiabetic agent. Diabetes 32:804-810, 1983

31. Fujiwara T, Yoshioka S, Yoshioka T, et al: Characterization of new oral antidiabetic agent CS-045. Studies in KK and ob/ob mice and Zucker fatty rats. Diabetes 37:1549-1558, 1988

32. Stevenson RW, Hutson NJ, Krupp MN, et al: Actions of novel antidiabetic agent englitazone in hyperglycemic ob/ob mice. Diabetes 39:1218-1227, 1990

33. Ikeda H, Takemori S, Sugiyama Y, et al: A new oral hypoglycemic agent, 5-[4-[2-(5-ethyl-2-pyrudyl)ethoxyl]benzyl] 2,4-thiazolidinedione (AD-4833, U-72, 102E, pioglitazone). I. Effects on glucose and lipid metabolism in normal and insulin resistant animals. Arzneimittelforschung 40:156-162, 1990

34. Gibbons GF, Burnham FJ: Effect of nutritional state on the utilization of fatty acids for hepatic triacylglycerol synthesis and secretion as very-low-density lipoprotein. Biochem J 275:87-92, 1991

35. Kobayashi M, Iwanishi M, Egawa K, et al: Pioglitazone increases insulin sensitivity by activating insulin receptor kinase. Diabetes 41:476-483, 1992

36. Suter SL, Nolan JJ, Wallace P, et al: Metabolic effects of new oral hypoglycemic agent CS-045 in NIDDM subjects. Diabetes Care 15:193-203, 1992

37. Saltiel AR, Olefsky JM: Thiazolidinediones in the treatment of insulin resistance and type II diabetes. Diabetes 45:1661-1669, 1996

38. Heydrick SJ, Ruderman NB, Kurowski TG, et al: Enhanced stimulation of diacylglycerol and lipid synthesis by insulin in denervated muscle. Diabetes 40:1707-1711, 1991

39. Saha AK, Kurowski TG, Colca JR, et al: Lipid abnormalities in tissues of the KKAy mouse: Effects of pioglitazone on malonyl-CoA and diacylglycerol. Am J Physiol 267:E95-E101, 1994

40. Kimball TJ, Childs MT, Applebaum-Bowden D, et al: The effect of training and diet on lipoprotein cholesterol, tissue lipoprotein lipase and hepatic triglyceride lipase in rats. Metabolism 32:497-503, 1983

41. Kiens B, Essen-Gustavsson B, Gad P, et al: Lipoprotein lipase activity and intramuscular triglyceride stores after long-term high-fat and high-carbohydrate diets in physically trained men. Clin Physiol 7:1-9, 1987

42. Tan MH, Sata T, Havel RJ: The significance of lipoprotein lipase in rat skeletal muscles. J Lipid Res 18:363-370, 1977

43. Ailhaud G, Grimaldi P, Negrel R: Cellular and molecular aspects of adipose tissue development. Annu Rev Nutr 12:207-233, 1992

44. De Vos P, Lefebvre AM, Miller SG, et al: Thiazolidinediones repress ob gene expression in rodents via activation of peroxisome proliferator–activated receptor gamma. J Clin Invest 98:1004-1009, 1996

45. Zhang B, Graziano MP, Doebber TW, et al: Down-regulation of the expression of the obese gene by an antidiabetic thiazolidinedione in Zucker diabetic fatty rats and db/db mice. J Biol Chem 271:9455-9459, 1996

46. Nolan JJ, Ludvik B, Beerdsen P, et al: Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone. N Engl J Med 331:1188-1193, 1994

47. Iwamoto Y, Kosaka K, Kuzuya T, et al: Effects of troglitazone: A new hypoglycemic agent in patients with NIDDM poorly controlled by diet therapy. Diabetes Care 19:151-156, 1996

48. Jacquez JA: Compartmental Analysis in Biology and Medicine. Ann Arbor, MI, University of Michigan Press, 1985

49. Press WH, Flannery BP, Teukolsky SA, et al: Numerical Recipes. Cambridge, UK, Cambridge University Press, 1986