A Tailored Therapy for the Metabolic Syndrome

The Dual Peroxisome Proliferator–Activated Receptor–α/γ Agonist LY465608 Ameliorates Insulin Resistance and Diabetic Hyperglycemia While Improving Cardiovascular Risk Factors in Preclinical Models

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A novel nonthiazolidinedione dual peroxisome proliferator–activated receptor (PPAR)-α/γ agonist, LY465608, was designed to address the major metabolic disturbances of type 2 diabetes. LY465608 altered PPAR-responsive genes in liver and fat of *db/db* mice and dose-dependently lowered plasma glucose in hyperglycemic male Zucker diabetic fatty (ZDF) rats, with an ED_{50} for glucose normalization of $3.8 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$. Metabolic improvements were associated with enhanced insulin sensitivity, as demonstrated in female obese Zucker (*fa/fa*) rats using both oral glucose tolerance tests and hyperinsulinemic-euglycemic clamps. Further characterization of LY465608 revealed metabolic changes distinct from a selective PPAR-γ agonist, which were presumably due to the concomitant PPAR-α agonism, lower respiratory quotient, and less fat accumulation, despite a similar impact on glycemia in male ZDF rats. In addition to these alterations in diabetic and insulin-resistant animals, LY465608 dose-dependently elevated HDL cholesterol and lowered plasma triglycerides in human apolipoprotein A-I transgenic mice, demonstrating that this compound significantly improves primary cardiovascular risk factors. Overall, these studies demonstrate that LY465608 beneficially impacts multiple facets of type 2 diabetes and associated cardiovacular risk, including those facets involved in the development of micro- and macrovascular complications, which are the major sources for morbidity and mortality in these patients. *Diabetes* 51:1083–1087, 2002

The World Health Organization estimated that by the year 2025, up to 300,000,000 people will suffer from type 2 diabetes (1). This alarming increase in the incidence of diabetes has been linked to several factors, including the current prevalence of obesity and sedentary lifestyles (2–4).

Although several treatment options exist, antihyperglycemic agents, in general, afford little protection from the eminent cardiovascular risk associated with type 2 diabetes (5). Therefore, a new approach aims to combine the insulin-sensitizing potential of a peroxisome proliferator–activated receptor (PPAR)-γ agonist, molecular target of the thiazolidinediones (TZDs) (6), with the beneficial lipid-modulating activities of a PPAR-α agonist, molecular target of the fibrates (7). Theoretically, this dual PPAR-α/γ agonist should be beneficial for addressing the major metabolic disorders of type 2 diabetic patients, hyperglycemia, insulin resistance, and dyslipidemia, and thus improve both complications resulting from elevated blood glucose and the cardiovascular risk associated with "syndrome X" (8).

Aside from improving dyslipidemia, cardiovascular risk may be further modified by a dual agonist because PPAR-α and -γ activation independently exert direct antiatherogenic effects in the vessel wall (9–16). Furthermore, the combined approach may diminish one of the undesirable side effects of selective PPAR-γ agonism, increased adiposity (17,18). Recently, we (G.J.E., B.A.O., C.L.B., unpublished observations) and others (19,20), have observed that PPAR-α agonists stimulate lipid oxidation and decrease adiposity in rodent models of obesity. Thus, by simultaneously stimulating both PPAR-α and -γ, the propensity for adipogenesis resulting from PPAR-γ activation may be offset by the propensity of PPAR-α activation to stimulate lipid catabolism.

The present study describes the effects of LY465608, a novel non-TZD dual PPAR-α/γ agonist. LY465608 was

tested in various rodent models of insulin resistance/type 2 diabetes, including the Zucker diabetic fatty (ZDF) rat, the *db/db* mouse, and the female obese Zucker (*fa/fa*) rat.

Lowering plasma triglycerides and elevating HDL cholesterol are primary clinical end points for PPAR-α stimulation and of paramount importance to reducing diabetic cardiovascular risk (21). Unfortunately, nature, as a result of species-specific differences in the regulation of apolipoprotein (apo)A-I, the major protein constituent of HDL cholesterol, has not provided an appropriate rodent model in which the latter PPAR-α–mediated response can be easily assessed (22). In humans, the apoA-I gene promoter is under positive control by PPAR-α, whereas in rodents, it is negatively regulated by PPAR-α. As a result of these differences, a PPAR-α agonist, such as fenofibrate, will elevate apoA-I protein and HDL cholesterol levels in humans, whereas in rodents, the same compound will lower apoA-I protein expression and HDL cholesterol levels. Thus, to examine clinically relevant PPAR-α agonist activities (HDL cholesterol–elevating potential) in a preclinical setting, the human apoA-I transgenic (apoA-I TG) mouse was used.

RESEARCH DESIGN AND METHODS

Animals. Male ZDF and female Zucker (*fa/fa*) rats were obtained from Genetic Models (Indianapolis, IN). In contrast to the Zucker (*fa/fa*) rat, the ZDF rat develops severe hyperglycemia resulting from peripheral insulin resistance and insulin deficiency (23). Male apoA-I TG mice and male C57BLKS/J-m+/+Lepr^{db} (*db/db*) mice were obtained from The Jackson Laboratories (Bar Harbor, ME). All animals were singly housed and maintained under standardized conditions (12-h light/dark cycle, 22°C) and provided free access to food and water.

Evidence for in vivo activation of PPAR-α and -γ. Male *db/db* mice (7 weeks of age) were randomly divided into four groups (vehicle control, 100 mg/kg fenofibrate, 30 mg/kg BRL49653, and 30 mg/kg LY465608; *n* = 10/ group). Animals were dosed once daily by gavage, and 6 h after the third dose, they were killed for tissue removal (liver and white adipose tissue). Total RNA was prepared from the tissue samples, and specific mRNA levels were quantified by real-time PCR.

Dose-response studies. Male ZDF rats and apoA-I TG mice were divided into five groups (vehicle control and 1, 3, 10, and 30 mg · kg^{-1} · day^{-1} LY465608 doses; *n* = 5/group). Rats (8 weeks of age) were dosed once daily by oral gavage in the morning for 14 days. The dosing regimen was similar for apoA-I TG mice, except for the study duration, which was 7 days. ZDF rat plasma glucose levels were assessed (conscious, tail bleed) 1 h after dosing on days 3, 7, 10, and 14 with a clinical chemistry analyzer (Monarch 2000 Multianalyzer; Instrumentation Laboratories, Lexington, MA). Serum was collected from apoA-I TG mice (CO$_2$ anesthesia, cardiac puncture) 3 h after dosing on day 7 for the assessment of HDL cholesterol and triglycerides. Human apoA-I protein levels in serum were assessed spectrophotometrically (catalog no. 356-125; Sigma, St. Louis, MO). For HDL cholesterol determination, serum samples for each treatment group were pooled and lipoprotein fractions separated by fast-protein liquid chromatography using a Superose 6 column (Amersham Pharmacia Biotech, Uppsala, Sweden). Cholesterol in the eluate was detected by spectrophotometry using an inline detection system.

Indirect calorimetry and body composition study. Male ZDF rats were divided into three groups (vehicle control, 10 mg · kg^{-1} · day^{-1} LY465608, and 10 mg · kg^{-1} · day^{-1} BRL49653; *n* = 5/group) based on starting plasma glucose levels (conscious, tail bleed) and body composition as assessed by dual-energy X-ray analysis (DEXA) scan. Rats were dosed orally by gavage once daily for 28 days. Body weight and food consumption was measured three times per week while the animals were maintained under standard husbandry conditions. Once weekly, plasma glucose levels were assessed, and rats were placed in a metabolic chamber (24-h period) for indirect calorimetry measurements (Oxymax; Columbus Instruments International, Columbus, OH). Food consumption was also assessed for the specific periods in which the animals were maintained in the metabolic chambers. At the end of the 28-day dosing period, body composition was reassessed.

Insulin sensitivity studies. Female obese Zucker (*fa/fa*) rats, 12 weeks of age, were divided into three groups (vehicle control, 10 mg · kg^{-1} · day^{-1} LY465608, and 30 mg · kg^{-1} · day^{-1} LY465608; *n* = 4/group). The rats were then
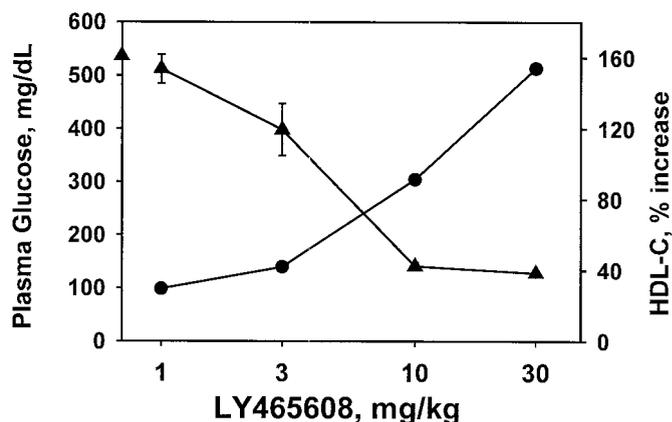

FIG. 1. LY465608 dose-response effects on plasma glucose levels (ZDF rat, ▲) and HDL cholesterol levels (apoA-I TG mouse, ●).

dosed by oral gavage once daily for 10 days at the indicated doses. After the last dose, the rats were fasted overnight and subjected to a second oral glucose tolerance test the next morning.

In a separate study, 10-week-old female *fa/fa* Zucker rats underwent surgical implantation of venous and arterial catheters. Two catheters were implanted in the jugular vein for infusion of ^{3}H-glucose, dextrose, and insulin, and one catheter was placed in the carotid artery for blood sampling during the clamp. Animals were allowed to recover from surgery at least 1 week before dosing with LY465608 or vehicle. LY465608 (10 mg/kg) or vehicle was administered by oral gavage for 14 days. After 14 days of dosing and an overnight fast, the animals underwent the hyperinsulinemic-euglycemic clamp procedure.

Statistical analysis. Results are expressed as means ± SE. Data were analyzed by ANOVA, with significant differences between means identified using Fisher's protected least-significant difference test. Differences were considered significant at $P < 0.05$.

RESULTS

Evidence for in vivo activation of PPAR-α and -γ. Liver acyl-coenzyme A oxidase appeared to be responsive to both PPAR-α and -γ because fenofibrate, BRL49653, and LY465608 increased mRNA levels 3.2-, 2.9-, and 3.8-fold over control levels, respectively. Liver apoC-III appeared to be more PPAR-α responsive as fenofibrate and LY465608 suppressed mRNA levels by 50 and 71% relative to control, respectively, whereas BRL49653 treatment resulted in 24% suppression.

In white adipose tissue, fatty acid–coenzyme A ligase long-chain 2 appeared to be a relatively selective PPAR-γ– responsive gene, as BRL49653 and LY465608 increased mRNA levels 3- and 3.7-fold over control, respectively, whereas fenofibrate increased fatty acid–coenzyme A ligase long-chain 2 mRNA by 33%.

Dose-response studies. As shown in Fig. 1, LY465608 dose-dependently lowered plasma glucose levels in ZDF rats. The ED$_{50}$ for half-maximal glucose lowering was calculated to be 3.8 ± 1.8 mg · kg^{-1} · day^{-1}, and glucose normalization occurred at 10 mg · kg^{-1} · day^{-1}. In apoA-I TG mice, a maximal increase in HDL cholesterol of 154% above control was observed at 30 mg · kg^{-1} · day^{-1}.

Along with HDL cholesterol changes, there were dose-dependent increases in serum total cholesterol (twofold at 30 mg · kg^{-1} · day^{-1}) and human apoA-I protein levels. Whereas fenofibrate (100 mg · kg^{-1} · day^{-1}) elevated human apoA-I protein levels 165% above control, LY465608 (1, 3, 10, and 30 mg · kg^{-1} · day^{-1}) increased human apoA-I protein 10, 76, 258, and 392%, respectively. LY465608 also
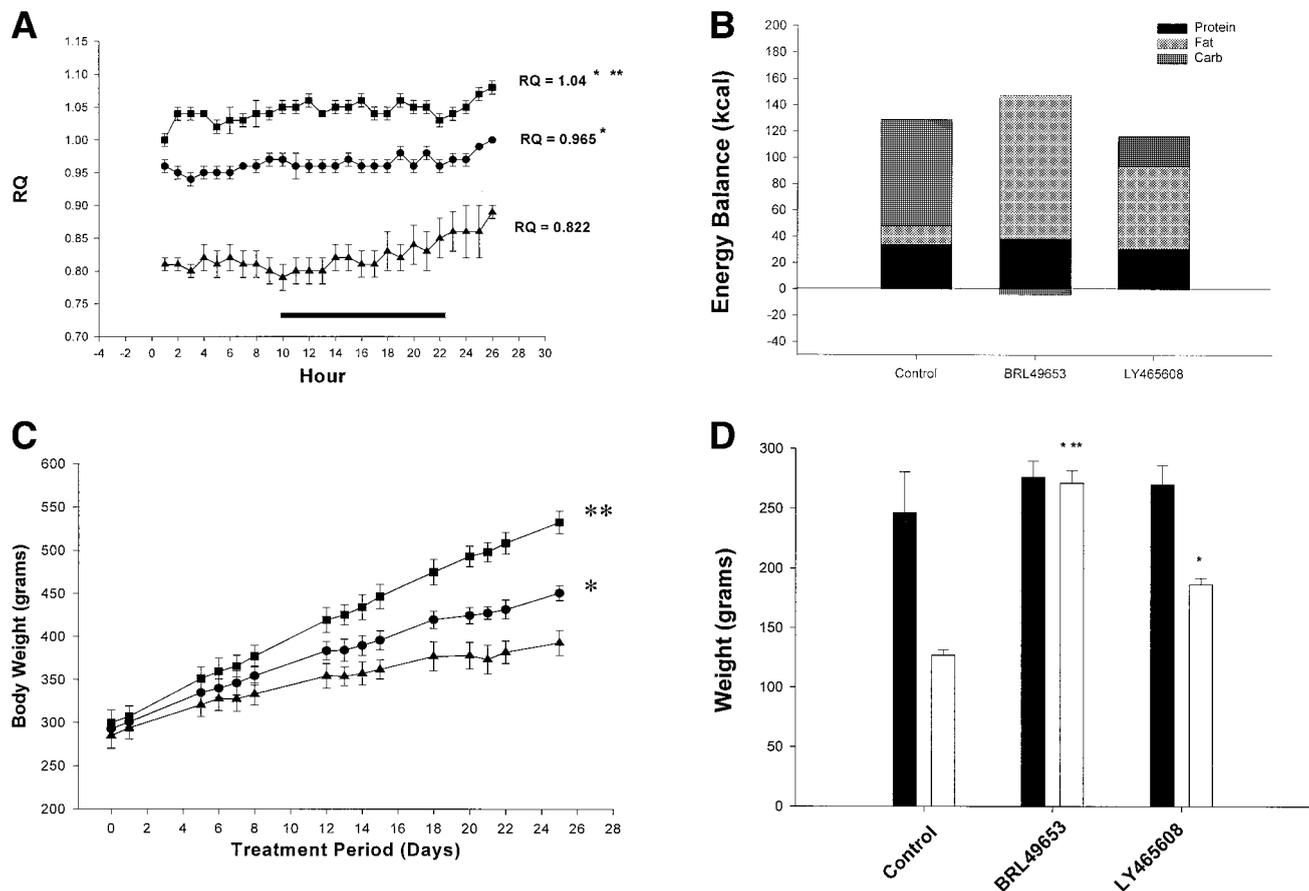
FIG. 2. *A*: Twenty-four hour RQ measurements in control (▲), BRL49653-treated (10 mg · kg⁻¹ · day⁻¹, ■), and LY465608-treated groups (10 mg · kg⁻¹ · day⁻¹, ●) on the 28th day of treatment. *B*: Energy balance (energy intake/energy expenditure) in control, BRL49653-treated (10 mg · kg⁻¹ · day⁻¹), and LY465608-treated (10 mg · kg⁻¹ · day⁻¹) animals after 28 days of treatment. *C*: Body weight in control (▲), BRL49653-treated (10 mg · kg⁻¹ · day⁻¹, ■), and LY465608-treated groups (10 mg · kg⁻¹ · day⁻¹, ●). *D*: Lean (■) and fat mass (□) in control, BRL49653-treated (10 mg · kg⁻¹ · day⁻¹), and LY465608-treated (10 mg · kg⁻¹ · day⁻¹) animals after 28 days of treatment. *Significantly different from control ($P < 0.05$); **significantly different from LY465608 ($P < 0.05$).

produced dose-dependent reductions in serum triglycerides (90% reduction at 30 mg · kg⁻¹ · day⁻¹), with an ED$_{50}$ calculated to be 3.1 ± 1.7 mg · kg⁻¹ · day⁻¹.

Indirect calorimetry and body composition study. Both LY465608 and BRL49653 normalized plasma glucose levels in male ZDF rats at the dose of 10 mg · kg⁻¹ · day⁻¹. This effect was noted after 1 week of dosing and persisted throughout the duration of the study for both compounds (data not shown). As seen in Fig. 2*A*, both LY465608 and BRL49653 shifted the pattern of energy metabolism toward carbohydrate use (as indicated by respiratory quotient [RQ] value near or above 1.0). The BRL49653 group displayed significantly higher RQ values (RQ = 1.04) than both the LY465608 (RQ = 0.965) and control (RQ = 0.822) groups. Average food intake during the five calorimetry measurement periods (24-h periods) was the same for all groups. Energy balance (intake/use) during the final calorimetry period for the three groups is displayed in Fig. 2*B*. Body weight measurements over the course of the study are displayed in Fig. 2*C*.

Whereas both treated groups showed significant weight gain relative to vehicle control, the LY465608 response was blunted relative to BRL49653. When measured under standard husbandry conditions over the course of the study, LY465608-treated rats consumed approximately the same amount of food as control rats, and BRL49653-treated rats consumed significantly more (~7 g more per day). DEXA scanning revealed that changes in whole-body mass in both treated groups were attributable to enhanced fat mass, with the BRL49653 group showing a significantly greater increase than the LY465608 group (Fig. 2*D*).

Insulin sensitivity studies. As shown in Fig. 3*A*, LY465608 (both at 10 and 30 mg · kg⁻¹ · day⁻¹) significantly improved (lowered) the glucose response to an oral glucose challenge. Furthermore, both fasting insulin levels and the insulin response to the glucose challenge were significantly reduced in the rats treated with LY465608 (Fig. 3*B*).

Moreover, in the glucose clamp study, LY465608 significantly increased the glucose infusion rate and the insulin-stimulated glucose disposal rate during the steady-state phase of the hyperinsulinemic-euglycemic clamp. The rate of steady-state hepatic glucose output was also significantly lower in the LY465608-treated group (Table 1).

DISCUSSION

Type 2 diabetic patients possess the same risk for mortality from myocardial infarction as nondiabetic patients with a previous history of myocardial infarction (24). The increased risk of coronary heart disease in diabetic patients
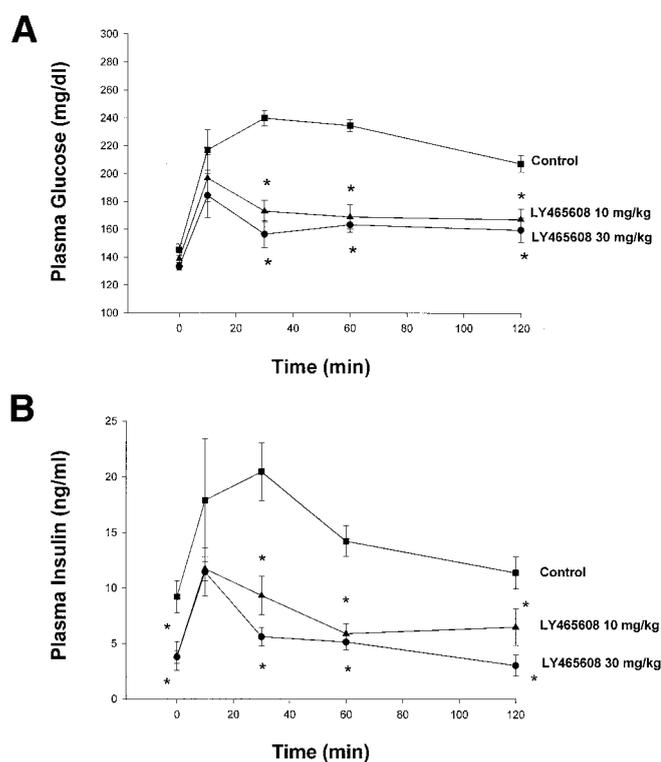
FIG. 3. *A*: **Effects of LY465608 on the plasma glucose response to an oral glucose challenge (2.5 g/kg).** *B*: **Effects of LY465608 on the plasma insulin response to an oral glucose challenge (2.5 g/kg). *Significantly different from control (*P* < 0.05).**

stems from the clustering of cardiovascular risk factors, including dyslipidemia (hypertriglyceridemia, low HDLs, and small dense LDLs), coagulopathy (elevated fibrinogen and plasminogen activator inhibitor-1), hypertension, and obesity, in addition to the hallmark problems of insulin resistance and hyperglycemia (21). Recent publications from the U.K. Prospective Diabetes Study have revealed that in type 2 diabetes, intensive glucose-lowering therapy is relatively ineffective at reducing macrovascular disease, despite decreasing microvascular complications (5). Thus, a clear goal for new antidiabetic therapies is not only to further enhance glycemic control but also to significantly improve the cardiovascular risk associated with diabetic dyslipidemia. This study describes one such tailored approach in which the insulin-sensitizing/glucose-controlling effects of PPAR-γ activation and the lipid/cholesterol-altering effects of PPAR-α activation have been combined within a single molecule, LY465608.

Two previous studies have described compounds that activate both PPAR-α and -γ. One of these compounds, KRP-297, is a TZD derivative, whereas the other, GW2331, is a fibrate derivative (25,26). In neither case, however, were the effects of these compounds examined in clearly diabetic animals or in animal models in which a humanized lipid/cholesterol response to PPAR-α was observable. Thus, it is difficult to speculate on the therapeutic utility of these compounds.

The structure and synthesis of LY465608, which binds with high affinity to both human PPAR-α (IC_{50} = 174 ± 17 nmol/l) and PPAR-γ (IC_{50} = 548 ± 72 nmol/l), has been previously documented (27) (compound 8). Moreover, in cell-based cotransfection studies, LY465608 was shown to

be an efficacious agonist at the human PPAR receptors (PPAR-α EC_{50} = 149.5, PPARγ EC_{50} = 882.0) as well as at the murine PPAR-α receptor (EC_{50} = 2,560 nmol/l). Incidentally, LY465608 also activates human PPAR-δ in cotransfection studies, although it is unclear what physiological significance this activity may impart. Potentially, activity at PPAR-δ, in addition to PPAR-α and -γ, may further improve cardiovascular risk, as it has been recently reported that selective agonists for PPAR-δ appear to increase HDL cholesterol in *db/db* mice (28) and in obese insulin-resistant nonhuman primates (29).

In vivo administration of LY465608-altered gene expression in a PPAR-α– and PPAR-γ–dependent manner in the *db/db* mouse, dose-dependently lowered plasma glucose in the male ZDF rat, improved insulin sensitivity in the obese insulin-resistant female Zucker (*fa/fa*) rat, and positively impacted lipid/cholesterol homeostasis (decreased serum triglycerides and elevated HDL cholesterol) in the apoA-I TG mouse. These results preclinically confirm that a dual PPAR-α/γ agonist approach results in alterations deemed necessary to prevent both micro- and macrovacular disease in type 2 diabetic patients.

The distinct pharmacological activities of LY465608, HDL cholesterol elevation and glucose lowering, which have been attributed to activation of PPAR-α and -γ, respectively, occurred within a very similar range, as noted by the significant overlap in dose-response curves. It may be speculated that LY465608-induced PPAR-α activation acts, in a coordinate fashion with PPAR-γ activation, to further ameliorate insulin resistance and improve glycemic control; we and others (19) have recently observed that selective PPAR-α agonists possess insulin-sensitizing/glucose-lowering potential independent of PPAR-γ activity. Thus, in addition to addressing diabetic dyslipidemia, combining the PPAR-α and -γ agonist tendencies for reducing insulin resistance may lead to improved clinical efficacy, or glucose control, relative to the approach using selective PPAR-γ agonists.

An additional advantage of the dual PPAR-α/γ agonist approach over existing PPARγ selective therapies, TZDs, also stems from the modifying aspects of PPAR-α activation. Whereas PPAR-γ receptor activation is associated with adipogenesis and increased weight gain (17,18), PPAR-α receptor activation is associated with enhanced lipid catabolism and loss of fat mass in rodent models (19,20). In the current study, it was observed that long-

TABLE 1
Effects of LY465608 in female obese Zucker (*fa/fa*) rats during euglycemic clamp

	Hepatic glucose output (mg · kg^{-1} · min^{-1})	Glucose disposal rate (mg · kg^{-1} · min^{-1})	Glucose infusion rate (mg · kg^{-1} · min^{-1})
Control			
Basal	9.1 + 0.8	8.9 + 0.8	—
Steady state	7.9 + 1.0	9.8 + 0.9	2.0 + 0.7
LY465608			
Basal	6.0 + 0.4*	6.1 + 0.4*	—
Steady state	4.3 + 0.9*†	13.6 + 0.8*†	9.6 + 0.8*

Data are means + SE. *Significantly different from control (*P* < 0.05); †significantly different from basal (*P* < 0.05).

term treatment (28 days) of ZDF rats with LY465608 did not enhance food consumption and resulted in significantly less fat accumulation and body weight gain compared with rats treated with BRL49653, despite the same level of glycemic control. The disparity in body composition observed for LY465608- and BRL49653-treated groups was predicted based on the differences in food consumption and RQ for these groups. Though still not "normal" (23), the LY465608-treated animals displayed greater balance in fuel use than the BRL49653-treated animals. Presumably, this difference stems from the propensity for PPAR-α agonism to stimulate lipid oxidation balancing, with the propensity for PPARγ agonism to stimulate carbohydrate disposal and adipogenesis. Regardless of the exact mechanism, the fact that similar levels of glycemic control can be obtained with significantly less weight gain and no augmentation of food consumption suggests that dual PPAR-α/γ agonists, such as LY465608, may hold even further advantages over selective PPARγ agonists as antidiabetic therapy.

In summary, the dual PPAR-α/γ agonist LY465608 dose-dependently lowers glycemia in the ZDF rat while simultaneously improving cardiovascular risk factors (reducing serum triglycerides and elevating HDL cholesterol) in the apoA-I TG mouse. This dual PPAR-α/γ agonist approach addresses risk factors for both the micro- and macrovascular disorders associated with type 2 diabetes and thus represents a "tailored" therapy for the major causes of morbidity and mortality associated with the disease.

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