AZ 242, a novel PPARα/γ agonist with beneficial effects on insulin resistance and carbohydrate and lipid metabolism in ob/ob mice and obese Zucker rats

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Abstract Abnormalities in fatty acid (FA) metabolism underlie the development of insulin resistance and alterations in glucose metabolism, features characteristic of the metabolic syndrome and type 2 diabetes that can result in an increased risk of cardiovascular disease. We present pharmacodynamic effects of AZ 242, a novel peroxisome proliferator activated receptor (PPAR)α/γ agonist. AZ 242 dose-dependently reduced the hypertriglyceridemia, hyperinsulinemia, and hyperglycemia of ob/ob diabetic mice. Euglycemic hyperinsulinemic clamp studies showed that treatment with AZ 242 (1 μmol/kg/d) restored insulin sensitivity of obese Zucker rats and decreased insulin secretion. In vitro, in reporter gene assays, AZ 242 activated human PPARα and PPARγ with EC_{50} in the μmolar range. It also induced differentiation in 3T3-L1 cells, an established PPARγ effect, and caused up-regulation of liver fatty acid binding protein in HepG-2 cells, a PPARα-mediated effect. PPARα-mediated effects of AZ 242 in vivo were documented by induction of hepatic cytochrome P 450-4A in mice. The results indicate that the dual PPARα/γ agonism of AZ 242 reduces insulin resistance and has beneficial effects on FA and glucose metabolism. This effect profile could provide a suitable therapeutic approach to the treatment of type 2 diabetes, metabolic syndrome, and associated vascular risk factors.— Ljung, B., K. Bamberg, B. Dahllöf, A. Kjellstedt, N. D. Oakes, J. Östling, L. Svensson, and G. Camejo. **AZ 242, a novel PPARα/γ agonist with beneficial effects on insulin resistance and carbohydrate and lipid metabolism in ob/ob mice and obese Zucker rats.** ***J. Lipid Res.*** **2002.** 43: **1855–1863.**

Supplementary key words hypertriglyceridemia • peroxisome proliferator activated receptor • type 2 diabetes

The metabolic syndrome and its associated increased risk of cardiovascular disease are responsible for a major worldwide health problem (1–3). Systemic excess of fatty acids (FAs) impairs the ability of insulin to stimulate glucose metabolism in skeletal muscle, thus contributing to the whole-body insulin resistance of the metabolic syndrome (4). Furthermore, oversupply of FAs increases hepatic triglyceride production, a key factor in the generation of the atherogenic lipoprotein profile of insulin resistance that is a major cardiovascular risk factor (5, 6). New knowledge about the transcription factors called peroxisome proliferator–activated receptors (PPARs) has opened possibilities for the treatment of insulin resistance associated with type 2 diabetes (7). PPARs are ligand-activated nuclear receptors that modulate the expression of genes involved in the transport and metabolism of lipids [for recent reviews see (8, 9)]. The relative distribution of PPAR subtypes and their transcriptional responses to activation vary in a tissue- and ligand-specific manner. PPARγ (NR1C3) is expressed mainly in adipose tissue, whereas PPARα (NR1C1) is most abundantly expressed in liver, skeletal muscle, and heart. Intact, postprandial insulin signalling is required, in concert with PPARγ-stimulated gene products, for storage of free fatty acids (FFA) primarily into adipose tissue triglycerides (TGs) and, to a lesser extent, to those of liver and muscle (10–12). Activation of PPARα, on the other hand, appears to mediate FA oxidation in muscle and liver, a condition that seems to be most important during fasting (13, 14).

In insulin-resistant hypertriglyceridemic animals, selective PPARγ agonists decrease FA exposure of nonadipose tissues, including the liver, by at least two mechanisms: enhanced suppression of FFA mobilization and increased diversion of FA into adipose tissue TGs (12). These agents also enhance glucose-metabolic insulin sensitivity, possibly

Abbreviations: CYP4A, cytochrome P_{450} 4A activity; FA, fatty acid; FCS, fetal calf serum; FFA, free fatty acid; GIR, glucose infusion rate; HI, human insulin; I, insulin infusion rate; IS, insulin secretion; L-FABP, liver fatty acid binding protein; PPAR, peroxisome proliferator activated receptor; RI, rat insulin; RIA, radio immunoassay; TG, triglyceride.

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secondary to reduced exposure to FA in skeletal muscle and hepatic tissues. These actions provide the rationale for the use of PPARγ agonists, like thiazolidinediones, for improvement of blood glucose control and dyslipidemia in patients with type 2 diabetes (15, 16). On the other hand, PPARα agonists in humans ameliorate the atherogenic lipoprotein profile of insulin resistance and reduce cardiovascular disease (17–20). Animal studies suggest that PPARα may have additional beneficial effects, including protection against obesity and the enhancement of insulin-mediated muscle glucose metabolism (14, 21). PPARα agonists also stimulate liver-specific genes required for secretion of apolipoprotein A-I (apoA-I) containing HDL and decrease apoC-III synthesis, thus improving VLDL triglyceride hydrolysis by lipoprotein lipase (22).

The important functions of PPARα and PPARγ in normal lipid and glucose metabolism have motivated the search for new agonists of these transcription factors that could decrease insulin resistance and its associated dyslipoproteinemia (23–25). The documented positive liver-centred effects of PPARα agonists on FA and lipoprotein metabolism and the established actions of PPARγ agonists on FA metabolism and improved insulin sensitivity have led to the hypothesis that substances with combined PPARα/γ effects could be superior to the individual specific ligands for the treatment of insulin resistance and its associated atherogenic dyslipidemia (24, 26, 27). The properties of the ligand-binding domains of PPARα and PPARγ have allowed development of substances that are combined agonists (23, 24, 28–31).

We report here the effects of AZ 242, a novel agent that binds and activates PPARα and PPARγ with similar high potency (28). To assess its potential for correction of lipid and glucose abnormalities associated with conditions of human insulin (HI) resistance, we studied the ability of AZ 242 to improve glucose control and ameliorate insulin resistance and hypertriglyceridemia in diabetic ob/ob mice and nondiabetic insulin-resistant obese Zucker rats. The results demonstrate that AZ 242 is potent and efficient in correcting the metabolic disorders in these disease models. To confirm functional activation of endogenous PPARα and PPARγ in relevant cell lines, AZ 242 effects were examined in human liver–derived HepG2 cells and 3T3-L1 murine pre-adipocytes. Evidence for specific PPARα activation in vivo was obtained by analyzing hepatic responses in lean mice. In normal mice, a number of enzymes for oxidizing FAs are under selective PPARα versus PPARγ control. This is in contrast to obese rodent models, where these same enzymes can also be regulated via PPARγ activation (32). The present in vivo and in vitro experiments provide evidence that the metabolic effects of AZ 242 are mediated by activation of PPARα and PPARγ.

MATERIALS AND METHODS

Animals and cell lines

Male lean (Ob/?) and obese, diabetic (ob/ob) mice, and lean B6C3F1 mice, 6-weeks-old, were bred and delivered by B&M A/S Breeding and Research Centre, Ry, Denmark. Male lean (Fa/?) and obese (fa/fa) Zucker rats, 8-weeks-of-age, were obtained from Charles River Wiga GmbH, Suffield, FRG, via Charles River Uppsala, Sweden. Animals were housed in the AstraZeneca Mölndal Laboratory Animal Resources Facility in transparent polycarbonate cages, with aspen wood chip bedding at a 12 h light/darkness cycle, a temperature of 21°C, and a relative humidity of 50% throughout the accommodation (at least 1 week) and dosing periods. Unless otherwise stated, all animals had free access to standard rodent chow (R3 Laktamin AB, Stockholm, Sweden) and tap water. All animal experiments were approved by the Local Ethics Review Committee on Animal Experiments, Göteborg Region. Cell lines 3T3-L1 and HepG2 were purchased from American Type Culture Collection (ATCC, Manassas, VA).

Materials

The compound AZ 242, AstraZeneca code AR-H039242XX, an enantiomer-pure di-hydro cinnamate derivative with the chemical name (*S*)-2-ethoxy-3-[4-[2-(4-methylsulphonyloxyphenyl)ethoxy]phenyl]propanoic acid (**Fig. 1**) was synthesized at Medicinal Chemistry, AstraZeneca, Mölndal (Andersson, K., patent application WO 9962872-A). The reference compounds (rosiglitazone and pioglitazone, PPARγ agonists; WY14,643, a rodent-selective PPARα agonist; and bezafibrate, a human and rodent PPARα agonist) were obtained from the same source. In all experiments, analytical grade reagents were used.

Animal experimental procedures

Potency and efficacy in obese, diabetic ob/ob mice. In vivo potency and efficacy were determined in groups of 7–10 ob/ob mice given a particular dose of test compound by gavage (10 ml/kg, vehicle 0.5% w/v methyl cellulose in water) once daily for 8 days. On the last day of dosing, food was removed and the final dose was given at 7 AM. Four hours later, blood was collected under inhalation anaesthesia from cut neck vessels and centrifuged. For each animal in the five test groups of an experiment, plasma levels of TGs, insulin, and glucose and the percentage weight gain during the test period were expressed as a percentage of those in the concurrent control group of 10–15 untreated ob/ob mice. In one experiment, a group of age-matched lean (Ob/?) mice were included for reference.

Clamp experiments on obese, insulin-resistant fa/fa Zucker rats. The effects of AZ 242 on insulin sensitivity were analyzed in euglycemic hyperinsulinemic clamp experiments in anesthetized obese fa/fa Zucker rats (n = 6) pre-treated for 1 week with a daily oral dose of AZ 242, 1 μmol/kg/d in 0.5% methyl cellulose, 2.5 ml/kg. Matched vehicle-treated obese (n = 4) and lean (Fa/?) (n = 3) Zucker rats served as controls. On the day of the clamp experiment, the final gavage was given at 07:00 and food was removed. The animals were anesthetized with 180 (obese Zuckers) or 120 (lean Zuckers) mg/kg intraperitoneal Na-thiobutabarbitol (Inactin®, RBI/Sigma, St. Louis, MO). Following tracheotomy, the

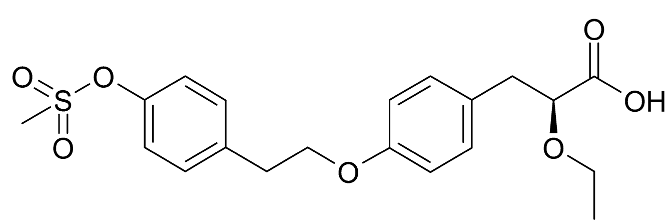


Fig. 1. Structure of the di-hydro cinnamate derivative AZ 242 [(*S*)-2-ethoxy-3- [4-[2-(4-methylsulphonyloxyphenyl)ethoxy]phenyl]propanoic acid].

spontaneously breathing animals were fitted with a carotid artery catheter for blood sampling and recording of arterial blood pressure as well as three catheters in a jugular vein for infusions of insulin and glucose and for top-up doses of anaesthetic, if needed, respectively. Rectal temperature was maintained at 38°C by means of external heating. Blood pressure, heart rate, and body temperature were monitored on a custom-made computerized recording system (PC-Lab, AstraZeneca, Sweden). The following protocol was used in all experiments. A stabilization period of at least 120 min elapsed between completion of the surgical preparation and commencement of the clamp, 5–7 h after removal of food. Blood glucose levels were determined every 5 min (YSI 2700 glucose analyzer, YSI, Inc., Yellow Springs, OH) using a blood sampling method allowing minimal sampling volumes (15 μl/sample). The hyperinsulinemic clamp was commenced once three successive blood glucose readings were within 10% of their mean value, which was used as the target glucose level for the subsequent clamp.

HI (Actrapid®Novo Nordisk A/S Bagsvaerd, Denmark), 10 mU/kg lean body mass/min, was infused using a syringe pump (CMA 1100, Carnegie Medicine, Solna, Sweden). Blood glucose was clamped to within 10% of the target glucose level by means of variable rate 20% (w/v) glucose (Glucos 200 mg/ml, Fresnius Kabi AB, Uppsala, Sweden) infusion, using another syringe pump (Model 22 I/W, Harvard Apparatus, Inc., South Natic, MA). A computer program (Gluclamp.V2.1A, AstraZeneca) was used to record blood glucose levels and the glucose infusion rate (GIR) and to set the glucose infusion pump according to the rate determined by the operator. The clamp period was defined as the earliest 30 min period during insulin infusion in which blood glucose (sampled once every 5 min) stayed within 10% of the target glucose level without any alteration in GIR. Immediately before insulin infusion was started and at the end of the clamp period, blood samples (200 μl) were collected from the arterial catheter directly into vials containing potassium-ethylenediaminetetraacetic acid (Microvette CB300, Sarstedt, Nümbrecht, Germany). Red blood cells were separated as rapidly as possible and plasma stored at −20°C for subsequent determination of TG, human and total insulin, C-peptide, glycerol and FFA.

To normalize for differences in body composition and plasma insulin levels, an "insulin sensitivity index" was calculated according to the following formula:

$$\frac{GIR}{blood \bullet \text{glucose}(\text{m}M)} \times \frac{bodyweight(kg)}{leanbodyweight(kg)} \times \frac{1}{plasmainsulin(nM)}$$

where GIR (μmol/min) is the steady state glucose infusion rate during clamp. The pancreatic insulin secretion, as assessed by the posthepatic insulin outflow rate, was estimated by the use of the selective insulin radio immunoassay (RIA) determination of exogenous HI and endogenous rat insulin (RI) plasma levels, based on the assumption that plasma clearance of HI and RI are equal in the rat. At steady state, the ratio of posthepatic insulin secretion (IS) and the entry of total insulin into the plasma [the sum of *IS* and exogenous insulin infusion rate (*I*)] would be as follows:

$$\frac{RI}{RI + HI} = \frac{IS}{I + IS}$$

During the clamp, *IS* could therefore be estimated, since *I* was known and *RI* and *HI* were measured. A basal insulin secretion rate IS_{Basal} could also be calculated assuming a plasma clearance equal to that obtained during the clamp.

$$IS_{Basal} = RI_{Basal}\left(\frac{I + IS}{RI + HI}\right)_{Clamp}$$

Induction of cytochrome P_{450} 4A activity in lean B6CJ3F1 mice. For determination of liver cytochrome P_{450} 4A activity (CYP4A), lean B6C3F1 mice were treated by gavage for 1 week once daily with AZ 242 (0.13 μmol/kg/d), the PPARγ agonist rosiglitazone (5 μmol/kg/d), and the PPARα agonist WY14, 643 (36 μmol/kg/d). The CYP4A-dependent lauryl ω-hydroxylase activity was measured in liver microsomes using ^{14}C-labeled lauric acid. Separation and detection of the 11- and 12-hydroxy metabolites were performed using reverse-phase HPLC coupled to a radioactivity detector (33).

In vitro experiments

Reporter gene assays. cDNAs containing the ligand binding domains of human PPARα or murine PPARα and PPARγ were amplified by PCR. Maintaining an open reading frame, the fragments were cloned 3′ to the GAL4 DNA binding domain and the nuclear localization sequence from T-antigen of Polyoma Virus in pSG5 (Stratagene, CA). A luciferase reporter plasmid was constructed by inserting five upstream activating sequences elements into the truncated SV40 promoter of pGL3-P (Promega, WI). U-2 OS cells (ATCC catalog no. HTB-96) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with resin-charcoal–stripped fetal calf serum (FCS) and transfected with the PPAR expression vectors and the luciferase reporter plasmid by electroporation using a Gene Pulser™ (BioRad, Hercules, CA). After electroporation, approximately 25,000 cells/well were seeded in triplicate 96-well plates in DMEM without phenol red. The test agents were added to the medium, and the plates were incubated for 40 h and then lysed using LucLite™ (Packard, CT) buffer. The luciferase signal was recorded in a Victor2™ plate reader (Wallach, Finland). The signals were normalized against plate-specific controls (DMSO for 0%; 16 μM pioglitazone, 4 μM WY14,643, 16 μM 5,8,11,14-eicosatetrayemoic acid for 100% activation of PPARγ, mPPARα, and hPPARα, respectively) and the values from the triplicate plates were averaged. Xlfit (ID Business Solutions) was used for fitting curves to the experimental points and to determine EC_{50}.

3T3-L1 adipocyte differentiation. Murine preadipocyte 3T3-L1 cells were cultured in DMEM with 25 mM glucose, 10% FCS and 2 mM L-glutamine at 10% CO_2. For the experiments, cells were seeded in 24-well plates, 0.5×10^4 cells/cm^2. After reaching confluence, usually after 5 to 6 days, cells were stimulated to differentiate by the addition of 0.05 mM 1-methyl-3-isobuthylxanthine (MIX) and 2 μg/ml dexamethasone (DEX), essentially as described (34). After 2 days, the DEX/MIX medium was removed, cells were washed three times with medium, and fresh medium with or without the test agent was added (duplicate wells for each drug concentration). Five days later, uptake of 2-deoxy-D-[^{3}H]glucose was measured as a marker of adipocyte differentiation (34). Cells were washed twice with 0.5 ml serum-free DMEM and incubated with 1 ml serum-free DMEM for 2 h. Thereafter, cells were washed twice with 0.5 ml Dulbecco's phosphate buffered saline (DPBS), and incubated with 1 ml DPBS in a water bath at 37°C for 10 min. Insulin (human; 1 μM) was added, and incubation continued at 37°C. After 20 min, 0.1 ml DPBS with 1 mM deoxyglucose and 6 μCi/ml 2-deoxy-D-[^{3}H]glucose was added, and incubation continued for another 10 min. Thereafter, cells were washed three times with 1 ml cold DPBS and finally solubilized with 0.75 ml 1% Triton X-100 at 37°C for 20 min. The radioactivity was determined by liquid scintillation counting using a 0.5 ml aliquot of the Triton/cell solution mixed with 0.5 ml Optiphase "Supermix" (Wallac, Turku, Finland).

HepG2 cell culture and proteomic analysis. Human liver derived HepG2 cells were seeded in a 12-well plate in triplicate at 1.5×10^5 cells/well and grown in Modified Eagle's Medium (MEM) supplemented with 10% FCS and 2 mM L-glutamine. Drug treatment was for 72 h. For metabolic labeling, medium was removed from the wells and 0.5 ml labeling medium (0.5 ml methionine-

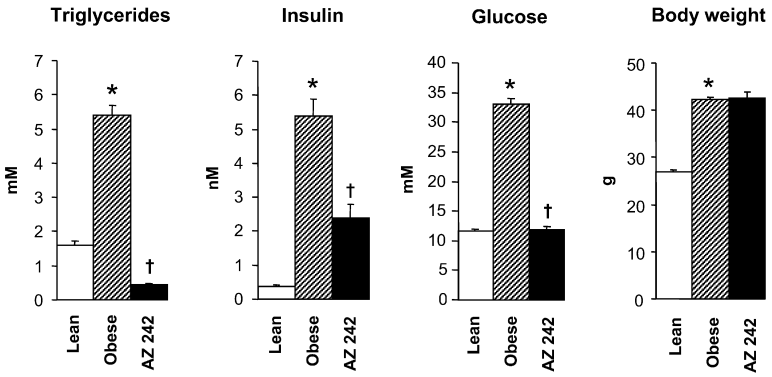


Fig. 2. Effects of AZ 242 on plasma triglyceride, insulin and glucose levels, and body weight. Age-matched lean (n = 11), obese control (n = 29), and obese AZ 242-treated ob/ob mice (n = 14) were dosed by gavage with 1 μmol/kg/d for 8 days. Control animals received vehicle alone. Values are mean ± SE. *Indicates a statistically significant difference ($P < 0.05$) between obese versus lean control; † between obese AZ 242-treated and obese control.

free MEM, supplemented with 10% FCS and 2 mM glutamine plus 21 μl [^{35}S]met Redivue PRO-MIX, [Amersham Pharmacia Biotech, Uppsala, Sweden]) was added to each well (corresponding to 0.3 mCi per well). Cells were lysed by adding 1 ml 2D-sample solution containing 8 M urea, 0.3% dithiothreitol, 0.5% IPG 3-10NL ampholytes, and 4% CHAPS (Amersham Pharmacia Biotech) to each well. Analysis of cellular HepG2 proteins by proteomics was done essentially as described using 3-10NL IPG strips in the first dimension in an IPG-Phor unit and a Hoeffer-Dalt tank for the second dimension (Amersham Pharmacia Biotech) (35). Gels were dried, and images of exposed screens were captured using an FX molecular imager (BioRad) and analyzed using PDQuest (BioRad). Protein spots were identified using mass fingerprinting and MALDI-TOF (Applied Biosystems, Framingham, MA) as described (32).

Analyses of plasma variables. Total plasma insulin was determined by RIA (Rat Insulin RIA Kit, Linco Research, Inc., St. Charles, MO). HI, administered in clamp experiments, was selectively determined with the Human Insulin RIA Kit (Linco Research, Inc.); plasma C-peptide concentrations were determined using a rat C-peptide RIA kit (Linco Research, Inc.). Colorimetric kit methods were used for the determination of plasma TGs, total protein, glucose (Glucose HK, Roche Diagnostics, Stockholm, Sweden), and plasma FFA (NEFA C, Wako, Richmond, VA). Photometric assays were performed using a centrifugal analyzer (Cobas Bio, F. Hoffman-La Roche & Co., Basel, Switzerland). CETP mass was measured by ELISA (Wako Chemie, Bad Homburg, Germany).

Statistics

Where appropriate, results were evaluated using paired parametric *t*-test. ANOVA was used when more than two groups were compared.

RESULTS

In vivo effects

Obese, diabetic ob/ob mice. The characteristically elevated plasma levels of TGs, insulin, and glucose, and increases in body weight in obese, diabetic (ob/ob) control mice, compared with those of lean (Ob/?) control mice after are shown in **Fig. 2**. Treatment with AZ 242 (1μmol/kg/d) for 1 week resulted in normalization of the hyperglycemia and a concomitant reduction in insulin levels, indicating greatly increased insulin sensitivity. At this dose,

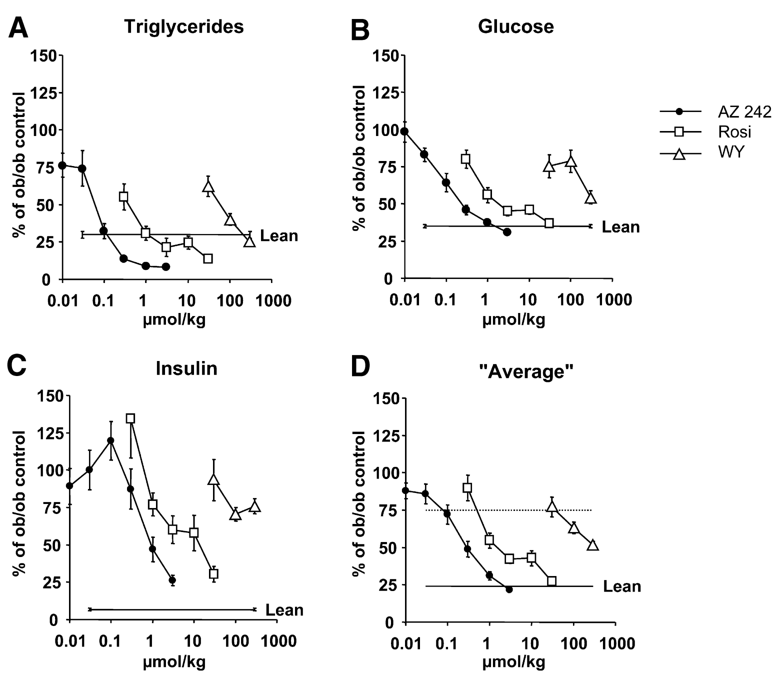


Fig. 3. Dose–response relationship between the effects of AZ 242, rosiglitazone, and WY 14,643 on plasma levels of triglycerides (TGs) (A), glucose (B), and insulin (C). Values are expressed as a percentage of control levels of concomitant placebo-treated ob/ob mice. The TG, glucose, and insulin percentage values are averaged for interpolation of ED_{25} (D). The bars indicate mean ± SE. Corresponding values for each parameter from age-matched lean animals are indicated (Lean).

TABLE 1. Insulin sensitivity in Zucker rats

	Lean		Obese Control		Obese-Treated	
	Basal	Clamp	Basal	Clamp	Basal	Clamp
Body weight (g)	380 ± 18		572 ± 11[a]		552 ± 16	
Lean body weight (g)	324 ± 14		345 ± 6		335 ± 8	
Blood glucose (mM)	4.0 ± 0.02	4.1 ± 0.03	4.4 ± 0.3	4.6 ± 0.2	5.0 ± 0.3	5.1 ± 0.3
Total insulin (nM)	0.6 ± 0.1	2.6 ± 0.1	6.4 ± 1.3[a]	9.4 ± 2.0[a]	2.0 ± 0.3[b]	3.8 ± 0.4[b]
Human insulin (nM)	<0.1	2.1 ± 0.1	<0.1	3.1 ± 0.4	<0.1	2.2 ± 0.1
C-peptide (nM)	1.2 ± 0.2	0.3 ± 0.04	5.0 ± 0.8[a]	4.4 ± 1.1[a]	2.7 ± 0.2[b]	1.8 ± 0.2[b]
TGs (mM)	1.9 ± 0.2	1.3 ± 0.2	6.8 ± 1.6	5.9 ± 1.3[a]	3.5 ± 0.3[b]	2.6 ± 0.3[b]
FFA (mM)	0.31 ± 0.04	0.05 ± 0.01	0.72 ± 0.17	0.49 ± 0.10[a]	0.66 ± 0.06	0.18 ± 0.03[b]
Glucose infusion rate (μmol/min)	–	27 ± 1.6	–	4.3 ± 31.9[a]	–	30 ± 3.3[b]

Plasma variables immediately before (Basal) and during the clamp.
Data represent mean ± SEM in lean untreated (n = 3), obese untreated (n = 4), and obese AZ 242-treated (n = 6) Zucker rats. Human insulin levels <0.1 indicate that values were below the limit of detection.
[a] $P < 0.05$; obese control versus lean control.
[b] $P < 0.05$; obese treated versus obese control.

plasma TG levels were lowered to a level below that of lean mice. There was no AZ 242 treatment–related effect on body weight. The oral in vivo potency and efficacy of AZ 242 in ob/ob mice following 1 week administration of graded doses was compared with that of the PPARγ agonist rosiglitazone and the rodent-selective PPARα agonist WY14,643 (**Fig. 3**). On the last day of dosing, 4 h after final gavage and removal of food, plasma levels of TGs, glucose, and insulin were measured. The values are presented as the percentage of the values obtained in control ob/ob mice receiving vehicle alone. In addition, an "average effect" was calculated as the mean percentage values for TGs, glucose, and insulin. This "average effect" was used for determination of the "potency" by interpolation of "ED_{25}", the oral dose causing a 25% reduction in the average effect. At any given dose, the response to each of the compounds was most prominent with regard to TG lowering (Fig. 3A), indicating that, at threshold doses, only TGs were reduced. This is compatible with PPAR-mediated effects primarily affecting lipid metabolism. Furthermore, at the high dose range, TGs were lowered below the values measured in lean untreated mice. The effects on plasma glucose levels (Fig. 3B) and basal insulin (Fig. 3C) were achieved dose dependently at higher doses than those required to lower TG. In Fig. 3D, the "average response" is expressed as a function of the administered doses. The dose causing a 25% reduction of this calculated variable was used during in vivo screening experiments in this murine model to evaluate the oral potency of new compounds during the development of AZ 242. The ED_{25} values obtained were 0.069 μmol/kg/d (AZ 242), 0.50 μmol/kg/d (rosiglitazone), and 36 μmol/kg/d (WY14,643), which correspond to a potency ratio of 1:7 versus rosiglitazone and 1:600 versus WY14,643. Figure 3 also shows the "average" for lean animals, expressed as a percentage of ob/ob controls. The intercepts of the dose response curves with the "average" line for the lean animals can be used for an approximate estimate of the doses of different PPAR agonists needed to correct the metabolic derangements of the ob/ob mice under the experimental conditions used.

Effects in obese, insulin-resistant fa/fa Zucker rats, clamp experiments. **Table 1** lists the basal levels of plasma variables measured in barbiturate-anesthetized lean (Fa/?), obese control (fa/fa), and obese treated (fa/fa) animals, 3 to 5 h after food removal and final gavage following 1 week treatment with either vehicle (lean and obese control) or 1 μmol/kg AZ 242. In addition, values recorded during euglycemic clamp conditions (10 mU/kg_{lbm}/min of HI) are also shown in Table 1. Compared with the lean Zucker rats, the age-matched untreated obese animals displayed basal hypertriglyceridemia and hyperinsulinemia but no hyperglycemia. Treatment with 1 μmol/kg/d AZ 242 for 1 week ameliorated but did not fully correct the basal hypertri-

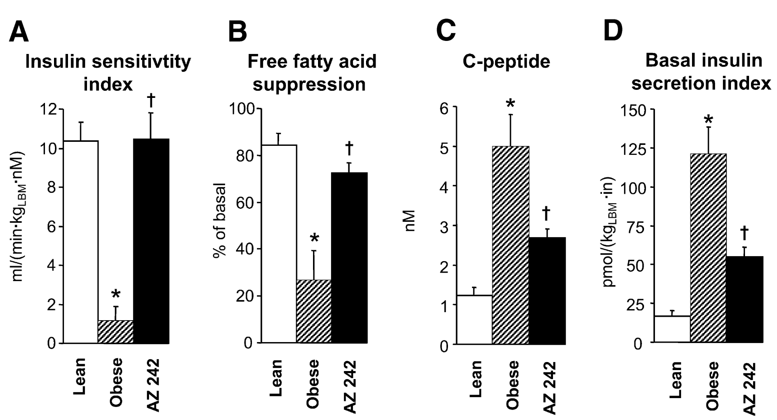


Fig. 4. Results from euglycemic hyperinsulinemic clamps in Zucker rats. Lean control (n = 3), obese control (n = 4), and obese AZ 242-treated animals (n = 6) were given vehicle or drug (1 μmol/kg/d) for 1 week prior to the clamp experiments. The insulin sensitivity index (A), clamp-induced fatty acid (FA) suppression (B), plasma C-peptide levels (C), and basal insulin secretion rate index (D), i.e., the posthepatic insulin entry into circulation, are compared with those of controls. Values are mean ± SE. * Indicates a statistically significant difference ($P < 0.05$) between obese versus lean control; † between obese AZ 242-treated and obese control.

TABLE 2. Induction of CYP4A in liver microsomes of lean B6C3F1 mice treated with PPAR agonists in vivo

Experiment	Substance	Dose	CYP4A Activity[a]	Fold Induction
		μmol/kg	*nmol/mg prot/min*	
1 (n = 3)	Control	–	0.5 ± 0.11	1
	AZ 242	0.13	7.1 ± 1.88	15
	WY14,643	36	10.7 ± 0.30	22
2 (n = 5)	Control	–	1.4 ± 0.30	1
	Rosiglitazone	5	1.5 ± 0.18	1.1

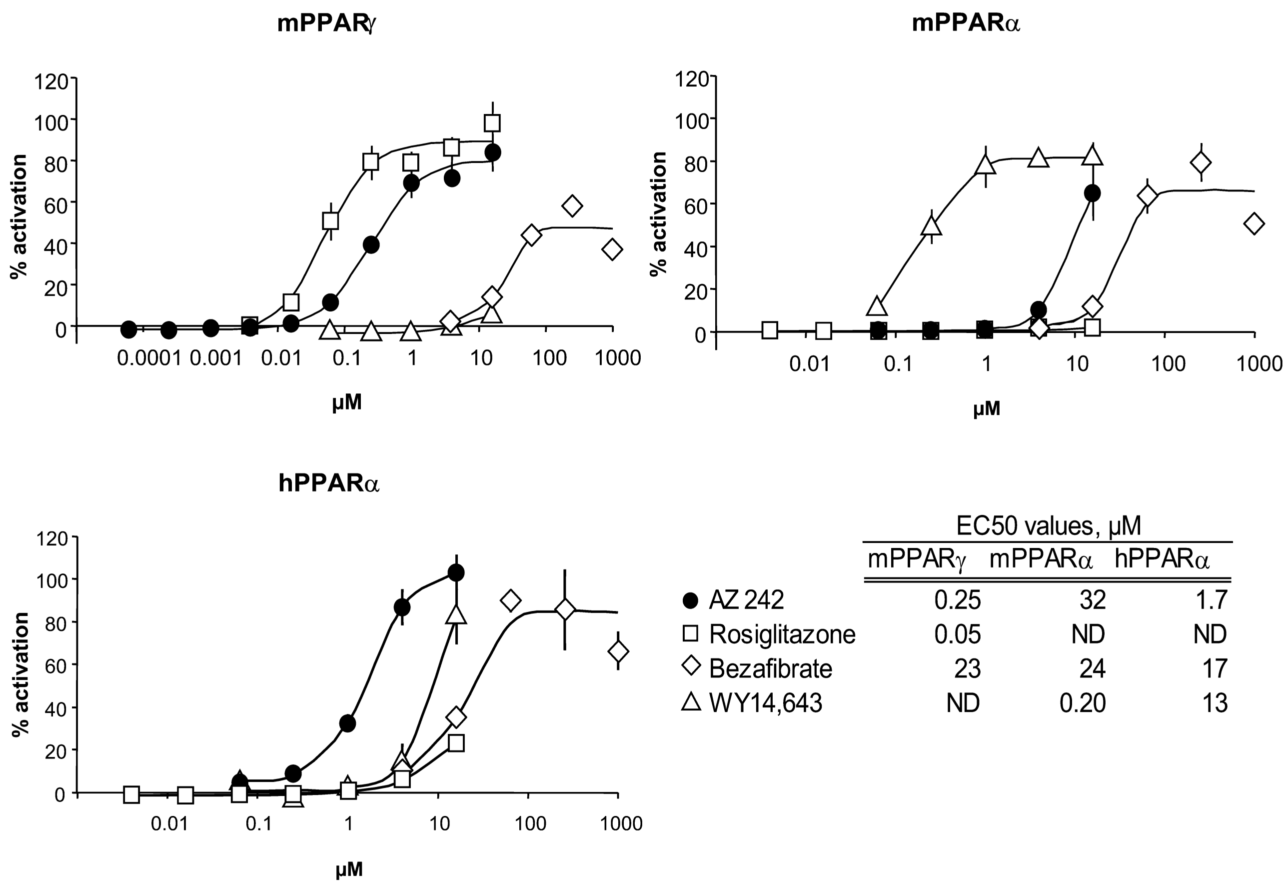
[a] Lean mice were treated for 1 week with the indicated doses. CPY4A-dependent lauric acid ω-hydroxylase activity was measured in isolated microsomes as described in Materials and Methods.

glyceridemia and hyperinsulinemia. However, the insulin sensitivity of severely insulin-resistant obese Zucker rats, as judged from the results obtained from the euglycemic hyperinsulinemic clamp experiments, was normalized by AZ 242 treatment with regard to the GIR (Table 1) as well as the insulin sensitivity index (**Fig. 4A**), where GIR has been normalized for lean body mass and elevation of total insulin level. Figure 4B illustrates that the impaired insulin-mediated FFA suppression of the control obese Zucker rats under clamp conditions is restored by AZ 242 treatment. Furthermore, basal insulin secretion, as judged by basal C-peptide levels (Fig. 4C), and the post-hepatic insulin appearance (Fig. 4D), assessed using HI as a tracer, were also markedly reduced by treatment with AZ 242.

Upregulation of CYP4A- in lean mice, a marker of PPARα activation. Since there is an overexpression of PPARγ in the livers of obese ob/ob mice, these animals do not provide a suitable model for investigating the activation of hepatic PPARα in vivo (32). Therefore, lean (B6C3F1) mice were treated once daily for 1 week with either AZ 242 (0.13 μmol/kg/d), the PPARγ agonist rosiglitazone (5 μmol/kg/d), or the PPARα agonist WY14,643 (36 μmol/kg/d). The doses used were in the lower "therapeutic range" for AZ 242 and WY14,643, based on the results obtained in ob/ob mice, whereas the dose of rosiglitazone corresponded to 10 × ED_{25} in ob/ob mice (Fig. 3). CYP4A-dependent lauric acid ω-hydroxylase activity was measured as a marker of PPARα activation in purified liver microsomes. **Table 2** shows that AZ 242 induced a response similar to that of WY14,643, whereas rosiglitazone failed to show any CYP4A induction, even at this high dose. The observed liver effects of AZ 242 were therefore PPARα mediated.

In vitro effects

Reporter gene assays. The selectivity and potency of AZ 242, rosiglitazone, WY14,643, and bezafibrate on murine PPARα (mPPARα), murine PPARγ (mPPARγ), and on hu-



	EC50 values, μM		
	mPPARγ	mPPARα	hPPARα
● AZ 242	0.25	32	1.7
□ Rosiglitazone	0.05	ND	ND
◇ Bezafibrate	23	24	17
△ WY14,643	ND	0.20	13

Fig. 5. Concentration-effect curves for AZ 242 (filled circles), rosiglitazone (open squares), bezafibrate (open diamonds), and WY14,643 (open triangles) in reporter gene assays. The symbols represent mean values ± SD (n = 3). The EC_{50} values calculated from the respective curves are summarized in the inserted table. AZ 242 activates both PPARα and PPARγ. ND, not determined ($EC_{50} > 16$ μM).

man PPARα (hPPARα) were determined in reporter gene assays in which the ligand-binding domain of the nuclear receptors was the primary target. Concentration-effect curves and EC_{50} values are presented in **Fig 5**. AZ 242 clearly activates both PPARα and PPARγ, whereas the other compounds are selective for either subtype. In similar reporter gene experiments on PPARδ, the three compounds did not show any agonistic effect in the concentrations tested (data not shown).

Effect of AZ 242 on insulin-mediated glucose uptake of 3T3-L1 preadipocytes. In 3T3-L1 cells, insulin-dependent glucose uptake increases with the extent of adipocyte differentiation and is efficiently promoted by PPARγ agonists (34, 36). 3T3-L1 cells therefore provide an intact cell model for PPARγ activity testing. Both AZ 242 and rosiglitazone, but not WY14,643, concentration-dependently increased adipocyte differentiation, as observed by microscopy (data not shown) and as indicated by the measured increase of insulin-stimulated glucose utilization with EC50-values of approximately 0.1 μM. WY14,643, however, had no effect in the concentrations tested (≤16 μM). The results provide strong evidence that AZ 242, like rosiglitazone, acts as a functional activator of PPARγ in murine adipocytes.

Proteomic evaluation of the effects of AZ 242 in HepG2 cells. Two-dimensional electrophoresis and mass spectrometry of proteins expressed in the human liver hepatoma cell line HepG2 were used to analyze the effects of AZ 242 and bezafibrate. These proteomics experiments showed that 8 μM AZ 242 and 71 μM bezafibrate induced qualitatively similar responses, characterized by 3- to 4-fold upregulation of one of the dominant cytosolic proteins. This protein was identified by mass spectrometry as L-FABP, a well-known target gene for PPARα activation in liver cells (**Fig. 6**). This observation thus confirms that AZ 242 activates endogenous PPARα, with a potency approximately 10-fold that of bezafibrate, in human liver derived intact cells.

DISCUSSION

The results of the current studies establish that AZ 242 is an orally active, potent, and efficient agent, improving insulin action and correcting the hypertriglyceridemia of ob/ob mice and obese Zucker rats. Furthermore, the results provide strong evidence that PPARα as well as PPARγ are activated in vivo. In ob/ob mice, the agent dose-

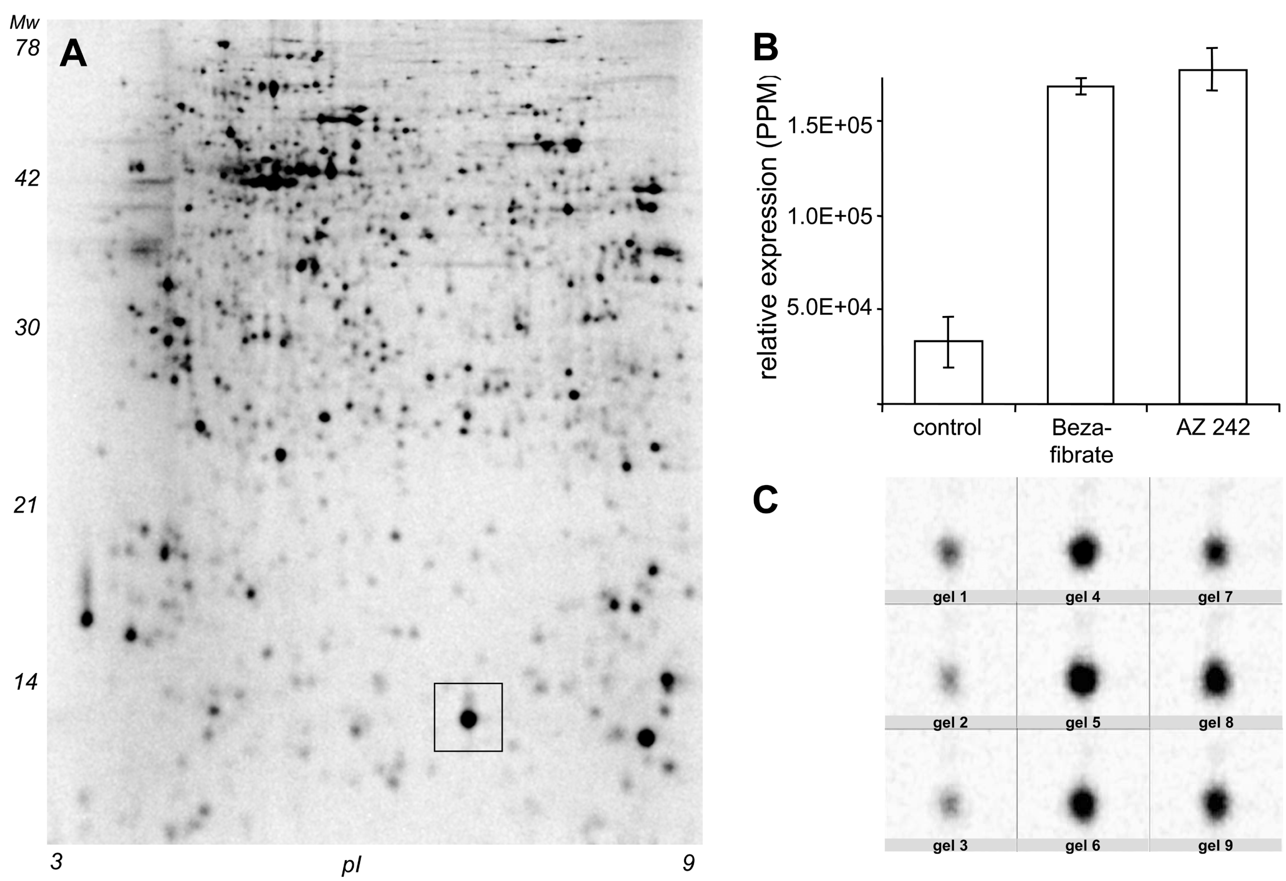


Fig. 6. Effects of AZ 242 and bezafibrate on human HepG2 hepatoma cells evaluated by proteomics. Cells were treated in triplicate with 8 μM AZ 242 or 71 μM bezafibrate for 72 h, which approximate to equi-effective concentrations in the reporter gene assay. A: Proteins labeled with [^{35}S]methionine, and extracted and separated by 2D-polyacrylamide gel electrophoresis. The box indicates the gel region with the liver fatty acid binding protein (L-FABP) spot, as identified by mass spectrometry. B: Relative expression of L-FABP in control cells, and cells treated with bezafibrate or AZ 242. C: Enlarged sections of triplicate gel sections with L-FABP from the three groups (ordered as described in B).

dependently ameliorated hypertriglyceridemia, hyperinsulinemia, and hyperglycemia. The dose–response curves show that AZ 242 abolished hyperglycemia, but no hypoglycemia was induced. The marked effect on plasma TG levels, even at doses less than 1 μmol/kg/day, is compatible with an AZ 242-induced enhancement of intracellular FA metabolism and utilization of plasma TG. In obese Zucker rats, under clamp conditions, the compound profoundly improved whole-body insulin sensitivity, as evidenced by the increased GIR required to maintain euglycemia and the increased insulin-mediated suppression of plasma FFA. In the basal state, AZ 242 ameliorated the hypertriglyceridemia and, probably secondary to the restored insulin sensitivity, also markedly reduced the hyperinsulinemia and insulin hypersecretion of the obese Zucker rat. It has previously been shown that long-term treatment with PPARγ-selective agents prevents the onset of diabetes in obese Zucker rats (37, 38). Two factors may contribute to this action. First, the treatment-induced reduction of pancreatic secretory burden (in the case of AZ 242, by 50%; Table 2, Fig. 4) may prevent pancreatic exhaustion. Second, as suggested by Unger and Orci, the reduced systemic lipid level is expected to reduce pancreatic lipid exposure and the associated apoptosis (39).

The above-mentioned AZ 242-induced improvements in whole-body glucose regulation and lipid levels are probably mediated by PPAR activation. Reporter gene assays established that AZ 242 is a potent agonist of PPARα and PPARγ in vitro (Fig. 5). In human hepatoma cells, AZ 242, like bezafibrate, showed clear PPARα effects by upregulating expression of L-FABP (Fig. 6). Since PPARγ agonists trigger adipocyte differentiation, the finding that AZ 242 potently induced 3T3-L1 differentiation and insulin sensitization provides further evidence of PPARγ agonism in intact cells (36). While PPARγ versus PPARα effects can be clearly differentiated in the in vitro context, this does not hold for all parameters in the in vivo situation. Specifically, insulin sensitization and lipid lowering have been reported for selective agonists of either PPARα or PPARγ (12, 40). Thus the relative contribution of PPARα versus PPARγ activation by AZ 242 to the observed effects in ob/ob mice and Zucker rats (Fig. 2, 4) is not possible to discern.

Unlike insulin sensitization and lipid lowering, other in vivo actions may be specifically related to PPARα activation. AZ 242 in therapeutically relevant doses in lean B6C3F1 mice increased the activity of CYP4A by more than 10-fold, as did the rodent-selective PPARα agonist WY14,643. In contrast, high doses of the selective PPARγ agonist rosiglitazone showed no effect on CYP4A activity (Table 2). There is compelling evidence that the regulation of hepatic CYP4A expression is under the control of PPARα in mice (41) and up-regulation of CYP4A is believed to be causally related to peroxisome proliferation (42). In conclusion, the results obtained in the ob/ob mouse and the obese Zucker rat demonstrate that AZ 242 is potent and efficient in correcting insulin resistance and glucose and FA metabolic abnormalities. Evidence is provided that these effects of the compound are mediated via PPARα and PPARγ. If these effects are translatable to man, AZ 242, acting as a combined PPARα and PPARγ agonist, will provide a useful treatment for the metabolic syndrome, including type 2 diabetes, and prevention of associated cardiovascular disease.

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