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PPARγ agonist induced cardiac enlargement is associated with reduced fatty acid and increased glucose utilization in myocardium of Wistar rats

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Abstract

In toxicological studies, high doses of peroxisome proliferator-activated receptor-gamma (PPARγ) agonists cause cardiac enlargement. To investigate whether this could be explained by a large shift from free fatty acid to glucose utilization by the heart, Wistar rats were treated for 2–3 weeks with a potent, selective PPARγ agonist (X334, 3 μmol/kg/d), or vehicle. X334 treatment increased body-weight gain and ventricular mass. Treatment lowered plasma triglycerides by 61%, free fatty acid levels by 72%, insulin levels by 45%, and reduced total plasma protein concentration by 7% (indicating plasma volume expansion) compared to vehicle animals. Fasting plasma glucose levels were unaltered. To assess cardiac free fatty acid and glucose utilization in vivo we used simultaneous infusions of non-β-oxidizable free fatty acid analogue, [9,10-^{3}H](*R*)-2-bromopalmitate and [U-^{14}C]2-deoxy-D-glucose tracers, which yield indices of local free fatty acid and glucose utilization. In anesthetized, 7 h fasted animals, left ventricular glucose utilization was increased to 182% while free fatty acid utilization was reduced by 28% ($P<0.05$) compared to vehicle. In separate studies we attempted to prevent the X334-induced hypolipidemia. Various dietary fat supplements were unsuccessful. By contrast, restricting the time during which the treated animals had access to food (promoting endogenous lipolysis), restored plasma free fatty acid from 27% to 72% of vehicle control levels and prevented the cardiac enlargement. Body-weight gain in these treated-food restricted rats was not different from vehicle controls. In conclusion, the cardiac enlargement caused by intense PPARγ activation in normal animals is associated with marked changes in free fatty acid/glucose utilization and the enlargement can be prevented by restoring free fatty acid availability.

Keywords: Cardiac hypertrophy; PPARγ; Fatty acid metabolism

1. Introduction

Thiazolidinediones are used clinically to improve blood glucose control in patients with type-2 diabetes, and are potent agonists of peroxisome proliferator-activated receptor-gamma (PPARγ). PPARγ agonists may be useful also for correcting other metabolic disturbances such as the dyslipidemia associated with the metabolic syndrome. Disturbances in fuel metabolism, especially lipid oversupply to the heart, may also be responsible for the so called diabetic cardiomyopathy (Zhou et al., 2000) and there is evidence that PPARγ agonists could play an important role in the correction of these disturbances (Berthiaume et al., 2004; Minoura et al., 2004; Oakes et al., 2001; Pickavance et al., 1999).

Numerous studies have documented the beneficial metabolic effects of PPARγ activation in animal models of the metabolic syndrome (Golfman et al., 2005; Zhou et al., 2000), including correction of hyperglycaemia and hypertriglyceridemia. Very little information is available in published form about findings in toxicological studies where high doses of PPARγ agonists are given to healthy animals. It has been shown that in this situation PPARγ agonists induce cardiac enlargement, increase plasma volume (Arakawa et al., 2004; Pickavance et al., 1999) and dramatically reduce plasma triglyceride concentration without causing hypoglycaemia (Berthiaume et al., 2004; Minoura et al., 2004). The eccentric cardiac enlargement induced in normal animals is accompanied with an increased left ventricular end diastolic pressure and has been considered a consequence of the increased plasma volume, leading to cardiac volume overload (Arakawa et al., 2004), rather than a direct hypertrophic effect on the cardiomyocytes (Bell and McDermott, 2005).

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We here hypothesize that in the normal animal, high doses of PPARγ agonists induce cardiac enlargement via extreme alterations in cardiac fuel metabolism caused by the ability of these agents to lower lipid availability. There are several situations where disturbances in fatty acid fuel metabolism are associated with cardiac enlargement and sometimes even cardiomyopathy. These include administration of fatty acid oxidation inhibitors (Litwin et al., 1990); fatty acid-free diets (Panos and Finerty, 1954); chronic insulin/glucose infusions (Belke et al., 2002; Holmang et al., 1996); genetic defects in oxidative metabolism (for review see (Antozzi and Zeviani, 1997; Eaton et al., 1996; Russell et al., 2005); and modulation of cardiac enzymes involved in oxidation (Sack et al., 1996; Schiffrin, 2005).

We have previously shown that PPARγ agonism ameliorated the 'lipid overload' condition in obese, dyslipidemic rats given therapeutic doses by: 1) increasing the ability of the adipose tissue to take up and store plasma free fatty acid, 2) enhancing insulin-mediated suppression of systemic free fatty acid mobilization, and 3) lowering hepatic triglyceride production and augmenting plasma triglyceride clearance (Oakes et al., 2001). No comparable information is available on the effect of these agents in healthy animals. This is especially important since the heart is thought to be highly dependant on circulating lipids (free fatty acid and triglyceride) for oxidative fuel metabolism. Thus induction of a severe hypolipidemia in metabolically healthy animals may seriously challenge normal cardiac energy metabolism and manifest itself much like the clinical situations of some inborn errors of metabolism. Therefore, the same pharmacologic effect (lipid lowering) could have very different consequences in healthy animals given high doses compared to animal models with the metabolic syndrome given therapeutic doses.

The aims of the present study were to document effects of intense PPARγ stimulation on in vivo cardiac fuel utilization and to investigate whether alterations in metabolism contribute to the cardiac enlargement in normal animals. For this purpose we studied the effects of a novel potent PPARγ agonist (X334) in Wistar rats, on cardiac free fatty acid and glucose uptake in vivo, circulating lipid availability, ventricular weight and plasma volume. The compound X334 induced profound hypolipidemia and to investigate whether this contributed to the observed cardiac enlargement we attempted to restore lipid availability using various interventions in combination with PPARγ treatment.

2. Methods

2.1. Animals and general procedures

Experimental procedures were approved by the Local Ethics Review Committee on Animal Experiments (Gothenburg Region, Sweden) and complied with the European Community guidelines for the use of experimental animals. Male Wistar rats (M and B Taconic, Ry, Denmark) were individually housed in a temperature (20–22 °C) and humidity (40–70%) controlled facility with a 12-h light/dark cycle (lights on 6:00 am). Unless otherwise stated the animals had free access to standard rodent diet (R3; Laktamin AB, Stockholm, Sweden) and tap water. The macronutrient composition of R3 chow, on a percent energy basis, consisted of 26% protein, 12% fat and 62% carbohydrate with a total energy content of ~12.6 kJ/g.

2.2. Drugs and solutions

The compound X334, AstraZeneca code AR-H049020XX, chemical name 3-{4-[2-(4-*tert*-butoxycarbonylaminophenyl) ethoxy]phenyl}-(*S*)-2-ethoxypropanoic acid was synthesized at Medicinal Chemistry, AstraZeneca, Mölndal. The selectivity and potency of X334 on murine PPARγ and murine PPARα was determined in reporter gene assays. Concentration–effect curves for X334 provided an EC_{50} value for PPARγ of approximately 20 nM vs. an EC_{50} of approximately 10,000 nM for PPARα. The compound is orally potent in obese, insulin resistant and dyslipidemic rodents (ob/ob mice and fa/fa Zucker rats), and has anti-hyperglycaemic, insulin and triglyceride lowering effects in these disease models. Hypertriglyceridemia was abolished in these models at oral doses of 0.03–0.3 μmol/kg/d for 1 week. In the present experiments X334 was given to metabolically healthy rats at doses 10–100 times this therapeutic dose.

2.3. Treatment groups and experimental protocols

Male Wistar rats were given vehicle or X334 at either 3 or 10 μmol/kg/d. Rats were dosed daily by gastric gavage between 7–8 am for a total of 2 weeks. Control rats were gavaged with an equal volume of vehicle solution (0.5% carboxymethyl cellulose, 2.5 ml/kg/day). In all studies body weight was recorded throughout the treatment period.

Unless otherwise stated, at the end of the dosing period, blood samples were collected directly from the tail vein of conscious animals into tubes containing potassium-EDTA (MICROVETTE® CB1000, Sarstedt, Nümbrecht, Germany). Blood samples were spun (1 min, 10 000 g, 4 °C) and plasma collected for analysis of triglycerides, free fatty acids, insulin and total protein concentration. Following collection of blood, the animals were anesthetized with isoflurane (FORENE®, Abbott Scandinavia AB, Solna, Sweden), sacrificed and the heart dissected carefully and left and right ventricles weighed. Results for "ventricular weight" represent the sum of left and right ventricular masses. In some experiments animals underwent additional acute experiments at the end of the dosing period before the collection and measurement of ventricular weight.

2.3.1. Anesthetized rat preparation

On the morning of the day of the study, food was removed at 7 am. Rats were anesthetized at 10 am with sodium thiobutabarbitol (120 mg/kg intraperitoneally, INACTIN®, Sigma, St. Louis, MO), and instrumented with tracheal, carotid artery and jugular vein catheters as previously described (Oakes et al., 2001). Body temperature was maintained at 37.5 °C and arterial blood pressure and heart rate recorded continuously. The acute experiment was started after a 1.5 h post-surgery stabilization period.

2.3.2. Conscious, chronically catheterised rat preparation — measurement of plasma free fatty acid and triglyceride concentration over 24 h

On days 7–9 of X334 (3 μmol/kg/d) or vehicle treatment, rats underwent surgery to implant a jugular vein catheter under isoflurane anesthesia as previously described (Oakes et al., 2001). Antibiotics were administered sub-cutaneously one day prior to surgery and for two days following surgery (ampicillin 150 mg/kg, Doktacillin®; AstraZeneca). Cannulae were exteriorized via a small cutaneous incision at the nape of the neck and patency was maintained by filling the lines with 45.5% (wt/wt) polyvinylpyrrolidone (Fluka Chemie AG, Buchs) dissolved in 0.9% sodium chloride, 20.6 mM sodium citrate solution. Oral X334 or vehicle administration was continued throughout this period. All rats included in the study were gaining weight and had recovered their pre-operative body weight by the time of the acute study. Before the study day, rats were acclimatised to the catheter coupling system that allowed jugular catheter sampling in virtually unrestrained animals. On the study day (days 14–15), the jugular catheter was unplugged and connected to the sampling system. In total, thirteen blood samples of 100 μl were taken over the 24-h period for measurements of plasma triglycerides and free fatty acids.

2.3.3. Measurement of plasma volume in anesthetized rats

Plasma volume was determined in animals treated for 14–20 days with X334 (3 μmol/kg/d) or vehicle. Instantaneous volumes of distribution of albumin were estimated based on plasma kinetics of radioactive labelled bovine serum albumin ([^{125}I] BSA, PerkinElmer Life Sciences, Boston, MA) in anesthetized animals. [^{125}I] BSA was prepared daily in a 2% BSA solution in normal saline. After a post-surgery stabilization period, a [^{125}I] BSA bolus (50 μl, $\sim 25\times10^6$ cpm/rat) was injected via the jugular vein. Blood samples (75 μl) were then taken via the carotid artery at 2, 4, 8, 16, 25, 35, 45, and 60 min. After centrifugation 25 μl of plasma was pipetted into a plastic vial for later determination of [^{125}I] activity using a LKB 1282 Compugamma-Universal gamma counter (LKB Wallac, Turku, Finland). Plasma [^{125}I] disappearance was well described by a double exponential function $C(t)=A\times e^{-\lambda1\times t}+B\times e^{-\lambda2\times t}$ where C is the concentration of ([^{125}I] BSA and t is time following bolus injection. Estimates of the 4 macroparameters (A, B, $\lambda1$ and $\lambda2$) for each experiment were obtained using non-linear regression analysis. Plasma volume (V) was calculated from the estimated value of $C(0)$ (equal to $A+B$), using the relation $V=$Injected [^{125}I] Dose$/C(0)$.

2.3.4. Proteomic analysis of hearts from PPARγ agonist treated rats

Protein expression patterns were studied in left ventricles from rats treated with either vehicle or X334 (10 μmol/kg/d) for 14 days using previously described methods (Lanne et al., 2001). In brief, 6–13 mg of tissue was homogenised (50 mg/ml) and protein expression patterns of left ventricles were obtained using 2-D gel electrophoresis (iso-electric focussing followed by SDS-PAGE, 200×200×1 mm). Regulated proteins were identified using mass spectrometry.

2.3.5. Assessment of whole-body and cardiac utilization of plasma free fatty acid and glucose in vivo

Cardiac free fatty acid and glucose utilization was assessed in vivo in 7-h fasted anesthetized Wistar rats treated with X334 (3 μmol/kg/d) or vehicle for 14–21 days. In an additional group of anesthetized, 7-h fasted rats ($n=4$), cardiac free fatty acid and glucose utilization were studied under a situation where plasma free fatty acid availability was lowered acutely with an infusion of nicotinic acid (~0.5 μmol/kg/min, Sigma, St. Louis, MO) begun 45 min prior to the beginning to the tracer experiment. Cardiac fuel utilization was assessed using a non-β-oxidizable free fatty acid analogue, [9,10-^{3}H]*R*-2-bromopalmitate and [U-^{14}C] 2-deoxy-D-glucose in methods previously described (Kraegen et al., 1985; Oakes et al., 1999). These tracer methods yield indices of local free fatty acid utilization and clearance, as well as glucose utilization.

2.3.5.1. Tracer infusates. Sterile saline solution containing [U-^{14}C]2-deoxy-D-glucose ([^{14}C]2DG, Amersham, Solna, Sweden), $\sim 60\times10^6$ dpm/ml was prepared. Each rat was given a bolus injection of $\sim 30\times10^6$ dpm. For infusion [9,10-^{3}H]*R*-bromopalmitate ([^{3}H]*R*-BrP, AstraZeneca R&D, Mölndal, Sweden) and unlabelled palmitate (Sigma, St. Louis, MO) were bound to BSA in normal saline solution as previously described (Oakes et al., 1999). Each rat received 45×10^6 dpm given in a 4 min infusion.

2.3.5.2. Experimental protocol. The carotid artery catheter was kept patent with a continuous infusion of sterile saline solution containing sodium citrate (20.6 mM, 10 μl/min). After a 90-min post-surgical stabilization period a 200 μl blood sample was taken for analysis of plasma free fatty acid, triglyceride, glucose and insulin levels. Immediately following a bolus injection of [^{14}C]2DG blood samples were taken at 2, 5, 10, 15, and 25 min. Starting at 30 min, a 4 min infusion of [^{3}H]*R*-BrP began and additional blood samples were taken at 31, 32, 33, 34, 35, 36, 38, 42 and 46 min. Blood samples were centrifuged at 4 °C and a 25 μl aliquot of plasma was added to 2 ml of lipid extraction mixture, and 10 μl added to a cellulose cone for subsequent combustion (see below).

After collection of the final blood sample (46 min) rats were given an overdose of thiobutabarbitol and the following tissues collected for analysis: red quadriceps, diaphragm, epididymal white adipose tissue, liver, and heart. The heart was carefully dissected into left and right ventricles and atria. Freshly collected tissue pieces (~100 mg) were weighed and placed in small cellulose cones for combustion.

2.3.5.3. Analysis of plasma and tissue samples. To discriminate [^{3}H]*R*-BrP and [^{14}C]2DG activities in plasma, a basic lipid extraction was performed based on a modified Hagenfeldt method (Hagenfeldt, 1966). The method involves an acid lipid extraction using a mixture of isopropanolol–hexane–1 M acetic acid (40:10:1).

Total tissue [^{3}H] and [^{14}C] activities were determined using a Packard System 387 Automated Sample Preparation Unit (Packard Instrument Co., Inc., Meriden, CT), which completely

oxidizes the sample and separates [^{3}H]H_20 and [^{14}C]CO_2 into separate scintillation vials for counting. Plasma and tissue [^{3}H] and [^{14}C] activities were measured using liquid scintillation spectrometry (Wallac 1409 counter; Wallac OY, Turku, Finland).

2.3.5.4. Calculations. Indices of tissue-specific free fatty acid and glucose utilization were calculated as previously described (Oakes and Furler, 2002; Oakes et al., 1999). The clearance rate of [^{3}H]*R*-BrP by the heart (K_f^*), an index of the ability of the tissue to utilize available free fatty acid, is defined as

$$K_f^* = \frac{m_B}{\int_0^T C_B(t)dt}$$

where T is the time of tissue collection (16 min), m_B is the tissue [^{3}H] content (at $t=T$), c_B is the arterial plasma concentration of [^{3}H]*R*-BrP. The integral in the denominator was assessed by measuring plasma tracer concentrations at multiple time points, performing curve fitting and analytic integration using the best fit parameters (Oakes et al., 1999).

An index of cardiac free fatty acid utilization rate (R_f^*) was calculated as:

$$R_f^* = C_P \cdot K_f^*$$

where C_P is the arterial plasma free fatty acid concentration.

Analogous expressions were applied to the [^{14}C]-tracer data to calculate the cardiac clearance rate of [^{14}C]2DG (K_g') and the cardiac glucose utilization rate index (R_g').

2.3.6. Dietary and physiologic interventions to restore endogenous and exogenous plasma free fatty acid supply in PPARγ agonist treated animals

To test whether the PPARγ induced cardiac enlargement could be due to extreme reductions in plasma lipid we investigated whether restoration of lipid availability in treated animals could prevent the hypolipidemia and cardiac enlargement. Two approaches were used in attempts to restore plasma lipid availability: (i) increasing exogenous lipid loading and (ii) increasing endogenous free fatty acid availability.

Firstly, we attempted to increase exogenous plasma fatty acids (triglyceride and/or free fatty acid) levels by two different high fat diets which differed only in their fatty acid composition. In one diet, fat was provided by beef tallow (270 g/kg food) consisting primarily of long chain saturated and mono-unsaturated fatty acids. In the other diet, medium-chain saturated fatty acids were provided by MIGLYOL® 810 (250 g/kg, CONDEA Chemie GmbH, Cranford, NJ) and a smaller amount of long chain poly-unsaturated fatty acids were provided by safflower oil (20 g/kg). Animals were placed on the diets one week before commencement of X334 (3 μmol/kg/d) or vehicle treatment. We also attempted to increase exogenous lipid availability by gavaging chow fed animals with oil. These animals were maintained on the standard high-carbohydrate/low fat diet (R3) and were gavaged with safflower oil (Sigma, St. Louis, MO) (supplement 6 ml/kg, equivalent to approximately 30% of the daily caloric intake). Safflower oil consists mainly of long chain poly-unsaturated fatty acids. Two groups of animals treated with X334 (3 μmol/kg/d) or vehicle were studied.

Our second approach to attempt to restore circulating lipid availability was to elevate endogenous lipolysis over the day. In the fed state insulin inhibits free fatty acid release from adipose tissue. This state is likely to prevail for much of the day in treated but not control animals due to the well-known effect of PPARγ agonists to increase food intake in rodents (Berthiaume et al., 2004; Larsen et al., 2003). To circumvent this treatment effect, and thereby raise endogenous free fatty acid availability, time restricted food access was imposed. Three groups of Wistar rats were studied: vehicle controls, X334 (3 μmol/kg/d) treated or X334 (3 μmol/kg/d) and food restricted. In the latter treatment group X334 treated animals were subjected to a restricted feeding protocol in which the animals had access to a pre-determined amount of food only between 4 pm and 7 am. In this group the amount of food offered daily to each rat was determined by its own average daily food intake in the control situation; determined over the three days immediately prior to the beginning of treatment. The vehicle group and the X334 treated, unrestricted group were given free access to food and all groups had free access to water. At the end of the treatment period blood samples were collected at 2 pm from tail vein of conscious animals. Following anesthesia left and right ventricles were separated and weighed as described above.

2.3.7. Analyses of plasma lipids, glucose and insulin and total protein

Colorimetric kit methods were used for the measurement of plasma free fatty acid (NEFA C, Wako, Richmond, Va, USA), triglycerides (GB, Boehringer Mannheim, IN, USA), glucose (Glucose HK, Roche, Stockholm, Sweden) and total protein (MPR3, Roche Diagnostic GmbH, IN, USA). Plasma insulin was determined using a rat insulin radioimmunoassay kit (Linco Research Inc., Charles MO, USA).

2.4. Statistical analyses

Where appropriate, results were evaluated using unpaired parametric *t*-test. A one-way analysis of variance (ANOVA) was used when more than two groups were compared.

The central measurement in this study was cardiac ventricular weight. To explore the influence of several factors on ventricular weight multiple linear regression analysis was performed (Astute, DDU software, Leeds, UK). The following factors were included in the model to account for variation in the ventricular weight at the end of the treatment period (Vw): pre-treatment body weight (BWs), body-weight gain over the treatment period (ΔBW) and treatment (Rx). The linear model applied to the data was:

$$Vw = A + B \times BWs + C \times \Delta BW + D \times BWs \times Rx$$

where A is the intercept, while B and C are partial regression coefficients for starting body weight and treatment period body-weight gain respectively. The final coefficient D, reflects the effect of X334 treatment on ventricular weight (beyond that associated with body-weight gain) which was implemented

Table 1
Body weight, ventricular weight, and plasma parameters in 7-h fasted Wistar rats treated with X334 or vehicle for 14 days

Dose μmol/kg/day	Body-weight start (g)	Body-weight gain (%)	Ventricular weight (g)	FFA mM	TG mM	Total protein (g/L)
0 (*n*)	361±12 (26)	8±1 (26)	0.848±0.02 (26)	0.598±0.06 (14)	1.34±0.11 (26)	63.9±0.92 (14)
3 (*n*)	373±10 (20)	13±1[a] (20)	0.990±0.03[a] (20)	0.165±0.04[a] (15)	0.53±0.03[a] (20)	59.4±0.65[a] (10)
10 (*n*)	310±8[a] (9)	23±1.3[a] (9)	0.989±0.03[a] (9)	0.227±0.03[a] (6)	0.42±0.03[a] (9)	51.4±0.50[a] (9)

Body weight, ventricular weight, plasma triglyceride (TG), plasma free fatty acid (FFA) and total plasma protein concentration following treatment (p.o.) with vehicle, X334 (3 μmol/kg/day), or X334 (10 μmol/kg/day), for 14 days. Blood sample taken from tail vein in 7-h fasted, conscious rats. Values are represented as mean±S.E.M. Number of observations in brackets, [a]$P=0.001$ compared to vehicle.

using a binary parameter Rx (=0 or 1, for vehicle and X334 groups, respectively) and was assumed to be dependent on the pre-treatment body weight of the animal.

Data is presented as mean±S.E.M. $P<0.05$ was considered statistically significant.

3. Results

3.1. Cardiac hypertrophy and hypolipidemia induced by high dose PPARγ agonist treatment in normal rats

Summary data describing the effect of two-week treatment of Wistar rats with the potent PPARγ agonist X334 on body weight, ventricular (left and right) weight and plasma clinical chemistry are presented in Table 1. Compared to vehicle treated animals, the groups X334 (3 μmol/kg/d) and X334 (10 μmol/kg/d) decreased plasma triglyceride concentration 61% and 69% respectively, and decreased plasma free fatty acid levels 72% and 62% respectively. PPARγ activation resulted in a significant increase in absolute ventricular weight (vehicle 0.848 g±0.02 vs. X334 (3 μmol/kg/d) 0.990±0.03 g, and X334 (10 μmol/kg/d) 0.989 g±0.03), and body-weight gain of the PPARγ treated animals (159% and 276% as compared to control). Ventricular weight normalized for body weight was also increased by 7% and 19% respectively in the X334 (3 μmol/kg/d) and X334 (10 μmol/kg/d) groups compared to vehicle animals (data not shown). Macroscopically both atria and ventricles appeared dilated without any marked wall thickening, consistent with eccentric cardiac hypertrophy. Total plasma protein concentration was reduced by approximately 7% X334 (3 μmol/kg/d) and 17% X334 (10 μmol/kg/d) compared to vehicle animals.

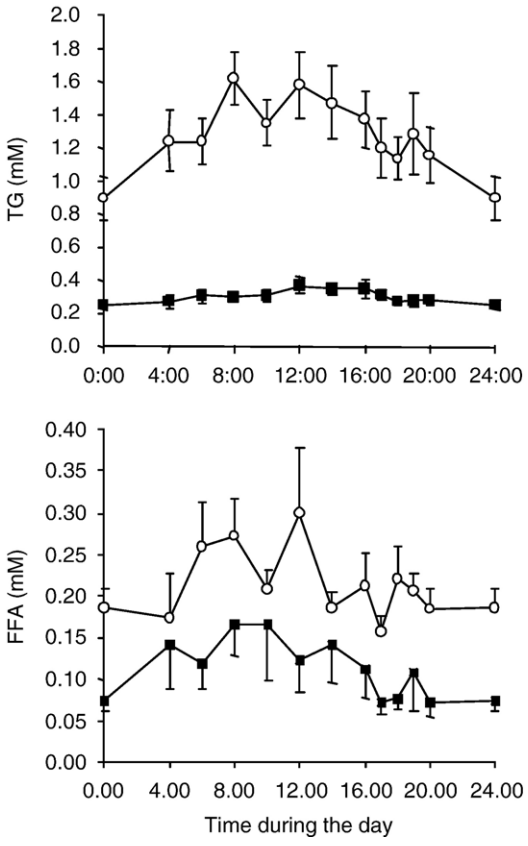

Fig. 1. 24-h plasma triglyceride (TG, upper panel) and free fatty acid (FFA, lower panel) concentration, measured at the end of 14-day treatment with vehicle ($n=8$, open circles) or X334 (3 μmol/kg/d, $n=7$, closed squares). Samples taken from conscious chronically catheterised rats with free access to food and water throughout the experiment. Values are represented as mean±S.E.M.

PPARγ agonist treatment significantly reduced plasma triglycerides and free fatty acids over the entire day (Fig. 1). Area under the 24-h plasma triglyceride curve was reduced by 76% by X334 treatment: from vehicle 30.4±2.5 to X334 7.2±0.7 mM h. Likewise the area under the 24-h plasma free fatty acid curve was reduced by 46% by X334 treatment: from vehicle 5.1±0.4 to X334 2.8±0.6 mM h.

The treatment induced cardiac enlargement was associated with a body-weight gain increase (Table 1). Since a body-weight increase, per se, would be expected to increase heart size we performed a multiple linear regression analysis (detailed in Methods) in order to reveal the effects of X334 on heart size beyond that which could be explained by body-weight gain. The following factors were included to account for variation in the ventricular weight at the end of the treatment period: pre-treatment body weight, body-weight gain over the treatment period and treatment.

Table 2
Multiple linear regression analysis of absolute ventricular weight changes following treatment with vehicle or X334 (3 μmol/kg/d) for 14 days

Factor	Regression coefficient	SE	*t*	P
Intercept (mg)	107.5	77.3	1.39	0.172
BWs (mg/g)	1.79	0.19	9.55	<0.0001
BW gain (mg/g)	3.38	0.67	4.98	<0.0001
Rx (mg/)	0.151	0.063	2.38	0.022

Dependence of ventricular weight at the end of the experiment on body weight at the start of the experiment (BWs), body-weight gain throughout the experiment (BW gain) and a PPARγ agonist treatment effect (Rx) on heart weight.

Table 3
Cardiac expression of significantly[a] regulated proteins after 14-day X334 (10 μmol/kg/d) treatment

Protein name	Swiss-Prot	Expression
		Treated/vehicle
Serum albumin[#]	P11382	1.74
Adipocyte fatty acid-binding protein (aFABP, ap2, albp)	P70623	3.68
Medium-chain specific mitochondrial acyl-CoA dehydrogenase (MCAD)	P08503	0.61
Annexin 6, lipocortin 6, Ca-binding protein CATA 65/67	P48037	1.69
α-crystalline B chain	P23928	1.34
Annexin 3, lipocortin 3, placental anti-coagulant protein 3, PAP-3, 35-α calcimedin	ANX_RAT	0.75
Mitochondrial fumarate hydratase	P14408	0.55

Protein name, Swiss-Prot database accession number and ratio of left ventricle protein expression between X334 treated and vehicle rats. [a]$P<0.05$ *t*-test. [#]Several albumin spots were identified but only one spot was up-regulated.

Results of this analysis are shown in Table 2. The ventricular weight data was well described by the linear regression model ($P<0.0001$). All regression coefficients were significant except for the intercept term, suggesting that absolute ventricular weight is significantly influenced by the body weight and body-weight change over the treatment period. Most importantly, however, this analysis strongly suggests that X334 treatment induced an increase in heart weight beyond its effect to increase body weight.

3.2. PPARγ agonist induced increase in plasma volume in anesthetized rats

In a separate study we investigated the effect of PPARγ stimulation (X334, 3 μmol/kg/d) on plasma volume in 7-h fasted, anesthetized animals using labelled albumin. Plasma volume was significantly increased in the X334 group: 41.4±1.7 ml/kg, $n=5$ vs. vehicle: 33±2.0 ml/kg, $n=6$, $P<0.05$). In these animals, total protein concentration was lower, 55.6±1.0 g/l in the X334 than in the vehicle group, 60.5±0.8 g/l ($P<0.05$). In all further experiments we have used the reduction of plasma protein concentration as a marker of PPARγ induced plasma volume expansion. Ventricular weight was significantly higher with PPARγ agonist treatment 0.875±0.03 vs. vehicle 0.773±0.02 g ($P<0.05$).

3.3. Effect of PPARγ agonist treatment on cardiac protein expression patterns

Using 2-D gel electrophoresis, protein expression patterns in left ventricles from rats treated with X334 (10 μmol/kg/d) or vehicle for 14 days revealed 9 proteins that were significantly regulated, of which 7 were successfully identified using mass spectrometry (Table 3). These included a number of proteins involved in glucose and fatty acid metabolism. Specifically, adipocyte fatty acid binding protein (aFABP) was up-regulated, while mitochondrial medium-chain specific Acyl-CoA dehydrogenase (MCAD; involved in β-oxidation of fatty acids) and fumarase (a Krebs cycle enzyme) were down-regulated (Table 3). Additionally, several serum albumin spots appeared on the gel. All were identified and quantified but only one of these was up-regulated. Of relevance for the heart, the calcium dependent phospholipid binding protein annexin 6 was also significantly up-regulated by X334 treatment.

3.4. Assessment of whole-body and cardiac utilization of plasma free fatty acid and glucose in vivo

Whole-body free fatty acid mobilization and an index of the combined ability of the tissues of the body to take up free fatty acid, plasma clearance, were estimated from the plasma tracer kinetics of [^{3}H]*R*-BrP (Table 4). In anesthetized animals fasted for 7 h, clearance of plasma free fatty acid was increased by 61% in treated animals (Table 4) while the rate of fatty acid appearance was not different between treated and vehicle animals, suggesting that there was no PPARγ mediated action to enhance free fatty acid mobilization in the fasted state in metabolically healthy animals. The net result of the lack of change in the rate of free fatty acid appearance and the enhanced clearance in the X334 (3 μmol/kg/d) treated animals was a 41% decrease in plasma free fatty acid level ($P<0.001$, Table 4).

Whole-body glucose utilization, representing the sum of the individual tissue utilization was estimated from the plasma tracer kinetics of [^{14}C]2DG (Table 5). Neither plasma glucose level nor whole-body glucose utilization was significantly

Table 4
Whole-body free fatty acid metabolism in anesthetized, 7-h fasted Wistar rats treated with vehicle or X334 (3 μmol/kg/d) for 14 days

Dose μmol/kg/day	Plasma FFA (mM)	Whole-body clearance [^{3}H]*R*-BrP (ml/kg/min) (K_p)	Index of whole-body rate of appearance of FFA (μmol/kg/min) (R_a)
0 ($n=7$)	0.61±0.04	29.6±1.6	18.0±1.5
3 ($n=8$)	0.38±0.03[a]	47.5±3.0[a]	16.6±1.7

Plasma free fatty acid levels (FFA) and indices of whole-body clearance of [^{3}H]*R*-BrP (K_p), and whole-body rate of appearance of free fatty acid (R_a). Data presented as mean±S.E.M. [a]$P<0.001$, *t*-test.

Table 5
Plasma parameters and whole-body glucose metabolism in anesthetized, 7-h fasted Wistar rats treated with vehicle or X334 (3 μmol/kg/d) for 14 days

Dose μmol/kg/day	Plasma glucose (mM)	Plasma insulin (nM)	Whole-body rate of disappearance (μmol/kg/min) (R_d)
0 ($n=7$)	8.2±0.7	0.57±0.08	41.4±5.9
3 ($n=8$)	8.1±0.3	0.32±0.04[a]	39.6±4.9

Plasma glucose and insulin concentration, and whole-body disappearance of [^{14}C]2DG (R_d). Data presented as mean±S.E.M. [a]$P<0.05$, *t*-test.

Table 6
Tissue-specific clearance (K_f^*) of [^{3}H]*R*-BrP tracer in anesthetized, 7-h fasted Wistar rats treated with vehicle or X334 (3 μmol/kg/d) for 14 days

Tissue	In vivo free fatty acid clearance rate index (K_f^*) μl/g tissue/min	
	Vehicle (n=7)	X334 (n=8)
Right ventricle	272±25	319±30
Atria	129±23	149±20
Red quadricep	13.2±1.5	15.2±2.3
White adipose tissue	10±1.1	19±1.6[a]
Diaphragm	41±7	48±7
Liver	595±46	712±38

Data presented as mean±S.E.M. [a]P<0.05, t-test.

Table 7
Tissue-specific utilization of plasma glucose (R_g') and free fatty acid (R_f^*) in anesthetized, 7-h fasted Wistar rats treated with vehicle (n=7) or X334 (3 μmol/kg/d, n=8) for 14 days

Tissue	In vivo glucose utilization index		In vivo free fatty acid utilization index	
	R_g' nmol/g tissue/min		R_f^* nmol/g tissue/min	
	Vehicle	X334	Vehicle	X334
Right ventricle	276±71	397±63	161±16	109±9[a]
Atria	232±41	484±67[a]	78±14	54±10
Red quadricep	49±9	39±4	7.8±0.8	5.5±1.1
White adipose tissue	21±4	17±2	6.1±0.8	6.4±0.5
Diaphragm	100±30	87±17	24±4	17±3
Liver	–	–	362±35	252±26[a]

R_g' data for liver are not included because of methodological limitations (phosphorylated 2-deoxyglucose is not trapped in hepatocytes). Data presented as means±S.E.M. [a]P<0.05, t-test

altered by X334 treatment. Insulin levels were, however, decreased by X334 suggesting an increase in insulin sensitivity (Table 5).

The local ability of the tissues to take up free fatty acid from plasma is represented by the tissue-specific clearances (K_f^*) of [^{3}H]*R*-BrP in Table 6. The increased whole-body clearance of free fatty acid from plasma can be largely explained by an increase in the sequestration of plasma free fatty acid by adipose tissue. However, the index of tissue plasma free fatty acid clearance in right ventricle, atria, red quadriceps and diaphragm were not significantly altered by treatment. There was a tendency for increased hepatic free fatty acid clearance by X344 treatment, although this variable did not reach statistical significance (p=0.068). Importantly, in the left ventricle, clearance of free fatty acid from plasma was not significantly altered by PPARγ agonist treatment (Fig. 2). Thus, in X334 treated animals, the absence of an increase in free fatty acid clearance into the heart in the face of a reduction in plasma free fatty acid levels led to a significant reduction in free fatty acid utilization equal to the product of the tissues' ability to take up free fatty acid from plasma and the concentration of free fatty acid in plasma (Fig. 2 and Table 7).

Tissue-specific glucose utilization in vehicle and X334 treated animals is shown in Table 7. In concert with the reduction in free fatty acid utilization, X334 elevated heart specific glucose utilization in vivo with significant increases in glucose utilization in the left ventricle and atria (Fig. 2 and Table 7). The increase in glucose utilization following PPARγ

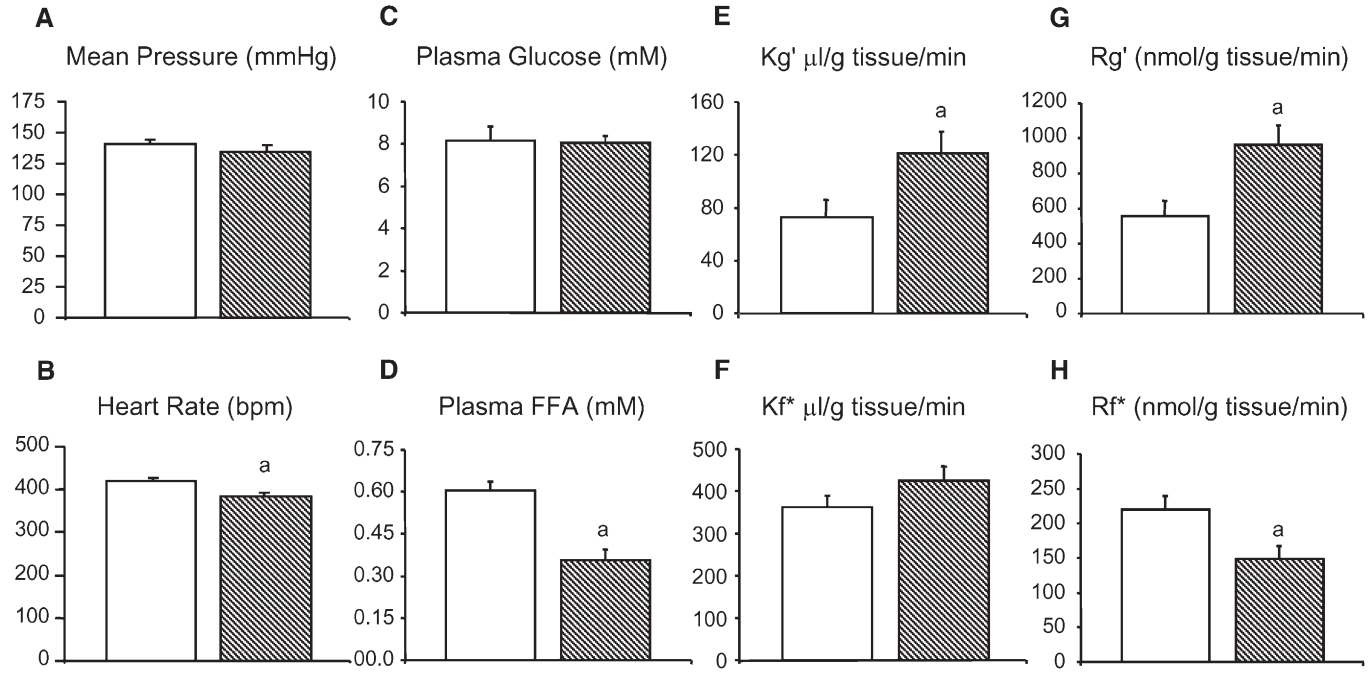

Fig. 2. Indices of left ventricular utilization of plasma free fatty acid and glucose in anesthetized, 7-h fasted, Wistar rats treated with Vehicle (white bars, n=7) or X334 (3 μmol/kg/d, n=8, striped bars). Anesthetized mean arterial pressure (A) and heart rate (B), plasma concentration of glucose (C) and free fatty acid (D), left ventricular clearance of [^{14}C]2DG and [^{3}H]*R*-BrP from plasma (K_g' and K_f^*, E and F respectively), and left ventricular utilization of plasma free fatty acid and glucose (R_g' and R_f^*, G and H respectively). Data presented as mean±S.E.M. [a]P<0.05 t-test.

Table 8
Body weight, ventricular weight and plasma parameters following treatment with vehicle or X334 plus dietary oil supplementation

	BW start (g)	BW gain (% of day 1)	Ventricular wt (g)	TG mM	FFA mM
Vehicle+oil (n=9)	344±3	8±1	0.839±0.02	0.79±0.066	0.436±0.06
X334 (3)+oil (n=10)	348±3	17±1[a]	0.986±0.0[a]	0.34±0.02[a]	0.173±0.0[a]

Body weight at start (BW start), body-weight gain (BW gain), ventricular weight (wt), plasma triglyceride (TG) and free fatty acid (FFA) concentration following 14-day treatment with vehicle or X334 (3 μmol/kg/d) plus dietary supplementation with safflower oil (6 ml/kg/d). Blood samples taken from the tail vein of conscious animals after 8-h fast where the animals had access to water only. Values are represented as mean±S.E.M. Number of observations in brackets. [a]P<0.001.

Table 10
Body weight, ventricular weight and plasma parameters in fat fed Wistar rats treated with vehicle or X334 for 21 days

	BW start (g)	BW gain (% of day 1)	Ventricular wt (g)	Plasma FFA (mM)	Plasma TG (mM)
Vehicle+fat diet (n=6)	349±4	17±1	0.907±0.02	0.30±0.03	4.36±0.39
X334 (3)+fat diet (n=6)	352±4	19±1.3	1.073±0.03[a]	0.18±0.03[b]	0.66±0.11[a]

Body weight at start (BW start), body-weight gain (BW gain), ventricular weight (wt), plasma triglyceride (TG) and free fatty acid (FFA) concentration following 21-day treatment of fat fed Wistar rats with vehicle or X334 (3 μmol/kg/d). Wistar rats were fed a high fat diet based on medium-chain saturated fatty acids (MIGYLOL® 810) for one week before and for the duration of the treatment period. Blood samples taken from the aorta of anesthetized animals after a 7-h fast where the animals had access to water only. Data is presented as mean±S.E.M. Number of observations in brackets. [a]P<0.001, [b]P<0.05.

treatment was specific to the heart whereas in white adipose tissue, red quadriceps muscle, and the diaphragm muscle it was not affected by treatment (Table 7).

Regional differences in cardiac metabolism were apparent (Table 7 and Fig. 2) Tissue free fatty acid utilization was 3 times greater in the left ventricle than in the atria, and 1.3 times greater in the left ventricle compared to the right ventricle. Similar to the local differences in free fatty acid utilization absolute rates of glucose utilization also differed in different parts of the heart being in the left ventricle, ~2-fold greater than in the right ventricle or atria. These relative regional differences in either free fatty acid or glucose use were not affected by PPARγ agonist treatment.

3.5. Tissue-specific free fatty acid and glucose parameters during acute nicotinic acid infusion

The results of the experiment above suggest that the reduction in cardiac free fatty acid utilization could largely be attributed to the fall in systemic free fatty acid availability rather than a reduction in the ability of the heart to take up available free fatty acid (cardiac clearance). In theory this need not be the case, as reductions in free fatty acid level could be compensated for by an enhanced cardiac clearance. To investigate this possibility and to examine the general dependence of cardiac free fatty acid uptake on free fatty acid level we investigated cardiac metabolic responses to acute free fatty acid lowering using nicotinic acid. For this purpose animals were anesthetized and fasted for 7 h. Acute infusion of nicotinic acid decreased plasma free fatty acid levels from 0.61±0.04 to 0.23±0.0 mM. Despite this 58% reduction in plasma free fatty acid levels, left ventricular clearance of free fatty acid from plasma was only modestly increased to 495±25 μl/g tissue/min compared from 362±27 μl/g tissue/min in vehicle treated animals. The relatively small increase in left ventricular free fatty acid clearance (36%) was not able to compensate for the large reduction in plasma free fatty acid availability during nicotinic acid infusion. Thus the index of free fatty acid utilization was 55% lower in the nicotinic acid infused animals compared to the controls: 99±14 nmol/g tissue/min vs. 220±20 nmol/g tissue/min, respectively.

The nicotinic acid induced suppression of cardiac free fatty acid utilization was associated with increased glucose utilization. In the left ventricle, glucose utilization increased by almost 580% compared to vehicle treated animals (3.2±0.27 vs. 0.56±0.09 μmol/g tissue/min respectively).

Table 9
Body weight, ventricular weight and plasma parameters in fat fed Wistar rats treated with vehicle or X334 for 21 days

	BW start (g)	BW gain (% of day 1)	Ventricular wt (g)	Plasma TG mM
Vehicle+fat diet (n=6)	334±9	19±1	0.872±0.02	2.54±.24
X334 (3)+fat diet (n=6)	329±5	32±1[a]	1.160±0.05[a]	0.80±0.04[a]

Body weight at start (BW start), body-weight gain (BW gain), ventricular weight (wt) and plasma triglyceride (TG) concentration following 2-day treatment of fat fed Wistar rats with vehicle or X334 (3 μmol/kg/d). Wistar rats were fed a high fat diet based on saturated fatty acids for one week before and for the duration of the treatment period. Blood samples taken from the aorta of anesthetized animals after a 7-h fast where the animals had access to water only. Data is presented as mean±S.E.M. Number of observations in brackets. [a]P<0.001.

3.6. Prevention of PPARγ induced cardiac enlargement by restoring endogenous plasma lipid availability

To test the hypothesis that the cardiac enlargement induced with X334 treatment was due to a chronic reduction in the lipid supply to the heart, we used a number of interventions in attempts to increase exogenous or endogenous free fatty acid availability in X334 treated animals. Various regimes to increase free fatty acid availability exogenously, either by supplementing fat in the diet or administering oil by gavage, did not prevent PPARγ induced cardiac enlargement (see Tables 8–11).

To elevate endogenous lipid availability we subjected X334 (3 μmol/kg/d) treated rats to a restricted feeding protocol, thus maximizing the time the treated animals spent in a fasted state throughout the day and maximizing the time for lipolysis to occur. X334 treated animals were restricted in the time the food was available and also in the amount of food they had access to.

Table 11
Composition of high fat diets used in studies

Ingredients	Amount (g/kg)	Brand	Supplier
Casein (purified high nitrogen)	160		ICN Biochemicals, Ohio, USA
Tallow (Beef Lard)*	270		ICN Biochemicals, Ohio, USA
or			
MIGLYOL®*	250		CONDEA Chemie GmbH, NJ, USA
+			
Safflower oil*	20		Sigma, St. Louis, MO
Corn starch	180	Maizena	Bestfoods Nordic A/S, Kristianstad, Sweden
Sucrose	160	Refined household	Danisco Sugar, Copenhagen, Denmark
Fructose (β-D-(−)-fructose)	130		ICN Biochemicals, Ohio, USA
Gelatin (flaked, 50 Bloom)	24		ICN Biochemicals, Ohio, USA
Mineral mix	53	AIN 93M	ICN Biochemicals, Ohio, USA
Vitamin mix	10	AIN 93-VX	ICN Biochemicals, Ohio, USA
Choline chloride	10		ICN Biochemicals, Ohio, USA
Methionine (DL methionine)	3		ICN Biochemicals, Ohio, USA

*MIGLYOL®+Safflower oil or Beef Lard were used as sources of fat in the diets. Animals were fed the high fat diets beginning one week prior to commencement of X334 or vehicle treatment.

Treated rats were provided with a pre-determined amount of standard rat chow made available between 4 pm and 7 am (see Methods). Total food intake over the treatment period was equal in the freely fed vehicle treated animals and the X334 treated, restricted group (Table 12). Animals treated with X334 with free access to food ate a significantly greater amount of food over the treatment period. Accordingly we found that the restricted feeding protocol in X334 treated rats increased plasma free fatty acid levels compared to treated rats that had been given free access to chow (vehicle group 0.35±0.04 mM, X334 freely fed group 0.10±0.02 mM and, X334-food restricted group 0.25±0.04 mM, Table 12). PPARγ agonist treatment induced increases in ventricular weight were attenuated by the food restriction protocol (Tables 12 and 13). Interestingly, the reduction in total plasma protein concentration seen in the X334 treated animals (and indicative of a plasma volume expansion) was still present in the X334-freely fed and food restricted groups (Total protein concentration: Vehicle group 62.6±0.9 g/l, X334-freely fed group 57.6±0.9 g/l and X334-food restricted group 56.4±0.7 g/l, Table 12).

4. Discussion

We observed that intense PPARγ activation for two weeks in metabolically healthy Wistar rats with the potent and selective PPARγ agonist X334, induced cardiac enlargement, as evidenced by increased cardiac mass resulting from an eccentric cardiac hypertrophy. This enlargement was greater than that which could be explained by the increase in body-weight gain which results from increased food consumption, a well known effect of PPARγ agonism, (Berthiaume et al., 2004; Larsen et al., 2003; Vasudevan and Balasubramanyam, 2004). In addition, X334 treatment induced plasma volume expansion, a dramatic reduction in plasma lipid availability, and caused a shift in cardiac fuel metabolism, reducing free fatty acid and increasing glucose utilization. To test whether fatty acid availability was involved in the cardiac enlargement we used a time restricted food access protocol, which restored plasma free fatty acid levels towards those found in control animals and prevented the cardiac enlargement.

Very few published observations are available concerning the effects of PPARγ agonists on in vivo fatty acid fluxes. In this study we used previously developed radioactive tracer methods to measure tissue-specific free fatty acid and glucose utilization in vivo (Kraegen et al., 1985; Oakes et al., 1999). The dramatic decrease found in systemic lipid availability was entirely due to effects in adipose tissue, increasing trafficking of plasma free fatty acid into adipose tissue, and thereby diverting free fatty acid away from other tissues including the heart. It is worth comparing the present results obtained in lean animals to our earlier study in obese, dyslipidemic rats (Oakes et al., 2001), where PPARγ treatment also enhanced adipose tissue fatty acid uptake. In contrast to the present results, where X334 treatment did not alter whole-body free fatty acid mobilization in the

Table 12
Body weight, ventricular weight, food intake and plasma parameters in Wistar rats

	BW start (g)	BW gain (%)	Total food intake (g)	Ventricular wt (g)	TG	FFA mM	Protein g/L
Vehicle (*n*)	328±7 (12)	7±1 (12)	298±8 (12)	0.781±.01 (12)	1.61±0.19 (12)	0.351±0.04 (12)	62.6±0.94 (12)
X334 (3) (*n*)	323±7 (11)	13±1[a] (11)	343±13[b] (11)	0.857±0.02[b] (11)	0.50±0.03[a] (11)	0.095±.02[a] (11)	57.6±0.86[b] (11)
X334 (3) restricted (*n*)	322±7 (12)	7±1 (12)	297±6 (12)	0.754±0.02 (12)	0.50±0.03[a] (12)	0.251±0.04 (12)	56.4±0.66[a] (12)

Body weight at experiment start (BW start), body-weight gain over the experimental period (BW gain), ventricular weight (wt), plasma triglyceride (TG), free fatty acid (FFA) and total plasma protein concentration following 14-day treatment with vehicle, X334 (3 μmol/kg/d), or X334 (3 μmol/kg/d) plus time restricted food access. Blood sample taken at 2 pm from tail vein of conscious animals where vehicle and X334 (3 μmol/kg/d) groups have had free access to food and water, while the X334 (3 μmol/kg/d) restricted group had access only to water (see Method section for time restricted food access protocol). Values are represented as mean±S.E.M. Number of observations in brackets. [a]$P<0.001$, [b]$P<0.05$.

Table 13
Multiple linear regression analysis of absolute ventricular weight changes following 14-day treatment with vehicle or X334 (3 μmol/kg/d plus restricted food access)

Factor	Regression coefficient	SE	*t*	P
Intercept (mg)	164.3	58.9	2.79	0.0081
BWs (mg/g)	1.66	0.14	11.88	<0.0001
BW gain (mg/g)	3.09	0.6	5.14	<0.0001
Txt_BWs (mg/g)	−0.023	0.04	−0.50	0. 6174

Dependence of ventricular weight at the end of the experiment on body weight at the start of the experiment (BWs), body-weight gain throughout the experiment (BW gain) and a PPARγ agonist treatment effect (Rx) on heart weight.

fasting state, PPARγ activation in the obese animals increased free fatty acid mobilization. In the fasting state therefore, a physiological situation where free fatty acid levels should be relatively high, PPARγ activation restricted free fatty acid availability in the lean but not in the obese animals.

The reduced cardiac free fatty acid utilization seen with X334 treatment may be a direct consequence of the action of PPARγ activation to lower systemic free fatty acid availability. Importantly, cardiac clearance of free fatty acid from plasma did not increase significantly to compensate for the lower free fatty acid level. This resulted in reduced cardiac free fatty acid utilization. Presumably to satisfy the energy demand, glucose utilization increased perhaps via operation of the Randle cycle (Randle et al., 1963). To test the general ability of the heart to compensate for decreased plasma free fatty acid level we examined the response to an acute nicotinic acid infusion, which decreased plasma free fatty acid levels. Nicotinic acid also increased glucose use as free fatty acid utilization was decreased.

Another possible explanation for the PPARγ agonist induced shift in fuel metabolism is increased insulin signalling causing increased glucose utilization and suppressed free fatty acid utilization in the heart due to increased insulin sensitivity (Jenkins et al., 1993; Randle et al., 1963; Sidossis and Wolfe, 1996; Vasudevan and Balasubramanyam, 2004). However, insulin efficiently lowers the ability of the heart to take up free fatty acid from plasma (Furler et al., 2000), reflected by the free fatty acid clearance parameter, which was not seen in the present study (Fig. 2). Therefore our results suggest that lowered plasma fatty acid availability, rather than increased insulin signalling, caused the shift in cardiac metabolism with PPARγ activation.

The notion that metabolic alterations could be involved in the cardiac enlargement seen in normal animals given high doses of PPARγ agonists is supported by several independent situations. Thus pharmacologic inhibition of β-oxidation and genetic mitochondrial enzyme deficiencies (Litwin et al., 1990; Sack and Kelly, 1998), which limit cardiac utilization of free fatty acid are associated with cardiac enlargement and even cardiomyopathy (Bressler and Goldman, 1993; Kusaka et al., 1995). It seemed reasonable then that the limited lipid utilization could also be involved in the X334 induced cardiac enlargement. To further test this idea, we attempted to restore free fatty acid availability in X334 treated animals using the unconventional approach of a restricted feeding protocol that increased endogenous lipolysis. Indeed, the food restriction intervention did succeed in restoring free fatty acid levels towards those of vehicle animals and prevented the cardiac enlargement. Furthermore, the prevention of cardiac enlargement by restoring free fatty acid availability occurred in spite of PPARγ induced plasma volume expansion indicated by similar levels of plasma proteins in both X334 treated groups (X334 treated-food restricted and X334-freely fed vs. vehicle, Table 12). This provides evidence against a role of plasma volume expansion in the cardiac enlargement observed in the current study.

We also attempted to restore lipid availability to treated animals using the more obvious approach of increasing exogenous fatty acid through dietary supplementation. We tried this using three very different protocols: oil supplementation by gavage, high fat diet based on saturated fatty acids, and a high fat diet based on medium-chain saturated fatty acids. Surprisingly, none of the interventions were successful in preventing cardiac enlargement. This might appear as evidence against a role for lipid availability in the cardiac enlargement; however, circulating lipid levels were dramatically lowered by X334 also in the fat supplemented animals, indicating a failure to restore myocardial lipid supply. We suggest that the heart was buffered against the increased exogenous lipid delivery predominantly by very effective PPARγ agonist induced trafficking of fatty acids into adipose tissue. Our clearance measurements show that this is the case for free fatty acid. In addition, increased adipose tissue lipoprotein lipase activity is an expected consequence of PPARγ agonism due to direct up-regulation of lipoprotein lipase gene expression (Laplante et al., 2003; Schoonjans et al., 1996) and prolongation of the post-prandial state (Laplante et al., 2003). It is generally held that effects on lipoprotein lipase expression results in increased trafficking of VLDL and chylomicron fatty acids into adipose tissue. In contrast, PPARγ agonism might also decrease utilization of fatty acids by the heart through a reduction of local lipoprotein lipase activity (Yu et al., 2005).

Even a high fat diet based on medium-chain fatty acids which enter the bloodstream as free fatty acids rather than chylomicron esterified fatty acids (Odle, 1997; Tso et al., 1995) was unsuccessful in preventing the cardiac enlargement. It is also possible that the X334 treatment may have restricted cardiac utilization of medium-chain free fatty acid as suggested by our observation that MCAD protein was down-regulated (Table 3). This follows because MCAD has an important function in the β-oxidation spiral and interestingly MCAD mRNA is down-regulated in the heart in rodent models of pressure overload hypertrophy (Sack and Kelly, 1998).

Proteomic analysis revealed several changes associated with the observed X334 induced cardiac enlargement. Down-regulation of fumarase (a Krebs cycle enzyme) and MCAD proteins could potentially limit fuel utilization and exacerbate the situation of low systemic fatty acid availability. However, in humans, genetic deficiencies in both of these enzymes are not associated with cardiomyopathy (Rustin et al., 1997; Saudubray et al., 1999). The protein annexin 6, a member of a family of calcium dependent-phospholipid binding proteins which are reported to regulate protein activities (Camors et al., 2005), was

modestly up-regulated by PPARγ stimulation. It is most abundantly expressed in the heart, where a 10-fold over-expression of the protein has resulted in cardiac dilatation and disrupted calcium homeostasis (Gunteski-Hamblin et al., 1996). The role for annexin 6 in human cardiomyopathy is unclear, since levels of this protein in human cardiac failure have been shown to be up-regulated (Benevolensky et al., 2000), or unchanged (Matteo and Moravec, 2000). The strongest regulation of any protein was the up-regulation of aFABP which provides evidence of a direct PPARγ mediated action in the heart (Coe and Bernlohr, 1998).

On the other hand, a recent study showed the persistence of PPARγ induced cardiac hypertrophy in cardiomyocyte-specific PPARγ knockout mice. Thus it is unlikely that any mechanism downstream of a direct cardiac PPARγ action (including aFABP up-regulation) can completely explain the cardiac enlargement (Duan et al., 2005). Our in vivo data supports this indirect mode of action on the heart via a primary effect in adipose tissue.

Insulin can exert a trophic myocardial effect as shown by induction of cardiac enlargement by chronic insulin infusions (Holmang et al., 1996) and the demonstration that deletion of cardiac insulin receptors in mice results in a smaller heart than in wild type mice (Belke et al., 2002). It is therefore possible that the increased cardiac size in the context of PPARγ activation might be the result of chronically increased insulin signalling due to increased insulin sensitivity in combination with prolonged post-prandial insulin elevations, a result of the hyperphagia.

We suggest that the result of excessive PPARγ agonist induced lipid lowering in healthy animals would be very different to the therapeutic application of PPARγ agonists in patients with the metabolic syndrome. In the patient context, metabolic disturbances including tissue lipid oversupply are expected to be ameliorated by PPARγ agonism. Indeed, in diabetic patients without any evidence of prior heart disease, there is very little evidence for an increase in left ventricular mass with thiazolidinediones treatment (Hirayama et al., 2001; Nesto et al., 2003), despite the fact that PPARγ agonist induced occurrence of oedema and plasma volume expansion are known side effects in patients (Nesto et al., 2003; Tang et al., 2003; Vasudevan and Balasubramanyam, 2004). Similarly, in isolated working hearts from Zucker diabetic rats, prior PPARγ agonist treatment has been shown to enhance myocardial glucose oxidation and improve contractile function (Golfman et al., 2005), while in vivo, troglitazone treatment lowered myocardial triglyceride content and prevented the loss of cardiac function in diabetic Zucker rats (Zhou et al., 2000).

In conclusion, we have investigated the relationship between cardiac enlargement and shifts in metabolism in response to intense PPARγ activation in healthy animals, at doses used in toxicological studies. The basic metabolic mode of action of PPARγ agonists to lower systemic lipid availability by effective sequestration into adipose tissue, which is beneficial in the context of the metabolic syndrome, results in a lipid deficit in metabolically healthy animals. We provide evidence that this exaggerated metabolic effect is involved in the cardiac enlargement seen in toxicological studies.

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References

Antozzi, C., Zeviani, M., 1997. Cardiomyopathies in disorders of oxidative metabolism. Cardiovasc. Res. 35, 184–199.

Arakawa, K., Ishihara, T., Aoto, M., Inamasu, M., Kitamura, K., Saito, A., 2004. An antidiabetic thiazolidinedione induces eccentric cardiac hypertrophy by cardiac volume overload in rats. Clin. Exp. Pharmacol. Physiol. 31, 8–13.

Belke, D.D., Betuing, S., Tuttle, M.J., Graveleau, C., Young, M.E., Pham, M., Zhang, D., Cooksey, R.C., McClain, D.A., Litwin, S.E., Taegtmeyer, H., Severson, D., Kahn, C.R., Abel, E.D., 2002. Insulin signaling coordinately regulates cardiac size, metabolism, and contractile protein isoform expression. J. Clin. Invest. 109, 629–639.

Bell, D., McDermott, B.J., 2005. Effects of rosiglitazone and interactions with growth-regulating factors in ventricular cell hypertrophy. Eur. J. Pharmacol. 508, 69–76.

Benevolensky, D., Belikova, Y., Mohammadzadeh, R., Trouve, P., Marotte, F., Russo-Marie, F., Samuel, J.L., Charlemagne, D., 2000. Expression and localization of the annexins II, V, and VI in myocardium from patients with end-stage heart failure. Lab. Invest. 80, 123–133.

Berthiaume, M., Sell, H., Lalonde, J., Gelinas, Y., Tchernof, A., Richard, D., Deshaies, Y., 2004. Actions of PPARgamma agonism on adipose tissue remodeling, insulin sensitivity, and lipemia in absence of glucocorticoids. Am. J. Physiol., Regul. Integr. Comp. Physiol. 287, 1116–1123.

Bressler, R., Goldman, S., 1993. A role of fatty acid oxidation in cardiac hypertrophy. Cardioscience 4, 133–142.

Camors, E., Monceau, V., Charlemagne, D., 2005. Annexins and Ca^{2+} handling in the heart. Cardiovasc. Res. 65, 793–802.

Coe, N.R., Bernlohr, D.A., 1998. Physiological properties and functions of intracellular fatty acid-binding proteins. Biochim. Biophys. Acta 1391, 287–306.

Duan, S.Z., Ivashchenko, C.Y., Russell, M.W., Milstone, D.S., Mortensen, R.M., 2005. Cardiomyocyte-specific knockout and agonist of peroxisome proliferator-activated receptor-gamma both induce cardiac hypertrophy in mice. Circ. Res. 97, 372–379.

Eaton, S., Bartlett, K., Pourfarzam, M., 1996. Mammalian mitochondrial beta-oxidation. Biochem. J. 320 (Pt 2), 345–357.

Furler, S.M., Cooney, G.J., Hegarty, B.D., Lim-Fraser, M.Y., Kraegen, E.W., Oakes, N.D., 2000. Local factors modulate tissue-specific NEFA utilization: assessment in rats using 3H-(R)-2-bromopalmitate. Diabetes 49, 1427–1433.

Golfman, L.S., Wilson, C.R., Sharma, S., Burgmaier, M., Young, M.E., Guthrie, P.H., Van Arsdall, M., Adrogue, J.V., Brown, K.K., Taegtmeyer, H., 2005. Activation of PPARgamma enhances myocardial glucose oxidation and improves contractile function in isolated working hearts of ZDF rats. Am. J. Physiol. Endocrinol. Metab. 289, E328–E336.

Gunteski-Hamblin, A.M., Song, G., Walsh, R.A., Frenzke, M., Boivin, G.P., Dorn II, G.W., Kaetzel, M.A., Horseman, N.D., Dedman, J.R., 1996. Annexin VI overexpression targeted to heart alters cardiomyocyte function in transgenic mice. Am. J. Physiol, Heart Circ. Physiol. 270, H1091–H1100.

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

Hirayama, H., Sugano, M., Abe, N., Yonemoch, H., Makino, N., 2001. Troglitazone, an antidiabetic drug, improves left ventricular mass and diastolic function in normotensive diabetic patients. Int. J. Cardiol. 77, 75–79.

Holmang, A., Yoshida, N., Jennische, E., Waldenstrom, A., Bjorntorp, P., 1996. The effects of hyperinsulinaemia on myocardial mass, blood pressure

regulation and central haemodynamics in rats. Eur. J. Clin. Investig. 26, 973–978.

Jenkins, A.B., Storlien, L.H., Cooney, G.J., Denyer, G.S., Caterson, I.D., Kraegen, E.W., 1993. Effects of blockade of fatty acid oxidation on whole body and tissue-specific glucose metabolism in rats. Am. J. Physiol: Endocrinol. Metab. 265, E592–E600.

Kraegen, E.W., James, D.E., Jenkins, A.B., Chisholm, D.J., 1985. Dose–response curves for in vivo insulin sensitivity in individual tissues in rats. Am. J. Physiol: Endocrinol. Metab. 248, E353–E362.

Kusaka, Y., Tanaka, T., Okamoto, F., Terasaki, F., Matsunaga, Y., Miyazaki, H., Kawamura, K., 1995. Effect of sulfo-N-succinimidyl palmitate on the rat heart: myocardial long-chain fatty acid uptake and cardiac hypertrophy. J. Mol. Cell. Cardiol. 27, 1605–1612.

Lanne, B., Potthast, F., Hoglund, A., Brockenhuus von Lowenhielm, H., Nystrom, A.C., Nilsson, F., Dahllof, B., 2001. Thiourea enhances mapping of the proteome from murine white adipose tissue. Proteomics 1, 819–828.

Laplante, M., Sell, H., MacNaul, K.L., Richard, D., Berger, J.P., Deshaies, Y., 2003. PPAR-gamma activation mediates adipose depot-specific effects on gene expression and lipoprotein lipase activity: mechanisms for modulation of postprandial lipemia and differential adipose accretion. Diabetes 52, 291–299.

Larsen, P.J., Jensen, P.B., Sorensen, R.V., Larsen, L.K., Vrang, N., Wulff, E.M., Wassermann, K., 2003. Differential influences of peroxisome proliferator-activated receptors gamma and-alpha on food intake and energy homeostasis. Diabetes 52, 2249–2259.

Litwin, S.E., Raya, T.E., Gay, R.G., Bedotto, J.B., Bahl, J.J., Anderson, P.G., Goldman, S., Bressler, R., 1990. Chronic inhibition of fatty acid oxidation: new model of diastolic dysfunction. Am. J. Physiol, Heart Circ. Physiol. 258, H51–H56.

Matteo, R.G., Moravec, C.S., 2000. Immunolocalization of annexins IV, V and VI in the failing and non-failing human heart. Cardiovasc. Res. 45, 961–970.

Minoura, H., Takeshita, S., Ita, M., Hirosumi, J., Mabuchi, M., Kawamura, I., Nakajima, S., Nakayama, O., Kayakiri, H., Oku, T., Ohkubo-Suzuki, A., Fukagawa, M., Kojo, H., Hanioka, K., Yamasaki, N., Imoto, T., Kobayashi, Y., Mutoh, S., 2004. Pharmacological characteristics of a novel nonthiazolidinedione insulin sensitizer, FK614. Eur. J. Pharmacol. 494, 273–281.

Nesto, R.W., Bell, D., Bonow, R.O., Fonseca, V., Grundy, S.M., Horton, E.S., Le Winter, M., Porte, D., Semenkovich, C.F., Smith, S., Young, L.H., Kahn, R., 2003. Thiazolidinedione use, fluid retention, and congestive heart failure: a consensus statement from the American Heart Association and American Diabetes Association. Circulation 108, 2941–2948 (October 7, 2003).

Oakes, N.D., Furler, S.M., 2002. Evaluation of free fatty acid metabolism in vivo. Ann. N. Y. Acad. Sci. 967, 158–175.

Oakes, N.D., Kjellstedt, A., Forsberg, G.B., Clementz, T., Camejo, G., Furler, S.M., Kraegen, E.W., Olwegard-Halvarsson, M., Jenkins, A.B., Ljung, B., 1999. Development and initial evaluation of a novel method for assessing tissue-specific plasma free fatty acid utilization in vivo using (R)-2-bromopalmitate tracer. J. Lipid Res. 40, 1155–1169.

Oakes, N.D., Thalen, P.G., Jacinto, S.M., Ljung, B., 2001. Thiazolidinediones increase plasma-adipose tissue FFA exchange capacity and enhance insulin-mediated control of systemic FFA availability. Diabetes 50, 1158–1165.

Odle, J., 1997. New insights into the utilization of medium-chain triglycerides by the neonate: observations from a piglet model. J. Nutr. 127, 1061–1067.

Panos, T.C., Finerty, J.C., 1954. Effects of a fat-free diet on growing male rats with special reference to the endocrine system. J. Nutr. 54, 315–331.

Pickavance, L.C., Tadayyon, M., Widdowson, P.S., Buckingham, R.E., Wilding, J.P., 1999. Therapeutic index for rosiglitazone in dietary obese rats: separation of efficacy and haemodilution. Br. J. Pharmacol. 128, 1570–1576.

Randle, P.J., Garland, P.B., Hales, C.N., Newsholme, E.A., 1963. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. Lancet 1, 785–789.

Russell, L.K., Finck, B.N., Kelly, D.P., 2005. Mouse models of mitochondrial dysfunction and heart failure. J. Mol. Cell. Cardiol. 38, 81–91.

Rustin, P., Bourgeron, T., Parfait, B., Chretien, D., Munnich, A., Rotig, A., 1997. Inborn errors of the Krebs cycle: a group of unusual mitochondrial diseases in human. Biochim. Biophys. Acta 1361, 185–197.

Sack, M.N., Kelly, D.P., 1998. The energy substrate switch during development of heart failure: gene regulatory mechanisms (Review). Int. J. Mol. Med. 1, 17–24.

Sack, M.N., Rader, T.A., Park, S., Bastin, J., McCune, S.A., Kelly, D.P., 1996. Fatty acid oxidation enzyme gene expression is downregulated in the failing heart. Circulation 94, 2837–2842.

Saudubray, J.M., Martin, D., de Lonlay, P., Touati, G., Poggi-Travert, F., Bonnet, D., Jouvet, P., Boutron, M., Slama, A., Vianey-Saban, C., Bonnefont, J.P., Rabier, D., Kamoun, P., Brivet, M., 1999. Recognition and management of fatty acid oxidation defects: a series of 107 patients. J. Inherit. Metab. Dis. 22, 488–502.

Schiffrin, E.L., 2005. Peroxisome proliferator-activated receptors and cardiovascular remodeling. Am. J. Physiol, Heart Circ. Physiol. 288, H1037–H1043.

Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A.M., Heyman, R.A., Briggs, M., Deeb, S., Staels, B., Auwerx, J., 1996. PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. EMBO J. 15, 5336–5348.

Sidossis, L.S., Wolfe, R.R., 1996. Glucose and insulin-induced inhibition of fatty acid oxidation: the glucose-fatty acid cycle reversed. Am. J. Physiol, Endocrinol. Metab. 270, E733–E738.

Tang, W.H., Francis, G.S., Hoogwerf, B.J., Young, J.B., 2003. Fluid retention after initiation of thiazolidinedione therapy in diabetic patients with established chronic heart failure. J. Am. Coll. Cardiol. 41, 1394–1398.

Tso, P., Karlstad, M.D., Bistrian, B.R., DeMichele, S.J., 1995. Intestinal digestion, absorption, and transport of structured triglycerides and cholesterol in rats. Am. J. Physiol.: Gastrointest. Liver Physiol. 268, G568–G577.

Vasudevan, A.R., Balasubramanyam, A., 2004. Thiazolidinediones: a review of their mechanisms of insulin sensitization, therapeutic potential, clinical efficacy, and tolerability. Diabetes Technol. Ther. 6, 850–863.

Yu, X., Burgess, S.C., Ge, H., Wong, K.K., Nassem, R.H., Garry, D.J., Sherry, A.D., Malloy, C.R., Berger, J.P., Li, C., 2005. Inhibition of cardiac lipoprotein utilization by transgenic overexpression of Angptl4 in the heart. Proc. Natl. Acad. Sci. U. S. A. 102, 1767–1772.

Zhou, Y.T., Grayburn, P., Karim, A., Shimabukuro, M., Higa, M., Baetens, D., Orci, L., Unger, R.H., 2000. Lipotoxic heart disease in obese rats: implications for human obesity. Proc. Natl. Acad. Sci. U. S. A. 97, 1784–178 9.