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The effects of rosiglitazone on fatty acid and triglyceride metabolism in type 2 diabetes

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Abstract *Aims/hypothesis:* We investigated the effects of rosiglitazone on NEFA and triglyceride metabolism in type 2 diabetes. *Methods:* In a double-blind, placebo-controlled, cross-over study of rosiglitazone in diet-treated type 2 diabetic subjects, we measured arteriovenous differences and tissue blood flow in forearm muscle and subcutaneous abdominal adipose tissue, used stable isotope techniques, and analysed gene expression. Responses to a mixed meal containing [1,1,1-¹³C]tripalmitin were assessed. *Results:* Rosiglitazone induced insulin sensitisation without altering fasting NEFA concentrations (−6.6%, $p=0.16$). Postprandial NEFA concentrations were lowered by rosiglitazone compared with placebo (−21%, $p=0.04$). Adipose tissue NEFA release was not decreased in the fasting state by rosiglitazone treatment (+24%, $p=0.17$) and was associated with an increased fasting hormone-sensitive lipase rate of action (+118%, $p=0.01$). Postprandial triglyceride concentrations were decreased by rosiglitazone treatment (−26%, $p<0.01$) despite unchanged fasting concentrations. Rosiglitazone did not change concentrations of triglyceride-rich lipoprotein remnants. Adipose tissue blood flow increased with rosiglitazone (+32%, $p=0.03$). Postprandial triglyceride [¹³C]palmitic acid concentrations were unchanged, whilst NEFA [¹³C]palmitic

acid concentrations were decreased ($p=0.04$). In muscle, hexokinase II mRNA expression was increased by rosiglitazone (+166%, $p=0.001$) whilst the expression of genes involved in insulin signalling was unchanged. Adipose tissue expression of *FABP4*, *LPL* and *FAT/CD36* was increased. *Conclusions/interpretation:* Rosiglitazone decreases postprandial NEFA and triglyceride concentrations. This may represent decreased spillover of NEFAs from adipose tissue depots. Decreased delivery of NEFAs to the liver may lead to lowered postprandial triglyceride concentrations. Upregulation of hexokinase II expression in muscle may contribute to insulin sensitisation by rosiglitazone.

Keywords Human metabolism · Insulin resistance · Integrative physiology · NEFA · Postprandial metabolism · Systems physiology

Abbreviations A-V difference: arteriovenous difference · FABP4: fatty acid binding protein 4 · FAS: fatty acid synthase · HSL: hormone-sensitive lipase · IR: insulin receptor · LPL: lipoprotein lipase · PDK4: pyruvate dehydrogenase kinase 4 · PI kinase: phosphatidylinositol-3 kinase · RLP-C: remnant lipoprotein cholesterol · V-A difference: venoarterial difference

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Introduction

The thiazolidinedione class of drugs is used to induce insulin sensitisation in insulin resistance. Despite their widespread use in type 2 diabetes, there is still debate over the physiological mechanisms by which thiazolidinediones exert their effects.

Some of the steps of thiazolidinedione action are known. Thiazolidinediones bind to and activate the peroxisome proliferator-activated receptor (PPAR γ) nuclear receptor [1]. As a transcription factor, PPAR γ regulates the expression of a variety of genes involved in metabolism [2]. A co-ordinated change in gene expression might be ex-

pected to increase the 'efficiency' of glucose handling and to increase insulin sensitivity.

The mechanisms of action of thiazolidinediones are less clear *in vivo* in humans: how do thiazolidinediones act on genes highly expressed in adipose tissue, yet induce insulin sensitisation in skeletal muscle? A commonly proposed answer is based on the interaction between adipose tissue and skeletal muscle: the glucose-fatty acid (Randle) cycle [3]. Glucose and fatty acids compete for use as a substrate by skeletal muscle. Thus, if circulating NEFA concentrations are increased, muscle glucose uptake would be less efficient. In this way, an increased output of NEFAs from adipose tissue in obesity would cause insulin resistance [4]. It is thought that thiazolidinediones decrease NEFA output from adipose tissue, thus modulating a signal of insulin resistance between adipose tissue and muscle [5, 6]. A recently proposed mechanism for this is the induction of glycerol kinase in adipose tissue by thiazolidinediones, thus establishing a futile metabolic cycle in adipocytes [7]. However, we have shown that this mechanism is not detectable *in vivo* in humans or *in vitro* in human adipocytes [8].

The concept that thiazolidinediones cause insulin sensitisation by affecting NEFA metabolism is supported by a large number of rodent *in vivo* studies. For example, in rodents, rosiglitazone and troglitazone decrease fasting NEFA concentrations by 40 to 90% [9, 10]. In contrast, in human studies, fasting NEFA concentrations increase, or fall by up to 30% [11–14], and these changes are often not statistically significant.

By reversing insulin resistance, do thiazolidinediones reverse the hypertriglyceridaemia of type 2 diabetes? Reports of the effects of different thiazolidinediones on triglyceride metabolism are inconsistent. Rosiglitazone has no effect on fasting triglyceride concentrations [15–19]. One study has looked at postprandial triglyceride concentrations and found no change with rosiglitazone [17]. In contrast, pioglitazone lowers fasting and postprandial triglyceride concentrations by approximately 15 to 25% [15, 20–22], which is presumably partly a result of its PPAR α activity [23]. Indeed, PPAR α activation by other drugs, such as the fibrate class of drugs [24], lowers fasting and postprandial triglyceride concentrations [25]. Troglitazone reduces fasting (–2 to –30% [11, 15, 26–30]) and postprandial triglyceride concentrations by 17 to 70% [11, 31]. However, it is unknown whether, like pioglitazone [23], troglitazone has any PPAR α activity in addition to its PPAR γ action. Rosiglitazone, a potent and specific agonist for PPAR γ , which dissociates the effects of triglyceride lowering and insulin sensitisation, seems to be a good tool to dissect the connections between insulin resistance and triglyceride metabolism.

In vitro studies examining the mechanisms underlying changes in postprandial lipid metabolism with thiazolidinediones have produced conflicting results in adipocytes and pre-adipocytes. Lipoprotein lipase (LPL) mRNA expression and mass increased [32, 33] or were unchanged [33, 34], depending on the dose of the thiazolidinedione and model used. The activity of LPL generally decreased

with treatment [33, 35], although this reduction was not always statistically significant [35].

We therefore set out to investigate the effects of rosiglitazone on fasting and postprandial NEFA and triglyceride metabolism. We used arteriovenous difference and stable isotope techniques in a double-blind, placebo-controlled, cross-over study design. To identify potential molecular mechanisms underlying the physiological responses, we augmented the metabolic studies with analyses of gene expression related to insulin signalling, and glucose and fat metabolism in skeletal muscle and adipose tissue biopsies.

Data on glycerol kinase from this study have been previously published and are not included here [8].

Subjects and methods

Patients Twenty-four patients with type 2 diabetes, treated with diet alone participated in the study. Entry criteria included an age of 30–70 years, a fasting plasma glucose of 7–12 mmol/l and a BMI greater than 24 kg/m². Patients previously treated with oral hypoglycaemic agents were excluded, as were those taking any medication known to affect glucose metabolism. Patients were in good health without cardiac, hepatic, renal or other chronic diseases, and without microvascular complications of diabetes as determined by history, clinical examination and routine blood investigations. Of the 24 subjects, one subject smoked (a pipe). This subject did not smoke for 12 h prior to the metabolic investigation days and did not change his smoking habits during the study period. All subjects had a residual insulin secretory capacity as defined by a fasting C-peptide concentration of 0.5 nmol/l or more. Measurements of weight, HbA_{1c}, and fasting insulin and glucose concentrations were taken at baseline and at each visit.

Study design This was a double-blind, placebo-controlled, cross-over study. Subjects were randomised into one of two groups: rosiglitazone 4 mg twice daily for 12 weeks followed by placebo for 12 weeks, or vice versa. Twelve of the 24 subjects had rosiglitazone as their first treatment and 12 had placebo as their first treatment. Compliance was assessed at regular intervals by tablet counts. At the end of each 12-week treatment period, patients attended the clinical research unit after a 10-h overnight fast for a full-day metabolic investigation. Vigorous exercise and alcohol were avoided for 24 h before each study.

The study was approved by the Oxfordshire Clinical Research Ethics Committee and all subjects gave their written informed consent.

Metabolic investigation protocol Tissue-specific arteriovenous techniques To assess the *in vivo* metabolism of adipose tissue and skeletal muscle in humans, we measured arteriovenous differences across these tissues. Differences between the composition of venous blood draining a particular tissue and arterialised blood reflect the metabolic activity of the specific tissue. Inclusion of

tissue blood flow in the calculations allows quantification of net metabolic activity. On the day of the study, serial blood samples were taken in the fasting state and for 6 h after metabolic provocation with a standardised mixed meal.

Blood sampling Arterialised blood was obtained from a vein draining a heated hand. Venous blood from muscle was taken retrogradely from a vein draining the deep structures of the forearm. Venous blood from adipose tissue was obtained from the superficial epigastric vein, as described previously [36]. This vein drains subcutaneous abdominal adipose tissue with negligible contribution from other tissues [37]. Oxygen saturation and ultrasonography were used to confirm adequate siting of the cannulae. Blood samples were taken simultaneously from all three sites. To prevent contamination of the blood from the forearm vein with blood from the hand, a wrist cuff was inflated to above the systolic blood pressure (either to 30 mmHg above the systolic blood pressure or to 200 mmHg, whichever value was higher) for 3 min before samples were taken. Two sets of baseline blood samples were taken 30 min apart. Patients then consumed a meal of a fat emulsion (40 g olive oil, 600 mg [1,1,1-¹³C]tripalmitin [CK Gas Products, Wokingham, UK], 400 mg emulsifier, flavourings), skimmed milk (200 ml) and Rice Krispies (Kellogg Company, Manchester, UK) containing 40 g fat, 40 g carbohydrate and 600 mg [1,1,1-¹³C]tripalmitin. All patients had been on a diet low in ¹³C prior to the study day. Further blood samples were taken for 6 h after the meal.

Tissue blood flow Subcutaneous abdominal adipose tissue blood flow was measured by ¹³³Xe washout [38]. Forearm muscle blood flow was assessed by strain-gauge plethysmography [39].

Tissue biopsies Skeletal muscle and adipose tissue biopsies were taken under local anaesthesia (1% lignocaine) at a standardised time (+300 min) in the postprandial period. Subcutaneous abdominal adipose tissue was taken with a 12-gauge needle, and muscle biopsies were taken from the vastus lateralis muscle using a percutaneous needle technique. Samples were immediately frozen in liquid nitrogen and stored at -70°C for later mRNA quantification.

Biochemical and gene expression analysis Plasma triglyceride, NEFA, glucose and insulin, and blood glycerol concentrations were measured as described previously [40].

The postprandial increase in ¹³C in the circulation was derived from the [1,1,1-¹³C]tripalmitin in the meal. In a representative subset of subjects (*n*=6), NEFAs and triglycerides were extracted from fasting and postprandial arterialised plasma samples for gas chromatography and gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Enrichments of [¹³C]palmitate in plasma NEFA and triglyceride fractions were measured. The results were multiplied by the concentrations of palmitate-NEFA and palmitate-triglyceride respectively, to give the concentrations of the tracer in these fractions, as previously described [40].

Remnant lipoprotein cholesterol (RLP-C) concentrations were measured by an immunoaffinity-based method as previously described [41].

Specific mRNAs were quantified by RT-cPCR and real-time PCR techniques, correcting for the housekeeping gene, cyclophilin, as previously described [42]. The mRNA of the PPAR γ -responsive gene, fatty acid binding protein 4 (*FABP4*) (*aP2*), was quantified in adipose tissue as a positive control for PPAR γ action in adipose tissue, along with the mRNA of genes involved in fatty acid metabolism in adipose tissue, such as *LPL*, hormone-sensitive lipase (*HSL*), fatty acid translocase (*FAT*)/*CD36*, fatty acid synthase (*FAS*) and *PPAR* γ 2. In muscle, expression of mRNA in genes involved in the key steps of glucose metabolism (*GLUT4*, hexokinase II, pyruvate dehydrogenase kinase 4 [*PDK4*] and glycogen synthase) and insulin signalling (phosphatidylinositol-3 kinase [PI₃ kinase] p85 α , p110 α , p110 β subunits, insulin receptor [*IR*], *IRS-1* and *IRS-2*) were measured.

Statistical analysis, power and calculations Adipose tissue blood flow was calculated as described previously [38]. Arteriovenous (A-V) and venoarterial (V-A) differences in metabolite concentrations were calculated. Absolute flux was calculated as the product of the A-V or V-A difference and adipose tissue blood flow [36]. LPL rate of action in vivo was calculated from triglyceride removal across each tissue [36]. The rate of action of HSL in adipose tissue in vivo (i.e. the rate of adipose tissue lipolysis) was calculated from the total adipose tissue glycerol release after subtraction of LPL rate of action [36]. The net inward flow of fatty acids from capillary to tissue (transcapillary flux) was calculated from the net rate of triglyceride removal and the release or removal of NEFAs across the tissue [43], and is calculated as: $3 \times (A-V)_{\text{triglyceride}} - (V-A)_{\text{NEFA}}$. This represents the net movement of fatty acids across the capillary wall, expected to be outward (negative) in the fasting state and inward (positive), representing fat storage, in the postprandial state. This net exchange includes fatty acids derived from triglycerides as well as plasma NEFAs. Insulin sensitivity in the fasting state was calculated by homeostasis model assessment (HOMA %S) [44].

The study was powered to detect a 30% difference in fasting NEFA output from adipose tissue with a power of 90% at a statistical significance level of 0.05.

Data were analysed using SPSS for Windows v10 (SPSS UK, Chertsey, UK) and statistical significance was set at *p*<0.05 for all tests. Variables are presented as means \pm SEM unless otherwise stated. Repeated measures ANOVA with time and drug (rosiglitazone or placebo) as within-subject factors was used to identify time effects, drug effects, and time and drug interactions. Potential carry-over effects were analysed using the Mann-Whitney *U*-test by comparing placebo data with data at randomisation, taking treatment order into account.

Results

Subjects Of the 24 subjects recruited (17 male), complete physiological data sets were available for 18 subjects (13 male), complete adipose tissue gene expression data for 23 subjects (16 male), and muscle gene expression data for 18 subjects (15 male). The age and sex distributions of the subgroups did not differ significantly from those of the whole group.

Baseline characteristics and general responses to treatment The mean age of the subjects at entry was 52.3 ± 10.1 years (mean \pm SD). After enrolment, all subjects had a range of baseline measurements recorded. These variables were not different from results after placebo treatment. There was no suggestion of a carry-over effect. As in other studies, rosiglitazone treatment was associated with an increase in BMI (Table 1) and a fall in haematocrit (Table 1). There was no difference in response to rosiglitazone treatment between male and female, or between older and younger subjects. As in other studies in humans, rosiglitazone significantly increased total cholesterol by 8% ($p=0.05$) and LDL cholesterol by 10% ($p<0.05$), whereas HDL cholesterol was unchanged ($p=0.12$).

Glucose and insulin responses As expected, rosiglitazone lowered HbA_{1c} (Table 1) and induced insulin sensitisation, as demonstrated by lowered fasting and postprandial plasma glucose and insulin concentrations (Fig. 1a, b) and an increase in the calculated median HOMA %S from 37.5 (interquartile range 23.8–50.3) with placebo treatment to 53.2 (43.5–63.9) ($p=0.01$) with rosiglitazone treatment.

Insulin sensitisation was also seen in muscle: the rate of forearm glucose uptake was maintained despite lower plasma glucose and insulin concentrations. There was a 30% increase in muscle glucose clearance per pmol/l of insulin (from 0.066 ± 0.017 to 0.086 ± 0.023 ml/hour per 1000 ml tissue per pmol/l of insulin, $p=0.05$), quantified as a time-averaged area under the curve for the fasting and postprandial periods. There was no significant difference

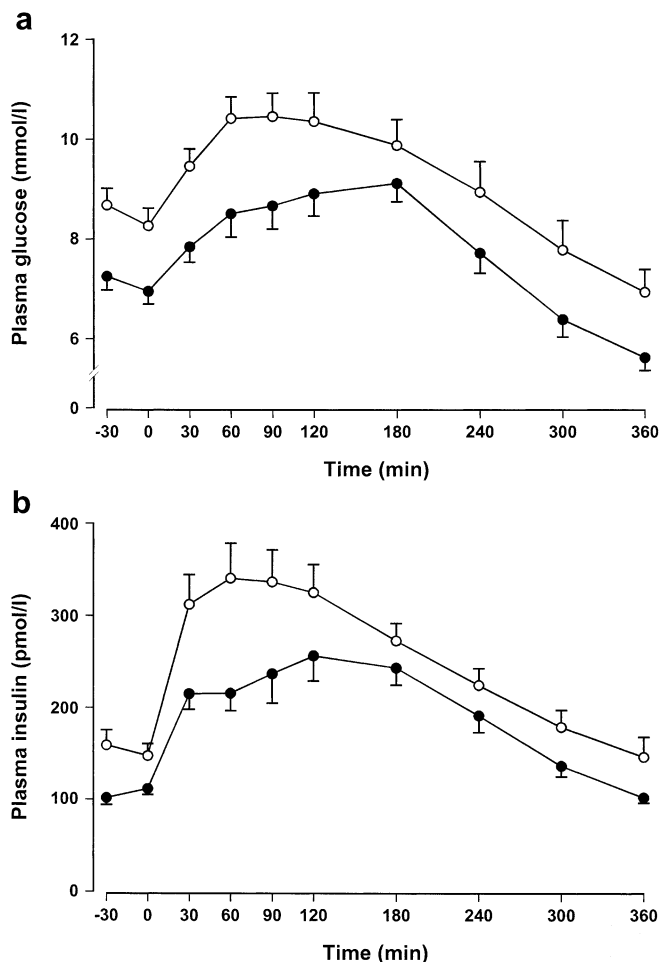


Fig. 1 Plasma glucose and insulin concentrations in response to a mixed meal (given at time 0) after placebo treatment (open circles) and rosiglitazone treatment (solid circles). **a** Plasma glucose concentrations (placebo vs rosiglitazone, $p<0.005$). **b** Plasma insulin concentrations (placebo vs rosiglitazone, $p<0.005$)

in glucose clearance by adipose tissue, assessed as a time-averaged area under the curve for the fasting and postprandial periods ($p=0.38$).

Changes in plasma NEFAs, triglycerides and RLP-C concentrations Fasting plasma NEFA concentrations were not significantly changed by thiazolidinedione treatment (633 ± 45 – 591 ± 38 $\mu\text{mol/l}$, $P=0.16$) (Fig. 2a). Postprandial plasma NEFA concentrations were reduced by 21% (time-averaged AUC from 483 ± 28 to 383 ± 20 $\mu\text{mol/l}$, $P=0.04$), primarily in the late postprandial period (Fig. 2a).

Fasting triglyceride concentrations were not altered by rosiglitazone (from 1.92 ± 1.06 to 1.98 ± 1.02 mmol/l, $p=0.86$) (Fig. 2b). Despite the lack of change in fasting concentrations, rosiglitazone reduced the postprandial rise in triglyceride concentrations compared with placebo by 26% (time-averaged incremental concentration from 855 ± 82 to 632 ± 99 $\mu\text{mol/l}$, $p<0.01$) (Fig. 2b). With rosiglitazone treatment, triglyceride concentrations began to decrease after 3 h, whereas during placebo treatment

Table 1 Characteristics of subjects

	Baseline	Placebo	Treatment
BMI (kg/m^2)	32.8 ± 1.0	32.9 ± 1.0	33.5 ± 1.1^a
HbA _{1c} (%)	7.5 ± 0.2	7.4 ± 0.2	7.0 ± 0.2^a
Fasting glucose (mmol/l)	8.93 ± 0.38	8.35 ± 0.31	6.99 ± 0.23^a
Fasting insulin (pmol/l)	133.0 ± 16.1	139.5 ± 12.7	97.3 ± 6.2^a
Haematocrit	0.44 ± 0.01	0.42 ± 0.01	0.40 ± 0.01^a
Total cholesterol (mmol/l)	5.54 ± 0.18	5.24 ± 0.19	5.68 ± 0.24^b
LDL cholesterol (mmol/l)	3.29 ± 0.17	3.19 ± 0.19	3.52 ± 0.23^b
HDL cholesterol (mmol/l)	1.29 ± 0.05	1.19 ± 0.05	1.23 ± 0.05
Triglycerides (mmol/l)	2.12 ± 0.21	1.93 ± 0.22	1.96 ± 0.22
NEFA ($\mu\text{mol/l}$)	Not analysed	633 ± 45	591 ± 38

Figures are means \pm SEM

^a $p<0.005$ vs placebo

^b $p<0.05$ vs placebo

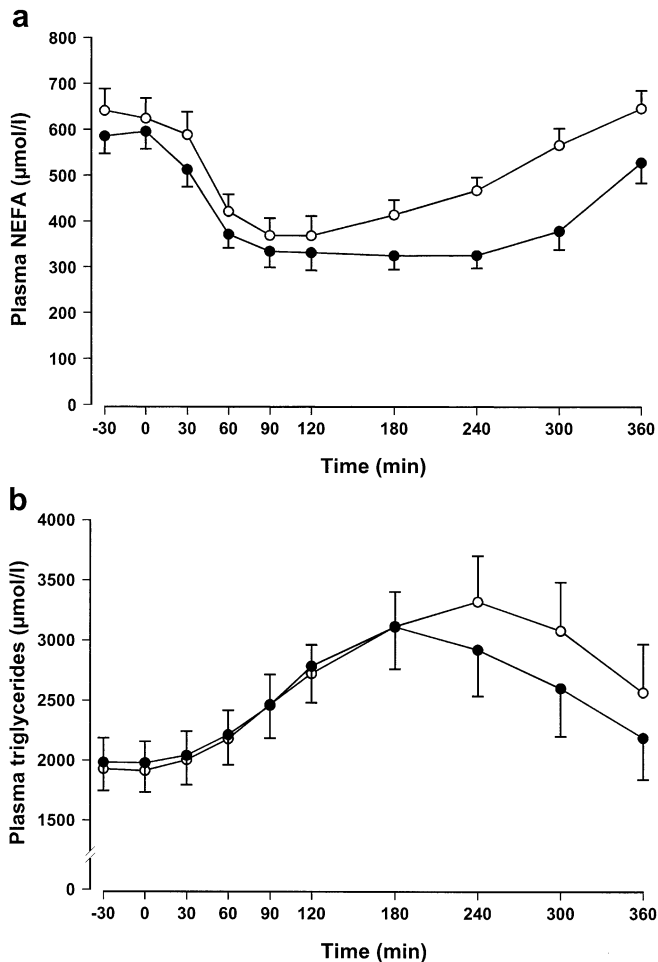


Fig. 2 Plasma triglyceride and NEFA concentrations in response to a mixed meal (given at time 0) after placebo treatment (*open circles*) and rosiglitazone treatment (*solid circles*). **a** Plasma NEFA concentrations (placebo fasting vs rosiglitazone fasting, $p=0.16$; placebo postprandial vs rosiglitazone postprandial, $p=0.04$). **b** Plasma triglyceride concentrations (placebo fasting vs rosiglitazone fasting, $p=0.86$; placebo postprandial vs rosiglitazone postprandial, $p<0.01$)

triglyceride concentrations peaked at 4 h before decreasing (Fig. 2b).

RLP-C was measured to give an estimate of the accumulation of remnants of triglyceride-rich lipoproteins after the meal. As would be expected, RLP-C concentrations increased from fasting to postprandial states (on placebo, from 0.38 ± 0.05 to 0.58 ± 0.08 mmol/l [$p<0.001$]; on rosiglitazone, from 0.46 ± 0.06 to 0.63 ± 0.11 mmol/l [$p<0.01$]). However, rosiglitazone did not significantly alter fasting or postprandial RLP-C concentrations ($p=0.68$).

Adipose tissue responses The net movement of fatty acids from capillaries to the adipocytes, the transcapillary flux, is shown in Fig. 3a. In the fasting state, there was a net release of fatty acids from adipose tissue into the circulation; this was increased by rosiglitazone treatment ($p=0.06$). Postprandially, there was a net uptake of fatty acids into adipose tissue, and this was not different

between treatment and placebo ($p=0.59$). The fatty acids taken up by adipose tissue are derived from two main sources: from the liberation of fatty acids from the intravascular hydrolysis of circulating triglycerides by LPL, and from the uptake of circulating NEFAs. The rate of action of LPL was unaltered by rosiglitazone treatment (Fig. 3b). Rosiglitazone increased fasting NEFA release by adipose tissue, although this was not statistically significant (+24%, $p=0.17$) (Fig. 3c). It was associated with an increase in the fasting HSL rate of action from 158 ± 31 to 346 ± 79 nmol/min per 100 g tissue, $p=0.01$) (Fig. 3d). Postprandial NEFA output and HSL rate of action were unchanged ($p=0.15$ and $p=0.18$, respectively) (Fig. 3d).

To explore the extent to which plasma concentrations of NEFAs were related to the output of NEFAs from subcutaneous abdominal adipose tissue, we plotted the graph of these two variables against one another (mean values are plotted in Fig. 4). Thus, there is no separate time axis on this graph; instead mean values of consecutive time points (-30, 0, 30, 60, 90, 120, 180, 240, 300 and 360 min) are connected with lines. At times -30 and 0 both subcutaneous abdominal adipose tissue and circulating NEFA concentrations were high. After the meal, both the plasma NEFA concentrations and the adipose tissue output of NEFA decreased (Fig. 4). At 120–180 min postprandially, adipose tissue output of NEFAs and plasma NEFA concentrations were both at their minimum (Fig. 4). After 240 min, plasma NEFA concentrations increased as adipose tissue NEFA output increased. The lines joining the consecutive time points formed a circular graph. The points of the circular graph whilst on rosiglitazone were distinct from and displaced downwards relative to the points of the circular graph on placebo. Using regression analysis, we calculated the gradients and slopes of the curve of each individual, comparing rosiglitazone and placebo. Rosiglitazone treatment increased the gradients ($p=0.05$) and lowered the y intercepts ($p<0.001$).

Muscle responses The transcapillary flux of fatty acids into muscle reflects fatty acids from two sources: those derived from LPL lipolysis of triglycerides and those taken up as NEFAs from the circulation. This transcapillary flux, or in other words the net uptake of fatty acids into skeletal muscle, was unaltered by rosiglitazone (Fig. 5a) (fasting, $p=0.56$; postprandial, $p=0.81$). However, the relative contributions of fatty acids from circulating NEFAs and from triglyceride lipolysis were altered. Rosiglitazone increased fasting uptake of fatty acids as NEFAs (+560%, $p=0.03$) (Fig. 5b), but did not significantly decrease fasting triglyceride lipolysis. Postprandially, rosiglitazone did not change NEFA uptake into muscle ($p=0.81$) (Fig. 5b). The rate of action of muscle LPL was unchanged by rosiglitazone (fasting, $p=0.12$; postprandial, $p=0.88$) (Fig. 5c).

Tissue blood flow Adipose tissue blood flow was not altered by eating, a finding consistent with previous results in insulin-resistant or obese subjects [45, 46]. However, rosiglitazone therapy increased both fasting and postprandial adipose tissue blood flow relative to placebo by 32%

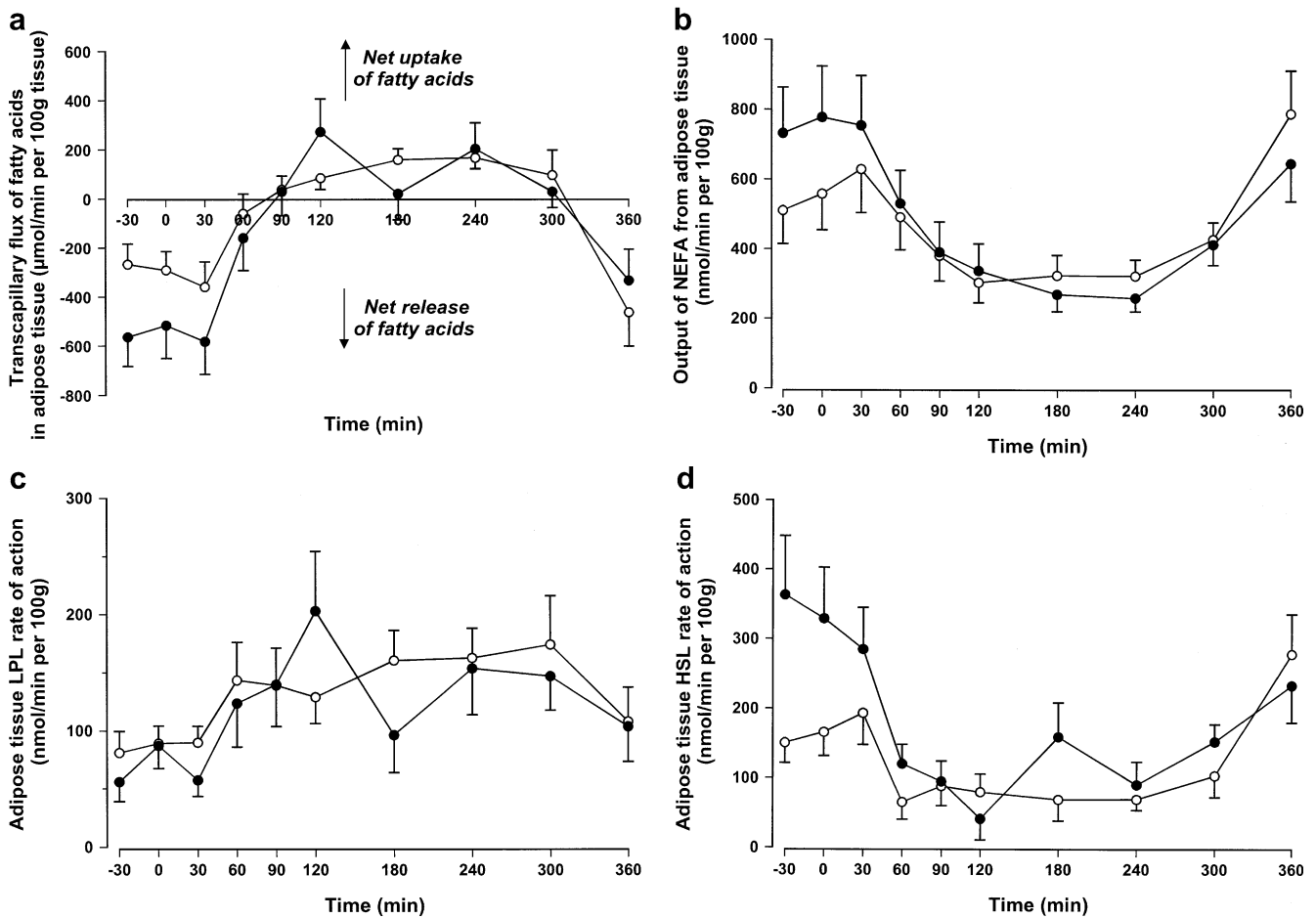


Fig. 3 Subcutaneous abdominal adipose tissue metabolism in response to a mixed meal (given at time 0), after placebo treatment (*open circles*) and after rosiglitazone treatment (*solid circles*). **a** Total flux of fatty acids into and out of subcutaneous abdominal adipose tissue ('transcapillary flux'), calculated from the V-A difference in NEFA concentrations across the depot and from the A-V difference in triglyceride concentrations across the depot, accounting for tissue blood flow. Negative values represent a net output of fatty acids from the tissue, whilst positive values represent a net uptake of fatty acids into the tissue (placebo fasting vs rosiglitazone fasting, $p=0.06$; placebo postprandial vs rosiglitazone postprandial, $p=0.59$). **b** NEFA output from adipose tissue,

calculated from the V-A difference in NEFA concentrations across the depot, accounting for tissue blood flow (placebo fasting vs rosiglitazone fasting, $p=0.17$; placebo postprandial vs rosiglitazone postprandial, $p=0.15$). **c** LPL rate of action in adipose tissue calculated from the removal of triglycerides across the tissue, accounting for tissue blood flow (placebo fasting vs rosiglitazone fasting, $p=0.50$; placebo postprandial vs rosiglitazone postprandial, $p=0.45$). **d** HSL rate of action in adipose tissue calculated from the V-A and A-V differences in glycerol and triglyceride concentrations across the depot, accounting for tissue blood flow (placebo fasting vs rosiglitazone fasting, $p=0.01$; placebo postprandial vs rosiglitazone postprandial, $p=0.18$)

(time-averaged AUC increased from 2.0 to 2.6 ml/min per 100 g of adipose tissue, $p=0.03$) (Fig. 6a). Forearm blood flow was unaffected by eating ($p=0.22$) or by rosiglitazone treatment ($p=0.20$) (Fig. 6b).

Stable isotope results To quantify the proportion of the circulating NEFA concentration that was derived from the intravascular lipolysis of circulating triglycerides, we measured concentrations of [$1\text{-}^{13}\text{C}$] palmitic acid in the circulation of a representative subset of subjects ($n=6$).

In this study, [^{13}C]palmitic acid both in the triglyceride fraction (Fig. 7a) and in the NEFA fraction (Fig. 7b) increased postprandially, as would be expected after a meal containing labelled triglycerides. Rosiglitazone did not alter the postprandial rise in [^{13}C]palmitic acid in the triglyceride fraction ($p=0.26$) (Fig. 7a), but decreased the

postprandial rise in [^{13}C]palmitic acid in the NEFA fraction ($p=0.04$) (Fig. 7b).

Gene mRNA expression in muscle and adipose tissue biopsy samples To supplement the physiological findings of the effects of thiazolidinediones on glucose and fatty acid metabolism, we measured the expression of genes important in these pathways in adipose tissue and skeletal muscle biopsies.

In skeletal muscle, hexokinase II mRNA was increased 2.2-fold ($p=0.001$) by rosiglitazone and *PDK4* was increased 1.5-fold ($p=0.04$). The expression of other genes involved in glucose metabolism was not significantly altered (Table 2). The expression of genes involved in insulin signalling (*IR*, *IRS-1/2*, *P85 α* , *p110 α* and *p110 β* subunits of PI₃ kinase) did not change (Table 2). LPL and

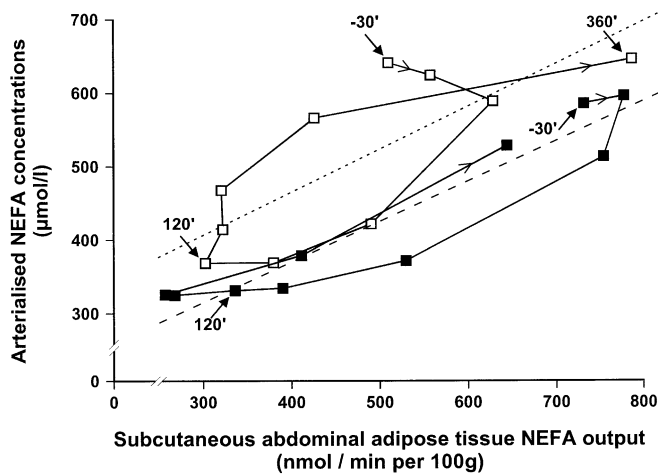


Fig. 4 Mean plasma NEFA concentrations plotted against mean NEFA output from adipose tissue. *Open squares* represent values after placebo treatment, and *solid squares* represent values after rosiglitazone treatment. *Solid lines* connect values from consecutive time points from 30 to 360 min postprandially, some of which are labelled with *arrows*. The *dotted line* represents the mean regression line for the time points on placebo. The *dashed line* represents the mean regression line for the time points on rosiglitazone. SEMs omitted for clarity (gradients of curve after placebo vs gradients of curve after rosiglitazone, $p=0.05$; y intercepts of curve after placebo vs y intercepts of curve after rosiglitazone, $p<0.001$)

FABP4 mRNA expression was upregulated, 2.1-fold ($p=0.006$) and 1.8-fold ($p=0.001$), respectively. *FATP1* was reduced by 30% ($p=0.02$). There was a statistically significant but modest increase in *UCP2* expression of 1.08-fold ($p=0.05$). Other genes involved in fatty acid metabolism were unchanged.

In adipose tissue the mRNA expression of the known PPAR γ target gene, *FABP4*, increased by 66% ($p<0.0001$) (Table 3). The expression in adipose tissue of *LPL* (+36%) and *FAT/CD36* (+15%) showed modest increases (Table 3).

Discussion

We set out to explore the association between fatty acid and triglyceride metabolism in response to rosiglitazone treatment in type 2 diabetes. We found that rosiglitazone decreased NEFA and triglyceride concentrations in the late postprandial phase, whilst leaving fasting concentrations unchanged. Mechanisms underlying these observations were explored using arteriovenous and stable isotope techniques.

The postprandial lowering of NEFA concentrations occurred more than 3 h after eating. In theory, this could be a result of either an increased removal or a decreased production of NEFAs. However, plasma NEFA concentrations generally reflect the rate of generation of NEFAs rather than their rate of removal [47, 48]. Here, we quantified the rate of NEFA removal by skeletal muscle and found that this was unchanged by rosiglitazone in the late postprandial period. Other authors have also addressed this issue by quantifying the actual rate of NEFA removal

in response to rosiglitazone. They found that rosiglitazone did not change the rate of NEFA removal [49]. Thus, NEFA removal is not likely to be altered by rosiglitazone, implying that the lowered postprandial NEFA concentrations are likely to be secondary to a lower rate of generation of NEFAs.

As PPAR γ is highly expressed in adipose tissue [50] and generation of NEFAs only occurs in adipose tissue, this seems to be a likely site of action of rosiglitazone. The adipose tissue depot that is the main source of circulating NEFAs is subcutaneous abdominal adipose tissue [51]. Therefore, we asked whether a reduced output of NEFAs from this depot could account for the lower postprandial NEFA concentrations. Using arteriovenous techniques in combination with measurements of adipose tissue blood flow, we found that rosiglitazone did not affect adipose tissue NEFA release postprandially. When the relationship between plasma NEFA concentrations and subcutaneous adipose tissue NEFA release was explored by regression analysis, the relationship was significantly changed by rosiglitazone. In effect, rosiglitazone lowered the intercept of the regression line towards zero, implying that NEFA generation from sources other than abdominal subcutaneous adipose tissue had been reduced in response to rosiglitazone.

Could different adipose tissue depots respond differently to rosiglitazone? One adipose tissue depot that may behave differently is visceral adipose tissue. A reduction in postprandial NEFA generation from this depot in response to rosiglitazone could account for the observed lowering of postprandial NEFA concentrations and would be consistent with the reduction of visceral adipose tissue mass with rosiglitazone found by other authors [52, 53].

By measuring plasma concentrations of stable isotopes derived from the meal containing [1,1,1- ^{13}C]tripalmitin, we were able to identify potential mechanisms underlying the postprandial lowering of plasma NEFA concentrations by rosiglitazone. NEFAs entering the circulation from adipose tissue can be derived from the lipolysis of either intracellular or intravascular triglycerides. The lipolysis of intravascular triglycerides by LPL produces NEFAs which are trapped in adipose tissue or spill over into the systemic circulation. We have previously shown that it is the spillover of NEFAs derived from chylomicron triglycerides that is particularly important in the late postprandial rise in plasma NEFA concentrations [54]. It is this process that we believe is affected by rosiglitazone treatment. [^{13}C] Palmitic acid in the triglyceride fraction is administered in the mixed meal, and so is found in chylomicron particles. The rate of appearance of [^{13}C]palmitic acid in the NEFA fraction decreased, despite no change in the rate of removal of [^{13}C]palmitic acid from the triglyceride fraction, implying that the rate of NEFA spillover from chylomicron triglycerides is lowered by rosiglitazone. Such lowered spillover of NEFAs with rosiglitazone could therefore account for the lowered postprandial NEFA concentrations. However, this mechanism did not seem to be active in subcutaneous abdominal adipose tissue. We suggest that this mechanism operates in visceral adipose

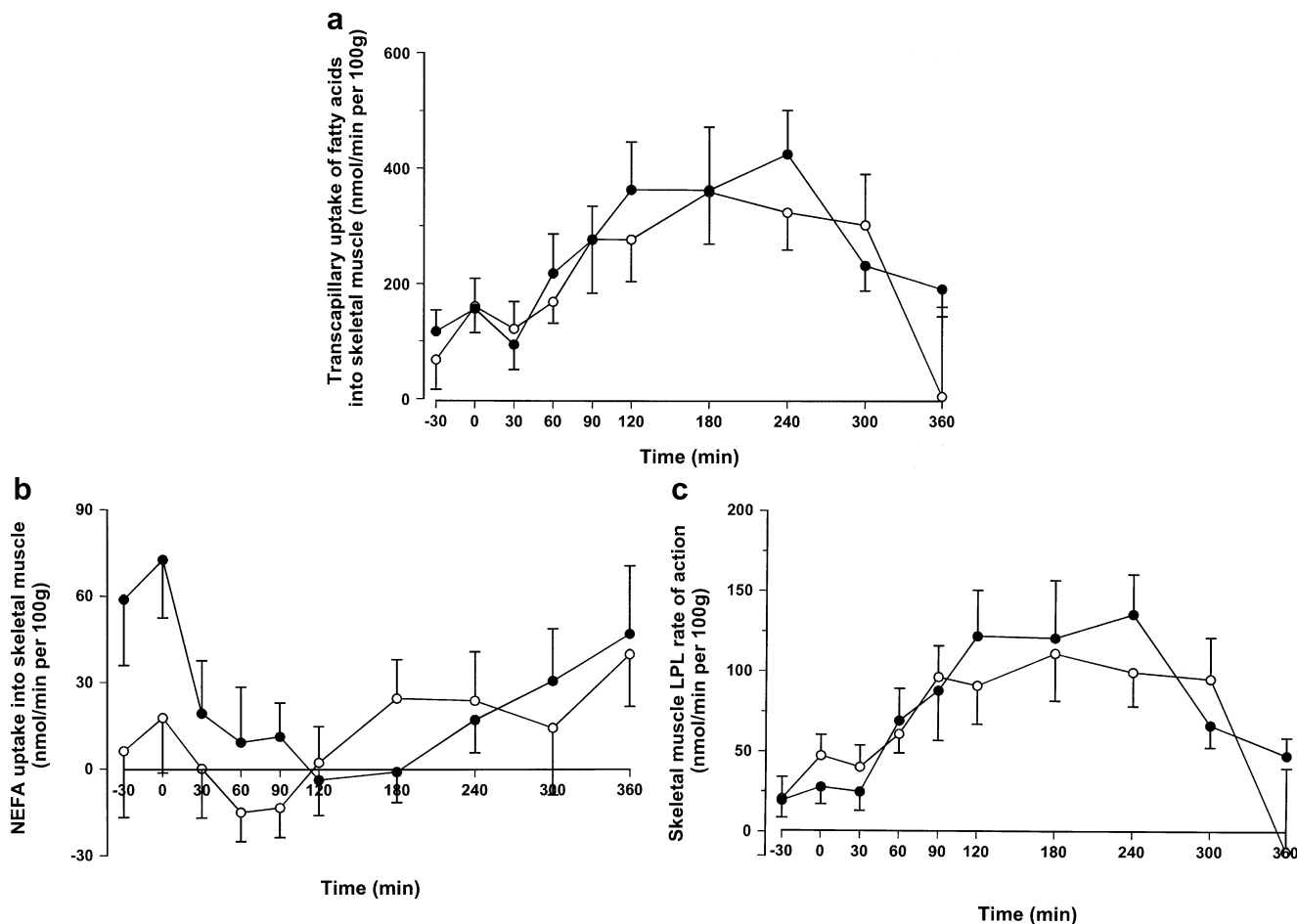


Fig. 5 Skeletal muscle metabolism in response to a mixed meal (given at time 0) after placebo treatment (*open circles*) and after rosiglitazone treatment (*solid circles*). **a** Total flux of fatty acids into and out of skeletal muscle ('transcapillary flux'), calculated from the V-A difference in NEFA concentrations across the depot and from the A-V difference in NEFA concentrations across the depot, accounting for tissue blood flow. Higher values represent increased uptake of fatty acids into muscle (placebo fasting vs rosiglitazone fasting, $p=0.56$; placebo postprandial vs rosiglitazone postprandial,

$p=0.81$). **b** NEFA uptake into skeletal muscle, calculated from the A-V difference in NEFA concentrations across the depot, accounting for tissue blood flow (placebo fasting vs rosiglitazone fasting, $p=0.03$; placebo postprandial vs rosiglitazone postprandial, $p=0.81$). **c** LPL rate of action in skeletal muscle, calculated from the removal of triglycerides across the tissue, accounting for tissue blood flow (placebo fasting vs rosiglitazone fasting, $p=0.12$; placebo postprandial vs rosiglitazone postprandial, $p=0.88$)

tissue in humans and accounts for the observed reduction in plasma NEFA concentrations.

In this study, rosiglitazone lowered triglyceride concentrations in an unusual pattern: late postprandial triglyceride concentrations decreased whilst fasting triglyceride concentrations were unaffected. Such an isolated postprandial reduction has only occasionally been described with any pharmacological agent [55–57]. In rodents, similar fasting-postprandial triglyceride effects have been noted after giving a non-thiazolidinedione PPAR γ agonist to male Sprague–Dawley rats [58]. In contrast to rosiglitazone, the other clinically used thiazolidinediones, pioglitazone and troglitazone, reduce both fasting and postprandial triglyceride concentrations. Rosiglitazone, in this and other studies [15–19], does not alter fasting triglyceride concentrations. There is only one previous report of the effect of rosiglitazone on postprandial triglyceride concentrations [17]. As that study was designed primarily

to examine glucose metabolism, blood samples were only taken until 180 min postprandially [17]. There was no change in the early postprandial period in plasma triglyceride concentrations in either the present study or that of Raskin et al. [17]. However, ours is the first study to examine triglyceride concentrations during the late postprandial period; we found that rosiglitazone consistently lowered triglyceride concentrations 3 h postprandially.

The postprandial lowering of plasma triglycerides was not simply secondary to an increased rate of removal of triglycerides by the two major tissues expressing LPL, adipose tissue and skeletal muscle: LPL rate of action was unchanged in both of these tissues. Could increased triglyceride removal by the liver account for the postprandial triglyceride reductions? The liver removes triglycerides by whole particle uptake of the remnants of triglyceride-rich lipoproteins. So an increase in the uptake of remnant particles by the liver should be reflected

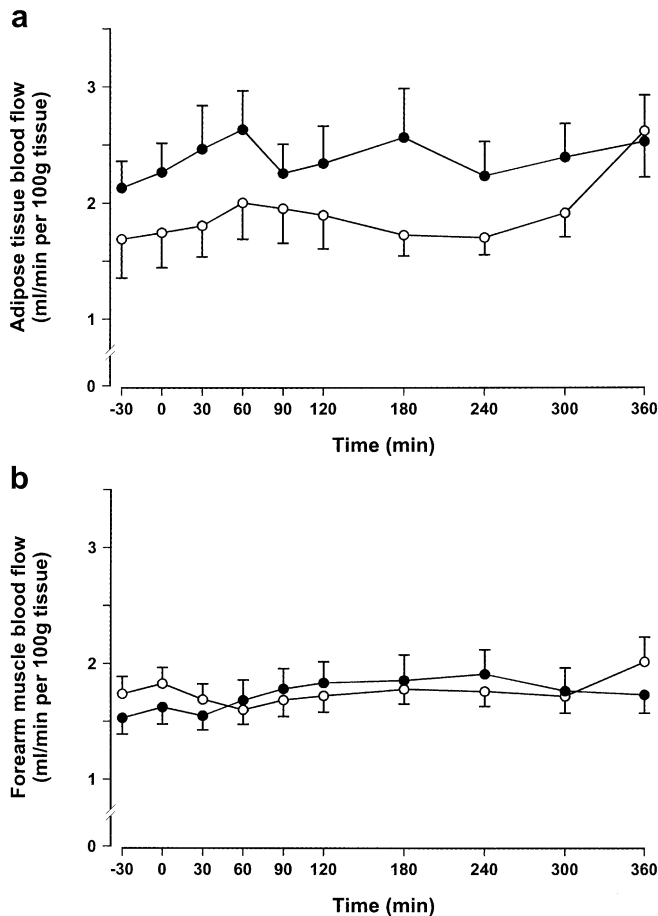


Fig. 6 Tissue blood flow in response to a mixed meal (given at time 0) after placebo treatment (*open circles*) and rosiglitazone treatment (*solid circles*). **a** Adipose tissue blood flow (placebo vs rosiglitazone, $p=0.03$). **b** Forearm muscle blood flow (placebo vs rosiglitazone, $p=0.20$)

by a decrease in RLP-C concentrations. We found that rosiglitazone did not alter RLP-C concentrations, thus increased triglyceride removal by the liver is unlikely to be the mechanism for the lowered postprandial triglyceride concentrations. An alternative explanation would arise from the decreased postprandial plasma concentrations of NEFAs. This would result in a decreased delivery of NEFAs to the liver, an effect that would be particularly marked if visceral adipose tissue were the main adipose tissue depot affected by rosiglitazone [59]. This in turn would lead to decreased liver fat content, an observation that has been made in rosiglitazone-treated patients [12]. Liver fat would serve as the immediate pool for the synthesis of VLDL triglycerides. Thus a reduction in liver fat might be expected to reduce VLDL triglyceride production by the liver, which would be observed as lower postprandial triglyceride concentrations. Reductions in NEFA concentrations have been previously shown to be associated with lower VLDL triglyceride liver output [60]. A second possible mechanism for lowered postprandial VLDL triglyceride concentrations would be the effects of insulin on VLDL triglyceride secretion. The acute inhibi-

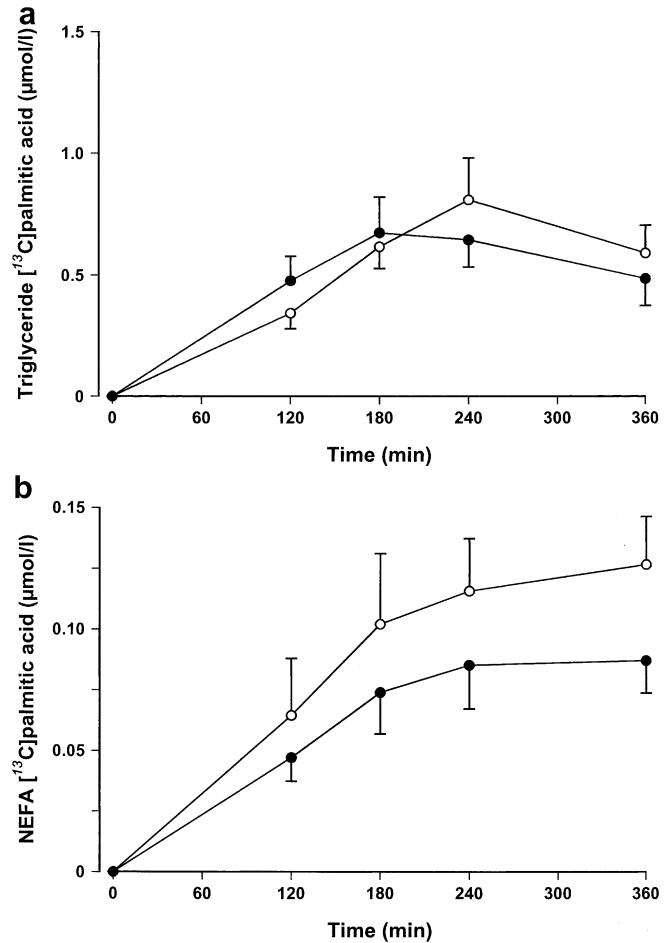


Fig. 7 Stable isotope concentrations in response to a mixed meal containing 600 mg of [1,1,1- ^{13}C]tripalmitin (given at time 0) after placebo treatment (*open circles*) and rosiglitazone treatment (*solid circles*). **a** Concentrations of postprandial [^{13}C]palmitic acid in the triglyceride fraction ($p=0.26$). **b** Concentrations of postprandial [^{13}C]palmitic acid in the NEFA fraction ($p=0.04$)

Table 2 mRNA expression in skeletal muscle in response to rosiglitazone treatment

Muscle	Fold increase		p value ^a
	Median	Interquartile range	
Insulin signalling pathway			
Insulin receptor	1.18	(0.89–1.70)	0.08
IRS-1	0.96	(0.75–1.57)	0.53
IRS-2	0.94	(0.61–1.33)	0.94
p85 α PI3K	1.21	(0.92–1.57)	0.09
P110 α PI3K	1.02	(0.63–1.94)	0.31
P110 β PI3K	0.95	(0.71–1.51)	0.64
Glucose metabolism pathway			
GLUT4	1.28	(0.88–1.72)	0.07
PDK4	1.51	(0.67–2.43)	0.04
Glycogen synthase	1.23	(0.77–1.55)	0.22
Hexokinase II	2.16	(1.38–2.82)	<0.001

^a Using Wilcoxon signed rank test

Table 3 mRNA expression in adipose tissue in response to rosiglitazone treatment

Adipose tissue	Fold increase		<i>p</i> value ^a
	Median	Interquartile range	
FABP4	1.66	(1.31–1.88)	<0.001
LPL	1.28	(1.10–1.66)	0.001
HSL	1.14	(0.91–1.25)	0.15
FAT/CD36	1.19	(1.05–1.38)	<0.001
FAS	1.31	(0.75–1.89)	0.17
SREBP1c	0.98	(0.75–1.26)	0.48
PPAR γ 2	1.28	(0.88–1.52)	0.31
Hexokinase II	1.07	(0.84–1.20)	0.86

^a Using Wilcoxon signed rank test

tion of VLDL triglyceride secretion by insulin is blunted in type 2 diabetes [61, 62]. It is therefore possible that rosiglitazone restores the effectiveness of insulin in inhibiting the secretion of VLDL triglycerides.

Adipose tissue blood flow is known to be decreased in obesity and type 2 diabetes [45]. Rosiglitazone increased adipose tissue blood flow by a small but consistent amount. This increase in adipose tissue blood flow with rosiglitazone has not been previously described but is consistent with vasodilatory effects attributed to rosiglitazone [63]. Indeed, adipose tissue blood flow is known to be important in the regulation of the tissue's metabolism [45], and a nutrient-responsive blood flow pattern has been associated with insulin sensitivity [64]. In contrast, muscle blood flow was not changed, suggesting that the effect of rosiglitazone on blood flow was tissue specific.

The activity of HSL, the enzyme responsible for adipose tissue lipolysis, was not decreased but significantly increased in the fasting state, as was the output of NEFAs from adipocytes as reflected in the net transcapillary flux of fatty acids ($p=0.06$). This finding is, however, entirely consistent with HSL simply responding to circulating insulin concentrations in the fasting state. As hyperinsulinaemia is reduced in the process of insulin sensitisation, HSL activity is consequently upregulated. Thus, adipose tissue may be seen as responding appropriately to ambient insulin concentrations. The postprandial changes in NEFA output and HSL activity in adipose tissue are also consistent with this hypothesis. Adipose tissue NEFA output and HSL rate of action in the postprandial state did not change with rosiglitazone therapy; both were suppressed by insulin after placebo and rosiglitazone treatment. To effectively suppress adipose tissue lipolysis, only a small increment in insulin concentrations above baseline is required [65]. Our data are consistent with those of Mayerson et al. [12] who used a combination of two-step hyperinsulinaemic–euglycaemic clamp and microdialysis techniques to assess in vivo adipose tissue lipolysis [12]. After rosiglitazone treatment, lipolysis was suppressed to a greater extent with a low dose of insulin.

Although the main aim of this study was to assess physiological changes in humans in response to rosiglitazone treatment, the measurement of mRNA expression in

biopsy samples allowed the investigation of potential mechanisms underlying the physiological changes observed in skeletal muscle and adipose tissue.

In skeletal muscle, we assessed the expression of genes involved in two pathways important in insulin sensitisation: glucose metabolism and insulin signalling. Rosiglitazone did not alter the expression of genes involved in insulin signalling in muscle, although mRNA concentrations of genes involved in insulin signalling may not directly reflect activity of the pathway. However, our data are consistent with the results of in vitro cultures of muscle cells exposed to rosiglitazone [66] and with the results of protein levels of IRS-1, IRS-2 and GLUT-4 measured in muscle biopsies [67, 68]. The entry of glucose into muscle cells has been shown to be a major controlling step of glucose disposal in type 2 diabetes [69, 70]. Although GLUT-4 mRNA expression was unchanged by thiazolidinedione therapy, this presumably reflects the fact that regulation of GLUT-4 activity occurs mainly at a post-transcriptional level [71]. An early step in skeletal muscle glucose metabolism, hexokinase II, responsible for phosphorylation of glucose on entry into the muscle cell, was induced 2.2-fold by rosiglitazone. This finding suggests a potential mechanism for the observed increased efficiency of forearm muscle glucose uptake with rosiglitazone treatment. Depression of basal skeletal muscle hexokinase II expression in type 2 diabetes was first demonstrated by Vestergaard and colleagues [72], and is supported by the in vivo work of Kelley and colleagues [70]. In healthy people, hexokinase II is induced by insulin to meet the increased demands for glucose handling by the tissue, and this induction fails to occur in type 2 diabetes [73–75]. This is the first study to demonstrate that insulin sensitisation by thiazolidinediones is associated with the normalisation of hexokinase II expression in type 2 diabetes in humans.

In adipose tissue, the expression of genes involved in fatty acid metabolism, such as *HSL*, *FAT/CD36*, *FAS* and *SREBP1c*, was not substantially altered, although a number of the changes observed were statistically significant. In contrast, *FABP4* (*aP2*), a gene known to be responsive to PPAR γ activation [76], was upregulated 1.7-fold in adipose tissue ($P<0.0001$).

The measurement of mRNA expression in the adipose tissue and skeletal muscle biopsies has limitations. We have demonstrated lower postprandial insulin and NEFA concentrations with rosiglitazone treatment. Thus, it is unclear whether the changes in mRNA concentrations are direct effects of rosiglitazone on skeletal muscle and adipose tissue or whether we are observing the consequences of lowered insulin concentrations. The use of mRNA expression also imposes limitations on the understanding of metabolic processes. For example, LPL is known to be primarily post-transcriptionally regulated and thus its expression does not necessarily correlate with its observed in vivo or ex vivo activity, as demonstrated here.

Conversely, a major strength of this study is that it assesses responses in co-ordinated integrated physiology and metabolism with thiazolidinedione therapy, not simply changes in gene or protein expression: the physiological

responses are the final integrated steps of the changes in mRNA and protein concentrations.

We propose that in humans, the main effect of rosiglitazone is not primarily to reduce fasting NEFA concentrations, but instead to reduce postprandial NEFA concentrations. This could be the mechanism of insulin sensitisation by rosiglitazone. This is also the first study to describe that rosiglitazone reduces postprandial triglyceride concentrations. We identify the potential underlying mechanisms for these observations. It is also possible that rosiglitazone has a direct effect on skeletal muscle glucose handling by increasing hexokinase II expression.

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