The PPARγ agonist rosiglitazone enhances rat brown adipose tissue lipogenesis from glucose without altering glucose uptake

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Running title: PPARγ activation and BAT lipid metabolism

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Abstract

We investigated mechanisms whereby PPARγ agonism affects glucose and lipid metabolism in brown adipose tissue (BAT) by studying the impact of PPARγ activation on BAT glucose uptake and metabolism, lipogenesis and mRNA levels plus activities of enzymes involved in triacylglycerol (TAG) synthesis. Interscapular BAT of rats treated or not with rosiglitazone (15 mg/kg/day, 7 days) was evaluated in vivo for glucose uptake and lipogenesis and in vitro for glucose metabolism, gene expression and activities of glycerolphosphate acyltransferase (GPAT), phosphatidate phosphatase-1 (PAP or lipin-1) and diacylglycerol acyltransferase (DGAT). Rosiglitazone increased BAT mass without affecting whole tissue glucose uptake. BAT glycogen content (–80%), its synthesis from glucose (–50%), and mRNA levels of UDP-glucose pyrophosphorylase (UDPG-PPL, –40%), which generates UDP-linked glucose for glycogen synthesis, were all reduced by rosiglitazone. In contrast, BAT TAG-glycerol synthesis in vivo and glucose incorporation into TAG-glycerol in vitro were stimulated by the agonist along with the activities and mRNA levels of glycerol 3-phosphate-generating phosphoenolpyruvate carboxykinase (PEPCK) and glycerokinase (GyK). Furthermore, rosiglitazone markedly increased the activities of glycerol 3-phosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT), but not that of lipin1-mediated phosphatidate phosphatase-1, enzymes involved in the sequential acylation of glycerol 3-phosphate and TAG synthesis. Because an adequate supply of fatty acids is essential for BAT non-shivering thermogenesis, the enhanced ability of BAT to synthesize TAG under PPARγ activation may constitute an important mechanism by which lipid substrates are stored in preparation for an eventual thermogenic activation.
Keywords

glycogen, glycerokinase, PEPCK, GPAT, lipin, DGAT

Abbreviations

COX4, cytochrome oxidase 4; CS, citrate synthase; DGAT, diacylglycerol acyltransferase; GLUT, glucose transporter; GPAT, glycerol 3-phosphate acyltransferase; GPD, glycerol 3-phosphate dehydrogenase; GyG, glycogenin; GyK, glycerokinase; GyS2, glycogen synthase 2; HEXO, hexokinase; IDH, isocitrate dehydrogenase; LDH, lactate dehydrogenase; LPA, lysophosphatidic acid; PAP, phosphatidate phosphatase-1; PDHα, pyruvate dehydrogenase subunit α; PEPCK, phosphoenolpyruvate carboxykinase; PFK, phosphofructokinase; PPARγ, peroxisome proliferator-activated receptor γ; TAG, triacylglycerol; TZD, thiazolidinediones; UDPG-PPL, UDP-glucose pyrophosphorylase; VLDL, very low density lipoproteins; WAT, white adipose tissue.
Introduction

Brown adipose tissue (BAT) plays an important role in rodent whole body energy homeostasis due its extreme ability under demand to dissipate energy mainly from fatty acid oxidation in the form of heat (4). This process, known as non-shivering thermogenesis, is positively regulated through norepinephrine (NE) release by the sympathetic nerves that densely innervate brown adipocytes. Upon sympathetic activation, NE interacts with β3 adrenergic receptors, which leads to lipase activation and hydrolysis of intracellular stores of triacylglycerol (TAG). As a result, lipolysis-derived fatty acids are directed to the mitochondria where they are either oxidized, releasing energy for heat production, or allosterically activate uncoupling protein (UCP) 1 and thus thermogenesis. Therefore, maintenance of adequate intracellular TAG stores is essential for BAT non-shivering thermogenesis as clearly supported by the marked increase in BAT TAG synthesis in rats acclimated to cold (4, 31). Beyond its importance in rodent energy metabolism, BAT has recently gained renewed attention since its identification in humans (32).

Peroxisome proliferator-activated receptor (PPAR) γ, a nuclear receptor highly expressed in BAT, is a master transcriptional regulator of brown adipocyte differentiation required for BAT development, function and survival (1, 11, 18, 19, 21, 36). Activation of PPARγ in vivo by the administration of synthetic, specific agonists results in BAT recruitment (increase in tissue mass and cell number) characterized by a marked elevation of UCP1 and other thermogenic genes which, however, do not translate into higher heat production due to reduced BAT sympathetic activity and thyroid status (16). In association with such tissue recruitment, PPARγ activation drastically increases BAT ability to take up and store fatty acids, especially those derived from lipoprotein-bound TAG through lipoprotein lipase (26). In fact, BAT appears to play an important role in the hypolipidemic action of PPARγ agonists in rodents (26). Of note is the fact
that isolated human white adipocytes can acquire features of brown adipocytes upon exposure to PPARγ agonists (43), raising the intriguing possibility that brown adipocytes may contribute to the metabolic actions of PPARγ agonists in humans as well.

In addition to lipids, BAT displays very high rates of glucose uptake per unit of weight, which means that even though BAT makes up only a small fraction of body weight in rodents, it can potentially constitute a significant glucose-clearing organ especially under sympathetic activation (4). In BAT, glucose is mainly used to synthesize glycerol 3-phosphate, the carbon backbone for fatty acid esterification and TAG synthesis (4). Along with glycerol 3-phosphate generation, significant amounts of glucose in BAT are also stored in the form of glycogen, converted to lactate by anaerobic glycolysis or used to synthesize fatty acids (4). Despite the well-known insulin sensitizing actions of PPARγ agonists and their remarkable BAT recruitment, little is known about the contribution of BAT to the improvement in whole body glucose homeostasis induced by these compounds, or how these compounds impact on the metabolic fate of glucose within BAT.

To address these issues, rats treated with the thiazolidinedione, rosiglitazone, were evaluated in vivo for glucose uptake and lipogenesis and in vitro for glucose oxidation, as well as in vitro for glucose oxidation and conversion to lactate, TAG and glycogen. These experiments were accompanied by extensive investigation of activities and mRNA levels of key enzymes of TAG synthesis.
Material and Methods

**Animals and treatment.** Animal experimental procedures were performed in accordance with the Canadian Guide for the Care and Use of Laboratory Animals and received prior approval of the Laval University animal care committee. Male Sprague-Dawley rats (Charles River, St. Constant, QC, Canada) were matched by weight and divided into control and rosiglitazone-treated groups that received a powdered rodent diet (Charles River #5075, Woodstock, ON, Canada) alone (control) or supplemented with the PPARγ agonist rosiglitazone (Avandia) at a dose of 15 mg/kg/day for 7 days. Rats were kept at 23 ± 1°C with a light/dark cycle of 12 h/12 h. The dose of rosiglitazone was chosen based on preliminary studies that showed its effectiveness to increase BAT mass within a short treatment (2, 16). Ground rosiglitazone was mixed with the powdered chow diet and the desired dose was achieved by adjusting the amount of drug to the average food consumption and body weight of rats every other day.

**In vivo glucose uptake.** The rate of glucose uptake by BAT was estimated *in vivo* essentially as previously described (13, 40). Briefly, 0.2 ml of a 2-deoxy-1-$^3$H-glucose (30 μCi, New England Nuclear, 11 Ci/mmol) in 0.9% NaCl solution was injected through a catheter inserted in the jugular vein and 0.2 ml of blood was collected at 1, 3, 5, 10, 20, 40 and 60 min after label injection for radioactivity and glucose estimation. Upon collection, blood was immediately deproteinized in Ba(OH)$_2$/ZnSO$_4$ as previously described (41) and centrifuged (2 min, 16000 × g). The supernatant was used for the determination of blood glucose with a glucose oxidase kit (Wako Chemicals, Richmond, VA) and 2-deoxy-1-$^3$H-glucose in a liquid-scintillation counter (Tri-Carb 2900TR, PerkinElmer, IL). After 60 min, rats were killed by overdose of ketamine/xylazine and tissues were used for determining the content of 2-deoxy-[1-$^3$H]glucose...
6-phosphate. Rates of glucose uptake were calculated as previously described (13). Blood glucose levels did not significantly change during the sampling period in either group, a requirement of the technique used.

**Tissue glycogen content.** Measurement of BAT glycogen content was performed exactly as previously described (28). Briefly, 30-50 mg of brown adipose tissue was digested with 500 µL 30% KOH saturated with Na₂SO₄ at 70°C for 30 min and transferred to an ice bath. After addition of 600 µL 95% ethanol, samples were centrifuged and the supernatant was discarded. Pellets were re-suspended in distilled water, incubated with 250 µL 5% phenol and 1 mL 96-98% H₂SO₄, and analyzed colorimetrically in a spectrophotometer at a wavelength of 490 nm.

**In vivo lipogenesis.** Fed, non-anesthetized rats were injected with ³H₂O (185 GBq/ml, Amersham, 3 mCi in 0.5 mL sterile saline solution, i.p.). One hour after injection, rats were killed by decapitation, blood samples were collected in EDTA-containing tubes, and the specific radioactivity of plasma water was determined after centrifugation (1500 × g, 15 min, 4°C). BAT was removed and processed for measurement of label incorporation into TAG. Tissue total lipids were extracted with 2:1 chloroform:methanol (17) and processed for the measurement of ³H incorporation into fatty acid component of TAG (3). Incorporation of ³H into TAG-glycerol was estimated by the difference between incorporation into total lipids and fatty acids. Rates of tissue fatty acid and glycerol synthesis were calculated assuming that each glycerol and each fatty acid incorporated into TAG contained 3.3 and 13.3 atoms of tritium, respectively (23, 44).

**In vitro glucose uptake and conversion to its metabolites.** Glucose conversion to its metabolites in vitro was measured as previously described (9). Briefly, explants (20-25 mg) of BAT were incubated in 1 ml of Krebs-Ringer bicarbonate buffer (in mmol/l): 118 NaCl, 4.8 KCl, 1.25 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃, 5.5 [U-¹⁴C]glucose (0.5 µCi/tube, Amersham,
3 mCi/mmol) supplemented with 2.5% fatty acid-free bovine serum albumin (Sigma, Oakville, ON, Canada), pH 7.4. Vials were closed with a rubber stopper, gassed with 5% CO2 and 95% O2 and incubated at 37°C for 1 h in the presence or absence of insulin (100 pmol/l). The insulin concentration was chosen to approximate to that found in vivo in the fasting state. At the end of the incubation, reactions were stopped with sulfuric acid and benzathonium hydroxide was injected with a syringe and needle into a strip of paper previously positioned in the rubber stopper to trap CO2. Explants were removed and destined to either lipid or glycogen extraction as described above and media were frozen for later lactate assay (BioVision Research Products, CA). Data for explants are expressed as µmol/ µg DNA/h to correct for cell number. The DNA content for each incubation well was determined using the Dneasy Tissue Kit (QIAGEN, Mississauga, ON, Canada) following manufacturer’s instructions.

Lipogenic enzymatic activities. Phosphoenolpyruvate carboxykinase (PEPCK) activity was assayed in 100,000×g supernatants obtained after homogenization of BAT in a buffer (pH 7.5) containing 20 mmol/l triethanolamine, 0.25 mol/l sucrose, 5 mmol/l mercaptoethanol and 1 mmol/l EDTA. PEPCK activity was determined by the method of Chang and Lane (6), based on the incorporation of H214CO3 (0.074 MBq) into acid-stable product as described previously (15). Total protein concentration in the homogenate was determined by the bicinchoninic acid method (39). The activity of glycerokinase (GyK) was measured following the recommendations of Newsholme et al. (33), in 2,000×g supernatants obtained after homogenization of BAT in ice-cold 10 g/l KCl and 1 mmol/l EDTA. The composition of the assay mixture, which contained [U-14C]-glycerol, and the isolation of labeled glycerol-3-phosphate were as previously described (24). Protein content of homogenates was determined by the method of Lowry et al. (29). Glycerol 3-phosphate acyltransferase (GPAT) activity was measured as previously described
(27, 38) with some modifications. BAT was homogenized in buffer A (10 mmol/l Tris-HCl, 250 mmol/l sucrose, 1 mmol/l EDTA and 1 mmol/l dithiothreitol, pH 7.5) and centrifuged at 600 x g, 4°C for 10 min. The supernatant was removed and centrifuged at 100,000 x g, 4°C, for 1 h. Pellets were re-suspended in buffer A and evaluated for GPAT activity after a 15 min incubation with or without 2 mmol/l (final concentration) N-ethylmaleimide on ice. The assay mixture was composed of 75 mmol/l Tris-HCl, pH 7.5, 4 mmol/l MgCl₂, 1 mg/ml BSA (essentially fatty-acid free), 8 mmol/l NaF, 100 mmol/l palmitoyl-CoA and 500 mmol/l of ¹⁴C-glycerol 3-P (0.5 μCi/tube, Amershan, 100 mCi/mmol). Reactions were stopped by adding 3 ml of chloroform:methanol (1:2, vol/vol) and 0.6 ml of 1% perchloric acid. After 5 min, 1 ml of chloroform and 1 ml of 1% perchloric acid were added, tubes were shaken vigorously, and the upper methanolic phase was discarded. The lower phase was washed 3 times with 1% perchloric acid, evaporated and radioactivity determined.

Diacylglycerol acyltransferase (DGAT) activity was measured (7) with some modifications. BAT was homogenized in buffer A and centrifuged twice at 12,000 x g, 4°C for 10 min. The supernatant was removed and centrifuged at 100,000 x g, 4°C, for 1 h. Pellets were re-suspended in buffer A and evaluated for DGAT activity. The assay mixture was composed of 150 mmol/l Tris-HCl, pH 7.5, 8 mmol/l MgCl₂, 1 mg/ml BSA, 100 mmol/l 1-[¹⁴C]palmitoyl-CoA (0.25 μCi/tube, Amersham, 50 mCi/mmol) and 0.8 mmol/l of 1,2-diacylglycerol in absolute ethanol. Reactions were stopped with 1.5 ml of propanol-2:heptane:water (80:20:2, v/v/v). After 5 min, 1 ml of heptane and 0.5 ml of water were added and tubes were vigorously shaken. The heptane layer was removed and washed twice with 0.5 N NaOH:ethanol:water (10:50:50, v/v/v), evaporated and counted.
Phosphatidate phosphatase-1 (PAP) activity and lipin-1 concentration. BAT was homogenized in 0.25 mol/l sucrose containing 2 mmol/l dithiothreitol (to stabilize PAP1 activity) and Protease Inhibitor cocktail (EDTA-free, Roche Diagnostics, Inc., Indianapolis, IN). The homogenates were then centrifuged at 4000 r.p.m. for 10 min at 4ºC and infranatants were collected. For the PAP1 assay Tween 20 (0.15%) was added to the homogenates to stabilize and increase the PAP1 activity. The assays were performed essentially as previously described (10, 30). The incubations contained in a final volume of 100 µl: 100 mmol/l Tris/maleate buffer, pH 6.5, 5 mmol/l MgCl₂, 2 mg/ml fatty acid poor bovine serum albumin and 0.6 mmol/l PA labeled with [³H]palmitate (about 1 x 10⁵ dpm/assay) which was dispersed in 0.4 mmol/l PC, and 1 mmol/l EDTA and 1 mmol/l EGTA. Reactions were stopped after incubation at 37ºC for 60 min with 2.2 ml of chloroform containing 0.08% olive oil as carrier and DAG was purified by using basic alumina (10, 30). Total PAP activities were calculated from measurements at three different protein concentrations to ensure the proportionality of the assay. Parallel incubations were performed in the presence of excess (5 mmol/l) NEM to inhibit PAP1 and to compensate for any lipid phosphate phosphatase activity in this assay.

Western blot analysis of lipin-1. Identical amounts of protein were electrophoresed in 8% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked with Odyssey: blocking buffer (Li-Cor Biosciences, Lincoln, NE) and then incubated with rabbit lipin-1 antibody (kindly supplied by Dr. Z. Yao, Ottawa Institute of Systems Biology) and IRDye 800 goat anti-rabbit IgG simultaneously or GADPH antibody. Images obtained at 800 nm were quantified using the Odyssey: Imager System (Li-Cor). Lipin 1 levels were corrected against GAPDH used as a loading control.
RNA isolation and analysis. BAT total RNA isolation, reverse transcription and quantification by real-time polymerase chain reaction (PCR) were carried out as previously described (16). Primers used for the PCR reactions are described in the Table 1. Results are expressed as the ratio between the target gene and the housekeeping gene 36B4, the expression of which is not significantly affected by rosiglitazone treatment.

Serum determinations. Serum glucose concentrations were measured with the YSI 2300 STAT Plus glucose analyzer (YSI, Yellow Springs, OH). Insulin levels were determined by RIA (Linco Research, St. Charles, MO) with rat insulin as standard. Serum TAG and nonesterified fatty acid (NEFA) levels were measured by enzymatic methods (Roche Diagnostics, Montreal, QC, Canada, and Wako Chemicals, Richmond, VA, respectively).

Statistical analysis. Data are expressed as means ± SEM. Simple effects of rosiglitazone treatment were analyzed by Student’s unpaired T test. P<0.05 was taken as the threshold of significance.
Results

Final body weight and body weight gain were significantly increased (7% and 28%, respectively) after 7 days of rosiglitazone treatment (Table 2). The higher body weight of rosiglitazone-treated rats was associated with an increase in food efficiency (20%) with the same food intake, increased BAT mass (2.2-fold) and DNA content (2.1-fold). Rosiglitazone significantly reduced plasma levels of insulin (−54%), NEFA (−47%), and TAG (−57%), but did not alter serum glucose levels. Rosiglitazone therefore exerted its expected metabolic actions on energy balance, BAT mass, indices of insulin sensitivity and lipemia.

The effects of rosiglitazone on the rates of glucose uptake in vivo and mRNA levels of glucose transporters (GLUT) 1 and 4 in BAT are illustrated in Fig. 1. Glucose uptake at the whole BAT tissue level was not significantly affected by rosiglitazone (Fig. 1A). When expressed per DNA content that corrects for brown adipocyte number, however, rosiglitazone markedly reduced BAT in vivo glucose uptake (−64%, Fig. 1A). This effect was associated with a marked down-regulation of tissue mRNA levels of GLUT4 (−60%, Fig. 1B), but no change in GLUT1.

In the face of such modulation of BAT glucose uptake by rosiglitazone, we investigated whether rosiglitazone altered the intracellular metabolism of the hexose. In an attempt to mimic in vivo conditions, in vitro determination of glucose metabolism was performed in the presence of low insulin concentration approximating fasting blood levels. Insulin-stimulated rates of glucose oxidation (Fig. 2A) and conversion to lactate in BAT (Fig. 2B) were not affected by rosiglitazone. This occurred despite a significant decrease in tissue mRNA levels of the glycolytic proteins phosphofructokinase A (PFK, −30%) and pyruvate dehydrogenase subunit α (PDHα, −70%). Rosiglitazone did not affect mRNA levels of hexokinase (HEXO), lactate
dehydrogenase (LDH), cytochrome oxidase 4 (COX4), citrate synthase (CS) and isocitrate dehydrogenase (IDH) 3 (Fig. 2C), which are involved in glycolysis, respiratory chain and Krebs cycle, respectively. Rosiglitazone markedly reduced BAT glycogen content (–80%, Fig. 2D), its synthesis from glucose in the presence of insulin (–50%, Fig. 2E), and mRNA levels of UDP-glucose pyrophosphorylase (UDPG-PPL, –40%, Fig. 2F), which generates UDP-linked glucose for glycogen synthesis. In contrast, BAT mRNA levels of glycogenin (GyG) and glycogen synthase 2 (GyS2) were not affected by rosiglitazone (Fig. 2F).

Because glucose is an important supplier of carbons for the synthesis of both the glycerol and fatty acid components of TAG in brown adipocytes, we next evaluated whether rosiglitazone would affect total and glucose-dependent lipogenesis in BAT. Total lipogenesis was measured *in vivo* by the incorporation of $^3$H from $^3$H$_2$O into the glycerol and fatty acid components of TAG. This technique allows the estimation not only of fatty acid synthesis from all carbon sources including those from glucose, but also glycerol synthesis from glucose via short glycolysis and glyceroneogenesis, although not that from glycerokinase (GyK). Rosiglitazone markedly increased BAT *in vivo* synthesis of the glycerol component of TAG (2.2-fold), without affecting *de novo* fatty acid synthesis (Fig. 3A). Similarly *in vitro*, rosiglitazone significantly increased insulin-stimulated glucose incorporation into BAT TAG as a result of the enhanced synthesis from glucose of the glycerol (68%), but not the fatty acid component of TAG (Fig. 3B). This was associated with a significant increase in BAT activities and mRNA levels of PEPCK (Fig. 3C and D) and GyK (Fig. 3E and F), which are key enzymes involved in the synthesis of glycerol 3-phosphate via glyceroneogenesis and from the direct phosphorylation of glycerol, respectively.

Synthesis of TAG occurs in a stepwise manner, beginning with the acylation of glycerol 3-phosphate to lysophosphatidic acid (LPA) by glycerol 3-phosphate acyltransferase (GPAT).
LPA is then acylated to form phosphatidate, which is dephosphorylated to diacylglycerol by PAP1 (lipin) and further acylated to TAG by diacylglycerol acyltransferase (DGAT). The effects of rosiglitazone on the activities and mRNA levels of GPAT, lipin and DGAT are illustrated in Figures 4, 5 and 6, respectively. Rosiglitazone increased total GPAT activity in BAT as a result of an increase in NEM-sensitive, but not –resistant activity (Fig. 4A). This was associated with a significant increase in BAT mRNA levels of GPAT isoform 3, but not 1 and 4 (Fig. 4B). In contrast, rosiglitazone did not affect BAT phosphatidate phosphatase-1 (PAP) activity (Fig. 5A), protein content or mRNA levels of lipin 1 (Fig. 5D and B, respectively), but modestly reduced lipin 1B mRNA levels (~35%, Fig. 5C). Finally, rosiglitazone treatment was associated with a significant 2-fold increase in BAT DGAT activity and mRNA levels of its isoform DGAT1, but not those of isoform 2 (Fig. 6A, B and C).
Discussion

In the present study we investigated the mechanisms whereby PPARγ agonism modulates glucose and lipid metabolism in BAT. The rosiglitazone-induced increase in BAT mass and DNA content was not associated with an increase in whole tissue glucose uptake. When corrected for cell number, rates of glucose uptake in BAT were robustly reduced by rosiglitazone along with BAT GLUT4 mRNA levels. Concomitantly, BAT glycogen content, its synthesis from glucose, and mRNA levels of UDPG-PPL were all reduced by rosiglitazone. In contrast, BAT TAG-glycerol synthesis in vivo was enhanced by rosiglitazone, likely the result of high provision of glycerol 3-phosphate from glycolysis and glyceroneogenesis associated with increased PEPCK and GyK activities. Such marked activation of glycerol 3-phosphate generation was followed by an elevation in the activities and mRNA levels of GPAT and DGAT, but not PAP activity catalyzed by lipin-1.

The study confirmed that the well-established positive actions of rosiglitazone on insulin sensitivity and lipemia occur despite increased body weight gain and a remarkable increase in BAT mass that is the result of brown adipocyte hypertrophy (increase in cell diameter) as well as hyperplasia (increase in cell number) (2, 16), the latter confirmed here by the higher tissue DNA content of rosiglitazone-treated rats compared to controls. Despite such increase in BAT mass, rates of glucose uptake at the whole tissue level were not affected by rosiglitazone (Fig. 1A). When corrected for cell number, however, rates of glucose uptake in BAT were markedly reduced by rosiglitazone in association with reduced tissue GLUT4 mRNA levels. This contrasts with the well-established PPARγ effects on glucose uptake by subcutaneous adipose tissue, skeletal muscle and the heart (47). The present findings therefore exclude glucose clearance by BAT as a contributor to the insulin-sensitizing effect of rosiglitazone found in vivo in rodents.
We have demonstrated that PPARγ activation results in an upregulation of glucose uptake in subcutaneous, but not visceral fat, which constitutes one of the many effects of PPARγ agonists that are depot-specific (Festuccia et al, unpublished observations). Here we extend this observation by demonstrating the existence of an adipose tissue type-specific effect of PPARγ agonism, with BAT responding differently from subcutaneous and visceral white adipose depots. The mechanism by which rosiglitazone reduces BAT glucose uptake has not yet been elucidated. It does not seem, however, to involve a direct effect of rosiglitazone on BAT as evidenced by the modest increase in glucose uptake and absence of change in GLUT4 mRNA levels in isolated brown adipocytes treated with rosiglitazone (20). Sympathetic innervation, through NE regulation of GLUT1 content and functional activation (8), and insulin, through stimulation of GLUT4 expression and translocation (42), are the major positive regulators of glucose uptake in BAT (4). It is, therefore, reasonable to suggest that the reduction in both BAT sympathetic drive and plasma insulin levels induced by rosiglitazone (16) might be involved in the reduction in BAT glucose uptake. Further research is clearly needed to verify this hypothesis.

Likely as an important consequence of such reduced glucose flux per brown adipocyte, rosiglitazone markedly reduced BAT glycogen content, its synthesis from glucose and the mRNA levels of UDPG-PPL, which generates UDP-linked glucose for glycogen synthesis. BAT stores significant amounts of glycogen that seems to be an important source of glucose during nonshivering thermogenesis as evidenced by the enhanced glycogen utilization in rats exposed to cold (12, 22). During nonshivering thermogenesis, significant amounts of glucose are converted to lactate by anaerobic glycolysis, producing ATP that might be important in compensating for reduced energy production due to the uncoupling of mitochondria (4). In addition, glucose seems to play a major role in replenishing citric acid cycle intermediates (anaplerosis) (34), which is
very important for keeping elevated levels of fatty acid oxidation (4). Thus the reduction in BAT glucose uptake and glycogen content induced by rosiglitazone might have some deleterious implication for BAT thermogenic activity.

In contrast to glucose homeostasis, we have demonstrated that BAT is an important component of the hypolipidemic action of rosiglitazone due its marked ability to take up and store fatty acids, especially those derived from lipoprotein-bound TAG through lipoprotein lipase (26). Here we extend this observation by showing that the enhanced BAT ability to store fatty acids is associated with a marked increase in TAG-glycerol synthesis estimated with the $^{3}$H$_{2}$O technique, suggesting an increase in glycerol 3-phosphate generation by short glycolysis and glyceroneogenesis. Confirming these findings, glucose incorporation into TAG-glycerol in vitro and PEPCK activity, the key enzyme of glyceroneogenesis, were significantly increased by rosiglitazone. In addition, rosiglitazone increased BAT GyK activity, which generates glycerol 3-phosphate by direct phosphorylation of glycerol taken up from the circulation or recycled from TAG hydrolysis. Interestingly, rosiglitazone upregulated GyK even in face of a significant reduction in BAT sympathetic drive (16), a major positive regulator of this enzyme in BAT (14, 24). Acclimation to cold and now PPAR$\gamma$ agonism are to date the sole known situations in which all three possible sources of glycerol 3-phosphate are concomitantly stimulated in BAT (14, 15, 31). Both situations have in common a marked increase in BAT LPL activity and fatty acid flux, which further illustrates the importance of PPAR$\gamma$ in controlling fatty acid esterification and TAG synthesis in BAT. Of note, rates of BAT fatty acid synthesis de novo were not affected by rosiglitazone, suggesting that most of the fatty acids used for TAG synthesis under these conditions are preformed. The latter likely originate from LPL-mediated hydrolysis of lipoprotein TAG and albumin-bound nonesterified fatty acids.
In addition to glycerol 3-phosphate generation, rosiglitazone activated TAG synthesis by stimulating the activities of GPAT and DGAT, which catalyze the first and last binding of fatty acids to the carbon backbone of glycerol 3-phosphate. Among the GPAT and DGAT isoforms identified to date, rosiglitazone specifically increased mRNA levels of GPAT3 and DGAT1, respectively, both located in the endoplasmic reticulum. The findings that GPAT3 and DGAT1 expression is markedly upregulated by PPARγ agonism during 3T3-L1 adipocyte differentiation (5, 46) and in BAT as shown here suggest direct transcriptional regulation of these enzymes by PPARγ, a hypothesis that remains to be tested. Surprisingly, BAT lipin levels and PAP activity were not affected by rosiglitazone in BAT. This contrasts with the marked stimulatory effect of rosiglitazone on lipin-1 levels in both subcutaneous and visceral WAT of rats (Festuccia et al, unpublished observations), and that of pioglitazone on lipin-1 expression in WAT of insulin resistant humans (45). Noteworthy, the specific activity of PAP (relative to protein) was 5-15-fold higher in BAT than in WAT (not shown) under basal conditions. Lipin-1 has a dual function: in addition to acting as an enzyme in glycerolipid synthesis, it also functions as a transcriptional coactivator both upstream and downstream of PPARγ (37). Lipin-1 coordinates the expression of adipogenic genes during pre-adipocyte differentiation (35), and it correlates with the expression of fatty acid oxidation genes in human and mouse WAT (10). The capacity for increasing TAG production can be expressed by the fatty acid-induced translocation of the cytosolic reservoir of lipin-1 to the endoplasmic reticulum, the site of TAG synthesis (37). The activation of the existing, abundant lipin-1 in BAT may be sufficient to accommodate the increased flux in the pathway of TAG synthesis brought by rosiglitazone without the need for increased lipin expression as is the case in white adipocytes.
The recent discovery of significant amounts of BAT in humans (32) makes it quite tempting to speculate on whether rosiglitazone-induced BAT recruitment shown in the rat also occurs in humans. Despite the attractive potential of BAT recruitment in terms of obesity treatment, at this point caution should be exerted in making such extrapolations mainly because of the absence of definitive proof of the thermogenic activity of human BAT and the difference in BAT localization between humans and rodents.

In conclusion, this study presents strong evidence that glucose handling in BAT does not directly contribute to the improvement in glucose homeostasis induced by rosiglitazone. This contrasts with its important role in the hypolipidemic action of the agonist, which may itself contribute to global insulin sensitization. Rosiglitazone, however, brings about a major shift in glucose utilization from glycogen storage toward lipogenesis in BAT, as summarized in Fig. 7. The rosiglitazone-induced increase in PPARγ activation results in an increased capacity to synthesize TAG due to enhanced glycerol 3-phosphate generation and GPAT and DGAT activities. This might be an important mechanism by which sufficient amounts of TAG are stored in BAT. Functionally, lipolysis of these TAG provides an adequate supply of intracellular fatty acids, which are essential for non-shivering thermogenesis. The so-called thrifty nature of PPARγ, therefore, extends to BAT and this appears to involve priming the organ for meeting future energy needs.

**Perspectives**

The recent finding of significant amounts of physiologically active BAT in adult humans (32) have renewed the attention toward the potential implication of this tissue in metabolic homeostasis. Despite its small contribution to whole body weight (approximately 2% in rodents),
BAT has a unique and extraordinary ability under demand to release energy from fatty acid and glucose oxidation as heat by nonshivering thermogenesis. It is estimated that during maximal activation, BAT is able to produce as much heat as all the rest of the body through markedly enhanced rates of fatty acid and glucose oxidation (4). Thus BAT can potentially constitute a significant glucose, fatty acid and TAG-clearing organ especially under sympathetic activation with possible implications in the treatment of dyslipidemias, hyperglycemia and insulin resistance. In this sense, PPARγ agonism, which is widely used in the treatment of insulin resistant states, has emerged as an interesting approach to recruit BAT alternatively to the sympathetic nervous system (16, 36). PPARγ agonists markedly increase rodent BAT mass and its ability to take up and store fatty acids in the form of TAG. In rodents, BAT significantly contributes to the marked hypolipidemic effects (reduction in triglyceridemia and NEFA levels) of PPARγ agonists (25, 26). Here we showed that such marked ability of BAT to synthesize TAG under PPARγ agonism is related to the enhanced generation of glycerol 3-phosphate and activities of GPAT and DGAT. A similar activation of TAG synthesis in BAT is only seen during cold exposure (31), the only difference being that, under this condition of increased sympathetic activity, newly synthesized TAG is rapidly hydrolyzed to supply fatty acids for thermogenesis, whereas under PPARγ agonism TAG is stored in BAT probably as a consequence of reduced sympathetic drive to BAT. In contrast to lipids, however, the present study shows that glucose handling in BAT does not directly contribute to the improvement in glucose homeostasis induced by rosiglitazone. In fact, glucose uptake per brown adipocyte is reduced by rosiglitazone, in striking contrast with the effects of the agonist in white adipose tissue and muscle. What are the mechanisms behind such tissue specific effect of the agonist on glucose uptake? Could excessive fatty acid clearance inhibit glucose uptake in BAT? These
unanswered questions and the present findings underline the remarkably wide range of PPARγ actions and the potential of this nuclear receptor as a means to impact energy metabolism in specific target tissues.
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References


**Legends to Figures**

Figure 1 – *In vivo* rates of glucose uptake per whole tissue (A) and per DNA (B) and mRNA levels of GLUT1 and GLUT4 (C) in brown adipose tissue of rats treated or not with rosiglitazone (RSG) for 7 days. Each column represents the mean ± SEM of 6-12 rats. *P* < 0.05 vs. untreated control.

Figure 2 – *In vitro* rates of glucose oxidation (A), conversion to lactate (B), tissue glycogen content (D), its synthesis from glucose (E) and mRNA levels of proteins involved in glycolysis (C) and glycogen synthesis (F) in brown adipose tissue of rats treated or not with rosiglitazone (RSG) for 7 days. Each column represents the mean ± SEM of 12 rats. *P* < 0.05 vs. untreated control.

Figure 3 – *In vivo* incorporation of $^3$H from $^3$H$_2$O into the glycerol and fatty acid components of triacylglycerol (TAG) (A), *in vitro* rates of glucose incorporation into triacylglycerol (TAG) and its fatty acid (FA) and glycerol components (B) and activities and mRNA levels of phosphoenolpyruvate carboxykinase (PEPCK, C and D, respectively) and glycerokinase (GyK, E and F, respectively) in brown adipose tissue of rats treated or not with rosiglitazone (RSG) for 7 days. Each column represents the mean ± SEM of 12 rats. *P* < 0.05 vs. untreated control.

Figure 4 – Total, NEM-resistant and NEM-sensitive glycerol 3-phosphate acyltransferase (GPAT) activities (A) and mRNA of the GPAT isoforms 1, 3 and 4 (B) in brown adipose tissue of rats treated or not with rosiglitazone (RSG) for 7 days. Each column represents the mean ± SEM of 6 rats. *P* < 0.05 vs. untreated control.
Figure 5 – Phosphatidate phosphatase-1 (PAP1) activity (A), mRNA levels of the lipin isoforms 1 (B) and 1B (C) and protein content (D) in brown adipose tissue of rats treated or not with rosiglitazone (RSG) for 7 days. Each column represents the mean ± SEM of 6 rats. Representative Western blots are shown for 3 controls (left) and 3 rosiglitazone-treated rats (right). Lipin levels were corrected against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels used as a loading control. * $P < 0.05$ vs. untreated control.

Figure 6 – Diacylglycerol acyltransferase (DGAT) activity (A) and mRNA levels of the DGAT isoforms 1 (B) and 2 (C) in brown adipose tissue of rats treated or not with rosiglitazone (RSG) for 7 days. Each column represents the mean ± SEM of 6 rats. * $P < 0.05$ vs. untreated control.

Figure 7 – Schematic representation of the enzymes in the pathways of glucose metabolism and triacylglycerol (TAG) synthesis affected by rosiglitazone in brown adipose tissue of rats. ↑, up-regulated; ↓, down-regulated; - not affected; ?, not determined.
### Table 1 – Pairs of primers used for quantitative real-time PCR

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Table 2 – Final body weight, cumulative food intake, food efficiency, brown adipose tissue (BAT) mass, and serum concentrations of insulin and metabolites in rats treated or not with rosiglitazone (RSG) for 7 days.

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<th>Control</th>
<th>RSG</th>
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<tr>
<td>Initial body weight (g)</td>
<td>240 ± 3</td>
<td>240 ± 2</td>
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<td>Final body weight (g)</td>
<td>316 ± 6.7</td>
<td>338 ± 4.1*</td>
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<tr>
<td>Body weight gain (g)</td>
<td>75.9 ± 5.7</td>
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<td>Food Intake (g)</td>
<td>228 ± 10</td>
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<td>Food efficiency (%) a</td>
<td>33.2 ± 1.3</td>
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<td>BAT (mg)</td>
<td>392 ± 42</td>
<td>853 ± 53*</td>
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<td>BAT DNA content (µg)</td>
<td>200 ± 32</td>
<td>552 ± 48*</td>
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<tr>
<td>Insulin (pmol/l)</td>
<td>228 ± 24</td>
<td>110 ± 12*</td>
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<tr>
<td>Glucose (mmol/l)</td>
<td>10.0 ± 1.5</td>
<td>12.2 ± 1.2</td>
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<tr>
<td>NEFA (mmol/l)</td>
<td>0.098 ± 0.007</td>
<td>0.051 ± 0.01*</td>
</tr>
<tr>
<td>TAG (mmol/l)</td>
<td>2.30 ± 0.20</td>
<td>0.99 ± 0.14*</td>
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</tbody>
</table>

Data are means ± SEM of 6 rats.* $P< 0.05$.

* Calculated as g body weight gain / 100 g food ingested.
Figure 3

A

$^3$H Incorporation (nmol/g of tissue, h)

- Control
- RSG

B

Glucose Incorporation (umol/ug of DNA, h)

- TAG
- TAG-FA
- TAG-Glycerol

C

PEPC Activity (nmol/mg protein, min)

- Control
- RSG

D

PEPC/36B4 mRNA

- Control
- RSG

E

GyK Activity (nmol/mg protein, min)

- Control
- RSG

F

GyK/36B4 mRNA

- Control
- RSG
Figure 4

A

B

Total
NEM-Resistant
NEM-Sensitive

Control
RSG

BAT GPAT Activity
(μmol/kg protein/h)

Relative mRNA levels

GPAT1
GPAT3
GPAT4

*
Figure 5

(A) PAP Activity (μmol/mg protein/min)

(B) Lipin 1/3βB4 mRNA

(C) Lipin 1B/3βB4 mRNA

(D) Lipin 1/GAPDH protein levels

Control

RSG
Figure 6

- DGAT Activity (nmol/μg protein ⋅ min)
- DGAT1/36B4 mRNA
- DGAT2/36B4 mRNA

Control vs. RSG comparison.
Glucose → Glut4 → Glycogen → UDPG-PPL → Glucose → Dihydroxyacetone → Glycerol-3-phosphate → Lysophosphatidic acid → AGPAT → Phosphatidic acid → Lipin → Diacylglycerol → DGAT1 → Triacylglycerol

Glycerol → GyK

Dihydroxyacetone → GyK

Glycerol-3-phosphate → GPAT3

Pyruvate → Fatty acid → Acetyl-CoA → Mitochondrion

Amino acids → PEPCK → Pyruvate

Lactate

Glucose → Dihydroxyacetone → Glycogen → Glut4

Glycogen → UDPG-PPL

Mitochondrion