PPARγ activation attenuates cold-induced upregulation of thyroid status and brown adipose tissue PGC-1α and D2

William T. Festuccia,1 Pierre-Gilles Blanchard,2 Thiago B. Oliveira,1 Juliana Magdalon,1 Vivian A. Paschoal,1 Denis Richard,2 Yves Deshaies.2*

1- Department of Physiology, Institute of Biomedical Sciences, University of São Paulo (USP), São Paulo, Brazil, 05508-900

2- Quebec Heart and Lung Institute Research Center, and Department of Medicine, Faculty of Medicine, Laval University, Quebec, Canada, G1V 4G5

Running head: BAT PPARγ activation and cold exposure

*Correspondent author: Dr. Yves Deshaies
Quebec Heart & Lung Institute Research Center,
Pav. d’Youville Y-3110
Quebec, QC, Canada, G1V 4G5
Email: yves.deshaies@phs.ulaval.ca
Phone: (418) 656-8711 ext 3738 Fax: (418) 656-494
Abstract

Here we investigated whether pharmacological PPARγ activation modulates key early events in brown adipose tissue (BAT) recruitment induced by acute cold exposure with the aim of unraveling the inter-relationships between sympathetic and PPARγ signaling. Sprague-Dawley rats treated or not with the PPARγ ligand rosiglitazone (15 mg/kg/day, 7 days) were kept at 23°C or exposed to cold (5°C) for 24 h and evaluated for BAT gene expression, sympathetic activity, thyroid status and adrenergic signaling. Rosiglitazone did not affect the reduction in body weight gain and the increase in feed efficiency, VO₂ and BAT sympathetic activity induced by 24-h cold exposure. Rosiglitazone strongly attenuated the increase in serum total and free T4 and T3 levels and BAT iodothyronine deiodinase type 2 (D2) and PGC-1α mRNA levels and potentiated the reduction in BAT thyroid hormone receptor (THR) β mRNA levels induced by cold. Administration of T3 to rosiglitazone-treated rats exacerbated the cold-induced increase in energy expenditure, but did not restore a proper activation of D2 and PGC-1α nor further increased UCP1 expression. Regarding adrenergic signaling, rosiglitazone did not affect the changes in BAT cAMP content and protein kinase A activity induced by cold. Rosiglitazone alone or in combination with cold increased CREB binding to DNA, but it markedly reduced the expression of one of its major co-activators, CREB binding protein (CBP). In conclusion, pharmacological PPARγ activation impairs short-term cold elicitation of BAT adrenergic and thyroid signaling, which may result in abnormal tissue recruitment and thermogenic activity.
**Keywords:** BAT recruitment; sympathetic activity; thyroid status; adrenergic signaling; thiazolidinediones

**Abbreviations:** BAT, brown adipose tissue; CART, cocaine- and amphetamine-regulated transcript; CIDEA, cell death-inducing DFF45-like effector A; D2, type 2 iodothyronine deiodinase; α-MT, DL-α-methyl-tyrosine ester; NE, norepinephrine; NETO, NE turnover rates; PGC-1α, peroxisome proliferator-activated receptor coactivator α; PPARγ, peroxisome proliferator-activated receptor γ; PRDM16, positive regulatory domain containing 16; SHP, small heterodimer protein (NROB2); T3, 3,5,3’-triiodothyronine; T4, thyroxine; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone; UCP1, uncoupling protein 1; UCP3, uncoupling protein 3.
Introduction

A possible utilization of brown adipose tissue (BAT) non-shivering thermogenesis as an alternative to treat obesity has gained renewed interest with the recent confirmation that a substantial number of adult humans possesses active BAT (6, 21, 31, 32, 37). Although current knowledge about the impact of BAT on human energy balance is still scarce, it is plausible to envisage that the efficacy of BAT-targeted therapies will depend upon the recruitment (increase in tissue mass and thermogenic capacity) of BAT in humans and the development of strategies to safely switch thermogenesis on and off.

In addition to chronic canonical sympathetic activation (5), BAT recruitment can be induced in rodents by administration of synthetic, specific ligands of peroxisome proliferator-activated receptor (PPAR) \( \gamma \). This nuclear receptor is highly expressed in BAT where it acts as a master transcriptional regulator of brown adipocyte differentiation required for tissue development, function and survival (1, 8, 15-17, 22). Similarly to chronic sympathetic activation, treatment of rodents with the PPAR\( \gamma \) ligand rosiglitazone is associated with a marked increase in BAT mass, content of the thermogenic uncoupling protein 1 (UCP1) and key enzymes involved in fatty acid oxidation and lipolysis (13, 29, 35). In a standard thermal environment, PPAR\( \gamma \)-induced upregulation of BAT lipolytic, oxidative and thermogenic machineries does not, however, translate into higher thermogenesis and energy expenditure \textit{in vivo} due to a rosiglitazone-induced down-regulation of BAT sympathetic activity and thyroid status, the major neuro-hormonal regulators of BAT function (14).

Further evaluation of BAT recruitment indicates that despite a similar increase in tissue mass and content of some thermogenic proteins, chronic sympathetic and PPAR\( \gamma \) activations induce somewhat divergent morphological and metabolic phenotypes in BAT. Indeed, whereas sympathetic stimulation results in an increase in the number of multilocular brown adipocytes with enhanced rates of glucose uptake and fatty acid oxidation, PPAR\( \gamma \)
activation is associated with an increase in unilocular brown adipocytes with reduced glucose uptake and unaltered oxidative rates (11).

It has been previously shown that, even in the face of a reduced thyroid status, the high thermogenic, oxidative and lipolytic potential induced by PPARγ activation (i.e. mRNA and protein content and concomitant increase in related processes when assessed in vitro) are actuated at the functional level in vivo by pharmacological β3-adrenergic stimulation (4, 26, 30). Because thyroid status was not evaluated in these studies, it was not possible to establish whether this actuation of BAT function induced by β3-adrenergic activation in PPARγ ligand-treated animals was associated with a re-establishment of normal thyroid function.

One interesting aspect of adrenergic elicitation of BAT thermogenic potential in PPARγ-treated animals was the absence of effect of the β3-adrenergic CL316,243 agonist on the expression of the adrenergically-regulated genes UCP1, lipoprotein lipase (LPL) and PPARγ coactivator 1α (PGC-1α) (26), which otherwise are direct targets of β3 stimulation. Supporting these findings, 24 h of cold exposure did not additively increase BAT UCP1 expression further than that induced by rosiglitazone alone in rats (10). The absence of additive effects of simultaneous PPARγ and adrenergic activation on BAT gene expression was quite unexpected in the face of both the reduced sympathetic drive to BAT found upon PPARγ ligand treatment of rats living in a standard thermal environment (14), as well as, our recent findings indicating that the optimal upregulation of UCP1 by PPARγ activation depends upon the presence of intact BAT sympathetic innervation and maintenance of minimal basal sympathetic tone (10).

Thus in the present study, in an attempt to further characterize the inter-relationships between sympathetic and PPARγ signaling in the regulation of key early events in BAT recruitment elicited by cold exposure, control and rosiglitazone-treated rats maintained at 23°C or exposed to cold (5°C) for 24 h were evaluated for determinants of energy balance,
serum metabolites, lipids and hormone levels, thyroid status, sympathetic activity and BAT gene expression profile of thermogenic proteins, and intracellular adrenergic signaling. Our main findings indicate a major failure of cold, in the presence of pharmacological PPARγ activation, to upregulate the thyroid status and expression of some specific adrenergic genes in BAT, which in turn might affect tissue recruitment and thermogenic function.
Research Design and Methods

Animals and treatment. Animal care and handling were performed in accordance with the Canadian and Brazilian Guides for the Care and Use of Laboratory Animals. All experimental procedures received prior approval of the Institute of Biomedical Sciences and Laval University animal care committees. Male Sprague-Dawley rats (Charles River Laboratories, St. Constant, Canada or Institute of Biomedical Sciences Animal Facility) were individually housed in stainless steel cages in a room kept at 23 ± 1°C with a light/dark cycle of 12 h/12 h (lights on at 0800). After a 4-day adaptation period, rats were matched by weight and divided into control and rosiglitazone-treated groups that were fed a nonpurified powdered rodent diet (Charles River Rodent Diet #5075; digestible energy content: 12.9 kJ/g) alone (control) or supplemented with the PPARγ agonist rosiglitazone (Avandia) at a dose of 15 mg/kg . day for 7 days. This dose was chosen based on preliminary studies that showed its effectiveness to increase BAT thermogenic proteins in a short period of treatment (e.g. 7 days). 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. At the seventh day of treatment, half of control and rosiglitazone-treated rats were transferred to a cold room (5°C) for 24 h with similar light/dark cycle and free access to water and food containing or not rosiglitazone. At the end of the 24-h cold exposure, rats were killed by decapitation and trunk blood and tissues were collected. A similar protocol was performed in which an extra group of rats treated with rosiglitazone received an i.p. injection of triiodothyronine (T3) at a dose of 10 μg/100 g of body weight before exposure to cold. This dose of T3 was previously shown to induce maximal saturation of thyroid hormone receptors in BAT (2).
Energy expenditure. O₂ consumption was determined in an open circuit system with an O₂ analyzer (Applied Electrochemistry, S-3A1). Energy expenditure measurements were carried out during 24 h after a 24-h adaptation period to the new cages. Data are presented as ml O₂/min.

NE turnover. Norepinephrine turnover rate (NETO), a reliable index of sympathetic activity in a given tissue, was estimated from the decline in tissue NE content after inhibition of catecholamine synthesis with dl-α-methyl-tyrosine ester (α-MT; Sigma, St. Louis, MO) as previously described (14). Rats kept at 23°C or exposed to cold (5°C) for 20 h were killed by anesthetic (ketamine/xylazine) overdose before or 4 h after i.p. injection of α-MT (350 mg/kg body weight). Interscapular BAT were rapidly removed, weighed, frozen in liquid nitrogen and stored at –80°C for later determination of NE content. Rates of NETO were calculated as the product of the fractional turnover rate (k) and the endogenous NE content at time 0 as previously described (3). Fractional turnover rate (k) was calculated by the formula: $k = \frac{\log [NE]_0 - \log [NE]_4}{0.434 \times 4}$, where [NE]₀ and [NE]₄ are the NE content at times 0 and 4 h, respectively.

Tissue NE content. Tissue NE content was measured as previously described (20). Briefly, tissues were homogenized in 0.2 N perchloric acid, 1 mM of EDTA, 1% sodium metabisulfite containing dihydroxybenzylamide as an internal standard, centrifuged, and the supernatant destined for catecholamine quantification was extracted with alumina. Catecholamines and dihydroxybenzylamide (internal standard) were eluted from alumina with the above homogenization solution and assayed by HPLC.
In situ hybridization for CART. Hypothalamic mRNA levels of cocaine- and amphetamine-related transcript (CART) was measured by in situ hybridization essentially as previously described (14). Briefly, hypothalamic sections were mounted onto poly-l-lysine coated slides, dehydrated in ethanol, fixed in paraformaldehyde, digested with proteinase K (10 µg/mL), acetylated with 0.25% acetic anhydride and dehydrated in ethanol gradient. Sections were incubated overnight with antisense 35S-labeled cRNA probe (10^7 cpm/ml) for CART at 60°C. Slides were rinsed with SSC, digested with RNAse-A, washed in descending concentrations of SSC, and dehydrated in ethanol gradient. Slides were defatted in toluene, dipped in NTB2 nuclear emulsion (Eastman Kodak), and exposed for 7 days before being developed. Slides were examined by darkfield microscopy using an Olympus BX51 microscope (Olympus America, Melville, NY). Images were acquired with an Evolution QEi camera and analyzed with ImagePro plus v5.0.1.11 (MediaCybernetics, Silver Spring, MD). The system was calibrated for each set of analyses to prevent saturation of the integrated signal. Mean pixel densities were obtained by taking measurements from both hemispheres of one to four brain sections and subtracting background readings taken from areas immediately surrounding the region analyzed.

RNA isolation and quantification. RNA was isolated from BAT and hypothalamus using QIAzol and the RNeasy Lipid Tissue Kit (QIAGEN). For cDNA synthesis, expand reverse transcriptase (Invitrogen) was used following manufacturer’s instructions and cDNA was diluted in DNase-free water (1:25) before quantification by real-time PCR. mRNA transcript levels were measured in duplicate samples using a Rotor Gene 3000 system (Montreal Biotech, Montreal, QC, Canada). The primers used for the PCR reactions are presented in Table 1. Chemical detection of the PCR products was achieved with SYBR Green I (Molecular Probes, Willamette Valley, OR). At the end of each run, melt curve analyses were
performed and a few samples representative of each experimental group were run on agarose gel to ensure the specificity of the amplification. Results are expressed as the ratio between the expression of the target gene and the housekeeping gene ARBP/36B4 (NM_022402), which was selected because no significant variation in its expression was observed between treatments.

*Western blot.* BAT was homogenized in lysis buffer (50 mM HEPES, pH 7.4, 40 mM NaCl, 2 mM EDTA, 10 mM β-glycerophosphate, 10 mM Na₄P₂O₇, 1% Triton 100X, 1% sodium deoxycholate 50 mM NaF, 1.5 mM Na₃VO₄, 0.1% SDS and a cocktail of protease inhibitors), subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, blocked for 1 h and incubated overnight at 4°C with primary antibodies (Cell Signalling Technologies, Beverly, MA). After washing, membranes were incubated with immunoglobulin G conjugated to horseradish peroxidase, washed again and the immunoreactive bands were detected by the enhanced chemiluminescence method. Densitometric analysis was performed with ImageQuant TL software (GE Healthcare, Little Chalfont, UK).

*CREB binding.* Nuclear extracts of BAT of rats treated or not with rosiglitazone maintained either at 23°C or exposed to cold for 24 h were analysed for CREB binding with a CREB (Phospho-Ser133) Transcription Factor Assay (Cayman Chemicals, Ann Arbor, MI). Briefly, 50 μg of protein from whole BAT homogenates prepared for western blot were used for the CREB DNA binding activity assay according to the manufacturer’s instructions (CREB (Phospho-Ser133)). The specificity of the method was verified by the use of a wild-type consensus oligonucleotide as a competitor for CREB binding which decreased the signal dramatically and data were expressed as percentage of positive control.
Serum determinations. Plasma glucose concentration was measured by the glucose oxidase method with the YSI 2300 STAT plus glucose analyzer. Plasma insulin, leptin, adiponectin (Linco Research, St. Charles, MO), total and free T3 and T4 (Coat-A-Count, DPC, Los Angeles, CA) and TSH (Biotrak rat TSH, Amersham Biosciences, Oakville, ON, Canada) were determined by radioimmunoassay. Plasma triacylglycerol and nonesterified fatty acid levels were measured by enzymatic methods (Roche Diagnostics, Montreal, QC, Canada, and Wako Chemicals, Richmond, VA, respectively).

Statistical analysis. Results are expressed as means ± SEM. Multifactorial ANOVA followed by the Newman-Keuls’ multiple range test were used to compare the effects of cold exposure and thyroid hormone treatment in control and rosiglitazone-treated rats. \( P < 0.05 \) was taken as the threshold of significance.
RESULTS

As shown in Table 2, rosiglitazone did not affect the reductions in body weight gain and feed efficiency and the increases in food intake and VO₂ induced by 24 h of cold exposure. Rosiglitazone significantly increased BAT and inguinal adipose tissue masses, an effect that was attenuated by cold exposure in the former but not the latter. Retroperitoneal white tissue mass was not affected by any of the treatments. Regarding serum parameters, rosiglitazone potentiated the reduction in insulin and triacylglycerol levels and attenuated the increases in NEFA induced by cold exposure. Furthermore rosiglitazone alone significantly reduced glycemia and increased serum adiponectin levels, such effects being completely blocked and attenuated by cold exposure, respectively. Finally, rosiglitazone in combination with cold significantly reduced serum leptin levels in comparison to other treatments.

As depicted in Figure 1A and B, pharmacological PPARγ activation did not affect the cold-induced increase in BAT glycerokinase (GyK), an enzyme that synthesizes glycerol 3-phosphate for triacylglycerol synthesis, and UCP1 mRNA levels. In contrast to those genes, rosiglitazone markedly attenuated the increase in iodothyronine deiodinase type 2 (D2), which converts T4 into biologically active T3, that of PGC-1α, an important regulator of BAT UCP1 transcription, and the reduction in cell death-inducing DFF45-like effector A (CIDEA, an attenuator of UCP1 activity) mRNA levels induced by cold exposure (Fig. 1C, D and E). Finally, rosiglitazone reduced mRNA levels of the positive regulatory domain containing 16 (PRDM16, a zinc-finger protein involved in brown adipogenesis that binds PPARγ and increases its transcriptional activity (25), an effect that was potentiated by cold exposure (Fig. 1F).

The inability of cold exposure to maximally activate BAT expression of the adrenergically regulated genes PGC-1α and D2 associated with PPARγ activation prompted us to investigate a possible effect of rosiglitazone modulating cold activation of BAT
sympathetic activity. There were no changes in BAT norepinephrine (NE) content, an index of the degree of sympathetic innervation rather than activity, by any of the treatments (Fig. 2A). As depicted in Figures 2B and C, rosiglitazone did not affect the increase in BAT k, NETO (Fig. 2B and C) and arcuate nucleus CART mRNA levels (Fig. 2D and E), a neuropeptide that has been previously demonstrated to be anatomically linked to and modulate BAT sympathetic nerves (9, 18).

Because thyroid hormones exert an important role in the amplification of intracellular adrenergic signaling in BAT, we next investigated whether rosiglitazone affects the activation of the hypothalamic-pituitary-thyroid axis associated with cold exposure. As depicted in Figure 3A, neither intervention altered hypothalamic mRNA levels of thyrotropin-releasing hormone (TRH). Serum TSH levels were not significantly changed by rosiglitazone, but combination of both cold and rosiglitazone induced a significant increase in TSH levels in comparison to control rats maintained at 23°C (Fig. 3B). On the other hand, rosiglitazone completely blocked the increases in serum total and free T4 and T3 and potentiated the decrease in THRβ mRNA levels induced by cold exposure (Figs. 3C, D, E, F and H). Finally, rosiglitazone significantly reduced BAT THRα1 mRNA levels, an effect that was not modulated by cold exposure (Fig. 3G).

The marked attenuation of thyroid axis activation by cold exposure induced by rosiglitazone prompted us to investigate more specifically whether their relative hypothyroid state was involved in the inability of cold to stimulate the expression of BAT PGC-1α and D2 (D2-generated T3 potentiates adrenergic signaling toward D2 expression in a feed-forward fashion). To this end, rats treated with rosiglitazone received, prior to cold exposure, an i.p. injection of T3. As depicted in Fig. 4, T3 administration markedly increased serum levels of free T3 (Fig. 4A), exacerbated the weight loss (Fig. 4B), the reduction in feed efficiency (Fig. 4C), and amplified the cold-induced increase in the mRNA levels of the T3 target genes small
heterodimer protein (SHP, a negative regulator of PGC-1α expression and energy expenditure in brown adipocytes (33)) and UCP3 in rats treated with rosiglitazone (Fig. 4G and H). Despite these effects, hormone therapy was not able to restore in rosiglitazone-treated rats the ability of cold exposure to increase BAT PGC-1α and D2 mRNA levels (Fig. 4D and E). Surprisingly, the strong increase in UCP1 mRNA levels brought by rosiglitazone alone was maximal and suggestive of a ceiling effect associated with PPARγ agonism as neither cold exposure alone nor in combination with T3 further increased UCP1 in rosiglitazone treated rats (Fig. 4F).

In an attempt to elucidate the mechanisms underlying the failure of the sympathetic nervous system to increase the expression of the adrenergically-regulated genes PGC-1α and D2 in BAT of rosiglitazone treated rats, we investigated the status of key steps of the intracellular adrenergic signaling pathway in this tissue including cAMP content, protein kinase A (PKA), CREB DNA binding and the expression and content of some coregulators of its transcriptional activity. As depicted in Figure 5A, rosiglitazone did not affect the increase in BAT cAMP content induced by cold exposure. Rosiglitazone alone significantly increased BAT PKA activity and CREB binding activity to its consensus DNA binding sequence and reduced CREB co-activator histone acetyltransferase CREB binding protein (CBP) mRNA levels (Fig. 5B, C and D). Among these effects, only the increase in PKA activity was attenuated by cold exposure. Finally, neither rosiglitazone nor cold nor their combination affected BAT protein content of the CREB regulated transcription co-activators (CRTC) 1 and 2 (Fig. 5E) and mRNA levels of other co-activators involved in the regulation of PGC-1α transcription (SIRT3, CHOP10 and ATF2; data not shown).
DISCUSSION

Our main findings indicate that despite having higher BAT mass and UCP1 content, rosiglitazone-treated rats displayed upon an acute cold exposure similar body weight gain, feed efficiency, VO₂ and BAT sympathetic activity than control, untreated rats. Despite those similarities, rosiglitazone markedly impaired cold ability to stimulate the thyroid axis and to properly increase BAT levels of the thermogenic genes D2 and PGC-1α, an effect associated with a marked reduction in the mRNA levels of THRβ and the CREB co-activator CBP.

Rosiglitazone-treated rats were challenged here with a short period of cold exposure (24 h) and evaluated for very early events involved in BAT recruitment aiming to unveil the interactions between PPARγ and adrenergic signaling and a possible potentiation of this process through tissue ‘priming’ by PPARγ activation. The markedly increased BAT mass (10, 11) and UCP1 content induced by rosiglitazone did not lead to higher energy expenditure upon cold exposure as estimated by body weight gain, feed efficiency and VO₂. These findings are in contrast with the additive increase in energy expenditure found upon concomitant treatment of mice with a PPARγ ligand and the β3 adrenergic receptor agonist CL-316243 (26). Such discrepancy between pharmacological vs. physiological elicitation of thermogenesis could be due to the duration of adrenergic activation (24 h of cold vs. 2 weeks of CL-316243), species differences (rats vs. mice) and the preferential recruitment of different thermogenic processes associated with pharmacological β3 adrenergic activation (nonshivering thermogenesis only) and acute cold exposure (shivering thermogenesis mainly) (5).

In contrast to energy expenditure, however, rosiglitazone markedly reduced cold ability to properly upregulate BAT mRNA levels of the adrenergically-regulated genes PGC-1α (23) and D2 (28) in BAT. Interestingly, this effect seems to be specific to some, but not all adrenergically-regulated genes, as the expression of GyK, a gene positively regulated by
the sympathetic nervous system and PPARγ in BAT (11, 12), was, as expected, upregulated by cold in rosiglitazone treated rats.

Based on our previous findings of a reduced BAT sympathetic tone and arcuate nucleus CART levels in rosiglitazone-treated rats (14), we tested the hypothesis that the impairment in cold-induced upregulation of D2 and PGC-1α by rosiglitazone was due to an impairment in the cold-induced activation of BAT sympathetic outflow due to a central action of rosiglitazone on arcuate CART levels. This hypothesis proved false as rosiglitazone did not impair cold activation of BAT sympathetic drive and upregulation of arcuate CART levels. Of note, the close association between arcuate CART levels and BAT sympathetic activity upon rosiglitazone and cold strongly supports a major involvement of CART in BAT regulation. Accordingly, arcuate CART-expressing neurons are anatomically linked to BAT sympathetic nerves (9) and their positive modulation increases BAT sympathetic tone (18).

The absence of impact of rosiglitazone on cold-mediated induction of BAT sympathetic tone led us to test whether the failure of cold to induce D2 and PGC-1α was due to a defect in intracellular adrenergic signaling. Because adrenergic signaling in brown adipocytes is strongly modulated by T3 (7, 27), an extensive analysis of thyroid status was performed. Cold exposure not only failed to increase serum total and free T4 and T3, but further reduced the already low BAT mRNA levels of THRβ in rosiglitazone-treated rats. The mechanisms by which rosiglitazone abolishes the upregulation of serum thyroid hormone by cold are unknown, but the absence of change in TRH and TSH indicates an action of the ligand on thyroid function. Furthermore, rosiglitazone impaired cold induced upregulation of both BAT D2, a local generator of T3 from T4 needed for the amplification of BAT adrenergic signaling (7), and PGC-1α, an important coactivator of THRβ activation of UCP1 transcription (23). These findings suggest an involvement of the thyroid status in the failure of cold to properly activate the expression of PGC-1α and D2 in rosiglitazone-treated rats.
To test the above hypothesis, we acutely administered T3 to rosiglitazone-treated rats prior to cold exposure at a dose shown to induce maximal saturation of thyroid hormone receptors in BAT (2) in an attempt to bypass the inability of cold to stimulate BAT D2 and thus local production of T3. In spite of the marked increase in energy expenditure (body weight loss and reduced feed efficiency) and the expected increase in the expression of T3 target genes (UCP3 and SHP) upon cold exposure, T3 did not restore the ability of cold to increase BAT D2 and PGC-1α, which may be due to a defect in T3 activation of BAT THRβ.

Accordingly, expression of THRβ, which is involved not only in T3 regulation of UCP1 and 3 (24) but also in the adrenergic activation of D2 expression (19), was markedly reduced in rosiglitazone-treated rats exposed to cold. A similar modulation may also apply to PGC-1α whose expression in the liver is upregulated by T3 (34), however, whether such regulation occurs in BAT and involves THRβ remains to be established.

To gain further insight into the mechanisms underlying the inability of cold to increase BAT D2 and PGC-1α in rosiglitazone-treated rats, we evaluated several aspects of BAT adrenergic intracellular signaling. The absence of effect of rosiglitazone on the increase in BAT cAMP induced by cold and the absence of major negative changes in maximal PKA activity exclude their involvement in the failure of cold to properly stimulate BAT D2 and PGC-1α under PPARγ activation. Rates of CREB DNA binding, on the other hand, were significantly increased by rosiglitazone in rats at 23°C or 5°C, indicating an exacerbation of CREB phosphorylation, translocation to the nucleus and interaction with its consensus DNA binding sequence. Although such increase in CREB binding seems counterintuitive to the failure of cold to upregulate BAT D2 and PGC-1α in rosiglitazone-treated rats, it does in fact indicate that PPARγ activation is perhaps affecting steps downstream of CREB binding to DNA. The binding of transcription factors such as CREB onto a promoter region can result in stimulation or inhibition of gene transcription depending on the recruitment and interaction
with co-activators or corepressors, respectively. Evaluation of BAT content of several CREB co-activators including CBP, CRTC-1 and -2, revealed a major effect of rosiglitazone decreasing mRNA levels of CBP, a protein lysine acetyltransferase that binds to phosphorylated CREB and acetylates histones loosening up chromatin structure for transcription initiation. Such reduction in CBP is compatible with reduced CREB efficiency, however, further experiments are required to establish whether reduced CBP is involved in the failure of cold to stimulate D2 and PGC-1α in BAT of rosiglitazone-treated rats.

In conclusion, pharmacological PPARγ activation is associated with an abnormal response of BAT thyroid and adrenergic signaling despite normal sympathetic activation induced by cold exposure and/or correction of relative hypothyroidism by T3 treatment. Due to the importance of thyroid and adrenergic signaling for the proper activation of BAT thermogenesis, it is very likely that the cold induction of a functional BAT thermogenic response may be partially impaired by rosiglitazone, a possibility that remains to be experimentally tested through the measurement of BAT temperature. Identification of the mechanisms underlying BAT abnormal response in rosiglitazone-treated rats to cold could help define better strategies to recruit BAT without affecting its thermogenic efficiency.
**Perspectives and Significance**

The identification of BAT in adult humans has opened up the opportunity for the development of strategies to take advantage of its unique thermogenic ability to treat obesity. For this, strategies not only to safely recruit BAT (i.e. increase functional mass and thermogenic capacity) in humans but also to turn thermogenesis on and off, will have to be developed. PPARγ activation has recently been recognized as a pharmacological alternative to adrenergic stimulation for BAT recruitment (22). Here, however, we report strong evidence indicating that BAT recruited by pharmacological PPARγ activation has an impaired thermogenic response upon an acute cold challenge. Whether such reduced BAT thermogenic ability persists following long-term cold exposure and whether it is dependent upon sustained continuation of rosiglitazone administration need to be investigated. Pharmacological PPARγ activation increases BAT mass by stimulating hyperplasia and lipid-associated hypertrophy. The fact that BAT recruitment is associated with a reduction in important markers of brown adipocytes such as PRDM16 and D2 along with an increase in the number of unilocular adipocytes, despite increased UCP1 levels, raises the possibility that PPARγ may be either inducing white adipocyte features in brown adipocytes or acting on the recently characterized “brite/beige” adipocytes (36). Characterization of the mechanisms underlying these phenotypes and the development of novel selective PPARγ modulators may help optimize PPARγ-mediated BAT recruitment at the functional level.
ACKNOWLEDGEMENTS

The authors are very grateful for the invaluable professional assistance of Yves Gélinas.

GRANTS

This work was supported by grants from the Canadian Institutes of Health Research (CIHR) and the Natural Sciences and Engineering Research Council of Canada (NSERC) to YD. WTF is a recipient of a Young Scientist Fellowship and Grant from the São Paulo Research Foundation (FAPESP 2009/15354-7 and 2010/10909-8). P-GB was the recipient of a Frederick Banting and Charles Best Canada Graduate Scholarship – Doctoral Award from CIHR.

DISCLOSURE

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

WTF designed and performed the study, analyzed the results and wrote the manuscript. PGB, TBO, VAP, JM performed the experiments, analyzed the results and revised the manuscript. YD designed and supervised the study and revised the manuscript.
REFERENCES


TABLE 1. Pairs of primers used for PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession #</th>
<th>5' Primer (5'-3')</th>
<th>3' Primer (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>36B4</td>
<td>NM_022402</td>
<td>TAAAGACTGGAGACAAGGTG</td>
<td>GTGTAAGTCAGTCTCCACAGA</td>
</tr>
<tr>
<td>CBP</td>
<td>NM_133381</td>
<td>CTGCTGGAAGAGGAAGGGGA</td>
<td>GGCACAGTGGACTGAAGTATTC</td>
</tr>
<tr>
<td>CIDEA</td>
<td>XM_214551</td>
<td>ACACCCCTGCTCGTCCTTCC</td>
<td>GGTGGCTTTGACATTGAGACAG</td>
</tr>
<tr>
<td>D2</td>
<td>NM_031720</td>
<td>ATGGGACTTCCTCAGCGTGA</td>
<td>GCACAGGAAGTCAAGAAG</td>
</tr>
<tr>
<td>GyK</td>
<td>NM_024381</td>
<td>CCTGTCCATTGAAATGTGTCATCC</td>
<td>GCCATGAAGCCATGACAATTAGTG</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>NM_031347</td>
<td>TCCTGTATACTATTATAGAATCAAGCC</td>
<td>AAACCATAAGCTGTCTCCATCATCC</td>
</tr>
<tr>
<td>PRDM16</td>
<td>NM_027504</td>
<td>GCAGACCCTGTGGAGTCTCCTGAAA</td>
<td>GCTCCCTGTGTGTCCTCCAGAT</td>
</tr>
<tr>
<td>SHP</td>
<td>NM_057133</td>
<td>CCTCTCTTCTGCTTTGGTT</td>
<td>ACACAATGCCCAGTGAGCCT</td>
</tr>
<tr>
<td>THRα1</td>
<td>NM_001017960</td>
<td>AAGTGGCTCTGCTGCAGGCT</td>
<td>TTGTCCTCTGCTCCAAAGCTG</td>
</tr>
<tr>
<td>THRβ</td>
<td>NM_012672</td>
<td>GAATGGGAGCTCCTCAAGACAGTCA</td>
<td>GGACATGATCTCCATGCAGCA</td>
</tr>
<tr>
<td>TRH</td>
<td>NM_013046</td>
<td>GTGCTAGAGACCTGGTGAA</td>
<td>TCTTGCCAGTGCTGGAGG</td>
</tr>
<tr>
<td>UCP1</td>
<td>NM_012682</td>
<td>TGTTGAGTTTCGAACCTTCC</td>
<td>GTGGGCTGCCAAATGAAATAC</td>
</tr>
<tr>
<td>UCP3</td>
<td>NM_013167</td>
<td>GAAGCAGGTTCTGCAACAGG</td>
<td>TGCAGGTGAAGCTGGTCAGG</td>
</tr>
</tbody>
</table>
TABLE 2. Effect of a 24 h cold exposure on body weight gain, food intake, feed efficiency, mass of white (WAT) and brown (BAT) adipose tissues, and serum concentrations of insulin, metabolites, adiponectin and leptin in rats treated or not with rosiglitazone (RSG).

<table>
<thead>
<tr>
<th></th>
<th>Control, 23°C</th>
<th>Control, 5°C</th>
<th>RSG, 23°C</th>
<th>RSG, 5°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (g)</td>
<td>10.03 ± 0.8a</td>
<td>2.59 ± 1.5b</td>
<td>8.45 ± 0.9a</td>
<td>-0.47 ± 1.37b</td>
</tr>
<tr>
<td>Food Intake (g)</td>
<td>28.7 ± 0.5a</td>
<td>37.2 ± 1.3b</td>
<td>31.8 ± 0.4a</td>
<td>38.1 ± 0.9b</td>
</tr>
<tr>
<td>Feed efficiency (%) a</td>
<td>34.8 ± 2.6a</td>
<td>3.68 ± 3.7b</td>
<td>29.8 ± 3a</td>
<td>-3.1 ± 3b</td>
</tr>
<tr>
<td>VO₂ (mL/min . kg bw.75)</td>
<td>12.2 ± 0.3a</td>
<td>16.9 ± 0.6b</td>
<td>13.0 ± 0.2a</td>
<td>17.7 ± 0.5b</td>
</tr>
<tr>
<td>BAT (mg)</td>
<td>399 ± 17a</td>
<td>421 ± 20a</td>
<td>871 ± 36b</td>
<td>770 ± 40c</td>
</tr>
<tr>
<td>Inguinal WAT (g)</td>
<td>1.85 ± 0.09a</td>
<td>1.91 ± 0.1a</td>
<td>2.7 ± 0.13b</td>
<td>2.5 ± 0.07b</td>
</tr>
<tr>
<td>Retroperitoneal WAT (g)</td>
<td>1.27 ± 0.09a</td>
<td>1.23 ± 0.07a</td>
<td>1.39 ± 0.74a</td>
<td>1.37 ± 0.12a</td>
</tr>
<tr>
<td>Insulin (pM)</td>
<td>234.8 ± 22.7a</td>
<td>134.8 ± 13b</td>
<td>115.6 ± 9b</td>
<td>75.5 ± 13c</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>10.1 ± 1.4a</td>
<td>12.4 ± 1.1a</td>
<td>6.1 ± 0.5b</td>
<td>9.7 ± 1.04a</td>
</tr>
<tr>
<td>NEFA (Eq/L)</td>
<td>96 ± 8a</td>
<td>168 ± 10b</td>
<td>52 ± 10c</td>
<td>97 ± 10a</td>
</tr>
<tr>
<td>TAG (mM)</td>
<td>2.34 ± 0.2a</td>
<td>1.61 ± 0.3b</td>
<td>0.95 ± 0.12c</td>
<td>0.49 ± 0.05d</td>
</tr>
<tr>
<td>Adiponectin (µg/mL)</td>
<td>3.85 ± 0.4a</td>
<td>4.38 ± 0.32a</td>
<td>16.77 ± 0.78b</td>
<td>14.39 ± 0.80c</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>4.56 ± 0.44a</td>
<td>3.98 ± 0.36a</td>
<td>5.24 ± 0.48a</td>
<td>2.22 ± 0.25b</td>
</tr>
</tbody>
</table>

Data are means ± SEM of 6-12 rats. Means not sharing a common superscript letter are significantly different from each other, *P*<0.05.

a Calculated as g body weight gain / 100 g food ingested.
FIGURE LEGENDS

Figure 1 – Brown adipose tissue (BAT) gene expression profile in rats treated or not with rosiglitazone (RSG) and exposed or not to cold for 24 h. Means not sharing a common superscript letter are significantly different from each other, \( P<0.05, \ n=12 \).

Figure 2 – Brown adipose tissue (BAT) norepinephrine (NE) content (Panel A), NE turnover rates (NETO, ng NE/ tissue. H, Panel B), NE fractional turnover rate (\( k \), %/h, Panel C) and arcuate hypothalamic nucleus mRNA levels of CART (Panel D and E) in rats treated or not with rosiglitazone (RSG) and exposed or not to cold for 24h. Optical density of CART mRNA hybridization signal is presented together with representative pictures of the \textit{in situ} hybridization. Means not sharing a common superscript letter are significantly different from each other, \( P<0.05, \ n=6 \).

Figure 3 – Hypothalamic TRH mRNA levels (A), serum TSH (B), total (C) and free (D) T4, total (E) and free (F) T3 and brown adipose tissue mRNA levels of THR\( \alpha \)1 and THR\( \beta \) in rats treated or not with rosiglitazone (RSG) and exposed or not to cold for 24h. Means not sharing a common superscript letter are significantly different from each other, \( P<0.05, \ n=12 \).

Figure 4 – Serum free T3 levels (A), body weight gain (B), feed efficiency (C), and brown adipose tissue (BAT) gene expression (D-H) in control and rosiglitazone treated rats exposed or not to cold for 24 h in association or not with an i.p. injection of T3 (10 \( \mu \)g/ 100 body weight). Means not sharing a common superscript letter are significantly different from each other, \( P<0.05, \ n=12 \).
Figure 5 – cAMP content, protein kinase A (PKA) activity, cyclic AMP responsive element binding protein (CREB) binding, CREB binding protein mRNA levels and CREB regulated transcription coactivator 1 and 2 protein content in brown adipose tissue of control and rosiglitazone treated rats exposed or not to cold for 24 h. Means not sharing a common superscript letter are significantly different from each other, $P<0.05$, n=4-12.
FIGURE 1

A  
23°C  
5°C  

Gyk/36B4 mRNA  
Control  RSG  

PGC-1α/36B4 mRNA  
Control  RSG  

CIDEA/36B4 mRNA  
Control  RSG  

PRDM16/36B4 mRNA  
Control  RSG
Figure 2

A) BAT NE Content (ng/tissue)

B) BAT NETO (ng NE/tissue·h)

C) BAT Fractional NE rates (%/h)

D) ARC CART mRNA

E) Control, 23°C vs. RSG, 23°C

Control, 4°C vs. RSG, 4°C
Figure 3

A. TRH/GAPDH mRNA

B. Serum TSH (μg/mL)

C. Total T4 (nM)

D. Free T4 (pM)

E. Total T3 (nM)

F. Free T3 (pM)

G. BAT THRα1/36B4 mRNA

H. BAT THRβ3/36B4 mRNA

Legend:

- 23°C
- 5°C

Significance:

- a significant difference between Control and RSG at 23°C
- b significant difference between Control and RSG at 5°C
- c significant difference between 23°C and 5°C
Figure 4

A  Free T3 Levels (pmol/L)  
- 23°C  
- 5°C  
- 5°C + T3

B  24h Body Weight (g)  
- Control  
- RSG  
- RSG

C  Feed efficiency (%)  
- Control  
- RSG  
- RSG

D  PGC-1α/36B4 mRNA  
- Control  
- RSG  
- RSG

E  D2/36B4 mRNA  
- Control  
- RSG  
- RSG

F  UCP1/36B4 mRNA  
- Control  
- RSG  
- RSG

G  SHP/36B4 mRNA  
- Control  
- RSG  
- RSG

H  UCP3/36B4 mRNA  
- Control  
- RSG  
- RSG
Figure 5

A. cAMP content

23°C

- Control
- RSG

5°C

- Control
- RSG

B. PKA Activity

CPM/μg protein/min

- Control
- RSG

C. CREB Binding

DNA binding (AU)

- Control
- RSG

D. CBP/36B4 mRNA

- Control
- RSG

E. Cold RSG

- - + - + - - + - - + - - + - +

CRTP2
CRTP1
p38MAPK

CRTC1/p38MAPK

- Control
- RSG

CRTC2/p38MAPK

- Control
- RSG