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Thyroid Hormone Synergizes with PPARγ and cAMP to Drive UCP1 Transcription and Brown-like Adipocyte Phenotype in 3T3-L1 Cells

Manal Malibary [123]

Abstract

Background: Brown adipose tissue expresses uncoupling protein 1(Ucp1), a mitochondrial protein essential for energy balance and non-shivering thermogenesis. This study aimed to show how cyclic adenosine monophosphate (cAMP), peroxisome proliferator-activated receptor γ (Pparγ), and triiodothyronine (T3) pathways together stimulate Ucp1 expression in white adipose tissue.

Methods: Differentiated 3T3-L1 cells, both transiently transfected and stably transduced with a UCP1 vector, were used to assess the effects of T3, Pparγ, cAMP agonists, and their combinations on Ucp1 expression.

Results: The results showed that treatment with T3, Pparγ agonists, and cAMP agonists significantly increased Ucp1 promoter activity in both undifferentiated and differentiated 3T3-L1 cells. In differentiated cells, combined treatment with rosiglitazone, T3, and forskolin led to a time-dependent increase in Ucp1 expression: 5-fold on Day 4, 7.5-fold on Day 8, and 10.5-fold on Day 12 ($P < 0.001$). *Prdm16* mRNA increased 1.5-fold on Days 4 and 8 ($P < 0.001$), and 3-fold on Day 12 ($P < 0.01$) after T3 and rosiglitazone treatment, with forskolin added in the final 12 hours.
Pgc1α expression peaked at a 2-fold increase on Day 12 ($P < 0.05$). *Cidea* expression was markedly upregulated, showing a maximum increase approximately 3 fold on Day 12 ($P < 0.001$). *Elovl3* doubled on Days 4 and 8, and increased approximately 3-fold by Day 12 ($P < 0.001$).

Conclusion: This study suggests that activating PPARγ, cAMP, and T3 pathways can induce browning of white adipose tissue, offering potential therapeutic strategies for obesity management.

Introduction

Adipose tissues play a crucial role in the regulation of thermogenesis. White adipose tissue (WAT) reserves surplus energy in lipid droplets, whereas brown adipose tissue (BAT) possesses the ability to dissipate energy as heat through non-shivering thermogenesis [1]. The transformation of white adipocytes into brite or beige adipocytes may be a therapeutic target for treating obesity. This process is affected by several factors including exposure to cold, hormones, diet, and physical activity. The transcriptional control of BAT formation requires upregulation of several genes [2] including those encoding peroxisome proliferator-activated receptor α (*Pparα*) and γ (*Pparγ*) [3], CCAAT-enhancer-binding proteins β (*C/EBPβ*) [4], proliferator-activated receptor gamma coactivator 1α (*Pgc1α*) [5], PR domain containing 16 (*Prdm16*) [6], cell death-inducing DNA fragmentation factor-like effector A (*Cidea*) [7], and uncoupling protein 1 (*Ucp1*) [8]. The principal mechanism for thermogenesis induced by cold exposure, leading to browning activation, is β3-adrenergic signaling. Additionally, several hormones, including Insulin-like growth factor 1 (IGF1) and thyroid hormone (TH), have been shown to enhance thermogenesis in BAT [9].

In C2C12 myogenic cells, treatment with rosiglitazone (Pparγ agonist), GW501516 (a Pparδ agonist), and bone morphogenetic protein (BMP)-7 for eight days significantly enhanced UCP1 expression in response to forskolin, an activator of the protein kinase A pathway. BMP7 enhanced the upregulation of UCP1 expression in the presence of forskolin when paired with rosiglitazone and GW501516; however, the presence of GW501516 was not essential for the induction of UCP1 [10]. Another study proposed that the function of β-alanine in specifically targeting HB2 adipogenic cells is associated with the forskolin-induced production of UCP1. This action is hypothesized to occur by enhancing the process of phosphorylation and activation of CREB, a transcription factor involved in the cAMP response element-binding pathway [11].

Thyroid hormone is a crucial regulator of energy balance and tissue growth. Numerous genes involved in adaptive thermogenesis depend on the conversion of thyroxine (T4) into T3 by the action of the iodothyronine deiodinase D2 enzyme (*Dio2*) [12]. Two nuclear thyroid hormone receptors (TRs), with different isoforms—TRα and TRβ—are encoded on chromosomes 17 and 3, respectively. These bind to thyroid response elements (TREs) either as homodimers or couple with the retinoid X receptor (RXR) as heterodimers [13]. However, the conversion of WAT to BAT in response to retinoic acid signaling appears to be time-dependent [14, 15].

Although the roles of TRα and TRβ may overlap, TRβ seems to be more important for the β3-adrenergic response, while TRα may be more significant for UCP1 induction. In hypothyroid mice, activation of TRβ induces UCP1 expression; however, treatment of mice with TRβ ligand is not enough to sustain body temperature upon cold stimulation indicating that UCP1 activation by TRβ alone is insufficient to enable thermogenesis [16]. In contrast, mutation of TRβ led to suppression of cAMP in the BAT, which implies that TRβ might participate in the β3-adrenergic response in mice [17].

Taken together, the above studies indicate that TH is involved in the stimulation of BAT gene markers. However, a direct link between TH, *Pparγ*, and cAMP has not been documented. This study aimed to investigate the combinatorial regulation of TH, *Pparγ*, and cAMP in differentiated white adipocytes.

Methods

Plasmid construction

Plasmids were constructed by amplifying the mouse *Ucp1* promoter-enhancer region using specific primers. The *Ucp1* gene (GenBankTMU63418) was used as a template for amplification. The sequences of the primers used are provided in Table 1. The amplified product was subcloned into the pGL3-basic vector to create the pGL3- Ucp1-Luc construct, which was used for transient transfection experiments. This cloning utilized the KpnI and SacI restriction enzyme sites present on the primers. The 3.1 kb PCR product and the pGL3-basic vector were digested with KpnI and SacI restriction enzymes to generate compatible ends and then purified using the QIAquick Gel Extraction Kit. The ligation products were transformed into E. coli competent cells via chemical transformation. Single colonies were picked, cultured in LB broth with ampicillin overnight, and plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (QIAGEN). Constructs were validated by restriction digestion and sequencing [18]. A separate construct, the Ucp1-Luc-P2 reporter, was used for stable transduction. The Luc-2P UCP1 reporter was kindly provided by Perehinec T.M. (Nottingham University, UK). Synthetic DNA sequences corresponding to the mouse *Ucp1* enhancer (-2530 to −2311) and proximal promoter (-284 to +120) regions were combined into a pLenti destination vector using the three-fragment Multisite Gateway (Invitrogen) cloning system. The integrity of these vectors was confirmed by restriction enzyme digestion and sequencing [19].

Cloning Primers	Sequence (5′ → 3′)
Forward Primer	GGGAGCTCCTGCAGAGCCACCTGGGCTAGG
Reverse Primer	GGGGTACCGTGCACACTGCCAAATCATCTC

Table 1: Cloning Primers used for Ucp1 gene amplification. This table presents the forward and reverse primer sequences used for amplifying the Ucp1 gene (GenBank™ U63418). Sequences are shown in the 5′ to 3′ direction.

Transient transfection

The 3T3-L1 cells were grown until they reached 80% confluence before being transfected with the reporter

pGL3 luciferase plasmid using Fugene 6 (Roche) according to the manufacturer's instructions. As an internal control, a Renilla pRL-SV40 construct (Promega) was co-transfected. After 24 h, the medium was replaced with DMEM containing 10 µM DMSO (control), 10 µM rosiglitazone, or 10 µM forskolin. A dual-luciferase assay kit was used to evaluate luciferase activity (Promega). The values were standardized to the Renilla luciferase signal.

Stable transfection

Constitutively expressing Ucp1-Luc-P2 vector-containing lentiviral particles were used to transduce 3T3-L1 preadipocytes. The 3T3-L1 cells were grown in a medium containing 10% FBS until they achieved 50% confluence. The lentiviral particles were diluted into 100 µl growth medium mixed with 6 µg/ml polybrene before being applied to the cells. For selection, the medium was replaced with new growth media containing 8 µg/mL of blasticidin. The cells were then transplanted to 24-well plates, and the medium was changed every three days until confluence was obtained.

Differentiation and luciferase assay

Stably transduced 3T3-L1 cells were allowed to grow until they reached confluence in six-well plates containing a growth medium. Two days later, 500 µM 3-isobutyl-1-methylxanthine (IBMX), 250 nM dexamethasone (Dex), and 167 nM insulin in 2 mL/well growth medium were added to 2 mL/well of the growth medium and incubated for 48 h. For the next 8–12 days, the cells were maintained in DMEM supplemented with 10% FBS and 167 nM insulin. Starting from Day 4 of the differentiation process, the cells were treated with 10 µM T3. Before performing a luciferase assay or RNA isolation, the cells were exposed to 10 µM rosiglitazone for 24 h, forskolin for the last 12 h, or both prior to luciferase assay. The luciferase activity was measured as above.

RNA extraction and quantitative real-time PCR

Total RNA was extracted using TRIzol (Invitrogen) from the differentiated 3T3-L1 cells. cDNA was synthesized using a cDNA synthesis kit (Qiagen) in accordance with the manufacturer's instructions. SYBR Green (Qiagen) was used for the quantitative real-time polymerase chain reaction (qRT-PCR) following the manufacturer's instructions. Table 2 contains the primer sequences. All gene expression levels were normalized to the cyclophilin levels.

Statistical Analysis

Software from SPSS Inc., Version 29.0 (IBM Corp., Armonk, NY, USA) was used to conduct ANOVA statistical analyses. All the provided p-values were two-tailed, and $p < 0.05$ was used to determine statistical significance.

Gene	Forward Primer	Reverse Primer
Cyclophilin	CATACAGGTCCTGGCATCTTGT	TGCCATCCAACCACTCAGTCT
Ucp1	GCCATCTGCATGGGATCAA	GGTCGTCCCTTTCCAAAGTG
Pgc1α	TGAGAGACCGCTTTGAAGTTTTT	CAGGTGTAACGGTAGGTGATGAAA
Elovl3	ATGAATTTCTCACGCGGGTTA	GCTTACCCAGTACTCCTCCAAAAA
Prdm16	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG
Cidea	CTTGGGAGACAACACGCATTT	TCTCGCTATTCCCGACCTCTT

Table 2: Primer sequences for real-time PCR. Table 2 lists the forward and reverse primer sequences (5′ to 3′ direction) used for real-time PCR analysis of target genes. Cyclophilin was used as a housekeeping gene for normalization. The primers were designed to amplify specific gene regions associated with brown and beige adipocyte markers, including Ucp1, Pgc1α, Elovl3, Prdm16, and Cidea.

Ethical Statement

The use of Escherichia coli competent cells for plasmid construction and propagation was conducted in compliance with the institutional biosafety guidelines of King Abdulaziz University. The E. coli strains used are non-pathogenic, standard laboratory cloning strains (e.g., DH5α, TOP10) classified as Biosafety Level 1 (BSL-1) organisms, and the work was performed in accordance with the NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules. This type of recombinant DNA research using non-pathogenic laboratory strains is exempt from specific Institutional Review Board (IRB) or Ethics Committee approval for human or animal subjects.

Results

Effect of T3 in combination with rosiglitazone, forskolin, or both on the UCP1-pGL3-Luc promoter in preadipocyte 3T3-L1 cells

An experiment was performed to determine whether T3, *Pparγ,* and cAMP agonists act synergistically to stimulate transcription from the full-length *Ucp1* promoter in 3T3-L1 cells. In preadipocyte 3T3-L1 cells, T3 treatment resulted in a significant increase in *Ucp1* promoter activity in preadipocyte 3T3-L1 cells (1.4-fold, $P < 0.001$). Combined addition of T3 and rosiglitazone enhanced *Ucp1* expression 3.5-fold ($P < 0.001$). The addition of T3 with forskolin increased *Ucp1* promoter-mediated luciferase expression about 4-fold ($P < 0.001$). However, combined treatment with T3, rosiglitazone, and forskolin achieved the highest increase in *Ucp1* promoter activity of about 5-fold ($P < 0.001$, Figure 1).

Response of the Ucp1-Luc-P2 vector to T3 in combination with rosiglitazone, forskolin, or both in differentiated 3T3-L1 cells

The 3T3-L1 cells were stably transduced with the Ucp1-Luc-P2 vector and differentiated for 8 days to investigate the influence of T3 activation on the *Ucp1* promoter in white adipocytes. The addition of 10 µM T3 on day 4 of differentiation increased the *Ucp1* promoter activity by four-fold ($P < 0.001$). The effect of PPARγ and cAMP

stimulation on *Ucp1* promoter activity was studied by combining T3 with 10 µM rosiglitazone for 24 h or 10 µM forskolin for 12 h. Combining T3 with *Pparγ* or cAMP agonists increased the activity of the *Ucp1* promoter by around five-fold (P < 0.001), as compared to the DMSO-treated group. However, the synergistic activation of the Ucp1-Luc-P2 reporter in comparison to the control was approximately eight-fold higher (P < 0.001; Figure 2) when cells were treated with the three drugs compared to non-treated cells.

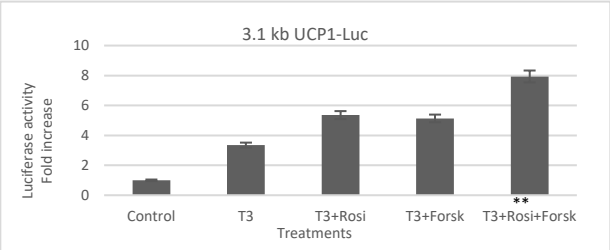

Figure 1: Effect of T3, rosiglitazone, and forskolin treatment on Ucp1 promoter in 3T3-L1 preadipocyte. Ucp1 reporter was transiently transfected into 3T3-L1 cells. Cells were treated in serum-free media with 10 µM DMSO (control), T3, rosiglitazone (Rosi), or both (10 µM) for 24 h, or forskolin (Forsk, 10 µM) for the final 12 h of rosiglitazone treatment before luciferase activity was evaluated. The data were presented as mean ± standard deviation (n = 3).

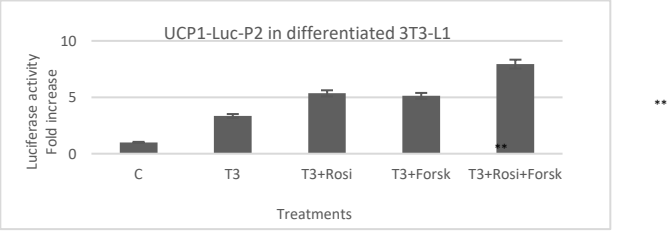

Figure 2: Stimulation of stable Ucp1-Luc-P2 vector activity in differentiated, stably transduced mouse 3T3-L1 cells. The Ucp1 reporter construct was stably transduced into 3T3-L1 preadipocyte cells. Cells were differentiated for 8 days before being treated for 24 h with 10 µM DMSO (control), 10 µM T3, Rosi, or both in serum-free media; Forsk (10 µM) was added for the final 12 h of Rosi treatment before luciferase activity was evaluated. The data were presented as mean ± standard deviation (n = 3). ** P < 0.001 versus untreated group.

Effect of T3 and forskolin on brown/beige adipocyte marker genes

To examine whether activation of rosiglitazone, T3, and cAMP influences the trans-differentiation of white to brown adipocytes, the expression of brown adipocyte markers in differentiated mouse 3T3-L1 cells was assessed by qRT-PCR. The transduced 3T3-L1 cells were differentiated for 12 days. Prior to RNA extraction, cells were treated with 10 µM *Pparγ* agonist rosiglitazone and 10 µM T3 for 24 h, with 10 µM forskolin added during the last 12 h of treatment. Results showed that the expression of Cyclophilin was unchanged (Figure 3a). However, treatment of the 3T3-L1 cells with rosiglitazone, T3, and forskolin resulted in a significant increase in *Ucp1* gene expression, approximately 5-fold, 7.5-fold, and 10.5-fold (all P < 0.001) on Days 4, 8, and 12, respectively (Figure 3b). Moreover, *Prdm16* mRNA expression was significantly upregulated compared to untreated cells, increasing about 1.5-fold on Days 4 and 8 (P < 0.001) and by 3-fold on Day 12 (P < 0.01; Figure 3c). The combination of rosiglitazone, T3, and forskolin enhanced *Pgc1α* expression, with a peak 2-fold increase observed on Day 8 and 12 (P < 0.05; Figure 3d). In addition, the expression of the brown/beige adipocyte marker genes *Cidea* and *Elovl3* was significantly enhanced by the treatment. *Cidea* expression was markedly upregulated at Day 4 and remained elevated at Day 8, and 12; P < 0.001; Figure 3e). In contrast, *Elovl3* expression showed a modest upward trend on Day 4 and 8, which became significant by Day 12 reaching approximately 3-fold increase relative to controls (P < 0.001; Figure 3f).

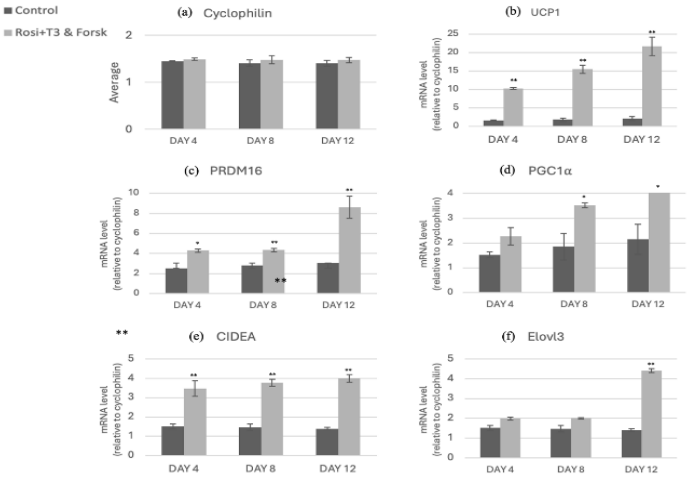

Figure 3: Expression levels of brown/beige adipocyte marker genes in differentiated 3T3-L1 cells. (a) Cyclophilin (housekeeping control). (b) Ucp1. (c) Prdm16. (d) Pgc1α. (e) Cidea. (f) Elovl3. Cells were treated with 10 µM rosiglitazone and 10 µM T3 for 24 h, with 10 µM forskolin added during the last 12 h. Data are mean ± SD (n = 3). *P < 0.05, **P < 0.001 versus untreated group.

Discussion

Role of Ucp1 Activation in Adipocyte Trans-Differentiation

The control of transcription factors that facilitate the trans-differentiation from white adipose tissue to brown adipose tissue is a primary research focus. *Ucp1* activation facilitates the conversion of white adipocytes into brown adipocytes in vitro [20]. Prior studies have examined the impacts of T3, rosiglitazone, and forskolin on adipocyte growth and functionality [21].

The synergistic activation of *Ucp1* by *Pparγ* and cAMP agonists has been well illustrated in both brown adipocytes and human adipocytes, due to the generation of endogenous *Pparγ* ligands that occurs as a result of cAMP-

induced lipolysis [22]. However, the present study is the first to demonstrate the three-way synergistic effect of T3, *Pparγ*, and cAMP on *Ucp1* expression in a white adipocyte model, highlighting a novel and potent regulatory signaling pathway for white adipose tissue browning.

The transient transfection of *Ucp1* reporter constructs in 3T3-L1 cells revealed that T3 could stimulate transcription from the *Ucp1* promoter; however, the most significant enhancement was observed when both rosiglitazone and forskolin were present. This may be due to the overexpression of *Dio2*, essential for brown adipose tissue thermogenesis; its absence in mice results in compromised thermogenic activity[23]. Bile acids also modulate TH production in BAT via the TGR5–cAMP–Dio2 pathway, underscoring the intricate interaction between T3 and cAMP in this mechanism [24]. T3 is crucial in the browning of white adipose tissue (WAT), since it upregulates thermogenic genes including *Ucp1* and promotes mitochondrial biogenesis and oxidative capability. It also regulates transcription factors, such as *Pparγ*, essential for browning, and operates synergistically with β-adrenergic signals, thereby enhancing this process [25]. Rosiglitazone promotes lipid accumulation in the 3T3-L1 cell line by enhancing PPAR protein expression on Day 14 of differentiation [26]. In adipocytes, rosiglitazone and T3 interact through a complicated mechanism that includes upregulating browning-specific transcription factors and activating pathways like phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) [27]. Additionally, T3 influences genes associated with thermogenesis and fat storage, promoting adipocyte differentiation and proliferation [28].

Effects of T3, Rosiglitazone, and Forskolin on UCP1 Expression in Differentiated White Adipocyte

The stable transgenic 3T3-L1 cell line expressing the UCP1 promoter was differentiated using several hormonal stimuli (T3*, a Pparγ* agonist*, and* β-adrenergic *stimulation*) to investigate the role of T3 in the upregulation of *Ucp1* in mature 3T3-L1 adipocytes. A recent study indicates that lobeglitazone, in comparison to other thiazolidinediones, significantly enhanced the generation of beige adipocytes and the expression of thermogenic genes [29]. The current results indicate that while T3 by itself significantly raises *Ucp1* levels, the greatest increase is achieved when T3 is combined with rosiglitazone and forskolin, demonstrating a synergistic effect. These three compounds strongly stimulated *Ucp1* and increased other brown/beige adipocyte marker genes (Figure 3). The cooperative effect of rosiglitazone and cAMP stimulation on *Ucp1* expression in differentiated primary brown adipocytes has been observed previously [30]. Moreover, the addition of both rosiglitazone and T3 during the differentiation process affects the responsiveness to β-adrenergic activation and increases *Ucp1* expression [31]. Furthermore, upregulation of *Ucp1* by T3 via the promoter TRE is required for the adrenergic receptor-induced stimulation of *Ucp1* [32]. There are positive correlations between parathyroid hormone levels and the whole-body content of lipids, BAT volume, and *Ucp1* content in humans [33]. In fully differentiated 3T3-L1 cells, cAMP increases the expression of brown and white adipogenic genes including *Ucp1*, *Pgc1α*, *Cidea*, *Pparγ*, and *C/EBPα/β* [34]. Moreover, stimulation of UCP1, *Prdm16*, and other brown adipocyte genes is regulated in part by the action of *C/EBPβ* on the cAMP-responsive element in the *Pgc1α* proximal promoter [35]. The role of rosiglitazone in inducing p38 mitogen-activated protein kinase (p38 MAPK) has previously been reported to stimulate the expression of *Ucp1* in fetal rat brown adipocytes [36].

Time-Dependent Effects of T3 on Thermogenic Gene Expression

The 3T3-L1 cells can differentiate into brown-like adipocytes in response to chronic treatment with a mixture of T3, IBMX, and rosiglitazone [31]. However, the time course of T3 effects in 3T3-L1 cells has not been extensively studied. In the current study, the effects of T3 treatment on differentiated 3T3-L1 cells were investigated, and mRNA levels were measured on Days 4, 8, and 12. In T3, rosiglitazone, and forskolin-treated cells, the expression of *Ucp1*, *Pparγ*, *Pgc1α*, *Cidea*, *Elovl3*, and *Prdm16* was significantly increased compared to untreated controls, with the maximal induction observed on Day 12 of differentiation (Figure 3). The *Ucp1* mRNA level was increased 10.5-fold on Day 12, confirming the strong browning effect. This substantial fold-increase in *Ucp1* expression, significantly higher than the 2-fold increase observed in the promoter activity assay, suggests that the synergistic effect is not limited to transcriptional initiation but also involves post-transcriptional stabilization of the *Ucp1* mRNA or other downstream regulatory mechanisms that amplify the final mRNA level. *Prdm16* and *Cidea* were also markedly increased, indicating a shift towards a brown-like phenotype. Moreover, a recent study comparing various browning agents in 3T3-L1 cells reported a maximum UCP1 induction of only 6-fold with a single agent [35], underscoring the exceptional potency of the 10.5-fold increase achieved by the combination of T3, Forsk, and rosiglitazone and further emphasizing the novelty of this synergistic approach.

Previously, it has been shown that incubation of differentiated 3T3-L1 cells with 5 nM T3 for 24 h increased *Ucp1* and *Cidea* mRNA levels [37]. In white adipocytes, *Ucp1* is activated by *Pparγ* and TRα in conjunction with *Pgc1α* [38]. Moreover, *Prdm16* functions as a *Ucp1* enhancer through activation of TR, and depletion of T3 reduces *Pparγ* expression in mouse embryonic fibroblasts. Interestingly, TRα can also facilitate *Prdm16*-dependent transcription in the absence of *Pparγ* [6]. Activation of the

Pparγ gene is induced via TREs dependent on deiodinase 3 during the proliferation stage and deiodinase 2 during the differentiation of preadipocytes, and this is directed by C/EBPs [39]. In human multipotent adipose-derived stem cells, treatment with 250 nM T3 activates *Ucp1*, *Pgc1α*, *Prdm16*, nuclear respiratory factor 1 (NRF1), mitochondrial transcription factor A (TFAM), *Cidea*, and *Elovl3*, all involved in cold-induced thermogenesis, as well as *UCP3*, which is required for lipid metabolism [40].

The mRNA level of *Elovl3* showed a progressive increase across Days 4, 8, and 12, consistent with its role in lipid recruitment and metabolism during brown adipocyte development. In parallel, *Cidea* mRNA was significantly upregulated by day 4 and remain elevated at day 8 and 12, supporting its function in maintaining thermogenic activity and sustaining *Ucp1* transcription in differentiated adipocytes [41, 42]. Previous study established that *Cidea* transcriptionally regulates the brightening of white adipocytes and thermogenesis by maintaining *Ucp1* transcription rather than initiating adipocyte trans-differentiation in human fat cells [43]. The sustained upregulation of *Cidea* from Day 4 to Day 12 is particularly significant, as *Cidea* is a key marker of thermogenesis-competent adipocytes and has been shown to directly regulate *Ucp1* transcription and thermogenic function in brite/beige adipocytes [36, 37]. Similarly, the progressive increase in *Elovl3* mRNA across Days 4, 8, and 12 is consistent with its established role in the elongation of very long-chain fatty acids (VLCFAs) and the necessary lipid remodeling that occurs during the maturation of brown adipocytes [38]. The co-regulation of these genes by the T3/PPARγ/cAMP signaling pathways highlights the comprehensive nature of the browning program induced by this combination.

The ultimate functional consequence of the observed transcriptional synergy is the massive upregulation of *Ucp1*, the molecular engine of thermogenesis. *Ucp1* functions by uncoupling the mitochondrial respiratory chain, dissipating the proton gradient across the inner mitochondrial membrane to generate heat instead of ATP. This profound, synergistic upregulation of *Ucp1* expression by the T3/PPARγ/cAMP pathways is the key finding of this study, directly highlighting the potential of this combination to drive a robust thermogenic phenotype in white adipocytes [44].

The use of 3T3-L1 cell lines as a model for investigating adipocyte development is the primary limitation of this work. Although these cells are helpful in studying adipocyte biology, it is possible that they may not fully represent human adipose tissue. Consequently, the findings from the study of 3T3-L1 cells may not directly apply to human physiology. Therefore, validating these results and their relevance for human obesity therapy will require in vivo investigations. Future studies should focus on validating this three-way synergy in vivo and determining the precise molecular interaction between the TR and the PPARγ/CREB complex at the *Ucp1* enhancer region to fully elucidate the mechanism of this novel regulatory pathway.

The study showed that thyroid hormone (T3), rosiglitazone, and forskolin work together to switch white adipocytes toward a brown-like, thermogenic state. This combined action not only boosts *Ucp1* but also engages a broader network of browning genes, pointing to a coregulated program of metabolic pathways. These results suggest that targeting multiple pathways at once may be a promising way to enhance energy expenditure. Looking ahead, in vivo studies will be essential to confirm these effects and to explore their potential in strategies against obesity and related disorders.

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Conflicts of Interest

The author declares no conflict of interest.

Author Contribution

Manal Malibary is the sole author of this manuscript. The author is responsible for all aspects of this work, including conceptualization, methodology, data curation, formal analysis, investigation, writing—original draft preparation, writing—review and editing, and project administration.

Data Availability Statement

The manuscript incorporates all datasets produced or examined throughout this research study, and the raw data presented in this study are available on request from the corresponding author.

Ethics Statement

This study did not involve any experiment on humans and animals. Therefore, approval from any ethics body was neither required nor sought.

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