Effects of ATF4 on PGC1α expression in brown adipose tissue and metabolic responses to cold stress

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Objective. We have shown previously that the expression of peroxisome proliferator activated receptor gamma coactivator (PGC1α) increases significantly in the white and brown adipose tissue of activating transcription factor 4 (ATF4) global knockout mice, which suggests that ATF4 is involved in the regulation of PGC1α expression. The goal of the current study is to investigate this possibility and elucidate the underlying cellular mechanisms.

Material/methods. The effects of ATF4 on PGC1α expression and on PGC1α promoter activity were analyzed in vivo and in vitro using mice, HIB-1B, and 293T cell line. The physiological functions of ATF4 in the regulation of PGC1α expression were confirmed by analysis of body temperature of Atf4−/− and Atf4+/+ mice in response to cold stress as well as expression of Complex I, II, III, and IV in BAT.

Results. In this study, we showed ATF4 to be a negative regulator of PGC1α expression through competitive binding with cAMP response element binding protein (CREB) at a cAMP response element (CRE) site in the PGC1α promoter. ATF4 was also found to influence the expression of mitochondria-related proteins, including Complex I, II, III, and IV through regulation of PGC1α. Finally, we showed that Atf4−/− mice have higher core body temperatures in reduced-temperature environments than control mice.

Conclusion. This study describes the mechanisms underlying ATF4 regulating PGC1α expression. We demonstrate a novel function of ATF4 in the regulation of thermogenesis. Taken together, these observations provide new insight into the physiological functions of ATF4, especially the regulation of thermogenesis and the response to cold stress.

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1. Introduction

Temperature control (thermoregulation) keeps organisms at their optimal operating temperatures. It is also an important adaptive metabolic response to environmental changes, including cold exposure. For example, when an animal is exposed to a cold environment, non-shivering and shivering thermogenesis is stimulated to maintain a constant core body temperature [1]. It has been shown that non-shivering thermogenesis, which burns fatty acids for heat production, occurs mainly in brown adipose tissue (BAT), which has recently been shown to be present in the human body [2–4].
Accumulated evidence has demonstrated that non-shivering thermogenesis is regulated by a marked, rapid induction of uncoupling protein (UCP1) and its upstream regulator peroxisome proliferator activated receptor gamma coactivator (PGC1α) in BAT at reduced temperatures [1,5]. PGC1α expression has been shown to be stimulated by activation of the sympathetic nervous system (SNS). This results in increases in cAMP levels, activation of protein kinase A (PKA), and phosphorylation of cAMP response element binding protein (CREB). Activated CREB then stimulates PGC1α expression via direct binding at the cAMP response element (CRE) site of the PGC1α promoter [6]. Much progress has been made in elucidating the positive regulators of PGC1α expression, including CREB, reactive oxygen species (ROS), AMP-activated protein kinase (AMPk) and sirtuin (silent mating type information regulation 2 homolog) 1 (SIRT1) [7]. However, except for serine/threonine protein kinase PKB/AKT and Twist-1, very few negative regulators of PGC1α expression have been identified [9-11]. This requires further investigation.

Activating transcription factor (ATF)4, also known as CREB2, belongs to a family of basic zipper-containing proteins [8,9]. It has been found to be expressed in a wide variety of tissues, including white adipose (WAT), BAT, liver, brain, and heart tissue [9,10]. It has been found to be involved in various physiological processes, such as long-term memory, osteoblast differentiation, endoplasmic reticulum (ER) stress, glucose metabolism, and metabolic responses to amino acid deprivation [11]. Our previous work revealed that the PGC1α mRNA level increases in the BAT of Atf4−/− mice [12]. This suggests a role for ATF4 in the regulation of PGC1α expression and the involvement of ATF4 in metabolic responses to thermogenesis. The aim of our current study is to investigate these possibilities and elucidate underlying mechanisms.

In the present study, we show that ATF4 is a negative regulator of PGC1α expression, which is mediated by competitive binding with CREB at a CRE site in the PGC1α promoter. Atf4−/− mice have higher core body temperatures than control mice in response to cold stress. Taken together, our study identifies a possible novel function for ATF4 in the regulation of responses to cold stress. It provides important information regarding negative regulation of PGC1α, which controls energy homeostasis and many other important functions.

2. Materials and Methods

2.1. Animal experiments

Homozygous (Atf4−/−) and wild-type (Atf4+/+) mice were produced by breeding heterozygous (Atf4+/−) mice, which were provided by Drs. Tim Townes (University of Alabama, AL, U.S.), Douglas Cavener (Penn State University, PA, U.S.), and Bob Paulson (Penn State University, PA, U.S.) [13]. For cold exposure, two- to three-month-old mice were exposed to 4 °C for 7 h or 3 h, as indicated [14]. Control mice were kept at room temperature. Core body temperature was measured using a rectal probe attached to a digital thermometer (Physitemp Inc, NJ, U.S.). Prior to the experiments, all mice were maintained on a 12-h light/dark cycle at 25 °C and provided with free access to commercial rodent chow and tap water. Following euthanasia, the interscapular BAT was quickly collected and frozen in liquid nitrogen for further analysis. All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Institution for Nutritional Sciences.

2.2. Cell culture

HIB1B cells were provided by Dr. Jae Bum Kim (Seoul National University, Korea). They were maintained in DMEM containing 10% fetal bovine serum (FBS). Stable Atf4-knockdown (ATF4 shRNA) cell lines were obtained by transfecting a pSilencer4.1-CMV-puro-ATF4 small hairpin RNA (shRNA) vector into HIB1B cells, and stable clones were selected in the presence of 1.0 mg/mL puromycin.

2.3. Plasmids

Primers amplifying the PGC1α promoter were designed according to EMBL/GenBank Accession No. AF106698. Sequences 5′-TATAGGTACCGTG- GCCAGGGGCTGC-3′ and 5′-TATAGGTACCGGATTTTGCAGACAGC-3′ were used as upstream primers to produce promoters with and without CRE sites. A single downstream primer (5′-GCCGATCCCTTT- CAAAGCTTTT- TCAACTC-3′) was used for amplification. PCR products were digested with Kpn I/Bgl II and inserted into the pGL3-Basic generating 168-luc for the PGC1α promoter with the CRE site (−65−69) and 96-luc for the PGC1α promoter without a CRE site (−96−69). pEGFP-ATF4 and pEGFP-CREB were provided by Dr. Zaiqing Yang (Huazhong Agriculture University, China) as templates for PCR amplification of the ATF4 and CREB cds sequences, respectively. pCMV-HA-ATF4 and pCMV-myc-CREB were generated by inserting the ATF4 or CREB cds PCR product into pCMV-HA (BD Biosciences Clontech, CA, U.S.) or pCMV-myc vector (BD Biosciences Clontech) for overexpression of ATF4 or CREB.

2.4. Transient transfections and luciferase assays

293T cells were maintained in DMEM containing 10% FBS, and transfection was performed with lipofectamine 2000 (Invitrogen Life Technology, Inc., Carlsbad, CA, U.S.) according to the manufacturer’s instructions. The pRL-SV40 carrying Renilla luciferase was also co-transfected as an internal control for monitoring the transfection efficiency. Forty-eight hours later, cells were harvested, and luciferase activities were analyzed using the Dual-Luciferase Assay Kit (Promega, Madison, WI, U.S.) according to the manufacturer’s instructions. Values are expressed relative to the control Renilla to exclude the effects of differences in transfection efficiency.

2.5. ChiP assays

ChiP assays were performed according to the manufacturer’s protocols (Millipore, U.S.). Briefly, the DNA–protein complexes in BAT were cross-linked with 1% formaldehyde. BAT was lysed and sonicated prior to immunoprecipitation with anti-ATF4 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.), anti-CREB antibody (Cell Signaling, Beverly, MA, U.S.) or normal
rabbit IgG (Santa Cruz Biotechnology) as a negative control at 4 °C overnight. DNA–protein immuno-complexes were collected using protein G magnetic beads. An immunoprecipitated PGC1α promoter was quantified using PCR with primers designed to amplify the region encompassing the 116 bp containing the CRE site (forward, 5′-GGGCTGCCTTGAGTTGACGTC-3′ and reverse, 5′-AGTCCCCAGTCATGACAAAAG-3′) or an upstream region encompassing 160 bp not involved in ATF4 or CREB response (forward, 5′-GGGCTGCCTTGAGTTGACGTC-3′ and reverse, 5′-TTTGATGGCAGCAACTCTAAACC-3′). Quantification of PCR products was calculated using Quantity One Analyzer (Bio-Rad Laboratories, Hercules, CA, U.S.).

2.6. RNA isolation and relative quantitative RT-PCR

Total RNA was prepared from mouse tissues and cells using TRIzol reagent (Invitrogen Life Technology, Inc.). One microgram of RNA was reverse transcribed with PrimeScript RT-PCR Kit (TaKaRa, Ohtsu, Shiga, Japan) and quantitative PCR was performed with a qPCR core kit for SYBR green I (ABI) in an ABI 7500 system (Applied Biosystems, Carlsbad, CA, U.S.). Primer sequences are available upon request.

2.7. Western blot

Whole-cell lysates from frozen tissues were isolated using RIPA lysis buffer with protease and phosphatase inhibitors. Primary antibodies, including anti-ATF4 antibody (Santa Cruz Biotechnology), anti-PGC1α antibody (Santa Cruz Biotechnology), anti-UCP1 antibody (Santa Cruz Biotechnology), and anti-Complex I, II, III, and IV antibody (Invitrogen Life Technology, Inc.), were incubated overnight at 4°C and specific proteins were visualized by ECL Plus (GE Healthcare, U.K.). Band intensities were measured using ImageQuant (Bio-Rad Laboratories) and normalized to β-actin or tubulin.

2.8. Statistical analysis

All values are presented as mean ± SEM. Differences between groups were analyzed by two-tailed Student t-test. Values of P<0.05 were considered statistically significant.

3. Results

3.1. Effects of ATF4 on PGC1α expression in vivo

We have shown previously that Pgclα mRNA levels are increased in the BAT and WAT of mice deficient for ATF4 [12]. In current study, we found that levels of Pgclα mRNA were also increased in the livers of Atf4−/− mice relative to those of Atf4+/+ mice (data not shown). The level of PGC1α protein was also significantly increased in these tissues (Fig. 1A). PGC1α protein was decreased in the livers of mice injected with adenoviruses designed for overexpression of ATF4 (Ad-ATF4) relative to control mice (Ad-GFP) (Fig. 1B). These results suggest a general role for ATF4 in regulating PGC1α expression.

3.2. Role of ATF4 in PGC1α expression in vitro

To determine whether ATF4 has any direct effect on PGC1α expression in vitro, we constructed a stable cell line with ATF4 expression knocked down by ATF4 shRNA in HIB1B. Levels of Pgclα mRNA and proteins were significantly increased in

![Fig. 1](image-url) - Effects of ATF4 deficiency on PGC1α expression in vivo. (A) Expression of PGC1α in brown adipose tissue (BAT), white adipose tissue (WAT), and the livers (LV) of ATF4 knockout (KO) and wild-type (WT) mice. (B) Protein levels of PGC1α and ATF4 in the LV of WT mice infected with adenoviruses expressing constitutively active ATF4 (Ad-ATF4) or control green fluorescent protein (Ad-GFP). Data are mean ± SEM of at least two independent experiments with mice from each group (n=6). *Effects of KO or Ad-ATF4 groups versus their respective control groups (two-tailed Student t-test, P<0.05). (A) PGC1α protein (top, Western blot; bottom, protein levels relative to actin). (B) PGC1α and ATF4 proteins (left, Western blot; right, protein levels relative to actin).
these cells relative to control HIB1B cells (Fig. 2A and 2B). PGC1α expression was significantly decreased in HIB1B cells infected with adenoviruses expressing constitutively active ATF4 (Ad-ATF4) relative to control green fluorescent protein adenovirus (Ad-GFP) (Fig. 2C and 2D).

3.3. Effects and mechanism of ATF4 on activation of PGC1α gene expression

To determine the mechanisms underlying ATF4-based regulation of PGC1α expression, we examined the effects of ATF4 overexpression on the activity of the PGC1α promoter with two constructs, one containing a CRE site (168-Luc) and one without it (96-Luc), in 293T cells transfected with pCMV-HA or vectors overexpressing ATF4 (pCMV-HA-ATF4). As expected, the basal activity of PGC1α was much higher in the construct containing the CRE site than in the one without. ATF4 overexpression significantly inhibited PGC1α promoter activity in both constructs, much more significantly in the one containing the CRE site (Fig. 3A). Because CREB positively regulates PGC1α expression via direct binding at CRE site of its promoter, we hypothesized that ATF4 may regulate PGC1α expression via competitive binding with CREB at the CRE site of PGC1α promoter [6]. As expected, CREB stimulation of PGC1α promoter activity was significantly repressed by ATF4 expression in a dose-dependent manner (Fig. 3B). Consistent with the inhibitory effect of ATF4 on CREB stimulation of PGC1α promoter activity, there was significantly less PGC1α promoter in ATF4-overexpressing cells subjected to ChIP with anti-CREB antibodies (as confirmed by Western blotting using ATF4 antibody, Fig. 3C) than in those infected with a blank retrovirus (Fig. 3D). Consistent with these results, the amount of PGC1α promoter was significantly increased when ChIP was performed with anti-ATF4 antibodies in cells transfected with retroviruses overexpressing ATF4 (Fig. 3D). The added chromatin showed similar numbers of bands amplifying both regions (Fig. 3D). Similar results were obtained when PCR products were quantified (Fig. 3D).

3.4. Effects of cold exposure on expression of ATF4 in BAT

When mice are placed in cold environments, nonshivering thermogenesis can be induced via increasing expression of PGC1α and its target gene, UCP1 [1]. Given the fact that PGC1α expression is regulated by ATF4 in vivo and in vitro, we speculated that ATF4 might be involved in the regulation of adaptive response to cold stress. A previous research team observed that increased PGC1α expression in BAT is a part of cold-induced thermogenesis [1]. Consistent with this, we found that Pgc1α mRNA and protein levels in BAT were significantly increased in response to cold stress (Fig. 4A and 4B). As a result, levels of Ucp1 mRNA and proteins were also significantly induced in BAT after cold exposure (Fig. 4A and 4B). However, Atf4 mRNA and protein levels in BAT were greatly decreased in response to cold stress (Fig. 4A and 4B).

![Figure 2](image-url) - Effects of ATF4 on PGC1α expression in vitro. Expression of PGC1α and ATF4 in stable ATF4 knockout HIB1B (ATF4 shRNA) and negative control cells (NC) (A and B). HIB1B transfected by adenovirus expressing constitutively active ATF4 (Ad-ATF4) or control green fluorescent protein adenovirus (Ad-GFP) (C and D). Data are mean ± SEM of at least two independent experiments. *Effects of ATF4 shRNA or Ad-ATF4 group versus their respective control groups (two-tailed Student t-test, P<0.05). (A and C) Pgc1α and Atf4 mRNAs; (B and D) PGC1α and ATF4 proteins (left, Western blot; right, protein levels relative to tubulin or actin).
3.5. Core body temperatures of Atf4−/− and control mice in reduced-temperature environments

To confirm the role of ATF4 in the regulation of adaptive response to cold stress, mice were exposed to 4 °C for 3 h. We found that Atf4−/− mice constantly displayed higher core temperatures than Atf4+/+ mice (Fig. 4D). To assess the possibility that ATF4 regulates cold stress responses by affecting PGC1α expression, we examined expression levels of PGC1α and its target gene, UCP1. Levels of PGC1α and UCP1 protein were much higher in the BAT of Atf4−/− mice subjected to cold stress than in wild-type mice subjected to the same conditions (Fig. 4E).

PGC1α has been shown to affect the expression of various proteins, which are involved in the regulation of mitochondrial biogenesis and play an important role in thermogenesis. We examined levels of protein complexes I, II, III, and IV in the BAT of mice exposed to a cold environment. We found that levels of complex I, II, III, and IV proteins were significantly higher in the BAT of Atf4−/− mice (Fig. 4F).

4. Discussion

PGC1α is one of the better-characterized metabolic regulators that control energy and nutrient homeostasis. We have previously shown that Atf4−/− mice display higher body temperatures, accompanied by increased PGC1α and UCP1 expression in BAT [12]. In the current study, we showed that overexpressing ATF4 decreases PGC1α expression in vitro and in vivo, suggesting that ATF4 is a
suppressor of PGC1α expression. Our study provides important information concerning negative regulation of PGC1α expression.

While investigating the mechanisms underlying ATF4 regulation of PGC1α expression, we first examined whether the CRE site at the PGC1α promoter is involved in this regulation, considering that ATF4 stimulates phosphoenolpyruvate carboxykinase (PEPCK) expression by binding to a particular subclass of asymmetric CREs in its promoter [9]. ATF4 significantly represses CRE-dependent transcription in CV-1 cells [8]. Consistent with this, we found that ATF4 suppresses PGC1α expression by binding at a CRE site on its promoter.

It has been established that PGC1α expression is induced by increased phosphorylation of CREB [6,15]. Activated CREB then binds to the CRE site on the promoter of PGC1α and stimulates transcription of this gene. Previous work has shown that ATF4 may suppress the CREB effect [16]. This raises the possibility that ATF4 regulates PGC1α expression by blocking the effects of CREB. Consistent with this possibility, we found that ATF4 decreases CREB stimulation of PGC1α promoter activity, as demonstrated by luciferase reporter
analysis and the amount of CREB binding at the CRE, which was in turn demonstrated by ChIP assay. The molecular mechanisms underlying the blocking effect of ATF4 on CREB activation of PGC1α expression, however, are unclear. It is not likely to be regulated via direct interaction with CREB, as shown by the fact that we failed to co-IP CREB with ATF4 in 293 cells in our preliminary experiments (data not shown). This is consistent with a previous report showing that ATF4 cannot form a dimer with CREB [8]. We speculate that ATF4 regulates PGC1α expression via competitively binding with CREB at the CRE site in the PGC1α promoter. The details of the underlying mechanisms will be investigated in the future.

In addition to investigating the molecular mechanisms underlying ATF4 regulation of PGC1α expression in BAT, we also explored the physiological significance of this regulation. Adaptive thermogenesis is an important metabolic change. It is required for the maintenance of energy homeostasis in response to cold stress. Compelling evidence has revealed that PGC1α and its target genes interact with BAT mitochondrial biogenesis, contributing to the metabolic response to cold stress. As expected, ATF4 was found to be involved in the metabolic response to cold stress. ATF4 expression was found to be suppressed in BAT and it dissociated from the CRE site in the promoter of PGC1α at reduced temperatures. Atf4−/− mice exhibited higher body temperatures than wild-type mice during cold exposure, suggesting that these mice are more resistant to cold stress. Our results further indicate that the differences between two strains of mice are caused by greatly increased PGC1α expression and its downstream targets, including UCP1 and complex I, II, III, and IV proteins in the BAT of Atf4−/− mice. These results collectively indicate that ATF4 is involved in the metabolic response to cold stress. Though the much higher expression of PGC1α and UCP1 protein levels in BAT of ATF4 KO mice relative to wild-type mice indicates the existence of non-shivering thermogenesis, because shivering thermogenesis is also induced by cold exposure, we can not exclude the possibility of a contribution of shivering thermogenesis to changes in body temperature in either ATF4 KO or wild type mice [17]. The relative magnitudes of the contributions of shivering and non-shivering thermogenesis to ATF4-dependent changes in body temperature during and after cold exposure will be investigated in the future.

Though we did not examine the browning of WAT following cold exposure in the current study, we speculate that it is unlikely to occur. We draw this conclusion based on our previous results, which showed no difference in UCP1 expression in WAT between ATF4 KO and wild-type mice [18]. However, the involvement of ATF4 in WAT browning was indicated by another study, which reported that UCP1 expression levels are significantly higher in the BAT of ATF4 KO mice than in that of the wild-type mice in the control group [19]. This possible role of ATF4 in the regulation of browning of WAT under cold stress conditions should not be ignored and will be investigated in the future.

It has been shown that ATF4 is constitutively expressed in a wide variety of tissues, including the brain, heart, WAT, BAT, liver, spleen, thymus, lung, and kidney [10]. Our current study shows that knocking down expression of ATF4 induces higher expression of PGC1α in the WAT, BAT, and liver. This suggests that ATF4 plays a general role in suppressing expression of PGC1α in different tissues. In addition to regulating thermogenesis in BAT, PGC1α has been shown to be intimately involved in regulating the switching of fiber types in muscle tissue and in glucose/fatty acid metabolism in the liver and WAT. This suggests that ATF4 may regulate these important processes via suppression of PGC1α expression. These possibilities will be investigated in the future.

In summary, we show that ATF4 is a negative regulator of PGC1α expression. We also show that this effect is mediated by competitive binding with CREB at a CRE site in the PGC1α promoter. This blocks activation of gene expression very effectively. Finally, we show that, in reduced-temperature environments, Atf4−/− mice maintain higher core body temperatures than control mice and are thereby more resistant to cold stress. Collectively, the results of our study identify a previously unknown function for ATF4: regulation of the cold stress response.

Authors contributions

Chunxia Wang performed all of the Western blotting, RT-PCR, and animal experiments. Tingting Xia helped to perform the ChIP assay. Ying Du and Bin Liu constructed and provided the adenovirus plasmid for overexpression of ATF4. Houkai Li and Qingshu Meng helped to perform the Western blot experiment. Shanghai Chen was responsible for providing Atf4−/+ and Atf4−/− mice. Chunxia Wang and Feifan Guo designed the experiments and wrote the manuscript. All the authors contributed to and approved the final manuscript.

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Conflict of interest

The authors have no conflicts of interest to disclose.

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