Biochemical and Biophysical Research Communications 421 (2012) 38–43

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

ATF3 inhibits adipocyte differentiation of 3T3-L1 cells

Min Kyung Jang [a], Cho Hee Kim [a], Je Kyung Seong [b], Myeong Ho Jung [a,*]

[a] School of Korean Medicine, Pusan National University, #30 Beom-eo ri, Mulguem-eup, Yangsan-si, Gyeongnam 609-735, Republic of Korea
[b] Department of Anatomy and Cell Biology, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Republic of Korea

ARTICLE INFO

Article history:
Received 19 March 2012
Available online 27 March 2012

Keywords:
Adipocyte differentiation
Activating transcription factor 3 (ATF3)
CCAAT/enhancer-binding proteins α (C/EBPα)
Promoter
Hypoxia

ABSTRACT

ATF3 is a stress-adaptive gene that regulates proliferation or apoptosis under stress conditions. However, the role of ATF3 is unknown in adipocyte cells. Therefore, in this study, we investigated the functional role of ATF3 in adipocytes. Both lentivirus-mediated overexpression of ATF3 and stably-overexpressed ATF3 inhibited adipocyte differentiation in 3T3-L1 cells, as revealed by decreased lipid staining with oil red staining and reduction in adipogenic genes. Thapsigargin treatment and overexpression of ATF3 decreased C/EBPα transcript and repressed the activity of the 3.6-kb mouse C/EBPα promoter, demonstrating that ATF3 downregulates C/EBPα expression. Transfection studies using mutant constructs containing 5′-deletions in the C/EBPα promoter revealed that a putative ATF/CRE element, GGATGTCA, is located between −1921 and −1914. Electrophoretic mobility shift assay and chromatin immunoprecipitation assay demonstrated that ATF3 directly binds to mouse C/EBPα promoter spanning from −1928 to −1907. Both chemical hypoxia-mimetics or physical hypoxia led to reduce the C/EBPα mRNA and repress the promoter activity of the C/EBPα gene, whereas increase ATF3 mRNA, suggesting that ATF3 may contribute to the inhibition of adipocyte differentiation in hypoxia through downregulation of C/EBPα expression. Collectively, these results demonstrate that ATF3 represses the C/EBPα gene, resulting in inhibition of adipocyte differentiation, and thus plays a role in hypoxia-mediated inhibition of adipocyte differentiation.

1. Introduction

Adipose tissue plays a major role in regulating metabolism by storing excess energy and mobilizing the stored lipid for energy supply. Furthermore, adipose also functions as an endocrine organ, secreting various adipokines and cytokines, such as leptin and adiponectin, to influence metabolism. Dysfunctional secretion of adipokines, together with dysregulated disposal of glucose and lipids, is a pivotal pathogenetic factor in the development of several metabolic disorders [1]. Significant changes in adipose mass or perturbation in adipocyte signaling can disrupt the overall metabolic homeostasis [2]. Adipocyte differentiation therefore has many implications for human diseases including obesity and its metabolic complications. A thorough understanding of the differentiation process is thus necessary for planning therapies against these diseases.

Adipocytes are thought to specifically arise from mesenchymal cells that undergo the commitment and differentiation process of adipogenesis [3]. Adipogenesis is a multi-step process involving a cascade of transcription factors and cell-cycle proteins that regulate gene expression and lead to adipocyte development. Several coactivators and negative regulators of this network have been elucidated in recent years [4,5]. Two transcription factor families have emerged as the key determinants of terminal adipocyte differentiation: CCAAT/enhancer-binding proteins C/EBPα, C/EBPβ and C/EBPδ, and peroxisome proliferator-activated receptor γ (PPARγ). As cells undergo the differentiation process in response to adipogenic signals, the initial event is the rapid induction of C/EBPβ and C/EBPδ expression. C/EBPβ and C/EBPδ proteins stimulate CDK inhibitor p21 expression, which leads to inhibition of CDK-mediated Rb phosphorylation. The induction of C/EBPβ and C/EBPδ is immediately followed by increases in PPARγ and C/EBPα. Once expressed, C/EBPα maintains expression of both PPARγ and C/EBPα genes via transactivation mediated by their respective C/EBP regulatory elements. Both PPARγ and C/EBPα play a critical role in the expression of most adipocyte-specific genes.

In addition to these two transcription factor families, several signaling pathways are reported to be involved in adipogenesis. The Wnt/β-catenin pathway negatively regulates adipocyte differentiation [6]. Members of the Kruppel-like factor (KLF) family are also implicated in adipogenesis and can either positively or negatively modulate adipocyte differentiation depending on the specific protein involved [7]. For instance, KLF4 transactivates CEBPβ promoter, initiating the adipogenic program [8]. KLF6 represses

Abbreviations: ATF3, activation transcription factor 3; ATF/CRE, ATF/CREB responsive element; C/EBP, CCAAT/enhancer-binding proteins; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; aP2, adipocyte fatty acid binding protein; HIF-1α, hypoxia inducible factor-1α; PPARγ, peroxisome proliferator activated receptor γ.

* Corresponding author. Fax: +82 51 510 8437.
E-mail address: jung0603@pusan.ac.kr (M.H. Jung).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.03.104

DLK1, which is a well-established inhibitor of adipogenesis, and therefore promotes adipose conversion [9]. In contrast, KLF2 can repress PPAR2 promoter activity, inhibiting adipogenesis in 3T3L1 cells [10].

Activating transcription factor 3 (ATF3) is a stress-inducible gene that encodes a member of the ATF/CREB family of transcription factors [11]. ATF3 is induced by signals such as proinflammatory cytokines, nitric oxide, and high concentrations of glucose, palmitate and ER stress. ATF3 regulates proliferation or apoptosis under stress conditions by down or upregulation of related genes [12]. We previously reported that ATF3 is increased in abdominal subcutaneous adipose tissue of obese mice, and negatively regulates adiponectin gene expression [13]. Furthermore, we demonstrated that ATF3 represses the expression of adiponectin receptors in adipocyte cells [14,15]. In a preliminary study, we conducted a genome-wide screen of ATF3-target genes in differentiated 3T3-L1 cells by chromatin immunoprecipitation-on-chip (ChIP-on-chip) to characterize the role of ATF3 in adipocyte cells. Analysis of functional annotation of the ATF3-target genes revealed enrichment in adipocyte differentiation genes, suggesting that ATF3 could regulate adipocyte differentiation.

Therefore, in this study, we investigated a potential role for ATF3 in regulating the differentiation of adipocytes and the mechanism involved in this event. Our data indicated that stress-induced ATF3 acts as a crucial inhibitor of adipocyte differentiation.

2. Materials and methods

2.1. Cell culture, differentiation, and treatments

Preadipocyte 3T3-L1 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (FCS, Invitrogen). Preadipocyte 3T3-L1 cells were differentiated into adipocytes in DMEM supplemented with 10% fetal bovine serum (FBS, Invitrogen) with differentiation medium containing 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, and 5 μg/ml insulin (Sigma–Aldrich Co., St. Louis, MO) for 2 d, followed by insulin for a total of 8 d. To investigate the effect of ATF3 on C/EBPα expression, fully differentiated 3T3-L1 cells were incubated with thapsigargin (0.5, 1.0 μM). To create a hypoxic experiment, fully differentiated 3T3-L1 cells were treated with $CoCl_2$ (50, 100 μM) (Sigma) for 24 h or placed in 2% O_2, 5% CO_2, 93% N_2 hypoxic chamber (Forma Scientific, Marietta, OH) for 1 or 2 d.

2.2. Construction of recombinant lentivirus containing ATF cDNA and infection

Recombinant lentivirus containing ATF3 cDNA was constructed previously [14]. To examine the effect of ATF3 on adipogenic differentiation, confluent preadipocyte 3T3-L1 cells (1×10^6 cells) were infected with recombinant lentivirus ATF3 (Lenti-ATF3) or control lentivirus (Lenti-GFP) at a multiplicity of infection 5. Two days later, the cells were differentiated as described previously. At d 8 after differentiation, the cells were fixed and stained with Oil red O. RNA transcripts for adipocyte differentiation genes, PPARγ, C/EBPα, and aP2, were measured by RT-PCR.

2.3. Oil red O staining

Cells were washed three times with PBS and then fixed for 2 min with 3.7% formaldehyde. Oil red O (0.5% in isopropanol, Sigma) was diluted with water (3:2), filtered through a 0.45-μm filter, and incubated with the fixed cells for 1 h at room temperature. Cells were washed with water, and the stained fat droplets in the cells were visualized by light microscopy and photographed.

2.4. Reverse transcription (RT)-PCR

Total RNA was extracted using Trizol reagent (Invitrogen). The mRNA in the samples was reverse-transcribed using a Superscript II™ First Strand Kit (Invitrogen). The resulting cDNA was amplified by PCR using the following primer pairs: mouse PPARγ F (5′-TTTTC AAGG GTGCCAGTTTC-3′) and R (5′-AATCCTTGGCCCTCTGAGAT-3′), C/EBPα F (5′-TTTGAGTCTGTGTCCTCACC-3′) and R (5′-CACAACTCAG CTTTCTGGTC-3′), aP2 F (5′-CCTCGAAGGTTTACAAAATGTGTGA-3′) and R (5′-AAACTCTTGTGGAAGTCACGCCTT T-3′), and ATF3 F (5′-A GTGAGTGCTTCTGCCATCG-3′) and R (5′-GCAGAGGTGCTTGTTCTG GA-3′). The housekeeping β-actin gene was amplified using the sense primer 5′-TAAAGACCTCTATGCCAACACAGT-3′ and the antisense primer 5′-CACGATGGAGGGGCCGGACTCATC-3′.

2.5. Promoter constructs

The region of the mouse C/EBPα promoter corresponding to nucleotides −3595 to +100 was amplified by PCR from mouse genomic DNA and inserted into the *KpnI*/*Xho*I sites in pGL3 basic (Promega, Madison, WI) using the sense primer 5′-GGTACCTCACT TCTGGAGGGTTTGGTAGG-3′ and antisense primer 5′-CTCGAGAGTT AGAGTTCTCCCGGCATGG-3′. Deletions in the 5′-flanking regions of the promoter were constructed by PCR using pairwise combinations of the sense primers 5′-GGTACCGCGGTGTCTGGCAACTG AGTC-3′ [for P(−3122)/Luc], 5′-GGTACCCCATTTCATGGGTGATGGTT TAC-3′ [for P(−2232)/Luc], 5′-GGTACCAGCTTCCTTCAACCCCAAGT CC-3′ [for P(−1962)/Luc], 5′-GGTACCTTAGTGCCCCCATGAATGA CAG-3′ [P(−1081)] and the antisense primer 5′-CTCGAGAGTTAGA GTTCTCCCGGCATGG-3′. Site-directed mutagenesis of the C/EBPα promoter [P(−1962)/Luc] was carried out using the Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The PCR was performed using 25 ng of P(−1962)/Luc as a template with primer 5′-GCATAAGCGA CGATGGCAGCCTCCTGCTA-3′. After digestion with *Dpn*I, the resulting plasmids containing the mutated P(−1962)/Luc were transformed into *Escherichia coli* XL10-Gold ultracompetent cells (Stratagene). The transformed cells were plated on LB ampicillin (100 μg/ml) agar plates and incubated at 37 °C overnight. The mutated P(−1962)/Luc was confirmed by sequencing analyses.

2.6. Transient transfection and luciferase assay

HepG2 cells grown in six-well plates were transiently transfected with mouse C/EBPα promoters and ATF3 expression plasmid using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. After transfection for 24 h, the cells were lysed in reporter lysis buffer (Promega) and the luciferase activity was measured using a Luciferase Assay System (Promega). To normalize the transfection efficiency, pCMV-β gal was included in each transfection as an internal control plasmid and the luciferase activity was normalized against the β-galactosidase activity. To see the effect of hypoxia on promoter activity of C/EBPα promoter, HepG2 cells were transfected with P(−3595)/Luc reporter and then incubated with $CoCl_2$ (50, 100 μM) or placed in 2% O_2 hypoxic chamber for 1 d.

2.7. Electrophoretic mobility shift assay (EMSA)

3T3-L1 cells were treated with thapsigargin for 24 h. Nuclear extract from the cells was prepared using Nuclear and cytoplasmic extraction reagent (Pierce, Rockford, IL) according to the manufacturer's instructions. A DNA probe for C/EBPα was generated by annealing biotin-labeled sense oligonucleotide (5′-TAAGCGAG-GATGTCAGCCTCCT-3′) and cold antisense oligonucleotide. For competition of probe binding, unlabeled oligonucleotides were added in 100-fold molecular excess relative to the respective

probe. DNA–protein binding reactions were carried out as previously described [14].

2.8. Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was conducted using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's protocol, modified as previously described [14]. 3T3-L1 cells were incubated in the presence of thapsigargin for 24 h or left unstimulated. Immunoprecipitated DNA was amplified by PCR using primers specific for the C/EBPα promoter, sense primer: 5′-TGATGACTGTAGTGTGTTCCAGC-3′ (−1981/−1959), antisense primer: 5′-TCTTTCTGCTGAGTTAAATATAATG-3′ (−1826/−1802).

2.9. Statistical analysis

All experiments were performed at least three times. The results are expressed as the mean ± SE. Statistical analysis was performed using Student's *t*-test. The data were considered statistically significant at $^{*}P < 0.05$.

3. Results

3.1. ATF3 inhibits adipocyte differentiation in 3T3-L1 cells

To determine whether ATF3 regulates adipocyte differentiation, we investigated adipogenesis in lentivirus-mediated ATF3-overexpressing 3T3-L1 cells. To this end, preadipocyte 3T3-L1 cells were infected with lentivirus containing ATF3 cDNA (Lenti-ATF3) or control virus (Lenti-GFP). Two days later, the cells were treated with dexamethasone and insulin for 2 d to induce differentiation. At 8 d after differentiation, we examined adipogenesis by oil red staining. The lentivirus-mediated overexpression of ATF3 was confirmed in 3T3-L1 cells by RT-PCR (Fig. 1A). ATF3-overexpressing cells exhibited less lipid accumulation by Oil red O staining and a markedly lower number of fat cells by light microscopy compared with control cells (Fig. 1A). We also examined PPARγ, aP2 and C/EBPα expression during differentiation using RT-PCR. As shown in Fig. 1A, the induction of adipogenic genes including PPARγ, aP2 and C/EBPα was greatly suppressed in ATF3-overexpressing cells (Fig. 1A). We further verified the inhibitory effect of ATF3 on adipocyte differentiation in stable ATF3-overexpressing 3T3-L1 cells. As shown in Fig. 1B, the oil red staining was dramatically reduced in ATF3-overexpressing cells compared with control cells. Consistent with reduced lipid droplets, the adipogenic genes (PPARγ, aP2 and C/EBPα) were also decreased in ATF3-overexpressing 3T3-L1 cells (Fig. 1B). Together, these results demonstrate that ATF3 inhibits adipocyte differentiation.

3.2. ATF3 downregulates transcription of C/EBPα in 3T3-L1 cells

For detailed analysis of the mechanism involved in ATF3-mediated inhibition of adipocyte differentiation, we investigated the effect of ATF3 on the expression of C/EBPα, one of the adipocyte differentiation master genes, in 3T3-L1 cells. To this end, we first examined the effect of an ATF3-inducer, thapsigargin, on C/EBPα expression in 3T3-L1 cells. As shown in Fig. 2A, treatment with thapsigargin decreased C/EBPα expression, whereas it increased ATF3 expression in the cells. To see the direct effect of ATF3 on C/EBPα expression, we measured C/EBPα expression in ATF3-overexpressing 3T3-L1 cells. To this end, we introduced a lentivirus vector carrying ATF3 into fully differentiated 3T3-L1 cells and analyzed the expression of C/EBPα by RT-PCR. As shown in Fig. 2A, overexpression of ATF3 repressed the expression of C/EBPα. Moreover, we examined the effect of ATF3 on the promoter activity of the C/EBPα gene to see whether repression of C/EBPα occurred at the transcriptional level. We isolated the 3.6-kb promoter of the mouse C/EBPα gene and cloned it into pGL3, designated P(−3595)/Luc. Then, the effect of ATF3 on its promoter activity was examined. Both thapsigargin treatment and ATF3 overexpression inhibited the promoter activity of C/EBPα in a dose dependent manner (Fig. 2B). Taken together, these results indicate that ATF3 inhibits adipocyte differentiation by downregulating C/EBPα expression.

3.3. ATF3 binds to the ATF/CRE site between −1921 and −1914 on the promoter of C/EBPα

Searching for a putative ATF/CRE site within the 3.6-kb promoter revealed several ATF/CRE elements in the 3.6-kb promoter

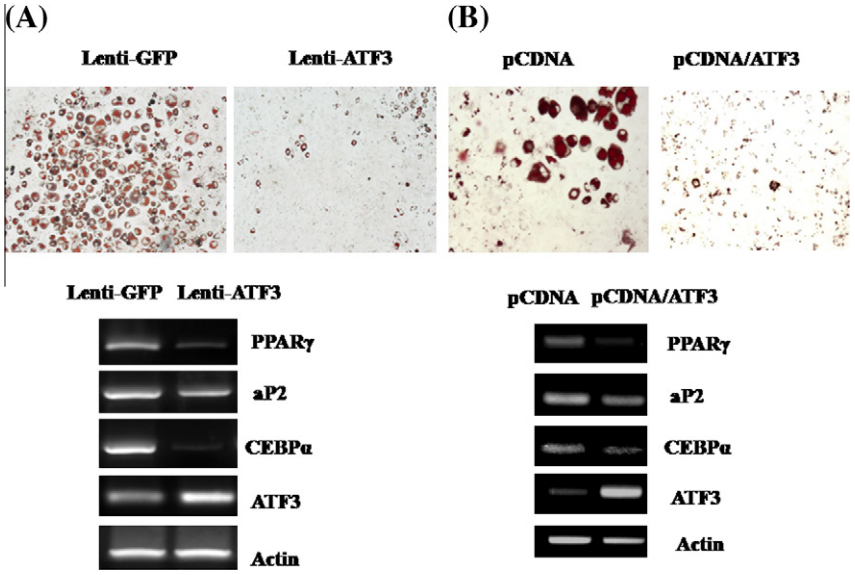

Fig. 1. ATF3 inhibits adipocyte differentiation in 3T3-L1 cells. (A) Preadipocyte 3T3-L1 cells were infected with lentivirus containing ATF3 (Lenti-ATF3), and differentiated in differentiation medium for 8 d. Oil red staining was used to measure adipogenesis. Expression of adipogenic genes (PPARγ, aP2, C/EBPα) was measured by RT-PCR using specific primers. (B) Preadipocyte 3T3-L1 cells stably transfected with ATF3 expression vector (pcDNA3-ATF3) were differentiated as described. Oil red staining was performed for measurement of adipogenesis. Expression of adipogenic genes was measured by RT-PCR using specific primers.

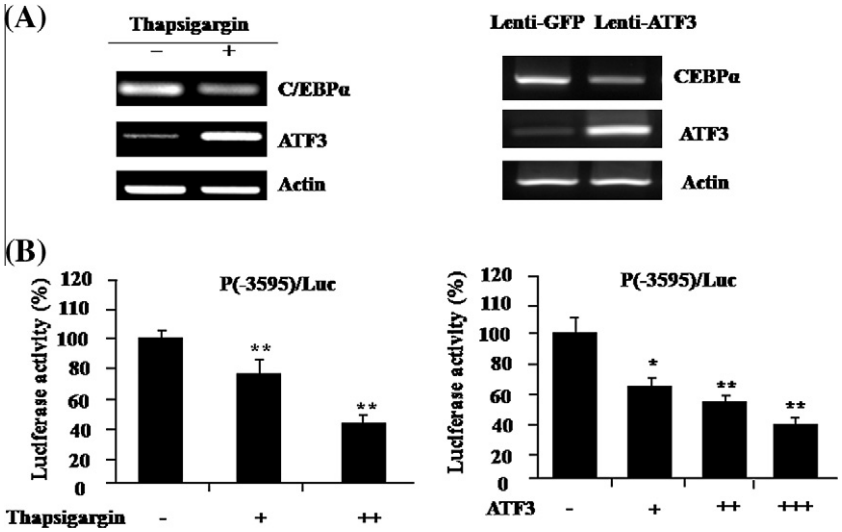

Fig. 2. ATF3 represses expression of C/EBPα. (A) Fully differentiated 3T3-L1 cells were incubated with 1.0 μM thapsigargin for 24 h and subjected to RT-PCR for C/EBPα expression. Fully differentiated 3T3-L1 cells were infected with lentivirus carrying ATF3 at a multiplicity of infection 5 and then incubated for 48 h. Expression of C/EBPα was measured by RT-PCR. (B) HepG2 cells were transfected with reporter containing mouse C/EBPα promoter P(−3567)/Luc and incubated with 0.5 or 1.0 μM thapsigargin for 24 h. HepG2 cells were transfected with P(−3567)/Luc reporter (0.2 μg) and ATF3-expressing vector (0.2, 0.4, 0.6 μg) for 24 h. Then, luciferase activities were measured and presented relative to no thapsigargin treatment or control vector without ATF3. All values represent the mean ± SE from three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.001$).

region of C/EBPα. To assess which DNA binding site is critical for repression of C/EBPα expression, we constructed several 5′-deleted promoters and measured the effects of ATF3 on their promoter activities. As shown in Fig. 3A, deletions between −3122 and −1962 in the promoter retained the ATF3-mediated repressive effect on promoter activity. However, deletions between −1962 and −1081 bp in the promoter abolished the repressive effect by ATF3, suggesting that the ATF3-responsive region is located in the region between −1962 and −1081. Within this region, a candidate ATF/CRE site (5′-GGATGTCA-3′) is present between −1921 and −1914 and could be involved in ATF3-mediated repression of C/EBPα expression. To verify that this element was critical in ATF3-mediated repression, we mutated the ATF3-responsive element (5′-GGATGTCA-3′) to (5′-CGATGGCA-3′) within the context of the P(−1962)/Luc. As shown in Fig. 3B, mutation of this element abolished the inhibitory effect of ATF3, suggesting that this element is critical for ATF3-mediated inhibition of C/EBPα promoter.

Next, to determine whether ATF3 was able to bind this element directly, EMSA was performed with biotin-labeled oligonucleotide probe (−1928/−1907) and nuclear extract from 3T3-L1 cells treated with thapsigargin. As shown in Fig. 3C, the probe produced a binding complex. A 100-fold excess of cold homologous oligonucleotide and ATF/CRE-consensus oligonucleotide competed for the binding, but binding was not abolished by heterogeneous oligonucleotide, suggesting that the binding complex is specific. An antibody supershift experiment was also conducted with ATF3 antibody. Co-incubation of ATF3 antibody produced supershifted bands (Fig. 3C). Furthermore, ChIP analysis was performed to identify binding of ATF3 to chromatin-associated C/EBPα promoter in vivo. The fully differentiated 3T3-L1 cells were treated (or not) with thapsigargin for 6 h and then with formaldehyde to cross-link DNA–protein complexes. After sonication, chromatin fragments were immunoprecipitated with ATF3 antibody, and the immunoprecipitated fragmented DNA was subjected to PCR to amplify C/EBPα promoter DNA (between −1981 and −1802) containing the ATF3 responsive element. As illustrated in Fig. 3 C, ATF3 antibody immunoprecipitated ATF3 binding element-containing promoter fragments from 3T3-L1 cells. Treatment with thapsigargin increased ATF3 binding. These results indicate a specific association of ATF3 with the C/EBPα gene promoter. Taken together, these findings indicate that ATF3 interacts with region from −1928 to −1907 in the C/EBPα promoter.

3.4. ATF3 plays a role in hypoxia-induced inhibition of adipogenesis of 3T3-L1 cells

To date, hypoxia in relation to adipose tissue function has been considered exclusively in terms of the effects of chronic, or continuous, O_2 deprivation [16]. Several studies have demonstrated that hypoxia induced by low O_2 tension or by incubation with $CoCl_2$ inhibits the differentiation of adipocyte cells [17,18]. To assess the role of ATF3 in hypoxia-mediated inhibition of adipogenesis via repression of C/EBPα gene expression, we investigated the expression of ATF3 and C/EBPα in 3T3-L1 cells treated with $CoCl_2$ or placed in 2% O_2 condition. First, we confirmed the hypoxia-mediated inhibition of adipocyte differentiation in 3T3-L1 cells. As shown in Fig. 4A, both $CoCl_2$ treatment and 2% O_2 condition inhibited adipogenesis as confirmed by oil red staining and decreased the expression of adipocyte differentiation genes including PPARγ and C/EBPα. Then, we investigated the effect of hypoxia on the expression of ATF3 and C/EBPα in fully differentiated 3T3-L1 cells. Both $CoCl_2$ treatment and 2% O_2 incubation increased ATF3 mRNA, whereas reduced C/EBPα mRNA (Fig. 4B). Furthermore, we examined the effect of hypoxia on the promoter activity of C/EBPα. $CoCl_2$ treatment repressed activity of the 3.6-kb promoter in a dose-dependent manner, and 2% O_2 incubation also inhibited the promoter activity (Fig. 4C). These results suggest that negative regulation of C/EBPα by ATF3 may play a role in hypoxia-mediated inhibition of adipogenesis.

4. Discussion

Identifying key factors that control adipocyte differentiation and metabolism is vital to understanding adipose biology and pathology. The transcriptional cascade controlling adipogenesis has been well characterized [4,5]. Transcription factors that control adipocyte differentiation act downstream of a complex signaling pathway that integrates signals from the surroundings. ATF3 is a stress-inducible transcription factor that regulates proliferation and apoptosis

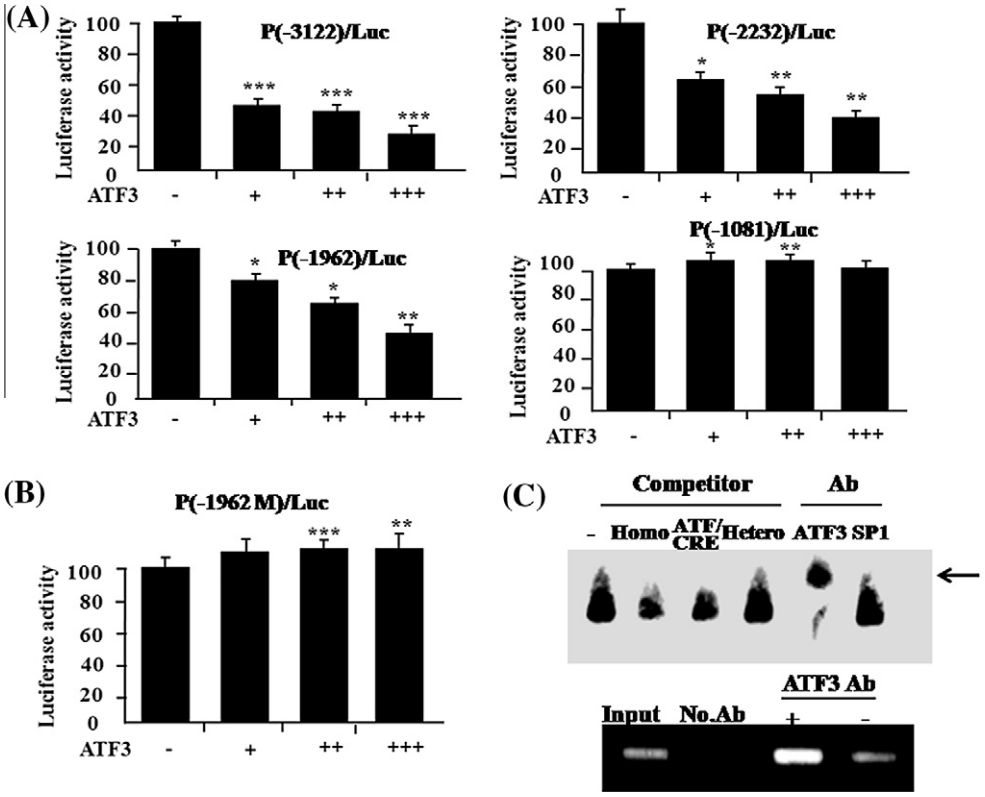

Fig. 3. ATF3 binds to the ATF/CRE element between −1921 and −1914. (A) 5′ serial deletions of mouse C/EBPα (0.2 μg) were transfected into HepG2 cells with ATF3 expression vector (0.2, 0.4, 0.6 μg), and then luciferase activities were measured. Luciferase activities are presented relative to the observable activity from control vector without ATF3. All values represent the mean ± SE from three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.001$, $^{***}P < 0.0001$). (B) The mutant reporter (0.2 μg) was transfected into HepG2 cells with ATF3 expression vector (0.2, 0.4, 0.6 μg) and then luciferase activity was measured. Luciferase activities are presented relative to the observable activity from mutant reporter without ATF3. All values represent the mean ± SE from three independent experiments ($^{**}P < 0.001$, $^{***}P < 0.0001$). (C) EMSA was performed on the ATF/CRE site (−1928 and −1907) using nuclear extract from 3T3-L1 cells. For the oligonucleotide competition experiments, a 100-fold excess of oligonucleotides was used (lane 1: no competition; lane 2: ATF/CRE oligonucleotides of C/EBPα; lane 3: consense ATF/CRE oligonucleotides; lane 4: SP1 oligonucleotides. The protein–DNA complex was supershifted by ATF antibody (lane 5), but not by SP1 antibody (lane6). Fully differentiated 3T3-L1 cells were incubated with 1 μM thapsigargin for 6 h, and ChIP was performed with ATF3 antibody. DNA that was immunoprecipitated with anti-ATF3 was amplified by PCR using primers specific for C/EBPα promoter.

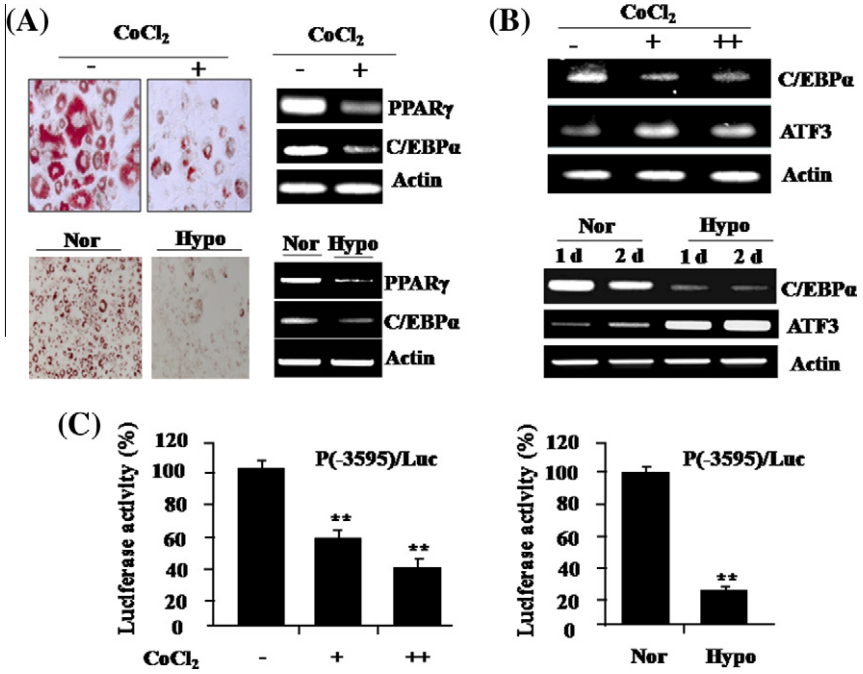

Fig. 4. ATF3 is associated with inhibition of adipocyte differentiation induced by hypoxia. (A) Preadipocytes 3T3-L1 cells were differentiated with differentiation medium for 2 d, and incubated with insulin in presence of $CoCl_2$ (100 μM) for 5 d or placed with insulin in 2% O_2 hypoxic condition for 5 d. (B) Fully differentiated 3T3-L1 cells were incubated with $CoCl_2$ (50, 100 μM) or placed in 2% O_2 hypoxic condition for 1, 2 d. (C) HepG2 cells were transfected with reporter containing C/EBPα promoter [P(−3567)/Luc] and incubated with $CoCl_2$ (50, 100 μM) for 24 h or placed in 2% O_2 hypoxic chamber for 1 d. Luciferase activities were presented relative to no $CoCl_2$ treatment or normoxia (21% O_2). All values represent the mean ± SE from three independent experiments ($^{**}P < 0.001$). Nor; normoxia, Hypo: hypoxia.

[11,12]. In our previous studies, we demonstrated that ATF3 represses expression of adiponectin and its receptors in 3T3-L1 cells, resulting in attenuation of adiponectin signaling in obesity or diabetic conditions, leading to insulin resistance [13–15]. However, the role of ATF3 in adipocytes is not well characterized. By the results of ChIP-on-chip assay for genome-wide screen of ATF3 target genes in differentiated 3T3-L1 cells, the ATF3-target genes revealed enrichment of adipocyte differentiation genes, suggesting that ATF3 could regulate adipocyte differentiation. Therefore, in this study, we investigated the role of ATF3 in adipocyte differentiation and the mechanism involved in this event.

Overexpression of ATF3 with lentivirus or stable transfection in preadipocyte 3T3-L1 cells inhibits the induction of proadipogenic genes and blocks adipogenesis, demonstrating that ATF3 inhibits adipocyte differentiation in 3T3-L1 cells. C/EBPα has been shown to play a critical role in certain aspects of adipogenesis [4]. C/EBPα is induced in direct response to the activation of PPARγ and its presence appears to be critical for differentiation of preadipocytes into mature fat cells. Specifically, the activation of PPARγ in fibroblasts lacking C/EBPα gives rise to immature adipocytes [4]. Therefore, in the current study, we investigated the effect of ATF3 on C/EBPα expression in 3T3-L1 cells to determine whether ATF3 mediates an anti-adipogenic effect by repressing C/EBPα expression. ATF3 induction using thapsigargin and overexpression of ATF3 repressed C/EBPα expression in fully differentiated 3T3-L1 cells. To investigate whether the downregulation of C/EBPα is mediated at the transcriptional level, we examined the effect of ATF3 on the promoter activity of the mouse C/EBPα gene. Both thapsigargin treatment and ATF3 expression efficiently repressed the activity of the promoter from −3569 to +100, demonstrating that ATF3 downregulates C/EBPα expression at the transcriptional level. These results indicate that ATF3 inhibits adipocyte differentiation by repressing transcriptional expression of C/EBPα.

Then, we identified the ATF3 responsive element in the C/EBPα promoter. ATF3 expression efficiently repressed the activities of promoters from −3569 to −1965. However, the repression was not observed in the −1081 promoter, suggesting that the putative ATF3 binding site is located between −1965 and −1081. A computer-assisted search of this 885-bp region using TFSEARCH revealed a candidate ATF/CRE site, GGATGTCA (−1921 and −1914) (Fig. 3B). EMSA and ChIP showed that ATF3 directly interacted with the C/EBPα promoter between −1921 and −1914. Furthermore, mutation of the ATF/CRE site abolished the repressive effect of ATF3 on promoter activity, indicating that the responsive element between −1921 and −1914 is critical for ATF3-mediated repression of C/EBPα. Consequently, decreased C/EBPα expression by ATF3 would have a significant impact on adipocyte differentiation.

A striking finding was that the number of preadipocytes capable of undergoing differentiation was reduced in obesity and negatively correlated with both the body mass index and the adipose cell size of the donor [16]. This finding suggests that excess lipids cannot be stored adequately in the subcutaneous adipose tissue in hypertrophic obesity and are consequently directed to other sites including the liver and skeletal muscle. Ectopic fat accumulation, a consequence of an inability to store excess lipids in the subcutaneous adipose tissue, clearly promotes insulin resistance in the liver and skeletal muscle. Therefore, it is important to clarify the mechanism for reduced adipocyte differentiation in the subcutaneous adipose tissue in hypertrophic obesity. We showed previously that ATF3 is increased in adipose tissue of hypertrophic obesity [13] and have demonstrated the anti-adipogenic effect of ATF3 in this study, suggesting that ATF3 may play a role in impaired adipocyte differentiation in the adipose tissue in hypertrophic obesity and in the promotion of insulin resistance.

A recent report showed that hypoxia inhibits adipogenesis by repressing PPARγ [16]. Expression of PPARγ and C/EBPβ is repressed by HIF-1α through induction of the basic helix-loop-helix transcription factor DEC1/Stra13, which represses PPARγ promoter activation and functions as an effector of hypoxia-mediated inhibition of adipogenesis [16]. Also, it has been reported that hypoxia prevents the nuclear translocation of GSK3β and the DNA binding capability of C/EBP/β, blocking the G1/S transition through HIF-1α-dependent induction of p27kip1 and an HIF-1a/p27-independent mechanism [17]. Our results that ATF3 induced by both $CoCl_2$ treatment and 2% O_2 incubation represses C/EBPα and inhibits adipogenesis of 3T3-L1 cells, suggest that ATF3 may participate in hypoxia-mediated adipogenic inhibition through downregulation of C/EBPα in adipocyte tissue. Therefore, our study proposes a HIF-independent mechanism in hypoxia-mediated inhibition of adipogenesis.

In conclusion, the stress-responsive transcription factor ATF3 is a negative regulator of adipogenesis in 3T3-L1 cells, acting to repress the transcription of a critical adipogenic transcription factor, C/EBPα.

Acknowledgment

This research was supported by a grant of the Korean Health Technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A101114).

References

[1] E.D. Rosen, B.M. Spiegelman, Adipocytes as regulators of energy balance and glucose homeostasis, Nature 444 (2006) 847–853.
[2] M.I. Lefterova, M.A. Lazar, New developments in adipogenesis, Trends Endocrinol. Metab. 20 (2009) 107–114.
[3] T.C. Otto, M.D. Lane, Adipose development: from stem cell to adipocyte, Crit. Rev. Biochem. Mol. Biol. 40 (2005) 229–242.
[4] S.R. Farmer, Transcriptional control of adipocyte formation, Cell Metab. 4 (2006) 263–273.
[5] E.D. Rosen, O.A. MacDougald, Adipocyte differentiation from the inside out, Nat. Rev. Mol. Cell Biol. 7 (2006) 263–273.
[6] T.C. Prestwich, O.A. Macdougald, Wnt/beta-catenin signaling in adipogenesis and metabolism, Curr. Opin. Cell Biol. 19 (2007) 612–617.
[7] C.W. Brey, M.P. Nelder, T. Hailemariam, R. Gaugler, S. Hashmi, Kruppel-like family of transcription factors: an emerging new frontier in fat biology, Int. J. Biol. Sci. 5 (2009) 622–636.
[8] K. Birsoy, Z. Chen, J. Friedman, Transcriptional regulation of adipogenesis by KLF4, Cell Metab. 7 (2008) 339–347.
[9] D. Li, S. Yea, S. Li, Z. Chen, G. Narla, M. Banck, J. Laborda, S. Tan, J.M. Friedman, S.L. Friedman, M.J. Walsh, Kruppel-like factor-6 promotes preadipocyte differentiation through histone deacetylase 3-dependent repression of DLK1, J. Biol. Chem. 280 (2005) 26941–26952.
[10] J. Wu, S.V. Srinivasan, J.C. Neumann, J.B. Lingrel, The KLF2 transcription factor does not affect the formation of preadipocytes but inhibits their differentiation into adipocytes, Biochemistry 44 (2005) 11098–11105.
[11] T. Hai, M.G. Hartman, The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis, Gene 273 (2001) 1–11.
[12] T. Hai, C.C. Wolford, Y.S. Chang, ATF3, a hub of the cellular adaptive-response network, in the pathogenesis of diseases: is modulation of inflammation a unifying component, Gene Expr. 15 (2010) 1–11.
[13] H.B. Kim, M. Kong, T.M. Kim, Y.H. Suh, W.H. Kim, J.H. Lim, J.H. Song, M.H. Jung, NFATc4 and ATF3 negatively regulate adiponectin gene expression in 3T3-L1 adipocytes, Diabetes 55 (2006) 1342–1352.
[14] H.J. Park, Y.M. Kang, C.H. Kim, M.H. Jung, ATF3 negatively regulates adiponectin receptor 1 expression, Biochem. Biophys. Res. Commun. 400 (2010) 72–77.
[15] I.U. Koh, J.H. Lim, M.K. Joe, W.H. Kim, M.H. Jung, J.B. Yoon, J. Song, AdipoR2 is transcriptionally regulated by ER stress-inducible ATF3 in HepG2 human hepatocyte cells, FEBS J. 277 (2010) 2304–2317.
[16] I.S. Wood, F.P. de Heredia, B. Wang, P. Trayhurn, Cellular hypoxia and adipose tissue dysfunction in obesity, Proc. Nutr. Soc. 68 (2009) 370–377.
[17] Z. Yun, H.L. Maecker, R.S. Johnson, A.J. Giaccia, Inhibition of PPAR gamma2 gene expression by the HIF-1-regulated gene DEC1/Stra13: a mechanism for regulation of adipogenesis by hypoxia, Dev. Cell 2 (2002) 331–341.
[18] Y.K. Park, H. Park, Prevention of CCAAT/enhancer-binding protein beta DNA binding by hypoxia during adipogenesis, J. Biol. Chem. 285 (2010) 3289–3299.