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ATF3 plays a role in adipocyte hypoxia-mediated mitochondria dysfunction in obesity

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ABSTRACT

Obesity-associated adipose tissue hypoxia plays a pivotal role in insulin resistance via impaired adipocyte dysfunction including mitochondria dysfunction. In this study, we investigated the involvement of hypoxia-inducible ATF3 in adipocyte hypoxia-mediated mitochondrial dysfunction. While HIF-1α and ATF3 were increased in white adipose tissue of high fat diet (HFD) obese mice compared with control lean mice, mitochondria-related genes were significantly reduced. Treatment with hypoxia mimetics $CoCl_2$ or incubation with 2% O_2 impaired mitochondria function as demonstrated by decreases in ATP production, NADH dehydrogenase activity, mitochondrial membrane potential, and reduced expression of mitochondria-related genes including NRF-1, PGC-1α, COX1 and SOD in 3T3-L1 adipocyte cells. Furthermore, overexpression of ATF3 in 3T3-L1 cells also decreased mitochondria function as well as expression of mitochondria-related genes. ATF3 knockdown in 3T3-L1 cells partly prevented the hypoxia-mediated decrease in mitochondria function and expression of mitochondria-related genes. The mitochondria-related genes were decreased in white adipose tissue of ATF3-overexpressing mice compared with wild-type mice. These results suggest that ATF3 may play a role in adipocyte hypoxia-mediated mitochondrial dysfunction in obesity.

1. Introduction

Obesity is a common disorder that predisposes individuals to type 2 diabetes, atherosclerosis, hypertension, and hyperlipidemia. The expansion of white adipose tissue during obesity development is caused by an increase in adipocyte size and total number of adipocytes due to preadipocyte differentiation [1]. Adipocyte size increases up to 140–180 μm in diameter during the development of obesity. However, the capacity for adipocyte hypertrophy is limited. The enlarged adipocytes endure less than adequate oxygen supply, since the diffusion distance for oxygen is utmost 100 μm. In situations where oxygen availability does not meet the demand of the surrounding tissue, hypoxia occurs. Adipocyte hypoxia was first proposed as a possible cause of inflammation in obesity in 2004 [2]. It has recently been demonstrated that white adipose tissue of obese mice is hypoxic, revealed both by pimonidazole staining and a markedly increased lactate concentration in adipose tissue [3]. These observations were also confirmed by measurements of hypoxia-inducible gene expression, which was elevated in obese animals [3].

Adipocyte hypoxia provokes adipocyte dysfunction, which plays a crucial role in the pathogenesis of obesity-related insulin resistance and type 2 diabetes [4]. Adipocyte hypoxia modulates the production of several inflammation-related adipokines, increasing interleukin 6 (IL-6), leptin and macrophage migratory inhibition factor production. However, it reduces adiponectin synthesis [5,6]. Furthermore, adipocyte hypoxia also inhibits the differentiation of preadipocytes [7] and impairs mitochondria dysfunction [8,9]. Increased glucose transport into adipocytes is observed with low O_2 incubation due to the up-regulation of GLUT-1 expression [10]. These findings suggest that cellular hypoxia may be a key factor in adipocyte physiology and the underlying cause of adipose tissue dysfunction contributing to insulin resistance associated with obesity.

Recently, the role of mitochondria in insulin sensitivity of adipocytes has been demonstrated [11,12]. Mitochondrial dysfunction caused by chemical treatment or genetic manipulation can lead to insulin resistance and decreased glucose utilization of adipocytes [12]. Mitochondrial dysfunction decreases the expression of adiponectin in adipocytes by activation of the JNK pathway [13]. The enhancement of mitochondrial biogenesis by overexpression of NRF-1 increased the expression of adiponectin in adipocytes [13]. These findings indicate that mitochondrial dysfunction not only

Abbreviations: ATF3, activating transcription factor 3; COX1, cytochrome C oxidase 1; HIF-1α, hypoxia inducible factor-1; HFD, high fat diet; MTT, 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide; NRF-1, nuclear respiratory factor 1; SOD, superoxide dismutase; TFAM, mitochondrial transcription factor 1; TMRE, tetramethylrhodamine ethylester.

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causes insulin resistance of adipocyte but also impairs secretion of adipokines, which compromises other tissues in terms of glucose utilization. Hypoxia inducible factor 1α (HIF-1α) is a major mediator of the hypoxia signal in the inhibition of mitochondrial function [8,9]. However, the HIF-1α-independent mediator in hypoxia-mediated mitochondria dysfunction is not characterized yet. Moreover, understanding hypoxic events in adipose tissue might be helpful to better understand the pathophysiology of obesity and to target involved pathways for the treatment of obesity-related diseases.

Activating transcription factor 3 (ATF3) is a stress-inducible gene that encodes a member of the ATF/CREB family of transcription factors [14]. ATF3 is induced by signals such as hypoxia, proinflammatory cytokines, nitric oxide, high concentrations of glucose, palmitate, and ER stress. ATF3 regulates proliferation or apoptosis under stress conditions by down or upregulation of related genes [15]. We previously reported that ATF3 is increased in white adipose tissue of obese mice and negatively regulates adiponectin gene expression [16]. Furthermore, we demonstrated that ATF3 represses the expression of adiponectin receptors in adipocyte cells and liver cells [17,18]. Very recently, we also reported that ATF3 inhibits differentiation of preadipocyte 3T3-L1 cells [19].

Since ATF3 is a hypoxia-inducible transcription factor and contributes to repression of adiponectin expression and inhibition of adipocyte differentiation, which are adipocyte dysfunctions induced by adipocyte hypoxia in obesity, we studied the role of ATF3 in adipocyte hypoxia-mediated mitochondria dysfunction in 3T3-L1 cells. ATF3 overexpression in 3T3-L1 cells decreased indicators of mitochondria function including ATP production, NADH dehydrogenase activity, mitochondrial membrane potential, and reduced expression of mitochondria-related genes. ATF3 knockdown in 3T3-L1 cells partially blocked hypoxia-mediated decrease in mitochondria function and expression of mitochondria-related genes, suggesting that ATF3 may be involve in adipocyte hypoxia-mediated mitochondria dysfunction.

2. Materials and methods

2.1. Cell culture, differentiation, and treatments

3T3-L1 pre-adipocyte cells, which were purchased from American Type Culture Collection (ATCC, CL-173TM; Manassas, VA), were maintained with Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT) containing 10% fetal calf serum (FCS; HyClone). The cells were differentiated for 2 days with M1 [DMEM containing 10% fetal bovine serum (FBS; HyClone), 5 μM insulin, 0.5 mM 3-isobutylmethylxanthine (IBMX), and 1 μM dexamethasone (DEX)] and for 8 days with M2 (DMEM containing 10% FBS and 5 μM insulin). To see hypoxic effects on mitochondria function and expression of mitochondria-related genes, differentiated 3T3-L1 cells were treated with $CoCl_2$ (50, 100, and 200 μM) or incubated in 2% O_2 hypoxic chamber (2% O_2, 5% CO_2) (Astec, Tokyo, Japan) for 24 h. Antibodies against ATF3 and HIF-1α were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Insulin, IBMX, DEX and $CoCl_2$ were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Animals

C57BL/6 mice (male, 4 weeks old) were purchased from Jung-Ang Lab. Animal, Inc. (Seoul, South Korea) and were fed normal chow diet or high-fat (HF) diet for 60 days. The HF diet contained 30% lard compared with the AIN93M-based control diet. Adipocyte-specific ATF3 transgenic mice were produced using aP2 promoter. Overexpression of ATF3 was confirmed by RT-PCR. All animal experiments were approved by the Pusan National University Animal Experiment Ethics Committee and were conducted in accordance with the institutional guidelines for care and use of laboratory animals.

2.3. MTT assay

Mitochondrial dehydrogenase activity in 3T3-L1 cells was detected using the MTT assay kit (MTT, Sigma, CA) in a 96-well plate. The culture medium was replaced by 100 μl of medium containing 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) solution, (5 mg/ml MTT in RPMI-1640 without phenol red) and cells were incubated for 4 h at 37 °C. The blue-colored tetrazolium crystals resulting from mitochondrial enzymatic activity on the MTT substrate were solubilized with 100 μl of 0.1 N HCl in anhydrous isopropanol. The absorbance was read at 540 nm on an ELISA microplate reader (MDS Analytical Technologies, Sunnyvale, CA).

2.4. Assay for mitochondrial membrane potential

Mitochondrial membrane potential was measured using tetramethylrhodamine ethylester (TMRE) kit (Abcam, Cambridge, MA). The 3T3-L1 cells in 96-well culture plates were incubated with 50 nM TMRE for 20 min at 37 °C in PBS containing 0.2% BSA. After the cells were washed, the TMRE fluorescence was measured with a microplate reader (Tecan, Salzburg, Austria). The excitation and emission wavelengths were set at 485 nm and 535 nm, respectively.

2.5. Assay for ATP production

Luciferase-based intracellular ATP contents were measured using the ATP determination kit (Molecular Probes, Eugene, OR) according to the manufacturer's recommendations. Briefly, 10 μl of cell lysates were mixed with 90 μl of the luciferin–luciferase reaction solution (1.25 μg/mL of firefly luciferase, 50 μM D-luciferin and 1 mM DTT in 1× Reaction Buffer). After a 15-min incubation, luminescence was measured using a Victor Light luminometer (Perkin–Elmer, Waltham, MA).

2.6. Plasmid and transfection

ATF3 expression vector (pcDNA3-ATF3) was previously described [16], and NRF-1 expression vector (pcDNA3.1-NRF-1) was a gift from Dr. Youngmi Kim Pak (College of Medicine, Kyung Hee University, South Korea). To see the effect of ATF3 on NRF-1 promoter activity, 2.5 Kb of mouse NRF-1 promoter (−2527/+17) was isolated with PCR and cloned into pGL3 (Promega, Madison, WI). Transfection of differentiated 3T3-L1 adipocytes with plasmid DNA (pCDNA, pCDNA3-ATF3, and pcDNA3.1-NRF-1) or siRNAs (ATF3siRNA or scramble) was carried out with a microporator (Digital Biotechnology, Suwon, South Korea) according to the protocol provided by the company. Briefly, differentiated 3T3-L1 were resuspended in resuspension buffer and incubated with the specified amount of plasmid DNA or siRNA. Microporation was performed using the following program: 1400 V (voltage), 30 ms (width), 1 pulse. After transfection, cells were maintained in growth medium without antibiotics for 24 h at 37 °C in a 5% CO_2 humidified atmosphere, after which the medium was replaced with complete medium. For hypoxic incubation, the transfected cells were incubated in a 2% O_2 hypoxic chamber for 24 h.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs from 3T3-L1cells or adipose tissue were extracted with 1 ml of Trizol™ (Invitrogen, Carlsbad, CA). The cDNAs were generated from 1 μg of total RNAs using Moloney Murine