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Research Paper

WNK4 is an adipogenic factor and its deletion reduces diet-induced obesity in mice

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ABSTRACT

The with-no-lysine kinase (WNK) 4 gene is a causative gene in pseudohypoaldosteronism type II. Although WNKs are widely expressed in the body, neither their metabolic functions nor their extrarenal role is clear. In this study, we found that *WNK4* was expressed in mouse adipose tissue and 3T3-L1 adipocytes. In mouse primary preadipocytes and in 3T3-L1 adipocytes, *WNK4* was markedly induced in the early phase of adipocyte differentiation. *WNK4* expression preceded the expression of key transcriptional factors PPARγ and C/EBPα. WNK4-siRNA-transfected 3T3-L1 cells and human mesenchymal stem cells showed reduced expression of PPARγ and C/EBPα and lipid accumulation. WNK4 protein affected the DNA-binding ability of C/EBPβ and thereby reduced PPARγ expression. In the WNK4$^{-/-}$ mice, PPARγ and C/EBPα expression were decreased in adipose tissues, and the mice exhibited partial resistance to high-fat diet-induced adiposity. These data suggest that *WNK4* may be a proadipogenic factor, and offer insights into the relationship between WNKs and energy metabolism.

1. Introduction

Metabolic syndrome (MetS), characterized by insulin resistance, dyslipidemia, hypertension, and central obesity, is a significant clinical problem associated with cardiovascular disease and diabetes (Alberti et al., 2009). Because white adipose tissue (WAT) is the main energy storage organ and secretes several humoral factors (Cristancho and Lazar, 2011), it has important roles in MetS. Core transcriptional factors regulating adipocyte differentiation include the master regulators, peroxisome proliferator-activated receptor gamma (PPARγ), and CCAAT/enhancer-binding protein alpha (C/EBPα) (Lefterova et al., 2008), and moderate reduction of PPARγ activity was shown to prevent obesity and improve insulin sensitivity (Kubota et al., 1999; Yamauchi et al., 2001; Jones et al., 2005).

The with-no-lysine kinases (WNKs) are a family of serine/threonine kinases composed of four human genes, *WNK1*, *WNK2*, *WNK3*, and *WNK4*. *WNK1* and *WNK4* were identified as being responsible for pseudohypoaldosteronism type II (PHAII; (Wilson et al., 2001), a hereditary hypertensive disease characterized by hyperkalemia, metabolic acidosis, and thiazide sensitivity (Gordon, 1986). We and others have clarified that WNK regulates the Na—Cl cotransporter (NCC) in the distal convoluted tubules of the kidney through phosphorylation of oxidative stress-responsive 1 (OSR1) and Ste20-like proline/alanine-rich kinase (SPAK) (Yang et al., 2007; Chiga et al., 2011). Recent studies have added further information that insulin phosphorylates WNK1 through protein kinase B (PKB)/Akt (Jiang et al., 2005), and that insulin is a powerful activator of the WNK4–OSR1/SPAK–NCC signaling cascade *in vitro* and *in vivo* (Sohara et al., 2011; Nishida et al., 2012; Takahashi et al., 2014). These data suggest that activation of the WNK–OSR1/SPAK–NCC signaling cascade caused by hyperinsulinemia may underlie the pathogenesis of salt-sensitive hypertension in MetS.

In extrarenal organs, recent studies have suggested that WNKs regulate cell growth, differentiation, and development. For example, WNK1 is required for mitosis in cultured cells (Tu et al., 2011). In addition, WNK2 is known to suppress cell growth (Hong et al., 2007), while WNK3 increases cell survival (Veríssimo et al., 2006), and WNK4 is required for the anterior formation of *Xenopus* embryos (Shimizu et al., 2013). However, the functions of WNKs in energy metabolism remain unknown.

In the present study, we reported a role of WNK4 as a regulator of adipocyte development. We found that WNK4 was expressed in the mouse adipose tissue. In primary preadipocytes and 3T3-L1 adipocytes, WNK4 was increased dramatically in the early phase of differentiation. The suppression of endogenous WNK4 decreased expression of PPARγ and C/EBPα, resulting in the impaired formation of mature adipocytes in 3T3-L1 cells and human mesenchymal stem cells. We also found that WNK4 was involved in cell cycle progression during mitotic clonal

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expansion (MCE), which might be the cause of decreased expression of PPARγ by C/EBPβ by WNK4 suppression. Consistent with the reduced expression of PPARγ, adult WNK4$^{-/-}$ (WNK4-KO) mice exhibited reduced adiposity and the decreased expression of adipogenic genes on high-fat diets (HFDs), suggesting the involvement of WNK4 in the development of obesity. These results suggested that the hypertension-causing gene *WNK4* not only is involved in the regulation of salt-sensitive hypertension, but also in energy metabolism.

2. Materials and Methods

2.1. Animals

The generation of the WNK4-KO mice, WNK4$^{D561A/+}$ knock-in mice, and WNK4 transgenic (WNK4-Tg) mice and their genotyping strategies were described previously (Takahashi et al., 2014; Wakabayashi et al., 2013; Yang et al., 2007). Studies were performed on each strain using littermates. The mice were raised under a 12-h day and night cycle, and were fed a normal rodent diet [6% kcal% fat] (Oriental Yeast, Tokyo, Japan) or high-fat diet [60% kcal% fat] (Research Diets, New Brunswick, Canada) and plain drinking water. This experiment was approved by the Animal Care and Use Committee of the Tokyo Medical and Dental University, Tokyo, Japan.

2.2. Cell Culture

3T3-L1 cells (ATCC; CL-173) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 U per ml penicillin, and 0.1 mg/ml streptomycin at 37 °C in a humidified 5% CO_2 incubator. The cells were induced to differentiate with Adipoinducer Reagent (Takara, Shiga, Japan) containing 10 μg/ml insulin, 2.5 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxantine (IBMX) (MDI). After 48 h of induction, 3T3-L1 cells were maintained in the medium containing DMEM supplemented with 10% FBS, penicillin/streptomycin, and 10 μg/ml insulin. For bumetanide stimulation experiments, the 3T3-L1 cells were exposed to MDI supplemented with 10 μM bumetanide/DMSO or DMSO as a negative control. The mpkDCT cells were maintained as described previously (Sohara et al., 2011). The human mesenchymal stem cells from adipose tissue (hMSC-AT) cells were purchased from PromoCell GmbH (Heidelberg, Germany). The hMSC-AT cells were cultured in Mesenchymal Stem Cell Growth Medium 2 (PromoCell), and were induced to differentiate with Mesenchymal Stem Cell Adipogenic Differentiation Medium 2 (PromoCell), according to the manufacturer's instructions. The preparation of stromal vascular fraction (SVF) from mice adipose tissue, culture of SVF cells, and adipogenic induction of SVF cells were performed as described previously (Suganami et al., 2005; Tanaka et al., 2014).

2.3. Transfection

3T3-L1 cells (3×10^5 cells/6-cm dish or 1×10^5 cells/3.5-cm dish) were transfected with the indicated amount of plasmid DNA or siRNA with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, Canada). For the knockdown experiments in 3T3-L1 cells, we used 20 nM cocktails of three duplexes of siRNAs. The oligonucleotide sequences of mouse si-WNK1, si-WNK4, si-SPAK and si-Nega were described previously (Sohara et al., 2011; Zeniya et al., 2013). The mouse si-OSR1 and si-C/EBPβ was obtained from Origene (MD, USA) and Santa Cruz Biotechnology (TX, USA), respectively. The hMSC-AT cells were transfected with the indicated amount of siRNA with Xfect RNA Transfection Reagent (Takara), as the manufacturer's instructions. For the knockdown experiments in hMSC-AT cells, we used 50 nM cocktails of two duplexes of siRNAs. The oligonucleotide sequences of human si-WNK4 were as follows: human si-WNK4-A ggaggacgacggcgagaagTT, human si-WNK4-B ugacagagugguugagugcTT. The differentiation of 3T3-L1 cells and hMSC-AT cells to adipocytes was induced 3 days after transfection.

2.4. Plasmids

Mouse WNK4 cDNA was isolated by PCR using mouse WNK4 vector (Ohno et al., 2011) and cloned into 3XFLAG-CMV10 vector (Sigma-Aldrich, MO, USA). Mouse C/EBPβ cDNA was isolated by PCR using mouse adipose tissue and cloned into T7-pRK5 vector. Sequences of the amplification primers employed were as follows: WNK4 sense, 5′-CTTGCGGCCGCGATGCTAGCACCTCGAAATAC-3′ and WNK4 antisense, 5′-ACCGATATCTCACATCCTGCCAATATC-3′; C/EBPβ sense, 5′-AGCAAATGGGTCGCGAGATGGAAGTGGCCAACTTCTACTACG-3′ and C/EBPβ antisense, 5′-CCAAGCTTCTGCAGGCTAGCAGTGGCCCGCCGA-3′. The mouse WNK4 cDNA was cloned into 3xFLAG-CMV10 vector (Sigma-Aldrich, MO, USA). The kinase-dead mutation (D318A) was introduced using a QuickChange Site-directed Mutagenesis kit (Stratagene, La Jolla, Canada) with the following primers: D318A sense, 5′-CTGTCAAAATCGGAGCCCTCGGACTGGCCA-3′, and D318A antisense, 5′-TGGCCAGTCCGAGGGCTCCGATTTTGACAG-3′ (Mutagenesis sites are underlined).

2.5. RT-PCR

Total RNA from the mouse adipose tissue or cultured cells was extracted using TRIzol reagent (Invitrogen). The total RNA was reverse-transcribed using Omniscript reverse transcriptase (Qiagen, Hilden, Germany). Sequences of RT-PCR primers employed were as follows: mouse C/EBPβ sense, 5′-GTTTCGGGAGTTGATGCAATC-3′, C/EBPβ antisense, 5′-AACAACCCCGCAGGAACAT-3′; mouse CUL3 sense, 5′-GGATGAGTTCAG GCAACATC-3′, mouse CUL3 antisense, 5′-GCATGTCTTGGTGCTGGTGG-3′; human perilipin sense, 5′- ACCCCCCTGAAAAGATTGCTT-3′, antisense, 5′-GATGGGAACGCTGATGCTGTT-3′; human PPARγ2 sense, 5′-GCAGGAGA TCTACAAGGACTTG-3′, antisense, 5′- CCCTCAGAATAGTGCAACTGG-3′; human β-actin sense, 5′-CCGTCTTCCCCTCCATCG-3′; antisense, 5′-GTCCCAGTTGGTGACGATGC-3′; the mouse C/EBPα, mouse PPARγ, mouse β-actin, mouse GAPDH, mouse FAS, mouse LPL, mouse FABP4, mouse WNK2, mouse WNK3, mouse WNK4, mouse KLHL2, mouse KLHL3, mouse F4/80, and mouse adiponectin primers were described previously (Zeniya et al., 2013; Madsen et al., 2003; O'Reilly et al., 2006; Dutruel et al., 2014; Suganami et al., 2005). Relative mRNA levels were normalized by endogenous reference genes (β-actin and GAPDH).

2.6. Indirect Calorimetry

Male mice at 9 weeks-old on a normal diet were monitored individually in a metabolism measurement system (Muromachi Kikai, Tokyo, Japan) for 48 h. Each cage was monitored for oxygen consumption and activity at 5 min intervals throughout the period. Total oxygen consumption was calculated as accumulation of oxygen uptake for each mouse divided by its body weight.

2.7. Immunoblotting

Extraction of protein samples and semiquantitative immunoblotting were performed as previously described (Yang et al., 2007). For immunoblotting, we used entire samples without the nuclear fraction ($600 \times g$) or the crude membrane fraction ($17{,}000 \times g$). To separate nuclear extracts of the cells, Nuclear and Cytoplasmic Extraction Reagents (PIERCE, AZ, USA) were used, following the manufacturer's instructions. The relative intensities of immunoblot bands were analyzed and quantified using ImageJ software (National Institutes of Health (NIH), MD, USA). Rabbit anti-FLAG (Sigma-Aldrich), rabbit anti-WNK1 (A301-516A; BETHYL), sheep anti-WNK3 (S346C), rabbit anti-mouse WNK4, rabbit anti-phosphorylated SPAK, rabbit anti-SPAK, rabbit anti-phosphorylated OSR1/SPAK, mouse anti-OSR1, anti-NKCC1, anti-

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