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TNFα-induced miR-130 resulted in adipocyte dysfunction during obesity-related inflammation

Chongtae Kim [a,1], Heejin Lee [a,1], Yoon Mi Cho [a], Oh-Joo Kwon [a], Wook Kim [b], Eun Kyung Lee [a,*]

[a] Department of Biochemistry, College of Medicine, Catholic University of Korea, Seoul 137-701, South Korea
[b] Department of Molecular Science and Technology, Ajou University, Suwon 443-749, South Korea

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ABSTRACT

Adipocytes are continuously stimulated by proinflammatory cytokines such as TNFα, which cause adipocyte dysfunction by facilitating the inflammatory response. Although miR-130 was reported to be an important regulator of adipogenesis by targeting *PPARγ* mRNA, little is known about the mechanisms regulating miR-130 expression during the proinflammatory response. Here, we examined miR-130 levels in white adipose tissue (WAT) from high-fat diet (HFD) mice and TNFα-stimulated adipocytes. Primary transcripts of miR-130 were increased after TNFα stimulation, indicating that induction of miR-130 during the pro-inflammatory response is regulated by a transcriptional event. A chromatin immunoprecipitation assay showed that p65 binding to the promoter regions of miR-130 was enhanced after TNFα treatment. Taken together, our findings suggest that induction of miR-130 by TNFα is responsible for adipocyte dysfunction.

1. Introduction

Inflammation is a key characteristic of metabolic syndromes such as obesity and type II diabetes. Adipocytes from obese subjects produce several chemokines and cytokines such as tumor necrosis factor alpha (TNFα), interleukin (IL)-1β, and IL-6. These proinflammatory cytokines facilitate the differentiation of macrophages by recruiting monocytes into the adipose tissue; this process is linked to adipocyte dysfunction [1]. Although critical factors related to adipocyte dysfunction in the obese have not been fully determined, the prevailing hypothesis is that cytokines generated by adipocytes are responsible for the recruitment of immune cells to obese adipose tissue and for the promotion of monocyte differentiation to M1 macrophages to initiate systemic inflammation [2]. Inflammatory responses in the adipose tissue could be induced by extracellular and intracellular stimuli from overnutrition, increased endoplasmic reticulum (ER) stresses, levels of free fatty acids, and apoptosis of adipocytes. The canonical inflammatory signaling pathway to activate nuclear factor κ B (NFκB) is also responsible for adipocyte dysfunction in obese subjects [3].

TNFα is a well-known pro-inflammatory cytokine that influences the expression of diverse immune and inflammatory response genes in various cell types through the activation of several DNA binding proteins including NFκB and activator protein 1 (AP-1) [4,5]. TNFα regulates multiple cellular processes such as proliferation, apoptosis, inflammation, and energy metabolism [6,7]. TNFα disrupts insulin signaling and sensitivity in adipose tissues. Adipocytes generate TNFα, IL-6, and IL-1β; the levels of cytokines were shown to be elevated in both adipose tissue and plasma in mice fed a high-fat diet (HFD) [8]. Furthermore, TNFα depletion prevented HFD-induced insulin resistance [8,9].

Several studies have established that TNFα affects adipogenesis and insulin signaling. TNFα-mediated down-regulation of peroxisome proliferator activator receptor gamma (PPARγ) is closely linked to adipocyte dysfunction [10]. PPARγ expression can be regulated at multiple levels including transcription, translation, mRNA stability, and turn-over of PPARγ by TNFα [11–18].

microRNAs (miRNAs) are a class of small non-coding RNAs that regulate gene expression in eukaryotes [19]. miRNAs interact with target mRNAs by forming incomplete base pairing and they negatively regulate the expression of target mRNAs by inhibiting translation or by destabilizing mRNAs. Several reports have indicated that miRNAs are involved in the regulation of adipogenesis and fat cell metabolism [20–25]. In a previous study, miR-130a and miR-130b (miR-130) were identified as novel regulators of adipogenesis by targeting PPARγ, a master regulator of adipogenesis. However, regulation of miR-130 by TNFα and its role in adipose tissues have not yet been elucidated.

Here, we report the molecular evidence for PPARγ regulation by miR-130 during TNFα-induced adipocyte dysfunction. We show

* Corresponding author. Address: 222 Banpodaero, Seocho-gu, Seoul 137-701, South Korea. Fax: +82 2 596 4435.
E-mail address: leeek@catholic.ac.kr (E.K. Lee).
[1] Equal contribution.

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that the expression levels of miR-130 were regulated by TNFα in adipocytes, and up-regulation of miR-130 resulted in adipocyte dysfunction through down-regulation of PPARγ.

2. Materials and methods

2.1. Cell culture, differentiation, transfection, and treatment

NIH-3T3-L1 cells were cultured in Dulbecco's modified essential medium (DMEM, Invitrogen), supplemented with 10% newborn calf serum and antibiotics (Gibco). To induce adipocyte differentiation, 3T3-L1 cells were incubated with 0.5 mM IBMX (Sigma), 1 μM dexamethasone (Sigma), and 167 nM insulin (Sigma) for 2 days, then incubated with 167 nM insulin for 2 days, and further incubated with DMEM/10% FBS with insulin for 2 more days. Differentiated 3T3-L1 cells were maintained in DMEM/10% FBS up to 8 days [26]. miRNAs (Ambion) and control siRNAs (Genolution) were transfected with lipofectamine (Invitrogen) or Nucleofector (Amaxa). Differentiated adipocytes were incubated with 10 μg/ml of TNFα or PBS overnight. White adipose tissues (WATs) from C57BL/6 mice fed a chow-diet (CD) or an HFD for 8 weeks were isolated.

2.2. RNA analysis

Total RNAs were prepared from whole cells or WATs by using Trizol (Invitrogen). After reverse transcription (RT) using random hexamers and reverse transcriptase (Toyobo), the abundance of transcripts was assessed by quantitative (q)PCR analysis using the SYBR green PCR master mix (Kapa Biosystems) and gene-specific primer sets. The primer sequences are listed in Table 1. RT-qPCR analysis was performed on the Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems). Individual microRNAs were further quantified using the Taqman microRNA detection assay (Applied Biosystems) or QuantiMir™ cDNA Kit (System Biosciences Inc.). For the Taqman microRNA detection assay, miRNA-specific primer sets supplied by the manufacturer were used in RT-qPCR. For QuantiMir™ cDNA analysis, forward primers were designed to be the exact sequences of the miRNAs in the miRBase database, and a universal reverse primer was used from the kit [20].

2.3. Chromatin immunoprecipitation (ChIP)

ChIP was done as previously described [27]. After crosslinking with 1% formaldehyde, cells were lysed in SDS lysis buffer containing 50 mM Tris–HCl (pH 8.0), 1% SDS, 10 mM EDTA, and protease inhibitor cocktail. Chromatin was fragmented by sonication (Bioruptor®) with 6 cycles (30 s on and 30 s off) and diluted in dilution buffer containing 16.7 mM Tris–HCl (pH 8.0), 167 mM NaCl, 1.2 mM EDTA, and 1.1% Triton-X. After pre-clearing, diluted supernatants were incubated with 1 μg of anti-polII (Millipore), anti-p65 (Santa Cruz Biotech), and control IgGs (mouse and rabbit, Santa Cruz Biotech) antibodies for overnight at 4 °C and further precipitated with protein A-Sepharose beads. Immune complexes were washed with washing buffer containing 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% Triton-X, and 0.1% SDS, and then incubated with protease K (Fermentas) at 55 °C. After centrifugation, the DNAs were precipitated with ice-cold EtOH at −20 °C. The quantitative analysis of purified DNA was assessed by PCR using specific primers for p65 binding promoter regions of miR-130a and miR-130b.

2.4. Western blot analysis

Whole-cell lysates and immunoprecipitants were prepared, separated by electrophoresis in SDS-containing polyacrylamide gels, and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). Incubation with primary antibodies to detect p65 (Santa Cruz Biotech) was followed by incubation with the appropriate secondary antibody conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotech); proteins were detected using the Clarity™ Western ECL Substrate (Bio-Rad).

3. Results

3.1. Proinflammatory signaling increased miR-130 expression

HFD-induced obesity recruits macrophages around the adipose tissue and promotes an inflammatory response that resulted in adipocyte dysfunction [10]. A previous study has shown that miR-130a and-130b could regulate adipogenesis by targeting PPARγ, a pivotal regulator of adipocyte differentiation and function [20]. To determine miR-130 levels during HFD-induced obesity, we analyzed the expression levels of miR-130 from WAT of CD or HFD mice. The levels of miR-130a and -130b were up-regulated to 3.9- and 2.9-fold, respectively, in WAT from HFD mice compared to CD mice (Fig. 1A). To examine whether up-regulation of miR-130a and miR-130b in the WATs from HFD mice is regulated transcriptionally, we examined the levels of their primary transcripts. As shown Table 2, miR-130a and miR-130b are encoded in chromosome 2 and chromosome 16 of the mouse genome, respectively. RT-qPCR results showed that their primary transcripts, pri-miR-130a and pri-miR-130b, in the HFD group were also 2.8- and 4.1-fold higher, respectively, than the levels in the CD group (Fig. 1B). Based on these data, we hypothesized transcriptional regulation of both miR-130a and miR-130b during HFD-induced obesity.

To examine the expression levels of miR-130 in a cell culture system, we used adipocytes differentiated from mouse preadipocyte NIH-3T3-L1 cells upon hormonal stimuli, as described in the Materials and Methods. TNFα is a well-characterized proinflammatory cytokine and is responsible for macrophage activation in adipose tissues [5]. We examined the levels of miR-130 in TNFα-stimulated adipocytes. Fully differentiated adipocytes were exposed to a low concentration of TNFα for 16 h to induce chronic inflammatory signaling cascades, which is similar to the environment of HFD-induced obesity. After RNA isolation, the expression levels of miR-130 and their primary transcripts were determined by RT-qPCR. TNFα treatment resulted in a 6.8- and 12.9-fold increase of both miR-130a and miR-130b, respectively, (Fig. 2A); their primary transcripts were also increased to 4.5- and 6.5-fold, respectively, in the TNFα-treated group (Fig. 2B). Taken together, these results suggest that both miR-130a and miR-130b are induced by the TNFα-mediated proinflammatory response in adipocytes.

3.2. TNFα mediated down-regulation of PPARγ and its target genes

PPARγ down-regulation by TNFα is closely linked to adipocyte dysfunction [10]. PPARγ expression could be regulated at both the transcriptional level and the post-translational level by TNFα [12,14].

To show TNFα-mediated adipocyte dysfunction, we measured the levels of *PPARγ* and its target genes, including *adipsin, aP2*, and *LPL* in WAT from HFD mice as well as in adipocytes stimulated with TNFα. As shown in Fig. 3A, *PPARγ* expression was significantly decreased in WAT of HFD mice compared to that of CD mice, thus resulting in the down-regulation of its target genes. A similar effect was observed in adipocytes exposed to TNFα (Fig. 3B). Chronic exposure of adipocytes to TNFα resulted in the decrease of *PPARγ, adipsin, aP2*, and *LPL*. These results indicate that the TNFα-mediated proinflammatory response is responsible for adipocyte dysfunction by down-regulating PPARγ expression.