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MicroRNA 132 Regulates Nutritional Stress-Induced Chemokine Production through Repression of SirT1

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Human adipose tissue secretes a number of proinflammatory mediators that may contribute to the pathophysiology of obesity-related disorders. Understanding the regulatory pathways that control their production is paramount to developing effective therapeutics to treat these diseases. Using primary human adipose-derived stem cells as a source of preadipocytes and *in vitro* differentiated adipocytes, we found IL-8 and monocyte chemoattractant protein-1 (MCP-1) are constitutively secreted by both cell types and induced in response to serum deprivation. MicroRNA profiling revealed the rapid induction of microRNA 132 (miR-132) in these cells when switched to serum-free medium. Furthermore, miR-132 overexpression was sufficient to induce nuclear factor-κB translocation, acetylation of p65, and production of IL-8 and MCP-1. Inhibitors of miR-132 decreased acetylated p65 and partially inhibited the production of IL-8 and MCP-1 induced by serum deprivation. MiR-132 was shown to inhibit silent information regulator 1 (SirT1) expression through a miR-132 binding site in the 3′-untranslated region of SirT1. Thus, in response to nutritional availability, induction of miR-132 decreases SirT1-mediated deacetylation of p65 leading to activation of nuclear factor-κB and transcription of IL-8 and MCP-1 in primary human preadipocytes and *in vitro* differentiated adipocytes. **(*Molecular Endocrinology* 23: 1876–1884, 2009)**

Historically, adipose tissue was thought to serve only as the storage site of excess energy; however, numerous studies have now identified a variety of proteins secreted from adipose, including both hormones and cytokines, which suggest a significant role for adipose in regulating whole-body energy metabolism and inflammation (1–9). These studies have led to the now generally accepted concept that adipose is a secretory organ with both endocrine and immunological activities (10). Obesity is a growing worldwide epidemic with type 2 diabetes and cardiovascular disease as common comorbidities. The development and progression of insulin resistance and atherosclerosis may result from a state of chronic inflammation within the adipose (11, 12). The pathogenesis of the inflammation is a matter of debate. Regardless of the cellular source of proinflammatory mediators within adipose, inhibition of their production may be therapeutically advantageous for the treatment of type 2 diabetes as well as cardiovascular disease. As a key transcription factor in inflammatory cascades in immune cells (13) and human adipocytes (14), nuclear factor-κB (NFκB) plays an important role in both innate and adaptive immunity. Chronic activation of NFκB *in vivo* has been shown to emulate the chronic inflammatory state observed with obesity and produces an insulin-resistant phenotype in IKKβ transgenic mice (15). However, the role of persistent activation of NFκB has not been directly assessed in human adipose. Furthermore, the mechanisms regulating the activation of NFκB and the production of proinflammatory mediators remain poorly characterized in human adipocytes.

MicroRNAs are small oligonucleotides that posttranscriptionally regulate gene expression by binding to the

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Abbreviations: CREB, cAMP response element binding protein; FBS, fetal bovine serum; IFNγ, interferon-γ; MCP-1, monocyte chemoattractant protein-1; miR-132, microRNA 132; MSD, MesoScale Discovery; NFκB, nuclear factor-κB; PPARγ, peroxisome proliferator-activated receptor-γ; siRNA, small interfering RNA; SirT1, silent information regulator 1; 3′-UTR, 3′-untranslated region.

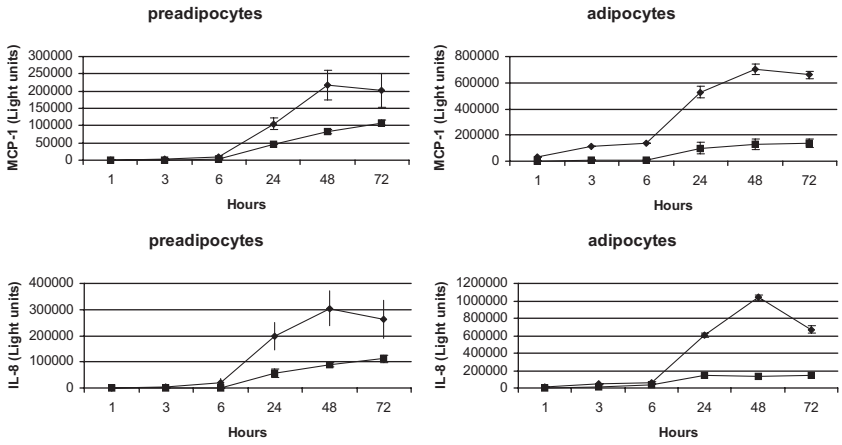

FIG. 1. Serum deprivation induces IL-8 and MCP-1. Human preadipocytes and *in vitro* differentiated adipocytes were incubated in serum-containing medium (■) or serum-free medium (♦) for the indicated times. After incubation, medium was collected, and the quantity of IL-8 and MCP-1 was determined using MSD assay kits. Proteins were quantitated by electrochemiluminescence detection and a MSD SECTOR Imager 6000. Data represent the mean of eight replicates ± SEM. These data are representative of three independent experiments.

3′-untranslated region (3′-UTR) of target gene sequences resulting in inhibition of transcription or translation (16, 17). Although over 700 microRNAs have been identified and various target predictions have been made, the function of these are just now being elucidated. Although the regulation of cell differentiation (18–20) by microRNAs and their dysregulation in various cancers (21, 22) is well documented, their role in signal transduction is less well understood.

To investigate the role of microRNAs in the production and secretion of proinflammatory mediators in human adipocytes, we profiled the expression of over 250 microRNAs in primary human preadipocytes and *in vitro* differentiated adipocytes under conditions of serum deprivation. We identified the induction of microRNA 132 (miR-132) and found it plays a key role in the response to serum deprivation in both preadipocytes and *in vitro* differentiated adipocytes. Furthermore, we demonstrate for the first time, the repression of silent information regulator 1 (SirT1) protein levels by miR-132 and propose a pathway where miR-132 represses the SirT1-mediated deacetylation of p65 resulting in NFκB activation and IL-8 and monocyte chemoattractant protein-1 (MCP-1) production.

Results

Serum deprivation induces IL-8 and MCP-1

To assess the production of proinflammatory mediators by primary human preadipocytes and *in vitro* differentiated adipocytes, cells were cultured in the presence or absence of serum, and at various times, the medium was analyzed for the presence of interferon-γ (IFNγ), IL-1β, IL-10, IL-12p70, IL-13, IL-2, IL-4, IL-5, IL-8, TNFα, and MCP-1. Serum deprivation induced a significant increase in the chemokines IL-8 and MCP-1 in both cell types compared with cells grown in serum-containing medium (Fig. 1). No other cytokine tested was significantly produced from either cell type under these conditions (data not shown). These data show both cell types constitutively express IL-8 and MCP-1 when grown in serum-containing medium. However, the *in vitro* differentiated adipocytes secrete higher basal levels of IL-8 and MCP-1 and, in terms of chemokine production, are more sensitive to the stress of serum deprivation compared with the preadipocytes. These data suggest the regulation of chemokine production by nutrient availability.

Serum deprivation induces miR-132

To identify pathways involved in the induction of chemokines by serum deprivation, we investigated the effect of serum deprivation on the expression of microRNAs in human preadipocytes and *in vitro* differentiated human adipocytes. Initially, we measured the expression of 255 microRNAs using real-time PCR (data not shown). We identified an increase in miR-132 expression and, in subsequent studies, confirmed this observation in both cell types in response to serum deprivation (Fig. 2). Serum deprivation rapidly induced the biphasic expression of miR-132 with peaks at 1 and 24 h in both cell types. The similarities between the miR-132 expression profile and chemokine production suggest a possible role for miR-132 in the regulation of chemokines in these cells.

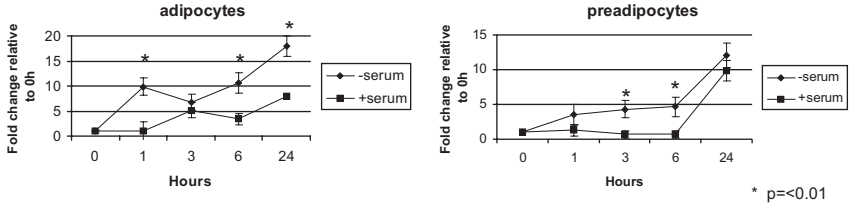

FIG. 2. Serum deprivation induces miR-132 expression. *In vitro* differentiated human adipocytes and human preadipocytes were treated with serum-containing medium (■) or serum-free medium (♦) for the indicated times. Cells were lysed and total RNA extracted as described in *Materials and Methods*. Expression of miR-132 was determined using real-time PCR. Data represent the mean of four replicates and are representative of three independent experiments. Data are expressed as fold change relative to 0 h and were normalized to input of total RNA into the reverse transcription reaction.

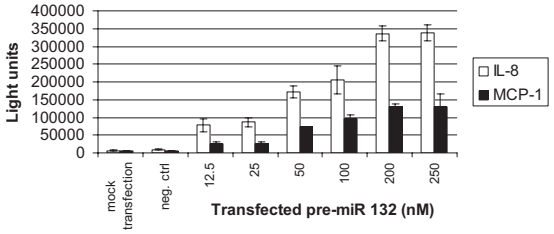

FIG. 3. MiR-132 induces IL-8 and MCP-1. Human preadipocytes were plated (3×10^4 cells per well) and transfected with varying concentrations of pre-miR-132 or a scrambled oligonucleotide as a negative control (250 nM). After 48 h incubation, medium was collected and the quantity of IL-8 (*white bars*) and MCP-1 (*black bars*) was determined using MSD assay kits. Proteins were quantitated by electrochemiluminescence detection and a MSD SECTOR Imager 6000. Data are expressed as light units of cytokine and represent the mean of eight replicates ± SEM. These data are representative of three independent experiments.

miR-132 induces IL-8 and MCP-1

To gain insight into the possible relationship between induction of miR-132 and IL-8 and MCP-1 production, we explored the role of miR-132 by overexpressing miR-132 in preadipocytes and *in vitro* differentiated adipocytes. Transfection of pre-miR-132 in preadipocytes for 48 h induced a concentration-dependent increase of IL-8 and MCP-1 (Fig. 3). No other cytokine tested (IFNγ, IL-1β, IL-10, IL-12p70, IL-13, IL-2, IL-4, IL-5, or TNFα) was significantly produced from either cell type under these conditions (data not shown). The induction of both chemokines appeared biphasic with concentration of transfected pre-miR 132 with an initial plateau at 12.5–25 nM and subsequently at 200–250 nM. However, miR-132 was more efficacious at stimulating IL-8 production. We next investigated whether the ability of miR-132 to induce IL-8 and MCP-1 was unique to the preadipocytes. Transfection of miR-132 for 48 h was similarly effective at inducing both chemokines in the *in vitro* differentiated adipocytes (data not shown). These data demonstrate that miR-132 overexpression is sufficient to induce IL-8 and MCP-1.

Inhibitors of miR-132 partially inhibit induction of IL-8 and MCP-1 by serum deprivation

To determine whether blocking endogenous miR-132 affects the induction of IL-8 and MCP-1 by serum deprivation, human preadipocytes were transfected with inhibitors of miR-132. Inhibitors to miR-132 decreased the induction of IL-8 in a concentration-dependent manner (Fig. 4A). At 50 nM transfected inhibitor, there was an approximate decrease of 33% ($P = 0.0002$). MiR-132 inhibitor was maximally effective at 250 nM (2-fold, $P = 0.000002$) compared with serum withdrawal alone (Fig. 4A). Likewise, miR-132 inhibitors partially blocked the induction of MCP-1 by serum deprivation (Fig. 4B). The maximally effective concentration of miR-132 inhibitor was 100 nM with a decrease of 33% compared with cells grown in serum-free medium alone. This brought the level of MCP-1 down to about 2-fold greater then the constitutive production seen in cells grown in serum-containing medium. These changes were specific to the inhibitor used because the negative control had no significant effect on the production of IL-8 and MCP-1.

Serum deprivation and miR-132 induces acetylation of p65 NFκB

NFκB plays a central role in the production of proinflammatory mediators, and IL-8 and MCP-1 are known transcriptional targets. Therefore, we initially assessed the role of NFκB by using 2-[(aminocarbonyl)amino]-5-(4-flurophenyl)-3-thiophenecarboxamide (TPCA1), a potent and selective inhibitor of IκB kinase 2 (IKK-2). Pretreatment of either preadipocytes or *in vitro* differentiated adipocytes with 50 nM TPCA1 blocked the induction of IL-8 and MCP-1 by serum deprivation, suggesting NFκB activation is necessary for chemokine production (data not shown). We also measured translocation of the transcription factor from the cytosol to the nucleus in human preadipocytes. After 1 h incubation in serum-free medium, the translocation of NFκB from the cytosol into the nucleus was seen (data not shown). Likewise, after 24 h transfection, pre-miR-132 induced a similar response. To investigate the

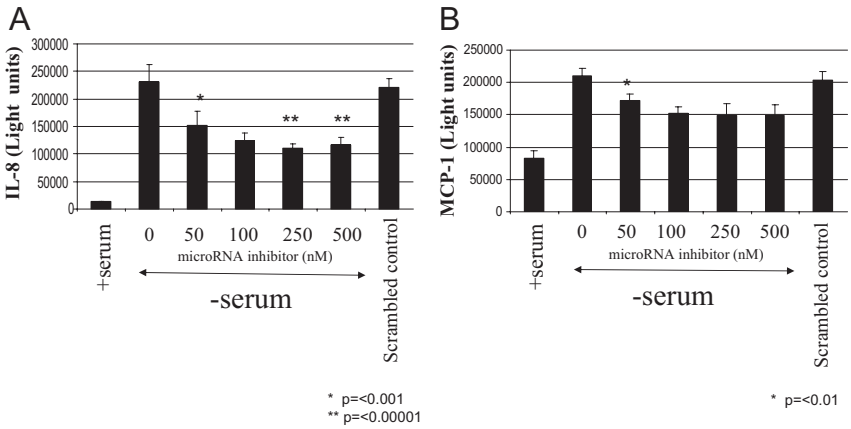

FIG. 4. MiR-132 inhibitor partially blocks IL-8 and MCP-1 production. Human preadipocytes were transfected for 24 h in DMEM/F12 containing 10% FBS with varying concentrations of miR-132 inhibitor or a scrambled oligonucleotide negative control (500 nM). Cells were then incubated with fresh serum-containing medium or with serum-free medium for 24 h as indicated. After incubation, medium was collected, and the quantity of IL-8 (A) and MCP-1 (B) was determined using MSD assay kits. Proteins were quantitated by electrochemiluminescence detection and a MSD SECTOR Imager 6000. Data are expressed as light units of IL-8 and MCP-1 and represent the mean of eight replicates ± SEM. These data are representative of three independent experiments.

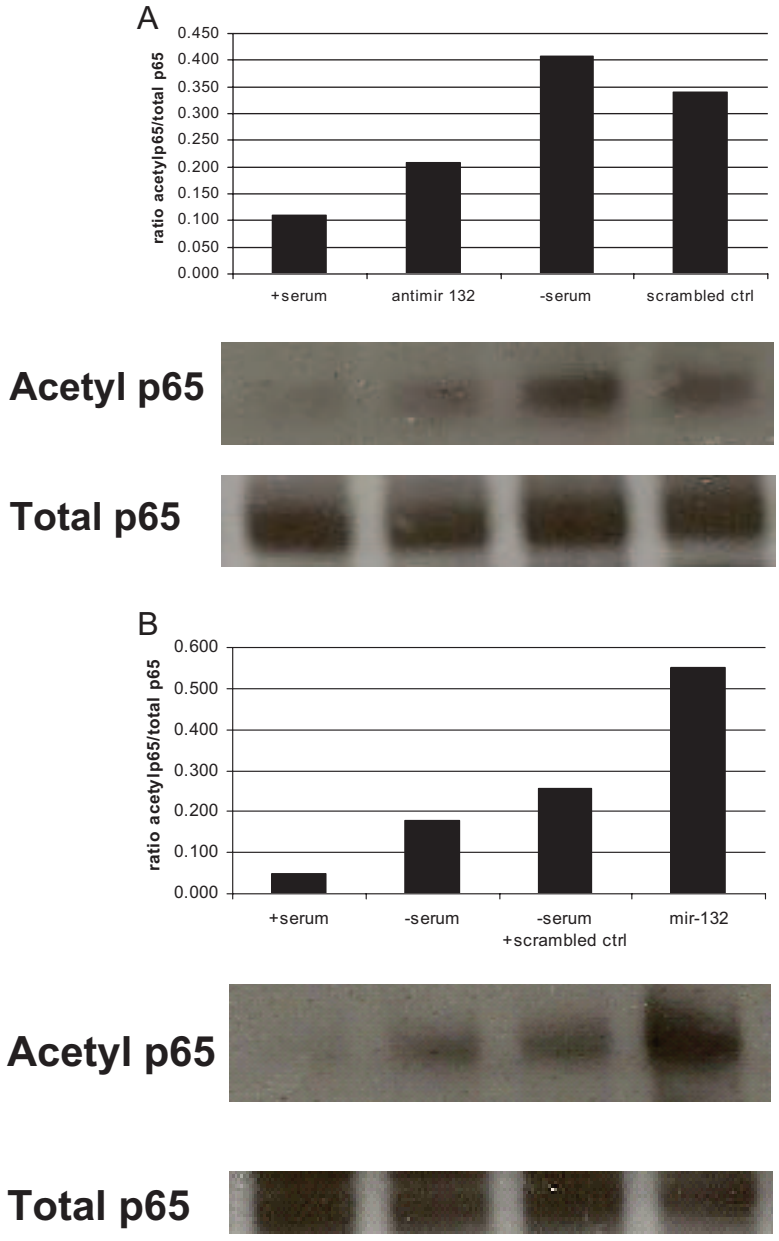

FIG. 5. Serum deprivation and miR-132 overexpression induces acetylation of lysine 310 p65 and miR-132 inhibitors decrease acetylation. Human preadipocytes (1×10^6 cells) were plated in 25-cm^2 flasks. Cells were transfected for 24 h with a miR-132 inhibitor or scrambled oligonucleotide (A) or pre-miR-132 or a scrambled oligonucleotide control (B) before addition of serum-containing medium or serum-free medium for 1 h. After incubation, cells were lysed and protein was immunoprecipitated overnight at 4 C using anti-total p65. Proteins were resolved by SDS-PAGE and blotted with anti-acetyl lysine 310 antibody. Enhanced chemiluminescence detection was used. These data are representative of two independent experiments.

mechanism of NFκB activation, we measured the acetylation of lysine 310 of the p65 component of the complex. After 1 h in serum-free medium, an increase in endogenous acetylated lysine 310 p65 was seen in the preadipocytes (Fig. 5A) and *in vitro* differentiated adipocytes (data not shown) that was not seen in cells grown in serum-containing medium. Transfection of a miR-132 inhibitor decreased the acetylation of lysine 310 p65, whereas a negative control oligonucleotide had no effect. Likewise, overexpression of miR-132 was sufficient to induce acetylation of lysine 310 p65 after 24 h transfection, whereas the transfection of a scrambled oligonucleotide control had no effect on acetylation of lysine 310 of p65 in cells grown in serum-free medium (Fig. 5B). Taken together, these data suggest the regulation of the acetylation/deacetylation pathway of NFκB by serum deprivation and miR-132.

Serum deprivation and miR-132 regulate SirT1 expression

Because miR-132 appears to regulate the acetylation of p65, we searched for protein targets that are known to be involved in acetylation/deacetylation pathways. The nicotinamide adenine dinucleotide-dependent deacetylase SirT1 was found as a predicted target of miR-132 in MiRBase (23–25) (http://microrna.sanger.ac.uk/). Thus, we assessed the protein levels of SirT1 in human preadipocytes and *in vitro* differentiated adipocytes in response to serum deprivation and miR-132 overexpression. A significant decrease in SirT1 protein levels were observed at 1 and 3 h in cells incubated in serum-free medium compared with cells grown in serum-containing medium (Fig. 6A). Likewise, overexpression of miR-132 for 24 h decreased SirT1 protein levels relative to cells transfected with a scrambled oligonucleotide control (Fig. 6B) while transfection of antimirs to miR-132 blocked the decrease in SirT1 seen with serum deprivation (data not shown). The decrease in SirT1 protein with miR-132 overexpression suggests the direct translational repression of SirT1 by miR-132 and the possible regulation of SirT1-mediated deacetylation of p65 by miR-132.

MiR-132 represses SirT1 expression through binding the 3′-UTR in the SirT1 gene

We first assessed whether serum deprivation or miR-132 overexpression affected SirT1 mRNA levels. Through gene expression analysis of SirT1 mRNA using real-time PCR, we found there was no change in SirT1 mRNA under any of these treatment conditions (data not shown). Thus, a luciferase reporter construct containing the 3′-UTR of the SirT1 gene and the predicted miR-132 binding site was prepared to assess whether the binding of miR-132 to the SirT1 3′-UTR mediates translational repression. After cotransfection of the reporter construct and pre-miR-132 or a scrambled oligonucleotide control in human preadipocytes, there was an approximately

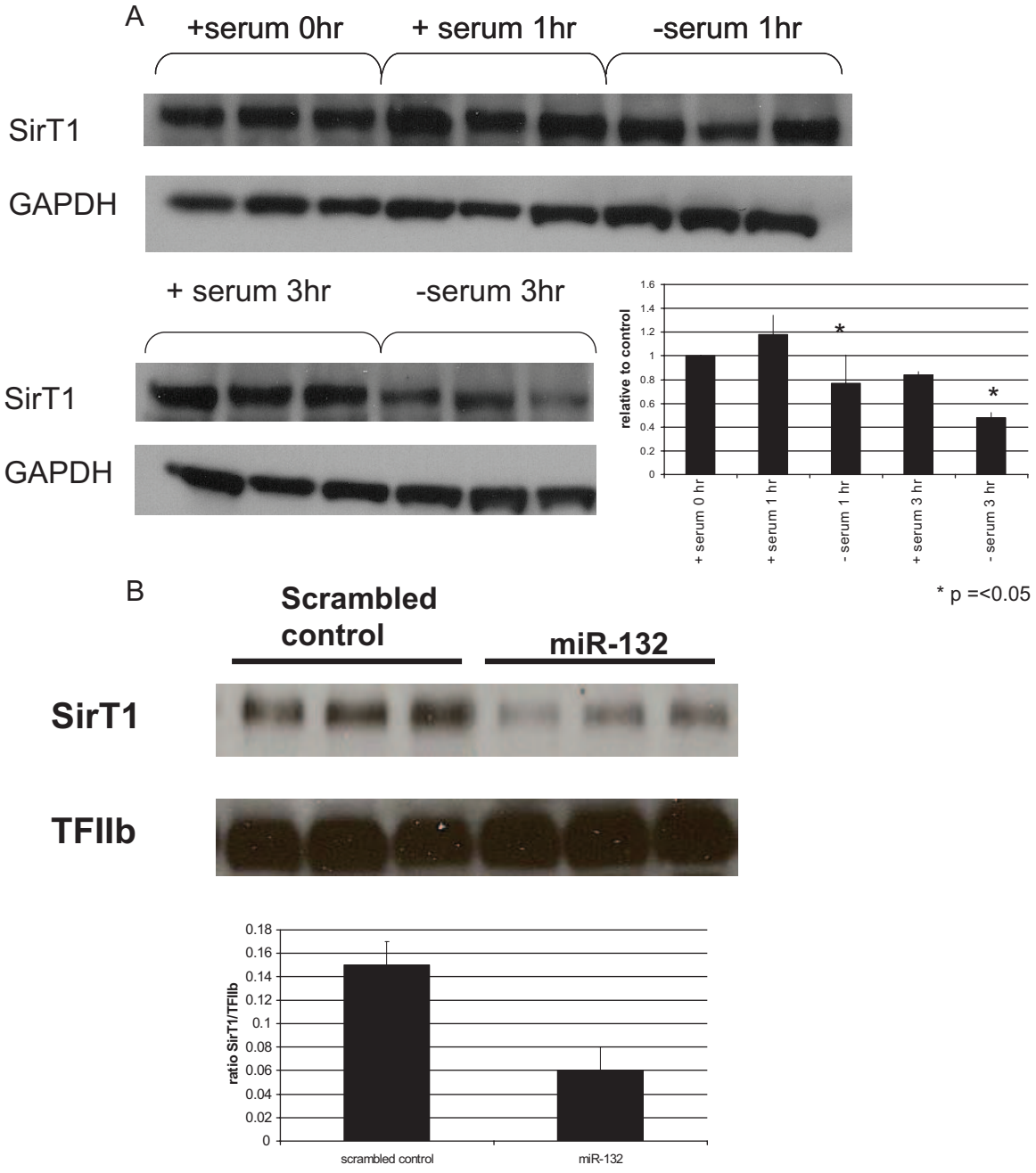

FIG. 6. Serum deprivation and miR-132 overexpression decreases SirT1 protein levels. Human preadipocytes were plated in 12-well plates. Cells were incubated in serum-containing medium or serum-free medium for various times (A) or transfected with pre-miR-132 for 24 h (B). Cells were lysed, collected, and analyzed as described in *Materials and Methods*. The *insets* show the quantitation of SirT1 protein by densitometry. These data are representative of three independent experiments.

2-fold decrease in luciferase activity of the reporter construct in cells cotransfected with pre-miR-132 compared with the scrambled oligonucleotide control (Fig. 7). These data suggest miR-132 represses SirT1 translation through directly binding the response element in the SirT1 3′-UTR.

Discussion

Studies of microRNAs have yielded unique insights into the regulation of important biological processes. Yamakuchi *et al.* (26) recently demonstrated the translational repression of SirT1 by miR-34a resulting in the activation of p53 and apoptosis, thus identifying miR-34a as a tumor suppressor. Our study is the first report to demonstrate the regulation of SirT1 by miR-132 and subsequent chemokine production through activation of NFκB. Previously, miR-132 was shown to be a transcriptional target of cAMP response element binding protein (CREB), which regulates neuronal morphogenesis through translational repression of p250GAP (27). Additionally, it's been

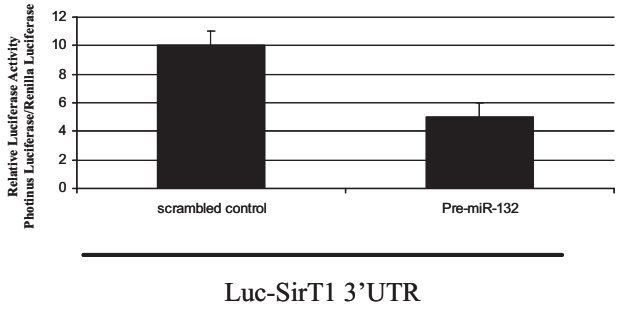

FIG. 7. miR-132 mediates translational repression of SirT1 through a binding site in SirT1 3′-UTR. Human preadipocytes (1×10^5 cells) were plated in 96-well plates and cotransfected with 200 ng pMIR-Report luciferase vector including the 3′-UTR of SirT1 containing the predicted miR-132 binding site and pre-miR-132 or scrambled oligonucleotide using an electroporator. Luciferase assays were performed using the dual-luciferase assay system 6–8 h after transfection. Data represent the mean of four biological replicates. The data are representative of two independent experiments.

shown to regulate the expression of methyl-CpG-binding protein (MeCP2) (28) and the angiotensin II type 1 receptor (AT1R) (29). MiR-132 was also reported to be induced by lipopolysaccharide in THP-1 cells (30), but a function was not ascribed. The biphasic expression pattern of miR-132 appears to parallel the biphasic activity profile of NFκB (31) and suggests miR-132 expression may be regulated by NFκB and represent a positive feedback loop. However, *in silico* promoter analysis failed to identify NFκB binding sites in the region of miR-132. As mentioned above, CREB has been shown to regulate miR-132 expression in neuronal cells (27). Additionally, CREB is regulated by high glucose in THP-1 cells where it has been shown to induce cyclooxygenase 2 (COX2), an enzyme that metabolizes arachidonic acid to prostaglandins (32). In our studies, CREB may regulate expression of miR-132 in response to the treatment conditions used where serum was absent and the concentration of glucose was relatively high (17.5 mM). Additional studies are needed to determine the exact mechanism of miR-132 transcription in human adipocytes.

SirT1 is highly regulated by nutrient availability. SirT1 protein levels *in vivo* are increased with starvation, fasting, and calorie restriction (33), whereas SirT1 protein decreases with age and senescence (33). Most interestingly, glucose appears to regulate SirT1 protein levels. Incubation of PC12 and HEK293 cells in the absence of both serum and glucose induces SirT1 protein expression through either an increase in transcription (34) or posttranscriptional regulation (35). In contrast, Nedachi *et al.* (36) showed low serum and high glucose represses SirT1 protein in C_2C_{12} cells. Likewise, Rodgers *et al.* (37) found high glucose mediated the posttranscriptional repression of SirT1 protein in mouse hepatocytes in conditions of low serum. Corroborating these findings, our study shows SirT1 protein levels decrease when human preadipocytes and *in vitro* differentiated adipocytes are incubated in serum-free conditions and high glucose (17.5 mM) and extends these studies by proposing the posttranscriptional regulation of SirT1 by miR-132. Inhibition of SirT1 in response to high glucose in adipocytes should also result in activation of peroxisome proliferator-activated receptor-γ (PPARγ), which allows the cells to store glucose during times of excess energy (38). However, our data suggest nutrient availability may result in persistent activation of NFκB and chemokine production.

SirT1 has recently been implicated in the regulation of inflammation. Recently, Zhu *et al.* (39) showed resveratrol, a SirT1 activator, decreased TNFα-induced MCP-1 secretion in 3T3-L1 adipocytes. Pfluger *et al.* (40) showed overexpression of SirT1 in mice resulted in a lower recovery of IL-6 and TNFα in serum of transgenic mice fed a high-fat diet and an attenuated response to TNFα-induced NFκB activation in transgenic mouse embryonic fibroblasts. Thus, increased SirT1 activity appears to be antiinflammatory in mice. In contrast, when inhibited, SirT1 appears to be proinflammatory. Kwon *et al.* (41) showed HIV Tat binds SirT1 and inhibits the SirT1-mediated deacetylation of p65 NFκB resulting in hyperactivated NFκB, a greater transactivation efficiency and an exaggerated immune response. Likewise, Yang *et al.* (42) recently showed that cigarette smoke extract caused a dose- and time-dependent decrease in SirT1 activity and protein and a concomitant increase in NFκB-dependent proinflammatory mediator release in MonoMac 6 cells. More recently, studies using small interfering RNA (siRNA) to knock down SirT1 reported an increase in TNFα-induced MCP-1 and other proinflammatory genes in 3T3-L1 adipocytes (43). The authors went on to show that knockdown of SirT1 led to an increase in acetylation of lysine 310 of p65. Taken together with the findings reported here, these data demonstrate a decrease in SirT1 activity increases activation of NFκB and transcription of proinflammatory mediators.

Reversible acetylation of signaling proteins has emerged as a key posttranslational modification regulating activity of p53 and Forkhead proteins. Several reports have shown the reversible acetylation of NFκB (44–47). Furthermore, Yeung *et al.* (44) demonstrated the attenuation of NFκB activity by SirT1 and histone deacetylases leading to the enhancement of TNFα-induced apoptosis. Acetylation of lysine 310 of the p65 component of the NFκB complex has been shown to be necessary for the full activation of NFκB (45) and affect the duration and the selectivity of gene transcription (46). Ito (47) reported the acetylated lysine 310 p65 complex preferentially bound the NFκB binding site on the promoter region of IL-8 but not granulocyte-mac-

rophage colony-stimulating factor. We found miR-132 inhibitors blocked the accumulation of endogenous acetylated lysine 310 p65 and partially inhibited the induction of IL-8 and MCP-1 in serum-deprived cells. These data confirm the importance of acetylated lysine 310 p65 on the transcriptional activation of NFκB and extends it to conditions of nutrient availability in human preadipocytes and adipocytes. MiR-132 indirectly regulates acetylation of NFκB through repression of SirT1. SirT1 also binds to and regulates p300 (48), the acetyltransferase involved in NFκB activation. The decrease in SirT1 protein may have a possible dual effect on acetylation of NFκB through both inhibition of deacetylation and activation of acetylation.

Previously, IL-8 and MCP-1 were shown to be constitutively secreted by both human preadipocytes and isolated adipocytes implying a normal biological function for these chemokines (49–52). Excessive production through dysregulated NFκB is thought to play a pathogenic role in metabolic diseases. Indeed, circulating levels of IL-8 and MCP-1 are higher in obese then lean individuals and are associated with parameters of insulin resistance, atherosclerosis, and cardiovascular disease (53–59). Our data support the central role of NFκB in the inflammatory process in adipose and implies the miR-132 repression of SirT1-mediated deacetylation of p65 could be a mechanism of NFκB dysregulation contributing to the chronic inflammatory state in adipose tissue. Preventing the decrease in SirT1 protein should block the activation of NFκB and may be a therapeutic alternative to activating SirT1.

Materials and Methods

Cell culture and transfection

Primary human adipose-derived stem cells were collected from the abdominal sc adipose of a 37-yr-old female with a BMI of 23.3 (Zen-Bio Biological Systems and Services, Research Triangle Park, NC) and used as a source for preadipocytes and differentiated adipocytes. Cells (passages 4–8) were cultured in sc preadipocyte medium (Zen-Bio) until treatment. To differentiate into adipocytes, the sc preadipocytes were plated in preadipocyte medium and then after 24 h were differentiated in DMEM/F12 plus 10% fetal bovine serum (FBS) medium (Invitrogen, Carlsbad, CA) containing 200 μM isobutylmethylxanthine, 20 nM dexamethasone, 20 nM GW347845X, a PPARγ agonist and 20 nM human insulin. After incubation for 7 d, the medium was changed to DMEM/F12 plus 10% FBS containing 20 nM insulin. All experiments on mature *in vitro* differentiated adipocytes were begun after d 14. Differentiation of cells was more than 90% as assessed by quantitating triglyceride accumulation using the Trinder assay (Sigma Chemical Co., St. Louis, MO) and real-time PCR analysis of fatty acid-binding protein 4 (FABP4 or ap2), PPARγ, and fatty acid synthase (FAS) mRNA, as markers of adipocyte differentiation. These results showed less than 5% interwell variability among differentiated cultures of adipocytes. Serum deprivation was carried out by incubating cells in DMEM/F12 plus 1% BSA-fatty acid free. Experiments done to overexpress or inhibit miR-132 were done using Dharmafect 2 siRNA transfection reagent and miRIDIAN microRNA 132 mimic (pre-miR-132) UAACAGUCUACAGCCAUGGUCG and mimic negative control UCACAACCUCCUAGAAAGAGUAGA (Dharmacon, LaFayette, CO) or miRCURY LNA knockdown probes CGACCATGGCTGTAGACTGTTA and scramble-miR control GTGTAACACGTCTATACGCCCA (Exiqon, Inc., Woburn, MA). The negative control oligonucleotides were tested at the highest concentration of the pre-miR-132 or inhibitor used in the experiment. Thus, we did not control for each concentration of microRNA used or transfect constant amounts of total microRNA. Uptake of microRNAs into cells was assessed using Cy-3-labeled pre-miR negative control and Cy-3-labeled anti-miR negative control (Ambion, Austin, TX) and Dharmafect 2 siRNA transfection reagent. Unless otherwise noted, all transfections were done in serum-containing medium.

mRNA gene expression analysis

Total RNA was isolated from cells using Promega total RNA isolation kit (Promega, Madison, WI). RNA was reverse-transcribed using the ABI high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Reactions containing 10 ng cDNA, SirT1 primers and probes (Assays on Demand; Applied Biosystems), and Universal PCR Master Mix (Applied Biosystems) were analyzed using a 7900HT sequence detector system (Applied Biosystems) as recommended by the vendor. The relative abundance of SirT1 mRNA was normalized to β-actin (Actb) mRNA.

MicroRNA gene expression analysis

Total RNA was isolated by removing the treatment medium, washing the cells with PBS, and adding 1 ml TRIzol reagent per well. A phenol-chloroform extraction was performed, and 1.25 vol 100% ethanol was added to the aqueous phase. The microRNAs were isolated according to the manufacturer's protocol using a mirVana microRNA isolation kit from Ambion (Austin, TX). The microRNAs were then converted to cDNA, and gene expression analysis was done using the TaqMan microRNA assays (Applied Biosystems).

NFκB translocation

Translocation of NFκB was assessed in human preadipocytes using the NFκB activation kit (ThermoScientific) and the Cellomics ArrayScan II high-content imaging system. Briefly, human preadipocytes (3×10^4 cells per well) were plated into 96-well plates. After incubation overnight, cells were treated by incubating in serum-free medium or transfected with pre-miR-132 (100 nM) (Dharmacon) for 24 h. After treatment for various times, cells were fixed and stained with anti-NFκB antibody followed by an AlexaFluor 488-conjugated secondary antibody. The ratio of fluorescence in the cytosol and nucleus was calculated as a quantitative measure of NFκB translocation.

Western blot analysis

Protein lysates were prepared using M-PER cell lysis buffer (Pierce, Rockford, IL) containing a protease cocktail inhibitor mix (Boehringer Mannheim, Mannheim, Germany). Protein lysates (10 μg total protein) were resolved by electrophoresis on

10% or 4–12% SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose (Invitrogen). Proteins were detected by immunoblotting using anti-acetyl lysine 310 p65, anti-NFκB p65, anti-SirT1(C14H4), anti-GAPDH, antirabbit IgG horseradish peroxidase-linked antibody, anti-TFIIB (Cell Signaling Technologies, Beverly, MA). All primary antibodies were used at 1:1000 dilution and incubated overnight at 4C. Antirabbit IgG HRP was used as the secondary antibody at 1:2000 dilution and detected using ECL reagents (Amersham, Piscataway, NJ).

Plasmid construction

A 1.7-kb DNA fragment containing the SirT1 3′-UTR and the predicted miR-132 binding site was PCR amplified from human liver cDNA (QUICK-Clone cDNA; Clontech, Mountain View, CA). Primers were designed based on Genbank NM_012238 and included a 5′ *Sac*1 site and a 3′ *Mlu*1 site to accommodate subcloning into the pMIR-REPORT plasmid (Ambion, Austin, TX).

Luciferase assays

Primary cultures of human preadipocytes (1×10^5 cells per well) were plated in 96-well plates. After 24 h, 200 ng pMIR-Report luciferase vector including the 3′-UTR of SirT1, miR-132, or scrambled oligonucleotide were transfected using a Nucleofector (Amaxa, Walkersville, MD). Luciferase assays were performed using the dual-luciferase assay system (Promega) 6–8 h after transfection.

Cytokine measurements

Media were collected at various times from preadipocytes and differentiated adipocytes to analyze secreted cytokines. MesoScale Discovery (MSD) assay kits were used according to the manufacturer's recommendations to quantitate IFNγ, IL-1β, IL-10, IL-12p70, IL-13, IL-2, IL-4, IL-5, IL-8, TNFα, and MCP-1 (MesoScale Discovery, Gaithersburg, MD) protein levels using electrochemiluminescence detection and a MSD SECTOR Imager 6000.

Acknowledgments

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