SIRT3 reduces lipid accumulation via AMPK activation in human hepatic cells

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OBJECTIVE: Sirtuin 3 (SIRT3) is a nicotinamide adenine dinucleotide (NAD)+-dependent protein deacetylase localized on mitochondria and regulates the adaptive thermogenesis in brown adipocytes. This study aims to investigate the role of SIRT3 in hepatic lipid accumulation, and whether the activation of adenosine monophosphate (AMP)-activated protein kinase (AMPK) is required.

METHODS: A retroviral system was used for overexpressing of SIRT3 in HepG2 cells, whereas a lentivirus-mediated vector encoding SIRT3 small interfering RNA (siRNA) was used to infect these cells for knocking down endogenous SIRT3 expression. The cells were treated with oleate to induce lipid accumulation and Nile red staining was used to assess the number of lipid droplets in HepG2 cells. The AMPK signaling pathway was facilitated with the administrating of isoproterenol and an immunoblot analysis was performed to assess the phosphorylation of AMPK and acetyl coenzyme A carboxylase (ACC). Compound C was adopted to inhibit AMPK activity.

RESULTS: The number of lipid droplets in HepG2 cells overexpressing SIRT3 was significantly lower than that in the control cells (P < 0.05). SIRT3-infected cells exhibited significantly more phosphorylation of AMPK and ACC (P < 0.05), which was reversed by the treatment of compound C, an inhibitor of AMPK. Knocking down SIRT3 downregulated phosphorylation of AMPK and ACC by 60–80% (P < 0.05) and promoted lipid accumulation. The activation of AMPK by SIRT3 was dependent on SIRT3 deacetylase activity.

CONCLUSION: SIRT3 reduces lipid accumulation via AMPK activation in human hepatic cells.

KEY WORDS: acetyl coenzyme A carboxylase, AMP-activated protein kinase, hepatic lipid accumulation, SIRT3.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver diseases worldwide. NAFLD is characterized by the excessive accumulation of triglyceride (TG) in hepatocytes,1,2 which results in hepatic steatosis in its most benign form, then progressing to intermediate lesions termed non-alcoholic steatohepatitis and eventually to cirrhosis.

The metabolic defect of free fatty acids (FFA) within hepatocytes leads to the excessive accumulation of TG. The three important sources of FFA are dietary fatty acids, fatty acids released from adipose depots by lipolysis and newly made fatty acids in the liver through de novo lipogenesis. Under physiological...
conditions, FFA not only can be utilized to synthesize TG which are secreted into blood as very low-density lipoproteins (VLDL) eventually, but also can be channeled towards the β-oxidation pathway. Therefore, increased fat delivery, increased fat synthesis, reduced fat oxidation, and/or reduced fat export in the form of VLDL result in excessive fat accumulation in the liver.

NAFLD is commonly associated with insulin resistance. Insulin resistance and the resulting hyperinsulinemia lead to de novo liver FFA synthesis, along with the increased delivery of FFA from adipose tissue to the liver and impairing of FFA β-oxidation. FFA synthesis is essential for the regulation of fat metabolism in the liver. FFA synthesis is mediated by seven enzymes, among which acetyl coenzyme A carboxylase (ACC) is the rate-limiting enzyme. It has been shown that these enzymes are transcriptionally regulated by the sterol regulator element-binding protein-1c (SREBP1c)\textsuperscript{3–5} and carbohydrate response element binding protein (ChREBP) in the liver.\textsuperscript{6–8} Thus, ACC, ChREBP and SREBP1c play the key role in FFA synthesis.

Recent studies demonstrated that AMP-activated protein kinase (AMPK) was involved in the regulation of FFA metabolism.\textsuperscript{9} AMPK is a heterotrimeric complex that serves as a sensor of the cellular energy level. AMPK is activated when the cellular energy store decreases, which leads to a rise in the cellular AMP level. Upon the activation of AMPK, the adenosine triphosphate (ATP)-utilizing pathway (such as FFA synthesis) is switched off and the ATP-producing pathway (such as FFA oxidation) is switched on. Two anti-diabetic drugs have been found that could activate AMPK and reduce liver fat accumulation.\textsuperscript{10,11}

Three independent mechanisms are involved in the reduction of lipogenesis in response to the activation of AMPK. ACC is phosphorylated by activated AMPK, which reduces malonyl-coenzyme A formation. The phosphorylation of ChREBP by activated AMPK will suppress lipogenic gene expression by inhibiting its entry into the nucleus. Activated AMPK will reduce SREBP1c expression through undefined mechanisms.

Recent studies have hinted that AMPK can be regulated by other protein factors besides through changes in the cellular energy status.\textsuperscript{12} In this study we report that the activity of AMPK in hepatocytes could be regulated by sirtuin 3 (SIRT3) \textit{in vitro}.

SIRT is a NAD\textsuperscript{+}-dependent protein deacetylase, which was first discovered in yeast and termed a silent information regulator 2 (SIR2).\textsuperscript{13,14} The mammalian SIR2 gene family sirtuin has seven members.\textsuperscript{15–17} SIRT1 has been shown to play important roles in adipocyte differentiation and cell apoptosis.\textsuperscript{18–20} Previous studies have demonstrated that SIRT3 was localized on the inner membrane of mitochondria and decreased the production of reactive oxygen species. In addition, it played an important role in adaptive thermogenesis in brown adipose tissue.\textsuperscript{21}

This study was designed to investigate the role of SIRT3 in hepatic lipid accumulation, and whether the activation of AMPK is required.

**MATERIALS AND METHODS**

**Cell culture and differentiation**

10T1/2, BOSC23 and HepG2 cells were cultured in Dulbecco’s Modified Eagle Medium (Gibco, Rockville, MD, USA) containing 10% bovine calf serum or 10% fetal bovine serum at 37°C. For 10T1/2 cell differentiation, cells at confluence were treated with 20 μmol/L 5-azacytidine (Sigma, St Louis, MO, USA) and 5 μg/mL insulin (Sigma, St Louis, MO, USA) for 3 days, and then refed with growth media containing insulin every 2 days. On day 8 of differentiation the cells were harvested.

**Plasmid construction**

A standard polymerase chain reaction-based cloning strategy was used to generate the human and murine PcDNA3.1-SIRT3 (PcDNA3.1-hSIRT3 and PcDNA3.1-mSIRT3) constructs. Site-directed mutagenesis was used to generate hSIRT3H248Y, where amino acid 248 had been changed from a histidine to a tyrosine to disrupt deacetylase activity.\textsuperscript{22} All the constructs were sequenced and then subcloned in a pBabe-puro vector as described previously.\textsuperscript{21}

**Retroviral infection**

BOSC23 cells were transfected with pBabe-puro-mSIRT3, pBabe-puro-hSIRT3 and its mutant by the calcium phosphate method. Forty-eight hours after transfection, the supernatants were saved and filtered through a 0.45 micron filter and then were used to infect 10T1/2 cells and HepG2 cells. Cells were then selected with 4 μg/mL puromycin.
Lentivirus-mediated SIRT3 small interfering RNA (siRNA)

For knocking down human SIRT3, specific siRNA (5'-GCC CAA CGT CAC TCA CTA CTT-3') targeting SIRT3 was designed. The BLOCK-iT lentiviral expression system was used to transduce siRNA to HepG2 cells according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). The knockdown of hSIRT3 was examined by Western blot analysis.

Postinfection procedure

HepG2 cells (purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) were treated with oleate at a concentration of 2 mmol/L for 6 days to induce lipid accumulation. One hour before harvesting, 1 μmol/L isoproterenol (Sigma, St Louis, MO, USA) was used to stimulate the cells for the phosphorylation of AMPK and ACC, and in another set of experiments infected cells were incubated with 2 mmol/L of oleate for 24 h for the staining with Nile red. Phosphorylation of AMPK and ACC was also examined in the presence of AMPK inhibitor compound C (CC) (Sigma, St Louis, MO, USA). HepG2 cells were pretreated with CC at 50 μmol/L for 24 h, followed by isoproterenol stimulation.

Northern blot

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The RNA samples (10 μg) were separated on 1% (w/v) agarose-formaldehyde gel and then transferred to a nylon membrane (Amersham Bioscience, Buckinghamshire, UK) by capillary blotting. The gene-specific probe was labeled with [a-32P]dCTP using the random primer method (Promega, Madison, WI, USA). Northern hybridization was carried out overnight at 65°C. The membrane was exposed to X-film (Eastman Kodak, Rochester, NY, USA) with two intensifying screens for 1–3 days.

Western blot

The HepG2 cells were washed in ice-cold phosphate-buffered saline and resuspended in a lysis buffer. Equal amounts of whole cell lysates were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The antibodies used for Western blot included anti-AMPKα1 and anti-pAMPK Thr-172 (Abcam, Cambridge, MA, USA), anti-hSIRT3 (Santa Cruz biotechnology, CA, USA), anti–ACCα and anti–pACC Ser 79 (Santa Cruz, CA, USA), and anti-actin (Abcam, Cambridge, MA, USA). The membrane was then incubated with appropriate secondary antibodies coupled to horseradish peroxidase and visualized by Enhanced Chemiluminescence (ECL) detection. The signal intensities were quantified by densitometric analysis. Actin was used as a control to standardize the expression level of protein.

Nile red staining

The HepG2 cells incubated with or without oleate were fixed with 3.7% paraformaldehyde, then the cells were stained with 0.1 mg/mL Nile red for 5 min in phosphate-buffered saline. Digitized fluorescent microscopy at excitation and emission wavelength settings of 543 and 598 nm, respectively, was used to quantified Nile red staining as reported previously.

Statistical analysis

Data were expressed as mean ± SE. The comparison of the two groups was performed using Student’s t test. P < 0.05 was considered to be statistically significant.

RESULTS

SIRT3 inhibits the differentiation of 10T1/2 into adipocytes

The role of SIRT3 in adipogenesis was studied in murine embryo pluripotent mesenchymal 10T1/2 cells by Northern blot analysis. Adipsin, which is an adipocyte-specific protein, was examined to assess adipogenesis. The result showed that the expression level of adipsin in cells overexpressing murine SIRT3 was lower than those without SIRT3 overexpression (Fig 1). This indicates that SIRT3 indeed inhibits adipogenic differentiation and raises the possibility that SIRT3 may play a role in fat metabolism-related diseases such as NAFLD.

Overexpression of SIRT3 reduces lipid accumulation and stimulates AMPK signaling pathway in HepG2 cells

Excessive hepatic fat accumulation is the hallmark of NAFLD. We investigated whether SIRT3 would influence lipid synthesis in hepatocytes. Retroviral vector expressing wild type SIRT3 was infected into the
human hepatoma cell line HepG2 cells, which were then treated with oleate and Nile red staining was performed. Nile red is an excellent stain for the detection of intracellular lipid droplets by a fluorescent microscopy or flow cytometry. The intensity of fluorescence represents the number of lipid droplets in cells. Our result showed that the number of lipid droplet in HepG2 cells overexpressing SIRT3 was significantly lower than in those without SIRT3 overexpression after treatment with oleate (P < 0.05) (Fig 2a and 2b).

Because ACC is the rate-limiting enzyme involved in FFA synthesis and ACC is the downstream effector of AMPK. ACC is inactivated upon its phosphorylation, which slows down FFA synthesis. The phosphorylation of AMPK and ACC in HepG2 cells was examined by Western blot analysis. As shown in Figure 2c,d, AMPK phosphorylation was potently stimulated by the overexpression of SIRT3 in HepG2 cells, and the SIRT3 infected cells also exhibited significantly more phosphorylation of ACC than the control cells. No changes in endogenous AMPKα1 and ACC proteins were detected. Therefore, these data suggest that SIRT3 has an inhibitory effect on lipid accumulation in the hepatocyte and AMPK may mediate the function of SIRT3 in hepatic lipid accumulation.

Figure 1. Sirtuin 3 (SIRT3) inhibits differentiation of 10T1/2 cell into adipocyte. 10T1/2 cell stably transfected with mSIRT3 was differentiated into adipocyte with 20 μmol/L azacytidine and 5 μg/mL insulin. On day 8 of differentiation, total RNA was extracted and adipsin (adipocyte specific protein) was detected by Northern blot. (EtBr) Ethidium bromide.

Figure 2. Overexpression of Sirtuin 3 (SIRT3) reduces lipid accumulation and stimulates the AMP-activated protein kinase (AMPK) signaling pathway in HepG2 cells; (a) retrovirus-mediated overexpression of SIRT3 in HepG2 cells was examined with Western blot analysis; (b) HepG2 cells infected with or without SIRT3 were treated with 2 mmol/L oleate or without oleate for 24 h, and stained with 0.1 mg/mL of Nile red. The staining was quantified with digitized fluorescent microscopy at excitation and emission wavelength settings of 543 and 598 nm, respectively. Vector; SIRT3; (c) and (d) HepG2 cells were treated with 2 mmol/L of oleate for 6 days and stimulated with 1 μmol/L isoproterenol for 1 h, then the cells were harvested. Vector; SIRT3; pAMPK phosphorylated AMPK; pACC phosphorylated ACC. © 2010 The Authors. Journal compilation © 2010 Chinese Medical Association Shanghai Branch, Chinese Society of Gastroenterology and Blackwell Publishing Asia Pty Ltd.
SIRT3 knockdown downregulates phosphorylation of AMPK and ACC and facilitates fat accumulation in HepG2 cells

To ascertain the role of SIRT3 in hepatic lipid accumulation, lentivirus-mediated vector encoding siRNA targeting human SIRT3 (vector encoding control siRNA was used as control) was used to infect HepG2 cells to shut down SIRT3 expression. After treatment with oleate and stimulation with isoproterenol, the phosphorylation of AMPK and ACC was examined by Western blot. As shown in Figure 3a, endogenous SIRT3 expression in cells infected with SIRT3 siRNA was remarkably suppressed compared with those infected with control siRNA. Notably, the knockdown of SIRT3 downregulated the phosphorylation of AMPK and ACC by 60–80% (Fig. 3b,c). Correspondingly, as shown in Figure 3d, SIRT3 knockdown significantly facilitated fat accumulation in HepG2 cells. Taken together with Figures 2 and 3, these results support the idea that SIRT3 has beneficial metabolic effects on hepatic lipids by stimulating the AMPK pathway. SIRT3 stimulates the phosphorylation of AMPK, which phosphorylates and then inactivates ACC and eventually inhibits lipid deposition in hepatocytes.

AMPK inhibitor reverses the effects of SIRT3 on the phosphorylation of AMPK and ACC and on fat accumulation in HepG2 cells

To explore the role of AMPK on the effect of SIRT3 on hepatic lipid accumulation, 50 μmol/L compound C (CC), which is an AMPK inhibitor, was used to pretreat HepG2 cells for 24 h before isoproterenol stimulation. Western blot analysis was...
carried out to detect the phosphorylation of AMPK and ACC. Consistent with the results showed in Figure 2c,d, overexpression of SIRT3 by a retroviral infection system increased the phosphorylation of AMPK and ACC significantly in HepG2 cells. It is worthy of note that CC treatment abolished this effect (Fig. 4a–c). A similar pattern was observed in fat accumulation (Fig. 4d). The overexpression of SIRT3 inhibited oleate-induced fat accumulation whereas SIRT3 lost its inhibitory effect in the presence of CC. These data strongly suggest that AMPK is necessary for the inhibiting effect of SIRT3 on hepatocellular lipid accumulation.

**SIRT3 activation of AMPK is dependent on its deacetylase activity**

Both the overexpression and knockdown of SIRT3 could modulate the phosphorylation of AMPK. Since SIRT3 is a NAD-dependent deacetylase, we detected whether the effect of SIRT3 stimulating AMPK activation is dependent on its deacetylase activity. In this study a SIRT3 mutant, SIRT3H248Y, was constructed, whose deacetylase activity was disrupted by changing the amino acid 248 from a histidine to a tyrosine. A retrovirus mediated vector encoding either wild type SIRT3 or SIRT3H248Y was used to infect HepG2 cells. After treatment with oleate and stimulated with isoproterenol, the phosphorylation of AMPK was examined by immunoblot. As shown in Figure 5, SIRT3 up-regulated the phosphorylation of AMPK, whereas SIRT3H248Y had no such capability and even acted as a negative modulator. These data indicate that SIRT3-stimulating AMPK phosphorylation is dependent on its deacetylase activity.

**DISCUSSION**

Reduced thermogenesis may contribute to the development of obesity in mice. SIRT3 could regulate adaptive thermogenesis by increasing mitochondria respiration. NAFLD, caused by lipid accumulation in hepatocytes, is a hepatic manifestation of the metabolic syndrome associated with obesity and insulin resistance. Whether SIRT3 plays a role in NAFLD remains to be identified. We focused our study on the function of SIRT3 on lipid metabolism in hepatocytes.

First, we found that the constitutive expression of murine SIRT3 in pluripotent mesenchymal 10T1/2 cells reduced adipogenesis when treated with azacytidine and insulin. The expression of adipins, which is an adipocyte-specific protein, was decreased in 10T1/2...
cells overexpressing murine SIRT3. This result raises the possibility that SIRT3 reduces lipogenesis in hepatocytes. Then we used the human hepatoma cell line HepG2 cells treated with oleate as a model to investigate whether human SIRT3 could inhibit lipid synthesis in hepatocytes. HepG2 cells overexpressing human SIRT3 were treated with oleate for 24 h. Nile red staining was performed to assess lipogenesis in the hepatocytes. We demonstrated that the number of lipid droplets in cells infected with human SIRT3 was significantly lower than that in cells without SIRT3 overexpression. We also examined lipogenesis by days after treatment with oleate, which showed that the difference in the number of lipid droplets between SIRT3 infected cells and control cells was gradually enlarged and culminated at the 6th day without any cellular morphological abnormality (data not shown).

It is well accepted that de novo fatty acid synthesis contributes to the development of hepatic steatosis. ACC is the rate-limiting enzyme involved in lipid synthesis and acts as the downstream effector of AMPK. Additionally, SIRT3 is expressed in brown adipose tissue and is localized on mitochondria. It modulates energy homeostasis by regulating the mitochondria function. AMPK is the sensor of cellular energy status. Therefore, we tried to figure out whether SIRT3 could phosphorylate ACC through the activation of AMPK. We failed to detect any significant difference in AMPK phosphorylation between cells with SIRT3 overexpression and control cells until the 6th day, whereas the enhanced phosphorylation of ACC could be detected during the whole 6 days (data not shown). This phenomenon could be rationalized by the fact that a minor change, usually undetectable by common methods, could trigger a series activating downstream factors and ultimate effectors, during which the signal could be exponentially amplified. This evidence suggests that the phosphorylation of AMPK by SIRT3 occurs at the early stage of signaling cascade.

To obtain further evidence, SIRT3 knockdown HepG2 cells were used to investigate the role of SIRT3 on hepatic lipid metabolism and AMPK activation. As expected, SIRT3 knockdown promoted lipid synthesis and decreased AMPK/ACC phosphorylation. Collectively, SIRT3 inhibited lipid accumulation in hepatocytes, thereby preventing NAFLD formation and development. The intriguing question is whether AMPK is required for the effect of SIRT3 on inhibiting hepatocellular lipid accumulation. The activity of AMPK could be inhibited by 6-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a] pyrimidine that is also known as compound C.27 It was shown that this compound could antagonize aminomimidazole-4-carboxamide ribonucleotide (AICAR) by blocking the uptake of AICAR into cells. HepG2 cells overexpressing SIRT3 and control cells were incubated with or without compound C.

SIRT3 is known as one of the class III histone deacetylases. Here we show that the capacity of SIRT3 to activate AMPK is dependent on its deacetylase activity, which raises the question as to what connects SIRT3 deacetylase activity to AMPK activation. Uncoupling protein 1 (UCP1) may be one reasonable candidate, based on the fact that SIRT3 promotes the expression of UCP1 in deacetylase-dependent manner and UCP1 has the potential to increase cellular AMP level, which thereby activates AMPK. Recently SIRT6, another member of the Siruin family, was shown to attenuate NF-kappa B signaling via H3K9 deacetylation at chromatin.28 Given that the NF-kappa B signaling pathway is implicated in fatty acid-induced NAFLD, the possi-

Figure 5. Sirtuin 3 (SIRT3)-stimulated AMP-activated protein kinase (AMPK) phosphorylation is dependent on its deacetylase activity; (a) and (b) HepG2 cells constitutively expressed human SIRT3 and its mutant, H248Y. Cells were harvested after treatment with oleate and isoproterenol and phosphorylations of AMPK and AMPKa1 were detected by immunoblot analysis (mean ± SE, n = 3). *P < 0.05.
bility was raised that SIRT3 may share the NF-kappa B pathway to inhibit NAFLD.

In conclusion, we have identified the role of SIRT3 in suppressing hepatic lipid accumulation, for which AMPK activation is required. Therefore, the activator of SIRT3 and/or AMPK could be a promising agent for the blockage and even reversal of NAFLD.

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