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**Basic Study**

Sirtuin1 attenuates acute liver failure by reducing reactive oxygen species *via* hypoxia inducible factor 1α

Cao P *et al.* Sirt1 attenuates ALF *via* HIF-1α

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Abstract

BACKGROUND
The occurrence and development of acute liver failure (ALF) is closely related to a series of inflammatory reactions, such as the production of reactive oxygen species (ROS). Hypoxia inducible factor 1α (HIF-1α) is a key factor that regulates oxygen homeostasis and redox and the stability of HIF-1α is related to the ROS level regulated by Sirtuin (Sirt) family. The activation of Sirt1 will lead to a powerful antioxidant defense system and therapeutic effects in liver disease. However, little is known about the relationship between HIF-1α and Sirt1 in the process of ALF and the molecular mechanism.

AIM
To investigate whether HIF-1α may be a target of Sirt1 deacetylation and their effects on ALF.

METHODS
Mice were administrated by lipopolysaccharide (LPS)/D-gal and exposed to hypoxic conditions as animal model and Resveratrol was used as an activator of Sirt1. The cellular model was established with L02 cells stimulated by LPS. N-acetyl-L-cysteine was used to remove ROS and the expression of Sirt1 was inhibited by Nicotinamide. Western blotting was used to detect Sirt1 and HIF-1α activity and related protein expression. The possible signaling pathways involved were analyzed by Immunofluorescent staining, Co-immunoprecipitation, Dihydroethidium staining and Western blotting.

RESULTS
Compared with mice stimulated with LPS alone, the expression of Sirt1 decreased and the level of HIF-1α acetylation increased in hypoxic mice, and the levels of carbonic anhydrase 9 and Bcl-2 adenovirus E1B interacting protein 3 increased significantly, which was regulated by HIF-1α, indicating an increase of HIF-1α activity. Under
hypoxia, the down-regulation of Sirt1 activated and acetylated HIF-1α in L02 cells. The inhibition of Sirt1 significantly aggravated this effect and the massive production of ROS. The regulation of ROS was partly through PPARα or AMPK. Resveratrol, Sirt1 activator, effectively relieved ALF aggravated by hypoxia, the production of ROS and cell apoptosis. It also induced the deacetylation of HIF-1α and inhibited the activity of HIF-1α.

CONCLUSION

Sirt1 may have a protective effect on ALF by inducing HIF-1α deacetylation to reduce ROS.

Key Words: Acute liver failure; Deacetylation; Hypoxia inducible factor 1α; Reactive oxygen species; Sirtuin 1

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Core Tip: Hypoxia inducible factor 1α (HIF-1α) is a transcription factor that regulates oxygen homeostasis. Under hypoxic conditions, HIF-1α translocates to the nucleus and binds to β-subunits, resulting in transcription of target genes. In acute liver failure, HIF-1α contributes to early liver cell necrosis. The activation of Sirtuin1 (Sirt1) will result in a powerful antioxidant defense system. This study examined the influence of Sirt1-mediated pathways on HIF-1α expression in vivo and in vitro, explored the relationship between Sirt1 and HIF-1α, and further explored its potential mechanism.
INTRODUCTION

Acute liver failure (ALF) refers to a large number of necrosis of liver cells or severe liver damage caused by various reasons\(^1\). ALF is often accompanied by coagulation dysfunction and progressive multiple organ failure due to liver metabolism disorders and decreased immune function\(^2\). The occurrence and development of ALF is closely related to a series of inflammatory reactions, such as the release of inflammatory cytokines and the production of reactive oxygen species (ROS)\(^3\).

HIF-1 consists of an oxygen-regulated subunit HIF-1\(\alpha\) and a constitutive expression subunit HIF-1\(\beta\). The activity and stability of the alpha subunit of HIF are regulated by its post-translational modifications such as acetylation\(^4\). Under hypoxic conditions, hypoxia-inducible factor (HIF)-1\(\alpha\) acts as a primary transcription factor to regulate hypoxia-related anti-inflammatory responses\(^5\). HIF-1\(\alpha\) is a key factor that regulates oxygen homeostasis and redox, and promotes effective adaptation to hypoxia\(^6\). During the development of liver diseases such as liver cancer, hypoxia is a common finding. Hypoxia promotes the stabilization of HIF-1\(\alpha\). HIF signal in innate immune cells and liver cancer cells is beneficial to the recruitment and maintenance of primordial tumorigenic immune cells, and promotes immune evasion\(^7\).

The monitoring of HIF-1\(\alpha\) activity by members of the Sirtuin (Sirt) family has been a topic of interest in recent years\(^8-10\). According to reports, HIF-1\(\alpha\) has been confirmed to be related to Sirt1, Sirt2 and Sirt3 in the Sirt family, the stability of HIF-1\(\alpha\) is related to the ROS level regulated by Sirt3 and the oxygen level regulated by Sirt6\(^11-14\). Sirt2 causes protein hydroxylation and ubiquitination by increasing the binding of HIF-1\(\alpha\) to propylamine hydroxylase\(^8\). However, the regulation mechanism of Sirt1 on HIF-1\(\alpha\) activity has always been a controversial topic.

Sirt1 in the sirtuin family is a NAD\(^+\)-dependent protein lysine deacetylase with diverse physiological functions such as anti-inflammatory, neuronal signaling, DNA repair, and stress response. Sirt1 has been shown to be an important target for the treatment of various diseases\(^15,16\) and its activation will lead to a powerful antioxidant defense system and therapeutic effects in liver ischemia reperfusion\(^17\). Studies have
shown that Sirt1 regulates HIF-1α through the formation of physical complexes between proteins, and Sirt1 may have a negative regulatory effect on HIF-1α[18]. Sirt1 has also been reported to regulate HIF-1α actively by stabilizing the protein[19]. Whether Sirt1 is used as a negative regulator or a positive regulator of HIF-1α, or depends on the experimental conditions or experimental models, remains to be further studied.

In this study, we examined the regulation of Sirt1 on HIF-1α activity in ALF and explored its possible molecular mechanisms.

MATERIALS AND METHODS

Mice
Male C57BL/6j wild-type mice aged 5-6 wk were purchased from Wuhan Biomedical Research Institute of Wuhan University. All mice were raised in the specific pathogen free (SPF) animal facility of Renmin Hospital of Wuhan University with conditions of light-controlled, room temperature 25 °C, humidity 55 ± 5 % and they were free to eat and drink. All animal operations were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University, China (Approval No. WDRY2021-K016).

Animal models
The mice were randomly divided into 6 groups with 6 mice in each group: Saline control group; Hypoxia group; Lipopolysaccharide (LPS) group; Hypoxia + LPS group; Resveratrol group and LPS + Hypoxia + Resveratrol group. Hypoxia group and Hypoxia + LPS group were cultured in COY Vinyl Anaerobic Chambers (COY, United States). To avoid pulmonary and cerebral edema caused by a rapid drop in oxygenation, gradually decreased the fraction of inspired oxygen (FiO2) (1%/day) from 21% normoxia (room-air oxygen) to 8% oxygen (severe hypoxia) over the course of 2 wk, followed by continual exposure to 8% oxygen for an additional 2 wk. On the 14th day after being exposed to 8% oxygen, Resveratrol (10 mg/kg; Sigma-Aldrich, United States)[20] was given intragastric administration in Resveratrol group and LPS +
Hypoxia + Resveratrol group while LPS (100 µg/kg; Sigma-Aldrich, United States) was administrated by intraperitoneal injection combined with D-Gal (400 mg/ kg) in LPS group and LPS + Hypoxia + Resveratrol group\textsuperscript{[21]}. 24 h after LPS administration, animals were quickly euthanized with inhaled CO\textsubscript{2}, followed by blood samples and liver tissues collection\textsuperscript{[21]}.

Cell culture
Human embryonic liver cell line L02 was purchased from China Center for Type Culture Collection (CCTCC). N-acetyl-L-cysteine (NAC) (Beyotime, Shanghai, China) (5 mmol/L\textsuperscript{[22]}, Nicotinamide (NAM) (Beyotime, Shanghai, China) (5 mmol/L\textsuperscript{[23]}, GW6471 (Sigma-Aldrich, United States) (3 µM)\textsuperscript{[24]} or Compound C (Sigma-Aldrich, USA) (10 µM)\textsuperscript{[25]} which were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, United States) were used to pretreat L02 cells for 1 h and followed by LPS (5 µg mL\textsuperscript{-1})\textsuperscript{[26]} treatment. Hypoxic conditions (1% O\textsubscript{2}) were obtained using humidified variable aerobic workstation InVivo2 400 (Ruskinn, Pencoed, United Kingdom)\textsuperscript{[27]}. For transient transfection, cells were transfected with 2 µg plasmid of pECE-flag-Sirt1 (Addgene, Cambridge, MA, United States) and pECE empty vector (Addgene).

Biochemical analyses
Blood samples were collected after mice were anesthetized. The level of malondialdehyde (MDA) (Cat.No. GM1134), superoxide dismutase (SOD) (Cat.No. GM1133) and glutathione peroxidase (GSH-Px) (Cat.No. GM1135) were determined with commercial kits (Servicebio, Wuhan, China) respectively according to the manufacturer's instructions.

Histopathological examination
The liver tissues were sliced completely and stained with haematoxylin-eosin (H&E). The pathological changes of liver tissue were observed and evaluated by light
microscope (Olympus, Japan). The degree of liver damage in the ALF models were assessed by the liver histology score.

**Immunofluorescent staining**
Liver tissue sections were intact and L02 cell suspensions were fixed on glass slides. Fixed with 4% paraformaldehyde for 30 min, and added 50-100 μL membrane rupture working solution and 3% hydrogen peroxide solution in sequence according to the manufacturer's instructions. Primary antibody against Acetyl-lysine or HIF-1α (Santa Cruz Biotechnologies, CA, United States) diluted 1:100 with 5% BSA was added on the slides and tissue sections, and incubated overnight at 4 °C in a wet box. Then, secondary antibody (1:50 dilutions, Beyotime, China) was used to incubate slides. Slides were imaged using fluorescent microscope (Olympus, Japan).

**Immunoprecipitation**
Approximately 1 mg of total protein was incubated with anti-Sirt1 antibody (Servicebio) or anti-HIF-1α antibody (Servicebio) overnight at 4 °C followed by precipitation with 20 μl of protein A/G-Plus-Agarose (Servicebio) for 4 h at 4 °C. The precipitated complex was immunoblotted with anti-Sirt1, anti-HIF-1α, or anti-acetyllysine.

**Detection of ROS production**
L02 cell suspensions were fixed on glass slides. Added 2 mL cell culture fluid and continue to culture for about 6 h. Added 1 mL dihydroethidium (Cat.No. GDP1018) which was dissolved in DMSO at a ratio of 1:1000 to each well and incubated in the dark. Added an appropriate amount of DAPI solution to the wells and stained. Then added a drop of anti-fluorescence quenching mounting plate into the hole, observed and took pictures under a fluorescent microscope (Olympus, Japan).

**Western blotting**
Extracted proteins from cells and tissues as directed by the radioimmunoprecipitation assay (RIPA) kit (Sigma-Aldrich, United States). Added an appropriate amount of concentrated SDS-PAGE protein loading buffer to the collected protein samples, and then added 5-10 μL of them to the SDS-PAGE gel sample holes. Low voltage constant pressure electrophoresis for the upper gel and high voltage constant voltage electrophoresis were applied, when bromophenol blue entered the lower gel. After electrophoresis, the proteins were transferred to polyvinylidene fluoride membranes. The following primary antibodies were used: Sirt1 (Cat.No. 9475, CST), PPARα (Cat.No. 23398R, Bioss), HIF-1α (Cat.No. 20398R, Bioss), AMPK (Cat.No. 32047, Abcam), p-AMPK (Cat.No. 131357, Abcam), Bnip3 (Cat.No. 109414, Abcam) and GAPDH (Cat.No. 8245, Abcam). Image Lab statistical software (Bio-Rad, United States) was used to evaluate band intensities on western blots.

Statistical analyses

Statistical analysis was performed using GraphPad Prism software version 8.0. The Y axis was labeled as fold of control mean. Data were expressed as the means ± SDs. Differences among multiple groups were evaluated using conventional Student’s t test or ANOVA. Statistical significance was considered at P < 0.05.

RESULTS

Hypoxia aggravated ALF and increased the expression of HIF-1α and its acetylation

The liver structure of each group was shown by histopathological examination. Compared with the control group, large-scale hepatocyte necrosis in the LPS and Hypoxia groups and the number of infiltrating inflammatory cells were significantly increased, while the inflammatory response was significantly more severe in the LPS + Hypoxia group (Figure 1A). Then, we tested the expression of some key proteins in ALF. As shown in the Figure 1B, compared with the control group, the expression of Sirt1 in the LPS group was significantly reduced and hypoxia aggravated this effect. The expression of Bcl-2 adenovirus E1B-interacting protein 3 (Bnip3) in the LPS +
hypoxia group was significantly increased, as same as carbonic anhydrase 9 (CA9), which were regulated by HIF-1α, suggesting that Hypoxia significantly increased the activity of HIF-1α in the LPS group. Of note, the expression of HIF-1α in the LPS + Hypoxia group was significantly increased in the form of acetylation. LPS significantly increased HIF-1α acetylation induced by Hypoxia (Figure 1C).

**Hypoxia reduced the expression of Sirt1 causing the activation and acetylation of HIF-1α**

Then, we detected the changes in the expression of Sirt1 in L02 cells during hypoxia. we measured the expression levels of Sirt1, HIF-1α and Bnip3 using western blotting. Compared to the control group, Hypoxia reduced Sirt1 expression and upregulated HIF-1α and Bnip3 expression in a time-dependent manner (Figure 2A-D). And through immunofluorescence experiments, we found that as the time of hypoxia increased, the expression of HIF-1α increased significantly in the form of acetylation (Figure 2E). We then analyzed the interaction between Sirt1 and HIF-1α. After hypoxia induced endogenous HIF-1α, Sirt1-HIF-1α binding was observed (Figure 2F). We next examined whether Sirt1 deacetylates HIF-1α. Immunoblotting with anti-acetyl-lysine in HIF-1α immunoprecipitates was used to detect the lysine acetylation level of HIF-1α. As shown in Figure 2G, Sirt1 overexpression significantly decreased HIF-1α acetylation, suggesting that Sirt1 regulated lysyl acetylation of HIF-1α. These results suggested that Hypoxia-induced enhancement of HIF-1α activity and lysine acetylation were related to the down-regulation of Sirt1.

**The inhibition of Sirt1 induced activation of HIF-1α and subsequently increased the production of ROS induced by hypoxia**

Next, we explored the possible molecular mechanisms of the interaction between Sirt1 and HIF-1α. As shown in the Figure 3A, LPS increased the expression of HIF-1α, and the expression of Sirt1 was further reduced after HIF-1α was increased by hypoxia in L02 cells. At the same time, the use of a specific Sirt1 inhibitor NAM to inhibit Sirt1
further aggravated this effect. Sirt1 appear to interact with HIF-1α in L02 cells. Studies have found that excessive production of ROS is considered harmful and related to hypoxia\textsuperscript{[28]}. Oxidative stress has been shown to promote inflammation during ALF\textsuperscript{[29]}. How oxidative stress is involved in inflammation during ALF remains unclear. Therefore, we examined the antioxidant effect of Sirt1 during Hypoxia. DHE staining showed that the level of ROS stimulated by LPS was significantly increased by Hypoxia, and this effect was enhanced when NAM was used to inhibit the Sirt1 signaling pathway (Figure 3B and C). Next, we found that the expression of Sirt1 was increased and the HIF-1α was opposite when NAC was used, which was an effective ROS scavenger (Figure 3D). At the same time, LPS-induced levels of ROS were significantly reversed by NAC (Figure 3E and F).

**The inhibition of Sirt1/PPARα signaling pathway increased hypoxia-induced ROS production in vitro**

Some studies have shown that liver PPARα expression is lower in patients with hepatitis C and advanced NAFLD, perhaps due to the inhibitory effect of multiple cytokines\textsuperscript{[30]}. This also shows that increasing PPARα may help reduce liver inflammation. In our study, as shown in Figure 4A, in the L02 cells stimulated by LPS, PPARα expression was decreased and aggravated after Hypoxia intervention, and its effect was further aggravated when NAM was used to inhibit the Sirt1 signaling pathway, suggesting that hypoxia-induced PPARα inhibition was closely related to Sirt1. In addition, Sirt1 expression was further reduced by the PPARα inhibitor GW6471, while HIF-1α was opposite (Figure 4B), and the levels of ROS were also improved (Figure 4C), suggesting that the inhibition of Sirt1/PPARα signaling pathway might increase hypoxia-induced ROS production in L02 cells.

**The inhibition of Sirt1/AMPK signaling pathway increased hypoxia-induced ROS production in vitro**
AMPK acts as a regulator of cellular energy metabolism and redox homeostasis. More and more evidence shows that AMPK plays a protective role by regulating the redox system[31]. Next, we further studied whether Sirt1 can regulate AMPK and its role in cell hypoxia in L02 cells. As shown in Figure 5A, the phosphorylation level of AMPK in L02 cells induced by LPS after Hypoxia treatment was significantly reduced, while NAM pretreatment aggravated this effect, indicating AMPK could be modulated by Hypoxia via Sirt1. In addition, AMPK inhibitor, Compound C further reduced the expression of Sirt1 and the expression of HIF-1α was further increased (Figure 5B) and the levels of ROS were also improved (Figure 5C). Therefore, these results suggested that Sirt1/AMPK signaling pathway might be involved in modulating ROS in LPS-stimulated L02 cells during hypoxia.

The activation of Sirt1 induced the inactivation and deacetylation of HIF-1α and subsequently rescued the progressive aggravation of ALF induced by hypoxia in vivo

Finally, to further determine whether Sirt1 attenuated the progressive aggravation of ALF induced by Hypoxia through the Sirt1/AMPK or the Sirt1/PPARα pathway, LPS-stimulated mice were exposed to Hypoxia with or without Resveratrol treatment, which was a Sirt1 activator. Compared with the LPS group, activation of Sirt1 by Resveratrol alleviated the severer liver tissue damage in the LPS + Hypoxia group (Figure 6A and B). And LPS + Hypoxia group mice showed lower activity of SOD and GSH-Px, while MDA levels were increased, indicating that hypoxia leaded to decreased antioxidant activity. However, Resveratrol treatment could significantly improve the activity (Figure 6C). As shown in Figure 6D, Resveratrol dramatically alleviated the Hypoxia-induced reduction levels of PPARα protein and the phosphorylation of AMPK in LPS-stimulated mice, suggesting that Sirt1 was a key regulator on the activation of PPARα and the phosphorylation of AMPK during Hypoxia in ALF. Finally, we demonstrated with animals whether Sirt1 has a regulatory effect on Hypoxia-induced HIF-1α lysine acetylation and HIF-1α activity. As shown in Figure 6E, with the intervention of Resveratrol, the expression of HIF-1α and the level of acetylation decreased.
significantly. These findings indicate that the activation of Sirt1 induced HIF-1α inactivation and deacetylation, thereby alleviated the progressive aggravation of ALF induced by hypoxia.

**DISCUSSION**

Recently, more and more studies have confirmed the effect of Sirt1 in liver disease. Sirt1 has been confirmed to have a protective effect in a variety of disease models, including liver fibrosis\cite{32}, drug-induced liver injury\cite{33}, non-alcoholic fatty liver disease\cite{34} and fatty liver\cite{35}. As well known, HIF-1α is a transcription factor that can promote the adaptive response of cells to hypoxia. Some reports have mentioned the connection between Sirt1 and HIF protein, but there are still many controversies about the results. According to reports, in hypoxic Hep3B or HEK293 cells, Sirt1 targeted HIF-2α and increased the transcriptional activity of HIF-2α, but not HIF-1α\cite{36}. On the contrary, another group of studies showed that Sirt1 interacted with HIF-1α, causing HIF-1α deacetylation to promote its activity in Hep3B and Huh7 cells\cite{19}. Therefore, the regulation of Sirt1 on the activity of HIF-1α and its expression seems to be cell-type-specific, which is currently unclear. It has not been reported that the beneficial effect of Sirt1 activation is related to its HIF-1α deacetylation against ALF.

In our research, we found that the activity of HIF-1α increased after acetylation and promoted hepatocyte apoptosis in ALF models and hypoxia models *in vitro*. In addition, we demonstrated that the expression of Sirt1 in L02 cells decreased in a time-dependent manner due to hypoxia, which was closely related to the activation and acetylation of HIF-1α. During hypoxia, with the decrease of the level of nicotinamide adenine dinucleotide (NAD\(^+\)), the activity of Sirt1 decreased and HIF-1α transcription activity further increased\cite{18,19}. Therefore, the insufficient expression of Sirt1 in the liver or the acetylation of HIF-1α might be the key mediators of ALF.

Next, we carefully evaluated Sirt1's regulatory effect on HIF-1α activity in ALF and explored its possible molecular mechanisms. ROS are by-products of normal metabolism in living cells, but excessive ROS accumulation can damage organelles,
leading to increased oxidative stress\textsuperscript{[37,38]}. ALF produces excessive amounts of ROS due to insufficient detoxification of toxic substances in the liver\textsuperscript{[39]}. Sirt1 has been reported to play an important role in anti-inflammatory and antioxidant processes\textsuperscript{[40]}. Here, we demonstrated that HIF-1α was over-activated in hypoxia due to increased level of ROS in the absence of Sirt1 and the effect was inhibited by the antioxidant NAC, indicating that ROS was involved in this activation.

In particular, PPARα is reported to be a potent inhibitor of NF-κB signaling pathway and inflammation\textsuperscript{[41]} and the positive effect of Sirt1 on the inflammatory pathway may be related to PPARα\textsuperscript{[42]} and the interference of PPAR transcriptional activity may disturb estrogen/androgen receptor expression and impair steroidogenesis and ROS metabolism\textsuperscript{[43]}. In addition, PPARα contributes to the protection of redox homeostasis\textsuperscript{[44]}. Previous studies have confirmed that Sirt1 can regulate AMPK, which was an important energy sensor\textsuperscript{[45]}. AMPK acts as a regulator of cellular energy metabolism and redox homeostasis. More and more evidence shows that AMPK plays a cardiovascular protective role by regulating the redox system\textsuperscript{[46]}. In diabetes, the activation of AMPK increases the expression of mitochondrial antioxidant enzymes and leads to a decrease in the production of mitochondrial ROS in endothelial cell\textsuperscript{[47]}

Our experiments revealed that the inhibition of PPARα and the phosphorylation of AMPK induced by Hypoxia were closely related to Sirt1 and the inhibition of Sirt1/PPARα or Sirt1/AMPK signaling pathway might increase hypoxia-induced ROS production in L02 cells. In order to determine whether the activation of Sirt1 induced inactivation of HIF-1α, subsequently rescued the progressive aggravation of ALF induced by Hypoxia in vivo, mice were treated with Resveratrol. As expected, the activation of Sirt1 significantly alleviated the degree of liver damage in ALF and enhanced antioxidant activity. And Resveratrol dramatically alleviated the Hypoxia-induced reduction level of PPARα protein and the phosphorylation of AMPK in LPS-stimulated mice. In addition, the activation of Sirt1 induced the deacetylation of HIF-1α compared to LPS-stimulated mice exposed to Hypoxia, the expression of HIF-1α and the level of acetylation decreased significantly.
One limitation of our study is that we did not use HIF-1α overexpressing mice *in vivo* to test whether the increase of Sirt1 activity can rescue ALF. We need to conduct further experiments to solve this problem.

**CONCLUSION**

In summary, we have demonstrated the role that Sirt1 reduced oxidative stress in ALF by regulating the activity and acetylation of HIF-1α achieved by normalizing the Sirt1/PPARα and Sirt1/AMPK pathway. Our research showed that the deacetylation and inactivation of HIF-1α induced by the activation of Sirt1 might have therapeutic benefits in reducing liver damage during ALF.
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