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SIRT1 attenuates palmitate-induced endoplasmic reticulum stress and insulin resistance in HepG2 cells via induction of oxygen-regulated protein 150

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ABSTRACT

Endoplasmic reticulum (ER) stress has been implicated in the pathology of type 2 diabetes mellitus (T2DM). Although SIRT1 has a therapeutic effect on T2DM, the mechanisms by which SIRT1 ameliorates insulin resistance (IR) remain unclear. In this study, we investigated the impact of SIRT1 on palmitate-induced ER stress in HepG2 cells and its underlying signal pathway. Treatment with resveratrol, a SIRT1 activator significantly inhibited palmitate-induced ER stress, leading to the protection against palmitate-induced ER stress and insulin resistance. Resveratrol and SIRT1 overexpression induced the expression of oxygen-regulated protein (ORP) 150 in HepG2 cells. Forkhead box O1 (FOXO1) was involved in the regulation of ORP150 expression because suppression of FOXO1 inhibited the induction of ORP150 by SIRT1. Our results indicate a novel mechanism by which SIRT1 regulates ER stress by overexpression of ORP150, and suggest that SIRT1 ameliorates palmitate-induced insulin resistance in HepG2 cells via regulation of ER stress.

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of character of obesity and insulin resistance in patients with type 2 diabetes mellitus (T2DM) [1,2]. In patients with obesity and NAFLD, hepatic endoplasmic reticulum (ER) stress plays a critical role in unvalancing metabolic homeostasis [3]. Connection between ER stress and metabolic dysregulation may represent a common pathological mechanism in obesity and T2DM [3,4]. This is significant to investigate the mechanisms that can serve as attractive therapeutic targets to ameliorate ER stress in metabolic syndrome.

When the ER, an organelle that control protein synthesis, is affected by extra cellular stress signals, an adaptive system, the unfolded protein response (UPR) starts to relieve protein misfolding stress in the ER. Protein kinase-like ER kinase (PERK) and eukaryotic initiation factor 2 alpha (eIF2 alpha) are activated in chronic ER stress and consequently attenuate new protein synthesis [5]. Inositol-requiring transmembrane kinase and endonuclease 1 alpha (IRE-1a) induces the splicing of X-box binding protein 1 (XBP-1), a transcription factor which can regulate ER stress genes, such as GPR78 [6]. Both PERK and IRE-1 alpha activate the main stress kinase JNK and this kinase induces serine phosphorylation of insulin receptor substrate-1 (IRS-1), leading to insulin resistance (IR) [7]. Chemically synthesized chaperones that attenuate ER stress can ameliorate the liver cell response to ER stress and protect against metabolic dysfunction [8]. This evidence suggests that the adaptive capacity of ER can be approved and that may provide potential novel therapeutic targets.

We have identified SIRT1 activation as a central mechanism by which polyphenols, such as resveratrol, prevent hyperlipidemia in low density lipoprotein receptor deficient ($LDLR^{-/-}$) type 1 diabetes model mice [9]. Other groups have recently investigated that both SIRT1 and AMPK pathways contribute to the beneficial effects of polyphenols on hepatic lipid metabolism in *in vitro* and *in vivo* [10].

Here, we report that SIRT1 ameliorates palmitate-induced IR through induction of ORP150 gene expression in HepG2 cells. Our study has found induction of ORP150 gene expression by SIRT1 activation and demonstrated that SIRT1 induced ORP150 through FOXO1. Furthermore, suppression of ORP150 blocked significantly the protective effect of resveratrol, a SIRT1 activator on palmitate-induced ER stress and IR. Therefore, we conclude that SIRT1 attenuates palmitate-induced ER stress and IR via modulation of ORP150 by FOXO1. This study also suggests that impaired expression of SIRT1 and ORP150 in hyperlipidemia may play a causal role in ER stress-induced IR in hepatocytes.

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2. Material and methods

2.1. Culture media and reagents

Commercially available HepG2 human hepatoma cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA). HepG2 cells were plated at a density of 10^5 cells/ml and cultured in DMEM medium containing heat inactivated 1% (v/v) fetal bovine serum (FBS) and 100 U/ml penicillin-100 μg/ml streptomycin. Palmitate and thapsigargin (TG) as a chemical inducer of the unfolded protein response (UPR) was purchased from Sigma (St. Louis, MO). Palmitate was conjugated to BSA at a 2:1 M ratio. Resveratrol was purchased from Sigma.

2.2. Transient transfection of human HepG2 cells

pcDNA SIRT1 was transfected into HepG2 cells with lipofectamin 2000 (Invitrogen, Grand Island, NY), performed according to a standard direction. Overexpression of SIRT1 was confirmed by Western blot analysis. Two hundred picomole ORP150 specific siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) and FOXO1a specific siRNA (Santa Cruz Biotechnology) were transfected in HepG2 cells with lipofectamin 2000, performed according to a standard direction. Suppressions of ORP150 and FOXO1a were confirmed by Western blot analysis.

2.3. Western blot analysis

This was performed using specific antibodies to p-PERK, PERK, p-eIF2 alpha, eIF2 alpha, p-Akt, Akt, FOXO1a, OR150, SIRT1, and beta actin at 1:1000 dilution. Horse radish peroxidase-conjugated secondary antibody (anti-rabbit at 1:10,000 dilution) was used and proteins were visualized by the chemiluminescence method. Equal loading of protein is normalized by beta actin as an internal standard.

2.4. Quantitative RT-PCR

Total RNA from treated cells was extracted with Trizol (Invitrogen) according to the manufacturer's direction. The mRNAs were reverse-transcribed with Super script RT kit ver. 3 (Invitrogen). Real-time PCR was performed with the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) on the ABI 7500 DNA Sequence Detection System with standard fluorescent chemistries. We used GAPDH as an internal standard. Human ORP150: forward, 5′-TGCA GTGATCACCGTGCCAG-3′ and reverse, 5′-TCTTTCCGGCGGAAGA-CACC-3′. Human CHOP: forward, 5′-ATGGCAGCTGAGTCATTGCCTT TC-3′ and reverse, 5′- AGAAGCAGGGTCAAGAGTGGTGAA-3′. Human GAPDH: forward, 5′-GAGTCAACGGATTTGGTCGT-3′ and reverse, 5′-GACAAGCTTCCCGTTCTCAG-3′.

2.5. Statistical analysis

The results are expressed as the mean ± SEM. The difference among groups was analyzed ANOVA with a post hoc analysis by Duncan's multiple test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. SIRT1 reduces endoplasmic reticulum (ER) stress-mediated IR in HepG2 cells

Hyperlipidemia has been reported to induce ER stress, which may lead to IR [11]. Therefore, we treated HepG2 cells with palmitate and

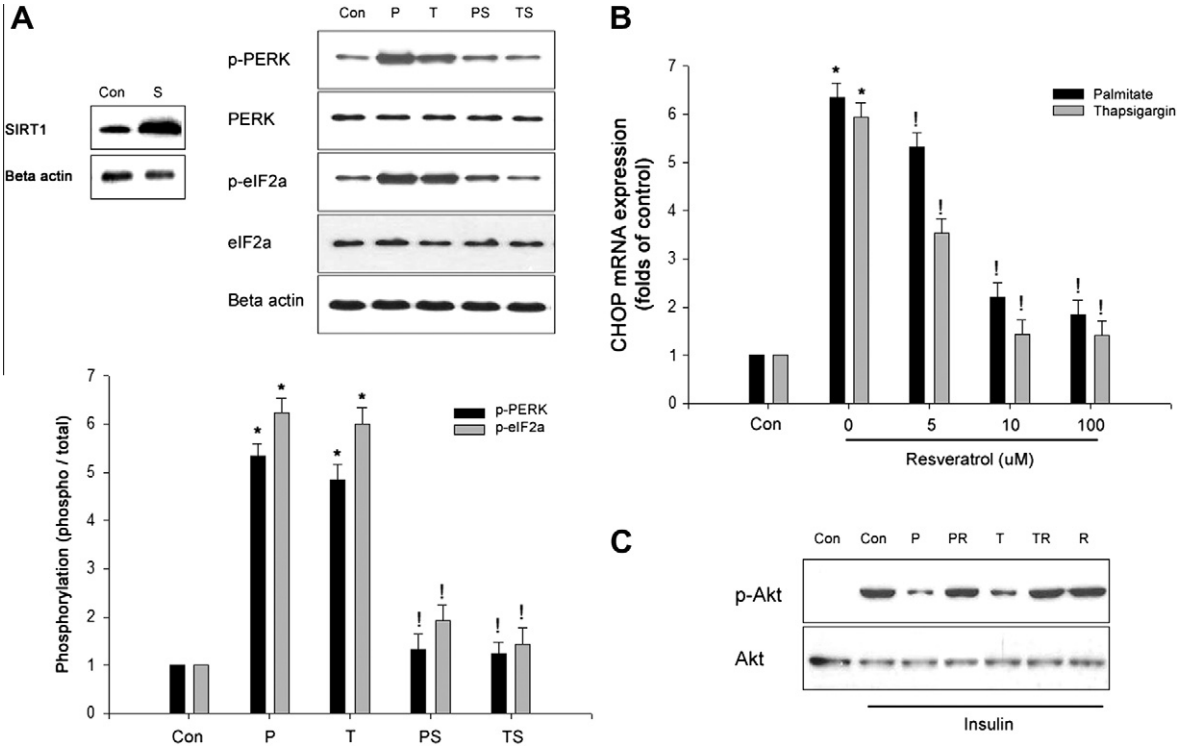

Fig. 1. SIRT1 attenuates palmitate- and thapsigargin-induced ER stress and IR in HepG2 cells. (A) Overexpression of SIRT1 was confirmed by Western blot analysis. Western blot analysis of ER stress markers (PERK and eIF2 alpha) is presented as te mean ± SEM ($n = 4$). (B) CHOP mRNA expression was measured by quantitative RT-PCR presented as the mean ± SEM ($n = 4$) (C) Western blot analysis of p-Akt and Akt from a total of three independent experiments. Treatments were carried out for 24 h. Con, transfected with an empty vector; P, 200 μM palmitate; TG, 200 nM thapsigargin; S, transfected with a SIRT1; R, resveratrol. *Significantly different from basal level ($P < 0.05$). ! Significantly different from the treatment with 200 μM palmitate or 200 nM TG ($P < 0.05$).

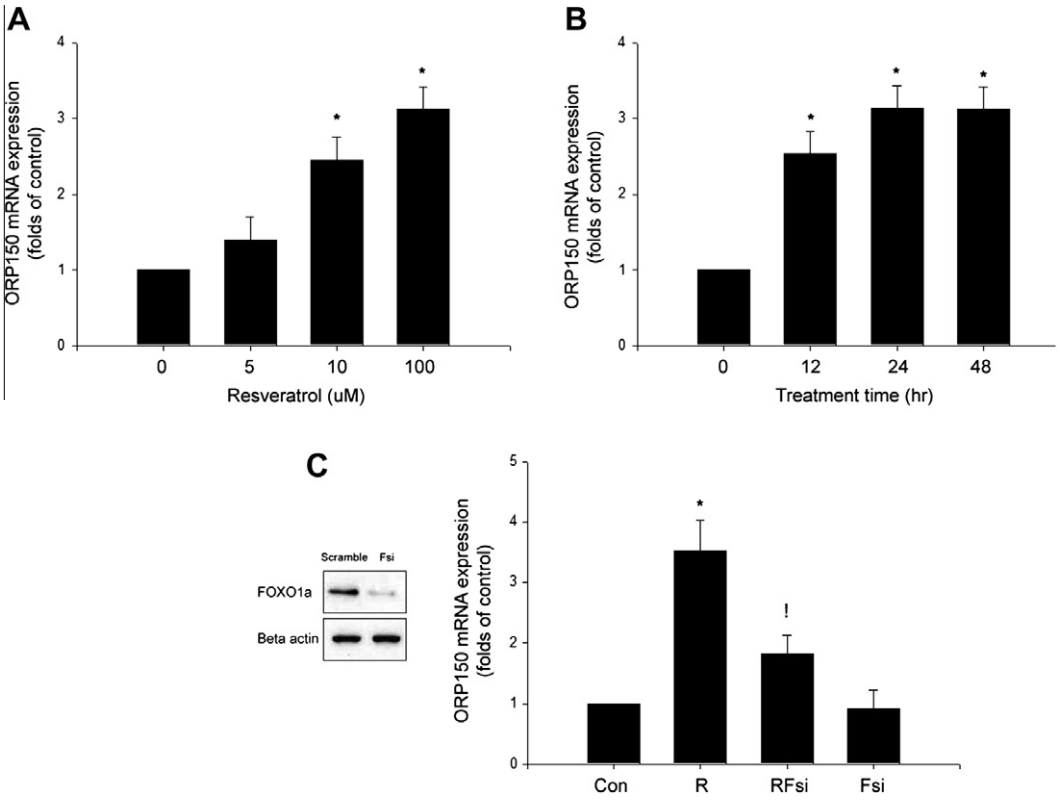

Fig. 2. SIRT1 induces ORP150 mRNA expression in dose and time dependent manners (A) ORP150 mRNA expression was measured by quantitative RT-PCR presented as the mean ± SEM ($n = 4$). This showed that resveratrol induces ORP150 mRNA expression in a dose dependent manner (0–100 μM). (B) ORP150 mRNA expression was measured by quantitative RT-PCR presented as the mean ± SEM ($n = 4$). This showed that resveratrol induces ORP150 mRNA expression in a dose dependent manner (0–48 h). (C) Suppression of FOXO1a was confirmed by Western blot analysis. ORP150 mRNA expression was measured by quantitative RT-PCR presented as the mean ± SEM ($n = 3$). This showed that FOXO1a played a critical role in the induction of ORP150 mRNA expression. Treatments were carried out for 24 h. Con, not treated or transfected with a scrambled siRNA; R, 100 μM, resveratrol; Fsi, transfected with a FOXO1a siRNA. *Significantly different from basal level ($P < 0.05$). ! Significantly different from the treatment with 100 μM resveratrol ($P < 0.05$).

thapsigargin to verify the protective effect of SIRT1 on chemically or palmitate-induced ER stress. Overexpression of SIRT1 and resveratrol inhibited thapsigargin (TG) and palmitate-induced ER stress and IR (Fig. 1).

3.2. SIRT1 induces ORP150 expression in HepG2 cells through FOXO1

We evaluated whether SIRT1 can increase ORP150 expression and treated HepG2 cells with resveratrol. Incubation of resveratrol promoted mRNA expression of ORP150 in dose and time dependent manners (Fig. 2). Suppression of FOXO1a using FOXO1a siRNA prevented the induction of ORP150 mRNA expression by resveratrol (Fig. 2).

3.3. Suppression of ORP150 expression abolishes the inhibition of palmitate-induced ER stress and IR by resveratrol

To further examine whether the protective effect of SIRT1 on ER stress and IR depends on ORP150. HepG2 cells were transfected with ORP150 siRNA or scamble siRNA as a control. ORP150 siRNA significantly suppressed ORP150 expression (Fig. 3). Suppression of ORP150 showed the inhibitory effects of resveratrol on ER stress genes (Fig. 3) and on IR (Fig. 3).

4. Discussion

Excessive ER stress contributes to hepatic IR during altered lipid metabolism [11]. Therefore, regulation of ER stress becomes critical in understanding its contribution to the pathogenesis of hepatic disorder including IR. Current study provides the novel mechanism that SIRT1 inhibits palmitate-induced ER stress and IR *in vitro* model. 1) We show that SIRT1 overexpression and activation by resveratrol protected markedly HepG2 cells against palmitate-induced ER stress and IR. 2) SIRT1 activation by resveratrol induces significantly ORP150 mRNA expression through FOXO1.3) Suppression of ORP150 in HepG2 cells abolished the protective effects of resveratrol on palmitate-induced ER stress and IR in HepG2 cells.

FOXO transcription factors are functionally modulated by phosphorylation, dephosphorylation, acetylation, and deacetylation [12]. SIRT1 deacetylates FOXO1, FOXO3, and FOXO4 and controls FOXO-target genes [13]. It has been reported that suppression of SIRT1 inhibits FOXO1 deacetylation and ORP150 expression [14]. In this study, we also demonstrated that knock down of FOXO1 impairs induction of ORP150 expression by resveratrol. This result suggests that FOXO1 plays a critical role in the link between SIRT1 and ORP150 expression for the protection against palmitate-induced ER stress.

ORP150 is an ER stress protein that serves as a chaperone and induced by extracellular stresses and signals such as starvation or oxidative stress [15]. In this study, we report that SIRT1 increases ORP150 mRNA expression for the first. This result was verified by SIRT1 agonist resveratrol. Induction of ORP150 shows protective roles in ER stress [16] and ER stress-induced dysregulation of calcium homeostasis and apoptosis [14,16], which supports the supposition that ORP150 expression could be a beneficial target against ER stress. We evaluated whether ORP150 plays an important role in the anti-ER stress effect of SIRT1. Suppression of ORP150 by ORP150

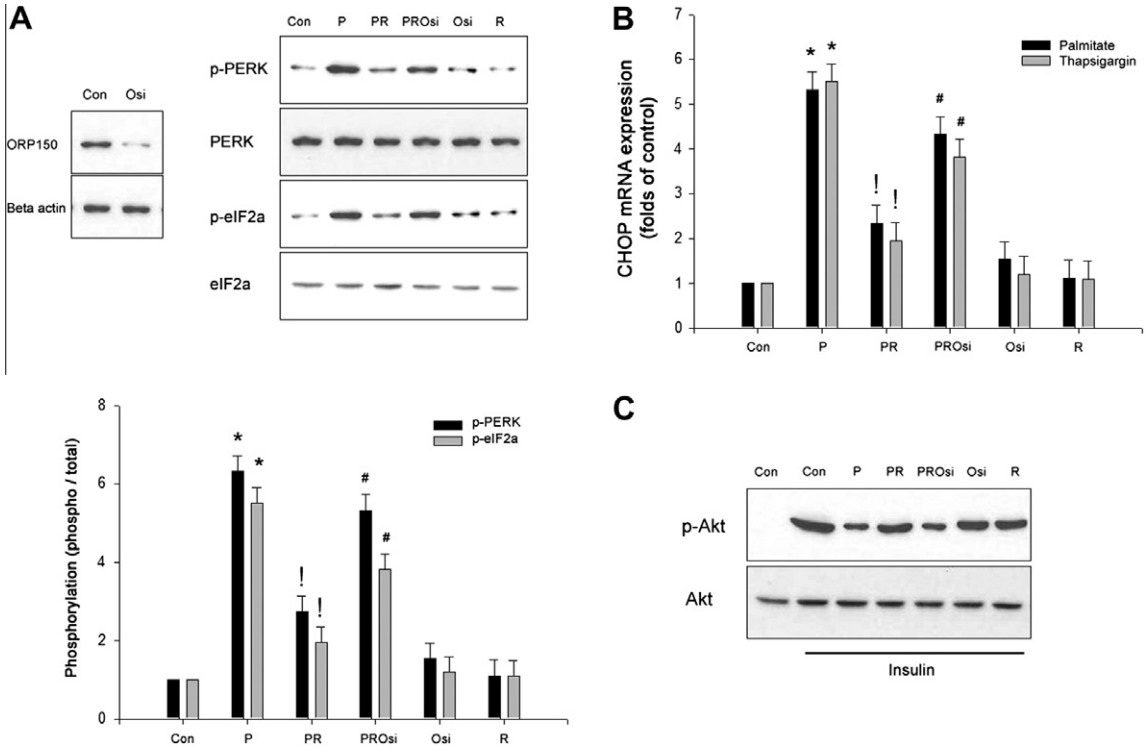

Fig. 3. SIRT1 dependent ORP150 ameliorates ER stress and IR in HepG2 cells. (A) Suppression of ORP150 was confirmed by Western blot analysis. Western blot analysis of ER stress markers (PERK and eIF2 alpha) is presented as te mean ± SEM (n = 3). (B) CHOP mRNA expression was measured by quantitative RT-PCR presented as the mean ± SEM (n = 3). (C) Western blot analysis of p-Akt and Akt from a total of three independent experiments. Treatments were carried out for 24 h. Con, transfected with a scrambled siRNA; P, 200 μM palmitate; Osi, transfected with an ORP150 siRNA; R, 100 μM resveratrol. *Significantly different from basal level ($P < 0.05$). ! Significantly different from the treatment with 200 μM palmitate ($P < 0.05$). # Significantly different from the treatment with 200 μM palmitate and 100 μM resveratrol ($P < 0.05$).

siRNA significantly blocked the effect of resveratrol on palmitate-induced ER stress markers and IR in HepG2 cells. Current results provide one of mechanisms for a direct link between SIRT1 and ER stress. The detailed mechanisms of protection against ER stress by SIRT1 require to be elucidated by further studies.

In conclusion, our results allow us to integrate SIRT1 into the transcriptional link that modulates ORP150 expression under pathologic (hyperlipidemic) condition. For the first, SIRT1 has been identified as a most direct and positive regulator of ORP150 through FOXO1a, and ameliorates palmitate-induced ER stress and IR in HepG2 cells. Therefore, SIRT1 agonist could be an effective therapeutic drug in the treatment of hyperlipidemia-induced hepatic IR.

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