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SIRT1 sensitizes hepatocellular carcinoma cells expressing hepatitis B virus X protein to oxidative stress-induced apoptosis

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ABSTRACT

We previously showed that SIRT1 deacetylase inhibits proliferation of hepatocellular carcinoma cells expressing hepatitis B virus (HBV) X protein (HBX), by destabilization of β-catenin. Here, we report another role for SIRT1 in HBX-mediated resistance to oxidative stress. Ectopic expression and enhanced activity of SIRT1 sensitize Hep3B cells stably expressing HBX to oxidative stress-induced apoptosis. SIRT1 mutant analysis showed that nuclear localization of SIRT1 is not required for sensitization of oxidation-mediated apoptosis. Furthermore, ectopic expression of SIRT1 and treatment with resveratrol (a SIRT1 activator) attenuated JNK phosphorylation, which is a prerequisite for resistance to oxidative stress-induced apoptosis. Conversely, suppression of SIRT1 activity with nicotinamide inhibited the effect of resveratrol on JNK phosphorylation, leading to restoration of resistance to oxidation-induced apoptosis. Taken together, these results suggest that up-regulation of SIRT1 under oxidative stress may be a therapeutic strategy for treatment of hepatocellular carcinoma cells related to HBV through inhibition of JNK activation.

1. Introduction

The human hepatitis B virus X (HBX) protein, encoded by human hepatitis B virus (HBV) genome, has been implicated with acute and chronic hepatitis, and is closely associated with liver cancer [1,2]. Several cell signal transduction pathways that regulate cell survival and proliferation can be activated by the HBX protein including the JAK1/STAT3 and PI3K pathways [3–6], the RAS/RAF/MAPK signaling cascade, which leads to NF-κB activation [7,8], and the WNT/β-catenin signaling pathway [9,10]. HBX also inhibits apoptosis by up-regulation of survivin, an antiapoptosis protein [11], and preventing nuclear translocation of p53, leading to abrogation of apoptosis [12]. In addition, HBX induces oxidative stress via calcium signaling and cellular kinases, resulting in activation of transcription factors NF-κB and STAT3 [3] and mitochondrial translocation of RAF-1 [13]. We also reported that hepatocellular carcinoma cells expressing HBX increase expression of forkhead box class O (FOXO) 4 through JNK to survive under oxidative stress [14].

JNK has been reported to be involved in signal transduction via growth factors, cytokines and cellular stresses, such as heat shock, UV irradiation and attack by reactive oxygen species (ROS). In particular, JNK and its downstream target c-Jun function as important regulators of cell proliferation and survival. JNK is activated by endoplasmic reticulum (ER) stress, which activates c-Jun, resulting in survival of hepatocytes. In contrast, heptocytes lacking c-Jun exhibit increased cell damage and apoptosis under ER stress [15]. In addition, JNK phosphorylates FOXO4 as a substrate, which is essential for induction of the transcriptional activity of antioxidant enzymes such as MnSOD and catalase, leading to a decrease of ROS level during oxidative stress [16]. A recent study revealed that resveratrol, a SIRT1 activator, can prevent high glucose-induced mesangial cell proliferation through inhibition of JNK and NF-κB activation [17].

SIRT1, a member of the class III histone deacetylases, has been conserved throughout evolution from yeast to man [18]. However, although the role of SIRT1 in extending the life span of organisms such as *Caenorhabditis elegans* and *Drosophila* is controversial [19], SIRT1 is implicated in the several biological processes, including metabolism, cell division, differentiation, survival, and senescence [20]. In particular, SIRT1 mediates the adaptation process to enhance the survival of an organism in the face of cellular stresses,

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such as nutrient deprivation and oxidative stress. SIRT1 can deacetylate p53, leading to attenuation of oxidation-induced apoptosis [21] and FOXO3A [22]. However, our recent study showed that overexpression of SIRT1, and enhancement of SIRT1 activity sensitizes Hep3B cells stably expressing HBX to doxorubicin-induced apoptosis [23].

Since we observed that HBX provides resistance to oxidation-induced apoptosis and other studies showed that SIRT1 is involved in the protection of cells from oxidative stress, we hypothesized that SIRT1 plays a role in protecting HBX-expressing hepatocellular carcinoma cells from oxidative stress. Here we tested this hypothesis and investigated the mechanisms involved.

2. Materials and methods

2.1. Cell cultures and transient transfection

Hep3B and Hep3B cells stably expressing vector (Hep3B-Vec) or HBX (Hep3B-HBX) were cultured in DMEM supplemented with 10% FBS, 1% penicillin, 1% streptomycin and puromycin (1 μg/ml). The cells were plated at 5×10^5 cells per well of a 6-well plate, 24 h before transfection, and then the cells were transfected with 2–4 μg of DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, USA).

2.2. Reagent, antibodies, and plasmids

Nicotinamide, resveratrol, PD98059 (ERK inhibitor), and SP600125 (JNK inhibitor) were purchased from Calbiochem (San Diego, USA). Hydrogen peroxide was obtained from Sigma–Aldrich (St. Louis, USA). Antibodies to SIRT1, GFP, FOXO3A and Actin were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Antibodies to JNK, phospho-JNK, poly (ADP-ribose) polymerase (PARP), and caspase 8 were obtained from Cell Signaling Biotechnology (Danvers, USA). For transient expression, pGFP-SIRT1-wild-type (WT), pGFP-SIRT1-NLS, and pGFP-SIRT1-NES vectors, which have been described elsewhere [24], were used.

2.3. Immunoblotting

Cells were harvested and lysed with lysis buffer [0.15 M NaCl, 1% Nonidet P-40, 50 mM Tris (pH 7.5)] containing 0.1 mM Na_2VO_3, 1 mM NaF, and protease inhibitors (Sigma–Aldrich). For immunoblotting, proteins from whole-cell lysates were resolved by 10% or 12% SDS–PAGE and transferred to nitrocellulose membranes. Primary antibodies were used at 1:1000 or 1:2000 dilutions, and secondary horseradish peroxidase (HRP)-conjugated antibodies (Santa Cruz) were analyzed at 1:2000 dilution in 5% nonfat dry milk. After a final washing, nitrocellulose membranes were exposed using a chemiluminescence assay kit (GE-Amersham, Piscataway, USA).

2.4. Statistical analysis

Data are presented as a means standard deviation. Student's *t*-test was used to compare groups and *p*-values less than 0.05 were considered significant.

3. Results

3.1. Up-regulation of SIRT1 protein and activity sensitizes Hep3B-HBX cells to oxidative stress-induced apoptosis

We have shown that, in Hep3B cells, HBX sequesters SIRT1 deacetylase, resulting in enhancing expression of β-catenin with oncogenic activity [23]. Moreover, since we reported that treatment with resveratrol, an activator of SIRT1, sensitizes Hep3B-HBX cells to doxorubicin-induced apoptosis, resveratrol is proposed to be an efficient therapeutic agent for the treatment of hepatocellular carcinoma cells related to HBV [23]. Based on these lines of evidence, we wondered whether resveratrol also sensitized Hep3B-HBX cells to oxidative stress-induced apoptosis. When we examined whether Hep3B-HBX cells are resistant to H_2O_2-induced apoptosis, we found that control Hep3B-Vec cells were sensitive to H_2O_2, while Hep3B-HBX cells were resistant to H_2O_2 (Fig. 1A). This result indicates that HBX contributes to resistance to H_2O_2 treatment, leading to survival of hepatocellular carcinoma cells under oxidative stress.

We next examined whether up-regulation of SIRT1 protein and activity enhances the sensitivity of Hep3B-HBX cells to oxidative stress-induced apoptosis. After Hep3B-HBX cells were transiently transfected with a SIRT1-GFP expression vector (pGFP-SIRT1-WT) and then treated with H_2O_2, the viability of the cells under oxidative stress was compared with that of Hep3B-HBX cells transfected with a GFP expression vector (pGFP). Cells transfected with pGFP-SIRT1-WT vector were clearly sensitive to oxidative stress, while cells transfected with pGFP vector were resistant (Fig. 1B). Furthermore, Hep3B-HBX cells treated with H_2O_2 alone and resveratrol (a SIRT1 activator) alone did not exhibit cell death until 48 h post-treatment, whereas cells treated with resveratrol and H_2O_2 exhibited cell death 24 h after treatment and the process was enhanced 48 h after treatment (Fig. 1C). We also confirmed that the cell death mediated by the combined treatment with resveratrol and H_2O_2 was attributed to apoptosis, because we observed up-regulation of cleaved caspase 8 and PARP in the cell lysates from cells administered the combined treatment compared to those in the cell lysates from cells treated with resveratrol alone or H_2O_2 alone (Fig. 1D). This result indicates that up-regulation of SIRT1 expression or SIRT1 activity can sensitize Hep3B-HBX cells to oxidative stress.

3.2. Nuclear localization of SIRT1 is not required for oxidation-induced apoptosis

Because SIRT1 can be localized in both the nucleus and the cytosol, depending on the cell context and developmental stage of cell [24], we wondered whether nuclear localization of SIRT1 is necessary for oxidation-induced apoptosis. To answer this question, we introduced SIRT1 localization mutants vectors, pGFP-SIRT1-NES, which localizes in the nucleus, and pGFP-SIRT1-NLS, which localizes in the cytosol, described fully elsewhere [24], into Hep3B-HBX cells. As controls, we used pGFP-SIRT1-WT vector as a positive control and pGFP as a negative control. GFP-conjugated WT and NES mutant SIRT1 were mainly localized in the nucleus while GFP-conjugated NLS mutant SIRT1 was detected in the cytosol (Fig. 2A). Transfection of Hep3B-HBX cells with pGFP-SIRT1-NLS, pGFP-SIRT1-WT, and pGFP-SIRT1-NES vectors sensitized oxidation-induced cell death of Hep3B-HBX cells to a similar degree (Fig. 2B). However, GFP alone did not provoke oxidative stress-induced cell death. This result suggests that nuclear localization of SIRT1 is not required for oxidative stress-induced apoptosis.

3.3. Ectopic expression and enhanced activity of SIRT1 attenuate JNK phosphorylation

Because the cellular response to oxidative stress has been implicated in activation of the JNK signaling pathway [25], we suggest that HBX-mediated activation of JNK confers resistance to oxidation-induced apoptosis in Hep3B-HBX cells. To test our hypothesis, Hep3B-HBX cells were administered by SP600125, a JNK inhibitor, and PD98059, an ERK inhibitor, and treated with H_2O_2 to

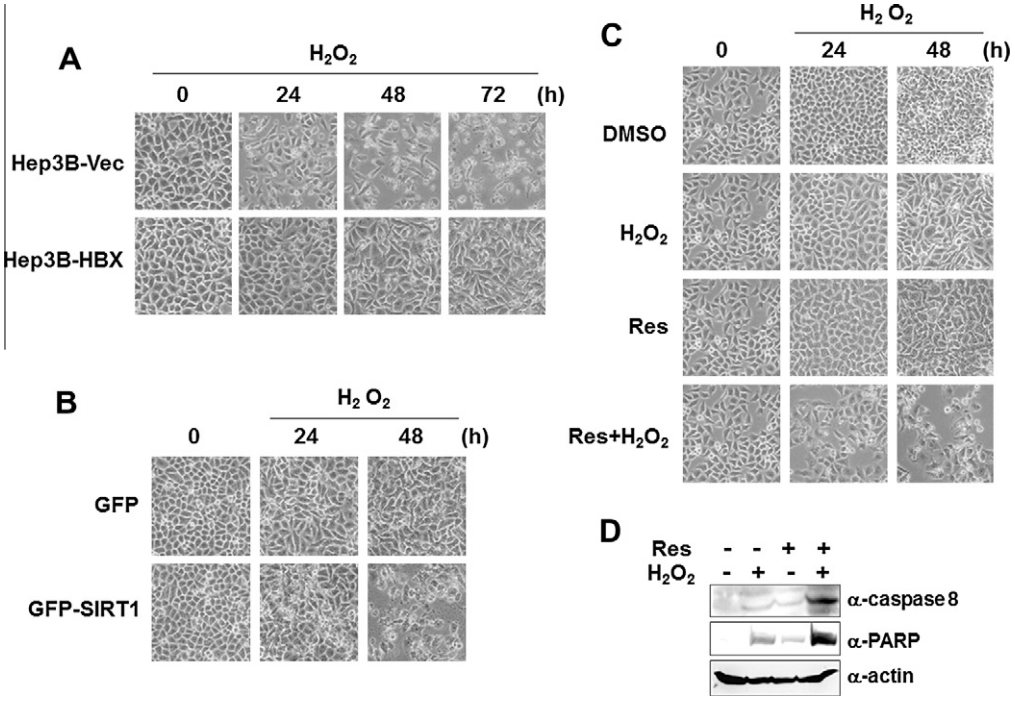

Fig. 1. Ectopic expression and enhanced activity of SIRT1 sensitizes Hep3B-HBX cells to oxidative stress-induced apoptosis. (A) Hep3B-Vec and Hep3B-HBX cells were treated with H_2O_2 (0.5 mM) and cells were observed under microscopy. (B) Hep3B-HBX cells were transfected with pGFP-SIRT-WT or pGFP vector as a control, and then treated with H_2O_2 (0.5 mM) for 48 h. Transfection efficiency was measured by GFP expression under fluorescence microscopy. Cell viability was recorded under light microscopy for 48 h. (C) Hep3B-HBX cells were treated with H_2O_2 (50 mM) alone, resveratrol (Res; 100 μM) alone, and H_2O_2 plus resveratrol and cell viability was recorded under light microscopy for 48 h. (D) Cell lysates from Hep3B-HBX cells treated as described above were prepared to observe apoptosis. Cleaved caspase 8 and PARP levels were evaluated by immunoblotting using the corresponding antibodies.

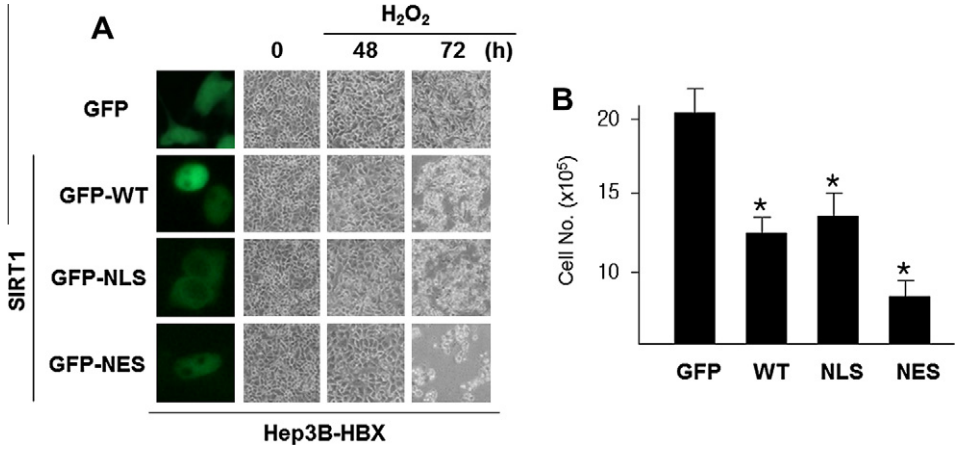

Fig. 2. Nuclear localization of SIRT1 is not necessary for oxidative stress-induced apoptosis (A) Hep3B-HBX cells were transfected with pGFP-SIRT1-NES (3 μg), pGFP-SIRT1-NLS (3 μg), pGFP-SIRT1-WT (3 μg), a positive control, and pGFP (3 μg), a negative control, vectors and then treated with H_2O_2 (0.5 mM) for 72 h. Transfection efficiency was measured as GFP expression under fluorescence microscopy. (B) Cell viability was also evaluated using Trypan blue exclusion. (*$p < 0.01$; GFP transfection *vs.* GFP-SIRT-WT, GFP-SIRT-NES, or GFP-SIRT-NLS transfection at 72 h under H_2O_2 treatment).

investigate the resulting sensitivity to oxidative stress. Cell death was not observed in Hep3B-HBX cells treated with the ERK inhibitor under H_2O_2, but was detected in the cells administered the JNK inhibitor under H_2O_2 treatment (Fig. 3A). This result indicates that activation of JNK is essential for resistance to oxidation-induced apoptosis in Hep3B-HBX cells. Of interest, one study has reported that resveratrol can also inhibit high glucose-induced activation of NADPH-ROS signaling pathway mediated by JNK/NF-κB signaling in renal mesangial cells [17]. Therefore, we examined whether resveratrol inhibited JNK activation, leading to sensitization of Hep3B-HBX cells to oxidation-induced apoptosis. Hep3B-HBX cells were treated with resveratrol or dimethyl sulfoxide (DMSO) under oxidative stress. We found that JNK phosphorylation level was not significantly increased in Hep3B-HBX cells during H_2O_2 treatment when treated with DMSO as a control (Fig. 3B). The result indicates that HBX mediated activation of JNK is maintained during oxidation stress. However, treatment of Hep3B-HBX cells with resveratrol significantly reduced the JNK phosphorylation level during oxidative stress (Fig. 3B). Furthermore, since we observed that ectopic expression of SIRT1 sensitized cells to oxidation-induced apoptosis (Figs. 2B and 3A), we examined whether the enhanced levels of SIRT1 protein might inhibit JNK phosphorylation as seen

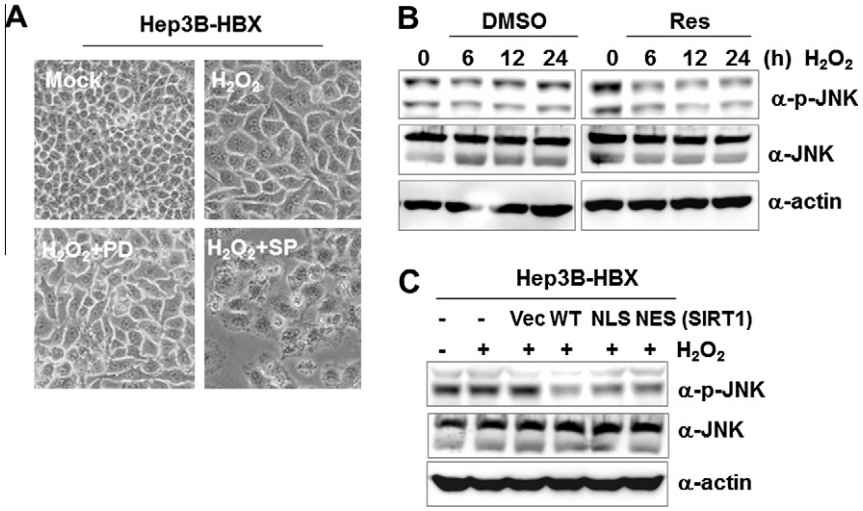

Fig. 3. JNK phosphorylation is attenuated by ectopic expression of SIRT1 and resveratrol (Res; 100 μM)treatment. (A) Hep3B-HBX cells were treated with PD98059 (PD; 30 μM) or SP600125 (SP; 20 μM) for 12 h followed by treatment with H_2O_2 for 36 h. Cells were observed by microscopy for 48 h after PD and SP administration. (B) Hep3B-HBX cells were treated with resveratrol for 12 h under H_2O_2 stress and cell lysates were prepared for detection of phosphorylated JNK. Proteins were separated by 10% SDS–PAGE, and JNK and phospho-JNK levels were examined by immunoblotting using the corresponding antibodies. (C) Hep3B-HBX cells were transfected with pGFP-SIRT1-NES, pGFP-SIRT1-NLS, GFP-SIRT1-WT, a positive control, and pGFP vector, a negative control, and then treated with H_2O_2 (0.5 mM) for 48 h. Transfection efficiency was measured as GFP expression by immunoblotting. After gel electrophoresis, JNK and phospho-JNK levels were examined by immunoblotting using the corresponding antibodies.

in the cells treated with resveratrol. To test our hypothesis, Hep3B-HBX cells were transiently transfected with pGFP-SIRT1-WT, pGFP-SIRT1-NLS and pGFP-SIRT1-NES vector, followed by H_2O_2 treatment. Twenty-four hours after H_2O_2 treatment, JNK phosphorylation was found to be reduced in cells transfected with pGFP-SIRT1-WT, pGFP-SIRT1-NLS and pGFP-SIRT1-NES vectors, compared to that in cells transfected with pGFP vector (Fig. 3C). This result suggests that overexpression of SIRT1 or activation of SIRT1 activity attenuates JNK phosphorylation in Hep3B-HBX cells, leading to enhancement of sensitivity to oxidative stress.

3.4. Inhibition of SIRT1 activity restores resistance to oxidation-induced apoptosis through JNK activation

We also investigated whether inhibition of SIRT1 activity blocks the effect of resvertrol, by reduction of JNK phosphorylation leading to sensitization to oxidation-induced apoptosis. Hep3B-HBX cells were treated with reveratrol alone, nicotinamide (a SIRT1 inhibitor) alone, or both resveratrol and nicotinamide under oxidative stress. Resveratrol treatment alone reduced the phosphorylation of JNK during oxidation stress as seen in Fig. 3B, whereas nicotinamide treatment alone restored the level of JNK phosphorylation to that in untreated cells (Fig. 4A). Furthermore, nicotinamide clearly abrogated the reduction in JNK phosphorylation level caused by resveratrol treatment (Fig. 4A). We examined FOXO3A protein level because FOXO proteins are known to be involved in resistance to oxidative stress [26–28]. We examined levels of FOXO3A protein in Hep3B-HBX cells after treatment with resveratrol alone, nicotinamide alone, and with resveratrol plus nicotinamide. We found that resveratrol reduced the FOXO3A protein level, while nicotinamide enhanced it in cells under oxidative stress compared to untreated cells under the same conditions (Fig. 4A). Combined treatment with resveratrol and nicotinamide partially restored the FOXO3A protein level (Fig. 4A). The result suggested that FOXO3A protein levels display a similar pattern to JNK phosphorylation levels. Resveratrol treatment sensitized Hep3B-HBX cells to oxidation-induced cell death; however nicotinamide treatment inhibited the effect of resveratrol on oxidation-induced cell death (Fig. 4B). Nicotinamide treatment blocked resveratrol-mediated apoptosis during oxidation stress (Fig. 4B), which was confirmed by a reduction in PARP and caspase 8 cleavage (Fig. 4C). These results suggest that SIRT1 activity plays a crucial role in the regulation of JNK activation, which eventually determines the sensitivity of Hep3B-HBX cells to oxidative stress.

4. Discussion

The role of SIRT1 in cell growth and survival is controversial. Some studies have reported that SIRT1 can promote cell growth by blocking cellular senescence through direct deacetylation of p53 [18], FOXO [29], and E2F1 [30]. In contrast, other groups have reported that increasing SIRT1 protein levels as a result of ectopic expression led to reduced cell proliferation and tumor formation in a colon cancer model [31], and that activation of SIRT1 activity by resveratrol also limited cell growth and reduced tumor formation in a breast cancer model [32]. Our recent study reported that SIRT1 plays an inhibitory role in cell growth by down-regulation of β-catenin, based on the finding that up-regulation of SIRT1 enhances sensitization of Hep3B-HBX cells to doxorubicin-induced apoptosis [23]. We herein showed that up-regulation of SIRT1 expression or its activity sensitizes Hep3B-HBX cells to oxidation-induced apoptosis through down-regulation of JNK activity, which supports the inhibitory role of SIRT1 in cell growth and survival. Therefore, our results indicate that up-regulation of SIRT1 protein or of its activity could be a therapeutic strategy for HBV-associated hepatocellular carcinoma.

Of interest, a recent study described a mechanism whereby oxidative stress regulates SIRT1 via JNK1. The study demonstrated that JNK1 functionally interacts with SIRT1 and phosphorylates SIRT1, leading to activation of SIRT1 during oxidative stress [33]. Since the phosphorylated SIRT1 was specifically able to deacetylate histone H3 protein but not p53 tumor suppressor, it was proposed that SIRT1 might induce expression of a set of genes that confer protection from oxidative stress by modifying chromatin [33]. Moreover, the further study showed that modulation of SIRT1 activity and protein levels did not alter JNK1 activity, indicating that SIRT1 seems to be a downstream molecule of JNK1 [33]. However, conversely, another study showed that SIRT1 activates the PTEN/JNK/FOXO signaling pathway [34]. In addition, another study supported the notion that modulation of SIRT1 affects JNK1 activity

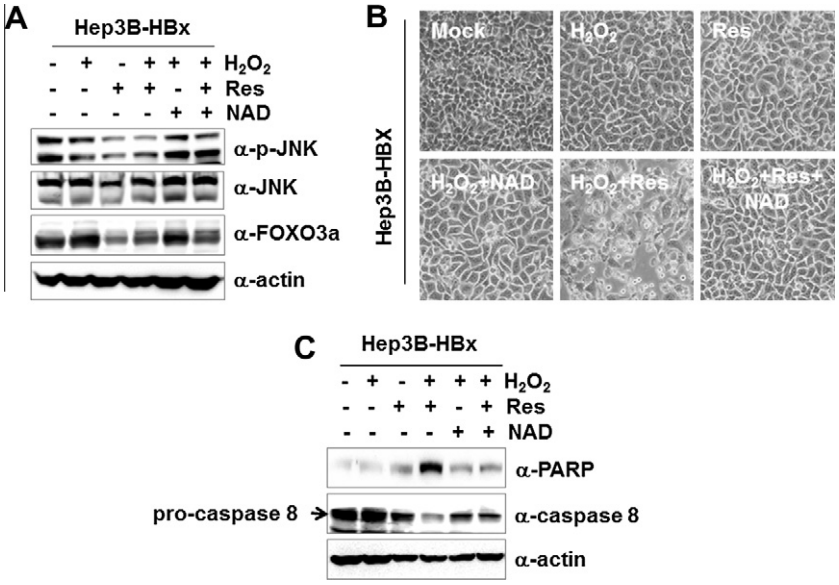

Fig. 4. Nicotinamide treatment inhibits the effect of reveratrol on JNK phosphorylation and apoptosis in Hep3B-HBX cells. (A) Hep3B-HBX cells were treated with resveratrol (Res; 100 μM) alone, nicotinamide (NAD; 5 mM) alone and resveratrol plus nicotinamide under H_2O_2 (0.5 mM) stress for 12 h. Cell lysates were prepared and proteins were separated by 10% SDS–PAGE then JNK, phospho-JNK, and FOXO3A levels were examined by immunoblotting with the corresponding antibodies. (B) and (C) Hep3B-HBX cells treated as above were observed for 48 h under a light microscopy and apoptosis was confirmed with immunoblotting using antibodies to caspase 8 and PARP. The arrow indicates pro-caspase 8 protein.

by demonstrating that resveratrol can prevent high glucose-induced mesangial cell proliferation through inhibition of JNK and NF-κB activation [17]. Consistent with the latter evidence, we observed that treatment with resveratrol, a SIRT1 activator, reduced the levels of phosphorylated JNK, while treatment with nicotinamide restored the resveratrol-mediated decrease in JNK phosphorylation (Fig. 4A). Furthermore, when we suppressed SIRT1 protein level expression with siRNA, we slightly observed enhanced phosphorylation of JNK under oxidative stress (data not shown). On the other hand, we suggest that the discrepant roles of SIRT1 in oxidative stress in the literature described above may be attributed to different cellular contexts and pathological conditions. We are currently investigating a molecular mechanism whereby up-regulation of SIRT1 reduces the level of JNK phosphorylation.

Acknowledgments

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