Research Article

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Suberoylanilide hydroxamic acid inhibits LX2 cells proliferation via decreasing yes-associated protein/transcriptional coactivator with PDZ-binding motif proteins

[Suberoylanilid hidroksamik asit, PDZ bağlayıcı motif proteinleri ile yes ilişkili protein/ transkripsiyonel koaktivatör azaltarak LX2 hücrelerinin proliferasyonunu inhibe eder]

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Abstract

Background: Hepatic fibrosis is a complex and dynamic process similar to "wound healing" that results in the progressive accumulation of connective tissue. We aimed to investigate the epigenetic control of liver fibrosis and Hippo pathway in human hepatic stellate cell (HSC) line. We examined the effect of Suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor on the LX2 cell line.
Material and methods: 2.5 μM SAHA was treated to LX2 cell line for 2 days. Cell proliferation and apoptosis measurement were performed by Muse Cell Analyzer. Yes-Associated Protein/Transcriptional Coactivator With Pdz-Binding Motif (YAP/TAZ) and alpha-smooth muscle actin (α-SMA) protein expression levels were measured by western blotting.
Results: In our study, we observed that the SAHA treatment reduced cell viability and induced apoptosis of LX2 cells statistically. We found that SAHA treatment decreased α-SMA, YAP and TAZ proteins levels statistically.
Conclusion: Decreased cell viability could be due to physiological, autophagical and also related to the apoptotical mechanisms. We thought that SAHA plays an important role in the creation of the fates of the LX2 cell line.

Keywords: hepatic fibrosis; hippo pathway; suberoylanilide hydroxamic acid; YAP/TAZ.

Öz

Amaç: Hepatik fibrozis, bağ dokusunun birikimi ile sonuçlanan "yara iyileşmesine" benzer karmaşık ve dinamik bir süreçtir. Bu çalışmada, insan hepatik stellat hücre (HSC) hattında karaciğer fibrozisinin ve Hippo yolağının epigenetik kontrolünü araştırmayı amaçladık. Histon deasetilaz inhibitörü olan Suberoylanilid hidroksamik asit (SAHA)'nın LX2 hücre hattı üzerindeki etkisini inceledik.
Materyal ve metod: LX2 hücrelerine 2 gün süreyle 2.5 μM SAHA uygulandı. Hücre proliferasyonu ve apoptoz ölçümleri, Muse Cell Analyzer cihazı ile gerçekleştirildi. Yes-associated protein/ transcriptional coactivator with PDZ-binding

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motif protein (YAP/TAZ) ve α-düz kas aktin (α-SMA) protein ekspresyon seviyeleri, western blot analizi ile belirlendi.

Bulgular: Çalışmamızda SAHA'nın, hücre canlılığını azalttığını ve LX2 hücrelerinin apopitozunu istatistiksel olarak indüklediğini gözlemledik. SAHA 'nın α-SMA, YAP ve TAZ protein düzeylerini istatistiksel olarak düşürdüğünü bulduk.

Sonuç: Hücre canlılığının azalması; fizyolojik, otofajik ve aynı zamanda apoptotik mekanizmalardan kaynaklanabilir. SAHA'nın LX2 hücrelerinin kaderinin belirlenmesinde önemli bir rol oynadığını düşünmekteyiz.

Anahtar Kelimeler: hepatik fibrozis; hippo yolağı; suberoylanilid hidroksamik asit; YAP/TAZ.

Introduction

Many of the biological events are controlled by various posttranslational modifications that realize in the amino tails of histones [1]. Among these modifications, the most studied histone modification is acetylation. Histone acetyl transferase (HAT) and histone deacetylase (HDAC) enzymes are controlled by histone acetylation. It effects important molecular events [2]. When the negatively charged acetyl group has interacted with the amino-terminal of the histone protein, positively charged lysine partially loses the amino acid charge and relaxes in chromatin. As a result of all these epigenetic regulations, the transcription factors facilitate access to the promoter regions of the genes and transcription occurs at this site [3, 4]. Acetylation is a reversible phenomenon and HDAC enzymes have been the target of varios drugs with the implication that they effect important cellular events [5, 6]. Compounds capable of inhibiting these enzymes termed HDAC inhibitor, are linked to the active sites of the corresponding enzymes so that histones remain in acetylated form and changes in gene expression occur [7]. HDAC inhibitors have an effect on basic cellular organization such as cell division and apoptosis. SAHA is a powerful reversible HDAC inhibitor [4, 8].

During biological events, proliferation, death and differentiation of cells are the main processes. Coordination of all these occurrences have role in different cellular regulations such as; physiological and pathological. The proliferation, death and differentiation of the cells are studied in detail and intensively. However, there are few studies on coordinated and interrelated research of these three processes. In recent years that has been reported that the Hippo signaling initiates cell death and differentiation and inhibits cell proliferation. For this reason, the Hippo pathway is thought to coordinate these processes as a key note [9,10]. In mammals, a pathway composed of YAP/TAZ and working with kinase cascade is defined [11]. TAZ plays an important role in Hippo pathway through TEA domain transcription factor (TEAD 1-4) [12]. YAP activation was detected during early activation of HSCs, which has a major affect in the fibrosis mechanism. It has even been suggested that YAP is required for the activation of HSCs [13].

The effect of the SAHA on the Hippo pathway and human hepatic stellate cell (HSC) activation has not yet been searched so far. In the present work, we tried to look at the epigenetic control of liver fibrosis and YAP/TAZ proteins in human HSC line. For this purpose, we analysed SAHA on the human HSC line (LX2).

Materials and methods

Reagent and chemicals

Dulbecco's modified Eagle's medium (DMEM hıgh gulatimine), fetal bovine serum (FBS), phosphate buffered saline (PBS) trypsin-EDTA, antibiotic and glutamine, were obtained from Biological Industries (Kibbutz Beit Haemek 25115 Israel). SAHA was purchased from Cayman Chemical (Michigan, USA) and Dimethyl sulfoxide (DMSO) purchased from Merck (Merck KGaA, Darmstadt, Germany). Annexin V δ Dead Cell Assay and Cell Viability Assay Kit (Merck Millipore, Billerica, MA, USA) were obtained from Merck Millipore Industries. YAP/TAZ, α-SMA, Beta Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Proteintecht (Rosemont, IL 60018, USA). Anti-mouse immunoglobulin and anti-rabbit immunoglobulin G horseradish peroxidase-conjugated antibodies, non fat dry milk and other buffers of the western blot were purchased from BIO-RAD (Hercules, California, USA). Radioimmunoprecipitation assay (RIPA) buffer, Phosphatase Inhibitor Cocktail 3, Phosphatase Inhibitor Cocktail 2 were obtained from Merck (Merck KGaA, Darmstadt, Germany).

Cell culture and treatment

LX2, a human HSC line, was provided kindly as a gift by Dr. Scott L. Friedman (Mount Sinai School of Medicine, New York). Human HSC line LX2 were cultured in DMEM, supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were grown at 37 °C SAHA was dissolved in Dimethyl sulfoxide at 1 mmol/L and SAHA was aliquoted and stored at −20. 2.5 μM SAHA applied for 48 h to LX2 cells.

Cell viability assay

LX2 cells were seeded 2.5×10^5 cell per well in a six well-plate in medium. SAHA was treated with different doses (0.5, 1, 2.5, 5, and 10 μM) to cells. After 48 h of incubation, the viability of the cells were measured by Muse Cell Analyzer. The IC_{50} value was determined as 2.5 μM and the formula below is used.

IC_{50} = 100 – [(control-treated group)/control × 100] (control is cell number in non treated group, treated group is the number of cells in the SAHA-treated group at different concentrations). After determination of SAHA concentration, we also evaluated cell proliferation depending on time (24, 48, 72, 96 h).

For this, LX2 cells were seeded 2.5 × 10^5 cell per well in a six well-plate in medium. Cells were treated 2.5 μM SAHA. After washing with phosphate buffered saline the cells were harvested by use of trypsin–EDTA, centrifuged at 300xg and assayed with Muse Count Viability Kit.

Apoptosis assay

LX2 cells were seeded 2.5 × 10^5 cell per well in a 6-well plate. 2.5 μM SAHA was used for the treatment of cells for two days. Briefly, cells were applied with trypsin, subjected to centrifugation at 350× *g* for 5 min, then washed once using PBS, and then resuspended with a kit. The cell suspensions are incubated Annexin V/Propidium iodide (PI) for 20 min at 25 °C and were analyzed by flow cytometry. All experiments were done in triplicate.

Western blotting analysis

LX2 cells were cultured into culture flasks containing medium then cells were treated with 2.5 μM SAHA for 48 h. Cells were harvested using cell scaper with Radioimmunoprecipitation assay buffer (cell lysis buffer) containing protease inhibitors (Phosphatase Inhibitor Cocktail3 and Phosphatase Inhibitor Cocktail2). Cells were centrifuged at 4 °C. Bradford method was used for the protein assesment. After loading the proteins, resolved on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane, blocked with non-fat dry milk at room temperature. Then membran was washed with tris buffered saline (TBS-Tween 20). Membrane incubated with primary antibodies (YAP, TAZ, α-SMA and β-actin) overnight at 4 °C. Then secondary antibodies were used and the corresponding protein was visualized in Biorad chemidoc using ECL (chemiluminescence marker Pierce Chemical Co., Rockford, IL). image software was used for calculation.

Statistical analysis

SPSS 19.0 statistical program was used for statistical analaysis. Student's *t*-test values are shown as mean ± SD. Differences between groups.

Results

Effect of SAHA on cell viability

After 24 h of incubation, cells were treated with different doses (0.5, 1, 2.5, 5, and 10 μM) of SAHA. The IC_{50} value was determined as 2.5 μM. After determination of SAHA concentration, we performed the cell viability assay as time-dependent manner for 4 days. SAHA effectively reduced the number of cells (Figure 1).

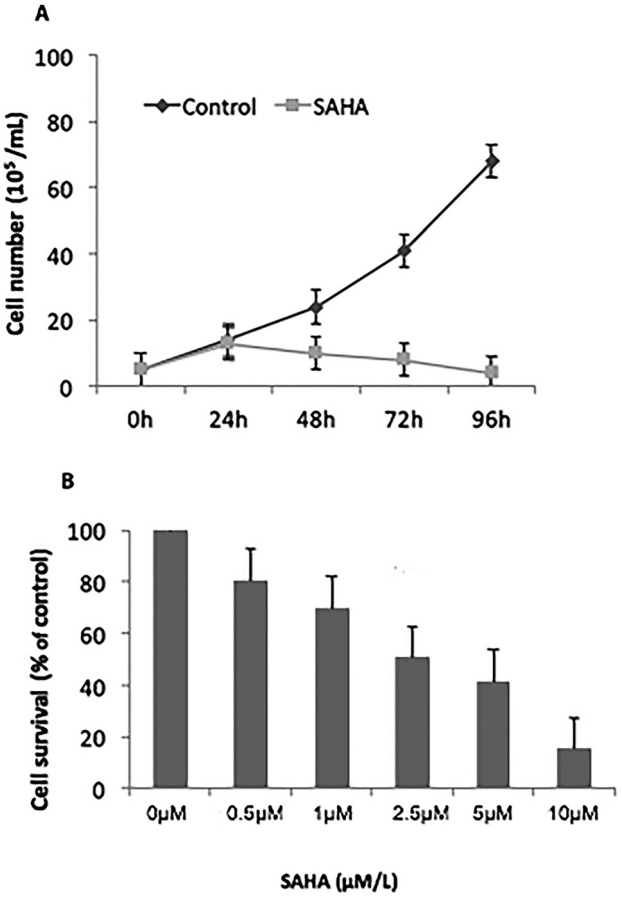


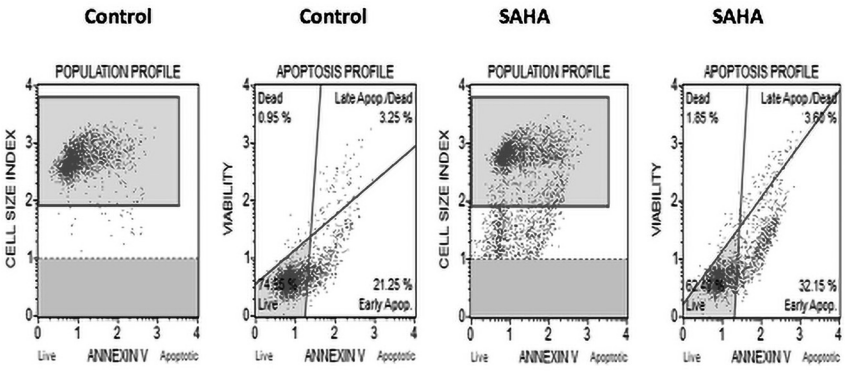
Figure 1: (A) SAHA has significantly reduced the number of cells depending on the time. Different concentration of SAHA were treated to LX2 cells for 48 h (B) There was a decrease in the number of LX2 cells due to dose in SAHA administration. The IC_{50} value was determined to 2.5 μM.

SAHA induces apoptosis in LX2 cell line

2.5 μM SAHA was used for the treatment of cells for two days. Annexin V/Propidium iodide (PI) for 20 min at 25 °C and were analyzed by flow cytometry. We found that SAHA induced total, early and late apoptosis compared the control group in LX2 cell line (Figure 2).

Effect of SAHA on YAP, TAZ and α-SMA protein levels

Cells were treated with 2.5 μM SAHA for 48 h. Membrane incubated with primary antibodies (YAP, TAZ, α-SMA and β-actin) overnight at 4 °C. Then secondary antibodies were used and the corresponding protein was visualized in Biorad chemidoc using ECL. Protein levels were measured with western blot to show effect of SAHA. In our analyses,



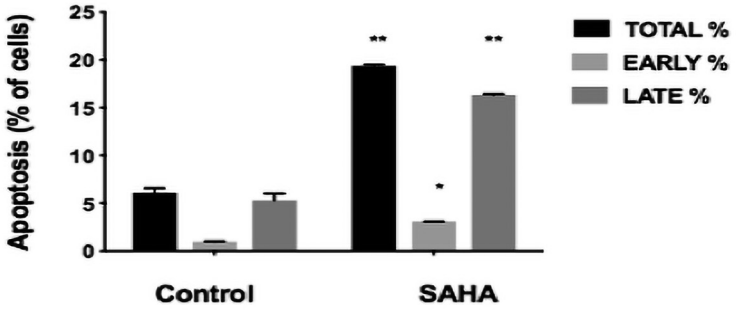


Figure 2: LX2 cells were exposed the Annexin V/Propidium iodide (PI) for 20 min and were analyzed by flow cytometry. SAHA induced total, early and late apoptosis compared the control group. Error bars point out Mean ± SD. * statistically significance at (*p<0.05; **p<0.01).

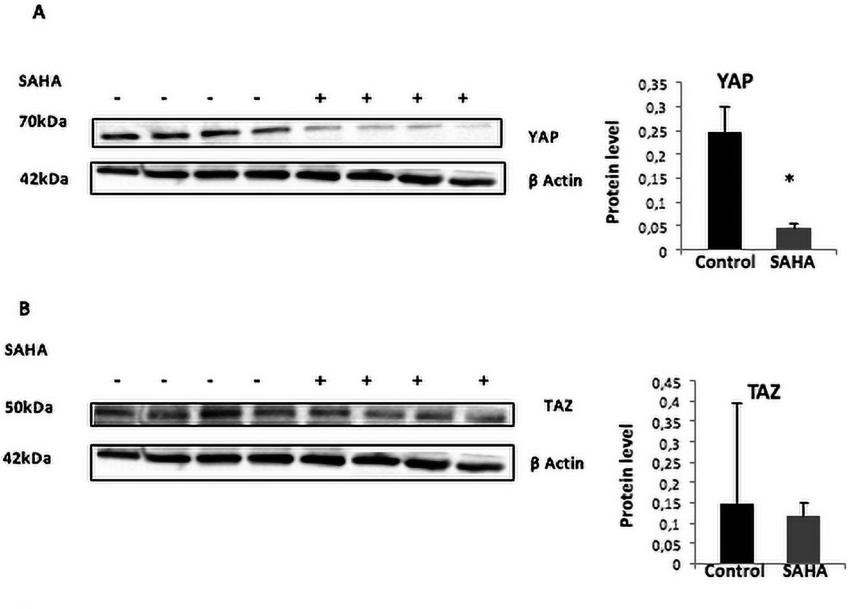


Figure 3: Western blot analysis of the LX2 hepatic stellate cell lines. Cell lysates were collected 48 h after treatment with SAHA (A). SAHA decreased YAP protein level (B). SAHA decreased TAZ protein level (C). SAHA decreased α-SMA protein expression. β-actin and GAPDH acted as a loading control protein. *, statistically significance at (*p<0.05).

we showed that SAHA decreased YAP, TAZ and α-SMA levels (Figure 3 A, B and C).

Discussion

YAP/TAZ proteins, which play a role in Hippo pathway, are thought to be new players as nucleolar mechanosensors during organ homeostasis and cancer formation [14]. In the SAHA-treated group, we found decreased cell viability compared to the control group. The cause of decreased cell viability could be many reasons, including physiological, autophagy and apoptosis. In the present work, we analysed the SAHA, an epigenetically regulating and HDAC inhibitor, on apoptosis in the in the human HSC cell line (LX2). We found that the SAHA induced apoptosis. We thought that SAHA has a crucial value in the creation of the fates of the LX2 cell line. In accordance with our study, it has been shown that SAHA is effective in suppressing the activation of LX2 and it has been reported that the suppressive effects of SAHA on HSC activation may play a role in the decrease of nuclear factor-kappa B1 (NFKB1) expression [8]. In another study, SAHA has been reported to have important effects on liver function such as suppressing liver fibrosis, and decreasing cell growth [15]. Then we wondered how the drug effects cell activation in the HSC line, so for this purpose, we evaluated α-SMA as hepatic activation marker. As a very important data, we have seen that the SAHA significantly reduces α-SMA in western blot analysis in LX2 cell line.

The Hippo pathway has important roles in cell function [16]. YAP behaves as an oncogene in several cancers [17], recent data suggest an interesting hypothesis that YAP/TAZ promote liver disease and cancer [18].

In the present study, SAHA reduced protein level of YAP and TAZ, induced apoptosis and decreased α-SMA protein level in LX2 cell line.

YAP activation was detected during the early activation process of HSCs. It has even been suggested that YAP/TAZ is required for the activation of HSCs [19, 20]. Studies on YAP/TAZ pathway and liver fibrosis are relatively new and limited [21] and YAP activation have an crucial role in liver fibrosis. Therefore, it has been emphasized that treatment protocols targeting YAP inhibition should be developed in the treatment of liver fibrosis [13].

In the process of fibrosis, the silent HSCs, transformed into myofibroblast-like cells by the degradation of the extracellular matrix [22]. Some complexities have been reported in mechanisms associated with regeneration of liver damage. YAP is a stem cell-associated factor that regulates liver proliferation and growth in normal mice, triggering the accumulation of ductus cells that appear reactive with liver progenitor capabilities with increased nuclear localization. The importance of YAP mediated signaling is unknown in liver disease [21]. Increasing of fibrotic lesions activates the cancer risk in different tissues, such as; liver and lung. Moreover, its mechanism is still unclear, especially relation with the fibrosis and cancer. The nuclear localization of YAP has been reported in stellate cells of carbon tetrachloride-induced mouse fibrotic liver and human cirrhotic livers caused by hepatitis C virus infection.

It has been reported *in vitro* that verteporfin-mediated YAP inhibition and the siRNA pathway silencing of the YAP protein hinder the binding between the YAP/TAZ proteins

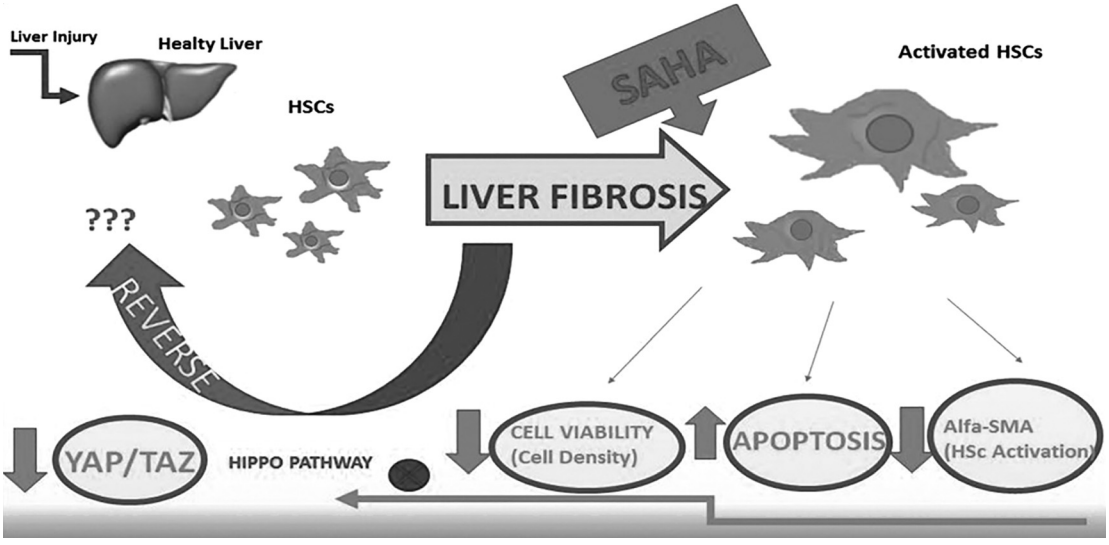


Figure 4: Possible mechanism of SAHA on LX2 cell line.

and the TEADs, thereby inhibiting the differentiation of HSCs into myofibroblast cells. In addition to support these informations, It has been reported that YAP activated by the hedgehog pathway transforms HSCs into myofibroblast cells [23].

It has been reported that TAZ levels are rised in human and mice livers with Nonalcoholic steatohepatitis (NASH). In addition, TAZ inhibiting after the development of steatosis reduces liver inflammation and fibrosis in fructose, palmitate, cholesterol fed mice [24].

In another study, it has been reported that ω-3 PUFA therapy improved liver fibrosis and inhibited the proliferation and activation of HSCs via increasing the degradation of YAP/TAZ [25].

In myofibroblastic HSCs, glutaminolysis (conversion of glutamine to alpha-ketoglutarate) is induced, and by inhibiting glutaminolysis, proliferative and myofibroblastic properties are reduced in these cells. They also has been showed that glutaminolysis is induced in chronically injured fibrotic livers in both mice and humans [26].

In HSCs of human and mouse livers with fibrosis have been reported to be found YAP/TAZ, and activation of these markers facilitates fibrogenic processes and regulates extracellular matrix (ECM) accumulation and tissue stiffness [27].

In diseases such as; pulmonary or liver fibrosis, microRNA-130/301 inhibition was stoped the YAP/TAZ, induction of extracellular matrix modification, and also downstream tissue fibrosis [28].

It has been reported that Morin (3, 5, 7, 2′, 4′-pentahydroxyflavone) activated Hippo signaling through significantly lowered transcriptional effectors YAP/TAZ expression was prevented by HSC activation [29].

Present study revealed that epidrug-SAHA might have protective effect on liver fibrosis by inhibiting hippo pathway as shown by decreased levels of YAP/TAZ proteins and inducing apoptosis in LX2 cell line (Figure 4).

Therefore, we thought that SAHA has an important role in the epigenetic regulation of the HSC cell line in this study. Our results indicated that SAHA possesses potent anti-fibrotic properties, which may be responsible for its effects on liver diseases. If the effect of SAHA is proven on decreasing the activation of HSCs with the other comprenhensive studies, it might be used in future treatment in patients with chronic liver and liver fibrosis.

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Conflict of interest: No conflict of interest was declared by the authors.

Informed consent: No informed consent is needed. (It is a cell culture study).

Ethics committee approval: Ethics committee approval is not needed. (It is a cell culture study).

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