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2014년 2월

서울대학교 대학원
의학과 내과학 전공
이 윤 빈

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February 2014

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지도교수 **윤 정 환**

이 논문을 의학석사 학위논문으로 제출함

2013년 10월

서울대학교 대학원

의학과 내과학 전공

이 윤 빈

이윤빈의 의학석사 학위논문을 인준함

2013년 12월

위 원 장 김용대 (인)

부위원장 윤정환 (인)

위 원 서경수 (인)

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by

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A Thesis Submitted to the Department of Internal Medicine
in Partial Fulfillment of the Requirements
for the Degree of Master of Philosophy in Medicine
at the Seoul National University College of Medicine

December, 2013

Approved by thesis committee:

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Abstract

Monosodium urate attenuates bile acid induced hepatocyte apoptosis by modulating ER stress and JNK activation

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Background: In cholestasis, accumulated bile acids within the liver tissue trigger hepatocyte apoptosis, primarily by activating proapoptotic signaling cascades. Monosodium urate (MSU) crystal is produced after release of uric acid during cellular death, and activates a cytosolic complex of proteins termed inflammasome via the sensory molecule NLRP3 leading to inflammation. This study aimed to investigate the effects of MSU in bile acid-induced hepatocyte apoptosis, and to identify the signaling pathway modulated by MSU.

Methods and Results: We performed in vitro studies using Huh-BAT cells, which are Huh-7 cells stably transfected with the sodium-dependent bile acid transporting polypeptide. Deoxycholate (DC) was used to induce hepatocyte apoptosis. Cell growth was assessed using MTS assay and apoptosis was quantified using 4′,6-diamidino-2-phenylindole dihydrochloride staining. The apoptotic and kinase signaling pathways were explored by immunoblot analysis and immunoprecipitation. MSU attenuated bile acid-induced hepatocyte apoptosis by reducing mitochondrial apoptotic signaling, such as caspase 9 and 7.

Augmentation of eIF2α phosphorylation and subsequent JNK activation induced by DC treatment were attenuated by MSU pretreatment. However, TRAIL-R2 oligomerization and caspase 8 recruitment to death-inducing signaling complex following DC treatment and TRAIL-induced activation of caspase 9 and 7 were not altered by MSU pre-treatemnt.

Conclusion: MSU attenuates bile acid-induced ER stress and subsequent JNK activation, leading to hepatocyte protection against bile acid-induced apoptosis. The antiapoptotic effect of MSU is independent of death receptor signaling pathway.

Key words: Monosodium urate, Bile acid, Apoptosis, Endoplasmic reticulum, c-Jun N-terminal kinase, Inflammasome

Student Number: 2012-21706

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Introduction

Cholestasis can be defined as an impairment of bile flow and cholestatic liver disease is caused by various conditions when bile acids are accumulated and hepatocytes are exposed to excessive bile acids.[1] If the cholestasis is not relieved with proper treatment, chronic cholestatic liver disease may progress to biliary cirrhosis or hepatic failure.[2] In cholestasis, toxic bile acids accumulate within the liver tissue and trigger hepatocyte apoptosis, primarily by activating proapoptotic signaling cascades.[3-5] In previous *in vitro* and *in vivo* studies, hydrophobic bile acids including deoxycholic and glycochenodeoxycholic acids were demonstrated to induce hepatocyte apoptosis.[6-8]

Monosodium urate (MSU) crystal, which is one of damage-associated molecular patterns (DAMPs), is produced after release of uric acid during cellular death, and activates the inflammasome via the sensory molecule NLRP3 leading to innate immune response, inflammation and gouty arthritis.[9,10] Inflammasome describes a molecular complex that is localized in the cytosol and cleaves and activates casepase-1 leading to the production of inflammatory cytokines, such as IL-1β and IL-18.[11,12] The role of inflammasome pathway was initially revealed in innate immune cells particularly macrophages. Previous studies identified the presence of inflammasome components in Kupffer cells and hepatic stellate cells, and that inflammasome activation is required for the development of alcoholic and non-alcoholic steatohepatitis and liver fibrosis.[13-15] Recently, it was demonstrated that

caspase-1 activation during hypoxia/reoxygenation promotes adaptive responses to oxidative stress and protect hepatocytes against cell death.[16]

The present study was designed to investigate the effects of MSU in bile acid-induced hepatocyte apoptosis, and to identify the signaling pathway modulated by MSU. Collectively, our study demonstrates that MSU attenuates bile acid-induced hepatocyte apoptosis by modulating endoplasmic reticulum (ER) stress and reducing subsequent c-Jun N-terminal kinase (JNK) activation, which suggests the role of DAMPs and inflammasome as an adaptive response to liver damage associated with cholestasis.

Materials and Methods

Cell line and culture

Huh-BAT cells, which are Huh-7 cells stably transfected with the sodium-dependent bile acid transporting polypeptide, were used in this study.[8] Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, streptomycin (100 mg/L), and penicillin (100,000 U/L). Cells were proliferated with 3% fetal bovine serum, and in other experiments, cells were serum starved overnight in order to avoid the effects of serum-induced signaling.

Reagents

MSU was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Deoxycholate (DC) and dantrolene, an ER stress reducer which inhibits the mobilization of Ca^{2+} from the ER to the cytosol, were obtained from Sigma-Aldrich (St. Louis, MO, USA). SP600125, an inhibitor of JNK, was obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Soluble human recombinant tumor necrosis factor related apoptosis inducing ligand (TRAIL) was obtained from Enzo Life Sciences (Plymouth Meeting, PA, USA).

Cell proliferation

The CellTiter 96 Aqueous One Solution cell proliferation assay (Promega, Madison, WI, USA) was used to measure cell proliferation. In this cell proliferation assay,

dehydrogenase enzymes convert 3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) -2H-tetrazolium salt (MTS) into soluble formazan only in metabolically active, proliferating cells. Following each treatment, 20 μL of CellTiter 96 AQueous One Solution reagent was added to each well of a 96-well plate. After 1 h of incubation, the 490 nm absorbance was measured with an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA).

Quantification of apoptosis

The degree of apoptosis was evaluated by using the nuclear binding dye 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) to quantify apoptotic cells with fluorescence microscopy (Leica Microsystems, Mannheim, Germany). DAPI was added to the treated cells for 30 min and the cells were examined by fluorescence microscopy. Apoptotic cells were defined as those containing chromatin condensation and nuclear fragmentation. The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells counted ×100. A minimum of 400 cells was counted for each treatment.

Immunoblot analysis

Cells were lysed for 20 min on ice in lysis buffer (50 mM Tris-HCl, pH 7.4; 1% Nonidet P-40, 0.25% sodium deoxycholate; 150 mM NaCl; 1 mmol/L EDTA; 1 mM phenylmethylsulfonyl-fluoride; 1 mM Na3VO4; 1 mM NaF; and 1 μg/mL each of aprotinin, leupeptin, and pepstatin) and then were centrifuged at 14,000 g

for 10 min at 4 ℃. Samples were resolved by SDS–PAGE, transferred to nitrocellulose membranes, blotted with appropriate primary antibodies, and incubated with peroxidase-conjugated secondary antibodies (Santa Cruz, CA, USA). Bound antibodies were visualized by using a chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL, USA) and exposed to Kodak X-OMAT film. The primary antibodies used were as follows: rabbit anti-caspase 7, rabbit anti-caspase 8, rabbit anti-caspase 9 and mouse anti-phospho-JNK, which were obtained from Cell Signaling Technology Inc (Danvers, MA, USA); mouse anti-phospho-eukaryotic initiation factor 2α (eIF2α), mouse anti-human Fas associated death domain (FADD) and goat anti-actin, which were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); goat anti-tumor necrosis factor related apoptosis inducing ligand receptor 2 (TRAIL-R2) from Enzo Life Sciences (Plymouth Meeting, PA, USA).

Immunoprecipitation

Cells were pretreated with 100 μg/mL MSU for 16 h prior to 200 μM DC treatment. Immunoprecipitation of the death-inducing signaling complex (DISC) stimulated by DC was performed. Recombinant human soluble FLAG-tagged TRAIL (sTRAIL; Enzo Life Sciences; 20 μg/mL) and anti-FLAG monoclonal antibody M2 (Sigma; 60 μg/mL) were premixed for 15 min on ice. Cells were left untreated or treated with 200 μM DC and stimulated in a final volume of 1mL with 50 ng/mL sTRAIL and 2 μg/mL anti-FLAG M2 antibody. Cell suspensions were

lysed in lysis buffer for 20 min on ice and then were centrifuged at 14,000 g for 30 min at 4 °C. The complexes were then precipitated by 35 μL of protein A/G (Sigma) beads overnight at 4 ℃. Beads were washed three times with the lysis buffer before the bound proteins were resuspended by boiling in SDS sample buffer. Equal amounts of precipitates were analyzed by immunoblot analysis.

Statistical analysis

All cell-based experimental data were acquired from at least three independent experiments from a minimum of three separate isolations and were expressed as the means ± standard deviations (SD). Statistical evaluations of numeric variables in each group were conducted using the Mann-Whitney U test. All statistical analyses were conducted using PASW statistical software version 18.0 (IBM, Chicago, IL). Statistical significance was defined as a P value less than 0.05.

Results

Attenuated bile acid-induced hepatocyte apoptosis by MSU

We first investigated whether DC-induced apoptosis was modulated in Huh-BAT cells by treating them with bile acid. MSU pretreatment significantly attenuated DC-induced hepatocyte apoptosis (Fig. 1A). When cells were pretreated with MSU, mitochondrial apoptotic signaling, such as caspase 9 and 7, was attenuated, whereas activation of caspase 8, which is an initiator caspase downstream of death receptor activation, was not altered (Fig. 1B). These findings implicate that MSU reduces bile acid-induced hepatocyte apoptosis by attenuating mitochondrial apoptotic signaling.

Mechanism of bile acid-induced apoptosis

To elucidate the possible mechanism of the attenuation of bile acid-induced apoptosis by MSU, we next explored which apoptotic signals were activated in DC-treated cells. Because MAPK signaling cascades regulate apoptotic pathways, we then assessed whether MAPK signaling cascades regulated bile acid-induced apoptosis. Out of many MAPK signals, we subsequently found that bile acid induced JNK activation (Fig. 2A). Bile acid-induced activations of caspase 9, 7 and 8 were diminished in cells pretreated with JNK inhibitor (Fig. 2B). These findings suggest that enhanced JNK activation in Huh-BAT cells following bile acid treatment is responsible for enhanced activation of apoptosis in DC-treated

cells. Since JNK activation may depend on ER stress, we next evaluated whether ER stress was activated in DC-treated cells. As shown in Fig. 3A, eIF2α phosphorylation, which reflects ER stress induction, was increased in DC-treated cells. When the cells were treated with bile acid in the presence or absence of dantrolene, ER stress reducer, bile acid-induced apoptosis was significantly reduced by dantrolene pretreatment (Fig. 3B). Enhanced eIF2α phosphorylation was not altered by JNK inhibitor pretreatment (Fig. 3C). Therefore, these findings overall indicate that bile acid induces upstream ER stress, thereby leading to JNK activation and apoptosis.

Mechanism of attenuated bile acid-induced apoptosis by MSU

We next evaluated whether MSU modulated bile acid-induced ER stress and JNK activation. When the cells were pretreated with MSU, bile acid-induced eIF2α phosphorylation JNK activation were attenuated (Fig. 4). This finding implicates that MSU reduces ER stress-dependent JNK activation in DC-treated Huh-BAT cells and thus, attenuates apoptotic cell death in these cells.

Since the attenuation of JNK activation may interfere with DISC activity, which is upregulated by bile acid, we then evaluated if DISC formation, which includes oligomerization of death receptor and recruitment of FADD and caspase 8, was modulated by MSU 2,4,8,17. DISC was immunoprecipitated by using anti-TRAIL-R2 antibody. As shown in Fig. 5A, TRAIL-R2 oligomerization and caspase 8 recruitment to DISC following DC treatment were not altered by MSU

pretreatment. When cells were treated with TRAIL in the presence or absence of MSU, the cellular growth was not altered in the cells pretreated with MSU (Fig. 5B). Moreover, TRAIL-induced activation of caspase 9 and 7 was not attenuated in cells pretreated with MSU (Fig. 5C). These observations collectively suggest that antiapoptotic signaling of MSU in bile-acid induced hepatocyte apoptosis is not through death receptor dependent signaling pathway.

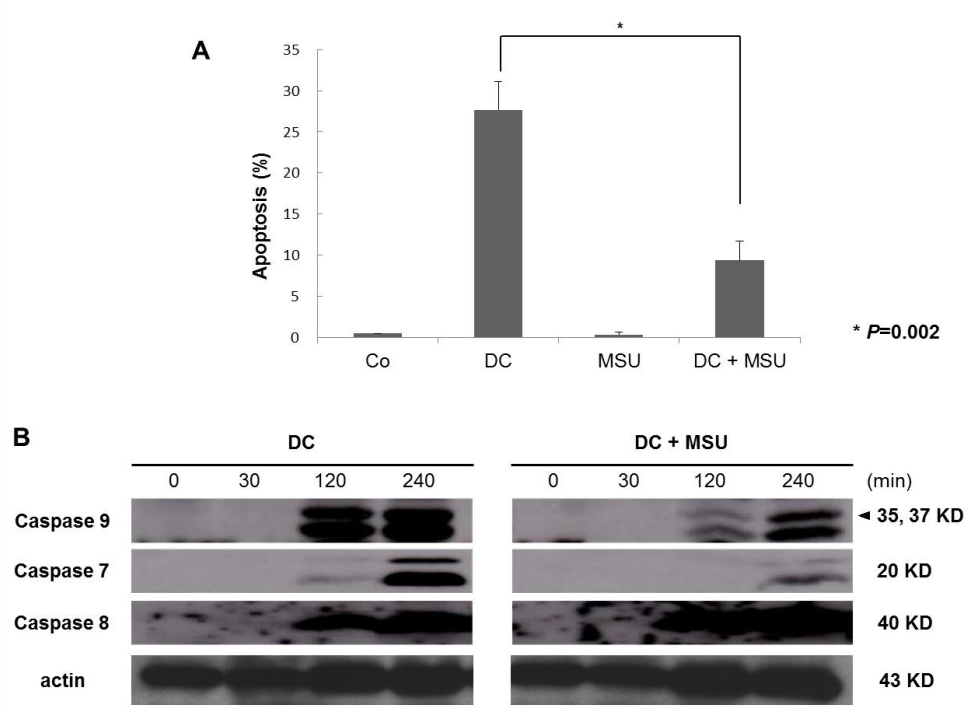

Figure 1. MSU attenuated bile acid-induced hepatocyte apoptosis. (A) Huh-BAT cells were pretreated with MSU (100 μg/mL) for 24 h prior to the addition of deocycholate (DC). Cells were then treated with DC (200 μM) for additional 5 h. Apoptosis was quantified by DAPI staining and fluorescent microscopy. (B) Huh-BAT cells were pretreated with MSU (100 μg/mL) for 24 h and then treated with DC (200 μM) for each indicated time period, lysed and immunoblot analysis was performed using anti-caspase 9, anti-caspase 7 and anti-actin antibodies. Data were expressed as the mean ± SD. *, P=0.002.

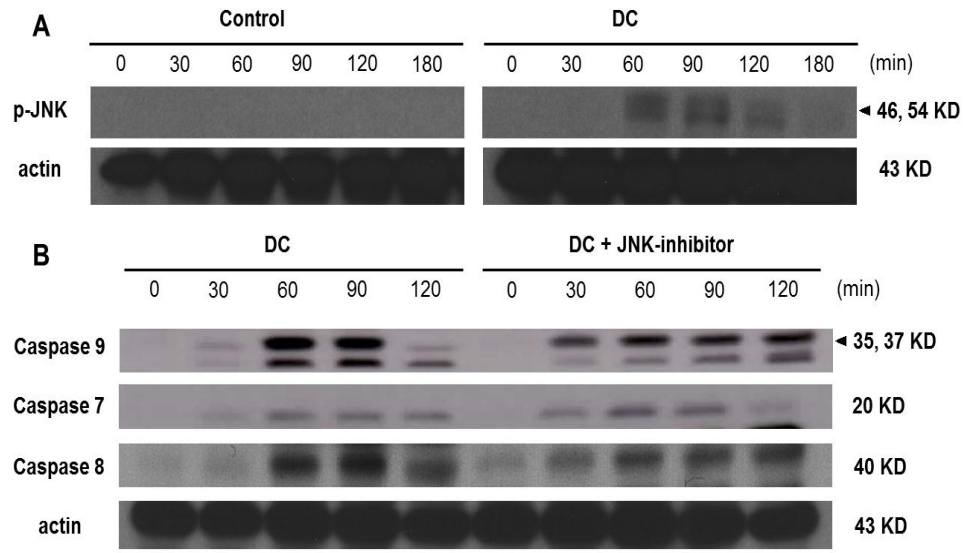

Figure 2. JNK activation in bile acid-induced hepatocyte apoptosis. (A) Huh-BAT cells were cultured with DC (200 μM) for the indicated time periods. Cells were lysed and immunoblot analysis was done with anti-phospho-eIF2α and anti-actin antibodies. (B) Huh-BAT cells were treated with DC (200 μM) in the presence or absence of JNK inhibitor (0 and 10 μM) treatment. Cells were lysed and immunoblot analysis was done with anti-caspase 9, anti-caspase 7 and anti-actin antibodies.

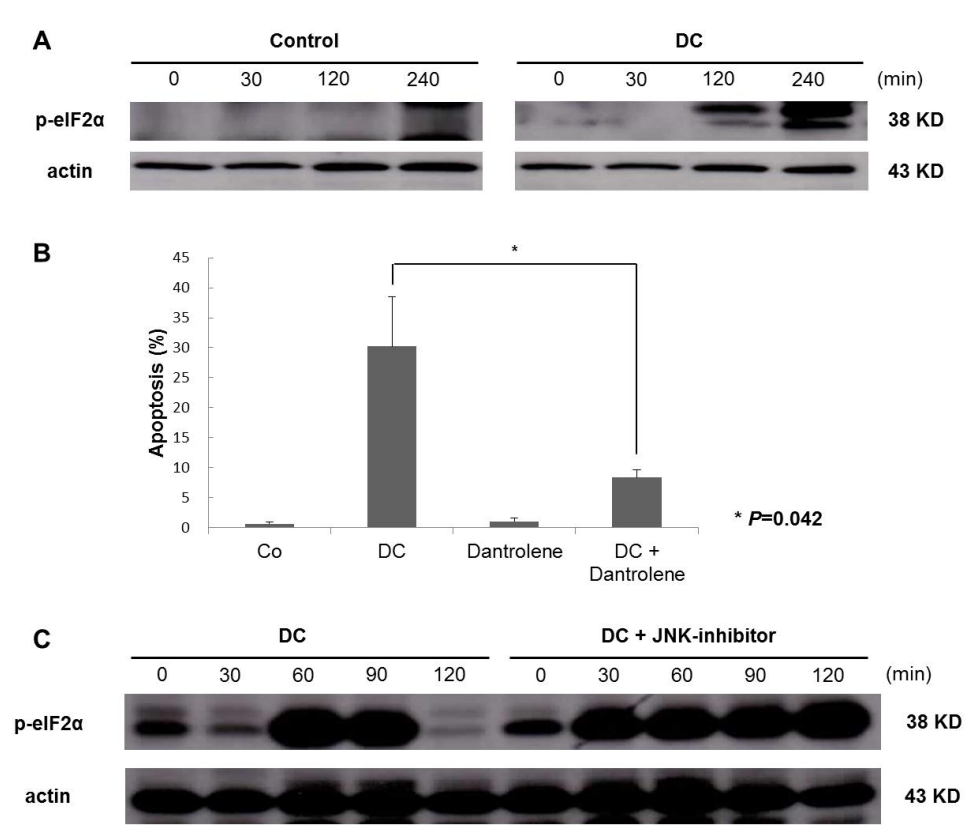

Figure 3. Enhanced ER stress in bile acid-induced apoptotic cell death. (A) Huh-BAT cells were cultured with DC (200 μM) for the indicated time periods. Equivalent amounts of proteins were immunoblotted with anti-phospho-eIF2α and anti-actin antibodies. (B) Huh-BAT cells were pretreated with dantrolene (30 μM) for 16 h, and then DC (200 μM) for additional 5 h. (C) Huh-BAT cells were treated with DC (200 μM) in the presence or absence of JNK inhibitor (0 and 10 μM) treatment. Cells were lysed and immunoblot analysis was done with anti-phospho-eIF2α and anti-actin antibodies. Apoptosis was quantified by DAPI staining and fluorescent microscopy. Data were expressed as the mean ± SD. *, P=0.042.

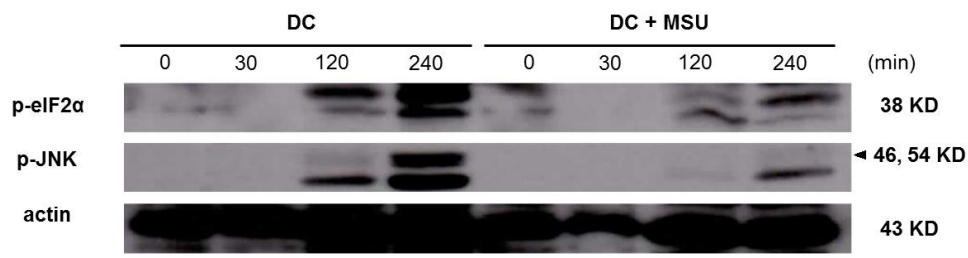

Figure 4. Attenuation of bile acid-induced ER stress and JNK activation by MSU. Huh-BAT cells were incubated with MSU (100 μg/mL) or media (control) for 24 h. Cells were then treated with DC (200 μM) for each indicated time period. Immunoblot analysis was performed on cell lysates employing anti-phospho-eIF2α, anti-phospho-JNK and anti-actin antibodies.

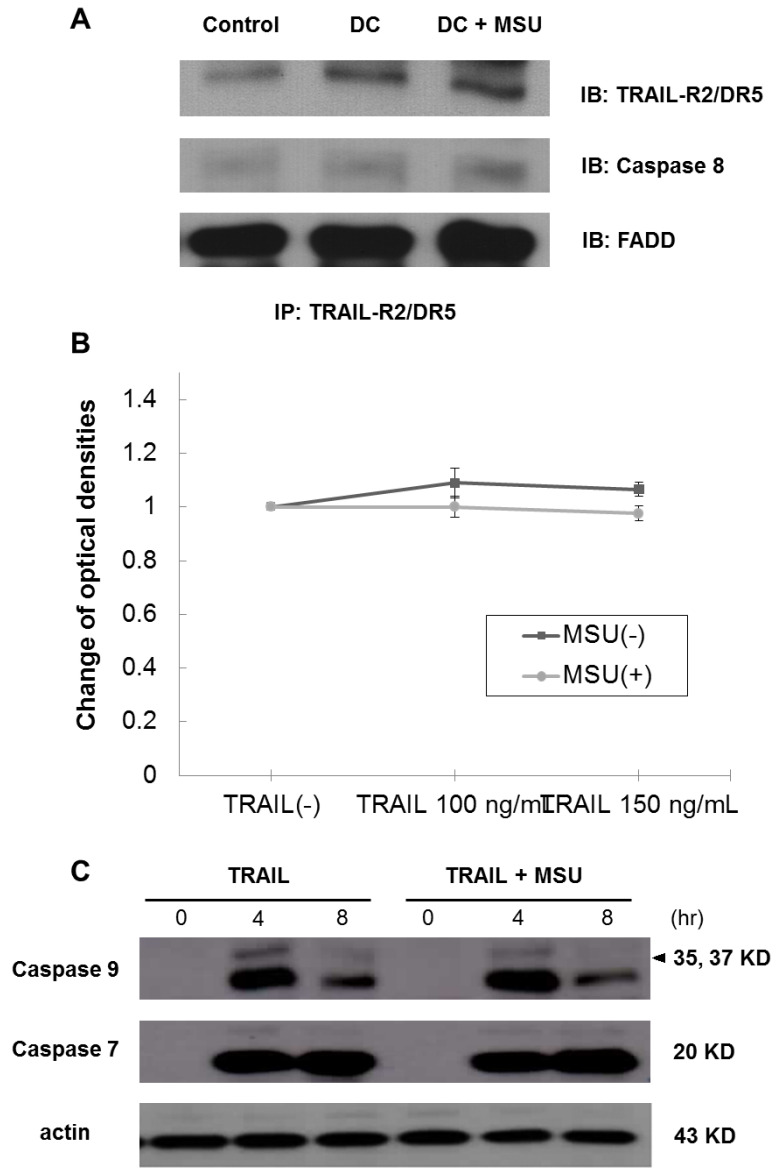

Figure 5. Antiapoptotic signaling of MSU independent of death receptor dependent signaling pathway. (A) Huh-BAT cells were treated with DC (200 μM) for 30 h and crosslinked recombinant FLAG-tagged soluble TRAIL (sTRAIL) for 30 min in the presence or absence of MSU (100 μg/mL) pretreatment for 16 h.

Cells were then lysed and DISC was immunoprecipitated using protein A/G beads and analyzed by immunoblotting for TRAIL-R2, caspase 8 and FADD. (B) Huh-BAT cells were pretreated with MSU (0 or 100 μg/mL) for 16 h prior to the addition of TRAIL. Cells were then treated with TRAIL (0, 100 or 150 ng/mL) for an additional 24 h and MTS assay was performed. Data were expressed as mean ± standard deviation of changes of optical densities compared to control cells. (C) Huh-BAT cells were pretreated with MSU (100 μg/mL) for 20 h, and then treated with TRAIL (100 ng/mL) for each indicated time period. Cells were lysed and immunoblot analysis was done with anti-caspase 9, anti-caspase 7 and anti-actin antibodies.

Discussion

The principal findings of this study are related to the antiapoptotic effect of MSU on bile acid-induced hepatocyte apoptosis. This study demonstrated that augmented ER stress induces JNK activation, leading to mitochondrial apoptotic pathway. Moreover, MSU attenuated bile acid-induced hepatocyte apoptosis, specifically by decreasing bile acid-induced ER stress and subsequent JNK activation. The antiapoptotic effect of MSU was shown to be independent of death receptor signaling pathway.

It is well known that accumulation of bile acids can induce hepatocyte apoptosis by activating death receptor signaling pathway or mitochondrial apoptotic pathway.[7,8,18] In previous studies, it was determined that bile acids can induce hepatocyte apoptosis by causing ER stress.[19] If bile acids accumulate within hepatocyte, bile acids cause Ca^{2+} release from ER and subsequent extracellular Ca^{2+} influx leading to the activation of calpain and caspase 12, which is specific to ER stress-mediated apoptosiss.[19] It was demonstrated that bile acids may activate both ER stress-mediated apoptosis and mitochondria-mediated apoptosis, thus implicating the cross-talk between ER and mitochondria.[20] Our study also showed the linkage between ER stress-mediated apoptosis and mitochondria-mediated apoptosis in bile acid-induced hepatocyte apoptosis, and furthermore, JNK activation is the downstream event of ER stress amplification.

MSU, which is a DAMP released and formed from damaged cells, was shown to

have antiapoptotic effect during bile acid-induced hepatocyte apoptosis. MSU reduced proapoptotic response of ER, thereby leading to the attenuation of subsequent JNK activation and apoptotic cell death. The activation of caspase 1 is a key step which can subsequently activate pro-cytokines pro-IL-1β and pro-IL-18, leading to inflammatory cytokines and development of inflammation.[21] A recent study suggested the protective role of caspase 1 activation against cell death after redox stress in hepatocytes.[16] In this study, it was shown that caspase 1 reduced mitochondrial respiration and limited reactive oxygen species (ROS) production in hepatocytes after hypoxia/reoxygenation. Although it was proposed that the NLRP3 inflammasome responds to ER stress via a previously uncharacterized signaling pathway distict from the unfolded protein response, the interaction between the inflammasome activation and ER stress is still unclear.[22]

Meanwhile, the mechanism affected by MSU was independent of death receptor signaling pathway. A previous study demonstrated that phagocytosis of MSU or other crystals leads to acidification and maturation of phagosomes into lysosomes, followed by lysosomal destabilization and rupture, thus resulting in the release of phagosomal contents, particularly cathepsin B.[23] However, it is still unknown how macrophages recognize and bind MSU or other crystals, and furthermore, how non-immune cells, such as hepatocytes, uptake crystalline materials. It was determined that accumulated bile acids can induce structural and functional damage to the basolateral membranes, cell organelle membranes in the setting of cholestasis.[24-26] Bile acid-induced injury of hepatocyte membrane may play a role

in the uptake of MSU crystal, however, this hypothesis needs to be verified.

This study demonstrated that MSU attenuates bile acid-induced ER stress and subsequent JNK activation, leading to hepatocyte protection against bile acid-induced apoptosis and that the antiapoptotic effect is independent of death receptor signaling pathway. Based on the results of our study, we propose a novel role for inflammasome activation induced by MSU in hepatocytes promoting compensatory response to cholestatic liver damage.

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요약 (국문 초록)

배경: 담즙 정체 상태에서 간 내 축적된 담즙산은 세포 자멸사 신호 전달 체계를 활성화시켜 간세포 자멸사를 촉진한다. Monosodium urate (MSU)는 세포사 후 요산이 방출되면 결정화되어 생성되는데, 이는 염증조절복합체라 명명된 세포질 내 단백 결합체를 활성화시킨다. 이 연구에서는 MSU가 담즙산에 의한 간세포 자멸사에 미치는 영향과 MSU에 의해 조절되는 신호 전달 체계를 밝혀내고자 하였다.

방법과 결과: 이 연구에서는 Huh-7 세포에 소디움 의존성 담즙 운반 폴리펩타이드를 발현시킨 Huh-BAT 세포를 이용하여 실험을 시행하였다. 간세포 자멸사 유발을 위해 deoxycholate (DC)를 사용하였다. 세포 증식 정도는 MTS 분석법을 이용하였고 세포 자멸사 정도는 4′,6-diamidino-2-phenylindole dihydrochloride 염색을 통해 정량화하였다. MSU의 작용에 관여하는 신호전달체계는 면역블롯법과 면역침강법을 이용하여 알아보았다. MSU는 caspase 9과 7과 같은 미토콘드리아 세포 자멸사 신호를 감소시켜 담즙산에 의한 간세포 자멸사를 억제하였다. 또한 담즙산에 의해 증강된 eIF2α의 인산화와 JNK 활성이 MSU에 의해 억제되었다. 반면, MSU에 의해 담즙산 처리 후 TRAIL-R2의 올리고머화 및 death-inducing signaling complex으로의 caspase 8 동원 정도는 변화되지 않았으며 TRAIL 처리 후 caspase 9과 7의 활성도 또한

변화되지 않았다.

결론: MSU는 담즙산에 의해 유발된 소포체 스트레스와 이에 이은 JNK 활성을 감소시켜 담즙산에 의한 간세포 자멸사를 억제함을 알 수 있었다. 또한 MSU의 세포 자멸사 억제 효과는 death receptor 연관 신호 전달 체계와는 독립적으로 작용함을 밝혀내었다.

주요어 : 요산염, 담즙산, 세포 자멸사, 소포체, 면역조절복합체

학번 : 2012-21706