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A study on the mechanism of liver fibrosis by mitochondrial uncoupler CCCP

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미토콘드리아 짝풀림제인 CCCP 에 의한
간섬유화 조절기전 연구

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ABBREVIATIONS

ActD	Actinomycin-D
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
α-SMA	alpha-smooth muscle actin
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
CHX	Cycloheximide
CQ	Chloroquine
HSCs	Hepatic stellate cells
MG132	Z-Leu-Leu-Leu-al
mtDNA	Mitochondrial DNA
mtROS	Mitochondrial reactive oxygen species
MMP	Mitochondrial membrane potential
PAI-1	Plasminogen activator inhibitor-1
PINK1	PTEN-induced kinase 1
Rho-123	Rhodamin-123

국 문 초 록

미토콘드리아 짝풀림제인 CCCP 에 의한 간섬유화 조절기전 연구

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지 도 교 수: 기 성 환

약학과

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간섬유화는 바이러스 간염, 알코올 등 여러가지 자극에 의해 간세포가 손상을 받게 되면 간실질세포 및 비실질세포들의 상호작용에 의해 간 조직중에 세포외기질 (ECM)이 축적되어 발생되는 상처 치유 반응 (wound-healing response) 이다. 하지만 간섬유화가 지속되는 경우에 비가역적 만성질환인 간경변으로 이행하기 때문에 간섬유화 단계에서 질병이 진행되지 않도록 하는 것이 매우 중요하다. 이러한 간섬유화 과정은 간성상세포 (hepatic stellate cell)가 주된 역할을 하게 된다. 간성상세포는 휴지기 상태에서의 비타민 A 저장 세포로써의 역할을 하다가 만성적인 간 손상 상태에 반응하여 콜라겐 등의 ECM 을 생성하는 근섬유아세포 형태로 분화된다. 미토파지 (mitophagy)는 자가포식작용 (autophagy)에 의해 손상되었거

나 불필요한 미토콘드리아를 선택적으로 분해하는 세포작용이다. 이러한 미토파지의 활성화는 여러 세포에서 미토콘드리아 기능을 조절하고 기능을 유지하는데 핵심적인 역할을 한다고 알려져 있다. 최근 본연구실에서는 간성상세포 활성화 및 간섬유화 과정에서 미토파지의 역할 및 관련 기전에 대한 연구를 수행한 바 있다. 하지만 미토콘드리아에 직접적으로 간섭하여 미토파지를 유도하는 미토콘드리아 짝풀림제 (uncoupler)에 의한 간성상세포 활성화 및 이를 제어하는 약물에 관한 연구는 전무한 실정이다.

본 연구에서 간성상세포에서 대표적인 미토콘드리아 짝풀림제인 CCCP (carbonyl cyanide m-chlorophenyl hydrazone)에 의한 간섬유화 조절기전을 규명하고 이를 조절함으로 간섬유화를 제어하는 약물을 발굴하고자 하였다. 간성상세포에서 미토콘드리아 짝풀림제인 CCCP 처리에 의해 PAI-1 의 단백질 발현이 증가함을 관찰하였다. CCCP 에 의한 발현 증가는 전사적인 수준에서 조절되지 않으며 전사후 수준에서 조절됨을 확인할 수 있었다. 리소좀 (lysosome) 억제제인 chloroquine 처리 시 CCCP 에 의한 PAI-1 의 증가가 보다 강화되었으나 프로테아좀 (proteasome) 억제제인 MG132 를 처리한 경우에는 유의한 발현 변화가 관찰되지 않았다. 이와 같은 결과는 CCCP 가 리소좀 기능을 통해 단백질 안정성에 영향을 주어 PAI-1 발현을 조절함을 시사한다. 그리고 CCCP 는 간성상세포에서 미토콘드리아에 직접적인 기능 장애를 유도하였고 자가포식작용을 활성화시켰다. 이와 더불어 CCCP 를 단회 또는 반복 투여한 동물 모델에서 간섬유화 및 미토파지 관련 단백질이 증가함을 관찰하였다. 이러한 결과들은 CCCP 에 의한 미토콘드리아 기능손상이 미토파지를 유도하고 더 나아가 간성상세포 활성화를 촉진시킴으로 간섬유화 진행을 촉진할 수 있음을 의미한다. 더 나아가, 미토파지를 조절하여 간성상세포 활성화를 제어하는 약물로 AMPK 활성제 AICAR 를 활용하여 평가하

였다. AICAR 는 CCCP 에 의해 증가한 PAI-1 발현을 감소시켰으며 CCCP 에 의한 미토콘드리아 기능손상을 및 미토파지를 억제하였다. 더 나아가, 또 다른 AMPK 직접적 활성제인 A769662 를 활용하여 AICAR 의 효과가 AMPK 의존적임을 확인할 수 있었다. 이러한 결과들을 바탕으로 AMPK 활성화제가 미토파지 매개 간성상세포 활성화를 억제함으로써 간섬유화 치료제로 활용될 수 있음을 제시한다.

I. INTRODUCTION

Liver fibrosis results from chronic liver injury caused by various etiologies, such as hepatitis B or C virus infection, alcohol abuse, and nonalcoholic steatohepatitis. Upon repeated injury, the liver undergoes a wound-healing response, leading to the excessive accumulation of extracellular matrix (ECM), and it can advance to end-stage cirrhosis, which is responsible for high morbidity and mortality worldwide [1-3]. Hepatic stellate cells (HSCs) is the primary effector cell, orchestrating the deposition of ECM in normal and fibrotic liver. Activation of hepatic HSCs in response to hepatic injury is a crucial step in hepatic fibrogenesis, and entails the transformation of quiescent vitamin-A rich cells into proliferative and contractile myofibroblasts [4, 5]. Thus, decrease of the number of activated HSCs is an attractive application for anti-fibrotic therapy. However, there is no established way to modulate HSC activation and fibrosis progression [6]. Hence, it is necessary to identify new regulatory targets and mechanisms involved in this process to treat liver fibrosis.

Liver is one of the organs abundant in terms of number and density of mitochondria [7]. Mitochondria are considered to be a main player in the regulation of hepatic cellular redox status, lipid metabolism, and cell death. It was well known that mitochondrial dysfunction is associated with both acute and chronic liver diseases progression [8, 9]. Autophagy is an evolutionarily conserved process by which cytoplasmic components are degraded in a lysosome-dependent manner.

Mitophagy, selective degradation of mitochondria by autophagy through lysosomal degradation, plays a key role in the liver's physiology [10, 11]. Mitophagy is regulated by several pathways, and the PINK1/Parkin pathway is the best characterized pathway. In normal mitochondria, PINK1 is imported into the mitochondria, where is degraded through the N-end rule pathway [12]. Upon mitochondrial depolarization, PINK is stabilized on the surface on the mitochondria. PINK1 accumulates on the damaged mitochondrial outer membrane and recruits

Parkin from the cytosol and activates Parkin's E3 ligase activity via phosphorylation. Afterward, Parkin ubiquitinates outer mitochondrial membrane proteins to trigger selective autophagy [13, 14].

In my lab, we found that TGF-β induced mitophagy through PINK1/Parkin pathway without mitochondrial dysfunction, which result in HSCs activation. However, there is still question whether mitochondria dysfunction-induced mitophagy leads to HSCs activation. Mitochondrial uncoupling is any process that dissociate between production of mitochondrial membrane potential and mitochondria-dependent ATP production. Mitochondrial uncoupling agent conducts proton inflow across the mitochondrial inner membrane without ATP synthesis [15-17]. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a typical mitochondrial uncoupler, is a protonophore that suppresses oxidative phosphorylation in mitochondria. CCCP induce mitochondrial permeability transition pore opening, conducting the disruption of mitochondrial membrane potential (ΔΨm) [18-21].

Thus, we currently explored whether mitochondrial uncoupler CCCP induce liver fibrogenesis through mitochondrial dysfunction-mediated mitophagy in HSCs. First, we examined CCCP-induced expression level of PAI-1, representative fibrogenesis marker, in HSCs. Then we determined regulatory mechanism associated with CCCP-induced PAI-1 expression. In addition, we tried to identify the candidate drug for liver fibrosis via regulation of mitophagy in HSCs. These current findings showed that mitochondrial dysfunction by mitochondrial uncoupler led to hepatic fibrogenesis via mitophagy induction, which might be promising target for prevention and treat the liver fibrosis

II. MATERIALS AND METHODS

1. Materials

Plasminogen activator inhibitor-1 (PAI-1) antibody was purchased from BD Bioscience (Mountain View, CA, USA). Anti-LC3B and anti-PINK1 antibodies were purchased from NOVUS Biologicals (Littleton, CO, USA). Anti-62 antibody was purchased from Abnova (Taipei, Taiwan). Anti-Parkin antibody was purchased from Abcam (Cambridge, MA, USA). Phospho-SMAD3, SMAD2/3 and SMAD7 antibodies were purchased from Cell Signaling Technology (Danvers, Massachusetts, USA). Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies, Mito-SOX were purchased from Invitrogen (Carlsbad, CA, USA). Rhodamine123 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Actinomycin-D (ActD), Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), α-SMA, and β-actin antibodies, Cycloheximide (CHX), Z-Leu-Leu-Leu-al (MG132), chloroquine (CQ), and Rotenone, AICAR, and A769662 were provided from Sigma (St. Louis, MO, USA). TGF-β was obtained from R&D Systems (Minneapolis, MI, USA).

2. Cell culture

LX-2 cells (Human Hepatic Stellate Cell Line) were generously obtained by Dr. S.L. Friedmann (Mount Sinai School of Medicine, NY, USA). HSC-T6 Cells (Rat Hepatic Stellate Cell Line) were obtained from Merck Millipore (Darmstadt, Germany). Cells were plated in 60 mm or 6-well (Thermo Scientific, Waltham, MA, USA) plates, and cells were grown to 70-80% confluence. Cells were maintained DMEM with 10% fetal bovine serum (FBS, Atlas Biologicals, Fort Collins, CO, USA), 50 U/mL penicillin/streptomycin at 37℃ in a humidified 5% CO2 atmosphere. Cells were then washed phosphate buffered saline (PBS), before sample preparation.

3. Primary Hepatic Stellate cells isolation

Hepatic stellate cells were isolated from the liver of 8-week-old ICR mice (Oriental Bio, Sungnam, South Korea). Briefly, after intubation in the portal vein, the liver were perfused in situ with Ca2+-free Hank’ s balanced saline solution (HBSS) at 37℃ for 20 min, and then further perfused with the solution containing 0.05% collagenase and Ca2+ for 20 min at a flow mice of 10 mL/min. The perfused livers were tear aparted, filtered through 70 μM cell strainer (BD Bioscience), and centrifuged at 50 g for 2 min to separate the parenchymal and non-parenchymal. HSCs were isolated according to the previously published method. Briefly, the supernatant was further centrifuged at 500 g for 15 min, resuspended in Ficoll with Percoll (1:10, GE Healthcare, IL, USA), and centrifuged at 1400 g for 5 min. HSCs were collected from the interface. Quiescent HSCs were cultured for 0 day.

4. Immunoblot analysis

Cell lysates, SDS-polyacrylamide gel electrophoresis and immunoblot analysis were performed as previously reported method [22]. The cell lysates were separated in 7.5% and 12% gel by electrophoresis, and then electrophoretically transferred to nitrocellulose membranes. After the membranes were blocked for 0.5 h, they were incubated with primary antibody at 4℃ overnight and then incubated with a horseradish peroxidase-conjugated secondary antibody. The immune reactive protein was visualized by ECL chemiluminescence detection kit. β-actin was used as control.

5. RNA isolation and RT-PCR analysis

Total RNA was extracted by TRIzol (Invitrogen) according to the manufacturer’s protocol. To obtain cDNA, total RNA (2 μg) was reverse-transcribed using an oligo (dT) 16 primer. The cDNA was amplified using a high-capacity cDNA synthesis kit (Bioneer, Daejeon, Korea) with a thermal cycler (Bio-Rad, Hercules, CA, USA). PCR amplified products were separated by

using 2% agarose gel, stained with ethidium bromide (Sigma, St. Louis, MO, USA), and visualized in gel documentation system (Fujifilm, Tokyo, Japan). The following primer sequences were used: human PAI-1 sense 5′ -CGCCAGAGCAGGACGAA -3′ , and antisense 5′ -CATCTGCATCCTGAAGTTCTCA -3′ ; human α-SMA sense 5′ -CGTGGCTATTCCTTCGTTAC-3′ , and antisense 5′ -TGCCAGCAGACTCCATCC-3′ ; human Col1A1 sense 5′ -CCTGGGTTTCAGAGACAACTTC -3′ , and antisense 5′ -TCCACATGCTTTATTCCAGCAATC -3′ ; human GAPDH sense 5′ -GAAGGTGAAGGTCGGAGTC-3′ , and antisense 5′ -GAAGATGGTGATGGGATTTC-3′ . GAPDH was used as a reference gene for normalization.

6. Luciferase assay

LX-2 cells in 12-well plates were serum starved for 4 h and transfected with Smad binding element (SBE)-Luc, activator protein 1 (AP-1)-Luc, hypoxia-inducible factor-1 response element (HRE)-Luc, NF-KB-Luc, or pRL-TK plasmid (a plasmid that encodes Renilla luciferase and is used to normalize transfection efficacy) using Lipofectamine^{TM}2000 reagent (Invitrogen) for 3 h. Next, transfected cells were allowed to recover in MEM containing 1% FBS overnight and then treated with CCCP for 12 h. The firefly and Renilla luciferase activities of cell lysates were assessed by the dual luciferase assay system (Promega, Madison, WI) according to the manufacturer's guidelines.

7. Mitochondrial ROS levl

Detection of intracellular superoxide in LX-2 cell. Cells were treated with and without CCCP (5 μM) at 37℃ for 0.25 h or a positive control, rotenone (10 μM) at 37℃ for 1 h, then treated with Mito-SOX (10 μM) at 37℃ for 0.5 h. Samples were harvested by trypsinization and washed as PBS. The intensity of the fluorescence in samples was measured using a Flow-

cytometry (Beckman-Coulter). Mito SOX fluorescence was used channel FL2-A.

8. Mitochondrial membrane potential assay

LX-2 cells were treated with or without CCCP (5 μM) at 37℃ for 24 h or a positive control, rotenone (10 μM) at 37℃ for 24 h, then treated with Rho-123 (0.05 ng/ml) at 37℃ for 0.5 h. Samples were harvested by trypsinization and washed as PBS. The intensity of the fluorescence in samples was measured using a Flow-cytometry (Beckman-Coulter). Rho-123 fluorescence was used channel FL1-A.

9. Measurements of mitochondrial DNA

Total mitochondrial DNA (mtDNA) was extracted from cells according to the manufacturer's protocol (Nucleogen, Siheung, Korea). Cytochrome C oxidase subunit II (mtCOXII) levels transcribed from mtDNA were measured by real-time PCR and normalized using nuclear-encoded receptor-interacting protein 140 (RIP140). The following primer sequences were used: human mtCOXII sense 5′-ACCTGCGACTCCTTGACGTTG -3′, and antisense 5′-TAGGACGATGGGCATGAAACTG -3′; human RIP140 sense 5′-GCTGGGCATAATGAAGAGGA -3′, and antisense 5′-CAAAGAGGCCAGTAATGTGCTATC -3′. Real-time PCR was performed using Step One (Applied Biosystems, Foster City, CA, USA) with a SYBR Green premix according to the manufacturer's guidelines (Applied Biosystems).

10. Measurements of ATP

The ATP was assessed using the EnzyLight ADP/ATP ratio assay kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's guidelines. Briefly, the ATP reagent from the kit was treated to cells grown in a 96 well plate. Then, Luminescence by ATP was measured using a luminometer (Promega, Madison, WI).

11. Animal experiments

Animal experiments were conducted under the guidelines of the Institutional Animal Use and Care Committee at Chosun University. For the acute model, CCCP (3 mg/kg), dissolved in 50% DMSO, was intraperitoneal injected to mice (n=9) and mice were sacrificed 6, 12, and 24 h after the treatment. For the subchronic model, CCCP (1 mg/kg/day), dissolved in 50% DMSO, was intraperitoneal injected to mice (n=3) for 2 weeks. Mice were sacrificed 24 h after the last injection. Blood and tissue samples were collected for the further analysis.

12. Blood biochemistry

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assessed using commercial kits (Asan Pharmaceutical, Seoul, Korea).

13. Statistical analysis

One-way ANOVA was used to assess significant differences between treatment groups. The Newman-Keuls test was used to assess the significance of the differences between the means of multiple groups. Results are expressed as mean ± standard Error (SE).

III. RESULTS

1. Mitochondrial uncoupler CCCP activated hepatic stellate cells

The activation of HSCs is a critical step in liver fibrosis. Therefore, to investigate whether CCCP can induce activation of HSCs, we analyzed PAI-1 expression level, representative fibrotic marker, in LX-2 cells (immortalized human HSC cells). First, we examined the time dependency of PAI-1 expression in response to treatment with 5 μM CCCP (Fig 1A). PAI-1 protein level was increased after 1-6 h of CCCP treatment. In addition, PAI-1 expression was gradually induced by CCCP stimulation up to 5 μM of CCCP (Fig 1B). PAI-1 induction by CCCP was confirmed in other hepatic stellate cell lines HSC-T6 and primary HSCs from mice (Fig 1C and D). These results suggest that CCCP can induce the activation of HSCs.

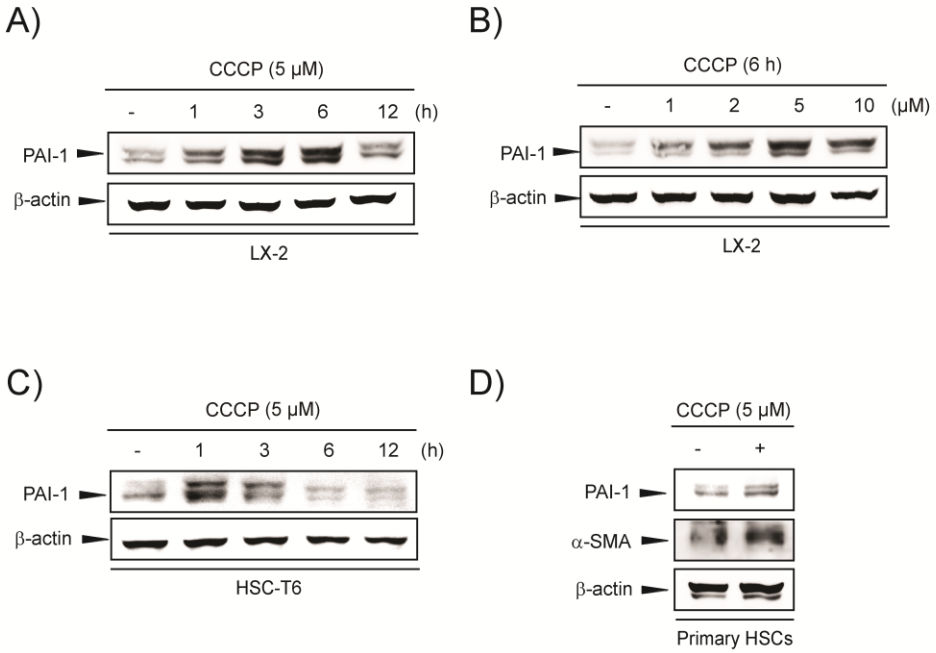
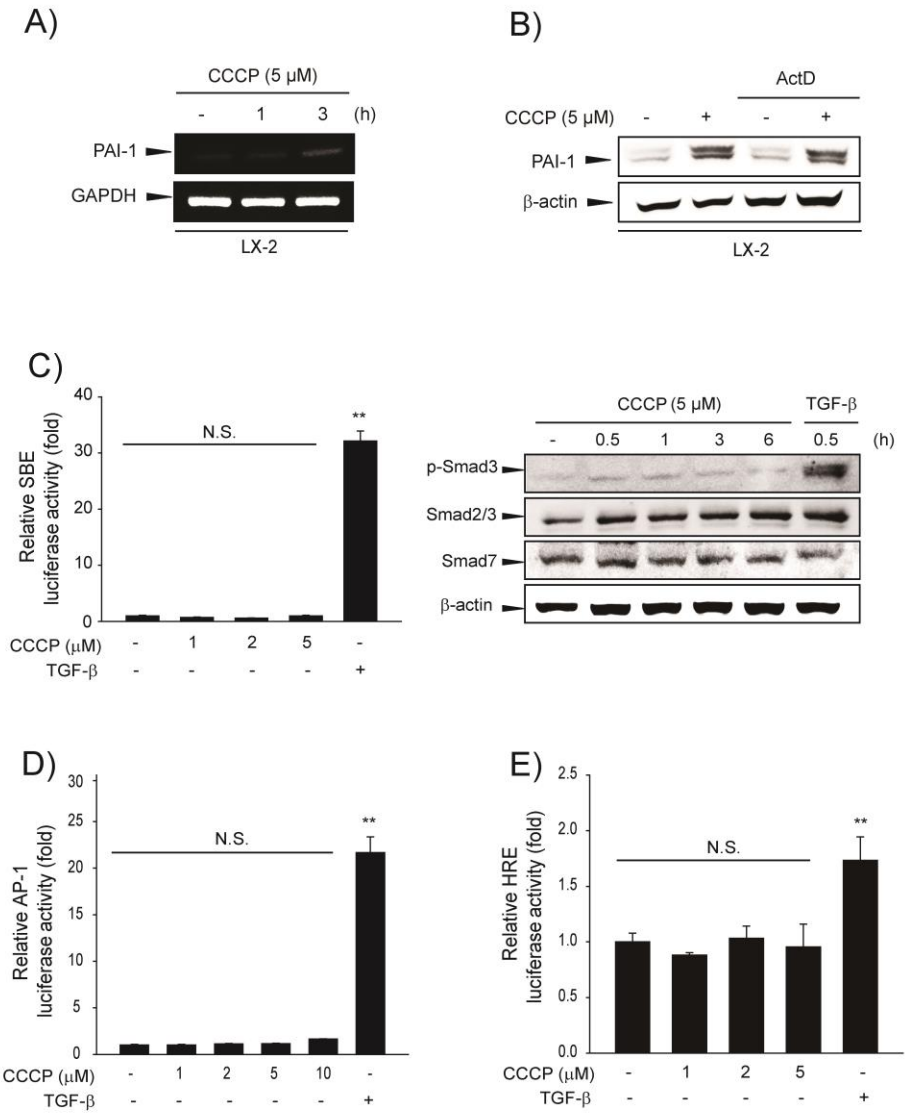


Figure 1. The effect on HSCs activation by CCCP in LX-2 cells

(A) The time courses of PAI-1 expression in LX-2 cells treated with CCCP. Immunoblot analyses for PAI-1 were carried out in cells treated with 5 μM CCCP for 1-12 h. (B) The effect of a diversity of concentration of CCCP on PAI-1 induction in LX-2 cells. LX-2 cells were incubated with 1, 2, 5 or 10 μM CCCP for 6 h, and then PAI-1 expression in the cell lysates were assessed by immunoblotting. (C) The effect of CCCP on PAI-1 induction in the other hepatic stellate cell line, HSC-T6 cell. (D) The effect of CCCP on PAI-1 induction in primary HSCs from mice. Primary HSCs were incubated with 5 μM CCCP for 6 h, and then PAI-1 and α-SMA expression in the cell lysates were assessed by immunoblotting.

2. PAI-1 induction by CCCP was not regulated in a transcriptional level

To verify whether CCCP-induced PAI-1 expression was due to increased transcription, we measured the PAI-1 mRNA level in CCCP treated LX-2 cells. The results showed that PAI-1 levels were not induced by CCCP treatment (Fig 2A). In addition, when the LX-2 cells were treated with the transcription inhibitor actinomycin-D (ActD) for 0.5 h before CCCP treatment, PAI-1 levels induced by CCCP was not altered by ActD treatment (Fig 2B). Furthermore, we examined Smad activity, which is key transcription factor for the fibrogenic gene expression, using Smad binding element (SBE) luciferase plasmid. However, SBE luciferase activity was not affected by CCCP treatment (Fig 2C-left). Consistently, Smad3 phosphorylation, Smad2/3 and Smad7 expression were not changed by CCCP treatment (Fig 2C-right). Then we also measured activity of other transcription factors associated with fibrogenic gene expression activator protein 1 (AP-1), hypoxia-inducible factor-1 (HIF-1) and NF-$_{K}$B luciferase activities, but their activities were not altered by CCCP (Fig 2D-F). These results support that PAI-1 expression was not transcriptionally regulated by CCCP.



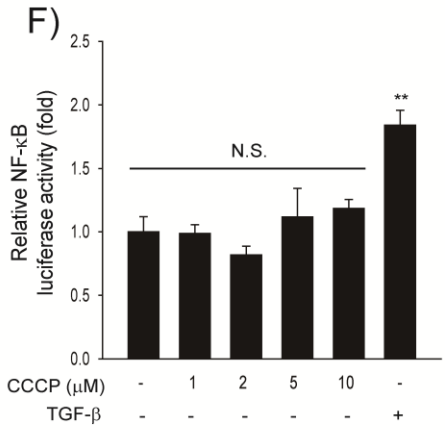


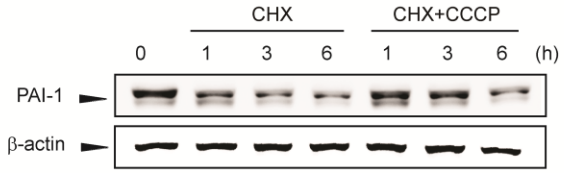
Figure 2. PAI-1 induction by CCCP was not transcriptionally regulated

(A) RT-PCR analysis. Cells were treated with 5 μM CCCP for 1, 3 h. mRNA levels of PAI-1 were assessed by RT-PCR using GAPDH as control. (B) The effect of actinomycin-D (ActD) on the PAI-1 upregulation by CCCP in LX-2 cells. The cells were treated with 5 mg/ml of ActD in the presence or absence of CCCP. The level of PAI-1 protein was monitored after CCCP treatment for 6 h. (C) The effect of CCCP on Smad binding element (SBE) luciferase activity by CCCP treatment. Cells were transfected with SBE luciferase reporter construct. Transfected cells were treated with CCCP for 12 h. Data represent the mean ± S.E. (n = 3, significant when compared with controls, $^{**}p<0.01$) (left). The effect of CCCP on Smad activity. LX-2 cells were incubated with CCCP for the indicated periods. Phosphorylation of Smad3, and expression of Smad2/3 and Smad7 in the cell lysates were assessed by immunoblotting (right). (D) The effect of CCCP on Activator protein 1 (AP-1) luciferase activity by CCCP treatment. Cells were transfected with AP-1 luciferase construct. Transfected cells were treated with CCCP for 12 h. Data represent the mean ± S.E. (n = 3, significant when compared with controls, $^{**}p<0.01$). (E) The effect hypoxia-inducible factor-1 (HRE) luciferase activity by CCCP treatment. Cells were transfected with HRE luciferase construct. Transfected cells were treated with CCCP for 12 h. Data represent the mean ± SE (n = 3, significant when compared with controls, $^{**}p<0.01$). (F) The effect NF-κB luciferase activity by CCCP treatment. Cells were transfected with NF-κB luciferase construct. Data represent the mean ± S.E. (n = 3, significant when compared with controls, $^{**}p<0.01$).

3. Regulatory mechanism of CCCP-induced PAI-1 induction

Therefore, we analyzed whether PAI-1 expression was modulated by post-transcriptional level. LX-2 cells were incubated with protein synthesis inhibitor, cycloheximide (CHX). Treatment on cells with CHX quickly decreased the amount of PAI-1. These results indicate that PAI-1 expression is affected by its degradation. Next, we investigated whether CHX-mediated PAI-1 decay was affected by CCCP treatment. Surprisingly, CCCP treatment delayed PAI-1 diminishes, suggesting that up-regulation of PAI-1 expression by CCCP is due to increased protein stability (Fig 3A). To identify whether delayed PAI-1 decrease by CCCP occurred via proteasomal or lysosomal protein degradation, we treated with a proteasomal inhibitor (MG132) or a lysosomal inhibitor (chloroquine, CQ) in LX-2 cells. We found that treatment of CQ with CCCP caused accumulation of PAI-1, more than CCCP alone. However, PAI-1 expression by CCCP was not changed by MG132 treatment (Fig 3B and 3C). Out results suggest that PAI-1 expression by CCCP was regulated by protein stability due to lysosome activity.

A)



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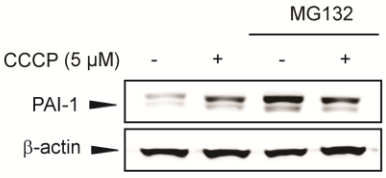

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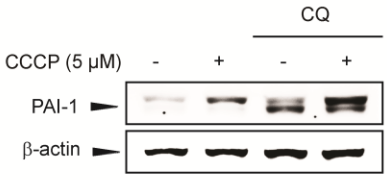
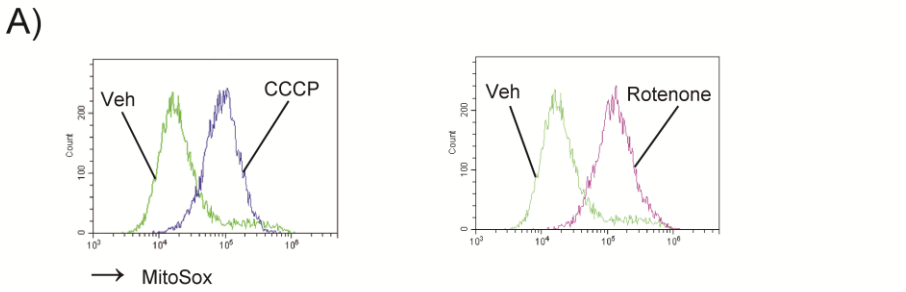


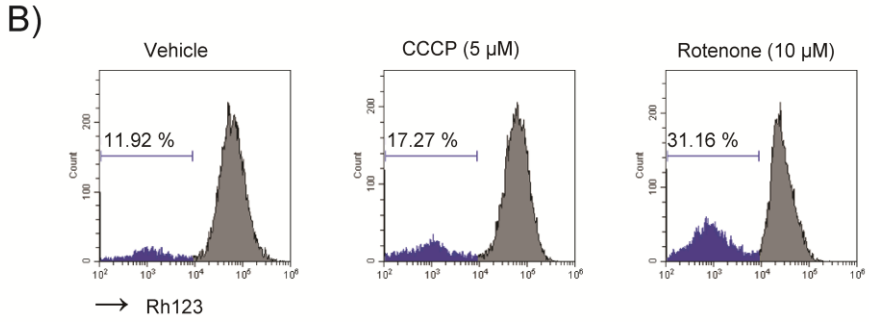
Figure 3. PAI-1 induction by CCCP was dependent on post-transcriptional mechanism

(A) The effect of chycloheximide (CHX) on PAI-1 expression by CCCP. LX-2 cells were incubated with 0.5 μg/ml CHX in the presence or absence of CCCP for the indicated periods. PAI-1 expression in the cell lysates were assessed by immunoblotting. (B) The effect of proteasomal inhibitor on PAI-1 induction by CCCP. LX-2 cells were incubated with MG132 in the presence or absence of CCCP for 6 h, and then PAI-1 expression in the cell lysates were assessed by immunoblotting. (C) The effect of lysosomal inhibitor on PAI-1 induction by CCCP. LX-2 cells were incubated with chloroquine (CQ) in the presence or absence of CCCP for 6 h, and then PAI-1 expression in the cell lysates were assessed by immunoblotting.

4. Mitochondrial dysfunction by CCCP stimulation

We investigated whether CCCP affects mitochondrial function in LX-2 cells. First, mitochondria ROS (mtROS) accumulation was measured using Mito-SOX. When we treated with CCCP (5 μM, for 0.25 h) in LX-2 cells, mtROS level was elevated (Fig 4A). Rotenone (10 μM, for 1 h), a mitochondria complex I inhibitor, was used as a positive control. Similarly, we examined mitochondrial membrane potential (MMP) change of the LX-2 cells through the use of Rhodamine 123 (Rho-123). When we treated with CCCP (5 μM, for 24 h) in LX-2 cells, disruption of MMP was increased (Fig 4B). In addition, CCCP in LX-2 cells significantly decreased levels of mtDNA, which presents numbers of mitochondria (Fig 4C). Moreover, we measured the cellular ATP levels. Treatment of CCCP reduced the levels of ATP compared with those in the control group (Fig 4D). Collectively these results strongly suggest that CCCP treatment induces mitochondrial dysfunction.





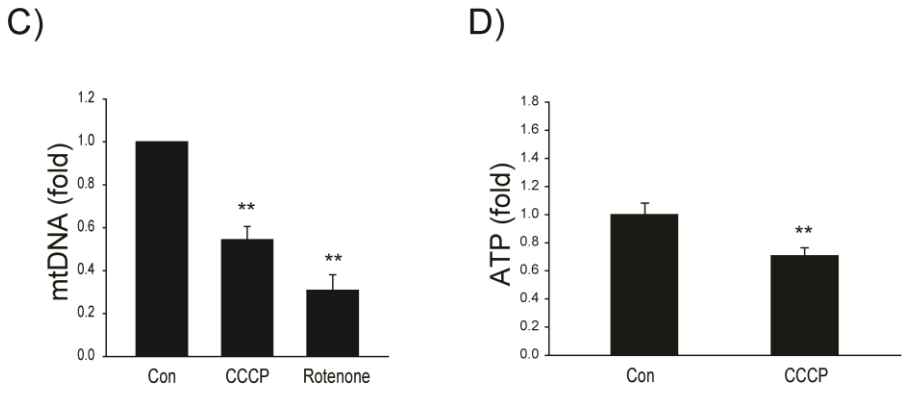


Figure 4. Effects of CCCP on mitochondrial function

(A) Measurement of mitochondrial ROS changes. LX-2 cells were treated with or without 5 μM CCCP for o.25 h, or 10 μM Rotenone for 1 h, and then incubated with Mito-SOX (10 μM) for 0.5 h. The cells were analyzed by FACS (green: vehicle, blue: CCCP, pink: Rotenone). (B) Measurement of mitochondrial membrane potential changes. LX-2 cells were treated with 5 μM CCCP or 10 μM Rotenone as a positive control for 24h, and then incubated with Rho-123 (0.05 ng/ml) for 0.5 h. The histogram showed a left shift of histogram peak representing the decrease of Rho-123 fluorescence intensity by the loss of MMP. Percentage of cells with reduced fluorescence intensity was calculated. (C) mtDNA contents. LX-2 cells were incubated with 5 μM CCCP for 24 h, and then performed to real-time PCR analysis used primers for the mtDNA region of COXII. Data represent the mean ± S.E. (n = 3, significant when compared with controls, **$p<0.01$). (D) Measurement of ATP. LX-2 cells were treated with or without 5 μM CCCP for 24 h. Data represent the mean ± S.E. (n = 3, significant when compared with controls, **$p<0.01$).

5. The effect of CCCP on mitophagy

It was reported that PINK1/Parkin pathway linking mitochondrial damage, ubiquitylation and autophagy in cells. To determine whether CCCP induce mitophagy in LX-2 cells, we examined representative autophagy and mitophagy markers. LC3B-II level was increased and p62 level was decreased in LX-2 cells exposed to CCCP compared with those in the control group. Moreover, expression of PINK1 and Parkin was increased by CCCP treatment. Thus, our data support that CCCP induced mitophagy in HSCs.

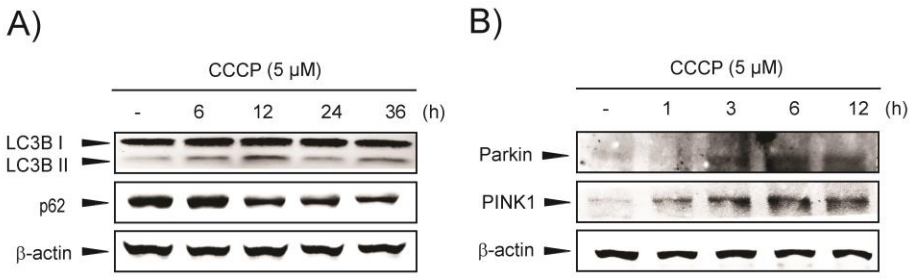


Figure 5. CCCP induces autophagy and mitophagy in LX-2 cell

(A) The effect of CCCP on autophagy activity. LX-2 cells were treated with for 6-36 h. LC3B and p62 expression in the cell lysates were assessed by immunoblotting. (B) The effect of CCCP on mitophagy. LX-2 cells were treated with for 1-12 h. Parkin and PINK1 expression in the cell lysates were assessed by immunoblotting.

6. CCCP induces fibrogenesis and mitophagy *in vivo*

To evaluate the whether CCCP treatment promoted liver fibrogenisis *in vivo*, we intraperitoneally injected CCCP to mice for 6-24 h at a dose of 3 mg/kg. First, we examined blood biochemistry analysis. Plasma ALT and AST levels, serum makers commonly used for liver injury, were significantly increased only 6 h after CCCP treatment (Fig 6A). Furthermore, we assessed protein marker of fibrogenesis and mitophagy, respectively, in mouse liver. PAI-1 expression was increased at 12-24 h. PINK1 levels were also upregulated in mouse liver sample (Fig 6B). Moreover, we also injected CCCP for 2 weeks via intraperitoneally into mice to examine subchronic effect of CCCP at a dose of 1 mg/kg. Plasma ALT and AST levels were not changed by CCCP treatment (Fig 6C). However, PAI-1 and PINK1 levels were increased by subchronic administration of CCCP (Fig 6D). These *in vivo* results support that CCCP causes liver fibrogenesis and mitophagy.

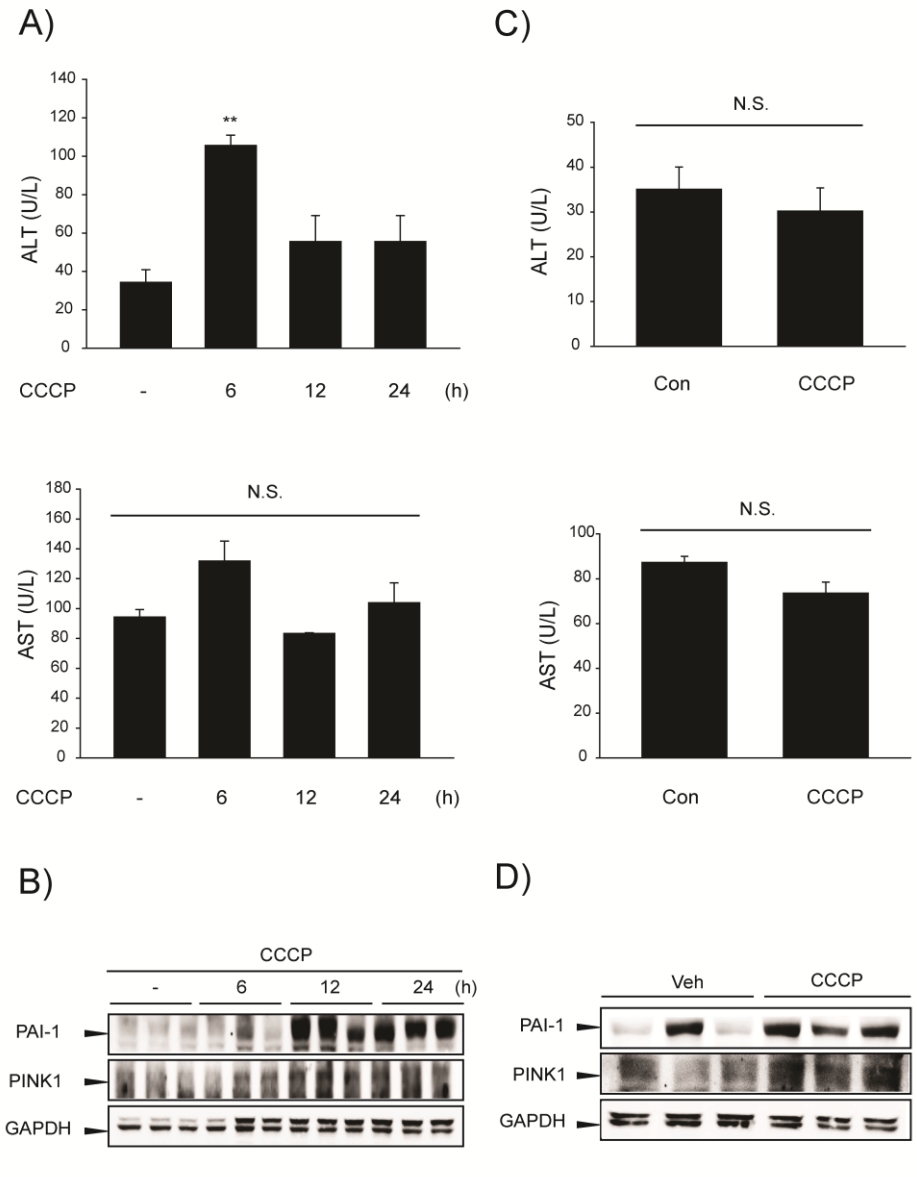


Figure 6. *In vivo* effect of CCCP treatment

(A) Single administration of CCCP. Mice were injected with vehicle or CCCP (3 mg/kg) for 6-24 h. ALT and AST levels were measured. Results represents the mean ± SE of 3 mice in each group (significant compared to vehicle-treated CCCP mice, $^{**}p < 0.01$). (B) Immunoblot analysis. PAI-1 and PINK1 expression was observed by immunoblotting in liver homogenates of injected with vehicle or CCCP (3 mg/kg). These results show data of 3 mice in each group. (D) Subchronic administration of CCCP. Mice were injected with vehicle or CCCP (1 mg/kg) daily for 2 weeks. ALT and AST levels were measured. Results represents the mean ± SE of 3 mice in each group (significant compared to vehicle-treated CCCP mice). (D) Immunoblot analysis. PAI-1 and PINK1 expression was observed by immunoblotting in liver homogenates of injected with vehicle or CCCP (1 mg/kg). These results show data of 3 mice in each group.

7. AICAR suppressed CCCP-induced mitophagy and HSCs activation

To examine the effect of AICAR on mitochondrial dysfunction induced by CCCP stimulation in LX-2 cells, we measured mitochondrial ROS production. We found that CCCP-induced mitochondrial ROS level was reversed by AICAR treatment (Fig 7A). In addition, CCCP-induced disruption of MMP was reversed by AICAR treatment (Fig 7B). Similarly, treatment of AICAR restored levels of mtDNA and ATP, which were decreased by CCCP treatment (Fig 7C and 7D). Next, to explore the effect of AICAR on CCCP-induced mitophagy in LX-2 cells, we examined representative autophagy and mitophagy markers. LC3B-II level was increased when treat with CCCP, while the up-regulation was reversed by the AICAR treatment (Fig 7E). Parkin and PINK1 levels were increased when treated with CCCP, which also reversed by the AICAR treatment (Fig 7F). Then we investigate the effect of AICAR on CCCP-induced fibrogenic gene expression in LX-2 cells. CCCP treatment of LX-2 cells resulted in increased PAI-1 expression, but this was significantly attenuated by AICAR treatment (Fig 7G). Taking together, these results indicate that AICAR protects LX-2 cells by inhibiting the mitochondrial dysfunction induced by CCCP stimulation, and then inhibits CCCP-induced mitophagy and liver fibrosis.

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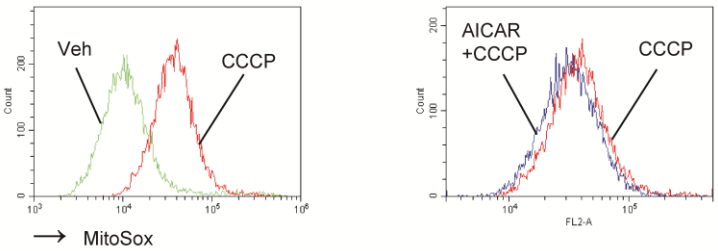

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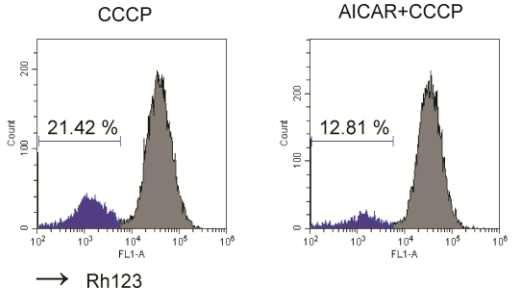

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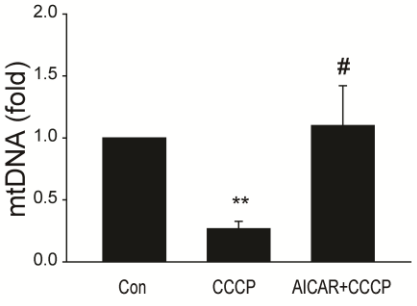

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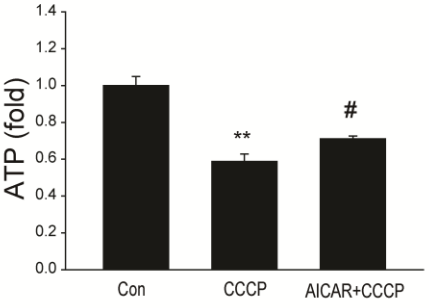

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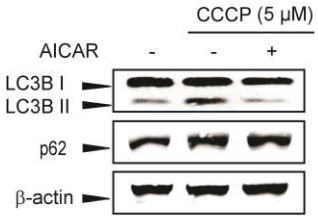

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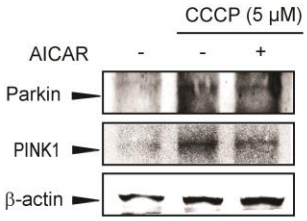

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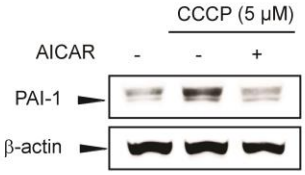


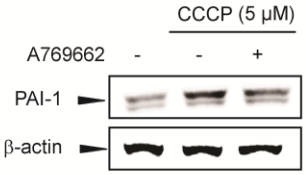
Figure 7. Effect of AICAR on CCCP-induced mitochondrial dysfunction

(A) Measurement of mitochondrial ROS changes. LX-2 cells were treated with AICAR (1 mM) and continuously incubated with CCCP (5 μM) for 15 min, then incubated with Mito-SOX (10 μM) for 0.5 h. The cells were analyzed by FACS (green: vehicle, red: CCCP, blue: AICAR+CCCP). (B) Measurement of mitochondrial membrane potential changes. LX-2 cells were treated AICAR (1 mM) and continuously incubated with CCCP (5 μM) for 24 h, and then incubated with Rho-123 (0.05 ng/ml) for 0.5 h. The histogram showed a left shift of histogram peak representing the decrease of Rho-123 fluorescence intensity by the loss of MMP. Percentage of cells with reduced fluorescence intensity was calculated. (C) mtDNA contents. LX-2 cells were treated AICAR (1 mM) and continuously incubated with CCCP (5 μM) for 24 h, and then performed to real-time PCR analysis used primers for the mtDNA region of COXII. Results represents the mean ± SE of data from at least 3 different replicates; significant compared to control, $^{**}p < 0.01$ or to CCCP alone, $^{\#}p < 0.05$. (D) Measurement of ATP. LX-2 cells were treated AICAR (1 mM) and continuously incubated with CCCP (5 μM) for 24 h. Results represents the mean ± SE of data from at least 3 different replicates; significant compared to control, $^{**}p < 0.01$ or to CCCP alone, $^{\#}p < 0.05$.

8. Effect of AICAR was dependent on AMPK activity

Next, we investigated the effect of AICAR on CCCP-induced mitochondrial dysfunction and fibrogenesis was dependent of AMPK activation. PAI-1 expression was increased when treated with CCCP, which was decreased by A769662 treatment (Fig 8A). Moreover, we found that CCCP-mediated disruption of MMP was reversed by A769662 treatment (Fig 8B).

A)



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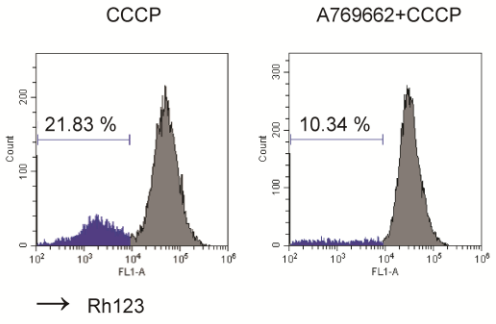

Figure 8. AMPK-dependent effect of AICAR on liver fibrogenesis

(A) The effect of A769662 on CCCP-mediated PAI-1 induction. LX-2 cells were treated with A769662 (10 μM) and continuously incubated with CCCP (5 μM) for 6 h. PAI-1 expression were assessed in the cell lysates by immunoblotting. (B) Measurement of mitochondrial membrane potential changes. LX-2 cells were treated A769662 (10 μM) and continuously incubated with CCCP (5 μM) for 24 h, and then incubated with Rho-123 (0.05 ng/ml) for 0.5 h. The histogram showed a left shift of histogram peak representing the decrease of Rho-123 fluorescence intensity by the loss of MMP. Percentage of cells with reduced fluorescence intensity was calculated.

IV. DISCUSSION

Mitochondria are essential organelles that regulate cellular energy homeostasis. Mitophagy plays a crucial role in the selective elimination of dysfunctional mitochondria to control mitochondrial quality, thus pathophysiologically modulating liver function. Recent studies have shown that mitophagy may participate in the pathogenesis of various types of liver diseases [23, 24]. Previously, we found that TGF-β, a central mediator of fibrogenesis, induced mitophagy through PINK1/Parkin pathway activation, which led to the activation of HSCs. In the present study, we explored whether mitochondrial uncoupler CCCP induced hepatic fibrogeneis in HSCs activation. Our current study showed that treatment with CCCP results in mitochondrial dysfunction, and promotes mitophagy in HSCs. CCCP induced PAI-1 expression, which was regulated by protein stability via lysosome activity, but not proteasome activity. Moreover, we previously reported that AMPK activation might protect mitochondrial injury. Treatment with CCCP increased mitochondrial dysfunction, which was restored by AMPK activator 5-aminoimidazole-4-carboxamide riboside (AICAR). We found that AICAR suppressed HSCs activation via mitophagy inhibition.

Our data have shown that mitochondrial uncoupler CCCP activates HSCs. PAI-1 induction by CCCP was confirmed in isolated primary HSCs from mice. Afterwards, we tried to investigate the mechanism of CCCP-induced PAI-1 expression. First, we examined whether CCCP-induced PAI-1 expression was regulated in a transcriptional level. PAI-1 mRNA levels were not increased by CCCP treatment. Moreover, Smad activity, which is a key transcription factor for the fibrogenic gene expression, using Smad binding element (SBE) luciferase plasmid were not affected by CCCP treatment. We thus also measured activity of other transcription factors associated with fibrogenic gene expression including activator protein 1 (AP-1), hypoxia-inducible factor-1 (HIF-1) and NF-KB using luciferase reporter plasmids, respectively. However, luciferase activities were not altered by CCCP treatment. These results

indicated that PAI-1 expression by CCCP was not transcriptionally regulated. Next, we explored whether CCCP-induced PAI-1 expression was regulated in post-transcriptional mechanism. Thus we first treated with protein synthesis inhibitor, cycloheximide (CHX). CHX-mediated PAI-1 decay was delayed by CCCP treatment. Next, we treated with proteasome inhibitor, MG132 and lysosome inhibitor, chloroquine (CQ) to identify role of proteasome or lysosome for the CCCP-mediated PAI-1 expression. Surprisingly, CCCP-induced PAI-1 levels were accumulated by treatment with CQ, but not by MG132. These results suggest that CCCP-induced PAI-1 expression was regulated by protein stability due to lysosome activity modulation. Further study is required and still going on how CCCP affects lysosome activity.

CCCP-induced hepatic fibrogenesis and mitophagy induction was confirmed in acute and subchronic treated mice. Moreover, we tried to find new agent regulating mitophagy for the liver fibrosis. AICAR is an adenosine analog which is taken up into cells by adenosine transporters on the cell membrane and subsequently phosphorylated, thus generating the AICAR monophosphate (ZMP), which can mimics the effect of AMP in AMPK activation [25]. Therefore, AICAR is commonly used to study AMPK signaling pathway. AMP-activated protein kinase (AMPK) plays critical roles in regulating cellular energy homeostasis. AMPK is activated in response to a variety of conditions such as nutrient starvation, hypoxia [26, 27]. In the liver, AMPK pathway is associated with various cellular processes, including protein synthesis, cell proliferation, and energy metabolism [28]. Recent studies have suggested that AICAR, an AMPK activator, improves liver injury. Activation of AMPK by AICAR attenuated bile duct ligation-induced inflammation in rats by protecting against liver injury [29]. Another study also showed that activation of AMPK by AICAR reverses drug-induced hepatocellular damage [30]. However, there is little studies about AICAR effect on chronic liver diseases such as liver fibrosis.

AICAR succeed to prevent mitochondrial dysfunction-mediated mitophagy and HSCs activation. To confirm whether AICAR-mediated effects are AMPK-dependent, we used

A769662, high specificity agent toward AMPK [31]. A769662 also inhibited PAI-1 expression and reversed mitochondrial dysfunction by CCCP. These results supposed that effect of AICAR was AMPK-dependent manner.

In conclusion, fibrotic gene expression by CCCP was regulated by protein stability due to lysosome activity regulation. Mitophagy induction via mitochondrial dysfunction by CCCP leads to HSCs activation, which was suppressed by AMPK activation. These findings present that AMPK activators act as a promising candidates for the prevention and treatment with liver fibrosis.

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