2016 년 2 월

석사학위 논문

The role of Lipin1 in the regulation of

hepatic fibrogenesis

조선대학교 대학원

약 학 과

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이 논문을 약학 석사학위신청 논문으로 제출함

2015 년 10 월

조선대학교 대학원

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2015 년 11 월

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List of abbreviations

ActD : actinomycin-D

α-SMA : alpha-smooth muscle actin

CHX : cycloheximide

CCl$_4$: carbon tetrachloride

CQ : chloroquine

HSC : hepatic stellate cell

MG132 : Z-Leu-Leu-Leu-al

Resveratrol : trans-3,4,5-tihydrixystilbene

SBE : smad binding element

TGF-β : transforming growth factor-beta

국 문 초 록

Lipin1 의 간섭유화 조절기전 연구

장 창 호

지 도 교 수 : 기 성 환

약학과

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간성상세포는 비타민 A 를 포함하는 세포로 간섭유화 진행에 있어서 주요한 역할을 한다. 이 세포의 분화 억제에 여러 지방세포 분화 관련 전사인자들의 활성화가 관여하는 것으로 알려져 있다. Lipin1 은 디아실글리세롤 포스파티테이즈로써 중성지질 (triglyceride) 합성에 관여하는 효소로 알려져 있으며 최근 연구 결과에 의하면 전사인자 활성화를 통하여 지방산 산화를 조절함이 보고되었다. 그러나 간성상 세포에서 TGF-β 신호 및 섬유화의 조절에 있어서 Lipin1 의 역할은 보고된 바 없다. 여기에서 우리는 간성상 세포의 활성화 또는 LX-2 세포에 TGF-β 처리시 Lipin1 의 발현이 하향 조절 됨을 관찰하였다. TGF-β 에 의한 Lipin1 발현 감소는 단백질의 안정성에 의존하였다. LX-2 세포에 프로테아좀 억제제인 MG132 를 처리했을 시 Lipin1 이 축적되었으나 라이소좀 억제제인 chloroquine 은 별다른 영향을 주지 않은 것으로 보아 Lipin1 의 분해는 프로테아좀 의존적임을 확인할 수 있었다. 실제 간성상 세포 및 HEK293 세포에서 Lipin1 의 ubiquitination 이 유의적으로 증가함을 관찰할 수 있었다. Lipin1 의 과발현은 TGF-β매개성 간섭유화 관련 유전자 발현의 유도를 억제하였으며 이는 Smad 인산화 및

전사 활성 억제에 기인하였다. 레즈베라트롤 (resveratrol)은 식물에서 발견되는 항산화물질인 폴리페놀 (polyphenol) 계열에 속하는 물질로 간섬유화를 억제할 수 있음이 동물 실험을 통하여 보고되었으나 정확한 기전에 대하여 보고된 바 없다. 레즈베라트롤은 간성상 세포에서 Lipin1 의 단백질 및 mRNA 를 증가 시켰다. 또한 레즈베라트롤이 Lipin1 의 ubiquitination 을 억제하여 단백질 안정화에 기인함을 규명하였다. 레즈베라트롤은 간성상 세포에서 TGF-1 에 의해 유도된 α-SMA, PAI-1 과 콜라겐 등의 간섬유화 관련 유전자 발현을 억제하였다. 또한 TGF-β에 의해 유도된 SBE-Luc luciferase 활성과 Smad3 인산화를 감소 시켰다. 레즈베라트롤의 TGF-β에 의해 유도된 PAI-1 발현 억제 효과는 Lipin1 의 siRNA 에 의하여 상쇄됨을 통하여 레즈베라트롤의 효능이 Lipin1 의 활성화가 요구됨을 규명하였다. 우리는 Lipin1 이 TGF-β/Smad 신호전달경로의 길항을 통해 간섬유화 진행 및 발병을 억제시키며 레즈베라트롤의 간섬유화 효능에 있어서 Lipin1 의 활성화가 필수적임을 본 연구결과를 통하여 제시한다.

Ⅰ. INTRODUCTION

Liver fibrosis is the result of chronic liver inflammation and cell death from various etiologies ranging from viral hepatitis and excessive alcohol intake, non-alcoholic fatty liver disease (NAFLD) and inherited diseases. It is characterized by an excessive accumulation of extracellular matrix (ECM) proteins, which lead to an impairment of liver function (3). Development and progression of liver fibrosis is orchestrated by a complex network of signaling pathways between many cell types.

Activation of HSCs in response to chronic liver injury is the critical step in the pathogenesis of liver fibrosis (15). In a healthy liver, HSCs are quiescent state and contain numerous vitamin A and triacylglycerols lipid droplets in the cytoplasm (34,35). Upon liver injury, quiescent HSCs lose lipid droplets and transdifferentiate into ECM-producing, highly proliferative myofibroblasts that express α-smooth muscle actin (α-SMA). Thus, Inactivation or elimination of myofibroblastic HSC became a primary target for anti-fibrotic therapy. It was reported that adipogenic or lipogenic transcription factors (C/EBPs, PPARγ, SREBP-1c and LXRα) were depleted in fibrogenesis (33,35). However, ectopic expression of these transcription factors reverses morphology and biochemical features of activated HSC to quiescent HSC (33). Moreover, treatment of HSC with agonists for PPARγ or LXRα effectively represses liver fibrosis and HSCs activation in both *vitro* and in *vivo* (5,25,26).

Transforming growth factor (TGF)-β is critically involved in HSC activation and ECM accumulation that leads to fibrosis. TGF-β stimulates the synthesis and deposition of various ECM components such as collagen of various types, laminin and fibronectin in the liver (7). The level of TGF-β significantly correlated with progression of liver fibrosis and is used as a non-invasive biomarker of hepatic fibrosis (20,24). Once activated, TGF-β binds to its

receptors that phosphorylate and activate Smads. Activated Smads are translocated into the nucleus and bind into the Smad binding element (SBE), leading to regulate transcription and expression of TGF-β-dependent genes (18).

Lipin1, Mg^{2+}-dependent phosphatidic acid phosphatase (PAP), has recently identified as a key regulator of lipid biosynthesis in several organs, including the liver (12). Lipin1 catalyzes the conversion of phosphatidate to diacylglycerol at the endoplasmic reticulum, which lead to the biosynthesis of triacylglycerol and phospholipids (10). In addition, Lipin1 can translocate into the nucleus and acts as a transcpritional coactivator to increase direct physical interactions with PPARα and PPARγ coactivator-1 alpha (PGC-1α), which facilitates mitochondrial fatty acid oxidation in the liver (13). Recent studies reveal expanding roles for Lipin-1 in the liver using knockout animal. Hepatocyte specific Lipin1 deficiency in mice exacerbates the development and progression of experimental alcohol-induced steatohepatitis (17). However, triglyceride synthesis was not affected in hepatocytes specific Lipin1 knockout mice after chronic feeding of the high fat diet and fructose diet (29). These controversial results suggest that more studies are needed to identify the pathophysiological role of Lipin1 in the liver. Moreover, the involvement of Lipin1 in the regulations of TGF-β signaling and fibrogenesis in HSCs is not investigated yet.

Resveratrol (trans-3,4,5-trihydroxystilbene) is a natural polyphenolic antioxidant compounds which is found in grapes and a variety of medicinal plants (4,27). A number of studies indicate that resveratrol possesses hepatoprotective properties and thus might be a preventive and therapeutic agent for a broad spectrum of liver diseases (2,6,19). Although resveratrol is known to inhibit liver fibrosis in chemical-induced or bile duct ligation injury animal model (8,9), its cellular and molecular mechanism of inhibiting hepatic fibrosis remains to be elucidated.

In this study, we sought to determine whether fibrogenic signaling regulates Lipin1 gene

expression in activated HSCs and whether Lipin1 regulates TGF-β signaling and hepatic fibrogenesis. Expression of Lipin1 is decreased in activated HSCs. The down-regulation of Lipin1 by TGF-β was due to ubiquitin-proteasome system. Ectopic expression of Lipin1 antagonized TGF-β-mediated cellular signaling and fibrogenic gene expression. Moreover, we found that treatment of HSC with resveratrol increased Lipin1 expression and inhibited TGF-β−activated fibrogenic gene expression via Lipin1 regulation, which contributes to the inhibition of TGF-β-mediated hepatic fibrogenesis. These data further elucidate the regulatory mechanisms by which Lipin1 regulates HSC activation and suggested that Lipin1 might be used as a promising therapeutic target against hepatic fibrosis.

Ⅱ. MATERIALS AND METHODS

1. Reagents and antibodies

Antibodies against Lipin-1, phospho-Smad3, Smad2/3 and ubiquitin antibodies were obtained from Cell Signaling Technology (Danvers, MA). PAI-1 antibody was obtained from BD (Bector, Dickinson and company). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies were purchased from Invitrogen (Carlsbad, CA). Resveratrol, Z-Leu-Leu-Leu-al (MG132), chloroquine (CQ), actinomycin-D (ActD), and α-SMA and β-actin antibody were from Sigma (St. Louis, MO). TGF-β was purchased from R&D Systems (Minneapolis, MN).

2. Cell culture

LX-2 cells (immortalized human activated HSCs) were kindly provided by Dr.S.L.Friedmann (Mount Sinai School of Medicine, New York, NY). HEK293 cell lines were purchased from ATCC (American Type Culture Collection, Manassas, VA). Cells were plated in 60 mm plates at 1×10^5 cells per well, and cells at 70-80% confluent. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; Hyclone, Logan, Utah), 50 units/ml penicillin, streptomycin at 37℃ in a humidified 5% CO_2 atmosphere.

3. Primary HSC isolation and culture

Livers from intact or CCl_4 (0.5 mg/kg, 24 h) treated mice are perfused using pronase/collagenase method and primary HSCs were isolated using gradient centrifugation as previously described (11). HSCs were cultured on uncoated plastic tissue culture dishes in DMEM containing 50 units/ml penicillin/streptomycin with 10% FBS at 37℃ in a humidified 5% CO_2 atmosphere.

4. Immunoblot analysis

Protein extraction and subcellular fractionation, SDS-polyacrylamide gel electrophoresis and immunoblot analyses were performed as previously described (36). Briefly, the cell lysates were separated by electrophoresis in 7.5% and 12% gels, and electrophoretically transferred to nitrocellulose. The nitrocellulose paper was incubated with the indicated primary antibody and then incubated with horseradish peroxidase-conjugated secondary antibody (Invitrogen, Carlsbad, CA). Immunoreactive protein was visualized by ECL chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK). Equal protein loadings were verified using β-actin.

5. siRNA knockdown experiment

Cells were transfected with non-targeting control siRNA (100 pmol) or siRNA directed against Lipin-1 (100 pmol)(Ambion®, Austin, TX) for 30 h using Lipofectamine™2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

6. RNA isolation and Real-time RT-PCR analysis

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To obtain cDNA, total RNA (2μg) was reverse-transcribed using and oligo(dT)$_{16}$ primer. The cDNA obtained was amplified using a high-capacity cDNA synthesis kit (Bioneer, Daejon, Korea) with a thermal cycler (Bio-Rad, Hercules, CA). Real-time PCR was performed with StepOne system (Applied Biosystems, Foster City, CA) using SYBR green premix (Applied Biosystems). The following primer sequences, which were synthesized by Bioneer, were used for the reactions: mouse Lipin-1 sense 5'-CGCCAAAGAATAACCTGGAA-3' and antisense 5'-TGAAGACTCGCTGTGAATGG-3',

mouse α-SMA sense 5'-TCCTCCCTGGAGAAGAGCTAC-3' and antisense 5'-TATAGGTGGTTTCGTGGATGC-3', mouse GAPDH sense 5'-TGCCCCCATGTTTGTGATG-3' and antisense 5'-TGTGGTCATGAGCCCTTCC-3', human Lipin-1 sense 5'-CCCGACCTTCAACACCTAAAAGT-3' and antisense 5'-TGGACTCTTTCATCTTGTGTGGA-3', human PAI-1 sense 5'-CGCCAGAGCAGGACGAA-3' and antisense 5'-CATCTGCATCCTGAAGTTCTCA-3', human Col1A1 sense 5'-CCTGGGTTTCAGAGACAACTTC-3' and antisense 5'-TCCACATGCTTTATTCCAGCAATC-3', human GAPDH sense 5'-GAAGGTGAAGGTCGGAGTC-3' and antisense 5'-GAAGATGGTGATGGGATTTC-3'. The relative levels of the PCR products were determined based on the threshold cycle value. A melting curve analysis was done after amplification to verify the accuracy of the amplicon.

7. Plasmid construction luciferase assay

To measure the activities of TGF-β/Smad signaling, cells were plated in 24-well plates overnight, serum starved for 6 h and transiently transfected with SBE luciferase plasmid and pRL-TK plasmid (a plasmid that encodes *Renilla* luciferase and used to normalize transfection efficacy) in the presence of Lipfectamine^{TM}2000 Reagent (Invitrogen, San Diego, CA) for 3 h. Transfected cells were allowed to recover in MEM with 1% FBS for overnight and then exposed to TGF-β for 6 h. Firefly and *Renilla* luciferase activities in cell lysates were measured using the dual luciferase assay system (Promega, Madison, WI) according to the manufacturer's instructions. Relative luciferase activities were calculated by normalizing firefly luciferase activities versus that of *Renilla* luciferase.

8. Immunoprecipitation

To assess the levels of ubiquitinated Lipin-1, cells were transfected with a plasmid encoding

His-tagged ubiquitin. Cell lysates (500 μg/ml) were incubated with the Lipin-1 antibody overnight at 4℃. The antigen-antibody complex was immunoprecipitated after incubation with protein G-agarose (Millipore, Temecula, CA) for 2 h at 4℃. Immune complexes were solubilized in 2×Laemmli buffer. Protein samples were resolved and immnoblotted with an anti-ubiquitin antibody.
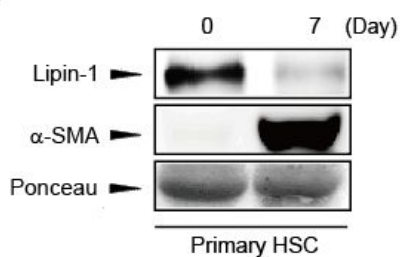
9. Statistical analysis

One-way ANOVA was used to determine the significance of the differences between treatment groups. The Newman-Keuls test was used to determine the significance of differences between the means of multiple groups. Results are expressed as means ± SDs.
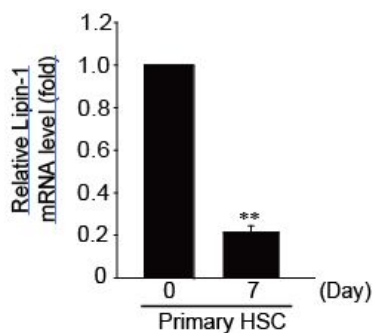
Ⅲ. RESULTS

1. Reduction of Lipin1 gene expression in activated HSC

First, we analyzed *Lipin1* gene expression during HSC activation in primary cultured murine HSCs. Lipin1 was abundant in quiescent HSCs as easily detected by western blotting and declined in fully activated HSCs, whereas the levels of α-SMA, the transdifferentiation marker, substantially increased (Fig. 1A). Real-time RT-PCR confirmed Lipin1 repression in activated HSCs (Fig. 1B). Next, we isolated HSC from vehicle treated mice and CCl_4-treated mice. Expression of Lipin-1 was significantly down-regulated in activated HSCs by CCl_4 (Fig. 1C). Treatment of LX-2 cells, immortalized human HSC cell lines, with TGF-β suppressed Lipin1 protein and mRNA expression levels, respectively (Fig. 1D and 1E). However, TGF-β treatment in LX-2 cells did not reduce Lipin-2 (Fig. 1E), which demonstrates that TGF-β specifically regulates Lipin1 gene expression. These results suggest the Lipin1 level was dramatically decreased along with the activation of HSC during liver injury.

A)

0   7   (Day)

Lipin-1

α-SMA

Ponceau

Primary HSC

B)

Relative Lipin-1 mRNA level (fold)

1.2
1.0
0.8
0.6
0.4
0.2
0

0   7   (Day)

**

Primary HSC

C)

Vehicle   CCl₄

Lipin-1

β-actin

Primary HSC

D)

TGF-β

-      3   6   (h)

Lipin-1

β-actin
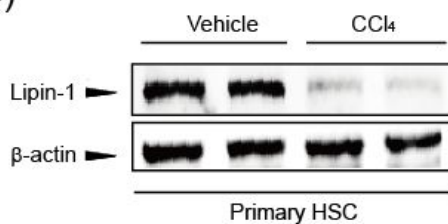
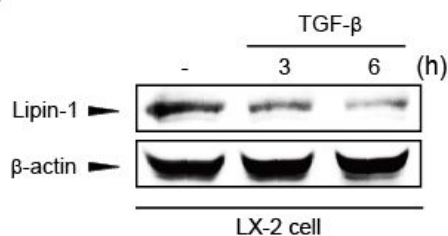LX-2 cell

E)

TGF-β

-      +
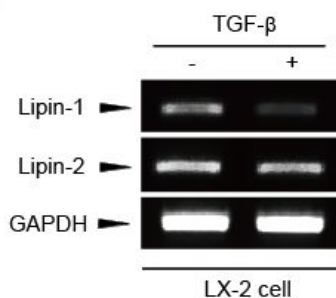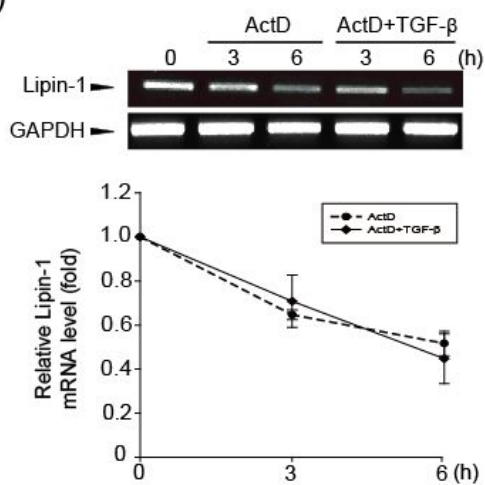
Lipin-1

Lipin-2

GAPDH

LX-2 cell

Figure 1. Down-regulation of Lipin1 during HSC activation

(A) Lipin1 expression in primary murine HSCs. Primary HSCs were isolated and were cultured in a growth medium for 0 or 7 days, and the cell lysates (20 μg each) were subjected to immunoblotting. The expression levels of Lipin1 and α–SMA were also determined in the lysates of isolated cells. α-SMA was detected as an activation marker of HSC activation and Ponceau-S (Ponceau) staining was carried out to confirm the equal protein loading. (B) Real-time PCR analysis. Lipin1 transcript was analyzed, with the mRNA level of GAPDH used as a housekeeping gene in freshly isolated (day 0) or culture-activated (day 7) in HSCs. The data are the means and standard errors of at least three separate experiments (significant different versus day 0: **p<0.01). (C) Lipin-1 expression in primary HSCs from CCl_4-treated mice. Mice were injected with 0.5 mg/kg CCl_4 for 24 h. Primary HSCs were isolated and protein lysates were accessed by immunoblotting. β-actin was used to confirm equal protein loading. (D) The effect of TGF-β treatment on Lipin1 down-regulation. LX-2 cells were treated with 2 ng/ml TGF-β for 3 or 6 h. (E) RT-PCR analysis. LX-2 cells were treated with 2 ng/ml TGF-β for 6 h. Transcriptional levels of Lipin1 and Lipin2 were determined by RT-PCR analysis.

2. The repression of Lipin1 expression by TGF-β is derived from the protein stability

Next we investigated whether Lipin1 repression during HSC activation is due to mRNA stability by TGF-β. RNA samples were prepared from cells treated with transcriptional inhibitors such as actinomycin-D (ActD) or ActD plus TGF-β. Treatment of LX-2 cells with ActD gradually decreased Lipin1 mRNA levels from 3 h. However, TGF-β treatment has no effect on Lipin1 mRNA stability (Fig. 2A). Sequentially, we analyzed the effect of protein stability on Lipin1. LX-2 cells were incubated with cycloheximide (CHX) to inhibit protein synthesis. Treatment of cells with CHX, the amount of Lipin1 expression quickly decreased. However, no reduction in the level of β-actin, which was used as a control, was detected. These results demonstrate that Lipin1 is rapidly turned over proteins. Subsequently, we investigated whether CHX-mediated Lipin1 suppression was affected by TGF-β treatment. Surprisingly, TGF-β treatment accelerated Lipin1 decay, suggesting that repression of Lipin1 expression by TGF-β is due to increased Lipin1 degradation (Fig. 2B).
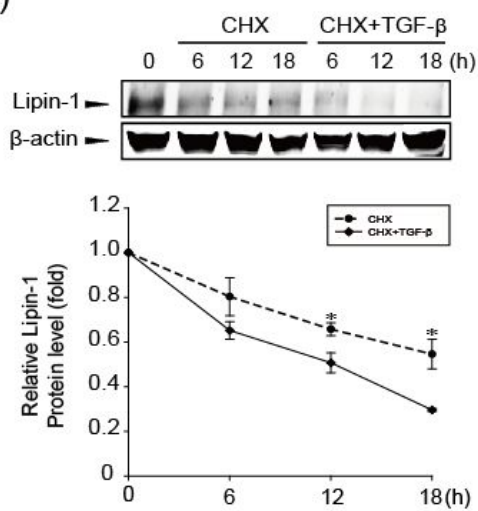
A)

B)

Figure 2. Post-translational regulation of Lipin-1 expression

(A) The effect of actinomycin-D (ActD) on the Lipin1 down-regulation by TGF-β in LX-2 cells. The cells were treated with 5 μg/ml of ActD in the presence or absence of TGF-β. The level of Lipin1 mRNA was monitored after TGF-β treatment for 3 or 6 h. The amount of Lipin1 mRNA was quantified. (B) The effect of cycloheximide (CHX) on Lipin1 expression. LX-2 cells were incubated with 0.5 μg/ml CHX in the presence or absence of TGF-β for the indicated periods. The amount of Lipin1 protein was quantified. The data are the means and standard errors of at least three separate experiments (significant as compared with CHX-treated groups at the same time, *p<0.05).

3. Lipin1 is polyubiquitinated and degraded *via* ubiquitin-proteasome system

To verify whether depletion of Lipin1 by CHX occurred via the proteasomal or lysosomal protein degradation, we treated LX-2 cells with a proteasomal inhibitor (MG132 [MG]) or a lysosomal inhibitor (chloroquine [CQ]), respectively. We found that MG, but not CQ, increased the basal level of Lipin1, indicating that Lipin1 degradation was regulated by proteasome pathway, but not lysosomal pathway (Fig. 3A). Moreover Lipin1 repression by TGF-β treatment was completely recovered by MG treatment, whereas CQ had no such effect (Fig. 3B and 3C). To identify the role of the ubiquitin-proteasome system in Lipin1 fate, HEK293 cells were transfected with expression vector for encoding His-tagged ubiquitin (Ubi) and the Lipin1 plasmid. After overnight incubation with or without MG, cells were lysed. The lysate was immunoprecipitated with an anti-Lipin-1 antibody, and then immunoblotted with an anti-ubiquitin antibody. In HEK293 cells cotransfected with Lipin1 and Ubi, Lipin1-ubiquitin complex were only detected in the presence of MG (Fig. 3D). Polyubiquitination of Lipin1 was confirmed in LX-2 cells (Fig. 3E). Taken together, these data strongly suggest that Lipin1 is polyubiquitinated and degraded by the ubiquitin-proteasome system.
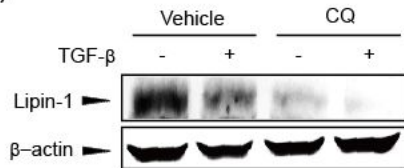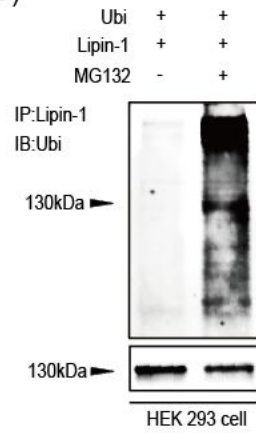
A)

Lipin-1

β-actin

B)

Lipin-1

β-actin

C)

Lipin-1

β–actin
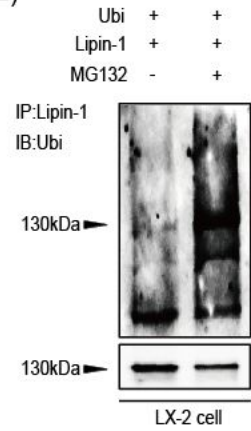
D)

HEK 293 cell

E)
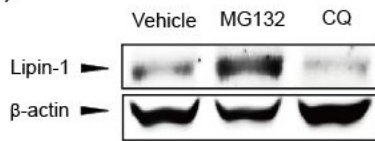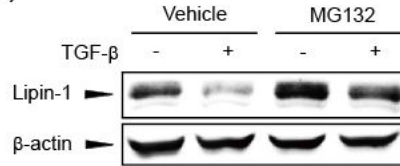
LX-2 cell

Figure 3. Role of ubiquitin-proteasome system on Lipin1 expression

(A) Lipin1 up-regulation by proteasome inhibitor. LX-2 cells were treated with lysosomal inhibitor chloroquine (CQ, 100 μg/ml) or the proteasomal inhibitor MG132 (MG, 10 μM) for 6 h, and then Lipin1 expression in the cell lysates was assessed by immunoblotting. (B) The effect of proteasome inhibition on Lipin1 down-regulation by TGF-β. LX-2 cells were preincubated with the MG for 6 h in the presence or absence of TGF-β, and then Lipin1 expression in the cell lysates was assessed by immunoblotting. (C) The effect of lysosomal inhibition on Lipin1 down-regulation by TGF-β. LX-2 cells were pretreated with the CQ for 6 h in the presence or absence of TGF-β, and then Lipin1 expression in the cell lysates was assessed by immunoblotting. (D) Polyubiquitination of Lipin1. HEK293 cells were transiently transfected with plasmids encoding His-tagged ubiquitin (Ubi) and Lipin-1 for 24 h. Cells were then treated with MG for 3 h. Polyubiquitinated Lipin-1 was immunopreciptitated with an anti-Lipin1 antibody, and then immunoblotted with an anti-Ubi antibody. (E) Ubiquitination of Lipin1 in LX-2 cells. Cells were transiently transfected and immunoprecipitated as described in D.

4. Inhibitory role of Lipin1 on fibrogenesis by TGF-β

Next, we determined the role of Lipin-1 on TGF-β-induced fibrogenic gene expression and Smad activation. LX-2 cells treated with TGF-β increased PAI-1, which is representative markers of HSC activation, in the MOCK-transfected cells, and this was significantly inhibited by Lipin1 overexpression (Fig. 4A). To address the downstream link between Lipin1 and TGF-β signaling, we assessed the inhibitory effect of Lipin1 on TGF-β-dependent Smad phosphorylation. The treatment of MOCK-transfected LX-2 cells with TGF-β enhanced Smad3 phosphorylation. However, Lipin1 overexpression decreased the phosphorylation of Smad3 by TGF-β (Fig. 4B). Consistently, Lipin1 inhibited the ability of TGF-β treatment or Smad3 transfection to induce luciferase activity from an SBE-driven reporter, respectively (Fig. 4C and 4D). Moreover, Lipin1 knockdown by siRNA markedly increased TGF-β-induced PAI-1 expression compare to control siRNA (Fig. 4E). The knockdown of Lipin1 was confirmed by immunoblotting. Our results suggest that Lipin1 inhibits TGF-β/Smad3 signaling and thus antagonizes Smad-dependent transcriptional responses.

A)

| | MOCK | | Lipin-1 | |
| TGF-β | − | + | − | + |

PAI-1 ▶

Lipin-1 ▶

β-actin ▶

B)

| | MOCK | | Lipin-1 | |
| TGF-β | − | + | − | + |

p-Smad 3 ▶

Smad 2/3 ▶

C)

Relative SBE Luciferase Activity (fold)

TGF-β

MOCK   Lipin-1

D)

Relative SBE Luciferase Activity (fold)

Smad 3

MOCK   Lipin-1

E)

| | siCon | | siLipin-1 | |
| TGF-β | − | + | − | + |

PAI-1 ▶

Lipin-1 ▶

β-actin ▶

- 18 -
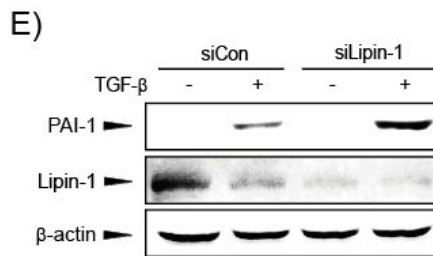
Figure 4. Inhibitory role of Lipin1 on fibrogenic gene expression and Smad3 phosphorylation

(A) Effect of Lipin1 on TGF-β-induced fibrogenic gene expression. LX-2 cells were transfected with pcDNA (MOCK) or pcDNA-Lipin1 (Lipin1) for 24 h, and 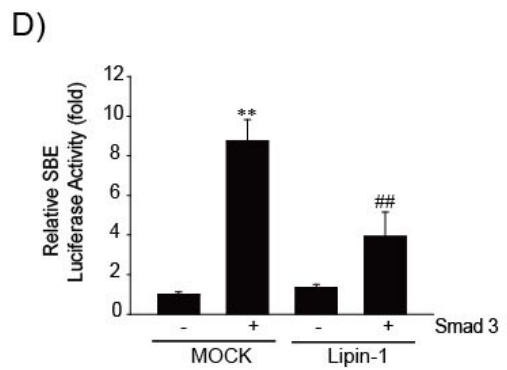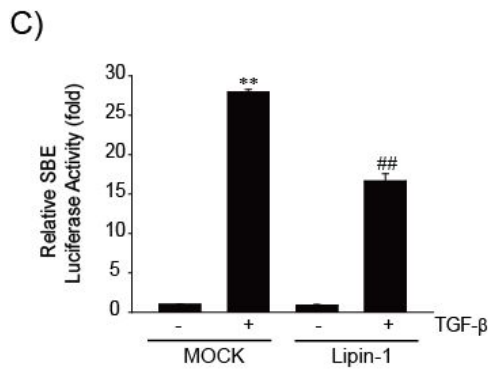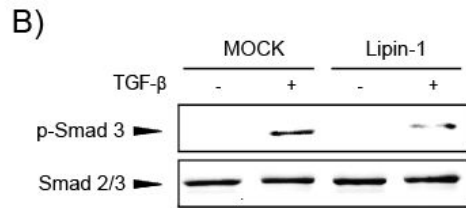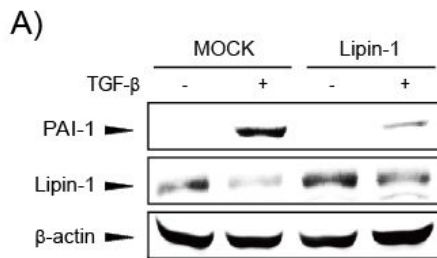then treated with TGF-β for 6 h. PAI-1 protein level was determined by immunoblotting. Lipin-1 expression was confirmed by immunoblotting with lysates from MOCK- or Lipin1-transfected cells. (B) Inhibition of TGF-β-mediated Smad3 phosphorylation by Lipin1. Cells were transfected as described above and treated with TGF-β for 30 min, and cell lysates were immunoblotted for examining Smad3 phosphorylation. Results were confirmed by repeated experiments. (C) The effect of Lipin1 on Smad3 activation by TGF-β treatment. Cells were transfected with a pGL-SBE luciferase construct. Transfected cells were treated with TGF-β for 6 h. Results represent the means ± SEs of three replicates (significant as compared with vehicle-treated controls, **p<0.01; significant as compared with TGF-β alone ##p<0.01). (D) Effect of Lipin1 on Smad3 overexpression by Smad3 transfection. Cells were transfected with plasmids encoding control (MOCK) or pcDNA-Smad3 (Smad3), and pGL-SBE luciferase construct. Transfected cells were treated with TGF-β for 6 h. Results represent the means ± SEs of three replicates (significant as compared with vehicle-treated controls, **p<0.01; significant as compared with Smad3 overexpression alone ##p<0.01). (E) Effect of Lipin-1 knockdown on TGF-β-mediated fibrogenic gene expression. LX-2 cells were transfected with control (CON) siRNA or Lipin1 siRNA for 24 h, and then treated with TGF-β for 6 h.

5. The effects of resveratrol on the expression of Lipin1

Resveratrol, which is a naturally occurring polyphenolic component, reduces liver fibrosis even though its molecular mechanism remains incompletely understood. Next we explored whether resveratrol increased Lipin1 expression in HSCs. We treated LX-2 cells with resveratrol at the concentrations of 10 or 30 μM and resveratrol treatment in cells induces Lipin1 expression (Fig. 5A). RT-PCR analysis clearly confirmed that Lipin1 mRNA level was significantly increased by resveratrol treatment (Fig. 5B). Moreover, resveratrol reverses TGF-β-driven Lipin1 down-regulation in LX-2 cells (Fig. 5C and 5D). In addition resveratrol inhibited polyubiquitination and degradation of Lipin1 in LX-2 cells (Fig. 5E). These data indicate that resveratrol up-regulates Lipin1 expression through inhibition of Lipin1 polyubiquitination in HSCs.

A)

Resveratrol (6 h)

| | - | 10 | 30 | (μM) |

Lipin-1 ►

β-actin ►

B)

Resveratrol (6 h)

| | - | 10 | 30 | (μM) |

Lipin-1 ►

GAPDH ►

C)

TGF-β

| Resveratrol (μM) | - | - | 10 | 30 |

Lipin-1 ►

β-actin ►

D)

TGF-β

| Resveratrol (μM) | - | - | 10 | 30 |

Lipin-1 ►

GAPDH ►

E)

Ubi	+	+	+
Lipin-1	+	+	+
MG132	-	+	+
Resveratrol	-	-	+

IP:Lipin-1
IB:Ubi

130kDa ►

130kDa ►
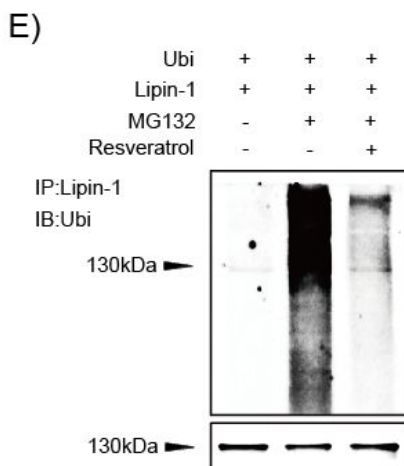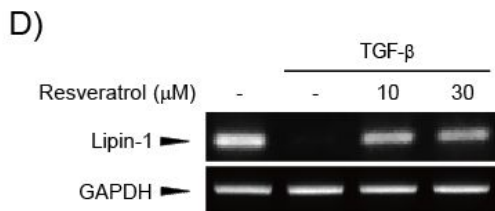
Figure 5. Lipin1 upregulation by resveratrol in LX-2 cell

(A) Lipin1 induction by resveratrol. LX-2 cells were treated with 10-30 μM resveratrol for 6 h. The Lipin1 protein levels in cell lysates were measured by immunoblotting. (B) RT-PCR analysis. Cells were treated with 10-30 μM resveratrol for 6 h. The Lipin1 transcript levels were determined by RT-PCR using GAPDH used as an internal control. (C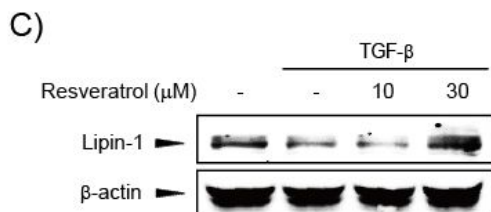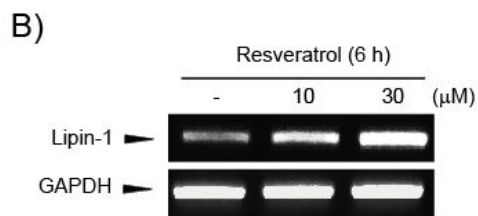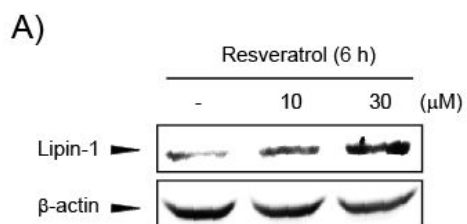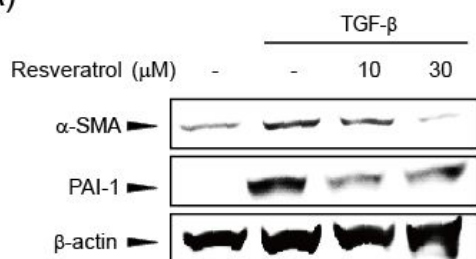) The effect of resveratrol on TGF-β-mediated Lipin1 down-regulation. Cells were treated with 10-30 μM resveratrol and continuously incubated with TGF-β (2 ng/ml) for 6 h. The Lipin1 protein levels were immunoblotted in the cell lysates. (D) RT-PCR analysis. Cells were treated with 10 or 30 μM resveratrol for 6 h, and then further incubated with TGF-β (2 ng/ml) for 6 h. The Lipin1 transcripts were analyzed by RT-PCR, with the mRNA level of GAPDH used as a housekeeping gene. (E) The effect of resveratrol on polyubiquitination of Lipin1. LX-2 cells were transiently transfected with plasmids encoding His-tagged ubiquitin (Ubi) and Lipin1 for 12 h. Cells were pretreated with MG132, then incubated with 30 μM resveratrol and continuously incubated with TGF-β (2 ng/ml) for 6 h. Polyubiquitinated Lipin1 was immunopreciptitated with an anti-Lipin1 antibody, and then immunoblotted with an anti-Ubi antibody.

6. Inhibition of the TGF-β/Smad signaling by resveratrol through Lipin1 regulation

Resveratrol effectively inhibited TGF-β-induced expression of α-SMA and PAI-1 (Fig. 6A). Real time-RT-PCR analysis showed that TGF-β markedly increased levels of PAI-1 and Col1A1 mRNA, which were significantly inhibited by resveratrol treatment (Fig. 6B). We confirmed the effect of resveratrol in isolated primary HSCs from mice. Resveratrol decreased protein and mRNA levels of α-SMA during HSC activation (Fig. 6C and 6D). Next we investigated whether anti-fibrogenic effect of resveratrol is likely due to inhibition of Smad3. TGF-β treatment significantly increased the levels of phosphorylated Smad3 and resveratrol treatment markedly inhibited the TGF-β-induced its phosphorylation (Fig. 6E). Consistent with the reduction in phosphorylated Smad3 by resveratrol, TGF-β-induced SBE-driven reporter gene activity was also reduced (Fig. 6F).
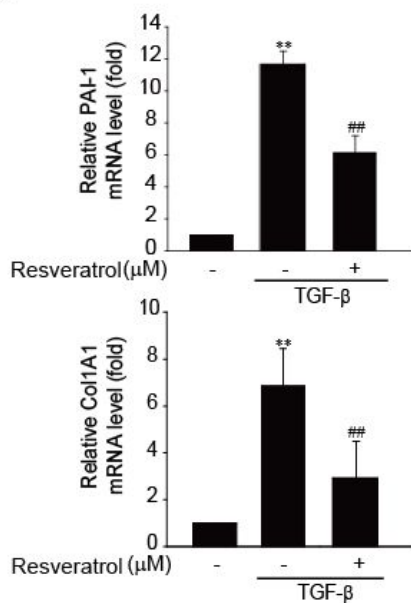
Figure 6. Inhibition of fibrogenic gene expression and Smad3 phosphorylation by resveratrol
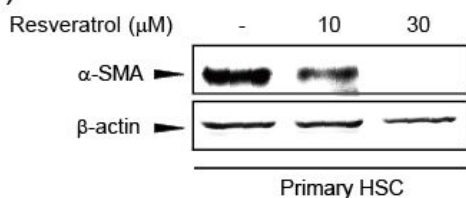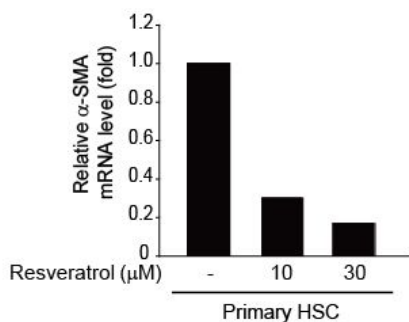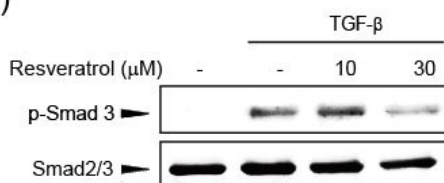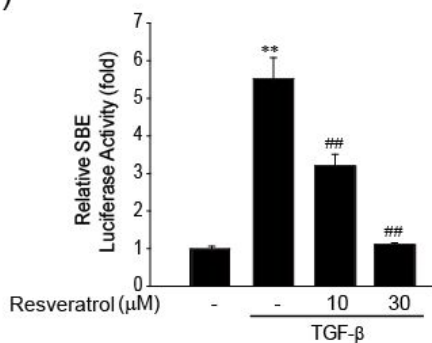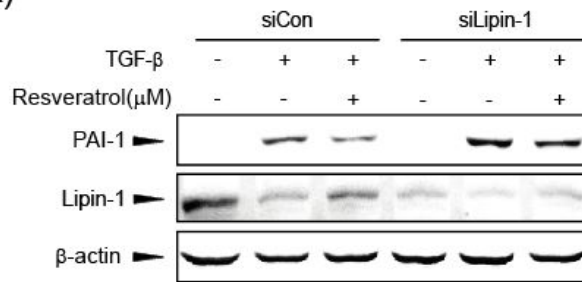
(A) Inhibitory effect of resveratrol on TGF-β-induced fibrogenic gene expression. Cells were treated with 10-30 μM resveratrol and continuously incubated with TGF-β (2 ng/ml) for 6 h. The levels of α-SMA or PAI-1 proteins were immunoblotted in the cell lysates. (B) Real-time PCR analysis. Cells were treated with 30 μM resveratrol, and then further incubated with TGF-β (2 ng/ml) for 6 h. The PAI-1 and Col1A1 transcripts were analyzed by Real-time RT-PCR with the mRNA level of GAPDH used as a housekeeping gene. Data represent the mean ±SE of three replicates; **P<0.01, significant versus vehicle-treated control; ##P<0.01, significant versus TGF-β alone. (C) The inhibitory effect of resveratrol in activated primary HSCs. Primary HSCs were cultured in a growth medium for 7 day with resveratrol (10-30 μM) and the cell lysates (20 μg each) were subjected to immunoblotting. (D) Real-time RT-PCR assays. Primary HSCs were cultured in a growth medium for 7 day with resveratrol (10-30 μM) and mRNA levels were analyzed by real-time RT-PCR. (E) The inhibitory effect of resveratrol on TGF-β-induced Smad3 phosphorylation. Cells were treated with 30 μM resveratrol, and then further incubated with TGF-β (2 ng/ml) for 30 min, and cell lysates were immunoblotted against phosphorylated Smad3 antibody. Results were confirmed by repeated experiments. (F) The effect of resveratrol on TGF-β-induced SBE luciferase activity. Cells were transfected with pGL-SBE luciferase construct. Transfected cells were preincubated with resveratrol (10-30 μM) and then treated with TGF-β for 6 h. Results represent the means ± SEs of three replicates; **p<0.01=significant versus vehicle-treated controls. ##p<0.01, significant versus TGF-β alone.

7. The inhibitory effect of resveratrol on fibrogenic gene expression through Lipin1 regulation

To verify the inhibitory role of resveratrol in TGF-β-mediated fibrogenesis through Lipin1 regulation, we measured PAI-1 expression under knockdown conditions of Lipin1 in LX-2 cells. Immunoblotting analysis shows that Lipin1 knockdown by siRNA abolished the antagonistic effect of resveratrol in TGF-β-mediated up-regulation of PAI-1 in LX-2 cells; this knockdown of Lipin1 was confirmed by immunoblotting (Fig. 7A). Our results suggest that the ability of resveratrol to repress TGF-β/Smad signaling is dependent on Lipin1 regulation (Fig. 7B).

A)

B)
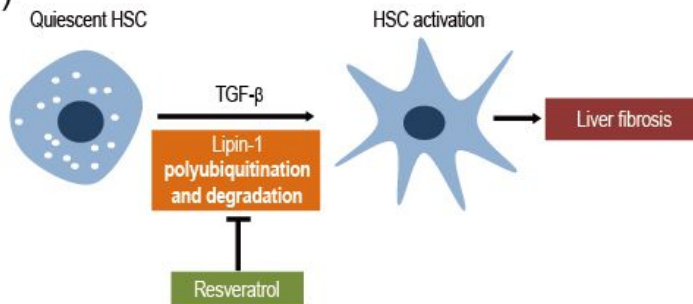
Figure 7. The role of Lipin1 in the inhibitory effect of resveratrol on fibrogenic gene expression

(A) LX-2 cells were transfected with control (CON) or Lipin1 siRNA for 24 h. Transfected cells were preincubated with resveratrol (10-30 μM) and then treated with TGF-β for 6 h. Results were confirmed by repeated experiments. (B) Schematic diagram illustrating the proposed mechanism of Lipin1 in the regulation of hepatic fibrogenesis.

Ⅳ. DISCUSSION

In this study, we demonstrated that Lipin1, which is known to have a PAP activity, is down-regulated during HSC activation with a loss of lipid droplets. Lipin1 expression is transcriptionally regulated by SREBP-1 (16) and PPARγ (21), which are key representative genes critical for lipid metabolism. The levels of all these transcription factors were reported to be decreased in activating or activated HSCs. Our result showing that Lipin1 expression was rich in quiescent HSCs, whereas its expression was decayed in activated HSCs (Fig. 1). Treatment of HSCs with TGF-β inhibited Lipin1 protein and mRNA expression levels. These result indicate that Lipin1 is downregulated when vitamin A and lipid-storing HSCs into a proliferative myofibroblastic phenotype.

Peterfy et al reported alternatively spliced gene products of the Lipin1 gene, referred to as Lipin1-α and -β. Lipin1 isoforms appear to have distinct roles in rodents (28). Lipin1-α is primarily localized to the nucleus and up-regulates expression of genes associated with adipocyte differentiation including PPARγ and C/EBP α. Whereas, Lipin1-β mainly resides in the cytoplasm and regulates the expression of genes involved in lipogenesis. We tried to compare the expression of Lipin1-α and -β in LX-2 cells after TGF-β treatment. RT-PCR analysis showed that the Lipin1-α and -β were expressed at comparable levels in TGF-β- treated HSCs (data not shown).

Treatment of HSCs with ActD resulted in decreased levels of Lipin1. Under these conditions, TGF-β did not alter the rate of Lipin1 mRNA degradation which suggests that TGF-β-mediated Lipin1 regulation is not due to mRNA stability (Fig. 2A). However, CHX treatment resulted in the decreased Lipin1 (Fig. 2B) and TGF-β treatment accelerated Lipin1 degradation by CHX. These results indicate that the steady-state stability of Lipin1 is an important determinant of cellular sensitivity to TGF-β. Proteasome inhibitor MG, but not

lysosome inhibitor CQ, increased the levels of Lipin1 protein (Fig. 3A-C). Moreover, Lipin1 is polyubiquitinated and degraded by the ubiquitin-proteasome system. However, we failed to detect to increase Lipin1 ubiquitination by TGF-β treatment (data not shown). Further studies are still required to understand and identify the concise mechanism and the physiological role of Lipin1 ubiquitination, and these are currently being investigated in our laboratory. Collectively, our results demonstrated for the first time that Lipin1 expression is regulated by the ubiquitin-proteasome system.

Resveratrol, a natural phytoalexin present in various plant species including grapes, has received much attention for several pharmacological efficacies such as anti-inflammatory and anti-oxidant properties, regulation of lipid metabolism and cancer prevention (1,14,30) However, further studies are still required to understand and determine the pharmacological mechanisms of resveratrol (32). Resveratrol's effects are explained by scavenging ROS, reducing DNA damage and lipid peroxidation in the cell membrane (23). Recently it was revealed that resveratrol protects against CCl_4- or DMN-induced liver fibrosis in animal models (9,22). However, its concise molecular mechanism against fibrogenesis in HSCs remains to be identified. Here we found that resveratrol significantly inhibited the TGF-β-induced fibrogenic gene expression and phosphorylation of Smad3 which is an essential transcription factor for the TGF-β signaling. Therefore, our results suggest that resveratrol increased expression of Lipin1 and the ability of resveratrol to repress TGF-β/Smad signaling is dependent on Lipin1 induction.

In conclusion, our study for the first time indicated that Lipin1 is down-regulated in HSC during liver fibrogenesis and its repression is regulated by ubiquitin-proteasome system. Furthermore, ectopic expression of Lipin1 inhibited TGF-β-induced HSC activation and TGF-β/Smad signaling. Lipin1 expression was increased by resveratrol, which contributes to the inhibition of TGF-β-induced hepatic fibrogenesis. Therefore, our study provides crucial

information to further refinement our understanding of the mechanisms by which the effect of

Lipin1 exerts health benefits especially for the hepatic fibrosis.

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ABSTRACT

The role of Lipin1 in the regulation of hepatic fibrogenesis

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Adipogenic transcriptional regulation was reported to inhibit transdifferentiation of hepatic stellate cells (HSCs), main fibrogenic cell type in the liver. Lipin1 exhibits dual function as enzymes catalyzing the conversion of phosphatidate to diacylglycerol and as transcriptional regulator. However, the involvement of Lipin1 in the regulations of TGF-β signaling and fibrogenesis in HSCs is not fully understood. Here we show that Lipin1 is down-regulated in activated primary HSCs or TGF-β treated LX-2 cells. The down-regulation of Lipin1 by TGF-β was not dependent on altered mRNA stability but rather due to protein stability. Treatment LX-2 cells with the proteasome inhibitor led to the accumulation of Lipin1. Moreover, we observed a significant increase in Lipin1 polyubiquitination in LX-2 and HEK293 cells. Over-expression of Lipin1 attenuated TGF-β-induced fibrogenic gene expression. In addition, Lipin1 inhibited TGF-β-mediated Smad phosphorylation and its transcriptional activity. Resveratrol, a well-known natural polyphenolic antioxidant, is known to inhibit liver fibrosis, although its concise mechanism of action remains elucidated. Our data showing that resveratrol significantly increased the levels of Lipin1 protein and mRNA in HSCs. Further investigation revealed that

resveratrol blocked the polyubiquitination of Lipin1. Resveratrol inhibited TGF-β-induced fibrogenic gene expression in HSCs. TGF-β-induced SBE-luciferase reporter activity was significantly diminished by resveratrol with a simultaneous decrease in Smad3 phosphorylation. Consistently, a gene knockdown of Lipin1 by siRNA abolished the inhibitory effect of resveratrol. We conclude that Lipin1 antagonized HSC activation through inhibition of TGF-β/Smad signaling and resveratrol affects Lipin1 gene induction and contributes to the inhibition of TGF-β-mediated hepatic fibrogenesis.