2015년 2월

박사학위논문

The Role of Sestrin2 as a Novel Therapeutic Target for the Liver Diseases

조선대학교 대학원

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양 지 혜

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신규 간질환 치료 표적으로써의 Sestrin2 역할연구

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이 논문을 약학 박사학위 논문으로 제출함.

2014년 10월

조선대학교 대학원

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2014년 12월

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Acronyms

a-SMA	Smooth muscle actin
Ad	Adenovirus
ActD	Actinomycin-D
AP-1	Activating protein-1
ATF	Activating transcription factor
BDL	Bile duct ligation (BDL)
BMDM	Bone marrow-derived macrophage
bZIP	basic region leucine zipper
CCl4	Chronic carbon tetrachloride
EMT	Epithelial-mesenchymal transition
ERK1/2	Extracellular-regulated protein kinases 1 and 2
Gal	D-Galactosamine
HSC	Hepatic stellate cells
H&E	Hematoxylin & Eosin
H_2O_2	Hydrogen peroxide
IL	Interleukin
IκB	Inhibitory κB

LPS	Lipopolysaccharide
LXR-α	Liver X receptor-α
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
NOX	NADPH oxidase
Prx	Peroxiredoxin
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SBE	Smad binding element
Sesns	Sestrins
Sesn2	Sestrin-2
Srx	Sulfiredoxin
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TNF	Tumor necrosis factor
4-HNE	4-hydroxynonenal

국문초록

신규 간질환 치료 표적으로써의 Sestrin2 역할연구

양 지 혜

지도 교수: 기 성 환

조선대학교 대학원 약학과

산화 환원 상태의 균형 변화에 의해 생성된 활성산소종 (Reactive oxygen species, ROS)은 생체 내에서 에너지 대사를 위해 산소를 이용하는 모든 생명체에서 발생하는 산소 부산물로써, 소량의 ROS는 세포 내 신호전달 물질로써 작용하여 세포 생존 및 성장, 염증 반응 등을 매개하여 생리 현상을 조절하는 기능을 한다. 하지만 다양한 외부 자극에 의한 과량의 ROS 생성은 산화적 스트레스를 유발하여 DNA, 단백질, 지질세포막을 포함한 주요 세포 내 거대분자와 반응하여 이들의 정상적 기능을 방해함에 따라 세포 사멸을 유도하여 여러 병리상태의 주요 원인으로 작용하게 된다. 이러한 ROS가 간 내에서 생성되게 되면, 초기에는 염증 신호를 자극하여 간염을 일으키게 되고, 또한 만성적이고 반

복적인 간 내 염증은 간세포 괴사를 일으키고 간조직 중에 섬유질을 형성하여 간섬유화, 간경변증, 간암 등과 같은 만성적 중증 간질환으로 이행 시키게 된다.

이와 같은 이유로 본 연구에서는 간 내에서 발생되고 있는 ROS을 억제하는 단백질인 Sestrin2의 항 염증, 항 섬유화 효능을 규명하고자 하였고, 나아가 Sestrin2의 발현을 조절할 수 있는 치료제를 발굴하여 간질환 치료제로서의 가능성을 탐색하고자 하였다.

첫 번째로, 본 연구에서는 동물 및 세포 염증 모델을 활용하여 염증 및 선천성 면역반응에 있어서 Sestrin2의 역할을 규명하였다. 또한, TLR 매개성 염증 신호에 대하여 Sestrin2의 항 염증 효능을 평가하였고, 그 분자적 기전을 탐색하였다. 대조군 mice에서 Gal/LPS에 의해 증대된 ALT/AST 수치와 염증반응이 adenovirus Sestrin2 투여에 의해 반전되었다. 그리고, Sestrin2 stable expression 대식세포에서도 LPS에 증가된 iNOS 발현과 사이토카인(TNF-α, IL-1β, IL-6)의 양이 억제되는 것을 관찰할 수 있었다. 이는 과도한 ROS/RNS로 인한 염증 반응 및 이로 인한 간염을 제어하기 위해 Sestirn2이 효율적으로 작용할 수 있음을 시사한다.

두 번째로, 본 연구에서는 만성 간질환 모델인 간 섬유화 모델에서 Sestrin2의 역할을 규명하였다. Adenovirus Sestrin2 투여 후, 간 섬유화 유도 물질인 사염화탄소 투여 또는 담즙 정체 간 손상인 담관 결찰 후에 ALT/AST 수치와 간 섬유화 지표들을 확인해 본 결과, 대조군 대비 Adenovirus Sestrin2 투여 mice군에서 유의적으로 억제되는 것을 관찰할 수 있었다. 또한, 간성상 세포주인 LX-2에 Sestrin2을 Sestirin2을 과발현 시킨 후 TGF-β 자극을 주었을 때, 간 섬유화 지표들(α-SMA, PAI-1, Col 1A1)이 현저히 억제되었다. 이는

Sestirn2이 간성상 세포 활성화 및 간섬유화 신호 억제를 통하여 간 섬유화의 발병 및 진행에 있어 치료표적이 될 수 있음을 시사한다.

세 번째로, 본 연구에서는 Sestrin2의 발현을 조절할 수 있는 후보물질을 도출하고자 천연물 library를 활용하여 스크리닝 하였고, methylated flavonoid인 Isorhamnetin이 Sestrin2 발현을 현저히 증가시킴을 관찰하였다. 그리고 Isorhamnetin에 의한 Sestrin2의 발현이 Nrf2를 매개로 하여 증가됨을 확인하였고, Isorhamnetin이 t-BHP에 의해 발생된 ROS 및 세포사멸을 감소시키는 것을 관찰하였다. 그 다음으로, 염증과 섬유화에서 치료제로써의 Isorhamnetin의 역할을 연구하였다. 대식세포에서 LPS로 인해 증가된 염증 반응들을 Isorhamnetin이 유의성 있게 감소시킴을 확인하였고, 간성상 세포주에서 TGF-β에 의한 간 섬유화 지표들을 현저하게 억제시키는 것을 밝혀냈었다.

결과적으로, 본 연구에서는 신규 스트레스 반응성 유전자인 Sestrin2 및 관련 신호조절 단백질, 하위 기능 단백질을 규명하고 Sestrin2의 간세포보호, 간염 및 간섬유화 억제 효능을 규명함으로써 간질환 치료 신규 타겟으로써의 Sestrin2의 가능성을 제시하였다. 또한 Sestrin2의 발현을 조절할 수 있는 물질로 Isorhamnetin의 역할을 규명함으로 Isorhamnetin이 간질환 치료 후보약물이 될 수 있음을 제시한다.

Ⅰ. Introduction

1. Reactive oxygen species and liver diseases

1.1 Reactive oxygen species

Reactive oxygen species (ROS) are generated from the metabolism of molecular oxygen, mainly in mitochondria and are important mediators of normal cell physiology (Halliwell, 1999; Rigoulet, Yoboue, & Devin, 2011), ROS can play a role as intracellular signaling molecules and are involved in regulation of diverse biological processes. Especially, hydrogen peroxide (H_2O_2) is a significant ROS in terms of cell signaling regulation (Veal & Day, 2011).

However, oxidative stress occurs when the equilibrium between ROS production and the antioxidant defense mechanism is skewed in favor of ROS production, and the immoderate ROS then causes direct damage to macromolecules, such as, lipids, nucleic acids, and proteins (Blumberg, 2004). Moreover, it is well known that oxidative damage is associated with various human diseases, such as, hepatitis, diabetes, cancer, and cardiovascular disease.

The peroxiredoxin (Prx) is consists of a family of thiol-dependent peroxidases that scavenge H_2O_2 and alkyl hydroperoxides, serve as antioxidant protein. Furthermore, the six mammalian Prx family members exhibit different tissue and organelle distributions (Immenschuh & Baumgart-Vogt, 2005). In addition, they are also associated with various cellular functions, such as, proliferation, differentiation, and apoptosis. (Immenschuh & Baumgart-Vogt, 2005;

Rhee, Woo, Kil, & Bae, 2012). The Prxs are classified into three subgroups, designated 2-Cys Prx, atypical 2-Cys Prx, and 1-Cys Prx. The 2-Cys Prx, existing as homodimers, contains two conserved cysteine residues for peroxide reduction. Under extremely oxidizing conditions, Prxs is deprived of peroxidase activity due to the overoxidation of cysteine to sulfonic acid (Cys-SO_3H) or sulfinic acid (Cys-SO_2H) (Rhee et al., 2012) in the active site. However, sufinylated Prxs are reactivated by sulfiredoxin (Srx), which decreases sulfinylated Prxs via an ATP-dependent mechanism (Chang et al., 2004; Jeong, Bae, Toledano, & Rhee, 2012; Woo et al., 2005).

1.2 The stage of liver diseases

Liver diseases include three conditions: fatty liver, hepatitis, and fibrosis/cirrhosis. Heavy alcohol drinking and excessive food intake can lead to fatty liver (Hiramine et al., 2011; Kargulewicz, Stankowiak-Kulpa, & Grzymislawski, 2014), or steatosis-the earliest stage of liver disease and the most common liver disorder. Steatosis is marked by an excessive buildup of fat inside liver cells. This condition can be reversed, however, when causes were withdrawn.

Hepatic steatosis may lead to a more severe, potentially fatal condition, hepatitis-an inflammation of the liver with symptoms including nausea, lack of appetite, vomiting, fever, abdominal pain and tenderness, jaundice, and, sometimes, mental confusion. If inflammation may continues, in some patients this inflammation eventually leads to fibrosis or cirrhosis, and end-stage hepatocellular carcinoma (HCC) for years or even decades in the liver, in which healthy liver cells are replaced by scar tissue, leaving the liver unable to perform its vital functions (Wynn, 2007). Liver cirrhosis is major cause of death in the United States, and

it was the 12th leading cause of death, in 2000. Cirrhosis mortality rates vary considerably among age groups. Whereas these are very low among young people, cirrhosis mortality rates increase considerably in middle age. In fact, cirrhosis is the fourth leading cause of death in ages of 45-54 (Scaglione et al., 2014).

1.3 Reactive oxygen species and liver diseases

The liver is a multifunctional organ that is responsible for detoxification and metabolic homeostasis. It has two blood supply sources: the hepatic artery delivers oxygenated blood from the general circulation and the portal vein supplies deoxygenated but nutrient-rich blood from the intestinal region (MacSween et al., 1979).

Many cell types compose the liver. The parenchymal cells, which are the most abundant in the liver, are hepatocytes (80% by volume) (Gershwin et al., 2003). The nonparenchymal cells such as endothelial cells, Kupffer cells, smooth muscle cells, hepatic stellate cells, and oval cells are other important cell components in the liver (Gershwin et al., 2003). All of these cells can modulate the progression of liver diseases and activate multiple signaling pathways. The liver is the first organ exposed to orally administered xenobiotics after absorption from the intestine, and it is a major site of biotransformation and metabolism.

Since the liver is a metabolically active organ, it is particularly susceptible to reactive oxygen species (ROS). ROS are produced in liver cells as byproducts of normal metabolism and detoxification. Therefore, a wide range of antioxidant systems have developed in the liver, so that when produced, ROS are rapidly destroyed (Casarett et al., 2008). However, sustained and excessive ROS cause cellular damage and have been linked to a variety of liver diseases. Viral hepatitis and alcoholic or nonalcoholic steatohepatitis are the 3 major causes of chronic

liver diseases, which are highly associated with oxidative stress, lead to liver fibrosis, cirrhosis, and end-stage hepatocellular carcinoma (HCC). Therefore, it is generally accepted that oxidative stress plays a key role in promoting the progression of these liver diseases (Zhu, Wang, Zhang, & Guo, 2012).

2. Sestrin2(Sesn2) as a novel antioxidant enzyme

2.1 The role of Sestirn2 as antioxidant enzyme

Sestrins(Sesns) are conserved proteins that accumulate in cells exposed to various stresses, and three human isoforms have been identified. Sesn1 (PA26) has been identified as a GADD (growth arrest and DNA-damage inducible genes) whose expression is regulated by p53 (Velasco-Miguel et al., 1999). Expression of Sesn2 (Hi95), which was identified as a PA26 homologue, is induced by hypoxia, DNA damage, and oxidative stress (Budanov et al., 2002). Sesn3 was named as a novel PA26-related gene through analysis of the PA26 gene structure (Peeters et al., 2003) and its expression is regulated by FOXO transcription factor (Nogueira et al., 2008).

The role of Sesn2 as a sulfinyl reductase is controversial (Woo, Bae, Park, & Rhee, 2009), but Sesn2 has been shown to have cytoprotective activity against various stresses such as hydrogen peroxideor ischemia through regeneration of over-oxidized peroxiredoxins (Budanov et al., 2002). Sesn2 induces autophagy through inhibition of mTOR signaling, which results in more efficient elimination of ROS-producing damaged mitochondria in stressed cells (Budanov & Karin, 2008; Maiuri et al., 2009). Our current data confirm the role of Sesn2,

which exerts beneficial effect on hydrogenperoxide-induced cell death. Treatment with SFN significantly increased cell viability from cell death induced by hydrogen peroxide, and this effect was reversed by Sesn2 knockdown. Sesn2 is a family of recently identified evolutionally conserved antioxidant protein that exhibit cysteine sulfinyl reductase activity and can protect cells from oxidative stress.

Sesn2 is up-regulated in response to a variety of stresses including hypoxia, DNA damage, oxidative stress, and energetic stress (Svegliati-Baroni, De Minicis, & Marzioni, 2008). It is reported that the expression of Sesn2 is regulated by p53 (Budanov & Karin, 2008), Nrf2 (Shin, Jin, Cho, & Ki, 2012), and HIF-1α (Essler, Dehne, & Brune, 2009) dependent manner. Moreover, Sesn2 induces autophagy by inhibiting mTOR signaling via an AMPK activation, which results in more efficient elimination of ROS-producing damaged mitochondria (Budanov & Karin, 2008; Lee et al., 2010; Maiuri et al., 2009).

2.2 Physiological role of Sestrin2

Furthermore, recent reports have revealed physiological roles of Sesn2 in the liver. Sesn2 protects liver from acute stimulation of lipogenesis associated with fasting and re-feeding through degradation of Keap1 and concomitant up-regulation of Nrf2 activity (Bae et al., 2013; Shin et al., 2012). Sesn2-mediated lipogenic gene repression is also explained by inhibition of liver X receptor-α (LXR-α) activity (Jin et al., 2013). Conversely, Sesn2 ablation exacerbates obesity-induced hepatosteatosis and insulin resistance via mTORC1-S6K activation and AMPK inhibition. However, the role of Sesn2 activation in the pathogenesis of liver diseases remains obscure.

3. The anti-inflammatory role of Sestrin2

3.1 Reactive oxygen species and pro-inflammatory signaling in liver

Toll like receptors (TLRs), mammalian homologues to the *Drosophila* Toll, comprise a family of transmembrane proteins that function in immunity and development. TLRs are ubiquitously expressed pattern recognition receptors, which are central to inflammatory response in a broad range of species. Furthermore, it is becoming apparent that a link exists between oxidative stress and TLR signaling. TLR4 activation by lipopolysaccharide (LPS) is capable of inducing ROS and NF-κB activation due to a direct interaction between TLR4 and NADPH oxidase (NOX) (H. S. Park et al., 2004). Moreover, Prx2 is critical for the regulation of LPS-induced inflammatory gene expression and for the activation of MAPKs and NF-κB through NOX and ROS signaling (C. S. Yang et al., 2007).

3.2 Inflammation and acute hepatitis

Kupffer cells, resident macrophages in the liver, have been implicated in the pathogenesis of the liver injury induced by hepatotoxin, chemical substances, and pharmacological agents (Winwood & Arthur, 1993). Activated Kupffer cells produce nitric oxide which result in oxidative stress through its interaction with ROS leading to the formation of peroxynitrite or it up-regulates the expression of pro-inflammatory cytokines or chemokines (Sass, Koerber, Bang, Guehring, & Tiegs, 2001). These inflammatory mediators directly regulate hepatocytes death or activate other cells such as hepatic stellate cells, sinusoidal endothelial cells as well as neutrophils. Kupffer cell activation by LPS is most important in Gal/LPS-induced acute

liver injury which is most widely used model for endotoxin-mediated hepatitis (Matsuo, Ukida, Nishikawa, Omori, & Tsuji, 1992).

4. The anti-fibrotic role of Sestrin2

4.1 Reactive oxygen species and TGF-β/Smad signaling

It is well established that generation of reactive oxygen species (ROS) plays a key role in liver fibrosis. TGF-β is produced by HSC in response to exogenous ROS, and vise versa. Elevated ROS production and resulting oxidative stress are commonly detected in livers from patients as well as in most types of experimental liver fibrosis models (Amara et al., 2010; Carnesecchi et al., 2011; Rhyu et al., 2005). Moreover, antioxidant therapy improves hepatic fibrosis in rodents and may exert beneficial effects in patients with chronic liver diseases (Fogden & Neuberger, 2003; Hanje, Fortune, Song, Hill, & McClain, 2006; Verma & Thuluvath, 2007). However, little is known regarding the function of the antioxidant system in activated HSC. In addition, ROS may have regulating effects on TGF-β-induced Smad2/3 activation. In cardiac fibroblasts, it was observed that TGF-β-induced phosphorylation of Smad2/3 was meaningfully inhibited by Nox4 gene silencing and by various antioxidant agents (Cucoranu et al., 2005). In addition to regulating phosphorylation of Smad, ROS may also modulate TGF-β signaling via Smad-independent mechanisms. In addition to Smad-transduced signals, TGF-β may activate other signaling pathways including the mitogen-activated protein kinase (MAPK) members JNK and p38 (Moustakas & Heldin, 2005). Of note, it is clear that

both JNK and p38 are redox sensitive, which can be activated by ROS in the cytoplasm (F. Jiang, Zhang, & Dusting, 2011).

4.2 TGF-β signaling and liver fibrosis

Liver fibrosis and cirrhosis are recognized as major causes of liver-disease-related morbidity and mortality worldwide with the potential to progress to liver failure (Wynn, 2007). Hepatic fibrosis results from chronic liver injury and inflammatory responses in conjunction with the accumulation of extracellular matrix (ECM) proteins, which eventually progresses to liver cirrhosis. Hepatic stellate cell (HSC) activation in response to liver injury is considered to be a key step in the liver fibrogenesis. Following liver injury, quiescent vitamin A storing HSC are converted into proliferative, fibrogenic, and contractile myofibroblasts with increased expression of smooth muscle actin (α-SMA) and biosynthesis of type I collagen. Thus, suppression of HSC activation is regarded as a principal target for the treatment of liver fibrosis. HSC activation is sophisticatedly orchestrated by a variety of signaling factors including cytokine, growth factors, and hormones. Among of them, TGF-β appears to be a key downstream mediator in liver fibrogenesis (Leask & Abraham, 2004). In HSCs, TGF-β is capable of triggering the transition to myofibroblast-like cells, stimulates the synthesis and contract ECM proteins. Strategies with the aim of disrupting TGF-β synthesis and/or its signaling pathways obviously attenuated experimental liver fibrogenesis (Branton & Kopp, 1999; Tian, Neil, & Schiemann, 2011). TGF-beta exerts its biological functions mainly via its downstream molecular signaling pathways, Smads. Smads regulate the signals from the receptors for TGF-β superfamily members to the nucleus. Catalytically active TGF-β receptor phosphorylates serine residues of receptor-activated Smad2 and Smad3. They, forms a hetero-

oligomers with Smad4 and translocated to the nucleus, where it initiates transcription TGF-β target genes.

5. Identification of isorhamnetin as a Sestrin2 modulator

5.1 Current therapeutics for liver diseases

Various reasons, such as viral infection, alcohol, fatty liver, lead to hepatitis, fibrosis and cirrhosis in liver. Although several specific therapies for patients who have different liver diseases have been successfully developed, specific and effective therapy of liver diseases remains not easily found. Therefore, the development of more effective and efficient therapeutics for the liver diseases is much required.

5.2 Isorhamnetin

Water dropwort (Oenanthe javanica, Umbelliferae), an herbal medicine for the treatment of jaundice, hypertension, diabetes, and abdominal pain (Ma et al., 2010; J. C. Park, Young, Yu, & Lee, 1995) as well as for food, has been widely used in Asian countries. Isorhamentin is a 3’ O-methylated metabolite of quercetin and one of a major constituent of O. javanica. It has been known several pharmacological activities such as anti-oxidative and anti-proliferative effects (Pengfei, Tiansheng, Xianglin, & Jianguo, 2009; Teng, Lu, Wang, Tao, & Wei, 2006). Nevertheless, it is not elucidated in detail that in vivo effect of isorhamnetin and its molecular mechanisms explaining how isorhamnetin suppresses the liver disease.

Ⅱ. Aims of the study

1. **Sestrin2 protects against TLR-induced Acute Hepatitis via Down-regulation of Pro-inflammatory Signaling**

2. **Sestrin2 protects against CCl4 or BDL-induced Hepatic Fibrosis via Down-regulation of TGF-β/Smad signaling**

3. **Identification of Isorhamnetin as a Sestrin2 Modulator and Therapeutic Function**

Ⅲ. Materials & Methods

1. Reagents and antibodies

Antibodies against iNOS, PARP1/2, Nrf2, Zeb1, Lamin A/C, Tubulin and IκBα were provided by Santa Cruz Biotechnology (Santa Cruz, CA). Sesn2 antibody was obtained from Proteintech (Chicago, IL). PAI-1 and N-cadherin antibodies were obtained from BD (Becton, Dickinson and Company). Phospho-ERK1/2, ERK1/2, phospho-p38, p38, phospho-JNK1/2, JNK1/2, phospho-c-Jun, c-Jun, phospho-c-Fos, c-Fos, phospho-Smad2, Smad2, phospho-Smad3, Smad3, Slug and caspage3 antibodies were obtained from Cell Signaling (Danvers, MA). Snail antibody was provided by Abcam. Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies were purchased from Invitrogen (Carlsbad, CA). SB203580 and SP600125 were purchased from Calbiochem (Billerica, MA). LPS (Escherichia coli 055:B5), peptidoglycan, poly (I:C), loxoribine, flagellin, ODN 1826, dimethylsulfoxide (DMSO), sodium nitrite, galactosamine, and α-SMA, β-actin antibody were from Sigma Chemicals (St. Louis, MO). TGF-β was purchased from R&D Systems.

2. Preparation of isorhamnetin

O. javanica was purchased from a home farm located in Chungdo, Gyeongsangbukdo, Korea. The 10 kg of air-dried stems and leaves of *O. javanica* were extracted three times with MeOH and then concentrated (1.2 kg). The methanolic extract was suspended in water and partitioned successively with $CHCl_3$ and *n*-BuOH. The *n*-BuOH fraction located onto a silica

gel column chromatography (15 × 80 cm, 70-230 mesh) was eluted with $CHCl_3$, then with a gradient of $CHCl_3$-MeOH. The fraction of $CHCl_3$-MeOH (25:1) was concentrated to give a dark brown residue (23 g). The obtained residue was further fractionated on silica gel column chromatography with a gradient of *n*-hexane-EtOAc [20:1 (5 L), 10:1 (3 L), 4:1 (3 L), 1:1 (2 L), each fraction volume was 250 ml]. Fractions 35-40 from this column were combined and evaporated to give a isorhamnetin mixture (3 g), and then successively washed with diethylether for further purification. Finally we obtained 2 g of isorhamnetin. An ultra performance liquid chromatography (UPLC) system equipped with BEH C18 column (1.7 μm, 2.1×100) and photodiode array detector (Waters ACQUITYTM, Milford, MA, USA) was used to evaluate the purity of purified isorhamnetin. The output signal of the detector was recorded using Empower Data System. The structures of the isolated isorhamnetin was confirmed on the basis of spectroscopic analyses including HPLC-ESI-MS (Agilent 6120 LC/MS system, Agilent Technologies, Palo Alto, CA) and NMR spectroscopy (data not shown), and verified by comparison with reported spectral data (Cao, Wei, & Ito, 2009). ^{1}H- and ^{13}C-NMR spectroscopy were carried out in a JEOL ECA-500 spectrometer (Tokyo, Japan) operating at 500 MHz and 125 MHz, respectively. Solvent signals (DMSO) was used as the internal standard.

3. Cell culture

RAW264.7 cells (a murine macrophage cell line) and HepG2 cells were supplied by the American Type Culture Collection (ATCC). LX-2 cells (immortalized human activated HSCs) were kindly provided by Dr. S. L. Friedmann (Mount Sinai School of Medicine, New York, NY), and. Cells were maintained in DMEM containing 10% fetal bovine serum, 50 units/ml

penicillin/ streptomycin at 37°C in a humidified 5% CO_2 atmosphere.

4. Animals

The protocols for the animal studies were approved by the Animal Care and Use Committee of Chosun University. Male ICR mice (6 wk old) were obtained from Oriental Bio (Sungnam, Korea) and acclimatized for 1 week. Mice (N=5/group) were housed at 20 ± 2°C with 12 h light/dark cycles and a relative humidity of 50 ± 5% under filtered, pathogen-free air, with food (Purina, Korea) and water available ad libitum.

5. Adenovirus preparation

For the generation of an adenoviral Sesn2 construct murine Sesn2 ORF was amplified by using attB-fused specific primers, and then inserted into pDONR^{TM}221 entry plasmid by BP recombination reaction (Invitrogen, Carlsbad, CA). The recombinant adenovirus was constructed and generated by using pAD/CMV/V5-DEST gateway plasmid according to the manufacturer's instructions (Invitrogen). The DNA sequences of recombinant adenovirus were verified by sequencing using the ABI7700 DNA cycle sequencer. Recombinant adenovirus for *in vivo* study was further purified by $CsCl_2$ density gradient centrifugation. Virus titer was calculated from $TCID_{50}$ method for *in vitro* study or optical intensity of 260 nm for *in vivo* study. Adenovirus which expresses LacZ (Ad-LacZ) was used as an infection control.

6. G1al/LPS-induced hepatitis

Acute hepatitis was induced by i.p. injection with 5 μg/kg LPS (Sigma-Aldrich) plus 700

mg/kg Gal (Sigma-Aldrich) and euthanized 8 h post-treatment. Adenovirus particles (1×10^9 pfu) suspended in PBS were injected in the tail vein 48 h prior to Gal/LPS injection.

7. CCl4-induced hepatic fibrosis

To induce liver fibrosis, CCl_4 dissolved in olive oil (10%) was intraperitoneally injected (0.5mg/kg) into the mice three times per week for 2 wk. And, before the mice were sacrificed, mice were induced by i.p. injection with CCl4 for 24 h. Adenovirus particles (1×10^9 pfu) suspended in PBS were injected in the tail vein 48 h prior to first i.p. CCl4 injection and adenovirus were injected into the mice in every five days for 2 wk.

8. BDL induced hepatitis

Male ICR mice were bile duct ligated as described (Kountouras, Billing, & Scheuer, 1984). BDL causes cholestasis, periductular inflammation, and fibrosis. Adenovirus particles (1×10^9 pfu) suspended in PBS were injected in the tail vein 48 h prior to BDL and adenovirus were injected into the mice in every five days for 2 wk.

9. Blood chemistry

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed using Spectrum®, an automatic blood chemistry analyzer (Abbott Laboratories, Abbott Park, IL).

10. Histopathology

Samples from liver were separated and fixed in 10% neutral buffered formalin, then embedded in paraffin, sectioned (3-4 μm) and stained with hematoxylin and eosin (H&E) for general histopathology or Sirius red for collagen fibers (Tipoe et al., 2010). After that the histopathological profiles of each sample were observed under light microscope (Nikon, Tokyo, Japan).

11. Establishment of a stable cell line expressing Sesn2

pCMV-SESN2 construct was generated as previously described (Essler et al., 2009). RAW264.7 cells were transfected with the plasmid pCMV-Tag3A (MOCK) or pCMV-SESN2 using Lipofectamine 2000, according to the manufacturer's instructions (Life Technologies). One day after transfection, cells were transferred to fresh DMEM medium containing 800 μg/ml G418 (Invitrogen) (the medium was replaced every three days). Two weeks later, Trypsin-EDTA was added to plates and colonies of G418 resistant cells were isolated under an inverted light microscope and grown further. Sesn2 expression was confirmed by western blotting using c-myc antibody.

12. Bone marrow-derived macrophage (BMDM) culture and differentiation

For the BMDM cultures, bone marrow was isolated from the femurs and tibias of mice and cultured in minimum essential medium alpha (α-MEM) supplemented with 10% FBS. The cells were plated and cultured overnight in the presence of macrophage colony-stimulating factor (M-CSF, 10 ng/ml) (Peprotech**,** Rocky Hills, NJ). The non-adherent cells were collected and

cultured for 3 days in the presence of M-CSF. The floating cells were removed, and the adherent cells were used as BMDMs.

13. Primary hepatocyte isolation

Primary hepatocytes were isolated and cultured as described previously (Kay et al., 2010). Briefly, ICR micewere anesthetized with Zoletil (Virbac, France) and the portal vein was cannulated under aseptic conditions. The liver was perfused in situ with Ca 148 2+-free Hank's balanced saline solution (HBSS) at 37 °C for 5 min. Livers were then perfused for 20 min with HBSS containing 0.05% collagenase and Ca 150 2+ at a perfusion flow rate of 10 ml/min. After perfusion, the livers were minced gently with scissors and suspended with sterilized PBS. The cell suspension was then filtered through a cell strainer and centrifuged at 50 ×g for 5 min to separate parenchymal and nonparenchymal cells. Viability of isolated hepato-cytes estimated by trypan blue staining is usually 80–90%. Isolated hepatocytes were plated on collagen-coated plate and cultured in DMEM containing 50 units/ml penicillin/streptomycin with 10% FBS.

14. HSC Isolation and Culture

Livers are perfused using pronase/collagenase method and Primary hepatic stellate cells (HSC) were isolated using gradient centrifugation. HSCs were cultured on uncoated plastic tissue culture dishes in DMEM containing 50 units/ml penicillin/streptomycin with 10% FBS at 37°C in a humidified 5% CO_2 atmosphere.

15. Recombinant Adenovirus Sesn2 Construct Infection

Cells were infected with adenovirus diluted in DMEM containing 10% FBS at a multiplicity of infection of 50 and incubated for 12 h. After removal of the viral suspension, cells were further incubated with DMEM containing 10% FBS for 2 days and then were treated with the indicated reagent. Ad-LacZ was used as an infection control. Efficiency of infection was consistently >90% with this method.

16. MTT assay

To measure cytotoxicity, cells were plated at in 96-well plates and treated chemicals for 12h or 24h., and viable cells were stained with MTT (0.2 mg/ml, 4 h). The media were then removed, and formazan crystals produced in the wells were dissolved with the addition of 200 μl of dimethyl sulfoxide. Absorbance at 540 nm was measured using an enzyme-linked immunosorbent assay microplate reader (Versamax, Molecular Device, Sunnyvale, CA). Cell viability was defined relative to untreated control [i.e., viability (% control) = 100 × (absorbance of treated sample)/(absorbance of control)].

17. Measurement of LDH level

LDH release into the media was measured using an LDH kit (Cayman, Ann. Arbor, MI) according to instructor's manual. Briefly, samples were incubated for 30 min at room temperature with an assay buffer containing LDH diaphorase, lactic acid, NAD+, and tetrazolium salt. Absorbances were then read at 490 nm using microplate reader (SpectraMAX, Molecular Device).

18 Assay of nitrite production

NO production was monitored by measuring nitrite content in culture medium as previously described (J. H. Yang et al., 2013). This was performed by mixing samples with Griess reagent (Sigma, St. Louis, MO) and a standard curve constructed using sodium nitrite (Sigma, St. Louis, MO). Absorbance at 548 nm was measured using an enzyme-linked immunosorbent assay microplate reader (Spectramax, Molecular Device) after incubation for 30 min.

19 Immunoblot analysis

Protein extraction and subcellular fractionation, SDS-polyacrylamide gel electrophoresis and immunoblot analyses were performed as previously described (Shin et al., 2012). Briefly, samples were separated by 7.5% or 12% gel electrophoresis and electrophoretically transferred to nitrocellulose paper. The nitrocellulose paper was incubated with the indicated primary antibody and then incubated with horseradish peroxidase-conjugated secondary antibody. Immunoreactive protein was visualized by ECL chemiluminescence (Amersham Biosciences, Buckinghamshire, UK). Equal protein loadings were verified using β-actin.

20. siRNA knockdown experiment

Cells were transfected with non-targeting control siRNA (100 pmol) or siRNA directed against Sesn2 (100 pmol) (ON-TARGETplus SMARTpool, Dharmacon Inc., Lafayette, CO) for 24 h using Lipofectamine 2000 according to the manufacturer's instructions.

21. 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 (2 μg) was reverse-transcribed using an oligo$(dT)_{16}$ primer. The cDNA obtained was amplified using a high-capacity cDNA synthesis kit (Bioneer, Daejon, Korea) using a thermal cycler (Bio-Rad, Hercules, CA). Real-time PCR was performed with STEP ONE (Applied Biosystems, Foster City, CA) using a SYBR green premix according to the manufacturer's instructions (Applied Biosystems). Primers were synthesized by Bioneer. The following primer sequences were used: mouse Sesn2 5'-TAGCCTGCAGCCTCACCTAT-3' (sense) and 5'-TATCTGATGCCAAAGACGCA-3' (antisense); iNOS 5'-CCTCCTCCACCCTACCAAGT-3' (sense) and 5'-CACCCAAAGTGCTTCAGTCA-3' (antisense); mouse TNF-α 5'-AAGCCTGTAGCCCACGTCGTA-3' (sense) and 5'-AGGTACAACCCATCGGCTGG-3' (antisense); mouse IL-1β 5'-TGGACGGACCCCAAAAGATG-3' (sense) and 5'-AGAAGGTGCTCATGTCCTCA-3' (antisense); mouse IL-6 5'-TCCATCCAGTTGCCTTCTTG-3' (sense) and 5'-TTCCACGATTTCCCAGAGAAC-3' (antisense); mouse $gp91^{phox}$ 5'-CCAGTGAAGATGTGTTCAGCT-3' (sense) and 5'-GCACAGCCAGTAGAAGTAGAT-3' (antisense); mouse $p47^{phox}$ 5'-GTGGAGAAGAGCGAGAGCGG-3' (sense) and 5'-GGTGGATGCTCTGTGCGTTG-3' (antisense); mouse $p22^{phox}$ 5'-TTCCTGTTGTCGGTGCCTGC-3' (sense) and 5'-TTCTTTCGGACCTCTGCGGG-3' (antisense); mouse α-SMA 5'-TCCTCCCTGGAGAAGAGCTAC-3' (sense) and 5'-TATAGGTGGTTTCGTGGATGC-3' (antisense); mouse PAI-1 5'-GACACCCTCAGCATGTTCATC-3' (sense) and 5'-AGGGTTGCACTAAACATGTCAG-3' (antisense); mouse Col 1A1 5'-

ACCTGTGTGTTCCCTACTCA-3' (sense) and 5'-GACTGTTGCCTTCGCCTCTG-3' (antisense); human Sesn2 5'-CAAGCTCGGAATTAATGTGCC-3' (sense) and 5'-CTCACACCATTAAGCATGGAG-3' (antisense); human α-SMA 5'-CGCATCCTCATCCTCCCT-3' (sense) and 5'-GGCCGTGATCTCCTTCTG-3' (antisense); human PAI-1 5'-CGCCAGAGCAGGACGAA-3' (sense) and 5'-CATCTGCATCCTGAAGTTCTCA-3' (antisense); human Col 1A1 5'-CCTGGGTTTCAGAGACAACTTC-3' (sense) and 5'-TCCACATGCTTTATTCCAGCAATC-3' (antisense); 18S 5'-GTAACCCGTTGAACCCCATT-3' (sense) and 5'-CCATCCAATCGGTAGTAGCG-3' (antisense). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or 18S was used as an endogenous control for RT-PCR.

22. Luciferase assay

To measure the luciferase activities of AP-1 or NF-κB, SESN2, SESN2(ΔSBE), SBE, MOCK- or SESN2-transfeced cells were re-plated in 24-well plates overnight, serum-starved for 6 h, and transiently transfected with AP-1, NF-κB, SESN2, SESN2(ΔSBE) or SBE luciferase plasmid and pRL-TK plasmid (a plasmid that encodes *Renilla* luciferase and used to normalize transfection efficacy) in the presence of Lipofectamine® Reagent (Invitrogen, San Diego, CA) for 3 h. Transfected cells were allowed to recover in DMEM for 3 h and then exposed to 1 μg/ml for 12 h. Firefly and *Renilla* luciferase activities in cell lysates were measured using the dual luciferase assay system (Promega) according to the manufacturer's instructions. Relative luciferase activities were calculated by normalizing firefly luciferase activities versus that of *Renilla* luciferase.

23. Assay of ROS generation

DCFH-DA is a cell-permeable, non-fluorescent probe that is cleaved by intracellular esterases and turns into highly fluorescent dichlorofluorescein upon reaction with H_2O_2. After chemical treatment, cells were stained with 10 μM DCFH-DA for 30 min at 37°C. H_2O_2 generation was determined by measuring dichlorofluorescein using fluorescence microscope (Zeiss, Germany) or fluorescence microplate reader (Jemini, Molecular Device) at excitation/emission wavelengths of 485/530 nm.

24. Enzyme-linked immunosorbent assay (ELISA)

TNF-α, IL-1β and IL-6 ELISA kits were purchased from BD (Becton, Dickinson and Company). TNF-α, IL-1β and IL-6 contents in culture medium or serum were measured by ELISA using anti-mouse TNF-α, IL-1β or IL-6 antibodies and biotinylated secondary antibody according to the manufacturer's instructions.

25. Gel shift assay

EMSA were performed as described previously (Jin et al., 2013). Double-stranded DNA probes for the consensus sequences of nuclear factor-κB (NF-κB, 5´-AGTTGAGGGGACTTTCCCAGGC-3´) and activator protein-1 (AP-1, 5-CGCTTGATG AGTCAGCCGGAA-3) were used for gel shift analysis after end-labeling probes with [γ-^{32}P]ATP and T_4 polynucleotide kinase. The reaction mixture contained 2 μl of 5× binding buffer (20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml poly(dI-dC), and 50 mM Tris·Cl (pH 7.5)), 8 μg of nuclear extracts, and sterile water in

a total volume of 10 μl. Incubations were initiated by adding 1 μl of probe (10^6cpm) and continued for 20 min at room temperature. Samples were loaded onto 4% polyacrylamide gels at 100 V. Gels were then removed, fixed, and dried, and subjected to autoradiography.

26. Determination of GSH content

The GSH contents in the cells were quantified using a commercial GSH determination kit (BIOXYTECH GSH-400, Oxis International). Cells were plated onto 6-well dishes and chemical treatment. And then, scraped cellswere lysed in buffer containing 5% metaphosphoric acid to precipitate proteins. After being centrifuged at 10,000 × g for 10 min, the supernatants were used to measure GSH concentration. Absorbance at 400 nm was measured using a microplate reader (SpectraMAX, Molecular Device, Sunnyvale, CA).

27. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the significances of differences between treatment groups. The Newman-Keuls test was used to determine the significances of differences between multiple group means. Results are expressed as means ± SDs.

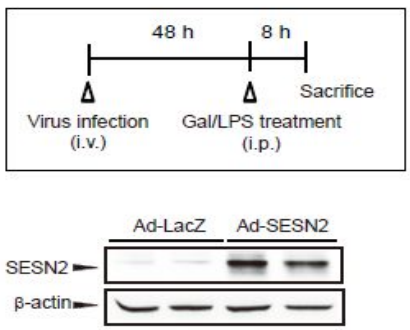
Ⅳ. Result

Part Ⅰ: Sestrin2 protects against TLR-induced Acute Hepatitis via Down-regulation of Pro-inflammatory Signaling

1. Protection of Ad-Sesn2 on Gal/LPS-induced Acute Hepatitis

Bacterial endotoxin has been implicated in the pathogenesis of acute fulminant hepatitis through its up-regulation of the pro-inflammatory signaling (Antoniades, Berry, Wendon, & Vergani, 2008). To investigate the effect of Sens2 on Gal/LPS-induced liver injury, we used a recombinant adenovirus expressing LacZ (Ad-LacZ) or Sesn2 (Ad-Sesn2), which cause robust exogenous gene expression in liver (Fig 1A). ALT and AST serum levels were significantly increased 8 h after Gal/LPS treatment in Ad-LacZ infected mice. However, elevated levels of ALT and AST by Gal/LPS were markedly decreased by Ad-Sesn2 (Fig 1B). Gal/LPS treatment results in increased the percentages of degenerative hepatic regions and numbers of inflammatory cell infiltrated in Ad-LacZ. These changes were attenuated by Ad-Sesn2 infection (Fig 2, Table 1). Biochemical and histological analysis data strongly support the protective effect of Sesn2 against Gal/LPS-induced acute hepatitis.

A)



B)

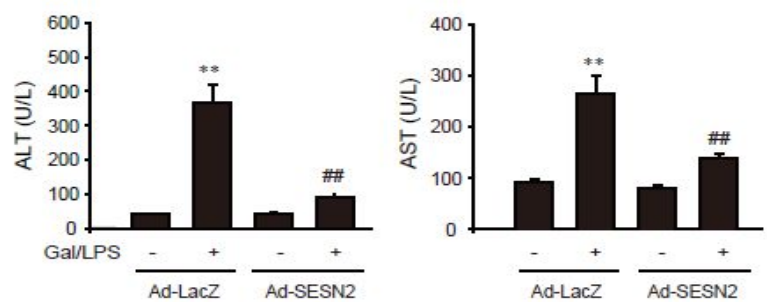


Figure 1. Protection of Ad-Sesn2 on Gal/LPS-induced acute hepatitis

A. Treatment schedule for Gal/LPS-induced acute hepatitis. Adenovirus particles (1×10^9 pfu) are infected via tail vein 48 h prior to injection with Gal (700 mg/kg)/LPS (5 μg/kg). Mice were sacrificed 8 h after Gal/LPS treatment (upper). Sesn2 expression was confirmed by immunoblotting in liver homogenates of infected with adenovirus LacZ (Ad-Lac) or Sesn2 (Ad-SESN2) (lower). *B*. The activities of ALT and AST were assayed by using an automated blood chemistry analyzer. All values were expressed as mean ± SD of 5 mice serum (significant as compared with vehicle control, **p<0.01; significant as compared with Gal/LPS alone, ##p < 0.01).

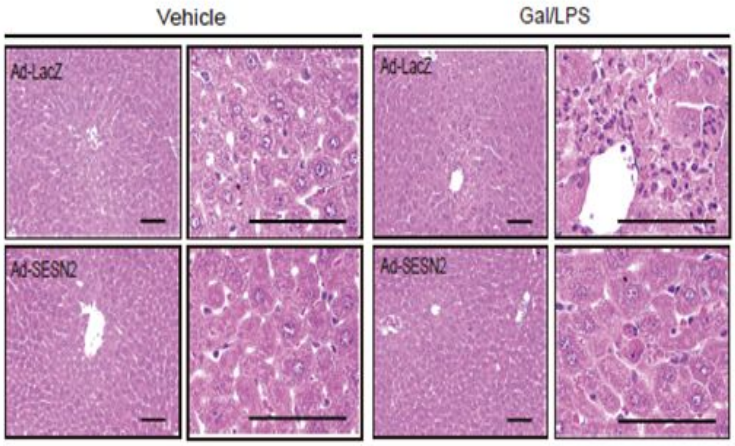


Table 1. Histomorphometrical analysis of hepatic tissues

Groups	Percentages of degenerative regions (%/mm^2 of hepatic parenchyma)	Mean numbers of inflammatory cells (cells/mm^2 of hepatic parenchyma)	Mean degenerative hepatocytes (cells/mm^2 of hepatic parenchyma)
Ad-LacZ vehicle	2.37 ± 1.79	7.00 ± 4.18	4.60 ± 1.82
Ad-LacZ Gal/LPS	37.91 ± 8.76**	243.60 ± 47.83**	169.80 ± 19.15**
Ad-SESN2 vehicle	2.28 ± 2.65	7.20 ± 3.70	4.40 ± 2.88
Ad-SESN2 Gal/ LPS	8.88 ± 1.69$^{**,\#\#}$	19.80 ± 2.39$^{**,\#\#}$	22.20 ± 10.35$^{*,\#\#}$

All values were expressed as mean±SD of five mice; *P < 0.05, **P < 0.01 = significant as compared to vehicle-treated group; $^{\#\#}$P < 0.01 = significant as compared to Gal/LPS-treated group.

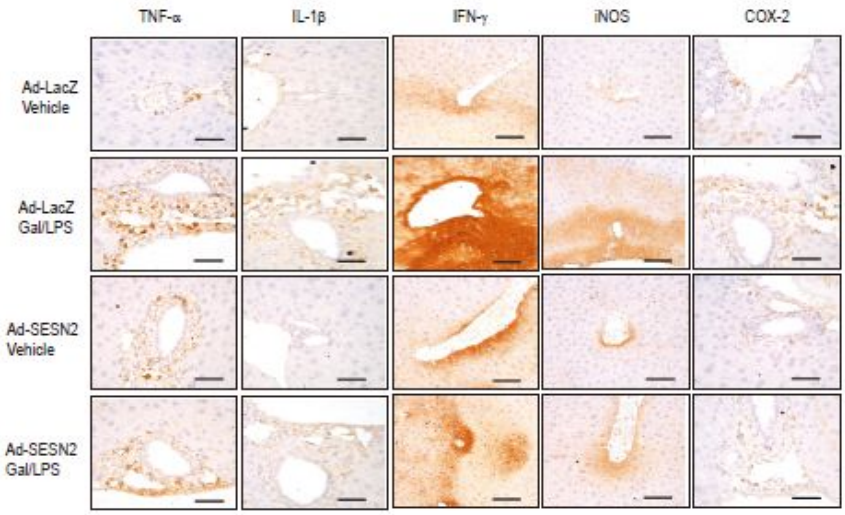
Figure 2. Inhibition of Gal/LPS-induced acute liver injury by Ad-Sesn2

Representative histological profiles of the liver. Samples from liver were separated and fixed in 10% neutral buffered formalin, then embedded in paraffin, sectioned (3-4 μm) and stained with H&E for general histological observations (Scale bar = 120 μm).

2. Inhibition of Gal/LPS-mediated Inflammatory Gene Induction by Ad-Sesn2

The cytokines including TNF-α has been recognized as an important regulator of the inflammatory response in animal models of fulminant hepatitis (Forman & Torres, 2002). TNF-α, IL-1β and IFN-γ-positive cells were increased in mice treated with Gal/LPS, but they were significantly attenuated by Ad-Sesn2. Moreover, Ad-Sesn2 decreased Gal/LPS-induced iNOS and COX-2 positive cells which produce nitric oxide (NO) and prostanoids, respectively (Fig 3A and B). Consistently, Gal/LPS-mediated TNF-α, IL-6, and IL-1β mRNA induction in liver tissue was suppressed by Ad-Sesn2 injection (Fig 4A). In addition, Ad-Sesn2 blocked the production of TNF-α, IL-6, and IL-1β in the plasma of mice challenged with Gal/LPS (Fig 4B). Furthermore, the antagonistic role of Sesn2 on Gal/LPS-induced iNOS expression is verified in mice (Fig 4C).These data demonstrate that Ad-Sesn2 inhibits Gal/LPS-induced inflammatory response during liver injury.

A)



B)

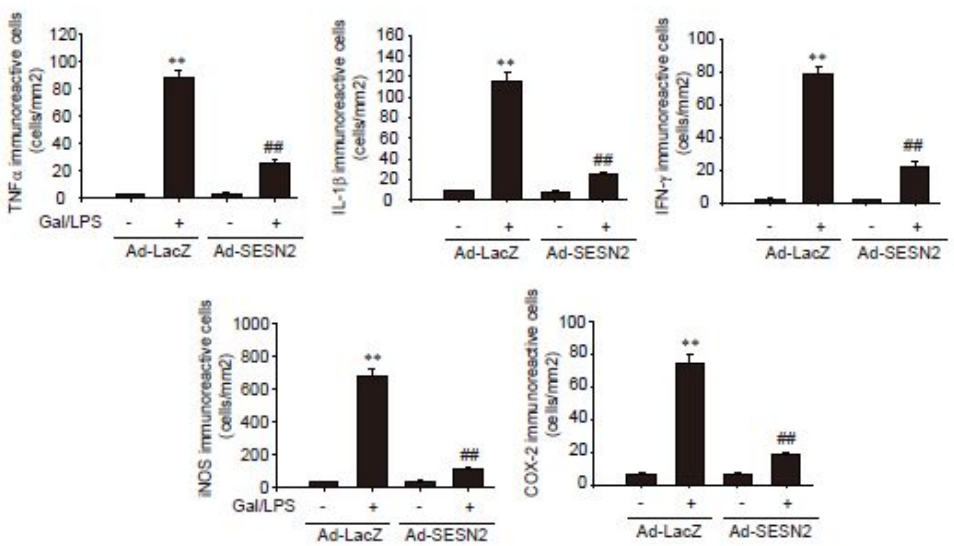


Figure 3. Representative immunohistochemistrical images

A, TNF-α, IL-1β and IFN-γ, iNOS and COX2 immunohistochemical staining of liver from mice treated with Gal/LPS or vehicle 48 h followed by adenovirus LacZ (Ad-LacZ) or adenovirus Sesn2 (Ad-SESN2) infection (Scale bar = 120 μm). *B,* The number of immunoreactive (positively stained) cells per/mm^2 was counted in liver. All values were expressed as mean ± SD of 5 mice liver (significant as compared with vehicle control, **p<0.01; significant as compared with Gal/LPS alone, ##p < 0.01).

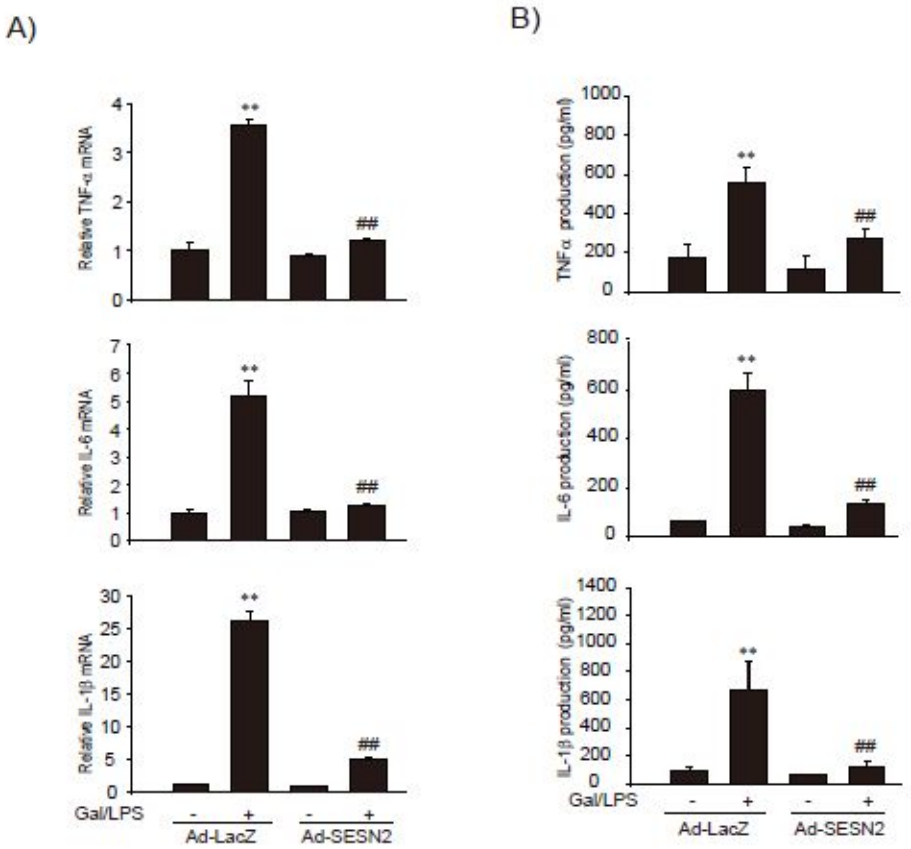


Figure 4. Inhibition of Gal/LPS-mediated inflammatory gene induction by Ad-Sesn2

A. RT-PCR analysis. The transcript levels of TNF-α, IL-6, and IL-1β were assessed by Real-time RT-PCR analysis. *B.* ELISA. TNF-α, IL-6, and IL-1β release into serum was determined by ELISA. *C.* The effect of Sesn2 on Gal/LPS-induced iNOS expression. Results are presented as means ± SD; **p<0.01; significant as compared with Gal/LPS alone, ##p < 0.01.

3. Inhibitory Role of Sesn2 on NO Production and iNOS expression

First, we generated a stable RAW264.7 cell line expressing Sesn2 to explore the effect of Sesn2 on TLR ligand (TLRL)-induced inflammatory response and the molecular mechanism involved. The expression of Sesn2 was confirmed by immunoblotting with c-myc or Sesn2 antibody (Fig. 5A). Although Sesn2 has previously been shown to be induced by various stimuli, Sesn2 is basally expressed weakly in macrophages. Indeed, long exposure of the western blot was required for detection of the Sesn2 protein in macrophages.

We found that LPS increased NO production in mock-transfected RAW264.7 cells (MOCK) as it did in RAW264.7 cells. In subsequent experiments, we examined whether Sesn2 expression affected LPS-induced NO production. Whereas NO production was elevated in MOCK cells by LPS, Sesn2 expression almost completely blocked the ability of LPS to induce NO production (Fig. 5B).

We next examined whether this lack of NO production by LPS was due to diminished iNOS expression in Sesn2 expressing cells. Sesn2 expression was found to prevent the induction of iNOS expression by LPS (Fig. 5C). Furthermore, RT-PCR revealed that LPS enhanced iNOS expression in MOCK cells but not in cells expressing Sesn2 (Fig. 5D).

To confirm the regulatory role of Sesn2 on NO production and iNOS expression, we measured NO production and iNOS expression after Sesn2 knockdown by Sesn2 siRNA. Sesn2 knockdown significantly increased LPS-induced NO production and iNOS expression compare to control siRNA (Fig. 5E and F). The knockdown of Sesn2 was confirmed by immunoblotting (Fig. 5F). Furthermore, we found that Sesn2 expression was markedly induced by LPS treatment (Fig. 5F). Further studies are needed to understand the molecular

mechanism and the physiological role of LPS-induced Sesn2 expression. In addition, the inhibitory role of Sesn2 on LPS-induced iNOS expression is confirmed by recombinant adenovirus Sesn2 (Ad-Sesn2) in RAW264.7 cells and bone marrow-derived macrophage (BMDM) (Fig. 5G and H). These observations suggest that Sesn2 might inhibit LPS-induced inflammatory responses.

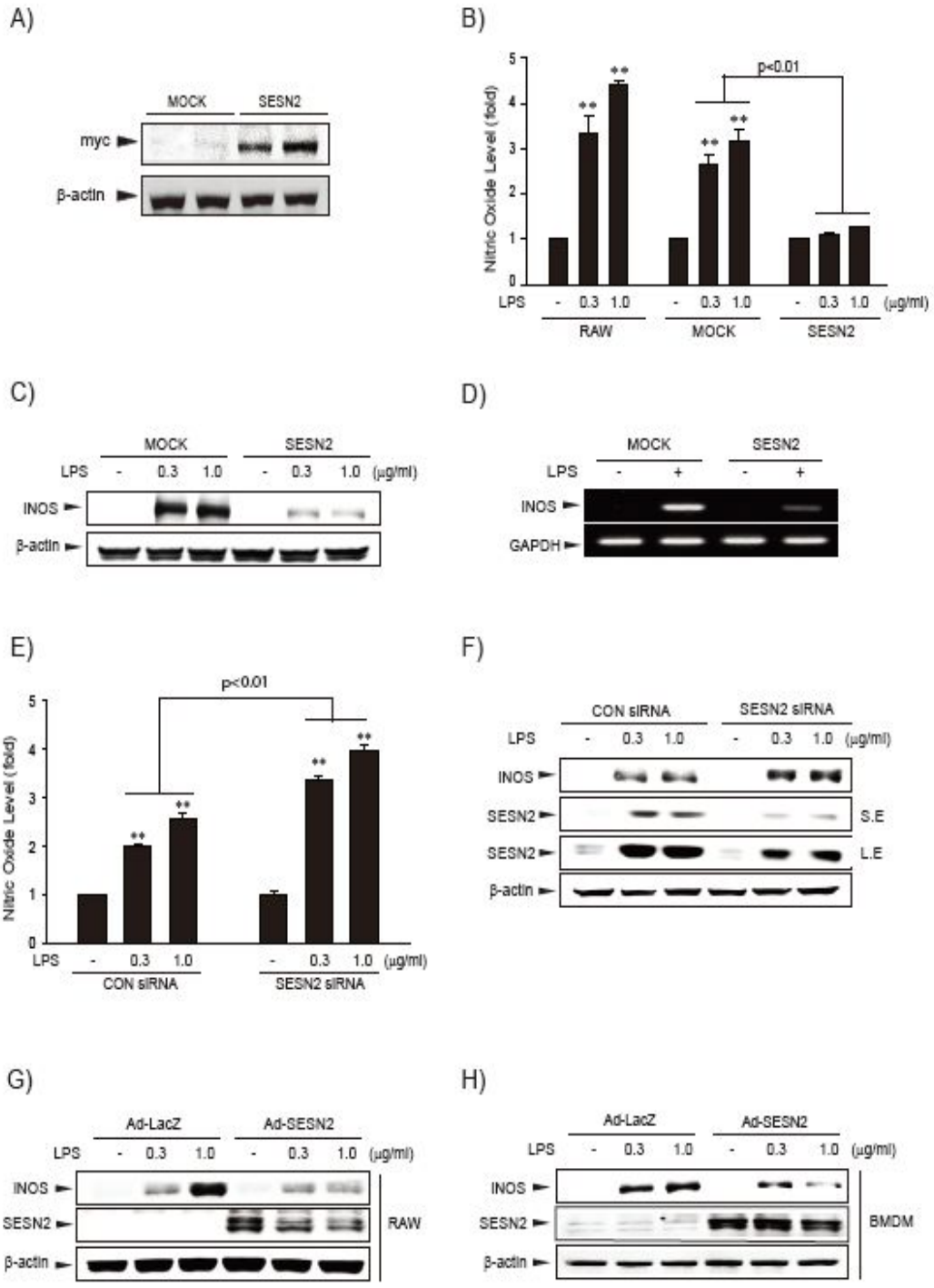


Figure 5. Inhibition of iNOS expression and NO production by Sesn2 in LPS-activated macrophages

A. Establishment of a stable cell line expressing Sesn2. Sesn2 expression was confirmed by immunoblotting in the cell lysates of pCMV-Tag3A-transfected RAW264.7 cells (MOCK) or pCMV-SESN2-transfected RAW264.7 cells (SESN2) with anti-c-myc or anti-Sesn2 antibodies. (S.E., short exposure; L.E., long exposure) *B*. NO production. NO concentrations in culture media treated with LPS (0.3 or 1 μg/ml) for 12 h were determined using Griess reagent. *C*. The effect of Sesn2 on LPS-induced iNOS expression. Cells were treated with 0.3 or 1 μg/ml LPS for 12 h. iNOS protein levels were determined by immunoblotting. *D*. RT-PCR analysis. Cells were treated with 1 μg/ml LPS for 3 h. iNOS transcript levels were analyzed by RT-PCR, using GAPDH as the internal control. *E*. Role of Sesn2 knockdown in LPS-stimulated NO production. NO concentrations in culture media treated with LPS (0.3 or 1 μg/ml) for 12 h were determined using Griess reagent. *F*. Effect of Sesn2 knockdown on iNOS induction by LPS. RAW264.7 cells were transfected with control (CON) siRNA or Sesn2 siRNA for 24 h, and then treated with LPS (0.3 or 1 μg/ml) for 12 h. S.E., short exposure; L.E., long exposure. *G*. Role of adenovirus Sesn2 (Ad-SESN2) on iNOS induction by LPS in RAW264.7 cells (RAW). *H*. Effect of Ad-Sesn2 on iNOS induction by LPS in bone marrow derived macrophages (BMDM). Results are presented as the means ± SDs of three replicates; **P<0.01 = significant versus vehicle-treated controls.

4. Inhibition of LPS-inducible Inflammatory Cytokines by Sesn2

Next, we verified the effect of Sesn2 on pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β. First we assessed the expression of TNF-α, IL-6, and IL-1β by Real-time RT-PCR analysis. LPS markedly induced TNF-α, IL-6, and IL-1β in MOCK-transfected cells, but not in RAW264.7 cells stably expressing Sesn2 (Fig. 6A). Next, release of cytokines into media was analyzed by ELISA in cells treated with LPS (0.3-1 μg/ml). Treatment of the MOCK-transfected cells with LPS substantially increased cytokine production, but as was observed for inflammatory gene expression, Sesn2 inhibited the release of cytokines by LPS (Fig. 6B).

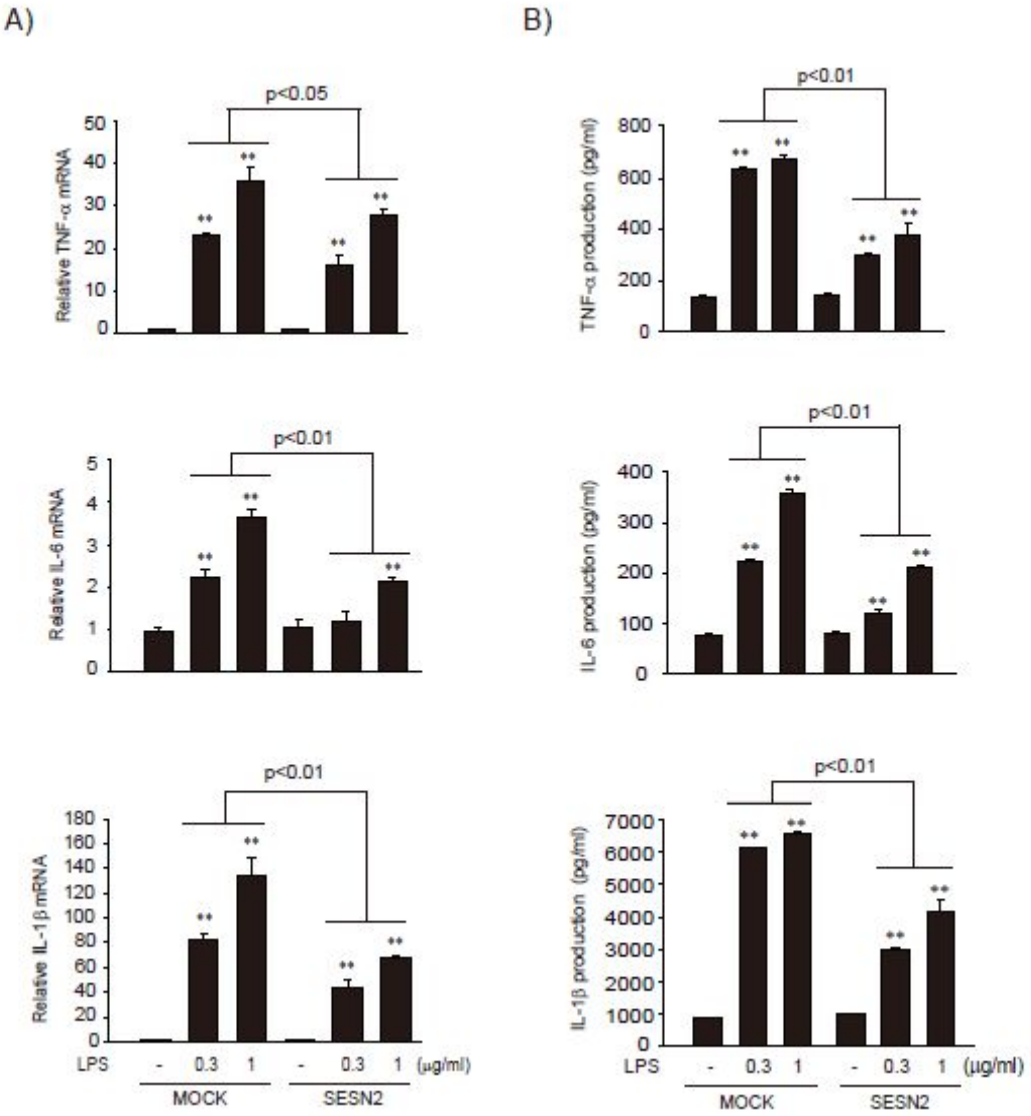


Figure 6. Inhibition of LPS-inducible inflammatory cytokines by Sesn2

A. RT-PCR analysis. The transcript levels of TNF-α, IL-6, and IL-1β were assessed by Real-time RT-PCR analysis. Cells were treated with 0.3 or 1 μg/ml LPS for 3 h. *B*. ELISA. TNF-α, IL-6, and IL-1β release into culture media was determined by ELISA. Results are presented as means ± SDs of three replicates; **P<0.01 = significant versus vehicle-treated controls.

5. Protective Role of Sesn2 on LPS-induced Oxidative Stress and Cell Death

ROS and reactive nitrogen species (RNS) production is stimulated by LPS in RAW 264.7 cells, and leads to cell death (Sanlioglu et al., 2001), and thus, we examined the effect of Sesn2 on the cytotoxicity elicited by LPS. Cell viability and plasma membrane permeability were assessed colorimetrically by MTT and by LDH release, respectively. Cells treated with LPS increased cell death in MOCK cells, whereas cells expressing Sesn2 significantly blocked cell death as measured by the MTT assay (Fig. 7A). Cytotoxicity was confirmed by quantifying the LDH released from the cytosol of damaged cells. MOCK cells treated with LPS significantly released LDH to medium, whereas Sesn2 transfected RAW264.7 cells did not (Fig. 7B). In addition, MOCK cells treated with LPS showed obvious increases in apoptosis markers, such as, PARP and caspase3, whereas Sesn2-transfected cells did not (Fig. 7C). Next, we examined whether Sesn2 affected LPS-induced ROS production. LPS increased ROS production, as shown by fluorescence microscopy or microplate reader, in MOCK cells, while Sesn2 almost completely prevented this increase in ROS (Fig. 8A upper and lower). To investigate whether the anti-oxidant effect of Sesn2 was caused by reduction of NADPH oxidase expression, the mRNA levels of NADPH oxidase components ($gp91^{phox}$, $p47^{phox}$, $p22^{phox}$) were examined by carrying out Real-time RT-PCR. LPS significantly induced the mRNA levels of $gp91^{phox}$, $p47^{phox}$, $p22^{phox}$ in MOCK-transfected cells, but not in RAW264.7 cells stably expressing Sesn2 (Fig. 8B). These results indicate that Sesn2 has a cytoprotective effect against LPS-induced ROS production and cell death through inhibition of NOX in macrophages.

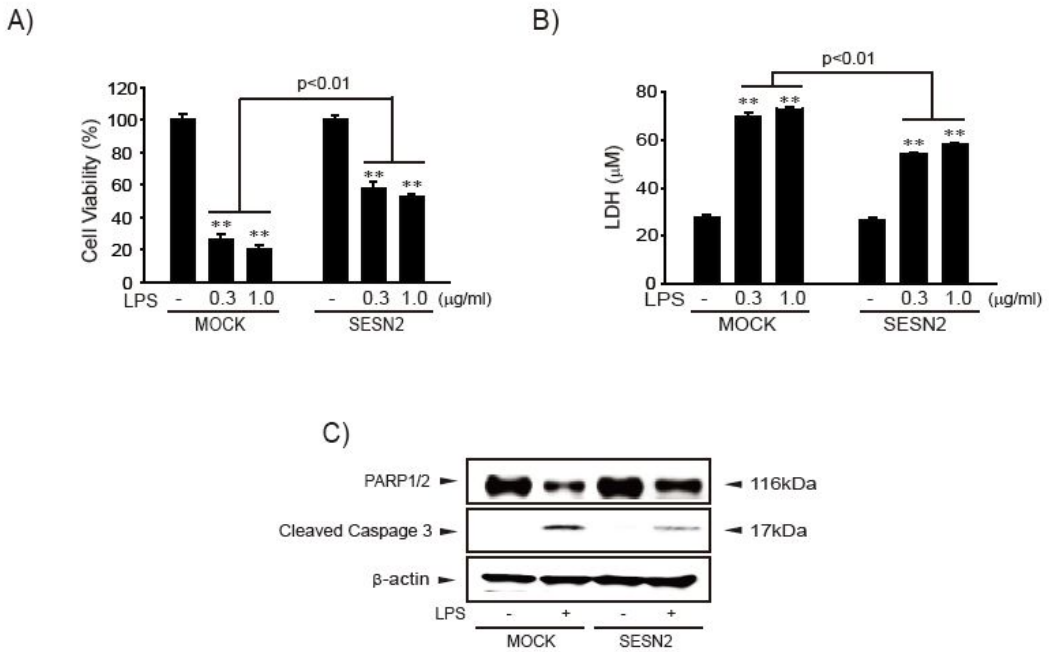


Figure 7. Protective role of Sesn2 on LPS-induced cell death

A. Effect of Sesn2 on LPS-induced cytotoxicity. Cells were treated with 0.3-1 μg/ml LPS for 24 h. Cell viabilities were assessed using an MTT assay. *B*. LDH assay. *C*. Sesn2 inhibited PARP1/2 reduction and caspase-3 cleavage by LPS. Cells were treated with 1 μg/ml LPS for 12 h. Precursor PARP and cleaved caspase-3 protein levels were immunoblotted in cell lysates. Results represent the means ± SDs of three replicates; * P <0.05, **P<0.01 = significant versus vehicle-treated controls.

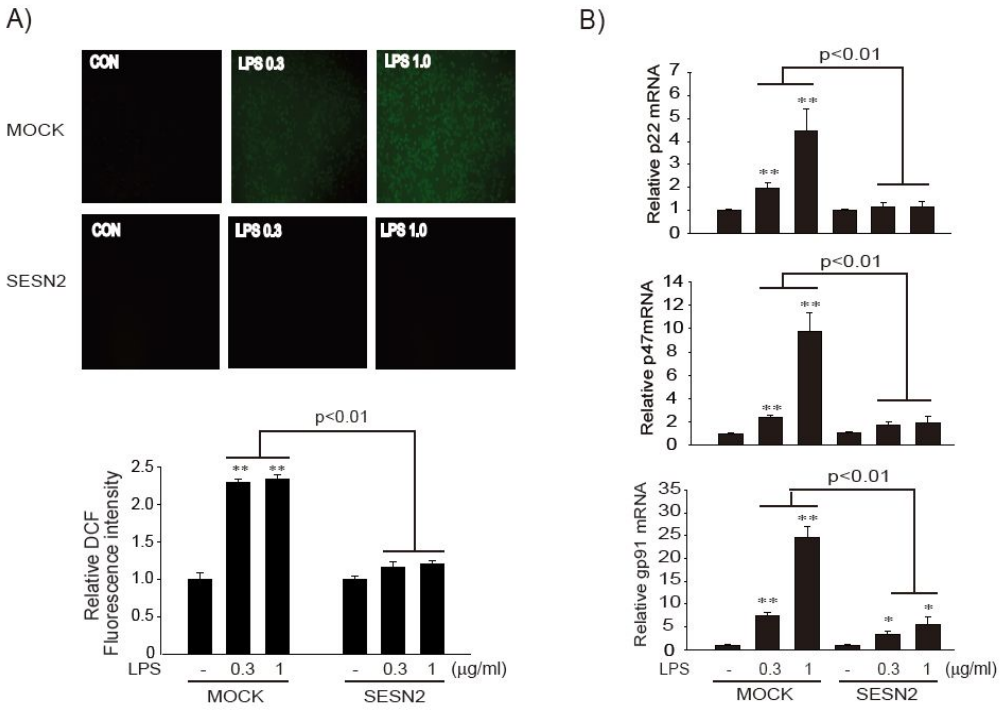


Figure 8. Protective role of Sesn2 on LPS-induced oxidative stress

A. The effect of Sesn2 on LPS-induced ROS production. Cells were stained with 10 μM DCFH-DA for 30 min at 37°C. H_2O_2 generation was determined by fluorescence microscopy (200X) (upper). Intracellular fluorescence intensities were measured using a fluorescence micro-plate reader (lower). *B*. The effect of Sesn2 on LPS-induced NOX expression. The transcript levels of $gp91^{phox}$, $p47^{phox}$, $p22^{phox}$ were assessed by Real-time RT-PCR analysis. Cells were treated with 0.3 or 1 μg/ml LPS for 3 h. Results represent the means ± SDs of three replicates; * P <0.05, **P<0.01 = significant versus vehicle-treated controls.

6. Specific Inhibition of LPS-inducible AP-1 activation by Sesn2

AP-1 and NF-κB are critical redox-sensitive transcription factors and interact with the upstream regions of inflammatory genes (de Vera et al., 1996). Both are activated in cells treated with LPS or subjected to other inflammatory stimuli and then regulate the transcriptional activations of inflammatory genes. To determine whether iNOS induction by LPS is accompanied by the activations of transcription factors, we first carried out gel shift assays. Treatment of MOCK cells with LPS resulted in increases in the band intensities of AP-1 DNA binding (Fig. 9A upper). However, Sesn2 expression completely abolished the formation of AP-1 DNA complex. Interestingly, the band intensity of NF-κB DNA binding complex by LPS was unaffected by Sesn2 expression (Fig. 9A lower). Immunocompetition assays using anti-c-Jun or anti-p65 antibody confirmed the specificity of AP-1 and NF-κB DNA binding (data not shown).

To confirm the role of Sesn2 in the activations of AP-1 and NF-κB, we carried out reporter gene analysis. LPS caused an increase in AP-1 luciferase activity in MOCK cells (Fig. 9B upper), whereas Sesn2 completely inhibited the ability of LPS to induce luciferase expression. In-line with our gel shift assay results, Sesn2 did not inhibit LPS-induced NF-κB luciferase activities (Fig. 9B lower) or IκB degradation (Fig. 9C). These data indicate Sesn2 specifically regulates AP-1 activation, but not NF-κB, in response to LPS, and that it inhibits inflammatory gene expression.

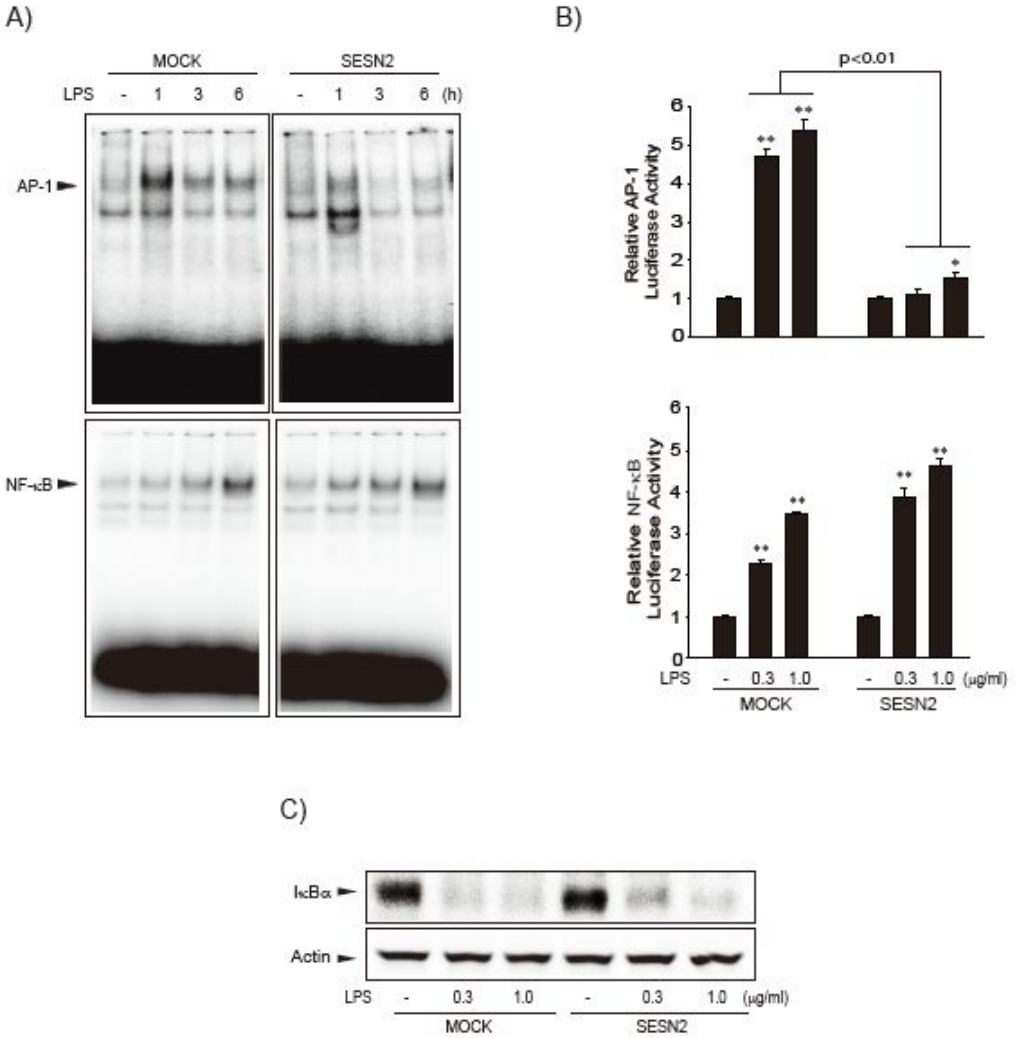


Figure 9. Specific inhibition of LPS-inducible AP-1 activation by Sesn2

A. EMSA. Cells were incubated with 1 μg/ml LPS for 1, 3 and 6 h, and nuclear extracts (protein content 8 μg) were incubated with a consensus AP-1 and NF-κB oligonucleotide both end labeled with [γ-^{32}P]ATP. Results were confirmed by repeating experiments. *B*. Inhibition of AP-1 luciferase activity by Sesn2. Cells were transfected with an AP-1 or NF-κB luciferase construct. Transfected cells were treated with LPS (0.3 or 1 μg/ml) for 12 h. *C*. The effect of Sesn2 on IκB degradation by LPS. Immunoblotting for IκB degradation. Cells were treated with LPS (0.3 and 1 μg/ml) for 30 min. Total IκB levels were determined by immunoblotting cell lysates. Results were confirmed by repeating experiments. Results represent the means ± SDs of three replicates; *P<0.05, **P<0.01 = significant versus vehicle-treated controls.

7. Role of Sesn2 in JNK or p38 dependent c-Jun Phosphorylation

AP-1 is a combination of Jun, Fos or activating transcription factor (ATF) and primarily acts as a heterodimer of Jun and Fos (Barnes & Karin, 1997). To investigate the inhibitory role of Sesn2 on LPS-induced AP-1 activation, we performed immunoblot analysis. LPS increased the phosphorylation of c-Jun in the lysates of MOCK cells but not in those of Sesn2 transfected cells, whereas LPS-induced c-Fos phosphorylation was unaffected by Sesn2 expression, indicating that Sesn2 specifically regulates c-Jun phosphorylation in response to LPS (Fig. 10A).

It has been well established that c-Jun activities are regulated by MAPKs, and primarily by JNK (Morton, Davis, McLaren, & Cohen, 2003). To determine which MAPK is a key player in the Sesn2-mediated inhibition of c-Jun phosphorylation, we examined the phosphorylations of extracellular-regulated protein kinases 1 and 2 (ERK1/2), p38 MAPK, and JNK. The phosphorylation of ERK1/2, p38, and JNK were dramatically increased in response to LPS in naïve control cells, whereas JNK phosphorylation, was markedly suppressed in Sesn2 transfected cells (Fig. 10B). Moreover, phosphorylation of p38 was also suppressed by the Sesn2 expression, albeit to a lesser extent than JNK (Fig 10B and C). These data suggest that the inhibitory effect of Sesn2 on AP-1 activation involves the selective JNK or p38 inhibition.

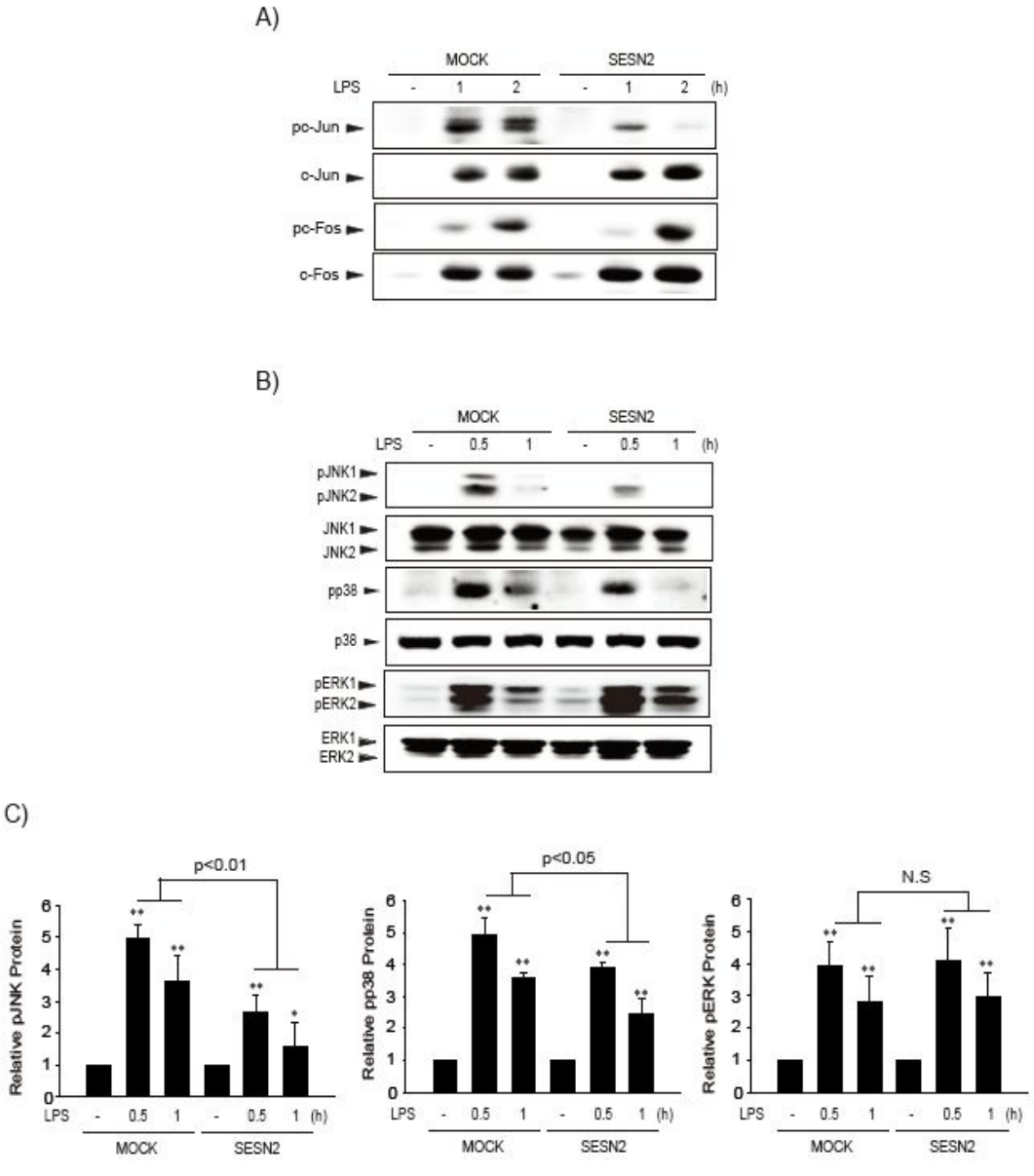


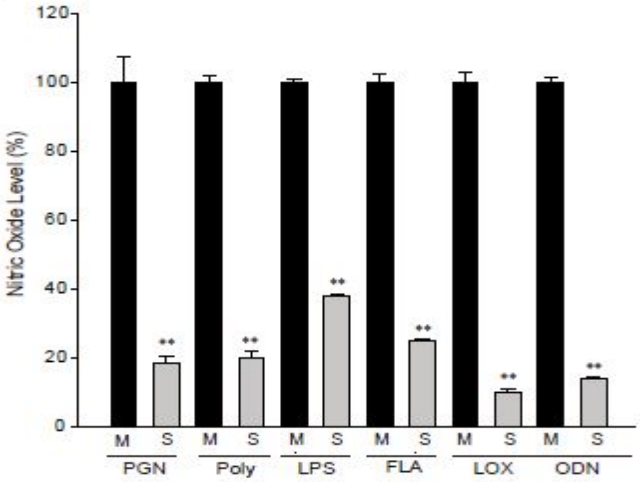
Figure 10. Effect of Sesn2 in JNK dependent c-Jun phosphorylation

A. Effect of Sesn2 on LPS-induced c-Jun phosphorylation in RAW264.7 cells. Cells were treated with LPS (1 μg/ml) for 1 or 2 h, and cell lysates were immunoblotted for examining c-Jun and c-Fos phosphorylation. Results were confirmed by repeated experiments. *B*. Effect of Sesn2 on LPS-induced phosphorylations of MAPKs. Cells were treated with LPS (1 μg/ml) for 30 min or 1 h, and cell lysates were immunoblotted. Results were confirmed by repeated experiments. *C*. Densitometric quantification of western blots shown in B. Results represent the means ± SDs of three replicates; *P<0.05, **P<0.01 = significant versus vehicle-treated controls.

8. Role of Sesn2 in TLR Ligands Induced-NO Production

Additional experiments were performed to investigate the effect of Sesn2 on NO production and iNOS expression activated by other TLRs as well as TLR4. Treatment of naïve cells with TLRLs, that is, peptidoglycan (TLR2 ligand), poly (I:C) (TLR3 ligand), LPS (TLR4 ligand), flagellin (TLR5 ligand), loxoribine (TLR7/8 ligand), or ODN (TLR9 ligand), all showed elevated NO production and iNOS expression, however Sesn2 transfected cells showed no increase in NO production and iNOS expression (Fig. 11A and B). Our results suggest that Sesn2 protects TLR ligand-induced proinflammatory gene expression.

A)



B)

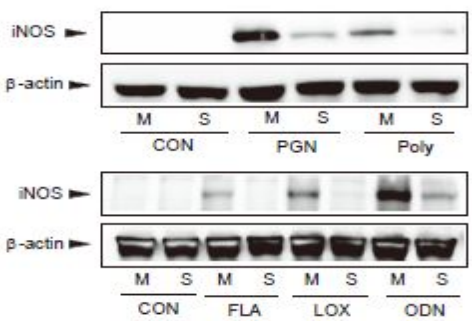


Figure 11. Effect of Sesn2 in TLR ligands Induced-NO production

A. NO production. Cells were treated with peptidoglycan (30 μg/ml), poly (I:C) (50 μg/ml), LPS (1 μg/ml), loxoribine (500 μM), FLA-ST (FLA, 1 μg/ml), or ODN 1826 (1 μM) for 12 h and NO levels were determined using Griess reagent. Results represent the means ± SDs of three replicates; **P<0.01 = significant versus vehicle-treated control. *B*. iNOS expression. Cells were treated with TLR ligands for 12 h and cell lysates were immunoblotted. Results were confirmed by repeated experiments. (Mock-M, SESN2-S)

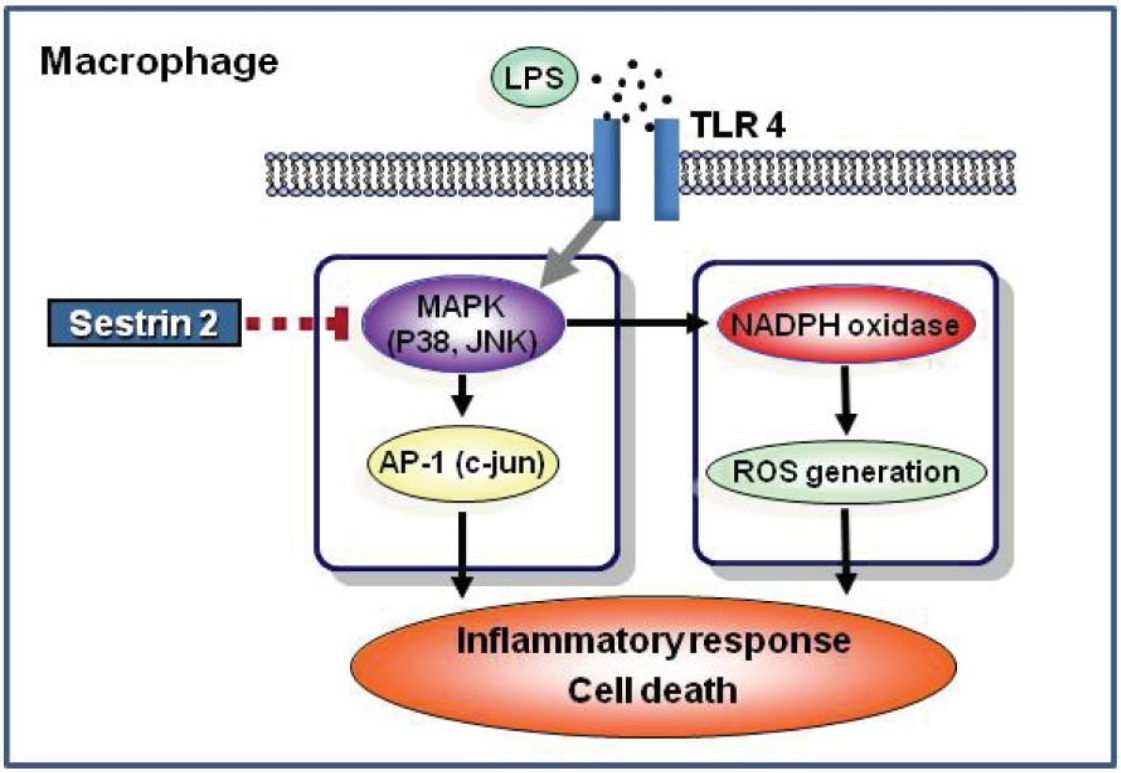


Figure 12. Role of Sestrin2 in the regulation of pro-inflammatory signaling

9. Discussion

Kupffer cells, resident macrophages in liver, produce and release ROS and RNS during phagocytosis and these species play important roles in inflammatory response as essential components of innate immune response against intracellular bacteria. However, excessive ROS accumulation in cells damages Kupffer cells and adjacent tissues, and these contribute to the pathogeneses of hepatic inflammatory diseases. Cells are protected from ROS by a sophisticated antioxidant defense system. Oxidative stress can also cause the apoptosis of macrophages, although the molecular mechanisms involved have not been well defined (Forman & Torres, 2002).

In the current study, we first found that Sesn2 protects endotoxin-induced acute fulminant hepatitis in mice using adenovirus expressing Sesn2 (Ad-Sesn2) (Fig. 1). Sesn2 inhibits liver injury induced by Gal/LPS and efficaciously suppressed inflammatory cytokines and mediators. Moreover, we identified the regulatory role of Sesn2 in pro-inflammatory and apoptosis signaling in TLRL-activated macrophages. Macrophages transfected with Sesn2 inhibited the effects of TLRs-induced inflammatory mediators and cytokines. These results were confirmed using Sens2 siRNA. Furthermore, Sesn2 prevented LPS-induced cell death by inhibiting ROS production (Fig. 7 and 8). Several reports have shown NADPH oxidase (NOX) complex is a major source of intracellular ROS generation in macrophages (Forman & Torres, 2002), and that NOX can be activated by TLRs (Kawai & Akira, 2007). The effect of LPS on macrophage cell death has been extensively studied and diverse molecular mechanisms are involved in its regulation. Especially, LPS-mediated TNF-α and NO production mainly contribute to macrophage cell death (Xaus et al., 2000). Moreover, LPS-induced ROS

production through activation of NADPH oxidase also results in apoptosis of macrophages (F. Jiang et al., 2011).We found here that LPS markedly induced the mRNA levels of $gp91^{phox}$, $p47^{phox}$, and $p22^{phox}$ in MOCK cells, but not in RAW264.7 cells stably expressing Sesn2 (Fig. 8B). These results indicate that Sesn2 inhibited LPS-induced cell death through inhibition of inflammatory mediators and/or ROS production.

The expressions of inflammatory genes are mainly regulated by transcription factors, such as, NF-κB and AP-1. Furthermore, NF-κB and AP-1 are essential transcription factors of many genes related to the regulation of inflammatory response (Aggarwal, Shishodia, Sandur, Pandey, & Sethi, 2006; Caamano & Hunter, 2002), and are regarded as redox-sensitive transcription factors (Surh, Kundu, Na, & Lee, 2005). AP-1 (dimeric basic region leucine zipper (bZIP) protein) is a transcription factor composed of homodimers or heterodimers of Jun, ATF, MAF, or Fos (Vesely, Staber, Hoefler, & Kenner, 2009). Of AP-1 family members, c-Jun is considered to play fundamental roles in inflammatory response. AP-1 activation is usually regulated by MAPKs. Especially, it is well known that the phosphorylation of c-Jun by JNK and p38 MAPK increases the transcriptional activities of AP-1 complexes. The JNK and p38 MAPK pathway is activated mainly by inflammatory cytokines and various environmental stresses (Keshet & Seger, 2010; Raingeaud et al., 1995). After activation, JNK and p38 MAPK regulates the expressions of proinflammatory genes via an array of transcription factors, such as, AP-1, ATF-2, and Smad (Gupta, Campbell, Derijard, & Davis, 1995; Hayes, Huang, Kambhampati, Platanias, & Bergan, 2003; Zarubin & Han, 2005; Zhang, Feng, & Derynck, 1998). In the current study, we found that JNK phosphorylation was almost completely blocked in Sesn2 expressing macrophages (Fig. 10B). Furthermore, phosphorylation of p38 MAPK was also suppressed by the Sesn2 expression, albeit to a lesser extent than JNK (Fig

10B and C). Reduced JNK or p38 MAPK activation by Sesn2 resulted in reduced c-Jun phosphorylation, whereas the phosphorylation of c-Fos was unaffected by Sesn2 (Fig. 10A). However, Sesn2 failed to inhibit LPS-induced NF-κB activation (Fig. 9), showing that selective inhibition of the MAPK/c-Jun pathway by Sesn2 is responsible for the suppressions of proinflammatory genes.

TLRs are expressed in macrophages, and play important roles in macrophage activation and defense against pathogens. In mammalian cells, TLR4 was first cloned as a homologue of dToll, to which TLR4 ligand (LPS) binds (Medzhitov, Preston-Hurlburt, & Janeway, 1997). Subsequently, other human TLRs were identified and characterized. Certain TLR ligands, such as, peptidoglycan, double-stranded RNA, flagellin, loxoribine, and bacterial CpG DNA, activate TLR2/6, TLR3, TLR5, TLR7/8, and TLR9, respectively (Barton & Medzhitov, 2003). TLR-ligand binding causes the activation of signal transduction pathways through the central adaptor proteins MyD88 and/or TRIF, and results in the downstream activations of NF-κB and AP-1. In the present study, all TLRLs induced NO production and iNOS expression in treatment naïve macrophages, and Sesn2 expression almost completely prevented LPS-induced inflammatory responses (Fig 11).

In summary, the study shows that Sesn2 antagonizes TLRs-mediated pro-inflammatory signaling and cell death *in vivo* and *in vitro*, and that Sesn2 specifically inhibits the TLRL-induced JNK, p38 MAPK and AP-1 pathway (Fig. 12). The results of this study provide insight of the roles of Sesn2 in innate immunity and inflammatory responses.

Part Ⅱ: Sestrin2 protects against CCl4/BDL-induced Hepatic Fibrosis via Down-regulation of TGF-β/Smad signaling

1. Induction of *Sesn2* gene expression in activated HSC

First, *Sesn2* gene expression during HSC activation in primary cultured HSC. Quiescent HSCs on day 0 exhibited the weak expression of Sesn2. After cultivation, the expression level of Sesn2 markedly increased, whereas the levels of α-SMA, the transdifferentiation marker, increased (Fig 13A). RT-PCR was carried out to determine whether the induction of *Sesn2* during HSC activation was due to increased transcription. The results showed that *Sesn2* mRNA levels were significantly increased by HSC activation (Fig. 13B). Treatment of LX-2 cells (immortalized HSC line) with TGF-β up-regulated Sesn2 expression in a dose-dependent manner and expression level peaked at 2-5 ng/ml of TGF-β (Fig 13C). We determined the time course of Sesn2 expression in response to TGF-β 2 ng/ml) and it was increased after 1-12 h of TGF-β treatment (Fig 13D).

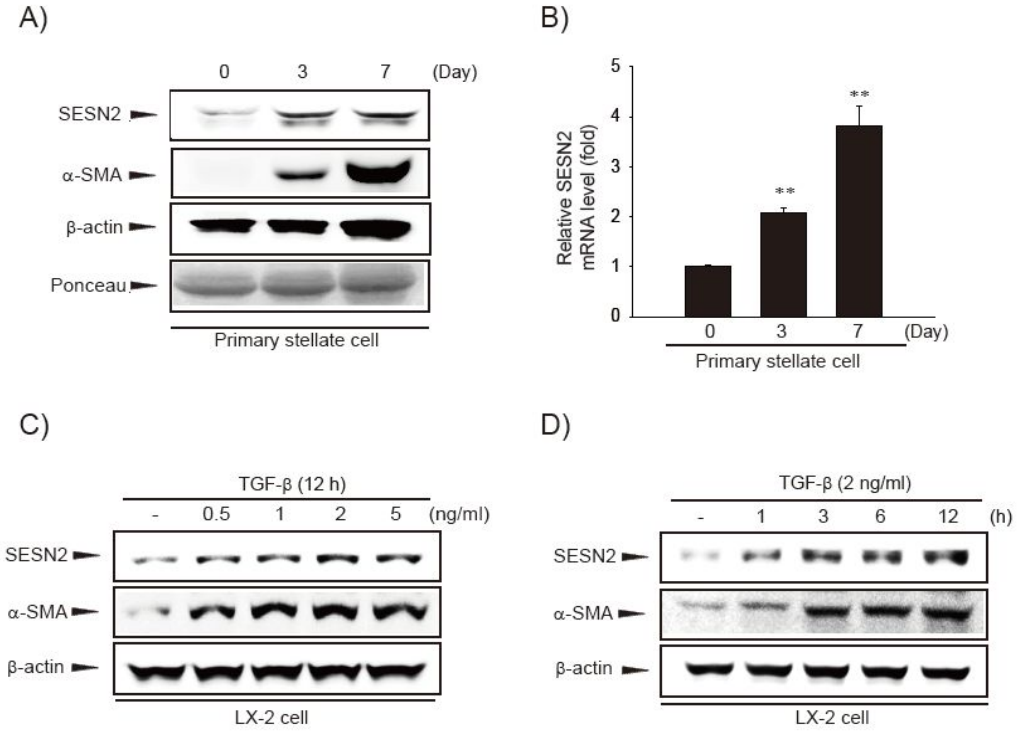
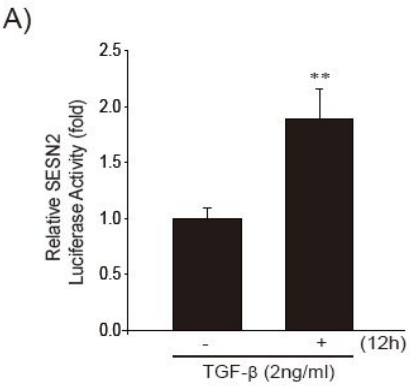


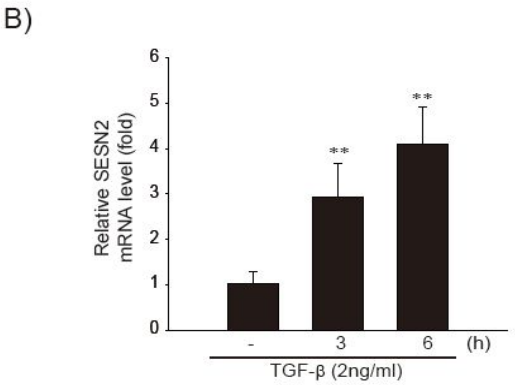
Figure 13. Sestrin2 (Sesn2) up-regulation during HSC activation

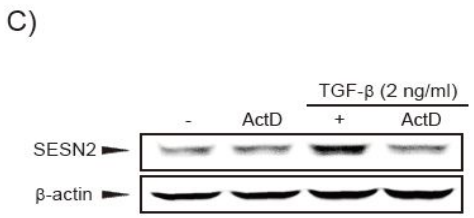
A. Sesn2 induction in HSCs. Primary HSCs were cultured in a growth medium for 3 or 7 days, and the cell lysates (20 μg each) were subjected to immunoblotting. The expression levels of Sesn2 were determined in the lysates of primary quiescent or activated HSC. The expression of HSC activation marker α-SMA and loading control β-actin was determined by Western blot analysis as indicated. Ponceau-S (Ponceau) staining of the Western blots was also used as the loading control. *B*. Real-time PCR assays. The data are the means and standard errors of at least three separate experiments (significantly different versus day 0: **P < 0.01). *C*. The effect of TGF-β treatment on Sesn2 induction. LX-2 cells were treated with 0.5-5 ng/ml TGF-β for 12 h. Sesn2 protein levels in cell lysates were measured by immunoblotting. *D*. Sesn2 expression was determined in cells treated with TGF-β (2 ng/ml) for 1-12 h.

2. Transcriptional Regulation of *Sesn2* gene Expression in Activated HSC

Quantitative Real-time RT-PCR analyses showed clear upregulation of Sesn2 transcription in TGF-β treated HSC. Sesn2 reporter gene assays were performed in HSC transfected with a construct containing the *Sesn2* promoter region from -1129 to +192 bp. Exposure of the transfected cells to TGF-β significantly increased luciferase activity of pGL4-phSESN2 (Fig. 14A). LX-2 cells were preincubated with the transcription inhibitor actinomycin-D (ActD) for 30 min before adding the TGF-β or vehicle, and then the levels of Sesn2 protein and mRNA were measured. Pretreatment of Actinomycin-D completely blocked the increase in Sesn2 protein and mRNA levels induced by TGF-β (Fig. 14C). These data suggest that the up-regulated Sesn2 by TGF-β was due to increased transcription.







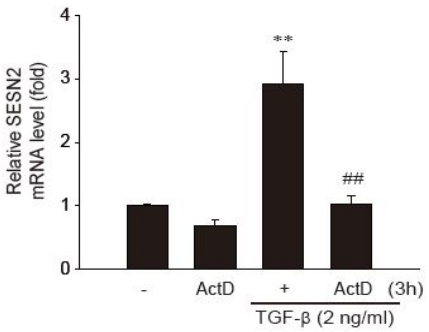


Figure 14. Transcriptional regulation of Sesn2 expression in HSC

A. Increase in Sesn2 transactivation by TGF-β Sesn2 luciferase assays were performed on the lysates of cells exposed 2 ng/ml of TGF-β in LX-2 cells. *B*. Real-time RT-PCR analysis. LX-2 cells were treated with TGF-β for 3-6 h. The transcripts of Sesn2 were analyzed by real-time RT-PCR assays, with the mRNA level of GAPDH used as a normalizing reference. *C*. The effect of actinomycin D (ActD) on the Sesn2 induction by TGF-β in LX-2 cells. The cells were treated with ActD in the presence and absence of TGF-β. The relative level of Sesn2 protein (left) and mRNA (right) were monitored after 12 h and 3 h TGF-β treatment, respectively. Data represent the mean ± SD of three separate experiments; the statistical significance of differences between each treatment group and the control (**P < 0.01); significant as compared with ActD alone, ##p < 0.01).

3. Role of Smad Activation on the Sesn2 up-regulation by TGF-β

TGF-β plays a variety of cellular functions mainly through its downstream signaling Smad pathway (Felici et al., 2003). To verify the functional role of Smad in TGF-β-mediated *Sesn2* induction, we first transfected cells with a plasmid that expresses Smad3 and a luciferase reporter gene. Expression of Smad3 increased the levels of Sesn2 luciferase activity (Fig. 15A). In the positive control, Smad3 expression enhanced smad binding element (SBE)-dependent luciferase activity (SBE-Luc). To identify putative SBEs in the *Sesn2* promoter, we examined the human *Sesn2* genomic locus in detail. The SBE [5'- CAGACA-3'] is a cis-acting element governing the regulation of many fibrogenic genes (Chen, Yuan, Lo, Trojanowska, & Varga, 2000), and detailed *in silico* analysis of the human *Sesn2* genomic locus revealed one potential SBE sequence in the human *Sesn2* promoter. This SBE sequence is located in the proximal promoter region, from approximately −964 to −956 bp. To examine the functional role of SBE in Sesn2 gene induction, we deleted the putative SBE in the *Sesn2* promoter. A specific disruption of the SBE in the promoter region of the *Sesn2* gene significantly decreased the ability of TGF-β or Smad3 over-expression to increase luciferase-reporter activity (Fig. 15B). These observations indicate that SBE in *Sesn2* promoter is functional and that it is involved in Smad-dependent regulation of the Sesn2.

In addition to Smad-transduced signals, TGF-β may activate other signaling pathways including the MAPK members JNK and p38 (Moustakas & Heldin, 2005). Interestingly, experimental evidence has demonstrated that JNK and p38 may in turn enhance the transcriptional Smad proteins activities by direct Smad3 phosphorylation or indirectly by

promoting Smad3 association with the transcriptional co-activator p300 (Moustakas & Heldin, 2005). Therefore, to explore whether MAPK are involved in TGF-β-mediated Sesn2 induction, we examined the effects of MAPK inhibitor on TGF-β-mediated Sesn2 induction. TGF-β-induced Sesn2 protein expression was significantly decreased by p38 inhibitor (Fig. 16A). These results suggest that Sesn2 induction is in part regulated by P38 in the HSC stimulated with TGF-β.

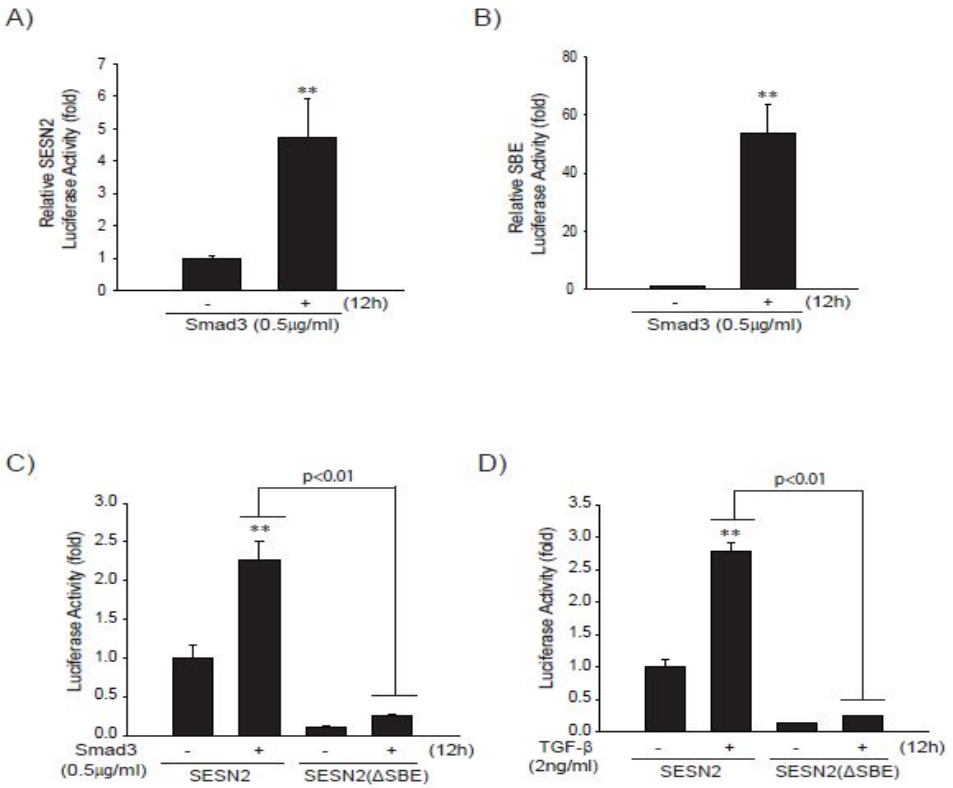
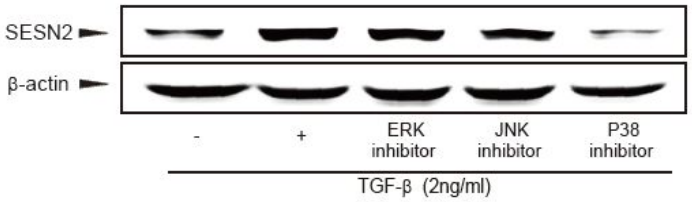


Figure 15. Smad-dependent stimulation of *Sesn2* gene expression in HSC

A and B. Smad-dependent regulation of Sesn2 expression. Sesn2-luciferase activation was determined in the lysates of LX-2 cells transfected with a Smad3 expression construct and Sesn2 (pGL4-phSESN2) (A) or Smad-dependent luciferase (SBE-Luc) reporter for 24 h (B). *C and D*. Effects of deletion mutation of putative SBE on the induction of luciferase activity. LX-2 cells were transfected with a Smad3 expression construct and pGL4-phSESN2or pGL3-phSESN2-ΔSBE (C). LX-2 cells were transfected with pGL4-phSESN2or pGL3-phSESN2-ΔSBE. Then, dual luciferase reporter assays were performed on the lysates of cells exposed to 2 ng/ml of TGF-β (D). Data represent the mean ± SD of four separate experiments; the statistical significance of differences between each treatment group and the control (**P < 0.01).

A)



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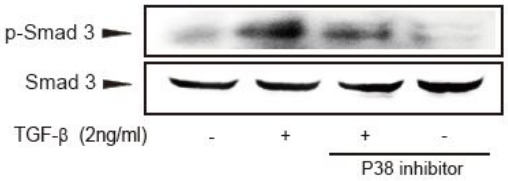
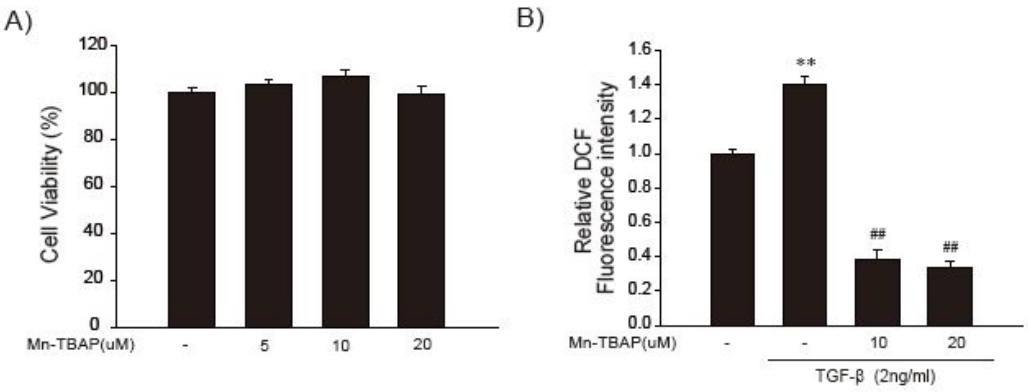


Fig.16. Association of MAPK with TGF-β-induced Sesn2 expression

A. LX-2 cells were exposed to TGF-β (2 ng/ml) for 12 h after preincubated in the absence or presence of 10μM ERK inhibitor, 10 μM JNK inhibitor or 10μM p38 inhibitor, and then the expression level of Sesn2 protein was analyzed. *B*.LX-2 cells were preincubated in the absence or presence of 10μM p38 inhibitor for 30 min and then were treated with 2 ng/ml of TGF-β for 30min. And the expression level of Smad3 phospholyation protein was analyzed. The result is representative of three independent experiments.

4. Redox regulation of TGF-β-mediated Sesn2 induction

TGF-β increases reactive oxygen species (ROS) production in HSC. This increased level of ROS, in turn, leads to increased production of ECM proteins (Barcellos-Hoff & Dix, 1996; Leonarduzzi et al., 1997). The intracellular ROS level was increased following TGF-β stimulation of HSC, but such ROS production diminished in the cells that had been pretreated with antioxidant Mn-TBAP (Fig. 17B). To explore whether ROS are involved in TGF-β-mediated Sesn2 induction, we examined the effects of antioxidants Mn-TBAP on TGF-β-mediated Sesn2 induction. TGF-β-induced Sesn2 protein expression was significantly decreased by Mn-TBAP (Fig. 17C). These results suggest that Sesn2 induction is in part regulated by ROS produced in the HSC stimulated with TGF-β.



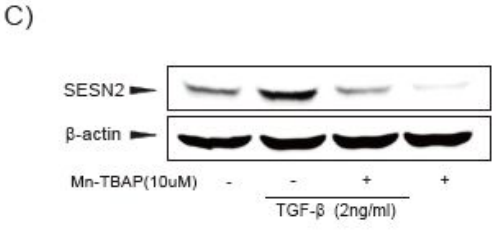


Figure 17. Involvement of ROS in TGF-β-mediated Sesn2 induction

A. MTT assay. LX-2 cells were preincubated in the absence or presence of 5-20 μM Mn-TBAP for 30 min and then were treated with 2 ng/ml of TGF-β for 24h. *B*. LX-2 cells were preincubated in the absence or presence of 10 or 20 μM Mn-TBAP for 30 min. After treated with 2 ng/ml of TGF-β for 24h, cells were stained with 10 μM DCFH-DA for 30 min at 37°C. H_2O_2 generation was determined by using a fluorescence microplate reader. Results represent the means ± SDs of three replicates; **P<0.01 = significant versus vehicle-treated controls. ##P < 0.01, significant versus TGF-β alone. *C*. LX-2 cells were exposed to TGF-β (2 ng/ml) for 12 h after pretreatment with 10μM Mn-TBAP or alone, and then the expression level of Sesn2 protein was analyzed. The result is representative of three independent experiments.

5. Inhibitory Role of Sesn2 on fibrogenic gene expression

Next, we explored the role of Sesn2 on TGF-β-induced fibrogenic gene expression. The exposure of LX-2 cells to TGF-β increased Plasminogen activator inhibitor-1 (PAI-1) and α-SMA in the MOCK transfected cells, and this was abolished by forced Sesn2 overexpression (Fig. 18A). Similarly, TGF-β-inducible mRNA level of PAI-1 and α-SMA was also reduced by Sesn2 in LX-2 cells (Fig. 18C). TGF-β was considered to be the most stimulus to epithelial to mesenchymal transition (EMT) and its molecular mechanism involved the process of EMT (Zeisberg et al., 2007). Moreover, forced expression of Sesn2 attenuated TGF-β-induced EMT markers including Slug, Snail, and N-cadherin. (Fig. 18B)

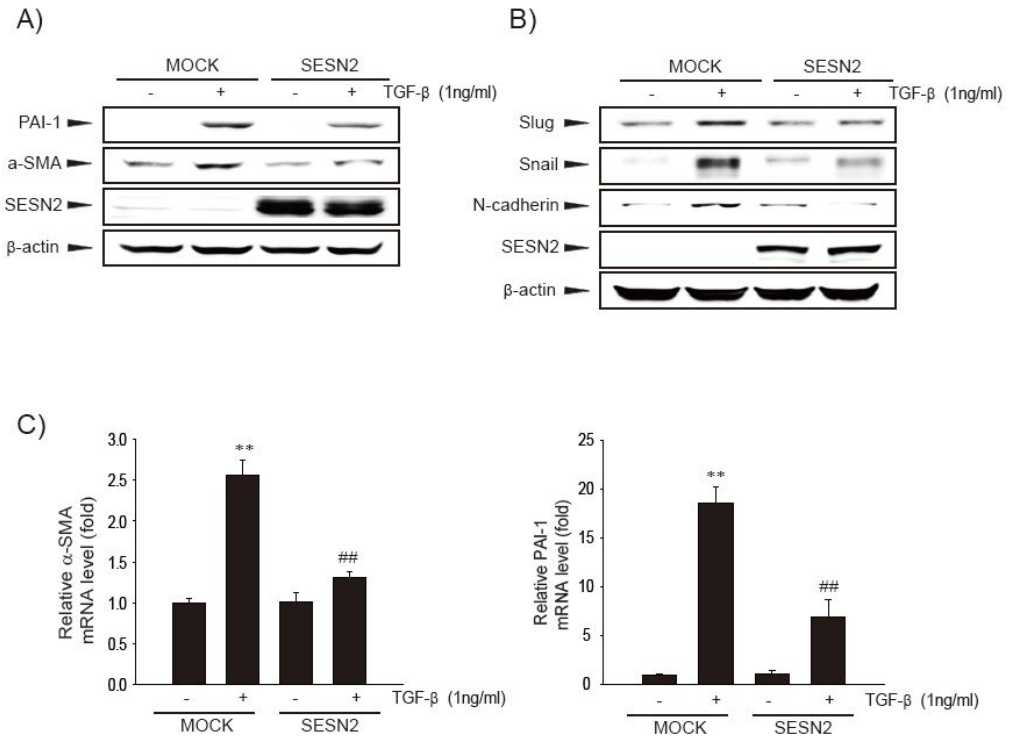


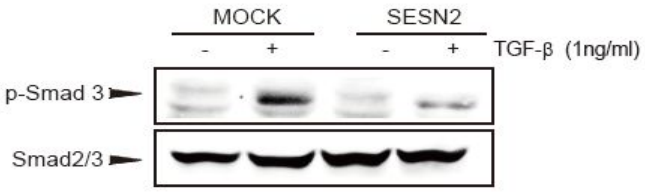
Figure 18. Inhibitory role of Sesn2 on fibrogenic gene expression

A. The effect of Sesn2 on TGF-β-mediated fibrogenic gene expression. LX-2 cells were transfected with pCMV-Tag3A (MOCK) or pCMV-SESN2 (SESN2) for 24 h, and then treated with TGF-β (1 ng/ml) for 12 h. PAI-1 and α-SMA protein levels were determined by immunoblotting. Sesn2 expression was confirmed by immunoblotting cell lysates of MOCK or SESN2 with anti-Sesn2 antibodies. *B*. The effect of Sesn2 on TGF-β-mediated EMT-related gene expression. Cells were treated as described in the legend *A*. EMT marker protein (e.g., Slug, Snail, or N-cadherin) levels were determined by immunoblotting. *C*. RT-PCR analysis. LX-2 cells were transfected as described in the legend *A* and treated with TGF-β (1 ng/ml) for 6h. α-SMA and PAI-1 transcript levels were analyzed by RT-PCR, using GAPDH as the internal control. Results represent the means ± SDs of three replicates; **P<0.01 = significant versus vehicle-treated controls. ##P < 0.01, significant versus MOCK-TGF-β alone.

6. Inhibitory Role of Sesn2 on TGF-β-mediated Smad Activation

To address the downstream link between Sesn2 and TGFβ1 signaling, we assessed the inhibitory effect of Sesn2 on TGF-β-dependent Smad phosphorylation. The treatment of mock-transfected LX-2 cells with TGF-β enhanced Smad phosphorylation. However, Sesn2 overexpression attenuated the phosphorylation of Smad3 (Fig.19A). As we expected, Sesn2 overexpression inhibited the ability of Smad3 to induce luciferase activity from an SBE-driven reporter (Fig.19B). Our results suggest that Sesn2 inhibits Smad phosphorylation and thus antagonizes Smad-dependent gene transcription.

A)



B)

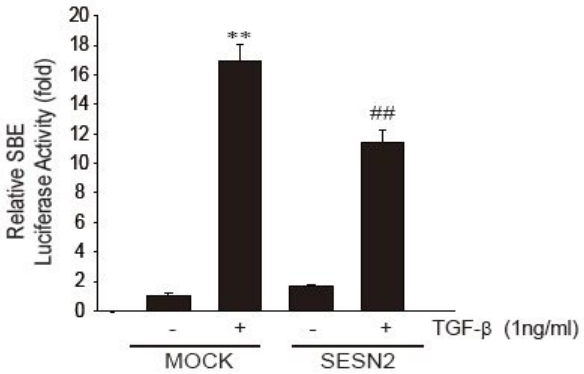


Figure 19. Inhibition of TGF-β-downstream signaling Smad by Sesn2

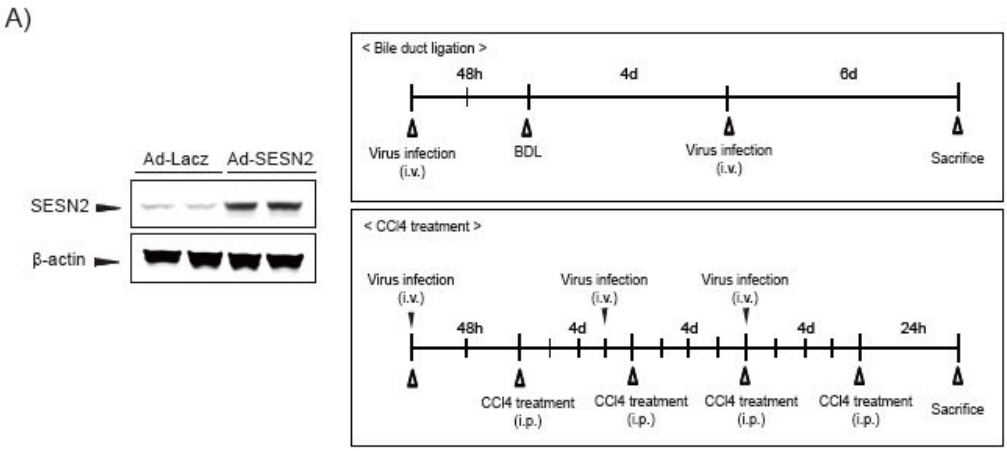
A. Cells were transfeced as described in the legend *A* and treated with TGF-β (1 ng/ml) for 30min, and cell lysates were immunoblotted for examining Smad3 phosphorylation. Results were confirmed by repeated experiments. *B,* Inhibition of Smad activation by Sesn2. Cells were transfected with a pGL-SBE luciferase construct. Transfected cells were treated with TGF-β (1 ng/ml) for 12 h. Results represent the means ± SDs of three replicates; **P<0.01 = significant versus vehicle-treated controls. ##P < 0.01, significant versus MOCK-TGF-β alone.

7. Inhibition of BDL- or CCl4-induced Hepatic Fibrosis by Ad-Sesn2

The most common methods used for inducing liver fibrosis in the experimental model were administration of carbon tetrachloride (CCl4) and bile duct ligation (BDL). To investigate the effect of Sens2 on hepatic fibrogenesis or fibrosis, we generated a recombinant adenovirus expressing LacZ (Ad-LacZ) or Sesn2 (Ad-Sesn2), which cause robust exogenous gene expression in liver (Fig 20A). Serum ALT and AST levels were significantly increased CCl_4-treated or BDL in Ad-LacZ infected mice. However, elevated levels of ALT and AST were markedly decreased by Ad-Sesn2 (Fig 20B).

To determine the hepatoprotective effects of Sesn2 against CCl_4 or BDL, we carried out histological examination of the extent of liver damage. First time, histopathological changes indicated the BDL-induced subacute cholestatic damages – focal hepatocellular necrosis, inflammatory cell infiltrations, bile duct hyperplasia and focal fibrosis were detected in the both BDL treated mice; Ad-LacZ-BDL and Ad-Sesn2-BDL at histopathological observation as compared with those of control mice, respectively. These are reconfirmed by histomorphometrical analysis; significant ($p<0.01$) increases percentages of degenerative regions, mean necrotic hepatocyte and infiltrated inflammatory cell numbers, bile duct and collagen fiber occupied regions were detected in Ad-LacZ-BDL and Ad-Sesn2-BDL groups as compared with those of each same adenovirus treated control mice, respectively. However, Ad-Sesn2-BDL treated mice showed significantly ($p<0.01$) lowered BDL-induced cholestatic subacute liver damages as compared with those of Ad-LacZ-BDL treated mice, in this experiment (Fig. 21 and 22). Secondly, Histopathological changes indicated the CCl_4-induced subacute liver damages – centrolobular necrosis including ballooning of hepatocytes, deposit

of lipid droplets in hepatocytes (fatty changed cells) and infiltration of inflammatory cells were detected in the both CCl_4 treated mice; Ad-LacZ infected mice and Ad-Sesn2 infected mice at histopathological observation with focal obvious centrolobular fibrosis in the lateral lobes, as compared with those of control mice, respectively. These are reconfirmed by histomorphometrical analysis; significant ($p<0.01$) increases percentages of degenerative regions, mean degenerative hepatocyte and infiltrated inflammatory cell numbers, collagen fiber occupied regions were detected in Ad-LacZ- CCl_4 treated mice and Ad-Sesn2- CCl_4 treated mice groups as compared with those of each same adenovirus treated control mice, respectively. However, Ad-Sestrin2-CCl_4 treated mice showed significantly ($p<0.01$) lowered CCl_4-induced subacute liver damages as compared with those of Ad-LacZ-CCl_4 treated mice, in this experiment (Fig. 21and 22).



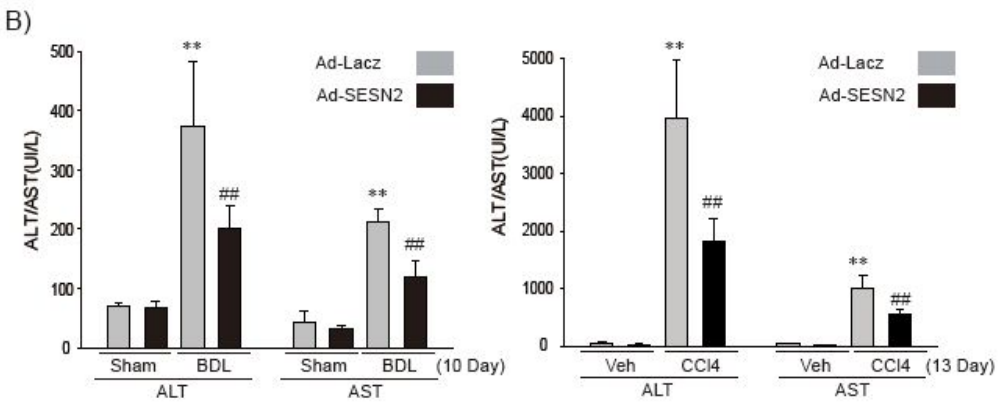


Figure 20. Protection of Ad-Sesn2 on BDL- or CCl4-induced hepatic fibrosis

A. Treatment schedule for CCl_4- or BDL- induced fibrosis. Sesn2 expression was confirmed by immunoblotting in liver homogenates of infected with adenovirus LacZ (Ad-Lac) or Sesn2 (Ad-SESN2). *B*. The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed by using an automated blood chemistry analyzer. All values were expressed as mean ± SD of 5 mice serum (significant as compared with vehicle control, **$p<0.01$; significant as compared with Ad-Lacz-CCl_4 or BDL, ##$p < 0.01$.

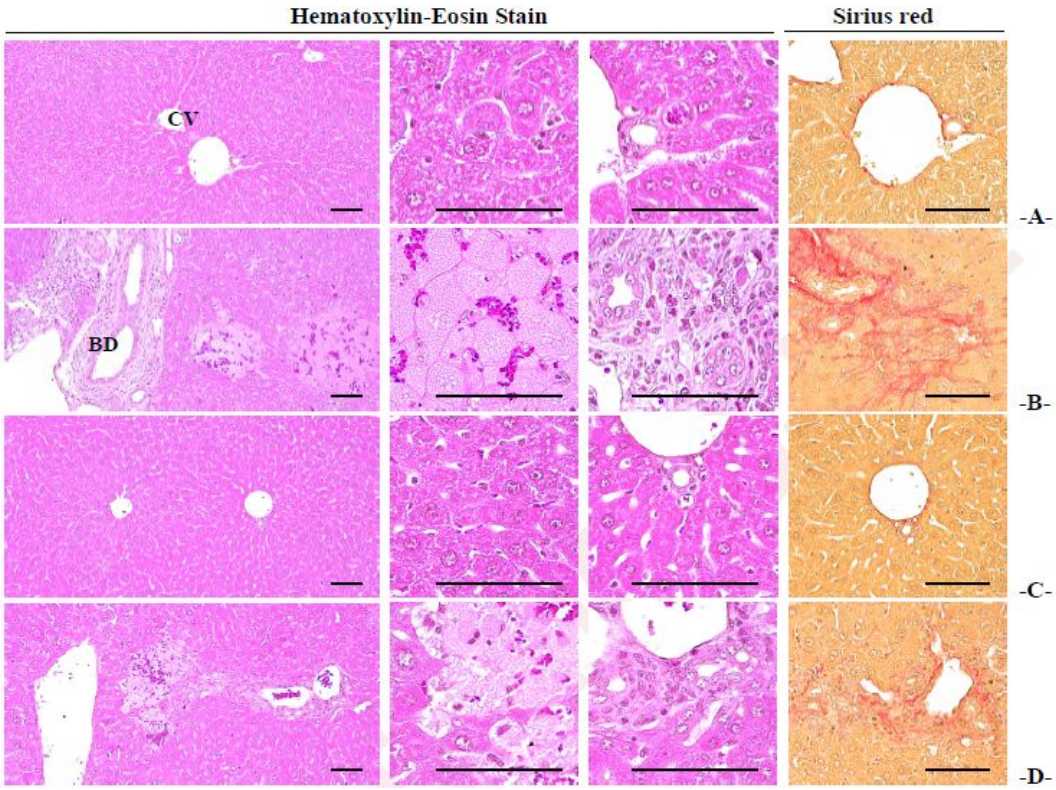


TABLE 1. Histomorphometrical Analysis of Hepatic Tissues, Taken form Vehicle or BDL-treated Mice

[Group Summary]

Index / Groups	Degenerative regions (%/mm^2)	Necrotic hepatocytes (cells/1000 hepatocytes)	Infiltrated inflammatory Cells (%/mm^2)	Bile duct occupied regions (%/mm^2)	Collagen fiber occupied regions (%/mm^2)
LacZ control	2.19±1.22	12.00±5.01	21.63±10.43	3.99±1.91	2.77±1.38
LacZ-BDL	56.11±6.13^{A}	499.63±95.75^{A}	918.38±235.58^{A}	28.46±3.38^{A}	37.67±5.15^{A}
Sestrin2 control	2.17±0.86	10.38±3.11	20.25±8.63	3.49±1.24	2.72±1.26
Sestrin2- BDL	30.46±4.26^{AB}	156.88±63.58^{AB}	213.63±129.45^{AB}	15.54±4.75^{AB}	17.49±5.47^{AB}

Values are expressed as mean ± SD of eight hepatic histological fields

LacZ: Recombinant adenovirus particles which expressed LacZ (1×10^9 pfu)
Sestrin2: Recombinant adenovirus particles which expressed Sestrin2 (1×10^9 pfu)
BDL: Bile duct ligation

^{A} $p<0.01$ as compared with each of same adenovirus treated control mice by MW test
^{B} $p<0.01$ as compared with LacZ-BDL by MW test

Figure 21. Inhibition of BDL -induced liver injury by Ad-Sesn2

Representative Liver Histological Images. Taken from LacZ control [A], LacZ-BDL [B], Sestrin2 control [C] and Sestrin2-BDL [D] mice. Histopathological changes indicated the BDL-induced subacute cholestatic damages – focal hepatocellular necrosis, inflammatory cell infiltrations, bile duct hyperplasia and focal fibrosis were detected in the both BDL treated mice; LacZ-BDL and Sestrin2-BDL at histopathological observation as compared with those of control mice, respectively. However, Sestrin2-BDL treated mice showed clearly lowered BDL-induced cholestatic subacute liver damages as compared with those of LacZ-BDL treated mice, in this experiment. LacZ: Recombinant adenovirus particles which expressed LacZ (1×10^9 pfu), Sestrin2: Recombinant adenovirus particles which expressed Sestrin2 (1×10^9 pfu), BDL: Bile duct ligation, CV: Central vein, BD: Bile duct, Scale bars = 120μm.

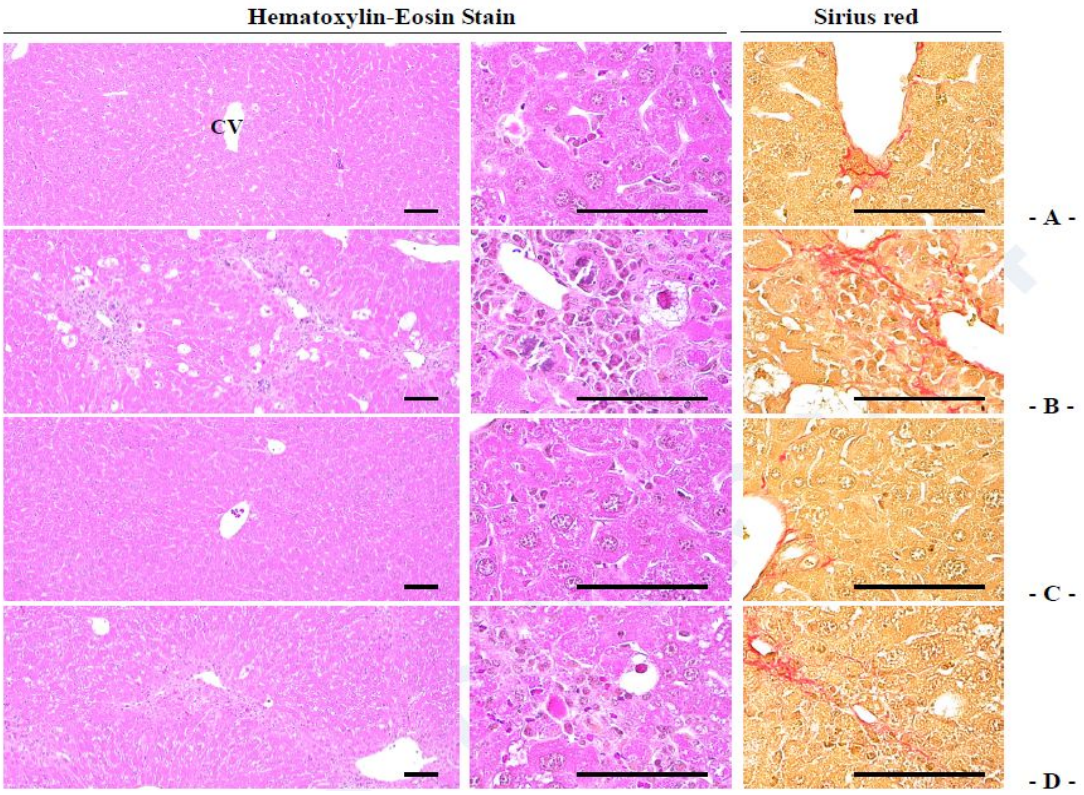


TABLE 2. Histomorphometrical Analysis of Hepatic Tissues, Taken form Vehicle or CCl4 -treated Mice

[Group Summary]

Index / Groups	Percentages of degenerative regions (%/mm^2)	Degenerative hepatocytes (cells/1000 hepatocytes)	Infiltrated inflammatory Cells (%/mm^2)	Collagen fiber occupied regions (%/mm^2)
LacZ control	3.18±1.81	22.63±8.81	20.50±14.03	4.35±2.67
LacZ-CCl4	47.15±5.82^{C}	382.38±74.92^{C}	625.88±123.40^{C}	30.57±4.21^{A}
Sestrin2 control	3.21±1.90	23.00±10.39	21.75±10.38	4.39±1.60
Sestrin2- CCl4	19.00±3.62^{CD}	79.63±16.22^{CD}	141.13±52.82^{CD}	15.48±3.93^{AB}

Values are expressed as mean ± SD of eight hepatic histological fields

LacZ: Recombinant adenovirus particles which expressed LacZ (1×10^9 pfu)
Sestrin2: Recombinant adenovirus particles which expressed Sestrin2 (1×10^9 pfu)
CCl4: Carbon tetrachloride

^{A} $p<0.01$ as compared with each of same adenovirus treated control mice by LSD test
^{B} $p<0.01$ as compared with LacZ-CCl4 by LSD test
^{C} $p<0.01$ as compared with each of same adenovirus treated control mice by MW test
^{D} $p<0.01$ as compared with LacZ-CCl4 by MW test

Figure 22. Inhibition of CCl4-induced liver injury by Ad-Sesn2

Representative Liver Histological Images. Taken from LacZ control [A], LacZ-CCl4 [B], Sestrin2 control [C] and Sestrin2-CCl4 [D] mice. Histopathological changes indicated the CCl4-induced subacute liver damages – centrolobular necrosis including ballooning of hepatocytes, deposit of lipid droplets in hepatocytes (fatty changed cells) and infiltration of inflammatory cells were detected in the both CCl4 treated mice; LacZ-CCl4 and Sestrin2-CCl4 at histopathological observation with focal obvious centrolobular fibrosis as compared with those of each same adenovirus treated control mice, respectively. However, Sestrin2-CCl4 treated mice showed obviously lowered CCl4-induced subacute liver damages as compared with those of LacZ-CCl4 treated mice. LacZ: Recombinant adenovirus particles which expressed LacZ (1×10^9 pfu), Sestrin2: Recombinant adenovirus particles which expressed Sestrin2 (1×10^9 pfu), CCl4: Carbon tetrachloride, CV: Central vein, Scale bars = 120 μm.

8. Inhibition of BDL- or CCl4-induced Fibrogenic Gene Induction by Ad-Sesn2

Nest, we determined the expressions of PAI-1 and α-SMA in the liver by immunoblotting (Fig23). Treatment with CCl_4- or BDL increased the protein levels of PAI-1 and α-SMA in liver tissue, which were completely inhibited by Ad-Sesn2 administration. Similarly, CCl_4- or BDL-inducible mRNA levels of PAI-1, α-SMA, and collagen were also reduced by Ad-Sesn2 infection (Fig24). Our results suggest that Sesn2 inhibits hepatic fibrogenesis and fibrosis via TGF-β/Smad mediated fibrogenic gene expression.

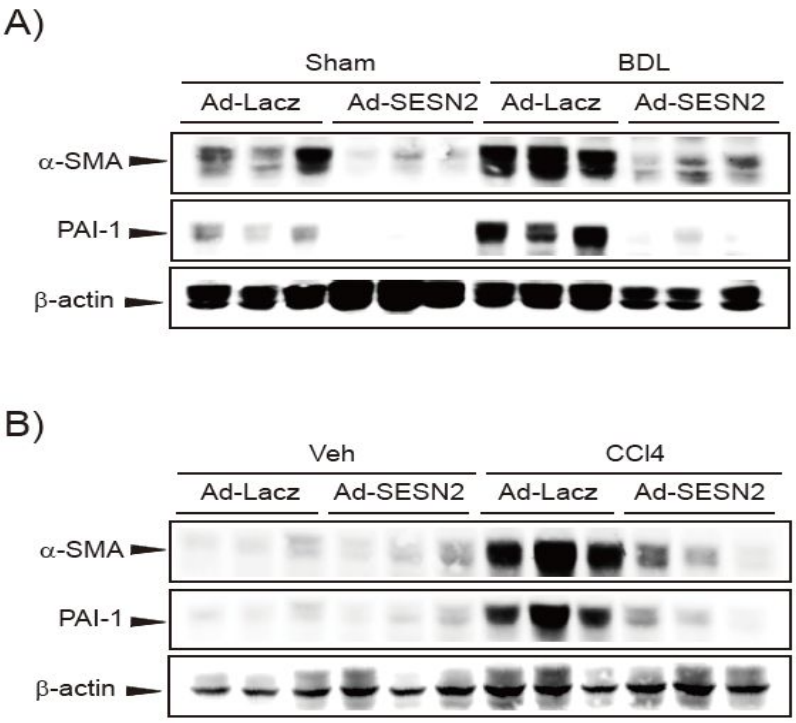


Figure 23. Inhibition of BDL- or CCl4--mediated α-SMA and PAI-1 induction by Ad-Sesn2

Western blot analysis. The protein levels of PAI-1 and α-SMA were assessed by Western blot analysis. *(A* and *B)* Results are presented as means ± SDs of three replicates

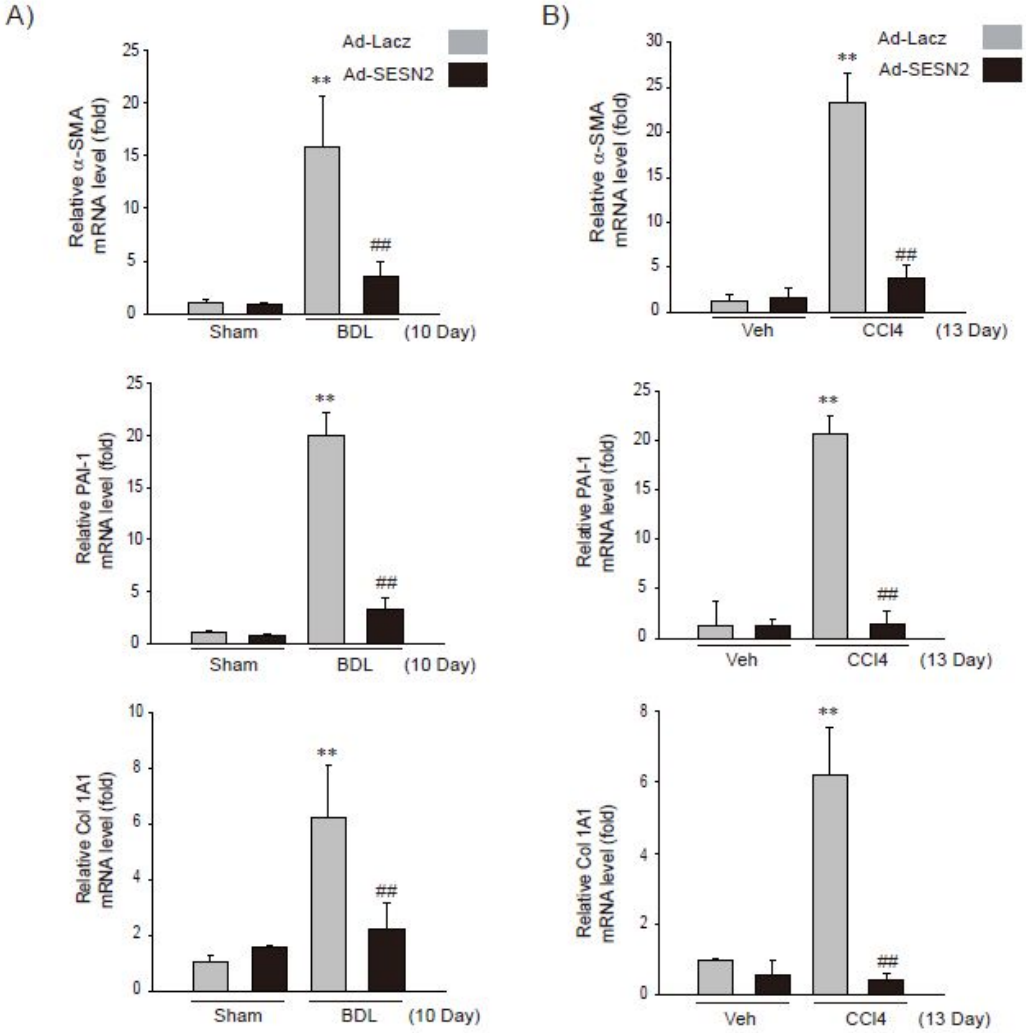


Figure 24. Inhibition of BDL- or CCl4-mediated fibrosis gene induction by Ad-Sesn2

RT-PCR analysis. The transcript levels of PAI-1, α-SMA and collagen 1A1 (COL 1A1) were assessed by Real-time RT-PCR analysis. *(A* and *B)* Results are presented as means ± SDs of three replicates; **P<0.01 = significant versus vehicle-treated controls; significant as compared with Ad-Lacz-CCl_4 or BDL, ##p < 0.01.

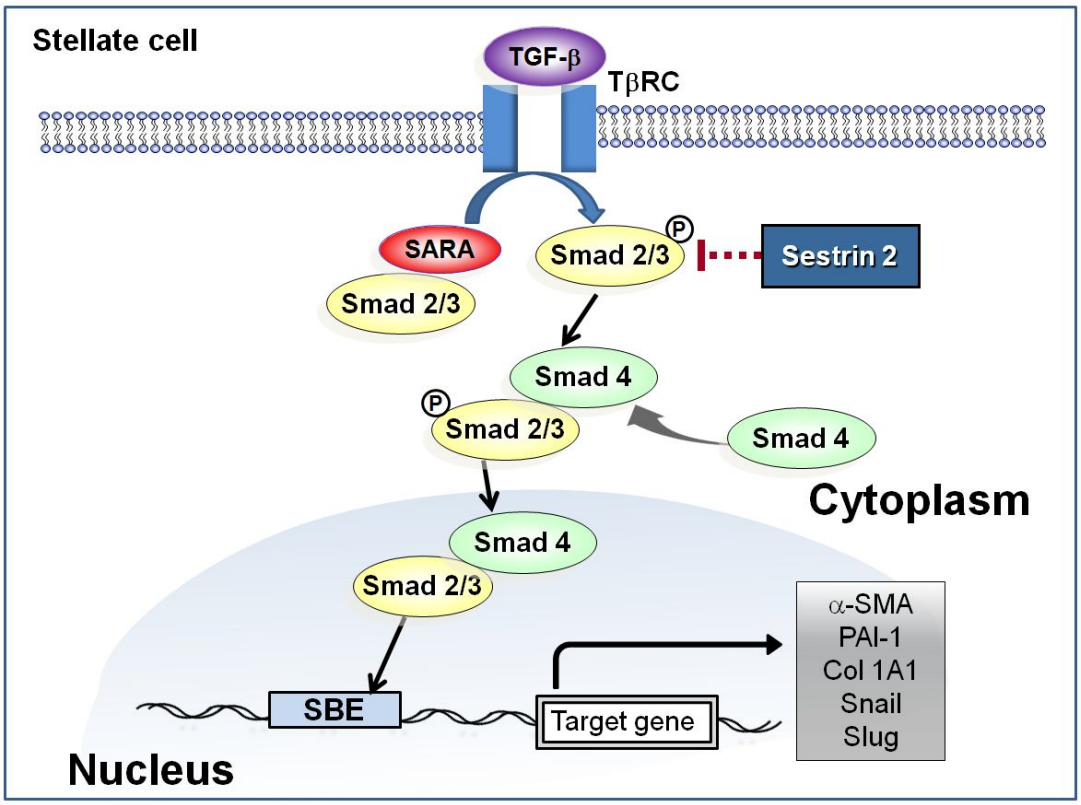


Figure 25. Role of Sestrin2 in the regulation of fibrosis signaling

9. Discussion

It is well established that generation of reactive oxygen species (ROS) plays a key role in liver fibrosis. TGF-β is produced by HSC in response to exogenous ROS, and vise versa. Elevated ROS production and resulting oxidative stress are commonly detected in livers from patients as well as in most types of experimental liver fibrogenesis models (Choi et al., 2014; De Minicis & Brenner, 2007; Zhan et al., 2006). Moreover, antioxidant therapy improves hepatic fibrosis in rodents and may exert beneficial effects in patients with chronic liver diseases (Gomes & Negrato, 2014; Y. Jiang et al., 2014; Sengsuk et al., 2014).

However, little is known regarding the function of the antioxidant system in activated HSC. In addition, the role of Sesn2 in the pathogenesis of hepatic fibrosis remains obscure. In the current study, we first investigated whether Sesn2 protects fibrogenesis in cultured hepatic stellate cells (HSC) or in mice treated with chronic carbon tetrachloride (CCl_4) or bile duct ligation (BDL). Activated primary HSC up-regulates the levels of Sesn2 protein and mRNA (Fig. 13). Transforming growth factor-β (TGF-β) also increased Sesn2 expression in cultured HSC, which was due to increased transcription. TGF-β signaling occurs primarily by activation of Smad proteins and overexpressed Smad3 increased Sesn2 luciferase activity (Fig. 15). *In silico* analysis of the 5' upstream region of *Sesn2* gene identified a putative Smad binding element (SBE) sequence. Deletion of the putative SBE demonstrated that SBE from −964 to −956 bp in the human *Sesn2* promoter was critical for the TGF-β-mediated response (Fig. 15).

Moreover, Sesn2 reduces stellate cell activation and epithelial-mesenchymal transition (EMT) markers, which is accompanied with a marked decrease in SBE luciferase activity and

Smad phosporylation (Fig. 18, 19). A recombinant adenovirus Sens2 (Ad-Sesn2) administration presents less severe hepatic injury as supported by decreases in CCl_4-or BDL-induced ALT/AST levels (Fig. 21). Furthermore, Ad-Sesn2 reduced liver injury and collagen accumulation (Fig. 23, 24). Collectively, our findings suggest that Sesn2 protects HSC activation and hepatic fibrosis via antagonizing TGF-β signaling.

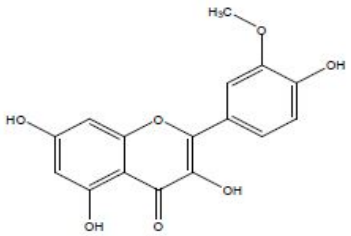
Part Ⅲ: Identification of Isorhamnetin as a Sestrin2 Modulator and therapeutic function

1. Sesn2 Activation by Isorhamnetin

Recently, we found that the novel antioxidant protein Sesn2 contains a functional ARE sequence in its promoter (Shin et al., 2012). As expected, isorhamnetin treatment in HepG2 cells induced the expression of Sesn2 (Fig. 27A, upper). To further elucidate the role of Nrf2 in the regulation of antioxidant gene expression by isorhamnetin, we deleted ARE in the Sesn2 promoter. Exposure of isorhamnetin resulted in a significant increase in the luciferase activity of pGL4-phSESN2. However, specific disruption of ARE in the promoter region of the *Sesn2 gene* completely abolished the ability of isorhamnetin to increase luciferase-reporter activity (Fig. 27A, lower).

Previously, we also found that Nrf2-ARE pathway regulates induction of Sestrin2 (Shin et al., 2012). To examine the effect of isorhamnetin on Nrf2 activity, we first treated HepG2 cells with various concentrations of isorhamnetin for 6 h and then examined the dose–response effect of isorhamnetin on the nuclear accumulation of Nrf2. Treatment with isorhamnetin gradually ncreased nuclear Nrf2 levels in a dose-dependent manner, which was comparable to that obtained with the same concentration of t-BHQ used as a positive control. Consistently, isorhamnetin effectively decreased he cytosolic Nrf2 (Fig. 27B)

A)



Isorhamnetin

B)

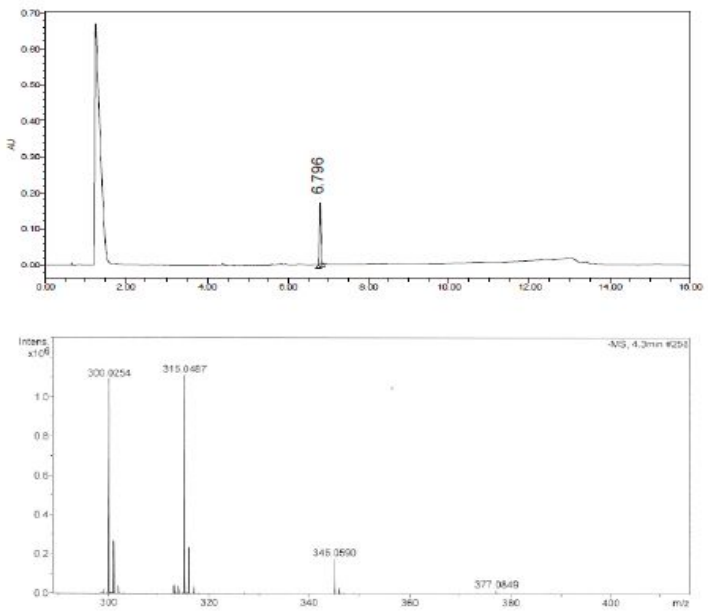


Figure 26. Chemical structure of isorhamnetin

A. Chemical structure of isorhamnetin. *B.* UPLC and LC–MS profiles of isorhamnetin. The purity of isorhamnetin from O. javanica was confirmed by UPLC analysis. The mobile phase was composed of 0.1% formic acid in water and 0.1% formic acid in acetonitrile with a gradient elution system at a flow rate of 0.4 mL/min. The detection UV wavelength was set at 254 nm (upper). Molecular weight to authenticate purified isorhamnetin was determined by HPLC–ESI–MS analysis (lower).

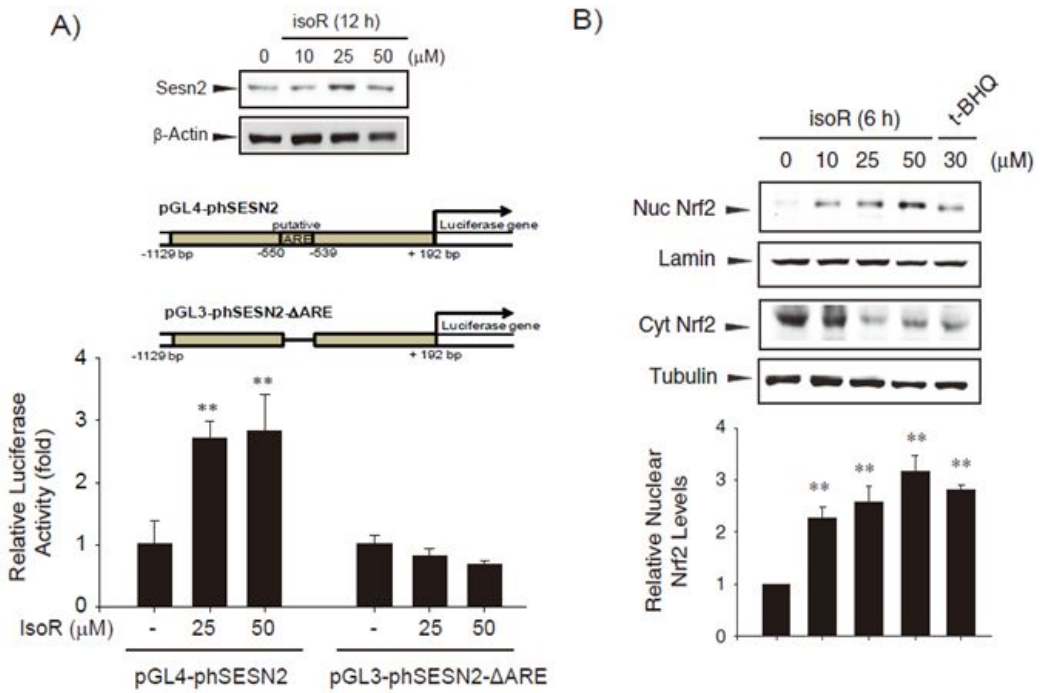


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Figure 27. Sesn2 activation by isorhamnetin

A. Sestrin2 (Sesn2) expression was examined in HepG2 cells treated with isorhamnetin (10–50 μM) for12 h (upper). The effects of a deletion of ARE on the induction of Sesn2 luciferase activity by isorhamnetin. Dual luciferase reporter assays were performed on the lysates of HepG2 cells that had been transfected with pGL4-phSesn2 or pGL3-phSesn2-ΔARE (deletion mutant of ARE in Sesn2). Activations of the reporter gene were calculated as a change in the ratio of firefly luciferase activity to Renilla luciferase activity (lower). *B*. The effect of varying concentrations of isorhamnetin on the nuclear translocation Nrf2 in HepG2 cells. Nrf2 protein was immunoblotted in the cytosolic or nuclear fractions of cells incubated with 10–50 μM of isorhamnetin for 6 h. t-BHQ (30 μM) was used as a positive control. Data represent the mean ± SD of three replicates; the statistical significance of differences between each treatment group and the control (**P < 0.01).

2. Inhibition of t-BHP-induced Oxidative Stress by Isorhamnetin

Because GSH biosynthesis is increased by isorhamnetin via Sesn2 activation, we next measured ROS production and GSH concentration in cells treated with t-BHP in concomitant treatment with isorhamnetin to determine whether isorhamnetin helps maintain redox-homeostasis. t-BHP induced ROS production as shown by fluorescence microscopy, whereas isorhamnetin pretreatment almost completely prevented ROS generation by t-BHP (Fig. 28A and B). Isorhamnetin alone did not affect ROS production (data not shown). In addition, t-BHP treatment markedly decreased GSH level, whereas these were maintained in cells exposed to t-BHP plus isorhamnetin (Fig. 28C). Both decreased ROS production and rescue of GSH by isorhamnetin suggest that isorhamnetin has antioxidant property against t-BHP-induced ROS production.

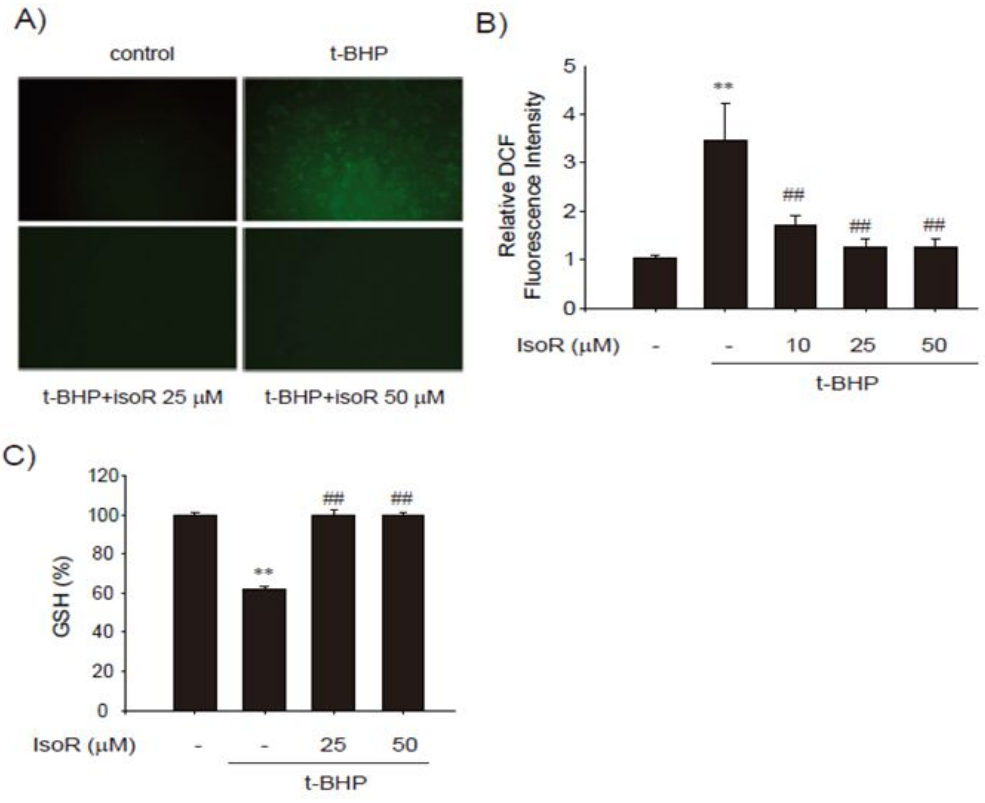


Figure 28. Inhibition of t-BHP-induced oxidative stress by isorhamnetin

A. The effect of isorhamnetin on t-BHP-induced ROS production. HepG2 cells were incubated with 500 μM t-BHP and/or 25–50 μM isorhamnetin for 5h. Cells were stained with 10 μM DCFH-DA for 30 min at 37 °C. H2O2 generation was determined by fluorescence microscopy (200×). *B*. Confirmation of reduced ROS production. Intracellular fluorescence intensities were measured using a fluorescence microplate reader. *C*. The GSH concentration was measured in lysates of cells treated with 500 μM t-BHP and/or 25–50 μM isorhamnetin for 12 h. Data represent the mean ± SD of four replicates; **P < 0.01, significant versus vehicle-treated control; ##P < 0.01, significant versus t-BHP alone.

3. Inhibition of t-BHP-elicited Cell Death by Isorhamnetin

High levels of ROS increase the susceptibility of cells to ROS-induced cytotoxicity. Next, we investigated the effect of isorhamnetin on the cytotoxic effects of t-BHP. Cell viability and cell membrane permeability were assessed colorimetric MTT and LDH release, respectively. Cells treated with t-BHP led to cell death, whereas the pretreatment with 50 μM isorhamnetin prevented t-BHP-induced cell death (Fig. 29A). Cytotoxicity was confirmed by quantifying the LDH released from the cytosol of damaged cells. Cells treated with t-BHP resulted in significant increases in LDH release, while pretreatment with 50 μM isorhamnetin was comparable to that seen in untreated (Fig. 29B). In addition, cells treated with t-BHP obviously increased markers of apoptosis, such as PARP cleavage and cleaved form of caspase-3 (Fig. 29C). However, isorhamnetin treatment prevented alterations in the levels of proteins associated with apoptosis. These results support the notion that the cytoprotective effect of isorhamnetin is due to its antioxidative effects.

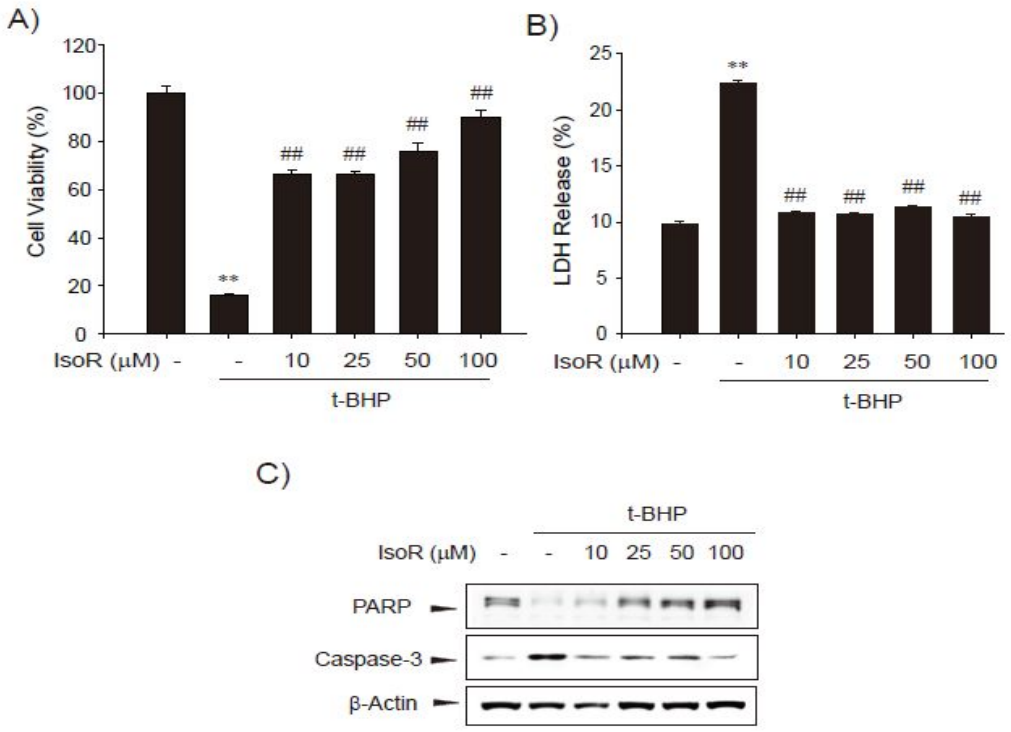


Figure 29. Inhibition of t-BHP-elicited cell death by isorhamnetin

A. MTT cell viability. The effect of isorhamnetin (10–100 μM, 1 h pretreatment) in the presence or absence of t-BHP (500 μM, 12 h) on cell viability was assessed using MTT assays. *B*. LDH assay. Cell membrane permeability was assessed by quantifying the amount of LDH released from the cytosol of t-BHP damaged cells. HepG2 cells were incubated with 500 μM t-BHP and/or 10–100 μM isorhamnetin. Data represent the mean ± SD of four replicates; **P < 0.01, significant versus vehicle-treated control; ##P < 0.01, significant versus t-BHP alone. *C*. Immunoblots of apoptotic proteins. Precursor PARP and cleaved caspase-3 were immunoblotted in the lysates of cells incubated with 10–100 μM isorhamnetin for 1 h, and then treated with 500 μM t-BHP for 12 h. Equal protein loadings were confirmed by immunoblotting for β-actin. Results were confirmed by three separate experiments.

4. Inhibition of LPS-induced Inflammation by Isorhamnetin

To further elucidate the anti-inflammatory effect and molecular mechanism of isorhamnetin, we used RAW264.7 cells, a murine macrophage cell line. We examined any possible toxicity of isorhamnetin in the cells. The MTT assay was performed and verified that cell viability was not affected by treatment with up to 100 μM isorhamnetin for 24 h (data not shown). Next, we tested the effects of sub-lethal concentration of isorhamnetin (10–100 μM) on LPS-induced iNOS expression in vitro (Fig. 30A). 30–100 μM of isorhamnetin treatment clearly blocked the iNOS induction by LPS, while 10 μM isorhamnetin minimally affected the iNOS expression. Therefore, we selected 30 or 100 μM of isorhamnetin for the subsequent experiments. To examine whether isorhamnetin transcriptionally regulated iNOS expression, we next monitored the mRNA levels of iNOS. RT-PCR analyses clearly showed that iNOS mRNA level was significantly increased by LPS. However, isorhamnetin treatment in the cells decreased iNOS expression (Fig. 30B). iNOS reporter gene analyses were conducted using RAW264.7 cells stably transfected with a construct containing the iNOS promoter region from _1588 to +165 bp. Consistent with the results of immunoblotting and RT-PCR analyses, luciferase induction by LPS was significantly prohibited by isorhamnetin treatment (Fig. 30C). As a result of iNOS inhibition, NO production was significantly blocked by isorhamnetin treatment (Fig. 30D).

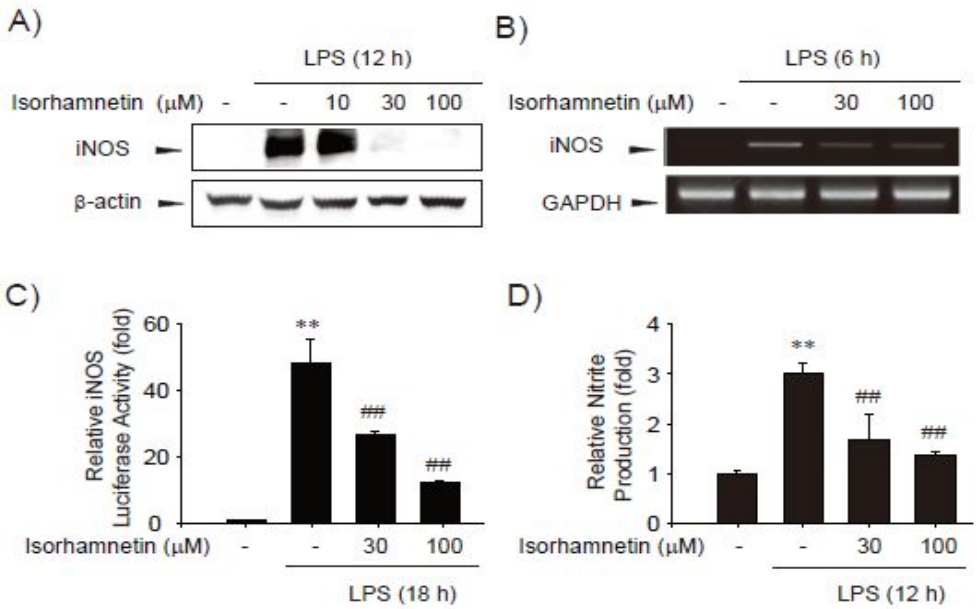


Figure 30. Inhibition of LPS-induced iNOS expression by isorhamnetin

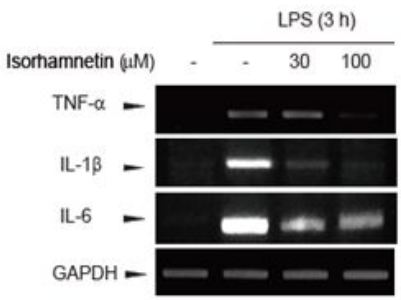
A. The effect of varying concentrations of isorhamnetin on LPS induced iNOS inhibition. Cells were treated with 10, 30 or 100 μM isorhamnetin and continuously incubated with LPS (1 μg/mL) for 12 h. iNOS protein levels were immunoblotted in the cell lysates. *B*. RT-PCR analysis. Cells were treated with 30 or 100 μM isorhamnetin for 1 h, and then further incubated with LPS for 6 h. The iNOS transcripts were analyzed by RT-PCR assays, with the mRNA level of GAPDH used as a housekeeping gene. *C*. iNOS luciferase assay. iNOS luciferase assays were performed in cells stably transfected with pGL-miNOS-1588, which contains murine iNOS promoter from -1588 to +165 bp and exposed to LPS and/or isorhamnetin in RAW264.7 cells for 18 h. *D*. NO production. NO concentration in culture media treated with LPS for 12 h was investigated using Griess reagent.

Data represent the mean ± SD of three replicates; **P < 0.01, significant versus vehicle-treated control; ##P < 0.01, significant versus LPS alone.

5. Inhibition of LPS-inducible Inflammatory Cytokines by Isorhamnetin

Next, we studied the effect of isorhamnetin on pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6. First we assessed the expression of TNF-α, IL-1β and IL-6 by RT-PCR analysis. LPS treatment markedly induced TNF-α, IL-1β and IL-6, whereas isorhamnetin treatment (30–100 μM) prevented gene expression (Fig. 31A). Next, the release of cytokines into the media was analyzed by ELISA in the media of RAW264.7 cells treated with LPS (1 μg/mL) alone or in concomitant treatment with isorhamnetin. Treatment of the cells with LPS substantially increased the production of the cytokines (Fig. 31B). In agreement with inflammatory gene expression, isorhamnetin treatment inhibited the release of cytokines by LPS in a concentration-dependent manner, demonstrating that isorhamnetin suppressed the expression of genes associated with the inflammatory process.

A)



B)

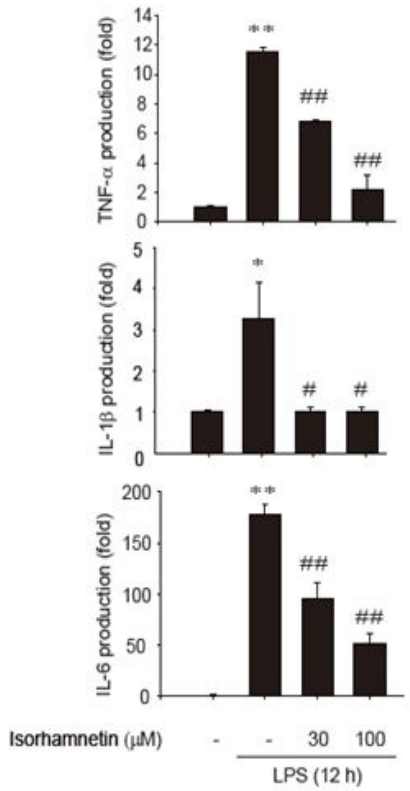


Figure 31. Inhibition of TNF-α, IL-1β and IL-6 by isorhamnetin

A. RT-PCR analysis. The transcripts of TNF-α, IL-1β and IL-6 were monitored by RT-PCR assays. Cells were treated with 30 or 100 μM isorhamnetin for 30 min and subsequently incubated with LPS for 3 h. *B*. ELISA. TNF-α, IL-1β and IL-6 release into culture media was determined by ELISA analysis. Data represent the mean ± SD of three replicates; *p < 0.05, **P < 0.01, significant versus vehicle-treated control; #P < 0.05, ##P < 0.01, significant versus LPS alone.

6. Inhibition of TGF-β-inducible Fibrosis Markers by Isorhamnetin

Next, we explored the effect of isorhamnetin on TGF-β-induced fibrogenic gene expression. The exposure of LX-2 cells to TGF-β increased plasminogen activator inhibitor-1 (PAI-1), α-SMA and EMT markers (Slug, Snail, Zeb1 and N-cadherin) in LX-2 cells, and this was clearly blocked by isorhamnetin (Fig. 32A). Similarly, TGF-β-inducible mRNA level of PAI-1 and α-SMA was also reduced by isorhamnetin in LX-2 cells (Fig. 32B).

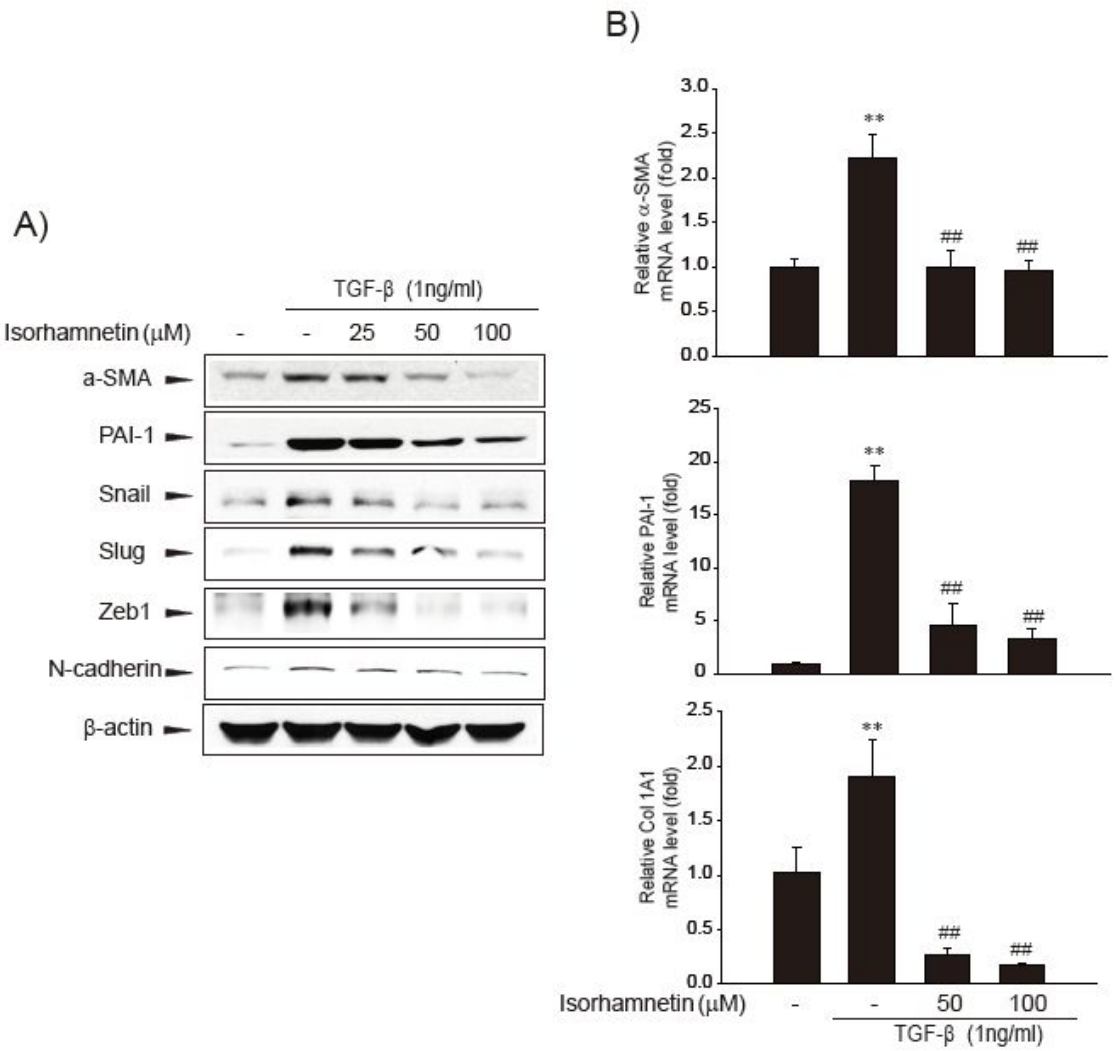


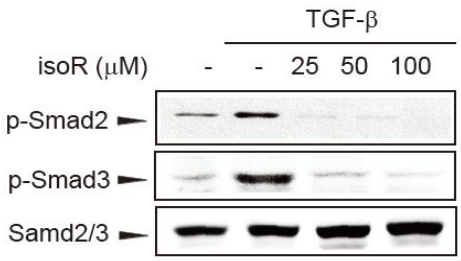
Figure 32. Inhibition of TGF-β-inducible Fibrosis markers by isorhamnetin

A. The effect of varying concentrations of isorhamnetin on TGF-β induced Fibrosis markers inhibition. Cells were treated with 25, 50 or 100 μM isorhamnetin and continuously incubated with TGF-β (1 ng/mL) for 12 h. Proteins levels were immunoblotted in the cell lysates. *B*. RT-PCR analysis. Cells were treated with 50 or 100 μM isorhamnetin for 1 h, and then further incubated with TGF-β for 6 h. The α-SMA, PAI-1 and Col 1A1 transcripts were analyzed by RT-PCR assays, with the mRNA level of GAPDH used as a housekeeping gene. Data represent the mean ± SD of three replicates; **P < 0.01, significant versus vehicle-treated control; ##P < 0.01, significant versus TGF-β alone.

7. Inhibition of TGF-β -inducible Smad3 Phosphorylation by Isorhamnetin

To address the downstream link between isorhamnetin and TGFβ signaling, we assessed the inhibitory effect of isorhamnetin on TGF-β-dependent Smad phosphorylation. TGF-β treatment markedly induced Smad phosphorylation. However, treatment with isorhamnetin attenuated the phosphorylation of Smad3 (Fig. 33A). As expected, isorhamnetin inhibited the ability of Smad3 to induce luciferase activity from an SBE-driven reporter (Fig. 33B). Our results suggest that isorhamnetin inhibits Smad phosphorylation and thus antagonizes Smad-dependent gene transcription.

A)



B)

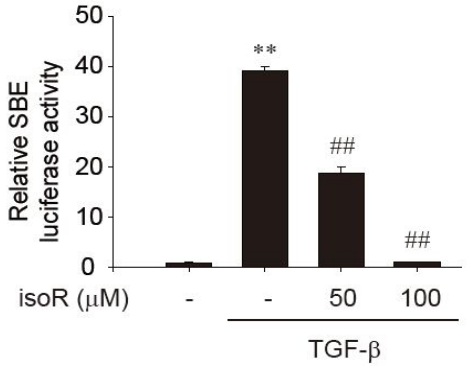


Figure 33. Inhibition of TGF-β -inducible Smad3 phosphorylation by isorhamnetin

A. Immunoblotting for Samd2 and Smad3 phosphorylation. Cells were treated with 25-100 μM isorhamnetin for 30 min before being incubated with TGF-β for 30 min. The cell lysates were immunoblotted and results were confirmed by repeated experiments. *B*. Inhibition of Smad activation by isorhamnetin. Cells were transfected with a pGL-SBE luciferase construct. Transfected cells were treated with 50 or 100 μM isorhamnetin and TGF-β (1 ng/ml) for 12 h. Data represent the mean ± SD of three replicates; **P < 0.01, significant versus vehicle-treated control; ##P < 0.01, significant versus TGF-β alone.

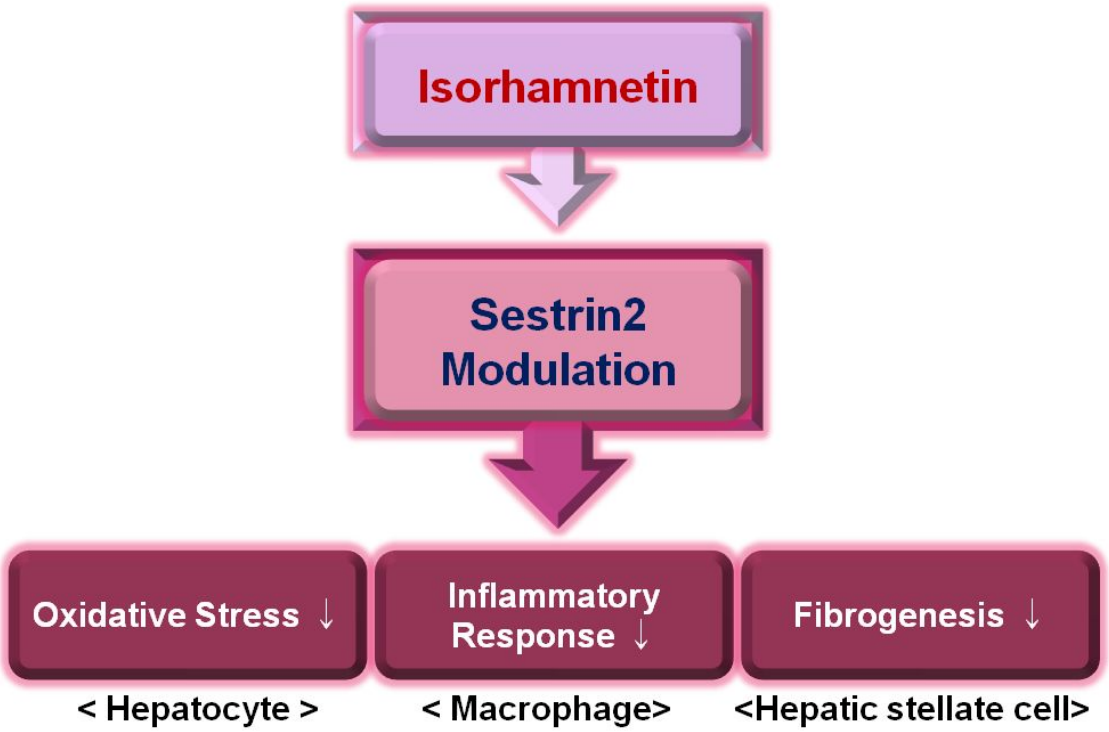


Figure 34. Effect of isorhamnetin as a Sestrin2 modulator and therapeutic function

9. Discussion

Sesn2 is a family of recently identified evolutionally conserved antioxidant protein that exhibit cysteine sulfinyl reductase activity and can protect cells from oxidative stress. Sesn2, up-regulated in response to a variety of stresses including hypoxia, DNA damage, oxidative stress, and energetic stress (Svegliati-Baroni et al., 2008), is recently reported physiological roles of Sesn2 in the liver. In addition, it is reported that Sesn2 protects liver from acute stimulation of lipogenesis associated with fasting and re-feeding through degradation of Keap1 and concomitant up-regulation of Nrf2 activity (Shin et al., 2012).

So, these results led us to investigate the effect of isorhamnetin on Sesn2 activation through a concomitant up-regulation of Nrf2 activity (Fig. 27). Furthermore, isorhamnetin significantly induced intracellular GSH levels and reduced ROS production and cell death induced by t-BHP (Fig. 28). In addition, our data showed that isorhamnetin inhibits acute inflammatory response in Raw264.7 cells. Consistently, isorhamnetin inhibited iNOS expression and NO production in LPS-activated macrophages (Fig. 30,31). It is well known that long-term treatment with LPS leads to cell death via ROS/RNS accumulation in Raw264.7 cells. Indeed, several lines of evidence suggest that cellular redox status regulates LPS-induced NF-κB activation (Li et al., 2008; Thimmulappa et al., 2006). In the current study, we also found that isorhamnetin protects against fibrogenesis in cultured hepatic stellate cells (HSC) (Fig. 32). Here, we found that isorhamnetin inhibits HSC activation and antagonizes TGF-β/Smad signaling (Fig. 33). Collectively, our findings strongly suggest that isorhamnetin treatment may become a promising therapeutics to effectively prevent or treat liver diseases.

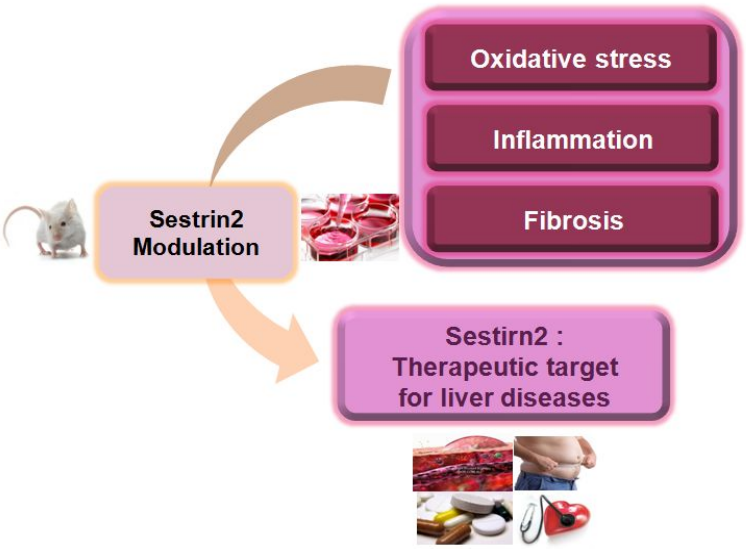
Ⅴ. Conclusions

From our studies we can conclude that:

1. The study shows that Sesn2 antagonizes TLRs-mediated pro-inflammatory signaling and cell death in vivo and in vitro, and that Sesn2 specifically inhibits the TLRL-induced JNK, p38 MAPK and AP-1 pathway. The results of this study provide insight of the roles of Sesn2 in innate immunity and inflammatory responses.

2. The study shows that Sesn2 protects against CCl4/BDL-induced hepatic fibrogenesis and fibrosis via inhibition TGF-β/Smad signaling.

3. The study shows that treatment of isorhamnetin as a Sestrin2 modulator may become a promising therapeutics to effectively prevent or treat liver diseases.



ABSTRACT

The role of Sestirn2 as a novel therapeutic target for the liver diseases

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Graduate School of Chosun University

A variety of diseases have been linked with excessive reactive oxygen species (ROS), which are mainly produced in the mitochondria as by-products of normal respiration and play a crucial role in the cell signaling pathways that control cellular homeostasis. Excessive ROS are promptly destroyed in the cell by a sophisticated antioxidant defense mechanism. At the molecular level, a series of studies have shown that oxidative stress is commonly induced in all forms of chronic liver injury and plays a crucial role in hepatic fibrogenesis and cancer development. It was recently reported that the sulfiredoxin (Srx) system plays an important role in antioxidant defense through catalysis of the reduction of cysteine sulfinicacid of hyperoxidized peroxired oxinsresulting in regeneration of peroxiredoxin. Sestrins were

recently identified, and they also have cysteine sulfinyl reductase activity and modulate peroxide signaling and anti oxidant defense.

However, the role of Sesn2 in acute hepatitis and the pathogenesis of hepatic fibrosis remains obscure.

First, we investigated whether Sesn2 regulates Toll like receptor (TLR)-mediated inflammatory signaling and sought to identify the molecular mechanism responsible. In cells expressing Sesn2, it was found that Sesn2 almost completely inhibited lipopolysaccharide (LPS)-induced NO release and iNOS expression. A gene knockdown experiment confirmed the role of Sesn2 in LPS-activated RAW264.7 cells. Consistently, pro-inflammatory cytokine (e.g., TNF-α, IL-6, and IL-1β release and expression were inhibited in Sesn2-expressing cells. Furthermore, Sesn2 prevented LPS-elicited cell death and ROS production via inhibition of NADPH oxidase. NF-κB and AP-1 are redox-sensitive transcription factors that regulate the expressions of diverse inflammatory genes. Surprisingly, Sesn2 specifically inhibited AP-1 luciferase activity and its DNA binding, but not those of NF-κB. AP-1 inhibition by Sesn2 was found to be due to a lack of JNK, p38 and c-Jun phosphorylation. Next, we investigated whether Sesn2 protects galactosamine (Gal)/LPS-induced liver injury in mice infected with a recombinant adenovirus Sesn2 (Ad-Sesn2). Ad-Sesn2 present less severe hepatic injury as supported by decreases in the ALT, AST and hepatocyte degeneration. Moreover, Ad-Sesn2 attenuated Gal/LPS-induced pro-inflammatory gene expression in mice. The study shows that Sesn2 inhibits TLR-induced pro-inflammatory signaling and protects cells by inhibiting JNK or p38-mediated c-Jun phosphorylation.

Next, we investigated whether Sesn2 protects fibrogenesis in cultured hepatic stellate cells (HSC) or in chronic carbon tetrachloride (CCl_4)-or bile duct ligation (BDL)-induced hepatic

fibrosis mice. Here, we found that Sesn2 is up-regulated during HSC activation and antagonized TGF-β/Smad signaling. Furthermore, recombinant adenovirus Sens2 (Ad-Sesn2) administration showed less severe hepatic injury and fibrosis in CCl_4-or BDL-induced fibrotic mice. Collectively, our findings suggest that Sesn2 protects hepatic fibrogenesis and fibrosis via inhibition TGF-β/Smad signaling.

Finally, we investigate the effect of isorhamnetin on Sesn2 activation through a concomitant up-regulation of Nrf2 activity. Furthermore, isorhamnetin significantly induced intracellular GSH levels and reduced ROS production and cell death induced by t-BHP. In addition, our data showed that isorhamnetin inhibits acute inflammatory response in Raw264.7 cells. Consistently, isorhamnetin inhibited iNOS expression and NO production in LPS-activated macrophages. It is well known that long-term treatment with LPS leads to cell death via ROS/RNS accumulation in Raw264.7 cells. Indeed, several lines of evidence suggest that cellular redox status regulates LPS-induced NF-κB activation. In the current study, we also found that isorhamnetin protects against fibrogenesis in cultured hepatic stellate cells (HSC). Here, we found that isorhamnetin inhibits HSC activation and antagonizes TGF-β/Smad signaling. Collectively, our findings strongly suggest that isorhamnetin treatment may become a promising therapeutics to effectively prevent or treat liver diseases. **Conclusion:** Taken together, our findings suggest that Sesn2 protects acute hepatitis and hepatic fibrosis. And isorhamnetin as Sesn2 Modulator may become a promising therapeutics of liver diseases.

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Free Radical Biology and Medicine 78 (2015) 156–167

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Role of sestrin2 in the regulation of proinflammatory signaling in macrophages

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ARTICLE INFO

Article history:
Received 12 June 2014
Received in revised form
1 October 2014
Accepted 3 November 2014
Available online 13 November 2014

Keywords:
Sestrin2
TLR
Inflammation
Oxidative stress
JNK
AP-1

ABSTRACT

Sestrins (Sesns) are conserved antioxidant proteins that accumulate in cells in response to various stresses. However, the regulatory roles of Sesn2 in the immune system and in inflammatory responses remain obscure. In the present study, we investigated whether Sesn2 regulates Toll like receptor (TLR)-mediated inflammatory signaling and sought to identify the molecular mechanism responsible. In cells expressing Sesn2, it was found that Sesn2 almost completely inhibited lipopolysaccharide (LPS)-induced NO release and iNOS expression. A gene knockdown experiment confirmed the role of Sesn2 in LPS-activated RAW264.7 cells. Consistently, proinflammatory cytokine (e.g., TNF-α, IL-6, and IL-1β) release and expression were inhibited in Sesn2-expressing cells. Furthermore, Sesn2 prevented LPS-elicited cell death and ROS production via inhibition of NADPH oxidase. NF-κB and AP-1 are redox-sensitive transcription factors that regulate the expressions of diverse inflammatory genes. Surprisingly, Sesn2 specifically inhibited AP-1 luciferase activity and its DNA binding, but not those of NF-κB. AP-1 inhibition by Sesn2 was found to be due to a lack of JNK, p38, and c-Jun phosphorylation. Next, we investigated whether Sesn2 protects galactosamine (Gal)/LPS-induced liver injury in mice infected with a recombinant adenovirus Sesn2 (Ad-Sesn2). Ad-Sesn2 present less severe hepatic injury as supported by decreases in the ALT, AST, and hepatocyte degeneration. Moreover, Ad-Sesn2 attenuated Gal/LPS-induced proinflammatory gene expression in mice. The study shows that Sesn2 inhibits TLR-induced proinflammatory signaling and protects cells by inhibiting JNK- or p38-mediated c-Jun phosphorylation.

Introduction

Reactive oxygen species (ROS) are essential mediators of normal cell physiology and are generated from the metabolism of molecular oxygen, mainly in mitochondria [1,2]. ROS can serve as intracellular signaling molecules and are involved in regulation of diverse biological processes. Moreover, hydrogen peroxide (H_2O_2) is a major ROS in terms of cell signaling regulation [3]. However, oxidative stress occurs when the balance between the ROS production and the antioxidant defense mechanism is skewed in favor of ROS production, and the excessive ROS then causes direct damage to macromolecules, such as lipids, nucleic acids, and proteins [4]. Furthermore, it is well known that oxidative damage is associated with various human diseases, such as cancer, diabetes, hepatitis, and cardiovascular disease.

The peroxiredoxins (Prxs) constitute a family of thiol-dependent peroxidases that scavenge H_2O_2 and alkyl hydroperoxides. In addition to antioxidant activities, they are also associated with diverse cellular functions, such as proliferation, differentiation, and apoptosis [5,6]. Furthermore, the six mammalian Prx family members exhibit different tissue and organelle distributions [6]. The Prxs are classified into three subgroups, designated 2-Cys Prx, atypical 2-Cys Prx, and 1-Cys Prx. The 2-Cys Prx exists as homodimers and contains two conserved cysteine residues for peroxide reduction. Under highly oxidizing conditions, Prxs lose peroxidase activity due to the over-oxidation of cysteine to sulfinic acid (Cys-SO_2H) or sulfonic acid (Cys-SO_3H) [5] in the active site. However, sulfinylated Prxs are reactivated by sulfiredoxin (Srx), which reduces sulfinylated Prxs via an ATP-dependent mechanism [7–9].

It was recently proposed that sestrins (Sesns) also have cysteine sulfinyl reductase activity and inhibit ROS production by regenerating

Abbreviations: Ad, adenovirus; ATF, activating transcription factor; BMDM, bone marrow-derived macrophage; bZIP, basic region leucine zipper; ERK1/2, extracellular-regulated protein kinases 1 and 2; Gal, D-galactosamine; IκB, inhibitory κB; LPS, lipopolysaccharide; NF-κB, nuclear factor-kappa B; NO, nitric oxide; NOX, NADPH oxidase; Prx, peroxiredoxin; RNS, reactive nitrogen species; ROS, reactive oxygen species; Sesns, sestrins; Sesn2, sestrin-2; Srx, sulfiredoxin; TLR, Toll-like receptor

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http://dx.doi.org/10.1016/j.freeradbiomed.2014.11.002
0891-5849/

European Journal of Pharmacology 740 (2014) 634–640

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and cellular pharmacology

AMPK activation by isorhamnetin protects hepatocytes against oxidative stress and mitochondrial dysfunction

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ARTICLE INFO

Article history:
Received 6 December 2013
Received in revised form
11 June 2014
Accepted 17 June 2014
Available online 24 June 2014

Keywords:
AMPK
Oxidative stress
Mitochondria
Isorhamnetin

Chemical compounds studied in this article:
Isorhamnetin (PubChem CID: 5281654)

ABSTRACT

Arachidonic acid (AA) is a ω−6 polyunsaturated fatty acid that is found in the phospholipids of membranes and released from the cellular membrane lipid bilayer by phospholipase A2. During this process, AA could produce excess reactive oxygen species and induce apoptosis and mitochondrial dysfunction by selectively inhibiting complexes I and III. Isorhamnetin, an O-methylated flavonol aglycone, has been shown to have cardio-protective, anti-adipogenic, anti-tumor, and anti-inflammatory effects. In the present study, we investigated the effects of isorhamnetin on hepatotoxicity and the underlying mechanisms involved. Our in vitro experiments showed that isorhamnetin dose-dependently blocked the hepatotoxicity induced by treatment with AA plus iron in HepG2 cells. Furthermore, isorhamnetin inhibited the AA+iron induced generation of reactive oxygen species and reduction of glutathione, and subsequently maintained mitochondria membrane potential in AA+iron treated HepG2 cells. In addition, isorhamnetin activated AMP-activated protein kinase (AMPK) by Thr-172 phosphorylation of AMPKα, and this was mediated with Ca(2+)/calmodulin-dependent protein kinase kinase-2 (CaMKK2), but not liver kinase B1. Experiments using CaMKK2 siRNA or its selective inhibitor, STO-609, revealed the role of CaMKK2 in the isorhamnetin-induced activation of AMPK in HepG2 cells. These results indicate isorhamnetin protects against the hepatotoxic effect of AA plus iron, and suggest that the AMPK pathway is involved in the mechanism underlying the beneficial effect of isorhamnetin in the liver.

1. Introduction

Oxidative stress promotes cellular damage and is a characteristic feature of several human diseases. For example, it has been shown that modification of membrane phospholipids by excess reactive oxygen species results in cell and tissue injury (Apel and Hirt, 2004; Bergamini et al., 2004; Reddy and Clark, 2004; Shah and Channon, 2004; Valko et al., 2006; Willner, 2004). During conditions of oxidative and/or inflammatory stress, reactive oxygen species and/or cytokines can induce the oxidative modification of fatty acids within membrane phospholipids, and during this process, arachidonic acid (AA; a ω-6 polyunsaturated fatty acid) is released from the cellular membrane lipid bilayer (Balboa and Balsinde, 2006; Gijon and Leslie, 1999). AA can induce cell death by promoting the uptake of calcium by mitochondria and the production of ceramide (Balboa and Balsinde, 2006; Gijon and Leslie, 1999). In particular, in the presence of iron, released AA stimulates cells to produce more reactive oxygen species, which can induce mitochondrial dysfunction and cell death (Fleming and Bacon, 2005; Galaris and Pantopoulos, 2008; Halliday and Searle, 1996; Neufeld, 2006). Therefore, treatment with AA plus iron offers a treatment model that could be useful for screening agents that protect mitochondria against severe oxidative stress.

AMP-activated protein kinase (AMPK) is a multifunctional cytosolic protein that plays important roles in energy homeostasis,

Abbreviations: AA, arachidonic acid; ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; CaMKK2, Ca(2+)/calmodulin-dependent protein kinase kinase-2; DCFH-DA, 2′,7′-Dichlorofluorescein diacetate; FITC, fluorescein isothiocyanate; GSH, glutathione; IsoRN, Isorhamnetin; LKB1, liver kinase B1; MMP, mitochondrial membrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PI, propidium iodide.

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http://dx.doi.org/10.1016/j.ejphar.2014.06.017
0014-2999/

Toxicol. Res.
Vol. 30, No. 1, pp. 19-25 (2014)
Open Access http://dx.doi.org/10.5487/TR.2014.30.1.019
pISSN: 1976-8257 eISSN: 2234-2753 **Original Article**

Toxicological Research
Official Journal of
Korean Society of Toxicology
Available online at http://www.toxrnet.or.kr

Licochalcone Suppresses LXRα-Induced Hepatic Lipogenic Gene Expression through AMPK/Sirt1 Pathway Activation

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(Received February 27, 2014; Revised March 24, 2014; Accepted March 26, 2014)

Licochalcone (LC), a major phenolic retrochalcone from licorice, has anti-inflammatory activity. This study investigated the effects of licochalcone A (LCA) and licochalcone E (LCE) on Liver X receptor-α (LXRα)-mediated lipogenic gene expression and the molecular mechanisms underlying those effects. LCA and LCE antagonized the ability of LXRα agonists (T0901317 or GW3965) to increase sterol regulatory element binding protein-1c (SREBP-1c) expression and thereby inhibited target gene expression (e.g., FAS and ACC) in HepG2 cells. Moreover, treatment with LCA and LCE impaired LXRα/RXRα-induced CYP7A1-LXRE-luciferase (CYP7A1) transactivation. The AMPK-Sirt1 signaling pathway is an important regulator of energy metabolism and, therefore, a potential therapeutic target for metabolic diseases, including hepatic steatosis. We found here that LCE increased AMPK phosphorylation and Sirt1 expression. We conclude that LC inhibits SREBP-1c-mediated hepatic lipogenesis via activation of the AMPK/Sirt1 signaling pathway.

Key words: Licochalcone A, Licochalcone E, Hepatic steatosis, Sterol regulatory element binding protein-1c, Liver X receptor-α

INTRODUCTION

Liver X receptor-α (LXRα), a member of the nuclear receptor superfamily, binds to the DR-4 motif known as the LXR response element (LXRE) in its target genes and acts as an important regulator of cholesterol, fatty acids, and bile acids (1). LXRα increased the efflux of free cholesterol as well as nascent and mature HDL through upregulation of the ATP binding cassette (ABC) sterol transporters, such as ABCA1 and ABCG1 (2). Activation of LXRα, however, is associated with increased lipogenesis, hypertriglycemia, and fat accumulation through *de novo* fatty acid synthesis in the liver due to the LXRα-induced increase in the expression of lipogenic genes, such as fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and stearoyl-CoA desaturase-1 (SCD-1) (3). Sterol regulatory element binding protein-1c (SREBP-1c) is an essential transcription factor for lipogenic gene expression (4). SREBP-1c is fundamental to the pathogenesis of metabolic diseases, including hepatic steatosis, and has been suggested as a potential therapeutic target (5).

Licorice root from *Glycyrrhiza inflata* (*G. inflata*) has been used in traditional and herbal medicines. This species contains unusual phenolic compounds, called retrochalcones, which include licochalcone A to E and echinatin. Licochalcone A (LCA; Fig. 1A upper panel) has been demonstrated to have a variety of pharmacological activities, including anti-bacterial, anti-cancer, and anti-inflammatory activities (6-9). Licochalcone E (LCE; Fig. 1A lower panel) has recently been isolated and characterized from *G. inflata* (10). The pharmacological efficacy of LCE has been studied extensively. Anti-diabetic (11), anti-inflammatory (12), and cytotoxic effects (13) have been reported for LCE. Moreover, recent report showed the anti-lipogenic effect of LCA in HepG2 cells and mice fed on high fat diet (14). They showed that the basal expression levels of lipogenic gene were decreased by LCA in HepG2 cells. In addition,

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Inflammation (© 2013)
DOI: 10.1007/s10753-013-9789-6

The Antioxidant Effects of Isorhamnetin Contribute to Inhibit COX-2 Expression in Response to Inflammation: A Potential Role of HO-1

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Abstract—Previously, we reported that isorhamnetin, a *3′-O*-methylated metabolite of quercetin, reduced inducible nitric oxide synthase (iNOS) expression and NO production. The present study further investigated the underlying mechanism of anti-inflammatory and antioxidant effects of isorhamnetin. Administration of isorhamnetin decreased the number of cyclooxygenase-2 (COX-2) positive cells in rats with carrageenan-induced paw edema. Isorhamnetin also suppressed lipopolysaccharide (LPS)-induced expression of COX-2 in cells. It is well known that LPS-induced reactive oxygen species (ROS) production leads to COX-2 induction. Isorhamnetin decreased LPS-induced ROS production and apoptosis. In addition, the basal expression of heme oxygenase-1 (HO-1) was increased by isorhamnetin treatment in agreement with the increase in nuclear translocation of NF-E2-related factor-2 (Nrf2), an essential transcription factor for the regulation of HO-1 expression. Moreover, pretreatment of tin protoporphyrin IX (SnPP), a chemical inhibitor of HO-1, reversed the ability of isorhamnetin to inhibit COX-2 expression. These results demonstrate that induction of HO-1 by isorhamnetin leads to a reduction in ROS production and its antioxidant property might contribute to the inhibition of COX-2 expression in response to inflammation.

KEY WORDS: isorhamnetin; anti-inflammation; cyclooxygenase-2; reactive oxygen species; heme oxygenase-1.

INTRODUCTION

Inflammation has been implicated in various diseases including sepsis, atherosclerosis, and cancer [1–3]. In response to foreign organisms, the immune response is provoked during inflammation. Histamine and serotonin produced from mast cells alter the vascular permeability, which results in infiltration of inflammatory cells [4]. Macrophages, as scavengers of foreign organisms and inflammatory mediator secretion cells, regulate immune responses [5]. However, the aberrant activation of macrophages causes cell damage and tissue injury. These detrimental responses affect cell membranes by modifying membrane phospholipids. As a result, arachidonic acid is released and then various eicosanoids are synthesized. These products cause deleterious outcomes and pain by affecting blood vessel, cells, and nerve endings [6, 7].

Cyclooxygenase-2 (COX-2), as an early response enzyme, is induced during the inflammatory response and facilitates inflammation through production of prostaglandins [8]. In macrophages, with inducible nitric oxide synthase (iNOS), the level of COX-2 is an important indicator for the evaluation of inflammatory extent. Therefore, many kinds of COX-2 inhibitors have been developed for therapy against various inflammatory diseases [8]. The

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ABBREVIATIONS: AMPK, AMP-activated protein kinase; CO, Carbon monoxide; COX-2, Cyclooxygenase-2; DCFH-DA, 2′,7-Dichlorofluorescein diacetate; HO-1, Heme oxygenase-1; IKK, IκB kinase; IκBα, Inhibitory κB α; iNOS, Inducible nitric oxide synthase; LPS, Lipopolysaccharide; MAPK, Mitogen-activated protein kinase; NAC, *N*-Acetylcystein; NADPH oxidase, Nicotinamide adenine dinucleotide phosphate oxidase; NF-κB, Nuclear factor-kappaB; Nrf2, NF-E2-related factor-2; PGE_2, Prostaglandin E_2; PMA, Phorbol 12-myristate 13-acetate; PPARγ, Peroxisome proliferator-activated receptory; ROS, Reactive oxygen species; SnPP, Tin protoporphyrin IX; TLR, Toll-like receptor; TNFα, Tumor necrosis factor alpha

Published online: 13 December 2013

Toxicology and Applied Pharmacology 274 (2014) 293–301

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Isorhamnetin protects against oxidative stress by activating Nrf2 and inducing the expression of its target genes

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ARTICLE INFO

Article history:
Received 12 August 2013
Revised 24 October 2013
Accepted 27 October 2013
Available online 7 November 2013

Keywords:
Isorhamnetin
Methylated flavonoid
Nrf2
Cytoprotection
ROS

ABSTRACT

Isorhamnetin is a 3′-O-methylated metabolite of quercetin, and has been reported to have anti-inflammatory and anti-proliferative effects. However, the effects of isorhamnetin on Nrf2 activation and on the expressions of its downstream genes in hepatocytes have not been elucidated. Here, we investigated whether isorhamnetin has the ability to activate Nrf2 and induce phase II antioxidant enzyme expression, and to determine the protective role of isorhamnetin on oxidative injury in hepatocytes. In HepG2 cells, isorhamnetin increased the nuclear translocation of Nrf2 in a dose- and time-dependent manner, and consistently, increased antioxidant response element (ARE) reporter gene activity and the protein levels of hemeoxygenase (HO-1) and of glutamate cysteine ligase (GCL), which resulted in intracellular GSH level increases. The specific role of Nrf2 in isorhamnetin-induced Nrf2 target gene expression was verified using an ARE-deletion mutant plasmid and Nrf2-knockout MEF cells. Deletion of the ARE in the promoter region of the *sestrin2* gene, which is recently identified as the Nrf2 target gene by us, abolished the ability of isorhamnetin to increase luciferase activity. In addition, Nrf2 deficiency completely blocked the ability of isorhamnetin to induce HO-1 and GCL. Furthermore, isorhamnetin pretreatment blocked t-BHP-induced ROS production and reversed GSH depletion by t-BHP and consequently, due to reduced ROS levels, decreased t-BHP-induced cell death. In addition isorhamnetin increased ERK1/2, PKCδ and AMPK phosphorylation. Finally, we showed that Nrf2 deficiency blocked the ability of isorhamnetin to protect cells from injury induced by t-BHP. Taken together, our results demonstrate that isorhamnetin is efficacious in protecting hepatocytes against oxidative stress by Nrf2 activation and in inducing the expressions of its downstream genes.

Introduction

Excessive reactive oxygen species (ROS) production is believed to cause a diversity of diseases. Furthermore, balance between the generation and elimination of ROS keeps cellular homeostasis (Rodriguez and Redman, 2005). Under normal physiological conditions, cells control ROS levels using a sophisticated antioxidant defense mechanism. However, under pathological conditions, such as in chronic inflammatory conditions, excessive ROS causes cellular dysfunction and remodeling (Finkel, 2011; Valko et al., 2007).

NF-E2-related factor 2 (Nrf2) is a member of the cap'n'collar family of bZIP transcription factors that bind to the antioxidant response element (ARE) and thereby regulate induction of genes encoding antioxidant proteins and phase II detoxifying enzymes such as γ-glutamylcysteine synthetase (GCS) (Jeyapaul and Jaiswal, 2000), hemeoxygenase 1 (HO-1) (Kweon et al., 2006), glutathione *S*-transferase A1/2 (Kwak et al., 2001), NAD(P)H:quinone reductase (NQO1) (Venugopal and Jaiswal, 1996) and sestrin2 (Sesn2) (Shin et al., 2012). It has been well established that Nrf2 activation in response to oxidative injury protects cells and tissues from oxidative stress. Under normal conditions, Nrf2 is localized in the cytoplasm where it binds with the Keap1, which functions as an adaptor for Cul3-based E3 ligase to regulate the proteasomal degradation of Nrf2 (Venugopal and Jaiswal, 1996). In fact, the Nrf2–Keap1 interaction leads to the rapid degradation of Nrf2 via Cul3 ubiquitin E3 ligase polyubiquitination (Kobayashi et al., 2004). However, after direct attack by ROS or resulting indirect actions, such as phosphorylation, Nrf2 dissociates from Keap1 and thereby translocates into the nucleus and transactivates its target genes through ARE. Diverse protein kinases have been implicated in the transduction of oxidative stress signals to ARE-mediated gene expression. Moreover, a number of reports have addressed the possible roles played by extracellular signal-regulated kinase (ERK1/2) (Zipper

Abbreviations: Sesn2, sestrin2; ARE, antioxidant response element; t-BHQ, *tert*-butylhydroquinone; ROS, reactive oxygen species; Nrf2, NF-E2-related factor-2; GCS, γ-glutamylcysteine synthetase; HO-1, hemeoxygenase 1; NQO1, NAD(P)H:quinone reductase; ERK1/2, extracellular signal-regulated kinase; PKC, protein kinase C; PI3K, phosphoinositide 3-kinase; AMPK, 5′ AMP-activated protein kinase.

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0041-008X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.taap.2013.10.026

Food and Chemical Toxicology 59 (2013) 362–372

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

O-methylated flavonol isorhamnetin prevents acute inflammation through blocking of NF-κB activation

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ARTICLE INFO

Article history:
Received 27 February 2013
Accepted 30 May 2013
Available online 15 June 2013

Keywords:
Anti-inflammation
IKK
Isorhamnetin
JNK
Nuclear factor-kappa B (NF-κB)

ABSTRACT

Here, we isolated isorhamnetin, a natural 3′-*O*-methylated flavonoid, from water dropwort (*Oenanthe javanica*, *Umbelliferae*) and investigated its ability to protect against acute inflammation *in vivo* and *in vitro*. To induce paw swelling, the hind paw of each rat was injected with a carrageenan 1 h after vehicle or isorhamnetin treatment. *In vitro* effect and mechanism studies were performed in lipopolysaccharide (LPS)-activated macrophages. Administration of isorhamnetin markedly inhibited the swelling volume and the thickness of hind paws. Moreover, isorhamnetin significantly reduced inflammatory cell infiltration and pro-inflammatory gene expression in rats. Isorhamnetin pretreatment inhibited inducible nitric oxide synthase (iNOS) expression and NO release in LPS-stimulated cells. Activation of nuclear factor-kappa B (NF-κB) and activating protein-1 (AP-1) is the key step in the *iNOS* gene induction. Isorhamnetin specifically inhibited NF-κB luciferase activity, but not AP-1. Pretreatment with isorhamnetin suppressed NF-κB nuclear translocation in accordance with decreased phosphorylation and degradation of inhibitory-κB. Consistently, TNF-α, IL-1β and IL-6 expression, representative NF-κB target genes, were almost completely prohibited by isorhamnetin. Furthermore, isorhamnetin inhibited LPS-induced JNK and AKT/IKKα/β phosphorylation. Our results suggest that isorhamnetin inhibited JNK, and AKT/IKKα/β activation, leading to NF-κB inactivation, which might contribute to the inhibition of the acute inflammatory response.

1. Introduction

Water dropwort (*Oenanthe javanica*, *Umbelliferae*) has been widely used in Asian countries as an herbal medicine for the treatment of jaundice, hypertension, diabetes, and abdominal pain (Ma et al., 2010; Park et al., 1995) as well as for food. Isorhamentin, 3′-*O*-methylated metabolite of quercetin and one of a major constituent of *O. javanica*, has been known several pharmacological activities such as anti-oxidative and anti-proliferative effects (Pengfei et al., 2009; Teng et al., 2006). Nevertheless, *in vivo* effect of isorhamnetin and its molecular mechanisms explaining how isorhamnetin suppress the inflammatory response are not elucidated in detail. In the current study, we successfully isolated isorhamnetin from *O. javanica* and examined its anti-inflammatory effect and its molecular mechanism in the carrageenan-induced paw edema animal model and in lipopolysaccharide (LPS)-activated RAW264.7 cells, respectively.

Acute inflammation is a normal protective response of a tissue to injury or destruction and is characterized by typical symptoms of increased blood flow to the tissue, swelling, redness, fever and pain. However, aberrant activation of the acute inflammatory response is thought to induce diverse tissue and organ damage without any benefits (Rankin, 2004). Nitric oxide (NO) exerts pleiotropic signaling as an effector molecule that regulates expression of pro-inflammatory mediators in the early response to invading pathogens (Ricciardolo et al., 2004). NO produced by constitutive nitric oxide synthase (cNOS) regulates crucial cellular functions; however, NO produced by inducible NOS (iNOS) during the inflammatory process plays an important role in the early inflammatory response (Lirk et al., 2002). In addition to NO, tumor

Abbreviations: LPS, lipopolysaccharide; NF-κB, nuclear factor-kappa B; NO, nitric oxide; NOS, nitric oxide synthase; AP-1, activating protein-1; IκB, inhibitory κB; MAPK, mitogen-activated protein kinase; H&E, Hematoxylin & Eosin; IL, interleukin; TNF, tumor necrosis factor; TLR, toll-like receptor; MPO, myeloperoxidase; PI3K, phosphatidylinositol 3-kinase; IKK, IκB kinase.

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http://dx.doi.org/10.1016/j.fct.2013.05.049

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2013, Article ID 763257, 9 pages
http://dx.doi.org/10.1155/2013/763257

Hindawi

Review Article

Role of the Nrf2-ARE Pathway in Liver Diseases

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Received 31 December 2012; Accepted 12 April 2013

Academic Editor: Mi-Kyoung Kwak

The liver is a central organ that performs a wide range of functions such as detoxification and metabolic homeostasis. Since it is a metabolically active organ, liver is particularly susceptible to oxidative stress. It is well documented that liver diseases including hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma are highly associated with antioxidant capacity. NF-E2-related factor-2 (Nrf2) is an essential transcription factor that regulates an array of detoxifying and antioxidant defense genes expression in the liver. It is activated in response to electrophiles and induces its target genes by binding to the antioxidant response element (ARE). Therefore, the roles of the Nrf2-ARE pathway in liver diseases have been extensively investigated. Studies from several animal models suggest that the Nrf2-ARE pathway collectively exhibits diverse biological functions against viral hepatitis, alcoholic and nonalcoholic liver disease, fibrosis, and cancer via target gene expression. In this review, we will discuss the role of the Nrf2-ARE pathway in liver pathophysiology and the potential application of Nrf2 as a therapeutic target to prevent and treat liver diseases.

1. Introduction

The liver is a multifunctional organ that is responsible for detoxification and metabolic homeostasis. It has two blood supply sources: the hepatic artery delivers oxygenated blood from the general circulation and the portal vein supplies deoxygenated but nutrient-rich blood from the intestinal region [1]. Many cell types compose the liver. The parenchymal cells, which are the most abundant in the liver, are hepatocytes (80% by volume) [2]. The nonparenchymal cells such as endothelial cells, Kupffer cells, smooth muscle cells, hepatic stellate cells, and oval cells are other important cell components in the liver [2]. All of these cells can modulate the progression of liver diseases and activate multiple signaling pathways.

The liver is the first organ exposed to orally administered xenobiotics after absorption from the intestine, and it is a major site of biotransformation and metabolism. Since the liver is a metabolically active organ, it is particularly susceptible to reactive oxygen species (ROS). ROS are produced in liver cells as byproducts of normal metabolism and detoxification. Therefore, a wide range of antioxidant systems have developed in the liver, so that when produced, ROS are rapidly destroyed [3]. However, sustained and excessive ROS cause cellular damage and have been linked to a variety of liver diseases. Viral hepatitis and alcoholic or nonalcoholic steatohepatitis are the 3 major causes of chronic liver diseases, which are highly associated with oxidative stress, lead to liver fibrosis, cirrhosis, and end-stage hepatocellular carcinoma (HCC). Therefore, it is generally accepted that oxidative stress plays a key role in promoting the progression of these liver diseases [4].

Elevated ROS and electrophiles induce a series of antioxidant genes through the activation of antioxidant response element (ARE) to protect cells against oxidative stress [5]. ARE-containing gene expression is primarily regulated by NF-E2-related factor-2 (Nrf2), a member of the cap'n'collar family of bZIP transcription factors [6]. Nrf2 is activated in response to oxidative stress and electrophiles in a variety of tissues and cells and plays a role as a multiorgan protector through target gene induction [7]. Keap1 is a negative regulator of Nrf2 and acts as an adaptor protein for functional E3 ubiquitin ligase complex with Cul3 and Rbx1 [8, 9]. In agreement with that, Nrf2 is constitutively accumulated in nuclei in Keap1-knockout mice [10].

Nrf2 activation is observed in nonparenchymal cells including hepatic stellate cells and Kupffer cells as well as in parenchymal hepatocytes [11, 12]. Moreover, many kinds of

Research Article
J Ginseng Res Vol. 37, No. 1, 45-53 (2013)
http://dx.doi.org/10.5142/jgr.2013.37.45

JGR Journal of Ginseng Research

Red ginseng extract protects against carbon tetrachloride-induced liver fibrosis

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Korean red ginseng, the processed root of *Panax ginseng* Meyer, has been frequently used for various therapeutic purposes in oriental medicine. The present study investigated the possible effect of Korean red ginseng extract (RGE) for the treatment of liver fibrosis in mice injected with carbon tetrachloride (CCl_4) for 4 wk. Liver injuries were assessed by blood biochemistry and histopathology in mice treated with CCl_4 alone or CCl_4+ RGE (30, 100, and 300 mg/kg). Concomitant treatment with RGE and CCl_4 (three times/wk for 4 wk) effectively inhibited liver fibrosis as evidenced by decreases in plasma alanine and aspartate aminotransferases, as well as by the percentages of degenerative regions, numbers of degenerative hepatocytes, and collagen accumulation in hepatic parenchyma. Treatment with CCl_4 for 4 wk increased mRNA levels of transforming growth factor β1 and plasminogen activator inhibitor 1 in fibrogenic liver, whereas RGE (30, 100, and 300 mg/kg) significantly blocked the induction of fibrogenic genes by CCl_4. Similarly, RGE also prevented transforming growth factor β1-mediated induction of fibrogenic genes in human hepatic stellate cell lines. More importantly, RGE markedly reduced the number of α-smooth muscle actin-positive cells in liver tissue. This study implies that RGE efficaciously protects against the liver fibrosis induced by chronic CCl_4 treatment, and may therefore have potential to treat liver disease.

Keywords: *Panax ginseng*, Korean red ginseng, Liver fibrosis, Transforming growth factor β1, α-smooth muscle actin

INTRODUCTION

Liver fibrosis is the pathophysiological response in the liver to a line of insults such as toxic, infectious, or metabolic agents [1]. Hepatic fibrosis is characterized by excessive accumulation of extracellular matrix (ECM) including collagen, which is found in most types of chronic liver diseases [1,2]. Accumulated ECM proteins form a fibrous scar that contorts the architecture of the liver. In general, chronic liver injury produces fibrosis of the liver, followed by cirrhosis, which is defined by the development of nodules of regenerating hepatocytes [1,2]. Cirrhosis induces hepatocellular dysfunction and may result in hepatocellular carcinoma, which is one of the most common cancers worldwide [3,4]. Despite the importance of liver fibrosis in human health, there is no efficient agent for clinical application to inhibit the progression of liver fibrosis yet.

It has been shown that carbon tetrachloride (CCl_4) has been used to induce liver injury in various animals

Received 17 Jul. 2012, **Revised** 11 Sep. 2012, **Accepted** 11 Sep. 2012

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Toxicology and Applied Pharmacology 271 (2013) 95–105

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Resveratrol inhibits LXRα-dependent hepatic lipogenesis through novel antioxidant *Sestrin2* gene induction

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ARTICLE INFO

Article history:
Received 24 January 2013
Revised 6 April 2013
Accepted 25 April 2013
Available online 5 May 2013

Keywords:
Resveratrol
Hepatic lipogenesis
Sterol regulatory element binding protein-1c
Liver X receptor-α
Sestrin

ABSTRACT

Liver X receptor-α (LXRα), a member of the nuclear receptor superfamily of ligand-activated transcription factors, regulates *de novo* fatty acid synthesis that leads to stimulate hepatic steatosis. Although, resveratrol has beneficial effects on metabolic disease, it is not known whether resveratrol affects LXRα-dependent lipogenic gene expression. This study investigated the effect of resveratrol in LXRα-mediated lipogenesis and the underlying molecular mechanism. Resveratrol inhibited the ability of LXRα to activate sterol regulatory element binding protein-1c (SREBP-1c) and thereby inhibited target gene expression in hepatocytes. Moreover, resveratrol decreased LXRα–RXRα DNA binding activity and LXRE-luciferase transactivation. Resveratrol is known to activate Sirtuin 1 (Sirt1) and AMP-activated protein kinase (AMPK), although its precise mechanism of action remains controversial. We found that the ability of resveratrol to repress T0901317-induced SREBP-1c expression was not dependent on AMPK and Sirt1. It is well established that hepatic steatosis is associated with antioxidant and redox signaling. Our data showing that expression of Sestrin2 (Sesn2), which is a novel antioxidant gene, was significantly down-regulated in the livers of high-fat diet-fed mice. Moreover, resveratrol up-regulated Sesn2 expression, but not Sesn1 and Sesn3. Sesn2 overexpression repressed LXRα-activated SREBP-1c expression and LXRE-luciferase activity. Finally, Sesn2 knockdown using siRNA abolished the effect of resveratrol in LXRα-induced FAS luciferase gene transactivation. We conclude that resveratrol affects *Sesn2* gene induction and contributes to the inhibition of LXRα-mediated hepatic lipogenesis.

Introduction

Nonalcoholic fatty liver disease (NAFLD), a rising worldwide problem, is one of the most common liver diseases and is defined as fat accumulation in more than 5% of hepatocytes. There are diverse causes of fatty liver; however the two most common factors are alcohol and metabolic syndrome, such as obesity, insulin resistance and cardiovascular disease. Although simple fatty liver itself may be considered a benign disorder, it can progress to hepatitis, fibrosis and eventually lead to irreversible end-stage liver diseases such as cirrhosis and liver cancer. To control the onset and progression of fatty liver, it is necessary to understand and determine the concise mechanism of lipogenesis in hepatocytes. In the regulation of lipogenesis, the nuclear receptor liver X receptor-α (LXRα), a member of the orphan nuclear receptor superfamily of ligand-activated transcription factors, serves as a lipid sensor that increases lipid synthesis in the liver (Joseph et al., 2002) and regulates insulin-stimulated lipogenesis (Chen et al., 2004).

LXRα activation regulates cholesterol homeostasis via its target gene expression (e.g., CYP7A1, ABCA1, ABCG1) associated with cholesterol conversion into bile acid and cholesterol efflux (Repa and Mangelsdorf, 2002; Zhao and Dahlman-Wright, 2010). In addition, LXRα activation results in hepatic steatosis and hypertriglycæmia through *de novo* fatty acid synthesis because this receptor increases the expression of lipogenic genes including fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC) and stearoyl-CoA desaturase-1 (SCD-1) (Grefhorst et al., 2002; Schultz et al., 2000). Sterol regulatory element binding protein-1c (SREBP-1c) is a very important target gene of LXRα activation (Repa et al., 2000) and is a key transcription factor of lipogenic gene expression as described above (Steffensen and Gustafsson, 2004). Therefore, the LXRα–SREBP-1c axis is an attractive target for the prevention and/or treatment of hepatic steatosis.

Resveratrol (3,4′,5-trihydroxystilbene) is a natural polyphenolic component synthesized in grapes and has diverse beneficial effects including antioxidant, anti-tumor and anti-inflammatory activities

Abbreviations: ACC, Acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; DN-AMPK, Dominant-negative AMP-activated protein kinase; FAS, Fatty acid synthase; FXR, Farnesoid X receptor; GSH, glutathione; HFD, High-fat diet; LXRα, Liver X receptor-α; LXRE, Liver X receptor elements; NAFLD, Nonalcoholic fatty liver disease; NASH, Nonalcoholic steatohepatitis; Nrf2, NF-E2-related factor 2; PXR, pregnane X receptor; ROS, Reactive oxygen species; RXR, Retinoid X receptor; SCD-1, Stearoyl-CoA desaturase-1; Sesn, Sestrin; Sirt1, Sirtuin 1; SREBP-1c, Sterol regulatory element binding protein-1c; T090, T0901317.

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http://dx.doi.org/10.1016/j.taap.2013.04.023

Food and Chemical Toxicology 56 (2013) 93–99

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Inhibitory effect of dihydroartemisinin against phorbol ester-induced cyclooxygenase-2 expression in macrophages

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ARTICLE INFO

Article history:
Received 17 September 2012
Accepted 6 February 2013
Available online 19 February 2013

Keywords:
Dihydroartemisinin
COX-2
NF-κB
C/EBP
AP-1
CREB

ABSTRACT

Dihydroartemisinin (DHA), a semi-synthetic derivative of artemisinin isolated from the traditional Chinese herb *Artemisia annua* L., has recently been shown to possess antitumor activity in various cancer cells. However, the effect of anti-inflammatory potentials of DHA in murine macrophage RAW 264.7 cells has not been studied. The present study investigated the effect of COX-2 and molecular mechanisms by DHA in PMA stimulated RAW 264.7 cells. DHA dose-dependently decreased PMA-induced COX-2 expression and PGE_2 production, as well as COX-2 promoter-driven luciferase activity. Additionally, DHA decreased luciferase activity of COX-2 regulation-related transcription factors including NF-κB, AP-1, C/EBP and CREB. DHA also remarkably reduced PMA-induced p65, C/EBPβ, c-jun and CREB nuclear translocation. Furthermore, DHA evidently inhibited PMA-induced phosphorylation of AKT and the MAP Kinases, such as ERK, JNK and p38. Taken together, our data indicated that DHA effectively attenuates COX-2 production via down-regulation of AKT and MAPK pathway, revealing partial molecular basis for the anti-inflammatory properties of DHA.

1. Introduction

Artemisinin, an effective antimalarial drug, is isolated from the traditional Chinese herb *Artemisia annua* L. dihydroartemisinin (DHA), the main active metabolite of artemisinin derivatives, has exhibited the strong anti-cancer effects among the derivatives of artemisinin (Dhingra et al., 2000; Zhou et al., 2005). Many studies have shown that DHA inhibits cell proliferation, induces cell cycle arrest, and promotes apoptosis in human cancer cell lines (Efferth et al., 2001; Lee et al., 2006; Singh and Lai, 2004). Recent hypothesis has been suggesting that artemisinin and its derivatives may be useful as anticancer drugs (Chaturvedi et al., 2010). Previous studies have shown that artemisinin inhibited the production of nitric oxide and the expression of several pro-inflammatory cytokines and matrix metalloproteinases (Hwang et al., 2010; Wang et al., 2009). However, the effect of these anti-inflammatory properties and the signaling pathway of DHA remain unknown.

In experimental practice, RAW 264.7 mouse macrophage cell line stimulated by PMA is widely used as the inflammatory cellular model to study the effect of anti-inflammatory drugs and herbs (Han et al., 2008). Macrophages play an important role in inflammatory disease through the release of cytokines such as TNF-α, IL-1β, IL-6, nitric oxide and other inflammatory mediators (Coussens and Werb, 2002). Cyclooxygenase (COX) catalyzes the conversion of arachidonic acid to prostaglandin E2 in the first step in the biosynthesis of prostanoids. There are two types of COX isoforms: COX-1 and COX-2. COX-1 is constitutively expressed in most tissues, while COX-2 expression is induced by several stimuli, such as mitogens, cytokines, and tumor promoters (Aggarwal et al., 2006). Inducible COX-2 may be responsible for the high prostaglandin levels frequently observed in inflammatory pathology (Jung et al., 2007).

Macrophages stimulated with phorbol 12-myristate 13-acetate (PMA) have been used as a model to overexpress the COX-2 role in the cell differentiation. PMA-induced COX-2 promoter activity requires several enhancer elements including nuclear factor-κB (NF-κB, −223/−214), CCAAT/enhancer-binding protein (C/EBP, −132/−124), and activator protein 1 (AP-1)/cyclic adenosine monophosphate (cAMP)-response element (CRE, −59/−53), which are important for regulating its transcription (Saunders et al., 2001; Schroer et al., 2002). In addition, COX-2 expression also activate mitogen activated protein (MAP) kinases (Han et al., 2008). MAPKs and Akt have been extensively studied relative to their regulation

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http://dx.doi.org/10.1016/j.fct.2013.02.017

Food and Chemical Toxicology 50 (2012) 1616–1622

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

1-Bromopropane up-regulates cyclooxygenase-2 expression via NF-κB and C/EBP activation in murine macrophages

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ARTICLE INFO

Article history:
Received 14 November 2011
Accepted 1 February 2012
Available online 14 February 2012

Keywords:
1-Bromopropane
COX-2
NF-κB
C/EBP
Akt
MAP kinases

ABSTRACT

1-Bromopropane (1-BP) has been used in industry as an alternative to ozone-depleting solvents. In the present study, we examined the effect of 1-BP on cyclooxygenase-2 (COX-2) gene expression and analyzed the molecular mechanism of its activity in murine RAW 264.7 macrophages. 1-BP dose-dependently increased COX-2 protein and mRNA levels, as well as COX-2 promoter-driven luciferase activity in macrophages. Additionally, exposure to 1-BP markedly enhanced the production of prostaglandin E_2 (PGE_2), a major COX-2 metabolite, in macrophages. Transfection experiments with several human COX-2 promoter constructs revealed that 1-BP activated the transcription factors nuclear factor-κB (NF-κB) and CCAAT/enhancer-binding protein (C/EBP), but not AP-1 or the cyclic AMP response element binding protein. Furthermore, Akt and mitogen-activated protein (MAP) kinases were significantly activated by 1-BP. These results demonstrated that 1-BP induced COX-2 expression via NF-κB and C/EBP activation through the Akt/ERK and p38 MAP kinase pathways. These findings provide further insight into the signal transduction pathways involved in the inflammatory effects of 1-BP.

1. Introduction

Cyclooxygenase (COX) catalyzes the synthesis of prostaglandins (PGs) from arachidonic acid. The two COX isozymes are encoded by separate genes, COX-1 and COX-2. The COX-1 isozyme is a housekeeping protein that is present in most tissues and catalyzes the synthesis of PGs for normal physiological functions. In contrast, COX-2 is not present under normal physiological conditions, but is rapidly induced by various tumor promoters, growth factors, cytokines, mitogens, and carcinogens in various cell types (Prescott and Fitzpatrick, 2000). Inducible COX-2 could be responsible for the high prostaglandins observed in much inflammatory pathology (Prescott and Fitzpatrick, 2000). In the COX-2 gene, promoter elements enable binding of nuclear factor-κB (NF-κB, −223/−214), CCAAT/enhancer-binding protein (C/EBP, −132/−124), and activator protein 1 (AP-1)/cyclic adenosine monophosphate (cAMP)-response element (CRE, −59/−53), which are important for regulating its transcription (Han et al., 2008a). NF-κB and C/EBP are known to regulate the transcription of the COX-2 gene in macrophage-like cells (Wadleigh et al., 2000). A CRE binding site is required for induction of COX-2 expression by viral oncogenes and mitogenic factors in murine cells (Thomas et al., 2000). AP-1 is a transcription factor that has been shown to play a critical role in promoting carcinogenesis (Han et al., 2008a). However, the relative contribution of the various promoter elements to COX-2 transcription in macrophages is not yet completely understood. Several inflammatory stimuli that induce COX-2 gene expression also activate mitogen-activated protein (MAP) and PI3K/Akt kinases (Pommery and Hénichart, 2005). MAP kinases are serine/threonine protein kinases that govern various cellular processes, including cell growth, proliferation, differentiation, and apoptosis (Whitmarsh and Davis, 1996). MAP kinases can be divided into three main subfamilies: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38. PI3K is a heterodimeric protein that consists of a catalytic subunit (p110) and a regulatory subunit (p85); PI3K signaling is frequently deregulated in carcinogenesis (Roymans and Slegers, 2001). The serine/threonine kinase Akt is activated downstream of PI3K and has a well-established role in promoting cell survival (Franke et al., 2003). Here, we hypothesized that the MAP kinases, Akt signaling pathways,

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doi:10.1016/j.fct.2012.02.006

ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY 32 (2011) 136–145

available at www.sciencedirect.com

ScienceDirect

Journal homepage: www.elsevier.com/locate/etap

Psidium guajava extract inhibits thymus and activation-regulated chemokine (TARC/CCL17) production in human keratinocytes by inducing heme oxygenase-1 and blocking NF-κB and STAT1 activation

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ARTICLE INFO

Article history:
Received 13 January 2011
Received in revised form
30 March 2011
Accepted 9 April 2011
Available online 17 April 2011

Keywords:
Psidium guajava
Atopic dermatitis
TARC
Heme oxygenase-1

ABSTRACT

Psidium guajava (*P. guajava*) is a food and medicinal plant with antioxidant, anti-inflammatory, and anti-allergic activities that support its traditional uses. The aim of this study was to determine the effects of *P. guajava* ethyl acetate extract (PGEA) on atopic dermatitis and to investigate the possible mechanisms by which PGEA inhibits cytokine-induced Th2 chemokine expression in HaCaT human keratinocyte cells. We found that PGEA suppressed the IFN-γ/TNF-α-co-induced production of thymus and activation-regulated chemokine (TARC) protein and mRNA in HaCaT cells. Additionally, PGEA inhibited the TNF-α/IFN-γ-co-induced activation of NF-κB and STAT1 and increased the expression of heme oxygenase-1 (HO-1) protein and mRNA. HO-1 inhibitor enhanced the suppressive effects of PGEA on TNF-α/IFN-γ-co-induced TARC production and gene expression. Collectively, these data demonstrate that PGEA inhibits chemokine expression in keratinocytes by inducing HO-1 expression and it suggests a possible therapeutic application in atopic dermatitis and other inflammatory skin diseases.

1. Introduction

Atopic dermatitis is a complex eczematous skin disease, accompanied by severe itching, that is affected by both genetic and environmental factors (Lee et al., 2007). Atopic dermatitis is estimated to affect approximately 8–25% of the human population worldwide, and its incidence is increasing (Weston and Howe, 2008). The inflammatory infiltrates in skin lesions consist not only of lymphocytes, but also of macrophages, eosinophils, mast cells, and Langerhans cells (Rudikoff and Lebwohl, 1998; Breuer et al., 2006).

Skin inflammatory processes are highly dependent on Th2 chemokine family (Pivarcsi and Homey, 2005). The chemokines are a superfamily of small cytokines that regulate trafficking of various types of leukocytes (Qi et al., 2009). Thymus and activation-regulated chemokine (TARC/CCL17), a Th2-type CC chemokine, is constitutively expressed in the thymus and is also produced by keratinocytes (Vestergaard et al., 1999), dendritic cells (Imai et al., 1996; Sallusto et al., 1998), endothelial cells (Campbell et al., 1999), bronchial epithelial cells (Sekiya et al., 2000), and fibroblasts (Yu et al., 2002). TARC is a ligand for CCR4, which is predominantly expressed on Th2 lymphocytes, basophils, and natural killer cells (Sallusto

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doi:10.1016/j.etap.2011.04.004

Food and Chemical Toxicology 49 (2011) 1416–1425

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Inhibitory effect of *Pleurotus eryngii* extracts on the activities of allergic mediators in antigen-stimulated mast cells

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ARTICLE INFO

Article history:
Received 7 February 2011
Accepted 19 March 2011
Available online 31 March 2011

Keywords:
Pleurotus eryngii
Anti-allergic activity
Mast cells
IL-4
NF-κB
COX-2

ABSTRACT

Pleurotus eryngii is an edible mushroom native to Europe, the Middle East, and North Africa, and is also grown in parts of Asia. The present study investigated the anti-allergy potential of *P. eryngii* extract (PEE) in antigen-stimulated RBL-2H3 mast cells. PEE inhibited allergy markers, including release of hexosaminidase and histamine, in antigen-sensitized RBL-2H3 cells. PEE also suppressed the expression and production of interleukin-4 and reduced antigen-induced NFAT and NF-κB transcriptional activity in antigen-sensitized mast cells. Moreover, PEE decreased the levels of proinflammatory cytokines and COX-2 and iNOS expression in antigen-sensitized mast cells. Finally, PEE suppressed antigen-induced signal protein phosphorylation of lyn, PLCγ2, PKC, Akt, and MAP kinases. Taken together, these results suggest that *P. eryngii* extract may provide insight into the prevention and treatment of allergic and inflammatory diseases.

1. Introduction

Mast cells are a major source of inflammatory mediators, including preformed mediators stored in secretory granules, cytokines, and lipid-derived eicosanoids (Thomas, 2001). After stimulation with antigen, mast cells release β-hexosaminidase, a marker of mast cell degranulation, and various allergic mediators such as histamine, cytokines, and arachidonic acid derivatives (Gilfillan and Tkaczyk, 2006). IgE-induced release of inflammatory mediators from mast cells causes the immediate symptoms of allergic diseases, including asthma, atopic dermatitis, and atopic eczema (Wedemeyer et al., 2000). Interleukin (IL)-4 is essential for IgE production (Kuhn et al., 1991) and promotes the switch from naïve T cells to Th2 cells (Hines, 2002).

The activation of mast cells is induced partially through the induction of nuclear factor κB (NF-κB) and nuclear factor of activated T-cells (NFAT) by antigen (Saito et al., 2009). NF-κB is a key transcription factor and regulates the expression of genes involved in immune and inflammatory responses (Marquardt and Walker, 2000). NFAT is a transcription complex and is required for the transcriptional regulation of immune responses and cytokines (Palanki, 2002).

The release of allergic mediators from mast cells can be stimulated through the antigen-induced aggregation of receptors with high affinity for IgE (FcεRI). This leads to the activation of signaling pathways that initially depend on an interaction of FcεRI with the Src kinase Lyn and subsequent downstream activation of Syk and other tyrosine kinases (Gilfillan and Tkaczyk, 2006). Degranulation of mast cells stimulated with IgE is markedly impaired, as is the activity of the downstream signaling molecules phosphatidylinositol 3-kinase (PI3-kinase) and Akt (Fukao et al., 2003). Furthermore, mitogen-activated protein kinase (MAP kinase) signaling cascades are important in the differentiation, activation, proliferation, degranulation, and migration of various immune cells, including mast cells (Duan and Wong, 2006). MAP kinase signaling modules are divided into at least three groups: extracellular signal-regulated kinase (ERK), p38 MAP kinase, and c-Jun NH_2-terminal kinase (JNK).

Several studies have reported that mast cells (Matsui et al., 2000) release reactive oxygen species (ROS) into the extracellular milieu in response to a range of stimuli, and ROS from mast cells are involved in allergic inflammation (Springer et al., 2007). Cyclooxygenase (COX) is the key enzyme responsible for the conversion of arachidonic acid, via oxygenation, to prostaglandins (PGs), which are pleiotropic mediators of immune responses (Hinz and Brune, 2002). COX-2 is inducible and plays a role in inflammatory cells such as macrophages, fibroblasts, and endothelial cells (Lim, 2010). Like COX-2, inducible nitric oxide synthase (iNOS) is also

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doi:10.1016/j.fct.2011.03.034

Food and Chemical Toxicology 49 (2011) 100–108

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Ethyl acetate extract of *Psidium guajava* inhibits IgE-mediated allergic responses by blocking FcεRI signaling

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ARTICLE INFO

Article history:
Received 24 July 2010
Accepted 2 October 2010

Keywords:
Psidium guajava
Anti-allergic activity
Mast cells
IL-4
NF-κB
Syk

ABSTRACT

Psidium guajava (*P. guajava*) is an important food crop and medicinal plant with antioxidant, anti-inflammatory, and anti-allergic activities, supporting its traditional uses. However, its precise effects remain unknown. We investigated the effects of *P. guajava* ethyl acetate extract (PGEA) on IgE-mediated allergic responses in rat mast RBL-2H3 cells. PGEA reduced antigen (DNP–BSA)-induced release of β-hexosaminidase and histamine in IgE-sensitized RBL-2H3 cells. In addition, it inhibited antigen-induced IL-4 and TNF-α mRNA expression and protein production in IgE-sensitized RBL-2H3 cells. PGEA also suppressed antigen-induced COX-2 mRNA and protein expression in these cells, as well as antigen-induced activation of NFAT and reactive oxygen species. Moreover, it inhibited antigen-induced activation of NF-κB and degradation of IκB-α. To identify the mechanisms underpinning the inhibition of degranulation and cytokine production by PGEA, we examined the activation of intracellular FcεRI signaling molecules. PGEA suppressed antigen-induced phosphorylation of Syk, LAT, Gab2, and PLCγ2 but not lyn, and inhibited antigen-induced phosphorylation of downstream signaling intermediates including MAP kinases and Akt. Collectively, the anti-allergic effects of PGEA *in vitro* suggest its possible therapeutic application to inflammatory allergic diseases, in which its inhibition of inflammatory cytokine production and FcεRI-dependent signaling events in mast cells may be hugely beneficial.

1. Introduction

Mast cells are secretory cells that are central to specific and innate immunity, allergic responses, and inflammation (McDermott et al., 2003; McLachlan et al., 2003). They also play an important role in initiating and perpetuating inflammatory responses in allergic reactions by secreting large amounts of cytokines such as interleukin-4 (IL-4) and tumor necrosis factor (TNF)-α (Bradding et al., 1994). In response to antigen stimulation, naive T helper cells differentiate into at least two types of effector cells, classified according to their distinct patterns of cytokine expression, as well as their different effects on ongoing immune responses (Kiani et al., 1997). The hallmark of T helper 1 (Th1) cells is the secretion of interferon-γ (IFNγ), which primarily promotes cell-mediated immunity, while Th2 cells secrete IL-4, IL-5, IL-10, and IL-13 and activate immunoglobulin E (IgE) and mediate eosinophilic responses. IL-4 is essential for IgE production (Kuhn et al., 1991) and promotes the differentiation of naive T cells into Th2 cells (Hines, 2002). It also acts as a mast cell growth factor *in vitro* and down-regulates high-affinity IgE receptor (FcεRI) expression on mouse bone marrow-derived mast cells (Bischoff et al., 1999). The activation of mast cells by antigen is partially realized through the induction of nuclear factor κB (NF-κB) and nuclear factor of activated T-cells (NFAT) (Saito et al., 2009). NF-κB is thought to play an important role in the regulation of proinflammatory cytokines, notably TNF-α, IL-6, and IL-8 (Salamon et al., 2005). It is a transcription factor that regulates the expression of genes involved in immune and inflammatory responses that involve inflammatory cytokine production (Marquardt and Walker, 2000). NFAT is a transcription complex believed to mediate the final step in the signal transduction pathway linking T-cell receptor engagement with the expression of IL-2 (Hutchinson and McCloskey, 1995).

In cultured mast cells, including the well-studied RBL-2H3 cell line, free arachidonic acid is rapidly metabolized to prostaglandin by cyclooxygenase (COX) during allergic reactions (Hundley

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doi:10.1016/j.fct.2010.10.003

Toxicology Letters 198 (2010) 331–341

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

The role of cyclooxygenase-2-dependent signaling via cyclic AMP response element activation on aromatase up-regulation by o,p′-DDT in human breast cancer cells

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ARTICLE INFO

Article history:
Received 25 June 2010
Received in revised form 24 July 2010
Accepted 26 July 2010
Available online 1 August 2010

Keywords:
o,p′-DDT
Aromatase
COX-2
Breast cancer

ABSTRACT

o,p′-Dichlorodiphenyltrichloroethane (o,p′-DDT) is a DDT isomer and xenoestrogen that can induce inflammation and cancer. However, the effect of o,p′-DDT on aromatase is unclear. Thus, we investigated the effects of o,p′-DDT on aromatase expression in human breast cancer cells. We also examined whether cyclooxygenase-2 (COX-2) is involved in o,p′-DDT-mediated aromatase expression. Treatment with o,p′-DDT-induced aromatase protein expression in MCF-7 and MDA-MB-231 human breast cancer cells; enhancing aromatase gene expression, and enzyme and promoter activity. Treatment with ICI 182.780, a estrogen receptor antagonist, did not affect the inductive effects of o,p′-DDT on aromatase expression. In addition, o,p′-DDT increased COX-2 protein levels markedly, increased COX-2 mRNA expression and promoter activity, enhanced the production of prostaglandin E_2 (PGE_2), induced cyclic AMP response element (CRE) activation, and cAMP levels and binding of CREB. o,p′-DDT also increased the phosphorylation of PKA, Akt, ERK, and JNK in their signaling pathways in MCF-7 and MDA-MB-231 cells. Finally, o,p′-DDT induction of aromatase was inhibited by various inhibitors [COX-2 (by NS-398), PKA (H-89), PI3-K/Akt (LY 294002), EP2 (AH6809), and EP4 receptor (AH23848)]. Together, these results suggest that o,p′-DDT increases aromatase, and that o,p′-DDT-induced aromatase is correlated with COX-2 up-regulation, mediated via the CRE activation and PKA and PI3-kinase/Akt signaling pathways in breast cancer cells.

1. Introduction

Breast cancer is one of the most common types of cancer among women throughout the world and it is the second leading cause of cancer deaths among women. Most breast tumors are hormone-dependent and express estrogen receptors; their growth is regulated by potent estrogenic hormones, such as estradiol. The enzyme responsible for the production of estradiol is aromatase, a member of the cytochrome P450 family of enzymes (Brodie et al., 1998). Aromatase is encoded by the CYP19 gene and is regulated in a complex, tissue-specific manner (Bulun et al., 2003, 2004; Simpson, 2004). In breast tumors, aromatase is stimulated through cyclic adenosine monophosphate (cAMP)-mediated pathways (Zhao et al., 1996; Sebastian et al., 2002; Zhou et al., 2001; Bulun et al., 2003, 2004; Simpson, 2004). Another factor that has been shown to regulate the activity of the aromatase enzyme is the bioactive lipid, prostaglandin E_2 (PGE_2). PGE_2 is formed through the activity of the cyclooxygenase-2 (COX-2) enzyme, which catalyzes the rate-limiting step in the biosynthesis of prostanoids from arachidonic acid (Hla et al., 1999; Simpson, 2004). Recent studies have demonstrated that COX-2 is produced in most human breast tumors with invasive characteristics, regardless of their hormone receptor status (McCarthy et al., 2006), suggesting that COX-2 may play a more central role in mediating breast tumor development and progression than was previously believed. The observation that human breast tumors overexpress COX-2 is consistent with the findings that a wide variety of human tumors of epithelial origin exhibit increased expression of COX-2 mRNA and protein and produce high levels of PGE_2. Furthermore, COX-2 is up-regulated in

Abbreviations: CRE, cyclic AMP response element; COX-2, cyclooxygenase-2; ER, estrogen receptor; o,p′-DDT, o,p′-dichlorodiphenyltrichloroethane; PGE_2, prostaglandin E_2.

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doi:10.1016/j.toxlet.2010.07.015