Free Radical Biology & Medicine 52 (2012) 671–682

Contents lists available at SciVerse ScienceDirect

Free Radical Biology & Medicine

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

Original Contribution

Sulforaphane attenuates hepatic fibrosis via NF-E2-related factor 2-mediated inhibition of transforming growth factor-β/Smad signaling

Chang Joo Oh [a,1], Joon-Young Kim [a,1], Ae-Kyung Min [a], Keun-Gyu Park [a], Robert A. Harris [a,b], Han-Jong Kim [a,*], In-Kyu Lee [a,*]

[a] Department of Internal Medicine and Department of Biochemistry and Cell Biology, Research Institute of Aging and Metabolism, WCU Program, Kyungpook National University School of Medicine, Daegu 700-721, Republic of Korea
[b] Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202-5122, USA

ARTICLE INFO

Article history:
Received 3 June 2011
Revised 12 October 2011
Accepted 9 November 2011
Available online 1 December 2011

Keywords:
Sulforaphane
Hepatic fibrosis
TGF-β/Smad signaling pathway
Nrf2
Antioxidant genes
Free radicals

ABSTRACT

Sulforaphane (SFN) is a dietary isothiocyanate that exerts chemopreventive effects via NF-E2-related factor 2 (Nrf2)-mediated induction of antioxidant/phase II enzymes, such as heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO1). This work was undertaken to evaluate the effects of SFN on hepatic fibrosis and profibrotic transforming growth factor (TGF)-β/Smad signaling, which are closely associated with oxidative stress. SFN suppressed TGF-β-enhanced expression of α-smooth muscle actin (α-SMA), a marker of hepatic stellate cell (HSC) activation, and profibrogenic genes such as type I collagen, fibronectin, tissue inhibitor of matrix metalloproteinase (TIMP)-1, and plasminogen activator inhibitor (PAI)-1 in hTERT, an immortalized human HSC line. SFN inhibited TGF-β-stimulated activity of a *PAI-1* promoter construct and $(CAGA)_9$ MLP-Luc, an artificial Smad3/4-specific reporter, in addition to reducing phosphorylation and nuclear translocation of Smad3. Nrf2 overexpression was sufficient to inhibit the TGF-β/Smad signaling and PAI-1 expression. Conversely, knockdown of Nrf2, but not inhibition of HO-1 or NQO1 activity, significantly abolished the inhibitory effect of SFN on $(CAGA)_9$ MLP-Luc activity. However, inhibition of NQO1 activity reversed repression of TGF-β-stimulated expression of type I collagen by SFN, suggesting the involvement of antioxidant activity of SFN in the suppression of Smad-independent fibrogenic gene expression. Finally, SFN treatment attenuated the development and progression of early stage hepatic fibrosis induced by bile duct ligation in mice, accompanied by reduced expression of type I collagen and α-SMA. Collectively, these results show that SFN elicits an antifibrotic effect on hepatic fibrosis through Nrf2-mediated inhibition of the TGF-β/Smad signaling and subsequent suppression of HSC activation and fibrogenic gene expression.

Hepatic fibrosis is a dynamic and progressive process characterized by excessive deposition of extracellular matrix (ECM)[2], including collagen and fibronectin, as a common consequence of most types of chronic liver disease [1–3]. This chronic process distorts hepatic architecture and impairs normal function, ultimately leading to cirrhosis, an end stage of liver disease that is associated with significant morbidity and mortality. Excessive ECM accumulation is the result of an imbalance between synthesis (fibrogenesis) and degradation of ECM (fibrolysis), i.e., an excess of fibrogenesis over fibrolysis [2–5].

Transforming growth factor (TGF)-β is considered the most prominent profibrotic cytokine that contributes to the development of hepatic fibrosis through modulation of both synthesis and degradation of ECM proteins [5,6].

TGF-β signals through a heteromeric complex of type I and type II serine/threonine kinase receptors that phosphorylate Smad2 and Smad3 (R-Smads) proteins, the canonical downstream effectors of TGF-β signaling [7,8]. Upon phosphorylation, R-Smads form a heteromeric complex with Smad4 (Co-Smad) and translocate into the nucleus, where they bind to and transactivate the "CAGA" consensus sequence in the promoters of the TGF-β-responsive fibrogenic genes such as collagen and fibronectin [8,9]. In fibrotic liver, enhanced TGF-β signaling stimulates transdifferentiation of hepatic stellate cells (HSCs) to α-smooth muscle actin (α-SMA)-positive myofibroblasts that are the main producers of excessive ECM proteins [4–6,9]. By contrast, TGF-β suppresses the activity of ECM-degrading proteases, such as matrix metalloproteinases, via upregulation of their inhibitors, including tissue inhibitor of matrix

Abbreviations: SFN, DL-sulforaphane; Nrf2, NF-E2-related factor 2; TGF-β, transforming growth factor-β; BDL, bile duct ligation; TIMP-1, tissue inhibitor of matrix metalloproteinase-1; PAI-1, plasminogen activator inhibitor-1; ECM, extracellular matrix; HSC, hepatic stellate cell; α-SMA, α-smooth muscle actin; EMT, epithelial–mesenchymal transition; ARE, antioxidant response element; HO-1, heme oxygenase-1; NQO1, NAD(P)H quinone oxidoreductase 1; ALK5, active activin-like receptor kinase 5.

* Corresponding authors. Fax: +82 53 426 6722.
E-mail addresses: jongikim@hotmail.com (H.-J. Kim), leei@knu.ac.kr (I.-K. Lee).

[1] These authors equally contributed to this work.

0891-5849/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2011.11.012

metalloproteinase (TIMP)-1 and plasminogen activator inhibitor (PAI)-1, leading to decreased ECM degradation [1–6,9,10]. The TGF-β-induced expression of these multiple fibrogenic genes is mediated primarily by a Smad-dependent mechanism. It has also been well documented that TGF-β increases reactive oxygen species (ROS), which mediate the profibrogenic effects of TGF-β via a Smad-independent pathway [11]. Therefore, inhibition of both Smad-dependent and Smad-independent TGF-β signaling pathways has been suggested as a promising therapeutic strategy for the treatment of hepatic fibrosis [11–13].

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a member of the cap'n'collar family of basic leucine-zipper transcription factors [14]. Nrf2 protects a variety of tissues and cells against oxidative stress and electrophiles through antioxidant response element (ARE)-mediated induction of diverse antioxidant and phase II detoxification enzymes such as heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), and glutathione *S*-transferase [15,16]. Studies in genetically modified mice have demonstrated that Nrf2 plays a protective role against hepatic diseases. Nrf2-deficient mice exhibited increased sensitivity and susceptibility to hepatic injury and fibrosis induced by hepatotoxins such as acetaminophen and CCl_4 [17–19]. Activation of Nrf2 by curcumin attenuated dimethylnitrosamine-induced liver injury and hepatic fibrosis in rats [20]. These proven hepatoprotective actions of Nrf2 were achieved mainly through the ARE-mediated induction of antioxidant/detoxifying enzymes. Thus, genetic or pharmacological activation of Nrf2 has been suggested to offer a novel therapeutic approach to the prevention and treatment of liver injury and fibrosis [21,22]. However, the additional role of Nrf2, beyond its antioxidant/detoxification properties, in protection against hepatic fibrosis has not been intensively investigated.

Sulforaphane (SFN) is an isothiocyanate derived from the myrosinase-catalyzed hydrolysis of glucoraphanin found in cruciferous vegetables, especially in broccoli [23,24]. Accumulating evidence suggests that SFN is a promising chemopreventive agent via Nrf2-dependent induction of phase II detoxifying enzymes. Moreover, recent studies suggest that the chemopreventive effect of SFN is mediated by multiple mechanisms, including induction of cell cycle arrest and apoptosis and inhibition of angiogenesis and metastasis [24–26]. Despite its proven chemopreventive efficacy, the potential effects of SFN on hepatic fibrosis and the profibrotic TGF-β/Smad signaling pathway have not been evaluated yet. In this study, we demonstrate a protective effect of SFN against hepatic fibrosis induced by bile duct ligation (BDL) in mice, along with reduced expression of α-SMA and type I collagen. We also demonstrate that the antifibrotic effect of SFN is attributed to the Nrf2-mediated but antioxidant-independent inhibition of TGF-β/Smad signaling, as well as inhibition of Smad-independent pathways in TGF-β signaling via its antioxidant activity.

Materials and methods

Reagents and plasmids

SFN, recombinant human TGF-β1, tin protoporphyrin IX (SnPP), and reduced L-glutathione (GSH) were purchased from Alexis Biochemicals (San Diego, CA, USA), R&D Systems (Minneapolis, MN, USA), Frontier Scientific (Logan, UT, USA), and Sigma–Aldrich (St. Louis, MO, USA), respectively. ES936, an inhibitor of NQO1 activity, was donated by Mazence (Suwon, Korea). The reporter plasmids $(CAGA)_9$ MLP-Luc and PAI-1-Luc (−850 to +20) were kindly donated by Drs. Jean-Michel Gauthier (Laboratoire GlaxoWellcome, France) and Bernd R. Binder (University of Vienna, Austria), respectively. The mammalian expression plasmids pcDNA3HA-ALK5TD, encoding constitutively active activin-like receptor kinase 5 (ALK5), and pcDNA3-Nrf2 were kind gifts from Drs. Carl-Henrik Heldin (Ludwig Institute for Cancer Research, Sweden) and Mi-Kyoung Kwak (Yeungnam University, Korea), respectively.

Preparation of recombinant adenovirus

Full-length mouse Nrf2 cDNA was inserted into the *Kpn*I and *Xho*I sites of the pAdTrack-CMV shuttle vector. As previously described [27], the recombinant adenoviral plasmid was generated, and recombinant adenoviruses were amplified in HEK-293 cells and purified.

Cell culture and transient transfection

hTERT, an immortalized human hepatic stellate cell line, was cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and antibiotics. AML12, a normal mouse hepatocyte cell line, was cultured in DMEM/F-12 medium (Invitrogen) supplemented with 10% FBS, a mixture of insulin–transferrin–selenium (Invitrogen), dexamethasone (40 ng/ml; Sigma–Aldrich), and antibiotics. Human hepatoma HepG2 cells were cultured in MEM supplemented with 10% FBS and antibiotics. For transient transfection assay, 24 h after being seeded in 24-well plates, HepG2 or AML12 cells were transfected with 300 ng/well of reporter plasmids and mammalian expression plasmids encoding Nrf2 (100 or 300 ng/well) or ALK5 (50 ng/well) using TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI, USA) or Lipofectamine Plus (Invitrogen), respectively. HepG2 cells were cotransfected with $(CAGA)_9$ MLP-Luc and 50 nmol/L Nrf2 small interfering RNA (siRNA) (sense—GCUUUUGGCG-CAGACAUUCdTdT and antisense—GAAUGUCUGCGCCAAAAGCdTdT) or control scramble siRNA (sense—CCUACGCCACCAAUUUCGUdTdT and antisense—ACGAAAUUGGUGGCGUAGGdTdT) duplexes (Bioneer Corp., Daejeon, Korea) using both TransIT-LT1 and TransIT-TKO transfection reagents (Mirus Bio). Cytomegalovirus–β-galactosidase plasmids were cotransfected as an internal control. Luciferase activity was normalized to that of β-galactosidase.

Semiquantitative reverse transcription (RT)-PCR and quantitative real-time (qRT)-PCR analyses

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions and semiquantitative RT-PCR analysis was performed as described previously [27]. Two micrograms of total RNA was reverse transcribed using the First Strand cDNA synthesis kit (Fermentas, Burlington, ON, Canada) according to the manufacturer's protocol. The first-strand cDNAs were amplified by PCR using gene-specific primers (Table 1) to determine mRNA expression levels. Quantitative real-time PCR was carried out using the Power SYBR Green PCR Master Mix with the StepOnePlus real-time PCR System (Applied Biosystems, Warington, UK). Gene-specific primer pairs used in qRT-PCR analysis are shown in Table 2. The expression levels of β-actin (semiquantitative RT-PCR) and GAPDH (qRT-PCR) were used as internal controls.

Western blot analysis

Western blot analysis was performed as described previously [27] using specific primary antibodies [anti-Nrf2 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), anti-PAI-1, anti-fibronectin (BD Biosciences, San Jose, CA, USA), anti-type I collagen, anti-α-SMA, anti-β-actin (Abcam, Cambridge, MA, USA), anti-phospho-Smad2(Ser465/467), anti-phospho-Smad3(Ser423/425), anti-Smad2, anti-Smad3, and anti-Smad4 antibodies (Cell Signaling, Beverly, MA, USA)]. For Western blot analysis of type I collagen, cell lysates were subjected to naive PAGE (nonheating and nondenaturing conditions).

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation assay was performed as described previously [28]. Briefly, HepG2 cells were fixed with 1%