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Sulforaphane protects against acetaminophen-induced hepatotoxicity

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ABSTRACT

Oxidative stress is closely associated with acetaminophen (APAP)-induced toxicity. Heme oxygenase-1 (HO-1), an antioxidant defense enzyme, has been shown to protect against oxidant-induced tissue injury. This study investigated whether sulforaphane (SFN), as a HO-1 inducer, plays a protective role against APAP hepatotoxicity *in vitro* and *in vivo*. Pretreatment of primary hepatocyte with SFN induced nuclear factor E2-factor related factor (Nrf2) target gene expression, especially HO-1 mRNA and protein expression, and suppressed APAP-induced glutathione (GSH) depletion and lipid peroxidation, which eventually leads to hepatocyte cell death. A comparable effect was observed in mice treated with APAP. Mice were treated with 300 mg/kg APAP 30 min after SFN (5 mg/kg) administration and were then sacrificed after 6 h. APAP alone caused severe liver injuries as characterized by increased plasma AST and ALT levels, GSH depletion, apoptosis, and 4-hydroxynonenal (4-HNE) formations. This APAP-induced liver damage was significantly attenuated by pretreatment with SFN. Furthermore, while hepatic reactive oxygen species (ROS) levels were increased by APAP exposure, pretreatment with SFN completely blocked ROS formation. These results suggest that SFN plays a protective role against APAP-mediated hepatotoxicity through antioxidant effects mediated by HO-1 induction. SFN has preventive action in oxidative stress-mediated liver injury.

1. Introduction

The liver, as the major site of drug metabolism, is also a major site of drug injury. Drug-induced liver injury continues to be a problem for many commonly used drugs, and represents a major challenge in designing potential therapies (Kaplowitz, 2005; Thames, 2004). Acetaminophen (APAP) is a widely used analgesic and antipyretic drug known to cause diffuse centrilobular hepatic necrosis at toxic doses (Mitchell et al., 1973a). At therapeutic dosage levels, the major elimination pathways of APAP are conjugation with glucuronide or sulfate in hepatocytes (Nelson, 1990). The remaining small amount of APAP is metabolized by the cytochrome P450 system. APAP toxicity is associated with the depletion of hepatic glutathione (GSH), followed by covalent binding of the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) (Corcoran et al., 1980; Ray et al., 1993). Under conditions of APAP overdose, the glucuronidation and sulfation routes become saturated and more extensive bioactivation of APAP occurs, leading to rapid depletion of hepatic GSH. As a result, NAPQI covalently binds to other cellular proteins, leading to the formation of APAP-protein adducts, and consequently, liver failure (Jollow et al., 1973). Previous reports have demonstrated that the sites of oxidative stress in the liver correlate with the sites of APAP toxicity (Hinson et al., 1998; Noh et al., 2013; Roberts et al., 1991). Furthermore, lipid peroxidation resulting from oxidative stress contributes to the initiation and progress of liver damage (Albano et al., 1985). These findings suggest that removal of oxidative stress is a protective mechanism against the development of APAP hepatotoxicity. Thus, natural compounds or detoxifying enzymes that contribute to enhancing intracellular antioxidant potential have importance in the protection or treatment of such injury. If APAP-induced liver injury is detected early, it can be readily treated with N-acetylcysteine (NAC), a cysteine prodrug and precursor of GSH, generally without significant liver injury (Rumack et al., 1981). NAC protects against rapid depletion of hepatic GSH, which supports the detoxification of NAPQI (Corcoran et al., 1985; Lauterburg et al., 1983). Unfortunately, many cases of APAP-induced hepatotoxicity are not diagnosed or seen early enough, leaving few treatment options to prevent liver injury.

Sulforaphane (SFN) is a dietary isothiocyanate, which is synthesized from a precursor found in cruciferous vegetables of the genus Brassica such as cauliflower, broccoli, kale, cole crops, cabbage,

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collards and Brussels sprouts, mustard, and cress, as well as in other genera such as the radish (*Raphanus* sp.) (Fahey et al., 2001; van Poppel et al., 1999). SFN has been widely studied since the 1980s given that it has chemotherapeutic properties, including anti-proliferative and anti-angiogenic properties (Guerrero-Beltran et al., 2012). In addition to its chemotherapeutic properties, previous studies have found that SFN induces nuclear factor E2-factor related factor (Nrf2)-mediated-phase 2 detoxification enzymes that elevate cell defense against oxidative damage (Hu et al., 2004; Yu et al., 1999). A large body of research has also demonstrated the protective effect of SFN on oxidative stress-damaged tissues including the brain, kidney, and heart, among others (Guerrero-Beltran et al., 2010; Ping et al., 2010; Priya et al., 2011; Song et al., 2009; Vauzour et al., 2010; Zhao et al., 2010). It is well known that increased oxidative stress occurs in APAP toxicity models, but the effect of SFN on APAP-induced liver injury remains unknown. Considering the potent antioxidant action of SFN, we hypothesized that SFN may have a protective role against APAP-induced hepatotoxicity. Therefore, the present study was conducted to investigate whether SFN attenuates APAP-induced oxidative stress damage by using *in vitro* and *in vivo* assays.

2. Materials and methods

2.1. Primary hepatocyte isolation and culture

Primary hepatocytes were isolated from whole liver tissues using collagenase perfusion methods as described previously (Nakagawa et al., 2008). Briefly, livers were perfused with ethylene glycol tetraacetic acid buffer followed by Hanks' balanced salt solution containing type I collagenase. Subsequently, the livers were removed and filtered through a 70-μm cell strainer (BD Biosciences). Cells were seeded into well plates of suitable size and allowed to adhere to the culture plates for 4 h before further experiments.

2.2. Cell viability

The cytotoxicity of APAP was evaluated in primary hepatocytes pretreated with SFN (dissolved in phosphate-buffered saline [PBS]) using a CCK-8 assay kit (Dojindo Molecular Technologies, USA). The primary hepatocytes were seeded in a 24-well plate for 24 h. The cells were pretreated with SFN (10 μM) for 6 h and the media were then replaced with new media containing APAP (15 mM) and incubated for 14 h. WST-8 reagent was then added to each well and the wells were further incubated for 1 h prior to the measurement of absorbance at 450 nm.

2.3. Measurement of malondialdehyde (MDA) production

MDA production was determined using the method described by Ohkawa et al. with a slight modification (1979). Sodium dodecyl sulfate (SDS, 8.1%) was added to both the cell lysates and liver homogenate, and then mixed with 20% acetic acid and a 0.8% aqueous solution of thiobarbituric acid, before being heated at 95 °C for 60 min. After cooling with tap water, both distilled water and a mixture of n-butanol and pyridine (15:1, v/v) were added and then the mixture was vigorously vortexed. Finally, the mixture was centrifuged at 3000 *g* for 10 min. The absorbances of the upper layers acquired from the centrifuged cells and the liver homogenates were measured at 535 nm. An MDA solution was made fresh by hydrolysis of 1,1,3,3-tetramethoxypropane (TMP). Lipid peroxidation was calculated using TMP as a reference standard.

2.4. Determination of GSH content

The total GSH content from cell lysates and the liver tissue was determined using the glutathione assay kit (Cayman, Ann Arbor, MI). In brief, the cells were collected by centrifugation (1000 *g* for 10 min at 4 °C). The cell pellet was sonicated in 1 mL ice-cold buffer and then centrifuged at 10,000 *g* for 15 min at 4 °C. The supernatants were removed and stored on ice. For determination of the hepatic GSH content, the liver tissues were homogenized in ice-cold buffer and then centrifuged at 10,000 *g* for 15 min at 4 °C. The supernatants were deproteinated before the assays. The GSH contents were calculated based on the slopes of standard curves and were expressed as micrograms per gram of liver tissue.

2.5. Real-time polymerase chain reaction (PCR) analysis

The total RNA from the cells and liver tissue was extracted and the samples were reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The resulting cDNA was amplified using the Exicycler 96 real-time PCR system (Bioneer, Daejeon, Korea) and the SYBR Premix Ex Taq (Bioneer) according to the manufacturer's instructions. The primers used are summarized in Table 1. The cycling conditions were 95 °C for 10 min, followed by 50 cycles of 95 °C for 10 s, and 60 °C for 1 min. To detect and eliminate possible primer–dimer artifacts, we generated a dissociation curve by adding a cycle of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Results were normalized using the reference gene, 18S RNA, and are represented as fold changes versus the reference gene.

Table 1
Nucleotide sequences of primers for real-time PCR of mRNA.

Gene	Direction	Sequence (5′–3′)
18S RNA	Forward	GACACGGACAGGATTGACAGATTGATAG
	Reverse	GTTAGCATGCCAGAGTCTCGTTCGTT
GCLM	Forward	TCACAATGACCCGAAAGAACTG
	Reverse	ACCCAATCCTGGGCTTCAAT
GCLC	Forward	TGGCTTTGAGTGCTGCATCT
	Reverse	ATCACTCCCCAGCGACAATC
Nrf2	Forward	GGCCTTTTTTGCTCAGTTTCA
	Reverse	ATGTGGGCAACCTGGGAGTA
NQO1	Forward	GGTTTACAGCATTGGCCACACT
	Reverse	AACAGGCTGCTTGGAGCAAA
HO-1	Forward	CAGCCCCACCAAGTTCAAAC
	Reverse	GGCGGTCTTAGCCTCTTCTGT
MnSOD	Forward	CCACACATTAACGCGCAGAT
	Reverse	TCGGTGGCGTTGAGATTGT
Cu/Zn SOD	Forward	GCGGTGAACCAGTTGTGTTG
	Reverse	CCCATACTGATGGACGTGGAA

GCLM and GCLC, catalytic and modifier subunits of γ-glutamyl cysteine ligase; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; NQO1, NAD(P)H:quinone oxidoreductase 1; HO-1, heme oxygenase-1; MnSOD, Manganese superoxide dismutase; Cu/ZnSOD, Copper/Zinc superoxide dismutase.

2.6. Western blotting

Cell lysates were obtained after SFN and APAP treatments and were homogenized with ice-cold lysis buffer. After three centrifugations at 12,000 *g* for 10 min, the amounts of soluble proteins were determined using Bradford assays. The proteins (25 μg) isolated from the liver tissues were separated using 10% SDS-polyacrylamide gel electrophoresis and were transferred to polyvinylidene difluoride membranes. Anti-heme oxygenase-1 (HO-1) and anti-glyceraldehyde 3-phosphate dehydrogenase (Cell Signaling Technology) antibodies were used for immunoblotting.

2.7. APAP-induced hepatotoxicity model

Male, 8-week-old C57BL/6 mice were maintained at the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). The mean body weights of the mice were 25.8 ± 1.2 g and they were housed in plastic cages in a temperature-controlled room (22 ± 1 °C) and maintained on a reverse 12 h light/dark cycle. Mice were continuously fed with normal chow diet ad libitum until the start of experiment. For experimental studies into the protective effects of SFN, mice were divided into three groups (vehicle, APAP and APAP+SFN; n = 5–6 per group), fasted overnight, and then 5 mg/kg of SFN (dissolved in PBS) was administered by oral gavage. Three hundred milligrams/kilogram of APAP was injected 30 min after treatment with SFN and the vehicle group was administered PBS only at the same time. Mice were then killed 6 h after exposure to APAP. The blood samples were collected for analysis of serum biomarkers (Aspartate aminotransferase [AST] and alanine transaminase [ALT]). All animal experiments were approved by the Institutional Animal Care and Use Committee and were performed in accordance with the institutional guidelines at the Korea Research Institute of Bioscience and Biotechnology.

2.8. Hematoxylin and eosin (H&E) and terminal deoxynucleotidyl transferase mediated dUDP nick end-labeling (TUNEL) staining and immunostaining

The liver was removed from the mice and immediately fixed in a buffer solution containing 10% formalin for pathologic analysis. Fixed tissues were processed for paraffin embedding, and 5 μm sections were prepared and stained with H&E. For the analysis of cell death, apoptotic cells were detected using TUNEL staining (Promega, Madison, WI, USA). As a marker of lipid peroxidation, 4-hydroxynonenal (4-HNE) was stained and then Alexa Fluor 488 nm goat anti-rat immunoglobulin G (1:200) was applied for visualization.

2.9. Reactive oxygen species (ROS) production

Liver tissue extracts were incubated with 20 μM 2′,7′-dichlorodihydrofluorescein diacetate (Invitrogen, San Diego, CA, USA) at 37 °C for 60 min. Fluorescence intensity was recorded using a Victor3 1420 Multilabel Counter (PerkinElmer, Palo Alto, CA, USA). The protein concentrations in the liver homogenates were quantified using