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Sulforaphane prevents microcystin-LR-induced oxidative damage and apoptosis in BALB/c mice

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Abstract

Microcystins (MCs), the products of blooming algae *Microcystis*, are waterborne environmental toxins that have been implicated in the development of liver cancer, necrosis, and even fatal intrahepatic bleeding. Alternative protective approaches in addition to complete removal of MCs in drinking water are urgently needed. In our previous work, we found that sulforaphane (SFN) protects against microcystin-LR (MC-LR)-induced cytotoxicity by activating the NF-E2-related factor 2 (Nrf2)-mediated defensive response in human hepatoma (HepG2) and NIH 3T3 cells. The purpose of this study was to investigate and confirm efficacy the SFN-induced multi-mechanistic defense system against MC-induced hepatotoxicity in an animal model. We report that SFN protected against MC-LR-induced liver damage and animal death at a nontoxic and physiologically relevant dose in BALB/c mice. The protection by SFN included activities of anti-cytochrome P450 induction, anti-oxidation, anti-inflammation, and anti-apoptosis. Our results suggest that SFN may protect mice against MC-induced hepatotoxicity. This raises the possibility of a similar protective effect in human populations, particularly in developing countries where freshwaters are polluted by blooming algae.

Keywords

Microcystin-LR (MC-LR); Sulforaphane (SFN); NF-E2-related factor 2 (Nrf2); Apoptosis; Reactive oxygen species (ROS)

Introduction

In the wake of rapid economic development and other environmental issues, algal blooms have become a frequent and problematic feature in many freshwater bodies over a wide geographic area. Species of the genus *Microcystis* exist worldwide and are the most dominant bloom-forming strains in China. *Microcystis* is widely known for producing the potent hepatotoxins called microcystins (MCs). MCs are a family of cyclic heptapeptides that have been implicated in the development of liver cancer, necrosis, and even fatal intrahepatic bleeding (Carmichael, 1994; Yoshida et al., 2001).

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Ultrastructural studies in rats (Hooser et al., 1990) and mice (Hermansky et al., 1993) have shown that MC-LR causes significant damage to intracytoplasmic organelles, such as mitochondria and the endoplasmic reticulum, and loss of microvilli and desmosomes in hepatocytes, thereby resulting in necrosis and hemorrhage. MC-LR also inhibits the activities of protein phosphatases 1 and 2A, while prolonged exposure to MC-LR induces inflammatory reactions and oxidative stress (Guzman and Solter, 1999). Also, the *in vivo* studies supported that MC-LR is a potent tumor promoter. Some intoxication episodes caused by toxic cyanobacterial blooms have been reported. Ueno et al. (1996) found a close correlation between the incidence of primary liver cancer (PLC) in Haimen City (Jiangsu Province) and MCs in drinking water through a two-year (1993–1994) epidemiological survey, and hypothesized that the MCs in the drinking water are one of the risk factors for the high incidence of PLC in this area.

Previous studies with both cell culture and animal models have shown that sulforaphane (SFN), derived from glucosinolates present in broccoli and other cruciferous vegetables, is effective in preventing cancer (Zhang et al., 1994; Cornblatt et al., 2007), inflammation (Lin et al., 2008), and skin damage (Talalay et al., 2007). Several mechanisms, including suppression of cytochrome P450 enzymes, activation of phase II enzymes via the Nrf2 transcription factor, and induction of tissue glutathione (GSH) levels, have been proposed to account for SFN-induced detoxification (Juge et al., 2007). Recently, using cell culture models, we found that SFN protects against MC-LR-induced cytotoxicity through activating the NF-E2-related factor 2 (Nrf2)-mediated defensive response in human hepatoma (HepG2) and NIH 3T3 cells (Gan et al., 2010a, 2010b).

Some other substances were reported to protect against acute hepatotoxicity, such as the antioxidant (Krakstad et al., 2006), grapefruit flavonoid naringin acting by altering intracellular protein phosphorylation (Blankson et al., 2000), and nostocyclopeptide-M1, as an atoxic and specific cyanobacterial inhibitor of MC uptake (Herfindal et al., 2011). In this study, we focused on investigating the protective effects of SFN against MC-LR-induced hepatotoxicity in mice. Here, we present evidence that SFN prevents MC-LR-induced liver damage and death in BALB/c mice through several defensive responses, including anti-cytochrome P450 induction, anti-oxidation, anti-inflammation, and anti-apoptosis.

Materials and methods

Chemicals and reagents

MC-LR was purified in our laboratory (Hu et al., 2009). SFN, chlomethiazole (CMZ), diallyl sulfide (DAS), and all other reagents were of the highest grade available and were obtained from Sigma-Aldrich, unless otherwise noted. MC-LR, SFN, CMZ, and DAS were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C until use.

Animal studies

Male BALB/c mice (6 weeks) were purchased from the Center for Disease Control and Prevention in Hubei (Wuhan, Hubei, P.R. China). The mice were kept in a barrier-sustained animal room controlled at suitable temperature ($24\pm 2^{\circ}\text{C}$), humidity ($55\pm 15\%$), ventilation (all-fresh-air system), and illumination (12-hour light/dark cycle). To examine the protective effect of SFN on MC-LR-induced hepatotoxicity, 70 mice were randomly assigned to the following 5 groups: (1) untreated control group; (2) 40 $\mu\text{g/kg}$ MC-LR daily group; (3) 50 $\mu\text{g/kg}$ MC-LR daily group; (4) 5 $\mu\text{mol/animal}$ SFN plus 40 $\mu\text{g/kg}$ MC-LR group; (5) 5 $\mu\text{mol/animal}$ SFN plus 50 $\mu\text{g/kg}$ MC-LR group. Compounds were administered through intraperitoneal (i.p.) injection. The mice in the fourth and fifth groups were injected with SFN 12 h before the injection of MC-LR. All mice were housed under identical conditions

in an aseptic facility and given free access to water and food (the mice food contained (%): wheat: 51.5; nonfat dried milk: 20.5; big meal: 11.5; vegetable oil: 10.0; beer yeast: 4.0; salt: 1.375; calcium hydrophosphate: 1.00; ferric citrate: 0.125). Two mice in each group were euthanized for histopathological analysis, RT-PCR, and Western blots at 4, 6, 8, 12, and 24 h after injection. The rest were treated for 10 days. All studies were approved by the Animal Study Committee of the Chinese Academy of Sciences.

Histopathology analysis

For each mouse, 3 specimens from different regions of the liver were collected and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH=7.4) at room temperature overnight. The paraffin-embedded tissue sections (4 μ M) were stained with hematoxylin and eosin using standard techniques.

RT-PCR assay

Total RNA was extracted from the cells by using the SV Total RNA Isolation System (Promega). A total of 3 μ g of RNA was used as a template for the first-strand cDNA synthesis using the ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA), with oligo(dT) as the primer. The assay was performed following the manufacturer's protocol. PCR primer sequences were as follows: CYP1A1, forward primer: 5' CTTGG ACCTC CTTGG AGCTG, and reverse primer: 5' CGAAG GAAGA GTGTC GGAAG; CYP1A2, forward primer: 5' CAATC AGGTG GTGGT GTCAG, and reverse primer: 5' GCTCC TGGAC TGTTT TCTGC; CYP2E1, forward primer: 5' ACCGG AGACA CCATT TTCAG, and reverse primer: 5' TCCAG CACAC ACTCG TTTTC; tumor necrosis factor- α (TNF- α), forward primer: 5' ACGGC ATGGA TCTCA AAGAC, and reverse primer: 5' GGTCA CTGTC CCAGC ATCTT. The primers for Nrf2; NAD(P)H dehydrogenase, quinone 1 (NQO1); and heme oxygenase-1 (HO-1) were similar to those used in a previous study (Gan et al., 2010a, 2010b): Nrf2, forward primer: 5' ACACG GTCCA CAGCT CATC, and reverse primer: 5' TGTC A TCAA ATCCA TGTCC TG; NQO1, forward, 5' AGGAA GAGCT AATAA ATCTC TTCTT TGCTG, and reverse, 5' TCATA TTGCA GATGT ACGGT GTGGA TTTAT; HO-1, forward, 5' AACTT TCAGA AGGGC CAGGT, and reverse: 5' CTGGG CTCTC CTTGT TGC; GAPDH, forward primer: 5' CGGAG TCAAC GGATT TGGTC GTAT, reverse primer: 5' AGCCT TCTCC ATGGT GGTGA AGAC. RT-PCR products were separated on a 1.2% agarose gel containing ethidium bromide and were visualized via ultraviolet light. The data presented are relative mRNA levels normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript levels, and the value of the untreated cells was set to 1.

TNF- α ELISA assay

Liver lysates were used to measure the concentrations of TNF- α via enzyme linked immunosorbent assay (ELISA) according to the Biosource (Camarillo, CA, USA) protocol. Twenty-five micrograms of hepatic protein was used in the ELISAs. Fifty microliters of incubation buffer was added to each well, and then 50 μ L of standard diluent buffer and 50 μ L of the samples were added. Then, 50 μ L of biotin conjugate was added and incubated for 90 min at 37 $^{\circ}$ C. The wells were washed 4 times with phosphate buffered saline-Tween (PBST), and then 100 μ L of streptavidin-HRP was added; the mixture was incubated for 30 min. The reaction was stopped by adding 50 μ L of 1 M H₂SO₄, and the absorbance was read at 450 nm in the automatic microplate reader.

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity

Serum samples were assayed for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by using commercially available enzymatic assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing City, P.R. China).

Liver lysate preparation and immunoblot analysis

After treatment, liver tissues were lysed in a buffer containing 50 mM Tris-HCl, 10 mM NaCl, 5 mM MgCl₂, 0.5% Tergitol-type NP-40 (NP-40), and 1 mM dithiothreitol (DTT) for 20 min on ice. Cytosolic fractions were obtained as supernatant after centrifugation at 15,000×g for 10 min at 4 °C. The pellet was resuspended in nuclear-extraction buffer (20 mM Hepes, pH 7.9, 0.5 M NaCl, 1 mM EDTA, 20% glycerol, 1 mM DTT) for 20 min on ice, followed by centrifugation at 15,000×g for 10 min at 4 °C. Proteins (20 µg) were loaded on an SDS-polyacrylamide gel, separated via electrophoresis, and electroblotted onto PVDF membranes (Millipore). Immunoblot analysis was performed using specific antibodies and enhanced chemoluminescence-based detection (Millipore). Antibodies against Nrf2, NQO1, and HO-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-GAPDH was purchased from Sigma (St. Louis, MO).

Caspase-3 activity

Hepatic tissues were placed in 0.9% normal saline and homogenized in a polytron homogenizer for 10 strokes. The resulting homogenate was centrifuged at 3000 rpm for 12 min and the supernatant fraction was used for caspase-3 activity measurement. Caspase-3 activity was measured using Ac-DEVD-Rhodamine 110 substrate (Roche) dissolved in assay buffer containing 50 mmol/L HEPES, 100 mmol/L NaCl, 10 mmol/L DTT, 1 mmol/L EDTA, and 0.1% CHAPS detergent (pH 7.4). Ten microliters of the cell lysate supernatant was mixed with 70 µL of assay buffer and 20 µL of 50 µmol/L DEVD-Rhodamine 110. The mixture was incubated at 37 °C in the dark for 1 h. The fluorescence associated with the released rhodamine (excitation at 485 nm, emission at 528 nm) was assayed in a spectrofluorometer (Max M2; Bio-Tek).

Lipid peroxidation assay

Lipid peroxides were quantified in fresh liver samples by using the thiobarbituric acid (TBA) method as previously described by Esterbauer and Cheeseman (1990). Values are presented as nmol TBARS/g tissue.

Measurement of GSH

The GSH concentration was measured using the Glutathione Assay Kit from Nanjing Jiancheng Bioengineering Institute (Nanjing City, P.R. China), following the manufacturer's instructions. All the experiments described in this section were performed in triplicate to obtain means and standard deviations.

Statistical analysis

All results are expressed as mean (standard error of the mean; SEM). One-way analysis of variance (ANOVA) and Student's *t* test were used to analyze the differences between the treatments. Statistical analyses were performed using GraphPad software. A *p*<0.05 was considered statistically significant.

Results

MC-LR induces liver injury, oxidative stress, and inflammation in BALB/c mice

On histological examination, the saline groups showed normal histology (Fig. 1A-a), however, the mice exposed to MC-LR alone had liver pathology showing hemorrhage (Fig. 1A-c), widespread apoptotic cells (Figs. 1A-d, e), and occasionally large necrotic areas (Fig. 1A-f). It is also shown that some cells surrounding the hemorrhage were swollen and had lost their granular appearance, and their nuclei turned faint or invisible so that the cells were most likely to be necrotic (data not shown). These *in vivo* results are consistent with our *in vitro* study that MC-LR causes significant damage on the liver by reactive oxygen species (ROS) generation as one of the likely causes.

A previous study indicated that induction of cytochrome P450 2E1 (CYP2E1) during MC-LR exposure is an important contributor to MC-LR-induced oxidative stress (Nong et al., 2007). To study whether CYP2E1 is induced by MC-LR treatment, we analyzed the mRNA levels of CYP2E1 and two other cytochrome P450 isoforms (CYP1A1 and CYP1A2). Fig. 1B showed that short-term exposures of 40 or 50 $\mu\text{g/kg}$ MC-LR to mice had a significant induction of CYP2E1 mRNA, but not that of CYP1A1 and CYP1A2; indicating that the induction is specific. Further studies with Western blots (Fig. 1B) showed that the level of immunoreactive CYP2E1 in the mice liver was increased after MC-LR exposure.

DAS and CMZ, two effective inhibitors of CYP2E1 expression, have been shown to inhibit ROS generation in liver cells (Nong et al., 2007). To study the relationship between CYP2E1 expression and MC-LR-induced ROS generation, we treated mice with MC-LR together with either DAS or CMZ. The results showed that both DAS and CMZ inhibited MC-LR-induced CYP2E1 expression (Fig. 1B) and ROS generation (data not shown), indicating that CYP2E1 overexpression by MC-LR underlies ROS generation.

Because increased expression of the proinflammatory cytokine TNF- α is also an important trigger for hepatic injury in response to MC-LR exposure (Yoshida et al., 2001), we next investigated the expression of TNF- α in MC-LR exposure in mice. RT-PCR results indicate that MC-LR exposure increased hepatic TNF- α mRNA accumulation in a time- and dose-dependent manner (Fig. 1C).

ELISA assay was used to measure the concentrations of TNF- α protein levels in liver lysates. Fig. 1D shows that hepatic TNF- α was 43% higher in MC-LR treated liver tissue than the untreated control group at 6 h time point, while the contrast was increased to 2.5-fold after 24 h, indicating that both ROS generation and TNF- α are dramatically induced in animal liver by MC-LR.

SFN prevents MC-LR-induced hepatotoxicity and animal death in BALB/c mice

Previously, SFN has been shown to be a potent competitive inhibitor of CYP2E1 (Barcelo et al., 1996). To further explore the underlying mechanisms by which SFN exerts its protective effect on MC-LR-induced hepatotoxicity, we examined CYP2E1 expression in livers of mice which were treated with and without SFN. Results (Fig. 2A) showed that SFN treatment dose-dependently inhibited MC-LR-induced expression of CYP2E1, including both mRNA and protein levels, in mice. Treatment with 5 μM SFN almost completely inhibited MC-LR-induced CYP2E1.

Several studies also show that SFN is effective in inhibiting TNF- α related signaling pathways (Kim et al., 2006; Jin et al., 2007). To further confirm the anti-inflammatory effect of SFN on MC-LR-induced TNF- α accumulation, mRNA level of TNF- α was examined in livers after mice were treated with 50 $\mu\text{g/kg}$ MC-LR for 24 h with or without SFN

pretreatment for 12 h. As demonstrated in Fig. 2B, SFN at 1 and 5 μ M significantly attenuated mRNA level of TNF- α .

The above results suggest that SFN may have an anti-inflammatory effect on MC-LR-induced hepatotoxicity in vivo. Thus, we treated mice with a single i.p. injection of SFN 12 h prior to MC-LR. Both ALT and AST activities in serum, indicators of liver damage, were examined 24 h after administration of MC-LR. Results (Figs. 2C and D) showed that both ALT and AST levels were very low in mice treated with only saline. However, both activities were significantly induced (28- and 14-fold, respectively) by MC-LR. In contrast, SFN pretreatment significantly inhibited the MC-LR-induced increase of serum ALT and AST levels at both 40 and 50 μ g/kg MC-LR treatments. However, the protective effect by SFN diminished at 60 μ g/kg dose of MC-LR treatment (Figs. 2C and D).

We also performed histological analysis on liver tissues from mice treated with MC-LR alone and with the combination of SFN and MC-LR. The results showed that SFN pretreatment preserved normal tissue structures with tight cellular contact and large round nuclei in most cells, and significantly reduced MC-LR-induced apoptosis and necrosis (Fig. 2E). We also observed the reduced levels of hemorrhage with SFN pretreatment. The results indicated that SFN is able to prevent hepatic damages by MC-LR in mouse models.

Finally, we studied whether SFN prevents MC-LR-induced mouse death. Mice were treated daily with a single i.p. injection of SFN 12 h prior to MC-LR for 10 days consecutively. Based on the result that the injection of 40 μ g/kg MC-LR did not induce mouse death, and five out of ten mice died with 50 μ g/kg of MC-LR injection, therefore, we chose 50 μ g/kg of MC-LR as the median lethal dose (LD₅₀) to investigate the protective efficiency by SFN. Fig. 3 shows that 5 of 10 mice treated with MC-LR alone died within 7 days of treatment. In sharp contrast, all mice treated with SFN and MC-LR survived ($p < 0.05$). The results strongly support the notion that SFN is protective to MC-LR-induced mouse death.

SFN activates Nrf2-mediated phase II enzymes and GSH in BALB/c mice

Previously, we found that SFN protects the HepG2 and NIH 3T3 cells from MC-LR-induced toxicity by inducing Nrf2-dependent phase II enzymes (Gan et al., 2010a, 2010b). To confirm our findings in vivo, we treated mice with a single i.p. injection with either saline or SFN and euthanized mice after 8 h. The liver samples were collected, fractionated, and analyzed by Western blot. Results (Fig. 4A) show the enrichment of Nrf2 in the nuclear fraction and the induction protein levels of NQO1 and HO-1 in the cytoplasmic fraction. The results confirm that SFN activates Nrf2 pathway in vivo.

To confirm the effects of SFN on Nrf2-related downstream targets, we analyzed liver samples from mice treated with MC-LR with and without pretreatment of SFN by RT-PCR and Western blot. Fig. 4B showed that SFN pretreatment caused dramatic increases in NQO1 and HO-1 mRNA levels. At protein level, both NQO1 and HO-1 were significantly induced by SFN alone and in the combined treatment.

MC-LR has been shown to induce lipid peroxidation in primary cultured rat hepatocytes (Boua cha and Maatouk, 2004). We therefore determined the lipid peroxide levels in liver with MC-LR at 50 μ g/kg body weight for 24 h with or without pretreatment with SFN (5 μ M) for 12 h. We found that MC-LR increased the liver lipid peroxide levels to nearly twofold, from 20.35 ± 2.37 to 38.93 ± 3.89 nmol TBARS/g tissue (Fig. 4C); however, pretreatment of mice with SFN abolished most of these effects. Additionally, we quantified the endogenous GSH levels in liver samples from mice treated either with MC-LR alone or the combination of SFN and MC-LR. SFN pretreatment completely blocked MC-LR-

induced GSH reduction (Fig. 4D). These results indicate that Nrf2 pathway was probably involved in the SFN-induced cytoprotection in vivo.

SFN blocks MC-LR-induced apoptosis in BALB/c mice

MC-LR has been shown to induce apoptosis as a way of toxicity (Bøe et al., 1991; Fischer and Dietrich, 2000). To determine if SFN is able to block MC-LR-induced apoptosis, we analyzed samples by immunostaining of PARP cleavage and measuring caspase-3 activity. As indicated in Fig. 5A, MC-LR at 50 µg/kg induced substantial PARP cleavage in BALB/c mice. Also, MC-LR caused a 2.7-fold increase in activity of caspase-3 at 24 h treatment (Fig. 5B). Both indicate that MC-LR induces apoptosis in mice liver. In contrast, pretreatment with SFN significantly reduced PARP cleavage and caspase-3 activity, blocking apoptosis induction.

Previous studies have shown that apoptosis induction by MC-LR is associated with changes in BCL-2 family members (Chen et al., 2005). For example, inductions of Bax and Bid are pro-apoptotic while inductions of Bcl-2 and Bcl-xL are anti-apoptotic (Chawla-Sarker et al., 2004; Bai et al., 2005). To investigate the role of these signaling proteins in apoptosis induction by MC-LR and cytoprotection by SFN, we measured the expression levels with 50 µg/kg MC-LR alone for 24 h treatment. As shown in Fig. 5C, Bid expression was moderately induced and Bcl-xL level was reduced, indicating induction of apoptosis. We did not observe significant differences in the protein levels of Bcl-2 and Bcl-xL with 6 h treatment with SFN alone (Supplemental Fig. 1). However, pretreatment with SFN was able to block the process by suppressing Bid induction and inducing Bcl-xL, agreeing with the inhibition of apoptosis induced by MC-LR.

Discussion

MCs are produced by the bloom-forming cyanobacterium *Microcystis* and are released into the surrounding water. Since algae blooming appears frequently in lakes and rivers in many areas all over the world, these water-dissolvable toxins, even at the trace amount level, are of special health and safety concern to both human beings and animals. MC-LR, one of the most potent cyanotoxins in MCs, can cause serious damages in various types of organs, including the liver (Matsushima et al., 1992). Because MCs are relatively chemically stable, completely removing them in the production of drinking water is both technically and economically challenging (Harada et al., 1996). Therefore, pragmatic approaches that protect people in MC-contaminated areas are urgently needed. Recently, we showed that SFN, a naturally-occurring isothiocyanate derived from cruciferous vegetables, can prevent MC-LR-induced cytotoxicity in cultured liver cells (Gan et al., 2010a, 2010b), suggesting that SFN is a promising agent in protecting livers from MC-LR-induced damages.

In the present study, we provide evidence with an animal model confirming the protective effect of SFN against MC-LR-induced hepatotoxicity. Our results showed that MC-LR-induced hepatotoxicity, reflected by inhibited ALT and AST activities and induced caspase-3 activity, was significantly blocked by pretreatment with SFN at physiologically attainable doses. Histopathology analysis of mice liver tissues also shows much reduced areas of hemorrhaging, necrosis, and more normal tissue structures in mice pretreated with SFN compared with the group treated only with MC-LR; supporting the protective role of SFN against MC-LR-induced cell morphological changes. More importantly, SFN significantly improves the survival rate of mice treated with MC-LR: all mice in the untreated control group and the SFN pretreatment group survived while a half of ten mice with MC-LR treatment alone died within 7 days. However, SFN is borderline effective to protect against MC-induced mouse death.

Oxidative stress has been implicated in the hepatotoxicity of MC-LR (Chen et al., 2005). A series of events induced by MC-LR, including ROS generation, cytochrome P450 CYP2E1 induction, lipid peroxidation activation, and lactate dehydrogenase activity release, can all be inhibited by ROS scavengers (Boua cha and Maatouk, 2004). The Nrf2-mediated phase II enzyme system has been reported to be one of the most important antioxidant defense mechanisms by up-regulating antioxidant response element-related detoxification enzymes (William and Thomas, 2008). In our previous study, we found that SFN protects cells from MC-LR-induced toxicity through activation of the Nrf2 pathway (Gan et al., 2010a, 2010b). In this study, we confirmed: a) Nrf2 translocation to the nucleus by SFN in mouse livers, b) induction of Nrf2 downstream target genes, NQO1 and HO-1, two phase II enzymes have been associated with anti-oxidative and anti-inflammatory effects (Blydt-Hansen et al., 2003), and c) induction of GSH, a major endogenous antioxidant, plays an important role in detoxification of MC-LR (Pflugmacher et al., 1998; Takenaka, 2001). These in vivo results confirmed our previous in vitro findings. Furthermore, we showed that SFN significantly reduced MC-LR-induced CYP2E1 expression in the liver. Since CYP2E1 induction has been found to be a potential cause of MC-LR-induced ROS generation (Nong et al., 2007), our results provided a new mechanism through which SFN protects MC-LR exposure.

Previous studies showed that vitamin E, a known dietary antioxidant, is moderately effective in protecting cells against MC-LR. For example, it decreases lipid peroxidation, suppresses ALT leakage, and inhibits GST activity induced by MC-LR (Gehring et al., 2003). However, it is not effective in inhibiting MC-LR-induced apoptosis, necrosis, and inflammation as assessed by histology (Gehring et al., 2003; Pinho et al., 2005; Prieto et al., 2009) suggesting that dietary supplements with only antioxidant activity offer limited protection against MC-LR-induced liver injury. In comparison, SFN is a versatile agent showing in this study that SFN offers not only antioxidant activity but also anti-inflammatory and anti-apoptotic functions.

SFN was reported to affect various molecular targets and pathways to prevent carcinogenesis, including phase I and II DME, Nrf2, NF κ B, cell cycle arrest, apoptosis, and receptors. Phase I and II DME, and Nrf2 were mediated with defensive response (Cheung and Kong, 2010). Kim et al. (2006) found that SFN alone did not induce any morphologic signs of cell death up to 10 μ mol/L, and treatment with 10 μ mol/L sulforaphane alone for 16 h did not induce any proteolytic processing of caspases. Our previous studies showed that SFN could significantly upregulate antioxidant genes and activate Nrf2 pathway as low as 5 μ mol/L SFN, but, Nrf2 protein levels decreased at doses $\geq$ 20 μ mol/L (Gan et al., 2010a, 2010b). Thus the present study indicated that the non-toxic dose of SFN was not pro-apoptotic.

Studies by Yoshida et al. (2001) indicated that inflammation also underlies hepatic injury by MC-LR. Therefore, anti-inflammatory agents are potential inhibitors of MC-LR-caused liver damages. Previously, Heiss et al. (2001) reported that SFN down-regulates lipopolysaccharide (LPS)-stimulated nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and TNF- α expression in raw macrophages. Also, Lin et al. (2008) found that SFN exerts its anti-inflammatory activity mainly via activation of Keap1-Nrf2 pathway in mouse peritoneal macrophages. In this study, we showed that SFN significantly decreased inflammatory cytokine TNF- α expression at both mRNA and protein levels induced by MC-LR in mice liver. We also found that SFN blocked MC-LR-induced liver cell apoptosis as evidenced by PARP cleavage and caspase-3 activity. Previous studies have shown that the Bid-Bax-Bcl-2 is the main pathway for MC-LR-induced apoptosis (Chen et al., 2005). In this study, we found that SFN inhibited pro-apoptotic Bid which was induced by MC-LR and induced anti-apoptotic Bcl-xL which was inhibited by MC-LR. Although the detailed mechanisms by which SFN blocks MC-LR-induced Bcl-2 family changes are unclear, the

results from the present study suggested that decreased oxidative damage and TNF- α expression by SFN may contribute to the anti-apoptotic activities of SFN.

Our previous studies indicated that MC-LR enhanced the levels of Nrf2 protein in a dose-dependent manner, but Nrf2 protein levels decreased at high doses of MC-LR ($>1 \mu\text{M}$) (Gan et al., 2010a, 2010b). It was also shown that treatment with a high dose of MC-LR inhibited Nrf2 activation and caused cells to undergo cell death (Gan et al., 2010a, 2010b). However, we did not find activation of Nrf2 by MC-LR in mice, it is still unclear whether the activation of Nrf2 by MC-LR is the main mechanism of MC-LR-induced liver tumor. Nrf2 could enhance chemoresistance to anticancer drugs (Homma et al., 2009). In this, we hypothesize that SFN will protect against liver cancer by prolonged exposure to low MC-LR, but the primary mechanism may not be Nrf2 activation pathway. Further preclinical and clinical studies are needed.

Taken together, the results presented in this mouse model study confirmed findings in our previous in vitro study that SFN is effective in cytoprotection against MC-LR-induced hepatotoxicity. Additionally, our study also provided some novel mechanistic insights of the protection. Although this small-scale animal study had some limitations, such as intake of SFN by mice was through i.p., not feeding, the encouraging results suggest that SFN warrants further exploration as a safe and potent means of preventing hepatotoxicity of MC-LR in further preclinical and clinical studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

MCs	microcystins
MC-LR	microcystin-LR
SFN	sulforaphane
Nrf2	NF-E2-related factor 2
ROS	reactive oxygen species
GSH	glutathione
CMZ	chlomethiazole
DAS	diallyl sulfide
DMSO	dimethyl sulfoxide
PBS	phosphate-buffered saline
ELISA	immunosorbent assay
TNF-α	tumor necrosis factor- α
NQO1	NAD(P)H:quinone oxidoreductase 1

HO-1	heme oxygenase-1
ALT	alanine aminotransferase
AST	aspartate aminotransferase
DTT	dithiothreitol
TBA	thiobarbituric acid
LPS	lipopolysaccharide
iNOS	nitric oxide synthase
COX-2	cyclooxygenase-2

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Fig. 1. MC-LR induces liver injury, oxidative stress, and inflammation in BALB/c mice. (A) Photomicrographs of hematoxylin and eosin-stained liver sections. Mice treated with 50 $\mu\text{g/kg}$ MC-LR and killed at 0, 4, 6, 8, 12, and 24 h. Livers were collected for histological examination. Tissues were sectioned at 4-mm intervals and then stained with hematoxylin and eosin for light microscopic analysis. The photomicrographs are of livers obtained on (a) control, (b) SFN alone treatment, (c) 4 h MC-LR treatment, (d) 8 h MC-LR treatment, (e) 12 h MC-LR treatment, and (f) 24 h treatment. (B) Expression of CYP1A1, CYP1A2, and CYP2E1. Mice treated with 40 and 50 $\mu\text{g/kg}$ MC-LR for 4 h. Total RNA was extracted from liver tissue and expressions of *CYP1A1*, *CYP1A2*, *CYP2E1*, and *GAPDH* genes were

analyzed by RT-PCR (up) according to Materials and methods. Immunoreactive CYP2E1 protein in liver lysates was assessed by Western blot analysis (down). GAPDH expression was analyzed as a loading control. All images are representative of at least 3 mice per treatment group. (C) Evaluation of TNF- α expression by RT-PCR. Total RNA was extracted from liver untreated or treated with MC-LR at different times (0, 6, 12, 24 h) and different doses (40, 50 and 60 $\mu\text{g/kg}$), while expressions of *TNF- α* and *GAPDH* genes were examined by semi-quantitative RT-PCR. *GAPDH* gene expression was analyzed as a loading control. (D) Secretion of TNF- α in liver. Each value represents the mean $\pm$ SEM values of 3 independent experiments. * $p<0.05$ versus control.

Fig. 2.

SFN prevents MC-LR-induced hepatotoxicity in BALB/c mice. (A) SFN significantly inhibited MC-LR-induced CYP2E1 upregulation. Mice were pretreated with the indicated concentrations of SFN for 12 h then treated with MC-LR for 4 h. Immunoreactive CYP2E1 protein in liver lysates was assessed by performing western blot analysis. (B) SFN inhibited MC-LR-induced TNF- α activity. Mice were pretreated with the indicated concentrations of SFN for 12 h then treated with MC-LR for 4 h. Total RNA was extracted from liver tissue and expression of TNF- α was analyzed by RT-PCR. (C) AST and (D) ALT activities were measured enzymatically. Mice were i.p. injected with SFN 12 h before being injected with MC-LR. Blood was collected after injection of MC-LR and transaminase levels were

assayed; hematoxylin and eosin staining was performed in liver sections as described in Materials and methods. Data are presented as mean±S.D. for 2 saline-, 5 MC-LR-, and 5 SFN+MC-LR-treated mice. Each value represents the mean±S.D. of three determinations. *p<0.05 versus control. (E) Twenty-four hours after MC-LR injection, livers were collected for histological examination. Tissues were sectioned at 4-mm intervals then stained with hematoxylin and eosin for light microscopic analysis.

Fig. 3.

SFN protects against MC-LR-induced mouse death. Comparison of animal survival in the SFN+MC-LR-treated group and the MC-LR group. The MC-LR group of 10 animals received a single i.p. MC-LR injection of 50 mg/kg and the SFN+MC-LR-treated group received a single i.p. injection of SFN 12 h before the injection of MC-LR. The course was followed for a period of 10 days. Each value represents the mean S.D. of three determinations. * $p < 0.05$.

Fig. 4. SFN stabilizes Nrf2 in vivo and inhibits lipid peroxidation and GSH reduction. (A) Immunoblot illustrating the increased expression of endogenous Nrf2 in liver tissue following i.p. injection of SFN. Mice were i.p. injected with either DMSO (lanes 1 and 2) or SFN (0.9 mg/animal, lanes 3 and 4) 8 h after injection and were then killed. The livers were removed and washed. The nuclear fractions were extracted and blotted for Nrf2 analysis and the cytoplasmic fraction was blotted for NQO1, HO-1, and GAPDH analysis. (B) SFN activates Nrf2 pathway in liver tissues. Mice were pretreated with SFN for 12 h and then treated with MC-LR for 8 h. Total RNA from liver tissue was extracted and reverse-transcribed into cDNA prior to RT-PCR analysis for detection of mRNA for Nrf2, NQO1

and HO-1 (top). Nuclear (N) and cytoplasmic fractions (C) were extracted for immunoreactive Nrf2, NQO1, and HO-1 by Western blotting (bottom). (C) SFN inhibits lipid peroxides. Two days after operation, fresh liver samples were collected and the level of lipid hydroperoxides was determined by using the TBA method. Each value represents the mean $\pm$ S.D. of three determinations. * p <0.05, ** p <0.01. (D) SFN inhibits MC-LR-induced GSH reduction. Mice were pretreated with SFN for 12 h and were then treated with MC-LR for another 24 h. The livers were collected and the glutathione contents were determined. Each value represents the mean $\pm$ S.D. of three determinations. * p <0.05, ** p <0.01.

Fig. 5.

SFN blocks MC-LR-induced apoptosis in BALB/c mice. (A) PARP cleavage. Liver lysates were prepared after 12 h pretreatment with SFN and treatment with MC-LR for 24 h to analyze the PARP cleavage. (B) Caspase-3 activity in liver samples subjected to SFN pretreatment and MC-LR treatment for 24 h. Each value represents the mean $\pm$ S.D. of three determinations. * p <0.05. (C) Western blots for Bax, Bid, Bcl-2, and Bcl-xL. Liver lysates were prepared after 12 h pretreatment with SFN and treatment with MC-LR for 24 h to analyze the Bax, Bid, Bcl-2, Bcl-xL, and GAPDH expressions.