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Activation of Nrf2 by Microcystin-LR Provides Advantages for Liver Cancer Cell Growth

Nanqin Gan, Xiaoyun Sun, and Lirong Song*

State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology,
CAS, Wuhan 430072, People's Republic of China

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Microcystin-LR (MC-LR) is a potent heptapeptide hepatotoxin at high doses, but its underlying mechanism of promoting liver cell proliferation at low doses is unclear. The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) is key in mediating the protective antioxidant response against various environmental toxicants, but emerging data suggest that constitutive activation of Nrf2 contributes to a malignant phenotype. The purpose of this study was to characterize the interactions and effects of Nrf2 activation on cell proliferation induced by MC-LR treatment. Treatment of HepG2 and Hep3B cells with MC-LR resulted in significant increases in Nrf2-ARE binding activities in the nuclear fractions and upregulation of its downstream genes *HO-1* and *NQO1*. A possible mechanism may be that MC-LR binds to the cytosolic regulator protein Keap1 to liberate Nrf2. Nrf2 knockdown inhibited MC-LR-induced cell proliferation and cell cycle progression. Together, these results indicate that MC-LR-induced upregulation of Nrf2 in cancer cells promotes liver cancer cell growth and suggest a positive role of Nrf2 in tumorigenesis.

Introduction

Microcystins (MCs) are a family of cyclic heptapeptide hepatotoxins produced by freshwater species of cyanobacteria which have been implicated in the development of liver cancer, necrosis, and even deadly intrahepatic bleeding (1). Microcystin-LR (MC-LR) is a strong protein phosphatase inhibitor that covalently binds to the serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A) and thereby influences the regulation of cellular protein phosphorylation (2). Previous *in vivo* and *in vitro* studies supported the contrasting responses to MC-LR exposure (3): low doses of MC-LR appear to increase liver cell survival and proliferation, while higher doses of toxin reduce cell viability (4).

MCs are known to inhibit PP1/PP2A and to induce apoptosis, and they have been reported to be linked to the generation of reactive oxygen species (ROS) (5–7). However, MC-LR can induce cellular proliferation. A possible mechanism for MC-LR-induced tumor growth could be due to the activation of the Akt and MAPK signaling pathway which is associated with the stimulation of cellular proliferation (8). However, the mechanisms of MC-LR-induced carcinogenesis and cellular proliferation are still unclear.

The transcription factor NF-E2-related factor 2 (Nrf2) has been identified as a key factor involved in the protection of cells from oxidative and electrophilic insults (9, 10). Many of its target genes are important in maintaining cellular antioxidant responses and xenobiotic metabolism. For example, NAD(P)H:quinone oxidoreductase 1 (NQO1) catalyzes the reduction of quinones, protecting cells against redox cycling and oxidative stress (11, 12), and reduces the toxic metabolites of acetaminophen back to the parent compound *in vitro* (13). Heme oxygenase-1 (HO-1) catalyzes the breakdown of heme into bilirubin, carbon monoxide, and iron. Bilirubin is an antioxidant,

and its generation following HO-1 induction reduces the cytotoxicity caused by oxidative stress (14). Glutathione S-transferases (GSTs) also facilitate the removal of toxic and carcinogenic chemicals by increasing their solubility and excretion (15, 16). Therefore, over the past decade Nrf2 was thought to be a good protein that protects humans from genotoxic damage caused by carcinogens.

However, Nrf2 can also exert negative effects, and recent studies have revealed its dark side. Constitutive activation of Nrf2 may contribute to a malignant phenotype (17), and increased expression and activity of Nrf2 have been observed in various tumor cells (18). Nrf2 promotes the survival of cancer cells under a deleterious environment and enhances chemoresistance to anticancer drugs (19). These inconsistencies suggest that Nrf2 exhibits contrasting roles in different stages of tumorigenesis. Thus, we focused in this study on investigating this pathway as a possible mechanism by which MC-LR promotes cell proliferation. Here, we present evidence for the first time showing that MC-LR can activate Nrf2 and promote the growth of liver cancer cells.

Experimental Procedures

Cells and Reagents. Human hepatocellular liver carcinoma cell lines HepG2 and Hep3B were obtained from the Cell Bank of Chinese Academy of Science and grown in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 unit/mL penicillin, and 100 μ g/mL streptomycin (Invitrogen) at 37 °C, 5% CO₂.

Nrf2-specific siRNA and control-siRNA were purchased from Qiagen (Valencia CA, USA). MC-LR was purified in our lab (20). Cycloheximide (CHX), MG132, and all other reagents were of the highest grade available from Sigma, unless otherwise noted.

Cell Lysate Preparation and Immunoblot Analysis. Cells were lysed in a buffer containing 50 mM Tris-HCl, 10 mM NaCl, 5 mM MgCl₂, 0.5% NP-40, 1 mM DTT, and protease inhibitors for 20 min on ice. Cytosolic fractions were obtained as supernatant after centrifugation at 15,000g for 10 min at 4 °C. The pellet was

* To whom correspondence should be addressed. Tel: 86-27-68780806. Fax: 86-27-68780871. E-mail: lrsong@ihb.ac.cn.

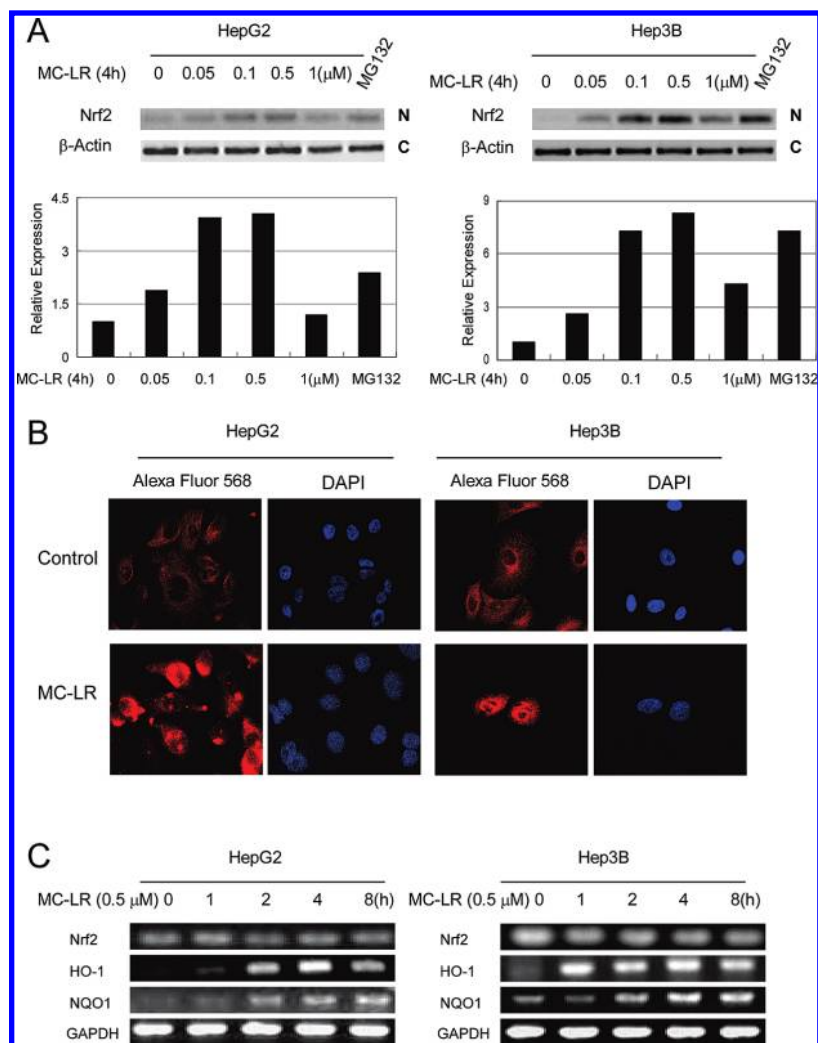


Figure 1. Activation of the Keap1/Nrf2 pathway by MC-LR in HepG2 and Hep3B cell lines. (A) Increase in nuclear Nrf2 protein levels in response to MC-LR. HepG2 and Hep3B cells were treated with MC-LR (0, 0.05, 0.1, 0.5, 1 μ M) for 4 h. Cytosolic (C) and nuclear (N) fractions were prepared from each cell line and analyzed by Western blot with anti-Nrf2 and β -actin antibodies. The intensities of immunoreactive Nrf2 bands were measured by image analysis software and expressed relative to actin. The values obtained from control cells were arbitrarily assigned a value of 1.0. (B) Nuclear translocation of Nrf2 protein induced by MC-LR. HepG2 and Hep3B cells were treated with MC-LR (0.5 μ M) for 4 h, and immunofluorescence staining was performed with anti-Nrf2 followed by Alexa Fluor 568-conjugated second antibodies (red). The nucleus was stained with DAPI (blue). (C) Induction of Nrf2 and its downstream target genes *NQO1* and *HO-1* by MC-LR. Total RNA was extracted from HepG2 and Hep3B cells untreated or treated with MC-LR for 1, 2, 4, and 8 h and expression of *NQO1*, *HO-1*, and *GAPDH* genes examined by semiquantitative RT-PCR. *GAPDH* gene expression was analyzed as a loading control.

resuspended in nuclear extraction buffer (20 mM Hepes, pH 7.9, 0.5 M NaCl, 1 mM EDTA, 20% glycerol, 1 mM DTT, and protease inhibitors) for 20 min on ice, followed by centrifugation at 15,000g for 10 min at 4 $^{\circ}$ C. Proteins (20 μ g) were loaded on a SDS–polyacrylamide gel, separated by electrophoresis, and then electroblotted onto PVDF membranes (Millipore). Immunoblot analysis was performed with specific antibodies and enhanced chemoluminescence-based detection (Millipore). Antibodies against Nrf2, Keap1, NQO1, HO-1, pRb, p21, and phosphor-pRb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti- β -actin was purchased from Cell Signaling Technology (Beverly, MA, USA).

Immunoprecipitation. Cell lysates were precleared with protein G-agarose (Invitrogen) for 1 h at 4 $^{\circ}$ C, followed by incubation with antibodies at 4 $^{\circ}$ C overnight with shaking. Immune complexes were precipitated by incubation with protein G-agarose at 4 $^{\circ}$ C for 1 h and brief centrifugation. The precipitates were washed extensively with PBST and were subjected to fractionation by SDS–PAGE. Protein bands were detected by immunoblotting with specific antibodies as specified in the figures. The anti-MC-LR antibody was from our laboratory (21).

Nrf2 Silencing. For Nrf2 silencing experiments, 20 nmol/L of control-siRNA and Nrf2-specific siRNA were used. Cells were

incubated with the siRNA in serum-free Opti-MEM without antibiotics using Oligofectamine transfection reagent (Invitrogen). After 4 h, Opti-MEM with 2 \times concentrated serum was added, resulting in a final concentration of 10% FBS, followed by incubation for 48 h before treatment.

Immunofluorescence. Cells were plated onto coverslips and fixed with 4% paraformaldehyde for 10 min at 37 $^{\circ}$ C. Subsequently, the cells were washed twice with PBS and then permeabilized with 0.5% Triton X-100 in PBS for 10 min. After the cells were washed with PBS three times, they were blocked with PBS plus 1% BSA and 0.05% Triton X-100 for 1 h at room temperature. Primary antibody (anti-Nrf2, Santa Cruz) was added to the cells at the dilution of 1:150 in PBS plus 1% BSA and 0.05% Triton X-100 and incubated at room temperature for 4 h. The cells were then washed with PBS/0.1% Tween 20 three times and further incubated with the secondary antibody, Alexa Fluor 568 conjugated donkey antirabbit IgG (Invitrogen), diluted 1:400 in PBS plus 1% BSA and 0.05% triton X-100 for 1 h at room temperature.

RT-PCR. Total RNA was extracted from cells 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 access RT-PCR system (Promega) and PCR as follows: Nrf2 [forward primer, 5'-ACACG GTCCA CAGCT CATC-3', and

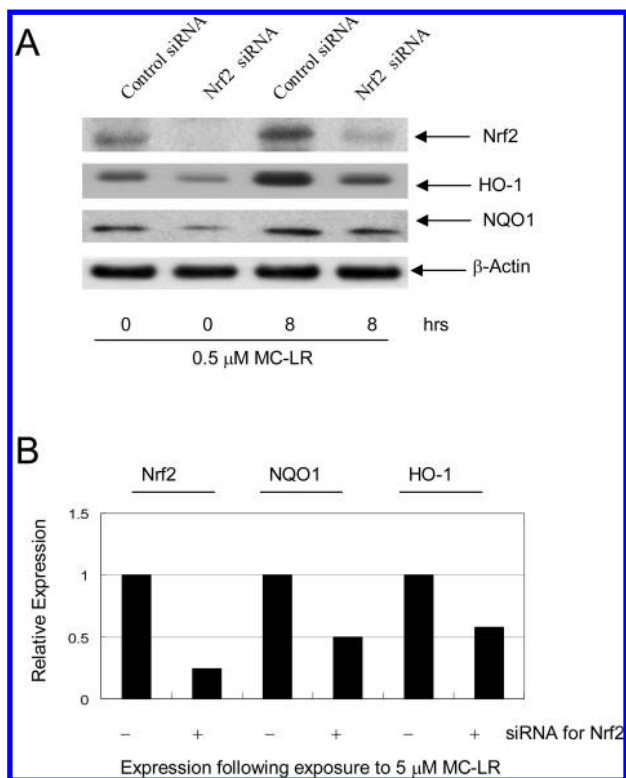


Figure 2. Increases in endogenous NQO1 and HO-1 mediated by MC-LR requires expression of endogenous Nrf2. (A) HepG2 cells were transfected with either control-siRNA or siRNA directed against Nrf2. After transfection, cells were exposed to 0.5 μ M MC-LR for 4 h, and then Western blot analysis was performed on the cell lysates with anti-Nrf2, anti-NQO1, anti-HO-1, and anti- β -actin. (B) Quantitation of Nrf2, NQO1, and HO-1 protein expressions. The intensities of immunoreactive Nrf2, NQO1, and HO1 bands were measured by image analysis software and expressed relative to that of β -actin. The values obtained from control cells were arbitrarily assigned a value of 1.0.

reverse primer, 5'-TGTCA ATCAA ATCCA TGTCC TG-3'], NQO1 [forward, 5'-AGG AAG AGC TAA TAA ATC TCT TCT TTG CTG-3', and reverse, 5'-TCA TAT TGC AGA TGT ACG GTG TGG ATT TAT-3'], HO-1 [forward, AAC TTT CAG AAG GGC CAG GT, and reverse, CTG GGC TCT CCT TGT TGC], GAPDH [forward primer, 5'-CGG AGT CAA CGG ATT TGG TCG TAT-3', and reverse primer, 5'-AGC CTT CTC CAT GGT GGT GAA GAC-3']. RT-PCR products were separated on a 1.2% agarose gel containing ethidium bromide and visualized by ultraviolet light.

CHX Chase Experiment. The post-transcriptional regulation of both the steady-state levels and half-life of the Nrf2 protein by CHX chase analysis was investigated. Cells were incubated in serum-free medium and treated with the protease inhibitor MG132 (10 μ M) for 2 h followed by washing with PBS three times. For analysis of steady state Nrf2 levels, the cells were treated with MC-LR (0.5 μ M), and then cell lysates were prepared at 0, 1, 2, and 4 h after MC-LR treatment. For CHX chase analysis, the cells were treated with CHX (100 μ g/mL) with or without MC-LR (0.5 μ M), and the cell lysates were prepared at 0, 10, 20, and 30 min after treatment. Whole-cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies against Nrf2.

Cell Staining for Cell-Cycle Analysis. After treatment with 0.5 μ M MC-LR for 16 h, cells were harvested and fixed with ice-cold 80% ethanol, treated with 500 μ g/mL RNase A (Sigma), and subsequently stained with 25 μ g/mL propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO). Cell-cycle distribution was measured by flow cytometry (Becton Dickinson, Mountain View, CA).

Cell Proliferation Assay. To measure cell proliferation, two different methods were used: MTT assay and cell-counting assay. For the MTT assay, cells were seeded in 24-well plates for MC-LR treatment. The medium and MC-LR were renewed every 48 h. Each day, cells were incubated with MTT reagent for 4 h, lysed with DMSO, and transferred to a 96-well plate for quantitation by a plate reader. For cell counting, 5×10^4 cells per well in 6-well plates were incubated in the presence or absence of MC-LR. The medium and MC-LR were renewed every 48 h. Numbers of viable cells were counted each day after staining with 0.4% trypan blue solution (Amresco).

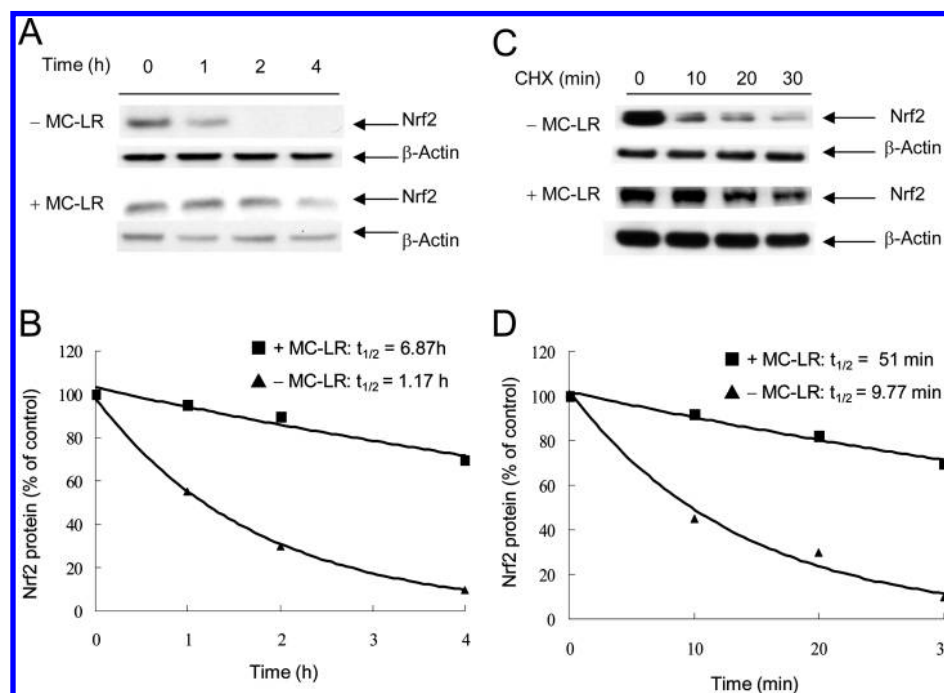


Figure 3. MC-LR inhibits Nrf2 protein turnover. (A and B) Steady state levels of Nrf2 protein. HepG2 cells were pretreated with MG132 (10 μ M, 2 h) followed by extensive washing to remove the MG132. The cells were then treated with MC-LR (0.5 μ M) for 0, 1, 2, and 4 h. Nrf2 protein was examined by immunoblotting. (C and D) CHX chase of Nrf2 protein. Cells were treated with CHX (100 μ g/mL) with or without MC-LR (0.5 μ M) for 0, 10, 20, 30 min. Nrf2 protein was examined by immunoblotting.

Data Analysis. At least three independent experiments were conducted for all analyses. Values are expressed as means \pm standard deviation. Student's *t*-test and Tukey's multiple comparison tests were used to estimate statistical significance, and *P* values of <0.05 were considered statistically significant.

Results

Activation of the Keap1/Nrf2 Pathway by MC-LR. We tested the activation of Nrf2 in HepG2 and Hep3B cells treated with various concentrations (0.05, 0.1, 0.5, and 1 μ M) of MC-LR for 4 h, and MG132 as the positive control. After cell washing in PBS, cytoplasmic and nuclear fractions were prepared, separated by SDS-PAGE, blotted, and probed with specific antibodies. MC-LR enhanced the levels of Nrf2 protein in a dose-dependent manner, with the highest level of induction observed at 0.5 μ M. Because Nrf2 regulates cellular survival responses, we hypothesized that treatment with a high dose of MC-LR could inhibit Nrf2, allowing cells to undergo programmed cell death. Therefore, we tested Nrf2 protein levels treated with high doses of MC-LR. Interestingly, at doses >1 μ M, Nrf2 protein levels decreased (Figure 1A). Immunofluorescence studies were used to confirm the increased nuclear localization of Nrf2 in cells subjected to MC-LR (Figure 1B). Nrf2 localization was examined by incubation with an anti-Nrf2 antibody; the location and integrity of the nucleus were determined by DAPI staining of the same cells. In control cells, low levels of Nrf2 expression were observed in the cytoplasm and the nucleus.

We further analyzed the endogenous expression of Nrf2 and its downstream target genes, *NQO1* and *HO-1*, using RT-PCR. Results (Figure 1C) showed that MC-LR treatment caused dramatic increases in *NQO1* and *HO-1* mRNA levels. In contrast, there were no changes in Nrf2 transcript levels after MC-LR treatment, indicating that activation of phase II enzymes *NQO1* and *HO-1* were due to nuclear accumulation of Nrf2, not Nrf2 expression change.

Increase in Endogenous *NQO1* and *HO-1* Mediated by MC-LR Requires the Expression of Endogenous Nrf2. An siRNA approach was used to determine if MC-LR-mediated expression of *HO-1* and *NQO1* were dependent upon Nrf2 (Figure 2). HepG2 cells were transiently transfected with either control-siRNA or siRNA directed against Nrf2. At 48 h after transfection, cells were exposed to MC-LR for 8 h, and then nuclear extracts were prepared for immunoblotting. The immunoblot was probed with antibodies directed against Nrf2, *HO-1*, *NQO1*, and β -actin. Exposure to MC-LR produced a robust increase in Nrf2 expression in cells transfected with control-siRNA. Gene silencing with siRNA directed against Nrf2 suppressed MC-LR-mediated expression of Nrf2. In cells transfected with control-siRNA, MC-LR also produced a robust increase in *NQO1* and *HO-1* expression. Transfection of siRNA directed against Nrf2 suppressed MC-LR-mediated expression of *NQO1* and *HO-1*. These experiments demonstrated a direct correlation between Nrf2, *NQO1*, and *HO-1* expression and supported the contention that MC-LR-mediated expression of *NQO1* and *HO-1* is Nrf2-dependent.

MC-LR Inhibits Nrf2 Protein Turnover. To compare the stabilization effects of Nrf2 protein with and without MC-LR treatment, protein decay and CHX chase experiments were performed to measure the half-life of the Nrf2 proteins. For analysis of steady state protein levels, Nrf2 was first induced by MG132 (10 μ M, 2 h) followed by extensive washing with fresh media. Figure 3A and B shows that the protein levels of Nrf2 decreased over time in the absence of an inducer with a

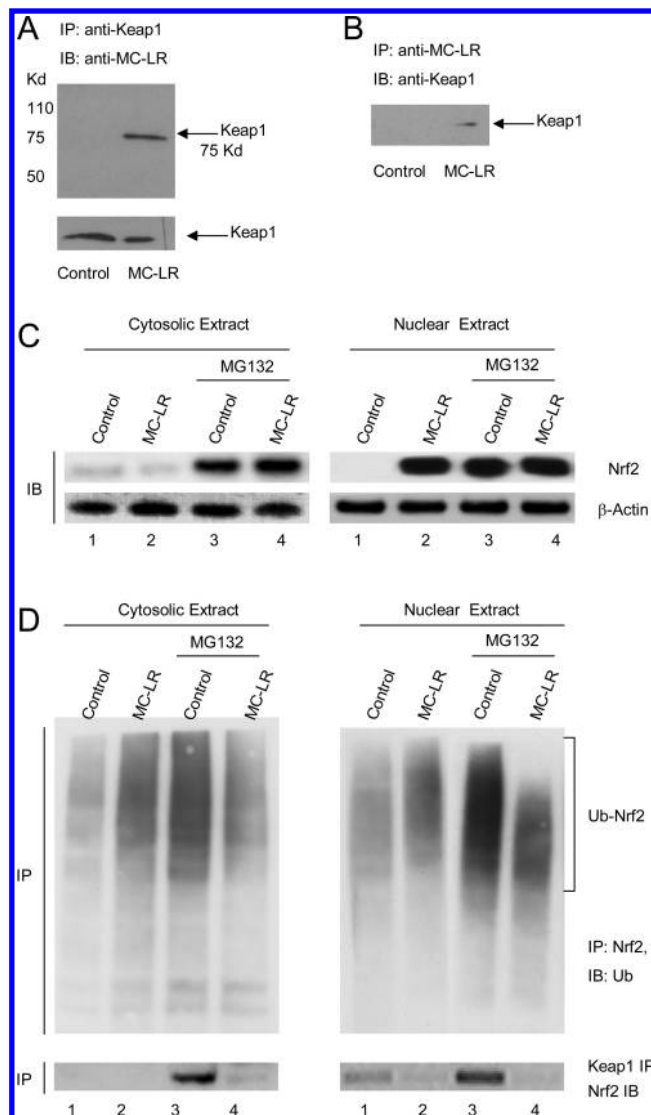


Figure 4. Interaction with Keap1 and inhibition of Nrf2 ubiquitination by MC-LR. (A and B) Binding of MC-LR to Keap1. HepG2 cells were incubated with vehicle or MC-LR (0.5 μ M) for 4 h. Subsequently, lysates were prepared and subjected to immunoprecipitation with either anti-Keap1 or anti-MC-LR antibodies. The precipitates were electrophoresed and probed. The precipitates were also probed with anti-Keap1 as a control to ensure that equal amounts of Keap1 were pulled down under each condition. (C and D) MC-LR inhibited the ubiquitination of Nrf2. HepG2 cells were treated with MC-LR (0.5 μ M) for 4 h or pretreated with MG132 for 2 h followed by MC-LR for 4 h. Cytoplasmic (left) and nuclear (right) fractions were prepared. (C) Immunoblotting of cytoplasmic (left) and nuclear (right) fractions. (D) Upper panel: Nrf2 was immunoprecipitated with anti-Nrf2 from cytoplasmic (left) and nuclear (right) fractions and then were detected by immunoblotting with anti-Ub. Bottom panel: cytoplasmic and nuclear fractions were immunoprecipitated with anti-Keap1 and immunoblotted with anti-Nrf2.

half-reduction time of 1.17 h, while treatment with MC-LR extended this period to 6.87 h. For the chase analysis, Nrf2 was induced, and then protein synthesis was blocked by treatment with CHX (100 μ g/mL). Figure 3C and D shows that, in the absence of MC-LR, the half-life of Nrf2 was 9.77 min, while treatment with MC-LR increased the half-life to 51 min in HepG2 cells. The results from our CHX chase experiments thus showed that MC-LR significantly stabilized Nrf2 degradation.

Interaction with Keap1 and Inhibition of Nrf2 Ubiquitination by MC-LR. To determine the mechanism of MC-LR in the activation of the Nrf2 pathway, we examined whether MC-LR could bind to the cytosolic regulator Keap1. Immuno-

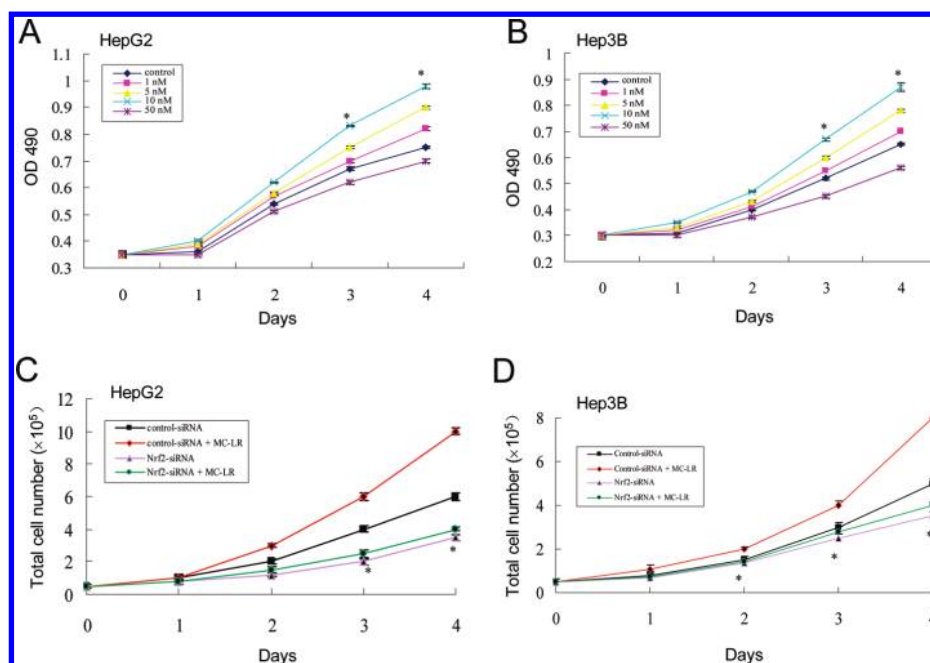


Figure 5. Nrf2 activated by MC-LR enhances cell proliferation in HepG2 and Hep3B cells. (A and B) HepG2 and Hep3B cells, respectively, were treated with the indicated doses of MC-LR for 1–4 days. Cell viability was determined by the MTT assay. (C and D) After 48 h of transfection with control-siRNA or siRNA directed against Nrf2, 5×10^4 HepG2 (C) and Hep3B (D) cells per well in 6-well plates were incubated in the presence or absence of MC-LR. The medium and MC-LR were renewed every 48 h. The numbers of viable cells were counted each day after 0.4% trypan blue solution staining. Mean cell numbers are plotted \pm SD for three independent experiments. * $P < 0.05$.

precipitation experiments were carried out to provide direct evidence for adduct formation between MC-LR and Keap1. HepG2 cells were treated with vehicle or 0.5 μ M MC-LR and lysed. The cell lysate was incubated with an anti-Keap1 antibody, and the immunoprecipitate was probed with anti-MC-LR antibody. A 75 kDa band which corresponds to the expected size of Keap1 was clearly detected with MC-LR treatment but not with vehicle (Figure 4A). To confirm this interaction, we performed the reverse pull-down experiment, i.e., precipitated with an anti-MC-LR antibody and then probed with an anti-Keap1 antibody. The immunoprecipitated protein was detected only in the cells treated with MC-LR (Figure 4B). These results demonstrated that MC-LR interacted with Keap1 in HepG2 cells.

We next investigated the mechanism of Nrf2 turnover by examining the ubiquitination of endogenous Nrf2 in the cytoplasmic and nuclear compartments in HepG2 cells. Treatment with MC-LR increased the Nrf2 protein levels in the nucleus (Figure 4C, right, lane 2) but not in the cytoplasm (Figure 4C, left, lane 2). Pretreatment with MG132 followed by MC-LR substantially increased Nrf2 proteins to a similar level in both fractions, reflecting the potent stabilizing effect of MG132 on Nrf2 proteins (Figure 4C, left and right, lanes 3–4). In the cytoplasm and nucleus, low but detectable levels of ubiquitinated Nrf2 were detected in MC-LR-treated cells (Figure 4D, lane 2). Pretreatment with MG132 dramatically increased the level of ubiquitinated Nrf2 (lane 3). However, this level was decreased by treatment with MC-LR (lane 4), reflecting the inhibition of Nrf2 ubiquitination since the total protein levels of Nrf2 were similarly high among the treatment groups (Figure 4C, right, compare lanes 2–4). These results indicated that MC-LR inhibited ubiquitination of Nrf2.

Inhibition of Nrf2 ubiquitination by MC-LR suggested the Nrf2/Keap1 complex as a target of MC-LR. Association of Nrf2 with Keap1 was examined by co-IP. Detectable association of Nrf2 and Keap1 was seen in the cytoplasm of untreated and MC-LR-treated cells consistent with the low protein levels of Nrf2 in the cytoplasm (Figure 4C, second panel, left, lanes 1–2).

MG132 markedly increased the association (lane 3). A low level of Nrf2/Keap1 association was observed with MC-LR and MG132 cotreatment, reflecting the inhibition of MC-LR on MG132-induced stabilization of Nrf2. In the nucleus, treatment with MC-LR reduced the association of Nrf2/Keap1 (Figure 4C, second panel, right, lanes 1–4). The findings revealed that MC-LR treatment markedly reduced Nrf2/Keap1 association.

Activation of Nrf2 by MC-LR Enhances Cell Proliferation in HepG2 and Hep3B Cells. It is well known that MC-LR increases liver cell survival and proliferation at low doses (22). To confirm that MC-LR could enhance liver cancer cell proliferation in our study, we treated HepG2 and Hep3B cells with different concentrations of MC-LR (0, 1, 5, 10, and 50 nM) and measured the total cell numbers using MTT assay. The results (Figure 5A and B) showed that initial rates of proliferation were similar in all samples; however, after 2 days the MC-LR-treated cells began to proliferate faster than the control. This result confirmed previous findings that MC-LR induces cell survival and proliferation.

To assess the role of the Nrf2 pathway in MC-LR-induced cell proliferation, we transiently knocked down Nrf2 by siRNA oligonucleotides in HepG2 and Hep3B cells and examined the total cell numbers after treatment with 10 nM MC-LR. Figure 2A (lane 1 and lane 2) shows the successful knockdown of Nrf2 mRNA in HepG2 cells. The knockdown of Nrf2 by siRNA potentially inhibited the proliferation of HepG2 and Hep3B cells, while MC-LR could not enhance the proliferation of these Nrf2-siRNA treated cells (Figure 5C and D). With reduced Nrf2, MC-LR lost the ability to stimulate cell proliferation. These results indicated that the Nrf2 activated by MC-LR enhanced the proliferation of liver cancer cells.

Activation of Nrf2 by MC-LR Alters the Cell-Cycle Distribution in HepG2 and Hep3B Cells. Finally, we tested the distribution of cell-cycle phases of HepG2 and Hep3B cells treated with MC-LR (Figure 6). The fraction of cells in the G₀/G₁ phase decreased from 58.55% to 31.24% in control-siRNA transfected HepG2 cells and from 39.5% to 22.02% in control-

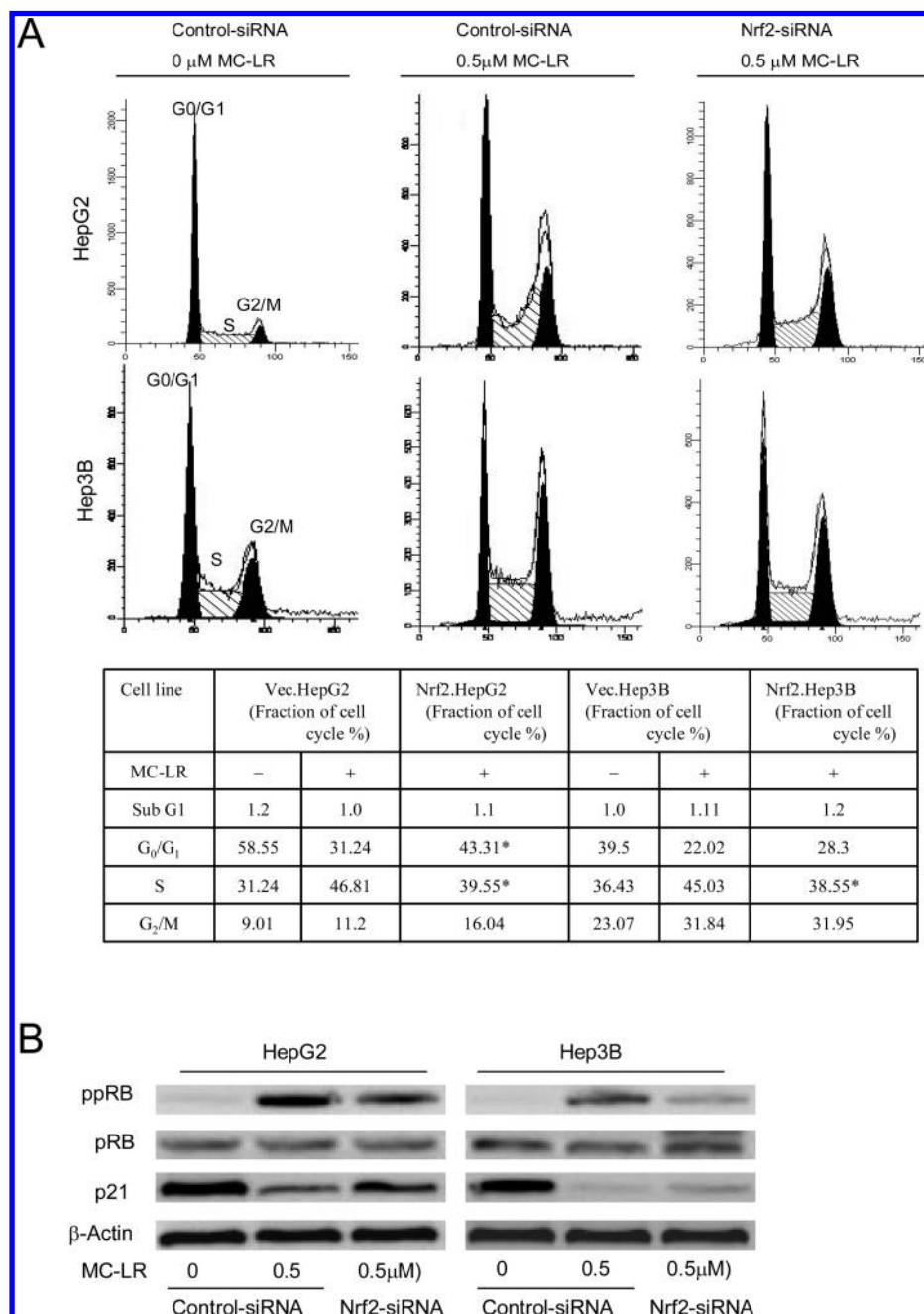


Figure 6. Effect of MC-LR on cell cycle distribution with or without Nrf2 knockdown. (A) HepG2 and Hep3B cells were transfected with control-siRNA or Nrf2-siRNA for 48 h. Cell cycle distribution was monitored before (left) and after 16 h of treatment with MC-LR by fluorescence-activated cell sorting with PI staining using a Coulter Epics XL flow cytometer. * $P < 0.05$, compared with control-siRNA transfected cells. (B) Protein levels of phosphorylated pRb and p21 in HepG2 (left) and Hep3B (right) cells. Cells were transfected with control-siRNA and Nrf2-siRNA and treated with or without MC-LR. Each protein level was determined by immunoblot analysis using antibodies against ppRb and p21. pRb and β -actin were used as the internal control of ppRb and p21, respectively.

siRNA transfected Hep3B cells. After MC-LR treatment, the fraction of HepG2 cells in S phase increased from 31.24% to 46.81% and that of Hep3B cells from 36.43% to 45.03%. After Nrf2 knockdown, the proportion of S phase cells decreased from 46.81% to 39.55% in HepG2 cells and from 45.03% to 38.55% in Hep3B cells with MC-LR treatment for 16 h. These results indicated that MC-LR could stimulate cell-cycle progression and that this stimulation was regulated by Nrf2.

We further assessed the phosphorylation of pRb and expression of p21 in HepG2 and Hep3B cells transfected with control-siRNA or Nrf2-siRNA and treated with or without MC-LR. The phosphorylated pRb was increased with MC-LR treatment in control-siRNA transfected cells but inhibited in Nrf2-siRNA transfected cells (Figure 6B, upper panel). However, the

expression of p21 was reduced with MC-LR treatment in control-siRNA transfected cells but increased in Nrf2-siRNA transfected cells (Figure 6B, bottom panel). Taken together, these results suggest that MC-LR regulates the G₁-S transition by the activation of Nrf2, which is consistent with a previous study showing that Nrf2 might regulate the G₁-S transition by modulating the expression of p21 and the phosphorylation of pRb (19).

Discussion

In the present study, we showed that MC-LR activated the Nrf2 pathway in a dose-dependent manner and enhanced the proliferation of liver cancer cells. The suppression of Nrf2 by

siRNA inhibited cellular proliferation, which could not be reversed by MC-LR. Moreover, MC-LR decreased the proportion of cells in the S phase after Nrf2 knockdown both in HepG2 and in Hep3B cells. These findings clearly indicate that Nrf2 contributes to MC-LR-induced cell proliferation.

Since the discovery of Nrf2 and its important role in the antioxidant response, it has been considered as a beneficial transcription factor that is essential in the protection from oxidative stress-related diseases. Paradoxically, the Nrf2 pathway is also induced by some toxic chemicals such as arsenic (23), hydrogen peroxide (24), chromium(VI) (25), and cadmium (26). This paradox may be explained by the balance between the induction of the Nrf2 defensive response and the toxic outcome elicited by a particular compound. It was reported that arsenic (23) and chromium(VI) (25) activate the Nrf2 pathway via the disruption of the Nrf2/Keap1 complex in the nucleus, while sulforaphane, a chemopreventive isothiocyanate, enables Nrf2 to escape Keap1-dependent degradation, leading to the stabilization of Nrf2, increased nuclear localization of Nrf2, and activation of Nrf2-dependent cancer-protective genes (27). However, it is still unclear whether naturally occurring beneficial compounds and harmful oxidative stress activate this pathway in a similar manner or through distinct mechanisms. In the present study, treatment of HepG2 and Hep3B cells with MC-LR resulted in a significant increase in Nrf2-ARE binding activity in the nuclear fraction. The possible mechanism for the Nrf2 activation by MC-LR may be that MC-LR binds to the cytosolic regulator Keap1 proteins and releases Nrf2. The Nrf2 proteins were able to avoid ubiquitination and proteasomal degradation, resulting in an increased stability of this transcription factor in the cytoplasm and its nuclear translocation. Therefore, it is possible that MC-LR binds to Keap1 through competition with Nrf2 and then liberates Nrf2. A similar mechanism was also reported by Satoh et al. who showed that electrophilic neurite outgrowth-promoting prostaglandin (NEPP) activates the Nrf2 pathway through binding to Keap1 (28). Similar to other Nrf2 activators, tBHQ and arsenic, MC-LR was able to block ubiquitination and degradation of Nrf2, resulting in the prolonged half-life of Nrf2. We also observed the subsequent induction of the Nrf2 target genes *NQO1* and *HO-1*, and the increase in endogenous NQO1 and HO-1 mediated by MC-LR required the expression of endogenous Nrf2.

The activation of Nrf2 has been reported to promote cancer cell growth and chemoresistance to anticancer drugs (19, 29–31). Ohta et al. found that there is a high incidence of loss of Keap1 function in patients with lung cancer, and the loss of Keap1 activity enhanced the nuclear accumulation of Nrf2 and elevated the expression of antioxidative and antixenobiotic stress enzymes and drug efflux pumps; these observations suggest that cancer cells with functionally down-regulated Keap1 acquired enhanced proliferation (29). Similar conclusions were also reached by Homma et al. through the knockdown of Nrf2 by siRNA (19). They found that the suppression of Nrf2 activity inhibited cellular proliferation and reduced the resistance to the anticancer drug in human lung cancer cells. In this study, the MC-LR-activated Nrf2 enhanced liver cancer cell proliferation, as evident by the transactivation of Nrf2, was further confirmed by the observation of abolished downstream activities in Nrf2-silenced cells. These findings indicated that activation of Nrf2 by toxins promotes the survival of cancer cells.

The role of Nrf2 in regulating the cell cycle was previously implicated (19). Homma et al. found that Nrf2 knockdown arrests the cell cycle in G₁ phase in Nrf2-rich lung cancer cells. To further explore the effect of Nrf2 on cell cycle distribution

with MC-LR treatment, we examined the cell cycles in Nrf2-silenced cells with or without MC-LR treatment and found that Nrf2 knockdown by Nrf2-siRNA inhibited the MC-LR-induced cell cycle through G₁ into S phase. The growth-inhibitory properties of Nrf2 suppression are likely attributable, at least in part, to its induction of cell cycle arrest at the G₁ phase as indicated in Figure 6.

In summary, we have shown that MC-LR is able to increase the stability of the Nrf2 transcription factor in the cytoplasm and its nuclear translocation via binding to the cytosolic regulator protein Keap1, while siRNA-mediated knockdown of Nrf2 mRNA can inhibit MC-LR-induced cell proliferation and cell cycle progression. Thus, MC-LR-induced up-regulation of Nrf2 in cancer cells enhances liver cancer cell growth. This study provides additional evidence to support a positive role of Nrf2 in cancer tumorigenesis.

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References

- (1) Carmichael, W. W. (1994) The toxins of cyanobacteria. *Sci. Am.* 270, 78–86.
- (2) Honkanen, R. E., Zwiller, J., Moore, R. E., Daily, S. L., Khatra, B. S., Dukelow, M., and Boynton, A. L. (1990) Characterization of microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatases. *J. Biol. Chem.* 265, 19401–19404.
- (3) Gehring, M. M. (2004) Microcystin-LR and okadaic acid-induced cellular effects: a dualistic response. *FEBS Lett.* 557, 1–8.
- (4) Sekijima, M., Tsutsumi, T., Yoshida, T., Harada, T., Tashiro, F., et al. (1999) Enhancement of glutathione S-transferase placental-form positive liver cell foci development by microcystin-LR in aflatoxin B₁-initiated rats. *Carcinogenesis* 20, 161–165.
- (5) Ding, W. X., Shen, H. M., and Ong, C. N. (2000) Critical role of reactive oxygen species and mitochondrial permeability transition in microcystin-induced rapid apoptosis in rat hepatocytes. *Hepatology* 32, 547–555.
- (6) Ding, W. X., Shen, H. M., and Ong, C. N. (2001) Pivotal role of mitochondrial Ca²⁺ in microcystin-induced mitochondrial permeability transition in rat hepatocytes. *Biochem. Biophys. Res. Commun.* 285, 1155–1161.
- (7) Fladmark, K. E., Brustugun, O. T., Mellgren, G., Krakstad, C., Bøe, R., Vintermeyr, O. K., Schulman, H., and Døskeland, O. (2002) Ca²⁺/Calmodulin-dependent protein kinase II is required for microcystin-induced apoptosis. *J. Biol. Chem.* 277, 2804–2811.
- (8) Zhu, Y. L., Zhong, X., Zheng, S., Ge, Z., Du, Q., and Zhang, S. Z. (2005) Transformation of immortalized colorectal crypt cells by microcystin involving constitutive activation of Akt and MAPK cascade. *Carcinogenesis* 26, 1207–1214.
- (9) Wild, A. C., Moinova, H. R., and Mulcahy, R. T. (1999) Regulation of gamma-glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. *J. Biol. Chem.* 274, 33627–33636.
- (10) Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., and Yamamoto, M. (1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* 13, 76–86.
- (11) Riley, R. J., and Workman, P. (1992) DT-diaphorase and cancer chemotherapy. *Biochem. Pharmacol.* 43, 1657–69.
- (12) Ma, Q., Kinneer, K., Bi, Y. Y., Chan, J. Y., and Kan, Y. W. (2004) Induction of murine NAD(P)H:quinone oxidoreductase by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin requires the CNC (cap ‘n’ collar) basic leucine zipper transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2): cross-interaction between AhR (aryl hydrocarbon receptor) and Nrf2 signal transduction. *Biochem. J.* 377, 205–213.
- (13) Moffit, J. S., Aleksunes, L. M., Kardas, M. J., Slitt, A. L., Klaassen, C. D., and Manautou, J. E. (2007) Role of NAD(P)H:quinone oxidoreductase 1 in clofibrate-mediated hepatoprotection from acetaminophen. *Toxicology* 230, 197–206.
- (14) Clark, J. E., Foresti, R., Green, C. J., and Motterlini, R. (2000) Dynamics of haem oxygenase-1 expression and bilirubin production in cellular protection against oxidative stress. *Biochem. J.* 348, 615–619.

- (15) Kobayashi, M., and Yamamoto, M. (2006) Nrf2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species. *Adv. Enzyme Regul.* 46, 113–140.
- (16) Zhang, D. D. (2006) Mechanistic studies of the Nrf2-Keap1 signaling pathway. *Drug Metab. Rev.* 38, 769–789.
- (17) Padmanabhan, B., Tong, K. I., Ohta, T., Nakamura, Y., Scharlock, M., Ohtsui, M., et al. (2006) Structural basis for defects of Keap1 activity provoked by its point mutations in lung cancer. *Mol. Cell* 21, 689–700.
- (18) Singh, A., Misra, V., Thimmulappa, R. K., Lee, H., Ames, S., et al. (2006) Dysfunctional Keap1-Nrf2 interaction in non-small-cell lung cancer. *PLoS Med.* 3, e420.
- (19) Homma, S., Ishii, Y., Morishima, Y., Yamadori, T., Matsuno, Y., et al. (2009) Nrf2 enhances cell proliferation and resistance to anticancer drugs in human lung cancer. *Clin. Cancer Res.* 15, 3423–3432.
- (20) Hu, C. L., Gan, N. Q., Chen, Y. Y., Bi, L. J., Zhang, X. E., and Song, L. R. (2009) Detection of microcystins in environmental samples using surface plasmon resonance biosensor. *Talanta* 80, 407–410.
- (21) Lei, L. M., Wu, Y. S., Gan, N. Q., and Song, L. R. (2004) An ELISA-like time-resolved fluorescence immunoassay for microcystin detection. *Clin. Chim. Acta* 348, 177–180.
- (22) Froscio, S. M., Humpage, A. R., Wickramasinghe, W., Shaw, G., and Falconer, I. R. (2008) Interaction of the cyanobacterial toxin cylindrospermopsin with the eukaryotic protein synthesis system. *Toxicon* 51, 191–198.
- (23) He, X. Q., Chen, M. G., Lin, G. X., and Ma, Q. (2006) Arsenic induces NAD(P)H-quinone oxidoreductase I by disrupting the Nrf2-Keap1-Cul3 complex and recruiting Nrf2-Maf to the antioxidant response element enhancer. *J. Biol. Chem.* 281, 23620–23631.
- (24) Li, J., Johnson, D., Calkins, M., Wrigh, L., Svendsen, C., and Johnson, J. (2005) Stabilization of Nrf2 by tBHQ confers protection against oxidative stress-induced cell death in human neural stem cells. *Toxicol. Sci.* 83, 313–328.
- (25) He, X. Q., Lin, G. X., Chen, M. G., Zhang, J. X., and Ma, Q. (2007) Protection against chromium (VI)-induced oxidative stress and apoptosis by Nrf2. Recruiting Nrf2 into the nucleus and disrupting the nuclear Nrf2/Keap1 association. *Toxicol. Sci.* 98, 298–309.
- (26) Chen, J., and Shaikh, Z. A. (2009) Activation of Nrf2 by cadmium and its role in protection against cadmium-induced apoptosis in rat kidney cells. *Toxicol. Appl. Pharmacol.* 241, 81–89.
- (27) Zhang, D. D., and Hannink, M. (2003) Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol. Cell. Biol.* 23, 8137–8151.
- (28) Satoh, T., Okamoto, S. I., Cui, J., and Watanabe, Y. (2006) Activation of the Keap1/Nrf2 pathway for neuroprotection by electrophilic phase II inducers. *Proc. Natl. Acad. Sci. U.S.A.* 103, 768–773.
- (29) Ohta, T., Lijima, K., Miyamoto, M., Nakahara, I., Tanaka, H., et al. (2008) Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth. *Cancer Res.* 68, 1303–1309.
- (30) Wang, X. J., Sun, Z., Villeneuve, N. F., Zhang, S., Zhao, F., et al. (2008) Nrf2 enhance resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2. *Carcinogenesis* 29, 1235–1243.
- (31) Lau, A., Villeneuve, N. F., Sun, Z., Wong, P. K., and Zhang, D. D. (2008) Dual roles of Nrf2 in cancer. *Pharmacol. Res.* 58, 262–270.

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