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Cinnamaldehyde Enhances Nrf2 Nuclear Translocation to Upregulate Phase II Detoxifying Enzyme Expression in HepG2 Cells

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ABSTRACT: Cinnamaldehyde has been demonstrated to stimulate glutathione production and the expression of phase II detoxifying enzymes in HepG2 cells. The mechanism underlying this cinnamaldehyde-mediated gene expression relies on Nrf2 transcriptional activity. Therefore, the molecular signaling events in cinnamaldehyde-mediated detoxifying enzyme expression were further investigated in this study. Cinnamaldehyde activated ERK1/2, Akt, and JNK signaling pathways, but not the p38 MAP kinase pathway, subsequently leading to Nrf2 nuclear translocation and eventually increasing phase II enzyme expression. In contrast, inhibition of ERK1/2, Akt, or JNK pathways attenuated Nrf2 nuclear translocation and phase II enzyme expression. Depletion of Nrf2 by small RNA interference (si-RNA) showed that the protein levels of phase II enzymes were no longer induced by cinnamaldehyde. A luciferase reporter assay and an electrophoretic mobility shift assay (EMSA) also demonstrated that cinnamaldehyde-activated signaling resulted in the increased transcriptional activity of Nrf2 through binding to the ARE4 enhancer sequence. Altogether, these data suggest that ERK1/2, Akt, and JNK pathways activated by cinnamaldehyde collectively control Nrf2 nuclear translocation and transcriptional activity, leading to the increase of phase II enzyme expression. Application of an appropriate chemopreventive agent such as cinnamaldehyde could potentially be an alternative strategy for cancer chemoprevention.

KEYWORDS: Chemopreventive agent, cinnamaldehyde, glutathione, glutathione *S*-transferase, glutamate-cysteine ligase, phase II detoxifying enzyme

INTRODUCTION

Cancer chemoprevention is the use of synthetic or naturally occurring compounds to prevent, inhibit, or reverse the process of carcinogenesis.[1] One of the intrinsic mechanisms for cancer chemoprevention is to induce the expression of antioxidant response element (ARE)-regulated genes, such as phase II and stress-responsive antioxidant enzymes, and deactivate carcinogenic molecules including ROS and electrophilic xenobiotics. ARE-regulated genes were induced through nuclear factor erythroid 2-related factor (Nrf2)−ARE signaling pathway.[2] The cis-acting ARE elements are found in the promoter or enhancer region of ARE-regulated genes, such as NAD(P)H: quinone oxidoreductase, glutamate-cysteine ligase (GCL), glutathione *S*-transferase (GST), UDP-glucuronosyltransferase, heme oxygenase-1 (HO-1), glutathione peroxidase (GPx), UDP glucuronosyl transferase (UGT)-1A, and NADPH/quinone oxidoreductase (NQO),[3] and they play a crucial role in the transcriptional upregulation of these genes by many electrophilic chemopreventive agents.[4] Nuclear transcription factor Nrf2 is a member of the basic leucine zipper family. Under uninduced conditions, Nrf2 is bound to a cysteine rich protein called Keap 1 (Kelch-like ECH-associated protein 1) and is sequestered in the cytoplasm. Once cells are exposed to oxidative stress or treated with electrophilic chemopreventive agents, the sequestered Nrf2 is released from Keap1 and translocated into the nucleus,[5] which associates with small Maf proteins to form a heterodimeric complex that finally induces ARE-mediated gene expression.

Cinnamaldehyde (Figure 1A), a major substance found in the essential oil from both the bark and leaves of some cinnamon varieties. GC-MS analyses showed that *Cinnamomum osmophloeum* (commonly known as pseudocinnamomum or indigenous cinnamon) contain cinnamaldehyde (90.6%) as the major component in the essential oil as demonstrated in our previous study,[6] which has been reported to have potential positive health effects[7] and may possess chemopreventive activity against cancer.[8] Cinnamaldehyde has been reported to exhibit several biological effects such as antiangiogenic effect[9] and anti-inflammatory activities.[10] Previous studies found that cinnamaldehyde induced apoptosis in a variety of tumor cell lines, including two human leukemia cell lines (HL-60 and K562)[6,11] and human hepatoma PLC/PRF/5 cells.[12] Cinnamaldehyde also exhibited antiproliferative activity on human colon-derived HCT 116 and mammary-derived MCF-7 carcinoma cells.[13]

Cinnamaldehyde is an electrophilic α,β-unsaturated aldehyde, which has been demonstrated to stimulate transcription of phase II detoxifying enzyme GCL resulting in observed increases of intracellular reduced glutathione (GSH) levels.[14] Studies conducted by Chew et al.[13] showed that cinnamaldehyde stimulated Nrf2-mediated downstream expression of thioredoxin reductase (TrxR) under sublethal doses. Furthermore, the ARE-dependent transcriptional activity was significantly stimulated by the treatment of cinnamaldehyde. The accumulated data propose that cinnamaldehyde is a Nrf2 activator, which induces transcriptional up-regulation of phase II enzymes and may function as a possible cancer chemopreventive agent at lower doses.[13]

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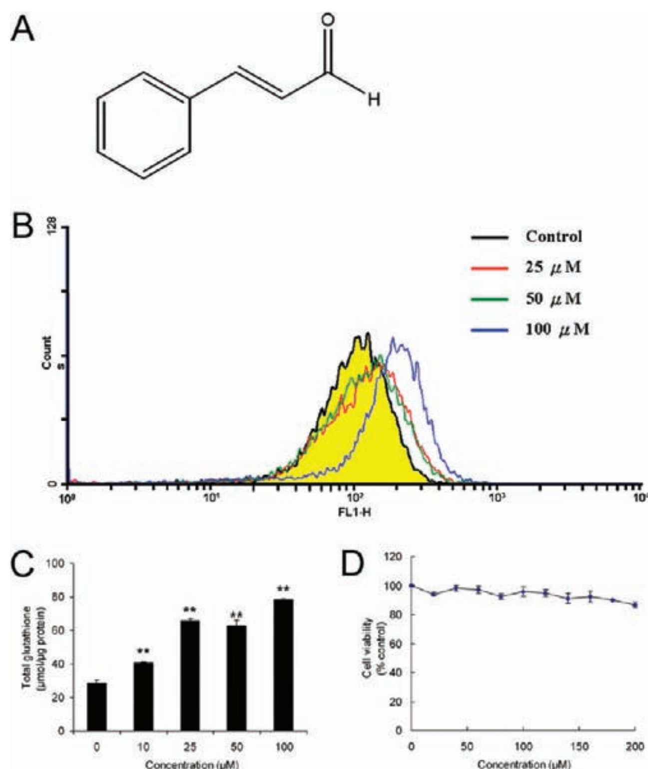

Figure 1. GSH generation is dose-dependently increased by cinnamaldehyde in HepG2 cells. (A) The structure of cinnamaldehyde. (B) Exponential growth of HepG2 cells were exposed to various doses of cinnamaldehyde for 6 h, and the intracellular DCF fluorescence intensity was measured using flow cytometry. (C) The content of GSH induced by various concentrations of cinnamaldehyde for 6 h was measured by ELISA and calculated from the average of three independent experiments. (D) Cells were exposed to various concentrations of cinnamaldehyde for 24 h before subjection to the MTT assay. Results (presented as the mean ± S.D.) are from three independently conducted experiments. The double asterisk indicates $P < 0.01$ as compared with the control.

Previous studies have indicated that phase II inducers not only covalently modify thiol groups of Keap1[15] but also are involved in the mechanism of the Nrf2−Keap1−ARE signaling pathway. Many upstream kinases, including phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC), and mitogen-activated protein kinases (MAPK), are assumed to involve the phosphorylation of Nrf2 causing the dissociation of Nrf2 from Keap1 and subsequent nuclear translocation of Nrf2.[16] One study indicated that treatment of prostate cancer PC-3 cells with phenethyl isothiocyanate (PEITC) induces Nrf2-dependent ARE activity and HO-1 gene expression through the activation of MAP kinases, including extracellular signal-regulated kinase (ERK) and c-Jun-NH2-kinase (JNK).[17] However, PKC, but not MEK/ERK, p38, or PI3K signaling pathways, is involved in Nrf2 nuclear translocation and its activation of NQO-1 gene expression in response to *t*-butylhydroquinone.[18] The role of signaling pathways in cinnamaldehyde-mediated detoxifying enzyme expression remains unclear. In this regard, the prime purpose of this series of experiments was to systemically study the regulatory role of signaling pathways in cinnamaldehyde-mediated gene expression using HepG2 cells as the model. As a result of these studies, the regulation of Nrf2 activation by specific signaling pathways in response to cinnamaldehyde would warrant further investigation.

MATERIALS AND METHODS

Cell Culture. Human hepatocarcinoma (HepG2) cells obtained from American Type Culture Collection (Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GibcoBRL; Grand Island, NY), 2 mM glutamine (GibcoBRL), and 1% penicillin/streptomycin (10000 units of penicillin/mL and 10 mg/mL streptomycin).

Chemicals and Antibodies. Cinnamaldehyde was purchased from Sigma-Aldrich (St. Louis, MO). Cell culture media were purchased from Invitrogen (Carlsbad, CA). PD98059, SB203580, SP600125, and LY294002 were purchased from Tocris Bioscience (Ellisville, MO). Antibodies against phospho-p44/42 MAPK (Thr202/Tyr204) (#9101), phospho-JNK (Thr183/Tyr185) (#9251), phospho-Akt (Ser473) (D9E), and ERK were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against Nrf2 and HO1, JNK, and lamin A were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 488-conjugated goat antirabbit antibody and Alexa Fluor 568-conjugated goat anti- rabbit antibody were purchased from Molecular Probes (Eugene, OR). A polyclonal antibody against GCLC was obtained from Lab Vision (Freemont, CA), while the polyclonal antibody against GST was obtained from BD Bioscience (San Jose, CA). Mouse monoclonal β-actin antibody and horseradish peroxidase (HRP)-conjugated goat antimouse IgG was from obtained from Chemicon (Temecula, CA). HRP-conjugated goat antirabbit IgG was from Zymed (South San Francisco, CA).

Viability Assay. Cells (1×10^4) were seeded in 96-well plates. After incubation for 24 h, cinnamaldehyde was added to each well by serial dilution, with the final concentration of the test extracts being 0−200 μM and then incubated for an additional 24 h. Cell viability was determined by a rapid colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) as described in the manufacturer's instructions (Sigma-Aldrich). The results were obtained from three independently conducted experiments.

Determination of Intracellular GSH Levels. For the measurement of intracellular GSH levels, flow cytometric analysis was performed as previously described.[19] HepG2 cells were treated with 0−100 μM cinnamaldehyde for the indicated times and stained with 50 μM chloromethylfluorescein-diacetate (CMF-DA) for an additional 30 min. The stained cells were analyzed immediately by a FACSCalibur flow cytometer using CellQuest program (BD, San Jose, CA).

Glutathione *S*-Transferase Assays. A modification of the method described by Jiang[20] was used to determine the activities of Glutathione *S*-transferase (GST). The reaction was carried out at 30 °C in 100 mM potassium phosphate (pH 7.0) containing 1 mM 1-chloro-2,4-dinitrobenzene (CDNB; Sigma−Aldrich), 2 mM GSH, and 3 μg of enzyme extract. After incubation for 5 min, the absorbance was measured at 340 nm. The concentration of conjugated CDNB with GSH was determined using the extinction coefficient 9.6 $mM^{-1}cm^{-1}$.

Immunofluorescence Microscopy. To detect the localization of Nrf2 in cinnamaldehyde-treated HepG2 cells, the HepG2 cells were seeded in a 6-well dish and grown to 80% confluence. Experimental cells were precultured with 100 μM cinnamaldehyde for 2 h. Then, cells having been precultured with cinnamaldehyde and control cells which were not precultured were fixed with methanol. After being washed three times with phosphate buffered saline (PBS), the cells were permeabilized with PBS containing 0.1% Triton X-100 for 5 min. After blocking with 5% bovine serum albumin for 1 h, the cells were probed with 200 μL of 1:300 diluted anti-Nrf2 antibody and then incubated in PBS containing 3% bovine serum albumin for 18−20 h at 4 °C. Cells were then washed with PBS and incubated with 50 μL of 1:500 diluted Alexa Fluor 488-conjugated goat antirabbit antibody. After 1 h of incubation at room temperature, 50 μL of 1:80 diluted propidium iodide (Sigma−Aldrich) was added, and the cells were incubated for another 30 min. The

fluorescent-labeled cells were analyzed using a laser confocal microscope (CARV II Confocal Imager; BD Biosciences).

Western Blot Analysis. HepG2 cells (1×10^6) were seeded in 6-cm plates and incubated at 37 °C for 16 h. Cells were treated with various concentrations of cinnamaldehyde for 0−12 h and then washed with cold PBS. Following centrifugation, cells were treated with 200 μL of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1% protease inhibitor cocktail, pH 7.4) and incubated at 4 °C for 1 h, after which the cell lysate was collected after centrifugation. Proteins in cell lysates were separated by subjection to sodium dodecyl sulfate−10% polyacrylamide gel electrophoresis (SDS−PAGE). The proteins on the gel were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore; Billerica, MA) with a Semidry transfer unit (Hoefer Inc.; Holliston, MA) and were probed with the primary antibodies at a dilution of 1:1,000 in 1× PBS-T buffer (0.05% Tween 20 in PBS buffer). After 1 h of incubation, HRP-conjugated secondary antibody (Zymed) at a 1:5,000 dilution was added, and the solution was incubated for another 1 h. Signals were developed by incubating the membrane with enhanced chemiluminescence (ECL) Plus Western Blotting Detection Reagents (GE Healthcare, UK) and exposed on X-Omat Blue XB-1 Film (Kodak, Rochester, NY) for autoradiography.

Transfection of siRNAs. The short interference RNA (siRNA) duplexes for this study were ordered from Sigma-Aldrich. The Nrf2-specific siRNA and control siRNA sequences have the following sequences: human Nrf2, CAAACAGAAUGGUCCUAAA; control, GAUCAUAC-GUGCGAUCAGA. For transient knockdown experiments, HepG2 cells (3×10^5) were transfected with 60 nM Nrf2-specific siRNA -(si-Nrf2) or control siRNA (si-control) using X-tremeGENE siRNA transfection reagent (Roche, Mannheim, Germany) according to the manufacturer's recommendation. After 24 h, the cells were treated with cinnamaldehyde and then subjected to Western blot analysis or the luciferase assay.

Transfection and Luciferase Reporter Assay. A DNA fragment containing three copies of the ARE4 elements from GCLC gene[21] (5′-CCCGTGACTCAGCGCTCCGTGACTCAGCGCTCCGTGA-CTCAGCGCT-3′; the ARE4 sequence is underlined) was subcloned into a pGL3-promoter vector to construct pGL3-ARE4-Luc. HepG2 cells were transiently transfected with a DNA mixture containing 2 μg of pGL3-ARE4-Luc and 0.5 μg of control plasmid pRL-TK (Promega, Madison, WI) using the TurboFect transfection reagent (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's published instructions. At 24 h following transfection, the cells were treated with 100 μM cinnamaldehyde for 0−24 h or pretreated with 10 μM of LY294002 (a PI3K inhibitor), SB203580 (a p38-MAPK inhibitor), SP600125 (a JNK inhibitor), and PD98059 (an MEK inhibitor) for 30 min and then treated with 100 μM cinnamaldehyde for 6 or 24 h. Whole-cell lysates were prepared, and luciferase activity was conducted utilizing the Dual-Luciferase Reporter Assay System (Promega) using a microtiter plate luminometer (LB940 Multilabel Reader, Berthold Technologies, Bad Wildbad, Germany) according to the protocol provided by the manufacturer. Renilla luciferase activity of pRL-TK was used to normalize the transfection efficiency.

EMSA (Electrophoretic Mobility Shift Assay). HepG2 cells were seeded for 24 h before treatment with 100 μM cinnamaldehyde for 6 h. Nuclear extracts were isolated using the NE-PER nuclear and cytoplasmic extraction kit (Pierce, Nepean, CA) following the manufacturer's published guidelines, and the protein concentration was measured using the Bradford assay (Bio-Rad, Richmond, CA). A double-stranded oligonucleotide containing three copies of ARE4 from the GCLC gene sequence was labeled with digoxigenin-11-ddUTP and terminal transferase using a DIG gel shift kit (Roche) according to the manufacturer's published protocol. The binding reaction was carried out in binding buffer containing 6 μg of nuclear extract and digoxigenin-labeled oligonucleotides at room temperature for 20 min and was then placed on ice. The reaction mixture was separated by native polyacrylamide (6%) gel electrophoresis. After electrophoresis, the gels were electroblotted onto positively charged nylon membranes. Digoxigenin-labeled complexes were detected with antidigoxigenin antibodies coupled to alkaline phosphatase (Roche). Signals were developed by incubating the membrane with substrate CSPD (Roche) and exposed on X-Omat Blue XB-1 Film for 10 min in an X-ray cassette.

Statistical Analysis. The data were analyzed using the SAS program (SAS Institute, Cary, NC). Student's *t*-test was used to examine statistically significant differences. *P*-values <0.05 were considered statistically significant.

RESULTS

Cinnamaldehyde Stimulates Glutathione Production in HepG2 Cells. GSH is the most powerful intracellular antioxidant and plays a role in the detoxification of a variety of electrophilic compounds and peroxides. Thus, we intended to investigate the effect of cinnamaldehyde on intracellular glutathione synthesis. The intracellular glutathione content in cinnamaldehyde-treated HepG2 cells was measured using flow cytometry. Interestingly, the intracellular GSH level was significantly enhanced by cinnamaldehyde treatment (Figure 1B). ELISA data also showed that the GSH levels were markedly increased by cinnamaldehyde (data not shown). After 100 μM cinnamaldehyde stimulation for 6 h, GSH production was about 2.7-fold higher in cinnamaldehyde-treated cells than in untreated cells (Figure 1C). The data from the ELISA assay also showed that cinnamaldehyde could enhance cellular GSH levels. After long-term exposure, the contents of GSH were not further increased, implying that the dynamic balance of the redox system would occur (data not shown). The relative cell survival rate of HepG2 cells treated with 100 μM cinnamaldehyde for 24 h achieved about 96% as analyzed by the MTT assay (Figure 1D), indicating that 100 μM cinnamaldehyde does not significantly contribute to cytotoxicity in mammalian cells. Taken together, this study has clearly demonstrated that cinnamaldehyde can promote GSH synthesis, suggesting that cinnamaldehyde has a cytoprotection role in response to stress.

Cinnamaldehyde Enhances Detoxifying Protein Expression. γ-Glutamylcysteine ligase calalytic subunit (GCLC), a catalytic subunit of γ-glutamylcysteine ligase (GCL), is the rate-limiting enzyme of GSH synthesis. Previous results have demonstrated that cinnamaldehyde can enhance GSH generation in HepG2 cells. Therefore, we next examined the involvement of GCLC expression in cinnamaldehyde-mediated GSH production. HepG2 cells were challenged by application of 100 μM cinnamaldehyde for different time periods, and GCLC protein levels were observed using Western blot analysis. As shown in Figure 2A (first row), the gradually increasing amount of GCLC proteins correlated to longer treatment with cinnamaldehyde. A significant increase of GCLC was observed after incubation with cinnamaldehyde for 12 h (Figure 2A, first row). As we know, γ-glutamylcysteine ligase is a member of the phase II detoxifying enzyme systems. We speculated as to whether cinnamaldehyde also contributes its stimulating effect on other phase II enzyme expression levels in HepG2 cells. Interestingly, cinnamaldehyde indeed enhanced glutathione-*S*-transferases (GST) and heme oxygenase-1 (HO-1), two of the phase II detoxifying enzymes, in a time-dependent manner (Figure 2A, the second and third rows). Dose-dependent analysis also indicated that the protein levels of GCLC, GST, and HO-1 were

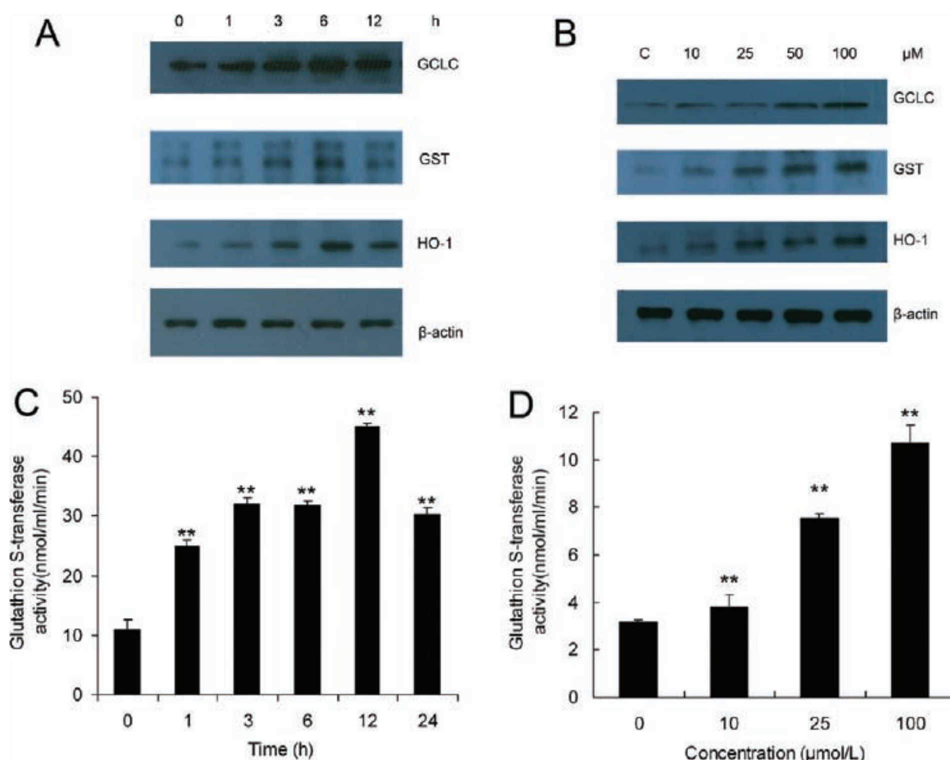

Figure 2. Induction of phase II detoxifying enzyme expression by cinnamaldehyde in HepG2 cells. (A) HepG2 cells were stimulated with 100 μM cinnamaldehyde for the indicated time periods, and the levels of GCLC, GST, and HO-1 were analyzed by Western blot analysis using specific antibodies against GCLC, GST, and HO-1. (B) The levels of GCLC, GST, and HO-1 induced by various concentrations of cinnamaldehyde were assayed by Western blot analysis. The GST activity induced by 100 μM cinnamaldehyde for 1−6 h (C) or by various concentrations of cinnamaldehyde for 24 h (D) was determined as described. Results (presented as the mean ± S.D.) are from three independently conducted experiments. The double asterisk indicates $P < 0.01$ as compared with the control.

remarkably augmented by increased concentrations of cinnamaldehyde (Figure 2B). To evaluate whether cinnamaldehyde also has an effect on the regulation of GST activity, HepG2 cells were treated with cinnamaldehyde for the indicated time periods, and GST enzyme activity was determined. The enzymatic activity of GST was time-dependently affected by 100 μM cinnamaldehyde for 1−24 h of exposure (Figure 2C), and a slight decrease of GST enzyme activity was observed at 24 h post-treatment. Furthermore, cinnamaldehyde could concentration-dependently enhance GST enzyme activity at various concentrations (10−100 μM) for 6 h. A 3.4-fold increase of GST activity in 100 μM cinnamaldehyde-treated cells was observed as compared with that of the untreated control cells (Figure 2D). On the basis of our data, it can be concluded that cinnamaldehyde could promote intracellular GSH production and activate the phase II detoxifying enzyme expression in response to various stresses by the use of GSH.

Cinnamldehyde Induces Nrf2 Nuclear Translocation. Several phase II detoxifying enzymes are regulated by transcriptional activation controlled by Nrf2. A desire to determine the mechanistic link promoted us to investigate the role of Nrf2 in cinnamaldehyde-mediated phase II detoxifying enzyme expression. To address this idea, activation of Nrf2 was detected in cinnamaldehyde-treated HepG2 cells by confocal laser microscopy. It has been well established that activated Nrf2 will translocate into the nucleus and bind to specific DNA elements to activate downstream gene expression. Therefore, HepG2 cells were treated with 100 μM cinnamaldehyde, and the intranuclear Nrf2 was stained with a specific antibody against Nrf2. Confocal microscope analysis revealed that fluorescently labeled Nrf2 had indeed accumulated in the nucleus of cells treated with cinnamaldehyde in a time-dependent profile (Figure 3A), indicating that a large body of Nrf2 was activated by cinnamaldehyde, whereas cinnamaldehyde did not cause increased mRNA expression of Nrf2 (data not shown). To further investigate the translocation of Nrf2 upon cinnamaldehyde treatment, cellular fractions were further separated into cytoplamic and nuclear fractions, and then the levels of Nrf2 in each fraction were evaluated by Western blot analysis. Figure 3B revealed that the levels of Nrf2 in the nuclear fraction gradually increased with prolonged cinnamaldehyde treatment, whereas decreased Nrf2 levels in cytoplasmic fractions with time were observed (Figure 3B), implying that Nrf2 might be, at least in part, the major transcription factor activated by cinnamaldehyde to regulate phase II enzyme expression. Taken together, our data indicate that cinnamaldehyde could activate phase II gene expression and consequently elevate the intracellular GSH level.

AKT, ERK, and JNK Signaling Pathways Participate in the Cinnamaldehyde-Induced Phase II Detoxifying Protein Expression. Signaling pathways regulate cellular biochemical processes, including gene expression, protein localization, protein turnover, cell growth and death, and responses to stress. Thus, from a regulatory viewpoint, it is important to delineate the effect of signaling pathways involved in the regulation of cinnamaldehyde-mediated Nrf2 activation and phase II enzyme expression. Several specific kinase inhibitors were used initially to assess

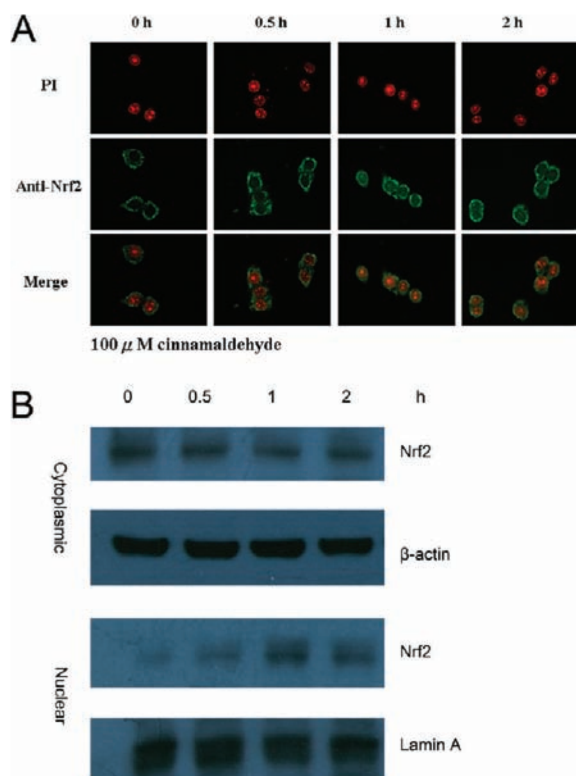

Figure 3. Cinnamaldehyde-induced Nrf2 nuclear translocation. (A) HepG2 cells were treated with 100 μM cinnamaldehyde for 0−2 h. HepG2 cells were fixed and immunostained with anti-Nrf2 antibody, whereas the nucleus was counterstained with propidium iodide (PI). The images of the cellular immunofluorescence were acquired using BD CARV II Confocal Imager. (B) The levels of Nrf2 in cytoplasmic and nuclear extracts from HepG2 cells treated with 100 μM cinnamaldehyde for 0−2 h were evaluated using Western blot analysis. Results are obtained from 3−5 independently conducted experiments.

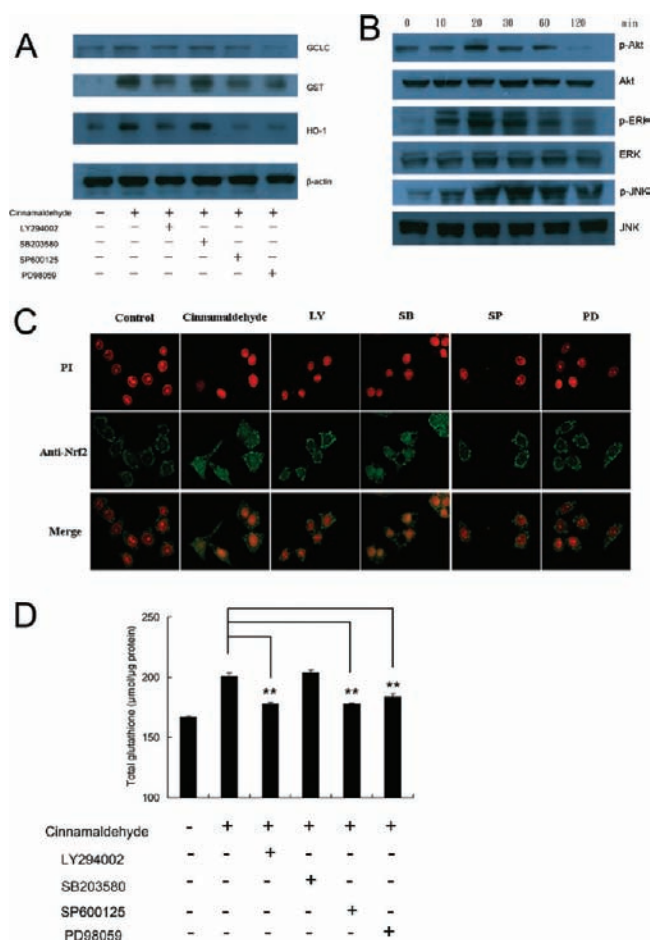

Figure 4. AKT, ERK, and JNK signaling pathways participate in the cinnamaldehyde-induced phase II protein expression in HepG2 cells. (A) Cells were treated with 10 μM LY294002, SB203580, SP600125, or PD98059 for 30 min and then challenged with 100 μM cinnamaldehyde for another 6 h. Total cell extracts were prepared and subjected to Western blot analysis for the detection of the levels of GCLC, GST and HO-1. (B) Cells were treated with 100 μM cinnamaldehyde for 0−120 min. Total cell extracts were prepared and subjected to Western blot analysis in order to detect the active phosphorylated forms of AKT, ERK1/2, JNK. (C) Cells were treated with 10 μM of the named inhibitors for 30 min and then cotreated with 100 μM cinnamaldehyde for another 6 h. The Nrf2 nuclear translocation was assayed as described in Figure 3A. (D) Cells were treated as described for panel A, and ELISA analysis was applied to measure the contents of GSH. Results (presented as the mean ± S.D.) are from 3−4 independently conducted experiments. The double asterisk indicates $P < 0.01$ using Student's *t*-test for comparison between cinnamaldehyde-treated cells and those exposed to the dose of cinnamaldehyde in the presence of kinase inhibitors.

whether a specific signaling pathway is involved in cinnamaldehyde-mediated Nrf2 activation and phase II enzyme expression. LY294002 (an inhibitor of the PI3K family), SB203580 (an inhibitor of p38 MAP kinase), SP600125 (an inhibitor of JNK1/2), and PD98059 (an inhibitor of MEK1/2) were used in the pretreatment of HepG2 cells for 30 min, and then cells were cotreated with 100 μM cinnamaldehyde for another 6 h. After treatment, cell extracts were prepared to determine the levels of GCLC, GST, and HO-1. As shown in Figure 4A, LY294002, SP600125, and PD98059 could effectively suppress the upregulation of these protein expressions by cinnamaldehyde, while SB203580 failed to repress cinnamaldehyde-mediated protein expression (Figure 4A). These results indicate that the AKT, ERK, and JNK signaling pathways, but not p38 MAP kinase, might be involved in the regulation of cinnamaldehyde-mediated phase II enzyme expression. Consequently, the extent of AKT, ERK, and JNK phosphorylation was further investigated following cinnamaldehyde treatment. The activated kinases were determined by Western blot analysis using antibodies specific to the forms of phosphorylated kinases. The results presented in Figure 4B reveal that cinnamaldehyde is able to activate AKT, ERK, and JNK signaling pathways, but not p38 MAP kinase, in a time-dependent manner (Figure 4B), consistent with the data in Figure 4A. The overall protein levels of these kinases were similar. It is conceivable that cinnamldehyde-enhanced phase II protein expression through AKT, ERK, and JNK signaling pathways regulates the enhancement of Nrf2 activation and translocation. To further investigate this possible role in Nrf2 activation, the translocation of Nrf2 stimulated by cinnamaldehyde was examined using a confocal microscope. The data clearly indicates that cinnamaldehyde induces abundant translocation of Nrf2 into the nucleus. However, the nuclear translocation of Nrf2 was specifically suppressed by the addition of inhibitors of the AKT, ERK, and JNK signaling pathways, but not the p38 MAP kinase inhibitor, further supporting the previously mentioned results

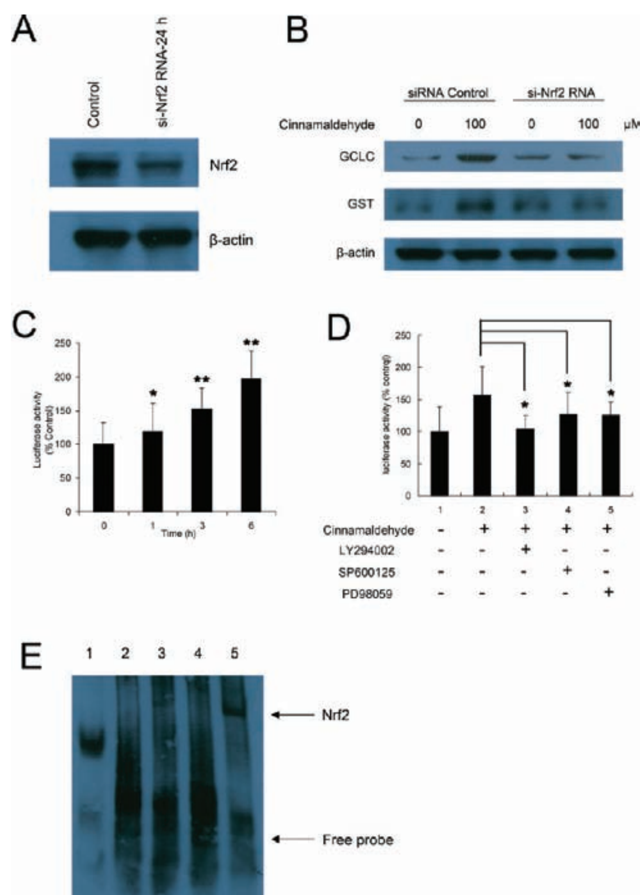

Figure 5. Cinnamaldehyde enhances phase II enzyme expression at the transcriptional level. (A) 60 nM duplex siRNA against Nrf2 (si-Nrf2) was transfected into cells, and expression was allowed to take place for 24 h. The levels of Nrf2 were evaluated using Western blot analysis. (B) After transfection of si-Nrf2 for 24 h, the cells were exposed to 100 μM cinnamaldehyde for 24 h, and then the levels of GCLC, GST, and HO-1 were measured. (C) The ARE4 element was cloned into the pGL3-Luc vector to construct pGL3-ARE4-Luc, which was then transfected into HepG2 cells. After 24 h, cells were exposed to 100 μM cinnamaldehyde for 1−6 h, and then luciferase activities were evaluated. The asterisk and double asterisk indicate $P < 0.05$ and $P < 0.01$ as compared with control. (D) Cells were transfected with pGL3-ARE4-Luc and were pretreated with the named inhibitors and cinnamaldehyde as described in Figure 4A; the luciferase activities were then conducted. The asterisk indicates $P < 0.05$ using Student's *t*-test for comparison between cinnamaldehyde-treated cells and those exposed to the dose of cinnamaldehyde in the presence of kinase inhibitors. (E) Electrophoretic mobility shift assay was performed using the oligomers carrying the ARE4 sequences. The nuclear extract obtained from cells treated with 100 μM cinnamaldehyde for 6 h (lane 5) demonstrated high affinity for ARE4-DNA binding, whereas untreated control cells (lane 4) did not bind to ARE4. A competition experiment was carried out by adding a 100-fold excess of nonlabeled wild type ARE4 oligomers (lane 3) to the reaction mixture containing the nuclear sample of lane 5 before adding the labeled probe. The negative control, a reaction mixture containing the labeled probe without nuclear proteins, is shown in lane 2. Lane 1 displays a positive control, a reaction mixture containing oligomers provided by manufacturer. Results (presented as the mean ± S.D.) are from three independently conducted experiments.

(Figure 4C). The GSH generation enhanced by cinnamaldehyde also was suppressed by the inhibitors of AKT, ERK, and JNK (Figure 4D). As a result of these findings, we conclude that AKT, ERK, and JNK signaling pathways are indeed involved in cinnamaldehyde-mediated Nrf2 activation and thus also are involved in phase II enzyme expression.

Cinnamaldehyde Enhances Phase II Detoxifying Enzyme Expressions at the Transcriptional Level. To determine whether Nrf2 participates in cinnamaldehyde-elicited phase II enzyme expression, we knocked down Nrf2 using a specific siRNA duplex (si-Nrf2 RNA). As shown in Figure 5A, si-Nrf2 RNA effectively attenuated Nrf2 protein levels. Interestingly, si-Nrf2 RNA significantly suppressed the cinnamaldehyde-enhanced GCLC and GST protein levels, whereas the si-control did not suppress the expression levels of these proteins (Figure 5B). These results provide further evidence indicating that cinnamaldehyde-elicited phase II enzyme expression is mediated by Nrf2. It has been shown that Nrf2 binds to ARE within the 5′-flanking region of phase II enzymes.[22] Therefore, the sequences around the ARE4 site were synthesized and subcloned into the reporter plasmid pGL3-luciferase. The specificity of cinnamaldehyde for the ARE sequences was then determined by transfecting HepG2 cells with the luciferase reporter construct harboring this element. At 24 h following transfection, cinnamaldehyde was applied and enhanced luciferase activity was observed and analyzed. The data presented in Figure 5C clearly indicates that cinnamaldehyde enhanced luciferase activity in a time-dependent manner. Moreover, pretreatment with AKT, ERK, and JNK signaling inhibitors returned the luciferase activity to the basal level, implying that cinnamaldehyde-elicited AKT, ERK, and JNK signaling pathways can indeed positively regulate phase II enzyme expression at the transcriptional level (Figure 5D). Consistent with the luciferase data, an EMSA further confirmed that cinnamaldehyde increased Nrf2 binding to the ARE4 site (Figure 5E). In concurrence with previous studies, we conclude that cinnamaldehyde can activate, at least in part, AKT, ERK, and JNK signaling pathways, which in turn enhance Nrf2 nuclear translocation and activation, consequently promoting phase II enzyme expression. In summary, cinnamaldehyde exerts antioxidative effects by inducing redox-responsive signaling pathways modulating redox-sensor Nrf2 activity.

DISCUSSION

As a redox-dependent transcription factor, Nrf2 controls the expression and coordinates the induction of a number of genes, including those that produce stress response proteins and detoxifying enzymes. Therefore, the nuclear abundance of Nrf2 is tightly regulated, achieved by the mechanism of nuclear export and degradation of Nrf2. It is conceivable that signaling pathways can play a distinct role in the regulation of Nrf2 protein subcellular distribution. Since Nrf2 has been proven to be the downstream transcriptional factor of cinnamaldehye regulating phase II detoxifying enzyme expression, the downstream regulatory signaling pathways of cinnamaldehyde remain unclear. In this study, we applied a cultured cell model using HepG2 cells to determine that low dose of cinnamaldehyde exerts its antioxidative effects by inducing the redox-responsive signaling pathways to modulate redox-sensor Nrf2 activity. Cinnamaldehyde stimulates phase II detoxifying enzyme expression in a manner dependent on Nrf2 nuclear translation and activation of gene expression through ARE binding. Most importantly, we have also identified that the signaling pathways MEK/ERK, PI3K/AKT, and JNK participate in the cinnamaldehyde-mediated phase II

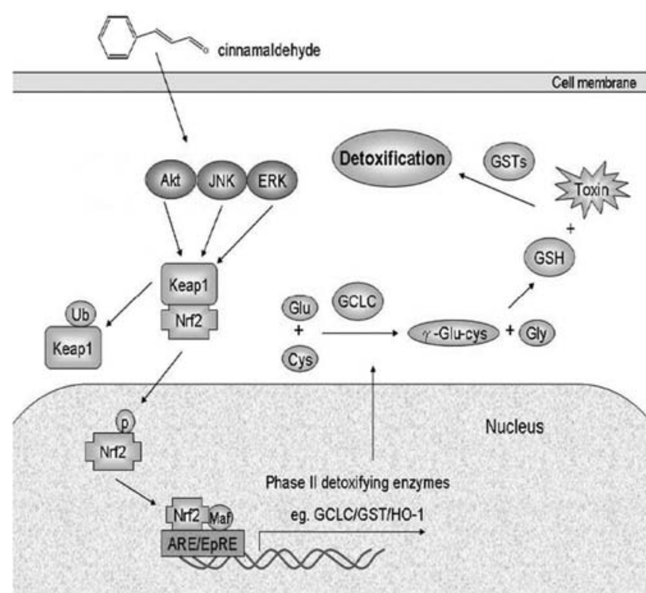

Figure 6. Diagram depicting the hypothesis of signaling regulation on phase II detoxifying enzyme expression through Nrf2 nuclear translocation following cinnamaldehyde exposure.

detoxifying enzyme expression. Furthermore, using specific kinase inhibitors and confocal microscopy, we have provided evidence that these pathways are involved in upstream signaling and that these pathways are essential for Nrf2 nuclear translocation and subsequent activation of downstream gene expression. In concurrence with previous findings, a working model of our hypothesis is illustrated in Figure 6.

Cinnamaldehyde is a natural active compound with electrophilic ability to modulate cellular redox homeostasis. Therefore, it is highly conceivable that redox-sensitive signaling pathways, for instance, MEK/ERK, JNK, and PI3K/AKT which were assayed in this study, are responsible for the signal transduction from cinnamaldehyde to Nrf2. Moreover, the PKC signaling pathway has been proven to directly phosphorylate Nrf2 on the Ser40 residue.[18] Fyn, a member of the Src tyrosine kinase family, also has been determined to phosphorylate Nrf2 on Tyr568 residue modulating Nrf2 nuclear translocation.[23] In agreement with previous studies, we demonstrate that MEK/ERK, JNK, and PI3K/AKT signaling modulated by cinnamaldehyde play a critical role in the regulation of Nrf2 nuclear localization and in the activation of phase II detoxifying enzyme expression. Since the phosphorylation of ERK1/2, JNK1/2, and PI3K/AKT are significantly stimulated by cinnamaldehyde, and the nuclear translocation of Nrf2 is specifically attenuated by inhibitors of these three signaling pathways, we postulate that these signaling pathways might directly phosphorylate Nrf2 in the cytoplasm and are required for the release of Nrf2 from Keap1 cytoplasmic sequestration. However, we cannot rule out the possibility that these signaling pathways might phosphorylate Keap1 directly to modulate the association between Keap1 and Nrf2. Moreover, it has been identified that the AREs contain internal AP-1 or AP-1-like binding sequences. AP-1 family members, such as JunD and c-Fos, can bind to the human NQO-1 ARE sequence.[24,25] Notably, AP-1 family members are the direct downstream effectors of the ERK and JNK signaling pathways. Therefore, we cannot rule out the possibility that the other transcription factors might also render an effect on the cinnamaldehyde-mediated phase II detoxifying enzyme expression except via the direct activation of Nrf2. Alternatively, previous reports have shown that phase II detoxifying enzyme inducers with electrophilic activity directly modify Keap1 on Cys273 and Cys288 residues, which in turn result in the release of Nrf2 in response to electrophiles and oxidative stress [26] Indeed, a number of studies have reported that cinnamaldehyde can target the thiols of cysteine residues on protein to form a cinnamaldehyde-protein conjugate, which can be implicated in the effect of the α,β-unsaturated carbonyl moiety in cinnamaldehyde.[27,28] Thus, except for the activation of signaling pathways, it is likely that cinnamaldehyde might, at least in part, directly attack Keap1 leading to Nrf2 disassociation and nuclear translocation. Further studies need to be performed to address this hypothesis.

Studies conducted by Chew et al.[15] showed that cinnamaldehyde stimulates Nrf2-mediated downstream expression of thioredoxin reductase (TrxR) and up-regulates ARE-dependent transcriptional activity under sublethal doses. On the contrary, cinnamaldehyde induces cell death at lethal doses. Therefore, cinnamaldehyde is an Nrf2 activator and might possess chemopreventive activity against cancer when used at a lower dose.[15] Using a cultured cell model in this study, we have demonstrated that cinnamaldehyde's chemopreventive effect at low doses might be a result from the enhancement of antioxidant response via Nrf2-mediated gene expression. Cinnamaldehyde induces the activation of three MAP kinases while it also possesses an inhibitory effect on LPS-mediated MAPK signaling activation in macrophages. Interestingly, cinnamaldehyde can induce three MAP kinase cascade activation to mediate apoptosis, whereas cinnamaldehyde exerts it cytoprotective effect via Nrf2-mediated phase II detoxifying protein expression. In this study, it was further demonstrated that the induction of Nrf2-mediated phase II detoxifying protein expression is required for MEK/ERK, JNK, and PI3K/AKT signaling pathways. As a result of our results presented here and their concurrence with previous reports, we speculate that the cinnamaldehyde possesses both cytotoxic and cytoprotective effects, depending on extracellular stress, the type of cell situation, and the whether it is in the context of signaling or dynamic redox balance. Most importantly, the different concentrations and duration of cinnamaldehyde exposure in distinct cell types determine the cell fate, especially since the balance of the signaling and redox state is always dysregulated in tumor cells. In summary, it is of great necessity to most effectively define the chemotherapeutic strategy for cancer therapy using dual effects of compounds that can act as effective chemotherapeutic and chemopreventive agents or adjuvants.

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