Isoalantolactone from Inula helenium Caused Nrf2-Mediated Induction of Detoxifying Enzymes

Article *in* Journal of medicinal food · October 2009

DOI: 10.1089/jmf2009.0072 · Source: PubMed

10 authors, including:

Soon Sung Lim
Hallym University
221 PUBLICATIONS **3,745** CITATIONS

SEE PROFILE

Jung Han Yoon Park
Hallym University
246 PUBLICATIONS **5,734** CITATIONS

SEE PROFILE

Jong-Sang Kim
Kyungpook National University
192 PUBLICATIONS **2,953** CITATIONS

SEE PROFILE

JOURNAL OF MEDICINAL FOOD
J Med Food 12 (5) 2009, 1038–1045

DOI: 10.1089/jmf.2009.0072

Full Communication

Isoalantolactone from *Inula helenium* Caused Nrf2-Mediated Induction of Detoxifying Enzymes

Ji Yeon Seo,[1] Jia Park,[1] Hyo Jung Kim,[1] In Ae Lee,[1] Ji-Sun Lim,[1] Soon Sung Lim,[2] Se-Jin Choi,[2] Jung Han Yoon Park,[2] Hui Jung Kang,[3] and Jong-Sang Kim[1]

[1]*Department of Animal Science and Biotechnology and School of Applied Biosciences, Kyungpook National University, Daegu;* [2]*Department of Food Science and Nutrition, Hallym University, Chuncheon; and* [3]*Department of Human Ecology, KyungSung University, Busan, Republic of Korea*

ABSTRACT Our previous study demonstrated that methanolic extract of *Inula helenium* (Elecampane) has the potential to induce detoxifying enzymes such as NAD(P)H:(quinone acceptor) oxidoreductase 1 (EC 1.6.99.2) (NQO1, QR) activity and glutathione *S*-transferase (GST) and found isoalantolactone and alantolactone as the active components. In this study we investigated the detoxifying enzyme-inducing potential of isoalantolactone, which is present in *I. helenium* and has a structure similar to that of alantolactone. The compound induced QR in a dose-dependent manner in both Hepa1c1c7 cells and its mutant BPRc1 cells lacking the arylhydrocarbon receptor translocator. Like with most phase 2 enzyme inducers, other phase 2 detoxifying enzymes, including GST, glutathione reductase, γ-glutamylcysteine synthetase, and heme oxygenase-1, were also induced by isoalantolactone in a dose-dependent manner in the cultured cells. Furthermore, isoalantolactone caused a proportionate increase in luciferase activity depending upon concentration and exposure time in the reporter assay in which HepG2-C8 cells, transfectants carrying antioxidant response element-luciferase gene, were used. The nuclear translocation of nuclear factor-E2-related factor 2 (Nrf2) was stimulated by the compound and attenuated by phosphatidylinositol 3-kinase inhibitors such as LY294002 and wortmannin. In conclusion, isoalantolactone is a candidate for chemoprevention and acts as potent phase 2 enzyme inducer by stimulating the accumulation of Nrf2 in the nucleus.

KEY WORDS: • ***Akt*** • ***antioxidant response element*** • ***chemoprevention*** • ***detoxifying enzymes*** • ***isoalantolactone*** • ***NAD(P)H:(quinone acceptor) oxidoreductase 1*** • ***nuclear factor-E2-related factor 2*** • ***phosphatidylinositol 3-kinase*** • ***sesquiterpenes***

INTRODUCTION

CHEMOPREVENTION HAS BEEN DEFINED as the use of pharmacologic or natural agents designed to prevent, suppress, or reverse the process of carcinogenesis before the development of malignancy.[1,2] One of the major mechanisms of chemical protection against carcinogenesis is the induction of phase 2 enzymes involved in detoxification of exogenous and endogenous mutagens and carcinogens. Antioxidant response element (ARE) is present in the promoter region of genes encoding for phase 2 detoxification/antioxidant enzymes such as heme oxygenase-1 (HO-1), NAD(P)H:(quinone acceptor) oxidoreductase 1 (EC 1.6.99.2) (NQO1, QR), and glutathione *S*-transferase (GST). In unstressed states, nuclear factor-E2-related factor 2 (Nrf2) is present in the cytoplasm in association with Kelch-like ECH-associated protein 1 (Keap1). Disturbance of the interaction between Nrf2 and Keap1, including covalent or oxidative modification of cysteine thiols in Keap1 by electrophiles or oxidative stress, results in Nrf2 release and its translocation into the nucleus. Binding of Nrf2 to the ARE sequence in genes encoding phase 2/antioxidant enzymes causes transcriptional activation of the relevant genes, promoting removal of reactive oxygen species or toxic chemicals. Many natural compounds such as curcumin, caffeic acid phenethyl ester, and sulforaphane are known to act as electrophiles in Nrf2/ARE activation.[3,4]

Our previous study demonstrated that sesquiterpenes isolated from *Inula helenium* (also known as Elecampane) induced NQO1 (QR), an anticarcinogenic marker enzyme.[5,6] In an attempt to elucidate the underlying mechanism of phase 2 enzyme induction by the sesquiterpene isoalantolactone, the effect of isoalantolactone (Fig. 1) on Nrf2/ARE activation was investigated.

MATERIALS AND METHODS

Materials

All cell culture reagents and fetal bovine serum were obtained from Gibco BRL (Gaitherburg, MD, USA). Hepa1c1c7 and BPRc1 cells were from the American Type

Manuscript received 16 March 2009. Revision accepted 30 June 2009.

Address correspondence to: Jong-Sang Kim, Ph.D., Department of Animal Science and Biotechnology, School of Applied Biosciences, Kyungpook National University, Daegu 702-701, Republic of Korea, E-mail: vision@knu.ac.kr

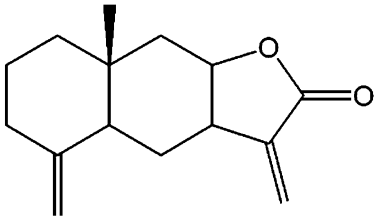

FIG. 1. Structure of isoalantolactone.

Culture Collection (Rockville, MD, USA). All other chemicals were of reagent grade. Roots of *I. helenium* L. were purchased from DeaGuang in Chuncheon, Republic of Korea, in 2006. A voucher specimen (number 325) is deposited at the Hallym University RIC Center in Chuncheon.

Cell culture

Hepa1c1c7 and its mutant (BPRc1) cells were plated at a density of, respectively, 3×10^5 and 5×10^5 cells per 100-mm-diameter plate (Nunc, Rochester, NY, USA) in 10 mL of α-minimum essential medium supplemented with 10% fetal bovine serum. The HepG2-C8 cell line established in the laboratory of Dr. A.N. Kong at Rutgers, The State University of New Jersey (Piscataway, NJ, USA) by transfecting human hepatoma HepG2 cells with pARE-TI-luciferase construct was used for the reporter assay.[7] HepG2-C8 cells were maintained in modified Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, GlutaMax (catalog number 35050-061, Gibco BRL), 100 units/mL penicillin, and 0.5 mg/mL G418. Cells were normally starved overnight in 0.5% fetal bovine serum-containing medium before treatment. The cells were normally incubated for 3–4 days in a humidified incubator in 5% CO_2 at 37°C. Cells were cultured for 48 hours and exposed to various concentrations of sample for another 24 hours, followed by biochemical assays.

Extraction and purification of QR inducer from I. helenium

Roots (1.5 kg) of *I. helenium* were air-dried followed by grinding in a Willey-Mill plant grinder. Ground plant material was soaked in *n*-hexane (8.5 L) for 24 hours. The solvent was decanted from the plant residue and evaporated *in vacuo* to yield 40.8 g of crude extract. A portion of the *n*-hexane root extract (5.2 g) was adsorbed to silica gel and applied to a silica gel chromatography column (particle size, 40–63 μm; 60×300 mm; 60 Å). Elution of the column was performed using increasing polarity mixtures of *n*-hexane/ethyl acetate in a series of three linear gradient steps. Step 1 consisted of 100/0 to 90/10 eluent using 2 L with step 2 consisting of 90/10 to 75/25 eluent using 1 L. Step 3 consisted of 75/25 to 0/100 eluent using 1 L, and the column was washed with 2 L of ethyl acetate. Column eluate was collected in 30-mL test tubes and, based on thin-layer chromatography similarities, recombined into 13 fractions: 1, tubes 1–18, 80 mg; 2, tubes 19–20, 85 mg; 3, tubes 21–28, 1.3 g; 4, tubes 29–33, 620 mg; 5, tubes 34–37, 45 mg; 6, tubes 38–41, 55 mg; 7, tubes 42–46, 86 mg; 8, tubes 47–52, 1.2 g; 9, tubes 53–62, 520 mg; 10, tubes 62–67, 52 mg; 11, tubes 68–72, 65 mg; 12, tubes 73–76, 96 mg; and 13, tubes 77–85, 1.1 g. Further purification of the bioactive fractions 8 and 13 was accomplished using repeated vacuum liquid chromatography procedures on silica gel to yield compound 1 (57 mg) and compound 2 (85 mg), respectively, with a purity of 98.8% as determined by high-performance liquid chromatography.

Identification of isoalantolactone

The identification of QR inducer was accomplished by comparison of spectroscopic data with those reported in the literature.[8,9] ^{1}H- and ^{13}C-nuclear magnetic resonance spectra were recorded in $CDCl_3$ on a Bruker (Billerica, MA, USA) Avance 400 MHz spectrometer. All ^{13}C multiplicities were deduced from 90° and 135° distortionless enhancement by polarization experiments. Infrared spectra were recorded on a FT-IR 4100 spectrometer (JASCO Inc., Easton, MD, USA). Mass spectra were obtained on a PolarisQ Ion Trap GC/MS^n (Thermo Fisher Scientific Inc., Waltham, MA, USA). Vacuum liquid chromatography separations were carried out on silica gel (Macherey-Nagel Kieselgel, Darmstadt, Germany). The analytical properties of the compound isolated (isoalantolactone) were as follows: Infrared υ_{max} cm^{-1}: 1,758 (γ-lactone), 1,448, 1,263. Electrospray ionization-mass spectrometry 70eV, m/z (relative intensity): 232 [M^+] (82), 217 [$M\text{-}CH_3$]$^+$ (40), 190 (100). Melting point: 111–113°C (lit. 111–112°C). ^{1}H nuclear magnetic resonance ($CDCl_3$): δ 0.75 (3H, s, H-14), 1.18 (1H, m, H-1), 1.30 (1H, d, $J=12.8$Hz, H-6), 1.42 (1H, d, $J=4.4$Hz, H-9), 1.48 (1H, m, H-1), 1.53 (2H, m, H-2), 1.67 (1H, dq, $J=6.8$, 2Hz, H-6), 1.78 (1H, d, $J=12.4$Hz, H-5), 1.93 (1H, $J=12.4$, 5.6Hz, H-3), 2.12 (1H, d, 15.6Hz, H-9), 2.26 (1H, dd, $J=13.6$, 2.0Hz, H-3), 2.92 (1H, q, $J=6.4$Hz, H-7), 4.37 (1H, d, $J=0.8$Hz, H-15), 4.43 (1H, br.t, $J=3.6$Hz, H-8), 5.52 (1H, s, H-13), 6.04 (1H, s, H-13). ^{13}C nuclear magnetic resonance ($CDCl_3$): δ 17.5 (C-14), 22.5 (C-2), 27.2 (C-6), 34.0 (C-10), 37.0 (C-1), 40.5 (C-7), 41.3 (C-3), 42.2 (C-9), 46.2 (C-5), 76.9 (C-8), 106.7 (C-15), 120.0 (C-13), 142.1 (C-11), 149.0 (C-4), 170.7 (C-12).

Biochemical assays

QR activity was measured by a spectrophotometric assay in which the rate of reduction of 2,6-dichlorophenolindophenol was monitored at 600 nm.[10] *tert*-Butylhydroquinone (20 μ*M* or 3.3 μg/mL), a known QR inducer, was used as a positive control in all biochemical assays. The specific activity of enzymes was normalized to the protein concentration, which was determined in triplicate using the assay of Lowry *et al.*[11] All data are reported as mean ± SD values whenever possible.

Assay of reporter gene activity

HepG2-C8 cells were plated in six-well plates at a density of 5×10^5 cells per well. After a 16-hour incubation, cells were cultured in fresh modified Dulbecco's modified Eagle's

medium with high glucose containing 0.5% fetal bovine serum for 12 hours before sample treatment. After cells were cultured for another 16 hours in the presence of various concentrations of sample, cells were collected, and the luciferase activity was determined according to the protocol provided by the manufacturer (Promega Corp., Madison, WI, USA).[7] In brief, after sample treatment, cells were washed twice with ice-cold phosphate-buffered saline and harvested in reporter lysis buffer. The homogenates were centrifuged at 12,000 *g* for 2 minutes at 4°C. A 20-μL supernatant was assayed for luciferase activity using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). Luciferase activity was normalized against protein concentration.

Preparation of nuclear protein extract

Nuclear and cytosolic protein extracts were prepared as previously described.[12] In brief, cells were cultured on 100-mm-diameter dishes to 90% confluence and treated with isoalantolactone for various times. After being washed, cells were harvested by scraping in ice-cold phosphate-buffered saline and collected by centrifugation at 500 *g* for 5 minutes. Cells were lysed with buffer A (10 m*M* HEPES-KOH [pH 7.9], 10 m*M* KCl, 0.1 m*M* EDTA, 0.5% Nonidet P-40, 1 m*M* dithiothreitol, and 0.5 m*M* phenylmethylsulfonyl fluoride) on ice for 20 minutes and then centrifuged at 14,000 *g* for 15 minutes at 4°C. The supernatants were saved as the cytoplasmic fractions. The nuclear pellets were washed three times with buffer A, resuspended in buffer B (20 m*M* HEPES, 0.5 *M* KCl, 1 m*M* EDTA, 1 m*M* dithiothreitol, and 1 m*M* phenylmethylsulfonyl fluoride, pH 7.9) for 30 minutes at 4°C on a rotating wheel, and then centrifuged at 14,000 *g* for 15 minutes at 4°C. One part of the nuclear fraction was subjected to immunoblot analysis using anti-Nrf2, anti-SAM68 antibody.

Western blot

Western blotting was performed on cytosolic fractions prepared from cultured cells to estimate the level of detoxifying enzymes according to a protocol described previously.[13,14] The primary antibodies, including anti-NQO1, anti-GST-pi, anti-HO-1, anti-γ-glutamylcysteine synthetase (γGCS), anti-glutathione reductase (GR), anti-Nrf2, anti-β-tubulin, and horseradish peroxidase-conjugated secondary antibody anti-goat or anti-rabbit immunoglobulin G, were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Statistical analysis

Statistical significance of QR enzyme activity and ARE-reporter assay data was tested by analysis of variance, followed by Duncan's multiple range test, using SPSS software (SPSS Inc., Chicago, IL, USA). $P < .05$ was considered to be statistically significant.

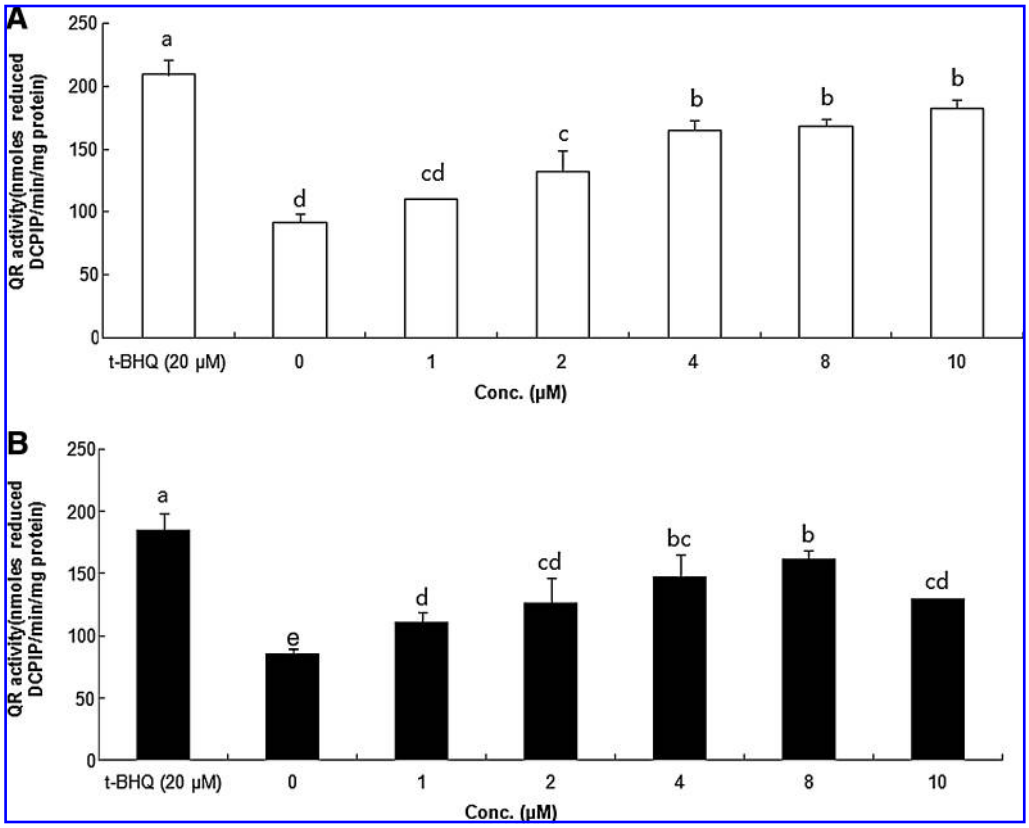

FIG. 2. QR-inducing activity of isoalantolactone in (**A**) Hepa1c1c7 and (**B**) BPRc1 cells. Hepa1c1c7 (□) and BPRc1 (■) cells were treated with various doses of isoalantolactone for 24 hours, followed by assaying for QR activity as described in Materials and Methods. Data are mean ± SD (bars) values. Means without a common letter differ significantly, $P < .05$. t-BHQ, *tert*-butylhydroquinone.

RESULTS

Effect of isoalantolactone on QR activity in murine hepatoma cells

Because QR is a biomarker enzyme for phase 2 detoxifying/antioxidant enzymes, we determined whether isoalantolactone induces QR activity in Hepa1c1c7 and its mutant BPRc1 cells lacking the arylhydrocarbon receptor nuclear translocator, which are typical murine hepatoma cell lines highly responsive to phase 2 enzyme inducers and thereby widely used for screening phase 2 enzyme inducers.[15–17] As shown in Figure 2, cytosolic QR enzyme activity in both Hepa1c1c7 and BPRc1 cell lines was increased in a dose-dependent manner in the range of 0–8 μM isoalantolactone. The enzyme activity of Hepa1c1c7 cells exposed to 10 μM isolantolactone was induced by 1.8-fold of the control, whereas the activity was increased by 2.1-fold in the cells incubated with 20 μM *tert*-butylhydroquinone, a known QR inducer.[18]

Isoalantolactone, at concentrations above 10 μM, suppressed QR activity, in particular, in BPRc1 cells, probably because of the cytotoxic effect of the compound. That is, the compound at the concentration that exerts cytotoxicity might inhibit cellular enzyme activity in a nonspecific fashion, although its expression was relatively little affected.

Effect of isoalantolactone on expression of phase 2 detoxifying enzymes in Hepa1c1c7 and BPRc1 cells

As isoalantolactone significantly induced the activity of QR in mouse hepatoma cells, we investigated the effect of isoalantolactone on the expression of other detoxifying/antioxidant enzymes. The compound enhanced the levels of GR, γGCS, GST-Pi, and HO-1 in a dose-dependent manner from 0 to 10 μM in both Hepa1c1c7 and BPRc1 cells (Fig. 3). For instance, isoalantolactone at the concentration of 8 μM showed a comparable induction potential for phase 2 detoxifying enzymes to *tert*-butylhydroquinone, a known QR inducer, at the concentration of 20 μM.

Dose–response effect on expression of pARE-TI-luciferase

To examine whether induction of phase 2 enzymes is mediated by ARE in the promoter region of genes encoding the enzymes, HepG2-C8 cells harboring pARE-luciferase gene construct were exposed to various concentrations of the compound for different times. As shown in Figure 4, the reporter assay showed that isoalantolactone increased luciferase gene expression proportionately with the increasing concentrations and exposure time to the compound. The compound (8 μM) activated the luciferase expression by 10- and 28-fold in HepG2-C8 cells treated for 12 and 24 hours, respectively,.

Translocation of Nrf2 into nucleus by isoalantolactone

We further investigated whether isoalantolactone stimulates nuclear translocation of Nrf2. The nuclear level of Nrf2 was enhanced with increasing dose of isoalantolactone. Accumulation of Nrf2 in the nucleus reached the highest level in the cells treated with isoalantolactone for 8 hours and then decreased with extended exposure to the chemical (Fig. 5).

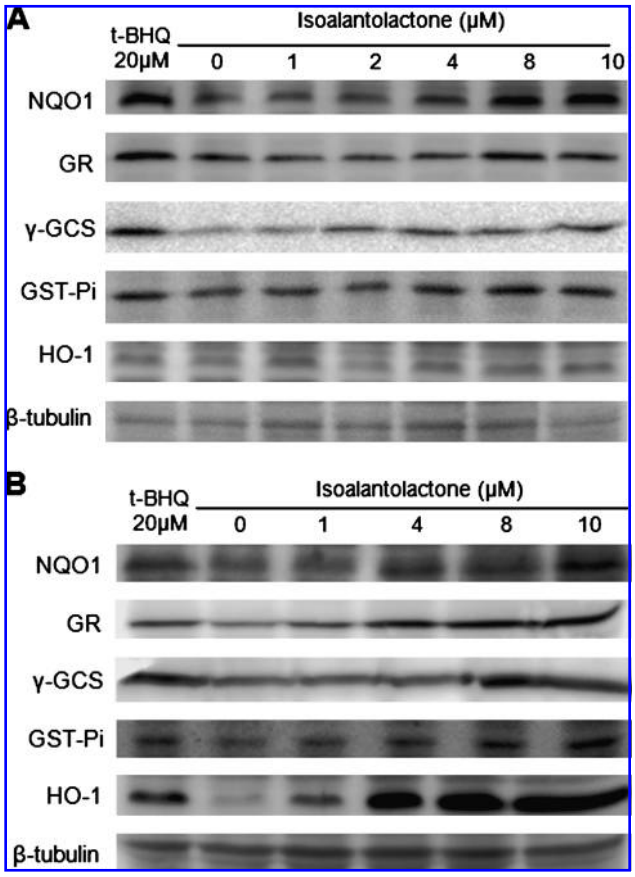

FIG. 3. Effect of isoalantolactone on the expression of phase 2 detoxifying enzymes, including NQO1, GR, GST-Pi, γGCS, and HO-1, in (**A**) Hepa1c1c7 and (**B**) BPRc1 cells. Cells were treated with various doses of isoalantolactone for 24 hours, followed by immunoblot analyses as described in Materials and Methods. t-BHQ, *tert*-butylhydroquinone.

Regulation of nuclear translocation of Nrf2 by kinase inhibitors

To determine whether nuclear translocation of Nrf2 by isoalantolactone is associated with some kinases such as protein kinase C (PKC), Jun N-terminal kinase (JNK), extracellular signal-regulated protein kinase (ERK) 1/2, p38 mitogen-activated protein kinase (p38 MAPK), and phosphatidylinositol 3-kinase (PI3K), Hepa1c1c7 and BPRc1 cells were treated with isoalantolactone with and without kinase inhibitors such as PD-98059 (ERK1/2 inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), staurosporine (PKC inhibitor), LY294002, and wortmannin (PI3K inhibitors), followed by examining nuclear accumulation of Nrf2 using western blotting. As shown in Figure 6, the PI3K inhibitors LY294002 and wortmannin

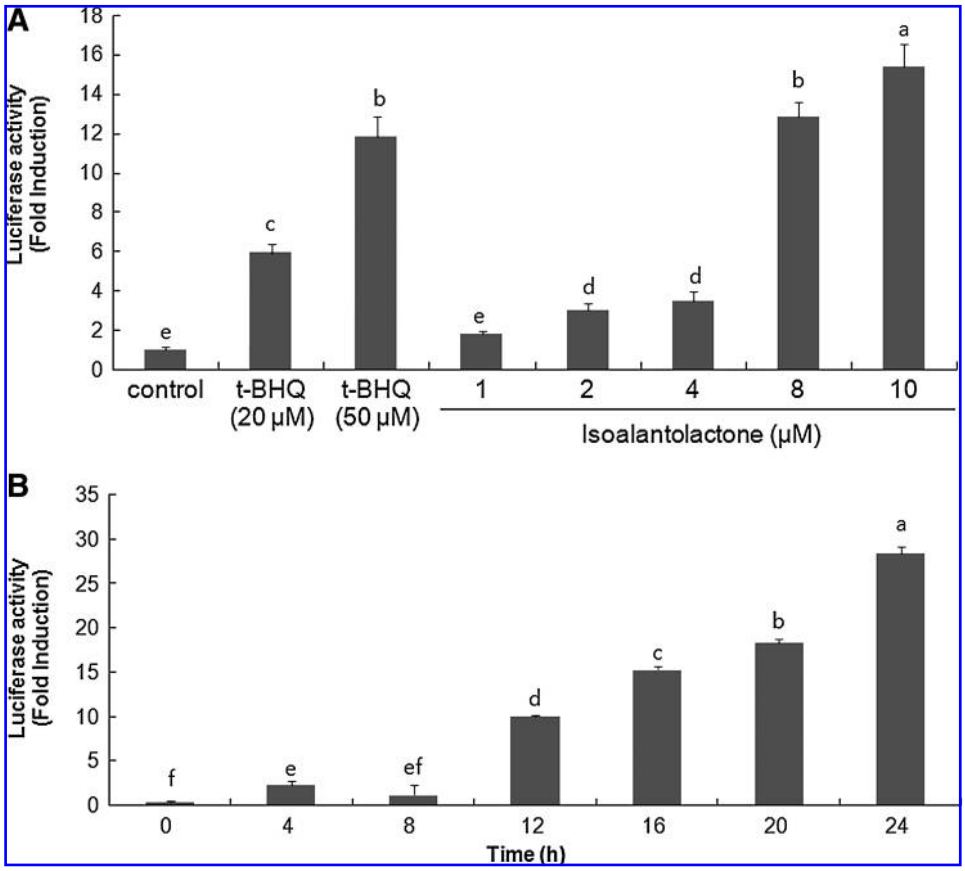

FIG. 4. (**A**) Dose- and (**B**) time-dependent induction of ARE-luciferase activities by isoalantolactone in HepG2-C8 cells that were generated by transfecting plasmid containing ARE-luciferase gene into HepG2 cells. Data are mean ± SD (bars) values. Means without a common letter differ significantly, $P < .05$. t-BHQ, *tert*-butylhydroquinone.

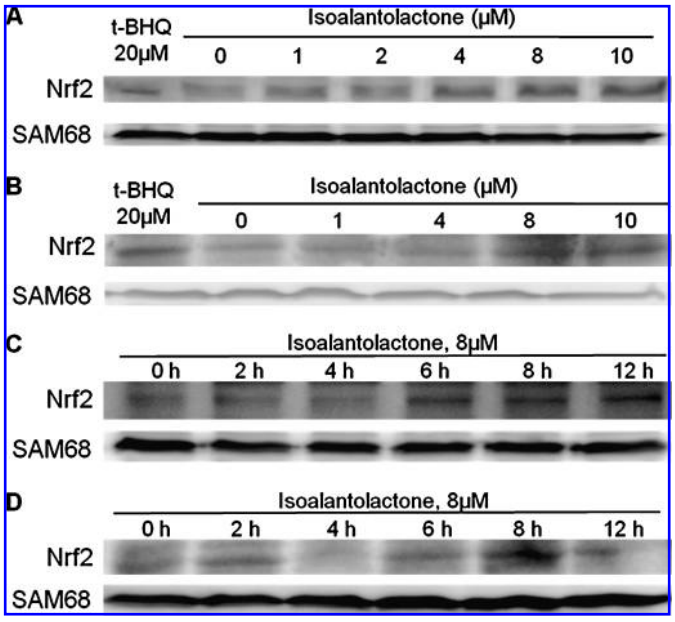

FIG. 5. Translocation into the nucleus of Nrf2 by isoalantolactone in Hepa1c1c7 and BPRc1 cells. (**A**) Hepa1c1c7 and (**B**) BPRc1 cells were treated with different concentrations of isoalantolactone for 24 hours. (**C**) Hepa1c1c7 and (**D**) BPRc1 cells were treated with 8 μM isoalantolactone for different periods. Antibody against Sam68 was used as a loading control for nuclear extract. t-BHQ, *tert*-butylhydroquinone.

significantly suppressed the nuclear accumulation of Nrf2 stimulated by isoalantolactone, whereas inhibitors for JNK, ERK, p38, and PKC did not bring about any significant repression of the compound-induced accumulation of Nrf2 in the nuclear compartment. Furthermore, inhibition of glycogen synthase kinase-3β (GSK3β) by lithium chloride caused a further increase in nuclear transport of Nrf2 (Fig. 7).

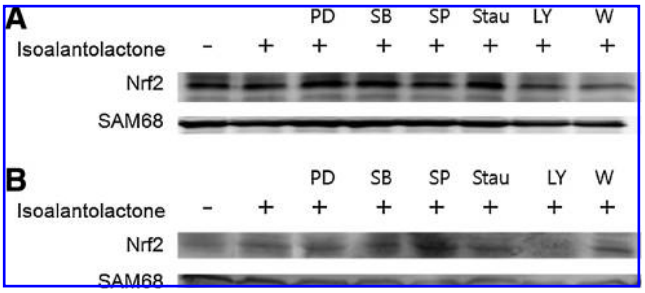

FIG. 6. Effect of kinase inhibitors on isoalantolactone-induced nuclear translocation of Nrf2. (**A**) Hepa1c1c7 and (**B**) BPRc1 cells were treated with isoalantolactone in the absence or presence of the kinase inhibitors PD98059 (PD) (20 μM), an ERK1/2 inhibitor, SB203580 (SB) (20 μM), a p38 MAPK inhibitor, SP600125 (SP) (20 μM), a JNK inhibitor, staurosporine (Stau) (0.3 μM), a PKC inhibitor, LY294002 (LY) (40 μM), a PI3K inhibitor, or wortmannin (W) (2 μM), a PI3K inhibitor, and subjected to western blotting.

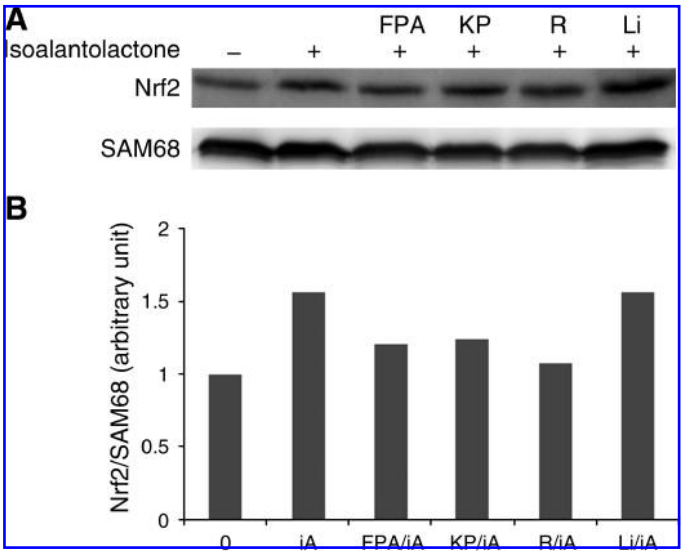

FIG. 7. Relationship between nuclear accumulation of Nrf2 and the Akt/mTOR/GSK3β signaling pathway in Hepa1c1c7 cells. See Figure 6 for details. (**A**) Hepa1c1c7 cells were treated with isoalantolactone (iA) in the absence or presence of the inhibitors FPA-124 (FPA) (25 μM), an Akt inhibitor, KP372-1 (KP) (60 nM), an Akt inhibitor, rapamycin (R) (400 nM), an mTOR inhibitor, and lithium chloride (Li) (25 mM), a GSK3β inhibitor, and subjected to western blotting. (**B**) Relative Nrf2/SAM68 expression.

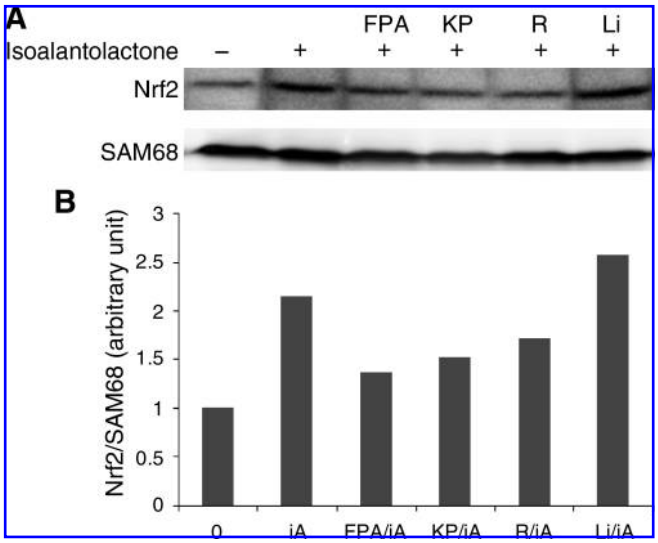

FIG. 8. Relationship between nuclear accumulation of Nrf2 and Akt/mTOR/GSK3β signaling pathway in BPRc1 cells. See Figure 6 for details. (**A**) BPRc1 cells were treated with isoalantolactone (iA) in the absence or presence of the inhibitors FPA-124 (FPA) (25 μM), an Akt inhibitor, KP372-1 (KP) (60 nM), an Akt inhibitor, rapamycin (R) (400 nM), an mTOR inhibitor, and lithium chloride (Li) (25 mM), a GSK3β inhibitor, and subjected to western blotting. (**B**) Relative Nrf2/SAM68 expression.

DISCUSSION

In our previous study in which more than 200 plant extracts were screened, the methanol extract of *I. helenium* was found to possess a significantly high capacity to induce QR activity.[5,19] The herb has been used in Oriental medicine as an expectorant and for treating tuberculosis, worms, and other infectious diseases such as tonsillitis.[20] We further fractionated the methanol extract of the plant and found that the hexane fraction was the most active in inducing detoxifying enzymes. A previous study also showed that sesquiterpenes present in the hexane fraction had various antitumor potentials, including antiproliferative activity.[19] In this study the mechanism leading to induction of phase 2 detoxifying/antioxidant enzymes by isoalantolactone was studied.

Sesquiterpenes lactones possessing an α-methylene γ-lactone functionality are electrophiles and are, thus, apt to interact with the ARE of phase 2 detoxifying enzyme genes and induce their expression.[21–24] Among the seven

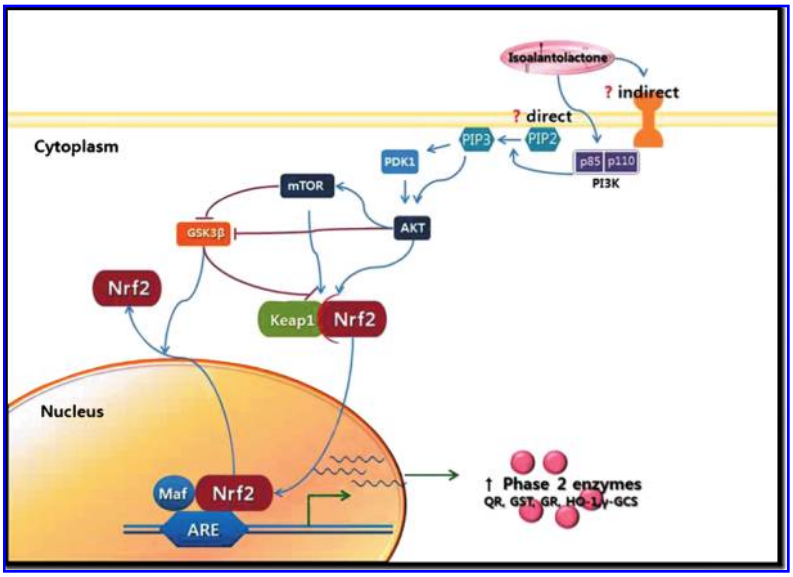

FIG. 9. Hypothetical mechanism for induction of phase 2 detoxifying enzymes by isoalantolactone. PDK1, pyruvate dehydrogenase kinase, isozyme 1; PIP2, phosphatidylinositol bisphosphate; PIP3, phosphatidylinositol trisphosphate. Color images available online at www.liebertonline.com/jmf.

sesquiterpenes examined in our previous study, three compounds (alantolactone, isoalantolactone, and 5α-epoxyalantolactone) showed strong QR induction, whereas the other sesquiterpenes were inactive or relatively low in activity. In particular, reduced forms of alantolactone and isoalantolactone did not induce QR, suggesting a significant role of the methylene group in the lactone ring in the activity (Fig. 1).[5,24] Opening of any of the three rings in the structure significantly reduced its potential for QR induction. In spite of structural similarity between isoalantolactone and alantolactone, a wide difference in cytotoxicity against several cancer cell lines was reported.[21] That is, most cancer cell lines were much more sensitive to alantolactone than isoalantolactone. However, the QR-inducing potential of isoalantolactone was higher than that of alantolactone (Fig. 2).[5] It has been suggested that alteration of the cellular redox status is due to elevated levels of reactive oxygen species and electrophilic species, and a reduced antioxidant capacity appears to be an important signal for triggering the expression of the detoxifying/antioxidant enzyme genes.[25,26]

The induction of many detoxifying/antioxidant enzymes such as QR in response to reactive chemical stress is regulated primarily at the transcriptional level. This transcriptional response is mediated by a *cis*-acting element, termed an ARE, initially found in the promoter of genes encoding the two major detoxification enzymes, GST A2 and NQO1 (QR).[25–27] More specifically, Nrf2 plays a major role in transcriptional activation of phase 2 detoxification enzymes through interacting with ARE sequence after translocation into the nucleus. Isoalantolactone, like most phase 2 enzymes inducers, stimulated nuclear translocation of Nrf2 in Hepa1c1c7 and BPRc1 cells and enhanced luciferase activity in HepG2-C8 cells transfected with ARE-luciferase gene construct at concentrations as low as 2 μM.

It is well established that Nrf2 activity is controlled, in part, by the actin-associated cytosolic protein Keap1. Activation of Nrf2 in response to altered redox status was thought to arise from dissociation from Keap1, followed by translocation of Nrf2 into the nucleus to exert its transcriptional activity.[25–28] Another possible mechanism of Nrf2 activation is phosphorylation by protein kinase(s) such as PKC and Akt. In fact, inhibition of AKT or PI3K repressed the nuclear accumulation of Nrf2 mediated by isoalantolactone, whereas inhibitors of ERK, p38, JNK and PKC did not affect the nuclear translocation of Nrf2 (Fig. 6), suggesting that the PI3K/Akt signaling pathway is associated with activation of Nrf2 by isoalantolactone. Several compounds such as brazilin, ursodeoxycholic acid, kahweol, and capsaicin have been reported to promote translocation of Nrf2 and reduced glutathione synthesis through an activation of the PI3/Akt/Nrf2 pathway.[29–32] Furthermore, the PI3K/Akt/mammalian target of rapamycin (mTOR) signaling pathway could be, in part, involved in the nuclear translocation of Nrf2 as inhibitors of Akt (FPA-124, KP372-1) and mTOR (rapamycin) suppressed the isoalantolactone-induced accumulation of Nrf2 in the nucleus (Figs. 7 and 8).[33] Inhibition of GSK3β by lithium chloride failed to further stimulate translocation of Nrf2 into nucleus, suggesting that GSK3β may not be associated with molecular modulation of detoxifying enzymes of isoalantolactone, as shown in Figures 7 and 8.[34]

In conclusion, isoalantolactone has strong capacity to control expression of genes whose protein products are involved in the detoxification and elimination of harmful reactive oxidants and electrophilic agents through conjugative reactions and by enhancing cellular antioxidant potential (Fig. 9).

ACKNOWLEDGMENTS

This study was supported by a grant from the Korea Research Foundation funded by the Korean Government (MOEHRD) (KRF-2005-F00055) and a grant (20070301-034-039-007-03-00) from the BioGreen 21 Program, Rural Development Administration, Republic of Korea.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

1. Balogun E, Hoque M, Gong P, Killeen E, Green CJ, Foresti R, Alam J, Motterlini R: Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. *Biochem J* 2003;371:887–895.
2. Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, Yamamoto M, Talalay P: Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc Natl Acad Sci USA* 2002;99:11908–11913.
3. Surh Y-J: Cancer chemoprevention with dietary phytochemicals. *Nat Rev Cancer* 2003;3:768–780.
4. Jeong WS, Jun M, Kong AN: Nrf2: a potential molecular target for cancer chemoprevention by natural compounds. *Antioxid Redox Signal* 2006;8:99–106.
5. Lim SS, Kim JR, Lim HA, Jang CH, Kim YK, Konishi T, Kim EJ, Park JH, Kim JS: Induction of detoxifying enzyme by sesquiterpenes present in *Inula helenium*. *J Med Food* 2007;10:503–510. Erratum in: *J Med Food* 2007;10:739.
6. Seo JY, Lim SS, Kim JR, Lim JS, Ha YR, Lee IA, Kim EJ, Park JH, Kim JS: Nrf2-mediated induction of detoxifying enzymes by alantolactone present in *Inula helenium*. *Phytother Res* 2008;22:1500–1505.
7. Kim BR, Hu R, Keum YS, Hebbar V, Shen G, Nair SS, Kong AN: Effects of glutathione on antioxidant response element-mediated gene expression and apoptosis elicited by sulforaphane. *Cancer Res* 2003;63:7520–7525.
8. Marshall JA, Cohen N: The structure of alantolactone. *J Org Chem* 1964;29:3727–3729
9. Mehra MM, Deshpanda KG, Ghatge BB, Bhattacharyya SC: Terpenoids-CV: transformation products of alantolactones. *Tetrahedron* 1967;23:2469–2479.
10. Benson AM, Hunkeler MJ, Talalay P: Increase of NAD(P)H: quinone reductase by dietary antioxidants: possible role in protection against carcinogenesis and toxicity. *Proc Natl Acad Sci U S A* 1980;77:5216–5220.
11. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265–275.

12. Nguyen T, Sherratt PJ, Nioi P, Yang CS, Pickett CB: Nrf2 controls constitutive and inducible expression of ARE-driven genes through a dynamic pathway involving nucleocytoplasmic shuttling by Keap1. *J Biol Chem* 2005;280:32485–32492.
13. Kim JH, Lee JS, Song KS, Kwon CS, Kim YK, Kim JS: Polyozellin isolated from *Polyozellus multiplex* induces phase 2 enzymes in mouse hepatoma cells and differentiation in human myeloid leukaemic cell lines. *J Agric Food Chem* 2004;52:451–455.
14. Kwak MK, Itoh K, Yamamoto M, Kensler TW: Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter. *Mol Cell Biol* 2002;22:2883–2892.
15. Seidel SD, Denison MS: Differential gene expression in wild-type and arnt-defective mouse hepatoma (Hepa1c1c7) cells. *Toxicol Sci* 1999;52:217–225.
16. Chandra S, de Mejia EG: Polyphenolic compounds, antioxidant capacity, and quinone reductase activity of an aqueous extract of *Ardisia compressa* in comparison to mate (*Ilex paraguariensis*) and green (*Camellia sinensis*) teas. *J Agric Food Chem* 2004;52:3583–3589.
17. Zhang Y, Talalay P, Cho CG, Posner GH: A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc Natl Acad Sci U S A* 1992;89:2399–2403.
18. Prochaska HJ, De Long MJ, Talalay P: On the mechanisms of induction of cancer-protective enzymes: a unifying proposal. *Proc Natl Acad Sci U S A* 1985;82:8232–8236.
19. Kwon CS, Kim JH, Son KH, Kim YK, Lee JS, Lim JK, Kim JS: Induction of quinone reductase, an anticarcinogenic marker enzyme, by medicinal herb extracts. *Nutraceut Food* 2002;7:358–366.
20. Chevallier A: *Encyclopedia of Medicinal Plants: A Practical Reference Guide to More than 550 Key Medicinal Plants and Their Use*. Dorling Kindersley, London, 2001, p. 109.
21. Konishi T, Shimada Y, Nagao T, Okabe H, Konoshima T: Antiproliferative sesquiterpene lactones from the roots of *Inula helenium*. *Biol Pharm Bull* 2002;25:1370–1372.
22. Wen J, You K-R, Lee S-Y, Song C-H, Kim D-G: Oxidative stress-mediated apoptosis—the anticancer effect of the sesquiterpenes lactone parthenolide. *J Biol Chem* 2002;277:38954–38964.
23. Iskander K, Jaiswal AK: Quinone oxidoreductases in protection against myelogenous hyperplasia and benzene toxicity. *Chem Biol Interact* 2005;153–154:147–157.
24. Jeong GS, Pae HO, Jeong SO, Kim YC, Kwon TO, Lee HS, Kim NS, Park SD, Chung HT: The alpha-methylene-gamma-butyrolactone moiety in dehydrocostus lactone is responsible for cytoprotective heme oxygenase-1 expression through activation of the nuclear factor E2-related factor 2 in HepG2 cells. *Eur J Pharmacol* 2007;565:37–44.
25. Dinkova-Kostova AT, Fahey JW, Wade KL, Jenkins SN, Shapiro TA, Fuchs EJ, Kerns ML, Talalay P: Induction of the phase 2 response in mouse and human skin by sulforaphane-containing broccoli sprout extracts. *Cancer Epidemiol Biomarkers Prev* 2007;16:847–851.
26. Nguyen T, Nioi P, Pickett CB: The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J Biol Chem* 2009;284:13291–13295.
27. Nguyen T, Rushmore TH, Pickett CB: Transcriptional regulation of a rat liver glutathione *S*-transferase Ya subunit gene. Analysis of the antioxidant response element and its activation by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate. *J Biol Chem* 1994;269:13656–13662.
28. Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M: Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev* 1999;13:76–86.
29. Okouchi M, Okayama N, Alexander JS, Aw TY: NRF2-dependent glutamate-L-cysteine ligase catalytic subunit expression mediates insulin protection against hyperglycemia-induced brain endothelial cell apoptosis. *Curr Neurovasc Res* 2006;3:249–261.
30. Arisawa S, Ishida K, Kameyama N, Ueyama J, Hattori A, Tatsumi Y, Hayashi H, Yano M, Hayashi K, Katano Y, Goto H, Takagi K, Wakusawa S: Ursodeoxycholic acid induces glutathione synthesis through activation of PI3K/Akt pathway in HepG2 cells. *Biochem Pharmacol* 2009;77:858–866.
31. Hwang YP, Jeong HG: The coffee diterpene kahweol induces heme oxygenase-1 via the PI3K and p38/Nrf2 pathway to protect human dopaminergic neurons from 6-hydroxydopamine-derived oxidative stress. *FEBS Lett* 2008;582:2655–2662.
32. Choi BM, Kim BR: Upregulation of heme oxygenase-1 by brazilin via the phosphatidylinositol 3-kinase/Akt and ERK pathways and its protective effect against oxidative injury. *Eur J Pharmacol* 2008;580:12–18.
33. Joung EJ, Li MH, Lee HG, Somparn N, Jung YS, Na HK, Kim SH, Cha YN, Surh YJ: Capsaicin induces heme oxygenase-1 expression in HepG2 cells via activation of PI3K-Nrf2 signaling: NAD(P)H:quinone oxidoreductase as a potential target. *Antioxid Redox Signal* 2007;9:2087–2098.
34. Salazar M, Rojo AI, Velasco D, de Sagarra RM, Cuadrado A: Glycogen synthase kinase-3beta inhibits the xenobiotic and antioxidant cell response by direct phosphorylation and nuclear exclusion of the transcription factor Nrf2. *J Biol Chem* 2006;281:14841–14851.