

Zerumbone Induces Heme Oxygenase-1 Expression in Mouse Skin and Cultured Murine Epidermal Cells through Activation of Nrf2

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Abstract

Zerumbone, a sesquiterpene derived from tropical ginger, contains an electrophilic α,β -unsaturated carbonyl moiety and was found to suppress chemically induced papilloma formation in mouse skin. Here, we report that topical application of zerumbone onto dorsal skin of hairless mice induces activation of NF-E2-related factor 2 (Nrf2) and expression of heme oxygenase-1 (HO-1). We compared the levels of HO-1 protein in the skin of zerumbone-treated Nrf2 wild-type and Nrf2 knockout mice, and *nrf2*-deficient mice expressed HO-1 protein to a much lesser extent than the wild-type animals following topical application of zerumbone. Treatment of mouse epidermal JB6 cells with zerumbone caused a marked increase of Nrf2 nuclear translocation followed by the promoter activity of HO-1, and also enhanced direct binding of Nrf2 to the antioxidant response element. Moreover, knockdown of Nrf2 in JB6 cells diminished the zerumbone-induced upregulation of HO-1. Notably, α -humulene and 8-hydroxy- α -humulene, the structural analogues of zerumbone that lack the α,β -unsaturated carbonyl group, failed to activate Nrf2 and were unable to increase HO-1 expression. Unlike zerumbone, these nonelectrophilic analogues could not suppress the 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced JB6 cell transformation and the intracellular accumulation of reactive oxygen species (ROS). Interestingly, when JB6 cells were treated with carbon monoxide-releasing molecule that mimics the HO-1 activity, the TPA-induced ROS production was markedly reduced. Taken together, these findings suggest that upregulation of HO-1 expression by zerumbone is mediated through activation of Nrf2 signaling, which provides a mechanistic basis for the chemopreventive effects of this sesquiterpene on mouse skin carcinogenesis. *Cancer Prev Res*; 4(6); 860–70. ©2011 AACR.

Introduction

Chemical stresses caused by environmental toxins, mutagens, and carcinogens have been known to cause many chronic inflammatory and degenerative diseases, including cancer (1, 2). The skin, which covers the body, is frequently subjected to the pathogenic effects of external chemical stresses, and thus must have efficient self-defense systems for the elimination or neutralization of toxic insults.

Among the cytoprotective proteins involved in cellular stress response, heme oxygenase-1 (HO-1) is of particular interest because its expression is commonly induced by a wide array of noxious stimuli (3, 4). The products generated by the upregulated HO-1 expression have important cytoprotective activities (5). HO-1 catalyzes the rate-limiting step in the degradation of potentially damaging free heme released from heme proteins under a variety of stress conditions. As the released free heme can generate highly toxic hydroxyl radical via the Fenton reaction in the presence of H₂O₂, induction of HO-1 expression is considered as a physiologically important adaptive survival mechanism for the stressed cells.

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One of the transcription factors that regulate the *Hmox1* gene transcription and induces the expression of HO-1 is NF-E2-related factor2 (Nrf2). Nrf2 is a member of the cap 'n' collar family of redox-sensitive bZIP (basic leucine zipper) proteins (6) and regarded as the major transcriptional regulator for the expression of a distinct set of genes encoding phase 2 detoxifying enzymes and other cytoprotective proteins (7). Nrf2 binds to antioxidant response element (ARE), a *cis*-regulatory DNA sequence located in the promoter of target genes. Under homeostatic resting conditions, Nrf2 is sequestered in cytoplasm by Kelch-like ECH-associated protein 1 (Keap1; ref. 8) and undergoes

proteasomal degradation following ubiquitination (9, 10). Under oxidative or electrophilic stress conditions, Keap1 cannot sequester and degrade Nrf2, but rather allows nuclear translocation of Nrf2 (11, 12). The importance of phase 2 detoxifying enzymes for inactivating chemical carcinogens was highlighted in a study with Nrf2-deficient mice. These mice were found to be more susceptible to experimental carcinogenesis (13) and were also highly sensitive to the acetaminophen-induced hepatotoxicity (14, 15).

The JB6 epidermal cells are derived from mouse skin and are regarded as an appropriate model for studying the molecular mechanisms underlying experimentally induced skin carcinogenesis and its chemoprevention. Numerous phytochemicals exert chemopreventive effects through induction of phase 2 detoxifying enzymes and cytoprotective proteins. Zerumbone, a major sesquiterpene found in the rhizomes of *Zingiber zerumbet* Smith (Zingiberaceae), has been shown to have strong antioxidant (16), anti-inflammatory (17), and anticarcinogenic (18, 19) properties in several *in vitro* and *in vivo* studies.

In this study, we investigated whether zerumbone could induce HO-1 expression in JB6 mouse skin epidermal cells in culture and also in mouse skin *in vivo*. Furthermore, we explored possible molecular mechanisms involved in HO-1 induction with special focus on Nrf2 signaling.

Materials and Methods

Chemicals, cells, and animals

Zerumbone was extracted from the rhizomes of *Zingiber zerumbet* Smith (Zingiberaceae) and its hydroxylated derivative 8-hydroxy- α -humulene (Zerumbol) was synthesized as described previously. The purity of both compounds is more than 95% (20). α -Humulene (purity >98%), dithiothreitol (DTT) and primary antibody for actin were obtained from Sigma-Aldrich. 12-O-Tetradecanoylphorbol-13-acetate (TPA) was purchased from Alexis Biochemicals. Antibodies against Nrf2 and Keap1 were purchased from Santa Cruz Biotechnology. Primary antibody for HO-1 was purchased from Stressgen. The mouse epidermal cell line, JB6 Cl41, was obtained from the American Culture Collection, and cell line characterization was done by monitoring cell morphology, karyotyping, and the cytochrome *c* oxidase I assay. The cells were maintained in minimum essential medium (MEM) containing 5% FBS and 25 μ g/mL gentamicin in an atmosphere of 95% air/5% CO₂ at 37°C. MEM, FBS, and gentamicin were purchased from Invitrogen. Female HR-1 hairless mice (6–7 weeks of age) were purchased from the Central Lab. Animal Inc. (Seoul, South Korea) and were housed in a climate-controlled quarters (24 \pm 1°C at 50% humidity) with a 12-hour light/dark cycle and with free access to food and water. Nrf2 knockout mice were kindly supplied by Dr. Jeffrey Johnson of the University of Wisconsin-Madison.

DNA and plasmid constructs

The plasmid pHO15-luc was kindly provided by Dr. J. Alam of Alton Ochsner Medical Foundation (New Orleans,

LA). The murine full-length *Hmox1* was amplified by reverse transcriptase PCR (RT-PCR) from the total RNA obtained from JB6 cells with primers 5'-TAA GGA TCC ATG GAG CGT CCA CAG CCC GAC (forward) and 5'-GCT CTA GAT TAC ATG GCA TAA ATT CCC ACT G (reverse), and subcloned into His-tagged pcDNA6 expression vector (Invitrogen) as *Bam*HI/*Xba*I fragment.

Western blot and immunohistochemical analysis

Female HR-1 hairless mice were treated topically on their backs with 10 μ mol of zerumbone or its analogs dissolved in 200 μ L of acetone and were killed by cervical dislocation at the indicated times. Proteins from the mouse epidermis were isolated as described previously (21). In another study, JB6 cells were treated with zerumbone or its analogs for the indicated durations. The treated cells were harvested, washed, and suspended in the lysis buffer as mentioned earlier. Cell lysates were centrifuged at 13,000 \times *g* for 15 minutes, and the aliquots collected from the supernatant containing protein were stored at -70°C. SDS-PAGE and Western blotting were carried out as described previously (21). Formalin-fixed and paraffin-embedded mouse skin tissues were prepared for immunohistochemical staining with HO-1 antibody according to the procedure described previously (21).

Preparation of nuclear proteins and immunocytochemical analysis

Nuclear extracts from JB6 cells were prepared as described previously (22). For the immunocytochemical analysis of Nrf2, cells were plated on the chamber slide and treated with zerumbone. After fixation with paraformaldehyde, samples were incubated with blocking agents (0.1% Tween-20 in PBS containing 5% bovine serum albumin), washed with PBS, and then incubated overnight in the presence of diluted (1:100) primary antibody. After washing with PBS, samples were then incubated with a fluorescein isothiocyanate-conjugated secondary antibody for 1 hour. Cells were also stained with propidium iodide and examined under a confocal microscope (Leika).

Reverse transcriptase polymerase chain reaction

Total RNA was isolated from JB6 cells by using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. To generate the cDNA from RNA, 1 μ g of total RNA was reverse transcribed with murine leukemia virus reverse transcriptase (Promega) for 50 minutes at 42°C and again for 15 minutes at 72°C. One microliter of cDNA was amplified in sequential reactions by using Maxime PCR PreMix Kit (iNtRON Biotechnology). For detection of HO-1 mRNA, 20 cycles of 94°C for 30 seconds, 53°C for 35 seconds, and 72°C for 30 seconds were conducted; for quantitation of actin mRNA, 20 cycle of 94°C for 30 seconds, 59°C for 35 seconds, and 72°C for 30 seconds were conducted. These PCR cycles were followed by a final extension for 7 minutes at 72°C. The primers used for each RT-PCR reactions are as follows: *HO-1*, 5'-TAC ACA TCC AAG CCG AGA AT-3' and 5'-GTT CCT CTG TCA GCA TCA

CC-3'; *Actin*, 5'-AGA GCA TAG CCC TCG TAG AT-3' and 5'-CCC AGA GCA AGA GAG GTA TC-3' (forward and reverse, respectively). Amplification products were analyzed by 2.0% agarose gel electrophoresis, followed by staining with ethidium bromide, and then photographed under UV light.

Transient transfection and luciferase reporter gene assay

For siRNA transfection, JB6 cells were seeded at a density of 4×10^4 cells/mL in 60-mm dishes and grown to 60% to 70% confluence in growth media. Nrf2 siRNA (Invitrogen) was transfected into JB6 cells with lipofectamine RNAi-MAX (Invitrogen) reagent according to the manufacturer's instructions. After 36-hour transfection, cells were treated with zerumbone for additional 6 hours, and the cell lysis was carried out with the lysis buffer for Western blot analysis. For the HO-1 luciferase assay, JB6 cells were cultured up to 50% confluence in 12-well plates in complete media that do not contain antibiotics. Cells were then transfected with 750 ng of pHO15-luc vector by using the Lipofectamine LTX according to the instructions supplied by the manufacturer (Invitrogen). In all cases, the total amount of transfecting plasmid DNA was quantitated and adjusted by using pcDNA3- β -galactosidase. After 24-hour transfection, cells were treated with zerumbone or its analogs for additional 24 hours, and the lysis of transfected cells was carried out by using the reporter lysis buffer. After mixing the cell extract with a luciferase substrate (Promega), the luciferase activity was measured by employing a luminometer (AntoLumat LB953; EG&G Berthold). The β -galactosidase assay was done according to the supplier's instructions (Promega β -galactosidase Enzyme Assay System) for normalizing the luciferase activity.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were done by using the EZ ChIP kit (Upstate Biotechnology) according to the instructions provided by manufacturer. PCR was done with 1 μ L of a ChIP sample, using the following primers (forward: 5'-GGG GCT AGC ATG CGA AGT GAG-3'; reverse: 5'-CAG GTC TGA CTT GGG AAT CCC-3').

Immunoprecipitation assay

JB6 cells were treated with 10 μ M/L biotinylated zerumbone for indicated time and cells were lysed in 250 mmol/L sucrose, 50 mmol/L Tris-HCl (pH 8.0), 25 mmol/L KCl, 5 mmol/L MgCl₂, 1 mmol/L EDTA, 2 μ M/L NaF, 2 μ M/L sodium orthovanadate, and 1 mmol/L phenylmethylsulfonylfluoride. Total protein (1 mg) was subjected to immunoprecipitation by shaking with Keap1 primary antibody at 4°C for 2 hours followed by the addition of protein G-agarose bead suspension (25% slurry, 20 μ L) and additional shaking for 2 hours at 4°C. After centrifugation at 3,000 rpm for 30 seconds, immunoprecipitated beads were collected by discarding the supernatant and washed with cell lysis buffer. The immunoprecipitate was then resuspended in 40 μ L of 2 \times SDS electrophoresis sample

buffer and boiled for 3 minutes. Supernatant (20 μ L) from each sample was collected by centrifugation and loaded on SDS-polyacrylamide gel. The incorporation of biotinylated zerumbone into immunoprecipitated proteins was visualized by Amersham streptavidin-horseradish peroxidase (HRP) conjugate (GE Healthcare).

Anchorage-independent cell transformation assay

The effects of zerumbone, α -humulene, and 8-hydroxy- α -humulene on TPA-induced cell transformation were investigated in JB6 cells. Cells (8×10^3 /mL) were exposed to TPA with or without each of these three compounds in 1 mL of 0.33% basal media Eagle (BME) agar containing 10% FBS or in 3.5 mL of 0.5% BME agar containing 10% FBS. The cultures were maintained for 14 days in a 5% CO₂ incubator kept at 37°C, and the cell colonies were scored by using a microscope equipped with the Image-Pro PLUS computer software program (Media Cybernetics).

Measurement of intracellular accumulation of reactive oxygen species

To measure the net intracellular accumulation of reactive oxygen species (ROS), a fluorescent probe 2',7'-dichlorofluorescein diacetate (DCF-DA; Molecular Probe) was used. Following a 2-hour treatment with TPA, cells were washed twice with HBSS solution (Cellgro) and loaded with 10 μ M/L of DCF-DA in a 5% CO₂ incubator kept at 37°C. After 30 minutes, cells were washed twice with HBSS solution, suspended in the complete media and examined under a microscope.

Results

Zerumbone induces HO-1 expression in JB6 cells

When JB6 cells were treated with zerumbone (10 μ M/L), HO-1 mRNA expression was increased in a time-dependent manner (Fig. 1A). HO-1 protein expression was also elevated by zerumbone treatment in time-dependent (Fig. 1B) and concentration-dependent manners (Fig. 1C). To investigate whether zerumbone-induced upregulation of HO-1 expression is mediated through the transcriptional activation of *Hmox1*, JB6 cells were transfected with 15-kb murine *Hmox1* promoter tagged with the luciferase reporter gene (pHO15-Luc), and the reporter luciferase activity was measured. As illustrated in Figure 1D, zerumbone significantly enhanced the pHO15-Luc activity.

Involvement of Nrf2-ARE in zerumbone-induced expression of HO-1 in JB6 cells

As Nrf2 is known to play a key role in the regulation of many antioxidant and cytoprotective proteins including HO-1, we examined the nuclear localization of Nrf2 in zerumbone-treated JB6 cells. As shown in Figure 2A, Nrf2 was translocated into nucleus, following zerumbone treatment as determined by Western blot analysis. It is also well documented that the inducer-dependent upregulation of mouse HO-1 expression is mediated by

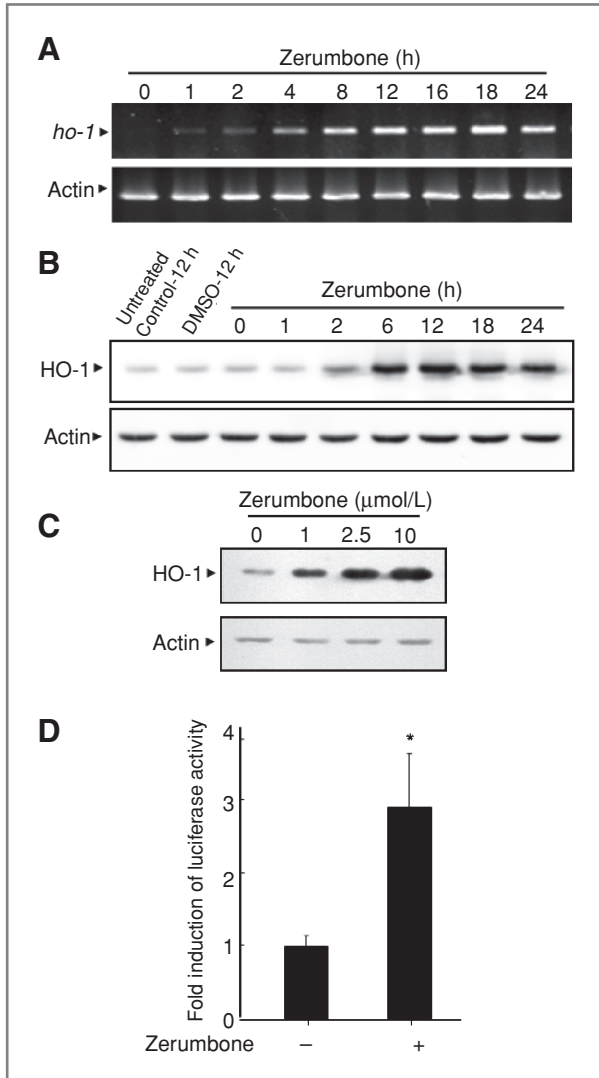


Figure 1. Zerumbone induces HO-1 mRNA and protein expression by stimulation of its promoter activity in JB6 cells. Cells were treated with zerumbone (10 $\mu\text{mol/L}$) for the indicated time, and total mRNA and protein were isolated and analyzed for the expression of HO-1 mRNA (A) and protein (B) expression by RT-PCR and Western blotting, respectively. C, cells were incubated with zerumbone at the indicated concentrations for 12 hours, and the levels of HO-1 and actin were measured by Western blotting. D, JB6 cells were transfected transiently with 750 ng of pHO15-luc and 24 hours later, cells were treated with zerumbone (10 $\mu\text{mol/L}$) for 24 hours. Cell lysates were then assayed for the luciferase activity. The experiment was done in triplicate and the data are presented as mean \pm SD. Statistical significance was evaluated by the Student's *t* test. *, *P* < 0.01, significantly different as compared with the dimethylsulfoxide (DMSO) control.

increased Nrf2 binding to the 2 distal promoter regions of *Hmox1*. These DNA sequence elements termed E1 and E2 contain 3 and 2 ARE-like StRE (stress-response elements), respectively (23). Thus, we examined whether the Nrf2 translocated into nucleus actually could bind to ARE by employing the CHIP assay. For this purpose, we used the

HO-1 primer which contains the most conserved ARE sequence in the E1 region. As shown in Figure 2B, JB6 cells treated with zerumbone exhibited the increased Nrf2-ARE binding activity. To determine whether HO-1 upregulation by zerumbone is mediated via Nrf2 activation, JB6 cells were transiently transfected with Nrf2 siRNA or its negative control vector, and the expression of HO-1 was compared by Western blotting. siRNA knockdown of Nrf2 gene abrogated the expression of HO-1 (Fig. 2C). This finding indicates that Nrf2 is essential for zerumbone-induced upregulation of HO-1 expression in mouse epidermal cells.

Zerumbone induces HO-1 expression in mouse skin in a Nrf2-dependent manner

Our previous study revealed that topical application of zerumbone inhibited chemically induced mouse skin papillomagenesis (19). This prompted us to examine whether zerumbone could induce HO-1 expression in mouse skin *in vivo*. When HR-1 hairless mouse skin was topically treated with zerumbone, HO-1 expression was increased in time-dependent (Fig. 3A) and dose-dependent manners (Fig. 3B).

To further verify whether zerumbone-induced upregulation of HO-1 in mouse skin also requires the presence of Nrf2, we treated topically the dorsal skin of Nrf2 wild-type (+/+) and knockout mice (-/-) with zerumbone and measured HO-1 expression by both Western blot (Fig. 4A) and immunohistochemical analyses (Fig. 4B). Topically applied zerumbone induced the expression of HO-1 in the epidermis of Nrf2 wild-type mice but not in the epidermis of Nrf2 knockout mice. Thus, it is evident that Nrf2 plays a pivotal role in zerumbone-induced HO-1 expression in mouse skin as well.

Zerumbone, but not α -humulene or 8-hydroxy- α -humulene (zerumbol), induces HO-1 expression and promotes Nrf2 nuclear translocation

In the resting cells, Nrf2 is localized in the cytoplasm and kept inactive by being tethered with Keap1. Murine Keap1 is known to contain 25 cysteine thiol residues. The cleavage of Nrf2-Keap1 complex occurs when a specific cysteine residue in Keap1 that serves as redox sensors is modified by electrophilic compounds or oxidized by ROS. Zerumbone bearing an α,β -unsaturated carbonyl moiety can serve as an electrophile. Figure 5A represents the chemical structures of zerumbone and two of its nonelectrophilic analogues, namely α -humulene and zerumbol. We hypothesize that zerumbone, being electrophilic in nature, may undergo Michael addition reaction with cysteine thiol residues present in Keap1. To clarify that zerumbone could directly modify Keap1, we utilized its biotinylated derivative. When JB6 cells were treated with biotinylated zerumbone (10 $\mu\text{mol/L}$) and immunoprecipitated with Keap1, the amount of biotinylated zerumbone directly bound to Keap1 was increased as detected with HRP-streptavidin (Fig. 5B). Co-incubation of cells with biotinylated zerumbone and thiol reducing agent DTT abrogated the

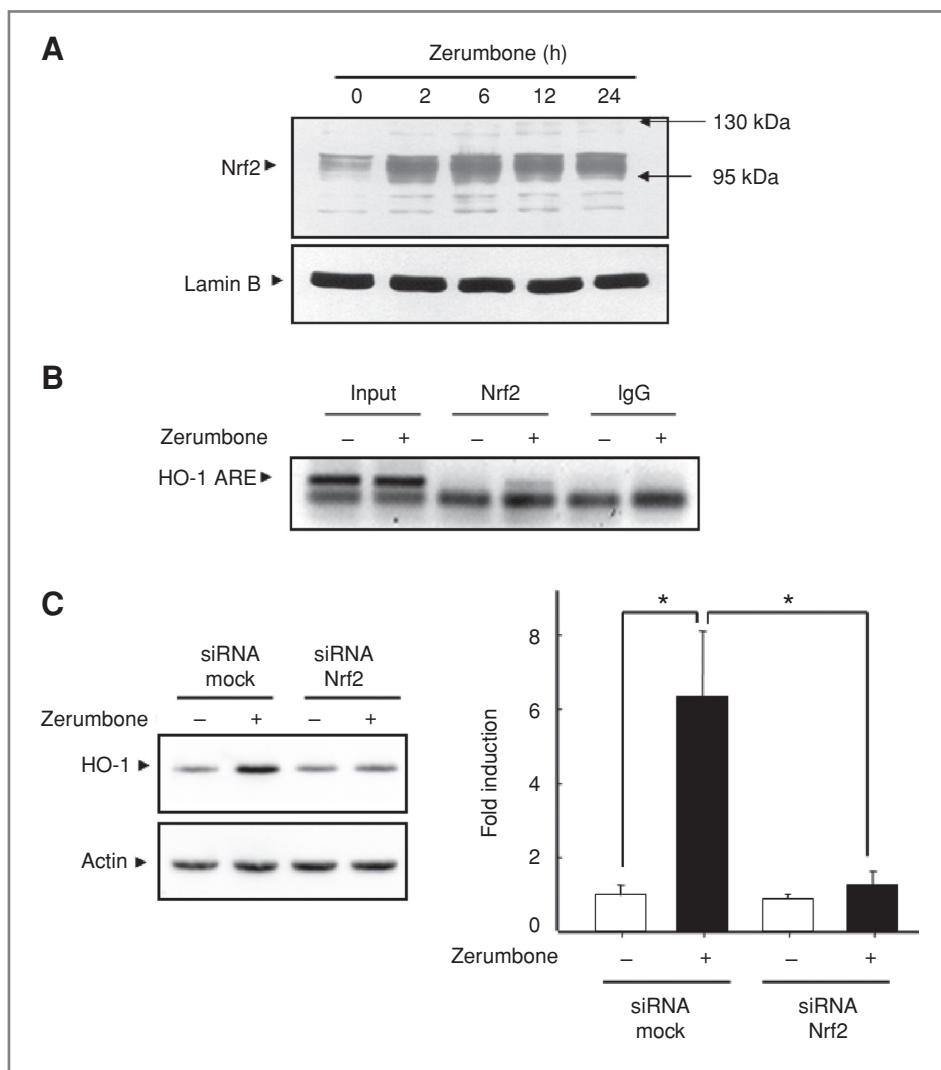


Figure 2. Zerumbone activates Nrf2-ARE signaling responsible for HO-1 expression in JB6 cells. **A**, nuclear translocation of Nrf2 is enhanced by zerumbone in JB6 cells. Cells were treated with zerumbone (10 $\mu\text{mol/L}$) and harvested at the indicated times. Nuclear fraction was isolated as described in Materials and Methods and analyzed to determine the nuclear Nrf2 levels by Western blotting. **B**, JB6 cells were incubated with zerumbone for 6 hours and the ChIP assay was conducted as described in Materials and Methods. **C**, cells were transiently transfected with siRNA-Nrf2 or control vector and treated with zerumbone (10 $\mu\text{mol/L}$) for 6 hours. Cell lysates were subjected to Western blotting to determine the levels of HO-1 expression. *, $P < 0.01$.

interaction of biotinylated zerumbone with Keap1. To further determine whether the electrophilic modification of Keap1 is important for Nrf2 activation and HO-1 expression, the effects of zerumbone on the nuclear translocation of Nrf2 and the promoter activity as well as expression of HO-1 were compared with those of its nonelectrophilic analogues. Although zerumbone promoted nuclear translocation of Nrf2 (Fig. 5C) and enhanced the *Hmox1* promoter activity (Fig. 5D), its nonelectrophilic analogues failed to induce both events. Likewise, only zerumbone that contains the reactive α,β -unsaturated carbonyl group, but not its nonelectrophilic analogues, induced HO-1 expression in JB6 cells (Fig. 5E) and mouse skin *in vivo* (Fig. 5F).

Role of zerumbone-induced HO-1 in protecting against TPA-induced ROS accumulation and transformation of JB6 cells

To confirm whether the induction of HO-1 expression provides chemopreventive effects against the mouse skin

tumor promotion, we examined the effects of zerumbone and its nonelectrophilic derivatives on TPA-induced growth of JB6 cells on the soft agar. Zerumbone, when treated to JB6 cells, significantly inhibited TPA-induced anchorage-independent cell growth (Fig. 6A). In contrast, treatment with α -humulene or zerumbol, which failed to induce HO-1 expression, did not show inhibitory effects on the TPA-induced cell transformation. To examine whether the inhibition of TPA-stimulated cell transformation by zerumbone is attributed to its blockage of ROS overproduction by TPA, we measured intracellular accumulation of ROS in cells treated with zerumbone or its nonelectrophilic analogues. As shown in Figure 6B, pretreatment with zerumbone attenuated TPA-induced ROS production in JB6 cells, whereas either α -humulene or zerumbol failed to affect TPA-induced ROS generation. In addition, when JB6 cells were transiently transfected with a plasmid harboring *Hmox1*, ROS accumulation induced by TPA was inhibited (Fig. 6C). To test whether CO, as a product of

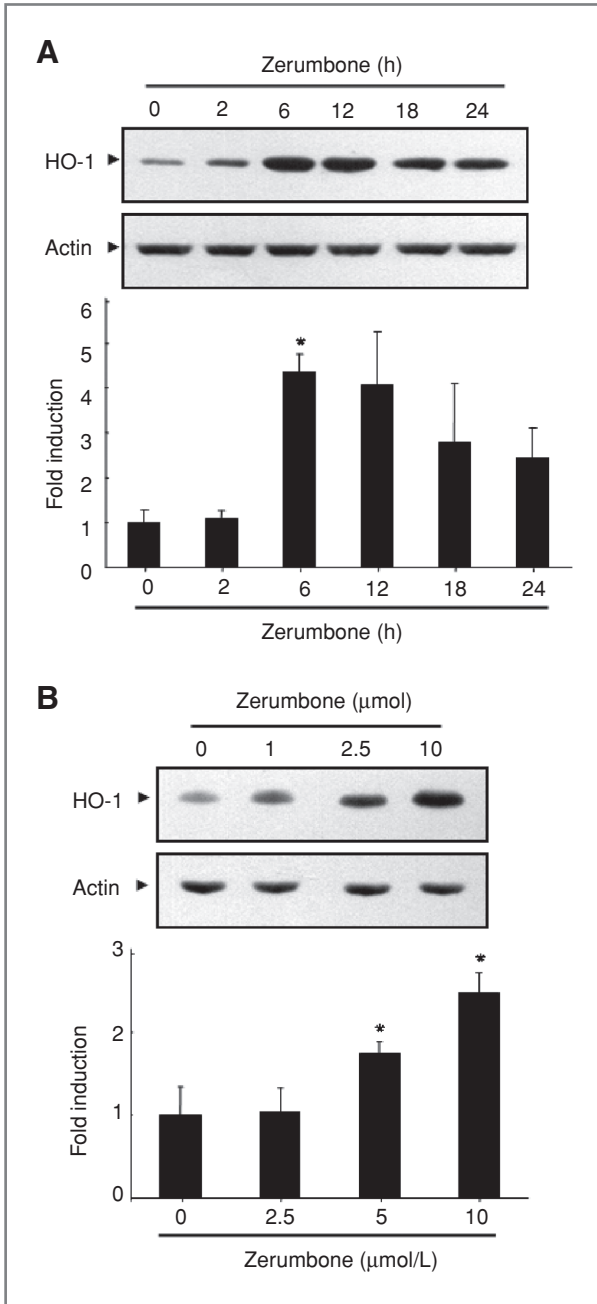


Figure 3. Zerumbone induces HO-1 protein expression in mouse skin *in vivo*. HR-1 hairless mice were treated topically with 10 μmol of zerumbone dissolved in 200 μL of acetone on the dorsal skin for the indicated durations (A) or with different doses (1, 2.5, and 10 μmol) of zerumbone for 6 hours (B). Tissue lysates were prepared, and the levels of HO-1 expression were determined by Western blot analysis. Graph indicates the mean values obtained from triplicate experiments with their SD. Significant differences were evaluated by the Student's *t* test. *, *P* < 0.05, significantly different as compared with the 0-hour control.

heme degradation catalyzed by HO-1, is responsible for inhibition ROS accumulation, we utilized the CO-releasing molecule tricarbonyldichlororuthenium (CORM). As shown in Figure 6D, treatment of JB6 cells with CORM

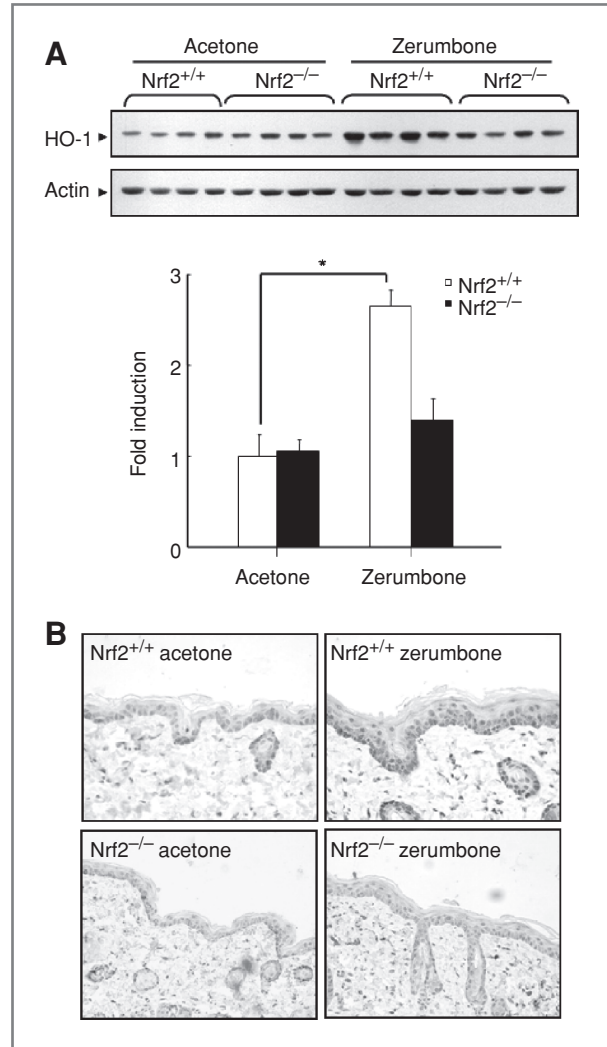


Figure 4. Nrf2 is required for zerumbone-induced induction of HO-1 expression in mouse skin *in vivo*. Dorsal skins of Nrf2 wild-type and Nrf2 knockout mice (*n* = 4 per treatment group) were treated with acetone or 10 μmol zerumbone, and the skin tissues were collected at 6 hours. A, tissue lysates were analyzed by Western blotting to measure HO-1 levels. Data are presented as mean ± SD. *, *P* < 0.01, significantly different from the acetone control in Nrf2 wild-type mice. B, formalin-fixed and paraffin-embedded skin sections were analyzed by immunohistochemistry and the levels of HO-1 were compared between Nrf2 wild-type and Nrf2 knockout mice.

(50 μmol/L) blunted the ROS accumulation caused by TPA treatment.

Discussion

Tumorigenesis is a multistage process that consists of at least 2 well-defined steps, initiation (DNA modification by carcinogens) and promotion (clonal expansion of initiated cells). During the initiation stage, carcinogens, in general, undergo phase I biotransformation to generate reactive intermediates that attack DNA, thereby inducing mutation

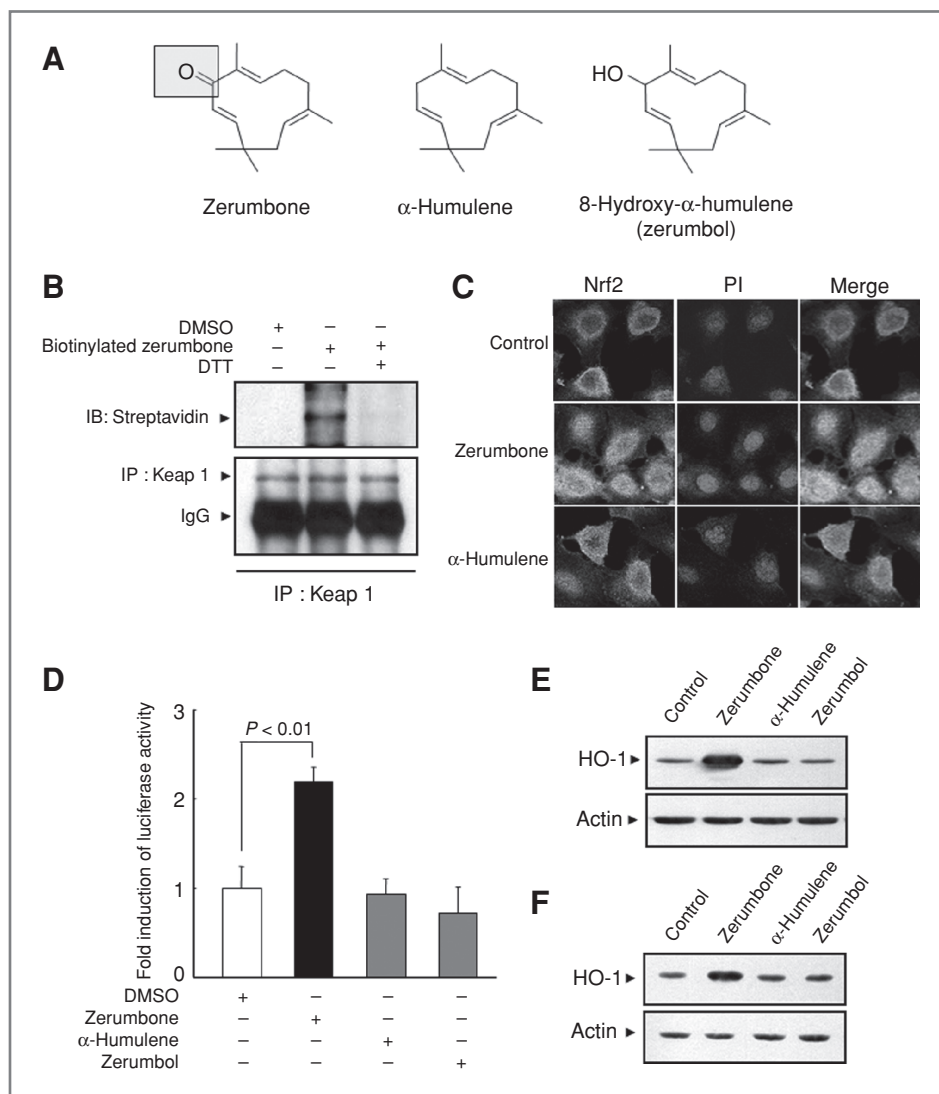


Figure 5. Zerumbone, but not α -humulene or zerumbol, induces HO-1 expression and Nrf2 nuclear translocation. **A**, chemical structures of zerumbone containing an α,β -unsaturated carbonyl group (marked by a square) and its nonelectrophilic derivatives not containing the electrophilic moiety. **B**, JB6 cells were treated with biotinylated zerumbone (10 $\mu\text{mol/L}$) for 6 hours. Immunoprecipitation was done by using Keap1 antibody and immunoprecipitates were subjected to Western blot analysis followed by detection with streptavidin–HRP conjugate. **C**, JB6 cells (1×10^4 per well) were seeded onto 4-chamber cover glasses immersed in 500 μL of 5% FBS-MEM. After 24 hours, cells were pretreated with zerumbone or α -humulene at 10 $\mu\text{mol/L}$ for 6 hours. Immunocytochemical analysis of Nrf2 was carried out as described in Materials and Methods. **D**, JB6 cells were transfected with 750 ng of pHO15-luc. Twenty-four hours after the transfection, cells were treated with 10 $\mu\text{mol/L}$ each of zerumbone, α -humulene, or zerumbol for 12 hours, and cell lysates were assayed for the luciferase activity. The experiment was done in triplicate and the data are presented as mean \pm SD. Statistical analysis was done by the Student's *t* test. **E**, cells were treated with zerumbone or its analogues (each at 10 $\mu\text{mol/L}$) for 12 hours, and the levels of HO-1 and actin were assessed by Western blotting. **F**, zerumbone and its derivatives (each at 10 μmol) were topically applied on the dorsal skin of HR-1 hairless mice for 6 hours, and the epidermal lysates were subjected to immunoblot analysis for the expression of HO-1. For both (E) and (F), mean values obtained from triplicate experiments are shown with their SD, and the significant difference was evaluated by the Student's *t* test (see Supplementary Data).

(24). Elimination of reactive species including electrophiles and ROS before they attack DNA is carried out by phase II detoxifying and antioxidant enzymes, such as glutathione-S-transferase, NAD(P)H:quinone oxidoreductase and HO-1 among others (25). Therefore, inducers of cytoprotective proteins have been proposed as potential candidates for the chemoprevention of carcinogenesis (26). In

this study, we found that zerumbone induced HO-1 expression in both cultured mouse epidermal JB6 cells and in mouse skin *in vivo*.

Induction of HO-1 expression is controlled at the transcription level partly through the ARE located in the promoter region of *Hmx1*. Mouse *Hmx1* promoter contains multiple copies of ARE sequences necessary for the

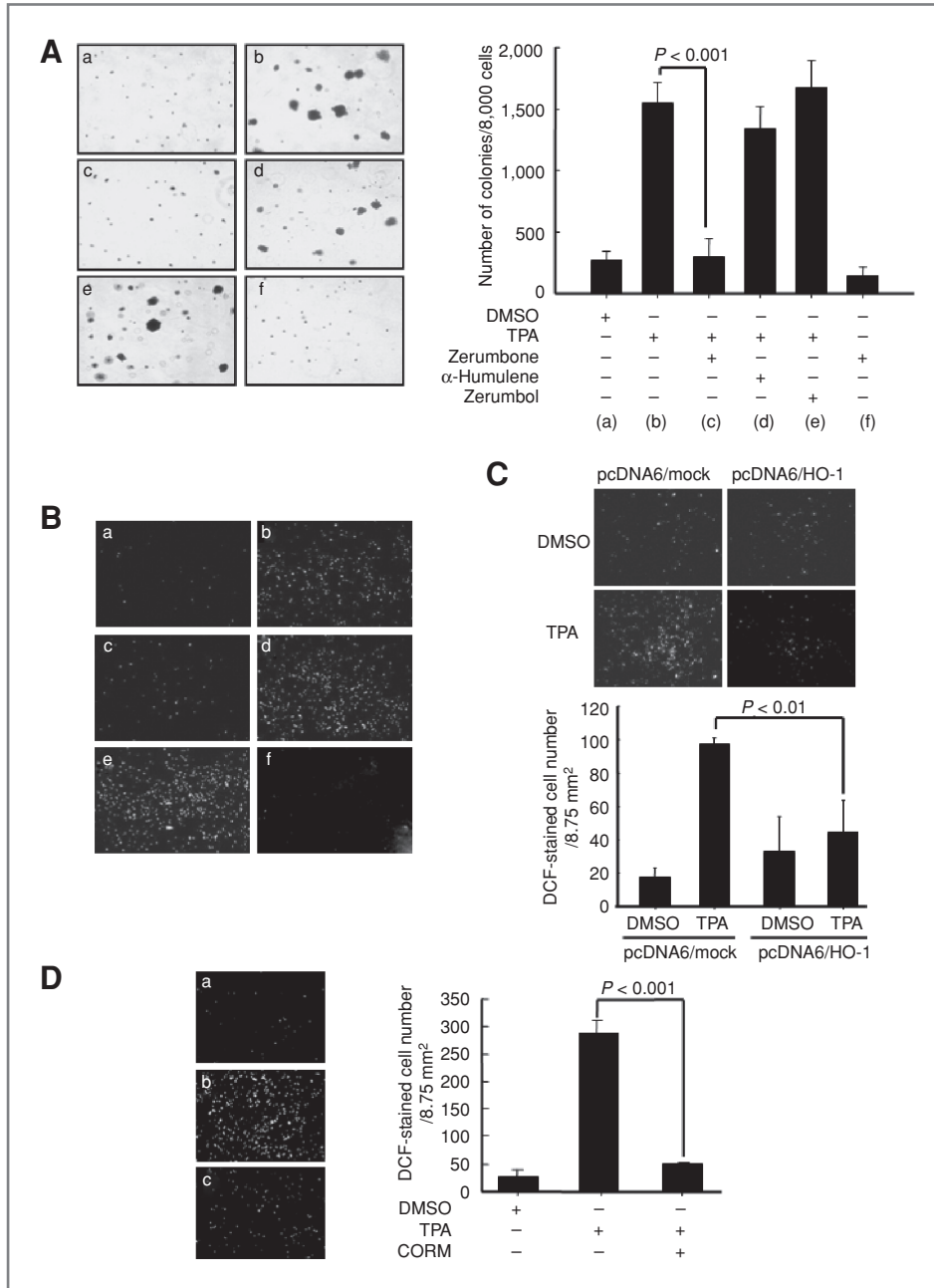


Figure 6. Role of zerumbone-induced HO-1 expression in protecting against TPA-induced transformation and ROS generation in JB6 cells. **A**, cells (8×10^3 per well) were exposed to 10 ng/mL TPA alone or together with zerumbone, α -humulene, or zerumbol at 10 μ mol/L each in 0.33% BME agar containing 10% FBS or 0.5% BME agar containing 10% FBS. Cell colonies were counted after 2 weeks of incubation at 37°C in 5% CO₂. Columns indicate mean values obtained from triplicate experiments with the bars as SD. **B**, cells were seeded (5×10^3 per well) into 8-chambered cover glasses immersed in 200 μ L of 5% FBS-MEM. After 24 hours, cells were pretreated with zerumbone α -humulene or zerumbol at 10 μ mol/L for additional 12 hours and then exposed to 10 ng/mL TPA for additional 2 hours. The intracellular ROS level was measured by DCF-DA staining as described in Material and Methods. DCF-stained cells were counted under a microscope with the aid of Image-Pro Plus Software (version 5.1): DMSO control (a), TPA alone (b), zerumbone and TPA (c), α -humulene and TPA (d), zerumbol and TPA (e), and zerumbone only (f). **C**, cells were seeded (5×10^3 per well) into 8-chambered cover glasses immersed in 200 μ L of 5% FBS-MEM and transiently transfected with pcDNA6/mock or pcDNA6/HO-1. At 24 hours after transfection, cells were treated with or without 10 ng/mL TPA for 2 hours. The intracellular ROS level was measured by DCF-DA staining as described earlier. Columns indicate mean values obtained from triplicate experiments with the bars as SD. **D**, cells were seeded (5×10^3 per well) into 8-chambered cover glasses immersed in 200 μ L of 5% FBS-MEM and were exposed to 10 ng/mL TPA alone or together with 50 μ mol/L CORM. Intracellular ROS level was measured by DCF-DA staining as described in Material and Methods. DCF-stained cells were counted under a microscope with the aid of Image-Pro Plus Software (Version 5.1): DMSO control (a), TPA alone (b), and CORM and TPA (c). Data are presented as mean \pm SD. Statistical analysis was done by the Student's *t* test.

activation of gene transcription by various inducers (23, 27). These ARE sequences are binding sites for the transcription factor Nrf2, a cytoplasmic redox-sensitive transcription factor tethered by Keap1. Thus, Nrf2 acts as the master switch in transcriptional activation of a battery of cytoprotective genes including *Hmox1* (28). Notably, the expression of *ho-1* mRNA induced by hyperoxia or butylated hydroxytoluene was significantly lower in Nrf2 knockout mice (Nrf2^{-/-}) as compared with that in wild-type (Nrf2^{+/+}) mice (29, 30). By utilizing Nrf2 knockout mice and Nrf2-siRNA, we were able to show that Nrf2 is essential for zerumbone-induced upregulation of HO-1 expression in mouse skin and JB6 cells, respectively.

The molecular mechanisms underlying Nrf2 activation that occurs in response to variety of stimuli including chemopreventive agents are not still fully defined (12, 31). However, the modification of Keap1, which tethers Nrf2 in the cytoplasm, is believed to be critical in nuclear accumulation of Nrf2. It is noticeable that Keap1 contains several conserved cysteine residues, and among these, C151, C257, C273, C319, C288, C297, and C613 are known to react with electrophiles and oxidants (32–36). Though the resulting modification of Keap1 is known to prevent Keap1 from binding to Nrf2 leading eventually to an induction of cytoprotective protein expression, Egger and colleagues have shown that modification of Keap1 cysteines is insufficient to hamper the interaction between Keap1 and Nrf2 (36). Although disruption of Keap1–Nrf2 interaction does not occur on cysteine modification of Keap1, it has been suggested that Keap1 modification by an ARE inducer results in Nrf2 activation through the disruption of Keap1–Cul3 interaction, alternative to the Keap1–Nrf2 complex (35, 37). Therefore, cysteine residues of Keap1 are considered to be potential targets for Nrf2 activation by zerumbone that retains electrophilic property.

Recently, Ohnishi and colleagues have reported that zerumbone containing the α,β -unsaturated carbonyl group can bind covalently to Keap1 but α -humulene, which lacks such an electrophilic structure, failed to do so (38). Our finding that biotinylated zerumbone directly interacted with Keap1 in mouse epidermal cells was in good agreement with the result of the aforementioned study. The finding that zerumbone, but neither α -humulene nor zerumbol, induced HO-1 expression and promoted Nrf2 nuclear translocation suggests that the α,β -unsaturated carbonyl structure of zerumbone plays a decisive role in Nrf2 activation and subsequent HO-1 expression through modification of critical cysteine residue(s) of Keap1. However, the identity of cysteine residue(s) of Keap1 directly modified by zerumbone needs to be clarified.

TPA, one of the most well-defined tumor promoters, can generate ROS. The important role of TPA-induced ROS overproduction in neoplastic transformation of JB6 cells has been reported (39). In this study, we found that zerumbone, but not α -humulene and zerumbol, inhibited TPA-induced ROS accumulation and also attenuated

the TPA-induced neoplastic transformation of JB6 cells. On the basis of these data, we speculate that zerumbone-induced expression of HO-1 is responsible for the inhibition of ROS accumulation in JB6 cells stimulated with TPA. Overexpression of HO-1 attenuated TPA-induced overproduction of ROS in JB6 cells, lending further support to this assumption.

It is likely that some products generated by induced HO-1 activity are responsible for the inhibitory effect of zerumbone on the TPA-induced ROS accumulation. It has been reported that zerumbone effectively suppressed TPA-induced superoxide anion (O₂⁻) generation by NADPH oxidase in HL-60 human leukemia cells (17). NADPH oxidase is a heme-containing membrane enzyme that catalyzes the production of O₂⁻ from oxygen and NADPH (40). Although NADPH oxidase has been originally identified as a unique enzyme of neutrophils, several NADPH oxidase-like heme-containing enzymes have been identified in non-neutrophil cells (41). Carbon monoxide (CO), which is produced solely by HO activity in mammalian cells, is known to bind and inhibit the heme-containing enzymes including NADPH oxidase (42–44). The activation of NADPH oxidase plays a key role in TPA-induced ROS generation (45). Moreover, CO has long been known to inhibit mitochondrial respiratory chain through binding with cytochrome *c* oxidase (46). TPA has also been reported to induce mitochondrial ROS in JB6 cells (47), which can be blocked by CO inhibition of cytochrome *c* reductase. As treatment of JB6 cells with CORM markedly inhibited TPA-induced ROS accumulation, zerumbone suppression of TPA-induced ROS production and tumor promotion is likely to be mediated by CO production as a consequence of HO-1 induction.

In conclusion, with the unique α,β -unsaturated carbonyl group, zerumbone activates Nrf2 and subsequently induces HO-1 expression in JB6 cells and mouse skin *in vivo*, which may partly account for its previously reported inhibitory effects on mouse skin tumor promotion.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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