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Capsaicin Induces Heme Oxygenase-1 Expression in HepG2 Cells *Via* Activation of PI3K-Nrf2 Signaling: NAD(P)H:Quinone Oxidoreductase as a Potential Target

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ABSTRACT

Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide), a major pungent ingredient of red pepper, is reported to have antimutagenic and anticarcinogenic properties. However, the mechanisms underlying its chemoprotective effects remain largely unresolved. In the present study, we found that capsaicin induced expression of heme oxygenase-1 (HO-1) in HepG2 cells. Capsaicin treatment resulted in a transient increase in the phosphorylation of Akt and subsequently nuclear translocation of NF-E2–related factor 2 (Nrf2), enhancing its binding to antioxidant response element (ARE). HepG2 cells treated with capsaicin exhibited increased production of reactive oxygen species (ROS). Prior exposure of cells to *N*-acetyl-L-cysteine blocked not only the ROS production but also the nuclear translocation of Nrf2 and its ARE binding, as well as HO-1 induction by capsaicin. Immunoblot analysis showed that whereas the level of HO-1 protein was elevated, that of NAD(P)H:quinone oxidoreductase (NQO1) was decreased after the treatment with capsaicin or the inhibitor of NQO1, dicumarol. We hypothesize that quinone metabolites or other reactive forms of capsaicin may bind covalently to NQO1 and thereby inhibit its activity, leading to production of ROS. This, in turn, would trigger the activation of Akt *via* phosphorylation, increase the nuclear translocation and ARE binding of Nrf2, and upregulate the expression of HO-1. *Antioxid. Redox Signal.* 9, 2087–2098.

INTRODUCTION

Peppers of *Capsicum* family are among the most commonly and frequently used spices throughout the world, particularly in Southeast Asian and Latin-American countries. Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide), a major pungent ingredient of hot chili pepper, has antiinflammatory and analgesic properties, and is currently used as a topical cream for arthritis pain relief (4). The compound also has antimutagenic, anticarcinogenic, and antioxidant activities (27, 31–33). However, the mechanisms underlying its chemopreventive and chemoprotective effects have not been fully understood.

As for the metabolism of capsaicin, it was proposed that capsaicin could undergo metabolic activation, most likely by hepatic cytochrome P450, to form an electrophilic epoxide (21). Capsaicin was found to interact covalently with hepatic proteins after *in vitro* incubation with rat liver microsomes or administration to rats (21). Other reactive species could also be generated. It was reported that hepatic cytochrome P450 2E1 (CYP2E1) activity is responsible for the conversion of capsaicin to the reactive phenoxyl radicals that bind irreversibly to CYP2E1 and inhibit the enzyme activity (26, 33). In this context, capsaicin appears to act as a suicidal or mechanism-based inhibitor of specific cytochrome P450 isozymes. In line with this notion, capsaics prolonged the pentobarbital sleeping time in rats (21) and suppressed the metabolic activation and cova-

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lent binding of benzo(*a*)pyrene to the DNA in murine keratinocytes (22). Based on these reports, it was proposed that bioactivation of capsaicin to electrophilic intermediates like epoxides, phenoxyl radicals, or semiquinone/quinone derivatives may be involved in the covalent modification and subsequent inactivation of enzymes like cytochrome P450s involved in the metabolic activation of carcinogens, which may account for its chemopreventive effects (26, 33).

In addition to inhibiting the cytochrome P450 activity, capsaicin was reported to inhibit the NAD(P)H-quinone oxidoreductase (NQO1) activity (35). Cytochrome P450–catalyzed metabolism of capsaicin could generate reactive quinonoids that bind covalently to cellular proteins/enzymes, especially those harboring cysteine residues, or could alternatively be trapped by cellular reduced glutathione (GSH). This will reduce the cellular GSH level or will inactivate the cysteine-containing enzymes like the flavoprotein reductases or NQO1, thereby inhibiting their activities. Such inhibition of NQO1 activity would block the two-electron reduction metabolism of quinones directly to hydroquinones and lead to production of $O_2^{\cdot -}$ and semiquinone radicals that cause oxidative stress to aerobic cells.

It is well recognized that induction of phase II detoxifying or antioxidant enzymes provides significant protection against oxidative and electrophilic stress (4). Heme oxygenase-1 (HO-1) is an enzyme with potent antioxidant, antiinflammatory, and antiproliferative functions. HO-1 is the inducible form of HO that catalyzes the rate-limiting step in the conversion of heme into biliverdin, carbon monoxide (CO), and free iron (20). Upregulation of HO-1 expression is recognized as a key event in cellular maintenance of antioxidant and chemoprotective defense capacity.

Excessive generation of reactive oxygen species (ROS) causes oxidative stress and depletes intracellular GSH, reducing the GSH-to-GSSG ratio (13). This, in turn, can serve as a signal to induce the expression of diverse phase II–detoxifying/antioxidant enzymes. Induction of antioxidant enzyme expression as an adaptive response to oxidative stress is mediated *via* activation of a major redox-sensitive transcription factor named NF-E2–related factor-2 (Nrf2) (9, 14). Under normal unstimulated conditions, Nrf2 is kept inactive in the cytoplasm by attachment to cytoskeleton *via* the cysteine-rich Keap1 adaptor protein. However, on exposure to prooxidative or electrophilic stimuli, cysteine residues of Keap1 are oxidized or covalently modified, and the Nrf2 is released from Keap1 (24). The released Nrf2 then translocates into nucleus and binds to specific DNA sequences known as stress-response elements (StREs) or antioxidant response elements (AREs) located in the promoter region of variety of genes encoding the phase II detoxification/antioxidant enzymes (12). Cellular levels of many of these cytoprotective enzymes, including those of NQO1 and HO-1, are, in general, tightly correlated with their mRNA levels, unless when the translated proteins undergo posttranslational modification degradation by proteases or covalent binding to reactive electrophilic metabolites. Ironically, some noxious chemicals and stimuli that cause oxidative stress oxidize specific cysteine residues in Keap1 and release Nrf2 to permit its translocation into the nucleus and binding to ARE, which leads to enhancement of the expression of phase II detoxifying/antioxidant enzymes.

In an attempt to explain the cytoprotective or chemoprotective effects provided by capsaicin, we examined its effect on the expression of HO-1 and the signaling pathways responsible for induction of this antioxidant enzyme. Results from this study indicate that capsaicin induces HO-1 expression by stimulation of phosphatidylinositol-3 kinase (PI3K)/Akt signaling that mediates nuclear translocation of Nrf2 and binding to ARE, leading to elevated transcription and translation of HO-1. Furthermore, the capsaicin-derived activation of upstream signaling factors responsible for induction of HO-1 expression is mediated by ROS produced as a consequence inhibition of NQO1 activity, most likely, by the electrophilic quinoids arising from capsaicin metabolism.

MATERIALS AND METHODS

Materials

Capsaicin, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), hemin, *N*-acetyl-L-cysteine (NAC), FAD, NADP, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 2-methyl-1,4-naphthoquinone (menadione), digitonin, and 3,3′-methylenebis(4-hydroxycoumarin) (dicumarol) were purchased from Sigma Chemical Co. (St. Louis, MO). Zinc protoporphyrin IX (ZnPP IX) was the product of OXIS International (Portland, OR). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). [γ-^{32}P]ATP was the product of NEN Life Sciences (Boston, MA). An antibody against HO-1 was obtained from Stressgen Biotechnologies Co. (Victoria, BC, Canada), whereas those against Akt and phospho-Akt were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against HO-2, ERK1/2, phospho-ERK1/2, and Nrf2 were products of Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Secondary antibodies for Western blotting were obtained from Zymed Laboratories Inc. (San Francisco, CA). LY294002 and SB203580 were purchased from Calbiochem (San Diago, CA), and U0126 was provided by TOCRIS (Ellisville, MO). pEF (control vector), pEF-dominant–negative Nrf2 (DN-Nrf2), and reporter gene-fusion constructs for luciferase (pTi-luciferase), wild-type ARE, and GC mutant ARE were kindly provided by Dr. Jeffery A. Johnson (University of Wisconsin–Madison, Madison, WI). Akt with the K179M mutation (kinase-dead Akt) was a generous gift from Dr. An-Sik Chung (Korea Advanced Institute of Science and Technology, Taejon, Korea).

Cell culture and measurement of cell viability

HepG2 cells (obtained from Dr. Han-Woong Lee, Younsei University, Seoul, Korea) were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were plated at appropriate densities, according to the scale of each experiment.

After incubation with capsaicin, cells were treated with MTT solution (1 mg/ml final concentration) for 2 h. The dark blue formazan crystals formed in intact cells were dissolved in DMSO, and the absorbance at 570 nm was read using a mi-

croplate reader. Results were expressed as the percentage of MTT reduction obtained in the treated cells, assuming that the absorbance of control cells was 100%.

Reverse transcriptase–polymerase chain reaction (RT-PCR)

Total RNA was isolated from HepG2 cells treated with capsaicin using Trizol (GibcoBRL, Grand Island, NY) by following the manufacturer's instructions. RT-PCR primers used in this study were as follows: (forward and reverse, respectively): *HO-1*, 5′-CAG GCA GAG AAT GCT GAG TTC-3′ and 5′-GAT GTT GAG CAG GAA CGC T-3′, 555 bp; *GAPDH*, 5′-AAG GTC GGA GTC AAC GGA TT-3′ and 5′-GCA-GTGGGTCTCTCTCCT-3′, 1,054 bp. To amplify the cDNA specific for HO-1, 25 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min were carried out, and to amplify the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to be used as an internal control, 26 cycles of 94°C for 1 min, 56°C for 2 min, and 72°C for 2 min were carried out. These amplification cycles were followed by a final extension at 72°C for 10 min. Amplification products were separated using 1.2% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV light. All primers were purchased from Bionics (Seoul, South Korea).

Western blot analysis

After treatment with capsaicin or other agents, cells (3×10^5 cells/3 ml in a 60-mm dish) were washed with phosphate-buffered saline (PBS), scraped, collected by centrifugation, and lysed. Dilution of primary antibodies including anti-HO-1 (1:2,000) (SPA-895; Stressgen, Victoria, British Columbia, Canada), anti-NQO1 (1:1,000) (SC 32793; Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-Akt (1:2,500) (CS 9272), and anti-phospho-Akt (1:500) (CS 9271L, Cell Signaling Technology, Beverly, MA), anti-Lamin B (1:1,000) (33-2000; Zymed Laboratories, South San Francisco, CA), anti-ERK (1:2,500) (SC 94) and anti-phospho-ERK (1:2,500) (SC 7383), anti-HO-2 (1:2,500) (SC 17786), and anti-Nrf2 (1:500) (SC-722, Santa Cruz Biotechnology Inc.), or anti-actin (Sigma Chemical Co.) was made in PBS containing 3% nonfat dried milk.

HO activity assay

The HO activity was measured by spectrophotometric determination of bilirubin produced from hemin added as the substrate. In brief, the microsomes obtained from HepG2 cells treated with capsaicin or other agents were incubated with the reaction mixture containing 0.8 m*M* NADPH, 2 m*M* glucose-6-phosphate, 0.2 U glucose-6-phosphate dehydrogenase, 20 μM hemin, 1 mg of rat-liver cytosol (source of biliverdin reductase), and 100 m*M* potassium phosphate buffer, pH 7.4. Reaction mixtures (1.0 ml total volume) were incubated at 37°C for 1 h in the dark and then placed on ice for 2 min to terminate the reaction. Bilirubin formed was determined by calculation from the difference in absorbance between 464 and 530 nm (extinction coefficient, 40 m*M*/cm for bilirubin). HO activity was expressed in picomoles of bilirubin formed per milligram microsomal protein per hour.

GSH/GSSG measurements and calculation of intracellular redox potential

Cells were washed twice with 1×PBS, detached by trypsinization, and treated with 10% (1:1, vol/vol) trichloroacetic acid to extract cellular GSH. The mixture was centrifuged at 13,000 *g* for 10 min to remove denatured proteins. The supernatant was assayed for the total GSH and GSSG concentration using the HPLC separation/fluorometric detection method of Neuschwander-Tetri and Roll (23). HPLC was performed using a PU-980 pump (Jasco Co., Tokyo, Japan) with a FP-920 fluorescence detector (Jasco Co.) and a 3.5-μm Symmetry C_{18} (4.6 × 75 mm) column (Waters Co., Milford, MA). Protein pellets were dissolved by overnight incubation in 1 *M* NaOH at room temperature. A protein assay was done according to the method of Lowery *et al*. (19).

NQO1 activity assay

The principle of the assay of quinone reductase is that glucose 6-phosphate and glucose-6-phosphate dehydrogenase continually generate NADPH, which is used by quinone reductase to transfer electrons to menadione. The resulting menadiol reduces MTT to blue formazan, which can be measured with an ELISA plate reader at 620 nm (15). After treatment with capsaicin, cells (4×10^3 cells/100 μl in 96-well plate) were washed with PBS and lysed by incubation at 37°C for 10 min with 50 μl of a solution containing 0.8% digitonin. The plates were then agitated on an orbital shaker for an additional 10 min at 25°C. Menadione (1 μl of 50 m*M* menadione dissolved in acetonitrile per milliliter of reaction mixture) was added just before the reaction mixture was dispensed into the microtiter plates. Measurements were made at 5-min intervals at 620 nm. NQO1 activity was determined by calculation of the difference in slope between samples and baseline (extinction coefficient, 11,300 M/cm at 620 nm).

Preparation of nuclear extracts

After treatment with 200 μM capsaicin, the HepG2 cells (3×10^5 cells/5 ml in 100-mm dish) were washed with cold PBS. Cells were collected by centrifugation and resuspended in ice-cold isotonic buffer A [10 m*M* Hepes (pH 7.9), 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 0.5 m*M* dithiothreitol (DTT), and 0.2 m*M* phenylmethylsulfonyl fluoride (PMSF)]. After incubation in an ice bath for 10 min, the broken cells were centrifuged again to collect the nuclear fraction. The nuclear fraction was then resuspended in ice-cold buffer C containing 20 m*M* Hepes (pH 7.9), 20% glycerol, 420 m*M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 0.5 m*M* DTT, and 0.2 m*M* PMSF, and lysed by 20-min incubation at 0°C. After a vortex mixing, the lysed suspension of nuclei was centrifuged again, and the collected nuclear extract was stored at −70°C for determination of Nrf2-ARE binding, as described later. The protein concentration of the nuclear extracts was determined by the Bradford method using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Electrophoretic mobility shift assay (EMSA) to detect Nrf2-ARE binding

The DNA–protein complexes were resolved by 6% nondenaturing polyacrylamide gel electrophoresis, as reported previously (17).

Transient transfection with ARE-luciferase reporter plasmid and luciferase assay

HepG2 cells were transfected with luciferase reporter plasmid, and the luciferase activity was measured using the Luciferase Assay System, as reported previously (17).

Measurement of intracellular ROS accumulation

Accumulation of ROS in HepG2 cells treated with capsaicin or dicumarol (for 3 h) was monitored using the fluorescence-generating probe DCF-DA. Cells (1×10^5 cells/600 μl in a four-well chamber slide) were rinsed with Kreb's Ringers solution and loaded with 10 μM DCF-DA. After 15-min incubation at 37°C, cells were examined under a confocal fluorescence microscope set at 488 nm for excitation and 530 nm for emission (Leica Microsystems, Heidelberg GmbH, Heidelberg, Germany).

Immunocytochemistry of Nrf2

To demonstrate the nuclear translocation of Nrf2, immunocytochemistry was performed, and the monoclonal antibody recognizing Nrf2 was used. Capsaicin-treated cells (1×10^5 cells/600 μl in a four-well chamber slide) were fixed in 10% neutral-buffered formalin solution for 30 min at room temperature. After a rinse with PBS, fixed cells were incubated in a fresh blocking buffer (0.5% Tween-20 in PBS, pH 7.4, containing 10% normal goat serum) for 1 h at room temperature. Cells were then incubated overnight at 4°C on addition of anti-Nrf2 primary antibody solution (diluted 1:100 in PBS with 1% bovine serum albumin). Afterward, the cells were washed 3 times with PBST (PBS containing 0.1% Tween-20) and then incubated for 1 h at room temperature on addition of FITC-goat anti-rabbit IgG secondary antibody diluted (1:1,000) in PBST with 3% bovine serum albumin. Cells were then rinsed with PBS, and the stained cells were analyzed under a confocal microscope.

Statistical analysis

When necessary, data were expressed as mean ± SD, and Student's *t* test was used to perform statistical analysis for single comparison. The criterion for statistical significance was $p < 0.05$ (Student's *t* test).

RESULTS

Capsaicin induces HO-1 expression and elevates HO activity

To determine the optimal dose range, HepG2 cells were treated with varying concentrations of capsaicin (≤500 μM) and for various durations (*e.g.,* 12 h, 24 h, and 36 h), and cell viability was determined using the MTT assay. About 86% of cells survived when exposed to 200 μM capsaicin for up to 36 h (data not shown).

When HepG2 cells were treated with different concentrations (5, 10, 25, 50, 100, or 200 μM) of capsaicin for 24 h, the maximal induction of HO-1 protein was achieved with 200 μM (Fig. 1A), and the expression of HO-1 protein began to be apparent at 12 h and continued to increase up to 36 h after incubation

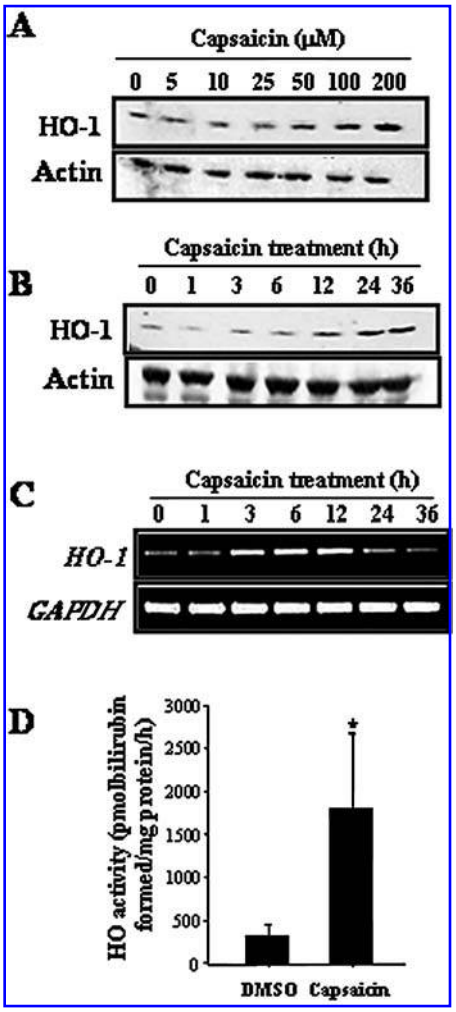

FIG 1. Induction of HO-1 expression and HO activity by capsaicin. (**A**) HepG2 cells were incubated with varying concentrations of capsaicin (0, 5, 10, 25, 50, 100, and 200 μM) for 24 h and harvested for Western blot analysis of HO-1 expression. (**B**) Cells were treated with capsaicin (200 μM) for the indicated durations. The same membranes were probed again with the antibody against actin to ensure equal loading of cellular proteins on the gel. (**C**) HepG2 cells exposed to 200 μM capsaicin were harvested at the indicated intervals, and total RNA was prepared. The RNA samples were analyzed by RT-PCR for the levels of *HO-1* mRNA, as described under Materials and Methods. *GAPDH* was used as a control for equal loading. (**D**) HepG2 cells were treated with capsaicin (200 μM) for 24 h and analyzed for HO activity by measuring the production of bilirubin. The data represent mean ± SD ($n = 3$). Significant differences between the compared groups are indicated (*$p < 0.05$).

with 200 μM capsaicin (Fig. 1B). The RT-PCR analysis showed that accumulation of HO-1 mRNA occurred only transiently between 3 and 12 h (Fig. 1C). The HO activity, as determined by production of bilirubin, was increased markedly in cells treated with capsaicin for 24 h (Fig. 1D). Thus, capsaicin upregulates HO-1 expression and increases the HO activity.

Capsaicin triggers ROS production that induces HO-1 expression

As shown in Fig. 2A, capsaicin caused marked production of ROS in HepG2 cells, which was abolished by pretreatment with NAC. Furthermore, the capsaicin-derived induction of HO-1 expression was also abolished by NAC (Fig. 2B). Assuming that ROS responsible for the capsaicin-mediated induction of HO-1 expression might be superoxide anion ($O_2^{\cdot -}$) that was overproduced as a consequence of inhibition of the NQO1 activity, we attempted to convert $O_2^{\cdot -}$ to H_2O_2 using MnTBAP, a synthetic membrane permeable SOD mimetic, thereby abolishing the capsaicin-dependent induction of HO-1 expression. In contrast to our expectation, the addition of MnTBAP enhanced the level of HO-1 protein accumulation above that induced by capsaicin alone (Fig. 2C). Combined, these results indicate that $O_2^{\cdot -}$ is not directly responsible for the induction of HO-1 expression. Instead of $O_2^{\cdot -}$ that cannot react rapidly with NAC or GSH, H_2O_2 capable of depleting cellular GSH and causing oxidative stress appears to trigger the signal to activate the events that lead to the upregulation of HO-1 expression by capsaicin. Alternatively, NAC could trap and inactivate the reactive quinone metabolites generated from capsaicin and prevent the overproduction of ROS (both $O_2^{\cdot -}$ and H_2O_2), thus abolishing the increased DCF-DA stain and the induction of HO-1 caused by capsaicin.

To examine the effects of capsaicin on the GSH/GSSG redox couple, HepG2 cells were treated with capsaicin for the indicated durations. The intracellular level of total GSH (combination of GSH and GSSG) was determined at various time intervals. The total GSH concentration was decreased at 3–6 h after the capsaicin treatment, but the level was almost completely restored in 12 h (Fig. 2D). After 24 h, the total GSH increased even above the basal level. Consistent with the kinetic

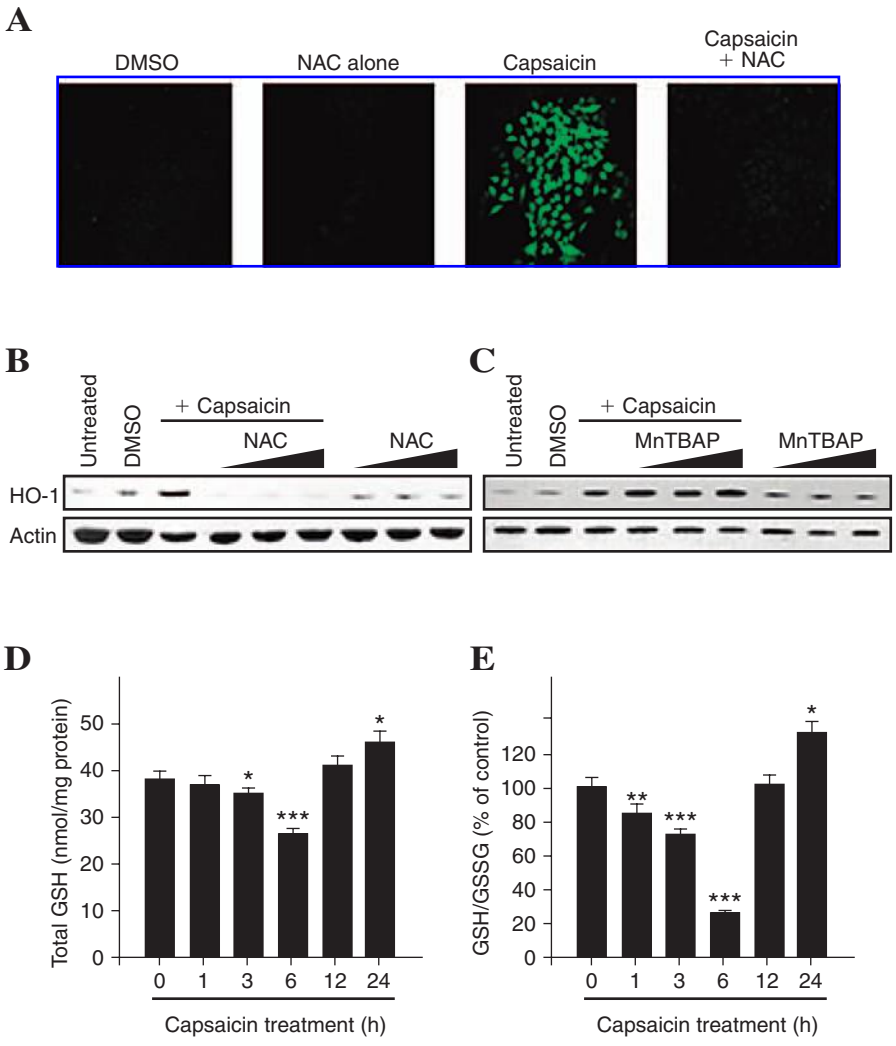

FIG. 2. Role of ROS in capsaicin-induced HO-1 expression. (**A**) Capsaicin stimulates ROS production in HepG2 cells. HepG2 cells were treated with 200 μM capsaicin in the absence or presence of NAC (5 m*M*) for 3 h. Cells were exposed to NAC for 1 h before the capsaicin treatment. ROS production was determined by the DCF-DA assay, as described in Materials and Methods. (**B, C**) Capsaicin-induced HO-1 expression was blocked by NAC, but not by MnTBAP. HepG2 cells were treated with 200 μM capsaicin in the presence of NAC (5, 7.5, 10 m*M*) or MnTBAP (100, 250, 500 μM) for 24 h. The HO-1 protein level in the cell lysates was determined by Western blot analysis. Actin was used as a control for equal loading. (**D**) Effects of capsaicin on total GSH levels in HepG2 cells treated with capsaicin. Cells were treated with capsaicin (200 μM) for the indicated periods. After treatment, cells were harvested, washed, and processed for GSH measurement by HPLC as described in Materials and Methods. (**E**) The ratio of GSH/GSSG in HepG2 cells treated with capsaicin. The experimental conditions and other details are described in Materials and Methods. The data represent mean ± SD ($n = 3$). Significant differences between the compared groups are indicated ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$).

profile of the total GSH levels, the GSH/GSSG ratio initially decreased on capsaicin treatment, but was restored eventually (Fig. 2E)

Capsaicin-induced HO-1 upregulation is mediated via Nrf2 signaling

As mentioned earlier, Nrf2 is a redox-sensitive transcription factor that becomes activated and translocates into the nucleus in response to stimuli that cause oxidative stress. After nuclear translocation, Nrf2 binds to ARE localized in the promoter region of genes encoding cytoprotective phase II detoxifying/antioxidant enzymes and stimulates their transcription. To explore whether capsaicin stimulates nuclear translocation of Nrf2 and enhances its DNA binding, as well as increases the transcriptional activity of ARE, Western blot analysis of nuclear extracts, the gel-shift assay to assess the Nrf2-ARE binding, and the luciferase reporter assay to measure the transcriptional activity of Nrf2 were conducted. As illustrated in Fig. 3A, capsaicin increased the nuclear translocation of Nrf2. Furthermore, capsaicin increased the Nrf2-ARE binding (Fig. 3B), consistent with the time course of HO-1 mRNA accumulation (see Fig. 1C). The increased Nrf2-ARE binding was accompanied by enhanced Nrf2 transactivation, as assessed by the luciferase reporter gene assay (Fig. 3C). The HepG2 cells transfected with the plasmid carrying mutation in the GC box of the ARE sequence exhibited markedly decreased luciferase reporter activity (Fig. 3C). Combined, these results suggest that capsaicin induces HO-1 expression by activation of Nrf2 signaling. In an effort to support this proposition, we conducted additional experiments using the dominant-negative mutant of Nrf2. Compared with the cells transfected with a functionally active vector harboring an intact Nrf2, cells transfected with the plasmid carrying a dominant-negative mutant form of Nrf2 with truncated N-terminal sequences expressed much lower levels of HO-1 on incubation with capsaicin (Fig. 3D). Co-incubation with NAC abrogated the nuclear translocation of Nrf2 induced by capsaicin, as illustrated by immunocytochemical analysis (Fig. 4A). In support of this immunocytochemical result, Western blot and EMSA analyses of nuclear extract also showed that NAC abolished the capsaicin-derived increase in the nuclear translocation of Nrf2 (Fig. 4B) and the Nrf2-ARE binding (Fig. 4C), respectively.

Increased PI3K activity and Akt phosphorylation mediate the capsaicin-induced enhancement of Nrf2-ARE binding and HO-1 expression

To elucidate the upstream signaling events that lead to activation of Nrf2 and induction of HO-1 expression, we examined the capsaicin-induced phosphorylation of representative signal-transducing kinases. We noted that phosphorylation of both Akt and ERK was increased significantly at 3 h after treatment with capsaicin (Fig. 5A). Next, to determine which signal-transducing kinase is most likely involved in HO-1 induction, we used selective inhibitors of representative signaling kinases, such as LY294002 for inhibition of PI3K, U0126 for inhibition of ERK, and SB203580 for inhibition of p38 MAPK. The result shown

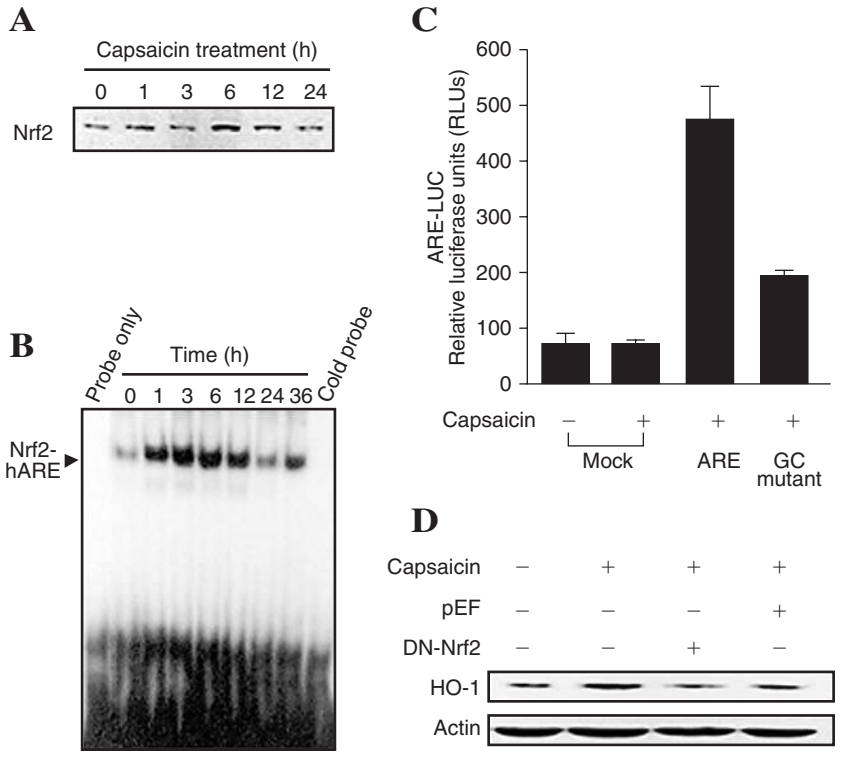

FIG. 3. Capsaicin-induced nuclear translocation, ARE binding, and transcriptional activity of Nrf2. (**A**) Effects of capsaicin on the levels of nuclear Nrf2. Nuclear extracts from HepG2 cells were prepared at the indicated intervals after treatment with 200 μM capsaicin. Immunoblots of nuclear lysates from treated cells were probed with the Nrf2-specific antibody. (**B**) Time course of capsaicin-induced activation of Nrf2-ARE binding. The nuclear extract isolated from capsaicin-treated cells was subjected to EMSA, as described under Materials and Methods. The competition assay for the Nrf2-ARE binding was carried out in the presence of 100-fold molar excess of unlabeled oligonucleotide. (**C**) The capsaicin-mediated transcriptional activation of ARE was measured by the luciferase reporter assay. The experimental details are described under Materials and Methods. The data are expressed as mean ± SD ($n = 3$). (**D**) Cells were transfected with pEF blank vector or dominant-negative mutant Nrf2 (DN-Nrf2) using the DOTAP transfection reagent. After 6-h transfection, cells were treated with 200 μM capsaicin and incubated for an additional 24 h. Protein from cell lysates was analyzed by Western blot probed with an HO-1–specific antibody.

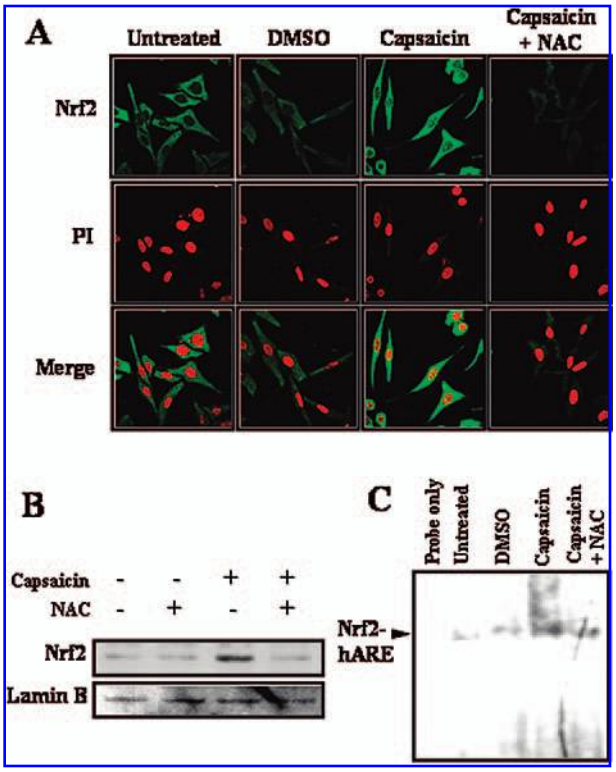

FIG. 4. Effects of NAC on capsaicin-induced Nrf2 activation. (**A**) Immunocytochemical analysis of Nrf2 translocation. HepG2 cells were treated with 200 μM capsaicin in the absence or presence of 5 m*M* NAC for 3 h. NAC was added 1 h before capsaicin treatment. (**B**) Nuclear extracts from HepG2 cells were prepared after treatment with 200 μM capsaicin in the absence or presence of NAC (5 m*M*) for 3 h. Immunoblots of nuclear lysates from treated cells were probed with the Nrf2-specific antibody. (**C**) Nuclear extracts from HepG2 cells treated with capsaicin for 3 h were incubated with [γ-^{32}P]-labeled oligonucleotides harboring the ARE consensus sequence. The Nrf2-ARE binding was analyzed with the gel-shift assay as described in Materials and Methods.

in Fig. 5B indicates that only the LY294002-mediated inhibition of PI3K/Akt activity is causally associated with abrogation of capsaicin-induced upregulation of HO-1 expression. Likewise, the Nrf2-ARE binding induced by capsaicin was attenuated by inhibition of PI3K activity (Fig. 5C). Further to verify that the PI3K/Akt pathway is indeed involved in the Nrf2-mediated induction of HO-1 expression, HepG2 cells were transfected with the plasmid carrying the kinase-dead Akt (Akt with K179M mutation) and then stimulated with capsaicin. As shown in Fig. 6, HepG2 cells transfected with functionally inactive kinase-dead Akt (KD-Akt) exhibited reduced expression of HO-1 (Fig. 6A) as well as Nrf2-ARE binding (Fig. 6B) on capsaicin treatment when compared with the cells transfected with the plasmid carrying functionally active hemaglutinin-tagged full-length Akt (HA-Akt). These findings indicated that the capsaicin-induced upregulation of HO-1 expression is mediated, at least in major part, by activation of the PI3K/Akt signaling pathway that enhances nuclear translocation of Nrf2 and increases its binding to ARE.

Capsaicin suppresses NQO1 expression and activity

NQO1 catalyzes the two-electron reduction of quinones to hydroquinones and competes with the one-electron reduction of quinones catalyzed by the cytochrome P450 reductase and other flavoprotein reductases that produce cytotoxic semiquinones or phenoxyl radicals. Although the hydroquinones produced by NQO1 can undergo conjugation to form glucuronides or sulfates and are eliminated by excretion, the semiquinone radicals produced by flavoprotein reductases undergo redox cycling in aerobic cells and remain in the cell to overproduce superoxide ($O_2^{\cdot -}$). Alternatively, quinoids can be

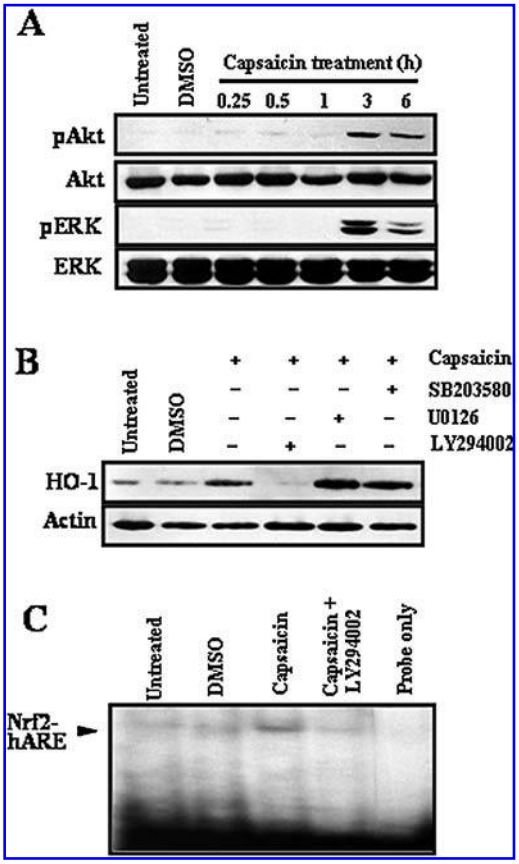

FIG. 5. Effects of pharmacologic inhibition of Akt on capsaicin-induced Nrf2 activation and HO-1 expression. (**A**) Effect of capsaicin on the phosphorylation of Akt and ERK. HepG2 cells were stimulated with 200 μM capsaicin for the indicated times, and the levels of phosphorylated and unphosphorylated Akt and ERK were measured by Western blot analysis. (**B**) HepG2 cells were preincubated for 1 h with 25 μM LY294002 (the inhibitor of PI3K, upstream of Akt), U0126 (the inhibitor of MEK1/2, upstream of ERK), or SB203580 (the inhibitor of p38) followed by capsaicin (200 μM) treatment for 24 h. (**C**) Effect of the pharmacologic inhibitor of PI3K (LY294002) on capsaicin-induced Nrf2-ARE binding activity. Nuclear extracts were prepared from HepG2 cells treated with 200 μM capsaicin in the absence or presence of LY294002 (25 μM) and subjected to EMSA for the measurement of Nrf2-ARE binding.

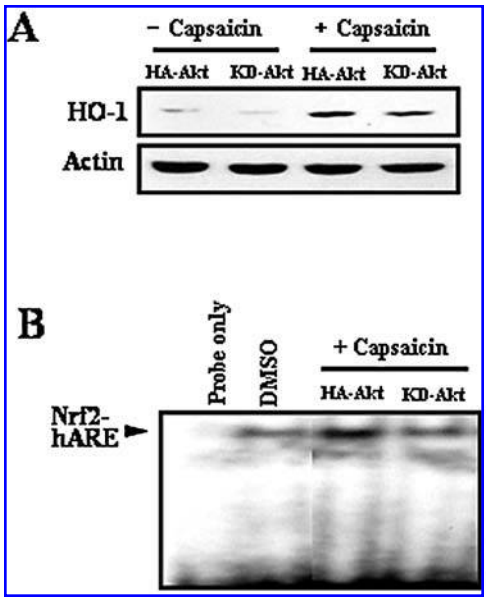

FIG. 6. Abrogation of HO-1 upregulation and Nrf2 activation by functional inactivation of Akt in capsaicin-treated HepG2 cells. (**A**) Cells were transfected transiently with functionally active hemaglutinin-tagged full-length Akt (HA-Akt) or kinase-dead (KD) Akt and then exposed to 200 μM capsaicin for 24 h. HO-1 expression was determined by Western blot analysis. (**B**) Effect of KD-Akt on capsaicin-mediated Nrf2-ARE binding activity measured by EMSA.

trapped in the cell by covalent binding with GSH or surrounding enzyme proteins, particularly those containing cysteine residues like NQO1, destroying or inhibiting its enzyme activity. Particularly when cellular GSH is depleted, destruction of NQO1 would be increased and $O_2^{\cdot -}$ will be overproduced. In response to such conditions causing oxidative stress, cells tend to induce the expression of several antioxidant enzymes. Capsaicin is known to undergo cytochrome P450-catalyzed oxidative metabolism and generates the reactive quinone metabolites (25, 26) and has been shown to enhance ROS production (15, and also Fig. 2A in this study). HepG2 cells were incubated with varying concentrations of capsaicin (25, 50, 100, and 200 μM) for 24 h and harvested for Western blot analysis to determine the levels of both cysteine-free HO-1 (29) and cysteine-containing NQO1 (18). Interestingly, although the content of HO-1 protein was increased, that of NQO1 was decreased in a concentration-dependent manner (Fig. 7A). Thus, further studies are needed to determine whether the decreased NQO1 protein level is causally associated with decreased transcription of *NQO1* mRNA, or alternatively, the result of covalent modification that hampers antibody recognition.

Capsaicin has been reported to inhibit the NQO1 activity (28, 34), and as shown in Fig. 7B, we confirmed that capsaicin treatment inhibited the NQO1 activity in HepG2 cells. The inactivation of NQO1 caused by capsaicin may contribute to the overproduction of ROS, which resembled that of dicumarol, a well-known inhibitor of NQO1 activity. Thus, HepG2 cells were treated with increasing concentrations of dicumarol (50, 100, 250 μM) for 3 h, a similar concentration range and the duration employed for the overproduction of ROS with capsaicin. The dicumarol-treated HepG2 cells showed a concentration-dependent increase of ROS production, as evidenced by the enhancement of DCF-DA fluorescence staining (Fig. 7C). Similar to the results obtained with capsaicin (see Fig. 1A), HepG2 cells treated with dicumarol exhibited an elevated HO-1 protein level comparable to that induced by capsaicin, but the levels of NQO1 protein present in these cells were decreased markedly when compared with those present in control cells (Fig. 7D). When the cells were exposed to increasing concentrations of dicumarol in the presence of NAC, the dicumarol-induced upregulation of HO-1 was attenuated significantly, but the dicumarol-induced depression of NQO1 expression was slightly reversed (Fig. 7D).

Capsaicin protects against SIN-1–induced cytotoxicity, which is abolished by HO-1 inhibition

Peroxynitrite produced by rapid interaction between $O_2^{\cdot -}$ and nitric oxide (NO) radicals is one of the most powerful oxidants. When the HepG2 cells were exposed to 5 mM SIN-1, a peroxynitrite donor releasing $O_2^{\cdot -}$ and NO simultaneously, nearly 40% of cells died (Fig. 8). However, when the cells were pretreated with capsaicin, a significant increase in the viability occurred, as determined by the MTT assay. The cytoprotective effect of capsaicin was abolished in the presence of the HO inhibitor ZnPP IX (see Fig. 8). ZnPP aggravated the cytotoxic effects of SIN-1 markedly. This finding suggests that the HO-1 expression induced by capsaicin may contribute to cytoprotection against SIN-1-induced cytotoxicity.

DISCUSSION

Intake of exogenous antioxidants to scavenge ROS and to eliminate other reactive species generated within the cell is considered to be an acceptable approach to reduce the oxidative stress implicated as the major cause of cellular injuries in a variety of chronic human diseases. Attention has been focused recently on the dietary phytochemicals, such curcumin, diallyl sulfide, and sulforaphane, which act as antioxidants (30). Many of antioxidative dietary phytochemicals have been demonstrated to provide cytoprotection, not only directly scavenging ROS but also *via* upregulation of antioxidant enzymes that include HO-1 (1, 2, 7, 10). Capsaicin and structurally related vanilloid analogues have been reported to possess antimutagenic, anticarcinogenic, and antioxidant activities (27, 31–33). Although the mechanisms underlying cytoprotective as well as antioxidant effects provided by capsaicin have been poorly understood, it may induce antioxidant gene expression, as do the aforementioned antioxidant phytochemicals.

Capsaicin and its vanillyl analogues are known to undergo oxidation and dehydrogenation catalyzed by cytochrome P450s to generate reactive quinone derivatives that can be trapped with GSH (25, 26, 33). The oxidized quinones arising either from capsaicin or other phenolic compounds are subjected to reduction either by NQO1 or by cytochrome-P450 reductase and even

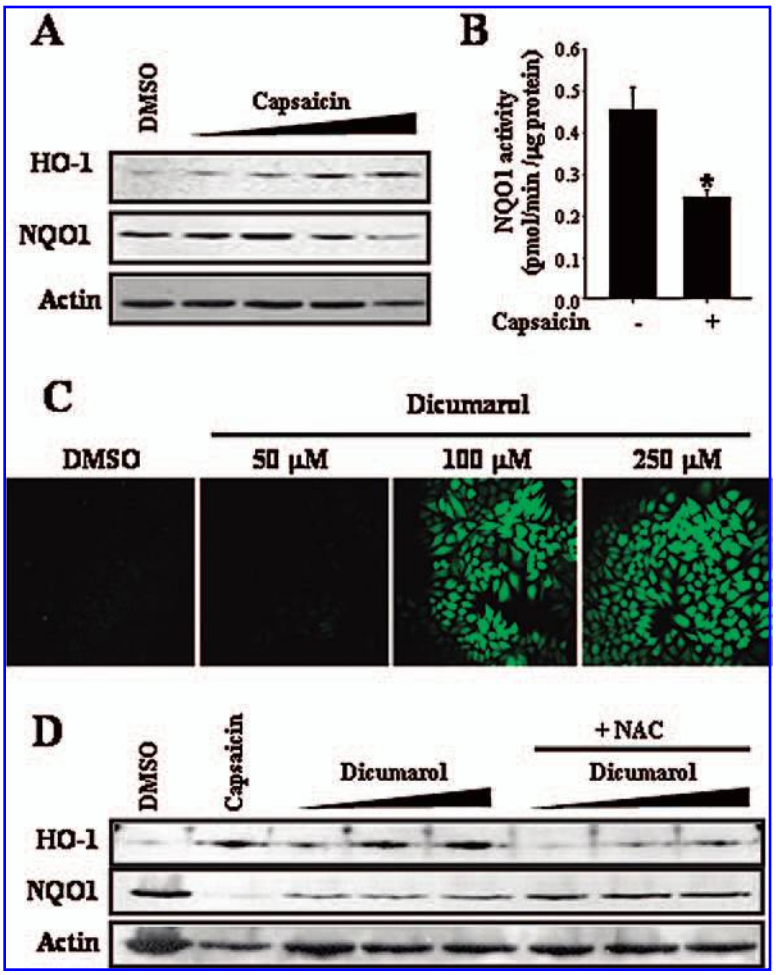

FIG. 7. Inhibition of NQO1 by capsaicin and dicumarol. (**A**) HepG2 cells were incubated with varying concentrations of capsaicin (25, 50, 100, and 200 μM) for 24 h and harvested for Western blot analysis of HO-1 and NQO1. The same membranes were probed again with the antibody against actin to ensure equal loading of cellular proteins on the gel. (**B**) HepG2 cells were treated with capsaicin (200 μM) for 24 h and analyzed for NQO1 activity. The data represent mean ± SD ($n = 3$). Significant differences between the compared groups are indicated ($^*p < 0.005$). (**C**) Inhibition of NQO1 stimulates ROS production in HepG2 cells. HepG2 cells were treated with dicumarol (50, 100, 250 μM) for 3 h. ROS production was determined by the DCF-DA assay, as described in Materials and Methods. (**D**) HepG2 cells were incubated with 200 μM capsaicin or varying concentrations of dicumarol (50, 100, and 250 μM) in the absence or presence of NAC (5 mM) for 24 h and harvested for the Western blot analysis of HO-1 and NQO1. The cells were preincubated for 1 h with or without NAC (5 mM) before the dicumarol treatment.

by other flavoprotein reductases. The phenoxyl radical and quinoid metabolites undergo covalent binding with GSH or proteins that contain cysteine residues and deplete GSH or inhibit the enzyme activity, respectively (3). Alternatively, the phenoxyl radicals are reoxidized to quinones in aerobic cells after donating one electron to molecular oxygen (O_2), thus producing $O_2^{\cdot -}$ (6, 15). Particularly when the GSH level is lowered, phenoxyl radicals undergo repeated redox cycling and overproduce $O_2^{\cdot -}$ and inactivate the cysteine-containing enzyme activities like NQO1, aggravating oxidative stress. In response to such prooxidant activity exerted by capsaicin, an upregulation of a battery of cytoprotective antioxidant enzymes including HO-1 could occur, thereby providing the general chemoprotective effects against the cytotoxicity caused by a wide variety of noxious stimuli. Although capsaicin does exert the prooxidant activity by generating ROS (see Fig. 2A), such oxidative stress is not considered to be detrimental to cells, as it is transient and is properly followed by *de novo* synthesis of antioxidant enzymes, such as HO-1. Thus, HepG2 cells exposed to capsaicin exhibited transient reduction in the GSH/GSSG ratio, but subsequently restored the GSH levels (see Fig. 2D and E). Induction of such transient, moderate oxidative stress appears to be

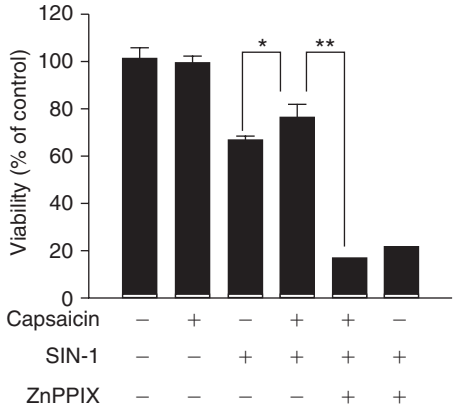

FIG. 8. Effects of capsaicin and/or ZnPP IX on SIN-1–induced cytotoxicity in HepG2 cells. HepG2 cells were pretreated with capsaicin (200 μM) in the presence or absence of 50 μM ZnPP IX for 24 h before exposure to SIN-1 (5 mM), a peroxynitrite donor, for additional 12 h. Cell viability was determined by the MTT reduction assay. The data represent mean ± SD ($n = 3$). Significant differences between the compared groups are indicated ($^*p < 0.05$; $^{**}p < 0.01$).

an important mechanism by which some chemopreventive and chemoprotective phytochemicals induce antioxidant gene expression. Thus, the ability of flavonoids to activate an ARE [or more correctly, electrophile response element (EpRE)]-mediated signaling has been reported to correlate with their redox properties characterized by quantum mechanical calculations (16). According to this study, flavonoids with a higher intrinsic potential to generate oxidative stress and redox cycling are the most potent inducers of EpRE-mediated antioxidant gene expression and that EpRE activation by flavonoids increased with decreasing GSH and *vice versa*, supporting an oxidative mechanism. The methoxy group present in the vanillyl ring moiety of capsaicin can be converted to the hydroxyl group by *O*-demethylase activity in the hepatocytes. The resulting catechol metabolite is anticipated to readily undergo redox cycling with concomitant generation of ROS to a greater extent than the parent compound. We found that the chemically synthesized *O*-demethylated catechol derivative of capsaicin strongly induced HO-1, even at a lower concentration than did capsaicin, whereas masking the *para*-hydroxyl group of capsaicin attenuated its HO-1–inducing activity (E.-J. Joung and Y.-J. Surh, unpublished observations).

Capsaicin has been shown to inhibit the NQO1 activity (28, 34). Although it has not been demonstrated that capsaicin directly interacts with NQO1, it is highly probable that an electrophilic species (Fig. 9) arising from capsaicin through its metabolism is responsible for the covalent modification and subsequent destruction of NQO1 that contains several cysteine residues (18). In any case, inhibition of NQO1 activity in aerobic HepG2 cells that contain relatively abundant cytochrome P450s and P-450 reductase would enhance the rate of ROS production.

In this study, we attempted to explore the possible molecular mechanisms underlying the chemoprotective as well as antioxidant effects of capsaicin, with prime focus on the upregulation of HO-1. Capsaicin upregulated the expression of HO-1 in a concentration- and time-dependent fashion without causing severe toxicity in HepG2 cells. Capsaicin enhanced the nuclear translocation of Nrf2 and its ARE binding and stimulated the transcriptional activity of Nrf2. Moreover, transfection of HepG2 cells with dominant-negative mutant of Nrf2 abolished the upregulation of HO-1 expression induced by capsaicin. These results thus indicate that Nrf2 is one of the essential regulators involved in the capsaicin-induced HO-1 expression, acting through ARE. Under normal unstressed physiologic conditions, Nrf2 is kept inactive in the cytoplasm in association with Kelch-like ECH associating protein 1 (Keap1), which contains six cysteine residues (8). On exposure of cells to chemicals that cause oxidative stress, cysteines in Keap1 are oxidized, and Nrf2 is released. Nrf2 dissociated from Keap1 then translocates to the nucleus, binds to AREs, and transactivates the genes encoding the phase II detoxifying and antioxidant enzymes, including HO-1.

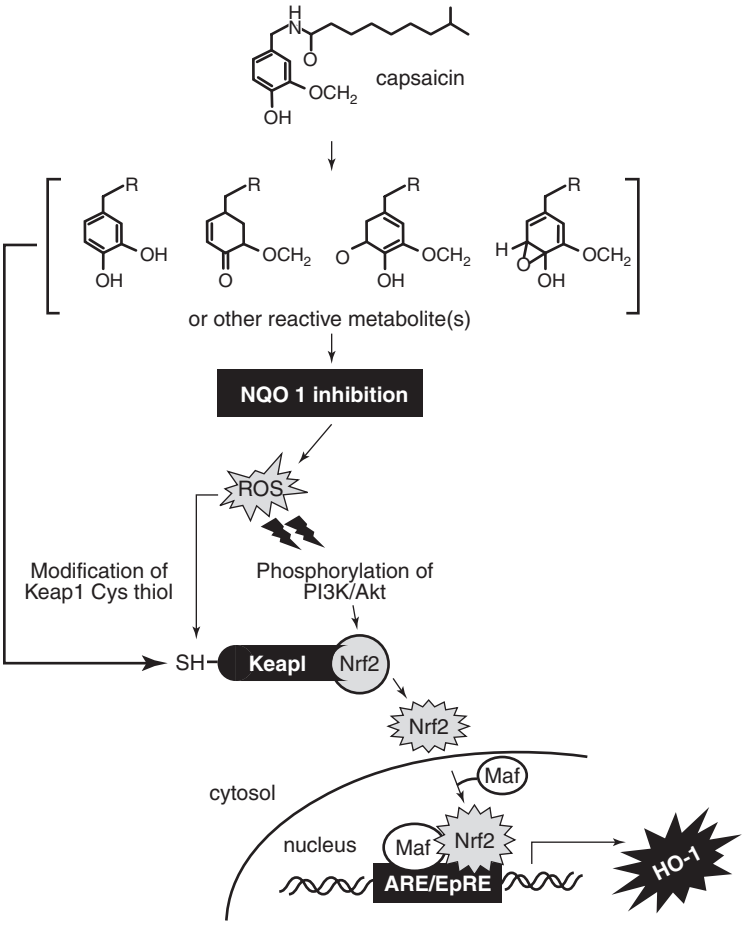

FIG. 9. A proposed pathway for capsaicin-induced Nrf2 activation and HO-1 expression. Capsaicin undergoes metabolism to produce reactive species that can interact with NQO1, thereby inactivating its enzyme activity. The resultant intracellular ROS accumulation can either stimulate PI3K-Akt signaling or oxidize the cysteine thiol of Keap1. Metabolically activated capsaicin can also modify Keap1 cysteine. All these events facilitate the dissociation of Nrf2 from Keap1 and its nuclear translocation for binding to ARE/EpRE, leading to induction of HO-1 expression.

Several upstream signaling pathways are known to activate Nrf2. In HepG2 cells, activation of both ERK and p38 MAPK pathways has been reported to mediate the *HO-1* gene activation induced by diallyl sulfide (7). In PC 12 cells, however, the induction of HO-1 expression caused by resveratrol has been reported to be mediated by activation of both ERK and PI3K/Akt pathways, but not via the JNK or p38 MAPK signaling (2). Conversely, arsenite has been shown to upregulate HO-1 expression through activation of Ras and JNK pathways, but not ERK signaling (11). In HepG2 cells exposed to capsaicin, although both ERK and Akt pathways were activated, the use of specific inhibitors of these signal pathways and another, such as LY294002, U0126, or SB203580, confirmed the involvement of Akt in HO-1 induction. This was supported by the results obtained using HepG2 cells transfected with functionally inactive Akt. Thus, it appears that different cells use a distinct set of signal-transduction pathways to induce HO-1 expression, depending on the nature of stimulus and intracellular redox microenvironment.

Capsaicin-induced HO-1 expression may be initiated by production of ROS that was abrogated by NAC, a cell-permeable cysteine that can bind electrophilic metabolites arising from capsaicin. The addition of NAC abolished not only the capsaicin-derived overproduction of ROS but also the nuclear translocation of Nrf2 and its ARE binding, as well as the upregulation of HO-1 expression. NQO1 is a homodimeric flavoprotein that contains cysteine residues and is present in aerobic cells ubiquitously. The enzyme catalyzes a direct two-electron reduction of quinones to hydroquinones. This reaction competes with the one-electron reduction of quinones catalyzed by cytochrome-P450 reductase and other flavoproteins generating semiquinone phenoxyl radicals that undergo redox cycling in aerobic cells to overproduce $O_{2\cdot}^{-}$ (15). Thus, it appears that capsaicin causes oxidative stress by production of ROS as a consequence of inhibition of NQO1 activity and this, in turn, would lead to activation of Nrf2 and upregulation of HO-1 expression via the PI3K/Akt signaling pathways, as schematically represented in Fig. 9. Although the mechanism of capsaicin-derived inhibition of NQO1 was not determined in the present study, the fact that a well-known inhibitor of NQO1, dicumarol, is also capable of inducing HO-1 expression as well as producing ROS and that this can be abrogated by NAC suggests that some connection exists between capsaicin-mediated inhibition of NQO1 and production of ROS that activates the signaling events, leading to upregulation of HO-1 expression. Oxidation or covalent modification of critical cysteine thiols present in Keap1 diminishes the affinity of this suppressor for Nrf2, and hence facilitates the nuclear translocation and subsequent ARE binding of Nrf2. As capsaicin generates ROS by inhibiting NQO1 and some of its electrophilic metabolites are capable of interacting with cysteine thiol, the possibility of Nrf2 activation through direct oxidation/modification of Keap1 cysteine thiol, independent of PI3K-Akt signaling, cannot be excluded (see Fig. 9).

In summary, capsaicin produces ROS, presumably by inhibiting NQO1, and the resulting oxidative stress may then activate the PI3K/Akt signaling pathway and promote the nuclear translocation of Nrf2 and Nrf2-ARE binding. Through this sequence of events initiated by capsaicin, expression of HO-1, a key detoxifying/antioxidant enzyme, is upregulated in HepG2 cells. Most notably, the prooxidant activity of capsaicin can contribute to its ability to induce HO-1. As NAC attenuated the HO-1–inducing activity of capsaicin, use of synthetic antioxidants that can directly scavenge ROS may mitigate the beneficial effects of some phytochemicals, such as capsaicin, whose chemoprotective effects are associated with their prooxidant properties responsible for EpRE-mediated synthesis of antioxidant enzymes. Although the same stress-responsive signaling cascades may be activated by the noxious stimuli, such as peroxynitrite, such response is very transient in general and can be readily overwhelmed by sustained or subsequent insults. Therefore, maintenance and even fortification of cellular defense capacity by the regular intake of natural phytochemicals capable of upregulating the antioxidant and other stress-responsive genes may represent one of the most practical and feasible ways to reduce the risk of a variety of disorders in which oxidative stress is frequently implicated.

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ABBREVIATIONS

ARE, antioxidant response element; CO, carbon monoxide; CYP, cytochrome P450; CYP2E1, cytochrome P450 2E1; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated protein kinase; GSH, glutathione; HepG2, human hepatoma cells; HO-1, heme oxygenase-1; Keap1, Kelch-like ECH-associating protein 1; MAPK, mitogen-activated protein kinase; MnTBAP, MN (II) tetrakis (4-benzoic acid) prophyrin; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; NAC, *N*-acetyl-L-cysteine; NO, nitric oxide; NQO1, NAD(P)H:quinone oxidoreductase; Nrf2, NF-E2–related factor 2; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol-3 kinase; ROS, reactive oxygen species; StRE, stress-response elements; SDS, sodium dodecylsulfate.

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