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ORIGINAL ARTICLE

Ajoene, a Major Organosulfide Found in Crushed Garlic, Induces NAD(P)H:quinone Oxidoreductase Expression Through Nuclear Factor E2-related Factor-2 Activation in Human Breast Epithelial Cells

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Background: NAD(P)H:quinone oxidoreductase-1 (NQO1) is a widely-distributed flavin adenine dinucleotide-dependent flavoprotein that promotes obligatory 2-electron reductions of quinones, quinoneimines, nitroaromatics, and azo dyes. This reduces quinone levels and thereby minimizes generation of excess reactive oxygen species (ROS) formed by redox cycling, and concurrent depletion of intracellular thiol pools. Ajoene is derived from crushed garlic. It is formed by a reaction involving two allicin molecules, and is composed of allyl sulfide and vinyl disulfide. Ajoene is present in two isomers, E- and Z-form.
Methods: Expression of antioxidant enzymes and nuclear factor E2-related factor-2 (Nrf2) was measured by Western blot analysis. NQO1 promoter activity was assessed by the luciferase reporter gene assay. ROS accumulation was monitored by using the fluorescence-generating probe 2′,7′-dichlorofluorescein diacetate. The intracellular glutathione levels were measured by using a commercially available kit.
Results: Z-ajoene significantly up-regulated the expression of representative antioxidant enzyme NQO1 in non-tumorigenic breast epithelial MCF-10A cells at non-toxic concentrations. Z-ajoene enhanced up-regulation and nuclear translocation of Nrf2, which plays a pivotal role in the induction of many genes encoding antioxidant enzymes and other cytoprotective proteins. Z-ajoene treatment also increased the activity of *nqo1*-promoter harboring antioxidant response element consensus sequences in MCF-10A cells. Silencing of Nrf2 by small interfering RNA abrogated ajoene-induced expression of NQO1. Z-ajoene activated extracellular signal-regulated kinase (ERK). Inhibition of ERK activation by U0126 abrogated ability of Z-ajoene to activate Nrf2 and to induce NQO1 expression. Intracellular ROS accumulation was observed after treatment with Z-ajoene, whereas the E-isoform was not effective. The inhibition of ROS by treatment with N-acetylcysteine, a radical scavenger, abrogated Z-ajoene-induced expression of NQO1 as well as activation of ERK and Nrf2, suggesting that Z-ajoene augments the Nrf2-dependent antioxidant defense via ROS generation and ERK activation.
Conclusions: Z-ajoene induces NQO1 expression in MCF-10A cells through ROS-mediated activation of Nrf2.
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Key Words: Ajoene, Garlic, Nuclear factor E2-related factor 2, NAD(P)H:quinone oxidoreductase 1, Extracellular signal-regulated kinase, Reactive oxygen species

INTRODUCTION

Reactive oxygen species (ROS) are recognized to play a key role in cell signaling. At the cellular level, oxidant injury elicits a broad spectrum of responses ranging from proliferation to growth arrest, to senescence, and to cell death [1]. Activation of mitogen-activated protein kinases (MAPK) is considered to be a pivotal step in ROS-induced signaling pathways. It has been shown that increased ROS production in leukemic cells leads to the activation of MAPK and cell death [2-5]. The MAPK pathways consist of three parallel kinase modules, that is, extracellular signal-regulating kinase (ERK1/2), c-Jun-N-terminal kinase (JNK),

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and p38 MAPK pathways. In general, JNK and p38 MAPK activation is associated with apoptosis induction, whereas ERK activation is cytoprotective [6].

Cells are equipped with a battery of cytoprotective enzymes, which are expressed in response to oxidative stress to protect against ROS-induced damage of cellular macromolecules, such as DNA, RNA, and proteins. These cytoprotective enzymes include NAD(P)H:quinine oxidoreductase-1 (NQO1), heme oxygenase-1 (HO-1), superoxide dismutase, glutathione (GSH)-*S*-transferase, GSH peroxidase, glutamate cysteine ligase, uridine diphosphate glucuronosyltransferase, etc. [7,8]. Among them, NQO1 plays an important role in protection against endogenous and exogenous quinines, thereby protecting the cell from undesired oxidative damage.

The proximal promoter regions of genes encoding many antioxidant and cytoprotective enzymes including NQO1 harbor a consensus sequence known as antioxidant response element (ARE) or electrophile response element (EpRE), which is a preferred target of the redox-sensitive transcription factor, nuclear factor E2-related factor-2 (Nrf2) [8]. Nrf2 is normally sequestered in the cytoplasm as an inactive complex with its cytosolic repressor, named Kelch-like ECH associated protein 1 (Keap1). As an adaptive response to mild oxidative or electrophilic insults, Nrf2 is dissociated from the inhibitory protein Keap1 and translocates to nucleus where it binds to cis-acting ARE or EpRE, leading to the transcriptional activation of antioxidant and cytoprotective genes [8,9].

Ajoene is most stable and abundant in macerate of garlic. It is present in two isomers, E- and Z-4,5,9-trithiadodeca-1,6,11-triene 9-oxide. Ajoene, an unsaturated disulfide, is formed from a chemical reaction involving two allicin molecules. It functions as an antioxidant by inhibiting the release of superoxide [10]. It also has antithrombotic (anti-clotting) properties, which helps prevent platelets from forming blood clots [11,12], potentially reducing the risk of heart disease and stroke in humans. Ajoene is also known to have effective broad-spectrum of antimicrobial (antibacterial and antifungal) properties [13,14]. Ajoene and several other organosulfur compounds from garlic have also been reported to inhibit the proliferation and to induce apoptosis of human mammary, bladder, and skin tumor cell lines [15-18]. Ajoene suppresses tumor cell growth by targeting the microtubule cytoskeleton of such cells [19].

The present study explored the antioxidant effects of ajoene and underlying mechanisms, with focus on its ability to activate Nrf2 and induce NQO1.

MATERIALS AND METHODS

1. Chemicals and biological agents

Crushed garlic bulb was incubated at the room temperature for 12 hours, and at 37°C for additional 2 hours, and then extracted with 80% ethanol. E- and Z-ajoene were purified by the repeated chromatography and their structures were identified by spectroscopic analysis. Dulbecco's modified Eagle's medium (DMEM) and FBS were obtained from Gibco BRL (Grand Island, NY, USA). Dithiothreitol (DTT), N-acetyl-L-cysteine (NAC), and anti-actin antibody were procured from Sigma Chemical Co. (St. Louis, MO, USA). Primary antibodies for Nrf2 and NQO1 were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA, USA). The ECL chemiluninescent detection kit was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Bicinchoninic acid protein assay reagent was a product of Pierce Biotechnology (Rockfold, IL, USA). Mouse monoclonal lamin B_1 antibody, human specific Nrf2-siRNA (sense 5′-AAGAGUAUGAG CUGGAAAAACTT-3′; antisense 5′-GUUUUUCCAGCUCAUACUCU UTT-3′), and Stealth™ universal RNAi negative control duplexes, and 2′,7′-dichlorofluorescein diacetate (DCF-DA) were purchased from Invitrogen (Carlsbad, CA, USA). Polyvinylidene difluoride (PVDF) membrane was supplied from Gelman Laboratory (Ann Arbor, MI, USA). The inhibitor of extracellular signal-regulated kinase (ERK; U0126) was purchased from Tocris (Ellisville, MO, USA).

2. Cells and culture

MCF-10A cells were purchased from the American Type Culture Collection. The cells were suspended in DMEM/F12 medium supplemented with 10 μg/mL insulin (bovine), 100 ng/mL choleratoxin, 0.5 μg/mL hydrocortisone, 20 ng/mL recombinant human epidermal growth factor, 0.5 μg/mL fungi zone, 2 mmol/L L-glutamine, 100 μg/mL penicillin/streptomycin/fungi zone mixture, and 5% heat-inactivated horse serum and maintained at 37°C in 5% CO_2. For isolation of embryonic fibroblasts, *nrf2*-null mice, in which the *nrf2* gene is disrupted by targeted gene knockout, were provided by Dr. Jeffery Johnson, University of Wisconsin, Madison, WI, USA. The $nrf2^{-/-}$, $nrf2^{+/-}$, and wild-type mice were maintained in the animal quarters in accordance with the university guidelines for animal care and were housed in a 12-hour light/dark cycle. They were fed standard rodent chow and given water ad libitum. Male and female $nrf2^{+/-}$ mice were paired and the pregnancies were monitored. Embryos

were obtained at the day 13.5 after pairing under aseptic conditions. The heads of the embryos were used to confirm the *nrf2* genotype by polymerase chain reaction, and the embryo bodies were minced into small pieces and cultured in high glucose DMEM supplemented with 10% FBS and kept at 37°C with 5% CO_2.

3. Preparation of cytosolic and nuclear extracts

MCF-10A cells were washed with cold PBS twice. The cells were pelleted by centrifugation and suspended in ice-cold isotonic buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.2 mM phenylmethylsulfonyl fluoride [PMSF]). After following incubation in an ice bath for 15 min, cells were centrifuged again and the supernatant was collected as a cytosolic fraction. The remaining cell pellets were resuspended in ice-cold buffer C (20 mM HEPES [pH 7.9], 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF) and were incubated in an ice bath for 2 hours. After vortex mixing, the resulting suspension was centrifuged, and the supernatant was collected as a nuclear extract and stored at −70°C.

4. Western blot analysis

Cell pellets were lysed in lysis buffer (0.5% Triton X-100, 20 mM Tris-HCl [pH 7.5], 2 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 50 mM β-glycerophosphate, 25 mM NaF, 1 mM Na_3VO_4, 2 μg/mL leupeptin, 2 μg/mL pepstatin A, 100 μg/mL PMSF, and 1 μg/mL antipain) for 1 hour at 4°C. Lysates were subjected to SDS-PAGE and transferred to PVDF membranes (Millipore Co., Bedford, MA, USA). After blocking at room temperature for 1 hour in TBS containing 5% skim milk and 0.1% Tween-20, the membranes were incubated with the following antibodies (diluted 1 : 1,000): rabbit anti-Nrf2, mouse anti-α-tubulin, goat anti-NQO1, mouse anti-p-ERK, rabbit anti-ERK, mouse anti-p-JNK, rabbit anti-p-p38 (Santa Cruz Biochemicals; Santa Cruz Biotechnology), rabbit anti-p-PKCδ (Cell Signaling, Danvers, MA, USA), rabbit anti-actin (Sigma Chemical Co.), or mouse anti-Lamin B (Invitrogen). Following three washes with TBS containing 0.1% Tween-20 (TBST), the blots were incubated with horseradish peroxidase-conjugated secondary antibody in 5% skim milk-TBST for 1 hour at room temperature. The blots were rinsed again three times with TBST, and the transferred proteins were incubated with ECL substrate solution for 1 minute according to the manufacturer's instruction and visualized with LAS 4000 (Fuji Film, Tokyo, Japan).

5. Transient transfection and the luciferase reporter assay

MCF-10A cells were seeded in 90-mm dishes and grown to 70% confluence in the complete growth medium. The cells were transfected with 6 μg of Nrf2-siRNA or negative control siRNA for 24 hours using WelFect-M GOLD transfection reagent (WelGENE, Gyeongsan, Korea). The transfected cells were treated with ajoene for additional 20 hours, followed by Western blot analysis. MCF-10A cells were seeded at a density of 2×10^5 per well in a six-well dish and grown to 60% confluence in the complete growth medium. The cells in each well were cotransfected with 2 μg of luciferase reporter plasmid construct harboring the ARE binding site and 0.5 μg of control vector pCMV-β-galactosidase using WelFect-M GOLD transfection reagent (WelGENE), and the cotransfection was carried out according to the instructions supplied by the manufacturer. After an 18 hours transfection, the medium was changed and the cells were further treated with Z-ajoene for 20 hours. The cells were then washed with PBS and lysed in 1× reporter lysis buffer (Promega Corporation, Madison, WI, USA). The lysed cell extract (20 μL) was mixed with 100 μL of the luciferase assay reagent, and the luciferase activity was determined using a luminometer (AutoLumat LB 953; EG&G Berthold, Bad Wildbad, Germany). The β-galactosidase activity was measured to normalize the luciferase activity.

6. Measurement of intracellular reactive oxygen species accumulation

Accumulation of ROS in MCF-10A cells treated with ajoene was monitored using the fluorescence-generating probe DCF-DA. Treated cells were rinsed with PBS and loaded with 10 μM DCF-DA for 30 minutes at 37°C to assess ROS-mediated oxidation of DCF-DA to the fluorescent compound DCF. Cells were washed once with Hanks' balanced salt solution (Gibco BRL) and filled with media. Cells were examined using a micromanipulator system (Leica Microsystems, Wetzlar, Germany) or FACSCalibur flow cytometer and Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

7. Glutathione assay

MCF-10A cells were plated in 90-mm dishes, allowed to grow for 24 hours then treated with 20 μM Z-ajoene for 24 hours. The pellets were suspended with cold 5% metaphosphoric acid and sonicated, and incubated on ice for 5 minutes. The suspension was transferred to a new tube and centrifuged for 5 minutes at 4°C. The oxidized GSH concentration was determined using GSH

detection kit (Enzo Life Sciences, Plymouth, PA, USA). The assay was carried out according to the instructions supplied by the manufacturer. The levels of reduced GSH and its oxidized form (GSH disulfide, GSSG) were determined according to the manufacturer's instructions. The rates of the reaction were compared to similarly prepared GSH and GSSG standard curves.

8. Statistical anaylsis

When necessary, data were expressed as means ± SD of at least three independent experiments, and statistical analysis for single comparison was performed using the Student's *t*-test. Significant differences are reported for $P < 0.05$, $P < 0.01$, and $P < 0.001$. All reported averages were calculated as arithmetic means.

RESULTS

1. Comparison of effects of E- vs. Z-ajoene on the expression of NAD(P)H:quinone oxidoreductase-1 in MCF-10A cells

The induction of antioxidant enzymes represents a primary cellular defensive mechanism against oxidative insult. NQO1, a representative cytoprotective enzyme, is known to play a key role in cytoprotection against oxidative damage [20]. According to International Union of Pure and Applied Chemistry, the ajoene molecule is named as 4,5,9-trithiadodec-1,6,11-thiene-9 oxide. The molecular structures of ajoene are shown in Figure 1A. We examined the effects of two isomeric forms of ajoene on the expression of NQO1 in human breast epithelial MCF-10A cells. Incubation of MCF-10A cells with Z-ajoene (20 μM) resulted in a time-dependent induction of NQO1 protein expression. In contrast, treatment of cells with E-ajoene (20 μM) poorly induced the expression of NQO1 (Fig. 1B). The expression of NQO1 was detected at 3 hours after treatment with Z-ajoene, which was

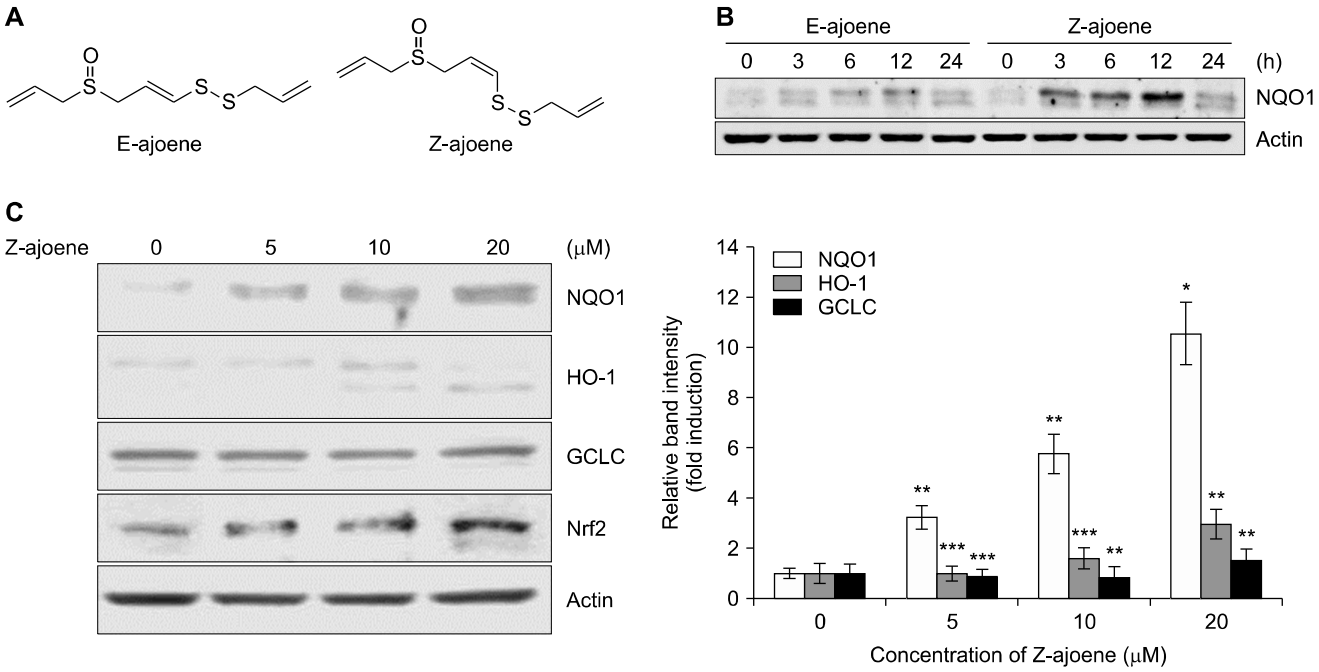

Figure 1. The molecular structures and the effects of ajoene on the antioxidant protein expression. (A) Naturally occurring ajoene isomers in garlic extracts. (B) MCF-10A cells were incubated with E- or Z-ajoene (20 μM each) for indicated periods. Whole cell lysates (40 μg protein) were separated by 12% SDS-PAGE and immunoblotted with antibodies of NAD(P)H:quinone oxidoreductase-1 (NQO1) and actin. Actin was measured to ensure equal protein loading. (C) MCF-10A cells were incubated with Z-ajoene at indicated concentrations for 20 hours. Whole cell lysates were performed SDS-PAGE and immunoblotted with antibodies of NQO1, heme oxygenase-1 (HO-1), glutamate—cysteine ligase catalytic subunit (GCLC), and nuclear factor E2-related factor-2 (Nrf2). Actin was used as the loading control. The band intensity of immunoblots for NQO1, HO-1, and GCLC was normalized to that of actin by GelPro image densitometry followed by statistical analysis. Bar graphs show relative numerical values of the band intensity of NQO1 (white columns), HO-1 (gray columns), or GCLC (black columns). Error bars represent means ± SD of three independent experiments. Asterisks indicate the statistically significant difference as compared with control (*$P <$ 0.05; **$P <$ 0.01; ***$P <$ 0.001).

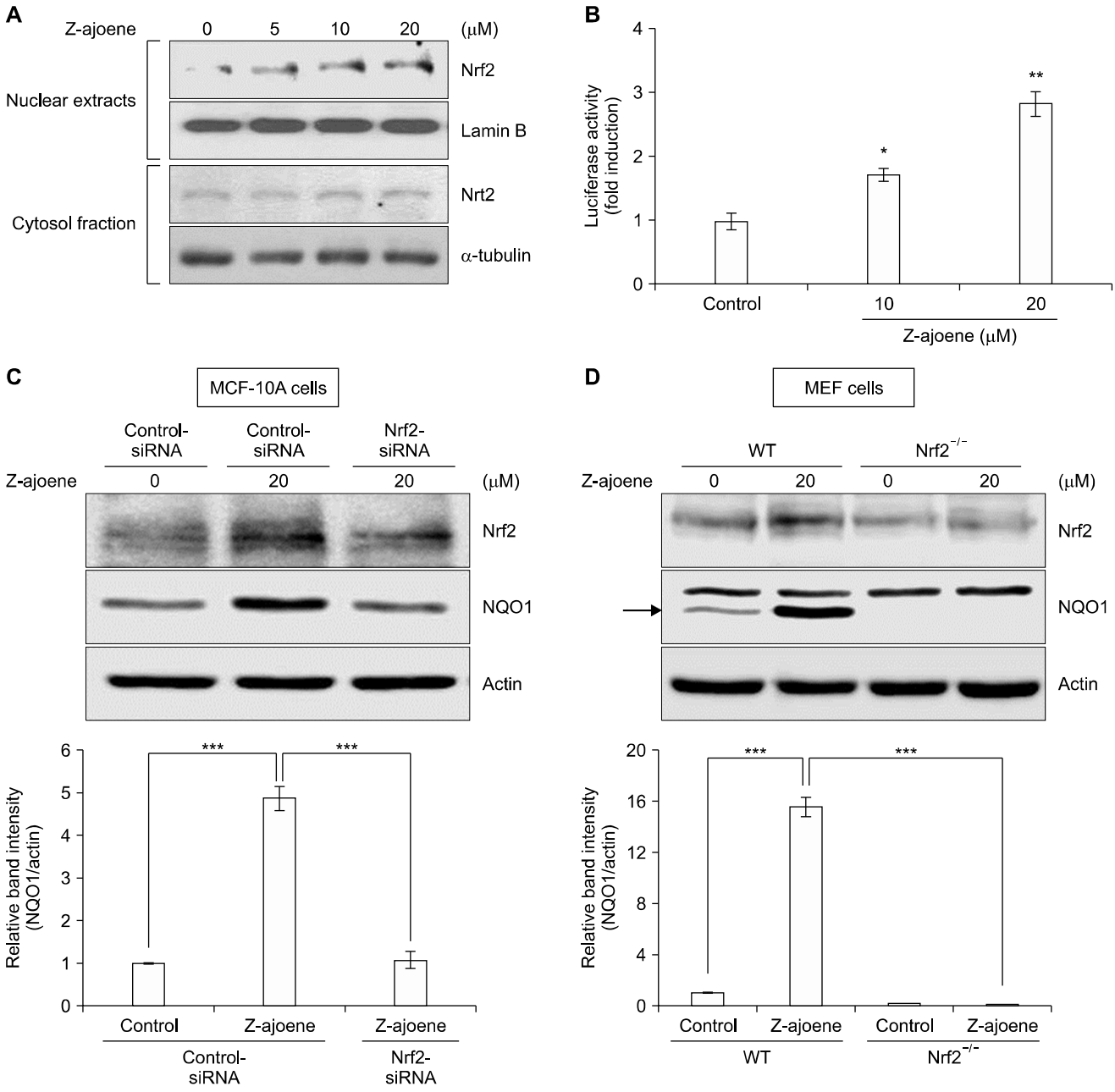

Figure 2. Nuclear translocation and transcriptional activity of nuclear factor E2-related factor-2 (Nrf2) and its implication in NAD(P)H:quinone oxidoreductase-1 (NQO1) upregulation induced by Z-ajoene. (A) Nuclear extracts and cytosol fraction from cells treated with Z-ajoene at indicated concentrations for 20 hours were subjected to Western blot (WB) analysis. Data are representative of three independent experiments showing a similar trend. (B) MCF-10A cells transiently transfected with *nqo1*-luciferase construct harboring antioxidant response element (ARE) sequences or mock vector were incubated with Z-ajoene (10 or 20 μM) for 20 hours as described in Materials and Methods. Transcriptional activity of *nqo1*-specific ARE was measured by the luciferase reporter gene assay with a luminometer. Error bars represent means ± SD of three independent experiments. Asterisks indicate the statistically significant difference as compared with control (*$P < 0.05$; **$P < 0.01$). (C) MCF-10A cells transiently transfected with Nrf2-siRNA or control siRNA were incubated with or without 20 μM Z-ajoene for 20 hours. Cell lysates were separated by SDS-PAGE (for Nrf2, 8%; for NQO1, 12%) and immunoblotted with NQO1 and Nrf2 antibodies. Actin was served as a loading control. ***Significantly different from the untreated control ($P < 0.001$). (D) MEF cells (wild type and Nrf2$^{-/-}$) were treated with or without 20 μM Z-ajoene for 20 hours. Cell lysates were conducted immunoblotting. The band intensity of immunoblots for NQO1 was normalized to that of actin by GelPro image densitometry followed by statistical analysis. Error bars represent means ± SD of three independent experiments. ***Significantly different from the untreated control ($P < 0.001$).

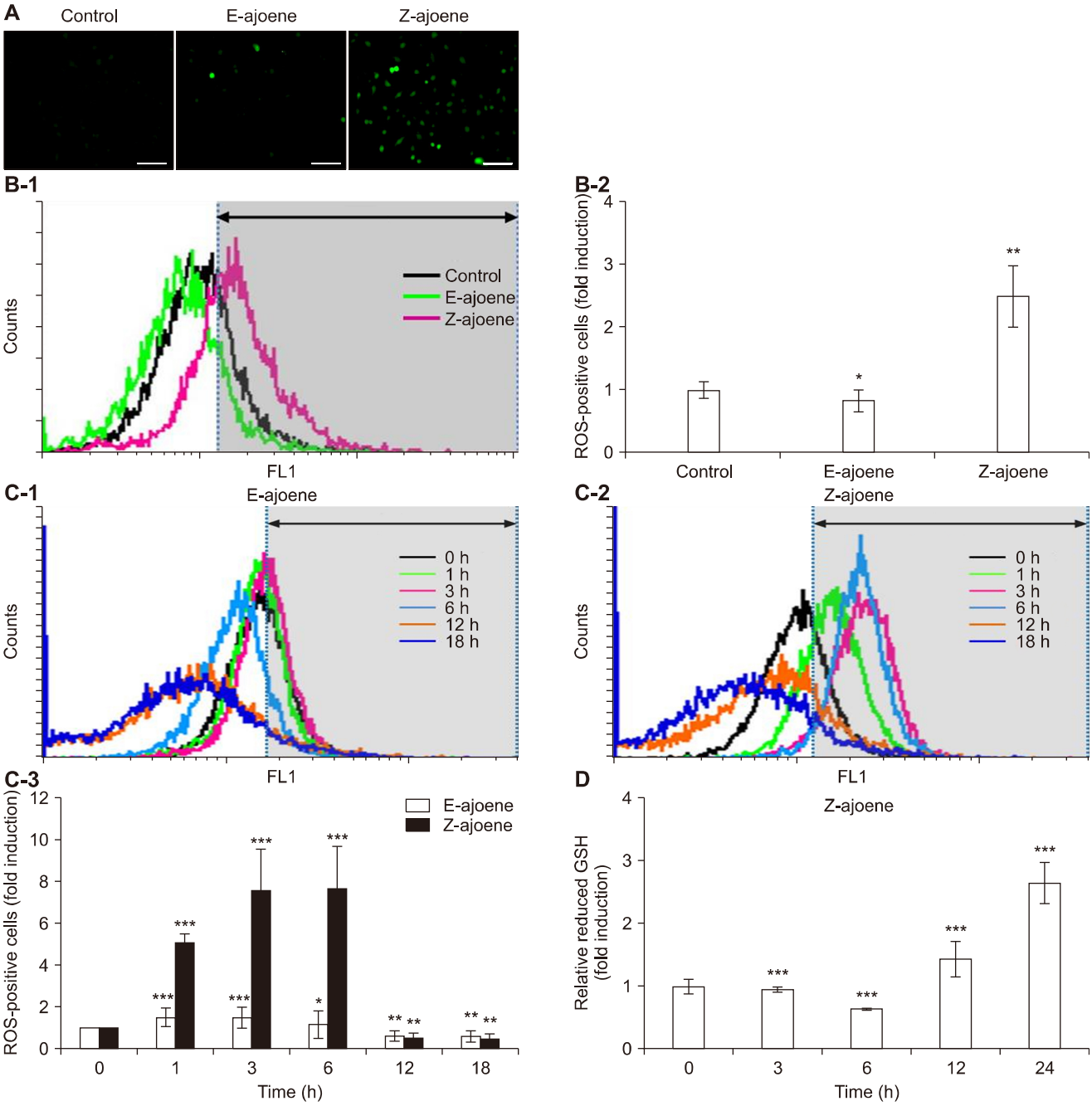

Figure 3. The effects of ajoene on the reactive oxygen species (ROS) generation and intracellular glutathione (GSH) accumulation. ROS generation by Z-ajoene was compared with that by E-ajoene. (A, B) MCF-10A cells were incubated with E- or Z-ajoene (20 μM each) for 6 hours and the generation of ROS was measured by the 2',7'-dichlorofluorescein diacetate (DCF-DA) staining followed by a fluorescence microscope (A) or flow cytometry (B-1) as described in Materials and Methods. Scale bar: 200 μm. (B-2) Bar graphs show relative numerical values of ROS-positive cells (mean values). Error bars represent means ± SD of three independent experiments. Asterisks indicate the statistically significant difference as compared with control (*$P < 0.05$; **$P < 0.01$). (C) MCF-10A cells were incubated with 20 μM each of E- (C-1) or Z-ajoene (C-2) for indicated periods and the generation of ROS was measured by the DCF-DA staining followed flow cytometry. (C-3) Bar graphs show relative numerical values of ROS generation by E-ajoene (white columns) or Z-ajoene (black columns). Error bars represent means ± SD of three independent experiments. Asterisks indicate the statistically significant difference as compared with control (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). (D) Changes in total intracellular GSH levels in response to Z-ajoene treatment in MCF-10A cells. The non-toxic concentration (20 μM) of Z-ajoene was treated to culture medium for indicated time periods, and total cellular GSH content was measured as described in Materials and Methods. Results are presented as the fold induction of intracellular GSH levels in Z-ajoene-treated cells compared with that in the control cells. Error bars represent means ± SD of three independent experiments. ***Significantly different from the untreated control ($P < 0.001$).

increased gradually to 12 hours. The Z-ajoene-induced NQO1 expression was concentration-dependent whilst expression of HO-1 and GCRC was much less prominent (Fig. 1C). Under the same experimental conditions, Z-ajoene induced Nrf2, a key transcription factor responsible for ARE-driven a majority of antioxidant enzymes (Fig. 1C).

2. Z-ajoene induces the expression of NAD(P)H: quinone oxidoreductase-1 through the activation of nuclear factor E2-related factor-2 in MCF-10A cells

To further determine whether the induction of NQO1 by Z-ajoene in MCF-10A cells was mediated through the activation of Nrf2, we examined the effect of Z-ajoene on the nuclear translocation and transcriptional activity of Nrf2. Nuclear extracts from MCF-10A cells incubated with or without Z-ajoene were subjected to Western blot analysis. Z-ajoene increased the nuclear accumulation of Nrf2 (Fig. 2A). We also determined the effect of Z-ajoene on the NQO1 promoter activity. Treatment of cells harboring the *nqo1*-promoter construct containing ARE sequences with Z-ajoene (10 or 20 μM) significantly induced NQO1 promoter activity as determined by the luciferase reporter gene assay (Fig. 2B). By exploiting small interfering RNAs (Nrf2 siRNA) and embryonic fibroblast cells from $Nrf2^{-/-}$ mice, we attempted to verify the role of Nrf2 in the induction of NQO1 expression in Z-ajoene-stimulated cells. Silencing Nrf2 by siRNA abrogated Z-ajoene-induced expression of NQO1 in MCF-10A cells (Fig. 2C). In addition, knockout of Nrf2 abolished the Z-ajoene ability to induce NQO1 protein expression (Fig. 2D). These results indicate that Z-ajoene induces the expression of NQO1 through the activation of Nrf2.

3. Z-ajoene-induced expression of NAD(P)H:quinone oxidoreductase-1 in MCF-10A cells is associated with the generation of reactive oxygen species

It has been demonstrated that the generation of ROS is involved in the activation of Nrf2 and its target genes [21]. To assess whether the induction of NQO1 by Z-ajoene mediated by ROS generation, we examined the effect of ajoene on intracellular ROS generation in MCF-10A cells using a fluorescence microscope and FACSCalibur. Treatment of MCF-10A cells with Z-ajoene (20 μM) increased the accumulation of intracellular ROS while E-ajoene did not (Fig. 3A and 3B). Furthermore, the generation of ROS was detected at 1 hour after treatment of Z-ajoene, which was sustained up to 6 hours (Fig. 3C). The intracellular GSH level is an indicator of oxidative stress [22,23]. As shown in Figure 3D, the GSH (reduced form) level in Z-ajoene-treated MCF-10A cells was decreased for 6 hours, but gradually restored thereafter. These changes of GSH levels are correlated with the generation of ROS by Z-ajoene in MCF-10A cells. Pre-treatment of cells with the antioxidant NAC (5 mM) diminished not only accumulation of ROS (Fig. 4A), but also expression of Nrf2 and NQO1 by Z-ajoene (Fig. 4B), suggesting that Z-ajoene-induced NQO1 expression is likely to be mediated by ROS. In addition, treatment of MCF-10A cells with Z-ajoene in the presence of a thiol reducing agent DTT (500 μM) also attenuated Z-ajoene-induced ROS generation (Fig. 4C) and expression of Nrf2 and NQO1 (Fig. 4D).

4. Reactive oxygen species-induced activation of mitogen-activated protein kinases/extracellular signal-regulated kinase mediates nuclear factor E2-related factor-2 activation and NAD(P)H: quinone oxidoreductase-1 expression in MCF-10A cells treated with Z-ajoene

To understand more in depth the molecular mechanism underlying Nrf2 activation by Z-ajoene, we examined upstream kinases in association with Nrf2 activation. Among the kinases that can phosphorylate Nrf2 (i.e., ERK, PKC δ, JNK, and p38), Z-ajoene treatment resulted in transient enhancement of ERK phosphorylation (Fig. 5A). Phosphorylation of PKCδ, JNK, and p38 was unaffected by Z-ajoene, suggesting that its effect on the ERK activation is relatively specific. Treatment with U0126 compound, a specific MEK/ERK inhibitor, abolished the translocation of Nrf2 into the nucleus and NQO1 expression elicited by Z-ajoene (Fig. 5B and 5C), indicating that ERK activation by Z-ajoene contributes to Nrf2 nuclear translocation and NQO1 expression. In addition, treatment of MCF-10A cells with NAC abrogated ERK phosphorylation and NQO1 expression (Fig. 5D). These results suggest that Nrf2 is activated and translocated into the nucleus via ERK activation elicited by Z-ajoene-induced ROS, resulting in NQO1 protein expression.

DISCUSSION

The biological activity of garlic is attributed to a group of water insoluble organosulfur compounds responsible for its characteristic odor. These compounds are not present in whole garlic, but are degra-dation products of allicin, the major compound produced when the clove is first crushed. One of the main degradation products of allicin is ajoene that exists in both E- and Z-isomeric forms. So far, most studies have been performed with the mixture of two isomers of ajoene. In vitro, Z-ajoene clearly

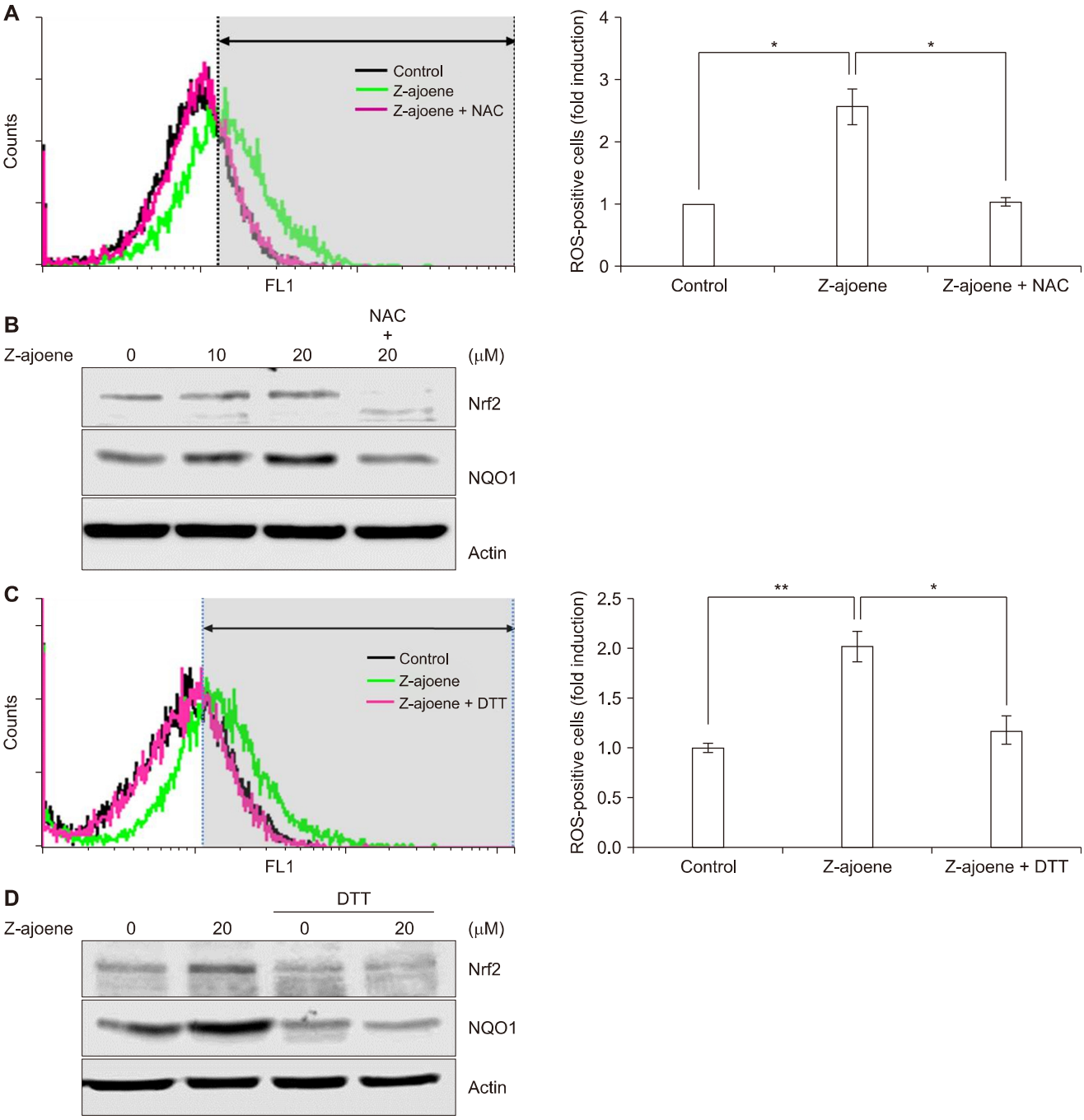

Figure 4. The effects of reactive oxygen species (ROS) by Z-ajoene on the expression of NAD(P)H:quinone oxidoreductase-1 (NQO1) and nuclear factor E2-related factor-2 (Nrf2). (A, B) MCF-10A cells were incubated with N-acetyl-L-cysteine (NAC) (5 mM) for 1 hour prior to the treatment with Z-ajoene at indicated concentrations for 6 hours (for measuring ROS) or 20 hours (for Western blot analysis). (C, D) Possible role of thiol modification in Z-ajoene-induced expression of NQO1. MCF-10A cells were incubated with a thiol reducing agent dithiothreitol (DTT) (0.5 mM) for 1 hour prior to treatment with Z-ajoene (20 μM) for 6 hours (for measuring ROS) or 20 hours (for Western blot analysis). (A, C) The ROS generation was measured by 2',7'-dichlorofluorescein diacetate assay followed by flow cytometry. Bar graphs show relative numerical values of ROS-positive cells (mean values). Error bars represent means ± SD of three independent experiments. Asterisks indicate the statistically significant difference as compared with control (*$P < 0.05$; **$P < 0.01$). (B, D) Cell lysates were subjected to Western blot analysis to examine the expression of NQO1 and Nrf2. Actin was used as a loading control.

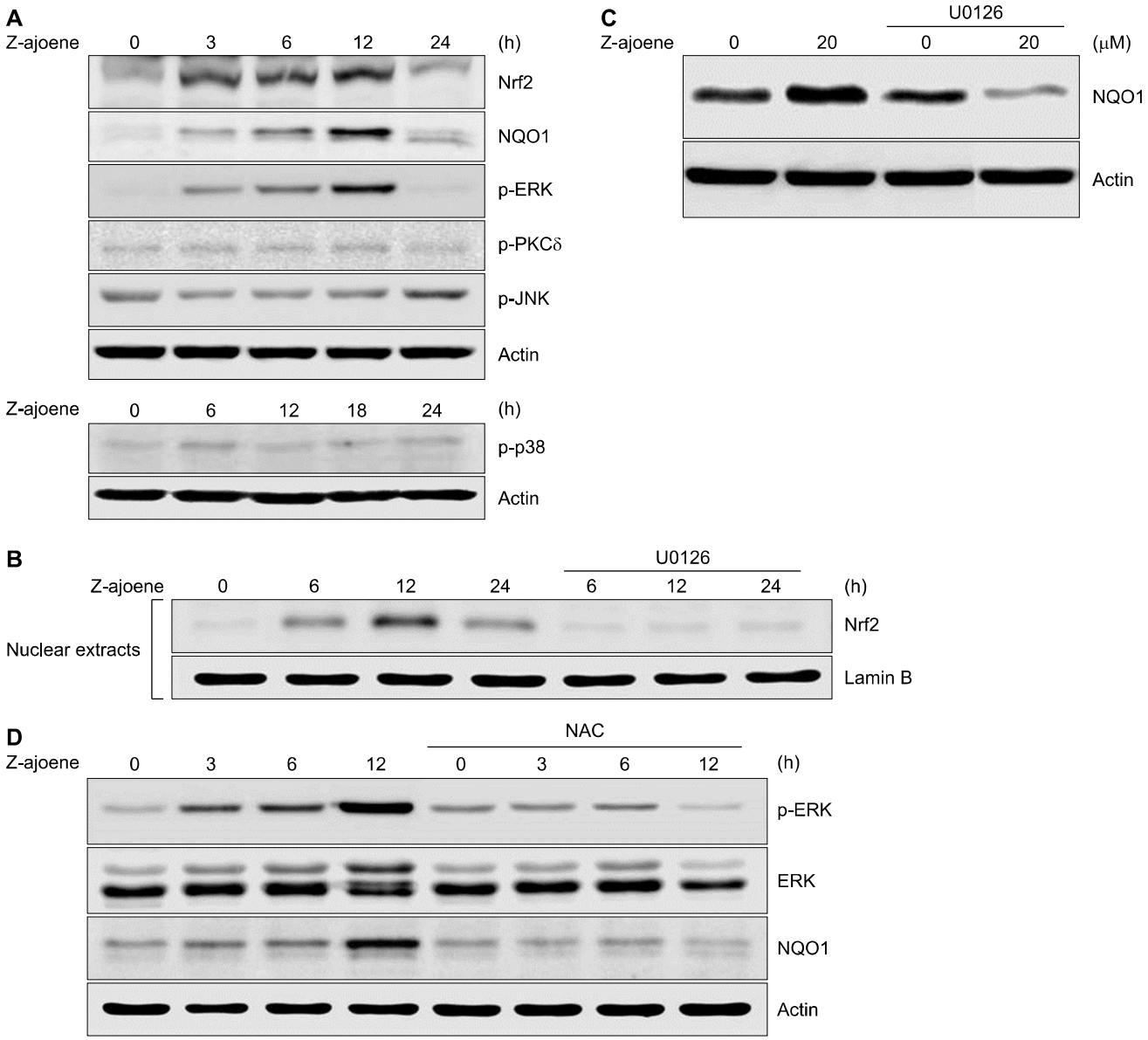

Figure 5. Involvement of extracellular signal-regulated kinase (ERK) in Z-ajoene-induced NAD(P)H:quinone oxidoreductase-1 (NQO1) expression in MCF-10A cells. (A) The effects of Z-ajoene on the phosphorylation of upstream kinases. Western blot analysis was performed to examine the expression of nuclear factor E2-related factor-2 (Nrf2) and NQO1 and the phosphorylation of ERK, PKCδ, JNK, and p38 in cells treated with 20 μM Z-ajoene. (B) Nuclear extracts from cells treated with U0126 (20 μM) for 1 hour prior to the treatment with 20 μM Z-ajoene were subjected to Western blot analysis of Nrf2. Lamin B was used as a nuclear protein marker. (C) MCF-10A cells were treated with U0126 for 1 h prior to the treatment with 20 μM Z-ajoene for 20 hours. Cell lysates were subjected to Western blot analysis to examine the expression of NQO1. (D) MCF-10A cells were incubated with N-acetyl-L-cysteine (NAC) (5 mM) for 1 hour prior to the treatment with 20 μM Z-ajoene for indicated periods. Cell lysates were subjected to Western blot analysis to examine the phosphorylation of ERK. Actin was used as a loading control.

showed a cell growth inhibition on several human cancer cell lines at non-toxic concentrations [24]. Ajoene has been shown to inhibit cell proliferation in a number of cancer cell lines including mammary, bladder, colorectal, hepatic, nasopharyngeal, gastric, prostrate, lung, pancreatic, lymphoma, leukaemia, and skin [24-26]. The Z-isomer is approximately 1.5 fold more active than the E-isomer in inhibiting the growth of cultured cancer cells [27].

Although the anticancer effects of ajoene have been studied in various experimental models, the antioxidant effects and the molecular mechanisms remain elusive. We compared the antioxidant effects of both isomers of ajoene in terms of their capability to induce expression of the representative antioxidant

enzyme, NQO1, and found that Z-ajoene effectively induced NQO1 upregulation. The transcriptional activation of genes encoding NQO1 is predominantly regulated by a redox-sensitive transcription factor, Nrf2. Under a condition of intracellular redox balance, Nrf2 remains tightly bound to its cytosolic repressor Keap1. In response to oxidative stress, Nrf2 is activated through oxidative or covalent modification of Keap1 and subsequently translocated to nucleus. The activation of Nrf2 can also be mediated via phosphorylation by a series of ROS-activated upstream kinases including ERK, PKC, JNK, phosphatidylionositol-3-kinase, protein kinase B/Akt, etc. Upon its nuclear translocation, Nrf2 binds to the cis-acting ARE/EpRE sequences located on the promoter regions of cytoprotective genes including that of NQO1 [9]. In the present study, we found the involvement of ERK in the enhanced nuclear translocation of Nrf2 and NQO1 upregulation by Z-ajoene in MCF-10A cells.

It is interesting to note that many chemoprotective phytochemicals possess pro-oxidant properties, which create a state of mild oxidative stress sufficient to induce stress-activated cytoprotective gene products as an adaptive response. Since the activation of Nrf2 requires transient disruption of cellular redox status shifted toward an oxidative stress, we have examined the effects of ajoene on the generation of ROS and GSH. GSH is the most abundant non-protein thiol in mammalian cells and has many critical functions, including defense against oxidative stress as a scavenger of ROS and electrophiles. Thus, GSH plays a role in maintaining cellular redox homeostasis by scavenging lipid peroxides, and detoxifying electrophilic intermediates of toxic xenobiotics [28]. Our study has revealed a significant decrease in the intracellular GSH level with a concomitant increase in an intracellular ROS level following Z-ajoene treatment. These findings were speculated that increasing ROS by Z-ajoene was related with the interaction between Z-ajoene and GSH reductase (GR). The attenuation of Z-ajoene-induced activation of ERK and Nrf2 and NQO1 expression upon pretreatment of cells with NAC suggests that Z-ajoene-induced ROS production plays a key role in turning on the ERK-Nrf2 signaling, thereby increasing the NQO1 expression.

Ajoene has been shown to inhibit human GR activity through covalent modification [10]. The outcome of this interaction was found to be formation of a mixed disulfide between the active site Cys58 and the CH_2 CH$-CH_2-$SO$-CH_2-$CH CH$-$S moiety of ajoene. We also predicted the X-ray crystal structure of ajoene-GR complex from the Protein Data Bank (PBD code 1bwc), and confirmed the interaction between the vinyl sulfur of ajoene and cysteine 58 of GR using the PyMol software (Supplemental Fig. 1).

Our present study demonstrate that Z-ajoene was found to induce NQO1 expression to a greater extent than did the E-isoform. Consistent with our findings, Z-isomer was found to be more active than the E-isomer in inhibiting cultured cancer cell growth, suggesting that regioselective protein interactions may be important for biological activities of ajoene [29]. The inactivation of GR by ajoene will interefere with regeneration of intracellular GSH from GSSG, thereby inducing mild oxidative stress. This, in turn, would provoke the adaptive antioxidant response by activating Nrf2 and inducing NQO1 expression. We found that Z-ajoene exhibited higher prooxidant activity than the E isoform in terms of generating ROS, which may account for the higher NQO1 induction by the former compound. It would be interesting to compare the inhibitory effects of both isoforms on GR activity as well as Nrf2 activation in the MCF-10A cells.

In our Density Functional Theory (DFT) calculations (Supplemental Fig. 2A), vinyl sulfur and allyl sulfur have positive (+0.142e) and negative (−0.002e) excess charge, respectively. This indicates that vinyl sulfur is more electrophilic than the allyl sulfur. It has been reported that the sulfoxide group of ajoene is hydrogen bound to the GR in the active site [29]. According to the DFT calculation, the oxygen atom has a negative excess charge of about −0.770e. Hydrogen atoms bonded to central double bonded carbon atoms have the largest positive excess charge (+0.156e and +0.157e) among all the hydrogen atoms in the molecule. These hydrogen atoms are expected to leave the molecule first in a proton transfer reaction. This may be related to potential antioxidant activity of ajoene. The bond lengths and the 3-dimensional plot of the electrostatic potential of the optimized ajoene are shown in Supplemental Figure 2B and 2C. These preliminary DFT calculation results strongly supported an antioxidant activity of Z-ajoene.

In conclusion, Z-ajoene inhibits GR activity and GSH synthesis, and creates a state of mild oxidative stress, and induces ROS generation and activation of ERK and Nrf2, thereby expression of NQO1.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.15430/JCP.2019.24.2.121.

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