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Phosphatidylinositol 3-Kinase Regulates Nuclear Translocation of NF-E2-Related Factor 2 through Actin Rearrangement in Response to Oxidative Stress

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ABSTRACT

Expression of phase II detoxifying genes is regulated by NF-E2–related factor 2 (Nrf2)-mediated antioxidant response element (ARE) activation. We showed previously that phosphatidylinositol 3 (PI3)-kinase plays an essential role in ARE-mediated rGSTA2 induction by oxidative stress. In view of the fact that the signaling pathway of PI3-kinase controls microfilaments and translocation of actin-associated proteins, the current study was designed to investigate the PI3-kinase–mediated nuclear translocation of Nrf2 and the interaction of Nrf2 with actin. *tert*-Butylhydroquinone (*t*-BHQ) caused Nrf2 to translocate into the nucleus in H4IIE cells, which was prevented by pretreatment of the cells with PI3-kinase inhibitors (wortmannin/LY294002). *t*-BHQ relocalized Nrf2 in concert with changes in actin microfilament architecture, as visualized by superposition of immunochemically stained Nrf2 and fluorescent phalloidin-stained actin. Furthermore, *t*-BHQ increased the level of nuclear actin, coimmunoprecipitated with Nrf2, which returned to that of control by pretreatment of the cells with PI3-kinase inhibitors. Cytochalasin B, an actin disruptor, alone stimulated actin-mediated nuclear translocation of Nrf2 and induced rGSTA2. In contrast, phalloidin, an agent that prevents actin filaments from depolymerization, inhibited Nrf2 translocation and rGSTA2 induction by *t*-BHQ. Subcellular fractionation and immunoblot analyses allowed us to detect both 57- and 100-kDa Nrf2. Immunoblot and immunoprecipitation assays showed that the 100-kDa protein comprised both Nrf2 and actin. The present study demonstrates that the PI3-kinase signaling pathway regulates rearrangement of actin microfilaments in response to oxidative stress and that depolymerization of actin causes a complex of Nrf2 bound with actin to translocate into nucleus.

Reactive oxygen species and electrophiles induce a battery of antioxidant genes through the activation of antioxidant response element (ARE), which involves NF-E2–related factor (Nrf) proteins and Maf family members (Bergelson et al., 1994; Wasserman and Fahl, 1997; Venugopal and Jaiswal, 1998). Nrf2 is essential for ARE-mediated induction of phase II detoxifying enzymes. Keap1 represses Nrf2 by binding to the amino-terminal Neh2 domain of Nrf2 and oxidative stress antagonizes Keap1 inhibition of Nrf2 activity (Itoh et al., 1999).

Among the groups of major phase II detoxifying enzymes, glutathione *S*-transferases (GSTs) display broad substrate specificity and play a critical role in providing protection against oxidative stress and electrophiles. GSTs catalyze a number of xenobiotics as substrates and produce glutathione conjugates (e.g., conjugation of reactive metabolic intermediates with GSH) (Bolton et al., 1993; Primiano et al., 1995; Nam et al., 1998). GSTs show noncatalytic binding properties (i.e., ligandins) and possess the capability to sequester nonsubstrate drugs and hormones. The level of GST expression is a crucial factor in determining the sensitivity of cells to a broad spectrum of toxic chemicals. Hence, the induction of GST families is a protective adaptive response to oxidative stress (Bergelson et al., 1994; Wasserman and Fahl, 1997; Venugopal and Jaiswal, 1998). In addition, GST serves as a regulatory molecule for cellular signaling pathway(s) and may affect cell proliferation and cell cycle control. A recent study revealed that GST inhibits formation of Jun-c-Jun NH_2-terminal kinase (JNK) complex and subsequently blocks mitogenic signaling induced by oncogenic ras-p21 (Chie et al., 2000; Villafania et al., 2000; Cho et al., 2001).

Phosphatidylinositol 3 (PI3)-kinase , a phospholipid kinase that phosphorylates phosphatidylinositols at the 3-position of the inositol ring, is activated by receptor tyrosine kinases

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K.W.K. and S.J.L. contributed equally to this work.

ABBREVIATIONS: ARE, antioxidant response element; Nrf2, NF-E2–related factor 2; GST, glutathione *S*-transferase; PI3, phosphatidylinositol 3; *t*-BHQ, *tert*-butylhydroquinone; PKC, protein kinase C; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; DTT, dithiothreitol; PI, propidium iodide; GSH, glutathione; PtdIns, phosphatidylinositide.

and forms complexes with phosphotyrosine sites in activated receptors. PI3-kinase activates cellular survival signals, mitogenesis, and cell transformation (Daulhac et al., 1999) and is involved in the regulation of the small GTPase Rac (Hawkins et al., 1995; Fritz and Kaina, 1999). Our studies showed that oxidative stress induced by *tert*-butylhydroquinone (*t*-BHQ) activates PI3-kinase and mitogen-activated protein kinases (Kang et al., 2001a). We and another group revealed that PI3-kinase served as an essential pathway for the induction of phase II enzymes including rGSTA2 and other phase II enzymes (Kang et al., 2001a,b; Lee et al., 2001a). The pathway of PI3-kinase was also involved in ARE-mediated rGSTA2 induction by oxidative stress such as sulfur amino acid deprivation (Kang et al., 2000). Other studies proved that protein kinase C (PKC) was responsible for phosphorylation of Nrf2 and its translocation into nucleus (Huang et al., 2000), which might be a critical event for nuclear translocation of Nrf2 and ARE activation in response to oxidative stress (Huang et al., 2000). Despite the identification of PI3-kinase as an essential enzyme for ARE-mediated *rGSTA2* gene expression (Kang et al., 2000, 2001a), the exact cellular signaling pathway for Nrf2/ARE-mediated GST induction by oxidative stress has not been clarified yet. Furthermore, the mechanistic basis and molecular steps directing the migration of Nrf2 into nucleus need to be established.

The activation of PI3-kinase leads to formation of PtdIns-3,4-bisphosphate and PtdIns-3,4,5-trisphosphate. These products are involved in the signaling for cytoskeletal rearrangement as well as for protein translocation (e.g., GLUT4) and DNA synthesis (Vollenweider et al., 1999). Rac downstream of PI3-kinase stimulated reorganization of actin filaments (Valgeirsdottir et al., 1998). In the present study, we used *t*-BHQ to further investigate the signaling pathway responsible for the translocation of cytoplasmic Nrf2 to nucleus. We found that Nrf2 colocalizes with actin in the cells treated with *t*-BHQ and the nuclear translocation of Nrf2 is dependent on actin rearrangement, which is controlled by the PI3-kinase pathway. We also verified the role of actin rearrangements in the ARE-mediated rGSTA2 induction with the experiments using cytochalasin B, an agent that inhibits actin polymerization. Cytochalasin B was capable of translocating cytoplasmic actin-bound Nrf2 to the nucleus, which led to the induction of rGSTA2. We used phalloidin, an actin-stabilizing agent, which prevents actin filaments from depolymerization, to further prove the role of actin in Nrf2 translocation. Because the level of 100-kDa protein cross-reacting with anti-Nrf2 antibody was increased by oxidative stress in the nuclear fraction prepared under the nonreducing condition, we were interested in the potential interaction of Nrf2 with actin. In this report, we describe for the first time that Nrf2 binds with actin and that the Nrf2-actin complex is translocated into the nucleus by oxidative stress for ARE activation.

Materials and Methods

Materials. Anti-rGSTA1/2 and anti-GSTα antibodies were supplied from Biotrin International (Dublin, Ireland) and Detroit R & D (Detroit, MI), respectively. Recombinant protein G-agarose and 5-bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium were obtained from Invitrogen (Carlsbad, CA). Anti-Nrf2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescein isothiocyanate (FITC)-conjugated or tetramethylrhodamine B isothiocyanate-conjugated anti-rabbit IgG antibodies were obtained from Zymed Laboratories (South San Francisco, CA). *t*-BHQ (97%) was purchased from Sigma-Aldrich (St. Louis, MO). LY294002 and phalloidin were obtained from Calbiochem (San Diego, CA). Other reagents in the molecular studies were supplied from Sigma-Aldrich.

Cell Culture. H4IIE rat hepatoma cell line was obtained from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C in humidified atmosphere with 5% CO_2.

Preparation of Nuclear and Cytoplasmic Fractions. Nuclear extracts were prepared essentially according to a method published previously (Schreiber et al., 1990). Briefly, the cells in dishes were washed with ice-cold PBS. Cells were then scraped, transferred to microtubes, and allowed to swell after the addition of 100 μl of hypotonic buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 2 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonylfluoride (M1 method). The lysates were incubated for 30 min in ice and centrifuged at 7,200*g* for 5 min at 4°C. Supernatants were used as cytoplasmic fractions for the assay of Nrf2 and stored at −70°C until use. Pellets containing crude nuclei were resuspended in 50 μl of extraction buffer containing 20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride and then incubated for 30 min in ice. The samples were centrifuged at 15,800*g* for 10 min to obtain supernatants containing nuclear fractions. In some experiments, subcellular fractionation was carried out using the lysis buffer without DTT (i.e., nonreducing condition) (M2 method). Nuclear fractions were stored at −70°C until use. To prepare cytosolic fractions for the analysis of rGSTA2, the cells were scraped after washing twice with PBS and sonicated to disrupt cell membranes. Cytosolic fractions were obtained by centrifuging cell lysates at 10,000*g* for 10 min and the fractions were used for rGSTA2 immunoblotting.

Immunoblot Analysis. SDS-polyacrylamide gel electrophoresis and immunoblot analyses were performed according to previously published procedures (Kim et al., 1997). The samples were fractionated by 7.5% (for Nrf2 and actin) or by 12% (for rGSTA1/2) gel electrophoresis and electrophoretically transferred to nitrocellulose paper. Equal loading of proteins in the samples was confirmed by Amido Black staining. The nitrocellulose paper was incubated with polyclonal rabbit anti-Nrf2 antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-rat rGSTA1/2 antibody (1:1000), followed by incubation with alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibodies, and developed using 5-bromo-4-chloro-3-indoylphosphate and nitro blue tetrazolium or enhanced chemiluminescence detection kit (Kim et al., 1997; Kang et al., 2000).

To further verify whether anti-Nrf2 antibody recognized Nrf2 protein, the immunoprecipitate of the nuclear fraction with anti-Nrf2 antibody was analyzed by matrix-assisted laser desorption ionization-time-of-flight mass spectrophotometry. The immunoprecipitate was fractionated by 7.5% gel electrophoresis and visualized with Coomassie blue. The band of the 57-kDa protein was excised, trypsinized, and subjected to matrix-assisted laser desorption ionization-time-of-flight mass spectrophotometry analysis (Applied Biosystems, Foster City, CA). The protein contained the following peptide masses and sequences, which are present in the Nrf2 sequence: 588.2, KPDTK; 590.2, GENDR; 607.3, AFNQK; 1431.3, EQFNEAQLALIR.

To determine the involvement of phosphorylation in the formation of 100-kDa Nrf2, the nuclear proteins prepared from cells treated with *t*-BHQ for 6 h were dephosphorylated in vitro. Briefly, the nuclear proteins were treated with calf intestinal alkaline phosphatase (4 units/ml) in a buffer (pH 9.5) containing 100 mM Tris-Cl, 5 mM $MgCl_2$, and 100 mM NaCl for 30 min, neutralized with hydrochloric acid, and then subjected to immunoblot analysis.

Immunocytochemistry of Nrf2. H4IIE cells were grown on Lab-TEK chamber slides (Nalge Nunc International Corp, Roches-

ter, NY) and incubated in serum-deprived medium for 6 h. The standard immunocytochemical method was used as described previously (Nancy et al., 1999). For immunostaining, the cells were fixed in 100% methanol for 30 min and washed three times with PBS. After blocking in 5% bovine serum albumin in PBS for 1 h at room temperature or overnight at 4°C, the cells were incubated for 1 h with polyclonal rabbit anti-Nrf2 antibody (1:100) in PBS containing 0.5% bovine serum albumin. The cells were incubated with FITC-conjugated goat anti-rabbit IgG antibody (1:100) after serial washings with PBS. Counter-staining with propidium iodide (PI, 2 μg/ml) verified the location and integrity of nuclei. Stained cells were washed and examined using a laser scanning confocal microscope (Leica TCS NT; Leica Microsystems, Wetzlar, Germany).

Staining of Actin with Fluorescein-Labeled Phalloidin. To selectively stain filamentous polymerized actin (F-actin), the cells were incubated with FITC-labeled phalloidin at a concentration of 0.2 U/ml for 30 min at 37°C (Herrington et al., 2000). Colocalization of Nrf2 and actin was determined using the combination of two fluorescent stainings. Actin was labeled with FITC-labeled phalloidin, whereas Nrf2 was stained with rabbit anti-Nrf2 antibody followed by tetramethylrhodamine B isothiocyanate-conjugated anti-rabbit IgG antibody. Stained cells were washed twice with PBS and examined using a laser-scanning confocal microscope.

Immunoprecipitation. To determine actin-bound Nrf2 contents, either nuclear fraction or total cell lysates (50 μg in 300 μl each) was incubated with polyclonal rabbit anti-Nrf2 antibody (Santa Cruz, CA) for 2 h at 4°C. The antigen-antibody complex was immunoprecipitated after incubation for 2 h at 4°C with protein G-agarose. Immune complexes were solubilized in 2× Laemmli buffer and boiled for 5 min. Samples were separated and analyzed using 7.5% SDS-PAGE and then transferred to nitrocellulose membranes. The samples were then immunoblotted with anti-actin antibody. Blots were developed using an ECL chemiluminescence detection kit for immunostaining, and 5-bromo-4-chloro-3-indoylphosphate and nitro blue tetrazolium were used to visualize immunoblots.

Results

Nuclear Translocation of Nrf2 by *t*-BHQ. Previous studies have shown that *t*-BHQ induces rGSTA2 through Nrf2/Maf-mediated ARE activation (Kang et al., 2000, 2001a). Immunocytochemistry of Nrf2 and a reporter gene study of ARE revealed that the PKC pathway plays a crucial role in Nrf2/ARE-mediated gene expression (Huang et al., 2000). To determine whether *t*-BHQ induced oxidative stress, the reduced GSH content was measured in H4IIE cells treated with *t*-BHQ (30 μM). The reduced GSH was decreased 3 to 6 h after *t*-BHQ treatment in a time-dependent manner (Fig. 1A). The GSH level was increased at the later time (i.e., 12 h), which might result from the adaptive cellular response. We next determined the time course of Nrf2 translocation by *t*-BHQ into the nucleus in H4IIE cells (Fig. 1B). Cells were treated with 30 μM *t*-BHQ for 3 to 12 h, fixed, and permeabilized. Immunocytochemistry showed that Nrf2 was located predominantly in the cytoplasm of control cells. Nrf2 has a perinuclear and nuclear localization at 3 to 6 h in the cells treated with *t*-BHQ, indicating that Nrf2 moved into the nucleus. At later time points (i.e., 12–24 h), Nrf2 redistributed in the cytoplasm. To verify this result, additional experiments were conducted with subcellular fractions. Western blot analyses showed that Nrf2 was detected predominantly in the cytoplasmic fraction of control cells. Conversely, a greater amount of Nrf2 was found in the nuclear fraction than in the cytosolic fraction of the cells treated with 30 μM *t*-BHQ for 3 to 6 h (Fig. 1C). The levels of nuclear and cytoplasmic Nrf2 returned to those of control at 12 h. To verify the purity of subcellular fractions, we assayed the level of glyceraldehyde-3-phosphate dehydrogenase, as a representative cytosolic enzyme, in the nuclear fraction, which was less than 3% of the amount present in cytoplasmic fraction. The changes in Nrf2 band intensity in cytoplasmic and nuclear fractions further supported redistribution of Nrf2 by *t*-BHQ.

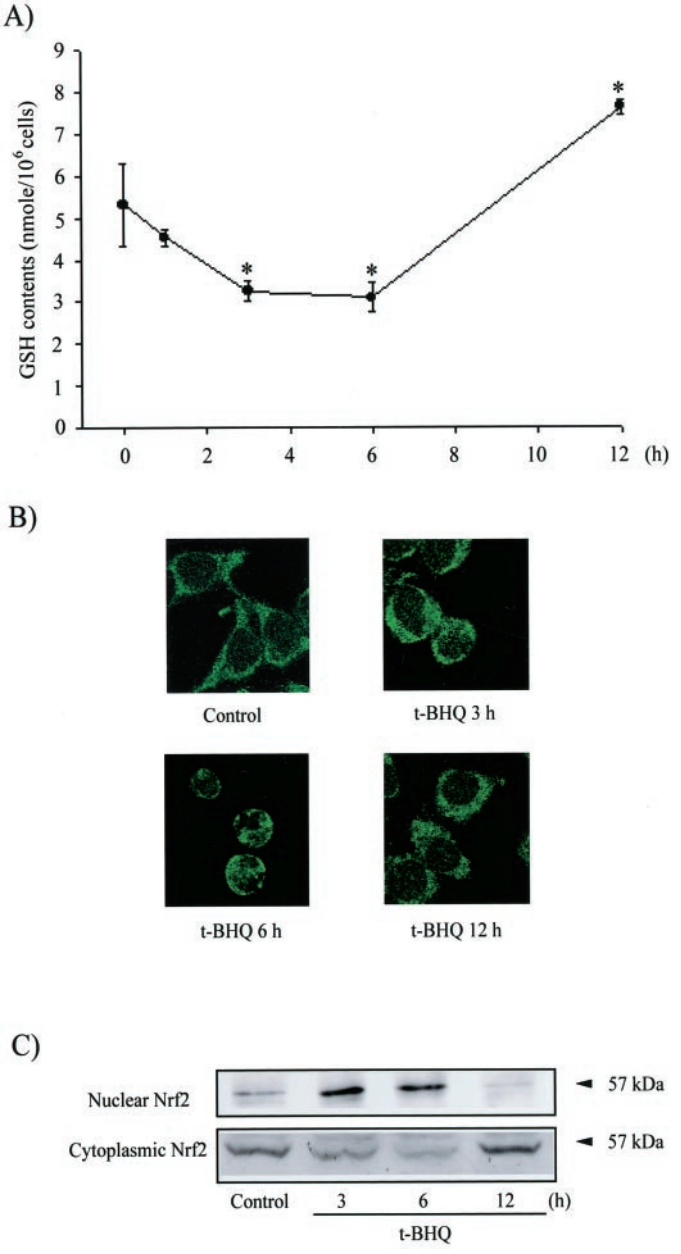

Fig. 1. Subcellular localization of Nrf2 in response to *t*-BHQ. A, the reduced GSH contents in H4IIE cells treated with *t*-BHQ (30 μM). Data represent the mean ± S.D. with three separate experiments (significant compared with control; *, $p < 0.05$). B, H4IIE cells were treated with 30 μM *t*-BHQ for 3 to 12 h. Nrf2 localization was immunochemically detected using anti-Nrf2 antibody. *t*-BHQ caused Nrf2 to migrate to the nucleus at 3 to 6 h, followed by returning to the cytoplasm at 12 h. C, Western blot analysis of Nrf2 in subcellular fractions. Cytoplasmic and nuclear fractions were obtained from cells treated with *t*-BHQ for 3–12 h and Nrf2 in each fraction was immunoblotted with anti-Nrf2 antibody. Results were confirmed by repeated experiments.

PI3-Kinase–Dependent Nrf2 Migration and rGSTA2 Induction. Previously, we showed that the activities of PI3-kinase and Akt were increased by *t*-BHQ for the first 6 h (Kang et al., 2001a). In the present study, we determined whether the PI3-kinase cascade was involved in the translocation of Nrf2. To probe the involvement of PI3-kinase in the nuclear translocation of Nrf2 by *t*-BHQ, the cells were treated with wortmannin or LY294002 in combination with 30 μM *t*-BHQ. Immunochemical staining provided the evidence that cytoplasmic Nrf2 was not translocated to nucleus in the cells treated with PI3-kinase inhibitors (Fig. 2A).

Immunoblot analysis revealed that the level of cytosolic Nrf2 was decreased by *t*-BHQ with a reciprocal increase in the level of nuclear Nrf2 (Fig. 2B). PI3-kinase inhibitors prevented changes in the band intensities of cytosolic and nuclear Nrf2 (Fig. 2B). The expression of rGSTA2 was dependent on PI3-kinase. Western blot analyses showed that wortmannin or LY294002 completely inhibited the induction of rGSTA2 by *t*-BHQ (Fig. 2B). Anti-rGSTA1/2 antibody preferentially recognized the induction of rGSTA2 because the protein subunit was inducible.

Colocalization of Nrf2 with Actin and the Role of PI3-Kinase. Nrf2 bound with Keap1 (Itoh et al., 1999) and Kelch protein, which is highly homologous to Keap1, is known to bind with actin (Way et al., 1995). Given the possible interaction of Nrf2 with actin-related proteins, we monitored whether *t*-BHQ changed cellular filamentous structure of actin using FITC-labeled phalloidin (Fig. 3A). The actin microfilament network was observed in the control cells. In all fields, actin had cytoplasmic localization with faint, filamentous nuclear staining. The cells treated with *t*-BHQ (6 h) had round shapes and smaller in size with shrunk nuclei. Treatment of cells with *t*-BHQ caused actin to have perinuclear and nuclear localization with reduced cytoplasmic areas. At 24 h, actin was relocalized in the cytoplasm with less intense nuclear staining. Superposition of the actin and Nrf2 images at 6 and 24 h after *t*-BHQ treatment showed a complete overlap in all fields (Fig. 3A).

We then analyzed the interaction of actin with Nrf2 in the nuclear extracts. *t*-BHQ increased the level of nuclear actin coimmunoprecipitated with anti-Nrf2 antibody in the cells treated with *t*-BHQ. The levels of Nrf2-bound actin in the nuclear fraction increased between the 1- and 6-h time points in a time-dependent manner, and the interaction between Nrf2 and actin disappeared at 24 h (Fig. 3B, left). The level of Nrf2-bound actin was not changed in the samples of total cell lysates (Fig. 3B, left). The samples precipitated after reacting with rabbit IgG in the absence of anti-Nrf2 antibody showed only negligible band (i.e., blank experiment) (Fig. 3B, right). Next, we assessed the effects of PI3-kinase inhibitors on the amounts of actin immunoprecipitable with Nrf2. Immunoblot analysis revealed that the level of nuclear actin interacting with Nrf2 returned to that of control by pretreating the cells with wortmannin or LY294002 (Fig. 3C), which was consistent with the result of immunocytochemistry. These results demonstrated that the PI3-kinase pathway indeed controlled cytoskeletal rearrangements (i.e., actin depolymerization and repolymerization) in H4IIE cells, which might be responsible for the nuclear migration of Nrf2.

Activation of Actin-Bound Nrf2/ARE and rGSTA2 Induction by Cytochalasin B. Cytochalasin B is an agent that inhibits actin polymerization and then disrupts filamen-

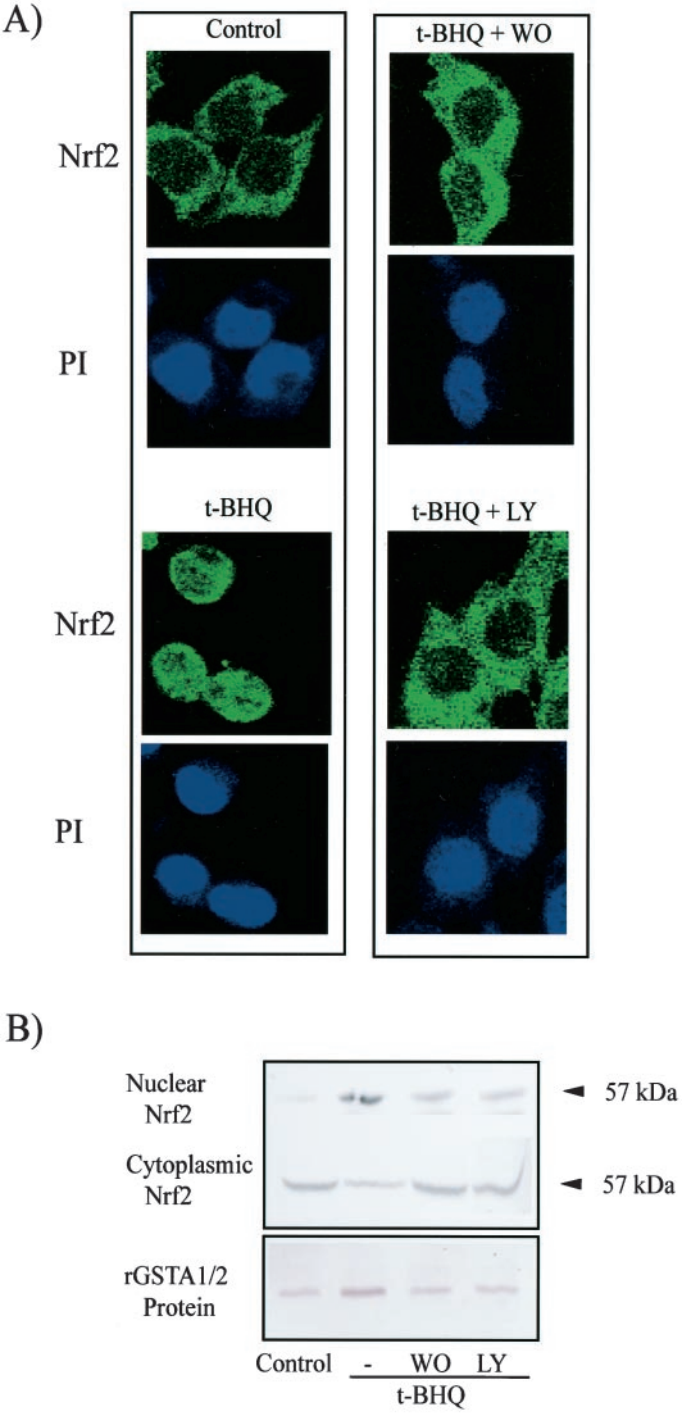

Fig. 2. The effects of PI3-kinase inhibitors on the subcellular localization of Nrf2. A, immunocytochemistry of Nrf2 in the cells treated with *t*-BHQ with or without a PI3-kinase inhibitor. The cells pretreated with wortmannin (WO, 0.5 μM) or LY294002 (LY, 25 μM) for 30 min were exposed to 30 μM *t*-BHQ for 6 h. The same fields were counter-stained with PI. B, the levels of nuclear and cytoplasmic Nrf2. Nrf2 localization was immunochemically assessed in the cells treated with *t*-BHQ (6 h) in combination with a PI3-kinase inhibitor. The effects of PI3-kinase inhibitors (wortmannin, WO, 0.5 μM; LY294002, LY, 25 μM) on *t*-BHQ–inducible rGSTA2 induction were determined by Western blot analysis, which confirmed nuclear translocation of Nrf2. The representative immunoblot shows the levels of rGSTA1/2 in the cells treated with 30 μM *t*-BHQ for 24 h in the presence or absence of PI3-kinase inhibitor. Each lane was loaded with 10 μg of cytosolic proteins. Results were confirmed by repeated experiments.

tous actin (Baumann, 2001). Cytochalasin B caps actin filaments and stimulates ATP hydrolysis on G-actin (Sotiropoulos et al., 1999). We were interested in determining whether depolymerization of actin by cytochalasin B induced nuclear translocation of Nrf2 and activated ARE in H4IIE cells (Fig. 4A). Treatment of cells with cytochalasin B (6 μM) caused the cells to become round and stimulated the migration of Nrf2 into nucleus. Immunocytochemistry revealed that the cells treated with cytochalasin B for 12 to 24 h changed; the actin microfilament network stained with fluorescein-labeled phalloidin had perinuclear and nuclear localization (Fig. 4A). The majority of stained actin localized inside the nucleus or underneath the nuclear membrane. Superposition of Nrf2 and actin fields showed a major overlap in the nucleus (i.e., intense yellow areas), with a greater extent of red Nrf2 fluorescence in the center. Although the superposition of actin and Nrf2 slightly differed from that in *t*-BHQ-treated cells, Nrf2 colocalized significantly with the cytochalasin B-treated actin microfilament system.

Studies were extended to confirm the interaction between Nrf2 and actin in the cells treated with cytochalasin B, an actin disruptor. The level of nuclear actin bound with Nrf2 gradually increased from 1 h after cytochalasin B treatment through 24 h (Fig. 4B). An intense actin band (43 kDa) was observed in the immunoprecipitate with anti-Nrf2 antibody 24 h after cytochalasin B treatment. Western blot analysis was performed to determine whether ARE activation by cytochalasin B led to an increase in the rGSTA2 protein level in H4IIE cells. Western blot analysis confirmed that rGSTA1/2 subunit was induced by cytochalasin B at 6–24 h (Fig. 4B). Northern blot analysis confirmed that the increase in rGSTA2 mRNA preceded the protein induction (data not shown).

Latrunculin B modifies G-actin and thus prevents actin polymerization (Sotiropoulos et al., 1999). We used latrunculin B to further confirm the effect of actin depolymerization on Nrf2-dependent rGSTA2 induction. Latrunculin B (1 μM) increased the nuclear Nrf2 level (12 h), compared with control, which led to the induction of rGSTA2 at 24 h (data not shown).

Inhibition of *t*-BHQ–Inducible Nrf2 Translocation and rGSTA2 Expression by Phalloidin. Phalloidin is an agent that prevents actin filaments from depolymerization (Lader et al., 1999; Sullivan et al., 1999). Phalloidin at the concentration of 2 μM actively inhibited actin depolymerization in H4IIE cells. To further confirm actin-mediated nuclear translocation of Nrf2, the effect of phalloidin on translocation of Nrf2 and rGSTA2 induction by *t*-BHQ was

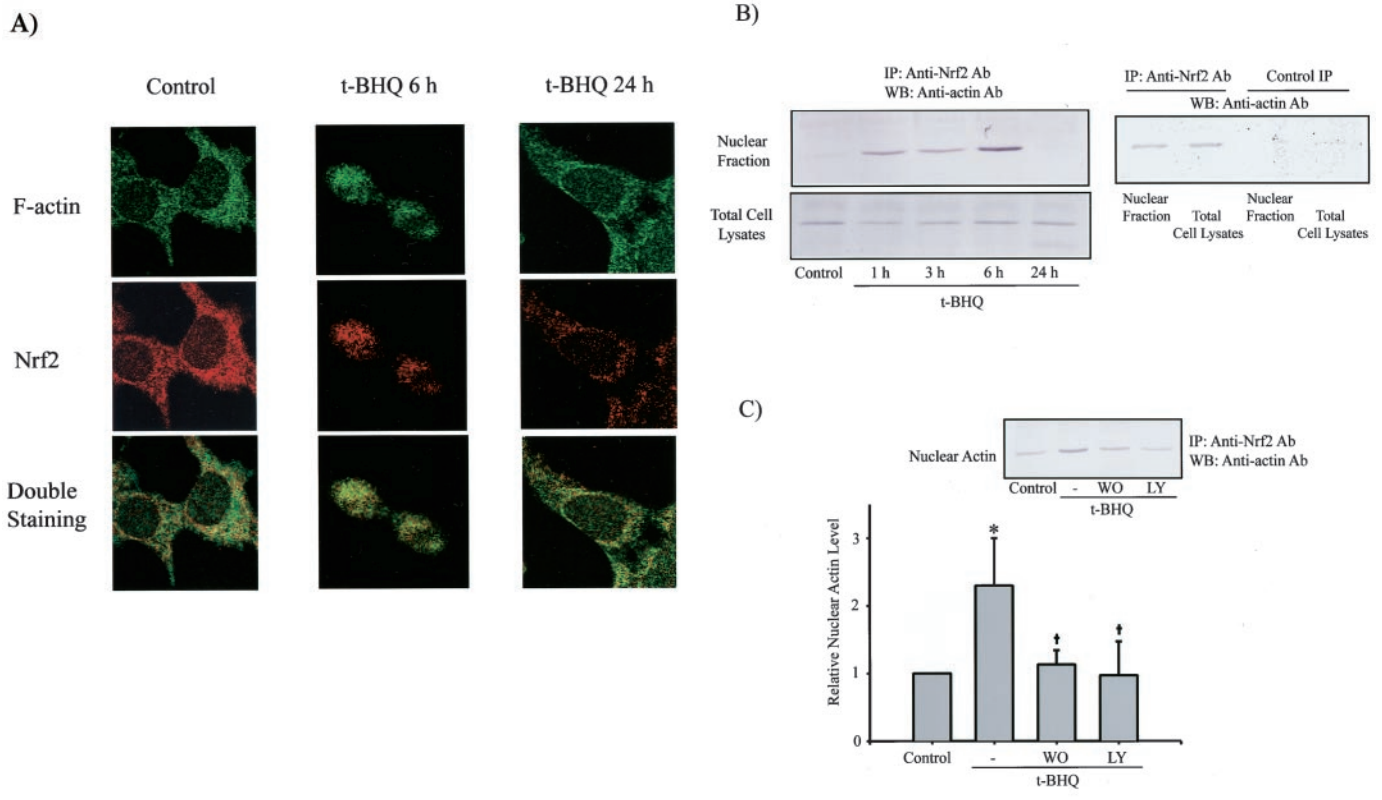

Fig. 3. PI3-kinase-dependent colocalization of actin and Nrf2. A, actin and Nrf2 localization in the cells treated with *t*-BHQ. To assess colocalization of Nrf2 with actin microfilaments, H4IIE cells treated with or without *t*-BHQ for 6 to 24 h were stained for actin with fluorescein-labeled phalloidin and for Nrf2 with anti-Nrf2 antibody and optically visualized using confocal laser-scanning microscopy. Corresponding images were superimposed to determine the degrees of overlap. Superposition of actin with Nrf2 showed a complete overlap of two staining patterns. B, immunoblot analyses of actin in the immunoprecipitates of nuclear extracts or cell lysates with anti-Nrf2 antibody (left). The nuclear extracts or lysates prepared from the cells treated with *t*-BHQ for 1 to 24 h were subjected to immunoprecipitation with anti-Nrf2 antibody and immunoblotted for actin. The blank experiment was performed with the samples (*t*-BHQ, 6 h) precipitated with protein G-agarose in the absence of anti-Nrf2 antibody (right). C, the levels of nuclear actin in the immunoprecipitate with Nrf2. The cells were exposed to *t*-BHQ for 6 h in the presence or absence of wortmannin (WO, 0.5 μM) or LY294002 (LY, 25 μM). Immunoprecipitation and immunoblot analyses were carried out as in Fig. 3B. Data represent mean ± S.D. from 4 independent experiments (*, $p < 0.05$, significant compared with control; †, $p < 0.05$, significant compared with *t*-BHQ alone). IP, immunoprecipitation; WB, western blot.

studied. Immunocytochemistry revealed that phalloidin (2 μM) inhibited the translocation of cytoplasmic Nrf2 to nucleus by *t*-BHQ (6 h) (Fig. 5A). Phalloidin partly suppressed the induction of rGSTA2 by *t*-BHQ, as evidenced by Western blot analysis (Fig. 5B). The increase in the level of nuclear actin coimmunoprecipitated with Nrf2 by *t*-BHQ was also abolished in the cells treated with phalloidin (Fig. 5C). These results further supported the conclusion that rGSTA2 induction by *t*-BHQ was dependent on actin rearrangements.

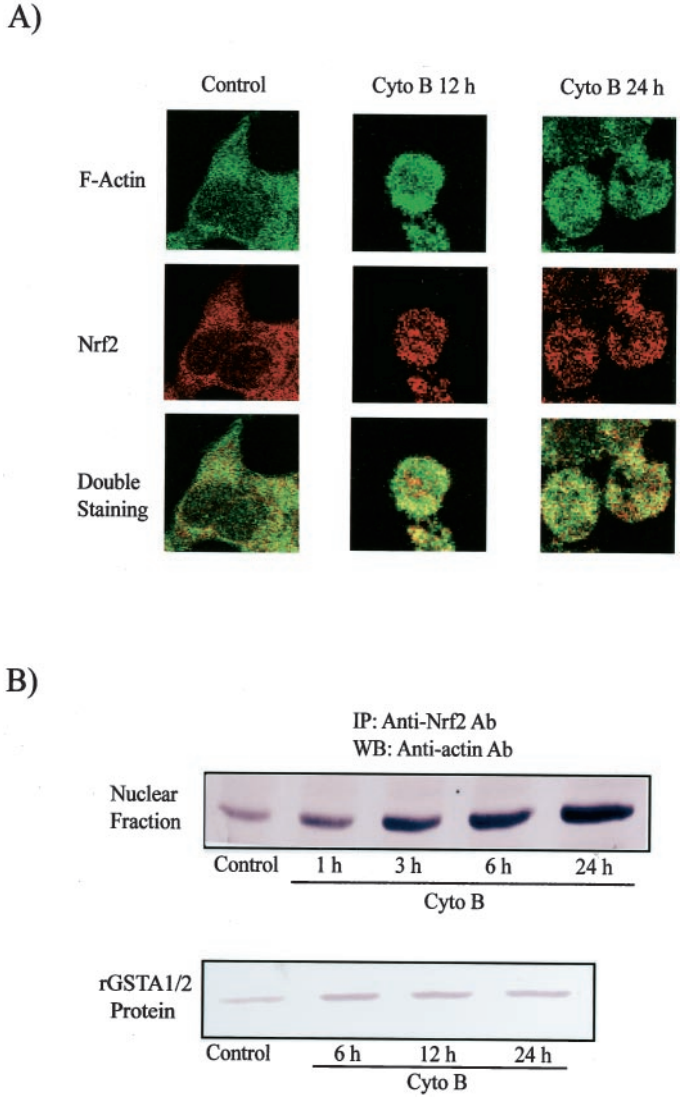

Fig. 4. Nrf2 and actin localization in H4IIE cells treated with cytochalasin B (Cyto B). A, actin and Nrf2 localization in the cells treated with cytochalasin B. To determine the effects of cytochalasin B on Nrf2 and actin organization, the cells were treated with 6 μM cytochalasin B for 12 to 24 h, stained for Nrf2 and actin with anti-Nrf2 antibody and fluorescent phalloidin, respectively, and optically examined by confocal laser-scanning microscopy. The corresponding images were superimposed to determine the degrees of overlap. Superposition of Nrf2 with actin showed major areas of overlap in the nucleus with only minimal overlap in the center. B, immunoblot analysis of actin and the effect of cytochalasin B on the expression of rGSTA2. The nuclear extracts prepared from the cells treated with 6 μM cytochalasin B for 1, 3, 6, or 24 h were subjected to immunoprecipitation with anti-Nrf2 antibody. The levels of actin in the immunoprecipitates were immunoblotted with anti-actin antibody. The rGSTA1/2 protein levels were measured in the cells treated with 6 μM cytochalasin B for 6 to 24 h as in Fig. 2. Results were confirmed by repeated experiments. IP, immunoprecipitation; WB, western blot.

Binding of Nrf2 with Actin. The apparent molecular mass of cytoplasmic and nuclear proteins immunoreactive with anti-Nrf2 antibody varies in the literature (Moi et al., 1994; Lee et al., 2001b). The apparent molecular mass of Nrf2, detected in the cytoplasmic proteins fractionated from cell lysates prepared with the buffer solution containing DTT (2 mM), was 57 kDa (M1 method, Fig. 6A). In cells treated with *t*-BHQ (6 h), 57-kDa Nrf2 was detected in the nuclear fraction, but not in cytoplasmic fraction (Fig. 6A). We also used the buffer solution lacking DTT for the preparation of cytoplasmic and nuclear fractions (M2 method, Fig. 6). Anti-Nrf2 antibody detected two bands with the apparent molecular masses of 100 and 57 kDa in the cytoplasmic fraction of control cells. The band of 100-kDa Nrf2 was much more intense than that of 57-kDa Nrf2. Treatment of cells with *t*-BHQ caused cytoplasmic 100-kDa Nrf2 to translocate to the nucleus (Fig. 6B). Because the proteins were fractionated in SDS-PAGE under the denatured condition, we raised the hypothesis that Nrf2 might covalently bind with actin.

Western blot analysis revealed that anti-actin antibody recognized both 100-kDa Nrf2 and 43-kDa actin in the subcellular fractions prepared under the nonreducing condition (Fig. 6C). In control cells, we immunochemically detected 43-kDa actin as a major band in both cytoplasmic and nuclear fractions. Western blot analysis showed that treatment of cells with *t*-BHQ caused cytoplasmic 100-kDa Nrf2, cross-reacting with anti-actin antibody, to be relocated to the nucleus (Fig. 6C).

To verify whether 100-kDa Nrf2 comprises Nrf2 and actin and the level of nuclear 100-kDa Nrf2 was affected by *t*-BHQ, 100-kDa Nrf2 immunoprecipitated with anti-Nrf2 antibody was immunoblotted with anti-actin antibody (Fig. 6D). Treatment of H4IIE cells with *t*-BHQ caused a time-dependent increase in the band intensity of 100-kDa Nrf2 (1–6 h). The protein level returned to that of control at 24 h. Conversely, the level of 43-kDa actin was not changed by *t*-BHQ at the time points examined. Because the concentration of cytosolic actin in nonmuscle cells is extremely high (i.e., 0.5 mM), the reverse immunoprecipitation analysis was not possible. These data provide evidence that Nrf2 is translocated into the nucleus as a complex bound with 43-kDa actin.

We previously proposed that PI3-kinase and Akt was involved in the phosphorylation step required for the activation of ARE (Kang et al., 2000, 2001a). In view of the role of phosphorylation of Nrf2 in ARE activation, we additionally performed an in vitro experiment with phosphatase to understand the molecular basis of Nrf2 interaction with actin. In the present study, treatment of 100-kDa Nrf2 with phosphatase yielded 57-kDa Nrf2 (Fig. 6E), which raised the possibility that 100-kDa Nrf2 was formed as a consequence of phosphorylation of Nrf2 and/or actin.

rGSTA2 Induction and Nrf2 Translocation by Insulin. Insulin activates PI3-kinase and Akt (Liao et al., 1998; Summers et al., 1999). It has been shown that insulin (0.1 μM) activates PI3-kinase in H4IIE cells (Liao et al., 1998). To further confirm whether Nrf2-mediated rGSTA2 induction resulted from PI3-kinase activation, we used insulin. In this study, insulin (0.1 μM) induced rGSTA2 at 12 to 24 h (Fig. 7, top). The extent of rGSTA2 induction by insulin was comparable with that by *t*-BHQ. Nrf2 translocated into the nucleus in cells exposed to insulin (3–24 h) (Fig. 7, bottom). The time course of Nrf2 translocation by insulin differed from that by

t-BHQ, indicating that the mechanistic basis of Nrf2 nuclear translocation by insulin (i.e., PI3-kinase activation without oxidative stress) may not be identical to that by *t*-BHQ (i.e., oxidative stress).

Discussion

Expression of phase II detoxifying enzymes is primarily regulated by the transcription factors, including the Nrf family, in the cells exposed to oxidative stress (Wasserman and Fahl, 1997; Venugopal and Jaiswal, 1998). Nrf2, as a nuclear transcription factor, plays an essential role in the ARE-mediated phase II enzyme expression. A previous study in this laboratory has shown that the transcription factors Nrf1/2 and small-Maf are involved in the induction of GST in the cells with decreased GSH by sulfur amino acid deprivation (Kang et al., 2000). The role of Nrf2 as one of major transcription factors for GST induction is further supported by the impairment of class α and μ GST induction by *t*-butyl-4-hydroxyanisole in Nrf2 knock-out mice (Bolton et al., 2000).

Previous studies have shown that oxidative stress such as sulfur amino acid deprivation and *t*-BHQ induces rGSTA2 and microsomal epoxide hydrolase with the activation of PI3-kinase and Akt (Kang et al., 2000, 2001a,b). The activation of PI3-kinase and Akt by *t*-BHQ was an essential step for the induction of rGSTA2. The crucial role of PI3-kinase pathway for ARE-mediated GST induction was proved by the chemical inhibitors of the enzyme (Kang et al., 2000, 2001a). We now demonstrated that cytoplasmic Nrf2 was not translocated into nucleus in the cells pretreated with PI3-kinase inhibitors, which prevented rGSTA2 induction. We also found that the activation of PI3-kinase and Akt by insulin caused Nrf2 to translocate to the nucleus and induced rGSTA2. This result further supports the notion that activation of PI3-kinase contributes to Nrf2-mediated rGSTA2 induction.

A series of enzymes would be assembled in the cell membrane to activate Nrf2 in a coordinated way. PKC is crucial in the phosphorylation of Nrf2 and the activation of ARE-mediated gene expression (Huang et al., 2000). Despite the finding that PKC-directed phosphorylation of Nrf2 activates ARE for phase II enzyme induction, the Nrf2 shuttling system has not been identified. The Nrf2 activity is repressed through interaction with Keap1 and is localized in the cytoplasm (Way et al., 1995; Robinson and Cooley, 1997). The PI3-kinase is activated by membrane receptor tyrosine kinase(s) and forms a complex with phosphotyrosine residues in the activated receptor. Relocation and rearrangement of cytoskeletal actin are dependent on the activities of these kinases (Heldman et al., 1996; Hooshmand-Rad et al., 1997). Induction of nitric oxide synthase and transforming growth factor-β was also dependent on actin cytoskeletal dynamics. (Hahn et al., 2000; Zeng and Morrison, 2001). The present study demonstrated for the first time that the pathway involving PI3-kinase was responsible for nuclear translocation of Nrf2 via actin cytoskeletal changes. The subcellular localization of Nrf2 completely depended on the actin microfilament network. A time course study revealed that *t*-BHQ translocated actin to the nucleus between 3 and 12 h, which was in agreement with Nrf2 migration. Immunocytochemistry revealed that Nrf2 perfectly colocalized with actin in the cells treated with *t*-BHQ. Colocalization of Nrf2 with actin and the role of PI3-kinase pathway for actin rearrangements strongly support

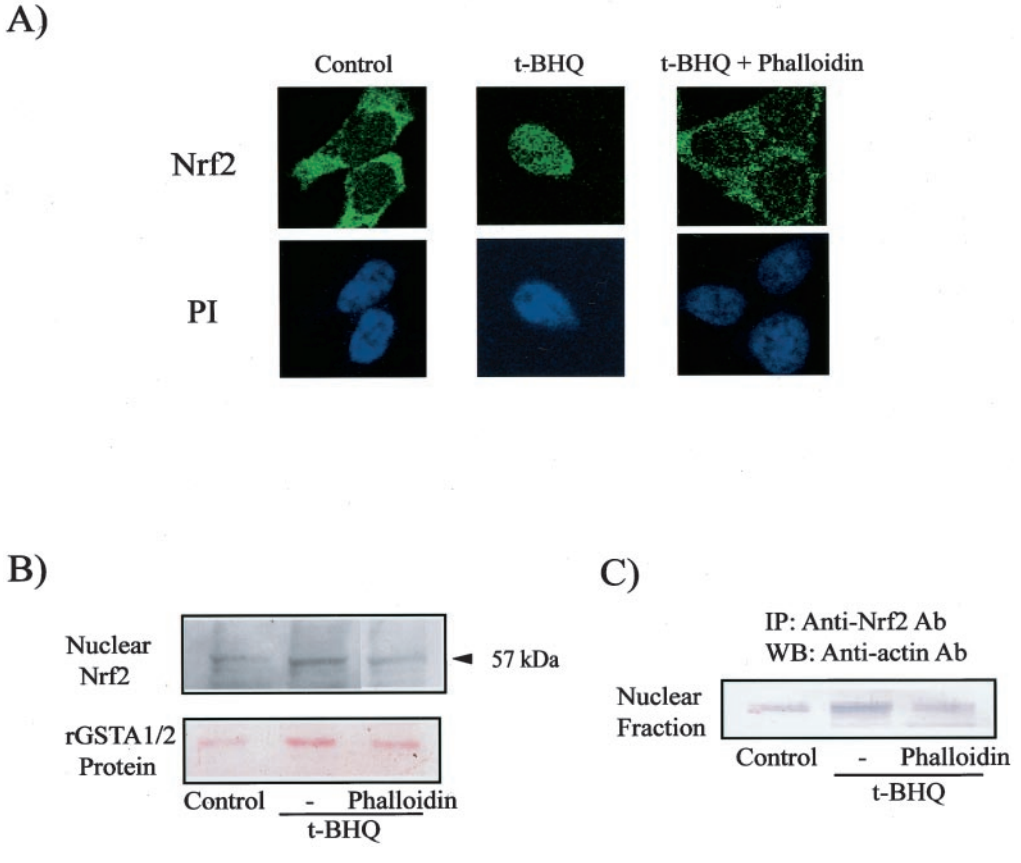

Fig. 5. Inhibition of *t*-BHQ-inducible Nrf2 translocation and rGSTA2 induction by phalloidin. A, the effect of phalloidin on the localization of Nrf2 in the cells exposed to *t*-BHQ. Immunocytochemistry of Nrf2 was performed in the cells treated with *t*-BHQ for 6 h in the presence or absence of 2 μM phalloidin. The same fields were counter-stained with PI. B, The effects of phalloidin on the levels of rGSTA1/2 protein in the cells treated with *t*-BHQ. Western blot analysis was carried out as in Fig. 2. C, the effect of phalloidin on the levels of nuclear actin coimmunoprecipiated with Nrf2. The cells were treated as described above. Immunoprecipitation and immunoblot analyses were performed as in Fig. 3B. IP, immunoprecipitation; WB, western blot.

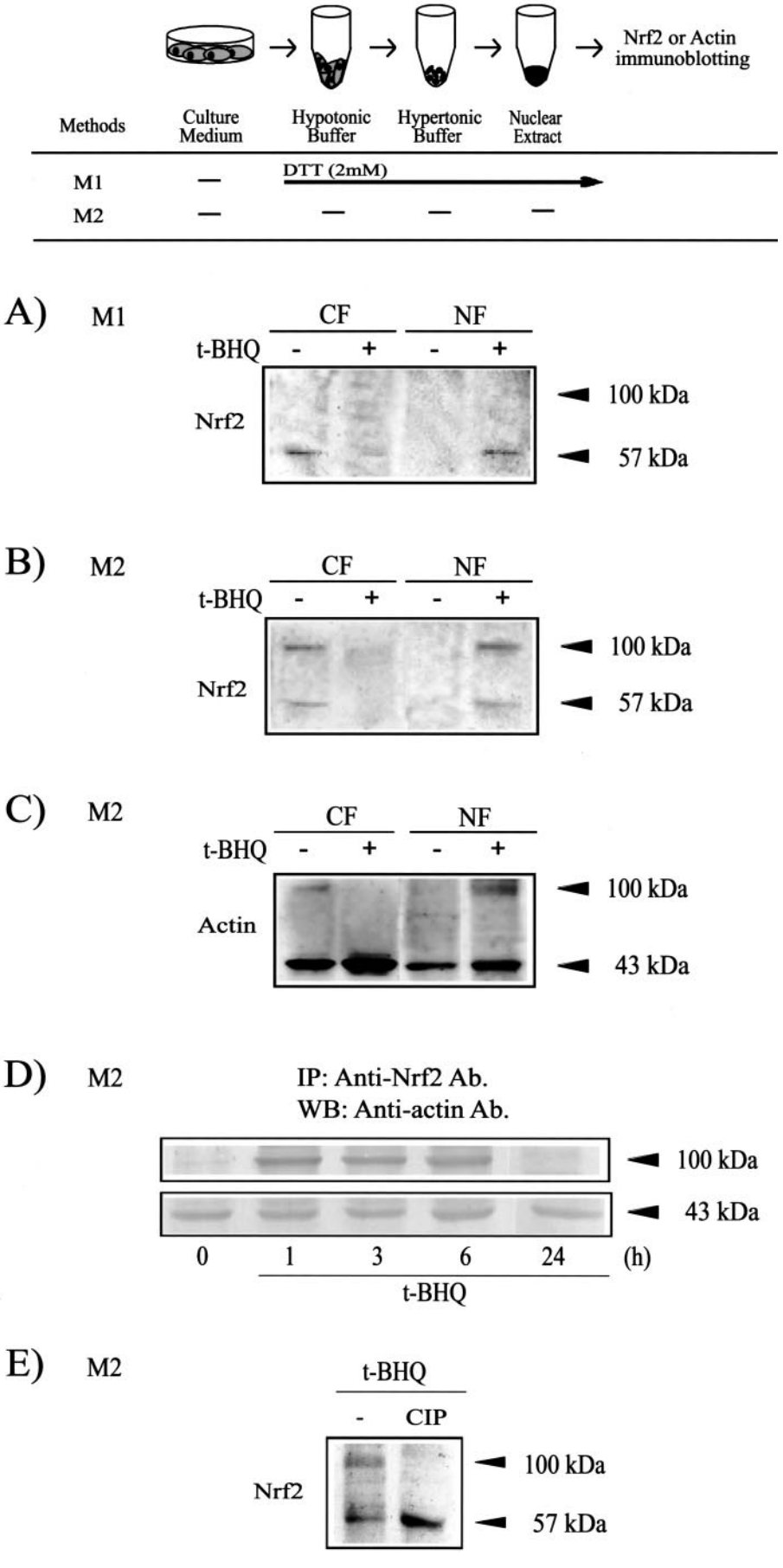

Fig. 6. Nuclear translocation of Nrf2 covalently bound with actin by oxidative stress. A, immunoblot analyses of cytoplasmic and nuclear Nrf2. The band intensity of nuclear Nrf2 was increased by treatment of H4IIE cells with *t*-BHQ for 6 h. The nuclear fraction was prepared by lysing untreated or *t*-BHQ–treated cells in the lysing buffer containing 2 mM DTT. The apparent molecular mass of nuclear Nrf2 was 57 kDa. Molecular mass was calculated from the R_f value using size markers. B, the levels of cytoplasmic and nuclear Nrf2 prepared in the absence of DTT. When nuclear lysate was prepared from cells in the absence of DTT, the molecular mass of immunochemically detectable Nrf2 in the nuclear fraction was 100 and 57 kDa. C, immunoblot analysis of actin. Anti-actin antibody recognized both 100-kDa protein and 43-kDa actin in the cytoplasmic fraction of cells, whereas *t*-BHQ induced nuclear translocation of the 100-kDa protein. Subcellular fractions were prepared according to M2 method. D, cross-reactivity of 100-kDa Nrf2 with anti-Nrf2 and anti-actin antibodies. The nuclear proteins prepared from cells treated with 30 μM *t*-BHQ for 1 to 24 h were immunoprecipitated with anti-Nrf2 antibody and then the precipitated protein was immunoblotted with anti-actin antibody. E, cleavage of 100-kDa Nrf2 to 57-kDa Nrf2 by phosphatase. The proteins in the nuclear fraction prepared from *t*-BHQ-treated cells for 6 h were incubated with calf intestinal phosphatase (CIP) in vitro (4 units/ml). Results were confirmed by repeated experiments. CF, cytoplasmic fraction; NF, nuclear fraction; IP, immunoprecipitation; WB, western blot.

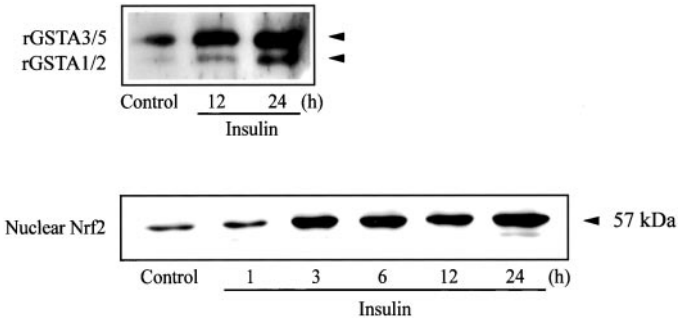

Fig. 7. rGSTA2 induction and Nrf2 translocation by insulin. Expression of rGSTA2 was monitored in cells treated with 0.1 μM of insulin for 12 to 24 h. Each lane contained 10 μg of cytosolic proteins. The levels of Nrf2 in the nuclear fractions (30 μg each) were determined by Western blot analysis. Results were confirmed by repeated experiments.

the possibility that the nuclear translocation of Nrf2 and subsequent ARE activation by *t*-BHQ are mediated with its actin binding and rearrangements. A time course study revealed that actin-bound Nrf2 translocated into the nucleus, which was supported by immunoprecipitation analysis with anti-Nrf2 antibody as well as with anti-actin antibody. Thus, the pathway of PI3-kinase is ultimately responsible for Nrf2 shuttling and ARE activation through rearrangement of actin.

Immunoblot analysis of actin in the nuclear extracts, immunoprecipitated with anti-Nrf2 antibody, revealed that Nrf2 covalently bound with actin in *t*-BHQ-treated cells. In strong reducing conditions (e.g., 2 mM dithiothreitol plus 20 μM β-mercaptoethanol) the apparent molecular mass of nuclear actin immunoprecipitated with anti-Nrf2 antibody was 43 kDa, as shown in the present study, while under weak reducing conditions the molecular masses of immunostained actin were 43 and 100 kDa. The 100 kDa protein may have resulted from binding of Nrf2 with actin. The binding of Nrf2 with actin might be covalent because the protein immunoprecipitated with anti-Nrf2 antibody was analyzed by SDS-PAGE under the denatured reducing condition. The addition of DTT to the Nrf2 protein bound with actin (100 kDa protein) failed to cleave the protein in vitro (data not shown). In the present study, treatment of 100 kDa Nrf2 with phosphatase yielded 57 kDa Nrf2. Hence, it is possible that the 100 kDa protein was formed as a consequence of phosphorylation of Nrf2 and/or actin.

Keap1 is the actin-binding protein that anchors Nrf2 in the cytosol. A previous study has shown that Keap1 is not found in the nucleus (Itoh et al., 1999). The authors speculate that the nuclear translocation of Nrf2 with actin would let Keap1 be released from Nrf2 in cells treated with *t*-BHQ. Once the Nrf2-actin complex translocated into the nucleus, the complex would polymerize in the nuclear compartment as a result of change in the redox state. Actin, a building block of microfilaments, may participate in the process of nuclear localization of Nrf2. Actin as a complex bound with Nrf2 may link the ARE-bound transcription factor to microfilaments in the nucleus and thus contribute to the stability of DNA protein complex.

We attempted to determine whether disintegration of cytoskeletal actin in the absence of oxidative stress could translocate Nrf2 into nucleus. Depolymerization and redistribution of actin by cytochalasin B induced translocation of Nrf2 to nucleus. Immunoprecipitation with anti-Nrf2 antibody and immunoblot analysis of actin demonstrated that nuclear actin bound with Nrf2 was increased by cytochalasin B in a time-dependent manner up to the time point of 24 h. However, the molecular mass of immunostained actin coimmunoprecipitated with Nrf2 was 43 kDa under the weak reducing conditions, which indicated that the actin noncovalently bound with Nrf2. Hence, rGSTA2 induction was dependent on the amount of actin-bound Nrf2. Superposition of Nrf2 and actin images almost completely overlapped in the cells treated with cytochalasin B. Although Nrf2 evenly distributed in the nucleus, actin had more intense localization in the inner areas of nuclear membrane. This differed from the complete overlap of Nrf2 and actin images in the cells treated with *t*-BHQ and may have resulted from noncovalent interaction of Nrf2 with actin.

The PI3-kinase catalyzes the phosphorylation of the 3′ position of the inositol ring of PtdIns-4,5-bisphosphate to PtdIns-3,4,5-trisphosphate, which, in conjunction with membrane-translocated activated phospholipase Cγ, would lead to hydrolysis of PtdIns-4,5-bisphosphate and generation of inositol-1,4,5-trisphosphate and diacylglycerol. Inositol-1,4,5-trisphosphate can stimulate the rise in $[Ca^{2+}]_i$, whereas diacylglycerol activates PKC. Hence, the PI3-kinase pathway may also be responsible for the mobilization of intracellular Ca^{2+} and the rise in $[Ca^{2+}]_i$ may be required for rGSTA2 induction in concert with stimulation of the pathway for Nrf2 activation. PKC with a rise in calcium would be activated after its relocation to the plasma membrane. Preliminary studies showed that the PI3-kinase pathway allowed Nrf2 to migrate to the plasma membrane before its translocation into the nucleus (K. W. Kang and S. G. Kim, unpublished observations). Thus, the essential PI3-kinase signaling pathway responsible for Nrf2 activation may also involve calcium increase for PKC activation.

In summary, we demonstrate that the PI3-kinase pathway regulates the rearrangement of actin microfilaments in response to *t*-BHQ and that depolymerization of actin allows the translocation of Nrf2 bound with actin to the nucleus and stimulates actin/Nrf2-dependent rGSTA2 induction.

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