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Forum Review

Molecular Mechanism of Nrf2 Activation by Oxidative Stress

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ABSTRACT

The capacity of cells to maintain homeostasis during oxidative stress resides in activation or induction of protective enzymes. Nuclear-factor-E2-related factor (Nrf)-2 as a member of bZIP transcription factors is expressed in a variety of tissues. Transcriptional activation of antioxidant genes through an antioxidant response element (ARE) is largely dependent upon Nrf2. The genes that contain a functional ARE include those encoding GSTA1, GSTA2, NAD(P)H:quinone reductase, and γ-glutamylcysteine synthetase heavy and light subunits that play a role in defense against oxidative stress. Previously, we showed that phosphatidylinositol 3-kinase (PI3-kinase) controls nuclear translocation of Nrf2 in response to oxidative stress, which involves rearrangement of actin microfilaments. Now, we report that PI3-kinase is responsible for the rise of cellular Ca^{2+}, which is requisite for nuclear translocation of Nrf2. Immunocytochemistry and subcellular fractionation analyses revealed that Nrf2 relocated from the cytoplasm to the plasma membrane prior to its nuclear translocation. We further found that CCAAT/enhancer binding protein-β (C/EBPβ), peroxisome proliferator-activated receptor-γ (PPARγ), and retinoid X receptor (RXR) heterodimer serve as the activating transcription factors for the phase II gene induction. Hence, PI3-kinase-mediated Nrf2 activation in combination with activating PPARγ-RXR and C/EBPβ contributes to antioxidant phase II enzyme induction via coordinate gene transactivation. *Antioxid. Redox Signal.* 7, 1664–1673.

APOPTOTIC CELL DEATH BY OXIDATIVE STRESS

Reactive oxygen species (ROS) derived from hydrogen peroxide (H_2O_2), superoxide (O_2^-), and peroxynitrite ($ONOO^-$) form powerful oxidants. ROS can be formed from O_2^- and H_2O_2 through a series of reactions (*e.g.,* Haber-Weiss reaction). Superoxide can form hydrogen peroxide either through spontaneous conversion or through enzymatic conversion by superoxide dismutase. Hydrogen peroxide may then be converted into hydroxyl radicals by Fenton reaction.

ROS are highly reactive and can lead to cell death (*e.g.,* apoptosis). Apoptosis (*i.e.,* programmed cell death) is explained by controlled autodegradation of cells and plays important roles in many biological processes including cellular damage, tumorigenesis, and teratogenicity (52). Mitochondria play a central role in apoptotic cell death through their ability to differentially regulate the movement of pro- and anti-apoptotic proteins such as Bcl-2, Bid, and Bax (8). ROS can directly stimulate the opening of the mitochondrial membrane transition pore and cause mitochondrial depolarization and cytochrome *c* leakage (2, 29). The release of cytochrome *c* to cytoplasm causes caspase-9 activation, which subsequently activates caspase-3 (55). In addition, some proteins such as p53 act in the surveillance of cell integrity and trigger apoptosis. ROS control the stability of the p53 protein (14).

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PHASE II ENZYME INDUCTION AS AN ADAPTIVE RESPONSE TO OXIDATIVE STRESS

To protect cellular damage induced by oxidative stress, mammals have novel antioxidant defense systems. The capacity of cells to maintain cellular homeostasis during oxidative stress resides in rapid activation or induction of protective enzymes, which decrease oxidative stress by reducing ROS. Antioxidants are substances that either directly or indirectly protect cells against harmful effects of toxic radicals. Antioxidants and antioxidant enzymes include ascorbic acid, α-tocopherol, β-carotene, polyphenol, flavonoids, GSH, superoxide dismutase, catalase, and glutathione peroxidase (40). Antioxidant agents can either scavenge ROS or stimulate the detoxification mechanism within cells, resulting in removal of ROS.

The phase II detoxifying enzymes including glutathione *S*-transferase (GST), microsomal epoxide hydrolase, and UDP-glucuronyl transferase are in general responsible for metabolic detoxification (13, 36). The conjugation reactions catalyzed by the phase II detoxifying enzymes allow highly reactive carcinogens or radical intermediates to be efficiently eliminated through excretion machinery and thus protect cells against redox-cycling and oxidative stress. Chemoprotective agents induce the enzymes that metabolize carcinogens to less reactive forms (41). In particular, induction of phase II enzymes may represent a protective adaptive response to oxidative stress. Studies provided evidence that antioxidants, prooxidants, and toxicants [*e.g., tert*-butylhydroquinone (*t*-BHQ), butylhydroxyanisole, thiazoles] were capable of inducing phase II enzymes (27, 36). Obviously, induction of phase II enzymes contributes to cytoprotection and potentially to self-repair of cells exposed to oxidative stress.

ROLE OF NUCLEAR FACTOR-E2-RELATED FACTOR (NRF) 2 ACTIVATION IN PHASE II ENZYME INDUCTION

The antioxidant response element (ARE) was identified as a *cis*-acting element responsible for the expression of phase II enzymes (54). AREs are widely found in the promoter regions of diverse detoxifying genes [*e.g.,* rat and mouse GSTA2 (10, 54), rat GSTP (46), rat and human quinone reductase/DT-diaphorase (9, 38)] and of the genes encoding antioxidant enzymes [*e.g.,* γ-glutamylcysteine synthetase (61), heme oxygenase-1 (51), and ferritin light and heavy chains (60)]. Because of the high similarity of the ARE binding site to that of activating protein-1 (AP-1) (G**TGAC**NNN**GC** vs. **TGAC**TCA), it was initially proposed that an AP-1 complex such as c-Jun and c-Fos was the transcription factor responsible for phase II enzyme expression (11, 25). The basic leucine zipper transcription factors including Nrf1 and Nrf2 heterodimerize with small Maf family proteins during oxidative stress, and bind ARE sequences (23, 58). Signals activated by oxidative stress stimulate transduction of Nrf2 activity and subsequent activation of ARE (44, 58). A series of studies from our laboratories have shown that a condition of GSH deficiency, endogenous ROS (*e.g.,* peroxynitrite), and prooxidants (*e.g.,* phenolic antioxidants) induce phase II detoxifying enzymes *via* Nrf2-mediated ARE transactivation (26–28). Because Nrf2 plays a role in ARE-dependent gene transcription, many of the genes encoding antioxidant enzymes are not inducible by electrophiles in Nrf2-deficient cells (17) or in Nrf2-knockout animals (53).

THE CELL SIGNALING PATHWAY OF NRF2 ACTIVATION

Signal transduction refers to the processes by which cells perceive the environmental and/or internal status. In many physiological responses, cellular signals are activated by the transducers attached to the cell surface plasma membrane in response to growth stimuli or chemical modulators. A number of laboratories have studied the signal transduction pathways that control activation of the transcription factors responsible for the expression of phase II detoxifying enzymes.

Phosphatidylinositol 3-kinase (PI3-kinase) is a lipid kinase that phosphorylates phosphatidylinositols at the 3 position of the inositol ring (4). PI3-kinase has been found to be associated with activation of cell survival signals by growth factors and has been implicated in mitogenesis and cell transformation (5). In addition, phosphorylated forms of phosphatidylinositol act as second messengers for several kinases including the serine-threonine Akt kinase and ribosomal S6 kinase (39). PI3-kinase is involved in the regulation of the small GTPase Rac by growth factors (*e.g.,* platelet-derived growth factor). Rac plays an important role in activation of c-Jun NH_2 terminal kinase (JNK) (12, 16). Our research group found that PI3-kinase regulates nuclear translocation of Nrf2 and Nrf2 binding to the ARE for GST induction (26, 27).

Toxic stimuli including oxidative stress engage the mitogen-activated protein (MAP) kinases and concomitantly induce transactivation of the target genes (1, 12). MAP kinase signaling pathways are generally initiated at the cell surface. Three distinct mammalian MAP kinase modules—JNK, extracellular signal-regulated kinase (ERK), and p38 MAP kinase—have been characterized (3, 56). An attempt to clarify the signaling pathway for the phase II enzyme induction was made by Yu *et al*. (62, 63). It has been claimed that treatment of human hepatoma cells (HepG2) or murine hepatoma cells (Hepa1c1c7) with *t*-BHQ or sulforaphane increased phosphorylation of ERK1/2 (62). Inhibition of ERK activation by PD98059, an MAP kinase kinase inhibitor, or by transient transfection with a dominant-negative mutant of MAP kinase kinase-1 blocked ERK activation and prevented the induction of quinone reductase and the ARE-linked reporter gene activity. Based on these observations, they proposed the possibility that induction of ARE-mediated phase II detoxifying enzymes was mediated by ERK1/2 through Raf-1 activation. They also proposed the hypothesis that p38 kinase negatively regulated ARE activation (63). In contrast, we found that PD98059 failed to inhibit the ARE binding activity (27). Rather, PD98059 treatment markedly increased GSTA2 in H4IIE cells in the absence of Nrf2 activation. Hence, other transcription factor besides Nrf2 appeared to account for the

enzyme induction (31). Our research results demonstrated that the flavone moiety, found in the chemical structure of PD98059, was responsible for the induction of GSTA2 through activation of the CCAAT-enhancer binding protein (C/EBP) response element in the promoter region of the gene (31). The lack of a role of MAP kinases in Nrf2 activation for phase II enzyme induction is consistent with the report by Huang *et al.* (21). In their study, neither MAP kinase kinase inhibitor nor p38 kinase inhibitor affected phosphorylation of Nrf2 in HepG2 cells. Taken together, these data support the conclusion that the ERK and p38 MAP kinase do not contribute to Nrf2 phosphorylation.

Protein kinase C (PKC) transduces signal into target molecules in response to extracellular stimuli (*e.g.,* inositol triphosphate-induced calcium release and *Ras* activation). The pathway involving PKC may serve as an initial triggering step for detection of change in the cellular redox state. The signal detection system that recognizes alterations in the redox state may exist in close proximity to the plasma membrane. The question as to what enzymes is responsible for Nrf2 phosphorylation has been recently resolved by Pickett's research group (21, 22). The PKC pathway appeared to be required for ARE activation, providing evidence that PKC-directed phosphorylation of Nrf2 is a critical event for its nuclear translocation in response to oxidative stress. Using a reporter gene assay, ARE-directed transcription was activated by phorbol 12-myristate 13-acetate, a PKC activator, but completely suppressed by PKC inhibition. Immunocytochemistry and western blot analyses revealed that both phorbol 12-myristate 13-acetate and *t*-BHQ promoted nuclear localization of Nrf2. They demonstrated that phorbol 12-myristate 13-acetate transiently activated Nrf2 phosphorylation, whereas *t*-BHQ or β-naphthoflavone led to persistent stimulation, which was abolished by the PKC inhibitor staurosporine, but not by U0126 and SB203580, the respective inhibitors of MAP kinase kinase and p38 kinase. In addition, purified Nrf2 could be phosphorylated *in vitro* by the catalytic subunit of PKC or by PKC that was immunoprecipitated from cell lysates. The phosphorylation site of PKC conferring Nrf2 activation, identified as Ser^{40}, plays a role in the signaling event for the ARE-mediated cellular antioxidant response (22). Recently, Yoshida's research group reported that 12-*O*-tetradecanoylphorbol 13-acetate-insensitive atypical PKC mediated Nrf2 activation in response to oxidative stress (45).

KEAP1/NRF2 BINDING

Investigations have revealed the role of the cytoskeleton-associated protein Keap1 in Nrf2 repression by its tight binding and localization of the complex in the cytoplasm as an inactive form (24, 37, 65). Six highly conserved regions were identified by comparison of the human and chicken Nrf2 amino acid sequences, and one of the regions, named Neh2, was shown to be required for the negative regulation of Nrf2 activity in HD3 erythroblasts (24). This led to the hypothesis that the Neh2 domain interacts with a cellular protein, Keap1. The most close homolog of Keap1 is a *Drosophila* actin-binding protein called Kelch, implying that Keap1 might be an Nrf2 cytoplasmic effector. They also showed that electrophiles antagonize Keap1 inhibition of Nrf2 activity *in vivo*, allowing Nrf2 to traverse from the cytoplasm to the nucleus and to potentiate the ARE-mediated response (24). Keap1 and Nrf2 may constitute a crucial cellular sensor for oxidative stress, and mediate a key step in transducing the signaling pathway that leads to transactivation of phase II enzyme genes by the Nrf2 nuclear shuttling mechanism.

It seems that the sulfhydryl group of Keap1 is the sensor regulating phase II enzyme induction (6). Electrophiles oxidize the most reactive cysteines of Keap1, located in the intervening region, whereas dithiothreitol treatment dissociates the binding of Keap1 and the Neh2 domain of Nrf2. In the resting state, cysteines C273 and C288 of the intervening region are in the reduced state. In this conformation, Keap1 sequesters Nrf2 in the cytoplasm. Upon exposure to oxidative stress, the reactive C273 and C288 form intermolecular disulfide bonds, and thus the two molecular residues of Keap1 are covalently linked. The resulting conformational change can liberate Nrf2 and allow its translocation to the nucleus (59). A mutation study from Mulcahy's research group also supported the key role of Keap1 in Nrf2 regulation (65). In their study, Keap1S104A, a mutational form of Keap1 in the BTB/POZ domain, failed to dimerize with Nrf2 and lacked its ability to sequester Nrf2 in the cytoplasm and to repress Nrf2 transactivation.

The function of Keap1 that controls the activation of Nrf2 is regulated by the scaffolding to the actin cytoskeleton (33). Using a series of Keap1 deletion mutants, it was shown that the double glycine repeat domain of Keap1 interacts with actin filaments and Nrf2 and that both the double glycine repeat the and C-terminal region act in cytoplasmic sequestration and activation of Nrf2.

ACTIN CYTOSKELETAL REORGANIZATION AS A FUNCTIONAL RESPONSE TO OXIDATIVE STRESS

In various cell types, including hepatocytes and fibroblasts, ROS cause disruption of cytoskeleton characterized by fragmentation and patching of F-actin. Regulation of actin polymerization and depolymerization in mammalian cells is a highly complex process that involves a number of actin-binding proteins, most of which are under the control of signaling pathways. Many of the observed alterations of cytoskeletal architecture by oxidative stress seem to be associated with oxidative modifications of cysteine sulfhydryls of actin (19, 47). Disruption of the normal organization of microfilaments coincides with a defect in sulfhydryl groups of actins after exposure of cells to ROS-generating agents such as menadione and diamide (42, 43). Interference of intracellular Ca^{2+} homeostasis appears to be related with disruption of cytoskeleton. A rise in Ca^{2+} concentration by ROS can promote dissociation of actin microfilaments and also activate certain proteases (*e.g.,* calpains) that cleave actin-binding proteins, which might be responsible for the loss of anchorage to the cytoskeleton complex (7).

We also have found that *t*-BHQ, a pro-oxidant, changed the cellular filamentous structure of actin. Immunocytochemical

analysis using fluorescein isothiocyanate-conjugated phalloidin to selectively stain cellular F-actin revealed that cellular localization of F-actin in response to *t*-BHQ paralleled that of Nrf2 (30). In this study, actin was disorganized and primarily detected close to the cell membrane 3 h after treatment with *t*-BHQ and the cytoskeletal actin was found to be reorganized at 24 h. This would represent dynamic depolymerization of F-actin into G-actin in response to oxidative stress.

ACTIN SCAFFOLD FOR NRF2 AND KEAP1

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 obviously depend on the activities of these kinases (18, 20). The induction process of certain genes (*e.g.,* nitric oxide synthase and connective tissue growth factor) involves actin cytoskeletal dynamics (15, 64). Recently, we reported that Nrf2 bound with actin and that the Nrf2–actin complex was translocated into the nucleus by oxidative stress for ARE activation (30). We found that Nrf2 colocalized with actin in cells and that nuclear translocation of Nrf2 was dependent on actin rearrangement, which was controlled by the PI3-kinase pathway. The role of actin rearrangements in the ARE-mediated gene induction was verified by the experiment using cytochalasin B, an agent that inhibits actin polymerization (30). Cytochalasin B was capable of translocating cytoplasmic actin-bound Nrf2 to the nucleus, which led to the induction of rGSTA2. Yamamoto's research group also examined the effect of actin disruption on localization of Neh2-green fluorescent protein (GFP) (*i.e.,* GFP fused to the Neh2 domain) in NIH 3T3 cells (33). Because the Neh2 domain is the interactive interface of Nrf2 with Keap1, they could use the fusion protein as a reporter for Nrf2 localization. Treatment with cytochalasin B resulted in severalfold increases in nuclear translocation of Neh2-GFP (33), supporting the role of actin architecture rearrangement in Nrf2 translocation.

In our experiment, the association between Nrf2 and actin on the ARE-DNA binding site was also analyzed by gel shift analysis. The band of the ARE-binding complex could be immunodepleted by the addition of anti-actin antibody (Fig. 1A). Also, addition of G-actin to the nuclear fraction retarded migration of the band of the Nrf2–DNA complex (Fig. 1B). These studies have led to the suggestion 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 the nucleus.

MEMBRANE LOCALIZATION OF NRF2 PRIOR TO ITS PHOSPHORYLATION

Convincing pharmacological and cell biological data indicated that the activities of PI3-kinase and Akt were increased by *t*-BHQ at early times (*i.e.,* the first 6 h) (27). Nrf2 was located predominantly in the cytoplasm of control cells. Nrf2 in cells exposed to *t*-BHQ moved into the nucleus at 3–6 h (Fig. 2A), whereas cytoplasmic Nrf2 was not translocated to the nucleus in cells pretreated with wortmannin or LY294002 for 30 min and subsequently exposed to *t*-BHQ (Fig. 2A) (30). When we inhibited the activity of PI3-kinase immediately after *t*-BHQ exposure, we were able to observe Nrf2 localization in the periplasma membrane (6 h) (Fig. 2A). Western blot analyses confirmed that the band intensity of Nrf2 in the plasma membrane fraction increased as compared to that in control cells or in cells treated with *t*-BHQ alone (6 h) (Fig. 2B). The differential distribution of Nrf2 in cells pretreated with the chemical inhibitors of PI3-kinase suggests that Nrf2 translocation to the plasma membrane is required prior to its nuclear translocation and that the PI3-kinase pathway may control cellular localization of Nrf2.

Because many of the cellular effectors require calcium for their function, we were interested in whether calcium content was changed in cells exposed to *t*-BHQ. Fluorescence analysis using Calcium Green revealed that *t*-BHQ rapidly stimulated the rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Fig. 3A). The rise in $[Ca^{2+}]_i$ continued for at least the first 200 s. Exposure of cells to thapsigargin (an endoplasmic reticulum

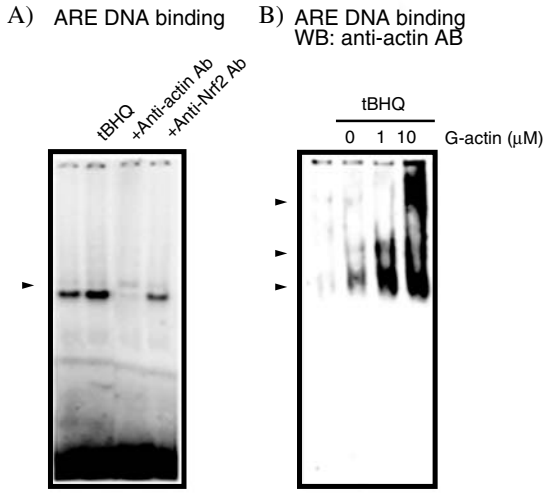

FIG. 1. Actin is a component of the Nrf2/ARE complex. (**A**) Gel shift analysis of the ARE transcription complex. Nuclear extracts were prepared from H4IIE cells incubated with 30 μ*M t*-BHQ for 6 h and subjected to gel shift analysis. All lanes contained 10 μg of nuclear extracts and 5 ng of labeled GSTA2 ARE DNA consensus sequence. Supershift experiments were carried out by incubating the nuclear extracts with the specific polyclonal antibody (Ab) directed against Nrf2 or actin for 1 h. Arrows indicate the ARE binding complex. (**B**) Binding of G-actin to the ARE transcription complex. G-actin (1–10 μ*M*) was incubated with nuclear extracts (10 μg) for 1 h, and the samples were subjected to gel shift-western blot (WB) analysis. The ARE transcription complex was fractionated by non-denaturing gel electrophoresis and electrophoretically transferred to nitrocellulose paper. The nitrocellulose paper was incubated with polyclonal rabbit anti-actin Ab (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with horseradish peroxidase-conjugated secondary Ab, and developed using the enhanced chemiluminescence detection kit.

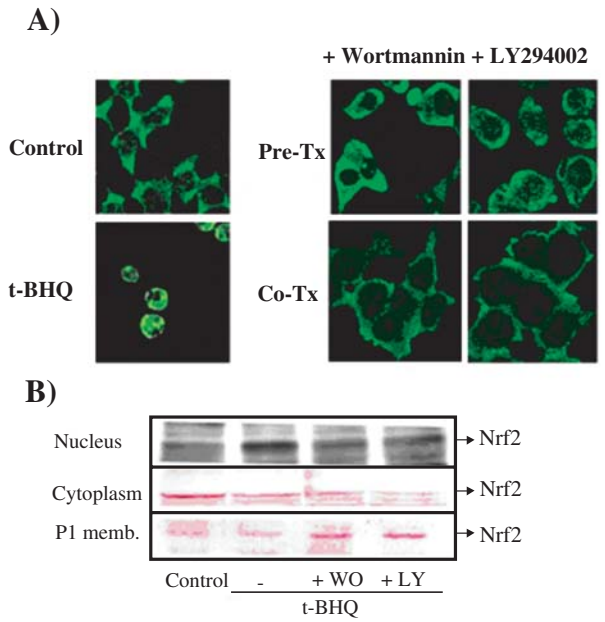

FIG. 2. Effects of PI3-kinase inhibitors on subcellular localization of Nrf2 in cells exposed to *t*-BHQ. (**A**) Immunocytochemistry of Nrf2 in cells exposed to *t*-BHQ with or without PI3-kinase inhibitor. Cells pretreated (30 min, Pre-Tx) or co-treated (Co-Tx) with wortmannin (WO) or LY294002 (LY) were exposed to 30 μ*M* *t*-BHQ for 6 h, and the cellular localization of Nrf2 was assessed by confocal microscopy. (**B**) Western blot analysis of Nrf2 in subcellular fractions. Plasma membrane (Pl memb.), nuclear, and cytoplasmic fractions were obtained from H4IIE cells treated with *t*-BHQ for 6 h, and the level of Nrf2 in each fraction was immunoblotted using anti-Nrf2 antibody.

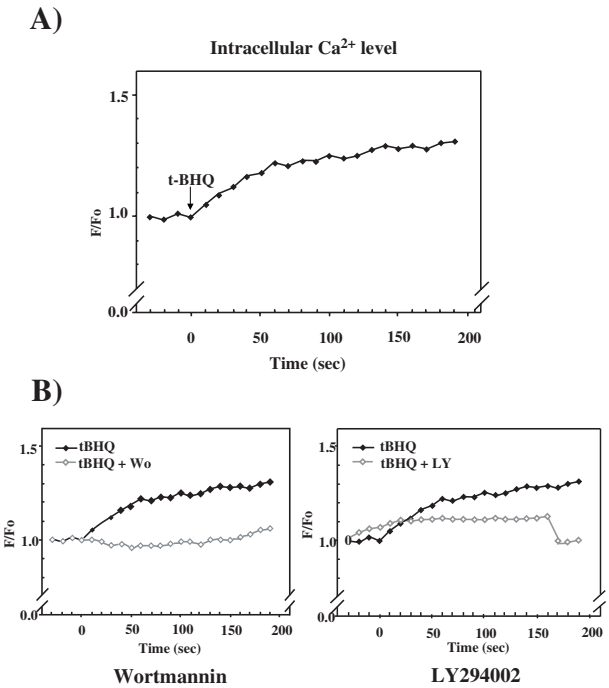

FIG. 3. Role of PI3-kinase in the increase in calcium by *t*-BHQ. (**A**) When cells were exposed to 30 μ*M* *t*-BHQ, cellular Ca^{2+} rapidly increased. Cellular Ca^{2+} was monitored using the fluorescence of Calcium Green-1 acetoxymethyl ester (5 μ*M*). (**B**) The inhibitory effects of wortmannin (Wo) or LY294002 (LY) on the rise in cellular Ca^{2+} by *t*-BHQ. Cells were exposed to *t*-BHQ following preincubation with Wo (0.5 μ*M*) or LY (25 μ*M*) for 30 min, and the cellular calcium content was monitored using a fluorescence dye. Data points represent means from three separate experiments.

Ca^{2+} depletor) or verapamil (a Ca^{2+} channel blocker) before *t*-BHQ treatment significantly inhibited the rise in cellular calcium (data not shown), which indicated that both the release of calcium from the intracellular storage and the influx of extracellular calcium contributed to the rise in $[Ca^{2+}]_i$. We were then interested in whether the PI3-kinase pathway was involved in the rise in calcium induced by *t*-BHQ. Pretreatment of cells with PI3-kinase inhibitors for 30 min inhibited the increase in cellular calcium by *t*-BHQ (Fig. 3B).

We next determined whether the rise in cellular calcium was associated with Nrf2 translocation. Immunocytochemistry revealed that Nrf2 failed to relocate to the plasma membrane in cells treated with 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis (acetoxymethylester) (BAPTA-AM), a cell-permeable calcium chelator, prior to *t*-BHQ treatment (Fig. 4A). Conversely, A23187, a calcium ionophore, allowed Nrf2 to migrate to the plasma membrane in cells treated with wortmannin + *t*-BHQ (Fig. 4B). A23187 increased cellular calcium, as confirmed by the fluorescence of Calcium Green. These results support the hypothesis that cellular calcium is necessary for migration of cytoplasmic Nrf2 to the plasma membrane. It is likely that oxidative stress causes translocation of Nrf2 as an actin-bound complex from the cytoplasm to the plasma membrane in a Ca^{2+}-dependent manner. Therefore, the PI3-kinase pathway may regulate the increase in cellular calcium in response to *t*-BHQ and the rise in $[Ca^{2+}]_i$ is required for shuttling of Nrf2 between the cytoplasm and the plasma membrane. PI3-kinase may also control the rise in cellular Ca^{2+}, which in conjunction with actin cytoskeletal rearrangements may regulate Nrf2 translocation into the nucleus through its activation at the plasma membrane presumably by PKC.

OTHER TRANSCRIPTION FACTORS FOR PHASE II ENZYME INDUCTION

In addition to Nrf2-mediated ARE activation, other transcription factors might be associated with the induction of phase II detoxifying enzymes. C/EBPα is a member of the protein complex interacting with the xenobiotic response element (XRE) in the GSTA2 promoter (49). It has been proposed that the activation of C/EBPα and the Ah receptor led to the induction of GSTA2 and quinone reductase via the C/EBP-containing XRE present in the upstream promoter regions of the genes (49, 57). Both XRE and ARE have been characterized as positive regulatory elements using reporter constructs linked to the promoter regions of GSTA2 in HepG2 cells. Recently, we demonstrated that oltipraz, a can-

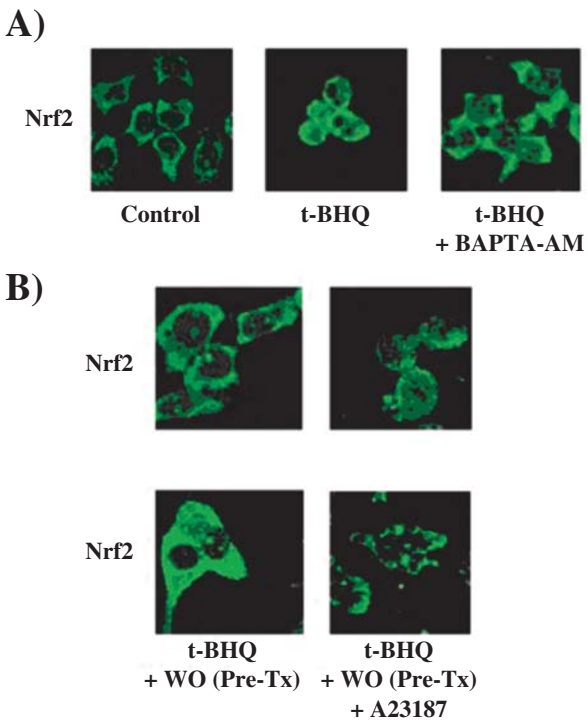

FIG. 4. Ca^{2+}-dependent relocation of Nrf2 to the plasma membrane. (**A**) The effect of calcium chelator on Nrf2 localization in cells exposed to *t*-BHQ, shown by immunocytochemistry of Nrf2 in untreated control cells or cells exposed to *t*-BHQ with or without BAPTA-AM (concomitant treatment, 20 μ*M*). BAPTA-AM inhibited the nuclear translocation of Nrf2 in cells treated with 30 μ*M t*-BHQ for 6 h. (**B**) The role of PI3-kinase in Ca^{2+}-dependent relocation of Nrf2 to the plasma membrane. H4IIE cells were treated with wortmannin (WO; 0.5 μ*M*) for 30 min (Pre-Tx) and then exposed to *t*-BHQ in the presence or absence of A23187 (0.1 μ*M*) for 6 h to monitor subcellular migration of Nrf2. The subcellular localization of Nrf2 was immunochemically assessed, as described previously.

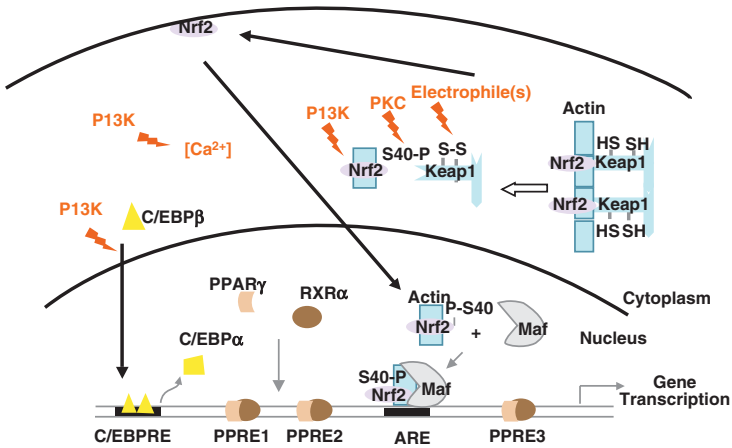

FIG. 5. Schematic diagram illustrating the proposed mechanisms by which oxidative stress activates Nrf2/ARE. Nrf2 is a member of the Cap'n'Collar (CNC) family of basic ZIP transcription factors. Transcriptional activation of antioxidant defense genes through AREs depends on Nrf2. Activation of ARE by ROS regulates phase II enzyme genes with the protein complex comprising Nrf2. The cytoskeleton-associated protein Keap1 represses Nrf2 by its tight binding and causes localization of the complex in the cytoplasm. Electrophiles antagonize Keap1 inhibition of Nrf2, inducing phosphorylation of Nrf2 at Ser40 by PKC. PI3-kinase (PI3K) controls nuclear translocation of Nrf2 in response to oxidative stress, which involves rearrangement of actin microfilaments. The PI3K pathway is also responsible for the rise of cellular Ca^{2+}, which was requisite for nuclear translocation of Nrf2. Nrf2 relocates from the cytoplasm to the plasma membrane prior to its nuclear translocation. In addition, C/EBPβ and the PPARγ and RXR heterodimer serve the activating transcription factors for the phase II gene induction. Hence, PI3K-mediated activation of Nrf2 in combination with activating PPARγ-RXR and C/EBPβ contributes to antioxidant phase II enzyme induction *via* coordinate gene transactivation, conferring cytoprotective effects against oxidative stress. C/EBPRE, C/EBPβ response element; PPRE, PPARγ-RXR response element.

cer chemopreventive agent, activated C/EBPβ and that the activating C/EBPβ was responsible for the strong induction of GSTA2 via the C/EBP response element (32). In general, the Ah receptor is essentially required for the activation of XRE in response to planar aromatic compounds (50). However, the Ah receptor was not a component in the C/EBP binding complex to the C/EBP response element within the XRE in the GSTA2 gene in cells treated with oltipraz, which would be an important biochemical point differentiating chemoprevention from carcinogenesis.

Peroxisome proliferator-activated receptors (PPARs), including PPARα, PPARβ, and PPARγ, constitute a subfamily of the nuclear receptor superfamily activated by a variety of natural and chemical ligands. The PPAR genes are involved in regulating diverse biological events such as lipid metabolism (34). The retinoid X receptors (RXRs), a member of the nuclear receptor superfamily, are believed to be heterodimeric partners for PPAR. Recently, we showed that PPARγ, which is sufficiently expressed in rat hepatocytes and hepatoma cells, in combination with ligand-activated RXR enhanced GSTA2 induction (48). Activation of PPARγ and RXR heterodimer may also contribute to the enzyme induction. Despite the weak induction of GSTA2 by retinoic acid alone, retinoic acid significantly potentiated the enzyme induction by PPARγ agonist, which suggested that RXR is required for the maximal activation of PPARγ in order to induce phase II detoxifying enzymes. When we deleted the ARE- or C/EBP-binding sites in the reporter construct of the GSTA2 promoter, the increase of the reporter activity in response to the combination treatment with PPARγ/RXR agonists was abolished. Furthermore, we found multiple PPAR-response elements in the GSTA2 promoter. Specific mutations of these multiple PPAR-response element sites resulted in the complete loss of their responsiveness to the agonists of PPARγ and RXR. In addition, we found that both Nrf2 and C/EBPβ are up-regulated in a PPAR- and RXR-dependent manner. These results suggest that the PPAR response module, which is activated by the ligand-bound PPAR and RXR heterodimer, plays an essential role in formation of the transactivation complex comprising Nrf2 and C/EBPβ.

CONCLUSION

Cellular signals are activated by the transducers attached to the cell surface plasma membrane in response to chemical modulators. The AREs bound with the protein complex comprising Nrf2 and Maf family members that are activated by reactive oxygens play an important role in the regulation of phase II enzymes. The pathway of PI3-kinase, whose activity is increased by oxidative stress, regulates rearrangement of actin microfilaments in response to oxidative stress, and then depolymerization of actin causes a complex of Nrf2 bound with actin to translocate into the nucleus via the plasma membrane (Fig. 5). In addition to the Nrf2/ARE activation signal, the role of C/EBP-mediated signaling pathway and of PPARγ and RXR heterodimer activation for PPAR-response enhancer module in the phase II enzyme induction was proved with identification of the enhancer element(s) of the genes. The signaling pathways regulating C/EBPβ activation and PPARγ and RXR heterodimer may activate distinct biochemical routes leading to cell survival and cytoprotection.

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ABBREVIATIONS

AP-1, activating protein-1; ARE, antioxidant response element; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethylester); *t*-BHQ, *tert*-butylhydroquinone; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; C/EBP, CCAAT/enhancer binding protein; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; GSH, glutathione; GST, glutathione *S*-transferase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; Nrf, nuclear factor-E2-related factor; PI3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; RXR, retinoid X receptor; XRE, xenobiotic response element.

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