Atypical protein kinase C mediates activation of NF-E2-related factor 2 in response to oxidative stress

Satoshi Numazawa, Makie Ishikawa, Aya Yoshida, Sachiko Tanaka, and Takemi Yoshida

Department of Biochemical Toxicology, School of Pharmaceutical Sciences, Showa University, Tokyo 142, Japan

Submitted 30 January 2003; accepted in final form 10 April 2003

NF-E2-RELATED FACTOR 2 (Nrf2), a member of the Cap’n’Collar (CNC) family of transcription factors, was originally found to regulate expression of the β-globin gene by binding the Maf recognition element (MRE) that resides on its enhancer region (35). Studies employing genetically modified animals (12, 19, 20) revealed that Nrf2 plays an important role in the transcriptional regulation of a set of genes induced by oxidative stress. Under unstimulated conditions, Nrf2 is sequestered in cytoplasm by binding its amino-terminal Neh2 domain with the actin-binding protein Keap1 (21), a homolog of the Drosophila Kelch protein. Oxidative stressors, such as electrophiles, liberate Nrf2 from the cytoplasmic complex, thereby translocating it into the nucleus (18, 21). Nrf2 binds to the heterodimer partner of certain basic leucine zipper (bZip) transcription factors, such as the small Maf family of proteins (33, 53) and Jun family members (54), and transactivates antioxidant response elements (AREs), which resemble the MRE sequence (20, 53). A set of genes of coding for enzymes induced by oxidative stress, such as heme oxygenase-1 (HO-1) (3), NAD(P)H-quinone oxidoreductase (NQO1) (23), glutathione S-transferase (11), cystine-glutamate transporter (48), and γ-glutamylcysteine synthetase (10, 55), possess AREs within their promoter/enhancer region. These enzymes, often called phase 2 proteins, share a protective function against electrophiles and reactive oxygen species (ROS), which can damage cellular macromolecules, although their mechanisms of protection differ. Consequently, the Nrf2-Keap1 complex is supposed to be a sensor for oxidative stress. Although the primary mechanism underlying the Nrf2-dependent gene expression of phase 2 proteins has been increasingly understood, the mechanism by which Nrf2 is liberated from the Nrf2-Keap1 complex remains controversial. Nrf2 regulation has been proposed to involve several different mechanisms, including 1) direct attack of the Nrf2-Keap1 complex by electrophiles or ROS (9, 15, 21, 60), 2) indirect actions, such as phosphorylation, which are involved in the dissociation (4, 18, 27), and 3) modulation of the stability of Nrf2 protein (2, 39, 49, 52). Furthermore, whether these modifications happen to Nrf2 (9, 17) or Keap1 (15, 60) has also been argued.

Because HO-1 is one of the most readily induced of the phase 2 enzymes, it has been proposed as a marker of oxidative stress (31). We have examined cellular signal transduction leading to HO-1 gene expression by...
using a model of oxidative stress in which cells are treated with phorone, an α,β-unsaturated carbonyl compound that causes the specific depletion of the reduced form of glutathione (GSH) (43, 44). During the course of these experiments, we found that specific inhibitors of protein kinase C (PKC) caused a substantial reduction in phorone-mediated induction of HO-1 gene expression. This observation is consistent with the findings of Huang et al. (18), who showed that the PKC pathway is involved in the nuclear translocation and activation of Nrf2 induced by 12-O-tetradecanoylphorbol-13 acetate (TPA) or tert-butylhydroquinone (tBHQ). However, we have observed that HO-1 inducibility of TPA is quite weak compared with other inducers, including heavy metals, heme, and electrophiles, even though it is a powerful PKC-activating agent. Moreover, tBHQ marginally induces HO-1, although it is a powerful inducer of the NQO1 gene. Consequently, it remains unclear whether PKC is actually involved in Nrf2 activation and HO-1 gene induction under oxidative stress conditions.

PKCs are a family of structurally related Ser/Thr protein kinases (42). The mammalian PKC isoforms can be divided into three groups—the classical PKC (cPKC), novel PKC (nPKC), and atypical PKC (aPKC)—by divergence of their structure in the amino-terminal regulatory domain (41). The cPKCs, including cPKCα, -βI, -βII, and -γ, have two common regions: C1, which consists of two cysteine-rich loops and binds diacylglycerol (DAG) and TPA; and C2, a calcium-binding domain. nPKCs, including nPKCδ, -ε, -η, and -θ, lack the C2 region, whereas the aPKCs, including aPKCζ and -λ, lack the C2 region and have only one cysteine-rich loop in the C1 region. These structural differences result in the requirement of distinct cofactors for each of the PKC isoforms. Calcium and DAG are required to activate cPKCs, whereas calcium is not required for nPKC activation. aPKCs do not require these factors when activated by certain lipid species, such as the products of phosphatidylinositol (PI) 3-kinase and ceramide (1, 16, 38, 50). A growing body of evidence indicates that cPKCs and nPKCs play divergent roles in controlling cell growth, differentiation, apoptosis, and carcinogenesis (40). aPKCs, on the other hand, have been shown to be involved in the growth, survival (14, 36, 58), and polarization (22, 24, 28) of cells; however, the precise function of these isoforms is not as well understood as that of the other subfamilies.

The present study indicates that the oxidative stress caused by phorone and 4-hydroxy-2,3-nonenal (4-HNE) induces nuclear translocation of Nrf2, but not the mutant in which Ser40 cannot be phosphorylated by PKC. Our in vivo results strongly support an idea originally introduced by Huang et al. (17), who showed in vitro that phosphorylation of Nrf2 at Ser40 by PKC liberates it from the complex with Keap1, thereby suggesting that PKC is involved in the nuclear translocation of Nrf2 and activation of ARE. Moreover, the present study reveals that the aPKC subfamily is responsible for the phosphorylation that is critical for Nrf2 activation and for the signal transduction leading to the induction of cytoprotective phase 2 proteins.

 MATERIALS AND METHODS

Materials. 4-HNE diethylacetal was obtained from Oxis International (Portland, OR) and dissolved in acetonitrile. A portion of the stock solution was evaporated; the residue was dissolved in 1 mM hydrochloric acid for 1 h, and the concentration was measured at 220 nm before use. Ro-31-8220, Gö-6976, and calphostin C were purchased from Calbiochem (La Jolla, CA). Phorone was obtained from Wako Pure Chemical (Tokyo, Japan). Anti-cPKCα and -nPKCζ antibodies were purchased from BD Transduction Laboratories (Lexington, KY) and anti-aPKCζ from Santa Cruz Biotechnologies (Santa Cruz, CA). The anti-aPKCζ antibody used for immunoprecipitation was purchased from BD Transduction Laboratories.

Cell cultures. WI-38 human fibroblast, COS-7 monkey kidney, and HepG2 human hepatoma cells were obtained from Riken Cell Bank (Tsukuba, Japan). WI-38 cells were maintained in Eagle’s MEM, and COS-7 and HepG2 cells were cultivated in DMEM, respectively; the cultures were supplemented with 10% fetal calf serum (FCS), 20 mM HEPES, and antibiotics in a humidified 5% CO2 atmosphere at 37°C.

Northern blot analysis. Total RNA samples (20 μg/lane) were size-fractionated by denaturing agarose gel electrophoresis before transfer to a Nytran membrane (Schleicher and Schuell, Dassel, Germany) as described (43). cDNA probes for HO-1 (59) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (30) were labeled by the multiprimer DNA reaction (Amersham Pharmacia Biotech, Amersham, UK) with [α-32P]dCTP (Institute of Isotopes, Hungary). Relative mRNA contents were measured with a Fuji BAS 3000 image analyzer (Fuji Photo Film, Kanagawa, Japan) and normalized with GAPDH mRNA.

Western blot analysis. Cells were washed and lysed by scraping into boiling 2× SDS-PAGE sample buffer. The lysates were boiled for 5 min and briefly sonicated. Samples were run on SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Pall BioSupport, Glen Cove, NY). Blots were blocked in 0.2% I-Block (Applied Biosystems, Foster City, CA) dissolved in Tris-buffered saline containing 0.2% Tween 20 (TTBS) for 1 h and probed with isoform-specific antibodies. After being washed, the blots were incubated with the secondary antibody coupled to peroxidase. Blots were developed using enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech) according to the supplier’s instructions.

Construction of plasmids. For construction of the ARE-Luc reporter plasmid used in the luciferase assay, a 120-bp fragment containing multiple AREs that reside 10 kb upstream of the transcription start site of human HO-1 gene (GenBank accession no. Z82244) was produced by PCR reaction using primers 5'-CGCCGATCCGTGTTTTTTCCTGAGTCACGGTCCCGAGGTCTATTTTTCGCTAAGTCACCGCCCCCGAGATCGTTTTTCGCTAG3' and 5'-CGCCGATCCCAATCTCTACCTGTAGCCTACGTTACCCGAGGTCTATTTTTCGCTAAGTCACCGCCCCCGAGATCGTTTTTCGCTAG3' (possessing the underlined 30-bp overlapping sequence), digested with BamHI, and inserted into the BamHI site upstream of the TATA-box promoter of the Herpes simplex virus thymidine kinase promoter of pTAL-Luc vector (BD Biosciences Clontech, Palo Alto, CA). A 1.8-kb Nrf2 and a 1.9-kb Keap1 cDNA fragment, both including the entire coding region, were PCR-amplified with a high-fidelity enzyme HF-2 (BD Biosciences Clontech) and primer sets of 5'-TATCTAGAC
CATGATGGACCTGGAGCTGCC-3' and 5'-TAGGATCCG-TTCTTCTTAACTCTTGCCTCTTAC-3' for Nrf2, and 5'-TATCTGACTTCTCTGCAGAC-3' and 5'-TAGGATCCACAGGTACATTCTGCTGCTTAC-3' for Keap1, using a human fetal brain cDNA library (Stratagene, La Jolla, CA) digested with XbaI and BamHI and inserted into the XbaI and BamHI sites of the pcDNA3.1 vector (Invitrogen). Wild-type and mutant Nrf2 fragments (residues 5–605) were PCR-amplified, digested with KpnI and EcoRV, and inserted in frame into the KpnI/EcoRV sites of pQB125 vector (Wako) to generate the expression vector for the fusion protein of Nrf2 and GFP (Nrf2/pQBI25). Primers for the PCR were 5'-gggat- tactGGAGGCTGCCGCGG-3' and 5'-CCGATATCGTTTTCTTAACTCTTGCCTCTTAC-3'. A 1.8-kb PKCδ cDNA fragment was PCR-amplified with a primer set of 5'-TATTCTAGAGCCGACCAGGAGGACAG-3' and 5'-TAGGATCCGACACATTCTTCTGCAGAC-3' using a human fetal brain cDNA library, digested with XbaI and BamHI, and inserted into the XbaI and BamHI sites of the pcDNA3.1. These constructs were confirmed by sequence analysis.

Site-directed mutagenesis. Site-directed mutagenesis was performed using the GeneEditor system (Promega, Madison, WI). The mutation primers used were as are follows (mutations are underlined): 5'-GAAGATTTTGACCTTGCAG-GACGGGAAAGA-3' [Nrf2(S40A)], 5'-GAAGATTTTGACCTTGCAG-GACGGGAAAGA-3' [Nrf2(S40G)], 5'-CTAGATAGTGGGGGCTGGAGAGGTCAAACAGAATGGTCC-3' [Nrf2(S597A)], 5'-CTAGATAGTGCCCTGGAGAGGTCAAACAGAATGGTCC-3' [Nrf2(S597E)], 5'-ACAAAAGACAAACATGCAAGCCGCTTGGAGGCTC-3' [Nrf2(S597E)], 5'-ACAAGGCAAGAACATGCAAGCCGCTTGGAGGCTC-3' [Nrf2(S447A)], 5'-ACAAAAGACAAACATGCAAGCCGCTTGGAGGCTC-3' [Nrf2(S447E)], 5'-CTTCTGTTGGTCCAAGAAGCATATGTTAAG-3' [Nrf2(S387A)], 5'-CTTCTGTTGGTCCAAGAAGCATATGTTAAG-3' [Nrf2(S387E)], and 5'-GTGTTCTCAGATGTTA AGATGTTAAG-3' [Nrf2(S387E)]. Site-directed mutagenesis was confirmed by sequence analysis.

Immunocomplex kinase assay. Cells were washed three times with ice-cold phosphate-buffered saline (PBS) and were lysed in 50 mM Tris, pH 7.4, containing 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, and 1 mM sodium vanadate, 50 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, 4 μg/ml leupeptin, and 4 μg/ml aprotonin. After centrifugation at 15,000 g for 10 min, cell extracts were incubated with 2 μg anti-aPKCδ antibody at 4°C for 60 min, followed by 2 μl of protein A agarose at 4°C for 60 min. The immunocomplex was washed twice with the lysis buffer containing 500 mM NaCl and once with the lysis buffer containing 500 mM NaCl and 0.5 M NaCl and 0.1 mM EGTA. The kinase reaction was carried out in 20 μl of the kinase buffer containing 1 μg of myelin basic protein, 5 μCi [γ-32P]ATP, and 100 μM cold ATP at 30°C for 10 min. The reaction was stopped by the addition of 5× sample buffer. Samples were boiled for 5 min and subjected to SDS-PAGE.

Reporter assay. HepG2 cells seeded on 24-multiwell dishes (0.8 × 105 cells/well) were transfected with ARE-Luc (0.24 μg), pRL-TK (0.08 μg, Promega), and expression vectors using Lipofectamine 2000 reagent (Invitrogen). The total amount of DNA was adjusted to 0.8 μg/well with the empty pcDNA3.1 vector. After 24 h, cells were lysed and firefly and Renilla luciferase activities were analyzed using the dual luciferase assay system (Promega). Firefly luciferase activity was normalized to the respective Renilla luciferase activity.

RESULTS

Phorone induces transient depletion of GSH and induces HO-1 gene expression in many cultured cell lines, including WI-38 cells (43) and in vivo systems such as rat liver (44). Northern blot analyses revealed that phorone-mediated HO-1 induction occurred in a dose-dependent manner (Fig. 1A, left) and was consid-
erably suppressed by pretreatment of cells with a PKC-specific inhibitor, Ro-31-8220, at a concentration (2 μM) sufficient to inhibit all PKC isozymes in cultured cells (50, 51). The inhibitory effect of Ro-31-8220 was dose dependent, and nearly complete inhibition was observed at 3 μM (Fig. 1A, right). The kinetics of the inhibition of HO-1 induction by Ro-31-8220 appear to be similar to the reported effect on aPKC (IC50 = 1 μM) (51). On the other hand, lower concentrations of c inhibitor, Ro-31-8220, at a concentration (2 μM) were shown to induce phase 2 proteins when administered exogenously (6, 29). 4-HNE induced HO-1 mRNA in WI-38 cells to a level comparable to that induced by phorone (Fig. 1B). Ro-31-8220 abrogated the effect of 4-HNE on HO-1 gene expression and had a similar effect on phorone-mediated induction (Fig. 1B). 4-HNE increased transcription of the luciferase gene controlled by the ARE enhancer, which resides about 10 kb upstream of the transcriptional start site of the human HO-1 gene in HepG2 cells (Fig. 1C), indicating that the cytotoxic lipid peroxidation product induced gene expression, at least in part, by an Nrf2-ARE-dependent mechanism. This effect of 4-HNE was also suppressed by Ro-31-8220 (Fig. 1C). Similar effects of Ro-31-8220 were observed when phorone was used as an inducer (data not shown).

Western blot analyses using isoform-specific antibodies revealed that WI-38 cells express cPKCα, cPKCγ, nPKCβ, and aPKCα, predominantly. Prolonged exposure of WI-38 cells to TPA at either 10 or 100 nM caused downregulation of cPKCα, cPKCγ, and nPKCβ, but had only a marginal effect on aPKCα levels even at 100 nM (Fig. 2A), as reported previously (46, 47). Phorone at a concentration of 0.25 mM induced HO-1 gene expression in TPA-pretreated WI-38 cells to a level similar to that of TPA-untreated cells, although the level of induction by 1 mM phorone was somewhat suppressed in the PKC downregulated cells (Fig. 2B). In addition, 4-HNE-mediated HO-1 induction was enhanced rather than inhibited by TPA pretreatment (Fig. 2C). These results suggest that Ro-31-8220-sensitive protein kinases, most likely certain PKC isozymes, are involved in phorone and 4-HNE-induced transactivation of the HO-1 gene. Moreover, these results suggest that TPA-sensitive PKC isozymes are unlikely to be involved. Therefore, the activity of TPA-insensitive PKC isoforms was measured using an immunocomplex kinase assay with anti-aPKCα antibodies in WI-38 cells treated with phorone or 4-HNE. These agents increased aPKCα activities in a dose-dependent manner and at doses comparable to those that resulted in the induction of HO-1 (Fig. 3).

The amino acid sequence of human Nrf2 revealed the existence of four possible sites for phosphorylation by PKC (8): Ser40, Ser387, Ser447, and Ser597 (Fig. 4A).

**Fig. 2.** Effect of PKC downregulation on oxidative stress-induced HO-1 gene expression. A: WI-38 cells were treated with 10 or 100 nM TPA for 24 h. Cells were washed and lysed in 2× SDS sample buffer. Western blot analysis was carried out using PKC isozyme-specific antibodies. B: WI-38 cells were pretreated with 100 nM TPA for 24 h and treated for 4 h with phorone at the concentrations indicated. The HO-1 gene expressions were normalized to the respective GAPDH expressions. Data are illustrated as percentages of HO-1 mRNA levels induced by 0.25 mM phorone and expressed as means ± SD (n = 3). C: WI-38 cells were pretreated with 100 nM TPA for 24 h and treated for 4 h with 10 μM 4-HNE. Data are illustrated as percentages of 4-HNE-induced HO-1 mRNA levels and expressed as means ± SD (n = 3).

Wild-type and mutant forms of Nrf2, in which a single Ala was introduced at either Ser residue, were inserted into the GFP expression vector, and the fusion proteins were transiently expressed in COS-7 cells. Analysis of the subcellular distribution of the GFP hybrid proteins by fluorescence microscopy demonstrated that the wild-type Nrf2 fusion protein was dispersed throughout all cell compartments (data not shown), as observed previously (9, 13, 21). The distribution pattern of the fusion proteins of mutant Nrf2 seemed to be identical to that of the wild type. Coexpression of Keap1 resulted in localization of the wild-type Nrf2-GFP fusion protein in the cytoplasm of the majority of transfected cells, and nuclear distribution was seen in a very small number of cells (Fig. 4, B and C). In contrast to the effect of Keap1 coexpression, treatment with 4-HNE increased nuclear distribution and decreased cytoplasmic localization of the fusion protein in the transfected cells (Fig. 4, B and C). On the other hand, in cells expressing Nrf2(S40A)-GFP and Keap1, 4-HNE failed to induce the nuclear translocation of the mutant protein (Fig. 4, B and C). GFP fusion proteins with the other Ser to Ala mutants of Nrf2 (S387A, S447A, and S597A) showed nuclear accumulation in response to 4-HNE, as was seen in the wild type (Fig. 4C).
4, B and C). Moreover, coexpression of the GFP fusion protein with Keap1 and the Nrf2(S40E) mutant, in which Ser40 was replaced with Glu to mimic constitutive phosphorylation, showed nuclear accumulation in the absence of stimulus (Fig. 4, B and C), although the Nrf2(S597E)-GFP dispersed mainly in the cytoplasm, as seen in the wild type (Fig. 4C).

Coexpression of aPKCα with Nrf2-GFP and Keap1 induced the nuclear accumulation of the fusion protein; however, the kinase inactive mutant aPKCα(K274W) did not affect the cytoplasmic localization of Nrf2-GFP (Fig. 5A). Forced expression of aPKCα, but not of the aPKCα(K274W) mutant, induced ARE-driven reporter activity, and Ro-31-8220 inhibited this effect (Fig. 5B).
Transfection of aPKC expression vector induced the reporter activity as well (data not shown). ARE transactivation by forced expression of Nrf2 was suppressed by cotransfection of Keap1 (Fig. 5C). This inhibitory effect of Keap1 was reversed by simultaneous expression of aPKC and treatment of the cells with 4-HNE, which were abrogated by Ro-31-8220 (Fig. 5C). It has been reported that forced expression of aPKC(K274W) suppresses aPKC-mediated cellular signals in a dominant negative fashion (26). ARE-driven reporter activity induced by 4-HNE was repressed by coexpression of aPKC(K274W) but, in contrast, was enhanced by coexpression of the wild-type aPKC (Fig. 5D).

DISCUSSION

The present study demonstrates signal transduction leading to HO-1 gene expression under oxidative stress conditions caused by GSH depletion or exposure to lipid degradation products. The PKC inhibitor Ro-31-8220 suppressed endogenous HO-1 gene expression and ARE-driven enhancer activity induced by phorone and 4-HNE, implying that PKC-dependent signal transduction plays a role in the HO-1 induction. These responses induced by phorone and 4-HNE also appeared in cells in which cPKC and nPKC were down-regulated by TPA. Inhibitors that suppress cPKC and nPKC did not affect the induction of HO-1. Furthermore, our results indicating ARE-mediated transactivation by forced expression of aPKC and aPKC(K274W) support the idea that this subfamily is responsible for Nrf2 activation, although the identity of the main enzyme involved was not explored. Ro-31-8220 is derived from staurosporin and, hence, inhibits PKC in a manner competitive to ATP (34, 56). Therefore, it may inhibit other kinases such as protein kinase A (PKA) at higher concentrations (34). However, the specific PKA inhibitor H-89 did not affect the phorone- and 4-HNE-mediated induction of HO-1 gene expression (data not shown). Therefore, PKA is unlikely to be involved in the oxidative stress-induced HO-1 gene expression. It has been shown that aPKC isoforms are less sensitive than cPKC and nPKC to Ro-31-8220 (34). The concentrations of Ro-31-8220 (2 or 3 μM) that inhibited HO-1 gene expression and ARE transactivation in the present study are comparable to those that were previously reported to inhibit aPKC in a cell culture system (32, 37, 51). The ARE activation by ectopic expression of aPKC was also suppressed by Ro-31-8220.
treatment. Moreover, the dominant-negative mutant of aPKC prevented 4-HNE-induced ARE transactivation, indicating that the aPKC isoforms are involved, at least in part, in HO-1 gene expression induced by oxidative stress.

The present study demonstrated that HO-1 induction by 1 mM phorone, but not by 0.25 mM phorone or 4-HNE, was somewhat suppressed in cells in which cPKC and nPKC were downregulated by TPA. We have already shown that phorone-mediated GSH depletion induces activations of ERK (43) and JNK (44), which could be involved in the machinery that enhances the HO-1 induction. Because cPKC and nPKC activate these MAP kinases (40), it is possible that these PKC subfamilies are involved in the activation of the HO-1 gene expression indirectly in cells treated with the high concentration of phorone. On the other hand, the shutdown of the ERK-mediated signal transduction by a MEK inhibitor PD-98059 did not inhibit, but rather enhanced, the 4-HNE-mediated HO-1 gene expression (unpublished observation). Such results were consistent with the present study showing that pretreatment of TPA enhanced the effect of 4-HNE on the HO-1 induction. Although it is not known at present why downregulation of TPA-sensitive PKCs and inhibition of the ERK-cascade caused enhancement of the 4-HNE-induced HO-1 gene expression, the results indicate that these signals are not involved at least in the positive regulation of HO-1 gene expression caused by the treatment of cells with 4-HNE or a low dose of phorone.

The present study indicated that ectopically expressed Nrf2-GFP fusion protein was sequestered in cytoplasm as a result of cotransfection with Keap1 and that treatment with stressors, such as phorone and 4-HNE, induced nuclear translocation of the fusion protein. However, cellular distribution of the mutant in which Ser40 cannot be phosphorylated (Nrf2(S40A)) was not substantially affected by these stressors, although the introduction of other Ser residues, which could be phosphorylated by PKC, to Ala mutations had no effect on 4-HNE-mediated nuclear translocation of the fusion protein. Moreover, Keap1 was unable to sequester the Nrf2(S40E) mutant, which mimics constitutive phosphorylation, suggesting that phosphorylation of the Ser40 residue affected the stability of the Nrf2-Keap1 complex. Huang et al. (18) reported that tBHQ and TPA induce phosphorylation by PKC and nuclear translocation of Nrf2, although relatively high concentrations were used. The present study demonstrated that TPA at a lower concentration (10 nM), which is able to activate cPKC and nPKC, did not induce nuclear translocation of the Nrf2-GFP fusion protein, although a higher concentration (100 nM) induced nuclear accumulation, as reported previously (18). More recently, it was further revealed that Nrf2 Ser40 can be phosphorylated by PKC in vitro (17). Our present results strongly support this possibility and suggest that the Ser40 is also phosphorylated in cells.

It has been shown that aPKC isoforms are activated by products of PI 3-kinase, such as PI 3,4,5-triphosphate (38), and its downstream kinase phosphoinositide-dependent kinase, PDK1 (5). In addition, it has been reported recently that the PI 3-kinase inhibitors wortmannin and LY-294002 inhibit nuclear translocation of Nrf2 and ARE-mediated transactivation induced by tBHQ (25, 27). Hence, it is possible that aPKC transmit signals originating from PI 3-kinase to cause nuclear translocation of Nrf2. On the other hand, HO-1 gene expression and ARE-mediated transactivation are not significantly affected by the PI 3-kinase inhibitors (unpublished observation). Therefore, under oxidative stress conditions, aPKC might be controlled by as yet undefined molecule(s) that reside upstream from aPKC and are distinct from PI 3-kinase. Formation of 4-HNE adducts on the PKC isoforms, as in the case of the c-Jun NH2-terminal kinase modification, and its nuclear translocation in hepatic stellate cells (45) is also possible; however, further studies are needed to determine the precise mechanism of aPKC activation.

It has recently reported that ARE activation involves redox changes of the reactive Cys thiols in Keap1 protein, which induce its conformational change and the subsequent release of Nrf2 (15). The author of that study presented a model in which reactive Cys residue-rich Keap1 orchestrates the events triggered by oxidative stress. On the other hand, it has been demonstrated that dimerization of Keap1 plays a role in the sequestration of Nrf2 in cytoplasm and that electrophiles disrupt Keap1 complex formation (60). Moreover, suppression of protein degradation is postulated to be involved in Nrf2-mediated ARE activation (49, 52). Thus, although it is widely accepted that Nrf2 is a transcription factor that plays a central role in the induction of phase 2 proteins, several conflicting models of Nrf2 activation and its subsequent nuclear translocation have been presented to date. Consequently, the possibility that the transcription factor is activated in a stimulus-dependent manner or that divergent mechanisms act simultaneously cannot be ruled out. The role of these mechanisms should be further examined in detail.

In conclusion, the present study revealed that oxidative stress induces ARE activation via phosphorylation of Nrf2 Ser40 by aPKC. The regulation by aPKC of intracellular signal transduction leading to the expression of genes coding for a set of proteins that act in the detoxification of electrophiles is consistent with previous findings that aPKC plays a role in cell survival (7, 14, 36, 57).

DISCLOSURES

A part of this study was supported by a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

REFERENCES


10. Chan JY and Kwong M. Impaired expression of glutathione synthetic enzyme genes in mice with targeted deletion of the Nrf2 basic-leucine zipper protein. Biochem Biophys Acta 1517: 19–26, 2000.


49. Sekhar KR, Yan XX, and Freeman ML. Nrf2 degradation by the ubiquitin proteasome pathway is inhibited by KIAA0132, the human homolog to INR2. Oncogene 21: 6829–6834, 2002.

50. Standaert ML, Bandyopadhyay G, Kanoh Y, Sajan MP, and Farese RV. Insulin and PI3P activate PKC-ζ by mechanisms that are both dependent and independent of phosphorylation of activation loop (T410) and autophosphorylation (T650) sites. Biochemistry 40: 249–255, 2001.


