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PKC Downstream of PI3-Kinase Regulates Peroxynitrite Formation for Nrf2-Mediated GSTA2 Induction

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The protective adaptive response to electrophiles and reactive oxygen species is mediated by the induction of phase II detoxifying genes including glutathione S-transferases (GSTs). NF-E2-related factor-2 (Nrf2) phosphorylation by protein kinase C (PKC) is a critical event for its nuclear translocation in response to oxidative stress. Previously, we have shown that peroxynitrite plays a role in activation of Nrf2 and Nrf2 binding to the antioxidant response element (ARE) *via* the pathway of phosphatidylinositol 3-kinase (PI3-kinase) and that nitric oxide synthase in hepatocytes is required for GSTA2 induction. In view of the importance of PKC and PI3-kinase in Nrf2-mediated GST induction, we investigated the role of these kinases in peroxynitrite formation for GSTA2 induction by oxidative stress and determined the relationship between PKC and PI3-kinase. Although PKC activation by phorbol 12-myristate-13-acetate (PMA) did not increase the extents of constitutive and inducible GSTA2 expression, either PKC depletion by PMA or PKC inhibition by staurosporine significantly inhibited GSTA2 induction by *tert*-butylhydroquinone (*t*-BHQ) a prooxidant chemical. Therefore, the basal PKC activity is requisite for GSTA2 induction. 3-Morpholinosydnonimine (SIN-1), which decomposes and yields peroxynitrite, induced GSTA2, which was not inhibited by PKC depletion, but slightly enhanced by PKC activation, suggesting that PKC promotes peroxynitrite formation for Nrf2-mediated GSTA2 induction. Treatment of cells with *S*-nitroso-*N*-acetyl-penicillamine (SNAP), an exogenous NO donor, in combination with *t*-BHQ may produce peroxynitrite. GSTA2 induction by SNAP + *t*-BHQ was not decreased by PKC depletion, but rather enhanced by PKC activation, showing that the activity of PKC might be required for peroxynitrite formation. LY294002 a PI3-kinase inhibitor blocked GSTA2 induction by *t*-BHQ, which was reversed by PMA-induced PKC activation. These results provide evidence that PKC may play a role in formation of peroxynitrite that activates Nrf2 for GSTA2 induction and that PKC may serve an activator for GSTA2 induction downstream of PI3-kinase.

Key words: Glutathione *S*-transferase, Peroxynitrite, PKC, PI3-kinase, Nrf2

INTRODUCTION

Glutathione S-transferases (GSTs) are an important family of detoxifying and cytoprotective enzymes. Studies from ours and other laboratories have shown that the expression of the *GSTA2* gene is transcriptionally regulated by Nrf2 activation (Nguyen and Pickett, 1992; Liu and Pickett, 1996; Kang *et al.*, 2000, 2001). GSTs are induced as part of the adaptive responses to electrophiles and reactive oxygen species. NF-E2-related factor-2 (Nrf2) plays a role in antioxidant response element -mediated GST induction in response to oxidative stress (Nguyen and Pickett, 1992; Liu and Pickett, 1996; Kang *et al.*, 2000, 2001). It appears that Nrf2 phosphorylation by protein kinase C (PKC) is a critical event for nuclear translocation of Nrf2 (Huang *et al.*, 2000). PKC-catalyzed phosphorylation of Nrf2 at Ser-40 is a signaling event leading to ARE-mediated cellular responses (Huang *et al.*, 2002). In addition, it has been shown that atypical PKC mediates activation of Nrf2 in response to oxidative stress (Numazawa *et al.*, 2003).

Previously, we have shown that peroxynitrite activates Nrf2 and Nrf2 binding to the ARE *via* the pathway of phosphatidylinositol 3-kinase (PI3-kinase) (Kang *et al.*, 2002). We also showed that PI3-kinase plays an essential

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role in the ARE-mediated GSTA2 induction by *tert*-butylhydroquinone (*t*-BHQ) a prooxidant chemical (Kang *et al.*, 2001). Nitric oxide (NO) regulates a number of physiological responses including stimulation of signal transduction pathways (Marshall *et al.*, 2000; Park *et al.*, 2000). Peroxynitrite, formed by reaction of NO with superoxide, is a potent oxidizing species and serves as a signal molecule in the cell. In our previous studies, constitutive nitric oxide synthase (cNOS) was required for Nrf2-mediated GSTA2 induction in H4IIE hepatocytes (Kang *et al.*, 2002), implicating that NO in hepatocytes reacts with other reactive oxygen species (ROS) for production of peroxynitrite. Nevertheless, the relationship between PKC and PI3-kinase in GST induction by peroxynitrite has not been clarified.

In view of the importance of PKC and PI3-kinase in Nrf2-mediated GST induction, this study investigated the role of these kinases in peroxynitrite formation for GSTA2 induction, examining the hypothesis that PI3-kinase controls PKC. In the present study, we used the chemical agents that produce distinct ROS to assess the effects of prooxidant, peroxynitrite and nitric oxide on GSTA2 expression in the H4IIE cells in which PKC was depleted or activated by phorbol 12-myristate 13-acetate (PMA).

MATERIALS AND METHODS

Materials

The antibody directed against GSTα was supplied from Detroit R&D (Detroit, MI). Horseradish peroxidase-conjugated rabbit anti-goat IgG was obtained from Zymed Laboratories Inc. (San Francisco, CA). PMA, staurosporine, 3-morpholinosydnonimine (SIN-1), *S*-nitroso-*N*-acetyl-penicillamine (SNAP) and LY294002 were purchased from Calbiochem (San Diego, CA). *tert*-Butylhydroquinone (*t*-BHQ, 97%) was obtained from Sigma-Aldrich (St. Louis, MO). Other reagents in the molecular studies were supplied from Sigma Chemical Co. (St. Louis, MO).

Cell culture

H4IIE cells, a rat hepatocyte-derived cell line, were obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS), 50 U/mL penicillin, and 50 μg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO_2. For PKC depletion and activation, cells were treated with 1 μM PMA for 18 h and 30 min, respectively. The cells were then exposed to *t*-BHQ (10 or 30 μM), SIN-1 (300 μM) or SNAP (100 μM) for 24 h. For some experiments, cells were incubated with 1 or 3 nM staurosporine or 30 μM LY294002 for 1 h and then subsequently exposed to 30 μM *t*-BHQ. Cells were washed twice with ice-cold phosphate buffered saline (PBS) before sample preparation.

Immunoblot analysis

After washing the cells with sterile PBS, the cells were scraped and sonicated for disruption. Cytosolic fractions were prepared by differential centrifugation. The subcellular preparations were stored at -70°C until use. Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis and immunoblot analysis were performed according to the previously published procedures (Kim *et al.*, 1997; Kang *et al.*, 2003). Briefly, samples were separated by 12% gel electrophoresis and electrophoretically transferred to nitrocellulose paper. The nitrocellulose paper was incubated with anti-rat GSTα antibody (1:1000) followed by incubation with horseradish peroxidase-conjugated secondary antibody, and developed using an ECL chemiluminescence detection kit (Kang *et al.*, 2003). Anti-rat GSTα antibody recognized both GSTA2 and GSTA3/5, which were clearly resolved by 12% gel electrophoresis. Equal loading of samples in the immunoblots was verified by SDS-PAGE and coomassie staining of replicate gels. Specificity of anti-GSTα antibody has been confirmed by the previous studies (Kim *et al.*, 1997; Kang *et al.*, 2003; Cho *et al.*, 2000).

Data analysis

Scanning densitometry was performed with Image Scan & Analysis System (Alpha-Innotech Corporation, San Leandro, CA). One way analysis of variance (ANOVA) procedures were used to assess significant differences among treatment groups. For each significant effect of treatment, the Newman-Keuls test was used for comparisons of multiple group means. The criterion for statistical significance was set at $p<0.05$ or $p<0.01$.

RESULTS

PKC plays a role in GSTA2 induction by *t*-BHQ

Previously, we have shown that peroxynitrite activates Nrf2 and Nrf2 binding to the ARE *via* the pathway of PI3-kinase and that cNOS in hepatocytes is required for the GSTA2 induction (Kang *et al.*, 2002). Prolonged treatment of cells with PMA (e.g. 18 h) resulted in depletion of PKC, whereas treatment of cells with PMA for a short period of time (e.g. 30 min) strongly activated PKC (Shah and Catt, 2002; Kang *et al.*, 2003; Vaughan *et al.*, 1997). In view of the role of PKC in Nrf2-mediated GSTA2 induction, we first determined whether PKC depletion or activation by PMA altered the extent of GSTA2 induction in H4IIE cells treated with *t*-BHQ (30 μM). PMA treatment of the cells for 18 h or 30 min resulted in no change in the constitutive expression of GSTA2 (Fig. 1A). Depletion of PKC by PMA pretreatment for 18 h prevented GSTA2 induction by 30

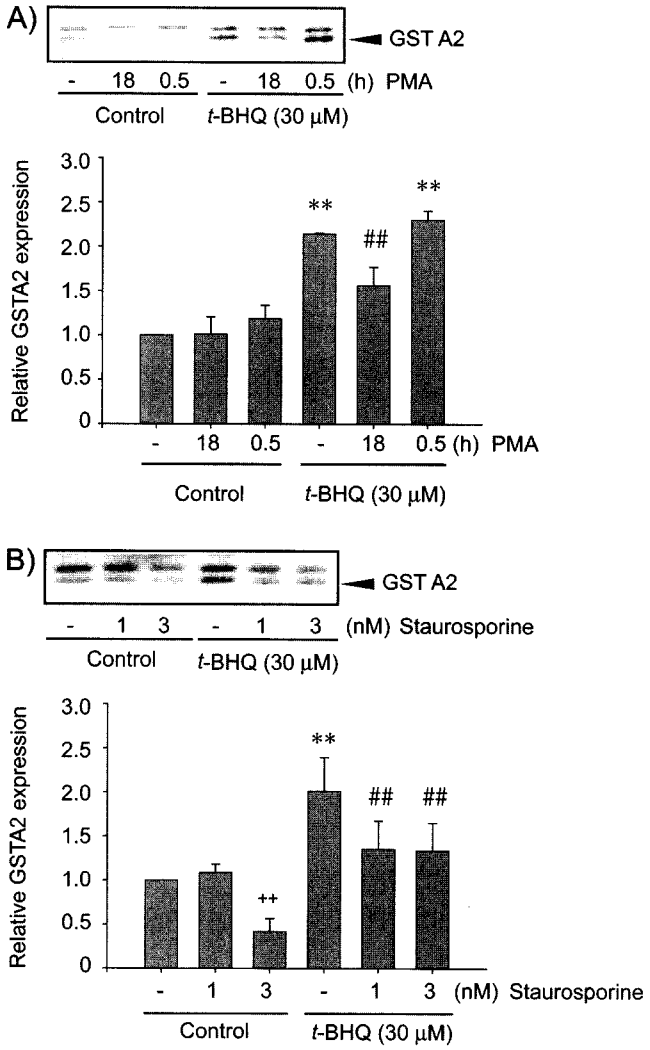

Fig. 1. Role of PKC in GSTA2 induction by *t*-BHQ. A) The effects of *t*-BHQ on the expression of GSTA2 in the cells pretreated with PMA. Immunoblot analysis shows the levels of GSTA2 in H4IIE cells that had been treated with PMA for 18 h or 30 min prior to *t*-BHQ treatment (30 μM, for 24 h). Each lane was loaded with 10 μg of cytosolic proteins. B) The effect of staurosporine on the constitutive and *t*-BHQ-inducible GSTA2 expression. Cells were treated with 30 μM of *t*-BHQ for 30 min in the presence or absence of staurosporine (1 or 3 nM). The level of GSTA2 was assessed by scanning densitometry of immunoblots. Data represent the mean ± S.D. with 3 separate experiments. One way analysis of variance was used for comparisons of multiple group means followed by Newman-Keuls test (significant as compared to control, $^{**}p<0.01$, $^{++}p<0.01$; significant as compared to *t*-BHQ alone, $^{\#\#}p<0.01$) (the level of GSTA2 in control cells = 1).

μM *t*-BHQ. In contrast, PKC activation enhanced *t*-BHQ induction of GSTA2 in H4IIE cells (Fig. 1A). To confirm the effect of PKC inhibition on the GSTA2 expression, cells were treated with staurosporine a chemical PKC inhibitor. Either the constitutive or the inducible GSTA2 expression was significantly inhibited by treatment of cells with 1 or 3 nM staurosporine (Fig. 1B). These results indicated that PKC was required for the induction of GSTA2 by the prooxidant.

PKC depletion did not change GSTA2 induction by SIN-1

SIN-1 under aerobic conditions decomposes NO and superoxide and thus yields mostly (i.e. ~76%) peroxynitrite (Klotz *et al.*, 2000). Previous studies have shown that Nrf2, located predominantly in the cytoplasm of untreated cells, translocated into the nucleus after treatment with SIN-1 and that activating Nrf2 bound to the ARE for GSTA2 induction (Kang *et al.*, 2002). In the current study, we confirmed that SIN-1 at the concentration of 300 μM induced GSTA2. GSTA2 induction by SIN-1, however, was not inhibited by PKC depletion, but enhanced by PKC activation (Fig. 2). The observation that GSTA2 induction by peroxynitrite was not changed by PKC depletion showed that PKC activity was required upstream of peroxynitrite formation. These data suggested that PKC serve as an activator for peroxynitrite production.

Our previous study showed that NO was required for GSTA2 induction, but NO alone was not sufficient to induce the enzyme (Kang *et al.*, 2002). This was evidenced by the observation that SNAP, an exogenous NO donor, alone did not increase GSTA2 mRNA although SNAP in combination with GSH depletion (i.e., oxidative stress) increased GSTA2 mRNA (Kang *et al.*, 2002). Similarly, SNAP plus a low concentration of *t*-BHQ (10 μM) induced

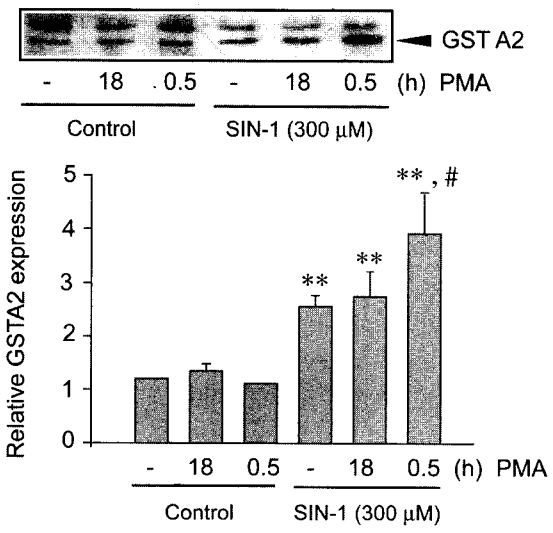

Fig. 2. The effects of PMA pretreatment on GSTA2 induction by SIN-1. Immunoblot analysis shows the levels of GSTA2 in H4IIE cells pretreated with PMA for 18 h or 30 min followed by treatment with 300 μM SIN-1 for 24 h. Immunoblot analysis was performed as described in Fig. 1. The level of GSTA2 was assessed by scanning densitometry of immunoblots. Data represent the mean ± S.D. with 3 separate experiments (significant as compared to control, $^{**}p<0.01$; significant as compared to SIN-1 alone, $^{\#}p<0.05$)(control level = 1).

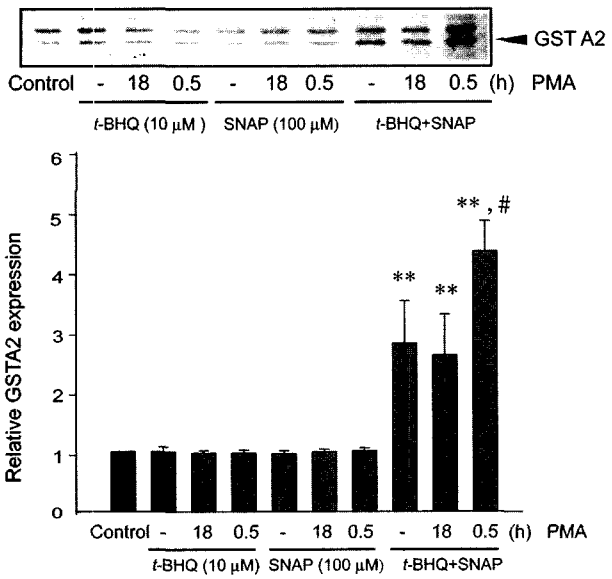

Fig. 3. The effects of PKC depletion or activation on GSTA2 induction by *t*-BHQ + SNAP. Immunoblot analysis of GSTA2 protein in the cells treated with PMA for 18 h or 30 min followed by exposure to 10 μM *t*-BHQ, 100 μM SNAP or *t*-BHQ + SNAP. The level of GSTA2 was assessed, as described in Fig. 1. Data represent the mean ± S.D. with 3 separate experiments. One way analysis of variance was used for comparisons of multiple group means followed by Newman-Keuls test (significant as compared to control, **p<0.0; significant as compard to *t*-BHQ+SNAP, #p<0.051) (control level = 1).

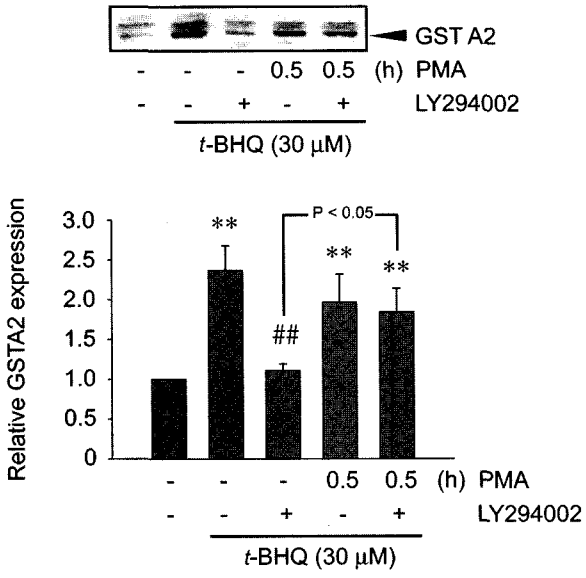

Fig. 4. Reversal by PKC activation of LY294002 inhibition of *t*-BHQ-inducible GSTA2 induction. H4IIE cells were treated with *t*-BHQ alone for 24 h or the cells pretreated with LY294002 (LY, 30 μM) for 1 h were continuously exposed to 30 μM *t*-BHQ + LY294002 for 24 h. To assess the effect of PKC activation, cells were treated with PMA for 30 min prior to treatment with *t*-BHQ or *t*-BHQ + LY294002. The levels of GSTA2 were assessed, as described in Fig. 1. Data represent the mean ± S.D. with 3 separate experiments (significant as compared to control, **p<0.01; significant as compared to *t*-BHQ alone, ##p<0.01) (control level = 1).

GSTA2 (Fig. 3). GSTA2 induction by SNAP plus *t*-BHQ was not inhibited by PKC depletion. In contrast, PKC activation by PMA treatment notably enhanced GSTA2 induction (24 h) (Fig. 3). These data further supported the hypothesis that PKC may be involved in activation process for peroxynitrite formation.

PKC is involved in GSTA2 induction downstream of PI3-kinase

We previously showed that oxidative stress activates PI3-kinase and increased phosphorylation of Akt and that inhibition of PI3-kinase activity prevented GSTA2 induction by oxidative stress (Kang *et al.*, 2000, 2001). We were interested in whether GST repression by PI3-kinase inhibition could be reversed by PKC activation. Treatment of H4IIE cells with LY294002 a PI3-kinase inhibitor completely inhibited GSTA2 induction by *t*-BHQ (30 μM) (Fig. 4). Repression of GSTA2 by LY294002 was significantly reversed by PKC activation with PMA. Thus, it is highly likely that PKC regulates GST induction downstream of PI3-kinase.

DISCUSSION

Previously, we found that peroxynitrite, a ROS derived from NO and superoxide, contributed to GSTA2 induction

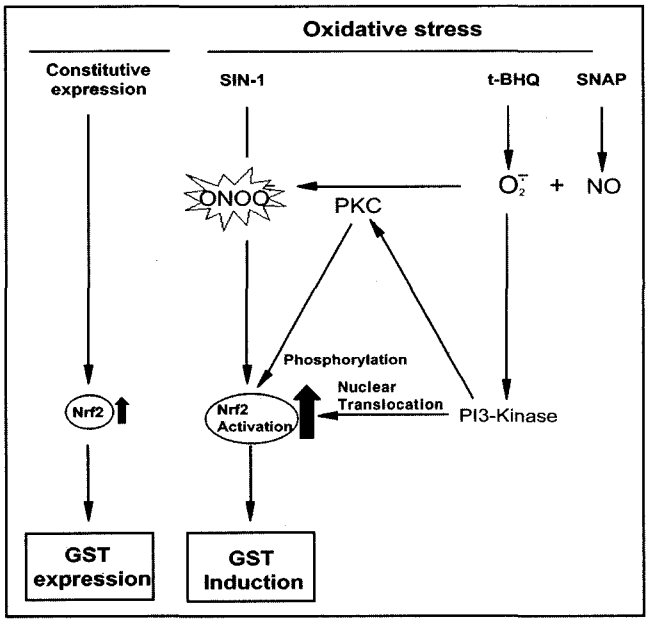

Fig. 5. Schematic diagrams illustrating the pathways by which peroxynitrite induces GSTA2. GSTA2 induction by peroxynitrite requires activation of Nrf2. PI3-kinase, which regulates nuclear translocation of Nrf2, is stimulated by oxidative stress. PKC, which works downstream of PI3-kinase, is responsible for peroxynitrite formation. Peroxynitrte is produced in cells by reaction of oxygen species with NO that is generated by cNOS in hepatocytes. PKC may activate cNOS in hepatocytes.

by GSH depletion and that PI3-kinase-mediated nuclear translocation of Nrf2 preceded transcriptional activation of the *GSTA2* gene (Kang *et al.,* 2000, 2001). In spite of the apparent contribution of peroxynitrite to Nrf2-mediated GSTA2 induction, the cellular signaling pathways have not been completely defined. In this study, we employed H4IIE hepatocytes pretreated with PMA for PKC depletion or activation. We also used staurosporine to confirm the effect of PKC inhibition on GSTA2 induction. Now, we report that PKC may play a crucial role in formation of peroxynitrite that activates Nrf2 for GSTA2 induction.

Transcription factor Nrf2 belongs to the cap-n-collar family of activators. Nrf2 binds to the ARE in the promoter regions of target genes (McMahon *et al.,* 2001; Itoh *et al.,* 2003). *t*-BHQ induces GSTA2 primarily through Nrf2 activation and binding of activating Nrf2 to the ARE (Kang *et al.,* 2001). Peroxynitrite, which serves as a signal molecule for activation of the transcription factor, stimulates ARE-dependent transcriptional activation of the phase II detoxifying gene (Kang *et al.,* 2002). SIN-1 is a chemical producing equimolar NO and superoxide, and hence generates peroxynitrite. SIN-1 activated PI3-kinase and Akt (Kang *et al.,* 2002; Klotz *et al.,* 2000) and actively translocated cytosolic Nrf2 to the nucleus for ARE activation (Kang *et al.,* 2002). Therefore, SIN-1 increased GSTA2 mRNA and protein. In the present study, we used SIN-1 to further assess the role of PKC in GSTA2 induction by peroxynitrite. No inhibition by PKC depletion of SIN-1 induction of GSTA2 strongly supported the hypothesis that PKC plays a crucial role in peroxynitrite formation for Nrf2-mediated GSTA2 induction. This hypothesis was further supported by the observation that PKC activation by PMA enhanced GSTA2 induction by SIN-1. Thus, it is highly likely that PKC is involved in peroxynitrite production presumably through the activating process of NO generation.

Depletion of GSH increases the susceptibility of animals to free radical-induced tissue damage and stimulates adaptive responses such as induction of phase II enzymes (e.g. GST). Previously, we observed that cNOS was highly expressed in H4IIE cells although the extent of NO production by cNOS is much less than that by inducible NOS. It appears that either inhibition of NOS by N^G-nitro-L-arginine methyl ester (L-NAME, a NOS inhibitor), or deficiency of L-arginine, an amino acid substrate required for NO production, reduced peroxides production by oxidative stress. Either L-NAME treatment or L-arginine deficiency inhibited Nrf2 translocation by oxidative stress. Consistently, GSTA2 induction by sulfur amino acid deprivation (i.e. GSH deficiency) was almost completely inhibited by inhibition of NOS (Kang *et al.,* 2002). These results indicated that NO physiologically produced by catalysis of cNOS contributed to peroxynitrite formation. The observation that SNAP enhances GSTA2 induction by GSH depletion (Kang *et al.,* 2002) also supported the conclusion that peroxynitrite produced by reaction of NO with ROS (e.g. superoxide) leads to GSTA2 induction.

Although SNAP, a NO donor, alone was inactive in inducing GSTA2 (Kang *et al.,* 2002), SNAP in conjunction with GSH depletion increased the levels of GSTA2 mRNA and protein. Requirement of both NO and other oxygen species was in agreement with the observation that treatment of cells with NO plus a low concentration of *t*-BHQ induced GSTA2 (Fig. 3). Because peroxides produced in cells include superoxide, SNAP plus *t*-BHQ would help produce peroxynitrite. In the present study, we observed that PKC activation by PMA notably enhanced GSTA2 induction by *t*-BHQ+SNAP. Hence, the rate of NO production may be the determining step for peroxynitrite production, in which PKC is likely to be involved. The amount of peroxynitrite may determine the extent of Nrf2 activation and thus Nrf2/ARE-mediated GSTA2 induction.

Inhibition by LY294002 of *t*-BHQ induction of GSTA2, which is mediated with Nrf2 binding to ARE (Kang *et al.,* 2000, 2001), verified the essential role of PI3-kinase in the GST gene regulation. LY294002 inhibition of GSTA2 induction was reversed in the cells in which PKC was activated, suggesting that PKC be involved in NO production possibly through activation of cNOS. Thus, peroxynitrite derived from NO may contribute to Nrf2 activation with the aid of PI3-kinase (Fig. 5), leading to Nrf2-mediated GSTA2 induction (Kang *et al.,* 2002). Peroxynitrite also activates PI3-kinase, as supported by the observation that SIN-1 increases the activity of PI3-kinase with Nrf2 activation (Kang *et al.,* 2002). There would be a certain degree of mutual activation between PKC and PI3-kinase, in which peroxynitrite may play a role as a limiting factor.

In conclusion, the present study provide evidence that PKC, which works downstream of PI3-kinase, may play a crucial role in peroxynitrite formation and that peroxynitrite is responsible for Nrf2-mediated GSTA2 induction, in which PI3-kinase regulates nuclear translocation of Nrf2.

ABBREVIATIONS

ARE, antioxidant response element; GSH, glutathione; GST, glutathione *S*-transferase; Nrf2, NF-E2-related factor-2; PI3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; PMA, phorbol 12-myristate-13-acetate; ROS, reactive oxygen species; SIN-1, 3-morpholinosydnonimine; SNAP, *S*-nitroso-*N*-acetyl-penicillamine; *t*-BHQ, *tert*-butylhydroquinone

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