p38 Mitogen-activated Protein Kinase Negatively Regulates the Induction of Phase II Drug-metabolizing Enzymes That Detoxify Carcinogens*

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Phase II drug-metabolizing enzymes, such as glutathione S-transferase and quinone reductase, play an important role in the detoxification of chemical carcinogens. The induction of these detoxifying enzymes by a variety of agents occurs at the transcriptional level and is regulated by a cis-acting element, called the antioxidant response element (ARE) or electrophile-responsive element. In this study, we identified a signaling kinase pathway that negatively regulates ARE-mediated gene expression. Treatment of human hepatoma HepG2 and murine hepatoma Hepa1c1c7 cells with tert-butylhydroquinone (tBHQ) stimulated the activity of p38, a member of mitogen-activated protein kinase family. Inhibition of p38 activation by its inhibitor, SB203580, enhanced the induction of quinone reductase activity and the activation of ARE reporter gene by tBHQ. In contrast, SB202474, a negative analog of SB203580, had little effect. Consistent with this result, interfering with the p38 kinase pathway by overexpression of a dominant-negative mutant of p38 or MKK3, an immediate upstream regulator of p38, potentiated the activation of the ARE reporter gene by tBHQ, whereas the wild types of p38 and MKK3 diminished such activation. In addition, inhibition of p38 activity augmented the induction of ARE reporter gene activity by tert-butylhydroxyanisole, sulforaphane, and β-naphthoflavone. Thus, p38 kinase pathway functions as a negative regulator in the ARE-mediated induction of phase II detoxifying enzymes.

Preexposure of human and rodent cells to some chemical compounds such as tert-butylhydroquinone (tBHQ),¹ tert-butylhydroxyanisole (BHA), and sulforaphane (SUL) prevents their ability to selectively induce phase II drug-metabolizing enzymes such as quinone reductase (QR), glutathione S-transferase (GST), UDP-glucuronosyl transferase, and epoxide hydrolase (3–7). Biochemical and genetic studies show that the induction of these detoxifying enzymes is primarily due to the transcriptional activation of genes and is regulated by an enhancer, called antioxidant responsive element (ARE) or electrophile-responsive element (8–11). Several nuclear proteins have been found to interact with ARE and to regulate phase II gene expression, although controversial results were obtained (12–17). However, the signal transduction pathways that relay the chemical signals to ARE-protein complex remain to be elucidated.

p38 is a member of the mitogen-activated protein kinase (MAPK) family that plays an important role in transducing extracellular signals into the nucleus. p38 MAPK was originally identified in endotoxic lipopolysaccharide-treated murine monocytes and macrophages as a homologue of high osmolality glycerol response 1 in yeast (18, 19). Independently, p38 was also identified in human cells as a cytokine-suppressive anti-inflammatory drug-binding protein (20) or as a reactivating kinase (21, 22). The kinase activity of p38 is regulated by phosphorylation on its threonyl and tyrosyl residues within the tripeptide motif TGY by dual specificity kinases, MKK3 (23) and MKK6 (24), which, in turn, are regulated by the upstream MAPK kinase kinases and small GTP-binding proteins, Rac1 and Cdc42 (25). The p38 pathway has been shown to be activated by various physiological and environmental stress signals such as proinflammatory cytokines (26), endotoxin (27), heat shock (21), UV radiation (26), and hyperosmolarity (18). Once activated, p38 can phosphorylate many transcription factors, such as ATF2 (23), Elk-1 (28), CHOP/GADD153 (29), MEF2C (30), and SAP-1 (31), leading to the changes of gene expression. The activated p38 can also phosphorylate MAPK-activated protein kinase 2 and 3, which subsequently phosphorylate a small heat shock protein, HSP27 (21, 22, 32). Although the exact physiological roles of p38 remain to be defined, this enzyme has been implicated in the production of cytokines (33), T cell proliferation (34), neuronal differentiation (35), platelet aggregation (36), and apoptosis (37). In this study, we demonstrated that p38 was involved in the regulation of phase II gene expression and acted as a negative regulator. We also showed that such a negative role of p38 was regulated by an upstream kinase, MKK3.

MATERIALS AND METHODS

Cell Culture, Antibodies, DNA Plasmids, and Chemicals—Human HepG2 and mouse Hepa1c1c7 hepatoma cell lines were obtained from...
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ATCC (Manassas, VA) and were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 2.2 g/liter sodium bicarbonate, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were normally starved overnight in serum-free medium before treatment, unless otherwise indicated. The rabbit anti-p38 polyclonal antibody was purchased from New England Biolabs Inc. (Beverly, MA). The anti-HA monoclonal antibody (12CA5) was purchased from Roche Molecular Biochemicals. GST-ATF2 (1-96)-expressing plasmid was kindly provided by Dr. J. Silvio Gutkind (National Institutes of Health, Bethesda, MD). pARE-TI-luciferase reporter construct (containing a single copy of the 41-base pair murine GST-Ya ARE and minimal TATA-

Inhibition of tBHQ-induced p38 activation by SB203580. HepG2 (A) or Hepa1c1c7 (B) cells were incubated with SB203580 (5 µM), SB202474 (5 µM), or solvent (0.1% Me2SO) for 1 h prior to challenge with tBHQ (100 µM) for an additional 1 h. Cells were harvested, and the endogenous p38 kinase activity was determined by immunocomplex kinase assays using 5 µg of GST-ATF2 (1-96) fusion protein as substrate. The protein level of p38 was determined by Western blotting. The experiment was repeated three times.

Bradford method, was added to the cuvette containing 1 ml of reaction buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 50 mM NaF, 100 µM Na2VO4, 5 µM ZnCl2, 30 mM Na3PO4, 2 mM iodoacetic acid, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100) and twice with kinase assay buffer (20 mM HEPES, pH 7.9, 10 mM MgCl2, 2 mM MnCl2, 0.1 mM Na2VO4, 50 mM β-glycerophosphate, and 10 mM p-nitrophenyl phosphate). Kinase reaction was performed by incubation of the immunoprecipitate with 5 µg of GST-ATF2, 2 µCi of [γ-32P]ATP, and 20 µM ATP in a 30-µl kinase assay for 30 min and terminated by Laemmli buffer. The phosphorylated product was heated to 95 °C for 5 min and analyzed by electrophoresis, autoradiography, and phosphor imaging.

Transient Transfection and Assays for Reporter Gene Activity—Selection for Stably Transfected HepG2 Cell Lines—HepG2 cells were transfected with pARE-TI-luciferase construct using a FuGENE TM 6 protocol (Roche Molecular Biochemicals). The transfection mixture was replaced with fresh medium 12 h after transfection. Cells were cultured for an additional 12 h prior to drug treatment. The β-galactosidase activity was determined as described previously (40). The luciferase activity was determined by the ECL system (Amersham Pharmacia Biotech).

RESULTS

Phase II Gene Inducer, tBHQ, Stimulates p38 Activity That Is Inhibited by SB203580—Human hepatoma HepG2 and mouse hepatoma Hepa1c1c7 have been used as the model cell lines for the study of phase II enzyme induction by various agents (41–43). Accordingly, we examined the effect of tBHQ, a commonly used phase II enzyme inducer, on p38 activity in the two cell lines. As shown in Fig. 1A, 100 µM tBHQ strongly induced p38 activity in HepG2 cells, as determined by immunocomplex kinase assays. Pretreatment with SB203580, a selective inhibitor of p38 (20, 44), attenuated the p38 activation by tBHQ, whereas SB202474, a negative analog of SB203580 (20), had little effect. Treatment with SB203580 alone also significantly decreased the basal activity of p38 as compared with the control cells. Similar results were obtained when Hepa1c1c7 cells were tested (Fig. 1B).

Inhibition of p38 Activity Augments the Induction of Quinone Reductase Activity by tBHQ—Phase II detoxifying enzymes include GST, UDP-glucuronosyl transferase, epoxide hydrolase, and QR. Experiments with Hepa1c1c7 cells showed that QR induction is a useful indicator of overall phase II enzyme induction (3, 41, 43). To understand the role of p38 activation in phase II enzyme regulation, we examined the effect of p38 on the induction of QR activity by tBHQ. Treatment of Hepa1c1c7 cells with tBHQ (100 µM) for 24 h resulted in a 4-fold induction of QR activity (Fig. 2). Surprisingly, co-treatment of tBHQ with SB203580 potentiated such induction. Furthermore, SB203580 alone also increased the QR activity. In contrast, the negative analog SB202474 had no effect on either.
SB203580. Hepa1c1c7 cells were treated with SB203580 (5 μM), SB202474 (5 μM), or solvent (0.1% Me2SO) for 1 h and then stimulated with tBHQ (100 μM) for 24 h. Cells were harvested and assayed for QR activity by measuring the reduction of 2,6-dichloroindophenol as described under “Materials and Methods.” The data shown are means of four independent experiments ± S.D.

the basal or tBHQ-induced QR activity. These results suggest that activation of p38 has a negative effect on the induction of phase II genes such as QR.

Inhibition of p38 Activity Also Enhances the Induction of ARE-dependent Reporter Gene Expression by tBHQ—Previous studies have shown that the induction of QR activity by tBHQ is transcriptionally regulated by an ARE DNA response element (45, 46). To examine if the inhibitory effect of p38 on tBHQ induction of QR activity is due to the changes in ARE-dependent gene expression, we performed transient and stable transfection assays. As shown in Fig. 3A, tBHQ induced more than 3-fold luciferase activity in HepG2 cells transfected transiently with ARE-luciferase reporter construct. SB203580 but not SB202474 significantly enhanced tBHQ-induced luciferase activity. Furthermore, SB203580 alone that inhibited the basal activity of p38 (Fig. 1A) stimulated luciferase activity (approximately 2-fold over control). In contrast, no induction of luciferase activity was observed when HepG2 cells were transiently transfected with the control vector (Ti-luciferase), indicating that the activation of reporter gene was specifically mediated by the ARE enhancer. Similar results were obtained in a HepG2-derived cell line (C4) that was stably transfected with an ARE-luciferase construct. SB203580 potentiated the induction of luciferase activity by tBHQ in a dose-dependent manner, whereas SB202474 had little effect even at 20 μM concentration (Fig. 3B).

Dominant Negative Mutant of p38 Potentiates tBHQ-induced ARE Reporter Gene Activity—To provide further evidence for the negative role of p38, we examined the effect of blockade of the p38 pathway with a dominant-negative p38 mutant on tBHQ-induced ARE reporter gene activity. HepG2 cells were transfected with ARE-luciferase reporter construct together with the plasmid encoding a dominant-negative p38 mutant, p38(AGF), or wild-type p38. After transfection, cells were treated with tBHQ (100 μM) for 24 h or left untreated as control. Overexpression of p38(AGF) substantially augmented the induction of ARE reporter gene by tBHQ in a dose-dependent manner (Fig. 4). Similar to SB203580, mutant p38 also raised the basal activity of ARE reporter gene in untreated cells. In contrast, forced expression of wild-type p38 decreased both basal and tBHQ-induced ARE reporter gene activities (Fig. 4). These data substantiate a negative role of p38 in the activation of ARE-dependent phase II genes.

Inhibition of p38 Potentiates the Activation of ARE-dependent Genes by BHA, SUL, and β-NF—Phase II detoxifying enzymes are induced by a variety of chemicals. We next asked whether p38 MAPK plays a similar role in the induction of phase II enzymes by other inducers. As shown in Fig. 5A, treatment with BHA, β-NF, and SUL stimulated the luciferase activity in the stably transfected C4 cells. The induction of luciferase activity by all three inducers was strongly enhanced by cotreatment with SB203580 but not with SB202474. Treatment with SB203580 alone also stimulated luciferase activity in the C4 cells. These results suggest that p38 also functions as a negative regulator of BHA-, β-NF-, and SUL-induced phase II
The induction of ARE reporter gene by tBHQ. HepG2 cells were transfected in duplicate with 0.5 μg of pCH110-β-gal, 0.5 μg of an ARE-luciferase reporter construct, and different amounts of the empty vector (Vec.) or the expression vectors for wild-type p38 or a dominant-negative p38 mutant, p38(AGF). After transfection, cells were incubated in culture medium for 12 h and then treated with tBHQ (100 μM) for 24 h, or they were left untreated as control. Luciferase activity was assayed and normalized against β-galactosidase activity. The amount of luciferase activity in untreated empty vector-transfected cells was arbitrarily set to 1. The data shown are means of three independent experiments.

gene expression. Interestingly, BHA and β-NF, like tBHQ, stimulated p38 activity that was inhibited by SB203580, whereas SUL is inactive (Fig. 5B).

Activation of ARE-dependent Genes by Inhibition of p38 Shows an Earlier Kinetics than That by tBHQ—The data above indicate that p38 negatively regulates both basal and inducible ARE-mediated gene expression. To obtain information on the potential target of p38, we compared the time courses of the effect of SB203580 and the effect of tBHQ on ARE-driven transcription in C4 cells. As shown in Fig. 6, no significant induction of luciferase activity was observed until 6 h after challenge with tBHQ (100 μM). However, the induction of luciferase activity by SB203580 (5 μM) appeared earlier and was detectable 3 h post-treatment. The stimulated luciferase activity by both agents accumulated as the treatments progressed. This result suggests that p38 may act directly on ARE-binding proteins or on the proximal components of the ARE-hinging proteins.

The Negative Role of p38 in ARE-mediated Gene Expression Is Regulated by An Upstream Kinase, MKK3—To understand the mechanism of p38 activation by tBHQ, we examined the roles of upstream kinases, MAPK kinases. Previous studies have shown that p38 activity can be regulated by both MKK3 and MKK4 (23, 24). However, MKK4 but not MKK3 can also activate c-Jun N-terminal kinase (23, 25). Given that tBHQ is a poor activator of the c-Jun N-terminal kinase pathway (47), we focused on the role of MKK3. As shown in Fig. 7A, while expression of MKK3 attenuated the induction of ARE reporter gene activity by tBHQ, dominant-negative MKK3 mutant, MKK3(ala), augmented the induction. Consistent with the luciferase assay result, wild-type MKK3 potentiated the activation of p38 by tBHQ, whereas MKK3(ala) showed the opposite effect (Fig. 7B). These data suggest that MKK3 is an upstream regulator for the negative role of p38 in tBHQ-induced phase II gene expression.

DISCUSSION

Phase II detoxifying enzymes are induced by a variety of chemicals. A number of studies suggest that this induction is regulated through the signal transduction pathways that are initiated by the inducers (48, 49). In the present study, we found that treatment of human or murine hepatoma cells with tBHQ stimulated p38 activity. Inhibition of tBHQ-induced p38 activity by its inhibitor, SB203580, potentiated the induction of QR activity and ARE-reporter gene expression by tBHQ, whereas SB202474, which did not inhibit p38 activity, was inactive. Interestingly, SB203580 also enhanced the basal and the inducible activities of ARE-reporter gene by BHA, β-NF, and SUL. Furthermore, overexpression of a dominant-negative mutant of p38 or its upstream kinase, MKK3, augmented the induction of ARE-driven transcription. However, overexpression of the wild-type p38 or MKK3 showed opposite effects. Therefore, this study, for the first time, unravels a signal transduction pathway that negatively regulates ARE-dependent phase II gene expression.

Although we provide good evidence that p38 inhibits basal and inducible ARE-mediated gene expression, the cellular target that mediates such a negative role of p38 is not clear. Since
the induced luciferase activity by SB203580 appears 3 h after treatment and shows an earlier kinetics than that by tBHQ, we speculate that p38 may directly act on ARE-binding proteins. p38 has been shown to phosphorylate several transcription factors, including ATF2 (23), Elk-1 (28), CHOP/GADD153 (29), MEF2C (30), and SAP-1 (31). However, these known substrates do not seem to be the mediators of negative action of p38, because we are unable to detect their presence in ARE-protein complex. Recently, using gel shift assays and cDNA transfection, several groups have identified various ARE-binding proteins, including the members of the basic leucine zipper transcription factor family, Nrf1 (15), Nrf2 (15, 17), and Maf (17, 49), and a non-basic leucine zipper nuclear protein, designated as ARE-BP-1 (16, 38). Whether these ARE-binding proteins can serve as substrates of p38 remains to be investigated. It is also possible that p38 phosphorylates a protein that is able to regulate the transcriptional activities of ARE-biding proteins. Indeed, a murine cytosolic protein, named Keap1, has been identified to suppress Nrf2 transcriptional activity by retaining Nrf2 in the cytoplasm (50). A human homologue of Keap1 has also been cloned independently (51). Sequence analysis of Keap1 and its human homologue reveals several potential MAPK phosphorylation sites. It will be interesting to examine the phosphorylation of Keap1 protein by MAPKs and the biological consequence of this event.

Activation of p38 by tBHQ and other phase II enzyme inducers is intriguing. p38 is preferentially activated by stress stimuli, such as UV radiation (26), heat shock (21), hyperosmolarity (18), oxidative stress (52), and proinflammatory cytokines (26). Genetic and biochemical evidence indicates that activation of p38 by these stress stimuli is mediated by upstream kinase, TAK1 or ASK1, that directly activates MKK3 or MKK6, which, in turn, activates p38 (53, 54). Since tBHQ, BHA, and β-NF are easily oxidized to reactive intermediates, resulting in chemical stress, it is therefore conceivable that a similar mechanism may also underlie the activation of p38 by these agents. Indeed, overexpression of a dominant-negative mutant of MKK3 inhibited p38 activation by tBHQ and attenuated the inhibitory effect of p38. Interestingly, expression of a dominant negative mutant of TAK1 or ASK1 also inhibited tBHQ-induced p38 activity; however, we were unable to detect any significant induction of TAK1 or ASK1 activity in tBHQ-treated HepG2 cells, implicating the involvement of a TAK1- or ASK1-related protein. The detailed mechanism by which tBHQ and other phase II enzyme inducers stimulate p38 activity is currently under investigation.

Protein kinases have been previously implicated in the activation of phase II genes. Okadaic acid, a potent inhibitor of serine/threonine-specific protein phosphatase, and Ras, a small GTPase that activates many kinase pathways, have been shown to potently induce ARE-mediated gene expression (55). Most recently, we demonstrated a role of extracellular signal-regulated protein kinase (ERK), another member of the MAPK family, in the induction of phase II detoxifying enzymes by chemicals (56). Inhibition of ERK2 activation by a specific MAPK kinase inhibitor, PD98059, or by overexpression of a dominant negative mutant of ERK2 attenuated the activation of ARE-dependent reporter gene as well as the induction of QR activity by tBHQ and SUL in HepG2 and Hepa1c1c7 cells.
Blockade of ERK pathway also attenuated Ha-Ras-induced ARE-reporter gene activity. Thus, in contrast to p38, ERK2 acts as a positive regulator of phase II gene induction. The opposite effects of p38 and ERK2 indicate that a complex mechanism may exist in regulating phase II gene expression. Differential activation of these positive and negative pathways, such as ERK versus p38, may determine the potency of a phase II enzyme inducer. Although tBHQ, BHA, and β-NF activate both ERK and p38 pathways, SUL stimulates ERK activity to a greater extent. Thus, in contrast to p38, ERK2 appears to act as a negative regulator of phase II gene induction. The association of MKK3 with p38 but not ERK2 might account for the differential effects observed in the present study. The blockade of ERK pathway also abrogated the inhibitory effects of SUL on both ERK and p38 pathways, SUL stimulates ERK activity to a greater extent. Thus, in contrast to p38, ERK2 appears to act as a negative regulator of phase II gene induction. This may provide a molecular basis for the future design of potential phase II enzyme inducers through structural refinement that enhances the activation of ERK but does not induce or even inhibit p38 activity.

In conclusion, we have demonstrated that a signaling kinase, p38, negatively regulates the induction of ARE-dependent phase II gene expression. Such a negative role of p38 is mediated by an upstream kinase, MKK3. Future studies will unravel the downstream effectors of p38.

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