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Role of PI3-kinase and MAP Kinases in the ARE-mediated Glutathione S-Transferase Induction by Phytochemicals: Comparison with the Oxidative Stress Caused by Decreased Glutathione

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ABSTRACT *: The expression of phase II detoxifying enzymes is affected by a variety of compounds and the induction of the enzymes plays an essential role in chemoprevention. A variety of phytochemicals such as sulfur-containing chemoprotective agents (SCC) may trigger cellular signals and activate phase II gene expression through ARE activation. SCC induces glutathione S-transferases. Studies were conducted to investigate the role of mitogen-activated protein (MAP) kinase and phosphatidylinositol 3-kinase (PI3-kinase) in the induction of GST (e.g. rGSTA2) by SCC. We also studied the MAP kinase pathway responsible for the GST expression by SCC and compared that with the pathway activated by oxidative stress as a result of sulfur amino acids deprivation (SAAD). SCC inhibited phosphorylation of ERK1/2 although the effect of SCC on JNK and p38 MAP kinase was minimal. Wortmannin and LY294002, PI3-kinase inhibitors, abolished the increases in rGSTA2 mRNA and protein levels by SCC. Deprivation of cystine and methionine caused oxidative stress in H4IIE cells, as evidenced by a decrease in the reduced glutathione and an increase in prooxidant production. Electrophoretic mobility shift assay revealed that the ARE complex consisting of Nrf-1/2 and Maf proteins was activated 12~48 h. The rGSTA2 mRNA and protein levels were increased by SAAD. Activation of ARE and induction of rGSTA2 were both completely inhibited by PI3-kinase inhibitors. Inhibition of p38 MAP kinase by SB203580 prevented the ARE-mediated rGSTA2 induction. The results of this study showed that PI3-kinase might play an essential role in the ARE-mediated rGSTA2 induction by SCC or SAAD and that the dual MAP kinase pathways were responsible for the enzyme induction.*

I. INTRODUCTION

The protective adaptive response to electrophiles and reactive oxygen species (ROS) is mediated by the induction of the phase II detoxifying enzymes. The role of antioxidant response elements (AREs) and activator protein-1 (AP-1) in the inducible expression of phase II enzymes (e.g. rGSTA2) by phenolic antioxidants has been extensively studied (Bergelson *et al.*, 1994; Wasserman and Fahl, 1997; Venugopal and Jaiswal, 1998). ARE coordinately regulates the expression of a battery of antioxidant genes.

Glutathione (GSH) as a nonprotein sulfhydryl molecule in cells plays a role as an intracellular protective substance and serves as an effective oxygen radical scavenger. A decrease in cellular reduced GSH content would increase oxidative stress. The GSH depleting agents such as buthionine sulfoximine and N-ethylmaleimide decrease cellular GSH levels by inhibiting the essential proteins involved in GSH synthesis or by direct conjugation. Because cysteine is a direct precursor of GSH, sulfur amino acids deprivation (SAAD) from the culture medium results in a decrease in the cellular GSH level, which subsequently elevate oxidative stress. Hence, SAAD experiment would serve as an appropriate model to assess the molecular events and the signaling pathway responsible for the phase II enzyme induction in response to a decrease in the cellular GSH *per se*, and to compare the effects with those caused by chemicals.

A variety of phytochemicals serve as chemopreventive agents. Many of chemopreventive agents may trigger cellular signals and subsequently activate phase II gene expression, which is important mechanism for chemoprotective activity (Bolton *et al.*, 1993). Chemoprotective phytochemicals include sulfur-containing agents such as diallyl sulfide (DAS) and dithiolthiones, which protect tissues and prevent carcinogene-

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sis (Fig. 1). Diallyl disulfide, diallyl sulfide or other organosulfur compounds, components of garlic oil and onions, have been extensively studied because of their potential protective effects against cancer or cytotoxicity. Treatment of animals with diallyl sulfide resulted in blocking the initial metabolic activation of certain chemical carcinogens through the inhibition of CYP2E1 catalytic activity and the expression of the enzyme (Brady *et al.*, 1991). The mechanism of chemoprevention (e.g. protection against chemical-induced carcinogenesis and radioprotection) is associated with the induction of phase II detoxifying enzymes (Bolton *et al.*, 1993, Kim *et al.*, 1994, Kim *et al.*, 1997). Treatment with the organosulfur compounds resulted in increases in hepatic GST activity as well. It appeared that the sulfur-containing compounds (SCC) were capable of elevating hepatic glutathione S-transferase levels through large increases in its mRNA with transcriptional activation (Kim *et al.*, 1996). Dithiolthiones are plant products with chemoprotective efficacy. Treatment of rats with oltipraz a derivative of dithiolthione resulted in dose-dependent increases in the rGSTA2, rGSTA3, rGSTA5, and rGSTM1 mRNA levels. Exposure of rats to both oltipraz and 3 Gy g-rays radiation resulted in an additive increase in rGSTA2, GSTM1/2 and rGSTA3/4 mRNA levels. Histopathological examinations revealed that exposure of rats to radiation caused mild to moderate hepatocyte degeneration with sinusoidal congestion whereas oltipraz was effective in blocking the radiation-induced liver injury. Studies from this laboratory showed that the expression of major GST genes is increased as a function of time after ionizing radiation, major GST subunits are coordiately expressed after exposure of animals to γ-rays, and that radiation-induced elevations in GST expression are further enhanced by oltipraz treatment. Thus, oxidative stress as well as chemoprotective phytochemicals can induce phase II enzymes.

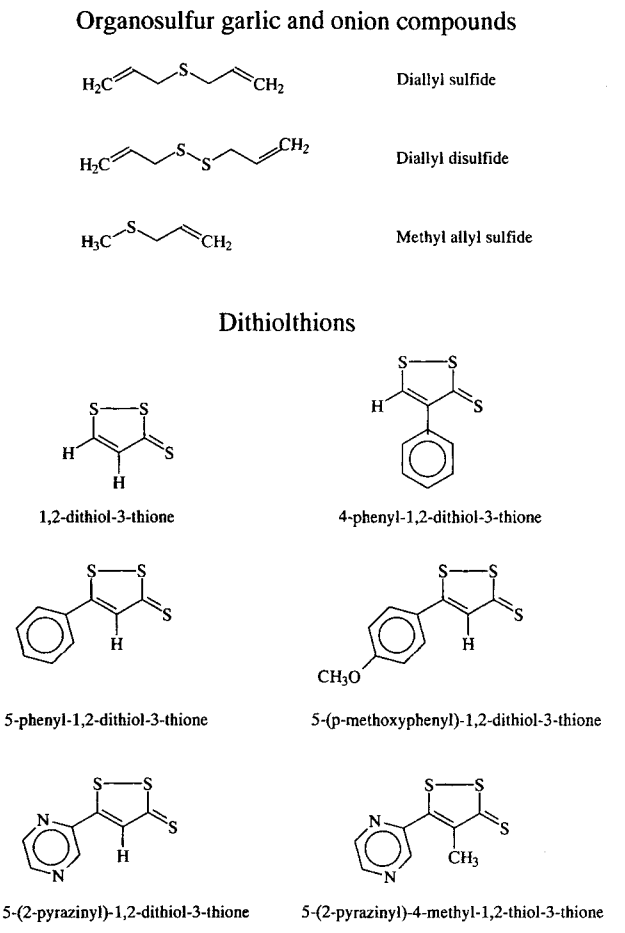

Fig. 1. Chemical structures of sulfur-containing chemoprotective agents including organosulfur garlic and onion compounds and dithiolthions.

Phosphatidylinositol 3-kinase (PI3-kinase) is a lipid kinase that phosphorylates phosphatidylinositols at the 3 position of the inositol ring. This enzyme has been found to be associated with the activation of cellular survival signals in response to several growth factors and has been implicated in the mitogenesis and cell transformation (Daulhac *et al.*, 1999). The phosphorylated forms of phosphatidylinositol act as second messengers for several kinases, including Akt (PKB) and ribosomal S6 kinase (Lin *et al.*, 1999). Recently, it has been reported that treatment of cells with hydrogen peroxide elevated Akt activity in diverse cells and that elevated Akt activity confered protection against oxidative stress-induced apoptosis (Wang *et al.*, 2000). Thus, cellular oxidative stress may cause the change in Akt activity and potentially in PI3-kinase activity. In view of the diverse biological effects of PI3-kinase, we investigated the role of PI3-kinase on the induction of rGSTA2 by SCC. We also studied whether PI3-kinase was activated by the oxidative stress following sulfur amino acids deprivation (SAAD) and PI3-kinase regulated SAAD-induced phase II enzyme induction in H4IIE rat hepatoma cells.

Oxidative stress activates the mitogen-activated protein (MAP) kinases (Wang *et al.*, 1998). Three distinct mammalian MAP kinase modules including extracel-

lular signal-regulated kinase (ERK), p38 mitogen-activated protein (MAP) kinase, and c-Jun NH_2-terminal kinase (JNK) have been characterized (Treisman *et al.*, 1996). ERK is stimulated predominantly by mitogens and growth hormones, and the activation of ERK induces proliferation or differentiation of cells. The p38 MAP kinase, a recently identified member of the MAP kinase family, is involved in apoptosis (Tan *et al.*, 1996). Stress-activated protein kinase cascade involves the activation of JNK, which consequently induces activator protein-1 (AP-1)-mediated transactivation of the AP-1 responsible genes (Wesselborg *et al.*, 1997). We investigated the effects of SCC and oxidative stress induced by decreased glutathione following SAAD on the rGSTA2 expression. Both phytochemicals and prooxidants induced phase II detoxifying enzymes. The current study was also designed to determine whether SCC or decreased glutathione (SAAD) activated the MAP kinases and to identify the MAP kinase(s) responsible for GST induction.

II. INDUCTION OF RGSTA2

Expression of GST is affected by oxidative stress (Pinkus *et al.*, 1996). Northern blot analysis was performed to determine whether the rGSTA2 mRNA was increased following SCC or SAAD treatment. SCC treatment induced rGSTA2. The mRNA level of rGSTA2 began to increase 6 h after SCC treatment, plateaued 12~24 h and gradually diminished at 48 h. The rGSTA2 subunit was assessed by Western blot analysis to confirm whether SSC led to induction of the GST subunit. rGSTA1/2 subunit began to be induced 6 h after SCC treatment, peaked at 12~24 h and extended up to 48 h. Anti-rGSTA1/2 antibody preferentially recognized the induction of rGSTA2 because the rGSTA2 subunit is inducible. The rGSTA2 mRNA significantly increased 24 h after incubation of the cells in the absence of sulfur amino acids, peaked at 48 h, and then returned to the basal level at 72 h. The rGSTA1/2 in the H4IIE cells was induced 48 h after SAAD and extended up to 72 h. These results demonstrated that either phytochemical or decreased glutathione increased rGSTA2 expression.

III. GLUTATHIONE CONTENTS AND PRO-OXIDANT PRODUCTION BY SAAD

Depletion of hepatic GSH increases the susceptibility of animals to free radical-induced tissue damage because the GSH plays a critical role in the detoxification of oxidative metabolites produced from endogenous and exogenous molecules. Deprivation of sulfur amino acids from the culture medium led to a decrease in the cellular GSH level, which subsequently elevated oxidative stress as evidenced by an increase in the fluorescence of DCF at 12 h (Fig. 2A). Because cysteine is a direct precursor of GSH, the lack of sulfur amino acids caused a time-dependent decrease in the reduced GSH with 50% decrease being noted at 12.6 h (Fig. 2B). The oxidative stress induced by the decrease in the intracellular GSH would affect the redox state

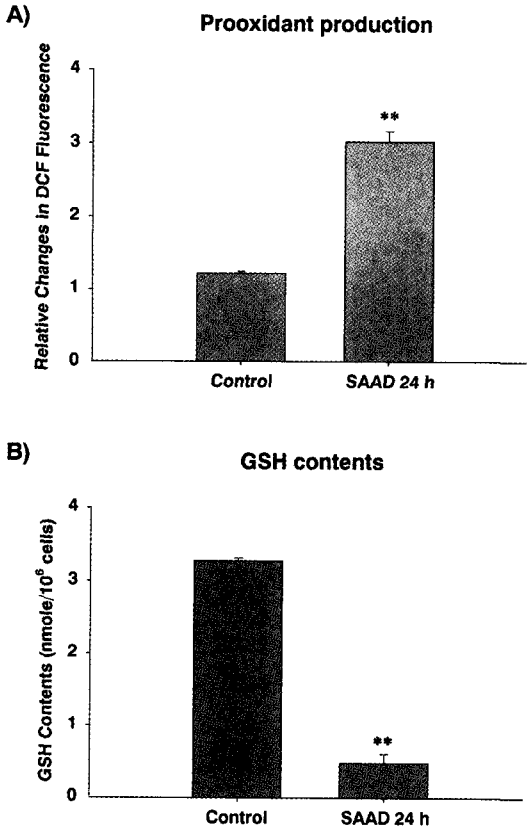

Fig. 2. (A) Relative dichlorofluorescein (DCF) fluorescence in H4IIE cells. H4IIE cells were cultured with or without sulfur amino acids for 12 h and loaded with DCFH-DA. Fluorescence was monitored at the excitation wavelength of 485 nm and the emission wavelength of 530 nm using a fluorescence plate reader. Data represent the mean±S.D. with 5 separate experiments, and are expressed as the relative changes to the initial fluorescence. (B) The reduced GSH contents in H4IIE cells cultured in deficiency of cystine and methionine. Data represent the mean±S.D. with 4 separate experiments. Significant as compared to the initial content, **$p<0.01$.

and the regulation of gene expression.

IV. ROLE OF PI3-KINASE ON THE RGSTA2 INDUCTION

H4IIE cells were incubated with SCC for 12 h in the presence of PI3-kinase inhibitors. Either wortmannin or LY294002 significantly inhibited the increase in rGSTA2 mRNA by SSC at 12 h. Immunoblot analysis also showed that either wortmannin or LY294002 prevented the induction of rGSTA1/2 by SCC. Hence, the change in the rGSTA2 mRNA level by inhibition of PI3-kinase activity paralleled with that in rGSTA2 protein. These results clearly showed that the activity of PI3-kinase was essential in the regulatory pathway leading to the ARE-mediated rGSTA2 induction by SCC.

To determine whether the PI3-kinase cascade is involved in induction of rGSTA2, H4IIE cells were incubated with 500 nM of wortmannin or 50 μM LY294002 for 24 h in the presence of SCC or in the culture medium without sulfur amino acids. To assess whether PI3-kinase inhibitors prevented rGSTA2 induction, the rGSTA2 mRNA level was monitored in the cells incubated with each PI3-kinase inhibitor. Either wortmannin or LY294002 completely inhibited the increase in rGSTA2 mRNA at 24 h after SAAD.

V. MAP KINASE ACTIVATION

Transcription factors such as c-Fos and c-Jun are phosphorylated by the MAPK family activated by a variety of stresses (Luo *et al.*, 1997; Hodge *et al.*, 1998). Previous studies in this laboratory revealed that ERK (Son *et al.*, 2000), JNK and p38 MAP kinase (Kang *et al.*, 2000) are activated at 1~12 h cultured in sulfur amino acids deprived medium. The levels of phosphorylated active JNK and p38 MAP kinase were not changed after treatment with SCC. In contrast to the activation of ERK by SAAD, the levels of active phosphorylated ERK were decreased 1~3 h after SCC treatment. In particular, active ERK completely disappeared 3 h after SSC treatment. We also measured the effect of SSC on the ERK activation stimulated by insulin. ERK is activated at 5 min after insulin treatment. The activation of ERK by insulin was significantly inhibited by treatment with SCC. These data showed that sustained activation of MAP kinases could result from oxidative stress induced by decreased glutathione, while SSC selectively inhibited the activation of ERK.

VI. DIFFERENTIAL EFFECT OF MAP KINASES ON THE RGSTA2 INDUCTION BY SCC AND SAAD

We were then interested in whether blockade of MAP kinase cascade led to a change in the rGSTA2 mRNA increase by SSC or SAAD. We studied the effects of PD98059 and SB203580 on the rGSTA2 mRNA increase by SCC. Concomitant treatment of cells with each of these inhibitors in combination with SCC failed to affect the increase in rGSTA2 mRNA. To establish the role of JNK on the rGSTA2 induction by SCC, cells were transfected with expression vectors of JNK1-dominant negative mutant. Transfection of H4IIE cells with a plasmid of JNK1-dominant negative mutant failed to inhibit the increase in rGSTA2 mRNA by SCC at 12 h. Neither SB203580 nor overexpression of JNK1 dominant negative mutant prevented SCC-inducible rGSTA2 expression. This supported the notion that neither p38 MAP kinase nor JNK was involved in SCC-inducible GST expression.

Cells were incubated with the specific MAP kinase inhibitor for 12 h. PD98059 (50 μM) did not affect the increase in rGSTA2 mRNA by SAAD. PD98059 rather significantly increased the gene expression by SAAD. SB203580, a specific p38 MAP kinase inhibitor, at the concentration of 10 μM significantly prevented the expression of rGSTA2. This result supports the conclusion that both PI3-kinase and p38 MAP kinase regulate the ARE-mediated rGSTA2 induction by SAAD and that ERK was not involved in the regulation of rGSTA2 expression by SAAD (Fig. 3).

The present study provided evidences that PI3-kinase might play an essential role in the ARE-mediated rGSTA2 induction by SCC or oxidative stress following SAAD. The PI3-kinase activity in conjunction with activation of Akt may represent a general essential pathway for the ARE-mediated rGSTA2 induction. Whereas activation of p38 MAP kinase by SAAD led to rGSTA2 induction, SCC induced rGSTA2 via

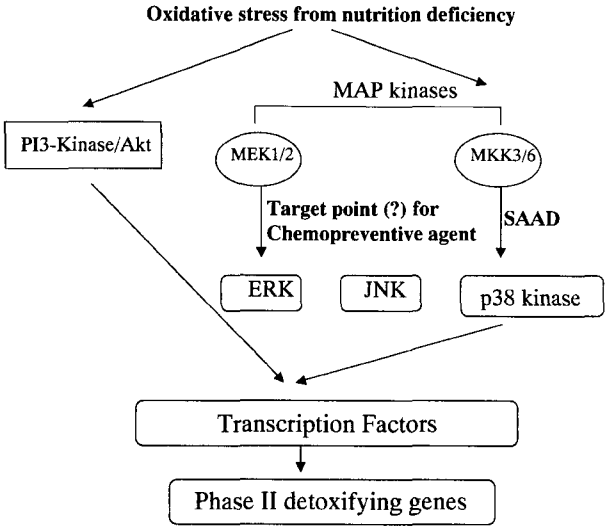

Fig. 3. The signaling pathways for the expression of phase II detoxifying enzymes.

activation of other MAP kinase.

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