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Advances in Brief

Redox Regulation of Glutathione *S*-Transferase Induction by Benzyl Isothiocyanate: Correlation of Enzyme Induction with the Formation of Reactive Oxygen Intermediates[1]

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Abstract

Here we report the molecular mechanism underlying the induction of glutathione *S*-transferase (GST) in rat liver epithelial RL34 cells treated with a cancer chemopreventive isothiocyanate compound, benzylisothiocyanate (BITC). BITC was found to significantly induce GST activity in RL34 cells. Northern and Western blot analyses demonstrated that BITC specifically enhanced the production of the class π GST isozyme (GSTP1). Our studies demonstrated for the first time that the addition of BITC to the cells resulted in an immediate increase in the reactive oxygen intermediates (ROIs) detected by a fluorescence probe, 2′,7′-dichlorofluorescin diacetate. The level of the ROIs in the cells treated with BITC (10 μM) was ~50-fold higher than those in the control cells. Furthermore, glutathione depletion by diethyl maleate significantly enhanced BITC-induced ROI production and accelerated the BITC-induced elevation of the GST activity, whereas pretreatment of the cells with glutathione inhibited both the ROI production and GST induction. The structure-activity relationship of the isothiocyanates also indicated that the ROI-producing activities closely correlated with their GST-inducing potencies. Moreover, the GSTP1 enhancer I-containing region was found to be essential for induction of the *GSTP1* gene by intracellular ROI inducers such as BITC and diethyl maleate. These data suggest the involvement of the redox regulation on the induction of GSTP1 by BITC.

Introduction

Xenobiotic metabolizing enzymes play a major role in regulating the toxic, oxidative, damaging, mutagenic, and neoplastic effects of chemical carcinogens. Mounting evidence has indicated that the induction of phase II detoxification enzymes (Ref. 1; *e.g.,* GST,[3] NAD(P)H:quinone oxidoreductase 1, UDP-glucuronosyltransferases, and epoxide hydrolase) by numerous compounds, including food phytochemicals, results in protection against toxicity and chemical carcinogenesis, especially during the initiation phase.

Among the phase II detoxification enzymes, GSTs are a family of enzymes that catalyze the conjugation of reactive chemicals with GSH and play a major role in protecting cells. After generating GSH conjugates, these are subsequently eliminated via a GSH-conjugate recognizing transporter. To identify and classify the inducers of the GSTs, we have recently developed a simple cell culture system using the rat liver epithelial RL34 cell line, which is sensitive to the already-known phase II inducers such as α,β-unsaturated aldehydes (2, 3). A number of studies support the fact that certain food phytochemicals protect against cancer. An important group of compounds that have this property are organosulfur compounds, such as ITCs. ITCs are compounds that occur as glucosinolates in a variety of cruciferous vegetables, such as *Brassica* species. Many ITCs are effective chemoprotective agents against chemical carcinogenesis in experimental animals. ITCs inhibit rat lung, esophagus, mammary gland, liver, small intestine, colon, and bladder tumorigenesis (4–8). Previous studies have also reported that ITCs inhibit phase I enzymes (cytochrome P-450) that are required for the bioactivation of carcinogens and increase the carcinogen excretion or detoxification by the phase II detoxification enzymes (9, 10).

ROIs are involved in many biological processes. Recent findings have revealed that both the *GST* gene expression and induction of the transcription factor, such as activated protein-1, might be related with intracellular oxidative stress (11, 12). In addition, it has been shown that JNK, which activates the transcriptional factor such as c-Jun, was induced by ITCs mediated by oxidative stress (13). The protective effect exerted by overexpressed Bcl-2 and antioxidants provide substantial evidence for the involvement of ROIs in the ITC-mediated JNK activation and apoptosis and point to oxidative stress as the probable mediator for signal transduction. However, the exact nature and the role of ITC-induced ROI production on GST induction are still not established and are the topics of this report.

In a recent study, we have screened a number of fruits for sources of GST inducers and described the isolation and identification of BITC as a major GST inducer from papaya.[4] In addition, we have also observed that, among a total of 20 isothiocyanates and their derivatives, BITC was found to be the most potent inducer of GST activity in the cells. Here we report the molecular mechanism underlying GST induction in the cells treated with BITC. Our studies demonstrated for the first time that the addition of BITC to cultured rat liver epithelial cells resulted in an immediate increase in ROIs detected by a

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[4] Y. Nakamura, Y. Morimitsu, T. Uzu, H. Ohigashi, A. Murakami, Y. Naito, Y. Nakagawa, T. Osawa, and K. Uchida. A glutamine *S*-transferase inducer from papaya: Rapid screening identification and structure-activated relationship of isothiocyanates, submitted for publication.

H_2DCF-DA fluorescence probe. Furthermore, the agent, such as DEM enhancing the BITC-induced ROI production, accelerated the GST induction by BITC, whereas the antioxidant GSH significantly inhibited them. These results suggested that ROIs are involved in the GST induction by BITC.

Materials and Methods

Materials. BITC and PITC were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). BSO were obtained from Aldrich Chemical Co., Ltd. All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). Antirat GSTP1 antisera was obtained from Biotrin International (Dublin, Ireland). Horseradish peroxidase-linked antirabbit IgG immunoglobulin was purchased from Dako (Glostrup, Denmark). H_2DCF-DA was obtained from Molecular Probes, Inc. (Leiden, the Netherlands). The protein concentration was measured using the BCA protein assay reagent from Pierce.

Cell Cultures. RL34 cells were obtained from the Health Science Research Resources Bank (Osaka, Japan; Ref. 14). The cells were grown as monolayer cultures in DMEM supplemented with 5% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.3 mg/ml L-glutamine, 0.11 mg/ml pyruvic acid, and 0.37% $NaHCO_3$ at 37°C in a atmosphere of 95% air and 5% CO_2. Postconfluency cells were exposed to the test compounds in the medium containing 5% fetal bovine serum.

Enzyme Assay. GST activity was measured using 1-chloro-2,4-dinitrobenzene as a substrate according to the method of Habig *et al.* (15).

Western Blot Analysis. The ITC-treated and untreated cells were rinsed twice with PBS (pH 7.0) and lysed by incubation at 37°C for 10 min with a solution containing 0.8% digitonin and 2 mM EDTA (pH 7.8). Each whole-cell lysate was then treated with the Laemmli sample buffer for 3 min at 100°C (16). The samples (20 μg) were run on a 12.5% SDS-PAGE slab gel. One gel was used for staining with Coomassie brilliant blue, and the other was transblotted on a nitrocellulose membrane with a semidry blotting cell (*Trans*-Blot SD; Bio-Rad), incubated with Block Ace (40 mg/ml) for blocking, washed, and treated with the antibody.

RNA Preparation and Northern Blot Analysis. The rat GSTP1 cDNA probe was obtained from the Japanese Cancer Research Resource Bank (17). The probe was labeled with [α-^{32}P]dCTP by random oligonucleotide priming (Amersham). The preparation of the total RNA from RL34 cells and Northern blot analysis were carried out as reported previously (9, 18).

GSH Assay. Measurement of GSH in the cells was spectrophotometrically performed using the commercial kit GSH-400 (Bioxytech). Confluent monolayer cells were exposed to test compounds for the indicated times, and at the end of the incubation period, cell monolayers were washed twice with PBS (pH 7.0) and extracted with the 5% metaphosphoric acid solution containing 5 mM EDTA. After centrifugation (10,000 × g for 20 min), 50 μl of 12 mM chromogenic reagent in 0.2 M HCl were added to the resulting supernatant (300 μl) and mixed thoroughly. After 50 μl of 7.5 M NaOH were added and mixed, the mixture was incubated at 25°C for 10 min, and then the absorbance was determined spectrophotometrically at 400 nm.

Intracellular Oxidative Products Determination. Intracellular oxidative products were detected by H_2DCF-DA as an intracellular fluorescence probe (19, 20). Briefly, the cells under confluency were treated with H_2DCF-DA (50 μM) for 30 min at 37°C. After washing twice with PBS, the test compound was added to the complete medium and incubated for another 30 min. A flow cytometer (CytoACE 150; JASCO, Tokyo, Japan) was used to detect DCF formed by the reaction of H_2DCF with the intracellular oxidative products. Experiments were repeated two times with similar results. The data are expressed as one representative histogram. Images of the cellular fluorescence were acquired using a confocal laser scanning microscope (Fluoro-View; Olympus Optical Co., Ltd., Tokyo, Japan) with a ×40 objective (488-nm excitation and 518-nm emission).

CAT Assay. A 3.0-kb fragment between −2.9 kb (*Eco*RI) and 59 bp (*Acc*I) of the *GSTP1* gene was inserted into the *Hin*dIII site of pSV0CAT and designated as ECAT (21). 1CAT was constructed from the ECAT using the appropriate restriction enzymes (21). RL34 cells were transfected with 10 μg of plasmid construct by the calcium phosphate coprecipitation procedure described by Chen and Okayama (22). Cells were harvested 48 h after transfection. Cell lysates were obtained after five freeze-thaw cycles in 0.25 M Tris-HCl (pH 7.5). The protein was equalized and used for the CAT assay. The degree of acetylation was determined by reading the intensity of the spots using the Fuji-BAS 2000 system (Fuji Photo, Tokyo, Japan).

Results

BITC Is a Potent Inducer of GSTP1. As shown in Fig. 1*A*, after exposure of the RL34 cells to 10 μM BITC for 24 h, the GST activity was increased 2.3-fold, compared with that of the BITC analogue, PITC. To examine the GST isozymes responsible for the increase in
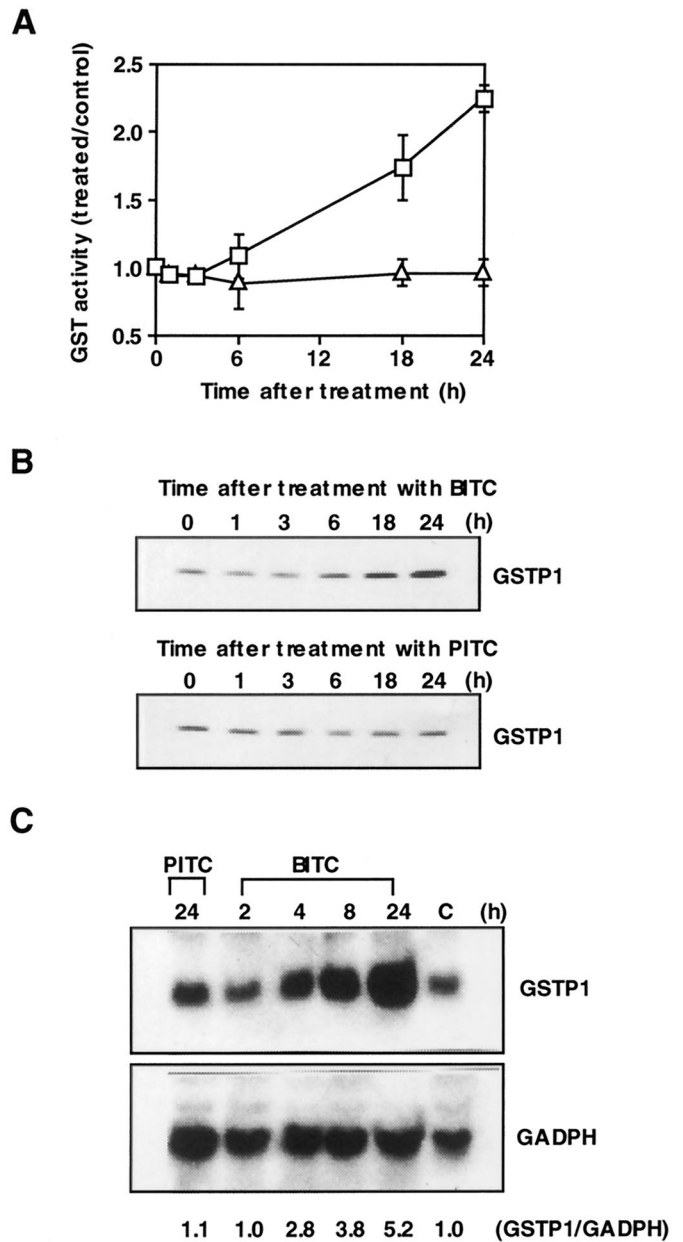

Fig. 1. Time-dependent effect of BITC and PITC on cellular GST activity, GSTP1 protein, and GSTP1 mRNA. The RL34 cells were incubated with 10 μM BITC or PITC in DMEM containing 5% FBS. *A*, GST activity. After incubation, the ITC-treated and untreated cells were lysed with 0.8% digitonin containing 2 mM EDTA, and the GST activities were then measured using CDNB as a substrate. □ (BITC) and △ (PITC), mean values of three independent experiments; *bars,* SD. *B*, immunoblot analysis of GSTP1. Each whole-cell lysate was treated with a Laemmli sample and was then run on a 12.5% SDS-PAGE slab gel. The gel was transblotted on a nitrocellulose membrane and treated with the antibody. *C*, Northern blot analysis of GSTP1 mRNA. Total cellular RNA (20 μg) was fractionated on 1% agarose/formaldehyde gel and transferred onto a nitrocellulose filter, and the RNA blot was then hybridized with a ^{32}P-labeled cDNA probe for GSTP1 (*top*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH; bottom*). The autoradiographs were scanned to quantify GSTP1 mRNA induction relative to control of the ratio (*GSTP1/GAPDH*).
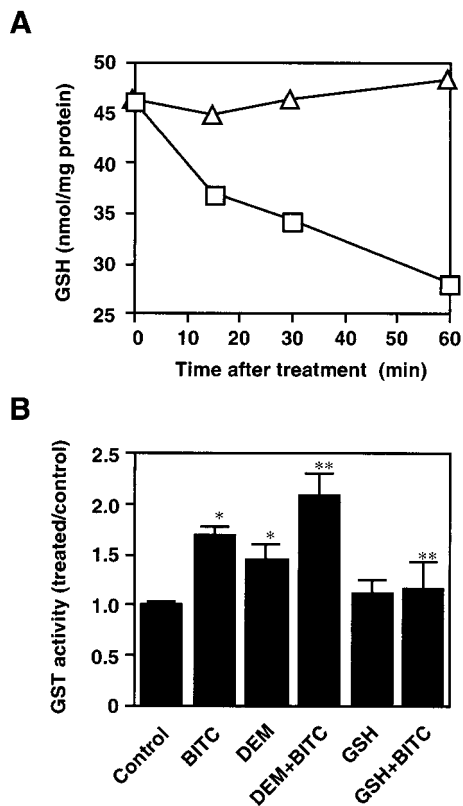
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A

B

Fig. 2. The effects of redox alteration on GST activity enhancement. *A*, BITC-induced intracellular GSH depletion. The cells were treated with 25 μM BITC ($\square$) or PITC ($\triangle$) for different time intervals. Intracellular GSH levels were colorimetrically measured as described in "Materials and Methods." *B*, effects of GSH and DEM upon short exposure of BITC-induced GST activity enhancement. The cells were pretreated with GSH (10 mM) or DEM (1 mM) for 1 h and treated with BITC (10 μM) or DMSO for 1 h. After washing out the BITC followed by another 16 h of incubation, the GST activity was determined. Data were the means of three independent experiments; bars, SD.

the GST activity of the BITC-treated RL34 cells, an immunoblot analysis was carried out. In RL34 cells, no detection of the class α GST isozyme, GSTA1, which is the most abundant GST isozyme in normal rat liver, and the constitutive expression of the class π GSTP1 have been reported previously (9). As shown in Fig. 1*B*, the protein level of GSTP1 significantly increased in the cells treated with BITC for >16 h. Another class α GST isozyme, GSTA3, was slightly induced (data not shown). These results indicated that the induction of GST activity by BITC mainly resulted from the enhanced expression of GSTP1. In addition, the Northern blot analysis of the total mRNA from RL34 cells treated with 10 μM BITC was performed using an oligonucleotide probe complementary to the GSTP1 mRNA sequence. As shown in Fig. 1*C*, the treatment of RL34 with BITC resulted in a 5.2-fold increase in GSTP1 mRNA after 24 h. Therefore, the increment in GSTP1 mRNA coincided with a substantial rise in the GSTP1 protein level and GST activity, whereas the effect of PITC on the GSTP1 protein and gene expression was almost negligible (Fig. 1*B* and *C*).

Redox Regulation on the GST Induction by BITC. The primary cellular target of ITCs is still unknown; however, there is clear evidence that the intracellular GSH level, regulating the redox state of the cell, may play an important role in the initiation of the cellular responses to numerous compounds (10). Hence we assessed whether the redox regulation is involved in the GSTP1 expression by BITC in the RL34 cells. The intracellular GSH level in RL34 was reduced by treatment of the active BITC but not by the inactive PITC (Fig. 2*A*). To assess the role of the oxidative stress in the induction of GST, we

examined the effects of the pretreatment of the cells with GSH or DEM prior to the BITC treatment. Because the maximal induction of the stress signaling pathways, such as JNK activation, was observed within 1~2 h after ITC treatment (13), the cells were exposed to 10 μM BITC for 1 h, and after washing out the BITC followed by another 16 h of incubation, the GST activity was measured. As shown in Fig. 2*B*, both GST activities were significantly elevated in the cells treated with BITC, even for 1 h. The GSTP1 protein was also significantly induced by BITC treatment (data not shown). Pretreatment of GSH for 1 h partially blocked the effect of BITC on the GST induction (Fig. 2*B*), whereas pretreatment before BITC stimulation as well as treatment alone of a potent thiol blocker, DEM, significantly and additionally enhanced the GST activity. These results suggested the involvement of redox regulation in the induction of GST by BITC.

Intracellular ROI Generation by BITC. To obtain additional support for the involvement of a redox mechanism during the GST induction by BITC, the intracellular ROI (hydrogen peroxide, lipid hydroperoxide, peroxinitrite, and so on) level was determined using H_2DCF-DA as an intracellular fluorescence probe. As shown in Fig. 3*A*, the BITC-induced increase in intracellular fluorescence, even at 10 min, was observed in the fluorescence images acquired by a confocal laser scanning microscope, whereas the control cells treated only with H_2DCF-DA showed a weak accumulation of DCF attributable to the ROIs produced by the basal oxidative metabolism. The level of ROIs induced by different concentrations of BITC was increased in a dose-dependent manner. The level at 10 μM BITC was ~50-fold higher than that of the control (Fig. 3*B*, *entries 1* and *3*). These data indicated that BITC is a potential inducer of intracellular ROIs.

We have observed recently that the order of GST induction potency is BITC > AITC $\gg$ PITC.[4] On the basis of this observation, we examined the relationship between the GST induction potency and ROI production. As shown in Fig. 4, weakly active AITC showed a slight production of ROIs, and inactive PITC did not produce ROIs. To determine whether the intracellular ROI accumulation was negatively regulated by the GSH level, we examined the effects of intracellular GSH modifiers, such as BSO, DEM, and antioxidants. BSO significantly reduced the GSH level (data not shown) but only slightly enhanced the ROI production, although reducing the GSH level almost comparable with BITC (data not shown), whereas the strong intracellular thiol blocker, DEM, significantly enhanced the ROI production. Moreover, the peroxide production by BITC was partially but significantly suppressed by GSH, quercetin, and the NADPH oxidase inhibitor, DPI, whereas the XOD inhibitor, allopurinol, was ineffective (Fig. 4). The intracellular production of ROIs was confirmed by the lack of inhibition by the membrane-impermeable, ROI-scavenging enzymes, catalase and superoxide dismutase.

GPEI Is the ROI Response Element. To confirm whether GPEI, a powerful enhancer, which is located about 2.5 kb upstream from the transcriptional initiation site of this gene (21), is the BITC- or intracellular ROI response *cis*-element, we used the construct including the 5'-flanking region of the *GSTP1* gene, *i.e.,* ECAT and a GPEI-deletion mutant 1CAT (Fig. 5*A*). We first tested the effect of BITC on both *ECAT* gene and *1CAT* gene expressions. As shown in Fig. 5*B*, BITC (5 μM) could enhance the activity of the *ECAT* gene 3.3-fold of the control in these cells, whereas BITC could not activate the GPEI deletion mutant 1CAT. Next, we examined the effects of the pretreatment of the cells with GSH or DEM prior to the BITC treatment. Pretreatment of GSH (10 mM) completely blocked the BITC-induced enhancement of the ECAT activity. However, DEM, which induced the accumulation of DCF attributable to the ROIs (Fig. 4), significantly enhanced not only the basal activity (3.8-fold) but also the BITC-stimulated activity of ECAT (1.4-fold; Fig. 5*B*). These results
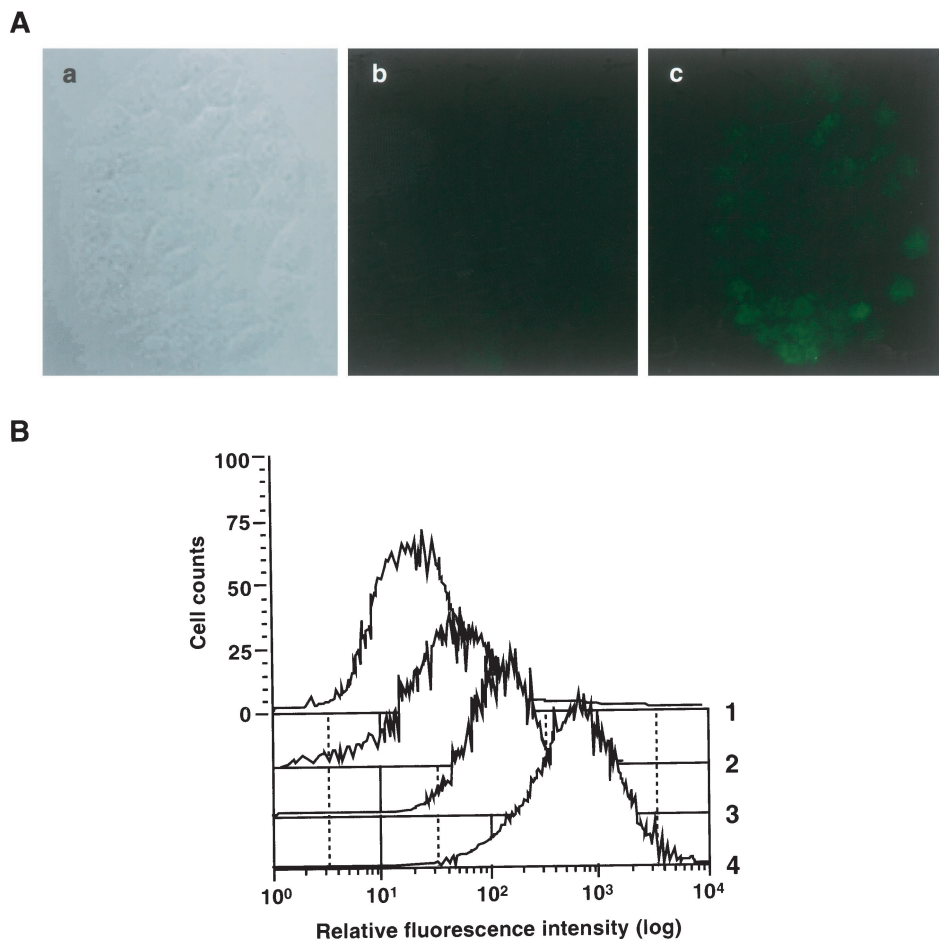
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A

Fig. 3. Changes in intracellular ROI levels after exposure of RL34 cells to BITC. The cells were incubated with H_2DCF-DA (50 μM) for 30 min and then treated with BITC. *A*, transition image (*a*) and fluorescence images (*b* and *c*) of a confocal laser scanning microscope. *b*, the cells before BITC stimulation; *c*, 10 min after BITC stimulation. *B*, flow cytometric analyses of BITC-induced ROIs. After BITC stimulation for 30 min, the cells were washed with PBS and resuspended in PBS containing 10 mM EDTA. The DCF fluorescence of >10,000 cells was monitored on a flow cytometer (CytoACE 150) with excitation and emission wavelengths at 488 and 510 nm, respectively. *entry 1*, untreated control treated only with DMSO; *entry 2*, BITC 5 μM; *entry 3*, BITC 10 μM; *entry 4*, BITC 25 μM.

B

suggest that GPEI is the ROI response element and that BITC may act on this element via production of the intracellular ROIs.

Discussion

ITCs have been shown to prevent chemical carcinogenesis in experimental animals. In mice, BITC blocked the neoplastic effects of diethylnitrosamine or benzo[*a*]pyrene on the lung and forestomach (2, 5, 23), and a variety of phenylalkyl ITCs reduced the pulmonary carcinogenesis of the tobacco-derived nitrosamine (4). The anticarcinogenic effects of ITCs may be closely associated with their capacity to induce phase II detoxification enzymes, including GSTs, which are involved in the metabolism of carcinogens. In fact, ITCs are known to induce the phase II enzymes in the rat liver (24). Moreover, we have recently isolated BITC as a principal inducer of GST activity from papaya fruits.[4]

The present results indicated that BITC elevated the GST activity in normal rat liver epithelial cells (RL34), even at the concentration of 10 μM (Fig. 1*A*). To determine which isozymes participate in the induction of the GST activity by BITC, an immunoblot analysis was done using the GST class-specific antibodies. Among the GST isozymes, GSTP1 significantly increased 16 h after the BITC treatment (Fig. 1*B*), suggesting that the increase in GST activity was largely attributable to the elevated synthesis of this protein. The increase in the GSTP1 protein level coincided with the substantial rise in the GSTP1 mRNA level (Fig. 1*C*). A recent study using transgenic mice lacking the class π GST demonstrated that this class of GST was involved in the metabolism of carcinogens, such as 7,12-dimethylbenz[*a*]anthracene, in mouse skin and had a profound effect on tumorigenicity (25). These results suggest that the class π GST isozyme could be one of the important determinants in cancer susceptibility, particularly in diseases where exposure to polycyclic aromatic hydrocarbons is involved.

It has been shown that the gene expression of GSTA1 is related to the intracellular oxidative stress presumably mediated by hydroxyl radicals ($\cdot$OH) or the pro-oxidative potential of GSTA1 inducers (11, 12). In addition to GSTA1, oxidative stress has been reported to enhance the expression of genes encoding other antioxidant enzymes, including the γ-glutamyl cysteine synthase (26), heme oxygenase (27), and heat shock protein 90 (28). Thus, it is increasingly recognized that an adequate amount of oxidative stress stimulates a variety of signal transduction pathways under circumstances that do not result in cell death. In a recent study, the treatment of RL34 cells with the major end product of the oxidized fatty acid metabolism results in GSTP1 induction (9) and shows a quick cellular GSH depletion, the generation of intracellular ROIs, and the activation of stress signaling pathways (10). The finding that at least 1 h of exposure to BITC was sufficient to evoke the elevation of GST activity (Fig. 2*B*) is consistent with the previous observation (13) that BITC induced the oxidative stress-dependent JNK activation within 1 h. These data suggest that ITCs may induce GST and/or other phase II detoxification enzymes through the stress signaling pathway involving oxidative stress and JNK or p38 cascade, similar to other stimuli, including hydrogen peroxide (10), UV light (29), osmotic stress (30), the DNA-damaging agent (31), inflammatory cytokine (32), and lipid peroxidation products (10).

In the present study, we showed that the BITC-induced enhancement of GST activity was blocked by the antioxidant GSH and enhanced by the thiol blocker DEM (Fig. 2*B*). These results suggest that the initial signal for GST induction is likely to be transduced to a plausible cytosolic
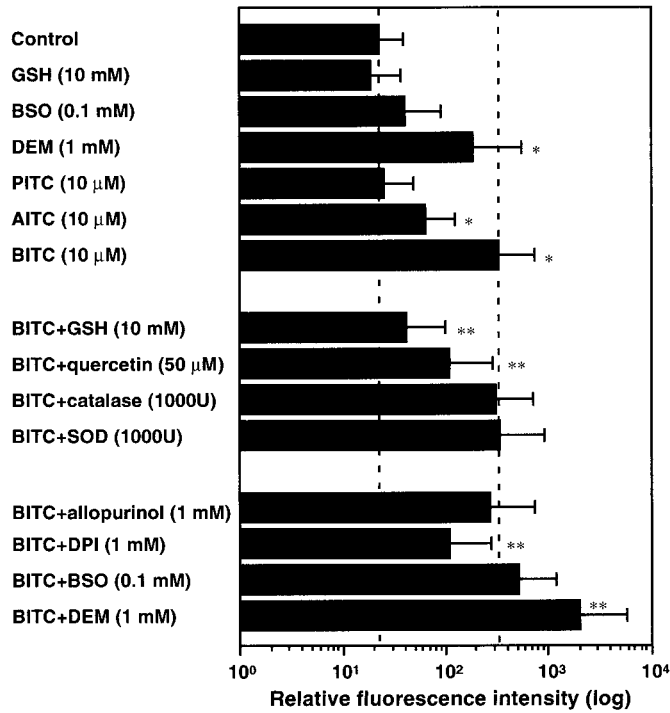
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Fig. 4. Effects of a variety of agents on intracellular ROI accumulation. The cells were incubated with H_2DCF-DA (50 μM) for 30 min and then treated with 10 μM ITCs for 30 min. In the case of GSH (10 mM), DEM (1 mM), quercetin (50 μM), catalase (1000 units), superoxide dismutase (1000 units), allopurinol (1 mM), and DPI (1 mM) were pretreated for 1 h before H_2DCF-DA treatment. BSO (0.1 mM) was pretreated for 6 h. The DCF fluorescence of >10,000 cells was monitored on a flow cytometer. *Bars,* SD.

sensor(s) or receptor(s). Talalay and Zhang (33) have reported recently that the total intracellular concentrations of the ITCs [ITC and the corresponding dithiocarbamate; R-NH-C(=S)-SG] correlated closely and was linear with their potencies as inducers of phase II detoxification enzymes. For example, BITC was quickly and significantly accumulated in Hepa 1c1c7 cells, but very little inactive PITC was detected within the cells (33). They have also suggested the possibility that intracellular GSH as a target of direct alkylation with ITCs plays a negative regulating role in the phase II detoxification enzyme induction, on the basis of the result that depletion of GSH by BSO increased the inducer potencies of several ITCs (33). We indeed observed that the treatment of BITC for 15 min resulted in a significant depletion of the intracellular GSH level (Fig. 2A). However, based on the observation that BSO, which reduces the intracellular GSH level, showed no GST-inducing potency (data not shown), it is unlikely that down-regulation of intracellular GSH is simply an initial signal for GST induction.

It is noteworthy that the treatment of BITC quickly and significantly enhanced the intracellular ROI production in RL34 cells detected by the fluorescence probe, H_2DCF-DA (Fig. 3). H_2DCF-DA reacts peroxidase dependently or spontaneously with some types of ROIs, including hydrogen peroxide, lipid hydroperoxide, hypochlorous acid, and peroxinitrite (34, 35). The experiments using the membrane-impermeable catalase and superoxide dismutase also suggested that the BITC-induced ROI generation might occur within the cells. In the structure-activity relationship study of ITCs, the ROI-producing activities correlated closely with their GST-inducing potencies (Fig. 4). These results provide clear evidence for the intracellular ROI production induced by BITC and suggest that intracellular ROIs may be involved in the BITC-stimulated GST induction.

The oxidative stress-inducible effect of BITC was blocked by the pretreatment of an antioxidant, such as GSH or quercetin (Fig. 4). In fact, RL34 cells exhibited superoxide dismutase- and XOD inhibitor-

inhibitable superoxide anion radical generation when ferric nitrilotri-acetate was treated for 24 h (36). It is unlikely that XOD is responsible for the BITC-induced ROI production because activation of XOD is required for the conversion time lag of xanthine dehydrogenase, a major form of XOD protein in healthy tissue (37) and because of the lack of interference by the enzyme inhibitor, allopurinol (Fig. 4). The treatment of BSO, only in part, mimicked the elevation of cellular oxidative stress (Fig. 4). It has been reported that BSO mainly depleted GSH by blocking GSH synthesis and GSH reductase but did not appreciably affect the mitochondrial GSH pools (38). On the other hand, the pretreatment of DEM, which blocks the intracellular thiol groups, including mitochondrial GSH (39), only produced ROIs and enhanced the BITC-induced oxidative stress (Fig. 4). Moreover, pretreatment of DPI, acting not only as a NAD(P)H oxidase inhibitor (40) but also an inhibitor of mitochondrial reactive oxygen species production
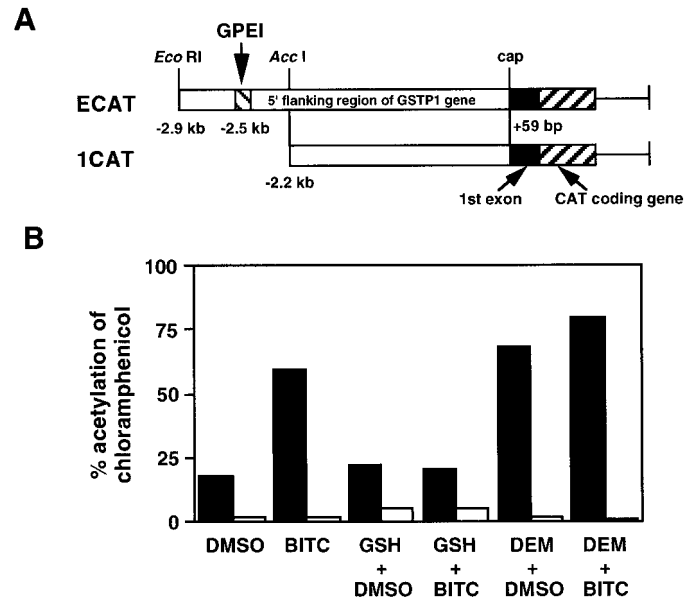

Fig. 5. Requirement of the GPEI-containing region for the induction of the *GSTP1* gene by BITC and redox alteration. *A,* structures of constructs of ECAT and 1CAT used for the transfection experiments. *B,* effects of BITC, GSH, and DEM on ECAT (■) or 1CAT (□) activity. The cells were pretreated with GSH (10 mM) or DEM (1 mM) for 1 h and then treated with BITC (5 μM) or DMSO for 1 h. After washing out the BITC followed by another 16 h incubation, the CAT activity was determined. *Column 1,* untreated control treated only with DMSO; *Column 2,* BITC; *Column 3,* GSH and DMSO; *Column 4,* GSH and BITC; *Column 5,* DEM and DMSO; *Column 6,* DEM and BITC.
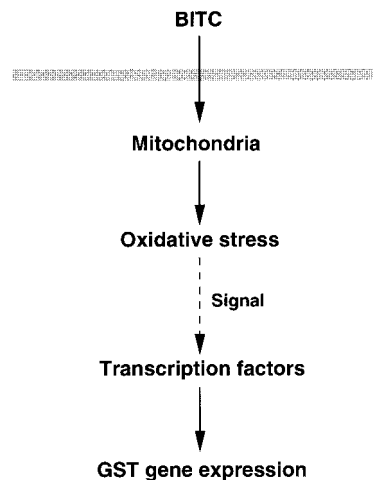

Fig. 6. A proposed mechanism for the BITC-induced *GSTP1* gene expression.

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(41), resulted in a significant decrease in the BITC-induced ROI accumulation (Fig. 4). These results strongly suggested that ROIs detected in the cells exposed to BITC may originate from the mitochondria, one of the major ROI-producing organella (Fig. 6). We have indeed observed that BITC enhances ROI production in isolated mitochondria.[5]

The mechanism of transcriptional regulation of GSTP1 has been revealed by a number of studies. As mentioned above, *GSTP1* gene expression is dominantly regulated by GPEI, containing a palindromic dyad of the 12-*O*-tetradecanoylphorbol-13-acetate responsible element-like sequence (21). This study revealed for the first time that GPEI is an essential *cis*-element required for the activation of the *GSTP1* gene through redox alteration by BITC or DEM (Fig. 5*B*). As mentioned above, the ITC-induced oxidative stress is reported to be involved in the ITC-induced activation of JNK (13), which phosphorylates transcriptional factors, such as c-Jun, ATF-2, and Elk-1, and strongly augments their transcriptional activity (42–45). Identification of the factor(s) that binds GPEI and activates the *GSTP1* gene expression is required for further understanding of the regulation of GSTP1 induction by BITC. In addition, it is noteworthy that the class π GST isozyme is closely related to the regulation of JNK signaling (46). It is within the range of possibility that the primary target of BITC or BITC-induced ROIs is GSTP1 itself, the modification of which may result in direct stimulation of the JNK signaling pathway. The observation that GST activity was inhibited by the incubation with BITC for 30 min[6] also supports this hypothesis.

In conclusion, intracellular ROIs are likely to mediate the BITC-induced *GSTP1* gene expression, based on the observations that: (*a*) a short exposure time (1 h) to BITC is sufficient to evoke the elevation of GST activity; (*b*) ROI-producing activities closely correlated with their GST inducing potencies; and (*c*) DEM enhanced the BITC-induced ROI production and accelerated both the basal and BITC-induced GST activities and the *GSTP1* gene expression, whereas the antioxidant GSH inhibited them. Although many researchers have confirmed that ITCs are promising and effective anticarcinogen candidates, some of the ITCs showed an enhancement of carcinogenicity or lack of chemopreventive effects in rat liver and kidney, especially dosed at the postinitiation phase (47, 48). Lee (49) has postulated that ITCs are oxidatively converted to the corresponding isocyanates, causing chromosome aberration, mutation, and cancer (50). These events may correlate with the intracellular ROI-producing potentials of the ITCs. In fact, cytotoxicity of ITCs at high dose can be inhibited by the antioxidant, *N*-acetylcysteine (51). As mentioned above, the profound relationship between GSTP1 and the stress signaling pathways (46) indicated that GSTP1 is one of the most important components that could influence key cellular functions, including growth, apoptosis, and transformation. Because the cancer preventive or promoting potential, threshold, and target organ of ITCs have to be distinguished in detail, further mechanistic studies on intracellular oxidative stress and the subsequent events induced by the ITCs are essential to provide supporting information.

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