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Temporal Effects of the Detoxification Enzyme Inducer, Benzyl Isothiocyanate: Activation of c-Jun N-terminal Kinase Prior to the Transcription Factors AP-1 and NFκB

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Benzyl isothiocyanate (BIT), a microconstituent found in cruciferous vegetables, is known to be a potent inducer of the detoxification enzyme, NAD(P)H: quinone reductase (QR). QR catalyzes a two-electron transfer to a wide variety of redox-cycling species, including quinones, transforming them into dihydrodiols, thereby preventing the mutation of DNA and reducing cancer risk. The upstream signaling mechanisms that lead to the induction of QR remain unclear. The 5′ promoter region of the human QR gene contains the *cis*-acting AP-1 and NFκB transcription factor binding sites. When HT29 human colon cells were exposed to 25μM benzyl isothiocyanate, AP-1 binding increased, beginning at 3 hours and increasing until 16 hours. NFκB binding also increased, reaching a maximum at around 6 hours. We also found that c-Jun N-terminal kinase (JNK), which phosphorylates c-Jun, a component of AP-1, was activated 9-fold over controls, beginning at 60 minutes. The temporal sequence of these events supports the idea that JNK is involved in the induction of QR and that this is an initial event preceding an increase in transcription factor binding and subsequent QR activity.

Several epidemiological studies have indicated that consuming a diet high in fruits and vegetables decreases the risk of developing colon cancer (1–3). A mechanism potentially responsible for this decrease in risk is the induction of phase II detoxification enzymes, including NAD(P)H:quinone reductase EC (1.6.99.2) (QR) and the family of glutathione-S-transferases (EC 2.5.1.18), by microconstituents present in dietary fruits and vegetables (4–6). The induction of phase II detoxification enzymes helps to eliminate carcinogens supplied by the diet, thereby decreasing the mutagenesis of DNA and preventing the initiation of carcinogenesis.

Strongly associated with decreased cancer risk is the family of cruciferous vegetables, which includes broccoli, cabbage, and kale (7,8). Cruciferous vegetables contain several microconsituents, including aromatic isothiocyanates, indoles, isoflavones, dithiolthiones, and coumarins. Many studies, performed in animal models, indicate that these microconsitituents have a chemoprotective action against carcinogenesis. Aromatic isothiocyanates, which arise in plants as the result of enzymatic cleavage of glucosinolates by myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1), have been implicated to be among the most potent chemoprotective agents known (7, for a review see 9). For example, Fischer rats treated with 8 mmol/kg phenethyl isothiocyanate (PEITC) showed only a 9% incidence of lung tumorigenesis upon treatment with the carcinogen 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK), compared to a 67% incidence in non PEITC-treated controls (10). Another study showed that in F344 rats, dietary phenethyl isothiocyanate completely inhibited N-nitrosomethyl benzylamine (NMBA)-induced esophageal tumors (11). An additional study demonstrated that 6-phenylhexyl isothiocyanate can inhibit NNK-induced lung tumorgenicity in strain A mice by >80% when administered at a 50-fold lower dose than NNK (12). Benzyl isothiocyanate has been found to be a potent inducer of the detoxification enzymes QR and GST (13–16). The detoxification enzyme, QR, is a ubiquitous enzyme that promotes the obligatory two-electron reduction of a wide variety of redox-cycling species, including quinones and other redox-cycling compounds, and thereby protects cells against cytotoxicity from these species and their metabolic precursors (14, 17). Detoxification enzymes, such as QR, induced by microconstituents contain in their upstream 5′ regulatory regions *cis*-acting regulatory elements, including the antioxidant response element (ARE) (18,19), the xenobiotic-

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responsive element/aromatic hydrocarbon-responsive-element (XRE/Ah) (20,21), AP-1 binding sites (22), and NFκB binding sites (23).

Although most of the individual regulatory elements in the promoter region have been identified, the sequence of signaling events involved in the transcriptional activation of QR have yet to be fully defined. One of the potential mediators of the upstream signaling involved in the induction of detoxification enzymes is c-Jun N-terminal kinase (JNK), also called stress-activated protein kinase (4). JNK is a subgroup of the mitogen-activated protein kinase family, which is comprised of three main subgroups: the extracellular signal-related kinases, ERKs, the JNKs, and p38 (24). Similar to the other subgroups, the activation of JNKs requires dual phosphorylation at conserved threonine and tyrosine residues by an upstream kinase, JNK kinase (JNKK/SEK/MKK4), which is itself regulated by the upstream kinase MEKK1. Recent studies have indicated the existence of at least three isoforms of JNK. These interact selectively with Jun, ATF2, and Elk-1 transcription factors (30). JNKs are activated by a variety of stresses, including ultraviolet irradiation, heat shock, protein synthesis inhibitors, inflammatory cytokines, and oxidative stress (31). Once activated, JNKs phosphorylate a limited number of transcription factors, including c-Jun, one of the components of the transcription factor AP-1. The phosphorylation of basal levels of bound c-Jun by JNK at serines 63 and 73 in the N-terminal, leads to the transactivation and increased expression of AP-1-regulated genes (for a review see Karin 1993 (44)).

QR also contains an NFκB binding site within its regulatory region (28). The activation of NFκB involves a post-translational mechanism involving the phosphorylation and subsequent degradation of the IkB inhibitory subunit by IkB kinase (29). The relationship between JNK, AP-1 and NFκB activation has yet to be fully understood, however, several studies have implicated that IkB kinase is activated in parallel with JNK (32–34). MEKK1, a MAP kinase kinase kinase upstream of MKK4, and JNK, activates IkB kinase in conjunction with the JNK pathway. The activation of NFκB and JNK by BIT seen in our study also support the idea that JNK and NFκB activation are governed by parallel signalling pathway(s).

A final point, relevant to the findings of this study, is that the binding of AP-1 and NFκB are known to be under redox control (35,36). Isothiocyanates cause a depletion of GSH within the cell because the major route of elimination of isothiocyanates is through conjugation with GSH (39). Also, enzyme induction by isothiocyanates has been associated with a modest oxidation of the GSH pool (37). Interestingly, the level of intracellular glutathione has been found to be a key regulator in the induction of stress-activated signal transduction pathways including JNKs (38). The common modulatory signal by BIT may therefore consist of an initial depletion of glutathione followed by a rapid activation of JNK and subsequent transcription factor binding.

This study investigates the temporal sequence of events that occur prior to an increase of QR activity and provides insight into the upstream mechanisms governing the regulation of QR expression by benzyl isothiocyanate. Due to the widespread occurrence of isothiocyanates within the diet and thus the chronic exposure of the gut to these compounds, we have used a moderately well-differentiated, minimal deviation, human colon cell line, HT29, as a model to investigate the ordering of the events leading to QR induction. We show here that QR is expressed in these cells and that QR activity is induced by the addition of benzyl isothiocyanate to cells. The initial event we measure is the activation of c-Jun N-terminal kinase which precedes an induction of AP-1 binding and a transient but definite induction of NFκB binding. These events correspond well with the induction of QR in these cells and our results help to confirm that these preliminary events are critical to the expression of QR in HT29 cells.

MATERIALS AND METHODS

Materials. Benzyl isothiocyanate was purchased from Aldrich (Milwaukee, WI). DMSO was purchased from Sigma (St. Louis, MO).

Cell culture. The human colon adenocarcinoma cell line HT29 was obtained from ATCC and cultured in McCoy's medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% FBS in 5% CO_2 at 37°C in a humidified incubator. Cells were plated at a density of 1×10^5 and grown for five to six days until 70% confluent at which time experiments were initiated. Media was replaced with serum-free media 24 hours before treating cells. Cells were treated with BIT in 0.2% DMSO or vehicle alone (0.2% DMSO).

NAD(P)H:quinone reductase activity. Cells were washed twice in ice-cold PBS, scraped, snap-frozen and stored at −70°C until use. Upon thawing cells were resuspended in PBS and lysed by freeze/thawing three times with liquid nitrogen. Then 10 ml of 0.25 M sucrose was added and the cells were centrifuged at 2,000×g for 5 mins. Twenty percent of the total volume of 0.1% of $CaCl_2$ was added to the supernatants and the samples were set on ice for 15 mins. The samples were spun at 4,000×g for 15 mins. The supernatants were then removed and assayed for QR activity. QR activity was measured by a spectrophotometric assay in which the rate of reduction of 2,6-dichlorophenol indophenol was monitored at 600 nm. Protein levels were measured by the Bradford assay (Bio-Rad, Richmond, VA) and results were normalized.

Nuclear extracts. Following treatment of cells, nuclear extracts were prepared by a modification of the method of Schreiber et al (40). Briefly, cells were harvested and washed twice in TBS. Buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) was added (400 μl/sample). Samples were placed on ice for 15 mins and 25 μl of 10% Triton-X 100 was added. Samples were vortexed for 10 secs, centrifuged and the supernatants were discarded. Cell pellets were resuspended in 50 μl of ice-cold buffer C (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and rocked vigorously for 15 mins at 4°C. The nuclear extracts were then centrifuged for 5 min at 4°C and the supernatants were measured for protein content by Bradford assay (Bio-Rad, Richmond, VA).

Electrophoretic mobility shift assays. The nuclear extracts were analyzed for AP-1 and NFκB binding activity by gel mobility shift assays following the manufacturer's protocol (Promega, Madison, WI). The AP-1 consensus oligonucleotide was 5′-CGCTTGATGAGTCAGCCGGAA-3′. The NFκB oligonucleotide was 5′-AGTTGAGGGGACTTTCCCAGGC. Briefly, 5 μl of nuclease-free water, 2 μl 5× gel shift binding buffer (20% glycerol, 5 mM $MGCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.25 mg/ml poly(dI-dC).poly(dI-dC)), 30 μg nuclear extract were incubated at 4°C for 10 mins. Then 1 μl of NFκB or AP-1 probe was added (greater than 50,000 cpm/ml) and the samples were incubated for a further 20 mins at 4°C. The samples were then run on a 10% polyacrylamide gel for 1hr 20 mins at 150V. The gels were dried and exposed to autoradiographic film with intensifier screen at −70°C overnight.

JNK assay. Jun kinase assays were carried out as described (24) using a GST-c-Jun (1-79) fusion protein coupled to glutathione-agarose beads as substrate, a kind gift of D.A. Brenner, University of North Carolina, Chapel Hill. The mutated version (GST-c-Jun Ser 63 and 73 mutated to Ala 63 and 73) was used as a control substrate. Briefly, HBB buffer containing proteases and phosphatase inhibitors was added to cell extracts. Cellular extracts (100 μg) were used as the kinase source. A GST-agarose-coupled c-Jun protein substrate was added and the mixture was rotated for 3 h at 4°C. The beads were purified by washing 4 times with HBB buffer plus phosphatase and protease inhibitors. A 'hot' reaction mix containing ^{32}P ATP was then added to samples for 20 mins at 30°C. Samples were run on a 10% SDS polyacrylamide gel which was dried and exposed to radiographic film overnight.

RESULTS

BIT Increases QR Activity 2–3-Fold Over Controls

In order to establish whether BIT increases QR activity in HT29 cells, we first measured the level of QR induction when cells were treated with 0–40 μM BIT (Fig. 1A). When cells were treated with a concentration of BIT as low as 10 μM for 24 hours, QR activity increased approximately 2-fold over controls. When cells were treated with 40 μM BIT for 24 hours, QR activity increased approximately 2.5-fold over controls, which was similar to the level of induction from treatment with 25 μM BIT (about 2.3 fold). In summary, BIT appeared to increase the induction of QR 2-2.5-fold over controls, starting at a concentration of 10 μM treatment for 24 hours. We then evaluated the time course of QR induction at 25 μM BIT.

To examine the time course of QR induction, the cells were dosed with 25 μM BIT from 0 to 24 hours. This concentration was found to be the most suitable for two reasons. First, 25 μM BIT approximates the cellular concentration to which cells are exposed to isothiocyanates in a typical diet, estimated to result from the ingestion of milligram quantities daily (9). Second, the treatment of HT29 cells with 40 μM BIT, even though inducing QR to a larger extent than 25 μM BIT, also led to more necrosis/apoptosis of the cells as evidenced by an increased number of floating cells in the media (data not shown). We therefore dosed cells with 25 μM BIT for 0 to 24 hours (Fig. 1B). QR induction stared increasing slightly at six hours and slowly increased to

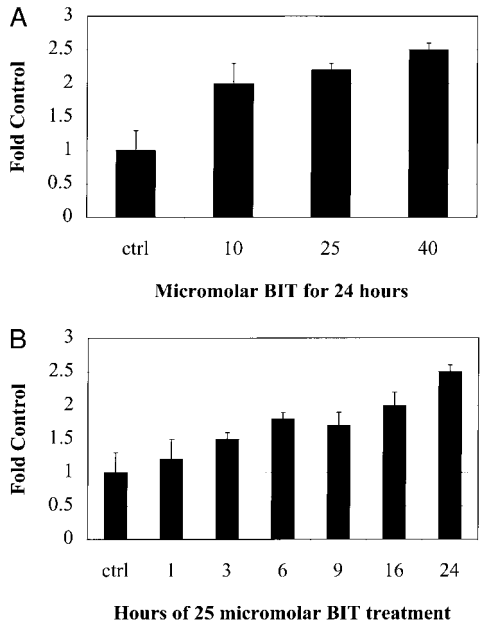

FIG. 1. (A) Dose-responsive induction of QR by BIT in HT29 cells. Cells were plated and allowed to reach a confluence of 70%, roughly 48 hours after plating. HT29 cells were treated for 24 hours, with fresh media containing 0-40 μM BIT and 0.2% DMSO. (B) Time course of QR induction by treatment of cells with 25 μM BIT. The cytosolic QR activities are expressed as percent of DMSO-treated controls. Each experimental point represents the mean determination of cells obtained from four plates.

2.5-fold over control at 24 hours. We were most interested in the earlier time points because of our focus on the events preceding the induction of QR. We therefore did not consider time points later than 24 hours, at which point the actions of phosphatases (25), changes in transcription factor binding, and other cellular mechanisms may interfere with the initial response to BIT treatment. From these data it appears that QR is induced beginning at approximately six hours and induction increases in a manner that correlates well with JNK activation and transcription factor binding, discussed below.

BIT Increases AP-1 Binding

Several studies have implicated the importance of AP-1 binding in the expression of QR (23, 26). We therefore examined the time course of AP-1 binding in HT29 cells in order to establish whether this event preceded the initial expression of QR activity. The electrophoretic mobility shift assay (EMSA) shown in Fig. 2, indicates that the binding of AP-1 in nuclear extracts of cells treated with 25 μM BIT from 0-16 hours increased beginning at 3 hours increasing until 16 hours. AP-1 binding, as measured by EMSA, preceded and corresponded with the increase in QR activity. An important point concerning these results is that the c-Jun gene contains an AP-1 binding site within its 5′ pro-

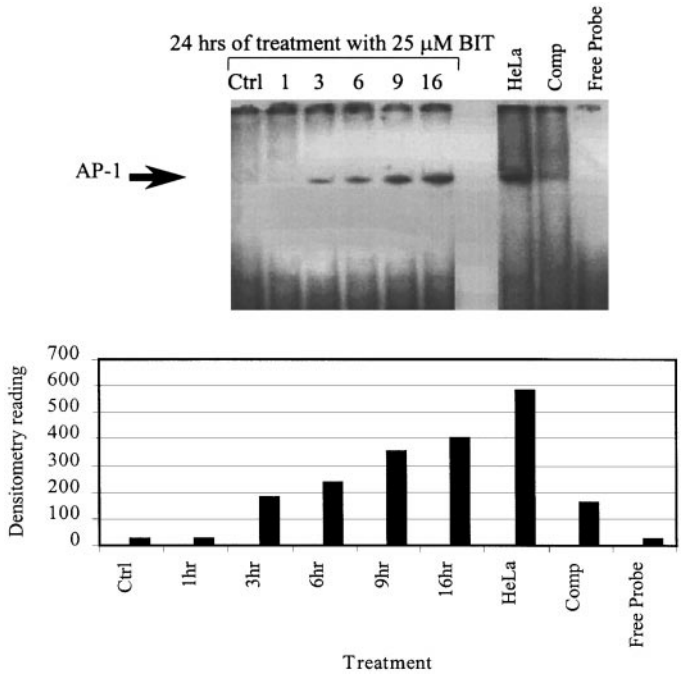

FIG. 2. AP-1 binding increases in response to 25 μM BIT treatment. EMSA of nuclear extracts isolated from HT29 cells treated with BIT in 0.2% DMSO for the indicated time points. Control cells were treated with DMSO alone. Nuclear extracts (30 μg) were incubated with radiolabelled oligonucleotide containing the consensus sequence for AP-1 in the presence of non-specific binding inhibitor poly (dI)-(dC). HeLa extract served as a positive control. Competitor indicates a 100-fold excess of cold oligonucleotide added to HeLa cell extract. DNA-protein complexes were resolved in 10% polyacrylamide gels. Gels were dried and exposed to autoradiographic film with an intensifier screen at −70°C overnight. This result is representative of three independent experiments.

moter region (27). It is possible that the increase in AP-1 at 16 hours is due to the increased presence of c-Jun protein which has been induced by the initial event of JNK activation, as discussed below.

BIT Increases NFκB Binding

As well as an AP-1 site, the QR gene contains within its 5′ promoter region an NFκB binding site. We were therefore interested in the temporality of NFκB binding and wished to examine its increased binding in relation to QR activity in HT29 cells. Many studies have indicated that the binding of the transcription factors, AP-1 and NFκB, are regulated by the redox state of the cell (36,41). BIT is known to generate a redox signal and deplete glutathione in HT29 cells (37). As shown in Fig. 3, nuclear extracts of cells treated with 25 μM BIT showed significantly increased binding at 6 hours but this binding did not occur at any other time point. One possible consequence of the increased NFκB transcription factor binding is its potential role in the increased expression of QR activity that is seen at later time points.

BIT Activates JNK Starting at 60 mins, and at 3 hours, 6 fold over Controls (Fig. 4)

In order to examine whether JNK activation preceded the increase of QR activity within the cells, we measured the time course of JNK activation by 25 μM BIT from 0 to 8 hrs. It should be noted here that the assay we used to measure JNK activity measured the total activity of all JNK isoforms, to the extent that they are capable of phosphorylating the GST-cJun substrate, and does not distinguish between the individual isoforms. We first saw an increase in JNK activation at 60 mins, which increased to a maximum at 3 hours and appeared to decrease slightly after that. JNK activation was minimal before one hour (data not shown). These results indicate that JNK activation by 25 μM BIT is rapid and occurs before the initial binding of AP-1 and NFκB. Once again, we were interested in the initial activation of JNK to see if activation occurred prior to initial AP-1 binding.

DISCUSSION

The precise upstream signaling mechanisms leading to the increase of QR within cells upon treatment with dietary microconstituents such as BIT, have yet to be fully clarified. To help establish the sequence of events leading to the induction of QR by BIT, we have examined the temporal relationship between JNK activation, and AP-1 and NFκB transcription factor binding, all of which were found to initiate prior to the induction of QR activity. We have attempted to clarify the sequence of events that lead to QR induction so as to gain insight into the mechanisms of cellular protection by diet.

We found 25 μM BIT to be a strong inducer of JNK activity, with activity similar to that seen with 10 ng/ml TNFα, a potent activator of JNK in HT29 cells. The activation of JNK is most likely cell-type specific and different forms of isothiocyanates have different effects within various cell lines. For example, phenethyl isothiocyanate (PEITC) activated JNK1 in a dose and time-dependent manner, with a maximal activation after 90 mins of treatment with 50 μM PEITC in HeLa cells (4). In this study, when the activation of JNK1 by BIT was compared with that by PEITC, the results showed that the activation of JNK by BIT was relatively weak in comparison. However, these results are likely to be cell-type specific. In addition, since the assay we used to measure JNK measures the phosphorylation of a GST-cJun substrate, we measured the activation of all the JNK isoforms that phosphorylate c-Jun, rather than JNK1 alone and this too may contribute to the differing results.

Our results show that BIT strongly activated JNK in HT29 cells and we can only speculate as to the cellular changes that lead to JNK activation. A possible candi-

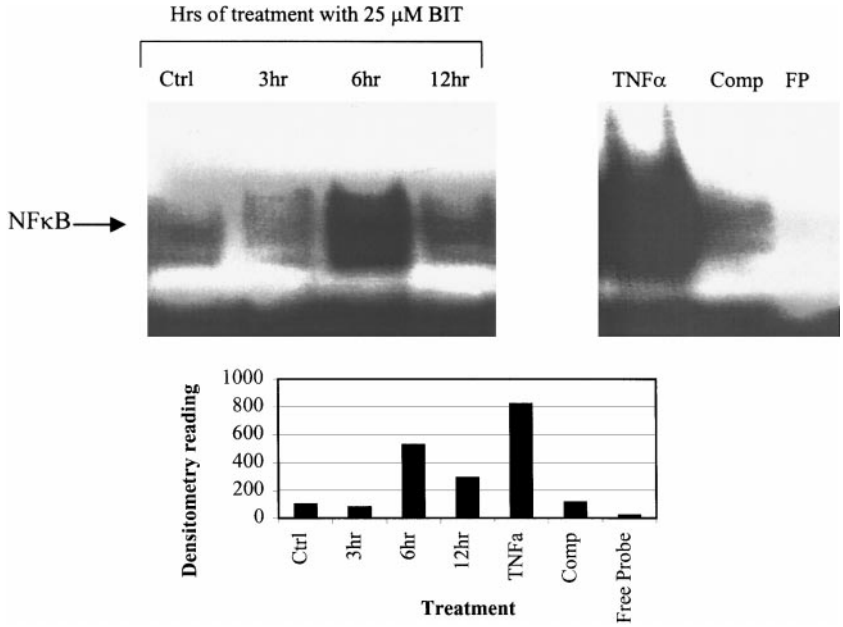

FIG. 3. NFκB binding increases in response to 25 μM BIT treatment. EMSA of nuclear extracts isolated from HT29 cells treated with a BIT in 0.2% DMSO for the indicated time points. Control cells were treated with DMSO alone. Nuclear extracts (30 μg) were incubated with radiolabelled oligonucleotide containing the consensus sequence for NFκB in the presence of non-specific binding inhibitor poly (dI)-(dC). Nuclear extract of cells treated with 10 ng/ml of TNFα served as positive control. Competitor indicates a 100-fold excess of cold oligonucleotide added to TNFα-treated cell extract. DNA-protein complexes were resolved in 10% polyacrylamide gels. Gels were dried and exposed to autoradiographic film with an intensifier screen at −70°C overnight. This result is representative of three independent experiments.

date for the cellular mechanism(s) leading to the activation of JNK is the depletion of reduced intracellular glutathione levels. In support of this idea, Wilhelm et al (38) demonstrated by modifying glutathione levels that the level of intracellular glutathione is a key regulator in the induction of JNK and p38 by alkylating agents (38). Indeed, many known activators of JNKs exert some type of oxidative stress on the cell. Other researchers have found that the depletion of glutathione and a change in cellular redox state plays an important role in AP-1-mediated QR expression (22,42).

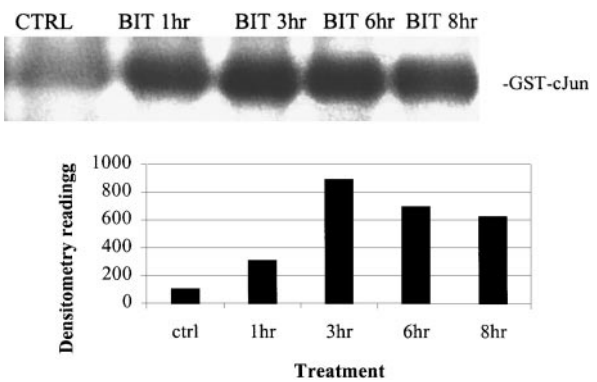

FIG. 4. JNK is activated upon treatment with 25 μM BIT. Cells were treated for 0–8 hours with 25 μM BIT. Cells were serum starved for 24 hours before treatment. Whole cell extracts were used for the kinase reaction with GST-c-Jun (1-79) as a substrate.

Since our results indicate that the initial activation of JNK by BIT takes place as soon as 60 minutes after the addition of BIT to cells, the rapid depletion of glutathione is a plausible mechanism for the activation of JNK.

In addition to AP-1 binding and JNK activation, BIT treatment of HT29 cells led to NFκB binding. Maximal binding occurred at 6 hours. However, the binding was transient and did not occur at any other timepoint. This may indicate that NFκB plays a less important role than AP-1 in sustaining the transcriptional regulation of QR. In support of this idea O'Dwyer et al studied the involvement of AP-1 and NFκB transcription factors in the control of QR expression induced by Mitomycin C treatment in HT29 cells. The specific involvement of AP-1 and NFκB was confirmed using mutational analysis and the effect of deleting the AP-1 element on both basal and induced transcription was more dramatic than deleting the NFκB element (23). Another possibility is that the probe used in our study was a consensus oligonucleotide optimized for NFκB binding, but which differed slightly from the sequence of NFκB site of the QR gene.

Recent studies have implicated that c-Jun and NFκB are involved in the same stress response pathway. In our study, the fact that BIT treatment of cells led to both JNK activation and NFκB binding supports the idea that these events may be subject to common regulatory mechanisms. Lee et al postulated that MEKK1, a key upstream regulator of the JNK pathway, also

induces the phosphorylation of the inhibitor of NFκB, IκBα, targeting this subunit for degradation (34). Another explanation for the dual activation of JNK and NFκB is that the common oxidative signal generated by BIT and the resulting depletion of glutathione may lead to the activation of AP-1 and/or JNK and NFκB which have been previously established to be under redox control (41,43).

One of the genes regulated by AP-1 is the c-*jun* protooncogene itself because of the presence of an AP-1 binding site within its own upstream regulatory region (27). The TPA-response element (TRE) in the c-*jun* promoter differs from the consensus TRE sequence by a 1-base pair insertion (27), and due to this change it is more efficiently recognized by c-Jun/ATF2 heterodimers than by conventional AP-1 complexes (44). Recent reports have indicated that p38 may play a more important role than JNK in the stimulation of c-*jun* transcription (45,46), however inhibition of p38 with SB203580 and subsequent effects of c-Jun expression seem to be dependent on the specific stimuli used. For example in C3H 10T1/2 cells, induction of early response genes, including c-*jun*, by TNFα or okadaic acid was not affected by the specific inhibitor of p38, SB 203580 (46). Therefore JNK and/or p38 may be involved in the stimulation of c-*jun* transcription by BIT. Phosphorylation of AP-1 by JNK may therefore lead to increased c-Jun levels and AP-1 dimerization, followed by increased binding of newly-synthesized AP-1 to its response element. Our measurement of the time course of AP-1 binding upon treatment of cells with 25 μM BIT supports this idea. An initial increase is seen at 3 hours which increases until 16 hours. We can therefore speculate that the initial AP-1 binding is due to the redox signal or glutathione depletion brought about by BIT treatment and that the further increase in AP-1 binding is the result of an increase in newly synthesized c-Jun leading to increased dimerization and binding of AP-1.

In this report we have studied the induction of QR. However, these results may also be indicative of the sequence of events leading to increases in the activity of glutathione-S-transferases and other AP-1-regulated genes. Previous studies have examined the induction of both QR and GST by AP-1 (18,22) and isothiocyanates have been shown to induce GST as well as QR (7,9). In summary, the findings of our study provide support for the idea that JNK is involved in the induction of detoxification enzymes such as QR. Further studies should be carried out to analyze these mechanisms in more detail so that a better understanding chemoprevention by diet can be achieved.

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